STUDIES ON VITAL STAINING

V. Double Staining with Brilliant Vital Red and Niagara Sky Blue. Correlation of Histological with Physiological Data

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(Received for publication, November 16, 1929)

INTRODUCTION

After repeated daily intravenous injections of brilliant vital red, the skin, the connective tissues and various internal organs of dogs take on a deep red coloration. This color is due in small measure to the diffuse staining of certain non-cellular elements, particularly of the elastic fibers, but in greater part to the deposition within certain cells of great numbers of fine granules of the dye. The staining of elastic fibers, though slight, is well seen by gross inspection of the walls of the larger arteries. Microscopic examination shows quite clearly that the elastic fibers so abundantly present are diffusely and uniformly tinged with dye, there being no evidence that the dye is deposited in granular form within these fibers. Bouffard (1), Goldmann (2), Schulemann (3), Pappenheim and Nakano (4) and many others have observed the diffuse staining of elastic fibers with trypan blue, though pyrrol blue gives no such staining (4), nor does lithium carmine (5) though both of these dyes are excellent "vital stains." Occasionally one can see that brilliant vital red has tinged the delicate ramifying elastic fibers present in the areolar connective tissues but a glance through the microscope shows that the dye, like the other dyes just mentioned, is stored largely in granular form in the phagocytic cells to which we have referred. This segregation of dye within living cells has been the subject of innumerable investigations. For many of the details we refer to the reviews by Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8).

Amongst the most active of these phagocytic cells are the Kupffer cells of the liver. Unlike many of the cells of connective tissues they lie in intimate contact with the blood stream and are directly exposed to foreign substances introduced into the circulating blood. Thus it is that these cells can take up India ink introduced into the blood whereas the phagocytic cells of the connective tissues generally have little opportunity to take up carbon particles which escape with such difficulty

through the capillary walls. Other cells like those which line the sinusoids of the spleen also have direct access to ink or dyes which may be present in the circulating blood. The endothelial cells of certain capillaries in the cortex of the adrenal and in the bone marrow also have phagocytic properties and may take up vital dyes, but the endothelial lining of blood vessels elsewhere seems to be almost entirely devoid of ability to retain these foreign substances.

Aside from these specific and well localized phagocytic types of endothelium, one finds in the connective tissues themselves enormous numbers of cells which are capable of taking up and storing in granular form a great variety of foreign substances which are sufficiently diffusible to pass through the capillary walls. A vast group of workers have shown that these cells of the connective tissues vary considerably in their morphology and there is still much uncertainty regarding the derivation of many of them.

Although many of the cells of connective tissues show little avidity toward acid colloidal dyes, still the majority can be stained under proper conditions of dosage and length of staining. Mast cells, lymphocytes and ordinary leukocytes show almost no tendency to take up these "acid dyes," but in the connective tissues are a vast number of "macrophages," "tissue phagocytes," "histiocytes," "clasmatocytes," or "resting wandering cells" as they are variously called, and these cells are highly phagocytic. They will take up particles of carbon or other finely granular material if this is injected directly into the tissues, but even more interesting is their capacity to take up various dyestuffs which are injected locally or which diffuse from the blood stream into the tissue fluids. In building up these intracellular granules, the dye is taken from solution and is greatly concentrated within the cell. It is obvious that the dye is not held there in simple solution, for the concentration is clearly many times that of a similar globule of a saturated solution of the dye. In the case of a few of the acid dyes, needle-like crystals may be formed but in no case is the exact nature of this concentrating mechanism understood. The literature of this question has been reviewed by Möllendorff (6). It remains an open question whether this process of dye storage differs fundamentally from the phagocytosis of finely particulate matter by these same cells. The former has been referred to as "dypsocytosis" (9) but this term has not gained wide acceptance. In the present experiments we have used the term "phagocytosis" to cover both. We have also used the term "vital staining" to refer to the dye storage, despite the fact that the word "staining" would seem to infer a reaction between dye and protoplasmic granules or other cytoplasmic constituents—a point still very much in dispute in the case of the "acid" dyestuffs.

Aside from these macrophages which are capable of ameboid motion, the fibroblasts of the connective tissues show an interesting behavior toward these acid vital dyes. Concerned as they are with the formation and maintenance of the fibers which bind the parts together, these cells have a much underdeveloped ability to take up foreign substances from the surrounding fluids. The faint

granular storage of lithium carmine by these cells is well shown in the plates of Ribbert (10) and Kiyono (11). Goldmann (2) showed that they will take up pyrrol blue. Evans and Scott (9) have placed particular emphasis upon the fact that they take up the dye much later than do the macrophages, and they have shown that granules do not appear in the fibroblasts until the animal has received large quantities of the dye. This sharp distinction in reaction has led Evans and Scott to set them aside as a distinct physiological class of cells.

These morphological details give us much valuable information concerning important physiological reactions of the tissues—reactions which would pass unobserved were it not that the tinctorial properties of these dyestuffs make their recognition easy. There is every reason to suspect that many products of normal metabolism in the body may be distributed in analogous ways, though they lack the color properties necessary for their demonstration.

It is to be regretted that the morphological studies with dyestuffs have always been so purely qualitative. It is most desirable that we know more about their distribution in the tissues from a quantitative point of view. Unfortunately histological methods are almost purely qualitative and chemical extraction of ingested dyestuffs is beset with serious difficulties.

At the present state of our knowledge it is perhaps as well that we restrict our quantitative studies to the phagocytic response of the body tissues as a whole. Adequate means for such studies exist, for, with certain restrictions (12), the rate at which a dye leaves the circulating blood stream is a measure of this tissue response, representing as it does the sum total of the absorptive power of the elastic tissue and of all of the "macrophages" and other phagocytic cells previously mentioned. The reason that this quantitative mode of attack has been neither popular nor successful in the past must be that many dyestuffs are not well suited for such studies. Many of them leave the blood stream in large part before there has been time for them to be thoroughly mixed with the circulating blood. Under these circumstances efforts to follow the rate of elimination from the blood stream must necessarily fail. Other substances, like India ink and many dyestuffs are precipitated by the body fluids, and the particles are mechanically caught and held for a time in certain of the capillaries. Obviously, here too, the rate of disappearance from the blood is no criterion of the phagocytic response of the tissue. With other dyes the elimination through the urine or the bile is so rapid that the tissues are afforded little opportunity to display their phagocytic properties, and here again the elimination rate gives none of the desired information concerning tissue absorption. Fortunately, certain dyestuffs are relatively free from these defects, and from this group we have selected two, brilliant vital red and Niagara sky blue, as being excellent representatives. A number of the physiological studies have been reported in the previous papers of this series. The results of those experiments will be reviewed in the light of the morphological studies about to be reported.

Methods

Many of the preliminary studies were made on rabbits, but typical experiments of all classes were made on dogs also, and these latter experiments are of particular interest because of the close relationship existing between them and the physiological experiments on distribution and elimination of dye and ink reported in previous papers of this series. In the case of both dogs and rabbits the ink was always given intravenously, but the rabbits in most cases received the dye into the peritoneal cavity, from which it is rapidly absorbed and carried with the circulating fluids to all parts of the body. The dyes and ink were given to the dogs by the intravenous method. The dogs received the regular mixed diet of hospital table scraps; the rabbits received hay, oats and cabbage.

The distribution of India ink can be studied to advantage in fixed tissues imbedded in paraffin and cut and stained by any of the ordinary methods. The two dyes, brilliant vital red and Niagara sky blue, are stored in granular form in many phagocytic cells scattered throughout the body, but the dye appears to be rather loosely fixed within the cell and after death it tends to diffuse, especially if the tissues are placed in any of the solutions ordinarily used in fixing and imbedding. Niagara sky blue suffers from this defect somewhat less than does brilliant vital red but in both cases the results in fixed tissues are quite unsatisfactory, and clear-cut pictures are to be obtained only by the study of freshly drawn films of tissues, teased preparations and fresh frozen sections of unfixed tissue. Even in these cases it is best to avoid adding water or saline to the preparation, and it is well to study the section immediately, without the addition of fluid or cover glass, though addition of glycerine and a cover-glass sometimes gives very satisfactory results. A drop of immersion oil may be placed directly upon preparations of omentum or mesentery and the tissue thus made available for study with the oil immersion lens.

It is desirable that several workers be concerned in making the fresh tissue preparations, for much valuable time may be saved and a maximum number of preparations may be prepared and examined before the cells die and the dye begins to diffuse away. Even so, it is rarely possible to make a detailed study of every organ while the tissues are fresh and much of the information is necessarily gained by piecing together the results of several similar experiments.

EXPERIMENTAL

The dye granules formed within the cytoplasm of living cells present great variations in size, shape and arrangement depending upon the dye employed and upon the amount of dye administered and upon the rapidity with which the staining is brought about. Much of the earlier work has been reviewed by Möllendorff (6). The magnitude and complexity of this subject is well brought out in an excellent monograph by Evans and Scott (9). Their experiments cover a wide territory

and they have formed certain general conclusions regarding the effect of dosage and the duration of the treatment with dye. It is our hope that much detail still lacking may be filled in and that it may be possible to bring such morphological studies more closely into relation with the physiological aspects of dye storage. We feel that the morphological studies gain greatly in significance if carried out in conjunction with quantitative measurements of the rate at which the cells remove the dye from lymph or plasma. Our previous studies (12, 13) on the rate at which brilliant vital red and Niagara sky blue pass out of the blood stream into the tissues have not been extended in detail to other dyes, and for this reason it seemed desirable that we confine our microscopic studies to these two dyes.

We have found interesting cytological details which vary with the amount of dye and with the way in which it is given. Much of this work must be presented at a later time. At present we can consider only those findings most directly related to our previous studies on the rate at which these dyes leave the circulating blood.

Experiment 22. Moderate Staining of Dog with Brilliant Vital Red.

Dog 28–266. Brown terrier, 8.2 kg.

May 20–23 inclusive, 6 cc. 2 per cent brilliant vital red daily. Eight more on 24th and 6 cc. on morning of May 25. Animal killed with ether several hours later. Tissues moderately stained. Skin and connective tissues delicate pink. Lungs almost free of dye. Aorta moderately stained. Liver dark red. Bile thick and deep cherry red. Lymphatics at hilus of liver distended with clear reddish lymph. Spleen not enlarged but rather red and firm. Cortex of kidney moderately red. Medulla pale pink. Urine from bladder clear and yellow. Moderate staining of ileo-cecal lymph glands. Central two-thirds of each is bright red. Choroid plexus dark red, but meninges and brain unstained.

Microscopically, a bit of omentum spread out on a slide shows enormous numbers of macrophages. Careful inspection shows that they contain very large numbers of extremely fine pink granules. No conspicuous large clumps of dye in any of these cells. All these cells are stained very much alike and all have round or slightly oval nuclei. The cell bodies are irregularly globular. No dye in fibroblasts. No dye in endothelium of vessels, though elastic tissue of walls of certain vessels is pale pink. Many Kupffer cells contain moderate numbers of dye granules. No large dye clumps. Many convoluted tubules of kidney contain numerous fine dye granules in epithelial cells but no granules in part of cell next to lumen. Many granules about the nuclei or at the basal side of the cells. Hypophysis faintly pink, and frozen sections show numerous irregular triangular cells containing fine dye granules. They appear to be between the epithelial cells. No dye seen in the latter. Epithelial cells of choroid plexus contain numerous dye granules which are extremely fine and uniform in size. A few cells, apparently macrophages, between these cells contain more abundant dye.

Experiment 23. Heavy Staining of Dog with Brilliant Vital Red.

Dog 28–93. Fat female poodle, 8.0 kg,

April 24–May 1, 24 cc. 2 per cent brilliant vital red daily. Dog somewhat weak on last day. Centrifuged plasma obtained five minutes after last dye injection contained 1030 mg. dye per liter; six hours later 830 mg. Dog then killed with ether. Connective tissues deep red, especially the fascia of the muscles. Liver, spleen and kidneys deep red. Aorta and other large vessels deeply stained. Microscopically, elastic tissue of aorta is pink. Bits of mesentery and omentum spread out on slides show many macrophages varying somewhat in size, the largest ones being about 30–40 micra in diameter. The granules of dye which they contain are decidedly irregular and angular and variable in size. No crystal formation in any of the cells. Apparently the central portions of certain of the larger granules are a trifle paler than the peripheral portions. Epithelial cells of liver contain no dye but the Kupffer cells are much more numerous than normal and contain many large and small dye granules. Many small angular cells, apparently endothelial cells, in adrenals contain dye granules but no dye seen in the epithelial cells. Fibroblasts in connective tissue films and in mesentery and omentum have distinct oval nuclei and there are many small spherical uniform dye granules in the cytoplasm. Such granules are present also in the ramifying processes of these cells. This staining of fibroblasts is very faint.

Experiment 24. Staining of Dog with Brilliant Vital Red. Examination of Connective Tissue Films from Thigh.

Dog 28–281. Short-haired brown adult mongrel, 12.6 kg.

July 1–4 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Preceding the injection on July 4 an area on inner side of hind leg was shaved and with aid of novocain a small incision in skin was made and bits of connective tissue removed from between muscles. Film preparations covered with glycerine and cover-glass were examined with oil immersion lens. There are moderate numbers of dye-laden cells, most being rather thin and somewhat elongated, but not so much so as fibroblasts. Some cells contain more dye than others. All granules are rather uniform in size and none are extremely large. Most cells contain between 30 and 50 granules each.

July 6–9 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Films again examined on July 10. Preparations rather variable but rather clearly there are more dye-laden cells than on July 4. The enlargement of individual cells is very stiking, most of them being several times as large as on July 4, and they are more rounded and many of them are much more than 30 micra in diameter. The dye granules are still uniform in size, though posssibly somewhat larger. The increase in number of granules per cell is very striking. Many contain well over 100, and some more than 150. The intensity of dye in individual granules appears unaltered. No change in shape of dye granules.

No more dye injections made, but during following days skin and mucous membranes became more pale. Films again made on July 17. There appear to be

as many dye-laden macrophages as before, and dye-containing fibroblasts are possibly more numerous. They contain many fine granules of dye but these granules are quite small. The macrophages appear unaltered in size and the granules are certainly not less numerous but perhaps slightly smaller. Very clearly the granules vary more in size and many macrophages contain 1–3 large irregular granules or clumps of dye. No evidence of change in shade or quality of color of dye granules, though a few appear a little paler than before.

The general distribution of brilliant vital red is rather similar to that of dyes studied by Ribbert (10), Goldmann (2), Schulemann (3), Kiyono (5) and others. Experiment 22 will serve to illustrate the findings where the tissues are but moderately stained with this dye. In contrast to this experiment the tissues in experiment 23 were heavily stained. This was quite evident on gross inspection of various organs and tissues.

Histological study shows in each case the formation of dye granules in widely scattered cells. Most brilliant deposits are seen in the Kupffer cells in the liver, in the sinus endothelium of the spleen and in the phagocytes of the splenic pulp. We recognize also the well-known deposits in the sinus endothelium of lymph nodes, in the endothelium of the capillaries in the adrenal cortex, and above all the deposits of dye in the macrophages scattered throughout the connective tissues generally. As Evans and Scott have shown, the fibroblasts take up dye much more feebly than do the macrophages, but in experiment 23 so much dye had been injected that the fibroblasts show slight though definite granules of dye. This participation on the part of new cell types helps explain the ability of the body to handle ever increasing amounts of dye. There is also reason to believe that large or often repeated smaller doses of dye stimulate proliferation on the part of the more active types of phagocytes.

Investigators have repeatedly stressed the fact that with chronic vital staining one sees increased numbers of Kupffer cells in the liver sinusoids. This is entirely in accord with our own observations. It is difficult to estimate an increase in the number of macrophages in the connective tissues, for they are always irregularly scattered and it is difficult to get representative preparations. Nevertheless it does seem that some such increase does occur. Cell proliferation on the part of phagocytes represents an adaptation in time of

stress, and the dye storage by fibroblasts must also help to rid the circulation of these foreign materials.

We must recognize that the cell itself has remarkable powers of adaptation. Thus, the heavily stained animal (experiment 23) has much larger dye granules in the cells than does one which is feebly stained (experiment 22). Along with this increase in size of granules it appears that the individual cells increase somewhat in size.

We must confess that frozen sections and teased preparations of the internal organs are not entirely satisfactory for the study of fine cytological details, especially in the case of dyes which so readily diffuse out of the cells after death. It is much easier to get satisfactory preparations from the subcutaneous tissues of the thigh. Evans and Scott (9) have made beautiful films of such tissue and we can agree with them that with proper precautions one can make out the finest details regarding the shape, size and even the number of granules within the cells. Our observations on these tissues show that cell granules may increase markedly in number during the earlier stages of staining and as representative of these observations we may cite experiment 24. We note that in this experiment the increase in size of the macrophages is quite evident as soon as they begin to show considerable deposits of dye granules. In fact it would seem that the increase in size is almost proportional to the increase in the amount of dye within the cell. The result is that the dye granules seem to be no more closely packed than they were during the earlier stages of staining. It is noteworthy that in this experiment there is very clear evidence that the dye granules have increased greatly in number. It would seem that after certain granules had reached a certain size, new foci are formed, but our inability to find numbers of these newer and smaller foci would suggest that relatively few active centers of growth occur at any one time but that these centers grow rapidly and newer ones are then formed.

This experiment appears to be out of agreement with experiments 22 and 23 just cited. These pointed to the conclusion that many small granules are present at first and that they all enlarge to reach the size seen in the more heavily stained animal. We are unable to state the factors which determine the growth by accretion on the one hand or the growth by new granule formation on the other. We believe that both processes occur. Perhaps each mode is character-

istic of certain cell types, but slight differences in mode of administering the dye must also be considered. The importance of the time factor is evident from experiment 24 just mentioned, for after the dye injections had been discontinued for one week we noted that each macrophage contained several large irregular granules as well as many smaller ones. There is rather obviously a decrease in the staining of the tissue with the passage of time and this can be seen in gross as well as from the microscopic study. The granules do not seem to have decreased greatly in number, though many of them appear smaller. It would seem that some had grown at the expense of others or that certain of the granules had fused to form these larger masses. We are carrying out further experiments in an attempt to clear up these and other closely related points. It is felt that such data gain greatly in significance if carried on together with studies of the rate at which the dye leaves the blood stream, for these latter data give us much important information concerning the functional activities from a quantitative point of view.

Of great interest from the physiological point of view is the distribution of the dye in organs such as liver and kidneys whose activities in secreting various dyestuffs is common knowledge. Brilliant vital red belongs to that group of dyestuffs which passes into the urine only in small amounts. Unless very large amounts of dye are injected the small amounts of dye present in the urine are so overshadowed by the normal urinary pigments that they would not be recognized by gross inspection, but if the urine be passed several times through filter paper the latter will be found to be stained a delicate pink color. This staining is dependent upon the selective adsorption of dye by cellulose.

That the kidneys secrete so little dye is distinctly surprising in view of the fact that certain of the convoluted tubules contain such beautiful and conspicuous granules of dye. On the other hand, the epithelial cells of the liver contain no granules of brilliant vital red and yet the dye passes into the bile in considerable quantities (15–30 per cent in 24 hours). These facts support the numerous investigations of recent years which are leading to the point of view that granule formation in the epithelium of glands is a process which may be quite unrelated to the question of whether or not the gland can excrete that particular dye. Very obviously the dye can pass through the epithelial cells without forming any granules in the cytoplasm. In fact a dye may gain passage quite rapidly, so that the amount in the cytoplasm at any one time may never be enough to produce even a noticeable diffuse staining.

In general the distribution of Niagara sky blue in the body is quite similar to that described for brilliant vital red. A few minor differences are seen. From the curves given in a previous paper (14) it is evident that during the first few hours Niagara sky blue leaves the blood stream distinctly more rapidly than does brilliant vital red, though an equilibrium partition between tissues and body fluids seems to be reached rather sooner, and at a time when relatively large amounts of the dye still remain in circulation. We have repeatedly observed that animals stained with the blue dye show conspicuous staining of skin and mucous membranes many days longer than do dogs stained with similar amounts of brilliant vital red. We have no way of knowing whether this is associated with differences in elimination from the body, for unfortunately the blue dye unlike the red one is partially decolorized on admixture with bile, so that it is impossible to compare the elimination of the two in fistula dogs. The blue dye passes into the urine somewhat more readily than does the red one, but the total lost in that way is not great.

The ability of the body to decolorize many dyes is common knowledge. Some are decolorized almost immediately. Teploff (15) shows that carmine granules within the cells gradually change in shade and become yellow or black in the course of weeks, indicating slow chemical transformation. We have studied connective tissue films removed from animals at intervals following a series of injections with brilliant vital red, but we have been unable to see any change in the shade of brilliant vital red with the passage of time. We have not yet made microscopic examination of the internal organs during this stage of regression, and the possibility remains that some of the dye may undergo such change in these organs. We do know that any decolorization which does occur is not great, for it is often possible to recover 50 to 85 per cent of the dye in the bile in the course of 5 or 6 days. After that the bile may still show a reddish tint, but the amount of dye present is too small to be measured in the presence of the bile pigments. We believe that most, if not all, of the dye gradually passes from the tissues back into the blood, from which it is removed and eliminated by the liver. It is only in a few places as in the centers of the lymph nodes that the dye is retained over a period of months. Certain of these cells seem to hold to the dye with the greatest tenacity, though in time they too lose their dye.

Experiment 25. Staining of Dog with Mixture of Brilliant Vital Red and Niagara Sky Blue.

Dog 27–107. Female mongrel, 9.0 kg.

Injection mixture made by mixing equal parts of 1.5 per cent brilliant vital red and 1.5 per cent Niagara sky blue. Five daily intravenous injections of 14 cc. each. No dye on 6th day. Animal killed with ether on 7th. No toxicity. Tissues uniformly purple. Endocardium of heart and walls of larger vessels bluish-purple. Lungs faintly stained, and in fibrous tissue frame-work are seen irregular cells containing purple granules of dye. Many phagocytic cells in splenic pulp. They contain reddish-purple dye granules. Liver purple. Kupffer cells contain purple granules. Epithelial cells free of dye. Epithelial cells of kidney contain many dark purple dye granules. No pure red or blue granules. Macrophages in areolar tissues and in mesentery also contain only purple granules.

In conclusion, no cells anywhere contain either pure red or blue granules though all granules in spleen were reddish-purple while those in liver and kidneys were bluish-purple.

Experiment 26. Staining of Rabbit with Mixture of Brilliant Vital Red and Niagara Sky Blue.

Female rabbit, 3.1 kg.

Injection mixture made by mixing equal parts of 1.5 per cent Niagara sky blue and 1.5 per cent brilliant vital red. On first day 3 cc. injected. None on second day, but on following days, 5th, 6th, 7th, 7 cc. respectively were given. No toxicity. On following day animal killed with ether. Skin and mucous membrane quite purple. Spleen, liver, and kidneys also. Macrophages in connective tissue of thigh and in omentum show many discrete reddish-purple granules. Some of those in phagocytes of spleen are purplish-blue; others bluish-purple. Epithelial cells of liver contain no dye granules. Kupffer cells contain many irregular purple granules. Epithelial cells of convoluted tubules of kidney are bluish-purple.

Data of great interest are to be obtained by administering two different dyes to the same animal. It is well that they be of widely different color in order that one may recognize each in the cells. We have already shown (16) that purple plasma containing a mixture of red and blue dye may be analyzed in the spectrophotometer and the amount of each dye present in the mixture determined, and with this method it was possible to follow the elimination of each dye from the blood stream even though both dyes were present together in the plasma. It was shown (14) that each dye is finally distributed between plasma and tissues in a way characteristic of that particular dye and at a rate quite independent of the presence of the other dye in the plasma and

tissues. In certain of these physiological studies the two dyes were injected simultaneously. In others a period of intensive staining with one dye preceded the injection of the second. Histological studies of various tissues are necessary as a basis for any attempt to interpret these results.

Dog 27–107 (experiment 25) received 105 mg. of each of the two dyes daily over a period of 5 days. Gross inspection of the skin and mucous membranes showed progressively increasing purple coloration, and the animal when killed with ether showed similar changes in the internal organs. It is of interest that the aorta, and larger blood vessels generally, show a distinct excess of the bluish coloration, but there is an element of red also. This is quite in agreement with our previous observation that elastic tissue shows greater affinity for Niagara sky blue than for brilliant vital red. Microscopical examination of the aorta always confirms the gross findings and shows that the color is due to diffuse staining of the elastic fibers. Other tissues show the phagocytic reaction in a very beautiful manner. The Kupffer cells of the liver, the endothelial cells and pulp cells of the spleen, the phagocytic cells of lymph nodes and the macrophages of the connective tissues contain beautiful deposits of dyes. We were rather surprised to find that almost without exception the granules within the cells contained a mixture of the two dyes, and as a result were purple.

In one or two experiments of this sort we have found an occasional granule which appeared to be either pure red or pure blue, but this is the exception. Experiments of this sort demonstrate that these two dyes are distributed within the body in almost identically the same manner. The existence of mixed granules indicates that even within the substance of the cell the protoplasm does not discriminate between the two. Experiment 26 shows practically identical results when the two dyes are given simultaneously to a rabbit. We have other experiments with dogs and with rabbits and all of them are in agreement.

Experiment 27. Staining of Dog with Niagara Sky Blue Followed by Staining with Brilliant Vital Red.

Young female mongrel dog, 5.5 kg. December 16, 20 cc. 2 per cent Niagara sky blue intravenously. Similar injections on following day. December 18th and again on 19th, 20 cc. 2 per cent brilliant vital red intravenously. No toxicity. Animal killed with ether on December 20th. Skin purple. Subcutaneous con-

nective tissues bluish-purple. Lungs very slightly stained. Endocardium of heart dark purple. Aorta purple, and microscopically elastic fibers in it are diffusely stained more blue than red. Many fibers from tough subperitoneal tissue are pure blue, others bluish-purple. Spleen purple. Malpighian bodies paler and grayer than pulp. Microscopically many large irregular cells contain granules varying greatly in size and shape. Many cells contain more red dye than blue and a few contain red ones only. A few small cells contain only blue granules. Many cells contain both types but no purple granules found. Liver deep bluish-purple. Epithelial cells contain no dye granules but Kupffer cells are very large and numerous. They contain many granules of both colors but blue ones are more numerous. A very small number of cells contain only red ones and these are rather less irregular in shape than the rest. No purple granules in any of the cells. Bile deep orange color. Kidney cortex stained but medulla pale. Microscopically many granules in cells of convoluted tubules. They vary greatly in size and shape. They are red, triangular masses and granules and rods with projecting knobs, some of these knobs being of a different color than the main mass, and often granules of different color are in contact but there are no purple granules. Urine olive color, clear. Mesenteric lymph nodes deep purple. Many phagocytes contain both dyes separately but no mixed granules. Very few cells contain only one type of dye. Mesentery contains many cells with granules more or less spherical. Red and blue granules about equally numerous. In places the two types of granules seem to be loosely in contact but in no place are they fused and in no case is a zone of one dye layered about the other.

Experiment 28. Rabbit Stained with Brilliant Vital Red Followed by Staining with Niagara Sky Blue.

Male rabbit, 2.7 kg.

Five daily intravenous injections of 3 cc. brilliant vital red (1.5 per cent). Two days later 3 daily injections of 4 cc. each of 1.5 per cent Niagara sky blue. Animal killed with ether forty-eight hours later. Mucous membranes, kidneys, liver, spleen quite purple. In liver and spleen there is a rather less amount of both dyes than usual but red dye is more abundantly present than blue. Some Kupffer cells contain only red dye but many contain granules of each. No purple granules. No granules in epithelial cells of liver. Epithelial cells of convoluted tubules of kidney contain red and blue granules but no purple ones. In general red granules are more in evidence in the part of the cells most distant from the lumen, while in the areas bordering lumen blue granules predominate. Beautiful double segregation of dye in macrophages in connective tissues and mesentery. Very few cells contain one type of dye only. No purple granules anywhere. The blue dye is rather more in evidence in these cells than in Kupffer cells of liver and phagocytes of spleen.

A very beautiful contrast to experiments of this sort is afforded if the two dyes be injected *in successive periods*. Thus, in experiment 27 we

gave 2 daily intravenous injections of 20 cc. of 2 per cent Niagara sky blue to a dog; this was followed by 2 daily injections of brilliant vital red. The tissues, at first blue, now took on an intense purple color. At autopsy the gross appearance of the organs was identical to that when the two dyes had been given simultaneously, but the microscopical picture was surprisingly different. There was little difference as regards the diffuse staining of the elastic fibers, but we were unprepared to find that in no instance were the two dyes associated to form purple granules. Quite in contrast, the phagocytic cells scattered throughout the body contained innumerable red granules as well as innumerable pure blue granules. In almost every case these two types of granules were clearly distinct from each other though here and there granules of the two types appear to lie in contact, but neither were they fused to form mixed purple granules nor was the red dye layered about the blue dye granules as one might expect from the mode of administration. Further experiments of this sort in dogs confirmed these results. In a number of experiments on rabbits similar results were obtained. The protocol of experiment 28 is typical.

These observations raise a number of important considerations regarding the mechanism by which dye granules are formed within cells and we shall return to this subject later. We may recall here our previous inability (14) to "block" the tissues with one dye against the entrance of another. Without detailed microscopic study one might have suspected that the two dyes were taken up by two entirely separate and distinct sets of phagocytic cells, but we have just seen that such is very clearly not the case, no matter whether the dyes are given simultaneously or in sequence. It is now obvious that in the case of these two dyes the cells may contain large quantities of one dye and still fully retain their ability to take up another at the normal rate. This is all the more remarkable when we realize that these two dyes are rather closely related in their chemical properties, and with the exception of their color in most of their physical properties also. If "blockade" is ever to be obtained, one would expect it in the case of such closely similar substances which are taken up by identically the same cells and, indeed, under certain circumstances brought intimately together within the same granules. Whatever the mechanism of the segregation of dye may be, it is at least clear that

the cells react toward each dye in a way which is independent of how much of the other dye may be within the cells, or in the surrounding fluids.

In this connection we wish to emphasize our observations (14) that the amount of either dye which may be taken up is related to the concentration of that dye in the surrounding fluids. Very evidently, each dye establishes its own partition between cells and fluids independently of the presence of the other. It is tempting to compare the partition of dyes between cells and fluids to the phenomenon of partition well known to physical chemists, where a substance may be distributed between two immiscible fluids and the relative amount in each layer may be very slightly or not all influenced by the presence of other similar substances which may undergo partition according to laws peculiar to themselves. We recognize the dangers of such analogies and we do not wish to pursue the comparison further, but it is significant that in the physical as well as in the biological phenomena several substances may undergo partition largely independently of each other.

We have injected dogs with Higgins' "American" India ink and have studied the influence of this procedure upon the rate at which brilliant vital red will leave the blood stream. A report already made (17) shows that in such animals there is a very marked retention of dye in the circulating blood, and at first we had thought that the ink previously given had "blocked" the phagocytic cells or had reduced their ability to take up dye. Studies of this sort made on bile fistula dogs showed that a different explanation is much more likely, for these experiments showed that during the period of observation almost no dye is excreted into the bile if the dog had received ink at the beginning of the experiment. Control experiments showed that normally such dogs would secrete rather large quantities of dye during the four-day period of observation. The failure of the ink-treated dogs to excrete the ordinary amount of dye through the liver seemed to account quite adequately for the longer retention of the dye in the circulating blood. Under these circumstances the dye is largely retained in the body, and throughout the course of the experiment more dye was present both in plasma and in tissues. Had we remained in ignorance of this altered liver excretion we might have fallen into the error of assuming a blockade on the part of the phagocytes. It seems quite possible that others have made such mistakes when studying "blockade." Many dyes are normally eliminated by liver or kidney much more rapidly than are brilliant vital red or Niagara sky blue, and, with those dyes especially, any disturbance in the excretory power of liver or kidney would pro-

duce even more striking retention in the blood stream. Any such retention must never be credited to defective phagocytosis until all of these other factors have been given the most careful attention. We feel that experiments indicating the presence of a "blockade" should be controlled by the study of elimination through liver and through kidneys.

We have made some little progress in determining how ink inhibits the excretion of the dye by the liver, but the experiments are not yet complete. Preliminary experiments indicate that some component of the ink other than the carbon is responsible, for experiment shows that the carbon can be removed from India ink without robbing the latter of its inhibitory power. Furthermore, finely ground graphite suspensions were shown (17) to be inert. Were it not for these experiments we might conclude that the failure of the liver to excrete dye was in some way associated with the fact that the liver comes to contain large quantities of carbon, deposited largely within the Kupffer cells of the liver sinusoids.

From a somewhat more restricted point of view, a study of the distribution of ink particles in the body offers much of interest, for these details are intimately concerned with the entire problem of phagocytosis and blockade of phagocytes. The distribution of the carbon particles in the body differs from that of our vital dyes in several interesting and fundamental respects, and it seems desirable that substances so radically different from the vital dyes should be studied in conjunction with the latter in order that we may learn the physiological response of the phagocytes to each and detect and differences which exist.

For the distribution in the tissues of ink or graphite suspensions introduced into the blood stream we refer particularly to the articles of Goldmann (2, 18), Kiyono (11), Nagao (19), Foot (20), Wislocki (21), Brickner (22), Palmer and Higgins (23), Higgins and Murphy (24).

DISCUSSION

Most recent workers agree that there are important differences between the behavior in living tissues of "acid" and of "basic" dyes. Perhaps the difference is associated with physico-chemical reactions involving the positively charged colored ion of "basic" dyes and the negatively charged colored ion of "acid" dyes. Möllendorff (6) and Herzfeld (25) have stressed the idea that the "basic" dyes unite with electro-positive structures normally existent in many cells. They have also stressed the view that the "acid" dyes do not unite with preexisting structures within the cell, but that their storage is a process closely related to the phagocytosis of larger particles, if not identical with it. It is common knowledge that these two classes of dyestuffs may enter the same individual cells, but in general the distribution in the body and the details of staining are profoundly

different. We draw attention to these facts lest our experiments with the "acid" dyes, brilliant vital red and Niagara sky blue, be confused with known facts obtained with basic dyes.

We do not propose to discuss the many differences which exist between the various "acid" dyes.

Special reference is made to the comparative studies of Schulemann (3, 26). He and other workers have reported experiments involving double staining with acid dyes. It would lead too far to discuss in any detail the confused literature dealing with the subject. We may refer to Steckelmacher (27), Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8) for literature. No really systematic study of the subject has been undertaken and it is no wonder that confusion prevails when the literature consists largely of isolated observations with the widest variation in dye, in species of animal and in mode of administration. Among the best controlled experiments on this subject are those of Steckelmacher (27). With the aid of lithium carmine and tolidine blue he observed double staining in liver and kidneys. His work on the frog and ours on mammals would seem to indicate that the mode of administration is of the greatest importance in the formation of mixed granules. This point of view was recognized by Schulemann (3) in certain of his earlier studies with trypan blue and vital new red. Separate injection of these two dyes gave him separate blue and red granules side by side within the cell. He encountered difficulty in producing purple granules when the two dyes were injected simultaneously, but he pointed out that vital new red is much more diffusible than trypan blue and that in all likelihood most of it is already anchored within the cells before much of the blue dye has had time to enter the cells. The result is as though the two dyes had been injected at separate intervals, and for this reason Schulemann was not surprised at his failure to find purple granules. Unfortunately he did not test out two dyes which were more nearly alike in regard to speed of penetration.

Our experiments as well as those of Schulemann and Steckelmacher all represent a rather acute type of staining. In no case did the process of staining extend over a period longer than a few days. In our own case this was felt desirable in order that the experiments might serve as controls on our previous measurements (12, 13) of the rate at which these dyes are taken up from the circulating blood.

Evans and Scott (9) report studies on vital staining in which the dyes were administered over a period of several weeks. They were particularly interested in the crystals of trypan blue which may form in the cytoplasm in these chronic types of experiments. They report finding separate deposits of trypan blue and T 148 even when the two dyes were given simultaneously, and when given

separately they found purplish granules as well as red and blue ones. The influence of the longer administration should be studied to compare the results with experiments like ours and Steckelmacher's. Admittedly the two dyes used by Evans and Scott differ considerably from each other and this must be kept in mind when attempting to correlate their results with ours.

The method of double dye injection should be of great service as an aid in the study of just how dye granules are built up within the cell. The difference in color makes it possible to follow the sequence of events. Our own experiments suggest that granules may be built up in several ways—either a few at a time, or many simultaneously and at nearly equal rate. Many more data must be secured before we can assign proper importance to the various factors which determine these finer cytological details. It should be evident that such information should be developed as a basis for physiological concepts and for physiological experiments on distribution of dye between tissues and surrounding fluids.

The experiments of a previous paper (12) showed that when large amounts of dye are given all at once or over a period of several days there is a well marked delay in the rate at which the dye leaves the blood stream. It was shown that *a priori* this may be due to a sluggish response on the part of dye-laden phagocytes, or to inefficient excretion of dye into the bile. Actual experimental study of these two possibilities showed that there is undoubtedly defective liver excretion when large doses of dye are given to the animal. In the course of 24 hours only 10 or 15 per cent of the dye may reach the bile, in contrast to 25–35 per cent when smaller doses are given. We expressed our belief that the longer retention in the blood stream may be due to this defective liver excretion alone, and not to faulty phagocytic power on the part of the tissues. If this is so we must accept the view that in the range of dosage studied, the distribution ratio of dye between plasma and tissues is constant, regardless of the dose employed. We may well be surprised at such a conclusion. It would seem to imply a vast reserve of phagocytic power not previously suspected. If we choose to believe that the tissues adapt themselves to the increased burden of larger dosage, we must also admit that this adaptation is almost instantaneous, for there seems to be no lag in the response when large doses of dye are injected or when a second dye is given simultaneously with the first (12).

Our experiments just outlined suggest a number of methods by which the tissues may adapt themselves. The increase in the number of phagocytes is one which we have observed, and this is commonly cited by others. We may also point out that increase in the size of the cell may help it to accommodate more dye without any loss of efficiency whatever. We may also call attention to the fact that with large dosage new types of cells, such as the fibroblast, take up part of the dye. This spread of the process of phagocytosis to an ever-widening group of cells may be of no little importance in maintaining the powers of the tissues to take up the large quantities of dye forced upon them. The existence of such compensating processes may cause less surprise than the fact that the process of compensation seems to be so prompt, with the result that we cannot even momentarily cause embarrassment to the phagocytic cells. We do not profess to know the relative importance of these various compensating mechanisms in relation to each other; nor may we overlook the possibility that there may be other important ways of maintaining the efficiency of this group of cells.

It may well be that methods may yet be devised to overload these cells. It may even be that refinements in the measurement of liver excretion will show that defective liver excretion will not account for quite all of the retention of dye in the plasma; but even so we cannot possibly deny the fact that the phagocytes have enormous reserve power available, or that they can adapt themselves almost perfectly to added labor within very wide limits.

The data already reported (12, 13) have given us a quantitative concept of the rate at which the body phagocytes will remove certain dyes from the circulating lymph and plasma. Those experiments on healthy living animals furnish information concerning the sum total response of all these cells combined. Details concerning the individual cells and concerning separate groups and classes of these cells are to be obtained by histological study. The attempt to combine the anatomical with the physiological approach has proved most stimulating. The results at present available represent but a beginning. Many data must still be gathered before we can know more than the elementary anatomical facts concerning the building up of dye granules within the cell. In certain instances it would seem that from the very

first the dye is deposited in a vast number of small foci within the cell, and that these tiny granules grow in size as more dye reaches the cell. In other cases it would appear that relatively few foci are active at any one time and that when the granules reach a certain size, new foci of deposition are formed within the cell. The results obtained when two dyes of different colors are given simultaneously or in sequence throw light upon these points.

Intimately connected with the theory of this granular dye deposition is the fact that as the dye deposits increase, the cell may enlarge so much that there may be no more crowding of the granules than in the earlier stages of staining when the cell is smaller and contains fewer granules. Yet, in other cases actual crowding undoubtedly does occur and with certain dyes the granules may agglutinate and needle-like crystals may even form. We believe that differences in dye employed and differences in administration account for many of these discrepancies to be found in the literature. A few of these variations are analyzed in the experiments here presented. All these anatomical details throw light upon our studies of the rate at which the dyes pass out of the plasma into the tissues. Very obviously it is desirable that we know whether the disappearance rate of dye from plasma is associated with increase in size and number of the phagocytic cells.

Our previous experiments have shown that a given dye passes from the surrounding fluids into the cell until a certain equilibrium point is reached. Dye accumulates within the cell and we find that the phagocytic activity comes almost to a standstill long before the fluids have given up all of their color. Control experiments show that dye-free phagocytes will function quite readily in the presence of plasma containing similar amounts of dye. We found also that these phagocytes may contain large amounts of this dyestuff and yet they can take up another closely related dyestuff in quite a normal manner. The histological details regarding the distribution of these two dyes form an interesting and important supplement to those observations.

SUMMARY

Vital staining reactions of brilliant vital red and Niagara sky blue are studied in dogs and in rabbits. Either dye alone is taken up to form red or blue granules within the cytoplasm of macrophages and of certain other cell types.

When the two dyes are injected simultaneously into the blood stream one finds that these cells build up granules which are purple from admixture of the two dyes. When several daily injections of one dye are followed by several daily injections of the other, one finds blue granules and red granules side by side within the cells, but no purple granules are found. This is thought to indicate that the dye is deposited in small foci which are active in a rather transitory way, and that the color of the granule is determined during its formative stage by the type of dye present in the fluids about the cell.

The enlargement of phagocytic cells and the increase in their number with large dosage, or with repeated offerings of the dye, represents a method by which the cells maintain their phagocytic powers at the normal level. Evidence is offered to indicate that these and perhaps other compensatory changes may take place with great rapidity, so that it has been impossible to "block" or even reduce noticeably the ability of these cells to take up additional quantities of dye.

Certain pitfalls in the experimental study of "blockade" are pointed out.

The authors of this and the 4 preceding papers of this series wish to express their appreciation to Dr. G. H. Whipple and to Dr. G. W. Corner for aid so kindly given in the preparation of the manuscripts.

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