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DESCRIPTION OF PLATE 34.

FIG. 1.—White Leghorn. Developmental capon. No reproductive tissue present.

FIG. 2.—White Leghorn. Developmental capon. One small testis present on the right side.

FIG. 3.—White Leghorn. Developmental capon. Two small testes present.

FIG. 4.—Silver Campine. Developmental poularde. Small ovary present.

FIG. 5.—Buff Orpington Crossbred. (Dwarf.) Developmental capon. Two testes present.

FIG. 6.—Rhode Island Red (Dwarf). Developmental capon with two testes present.

(It should be noted that the figures do not indicate the relative sizes of the birds.)

A Graphite Suspension for Intravital Injection of Capillaries.

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(Communicated by Dr. H. H. Dale, Sec. R.S.—Received April 5, 1927.)

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[PLATE 35.]

In the ordinary use of injection masses the observer expects to fill and obstruct vessels, and the injection is considered successful in the degree to which it seems to fill the maximal number of available paths.

In contrast to this relatively simple procedure is found an increasing number of attempts to inject a living tissue in such a manner as to fill all the vessels transporting blood at the moment when the normal circulation is interrupted. Noteworthy among such applications of injection technique to physiology are the contrasting injections of resting and active muscle made by Krogh (1) in 1919. In these experiments frogs and guinea-pigs received a suspension of India-ink by vein, and this material, mixed with the blood of the animal, was

pumped to the capillaries by the heart. Results obtained in this way constitute single terminal observations, since capillary obstruction begins to take place practically at once.

It became desirable in perfusion experiments upon the frog to make injections which could be removed at will, with return to a clear perfusion fluid carrying substances under examination for their effects upon the capillaries. India-ink is entirely unsuitable for such work, since it has, to a conspicuous degree, a tendency to adhere to vessel walls, an adhesive power which leads to obstructive injections. In order to obtain a true picture of the vascular condition of a tissue at a given moment, it is obvious that a non-obstructive injection will be greatly superior. Indeed, we may consider that the ideal injection mass would be one transported by the capillaries exactly as is the blood of the animal, but so coloured as to permit counts of capillary number, measurement of individual capillaries, etc.

At the suggestion of Prof. Krogh, an ink has been made in which graphite is utilized as the pigment. The results obtained with this ink so far excel those secured with other masses that we have thought it worth while to offer, in advance of further papers dealing with the actual experiments in which it has been employed, a description of this new preparation.

In order to make the ink, one obtains Hydrokollag 300 from E. de Haën, Seelze bei Hannover, Germany. Hydrokollag is a colloidal preparation of graphite containing ammonia and a small amount of cherry gum as a protective colloid. We have obtained it in 100, 500 and 1,000 gm. tins. Number 300 mixes freely with blood plasma, blood serum, and acacia without agglutinating. Other samples of colloidal graphite, prepared and supplied by de Haën, but containing different protective colloids, give gross agglutination or sufficient agglutination to cause their rejection.

The graphite, when received, is a fairly thick syrup with sediment at the bottom. In preparing the ink, this material is first thoroughly stirred and shaken; a portion in grams is then mixed with double the number of cubic centimetres of water, to which has been added enough sodium hydroxide to give a Ph of 8·5. This is sufficient alkali to enable one to drive off the ammonia with an air blast. When ammonia-free, the suspension will show a moderate number of small agglomerations of particles, as it did at the start, and it will have become somewhat concentrated, the degree of concentration depending upon the manner in which the air blast has been employed. In practice it is convenient to dilute 12 gm. of Hydrokollag with 25 c.c. of the sodium hydroxide solution and to allow air to pass through during the night. Next morning, one

has from 25 to 30 c.c. of ammonia-free suspension, and to this one adds an equal quantity of 6 per cent. acacia plus 1·3 per cent. sodium chloride, thus securing a final product which contains 3 per cent. acacia and 0·65 per cent. sodium chloride and is suitable for use in frogs. Acacia solutions are ordinarily somewhat acid, and when added in equal amount cause the slightly alkaline suspension to become very nearly neutral. Buffering with phosphates does not affect the suspension. If the ink is to be used in mammals, 12 per cent. acacia and 1·8 per cent. sodium chloride should be employed.

It is next essential to get rid of the aggregates which the preparation contains. The most satisfactory method for doing this is by settling, followed by filtration. The material is placed in high graduates or test-tubes, and is allowed to stand in the ice box for at least twenty-four hours. One may then pipette off the upper portions of the suspension and obtain a material containing comparatively few particles capable of causing embolism. In order to get rid of the few remaining oversize particles, the final product is filtered at least four times through an alundum crucible (R.A. 98, Norton Co., Worcester, Mass., U.S.A.). This crucible is a coarse filter which happens to be exactly right for the final preparation of the ink. It is convenient in that it may be used repeatedly after having been cleaned by firing in the blast flame. No doubt, however, other filters of the required degree of permeability can be found. It is desirable that the filter used should not be too tight. Graphite does not build up easily upon the filter, but even a small degree of plugging makes the final filtration very slow and brings about dilution of the ink.

After four passages through the alundum crucible, the suspension is mixed with an approximately equal quantity of blood serum, blood serum plus acacia solution, or acacia solution alone, the exact degree of dilution desirable being a matter for trial in the region under examination. The final product should show particles of very uniform size and no agglutination.

The size of the individual particles in the mass which we have used, in comparison with human erythrocytes, is shown in fig. 1. The largest particles in such a suspension as this have proved non-obstructive in the frog, but, if desirable, even these may be practically eliminated by lengthening the period of settling. Judicious use of the centrifuge would undoubtedly accomplish the same result, but since our experiments have not required better suspensions than the one illustrated, no attempt has been made to carry the process of sizing to its possible limit.

Plate 35, consisting of four microphotographs of the web of a frog's foot, illustrates the use of the ink. Perfusion of the web illustrated began at 11 a.m.,

the perfusion fluid being 3 per cent. acacia *plus* 20 per cent. horse serum, with sodium chloride to 0·65 per cent. Picture 1 was taken at 11.25; picture 2 at

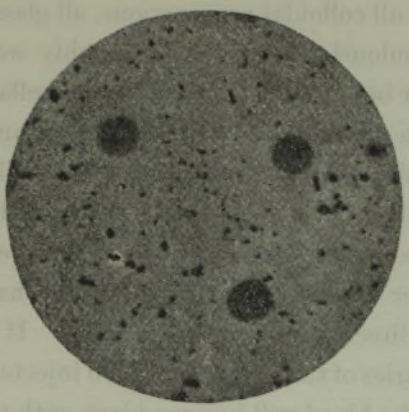


FIG. 1.—Microphotograph of a film of diluted graphite ink suspension mixed with human blood, fixed with methyl alcohol and stained with eosin. Magnification × 286.

11.41, three minutes and forty-five seconds after turning on the graphite ink; picture 3, with lower magnification, at 11.42; and picture 4 at 11.50, nine minutes and thirty seconds after turning off the injection mass and returning to the acacia-serum solution. The melanophores can be seen to have contracted slightly during the period of observation, there having been practically no pituitrin in the solution used. These microphotographs show clearly that the graphite ink has caused no capillary obstruction, and that it has rendered the capillaries beautifully visible.

Perfusion experiments on the frog's web, lasting just short of three hours, have been performed, during the course of which the injection has been made and removed nine times, without tendency for the graphite to block vessels or to adhere to capillary walls until the last injection, when edema had begun to appear. The longest time that the injection has been kept on in any one experiment is fifteen minutes. This time could, undoubtedly, be exceeded and be followed by complete washing out. When the graphite ink causes obstruction, the result seems rather to express an abnormal change in the capillaries than to indicate any untoward property of adhesion inherent in the graphite. Results of the sort described cannot be obtained by means of any other substance with which we are familiar.

The fact that this preparation of ink contains a small amount of cherry gum has been noted. This gum may be removed by washing with sodium hydroxide solution, Ph 8·5, a washing which will also remove the ammonia. Alundum crucible R.A. 360 worked satisfactorily when used with a pressure of one

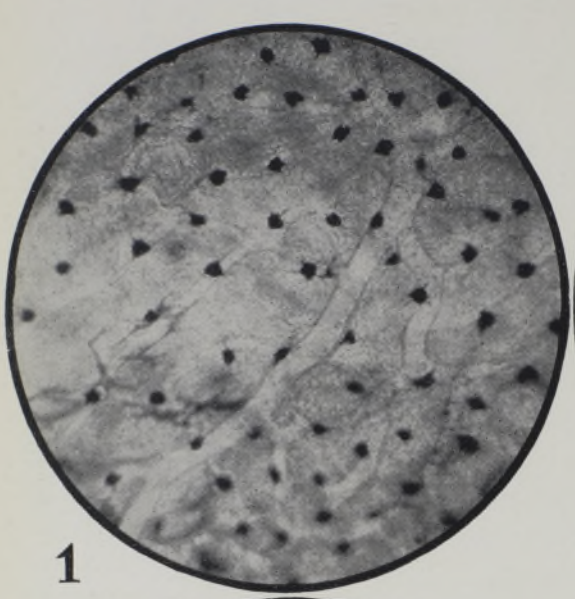
atmosphere and constant stirring. The procedure is troublesome, and, since cherry gum has proved inert, it may be omitted.

As in the handling of all colloidal preparations, all glassware used in preparing the ink must be scrupulously clean, *i.e.* thoroughly washed in tap-water and rinsed in distilled water before drying. The Hydrokollag, as received, is sterile because of its ammonia content. If handled in a cleanly fashion, mixed with sterile acacia and freshly distilled water, and not allowed to become acid, the final preparation will keep in the ice box unchanged for at least two months.

In addition to the perfusion experiments which have dealt with the foot of the frog, and no other tissues, some observations have been made on the graphite ink injected directly into the circulation. If one arranges a frog so as to observe the capillaries of the tongue and then injects the graphite suspension into the femoral vein, the blood will become black with the particles of graphite, which can be seen running with the corpuscles in the capillaries. No agglutination or blockage is visible, and one apparently has made an ideal inert addition to the blood. But after about ten minutes the graphite particles begin to collect in irregular masses, which appear as if held together by a fine coagulum. These increase in size and soon constitute emboli. The phenomenon is precisely that described by Cohnheim and Litten (2) in 1875 for aniline blue injections observed in the tongue capillaries.

No *in vitro* agglutination of the graphite takes place if the suspension is mixed with heparinized frog's blood and allowed to stand overnight. On the other hand, frogs completely heparinized before the graphite injection show intravascular agglutination, with the same speed and in the same manner as animals whose blood is completely coagulable. The removal of the liver or spleen has no effect in preventing the agglutination. In hepatectomized frogs the number of particles in the circulation remains high for a long time, and one not only has a beautiful view of the agglutination process, but may also follow the gathering of particles by the phagocytes.

The existence of this agglutinative reaction makes it impossible to introduce graphite ink into the circulation with the expectation that matters will remain entirely normal for more than five minutes. In perfusion experiments such as those recounted above, the particles are not recirculated, and the steady washing out seems to avoid the appearance of an effect which would nullify the usefulness of the graphite injections.



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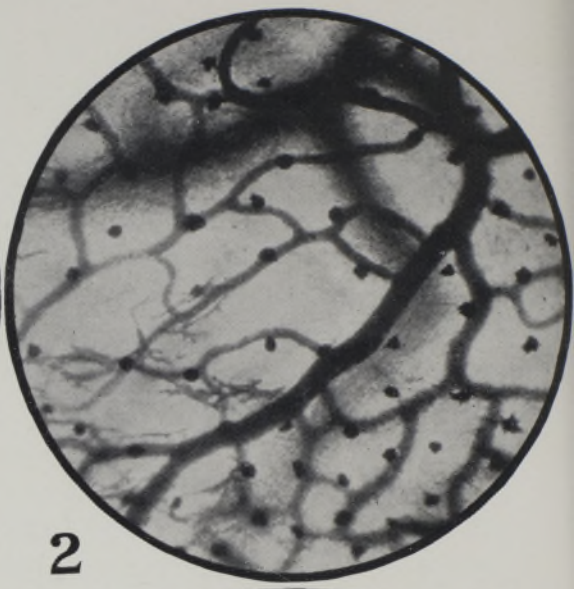

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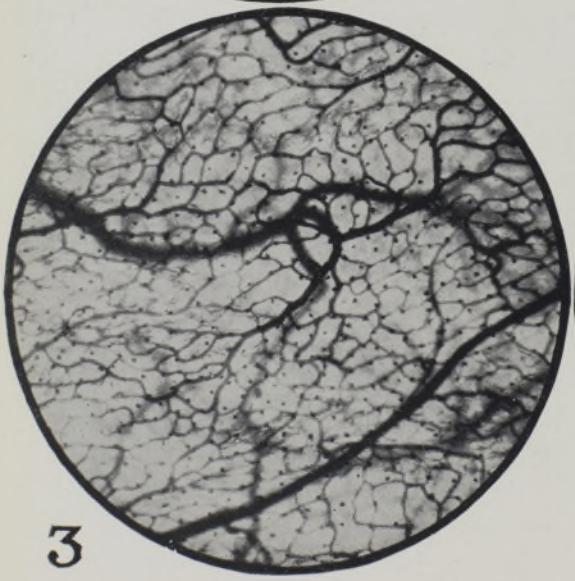

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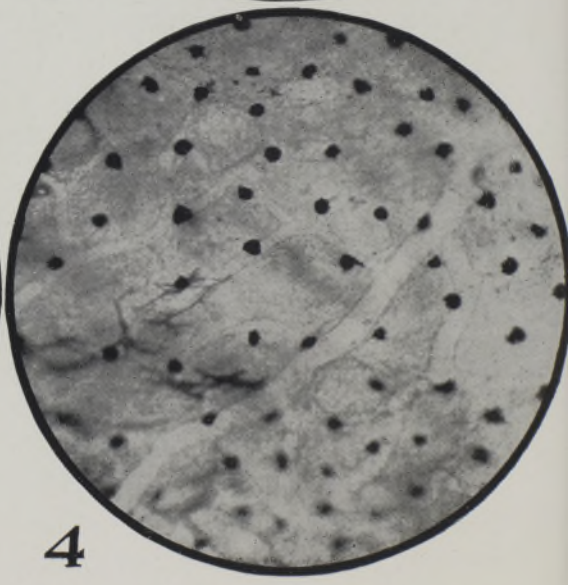

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(*Facing p.* 467.)

Summary.

1. A method for preparing a graphite injection fluid is described, and evidence is submitted showing that this fluid possesses qualities essential for physiological injections if employed in perfusion experiments.

2. These qualities consist essentially in the ability of the graphite particles to mix with blood without agglutinating and to pass through the capillaries without sticking to the walls.

3. When injections of the graphite fluid are made in intact animals, intravascular agglutination of the particles begins in about ten minutes and embolism takes place.

It is a pleasure to express our gratitude to Prof. Krogh and Dr. Rehberg.

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DESCRIPTION OF PLATE 35.

Microphotographs of the web of a brown frog. No. 1, taken at 11.25 a.m., after twenty-five minutes of perfusion with an acacia-serum solution, during which a graphite ink injection had been made and washed out; No. 2, taken at 11.41, three minutes and forty-five seconds after turning on the graphite ink; No. 3, taken at 11.42; No. 4, taken at 11.50, nine minutes and thirty seconds after turning off the ink and returning to the clear perfusion fluid. Magnification, Nos. 1, 2, and 4, × 48·4; No. 3, × 16·9.