# LOCALIZATION OF ANTIGEN IN TISSUE CELLS

## V. Capsular Polysaccharide of Friedländer Bacillus, Type B, in the Mouse*

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Plates 1 and 2

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In previous studies from this laboratory a method has been described for the detection of antigenic material in tissues (1), utilizing antibody conjugated with fluorescein as a specific cytochemical reagent. The resulting fluorescent microprecipitate can be visualized under the fluorescence microscope. This method has been applied to studies of the distribution of pneumococcal polysaccharides (2) and to the detection of mumps and rickettsial antigens (3).

In this paper the results of studies on the distribution of the capsular polysaccharide of the Friedländer bacillus, type B, are reported. This polysaccharide is similar in its chemical and immunological properties to the pneumococcal polysaccharides previously studied (4).

Observations were made on bones and joints as well as on most of the organs studied in the experiments with pneumococcal polysaccharide. In the lungs, the fate of intravenously administered polysaccharide was compared with its distribution when given by inhalation.

### *Materials and Methods*

Capsular polysaccharide was prepared by the method of Heidelberger, Goebel, and Avery (5) from a strain of Friedländer bacillus, type B,[1] recently isolated from a patient with pneumonia. Solutions of polysaccharide for injection were prepared by moistening the dry material with distilled water, allowing the imbibition of water to proceed for several hours in the cold, and then adding 0.85 per cent saline and sufficient carbonate buffer (pH 9.0) to bring the pH to 8.0. The material went completely into solution after 10 minutes' shaking; the pH was brought back to neutrality by the addition of N/10 hydrochloric acid.

Friedländer B antiserum was prepared in rabbits by immunization with formalin-killed organisms of the strain used in preparing the polysaccharide. The serum was harvested and

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[1] Kindly supplied by Dr. Maxwell Finland.

a crude globulin fraction separated by 50 per cent saturation with ammonium sulfate. The polysaccharide reacted with this fraction (protein concentration, 3.38 per cent) in dilutions up to 1 in 8 million. A portion of the fraction was conjugated to fluorescein, purified, and absorbed with mouse liver powder by methods previously described (1).

A single 2 mg. dose of Friedländer B polysaccharide was injected intravenously into each of a number of mice. Both male and female mice, of the Schwentker strain, weighing between 15 and 20 gm. were used. These mice were killed in pairs with ether 4 hours, 24 hours, 2, 4, 8, 16, and 33 days after injection. In addition, two animals receiving 3 mg. were killed after longer intervals, one at 3 months and one at 5. These two animals will be considered in a separate section of the results. The organs examined as routine were heart, thymus, axillary lymph node, liver, spleen, kidney, skeletal muscle, lung, and suprarenal gland. A limited number of observations were also made on the testis, bone, bone marrow, cartilage, and joints. The ovary and uterus were examined from a female mouse which had received 5 mg. of polysaccharide, and the joints were studied in a 12 day old rat which had received 5 mg. also. In 5 mice 0.05 ml. of a solution containing 0.1 mg. polysaccharide was administered by inhalation under ether anesthesia. These animals were killed 2 days later and the lungs and adjacent tissue were studied. Precipitin tests for polysaccharide were made on the bile and on the urine in several instances.

*Preparation of Tissues for Examination.*—Preliminary experiments were made on sections prepared from unfixed, frozen organs by the technique of Linderstrøm-Lang and Mogensen (6) as described in a previous paper (1). Difficulty was experienced with this method, due to the polysaccharide's dissolving out of the section and forming a precipitate in the supernatant antibody-fluorescein conjugate; particles of this precipitate then settled at random on the section.[2] This difficulty had also been experienced to a lesser extent with the larger doses of pneumococcal polysaccharides. Tissues which were preserved in the cold in Rossman's glycogen fixative (picric acid–formalin–alcohol) were found to give more satisfactory results, just as in the case of pneumococcal polysaccharides. Since there was no apparent reduction in the intracellular concentration of polysaccharide from the fixation, the observations to be described were made on tissues treated in this way. Paraffin sections were cut at 5$\mu$ and exposed to antibody-fluorescein conjugate according to the method previously described (2). Specimens of joint were fixed in the same way. Some were then decalcified in the cold with daily changes of 5 per cent trichloracetic acid for 6 to 10 days before embedding; one was examined without decalcification.

Controls of specificity consisted of: (1) absence of reaction between normal tissues and the fluorescein-antibody, and (2) specific inhibition of the reaction by previous exposure of the section to unconjugated homologous antibody. The reaction was found to be regularly specific. Under the fluorescence microscope, the bright yellow-green deposit of fluorescein stood out in sharp contrast to the bluish grey autofluorescence of the fixed tissue elements.

## *Distribution of the Antigen*

The time interval between the injection of polysaccharide and its first appearance in the cells varied from one location to another. On the basis of the length of this interval, the tissues mainly involved in the taking up of the material can be separated into several groups. After the shortest period of

[2] This method is, however, essential in the study of less stable antigens. In the case of plasma proteins, for example, where the antigen also tends to dissolve out of the section, its solubility can be depressed, without destroying its antigenic activity, by brief fixation of the section in 95 per cent ethanol.

observation (4 hours) polysaccharide was already present in high concentration in the Kupffer cells of the liver sinusoids and in the macrophages of the red pulp of the spleen and of the sinuses of lymph nodes. It was also found in the endothelium of capillaries and larger blood vessels, and (in rather smaller amounts) in the lymphoid follicles of the spleen and lymph nodes. After 24 hours the hepatic cells and additional macrophages in connective tissue had taken up the material. Forty-eight hours after injection, specific fluorescence could be demonstrated in the cells of the juxtaglomerular segment of the distal renal tubule, and 4 days after injection some of the cells of the suprarenal cortex had taken up polysaccharide.

ENDOTHELIUM, CONNECTIVE TISSUE, AND MUSCLE.—

Following its early appearance in the vascular endothelium, polysaccharide had disappeared from that of the larger vessels 24 hours after injection and had become generally much less concentrated in that of the capillaries. It remained in the capillary walls of some organs (see below) for the 33 days of the experiment, however, and was still abundant at that time in those of the renal medulla. Within the blood plasma, fluorescent precipitate was abundant at 4 hours, reduced in amount at 48 hours, present only in traces at 4 days, and not detectable thereafter.

In connective tissue the macrophages were conspicuous as early as 24 hours because of their taking up large amounts of the material, while the fibroblasts took it up scantily. During the subsequent few days, polysaccharide-containing macrophages increased in number and size. Later their number diminished, but polysaccharide could still be detected in many of them 33 days after injection. In addition to its intracellular location, polysaccharide appeared to be adsorbed to collagenous fibres throughout the body. In many sites it persisted throughout the experimental period.

In cardiac and skeletal muscle only endothelia and connective tissue elements took up polysaccharide. Its penetration into the muscle fibres could not be definitely established. The stromal macrophages displayed polysaccharide in both types of muscle at 24 hours. The concentration of polysaccharide in the capillary walls was greater in the heart than in skeletal muscle: fluorescence was still detectable after 33 days in the capillary endothelium of cardiac muscle, but had disappeared from that of skeletal muscle after 4 days. In the heart, the endocardium and pericardium both took up the material within 4 hours. It had almost disappeared from the endocardium by 24 hours, but persisted somewhat longer in the pericardium.

RETICULO-ENDOTHELIAL SYSTEM.—

*Spleen.*—In the spleen the red pulp showed the most prominent fluorescence at all times. At 4 hours the large stellate macrophages already contained a

high concentration of polysaccharide; they were seen mainly in a narrow zone encircling the lymphoid nodules. At later periods these fluorescent cells were scattered diffusely throughout the red pulp. It was impossible to say whether these cells were exclusively macrophages or included monocytes and reticulo-endothelial cells. At 4 hours there was some polysaccharide in those cells of the white pulp which occupied the central areas of the nodules. By their stellate shape these appeared to be reticulo-endothelial cells. Traces of fluorescent material also appeared in or around the small lymphocytes, both in the red and the white pulp. Because of the granular nature of this particular antigen-antibody precipitate, it was never possible to satisfy ourselves with certainty that the material on the lymphocytes was intracellular, as had been possible in the case of type II pneumococcal polysaccharide (2, Fig. 1).

*Lymph Node.*—In all the specimens of axillary lymph nodes, from 4 hours to 33 days after the injection of polysaccharide, the most striking feature was the occurrence of wedges of brilliantly fluorescent macrophages which extended from the capsule to the hilus (Fig. 1). The concentration of polysaccharide in such macrophages decreased somewhat between the 16th and 33rd day, but was still very high at the end of the latter period. Many lymphocytes in the lymphoid nodules also appeared to contain traces of fluorescent material at 4 and 24 hours, but after longer periods only those immediately adjacent to polysaccharide-containing macrophages were definitely fluorescent. During the first 2 weeks, antigen also occurred in the efferent lymph, in or on the lymphocytes.

*Thymus.*—Polysaccharide was prominent in the capsule and stroma of the thymus at all periods. By 24 hours after injection, macrophages along the course of vessels contained considerable amounts of material, but those within the lymphoid tissue displayed very little. The take up by these cells was even more prominent at 48 hours and was still apparent at 33 days. No fluorescence in lymphocytes was observed at any time.

*Bone Marrow.*—Bone marrow was examined in those preparations described below in the section on joints. There was a large amount of fluorescent material in the marrow (Fig. 2), principally in what appeared to be reticular cells; further identification of other cellular elements was not attempted.

BONE, CARTILAGE, AND JOINTS.—

The specimens of joints examined were decalcified knee joints from mice injected intravenously with 2 mg. of polysaccharide and killed 4, 8, 16, and 33 days later, and from a 12 day old rat killed 48 hours after an intraperitoneal injection of 5 mg. of polysaccharide. In addition, an interphalangeal joint from the same immature rat was fixed and sectioned without decalcification.

Polysaccharide penetrated freely into the joint cavity. In the undecalcified

preparation, diffuse fluorescence filled the joint space. In the decalcified specimens, the material appeared to have been precipitated on the synovial and cartilaginous joint surfaces, which were covered with a layer (sometimes discontinuous) of brightly fluorescent granules (Fig. 5). In both the undecalcified and decalcified specimens the cells of the synovial membrane contained large amounts of polysaccharide, and small fluorescent granules could also be seen between the cells. The concentration of polysaccharide in the joint tissues was reduced after periods longer than 48 hours, but the material could still be detected in some synovial cells after 16 days.

In some specimens, granules of fluorescent material were observed in the cartilage cells adjacent to the joint. None, however, occurred in the epiphyseal cartilage (Fig. 2). In the forming bone spicules, polysaccharide appeared in the osteoblasts and in occasional osteocytes.

LUNG.—

After intravenous administration of the antigen, the macrophages of the lung (the "dust" and septal cells) contained no detectable polysaccharide in 4 hours, but became faintly fluorescent by 24 hours. They were possibly a little more conspicuous at 48 hours and at later periods, though again very faint by 33 days. There was no other detectable take up of polysaccharide by lung elements except, in the early stages, by the capillary endothelium.

When given by inhalation, polysaccharide was far more concentrated in the lungs than it was when administered intravenously. Five mice were given 0.1 mg. of polysaccharide in 0.05 ml. of saline intranasally under ether anesthesia and killed 48 hours later. In these lungs, there were small areas of consolidation visible in the gross. Histological examination showed areas of frank consolidation, with exudate in the alveolar lumens, and marked infiltration with macrophages, lymphocytes, and a few polymorphonuclear leucocytes. When the sections were examined for antigen, polysaccharide was concentrated in the macrophages, both in consolidated and unconsolidated parts of the lung (Figs. 3 and 4). Its distribution was irregular, however, and some areas contained little or none. In other sites, especially in the areas of consolidation, the concentration was high, far higher than that attained following intravenous administration of much larger amounts. In these areas, polysaccharide was present not only in macrophages, but also in the endothelium of an occasional capillary loop, and in small bits of cytoplasm often containing basophilic granules, on the alveolar walls and in the alveolar lumens. Occasional small cells, apparently lymphocytes, also displayed cytoplasmic fluorescence. No fluorescence occurred in the bronchial epithelium, but exudate in the lumens of the main bronchi contained a considerable amount of polysaccharide, some of it in free cells. In these five animals, the macrophages in the hilar and tracheal lymph nodes displayed small amounts of polysaccharide.

LIVER, KIDNEY, AND UTERUS.—

*Liver and Gall Bladder.*—At 4 hours the Kupffer cells of the liver sinusoids already contained large amounts of polysaccharide and this persisted throughout the whole period of observation. After the first day or two, they increased in size and at 33 days they were large and as brightly fluorescent as at any earlier period. The rest of the sinusoidal endothelium also contained polysaccharide at all times, though in lower concentration. The hepatic cells were not fluorescent until 24 hours, when some polysaccharide made its appearance in the cells of the portal and intermediate zones. At 4 and 8 days the concentration of polysaccharide in the hepatic cells had increased (Fig. 8) but by 16 days it was diminishing again, and at 33 days had virtually disappeared (Fig. 9). Fluorescence tended to persist longer near the portal areas than centrally.

Apparent reaction within the bile capillaries was noticed first at 24 hours and was still present after 33 days. Confirmatory evidence of the excretion of polysaccharide *via* the bile was obtained by testing samples of bile from the gall bladder. The bile from animals injected intravenously with 2 mg. of polysaccharide contained precipitable antigen at 5 and 17 days, but not at 33 days.

One gall bladder, examined 24 hours after the intravenous injection of 2 mg. of polysaccharide, showed fluorescent material in the capillary walls and in macrophages in the submucosal layer, but none in the epithelium.

*Kidney.*—At 4 hours there was very bright fluorescence of the peritubular capillary plexuses of the kidney. This occurred in all areas, but was most marked in the juxtamedullary zone of the cortex. Thereafter the concentration of polysaccharide in the cortical capillaries decreased progressively (Fig. 6). At 24 hours the amount in the capillaries of the medulla exceeded that in those of the cortex, and at this time uptake by stromal macrophages, especially in the medulla, made its appearance. By 33 days the cortex displayed only occasional flecks of fluorescence in stromal macrophages, whereas in the medulla large amounts of polysaccharide persisted in both capillary walls and macrophages.

At 4 hours, a considerable amount of polysaccharide was present in the glomerular tufts. Fluorescence in these sites was much reduced at 24 hours and occurred in only a few cells, probably macrophages. The number of these polysaccharide-containing cells became progressively fewer, till at 16 days they were seen only in a small proportion of glomeruli and at 33 days had disappeared entirely.

Polysaccharide was observed in casts within the collecting tubules and was demonstrated in the urine of several mice by the precipitin reaction. At 48 hours the presence of polysaccharide within epithelial cells of those segments of the distal tubules in apposition to the glomeruli first became apparent. It was sometimes present in all the cells (Figs. 6 and 7), sometimes mainly in those lying opposite the macula densa. Apart from this limited region of the distal tubule no polysaccharide was detected in the tubular epithelium. At

the same time fluorescence in the walls of some of the glomerular arterioles close to the same segments of the distal tubules was seen. It was not possible to say whether these were afferent or efferent vessels. Polysaccharide in the distal tubules and glomerular arterioles was a constant feature at each subsequent period, though it gradually diminished in quantity.

*Uterus and Oviduct.*—In the uterus of a mouse killed 48 hours after the intravenous injection of 5 mg. of polysaccharide, the stromal cells in the endometrium and scattered macrophages in the muscularis were found to contain high concentrations of the material. Traces were also observed in occasional epithelial cells, especially in the deeper glands. In the oviduct polysaccharide was present only in the macrophages of the stroma.

SUPRARENAL, TESTIS, AND OVARY.—

*Suprarenal.*—At 4 hours, fluorescence within the suprarenal was mainly in the lumens of the blood vessels, particularly in the zona reticularis and medulla; macrophages in the walls of these vessels had also taken up antigen at this time and remained fluorescent at 33 days. Also at 4 hours fluorescent granules appeared to be adsorbed on the surfaces of the cells of the whole cortex, more concentrated in the zona glomerulosa than elsewhere. It was impossible to say whether this polysaccharide was on the reticulum or within the cells. This concentration of granules in the glomerulosa was more pronounced after 24 and 48 hours, and at 4 days the presence of polysaccharide actually within the cells of this zone first became assured. At the same time a smaller amount of polysaccharide was also observed in cells of the zona reticularis. Fluorescence persisted in both these zones throughout the month's period of observation, although diminishing somewhat. Only traces of polysaccharide could be detected at any time in the cells of the zona fasciculata. The cells in the suprarenal medulla, other than the macrophages and capillary endothelium, failed to take up polysaccharide.

*Testis.*—No polysaccharide was detected at any time in the seminiferous tubules, but antigen appeared in some of the interstitial cells after 24 hours and was still apparent after 33 days.

*Ovary.*—Forty-eight hours after an intravenous dose of 5 mg., polysaccharide was found in the cells of the theca interna of the follicles in the ovary and also in the interstitial cells. It occurred in low concentration in the lutein cells (principally those at the periphery) and in larger amounts in occasional macrophages in the sinusoids of the corpus luteum.

### *Persistence of the Antigen*

Liver, spleen, kidney, thymus, suprarenal, heart, and skeletal muscle were examined of the mouse which was killed 3 months after a single injection of 3 mg. of polysaccharide. Polysaccharide persisted in considerable concentration in the Kupffer cells and to a far less degree in the sinusoidal endothelium

of the liver. Traces also remained in the renal stroma, especially in the medula, and in macrophages in the red pulp of the spleen. None persisted in cells in the other organs studied.

Liver, spleen, thymus, lymph node, testis, heart, and skeletal muscle were studied of the mouse killed after 5 months. In these, antigen had all but disappeared. Traces were still present in a few Kupffer cells in the liver, and even smaller amounts in a few macrophages in the spleen.

## DISCUSSION

These results with Friedländer B polysaccharide extend findings previously reported (2) on the distribution of pneumococcal polysaccharides types II and III after intravenous injection into the mouse. The distribution of all three of these acid bacterial polysaccharides was almost completely parallel. Their storage for long periods is also a striking feature of the behavior of these antigens and indicates that their degradation *in vivo* is either difficult or impossible (7). Evidently they are slowly eliminated by the cells into which they find their way, either to be excreted in the urine or bile, or to be delayed in their passage by engulfment by another cell, or to drift into other areas of the tissue spaces.

The cells involved in this storage are primarily the phagocytic cells throughout the body. The substances fail to become associated with epithelial cells generally, though there are some exceptions: (1) secretory epithelia, *i.e.* the hepatic cells, the juxtaglomerular segments of the distal renal tubules, and some of the uterine gland cells; and (2) cells in the steroid-secreting areas of the suprarenal, ovary, and testis. Cardiac muscle cells and an occasional skeletal muscle cell were found to take up pneumococcal polysaccharide, but this could not be demonstrated for Friedländer B polysaccharide.

The taking up of pneumococcal polysaccharide by the epithelium of the distal renal tubule was reported in a previous paper (2) but attention was not drawn to the consistent nature of this localization and to the fact that it was confined to the juxtaglomerular segment. Reexamination of sections of kidneys from mice injected with pneumococcal polysaccharide, type III, has shown that the area of deposition was identical with that now described.

In the lungs, the macrophages of the alveolar walls took up a relatively small amount of polysaccharide after it had been administered intravenously but contained high concentrations after a much smaller dose had been given by inhalation. The intranasal dose used was the largest employed by Horsfall and McCarty (8) in demonstrating the inhibiting effect of this polysaccharide on the multiplication of PVM virus in the lungs of mice; they found that smaller doses were also effective. The low take up of polysaccharide in the lungs after its administration intravenously is consistent with the observation by these workers that inhibiting substances were ineffective when given by this route rather than by inhalation.

In the mice receiving polysaccharide by inhalation, antigen was likewise found in the macrophages, but was not present consistently in other lung elements. In those areas containing the most polysaccharide, however, and which were likewise the seat of an inflammatory reaction, there were small bits of cytoplasm in the alveolar lumens which fluoresced brightly. The origin of this cytoplasm is, of course, obscure. For this reason, as well as because of the minute doses of polysaccharide effective in the inhibition of PVM virus by Friedländer B polysaccharide, it is not possible from present data to identify the probable cell type involved in this interesting effect of the polysaccharide. A careful serial study of the fate of the polysaccharide after inhalation, as well as a study of the localization of the virus itself, if possible, would probably be necessary to approach this question.

It is a matter of interest that the polysaccharide penetrated into the synovial fluid and that comparatively large amounts were taken up by the synovial membrane. Cartilage cells near the joint were sometimes found to contain the material, which had presumably diffused in from the joint space.

## SUMMARY

The fate of the capsular polysaccharide of Friedländer B bacillus in the mouse after its intravenous administration was studied by means of homologous antibody labelled with fluorescein. The results indicate that this acid polysaccharide, like pneumococcal polysaccharide, types II and III, was rapidly taken up by phagocytic cells throughout the body, where it persisted in decreasing concentration for more than 33 days. It was widely distributed in the capillary endothelium and on collagenous fibres in all organs. It made a transient appearance in or on lymphocytes in the spleen and lymph nodes. It was found in the hepatic epithelium and in the bile; in the juxtaglomerular segment of the distal renal tubule and in an occasional cast in the lumens of collecting tubules; in the epithelium of some uterine glands; and in cells in the steroid-secreting tissues of the ovary, suprarenal cortex, and perhaps of the testis.

In joints the synovial membranes contained large amounts of antigen, and some also penetrated into cartilage cells adjacent to the joint cavity. Osteoblasts and a few osteocytes also took up the polysaccharide.

When administered by inhalation, the polysaccharide was found in high concentration in the pulmonary macrophages but could not be found constantly in other lung elements.

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## EXPLANATION OF PLATES

All photomicrographs are of organs from animals to which Friedländer B capsular polysaccharide had been administered. The tissues were fixed in picric acid–formalin–alcohol, embedded in paraffin, sectioned at 5$\mu$, and exposed to fluorescein-labelled homologous antibody. Except in the case of Fig. 7, the sections were photographed under the fluorescence microscope. The lightest areas represent the yellow-green fluorescence of the precipitated fluorescein-antibody, while the histological topography of the organs is made visible by a faint bluish autofluorescence of the tissues.

### PLATE 1

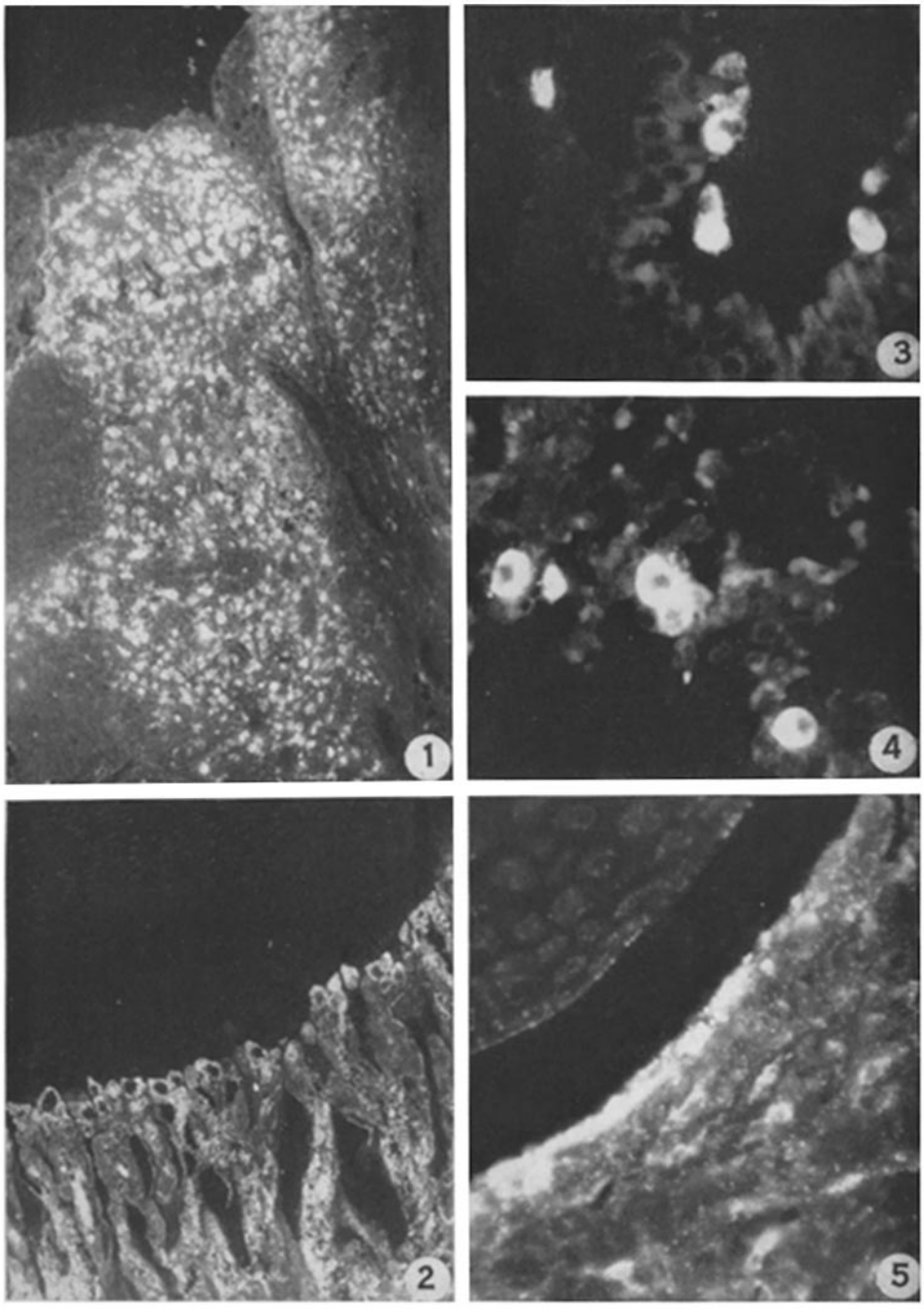
FIG. 1. Axillary lymph node of mouse (2 mg. polysaccharide intravenously; killed after 16 days). Large accumulations of macrophages beneath the capsule and between the lymphoid nodules contain high concentrations of polysaccharide. × 140.

FIG. 2. Upper end of tibia of 12 day old rat (5 mg. polysaccharide intraperitoneally; killed after 48 hours; bone decalcified after fixation). The epiphyseal cartilage occupies the upper part of the figure, the newly formed bone the lower part. The marrow spaces, occupied by reticular cells and osteoblasts, contain large amounts of polysaccharide. None is visible in the cartilage. × 140.

FIG. 3. Bronchiole of mouse (0.1 mg. polysaccharide by inhalation; killed after 48 hours). Several macrophages containing polysaccharide are free in the lumen of a respiratory bronchiole. The epithelium of the bronchiole contains no polysaccharide. × 560.

FIG. 4. Lung from same mouse. Several septal cells both in the walls of alveoli and free in the lumen contain large amounts of polysaccharide. The relative brightness of the red blood cells in the capillary plexus is due to non-specific fluorescence. × 560

FIG. 5. Knee joint of a 12 day old rat (same animal as Fig. 2). The synovial membrane contains large amounts of polysaccharide in synovial cells, with a notable concentration on the surface. Some of that on the surface probably resulted from precipitation from the joint fluid by the trichloroacetic acid used in decalcification. Small granules are also scattered between the cells. The portion of semilunar cartilage visible at the upper left corner of the figure shows minute granules of polysaccharide in the cartilage cells. × 560.




(Hill *et al.*: Localization of antigen in tissue cells. V)

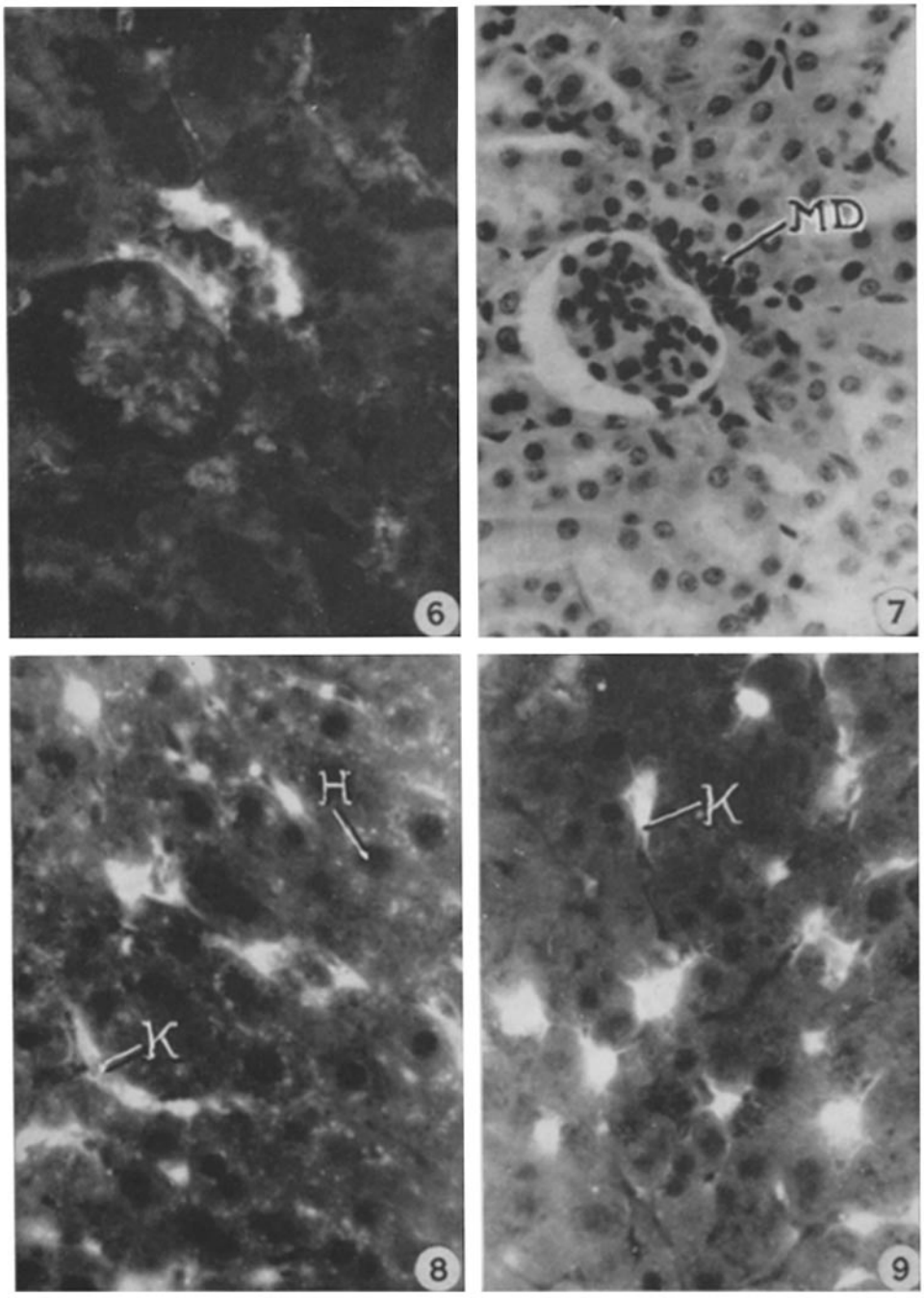
PLATE 2

FIG. 6. Renal cortex from mouse (2 mg. polysaccharide; killed after 48 hours). Large amounts of polysaccharide occur in the epithelium of the juxtaglomerular segment of a distal tubule. Traces can also be seen in the glomerulus, and in the peritubular stroma and capillaries. × 560.

FIG. 7. Renal cortex. Identical field of the same section, stained with hematoxylin and eosin after the photograph shown in Fig. 6 had been taken. The site of the fluorescence in Fig. 6 is seen to have included the macula densa (MD). × 560.

FIG. 8. Liver of mouse (2 mg. polysaccharide intravenously; killed after 8 days). Enlarged Kupffer cells (K) contain big amounts of polysaccharide; fluorescent granules are also clearly visible in the cytoplasm of many hepatic cells (H). × 560.

FIG. 9. Liver of mouse (2 mg. polysaccharide intravenously; killed after 33 days). The Kupffer cells (K) still contain large amounts of polysaccharide, but none is now visible in the hepatic cells. × 560.




(Hill *et al.*: Localization of antigen in tissue cells. V)