FIXING AND STAINING METHODS FOR LEAD AND COPPER IN TISSUES *

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Lead and copper are of great importance clinically, especially lead, owing to their poisonous properties. Unfortunately there are no specific differential stains for these metals in tissues as there is, for example, for iron. Therefore it has not been possible to study them microscopically in direct connection with the lesions they produce. Both metals will stain gray to black if thin pieces of tissue are placed in a solution of hydrogen sulphide, but the test is not at all delicate and microscopically is useless. The object of this communication is to call attention to two simple staining methods which although only partially differential are very delicate and histologically of value.

STAINS FOR LEAD

Hematoxylin Stain: Hematoxylin and its ripened derivative hematein unite with a number of metals to form colored compounds, some of which have been found very useful as stains for nuclei and other tissue elements. As a rule hematein is required to make the stain effective. For this reason an alum hematoxylin solution must be ripened by the aid of light, heat or an oxidizing reagent. For staining lead, hematoxylin itself is essential and ripening of the staining solution must be prevented as far as possible. On this account a solution in dibasic potassium phosphate has been found most useful.

Fixation: Tissues to be examined for lead must be fixed in 95 per cent or absolute alcohol. Formalin is worthless and therefore tissues fixed in this reagent in the past are useless.

Method of Staining: Stain celloidin sections in the following solution in the paraffin oven (about 54° C.) for 2 to 3 hours, rarely for longer. Paraffin sections are loosened from the slide.

Dissolve 5 to 10 mg. (but not more) of hematoxylin in a few drops of absolute or 95 per cent alcohol and add 10 cc. of a freshly filtered 2 per cent aqueous solution of dibasic potassium phos-

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phate. After staining, wash the sections in several changes of tap water for 10 minutes to 1 hour, dehydrate in 95 per cent alcohol, clear in terpineol and mount in terpineol balsam.

Results: By this method lead is stained a light to a dark grayish blue, and the nuclei (which owe their staining properties to the presence of metals) take a deep blue color.

Other solutions of hematoxylin ripen so quickly that hematein or some intermediate product is formed and stains the lead brownish instead of blue and are therefore useless.

The best tissue for studying the effect of acute poisoning by lead is obtained by feeding rats with dog chow thoroughly soaked with a saturated (about 1 per cent) solution of lead chloride and giving them the same solution to drink. Pulverized metallic lead and lead carbonate and phosphate may also be used but act more slowly. In 8 weeks the cytoplasm of the liver cells contains numerous small, round and irregularly shaped granules which tend to fuse together to form networks and which stain clear blue by the hematoxylin method given above.

Excellent tissue for study can also be obtained by feeding *Macacus rhesus* monkeys with lead chloride but they are much more susceptible to lead poisoning than rats. A monkey given 25 cc. of a saturated solution daily on its food became partially paralyzed in its hind legs and unsteady on its feet in 8 weeks. Microscopically many liver cells were necrotic, mitotic figures were numerous and small foci of regenerated liver cells occurred here and there. In the old cells were granules of different sizes which tended to fuse together to form networks. Both the granules and the networks stained blue in the hematoxylin solution recommended above. The liver tissue showed all the microscopic changes of an early beginning cirrhosis. A smaller dose and longer time seemed all that was necessary to produce a typical cirrhosis but much time (months or years) would evidently be required.

Methylene Blue Stain: Using sections from these same experimental lesions produced by lead, it was found that the granules which stain with hematoxylin stain even more intensely by methylene blue. Staining 10 to 20 minutes in a 0.1 per cent solution in 20 per cent alcohol is sufficient and decolorization in 95 per cent alcohol takes about the same length of time. For microphotographic purposes this stain is of value.

These two staining methods were tried on sections of alcohol-fixed livers from numerous cases of alcoholic cirrhosis. The old hyalin, which is characteristic of this type of cirrhosis, was stained slightly or not at all by both methods, probably owing to the disappearance of the lead. On the other hand, many of the younger regenerated liver cells contained numerous granules and small networks which stained intensely blue and resembled closely those produced in rats and monkeys by acute poisoning with lead.

The methylene blue solution was then tried on paraffin sections of Zenker-fixed livers from rats and monkeys poisoned with lead. The granules and beginning networks stained intensely blue. The same was true of sections of livers from human cases of alcoholic cirrhosis and if the usual staining method of phloxine followed by methylene blue was employed and the methylene blue was allowed to act long enough so as to stain deeply, the old hyalin was colored various shades of red while the granules and beginning networks were stained deep blue.

The probable explanation is that the lead unites with the chrome salt present in the Zenker's fluid and the lead chromate formed is not soluble in the acetic acid and therefore persists.

Stains for Copper

Copper is fixed well by both alcohol (95 per cent or absolute) and neutral formalin, and is stained intensely blue by hematoxylin and by hematein. The simplest method is to use the same solution recommended for staining lead. The iron so commonly associated with copper stains black after alcohol fixation, but light to dark brown after formalin fixation.

Rats were poisoned with copper acetate (normal cupric) by giving them each 2 cc. of a 5 per cent aqueous solution daily on their food. The dosage was a little too large because they began to die after 4 months. Two organs, the liver and the kidney, showed marked lesions. In 2 to 3 months all the liver cells contained small granules which stained intensely blue to blue-black with hematoxylin. After that length of time the liver cells began to undergo necrosis and regeneration took place. The resulting picture after 6 months was very much like that seen in the liver from an early active case of hemochromatosis but the pigment present was copper hemofuscin, not hemosiderin, and the granules

stained blue, not black. The beginning elimination of copper and transformation of hemofuscin to hemosiderin seem to require about 7 months.

The lesion in the kidneys was equally marked. Copper hemofuscin was deposited abundantly in the cells lining the convoluted tubules and caused necrosis. The desquamated necrotic cells filled the lumens of the tubules and active regeneration occurred along the walls.

When the hematoxylin staining method was tried on sections of livers from cases of hemochromatosis it was found that the hemosiderin in the pigment stained black after alcohol, but light to dark brown after formalin fixation. In the islands of regeneration, when present, where the pigment was being laid down and was therefore freshly formed, the granules stained blue to blue-black, strongly suggesting copper.

Frequently inspissated bile was found in dilated bile capillaries. It was colored deep blue by hematoxylin, indicating copper, and clearly explains the manner in which copper gains entrance to gall stones where its presence was pointed out by Schönheimer and others.

In the kidneys from cases of hemochromatosis the lesion was much less marked than in the animals poisoned with copper, but hemosiderin was found in some of the cells of the convoluted tubules and in a few cases casts in the collecting tubules stained blue with hematoxylin, indicating copper.

When the methylene blue stain was used on sections of the experimental lesions produced in rats the copper stained blue but to only a moderate degree. The same was true of the copper in the islands of regeneration in the liver in hemochromatosis. For staining the latter lesions methylene blue has, however, one great advantage. Hemosiderin does not take the stain and therefore appears yellow to light brown. As a result the copper, in spite of its light blue color, shows up quite clearly. When the pigment granules contain both copper and iron they stain green.

Comment

For many years we have been working primarily on the subject of chronic lead poisoning and its relation to alcoholic cirrhosis, and to a less extent on chronic copper poisoning and its relation

to hemochromatosis. It seems advisable to publish at the present time the methods found useful for fixing these metals, especially lead, in the tissues and for demonstrating them by special stains. Because the staining methods are only partially differential and therefore not diagnostic, they have to be supported by a certain amount of histological and experimental work. As a result a brief summary of our results to date is presented.

Both metals, but especially copper, are very slowly acting, chronic poisons. To produce experimentally all the steps of the various changes which occur in man would require a number of years. In the meantime much valuable pathological material might be lost if not properly preserved. Alcohol is the best fixative for both metals and is absolutely necessary for demonstrating lead. Formalin is about as useful as alcohol for copper. Zenker's fluid is the best fixative for tissues to be studied histologically and preserves both metals but cannot be considered as an ideal fixative for them because the hematoxylin staining method is useless after it. The wisest procedure is to preserve tissues in all three fixatives from all important cases.

The liver would seem to act as a temporary storehouse for lead which causes little or no evident damage unless the amount absorbed exceeds a certain minimum.

Hyaline bodies were found in the nuclei of liver cells in the monkey and the hog, as in children, but were not found in the rat. They stained blue with hematoxylin like the granules in the cytoplasm of the liver cells but slightly less intensely.

The decision whether or not a case of cirrhosis is due to lead, copper or some other agent must be made by a pathologist using the best differential stains available. The patient may have had hemochromatosis and yet not have imbibed any amount of copper for months or years before death and it might have been largely or entirely eliminated during that time. Under these conditions the chemist is helpless and his results valueless unless his work is controlled by careful microscopic examination to show whether lead or copper is present or not. On the other hand, an individual may have a normal appearing liver and yet may have imbibed a large amount of lead or copper during several months preceding death, and therefore show much of one or the other metal present.

The hematoxylin staining method given here, when applied to

the central and peripheral nervous system, brings out, apparently, the same structures as the microincineration method, namely nuclei and nucleoli, Nissl bodies, and the myeloaxostroma of nerves, owing to the presence of metals in them. If lead is deposited in the nervous system in lead poisoning it would seem to occupy the same situations and simply increase the amount of mineral present.

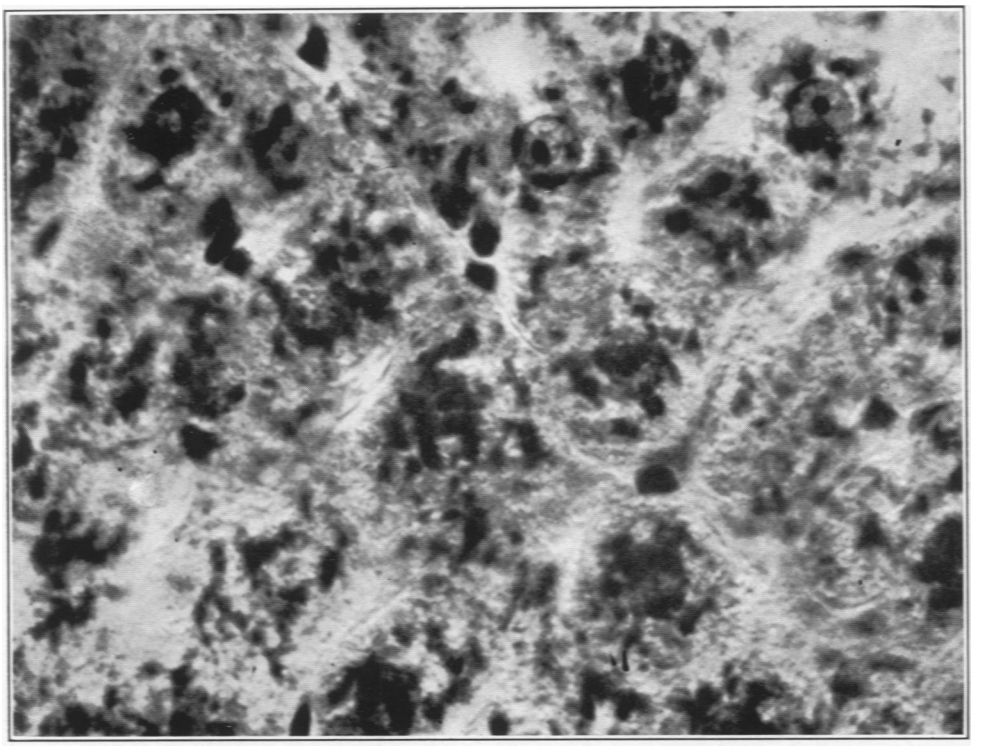
NOTE: We are indebted to Mr. Frank Dinsmore for technical assistance in making the microphotographs.

DESCRIPTION OF PLATES

PLATE 83

FIG. 1. Liver of a rat fed a maximum dose of lead chloride daily for 8 weeks. The liver cells contain granules and early hyaline networks stained blue with methylene blue. Fixation in alcohol. × 1000.

FIG. 2. Liver of a child poisoned with lead. The liver cells contain coarse granules and young hyaline networks stained deep blue with hematoxylin. Fixation in alcohol. × 1000.



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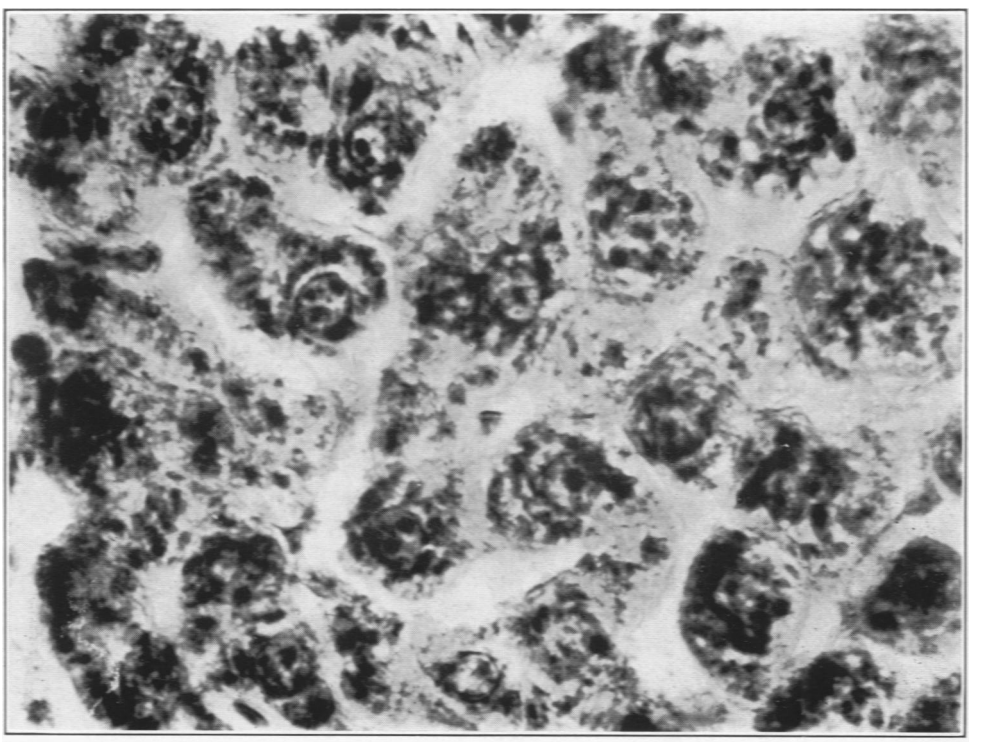

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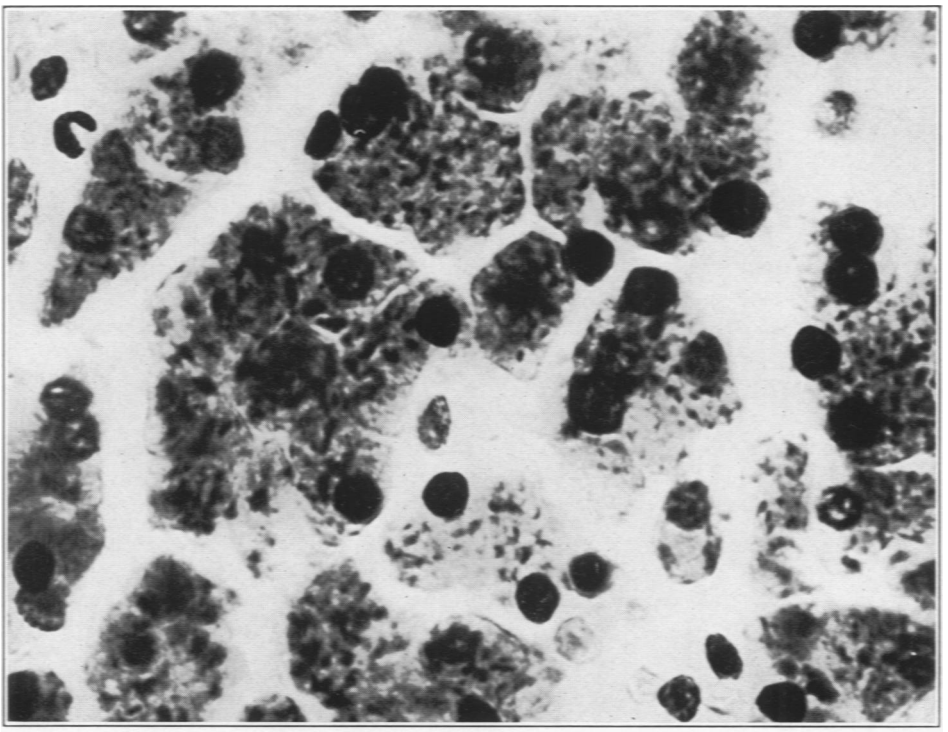
PLATE 84

FIG. 3. An island of regeneration from an active case of alcoholic cirrhosis containing much old hyalin. The liver cells are filled with granules and young hyaline networks stained intensely blue. Fixation in Zenker's fluid. Phloxine-methylene blue stain. × 1000.

FIG. 4. A normal peripheral nerve showing the myeloaxostroma as stained by the hematoxylin method recommended. × 1100.



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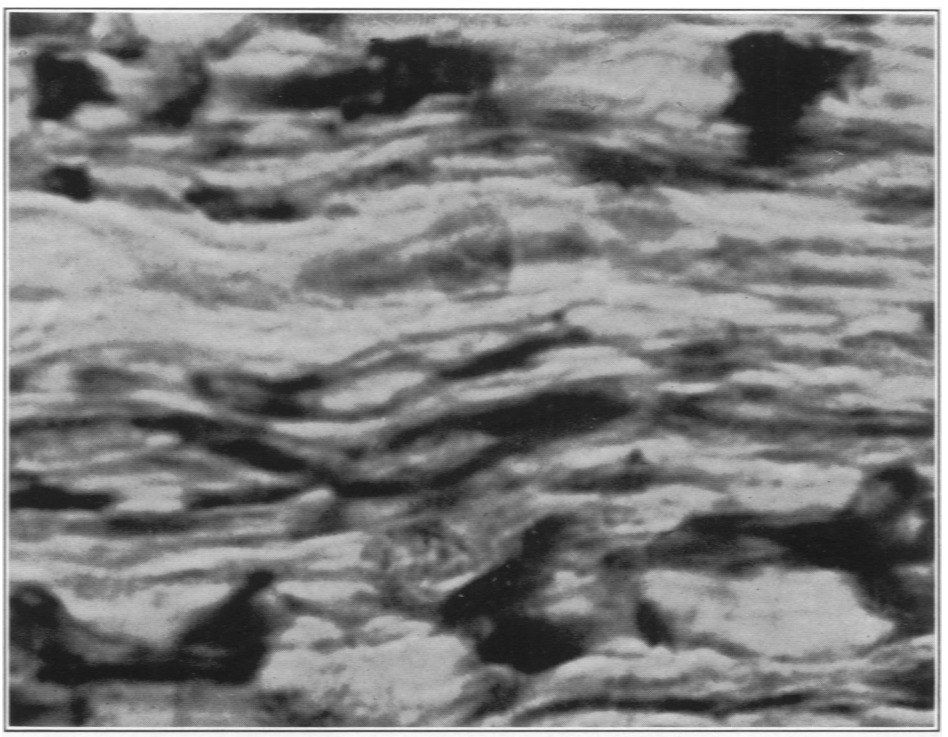

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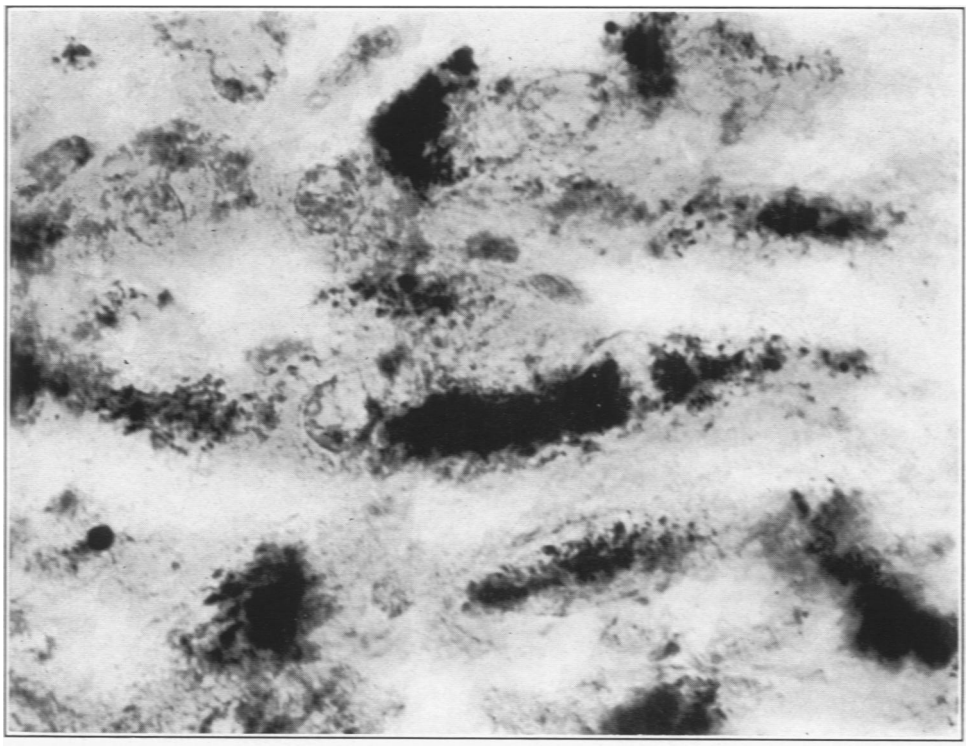
PLATE 85

FIG. 5. Liver of a rat fed cupric acetate for 6 months. The liver cells are filled with masses of granules containing copper stained a deep blue by hematoxylin. Fixation in alcohol. × 1000.

FIG. 6. Kidney of a rat fed cupric acetate for 6 months. The convoluted tubules are distended with necrotic cells filled with granules containing copper stained deep blue with hematoxylin. Fixation in alcohol. × 1000.



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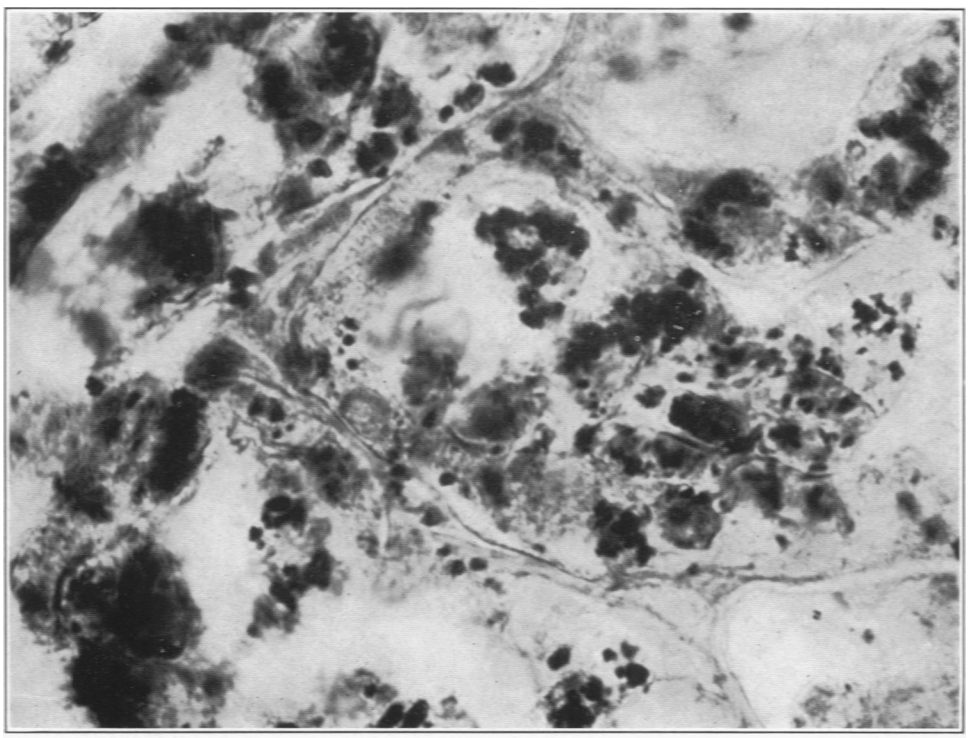

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