PHYSIOLOGICAL CONDITIONS EXISTING IN CONNECTIVE TISSUE

II. The State of the Fluid in the Intradermal Tissue

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The findings of the preceding paper indicate that substances of large molecule extend through connective tissue along connective tissue fibers (1). A vital dye, escaping from the lymphatics of the ears of living mice, could first be seen outside the channels as bristly lines of color extending from them apparently along the fibers. These lines of color can be bent and twisted by pressure with a micro probe and spring back to their original positions when the pressure is removed, as if the dye were fixed upon or between the fibers. Its subsequent movement seemed to be determined by their form and structure. The removal of body fluid from the animals by bleedings did not change the way in which the dye extended. By contrast it was completely changed by the presence of free fluid or edema in the tissues. Dye escaping from lymphatics into edematous tissue took the form of a diffusely colored band easily movable in the edema fluid when pressed upon by a micro probe.

Because of these facts, and because our observations failed to disclose the presence of free interstitial fluid in normal connective tissue it seemed important to study the movements of other dyes through normal tissues and those containing free fluid. Will dyes, differing in diffusibility and in chemical constitution from the one already employed, show the same phenomena in passing through the tissues; that is to say, will they take the same path, along the connective tissue fibers? Can fluid be demonstrated in normal connective tissue by means of highly diffusible dyes? Will mechanical forces which rub or squeeze the fibers together, enhance the spread of dye in the tissues? These questions were answered by experiments now to be detailed.

Methods

The methods employed for the present work were like those described in the preceding paper. Besides the vital dye pontamine sky blue a more diffusible dye, patent blue V was used. The nature of this dye and its preparation will be discussed below. In addition to the vital dyes two indicator dyes, brom thymol blue and brom cresol purple, were chosen. These indicators differ profoundly from the vital dyes in chemical constitution; they are far more diffusible than the latter and further are highly irritating to the tissues when used in strength, eliciting a prompt and marked edema. They were chosen because of the pH range of their color change. Properly adjusted they can change color within the tissues from blue or purple to buff. The thought suggested itself that these dyes, injected into the lymphatics and escaping through the tissues would practically disappear in the latter after changing to a buff color, but not before their irritant action had changed the fluid content of the normal tissue inducing an edema. At this stage dye in the blue form could be reinjected into the same lymphatics and its passage through the changed tissues observed. To obtain the indicators in such a form that they were capable of changing color, from purple or blue to buff, within either the lymphatics or the tissues, they were made up in 4 per cent aqueous solutions. These solutions, as shown by freezing point determinations with the Beekman apparatus, were isotonic with blood. Sufficient N/10 NaOH was then added to bring the pH of the brom cresol purple solution to 6.3 and that of the brom thymol blue to 7.0, with result that both were approximately 50 per cent dissociated. Very little NaOH was required. The solutions were injected intradermally into the ears of the anesthetized mice in the usual manner (1) and appeared promptly in the lymphatics.

The Effects of Free Interstitial Fluid upon the Movement of Dye through the Tissues.—As described in the previous paper, the vital dye pontamine sky blue after escaping from the lymphatics into the tissue, called out free fluid into the latter within a few minutes. The bristly colored projections of escaping dye disappeared and the diffuse blue color that took their place could be moved about at will by micro probes. The influence of the presence of free fluid in the tissues was much better shown by the experiments done with the two indicator dyes.

A description of some typical findings with brom thymol blue will suffice. In less than 1 minute after injecting it as a blue fluid into the lymphatics typical blue hair-like projections appeared outside the channels. They maintained their contours even when bent and twisted by the micro probe. Almost at once, however, diffusion from them began and within a minute and a half after dye first ap-

peared outside the lymphatics, the colored projections showed the "second phase" of dye escape (1), that is to say, diffuse staining between them which could not be dislodged by the micro probe. Within another 2 or 3 minutes the color could easily be dislodged and the hair-like projections were scarcely visible. The irritant indicator had rapidly produced a local edema, as shown by the demonstration of free fluid. In another minute and a half, or even less, the diffuse blue coloration turned to buff. The color within the channels changed as well,—a phenomenon to be taken up in future studies.

In 44 experiments the lymph channels were gently reinjected with more of the indicator in the blue form after the change to buff had taken place outside, and now within a few seconds dye passed through the channel wall to form an evenly colored blue band outside it, with little or no indication of the hair-like projections as the dye spread outward through the tissue. Later the band turned buff. Pressure with the micro probe, exerted at once after the appearance of the blue color outside the channels, left a clear, colorless spot showing that the dye had been dissolved in the free fluid already present in the tissues which was squeezed away by the pressure. This occurred even before the blue band turned to buff. The free fluid induced by the previous injection had completely changed the state of affairs as concerned the movement of the dye. The experiments excluded all possibility that the appearance of the bristly and wavy colored projections, following dye injection into lymphatics, were due to the very slight pressure exerted when introducing the fluid. For dye was reinjected into the lymphatics at the same pressure in all of the experiments but its manner of transport through the tissues differed.

In the experiments described in the preceding paper the vital dye pontamine sky blue escaped from the lymphatics into tissue previously normal. The hair-like projections of color, seen under these circumstances, were also observed when the indicators first passed from the lymphatics into normal tissue. That is to say, these dyes, far more diffusible than pontamine sky blue and of a greatly different chemical constitution, seemed to move, like it, along the connective tissue fibers of normal tissues. When the irritant effect of the indicators had called out free fluid into the tissues in demonstrable amounts and the indicators were reinjected into the lymphatics the

bristles of color were no longer observed, as the dye, escaping from the channels, moved outward through the tissues. Clearly a change in the state of the tissues had occurred which modified the manner in which the indicator dyes moved through them.

The same phenomenon was observed in eight experiments in which 2 or 10.8 per cent solutions of pontamine sky blue were introduced into lymphatics previously injected with one of the indicators. In these tests sufficient time was given to permit the escape of the indicator into the tissues, and its color change there before the pontamine sky blue was injected. Under these conditions the vital dye, escaping from the lymphatics, appeared in the tissues as a diffuse blue cloud: no bristles of color were seen. The findings indicate that the changed state of the tissues, in which fluid had accumulated, affected the mode of transport of pontamine sky blue and the indicator dyes, of widely different constitution and diffusibility.

Evidence on the Condition of Fluid in the Intradermal Tissues.—Although, in the previous work (1), we had been unable to demonstrate the presence of free fluid in the normal tissues of the mouse ear, the possibility remained that it was present there in amounts which defy detection. The diffusion of pontamine sky blue, escaping from the lymphatics, might have been so slow that it failed to reach the free fluid in the tissues before its own irritant action had called forth an edema.

To throw light on this point experiments like those described in the preceding paper were made with another dye, patent blue V,[1] which is much more diffusible than pontamine sky blue and like it tolerated in large doses when given intravenously, and calling forth a mild edema in about the same time. It was assumed that, being far more diffusible than pontamine sky blue, patent blue V should demonstrate sooner the presence of any free fluid within the tissues.

Patent blue V[1] has been used by us in many previous studies of the physiology of the lymphatics of mice, rabbits, and human beings (2–6). Like pontamine sky blue it is an acid vital dye and does not stain the formed elements of the tissue, during the periods required for the experiments. It is much more diffusible than pontamine sky blue having a molecular weight of about 585 (7).

Preparation of Solutions of Patent Blue V.—The dye was obtained in solid form

[1] General Dyestuffs Company.

as a crude technical dye, soluble to about 1 per cent in water and toxic to animals. It was converted to a more soluble, relatively non-toxic sodium compound. A weighed quantity of dye was suspended in distilled water and heated on a water bath. N/1 NaOH was slowly added with constant stirring till almost all the dye had gone into solution. The reaction started at once and one could perceive readily when almost all the dye had dissolved. The suspension of dye had a fluorescent appearance and the dissolved one a beautifully clear blue color. The calculated amount of N/1 NaOH to be added, approximately 150 cc. for each 100 gm. of dye, was not employed; instead we added only 80 to 130 cc., for the dye was not in pure form. (This precaution was taken to avoid an excess of NaOH.) After heating the mixture for ½ hour more, it was cooled, filtered twice and evaporated to a concentrated solution. The latter was dried in an oven *in vacuo* at less than 70°C. The dried material was then extracted three times with absolute alcohol. The latter when evaporated to dryness gave a good yield of non-toxic dye.

The preparation of the dye seemed to be better when small batches of the crude material were used. As determined by the freezing point method, aqueous solutions, 11 gm. in 100 cc. of water, were isotonic with blood.

The procedure as outlined above was employed to prepare the dye solutions used in this and in our preceding work. More recently a batch of dye has been received which, in the crude form, is more soluble in water than that obtained previously. Purification of the latest batch was carried out as follows: The dye was mixed in a mortar with dry, anhydrous sodium carbonate, 10 parts dye to 1 of the carbonate. Enough distilled water was added to make a paste and the mixture ground for 5 minutes. A little more water was added and the paste ground again for 5 minutes. Enough absolute alcohol was then poured on to make a final 95 per cent alcohol mixture; that is to say, the water previously added representing 1 part, 19 parts of absolute alcohol were added. After shaking in a mechanical shaker 20 minutes, the alcohol was filtered off through two layers of paper. The alcoholic solution evaporated to dryness yielded seemingly pure dye. This dye has not yet been used for biological experiments and it is suggested that others, employing patent blue V, may find other methods of purification necessary.

Aqueous, 11 per cent, solutions of patent blue V, isotonic with blood, were made up, and subsequently diluted with equal parts of Tyrode's solution or Locke's solution. The resulting 5.5 per cent solution had a tinctorial value about like that of a 2 per cent solution of pontamine blue and elicited edema in the tissues in about the same time. It was in approximately equimolecular concentration with a 10.8 per cent solution of the latter.

Observations like those described in the preceding paper were now made with both the diffusible patent blue V and the indiffusible pontamine sky blue. In escaping from the lymphatics and moving through the tissues both dyes showed the same sequence of events,

already described as observed with pontamine sky blue. As will be seen below, only some of these events, but not all of them, occurred in shorter time when the more diffusible dye was used.

The 5.5 per cent solution of patent blue V, prepared as described, was injected into the lymphatics of the ears of 60 mice. With a stop watch the intervals were recorded at which the bristly colored hairs first appeared as also that at which they were first seen to broaden. In addition the time was taken at which a homogeneous blue color appeared between the bristles but could not be dislodged by prodding (the second phase), and the moment was recorded when the color was first found freely movable in the tissues, indicating the presence of free fluid in demonstrable amounts.

The lymphatics in the ears of 20 mice were injected with a 2 per cent solution of pontamine sky blue, and its movement through the tissues watched in the same manner. This dye solution possessed about the same tinctorial value as the patent blue V solution and the experiments served as controls.

We wish to stress at the outset that the judgments required for an experiment of the nature described were difficult and liable to subjective error. Yet in the absence of a better way to proceed, we feel the results to be worthy of consideration. They have been summarized in Table I, columns 2 and 4, together with the findings from another experiment, yet to be described. The table shows, in the appropriate places, the times at which the various phenomena were first observed in the tissues of the mid-third of the injected ears. It is to be noted that the various phenomena were often coexistent in any one ear, for example, in certain portions of an ear the second phase of dye escape might be seen while free fluid, a later event in the sequence, could be demonstrated elsewhere. This will be evident from the figures in the table.

The highly diffusible dye, patent blue V, escaped from the lymphatics rapidly. The bristly or wavy projections of color became wide sooner than in the control experiments, no doubt because the highly diffusible dye moved away from the bristles more rapidly than did the poorly diffusible one. Very shortly after injection one could see homogeneous coloration between the blue bristles, which resisted displacement by pressure; there existed in other words the second phase of dye escape. But freely movable color, indicating the presence of free fluid, did not appear much sooner than in the controls with pontamine sky blue. This observation indicates that there

was no demonstrable amount of free fluid in the normal tissue which had remained unrecognized in the control experiments, and in our earlier tests, too, because of the indiffusibility of the dye pontamine sky blue.

TABLE I

The Time at Which the Phenomena of Dye Escape Occur, as Influenced by an Irritant in the Dye Solution and by Diffusibility of the Dye

	Poorly diffusible dye (control experiments)	Poorly diffusible dye (after addition of irritant)	Readily diffusible dye (no irritant)
Injected fluids	2 per cent pontamine sky blue	2 per cent pontamine sky blue plus ammonia water	Patent blue V
	min.	*min.*	*min.*
First appearance of colored bristles	Average: 4½ Extremes: 2–7	2 1–3	1¾ ¾–3
First definite widening of colored bristles	Average: 7½ Extremes: 5–10	4½ 3–7	3½ 3 –4¾*
Second phase (first appearance of diffuse color between bristles; color cannot be dislodged by pressure)	Average: 7¾–13⅓ Extremes: 5–15†	In only one of 20 instances could this phase be identified	4 –7½ 3½–13‡
Free fluid (first demonstration of freely movable color)	Average: 10½ Extremes: 8–14½	5⅓ 2¾–9⅓	9¼ 8 –10§

The data of the table are explained in the text. In the control experiments (second column) the sequence of events took place more slowly than in tests in which 10.8 per cent dye solutions were used (Table I of the preceding paper).

* The colored projections became wide in a shorter time after injection than in the other experiments because of the diffusibility of patent blue V.

† This phase endures in some portions of the ear after free fluid has made its appearance elsewhere.

‡ The homogeneous coloration between the bristles was not displaced by pressure and remained just as long as in the controls.

§ Free fluid not demonstrated sooner than in the controls although the dye was more diffusible.

Another experiment was made to test the point. A tissue irritant was added to the solution of relatively indiffusible dye, pontamine sky blue, to bring free fluid rapidly into the tissues after injection of the mixture. We wished to see whether or not free fluid appearing in the tissues sooner than in the control experiments, would also be demonstrated sooner by the relatively indiffusible dye.

Varying amounts of C.P. ammonia water were added to the 2 per cent solution of pontamine sky blue in Locke's solution and injected into the lymphatics of the mouse ear in the usual manner. By trial and error it was found that the addition of 0.005 cc. of the ammonia water to each cc. of the dye solution brought demonstrable amounts of free fluid into the tissues rapidly. Intradermal injections of this mixture were made into the ears of 20 mice and its movement through the tissues watched, as first described, and compared with the control experiments.

The findings are summarized in Table I, column 3. When the ammonia in this experiment, acting as an irritant, brought fluid rapidly into the tissues, the poorly diffusible pontamine blue called attention to the presence of this fluid much sooner than in the control experiments. From this it was plain that the time required by pontamine sky blue to disclose the presence of fluid, in the preceding experiments, was not due to slow diffusion of the dye into fluid already present in the tissues, but to one of the two other possibilities discussed above: either free fluid was present in the normal tissues in amounts too small for dye dissolved in it to be visible, or else it was lacking.

The Spread of Dye through Tissue as Influenced by Slight Intermittent Changes in Pressure

The findings so far described in this and in the preceding paper suggest that connective tissue fibers play, indirectly, an important part in the transport of certain substances through resting tissues. Dyes of differing diffusibility and molecular size seem to move through tissues along or next to the fibers, but not as a diffusely colored cloud. The fact that free fluid of the connective tissues is present only in small amounts, or is lacking adds to the importance of the fibers as a pathway of interstitial transport. This being so, changes in pressure which rub or squeeze the fibers together should spread dyes more rapidly through the tissues. The mechanical effects of pulsation of blood vessels within a tissue increases the movement of fluid through it, as shown by increased lymph formation (8), and enhances the spread of dye as well (6). That slight changes in external pressure increase the interstitial spread of dyes has also been shown (5).

Attempts were next made, under high magnification, to find out what really happens when dye spreads through tissues subjected to slight intermittent changes in external pressure.

A tambour was constructed, as described elsewhere (5). After injecting the lymphatics with pontamine sky blue in the usual way, the ears of mice were placed over the tambour, between it and a glass cover slip for observations under the microscope.

After the bristly projections of color extending from the lymphatics became visible, intermittent pressure, in controlled amount, equivalent to a column of water 2 to 8 cm. in height, was brought to bear upon the ear by the tambour. Pressure was exerted for 1 second, with a period of relaxation of 2 seconds.

With each squeeze by the tambour the closely interweaving projections of dye could be seen bending, twisting, and bearing upon one another. They extended outwards with great rapidity and yet when the pulsations of the tambour were stopped and pressure was made upon them with the micro probe, the color could not be dislodged. This state of affairs lasted during only a minute or so of the intermittent pressure. Then a sequence of events took place like that already described in the preceding experiments, but more rapidly; the contour of the hair-like projections became less clear and free fluid became demonstrable in the usual way.

After more than one minute of intermittent pressure, the colored projections of dye could no longer be seen; and now when pressure was discontinued and the tissue prodded, the color was found freely movable, demonstrating the presence of free fluid.

The experiments showed that mechanical pressure, like that of massage, but far more mild, not only increased the rate at which dye spread through the tissues, but spread it through them in bristly or wavy colored lines. The path of dye movement seemed to be along or between the connective tissue fibers. The combined action of the dye and of the pressure changes brought free fluid into the tissues sooner than usual.

The Interstitial Movement of Dye Merely Brought in Contact with Connective Tissues

In the experiments so far described the passage of dye through the tissues was studied only after its escape from lymphatics while it moved in a direction opposite to that presumably taken by the fluids which form lymph. What can be said about the movement of dye that is not injected but merely brought in contact with connective tissue?

A method, described in an earlier paper (5) was available by which minute portions (0.0001 to 0.00005 cc.) of the isotonic 2 per cent solutions of pontamine sky blue were brought into contact with the tissues, through micro pipettes inserted into tiny micro puncture wounds. The fluids passed in by capillarity, no pressure being exerted, and formed little spots of dye, micro maculae as we have termed them (5, 6). Scores of micro maculae were made and their margins observed at high magnifications. Only a few colored hair-like projections were ever seen at the margins of these spots and then only for the first minute or so after the dye was instilled.

The advancing edge of color had a very different appearance from that of dye escaping from a lymphatic. The reason for this was not far to seek. In the experiments in which the lymphatics were injected, minute amounts of dye passed slowly into the tissues, accompanied by very minute amounts of water at most. When micro maculae were made the dye was present in a relatively large amount of free fluid, a state of affairs like that when it was present in edema fluid. Further, the dye itself causes edema, so that a local collection of edema fluid occurred at the margins of the maculae. Under such circumstances no bristles of color were to be seen, nor could they be expected to form. The presence of free fluid was demonstrable at all times by displacing the color with micro probes.

In other experiments of the same sort the intermittent pressures were brought to bear after dye maculae had been introduced into the tissues in the manner described. As already stated, their margins showed no signs of bristling, hair-like projections of color. But when pressure was exerted intermittently upon them, blue, hair-like projections suddenly made their appearance and were then seen to bend and rub together with each pressure change. As in the experiment just described, the blue projections elongated with great rapidity, while retaining their contours. But in less than a minute they faded from view, the color having become a displaceable cloud. If the intermittent pressure was stopped sooner, that is to say before the hair-like projections disappeared, this secondary change took place later.

These experiments with the tambour indicate that the rôle of the connective tissue fibers, in promoting dye spread, is even greater in tissues subjected to changing external pressure than in resting tissues.

DISCUSSION

The findings reported here confirm and extend those given in the preceding paper. There it was shown that the vital dye, pontamine sky blue, moves interstitially through the connective tissue of the mouse ear along or between the connective tissue fibers. Here it has been demonstrated that other dyes, differing from pontamine sky blue in chemical constitution and in diffusibility, apparently move through the normal tissues of the ear in a similar manner, that is to say along or between the connective tissue fibers. Dye, spreading through the ear more rapidly than usual, under the stress of changing external pressures, also seems to move along or between the fibers as they are bent and squeezed upon one another by the pressure.

Experiments designed to demonstrate the presence of free interstitial fluid have failed. This being so, the function of connective tissue fibers as pathways of interstitial transport becomes of greater importance.

What can one infer about the movement of other substances or of water through the tissue? What does our work show about the state of interstitial fluid or about the nature of the interstitial spaces? Do the latter indeed exist? To discuss these questions it will be necessary to recall from time to time evidence which has been presented recently in several papers from this laboratory.

First it may be recalled that pontamine sky blue (mol. wt. about 990) and patent blue V (mol. wt. about 585) are acid vital dyes. They do not become fixed upon the formed elements of the tissues during short periods of time such as were employed in our experiments. One of the experiments reported here showed that the diffusible dye spread more rapidly through the tissues of the mouse ear than the indiffusible one, a fact supporting the view that their spread through the tissues is probably like that of other molecules of the same size and of similar physicochemical constitution.

From the behavior of the dyes we can make no attempt to judge of the movement of fluids; it may be faster or slower than that of the colored particles or even in the opposite direction. For example, we cannot say whether or not fluid moved outwards from the lymphatics as the dye escaped from them. On the other hand, we can judge

from the behavior of the particles whether or not there is more or less water in the tissues and whether the water can move freely in them. This point need not be labored here—it has been amply discussed in the body of the paper. The presence of visible amounts of fluid stained blue by the dye could be recognized by exerting pressure on the tissues with the micro probe, the colored fluid moving interstitially as result.

But why do dyes travel along or between fibers? Are they adsorbed on them? Is there an interstitial space about each fiber,—a crack, so to speak,—between it and an interstitial ground substance? Is there a relatively thick layer of water about the fibers or is there a thin film? Or is there merely an interface between two adjacent surfaces? Adsorption of dye, or of other substances of large molecule, upon the fibers will not explain all the observed phenomena. If it occurs, it must enhance the rôle of the fibers in the transport of substances through tissues, as will be seen below.

It is universally agreed that the appearance of tissues in fixed microscopic sections is misleading. The spaces which appear between the formed elements are produced chiefly by shrinkage in the process of dehydration. All workers using methods of microinjection have been struck by the fact that the connective tissues resist the introduction of fluids under pressure and behave as though the cells and connective tissue fibers were imbedded in a continuous ground substance. In this laboratory we have frequently observed that the tissues of the ear of the mouse offer a strong resistance to the injection of dye solutions through a small pipette (0.5 mm. in external diameter and about 0.2 mm. in bore). Fluid under slight pressure does not enter from the pipette until the latter is partially withdrawn, leaving a space previously occupied by its shaft. Dye solution then rushes at once from the pipette to fill this space, but as soon as this has happened resistance is encountered again. If the pipette is once more partially withdrawn, more dye promptly escapes into the newly formed space. As result there appears in the tissue a dye-stained track looking like that obtained when a wire dipped in dye solution is thrust into a block of gelatin or agar and rapidly withdrawn. The concept of wide tissue spaces like those seen in microscopic sections, but filled with free fluid, is inadequate to explain these phenomena.

During the present work suggestive evidence has been found of the presence of a matrix in the interstices between the formed elements. The possibility of the existence of such a matrix has been fully discussed by others (9–13). In most of our experiments dye, after appearing as discrete, hair-like projections between or upon the connective tissue fibers, spread from them and colored the neighboring tissues a diffuse blue. The fact that pressure over these diffuse blue areas failed to squeeze away the color showed that it was not dissolved in free fluid. When pressure was brought to bear the paling in color that took place was like that seen *in vitro*, under the microscope, when blocks of agar containing the dye are pressed upon, and in this way thinned. The change was seen in the ears whether diffusible or indiffusible dye was used. Furthermore the interval elapsing between the time of injection and the moment at which colored free fluid became demonstrable was the same whether the diffusible or indiffusible dyes had been employed. Had free fluid been already present in the tissue, it would have become demonstrable sooner with the highly diffusible dye capable of moving into it more rapidly. An irritant, ammonia, called out fluid so quickly that the stage of diffuse staining, not due to free fluid, the second phase, so called, was not recognizable. From this observation we can conclude that the diffuse blue coloration with no demonstrable free fluid, to be noted under ordinary circumstances, was due to dye distributed through some sort of matrix which could not be displaced by pressure.

It is conceivable that our method is adequate to demonstrate fluid only when edema exists. Since the method can and does demonstrate microscopic amounts of fluid, the objection, if valid, serves to emphasize a point we wish to bring out in these papers, that at most only submicroscopic quantities of fluid can be free in the tissue. If interstitial pools of fluid actually exist, through which there is a movement of fluid, they must be far smaller than the usual histological section would lead one to believe, so small as not to be demonstrable on sensitive test with a dye. How does fluid move through tissues if not through spaces? The rôle of connective tissue as a pathway for the spread of dyes has been stressed. It is possible that fluid movement through tissues may take place between or surrounding connective tissue fibers and cells like the dye movement, perhaps in thin

films, so thin that the water or fluid is no longer free in the usual meaning of the word, but is held to the fiber firmly by surface forces. Something of this sort will account for the finding that dye cannot be dislodged by pressure from its position along or between the fibers until its presence has called forth excess fluid, that is to say an edema. The state of tissue fluid may very well be analogous to that of a film of water caught between two pieces of glass, to all purposes captured, unable to move freely this way or that, but still chemically capable of behaving as fluid,—to diffuse into cells, to transport ions, to permit the exchange of solutes through it. The term "captured" has been used to avoid the concept of chemically "bound" water. The captured films of water, if they exist, must be so thin that they are practically a part of the connective tissue, not interstitial pools of fluid. This concept of fluid captured by capillary forces would explain the fact that fluid does not normally seep through the tissues and collect in the dependent portions of the limbs.

It is almost needless to point out that these remarks do not in any way contradict the known fact that 20 to 30 per cent of the body fluid is extravascular and extracellular, as shown by the work of Lavietes, Bourdillon, and Klinghoffer (14) on the distribution of sulfocyanate between the blood and tissues; by the work of Bourdillon and Lavietes (15) on the distribution of sulfate; and by that of Harrison, Darrow, and Yannet (16), Hastings and Eichelberger (17), and others (18). Our findings, here reported, yield no information on the amount of fluid in normal tissues, they indicate only that the interstitial fluid is not freely movable fluid lying in pools or lacunae, the so called tissue spaces.

It is to be noted that our studies have been made on the connective tissue of the ear of the mouse. In this tissue the presence of a large amount of extravascular, extracellular fluid can be accounted for by its existence on surfaces. As the diagrams and photographs of the preceding paper indicate poorly, the colored projections we observed are extremely numerous, constituting a fuzz on every lymphatic capillary. The connective tissue fibers about blood capillaries are equally numerous (19). The vascularization of the connective tissue of the mouse ear is exceedingly rich (20). It is important for the purposes of this discussion that the reader should inspect Fig. 10

of the article just referred to. Only from the photograph there shown can one appreciate the richness of the capillary plexuses of the ear. The millions of fibers attached to vessels in the connective tissue must present a great surface area. To what extent our conclusions are applicable to other, less vascular tissues, cannot be said at present.

We are not alone in the belief that there is no substantial amount of free interstitial fluid, to use the word "free" in the sense in which it is usually understood. Clark and Clark (21), in studies of the blood capillaries in the tails of amphibia, and in transparent chambers in the ears of rabbits, have noted that particles and cells present in the tissues outside the vessels show no brownian movement and they infer that normally there is no free fluid present. Occasionally, in states of inflammation, they have observed spaces existing about blood vessels which sometimes contained free fluid as shown by the brownian movement of particles in it.

One other point deserves mention. Earlier work from this laboratory (22, 2) has shown that blebs of dye solution resulting from the forcible injection of the colored fluid into the ear of the mouse spread chiefly from the periphery of the ear toward its base. The dye maculae of the present work also spread in this manner although they were not under pressure. This spread of the dye toward the ear base occurred in ears lying horizontally upon porcelain placques and cannot be attributed to gravity. Recently Peters (23) has referred to the work as evidence for the presence of free fluid in the tissues under normal circumstances. Formerly we too believed this to be the case, but our later studies have shown that the dyes we used elicit an edema, the condition being very different from the normal as the present work sufficiently attests.

The phenomena here reported offer an explanation for many of the findings of our previous studies (5, 6, 8) on the factors governing the spread of substances through tissues. These have shown that mechanical forces have major importance in promoting the interstitial spread of dyes and of fluids. Slight changes in external pressure yielded the most rapid interstitial spread of dye that we have ever observed (5). In quiet, resting tissue, through which dye moves in hair-like projections, the mechanical effect of the pulse increases the interstitial spread of dye (6). It also increases the formation and

flow of lymph (8), indicating that the movement of fluid through the tissues must be increased as well as the movement of dye. The appearance of edema in tissues perfused with a non-pulsating current of blood does not lead to an increased spread of dye (6) but if a pulsating flow is used dye spread is increased. In normal tissues dye spread is greater than in edematous tissue (5). In hyperemic normal tissue becoming edematous, but not yet boggy, dye spread is still greater. Large amounts of free fluid in the tissues fail to increase dye spread.

Dye spread is greatest at those times at which one can observe the extension of colored projections of dye along or between the formed elements of the tissues. Our studies on the mechanical effects of the pulse and on changes in external pressure indicate that substances move most rapidly through the tissues while the formed elements are close together and not separated appreciably by edema fluid. At such times the periodic changes in caliber of the vessels, brought about by the pulse, or the mechanical effects of changes in external pressure seem to increase the interstitial spread of dyes by squeezing together or weaving the fibers and formed elements of the tissues. It has just been mentioned that in normal hyperemic tissues not as yet demonstrably edematous but on the way to becoming so, dye spread is greatly increased (5). So too is the formation and flow of lymph (6, 8). These findings indicate that the movement of fluid from the blood through the tissues to the lymph is also enhanced at those times at which the formed elements are not appreciably separated by edema fluid. From this it would seem that the movement of the dye is probably increased by that of the fluid. This is further shown by the fact that the perfusion of edematous tissue (6) by large volumes of blood at high, unvaried pressure leads to but little dye spread and to little formation or flow of lymph (8).

It is conceivable that a perifibrillar movement of substances may be the method of supply of nutriment to tendons, structures which are notably avascular. One may even hazard a guess that the transport of nutritive substances for the central nervous system takes place to a large extent along fibers. It has long been recognized that in nervous tissue the vascular feet of the astrocytes form the connection between capillary walls and the interstitial tissue of the cen-

tral nervous system (24). Further, as shown in some of the photographs in the previous paper we have seen dye, after its escape from the lymphatics, spread out along the sheaths of peripheral nerves. These and other problems must wait for future work. Whether dyes escape from blood capillaries along fibers is now under investigation.

SUMMARY

The interstitial movement of several dyes of widely different chemical constitution and diffusibility, in the connective tissues of the mouse ear, has been observed at high magnification. Dye extension seems to be conditioned by the form and structure of the connective tissue fibers. After escaping from the lymphatics of the ears of living mice, each dye appeared first in the tissues as bristly projections of color. These bent and twisted when pressed upon by a micro probe but sprang back into place when the pressure was removed. The present work and the preceding have shown that the lines of color are formed by dye along or between connective tissue fibers.

Intermittent external pressure applied to the tissue, squeezes and bends the fibers together and greatly increases the spread of dye along them. The connective tissue fibers assume an important rôle in the spread of substances through tissues subjected to pressure changes.

The experiments have given evidence of the existence of a tissue matrix in the organ but none of the presence of free interstitial fluid. In tissue subjected to irritant stimuli and in frankly edematous tissue, free fluid is readily demonstrated. When it is present the method of extension of dye is completely changed. Dye appears in the tissues as a diffusely colored cloud which can be freely moved by pressure with a micro probe.

The bearing of this evidence upon the condition of interstitial fluid and the nature of the interstitial spaces is discussed.

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