VITAL STAINING OF THE CONNECTIVE TISSUES

By LESTER S. KING, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

PLATE 1

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When trypan blue is injected intravenously, the animal appears blue within a few minutes, not only in its integument, but in the internal organs as well. 12 or more hours after the administration of the dye, there are innumerable granules within macrophages and other colloidopexic cells, easily demonstrable by ordinary histological technique. But the animal is blue long before such granules are formed. In the extensive literature on vital staining with the acid colloidal dyes, most of the attention has been focused on the formation of intracellular granules and on the problems of the reticulo-endothelial system. The present investigation is concerned with the problem, where, apart from the blood stream, is trypan blue before it is flocculated?

After trypan blue is introduced into the circulating blood, some is excreted almost at once, while much of it passes into the tissues with equal rapidity. Once the dye has passed the endothelium, its further course has not been adequately studied. Petroff (1) found that, in the mesentery of the living frog, the walls of the vessels may become colored in less than 20 minutes after the dye is administered. He concluded that such staining of the vessel walls is due to the specific capacity of the elastic elements to adsorb the dye in question. In this and several similar investigations (2–6) interest has focused chiefly on the passage of substances directly from the blood through the endothelium of the larger blood vessels, and on the possible relations of these data to the problems of atherosclerosis. Apart from the vital staining of the elastic tissue in the blood vessel walls, however, the locus of the dye, before the formation of granules, is not satisfactorily explained. Cappell (7), for example, says that, after an intravenous injection, when the animal becomes blue in a few minutes, "thorough microscopic examination of the tissues at this time shows no evidence of the dye, which is located in the body fluids."

Methods

In the present study, guinea pigs and white mice were used. Trypan blue was administered intravenously, in varying dosages, and tissue was examined from 10

minutes to 72 hours after the injection. In some instances small portions of tissue were placed directly into glycerin, and examined immediately under the microscope. This sufficed to show all the principal features, but for more detailed study fixation and sectioning were necessary. Several fixatives were tried, but by far the most satisfactory was the Heidenhain's Susa mixture (formalin 20, corrosive sublimate 5, NaCl 0.5, acetic acid 4, trichloracetic 2, aq. 80). Fixation for 2 hours was adequate. The tissue, without washing, was then rapidly dehydrated in several changes of 95 per cent and absolute alcohol within a period of 4 to 5 hours, cleared in chloroform, and embedded in paraffin. Sections were cut at 30 microns, and, after removal of mercury precipitate, were examined without counterstain. Nuclei could be very readily seen by closing down the substage diaphragm.

In the study of the brain, the method of Spalteholz was utilized in some instances. The brains (entire) were fixed for 3 hours at 37°C. in absolute alcohol, followed by 2 to 3 hours in absolute-benzol, cleared in benzol overnight, and in oil of wintergreen the next day.

Mice proved to be far more satisfactory than guinea pigs for intravenous injection of trypan blue. 5 mg. of the dye (0.5 cc. of a 1 per cent solution) are supported by an 18 gm. mouse without difficulty, and produce a deep coloration. A 300 gm. guinea pig, on the other hand, has often succumbed within an hour to 15 mg. of the dye, even when injected very slowly. Even if it survived, the resulting coloration was not intense. To produce a satisfactory degree of vital staining in a guinea pig with a single injection, 30 to 40 mg. are necessary, and in the great majority of cases this results in death within a few hours or less. At such time the features described below are vividly discernible, but in order to compare such findings with those in animals 24 to 72 hours after injection, mice were used for the most part.

In mice, on different occasions, as much as 15 mg. have been given intravenously (0.75 cc. of a 2 per cent solution) without causing death. Very rarely a mouse receiving 5 mg. intravenously dies 24 to 36 hours after injection, but this is in marked contrast to the behavior of guinea pigs, in which death occurs in a few hours.

Many of the animals had previously been used in experiments with equine encephalomyelitis, but had survived without symptoms. The findings in such survivors, however, were identical in every respect with those in normal animals.

Out of the large number of mice and guinea pigs studied, a series of 30 mice, 5 to 6 weeks of age, and about 17 to 19 gm. in weight, were treated in the following manner. Half were given 2.5 mg. of dye intravenously, the other half 5 mg. Animals were sacrificed at intervals of 10 minutes, 1, 4, 24, and 72 hours. The following organs were prepared for examination as described above: bladder, small intestine, mesentery, kidney, liver, spleen, diaphragm, chest wall, lung, aorta, esophagus and trachea, ear, and brain. Practically all the other organs have been studied at one or another time, but for systematic observation the foregoing seemed an adequate selection. The coloration of animals receiving the smaller dose

differed only in intensity. 2.5 mg. of dye were adequate to show all the features described below, but twice that dose made examination and photography simpler.

General Features of the Staining

A mouse of 18 gm., examined 10 minutes after the intravenous injection of trypan blue, already shows the fixation of the dye by the intercellular connective tissue elements. These elements are three in number,—collagen, reticulin, and elastic tissue.

Collagen.—Union of dye and tissue is most brilliantly in evidence in hollow viscera with considerable connective tissue beneath the epithelium, that is, with a well developed lamina propria; *e.g.*, the urinary or gall bladder, ureter, vas deferens, esophagus, to a lesser extent the intestines, and the like. Such organs are, macroscopically, a deep blue. Microscopic examination (Fig. 1, esophagus) shows an unstained epithelium but a very deeply stained tunica or lamina propria. The muscle fibers themselves are colorless, although connective tissue septa stand out with a prominence proportional to their size. The adventitia stains, but somewhat less intensely.

A second example, here illustrated (Fig. 3), is the abdominal wall of a mouse. (The anterior abdominal wall was rolled up before fixation, and the architecture is consequently distorted.) The fascial planes are intensely blue, while the connective tissue septa are stained somewhat less deeply. Excellent preparations may also be made from the diaphragm, where the pleural and peritoneal surfaces stand out vividly against the colorless muscle fibers.

The connective tissue partitions of all the organs are stained in a similar fashion. Such glands as the thyroid, submaxillary, or testis, with abundant collagen, or the liver, with proportionally less, as well as the connective tissue coats of the hollow viscera or of the skin, all exhibit the same selective vital staining of the collagen. This staining is diffuse and not particulate or granular. The collagen bundles are stained in their entirety.

This staining of collagen is independent of the density of the tissue or the degree of vascularization. The compact connective tissue, such as dura mater, capsule of the kidney, periosteum, endosteum and perichondrium, all fix the dye intensely after but a few minutes. Tendon acts in a similar fashion, although the stain is not so deep.

For example, the central tendon of the diaphragm, or the Achilles tendon, binds trypan blue in a diffuse manner, easily visible in both fixed and unfixed preparations, long before there are any granules formed intracellularly.

Reticulin.—Reticulin may be defined as the connective tissue fibers which stain black with ammoniacal silver solutions, in contrast to collagen which stains brown (or rose to rose-purple, if the section is toned with gold). These argyrophile fibers, which have a very wide distribution in the body, differ markedly among themselves in size. They differ as well in their capacity to fix trypan blue. In fat depots, such as in the mesentery, or more especially the pelvis of the kidney, the intercellular connective tissue shows a vivid degree of vital staining. On the other hand, the network of fine argyrophile fibers which surround skeletal muscle fibers remains completely uncolored. The reticulin of the spleen or bone marrow stains but little or not at all. In the spleen some of the scanty reticulin within the follicles may at times assume a blue color, but a similar staining in the reticulin of the pulp has not been observed. There are undoubtedly several unknown factors involved, of which one may be that of sheer mass.

Elastic Fibers.—The staining of elastic fibers in the blood vessels has been adequately treated by previous investigators. A coloration of elastic fibers in the walls of pulmonary alveoli is readily observable, but this is most evident 24 hours after injection. Special note should be given to the capsule and trabeculae of the spleen. These are composed of dense collagen very rich in elastic fibers, and stain with great brilliance (Fig. 2). The coloration, however, is due to fixation of the dye by both collagenous and elastic fibers, and not to either separately.

The fate of the dye which diffusely stains the connective tissues is readily followed. It is slowly given up and at the same time is aggregated into intracellular granules by colloidopexic cells. As the diffuse staining diminishes, the granules in the histiocytes and other cells progressively increase in number. This is most noticeable in adipose tissue. At 10 minutes after the dye is given, the reticulin framework is clearly outlined in blue. At 4 hours, practically all the dye has been aggregated by the histiocytes and very little remains of the diffuse coloration. Collagen, however, holds the dye for a much longer time.

At 4 hours, a few histiocytes contain granules, but the general intensity of the diffuse staining is not much less than at 10 minutes. By 24 hours most of the diffuse coloration has disappeared from the looser types of collagenous tissue, while, correspondingly, intracellular granules have increased enormously in number. Where the connective tissue is dense, as, for example, in the periosteum, perichondrium, central tendon of the diaphragm, basement membrane of the trachea, and the like, the diffuse color persists even for 72 hours. In such regions not only are the histiocytes and fibroblasts filled with granules (these two cell types are, of course, readily distinguishable on the basis of vital staining), but the intercellular substance maintains a pale blue homogeneous tint. The elastic tissue elements in the blood vessels hold the dye for much longer periods, even after it has disappeared from collagen.

Special Instances

A few organs and tissues deserve special comment.

Kidney.—The dye is excreted very rapidly. Within 2 minutes after an intravenous injection a bluish red urine has been seen. The lumina of the kidney tubules, especially the loops of Henle and the collecting tubules, often contain large masses of precipitated dye. In the kidney substance proper, the reticulin framework and the basement membrane of the tubules are outlined in blue after 10 minutes. This disappears at the end of 4 hours. The first granules seen in the kidney are in the proximal convoluted tubules, that is, in parenchymatous cells. The granules appear after an hour and are situated between the nucleus and the free border of the cell. This occurs long before any granules in histiocytes are visible.

Liver.—Granules of trypan blue are seen within Kupffer cells after 10 minutes. At this time the dye is visible as small spherical masses, and only later do the typical crystal-like flecks and granules appear. The capsule and connective tissue septa, vividly blue after 10 minutes, fade rapidly. At 24 hours, there is but little diffuse staining, and at 72 hours none. During this interval, of course, the Kupffer cells and interstitial phagocytes become heavily crammed with granules, while the endothelial cells contain fewer and smaller dye particles. The liver cells of the mouse contain few or no granules, although the dye is very rapidly excreted into the bile.

Spleen.—The spleen forms a marked contrast to the liver. The capsule and trabeculae maintain their diffuse staining, with but little diminution in intensity at the end of 72 hours. In the pulp the reticulin does not stain. Some in the center of the follicles may be colored after 10 minutes to an hour, but this does not

last. Phagocytized granules are surprisingly scarce at 4 hours, and not very prominent even at 24 to 72 hours. In the parenchyma the intracellular granules are situated almost exclusively in the pulp, and are not nearly so vivid as in the other organs.

Blood Vessels.—The veins show a diffuse staining of their entire walls, which for the most part has quite disappeared at 24 hours. In the arteries the internal elastic laminae and the adventitia stand out sharply, even after 10 minutes. The muscle fibers are unstained. In the medium sized arteries some of the fine elastic fibers of the media appear a pale blue. In the aorta the internal elastic lamella stains almost at once, as do the external lamellae and adventitia, but the elastic fibers of the media are colored only after 24 hours. Figs. 5 and 6 show an aorta at 1 hour and 72 hours, respectively. The reason for the long time required to stain the media is not altogether clear. However, the elastic fibers, once stained, hold the dye very tenaciously.

Brain.—The brain tissue proper, which has no intrinsic connective tissue stroma, does not stain with trypan blue.[1] However, in the brain the dura mater, the stroma of the pineal gland, and the choroid plexuses stain at once, just as does connective tissue in other parts of the body. The leptomeninges can be seen diffusely stained only at times, where the trabeculae are concentrated in sufficient mass. Thus, diffuse staining in the adventitia of the larger blood vessels of the pia is quite readily seen, although color could not be detected in the finer reticulin strands. However, the dye must have attained even the finer meshes, for 24 to 48 hours after a single intravenous injection there are myriads of fine granules within the cells of the pia-arachnoid. Obviously, dye must have been present in order that granules be formed.

The cerebral blood vessels present an unusual feature (Fig. 4). Whereas the internal elastic membrane of other arteries is distinctly colored after 10 minutes, in the arteries of the brain such coloration is not very pronounced until 24 hours have elapsed. Then the elastic membranes appear very distinctly, even after a small dose of dye. The best method of demonstration is as follows: 24 to 72 hours after a single intravenous injection, the mouse is killed, the head perfused with saline through the aorta, the entire brain fixed and cleared by the method of Spalteholz, and examined under the low power of the microscope. The major arteries are clearly outlined by the elastic membranes, and can be followed for considerable distances. Adequate preparations can be made after an interval of 1 hour, but the results are more satisfactory after 24 hours. At the same time the wealth of granules in the pia can be seen to good advantage.

The dye passes through the vascular endothelium with ease, as evidenced by the granular vital staining of the leptomeninges. Why the elastic fibers are late in staining is not clear at present.

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[1] Certain exceptions to this statement, irrelevant here, are discussed elsewhere (8).

The problem of the "blood-brain barrier" in relation to vital staining is discussed in a separate communication (8). Attention should be called to the diffuse vital staining of the infundibular region which is considered more fully in a previous paper (9).

The union of trypan blue and connective tissue is not restricted to cases where the dye is given intravenously. In mice (20 to 25 gm.) receiving 5 mg. of the dye subcutaneously, the vital staining of the intercellular connective tissue is quite vivid after 2 hours, but not as sharp as with the intravenous route. The only intrinsic difference between these two modes of administration is in the speed and concentration with which the dye reaches the circulating blood.

There are certain artifacts which appear in the method of examination described above. At times, if fixation is unduly prolonged, certain nuclei will be outlined in blue. The staining affects the chromatin masses, which are as clearly outlined as if hematoxylin had been applied. This is quite different from the true vital staining of pyknotic or degenerated nuclei, so familiar in vital staining of injured areas. At other times the cytoplasm of certain cells will stain, along with the nuclei, as, for example, some striated muscle fibers, or some of the renal epithelium. Such staining is considered artifact for the following reasons: Staining of nuclear chromatin, when present, invariably occurs in the midst of heavy accumulations of trypan blue in the connective tissue, for example, in the periosteum. Again, the fecal contents of the small intestine are often deeply stained with the dye. Sometimes the epithelial cells in contact with this fecal mass will show a staining of both cytoplasm and nucleus, whereas cells more basally placed in the villi are unstained.

One preparation of the kidney, 24 hours after the administration of the dye, was very instructive. Ordinarily at this time there is no diffuse staining in the kidney except in the blood vessels; but the proximal convoluted epithelium is heavily crammed with dye granules, while the nuclei are totally unstained. In this particular preparation, there were practically no dye granules at all, but the epithelial cells, both nuclei and cytoplasm, were colored a distinct and diffuse blue. When animals are not perfused, there is abundant dye in the blood plasma, which appears as irregular blue granular masses in fixed preparations. In sections, the nuclei of the leucocytes are often stained. This type of cellular staining is quite regularly seen in embedded preparations when an unsuitable fixative, such as Carnoy's, is employed.

It seems probable that the bond between trypan blue and the connective tissue is not sufficiently strong to withstand improper or overlong fixation. There is some postmortem solution of the dye and diffusion into adjacent cellular elements which then exhibit a nuclear and cytoplasmic staining. Rapid and suitable fixation is necessary for the preservation of the color in its original site.

DISCUSSION

It seems clear that trypan blue injected into the blood stream is fixed within a very few minutes by the intercellular connective tissue all over the body. It occurs somewhat more slowly when the dye is given subcutaneously. This fixation, or bond of union between dye and tissue elements, is antecedent to any granular storage or colloidopexic cellular action and is the factor responsible for the macroscopic color a few minutes after the dye is administered. The almost immediate diffuse union between dye and connective tissue is observable in fresh organs as well as in those subjected to proper histological fixatives. It is very readily visible with small doses of dye, as, for example, 2.5 mg. for an 18 gm. mouse. Furthermore, the color remains even if the blood vessels are perfused before examination.

With trypan blue, "affinity" between dye and tissue elements is primary, while intracellular storage by histiocytes is secondary. The ability of certain cells to form intracellular granules is, so to speak, an accidental feature of vital staining. Tissues which are rich in histiocytes will show many granules after 24 to 48 hours, tissues poor in histiocytes will show but few; yet the preliminary union between dye and connective tissue is the same in both instances. This bond of union, whatever its nature (perhaps it is an adsorption phenomenon, as Petroff suggests for elastic tissue) is moderately firm. The elastic fibers hold the dye with greatest intensity, reticulin with the least. Dense collagen is closer to elastic tissue, loose collagen to reticulin. As the dye is given up, it is flocculated into granules by colloidopexic cells, such as histiocytes, which happen to be in the neighborhood.

Roman (10), working with a dye derived from atophan (cinchophen), reported results somewhat similar to those described above. He found a light diffuse staining of collagen, and a deeper staining of elastic fibers, as well as a granular storage in the customary loci. Neither the dye, thienyl-quinoline-carbonic acid, which appears to have been impure, nor the results reported in that publication, have received significant attention in the literature on vital staining.

It seems more than coincidence that histiocytes, which are capable of flocculating the trypan blue, occur in the connective tissue which binds the dye in the first place. These cells seem specialized to segregate the dye which has been adsorbed (let us provisionally use this

term) by the intercellular matrix. Since evolutionary specialization could scarcely take place for the purpose of dealing with trypan blue, the following suggestion seems plausible: that a preliminary bond of union with the connective tissue is a general property of noxae which are dealt with by the reticulo-endothelial system. In this sense trypan blue is merely a prototype which happens to be visible.

SUMMARY

Trypan blue injected intravenously is bound almost at once by the intercellular connective tissue elements all over the body,—by collagen, reticulin, and elastic fibers.

This union of dye and tissue elements is the factor responsible for the early macroscopic blue color and is antecedent to cellular colloidopexic action.

Different examples of connective tissue differ among themselves in their ability to hold the dye.

Diffuse staining of elastic fibers noted by previous observers is merely a special case of the general affinity of connective tissue for the dye.

The evidence suggests that the histiocytes are cells specialized to segregate noxae that become diffusely bound to the intercellular connective tissue matrix.

BIBLIOGRAPHY

1. Petroff, J. R., *Beitr. path. Anat. u. allg. Path.*, 1922, **71**, 115.
2. Okuneff, N., *Virchows Arch. path. Anat.*, 1926, **259**, 685.
3. Glasunow, M., *Virchows Arch. path. Anat.*, 1926, **261**, 837.
4. Hackel, W., *Z. ges. exp. Med.*, 1930, **72**, 762.
5. Duff, G. L., *Am. J. Path.*, 1932, **8**, 219.
6. de Vecchi, B., and Costa, A., *Ann. Anat. Path.*, 1931, **8**, 1073; *Pathologica*, 1932, **24**, 145.
7. Cappell, D. F., *J. Path. and Bact.*, 1929, **32**, 595, 629.
8. King, L. S., *Arch. Neurol. and Psychiat.*, in press.
9. Wislocki, G. B., and King, L. S., *Am. J. Anat.*, 1936, **58**, 421.
10. Roman, B., *Cor.-Bl. schweiz. Aerzte*, 1918, **48**, 1638.

EXPLANATION OF PLATE 1

All illustrations are unretouched photographs of organs of mice that had received 5 mg. of trypan blue intravenously. Figs. 1, 2, 3, 5, 6 are of vitally stained tissues fixed in Susa, embedded in paraffin, sectioned at 30 microns, and mounted without counterstain.

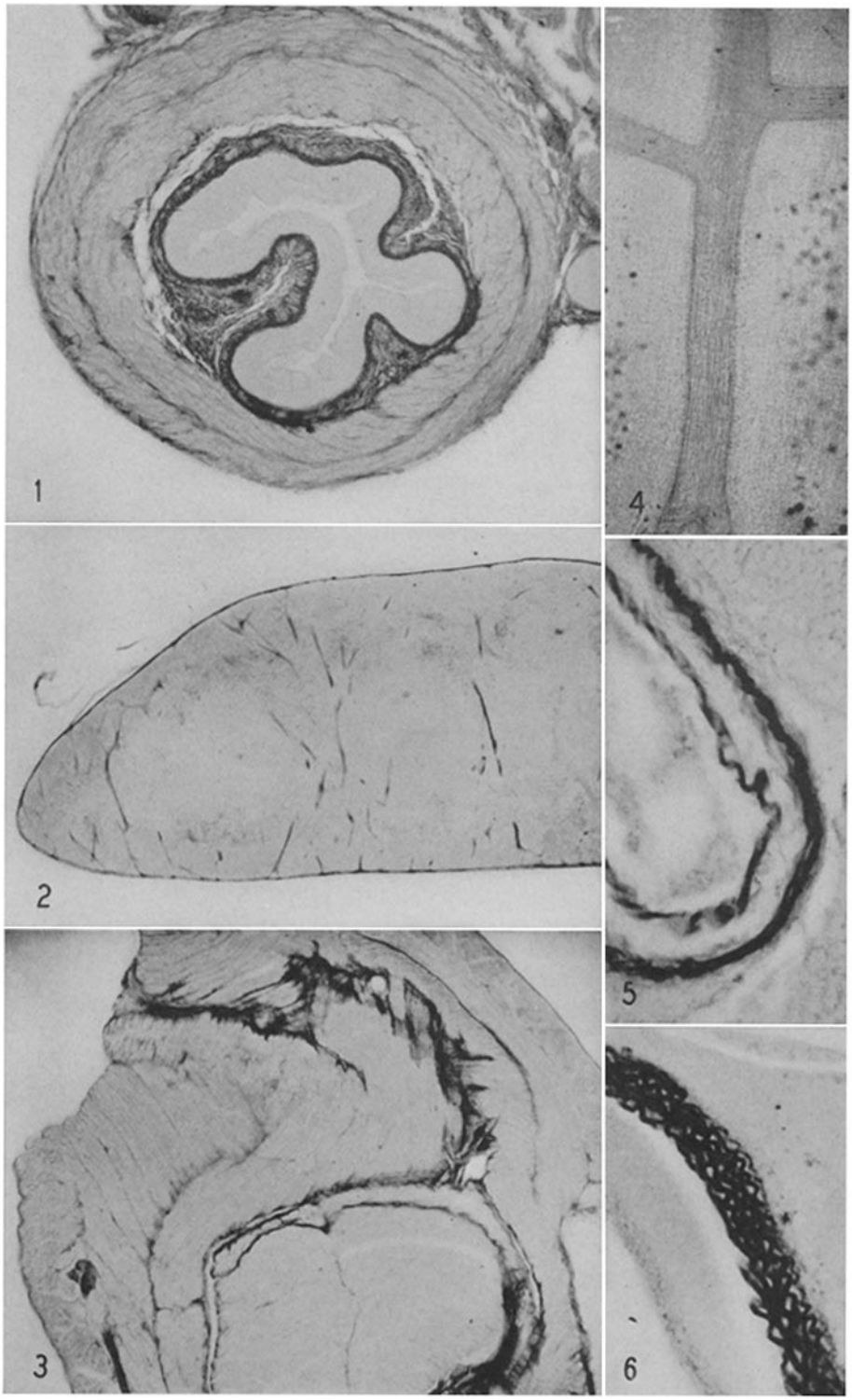
FIG. 1. Esophagus. Mouse killed after 1 hour. × 73.5.

FIG. 2. Spleen. Mouse killed after 1 hour. Note the vital staining of the central arteries of the follicles. In the pulp are masses of red cells, which appear dark in the photograph, although under the microscope there is no possibility of confusion with the vitally stained elements. × 31.5.

FIG. 3. Anterior abdominal wall, rolled up before fixation. Mouse killed after 1 hour. × 55.

FIG. 4. Base of a brain (entire) cleared by the method of Spalteholz, showing the basilar artery and branches vitally stained by the trypan blue. The black masses at the sides are refractive artifacts due to air. The enormous number of fine granules in the meshes of the pia-arachnoid are not visible in the photograph. Mouse killed after 48 hours. × 26.

FIGS. 5 and 6. Thoracic aorta. Fig. 5 taken after 1 hour, Fig. 6 after 72 hours. Red blood cells within the lumina may be compared with Fig. 2. × 147.5.



Photographed by J. A. Carlile

(King: Vital staining of connective tissues)