LOCALIZATION OF ANTIGEN IN TISSUE CELLS

III. Cellular Distribution of Pneumococcal Polysaccharides Types II and III in the Mouse*

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Plates 1 to 4

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Previous studies from this laboratory (1, 2) have described a method for the cytochemical detection of antigen in tissue sections by means of fluorescein-labelled antibody. This technique has been employed in the present study for the localization of pneumococcal capsular polysaccharides, types II and III, in various tissues and organs of the mouse.

Evidence of the presence of the pneumococcal polysaccharides in tissues was first obtained from examination of extracts of organs from patients with lobar pneumonia. Nye and Harris (3) tested extracts of organs from fatal cases of pneumonia for their content of polysaccharide by the precipitin reaction, and found large concentrations in lung and smaller amounts in liver and spleen. Frisch and colleagues (4) reported large amounts of polysaccharide in fluid expressed from lung tissue in types I, II, III, VII, and VIII lobar pneumonia. Felton (5) examined extracts of organs from patients without any history of pneumonia and demonstrated traces of one or more types of polysaccharide in the liver, spleen, and kidney. The presence of such traces was shown by the specific antigenic action of the extracts when injected into mice.

Recently Felton and coworkers (5, 6) have made an extended investigation of the localization of polysaccharides in the tissues of mice in connection with the so called "immunological paralysis" produced by these substances. They measured by the precipitin reaction the amount of polysaccharide in extracts of pooled organs taken from mice injected intraperitoneally with each of the polysaccharides, types I, II, and III. One month after injection, these antigens could be detected in extracts of each of the following organs: liver, spleen, kidney, muscle and bone heart, lung, stomach and intestine, blood, and skin.

In the present work, the distribution of polysaccharides, types II and III, in the tissues of the mouse was followed histochemically by means of the fluorescein-antibody technique. It has thus been possible to identify the tissue

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cells in which these polysaccharides became fixed after intravenous injection. The most regular and striking concentrations were found in (1) the cells of the reticulo-endothelial system, (2) the capillary endothelium, (3) the connective tissue cells and fibers, (4) the lymphocytes, (5) steroid-forming cells in the adrenal cortex, testis, and ovary, (6) epithelium of the liver and uterus, and (7) the cardiac and smooth muscle cells. In addition, data are presented describing the relative persistence of polysaccharide, type III, in these cells, as well as its relative uptake by and disappearance from the tissues.

Materials and Methods

Polysaccharides.—Both of the pneumococcus capsular polysaccharides, types II and III,[1] employed in the present work were antigenic in mice as tested by the procedure described by Avery and Goebel (7). In the case of each product, a dosage of 0.5 microgram resulted in the production of resistance 10 days later to 1000 to 10,000 minimal lethal doses of homologous pneumococcal culture.

Injection of Mice.—The polysaccharides were dissolved in sterile saline to the desired concentration, and 0.5 ml. was injected into the tail vein of white mice weighing 20 gm. The dosages administered ranged from 0.008 mg. to 8 mg. Each mouse received a single injection. For the detailed description of polysaccharide distribution in the tissues, 4.0 mg. was injected, and the mice were killed after 1, 2, or 4 days. In the case of the type III polysaccharide, the distribution was followed in a number of organs for as long as 75 days. In this experiment, at least 2 mice were killed and examined at each of the following intervals: 1, 2, 4, 8, 16, 32, 50, and 75 days. In addition, one mouse received 8 mg. of type III polysaccharide and was killed after 6 months. Blood samples were taken at the time the animals were killed, and the sera were tested for polysaccharide by the precipitin reaction.

Histological Procedures.—Organs removed for routine examination included the spleen, axillary and inguinal lymph nodes, thymus, lung, liver, kidney, heart, and adductor muscle of the right hind leg. From occasional mice the following organs were studied as well: the stomach, duodenum, colon, adrenal, testis, ovary, oviduct, uterus, skin, sternum, brain, and spinal cord.

Of various fixatives tested, the picric acid–alcohol–formalin mixture employed by Rossman (8) for glycogen was found best for preserving the polysaccharide within tissue cells. The tissues were fixed in the cold (5°C.) for 12 to 18 hours. To minimize hardness and to facilitate sectioning, small pieces of tissue were employed and the periods of dehydration and infiltration with paraffin were made as brief as possible. Sections were cut at 5 μ. After mounting on gelatinized slides, the sections were dried overnight at 37°C., and then stored in the refrigerator until ready for staining.

Sections were also prepared from unfixed, frozen organs by the Linderstrøm-Lang technique (9), as described in the preceding paper (2).

Use of the Fluorescein-Antibody Conjugates.—Fluorescein-antibody conjugates of antipneumococcal sera, types II and III, were prepared as described in the preceding paper (2). The conjugates were absorbed twice with equal volumes of a suspension of mouse liver cells or with mouse liver powder. The controls employed for establishing specificity of staining were (*a*) absence of reaction with normal mouse tissues; (*b*) specific inhibition; (*c*) absence of reaction with specifically absorbed conjugate.

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The procedure for staining the paraffin sections was as follows: The sections were deparaffinized in xylene, then hydrated through graded alcohols. It was important to wash out all the picric acid from the sections to prevent non-specific fixation of fluorescein-labelled protein on the sections during staining. To this end, the slides were washed, with continuous agitation, for 20 minutes in 95 per cent alcohol and then for 30 to 40 minutes in 70 per cent alcohol until the sections were perfectly colorless. This procedure removed the picric acid completely without detectable loss of polysaccharide. The slides were then immersed in 0.85 per cent saline and agitated for only 30 seconds in order to remove the alcohol and yet avoid leaching out the polysaccharide. The fluorescein-antibody preparation was applied immediately to the section.

When frozen, unfixed sections were used, the conjugate was applied directly to the dry section.

The reaction was carried out by layering 2 drops of the fluorescent antibody over the section and allowing it to stand at room temperature for 20 minutes. The excess conjugate was then removed by immersing the slide in physiological saline buffered at pH 7.0 and washing with continuous gentle agitation for 10 minutes. The sections were mounted in glycerol buffered at 7.0.

Examination of the sections under the fluorescence microscope was carried out preferably on the same day they were prepared, although, when stored in the refrigerator, the slides showed no appreciable loss of fluorescence for 2 or 3 days. For identification of tissues and cells, other sections of the material were stained with hematoxylin and eosin.

Periodic Acid–Schiff Method.—An alternative method for staining the polysaccharide was the periodic acid–Schiff method described by McManus (10) for the staining of mucoid substances. This method has also been employed by Hotchkiss (11) for visualizing several bacterial polysaccharides.

Tabulation of Polysaccharide Distribution.—In order to compare the distribution of polysaccharide in different cell types, particularly under conditions of varying dosage and duration of treatment, the following arbitrary scheme was devised. The proportion of cells of a given type in which polysaccharide was definitely detected was graded by the numbers 0, 1, 2, 3, 4, as follows: 0, no cells containing polysaccharide; 1, few cells; *i.e.*, less than 5 per cent of the cells seen in the section; 2, some cells; *i.e.*, 5 to 25 per cent; 3, many cells; *i.e.*, 25 to 75 per cent; 4, nearly all; *i.e.*, 75 to 100 per cent.

RESULTS

Distribution of Pneumococcal Polysaccharides, Types II and III, in the Mouse

In the following section a detailed description is given of the localization of polysaccharide in the organs studied and its variation with time. The dose given in most instances was 4.0 mg. in a single injection. Polysaccharide was detected in the sera of all these mice when they were killed. No difference was noted in the kinds of cells in which the type II or III polysaccharide could be detected; consequently, reference to polysaccharide includes both substances.

Spleen.—Polysaccharide was located in both the red and white pulp of the spleen (Figs. 1 to 4). In the red pulp (Fig. 4) it occurred in the cytoplasm both of the reticulo-endothelial elements and of the lymphocytes. The larger cells (for the most part macrophages and monocytes) contained such dense concentrations of the material that little detail except the nuclear shadow could be made out. The smaller cells (lymphocytes) contained less material, and here

it occurred either as discrete granules in the cytoplasm or sometimes as a diffuse "wash."

TABLE I

*Effect of Time on the Distribution of Injected Pneumococcal Polysaccharide, Type III, in the Tissue Cells of the Mouse**

Cell types	Time after injection of polysaccharide, *days*							
	1	2	4	8	16	32	50	75
Spleen macrophages	3‡	4	4	4	3	3	2	3
Spleen lymphocytes	2	3	2	2	1	1	1	1
Inguinal node macrophages	3	4	4	4	4	3	3	3
Inguinal node lymphocytes	2	2	1	1	1	1	1	1
Axillary node macrophages	4	4	4	4	4	4	3	3
Axillary node lymphocytes	2	2	2	2	1	1	1	1
Thymus macrophages	2	3	3	2	3	2	1	2
Thymus lymphocytes	2	2	2	1	2	1	1	1
Lung septal cells	4	4	3	3	3	2	2	2
Liver Kupffer cells	3	4	4	4	4	3	3	3
Liver hepatic cells	1	2	3	2	2	2	2	1
Kidney capillary endothelium	4	4	4	4	3	3	2	2
Heart macrophages	4	4	4	3	3	3	2	2
Heart myocardial cells	2	2	2	1	1	1	1	1
Skeletal muscle cells	0	1	1	0	1	1	0	0

* Amount of polysaccharide administered intravenously was 4.0 mg. in a single dose.

‡ The relative proportion of the cells of a given type which clearly contained polysaccharide was graded by the numbers 0, 1, 2, 3, 4 as described in the text (p. 17). Results given are the average of the distribution in two mice for each period.

For the spleen, the macrophages graded lay in the red pulp, the lymphocytes in the white pulp; for the lymph node, the macrophages considered were in the subcapsular sinus, the lymphocytes in both the cortex and medulla; for the kidney, the capillary endothelium of the peritubular plexuses in the medulla was considered.

Polysaccharide remained fixed in most of the macrophages of the red pulp for longer than 75 days (Table I).

Lesser amounts of polysaccharide occurred in white pulp, the lymphoid nodules of the spleen. Here it was found both in the round cells of the lymphocyte series and in the phagocytic reticular cells, or macrophages (Figs. 1 to 3).

At first there was a definite pattern of distribution of polysaccharide within the lymphoid nodule. During the first 2 days after injection, it occurred principally in those cells near the outer edge of the nodule and in those near the artery. By 4 days, polysaccharide was uniformly distributed. At this time, the fluorescent staining of the cytoplasm of the lymphocytes was quite bright. After 8 days, however, only a faint fluorescent "wash" could be detected.

Thymus.—In the thymus, polysaccharide was detected principally in macrophages and to a lesser extent in lymphocytes (Fig. 5). The areas of uptake were clearly associated with the blood vessels passing through the gland; *i.e.*, near the afferent arteries and the medullary veins. Areas lacking large blood vessels contained little or no polysaccharide. As in the other lymphoid organs, the antigen persisted in the macrophages for a considerably longer time than in the lymphocytes (Table I).

Lymph Nodes.—The following description applies to both the inguinal and axillary lymph nodes. The over-all concentration of polysaccharide in the lymph nodes appeared to be lower than that in the spleen and similar to that in the thymus. The principal concentrations were in the reticulo-endothelial cells of the subcapsular sinus (Figs. 6 and 7), the medullary sinuses, and the efferent lymphatics at the hilus. Some of these cells contained large concentrations for the entire 75 days of the experiment. In addition, smaller quantities of polysaccharide occurred within some of the lymphocytes (Fig. 6). The lymphocytes which contained most antigen were situated in the medullary cords near the hilus and in the efferent lymphatics. In a few specimens, however, considerable amounts of material were found in the cells within the cortical nodules (Fig. 6). In general, polysaccharide persisted in significant numbers of lymphocytes only for the first 8 days after injection (Table I).

The capsule of the node appeared to have a considerable amount of polysaccharide absorbed onto its fibers. In addition, the adipose tissue surrounding the node contained much material which occurred principally in the fibroblasts and on the reticulum. Some was detected also in the cytoplasm of the fat cells. Uptake by fat and connective tissue was observed in all organs studied; these locations will not be reiterated below.

Heart.—The greatest concentration of polysaccharide in the heart was found in macrophages and on the reticulum between the muscle fibers (Fig. 8). These were the major sites of antigen fixation after 75 days. Some occurred also in the endothelium of the capillaries supplying the muscle and in the endocardium. In addition, traces of antigen were sometimes revealed within the muscle fibers. Generally, this material appeared as a diffuse "wash" of fluorescence; occasionally granules were scattered between the myofibrils. Intramuscular polysaccharide usually occurred in the vicinity of heavily laden macrophages (Fig. 8).

Adductor Muscle.—Polysaccharide was usually limited to the stroma of skeletal muscle. Here it occurred in greatest concentration in macrophages and the

endothelium of capillaries, and to very slight degree on the reticulum. Traces were observed only occasionally within the muscle fibers (*cf.* Fig. 9).

Kidney.—Most of the polysaccharide in the kidney was found in the endothelium of the glomeruli and the capillaries serving the tubules (Fig. 10). It was particularly striking in the capillaries of the kidney medulla (Fig. 11); in this location, the cells and fibers of the supporting stroma also contained antigen. In the medullary sites, it persisted for longer than 75 days. In addition, the walls of the arterioles immediately adjacent to glomeruli sometimes contained large amounts of the fluorescent material; the walls of the larger vessels usually contained none.

The tubular epithelial cells were usually free of polysaccharide; occasionally, however, a few cells took it up, particularly in the distal convoluted tubules adjacent to the glomeruli (Fig. 10). Polysaccharide also occurred as droplets in the lumens of the tubules, and as a component of casts occurring in the distal tubules and collecting ducts.

Gastrointestinal Tract.—Sections of the stomach, duodenum, appendix, and colon were examined for the distribution of polysaccharide. The greatest amounts were found in the macrophages and reticulum in the submucosa and the lamina propria of the mucosa, and in the endothelia of blood and lymph capillaries. Occasionally, polysaccharide could be detected in segments of the smooth muscle wall and in the cells of the serous coat. No material could be found in the mucosal epithelium or in the contents of the lumen. In the lymphoid nodules of the intestinal wall, polysaccharide occurred principally in macrophages, but occasionally in lymphocytes as well.

Liver.—The principal accumulation of polysaccharide in the liver occurred in the Kupffer cells lining the sinusoids (Fig. 12). Fluorescent material could be found in most of these cells during the entire 75 day period. Lesser concentrations occurred within the hepatic cells, with most appearing in those lying in the peripheral and intermediate zones of the lobule. In favorable preparations, polysaccharide could also be distinguished within the bile capillaries (Fig. 12). As shown in Table I, the hepatic cells took up polysaccharide at a gradual rate, maximal accumulation occurring at 4 days. Following this there was a gradual disappearance of material from the parenchymal cells.

Lung.—In the lung, the lining of the alveoli (capillary endothelium) contained small amounts of polysaccharide (Fig. 13); the epithelium of the bronchi and bronchioles contained none. The principal concentrations occurred in the macrophages of the alveolar walls, the so called septal cells. It persisted in these cells for as long as 75 days. In addition, traces of polysaccharide occurred in the endothelium of arterioles and venules. In isolated specimens, it was detected in the smooth muscle cells in the walls of bronchi, bronchioles, and the larger blood vessels. Material also appeared to be adsorbed onto collagenous fibers in the walls of both the blood vessels and the bronchial tree.

Testis.—Polysaccharide occurred solely in the interstitial tissue of the testis—both in the cells and in the walls of the small blood vessels (Fig. 14). All the interstitial cells contained some antigen, although the amount varied. None of the material occurred in the seminiferous tubules or in the sperm.

Ovary.—Polysaccharide was found especially concentrated in macrophages of the stroma, in the cells of the interstitial glands, and in isolated macrophages lying along the sinusoids in the corpora lutea. In addition, lesser concentrations were observed within the lutein cells and the cells of the theca interna of the follicles. None occurred in the granulosa cells of the follicles.

Oviduct and Uterus.—No polysaccharide whatsoever occurred in the mucosal epithelium of the oviduct, but extremely large amounts occurred in the submucosa. Here it was most concentrated in scattered macrophages, but some also occurred in the fibroblasts and on the fibers. Likewise polysaccharide was present in the macrophages lying within the muscle layers but was usually absent from the muscle fibers proper.

In the uterus, unlike the oviduct, fine particles of polysaccharide were detected within the epithelial cells, covering the surface and lining the glands. Traces were present in the lumen of the uterus as well. As in the oviduct, large concentrations occurred in macrophages lying in the endometrial stroma and amongst the muscle fibers. Occasionally some occurred in the smooth muscle.

Adrenal Gland.—Polysaccharide was regularly present in the form of minute droplets within the cortical cells of the zona glomerulosa (Fig. 15). It appeared occasionally in the zona reticularis and was usually absent from the zona fasciculata. In addition, occasional macrophages in the endothelium of the cortical sinusoids contained accumulations of the antigen. In the medulla, the material was limited to macrophages lying interspersed among the groups of medullary cells.

Skin.—No polysaccharide was present in the epidermis. In the dermis, there was considerable material in the connective tissue, both within the fibroblasts and macrophages and adsorbed onto the fibers. Polysaccharide could be detected in the endothelium of the blood and lymph capillaries. In the subcutaneous tissue, the fat cells and other connective tissue cells were brightly fluorescent, as were the cells in the fascia surrounding the subjacent skeletal muscle bundles. The skeletal muscle fibers themselves contained no polysaccharide.

Nervous System.—In the central nervous system, polysaccharide was limited to the walls of the blood vessels of brain and cord, to the chorioid plexuses, and to the meninges. In the posterior pituitary, polysaccharide occurred only in the walls of the capillaries. In the anterior part of the gland, it occurred throughout the sinusoidal endothelium, particularly in the macrophages, and in some of the epithelial cells as well. In the sciatic nerve, polysaccharide occurred within what appeared to be the sheaths of Schwann enveloping the

axis cylinders. In addition, some occurred on the fibers of the enmeshing reticulum.

Sternum.—It was found that considerable amounts of polysaccharide remained *in situ* during decalcification of the bone by trichloracetic acid. Polysaccharide was detected in a large number of cells in the marrow, which appeared from their distribution to be cells of the supporting reticulo-endothelium. More precise identification of these cells was impossible under the fluorescence microscope. In such sections, polysaccharide could also be seen in the periosteum, in the stroma of the Haversian canals, in the macrophages of the attached muscles, and in the marrow fat.

Effect of Dosage of Injected Polysaccharide Type III on Distribution

The experiment was carried out as follows: 6 groups of 3 mice were injected with the following amounts of type III polysaccharide: 0.008, 0.03, 0.125, 0.50, 2.0, and 8.0 mg. Twenty-four hours later the mice were killed, the tissues removed for examination, and the sera tested for polysaccharide by the precipitin reaction. Tissues were studied in parallel after fixation in picric acid–alcohol–formalin and after freezing and sectioning by the Linderstrøm-Lang method. Sections were made of the following organs: lung, liver, kidney, heart, adductor muscle, spleen, and axillary lymph node.

With the 0.008 mg. dose, polysaccharide could not be detected in the serum, nor could any be detected in the organs histochemically, either in frozen or fixed preparations. At the 0.03 mg. dose, however, the serum gave a positive precipitin reaction; and polysaccharide could be found in the Kupffer cells of the liver, the macrophages of the spleen and lymph nodes, the endothelium of the capillaries in the kidney, the macrophages of the heart, and the septal cells of the lung (Table II). No polysaccharide could be detected at this dose in the hepatic cells, lymphocytes, or myocardial cells. The results were identical with both methods of tissue preparation.

The effect of increasing the dosage to 0.125 mg. was to increase relatively the proportion of each cell type taking up polysaccharide. In addition, at this dose, polysaccharide could be detected also in the hepatic cells, myocardial cells, and lymphocytes. With the highest dosage used, namely, 8.0 mg., polysaccharide uptake was increased to a point where the majority of all these cells contained polysaccharide, as well as occasional skeletal muscle cells (Fig. 9).

Distribution of Polysaccharide Type III 6 Months after Injection

In one mouse, the distribution of polysaccharide was examined 6 months after a single injection of 8 mg. of type III antigen. In this animal, polysaccharide was found principally in macrophages in the spleen (Fig. 16), Kupffer cells in the liver (Fig. 17), endothelium of the capillaries around Henle's loops in the kidney (Figs. 18 and 19), macrophages in the heart (Fig. 20), and adrenal cortical cells (Fig. 21). Only traces persisted in the axillary lymph node, lung,

skeletal muscle, and testis. The serum gave a faintly positive precipitin reaction for circulating polysaccharide.

Distribution of Polysaccharide as a Result of Infection

Two groups of 3 mice each were infected with 0.1 ml. of 2 different dilutions (10^{-4} and 10^{-5}) of a highly virulent 6 hour culture of pneumococcus type III. One mouse from each group was killed when moribund according to the following plan. When one in each group had

TABLE II

*Effect of Dosage on the Distribution of Pneumococcal Polysaccharide Type III**

Cell types	Amount of polysaccharide injected, *mg.*					
	0.008	0.031	0.125	0.50	2.0	8.0
Spleen macrophages	0	1	2	3	3	4
Spleen lymphocytes	0	0	0	1	2	3
Lymph node macrophages	0	1	2	2	3	4
Lymph node lymphocytes	0	0	1	1	2	2
Lung septal cells	0	1	2	2	3	4
Liver Kupffer cells	0	2	3	3	4	4
Liver hepatic cells	0	0	1	2	2	3
Kidney capillary endothelium	0	1	2	3	4	4
Heart macrophages	0	1	2	3	4	4
Heart myocardial cells	0	0	1	2	2	3
Skeletal muscle cells	0	0	0	0	0	1

* The mice were killed 24 hours after injection. The relative proportion of the cells of a given type which clearly contained polysaccharide was graded by the numbers 0, 1, 2, 3, 4, as described in the text (p. 17).

died, the sicker of the 2 survivors was sacrificed. This was approximately 40 hours after infection. In each case the remaining mouse in each group died within 3 hours. The sacrificed mice were autopsied, frozen sections of the organs prepared, and examined. While it is possible that some of the fluorescent staining observed may have been due to pneumococcal antigen other than polysaccharide, the amount of this was probably small by comparison.

The most prominent features were the large amounts of specific staining both of organisms and of free antigen in the serosal cells and exudate covering the surfaces of the liver, spleen, heart, lung, and thymus. There were many organisms visible in the vessels of the liver, heart, and kidney, and in the red pulp of the spleen. The Kupffer cells of the liver, the macrophages of the splenic

red pulp, and the macrophages of the subcapsular sinuses of the lymph nodes contained large amounts of antigenic material, which consisted both of bacterial bodies and of material spread diffusely in the cytoplasm of these cells. An occasional vessel in the myocardium showed organisms lined up along it, with free antigen in the endothelium and in the exudate around the vessel.

Aside from the phagocytic cells, the only other cells containing antigen were occasional groups of lymphocytes in the lymph node. The hepatic cells, myocardial cells, and renal tubular cells were free of antigen. The adrenals were not examined.

Compared with the mice injected with known doses of polysaccharide, it was estimated that the generalized distribution of antigen in these infected mice corresponded to a dose of about 0.5 mg., although, of course, the large localized concentrations in the serous membranes as a result of peritonitis and pleuritis were greater than those seen following intravenous injection of free polysaccharide. It appeared that such an acute infection in a highly susceptible animal results in death before large amounts of antigen can become widely distributed.

Localization of Polysaccharide, Type III, by Means of the Periodic Acid–Schiff Method

Attempts to detect pneumococcal polysaccharide, type III, in the tissues by means of the periodic acid–Schiff method were complicated by the relative non-specificity of the staining reaction. Staining occurred not only with the pneumococcal polysaccharide but also with normal tissue components such as reticular fibers, collagen, mucus, cartilage matrix, and glycogen (12). It was possible to rule out glycogen by first digesting the preparations with saliva. However, because of the rather weak staining obtained and because of the lack of specificity, this method served only to corroborate the results of the fluorescein-antibody technique (compare Figs. 6 and 7).

DISCUSSION

This investigation of the fate of injected pneumococcal capsular polysaccharide has served a twofold purpose. It has offered an opportunity for testing and improving this method for the histochemical localization of antigens (2); and second, it has uncovered the widespread distribution of these polysaccharide antigens in the tissues of the mouse. The fate of these substances is of interest not only from the point of view of antigen localization but also because of their importance in the virulence of the organisms producing them.

Earlier studies of the distribution of antigens have been confined almost entirely to their demonstration in organ extracts (see reference 2 for summary). Some evidence, however, has been obtained of their cytological distribution by the use of dye-labelled proteins. Thus, Sabin (13) demonstrated the presence of dye-labelled egg albumin in phagocytic cells, that is, in macrophages, neutro-

philes, lymphatic endothelium, monocytes, and Kupffer cells. Smetana (14) described the localization of similarly tagged protein in the parenchyma of the convoluted tubules of the kidney.

In the present work, pneumococcal polysaccharide has been identified in the following sites: the reticulo-endothelial elements (fixed and free macrophages, monocytes, and reticular cells), fibroblasts, capillary endothelium, lymphatic endothelium, endocardium, lymphocytes, hepatic cells, steroid-producing cells in the ovary, testis, and adrenal cortex, and epithelium of the uterus. Small amounts were detected also in cardiac, smooth, and skeletal muscle cells. In addition, the material appeared to be adsorbed onto reticular and collagenous fibers.

It is interesting that this distribution is similar in many respects to that of acid vital dyes (*e.g.*, trypan blue, isamine blue, pyrrol blue) and also in some respects to certain suspensoids (*e.g.*, saccharated oxide of iron, carbon (15–20)). Such substances have long been used as models for the study of the protective mechanisms against foreign materials, particularly infectious agents. However, just as these substances vary among themselves in the sites they come to occupy and in the relative rates at which they are taken up and released from various tissues (15, 16) so these polysaccharides resemble no one of them exactly in this distribution, either qualitatively or quantitatively. Polysaccharide resembles trypan blue (15, 17), for example, in that it rapidly penetrates a large variety of tissues throughout the body, but differs by persisting in these locations for a long time. This persistence is particularly remarkable in the capillary walls and on the collagenous fibers—sites where trypan blue is only temporarily visible (15, 18). Polysaccharide also penetrates lymphocytes, like saccharated oxide of iron (16); the latter material, however, even though a suspensoid, disappears from these cells much more rapidly. It may be said that, in general, polysaccharide behaves like the acid colloidal dyes in its rapid and widespread penetration, and the suspensoids in its long persistence. This behavior may depend on the facts that both the type II and type III polysaccharides are acidic; they are of macromolecular dimensions with molecular weights of the order of 150,000 to 500,000 (21); and they appear not to be metabolized by mammalian tissues (22).

In some respects polysaccharide differs quite markedly from these other foreign materials. Despite its occurrence in the lumens of the kidney tubules and its presence in the urine (7, 23, 24), it is reabsorbed only in a narrow range of the tubular cells, unlike other substances of large molecular size which pass through the glomerulus (15, 25, 14). Likewise, polysaccharide does not appear to enter the cells of the posterior pituitary, as do trypan blue and certain other acid dyes (19). These facts suggest a certain selectivity by these cells in their uptake of foreign materials and emphasize the limitations of using any one substance as a model.

Since the present work was undertaken primarily as an approach to the

problem of antigen distribution, some consideration should be given to the possibility that antigens, like other substances, may vary among themselves in their distribution. There is indirect evidence, derived from the study of serum disease, that the injection of different antigens results in lesions in differing locations. Hawn and Janeway (26), using purified bovine albumin and γ-globulin, found that lesions produced by albumin were limited to arterial walls, whereas those from γ-globulin occurred in the glomeruli and the myocardial connective tissue. While it would appear that direct evidence on the distribution of these different protein antigens is necessary before the pathogenesis of serum disease can be approached, nevertheless, it is of some interest that pneumococcal polysaccharide was regularly present in all three of these locations.

It should be also pointed out that these capsular polysaccharides evidently occupy a somewhat unusual position in the spectrum of antigenic materials. They will not stimulate antibody production in the rabbit unless coupled to protein (7, 27). Injected into mice in doses of 0.1 to 5 micrograms, they actively immunize against fatal challenge (7, 28, 29). However, at 50 to 100 times these doses, they not only fail to immunize, but they make immunization by subsequent smaller doses impossible for a year or longer. This problem was studied in detail by Felton and collaborators (5, 6), who found that this was not a general toxic effect on the antibody-producing mechanism, but that interference with immunization was type-specific. They demonstrated the long persistence of these pneumococcal polysaccharides in mice and suggested that the "immunological paralysis" induced by large doses was brought about by chronic fixation of polysaccharide in the reticulo-endothelial cells, where it was assumed to interfere in some way with the formation of antibody.

Although the present work demonstrates that polysaccharide is taken up by reticulo-endothelial cells, this does not necessarily indicate that antibody formation is inhibited even if it be assumed that these cells are the site of antibody formation. Six months after injection, many reticulo-endothelial cells no longer contain polysaccharide—especially true in the lymph node—yet at this time "immunological paralysis" is still marked. A simpler explanation is at least possible, namely, that newly formed antibody is continuously neutralized as a result of the persistence of the antigen both in the tissues and in the circulating blood.

The question of the cell type or types responsible for the formation of antibody cannot be answered by a study of the distribution of an antigen. But those cells which can be shown to take up antigenic material are possible sites of synthesis. In view of the recent evidence of Ehrich and Harris (30) and Dougherty, Chase, and White (31, 32) suggesting that the lymphocyte is a source of antibody, it is interesting that polysaccharide is taken up by cells of the lymphocyte series. However, other sites, such as the reticulo-endothelial

cells (13) and the plasma cells (33, 34) have also been considered as sources of antibody. In this work we have not attempted to trace polysaccharide to plasma cells, since the identification of individual cell types in mixed cell populations is difficult under the fluorescence microscope. This might be possible by subsequently staining the fluorescence preparation for the identification of the cells as illustrated in Figs. 1 to 3.

A possible approach to the problem of where antibodies are formed is the reversal of the present method by using a labelled protein antigen. Use of such a labelled antigen to detect antibody *in situ* would perhaps throw some direct light on the problem.

Finally, it is curious that these capsular polysaccharides which are known to be of paramount importance in determining the virulence of the pneumococcus and which have been shown to be widely disseminated throughout the body of the mouse, a highly susceptible animal, have little or no toxic effects (35). Doses of the material ten times greater than the amount present in mice dying of infection are tolerated without difficulty. This apparent lack of toxicity should not be taken to indicate that such polysaccharides are inert, however. Evidence has been obtained showing that polysaccharide enhances the invasiveness of pneumococci in normal animals presumably by interfering with their defenses (35, 36). Previous workers (7, 37–39) have reported purpura following their injection into mice, and we have frequently noted small hemorrhages in the lungs, liver, kidneys, and myocardium which may perhaps be related to the presence of polysaccharide in the vascular endothelium.

That the presence of polysaccharide in cells may result in interference with cellular function seems of interest particularly in relation to the recent work of Ginsberg, Goebel, and Horsfall (40) who demonstrated that Friedländer polysaccharide, type B, inhibits the multiplication of mumps virus in the allantoic sac of the chick embryo and PVM virus in the mouse lung, both apparently the result of an effect on the host cell rather than upon the virus. In this connection, it would seem worthwhile to examine more closely the problem of whether fixation of these polysaccharides in cells may result in an alteration of their function.

SUMMARY

The cytological distribution of the pneumococcal polysaccharides, types II and III, was followed in the tissues of the mouse.

The most constant and striking concentrations of these polysaccharides were found in the cells of the reticulo-endothelial system, the ordinary capillary endothelium, and fibroblasts throughout the body. In addition, polysaccharide was detected in monocytes and lymphocytes, hepatic cells, cardiac and smooth muscle cells, uterine epithelium, and in steroid-forming cells in the adrenal cortex, testis, and ovary.

Studies of the persistence of polysaccharide, type III, in the tissues were carried out after an injection of 4.0 mg. The polysaccharide remained for at least 75 days in the macrophages of lymphoid organs, the Kupffer cells of the liver, the interstitial macrophages in the myocardium, the lung septal cells, the capillary endothelium, and the renal glomerulus. After a single injection of 8 mg., it persisted for at least 6 months in the macrophages of the spleen, liver, and heart and in the endothelium of peritubular capillaries in the kidney.

The smallest dose of polysaccharide which produced detectable amounts in any cells 24 hours after injection was 0.03 mg.

The distribution of polysaccharide is compared with that of acid vital dyes and suspensoids, and the significance of its fixation in relation to its antigenicity and possible toxicity in mice is discussed.

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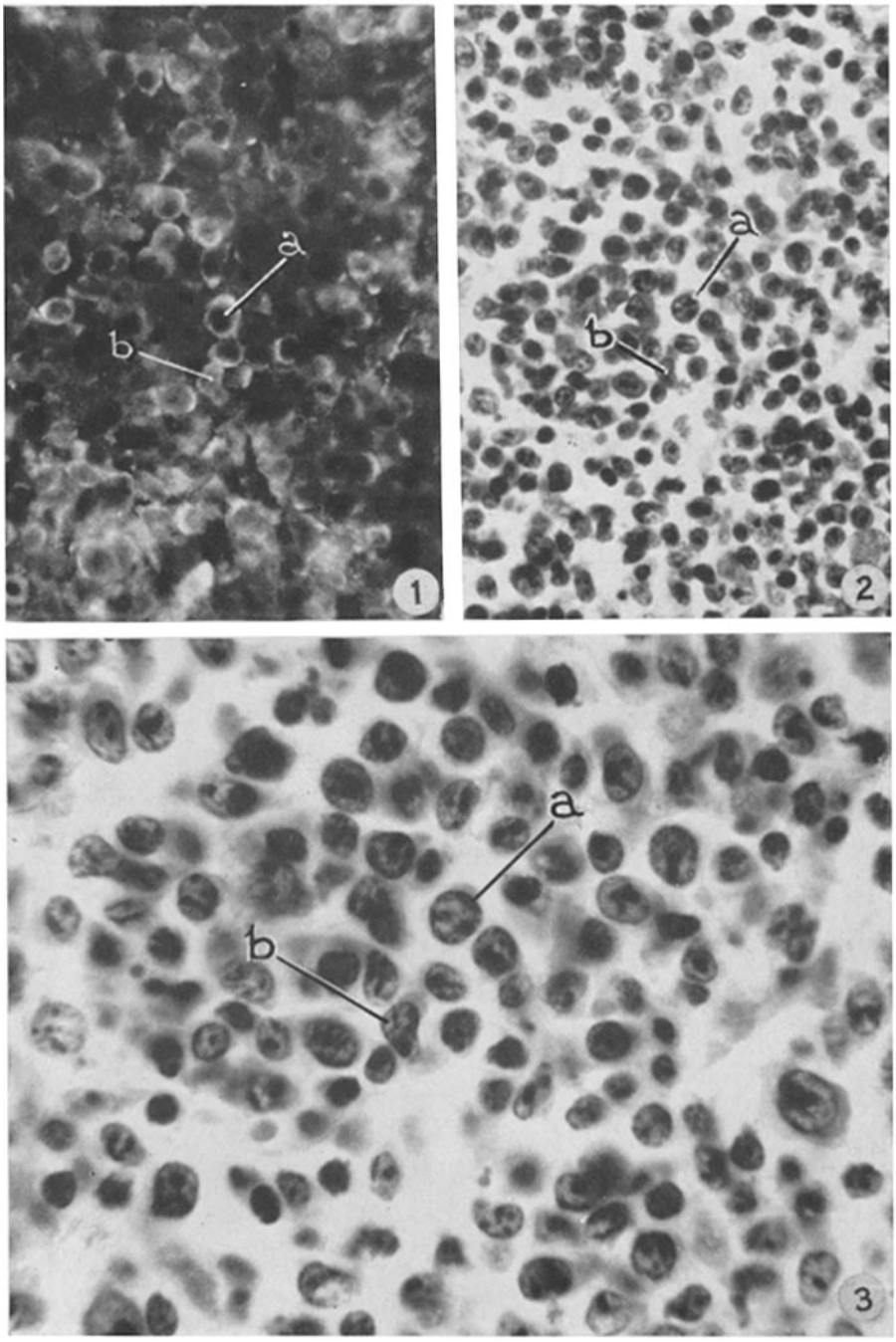
EXPLANATION OF PLATES

All photomicrographs are of organs from mice which received pneumococcal polysaccharide. All figures, excepting Figs. 2, 3, and 7, are fluorescence micrographs of sections which were treated with homologous antibody conjugated with fluorescein. The lightest areas represent the yellow-green fluorescence of the deposited fluorescein-antibody; the topography of the organs is made visible by the faint blue autofluorescence of the normal tissue.

PLATE 1

FIGS. 1–3. All three photomicrographs are of the same field from the white pulp of the spleen of mouse 91 (4 mg. polysaccharide type II; killed after 4 days). Fixed in picric acid–alcohol–formalin. After the fluorescence was photographed (Fig. 1, × 600), the cover slip was floated off and the section was stained with Harris' hematoxylin and eosin. (Fig. 2, × 600, and Fig. 3, × 1100.) Polysaccharide is demonstrated in the cytoplasm of many of the cells; by far the majority of cells present are lymphoid (*a*) in nature; a few are reticular cells (*b*).



(Kaplan *et al.*: Distribution of pneumococcal polysaccharides)

PLATE 2

FIG. 4. Spleen from mouse 76 (4 mg. polysaccharide type III; killed after 2 days). Fixed in picric acid–alcohol–formalin. Polysaccharide occurs principally in the reticulo-endothelial cells of the red pulp (*r*); in this instance, only traces are present in the white pulp (*w*). × 150.

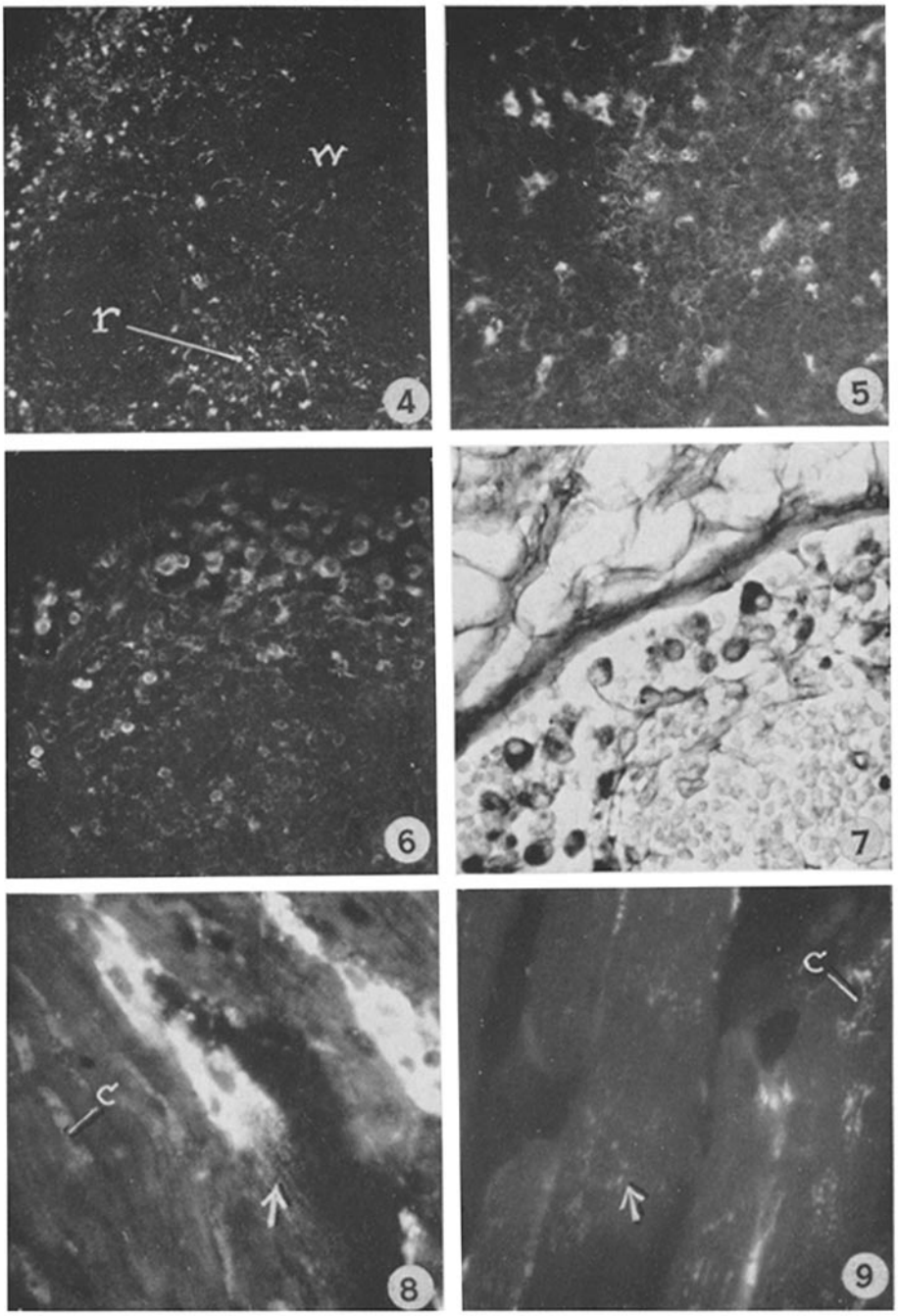
FIG. 5. Thymus from mouse 76 (4 mg. polysaccharide type III; killed after 2 days). Fixed in picric acid–alcohol–formalin. Dense accumulations of antigen are revealed in the stellate macrophages, and smaller amounts within many of the lymphocytes. × 300.

FIG. 6. Subcapsular region of axillary lymph node from mouse 77 (4 mg. polysaccharide type III; killed after 2 days). Fixed in picric acid–alcohol–formalin. Polysaccharide occurs principally in macrophages which lie in the subcapsular sinus and within the cortex of the node; traces may be detected in the cytoplasm of the cortical lymphocytes. × 300.

FIG. 7. Capsule and subcapsular sinus of axillary lymph node from mouse 30 (4 mg. polysaccharide type III; killed after 1 day). Fixed in picric acid–alcohol–formalin; stained by the periodic-Schiff method. The cytoplasm of the macrophages in the subcapsular sinus stains intensely. Normal macrophages give no such reaction, and the staining was unaffected by previous digestion with saliva. Reticulum normally gives the Schiff reaction. × 450.

FIG. 8. Cardiac muscle from mouse 91 (4 mg. polysaccharide type II; killed after 4 days). Fixed in picric acid–alcohol–formalin. Polysaccharide is concentrated in the cytoplasm of macrophages lying among the muscle fibers. In addition, it is seen in the lining of the capillary walls (*c*) and apparently adsorbed onto the cross-striations of the muscle fiber immediately below the large macrophage (arrow). × 600.

FIG. 9. Skeletal muscle from mouse 10 (8 mg. polysaccharide type III; killed after 1 day). Frozen section. The antigen is revealed principally in the walls of the capillaries (*c*) but appears to be present also within the muscle fibers (arrow). × 400.



(Kaplan *et al.*: Distribution of pneumococcal polysaccharides)

PLATE 3

FIG. 10. Kidney cortex from mouse 76 (4 mg. polysaccharide type III; killed after 2 days). Fixed in picric acid–alcohol–formalin. Fluorescence characterizes the glomerulus (*g*) and the walls of the peritubular plexuses. In addition the cells of the distal tubule (*t*) immediately adjacent to the glomerulus contain large amounts of the material. × 300.

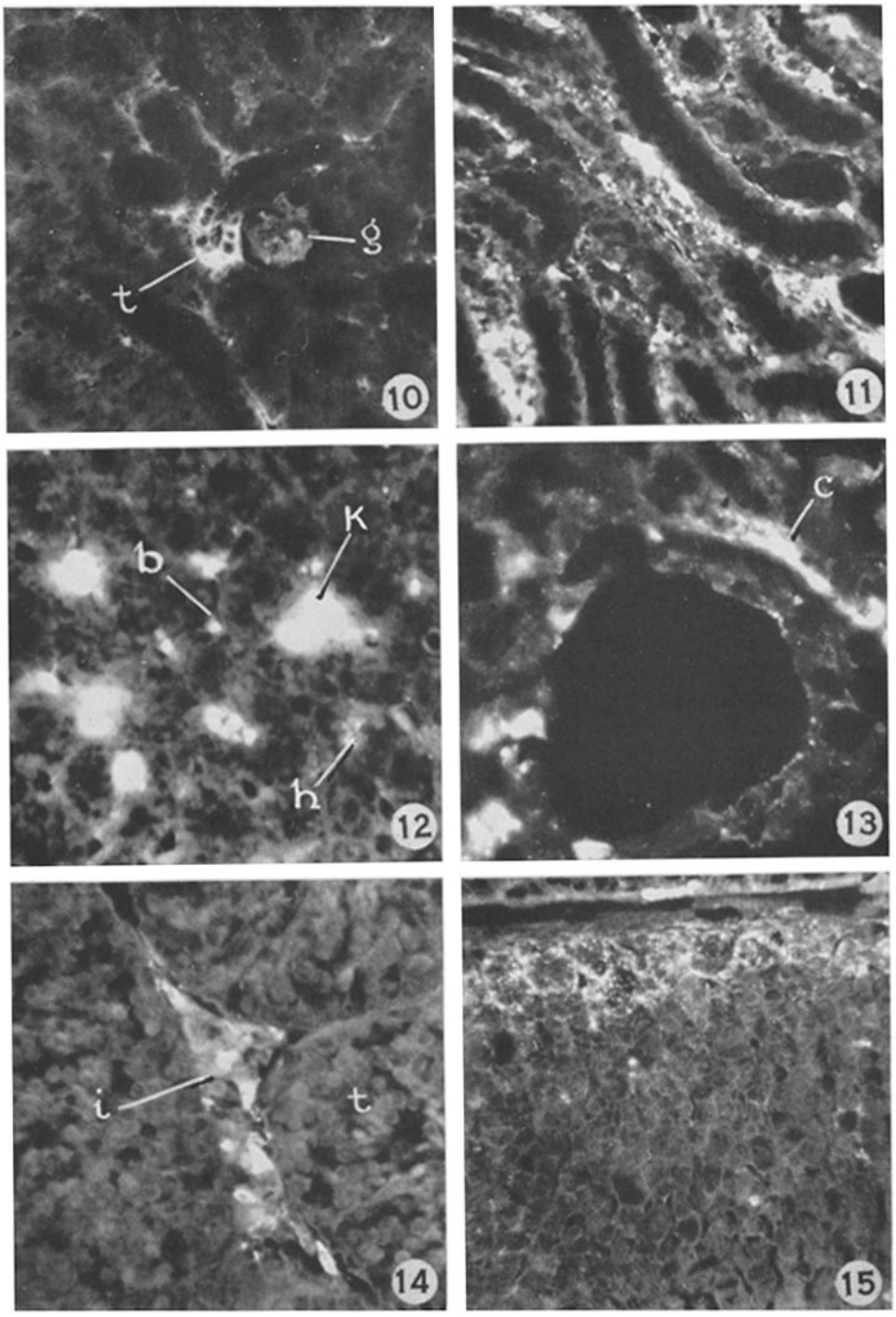
FIG. 11. Kidney medulla from mouse 91 (4 mg. polysaccharide type II; killed after 4 days). Fixed in picric acid–alcohol–formalin. Granules of polysaccharide occur in the walls of the capillary plexuses surrounding the loops of Henle as well as in several macrophages. × 300.

FIG. 12. Liver from mouse 176 (2.5 mg. polysaccharide type III; killed after 4 days). Fixed in picric acid–alcohol–formalin. Large quantities of polysaccharide are present within the stellate cells of von Kupffer (*k*). In addition, the reaction is seen in the intercellular bile capillaries (*b*) and in the cytoplasm of occasional hepatic cells (*h*). × 600.

FIG. 13. Lung from mouse 75 (4 mg. polysaccharide type III; killed after 1 day). Fixed in picric acid–alcohol–formalin. Antigen occurs principally in the capillary walls (*c*) and in the septal cells; tiny granules are also visible along the walls of the alveolus. × 400.

FIG. 14. Testis from mouse 176 (2.5 mg. polysaccharide type III; killed after 4 days). Fixed in picric acid–alcohol–formalin. Polysaccharide is limited to the capillaries and the interstitial cells (*i*) between the seminiferous tubules (*t*). × 300.

FIG. 15. Adrenal cortex from mouse 177 (2.5 mg. polysaccharide type III; killed after 4 days). Fixed in picric acid–alcohol–formalin. Fluorescent material occurs both in the periadrenal fat and in granules within the cortical cells of the zona glomerulosa. (The fluorescent lipids of the cortical cells are all removed by fixation and paraffin embedding.) × 300.



(Kaplan *et al.*: Distribution of pneumococcal polysaccharides)

PLATE 4

All figures on this plate are of organs from mouse 156, which received a single injection of 8 mg. of polysaccharide type III and was killed 6 months later. Fixed in picric acid–alcohol–formalin. × 300.

FIG. 16. Spleen. White pulp on the left (*w*), red pulp on the right (*r*). Polysaccharide persists in histiocytes and reticular cells in both sites.

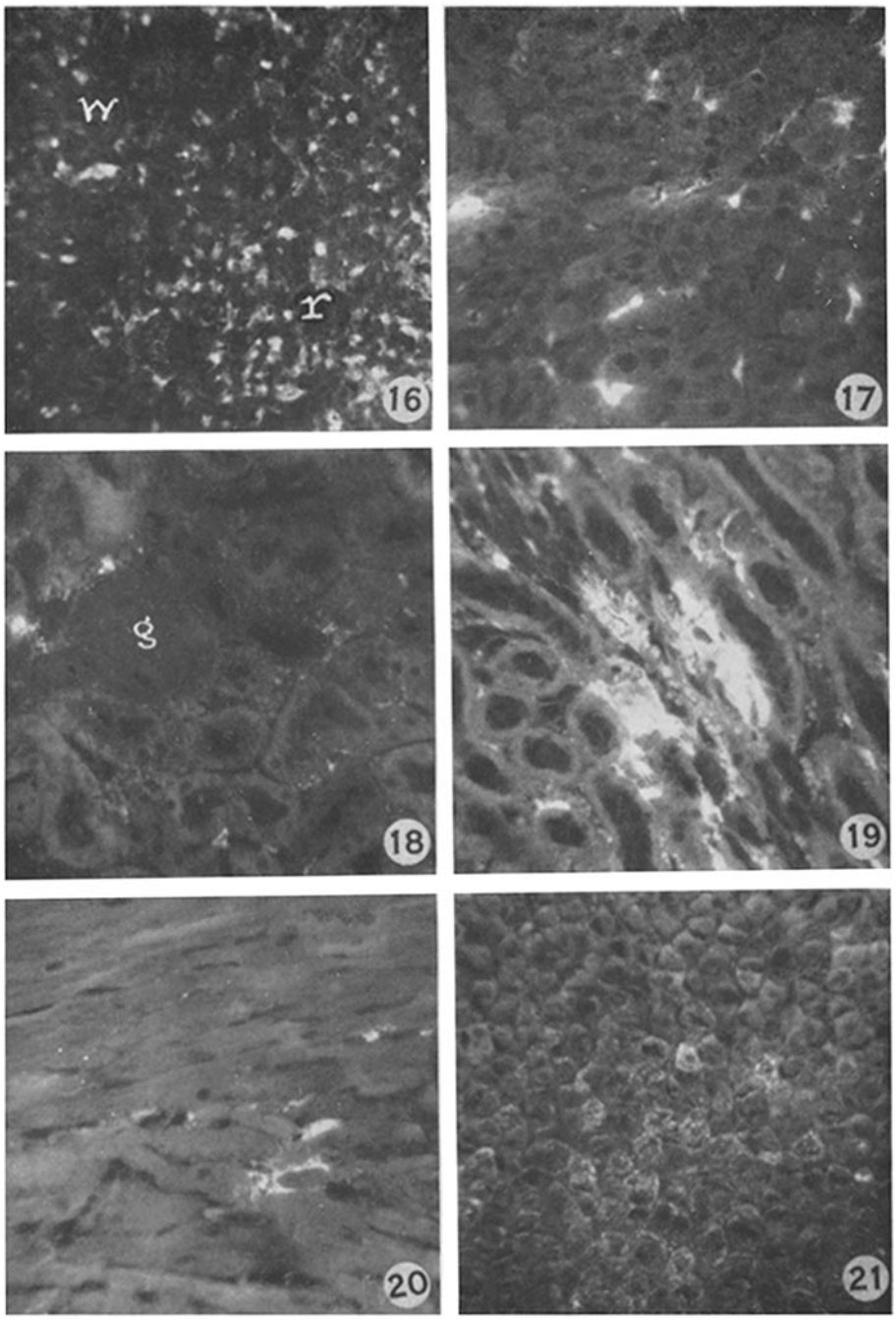
FIG. 17. Liver. Fluorescent material is limited to the Kupffer cells.

FIG. 18. Kidney cortex. Only traces of polysaccharide remain in the walls of the peritubular plexuses, and none in the glomerulus (*g*).

FIG. 19. Kidney medulla. Polysaccharide persists in the stromal macrophages and in the walls of the peritubular capillaries.

FIG. 20. Heart. Polysaccharide occurs in isolated macrophages between the cardiac fibers.

FIG. 21. Adrenal cortex—zona fasciculata. Traces of polysaccharide remain as granules within the adrenal cortical cells.



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