

THE APPLICATION OF HISTOCHEMICAL METHODS
IN THE STUDY OF THE SKIN IN LEPROSY
DEMONSTRATION OF BACILLUS-CONTAINING CAPILLARIES AND AXONS
BY TREATMENT FOR PHOSPHATASES

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Histochemical methods have been devised to demonstrate certain metabolic activities of cells. By these techniques certain structures in a particular tissue are made to stand out in contrast to the other constituents. We have applied the methods for demonstrating alkaline and acid phosphatase activity to the study of the skin in leprosy.

Pearse (5) has pointed out that these methods depend on incubating the tissue with a substrate which contains an organic phosphate (sodium-B-glycerophosphate being usually used). Phosphate will be liberated at the site of phosphatase activity. If the pH of the solution is 9.0 or above, alkaline phosphatase is responsible for the phosphate liberated. If the pH of the solution is between 5.0 and 6.0, acid phosphatase effects the liberation of phosphate.

To fix the phosphate where it is liberated, a calcium salt is added to the substrate used for alkaline phosphatase activity, which precipitates the phosphate as calcium phosphate. That product is then replaced by brownish-black cobalt sulphide by first placing the tissue in cobalt nitrate and then in a solution of yellow ammonium sulphide. Structures which contain alkaline phosphatase can be recognized in the tissues by their brownish-black color. In the acid solution, however, calcium phosphate will not precipitate as it is soluble in those conditions. Lead nitrate therefore replaces the calcium salt in the incubation mixture, and lead phosphate is precipitated at the site of acid phosphatase activity. The lead phosphate is then converted into black lead sulphide by treatment with yellow ammonium sulphide.

PRESENT INVESTIGATION

Skin biopsy specimens were taken from leprosy patients after infiltrating the skin with 1 per cent procaine, care being taken to prevent the solution from getting into the tissues to be examined. The specimens were fixed immediately after removal in 2 per cent neutral formalin at 4°C. in a refrigerator for 4 hours. Gomori (3) states that chilled acetone causes the least inactivation of the enzymes, and recommends it for general use. He says that neutralized formalin destroys over 75 per cent of

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the alkaline phosphatase in less than 24 hours at room temperature, but that there is little effect at ice box temperature. Both Gomori and Pearse believe that formalin fixation is of no use if the tissue is later to be imbedded in paraffin, but Pearse recommends cold-formalin-fixed frozen sections for estimation of both acid and alkaline phosphatase activity. We have found that when skin is fixed in cold absolute acetone it is very difficult to cut, as it becomes brittle and readily fragments. We have obtained satisfactory results with a weak solution of formalin used for a short time.

Frozen sections were cut at 15 microns thickness, washed in distilled water, and drawn onto clean slides without egg albumin. The slides were dried at room temperature for one-half hour. The sections were then dehydrated in absolute alcohol and covered with a 0.5 per cent celloidin (in equal parts of ether and alcohol), to prevent them from detaching.

Alkaline phosphatase.—The sections were now incubated in a substance with a pH of 9.2-9.3. The solution used was a slight modification of that recommended by Gomori. We preferred to weigh out the sodium-B-glycerophosphate, as it tended to develop a fungus growth when kept in solution.

| | |
|-----------------------------------|----------|
| Sodium-B-glycerophosphate, | 0.5 gm. |
| Magnesium chloride, 20% solution, | 5 cc. |
| Calcium chloride, 2% solution, | 12.5 cc. |
| Sodium barbital, 10% solution, | 7.5 cc. |
| Distilled water to | 100 cc. |

The sections were incubated at 37°C. for different periods ranging from 2 to 48 hours, the best results being obtained at 16-24 hours. After washing in running tap water the slide was treated with 2 per cent cobalt nitrate for 5 minutes. Then it was washed with distilled water and treated with dilute yellow ammonium sulphide (one drop of the ammonium sulphide to 10 cc. distilled water), 1-2 minutes until light brown. Then followed alcohol dehydration, xylol clearing, and Canada balsam mounting. This method showed the capillaries standing out as brownish-black tube-like structures. The epithelium was also stained the same color.

We decided to endeavor to demonstrate the presence of acid-fast organisms in sections which had been treated for alkaline phosphatase activity.

After the brown color had developed the slide was washed well in distilled water and then treated with 0.5 per cent periodic acid to partially bleach the deposit of cobalt sulphide. The bleaching had to be controlled under the microscope, otherwise the section would become completely bleached. The sections were then washed and stained by the Ziehl-Neelsen method: carbol-fuchsin 15 minutes, decolorization with acid alcohol (2% sulphuric in 70% alcohol), counterstaining lightly with hemaxotylin, then acid alcohol, dehydration, clearing and mounting as usual.

In sections stained with hematoxylin and eosin, we had noticed vacuoles in the giant cells of tuberculoid leprosy, and decided to treat a paraffin section from such a specimen for evidence of alkaline phosphatase activity by the method described. For this tissue there had been no special fixation or refrigeration prior to paraffin imbedding; it was fixed in 10 per cent neutral formalin, dehydrated in graded alcohol, cleared in xylol and imbedded in paraffin. The vacuoles showed the presence of alkaline phosphatase activity.

Acid phosphatases.—To demonstrate the presence of acid phosphatases we used the following solution in which to incubate the sections.

| | |
|--|---------|
| Sodium-B-glycerophosphate, | 0.5 gm. |
| Lead nitrate, | 2.0 cc. |
| M/1 acetate buffer, | 5.0 cc. |
| Distilled water to | 100 cc. |
| (The M/1 acetate buffer consisted of 6% acetic acid, 30 cc., and 13.6% sodium acetate, 70 cc.) | |

The sections were incubated for 16, 24, and 48 hours at 37°C.; the maximum enzymic activity was found at 48 hours. The section was then treated with dilute yellow ammonium sulphide, and a black color developed in the axons and nuclei of the tissue. Acid-fast bacilli were stained as described above except that the bleaching with periodic acid was omitted. When periodic acid was used the whole tissue was very rapidly and completely bleached.

RESULTS

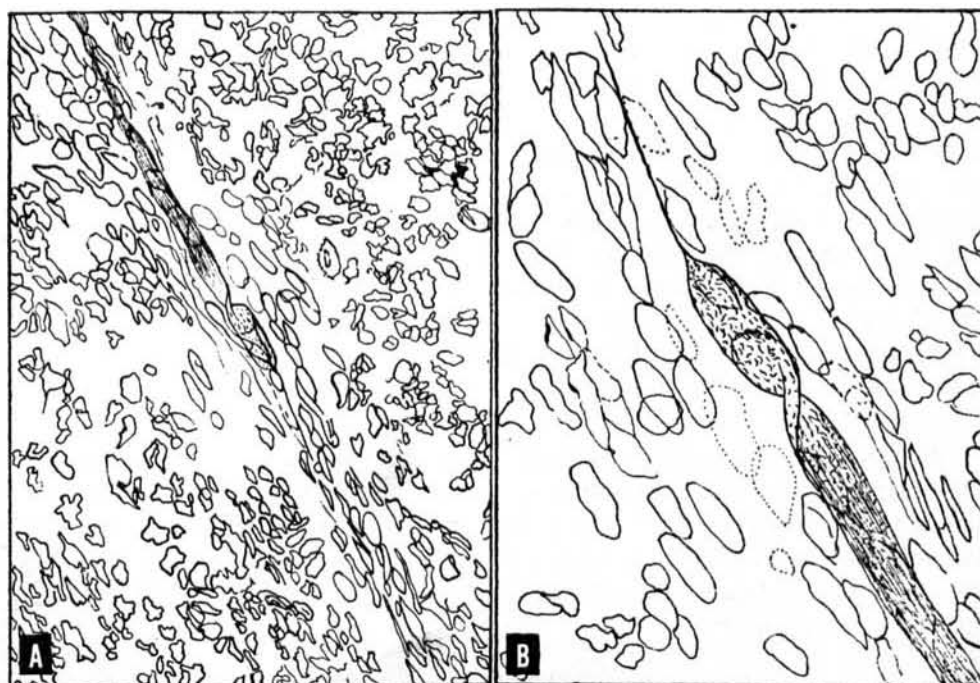
Alkaline phosphatase activity in tuberculoid and lepromatous leprosy.—The skin sections show the presence of tube-like structures which branch irregularly in the dermis, and which we believe to be capillaries. These are well marked in the infiltrated areas, much more numerous than in the skin of normal persons.

In tuberculoid leprosy these structures tend to surround the areas of infiltration. In some cases those areas are broken up into small foci, each focus being surrounded by capillaries (Figs. 1 and 2).

In lepromatous leprosy the vessels are wider, without the twistings seen in the tuberculoid type of lesion (Fig. 3). Under a higher magnification, clear spaces can be seen in the capillaries (Fig. 4). When stained for acid-fast bacilli, these spaces were found to be filled with them (Fig. 5).

Alkaline phosphatase activity in giant cells.—We were interested to find that the vacuoles which we had noticed in the giant cells of tuberculoid leprosy showed a strong reaction for alkaline phosphatase (Fig. 6.). No other structures in the section showed a similar activity. This enzymic activity was preserved even though the tissue had been fixed in unchilled formalin, followed by paraffin imbedding. Acid-fast staining did not show any bacilli in these vacuoles.

Acid phosphatase activity.—A piece of normal radial nerve from a nonleprous patient, when treated by the method described, showed a black precipitate in the axons and in the nuclei of the neurilemmal cells (Fig. 7). We also found that this method will reveal even the finest nerve endings in the skin of the finger tip of a healthy person. These fine fibers were more delicate than any we had been able to demonstrate with silver stains. When the method was applied to skin specimens from patients with lepromatous leprosy we were able to demonstrate nerve fibers running through areas of infiltrate, as had been shown with silver stains. When this method was combined with the carbol-fuchsin stain, we found bulbous swellings on the axons filled with bacilli. In certain places the axons appeared to split, swell, twist, and rupture (Figs. 8 and 9 and Text-Fig. 1).



TEXT-FIG. 1. Schematic diagram showing Figures 8 and 9 in greater detail. A-Fig. 8, B-Fig. 9.

The normal axon was seen running up to this swelling; and unswollen axons could be seen running near a swollen one filled with acid-fast bacilli (Fig. 10). In one area we found a single axon with a small swelling in it which contained two acid-fast bacilli. In another area a few bacilli were lying in line with the faint outline of a fiber linking them together.

DISCUSSION

The demonstration of alkaline phosphatase activity in the capillary wall is well known. Pearse (6) states that it is a useful method for showing capillaries in a tissue, as they stand out clearly against the background of an inflammatory infiltrate. Arterioles and venules are usually not displayed by this method.

The demonstration of acid-fast bacilli in association with the capillaries in lepromatous leprosy is interesting. It has long been known that the bacilli can readily invade the blood stream in leprosy. They have been demonstrated in the circulating blood, and they are removed by the reticuloendothelial cells throughout the body. We cannot be sure of their exact location in the capillaries, but they are most probably in the endothelial cells. If they were in the lumen, it does not seem very likely that they would have remained massed as we have found them.

The reason for the presence, in the giant cells of tuberculoid lesions, of vacuoles which display alkaline phosphatase activity is not known. The observation is simply recorded as of interest.

Acid phosphatase activity in nerves provides an excellent method of showing axons and neurilemmal cells in tissues. The myelin sheath remains relatively unaffected. This method, followed by carbol-fuchsin staining, provides a satisfactory technique to show where the acid-fast bacilli are and what changes they effect. We have found large numbers of granular and rod shaped bacilli in the swollen axons. Similar balloon-like structures containing bacilli were seen when lepromatous tissue was stained for myelin and acid-fasts in these laboratories (1). Also, by silver impregnation methods there was demonstrated ballooning of axons at intervals (2), which was taken to be a sign of degeneration.

We are not in a position to discuss how the bacilli reach the axons or what happens to them. Khanolkar (4) has brought forward the interesting theory that they invade and then travel up the axons. Our findings confirm the presence of bacilli in swollen and apparently degenerated axons, but we are not able from our material to prove that the bacilli had actually travelled along the axon.

SUMMARY

Capillaries and nerves have been shown to contain sufficient alkaline and acid phosphatase, respectively, for these structures to be outlined by histochemical methods. Carbol-fuchsin staining combined with these methods have shown the presence of bacilli in the capillaries and in the axons.

RESÚMEN

Por medio de métodos histoquímicos, que se describen a fondo, se estudiaron lesiones leprosas de forma tuberculoidea y lepromatosa en cuanto a actividad de la fosfatasa alcalina y la ácida. Revela la primera los capilares sanguíneos y la segunda las fibras nerviosas. Algunas veces se combinaron esas técnicas con la coloración con carbol-fuchsin para el descubrimiento de bacilos ácidosresistentes en relación con dichos tejidos.

En las lesiones tuberculoideas, los capilares tubulares se ramifican irregularmente y suelen circundar los distintos focos del infiltrado. Las vacuolas observadas en las células gigantes revelaban, según se notó, notables signos de fosfatasa alcalina.

En las lesiones lepromatosas, los capilares son anchos y no muestran torsión. Con mucho aumento, cabe observar en ellos espacios despejados, que, según se ha notado, contienen grupos de bacilos.

El procedimiento de la fosfatasa ácida se mostró capaz de revelar finas fibras nerviosas, más delicadas que las observadas después de la coloración argéntica. In las lesiones lepromatosas, esas fibras aparecen atravesando zonas de infiltración, pareciendo en algunos sitios henderse, inflarse, torcerse y romperse. Los cilindros-ejes inflados resultaron estar llenos de bacilos ácidosresistentes. (No se mencionan los hallazgos en las lesiones tuberculoideas.) Aunque los AA. confirman, pues, el aserto de Khanolkar en el sentido de que existen bacilos en el cilindro-eje, no pueden afirmar que hayan realmente transitado los últimos por dicho elemento.

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DESCRIPTION OF PLATES

PLATE (5)

Photomicrographs illustrating the findings in sections of leprosy lesions treated for alkaline phosphatase activity. (The actual magnifications are 12% less than stated, because of reduction in reproduction.)

FIG. 1. Photomicrograph of a skin section from a case of tuberculoid leprosy showing lobulated areas outlined by brownish-black-staining capillaries, the result of alkaline phosphatase activity (L.216). $\times 27$.

FIG. 2. A higher magnification of an area in Fig. 1. $\times 460$.

FIG. 3. A skin section from a case of lepromatous leprosy, showing capillaries less numerous and less irregular than in tuberculoid lesions (L.244). $\times 27$.

FIG. 4. High-power photomicrograph showing clear spaces in capillary wall in lepromatous leprosy (L.243). $\times 460$.

FIG. 5. Same as Fig. 4, stained to show acid-fast bacilli in the capillary structure, higher magnification. $\times 900$.

FIG. 6. A tuberculoid lesion, showing giant cells of the Langhans type containing rounded black deposits, the results of alkaline phosphatase activity (L.216). $\times 460$.

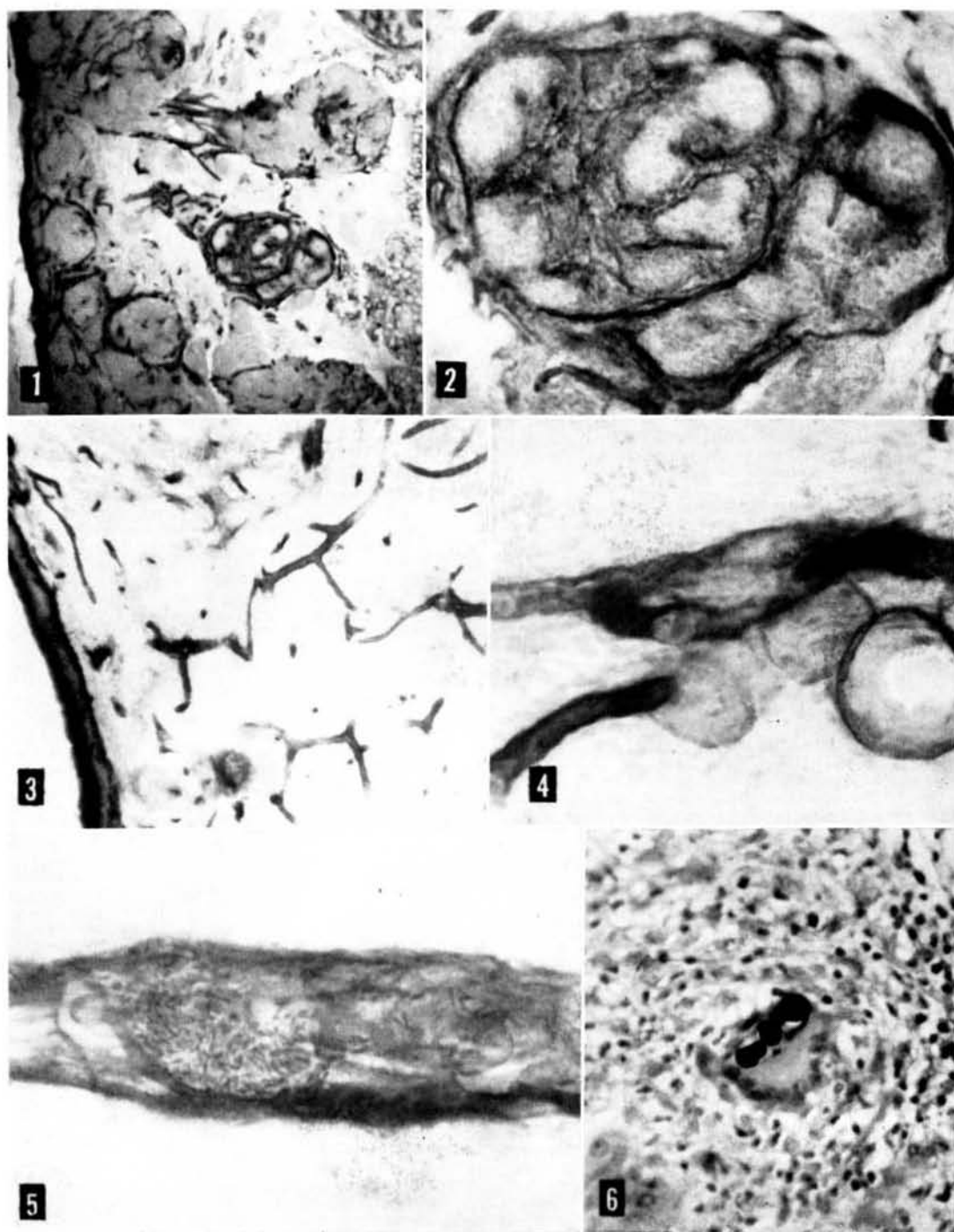


PLATE 5.

PLATE (6)

Photomicrographs illustrating the findings in sections of lepromatous leprosy lesions treated for acid phosphatase activity, together with a normal control. (No reduction in reproduction.)

FIG. 7. A normal radial nerve from a nonleprous patient, treated for acid phosphatase activity. $\times 460$.

FIG. 8. The same skin as in Fig. 3, treated for acid phosphatase activity plus acid-fast stain (L. 244). Running across the section can be seen a nerve fiber which widens out and twists on itself. $\times 460$.

FIG. 9. Higher magnification of Fig. 8, showing the fiber widening out and twisting on itself. The swollen part contains many acid-fast bacilli. $\times 900$.

FIG. 10. Another area of the same section, showing a swollen axon containing acid-fast bacilli, and alongside it axons which are of apparently normal caliber. $\times 900$.

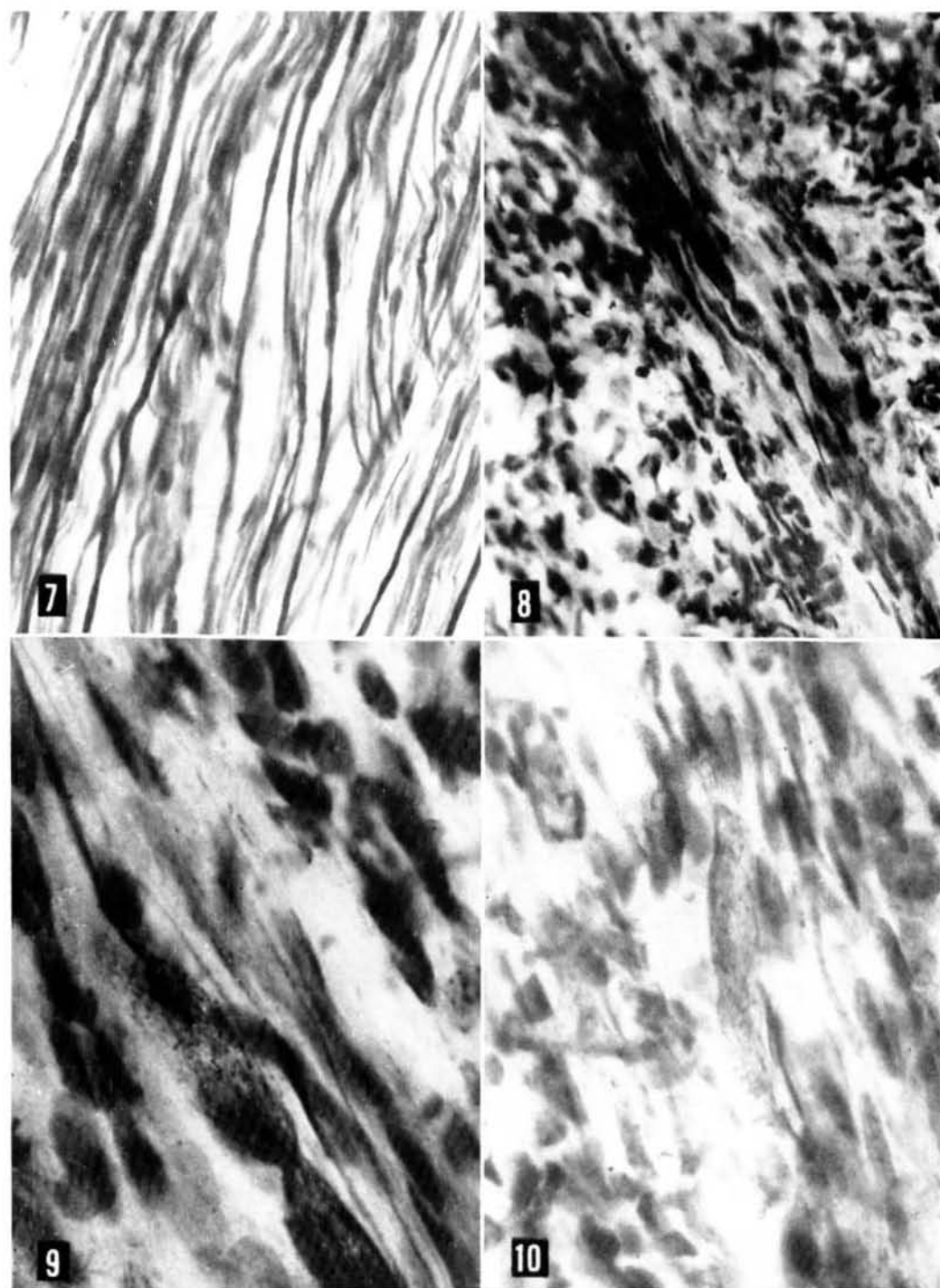


PLATE 6.