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*Proceedings of the 28th International Congress of Physiological Sciences Budapest 1980*

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Volume 7

# Cardiovascular Physiology Microcirculation and Capillary Exchange

*Editors*

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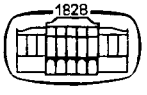


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# FOREWORD

This volume is one of the series published by Akadémiai Kiadó, the Publishing House of the Hungarian Academy of Sciences in coedition with Pergamon Press, containing the proceedings of the symposia of the 28th International Congress of Physiology held in Budapest between 13 and 19 July, 1980. In view of the diversity of the material and the "taxonomic" difficulties encountered whenever an attempt is made to put the various subdisciplines and major themes of modern physiology into the semblance of some systematic order, the organizers of the Congress had to settle for 14 sections and for 127 symposia, with a considerable number of free communications presented either orally or as posters.

The Congress could boast of an unusually bright galaxy of top names among the invited lecturers and participants and, naturally, the ideal would have been to include all the invited lectures and symposia papers into the volumes. We are most grateful for all the material received and truly regret that a fraction of the manuscripts were not submitted in time. We were forced to set rigid deadlines, and top priority was given to speedy publication even at the price of sacrifices and compromises. It will be for the readers to judge whether or not such an editorial policy is justifiable, for we strongly believe that the value of congress proceedings declines proportionally with the gap between the time of the meeting and the date of publication. For the same reason, instead of giving exact transcriptions of the discussions, we had to rely on the introductions of the Symposia Chairmen who knew the material beforehand and on their concluding remarks summing up the highlights of the discussions.

Evidently, such publications cannot and should not be compared with papers that have gone through the ordinary scrupulous editorial process of the international periodicals with their strict reviewing policy and high rejection rates or suggestions for major changes. However, it may be refreshing to read these more spontaneous presentations written without having to watch the "shibboleths" of the scientific establishment.

September 1, 1980

J. Szentágothai

President of the
Hungarian Academy of Sciences

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*A. G. B. Kovách, J. Hamar, L. Szabó (eds)*

# COORDINATION OF MICROCIRCULATORY FUNCTION WITH OXYGEN DEMAND IN SKELETAL MUSCLE

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Local regulation of the peripheral circulation is most commonly thought of in terms of regulation of blood flow, and processes such as autoregulation, functional hyperemia, and reactive hyperemia are all taken as indices of the coupling between peripheral circulatory function and tissue metabolism. When other variables such as hormonal influences and neuronal control mechanisms are excluded, a tight parallelism is usually found between flow and the metabolic needs of tissues. This relation has in the past led to the virtual exclusion of other elements of microvascular function from consideration in local regulatory processes. In this discussion I will examine some of the facts showing how microcirculatory parameters other than bulk flow of blood to the tissues may be controlled and, in addition, how various microvessel elements may interact with one another.

The focus of the discussion will be on the ways in which oxygen delivery to cells is regulated. This is not intended to indicate that oxygen is the sole substance of interest in regulation of the peripheral circulation, but rather, is simply the focus for a line of investigation which we have been following for several years. For analytical purposes the problem of relating cellular oxygen delivery to microcirculatory function can be broken down into a schema such as that shown in Figure 1.

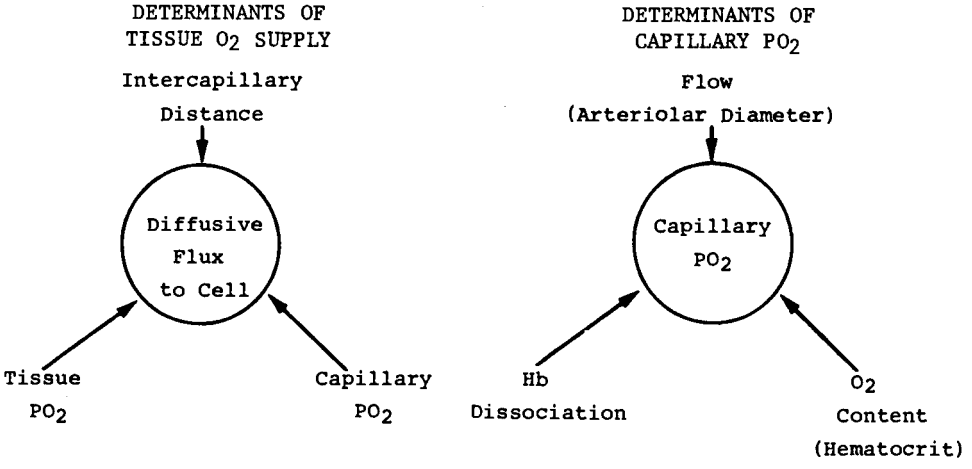


Figure 1. Determinants of Cellular $O_2$ Delivery.

The left side of the figure shows the variables which must be known in order to specify the diffusive flux of oxygen from a capillary to a cell. Assuming a constant diffusion coefficient for oxygen, simple diffusion theory can predict $O_2$ delivery from a knowledge of diffusion distance (capillary density), and the difference between the $PO_2$ of the cell and the capillary. The right side of the figure shows a number of variables which may determine the capillary $PO_2$. Capillary $PO_2$ would, of course, also be influenced by the state of arterial oxygenation and by phenomena such as diffusional shunting above the level of the capillary bed. However, the essence of the findings to be presented here is that the variables shown in the left side of the figure can be measured directly using appropriate microvascular methodology, and thus one need not know what factors contributed to the capillary $PO_2$, only that the capillary $PO_2$ in the vicinity of the metabolizing cells had a certain value. For the purposes of the present discussion, it is also assumed that the tissue is in the steady state and that $O_2$ diffusion to the cells is equal to the consumption rate.

I will focus on four variables and how they are inter-related by microvascular control systems to regulate cellular oxygen delivery. These are: arteriolar diameter (flow control), capillary density (diffusion distance), $O_2$ content (hematocrit), and cell $PO_2$. Within this context, two broad issues will be addressed. First, how do the microvascular elements (diameter, density, and hematocrit) vary and interact during regulation of blood flow? Second, is available evidence generally consistent with the idea that the system is designed to regulate tissue $PO_2$ within relatively narrow limits? An additional point which will be emphasized is that it may not be a simple matter to recognize a regulated process in a system as complex as this one.

A large part of the work done to date on the local regulation of circulatory function has been carried out on perfused organs of various sorts and microvessel behavior is inferred from measurements of flow and the capillary filtration coefficient or permeability surface area product. Behavior of the tissue oxygen consuming processes has been inferred from analysis of mixed venous blood. All of these measurements provide indirect estimates of the variables shown in Figure 1. Additional factors such as: variable behavior of series elements in the microvasculature, regional heterogeneities within the tissues, and possible shunting of gases between arterioles and venules, present difficulties in making clear statements of conditions at the level of individual cells (Duling & Klitzman, 1980).

We have approached this research by utilizing relatively recent improvements in techniques for studying the microcirculation (Johnson, 1972) to examine the relations among microcirculatory parameters and striated muscle contraction. The basic experimental paradigm has been to attempt to focus measurements on small, reasonably well defined units, consisting of arterioles, capillaries, and associated striated muscles, and to examine how these elements interact. The net result of oxygen consumption by tissue and microcirculatory oxygen supply has been assessed using the oxygen microelectrode developed by Whalen et al. (1967). With this electrode we can measure $PO_2$ at locations confined to a few microns in diameter and, when combined with appropriate microscopy, the exact position of the electrode relative to microvascular elements and striated muscle cells can be ascertained.

Typically, we have chosen the minimum tissue $PO_2$ as a parameter to be measured; this is obtained by visually selecting a site for study which is at the venous end of a capillary and midway between a pair of capillaries,

thus approximating Thews' "lethal corner" (Thews, 1960). This point was chosen as it may have important implications in regulation of flow and metabolites since it will be the first part of the tissue whose function is limited by $O_2$ availability (Honig et al., 1971).

## DIAMETER CHANGES DURING MUSCLE CONTRACTION

The relations between oxygen supply and demand have been varied experimentally in two ways, either by changing the tissue metabolic rate by stimulation of the striated muscle cell, or by changing the apparent $O_2$ consumption by varying the $PO_2$ of a superfusion solution covering the tissue. An increase in superfusion solution $PO_2$ is used to mimic a decrease in oxygen consumption by the tissue, since a fraction of the oxygen can be supplied from the solution and need not be supplied by vascular means.

Figure 2 shows how arteriolar diameter is influenced by changes in superfusion solution $PO_2$. Elevation of the superfusion solution $PO_2$ results in an increased tissue $PO_2$ (Fig. 6A) and a corresponding decrease in arteriolar diameter. The decrease in arteriolar diameter reduces flow and minimizes the change in $PO_2$ which is induced by superfusion with a solution containing high oxygen (Duling, 1972; Gorczynski & Duling, 1978).

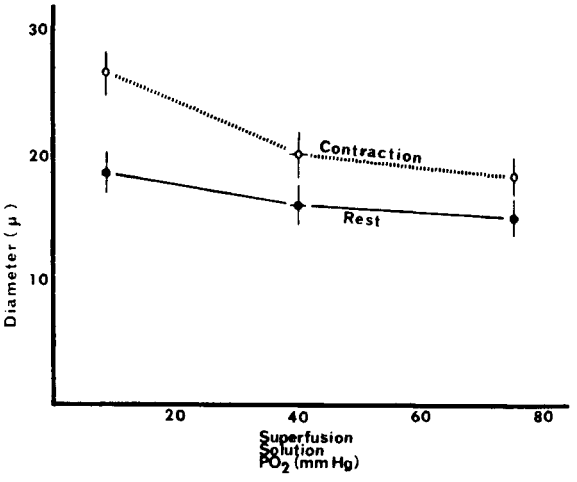


Figure 2. Effect of Changes in Superfusion Solution $PO_2$ on Arteriolar Diameter in Resting and Contracting Striated Muscle.

Stimulation of the striated muscle can be combined with alterations in superfusion solution $PO_2$ to permit independent variation of tissue $PO_2$ and muscle work (Fig. 2). In the experiment depicted here, superfusion solution $PO_2$ was varied either during resting conditions or during stimulation of the striated muscle at 1 Hz. Stimulation of the striated muscle resulted in a vasodilation and a fall in tissue $PO_2$. However, even during contraction, the striated muscle microcirculation continued to constrict in response to elevations in superfusion solution $PO_2$.

The difference in arteriolar diameter at rest and during contraction reflects the microvessel equivalent of functional hyperemia, and this

functional dilation varies with both the initial state of the microcirculation and with the initial tissue oxygen tension. As superfusion solution and tissue oxygen tension are elevated, functional dilation is diminished. Using the relation between vascular conductance and the fourth power of the diameter, we have estimated that the findings are consistent with a progressively smaller conductance change, i.e., functional hyperemia, as superfusion solution $PO_2$ is elevated (Damon, unpublished).

Both tissue $PO_2$ and diameter increment are observed to change during muscle stimulation, and we have attempted to establish a cause-and-effect relationship between these two variables (Gorczynski & Duling, 1978). This was done by raising superfusion solution $PO_2$ during striated muscle contraction so as to return the tissue $PO_2$ toward the resting value. If variation of tissue $PO_2$ was the sole controller of arteriolar diameter, then restoration of tissue $PO_2$ to resting levels during continued contraction should have restored diameter to control. In fact, when tissue $PO_2$ in the contracting muscle was restored to resting levels, the vasodilation during contraction was reduced by only about 50%. These and other findings suggested that as much as 50% of the vasodilation during functional hyperemia was associated with the production of some vasodilator metabolite not linked to the tissue levels of oxygen.

A major area of concern in studies on local regulatory mechanisms has historically been the nature of the vasodilation which produces functional hyperemia. A consideration of this problem is beyond the scope of the present presentation but, in my judgement at this time, there is no unequivocal demonstration as to the chemical nature of either the oxygen linked or the oxygen independent vasodilator associated with muscle contraction. An interesting sidelight on the experiments shown in Figure 2 is that the vessels will continue to constrict when tissue $PO_2$ is elevated to very high levels by increasing superfusion solution $PO_2$; half maximal contraction is obtained at a superfusion solution $PO_2$ of 23 mm Hg, a value far in excess of the $K_m$ for mitochondria (Chance et al., 1969). This would suggest that the sensor for altered tissue $PO_2$ is not cytochrome $a_3$ or that the in situ $K_m$ for this enzyme is much higher than reported in vitro.

## INTERACTION OF CAPILLARIES AND ARTERIOLES

As pointed out in the discussion of Figure 1, the $O_2$ supply to tissues can be varied by both increases in flow and by diminutions in the diffusion distance. We have therefore examined the effect of increases in superfusion solution $PO_2$ and stimulation of the striated muscle on capillary density and found, as have others (Lindbom et al., 1980; Prewitt & Johnson, 1976), a behavior pattern similar to that observed for the arterioles. Increasing the solution $PO_2$ causes a reduction in the number of perfused capillaries and stimulation of the striated muscle causes capillary recruitment.

The major subject of this manuscript is not the regulatory process per se, however, but how various elements in the microcirculation interact. It has been known for some time that the response of the vasculature to external stimuli (Myers & Honig, 1969) and to local regulatory stimuli (Jones & Berne, 1965) may depend on the conditions which exist at the time of stimulation. More recently, Granger and colleagues have shown that not only is the flow response to various stimuli a function of the initial conditions, but also capillary recruitment appears to be, at least in part, determined by conditions at the time stimuli are applied (Granger et al., 1976). They have reported that a high oxygen availability at the time

muscle contraction is initiated will influence the vascular bed in such a way that it accomplishes the necessary augmentation in supply of oxygen largely by an increase in capillary density and oxygen extraction from the blood. On the other hand, they found that, under conditions in which oxygen availability was low at the time of muscle stimulation, oxygen supply was increased to meet the new demand to a larger extent by arteriolar vasodilation and flow increases.

When these data are viewed from the perspective of the fact that recent investigations have failed to disclose the presence of a precapillary sphincter in the microcirculation of striated muscle (Eriksson & Myrhage, 1972; Gorczynski et al., 1978; Lindbom et al., 1980), their meaning is difficult to interpret. Lacking a precapillary sphincter, one must propose that capillary patency is controlled by arterioles. However, arterioles are also thought to control flow. Therefore, one is faced with the proposition that there may be differential control of blood flow and the number of open capillaries, but the same structure, the arteriole, presumably controls both processes. In view of this problem in understanding interactions between flow control and capillary density control, and in view of the fact that capillary density in Granger's experiments was determined by the measurement of capillary filtration coefficient, which is an indirect estimate of capillary recruitment, we decided to compare the ways in which altered oxygen availability at the time of stimulation of striated muscle would influence capillary recruitment and changes in arteriolar diameter.

As mentioned, capillary density in both the resting and contracting striated muscle was sensitive to changes in superfusion solution $PO_2$. However, resting capillary density was somewhat more sensitive to changes in solution oxygen tension than was capillary density in the contracting muscle, and thus, the increment in capillary density during contraction (capillary recruitment) was greater as superfusion solution $PO_2$ was equilibrated with progressively higher fractions of oxygen up to 10%.

In contrast, as indicated previously, the calculated change in conductance during striated muscle stimulation, based on measurement of arteriolar diameter, decreased as superfusion solution $O_2$ content was raised. Figure 3 shows a comparison of the effect of altered superfusion solution $PO_2$ on both capillary recruitment and functional dilation in the cremaster muscle during exercise. Whereas functional dilation decreases substantially between a tissue $PO_2$ of 12 mm Hg and 35 mm Hg, capillary recruitment increases correspondingly, as observed by Granger et al. (1976).

Thus, our data are consistent with the idea that initial oxygen availability can have differential effects on capillary density and arteriolar diameter. This apparent interaction might be the result of some form of interplay between the capillaries and arterioles, coordinated by tissue events such as changes in tissue $PO_2$. Alternatively, the interaction might be secondary to the fact that the vasculature is initially constricted at the high $PO_2$'s and thus might be a nonspecific effect of constriction. Support for the idea that the effect of altered superfusion solution $PO_2$ may be the result of the constriction per se rather than some purposeful regulatory process is provided by the observation that much of what has been described as reciprocal changes in capillary recruitment and functional dilation can be mimicked by application of norepinephrine rather than by vasoconstriction with $O_2$. When norepinephrine is applied to the cremaster, similar arteriolar vasoconstrictions and/or capillary density alterations can be induced with opposite changes in tissue $PO_2$ (Klitzman, 1979). Tissue $PO_2$ is raised by $O_2$ application and lowered by norepinephrine application. Functional dilation during striated muscle stimulation is diminished by an initial vasoconstriction with norepineph-

rine, but capillary recruitment is enhanced. These effects appear to correlate very well with the change in the initial diameter of the arteriole, not with an attempt of the tissue to precisely regulate some variable related to tissue metabolism or tissue $PO_2$.

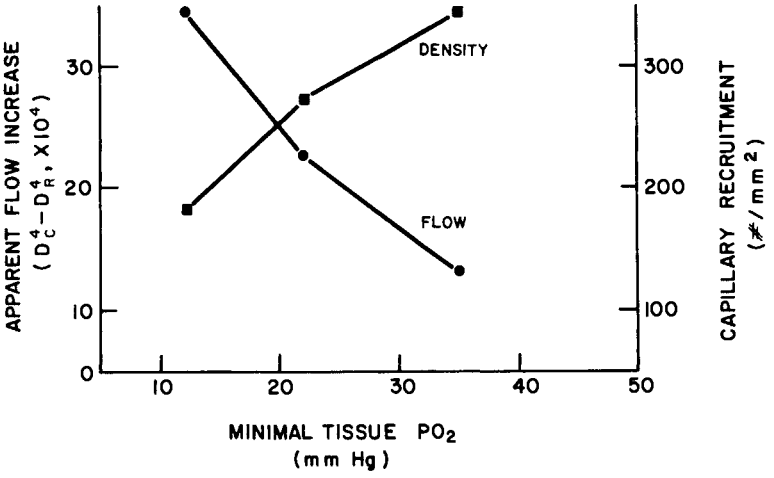


Figure 3. Influence of Altered Tissue $PO_2$ on Changes in Estimated Flow and Capillary Density in Contracting, Striated Muscle. Flow was estimated by computing conductance by raising observed diameter of the arterioles to the fourth power.

How can the effects of initial constriction on capillary recruitment and functional dilation be explained in the absence of a precapillary sphincter? Capillaries originate from relatively narrow orifices in the wall of arterioles. Obviously, as the size of the arteriole changes, the size of the capillary orifice must change in some proportional way. If it is assumed that the size of the orifices in a dilated arteriole are in the range of the size of the red cell, then a relatively simple model can be proposed to explain the observed data. No quantitative data are available which relate the size of the capillary orifice to arteriolar diameter and to red cell size, but the relations shown in Figure 4 are based on the reasonable assumption that, in the maximally dilated vascular bed, all of the capillary orifices are large enough to permit red cell entry. Figure 4 has been drawn with both diameter and capillary density normalized. It is assumed that, as the arteriole constricts, capillary orifice size decreases, but over the upper end of the diameter range, the capillary orifices are substantially larger than the red cell, and therefore, most or all of the capillaries remain perfused. In some critical region of the diameter range, it is assumed that a fairly large fraction of the capillary orifices approach the diameter of the red cell and, in this region, reduction in diameter results in closure of a relatively large fraction of the capillaries. Finally, at very small arteriolar diameters, few of the

capillaries are patent because the vast majority of the capillary orifices are too small to permit red cell entry. Given such a relationship between orifice size and the arteriolar diameter, one can predict the effect that a given change in diameter would have when starting from various initial levels. In cases where the striated muscle contraction is initiated from a relatively large initial diameter (low $O_2$, right end of the curve), functional dilation of the arteriole will result in a small effect on capillary density since most of the capillary orifices are already large enough to permit red cell entry. On the other hand, in the steep portion of the curve, the same change in diameter will result in a relatively large fraction of capillary orifices opening sufficiently to permit red cell entry as the arteriolar diameter increases.

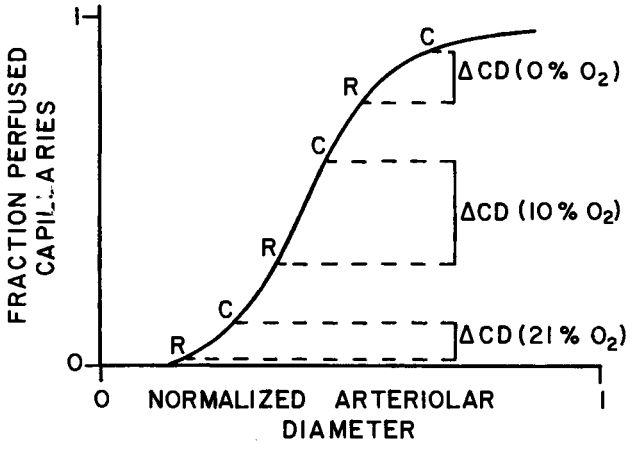


Figure 4. Hypothetical Relation Between Capillary Patency and Arteriolar Diameter. R = resting diameter, C = diameter during contraction.

The model which we propose is largely hypothetical; however, two pieces of unpublished data would support the idea that this may be a realistic model. First, using cannulated and pressurized isolated arterioles from the brain (Duling et al., 1978), we have observed the behavior of the size of orifices in the arteriolar wall as a function of changes in lumen diameter induced by changes in intraluminal pressure. This was done in a vessel with no vasomotor tone, and therefore, changes in orifice size must have reflected passive behavior. In fact, it was observed that orifice size changed little in the maximally dilated vessel at a pressure which would correspond to approximately the physiological range. As pressure was reduced, the arteriolar diameter decreased and the curve relating orifice diameter and microvessel diameter became progressively steeper. Thus, the model appeared to be at least qualitatively correct.

A second piece of evidence supporting the passive sphincter model is shown in Figure 5.

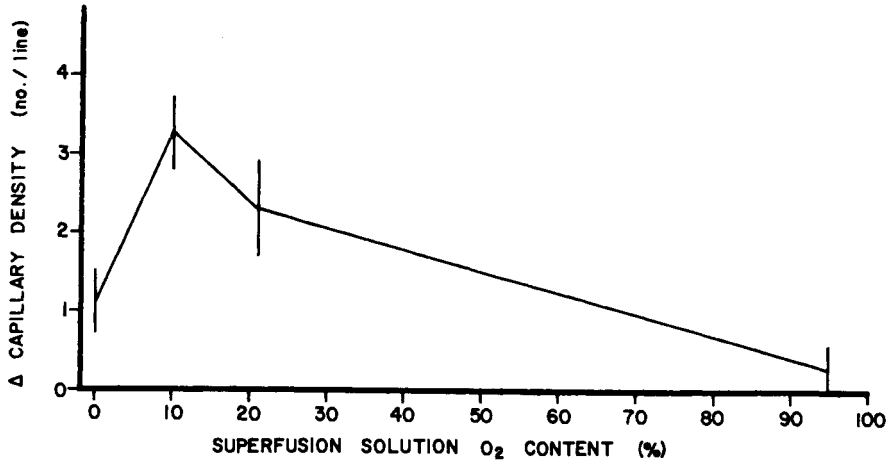


Figure 5. Capillary Recruitment in Contracting Striated Muscle. Stimulation was at 1 Hz. Capillary count expressed as number of capillaries intersecting a 250 μm long reference line in the microscope field.

This figure shows the changes in capillary density during stimulation of striated muscle plotted as a function of superfusion solution $PO_2$, but the range of $PO_2$'s studied has been extended very much beyond those referred to previously. As mentioned, the arterioles continue to constrict in the high $PO_2$ range, and correlated with this is a major reduction in capillary recruitment with muscle contraction once the superfusion solution $PO_2$ is raised above approximately 80 mm Hg. Examination of Figure 4 shows that this might have been predicted from the model since, at very small diameters, a given increase in diameter induced by stimulation of the striated muscle might be expected to increase only a few of the capillary orifices above the diameter of the red cell. Thus, capillary recruitment would have been predicted to be smaller at the smaller diameter, as is actually observed (Fig. 5). Therefore, the two pieces of indirect data are consistent with the idea that capillary patency is determined by arteriolar wall mechanics, and not by a complex interaction between tissue demands and control elements of capillaries and arterioles. However, the quantitative significance of the hypothesized mechanism can be evaluated only when size distribution data for red cells and capillary orifices are known and when the latter are related to the diameter of the arteriole.

## CAPILLARY BLOOD OXYGEN CONTENT

Returning to Figure 1, we see that capillary hematocrit should have an impact on cellular oxygen delivery. Recently, it has become apparent that changes in microvessel hematocrit may influence tissue $O_2$ delivery. We have observed, as have others before us, that the hematocrit in microvessels of striated muscle is very low (Johnson et al., 1971; Klitzman & Duling, 1979; Lipowsky & Zweifach, 1977); capillary hematocrit may be on the order of one-fifth of systemic hematocrit. More important than the fact that capillary hematocrit is low is the fact that capillary hemato-

crit varies both spontaneously (Johnson et al., 1971) and with a wide variety of stimuli of physiological interest (Klitzman & Duling, 1979). Increasing superfusion and/or tissue $PO_2$ will result not only in arteriolar constriction and reduction in capillary density, but also in a decrease in capillary hematocrit. Similarly, stimulation of striated muscle results in an increase in capillary hematocrit. The magnitude of the increase in capillary hematocrit with muscle stimulation has been shown to be relatively independent of the initial level of tissue oxygenation over the range of tissue $PO_2$'s from 10 to 40 mm Hg and during one Hz stimulation of the striated muscle. Interestingly, the increase in capillary hematocrit closely parallels the increase in capillary velocity observed during functional hyperemia (Klitzman, 1979).

The mechanism for the increase in capillary hematocrit associated with functional hyperemia is not known, but it is of obvious importance to determine the mechanism in order to understand the potential role of such an increase in hematocrit in augmenting tissue oxygenation during muscle contraction. In the working cremaster muscle, we have determined that the increase in capillary hematocrit is not the result of decreased shunting of red cells through some non-capillary pathway in the muscle. The increase in capillary hematocrit appears to reflect a true increase in the number of red cells per unit time which traverse the capillary.

We have examined a number of possibilities consistent with conservation of mass of red cells passing through tissue, which might explain the capillary hematocrit variations. The only explanation which is consistent with all of the data we have obtained to date is that an annulus of relatively motionless plasma exists within the capillaries, leaving a core of relatively rapidly moving blood with a higher hematocrit in the center. This model is, of course, consistent with earlier observations of plasma layers in microvessels and with the fact that hematocrits obtained by indicator dilution techniques using simultaneous measurement of red cell volume and plasma volume suggest the existence of a pool of noncirculating plasma within the peripheral circulation (Gibson et al., 1947). In order to explain the data which we have collected quantitatively, the relatively stationary plasma layer would have to be on the order of one micron in thickness.

The physiological importance of the alteraction in hematocrit remains to be determined. If the low hematocrit represents simply a stationary plasma layer within the capillaries, whose thickness changes with functional demand, then the hematocrit changes are not likely to be of major importance with regard to determining the delivery of oxygen to tissue, since the relevant hematocrit should be the core hematocrit which is substantially higher and may not be as variable. On the other hand, if the change in hematocrit reflects some other mechanism for increasing the number of red cells in each unit of blood entering the capillary, then it may have substantial importance in determining oxygen delivery to tissue. Only further investigation of the hemodynamics of this process will allow us to accurately assess the importance of microvessel hematocrit changes during exercise.

## TISSUE $PO_2$ DURING FUNCTIONAL HYPEREMIA

Cellular oxygen tension reflects the net result of oxygen consumption and oxygen delivery and as such it is an important variable in understanding the integration of tissue and microvessel function (Fig. 1). In analyzing the behavior of the microvessel regulatory system in striated muscle, tissue $PO_2$ may be viewed from two perspectives. The most common

view is that tissue $PO_2$ is a regulated variable, with the combined activity of the control elements acting to constrain the $PO_2$ within a relatively narrow range (Granger & Shepherd, 1979). Alternatively, tissue $PO_2$ may be viewed as a determinant of tissue $O_2$ supply, since it represents the lower end of the diffusion gradient from capillary to cell. We have examined these two possibilities by varying superfusion solution over a wide range and by observing tissue $PO_2$ during stimulation of striated muscle. The results of such experiments are shown in Figure 6.

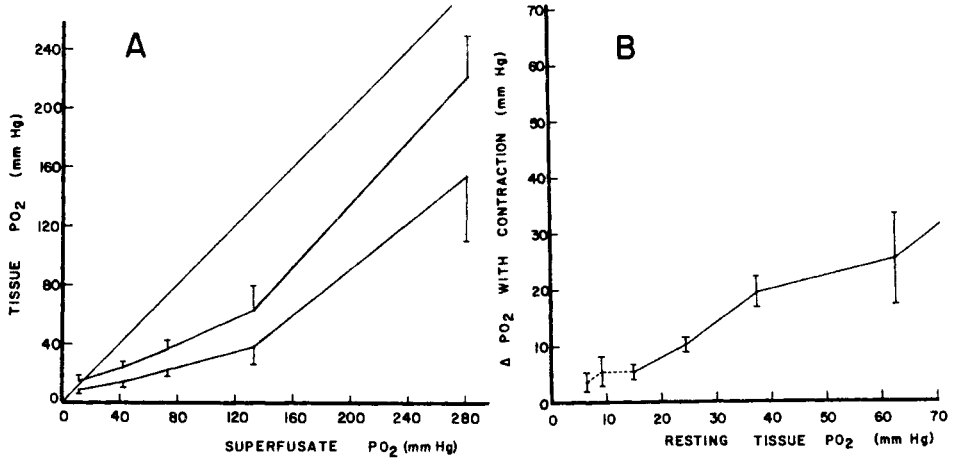


Figure 6.A. Relations Between Tissue $PO_2$ and Superfusion Solution $PO_2$ in Resting (upper line) and Contracting (lower line) Cremaster Muscle. In Panel B, the fall in tissue $PO_2$ which occurred during the period of muscular contraction is plotted against the $PO_2$ in the resting muscle at the time contraction was initiated. The points in the lower left corner of Figure 6B (dotted line) were obtained by applying sufficient norepinephrine to match the arteriolar constriction induced by 5 and 10% oxygen.

If there were no regulation of tissue $PO_2$, and constant oxygen consumption by the tissue, one would expect the tissue $PO_2$ to run in parallel with, and lower than the superfusion solution $PO_2$ down to a point where $PO_2$ in the tissue reaches a limiting value for $O_2$ consumption. From there on, tissue $PO_2$ and solution $PO_2$ should converge with further reductions in solution $PO_2$. On the other hand, if the system were behaving as a precise regulator of tissue $PO_2$, then one would expect little change in the tissue $PO_2$ over a rather broad range. In fact, neither of these patterns was observed.

Only in the resting muscle at very high $PO_2$'s (considerably in excess of normal), was any parallelism between tissue $PO_2$ and superfusion solution $PO_2$ observed. Below a tissue $PO_2$ of about 50 mm Hg, the resting muscle

has a different slope from the line of identity, apparently due to the regulatory processes described above. At the low end of the curve, the tissue $PO_2$, instead of remaining constant, falls as a relatively constant fraction of the solution $PO_2$.

Not only did the tissue $PO_2$ change during alterations in the superfusion solution $PO_2$, but stimulation of the striated muscle caused a reduction in the tissue $PO_2$, the magnitude of which was proportional to the tissue $PO_2$ at the time of the stimulation (Fig. 6A & B). This observation is not easily reconciled with a model in which the tissue $PO_2$ is regulated around a "set point", although it is possible to view the variations in $PO_2$ of the tissue as "offsets" in a proportional control system.

Whereas Figure 6 does not provide much support for the idea that the tissue $PO_2$ is a tightly regulated parameter, the data presented are consistent with the concept that variations in tissue $PO_2$ are important determinants of the diffusion gradient driving oxygen to the cells. Tissue $PO_2$ is reduced by reduction in superfusion solution $PO_2$ and by muscle work. Both experimental manipulations call for an increased delivery of oxygen by the vessels, and the reduced tissue $PO_2$ will increase the diffusion gradient from capillary to cell, thus enhancing cellular oxygen delivery.

An additional interesting aspect of these findings is that the tissue $PO_2$ falls during contraction by an amount proportional to the resting tissue $PO_2$. This is not expected from a set point model of regulation, nor from some form of regulation based on the tissue operating close to a $PO_2$ which limits oxidative phosphorylation. However, the behavior pattern shown in Figure 6B can easily be viewed as functional, given that reducing the tissue $PO_2$ is one way of augmenting the tissue $O_2$ supply. In this context, the larger reduction in tissue $PO_2$ which is observed when the oxygen availability from the solution is high reflects the fact that the tissue accomplishes the necessary increment in oxygen delivery to cells by the simple expedient of reducing the tissue $PO_2$. So long as the tissue $PO_2$ remains above a value which limits the activity of oxygen consuming enzymes, this is an effective way of augmenting supply.

It was mentioned previously that many elements of the vasomotor changes induced by elevation of the superfusion solution $PO_2$ could be mimicked by application of norepinephrine in a dose sufficient to produce constrictions equivalent to those observed with oxygen. A similar observation has been made on the effect of initial constriction on the tissue $PO_2$ response to contraction. The dotted lines shown in the lower left portion of Figure 6B are the tissue $PO_2$ data obtained during such superfusions. A reduction in $PO_2$ induced by vasoconstriction with norepinephrine results in lower rest $PO_2$ and a smaller increment in tissue $PO_2$ during muscle stimulation. These data also fail to support the concept that the system is organized so as to regulate tissue $PO_2$ about a single, narrowly defined value. Instead, the norepinephrine data, as well as the other findings shown in Figure 6, suggest that tissue $PO_2$ is one element in the determination of the altered supply of oxygen to striated muscle during contraction.

This paper has focused on the integration of microvessel elements in the regulation of tissue oxygenation. In this context, the variation in the amount which the tissue $PO_2$ decreases during contraction is quite significant, since it means that the initial state of the vasculature determines the contribution of a change in tissue $PO_2$ to the overall augmentation of tissue $O_2$ supply. Taken with findings described earlier, this means that, at high levels of oxygenation, the oxygen required for contraction is supplied to a greater extent by capillary recruitment (Fig. 3), and also by a reduction in tissue $PO_2$ (Fig. 6B). In contrast, under con-

ditions in which tissue $O_2$ availability is low, tissue oxygen supply is augmented during contraction to a larger degree by arteriolar dilation and a flow increase (Fig. 3).

It is of interest to consider how the design of the microcirculation itself may influence the changes which we observe in these experiments. Proctor has reported that the tissue $PO_2$ of the rat cremaster is unaltered during stimulation of the muscle (Proctor, 1980). His observation in the rat and ours from the hamster are plotted in Figure 7.

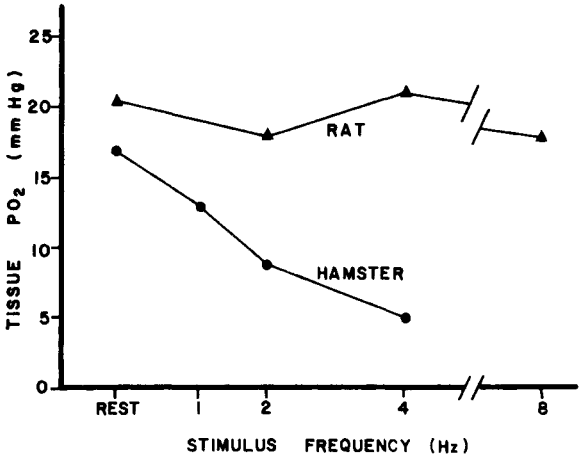


Figure 7. Correlations Between Tissue $PO_2$ and Stimulus Frequencies in Hamster and Rat. Data for the rat are the results of Proctor (1980).

In the hamster cremaster muscle, tissue $PO_2$ falls progressively as stimulus frequency is increased to 4/second. In contrast, in the rat, the data show relatively constant tissue $PO_2$ over the entire range of stimulus frequencies studied. One might suggest that this reflects less precise regulation of tissue $PO_2$ in the hamster, and more precise regulation in the rat. However, this neglects the fact that rats used in microcirculatory work are typically on the order of 30 to 50 days in age, while hamsters are typically on the order of 60 to 100 days of age, and thus are more mature animals.

Maturity influences the capillary density of striated muscle. Figure 8 shows variations in capillary density which we have observed to correlate with maturation of the hamster (Sarelius, unpublished observations). The figure shows that, over the period of 35-132 days, there is a progressive decrease in the capillary density of the cremaster muscle; similar findings have been reported in other muscles of the rat (Sillau & Banchero, 1977). Thus, comparing a 30 day old rat with a 100 day old hamster as in Figure 7 is likely to be a comparison of animals with quite different capillary densities. The change in capillary density has also been reported to occur in parallel with changes in muscle fiber type (Hudlicka, 1973; Sillau & Banchero, 1977).

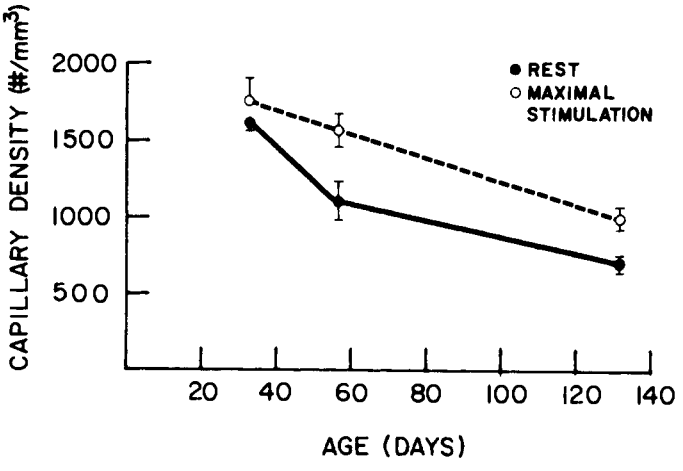


Figure 8. Influence of Age on Capillary Density of the Hamster Cremaster Muscle. Maximal dilation was obtained with $10^{-4}$ M adenosine and 8 Hz electrical stimulation.

The change in capillary density with development may have direct bearing on the question of the precision of regulation of tissue $PO_2$, referred to previously. Figure 9 represents a very simple model of what would be expected to happen to tissue $PO_2$ under conditions in which metabolic rate is doubled, and flow is doubled to match metabolic rate. This is assumed to occur under two different conditions: a condition with a high capillary density, indicated on the figure by a half intercapillary distance (R) of 15 microns, and a condition with a low capillary density, with a half intercapillary distance of 30 microns. These values would be appropriate for the cremaster vessels of the young and the mature hamsters.

Regulation is assumed to be identical in the two cases; that is, both flow and metabolism are assumed to be doubled and, for the purposes of the model, this is hypothesized to occur without change in capillary density. This would result in a constant capillary $PO_2$, and therefore, in order to deliver the increased flux of oxygen required by the doubling of metabolism, tissue $PO_2$ need only fall 2 mm Hg with the high capillary density, and would have to fall 10 mm Hg with the low capillary density.

Measurement of tissue $PO_2$ in contracting muscle is influenced by changes in position of the electrode relative to capillaries and by stimulus artifacts. Therefore, substantial uncertainty is induced in the $PO_2$ measurement. In our experience, a change of 2 mm Hg is likely to be too small for an oxygen electrode measuring $PO_2$ at a tissue site to detect, while 10 mm Hg is a sufficiently large change that a difference usually would be detected. Therefore, the simple model shown in Figure 9 describes a situation in which differences in capillary density which correlated with the age of the animal, coupled with the resolution of the electrode, would lead one to believe that tissue $PO_2$ was a relatively tightly regulated variable in the case of the animal with the small intercapillary distance, and poorly regulated in the case of the animal with the large intercapillary distance. This, in spite of the fact that the hypothetical level of regulation was identical in the two cases, i.e., flow was doubled to match metabolism. Thus what appears to be tighter regulation is a simple outcome of the geometry of the system which

changes with the muscle type and age of the animal.

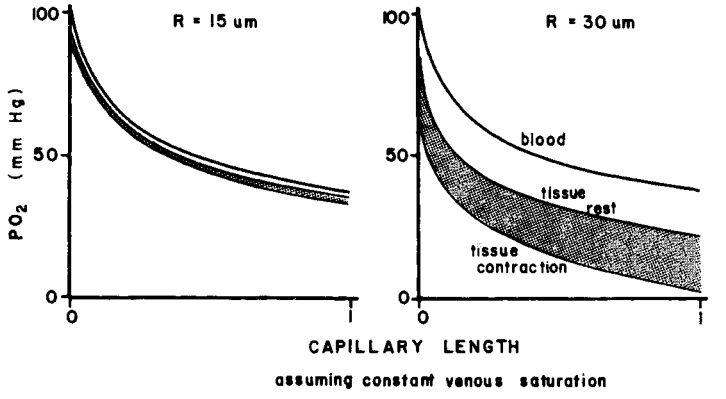


Figure 9. Hypothetical Influence of Capillary Density on Tissue $PO_2$ in Resting and Contracting Striated Muscle. The line labeled "blood" is calculated from the hamster dissociation curve, and the "rest" and "contraction" tissue $PO_2$ lines are based on the gradient predicted by the Krogh equation. The shaded area represents the reduction in tissue $PO_2$ to be expected with muscle contraction.

Several points should be made in summarizing the ideas presented here. First, it is clear that measuring one variable in the composite of parameters which determine the supply of oxygen to the tissue may yield misleading results as to the efficacy of the regulatory process. When oxygen availability is high, both our work and Granger's work show that measurement of flow alone would lead to the conclusion that regulation was rather incomplete since capillary recruitment and reduced tissue $PO_2$ are relatively more important. On the other hand, as oxygen availability becomes lower and lower, flow regulation becomes more and more precise. Under these conditions, we find relatively more constant tissue $PO_2$ (Fig. 6) which, if evaluated only in the range of low tissue $O_2$ availability, would lead one to believe that tissue $PO_2$ was a regulated variable. Evaluation at the high end of the tissue $O_2$ availability range discloses no evidence of regulation of tissue $PO_2$, however.

A second point to be made, based on the observations reported in Figures 6, 7, 8, and 9, is that the finding of a constant $PO_2$ in the tissue during contraction of the striated muscle need not be evaluated solely in terms of regulation of tissue $PO_2$. Low initial tissue $PO_2$'s and differences in capillary density in the young rat and the adult hamster would induce differences in the behavior of the tissue $PO_2$ which could easily be interpreted as differences in the regulatory process.

Finally, it is apparent that explanations of such complex interactions as described in the preceding paragraphs need not invoke the existence of specific control elements responsible for either flow control or capillary density control. The passive sphincter model of capillary den-

sity control, whose behavior is illustrated in Figure 4, appears to be consistent with much of the existing data, and almost certainly must have some impact on capillary recruitment in striated muscle. Its behavior is determined not by independent control elements for capillary density and arteriolar diameter, but rather by the geometry inherent in the design of the microcirculation. Similar comments might be made regarding the behavior of tissue $PO_2$ predicted by Figure 9. This is not meant to imply that either the passive operation of the precapillary sphincter or the influence of different capillary densities are the sole explanations for the behavior observed. Rather, in my view, these phenomena are likely to be a substrate upon which more active control processes may be superimposed.

A general principle which may be derived from our experiments is that regulation exists on both a short term and a long term basis. Physiologists are accustomed to observing phenomena such as functional hyperemia and capillary recruitment, which occur over a short period of time. However, it is perhaps appropriate to devote more attention to regulation on a different scale. This would involve the study of how the intrinsic nature of microvessel structure takes part in the regulation of tissue metabolism and blood supply.

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# THE ROLE OF INTRAVASCULAR PRESSURE IN REGULATION OF THE MICROCIRCULATION

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## Introduction

Regulation of blood flow in the microcirculation is carried out by a variety of mechanisms involving neural, metabolic and myogenic factors. The importance of the first two of these, neural and metabolic mechanisms is scarcely open to question. It can be shown in a number of ways that vasomotor nerves have an important influence, and in emergency states an overriding influence, on local blood flow to the tissues. This Congress has already heard a number of new reports which further emphasize the importance as well as the complexity of this control mechanism. Similarly, it has been shown many times and in many ways that blood flow and tissue metabolism are very closely related. Teleology alone dictates that a relationship must exist between blood flow and tissue nutritive requirements. However, it must be noted that the pathways involved in this control system are still far from clear and require much additional work. By contrast with the first two topics, the role and contribution of the myogenic response to flow regulation is much less clear.

## Response to Dynamic Stretch

It has long been appreciated that the application of force to muscle, particularly smooth muscle, leads to a contractile response. For example, quick stretch of a variety of smooth muscle types was shown by Burnstock and Prosser to lead to contraction [9]. This response is typically transient in nature. Similar behavior can be elicited in other muscles as well. The cardiac pacemaker cells of the snail are sensitive to applied force, sudden stretch increases frequency of contraction by 50 to 100% [1]. In the peripheral blood vessels transient force also causes a contractile response [8]. Unfortunately this response is not so easily elicited or studied in the blood vessels as in other types of smooth muscle, perhaps because vascular smooth muscle is very sensitive to the trauma of preparation. A quick stretch response has been shown in human umbilical artery and occasionally in artery segments from other tissues [38]. It appears that the quick stretch response is a feature of venous blood vessels as well. Stretch of the rat portal vein strip causes increased frequency of contraction and electrical spike activity during dynamic stretch [22]. However, whole organ studies and studies of isolated vein segments do not reveal venous response to quick stretch in other beds [25]. Thus, the extent and importance of this response in the venous system is as yet unclear.

A key question so far as local regulation is concerned is whether this behavior extends to the arterioles. Several lines of evidence suggest that it does. In the course of studies on reactive hyperemia we examined the flow pattern in individual capillaries of sartorius muscle [11]. A substantial number showed an initial hyperemia which was followed by a secondary hypoemia or flow stoppage. The latter is apparently due to constriction of the arteriole following the sudden pressure rise. This effect is even more pronounced in mesenteric capillaries. In the latter bed we studied the effect of brief (3-10 second) arterial pressure pulses of 40 to 60 mmHg amplitude starting from a low normal pressure (80 mmHg) [35]. Flow was normal at the low pressure because of autoregulation and did not increase with the brief pressure pulse. Nonetheless, flow decreased and stopped several seconds after the pressure pulse. This appears to represent an arteriolar response to the transient pressure increase. If pressure is increased slowly, there is an autoregulatory compensation keeping flow constant but there is no transient flow stoppage.

It has been suggested that the transient response of the arterioles to quick stretch may provide a substantial fraction of basal vascular tone due to the actions of the normal arterial pulse pressure on the arterioles. Johansson and Mellander [22] calculate that a 1% stretch of the arteriole is likely to occur at normal pressure pulse amplitude. At normal pulse frequency the rate of stretch of the arteriole during the anacrotic phase of the pressure pulse is 200%/min. A stretch of this rate is known to produce maximal electrical and contractile activity in the portal vein preparation. It has been suggested that if the arteriole behaves as does the portal vein, the normal pulse pressure will cause a continued mechanical stimulus and sustained contraction of the arteriole. In support of this postulate, Mellander and Arvidsson [34] and Rovick and Robertson [36] both found that vascular resistance in isolated vascular beds was elevated during pulsatile perfusion as contrasted to pulseless perfusion at the same mean pressure. Of course the caveat must be noted that pulsatile perfusion might provide a more uniform distribution of capillary flow and increase tissue $PO_2$, which could lead to increased vascular resistance.

In further studies of the dynamic stretch response in the skeletal muscle vascular bed, Mellander and co-workers have described in detail the vascular resistance response to ramp changes in transmural pressure [5, 18, 19, 20]. The latter were induced by changing pressure in a plethysmograph surrounding the muscle. Rapid decrease of ambient pressure by 40 mmHg at a rate of 2.7 mmHg/sec caused an increase in total vascular resistance which could be localized to vessels in the microvascular network less than 25 microns i.d. as shown in Figure 1. When ambient pressure was maintained at -40 mmHg the resistance returned toward control but remained elevated by about 20% in the steady state. Thus it appears that in this vascular bed there is a very pronounced dynamic component of the myogenic response. Other studies by this group suggest that about one-third of the basal vascular tone in the microvessels is attributable to the normal arterial pulse. Since the larger arterial vessels do not respond to any substantial degree to pulsatile pressure, their basal tone must be attributed to other mechanisms.

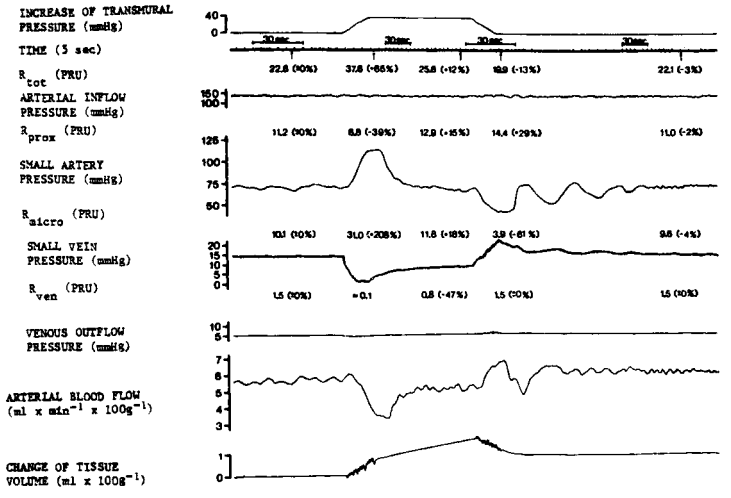


Figure 1. Response of skeletal muscle vascular bed to increase of transmural pressure by 40 mmHg. Note the large increase in small artery pressure which reflects a large increase in resistance downstream to the catheter tip. Pressure recorded at this site is believed to represent arterioles of approximately 25 microns diameter based on the observed level of pressure in the control state. From Grande, P. -O., J. Lundvall and S. Mellander [19], reproduced by permission.

In summary of this section, it may be said that response of the arterioles to dynamic stretch appears to be of importance in providing a certain fraction of the normal vascular tone. It may be noted also that change of heart rate would perhaps change the quick stretch stimulus and decrease peripheral resistance when heart rate is slowed. This may be of some homeostatic significance but more work needs to be done on this topic.

## Myogenic Response to Sustained Force

The main body of knowledge and greatest source of controversy regarding the myogenic response is the effect of sustained force of pressure on the smooth muscle. It was first proposed by W. M. Bayliss in 1902 [4] that the normal level of sustained intravascular pressure provides a stimulus to the vessels which causes a tonic contraction of the vessel. Bayliss related this behavior to the quick stretch response of smooth muscle cells as follows: "Bearing in mind the well-known reaction of muscle in general to stretching, instances of which are, amongst others, the increased force of the heartbeat produced by intravascular pressure,

the effect of tension on the snail's heart, and the contraction of the body walls of the earthworm to pull, it is natural to suppose that the muscular coat of arteries reacts in a similar way to intravascular pressure ... ." It appears however, that for the most part, Bayliss' analogies related to transient rather than steady state phenomena. The principal and in fact the most difficult issue to resolve is whether the sustained application of force leads to a sustained shortening of the smooth muscle cell. In respect to this issue data from non-vascular smooth muscle is of less help. On the one hand, sustained application of force to non-vascular smooth muscle can lead to a sustained active response and depolarization of the cell membrane. For example, taenia coli responds to sustained application of force with membrane depolarization and increased frequency of spike activity [7]. On the other hand, this response is associated with increased length of the cell. It appears that increased contractile activity is generated by application of external force but it does not prevent an increase in length.

Sustained application of force also leads to increased electrical and contractile activity of vascular smooth muscle. For example, isolated portal vein strips [22], the bat wing vein [3], 43] and lymphatic channels [21] studied in vivo all respond to increased force or pressure with an elevation of the rate of electrical discharge and/or increased frequency of spontaneous vasomotion. However, in all cases it appears that this increased contractile activity is associated with elongation of the smooth muscle cell. Thus, it could be argued that the sustained response is due to elongation and deformation of the cell membrane.

We will return to this point later when the underlying mechanism of the myogenic response is examined in more detail.

Experimental findings from whole organ studies suggest that when intravascular pressure is elevated, the smooth muscle of the arterioles does not merely oppose elongation, rather those vessels become smaller at elevated pressure. This phenomenon has been attributed to a myogenic response to elevated intravascular pressure [14, 15]. Other explanations such as changes in tissue vasodilator metabolites are difficult to rule out when flow changes simultaneously with the alteration of intravascular pressure and in fact appear to play a very major role in certain vascular beds when arterial pressure alone is altered. In whole organ studies the principal observation in support of the sustained myogenic response is the constriction of pre-capillary vessels with venous pressure elevation as shown in Figure 2 from studies on the small intestine [25]. In this instance flow falls and a metabolic mechanism should cause relaxation of the precapillary vessels. Various sympathetic blocking agents do not abolish the response [24]. Therefore, elimination of other possibilities leads us to consider that a direct response of the arterioles to intravascular pressure elevation is involved.

Direct studies of the arterial blood vessels have provided further evidence in support of the myogenic theory. At the level of the arterioles a dilation in response to reduction of arterial pressure and constriction in response to venous pressure elevation has been reported. In respect to the latter, Baez and co-workers [3] reported in the rat mexoappendix preparation that the response is graded, that it is strongest in the smallest pre-capillary vessels, moderate in mid-sized arterioles and weakest in the large arterioles. This observation is consistent with the

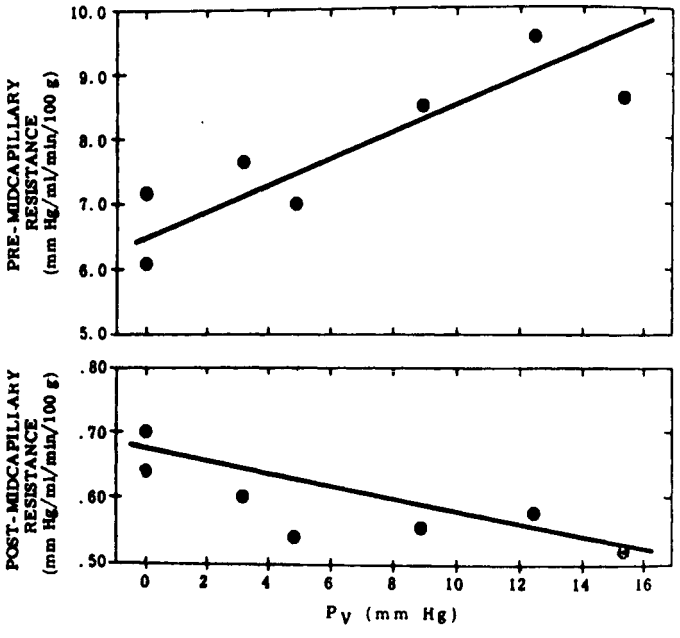


Figure 2. Relation between venous pressure and vascular resistance in the isolated small intestine. Total vascular resistance was divided into pre- and post-midcapillary resistance by an estimate of mean capillary pressure using the isogravimetric techniaue. From Johnson [25].

concept that a myogenic response is involved since it would be expected that the pressure change would be greatest in the small arterioles immediately upstream from the capillaries.

Burrows and Johnson [10] have found that elevation of venous pressure in the cat mesentery caused constriction of the arterioles. In an earlier study, Johnson and Wayland [30] found that capillary flow was sensitive to small changes in venous pressure -- in many vessels a small increase in pressure caused a disproportionate reduction in flow. Also, a small increase in pressure could initiate periodic vasomotion as well as a pronounced flow reduction suggesting that a threshold of sorts exists for the response to intravascular pressure elevation.

Some investigators have examined the effects of transmural pressure by altering ambient pressure around the tissue. For example, Bouskela and Wiederhielm [6] studied the reactions of the arterioles in the bat wing when the body of the animal was placed in a pressure chamber from which the wing protruded. Changes in ambient pressure in the box produced equivalent changes in intravascular pressure throughout the wing vasculature. Increasing intravascular pressure caused substantial constriction of the

arterioles ( a 50 mmHg increase in pressure caused a 40% decrease in diameter) and at high pressures flow ceased even though the arterial-venous pressure gradient was not altered. Reduction of intravascular pressure caused pronounced dilation of the arterioles, especially when pressure was reduced by about 50 mmHg. An interesting feature of the response in bat wing arterioles is an apparent fatigue of the vessels after several minutes of sustained response to pressure, causing partial relaxation.

The response of the arterioles has also been studied in vivo under conditions of no flow. Baez [2] was the first investigator to report this type of study. He found that static pressure elevation in the rat mesoappendix preparation caused constriction of an arteriole under study. A similar study in cat mesentery by Johnson and Intaglietta [29] led to similar findings. In both studies the duration of the study was fairly brief and the preparation may not have tolerated the total ischemia well. In Baez' study the response was seen only when pressure was raised from 40 to 80 mmHg, the arteriolar lumen decreasing from 20.0 microns to 18.4 microns. The vasculature was perfused with a Ringer's solution with 3% albumin added. In the study by Johnson and Intaglietta, the preparation was blood-perfused by the animal. Arteriolar diameter decreased from 21.5 microns to about 17 microns in seven preparations when intravascular pressure was increased from 12 to 44 mmHg. In the latter study the contraction appeared to weaken during the third minute of pressure elevation and gradual relaxation may have also occurred during the control period.

In a related experiment, Johnson and Intaglietta stopped flow in individual arterioles by means of a micro-occlusion pipette and measured arteriolar diameter changes while perfusion pressure was altered to the mesentery. As shown in Figure 3, reduction of arterial pressure from 90 to 60 mmHg caused dilation of the arteriole from 24 microns to 31 microns. Restoration of pressure caused constriction to 23 microns. Constriction with pressure elevation was observed with repeated step changes from 60 to 90 mmHg and in this case the response did not appear to wane with time. The sustained myogenic response may not be limited to arterioles. Speden has shown that the isolated perfused rabbit ear artery constricts with intravascular pressure elevation in the pressure range 30-80 mmHg when the preparation has been pre-treated with adenaline [39].

## Mechanism of the Myogenic Response

The mechanism of the myogenic response is still largely a matter of speculation. A conceptual problem which has defied easy explanation is how the cell can shorten to less than its original length and remain shortened when the intravascular pressure is elevated [14, 15]. If deformation or stretch of the cell membrane is the stimulus, it would appear that the stimulus for contraction would disappear when the smooth muscle cell returns to its original length. A schematic of this model is shown in Figure 4. When the cell is elongated by an increment $\Delta L_i$, the cell membrane is stretched and it is postulated that electrical activity or ionic permeability increase, causing activation of the contractile machinery. The cell will shorten until an equilibrium value is reached at which the cell is elongated by an amount $\Delta L_f$, which provides adequate error signal to maintain the activation of the contractile machinery.

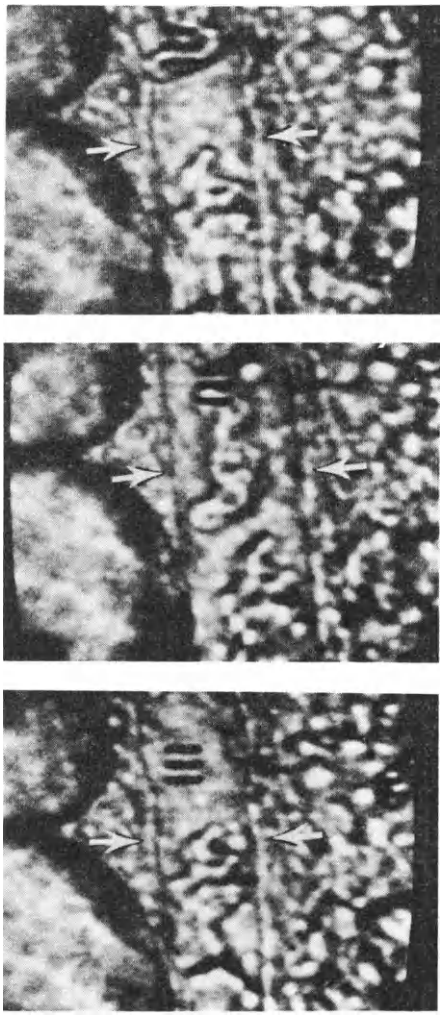


Figure 3. Diameter changes in an arteriole of cat mesentery during changes in perfusion pressure to the isolated, perfused mesentery preparation. A micropipette was placed on the vessel immediately downstream from the site shown and flow was stopped by application of pressure to the vessel. Subsequently, arterial perfusion pressure to the mesentery was changed in a stepwise manner from 90 to 60 mmHg for two minutes followed by return to 90 mmHg. Details of the experimental procedure are described by Johnson and Intaglietta [29].

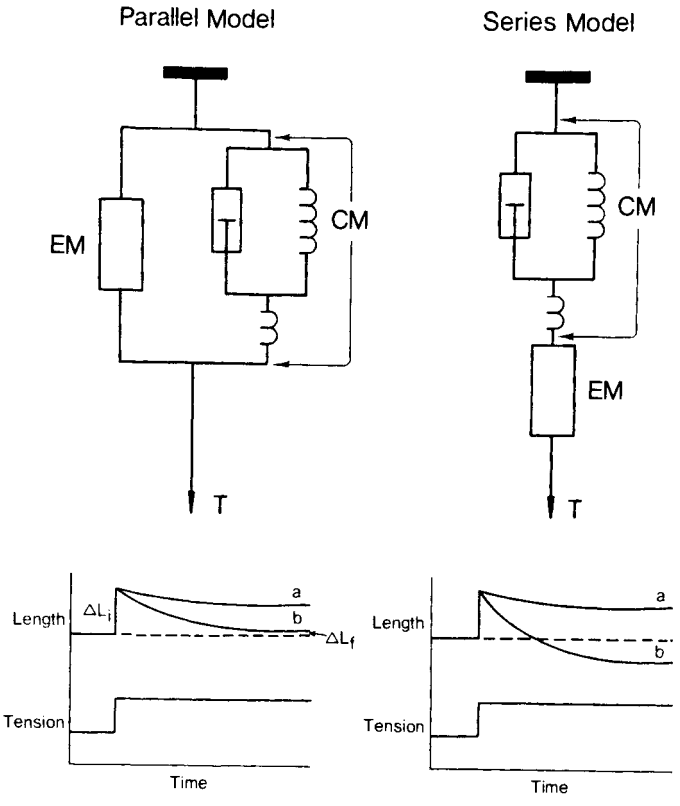


Figure 4. Hypothetical models of the myogenic mechanism. The parallel model shown in the left panel consists of an excitable membrane (EM) in parallel with the contractile apparatus (CM), shown here as consisting of viscous and elastic elements. The series model shown in the right panel consists of the same two elements connected in series. The lower section of each panel shown the expected response under conditions of low and high gain with critical damping. From Johnson [27] by permission.

Several investigators however, have suggested that circumferential wall tension rather than cell length may be the controlled variable [26, 30, 32, 41]. According to the well known Laplace relationship, Tension = pressure x radius. Alternatively, wall stress (tension per unit cross/sectional area of the wall) may be considered as the controlled variable. In the Laplace relationship, stress = pressure x radius/wall thickness. The circumferential force model of the myogenic response may be conceptualized as a contractile element in series with an excitable membrane. This is in contradistinction to the parallel model in which the excitable cell membrane is in parallel with the contractile machinery. The essential features of this model and its behavior is also shown in Figure 4.

In the instance of the series model a step increase in tension would provide a fixed deformation or stretch of the excitable membrane. The deformation would not be reduced as contraction occurred. That is, there is no negative feedback to provide a control of the system. The cell would shorten to the extent it is able under the conditions extant at that time which is characterized here as the gain of the system. If we apply these models to the arteriolar network we may then determine whether either can possibly explain the myogenic behavior observed in those vessels. It would appear that the parallel model could explain the response to quick stretch, but only if there is a time delay in the feedback loop. As shown in Figure 4, the muscle cell shortens in a manner which is critically damped. However, if the system were underdamped, overshoot could occur and provide a transient reduction of length below the equilibrium value. The series model in the form presented here (as it would be for a muscle strip) would not appear to explain the quick-stretch response since there is no feedback to limit the response. In respect to this point, it should be noted, however, that Sigurdsson, Johansson and Mellander [37] found that the excitatory response of the rat portal vein to dynamic stretch appeared to be more closely related to the rate of increase of force than to the rate of increase of length.

The series model must be considered in cylindrical geometry rather than linear geometry when applying it to blood vessels in vivo. In this case, as shown in Figure 5, the series model as applied to the arteriole includes the Laplace relationship. With the addition of the cylindrical geometry, a negative feedback component is added beacuse the wall tension is directly proportional to vessel radius. As pressure is increased, tension is elevated initially but begins to fall as the vessel constricts and radius decreases. When the vessel has returned to its initial radius there is still a substantial error signal because the wall tension is still elevated and the excitable membrane or sensor element is still deformed. However, with further contraction the membrane should return to its original conformation and ultimately the vessel will stabilize at a diameter less than the control value which still provides some error signal for the maintained contraction of the smooth muscle cell.

To test the hypothesis that wall tension is the controlled variable the relation between intravascular pressure, arteriolar diameter and calculated wall tension has been examined as pressure is altered. Burrows and Johnson [10] found that wall tension tended to be maintained as arterial pressure to the cat mesentery preparation was reduced but a substantial error signal was evident. That is, the dilation with pressure reduction was not sufficient to return circumferential tension to its initial value. In particular these authors examined the question of whether an over-reaction occurred, since such behavior would negate the hypothesis. Even in the most reactive vessels, wall tension returned to control levels at best with two examples 6% overshoot or less. When venous pressure was increased in the same preparation the response was, on the average, greater for a given increase in intravascular pressure but the difference was not significant. In two instances the calculated wall tension decreased on the order of 25% when intravascular pressure was elevated by raising venous pressure. These values seem to be outside the range of experimental error. However, wall tension is calculated from measured vessel diameter of the lumen while the tension we would like to consider is in the smooth muscle layer, which has a slightly larger radius (on the order of 1 micron) than the lumen.

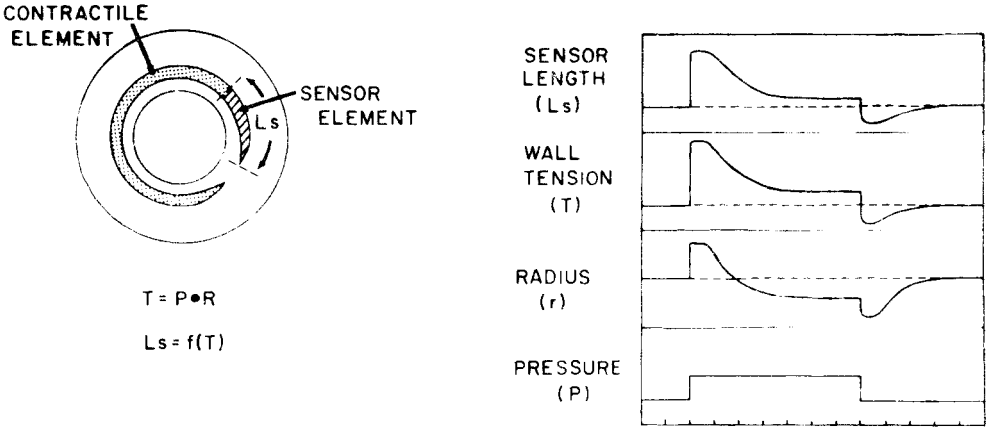


Figure 5. Diagram of the series model of the myogenic mechanism applied to cylindrical geometry of the arteriole. The sensor element is assumed to be a region of the cell or wall membrane which is connected in series with the contractile element. The sensor is hypothesized to be passively distensible. From Johnson [26] reproduced by permission.

This correction would add about 10-15% to the initial radius and even more to the constricted radius as both the smooth muscle and the endothelial cell layers get thicker when the vessel narrows.

Bouskela and Wiederhielm [6] also calculated wall tension in the arterioles of the bat wing as transmural pressure was changed. They found tension was, on the average, well maintained over a substantial range of intravascular pressures as shown in Figure 6.

These findings appear to be quite consistent with the myogenic hypothesis with tension as the regulated variable. But the questions of what structure or structures within the cell might constitute the sensor and how the excitation might be brought about are unanswered. In respect to the first question we note that the contractile machinery in the smooth muscle cell is anchored to dense bodies in the cytoplasm and on the sarcolemma. Fay [13] has described indentations which appear in the cell surface as the muscle contracts and the dense bodies are pulled inward and toward each other. It seems logical to suppose that the areas of the cell membrane in the vicinity of the dense areas may be under tension and may bear the brunt of the force which is transmitted from the contractile apparatus to the surrounding tissue outside the smooth muscle

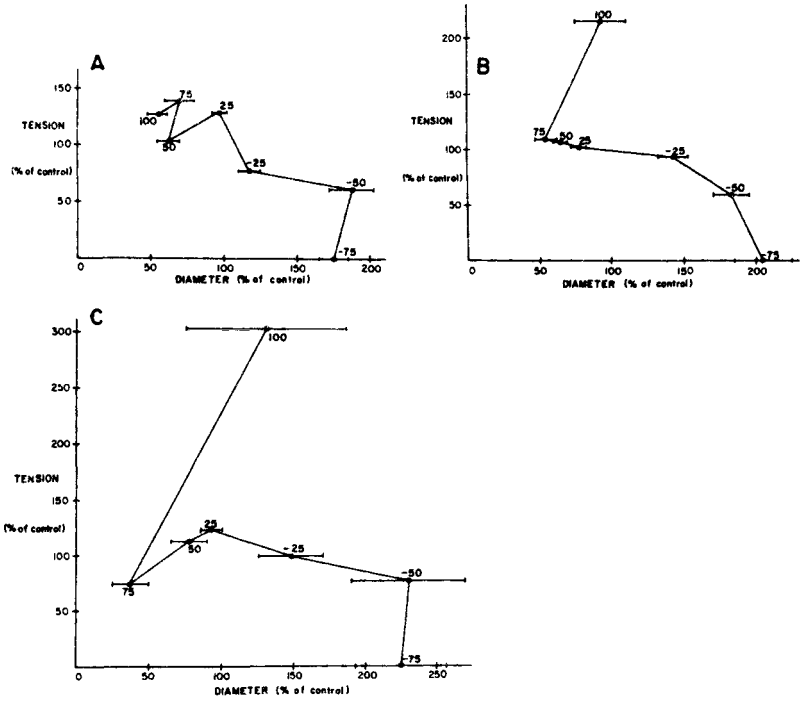


Figure 6. Plot of tension versus diameter for an arteriole (A) terminal arteriole (B) and pre-capillary sphincter (C) in the wing of the bat. The numbers in the figure refer to ambient pressure around the bat in mmHg. From Bouskela and Wiederhielm [6] reproduced by permission.

cell. It is therefore conceivable that the sarcolemma in this region may function as a tension sensor. In this regard it may also be noted that in studies of taenia coli [7] and strips of snail heart [1], it has appeared that the magnitude of the contractile or electrical activity was better correlated with tension than with muscle length. In the case of taenia coli the length-tension relationship has considerable hysteresis. When membrane potential is plotted against length this hysteresis is also present but hysteresis disappears when membrane potential is plotted against tension.

In respect to vascular smooth muscle, the morphology is somewhat similar to that of other smooth muscles. The dense bodies are quite numerous in the cell and on the sarcolemma. In electron microscopy studies we have noted (Carlson, Burrows and Johnson, unpublished data) that the dense bodies are about three times as numerous along the adventitial surface of the cell as along the luminal surface. This suggests that the contractile machinery is also largely anchored along the outer surface of the cell. This arrangement may have important implications for the manner in which force is distributed inside and

outside the smooth muscle cell. However, further study is required before the full implications can be appreciated. The areas of the dense bodies themselves are thickened and it is not clear that they would be suitable structures for receptor type activity.

This brings us to the next question, namely, the possible nature of the receptor process in the myogenic response. In sensory receptors a deformation of the membrane is usually considered to initiate depolarization [12, 33]. It is tempting to consider membrane depolarization as the mechanism of sensor activation in the myogenic model. This appears to be the means by which the guinea pig taenia coli transduces the applied force and elicits a mechanical response [7]. Also the rat portal vein shows increased bursts of electrical activity, closely correlated with mechanical activity, when stretched [22]. However, there is ample evidence that membrane depolarization is not necessary to elicit contraction in arterial smooth muscle [23]. Perhaps a local change in membrane permeability in the region of the dense body is brought about by application of external force and stretch of that area. If permeability to a specific ion such as calcium were increased for example, it is possible that the contractile activity could be increased without membrane depolarization. While such possibilities remain highly speculative, the unusual nature of the so-called pharmaco-mechanical coupling in arterial smooth muscle makes such possibilities worth some consideration.

## Interaction of Myogenic and Metabolic Factors

The role of the myogenic mechanism in overall regulation of the microcirculation is a matter which requires intensive further study. As noted earlier, the myogenic response may contribute to autoregulation of blood flow, a phenomenon of rather widespread occurrance in the various organs of the body. As arterial pressure is reduced, relaxation of the arterioles occurs in a generalized manner, leading to reduction of vascular resistance and restoration of flow. It has been proposed that this phenomenon is due to a myogenic response, or more precisely, a reduction of myogenic activity with loss of the pressure stimulus. It has also been suggested that accumulation of metabolites plays a role in causing the arterioles to relax at reduced perfusion pressure and flow. A precise dissection of the relative contribution of the two factors is difficult and many investigators have been content to resolve the matter of which mechanism is dominant by elevation of venous pressure, which reduces flow while elevating intravascular pressure. By this criterion, the myogenic factor is dominant in small intestine, liver, spleen and colon, while the results are mixed or favor the metabolic mechanism in skeletal muscle [27]. However, other studies have assessed the relative contributions to autoregulation by changing flow alone or pressure alone. Johnson and Intaglietta [29] performed partial or complete occlusions of individual arterioles and examined diameter changes immediately upstream. Pressure changes were assumed to be minimal. Modest dilation of 1 to 2 microns was seen with local flow reduction by this means. When flow was stopped completely, reduction of intravascular pressure elicited further dilation of the arteriole by 3 to 4 microns. From these experiments it was deduced that flow sensitivity was responsible for about 25% of the arteriolar dilation seen with arterial pressure reduction.

Another approach to this problem has been to alter the oxygen level of the tissue with a suffusing solution. Sullivan and Johnson [40] found

that elevation of tissue $PO_2$ by this means caused arteriolar constriction accompanied by substantial reduction in flow in many vessels and complete flow stoppage in others. Moreover, autoregulation of blood flow was abolished under elevated oxygen. These findings suggest that the mechanism is by far the dominant means of blood flow autoregulation in skeletal muscle micorcirculations. However, as noted above, increased transmural pressure can cause myogenic constriction in skeletal muscle [19]. The myogenic mechanism is apparently somehow overriden under conditions of elevated oxygen. The latter mechanism would require that elevating tissue oxygen does more than simply lower the level of vasodilator substances for that action alone would not prevent the loss of myogenic tone with pressure reduction.

## Interaction of Myogenic and Neural Factors

Relatively little is known at the present time regarding the interaction of myogenic and neural factors. Some experiments indicate that the two control mechanisms can co-exist without obvious interference. The studies by Bouskela and Wiederhielm [6] which demonstrate a strong myogenic response were carried out on intact, unanesthetized animals. The extent of innervation of the vessels studied is, however, unclear. Speden [39] found that rat ear artery actively constricted to intravascular pressure elevation only in the presence of adrenaline. However, studies in cat skeletal muscle indicate that beta adrenergic activity antagonizes the myogenic response [17].

It may also be that the myogenic response acts in opposition to neural control under certain conditions. For example, Wiedeman [42] noted that sections of the vasomotor nerves of the bat wing caused dilation of the larger arterial vessels which are innervated. After a delay, the small downstream arterioles which are not innervated constricted, possibly as a consequence of the elevated intravascular pressure. Alternatively, a washout of vasodilator metabolites could have been responsible. The observation that vessels of different size and location behave differently is an important finding which has implications for overall understanding of the behavior of the arteriolar network.

## Integration of Arteriolar Behavior in A Network Model

In considering the response of an arteriole to changes in pressure the vessel's location in the arteriolar network may be important for several reasons. First, as described by Gore [16], the larger and smaller arterioles may be located differently on the length-tension curve as a consequence of the different intravascular pressures in vessels. Second, when a change is induced, it could affect large and small arterioles differently. For example, elevation of arterial pressure raises pressure rather uniformly in large and small arterioles on a percentage basis. By contrast, elevation of venous pressure increases pressure more in the small arterioles than in the large vessels on an absolute basis and even more disproportionately on a relative or percentage basis. Changes in ambient pressure influence transmural pressure in large and small arterioles by the same absolute amount.

If the pressure change is initiated from the venous side it is to be expected that the small arterioles will be affected sooner and to a greater degree than large arterioles. It is perhaps worth noting also

that capillary filtration coefficient is greatly reduced when venous pressure is elevated, perhaps reflecting the strong constriction of the small precapillary vessels [28]. The reduction in flow secondary to small arteriole constriction will influence tissue metabolite levels which will further reduce any constrictor influence of the intravascular pressure itself in the large arterioles. When the pressure change is induced from the arterial side, the response of the larger arterioles will tend to reduce the magnitude of the pressure change in the smaller vessels downstream. In the case of a very reactive vascular bed it is quite possible that small changes in arterial pressure could be completely compensated by adjustments in the large arterioles alone. In this case, the pressure in the small arterioles would not change and pressure and flow in the capillary network would be unaltered. Therefore it appears that a purely myogenic mechanism has the potential to maintain constant pressure and flow over some range of arterial pressures. This behavior would require that the large and small arterioles behave independently as a series-coupled network of myogenic effectors. We do not know at the present time whether such behavior exists on a broad scale.

## Conclusions

Available evidence suggests that the arteriolar network possesses a sensitivity to intravascular pressure and that this myogenic behavior is an important control mechanism in the microcirculation. While data in a few vascular beds appears to be convincing, the importance of this mechanism in many beds remains to be determined and requires further study. Studies to date suggest that the smooth muscle cell may respond to changes in applied force in such a manner as to keep circumferential tension constant. However, we know very little about the myogenic mechanism at the cellular level. Also, we can only speculate as to the detailed manner in which an arteriolar network composed of myogenically active vessels might behave. It is possible that such a system would provide a well-regulated blood flow over a range of arterial perfusion pressures.

In looking to the future we can see the need for much additional work on this fascinating, if elusive, mechanism. I would suggest that our greatest need at this moment is for a better understanding of these mechanisms at the cellular level. At the present time we simply do not know how the smooth muscle cell senses and controls its response to a change in intravascular pressure. That is our challenge for the future, and a formidable challenge it is indeed!

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# ARACHIDONIC ACID METABOLITES IN THE CARDIOVASCULAR SYSTEM

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Platelet aggregation induced by ADP or collagen in vitro is inhibited by aspirin (Weiss and Aledort, 1967, O'Brien, 1968), an inhibitor of prostaglandin biosynthesis (Vane, 1971). Therapeutic oral doses of aspirin also prolong bleeding time (Quick, 1966) and inhibit prostaglandin production in platelets (Smith and Willis, 1971). Hence Smith and Willis (1971) suggested that the inhibitory effect of aspirin on platelet aggregation was attributable to inhibition of prostaglandin biosynthesis. At that time, however, it was difficult to reconcile this with the fact that the known prostaglandins did not induce platelet aggregation. The isolation of the unstable endoperoxides ($PGG_2$ and $PGH_2$) formed during the cyclo-oxygenase reaction (Hamberg and Samuelsson, 1973; Hamberg et al, 1974; Hamberg et al, 1975) and the demonstration that they caused platelet aggregation overcame this difficulty.

Hamberg et al (1974) and Smith et al (1974) showed that prostaglandin endoperoxides were released during platelet aggregation; their further metabolism leads to formation of an even more unstable compound thromboxane $A_2$ (Hamberg et al 1975) with a half life of 30 seconds. Thromboxane $A_2$ is more potent than the parent endoperoxide in inducing platelet aggregation (Hamberg et al, 1975; Moncada and Vane, 1977) and in constricting arterial smooth muscle (Bunting et al, 1976a; Needleman et al, 1976). Thromboxane $A_2$ undergoes spontaneous degradation to thromboxane $B_2$, a stable substance which does not affect platelet aggregation.

The involvement of endogenous cyclic endoperoxides and thromboxane $A_2$ in platelet aggregation and prevention of their formation by the inhibition of cyclo-oxygenase explains the inhibition of collagen induced aggregation and the second phase of ADP induced platelet aggregation in vitro by aspirin-like drugs.

In 1976, the Wellcome group discovered that a microsomal enzyme from blood vessel walls converts prostaglandin (PG) endoperoxides to an unstable substance which relaxes isolated blood vessel strips and potently inhibits platelet aggregation (Moncada et al, 1976a; Bunting et al, 1976b; Gryglewski et al, 1976; Moncada et al, 1976b). Structural determination and synthesis (Johnson et al, 1976) of this compound showed it to be an intermediate in the formation of the previously-described 6-oxo-$PGF_{1\alpha}$ (Pace-Asciak, 1976; Dawson et al, 1976). The compound, first known as PGX was renamed prostacyclin with the abbreviation of $PGI_2$ (Johnson et al, 1976).

The stability of prostacyclin depends on temperature and pH; it has a half-life of 10 minutes at 22°C and neutral pH. Boiling destroys its activity within 15 seconds. In blood at 37°C prostacyclin has a half-life of 2-3 minutes (Dusting et al, 1977a). It is unstable because of the 5,6 enol ether linkage. At pH 10-11, prostacyclin is virtually stable.

Prostacyclin is the main metabolite of arachidonic acid (AA) in isolated vascular tissues (Johnson et al, 1976; Salmon et al, 1978) and is produced by blood vessels of all species so far studied including man (Bunting et al, 1976b; Dusting et al, 1977b; Moncada et al, 1977a). The importance of prostacyclin in the maintenance of vascular homeostasis, its relevance to certain disease states and its potential therapeutic use will be considered in this review.

Anti-thrombotic and haemostatic effects of prostacyclin

Prostacyclin is the most potent naturally-occurring inhibitor of platelet aggregation known. It antagonises the action of all the common platelet aggregating agents and is 30-40 times more potent than $PGE_1$ (Moncada and Vane, 1977) and 1000 times more potent than adenosine (Born, 1962) in preventing ADP-induced aggregation in human platelet-rich plasma (PRP). Given intravenously to rabbits it inhibits electrically-induced thrombus formation in the carotid artery, at the same time prolonging bleeding time (Ubatuba et al, 1979). It also prevents sudden death induced by intravenously infused arachidonic acid (Bayer et al, 1979). Local or systemic administration to dogs inhibits thrombus formation in a partially occluded coronary artery (Aiken et al, 1979). Prostacyclin also disaggregates platelet clumps *in vitro* (Moncada et al, 1976b; Ubatuba et al, 1979), in extracorporeal systems where blood is perfused over collagen strips (Gryglewski et al, 1978a), and *in vivo* in the circulation of man (Szczeklik et al, 1978a).

Treatment of vascular tissue, microsomes or endothelial cells with indomethacin or aspirin does not prevent the generation of prostacyclin-like activity when the tissue is incubated with platelets (Bunting et al, 1976b; Gryglewski et al, 1976; Bunting et al, 1977; Marcus et al, 1979). This observation led to the proposal (Bunting et al, 1976b; Gryglewski et al, 1976) that the vessel wall can synthesise prostacyclin from PG endoperoxides provided by the platelets, as well as from its own endogenous precursors. This hypothesis has proved to be controversial and has been opposed by Needleman et al. (1979) and Hornstra et al. (1979). However, Marcus et al. (1979) have shown that feeding of endoperoxides to endothelial cells suspended in PRP only occurs *in vitro* when the platelet number approximates to that of normal blood. Too high a platelet concentration induces a platelet-platelet interaction which limits the platelet-endothelial cell reaction. It is still not known whether prostacyclin production by endothelial cells from endoperoxides of stimulated platelets occurs *in vivo*, but clearly, close proximity of the two cells would be necessary, as occurs when the platelet adheres to the vessel wall.

Prostacyclin inhibits aggregation by activating platelet adenlyate cyclase, thus leading to an increase in cAMP (Gorman et al, 1977a; Tateson et al, 1977). Prostacyclin is the most potent stimulator of adenylate cyclase yet found and the duration of this effect is longer than that seen with $PGE_1$ or $PGD_2$ (Gorman et al, 1977a). Prostaglandin endoperoxides and $TXA_2$, on the other hand, inhibit $PGE_1$-stimulated cAMP accumulation and elevate cGMP levels, although they do not alter basal cAMP levels (Miller and Gorman, 1976). Thus, PG endoperoxides have a pivotal role in platelet function, for they can either be converted by thromboxane synthetase in the platelets into the pro-aggregatory, vasoconstrictor $TXA_2$, or alternatively by the vessel wall to form prostacyclin which is anti-aggregatory and

vasodilator (Moncada and Vane, 1979 a & b).

Prostacyclin also elevates cAMP concentrations in fibroblasts (Gorman et al, 1979; Hopkins et al, 1978), suggesting that the prostacyclin/$TXA_2$ system may be significant in cells other than platelets. Stimulation of adenylate cyclase by prostacyclin in vascular tissue has been proposed as the mechanism of its vasodilator effects, since prostacyclin in the presence of a phosphodiesterase inhibitor causes an increase in cAMP levels in bovine coronary artery rings (Dembinska-Kiec et al, 1979), and relaxation of smooth muscle cells has been associated with increased cAMP levels (Andersson, 1973).

In blood vessel walls, prostacyclin synthetase is most highly concentrated in the intimal surface and decreases in activity towards the adventitial surface (Moncada et al, 1977b). Pro-aggregating materials, on the other hand, progressively increase in concentration from the intimal to the adventitial surface (Moncada et al, 1977b). Comparison of prostacyclin production by cultured cells from vessel walls shows that endothelial cells are the chief producers of prostacyclin (Harker et al, 1977; MacIntyre et al, 1978; Weksler et al, 1977a), although the subendothelium can also produce significant amounts (Silberbauer et al, 1978).

The ability of the vascular wall to synthesise a material which can actively prevent platelets from clumping explains the long-recognised fact that platelets do not stick to healthy vascular endothelium. When injury occurs to the vascular wall platelets adhere to the exposed subendothelial layers and in doing so release pro-aggregatory materials which cause other platelets to aggregate and form a thrombus. Production of prostacyclin by the endothelial cells surrounding the injury, together with circulating prostacyclin, limits the size of the thrombus. The more severe the injury, the greater will be the propensity for thrombus formation for not only will the damage to the prostacyclin-producing endothelial cells be greater, but also the more pro-aggregating layers of the vascular wall will be exposed (Moncada and Vane, 1979 a & b).

Prostacyclin inhibits platelet aggregation (platelet-platelet interaction) at much lower concentrations than are required to prevent platelet adhesion (platelet-collagen interaction) (Higgs, E.A. et al, 1978). Thus at low, perhaps physiological, concentrations prostacyclin does not prevent platelets from interacting with damaged vascular tissue, possibly allowing the platelets to feed endoperoxides to prostacyclin synthetase in the surrounding intact tissue, but at the same time limiting further platelet attachment to the monolayer. Clearly, prostacyclin plays an important part in the control of platelet aggregability, even though other factors may contribute to the thrombo-resistant properties of vascular endothelium.

Prostacyclin - Thromboxane $A_2$ balance and anti-thrombotic therapy

Arachidonic acid is converted into PG endoperoxides by cyclo-oxygenase for which a number of specific inhibitors exist, the chief of which are the aspirin-like drugs (Ferreira et al, 1971; Smith and Willis, 1971; Vane, 1971). Specific inhibitors of thromboxane synthetase have also been described (Blackwell et al, 1978; Moncada et al, 1977c; Gorman et al, 1977b) and treatment with these should achieve an anti-thrombotic effect, with a prolonged bleeding time and inhibition of thrombus formation, whereas inhibition of prostacyclin synthetase should lead to a prothrombotic state.

Aspirin inhibits platelet aggregation and prolongs bleeding time (Smith and Willis, 1971; Jobin, 1978) and has been tested over a number of years in the treatment of thrombosis (Majerus, 1976). The fact that formation not only of the prothrombotic $TXA_2$ but also the anti-thrombotic prostacyclin is dependent on cyclo-oxygenase provides an explanation for the contradictory experimental and clinical results obtained when aspirin is used as an anti-thrombotic agent (Turpie and Hirsh, 1978; Verstraete, 1978). However, much evidence has accumulated recently to suggest that aspirin treatment may be manipulated so that the net effect is anti-thrombotic. Firstly it seems that aspirin sensitivity of cyclo-oxygenase varies with the tissue of origin (Burch et al, 1978a; Patrono et al, 1976) and that platelet cyclo-oxygenase is more sensitive to aspirin inhibition than that of endothelial cells (Burch et al, 1978a). In addition, the inhibitory effect on platelets is longer lasting because aspirin acetylates the active site of the enzyme, leading to irreversible inhibition (Roth and Majerus, 1975; Roth and Siok, 1978). Platelets are unable to synthesise new protein (Marcus, 1978) so that cyclo-oxygenase activity in the platelets is only restored when new platelets, formed from unaffected megakaryocytes, enter the circulation to replace those affected by the aspirin block (Burch et al, 1978b) - a period dependent upon platelet turnover. Endothelial cells, on the other hand, are able to regenerate cyclo-oxygenase (Czervionke et al, 1978; Kelton, et al, 1978); this ability is prevented by treatment with the protein synthesis inhibitor, cycloheximide (Czervionke et al, 1979).

These in vitro observations have been supported by in vivo experiments which show that low doses of aspirin given to rabbits are more effective in blocking $TXA_2$ formation than prostacyclin formation (Amezcua et al, 1978; Korbut and Moncada, 1978). Selective blockade of platelet cyclo-oxygenase by aspirin also occurs in humans (O'Grady and Moncada, 1978; Rajah et al, 1978). A single low dose of aspirin (0.3 g) increases bleeding time 2 hours after ingestion, whereas a high dose (3.9 g) has no effect on this parameter. After the high dose, however, $TXA_2$ formation and platelet aggregation are impaired and complete recovery does not occur until 168 hours after ingestion. This contrasts with the effect of this dose on bleeding time, which is unchanged 2 h after ingestion but increases at 24 and 72 h and recovers to pretreatment levels by 168 h (O'Grady and Moncada, 1978). Masotti et al. (1979) have measured the effect of aspirin on platelet aggregation and blood levels of prostacyclin and malondialdehyde (MDA), which is an index of $TXA_2$ formation. They showed that a low dose of aspirin (approx. 3.5 mg/kg) blocks platelet aggregation and MDA formation with only a slight effect on prostacyclin production, and that inhibition of platelet aggregation and MDA formation was still present after 72 h, whereas prostacyclin inhibition reverses within 24 h, even after higher doses of aspirin (8-10 mg/kg).

Cutaneous bleeding time decreases with age, perhaps because of a reduced ability to synthesise prostacyclin, and hence a predominance of $TXA_2$ generation (Jorgensen et al, 1979). Thus, the response to aspirin is dependent upon age, so that a low dose prolongs but a high dose has no effect on bleeding time in the young, whereas both doses lead to an increase in bleeding time in the old (Jorgensen et al, 1979). This may explain why some workers have failed to demonstrate the differential effect of low and high doses of aspirin on bleeding time (Godal et al, 1979).

These results, together with the observations that thrombin-induced platelet adherence to endothelial cells is enhanced by aspirin treatment (Czervionke et al, 1978) and that treatment of arterial tissue in vitro with aspirin enhances its thrombogenicity (Baumgartner and Tschopp, 1979), strongly indicate that the use of aspirin as an anti-thrombotic must be regulated so that a situation is achieved

which gives maximum inhibition of thromboxane synthesis with minimum impairment of prostacyclin production. Only in this way will the overall effect be anti-thrombotic. This may be accomplished by low doses of aspirin every day or every two days, or alternatively by infrequent large doses, thus utilising the irreversible inhibition of platelet cyclo-oxygenase.

The anti-thrombotic activity of dipyridamole can be attributed to an effect on the balance between $TXA_2$ and prostacyclin. Dipyridamole inhibits phosphodiesterase thereby potentiating the increase in cAMP caused by circulating prostacyclin (Moncada and Korbut, 1978), which may account for the ability of dipyridamole to inhibit platelet aggregation in vivo. Dipyridamole has been shown to be most effective after a small dose of aspirin or 24 h after a high dose, at which time prostacyclin dominates in the $TXA_2$-prostacyclin balance (Moncada and Korbut, 1978). The synergistic effect of low doses of aspirin with dipyridamole has long been recognised in models of thrombosis and in clinical results (Harker and Slichter, 1972; Honour et al, 1977) and it would seem rational to use dipyridamole in combination with a dose of aspirin selected to achieve an anti-thrombotic state. Not all anti-thrombotic agents act in this way; levels of sulphinpyrazone (Gordon and Pearson, 1978) or ticlopidine (Ashida and Abiko, 1978) which inhibit platelet aggregation have little effect on prostacyclin production.

In summary, anti-thrombotic therapy may be achieved by altering the prostacyclin-$TXA_2$ balance in one of a number of ways - by selecting an aspirin treatment regime which inhibits $TXA_2$ formation without affecting prostacyclin production, by inhibiting thromboxane synthesis, or by potentiating either the synthesis or the action of prostacyclin.

In contrast, agents which create a situation in which $TXA_2$ is the dominant influence have been found to lead to a prothrombotic state. Tranylcypromine, an inhibitor of prostacyclin synthesis, enhances platelet aggregation in a model of thrombosis in the mouse brain microcirculation (Rosenblum and El-Sabban, 1978). Hydrocortisone, which prevents the release of arachidonic acid from cell membranes (Flower, 1978) inhibits prostacyclin production in vessel walls and reduces bleeding time in normal or thrombocytopenic rabbits (Blajchman et al, 1979). The toxic effects of cadmium, which causes thrombosis and arterial hypertension, have been attributed to selective damage to vascular endothelium resulting in impaired ability to produce prostacyclin (Caprino and Togna, 1979).

Cardiovascular effects of Prostacyclin

It is also possible that formed elements of blood such as the white cells, which produce endoperoxides and $TXA_2$ (Davison et al, 1978; Goldstein et al, 1977; Higgs et al, 1976), interact with the vessel wall to allow formation of prostacyclin, as do the platelets. This suggestion, coupled with the fact that prostacyclin may modulate white cell behaviour (Higgs et al, 1978; Weksler et al, 1977b) could well mean that prostacyclin plays a role in the control of white cell migration during the inflammatory response.

Unlike other prostaglandins, such as $PGE_1$ and $PGF_{2\alpha}$, prostacyclin is not inactivated on a passage through the pulmonary circulation (Dusting et al, 1978a), and this is probably due to the fact that prostacyclin, although a good substrate for lung prostaglandin dehydrogenase, is not a substrate for the uptake mechanism responsible for transport from the circulation to the intracellular enzyme (Hawkins et al, 1978). Indeed, the lung can constantly release small amounts of prostacyclin into the circulation (Gryglewski et al, 1978a; Moncada et al, 1978). The concentration of prostacyclin is higher in arterial than in venous blood due to

overall inactivation of about 50% in one circulation through peripheral tissues (Dusting et al, 1978a). These results, originally obtained in experimental animals, have now been confirmed in man where Hensby and colleagues (1979) have shown that the concentrations of 6-oxo-$PGF_{1\alpha}$ are higher in arterial than in venous blood in patients prepared for cardiac catheterisation (Hensby et al, 1979). Thus, platelet aggregability in vivo can be modulated by circulating prostacyclin which will reinforce the actions of locally-produced prostacyclin throughout the vasculature. The kidney may also release $PGI_2$ into the circulation as a result of a specific stimulus, such as bradykinin (Muliane et al, 1979a).

Prostacyclin relaxes in vitro most vascular strips including rabbit coeliac and mesenteric arteries (Bunting et al, 1976b), bovine coronary arteries (Dusting et al, 1977b; Needleman et al, 1978) human and baboon cerebral arteries (Boullin et al, 1979) and lamb ductus arteriosus (Coceani et al, 1978). Exceptions to this include the porcine coronary arteries (Dusting et al, 1977c), some strips of rat venous tissue and isolated human saphenous vein (Levy, 1978), which are weakly contracted by prostacyclin. Whether these same effects are induced in the corresponding circulations in the intact animal or man has not been studied. In the human umbilical arterial strip, prostacyclin induces a dose-dependent relaxation at low concentrations and a dose-dependent contraction at higher concentrations (Pomerantz et al, 1978).

In isolated Langendorff-perfused hearts of the guinea pig, rabbit and rat, not only is prostacyclin a potent vasodilator but it is also the predominant metabolite of arachidonic acid (Needleman et al, 1978; Schror et al, 1978; De Dekere et al, 1977). We, and others, have investigated the coronary actions of prostacyclin in the isolated Langendorff perfused heart of the rabbit and guinea pig where it is a powerful dilator (Schror and Moncada, 1979; Wennmalm, 1979) and in the intact heart of open chest dogs (Armstrong et al, 1977; Dusting et al, 1978b; Hyman et al, 1978). Local injection of prostacyclin (50-500 ng) into the coronary circulation increased coronary blood flow without systemic effects and it was a more potent coronary dilator than $PGE_2$. Furthermore, in dogs, prolonged coronary vasodilatation was elicited by prostacyclin (20-100 μg) absorbed through the myocardium after dripping a solution on to the surface of the left ventricle (Dusting et al, 1978b).

The effects of prostacyclin on cardiac arrhythmia and infarct size after coronary occlusion have been studied by several workers and at the moment there is no agreement as to the potential beneficial or detrimental effect. $PGI_2$ has been reported to have variable effects on the cardiac rhythm of isolated rabbit and rat hearts depending on the dose and the experimental procedure (Karmazyn et al, 1978; Mest and Forster, 1979). In vivo, however, there are reports indicating that $PGI_2$ produces an increase in arrhythmias in the rat and cat after acute coronary artery occlusion (Au et al, 1980; Kelliher et al, 1979; Dix et al, 1979). However, in the anaesthetised dog an infusion of $PGI_2$ (320 ng/kg/min) for 6 h starting 17 min after coronary occlusion did not alter infarct size (Ribeiro et al, 1979). $PGI_2$ infusion at 420 ng/kg/min for 6 h in anaesthetized dogs starting 3 min after occlusion reduced the infarct size (Jugdutt et al, 1979). Doses of $PGI_2$ which increased arrhythmia and ventricular fibrillation in the rat following coronary occlusion decreased infarct size in the survivors (Au et al, 1980).

In dog, rabbit or rat prostacyclin is hypotensive (Armstrong et al, 1977) and is more potent than $PGE_2$. Prostacyclin is at least 100 times more active than its degradation product, 6-oxo-$PGF_{1\alpha}$ (Armstrong et al, 1977). In the cat, $PGI_2$ has been shown to be a potent pulmonary vasodilator where it also inhibited the hypertensive and platelet aggregation effects of infused ADP (Hyman and Kadowitz, 1979).

In the dog, prostacyclin infused intravenously reduces renal vascular resistance and increases renal blood flow and urinary excretion of sodium, potassium and chloride ions at doses below those needed for a systemic effect (Bolger et al, 1978; Hill and Moncada, 1979). There is increasing evidence that prostacyclin mediates the release of renin. Arachidonic acid, prostaglandin endoperoxides or prostacyclin all stimulate renin release from slices of rabbit renal cortex, but $PGE_2$ has no such effect (Weber et al, 1976; Wharton et al, 1977a). Furthermore, indomethacin reduces renin release in animals and man (Data et al, 1976; Frolich et al, 1976; Larsson et al, 1974). Prostacyclin-like activity and 6-oxo-$PGF_{1\alpha}$ have been identified in incubates of $PGG_2$ or $PGH_2$ with renal cortical microsomes (Remuzzi et al, 1978; Wharton et al, 1977b; Zenser et al, 1977). Thus, prostacyclin may be the obligatory endogenous mediator of renin secretion by the kidney. Indeed, prostacyclin induces renin release (Gerber et al, 1978) and increased concentrations of angiotensin II in arterial blood (Hill et al, 1978) when infused intrarenally into dogs (Gerber et al, 1978). 6-oxo-$PGF_{1\alpha}$ is also formed by collecting tubule cells isolated from rabbit papillae (Grenier and Smith, 1978). Interestingly, angiotensin II releases prostacyclin from the rat kidney in vitro (Silberbauer et al, 1979) and the dog kidney in vivo (Mullane et al, 1979a).

Prostacyclin is also a strong vasodilator in the mesenteric and hind limb circulations of the dog (where $TXA_2$ is a vasoconstrictor) (Dusting et al, 1978c) and on the precapillary side of the microcirculation of the hamster cheek pouch (Higgs et al, 1979), where it also reverses epinephrine-induced vasoconstriction. In this preparation 6-oxo-$PGF_{1\alpha}$ had 1/20th the vasodilator activity of prostacyclin and was more potent than $PGE_2$. In the pulmonary circulation of the dog, prostacyclin is the only product of arachidonic acid which produces strong vasodilatation (Kadowitz et al, 1978; Mullane et al, 1979b). It also dilates the pulmonary vascular bed of the foetal lamb where its potency is greater than $PGE_1$ but less than $PGE_2$ (Leffler and Hessler, 1979).

Effects of prostacyclin in man

Prostacyclin also induces vasodilatation and hypotension in man when given either intravenously or by inhalation (Gryglewski et al, 1978b; O'Grady et al, 1979; Szczeklik et al, 1978b). Intravenous infusion of prostacyclin in man inhibits platelet aggregation measured ex vivo (Szczeklik et al, 1978a; O'Grady et al, 1979; Fitzgerald et al, 1979) and at higher doses disperses circulating platelet aggregates (Szczeklik et al, 1978a). Template bleeding was significantly increased during prostacyclin infusion in one study (Szczeklik et al, 1978a) but not in another (O'Grady et al, 1980), this may reflect the higher dose administered in the former study. Prostacyclin has vasodilator effects in man producing facial flushing, increased peripheral skin temperature, increased heart rate and, at higher doses, reduction in diastolic blood pressure (Szczeklik et al, 1978a; O'Grady et al, 1979; Fitzgerald et al, 1979). Platelet count, platelet factor III, accelerated partial thromboplastin time, prothrombin time, euglobin clot lysis time and fibrinogen degradation products are unchanged by prostacyclin infusion (Szczeklik et al, 1978a; O'Grady et al, 1979). Prostacyclin has more persistant effects on platelet aggregation than on the cardiovascular system, the cardiovascular effects reversing within five minutes of discontinuing infusion while partial inhibition of platelet aggregation persists in some instances for nearly two hours (O'Grady et al, 1979). Possibly this reflects persistant elevation of platelet cyclic AMP after prostacyclin is discontinued, or the stabilization of prostacyclin by plasma (Pederson, 1978).

It was originally suggested (Szczeklik et al, 1978a) that prostacyclin had

direct positive chronotrophic and inotropic effects in man. Further assessment of the cardiovascular effects by non-invasive methods did not support the suggestion that prostacyclin had direct chronotropic effects though a minor inotropic effect could not be excluded (Warrington and O'Grady, 1980).

Prostacyclin and Extracorporeal Circulations

The circulation of blood through extracorporeal systems involves the blood coming into contact with artificial surfaces which are unable to generate prostacyclin. In the course of such procedures thrombocytopenia and loss of platelet haemostatic function occur and make an important contribution to the bleeding problems following, eg. charcoal haemoperfusion and prolonged cardiopulmonary bypass in man (Friedenberg et al, 1978; Moriau et al, 1977; Weston et al, 1977). Formation of microemboli during cardiopulmonary bypass may also contribute to cerebral complications which sometimes follow this procedure (Patterson and Kessler, 1969). In animals subjected to experimental renal dialysis (Woods et al, 1978), charcoal haemoperfusion (Bunting et al, 1979) and cardiopulmonary bypass (Longmore et al, 1979) infusion of prostacyclin during the procedure prevented this platelet damage and thrombocytopenia thus increasing the biocompatability of the procedure. These findings have been confirmed in patients with fulminant hepatic failure undergoing charcoal haemoperfusion (Gimson et al, 1980). Prostacyclin infusion prevented the fall in platelet count and elevation of β thromboglobulin seen in the control patients. In addition two of the control patients developed marked hypotension during the procedure, in one associated with a marked rise in Swank Screen filtration pressure, while this did not occur in the prostacyclin treated patients. A study of serial haemoperfusion with prostacyclin on the survival rate of patients with fulminant hepatic failure is now in progress.

During cardiopulmonary bypass in man preliminary indications (Bunting et al - unpublished results, 1980) are that in patients receiving prostacyclin during bypass platelet number and function are better preserved and marked rises in Swank Screen filtration pressure prevented.

Prostacyclin and Heparin

During the course of studies on extracorporeal circulation systems it was observed that prostacyclin potentiates the effects of heparin (Bunting et al, 1979). Further studies on this interaction demonstrated that prostacyclin has also a small indirect anticoagulant effect. Indeed platelets stimulated by low doses of aggregating agents accelerate clotting by providing a surface upon which coagulation factors can combine and react more efficiently (see Marcus, 1978). Prostacyclin, by preventing platelet activation, inhibits the shortening of clotting time produced when either kaolin or collagen are incubated with platelet rich plasma (Bunting and Moncada, 1980). Platelets release antiheparin activity which in vitro reduces the anticoagulant effect of heparin. Prostacyclin by inhibiting this release and by preventing the development of pro-coagulant activity can potentiate the action of heparin as much as one hundred per cent (Bunting and Moncada, 1980). These in vitro findings agree with the observations in extracorporeal circulations (Bunting et al, 1979).

Perspectives in the development of new antithrombotic therapy

Greenland Eskimos have a very low incidence of coronary arterial disease (Bang et al, 1971). It has been suggested that this might be attributable to their low level of cholesterol, triglyceride, low density lipoprotein and very low density

lipoprotein in the blood, together with high levels of high density lipoprotein in male Eskimos (Bang et al, 1971; Bang and Dyerberg, 1972). Epidemiological studies (Dyerberg et al, 1975; Bang et al, 1976) have shown these lipid patterns to be dietary rather than genetically determined. A bleeding tendency in Eskimos has been well documented (Bertelsen, 1940; Christensen et al, 1953; Dyerberg and Bang, 1979). The plasma-lipids of the Greenland Eskimos contain only small amounts of arachidonic acid and relatively high amounts of eicosapentaenoic acid, the reverse of the situation in Caucasian Danes (Miller et al, 1977). Dyerberg and Bang, (1978) suggested that the low incidence of myocardial infarction in Eskimos might be due to a relative lack of a substrate required for generation of pro-aggregatory prostaglandin or thromboxane in the platelet. In fact eicosapentaenoic acid (C20:5ω3) is transformed in the platelet to thromboxane $A_3$ which is less proaggregatory than thromboxane $A_2$ derived from arachidonic acid (Raz et al, 1977; Gryglewski et al, 1979). However the vessel wall can utilize eicosapentaenoic acid to synthesise a potent anti-aggregatory agent which is probably $PGI_3$ (Dyerberg et al, 1978). Hence the relative immunity of the Eskimos to acute myocardial infarction could be attributed to both their lipid profile and an increased anti-aggregatory activity which would also explain the bleeding tendency in this population (Dyerberg and Bang, 1979). Eicosapentanoic acid is chiefly present in marine mammals and fish: Cod liver oil contains 10 percent, the oil of most fish between 8-12 percent and that of oysters and caviar more than 20 percent. A comparable intake of eicosapentaenoic acid to the Eskimos would require almost 80 ml cod liver oil, equivalent to 700 calories per day. The importance of polyunsaturated fats in cardiovascular disease remains controversial: what may be important is not polyunsaturated fats as a whole but specific polyunsaturated fatty acids. Margarine for example contains 9-48 percent linoleic acid, the remainder of the fats being C16:0 (4-18%), C18:0 (1.5-14%), C18:1ω9 (8-53%) and C18:3ω3 (0.1-5%) (Weihrauch et al, 1977). Of these only linoleic acid (C18:2ω6) can, when in the cis form be transformed into a precursor for prostacyclin, and a considerable amount of the linoleic acid in margarine is in the trans form (Weihrauch et al, 1977). There is no margarine available with a high proportion of eicosapentaenoic acid (C20:5ω3). Perhaps, therefore, the debate about polyunsaturated fats should concern the merits of fats derived from fish rather than meat instead of concentrating on the relative merits of butter and margarine. The possibility that medication or dietary supplementation with eicosapentaenoic acid would provide prophylaxis against arterial thromboembolic disease remains to be explored.

Prostacyclin and prostacyclin-like compounds, by elevating platelet cyclic AMP, inhibit platelet aggregation induced by most stimuli. This is in contrast to aspirin and thromboxane synthetase inhibitors which block aggregation to some agents but not all. Hence prostacyclin analogues could offer superior anti-thrombotic properties and such agents will no doubt be studied in the not too distant future.

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# PROSTACYCLIN AND ATHEROSCLEROSIS

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Prostacyclin is a biologically active lipid with potent antiaggregatory and vasodilatory properties ( Gryglewski et al.1976, Moncada et al.1976). In one of the pioneering papers on the discovery of prostacyclin ( Gryglewski et al. 1976) we have described that 15-hydroperoxyeicosatetraenoic acid (15-HPETE, 15-HPAA, $IC_{50}$ = 1.5 μM) and tranylcypromine ( $IC_{50}$ = 600 μM) are inhibitors of prostacyclin synthetase in porcine aortic microsomes. 15-HPETE inhibits prostacyclin generation also in rabbit arterial slices ( Moncada et al. 1976) and in cultured human endothelial cells ( Marcus et al. 1978). 15-HPETE shares its destructive action on prostacyclin synthetase with a vast number of other lipid peroxides ( Salmon et al.1978). It has been proposed that 15-HPETE suppresses the formation of prostacyclin ($PGI_2$) as a consequence of the peroxidatic reduction of this hydroperoxide and the release of $[O_x]$ ( Kuehl Jr.et al.1980).

The above in vitro findings prompted us to put forward a hypothesis that an increase in lipid peroxidation promotes the development of atherosclerosis owing to the selective removal of prostacyclin from the body ( Dembińska-Kieć et al. 1977, Gryglewski et al.1978 a) and subsequent activation of blood platelets ( Żmuda et al.1977). We believe that prostacyclin is a natural antiatherosclerotic hormone ( Gryglewski 1979).

Indeed, feeding rabbits a diet high in oleic acid and in cholesterol leads to a dramatic suppression of prostacyclin

generation by aorta, mesenteric arteries, heart ( Dembińska-Kieć et al.1977, Gryglewski et al.1978 a), lungs and kidneys ( Dembińska-Kieć et al.1979). This suppression is observed as early as one week after feeding the rabbits an atherogenic diet ( Masotti et al.1979). The selective blockade of prostacyclin generation by lipid peroxides which are formed during hyperlipidemia may divert arachidonic acid metabolism from prostacyclin to prostaglandins in arteries ( Berberian et al. 1976) and kidney ( Dembińska et al.1979) and to thromboxane $A_2$ in platelets ( Żmuda et al.1977). This last phenomenon has been also observed in atherosclerotic patients ( Szczeklik and Gryglewski 1979, Szczeklik et al.1978 a).

Until now, we have not been able to produce a direct evidence that human atherosclerosis is casually associated with an increased lipid peroxidation. Nonetheless, lipid peroxides have been found in human atherosclerotic arteries ( Glavind et al.1952), in ceroid atheromatic plaques ( Hartroft et al.1965) and in retina during ocular siderosis ( Hiramitsu et al.1976). Human atheromatic plaques hardly generate prostacyclin ( Angelo et al.1978). Low-density lipoproteins were reported to inhibit the generation of an antiaggregatory principle by cultured human endothelial cells ( Nordøy et al. 1978) and to damage them (Henriksen et al.1979) while high-density lipoproteins were found to prevent the deleterious action of low-density lipoproteins.

We have recently found (Szczeklik and Gryglewski,1980) that serum lipid peroxide levels rise in common types of hyperlipoproteinemias from 2.5±0.1 to 3.8±0.3 nmoles malondialdehyde $ml^{-1}$. The sum of lipid peroxide concentrations in the lipoprotein fractions is several times higher (10.8±3.0 nmoles MDA $ml^{-1}$ in healthy volunteers and 26.0±3.3 nmoles MDA $ml^{-1}$ in hyperlipoproteinemia type V) than the corresponding serum lipid peroxide concentrations. Most of serum lipid peroxides are accumulated in low-density lipoproteins (LDL). In common types of hyperlipoproteinemias lipid peroxides may also appear in very low-density lipoproteins (VLDL) and in chylomicrones, but never in high-density lipoproteins (HDL).

HDL when mixed with LDL seem to protect a hydroperoxy moiety in LDL from being detected by the thiobarbituric acid method or by the iodometric titration. LDL but not HDL inhibit the release of prostacyclin from superfused bovine coronary artery and from the incubated slices of rat aorta. Administration of vitamin E ( 300 mg daily for a week) substantially suppresses lipid peroxide levels in LDL and chylomicrones of patients with hyperlipoproteinemias.

The above evidence for involvement of lipid peroxidation and prostacyclin deficiency in pathogenesis of atherosclerosis prompted us to begin clinical trials in which synthetic prostacyclin was administered into atherosclerotic patients in a hope that it will replace in their body the lacking endogenous hormone.

## CLINICAL TRIALS

The first report on pharmacological effects of prostacyclin in healthy men came from Kraków ( Gryglewski et al.1978 b, Szczeklik et al.1978 b). We have learnt by then that pharmacologically active doses of prostacyclin are at a range of 1 - 20 ng $kg^{-1}min^{-1}$, i.v., while higher doses may result in syncope. Moderate lowering of diastolic blood pressure, elongation of bleeding time, reddening of face and palms as well as inhibition of platelet aggregates were the most prominent symptoms of pharmacological action of prostacyclin. A year later prostacyclin was administered into first five patients suffering from advanced arteriosclerosis obliterans ( Szczeklik et al.1979 ). Presently, we report on the effect of prostacyclin therapy in fifty patients suffering from peripheral obstructive vascular disease. The observation period was from 4 to 16 months.

### Patients and methods

Fifty patients (44 men and 6 women) were treated with prostacyclin. Arteriosclerosis obliterans was diagnosed in 36 patients (46 - 76 years old) and thrombangiitis obliterans in 12 patients (33 - 44 years old). Two women (24 and 26

years old) suffered from Takayasu disease of lower extremities. In all but 3 patients the diagnosis was confirmed by angiographic examination.

Ischemia at rest was recorded in 44 patients as evidenced by rest pain, ischemic ulceration or necrosis. Only in 6 patients physical exercise ( walking) was necessary to induce pain. Out of 50 patients 7 had undergone vascular reconstructive surgery, 8 perivascular sympathectomy, 5 amputation of toe or foot and 5 amputation of leg beneath knee.

Sodium salt of prostacyclin ( Upjohn Co., Kalamazoo,U.S.A. and Wellcome Research Laboratories,Beckenham,U.K.) was dissolved in 0.1 M glycine buffer pH 10.5 and infused into femoral artery (33 patients) or subclavian vein (17 patients) at a dose of 2 - 10 ng $kg^{-1}min^{-1}$ for 72 hours. In 20 patients the prostacyclin therapy was repeated 2 - 4 times every 1 - 20 weeks. The total observation period for 50 patients studied was 3 - 17 months. Apart from prostacyclin no other pharmacological treatment was prescribed.

## Results

Infusions of prostacyclin made the affected leg to become dry and hot. Erythema usually appeared. Platelet aggregability was suppressed even 3 hours after the infusion of prostacyclin was terminated.(Fig.1) Infused dose of prostacyclin was maintained as high as possible. Side effects which were responsible for lowering of the dose of prostacyclin occured in the diminishing order of frequency: pain in the infused leg, headache, articular pain, nausea, hipotonia, cardiac arrhythmia. Moderate hyperglycemia was recorded in several patients, especially in those with otherwise balanced diabetes. Because of rest pain, 23 patients had to rely on narcotic or non-narcotic pain-killers administered several times daily. In 15 out of those 23 patients pain extinguished for a period of 4 weeks to 16 months, next day after termination of prostacyclin infusion. in a half of 32 patients with ischemic ulcers partial or complete healing was observed while in 7 patients with deep penetrating necrosis no improve-

ment occured.(Table 1) In 5 out of 6 patients with intermittent claudication prostacyclin caused a sustained increase in walking distance ( 4 km $h^{-1}$) by at least 50 per cent.

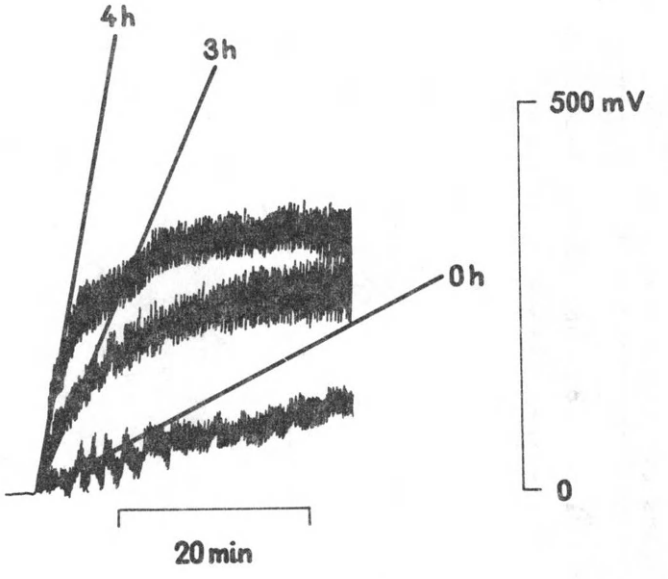


Fig.1. Aggregation of platelets on a collagen strip that was superfused with heparinized blood(1 ml $min^{-1}$) withdrawn from femoral artery of a 24 yrs old woman suffering from Takayasu disease. Aggregation was recorded immediately after termination of i.a.infusion of prostacyclin (+ ng $kg^{-1}min^{-1}$ for 72 hrs) (0 h) as well as 3 and 4 hrs later. The slope of increase in weight of a collagen strip (0-500 mV) is inversely proportional to the degree of suppression of platelet aggregability by circulating prostacyclin.

Table 1. Results of prostacyclin therapy

| Diagnosis | Number of patients $PGI_2$ infusion single/multiple | Intermittent claudication | Symptoms of ischemia at rest | | | Results of therapy | | |
|---|---|---|---|---|---|---|---|---|
| | | | Pain | Ulceration | Necrosis | long-term (2-16 mnth) improvement | short-term improvement | no |
| A.o. | 36<br>25/11 | 5 | 15 | 19 | 7 | 15 | 10 | 11 |
| T.o. | 12<br>6/6 | 1 | 6 | 11 | 3 | 4 | 4 | 4 |
| D.T. | 2<br>0/2 | 0 | 2 | 2 | 1 | 1 | 1 | 0 |
| Total | 50<br>31/19 | 6 | 23 | 32 | 11 | 20 | 15 | 15 |

A.o. = arteriosclerosis obliterans

T.o. = thrombangiitis obliterans

D.T. = Takayasu disease

## DISCUSSION

The results of our open clinical trials with prostacyclin will be presented elsewhere ( Szczeklik et al.1980). In summary , single or repeated courses of prostacyclin therapy resulted in a long-term clinical improvement in 40 per cent of patients with peripheral vascular disease, mostly with arteriosclerosis obliterans. It should be mentioned that the majority of patients suffered from the advanced form of the disease (Table 1). In those patients fibrinolytic, anticoagulant or vasodilatory therapy is of little value (Coffman,1979). Therefore, we assume ex iuvantibus that prostacyclin did some thing special to those patients, i.e.substituted the lacking anti-atherosclerotic endogenous hormone with synthetic prostacyclin. We believe that prostacyclin is necessary to keep platelets in a non-aggressive state. When the protective anti oxidant mechanisms ( Dormandy 1978) fail to function and lipid peroxides accumulate in the body killing prostacyclin synthetase, then deficiency of the hormone is followed by deficiency of its second messenger - cyclic AMP - both in platelets and in vascular walls, thus increasing platelet aggregability ( Gorman 1979) and endothelial permeability ( Numano 1977). Activated platelets adhere to the defenseless arterial wall and, finally,they form mural micro-aggregates which release pro-inflammatory mediators and enzymes. A focal damage made to endothelium promotes a massive aggregation of platelets. Platelet-derived growth factor(PDHF) is released (Ross et al.1974, Antoniades et al.1979), migration of myocytes is started and formation of atheromatic plaque is accomplished (Ross and Harker 1976).

Inhibition of pathological peroxidation of body lipids would be the most desirable line of defense against atherosclerosis. Another possibility is to administer prostacyclin or its analogs into atherosclerotic patients or to stimulate generation of endogenous prostacyclin (Gryglewski 1979). In this respect angiotensin II ( Gryglewski et al.1979, Silberbauer et al.1979 b), bradykinin (Gryglewski et al.1979), cholinergic stimulators ( our unpublished data), BAY g 6575

(Chamone et al.1979), Seuter et al.1979) and almitrine ( Gryglewski 1980) were claimed to trigger production of the hormone. Apart from atherosclerosis, generation of prostacyclin is also suppressed in diabetic patients ( Silberbauer et al. 1979 a). A link that exists between these two diseases is well known. On the other hand, the studies on secretion of prostacyclin in patients with renal ( Remuzzi et al.1977, VanHoff 1979) and hepatic (VanHoff) failure may lead to isolation of an endogenous stimulator of prostacyclin release.

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# PROSTACYCLIN RELEASED BY ANGIOTENSINS FROM LUNGS AND ISOLATED VASCULAR TISSUE

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## INTRODUCTION

The generation of prostaglandins (PGs) within the walls of blood vessels is probably an important mechanism for local modulation of vascular smooth muscle tone. McGiff and coworkers (1970) suggested that renal blood flow is regulated by the interaction between the constrictor action of angiotensin II (AII) and the release of vasodilator PGs which it induces. The release of PGs by bradykinin may also be an important component of the vasodilator and intrarenal actions of this peptide (McGiff et al., 1972). In support of a general role for PGs as modulators or mediators of vascular actions of these peptides, substances resembling PGE have been found in renal venous blood (Aiken & Vane, 1973) and in perfusates of renal, coronary and mesenteric vascular beds (Needleman et al., 1975; Blumberg et al., 1977) following stimulation with AII. Furthermore, AII stimulates generation of an immunoreactive PGE-like substance by human umbilical endothelial cells in culture (Gimbrone & Alexander, 1975). The proposed role of endogenous PGs as modulators of AII activity was further supported by the demonstration that indomethacin and meclofenamate, substances which inhibit biosynthesis of PGs (Vane. 1971), enhanced vascular responses to AII (Aiken & Vane, 1973; Aiken, 1974; Messina et al., 1976; Blumberg et al., 1977).

PGE-like substances in the above studies were identified by bioassay, using gastrointestinal tissues, or chromatographic mobility of the substance on thin layer gels compared with standard $PGE_2$. It is now known that these bioassay tissues do not easily differentiate between $PGE_2$ and prostacyclin ($PGI_2$), or its stable degradation product 6-oxo-$PGF_{1\alpha}$, (Omini et al., 1977). Prostacyclin is too unstable to be detected by chromatography, while the chromatographic mobility of 6-oxo-$PGF_{1\alpha}$ is very similar to $PGE_2$ and $PGF_{2\alpha}$ in most commonly used solvent systems (Cottee et al., 1977). It now seems likely that prostacyclin, and not $PGE_2$, is the predominant metabolite of arachidonic acid synthesized by isolated blood vessels (Moncada & Vane, 1978).

In 1978 Gryglewski and colleagues (1978a,b) suggested that the huge endothelial cell surface of the lungs might constantly release small amounts of prostacyclin into the passing blood. Their demonstration that the levels of prostacyclin in arterial blood were higher than in mixed venous blood of cats was confirmed by Moncada et al. (1978) in rabbits,

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using an antibody for 5,6 dihydro-$PGI_2$, which cross-reacted with prostacyclin. Indeed, prostacyclin is potentially a circulating hormone for, unlike $PGE_2$ and $PGF_{2\alpha}$, it escapes the pulmonary inactivation process and recirculates (Dusting et al., 1978b). Furthermore, arachidonic acid is transformed into prostacyclin as it passes across the lungs in vivo (Dusting et al., 1978a; Mullane et al., 1979). Since AII probably releases prostacyclin from local vascular beds such as the kidney, it seemed important to know whether it also affected release of endogenous prostacyclin in the lungs. In this paper I have reviewed our evidence for regulation of prostacyclin release by angiotensins both in vivo in dogs using bioassay, and in an isolated, perfused vascular preparation of rats, using bioassay and radioimmunoassay. Gryglewski (1979) has recently reviewed similar evidence for release of prostacyclin by angiotensins obtained in cats, and there appear to be some differences between these species.

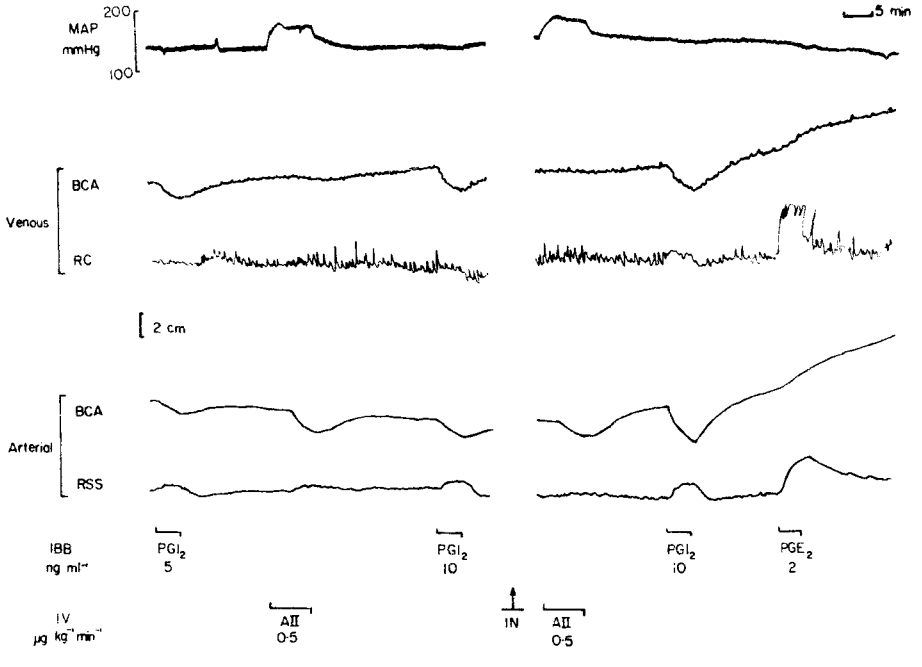


Fig 1. Simultaneous bioassay of prostacyclin ($PGI_2$) in reoxygenated right atrial (venous) blood and arterial blood withdrawn from an anaesthetized dog. Mean arterial pressure (MAP) is shown in addition to recordings from two bovine coronary arteries (BCA), a rat colon (RC) and a rat stomach strip (RSS). $PGI_2$ and $PGE_2$ were infused directly into the bathing blood (IBB). Intravenous (IV) infusion of angiotensin II (AII) caused much greater relaxation of BCA bathed in arterial blood, and little effect on other tissues. After indomethacin (IN, 10 mg/kg) AII still caused BCA relaxation, and the tissue is more sensitive to $PGI_2$.

## PROSTACYCLIN RELEASED FROM LUNGS BY ANGIOTENSINS

We have measured prostacyclin by direct bioassay in blood withdrawn from chloralose-anaesthetized dogs (Vane, 1964) using bovine coronary artery, rat stomach strip and rat colon. The selectivity of these assay tissues for prostacyclin is increased by treating them throughout the experiments with phenoxybenzamine, propranolol and ($Sar^1$-$Ile^8$)-angiotensin II (Mullane et al., 1979), with additional antagonists for muscarinic, 5-hydroxytryptamine, and histamine ($H_1$- and $H_2$-) receptors (Dusting, 1980) Intravenous infusions of AII (0.2-1.0 µg/kg per min) increased systemic and pulmonary arterial pressures, relaxed bovine coronary artery and contracted slightly rat stomach strip, when these tissues were bathed in arterial blood. The effects on the bioassay tissues were matched by prostacyclin (2-20 ng/ml), but not by $PGE_2$ or $PGF_{2\alpha}$ (Fig. 1). None of the following substances caused relaxation of the bovine coronary artery : AI (10 ng/ml), AII (20-200 ng/ml), (des-$Asp^1$) AII (0.1-1 µg/ml), (des-$Asp^1$-$Arg^2$) AII (1-5 µg/ml), adrenaline (5-100 ng/ml), adenosine (100-200 ng/ml) bradykinin (10-200 ng/ml). The $PGI_2$-like substance released by AII disappeared during incubation of arterial blood for 6 min, as does standard prostacyclin (Dusting et al., 1978b). Much less of the $PGI_2$-like substance appeared in blood withdrawn from the right atrium (Fig. 1) or pulmonary artery, indicating that it originates from the lungs. Furthermore, infusion of noradrenaline (2-5 µg/kg per min) did not have any effects on the bioassay tissues, indicating that the release induced by AII was not a consequence of the rise in systemic or pulmonary blood pressure.

AI, infused or injected intravenously (1-3 µg/kg and 1 µg/kg per min) also released a $PGI_2$-like substance into arterial blood (Fig. 2). The

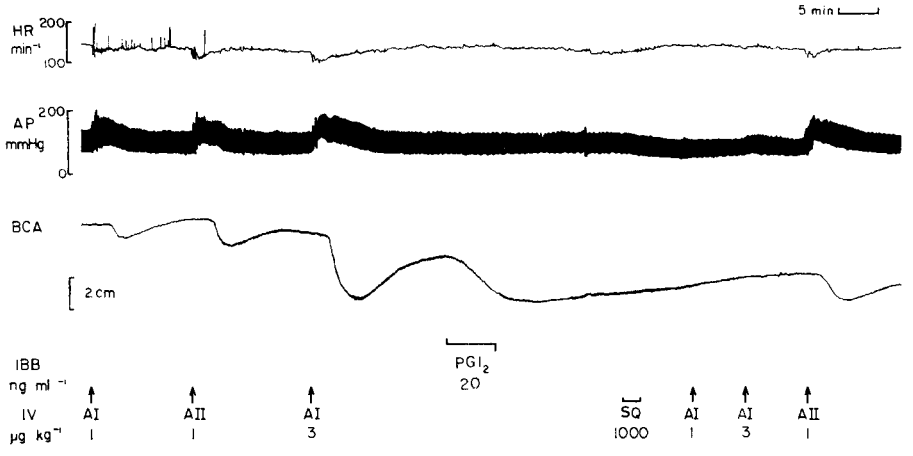


Fig 2. Anaesthetized dog. Release of the $PGI_2$-like substance by angiotensin I (AI) and AII, infused IV at the points indicated beneath the records. Records are heart rate (HR), arterial pressure (AP) and a bovine coronary artery (BCA) bathed in arterial blood. After captopril (SQ, 1 mg/kg) effects of AI were abolished, whereas those of AII were not altered.

effects of AI, but not of AII, were abolished by captopril (1 mg/kg i.v.). Therefore, AI releases prostacyclin only after it is converted in the lung to AII. A similar conclusion was reached by Gryglewski (1979) in his studies using cats. However, in contrast to dogs, intravenous AII also released the $PGI_2$-like substance from other organs (Gryglewski, 1979) since the concentrations detected in right atrial and systemic arterial blood were similar in cats. Gryglewski (1979) suggested kidney and brain may be additional sources of circulating prostacyclin in this species.

In our experiments in dogs, acute treatment with indomethacin (2-10 mg/kg i.v.) aspirin (100 mg/kg) or meclofenamate (2 mg/kg) reduced, but did not eliminate, release of the $PGI_2$-like substance by AII (Fig. 3) and enhanced the pressor response. Moreover, the residual bovine coronary relaxation induced by AII infusion in indomethacin-treated dogs was not further reduced by treating the bioassay tissue with hyoscine (0.1 µg/ml), methysergide (0.2 µg/ml), cimetidine (10 µg/ml) and mepyramine (0.1 µg/ml). AII-induced bovine coronary relaxation was, however, abolished by intravenous infusion of $(Sar^1\text{-}Ile^8)$-angiotensin II (0.5-1.0 µg/kg per min). Therefore, either an unidentified, $PGI_2$-like substance released from the lungs by AII contributes to the bovine coronary relaxation, or the pulmonary site of prostacyclin biosynthesis may be somewhat resistant to inhibition by acute intravenous treatment with non steroidal anti-inflammatory drugs. An analogous finding that acute treatment with indomethacin had only a marginal effect on the low endogenous levels of PGE's and PGF's in renal venous blood of conscious dogs was made by Terragno et al. (1977).

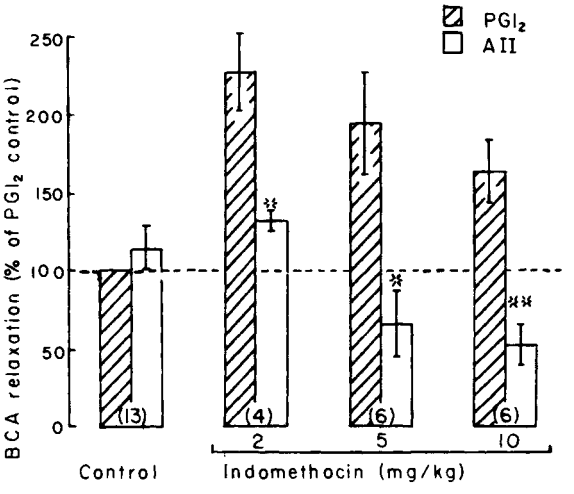


Fig. 3. Results from 13 anaesthetized dogs. Mean responses of bovine coronary artery (BCA) bathed in arterial blood before and after three doses of indomethacin. In each experiment, responses to $PGI_2$ (5-20 ng/ml, infused over the tissues) and those induced by AII (0.2-1.0 µg/kg per min, intravenously) are expressed as percentage of the control response to $PGI_2$ before indomethacin. The bars represent s.e. of the means, and the number in brackets represents the number of dogs at each dose level (3 dogs received two doses of indomethacin). AII causes significantly less BCA relaxation than $PGI_2$ after all doses of indomethacin (by paired t-test, $*P<0.05$, $**P<0.01$).

In summary, AII releases a $PGI_2$-like substance into the circulation mainly from the lungs in dogs, but possibly also from other vascular beds, notably the kidneys and brain in cats. Since the blood flow in cerebral and coronary vascular beds is not reduced by systemic infusion of AII (Bünag, 1974), prostacyclin released from the lungs during powerful activation of the renin-angiotensin system may help preserve blood flow to the heart and brain. Certainly, the systemic pressor response to AII is enhanced by indomethacin, which reduces prostacyclin release. Furthermore, prostacyclin released by AII has an additional important role in protecting the arterial vasculature against deposition of platelet thrombi, particularly in coronary and cerebral vascular beds.

## PROSTACYCLIN RELEASED FROM OTHER VASCULAR BEDS BY ANGIOTENSINS

There is now substantial evidence for the existence of local vascular renin and angiotensin generating systems. Recently, intrarenal infusion of AII has been demonstrated to release a $PGI_2$-like substance from the kidneys in dogs (Shebuski & Aiken, 1980; Mullane et al., 1980) and cats (Gryglewski, 1979). The $PGI_2$-like substance was characterized by bioassay techniques using bovine coronary artery and by in vivo inhibition of platelet clumping in a partially constricted coronary artery (Shebuski & Aiken, 1980). We have also found evidence for conversion of angiotensin and consequent release of prostacyclin in a simple, isolated arterial vascular preparation of the rat mesenteric bed (Dusting & Mullins, 1980; Dusting et al., 1980a). The vasculature was perfused with Krebs' solution via the mesenteric artery (McGregor, 1965), and PGs in the mesenteric effluent were bioassayed on bovine coronary artery, rat stomach strip and rat colon. The bioassay tissues were treated with a mixture of antagonists (Gilmore et al. 1968), ($Sar^1$-$Ile^8$)-AII and indomethacin to increase the sensitivity and specificity of the assay and to prevent direct actions of angiotensins. Aliquots of the mesenteric effluent were also collected for radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ and $PGE_2$. Injection of arachidonic acid (1-10 µg), AI (0.05-2 µg) or AII (0.025-1 µg) through the mesentery was followed in each case by release of a $PGI_2$-like substance (Fig. 4). 6-oxo-$PGF_{1\alpha}$ levels in the mesenteric effluent increased by about 4 ng/ml following stimulation with AII (0.025 or 0.5 µg), whereas $PGE_2$ increased by less than 1.5 ng/ml in the same experiments (Dusting et al., 1980a). Cross reactivity of $PGE_2$ with the 6-oxo-$PGE_{1\alpha}$ antibody was 0.1%, and 6-oxo-$PGE_{1\alpha}$ cross reactivity with $PGE_2$ antibody was 0.3%. Full details of the radioimmunoassay will be published elsewhere (Nolan, Dusting & Martin). Infusion of indomethacin (1 µg/ml) through the mesenteric vasculature abolished the effects of AI, AII and arachidonic acid on the bioassay tissues.

## MECHANISM OF ANGIOTENSIN-INDUCED RELEASE OF PROSTACYCLIN

In the rat isolated mesentery preparation, AI and AII, in doses which released prostacyclin, did not increase perfusion pressure. Noradrenaline (0.05-0.5 µg) sharply increased perfusion pressure in this preparation, but did not release the $PGI_2$-like substance (Dusting & Mullins, 1980). Therefore, AII-induced release of prostacyclin is unlikely to be a direct consequence of the mechanical effects of vasoconstriction. Prostacyclin release induced by AI and AII was however, abolished by ($Sar^1$-$Ala^8$)-AII, (0.03 µg/ml) indicating that stimulation of prostacyclin biosynthesis involves activation of the angiotensin receptor. Prostacyclin release induced by AII was also abolished by mepacrine (Fig. 4) and was inhibited after perfusion for 1-3 h with dexamethasone (Dusting et al., 1980a).

These treatments did not affect conversion of exogenous arachidonate into prostacyclin (Fig. 4). Mepacrine and dexamethasone have been shown to inhibit phospholipase $A_2$ in guinea pig lungs (Vargaftig & Dao Hai, 1972; Blackwell et al., 1978). Therefore, it appears that AII stimulates biosynthesis of prostacyclin in vascular endothelium by activating an AII receptor linked to a phospholipase.

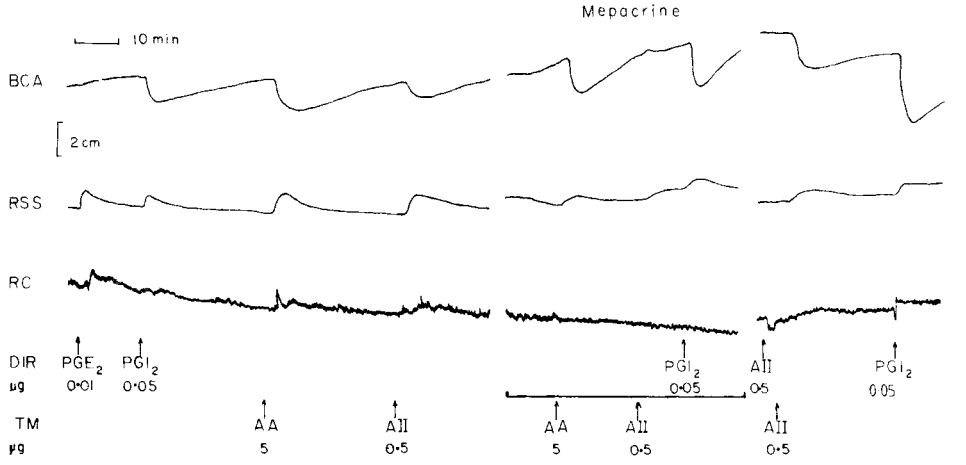


Fig. 4. Bioassay of $PGI_2$ in the effluent of rat isolated, perfused mesenteric vasculature. Arachidonic acid (AA), and AII are injected through the mesentery (TM) in the doses indicated. Bovine coronary artery (BCA), rat stomach strip (RSS), and rat colon (RC) are superfused with the mesenteric effluent containing indomethacin and antagonists (see text). $PGI_2$ and $PGE_2$ are injected directly (DIR) over the tissues. In the centre panel mepacrine (33 µg/ml) is perfused through the mesentery, and $PGI_2$ release induced by AII is inhibited, whereas AA conversion is not altered. The effects of mepacrine are reversed within 30 min after terminating the infusion, as indicated in the right hand panel.

The incubation of AII (0.2-0.5 µg/ml) with extravasated blood from dogs did not induce biosynthesis of prostacyclin, thromboxane $A_2$ or stable PGs (Dusting, 1980), although arachidonic acid is readily converted to thromboxane $A_2$ and stable PGs in this system (Mullane et al., 1979). Platelets are probably the major source of cyclo-oxygenase in blood, and it has been suggested that its substrate in platelets is derived from coupled actions of an inositol-specific phospholipase C and diglyceride lipase (Rittenhouse - Simmons, 1979; Bell et al., 1979). Therefore, while AII probably activates phospholipase $A_2$ in vascular tissues, and perhaps also in non-vascular pulmonary tissues, it does not appear to activate phospholipase C or diglyceride lipase in platelets.

## ANGIOTENSIN CONVERSION AND PROSTACYCLIN RELEASE

Both AI and AII release prostacyclin from the lungs *in vivo* and from isolated mesenteric vasculature. The relationship between angiotensin

conversion and prostacyclin release was examined more closely in the mesenteric vasculature preparation. Prostacyclin was assayed as before using bovine coronary artery and rat stomach strip, and AII in the mesenteric effluent was assayed simultaneously using rabbit aorta treated with captopril (1 µg/ml), to inhibit intramural conversion of AI. Under these conditions, AI (0.1-10 µg) had no direct effect on the rabbit aorta, but caused contraction when injected through the mesentery, indicating between 3 and 8% conversion of the AI in the perfusion fluid. However, AI appeared to be one fifth as potent as AII in releasing prostacyclin (Dusting et al., 1980b). Indeed, when contractions of rabbit aorta produced by AII were matched by an appropriate amount of AI, the latter caused significantly greater relaxation of the bovine coronary artery than the former, indicating greater release of prostacyclin by the decapeptide. Therefore, either AI has some additional direct action in releasing prostacyclin or local conversion of AI to AII in the vasculature is a more effective

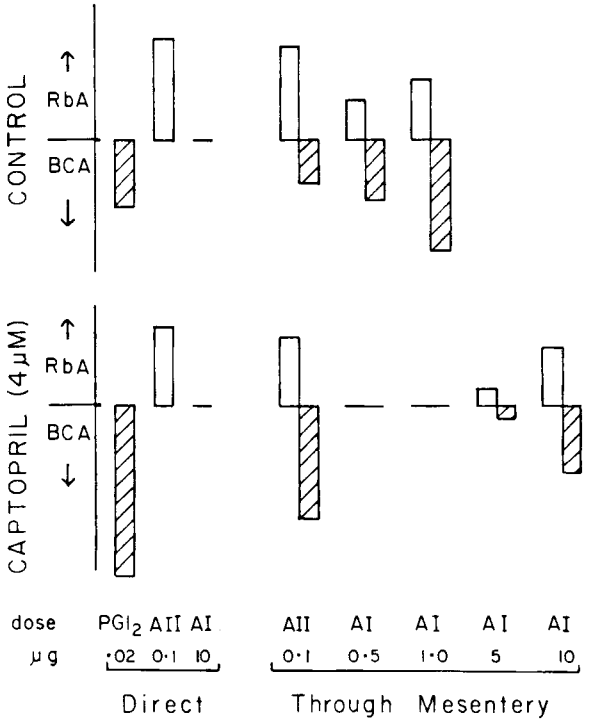


Fig. 5. Rat mesenteric vasculature. Diagrammatic representation of responses of rabbit aorta (RbA, open columns) and bovine coronary artery (BCA, hatched columns) before (upper panel) and during (lower panel) perfusion of the mesenteric vasculature with captopril (1 µg/ml). The responses are measured from a typical record, but the order of doses is changed for clarity. The doses represented are shown beneath the columns. Contractions of RbA produced by low doses of AI, indicating partial conversion to AII, are abolished during treatment with captopril, as are relaxations of BCA, which indicate $PGI_2$ release. AII-induced effects are not altered by captopril.

stimulus for prostacyclin release than AII in the perfusion fluid. The latter explanation appears more likely because captopril inhibited conversion of AI to AII in the mesentery to prevented release of prostacyclin by low doses of AI (0.2-1.0 µg, Fig. 5). Higher doses of AI (2-10 µg) did result in some prostacyclin release despite the presence of the inhibitor, but this was always accompanied by contraction of rabbit aorta (Fig. 5), indicating residual conversion of AI and AII. Therefore, stimulation of prostacyclin biosynthesis by AI in lungs and mesenteric vasculature results from its conversion to AII.

We could not examine vascular reactivity in the mesenteric vasculature since the smooth muscle appears to be insensitive to angiotensins, even in the presence of indomethacin. However, there is evidence in this vascular bed and others that angiotensins and PGs may have local regulatory roles. The mesenteric vasculature is capable of generating AI from the precursor tetradecapeptide (Malik & Nasjletti, 1976), and it can convert AI to AII. We have now shown it can subsequently synthesize prostacyclin and possibly $PGE_2$. It is difficult to assess the concentration of AII reaching the receptor sites on vascular smooth muscle and those necessary for activation of prostacyclin biosynthesis, but the site of enzymatic conversion of AI to AII may be closely juxtaposed to, and perhaps even coupled to, the enzymes of the prostacyclin biosynthetic pathway. Indeed the biosynthesis of prostacyclin in the vascular wall occurs mainly in the endothelium (Moncada et al., 1977), and fluorescein-labelled antibodies for converting enzyme have shown it is localized in the luminal surface of vascular endothelium (Caldwell et al., 1976). Therefore, local concentrations of AII at the site for prostacyclin biosynthesis may be higher than at the receptor sites in vascular smooth muscle, and provide an ideal setting for modulation of AII vasoconstrictor activity by vasodilator prostacyclin. Furthermore, prostacyclin released from the endothelial surface has an additional important role in preventing total obstruction of capillary blood flow by platelet emboli, under conditions where it may already be compromised by angiotensin-induced arteriolar constriction.

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# EFFECT OF PROSTACYCLIN ON THE SYMPATHETICALLY-MEDIATED NOCICEPTIVE CARDIOVASCULAR REFLEX

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Bradykinin when applied to the epicardium of the left ventricle of anaesthetized dogs stimulates cardiac receptors with nonmedullated afferents in the cardiac sympathetic nerves (Uchida & Murao, 1974) and elicits an excitatory chemoreflex which includes a rise in blood pressure, tachycardia, renal vasoconstriction and muscular vasodilatation (Staszewska-Barczak et al., 1976). The pressor effects and tachycardia are mainly due to an increased efferent sympathetic discharge to the heart and blood vessels, since these effects are little affected by vagotomy but are selectively suppressed by $\alpha$- and $\beta$-adrenoreceptor blockade with phenoxybenzamine and propranolol, respectively (Staszewska-Barczak & Dusting, 1977). This sympathetic chemoreflex induced by bradykinin is potentiated by concomitant topical application of prostaglandin $E_1$ or $E_2$ to the heart and reduced after inhibition of prostaglandin biosynthesis with indomethacin. On the basis of these and other observations, we proposed that bradykinin and prostaglandins when released locally by the ischaemic heart can act in concert to stimulate sensory receptors that signal the pain of ischaemia and mediate the sympathetic reflex mechanims responsible for the cardiovascular events accompanying anginal attack (Staszewska-Barczak et al., 1976).

There is now increasing evidence indicating that some of the biological effects attributed previously to classical prostaglandins may in fact be mediated by more recently discovered unstable substances, prostacyclin ($PGI_2$) and thromboxane ($TxA_2$) (Hamberg et al., 1975 ; Moncada & Vane, 1977, 1978). Prostacyclin is the principal product of arachidonic acid metabolism in the vascular endothelium (Gryglewski et al., 1976). It has also been identified as the major arachidonate derivative formed in the pericardium (Herman et al., 1979) and cardiac myocytes (Vahouny et al., 1979) and released from the heart in several species (DeDekere et al., 1977; Schrör et al., 1978). Although the principal effects of prostacyclin are to cause vasodilatation and inhibit platelet aggregation (Moncada & Vane, 1978), it is also known to have a role in nociception (Ferreira et al., 1978; Higgs et al., 1978) and to activate cardiac vagal receptors resulting in bradycardia (Chapple et al., 1980; Hintze et al., 1979). The current study describes the action of prostacyclin on the sympathetic cardiac chemoreflex and compares it with the effects produced by prostaglandin $E_2$. The findings regarding the effect of prostacyclin on vagally-mediated depressor cardiac chemoreflex are also reported.

ACTIONS OF PROSTACYCLIN ON BRADYKININ-INDUCED SYMPATHETIC CHEMOREFLEX

Prostacyclin can exert its action on the heart when released locally into the coronary blood stream from vascular endothelium (Dusting et al., 1977), and also when released from extravascular tissues, such as cardiac myocytes (Vahouny et al., 1979) or pericardial (Herman et al., 1979), and presumably also epicardial, mesothelium. Since prostacyclin is a circulating hormone (Dusting et al., 1978b) it can also reach the heart when released into the blood stream from the endothelial cells in the peripheral and pulmonary vascular beds (Gryglewski et al., 1978; Moncada et al., 1978). All these possibilities were taken into account while examining the action of prostacyclin on the sympathetic and vagally-mediated chemoreflexes elicited from the heart with bradykinin or nicotine.

Effects of topical applications of prostacyclin and prostaglandin $E_2$ to the heart

In chloralose-anaesthetized, artificially ventilated, open-chest dogs, topical applications to the left ventricular epicardium of bradykinin in doses of 0.01, 0.1 or 1 µg elicited dose-dependent reflex rises in mean arterial pressure and heart rate (Fig. 1A, first panel). With prolonged application at a rate of 1 µg/min for a period of 8 min, the pressor effects and tachycardia were usually maintained although at a lower level than the initial response (Fig. 2, first panel). Treatment with indomethacin (2-5 mg/kg, i.v.) reduced the pressor responses and sometimes the reflex tachycardia produced by epicardial bradykinin (Fig. 1A). The magnitude of the pressor effects produced by 0.01 µg of bradykinin was reduced from 10.3 ± 1.2 mmHg to 6.8 ± 0.8 mmHg (mean ± SEM, n = 3), i.e., by 34 percent, and that of 0.1 and 1 µg bradykinin from 12.7 ± 0.9 to 7.1 ± 0.1 mmHg (mean ± SEM, n = 9) and from 19.3 ± 2.7 to 9.8 ± 1.3 mmHg (mean ± SEM, n = 6), i.e., by 46 and 48 percent, respectively.

Prostacyclin (0.1-0.3 µg/min) or prostaglandin $E_2$ (0.1-0.3 µg/min), when dripped as solutions on to the epicardium of the left ventricle had no reflexogenic effects by themselves, but increased on the average by 50.8 percent (9.1 SEM, n = 12) and by 35.7 percent (8.8 SEM, n = 9), respectively, the magnitude of reflex pressor effects induced by epicardial application of 0.01-1 µg of bradykinin. In dogs in which pressor responses to bradykinin had been reduced by indomethacin, the potentiating effects of both prostaglandins were much greater; prostacyclin increased the responses by a mean of 107 percent (27 SEM, n = 11) and prostaglandin $E_2$ by 119 percent (37 SEM, n = 7). With prostacyclin, the potentiating effect was quick in onset (within 60-90 s of application), but short-lived; it disappeared as soon as prostacyclin was removed from the heart (Fig.1A). In contrast, the potentiating effect of prostaglandin $E_2$ was usually slower in onset but longer lasting; increased pressor responses to bradykinin persisted for up to 30 min after removal of prostaglandin $E_2$ from the heart (Fig. 1B).

In experiments, where bradykinin was dripped on to the heart at a rate of 1 µg/min, the pressor response and tachycardia gradually declined to a level 50-60 percent lower than the initial response. This decline in responses could be reversed when prostacyclin (0.3 µg/min) or prostaglandin $E_2$ (0.3 µg/min) were dripped onto the heart during the last 3 min of bradykinin stimulation. The prostacyclin metabolite, 6-oxo-$PGF_{1\alpha}$ when dripped on to the heart at a rate of 1 µg/min was ineffective in

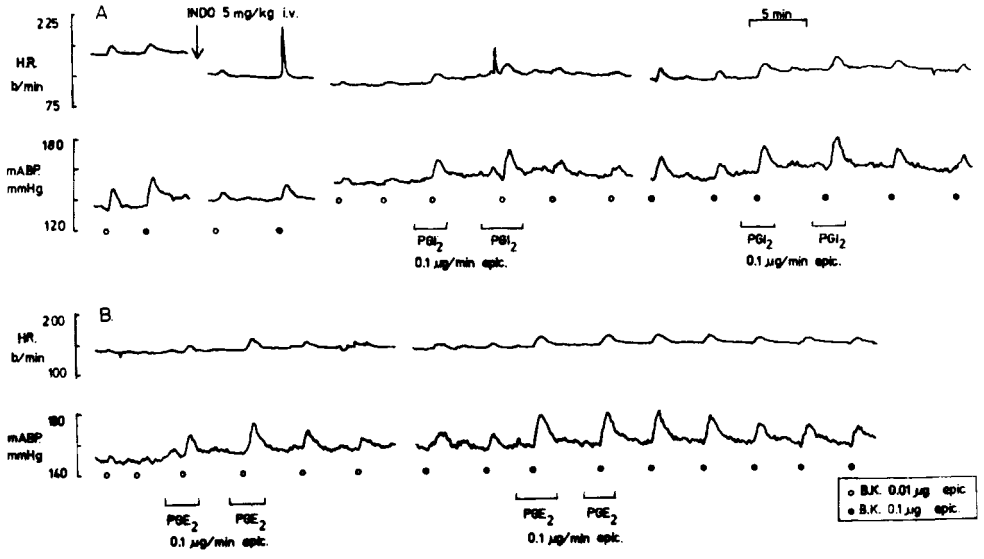


Fig. 1. The reflex pressor effects and tachycardia induced by epicardial bradykinin are inhibited by indomethacin and potentiated by concomitant epicardial applications of prostacyclin or prostaglandin $E_2$. Tracings of the heart rate (HR) and mean arterial blood pressure (mABP) of the dog show in Panel A: dose-dependent pressor effects and tachycardia produced by two doses (0.01 (o) and 0.1 (•) µg)) of bradykinin given to the left ventricular epicardium (epic); these responses are reduced after indomethacin (INDO, 5 mg/kg, i.v.) and are subsequently transiently restored during superfusion of the epicardium by prostacyclin ($PGI_2$) at a rate of 0.1 µg/min for 3 min.

Panel B shows that the heart rate and blood pressure responses to epicardial bradykinin are also restored by superfusing the heart surface with prostaglandin $E_2$ ($PGE_2$) at a rate of 0.1 µg/min for 3 min periods. Note, that with prostacyclin the responses were greater only during superfusion, whereas with $PGE_2$ potentiation was long-lasting. Vertical scales: beats/min for HR and mmHg for mABP; time scale: 5 min.

restoring the reflex pressor and heart rate responses to bradykinin superfusion (Fig. 2).

Effects of intra-coronary administration of prostacyclin and prostaglandin $E_2$

When infused into the anterior descending coronary artery in doses which were without systemic effects, prostaglandin $E_2$ (0.1-0.3 µg/min) again increased by a mean of 31 percent the reflex pressor effects of epicardial bradykinin. In contrast, intra-coronary infusion of prostacyclin (0.1-0.3 µg/min) was associated with nearly 30 percent reduction

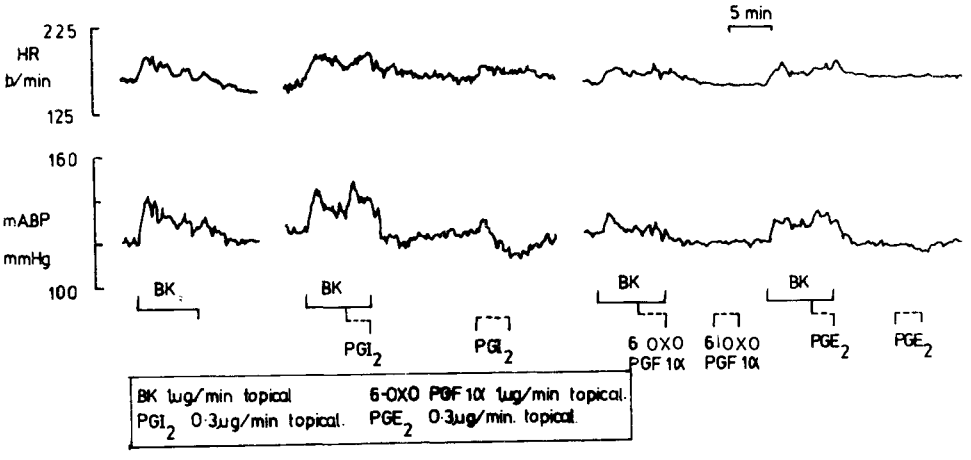


Fig. 2. Potentiation of the reflex pressor responses and tachycardia to epicardial superfusion of bradykinin (BK) by prostacyclin ($PGI_2$) and prostaglandin $E_2$ ($PGE_2$). The records of heart rate (HR) and mean arterial pressure (mABP) from the dog show that BK when dripped onto the surface of the left ventricle at a rate of 1 μg/min for 8 min, elicits elevations in the heart rate and blood pressure that become less pronounced with prolonged stimulation. This decrease in responses is reversed when $PGI_1$ or $PGE_2$ are applied topically at rates of 0.3 μg/min to the same area of the left ventricle during the last 3 min of bradykinin stimulation. 6-Oxo-$PGF_{1\alpha}$ applied in the same manner at a rate of 1 μg/min is without effect. The tracing also shows that neither $PGI_2$ , $PGE_2$ nor 6-oxo-$PGF_{1\alpha}$ elicit reflexogenic effects when applied to the heart in the absence of bradykinin. Time and vertical scales as in Fig. 1.

in the magnitude of bradykinin-induced pressor effects. A comparison of the effect produced by epicardial and intracoronary applications of prostacyclin and $PGE_2$ on bradykinin-induced sympathetic chemoreflex is shown in Fig. 3.

Effect of intravenous administration of prostacyclin

In several experiments, prostacyclin was infused intravenously at a rate of 10-20 ng/kg per min. At these rates of infusion the mean arterial pressure was either unchanged, or fell at most by 5-10 mmHg. The reflex pressor responses to epicardial applications of 0.1 and 1 μg of bradykinin were either not changed, or slightly reduced.

ACTIONS OF PROSTACYCLIN ON NICOTINE-INDUCED VAGALLY-MEDIATED CHEMOREFLEX

Nicotine (10 μg) when applied to the left ventricular epicardium, caused a prompt fall in mean arterial pressure by a mean of 20.6 mmHg (2.7 SEM, n = 8) and a reduction in heart rate by 5.8 beats/min (mean ±

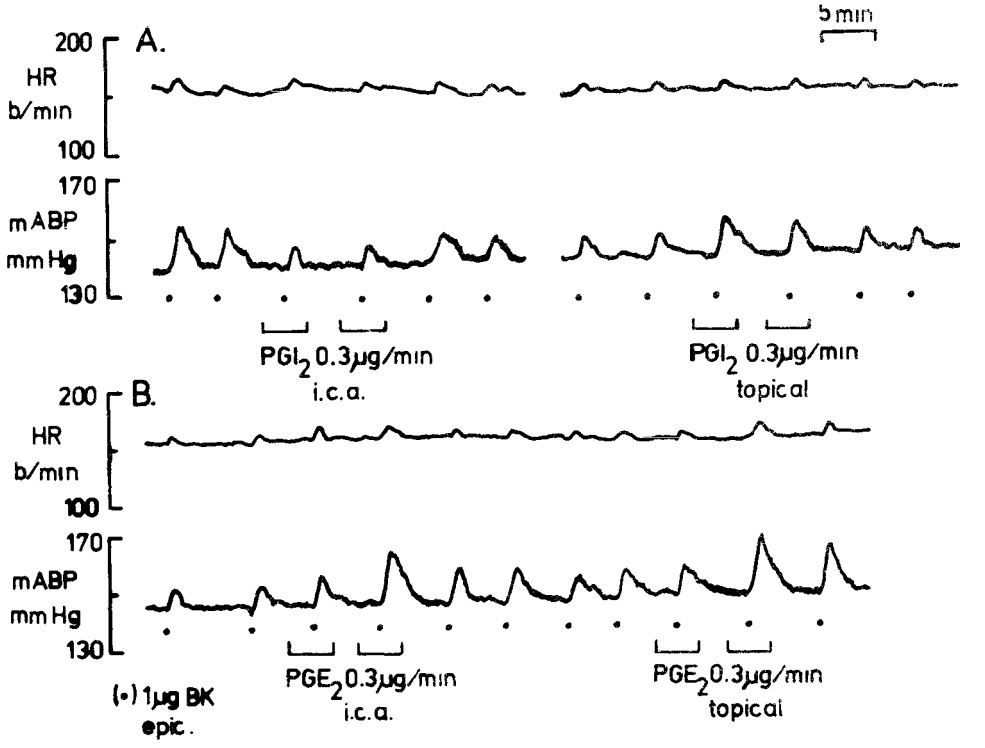


Fig. 3. Prostacyclin ($PGI_2$) when infused into the coronary artery (i.c.a.) of the dog's heart reduces, but when applied topically to the left ventricle, potentiates the reflex pressor responses to epicardial bradykinin (1 µg) (Panel A). Prostaglandin $E_2$ ($PGE_2$) enhances bradykinin-induced pressor effects during both intracoronary and topical application (Panel B). Time and vertical scales as in Fig. 1).

2.4 SEM, n = 6). These depressor effects of nicotine were potentiated on the average by 27 percent during intravenous infusion of prostacyclin (10-20 ng/kg per min) and by 32 percent during intracoronary infusion of 0.3 µg/min of prostacyclin. With epicardial application of prostacyclin the results were rather inconsistent: out of 7 experiments in which this effect was tested, potentiation by about 30 percent occurred in 3, no change in 2, and a reduction of depressor response to nicotine in 1.

## DISCUSSION AND CONCLUSIONS

In the present study, prostacyclin when applied in low doses to the left ventricular epicardium had no reflexogenic activity by itself, but increased on the average by 50 percent the magnitude of the reflex pressor effects elicited from the heart with bradykinin. This potentiating effect was quick in onset but short-lasting. In this respect, the action of prostacyclin differed from that of prostaglandin $E_2$, whose potentiating effects were relatively slow in onset but prolonged. Since the sympathetic

chemoreflex activated by bradykinin is nociceptive in nature (Uchida & Murao, 1974; Staszewska-Barczak et al., 1976), our results suggest that prostacyclin when formed in, and released from the extravascular tissues within or close to the heart, e.g., from cardiac muscle cells (Vahouny et al., 1979) and epicardial or pericardial mesothelium (Herman et al., 1979) can act to sensitize the sympathetic chemosensitive afferents to bradykinin and thereby contribute to the onset of pain and reflex hypertension during anginal attacks and possibly also during pericarditis. That prostacyclin causes marked but short-lasting hyperalgesia had been previously demonstrated in the rat paw and the dog's knee joint with modified Randall-Selitto technique (Ferreira et al., 1978; Higgs et al., 1978).

Coronary vessels convert arachidonic acid mainly into prostacyclin (Dusting et al., 1977). When released into the coronary blood stream, prostacyclin may cause vasodilatation (Dusting et al., 1978a) and prevent platelet aggregation (Aiken et al., 1979; Gryglewski et al., 1976), and also to reduce sympathetic discharge to the heart (Khan & Malik, 1980). Recently, Hintze et al. (1979), Dusting et al. (1978a) and Chapple et al. (1980) have reported that large doses of prostacyclin (up to 30 µg or 1 µg/kg) when administered into the left heart chambers, or by intravenous, intra-arterial pulmonary or intracoronary routes, produce hypotension that is often accompanied by bradycardia. In contrast, hypotension induced by administration of large doses of $PGE_1$ or $PGE_2$ (50 µg) is closely followed by a baroreceptor-mediated increase in heart rate. Since vagotomy reverses prostacyclin-induced bradycardia and reduces its depressor effects, it has been proposed that slowing of the heart rate and at least part of the hypotensive effect are due to prostacyclin-induced activation of vagally-mediated reflex mechanisms (Chapple et al., 1980; Hintze et al., 1979). It is highly unlikely, however, that concentrations of prostacyclin needed to elicit the depressor vagal chemoreflex may be present in the circulation under any physiological or pathological conditions, or even during therapeutic use of prostacyclin. Our findings that sub-depressor doses of prostacyclin when administered into the coronary or systemic circulation potentiate the reflex depressor effects of nicotine suggest that the physiological role of circulating prostacyclin is to sensitize vagal receptors in the heart to chemical and probably also mechanical stimulation and thereby to facilitate the reflex depressor mechanisms. A predominance of vagal sensory input over the sympathetic afferent input into the central nervous system may explain why prostacyclin reduced, while $PGE_2$ which has no vagal effects (Hintze et al., 1979), potentiated the reflex pressor effects to bradykinin during intracoronary administration.

In conclusion, the results suggest that prostacyclin when released from the extravascular tissues within or close to the heart can act to sensitize sympathetic chemosensitive nerve endings to bradykinin and thereby contribute to the signalling of pain and initiation of the reflex hypertension and tachycardia that may accompany anginal attacks. However, when released into the coronary or systemic circulation, prostacyclin can act to sensitize vagal receptors in the heart to chemical and possibly also mechanical stimulation and hence facilitate the reflex depressor mechanisms. This action of circulating prostacyclin, in addition to its direct vasodilator and anti-aggregatory effects, may be of special importance in ischaemic heart disease, since the predominance of vagal over sympathetically mediated reflex activity would result in reduction of workload and oxygen demand by the heart.

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# ROLE OF PROSTAGLANDINS IN THE REGULATION OF BLOOD PRESSURE AND BLOOD FLOW

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Prostaglandins (PG) generated locally within blood vessels have been shown to act directly by affecting the function of vascular smooth muscle (Vane and McGiff, 1975) and indirectly by modifying the responsiveness of small blood vessels to a variety of vasoactive agents (Messina et al., 1975). Recently it was also demonstrated that some of the prostaglandins have the ability to stimulate cardiopulmonary receptors with vagal afferents to produce a reflex vasodepressor response, accompanied by bradycardia (Hintze et al., 1979a; Chapple et al., 1980). This paper presents some of the recent findings that relate to the local as well as the reflex cardiovascular actions of prostaglandins.

## MICROCIRCULATORY STUDIES WITH PROSTAGLANDINS

Microcirculatory studies were performed in the exteriorized rat cremaster muscle preparation (Baez, 1973) to study the vasomotor effects of arachidonic acid (AA), the precursor of the bisenoic prostaglandins and $PGH_2$, and to compare their actions to two of the major prostaglandins synthesized within blood vessels, namely prostacyclin ($PGI_2$) and $PGE_2$.

All four substances were applied topically to the cremaster muscle in 100 µl aliquots in concentrations ranging from $0.5X10^{-10}$ to $0.5X10^{-3}$M. Dose-dependent, arteriolar dilator responses were obtained with AA, $PGH_2$, $PGI_2$ and $PGE_2$. However, the threshold responses and the $ED_{50}$ for the agents did vary (Figure 1). No vasoconstrictor responses were observed in response to any of the agents. The $ED_{50}$ for AA was $1.0X10^{-5}$M; for $PGH_2$ and $PGI_2$ $0.8X10^{-6}$M and for $PGE_2$ $0.5X10^{-6}$M. Responses to $PGE_2$ and $PGI_2$ were similar up to a concentration of $0.5X10^{-5}$M; however, above this dose dilator responses to $PGI_2$ were significantly less than those to $PGE_2$. In regard to threshold doses, approximately ten times more $PGH_2$ and 100 times more AA had to be administered to obtain responses equivalent to $PGI_2$ and $PGE_2$. Since AA has no vasoactivity of its own it has to be converted to prostaglandins to cause local vasodilation. From these data it cannot be ascertained which of the prostaglandins ($PGE_2$ or $PGI_2$) is produced. The cremasteric microvascular compartment nevertheless has the enzymes necessary to convert AA to vasodilator metabolites.

The direct vasodilator activity of prostaglandins is not the sole microcirculatory action of these compounds, as was earlier demonstrated for $PGE_1$ in the rat mesentery and cremaster muscle (Messina et al., 1974). In

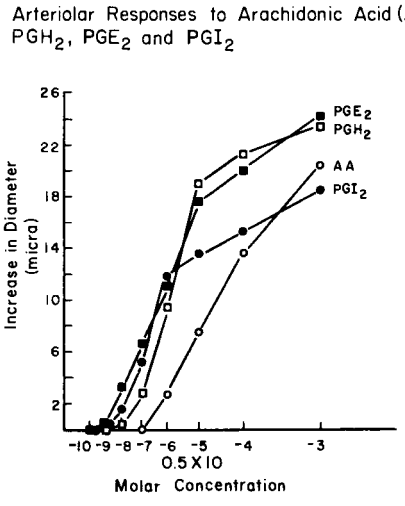


Figure 1. Arachidonic Acid and its metabolites cause dose-related increases in the diameters of rat cremaster muscle arterioles.

that study it was shown that $PGE_1$ inhibits arteriolar constrictor responses to catecholamines, angiotensin and vasopressin, independent of its own vasodilator action. In the present study we have assessed the ability of $PGI_2$ and $PGE_2$ to alter arteriolar reactivity to norepinephrine (NE) in the rat cremaster preparation. Arteriolar responses to topical application of either 5 or 10 ng of NE were tested before and after topical administration of $0.5X10^{-4}M$ of either $PGI_2$ or $PGE_2$. After the dilator response to $PGE_2$ or $PGI_2$ had subsided responses to NE were retested at five minute intervals. Figure 2 is a record of an actual experiment depicting the protocol followed and the results obtained with NE before and after $PGE_2$.

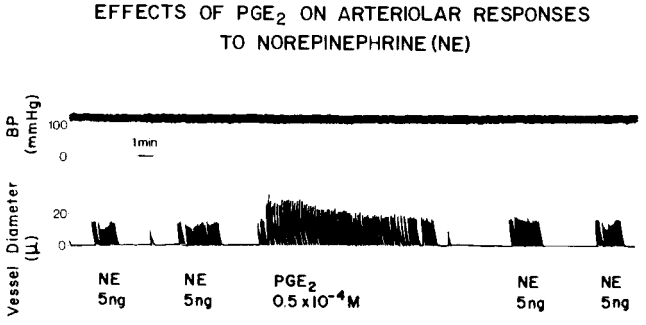


Figure 2. The top panel is the systemic arterial blood pressure of the rat; the bottom panel is the actual arteriolar diameter as measured by image-shearing. An increase in the height of the record reflects dilation; a decrease, return to control or constriction. Five minutes after the application of $PGE_2$ the vasoconstrictor response to NE is still repressed.

Table 1 summarizes the data obtained for both $PGI_2$ and $PGE_2$. Prostacyclin did not alter the arteriolar responses to either dose of NE. In contrast $PGE_2$ inhibited the constrictor response to both 5 and 10 ng of NE. Apparently $PGI_2$ is unlike either $PGE_1$ or $PGE_2$ in that it does not reduce vascular reactivity to catecholamines. The reasons for or the implications of these differences are at the present time unknown.

Table 1. Effects of $PGI_2$ and $PGE_2$ on arteriolar constrictor responses to norepinephrine (NE).

| PGs $0.5X10^{-4}M$ | NE Dose | Control Diameter (Micra) | Decrease in Diameter Before PGs | Decrease in Diameter After PGs 5min | 10min |
|---|---|---|---|---|---|
| $PGI_2$ | 5ng (9) | 16.0 ± 1.2 | 7.2 ± 0.8 | 6.5 ± 0.7 | 6.9 ± 0.7 |
| $PGI_2$ | 10ng (10) | 16.0 ± 0.9 | 10.7 ± 1.0 | 10.3 ± 1.0 | 11.0 ± 0.7 (8) |
| $PGE_2$ | 5ng (7) | 16.4 ± 1.6 | 5.1 ± 0.7 | 1.2 ± 0.7* | 5.3 ± 1.1 |
| $PGE_2$ | 10ng (8) | 15.3 ± 1.0 | 11.4 ± 0.9 | 4.8 ± 1.7** | 10.2 ± 1.3 |

Values are means ± standard error. *$P < .005$; **$P < .01$ comparing responses before and after $PGE_2$. Numbers in parentheses are numbers of animals used in the study.

REFLEX CARDIOVASCULAR EFFECTS OF PROSTAGLANDINS

In previous studies we have demonstrated that, in contrast to other vasodepressor agents, when $PGI_2$ or AA is injected into the cardiopulmonary region (but not into the carotid artery or the aorta) of both open and closed-chest anesthetized dogs, the fall in blood pressure is followed by a reduction in heart rate (Hintze et al., 1979a). Vagal section or atropine eliminated the bradycardia, thus establishing the reflex nature of the response. Since prostaglandins have previously been shown to stimulate chemically sensitive vagal c-fiber endings in the heart (Baker et al., 1979) it is quite likely that the Bezold-Jarisch like, reflex depressor response to $PGI_2$, that we and others (Chapple et al., 1980) observed, is mediated by ventricular receptors subserved by vagal afferent fibers (Hintze et al., 1979b; Kaley et al., 1980).

The purpose of the present experiments was to investigate further the reflex cardiovascular responses that follow intravenous infusions of $PGI_2$ into anesthetized and conscious dogs and to compare the effects of $PGI_2$ with those of $PGE_2$ and nitroprusside.

Anesthetized dogs.

Male mongrel dogs (N=7) were anesthetized with a mixture of equal amounts of Dial-urethane (Ciba-Geigy) and Nembutal (Abbott). Surgery and preparation of animals followed previously established procedures (Hintze et al., 1979a). Intravenous infusion of $PGI_2$ produced the expected fall in blood pressure that was followed, in some dogs, by a reduction in

heart rate (Figure 3). In spite of the acute drop in both systolic and

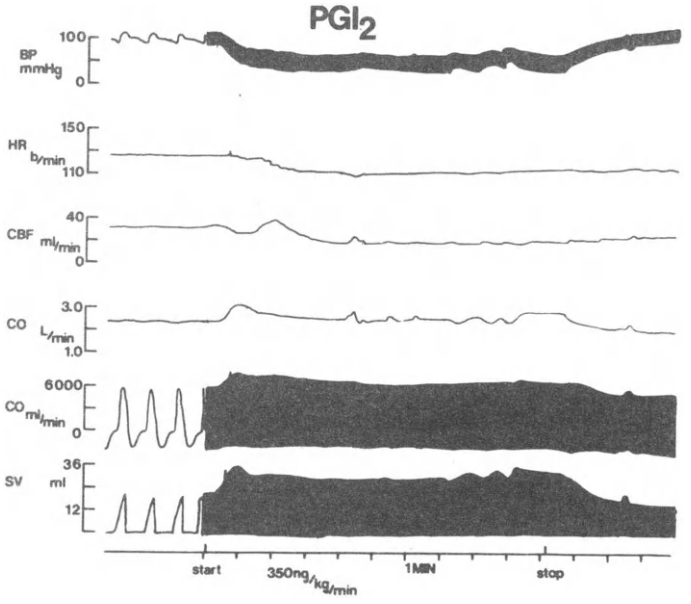


Figure 3. Actual record of an anesthetized, open-chest dog showing the effects of an intravenous infusion of $PGI_2$. Arterial blood pressure (BP); heart rate (HR); circumflex coronary blood flow (CBF); cardiac output (CO); instantaneous aortic flow (CO ml/min); stroke volume (SV).

diastolic blood pressure, throughout the period of the infusion, no baroreflex induced increase in heart rate appeared. Cardiac output increased, as stroke volume became elevated significantly during the infusion of $PGI_2$. There was a decrease in myocardial blood flow, most likely the result of the hypotension that tends to offset the effect of $PGI_2$ on coronary resistance, through its direct, vasodilator action.

Summary data of the effects of infusions of $PGI_2$ on cardiovascular function in anesthetized dogs are given in Figure 4, to be compared with those of nitroprusside. For a given drop of blood pressure $PGI_2$ induced a much smaller increase in heart rate (or an actual reduction in heart rate) than nitroprusside, indicating a blunting of baroreflex activity, perhaps by stimulation of an antagonistic, vagally-mediated depressor reflex. Infusions of nitroprusside, a drug whose systemic and pulmonary vasodilator activity is similar to that of $PGI_2$, consistently induced the expected baroreflex mediated increase in heart rate. The significantly greater increase in cardiac output (stroke volume) to $PGI_2$ over that to nitroprusside occurs even when these two agents cause similar reductions in afterload and is perhaps related to differential effects on capacitance vessels. Interestingly, as also noted by other investigators (Jentzer et al., 1979), nitroprusside increases coronary blood flow significantly more than $PGI_2$ for given decrements of mean arterial blood pressure.

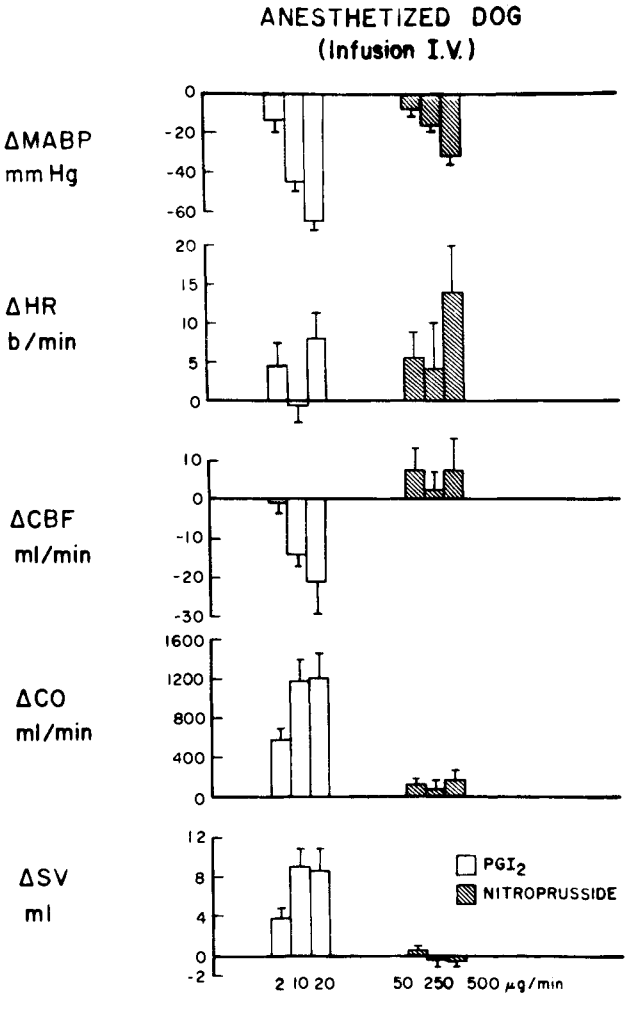


Figure 4. Effects of infusions of $PGI_2$ and nitroprusside on cardiovascular function of anesthetized dogs (N=7). The bars indicate the actual mean changes from control for all functions, as recorded at a steady-state point (8 minutes) during the 10 minute infusion of the drug. Lines are standard errors of the mean. Mean arterial blood pressure (MABP); heart rate (HR); coronary blood flow (CBF); cardiac output (CO); stroke volume (SV).

Conscious dogs.

Resting, conscious dogs, with exteriorized carotid loops (N=5) for the recording of blood pressure and heart rate (Ito and Scher, 1978) were

given infusions of $PGI_2$, $PGE_2$ and nitroprusside (Figure 5). Even at the

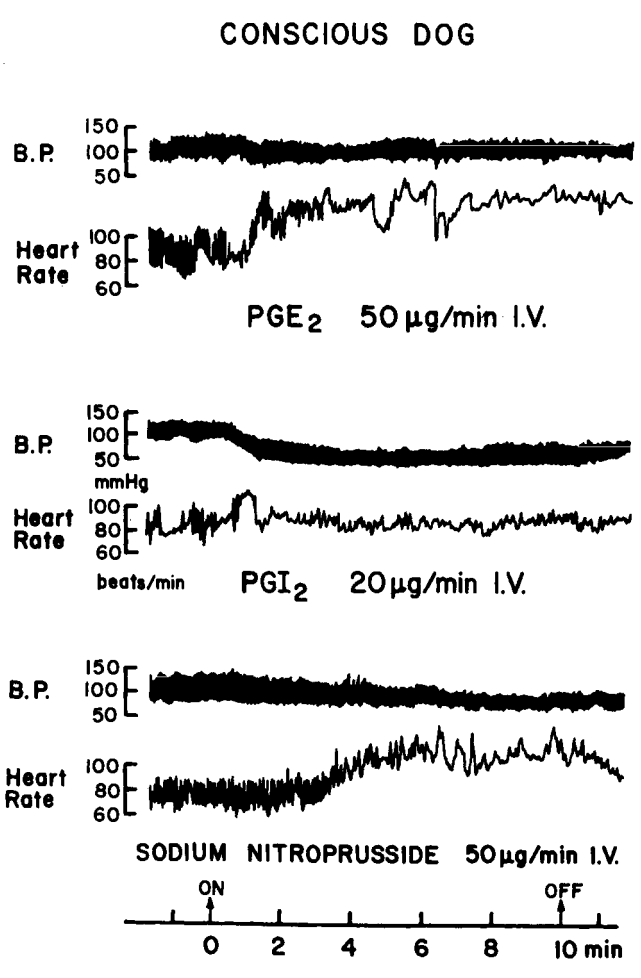


Figure 5. Actual recording showing the effects of 10 minute infusions of $PGE_2$, $PGI_2$ and nitroprusside on arterial blood pressure (BP in mmHg) and heart rate (HR in beats/min) in a conscious dog, trained to lie quietly on the table. Apart from a transient tachycardia there is no significant change in heart rate during the infusion of $PGI_2$, despite the fact that the blood pressure fall to $PGI_2$ is greater than that to $PGE_2$ or nitroprusside.

slow resting heart rate of conscious dogs, the infusion of $PGI_2$ frequently induced bradycardia, or at least significantly tempered the baroreflex mediated increase in heart rate that always occurred following the infu-

sions of the other vasodepressor agents ($PGE_2$ and nitroprusside) that were studied. This implies that $PGI_2$ interferes with normal baroreflex modulation of hypotension.

In other experiments, done in anesthetized dogs, it was also shown that a significant portion of the vasodepressor effect of $PGI_2$ is a result of a reflex reduction in peripheral resistance (Hintze et al., 1979a; Chapple et al., 1980). The specific reflex cardiovascular effect of $PGI_2$, that together with its direct vasodilator effect (vida supra) accounts for the hypotension caused by this agent, may be important in overall cardiovascular regulation. In addition, the reflex bradycardia and hypotension that may follow the ischemia induced release of $PGI_2$ within the myocardium, together with the other multifaceted actions of this agent (Moncada and Vane, 1979), may prove to be an important and beneficial aspect of the response of the myocardium to injury.

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# CARDIOVASCULAR ACTIONS OF TWO THROMBOXANE $A_2$ ANALOGS*

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Thromboxane $A_2$ ($TA_2$), an arachidonic acid metabolite generated from platelets, is reported to be a potent constrictor of blood vessels and aggregator of platelets (1). Recent evidence suggests that it is involved in the pathogenesis of myocardial ischemia, leading to cell death and extension of ischemic myocardial damage (2). However, $TA_2$ with a half-life of only 30 seconds at pH 7.4 and 37°C (3) has been difficult to evaluate in biological preparations due to its extreme instability and rapid metabolic degradation (4, 5) in biological preparations.

Although $TA_2$ has not been isolated or chemically synthesized, Samuelsson et al. (6) have proposed its structure on the basis of its chemical properties. Recently, Nicolaou and coworkers (7, 8) have synthesized two stable analogs of $TA_2$ termed carbocyclic thromboxane $A_2$ ($CTA_2$) and pinane thromboxane $A_2$ ($PTA_2$). Figure 1 illustrates the chemical structures of $TA_2$ and its two synthetic analogs. $PTA_2$ and $CTA_2$ are very close structures to $TA_2$, differing only in the ring substituents. $PTA_2$ is different in that the oxygen atoms in both rings are replaced by carbon atoms and that there are 2 methyl groups present on the bridge carbon in the four-membered ring. $CTA_2$ is different only in substituting carbon atoms for the two oxygen atoms in the ring structures.

Table 1 summarizes the known biochemical and biological properties of $PTA_2$ and $CTA_2$. Neither agent exerts any significant effect on cyclooxygenase or prostacyclin synthetase, thus not altering total arachidonic acid induced formation of endoperoxides (e.g., $PGG_2$ or $PGH_2$) nor the synthesis of prostacyclin ($PGI_2$). However, both $CTA_2$ and $PTA_2$ are potent inhibitors of thromboxane synthetase, and markedly inhibit thromboxane $A_2$ production at 50 μM. Thus, both analogs are selective thromboxane synthetase inhibitors. Neither analog alters cardiac contractility. Moreover, neither analog is an effective inducer of platelet aggregation either in vivo or in vitro up to concentrations of 200 μM. Both analogs effectively inhibit platelet aggregation to arachidonic acid and $PGH_2$ analogs at concentrations of 5 μM.

However, the two analogs are very different with regard to their biological properties. $CTA_2$ acts to labilize (i.e., increase the leaki-

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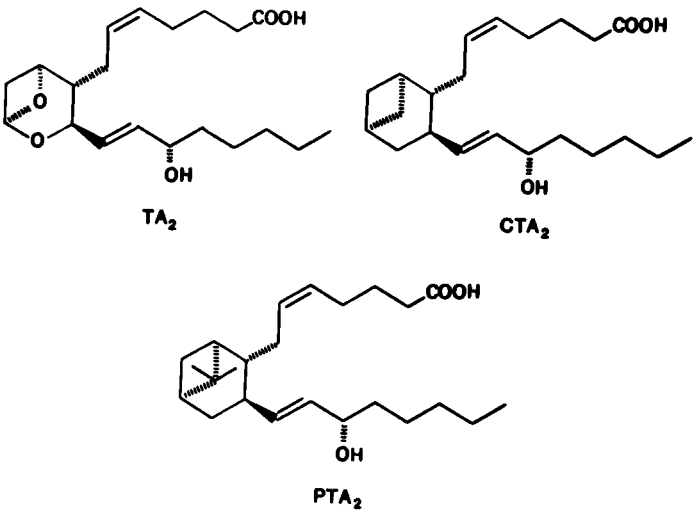


Figure 1. Structural formulas of thromboxane $A_2$ ($TA_2$), carbocyclic thromboxane $A_2$ ($CTA_2$) and pinane thromboxane $A_2$ ($PTA_2$).

ness of) liver and myocardial lysosomes at 200 nM, whereas $PTA_2$ is a lysosomal stabilizer at 1 μM. Moreover, $CTA_2$ is a very effective coronary vasoconstrictor at concentrations as low as 0.2 nM, whereas $PTA_2$ has no effects on coronary vasoactivity up to concentrations of 1 mM. Furthermore, $CTA_2$ does not modulate the coronary vasoactivity of other agents (e.g., angiotensin, $PGH_2$ analogs, norepinephrine), whereas $PTA_2$ markedly antagonizes the coronary vasoconstrictor effects of $CTA_2$ and $PGH_2$ analogs. Thus $PTA_2$ is a thromboxane receptor antagonist as well as being a thromboxane synthetase inhibitor. As such it has potential usefulness as a valuable therapeutic agent. In contrast, $CTA_2$ is a thromboxane agonist on blood vessels without contributing to $TA_2$ release. Moreover, it dissociates the vascular from the platelet actions of thromboxanes, and as such is a useful tool to probe the circulatory actions of thromboxanes.

Table 2 summarizes the pathophysiologic actions of $CTA_2$ as well as the therapeutic effects of $PTA_2$ in a variety of intact animal preparations. In the anesthetized open-chest cat, $PTA_2$ failed to exert any direct effect on nutritive blood flow to heart, lung, splanchnic viscera and skeletal muscle as measured by radiolabelled 15 μm microspheres. In

Table 1. Biochemical and Biological Properties of Stable Thromboxane Analogs

| Properties | Pinane Thromboxane $A_2$ ($PTA_2$) | Carbocyclic Thromboxane $A_2$ ($CTA_2$) |
|---|---|---|
| Direct inotropic effect | None | None |
| Direct coronary vasoactive agent | None | Potent vasoconstrictor |
| Modulation of coronary vasoactivity | Antagonizes endoperoxide and $CTA_2$ induced vasoconstriction | None |
| Direct platelet aggregatory effect | Very weak | Very weak |
| Modulation of platelet aggregation | Antagonizes arachidonic acid and endoperoxide induced aggregation | Antagonizes arachidonic acid and endoperoxide induced aggregation |
| Lysosomal membrane | Stabilizes lysosomal membranes | Labilizes lysosomal membranes |
| Cyclo-oxygenase | No effect | No effect |
| Prostacyclin synthetase | No effect | No effect |
| Thromboxane synthetase | Inhibits | Inhibits |

contrast, $CTA_2$ at 2-5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ decreased myocardial flow by 32-35% and blood flow to the liver by 46%, the intestine by 54% and the pancreas by 82% (9). $PTA_2$ was able to prevent these decreases in blood flow when given prior to $CTA_2$ infusion. $CTA_2$ did not affect bronchial, renal, adrenal or skeletal muscle flow. It is of considerable interest that the regions rendered ischemic by $CTA_2$ are precisely the same areas that dilate in response to prostacyclin (10). Whether this is pure coincidence or represents a subtle dilator-constrictor control system, cannot be stated with any degree of certainty at present.

Table 2. Pathophysiologic and Therapeutic Actions of Stable Thromboxane Analogs

| Effect | Pinane Thromboxane $A_2$ ($PTA_2$) | Carbocyclic Thromboxane $A_2$ ($CTA_2$) |
|---|---|---|
| Coronary blood flow (cat) | Prevents $CTA_2$ induced coronary constriction | Coronary constriction |
| Traumatic shock (rat) | Protects, improves survival | Enhances lethality |
| Myocardial ischemia (cat) | Protects, retards spread of ischemic damage | Exacerbates spread of ischemic damage |
| Sudden death (rabbit) | Protects against $CTA_2$ induced sudden death | Induces sudden death (coronary vasospasm) |

In Noble-Collip drum trauma shock in anesthetized rats producing a $PTA_2$ at a rate of 1 $\mu mole \cdot kg^{-1} \cdot h^{-1}$, significantly prolonged survival from 1.3 ± 0.4 to 3.4 ± 0.5 h ($p<0.01$). Moreover, $PTA_2$ prevented the accumulation of thromboxane $B_2$ in the blood as well as prevented the appearance of the lysosomal protease, cathepsin D and the cardiotoxic peptide, myocardial depressant factor (MDF). $CTA_2$ only exacerbated the pathophysiological state of traumatic shock, slightly shortening survival time (11).

In acute myocardial ischemia (MI) in anesthetized open-chest cats rendered ischemic by LAD coronary artery occlusion, $PTA_2$ proved to be extremely beneficial (12). Not only did $PTA_2$ infusion, at a rate of 0.5 $\mu mole \cdot kg^{-1} \cdot h^{-1}$ starting 30 min post-occlusion prevent the extension of ischemic damage, $PTA_2$ also proved to be anti-arrhythmic. $PTA_2$ dramatically prevented the loss of myocardial creatine phosphokinase (CK), as well as the increase in plasma CK. $PTA_2$ also reversed the elevation in S-T segment of the electrocardiogram as well as the incidence of premature ventricular contractions (PVC) after induction of MI. Part of the mechanism of this protective effect was the prevention of circulating thromboxane $B_2$, antagonism of thromboxane action and preservation of lysosomal integrity in ischemic myocardial tissue (12). Whether this last effect is due to the first two actions or is independent is not known.

$CTA_2$ when given to anesthetized cats subjected to MI at a dose of 1 $\mu g \cdot kg^{-1}$, enhanced myocardial ischemia (without induction of platelet aggregation). $CTA_2$ produced a reduction in myocardial CK of 44% in addition to a decrease of 26% induced by the coronary artery ligation. Comparable decreases were observed with respect to myocardial cathepsin D, a lysosomal protease. Thus, $CTA_2$ clearly exacerbates the myocardial cellular damage resulting from acute coronary ligation (13).

When injected intravenously at a dose of 100-125 $\mu g \cdot kg^{-1}$ into anesthetized or conscious rabbits, $CTA_2$ produced acute sudden death in 8 to 11 minutes (9). The sudden death was characterized by a rapid decrease in mean arterial blood pressure (MABP) after a transient increase in MABP. Within 1 minute, there is a dramatic increase in S-T segment and by two minutes there is a large broad T-wave, changes indicative of acute myocardial ischemia. Respiration becomes rapid and shallow 2-3 minutes after $CTA_2$ injection and eventually respiration ceases to be effective as MABP falls below 30-35 mm Hg. $PTA_2$ can prevent these changes quite effectively as can imidazole, a thromboxane synthetase inhibitor. Thus, $CTA_2$, a thromboxane vascular agonist can produce death, which $PTA_2$, a thromboxane synthetase inhibitor and receptor antagonist, can prevent. These findings suggest an important pathophysiological role of thromboxane $A_2$ in ischemia and shock. Recently, Ingerman et al. (14) has shown that thromboxane $A_2$ may be released from blood vessels as well as platelets. This may intensify the role of thromboxane $A_2$ in these ischemia-shock states.

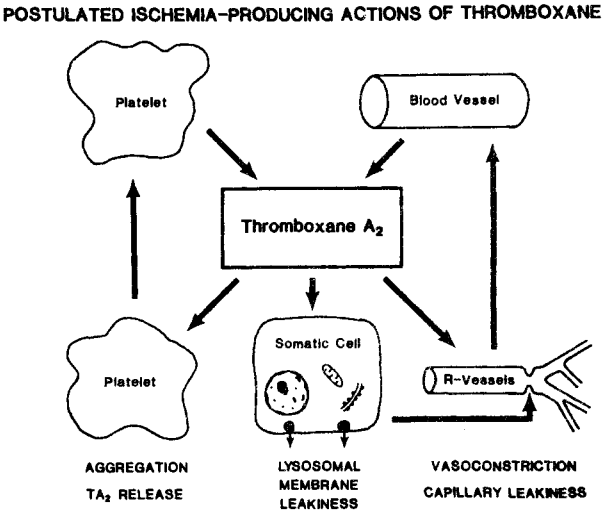


Figure 2. Postulated Ischemia-Producing Actions of Thromboxane $A_2$. Platelets and blood vessels are the major sources of release of thromboxane $A_2$ which acts on platelets to induce their aggregation, on lysosomal membranes in somatic cells to induce release of hydrolytic enzyme and on resistance (R) vessels to produce vasoconstriction. These actions produce positive feedback effects resulting in the formation of additional thromboxane $A_2$.

Figure 2 summarizes the postulated ischemia-producing actions of thromboxane $A_2$ and their potential sites of action. Thus, a variety of noxious stimuli including hypotension, hypoxia, mechanical trauma and humoral agents (e.g., thrombin, epinephrine, collagen, etc.) can release thromboxane $A_2$ from platelets and blood vessels, where it acts locally in the microcirculation.

The major actions appear to be upon platelets to induce aggregation, on resistance vessels to produce vasoconstriction and on somatic cell lysosomes (e.g., in myocardium) to release their acid hydrolases (e.g., cathepsins and phospholipases). These acid hydrolases can damage the microcirculation (15) and particularly sensitize the myocardium to cardiodepressant influences including MDF (16) although they do not induce platelet aggregation directly (17). The subsequent vasoconstriction can further produce local conditions favoring more thromboxane $A_2$ release, just as aggregating platelets produce $TA_2$ which can further promote other platelets to release additional thromboxane $A_2$. In this manner, thromboxanes may locally exacerbate the already existing ischemia by stimulating these positive feedback mechanisms. Thus, thromboxanes may be important humoral mediators of ischemia and shock contributing significantly to their pathophysiology.

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# COMPARISON OF VASODEPRESSOR AND VASODILATOR EFFECTS OF PROSTAGLANDINS $E_1$, $E_2$, $I_2$ AND 6-KETO-$E_1$ *

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## INTRODUCTION

Prostaglandins (PGs) $E_1$, $E_2$ and $I_2$ reduce blood pressure and increase blood flow in most peripheral vascular beds. In particular, these substances produce renal and mesenteric vasodilation when they are administered either intravenously (I.V.) or by direct, close-arterial injection or infusion (1-3). Recently, a novel metabolite of $PGI_2$ has been identified which appears to possess properties which are similar to those of prostacyclin ($PGI_2$). This substance, 6-Keto-$PGE_1$, inhibits platelet aggregation (4) and lowers blood pressure and renal vascular resistance in the rat (5) and it has been suggested that 6-Keto-$PGE_1$ may mediate some of the effects of $PGI_2$. Therefore, one of the aims of this study was to compare the activity of 6-Keto-$PGE_1$ with that of $PGI_2$ on blood pressure and renal and mesenteric hemodynamics in the dog, a species in which the effects of 6-Keto-$PGE_1$ have not been studied.

Secondly, although $PGI_2$ and $PGE_2$ produce similar effects in peripheral vascular beds when administered by local injection or infusion, they have not previously been investigated when administered into the arterial circulation via infusion into the left ventricle, with the effects on blood pressure and regional blood flows simultaneously determined. Infusion into the left heart allows the prostaglandins to be distributed to the periphery without passage through the lung, an organ which selectively inactivates $PGE_2$ but not $PGI_2$ or 6-Keto-$PGE_1$ (6,7). We infused $PGE_2$, $PGI_2$, $PGE_1$ and 6-Keto-$PGE_1$ into the left ventricle and compared the effects on aortic pressure, renal and mesenteric blood flows. The results show that all four prostaglandins have similar effects on pressure and renal vascular resistance. In the intestine, however, the response to $PGE_2$ was unique in that it was transient in nature whereas responses to the other prostaglandins were maintained throughout the duration of infusion.

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## METHODS

Twenty-five mongrel dogs of either sex weighing 16 - 26 kg were anesthetized with pentobarbital sodium (30mg/kg i.v.) and supplemental doses were given as needed. A cuffed endotracheal tube was inserted and the dogs were allowed to spontaneously breathe room air, although in some animals respiration was assisted by a Harvard respirator.

Surgical procedures used to isolate the renal and superior mesenteric arteries for electromagnetic flow measurement (Carolina flowmeter and probes) have been previously described (2,3). Systemic pressure was measured from a catheter whose tip was advanced into the aorta from a femoral artery using a P23 dB Statham pressure transducer. All pressure and flow signals were filtered to yield mean values. Infusion of prostaglandins into the left ventricle was carried out through a 7F Cordis pigtail catheter passed into the left ventricle from the left carotid artery.

All animals received 2.5 mg/kg indomethacin (Merck) (dissolved in 100mM sodium carbonate to a concentration of 2.5 mg/ml) to reduce endogenous prostaglandin synthesis and efficacy of the blockade was assessed by observing vascular responses to 1 mg arachidonic acid (Sigma 99% or NuChek) injections into the ventricle. Blood pressure as well as renal and mesenteric vascular responses to arachidonate were substantially reduced (>80%) for the 4-5 hr duration of the experiments. $PGE_1$, $E_2$ and 6-Keto-$E_1$ (Upjohn) were dissolved in 100% ethanol and stored at $4^0$C in brown bottles. Aliquots of these stock-solutions were diluted with saline to the appropriate concentrations for infusion (50, 100 or 200 ng/kg/min at a rate of 0.2ml/min) using a Harvard syringe infusion pump. $PGI_2$ (Upjohn) was stored as a powder at $-70^0$C and a small quantity was weighed out and dissolved in 20mM Tris (pH 9.0). This stock was further diluted with Tris buffer (pH 8.5) for infusion. Administration of vehicles (ethanol-saline, Tris buffer, and saline) produced little or no effect on the cardiovascular system.

Since infusions produced changes in blood pressure, all flow data were converted to resistance units by dividing pressure by flow and results are reported as percent change from control. Calculations were carried out on a DEC PDP8E computer and analyzed by analysis of variance and the Neuman-Keuls test (8).

## RESULTS

Dogs received infusions of $PGE_2$ and $PGI_2$ at doses of 50 (n=5), 100 (n=8) or 200 (n=7) ng/kg/min, with the order of infusion randomized from dog to dog. An additional 5 animals received infusions of $PGE_1$ and 6-Keto-$PGE_1$ at a dose of 200 ng/kg/min.

### Aortic Pressure

Changes in aortic pressure produced by prostaglandin infusions are summarized in Table 1. The effects of $PGE_2$ and $PGI_2$ were similar at every dose. At 200 ng/kg/min, 6-Keto-$PGE_1$ produced somewhat smaller decreases in pressure than $PGE_2$ or $PGI_2$, whereas $PGE_1$ produced considerably larger reductions in pressure than the others.

Table 1. Effects of 14 minute prostaglandin infusions on aortic pressure.

| PG | DOSE (ng/kg/min) | CONTROL (R.U.)[a] | MINIMUM (R.U.) | % CHANGE |
|---|---|---|---|---|
| $I_2$ | 50 | 136±6 | 126±3* | 7±2 |
| $E_2$ | 50 | 158±12 | 142±14* | 10±3 |
| $I_2$ | 100 | 166±7 | 137±9* | 18±3 |
| $E_2$ | 100 | 154±8 | 122±9* | 20±3 |
| $I_2$ | 200 | 153±5 | 104±14* | 32±9 |
| $E_2$ | 200 | 161±9 | 114±14* | 30±6 |
| 6-Keto-$E_1$ | 200 | 166±10 | 130±10* | 22±2 |
| $E_1$ | 200 | 165±10 | 88±10* | 47±4 |

* P<.05 compared to control

Renal Vascular Resistance

Each of the prostaglandins reduced renal vascular resistance. Table 2 summarizes the results and shows that again $PGI_2$ and $PGE_2$ produced similar changes, whereas $PGE_1$ produced the largest and 6-Keto-$PGE_1$ produced the smallest responses.

Table 2. Effects of 14 minute prostaglandin infusions on renal vascular resistance.

| PG | DOSE (ng/kg/min) | CONTROL (R.U.)[a] | MINIMUM (R.U.) | % CHANGE |
|---|---|---|---|---|
| $I_2$ | 50 | 1.3±0.20 | 0.9±0.1* | 25±9 |
| $E_2$ | 50 | 1.2±0.20 | 1.0±0.2* | 19±2 |
| $I_2$ | 100 | 1.6±0.2 | 1.0±0.1* | 36±6 |
| $E_2$ | 100 | 1.2±0.2 | 0.8±0.1* | 37±4 |
| $I_2$ | 200 | 2.2±0.43 | 1.2±0.2* | 42±6 |
| $E_2$ | 200 | 1.26±0.23 | 0.8±0.1* | 42±2 |
| 6-Keto-$E_1$ | 200 | 2.0±0.5 | 1.3±0.3* | 32±5 |
| $E_1$ | 200 | 2.2±0.6 | 1.0±0.3* | 56±5 |

[a] R.U. = Resistance Units (mmHg/ml/min)

* P<.05 compared to control

Mesenteric Vascular Resistance

Responses of the mesenteric vascular bed to $PGI_2$, $E_1$ and 6-Keto-$E_1$ were different from those observed with $PGE_2$ Figure 1 shows that whereas $PGI_2$ produced sustained changes in the observed measurements for the duration of the infusion, $PGE_2$ effects on mesenteric blood flow were not constant even though the infusion was maintained for the entire length of time the recordings were made. $PGE_1$ and 6-Keto-$PGE_1$ produced tracings similar to that shown for $PGI_2$ and not $PGE_2$.

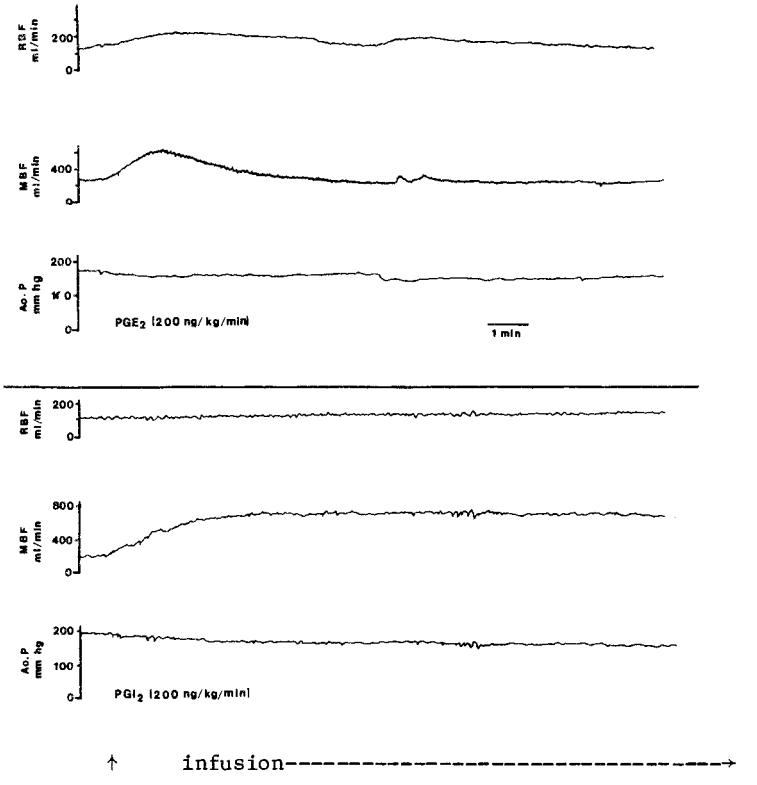


Figure 1. Comparison of tracings taken during a 14 minute infusion of $PGE_2$ (top) and $PGI_2$ (bottom).

Table 3 summarizes the results of prostaglandin infusion on mesenteric vascular resistance. Data involving $PGE_2$ are taken at the peak of the response.

Table 3: Effects of 14 min PG infusions on mesenteric vascular resistance

| PG | DOSE (ng/kg/min) | CONTROL (R.U.)[a] | MINIMUM (R.U.) | % CHANGE |
|---|---|---|---|---|
| $I_2$ | 50 | 0.65±0.17 | 0.30±0.06* | 41±10 |
| $E_2$ | 50 | 0.80±0.23 | 0.49±0.11* | 32±9 |
| $I_2$ | 100 | 0.83±0.17 | 0.24±0.02* | 63±8 |
| $E_2$ | 100 | 0.61±0.10 | 0.29±0.04* | 49±5 |
| $I_2$ | 200 | 0.80±0.19 | 0.22±0.04* | 67±8 |
| $E_2$ | 200 | 0.72±0.10 | 0.31±0.03* | 56±3 |
| 6-Keto-$E_1$ | 200 | 0.80±0.18 | 0.3 ±0.08* | 63±4 |
| $E_1$ | 200 | 0.85±0.14 | 0.18±0.03* | 77±6 |

[a] R.U. = Resistance Units (mmHg/ml/min)

* $P < .05$ compared to control

The actual values of control mesenteric and renal blood flows from which the resistances were calculated are given in Table 4.

Table 4: Initial Blood Pressure and Blood Flow Rates in the Renal and Mesenteric Vascular Beds.

| PG | DOSE (ng/kg/min) | PRESSURE (mmHg) | MESENTERIC BLOOD FLOW (ml/min) | RENAL BLOOD FLOW (ml/min) |
|---|---|---|---|---|
| $I_2$ | 50 | 136±6 | 304±62 | 112±11 |
| $E_2$ | 50 | 158±12 | 258±59 | 139±15 |
| $I_2$ | 100 | 166±7 | 259±49 | 113±14 |
| $E_2$ | 100 | 154±8 | 288±38 | 136±15 |
| $I_2$ | 200 | 152±5 | 233±40 | 95±14 |
| $E_2$ | 200 | 161±9 | 234±21 | 152±20 |
| 6-Keto-$E_1$ | 200 | 166±10 | 249±48 | 114±17 |
| $E_1$ | 200 | 165±10 | 210±34 | 96±22 |

Recovery Times

The time interval between cessation of infusion and return to baseline of flow and pressure are summarized in Table 5. Recovery times following $PGI_2$ were very short compared to the other prostaglandins and recovery from each infusion was observed in every animal.

Table 5: Recovery Times: Interval between the end of infusion and return to baseline of flow and pressure.

| PG | DOSE (ng/kg/min) | MEAN AORTIC PRESSURE (min) | MESENTERIC BLOOD FLOW (min) | RENAL BLOOD FLOW (min) |
|---|---|---|---|---|
| $I_2$ | 50 | 3.8±0.8 | 3.4±0.9* | 3.2±1.1 |
| $E_2$ | 50 | 11.2±1.0 | | 9.2±1.7 |
| $I_2$ | 100 | 6.7±1.3 | 2.6±0.4 | 3.9±0.9 |
| $E_2$ | 100 | 26.3±3.3 | --- | 20.9±3.1 |
| $I_2$ | 200 | 5.3±1.4 | 3.7±1.3 | 3.3±1.9 |
| $E_2$ | 200 | 37.8±8.3 | --- | 31.5±7.3 |
| 6-Keto-$E_1$ | 200 | 23 ±6 | 23 ±6 | 23 ±6 |
| $E_1$ | 200 | 30 ±6 | 30 ±6 | 30 ±6 |

*Effect of $PGE_2$ was transient during the infusion and no change in mesenteric blood flow was observed when infusion was discontinued.

## DISCUSSION

These results demonstrate that prostacyclin and $PGE_2$ produce similar effects on blood pressure and renal vascular resistance when infused into the arterial circulation. In addition, we found that $PGE_1$ was somewhat more potent than $PGI_2$ and 6-Keto-$PGE_1$ was less potent in reducing blood pressure, renal and mesenteric vascular resistance than $PGI_2$. An unexpected result was that the effect of $PGE_2$ in the gut, although potent, was transient and mesenteric blood flow returned to control levels despite the fact that infusion was continued.

6-Keto-$PGE_1$ appears to be a naturally occurring product of prostacyclin metabolism (9) which inhibits platelet aggregation (4), escapes pulmonary inactivation (10) and lowers rat renal vascular resistance (5); properties which it shares with $PGI_2$. In addition, 6-Keto-$PGE_1$ was similar to, but less potent than $PGI_2$ in dilating the feline pulmonary vascular bed and in reversing the pulmonary hypertensive effect of ADP (10). In the present study, 6-Keto-$PGE_1$ was less potent than $PGI_2$ in reducing aortic pressure, renal and mesenteric vascular resistance. However, the effects of 6-Keto-$PGE_1$ persisted for 5-7 times as long as those of $PGI_2$. Therefore, if significant amounts of 6-Keto-$PGE_1$ are produced *in vivo* from $PGI_2$, it is possible that this metabolite may contribute to the action of prostacyclin. Additionally, these results demonstrate that the vascular activity of 6-Keto-$PGE_1$ in the dog is similar to that of $PGI_2$ and $PGE_1$, which agrees with previous reports from studies using cats and rats.

In the present study, infusions of $PGE_2$ and $PGI_2$ produced quantitatively similar reductions of mean aortic pressure and renal vascular resistance over a range of dose of 50-200 ng/kg/min. These results agree with those of Gerber et al (11) who reported that infusions of $PGE_2$ and $PGI_2$ into the renal artery of dogs at dose rates of 10 and 100 ng/kg/min produced comparable increases in renal blood flow. However, when $PGE_2$ and $PGI_2$ were infused into the aortic arch of anesthetized dogs,

Gerkins et al (12) showed that $PGE_2$ (0.3-3ng/kg/min) produced responses that were similar to those produced by $PGI_2$ (3-30ng/kg/min). Thus, at doses of 0.3-30ng/kg/min $PGE_2$ appears to be 3-10 times as potent as $PGI_2$, whereas in the present study, at doses of 50-200 ng/kg/min $PGI_2$ and $PGE_2$ appear to possess similar activity.

A novel finding in the present study is that the response of the mesenteric vascular bed to increased levels of $PGE_2$ was transient. This finding has not been explained but we could not modify the response with phenoxybenzamine (3 aminals) or hexamethonium (3 animals). Repeated injections of $PGE_2$ into the mesenteric artery does not produce tachyphylaxis (Feigen, unpublished observations), nor has tachyphylaxis to the response to $PGE_2$ been reported in any other organ to the best of our knowledge. We have called this transient response to $PGE_2$ "escape" and suggest that this effect is related to the autoregulatory escape that the mesenteric vascular bed exhibits when presented with vasoconstrictor stimuli.

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# CAPILLARIES AS THE SOURCE OF PROSTACYCLIN AND PROSTAGLANDIN SYNTHETASES IN BRAIN

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ABSTRACT

Capillaries were isolated from cerebral cortex of guinea-pigs and rats. The synthesis of prostacyclin and prostaglandins (PG-s) was determined in the isolated capillaries in vitro applying 1-$^{14}$C-arachidonic acid as substrate. It has been found that the cerebral cortex capillaries of guinea-pigs and rats were capable of synthesizing prostaglandins from the labeled precursor, however, the results suggested a species difference in the distribution of PG-s produced by brain capillaries. A species dependent rate of conversion of 1-$^{14}$C-arachidonic acid to PG fractions was also observed. The main PG-s synthesized by guinea-pig capillaries were $PGD_2$ and $PGE_2$, respectively. The $PGD_2$ was formed in largest quantity in the rat brain capillaries. The total conversion of arachidonic acid to prostacyclin and PG-s was found to be 16.44 - 21.1 % in guinea-pigs and 7.64 - 9.16 % in rats. A slight PG formation was observed in the capillaries in vitro without addition of norepinephrine and glutathione. Glutathione (reduced form) did not alter alone the arachidonate cascade of capillaries, while the norepinephrine elicited an expressed elevation of the biosynthesis of $PGF_{2\alpha}$ and $PGE_2$, respectively. More $PGE_2$ than $PGF_{2\alpha}$ was produced by the capillaries in the presence of glutathione and norepinephrine. The biosynthesis of PG-s was also determined in glomeruli of kidney rich in capillaries. The distribution of synthesized PG fractions and the conversion of arachidonic acid in glomeruli showed a similar pattern as it was found in brain capillaries. The results suggest that the capillaries not only react to prostacyclin and PG-s but they are able to synthesize these components of arachidonate cascade.

## INTRODUCTION

Prostaglandins are synthesized by vascular tissues (Raz et al. 1977) and they might contribute to the regulation of vascular tone (White et al. 1971, Denton et al. 1972) and to the ability of endothelial surface to repel the deposition of platelets (Bunting et al. 1976, Gryglewski et al. 1976).

According to Terragno et al. (1978) prostacyclin is the major product of arachidonic acid metabolism in vascular tissue. It has previously been shown that blood vessels in general possess only limited ability to convert arachidonic acid to prostaglandins (Gryglewski et al. 1976, Skidgel and Printz 1978). Recently,Gerritsen et al. (1979) found that microvessels of bovine brain synthesize certain PG-s *in vitro*. It remained unclear, however, whether the guinea-pig and rat brain capillary wall itself has the capacity for synthesizing prostacyclin and prostaglandins or it might respond only to these molecules released from elsewhere of the organism. To elucidate this important point the synthesis of prostacyclin and prostaglandins was assayed in isolated brain capillaries of guinea-pigs and rats. Experiments were carried out to investigate the significance of cofactors (norepinephrine, glutathione) in the activation of arachidonate cascade. For comparision the profile of PG formation in capillaries rich glomeruli of guinea-pigs and rats has also been determined.

## MATERIALS AND METHODS

### Animals

Male and female guinea-pigs (five in each experiments, 65 altogether) weighing 350-450 g and Sprague-Dawley CFY male rats (ten in one series, 60 altogether) weighing 170-190 g were used.

### Materials

Arachidonic acid (grade I) was purchased from Sigma Chemical Co., St. Louis, Mo. 1-$^{14}$C-arachidonic acid (2035 MBq/mM spec. act.) was obtained from Amersham, England. Glutathione, reduced form (research grade) was product of Serva, Feinbiochemica, Heidelberg, Germany. Norepinephrine bitartarate was purchased from Rhône-Poulenc S.A., France. $PGE_2$, $PGF_{2\alpha}$, $PGD_2$ 6-oxo-$PGF_{1\alpha}$ were generously provided by Dr. J.E. Pike of the Upjhon Co., Kalamazoo, Mich.

### Isolations of brain capillaries and glomeruli

The brain of guinea-pigs and rats were perfused with NaCl solution (0.9 %) under light ether anaesthesia. The fraction of isolated brain capillaries was prepared by a micromethod described earlier (Joó and Karnushina 1973). The cerebral cortex was freed of myelin and pial membranes and homogenized manually through nylon sieves of different pore size at 4°C. Suspensions were made in 10 vol. of 50 mM Tris/HCl buffer (pH 7.2) containing 0.25 M sucrose and 5 mM EDTA. The mixture was centrifuged at 3500 g for 10 min at 4°C. The pellet was resuspended in the same buffer and placed on a stepwise sucrose gradient (1.0 M, 1.3 M, 1.5 M; 6 ml each) and centrifuged in a Beckman SW 25.1 rotor at 58 000 g for 60 min at 4 °C. The capillaries were recovered from the bottom of the tubes. The capillaries were resuspended in 10 vol. of Tris/HCl buffer (50 mM, pH 7.2) and centrifuged at 3500 g for 10 min at 4 °C.

The isolation of glomeruli of kidneys was carried out by the method of Richterich and Franz (1960).

Assay of prostaglandin synthesis

Prostacyclin and prostaglandin synthesis in brain capillaries and in glomeruli of guinea-pigs and rats were determined with 1-$^{14}$C-arachidonic acid (0.74 kBq) in the presence of reduced glutathione (2 mM) and norepinephrine (0.1 mM - 1.0 mM) as co-factors. Capillaries (0.5 - 1.2 mg protein) and glomeruli (0.9 - 1.8 mg protein) were the enzyme sources in the assay systems. Incubations (triplicate from each sample) were performed in Tris/HCl buffer (50 mM, pH 7.4) at 37 °C for 30 min. The reaction was stopped by cooling at 4 °C and by acidification (pH 3) with formic acid. The extraction and separation of prostacyclin and prostaglandins were carried out by the method described earlier (Gecse et al. 1979). Prostaglandins and 6-oxo-PGF$_{1\alpha}$ were immediately extracted with ethyl acetate (twice 3 ml). The organic phases were collected and evaporated under $N_2$ streem. The residues were reconstituted in 300 ul ethyl acetate and quantitatively applied to silica-gel G t.l.c. plates. The plates were developed to a distance of 15 cm in the organic phase of ethyl acetate: acetic acid: 2,2,4-trimethyl pentane: water (110: 20: 30: 100 ). Each 5 mm band of chromatograms was scraped off and the radioactivity was determined in an LKB 81 000 liquid scintillation counter using 5 ml scintillant toluene containing 0.4 % w/v PPO, 0.02 % w/v POPOP, 10 % v/v ethanol. The radiolabeled products formed from arachidonic acid were identified with unlabeled authentic PG standards which were detected with anisaldehyde reagent according to Kiefer et al. (1975).

Protein determinations were done by the microbiuret method of Goa (1953).

Statistical analysis was made by Student's t test.

RESULTS

The stable metabolite of prostacyclin (6-oxo-PGF$_{1\alpha}$ ) and the so-called "classical" prostaglandins ($E_2$, $D_2$, $F_{2\alpha}$ ) were identified in the incubation mixture of capillaries and glomeruli isolated from guinea-pigs and rats. An unidentified peak of hydroxy- or hydroperoxide-fatty acids was also found.
Cerebral cortex capillaries of guinea-pigs and rats were able to synthesize prostaglandins from 1-$^{14}$C-arachidonic acid, however a difference was observed in the distribution of prostaglandins produced by guinea-pig or rat brain capillaries. The main PG-s synthesized by guinea-pig capillaries were $PGD_2$ and $PGE_2$, respectively. Much less $PGF_{2\alpha}$ and prostacyclin metabolite, 6-oxo-PGF$_{1\alpha}$ were formed. The distribution of synthesized PG-s by rat brain capillaries have shown a different pattern compering to that of the guinea-pigs. $PGD_2$ was formed in largest quantity, while the amount of other fractions ($PGF_{2\alpha}$, $PGE_2$, 6-oxo-PGF$_{1\alpha}$) was significantly less. The production of 6-oxo-PGF$_{1\alpha}$ was similar in both two species (Fig. 1.). The total conversion of arachidonic acid to PG-s, as well as the percentage distribution of PG-s synthesized by guinea-pig or rat brain capillaries were different. In guinea-pigs the conversion of labeled arachidonic

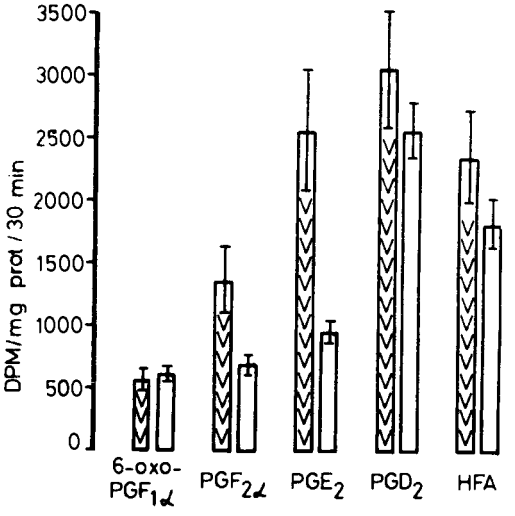


Fig. 1. Formation of prostacyclin and prostaglandins in isolated brain capillaries of guinea-pigs and rats. The incubation mixture contained 1-$^{14}$C-arachidonic acid (0.74 kBq), glutathione (2 mM), norepinephrine (1 mM), capillaries isolated from guinea-pigs (▨) or rats (□). The incubations were performed at 37 °C for 30 min in Tris/HCl buffer (50 mM, pH 7.4).

acid to prostacyclin and prostaglandins was found to be 16.44 - 21.10 %, while in the rats it was found to be 7.64 9.16 % only.

On the basis of the results obtained in isolated brain capillaries it was worthwhile to investigate the arachidonate cascade in an other tissue being rich in capillaries. Therefor the biosynthesis of PG-s was determined in glomeruli of kidney. To clarify, whether or not a species difference exist in the PG formation, glomeruli of guinea-pigs and rats were isolated and applied as enzyme source. The synthesis of prostaglandins was much higher in the glomeruli of guinea-pigs than in that of rats. The main PG fractions formed by guinea-pig glomeruli were $PGE_2$ and $PGF_{2\alpha}$. Much less $PGD_2$ biosynthesis was obtained. The formation of 6-oxo-$PGF_{1\alpha}$ - the stable metabolite of prostacyclin - was negligible. In the glomeruli of rats the $PGD_2$ production was the most expressed (Fig. 2.). The conversion of precursor labeled arachidonic acid to prostacyclin and PG-s by glomeruli of kidney was found to be 6.12 - 9.25 % in rats and 21.08 - 25.32 % in guinea-pigs.

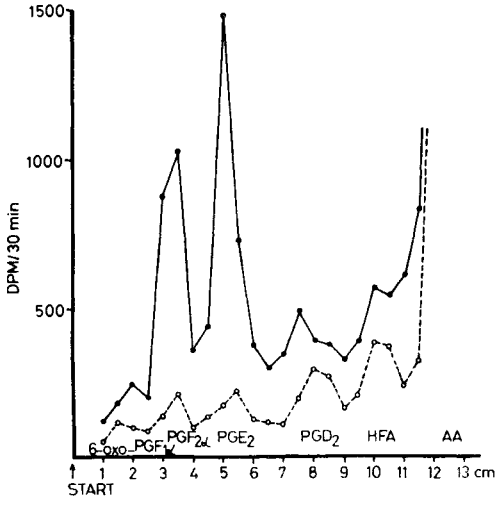


Fig. 2. Prostaglandin synthesis in the isolated glomeruli of guinea-pigs and rats. Typical radiochromatogram of arachidonate cascade obtained after incubation of 1-$^{14}$C-arachidonic acid (0.74 kBq)with glomeruli of guinea-pigs (●—●) or rats (o--o) in the presence of cofactors (glutathione, reduced form 2 mM and norepinephrine 1 mM). Incubations were performed at 37 $^{o}$C for 30 min in Tris/HCl buffer (50 mM, pH 7.4). The protein content of glomeruli was 0.98 mg (●—●) or 1.02 mg (o--o). HFA = hydroxy-, hydroperoxide fatty acids; AA = arachidonic acid.

The fact that the isolated brain capillaries of guinea-pigs or rats consist of morphologically intact cells suggested that the enzymes involved in the synthesis of prostaglandins are in active form in the cell membranes, so they do not need additional cofactor or cofactors in the incubation mixture of capillaries. Therefore, some of the experiments were devoted to give more informations on the role of norepinephrine and glutathione - which are generally accepted activators of

arachidonate cascade - in the PG synthesis of capillaries. The incubation of capillaries with arachidonic acid without addition of cofactors also resulted in the production of 6-oxo--$PGF_{1\alpha}$ and prostaglandins, however, the amount of PG-s reflected to a small enzyme activity. The synthesis of $PGF_{2\alpha}$ and $PGE_2$ by capillaries was found to be elevated when norepinephrine (1 mM) was added to the incubation mixture. The production of $PGD_2$ and 6-oxo-$PGF_{1\alpha}$ was only moderately increased. The formation of hydroxy- or hydroperoxide fatty acids was not significantly altered (Fig. 3.).

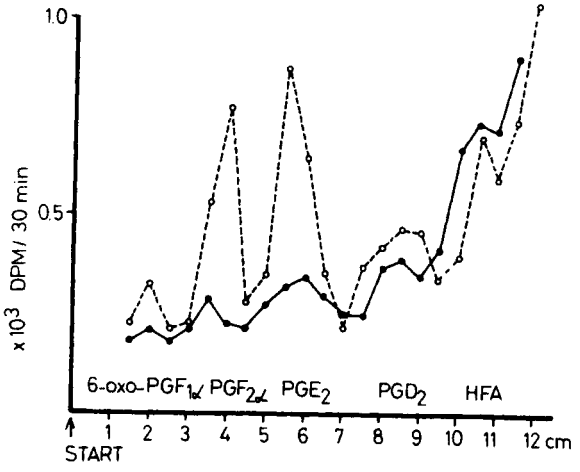


Fig. 3. Cofactor dependent prostaglandin synthesis in brain capillaries isolated from guinea-pigs. Typical radiochromatogram. One series of incubation mixtures contained 1-$^{14}$C--arachidonic acid (0.74 kBq), capillaries as enzyme source (1.07 mg protein) and norepinephrine (1 mM, o--o); the other samples were incubated without norepinephrine cofactor (●—●), the protein content was 1.02 mg. The incubations were performed at 37 °C for 30 min in Tris/HCl buffer (50 mM, pH 7.4). The amount of formed PG fractions is expressed in DPM/30 min on the ordinate. The distance from the origin is presented in cm on the abscissa. HFA = hydroxy- or hydroperoxide fatty acids.

Reduced glutathione did not modify the synthesis of prostaglandins in the capillaries as compared to the samples free of cofactors (Fig.3. and Fig. 4.). The combined application of glutathione and norepinephrine in the incubation mixtures resulted in an increased enzyme activity of arachidonate cas-

cade, inducing an elevated production of each PG fractions, especially that of $PGE_2$ (Fig. 4.).

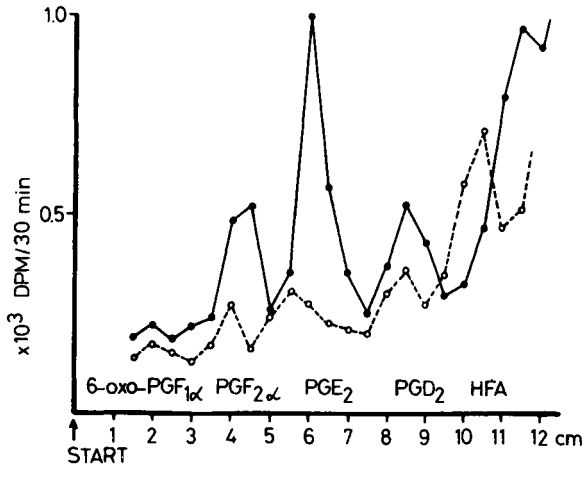


Fig. 4. The effect of glutathione on the synthesis of prostaglandins in isolated brain capillaries of guinea-pigs. Typical radiochromatogram. One series of incubations contained 1-$^{14}$C-arachidonic acid (0.74 kBq), capillaries as enzyme source (0.97 mg protein), glutathione (reduced form 2 mM) and norepinephrine (1 mM) ●—●; the other series of samples were incubated without norepinephrine o--o, the protein content of enzymes was 1.1 mg. The incubations were performed at 37 °C for 30 min in Tris/HCl buffer (50 mM, pH 7.4). HFA = hydroxy- or hydroperoxide fatty acids.

## OISCUSSION

Moncada et al. (1977) and Herman et al. (1977) proposed that the endothelium is the primary site for the synthesis of prostacyclin in blood vessels. The brain microvessels consist of endothelial cells and the glomeruli of kidney are rich in capillaries, therefore in these vessels the main product of arachidonic acid cascade supposed to be prostacyclin. In accordance with observations of Gerritsen et al. (1979) the authors found only a slight 6-oxo-$PGF_{1\alpha}$ synthesis in brain capillaries and in glomeruli of kidney.

The $PGE_2$ and $PGD_2$ were the main products synthesized by guinea-pig brain capillaries without addition of cofactors (norepinephrine and glutathione reduced) in the incubation mix-

tures, at the same time much less $PGF_{2\alpha}$ was also produced. In the presence of norepinephrine and glutathione the amount of $PGE_2$ and $PGF_{2\alpha}$ was significantly elevated in comparison with the cofactor free samples. Cofactors induced a moderate increase in the synthesis of $PGD_2$. Gerritsen et al. (1979) reported that $PGE_2$ is the major product of endoperoxide metabolism in bovine brain microvessels. Our observations are in accordance with this finding. Beside $PGE_2$, however, we detected $PGF_{2\alpha}$ and $PGD_2$ formation by guinea-pig and rat brain capillaries; these data might reflect a species difference in the PG synthesis by microvessels of the brain.

In our hands the brain microvessels of guinea-pigs and rats showed a PG profile very similar to the veins reported by Skidgel and Printz (1978), who demonstrated that the capacity of blood vessels to synthesize PG-s and prostacyclin varies widely.

The prostacyclin biosynthesis by brain capillaries was different from large arterial blood vessels (Moncada et al. 1977, Herman et al. 1977, Skidgel and Printz 1978), since we found less than 10 % conversion of arachidonic acid to prostacyclin.

Cotte et al. (1977) using ram seminal vesicle cyclooxygenase found that the prostacyclin synthesis was inhibited by reduced glutathione. On the contrary, the prostacyclin formation of brain microvessels in guinea-pigs or rats - in our experiments - was not influenced by glutathione (reduced).

Our results suggest that prostacyclin and prostaglandins produced in the capillary endothelium could be involved in the activity dependent regulation of regional blood-flow in the brain microvessels.

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# CONCLUDING REMARKS ON CIRCULATORY ACTIONS OF PROSTACYCLIN AND THROMBOXANE

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Dr. Vane was asked the present status of the endoperoxide "steal" hypothesis which postulated that peroxides liberated by the platelets could be utilized by prostacyclin synthetase in the vessel wall. He explained that although the hypothesis had been criticised by Phil Needleman and Gerard Hornstra, a more recent paper from Marcus's lab had shown that endoperoxide utilization depended upon the concentration of platelets in the platelet-rich plasma. Utilization of endoperoxides liberated from the platelets by endothelial cells could be demonstrated when the number of platelets approximated to that in normal plasma.

Professor Gryglewski was asked whether he intended to test prostacyclin on a double blind basis in peripheral vascular disease. He said that he did not, for he did not regard it as being ethically justified. The Chairman pointed out that other groups were testing prostacyclin against a vasodilator and that the results should be available early in 1981.

Dr. Janina Staszewska-Barzak and Dr. Gabor Kaley had a vigorous discussion as to the relevance of the vagal reflex slowing of the heart induced by prostacyclin in dogs. Dr. Staszewska-Barzak thought that the effects demonstrated by Dr. Kaley were of a pharmacological nature, the dose of prostacyclin being much higher than that likely to be found in the circulation. Dr. Kaley could not agree and thought that the reflex may well have a physiological role.

The paper by Lefer et al was not presented.

Dr. Feigen was questioned about the apparent tachyphylaxis to prostaglandin $E_2$ which he demonstrated on intestinal blood flow. He did not think it was due to the release of an easily-identified vasoconstrictor substance.

Dr. Gecse presented evidence that biochemical preparations of brain capillaries synthesized several different prostaglandins and that co-factors such as glutathione and noradrenaline substantially increased the production. Dr. Vane

suggested that the addition of co-factors such as glutathione might redirect the biosynthetic pathway away from prostacyclin towards a more stable prostaglandin such as $PGE_2$, $PGF_{2\alpha}$ and $PGD_2$. Professor Gryglewski referred to the very low percentage conversion of arachidonic acid to prostanoids and thought that incubation of $PGH_2$ with the capillary preparation would be a better indication of the physiological situation.

*Adv. Physiol. Sci. Vol. 7. Cardiovascular Physiology. Microcirculation and Capillary Exchange*
*A. G. B. Kovách, J. Hamar, L. Szabó (eds)*

# INTRODUCTION TO ROUTES OF TRANSCAPILLARY TRANSPORT: CORRELATION OF STRUCTURE AND FUNCTION

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This symposium is put together with the purpose of reviewing and re-evaluating present concepts of transendothelial-transcapillary transport routes as they relate to the fine structure of the vascular endothelium of mammals. There are essentially two schools of thought with respect to transendothelial transport routes of which one believes more in plasmalemmal vesicular channels, and the other in intercellular clefts. Each school of thought does admit that both transendothelial transport systems may function from time to time. An attempt will be made to reconcile these points of view, and to discuss further the assumption that the large-pore system used by high molecular weight substances is represented by endothelial plasmalemmal vesicles, whereas the small-pore system used by low molecular weight substances is represented by slit-like spaces between adjacent occluded areas of tight junctions of the interendothelial clefts.

It is recognized that the fine structure of the endothelium varies considerably depending on the organ and tissue, and also on the vascular segment in which it is present. In blood capillaries, the endothelial cells are relatively thin and may contain gaps or holes, open or closed fenestrations, as well as vesicles connected either to the lumenal or the ablumenal cell membrane, or forming a continuum of several merging vesicles, having the shape of beaded tubular channels across the endothelium. In each case, the question arises which size molecules take advantage of which transcapillary transport route? In arteries and veins, the endothelial cells are thicker than in most capillaries, and gaps, holes and fenestrations do not occur, whereas the vesicular elements are ubiquitous. Because of the height of the endothelial cells, the vesicles rarely form channels. Interendothelial clefts have been considered potential transendothelial transport routes, particularly in these types of blood vessels. The design of the junctional areas, holding endothelial cells together, has been the focus of interest and analysis, since the architecture of these areas may determine which size molecules, if any, will penetrate from or to the vascular lumen. It has been demonstrated that the junctions are of two types: gap junctions and tight junctions. The gap junctions are focal points of contact which serve as mediators of chemical and electrical transmission between adjoining cells. The tight junctions serve as areas with increased cellular adhesion. Their design varies depending on the vascular segment in which they are present. In venules and veins, they consist of a small number of granular membrane strands and points of contacts, whereas there seems to be a larger number of these features in

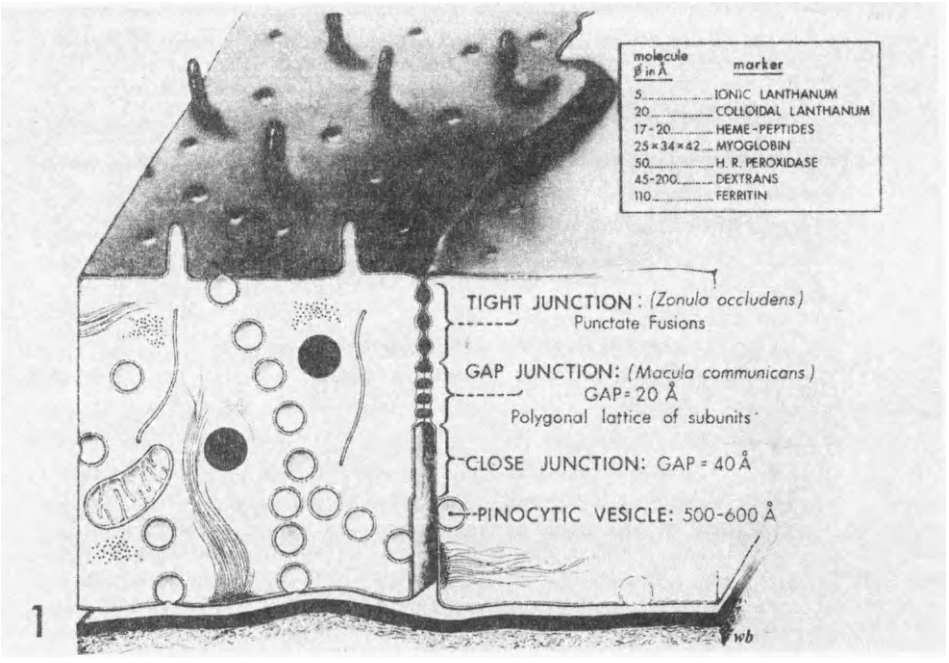


Fig. 1. Summary of general appearance of the several types of cell junctions between endothelial cells as seen in sectioned material. The most commonly used markers for testing transendothelial transport in electron microscopy are indicated in box together with approximate molecular diameter ($\phi$). Tight junction appears as punctate fusions of outer leaflets of apposed cell membranes, and is believed by most investigators to allow penetration of only ionic lanthanum. Gap junction in sections also appears to consist of cell membrane fusions, but has been shown to allow penetration of colloidal lanthanum and heme peptides with an average diameter of about 20 Å, therefore qualifying this junction as a gap junction, 20 Å wide. The close junction does not show a membrane fusion and allows penetration of myoglobin with a molecular diameter up to 40 Å. The pinocytic vesicles have been demonstrated to transport all tracer substances listed, particularly horseradish peroxidase, dextrans, and ferritin particles. Note that size of junctions has been slightly exaggerated in the drawing to explain more readily their substructure. From: RHODIN, J.A.G. Architecture of the vessel wall. Handbook of Physiology, Sect. 2. The Cardiovascular System. Vol. II. Vascular Smooth Muscle. The American Physiological Society, Bethesda, Maryland, 1980. pp. 1-31.

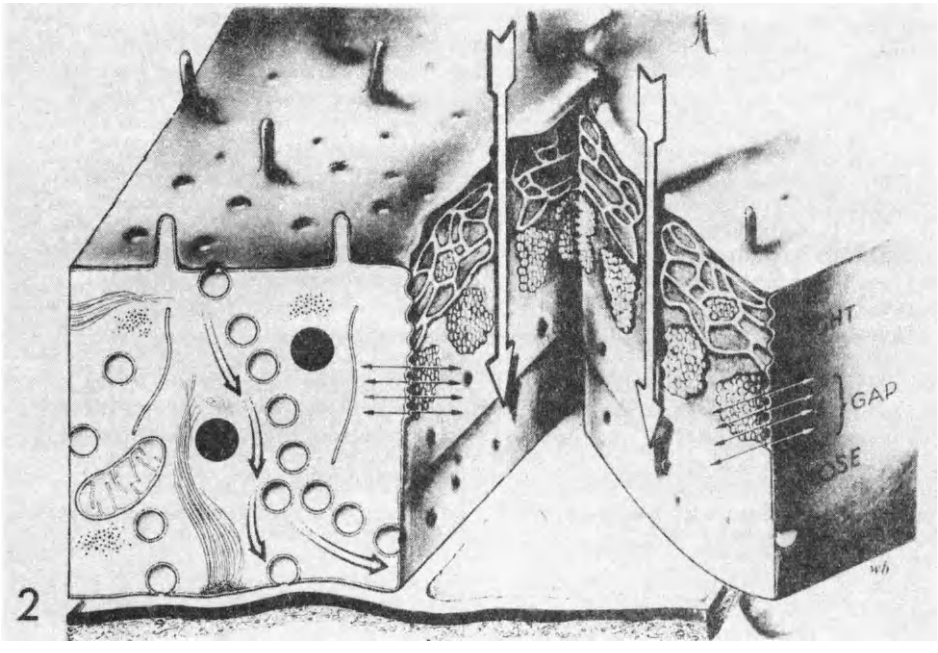


Fig. 2. Summary of general appearance of the several types of cell junctions between endothelial cells as seen in freeze-cleaved material. It is assumed that by using this preparation technique, one has been successful in separating the two adjoining cells shown in Figure 1, so that it is possible to examine the apposing cell surfaces. In reality, the freeze-cleavage technique splits along the central plane of cell membranes and other membranes, It now appears that the punctate fusions of the tight junction are in fact derived from a system of ridges formed by minute particles within the cell membranes. Large arrows indicate that some investigators have demonstrated penetration of colloidal lanthanum across these junctions in some segments of the cardiovascular system, which is assumed to prove that these ridges are interrupted at some points along the tight junctions, either permanently or momentarily. Gap junctions are, in fact, patchlike areas (maculae) with minute membrane particles forming small linkages, which serve as areas for transfer of ions and metabolites from one cell to the next, indicated by small double-headed arrows. Double arrows indicate movement of pinocytic vesicles. This movement can also be assumed to occur in the opposite direction. From: RHODIN, J.A.G. Architecture of the vessel wall. Handbook of Physiology, Sect. 2. The Cardiovascular System. Vol. II. Vascular Smooth Muscle. The American Physiological Society, Bethesda, Maryland, 1980. pp. 1-31.

arterioles and arteries. However, sites have been identified in arterial endothelium where there exists a deficiency in the tight junctional complex, even under normal conditions, consisting of slit-like spaces between adjacent occluded areas. It has been suggested that these slits serve as a selective pore system for low molecular weight substances.

Certain pathological conditions seem to utilize the interendothelial clefts as a means of achieving a higher permeability than exists normally. In the initial phases of experimental hypertension, there is an increased permeability to horseradish peroxidase in arterioles and arteries, as indicated by the presence of this low molecular weight marker in the clefts between endothelial cells and the subendothelial space. There is also a simultaneous increase in plasmalemmal vesicular transport, as evidenced by an increased transport of ferritin markers by the vesicles. In inflammation, circulating large molecular weight markers such as carbon black particles leave the vascular lumen of venules and veins through dilated interendothelial clefts. Experimental hypertension and inflammation will, therefore, be introduced in this symposium as examples of increased endothelial permeability in an attempt to elucidate further the discussion of transcapillary-transendothelial routes of transport under normal conditions.

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# VESICLES AND/OR CONTINUOUS CHANNELS

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This presentation reviews available evidence that plasmalemmal vesicles of capillary endothelial cells represent subcellular structures which move water, small solutes and macromolecules across the endothelium. It also reviews the evidence in favor of the hypothesis that the plasmalemmal vesicles of some endothelial cells form transendothelial channels or fenestrations by a fusion of several vesicles. The basis for this work is summarized in Palade et al., 1979, and represents a long series of impressive investigative efforts by Drs. Palade, Maya and Nicolae Simionescu, as well as Drs. Clementi and Bruns. In order to balance the review, evidence will also be presented which counters some of the data published by the Palade group in which the influence of the fixatives on the formation of the endothelial vesicles, as well as their possible role as strictly phagocytic, will be presented.

Basically, two types of endothelial cells are present in mammalian tissues: 1) cells with continuous endothelial cytoplasm, and 2) cells with fenestrated endothelial cytoplasm. The continuous type of endothelial cells are present in skeletal muscle, lung and nervous tissue. The fenestrated type of endothelial cells are found in most viscera, more precisely the intestines, kidney, and endocrine organs. There is a third type of endothelial cytoplasm found mainly in the liver and bone marrow, which has large openings and which allows plasma to leave the vascular lumen freely. This type is not considered here. As a general statement, Palade et al. (1979) report that endothelial cells of arterioles have an average of about 200 vesicles per $\mu m^3$; capillaries have about 400 $\mu m^3$, and that postcapillary venules have about 600 vesicles per $\mu m^3$. However, depending on the organ, there is a variation in the number of vesicles. According to Rees and Karnovsky (1967), brain capillaries have the smallest population. Lung capillaries also have a limited number of vesicles (Weibel, 1970). The capillaries of the intestines have a high number of vesicles (Clementi and Palade, 1969), and muscle capillaries seem to have the largest population of vesicles (Palade et al., 1979).

In skeletal muscle capillaries, the continuous endothelium contains a large number of plasmalemmal vesicles (also referred to as pinocytotic or micropinocytotic vesicles). Of these vesicles, roughly one-third are connected to the lumenal cell membrane, and thus open on the blood front. One-third of the vesicles open on the tissue, (ablumenal front) and a

third group of vesicles appears to be free within the cytoplasmic matrix.

According to Palade et al. (1979), most of the vesicles do not participate in the process of intake and delivery to the lysosomal system of endothelial cells, as would be the case in fibroblasts and macrophages. Rather, their intracellular pathway is different, delivering the content of the vesicle which is picked up at one cell surface onto the the opposite cell surface. In other words, the working hypothesis of the Palade group is that endothelial cells are specialized in bulk transport of water, solutes and macromolecules via the system of vesicles. The vesicles would, therefore, serve as independent carriers across the endothelial cells. The forces which move the vesicles would be either diffusion or a Brownian motion in the cell-sol. It is assumed that the thermal kinetic energy is sufficient to account for the vesicle movement. However, the energy required for the interaction (fusion-fission) between the vesicles and the plasma membrane has not be determined.

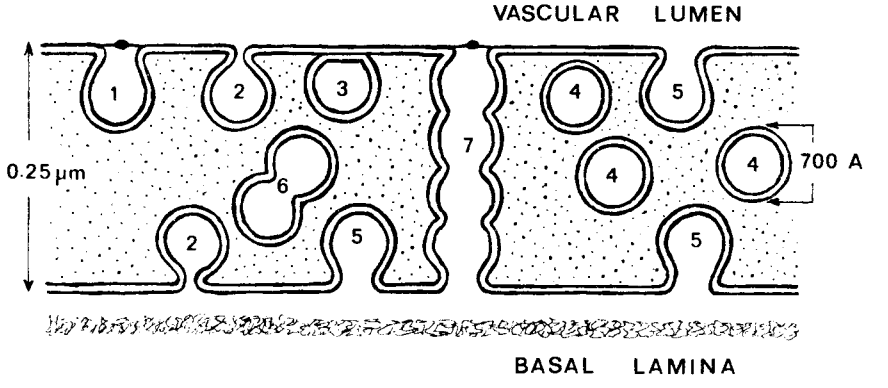


Fig. 1. Schematic diagram of the endothelium of blood capillaries and the several modulations of pinocytotic (plasmalemmal) vesicles observed by several investigators, a summary of which was published by Palade et al. in 1979. Plasmalemmal vesicles, some with stomatal diaphragms (1) at the plasma (lumenal) front are assumed to go through stages of fission (2,3) and eventually become detached from the plasma membrane, occurring freely in the cytoplasm (4) and fusing with the membrane of the tissue (ablumenal) front (5), perhaps going through the same stages (in reverse order) seen in fission (2,3). Cytoplasmic free vesicles (4) may become joined to each other by fusion (6) and they may also form transendothelial channels (7), some of which are provided with stomatal diaphragms at one or both ends. The thickness of the capillary endothelium ranges between 0.1 µm to 0.3 µm in the thinnest parts of the cytoplasm. The pinocytotic (plasmalemmal) vesicles average 700Å in diameter.

The vesicles have been shown to hold, and presumably, also to transport experimentally administered tracer substances. The following tracer substances have been used: colloidal gold, 300Å (Palade 1960, 1961); dextrans and glycogen, 300-100Å (Simionescu et al. 1972); ferritin, 100Å (Bruns & Palade 1968; Clementi & Palade 1967; Casley-Smith & Chin 1971); peroxidase (HRP), 50Å (Karnovsky 1967; Williams & Wissig 1975; myoglobin, 33Å (Simionescu et al. 1973); cytochrome C, 30Å (Karnovsky 1970); heme-undeca-peptide (microperoxidase), 20Å and heme-octa-peptide, 70Å (both

Simionescu et al. 1975). Estimates of the times required for the transport of some of these tracers have been made. Thus, it seems that ferritin requires about 10 minutes across the endothelium, myoglobin about 60 seconds, and the heme-peptides about 45 seconds.

Not infrequently, plasmalemmal vesicles are seen to fuse within the cytoplasm, and often form chains of fused vesicles giving rise to transendothelial channels across the cytoplasm, connecting the lumenal and the ablumenal plasma membranes. The channels could also be formed by a single plasmalemmal vesicle, but most often by a chain of two or more vesicles which open simultaneously on the blood front and tissue front of the endothelium. The channels are considered unstable, and their transient existence estimated to be less than 30 seconds. The majority of these channels are suggested to represent the small-pore system of skeletal muscle capillaries. Some chains of vesicles, which are free of size-limiting structures such as diaphragms and strictures, are suggested to represent part of a large-pore system.

In intestinal mucosal capillaries, the endothelium contains a large number of fenestrations. These are, broadly speaking, considered modifications of a plasmalemmal vesicle, or a derivation of two merging vesicles. It was demonstrated experimentally that the fenestrations represent the large- and small-pore systems of these capillaries. The fenestrations were considered reasonably stable structures within time limits of 1-5 seconds. It was concluded that endothelial channels and fenestrations are derived from fusing plasmalemmal vesicles, and in fact, are variants of a common process.

In summary, Palade et al. (1979) maintain that the fenestrae of the visceral capillaries represent a combination of the large- and small-pore systems. In muscle capillaries, as well as pulmonary capillaries, the transendothelial channels, formed by interconnected rows of vesicles, could represent the small-pore system, and perhaps also the large-pore system, provided these channels are free of size-limiting structures below 500Å, such as stomatal diaphragms.

During the last couple of years, there has been an increasing concern among some physiologists and cell biologists as to the correctness of the hypothesis presented above (Palade et al. 1979) that the vesicles and/or channels of vesicles serve as the basis for transendothelial transport. The physiologists have difficulty in accepting the vesicles/channels, since they believe more in a mechanism of filtration/diffusion through pores than transport in bulk by vesicles. The cell biologists have suggested that the fixatives may artefactually form vesicles/channels. It has been suggested that the vesicles are part of a pinocytic cell defense mechanism which responds to normal and artificial substances, rather than serving the specific function of carrying substances and/or fluid across the endothelial cells.

Using labelled albumin as a tracer in the maximmally vasodilated perfused rat hindleg muscle vascular bed, Rippe et al. (1979) measured the accumulation of this tracer and of fluid in the muscle interstitial fluid during one hour of tracer perfusion in three different situations: tissue cooling, varying filtration rates, and different colloidal osmotic pressures of the perfusate. They concluded that microvesicular transport is of no quantitative significance for the transcapillary passage of macro-

molecules. Their findings suggested that filtration, and to a minor extent, diffusion through large pores is in essence responsible for this macromolecular transfer. It is known that micropinocytosis in fibroblasts is greatly influenced by cooling of the tissue. Thus, the pinocytotic activity at 14°C is about 10% of that at 37°C (Steinman et al. 1974). In the experiments by Rippe et al. (1979), capillary permeability characteristics did not change significantly during cooling, findings which are incompatible with the concept that vesicular transport should normally be a major mechanism for macromolecular transport across capillary walls. Rippe et al. (1979) concluded that "the fact that tracers rapidly enter both pinocytotic vesicles and the interstitial fluid by no means proves the tracer is transported to the interstitial space via micropinocytosis, it may as well have arrived there by pre-bound filtration and diffusion".

There has recently appeared additional support for the hypothesis that the vesicles have a phagocytic function other than being solely involved in bulk transporting activity. Wagner et al. (1980) isolated epididymal fat capillaries, and in a bath, exposed them to ferritin tagged to rhodamine, or to rhodamine tagged to serum albumin. After washing and homogenization, the homogenate showed strong rhodamine fluorescence in the case of the ferritin experiments, but virtually none in the albumin experiments. An electron microscope analysis showed that the ferritin particles were inside the vesicles. It was assumed that the ferritin, being a foreign molecule, was taken up by the vesicles in an attempt to phagocytize it, while the "biological molecule" albumin was not. This leads to the question as to whether such tracers as peroxidase and microperoxidase might also be "irritants".

For some time investigators have been concerned about the effect of fixatives on endothelial vesicles, as well as the ability of the fixative to capture instantaneously the real image, number and distribution of vesicles. Wolff (1966) pointed out that there might be a relationship between vesicle distribution and perfusion pressure, since he found a higher frequency of vesicles in collapsed, as opposed to open capillaries. In the rat skeletal muscle, Johansson (1979) noticed that the number of ablumenal vesicles was reduced in the capillary endothelial cells during elevated venous pressure.

In our own laboratory, we have recently compared the effects of two types of fixation on endothelial vesicles in small arterioles of the rat mesentery. Glutaraldehyde was administered either by a superfusion of the mesentery, or by an intra-arterial perfusion under high pressure. The direct effect on the microvessels was observed by intravital microscopy and recorded on videotape. Subsequently, the identical vascular segment which was recorded in vivo, was analyzed by electron microscopy. With regard to the number of endothelial vesicles, there were considerably fewer vesicles in the arterioles fixed by perfusion under high pressure compared to the microvessels fixed by superfusion. Our findings suggest that the perfused fixative, under high pressure, reaches the endothelial cell membrane much faster, and seems to have a more rapid effect on the vesicles than the superfused fixative. This may suggest that a slow process of fixation brings out a larger number of vesicles than a rapid process of fixation under high pressure, perhaps in response to an irritant.

In a recent investigation, Mazzone and Kornblau (1980) used trans-

mission electron microscopy to examine the size, number and distribution of capillary endothelial vesicles in rabbit lungs fixed either by vascular perfusion or rapid freezing. In the perfusion experiments, the lungs were first perfused in situ with Ringer's solution followed by glutaraldehyde perfusion. In the freezing experiments, the lungs were perfused in situ with autologous heparinized blood and then rapidly frozen with a 70% ethylene glycol solution cooled to -80°C. Samples of the lung tissue were then placed in a fresh bath of 70% ethylene glycol containing glutaraldehyde and paraformaldehyde at -80°C, and subsequently put through a freeze substitution procedure according to details published earlier (Mazzone et al. 1978, 1979). The results showed that there was a significantly greater number of vesicles in the perfused lungs than in lungs fixed by rapid freezing. Thoroughfare channels were not abundant in the pulmonary capillary endothelium fixed by either method. Furthermore, vesicles were significantly larger in lung fixed by vascular perfusion, and there were also a high percentage of joined vesicles observed to be free in the cytoplasm after the perfusion fixation, and more free vesicles in the endothelial cells of the rapidly frozen lung.

It was concluded from this study that the size, density and distribution of pinocytotic vesicles in the pulmonary capillary endothelium is influenced by the method of fixation, perhaps because enough time is available for vesicles to be affected prior to actual chemical fixation, whereas rapid freezing and freeze substitution, which is a very rapid mode of fixation, results in a more physiological preservation.

Support for this interpretation of the results obtained by Mazzone and Kornblau (1980) is offered by Casley-Smith (1979) who was the first to compare conventional chemical fixation with freeze-substitution fixation during his study of capillaries of the mouse diaphragm. Acetone at -80°C was used as a subtitution medium. The results showed a complete absence of thoroughfare channels. The number of cytoplasmic vesicles was similar in both types of fixation, but many more vesicles were in the fusion-mode, joining the ablumenal cell membrane, rather than in the fission-mode at the lumenal cell membrane. It was concluded that thoroughfare channels are artefacts of chemical fixation, and that the relative slowness of chemical fixation would allow many vesicles to fuse with the cell membrane before being fixed.

There is no doubt that the high resolution electron microscope studies of endothelial vesicle modulations by the Palade group are correct, since a generally accepted method of chemical fixation was used, a method which has yielded an enormous amount of information about cell ultrastructure during the last decade. However, the introduction of rapid freezing-substitution methodology seems to question the reliability of some of the structural information gained from chemical fixation, particularly with reference to the actual position of endothelial vesicles at the moment of fixation. In addition, the function of these vesicles has also been questioned. Further investigations are required to prove their role as trans-endothelial carriers, members of a generalized cell defense mechanism against irritants, or both.

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# FUNCTIONS OF ENDOTHELIAL JUNCTIONS

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The capillaries of muscle are lined by two continuous layers, an inner cellular layer, the endothelium, and an outer acellular layer, the basement membrane (Bruns and Palade, 1968a). At this point let us consider only the inner cellular layer. The endothelium resembles a thin, simple squamous epithelium (Fig. 1). It is characterized by having an abundance of small vesicles in its cytoplasm. Many of the vesicles are open at either the lumenal or interstitial surface of the cell; the rest appear to be free in the cytoplasm. Somewhere along the perimeter of the capillary, the endothelial cell contacts either itself or an adjacent endothelial cell to form an endothelial cleft (Fig. 1). The cleft can be viewed as a potential passageway through which water and solutes may exchange between blood plasma in the capillary lumen and interstitial fluid in the pericapillary space. The point of this paper will be to review the experimental evidence which provides insight into the extent to which the cleft serves this purpose.

The general permeability characteristics of muscle capillaries have been determined by physiologic investigation (Pappenheimer 1953, Renkin 1964). Molecules up to 10,000 daltons in molecular weight diffuse across the capillary wall with little restriction. Larger molecules up to 40,000 daltons in molecular weight cross the wall with sharply increasing restriction. Molecules above this size cross the wall slowly at a rate that seems to be independent of their molecular weight. Although this behavior of the capillary can be explained, theoretically, by assuming that its wall is an inert membrane permeated by a set of small and a set of large pores (Landis and Pappenheimer 1963), the ultrastructural evidence we have just seen informs us that the structure of the wall is, in fact, more complex. To gain a fuller comprehension of this complexity, let us examine the structure of, specifically, the endothelial clefts in more detail.

The diameter of capillaries of the mouse diaphragm is approximately 4.5 μm (Bundit 1979). From this dimension, their average circumference is calculated to be 14 μm. When cross sections of capillaries were examined, the number of clefts observed in any section varied from 0 to approximately 3 (Williams, 1971). In 25% (i.e., 35 out of a total of 138) of the cross sections, the endothelial lining lacked a cleft. In such capillaries, the endothelial lining had, therefore, the configuration of a seamless tube. Most frequently, however, a single cleft was present. These data suggest that the capillary tubes are usually formed by a single endothelial cell whose lateral borders are joined to one another (Fig. 1). The length of endothelial cells in capillaries of muscle has not yet been determined.

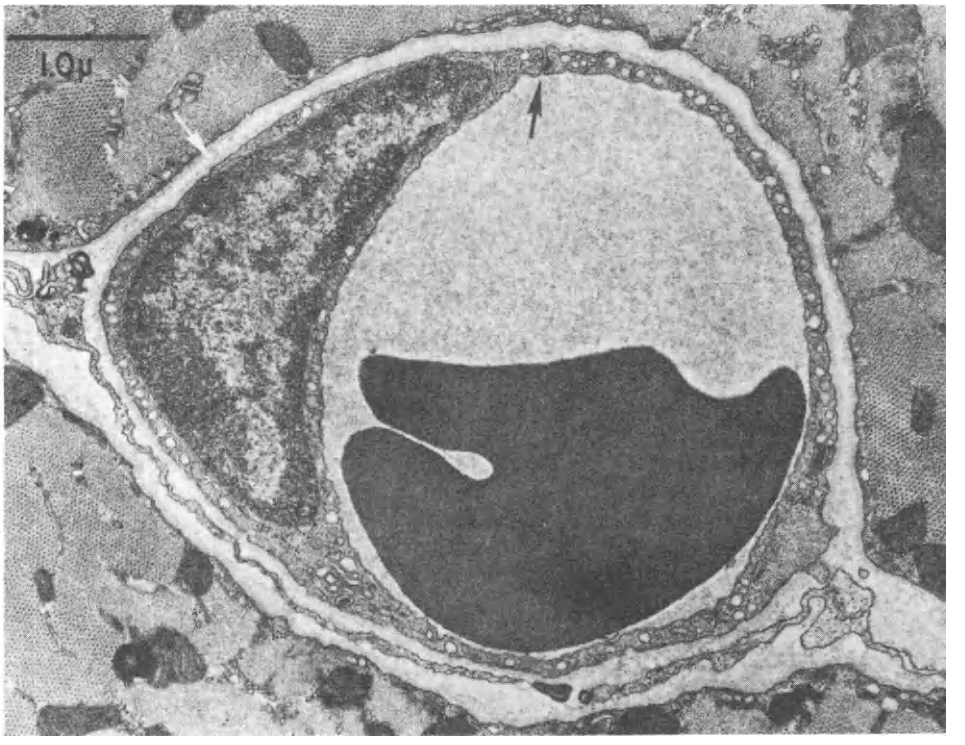


Figure 1. Continuous capillary in the mouse diaphragm. The endothelial lining of the capillary is formed by a single endothelial cell. The endothelial cleft where the lateral borders meet is indicated by a black arrow. The endothelial cell is surrounded by the thin capillary basement membrane (white arrow).

Within endothelial clefts viewed in thin sections, the apposed plasma membranes of the endothelial cells usually contact one another at one to three discrete sites (Fig. 2). At such sites we cannot discern, from their appearance, whether the plasma membranes have fused together, obliterating the intercellular space, or whether they are merely closely apposed to one another. Only 4% (2 out of a total of 57) of the clefts lack a site of membrane contact (Table 1). In such clefts the endothelial plasma membranes come no closer than 3 nm of one another. It is the sites of membrane contact that are of paramount interest to us because they undoubtedly determine whether or not the clefts are accessible as a pathway for the exchange of water and solutes between plasma and interstitial fluid. My colleagues and I (Williams and Wissig 1975, Wissig and Williams 1978, Wissig 1979, Bundit and Wissig 1978, Bundit 1979) have tested the barrier capability of the sites by introducing tracers into the circulation and assessing whether the tracers can penetrate through the clefts. Similar studies had been carried out earlier by other investigators, but their findings conflicted with one another. For example, one investigator stated that the clefts were freely permeable to horseradish peroxidase, a tracer of 40,000 daltons molecular weight (Karnovsky 1967), whereas other investigators stated that the clefts were impermeable to microperoxidase, a tracer of only 1,900 daltons molecular weight, and to larger tracers as well (Simionescu et al. 1973, 1975a; Themann et al., 1971). I shall sum-

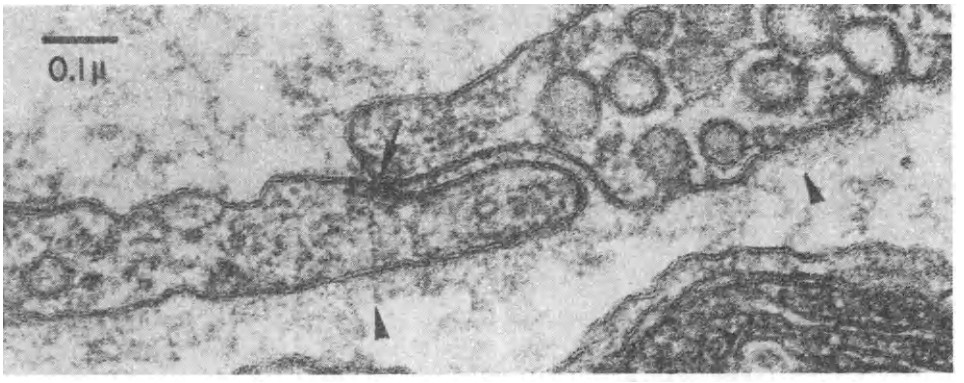


Figure 2. In the center of the field is an endothelial cleft where lateral endothelial borders meet. At the site indicated by an arrow, the endothelial plasma membranes contact one another. Elsewhere in the cleft the membranes are separated by a narrow space. Arrowheads indicate the finely filamentous basement membrane underlying the endothelium.

marize the findings from our own studies with microperoxidase and horseradish peroxidase.

We assessed the patency of endothelial clefts to electron-dense, mass tracers such as the peroxidases by examining the localization of the tracer within the clefts only during that period, after intravenous injection of the tracer, when the concentration of the tracer in the capillary lumen was high and its concentration in the pericapillary interstitium was low. Under such conditions, when we observed that the tracer did not penetrate beyond the first site of membrane contact (Fig. 3A), we concluded that the cleft was relatively impermeable or 'closed' to the tracer. When we observed that the tracer was present in the cleft distal to the last site of membrane contact at a concentration higher than in the interstitium (Fig. 3B), we concluded that the cleft was permeable or 'open' to the tracer. When penetration of the tracer through the cleft was arrested at some intermediate point (Fig. 3C), we concluded that the cleft was partially permeable or 'partially open' to the tracer.

We examined the localization of horseradish peroxidase within endothelial clefts of capillaries of the mouse diaphragm at 2, 5, and 10 minutes after the tracer was injected intravenously (Table 2). The pro-

Table 1. Number of Membrane Contacts in Endothelial Clefts of Capillaries of Mouse Diaphragm*

| Number of Clefts Examined | Clefts Without Any Contact | Clefts With One Contact | Clefts With Two Contacts | Clefts with Three or More Contacts |
|---|---|---|---|---|
| 58 | 2 (0.03)** | 30 (0.52) | 20 (0.35) | 6 (0.10) |

* Data from Williams 1971

** Proportion of the total number (n=58) of clefts.

portion of endothelial clefts that was either open, partially open, or closed to the tracer was roughly similar after each of the three intervals. This is not surprising because the distribution of the tracer in the clefts should reach equilibrium shortly after it is injected and remain stable as long as the concentration of tracer in the capillary lumen does not diminish appreciably and a substantial concentration of tracer does not accumulate in the interstitium. Approximately 10 percent of the clefts were open, 45 percent were partially open, and 45 percent were closed to the tracer. These findings indicate that the endothelial clefts restrict passage of horseradish peroxidase, a molecule of 40,000 daltons molecular weight with a Stokes diameter of 5 nm.

Our findings with microperoxidase were quite different. To begin with, after this tracer was injected intravenously, it accumulated rapidly in the pericapillary interstitium. In order to meet one of the criteria of our study, i.e., to examine the clefts before the interstitium contains a dense concentration of tracer, it was necessary to fix the diaphragm quickly, within 45 sec after initiation of the injection of the tracer. After this interval we observed that approximately 60% of the clefts were open, 25% were partially open, and 15% were closed to the tracer. These findings indicate that the clefts are relatively permeable to microperoxidase, a molecule of 1,900 daltons molecular weight with a Stokes diameter of $\sim$ 2 nm.

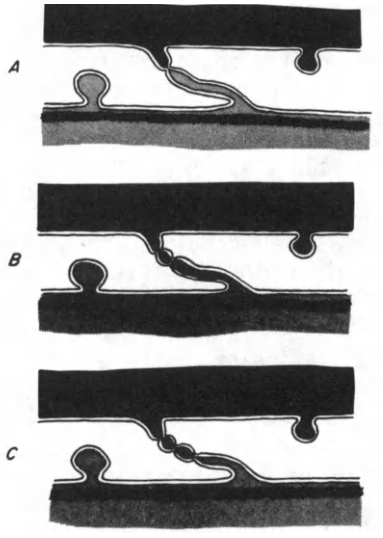


Figure 3. Three patterns of localization (A,B, and C) of peroxidase in endothelial clefts at a short interval after the tracer was injected intravenously. In each case, the capillary lumen contains dense reaction product, and there is little reaction product in the interstitium. In A, dense reaction product does not penetrate beyond the first membrane contact within the cleft. In B, dense reaction product penetrates the length of the cleft to its interstitial terminus. In C, dense reaction product does not penetrate beyond the last membrane contact in the cleft.

If we compare our findings obtained with microperoxidase and horseradish peroxidase, we note, first of all, that the smaller tracer passes much more quickly across the capillary wall than does the larger. This is precisely what we should expect to see on the basis of earlier physiologic findings. Second, we note that the endothelial clefts are relatively permeable to a tracer of less than 10,000 daltons molecular weight, whereas they are much less permeable to a tracer of 40,000 daltons molecular weight. Interestingly enough, these functional attributes of the endothelial clefts correspond to those predicted, from physiologic data, for the small pore of capillaries in muscle. It is likely, therefore, that the clefts are the morphologic counterparts of the small pore (Wissig 1979). The likelihood of the clefts being the small pore would be further strengthened if we could exclude from consideration other potential candidates for the small pore, e. g., the small cytoplasmic vesicles either acting as shuttles (Bruns and Palade 1968b) or linked with the plasma membrane of the endothelial cell to form continuous channels across the endothelium (Simionescu et al. 1975a). It is unlikely that vesicles

Table 2. Proportion of Capillary Endothelial Clefts Permeable to Horseradish Peroxidase*

| Number of Mice | Amount of Tracer Injected (mg) | Time** Interval (sec) | Open*** Clefts | Partially Open Clefts | Closed Clefts |
|---|---|---|---|---|---|
| 1 | 2.5 | 2 | 0.25 | 0.44 (n=32) | 0.31 |
| 1 | 2.5 | 5 | 0.03 | 0.47 (n=30) | 0.50 |
| 1 | 2.5 | 10 | 0.10 | 0.46 (n=39) | 0.44 |

*Data from Bundit 1979.
**Interval between initiation of tracer injection and initiation of fixation.
***Proportion of total clefts (n) assessable for tracer localization.

acting as shuttles could account for the rapid movement of microperoxidase across the capillary wall because a substantial amount of the tracer has already accumulated in the interstitium within the time it takes for a vesicle to cross the endothelial cell (Simionescu et al. 1973). Vesicles arranged as channels should not account for our findings regarding the passage of horseradish peroxidase and microperoxidase across the wall of capillaries of the mouse diaphragm because, after extensive examination of thin sections, we have observed transendothelial channels only in venules and not in capillaries (Wissig 1979). In the microcirculation of the diaphragm, capillaries and venous vessels are located in different regions of bipolar microvascular fields so that the two can be examined individually without their being confused with one another (Simionescu et al. 1978a, Bundit and Wissig, 1978). Thus we have learned that only the endothelial clefts of capillaries in muscle possess critical functional attributes of the small pore and serve as the principal diffusion pathway by which small solutes cross the capillary wall.

The membrane contacts which we observe bridging endothelial clefts in thin sections are similar, structurally, to the occluding junctions that link the plasma membranes of neighboring cells in so-called 'leaky' epithelia (Claude and Goodenough 1973). However, in contrast to their endothelial counterparts, the epithelial junctions are impermeable to

Table 3. Proportion of Capillary Endothelial Clefts Permeable to Microperoxidase*

| Number of Mice | Amount of Tracer Injected (mg) | Time ** Interval (sec | Open*** Clefts | Partially Open Clefts | Closed Clefts |
|---|---|---|---|---|---|
| 4 | 10 | 45 | 0.62 | 0.25 (n=71) | 0.13 |

*Data from Bundit 1979.
**Interval between initiation of tracer injection and initiation of fixation.
***Proportion of total clefts (n) assessable for tracer localization.

lipid-insoluble solutes larger than ions. We can postulate that microperoxidase is able to leak through endothelial clefts either because the contact between membranes of adjacent endothelial cells does not form a tight seal, or because the membrane contacts are discontinuous in a manner that is not easily detectable in thin sections. At present we do not know which explanation is the correct one, but we can cite pieces of evidence which offer support for both of them.

In replicas of endothelial plasma membranes prepared with the freeze-fracture technique, the membrane contacts between adjacent endothelial cells appear as mirror-image networks of ridges on the protoplasmic half and grooves on the external half of the membrane (Fig. 4). The appearance of the endothelial junction in the replicas is superficially similar to that of 'leaky' epithelia, but the two differ in certain respects. In the endothelial junction, the ridges on the protoplasmic half of the membrane show minute discontinuities, whereas the corresponding ridges of the epithelial junction are continuous. The grooves on the external half of the endothelial membrane, often contain particles, whereas the grooves of the epithelial junction are devoid of particles. Moreover, in venous capillaries, the ridges on the protoplasmic half of the membrane become progressively less well developed. They are low in height and are surmounted by a row of particles (Fig. 5). These differences suggest that the structural properties of the membrane fusion between the endothelial and the epithelial membranes differ at the macromolecular level, and such differences might account for their difference in permeability. In addition, the components of the endothelial network have been described as 'staggered', implying that the network is discontinuous (Simionescu et al. 1975b). The discontinuity of the network becomes obvious near the venous end of the microvascular bed (Fig. 6). The discontinuities in the network could provide narrow, slitlike pathways between the endothelial plasma membranes through which tracers could cross the junction (Fig. 7). The pathways would be tortuous and, for that reason, would be difficult to detect in thin sections. The narrow width of such pathways might allow them to be relatively accessible to microperoxidase (Stokes diameter $\sim$ 2 nm) but relatively inaccessible to horseradish peroxidase (Stokes diameter 5 nm).

At the beginning of this paper, I pointed out that the wall of muscle capillaries is bilayered, the second layer being the basement membrane. Because the basement membrane is continuous, it is conceivable that it, like the endothelium, functions as a permeability barrier. Viewed in thin sections, the basement membrane has a finely fibrillar texture (Fig. 2) and is approximately 50 nm thick (Bruns and Palade 1968). It has three

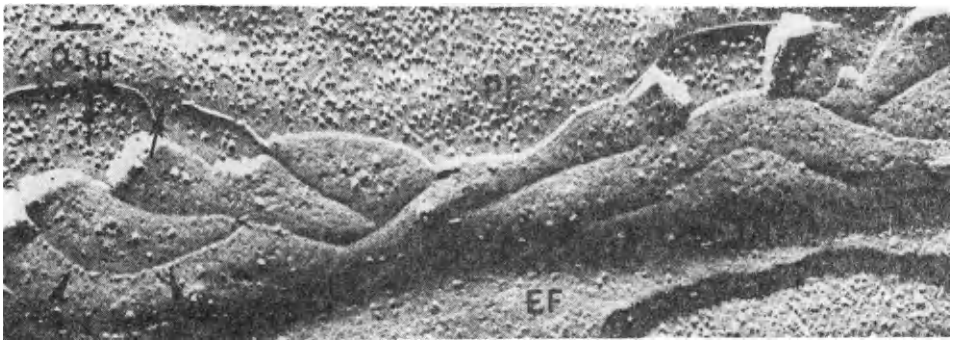


Figure 4. Freeze-fracture replica of a capillary endothelial junction. Linear ridges marking lines of membrane contact appear on the protoplasmic half (PF) of one plasma membrane, and grooves also marking lines of membrane contact appear on the external half (EF) of the apposed plasma membrane. The ridges are interrupted at localized sites (arrows). Some grooves contain a row of intramembrane particles (arrowheads). The network formed by a combination of the ridges and grooves appears to be relatively continuous in this region.

strata: a central lamina densa which is bordered on either surface by a lamina rara interna or externa (Katsuyama et al. 1977). The composition of several basement membranes has been examined with a variety of techniques, including gel electrophoresis of components from isolated basement membranes, cytochemistry and immunocytochemistry. The results indicate that basement membranes have four principal constituents: type IV collagen (Kefalides 1971, 1975); two glycoproteins, fibronectin (Yamada and Olden 1979) and laminin (Timpl et al. 1979); and glycosaminoglycan (Jones 1969, Katsuyama et al. 1977, Kanwar and Farquhar 1979a,b,c).

Thus far, involvement of the basement membrane in permeability phenomena of capillaries has been documented primarily in fenestrated capillaries. In the renal glomerulus, it has been shown that the principal permselective barrier of the glomerular filter is the basement membrane, and the capability of the basement membrane to restrict passage of anionic molecules down to 40,000 daltons in molecular weight is based partly upon the porosity and partly upon the net negative charge of the membrane (Brenner et al. 1978, Rennke et al. 1978). The constituent of the glomerular basement membrane that bears the negative charge has been identified as the glycosaminoglycan, heparan sulfate (Kanwar and Farquhar 1979a,b,c). In contrast, the endothelium lining hepatic sinusoids is permeated by patent fenestrae 100 nm in diameter, and it is the absence of a basement membrane underlying the endothelium (Wisse, 1970) which permits large lipoprotein particles to exchange freely between the sinusoidal lumen and the space of Disse (Naitoh and Wisse, 1978). In fenestrated capillaries of the intestinal mucosa, the endothelium is slightly permeable to dextran and glycogen particles. After having penetrated the endothelium, the escape of the particles into the pericapillary interstitium is restricted by the capillary basement membrane (Simionescu et al. 1972).

Experimental findings which suggest that the basement membrane of continuous capillaries is involved in permeability phenomena are fewer in number. We are familiar with the phenomenon in which the tracer, col-


Figure 5. Freeze-fracture replica of a capillary endothelial junction. The junctional components appear principally on the protoplasmic half (PF) of one endothelial plasma membrane. The external half (EF) of the adjacent plasma membrane appears in the lower portion of the figure. The ridges on the protoplasmic half of the membrane are low and poorly developed. They are surmounted by a discontinuous row of intramembrane particles. The network formed by the ridges appears to be discontinous.


Figure 6. Freeze-fracture replica of an endothelial junction of a venous capillary or small venule. Empty grooves marking the sites of lines of membrane contact within the junction appear on the external half (EF) of one endothelial plasma membrane. The protoplasmic half (PF) of the adjacent endothelial plasma membrane appears in the lower portion of the figure. The grooves on the external half of the membrane are widely separated from one another and appear to form a highly discontinuous network.

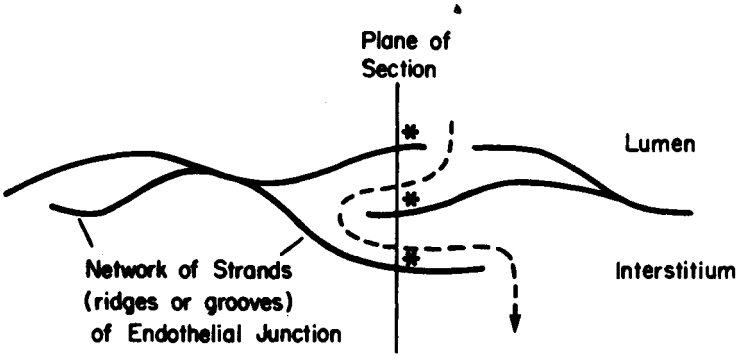


Figure 7. Diagram of the freeze-fracture image of an endothelial junction in a muscle capillary. The curved lines illustrate the network formed by lines of contact between apposed plasma membranes of adjacent endothelial cells. A dashed line traces a pathway through a region of discontinuity in the network. The straight line represents the plane of a vertical thin section through the region of discontinuity. In the thin section, the endothelial cleft would be bridged by the three sites of membrane contact marked with asterisks, and the tortuous pathway through the junction would not be detected.

loidal carbon, having penetrated into venular endothelial clefts opened by the action of autacoids such as histamine and serotonin, is confined temporarily within the clefts by its inability to pass through the basement membrane (Majno and Palade 1961, Fig. 8). Investigators have shown that the smaller tracer, ferritin, may also have difficulty penetrating the basement membrane under the same experimental conditions (Simionescu et al. 1978b). Moreover, when ferritin is injected into the interstitium of muscle, the capillary basement membrane appears to restrict ferritin from free access to the interstitial surface of capillary endothelial cells (Johansson 1978).

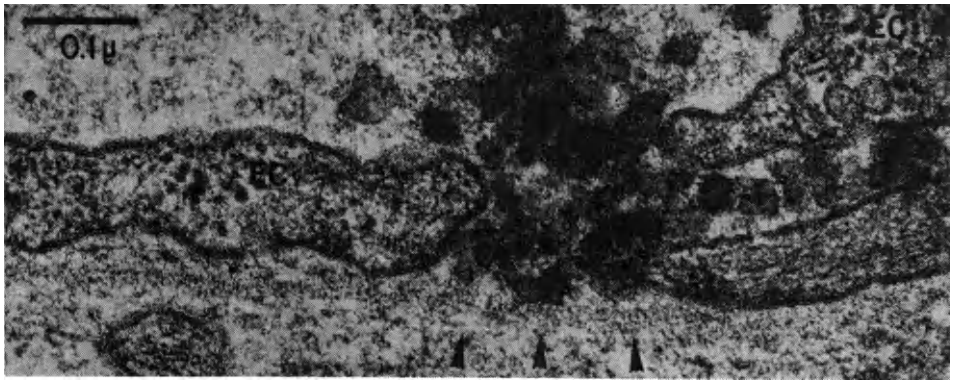


Figure 8. Endothelial cleft of a postcapillary venule from the diaphragm of a mouse injected with colloidal carbon and histamine. The lateral borders of the endothelial cells (EC) are separated from one another, and the intervening space is filled with carbon particles. The particles are restrained from entering the interstitium by the basement membrane (arrowheads).

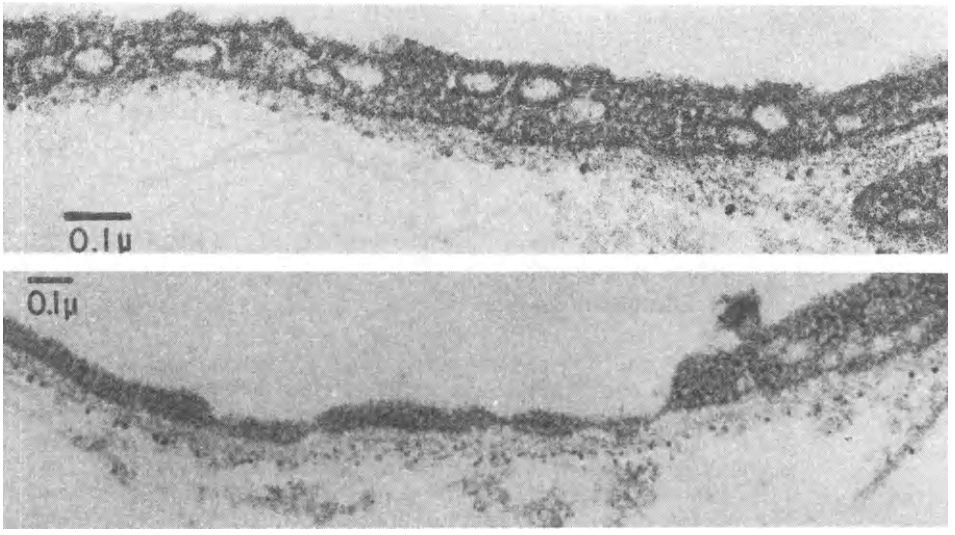


Figure 9.(above) and 10 (below). Continuous capillary from the mouse diaphragm (Fig. 9) and fenestrated capillary from the mouse intestine (Fig. 10), both stained with ruthenium red according to the technique of Kanwar and Farquhar (1979c). In each capillary the finely filamentous basement membrane underlying the endothelium is marked by particles of ruthenium red. The particles are located in the stratum of the basement membrane that corresponds to the lamina rara externa.

The experimental observations I have just cited suggest that the basement membrane of fenestrated and continuous capillaries has a porous structure that can restrict passage of large proteins and colloidal particles. In addition, my colleague, Aristides Charonis, recently learned that the basement membrane of both these types of capillaries stains with ruthenium red in the same manner as does the glomerular basement membrane (Kanwar and Farquhar 1979c) indicating that their basement membranes also possess a fixed negative charge (Figs. 9 and 10). Thus, the basement membranes of fenestrated and continuous capillaries may be able to restrict passage of large molecules not only on the basis of their porosity but perhaps also on the basis of their fixed negative charge. The fixed negative charge should not affect appreciably the passage of neutral or cationic proteins, but could hinder the diffusion of anionic proteins that have diffused through endothelial fenestrae or clefts. In the case of continuous capillaries, the charged basement membrane might reject anionic proteins transferred across the endothelium by vesicles and increase the likelihood of their being returned back to the capillary lumen within vesicles. By the same token, a fixed negative charge on the capillary basement membrane could also ensure that anionic plasma proteins that do enter the perivascular interstitium are returned to the circulation via the lymphatic system and not by vesicular transfer or diffusion back across the endothelium.

In this paper I have summarized evidence, obtained in my laboratory, which indicates that endothelial clefts of capillaries in muscle allow

rapid passage of a tracer with a molecular weight of 1,900 daltons and restrict passage of a tracer with a molecular weight of 40,000 daltons. This behavior of the cleft parallels the behavior of the postulated small pore. Although the cleft is bridged by a network of lines of contact between the plasma membranes of adjacent endothelial cells, the network forms an imperfect barrier, either because the membranes along the lines of contact are incompletely fused or because the network is discontinuous. Any molecule that has crossed the capillary endothelium by vesicular transfer or by permeation through clefts encounters a second continuous barrier, the basement membrane, which is negatively charged and has limited porosity. The overall permeability of capillaries in muscle may be determined by a combination of the properties of the endothelium and basement membrane, and not simply by those of the endothelium alone.
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# THE FROG MESENTERIC CAPILLARY AS A MODEL OF MAMMALIAN CONTINUOUS CAPILLARIES

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One of the major problems which has complicated correlation of structure and permeability of capillaries is the structural and functional heterogeneity of the microvessels. Permeability data have been derived from whole organ studies and therefore represent the average of a range of permeabilities. Correspondingly, most ultrastructural studies have been on specimen of unknown topographical localization within the microvascular bed. However, the recent introduction of techniques for recording physiological properties of single capillaries has significantly reduced the problems of heterogeneity. Permeability measurements using microelectrode techniques have been performed directly in identified capillary segments (Crone et al. 1978; Crone 1980). This has provided us with a unique opportunity to examine the ultrastructure of part of the microvasculature that is well defined physiologically. This paper summarises work undertaken by drs.C.Crone, O.Christensen, J.Frøkjær-Jensen and the author.

The microvasculature of the frog mesentery has been used for single capillary studies because of the large capillary diameter, and accessibility of well defined capillary segments in the exposed mesentery. Permeabilities and hydraulic conductivities have been determined throughout this capillary bed (Crone et al. 1978: Crone 1980; Curry et al. 1976; Michel 1980), highlighting the need for quantitative morphological study of the preparation. We have therefore undertaken a structural study of consecutive segments of frog mesenteric microvessels. In order for findings from frog mesenteric capillaries to be compared with those from continuous mammalian vessels, it is necessary to show that the two vessel types have comparable morphology; therefore our primary aim was to assess the similarity of the frog vessels to mammalian continuous capillaries. Secondly, we aimed to make quantitative observations on structures which might serve as permeability pathways, in order to decide which of possible alternative routes is the most likely to be important for the penetration of hydrophilic solutes.

Materials and methods.

Adult male frogs (Rana temporaria) were used for the experiments. The animals were anesthetized by immersion in 5% urethane. The mesentery was exposed and spread out over the surface of a perspex pillar for transillumination. The mesenteric microvasculature was photographed at a magnification of x 40 and the direction of the blood stream in the capillaries was indicated by arrows on the prints. Then the mesenteries were initially fixed by superfusion with a mixture of buffered aldehydes and after subsequent preparation for electron microscopy flat imbedded in Epon. Thin sections of the capillaries were examined in a Zeiss 10B electron microscope equipped with a $\pm 60^{\circ}$ goniometer stage.

On the photographs taken in vivo the mesenteric capillaries were marked as arterial capillaries, midcapillaries and venous capillaries according to the following criteria. The segment between a diverging and a converging dichotomy was defined as a midcapillary. Going upstream the segment between the first and the third diverging dichotomy is called an arterial capillary. A venous capillary is the segment between the first and the third converging dichotomy on the venous side of a midcapillary. These marked segments were readily recognized in the flat imbedded mesenteries.

Thin sections of the three classes of capillaries were selected randomly for the quantitative measurements. The entire wall was photographed at an electronic magnification of x 25000. The final magnification of the electron micrographs was x 61600. Linear dimensions on the prints were measured with a cartographer's wheel. Areas were determined with a Zeiss-planimeter. Length densities, surface densities and volume densities were determined according to current stereological principles (Weibel 1973).

Results.

The frog mesenteric capillaries are situated in collagenous connective tissue between the two leaflets of the mesothelium (Fig. 1). The general organization of the vascular wall was the same in the three groups of capillaries. It consisted of flattened endothelial cells, a basement membrane (often ensheathing pericytic processes), and a poorly defined adventitia. The endothelial cytoplasm had a high content of plasmalemmal vesicles with an average outer diameter of 750 Å. Observations in gallotannin treated tissue (Fig. 2) indicated that the majority of vesicles are elements in invaginations of either luminal or abluminal plasmamembrane (Bundgaard et al. 1979b). This view has been substantiated by a three dimensional reconstruction of segments of the endothelium based on ultrathin serial sectioning (Frøkjær-Jensen 1980). However, transendothelial channels created by chains of fused vesicles were not encountered. The usual organelles could be observed in the thicker parts of the endothelium; for example, mitochondria occupied approximately 2.5% of the volume, a figure comparable to mammalian continuous capillaries (Oldendorf et al. 1977). The junctional complexes between neighbouring endothelial cells appeared as 1-3 punctate contacts or fusions between

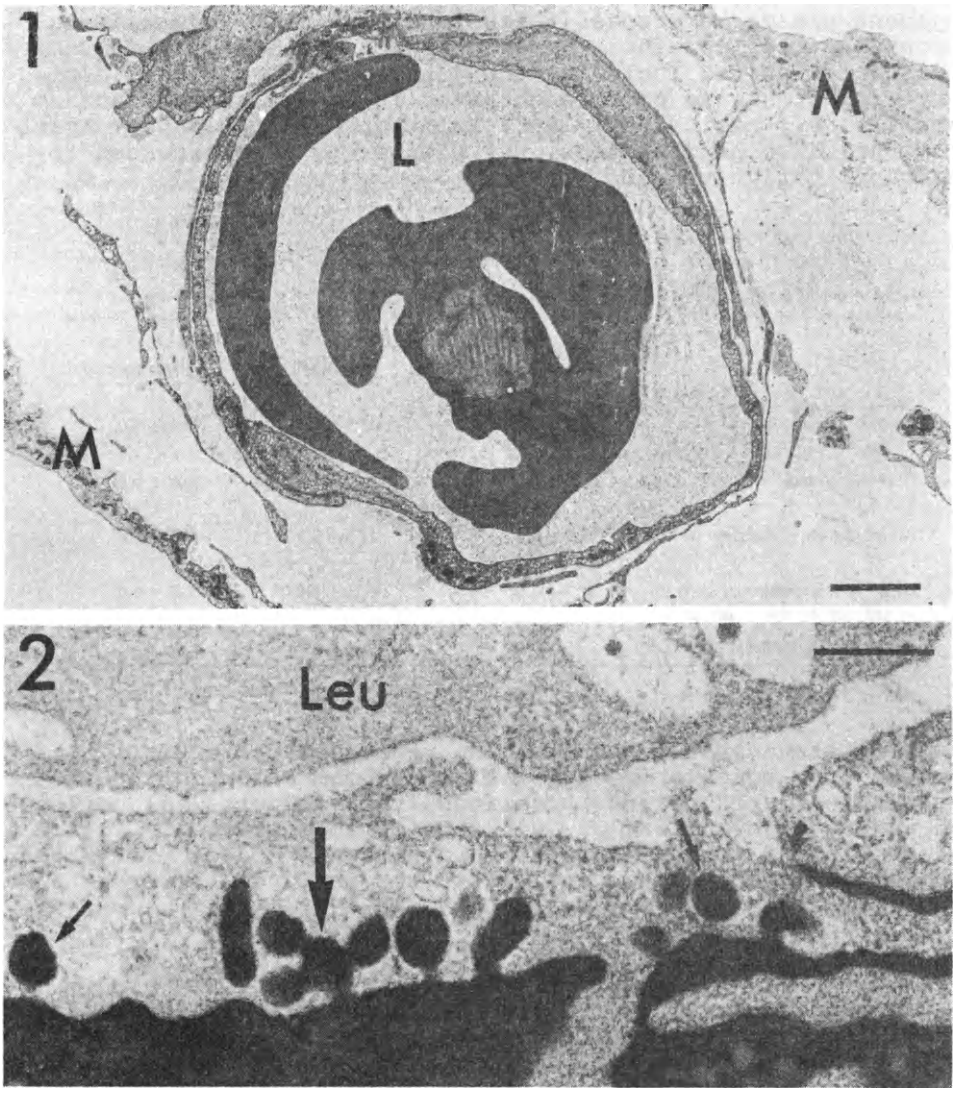


Fig. 1. Midcapillary from the frog mesentery. L, capillary lumen; M, mesothelium. Bar, 2 µm.

Fig. 2. Capillary endothelium from a frog mesentery treated with tannic acid after osmium fixation. The mordant which did not penetrate the abluminal plasmalemma of the endothelium caused an intense staining of the pericapillary space. Half of the apparently free vesicles were labelled (small arrows )as the pericapillary space indicating a continuity between the interior of these vesicles and the interstitium. All the labelled vesicles are probably elements in racemose invaginations of the type marked by the large arrow. Curved arrow indicates an interendothelial junction. Leu, leucocyte; Bar, 0.2 µm.

adjacent plasma membranes. A few fenestrae were observed in venous capillaries. These general sturctural features of the endothelial cells, including the junctional morphology, are very similar to the picture observed in mammalian continuous capillaries (Rhodin 1967, 1968; Bruns and Palade 1968a). Apart from the vascular diameters, the similarity also extended to the quantitative results (Table 1).

**TABLE 1**

MORPHOMETRIC DATA ON FROG MESENTERIC CAPILLARIES

| | Arterial capillaries | Midcapillaries | Venous capillaries |
|---|---|---|---|
| Diameter (μm) | 19.8 ± 4.4 | 13.5 ± 1.9 | 19.5 ± 4.0 |
| Thickness of endothelial cells (μm) | 0.27 ± 0.02 | 0.21 ± 0.01 | 0.23 ± 0.04 |
| Volume density of vesicles (%) | 11.6 ± 0.4 | 12.6 ± 0.9 | 11.9 ± 0.4 |
| Length of interendothelial clefts per $\mu m^2$ capillary wall ($\mu m/\mu m^2$) | 0.27 ± 0.04 | 0.18 ± 0.02 | 0.18 ± 0.03 |
| Depth of interendothelial clefts (μm) | 0.40 ± 0.03 | 0.70 ± 0.08 | 0.98 ± 0.11 |

Mean ± S.E.M.

However, structural features which represent differences between frog mesenteric capillaries and mammalian continuous capillaries were present. Occasionally the endothelial cells had a very high content of 70 Å fibrils and especially in the endothelium of venous capillaries many dense bodies (150-250 nm in diameter) were encountered. Also the pericytic coverage of the capillaries was remarkably high (50-60%).

Discussion.

Frog mesenteric capillaries have been classified as continuous on morphological grounds (Mason et al. 1979). We have extended the morphological approach and obtained new quantitative information; our demonstration of extensive structural similarities in general and quantitative morphology between the capillary beds of the frog mesentery and mammalian skeletal muscles confirm that a 'continuous' classification is justified. The structural features peculiar to frog mesenteric capillaries, bundles of microfibrils, dense bodies, and an incomplete pericytic coverage, are unlikely to be of major significance in the transcapillary passage of small solutes. This preparation can therefore be assumed to be a useful model of continuous capillaries in general.

Our morphometric results when combined with determinations of potassium permeabilities on segments of these same capillaries (Crone et al. 1978), now for the first time permit an attempt to correlate quantified ultrastructure and permeability to small solutes of a well defined segment of the microvascu-

lature; furthermore, they give additional insights into the continuing problem of the nature of the permeability pathway. Only two structures need to be seriously considered in this context, the interendothelial cleft and transendothelial vesicular channels. The interendothelial clefts were for many years regarded as the probable main pathway (Starling 1908; Chambers and Zweifach 1947) but the classification of the interendothelial junctions as zonulae occludentes initiated a search for an alternative pathway (Bruns and Palade 1968a,b). This led to the proposal that small solutes permeate to a significant extent via transendothelial channels created by chains of fused vesicles (Simionescu et al. 1975b, 1978), structures known since 1966 (Wolff 1966).

Our morphological studies do not favour permeation via transendothelial channels. Bundgaard et al. (1979a) have recently assessed the role of transendothelial channels in potassium permeability of frog mesenteric capillaries. The permeability data predicted that about 0.25% of the capillary wall had to be available for free diffusion. It was assumed that the channels were the main pathway and their least diameter 200-250 Å. This implied that there should be 5-8 channels per $\mu m^2$, equivalent to around 20 per capillary cross section of 600 Å thickness. About 700 capillary cross sections were examined. This corresponded to about 1900 $\mu m^2$ capillary wall where 10000-15000 channels should be expected according to the assumptions. However, no channels were identified in the 700 sections. It was realised that if the supposed channels followed a tortuous course within the cytoplasm most channels would only partially be included in a single thin section and consequently not identified. On the other hand, in the attenuated peripheral zone of the endothelial cells the tortuousity of the channels was limited since one or two vesicles were sufficient to create a channel. The absence of channels even in this part of the endothelium led to the conclusion that transendothelial channels were too rare to play an important role as pathway for small hydrophilic solutes in this capillary bed.

A similar approach can be applied to the alternative pathway - the interendothelial clefts - in the following way. The assumption that the clefts are the main pathway to potassium ions will lead to the equation, $P_K = (D_K \times W \times L)/\Delta X$. $P_K$ is the potassium permeability ($67 \times 10^{-5}$ cm/sec; Crone et al. 1978), $D_K$ is the diffusion coefficient of potassium at 20°C ($1.8 \times 10^{-5}$ $cm^2$ $sec^{-1}$), W is the average width of the clefts, L the average length of the cleft openings per unit area capillary wall and $\Delta X$ is the average cleft depth. L and $\Delta X$ have been estimated in the present study, and the cleft width, W, can then be deduced. The result is about 120 Å, which represents the necessary average effective width of the clefts if potassium permeates exclusively between the endothelial cells. That this figure is compatible with an endothelial cleft pathway is clear when it is realised that a cleft of e.g. 200 Å narrowing to 10 Å at junctions would behave as a channel of this sort of average width (Lassen and Trap-Jensen 1969). At present, this is as far as our discussion can go, as current electromicroscopic techniques do not permit greater resolution

of the cleft and junctional dimensions, particularly as dimensions in this range are quite possibly disturbed during the preparative procedures (Moretz et al. 1969; Schultz and Karlsson 1972). In addition, the three-dimensional organization of the junctions, and therefore the diffusion path length in this region, is completely unknown (Simionescu et al. 1975a). Moreover, the structure of the endocapillary layer and the extracellular content of the cleft itself may strongly influence diffusion via this pathway (Bundgaard 1980; Curry 1980). In summary, our morphometric data can be used in conjunction with the known permeability properties of these well defined capillaries to deduce an average effective width of 120 Å for the permeability pathway, which, in our view, is likely to be via interendothelial clefts rather than transendothelial vesicular channels. Recent electron microscopical tracer studies by Wissig et al. (1979) have led to a similar conclusion for capillaries in mouse skeletal muscle.

The endothelial plasmalemmal vesicles are assumed to play an important role in transendothelial transport of macromolecules (Majno and Joris 1978). However, with our finding that almost all vesicles are elements in invaginations of the endothelial plasmalemma (Bundgaard et al. 1979; Frøkjær-Jensen 1980) we have questioned the concept of vesicular transport, since this theory implies that a substantial fraction of the vesicles are in transit in the cytoplasm. A reexamination of current data on the vesicles led us to the view that vesicular labelling observed in most tracer studies might be explained by diffusion into cisternal systems driven by a concentration gradient. In addition, the fact that electron microscopical tracers of increasing molecular dimensions appear on the tissue side with increasing delay suggests diffusion rather than vesicular transport (Bundgaard 1980). Also different physiological studies have indicated that macromolecules permeate the capillary wall exclusively by diffusion and convection (Rippe et al. 1979; Noer and Lassen 1979).

In conclusion, we propose that endothelial plasmalemmal vesicles are part of semipermanent invaginations of the plasmamembrane. This view implies that transendothelial passage of macromolecules by vesicular transport is absent or of very limited importance. Possible diffusion pathways for macromolecules in continuous capillaries are rare fenestrae, transendothelial vesicular channels and focal widenings of the interendothelial clefts (leaks).

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# INCREASED PERMEABILITY OF THE ARTERIO-CAPILLARY SEGMENT IN HYPERTENSION

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Hypertension is known to affect the permeability of endothelium primarily in arterial segments (Jellinek et al. 1969; Giacomelli et al. 1970; Hüttner et al. 1970, 1973c). Although a relationship has been reported in various vascular beds between the level of intravascular pressure and the passage of ultrastructural protein tracers through capillary endothelium (Pietra et al. 1969; Schneeberger and Karnovsky, 1977; Boutet et al. 1974; Rona et al. 1977) autoregulatory mechanisms prevent, in most hypertensive situations, an increased blood flow and permeability changes in capillaries (Nagy et al. 1979a, 1979b). Differences in the organization of endothelium may further influence the permeability of different vascular segments (Simionescu et al. 1975b, 1976). While the role of hemodynamic factors in this phenomenon is well established, the structural basis of increased permeability in arterial segments is not fully elucidated.

In the present study, the endothelium of rat thoracic aorta was used as a model system to approach this problem. Permeability of endothelium to horseradish peroxidase (HRP) and organization of endothelial cell junctions were studied in the rat thoracic aorta: a) during periods of acutely induced high blood pressure and b) in models of chronic experimental hypertension. Acute high blood pressure was produced by intravenous infusi n of norepinephrine while chronic hypertension was induced by three experimental procedures: 1) aortic ligature between renal arteries, 2) uninephrectomy and NaCl-rich diet, 3) uninephrectomy, 0.9% NaCl as drinking fluid and subcutaneous administration of desoxicorticosterone acetate (DOCA). While monitoring blood pressure on a polygraph, HRP was injected intravenously and localized subsequently in the aortic intima by light microscopy and thin-section electron microscopy. The permeability changes observed in various experimental settings were correlated with organization of endothelial cell junctions as revealed by freeze-fracture electron microscopy.

## Acute arterial hypertension

Norepinephrine was infused through the jugular vein of Sprague-Dawley and Wistar rats in a dose of 4 to 6 g body weight per minute, which resulted in abrupt elevation of systolic and diastolic blood pressures up to a 240 mmHg systolic level. During periods of high blood pressure, parallel with normotensive controls, the animals were injected via the femoral vein with 10 mg per 100 g body weight of HRP (Sigma,

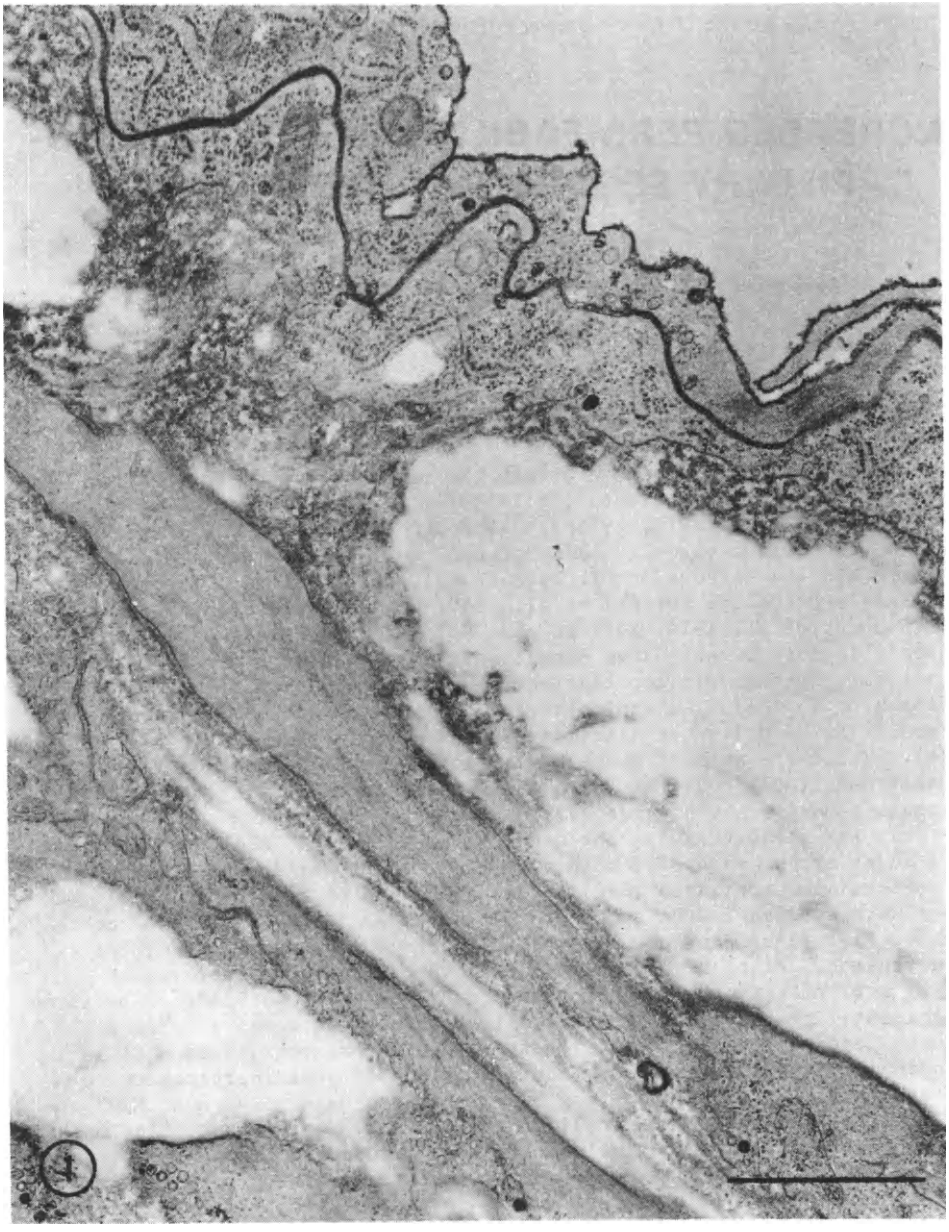
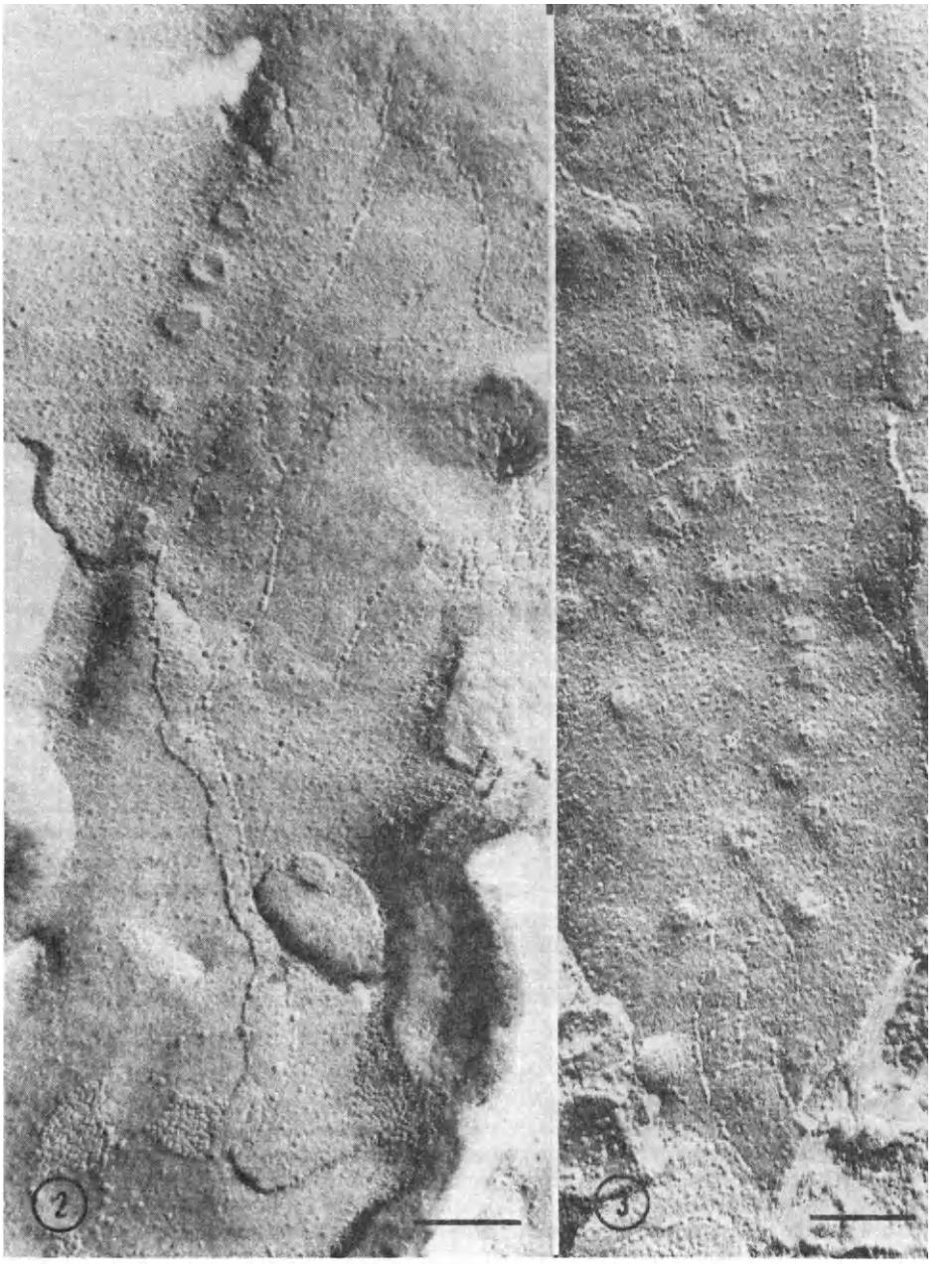


Fig.1. Thin-section electron micrograph of aortic endothelium from Sprague-Dawley rat with norepinephrine induced high blood pressure at 60 seconds after i.v. injection of horseradish peroxidase. Peroxidase reaction product is localized throughout the entire length of inter-endothelial cleft, in plasmalemmal vesicles communicating mainly with the cleft and with a lower density in the subendothelial space. Lead citrate stained preparation. Bar:1 μm.




Figs. 2 and 3. Freeze-fracture electron micrographs of lateral endothelial plasma membranes from control Wistar rat aorta. The fracture plane visualizes double-stranded zonular tight junction with interpolated gap junctions on Fig. 2, and discontinuous loop forming strands of macular tight junction on Fig. 3. Bars:0.2 μm.

type II) dissolved in 0.5 ml of saline per 100g body weight for 60 seconds or 6 minutes then the aortae were fixed by perfusion. Development of HRP reaction product, processing of tissues for light and thin-section electron microscopy ( Hüttner et al. 1973a, 1973b, 1973c) as well as for freeze-fracture electron microscopy (Hüttner and Peters, 1978) were carried out as described previously.

Compared to normotensive controls, HRP reaction product appeared earlier in the subendothelial space of Sprague-Dawley rats during periods of norepinephrine induced high blood pressure (Hüttner et al. 1973c). The interendothelial clefts were labelled to different degrees along their length. In some of the clefts the concentration of HRP throughout their length was the same, and exceeded the concentration of the tracer in the subendothelial space (Fig. 1). In other clefts, the concentration of HRP decreased stepwise in consecutive junctional compartments, as traced from the lumen to the subendothelial interstitium. In still other clefts, the concentration of HRP was lower in junctional compartments than in the adjacent subendothelial space. Plasmalemmal vesicles in endothelial cells were commonly labelled; they were localized preferentially near to, or communicated with labelled segments of intercellular clefts. The density of HRP reaction product however was higher in some vesicles than in the adjacent interendothelial or subendothelial space. This latter pattern of localization may be interpreted as evidence for transport of HRP by the vesicles (Simionescu et al. 1975a; Westergaard et al. 1977), however, we cannot rule out the possibility that HRP was picked up and concentrated by the vesicles after it had traversed focally opened interendothelial junctions (Karnovsky, 1967; Cotran and Karnovsky, 1968; Nagy et al, 1979a, 1979b). Compared to Sprague-Dawley rats, the pattern of HRP localization was similar in the aortic endothelium of Wistar rats with norepinephrine induced high blood pressure, however the tracer labelled the intercellular clefts and subendothelial space in lower density and reaction product was not detected in these compartments of the normotensive control animals with the HRP dose used. The cause of difference in the permeability of aortic endothelium between the two animal strains is not clear and awaits elucidation.

Heterogeneous labelling of interendothelial clefts by HRP correlated well with heterogeneous organization of cell junctions visualized by freeze-fracture replicas of aortic endothelial cells prepared both from normotensive controls and from animals with norepinephrine induced high blood pressures. While fracture faces of the lateral plasma membrane of some endothelial cells showed continuous single- or double-stranded zonular tight junctions, other endothelial cells were interconnected by focal macular type tight junctions (Figs. 2 and 3). In these macular tight junctions, the individual junctional strands formed loops or appeared as free-ending elements and were found far away from each other (Hüttner and Peters, 1978). We propose that sites of preformed discontinuities in the line of tight junctions between some endothelial cells may represent preferred interendothelial routes of increased permeability during periods of acute hypertension.

Chronic arterial hypertension

The three models of chronic hypertension produced in Wistar rats and selected for these studies are associated with different plasma renin and plasma mineralocorticoid levels (Gabbiani et al. 1979): 1) Aortic ligation between the renal arteries results in high plasma renin and high

plasma aldosterone levels during the early (7-10 day) phase of hypertension but normal level of these hormones during the late (40 day) phase of hypertension; 2) unilateral nephrectomy and NaCl-rich diet results in continuously low levels of plasma renin and aldosterone; 3) unilateral nephrectomy, 0.9% NaCl as drinking fluid and subcutaneous administration of DOCA again results in continuously low levels of plasma renin and aldosterone, however, the level of the circulating exogenous mineralocorticoid (DOCA) is obviously high during both the early and late phases of hypertension.

In the three selected models of hypertension, endothelial cell changes were strikingly different (Gabbiani et al. 1979): 1) Early after aortic ligation endothelial cells were hypertrophic and the permeability of aortic endothelium to HRP was markedly increased as compared to controls. Late after aortic ligation endothelial cell hypertrophy and increased permeability to HRP regressed in spite of persisting high blood pressure; 2) both early and late after unilateral nephrectomy and NaCl-rich diet the endothelial cells were similar to controls and there was no evidence of increased permeability to HRP; 3) early after unilateral nephrectomy, 0.9% NaCl as drinking fluid and subcutaneous administration of DOCA, endothelial cells were hypertrophic and the permeability of endothelium to HRP increased similar to that found early after aortic ligation. These changes however did not regress but rather progressed by the late stage of this hypertensive model. Summerizing the observations on the three chronic hypertensive models it is evident that hypertrophy and increased permeability of aortic endothelium to HRP was more marked during hypertensive situations associated with high level of circulating endogenous or exogenous mineralocorticoids than during those associated with low level of these hormones (Gabbiani et al. 1979). Preliminary experiments indicate furthermore, that in these situations there is a higher turnover of endothelial cells when compared to controls or to other hypertensive models (Schwartz and Benditt, 1977; Gabbiani et al. 1980), suggesting that different mechanisms of hypertension produce different rates of endothelial cell replication, which in turn result in morphologically and functionally different endothelial cell layers.

On the basis of these observations it is logical to postulate that circulating mineralocorticoids and/or increased turnover of endothelial cells may affect cell junction morphology and the barrier function of endothelium. Freeze-fracture electron microscopy indeed revealed differences in the organization of tight junctions between various hypertensive groups (Hüttner et al,. 1979). Early after aortic ligature, (a hypertensive situation associated with increased endothelial cell turnover and increased permeability), lateral endothelial plasma membranes contained many small linear aggregates of intramembrane particles. These structures probably represent early steps in cell junction assembly (Montesano et al. 1975; Porvaznik et al. 1979), thus their presence was consistent with neoformation of cell junctions in hypertension. Tight junctions furthermore were commonly macular type although multistranded at this time. In contrast, late after aortic ligature and late after uninephrectomy and NaCl-rich diet, (hypertensive situations associated with normal endothelial cell turnover and absence of increased permeability), zonular multistranded tight junctions dominated the lateral endothelial plasma membranes. In the uninephrectomy, 0.9% NaCl as drinking fluid and DOCA model, (characterized by progressive permeability changes), small aggregates of intramembrane particles interpreted as developing cell junctions were again particularly numerous (Fig. 4) and tight junctions were commonly discontinuous and poorly organized.

Fig. 4. Lateral plasma membrane of aortic endothelial cell from hypertensive Wistar rat at 40 days after unilateral nephrectomy, 0.9% NaCl in drinking fluid and DOCA. Honey-comb-like aggregates of intramembrane particles visualized on P-face represent a step in cell junction assembly. Bar: 0.2 μm.

It may be hypothesized that an increase in tight junction formation in aortic endothelium is an adaptative change in chronic hypertension; this increase may contribute to the barrier function of endothelium in certain hypertensive situations, but it is not efficient to produce a "tight" endothelium when the junctions are mostly developing or discontinuous macular type. This proposal is consistent with results of comparative studies on various epithelia establishing correlation between intercellular permeability and the complexity of tight junctional network under both normal (Friend and Gilula, 1972; Claude and Goodenough, 1973) and experimental conditions (Wade and Karnovsky, 1974; Humbert et al., 1976).

Discontinuous organization of tight junctions however is probably not the sole factor explaining increased permeability to HRP of the aortic endothelium in hypertension. Intramembrane networks of tight junctions visualized by freeze-fracture electron microscopy may simply represent a scaffold for adjacent membrane sites interconnected by chemical bonds. Permeability changes have been reported in epithelia in face of structurally unaltered tight junctional networks (Martinez-Palomo and Erlij, 1975). The result of our studies nevertheless suggest that increased permeability of the arterio-capillary segment in hypertension is based on opening of interendothelial routes preferentially at sites of structurally discontinuous cell junctions. Discontinuities in the line of tight junctions are preformed structures between some arterial endothelial cells that may change in size during periods of acutely elevated blood pressure or they represent sites of defective de novo tight junction assembly that may serve as routes of intercellular permeability in some chronic hypertensive situations.

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# MICROCIRCULATION AND PERMEABILITY OF MICROVESSELS UNDER THE CONDITION OF PHYSICAL LOAD

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The problem of hemodynamic performance in the terminal parts of the vascular bed /along with the traditional analysis of systemic hemodynamic mechanisms/ attracts nowadays considerable attention to studies of blood circulation. This is largely due to the understanding of the fact that systemic mechanisms of hemodynamics play only a very general role in circulation, i.e. the role of distribution of blood among working organs in the body; these systemic mechanisms alone are unable to regulate the interaction between blood and parenchyma of organs /Kuprianov, Karaganov, Kozlov, 1975; Chernukh et al., 1975/. Multiple studies demonstrated that the ability of local blood flow regulation belongs to the system of microvessels in the organs, i.e. the very system of vessels which ensures the process of blood microcirculation in the body /Zweifach, 1961, Kuprianov et al., 1975/.

In the present report two main points are being discussed. First, how high the lability of the microcirculatory system in man is and how strong the reaction of its particular sections is when the organism is subject to extreme stress. Second, what happens to the microcirculation in muscles under exercise hyperemia.

Different states of blood microcirculation /in comparison with changes in parameters of central hemodynamics/ were studied in ordinary men 17 to 25 years old /control group/ and in sportsmen of high qualification /stayers and sprinters/ after submaximum physical load. The test load was given on veloergometer; the load rate was increased stepwise. The states of central hemodynamics were controlled by the pulse rate and the velocity of blood circulation, measured by oxyhemogramm. In studies of the microcirculation the microphotocapillarymetry of the finger nailfold hand and the forearm skin was used. The biomicrophotoscopy of vessels in the in the conjunctiva was also employed. The main parameters of microvessels, i.e. their diameter and density, were calculated in order to estimate the changes taking place in them.

In both groups number of bloodperfused capillaries in skin and conjunctive bulbi decreased in response to submaximum load; this phenomenon was stronger in sportsmen than in nontrained persons. Along with the decrease of functioning capillary

density under physical load the phenomenon of vasodilatation was observed; its average value was about 13% over status control. In the recovery period the venous part of the skin capillaries was found to be most labile.

The data of biomicroscopy of conjunctiva demonstrated that after submaximal physical load the diameter of the vessels increased in all parts of the microcirculatory bed /Fig. 1/. Thus, the diameter increased by 19.5%, 19.8%, 10.6%, 36.3% and 32.2% of the arteriols, precapillaries, capillaries, venules and veins, respectively. It should be noted that vasodilatation of venous microvessels was more pronounced which speaks for the significant role of capacity vessels in performing a response reaction of the organism to physical load.

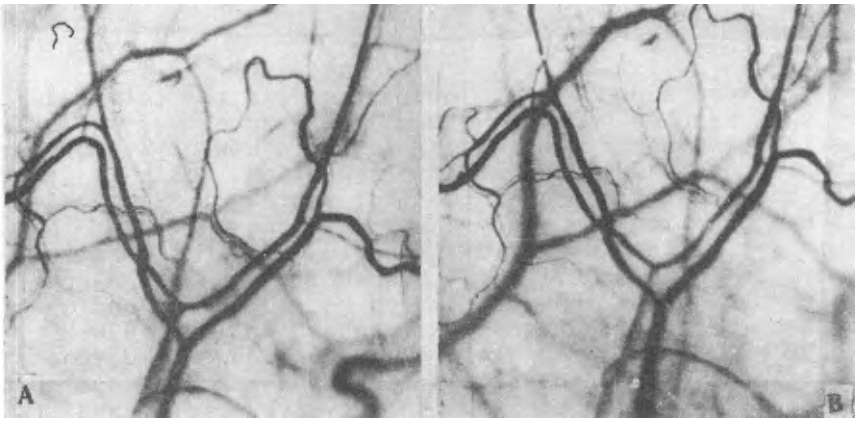


Fig. 1: Microvessel changes in conjunctiva bulbi of man under the condition of physical load. /Biomicrophoto; ob. x 4.5/.
A - at rest; B - after submaximum physical load.

Changes in central hemodynamic parameters closely correlate with changes in microcirculation /Fig. 2/. Within the restitution period, the restoration of the initial level of central hemodynamic parameters occures heterochronically to changes in the microcirculation. Such heterochronism is a typical peculiarity of the restitution period.

The comparative analysis of microcirculatory changes in the control group and in sportsmen /Fig. 3/ demonstrate directed morphofunctional transformation of the microvascular system in the latter. This transformation is based on changes in structural parameters of microvessels and also on modification of specific features of their reaction to extreme influence.

It is widely accepted nowadays that the work hyperemia in skeletal muscle is characterised by the vasodilatation of intraorgan vessels, by increased number of functioning capillaries and by higher rate of blood flow through the organs /Folkow, Halicka, 1968; Folkow, Neil, 1971/. All the same,

a common concept of quantitative changes in the microvascular bed under development of exercise hyperemia does not exist.

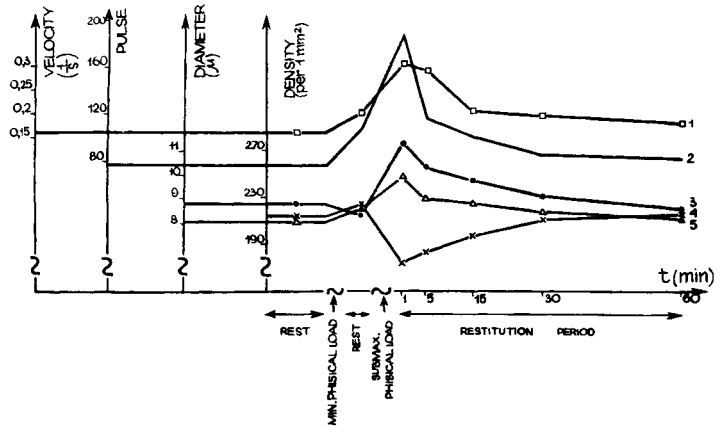


Fig. 2: Dynamics of changes in microvessels and central hemodynamic parameters in man under the condition of physical load.
1 - velocity of blood flow; 2 - pulse; 3 - diameter of nailfold capillaries; 4 - density of functioning capillaries in the skin; 5 - diameter of capillaries in conjunctive bulbi.

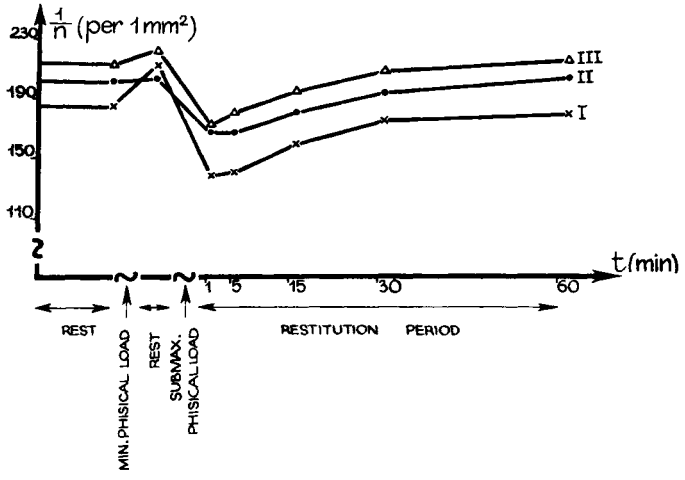


Fig. 3: Changes in skin capillary density under condition of physical load.
I - control group; II - sprinters; III - stayers.

Studies of intramuscular microvessels, under the condition

of rest and during exercise hyperemia, were undertaken in m. quadriceps femoris of cat and white rat. Changes in parameters of microvessels under exercise hyperemia were observed by biomicrophotography and by traditional angiologic methods.

The biomicroscopic data demonstrated that immediately after contraction of muscle vasodilatation in all parts of the microvascular bed appeared; simultaneously, the blood supply of the vessels and transorgan blood flow increased /Fig. 4/. Changes in diameter of microvessels show that already 30 sec after muscle contraction maximal arteriolar dilatation occurs; a little later /at the end of the first minute/ maximal dilatation of capillaries and venules appears. Then the vasodilatation gradually fades, first in arterioles and later in venules /Fig. 5/.

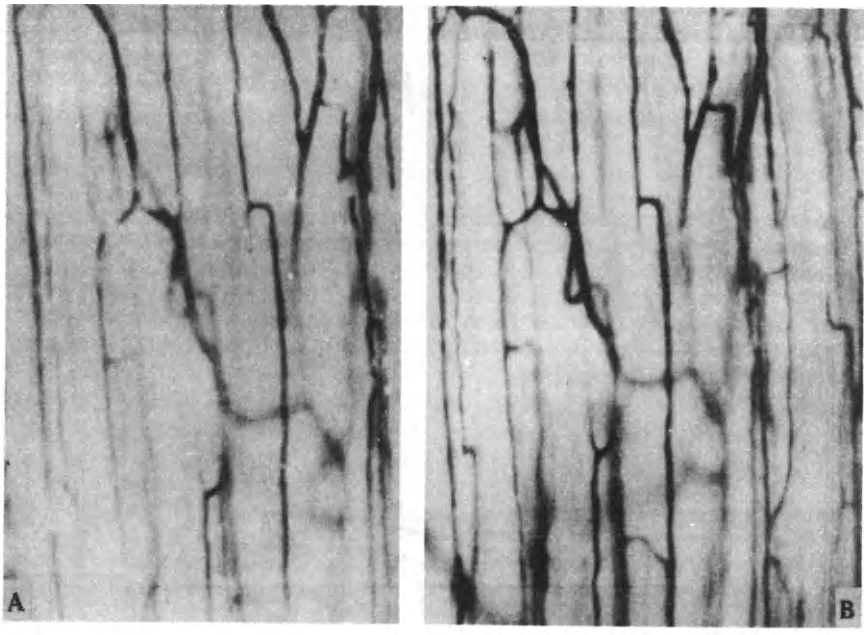


Fig. 4: State of microcirculation in m. quadriceps of rat at rest /A/ and under the condition of work hyperemia /B/. /Biomicrophoto, ob. x10, oc. x10/.

The degree of changeability of vessels in arterial and venous parts of the microvascular bed are not the same. Thus, from 14 arterioles, having an initial diameter of 30 to 45 $\mu$m, 11 vessels were found dilated and the average increase of the diameter was about 14.5%. From 30 venular vessels, with diameters of 35 to 50 $\mu$m, 28 demonstrated an average diameter increase of 28% within the postcontraction period.

In vivo information about the dynamics of the density of functioning capillaries was obtained only from the surface layers of the muscle /the projectional morphometric plane was oriented along the muscle fibers/. Under conditions of rest

there were $42 \pm 5.2$ capillaries per sq. mm in m. quadriceps; under exercise hyperemia this figure grew up to $64 \pm 6.1$; i.e. by 51%. The increase in functioning capillary density reached its maximum already within the first minute of the post-contraction period and stayed at that level for approximately 5 min, after which it began to decrease gradually, coming back to its initial level in 20 min.

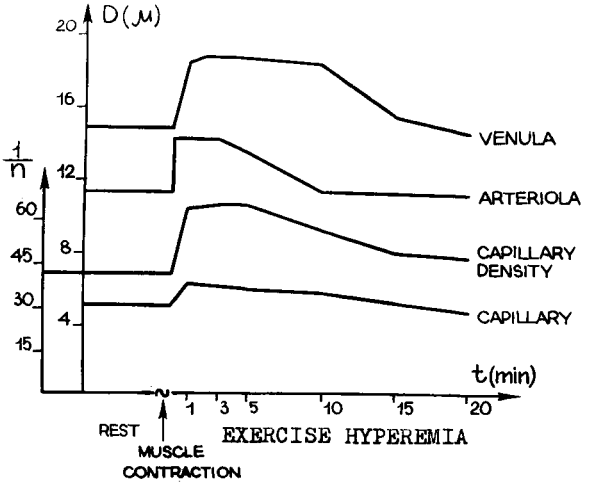


Fig. 5: Dynamics of changes in microvessel parameters in m. quadriceps of rat under condition of exercise hyperemia.

The maximal changeability of flow in muscles under rest was observed in the so called transversal short capillaries; this changeability is manifested in decreased or increased velocity of erythrocyte movement as well as in the reversal of flow direction. This phenomenon can be explained by a special strategic state of transversal capillaries.

Injection methods, revealing all capillaries in a muscle, and staining muscle cross-sections with benzidine, revealing only functioning capillaries, make it possible to estimate reserve abilities of the capillary bed in a muscle under rest. Calculations of the capillary density in m. quadriceps of the white rat demonstrated that the number of capillaries in the zone of red muscle fibers /RMF/ is 2184 per sq. mm of a cross-section area, while in the zone of white muscle fibers /WMF/ this number is 1092 per sq. mm. The density of functioning capillaries in the same muscles under rest was considerably lower, in RMF 896 per sq. mm, while in WMF 336 per sq. mm. It means that reserve abilities of the capillary bed in a skelatal muscle are rather broad, which ensures a comparatively high lability and adaptation of the capillary flow to changing working conditions of the muscle.

During exercise hyperemia functional capillary density in WMF of white rat rises by 57%, while in RMF by 19% /Fig. 6/. In m. quadriceps of the cat similar changes are more signifi-

cantly expressed.

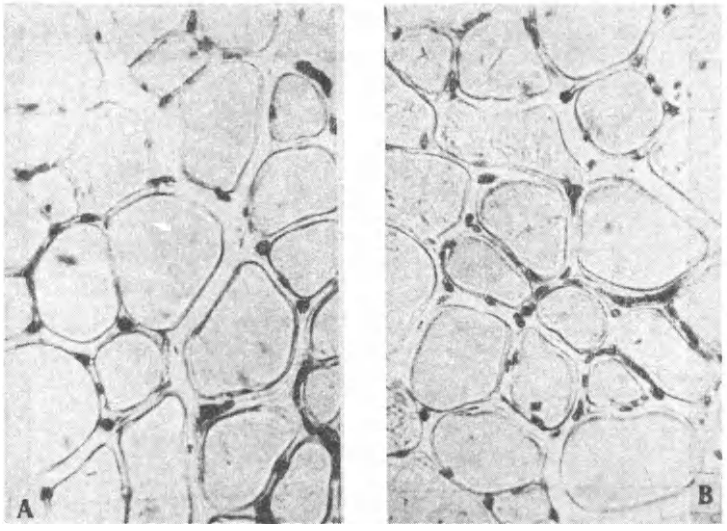


Fig. 6: Vascularisation of red muscle fibred in m. quadriceps of rat at rest /A/ and during exercise hyperemia /B/. /Benzidine: ob. x40, oc. x8/.

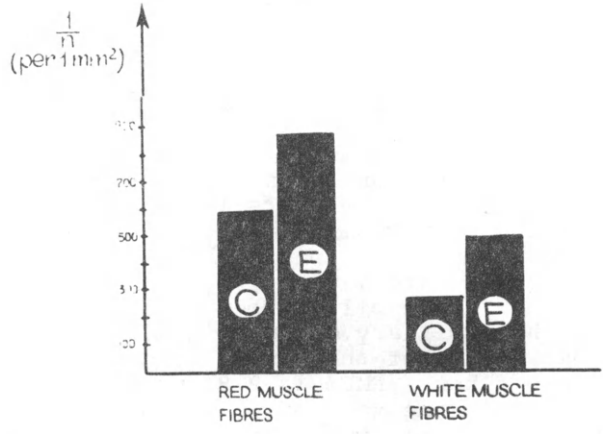


Fig. 7: Functional capillary density /$^1/_n$/ in m. quadriceps of cat at rest /C/ and during exercise /E/ hyperemia.

From hemodynamic point of view, the microvascular bed of a muscle represents a complex multichannel system, composed of vessels of different diameters, which are interconnected with parallel and consecutive links. The most typical pecu-

liarity of hemodynamics in microvessels is the high lability of capillary blood flow in vivo. This changeability of capillary blood flow, as it was shown in our previous studies /Kozlov et al. 1979/, is a necessary condition for microcirculatory adaptation to different needs of organs during different functional states. Since the volume of the microcirculatory bed is larger than the volume of blood circulating in it, it is possible to consider this on the basis of spontaneous lability of the capillary blood flow by which adaptive reactions of the microvessels system take place. There is a rival manner of interaction between different capillaries. The biomicroscopy data demonstrate that the involvement of additional reserve capillaries into the blood flow leads to a temporal stabilization of the circulation and flow direction in longitudinal capillaries, because there exists a limited spontaneous lability of blood flow in transversal capillaries /Fig. 8/.

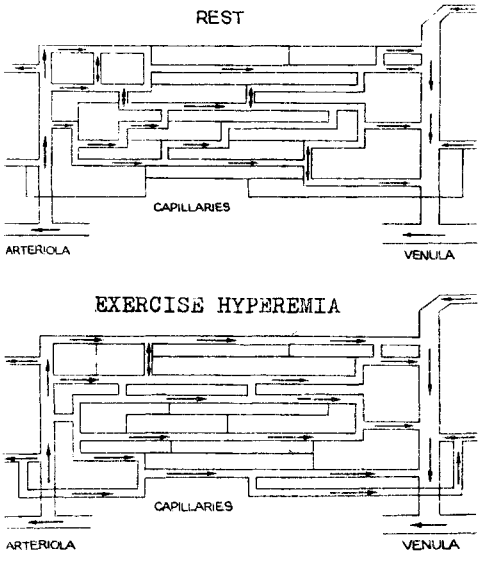


Fig. 8: Alteration of blood flow direction in skeletal muscle under condition of exercise hyperemia.

The process of transcapillary blood flow is closely connected with the transcapillary transport substances. The latter is one of the most important resulting factors of transorgan circulation of blood.

The permeability of microvessels in m. quadriceps of white rat was studied with luminescent biomicroscopy during rest and exercise hyperemia. The fluorescein-labelled bovine globulin /FG/ was used as an indicator of permeability. The quantitative luminescent analysis was performed with a microspectrofluorometer.

It was shown that the FG concentration in the muscle during exercise hyperemia was 40 to 45% higher than that during rest; these data demonstrate the increase of transendothelial transport of substances. Measurements of the FG concentration close to a single capillary demonstrate that the permeability

of microvessels increases during exercise hyperemia /Fig. 9/.

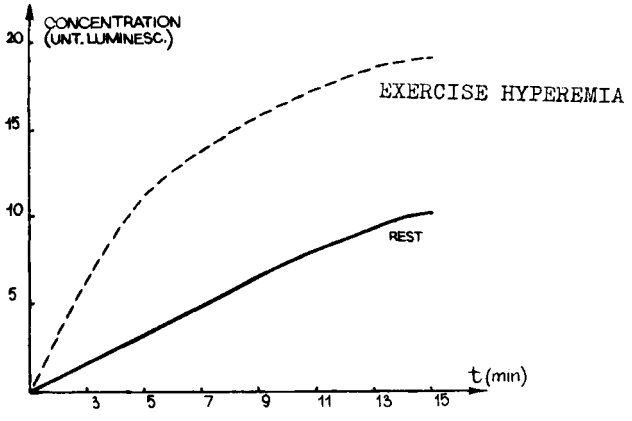


Fig. 9: Changes in concentration of globulin labelled with fluoresceine isotiocynate near a single capillary in m. quadriceps of rat.

The obtained experimental data permit to consider exercise hyperemia not only as increased perfusion of dilated intramuscular microvessels with arterial blood. Exercise hyperemia proves to be a directed process of urgent adaptation of the microcirculation in skeletal muscle; this process is based on changes in transendothelial transport of substances in accordance with grown needs of an organ, caused by intensification of transcapillary blood flow and increase of the microvessel permeability.

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# TRANSCAPILLARY FLUID FLUX INDUCED BY TRANSIENT HYPERTONIC DISTURBANCES

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A change in the osmotic pressure of the plasma effects a flow of tissue fluid. Effros used an in vivo procedure to quantify the osmotic extraction by a transient hypertonic disturbance. As discussed later, the filtration coefficient so computed from the concentration changes in venous blood samples may be 3 to 4 times the coefficient obtained by the alteration of hydrostatic pressure. Is this large difference attributable to the opening of more pores by the hypertonic disturbance for the flow of tissue fluid? Could the computation scheme overestimate the osmotic transient level? To avoid the latter uncertainty, we measured the osmotic transient in an isolated lobe preparation to quantify the flow of tissue fluid at the capillary level to evaluate directly the filtration parameters of the endothelium. A model analysis was developed to simulate the action by the hypertonic bolus on the initial fluid extraction and the role of tissue dehydration on the weight recovery.

## METHOD

Mongrel dogs with a weight 15 to 20 Kg were anesthetized by Pentobarbital (30 mg/Kg) and then exsanguinated. After the isolation of the left lower lobe, its artery, vein, and bronchiole were cannulated. The weight of this lobe was measured and identified as the wet tissue weight Wt.

The system to perfuse the lobe had an arterial reservoir to maintain a constant arterial pressure of 17cm$H_2O$ and a venous reservoir for a venous pressure of 3cm$H_2O$. The temperature of the perfusing blood is maintained at 37°C. Any transient changes in the arterial and venous pressure at sites 3 cm away from the lobe were monitored by Statham pressure transducers. The lobe was placed on a lucite stage for continous monitoring of the weight by a Statham force transducer. After the lobe was connected to the perfusion system it was inflated and maintained at an alvelolar pressure of 8cm$H_2O$.

A thin catheter was inserted through the arterial catheter to a distance 7 cm away from the artery. This thin catheter was connected to a 1ml syringe mounted on a Sage Automatic Injector. As the injector was activated, it infused rapidly 0.85ml of solution into the flowing arterial blood. This rapid infusion raised the pressure and induced a transient weight change as shown on the left panel of Fig. 1. To reduce this change which is about half of the maximum weight change induced by hypertonic disturbance, another catheter was inserted 5cm upstream of the one for injection to withdraw the same volume of blood from the arterial catheter. Both the injection and withdrawal syringes were operated by the same Sage

injector. This compensation maneuver significantly reduced the weight perturbation by the injection procedure as shown on the right panel of Fig. 1. The middle curve shows the weight change resulted from the injection of 0.85ml of saline and the withdrawl of 0.85ml of blood. The persistant weight change after the initial gain might be related to the lower viscosity of the saline bolus. A slight decrease in venous pressure (less than 0.5cm$H_2O$) was observed.

A 2volume of 0.85ml of 0.8M NaCl or 1.6M urea was injected in a way similar to the saline injection after an ½ hr. of stabilization of perfusing the lobe. The amount of the hypertonic disturbance over the isotonic level, $\delta$, is 1100ml·mosmol/Kg. Fig. 2 shows the weight change after the injection of a hypertonic NaCl bolus. Superposed on the same figure is the weight change associated with the saline injection. The difference between these two curves is taken as a measure on the flow of tissue fluid by the osmotic transient.

Right after the injection of the hypertonic bolus, some 60ml of the outflow from the venous reservoir was collected to prevent the recirculation of the hypertonic bolus and the elevation of the tonicity of the perfusion. The amount being collected divided by the time of collection yielded the flow. A 60 ml of normal blood was added to maintain the blood volume.

The hypertonic disturbance of NaCl and urea was performed every ½ hr. for 3 hours until all reserved blood was exhausted in supplementing the blood volume.

## THEORETICAL ANALYSIS

The fluid flux Jf induced by the hypertonic disturbance is assumed to be governed by the generalized version of the Starling hypothesis:

$$Jf = KS(Pc - Pt - \sigma(\Pi pl - \Pi t)) \qquad (1)$$

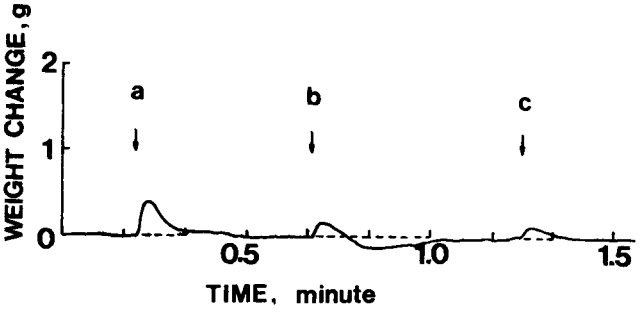


Figure 1. The weight change induced by the injection procedure: a) the injection of 0.85ml of blood; b) the injection of 0.85 ml of saline and the simultaneous withdrawal of .85ml of blood 5 cm upstream of the injection site; and c) the injection of .85ml of blood withdrawal. The arrow indicates the time of injection These changes reflect changes in vascular volume which are reduced by the compensating withdrawal.

where K is the filtration coefficient, S is the filtration surface area, and $\sigma$ is the reflection coefficient. Pc is the hydrostatic pressure in the capillary and Pt is the interstitial fluid pressure. The responsible osmotic pressure $\Pi pl$ and $\Pi t$ are those of the plasma and the tissue fluid respectively.

In the present transient experiment, the perturbation on arterial and venous pressures by the injection are less than 1 $cmH_2O$. Therefore Pc is considered as constant in the analysis of the fluid flux. To account for the effect of volumetric change of a compliant tissue compartment, we set the change in tissue pressure as

$$Pt - Pto = E(W-Wo)/Wf \qquad (2)$$

where W - Wo reflects the increase in tissue volume and E describes the rigidity of the interstitial space Wf from which the fluid is extracted.The o subscript of a quantity refers to its baseline value.

As the injected hypertonic bolus flows through the arterial branching system, it is dispersed by the nonuniform flow. Based on our indicator dilution study on the mesenteric microvasculature (Nellis and Lee) we take the change of plasma osmotic pressure at the capillary level as

$$\Pi pl - \Pi plo = A\, e^{-\beta(t-ta)}\, 1(t-ta) \qquad (3)$$

where ta is the time between the injection of the hypertonic bolus and its appearance at the capillary and 1(t) is a unit step function. The decay constant $\beta$ characterizes the dispersion of the hypertonic bolus.

Effros showed that the flow of tissue fluid is of a very hypotonic solution. As a first approximation, we may consider that the flow carries no solute. Thus the total solute in the tissue compartment, Wf, remains constant, i.e.

$$\Pi t\ (Wf + W - Wo) = \Pi to\ Wf$$

where the osmotic pressure is considered as linearly dependent on the solute concentration. As the fractional weight change is small, we linearize the equation above to

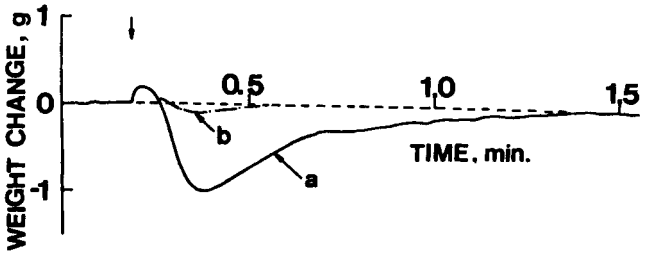


Fig. 2. The change in weight following the injection of 0.85ml of .8M NaCl. The saline curve is superposed in the figure to indicate the change in vascular volume.

$$\Pi t - \Pi to = \Pi to\ (W - Wo)/Wf \qquad (4)$$

For the base line condition ($t<ta$) that the weight is constant before the hypertonic injection, we have

$$W = Wo \qquad (5)$$

and $dW/dt = KS(Pc-Pto - \sigma(\Pi plo - \Pi to)) = 0$

Substituting Eq. (2)-(4) into Eq. (1), and recognizing $\rho Jf = dW/dt$, we obtain the following first order differential equation on the change of the lobe weight.

$$dW/dt + \alpha(W - Wo) = -\sigma KSA\rho\ e^{-\beta(t-ta)}\ 1(t-ta) \qquad (6)$$

where $\alpha = (E + \sigma\Pi to)\rho KS/Wf$

With the initial condition that $W = Wo$ at $t = ta$, the solution of the governing equation is

$$W - Wo = \sigma KSA\rho(e^{-\alpha(t-ta)} - e^{-\beta(t-ta)})\ 1(t-ta)/(\alpha-\beta) \qquad (7)$$

As $\delta$ is the amount of hypertonic disturbance injected at the pulmonary artery, the conservation of solute leads to

$$\delta = Q(1-H) \int_{ta}^{\infty} A\ e^{-\beta(t-ta)}\ dt = Q(1-H)A/\beta \qquad (8)$$

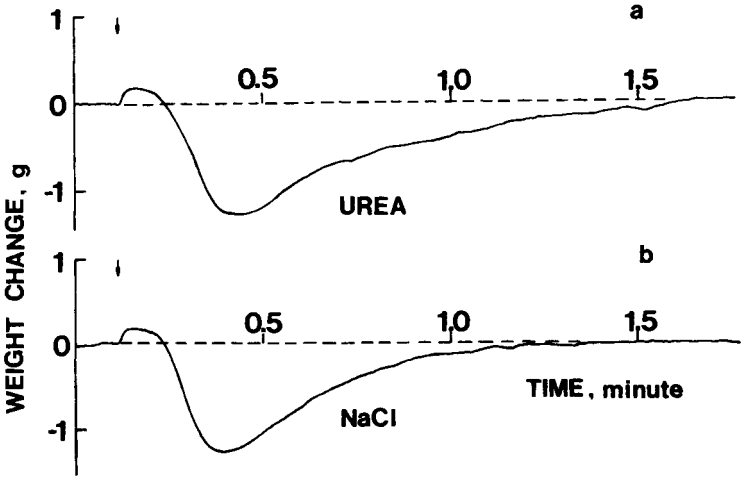


Fig. 3. Similar weight changes resulted from the injection of 0.85 ml of .8M NaCl and of 1.6M urea.

where Q is the total blood flow and H the hematocrit. This equation determines A.

As the maximum weight change is a readily identifiable quantity from the experimental record, Eq. (7) and (8) yield a relation between $\Delta Wm$ and $\delta$

$$\Delta Wm = \frac{\sigma KS\,\varsigma\delta}{Q(1-H)} \quad (e^{-\alpha tm} - e^{-\beta tm})/(1 - \alpha/\beta) \qquad (9)$$

where tm is the time from the initiation of weight change to the maximum one. For the range of $\alpha tm$ and $\beta tm$ encountered in our experiment, the ratio of the last two factors in the equation above is approximately 1.4. We have a simplified equation to compute the filtration coefficient

$$\sigma KS/Wo = 1.4\Delta Wm\ Q\ (1 - H)/\ (\delta Wo\,\rho) \qquad (10)$$

## RESULTS

Fig. 3 shows the weight change due to NaCl disturbance and that of the urea one. No significant difference is indicated. This is in contrast to the finding of Effros who found the NaCl hypertonic bolus might alter the deformability of red blood cells and impede the blood flow. It is noted that the present hypertonic disturbance over the istotonic level is only one third of that used in Effro's experiment.

From these experimental records, we identified the maximum weight change during the osmotic transient, $\Delta Wm$, the time from an initial weight change of the transient to the maximum, tm, and the time for which the weight change is $\Delta Wm/e$, te. These measured quantities for seven lobes are presented in the first three rows of Table 1. Based on these values, we used an iterative procedure to find $\alpha tm$, $\beta tm$ which made Eq. (7) fit best with the experimental data. The filtration coefficient expressed in terms of $\sigma KS/Wt$ and the maximum hypertonic solution disturbance at the capillary level A were computed. These computed values are presented in the last four rows of Table 1. The flow rate of these experiments was 140± 14ml/min. and the wet tissue weight Wt is 28 ±3g.

A comparison between the experimental measured curve and its theoretical matching is shown in Fig. 4. For all comparisons, the correlation coefficient is .97 or better.

Based on this modeling, the effect on the weight during passage of the hypertonic disturbance and its subsequent recovery by the dehydrated tissue is resolved in two curves as shown in the third panel of Fig. 4. A complete passage of the hypertonic bolus within the first 10 seconds is indicated. The fourth panel shows the rapid decrease in the flow of tissue fluid and its trend in the recovery of tissue weight.

The value of $\Delta Wm/Wt$ measured at the end of the experiment increases slightly to 3.7 ± 0.6 (NaCl) and 3.0 ± 0.8 (urea). te/tm decreases to 3.3 ± .6 and 4.1 ± .7 respectively. However, these changes are not statistically different ($P>0.05$) from the initial values given in Table 1. Together with a slight decrease in flow, these findings suggest an increase in filtration coefficient.

## DISCUSSION

The relation for $\alpha$ in Eq. 6 allows us to evaluate the ratio Wf/Wo. Let us take E = 100cm $H_2O$ (Mitzner and Robotham), $\Pi to$ = 300mosmol/kg, and $\sigma$ = o.3. From the experimentally determined value of $\alpha tm$ and $\sigma KS/Wo$ we find that the fluid space in the tissue available for the osmotic extraction by NaCl disturbance is 34%. This value is comparable to the chloride space of dog lungs found by long term tracer experiments (Levine

Table 1, The Filtration parameters describing the Osmotic Transient.

| | NaCl | Urea | Unit |
|---|---|---|---|
| $\Delta Wm/Wt$ | 3.2 ± .07 | 2.7 ± .03 | % |
| tm | 8.8 ± 1.2 | 9.2 ± .7 | s |
| te/tm | 4.0 ± 1.0 | 4.3 ± 1.0 | |
| $\sigma KS/Wt$ | 51 x $10^{-6}$ | 45 x $10^{-6}$ | ml/(s·mosmol/Kg·g) |
| $\alpha tm$ | .41 | .36 | |
| $\beta tm$ | 1.87 | 2.15 | |
| A | 180 | 200 | mosmol/Kg |

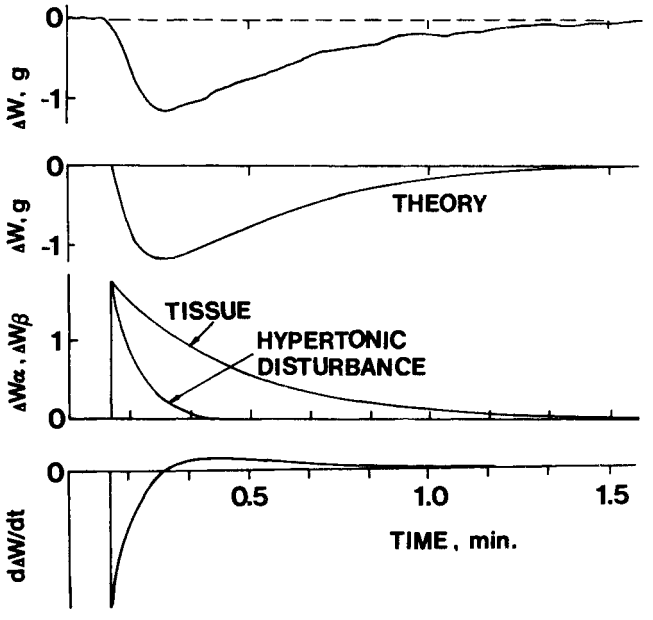


Fig. 4. The Comparison Between Experimental and Theoretical Results. a) Experimental weight change, b) The theoretical prediction: $\sigma KS/Wt=40x10^{-6}$ml/(s·mosmol/Kg·g), $\alpha tm=.44, \beta tm=1.90$, tm=8.7 sec., c) The two exponential functions in Eq. 7 whose difference is the weight change., d) The flow of tissue fluid induced by the osmotic extraction

et al.). Thus the extraction space of the tissue, Wf, may indeed be a significant part of the interstitial fluid space.

Let us compare the filtration characteristics obtained by the osmotic transient with that of the alteration in hydrostatic pressures. The endothelium barrier and cell junctions for both filtrations are idealized as a membrane with heterogenous pores. When there is a gradient in the hydrostatic pressure across the membrane, all pores having a diameter larger than water molecules will allow the passage of water. Thus the filtration coefficient Kf identified by a change in hydrostatic pressure reflects the contribution from all heterogenous pores. In the osmotic transient, the pores having a diameter much larger than the solute molecule do not exhibit a fluid flow because their reflection coefficient is zero. Thus the computed filtration K should reflect the contribution from a fraction of the total heterogenous pores and hence be smaller than Kf. Taking $\sigma$ = .3, we find that the value of KS/Wt from our results is 2.4 ml/(h·cm$H_2O$·100 g wet lung tissue). This value is slightly smaller than the filtration coefficient reported by Gaar et al. (3.1 ml/(h·cm$H_2O$·100g) and our previous result (Lee and Lee, 5.1ml/(h·cm$H_2O$·100g)). To convert Effros' result, we take the wet tissue weight of the lung to be 173g which is 71% (Staub) of the reported lung weight. The value of $\sigma$KS/W(=.216x$10^{-3}$ ml/(s·mosmol/Kg·g)) from Effros' work yields a filtration coefficient KS/Wt to be 14 ml/(h·cm$H_2O$·100g). This larger value may indicate either the opening of new pores in Effros' experiment or some uncertainties in computing the filtration characterisitcs of the capillary from venous samples.

For our experiments, the maximum hypertonic disturbance imposed by the sample at the injection site is 1,300mosmol/kg. Due to the dispersion through the arterial system, this maximum is reduced to 180 mosmol/Kg for NaCl disturbance. We may use similar procedure to evaluate the transport of hypertonic bolus in Effros' experiment. It is noted that the mean transient time for the arterial system is ta+1/$\beta$. Thus the product $\rho$Q/($\beta$Wt) is directly related to the fractional volume of arterial vasculature. This product is .39 for the NaCl experiment. If this fractional volume is similar to that of Effros' experiment, we estimate from his results that $\beta = 0.54s^{-1}$. The correspondent maximum hypertonic disturbance, A, is 367 mosmol/Kg at the level of capillary. Thus the transport of the hypertonic disturbance can be described by a reduction in the osmotic pressure from an original maximum of 3,700mosmol/Kg to 367mosmol/Kg at the capillary level and then to 80mosmol/Kg in venous samples as the bolus dispersed through the arterial, capillary,and venous system.

From our theoretical analysis, the maximum fluid flux is estimated to be 0.01ml/(s·g of wet lung tissue). The correspondent osmotic pressure is 180 mosmol/Kg. The maximum flux computed from the venous sample by Effros is 0.02ml/(s.g). If we assume that the dispersion imposed on the dilution by the flow of tissue fluid is similar to the dilution of the osmolality from 367 to 80mosmol/Kg, the maximum fluid flux at the capillary can be as high as 0.09ml/(s.g). It is noted that the flow rate in Effros' experiment is 0.21ml/(s.g ). It is probable that this large flow of tissue fluid can affect the vascular flow and the dispersion of indicators.

In the present model analysis, we assume a minimal solute exchange across the capillary endothelium. Two observations support this assumption. First it takes less than 10 seconds for the hypertonic bolus to pass through the capillary. Even though the maximum osmotic pressure is 180mosmol/Kg, the short duration limits significantly the solute flux. Secondly, the maximum change in tissue osmotic pressure due to the disturbance is at most 20mosmol/Kg (Eq. 4). It is probable that the solute flux induced by this small pressure may be negligible. For the experiment

on sustained hypertonic infusion (Taylor and Gaar), the time scale is much longer than the present transient experiment. The incorporation of Kadem-Katchalsky equation to account for the long term solute flux into the present model may provide a more accurate estimation on the reflection coefficient from their experiment.

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# THE EFFECT OF ALBUMIN ON THE SIZE-LIMITING STRUCTURES DETERMINING TRANSCAPILLARY EXCHANGE

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## INTRODUCTION

The removal of plasma proteins from the perfusate causes an increased permeability and decreased selectivity of the walls of the continuous capillaries of mammalian muscle and frog mesentery (Landis and Pappenheimer 1963, Mason et al. 1977). This observation is an important clue to the nature of the size-limiting structures within the pathways for water and hydrophilic solutes across the capillary wall.

In individually perfused capillaries of frog mesentery we have shown that a fivefold increase in the hydraulic conductivity of the capillary wall is rapidly reversed by the addition of albumin, γ globulin or hemoglobin to the capillary perfusate (Mason et al. 1977). We have also examined the ultrastructure of capillaries perfused without albumin and found no conspicuous abnormalities (Mason et al. 1979). We concluded that albumin combines reversibly with some electron-lucent component of the capillary wall to increase the frictional resistance of transcapillary channels to water and hydrophilic solutes.

Two hypotheses have been proposed to account for the interaction of albumin with the capillary wall. One states that the protein is absorbed onto the walls of transcapillary pathways thereby reducing the effective channel dimensions (Fig. 1A) (Landis and Pappenheimer 1963, Mason et al. 1977). The second hypothesis states that albumin is a component of a three dimensional network of fibrous molecules which lies within all or part of the transcapillary pathway and determines the selectivity of the capillary wall (Fig. 1B) (Curry and Michel 1980). Such a network has been demonstrated on the surface of the endothelial cell using appropriate staining for acidic glycosaminoglycans (Luft 1966, Shirahama and Cohen 1972). The presence of a three dimensional network formed by the glycoproteins which are part of the plasma membrane of the endothelial cell would also account for the exclusion of ferritin from the lumenal vesicles and lumenal surface of endothelial cells demonstrated by Michel and his colleagues (Loudon et al. 1979). To distinguish between the two hypotheses, I have made measurements of the permeability of the capillary wall to colored solutes in capillaries perfused with, and without albumin in the perfusate, and interpreted the results in terms of the pore and fiber matrix theories of capillary permeability outlined below.

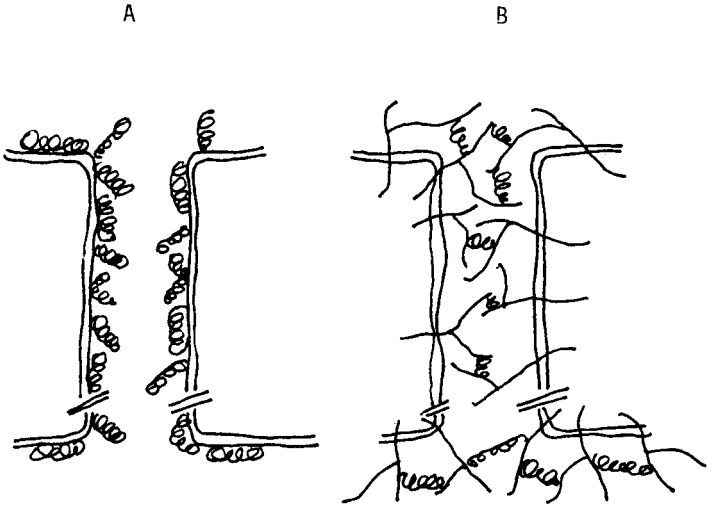


Figure 1. Two mechanisms proposed to explain the effect of albumin on transcapillary pathway structure. A. The absorption of albumin onto the walls of the pathways. B. Interaction of albumin with an endocapillary layer which lines the cell surface including lumenal vesicles and the wide regions of the intercellular cleft and appears to merge with the basal laminae.

## THEORY

Pore Model. The permeability coefficient (P) of a solute with a Stokes-Einstein radius a in a membrane penetrated by cylindrical pores, radius R and surface density n, can be written as:

$$P = \frac{D}{\Delta x} \cdot n\pi R^2 \frac{As}{Ap} \qquad (1)$$

D is the free diffusion coefficient, $\Delta x$ the membrane thickness and As/Ap a measure of the exclusion and hydrodynamic drag on the solute within the membrane (Landis and Pappenheimer 1963). The hydraulic conductivity (Lp) of the membrane is given as:

$$Lp = \frac{n\pi R^4}{\Delta x \eta} \qquad (2)$$

where $\eta$ is water viscosity. It follows from Eq. 1 that:

$$\frac{P^o}{P} = \frac{R^{o\,2}}{R} \cdot \frac{(As/Ap)^o}{(As/Ap)} \qquad (3)$$

where the superscript o designates measurements made in a capillary with

no albumin in the perfusate. The number of pathways (hence n) is assumed to remain constant.

The corresponding relation for the hydraulic conductivities is:

$$\frac{L_p^o}{L_p} = \left(\frac{R^o}{R}\right)^4 \qquad (4)$$

assuming the viscosity change due to the removal of albumin is negligible.

Fiber matrix model. Expressions describing the capillary membrane coefficients in terms of the fiber radius, $r_f$, and fiber length per unit volume, $\ell$, have recently been developed based on Ogston's relations for the exclusion and diffusion of a spherical solute in a fibrous network (Curry and Michel 1980, Curry 1980). Specifically:

$$P = \frac{Ap}{\Delta x} \cdot \phi \cdot Dm \qquad (5)$$

where the membrane partition coefficient

$$\phi = \exp - (1-\varepsilon)\left(\frac{2a}{r_f} + \frac{a^2}{r_f^2}\right)$$

and the membrane diffusion coefficient

$$Dm = D \exp - (1-\varepsilon)^{0.5}\,(1 + a/r_f).$$

The porous region of the capillary has an area Ap whose geometry need not be specified and $\varepsilon$ is the void volume equal to $(1-\pi r_f^2 \ell)$.

The hydraulic conductivity of the membrane is given as:

$$Lp = \frac{Ap}{\Delta x} \cdot \frac{\varepsilon^3}{(1-\varepsilon)^2} \cdot \frac{1}{\eta} \cdot \frac{r_f^2}{4K} \qquad (6)$$

where K is a parameter dependent upon fiber geometry. In the calculations described below its value is taken as 5 (Curry and Michel 1980).

Our calculations using the fiber matrix model have shown that a network of fibrous molecules 0.6 nm in radius, occupying 5% of the fiber volume, will account for the permeability properties of frog mesenteric capillaries to water and compact solutes ranging from 0.2 to 3.5 nm in radius.

## METHODS

To measure P, a capillary in frog mesentery is perfused using two micropipettes to enable rapid switching from a clear perfusate to one containing the colored test solute. A portion of the perfused

capillaries lies along the centerline of a rectangular window (100 μm wide and 400-600 μm long) defined by a substage diaphragm. The light intensity within the window is measured using a photomultiplier tube attached to the camera eye-piece of a dissecting microscope. As colored solute enters the capillary lumen there is a step increase in optical density ΔOD; subsequent diffusion of solute across the capillary wall and into the tissue is measured as a further, initially linear increase in OD with time $(dOD/dt)_o$. The permeability coefficient is calculated as:

$$P = \left(\frac{dOD}{dt}\right)_o \cdot \frac{1}{\Delta OD} \cdot \frac{r}{2}$$

where r is the capillary radius (Michel 1978). The test solutes were Patent Blue V (PBV) measured at a 620 nm, and Dextran 3000 ($D_3$) labelled with F.I.T.C. measured at 490 nm.

RESULTS

In 13 capillaries $P_{PBV}$ was measured in a capillary perfused first with 1 gm% albumin in the frog Ringer perfusate, then with Ringer alone. The results are given in Table 1.

Table 1. PBV Permeabilities

| Capillary No | $P_{PBV}$ | $P^o_{PBV}$ | $P^o_{PBV}/P_{PBV}$ |
|---|---|---|---|
| | cm $sec^{-1}$ x $10^5$ | cm $sec^{-1}$ x $10^5$ | |
| 1 | 3.8 | 5.8 | 1.54 |
| 2 | 7.1 | 13.0 | 1.82 |
| 3 | 2.6 | 3.1 | 1.18 |
| 4 | 8.4 | 17.3 | 2.04 |
| 5 | 3.8 | 7.0 | 1.84 |
| 6 | 5.2 | 7.4 | 1.41 |
| 7 | 3.4 | 7.8 | 2.26 |
| 8 | 43.1 | 68.9 | 1.59 |
| 9 | 3.3 | 3.3 | 0.97 |
| 10 | 3.8 | 8.5 | 2.24 |
| 11 | 7.9 | 6.0 | 0.75 |
| 12 | 2.8 | 3.92 | 1.38 |
| 13 | 0.83 | 1.54 | 1.82 |
| Mean | | | 1.60 |
| ± SEM | | | 0.128 |

There is no relation between individual values of was $P^o_{PBV}/P_{PBV}$ and the magnitude of $P_{PBV}$.

In a second series of experiments measurements of P's for both PBV and D3 were made on each of 9 capillaries perfused with 1 gm% albumin in the perfusate. The mean of the individual values of $P_{PBV}/P_{D3}$ was 7.74 ± 1.04 (SEM). In another 6 capillaries perfused with Ringer alone, the mean ratio $(P_{PBV}/P_{D3})^o$ was reduced to 3.52 ± 0.46*.

*Presented to The Physiological Society, Cambridge meeting, July 1980.

## DISCUSSION

The two parts of Table 2 summarize calculations to test the hypothesis that albumin absorbs onto the walls of transcapillary channels.

Table 2. Pore-Protein Interaction.

A. Removal of albumin increases pore radius from 4.5 to 5.7 nm.

| Permeability Ratio | Calculated | Measured |
|---|---|---|
| $L_P^o/Lp$ | 2.5 | 5 |
| $P_{PBV}^o/P_{PBV}$ | 1.60 | 1.60 |
| $\frac{(P_{PBV}/P_{D3})^o}{(P_{PBV}/P_{D3})}$ | $\frac{6.8}{7.7}$ | $\frac{3.5}{7.7}$ |

B. Removal of albumin increases pore radius from 4.5 to 10 nm.

| Permeability Ratio | Calculated | Measured |
|---|---|---|
| $L_P^o/Lp$ | 25 | 5 |
| $P_{PBV}^o/P_{PBV}$ | 4.9 | 1.60 |
| $\frac{(P_{PBV}/P_{D3})^o}{(P_{PBV}/P_{D3}}$ | $\frac{3.7}{7.7}$ | $\frac{3.5}{7.7}$ |

The increase in pore radius by 1.2 nm in Table 2A is the maximum consistent with the measured increase in permeability to PBV (Row 2). It is calculated from Eq. 3 neglecting a change in As/Ap. Row 1 shows that the corresponding increase in Lp calculated from Eq. 4 is only one half of the measured value. Similarly the calculated decrease in permeability of $P_{PBV}$ relative to D3 (Row 3) is very much smaller than the measured decrease. No consistent description of the change in the structure of transcapillary pathway is obtained.

The larger increase in pore radius in Table 2B was calculated from Eq. 1 to account for the measured value of $(P_{PBV}/P_{D3})^o$ (Row 3). Rows 1 and 2 of Table 2B show that the corresponding increases in the ratio $P_{PBV}^o/P_{PBV}$ and $L_P^o/Lp$ are respectively 3 and 5 times as great as the measured values. Results very similar to those described in Table 2B are obtained if the osmotic reflection coefficients of the capillary wall to Ficoll 70 measured in the presence and absence of albumin (Michel and Phillips 1979) are analyzed in terms of the pore theory (Michel, personal communication). It is concluded from the analyses presented in Table 2 that albumin does not simply absorb onto the walls of transcapillary channels to maintain the normal permeability properties of the capillary wall.

The alternative hypothesis that albumin modifies the structure of a

fibrous network within transcapillary channels was tested using the fiber matrix model of capillary permeability. Two internally consistent descriptions of the permeability changes in the capillary wall when albumin is removed from the perfusate are summarized in Table 3.

Table 3. Fiber-Protein Interaction.

A. Removal of albumin reduces the fractional fiber volume 8.5% - 3.5% without a change in fiber radius.

| Permeability Ratio | Predicted | Measured |
|---|---|---|
| $L_P^o/L_P$ | 6.8 | 5 |
| $P_{PBV}^o/P_{PBV}$ | 1.43 | 1.6 |
| $(P_{PBV}/P_{D3})^o$ | 4.0 | 3.5 |
| $(P_{PBV}/P_{D3})$ | 7.5 | 7.7 |

B. Removal of albumin increases fiber radius from 0.6 to 1.2 nm keeping fractional fiber volume constant.

| Permeability Ratio | Predicted | Measured |
|---|---|---|
| $L_P^o/L_P$ | 4 | 5 |
| $P_{PBV}^o/P_{PBV}$ | 1.34 | 1.6 |
| $(P_{PBV}/P_{D3})^o$ | 3.8 | 3.5 |
| $(P_{PBV}/P_{D3})$ | 7.5 | 7.7 |

As shown in Table 3A a reduction in the fractional fiber volume to approximately one half its value in the presence of albumin will account for the decreased selectivity of the capillary wall measured as the ratio of $P_{PBV}$ to $P_{D3}$ (Row 3, calculated from Eq. 5). The corresponding increase in either Lp (Row 1, Eq. 6) or $P_{PBV}$ (Row 2, Eq. 5) is reasonably consistent with measured values. These model calculations suggest that albumin molecules occupy about one half of the network volume when they are present. It is interesting to note that the fractional fiber volume which accounts for the normal selectivity of the capillary wall to flexible dextrans is larger than the 5% which accounts for the selectivity of the capillary wall to albumin and myoglobin (Curry and Michel 1980).

The figures in Table 3B provide another description of the interaction of albumin with the fibrous network. The effect of albumin removal is described in terms of a doubling of fiber radius, keeping the fiber volume constant. The simplest interpretation of these calculations is that very small amounts of albumin are necessary to spread the "small radius" fibers on the glycoprotein side chains within the transcapillary pathway. The mechanism may involve the formation of cross linking bonds between adjacent side chains or the absorption of albumin onto sites which would otherwise cause collapse of the network into a more compact

configuration with shorter and thicker fibers. This model indicates that further investigations of the chemical interactions between endothelial cell surface glycoproteins and plasma proteins are required.

## CONCLUSION

Although the specific details of albumin's interaction with the fibrous network remain to be clarified, the hypothesis that albumin is a component of a size limiting fibrous network within transcapillary channels conforms to the experimental data described above. The physical and chemical properties of this network must be considered in future attempts to correlate the structure and function of the capillary wall.

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*A. G. B. Kovách, J. Hamar, L. Szabó (eds)*

# CONCLUDING REMARKS ON TRANSCAPILLARY TRANSPORT: CORRELATION OF STRUCTURE AND FUNCTION

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The reviews, presentations, and discussions of this symposium have, in great detail, dealt with the several structural alternatives that are presently known to exist, and which might conceivably be related to and engaged in transendothelial transport.

There seemed to be a rather solid unanimity in favor of the fact that transendothelial transport of solutes and macromolecules occurs by diffusion and convection via the interendothelial cell junctions. It was suggested that this may take place at all junctions of every segment of the microvascular bed. If this is so, then the present preparation techniques in electron microscopy are not able to preserve and reveal the intercellular gaps and channels necessary for this process. Another possibility was suggested, namely that these processes may occur only at certain points or segments of the microvascular bed, notably the venous ends of the capillaries and/or in the postcapillary venules, where there is ultrastructural evidence at hand that gaps do exist in the tight regions of the interendothelial junctions.

The possibility that transendothelial transport may occur via vesicles or through channels, derived from fusions of vesicles, encountered considerable criticism. The role of the so-called pinocytotic (or plasmalemmal) vesicles is considered to be more of a participation in endothelial metabolism. Conceivably, vesicles might pick up nutrients at the cell surface, and discharge metabolic end products at the same or another surface of the cell, as would be the case in the smooth muscle cell, where identical vesicular structures are present. The vesicles are also considered to represent invaginations of the cell membrane, extending the cell surface and associated functions into the cell, but without the postulated fission of vesicles from the cell membrane and subsequent movement into the cell or across the endothelium. The fusion of vesicles to form transendothelial channels seemed to be unacceptable to several investigators, and considered artefacts as the result of a slow influence by the chemical fixatives. Strong support for the correctness of this interpretation has been presented by those investigators who used a freeze-substitution method of fixation.

It was concluded that the discussions of transendothelial transport routes had resulted in a return to the early assumptions that most of what is transported across the endothelial cells, in terms of solutes and macromolecules, takes the interendothelial cell route, and that the structural evidence for such potential, interendothelial channels or "pores" would have to await new preparation techniques in electron microscopy.

*Adv. Physiol. Sci. Vol. 7. Cardiovascular Physiology. Microcirculation and Capillary Exchange*
*A. G. B. Kovách, J. Hamar, L. Szabó (eds)*

# INTRALYMPHATIC VS. TISSUE PRESSURE IN THE EDEMATOUS BAT WING*

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The mechanism of lymph formation was not seriously considered until the early part of this century. Prior to that time, interstitial fluid and lymph were usually considered to be one and the same. It was assumed that interstitial fluid moved down a pressure gradient into the terminal lymphatics to become lymph. Studies by McMaster (1947) using the needle method of recording interstitial pressure (IP) and lymphatic pressure (LP) apparently confirmed these suppositions.

The advent of Guyton's negative interstitial fluid pressure hypothesis (Guyton, 1963) made it necessary to reevaluate the mechanism of lymph formation. Although IP on the order of 4 to 6 mmHg below atmospheric pressure was being recorded with the wick (Scholander et al., 1968) and capsule techniques, terminal lymphatic pressure was still found to be near atmospheric pressure (Wiederhielm and Weston, 1973; Zweifach and Prather, 1975). Prima facie this appears as a paradox, as it requires interstitial fluid to flow uphill (against a pressure gradient) in order to enter the lymphatics.

Not all researchers accept the validity of the negative IP theory (Stromberg and Wiederhielm, 1970) and postulate a passive mechanism of lymph formation instead. Studies by Wiederhielm and Weston (1973) in the wing of the Mexican free-tailed bat showed no significant difference between average IP and average LP as measured with the servo-nulled micropressure technique. They suggest, consequently, that water and solvents passively enter the terminal lymphatics from the interstitium.

The bulk of current evidence, however, favors the negative IP hypothesis (Guyton et al., 1971). Therefore, one must postulate an active mechanism of lymph formation in order that interstitial fluid may be moved from the tissue space (below atmospheric pressure) into the terminal lymphatics (essentially at atmospheric pressure). Two hypotheses have been proposed in this regard: 1) the osmotic pull hypotheses of Casley-Smith

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and 2) the lymphatic suction hypotheses. Casley-Smith argues that compression of the terminal lymphatics expresses water, and thereby concentrates the contained protein (Casley-Smith, 1977). Upon release of the compressive force, he suggests that the concentrated lymph exerts an osmotic force on the surrounding water, moving it into the lymphatics. The interstitial proteins would be carried along. Casley-Smith's theory has not found wide support due to questions concerning its theoretical viability (Michel, 1974) and opposing experimental evidence (Nicolaysen et al., 1975). Lymphatic suction of interstitial fluid, on the other hand, would occur due to the passive re-expansion of the terminal lymphatics following active contraction or external compression. The stretching of the interstitial tissue elements during lymphatic compression would generate a tension in the lymphatic walls during relaxation. The result would be a suction upon the interstitial fluid. This has been proposed by several workers (Reddy et al., 1975; Guyton et al., 1975), but has never been demonstrated experimentally. The purpose of this study was to evaluate the lymphatic suction theory of lymph formation.

## METHODS

The terminal lymphatics in the wing membrane of the bat Myotis lucifugus were used as a model in this study of lymph formation. The microcirculation in the bat wing may be viewed without anesthesia or surgery, and the two dimensional lymphatic distribution of the wing lends itself ideally to such a study. Figure 1 is a diagram of the distribution of the terminal lymphatics (l.c.) in the bat wing. They can be described as extended chains of flattened saccules distributed sparsely throughout the interstitium, usually at some distance from the major blood vessels. In the non-edematous wing, the terminal lymphatics show no contractility and, in fact, cannot be seen at all as the thin-walled vessels blend imperceptibly with the interstitial tissues. In order to discern the terminal bulbs, it was necessary to create a mild local edema. A few nanoliters of modified Ringer's solution, buffered with HEPES, were infused into the interstitium of the wing. Within minutes, filled and actively contracting terminal lymphatic bulbs could be detected. If allowed to run their course, the lymphatics would remain contractile for 30 minutes or more and then gradually disappear.

Pressure measurements within the contracting terminal lymphatics and in the adjacent interstitium were made with a dual-channel servo-nulled micropressure system built in this laboratory. Accuracy greater than 0.1 $cmH_2O$ was obtained with little drift over the course of any one measurement. Atmospheric reference (zero) levels were established by placing the microelectrode/transducer tip in a pool of the infusion solution on the wing surface immediately adjacent to the area where pressure was to be measured. Zero values were checked before and after each measurement, and were recorded along with the data on FM instrumentation tape. Microelectrode placement was carried out under 640X magnification on an American Optical Research No. 5 microscope using a Leitz 32X long working distance objective.

Terminal lymphatic luminal pressure and pressure in the adjacent interstitial space were recorded simultaneously. Two- to four-minute records of pressure at each site were recorded on FM instrumentation tape along with pre-record and post-record zero levels on each channel. The data were subsequently digitized at 20 points/second and the digitized

data were normalized to correct for drift (linear interpolation between beginning and ending zero levels) and gain differences between channels. Average lymphatic pressure (LP), average interstitial pressure (IP), percent of time IP exceeded LP, and average pressure gradient when IP > LP were calculated from the normalized data.


Fig. 1 Diagram of the lymphatic distribution in the wing web of the bat, Myotis lucifugus. Transport lymphatics (t.l.) and collecting lymphatics (c.l.) are contractile vessels containing bicuspid valves, which are found in association with the larger arterioles and venules. The terminal lymphatics (or lymphatic capillaries, l.c.) are sparsely distributed throughout the tissue space. These chains of flattened saccules are also contractile but lack bicuspid valves. (Taken from Webb and Nicoll (1944) by permission of Wistar Institute Press.)

## RESULTS

Figure 2A is a diagram of the placement of the pressure-sensitive micropipettes relative to a contracting terminal lymphatic bulb. Pipette I records interstitial pressure immediately outside the wall of tne bulb, while Pipette II records intraluminal lymphatic pressure simultaneously. Figure 2B is a short segment of data recorded from the lymphatic bulb diagrammed in Fig. 2A. Channel II nicely demonstrates the rhythmic contractions of the bulb. This particular bulb produced 8.5 contractions/minute with the average for all experiments being 9/minute. Channel I (IP) shows very little modulation, although in some experiments IP near the bulb wall varied a small amount synchronously with the bulb contractions.

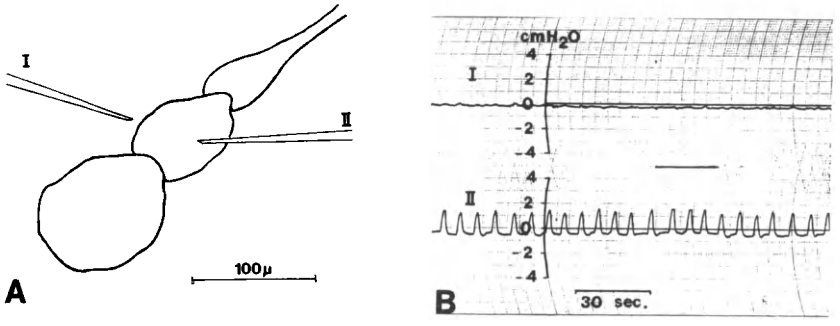


Fig. 2 A) placement of pressure pipettes for simultaneous recording of intraluminal lymphatic pressure (II) and adjacent interstitial pressure (I). B) Sample recording demonstrating contractile nature of lymphatics. (From Hogan (1980) by permission of Williams and Wilkins Co.)

It is not immediately obvious from the analog record in Fig. 2A that IP exceeds LP at any time. The zero line and scales on this record are only nominal, however, without correction for drift. After digitizing these data, and normalizing as described previously, a segment of Fig. 2B (represented by central black line) was displayed on a digital oscilloscope as shown in Fig. 3. In this figure both IP and LP are shown on the same scale, and it is immediately obvious that LP falls below IP for a significant portion of the contractile cycle. The two white dots at either end of the IP signal are the atmospheric zero reference. Thus, LP not only falls below IP during relaxation, but it also falls below atmospheric pressure.

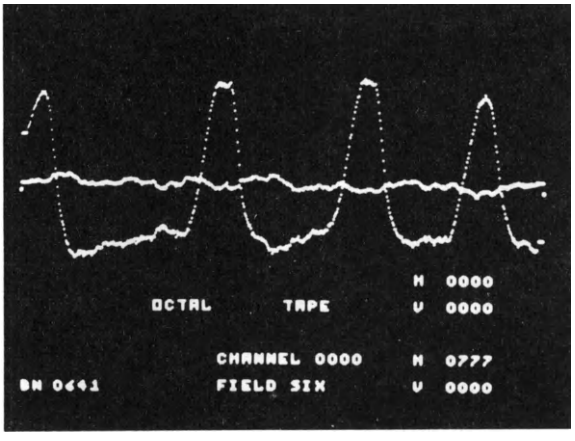


Fig. 3 Digital display of a data segment from Fig. 2B after elimination of differences in baseline drift and gain between channels. Lymphatic pressure falls below interstitial pressure during relaxation producing a "filling phase." (From Hogan (1980) by permission of Williams and Wilkins Co.)

The experiment represented by Figs. 2 and 3 was carried out 20 times in 16 different animals. Four records were discarded because post-record zero levels could not be obtained, leaving a final sample of 16 records from 12 animals. The results are listed in Table 1. Each entry represents an average over 2 to 4 minutes encompassing many contractile cycles. The average interstitial pressure for all experiments was 0.0 ± 0.2 $cmH_2O$, which was exceeded by the average lymphatic pressure of 0.4 ± 0.6 $cmH_2O$. The significance of the difference was low ($p > 0.2$) due to the high variance of the LP measurements. The lymphatic "filling phase" was defined as that part of the cycle when IP exceeded LP. This is shown in Table 1 as "Percent of cycle IP>LP". In all cases IP did exceed LP for some part of the contractile cycle ranging from 1 to 86 percent (average 43 ± 24 percent). During the filling phase the average gradient for fluid inflow into the lymphatic lumen was 0.3 ± 0.2 $cmH_2O$. This value is significantly greater than zero ($p < 0.01$) and easily within the accuracy of the equipment.

Table 1 - Interstitial vs. Intralymphatic Pressures

| Expt. | Average Pressure ($cmH_2O$) Interstitial (IP) | Lymphatic (LP) | Percent of Cycle IP > LP | Average gradient during filling phase ($cmH_2O$) |
|---|---|---|---|---|
| 1/16-1 | 0.16 | 0.59 | 62 | 0.50 |
| 1/17-1 | -0.18 | -0.20 | 61 | 0.24 |
| 1/18-2 | -0.09 | -0.01 | 46 | 0.70 |
| 1/18-3 | -0.10 | -0.26 | 62 | 0.48 |
| 1/18-4 | -0.21 | 0.63 | 30 | 0.23 |
| 1/23-1 | 0.11 | 0.19 | 60 | 0.35 |
| 1/23-2 | -0.13 | 0.65 | 24 | 0.21 |
| 1/30-1 | 0.03 | 0.40 | 48 | 0.18 |
| 1/31-1 | -0.36 | -0.13 | 27 | 0.25 |
| 1/31-2 | 0.15 | 0.53 | 21 | 0.20 |
| 2/07-1 | 0.27 | 0.32 | 55 | 0.20 |
| 2/08-5 | 0.57 | 1.90 | 6 | 0.14 |
| 2/14-1 | 0.01 | -0.58 | 86 | 0.73 |
| 2/28-2 | 0.13 | 0.16 | 66 | 0.36 |
| 3/06-2 | 0.19 | 1.11 | 1 | 0.08 |
| 3/08-1 | -0.07 | 1.00 | 38 | 0.15 |
| Minimum | -0.36 | -0.58 | 1 | 0.08 |
| Maximum | 0.57 | 1.90 | 86 | 0.73 |
| Average | 0.03 | 0.39 | 43 | 0.31 |
| Std. Dev. | 0.22 | 0.61 | 24 | 0.20 |

Results of 16 simultaneous measurements of interstitial (IP) and lymphatic pressures (LP) associated with contracting terminal lymphatic elements. Each value represents an average from a two to four minute data segment encompassing numerous lymphatic contractile cycles. In all experiments IP exceeded LP for some fraction of the contractile cycle (average = 43 ± 24 percent) in spite of the fact that average LP (0.4 $cmH_2O$) was greater than IP (0.0 $cmH_2O$).

## DISCUSSION

These data indicate that the contractile terminal lymphatics in the bat wing generate a small but significant suction upon the interstitium during the relaxation phase of their contractile cycle. The pressure gradient thus established should be adequate to move interstitial fluid into the terminal lymphatics in the process of lymph formation. A question remains, however, as to whether this suction by the lymphatics is adequate, by itself, to account for net lymph formation. This study has shown that, although there is an inwardly directed pressure gradient across the lymphatic wall during part of the contractile cycle, the average pressure gradient over the entire cycle is such that net flow should be in the retrograde direction. Therefore, if lymphatic suction is to be a viable mechanism for lymph production, a mechanism must be supposed which would allow for the preferential movement of lymph downstream, rather than back through the walls, during contractions. There is considerable anatomical evidence that endothelial gap junctions in the terminal lymphatic walls may act as flap-like valves producing the necessary rectifying action. It has been shown that the lymphatic anchoring elements attach to the abluminal, but not the luminal, flap of the frequent overlapping gap junctions in the lymphatic wall (Leak, 1976). The luminal flap is free to widen the junction if interstitial pressure exceeds intraluminal lymphatic pressure, or to close tightly against the stabilized abluminal flap if the gradient is in the opposite direction. A directionally biased hydraulic resistance of the terminal lymphatic wall is the result. This is only theory at present.

It should be noted in the interpretation of these results that all measurements were made in a tissue in which an excess of fluid was present. Only a very mild edema was created by the artificial infusions, as demonstrated by average interstitial pressure essentially at the atmospheric reference level (IP = 0.03 ± 0.22 $cmH_2O$). It cannot be proven from these data that the same mechanism, lymphatic suction, would operate at lower levels of ambient interstitial fluid pressure. However, there is no theoretical reason why the same mechanism would not be operable at subatmospheric IP levels. In fact, a recent computer simulation by Granger and Shephard (1979) of lymphatic dynamics produced graphs of lymphatic and interstitial pressure remarkably similar to Fig. 3, but with the IP level nearly 5 mmHg below atmospheric pressure. It is, therefore, mathematically plausible for lymphatic suction to produce lymph at subatmospheric levels of fluid pressure in the interstitium.

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# CONCENTRATION OF MACROMOLECULES IN THE TISSUE AND LYMPHATICS

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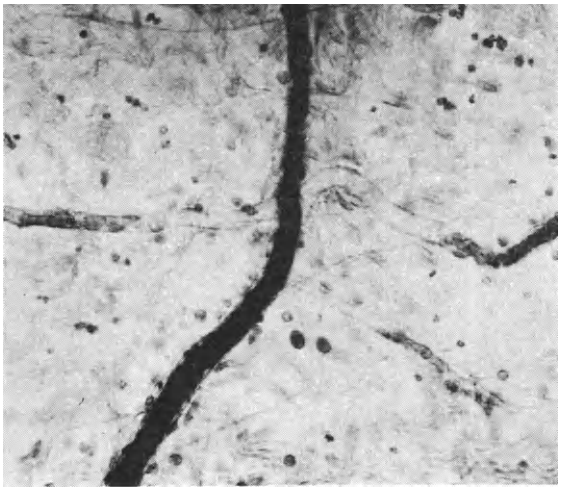
We use two microphotometric techniques for intravital microscopy in order to measure plasma proteins in situ , the ultramicrospectrophotometry (UMSP) and the scanning microfluorometry (SMF). With UMSP the monochromatic absorbance of proteins in the ultraviolet wave length range at 280 nm is measured. For technical reasons a thin tissue like the mesenteric plate must be used. It is exposed into a transparent superfused chamber. In the ultraviolet microscope we see the absorbance of proteins within cells and in the circulating blood plasma. The interstitial tissue shows an absorbance which is not homogeneous but structured in tiny streaks and a net-like arrangement. These structures correspond to the preformed fibrous components of the connective tissue (Fig. 1).



We must keep in mind this arrangement when computing protein concentrations of the extravascular compartment.

Lymph vessels drainy the intestinal tissue have various protein absorbance pointing to their high or low protein content in comparison to the perivascular space (Fig. 2 and 3).

Fig. 1. Exposed rat mesentry, ultraviolet microscope, monochromatic absorbance at 280 nm. Objective ultrafluar 10:1, n.A. 0,2, Ocular 8x. 3 capillaries and a venule with perivascular space.

Fig. 2. Exposed rat mesentery, ultraviolet microscope, monochromatic absorbance at 28o nm. Objective 1o : 1, n. A. o,2, Ocular 8 x.
Lymph vessel branch, drainig the intestinal tissue, low protein absorbance.

Fig. 3. Similar object as Fig. 2. Lymph vessel with high protein absorbance.

Measurements of the protein absorbance are made using a round aperture giving a measuring field in the object of 227 $\mu m^2$, therefore comprising a lot of tissue elements with prefered protein absorbance as mentioned. We found in the cell- free perivascular connective tissue a protein content between 1o and 8o pg per measuring field (Witte and Zenzes-Gepräqs 1977, 1978). The lowest values represent the tissue remote from any capillary vessels, the highest values represent the perivascular area near venular blood vessels. The differences between different kinds of microcirculatory vessels are significant, as are the differences between different distances outside the vessel wall (Table 1).

Table 1. Mean values of protein absorbance at 28o nm in exposed rat mesentery within a measuring field of 227 $\mu m^2$ of the perivascular connective tissue. Calculation of protein concentration of the extravascular space with regard to the microcirculatory vessels.

| Vessel | Distance outside vessel µm | n | $m_A$ 28o | $m_{protein}$ pg | extravascular plasmaprotein $mg \cdot ml^{-1}$ tissue |
|---|---|---|---|---|---|
| arteriole | 1o | 1oo | o,245 | 52,5 | 4,58 |
| | 3o | 1oo | o,212 | 3o,o | 2,62 |
| capillary | 1o | 31 | o,222 | 37,o | 3,23 |
| | 3o | 31 | o,182 | 1o,o | o,88 |
| venule | 1o | 1o4 | o,284 | 78,5 | 6,85 |
| | 3o | 1o4 | o,237 | 47,o | 4,1o |

In order to calculate protein concentrations we must know the thickness of the object we measure. For this purpose we developed a device with differential interference contrast optics. On the basis of these data we calculated the protein content of 1 ml interstitial tissue with 3,4 mg as a

mean value with high topographical variations as shown in table 1. In comparison to the circulating blood plasma these values correspond to 8 % (interstitial space remote from any blood vessels) until 6o% (perivenular tissue). Note, that the phenomena of the so called exclusion volume effect within the connective tissue are not measurable due to the dimensions of the measuring frame used in the microscope.

The lymph protein was measured in lymph vessels situated within the fat free parts of the mesentery. They drain the periintestinal tissue. Their protein content represents therefore the interstitial protein of the intestinal tissue. We found rather high individual variations of the lymph protein content between different lymphatic vessels under normal experimental conditions (Compare Fig.2 and 3). These variations lead to large standard deviations of the mean values found in 4 experiments (Tab. 2). In each animal the same lymph vessel area was measured seven times during 2o minutes after exposure of the mesentery loop into the measuring chamber. The lowest absorbance found corresponds to a protein content of 1o pg protein per measuring field, the highest absorbance corresponds to 5oo pg protein per measuring field of the same size in the same animal. The lymph protein of individual mesenteric lymph vessels may vary therefore between 1 to 5o. Most values, however, were found in the range of 15o - 25o pg protein per measuring field. The volumes of the measured lymph vessels did not contribute significantly to the various protein contents.

Table 2. Mean values and standard deviations of all measurements in lymph vessels at 28o nm during initial observation period (before any i.v.-injections).

| Number of consecutive measurements | n | $m_{A\ 28o}$ | SD ± | Protein pg/1oo $\mu m^2$ |
|---|---|---|---|---|
| 1 | 77 | o,527 | o,152 | 1o6,5 |
| 2 | 76 | o,517 | o,155 | 1o3,6 |
| 3 | 48 | o,481 | o,146 | 92,9 |
| 4 | 14 | o,519 | o,151 | 1o4,2 |
| 5 | 7 | o,569 | o,12o | 119,o |
| 6 | 4 | o,591 | o,o7o | 12o,5 |
| 7 | 4 | o,574 | o,o82 | 12o,5 |

Since we found the same thickness of the mesenteric tissue having lymph vessels as in perivenular regions we are allowed to compare the protein content of both regions measured by absorption photometry. On the basis of this assumption the protein concentration of the lymph is at least two times that of the interstitial region with the highest protein concentration, that is the perivascular tissue around venules. It should be kept in mind, however, that the interstitial protein concentration of the connective tissue probably will be lower than that of the intestinal wall drained by the lymphatics under study.

In very rare instances, however, very tiny lymph vessels can be found in the mesenteric connective tissue with a blind end. They are in all probability real terminal or initial lymphatics draining the connective tissue. They are functionally the own initial lymph vessels of the mesenteric plate, distinguishable from the intestinal collecting lymph vessels. The absorbance within these initial lymphatics shows clear variations, too. The absorbance was found as quite the same as in the surrounding connetive tissue (Fig. 4), sometimes the absorbance was considerably higher (Fig. 5). Therefore, even within initial lymphatics, the protein concentration varies and can reach higher values than in the interstitial tissue.

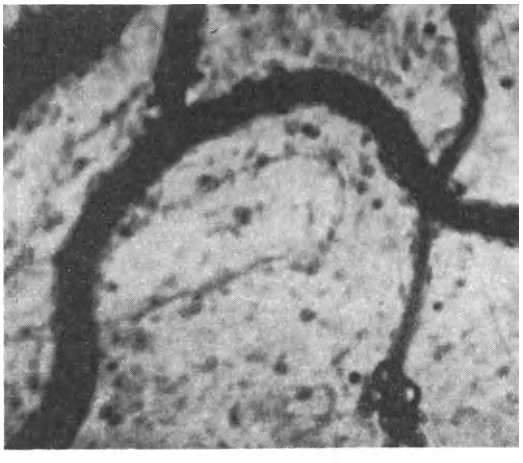


Figure 4. Exposed rat mesentery, ultraviolet microscope, monochromatic absorbance at 280 nm.
Blind end of an initial lymphatic with a little less absorbance of the lymph than in the surrounding interstitial connective tissue.
Objective ultrafluar 10:1
Ocular 8 x

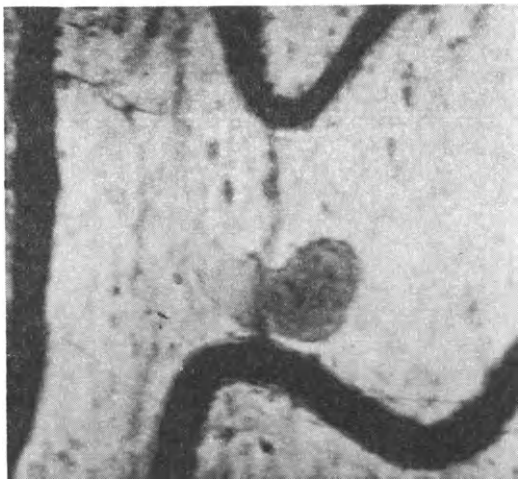


Figure 5. Similar situation as in figure 4.
Initial lymphatic with a distinctly higher protein absorbance in the blind end than in the surrounding connetive tissue.

The scanning microfluorometry (SMF) makes use of the fluorescent labelling of macromolecules (Witte 1967). After their intravenous injection in small amounts, their passage through the wall of microcirculatory blood vessels into the perivascular tissue, the transport within the interstitial space and their appearence in the lymph is measured by fast line - scanning device (Witte 1979). In this way we get continuous quantitative information about the dynamics of protein permeability, starting from time zero. After intravenous application of the labelled proteins we see the fluorescent circulating plasma and after some time a passage through the vessel wall mostly at venular vessels and a spreading into the perivascular tissue (Witte 1965). It proceeds normally under the form of fine streaks corresponding to fibrillar structures of the connective tissue (Fig. 6). These findings confirm therefore our statements, that the surfaces of such structures serve as channels for the macromolecular transport in the interstitial space.

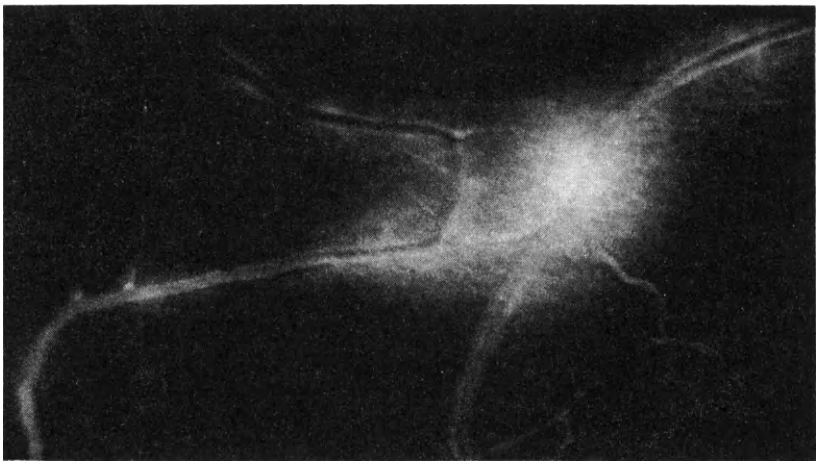


Figure 6. Exposed rat mesentery, fluorescence microscope, blueviolet fluorescence illumination. Objective neofluar 1o:1, Ocular 8x. Labelling of plasma proteins by intravenous injection of Brillantsulfoflavin. Penetration of the label through the vascular wall at a branching of venular blood vessels and spreading into the surrounding connective tissue in a network like fashion forming streaks in the connective tissue. Demonstration of channels for macromolecular transport in the tissue.

By our ultra-fast scanning fluorometry the amount of labelled protein within the circulating blood and the amount in the perivascular compartment are computed. The distribution in the extravascular pool is different depending on the kind of vessels, the proteins labelled and the permeability state of the animal. Finally the appearence of the label in the lymph can be measured. Normally, the marked serum proteins begin to appear in the intestinal lymph 5-10 minutes after the intravenous injection. After one hour the total increase of the labelled lymph protein content may equal the concentration of the labelled protein of the circu-

lating blood (Fig. 7- 12). In other instances we find a rapid appearing peak of the labelled protein in the lymph with a similar rapid decrease (Fig. 13).

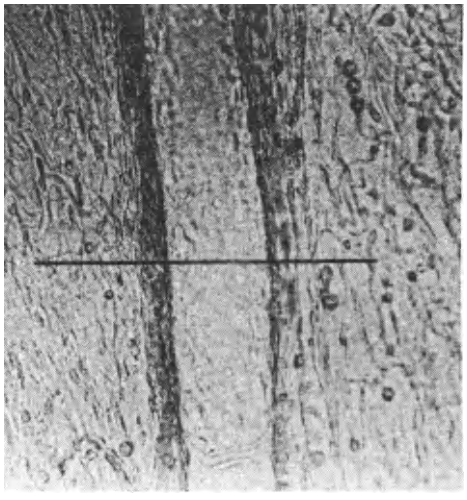


Figure 7. Exposed rat mesentery, scanning microfluorometry. Objective Neofluar 1o : 1, n.A. o,3, Ocular 8 x. Lymph vessel with small arterioles at both sides. Bright field illumination.

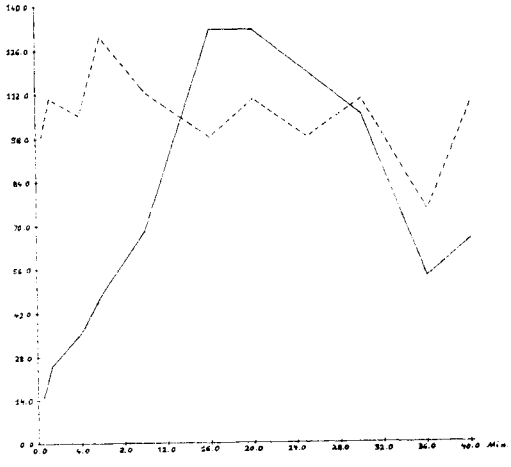


Figure 8. Same field as figure 7. Scanning fluorometry after i.v. injection of FITC-rat serum, o.25 ml/1oo g body weight. Maximal fluorescence intensities within a arteriole (dotted line) and the lymph (solid line) during 4o min after the injection of the labelled protein.

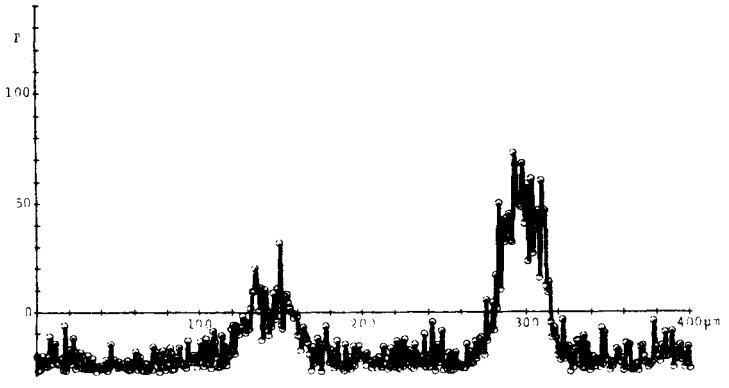



Figure 9. Same experiment as figure 7-8. Example of a scanning line as indicated in figure 7.
Values 3o seconds after i.v.injection of the label.
Fluorescence within both arterioles, no fluorescence in the lymph (between the arterioles).
Abscissa: dimesion of the scanning line, distance between each measurement= 1 micron.
Ordinate: Fluorescence intensity.

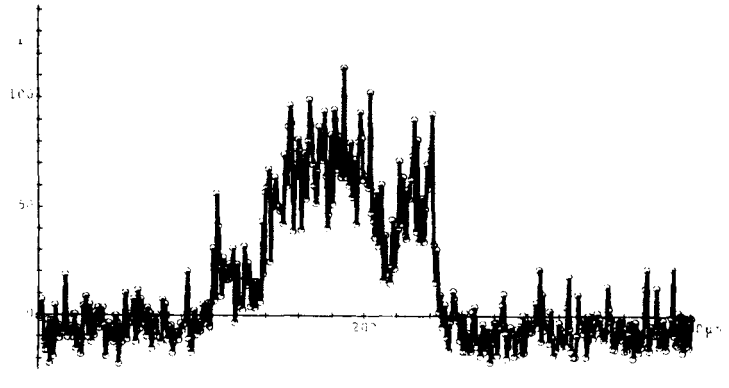


Figure 1o. Same experiment as figure 9. Fluorescence intensities 2o minutes after i.v.injection of the label.
Fluorescence mostly within the lymph, less fluorescence within the arterioles.

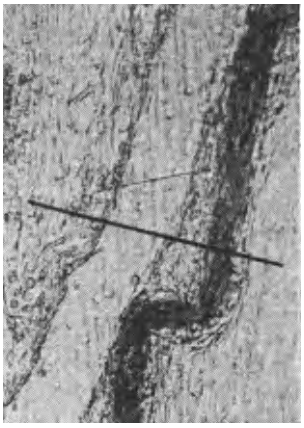


Figure 11.

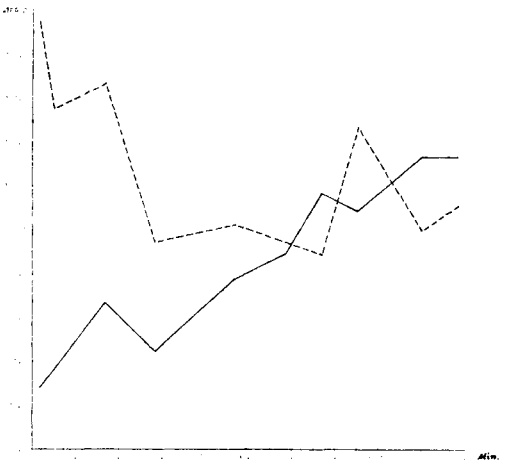


Figure 12.

Figure 11 and 12. Scanning microfluorometry of a lymph vessel and a venule (Figure 11). Optical conditions see Fig.7. Figure 12. Maximal fluorescence intensities within the lymph (solid line) and the venule (dotted line) 1 to 60 minutes after i.v.injection of FITC- rat serum, 0.25 ml/100 g body weight.

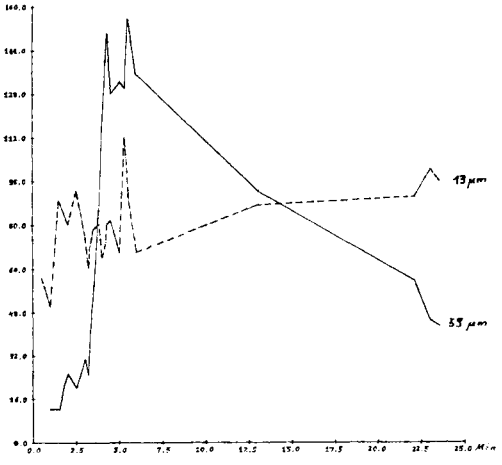



Figure 13. Scanning microfluorometry after i.v. injection of FITC-rat serum, 0.25 ml/100 g body weight. Maximal fluorescence intensities of the lymph /solid line/ and an arteriole nearby /broken line/ 1/2 to 25 minutes after application of the label.

A passage of labelled lymph proteins back into the interstitial tissue was never observed. The permeability of the lymph vessels for proteins seems to be unidirectorial, only from the interstitium into the lymphatics.

In the last example a rare finding with a terminal lymphatic of the connective tissue is shown (Fig. 14). 6 minutes after the intravenous injection of FITC labelled rat serum the label appears in this initial lymphatic, at the same time as in collecting lymph vessels draining the intestine. 1o minutes after the injection the initial lymphatic is filled with a marked fluorescence (Fig. 15). So even in such initial lymphatics an increase of protein concentration is possible speaking for a restricted and unidirectorial permeability of these terminal lymph vessels for plasma proteins.

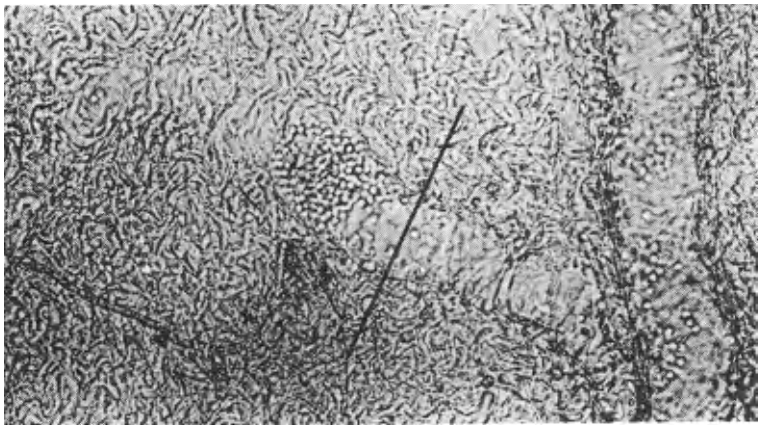


Figure 14.Initial lymphatic vessel (similar to fig. 4 and 5), coming from left to right, partially filld with lymphocytes.

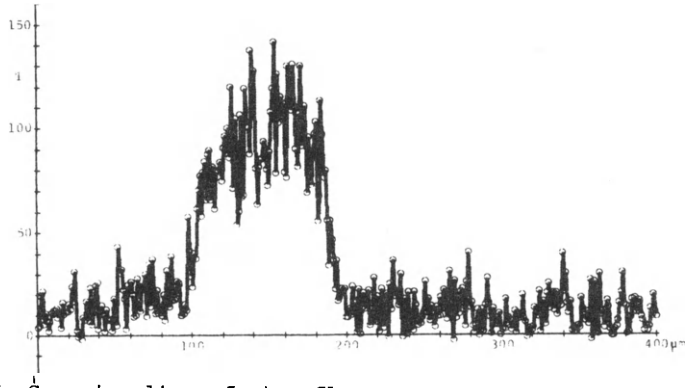



Figure 15. Scanning line of microfluorometry through to initial lymphatic of fig. 14, 1o min after i.v.injection of FITC-rat serum.

In conclusion: We have found that permeability processes for macromolecules are topographically defined phenomena. They are related to the type of microcirculatory vessels. The fibrous structure of the interstitial connective tissue serves as channels for the macromolecular transport in the extravascular space. The permeability for macromolecules in lymph vessels, even the initial lymphatics, is unidirectorial, only from the interstitium into the lymph. A concentration of macromolecules compared to the interstitial tissue can occur.

Acknowledgments.

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# RELATIONS BETWEEN TISSUE FLUID AND LYMPH IN THE LUNGS: THE MACROSCOPIC ORGANIZATION OF THE INTERSTITIUM

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In the course of evolution, according to Rodbard (1969), the interstitial tissue of complex organs developed as two compartments in series; namely, the interstitium immediately adjacent to the parenchymal cells and the loose connective tissue surrounding large clusters of cells. Lymphatic vessels are limited to the latter compartment (Figure 1). Van Hayek (1960) used the same principle in describing the lung's interstitium. He envisioned the alveolar wall (perimicrovascular) interstitium to be in series with the loose connective tissue of the bronchovascular sheaths, the interlobar septae and the pleura. There are no lymphatic vessels in the alveolar wall; only in the loose connective tissue (Staub, 1974). It was shown by us many years ago that the loose connective tissue spaces serve as sumps for fluid draining from the alveolar wall (perimicrovascular) space during interstitial pulmonary edema (Staub, 1967).

These anatomically defined interstitial spaces of the lung are also functionally different in terms of their relationship to alveolar pressure. In current physiologic jargon, the perimicrovascular interstitium and the loose connective tissue interstitium are situated within the alveolar and extra-alveolar compartments, respectively, as shown in Figure 2. The effectiveness of the loose connective tissue compartment as a sump requires that the pressure there (Pct) is normally less than that of the alveolar wall interstitium (Ppmv) and that the distensibility of the loose connective tissue space is greater than that of the alveolar wall interstitium. Thus, substantial amounts of fluid can accumulate without raising tissue fluid hydrostatic pressure to high levels.

Gee and Williams (1979) measured the volume of fluid that can be retained in the perivascular fluid cuffs of isolated dog lungs at different distending pressures. The cuffs appeared to be very distensible at low pressures, achieving nearly 25% of their maximum volume at a transpulmonary pressure of 2 $cmH_2O$. Gee and Havill (1980) have also found that, during pulmonary edema, fluid entering the lymphatics came from the perimicrovascular filtrate of the alveolar wall rather than from the fluid that had accumulated in the bronchovascular cuffs. They did a very simple experiment in which they produced interstitial edema using alloxan in anesthetized dogs, then injected Evans Blue Dye intravenously to label the plasma proteins. The lung lymph turned blue within a few minutes, but the bronchovascular cuff fluid remained clear.

New measurements of hydrostatic pressure (see Table) in the loose bronchovascular connective tissue spaces at the hilum of the lung confirm the fact that the pressure there is less than alveolar pressure and pleural pressure (Goshy, 1979; Inoue, 1980). The absolute value of the pressure is dependent both on lung volume and on pulmonary vascular pressures. The measurements by Meyer (1963) and by Parker (1978) of subatmospheric pressure in implanted plastic capsules and in fluid-filled segments of lung, is probably related to the pressure (Pct) in the loose connective tissue (extra-alveolar) space; not the alveolar wall perimicrovascular interstitial fluid pressure (Ppmv) at the major sight of fluid filtration.

Bhattacharya in my laboratory has measured microvascular pressure in vessels at the pleural surface of the isolated, perfused dog lung by direct micropuncture (Bhattacharya, 1980). He is currently measuring pressures in the fluid cuffs around small subpleural arteries and veins. At these locations, just beneath the pleura, close to the alveolar wall and far from the loose connective tissue at the hilum of the lung, he finds the fluid pressure to be above pleural pressure but slightly negative relative to alveolar pressure. Puncturing different locations in the same lobe, he finds that loose connective tissue pressure (Pct) is equal to or greater than alveolar pressure near the bottom of the lung compared to the top. This is to be expected since, when interstitial pulmonary edema develops, there is a column of fluid within the extra-alveolar compartment extending from the top to the bottom of the lung. His findings account for the fact that alveolar flooding occurs in the lower part of the lung first, since that is where interstitial cuff fluid pressure exceeds alveolar pressure.

The lung has another large extravascular compartment; namely, the alveolar gas space. Fluid enters and leaves the alveolar space in edema through the alveolar barrier (alveolar epithelium or terminal airway eqithelium).

Humphreys (1967) found that after birth, with the onset of air breathing, fetal lung alveolar fluid was cleared from the lung quite rapidly, 40% via the lymphatics and 60% by reabsorption into the blood. Bland (1977 and personal communication) finds even less lymph flow increment in lambs with chronic lung lymph fistulas implanted 10 days prior to birth. The reason most of the fetal lung fluid is reabsorbed into the blood rather than being transported as lymph is that it does not contain protein. Matthay (1980) in my laboratory measured the clearance of fluid placed in the alveoli of anesthetized sheep. He found that isosmotic electrolyte solutions are removed at the rate of about 20%/h without causing any change in lung lymph flow. Isosmotic pretein-containing solutions, however, are removed at a slower rate of about 10%/h but these always cause an increase in lung lymph flow.

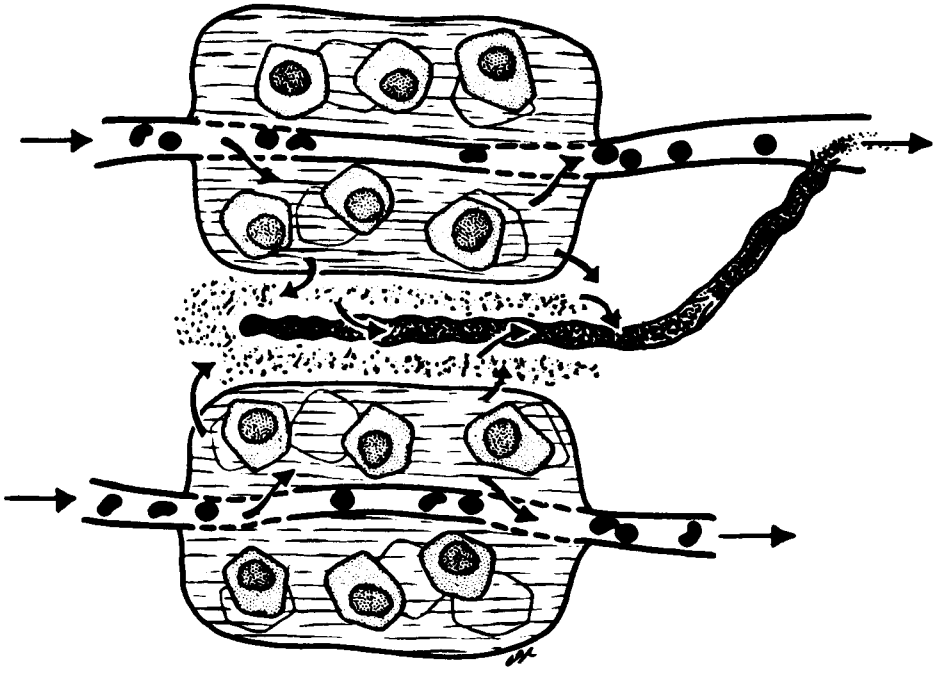
The macroscopic organization of the interstitial tissue as conceived by Rodbard and by Von Hayek is substantially correct. Fluid filtering from alveolar wall microvessels enters the perimicrovascular interstitial compartment where the hydrostatic pressure (Ppmv) is approximately equal to alveolar pressure. From there, it drains along a hydrostatic pressure gradient (partially due to breathing movements of the lung) into the bronchovascular loose connective tissue where the pressure is low (Pct). Normally, the filtrate enters the lymphatic capillaries and is actively pumped back into the venous circulation at the base of the neck. When the

rate of fluid filtration exceeds the filling capacity of the lymphatics, the excess fluid accumulates in the bronchovascular fluid cuffs.

Thus, the lymphatic vessels and the extra-alveolar loose connective tissue space are in series with the alveolar wall perimicrovascular interstitium but in parallel to each other (Figure 3). Lung lymph represents recent microvascular filtrate rather than bronchovascular cuff fluid. The alveolar gas space is in series with all three interstitial compartments across the alveolar barrier. Alveolar fluid appears to exchange mainly with the extra-alveolar compartment. During alveolar fluid clearance large bronchovascular cuffs form but lymph flow may not be affected at all.

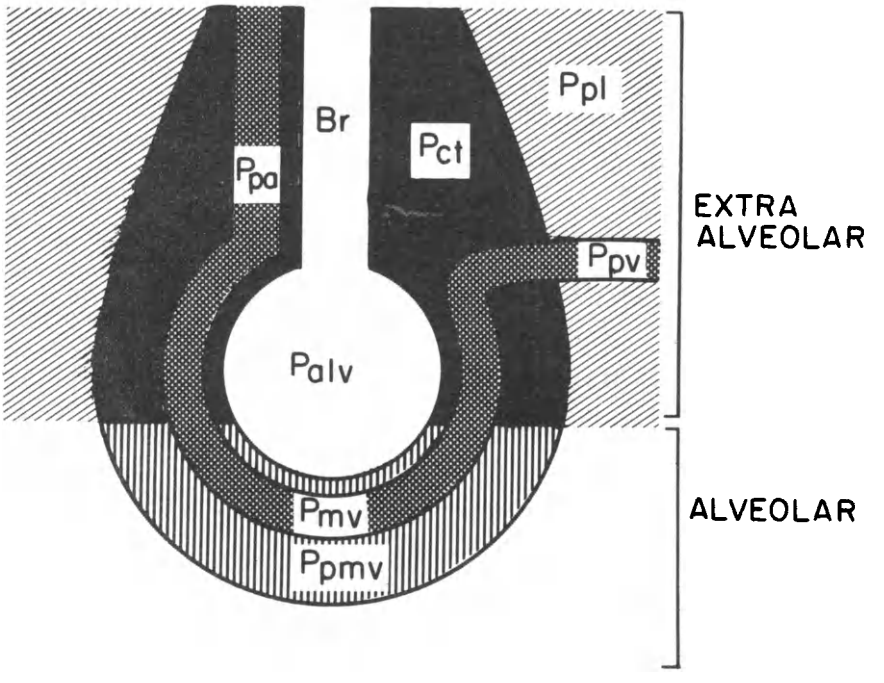
Physiologists investigating lung fluid balance or lung interstitial mechanics need to pay careful attention to the distinct anatomic regions of the interstitium. One cannot simply refer to "interstitial fluid pressure", as if there is a single-well-mixed compartment characterized by a single hydrostatic pressure.

Figure 1



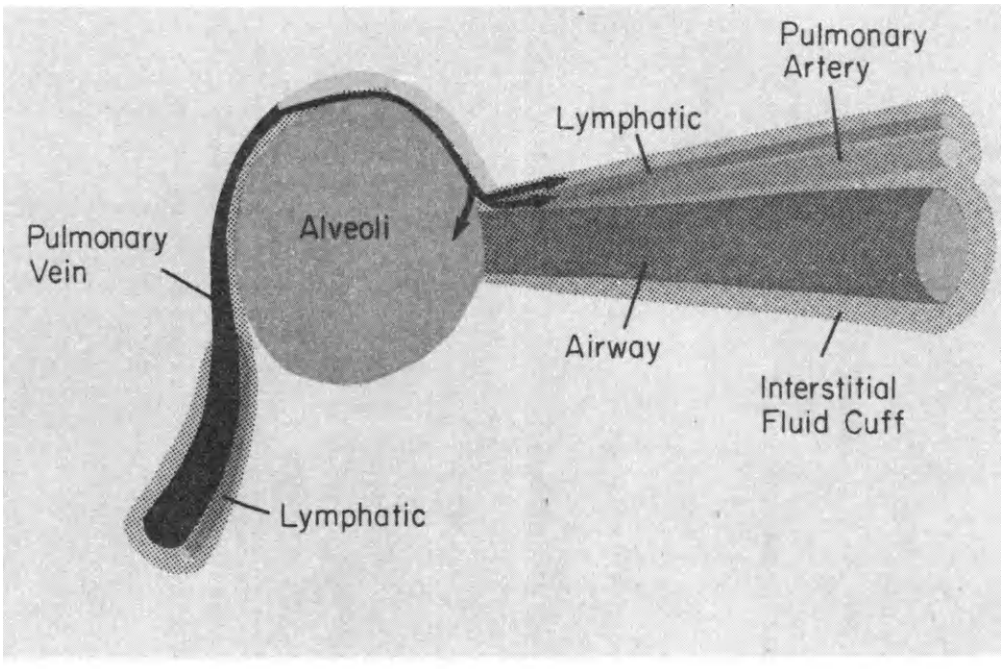
The general concept of two interstitial compartments arranged in series. The parenchymal cells are bathed by their own microvascular filtrate which slowly seeps into the surrounding loose connective tissue where the lymphatic vessels are located. (From S. Rodbard, 1969)

Figure 2




In terms of fluid filtration and fluid storage, the lung consists of two parts, depending on how hydrostatic pressures change with respect to alveolar pressure (Palv). When lung volume increases, pleural pressure (Ppl) decreases as does pressure in the extra-alveolar compartment (loose connective tissue, Pct). Pulmonary artery pressure, Ppa, and pressure in that portion of the microvessels (Pmv) that is extra-alveolar, generally decreases slightly with respect to Palv but increases relative to Ppl and Pct. Pulmonary venous pressure, Ppv, reflects changes in Ppl. In the alveolar compartment the pressures (microvascular, Pmv, and perimicrovascular, Ppmv) rise in parallel to, although less than, Palv. In terms of fluid filtration, changes in lung volume have only minor effects, but the storage capacity in the extra-alveolar connective tissue space is increased (Staub, 1978).

Figure 3




Schema of extravascular, extracellular water pathways in the lung. The lymphatics and loose connective tissue (bronchovascular fluid cuffs) are arranged in parallel. Both are extra-alveolar and in series with the alveolar wall (perimicrovascular) interstitium. Ordinarily, fluid filtering from microvessels moves to the initial lymphatics which respond to passive filling by actively pumping fluid out of the lungs. As filtration rate increases, the lymphatics cannot transport all of the fluid so that the interstitial cuffs fill. As the cuffs expand, pressure in them rises until it reaches or exceeds alveolar pressure at which point fluid begins to flood the alveoli. This final point is still unproven (Staub, 1980).

TABLE

LOOSE CONNECTIVE TISSUE PRESSURE IN THE DOG LUNG

| | Location | Pressure (Pct)* Relative to Pleural Pressure ($cmH_2O$) | Method | Reference |
|---|---|---|---|---|
| 1. | Center of Lobe | -3 to -9<br>+3 (edema) | Implanted Capsule | Meyer, 1968 |
| 2. | Sublobar Unit | -5 to -6 | Fluid-filled Segment | Parker, 1978 |
| 3. | Bronchovascular Sheath at Hilum | -2 to -3 | Wick Catheter | Goshy, 1979 |
| 4. | Bronchovascular Sheath at Hilum | -2 to -8<br>0 (edema) | Wick Catheter | Inoue, 1980 |
| 5. | Subpleural Fluid Cuffs and Septae | +4 to +7<br>(edema) | Micropuncture | Bhattacharya (personal communication) |

* The numbers are for Ppl = 0 in isolated lobes and estimated to be -5 $cmH_2O$ in intact animals. Comparable vascular pressures, in so far as possible to decipher original sources. Pct is called Pif, Px and Px(f) by some workers.

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# THE INITIAL LYMPHATIC CYCLE AND THE FORCES RESPONSIBLE FOR IT

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## THE MORPHOLOGY OF THE INITIAL LYMPHATIC CYCLE

The essential features of the cycle are that the initial lymphatics fill via open endothelial intercellular junctions during tissue relaxation (i.e. when the tissues are not compressed); they empty during tissue compression (due to the actions of adjacent muscles, etc.), at which time the open junctions become temporarily closed - at least to macromolecules [7, 10, 11]. Allen and Vogt [1] originally suggested that von Recklinghausen's [41] stomata might function in this way. The stomata were confirmed by electron microscopy [2], as were their opening and closing [7, 9, 11], and that these vessels empty during tissue compression and fill with its relaxation [47]. The inlet valves in their walls (the openable junctions), and the intra-lymphatic outlet valves, let them act as millions of tiny force-pumps.

The other essential feature of the initial lymphatics is the presence of many anchoring filaments which join the endothelium to the interstitial tissue [40]. These explain why they are still held open in oedema, when the increased total tissue pressure closes the blood vessels. The anchoring filaments link the initial lymphatics to the interstitial tissue, especially the collagen fibres [16, 29, 30]. While they attach to the endothelium at many places, they do not attach to the inner of the two cells at a junction - thus allowing this to be opened by inflowing fluid. It will be shown that normally the filaments are unimportant, but they are essential for the functioning of the system in oedema. Exceptionally, the vessels can be collapsed, if the oedema is severe [48], or if hyaluronidase is used to destroy the filaments' unions with the tissues [3].

## POSSIBLE FORCES CAUSING THE ENTRY OF FLUID, ETC., INTO LYMPHATICS

### Hydrostatic Pressure Gradient

In oedema, and in certain organs which have tight capsules and positive tissue hydrostatic pressures, everyone is agreed that these are likely to be greater than the intralymphatic pressure [10]. Hence there will be a hydrostatic pressure gradient directed from the tissues into the vessels. Here, these probably act more like conduits than pumps [10] - although it has been suggested [19] that the hypothetical colloidal osmotic pressure mechanism (see below) may still function. This concensus does not, however, apply to opinions about the forces responsible for lymphatic filling in most normal tissues. A number of possible mechanisms have been suggested.

In these tissues, it is highly likely [21, 22] that the tissue hydrostatic pressure is negative (i.e. less than atmospheric). Contrasting with this is the fact that pressures in the initial lymphatics, where these have been measured, have been found to be much higher, approximating atmospheric pressure [18, 23, 25, 31, 32, 33, 35, 37, 50, 53]. Two points should be emphasised. One is that while Hogan [25] and Nicoll and Hogan [35] did find that the tissue hydrostatic pressures were slightly greater than the intralymphatic pressures during the filling-phase, this was in bat wing which had been made locally oedematous in order to visualise the lymphatics - hence this was an oedematous situation, not the normal one. In addition, these initial lymphatics are unique, in that they are contractile. This oedema, and the uniquely contractile nature of these initial lymphatics, completely negates attempts [36] to generalise these findings. In normal tissue there is no extra-lymphatic space, left by the contracting initial lymphatic, into which fluid might be "sucked". Also, in mesentery the hydrostatic pressure gradient is outwards from these vessels [18].

The second point is that all these intra-lymphatic pressure measurements have been made in immobilised tissue. It will be shown later how essential movement is (with its varying total tissue pressure) for the functioning of the initial lymphatic cycle, and how errors in measurements of lymph concentration have almost certainly arisen from neglecting this. Thus, it is obvious that there is a major difficultly in explaining the filling of the initial lymphatics by a simple hydrostatic pressure gradient [18, 21, 22, 43, 54, 55]: it is usually in the wrong direction!

Suction into the Initial Lymphatics

It is sometimes suggested that tissues are distorted during the emptying--phase of the initial lymphatics, so that when they are released from pressure (during the filling-phase) the anchoring filaments are under tension and they pull the initial lymphatics open. This argument ignores the fact that these filaments are quite infrequent in some tissues (e.g. the intestinal villi and testis [10]), yet these tissues certainly form much lymph. It has been disproved [Table 1 - 12] by the differences in conformation of the initial lymphatic walls during oedema (when the filaments are pulling on the vessels) and in normal tissues during the

TABLE 1

The Conformations of the Walls of the Initial Lymphatics

| | "PULLED-OUT" | | | FLAT | "PUSHED-OUT" | | | TOTAL |
|---|---|---|---|---|---|---|---|---|
| | Very | Mod. | Slight | | Slight | Mod. | Very | NUMBERS |
| Diaphragms - uninjured, both phases | | | | | | | | |
| | 5 | 13 | 15 | 73 | 65 | 44 | 35 | 250 |
| " - injured | 32 | 29 | 29 | 26 | 19 | 8 | 7 | 150 |
| Jejunum - uninjured, both phases | | | | | | | | |
| | 3 | 6 | 5 | 15 | 21 | 19 | 11 | 80 |

These appearances were estimated, "blindly", on electron micrographs of mouse diaphragms and puppy jejuna. The former were fixed at various phases of the cycle, the latter had these mimicked by drugs.

filling and emptying of the initial lymphatics. Pulling-out of the walls will produce a characteristic conformation, which will be quite different from that caused by the pushing-out of the walls by an intra-lymphatic hydrostatic pressure which is greater than the tissue hydrostatic pressure. The vessels are much more "pushed-out" than "pulled-out" in normal tissue (in both phases of the cycle); in oedema the reverse occurs. In fact no significant differences were found between the two phases. The differences between the normal tissues and the injured one were very significant [12].

An alternative [42, 46] is that the collecting lymphatics might be attached to the tissues, which would be distorted during the emptying of these vessels and hence cause suction on them during their filling. This could be transmitted to the initial lymphatics. However this can not be true: during the filling of the initial lymphatics, the pressures in them are slightly lower than in the collecting lymphatics [50, 53]. Also, unlike with the initial lymphatics, anchoring filaments do not attach to the walls of the collecting ones [10], so the tissues do not have these to pull on.

Pumping by Endothelial Vesicles

Occasionally, some workers hold that vesicles might actively pump fluid, proteins, etc. into the vessels [10]. This ignores the facts that it has repeatedly been shown that almost all material enters the vessels via the open junctions (even though these are sometimes infrequent - 18), that the endothelium is quite unlike active cells (e.g. epithelium), and that vesicles are passive structures moved by Brownian motion [10, 49]. They transport material down a concentration gradient, i.e. diffusion in quanta.

Colloidal Osmotic Pressure of the Lymph

It has been suggested [4, 10] that fluid and proteins could be caused to enter the vessels if the lymph had a higher protein concentration than that of the tissue fluid, if this state were periodically renewed - against the dilution of the lymph by this entering fluid, if the colloidal osmotic pressure difference could be effective across the widely open junctions, and if the resulting flow of fluid could sweep the proteins up their own concentration gradients. It has been shown (above) that other alternative mechanisms have evidence against them: the various prerequisites for this one have increasing evidence in their favour (below). Details have been discussed elsewhere [4, 7, 8, 9, 10, 19]; the essentials are outlined below:

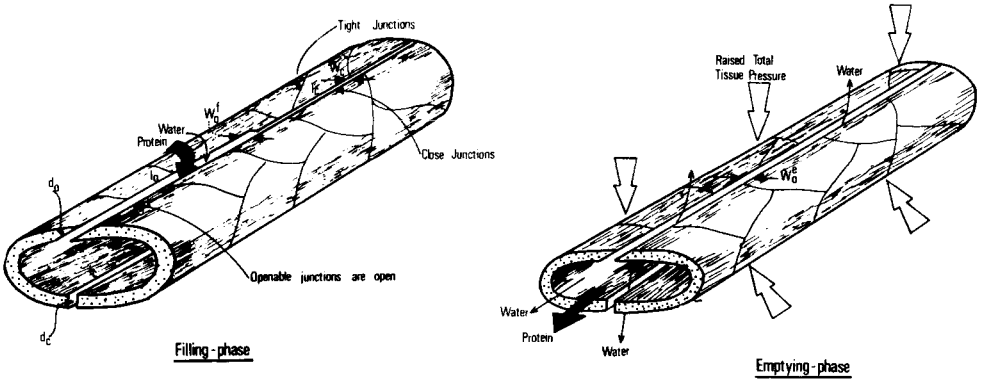


Figure 1. Filling- and emptying-phases of initial lymphatic cycle [19].

Basically, the hypothesis is that the the concentrated lymph's colloidal osmotic pressure causes fluid and protein to enter the initial lymphatics during the filling-phase. Once the lymph is so diluted that there is an equilibrium of all the hydrostatic and osmotic pressures, the junctions close under the influence of the former; the intermediate-phase begins. The emptying-phase starts when the total tissue pressure rises. This will be transmitted almost in toto to the lymph, and is considerably greater than the (increased) tissue hydrostatic pressure. Thus fluid is ultrafiltered out of the the junctions - the normally close (narrow) ones and those which were open, but which are temporally closed to proteins. Thus the lymph is reconcentrated. Simultaneously, some lymph is forced into the adjacent collecting lymphatics. These are subjected to similar total tissue pressures, etc., so the lymph in them would not alter, but would represent the mean of that in the initial lymphatics. Once it passes to the remote collecting lymphatics, outside the region, the concentrated lymph would be rediluted (because of its high osmotic pressure) to approximately equal the tissue fluid outside these vessels. Even then it is likely to be altered by being increasingly concentrated by the raised hydrostatic pressures, caused by the contractions of the vessels' walls [23]. Other changes will occur due to lympho-venous communications and at lymph-nodes [10, 13, 20].

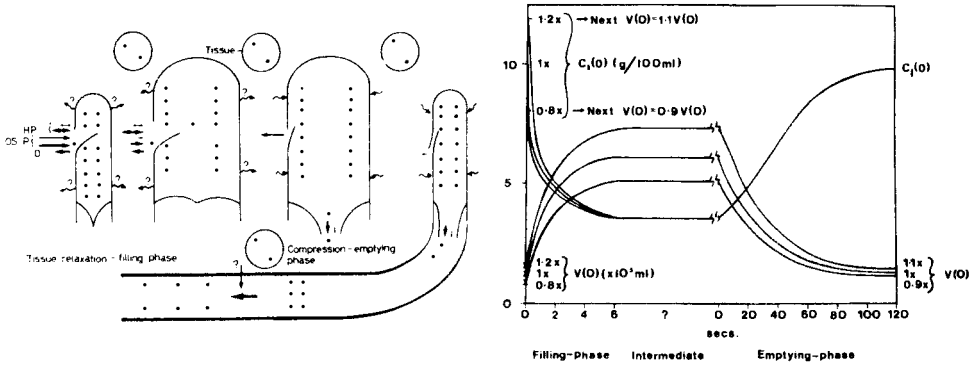


Figure 2. A diagram (left) shows the protein concentrations, the tissue pull (in edema) and push (wavy arrows), the directions and magnitudes of flux of fluid & protein (thin & thick arrows) from hydrostatic pressure, osmotic pressure and diffusion. On the right are lymph volume & concentration, in a mathematical model [19], showing their negative-feedback.

## EVIDENCE CONCERNING THE COLLOIDAL OSMOTIC PRESSURE HYPOTHESIS

### The Concentration of Lymph, and its Variation during the Cycle

It is vital that the distinction between initial lymphatic lymph and that in the remote collecting lymphatics be clearly appreciated. The latter is what is almost always sampled; its possible variations have been discussed. Initial lymphatic lymph has only been sampled twice [23, 44]. Unfortunately in neither case were there varying total tissue pressures; thus this hypothetical mechanism could not have been operating.

Tissue fluid is still more difficult to sample, except from relatively huge volumes of tissue (even with micromethods). This has little relation to the minute amounts near the initial lymphatics [10, 13, 20]. Even so, when a sample has been compared with initial lymphatic lymph [43, 44], no varying total tissue pressure was present - so that this hypothesis was not tested.

Intravital light microscopy has indeed suggested that initial lymphatic lymph is more concentrated than tissue fluid [24, 28, 51, 52] but, because of possible exclusion of macromolecules from the interstitial tissue, this is inconclusive. Observations on sections with the light microscope also suggest this, but again such exclusion is possible [26, 27].

The electron microscope allows one to avoid cells and fibres in the tissue, so that only the exclusion by the ground substance is at present unknown and unavoidable. Calculations [7, 17] suggest that it is not significant, in view of the considerable increases (about 3 times) in protein concentrations observed in the lymph compared with those in the tissues. These have been found in the mouse ear, diaphragm and jejunum, and the puppy jejunum, by counting ferritin molecules and lipoproteins, using mass-densitometry on native plasma proteins, and by autoradiography with RISA [7, 9, 11, 17]. Oedematous tissue showed similar concentration differences, yet minimised possible exclusion effects [7]; using the diaphragm (with the tracers being injected into the peritoneal cavity) avoided them completely [7, 11].

These studies not only showed that the mean protein concentrations in the initial lymphatics, taken randomly over the whole cycle, was some three times that in the tissues. More significantly, the variations in the concentrations of both plasma proteins and ferritin during the various stages of the cycle were just as suggested by the hypothesis, and as would be essential for its functioning [7, 9, 11]. This was in the diaphragm and the jejunum, stopping the cycle by freezing as well as by chemical fixation. It can be seen that dilution occurs with filling; while reconcentration occurs during emptying:

TABLE 2

RATIOS OF PROTEIN CONCENTRATIONS IN THE LYMPH / TISSUE FLUID

| | Time after start of phase | Mean Ratio | Standard Error |
|---|---|---|---|
| EMPTYING-PHASE | 2 seconds | 3.19 | (0.291) |
| (inspiration) | 10 | 5.21 | (0.413) |
| FILLING-PHASE | 2 | 3.17 | (0.287) |
| (expiration) | 10 | 1.78 | (0.209) |
| | 30 | 1.05 | (0.136) |

These were freeze-substituted mouse diaphragms [11]. The protein concentration was measured by mass-densitometry in the electron microscope, with 80 observations per group.

The initial lymphatics in the jejunum receive a relatively uniform dose of the tracers [7, 9, 17]. Thus the redilution of the lymph in the remote collectors (i.e. those just outside the villi) is evident, without its possible artefactual dilution by unlabled lymph. As predicted by the hypothesis, this remote-collector lymph was greatly diluted relative to that in the initial lymphatics, and that in the adjacent collectors was not [7]. The latter has also been found by others [34]. They held it invalidated the hypothesis, but the signficance of the remote- vs. the adjacent-collectors had not been realised.

## Colloidal Osmotic Pressures across Large Pores

There is increasing theoretical support for the concept that macromolecules can exert nearly all their colloidal osmotic pressures across pores many times wider than themselves, and that the fluid-flow thus caused can sweep some of them along with it, into the more concentrated solution [5, 6, 37, 39, 54]. I.e. they can climb up their own concentration gradients - the "bootstraps effect" [39]. Perhaps their diffusion coefficients are so low that the flow of fluid largely prevents their outwards diffusion. Thus, a "virtual membrane" may occur at the pore - just as if a true semi-permeable membrane were there, except that this one is freely permeable to inflowing protein as well as fluid [5]. The functioning of fenestrae in blood capillaries also supports this idea [6, 10]. Here the same mechanism probably occurs, giving rise to a large local - tissue - circulation of fluid and proteins, with a small (but very important - 6) part entering lymphatics.

There is also in vitro evidence. Considerable colloidal osmotic pressures can be produced even across a piece of filter paper [15]! Not only have the colloidal osmotic pressures been confirmed, but the nett uptake of macromolecules has been demonstrated, in vitro, across pores similar in size to both fenestrae and the open junctions of initial lymphatics [Fig. 3 - 14]. While, for the colloidal osmotic pressure to be maintained, the solute must be continuously replaced (preventing local, unstirred-layer effects), it seems feasible for this effect to occur for long enough to fill the initial lymphatics - given the large concentration differences.

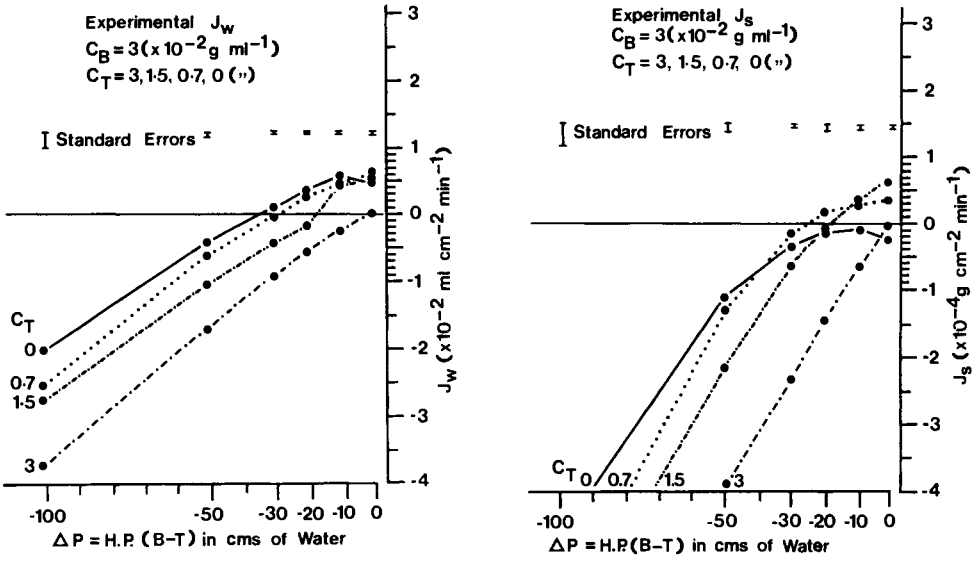


Figure 3. Fluid ($J_w$) and protein ($J_s$) fluxes across a membrane (50 nm pores), with 3 g/100 ml of dextran (70,000) as the concentrated solution ($C_B$) and the dilute one ($C_T$) varying. H.P. = hydrostatic pressure; -ve. = from B to T; fluxes into B are positive [14].

## Ultrafiltration of the Initial Lymphatic Lymph

It has been shown [8] that only some 1% of the fluid entering the initial lymphatics actually passes to the collecting ones. While some lymph may

well be lost before it reaches ducts large enough to cannulate [20], most of the other 99% is presumably ultrafiltered to the tissues. This water will not just dilute the local tissue fluid - which would vitiate the hypothesis [45, 46] - but, because it causes a local increase in tissue hydrostatic pressure, will pass into the ground substance and thus to the blood. Much also leaks out of junctions other than those which open [19].

### The Permeabilities of the Lymphatic Walls

It is well known that both small and large molecules enter the vessels via the open junctions, and that the close (narrow) junctions are permeable to small molecules [10]. It has frequently been shown that the walls of the collecting lymphatics are quite permeable to small molecules, but not to large ones [10]. Hence the selective mechanisms exist for filling the initial lymphatics, for ultrafiltering the fluid in them, and for the redilution of the lymph in the remote collecting lymphatics.

### Modelling the Initial Lymphatic Cycle

A mathematical model of the cycle [19] shows that the whole process is physically possible (Fig. 2) and the inevitability of the intermediate--phase. It also shows that it possesses negative-feedback. Disturbing the cycle (by variations in the amounts of fluid or protein reaching the vessels, or in the lengths of the phases of the cycle), causes it to adjust itself so that these effects are minimised.

## CONCLUSION

In oedematous tissues (and a few normal ones) the initial lymphatics probably fill via an inwardly-directed hydrostatic pressure gradient. In most normal tissues the colloidal osmotic pressure hypothesis offers the only ready explanation for how material enters these vessels. There is considerable in vitro and in vivo evidence in its favour. It is however equally evident that considerably more information is needed, especially intravital studies using varying total tissue pressures.

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# ARE PRESSURES IN IMPLANTED CAPSULES A VALID MEASURE OF INTERSTITIAL FLUID PRESSURES?

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Before 1960, almost all research workers used the following method in an attempt to measure the interstitial fluid pressure: A small syringe needle was inserted into the tissue, and then a minute amount of fluid was injected at the tip of the needle. While the injected fluid was still at the tip, the pressure was measured by a low compliance pressure measuring device. However, as the fluid became absorbed, the pressure measurements became erratic, and then additional amounts of fluid had to be injected. This method almost universally measured a slightly positive pressure in loose subcutaneous tissue and somewhat higher positive pressures in tight tissues.

However, in the last twenty years the needle method has been employed with progressively less frequency. In its place are three other methods: (1) the implanted perforated capsule method that we introduced in 1960[1], (2) the wick method that was introduced by Scholander and his co-workers in 1968[2], and (3) the microcapillary pipette method introduced by Wiederhielm also in 1968[3]. In general, the pressures measured in loose subcutaneous tissue using the implanted capsule technique range from -4 to -7 mm Hg (when the surrounding atmospheric pressure is the zero reference pressure). The wick method gives values ranging from -1 to -3 mm Hg, and the microcapillary pipette method gives values from -2 mm Hg to +2 mm Hg. Since these pressure values differ from each other, it is questionable which one of them most nearly represents true interstitial fluid pressure. Furthermore, there are not many ways for testing the validity of the different methods, because the true values are not known.

When we first introduced the implanted perforated capsule method, we were quite aware of this problem. Therefore, we set up a number of different tests to determine whether or not the pressures measured by the capsule method were reasonable in different types of experimental conditions. For instance, would the capsule pressures change in the appropriate direction and quantitatively by appropriate amounts when tissue fluid volumes were increased or decreased? Also, would the measured capsule pressures change the way that one would expect interstitial pressure to change when capillary pressure is raised or decreased? And, finally when the colloid osmotic pressure of the circulating plasma is altered, are the changes in pressures those that would be expected? In other words, are the recorded changes those that one would expect on the basis of a reasonable understanding of the architecture of the tissues themselves, and do the pressures change in the appropriate direction and by appropriate quantitative amounts to fit with Starling's law of the capillaries? Some

of the tests for validating the capsule technique were the following:

## RE-EQUILIBRATION OF CAPSULE PRESSURE MEASUREMENTS AFTER PERTUBATIONS

Figure 1 illustrates two ten-minute periods of capsule pressure measurements under two separate conditions [4]: First, in the upper record a minute amount of fluid was injected into the capsule to raise the pressure instantaneously from -6 mm Hg to +3 mm Hg. During the subsequent minutes, this extra fluid disappeared from the capsule, and the pressure returned almost exactly back to the -6 mm Hg that had been recorded at the beginning of the record. In the lower record, the opposite effect was observed, a minute amount of fluid was withdrawn from the

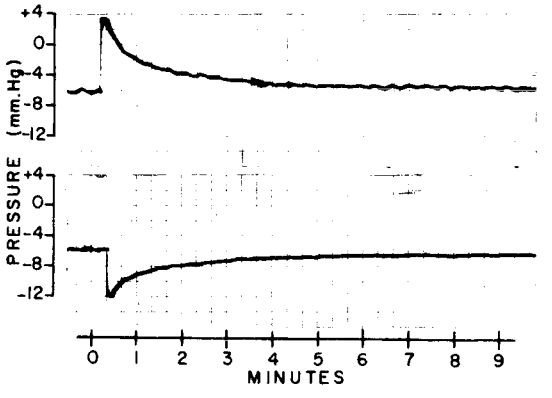


Figure 1. Re-equilibration of pressure in a capsule after the fluid volume in the capsule was changed. During the upper record, 1.5 $mm^3$ of fluid was injected into the capsule. During the lower record, 1.0 $mm^3$ of fluid was removed from the capsule. Note the asymptotic approaches of the pressure back toward its original level. (Reprinted from Guyton, Circ. Res. 12:399, 1963, with permission of the American Heart Assn., Inc.)

capsule, reducing the pressure from -6 to -12 mm Hg. Again, during the ensuing few minutes the pressure returned almost exactly to the previous level. From these records, it is clear that the measured pressures in the capsule are dynamically established and are not a sequested type of pressure.

Similar studies were performed in implanted capsules in dead dogs. Almost identically the same types of records were recorded. This indicated that the fluid movement was not between the cavity of the capsule and flowing blood in the capillaries. Instead, the movement was presumably through the perforations between the capsule cavity and the surrounding interstitial fluid. Furthermore, the dye T-1824, which attaches to plasma proteins, was injected into capsules and was observed over a period of hours to diffuse through the perforations of the capsules. This indicated that there is reasonably free movement of proteins through the interstitial spaces that communicate between the capsule cavity and the surrounding interstitium. In a later study, Granger [5] measured the reflection coefficient of the capsule membrane for albumin and showed

this to be only 0.23, indicating once again that proteins diffuse through the connecting passageways between the cavity and the surrounding interstitial fluids almost as easily as the diffusion of water itself.

## MEASUREMENT OF CHANGES IN CAPSULE PRESSURE AND SIMULTANEOUS CHANGES IN NEEDLE PRESSURE IN VERY EDEMATOUS TISSUE

When large amounts of edema fluid are present in a tissue, the fluid is so mobile in the tissue that its pressure is the same in widespread areas. Also, fluid can sometimes actually be withdrawn from the edematous tissue through an inserted needle. To measure fluid pressure in the edematous tissue using a needle under these conditions does not require preliminary injection of a small bolus of fluid at the tip of the needle. Furthermore, pressing on the edematous tissue will raise the pressure in the edema fluid, and corresponding changes in pressure can be recorded from a needle or from an implanted capsule. The upper tracing of Figure 2 illustrates such needle pressure measurements and the lower tracing

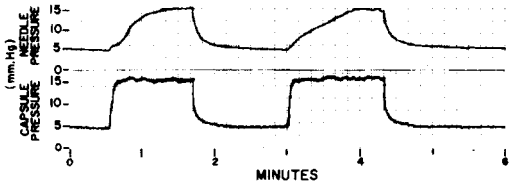


Figure 2. Change in needle pressure and capsule pressure in an edematous dog leg when the edematous tissue was compressed from the outside. This record shows rapid elevation of the capsule pressure and much slower elevation of the needle pressure, but equal total change in both measured values. (Reprinted from Guyton, Circ. Res. 12:399, 1963, with permission of the American Heart Assn., Inc.)

simultaneous capsule pressure measurements [5]. Note that when the edematous tissue was compressed the pressure rose from +5 mm Hg to +15 mm Hg, as recorded by both methods. However, the pressure recorded by the capsule rose to its new equilibrium level within a few seconds, though as long as one minute was required for full response of the pressure recorded by the needle. These records illustrate that the pressure measured in the cavity of the capsule is a true representation of the pressure in the fluid outside the capsule (as measured by a needle). The records also illustrate that there was no osmotic pressure difference between the two pressure measurements, indicating that the pressure inside the capsule, at least normally, is not caused by an osmotic pressure

differential at the lining of the capsule. Unfortunately, similar comparisons between capsule and needle measurements could not be made in the negative pressure range because the needle will not measure pressure changes in that range.

## Measurement of Transmembrane Fluid Movement Through Tissue Capillaries Under the Influence of Different Starling Forces.

If the capsule method gives a reliable measurement of interstitial fluid pressure, then it should be possible also to use the capsule procedure for studying the relationship between interstitial pressures and rates of fluid exchange across the capillary membranes of the tissues lining the inner surfaces of the capsules. Figure 3 illustrates such an experiment in which fluid was sucked from a needle inserted into a capsule cavity [6]. In Panel A, the venous pressure was kept constant at +7 mm Hg while the interstitial pressure (the pressure inside the cavity of the capsule) was decreased in steps from -6 mm Hg down to -36 mm Hg. The upper tracing of this panel shows the volume of fluid movement from the capillaries into the capsule cavity and thence into the

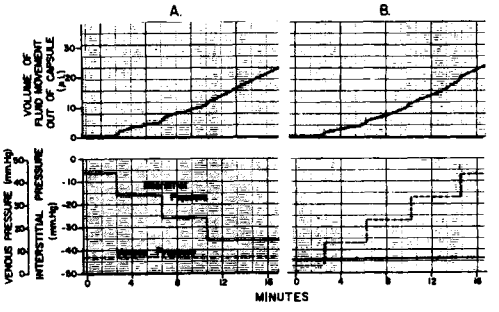


Figure 3. Movement of fluid through the tissue capillary membrane as measured by fluid movement out of the capsule caused by: A. progressive decrease of interstitial fluid pressure to more negative values, and B. progressive increase in venous pressure to more positive values. (Reprinted from Guyton et al.: Circ.Res. 19:1022, 1966, with permission of the American Heart Assn., Inc.)

needle. Note that the slope of the fluid volume curve (which was a measure of the rate of fluid movement) became progressively steeper as the interstitial pressure was decreased. Furthermore, the increase in fluid movement was linearly related to the decrease in interstitial pressure below the initial value of -6 mm Hg, which was the value that gave zero fluid outflow.

In Panel B of Figure 3, the interstitial pressure was maintained at a constant value of -6 mm Hg while the venous pressure was raised from an initial value of +3 mm Hg up to a final value of +43 mm Hg. After each step of venous pressure rise, the rate of fluid movement from

the capillaries into the capsule again increased progressively and linearly. Furthermore, the rate of increase of fluid movement out of the capillaries was almost exactly the same for a given rise in venous pressure as for a given decrease in interstitial pressure. Thus, it was clear that pressures measured in the cavities of the capsules caused movement of fluids through the local capillary membranes in a manner that would be predicted by applying the principles of Starling's law of the capillaries.

## SHAPE OF THE INTERSTITIAL FLUID VOLUME-PRESSURE CURVE AS MEASURED USING THE CAPSULE METHOD

If normal subcutaneous tissues have a negative interstitial fluid pressure, then one would expect the solid elements of the tissues (the collagen fibrils, etc.) and the semi-solid elements of the tissue (the tissue gel and the tissue cells) to be conpacted against each other. Therefore, very slight changes in the amount of fluid in the tissue spaces should change the interstitial pressure considerably. Yet, once edema should occur, one would expect the great excess of fluid in the tissues to push the solid and semi-solid elements of the tissues apart, an effect that has actually been observed by Gersh and Catchpole [7]. Furthermore, since the tensile elements of the subcutaneous tissues are not strong, one would expect that in the edematous state a large change in interstitial fluid volume would cause very little change in pressure. Figure 4 illustrates four volume-pressure curves recorded from four separate dog

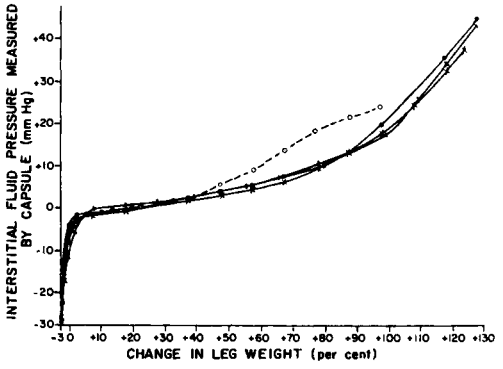


Figure 4. Relationship of interstitial fluid pressure to change in leg weight during progressive increase in interstitial fluid volume. Each curve represents results from a separate leg. (From Guyton, Circ. Res. 16:452, 1965, with permission of the American Heart Assn., Inc.)

legs [8] in which the following measurements were made: First, the leg was perfused with 10 per cent Dextran solution which reduced the weight of the leg by 3 per cent. The initial average capsule pressure measured in the leg was -7 mm Hg and this decreased to an average of -27 mm Hg as a result of the 3 per cent loss in leg weight. Then the leg was made progressively more edematous by perfusing the leg vasculature with saline

having no Dextran, and perfusing at a high pressure. The curves illustrated in the figure are those generated during this progressive increase in fluid in the leg. Note that, so long as the measured interstitial fluid pressures were below atmospheric pressure, the pressures recorded in each of the capsules rose extremely rapidly for very slight changes in leg weight. However above atmospheric pressure, tremendous changes in leg weight occurred for very little change in measured interstitial pressure. Indeed, one is impressed for each curve with the sharpness of the change in tissue compliance that occurred almost exactly when the pressure in the capsule reached atmospheric pressure level.

It is clear, therefore, from the curves in Figure 4 that the compliance of normal tissues is far less than the compliance of edematous tissues. This fits with the idea that normal tissues function in a compression mode in which the solid elements of the tissues are compacted together, and it fits with the idea that in the edematous state the structures of the tissues can be pushed apart quite easily, thus giving a much higher compliance.

## MEASUREMENT OF CAPSULE PRESSURES WHEN THE PLASMA COLLOID OSMOTIC PRESSURE IS GREATLY INCREASED

When concentrated Dextran solution or any other highly oncotic solution is injected into the circulating blood, one would expect absorption of fluid from the interstitium into the circulating blood and corresponding reduction of the volume of fluid in the interstitium. The reduced volume of fluid in the interstitium should then decrease the interstitial fluid pressure. Such an experiment was performed 44 times in dogs [8]. The plasma colloid osmotic pressure was increased by injecting 20 per cent Dextran-70 solution intravenously. Figure 5 illustrates a typical experiment in which 50 ml of 20 per cent Dextran were injected three separate times, followed by three injections of 150 ml of isotonic salt solution, while tissue capsule pressure was being recorded. Note the marked decrease in pressure (to a greater degree of negativity) each time the tissues were dehydrated by injecting the Dextran. Also, note the marked increase in pressure each time the tissues were hydrated by injecting saline. Two explanations have been offered for the marked decrease in capsule pressure: (1) that this results from increased oncotic pressure in the interstitial fluid, which then causes osmotic withdrawal of fluid from the capsule, (2) that this results from decreased interstitial hydrostatic pressure, which is measured directly by the capsule pressure. In 8 of the experiments, 8 ml per kg of 20 per cent Dextran was injected into dogs with a normal negative capsule pressure averaging −5 mm Hg, and the pressure fell to an average of −11 mm Hg, a decrease of 6 mm Hg. Addition of the 8 ml of 20 per cent Dextran to the plasma could theoretically pull a maximum of 24 ml of fluid from the interstitium for each kg of tissue if there were no rise in capillary pressure. However, the blood volume would increase from 70 to 102 ml, an increase of 46 per cent which would certainly increase the capillary pressure tremendously. Therefore, it is doubtful that more than about 8 ml of fluid could be removed from the interstitium. Since the interstitial volume per kg is 160 ml, this would cause 5 per cent interstitial fluid dehydration or an increase in subcutaneous tissue interstitial fluid oncotic pressure of only 0.25 mm Hg. Since the actual decrease in the recorded capsule pressure was 24 times this much, the concept that interstitial fluid oncotic pressure changes caused osmotic removal of fluid from the capsule is not a valid explanation for the decrease in pressure. On the other

hand, a decrease in interstitial hydrostatic pressure is a viable explanation. Furthermore, the 6 mm Hg decrease in pressure that was recorded is approximately the hydrostatic pressure change one would predict from measurements of interstitial space compliance made in other ways.

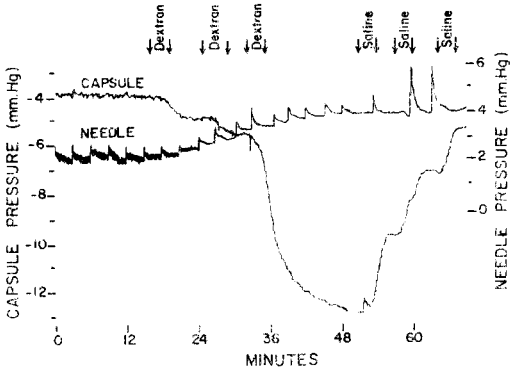


Figure 5. Effect on capsule and needle pressures caused by injecting intravenously 20 per cent Dextran solution and subsequently isotonic saline solution. Each injection of Dextran was 50 ml, and each injection of saline was 150 ml. Note that the Dextran, which dehydrated the interstitial spaces, caused the capsule pressure to fall, while saline, which hydrated the interstitial spaces, caused the capsule pressure to rise. These responses are those predicted by the principles of Starling's equilibrium of the capillaries. The needle pressures, however, failed to respond in the same direction, illustrating that these are not measurements of interstitial fluid pressure. (Reprinted from Guyton, Circ. Res. 12:399, 1963, with permission of the American Heart Assn., Inc.)

## COMMENT

In the above paragraphs we have recounted some of the procedures employed to show that recorded capsule pressures change directionally and approximately quantitatively as one would predict from knowledge of the physical nature of the interstitium itself and knowledge of movement of fluids through capillary membranes in response to changes in Starling capillary forces. The other methods for measuring interstitial pressure either have not been substantiated in similar ways or have been substantiated only partially. However, simultaneous recordings of tissue pressure, by the capsule method and the wick method were made by Prather and his colleagues [9]. They showed that when various Starling forces were changed, the changes in pressures recorded by the capsule were 4 or more times as great as those recorded by the wick, and the rapidity with which these changes occurred was 3 to 10 times as great. Nevertheless, the wick method did give changes in the proper direction, though changes that were considerably less than those that would have been pre-

dicted from our knowledge of Starling's law of the capillaries.

Therefore, we conclude that the recorded pressure measurements made using the capsule method do change in appropriate directions and approximately quantitatively in accord with known principles of fluid exchange through the capillary membranes in tissue spaces.

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*Adv. Physiol. Sci. Vol. 7. Cardiovascular Physiology. Microcirculation and Capillary Exchange*
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# DETERMINANTS OF LYMPH FLOW AND COMPOSITION

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According to Starling's Hypothesis, the rate of fluid movement across capillary walls depends on the balance of hydrostatic (P) and colloid osmotic ($\Pi$) forces. Under normal conditions, a small net loss of fluid from the capillaries is compensated for by lymphatic drainage of leaked fluid and solutes from the interstitium. Filtration rate and lymph flow can be increased by raising capillary hydrostatic pressure or by lowering plasma colloid osmotic pressure, or by combinations of these procedures. Two questions may be asked about the results: 1) Are the increments of lymph flow produced by comparable changes in P and $\Pi$ the same? 2) Are the changes in lymph:plasma protein concentration ratios (R) the same? The answers may throw light on the nature of pathways in the capillary wall through which fluid and macromolecular solutes penetrate. Specifically, we might expect changes in $\Pi$ to have less effect on both fluid and pro ein transport if transport takes place through large transcellular or inte - cellular openings which have low reflection coefficients for plasma proteins.

## EXPERIMENTAL

We compared the effects of plasma protein dilution by plasmapheresis with those produced by venous congestion on the flow and composition of lymph from the paws of anesthetized dogs. Lymphatics of both hind paws were cannulated. The paws were flexed mechanically to promote lymph drainage. Lymph samples were collected at 30 minute intervals, and flows determined by weight/time. Arterial blood samples were taken every half hour. Samples were analyzed for total plasma protein ([P]) and for individual components as described in previous publications (Renkin et al. 1977). Experiments were done on 7 dogs. In 5 dogs, one paw was subjected to venous congestion, either before (3) or after (2) plasmapheresis. In one dog, only venous congestion was done. In all experiments, these manipulations followed a period in which control data for both paws was obtained.

## RESULTS

Parameters measured directly are summarized in table 1 below.

Table 1 Parameters measured directly (mean±SEM)

| | [P]P mg/ml | PV mmHg | L mg/min | [P]L mg/ml | R |
|---|---|---|---|---|---|
| Control (12) | 60.7 ± 1.4 | 6.2 ± 0.4 | 10.3 ± 1.2 | 18.3 ± 2.5 | 0.30 ± .04 |
| Incr PV (4) | 61.8 ± 4.0 | 27.6 ± 1.9 | 31.2 ± 5.2 | 10.5 ± 3.6 | 0.16 ± .05 |
| Decr [P]P (6) | 31.6 ± 1.6 | 6.7 ± 0.8 | 30.4 ± 6.0 | 6.7 ± 1.0 | 0.21 ± .03 |
| Both (5) | 31.8 ± 1.9 | 24.5 ± 2.4 | 52.3 ± 6.7 | 4.0 ± 0.9 | 0.13 ± .03 |

On the average, plasma protein concentration ([P]P) was reduced to nearly half the control level. This produced an almost 3-fold increase in lymph flow (L) at normal venous pressure. The degree of venous pressure (PV) elevation was chosen to give about the same lymph flow increase. Superposition of both procedures resulted in an increase in L close to the sum of those produced by either one alone. Lymph protein concentration ([P]L) decreased after venous congestion, decreased more after plasma dilution, and still further after both procedures. Reduction of lymph: plasma ratio for total protein (R) was parallel to the increase of lymph flow, but less than proportional to it. The magnitude of change in R was similar for plasmapheresis and venous congestion.

Colloid osmotic pressures of plasma ($\Pi P$) and lymph ($\Pi L$) were calculated from total protein concentrations and albumin/globulin ratios by the equations of Navar and Navar (1977).

Table 2 Calculated Osmotic Pressures (mmHg)

| | $\Pi P$ | $\Pi L$ | ($\Pi P$-$\Pi L$) |
|---|---|---|---|
| Control (12) | 18.4 ± 0.6 | 3.8 ± 0.7 | 14.7 ± 0.6 |
| Incr PV (4) | 18.2 ± 1.8 | 2.0 ± 0.8 | 16.2 ± 1.2 |
| Decr [P]P (6) | 7.1 ± 0.4 | 1.1 ± 0.3 | 5.9 ± 0.4 |
| Both (5) | 7.0 ± 0.4 | 0.7 ± 0.2 | 6.3 ± 0.4 |

The mean reduction in $\Pi P$ after plasmapheresis was about 11 mmHg. This is scarcely more than half the elevation of PV required to produce the same increase in L by venous congestion (about 21 mmHg, table 1). However, several factors act to modify the actual change in the balance of Starling forces in each case. First, the fall of lymph protein concentration with either maneuver acts to increase $\Pi P$-$\Pi L$, and thus to diminish the force favoring filtration. This effect is larger at the high [P]P and [P]L levels in the venous congestion experiments than at the low [P]P and [P]L levels after plasmapheresis. Second, the elevation of peripheral venous pressure is not transmitted fully to the capillaries. If postcapillary resistance is 0.2 total vascular resistance, the change in capillary hydrostatic pressure will be 0.8 time the change in venous pressure. Third, if the capillary reflection coefficient ($\sigma$) for plasma protein is less than one, the effect of changes in $\Pi P$-$\Pi L$ will be proportionally reduced. For the capillaries of the dog's paw, $\sigma$ probably lies between 0.8 and 1.0 (Renkin 1977). The fourth factor is increase of tissue hydrostatic pressure. It was not measured in our experiments, but is probably

proportional to the observed increase in lymph flow, and therefore would not contribute appreciably to the difference in Starling force balance between venous congestion and plasmapheresis, since lymph flows were closely similar.

The net force tending to increase filtration and lymph flow ($\Delta PF$) may be estimated by the relation $\Delta PF = 0.8\ \Delta PV - \sigma\ \Delta(\Pi P - \Pi L)$. The deltas refer to changes from control values. Table 3, below, gives $\Delta PF$'s for $\sigma$'s of 0.8 and 1.0.

Table 3 Filtration force changes, neglecting tissue pressures (Exp - Control), (mmHg)

| | $0.8\ \Delta PF$ | $\Delta(\Pi P - \Pi L)$ | $\Delta PF(\sigma=1)$ | $\Delta PF(\sigma=0.8)$ |
|---|---|---|---|---|
| Incr PV (4) | 17.4 ± 2.0 | 3.3 ± 1.7 | 14.2 ± 3.3 | 14.8 ± 3.0 |
| Decr [P]P (6) | 0.2 ± 0.2 | -9.7 ± 0.4 | 9.9 ± 0.6 | 8.0 ± 0.4 |
| Both (5) | 14.8 ± 2.1 | -7.2 ± 1.4 | 22.0 ± 2.4 | 18.8 ± 1.6 |

If $\sigma=1$, $\Delta PF$ for venous congestion is 40% greater than for plasmapheresis; if $\sigma=0.8$, about 85% greater. Calculation of the proportionality coefficients, $\Delta L/\Delta PF$ yields correspondingly greater values for plasmapheresis relative to venous congestion. Values for the combined procedures are intermediate.

Blood-lymph protein transport, expressed as plasma clearance of total protein (LR) increased with lymph flow after all these experimental procedures (figure 1 below). The straight lines show coupling (solvent-drag) ratios for reflection coefficients of 0 and 0.9 (slope = $1-\sigma$). The heavy dashed line is the relation of LR to L observed in an earlier series of 8 preparations in which PV was raised in graded increments (Renkin et al. 1977). The slope of the straight part is about 0.1. The observed increment in LR when L was raised by plasmapheresis was approximately twice as large as when L was raised to the same level by venous congestion.

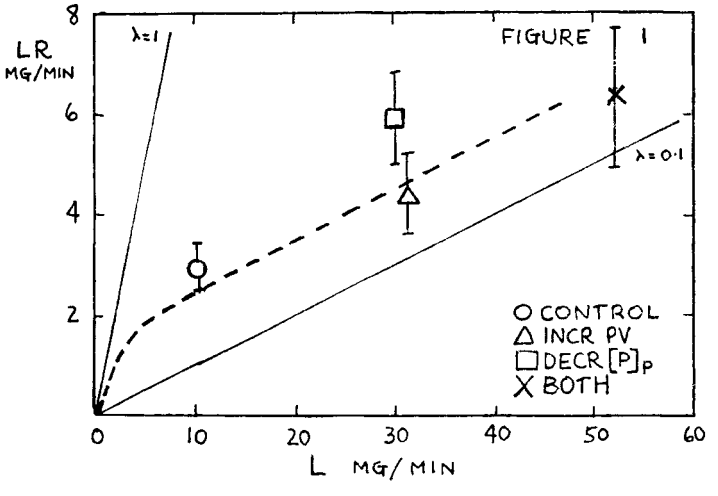


Figure 1. Changes in the relation of blood-lymph clearance of plasma protein (LR) to lymph flow (L).

Our results show that the increase in lymph flow for a given decrease in transcapillary colloid osmotic pressure difference is 1.4 to 1.8 times as great as that produced by an equivalent increase in hydrostatic pressure. Furthermore, for a given increase in lymph flow, decreasing colloid osmotic pressure produces a larger increase in blood-lymph protein clearance. Both these observations are contrary to expectation on the basis of fluid and protein transport through large pores or channels, with low reflection coefficients for plasma proteins.

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# MACROMOLECULAR REMOVAL VIA BLOOD AND LYMPH

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According to the time honored concept macromolecules, including extravasated plasma proteins, are removed from the tissue spaces by the lymphatic vessels. The amount of protein transported in 24 hrs by the lymphatic system is about equal to the total mass of circulating plasma protein /Wassermann and Mayerson, 1951; Yoffrey and Courtice, 1956; Rusznyák et al. 1961/. One of the principal supports for the view that protein from the tissue fluid is returned to the blood stream only by the way of lymphatic vessels was just the assertion that in man and in experimental animals a constant fraction of circulating protein leaves the vascular compartment and an equivalent amount is returned to the plasma in the lymph /Landis and Pappenheimer, 1963/. A calculation made on the basis of the available data /Rusznyák et al., 1961/ shows that this is not correct. For example in dogs the average lymph flow in the thoracic duct is about 2 ml/kg/hr and the albumin concentration of this lymph is about 75 % of plasma concentration. Assuming a plasma volume of 45 ml/kg the albumin fraction transported by the lymphatics is 0.033/hr or 0.00055/min. In dogs with thoracic duct fistula the fraction of injected radioalbumin leaving the circulation was 0.00118/min. Accordingly the ratio of albumin escape versus lymphatic return is 2.15, i.e. less than half of the albumin leaving the circulation is returned by the lymphatics.

We became interested in the problem of macromolecular removal from the tissue spaces via the blood stream when we were attempting to stain the renal lymphatics by injecting Evans-blue or India ink into the renal cortex of dogs. The renal lymphatics were not stained, nonetheless the injected substances disappeared rapidly from the tissue. This observation lead to a systematic study of macromolecular removal from various tissues. The protocols of these experiments are summarized in table 1. Space limitation allows here to report only some selected results.

Tab. 1. Experiments for the study of macromolecular removal via blood and lymph

1. Transport of colloids injected into the tissues
   a/ Injected substances: $I^{131}$-albumin, $I^{125}$ PVP, $Au^{198}$ colloid volume: 0.04 - 0.08 ml
   b/ Tissues: kidney, liver, gut, myocardium, striated muscle, skin
   c/ Methods: external counting, measurement of amounts present in plasma and lymph

2. Transport of cellular enzymes from various tissues
   a/ Enzymes: LDH, MDH, GOT, CPT, Gl.D.H., CPK, Phosphatases, LAP
   b/ Tissues: skeletal muscles, myocardium, kidney
   c/ Methods: muscle activity, local anoxia, measurement of enzyme activities in plasma, thoracic duct, femoral, renal and cardiac lymph

3. Absorption and transport of protein from serous cavities
   a/ Introduced substance: $^{131}$I-albumin
   b/ Sites of introduction: peritoneal cavity /diaphragm, liver, gut/, pleural cavity, pericardial sac /epicardium and parietal pericardium/
   c/ Methods:
      - Injection of diluted plasma, direct $^{131}$I-albumin application on serous surfaces
      - External counting and collection of plasma, thoracic, right duct and cardiac lymph

Starling /1896/ has observed large regional differences in the protein content of lymph and already postulated corresponding differences in the permeability of the capillary wall. It was to be expected, that these differences are reflected not only in the movement of macromolecules out from the blood stream but also in their return to it. Actually the renal cortex and the inactive striated muscle proved to be the two extremes /Szabó et al., 1973c/. Injected 131-I-albumin /Fig.1./ disappeared from the renal cortex of dogs with a T 1/2 of about 4 sec

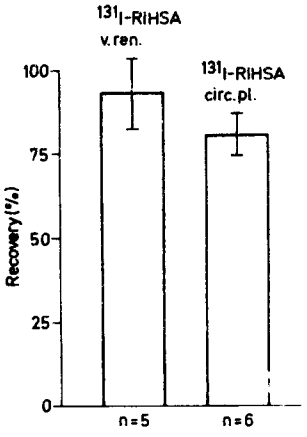


Fig. 1. Recoveries in a renal venous blood and circulating plasma of 131-I-albumin injected into the renal cortex

and 94 % of the injected radioactivity was recovered within 1 min in renal venous blood. After 5 min 80 % of the labelled protein was present in the circulating blood and the thoracic duct lymph collected in the first 30 min contained only 0.6 %. When labelled albumin was injected into the resting quadriceps muscle of dogs in 6 hrs 15 % disappeared from the site of injection, but total recovery in circulating plasma was 3.1 % and in lymph only 0.8 %. However, from actively contracting muscle 57.5 % was cleared in 6 hrs and more than 40 % recovered in thoracic duct lymph. The amount removed by the blood stream remained the same as in inactive muscle. This shows that muscle capillaries are practically impermeable to macromolecules /Fig.3./.

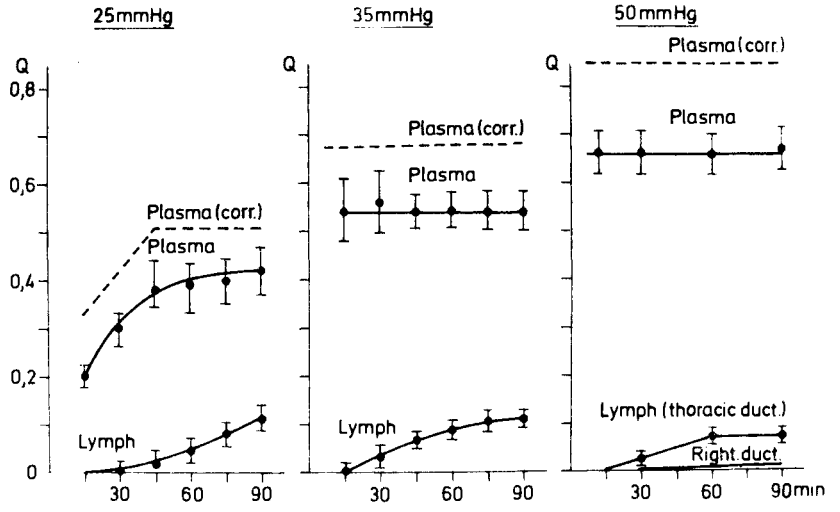


Fig. 2. Recoveries in plasma and lymph of labelled albumin infused into the common bile duct at various pressures

In the liver large intercellular gaps are present in the sinusoids and these capillaries do not restrict the leakage of macromolecules. Protein removal from the liver was studied by the infusion of labelled albumin into the common bile duct of dogs at various pressures /Szabó et al., 1975b/. At increased biliary tract pressure water and solutes leak into the liver interstitium, and are consequently removed both by the lymph and blood stream. After equilibration a constant fraction of the infused labelled albumin was found in circulating plasma. The plasma fraction increased with rising infusion pressure and at the same time the fraction recovered in thoracic duct lymph decreased /Fig. 2./. Accordingly the ratio of non-lymphatic versus lymphatic transport varies between 3.7 and 11.0.

The blood capillaries play also a major part in the removal of macromolecules from the myocardium. In 6 hrs 55 % of the labelled albumin and 31 % of 198-Au-colloid /particle size 30 nm/ injected into the left ventricular myocardium was cleared from the injection site, 34.6 % of albumin was found in circulating plasma, but only 8.6 of the gold colloid was recovered in blood and in the Kupffer cells of the liver, i.e. gained

access to the blood stream. On the other hand, 5.4 % of the albumin was recovered in cardiac lymph and 11.1 % of the gold colloid was trapped in the regional lymph nodes. In these experiments the lymphatic transport of albumin was probably underestimated. It can be assumed, that the total lymphatic transport of protein is at least 12 %. The amount recovered in blood plasma should be also corrected for secondary loss. With this correction its value is about 35 %. Accordingly the blood capillaries remove from the myocardium about 3 times as much protein and nearly as much gold colloid as the lymph vessels /Fig. 3. and 4./.

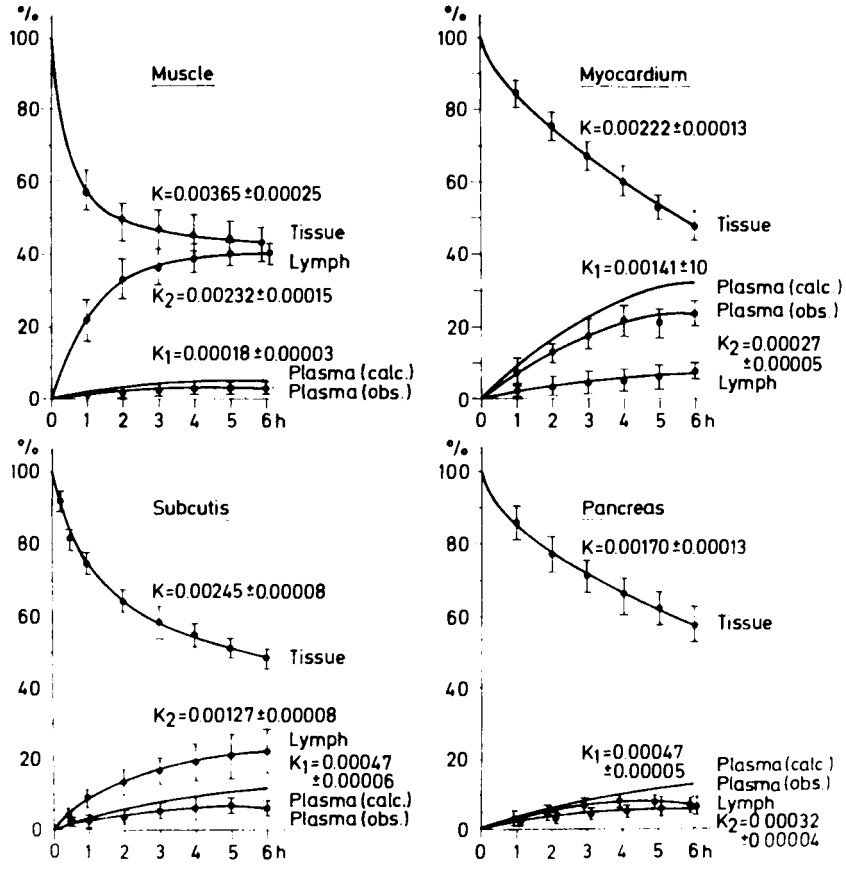


Fig. 3. Tissue clearances and recoveries in plasma and lymph of labelled albumin injected into various tissues

From the pancreas 37 % of albumin and 20 % of gold colloid was cleared in 6 hrs; 12.5 % of albumin was recovered in circulating plasma and 7.2 % in thoracic duct lymph. Of the gold colloid 7.0 % was found in lymph and lymph nodes and only 1.2 % gained access directly into the blood stream. In consequence of the breakdown of the injected protein the clearance of labelled albumin from the gut wall could not be investigated.

The removal of 125-I-labelled PVP /mol. mass 30.000 Dalton/ was measured instead. In 6 hrs 33 % of the label was recovered in plasma and urine, the lymphatic transport was 42 %. The 6 hrs clearance of gold colloid was 26 %; 16 % was recovered in the lymphatic system and 1.4 % was found in blood and liver tissue.

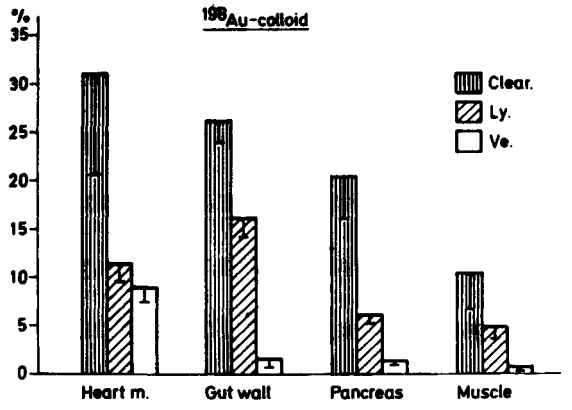


Fig. 4. Tissue clearances and recoveries in lymph/ly/ and in the circulation /Ve/ of gold colloid injected into various tissues

Macromolecular removal from the skin was studied in the hindlimb of dogs. The 6 hrs clearance of labelled PVP from the extremity which was passively moved was 53 % and that of albumin 52.7 %; 14 % of PVP and 11 % of albumin gained access directly into the circulation. The lymphatic transports were 26 % and 21.6 % respectively /Fig. 3./.

In all studies reported here evidence was obtained for the removal of macromolecules from the tissues both via lymph and via blood stream. It can be objected that in these experiments macromolecules might be driven by the injection pressure through open junctions in the microvascular wall or even through openings arising in consequence of injury. Experiments were undertaken by a method not involving intratissular injection. In these investigations the concentrations of intracellular enzymes were measured in arterial and regional venous blood as well as in local lymph. A substantial leakage of cellular enzymes into the extracellular fluid was attained by regional anoxia. In the myocardium of dogs this was elicited by 6 hrs ligation of the descending branch of the left coronary artery /Szabó et al., 1974b/. Normal concentrations of most cellular enzymes were substantially higher in cardiac lymph than in blood plasma, and during anoxia lymphatic concentrations rose before plasma concentrations. However, for some enzymes /LDH, MDH, GOT/ already after 6 hrs, for others /e.g. CPK/ after 24 hrs significant arterio-venous difference could be detected in coronary venous blood /Fig. 5./. The ratios of venous versus lymphatic transport were calculated from average cardiac lymph and coronary blood flow rates, arterio-venous differences and lymphatic enzymes concentrations. They were 1.22 /LDH/, 1.35 /MDH/ and 3.5 /GOT/ respectively.

Renal anoxia was produced by clamping both renal arteries for 30 min or 2 hrs /Szabó et al., 1974a/. A 30 min anoxia lead only to mild ischaemic damage with a rise of LDH and MDH activities in urine, renal and thoracic duct lymph. A 2 hrs ischaemia increased the concentrations of all enzymes /LDH, GOT, GPT, LAP, phosphatases/ in urine and renal lymph. Plasma and thoracic duct lymph activities of some enzymes were also increased /Fig. 6./. There was an arterial-renal venous concentration

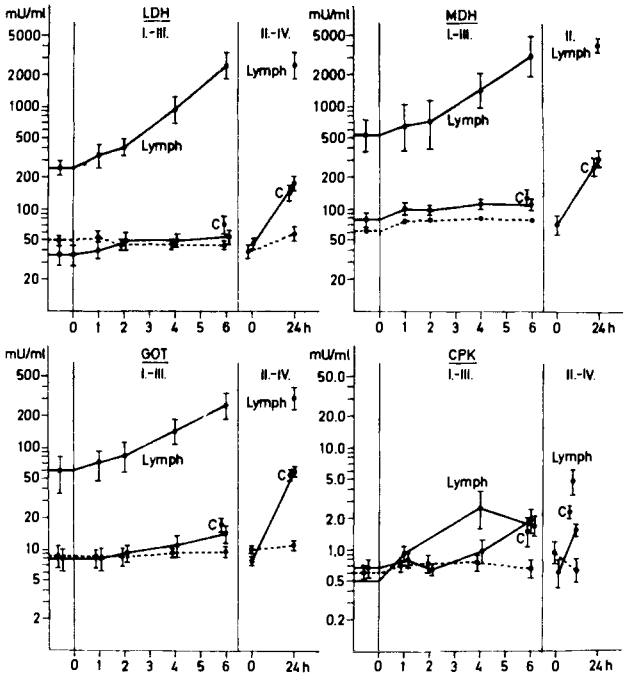


Fig. 5. Enzyme concentrations in cardiac lymph and blood plasma after coronary occlusion. Solid lines: concentration in cardiac lymph and arterial blood plasma of the infarct animals. Dashed lines: concentrations in sham-operated animals. C: enzyme concentrations in coronary sinus blood plasma

difference of LDH and GOT. Calculations were made on the importance of lymphatic and venous transport routes. For LDH and GOT venous/lymphatic transport ratios of 60 and 94 respectively were obtained.

Finally in dogs blood flow to both hindlimbs was occluded for 3 1/2 hours. The ischaemia produced 1 and 2 hrs after tourniquet release a substantial increase of LDH activity in the crural lymph and a significant difference between enzyme levels in femoral venous and arterial blood /Szabó et al., 1972/. In dogs with thoracic duct fistula lymphatic and arterial plasma LDH activities rose also after muscle activity. In these experiments the rise of plasma concentration can be interpreted as a sign of direct access of the enzyme to the blood stream.

The removal of macromolecules from the serous cavities was

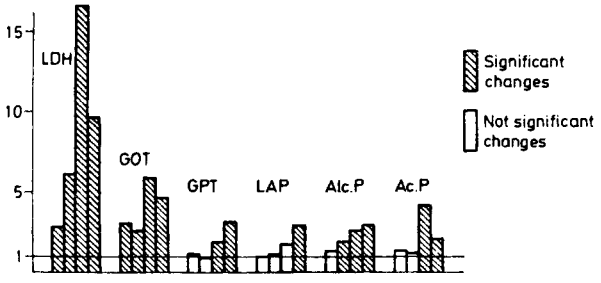


Fig. 6. Effect of 2 hr renal ischaemia on enzyme concentrations in serum /1st columns/, thoracic duct lymph /2nd columns/, renal lymph /3rd columns/ and urine /4th columns/. Enzyme activities before occlusion of renal artery = 1.

investigated by various methods /Szabó et al., 1973a/. In the first series diluted homologus plasma containing 131-I-albumin was injected into the serous cavities. With external detection it was found that the labelled protein was absorbed most rapidly from the peritoneal cavity. The rates of absorption from the thoracic cavity and from the pericardial sac were similar /Fig. 7./. The right duct or, from the pericardial sac the cardiac lymph vessel, transported more protein than the thoracic duct /Fig. 8./. In all experiments substantial amounts of labelled protein were found in blood plasma /Szabó et al., 1973b/. To eliminate the disadvantage of the injection of various amounts of fluid and protein into the serous cavities in a second series of experiments a standard amount of protein was applied to a standard surface. A filter paper with a surface of 1 $cm^2$ containing 1 mg of labelled albumin was placed directly on the serous membranes. In the peritoneal cavity in 6 hrs 50.5 % of the protein applied on the liver capsula was found in plasma and lymph, but only 12 % was removed from the abdominal surface of the diaphragm. Absorption from the intestinal serosa was insignificant. Substantial amounts of the labelled protein were recovered in blood plasma: 34.1 % after application to the liver capsula and 8.4 % after diaphragmatic application. In experiments with cannulated thoracic duct and ligated right side lymphatics these values dropped to 18.7 % and 4.7 % respectively. This may represent the true venous transport. The total amount of protein transported by the lymphatics on the right side of the neck was calculated accordingly from the differences in plasma recoveries in the two types of experiments. It was found that 5.8 %, or 58 mg albumin/$cm^2$ is transported in 6 hrs from the diaphragmatical surface by the lymphatics of the right side, 2 % or 20 $\mu g/cm^2$ by the thoracic duct and 4.7 % by the blood capillaries. On the liver capsula 25 % (250 $\mu g/cm^2$) is transported by the right duct and other lymph vessels of the right side, 7 % (70 $\mu g/cm^2$) by the thoracic duct and 19 % (190 $\mu g/cm^2$) by blood vessels /Fig. 9./. The ratio of non-lymphatic/lymphatic transport is about 0.60. In experiments where diluted plasma was injected into peritoneal cavity this ratio is significantly lower: 0.28. In the pleural cavity the computed ratio is 0.53.

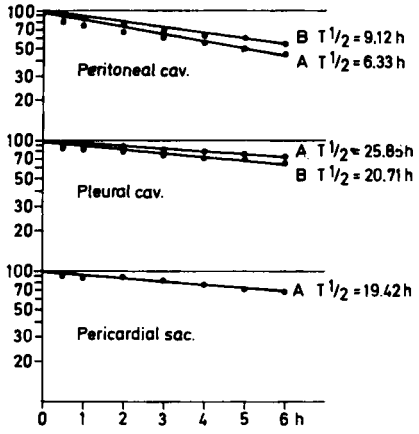


Fig. 7. Decrease of locally measured radioactivity after introduction of a fluid containing labelled albumin into the serous cavities. A: lymphatics on the right side not occluded. B: lymphatics on the right side occluded

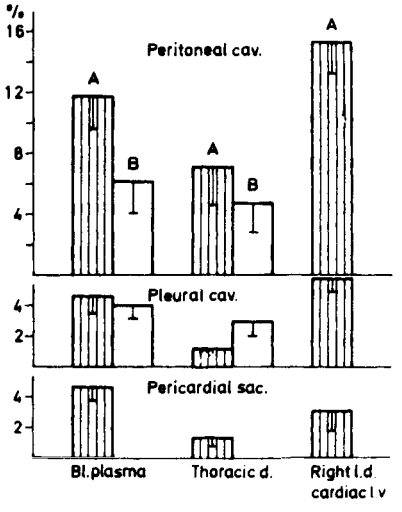


Fig. 8. 6 hrs recoveries of labelled albumin after injection of diluted homologous plasma labelled with 131-I-albumin into the serous cavities

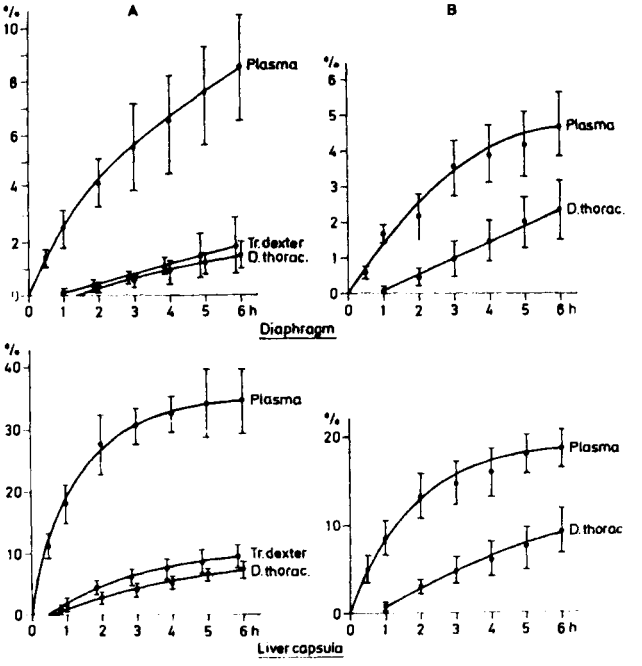


Fig. 9. Recoveries in plasma and lymph of labelled albumin applied directly to the serous membranes.
A: cannulated right lymph duct.
B: all lymphatics-venous connections of the right side occluded

In the pericardial sac absorption was studied on the epicardium and parietal pericardium /Szabó et al., 1975a/. On the epicardium total 6 hrs recovery in plasma, thoracic duct and cardiac lymph was 19 % (190 µg/cm$^2$) and on the parietal pericardium 6.3 % (63 µg/cm$^2$). The cardiac lymph vessel transported 7.1 % from the epicardium, but only 1.0 % from the parietal pericardium. Transport by the thoracic duct lymph was a few cases excepted negligible. Significant amounts, 10.7 % and 6.4 % respectively, were found in circulating plasma. The occlusion of the lymph channels on the right side of the neck decreased plasma concentration by 40 % /Fig. 10./. It follows that about this amount of cardiac lymph is carried to the cervical veins by lymphatic channels other than the so called cardiac lymph vessel. Finally the venous versus lymphatic transport ratio was found on the visceral pericardium to be 0.75.It must be stressed again that in experiments made in animals where every connection between the venous and lymphatic systems was interrupted either by cannulating or by ligating the thoracic duct and the right lymph trunk substantial amounts of the labeled protein introduced into the serous cavities were found in circulating plasma.

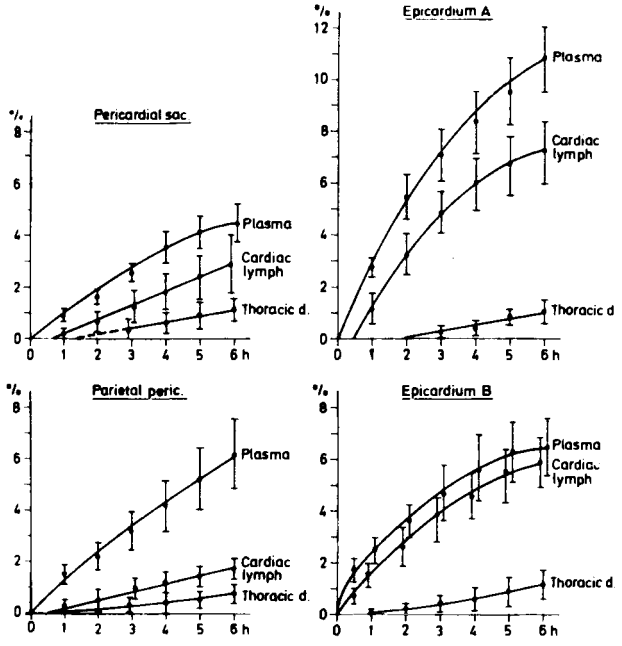


Fig. 10. Recoveries of labelled albumin injected into the pericardial sac or applied directly on the visceral or parietal pericardium. Type B of experiments: occlusion of all lymphatics on the right side of the neck

The relationship between lymphatic and non-lymphatic transport of macromolecules is different in the individual tissues, and it varies also with particle size and probably with their shape and electrical charge. The non-lymphatic versus lymphatic transport ratios found in various tissues with several macromolecules are presented in Table 2.

Very little is known about the transport pathways of macromolecules from the interstitial spaces into the microvasculature. Most of the pathways open to the macromolecules leaving the microvessels may allow their transport in the reverse direction. The passage into the blood capillaries of macromolecules introduced into the tissues, e.g. labelled colloids, or formed there, e.g. cellular enzymes, may occur by any of these mechanisms.

Tab. 2. Venous versus lymphatic transport ratios of macromolecules

| | $I^{131}$-albumin | Enzymes | | $Au^{198}$-colloid |
|---|---|---|---|---|
| Kidney cortex | 166 | LDH | 60 | 166/$Cr^{51}$-rbc/ |
| Liver | 3.7 - 11.0 | GOT | 94 | |
| Myocardium | 2.0 | LDH | 1.22 | 0.77 |
| | | MDH | 1.35 | |
| | | GOT | 2.50 | |
| Pancreas | 1.45 | | | 0.17 |
| Intest. wall | 0.80 /$I^{125}$-PVP/ | | | 0.09 |
| Muscle active | 0.08 | | | 0.09 |
| inactive | 2.10 | LDH | 1.00 | 0.09 |
| Skin | 0.38 | | | |
| Peritoneum | 0.28-0.60 | | | |
| Pleura | 0.53 | | | |
| Pericardium | 0.75 | | | |

There is also evidence suggesting that a major part of the extravasated plasma proteins returns by a mechanism yet unknown, but most probably by dissipative transport into the vasculature. The controversial question is whether a net flux of these proteins is possible or not. A net fluw would mean that proteins move not only against a hydrostatic pressure gradient, but against a protein concentration gradient as well. While this seems unlikely it must be conceded, that nothing in our observations excludes it. But, on the other hand, experiments where the passage of macromolecules was investigated in the direction of a concentration gradient cannot be interpreted as evidence for a net flux against the gradient.

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# INTERSTITIAL-TO-BLOOD MOVEMENT OF PLASMA PROTEINS

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## INTRODUCTION

It is generally accepted that diffusion, convection and vesicular exchange are the principal processes involved in the movement of plasma proteins across the capillary wall (1). When capillaries are in a state of net fluid filtration the input of macromolecules into the interstitium result partly from dissipative processes and partly by convection since the flow of solvent and concentration gradient are both directed towards the interstitium. There are, however, experimental and physiological conditions which will directionally uncouple convective and diffusive transport of macromolecules across the capillary wall. Experimentally one can reverse the diffusive process by reversing the concentration gradient of the macromolecule across the capillary wall, i.e., from interstitium to blood (2). Likewise, convective transport can be directionally uncoupled from diffusive exchange by imposing a state of net fluid absorption across the capillary wall (3, 4). In this treatise, we present the results of experiments designed to address the question of whether the diffusive and convective fluxes of macromolecules across the capillary wall can be directionally uncoupled. Two approaches were used to assess transcapillary solute fluxes: the lymphatic protein flux method and vascular efflux rate of $^{125}$I-labelled gamma globulin. The small intestine was used in these studies since diffusive and convective fluxes could be directionally uncoupled by simple experimental and physiological maneuvers.

## METHODS

### Lymph Protein Flux

The experimental values used in the lymph protein flux analysis were derived from previously published studies from this laboratory (5, 6). In brief, total lymph flow, ($J_{V,L}$) lymph protein concentration ($C_L$) and plasma protein concentration ($C_P$) were measured in isolated autoperfused cat ileum preparations. In one series of experiments capillary filtration rate (and lymph flow) was enhanced in a graded fashion by stepwise elevation of venous pressure (5). Steady-state values of $C_L$, $C_P$, and $J_{V,L}$ were acquired at each venous pressure under isovolumetric conditions. When $C_L$, $C_P$, $J_{V,L}$ and intestinal volume are in a steady-state it is assumed that lymph flow rate ($J_{V,L}$) equals net transcapillary volume flow ($J_{V,C}$), and lymph protein concentration ($C_L$) equals the interstitial protein concentration ($C_I$).

In another series of experiments $C_L$, $C_P$, and $J_{V,L}$ were determined at various transmucosal volume absorption rates (6). Net transmucosal volume absorption was stimulated by intralumenal placement of a nutrient solution, and the rate of water absorption ($J_{V,M}$) was determined using a volume recovery method. Steady-state values of $C_L$, $C_P$ and $J_{V,L}$ were acquired at each absorption rate. Under steady-state conditions it is assumed that net transcapillary volume flow ($J_{V,C}$) equals the difference between net transmucosal volume absorption rate and lymph flow, i.e., $J_{V,C} = J_{V,L} - J_{V,M}$. An inherent assumption in the estimation of $J_{V,C}$ from $J_{V,M}$ and $J_{V,L}$ is that the rate of increase in interstitial fluid volume during absorption is small relative to $J_{V,M}$ and $J_{V,L}$.

Resolution of convective and diffusive components of transcapillary protein transport in the absorbing and nonabsorbing bowel was achieved by applying the data to phenomenological equations based on the irreversible thermodynamics of solute-solvent coupling. The Kedem-Katchalsky (7) equation, which relates convective and diffusive fluxes to the net flux of molecules, states that:

$$J_{P,n} = \underbrace{J_{V,C}\,(1-\sigma_f)\,\bar{C}_P}_{\text{convective}} + \underbrace{PS\Delta C}_{\text{diffusive}} \qquad \ldots\ldots 1$$

where $J_{P,n}$ is the net transcapillary protein flux, $\sigma_f$ is the solvent drag reflection coefficient, $\bar{C}_P$ is the average protein concentration across the capillary wall, i.e., $(C_L + C_P)/2$, PS is the permeability-surface area product, and $\Delta C$ is the transcapillary protein concentration gradient ($C_P - C_L$).

The net transcapillary protein flux was estimated from the product of lymph flow and lymph protein concentration, i.e., $J_{P,n} = J_{V,L} \times C_L$. Although values of $C_P$ and $J_{V,C}$ were readily estimated from the raw data, estimation of the convective flux ($J_{P,C}$) required the determination of $\sigma_f$. The solvent drag

reflection coefficient ($\sigma_f$) was determined using the cross-point method recently described by Taylor et al. (8). With this method the solvent drag reflection coefficient ($\sigma_f$) can be estimated from the intersection of two linear equation equations relating $1-C_L/\bar{C}_p$ and $(J_{V,C} \times C_L)/(C_p - C_L)$. Each linear equation is derived from a single flux state. Solutions for $\sigma_f$ were obtained by both algebraic and graphical analysis. Having solved for $\sigma_f$ at the various flux states, the convective flux was determined using $J_{V,C}$, $C_L$, $C_p$, and $\sigma_f$, and equation 1.

Once the net transcapillary protein flux and the convective flux were determined, the diffusive protein flux ($J_{P,D}$) was estimated using the difference between the net flux and the convective flux, i.e., $J_{P,D} = J_{P,n} - J_{P,C}$.

Protein Efflux

Rabbits were injected intravenously with $^{125}I$- labelled bovine gamma globulin 16 to 18 hours prior to commencing the experiment. The animals were anesthetized with sodium pentobarbitol and urethane and a segment of jejunum was vascularly isolated except for the main artery and vein supplying the segment. The tissue was perfused by means of a roller pump with a Ringer-phosphate solution at 37°C containing 2.4% albumin and 3% Dextran 70. Prior to cannulating the vessels either $^{59}Fe\ Cl_3$ ( in an amount less than the free iron binding capacity of plasma) or $^{51}Cr$-labelled autologous red cells were injected as a vascular tracer. The tissue was perfused at a mean pressure of 75 mmHg for approximately 10 minutes and the venous effluent samples collected at 5 to 60 second intervals. At the end of the period of perfusion the loop of small intestine was removed and counted to determine the total radioactivity remaining in the tissue.

To examine if any change had occurred in the physicochemical characteristics of the labelled gamma globulin in venous washout, aliquots of the samples were subjected to ultrafiltration through Diaflo ultrafiltration membranes XM50 to UM2. These filters retain molecules with molecular weights greater that 50,000 daltons (XM50) to greater that 2,000 daltons (UM2).

Efflux ($\eta$), that is, the proportion of tracer leaving the tissue per unit time, was determined by a method of graphical analysis (2). The total $^{125}I$-gamma globulin in the tissue prior to perfusion was obtained by counting the loop of intestine after perfusion and adding the activity washed out of the tissue (excluding vascular washout) and collected in the venous samples during the perfusion. The total activity was plotted on a log scale as a function of time of perfusion. The slow rate of escape of gamma globulin from the tissue ($k_1$) was obtained by fitting a straight line to the points obtained during the latter part of the perfusion. By subtracting the values on this line from the initial curve the fast component of efflux ($k_2$) was obtained (see Figure 4).

## RESULTS

### Lymph Protein Flux

We were able to calculate the convective flux of proteins across intestinal capillaries under conditions of net capillary filtration and absorption since values for $\sigma_f$, $C_L$ and $C_p$ were acquired at each capillary volume flow. Figure 1 presents the relationship between convective protein flux and transcapillary volume flow. The results in Figure 1 indicate that the convective protein flux increases as capillary filtration rate is increased by venous pressure elevation. When the intestinal capillaries are converted from filtering to absorbing vessels by enhancing transmucosal water movement, the convective movement of proteins is in the direction of interstitium to blood. As illustrated in Figure 1, the convective movement of proteins from interstitium to blood increases in a nonlinear fashion with capillary absorption rate. These results indicate that the convective flux of macromolecules during absorption is directionally uncoupled from diffusive transport.

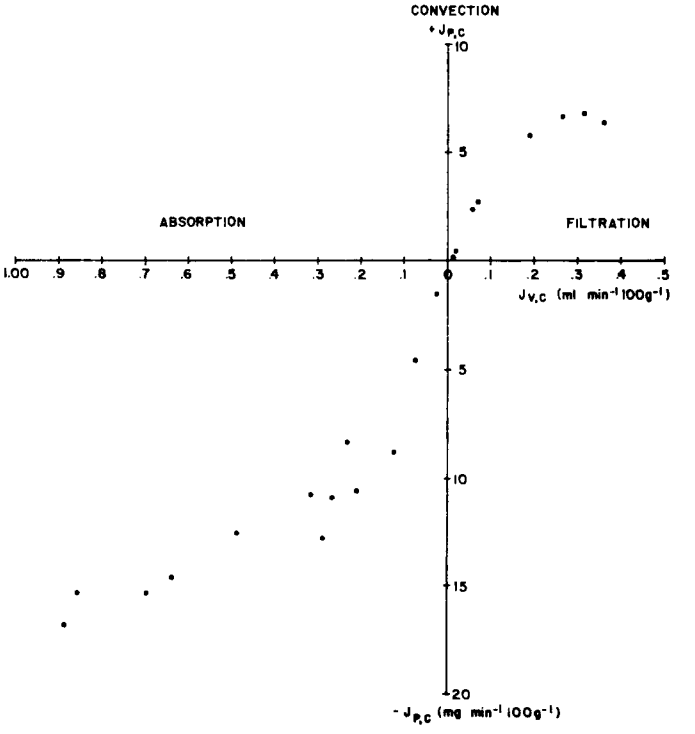


Figure 1: Relationship between transcapillary convective protein flux ($J_{P,C}$) and net transcapillary volume flow ($J_{V,C}$). Note that the convective flux is in the direction of interstitium to blood in absorbing capillaries.

The relationship between the diffusive flux of proteins and the net transcapillary volume flow under conditions of net capillary filtration and absorption is presented in Figure 2. The relationships acquired indicate that increasing the net transcapillary volume flow in either direction enhances the diffusive flux; however, the diffusive flux is more greatly affected by capillary absorption than filtration.

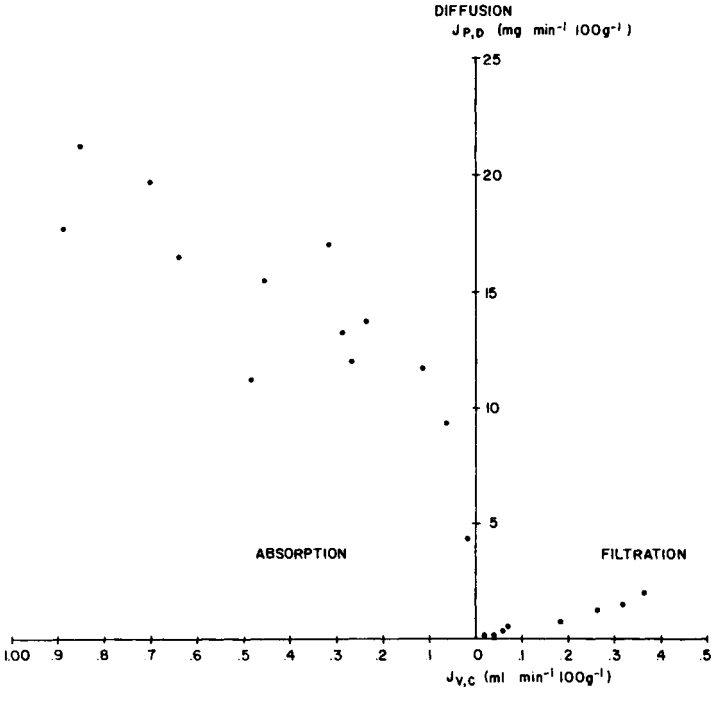


Figure 2: Relationship between transcapillary diffusive flux of proteins ($J_{P,D}$) and net transcapillary volume flow ($J_{V,C}$) in absorbing and non-absorbing small bowel.

From the data presented in Figures 1 and 2 it is evident that net transcapillary protein flux (lymphatic protein flux) increases as the net transcapillary volume flow is increased in either direction. Table I presents the values for net, diffusive and convective fluxes under control conditions, during enhanced capillary filtration, and during capillary absorption. The results presented in Table I demonstrate that, for a given rate of fluid movement, net transcapillary protein flux is more markedly affected by capillary filtration than absorption.

TABLE I

Convective, diffusive and net transcapillary protein fluxes across filtering and absorbing capillaries.

| Net transcapillary volume flow (ml/min x 100g) | Diffusive protein flux (mg/min x 100g) | Convective protein flux (mg/min x 100g) | Net protein flux (mg/min x 100g) |
|---|---|---|---|
| + .03* | 0.20 | 1.10 | 1.30 |
| + .32 | 1.20 | 7.00 | 8.20 |
| - .32 | 14.10 | -10.50 | 3.70 |

*positive value indicates net fluid filtration and negative value indicates net fluid absorption.

Protein Efflux

The results of an individual experiment on the small intestine are shown in Figures 3 and 4. The relative concentration of the gamma globulin was consistently greater than that of the vascular tracer indicating movement of the gamma globulin from the interstitium of the small intestine into the capillary lumen in addition to that washed out of the blood vessels. The efflux was divided into two distinct components of washout, an initial rapid component $k_2$, followed by a slower component $k_1$. The mean ($\pm$ SEM) for $k_2$ from seven experiments was $1.08 \pm 0.18$ $min^{-1}$ and was associated with the loss of 20% of the activity from the small intestine. The remaining 80% of radioactivity left the intestine at a slower rate of $0.015 \pm 0.001$ $min^{-1}$.

There was no consistent or significant effect of plasma flow rate on either the fast or slow efflux of gamma globulin. The nature of the vascular tracer, either $^{59}Fe$-labelled transferrin or $^{51}Cr$-labelled red cells, had no significant effect on the observed efflux. The amount of membrane surface area available for exchange will influence the value for efflux obtained in a given tissue. In order to facilitate comparisons with data obtained previously in other tissues, efflux was also calculated per $cm^2$ of capillary surface area. Estimates of intestinal capillary surface area (S) are not available for the rabbit, however, a value of 125 $cm^2$ $g^{-1}$ was observed for capillary surface area in the cat jejunum (9). When expressed per unit of surface area the fast component of efflux ($k_2/S$) was $8.6 \times 10^{-3}$ $min^{-1}$ $cm^{-2}$ and the slow component ($k_1/S$) was $120 \times 10^{-6}$ $min^{-1}$ $cm^{-2}$.

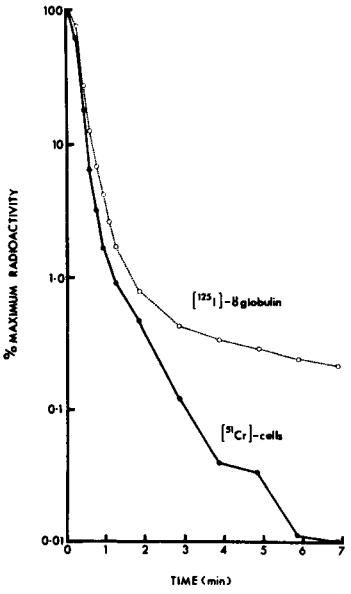


Figure 3: Radioactivity in venous samples collected during perfusion of the small intestine with tracer-free artifical plasma. Values are expressed as a percentage of the radioactivity recirculating in the animal immediately prior to perfusion. $^{51}Cr$-labelled red cells (•——•)indicate washout of intestinal vasculature and $^{125}I$-gamma globulin (o- -o) gives the combined washout of vasculature and efflux of gamma globulin.

## DISCUSSION

It has previously been shown that macromolecules can move in significant quantities across skeletal muscle capillaries in the direction of interstitium to blood (2). Perry and Garlick, using the protein efflux method, demonstrated that gamma globulin entered tracer free perfusate initially at a fast rate followed by a slower, more prolonged rate of efflux. The slower component was .007 $min^{-1}$, and considered to represent transcapillary exchange of the intact gamma globulin. The results of the present study indicate that interstitial to blood movement of macromolecules (e.g. gamma globulin) also occurs in the nonabsorbing small intestine under similar experimental conditions. The slow efflux of gamma globulin from the small intestine was essentially twice the rate reported for skeletal muscle. However, when the efflux is expressed per unit of surface area the values for skeletal muscle and small intestine become indistinguishable, i.e., $\cong 120 \times 10^{-6}$ $min^{-1}$ $cm^{-2}$. Since

these two tissues represent continuous and predominatly fenestrated capillary beds, the similarity in the efflux rates suggests that the limiting barrier governing the movement of macromolecules is not the endothelium.

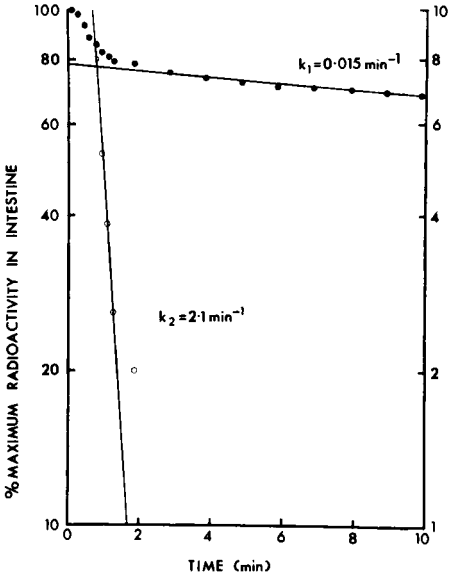


Figure 4: The percentage of $^{125}I$ radioactivity in the rabbit small intestine (excluding activity contained in the vasculature) expressed as a function of time of perfusion. The slow efflux of $^{125}I$-gamma globulin ($k_1$) was obtained by fitting a straight line to the latter portion of the relationship. Subtraction of values on this line from the points representing total $^{125}I$ radioactivity in the intestine gave values (o---o) from which the fast rate of efflux of $^{125}I$-gamma globulin ($k_2$) was obtained. The values for $k_2$ are expressed on a scale of 1 to 10%.

In another series of studies we estimated the diffusive and convective fluxes of macromolecules across intestinal capillaries by applying steady-state lymphatic protein flux data to phenomenological transport equations. This approach was applied to data acquired when intestinal capillaries were either filtering as a result of venous pressure elevation, or absorbing due to enhanced water transport across the mucosal membrane. The results acquired using this approach suggest that convective transport is the predominant process tending to move proteins from blood to interstitium across filtering capillaries. During net fluid absorption the flow of solvent across the capillary wall is directionally opposing the protein

concentration gradient; therefore, the transcapillary convective flux of proteins is directionally uncoupled from the diffusive flux. This study provides an example of significant interstitial to blood movement of macromolecules during a normal physiological process, i.e., net transmucosal water absorption.

Comparison of the data obtained with the two approaches is difficult since the diffusive and convective processes were uncoupled by different methods. However, it is interesting to note that the flux of gamma globulins predicted from the efflux studies is approximately 6-13 times greater than the diffusive flux of proteins predicted for normal filtering capillaries using the lymphatic protein flux method. The basis for this descrepency is not readily apparent at this time; however, it does underline the need for development of analyses which more clearly separate diffusive and convective exchange. Simultaneous measurements using the two approaches should prove beneficial in defining these processes.

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# CONCLUDING REMARKS ON THE LYMPHATIC SYSTEM: FUNCTIONING AND INTERRELATIONSHIPS

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Unfortunately, C.A. WIEDERHIELM was unable to be present, but parts of his work were presented by the Chairman, and other parts by R.D. HOGAN, who had recently collaborated with him. The latter stated that they had indeed found negative tissue hydrostatic pressures in some species of bat; with positive ones in others - both being measured with micropipettes. /It appeared that the bats with the more frequently contractile initial lymphatics had the lower pressures./ It was brought out by A.C. GUYTON that they had made many attempts to measure these pressures, only a few of which were successful; it was suggested that the unsuccessful attempts may have been those which were negative. Many agreed that these pressure must vary considerably, both with space and with time. After R.D. HOGAN's presentation, it was pointed out that these bat wing initial lymphatics were quite unique, since none others were contractile. CASLEY-SMITH also emphasised that this preparation was edematous - as HOGAN stated - and that therefore these findings, while important in edema, were not applicable to normal tissue; here the weight of the evidence indicated that the intra -lymphatic hydrostatic pressure was higher that the tissue hydrostatic pressure.

Many speakers pointed out that S. WITTE's techniques,while certainly showing considerably higher concentrations of proteins in the initial lymphatics than in the tissues, could not be accepted unequivocally since the exclusion by both the tissue fibres and the ground substance was not measurable.

However, some agreed that these were not likely to account for all his findings.

A number of speakers - G. RUTILI, G. SZABÓ and others in the literature - suggested that lymph and tissue fluid were identical in protein concentration. A number of speakers pointed out that their findings were quite inapplicable to the living animal because their tissues were often immobilised for at least 30 minutes and sometimes up to 3 hours. Under these conditions it was absolutely impossible for the initial lymphatic pumps /and often the collecting lymphatic ones/ to function. Thus, *e.g.*, the hypothesis of CASLEY-SMITH could not have been tested by these experiments. This indeed was one of the main themes to emerge during the symposium: the necessity for variations in total tissue pressure to occur if lymphatic function were to be studied in anything approaching normal conditions.

N.C. STAUB presented very convincing evidence that in the lung /and, from the many discussants and in the literature, in other organs/ the initial lymphatics drain only the fine connective tissue lining adjacent to the tissue of functional importance - here, the alveoli. There are large collections of interstitial tissue, here, the cuffs - which are in parallel with the initial lymphatics and are only indirectly drained by them. Thus it was emphasised that the interstitial tissue is composed of a number of compartments. This was an extension of RODBARD's "capillarion" concept, and agreed well with what a number of investigators - including G. SZABÓ - were now suggesting.

J.R. CASLEY-SMITH presented new evidence in favour of the hypothesis that fluid - and macromolecules - enter the initial lymphatics because of the concentrated lymph exerting its colloidal osmotic pressures across the open junctions; this included proof that these vessels are not dilated by the anchoring filaments <u>normally</u> and freeze-substitution studies confirming that the initial lymphatic lymph is concentrated to some 5 times the tissue fluid during the emptying-phase and diluted during the filling-phase. For once, this went almost unchallenged.

A.C. GUYTON showed how sensitive his capsules are to variations in the collodial osmotic pressure of the blood. His calculations which implied pumping and concentrating by the lymphatic system were easily accomodated by the previous speakers' hypothesis. Discussion brought out the responsiveness of his techniques. It was also pointed out that the capsules allow movements in the tissues - with which the capsules are in intimate hydraulic contact - while the alternative methods of measuring the tissue hydrostatic pressure do not readily permit movement. This may well explain why the capsules usually show more negative pressures than the other techniques.

E.M. RENKIN discussed the flow of lymph and its alterations when the plasma oncotic pressure, or/and venous pressure are altered. It showed that much of the flow is convective, not diffusive, and that considerable resistance occurs in the tissues. Again, this agreed with the conclusions of a number of discussants.

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# THE BLOOD-BRAIN BARRIER REVISITED

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25 years ago the blood-brain barrier was a rather confused subject which was hardly worth the attention of physiologists. The main reason for this state of affairs was an uncertainty about the very structure one was talking about and lack of proper experimental techniques. The early 1960's were further haunted by another spectre in the shape of morphological findings claiming that the brain had no interstitial space.

One way out of this was to take a simplistic attitude and regard the blood-brain barrier as a special problem within the general area of capillary permeability. It was known that capillaries varied in structure ranging from unselective fenestrated to continuous capillaries with different degrees of tightness (Bennett et al. 1959).

Pappenheimer et al. (1951) had formulated an equivalent model of the capillary membrane which was strictly quantitative and potential useful in a quantitative analysis of brain capillaries. Krogh - many years earlier - had indicated how to reduce the complexities of blood-tissue exchange to a problem of exchange between one straight capillary and its surrounding tissue cylinder (1919). This simplification made it possible to handle capillaries in a whole organ by assuming that the organ was composed of a multitude of similar units (Krogh cylinders). What was now lacking was a proper quantitative method applicable to brain vessels.

In essence there were two possibilities:

1) Measurement of the early unidirectional loss of a test

solute from the capillaries during one transit through the brain.

2) Determination of the uptake in brain tissue of a test solute added to the blood.

The first principle materialised in the form of the 'indicator diffusion' method (Crone 1963, 1965), while the second principle came into use as socalled 'integral' techniques (Ohno et al. 1978; Gjedde 1980; Amtorp 1980).

The two methods are mirror images and each has its special advantages. The indicator diffusion method is useful for test solutes with high or intermediate permeabilities, while the tissue uptake method can handle slowly permeating substances.

Oldendorf (1970) created a very useful hybrid between the two methods by using a highly diffusible reference substance and expressing brain uptake rates relative to a solute with complete distribution in the brain during a single transit (THO or ethanol).

Supplied with these three powerful methods physiologists began a broad quantitative attack on the barrier and to-day permeabilities of the blood-brain barrier are given in accurate figures. Interestingly, as the techniques have improved, figures for non-electrolyte permeability have become lower and lower. The barrier has become tighter and tighter as it were.

A very great help in the mental clarification process was the contribution by eminent morphologists who showed that electron-dense tracers (down to a molecular weight of 2000) did not permeate through the brain endothelium (Brightman and Reese, 1969; Reese et al. 1971) while the glial foot processes constituted an incomplete envelope.

What has come out of the many experiments?

1) A picture of the brain capillary with endothelial cells united by true tight junctions, implying that access to the brain is more or less synonymous with transendothelial passage.

2) Hydrophilic solutes which permeate the blood-brain barrier rapidly employ special facilitating transport mechanisms

(Lund-Andersen 1979).

3) Most hydrophilic solutes permeate the blood-brain barrier at rates which are about 1000 times slower than in other continuous capillaries, such as muscle, lung or skin (cf. table 1).

4) The transport system in the endothelial cells can adapt to lasting changes in plasma concentrations (Gjedde & Crone 1975; Cremer et al. 1976).

Table 1. Passive diffusional permeabilities of various biological membranes

| | Erythrocytes | Brain[1] | Muscle[2] |
|---|---|---|---|
| | $10^{-10}$ cm·sec$^{-1}$ | $10^{-8}$ cm·sec$^{-1}$ | $10^{-5}$ cm·sec$^{-1}$ |
| Inulin | - | 1.0 | 0.3 |
| Sucrose | - | 1.4 | 0.7 |
| Mannitol | 4[3] | 8.4 | 1.4 |
| $K^+$ | 5[4] | <30.0[5] | 5.0 |

Amtorp (1980)[1] Crone (1963, 1973)[2] Bowman & Levitt (1977)[3] Bjerrum (1979)[4] Hansen et al. (1977)[5]

The table shows permeabilities of various biological membranes to small hydrophilic solutes. It is seen how close the permeability of the blood-brain barrier is to that of a cell membrane. It is three orders of magnitude lower than that of muscle capillaries.

This picture of the blood-brain barrier is a more or less complete confirmation of Krogh's postulate (1946) that the barrier has characteristics of a cellular membrane. Also, Collander's systematic studies (1937) of cell permeability to non-electrolytes have proven to be immediately applicable to the blood-brain barrier. In both cases wide generalizations have given drive to research efforts.

## THE BLOOD-BRAIN BARRIER AS AN EPITHELIUM

Is it possible to get inspiration for continued experimental work by generalising further? The idea which offers itself is that of the blood-brain barrier as a secreting, tight epithelium (Crone 1977, 1981).

The morphological structure of the brain capillary endothelium has many similarities with epithelia. Flat cells resting on a basement membrane - kept together by tight junctions. Could it be that the brain endothelium like other tight epithelia has the capacity of performing solute-linked fluid transport? If so, sodium ions might be pumped into the brain interstitial fluid with chloride following passively, by the mechanism postulated by Koefoed-Johnsen and Ussing (1958). The inward pumping of sodium might be coupled to potassium transport in the opposite direction in accordance with the findings of Bradbury and Stulcova (1970).

A mechanism of solute-linked water flow might explain extra-choroidal formation of cerebrospinal fluid, observed many times. The requirements to the endothelial cells would be small since the pumping rate probably is rather slow and because the total capillary surface area is so large. Furthermore, the capacity of executing active transport, depends on oxidative metabolism. As shown by Oldendorf & Brown (1975) the content of mitochondria in endothelial cells in brain capillaries is larger than in other capillaries. The DC-potential across the blood-brain barrier - with a positive potential in the brain interstitial fluid,could be generated as a result of active sodium transport, leading to diffusion potentials across the membrane.

Looking back on the developments of the physiology of the blood-brain barrier one can say that application of rigorous quantitative methods has paid off. The blood-brain barrier has now risen to the status of a well-characterised biological membrane. It has been deprived of some its mysteries and with continued application of rigorous techniques it will reveal many hidden secrets.

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# STRUCTURAL ELEMENTS COMPRISING THE BLOOD-BRAIN BARRIER

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Ever since the blood-brain barrier was recognized in the original work of Ehrlich (1885), attempts have been made to provide the specific case of permeability, determining the rate of entry of different substances from the blood stream into the brain, with a structural basis. Electron microscopy, in combination with appropriate tracer techniques, has provided by now clear evidence for the primary importance of the cerebral capillary endothelium in the maintenance of the blood-brain barrier.

So far, several valuable reviews and books have dealt in great detail with the ultrastructural characteristics of cerebral microvessels in normal and pathological situations (Rapoport 1976, Wolff 1977, Bradbury 1979, Bär 1980 and van Deurs 1980). Therefore, my intention is to point out - without meeting every requirement - only the main structural features and changes being related to the blood-brain barrier.

## 1. Fine structural features of the capillary endothelium

The unique ultrastructure common to all the vertebrate blood capillaries in the brain proper with the blood-brain barrier consists of a continuous endothelium, a complete basal lamina and the surrounding glial and neuronal elements without a pericapillary connective tissue space. No fenestration occurs in the endothelium except for some pathological cases (Hirano and Zimmerman 1972).

### 1.1. The luminal plasma membrane

Since the blood-borne trypan blue (Broman 1955 and Fig.1), fluorescein tracers (Klatzo and Otenasek 1962), some metabolites (Crone 1965) and other substances (Bakay 1956) were shown not to enter freely from the blood stream into the brain parenchyma, it seems to be still reasonable that the actual localization of the blood-brain barrier in the endothelium has been placed in the luminal plasma membrane by Rodriquez (1955) and Rodriquez-Peralta (1957).

### 1.2. The pinocytotic vesicles

In the cerebral capillaries, the number of micropinocytotic vesicles is low (Reese and Karnovsky 1967 and Fig. 2).

In freeze-fracture preparations of the cerebral capillaries, the actual number of endothelial vesicles was 5 per $um^2$ at the luminal endothelial front, whereas 30-40 per $\mu m^2$ at the abluminal front (Connel and Mercer 1974 and Tani et al. 1977). According to van Deurs and Amtorp (1978), there exists a population of vesicles in the endothelial cytoplasm, which is not involved in transport processes for macromolecules. Fig. 3 shows the barrier to ferritin particles in brain capillary.

### 1.3. The tight junctions

Cerebral capillary junctions were found in studies with horseradish peroxidase (molecular weight 39.800, diameter 5-6 nm) to be tighter than those in noncerebral tissue (Brightman and Reese 1969). In freeze-fracture replicas, the cerebral endothelial tight junctions typically resemble those of noncerebral vessels, namely they consist of a network of "ridges" composed of approximately 10 nm particles (Connel and Mercer 1974, Dermietzel 1975 and Yamamoto et al. 1976). In the internal composition of tight junctions, high degree of variability was found (Tani et al. 1977). In the light of our present knowledge, it is still difficult to explain why the cerebral endothelial tight junctions are tighter than those of other noncerebral blood vessels. In this context, the recent data of Spatz et al. (1980) should be mentioned, who found the formation of gap junctions between the endothelial cells in the absence of glial elements in cultured brain capillaries. This important observation elucidates how the presence of glial influence can contribute to the development of tightness of intercellular junctions connecting brain capillaries. Induction of a capillary enzyme by glial cells was shown recently (DeBault and Cancilla 1980).

### 1.4. The basal lamina

The basal lamina itself seems not to take an effective part in the transport processes directed from brain to blood. Studies with tracer substances indicated that, after intraventricular or intracerebral administration, the basal lamina was readily reached and the tracers were arrested by the interendothelial tight junctions, only (Becker et al. 1968 and Brightman 1968).

### 1.5. The pericapillary glial processes

The astrocytic end-feet system surrouding the capillary basal lamina has long been regarded as the anatomical site of the blood-brain barrier (Edström 1964). This theory has received much support during the 1950's and 1960's, but since the study of Bodenheimer and Brightman (1968), in which the blood-brain barrier was detected in Necturus maculosus where the capillaries were surrounded by perivascular connective tissue spaces instead of glial processes, it has attracted less attention. Glial cells certainly can not be regarded as the only anatomical substrate for the blood-brain barrier mechanism and for the transport system, but it is certain that, in an important supplemental way, they take part in the function and in the active transport of certain substances (see for example DeBault and Cancilla 1980).

## 2. Fine structural changes of the capillary endothelium in pathological circumstances

The opening of the blood-brain barrier, mainly for macromolecules, was seen in many different experimental and pathological situations. The cellular mechanism underlying the extravasation, possibly because of the complexity of events, is still a question under debate.

### 2.1. The luminal plasma membrane

In cases of enhanced permeability of brain capillaries, the formation of micropinocytotic (Raimondi et al. 1962 and Tani and Evans 1965) and the so-called "coated vesicles" (Joó 1971) was observed. To understand the permeability of this cell membrane related to the blood-brain barrier requires a knowledge of its molecular organization, enzymatic content, metabolic activity and the interaction between the intrinsic and exstrinsic factors in various regions of the central nervous system. The availability of a subcellular fraction (Joó and Karnushina 1973) being enrinched in membranes deriving from brain capillaries seems to be a reasonable experimental system to study those factors, which are involved in the regulation of permeability in relation to the blood-brain barrier.

### 2.2. The vesicular transport

Results of many ultrastructural studies indicate the activation of a transendothelial vesicular transport in cases of opened blood-brain barrier (Joó 1971, Westergaard and Brightman 1973, Westergaard 1975, Beggs and Waggener 1975 and Povlishock et al. 1978). As to the possible cellular mechanism of endothelial activation, the involvement of cyclic AMP in transendothelial vesicular transport should be noted (Joó 1972 and Joo et al. 1975a and Figures 4-6).

### 2.3. The transendothelial channels

The presence of transendothelial channels in the cerebral endothelium was evidenced in several studies (Hashimoto 1972, Hanson et al. 1975 and Beggs and Waggener 1975). Recently, Brightman (1977) reported on the formation of transendothelial channels after infusions of hyperosmotic solutions, which were formed by confluent pinocytotic vesicles.

### 2.4. The tight junctions

Although the majority of authors presented data in favour of the enhanced transendothelial vesicular transport (vanDeurs 1976 and Westergaard et al. 1977), there are some other reports, in which evidence was provided for the opening of tight junctions (Giacomelli et al. 1970 and Nagy et al. 1979a and 1979b). Such opening of tight junctions in brain capillaries was first demonstrated by Brightman et al. (1973), after infusions of hyperosmotic urea solution in an ultrastructural study. The opening of junctions was found to be reversible (Rapoport et al. 1971). There are several other findings (for details see van Deurs 1980), in which opening of junctions was seen together with enhanced vesicular transport. As regards the possible cellular mechanism responsible for the opening of junctions, first of all the contraction of endothelial cells should be taken into

account (Owman et al. 1977, Le Beux and Willemot 1978 and Nag et al. 1978).

Contradictorily to the above-mentioned interpretations, Westergaard et al. (1977) stated that, in studies with horseradish peroxidase, the micropinocytotic vesicles could also transport the tracer into the closed tight junction.

### 2.5. The basal lamina

Short after intravenous injections of nickel chloride or mercuric chloride, together with the development of enhanced macromolecular permeation through the cerebral capillaries, collagen-like fibre formation was observed in the basal lamina of brain vessels (Joó 1968, 1969). It was assumed that, similarly to the function of ATPase in the red blood cell (Gárdos et al. 1966), the ATPase activity takes part in the maintenance of high molecular organization of the tropocollagen fibres in the basal lamina (Joó 1979). When the ATPase activity was blocked by the above-mentioned treatments and the molecular arrangement was changed, the permeability was increased indicating how the basal lamina could participate in the regulation of macromolecular transport through the brain capillaries. Collagenase treatment also increased the permeability of the blood-brain barrier (Robert and Godeau 1974 and Robert et al. 1977).

## 3. Enzymes confined to the capillary endothelium

In histochemical studies, several enzymes have been detected so far in the cerebral capillary wall. Many of them seems to take part in some way in the regulation of permeability in brain vessels. For example, the nonspecific alkaline phosphomonoesterase (EC 3.1.3.1.) activity was found to be increased in cases of enhanced vascular permeability (Samorajski and McCloud 1961). The capillary butyrylcholinesterase (EC 3.1.1.8.) activity, described originally by Koelle (1954), could be demonstrated at the light microscopic level in some species in those brain areas that were protected by the blood-brain barrier (Joó and Csillik 1966 and Flumerfelt et al. 1973). "Extraneuronal" dopa-decarboxylase (EC 4.1.1.26.) was also found in the wall of brain capillaries, providing an enzyme trapping mechanism for monoamine precursors (Betler et al. 1964, 1966). GABA-transaminase (EC 2.6.1.1.) positivity of brain vessels has been interpreted in relation to the regulation of capillary GABA transport (Van Gelder 1965, 1966). The presence of adenosine triphosphatase (ATPase) activity (EC 3.6.1.3.) in brain capillaries was reported by Torack and Barrnett (1964) and its possible functional significance was discussed earlier. In addition, γ-glutamyl transpeptidase (EC 2.3.2.2., Albert et al. 1966 and Orlowski et al. 1978), aminopeptidases (Shaw and Cook 1978) and $Na^+$-$K^+$-ATPase (Firth 1977) were detected in the brain capillaries. All these enzymes seem to be involved in some functions of the blood-brain barrier, the latter enzyme in the maintenence of the composition of extracellular fluid in brain (Bito 1969 and Eisenberg and Suddith 1979). Recently, the polarity of the blood-brain barrier was recognized (Betz et al. 1978, 1980) and certain differences were observed in the distribution of enzymes in the luminal and antiluminal membranes.

As cyclic AMP was shown to be able of increasing the pinocytosis and permeability in brain capillaries (Joó 1972 and Joó et al. 1975a), it was of interest to see if the synthesizing enzyme, the adenylate cyclase (EC 4.6.11.) and its counteracting system with the guanylate cyclase(EC 4.6.1.2.) operate in brain capillaries.

3.1. The capillary adenylate cyclase

According to our histochemical findings (Joó et al. 1975b), the brain capillaries exhibit adenylate cyclase activity (Fig. 7). Under the electron microscope, the reaction product was observed in the luminal and basal membranes of capillaries (Figs. 8-10). In biochemical measurements, performed in a capillary-rich subcellular fraction, the basal activity of adenylate cyclase was 63.2±3.0 pmol cyclic AMP.mg$^{-1}$.min$^{-1}$. Vasoactive substances seem to have effect on the capillary adenylate cyclase (Joó et al. 1975a, Baca and Palmer 1978, Huang and Drummond 1979 and Karnushina et al. 1980a). These findings indicate the presence of mixed population of hormone receptors in the membranes of cerebral capillaries, which most likely take an active part in the regulation of transendothelial transport systems.

In agreement with our data (Joó et al. 1976), recently, Rosengren and Persson (1979) have provided evidence, in experiments with chlorpromazine treatment of cerebral stab wound, for the involvement of adenylate cyclase in the pathogenesis of brain oedemas.

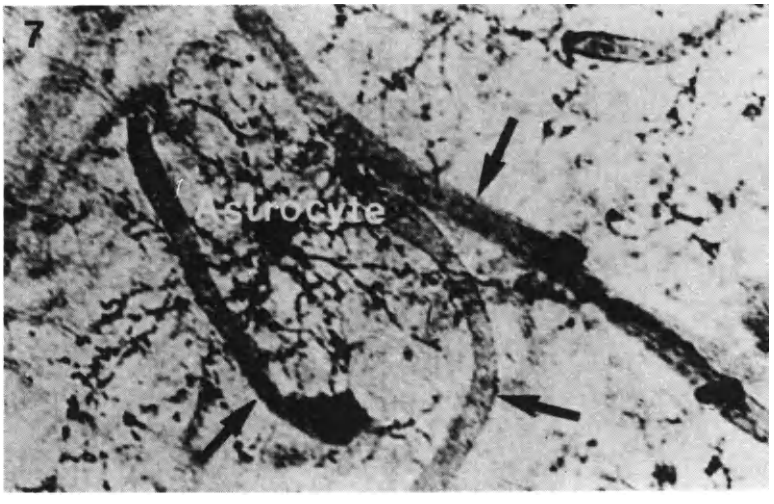



Fig. 7. Adenylate cyclase activity of capillaries (arrows) and an astrocyte under the light microscope.

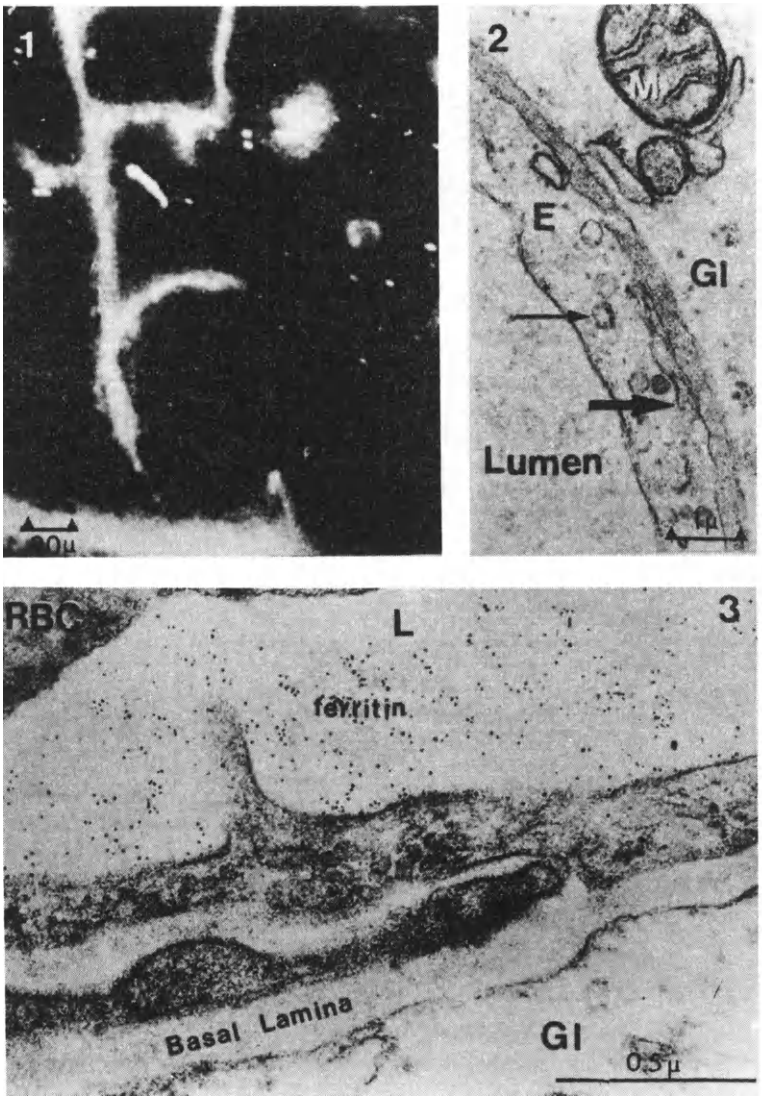



Fig. 1. Lack of macromolecular transport of trypan blue under fluorescent microscope.

Fig. 2. Detail of the capillary wall under the electron microscope. A few microvesicles (thin arrow) and tubular structures (thick arrow) are present in the endothelium E. Gl=glial end-feet system, M=mitochondrion.

Fig. 3. Ferritin particles, as marker substances, cannot penetrate brain capillaries in the normal condition. L=lumen, RBC=red blood corpuscule, Gl=glial cell cytoplasm.

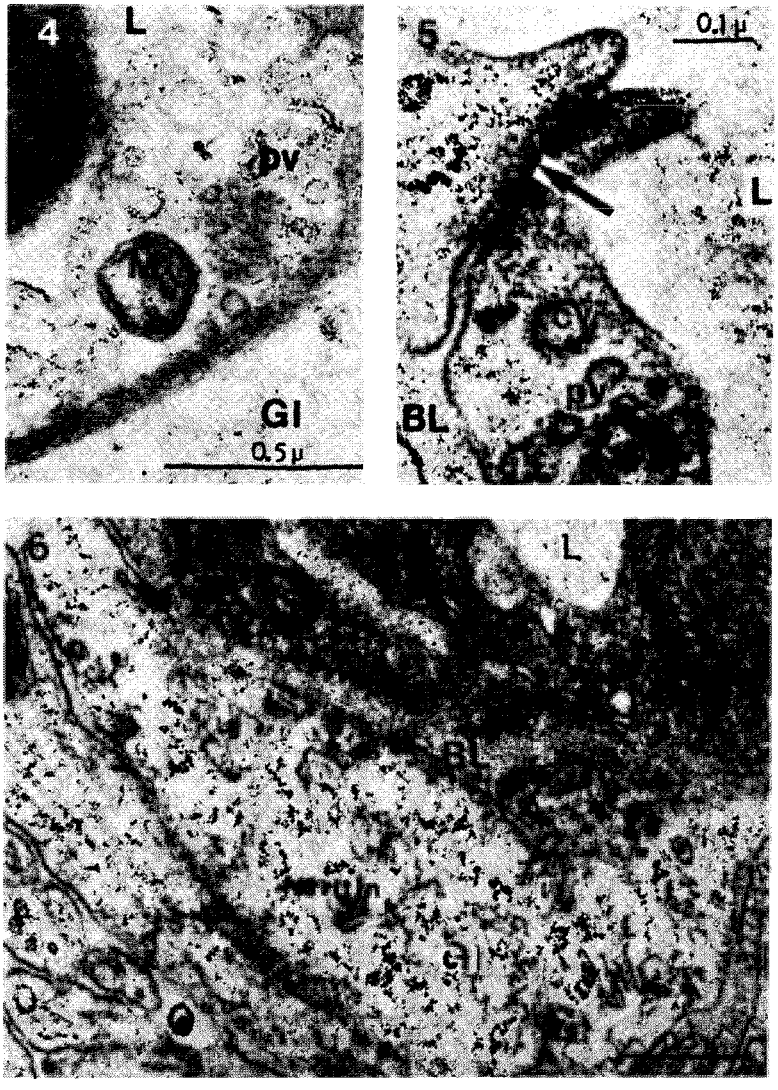
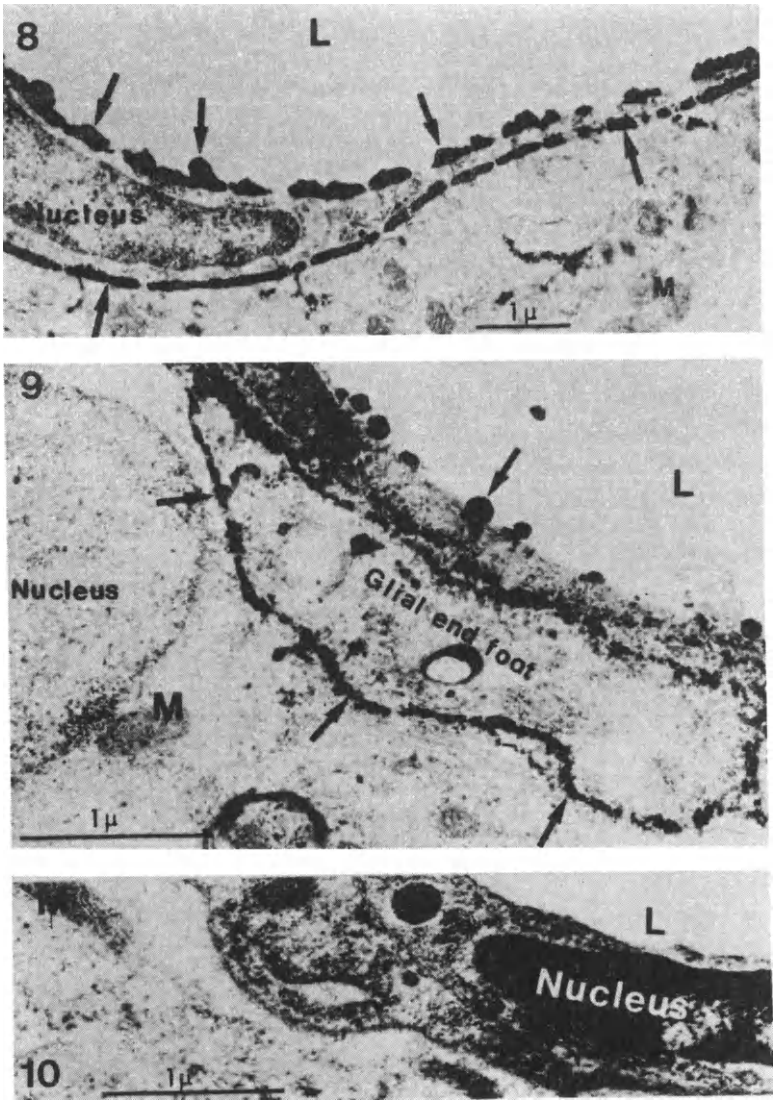



Fig. 4. The number of pinocytotic vesicles (pv) was increased 5 minutes after dibu-cAMP administration. L=lumen, M=mitochondrion, Gl=glial cell cytoplasm.

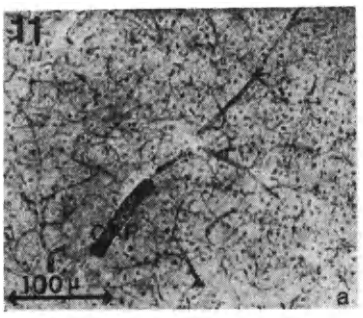
Fig. 5. Tight junctions (arrow) between the endothelial cells remained closed 5 minutes after dibu-cAMP treatment. L=lumen, cv=coated vesicle, pv=pinocytotic vesicles, BL=basal lamina.

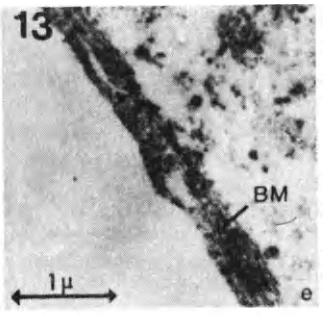
Fig. 6. The exogenous ferritin has penetrated through the capillary wall and accumulated in the glial end-foot after dibu-cAMP treatment.

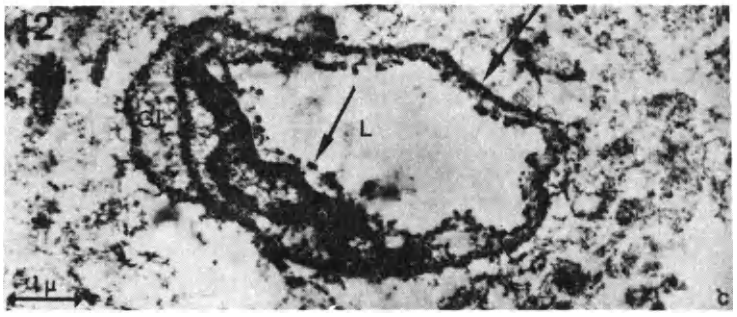


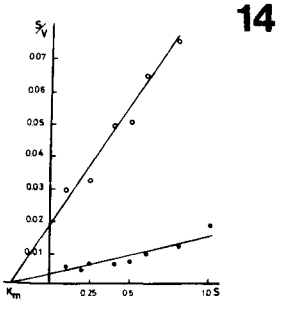

Figs. 8 and 9. Adenylate cyclase activity under the electron microscope. Luminal membrane and the basal lamina show (arrows) strong enzyme activity.

Fig. 10. Substrate-free control.



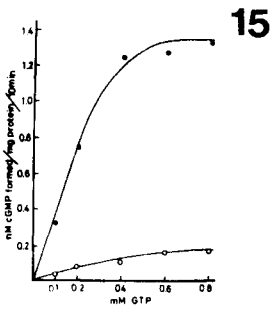




Fig. 11. Guanylate cyclase activity under the light microscope.

Fig. 12. Guanylate cyclase activity under the electron microscope. L=lumen, arrows indicate the localization of reaction end products.

Fig. 13. Substrate-free control.

Fig. 14. Biochemical determination of the guanylate cyclase activity in the capillary-rich subcellular fraction. The $K_m$ value of the activated enzyme (●——●) is the same as that of the control (○——○).

Fig. 15. Guanylate cyclase activity could be activated by Triton X-100 treatment (○——○). Basal activity, (●——●).

### 3.2. The capillary guanylate cyclase

With histochemical methods, the reaction end product indicating the presence of guanylate cyclase was detected in brain capillaries (Fig. 11). Under the electron microscope, the dense reaction product was present in the luminal and basal membranes of capillaries (Fig. 12). Fig. 13 shows the substrate-free control. According to our biochemical measurements, the basal activity of guanylate cyclase in the capillary-rich fraction was $20.1 \pm 1.7$ pmol cyclic GMP.$mg^{-1}$.$min^{-1}$. The cyclic GMP accumulation was found to be linear in the course of incubation up to 15 min and showed a linear dependence on the protein concentration varying from 50 µg/tube to 200 µg/tube in the incubated samples. The $K_m$ value of the enzyme was found around 0.25 mM (Fig. 14). Several attempts were made to activate the enzyme with acetylcholine, histamine and enkephaline ($10^{-4}$ M) and failed to obtain any significant rise. Only Triton X-100 (Fig. 15) was found to be able of activating the enzyme up to 6 times ($120 \pm 16.3$ pmol cyclic GMP.$mg^{-1}$.$min^{-1}$). The $K_m$ value seemed to remain unchanged (Karnushina et al. 1980b).

### 4. Practical considerations

The blood-brain barrier effect is a very complex phenomenon and due to its great clinical significance, it would be necessary to have a unitary concept, which could explain the majority, if not all, of the complex processes. A multimodal concept, proposed by Lee (1971), may meet this requirement. The concept includes an anatomical-structural-substrate as a barrier basis, a fraction of passive diffusion for small molecules, a carrier-mediated transport mechanism to facilitate the diffusion of small and large molecules and an active transport system with a regulatory function. It has to be realized that, in such a complex system, only those studies can be expected to lead to the better understanding and higher efficiency of therapeutic treatment of clinical cases, which elucidate the molecular interactions taking place during normal functioning and pathological circumstances.

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# INSULIN AND THE BLOOD-BRAIN BARRIER

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The blood-brain barrier (BBB) is the interface between the neurones, with their carefully regulated environment, and the blood on which they so much depend for a rapid and constant supply of glucose and oxygen. The function of the BBB seems to be to regulate the neuronal environment, maintaining homeostatis with respect to pH, ions and neurotransmitters, and, at the same time facilitate the passage of metabolically important substances (Barry et al. 1980). Glucose crosses the BBB by carrier mediated transport (Crone 1965; Yudilevich and De Rose 1971) and as glucose transfer across brain cell membranes is extremely rapid (Lund Andersen 1979), we can assume that glucose supply to the brain is governed by transfer across the BBB.

Insulin plays a central role in the regulation of glucose turnover, promoting synthesis of energy reserves (glycogen and lipids) and increasing sugar transport into muscle and adipose tissue (Narahara and Ozand 1963; Vinten 1976): accordingly insulin is of great indirect importance to the brain which depends almost entirely on glucose metabolism. In any discussion of the action of insulin on a particular tissue, it is important to distinguish between the direct and indirect effect of insulin on that tissue. This is nowhere more important than when investigating the action of insulin on the central nervous system (Rafaelsen 1961, 1967). Whether insulin exerts any direct influence on the brain in vivo is a controversial matter. It passes very slowly into CSF and brain extracellular fluid (Woods and Porte 1977) and any immediate regulation of transport and metabolism in the brain parenchyma is therefore excluded. However, a more slow action once it has crossed the BBB is of course still possible (Rafaelsen 1961) and in this context it is important to note that insulin receptors are abundant in the brain (Havrankova et al. 1978).

Specific receptor binding sites for insulin have recently been demonstrated on the brain capillary endothelium (van Houten and Posner 1979), and as the BBB glucose carrier is almost definately located on the capillary endothelium one might suppose that insulin could have an effect on BBB glucose transport. On

this point, however, the literature is inconclusive, partly because many authors fail to make a clear distinction between a direct effect on unidirectional flux across the BBB and an effect on net uptake secondary to a change in brain metabolism. Furthermore, when studying brain glucose uptake and unidirectional flux it is important to maintain steady state conditions with respect to blood/brain glucose concentrations (Lund-Andersen 1979), partly because there is a timelag between change in blood-glucose level and cerebral-glucose concentration.

The conflicting results obtained with earlier studies of the effect of insulin on BBB transfer and brain uptake of glucose can be explained to some extent by the consequences of failing to maintain blood-glucose level constant during the study. For example, the finding of Butterfield et al. (1966) that cerebral glucose uptake in human brain fell after insulin administration can be explained by reduction in glucose influx caused by the insulin-induced hypoglycemia whilst the glucose efflux is still high due to the relatively high cerebral glucose concentration. Similarly the finding of Daniel et al. (1975) that insulin increased net uptake of glucose in the rat can be explained by non-steady state conditions at the time of uptake measurement: they tried to avoid the hypoglycemic effects by giving glucose injections 14 min after insulin and 2 min before uptake measurement, thus performing their uptake measurement at a time when brain glucose concentration is still subnormal because of the preceding 14 min of hypoglycemia, but blood glucose was high.

Gottstein et al. (1965) investigated the effect of insulin on the glucose uptake of the human brain and found that when the blood-sugar level was raised by glucose infusion, brain glucose uptake increased, but that the increase was much more pronounced when insulin was infused together with the glucose. In diabetics, reduction of the blood-sugar level by insulin also elicited an increase in brain glucose uptake (Gottstein and Held 1967). As cerebral oxygen consumption remained constant in both cases, indicating that metabolism was unchanged, these findings were interpreted as evidence of an effect of insulin on the transfer of glucose into brain (Gottstein 1975).

As with brain uptake, earlier studies of the effect of insulin on unidirectional flux of glucose across the BBB (Crone 1965; Betz et al. 1973; Daniel et al. 1975) have been inconclusive and can be criticised for various reasons which include failure to maintain steady state conditions (further discussed in Hertz et al. 1980). However, in a recent study we have demonstrated an unequivocal effect of insulin on BBB glucose transport: insulin augmented unidirectional flux across the BBB under conditions in which the net uptake by the brain remained constant (Hertz et al. 1980). This study, which was performed in man, will be briefly described.

## The effect of insulin on BBB transport of glucose in man

The effect of insulin on both the unidirectional flux of glucose across the BBB, and the net uptake of glucose by the brain, was studied in seven patients hospitalised for cerebral disorders necessitating carotid angiography. (The patients were studied in connection with the angiography, informed consent having been obtained). Unidirectional glucose flux was determined with the indicator dilution technique (Crone 1963; Yudilevich and De Rose 1971) as used in human studies (Lassen et al. 1971 and Paulson et al. 1977). Net uptake of glucose was determined from the difference in glucose concentration of arterial and sagittal sinus blood. Cerebral blood flow (CBF) was measured with the 133-Xenon injection method.

The unidirectional flux and net uptake of glucose across the BBB were determined six times in each patient, twice before, twice during and twice after an intravenous infusion of insulin (0.4 U/kg over 30 min). The blood glucose concentration was continutally monitored (using a glucose electrode and maintained constant throughout the study by variable infusion of 50% glucose. This was essential in order that the effects of insulin per se could be distinguished from those of the hypoglycemia that it induces.

The blood glucose level was maintained relatively constant throughout the study (fig. 1), despite the presence of high plasma insulin levels during and after the insulin infusion (fig. 2 - above), and the arterial glucose concentration (fig. 2 - center) was almost identical at each measurement of flux/uptake. This is in fact an important aspect of the study.

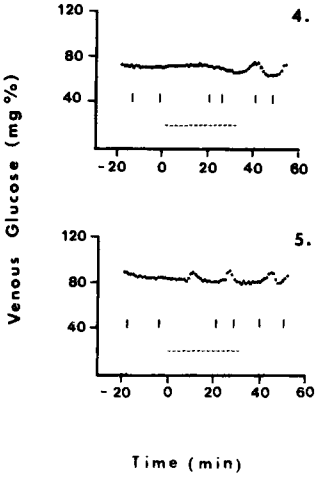


Fig. 1:
Venous glucose concentration in 2 patients at each minute during the investigation.
The broken line represents the duration of insulin infusion and the vertical bars indicate each measurement of flux/uptake.

The unidirectional flux of glucose across the BBB, as calculated from the product of CBF x arterial glucose concentration x glucose extraction, E, (measured with the indicator dilution method) increased significantly from a resting level of 0.46 µmol/g/min to 0.68 at 20 min and 0.66 µmol/g/min at 30 min after starting the insulin infusion (fig. 2). 10 min after the end of the insulin infusion, when plasma insulin was much lower, the unidirectional flux was still higher (0.56 µmol/g/min) than the resting level, but not significantly so. In contrast to the effect of insulin on the unidirectional flux across the BBB, the net uptake of glucose by the brain did not change significantly, being 0.30 µmol/g/min prior to insulin infusion, 0.32 and 0.35 µmol/g/min during the infusion, and 0.32 µmol/g/min afterwards (fig. 2).

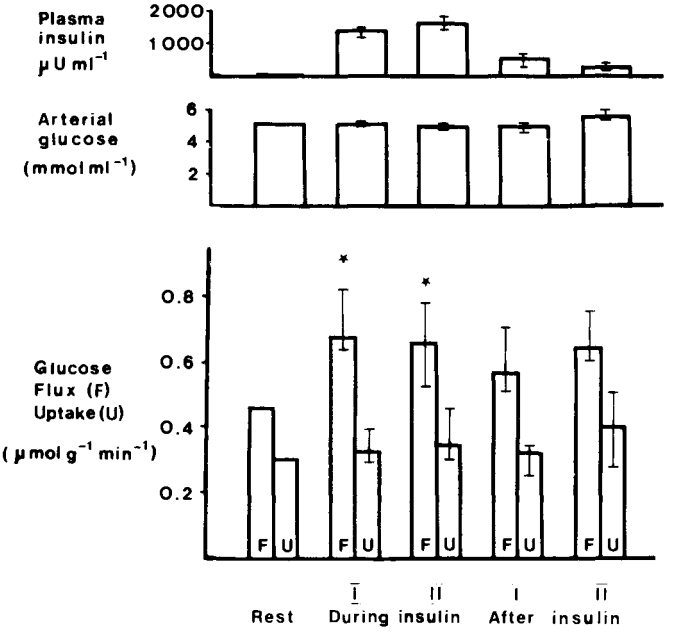


Fig. 2: The unidirectional flux and the net uptake of glucose into brain are shown in relation to plasma insulin and arterial glucose concentration. The columns are the median values, the bars indicate the quartiles of the difference between the resting and experimental values.

The additional glucose that crosses the BBB under the influence of insulin is not retained by the brain, but returns to the blood as backflux, indicating that the insulin stimulates the transport of glucose across the BBB in both directions (i.e. blood to brain and brain to blood) (fig. 3).

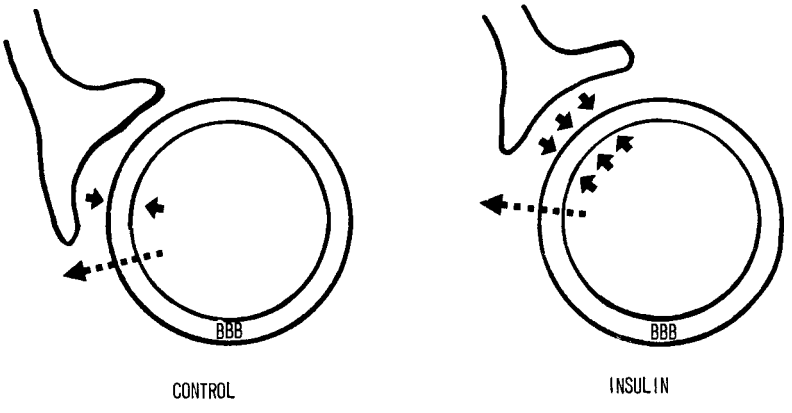


Fig. 3: A diagramatic representation of the direct effect of insulin on the blood-brain barrier (BBB). Insulin stimulates unidirectional flux (◄) of glucose across the BBB in both directions (blood-brain and brain-blood) whereas net flux (◄▪▪▪▪▪▪) remains constant.

It must be stressed that the present discussion pertains only to the question of the direct effects of insulin on BBB glucose transport, and in this respect it is irrelevant whether or not insulin has any effects on brain metabolism which could have secondary effects on glucose transport. However, a clear distinction must be made between BBB transport and brain uptake. When such a distinction is made, and when the appropriate techniques are used to study both parameters simultaneously, as in the study outlined above (Hertz et al. 1980), it becomes apparent that insulin can be shown to have a direct effect on BBB glucose transport (i.e. unidirectional flux). In that study, a high plasma insulin level was maintained for 30 min, after which the insulin infusion was stopped and plasma insulin fell rapidly. However, the unidirectional flux remained higher than normal, which may be because sufficient insulin is bound to the brain capillary insulin receptors (van Houten and Posner 1979). There was a tendency towards an increase in brain uptake of glucose 30 min after the insulin infusion, which might reflect a metabolic effect as some insulin would have penetrated the BBB by this stage.

The mechanism whereby insulin accelerates glucose transport remains to be elucidated - it may involve speeding up the carrier - although the effect is probably mediated by the insulin receptor, which is present in some 20 different cell types (Kahn and Roth 1976), including the BBB (van Houten and Posner 1979).

Apart from the glucose transport, there are other aspects of insulin and the BBB which should be mentioned. It is well known that insulin causes hyperpolarisation of certain biological membranes (Zierler 1972) and one might speculate as to whether ion fluxes into or across the brain capillary endothelium could be effected. However, Thurston et al. (1977) produced some evidence that there is no effect of insulin on $K^+$ and $Na^+$ transport in normal brain. Insulin may have indirect effects on amino acid transport across the BBB due to insulin induced changes in plasma amino acid levels, and hence competition for amino acid carriers (Fernando et al. 1976; De Montis et al. 1978).

Finally there is one possible effect of insulin on the BBB which (as far as we are aware) has not been examined as yet: could insulin stimulate the brain capillary endothelium to form micropinocytotic vesicles, a process that we(Barry et al. 1980) consider to constitute breakdown of the BBB,which is normally devoid of vesicles. This may not be such a remote possibility as it may seem. It has recently been demonstrated that insulin stimulates vesicle formation in the capillary endothelium of striated muscle in diabetic rats: these rats have a subnormal density of vesicles which returns to near normal after administration of insulin (Østerby et al. 1978). These findings may be the morphological counterpart of the decrease in plasma volume and in the intravascular pool of albumin in diabetic patients after insulin injection (Gundersen and Christensen 1977). Whether a similar stimulation of vesicle formation could occur in the BBB of diabetic patients is unknown, although it would seem unlikely in normal human brain as evidenced the normal barrier function during insulin infusion in our study (Hertz et al. 1980). However, such a possibility can not be dismissed outright.

It can be concluded that insulin, at least with the relatively high dose used in our study (Hertz et al. 1980), exerts an effect on BBB glucose transport. Whether this is also true at more physiological insulin levels requires further study. However, the finding of an abundance of insulin receptors on the BBB (van Houten and Posner 1979), together with our finding that insulin speeds up BBB glucose transport, opens up the possibility that insulin may play a role in the long-term regulation of transport, perhaps by influencing the number of carrier sites, as has been shown for adipocytes (Olefsky and Kobayashi 1978).

x)
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# PROPERTIES OF TRANSPORT PROCESSES OF THE BLOOD-BRAIN BARRIER DURING DEVELOPMENT

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In this paper data will be presented to demonstrate the selective nature of the modifications to the transport processes between the blood and brain that occur during the period of post-natal development.

A substance such as glucose enters the brain much faster than could be achieved by simple diffusion through the plasma membranes of capillary endothelial cells. Crone (1965) was the first to show that in addition to a small diffusional component there is a faster uptake that is saturable. The saturable nature of the transport together with the characteristics of stereospecificity (D-glucose is transported faster than L-glucose, Oldendorf, 1971) and of competition between similar substrates (glucose analogues inhibit the transport of glucose, Oldendorf, 1971; Cremer & Cunningham, 1979), are indicative of a specialized diffusion process. Such a process in brain probably involves a particular protein localised in the plasma membrane of capillary endothelial cells.

Glucose is not the only small molecular weight, water soluble substance to move into brain at high rates. There seem to be separate "carriers" for groups of amino acids, for monocarboxylic acids and for several other classes of compounds (Pardridge & Oldendorf, 1977). The properties of these various transport processes have been well characterised. Most data have been obtained in adult animals and only more recently has it been established that some of these characteristics differ in animals of a younger age.

There has been some controversy about the tightness of the barrier to small molecular weight polar solutes in the young. However, a recent study (Johnanson, 1980) has confirmed that even in the newborn rat entry into brain of a solute such as mannitol is very limited (Table 1). Nevertheless, even at a young age the rate of entry of glucose into the brain is several times greater than the rate of mannitol

Table 1. $^{3}H$ Mannitol spaces in rat tissues at one hour after intraperitoneal injection.

| Age (weeks) | Heart | Liver | Brain |
|---|---|---|---|
| 0.5 | 24.6 | 66.3 | 6.4 |
| 1 | 24.6 | 67.8 | 5.4 |
| 2 | 25.4 | 68.2 | 5.0 |
| 3 | 22.8 | 66.6 | 4.6 |
| Adult | 20.1 | 67.6 | 3.9 |

Data are taken from Johanson (1980) and are given as volume of distribution space:
100 x (dpm per mg tissue/dpm per mg plasma water).

which implies the presence of a selective glucose carrier system (Cremer et al, 1976; Daniel et al, 1978). The uptake of glucose in 20-day old rats has been shown to be inhibited by a non-metabolizable glucose analogue (Fig. 1) which is similar to the situation in adults (Cremer & Cunningham, 1979). This evidence of competitive inhibition lends further support to the presence of a specific transport process in the capillary endothelium of the developing rat brain.

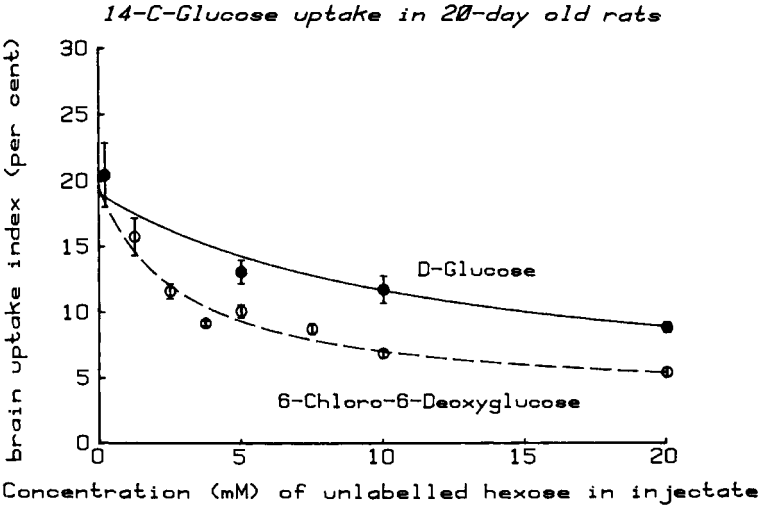


Fig. 1. The brain uptake index for [$^{14}C$]glucose using the technique of Oldendorf (1971).

A detailed analysis of the kinetic characteristics of glucose influx into the brain of rats of different ages was

made by Cremer et al (1979). Essentially the rate of total unidirectional influx, i.e. saturable plus non-saturable components, is given by the relationship, $v_{total} = (E)(F)(S)$, where E is the fractional extraction of unidirectional influx, F is the rate of cerebral blood flow and S is the concentration of glucose in the blood. The saturable component is described by the Michaelis-Menten equation:

$$v_o = \frac{V_{max}\,[S]}{K_m + [S]} \qquad \text{Eq. (1)}$$

The non-saturable component is equal to $K_d\,[S]$, where $K_d$ is a constant of free diffusion. A combination of the terms gives:

$$v_{total} = \frac{V_{max}\,[S]}{K_m + [S]} + K_d\,[S] \qquad \text{Eq. (2)}$$

Rates of glucose influx computed from the kinetic constants determined by Cremer et al (1979) are shown in Figure 2. It can be seen that the saturable component in rats 14 to 19 days old had less than half the capacity of adult rats. There was less difference with age in the total influx since the non-saturable component was greater in the younger animals.

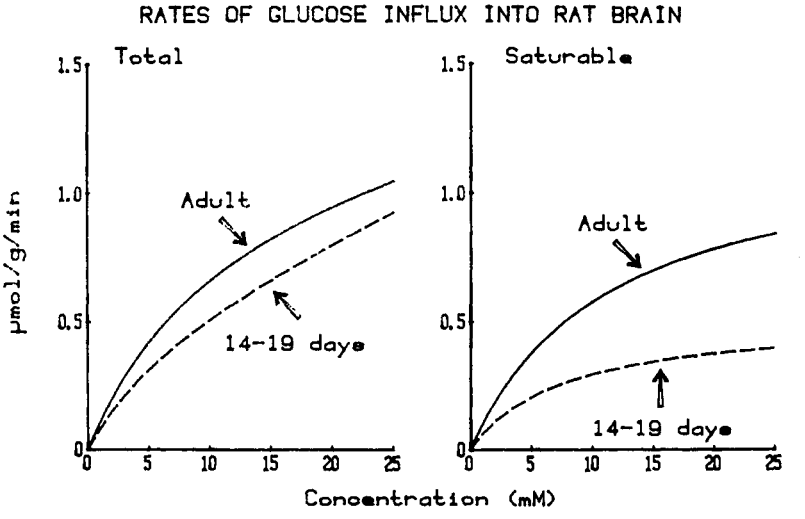


Fig. 2. Rates of glucose influx computed from the kinetic constants determined by Cremer et al, (1979).

Similar studies were made on lactate and pyruvate transport. The changes with age were very striking and were opposite to those for glucose. The rates of total influx were more than ten times greater in young animals compared to adults (Figs. 3 & 4). The differences with age in the saturable components were even greater. It seems likely that lactate and pyruvate share the same carrier mechanism.

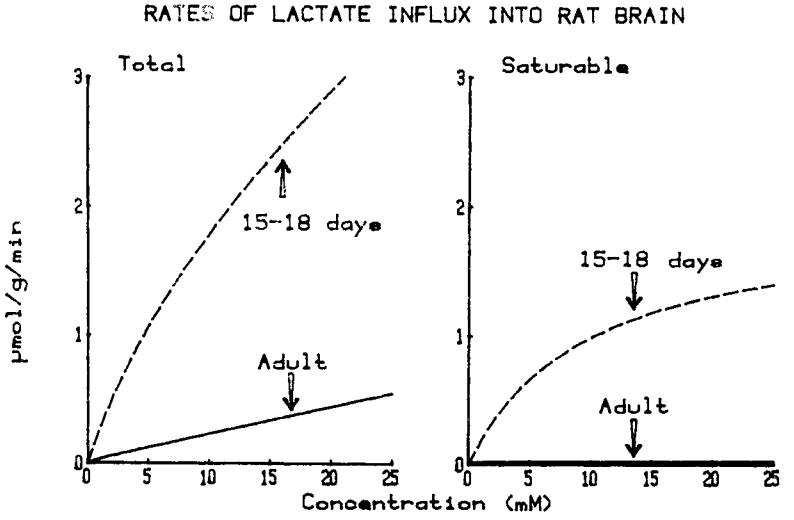


Fig. 3. Rates of lactate influx computed from the kinetic constants of Cremer et al, (1979).

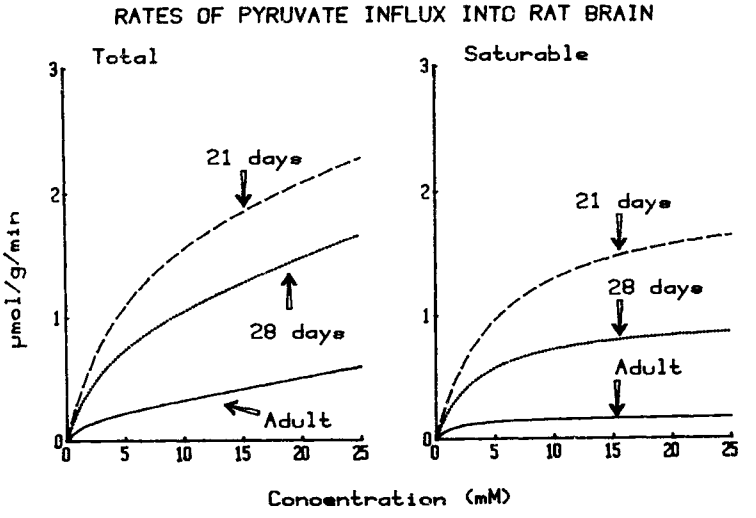


Fig. 4. Rates of pyruvate influx computed from the kinetic constants of Cremer et al, (1979).

The high permeability to lactate in young animals would support the suggestion that, in the newborn particularly, if there is an episode of hypoglycaemia then lactate can replace glucose as an important fuel for the brain (Levitsky et al, 1977).

Other substrates alternative to glucose are the ketone bodies, 3-hydroxybutyrate and acetoacetate. Hawkins et al, (1971) were the first to show that the net rate of ketone body utilization by the brain was several times greater in young suckling rats compared to adults. The arterio-venous difference values they obtained are shown in Fig. 5. Several subsequent studies have shown changes with age in the transport process (Moore et al, 1976; Cremer et al, 1976; Daniel et al, 1977). A recent kinetic study on 3-hydroxybutyrate transport has shown a remarkably close agreement between the rates of total and net influx in suckling animals (Fig. 5). This indicates that the rate of transport into the brain is a major determinant of the rate at which the ketone bodies are metabolised.

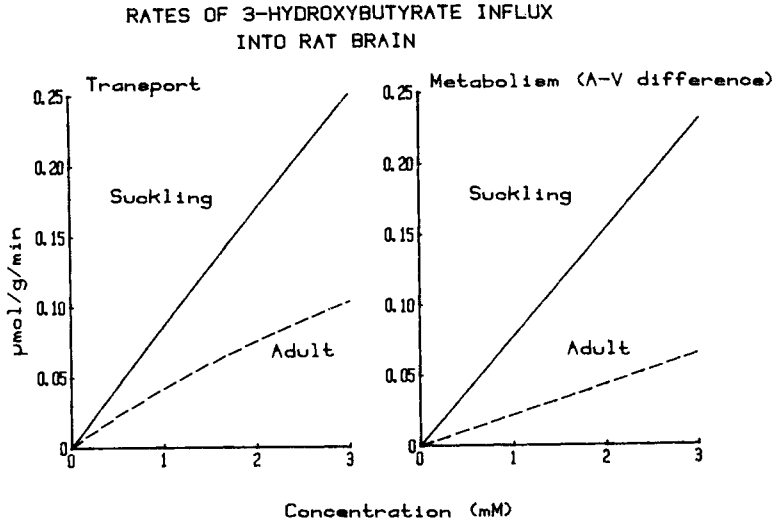


Fig. 5. Rates of influx of 3-hydroxybutyrate into rat brain. "Transport" represents total influx and "Metabolism" represents net influx. Data are from Hawkins et al (1971); Gjedde & Crone (1975) and Cremer & Cunningham unpublished observations.

Very similar observations have been made in man where the net uptake of ketone bodies by the brain, as determined by

arterio-venous difference measurements was found to be several times higher in infants and young children compared to adults (Kraus et al, 1974; Settergren et al, 1976).

If increased transport into the brain can be taken as indicative of nutritional requirements of the growing brain then the recent findings of Braun & Oldendorf (1980) in newborn rabbits are of considerable importance. Not only was a saturable process demonstrated for the transport of glucose but also for L-arginine, adenine and choline the capacities of which were many times greater than in adult animals (Table 2).

Table 2. Blood-brain barrier uptake of metabolic substrates in the newborn and adult rabbit.

| Compound | Brain Uptake Index Newborn | Adult |
|---|---|---|
| D-Glucose | 21 | 20 |
| L-Arginine | 50 | 10 |
| Adenine | 30 | 4 |
| Choline | 47 | 18 |

Data are taken from Braun & Oldendorf (1980).

One of the most interesting aspects of the changes with age in the transport processes of the blood-brain barrier is the selective nature of the changes. This is exemplified by a range of substrates tested in the rat and listed in Table 3. These show unequivocally that the changes with age cannot be explained by a general leakiness of the barrier. Rather, they are selective for different groups of substances, some of which are increased, others decreased, while yet others are similar at all ages.

Table 3. Blood-brain barrier uptake of metabolic substrates in the suckling and adult rat.

| Compound | Brain Uptake Index Suckling | Adult |
|---|---|---|
| D-3-hydroxybutyrate | 26 | 7 |
| L-Lactate | 58 | 10 |
| D-Lactate | 23 | 3 |
| Pyruvate | 87 | 40 |
| D-Glucose | 20 | 29 |
| Leucine | 40 | 50 |
| Glycine | 2 | 2 |
| Lysine | 19 | 16 |

Data are from Cremer et al, (1976) and Cremer & Teal, unpublished observations.

The question remains "what are the factors that are responsible for the selective changes?" The concentration at which a substance is chronically sustained in the blood may play a prominent role. The studies on ketone body transport have shown that when the blood concentrations are kept high over a period of days then there is a high maximal transport rate (Gjedde & Crone, 1975; Cremer et al, 1976; Daniel et al, 1977). Furthermore, a gradual decline in the blood concentration of ketone bodies is followed by a fall in the transport capacity. The possibility of an association between sustained blood concentration and transport capacity may well have clinical relevance. This would be particularly so in the nutritional care of babies and young children to ensure adequate growth of the brain and to protect it from harmful influences on transport processes.

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# REGULATION AND ADAPTATION OF SUBSTRATE TRANSPORT TO BRAIN

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The concept of the cerebral capillary endothelium as a major regulatory structure, guarding the extracellular space of the brain, is the natural consequence of the finding that the glial envelope is very permeable, even to macromolecules.

Most substrates that pass the cerebral capillary endothelium do so by facilitated diffusion, mediated by carriers in the endothelium at rates that are non-linearly dependent on the substrate concentration in blood (plasma). If concentrations in the extracellular space are to be kept constant, the regulation must be a function of the concentration in the plasma or in the interstitial fluid.

In cases where the rate of metabolism approaches the rate of transport (cf. Table 3), it is unclear how substrate concentration in plasma is varied in response to varying metabolism.

## Substrates

D-glucose is the most completely studied example of a solute that passes the cerebral capillary endothelium by facilitated diffusion (CRONE 1960). The facilitated nature of its diffusion has been documented in a large number of species, using a variety of methods (LUND-ANDERSEN 1979). Hence D-glucose naturally lends itself to an attempt to describe the relationship between transport and metabolism. However, D-glucose is not the only nutrient of importance to brain; ketone bodies and lactate have attracted attention as alternative substrates in special circumstances, including the fetal, neonatal and immature periods (GREGOIRE & al. 1978, CREMER & al. 1979) and starvation (OWEN & al. 1967, GJEDDE & CRONE 1975, DE LA MONTE & al. 1977).

## Methods

D-glucose and monocarboxylic acid transport across the cerebral capillary endothelium can be expressed as extraction fractions, rate constants, apparent permeability, total fluxes, or by the operational "kinetic" constants Tmax and Km, dependent on the choice of method. If transport is to be expressed independently of the substrate concentration, calculation of Tmax and Km is necessary. The calculation is based on the un-

Table 1: Effect of reduced metabolic rate on blood-brain glucose transfer

| condition | CMR | Tmax | CBF | Kin |
|---|---|---|---|---|
| | (μmol/100g/min) | | (ml/100g/min) | |
| awake | 107 | 409 | 116 | 19.5 |
| barbiturate anesthesia | 65 | 235 | 58 | 13.2 |

(From SOKOLOFF & al. 1977, GJEDDE & RASMUSSEN 1980)

certain assumption that the kinetic constants are not directly or indirectly influenced by the substrate concentration in plasma, and is further complicated by the possibility of multi-affine transport (GJEDDE & CRONE 1975, GJEDDE 1980b).

The observations to be discussed below were made by intracarotid (OLDENDORF 1970) or intravenous (GJEDDE & al. 1980) injection of labeled tracers in rat. The intravenous (integral) injection method has the advantage that it is possible to determine blood flow (CBF) and tracer uptake ($\bar{J}$) simultaneously and therefore to calculate tracer flux rather than extraction fraction. The constants Tmax and Km were calculated from unidirectional fluxes at various plasma concentrations, obtained by systemic administration of glucose or insulin (GJEDDE 1980a) or by adding unlabeled substrate to the intracarotid injectate. Metabolic rates (CMR) were determined by arteriovenous deficits or by the regional method of SOKOLOFF & al. (1977).

## Acute change of metabolic rate

In barbiturate anesthesia, the cerebral metabolic rate falls in proportion to the level of anesthesia, as does blood flow. Blood-brain glucose transfer falls in approximate proportion to the blood flow, as shown in Table 1, both with respect to the rate constant for the initial rate of unidirectional transfer, Kin, and Tmax for glucose transport.

The relationship between cortical metabolic rate and unidirectional blood-brain glucose transfer in situations that range from deep anesthesia to post-ischemic hypermetabolism, is summarized in Figure 1, from which it is evident that glucose transport is closely related to metabolic rate.

The couple between metabolic rate and blood flow is well-established but remains unexplained. None of the mediators suggested, i.e. oxygen, the hydrogen ion, potassium, adenosine, ATP, and hyperosmolarity, has been definitively shown to be the link between metabolism and blood flow. Hence, a proper attempt to explain the couple between metabolism and glucose transport must await clarification of the flow-metabolism couple. However, a tentative explanation is perhaps suggested by the possible

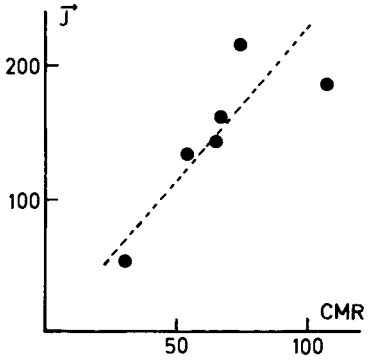


Figure 1. Couple between glucose metabolism and unidirectional blood-brain glucose transfer. Abscissa: Cortical glucose consumption (µmol/100g/min). Ordinate: Unidirectional blood-brain glucose transfer in the steady-state (µmol/100g/min). Points represent conditions that range from deep anesthesia to post-ischemic hypermetabolism (adapted from GJEDDE & al. 1980, SIEMKOWICZ & GJEDDE 1980, and GJEDDE & RASMUSSEN 1980).

presence of intermittent capillary perfusion in other organs. According to this suggestion, the capillary surface available for transport of glucose varies in proportion to the number of perfused capillaries. Such a mechanism can link transport, flow and consumption rates.

## Acute change of plasma concentration

Hypoglycemia dramatically affects cerebral function. The effect, however, does not appear to be a simple result of substrate shortage. Thus, it has been shown that acute interruption of the substrate supply to the brain interferes with brain function before significant reductions of energy-stores can be measured (DUFFY & al. 1972, GORELL & al. 1977).

Whole-brain glucose consumption was measured during progressive reduction of the glucose concentration in plasma, as shown in Table 2. Glucose consumption fell gradually with the reduction of blood-brain glucose transfer, despite the observation that sufficient glucose appeared to enter brain during moderate hypoglycemia. Only plasma glucose levels below 3mM resulted in blood-brain glucose transfer rates that were obviously limiting for the metabolism.

The reason for the gradual reduction of glucose consumption is unknown. At the lowest plasma glucose concentrations, regional blood flow distribution changes significantly. Regional blood flow increases in the brain stem and midbrain but decreases in the basal ganglia and cortical regions, as shown in Figure 2, in conjunction with a proportional redistribution of glucose transfer. The redistribution of blood flow results in a

Table 2: Effect of hypoglycemia on cerebral metabolic rate and blood-brain glucose transfer

| [glc]a (mM) | $\vec{J}$ (µmol/100g/min) | CMR | CBF* (ml/100g/min) |
|---|---|---|---|
| 30.8 | 205 | 74 | 87 |
| 10.4 | 159 | 67 | 129 |
| 6.4 | 129 | 43 | 137 |
| 3.2 | 86 | 35 | 92 |
| 2.0 | 66 | 37 | 134 |
| 1.3 | 37 | 39 | 142 |
| 0.6 | 11 | 15 | 95 |

*halothane anesthesia (adapted from GJEDDE & SIEMKOWICZ in preparation).

relative preservation of the glucose supply to the lower brain regions at the expense of the higher brain regions. The redistribution coincides with the onset of deep coma. The brain therefore appears capable of regulating the glucose supply to different regions of brain, and to differentiate between vital and less vital regions. A similar pattern has been observed in post-ischemic recovery of brain function (HANSEN & al. 1980).

Table 3: Effect of starvation on blood-brain ketone body transfer and metabolic rate

| days starved | [βHBA]a (mM) | Tmax | $\vec{J}$ (µmol/100g/min) | CMR |
|---|---|---|---|---|
| 0 | 0.08 | 16 | 0.5 | 0.6 |
| 5 | 1.3 | 96 | 11 | 12 |

(Adapted from GJEDDE & CRONE 1975, DE LA MONTE & al. 1977).

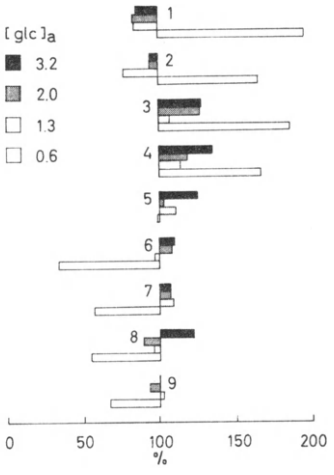


Figure 2. Regional blood flow (and blood-brain glucose transfer) in 9 regions of brain, expressed as a percentage of whole-brain values. The regions are numbered in the caudal-rostral directions and include: 1, cerebellar hemispheres; 2, vermis; 3, medulla and pons; 4, midbrain; 5, basal ganglia; 6, parietal cortex; 7, occipital lobe; 8, temporal lobe; 9, frontal lobe. Plasma glucose concentrations are given to the left, the whole-brain average in Table 2.

## Chronic change of plasma concentration

Permanent changes of substrate concentration in plasma occur in several situations, the most common of which is perhaps poorly regulated diabetes mellitus. Other conditions of considerable interest include starvation and congenital hypergalactosemia. Both chronic hyperglycemia and classic galactosemia may lead to serious disturbances of brain function but curiously, neither starvation nor the galactokinase deficient variety of hypergalactosemia (in which no galactose-1-phosphate is accumulated) affect the brain (KALCKAR & al. 1973).

One reason for the absent cerebral symptoms in uncomplicated starvation seems to be the increased cerebral consumption of ketone bodies which substitute for glucose. The brain consumes all the ketone body transported into the tissue, as shown in Table 3, and the ketonemia of starvation induces an increase of the transport capacity which allows ketone body consumption to increase 20-fold or more. In the absence of induction, only half of this increase could have been sustained.

The Km of ketone body transport increases as well. This observation led to the speculation that several transport mechamisms of differing affinities towards the substrate may be present in the cerebral capillary endothelium (GJEDDE & CRONE 1975).

Table 4: Effect of chronic hyperglycemia on blood-brain glucose transfer

| condition | [glc]a (mM) | CBF (ml/100g/min) | Kin | $\vec{J}$ (µmol/100g/min) |
|---|---|---|---|---|
| control (<10mM) | 12.3* | 57 | 12.5 | 199 |
| | ±1.1 | ±3 | ±0.4 | ±19 |
| chronic hyperglycemia for 2 wks (>20mM) | 12.6** | 48 | 9.9 | 156 |
| | ±1.1 | ±0.3 | ±0.2 | ±14 |

*Elevated by i.p. glucose. **Reduced by i.p. insulin. Values are means ± S.E.M. of 4 observations.

Chronic hyperglycemia was introduced in rats by a single i.p. dose of streptozotocin. Glucose transport was tested after 2-3 weeks and compared to that of control rats. The transport was compared at the same glucose concentrations in plasma, as shown in Table 4. Hyperglycemia for 2-3 weeks resulted in a reduction of the rate constant for the initial rate of unidirectional glucose transfer, as well as for the steady-state unidirectional transfer at a given concentration. The Tmax and Km

Table 5: Effect of chronic hyperglycemia on bi-affine blood-brain glucose transfer

| condition | Tmax(1) (µmol/100g/min) | Km(1) (mM) | Tmax(2)/Km(2) (ml/100g/min) |
|---|---|---|---|
| control* (<10mM) | 160 | 1.1 | 5.2 |
| | ±10 | ±0.5 | ±0.6 |
| chronic hyperglycemia for 2 wks (>20mM) | 94 | 0.7 | 7.0 |
| | ±6 | ±0.2 | ±0.3 |

*From GJEDDE 1980c. Values are means ± S.E.M. of 40 observations.

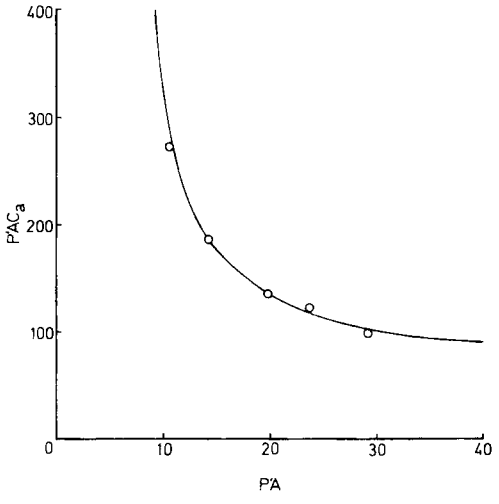


Figure 3. Eadie-Hofstee ("transport-vs-permeability") plot of blood-brain glucose transfer in chronic hyperglycemia. Abscissa: Apparent permeability (ml/100g/min). Ordinate: Unidirectional blood-brain glucose flux in steady-state (µmol/100g/min). Points represent 5-10 observations at the same plasma glucose concentration. Curve was drawn from kinetic constants given in Table 5.

of glucose transport in hyperglycemia were estimated from the unidirectional transfer at different glucose concentrations in plasma, as shown in Figure 3. In analogy to the Scatchard-plot of receptor binding, a "transport-vs-permeability" plot yields a straight line in the case of a kinetically single transfer mechanism. As seen in Figure 3, it was impossible to fit a single line to the points. An acceptable fit required at least two sets of kinetic constants, compatible with both high and (very) low affinity transport of glucose in the cerebral capillary endothelium.

Two transport systems for glucose were recently reported also in control (anesthetized) rats (GJEDDE 1980c), as shown in Table 5. The affinity of the low affinity mechanism is so low as to preclude separate determination of meaningful values for Tmax(2) and Km(2). The high affinity mechanism revealed a reduction of Tmax(1) in hyperglycemia but no change of Km(1). Mutual adjustment of the two transport systems caused the "effective" Km to rise from a value close to the normal glucose concentration in plasma to value close to the concentration in hyperglycemia. The benefit of such an adjustment is not immediately clear but may help limit the increase of the glucose concentration in the extracellular space. Also, in this Symposium, Dr. OLDENDORF has emphasized the observation that Km values of transport systems in the blood-brain barrier tend to follow

the substrate concentrations. The differential adaptability of two transport mechanisms may accomplish just this.

Rapid reduction of plasma glucose in chronic hyperglycemia can bring the blood-brain glucose flux below the level sufficient to sustain normal brain function, and may explain the serious cerebral disturbances seen in late, poorly regulated diabetes mellitus, the complications of which are now believed to be related to hyperglycemia. A syndrome of "relative cerebral hypoglycemia" has also been described by WYKE (1969) and ascribed to normalization of plasma glucose in otherwise normal subjects with chronic moderate hyperglycemia.

## Conclusions

The observations summarized above lead to the conclusion that substrate transport across the cerebral capillary endothelium is highly variable but linked to changes of cerebral metabolic rate, perhaps by blood flow. This conclusion explains how the brain interstitial fluid concentration of glucose can be relatively low and still sustain considerable variation of metabolic rate.

Chronic changes of substrate concentration, on the other hand, induce compensatory changes of transport that tend to prevent major fluctuations of substrate supply. In addition, the adaptability of the blood-brain barrier substrate transport is an important factor to be considered in the pathogenesis of symptoms of inborn errors of metabolism and endocrine diseases.

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*Adv. Physiol. Sci. Vol. 7. Cardiovascular Physiology. Microcirculation and Capillary Exchange*
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# IN VITRO INVESTIGATIONS OF THE BLOOD-BRAIN BARRIER: ISOLATION AND CULTURE OF BRAIN CAPILLARY ENDOTHELIAL CELLS

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The capillary endothelium of the brain plays an important role in homeostasis within the central nervous system by limiting the blood to brain passage of some solutes while facilitating exchange of others. This selective permeability barrier, termed the blood-brain barrier, is produced by a combination of specific membrane transport systems, a low rate of pinocytosis, and the presence of continuous intercellular tight junctions which seal adjacent endothelial cells together /Rapoport, 1976/.

It is difficult to study properties of brain capillary endothelial cells in vivo because they represent a small fraction of the total cells in the brain. In recent years several investigators have used isolated brain capillaries as a model system for short-term studies /Goldstein, et al., 1975; Betz and Goldstein, 1978; Betz, et al. 1979; Goldstein, 1979; Hjelle, et al. 1978; Herbst, et al. 1979; Brendel, etal. 1974; Eisenberg and Suddith, 1979/. Although much useful information has come from this work, the desirability of a cell culture system for brain capillary endothelial cells is apparent. Several methods for culture of these cells have been described /Debault, et al. 1979; Panula, et al. 1978; Phillips, et al. 1979/. However, the resulting cells do not retain properties which are important for blood-brain barrier function in vivo.

In this report we describe the successful culture of capillary endothelial cells derived from rat brain. Primary cultures retain both endothelial cell markers and some unique features typical of brain capillary endothelial cells in vivo. Our method provides a large number of endothelial cells which whould be useful for studies of brain capillary function. Preliminary reports of this work have been published /Bowman, et al. 1979; Penney, et al. 1980/.

Rat brain capillaries were isolated as described by Betz, et al. /1979/. In order to remove the basement membrane and adhering pericytes, isolated capillary segments were suspended in collagenase/dispase and agitated overnight. At the end of the dissociation period, the cells were layered over a 50% Percoll gradient and banded by centrifugation. Cells derived

from capillaries were routinely cultured in Nutrient Mixture F12 /Gibco/ containing 10-20% CS and 1x Antibiotic-Antimycotic mixture /Gibco/. They were seeded onto collagen coated plastic surfaces in T25 flasks. Cultures were maintained in a 2% $CO_2$ air incubator, and medium was changed every 2-3 days.

The cultured brain capillary endothelial cells produce factor VIII antigen which is currently the only reliable marker for an endothelial origin of cells in culture /Jaffe, et al. 1973/. Our primary cultures also retained other less specific endothelial markers including the presence of angiotensin converting enzyme activity and a cell surface which does not bind platelets. In addition, cultured cells had intimate intercellular attachments including tight junctional complexes. In most of our transmission electron micrographs there were few pinocytotic vesicles as would be expected for an endothelial cell derived from brain capillaries.

It is likey that there are fundamental differences between endothelial cells derived from capillaries and those derived from large vessels. These differences should preclude use of large vessel endothelial cell cultures as a model system for the study of microvessel function and pathology. In addition, there appear to be important differences between capillary endothelium from various organs /Wolff, 1977/. Thus, endothelial cells derived from brain capillaries should be the best culture system for studies of blood-brain barrier function.

Little is know about the metabolism of the capillary endothelium, possible because it is difficult to prepare a pure population of endothelial cells from most organs. A distinct advantage of studying the brain capillary endothelium is the ease with which it can be freed from surrounding tissue. Although brain capillaries initially contain two cell types, the endothelial cells can be separated from the pericytes by gradient centrifugation. Isolated brain capillaries can be obtained in large numbers from several different species and easily provide ten million or more endothelial cells when prepared from rat brain according to our method. Primary cultures of these cells should be suitable for studies of normal brain capillary function. In addition, they provide a new model system for the study of capillary injury.

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# EFFECT OF HISTAMINE ON THE PERMEABILITY, FINE STRUCTURE AND ADENYLATE CYCLASE ACTIVITY OF BRAIN CAPILLARIES

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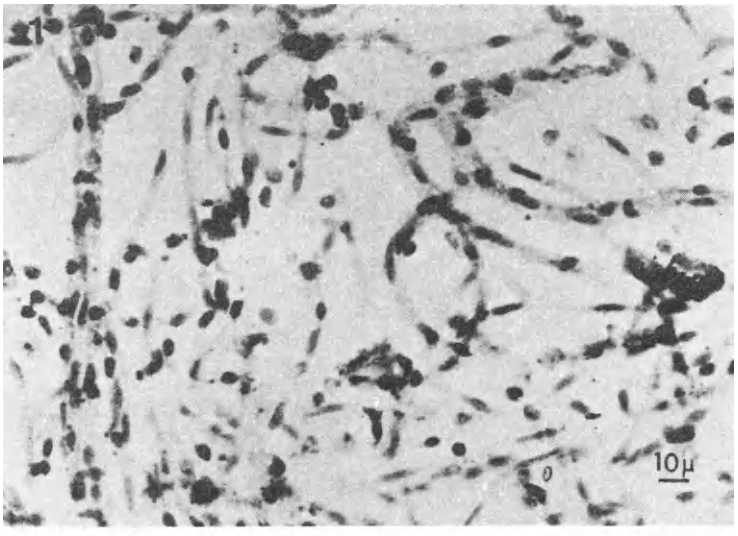
It is well known that the histamine, among other endogenous vasoactive substances, has a definite role in the regulation of permeability in the peripheral microvessels. Although several studies have been performed in capillary enriched fraction of brain tissues in order to throw some light on the possible cellular mechanisms regulating the permeability of the blood-brain barrier, the possible role of histamine in these processes has not been clarified yet. Similarly to the major part of vascular wall (El-Ackad and Brody 1975) histamine could also be detected in appreciable amount in the capillary-rich fraction of brain tissue (Jarrott et al. 1979, Karnushina et al. 1979). It remained to be elucidated if histamine had any effect on the transport processes of brain capillaries.

In our experiments, we used a capillary enriched fraction to study the effect of histamine on the adenylate cyclase in vitro, and in other, in vivo studies, we looked at the effect of histamine on macromolecular transport and on the fine structure of brain vessels.

For the biochemical investigations, we prepared the capillary enriched fraction according to the micromethod described by Joó and Karnushina (1973).The purity of the capillary-rich fraction prepared from guinea pig cerebral cortex was regularly examined under light microscope using toluidine-blue staining (Fig. 1). The microvessel fraction consisted mainly of pure capillaries, but rarely arterioles and venules of larger size were also present. Only a negligible contamination by nuclei and cell debris could be observed. Morphometric evaluation of the fraction performed on electron micrographs ( Weibel et al. 1966) showed that capillaries were present more than 95 per cent (Fig. 2).

Adenylate cyclase activity was measured according to Hegstrand et al. (1976), and the cyclic AMP content was determined by the protein-kinase binding method of Brown et al. (1971). The effect of increasing concentrations of histamine on adenylate cyclase activity of a capillary-rich fraction is illustrated in Fig. 3. The concentration required for half-maximal stimulation was around $5 \times 10^{-6}$M, whereas maximal stimulation was achieved at about $10^{-4}$M. In various preparations the maximal stimulation by histamine ranged from 1.4 to 2.4 fold of basal enzyme activity.



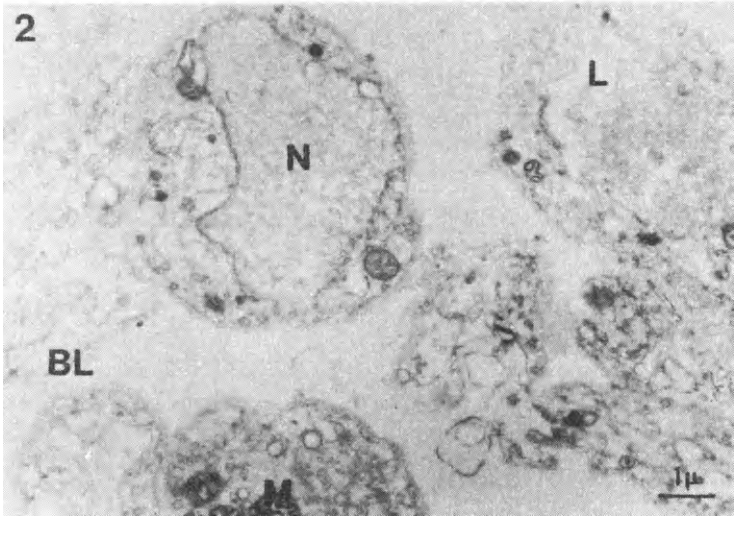




Fig. 1. Light microscopic appearance of the capillary-rich fraction after toluidin -blue staining. In addition to the capillaries, few arterioles and venules were occasionally also present.

Fig. 2. A typical ultrastructural picture of the capillary-rich fraction. N : nucleus of an endothelial cell, BL : basal lamina, L : lumen of the capillary.

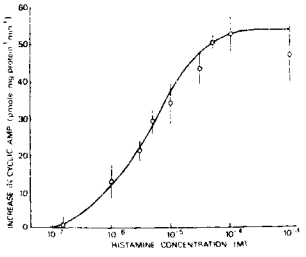


Fig. 3. Stimulation by histamine of the adenylate cyclase activity in the capillary-rich fraction of the guinea-pig cerebral cortex. Results are expressed as increases in cyclic AMP over the basal level ( $55.4 \pm 2.9$ pmoles x mg protein$^{-1}$ x min$^{-1}$ . Each value is the mean $\pm$ S.E.M. of data from triplicate incubations.

To clarify the nature of the receptors ($H_1$ or $H_2$) linked to the histamine-sensitive adenylate cyclase, we characterized the effect by means of various agonists and antagonists. Concentration response curves of two $H_1$-receptor agonists ( 2-methylhistamine and 2-thiazolylethylamine ) and two $H_2$-receptor agonists ( dimaprit and 4-methylhistamine ) are compared to that of histamine (Fig. 4A and B).In both cases the curves of $H_2$-receptor agonists are closer to the effect of histamine. The $H_1$-receptor agonists could stimulate the basal enzymatic activity only in larger dosis.

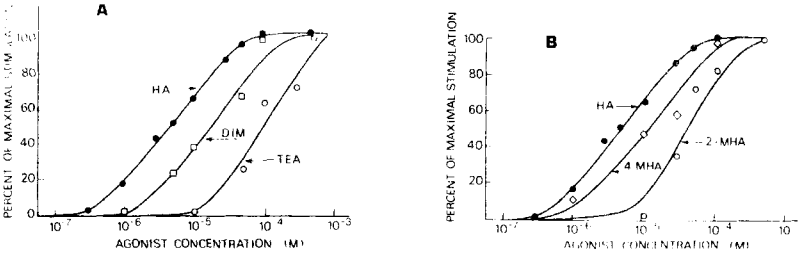



Fig. 4. Stimulation by histamine and various $H_1$- and $H_2$-receptor agonists of the adenylate cyclase activity in the capillary-rich fraction of the guinea-pig cerebral cortex. Results for histamine (HA), dimaprit (DIM) and 2-thiazolylethylamine (TEA) in Fig. 4A, as well as for 4-methylhistamine (4-MHA) and 2-methylhistamine (2-MHA) in Fig. 4B, are expressed as percentages of maximal stimulation elicited by each agent. Means from 6 incubations in two separate experiments.

Mepyramine and cimetidine were employed as $H_1$ or $H_2$-receptor antagonists. Fig. 5 shows the stimulation of adenylate cyclase activity in capillaries induced by increasing concentrations of histamine when assayed successively in the presence of cimetidine ($H_2$-receptor antagonist) in two different concentrations. The parallel displacement of the concentration-response curves toward the right side, without change in the maximal response to histamine, indicates the competitive inhibition induced by this type of antagonists. The inhibition constant was calculated to Cheng and Prusoff (1973) and was found at the value of $7 \times 10^{-7}$ M. The inhibition constant of mepyramine was higher ( $5 \times 10^{-6}$ M). The data obtained by agonists and antagonists suggest that the effect of histamine on the adenylate cyclase activity is linked to the $H_2$-receptors. Other details of our results were published recently (Joó et al. 1975 and Karnushina et al. 1980).

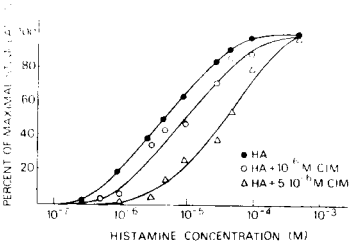



Fig. 5. Inhibition by cimetidine of the histamine-induced stimulation of adenylate cyclase activity in the capillary fraction of the guinea-pig cerebral cortex. Homogenates of the fractions were incubated in the presence of various concentrations of histamine (HA), alone (•——•) or together with $10^{-6}$ M (o——o) or $5 \times 10^{-6}$ M (Δ——Δ) cimetidine (CIM). Results are expressed as percentages of the maximal response to histamine.

Having seen the in vitro effect of histamine on the capillary adenylate cyclase, we wanted to check the effectiveness of histamine administered in vivo. For this reason, rats were anaesthetized with pentobarbital ( 40 mg/kg i.p.) and fixed in a position lying on their backs. The right carotid artery was prepared in the lower part of the neck, and thin plastic canule was inserted into the vessel. Histamine solutions were made up by Krebs-Ringer solution in concentrations of 60 μg/ml and 0.5 mg/ml. The histamine was perfused via carotid artery by an Infumat driver (KUTESZ, Hungary). Every animal was given 1 ml in 15 minutes. The controls were perfused by Krebs-Ringer solution under the same circumstances. After the infusion the animals were immediately decapitated and 1-2 $mm^3$ pieces of the parietal cortex were fixed by immersion either in Bouin solution for light microscopic immunohistochemical detection of albumin or in Karnovsky solution for electron microscopy. For the visualization of albumin, the original method of Wilmes and Hossmann (1979) was applied according to the peroxidase-antiperoxidase (PAP) procedure (Sternberger 1974).

After postfixation with osmic acid and dehydration the samples

for electron microscopy were embedded in Durcupan (Fluka). Sections of gold and silver interference colours were cut by Porter-Blum ultramicrotome and were stained with uranyl acetate and lead citrate and viewed under a JEOL 100B electron microscope.

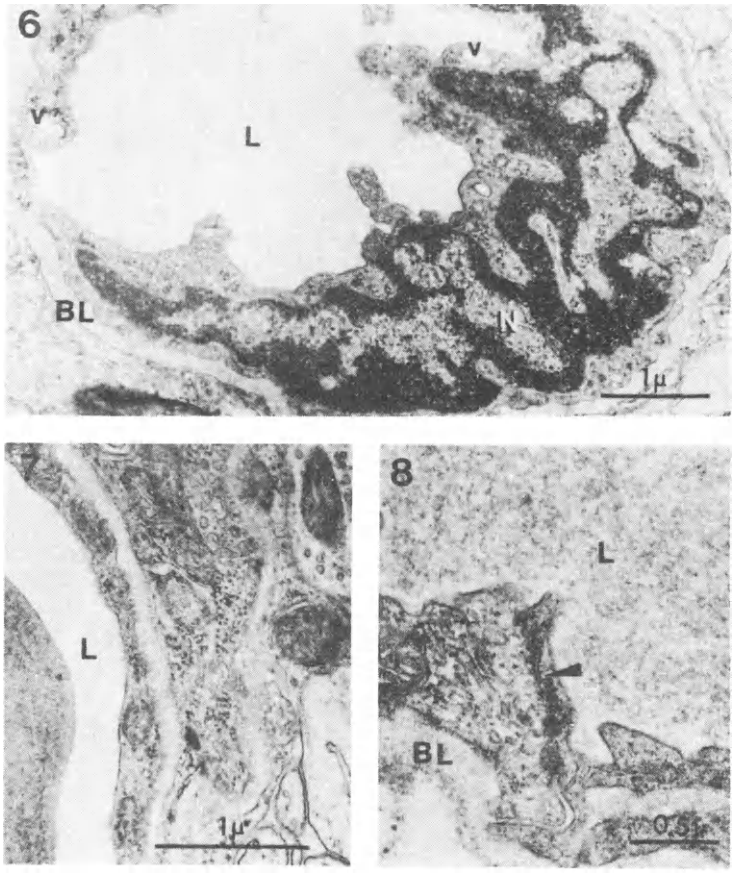
Figs. 6-8 show the typical features of the cerebral capillaries in control perfused animals. Apart from contractions of some endothelial nuclei and appearance of large vacuoles in the cytoplasm ( Fig. 6), no sign of enhanced pinocytosis and opening of the tight junctions was seen (Fig. 7-8).

After 0.5 mg/ml histamine perfusion, ultrastructural changes could be detected in the capillary wall. Oedematous alterations of the glial end feet system were frequently found (Fig. 9). The characteristic enhancement in numbers of pinocytotic vesicles indicates the increased transport through the capillary wall (Fig. 10). The tight junctions between the adjacent capillaries seemed to have changed in the following way : The pools between the fusion points of the tight junctions have extended and the interendothelial fusion points have disappeared. The only fusion point, which seemed to have remained closed was seen at the luminal surface (Fig. 11). This finding is in agreement with our earlier finding (Joó 1972), when the transendothelial vesicular transport has been found to take part in the enhanced permeability in the way how Hansson et al (1975) have demonstrated it.

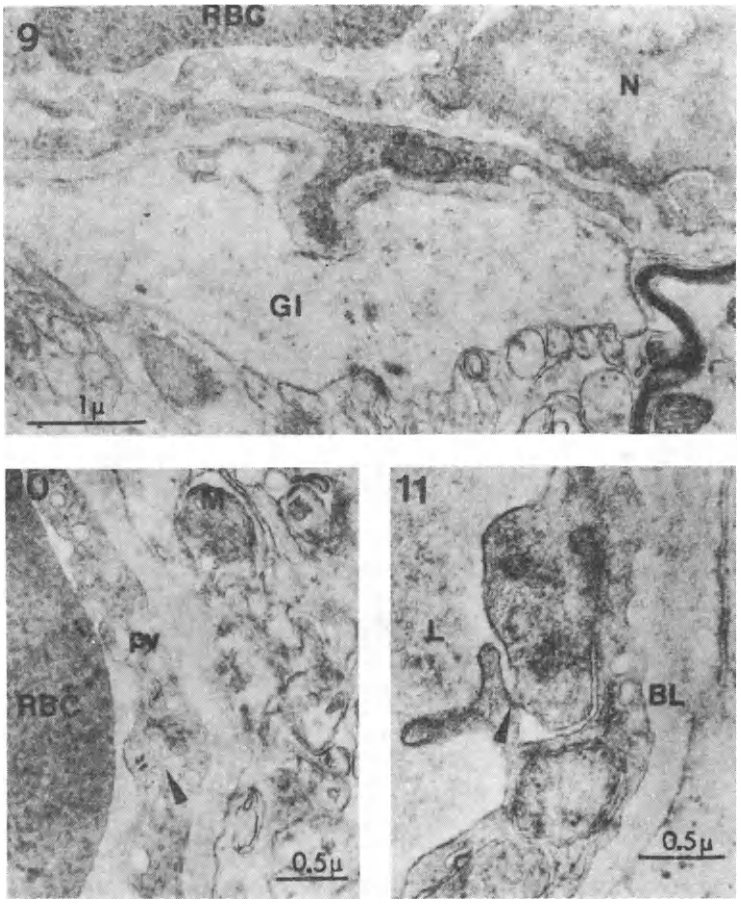
Permeation of serum albumin on the effect of histamine was checked by the PAP immunohistochemical method. In the controls, no sign of extravasation was seen (Fig. 12). After histamine perfusion ( 60 µg/ml) serum albumin has leaked out from the majority of capillaries (Fig. 13) and accumulated in astrocytic processes (Fig. 14) and occasionally in some glial cells (Fig. 15) as well.

According to the general opinion of earlier studies, histamine has no effect on the brain capillaries (Broman and Lindberg-Broman 1945, Ashton and Cunha-Vaz 1965, Westergaard and Brightman 1973, Wolff et al. 1975). In our studies, performed on isolated brain capillaries, we found an activation of the capillary adenylate cyclase by histamine. This finding has indicated the possible in vivo effect of histamine on the permeability of brain microvessels. According to our expectation, contrary to the above mentioned negative data of literature, we found with the use of the sensitive PAP immunohistochemical method, the permeability increasing effect of histamine on brain capillaries. In agreement with our present results, Gross and co-workers (1980) have recently published that the intra-arterial histamine increased the cerebrovascular permeability for $^{14}C$-sucrose. They found that this effect of histamine could be reduced or blocked by $H_2$-receptor antagonist, metiamide.

Summarizing the above-mentioned results, it seems that histamine can increase the transport processes in the cerebral capillaries. Thus, histamine, like in peripheral tissues, may take part in certain oedematous alterations of the brain.




Figs 6-8. The ultrastructure of a cerebral capillary after perfusion with Krebs-Ringer solution. L : capillary lumen, BL : basal lamina, N : nucleus of the endothelial cell, M : mitochondrion. The arrowhead shows the junction between two adjacent endothelial cells.




Figs 9-11. The changes in the ultrastructure of capillary endothels after histamine perfusion ( 0.5 mg/ml). N : nucleus, RBC : red blood cell, Gl : glial process, pv : pinocytotic vesicles, M : mitochondrion, BL : basal lamina, L : capillary lumen. The arrowhead in Fig. 10. points at a possible channel formation from the pinocytotic vesicles. In Fig. 11. the arrowhead shows the remained last fusion point of a tight junction of two adjacent endothelial cells.

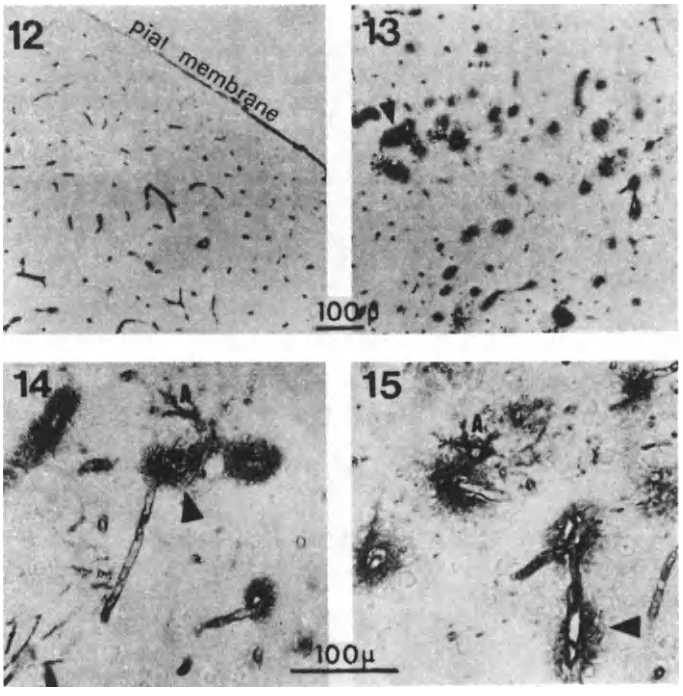



Fig. 12. The peroxidase-antiperoxidase (PAP) reaction after perfusion with Krebs-Ringer solution.
Figs 13-15. PAP-reaction performed for the visualization of exuded albumin (arrowheads) after intracarotic histamine infusion ( 60 µg/ml). A : astrocytic elements.

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# HOMEOSTASIS OF THE COMPOSITION OF CEREBRAL INTERSTITIAL FLUID

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Physiologists, preceding Claude Bernard, had considered the existence of compensatory adjustments neutralizing disturbances in the living body. However, it was the physiological insight and experimental approach of Bernard which led him to frame the concept of a controlled internal environment (1878). He regarded it as the precondition for a free life. Of his conclusion that all the vital mechanisms, varied as they are, have only one object, that of preserving constant the conditions of life in the internal environment, Haldane (1922) said that no more pregnant sentence was framed by a physiologist. Haldane, himself, established the remarkable constancy of the partial pressure of $CO_2$ in alveolar air, based on respiratory adjustments, and realized that the control of alveolar $CO_2$ was directed towards maintaining constant the concentration of carbonic acid and hence to some extent the concentrations of hydrogen and hydroxyl ions in the body fluids.

Cannon (1929) coined the term homeostasis and considered some of the physiological entities controlled, together with possible mechanisms. He also listed some of the serious consequences arising when the acceptable limits of variation in the internal environment are exceeded, as in hypoglycaemia, water intoxication, salt, calcium and pH disturbance, and hypo- and hyperthermia. Barcroft (1932) drew attention to the organ system which is most frequently affected when the limits are transgressed and hence argued that control of the internal environment is particularly directed towards maintaining optimum function of this system. 'If the limits are transgressed something happens to impair the efficiency of the organism. Look down the list of disabilities produced by alterations in the internal milieu and you will see almost no reference to the grosser bodily functions, nothing about muscular contraction as such, nothing about the heart as such, nothing about the kidney, the liver or the pancreas. In almost every case the blow is to the nervous system; we can go further, in almost every case it is to the central nervous system; in almost every case it is to the higher parts of the central nervous system. And so far as our investigation has shown us anything it has shown that the fixity of internal environment is controlled by the upper part of the central nervous system, and it is as a general rule the upper part of the central nervous system which suffers if the environment alters beyond physiological limits. The fixity of the internal environment is the condition of mental activity.

In the late twenties and early thirties of the 20th century good evidence was published that the concentrations of magnesium, potassium and calcium in human cerebrospinal fluid (CSF) are held remarkably constant in the face of much wider variations in their respective concentrations in blood serum (see Bradbury, 1979, for detailed references). It is surprising that none, to my knowledge, of the workers involved speculated that the constancy they had demonstrated in cerebrospinal fluid might reflect a powerful homeostasis of the extracellular environment of the central neurons - a second milieu intérieur with a finer secondary control beyond that of the blood and the general extracellular fluid. Possibly, ideas of free exchange between CSF and interstitial fluid of brain were not sufficiently developed at that time for them to realize the significant meaning of their findings namely that a constancy of the ionic composition of CSF reflects a similar condition in the interstitial fluid of the central nervous system.

The homeostasis of ions in CSF was later demonstrated more rigorously by intravenous infusion of solutions of inorganic cations to considerably raise plasma levels, e.g. for potassium (Bekaert & Demeester, 1951) and for magnesium and calcium (Kemény et al, 1961). It was for Pappenheimer (1965) and his colleagues to prove that the homeostasis of alveolar $P_{CO_2}$, discovered by Haldane, was determined by cells of the respiratory centre in the medulla responding to the pH of cerebral interstitial fluid and thus must be directed towards keeping a near constant concentration of hydrogen ions in this field. Numerous physiological experiments have fully confirmed the homeostasis of various cations in both CSF and whole brain in both acute and prolonged experiments. More recently direct measurements of the activity of certain cations in the interstitial fluid of brain with ion-selective electrodes have demonstrated a relative independence of concentrations here from those in blood, e.g. for potassium (Hansen et al, 1977).

## MECHANISMS OF HOMEOSTASIS

What might be the mechanisms of this ionic homeostasis? Obviously, the presence of a tight blood-brain barrier is a good basis. Maintenance of solute gradients across leaky cerebral capillaries would involve excessive and unnecessary expenditure of energy. Since, however, the cerebral capillaries do have a finite permeability to ions, the stability in each case must depend on a dynamic equilibrium. If the amount of a substance in a compartment is to be stable, inflow and outflow must be equal. If the amount is disturbed in the direction of excess, the compensation could be achieved by a reduction in inflow or an increase in outflow or both. Similarly, if there is a depletion, the converse must occur for homeostasis. Whilst the above principles must always apply, a number of different special mechanisms are active in the brain-CSF system.

1). Homeostasis of solutes in blood and the general extracellular fluid (ECF) brought about by the kidney and other organs provides a primary basis on which the mechanisms at the blood-brain barriers can act. Fine temperature control can be most readily maintained in a water-bath, if the temperature of the room in which it is sited is also controlled.

2). As Barcroft's analysis (1932) indicated much general homeostasis is determined by negative feed-back loops (neural or endocrine or both) for which the receptor for a disturbance (error signal) is within the central

nervous system (CNS) itself. Thus function of organs or organ-systems outside the CNS is frequently directed towards homeostasis of the cerebral interstitial fluid. Good examples are the role of interstitial fluid pH in determining ventilation, the role of its osmolality in determining drinking and water excretion and indeed the role of hypothalamic thermoreceptors in controlling heat losing and heat conserving mechanisms.

The question of there being neural or endocrine mechanisms leading to solute homeostasis of cerebral ECF's for which receptor and effector cells lie totally within the brain-CSF system was raised by Bradbury & Davson (1965). No evidence has yet been produced for the hypothesis but it remains a possibility.

3). In the short term, homeostasis of a fluid can be achieved by means of a reservoir of the solute controlled which allows release at periods of depletion and re-uptake at times of excess, i.e. 'buffering'. Homeostasis of the pH of CSF has been speculated as being partly due to the buffering action of proteins and other weak acids in brain, i.e. to the brain acting as a giant red cell. If such were true the brain cells should take up chloride during extracellular acidosis and release it during alkalosis. The evidence for this is dubious. Certainly, glial cells can take up large amounts of potassium by both passive and active mechanisms, when the concentration of this ion is raised in the interstitial fluid (Abbott & Pichon, 1976). The mechanism appears to be important in restoring resting levels of interstitial potassium after frequent firing of neurons. It seems not unlikely that cells in brain could also temporarily 'buffer' extracellular calcium and magnesium in this way, since they partly control their own intracellular fluid on the basis of exchanges with large intracellular stores of unionized calcium and magnesium.

4). Hydrogen ion is the only cation subject to metabolic control in brain. In alkalosis, relatively large amounts of lactic and other organic acids are released by enhanced glycolysis into the cerebral extracellular fluids (Plum & Posner, 1967; Siesjö, 1973). The mechanism appears to be a direct effect of pH on the enzyme phosphofructokinase. The transmembrane potential of brain cells favours movement of the lactate into the interstitial fluid where it will replace bicarbonate ions which simultaneously pass into the cells to react with the $H^+$ ions from the lactic acid to form carbonic acid and hence $CO_2$. Another example of metabolic control in the brain is the creation of osmotically active solute, probably largely amino acids, in brain during prolonged hyperosmolar states (Baxter, 1967). This contributes to the restoration of brain volume to normal after osmotic shrinkage.

5). The basis for homeostasis may be rendered simpler in a flowing system. Bradbury & Sarna (1977) have demonstrated how a flowing CSF might regulate ion concentration in cerebral interstitial fluid. The following are required. The blood-brain barrier must be impermeable to the solute in question or must transport it at a constant net influx or efflux. Cerebrospinal fluid must be secreted at a constant rate and must contain a constant concentration of the solute in question. There is some evidence that such a system might play a role in the homeostasis of calcium and magnesium in cerebral interstitial fluid, but not of potassium.

6). In the case of potassium and probably of other ions, the kinetic properties of mechanisms sited between cerebral extracellular fluids and blood are crucial. If one considers solute movement across the blood-brain barrier, no volume flow assumed, a degree of homeostasis will ensue if during a disturbance in plasma concentration $\Delta C_{pl}$, the change in influx into interstitial fluid $\Delta J_{in}$ is less than the corresponding relation of $\Delta J_{out}$ to $\Delta C_{isf}$, the change in concentration in interstitial fluid, i.e. if

$$\Delta J_{in} / \Delta C_{pl} < \Delta J_{out} / \Delta C_{isf}$$

Bradbury (1971) summarized results from in vivo experiments demonstrating that exchanges of radioisotopic potassium conformed to this prediction. In particular, these experiments indicated the presence of a ouabain-sensitive sodium pump with a steep sigmoid relation between potassium efflux and the concentration of this ion in cerebral extracellular fluid. The active transport of potassium was directed towards blood and the site of the pump was thought to be at the blood-brain barrier. Firth (1977) developed a specific histochemical technique for Na-K dependent ATP-ase so that the site of high concentrations of this enzyme could be visualized in electron micrographs of brain. A high density of the enzyme occurred in the abluminal plasma membranes of capillary endothelial cells but not in the luminal cells. Isolated cerebral capillaries in vitro have also been shown by Goldstein (1979) to accumulate radioactive potassium and rubidium ions by a ouabain sensitive pump with similar characteristics to that demonstrated in vivo.

## CONCLUSIONS

There is a high degree of homeostasis of the milieu interieur of the brain. Amongst solutes controlled in the cerebral extracellular fluids are the cations of hydrogen, potassium, calcium and magnesium. A multiplicity of mechanisms are involved, as one might anticipate, for a system where control is so important for function. Recent techniques have localized the site of an important component of the potassium homeostatic mechanism to within the capillary endothelium of brain. Constancy of the internal environment of the brain is a condition for ordered nervous activity and is obtained by mechanisms acting both on the fluids of the body as a whole and on those of the brain in particular. In each case, the error signal which activates the negative feed-back control is often derived from within the central nervous system or its extracellular fluids, thus ensuring that this is the region where maximum homeostasis is achieved.

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# CONVECTION OF BRAIN INTERSTITIAL FLUID

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The proposal has been made that the blood-brain barrier can be viewed as a secretory epithelium with a NaCl-coupled fluid transport from capillary plasma into brain (Bradbury, 1975; Crone, 1980). We have estimated the rate of interstitial fluid (ISF) drainage from brain in two mammalian species, rat and rabbit. On the assumption that the volume of brain ISF remains constant, with a balance between the rates of fluid production and removal, our estimate of drainage rate can be taken as the rate of net fluid production within the brain. By what mechanism is this fluid produced? By NaCl-coupled fluid secretion at the blood-brain barrier, as has been suggested? By filtration at the capillary wall through the balance of Starling forces? Or, by metabolic water production? The purpose of this paper is to evaluate these mechanisms using measured parameters of the system with the aim of providing new insights into the proposal that the blood-brain barrier can be viewed as a secretory eptithelium.

First, before considering the mechanism of ISF production, it is appropriate to review our methods for studying convection of brain ISF and the evidence that the rate of drainage of this fluid from the central nervous system can be estimated. Convection is studied by following the removal of extracellular tracers from brain following microinjection into the interstitium. Anatomical tracers are used to outline the pathways of flow and radiolabelled extracellular tracers to study the dynamics of exchange. Each animal is provided with a stainless steel guide cannula (26 gauge) implanted stereotaxically under pentobarbital anesthesia with the tip located in the caudate nucleus. One week later, the animal is reanesthetized and 0.5 µl (rats) or 1.0 µl (rabbits) of a test solution containing one or more of the test compounds infused slowly into the caudate nucleus using a stainless steel injection cannula (33 gauge) attached to an infusion pump. The tip of the injection cannula extends 1 mm below the end of the guide cannula. The amount of tracer injected into brain can be determined accurately by measuring the linear displacement of a small air bubble in calibrated infusion tubing. The distribution of anatomical tracers is followed using routine histological techniques, the distribution of radiolabelled tracers by assaying tissue and fluid samples for radioactivity.

With this tissue clearance technique, we have obtained two independent lines of evidence consistent with the classical view that in the brain, as in other tissues, there is convection of the ISF (Weed, 1914). First, in agreement with earlier workers (e.g. His, 1865), we find that anatomical

tracers move through the tissue along preferential channels, yielding a pattern of distribution consistent with convection rather than diffusion; and, second, the kinetics of removal from whole brain of radiolabelled extracellular tracers provide new evidence of the turnover of cerebral ISF. These results are considered in more detail below. The recent results of Rosenberg et al. (1980) also indicate a volume flow of ISF in normal brain tissue.

## Intracerebral channels of flow

Pathways of flow were identified by following the distribution of colored dextran and of horseradish peroxidase through the interstitium following microinjection into brain (Cserr & Ostrach, 1974; Cserr et al., 1977). Results indicate that ISF flows in a system of extracellular channels including the spaces around blood vessels (perivascular spaces), between fiber tracts and in the subependymal layer of the ventricular ependyma. With respect to the importance of perivascular spaces in the drainage of ISF, our results support the hypothesis (His, 1865; Weed, 1914) that these channels serve as preferential pathways of flow analogous to the lymphatic channels of systemic tissues.

## Removal of radiolabelled extracellular tracers from brain

In order to obtain quantitative information concerning fluid removal from brain we injected radiolabelled extracellular tracers into the caudate nucleus and followed their subsequent rate and route of efflux from brain (Cserr et al., 1977; Cserr et al., submitted). Three compounds were used: $^{125}$I- or $^{131}$I-serum albumin (RISA, 69,000 daltons), $^{14}$C-polyethylene glycol (PEG, 4,000 daltons) and $^{3}$H-PEG (900 daltons). The diffusion coefficients for these compounds range from 0.85 x $10^{-6}$ $cm^2$ $sec^{-1}$ for RISA to 4.0 x $10^{-6}$ $cm^2$ $sec^{-1}$ for the 900 dalton PEG, providing an approximately five fold range in diffusion coefficient. The amount of isotope remaining in whole brain was determined 1, 4, 18 and 24 hours after injection in rats. For all three test compounds, clearance from brain approximates a single exponential decay. Thus,

$$N_b = N_i e^{-kt}$$

where $N_b$ is the total radioactivity remaining in brain at the end of the experiment (dpm), $N_i$ the total radioactivity injected into brain (dpm) and k the first order rate constant for total isotope efflux from brain ($hr^{-1}$). Values for k (means $\pm$ SE) determined by a nonlinear least squares fit of the data for RISA, PEG-4,000 and PEG-900 were .057 $\pm$ .003 $hr^{-1}$, .055 $\pm$ .002 $hr^{-1}$ and .048 $\pm$ .002 $hr^{-1}$, respectively. The corresponding half-times of disappearance from brain are 12.2 hr, 12.6 hr and 14.4 hr, respectively. Similarity in efflux rate despite the five fold difference in diffusion coefficient is consistent with convective rather than diffusive losses from brain.

The conclusion that the polyethylene glycols and RISA are removed from brain by convection would be strengthened if the pathways of drainage were identified. From the perivascular spaces it has generally been considered that ISF flows into the subarachnoid space with subsequent drainage into the dural venous sinuses (Weed, 1923). More recent evidence suggests that the cervical lymphatics also contribute to the clearance of ISF from the central nervous system (Foldi et al., 1968). To examine this problem, tracer efflux into cerebrospinal fluid (CSF, in rats and rabbits)

and into lymph (rabbits only) was estimated following microinjection of RISA into the brain. Samples of large cavity CSF were withdrawn from the cisterna magna at the end of the experiment. Lymph was collected for 5 to 8 hours from either one or both cannulated jugular lymph trunks. Results are summarized in Table 1. In rats, analysis of tracer concentration over time in the sample of cisternal CSF indicates that only 10-20% of tracer efflux is into this compartment. In rabbits, on the other hand, the major fraction of RISA efflux from brain can be accounted for by drainage into CSF and lymph (Bradbury, Cserr & Westrop, submitted). Lymph collection was not attempted in rats due to technical considerations. The pathway of ISF drainage into lymph in rabbits, outlined with labelled albumin, includes passage by way of the arachnoid sheath of the olfactory nerve to the nasal submucosa.

Table 1. Flow of cerebral ISF compared with lymph

| Tissue | Drainage rate, µl/g tissue per min | Percent drainage via various pathways | |
|---|---|---|---|
| | | CSF | Lymph |
| Brain, rat | 0.11 | 10 - 20 | -- |
| Brain, rabbit | 0.08 | 30 | 50 |
| Systemic tissues | 0.02 - 0.04* | 0 | 100 |

*Flow rate estimated on the basis of lymph flow through the thoracic duct.

The fluid in the subarachnoid space over the convexities of the cortex (near the site of isotope injection) mixes slowly with large cavity CSF (Weed, 1923) and would not be expected to be represented in the sample of fluid withdrawn from the cisterna magna. Thus, a fraction of tracer efflux may cross the subarachnoid space without being detected by our technique. Conceivably, this could account for the additional components of efflux not accounted for by our analyses of lymph and large cavity CSF.

## Estimation of the rate of ISF production

The rate constant for efflux from brain, k, is for total tissue clearance and may describe losses by a number of pathways (including CSF, lymph and the blood-brain barrier) and by a number of mechanisms (including bulk flow and diffusion). Evidence summarized above is consistent with the conclusion that albumin and the two polyethylene glycols are cleared from the interstitium principally by convection into CSF and lymph. In addition, there must also be a small diffusive component of efflux across the blood-brain barrier into capillary plasma. Following correction for this diffusive efflux, the clearance data for the extracellular tracers can be used to estimate the rate of ISF production by brain (Cserr et al., submitted). This calculation assumes that there is a steady-state between drainage and production of ISF. Since total efflux may include additional non-convective components of efflux not identified in our experiments, this estimate should be viewed as a maximal estimate of the rate of ISF production.

The rate constant for albumin exchange across the blood-brain barrier has been measured (Blasberg, Fenstermacher and Patlak, personal communication). Correcting the rate constant for total efflux of albumin from rat brain (.057 $hr^{-1}$) for transfer across the blood-brain barrier (.001 $hr^{-1}$) yields a rate constant for fluid drainage from brain of .056 $hr^{-1}$ (t 1/2 = 12.4 hr). For an extracellular fluid volume of 16% (160 µl per g brain) it follows that the rate of ISF production by rat brain is 0.11 µl/g brain per min (160 µl per g brain/2/12.4 hr). For rabbit, the comparable value is .08 µl/g brain per min. These values, plus similar data for lymph, are included in Table 1.

The mechanism of ISF production

Now that we have an estimate for the rate of ISF production, we can consider the proposal that this fluid is produced by active secretory processes at the blood-brain barrier. ISF may be produced at the blood-brain barrier either by filtration of capillary plasma as in systemic tissues or by secretion through the coupled transport of solutes and water. The possible contribution of metabolic water to ISF production has also been suggested (e.g., Rapoport, 1976); however, since free water would diffuse rapidly down its concentration gradient from brain into capillary plasma, it is unclear how metabolic water could contribute to ISF production unless its formation in brain were accompanied by the formation of an isosmolar equivalent of metabolites. Furthermore, the rate of ISF production estimated on the basis of our data (0.11 µl/g brain per min) is more than three times the rate of metabolic water production by the brain (0.03 µl/g brain per min) (Rapoport, 1976). The possibility that the ISF is produced at the cerebral capillary by the process of filtration can also be evaluated on the basis of our data plus experimental values for the hydraulic conductivity of the cerebral vascular bed. Thus, given values for the hydraulic conductivity of the blood-brain barrier (µl/cm $H_2O$ per g brain per min) in rabbit (Fenstermacher & Johnson, 1966) and in man (Paulson et al., 1977) of $4.6 \times 10^{-3}$ and $4.3 \times 10^{-3}$, respectively, transcapillary pressure gradients required to produce ISF at the rate of 0.11 µl/g brain per min can be estimated as 24 cm $H_2O$ (0.11/0.0046) and 26 cm $H_2O$ (0.11/0.0043), respectively. These estimated values for the transcapillary pressure gradient are roughly 60 times the normal net filtration pressure in systemic capillaries of 0.4 cm $H_2O$ (Guyton et al., 1971). The normal transcapillary pressure gradient in cerebral capillaries is not known; however, it seems extremely unlikely that it could approach 25 cm $H_2O$, the value required to explain the estimated rate of ISF production on the basis of filtration. Based on this analysis I suggest that ISF production in the brain can not be explained solely by filtration of plasma or by metabolic water production. Although there is no direct evidence for secretion of ISF at the blood-brain barrier, Bradbury (1975) and Crone (1980) have hypothesized net fluid secretion on the basis of structural and functional similarities of the blood-brain barrier to secretory epithelia. If correct, this could easily explain the estimated rate of ISF turnover. Thus, this analysis of the rate of ISF production supports the proposed analogy of the blood-brain barrier to a secretory epithelium.

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# PATHOLOGICAL OPENING OF THE BBB

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In pathological opening of the BBB two aspects deserve consideration. The one concerns the abnormal passage of various substances across the endothelial barrier, the second relates to the fate of extravasated substances in the brain parenchyma.

According to modern concepts, the BBB resides in the endothelium and is regarded as a conglomerate of various "barrier systems" which homeostatically regulate the biochemical environment optimal for the brain function. It would appear likely that in various pathological conditions the individual vulnerability among these systems may vary and be selective. Indeed, such selective vulnerability of the BBB has been clearly demonstrated in the cerebral ischemia due to obstruction of a major arterial branch. In these studies [16, 17] the abnormal passage of micromolecular substances, such as sucrose or sodium fluorescein preceded and lasted much longer than the leakage of serum proteins, which was evident between 5-15 hours after release of arterial occlusion. At the same time, after the same ischemic insult the peak of abnormal passage for exogenous noradrenaline, serotonin and dopamine was observed after a 3-day interval [3].

The feature of differential passage in a given BBB injury is relevant to interpretation of the most commonly used dye-protein tracers such as sodium fluorescein (NaFl) and Evans Blue (EB). The differences in the behavior of these tracers could be ascribed to differences in the degree of their binding to serum proteins [24]. The NaFl being to a considerable degree unbound acts as a micromolecular indicator, whereas the abnormal penetration of EB is primarily related to the albumin moiety of the tracer.

The other factors relevant to interpretation of tracer passage across the endothelial barrier are timings of BBB opening and of tracer circulation in the blood. It is obvious that if the opening of the BBB and adequate levels of tracer concentration in the blood are separated in time, falsely negative interpretations may be entertained in conditions clearly associated with BBB changes. For example, testing with a tracer introduced after a short-lasting BBB opening has already terminated will, obviously, give negative results. Similarly, negative findings will be observed when the level of a tracer becomes reduced to such a degree that it is not adequate to demonstrate late occurring BBB damage or when the tracer evaluation is carried out before a delayed BBB opening.

The ways by which BBB regulatory systems, residing in the endothelium, can be affected are largely unknown. Disturbances concerning the energy-dependent active transport systems may be directly related to energy supply deficit, as it occurs, e.g., in ischemia. Disarrangements in facilitated transport systems can be due to binding of SH-groups in cell membranes or due to interference with a specific carrier. Otherwise, the latter systems can be affected to a different degree resulting either in increased or in decreased facilitated transport. This was demonstrated with regard to passage of $^{14}C$ methyl-O-glucose after exposing the cerebral vasculature to $HgCl_2$ [19, 20]. In these experiments a slight BBB injury resulted in inhibition of glucose transport, whereas a severe mercurial BBB damage led to an increased entry of the glucose. Since the endothelial barrier for biogenic amines is regarded now as being of enzymatic nature, it can be assumed that a suppression of such specific enzymatic activities may produce an abnormal passage of exogenous biogenic amines into the brain parenchyma, and this has been supported by recent studies [1].

The best studied, perhaps, has been the leakage of proteins across the endothelium. Using the electron microscopically demonstrable horseradish peroxidase (HRP) as the tracer, the abnormal passage of proteins was related in many pathological conditions to a marked stimulation of vesicular pinocytotic transport, which is mostly insignificant in normal cerebral endothelial cells. Although the mechanism for induction of pinocytotic transport remains enigmatic, there are indications that adenylate cyclase, cyclic AMP and some neurotransmitters may play an important role in stimulation of pinocytotic activity in the endothelium [7]. The pinocytotic transfer of HRP across the endothelium has been described in cerebral ischemia [21], in acute hypertension [22], cold injury [11] and in epileptic seizures [12]. In microembolization, in addition to vesicular uptake, a formation of spherical and tubular channel-like structures was described as providing pathways for the HRP passage across the endothelium [13]. The microembolization experiments revealed, independently from the foci of protein extravasation, also areas in which an acute entry of water into the extracellular compartment could be surmised from the appearance of widely dilated clear extracellular spaces around the blood vessels in areas in which the cellular elements failed to show any evidence of swelling and there was no abnormal passage of HRP [2]. The presence of such clear extracellular spaces without HRP extravasations was demonstrable only for a few hours after cerebral microembolism. It might be speculated that such rapid flooding of the extracellular compartment could possibly be of neurogenic origin, since Raichle et al. [18] demonstrated that stimulation of noradrenergic centers in the locus coeruleus can produce an increased permeability of cerebral vasculature to water, associated with a concomitant decrease in the blood flow. The fact that following the microembolism the extracellular water does not seem to remain for long in the areas not visibly affected by ischemic injury could be explained by the absence of extravasated serum proteins which were shown to be responsible for the retention of water in vasogenic brain edema [10].

The fate of substances after passing the endothelial barrier into brain parenchyma is of significance: firstly, for proper interpretation of findings observed with BBB tracers and secondly, in view of the potential effect of various extravasated substances on the brain tissue.

In studies on vasogenic brain edema produced by cold lesion the fate of the extravasated EB was studied separately with regard to its dye and albumin moieties [10]. Comparing the visual and fluorescence observations on the dye with immunocytochemical demonstration of serum proteins by peroxidase-antiperoxidase (PAP) method, it was established that after some time the protein moiety of the tracer disappears, mostly due to intraglial digestion, whereas the EB dye becomes attached to the cellular constituents and remains in the tissue for a considerable period of time. The uptake and lysosomal digestion of extravasated proteins by astrocytes and to a lesser degree by microglia coincided with significant reduction in the water content of the previously edematous tissue, as could be ascertained by specific gravity measurements. Thus, a hypothesis can be formulated which suggests that the resolution of the vasogenic brain edema is primarily due to removal of extravasated serum proteins from the extracellular spaces, and this re-establishes the normal osmotic balances and water movement relationships between blood and brain by releasing the water previously bound by proteins, allowing it to diffuse away.

The possibility of tracing the extravasation of the animal's own serum proteins by immunocytochemical method (PAP) allows us to obtain a closer insight into BBB disturbances characterized by chronic, intermittent openings of the barrier. In the stroke-prone spontaneously hypertensive rats (SPRSH) the PAP method revealed in animals, some of which showed no clinical symptoms, an extensive spreading of extravasated proteins throughout the white matter [3]. In the adjacent areas of the gray matter, such as the lower cortical layers, there was a striking localization of serum proteins in the cytoplasm of the neurons, which with Nissl staining revealed no obvious abnormalities. Testing the vascular permeability in these rats by a single injection of EB and sacrificing them one hour later revealed either no BBB changes or only a few, small foci of the EB extravasation. Such chronic leakage of serum proteins recognizable only by immunocytochemical methods is likely to account for the vasogenic brain edema which appeared to be present in the white matter. On the other hand, a remarkable presence of serum proteins in the cytoplasm of the neurons seemed not to have any appreciable adverse effect.

Besides the extravasation of serum proteins which may cause retention of water in the tissue, an abnormal leakage of other plasma constituents, such as exogenous biogenic amines (e.g., in cerebral ischemia) might conceivably exert some effects on the brain parenchyma. Otherwise, the brain tissue may suffer from a deficit in some essential substance due to faulty transport across the endothelial barrier. This was shown to occur by inhibition of the facilitated transport of a particular compound due to an excessive accumulation of related other compounds which compete for the same carrier system. Such situations have been described in inborn metabolic errors, such as in galactosemia characterized by enzymatic deficiency, an excessive accumulation of galactose and its metabolic derivatives associated with a competitive inhibition of glucose transport resulting in adverse effects on brain function [8, 15]. Similarly, in phenylketonuria due to deficiency of phenylalanine hydroxylase [6, 23] there is an excessive accumulation in the blood of phenylalanine which, by drastically changing its concentration ratio to other essential amino acids, inhibits their transport into the brain. In maple syrup disease, a condition associated with inborn aminoaciduria, a deficient metabolism of leucine, isoleucine and valine leads to interference by these excessively accumulated compounds with the transport of other important amino acids [14].

In the differentiated response of complex mechanisms involved in the pathological opening of the BBB a "latent" period, i.e., time between an insult and an abnormal passage of a substance, appears not to be constant but to reflect the principle of the "maturation phenomenon" [9]. According to this phenomenon, the maturation or the progression of injury, expressed in various parameters, is related to the intensity of an insult, a lesser intensity resulting in slower development of the lesions. In cerebral ischemia the maturation phenomenon was described with regard to extravasation of serum proteins [5], and the well-known delay in clinical appearance of positive scanning using radioactive protein tracers may be related to a gradual maturation of BBB injury in these patients. As explanation of the maturation phenomenon one can speculate along the following lines: An insult might affect the synthesizing machinery of a cell. When this becomes impaired - and the degree of impairment would be proportional to the intensity of an insult a cell, such as the endothelial type, will be able to carry on for some time with existing supplies of enzymic and structural compounds. It is only when with increasing deficit the supplies become almost totally exhausted that a cell will succumb, and this will occur after a delay the length of which will be related to the degree of impairment of the synthesizing machinery.

In conclusion, it can be stated that the elucidation of changes in cerebrovascular permeability related to a complex pathophysiology of cerebral endothelium, should contribute essentially to better understanding of many brain disorders.

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# SPECULATIONS ON FUNCTIONS OF THE BLOOD-BRAIN BARRIER

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Professor Crone, who has himself contributed so much to this field, has honored me by asking me to speculate on the teleology of the blood-brain barrier (BBB). Accepting such an invitation is immediately presumptuous, because it implies that we are capable of understanding the teleology of the organism. Perhaps we can lessen this presumption by assuming that there is some reason for the entire organism to be in existence and that we are simply studying a submechanism within the organism which makes the entirety work more efficiently.

## INTRODUCTION

The BBB refers to the uniquely selective permeability of the capillaries of the central nervous system (CNS). In the remainder of the body, the walls of capillaries are freely permeable to all small molecules (less than 20,000-40,000 daltons), but brain and spinal cord capillaries demonstrate a wide range of selective permeabilities to these molecules. This permeability is based almost entirely upon specific molecular configurations and hardly at all on molecular size.

In the entire body, about one-fifth of the tissue volume is extracellular fluid (ECF), and of this, about one-fifth is pumped through permeable microvasculature to keep the entire ECF uniform in composition. Provided that this small fraction of moving ECF is brought close enough to the more static interstitial ECF so that efficient diffusional exchange over short distances can result, only this fraction need be moved, thus reducing the total energy required. In this way, the composition of the ECF is made uniform throughout the body and the effective diffusional distance between any two cells in the body is brought down to a few microns rather than the many centimeters which may actually separate organs. Thus the general microcirculation seems to perform a relatively simple and nonselective function of rendering the general ECF uniform, and the wall of the general capillary is not regionally effective in causing any nonuniformity in the distribution of ECF solutes. A major mechanism for specific distribution of substances in the general body appears to be highly specific receptor sites on certain target cells which can result in localized concentrations of substances originating remotely.

In the general body the major routes of exchange through the capillary wall appear to be extracellular, via intercellular clefts, and (to a lesser extent) pinocytosis. There is now widespread agreement that

these nonspecific routes are virtually absent in the brain and that almost all exchange of substances through the walls of brain capillaries seems to be transcellular, with the substances passing directly through the plasma membranes and cytoplasm of the brain capillary endothelial cell. Whether or not transcellular transport also takes place in general capillaries is not clear. Our attempts to study such transport in rabbit skin capillaries were frustrated by the efficient simultaneous nonspecific transport which exists through intercellular clefts. Presumably some substances (such as blood gases) do readily penetrate the general capillary cells through selective exchange, but we were unable to see this against the background of the large nonspecific extracellular exchange available to all small molecules.

However, the brain ECF is in a special relationship with the general ECF in that there is a selective barrier (the BBB) between the two. I shall not attempt to give a comprehensive review of the possible functions of the BBB but rather will confine myself to certain aspects of the BBB which have become of interest to us during the course of the last decade. During this time we have studied BBB permeability to approximately 200 substances, most of which have been examined under various conditions of transport competition. These studies for the most part have concerned the fractional extraction of radiolabeled test substances following carotid arterial injection during a single microcirculatory passage in the pentobarbital-anesthetized rat. After carrying out such studies on approximately 10,000 experimental animals, we feel in a position to make some generalizations, one of which is that the BBB evidently performs many functions.

## SPECIFIC POSSIBLE FUNCTIONS

### Exclusion of Blood-Borne Toxins

The first suggestion one might logically make concerning the BBB's function would be that it protects the brain from potentially toxic substances in blood. A good example of such a substance is bilirubin, which is strongly bound to plasma protein and thus is kept out of the brain even when present in the blood in high concentrations during clinical jaundice. Bilirubin is lipophilic and neurotoxic, and keeping it out of the brain clearly serves a useful function. The occasional patient with jaundice in later life is presumably made less symptomatic because of this, and the brains of infants with neonatal jaundice are also protected against bilirubin in most cases.

### Systemic Neurotransmitters

The BBB is highly impermeable to the known centrally active neurotransmitter agents such as norepinephrine. These agents may undergo strikingly rapid transient changes in concentration in the systemic blood, but the presence of the BBB prevents these wide fluctuations from being transmitted into the brain ECF where they could bring about unwanted changes in CNS activity. I would expect, for example, that if an individual were to jump into cold water, the resulting burst of systemic norepinephrine might result in a disabling cerebral excitation if there were no BBB.

Conversely, the impermeability of the BBB to central neurotransmitters serves to prevent their loss to blood plasma after their synthesis within the brain. This not only conserves these

neurotransmitters but confines them near their site of origin. For example, norepinephrine released into a cortical synaptic cleft, is removed by several mechanisms, such as diffusion from the synapse and re-uptake into the nerve ending. If the BBB were freely permeable to norepinephrine, the blood (which would now be acting as a sink) would become an additional major source of removal; it would compete for nerve ending re-uptake and require the formation of a considerably greater amount of norepinephrine. After passing into the blood, the norepinephrine might also reappear elsewhere in the brain. Then the ability of the brain to restrict its neurotransmitter activity to small discrete regions would be impaired.

An analogous situation would be a household in which the occupants communicated by olfaction. If the house were of an open frame construction in which external wind blew through freely, then the olfactory substances necessary for communication would be blown away, perhaps spreading to other parts of the house en route to the outside. A great deal of these olfactory substances would then be required for communication in the face of this great loss. If the turnover of air in the house were optimally low, however, it would be possible to achieve high local concentrations of the communicating substances with production of only a relatively small amount of each.

Modulation of Substrate Entry by Carrier Transport Systems

The BBB contains a number of specific transport systems which exhibit saturation, thereby limiting the rate at which metabolic substrates in plasma can enter brain. These carriers facilitate the entry into brain of polar metabolic substrates which would otherwise not be able to enter. The Km's of most of these appear to be approximately that of their respective concentrations in blood plasma. (This is similar to most of the enzymes in the body, whose substrate concentrations also approximate their Km.) Thus the BBB transport systems serve very efficiently to stabilize the rate of flux entry in the presence of changes in substrate blood levels.

The entry of glucose into brain is almost entirely carrier-mediated and is held relatively constant in the presence of hyper- or hypoglycemia. Lactate's penetration of the BBB is accelerated by a carrier shared by several other short-chain monocarboxylic acids. The level of blood lactate may fluctuate greatly in brief periods of time; a substantial rise can occur, for example, after bursts of extreme muscular activity. It would seem advisable that such rapid fluctuations not be transmitted into brain without some modulation. The short-chain monocarboxylic acid BBB carrier is about half saturated in the presence of usual blood lactate and pyruvate levels. In the presence of a sudden elevation of blood lactate, we can presume that the rate of entry of this lactate into brain ECF is less than proportional to the plasma-brain concentration gradient and that the brain is partially protected from the full lactate influx.

Brain Capillary Endothelial Cell Enzymes

The requirement that substances passing from plasma to brain pass through the brain capillary endothelial cells exposes them to the cytoplasmic enzymes of these cells. For some substances, this may enhance the effective BBB by making substances which get through the inner endothelial cell membrane more polar (by cytoplasmic enzyme actions) and thus preventing them from passing through the external endothelial cell membrane. Such a mechanism is probably effective with the monoamine

transmitter agents, which are oxidized to an unknown extent within the endothelial cell cytoplasm and whose overall rate of penetration through the BBB is thereby impaired. In such cases, there is an enzymatic enhancement of the BBB. Since monoamine oxidase is largely confined to the surface of mitochondria, the large number of endothelial cell mitochondria in brain makes these cells several times as effective as sources of monoamine oxidase and thus should further enhance the enzymatic component of the BBB.

While these substances are in the cytoplasm, there may be metabolically useful enzymatic transformations, as in the case of dihydroxyphenylalanine (DOPA), which is probably converted (to a substantial degree) to dopamine. This DOPA is then free to diffuse either into the blood or the brain ECF. That dopamine which diffuses into the brain results in a general level of extracellular dopamine which might not readily be obtained by intracellular CNS decarboxylation. Such an endothelial cell enzymatic mechanism may enable the BBB to provide brain ECF with potentially useful metabolic intermediates. Endothelial cytoplasmic dopamine resulting from decarboxylation no longer has an affinity for the BBB neutral amino acid carrier; thus its rate of movement in either direction is greatly reduced relative to DOPA.

The Large Apparent Metabolic Work Capacity of the BBB

It has been shown that the mitochondrial content of the brain capillary endothelial cell is approximately four times that of non-neural capillary endothelial cells; approximately 10-11% of the cytoplasm is mitochondria in brain and spinal cord capillary endothelial cells, and about 2.5-3% in other tissues. The BBB's relatively large mitochondrial content suggests that it is capable of a correspondingly larger workload. Since the workload would be approximately four times that of the usual general capillary, it suggests that about one-fourth of the workload of the brain capillary cell is shared by the general capillary cell and three-quarters is not; this excess may well be concerned with pumping ions in and out of brain ECF.

There would be no point in having a mechanism within the wall of the general capillary which would attempt to create a concentration gradient across this wall. Any such gradient would immediately be abolished by diffusion through the nonspecific routes in the capillary wall. Where such nonspecific diffusion does not exist, however, such as in the BBB, it would be possible to maintain a concentration gradient. The logical place for the pump required to create and maintain this gradient between blood plasma and brain ECF would be within the capillary wall, since this is where the gradient occurs. In the case of potassium ion, for example, the concentration of ECF in brain is approximately 40 percent lower than in blood plasma. This undoubtedly serves to hyperpolarize and thus stabilize the neuron. To maintain this gradient, which must be across the capillary wall, requires the expenditure of energy, and this could well be one of the functions being carried out by the BBB's apparent excess of mitochondria.

The BBB's apparent excess work capacity could also be related to the apparent capability of the brain parenchyma to generate some cerebrospinal fluid (CSF). Although it is generally believed that most of the CSF is formed by choroid plexus under normal circumstances, several workers have shown that under experimental (and thus abnormal) circumstances, the brain

parenchyma is evidently capable of generating some measurable CSF. Some of the BBB's energy may be used to pump sodium into the brain ECF and thereby create the ionic imbalance necessary for CSF formation.

The carotid injection of hyperosmotic solutions results in a transient loss of the BBB, presumably by some effect of the osmotic shock on the capillary endothelial cell. This happens in a clinical setting when patients are subjected to the rapid carotid injection of approximately 1.6 molar solutions during cerebral angiography. There is no obvious change in cerebral function, suggesting that a brief (few hours) interruption of BBB function is not greatly deleterious to cerebral function. It suggests further that the benefits derived from having a BBB are long-range, such as protecting brain from growth hormone and other hormonal effects.

How is the BBB Maintained?

It is interesting to speculate on the mechanism by which the BBB is maintained. How does a capillary cell know that it is in brain and that it should alter its fine structure in a way which will result in the BBB? It is attractive to propose that the CNS generates some humoral influence which causes nonspecific trans-endothelial transport to shut off. Such a humoral influence could be produced by the astrocytes which immediately surround CNS capillaries. The BBB appears to be a result of some ongoing function of healthy brain, since almost any significant abnormality of brain tissue results in a loss of BBB. In such lesions the unique brain capillary cell characteristics, which cause the BBB, disappear, and the lesion's capillaries come to resemble the nonspecifically permeable capillaries in other tissues. This could be the result of malfunctioning astrocytes no longer able to produce the proposed humoral influence which normally maintains the BBB by inhibiting pinocytosis, fenestra formation, and tight inter-endothelial cell junction formation. These all represent specific characteristics of the plasma membrane of the endothelial cells and could be closely interrelated. A single substance created by the astrocyte could conceivably bring about all three of these effects. How the mitochondrial content of these cells is increased is quite beyond my speculating abilities, however.

BBB as a Second-Order Homeostatic Mechanism

Each of the many specialized cells in the total organism contributes something toward the optimization of the general ECF, and their concerted action results in a considerable homeostasis of the ECF in the presence of a changing external environment. Thus the internal temperature, pH, osmolarity, glucose concentration, oxygen concentration, etc. are held within narrow optimum limits despite changes in these parameters in the immediate environment of the organism. The neuronal environment appears to enjoy a substantially higher degree of homeostasis than does the general ECF and, in several instances, optimization is at a different concentration from the general ECF. The BBB appears to offer a second-order homeostatic mechanism further optimizing the neural extracellular fluid environment as an ultrastable subcompartment of the general ECF.

# INDEX

The page numbers refer to the first page of the article in which the index term appears.