NORMAL AND PATHOLOGICAL FACTORS INFLUENCING THE SPREAD OF A VITAL DYE IN THE CONNECTIVE TISSUE

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The pulsation of blood vessels increases the spread of vital dye through a tissue (1). Further, in promoting interstitial dye spread, the mechanical action of the pulse seems to be more important than the pressure of the blood or its rate of flow. The pulsation of blood vessels also favors the movement of fluid through tissues, as indicated by increased formation and flow of lymph (2).

The mechanism of the interstitial movement of fluid or other substances is not understood. It has seemed reasonable to assume that a study of the variations in the rate or character of the spread of vital dyes through tissues, under differing conditions which change the total fluid content of the body, may give evidence bearing upon the problem.

In the present work we have studied in living tissues, under varying physiological and pathological conditions, the spread of a vital dye, pontamine sky blue,[1] introduced without pressure, and in solution isotonic with blood. This dye is the most indiffusible one we have found and it has been employed by us in previous work on the permeability of the lymphatics and on lymph flow (1–8). The dye does not stain the formed elements of the tissues during the short time of the experiments.

Methods

The purification and preparation of the dye solution is simple. The crude dye, obtained in solid form as a sodium salt with various impurities, is dialyzed for 2 or 3 days through parchment, against hot running water. From time to time the

[1] Du Pont Chemical Company.

flow of water is shut off and after a few minutes samples taken close to the membrane are tested for chlorides. If chlorides are present the dialysis is continued against hot tap water until the test becomes negative. For 2 more days further dialysis is done against many changes of distilled water. This is carried out in the ice box to inhibit bacterial growth.

As shown by freezing point determinations, a 21.6 per cent aqueous solution of this dye was isotonic with blood. A 2 per cent solution, also isotonic with blood, was prepared by suitably diluting the strong dye mixture with Tyrode's or Locke's solution.

In earlier work (1), a method was devised by which we could bring test fluids into the superficial connective tissue without the exertion of pressure. This method, improved and modified, has been employed for the present studies. Mice of about 25 gm. body weight were anesthetized with sodium luminal, injected intraperitoneally, in 2 per cent solution, 0.125 cc. for each 10 gm. of body weight. They were then placed upon plasticine moulds so made that the animal lay with its ears spread horizontally on white porcelain plaques under the binocular microscope (9, 3). With exceedingly fine dissecting needles minute punctures were made through the epidermis into the subpapillary layer of the corium, opening a direct pathway to the tissue, and under a binocular microscope minute amounts of the isotonic 2 per cent pontamine sky blue solution in Tyrode were instilled into the puncture wounds from a micro pipette. To introduce it into the tissues the tip of the pipette was brought into gentle contact with the stab wound allowing the colored solution to drain by capillarity into the wound until it was just filled. Under these circumstances the dye promptly appeared as a superficial spot of color situated just beneath the epidermis, lying in the tissues under no pressure other than that incidental to osmosis and diffusion. As will be seen below, when first observed these spots varied from 0.8 to 1.2 mm. in diameter and were approximately 3 times larger than the puncture wounds which were 0.3 to 0.4 mm. in diameter. The dye lay in a shallow layer with sharply defined, smooth margins, which gradually paled, as the dye spread through the tissues, although the margins remained well defined for over an hour. Gradually the whole spot of dye became paler throughout. For these reasons experiments were carried on only while the margins of color were well defined. These minute pools of color will be referred to as dye spots or maculae. In size and shape they have much general resemblance to the common pigment maculae of skin.

Standard Micro Maculae.—In our first experiments scores of dye maculae were placed in the ears of mice, but one in each ear, and their spread measured as will be detailed below. We tried to make them the same size by simply holding the micro pipette against the tissue, until enough dye had entered by capillarity to yield a spot of the desired size, as observed through the binocular microscope. Despite all efforts, maculae of differing sizes developed. Later work showed that the smaller maculae spread more, in relation to their original size, than did the larger ones.

Means were sought to form maculae of the same size, containing equal quantities

of dye solution. A micro pipette, of about 0.1 mm. external diameter and 0.07 mm. internal diameter, was prepared, with an even bore near the tip. In the ears of anesthetized mice many micropuncture wounds were made under the microscope, in the usual way, and filled with dye from the pipette until the colored fluid was flush with the surface. To form the average dye spot, 0.8 to 1.2 mm. in diameter in a puncture 0.3 to 0.4 mm. in diameter, the upper meniscus of the column of dye solution in the pipette, 0.07 mm. in diameter, moved on the average 6.3 mm. toward the tip, requiring therefore a volume of 0.024 c.mm. When the subsequent spread of these dye spots was determined it was found that those measuring from 0.8 to 1.2 mm. in diameter spread with great regularity. Thereafter to form maculae of dye we endeavored to use for each spot the same amount of dye solution as indicated above, and except where specially mentioned we subsequently measured only those which fell within the limits of size that are also indicated above. Dye spots made in this way will be called standard micro maculae. Subsequently many similar micro pipettes were made and calibrated so that standard micro maculae could be used for all experiments.

Measuring the Spread of Dye.—Any method of measuring the interstitial spread of dye is open to certain objections. For example, in the ears of mice dye did not spread uniformly in all directions. Movement was greatest toward the base of the ear; it was less in both directions across the ear and least toward the tip. For this reason, in every instance we measured two diameters of each spot, the first in the direction of the longitudinal axis of the ear, that is to say from tip to base,—the other transversely in a direction at right angles to this. At intervals of 5, 20, 40, and 60 minutes after forming the dye spots, these measurements, which will be called longitudinal and transverse diameters of the maculae, were taken through an eyepiece containing an ocular micrometer scale. At the magnifications employed, the spread of the colored margin of a dye spot over one of the smallest linear divisions of the scale, which will be called micrometer units, represented an actual spread of 0.33 mm. in the observed ear.

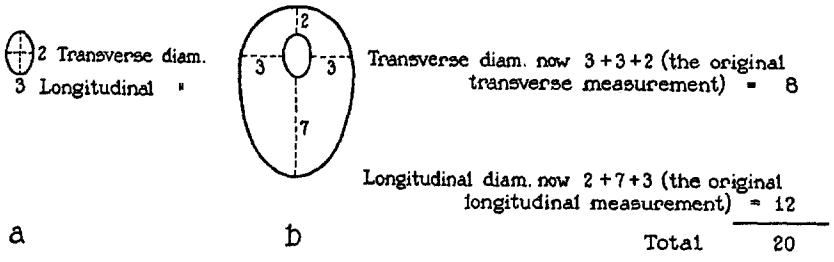
Within 30 seconds to 1 minute after the introduction of the dye and 5, 20, 40, and 60 minutes later, the outlines of the maculae were drawn with a camera lucida. The areas of the drawings were later determined with a planimeter.

We have indicated the spread of dye in several ways. Two series of charts have been drawn, the first showing in micrometer units the sums of the measurements of the diameters of the spots, the second, in planimeter units, the areas of the spots. The spread of standard micro maculae is shown by continuous lines. As will be seen below, the slope of the curves in both series of charts indicates the rate of dye spread under the varying physiological conditions studied.

In the previous work (1) the spread of dye, observed in the ears of rabbits perfused with pulsatile and non-pulsatile streams of blood, was indicated by dividing the final measurements of the areas of the spots by their initial measurements. One could thus say how many times the dye spot had increased its initial size. In the present work, for comparison, we have calculated the spread of dye in this

way too, using not only the measurements of area but also the sums of the diameters of the spots.

We have further calculated, from the measurements described above, the actual extension of dye through the tissues under differing physiological conditions, to be described below. An example will show best how this has been done. In Text-fig. 1 *a*, a small oval has been drawn to represent a greatly magnified dye spot, with transverse and longitudinal diameters of 2 and 3 micrometer units respectively, their sum equalling 5 units. In the diagram each micrometer unit, as defined above, is represented by 2 mm., the long or longitudinal diameter of the spot in the longitudinal axis of the ear, the short one in the transverse axis. In Text-fig. 1 *b*, the spot as it appeared originally is drawn within the large oval



TEXT-FIG. 1

which represents the dye spot after spreading for an hour. The total transverse diameter of the spot, which of course includes the original transverse measurement, has now become 8 units in length, adding to the original 2 units two extensions of 3 units each. The total longitudinal diameter has become 12 units in length, adding to the original 3 units, an extension 2 units long toward the tip of the ear and one 7 units long toward the base. The sum of the diameters has become 20 units. The extension of dye through the tissues, toward the four cardinal points, after the spot was first measured is represented by the dotted lines; 3 units to the right, 3 to the left, 2 upwards, 7 downwards. The sum of these (15 units) expresses better than the sum of the diameters of the entire spot (20 units) the actual spread of dye through the tissues, for it is not dependent upon the initial size of the spot. Calculations of dye spread made in this way will be termed radial spread. Radial spread can be determined by subtracting the initial sum of the diameters from the final diameter sum. Lastly, we have calculated the area of the region of tissue invaded by the color of a spreading dye spot after its formation, by subtracting the original area from the final area, as for example in Text-fig. 1, by subtracting the area of the small oval from that of the large one. The spread of dye expressed in this manner will be called areal spread. We have used this term to express the additional fact that the dye spots are rarely perfectly circular in form to begin with and that they do not, as a rule, spread equally in all directions. Were this so the area of spread could be called annular.

TABLE I

The Spread of Dye through the Connective Tissue of the Mouse Ear in 1 Hour

(2 per cent solution of pontamine sky blue)

	For individual variations see curves in charts	Initial measurement of spots				Ratio of: Final measurement of spots / Initial measurement of spots		Actual increase in size			
		Sums of diameters		Areas				Radial spread Final sum of diameters minus initial sum of diameters		Areal spread Final areas minus initial areas	
		Micrometer units	mm.	Planimetric units	sq. mm.	Final diameter sums / Initial diameter sums	Final areas / Initial areas	Micrometer units	mm.	Planimetric units	sq. mm.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
Normal luminal	2*a*, 2*b*	5.8	1.7	91	8.1	2.7	3.9	9.7	2.9	267	23.5
Normal ether (hyperemia)	3*a*, 3*b*	6.3	1.9	111	9.2	3.1	4.9	13.2	4.0	434	38.2
Normal, actively moving	4*a*, 4*b*	6.9	2.1	99	8.7	3.6	8.5	17.4	5.3	739	65.0
Bled luminal	5*a*, 5*b*	7.1	2.2	115	10.1	2.0	2.9	6.8	2.1	218	19.2
Dead	6*a*, 6*b*	6.0	1.8	92	8.1	1.8	1.8	5.1	1.5	77	6.7
Forming edema	7*a*, 7*b*	6.2	1.9	88	7.8	3.6	5.6	16.0	4.9	404	35.8
Bled edema	8*a*, 8*b*	6.2	1.9	130	11.4	2.7	3.4	12.7	3.9	316	27.8
Edema already formed	9	6.3	1.9	98	8.6	2.7	4.0	10.8	3.3	297	26.1
Dead edematous	10	6.1	1.9	118	10.3	2.9	3.3	11.5	3.5	270	23.8

Averages of the measurements of the spread of dye spots in each group of experimental animals. The data given in the table are fully described in the text. The figures in column 7 are derived by adding the figures in column 3 to those in column 9 and dividing by the former. The figures in column 8 are derived by adding the figures in column 5 and 11 and dividing by the former.

As will be described below, several groups of experiments were done under physiological conditions differing for each group. We have averaged our calculations from each group of experiments and have expressed in Table I the spread of the dye spots in all the ways mentioned above. This has been done to show that each method of calculation brings out roughly the significant differences in the spread of dye spots under the differing physiological conditions. The table should be used in conjunction with the descriptions of the experiments to be reported below. In the table columns 3 and 4 give the averages of the initial measurements of the sum of the diameters of the spots in micrometer units and millimeters. The averages of the initial areas, in planimeter units and square millimeters, are given in columns 5 and 6. In columns 7 and 8 the average increase in the size of the dye spots in each group of animals is expressed by dividing the final measurements by the initial ones. For example, the figures in the first line in columns 7 and 8 show that the spots of dye in normal animals, under luminal anesthesia, were 2.7 times larger at the end of the hour than at the beginning, as expressed in terms of the increase in diameter sums, and 3.9 times larger in area. In columns 9 and 10, the averages of the radial spread of dye spots in the ears of mice subjected to the various experimental procedures is shown both in micrometer units and in millimeters, and in columns 11 and 12 the averages of areal spread of the maculae in planimeter units and in square millimeters. It has just been noted that all the methods to calculate dye spread show a rough agreement. From this we feel that the method employed in our previous work (1) adequately expressed the differences in dye spread that were observed there.

In a few experiments spots of dye were also made with the aid of uncalibrated pipettes and their spread measured in the usual way. As already mentioned, they varied much in size. The initial measurements of some of the maculae made with uncalibrated pipettes matched those of the standard dye spots, and when this occurred the former spread at the same rate as the latter. Under similar conditions, as will be seen below, the differences in the rate of spread of maculae of equal original size, whether standard spots or those formed with uncalibrated pipettes, were never as great as those caused by alterations in the experimental conditions. As result the spread of both kinds of maculae has been plotted on some of the charts, the standard spots by continuous lines, the others by dotted lines. These charts indicate that the spread of maculae of the same size is similar.

The dotted lines have been included in the charts of this report to show further that, in the preceding work, we were justified in comparing the spread of spots of similar size but made with uncalibrated pipettes.

In earlier papers we have noted that intradermal injections of dye, when made with a hypodermic needle, are to a large extent intralymphatic (6, 7). Superficial lymphatic capillaries are torn or ruptured by the needle and dye enters them directly. In the present work, using a micro dissecting needle and micro pipettes, the puncture wound in the skin could be made in the ear of the mouse about three times out of five without rupturing a superficial lymphatic capillary. All instances were discarded in which dye placed in the puncture wound appeared in a lymphatic.

In other experiments, detailed below, in which edema was produced, droplets of fluid frequently escaped from the tissues through the puncture wound, carrying with them the dye solution. These instances too were discarded.

A single spot of dye was placed in the connective tissue of each ear and in the same relative location in all the experiments. This was done in order to keep the results comparable in every possible way, for we had observed that dye injected into the skin of various portions of the body spread with unequal rapidity. The rate was especially fast where the skin was subjected to the respiratory movement. The ears of mice were ideal to work with, for they could be kept completely at rest, while furthermore their tissues and even the finest blood capillaries were readily observable in light from a carbon arc filtered and cooled by passage through 4 cm. of Mohr's solution and reflected onto the ears by a concave mirror. The organs were constantly watched for evidence of circulatory changes and for edema. Varying degrees of reflex hyperemia followed upon the making of the puncture wounds. Under the binocular microscope, areas distant from the maculae were gently prodded from time to time with a micro needle for evidence of pitting in the skin, and at the end of the experiment this was done to the tissue immediately about them. The onset of edema could readily be seen. All findings from ears showing signs of it will be considered separately, as will those from hyperemic ears.

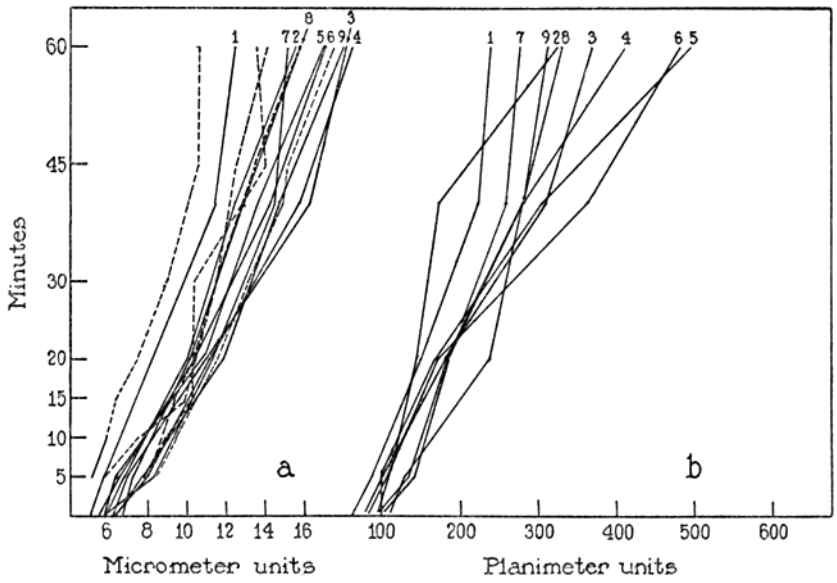
The Spread of Dye through Tissues as Affected by Changes in Their Physiological State

The spread of 2 per cent solutions of pontamine sky blue was tested in the ears of normal mice and in those of animals subjected to various procedures affecting the total body water content.

A 21.6 per cent aqueous solution, isotonic with blood, was employed, diluted to 2.0 per cent with Tyrode's solution. The spread was first measured in mice of about 25 gm. anesthetized by the intraperitoneal injection of 0.25 cc. of a 2 per cent solution of sodium luminal. In Text-fig. 2 *a* the spread of 9 standard micro maculae in the ears of the luminalized mice have been plotted in continuous lines representing the spread as shown by the sum of the diameters for each 20 minutes of an hour. The chart shows further the spread of 5 maculae which happened to be of about the same size, made under similar conditions with uncalibrated pipettes. The average of the initial measurements of the sums of the diameters of these maculae was 5.8 units and at the end of the hour 15.8 units. The size of the spots, as expressed by these measurements, had increased 2.7 times.

In Text-fig. 2 *b* the continuous lines, Nos. 1 to 9, represent the spread of the 9 quantitative maculae designated by the same numbers in Text-fig. 2 *a*, but plotted in terms of planimetric units determined as already described. The curves represent increase in area. The average of the area determinations at the end of the hour was 3.9 times that of the average of the initial measurements.

The averages of the actual movement of dye through the tissues, in these and subsequent experiments (radial spread and areal spread), are presented in Table I, columns 9 to 12. The radial spread averaged 9.7 units, or about 2.9 mm., in the experiments just described.



TEXT-FIGS. 2 *a* and 2 *b*. The spread of dye in the ears of normal mice anesthetized with luminal.

Text-figs. 2 *a*, 3 *a*, . . . 8 *a*, inclusive depict the spread of dye maculae in the ears of mice during periods of 1 hour, under the varying conditions signified by the legends. The spread has been determined by measurements of the transverse and longitudinal diameters of the spots, through an ocular micrometer scale. It is plotted in units of the micrometer scale as described in the text. The spread of "standard micro maculae" is shown by the continuous lines, that of other maculae by the dotted lines.

Text-figs. 2 *b*, 3 *b*, . . . 8 *b*, inclusive show measurements of the areas of the same standard micro maculae, the spread of which has been plotted in terms of increase in diameters in the corresponding charts of the *a* series. The areas of the dye spots were determined with a planimeter and have been plotted in planimeter units as described in the text.

Text-figs. 9 and 10 show the spread of dye maculae under the conditions described in the text for each respectively. Spread was determined as in the instances shown in the *a* series, and is plotted in units of the micrometer scale.

The Effect of Hyperemia on the Spread of Dye through the Tissues.— The depth of anesthesia varied much in the different mice, although the dose of anesthetic was the same. The maculae of dye in the ears of the animals lightly under anesthetic, and responsive to the experimental insult, spread more rapidly than in those of the animals deeply under, and unresponsive. In the former a reflex vasodilatation in the ear occurred promptly after the first prick of the micro needle, the circulating blood was brighter and the blood pressure probably higher. In the deeply anesthetized animals there was no obvious vascular response to the experimental manipulations.

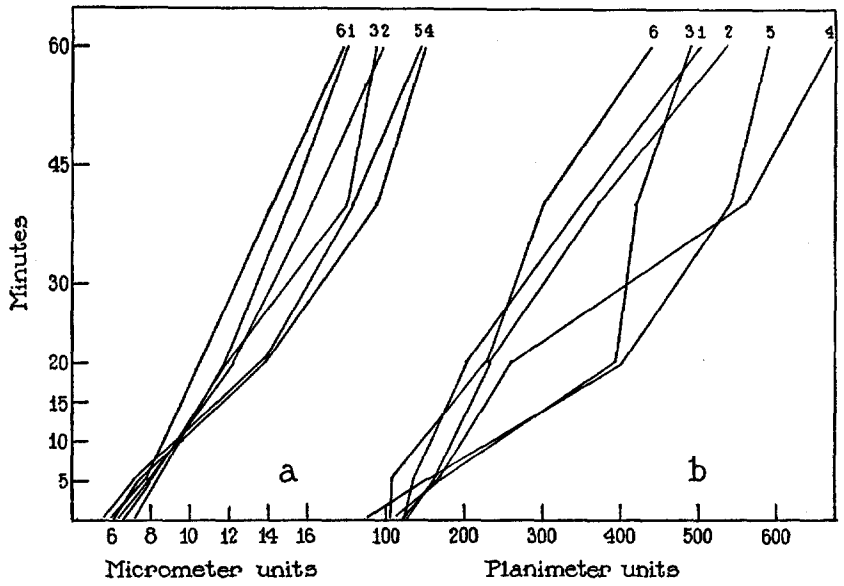
A few experiments were done to test the effect of the reflex hyperemia upon the spread of dye through the tissues.

Other work done in this laboratory for a different purpose has shown that the circulation in the ears of etherized mice is far more active than in those receiving luminal. Consequently to obtain hyperemic ears mice were given just enough ether to keep them quiet during the experimental procedures. The ears of these animals were pink, the vessels filled with bright blood, and far more reactive to the puncturing of the epidermis and the instillation of the dye than the ears of luminalized animals. An especially sharp watch was kept for developing edema, and this involved frequent prodding of the ear with a micro needle for signs of microscopic pitting on pressure. All instances showing it were ruled out. Text-figs. 3 *a* and 3 *b* show the spread of maculae of 2 per cent dye solution in the etherized animals. In Text-fig. 3 *a* the spread is expressed in terms of the increasing sums of the diameters of the maculae for a period of one hour, and in Text-fig. 3 *b* in terms of the increasing areas during the same period. The maculae spread on the average 3.1 times their initial size in terms of the diameter measurements, as compared with 2.7 times in the luminalized animals. In terms of area they spread 4.9 times (Text-fig. 3 *b*) instead of 3.9 (Text-fig. 2 *b*). The average radial spread amounted to 13.2 units or 4.0 mm. In many of the experiments edema occurred. The results in these will be considered separately.

The Effect of Movement upon the Spread of Dye through the Tissues.— In the experiments just reported the average spread of dye through the tissues was greater than that observed in luminalized mice. The slope of the charted curves is less steep. The animals, though lightly under ether, were perfectly quiet and the reactive hyperemia was intense, resulting probably in a blood circulation greater than in the animals previously observed, and at a higher pressure. Next, it became a matter of interest to see what would be the spread of the

dye through the tissues of animals that were moving about. An attempt to approach the normal conditions of movement was made in the following manner.

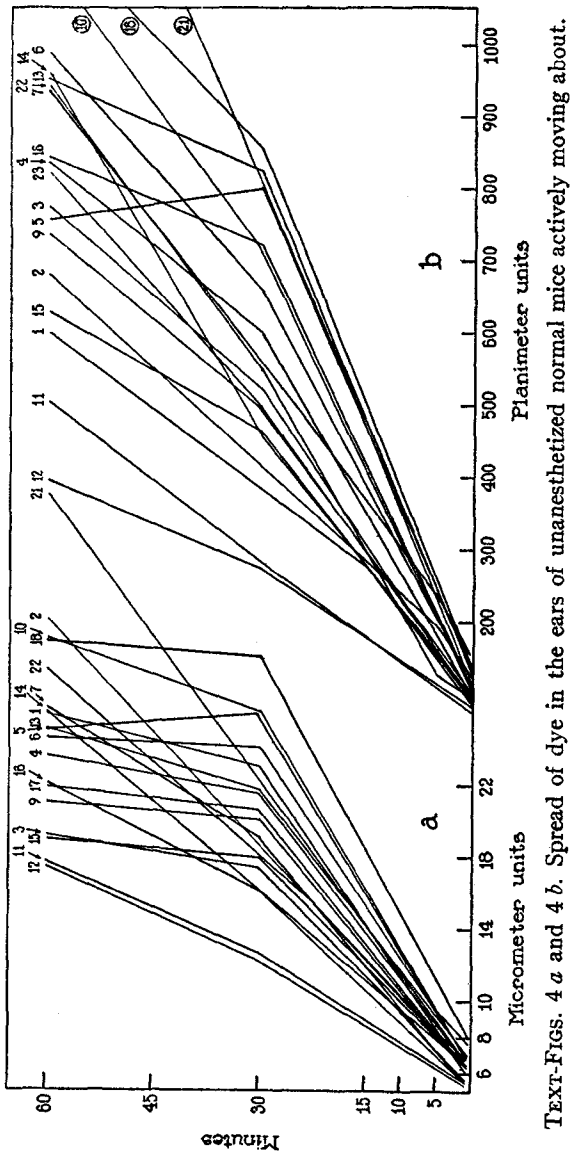
Just enough ether was given to keep the animals quiet while the 2 per cent dye solution was introduced into the skin. Ether was used so that the circulatory effects would be as much as possible like those of the mice in the experiments just described. Stiff pasteboard collars 4 cm. in diameter, were placed about the necks to prevent the ears being scratched or rubbed against the sides of the cages. As soon as the necessary measurements of the dye spots had been made, the ani-



TEXT-FIGS. 3 *a* and 3 *b*. Spread of dye in the hyperemic ears of normal mice lightly anesthetized with ether.

mals were allowed to come out of the anesthetic and within 5 or 10 minutes they were running about the cages in a normal fashion. Further measurements of the maculae of dye were taken at the half hour period and again after an hour with the administration of just enough ether to keep the mice still, while the required observations were made. In Text-figs. 4 *a* and 4 *b* the findings have been charted in the usual way.

In these animals the reactive hyperemia was as great but apparently no greater than that of the mice kept at rest under ether during the entire time of the experiment. A few showed traces of edema of the



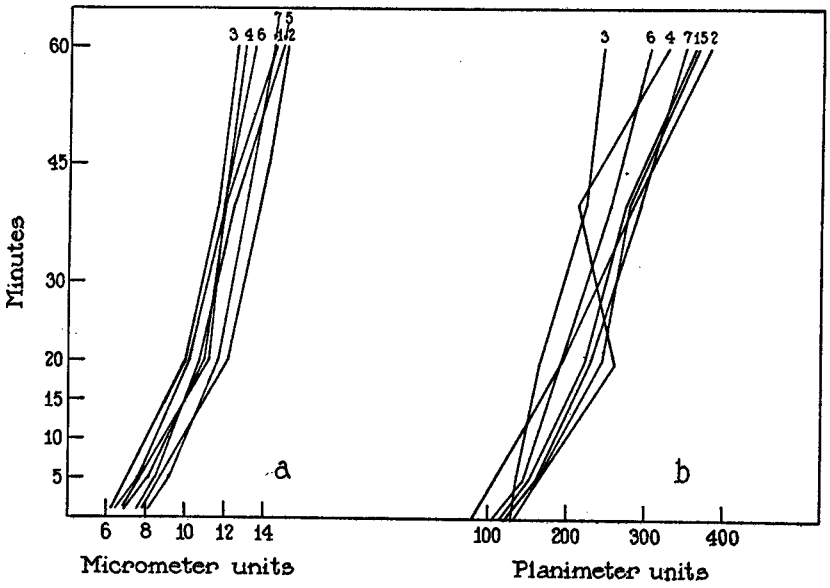
TEXT-FIGS. 4 *a* and 4 *b*. Spread of dye in the ears of unanesthetized normal mice actively moving about.

ear on prodding, though free fluid could never be demonstrated by pricking the skin. These instances were ruled out. The animals varied much in their activity after the spots of dye were placed and the results varied correspondingly from instance to instance. During the first half hour, the dye spread in these relatively normal animals was the greatest that we observed. The average of the measurements of the diameter sums increased 2.9 times after the lapse of 30 minutes and 3.6 times after a full hour. In area the spots of dye increased 5.8 and 8.5 times in the same periods. As the charts show, the slope of the curves in the latter period became much steeper, indicating that the spots were increasing their size more slowly probably because the dye was being absorbed. Table I shows the figures for average radial spread and areal spread. It would seem that normal conditions are optimal for the spread of substances through the tissues and hence for their nourishment. More will be said of this below when other data have been considered.

The Immediate Effects of Hemorrhage upon the Spread of Dye through the Tissues.—It is well known that, following massive hemorrhage, the tissues give up fluid to the blood. Starling (10–12) and Leathes (13) first showed that the fluid shift under these circumstances occurs with great rapidity. As we wished to test the rate of the spread of dye in tissues which were relatively dehydrated, 22 mice of about 30 gm. body weight were bled approximately one-third or one-half their blood volume, that is to say 0.7 to 1.0 cc., by a method now to be detailed.

To 200 cc. of N/10 HCl in a beaker, 1 cc. of the pooled blood of 10 mice, bled for other purposes, was added and the hemoglobin converted to acid hematin. The resulting amber mixture served as a "standard" for the color comparisons which were made in the experiments to be described below. Its hemoglobin content was determined by the method of Newcomer (14) and thereafter the fluid was kept in a beaker sealed with heavy rubber sheets, to prevent evaporation, there being no air space above the fluid. When not in use for color comparisons the fluid was kept in the ice box. All the hemoglobin readings described below were done in a period of about 8 days. Mice of 28 to 30 gm. body weight, anesthetized with luminal, were placed on the plasticine moulds as usual, with the ears resting on porcelain plaques and the tail immersed in 200 cc. of warm N/10 HCl in a beaker similar to that containing the standard solution. The tail was snipped with sharp scissors and blood allowed to collect in the beaker until the

rapidly stirred contents assumed a color like that of the standard. In this manner, approximately 1 cc. of blood was removed from each mouse in 4 to 8 minutes. The fluids so obtained were placed in closed containers to prevent evaporation and an hour later, after the hemoglobin had been given ample time to be converted to acid hematin, their colors were compared with that of the standard in the Dubosq colorimeter and read together with the latter against Newcomer's glass standard (14). If the hemoglobin reading of the sample taken from the bled animal showed that less than approximately 0.7 cc. of blood had been taken, the experiment was discarded.



TEXT-FIGS. 5 *a* and 5 *b*. Spread of dye in the ears of mice after severe hemorrhage.

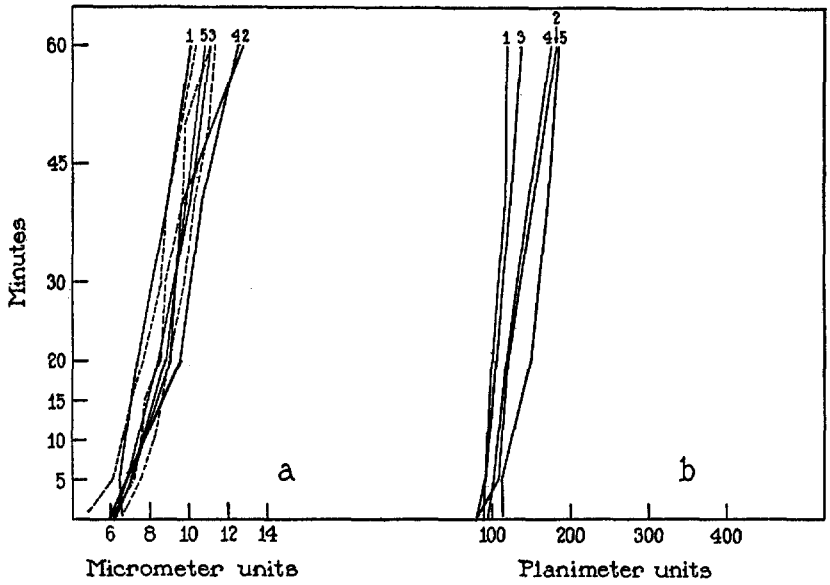
Within a minute after bleeding the animals, maculae of the 2 per cent pontamine sky blue were placed in the ears as usual. This required less than a minute.

Text-figs. 5 *a* and 5 *b* show the spread of 7 standard dye maculae in the ears under these conditions, the first expressing the increase in the sums of the diameters, the second the increase of the areas of the same maculae, numbered similarly in both charts. An average spread of 2.0 times the initial size, in terms of diameters, and 2.9 times in area was obtained in this experiment, far less than that occurring in the luminalized mice (2.7 and 3.9 times respectively). The radial

and areal spreads averaged 6.8 units (2.1 mm.) and 218 units (19.2 sq. mm.).

In this experiment despite the severe bleeding the animals had undergone, about half showed a formation of edema of the ears after pricking the skin and inserting the dye. The findings in these instances will be outlined below, together with the findings in the edema occurring under other circumstances.

Dye Spread in the Ears of Dead Mice.—Mice of 20 gm. body weight were given luminal in the usual manner and then killed with ether



TEXT-FIGS. 6 *a* and 6 *b*. Spread of dye in the ears of mice after death.

or chloroform just before placing maculae of 2 per cent pontamine sky blue in their ears. Under these circumstances there was very little spread of dye in the tissues. Text-figs. 6 *a* and 6 *b* show the spread from 5 standard maculae and 3 others, made with uncalibrated pipettes, (dotted lines) which happened to be the same size. They spread on the average but 1.8 times in diameter sum and 1.8 times in area. The average radial spread of only 5.1 units (1.5 mm.) and the areal spread of 77 units, only 6.7 sq. mm., were the smallest ever observed. The spread of dye through an edematous ear of a dead animal was far greater, as will be demonstrated further on.

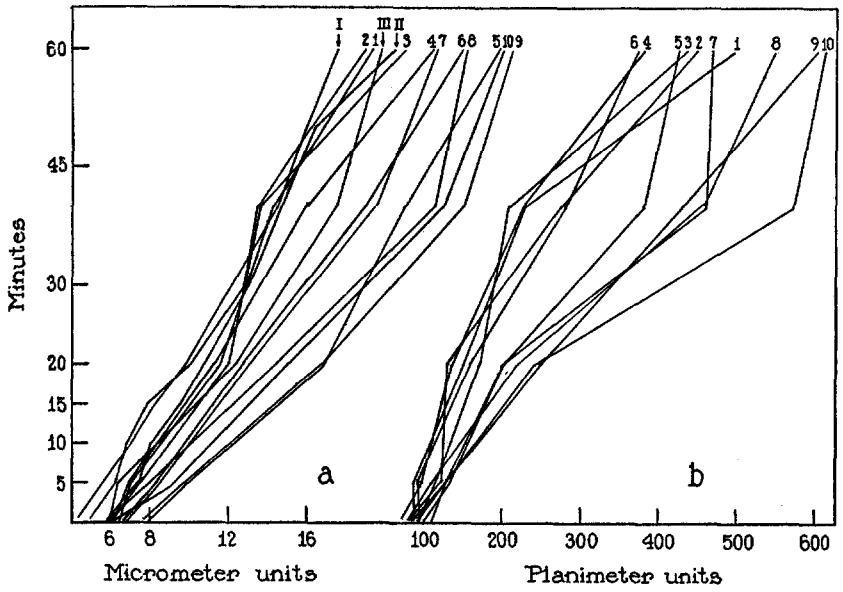
The Influence of a Forming Edema upon the Spread of Dye through the Tissues.—As already mentioned, some of the normal mice anesthetized with luminal, and even some of those which were anesthetized with luminal and then bled, developed slight edema in the tissues after instilling dye into the ear. The edema became demonstrable under the binocular microscope by an obvious increase in the thickness of the ear tissue or by pitting of the skin when pressed upon by a micro needle. These instances, excluded from the foregoing comparisons, will now be considered. In all, the edema formed as the dye spread, and became visible about 15 minutes after making the spot of dye. In all the instances too the appearance of edema was preceded by reflex hyperemia which followed the manipulations necessary to form the puncture wound and instil the dye. Whether or not the edema was due in part to the introduction of the dye we do not know. The ears seemed to become more edematous as the period of observation continued.

The rate of dye spread was increased in all, as evidenced by Text-figs. 7 *a* and 7 *b*, and 8 *a* and 8 *b*. Text-figs. 7 *a* and *b* present the measurements of the dye spots in the ears of animals treated as in the experiment from which Text-figs. 2 *a* and 2 *b* were taken, that is to say, in quietly resting mice anesthetized with luminal. In these instances, however, the ears became edematous during the period of observation. The charts show the spread of the dye spots in terms of the sums of the diameters and of their areas, the lines which bear the same numbers corresponding to the same spots, as usual. During an hour the averages of these measurements increased 3.6 and 5.6 times respectively. The spread was far more than that in luminalized mice, when there was no edema. The radial spread of 16.0 units (4.9 mm.) and the areal spread of 404 units (35.8 sq. mm.) show a marked difference too.

In Text-figs. 8 *a* and 8 *b* the measurements of dye spread have been charted for the ears of those bled animals which developed edema despite the hemorrhage. An average increase of 2.7 and 3.4 times is greater than that occurring in the ears of bled animals which did not develop edema (Text-figs. 5 *a* and 5 *b*). The average radial and areal spreads (Table I) are even greater than was observed in the normal ears of quiet animals under luminal.

The findings show that dye spread increased in the ears becoming edematous. Great irregularities appeared owing, no doubt, to the fact that edema occurred in a hit-or-miss manner, the degree of edema differing with the intensity of the reaction of the individual animals.

Because of this irregularity experiments were next planned to study the spread of dye in ears rendered edematous under controlled circumstances.



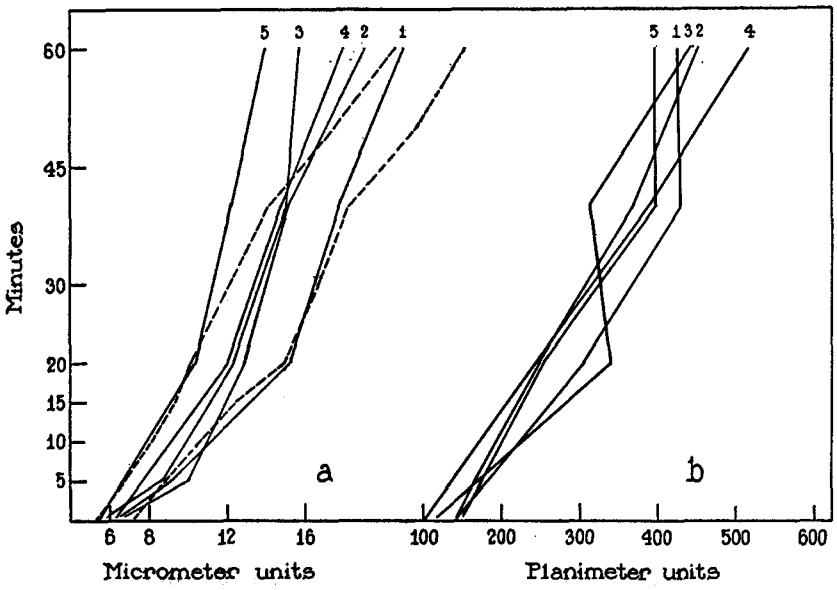
TEXT-FIGS. 7 *a* and 7 *b*. Spread of dye in the ears of normal mice during the formation of edema.

With a camel's hair brush xylol was repeatedly applied to the ears of mice anesthetized with ether. A reactive hyperemia developed almost at once and within a few minutes an intense edema appeared. The ears, which pitted on pressure, assumed a ground-glass appearance under the microscope, and droplets of free fluid escaped when the skin was punctured by the micro needle. Maculae of dye were placed as usual in such ears to study the effect of the rapidly appearing edema.

These experiments were not successful. In almost every instance free fluid began to escape from the puncture wounds within 5 or 10 minutes after the instillation of the dye. The free fluid was deeply colored by the pigment and the maculae spread for only a few minutes

and then rapidly decreased in area as the dye was forced to the surface, or perhaps taken up by the blood, for the ears were intensely hyperemic.

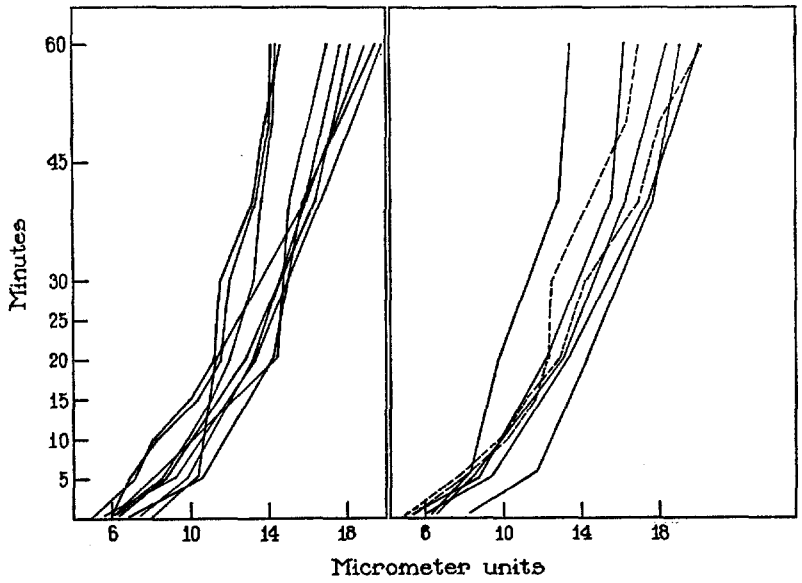
Dye Spread through Tissues Already Edematous.—What can be said of the effect of an excess of fluid already present in a tissue, upon the spread of dye through it? To test the point, maculae of dye were placed in the ears of mice, in the manner described, at intervals from 2 to 18 hours after inducing edema by paintings with xylol. In these



TEXT-FIGS. 8 *a* and 8 *b*. Spread of dye in the ears of mice during the formation of edema but after hemorrhage.

ears, already edematous, the dye often oozed from the puncture wounds and measurements could not be satisfactorily made. However, in some of the experiments this difficulty was not encountered. The spread of these maculae was far less than that of similar dye spots during the formation of edema (Text-figs. 7 *a* and 7 *b*) and was about like that seen in the normal ear. For brevity only one chart is reproduced, Text-fig. 9, to show the spread of those dye spots which were placed in ears 3 to 4 hours after the edema was induced. No oozing of colored fluid took place in these instances. The curves are

plotted in terms of the measurements of the sums of the diameters of the maculae. It will be seen at once that their slope is like that of the curves in Text-fig. 2 *a*. At the end of the experiments the dye spots were, on the average, 2.7 times their initial measurements. The average of the areas, not shown in any chart, increased 4.0 times (Table I). The radial and areal spreads, Table I, were 10.8 units (3.3 mm.) and 297 units (26.1 sq. mm.) respectively. Clearly the



TEXT-FIG. 9 TEXT-FIG. 10

TEXT-FIG. 9. Spread of dye in the edematous ears of mice after the edema had formed.

TEXT-FIG. 10. Spread of dye in the edematous ears of mice killed after the edema had formed.

presence of an edema already formed did nothing to speed up the spread of substances through the tissue even though free fluid was present in excess, as shown by the frequent appearance of droplets of fluid at the surface of the minute puncture wounds. This finding will be fully discussed in a following paper after presenting further data which bear upon the theme.

The Spread of Dye through the Edematous Tissues of Dead Animals.— The ears of mice were painted with xylol as in the preceding experi-

ments and the animals killed 3 hours later, after the edema had become intense. Spots of dye were placed in the ears in the usual way. In Text-fig. 10 we have plotted the spread of these spots as determined by their diameter measurements. The slope of the curves is very similar to those of Text-fig. 9, showing that dye spreads with equal rapidity in the ears of dead and living animals when edema of the ears is present. The figures given in Table I bear this out, too. Dye spread was much faster than in the non-edematous ears of dead animals, Text-fig. 6 *a*, and closely approached that observed in normal living ears, as a comparison with Text-fig. 2 *a* and a reference to Table I will show.

The Effect of Mechanical Stresses upon the Spread of Substances through the Tissues

In actively moving unanesthetized animals the dye spread through the tissues with great rapidity. For this reason it seemed wise to test the effect of slight changes in external pressure upon dye spread.

The ears of 20 mice were subjected to mild intermittent changes in external pressure after maculae of dye had been placed in them. The ears lay upon a soft rubber tambour and beneath a glass cover slip while pressure changes were exerted by the tambour 20 times a minute. Pressures of only 2 to 8 cm. of water were used.

By fixing thin rubber tissue over the end of a glass tube 3 mm. in diameter and connected by rubber tubing to a levelling bulb which could be mechanically raised or lowered at will, a small tambour was constructed. The glass tube was filled with water, the remainder of the apparatus with mercury. After forming dye maculae in the usual manner, the ears of mice were placed over the tambour, between it and a glass cover slip, in such a way that dye spots could be observed under the microscope. Intermittent pressures, equivalent to a column of water 2 to 8 cm. of water in height, were brought to bear upon the ear by the tambour. Each period of pressure endured but 1 second, the periods of relaxion, 2 seconds.

The mild pressure changes produced an enormous increase in the rate and extent of the dye spread. The color extended through the ears so far and so fast that repeated measurements could not long be made, for the margins of the maculae became too indistinct. In many instances, after 2 or 3 minutes of application of the intermittent pressure the whole ear became colored by the dye. Slight changes

in external pressure, then, caused far greater differences in dye spread than any of the other factors studied. This finding, too, will be discussed below.

DISCUSSION

The spread of dye in a tissue is subject to many influences. Our experiments have given full play to some of these and ruled out others, demonstrating to what degree they enhance or hinder spread. The greatest spread of dye appeared in the ears which were subjected to intermittent changes in external pressure, the next greatest in those of relatively normal, actively moving animals. In resting, quiet mice the spread of color was most pronounced in those previously normal ears which became frankly edematous during the period of dye spread. It was almost as great in the hyperemic ears of etherized mice. It was less in ears frankly edematous to begin with, and in the ears of normal luminalized mice, but much less in the dehydrated tissues of bled animals and least in the non-edematous ears of recently killed animals.

Histological studies of the tissue changes incident to edema in the ear of the mouse have been reported by Pullinger and Florey (15). As a result of the collection of fluid the connective tissue fibers and other formed elements of the ear are widely separated. In our experiments, puncture of the skin of edematous ears of either living or dead mice led to the appearance of droplets of free fluid at the surface of the organ, from which it seems probable that the wide spaces of the edematous ear are filled with free fluid. The spread of dye taking place through living ears that are already edematous and boggy before the dye is introduced (Text-fig. 9) is about like that taking place in the edematous ears of dead animals in which there can be no fluid movement. The spread in the latter instances must represent that consequent on diffusion alone through the free fluid in the tissues of the ear. From this one can infer that in marked edema in the living animal diffusion is the chief factor in extravascular spread.

In the non-edematous ear of the dead animal spread of dye is very slight, from which one can infer that the main factors responsible for spread in normal ears cease to act after the circulation has ceased. There must be mechanisms operative in the normal living ear which in some way overcome the barriers to spread.

In some of the experiments maculae of dye were placed in normal ears which, during the hour period of observation, became edematous. In other experiments edema and free fluid were already present in the tissues before the dye was introduced. Dye spread was increased under the first set of circumstances and not under the second. In the ears of those animals in which edema occurred after the spots of dye had been made, there were periods of a few minutes during which fluid was collecting imperceptibly between the formed elements or connective tissue fibers. Something evidently happened to increase the spread of dye through the tissues during those periods before one could recognize edema, and while the cells and connective tissue fibers were still in close approximation or forced apart but slightly by the increasing tissue fluid. That is to say the increase in the rate of dye spread occurred before the tissues became boggy, before the formed elements became widely separated by fluid and edema became visible.

The spread of dye along or between tissue elements not widely separated should be favored by any mechanical forces which tend to rub or squeeze the formed elements together. As shown above, slight intermittent changes in external pressure have this effect and greatly increase the spread of dye through the tissues of normal ears. A similar mechanical effect by the pulsation of blood vessels has been demonstrated in preceding work (1). One would expect the pulse, acting in this way, to increase the spread of the dye through the tissues only during the period in which an edema is forming, for when the formed elements have become widely separated by fluid, their movements will no longer tend to squeeze fluid from between them. The experiments reported here favor this point of view for, as just described, it actually has been observed that dye spread is not as great in a quiet, edematous tissue after it has become boggy, as in a tissue just beginning to become edematous. Further discussion of this problem must be deferred to the following paper, which reports data on the manner of the interstitial movement of substances.

SUMMARY

A method has been devised to measure the spread of vital dyes in the skin of mice. Spread is greatly influenced by physiological and pathological changes which add fluid to the tissue or abstract it.

Spread is greater in the quiet, living ear than in the ear of an animal just killed. It is equally considerable in the frankly edematous ears of living and dead animals, and not greater in either case than in normal, quiet tissues. During the early stages of edema formation on the other hand, dye spread is notably rapid. It is still greater in the ears of normal animals actively moving about, and is greatest in tissues subjected to very gently intermittent changes in external pressure.

The significance of these findings is discussed.

BIBLIOGRAPHY

1. McMaster, P. D., and Parsons, R. J., *J. Exp. Med.*, 1938, **68,** 377.
2. Parsons, R. J., and McMaster, P. D., *J. Exp. Med.*, 1938, **68,** 353.
3. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1932, **56,** 223.
4. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **56,** 239.
5. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1934, **60,** 479.
6. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1933, **57,** 751.
7. McMaster, P. D., *J. Exp. Med.*, 1937, **65,** 347.
8. McMaster, P. D., *J. Exp. Med.*, 1937, **65,** 373.
9. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **55,** 417.
10. Starling, E. H., *J. Physiol.*, 1894, **16,** 224.
11. Starling, E. H., The fluids of the body, Chicago, Keener, 1908.
12. Starling, E. H., in Schaeffer, E. A., Text book of physiology, Edinburgh-London, Pentland, 1898.
13. Leathes, J. B., *J. Physiol.*, 1895, **19,** 1.
14. Newcomer, H. S., *J. Biol. Chem.*, 1918, **23,** 119.
15. Pullinger, B. D., and Florey, H. W., *Brit. J. Exp. Path.*, 1935, **16,** 49.