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Research Article

STUDIES ON GANGRENE FOLLOWING COLD INJURY. IV. THE USE OF FLUORESCEIN AS AN INDICATOR OF LOCAL BLOOD FLOW: DISTRIBUTION OF FLUORESCEIN IN BODY FLUIDS AFTER INTRAVENOUS INJECTION [1]

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The use of fluorescein as a means of determining the adequacy of blood flow and the degree of capillary permeability in both normal individuals and in those manifesting various pathological states has been described in a series of papers by Kurt Lange and his associates (1, 2, 3). In general, the method depends upon the introduction of fluorescein into the blood stream and its detection by exposing the region under study to ultraviolet light. Data have been obtained by direct observations, photographic recording, and photoelectric registration of the yellow-green glow which results from the excitation of fluorescein in the tissues. It is obvious that the appearance of fluorescence in tissues remote from the site of injection of fluorescein demonstrates the existence of circulating blood within the area under observation. However, the interpretation of the significance of gradations in the intensity of fluorescence, the rate at which maximum intensity is reached, and the rate at which it diminishes requires detailed knowledge of the properties of fluorescein and the manner in which it becomes distributed in blood and tissue fluid.

Changes in the time-intensity relationships of fluorescence after the intravenous injection of fluorescein have been used recently as the basis for explanations of certain abnormalities present in myxedema and in tissues subjected to severe injury by cold (4, 5). In both of these widely different abnormal states, certain of the divergences from normal in the distribution of fluorescein were attributed to changes in capillary permeability. While there is no doubt that the permeability of the capillary membranes must play an important part in determining the distribution of dye between the blood and the interstitial fluid, the influence of other factors affecting the blood-interstitial fluid equilibrium should not be overlooked. The recent important contributions of Zweifach and his coworkers (6) serve to illustrate how changes in the pattern of local blood flow may first alter, in a striking way, the freedom of exchanges between the flowing blood and the interstitial fluid and later give rise to stasis and changes in capillary permeability as secondary phenomena.

The experiments to be described below were undertaken to study in normal rabbits the distribution of intravenously injected fluorescein in blood and other body fluids, some of the factors influencing fluorescein equilibrium, and the extent of penetration of cells by the dye.

METHODS

Fluorescein was administered to normal adult rabbits by injection into the marginal ear vein. The dose used was 75 mgm. per kgm. body weight and was given as a 5 per cent solution of sodium fluorescein in distilled water. White rabbits were used in order to avoid errors due to skin pigment, and all areas for study were closely clipped. The source of the ultraviolet light used for the excitation of fluorescein was a Shannon unit No. 92 consisting of a mercury vapor spot lamp (HGCH4) mounted in a holder with adjustable trunnions on a transformer and fitted with two glass filters: a UV heat-resisting Red-Purple and a UV Blue Purple Ultra.

Photoelectric measurement of the intensity of skin fluorescence was accomplished by means of a photometer constructed from a General Electric light sensitive cell of the barrier layer type connected through a tap switch to a reflecting galvanometer. The cell was mounted in a light-shielded housing set at a distance of 10 cm. from the surface of the skin area to be studied. A metal shield, set in the plane of the skin surface, restricted the area, which served as the source of light falling on the cell, to a circle 37 mm. in diameter. The ultraviolet lamp

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

was held in a clamp with the surface of the globe at a distance of 11 cm. from the shield with the incident light directed on the skin at an angle of 45°, and at 90° to the path of the visible light from the skin surface to the light-sensitive cell. Light falling on the photo cell was restricted to the yellow-green region of the spectrum by means of a Wratten No. 61 filter mounted in the photocell housing.

The intensity of fluorescence was expressed in units of galvanometer deflection. Galvanometer readings, taken when the skin was exposed to ultraviolet light before fluorescein injection, were subtracted from those after fluorescein to yield the change due to the presence of dye. The blank reading amounted to about 5 per cent of the maximum deflection under full fluorescence in normal animals.

The concentration of fluorescein in blood and peritoneal fluid in most of the experiments was measured by adding 1 ml. of the fluid in question to 6 ml. of 95 per cent ethyl alcohol and separating the precipitated protein by centrifugation. The fluorescein dissolved freely in the alcohol used for dilution and precipitation. The resulting yellow color was measured in a Klett Summerson photoelectric colorimeter with a Wratten No. 44a filter. The concentration of the dye was calculated by the comparison of colorimeter readings with values obtained from a standard curve plotted from colorimeter readings made on alcoholic solutions of fluorescein of known concentration. Blanks were prepared from a mixture of alcohol and fluorescein-free blood to obtain a zero setting of the colorimeter. Comparison of readings from solutions containing known amounts of fluorescein dissolved in alcohol with readings obtained upon alcoholic extracts of blood to which known amounts of fluorescein had been added showed that the yield of fluorescein was complete and that no interfering pigment was retained in the extract.

A few of the analyses were carried out without extraction. Twenty cu.mm. of the blood or other fluids were diluted to 50 ml. with phosphate buffer at pH 7.0, and the concentration of fluorescein was measured in a Coleman Photofluorometer against solutions of known fluorescein concentration. Good agreement was obtained between the results from both methods of analysis.

RESULTS

1. *The relation between blood concentration of fluorescein and the fluorescence of the skin.*

The gross appearance of fluorescent skin under ultraviolet light after the intravenous injection of fluorescein indicated an initial distribution of dye in close relation to blood vessels. Within a very brief time fluorescence was uniform over the whole area in a manner consistent with the free passage of dye between the blood and the extravascular fluid. The time relations of this equilibrium were explored in normal rabbits by making serial measurements of both the concentration of fluorescein in the blood and measurements of the intensity of skin fluorescence. Figure 1 shows curves plotted from representative data from one animal. The curve describing the rate of disappearance of fluorescein from the blood is of the "die-away" type and indicates the dependence of rate of removal upon concentration gradients. Since fluorescein is excreted freely in the urine (3) at the same time as it is being moved from the blood stream to the extravascular fluid, this curve represents the change in concentration resulting from loss of dye as well as its dilution in the entire extracellular phase. The maximum intensity of skin fluorescence was reached at a time when the blood concentration was falling rapidly. Fluorescence declined very little over the following period of about 15 minutes. In this short interval the blood concentration of dye decreased sharply by 18 to 20 mgm. per 100 ml. In the period from 30 minutes after the injection of fluorescein up to 140 minutes, the decline of intensity of fluorescence exhibited an orderly decrease in rate with time. Thus, the curve describing decreasing fluorescence from 30 minutes after injection is qualitatively similar to that describing the change in blood concentration of dye.

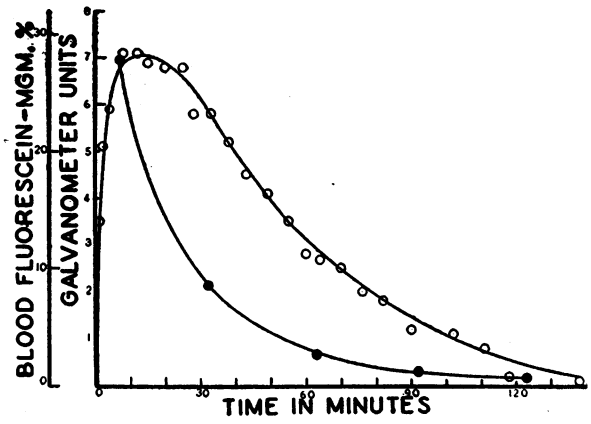

FIG. 1. CHANGE IN INTENSITY OF FLUORESCENCE IN NORMAL RABBIT'S EAR WITH TIME COMPARED TO DISAPPEARANCE OF FLUORESCEIN FROM THE BLOOD

Representative data from one animal. Open circles—fluorescence, in galvanometer units. Solid circles—concentration of blood fluorescein.

The regularity of the rate of disappearance of fluorescein from the blood of rabbits is illustrated

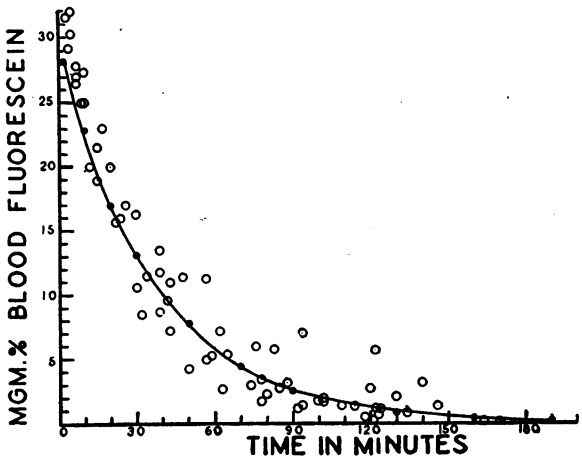

FIG. 2. CHANGE IN BLOOD FLUORESCEIN CONCENTRATION WITH TIME IN NORMAL RABBITS

Data from determinations in 14 animals after intravenous injection of 75 mgm. per kgm. sodium fluorescein. Open circles—individual determinations. Curve with solid circles fitted to data on a semi-log plot by method of least squares (Figure 3).

by the curve in Figure 2. The open circles represent individual measurements of blood fluorescein on 14 animals. The same data were plotted as the logarithm of the blood concentration against time in Figure 3. The rectilinear fit in Figure 3 was calculated by the method of least squares. The line drawn through the solid circles in Figure 2 was derived from the least square fit by the general formula

$$\log y = a + bx.$$

The constants for the line drawn in Figure 3 are: $a = 2.48$ and $b = 0.0118$.

A similar regularity in the rate of decrease of fluorescence is illustrated in Figure 4. This graph shows the distribution of the measurements on the ears of six normal rabbits of fluorescence intensity expressed as the log of the intensity in galvanometer units plotted against time. Each point represents an individual measurement. The straight line was fitted by the method of least squares. The constants are $a = 1.99$ and $b = 0.0087$. Statistical analysis of the slopes of the lines in Figures 3 and 4 showed that they are not significantly different.

These observations show that the maximum skin fluorescence does not coincide in time with maximum concentration of dye in the blood but rather occurs at a time when the blood fluorescein concentration has been sharply reduced. Thus, an important influence upon the rate of loss of dye from the blood stream is the rate at which it accumulates in the extravascular fluid, and the intensity of fluorescence in the skin must be considered an expression of extravascular fluorescein concentration rather than an expression of either blood flow rate or the concentration of fluorescein in the blood. However, it is clear that the rate at which the dye may enter or leave the extravascular fluid is dependent in some way upon concentration gradients. These data do not permit a decision as to the relative importance of diffusion or filtration as factors determining the rate of transfer.

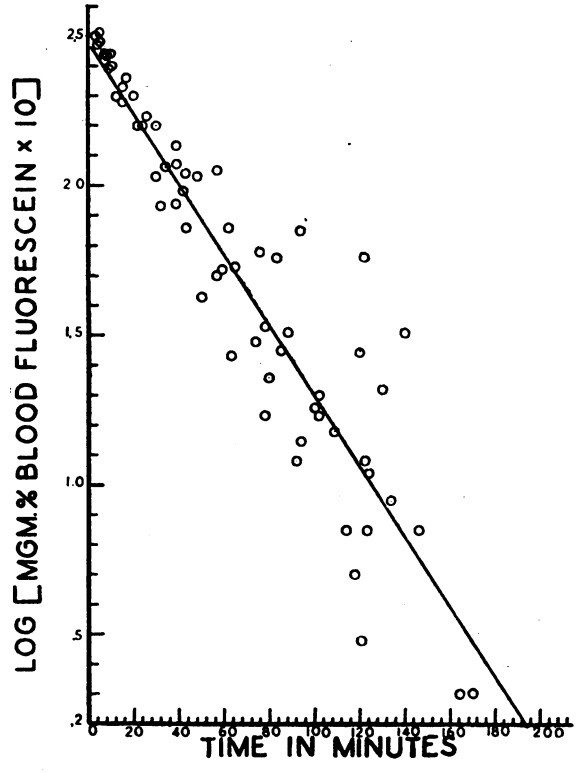

FIG. 3. CHANGE IN BLOOD FLUORESCEIN CONCENTRATION WITH TIME IN NORMAL RABBITS

The logarithm of blood fluorescein concentration is plotted as a function of time for various intervals after intravenous injection of 75 mgm. per kgm. sodium fluorescein. Straight line calculated by the method of least squares. Each point represents a single determination. Data from 14 animals.

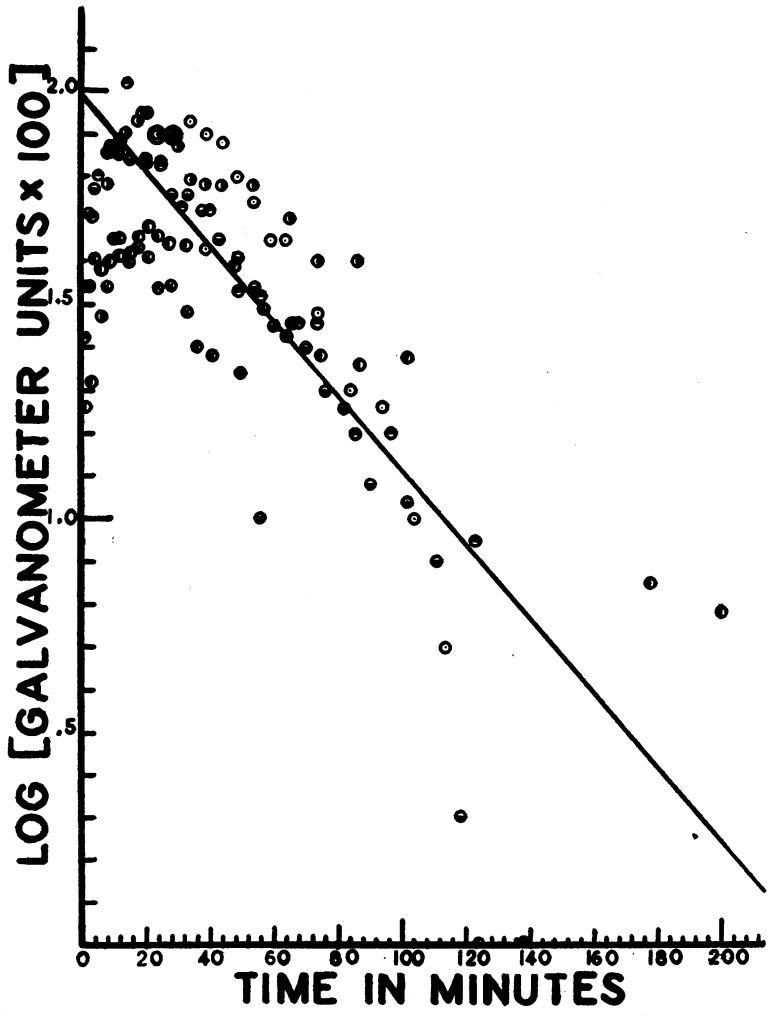

FIG. 4. INTENSITY OF FLUORESCENCE OF RABBITS' EARS EXPRESSED AS LOG GALVANOMETER UNITS

Straight line calculated by the method of least squares. Data from 6 animals. Each symbol represents 1 animal. Fluorescein injected at zero time.

2. *The diffusion of fluorescein from the blood against a filtration gradient*

Although Lange and Boyd (3) have studied the role of diffusion and filtration in exchanges of fluorescein across membranes, their experiments were carried out *in vitro* with collodion membranes. In order to demonstrate *in vivo* the free passage of dye from the blood into extravascular fluid under circumstances where the net filtration gradient was bringing about a movement of fluid into the blood stream, fluorescein exchanges were studied in three rabbits which had received intraperitoneal injections of Ringer's solution before dye was administered.

Each animal received 50 ml. per kilogram body weight of Ringer's solution containing 0.2 per cent glucose. The Ringer's solution was injected intraperitoneally, and, immediately afterward, the usual dose of fluorescein was injected intravenously. Samples of blood and of fluid aspirated from the peritoneal cavity were taken at intervals and the concentration of fluorescein was determined. After 107 to 140 minutes, the animals were killed and the volume of fluid remaining in the peritoneal cavity was measured. Estimation of the protein concentration of plasma and of peritoneal fluid was made by the method of Barbour and Hamilton (7).

TABLE I

Changes in fluorescein concentration in blood and peritoneal fluid

75 mgm. per kgm. fluorescein injected intravenously
50 ml. per kgm. Ringer's solution injected intraperitoneally

Animal number	Weight	Time of sampling. Minutes after dye injection		Plasma protein	Fluorescein	
		Blood	Peritoneal fluid		Blood	Peritoneal fluid
	kgm.			*grams per 100 ml.*	*mgm. per 100 ml.*	
23	2.20	15	12	5.78	21.5	0.70
		42	39	5.37	9.6	0.80
		85	82	5.48	2.8	1.30
		122	118	5.52	1.2	1.45
		146	148	5.34	0.7	1.40
20	2.46	10	21	4.99	27.4	0.90
		39	45	5.03	13.5	1.20
		75	82	5.41	5.8	1.40
		122	126	5.07	2.1	1.30
28	1.80	11	16	4.45	23.0	1.10
		39	43	4.66	11.8	2.40
		65	71	4.52	5.4	3.20
		102	107	4.39	1.7	2.90

TABLE II

Diffusion of fluorescein against a filtration gradient

Terminal fluid volume and fluorescein concentrations after intraperitoneal injection of 50 ml. per kgm. body weight of Ringer's solution and intravenous injection of 75 mgm. per kgm. fluorescein.

Animal number	Mgm. dye injected at 75 mgm. per kgm.	Ringer's solution			Total time	Dye in peritoneal fluid		
		Injected	Recovered	Recovery		Final conc.	Total	Injected dye
		ml.	*ml.*	*per cent*	*min.*	*mgm. per 100 ml.*	*mgm.*	*per cent*
20	181	123	53	43	126	1.3	0.69	0.38
23	165	110	72	65	148	1.4	1.10	0.67
28	135	90	50	56	107	2.9	1.45	1.07

Data obtained from three animals are presented in Tables I and II. Figure 5 shows curves describing the time course, in a representative experiment, of the changes of dye concentration in the blood and in the Ringer's solution withdrawn from the peritoneal cavity. The intersection of the curves at 110 minutes after the injection of fluorescein shows the time at which the concentration of the dye was equal in blood and peritoneal fluid. For approximately 40 minutes thereafter, the concentration of dye in blood continued to decrease while that of the fluid in the peritoneal cavity decreased comparatively little. Since the change in fluid volume in the peritoneal cavity of all three animals (Table II) showed vigorous absorption during the time that dye was passing into this fluid, the net change in fluorescein concentration was one largely determined by diffusion pressure. The permeability of capillaries within the peritoneal cavity during the experiments was assumed to undergo no significant change, since the highest specific gravity measured on peritoneal fluid did not exceed 1.0066. The data in Table I show that the concentration of dye in the peritoneal fluid reached and exceeded the concentration in the blood in two of the animals tested. In the remaining animal (No. 20), the concentration of dye in the peritoneal cavity began to decline before it reached equilibrium with the blood.

Teorell (8) has made an extensive theoretical analysis of the kinetics of distribution of substances introduced into the body on the basis of the following relationships derived from Fick's law:

(a) the total *amount* of substance transferred between blood and the tissue is:

$$\text{amount} = \underset{-dN}{\text{diffusion coeff.}} \times \underset{D}{\text{conc. gradient}} \times \underset{dc/dx}{} \times \underset{A}{\text{surface}} \times \underset{dt}{\text{time}} \quad (1)$$

and

(b) since the diffusion coefficient is related to the net value of the friction coefficients encountered, it will be incorporated with the effective permeation surface (A in Eq. 1) and the boundary thickness in a "permeability coefficient" as k'_n in the following:

$$\text{Amount across boundary in the time unit} = \underset{\frac{-dN}{-dt}}{} = \underset{k_n'}{\text{perm. coeff.}} \times \underset{\frac{N_i}{V_i} - \frac{N_0}{V_0}}{\text{conc. diff.}} \quad (2)$$

where N_i, V_i and N_o, V_o are the amounts of the substance and the fluid volume respectively inside and outside the boundary, and "amount" is the number of gram molecules or grams or any unit.

Where it may be assumed safely that the dis-

tribution of the substance introduced into the blood is not complicated by selective accumulation in depots, reaction with blood constituents, by tissue inactivation or by changes in volumes of fluid concerned, Teorell's analysis, by the simple formulation given above, may be applied. His calculations show that, after the concentration of substances in the blood and in the extravascular phase have become equal, the removal of substances from both phases occurs at about the same rate but that the concentration of substances in tissue fluid remains persistently above that in the blood.

The conditions of the experiments reported here differ from those in Teorell's analysis in two ways: the dye leaves the blood and enters a large amount of fluid which is continuously changing in volume, and in addition, the blood is being continuously diluted by the fluid which is being absorbed. Zweifach (9) has pointed out that transfers of fluid across capillary membranes are "transfers of fluid in bulk." It may be assumed that the transfer of fluid from the peritoneal cavity involves also the removal of at least some of the fluorescein which had previously passed into this fluid from the blood stream.

The course of events in animal No. 20 serves to emphasize the effect of fluid filtration from the peritoneal cavity to the blood upon final concentration relationships. A simplified statement of the factors determining the concentration of dye in peritoneal fluid may be presented as follows:

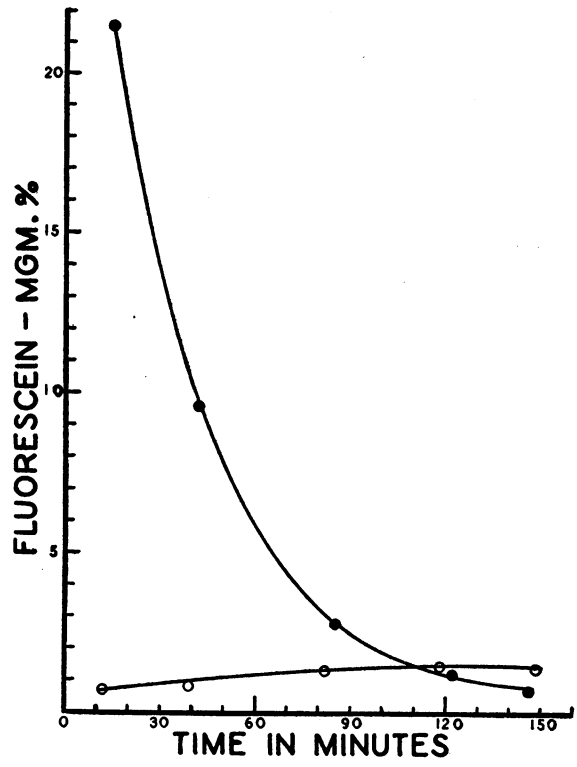

FIG. 5. TIME COURSE OF CHANGES IN FLUORESCEIN CONCENTRATION IN BLOOD AND PERITONEAL FLUID

Representative data from 1 animal. Fluorescein injected at zero time. Solid circles—concentration of fluorescein in blood. Open circles—concentration of fluorescein in peritoneal fluid.

$$\frac{\text{(dye entering mgm. per min.)} - \text{(dye leaving mgm. per min.)}}{\text{(fluid entering ml. per min.} - \text{(fluid leaving ml. per min.)}} = \frac{\text{mgm. gain or loss of dye}}{\text{ml. gain or loss of volume}} = \text{net change}$$

in conc. of dye and the concentration of dye in the peritoneal fluid may be expressed as

$$\text{mgm. of dye per 100 ml.} = \frac{\text{net change in conc. of dye}}{\text{volume of fluid in the peritoneal cavity}} \times 100.$$

Where the rate of fluid filtration from the peritoneal cavity is high, as it was in the case of animal No. 20, the rate of removal of dye along with the fluid may be sufficiently great to bring about a decline of fluorescein concentration in the peritoneal cavity before concentration equilibrium is established. We have no reason to believe that there was any qualitative difference in the processes involved in fluorescein exchange among the three animals tested in the manner described. The attainment of fluorescein equilibrium between blood and the Ringer's solution in the peritoneal cavity may not safely be considered to represent simple relationships of diffusion pressure and permeability. The magnitude of the equilibrium concentration and the time at which this concentration was reached depends upon the rate and direction of fluid exchange as well as upon the rate and direction of transfer of dye.

These observations show that, while fluorescein may diffuse out of the blood into extravascular fluid at the same time as fluid is being actively

taken into the blood in the same region, the movement of dye is not independent of the movement of water.

3. *The extent of cell penetration by fluorescein.*

A further examination of the distribution of fluorescein after intravenous injection was carried out by the analysis of blood and skeletal muscle from animals subjected to previous ligation of the renal pedicles. Three rabbits were anesthetized by the intraperitoneal injection of dial, 90 mgm. per kgm. body weight. After supplementary administration of ether the renal pedicles were ligated acutely either through lumbar incisions or by an abdominal approach. The wounds were closed and control blood samples were taken. An intravenous injection of fluorescein, 75 mgm. per kgm. body weight, was given. At intervals approximately one-half hour apart, three blood samples were taken. Following the last sample, the animals were killed and samples of skeletal muscle were excised. Plasma was analyzed for fluorescein by the methods described previously. The muscle samples were weighed, homogenized in a Waring Blendor and subjected to extraction for the determination of fluorescein. The muscle from the first animal studied was extracted directly with 95 per cent alcohol in the proportion of 1 gram of minced muscle to 6 ml. of alcohol. Although the muscle was grossly yellow in appearance this method of extraction yielded no detectable fluorescein when the alcoholic extract was subjected to colorimetric comparison. In the remaining two animals the muscle samples weighing approximately 50 grams each were extracted directly in 200 ml. of cold phosphate buffer at pH 7.0 or were subjected to repeated freezing and thawing before final separation of the extracting medium by centrifugation. The shapes of the curves describing the "dilution" of injected fluorescein indicated that even at the end of 150 minutes the blood concentration of the dye continued to fall slowly. Calculation of the volume of fluid in which the fluorescein would have to be dissolved, if solution alone were to account for the reduced concentration with time, showed that the apparent distribution volume of dye amounted to 39 to 45 per cent of the animal's body weight. Various investigators (10, 11) have found that the extracellular phase of most mammals including rabbits comprises between 20 and 30 per cent of the body weight. Therefore, the apparent distribution volume of fluorescein observed in the present studies must be accounted for by an appreciable penetration into the cellular compartment, or loss by some other route.

The analyses of muscle samples from the same animals yielded values for fluorescein concentration of 1.03 to 1.92 mgm. of dye per 100 grams of fresh muscle. On the assumption that even distribution of fluorescein existed throughout the extracellular phase, the comparison of plasma fluorescein with muscle fluorescein showed that the distribution volume of dye amounted to 8.4 to 11.4 per cent of the muscle weight. Others have reported that the extracellular phase of rabbit muscle is approximately 16 per cent by weight (10). It, therefore, seemed unlikely that the large total distribution volume of dye could be accounted for on the basis of a general penetration of dye into cells.

All of the animals were observed to have brightly fluorescent yellow dye in the gallbladder bile and in the lumen of the intestine. Analysis of the intestinal fluid for fluorescein in one animal showed that the concentration of dye was 45.75 mgm. per 100 ml. The apparent dilution volume of dye, estimated from the amount injected and the final concentration, was greatly in excess of the extracellular phase volume calculated as 25 per cent of the body weight; 925 ml. compared to 500 ml. The amount of dye which would be dissolved in the volume representing the difference between the two values given above at the final blood concentration of 16.25 mgm. per 100 ml. was

$$425 \text{ ml.} \times \frac{16.25 \text{ mgm.}}{100 \text{ ml.}} = 69.1 \text{ mgm.}$$

If all of this "lost dye" were in the intestinal tract at a concentration of 45.75 mgm. per 100 ml., the volume of fluid necessary to accommodate it would be:

$$\frac{69.1 \text{ mgm.}}{V} = \frac{45.75 \text{ mgm.}}{100 \text{ ml.}}$$

$$V = 151 \text{ ml.}$$

Since the brilliant fluorescence of gallbladder bile

indicated a concentration of dye even greater than that in the fluid in the lumen of the intestine, a dilution volume much less than the estimated volume of 151 ml. would actually accommodate the amount of "lost dye" indicated above. Carmichael, Strickland, and Driver (12) report data from which the water in the contents of various parts of the gastro-intestinal tract of rabbits may be calculated. The average water content of the small intestine, cecum and colon from six rabbits, weighing approximately 2 kilograms each, was 114 ml. Thus, it is reasonable to assume that the disparity between the measured distribution volume of fluorescein and that predicted for the extracellular phase may be accounted for by the amount of dye excreted into the bile and the lumen of the intestine.

The distribution of fluorescein between plasma and red blood cells was studied on blood samples from four rabbits by equilibrating heparinized whole blood *in vitro* at 37° C. with known amounts of fluorescein. The fluorescein concentration of the separated plasma was determined at intervals, and the distribution of dye between the cells and the plasma phase was calculated from these measurements and the hematocrit values. Within one minute after the addition of dye to the blood, only about 85 per cent of it could be found in the plasma, and over a period of 2 hours the plasma fluorescein diminished to 82.7 per cent of the amount predicted from the quantity of dye originally added to the sample. Thus, either penetration of fluorescein or its adsorption upon the surface of red blood cells removed from 15 to 17.3 per cent of it from the plasma.

When red cells were subjected to alteration of their normal surface volume relationship by suspending them in solutions of sodium chloride varying in concentration from 1.8 per cent to 0.54 per cent before equilibrating them with fluorescein, no differences were noted in the amount of dye recovered from the non-cellular phase of the suspension. Since there is little or no change in cell surface area and large change in cell volume under the above conditions, it seems likely that the disappearance of fluorescein from the plasma phase may be ascribed to surface adsorption rather than cell penetration.

CONCLUSION

1. The intensity of fluorescence of regions exposed to ultraviolet light after the intravenous injection of fluorescein depends more directly upon the amount of dye in the interstitial fluid than upon the concentration of dye in the blood.

2. The amount of fluorescein present in the interstitial fluid depends to an important degree upon filtration processes involved in the exchanges of plasma ultrafiltrate across capillary membranes as well as upon simple diffusion exchanges. The movement of dye across capillary membranes should not, therefore, be considered to be independent of the movement of water.

3. The distribution of fluorescein in the various water compartments of the body after intravenous injection extends throughout the extracellular phase. The dye is excreted in large amounts in the bile and small quantities, 15 to 17 per cent, become associated with the cellular fraction of the blood in a manner which suggests surface occlusion rather than penetration of the cells.

SUMMARY

The distribution of injected fluorescein, a fluorescent dye, was studied in normal rabbits. A consideration of the relation of dye concentration in the blood and the intensity of fluoresence of the animal's skin under ultraviolet light indicated that maximum intensity of fluorescence is reached at a time when the concentration in the blood is falling rapidly. Thereafter, the disappearance of dye from the blood and the decline of intensity of skin fluorescence occurred at about the same rate.

The exchanges of fluorescein injected into the blood stream with Ringer's solution introduced into the peritoneal cavity of rabbits showed that the concentration of dye rose in the fluid within the peritoneal cavity during the time that the fluid was being actively absorbed into the blood. The concentration of dye in the fluid within the peritoneal cavity at any time after injection depends upon the amount of it entering and leaving the peritoneal cavity as well as upon the direction and volume of fluid exchange. Therefore, in circumstances where the volume of extravascular fluid may be changing, the movement

of dye is not independent of the movement of water.

The distribution of intravenously injected fluorescein was tested in rabbits after ligation of the renal pedicles. Calculations of the apparent dilution volume of the dye yielded values equivalent to from 39 to 45 per cent of the animals' body weight. Determinations of the fluorescein content of samples of skeletal muscles from the same animals showed no evidence that the dye penetrated the intracellular phase. If the amounts of dye in muscle were assumed to be in equilibrium with the fluorescein in the blood, the volume of muscle water in which the dye was dissolved equalled 8.4 to 11.4 per cent of the total weight of muscle. Sufficient amounts of fluorescein were found in the bile and in the lumen of the gut to approximate the amount which had disappeared from the extracellular phase.

When heparinized whole blood was equilibrated for 2 hours *in vitro* at 38° C. with known amounts of fluorescein, approximately 83 per cent of the amount of added dye could be recovered from the separated plasma. Since osmotic manipulation of red cell volume without change in the surface area did not change the amount of dye recovered from the fluid phase, the loss of dye was tentatively attributed to surface adsorption rather than cell penetration.

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