LOCALIZATION OF ANTIGEN IN TISSUE CELLS

VI. The Fate of Injected Foreign Proteins in the Mouse*

By ALBERT H. COONS, M.D., ELIZABETH H. LEDUC, Ph.D., and MELVIN H. KAPLAN

With the Technical Assistance of Jeanne M. Connolly

(From the Departments of Bacteriology and Immunology, and of Anatomy, Harvard Medical School, Boston)

Plates 3 to 5

(Received for publication, September 22, 1950)

Earlier studies in this series have described the distribution among the cells of the mouse of three bacterial polysaccharides after intravenous injection (1, 2). In this paper similar studies with crystalline egg albumin, crystalline bovine albumin, and human γ-globulin are reported.

A brief summary of previous accounts of the distribution and time of disappearance of antigenic material after its injection has been presented in an earlier paper (3), and only those bearing on the cell types which take up proteins will be mentioned here. All the available data have been collected by coupling proteins to dye molecules, injecting the colored product, and examining tissue sections for the presence of the dye. Sabin (4) found that R-salt-azo-benzidine-azo-egg-albumin was taken up by Kupffer cells, macrophages, neutrophiles, lymphatic endothelium, monocytes, adventitial cells of the bone marrow, and fibroblasts. Smetana (5) reported that R salt coupled with egg albumin, and serum albumins and globulins both foreign and native were visible after injection in Kupffer cells, other reticulo-endothelial cells, and the cells lining the proximal convoluted tubules of the kidney; they persisted in these sites in the mouse for longer than 28 days, with the exception that dog serum globulin-R-salt had disappeared from the renal tubules by that time. Kruse and McMaster (6) carried out careful experiments with azoproteins (horse serum and bovine γ-globulin) of an intense blue color. These azoproteins were taken up by the reticulo-endothelial system, and by fibroblasts in all organs, as well as by the renal tubules; they were also found in smooth muscle. They could not be found in lymphocytes, nor in any part of the brain. They were not detected in cell nuclei. Kruse and McMaster demonstrated by complement fixation that antigen was still present in extracts of the livers of such animals 2 days after injection, and later McMaster and Kruse (7) reported the persisting antigenic activity of bovine γ-globulin in the liver as long as 70 days after injection. Recently, Gitlin (8) has also confirmed the take-up of azoproteins by macrophages and renal tubular epithelium.

* This work was supported by a grant-in-aid from the Helen Hay Whitney Foundation.

The present studies have been carried out by taking advantage of the fortunate fact that the antibody molecule retains its specificity after conjugation under gentle conditions with aromatic compounds through the azo (9) and the carbamido (10) linkages. This makes possible the use of antibody coupled with fluorescein isocyanate as a specific stain or fluorochrome which deposits an immune precipitate over those areas of a tissue section containing the homologous antigen. The precipitate is brightly fluorescent under the fluorescence microscope and indicates the presence of the antigen. The method has been described in detail previously (3). Its use makes possible studies of the distribution in animal tissues of foreign and native substances without prior labelling, provided only that they are or can be made sufficiently antigenic to stimulate the production of the requisite antiserum in animals.

The data presented in this report extend the observations summarized above, and present evidence that with reasonable certainty these large molecules, or fragments of them,[1] penetrate the nuclear membrane of several cell types and can be found in the nuclear sap.

EXPERIMENTAL

Materials and Methods

Antigens.—(*a*) Egg albumin was crystallized five times by the method of Sørensen (12), redissolved, and dialyzed to remove ammonium sulfate. A portion was lyophilized. Crystalline egg albumin was also purchased from Armour and Company (no differences were noted between these preparations). (*b*) Crystalline bovine albumin was purchased from Armour and Company. (*c*) Human plasma γ-globulin was obtained from the Department of Physical Chemistry through the courtesy of Professor J. L. Oncley. It was supplied in solution containing 17.7 gm. of protein per 100 ml. in 0.3 molar glycine. By electrophoretic analysis it contained 99 per cent γ-globulins.

Preparation of Antisera and Conjugates.—For immunizing rabbits, portions of egg albumin and human γ-globulin were precipitated with alum by the method of Heidelberger and Kendall (13). Bovine albumin was injected as a solution. Groups of three rabbits were immunized with each antigen respectively, following the dosage and schedules suggested by Heidelberger and Kendall. The titers as determined by the ring test were:—

Antiegg albumin: 1/500,000; antibovine albumin: 1/500,000; antihuman γ-globulin: 1/100,000.

The serum from each group of rabbits was pooled, a crude globulin fraction prepared by precipitation with 1/2 saturated ammonium sulfate, conjugated with fluorescein isocyanate, and purified (3).

Mice.—White Swiss male mice of the Schwentker strain weighing between 18 and 22 gm. were injected intravenously in a tail vein with various amounts of one of the proteins under study contained in volumes of saline ranging from 0.1 to 1.0 ml. per dose; the distribution of the protein did not vary with the volume in which it was administered. Only one injection

[1] There are apparently no very precise data on the size of the smallest molecule which will still precipitate antibody. Landsteiner (11) reported that hydrolytic products from silk fibroin, while inhibitory, failed to precipitate antibody. He estimated that these cleavage products had a molecular weight of about 1000.

was given to each mouse. They were sacrificed after varied time intervals by decapitation while under light ether anesthesia. This method served to drain out much of the blood which otherwise tended to obscure the cytological findings. A few mice were decapitated after being stunned by a light blow; no difference in the subsequent findings attributable to ether was found. The organs to be examined were removed, quick-frozen at −70°C. within 20 minutes after death, and stored at −20°C. until frozen sections could be prepared, usually within a few days.

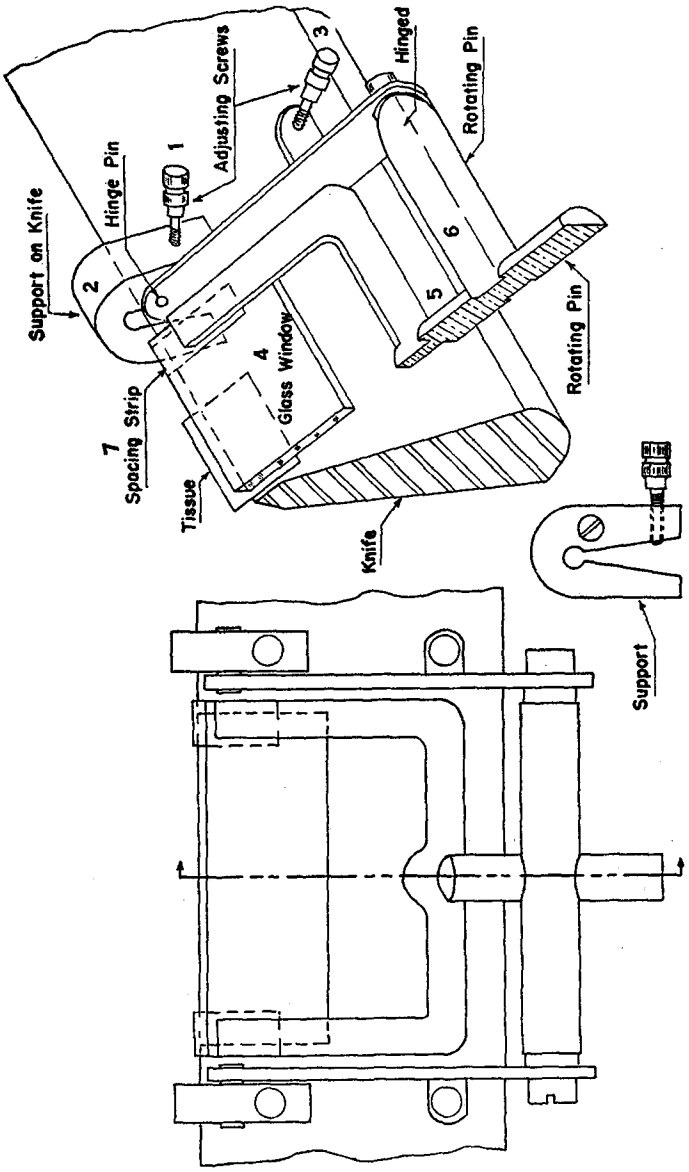
The disappearance of human γ-globulin was studied in pairs of mice injected with 4 mg. intravenously and sacrificed at intervals ranging from 30 minutes to 8 days. That of bovine plasma albumin and egg albumin was followed in the same way after doses of 10 mg. until it was complete. In addition single mice injected with human γ-globulin were sacrificed at intervals after the injection of 10 mg. for purposes of comparison, and a series of pairs of animals was followed after 5 mg. of bovine albumin. The organs examined as a routine were kidney, liver, spleen, thymus, axillary lymph node, adrenal, heart, lung, and skeletal muscle. Testis and duodenum were frequently examined. The central nervous system was omitted from this study.

Frozen Sections.—The method used was that of Linderstrøm-Lang and Mogensen (14), modified in small ways which, however, greatly increase its convenience for routine use.

The essence of the method is to prepare frozen sections in an atmosphere below freezing so that they can be lifted from the knife and placed on a slide before they thaw. It is virtually impossible to carry out this 10-second operation with an ordinary freezing microtome at room temperature. Linderstrøm-Lang and Mogensen solved this problem by placing their microtome in an insulated box cooled with dry ice and provided with gloved armholes, windows, and adequate lighting. Sections cut in such a cryostat from frozen tissue remain in a tight roll at the top of the knife, and cannot be unrolled. To overcome this difficulty they devised an attachment which held a glass guide with its top edge at the edge of the knife blade, and its plane parallel to the knife. It was held 50 μ away from the knife by strips of interposed cellophane to provide clearance for the tissue section to slide between the guiding planes of the knife and the glass. This effectively prevented the sections from rolling up. The glass could be swung back on a hinge, and the flat section lifted off and placed on a slide with small forceps.

The manipulation of 4 μ thick slices of ice is a delicate and frustrating exercise, so that it seems useful to describe the modifications of the method which have been made in this laboratory, and in our hands have made it an easy one to use. The cryostat[2] is illustrated in Fig. 1. The interior is about 6 cubic feet in volume, cooled by coils soldered to the inside walls with the exception of the tapered front section. The compressor, located below, is powered with a ⅓ h.p. motor, and the refrigerant circulation is regulated by an adjustable expansion valve, and a back-pressure regulating valve. Thermostatic control is unnecessary. The tapered front section is provided with gloved armholes, insulated glass window, and a door. Air inside the box is mixed by a small fan at the back, which is provided with a foot switch so that the fan can be momentarily stopped during the manipulation of each section, as otherwise the section will be blown away. The interior is lighted by an 8 watt fluorescent cylinder along the top front inside, and by light from an external lamp admitted through an insulated glass port at the left rear and thrown on the working area by an adjustable mirror at the upper left front. The well insulated walls are pierced with tubes admitting the wiring to fan and light, and others (plugged when not in use) which can be used for the admission of suc-

[2] The construction of the cryostat was made possible by a grant-in-aid from the Life Insurance Medical Research Fund. It was built by the Harris Refrigeration Co., Cambridge, Massachusetts.



FRONT VIEW AND PICTORIAL SECTION THROUGH CENTER OF GLASS WINDOW

TEXT-FIG. 1

tion lines, etc., making the box adaptable for chemical manipulations if desired. The working temperature for cutting frozen sections is from −16° to −18°C., although it should be possible to lower it to about −25°C.

A diagram of the device[3] holding the glass guide is reproduced in Text-fig. 1. The essential modification in its design is to suspend it from the top of the knife. Regulation of the adjusting screws (*1*) through the support (*2*) allows the raising or lowering of the upper edge of the glass to the exact edge of the knife edge; this adjustment is smooth if the cross-section of the knife is triangular. The lower pair of adjusting screws (*3*) holds the glass window (*4*) away from the knife at the bottom, allowing its adjustment into the plane of the bevel of the knife edge. Finally, the vertical (*5*) and horizontal (*6*) rotating pins supporting the frame holding the glass allow it to fall into the vertical plane of the knife facing the operator, supported at its upper corners by narrow strips of cellophane (*7*). The horizontal (*6*) bar at the bottom has pins at each end so that the glass guide and its holder can be swung back toward the operator, allowing the section to be removed. A photograph of the device in use is reproduced in Fig. 2.

The exact dimensions of this device are not indicated as its width must be adapted to the distance between the clamps holding the microtome knife, and its height to the width of the knife blade.

The rotary microtome should be sturdy, reliable, and easy to dismantle. It must be disassembled, wiped dry, and oiled with light oil after each use to prevent rusting.

In use, the temperature should be steady between −16 and −18°C., the knife sharp and set at an angle of about 20° from the vertical, and the top of the glass guide at the edge of the knife. The distance between the guide and the knife should not be too great, as then the sections will roll up as though the guide were not in place.

With a little practice, sections of unfixed frozen tissue 4 micra thick may be cut as a routine.

Fixation and Staining of Frozen Sections.—The principal difficulty encountered in the course of these studies was the development of a method for handling the sections after they had been cut, for it was discovered at the outset that these protein antigens dissolved out of the tissue slice with ease during the period of exposure to the fluorescent antibody solution. They were then free to react in the supernatant drop, and the resulting precipitate settled upon and clung to the tissue; this produced scattered fluorescent precipitate which defeated the plan. An attempt was therefore made to depress their solubility, without at the same time destroying their capacity for specific reaction with antibody. It was found that 95 per cent ethanol[4] acting for 30 minutes at 37°C. would accomplish this for both egg albumin and human γ-globulin. With bovine albumin it was necessary to put the slides in acetone at room temperature for 15 minutes following treatment with alcohol, and even then there was some scattered precipitate.

Each section as cut was placed on a cold gelatinized slide, removed from the cryostat, and thawed by placing a finger under it. It was then placed in the air stream from a fan, and allowed to dry at room temperature for about 1 hour. As a routine, these slides were then stored in a refrigerator at 4°C. overnight. The following morning they were placed in Coplin jars containing 95 per cent ethanol preheated to 37°C. and kept at that temperature for 30 minutes in a water bath. They were then removed from the ethanol, and allowed to dry in a nearly vertical position in an incubator at 37°C. for 30 minutes. This step removes the alcohol without exposing the section to lower concentrations of it. A drop of the appropriate fluorescein-antibody solution was then placed over each section and the reaction allowed to take

[3] Made by Mr. J. F. Palmborg, Boston.

[4] This concentration is optimum. Absolute ethanol fails to depress the solubility; 90 per cent and lower ethanol concentrations (by volume) impair the antigenicity.

place for 30 minutes. After washing in buffered saline for 10 minutes, they were mounted in buffered glycerol and examined visually under the fluorescence microscope. The details of these procedures and solutions have been described previously (3).

Tests of Specificity of Staining.—Controls of specificity consisted of failure of the conjugate, after purification, to stain normal mouse tissue, and specific inhibition of the staining by previous exposure of the section to unconjugated homologous antiserum. These controls were carried out during the course of investigation of the distribution of each antigen; they were not performed as a routine. The disappearance of staining with time after injection also served as a control.

Identification of Cells Containing Antigen.—Photographs were made of the antigenic distribution as revealed under the fluorescence microscope, and a record made of the bearings of each field on a mechanical stage equipped with an accurate vernier. The coverslip was then removed by immersing the slide in saline in a Coplin jar until it dropped off. The slide was put in 10 per cent formalin for 10 minutes, then washed, and stained with hemotoxylin and eosin. Direct comparison was then made of the stained field with the fluorescence photograph, and when desired a comparison photograph taken of the stained preparation.

These preparations were more difficult to photograph than those of the polysaccharides studied previously (1, 2), because the fluorescent antibody was apparently deposited in smaller amounts, and because the frozen sections were not as crisp and the tissue elements had less autofluorescence than those prepared after picric acid–alcohol–formalin fixation.

RESULTS

The sites of deposition of the three proteins were very similar despite the differences in properties which they exhibit as molecular species. The fates of bovine albumin and egg albumin differ only in a few details from that of human γ-globulin as described below. These points of difference will be described later. The evidence for their penetration of nuclei will be collected in another section of the paper; that they are present in nuclei has been accepted in the following description. This description is that of a single mouse; but the illustrations have been drawn from the whole material.

Distribution of Human Plasma γ-Globulin in the Mouse.—Mouse 399 was injected intravenously with 10 mg. of human γ-globulin and killed 30 minutes later.

Kidney.—The blood plasma and connective tissue stroma were very bright (Figs. 3 and 4). Some of the cells in the stroma exhibited cytoplasmic fluorescence, and prominent collections of nuclear antigen. These latter were usually concentrated in an elongated rod-like mass that stood out distinctly against the oval shadow of the nucleus (Fig. 17), sometimes in its long axis, sometimes well toward its periphery. In the renal cortex, the concentration of the antigen was higher around the vascular bundles. The glomeruli contained moderate amounts of antigen, the largest amounts in the form of bright rings which appeared to be coextensive with the nuclear membranes. Inside the nuclei there were irregular patches of somewhat fainter fluorescence (Fig. 5). The cytoplasm of the cells in the capillary loops contained smaller amounts of diffusely distributed antigen. Traces of fluorescence were visible in the cytoplasm of some of the cells of the convoluted tubules just outside the nuclear shadow. But the most striking aspect of the tubular epithelium was the appearance of their nuclei, more than half of which showed moderate amounts of fluorescence (Figs. 7 and 8, note that this is egg albumin, *vide infra*). In the medulla there was some scattered pre-

cipitate from the large amount of antigen in the blood vessels. The lumina of the collecting tubules contained antigen. The brightest object in the section was the papilla, where the stroma, the vessels, and the lumina of the tubules combined their display.

Liver.—There was a large amount of green fluorescence visible. The blood plasma in the large vessels and sinusoids stood out brilliantly. The antigen had penetrated some of the cells both of the hepatic parenchyma and reticulo-endothelium. The cytoplasm of the Kupffer cells was diffusely stained, and occasionally some fluorescent material could be seen concentrated in a small spot within the nucleus. Many (perhaps a quarter) of the hepatic cells, distributed at random throughout the lobule, were mottled with cytoplasmic fluorescence (Fig. 10) which sometimes took the form of tiny bright rings, sometimes that of brilliant masses which were demonstrated to be vacuoles by subsequent staining with hemotoxylin and eosin. Perhaps half of the hepatic cell nuclei (Fig. 9) showed irregular patches of antigen occupying about the middle two-thirds, and within these patches several nucleoli could often be made out as dark shadows.

Spleen.—Bright green fluorescence was observed in the red pulp, both in the blood and in the cells around the venous sinuses and blood vessels. The reticular cells stood out quite brilliantly with diffusely distributed antigen in the cytoplasm and irregular concentrations of fluorescent material in the middle of the nuclei. Most lymphoid cells of the red pulp also contained both cytoplasmic and nuclear antigen. In the white pulp the antigen was present in moderate amount in the cytoplasm and nuclei of all cells in the central two-thirds (Fig. 11) and in a narrow zone around the periphery of each lymphoid nodule.

Lymph Node.—The axillary lymph node contained much less antigen than the spleen, chiefly in the cells lining the lymphatic sinuses, and in areas two or three cells thick around the sinuses and around the blood vessels. The lymph nodules were free of antigen.

Thymus.—The thymus was free of antigen except for the connective tissue around the interlobular septa, and a few reticular and lymphoid cells around the blood vessels.

Skin.—The connective tissue of the dermis was brilliantly fluorescent. It would appear that the antigen present in the tissue fluids had become adsorbed onto the collagenous fibers. The cells of this layer were obscured by the intense fluorescence of the fibers. The epithelial cells of the epidermis and hair follicles were negative.

Cardiac and Skeletal Muscle.—Only the thin fibers and the cells of the connective tissue stroma exhibited the presence of antigen; the muscle fibers and their nuclei were negative.

Adrenal.—The cortical and medullary cells were free of antigen. Moderate fluorescence was observed in the cytoplasm and nuclei of the cells lining the sinusoids of the cortex. The highest concentration was in the sinusoids at the corticomedullary junction, but the antigen was also visible in the lining cells in all three layers of the cortex. The blood plasma in the medullary sinuses was bright.

Duodenum.—The smooth muscle showed traces of antigen between its bundles, apparently around small blood vessels, but none in the smooth muscle cells. The basement membrane was bright. The stroma extending into the villi showed smaller amounts of antigen among the connective tissue fibers, in the cytoplasm of the connective tissue cells, and in a few of their nuclei. The epithelium covering the villi was free. Most striking was the high concentration of antigen in a few of the Paneth cells in the glands at the base of the villi (Fig. 12). Brilliant fluorescence filled the nuclei so that the nucleoli stood out as dark shadows. In the cytoplasm it appeared as large bright masses distributed throughout the cytoplasm. It was not possible to determine the relation of the antigen to the secretory granules in this preparation.

Testis.—The interstitial tissue was stained only in part. The cytoplasm of some of its cells showed diffuse antigen, and an occasional antigen-containing nucleus in a cell without antigen in its cytoplasm was seen. The tubules were free.

Lung.—(Frozen sections of the lung have not been very satisfactory in our hands. It is necessary to cut them at 8 micra; also they tend to be compressed, obliterating many of the alveoli.) There was much antigen, with much scattered precipitate (the thickness of the section of course influences the amount apparent). Some capillaries with bright endothelium were visible, and the cytoplasm and nuclei of many cells of different size, probably histiocytes, contained antigen. The adventitia of the veins and arteries was very bright, that of the bronchi less so.

The description of the findings in this mouse can be supplemented by a few comments. In cells with a nucleus that contained antigen, the amount apparent there was often more than that in the cytoplasm of the same cell; in the liver and kidney there was usually *no* cytoplasmic antigen in the many epithelial cells with fluorescent nuclei. In the connective tissue the amount of antigen present in a given nucleus was more closely related to the amount of antigen in the surrounding tissue and in the cytoplasm of its cell, although here too the nuclear antigen was often brighter than the surrounding elements.

The most prominent part of the connective tissue in sections stained for antigen was the arterial adventitia, especially of the kidney (Fig. 13) and this did not appear to vary among the three antigens studied. No special attempt was made to study the variation in the amount of antigen deposited in the arterial adventitia from organ to organ, but it was noted that the small arteries in the kidney invariably had the most, whereas those in the myocardium were less often prominent.

A rather constant finding was the presence of all three proteins in the endothelial lining of veins in the kidney and the liver. The endocardium was less constantly involved, although antigen was usually visible there. Likewise, the serosal cells of the peritoneum and pleura often contained antigen.

Occasionally in the myocardium, and on a few occasions in skeletal muscle there was the suspicion that some antigen had penetrated the muscle cells, but this could not be confirmed photographically.

Differences in Distribution and Disappearance.—The only obvious differences in the behavior of these three proteins after injection were their distribution in the kidney, and their rates of disappearance.

In the kidney, there was a marked difference in localization of egg albumin as compared with that of human γ-globulin and bovine albumin. At all periods, the cells of the glomeruli were empty of egg albumin, nor could any be detected in whatever blood may have been present (the blood in larger vessels was bright). In one mouse there was the faintest possible trace of egg albumin in one or two cells of the capillary loop, or in the plasma in it. By contrast, the cell cytoplasm and nuclei of the glomeruli both contained the other two antigens, the nuclei invariably more than the cytoplasm. In addition, egg albumin was the only protein of the three to appear in the cytoplasm of the tubular epithelium in which it was apparently limited to that of the proximal tubule. All three were regularly found in the tubular nuclei and in the lumina. Bovine albumin seemed to gain access to fewer tubular nuclei than did the others.

In the adrenal, each of the proteins made an appearance in the cytoplasm and nuclei of the cortex in small amounts 10 minutes after the injection of 10 mg., but it did not persist.

The rate of disappearance of each protein is tabulated in Tables I to III. In these tables, no attempt at quantitation has been made except to indicate those instances in which only the barest detectable amounts were present (tr).

TABLE I

Disappearance of Egg Albumin after Injection of 10 Mg.

	10 min.	30 min.	2 hrs.	4 hrs.	1 day
Epithelium					
Adrenal	+	0	0		0
Kidney glomeruli	0	tr	0	0	0
tubules	+	+	+	tr	0
Liver	+	+	tr	tr	0
Lymphocytes					
Node	tr	0	0	0	nd
Spleen	0	0	0	0	0
Reticulo-endothelial system					
Kupffer cells	+	+	tr	tr	tr
Spleen	+	+	tr	tr	nd
Node	tr	tr	tr	tr	nd
Connective tissue					
Lung	+	+	+	tr	0
Heart	+	+	0	tr	0
Kidney	+	+	tr	+	0
Muscle	+	+	tr	tr	0

tr = trace; nd = not done.

It can be seen that while egg albumin was practically gone at the end of 4 hours, bovine albumin persisted in traces for 2 days, and human γ-globulin in a smaller dose could still be detected for 6 days. The latter substance was still present in the glomeruli 8 days following a 10 mg. dose (Fig. 6). The highest concentrations of all three were attained in the fixed phagocytic cells, particularly reticulum cells, and in these cells antigen tended to persist in traces. However the connective tissue in many locations took up moderate amounts of antigen, which persisted quite as long as that in the reticulo-endothelial system.

The Evidence for the Intranuclear Penetration of Antigen.—Repeated observations with human γ-globulin and with egg white have been made by the method of photographing the deposited fluorescent antibody, restaining the slide with

hemotoxylin and eosin, and reexamining the same field in visible light. In this way it could be assured that portions of many nuclei had been made fluorescent by deposited antibody. The conjugate used was tested to be sure it did not stain normal mouse tissue nuclei. Specific inhibition (3) (see Materials and Methods) of the nuclear fluorescence was successful. There seems no doubt that the fluorescent antibody had reacted specifically with its homologous an-

TABLE II

Disappearance of Bovine Albumin after Injection of 10 Mg.

	10 min.	30 min.	2 hrs.	4 hrs.	8 hrs.	1 day	2 days	4 days
Epithelium								
Adrenal	+	d	0	0	d	0	0	0
Kidney glomeruli	+	+	+	+	d	0	0	0
tubules	+	tr	+	+	0	0	0	0
Liver	+	+	+	+	+	0	0	0
Lymphocytes								
Node	+	+	+	tr		tr	0	0
Spleen	+	+	+	+	tr	tr	0	0
Reticulo-endothelial system								
Kupffer cells	+	+	+	+	+	tr	0	0
Spleen	+	+	+	+	+	+	tr	0
Node	+	+	+	+	+	+	+	0
Connective tissue								
Lung	+	+	+	+	+	+	tr	0
Heart	+	+	+	+	+	+	tr	0
Kidney	+	+	+	+	+	+	tr	0
Muscle	+	+	+	+	+	+	tr	0

d = doubtful.

tigen in the nuclei (Figs. 7 to 17). The only question, therefore, was whether this appearance reflected the true state of affairs in the living animal, or whether it was a regular artifact which resulted from our methods of handling the tissues after the animal was killed.

Although the removal of several organs from an animal takes an appreciable time, care was taken on several occasions to freeze the kidney within 1 to 2 minutes after death. The freezing was complete in about 30 seconds. It seems unlikely that the protein had shifted from the cytoplasm to the nucleus during this period, or that it shifted during storage, particularly since in several cases the sections were cut almost immediately. The regular mechanical shifting of

antigenic material in so precise a fashion during the cutting of the frozen tissue seems impossible. There remains however the possibility that this shift occurred during the thawing of the tissue on the slide. Each section as cut was thawed on the slide, and the melting tissue fluid could have spread the antigen, especially since the tissue fluids contained appreciable quantities of it. After the sections were dried, they were fixed in 95 per cent alcohol, which apparently effectively prevented subsequent spread during staining.

TABLE III

Disappearance of Human γ-Globulin after Injection of 4 Mg.

	30 min.	2 hrs.	4 hrs.	8 hrs.	1 day	2 days	4 days	6 days	8 days
Epithelium									
Adrenal	0	0	0	0	0	0	0	0	0
Kidney glomeruli	+	+	+	+	+	+	tr	tr	0
tubules	+	+	+	+	tr	tr	0	0	0
Liver	+	+	+	+	+	tr	0	0	0
Lymphocytes									
Node	+	+	+	+	+	tr	0	0	0
Spleen	+	+	0	0	0	0	0	0	0
Reticulo-endothelial system									
Kupffer cells	+	+	+	+	+	+	tr	tr	0
Spleen	+	+	+	+	+	+	tr	tr	0
Node	+	+	+	+	+	+	+	tr	0
Connective tissue									
Lung	+	+	+	+	+	+	+	+	0
Heart	+	+	+	+	+	+	+	+	0
Kidney	+	+	+	+	+	+	+	+	0
Muscle	+	+	+	+	+	+	+	tr	0
Circulating blood plasma								+	0

There are three points against the presence of antigen in the nuclei being the result of spread during manipulation. In the first place, although the amount of nuclear precipitate in general was higher in those areas where the amount of extranuclear antigen was highest, this was not always the case. In some areas of the kidney, and frequently in the liver, areas could be found where perhaps half of all the nuclei contained antigen and collections of extranuclear antigen were distant. Then too, 10 minutes after injection of the antigen (and 30 minutes as well in the case of γ-globulin) but not later than that, there

were bright rings around the nuclei in the renal tubules and glomeruli which looked as though the actual penetration of the antigen through the nuclear membrane was being observed. Further, egg albumin was never observed in the nuclei of the glomerular tufts, although under the same conditions both bovine albumin and human γ-globulin were regularly found in these nuclei. But egg albumin *was* found in other nuclei just as were the other two antigens. The intrinsic evidence therefore points against the existence of an artifact.

The second point is experimental. If one assumes, for example, that human γ-globulin in the tissue fluid of the mouse has a special affinity for the nuclei in the cut section, then the same effect should be created if a solution of the protein were spread over freshly cut sections from a normal mouse and allowed to dry, the slides thereafter being processed as usual. When this was tried, indiscriminately scattered precipitate resulted, which was without pattern and which did not pick out the nuclei.

Finally, paraffin sections (picric acid–alcohol–formalin fixation) of mice injected with pneumococcal II polysaccharide and Friedländer B polysaccharide remaining from previous studies (1, 2) were stained and examined to determine whether these substances too could be found in nuclei. This had not been observed earlier when nuclei were not objects of special concern. A few connective tissue cells were found in the kidney showing the same inclusions in their nuclear shadows as were seen with the proteins (Figs. 15 and 16). In the lung of a mouse to which Friedländer B polysaccharide had been administered by inhalation, numerous cells in the inflammatory exudate, both macrophages and lymphocytes, were found with intranuclear antigen. Although there were occasional suspicious appearances, there was no *clear* demonstration of these two polysacchardies in the nuclei of lymphocytes, or hepatic or renal epithelium. Since these were paraffin sections, the possibility of drift due to thawing is eliminated, although of course diffusion during fixation cannot be excluded. We were unfortunately not equipped to prepare paraffin sections from tissue dried from the frozen state after the method of Packer and Scott (15). Fixation of tissue containing human γ-globulin or bovine albumin in acetone, and imbedding in paraffin destroyed the antigen.

When a comparison was made between photographs of fluorescent antibody deposit and the same field stained with hemotoxylin and eosin, it could often be seen that the area of antigenic inclusion in the nucleus did not stain or stained weakly with hematoxylin. In nuclei with nucleoli, the shadows of the nucleoli could be seen in the midst of the fluorescence, and it seemed clear that the antigen was present, at least for the most part, in the nuclear sap.

What of the possibility that the material apparently in the nucleus was actually on the outer surface of the nuclear membrane? Against this is the appearance, soon after injection, of thin sharp rings around the nuclear shadow, as described above. This appears to be the morphological representation of

staining on, or within the thickness of, the nuclear membrane. Later it disappears; the intranuclear inclusions remain.

DISCUSSION

The observations reported amply confirm the previous studies of the function of the reticulo-endothelial system in taking up antigenic material in solution (4–8). In addition, they extend the previous findings (1, 2) on the distribution of acid bacterial polysaccharides to three proteins of differing molecular weight. Both these classes of foreign materials were found after intravenous injection in the vascular endothelium, in connective tissue cells and adsorbed on collagen fibers, in lymphoid cells, and in the epithelium of the adrenal cortex, the kidney, and the liver. Although their rates of disappearance differ markedly, both kinds of material remain longest in the reticulo-endothelium and in the connective tissue.

The mechanisms responsible for the disappearance of the three proteins are not clear. All three could be seen in the lumina of the collecting tubules, but undoubtedly their differing rates of disappearance are partly due to the relative ease with which they pass through the glomerular filter. The rapid excretion of egg albumin (16) and bovine plasma albumin (17) has been demonstrated after massive doses in rats. Two additional possibilities are that they gradually undergo degradation in the cells, and that they are masked in the tissues by the gradual appearance of antibody formed in response to their injection. It seems unlikely that enough generally distributed antibody could have been produced to be a serious factor in causing the almost complete disappearance of egg albumin from tissue cells in 4 hours. Of course, in local areas such as the lymph node it may have played a role. More likely in the clearing of this substance are excretion and proteolysis. No doubt all three mechanisms operate, their relative importance varying with the substance involved.

The failure to find egg albumin in the glomeruli, whereas the other two proteins were prominent there, is a curious outcome. Egg albumin was undoubtedly passing through the glomeruli at the time (16). On the other hand bovine albumin is also excreted in impressive amounts by the kidney (17). Is it possible that the smaller molecules, represented in this case by egg albumin, pass through pores, whereas the larger molecules pass through the substance of the plasma membrane (*vide* reference 18)? This is supported by the intracellular presence of the larger molecules.

The long persistence of proteins coupled with azo dyes reported by Smetana (5), and by McMaster and Kruse (7) is difficult to reconcile with our findings, particularly in view of the finding of the last two of antigenic activity in the liver of mice 70 days after the injection of 5 mg. of bovine γ-globulin. This does not agree very well with our data on the disappearance of a similar dose of human γ-globulin; but the disappearance of human γ-globulin in larger

doses was not followed to the end, and bovine globulin may well differ. More work is obviously called for in this direction.

The most unexpected finding during the course of these experiments was the presence of foreign protein in the nuclei of several cell types. Kruse and McMaster (6) failed to find colored azoproteins in the nuclei. Goldstein and Harding (19) studied isolated nuclei from oocytes of the frog, observing their changes in volume after immersion in solutions of various substances. They found that egg albumin failed to penetrate. These discrepancies probably depend on marked differences in the sensitivities of the methods employed. If our observations are not the result of an artifact, the possibility of which has already been discussed, they indicate a heretofore unsuspected intimacy between the circulating blood and the cell nucleus of some cell types (but *cf.* reference 20). The nuclei of the liver and kidney, particularly, contained antigen in higher concentrations than the cytoplasm, and more frequently antigen when *none* was detectable in the cytoplasm. On the other hand, on a few occasions, large vacuoles containing antigen were observed in liver cells when their nuclei were empty. On the whole, the observation indicates, if sound, a rapid interchange of material between the cytoplasm and the nucleus. The higher concentrations in the nucleus are difficult to interpret; perhaps they indicate a higher rate of protein degradation in the cytoplasm.[5]

Burnet and Fenner (22) have proposed the hypothesis that the presence of antigenic material in an antibody-producing cell modifies the synthesis of globulin in a way which can be transmitted to its descendants, in order to account for the continuing production of antibody after the antigen has disappeared, and in explanation of the difference between the first and subsequent doses of antigen. The finding of antigen in the nucleus lends a certain indirect support to this hypothesis even though its authors do not require this, since they postulated the existence of a transmissible cytoplasmic gene product as the substance undergoing such modification. Moreover, the proteins under discussion were found in both the cytoplasm and the nucleus of macrophages, reticulum cells, and lymphocytes, all of which are suspected of having a hand in antibody production. On the basis of antigenic distribution one cannot choose among them as producers of antibody, but by the same token it is not necessary to postulate the transfer of antigen from one to the other. However, the actively phagocytic cells might well perform the function of solubilizing particulate antigenic complexes such as bacteria, as suggested by Harris and Ehrich (23).

The involvement in serum disease of the connective tissue of the heart and vessels, and of the glomeruli, is evidently governed less by selective localization (24) than by focal persistence; the antigen must remain in those locations in high enough concentration and long enough for sufficient antibody produc-

[5] Rather (21) has presented evidence of hemoglobin breakdown in the cytoplasm of the renal tubule.

tion to take place. This perhaps accounts for the massive doses of antigen required to elicit serum disease.

SUMMARY

The fate of three proteins, crystalline hen's egg albumin, crystalline bovine plasma albumin, and human plasma γ-globulin, was traced after intravenous injection into mice. This was done by preparing frozen sections of quick-frozen tissue, allowing what foreign protein might be present in the section to react with homologous antibody labelled with fluorescein, and examining the section under the fluorescence microscope. By this means, which employs the serological specificity of the protein as a natural "marker," all three of these proteins were found in the cells of the reticulo-endothelial system, the connective tissue, the vascular endothelium, the lymphocytes of spleen and lymph node, and the epithelium of the kidney tubules, the liver, and in very small amounts in the adrenal. The central nervous system was not studied.

All three persisted longest in the reticulo-endothelial system and the connective tissue, and in the doses employed egg white (10 mg.) was no longer detectable after 1 day, bovine albumin (10 mg.) after 2 days, and human γ-globulin (4 mg.) after 6 days, although in a somewhat higher dose (10 mg.) human γ-globulin persisted longer than 8 days. Egg albumin differed from the others in not being detectable in the cells of the renal glomerulus.

It was found that each of the three proteins was present in the nuclei of each cell type enumerated above, often in higher concentration than in the cytoplasm. Further, some of the nuclei not only contained antigen, soon after injection, but were also surrounded by a bright ring associated with the nuclear membrane. By means of photographic records under the fluorescence microscope of sections stained for antigen, and direct observation under the light microscope of the same field subsequently stained with hematoxylin and eosin, it could be determined that the antigen was not adsorbed to chromatin or nucleoli, but was apparently in solution in the nuclear sap.

As this paper was being submitted for publication, Crampton and Haurowitz[6] reported the detection, after intravenous injection, of radio-iodo-ovalbumin and radio-iodo-bovine γ-globulin in the nuclear fractions of rabbit liver homogenates.

The senior author desires to record a debt to the late Dr. L. R. Morrison for his friendly advice and help in connection with the preparation of frozen sections.

BIBLIOGRAPHY

1. Kaplan, M. H., Coons, A. H., and Deane, H. W., *J. Exp. Med.*, 1950, **91**, 15.
2. Hill, A. G. S., Deane, H. W., and Coons, A. H., *J. Exp. Med.*, 1950, **92**, 35.
3. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, **91**, 1.

[6] Crampton, C. F., and Haurowitz, F., *Science*, 1950, **112**, 300.

4. Sabin, F. R., *J. Exp. Med.*, 1939, **70,** 67.
5. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
6. Kruse, H., and McMaster, P. D., *J. Exp. Med.*, 1949, **90,** 425.
7. McMaster, P. D., and Kruse, H., *Fed. Proc.*, 1950, **9,** 387.
8. Gitlin, D., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 138.
9. Marrack, J., *Nature*, 1934, **133,** 292.
10. Coons, A. H., Creech, H. J., and Jones, R. N., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47,** 200.
11. Landsteiner, K., *J. Exp. Med.*, 1942, **75,** 269.
12. Sørensen, S. P. L., and Hoyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, 1917, **12,** 12.
13. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62,** 697.
14. Linderstrøm-Lang, K., and Mogensen, K. R., *Compt.-rend. trav. Lab. Carlsberg, série chim.*, 1938, **23,** 27.
15. Packer, D. M., and Scott, G. H., *J. Tech. Meth. Bull. Internat. Assn. Med. Museums*, 1942, **22,** 85.
16. Oliver, J., *J. Mt. Sinai Hosp.*, 1948, **15,** 175.
17. Rather, L. J., *Stamford Med. Bull.*, 1948, **6,** 117.
18. Hoeber, R., Physical Chemistry of Cells and Tissues, Philadelphia, Blakiston Co., 1945.
19. Goldstein, L., and Harding, C. V., *Fed. Proc.*, 1950, **9,** 48.
20. Schechtman, A. M., and Nishihara, T., *Science*, 1950, **111,** 357.
21. Rather, L. J., *J. Exp. Med.*, 1948, **87,** 163.
22. Burnet, F. M., and Fenner, F., The Production of Antibodies, Melbourne, Macmillan, 2nd edition, 1949.
23. Harris, T. N., and Ehrich, W. E., *J. Exp. Med.*, 1946, **84,** 157.
24. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85,** 571.

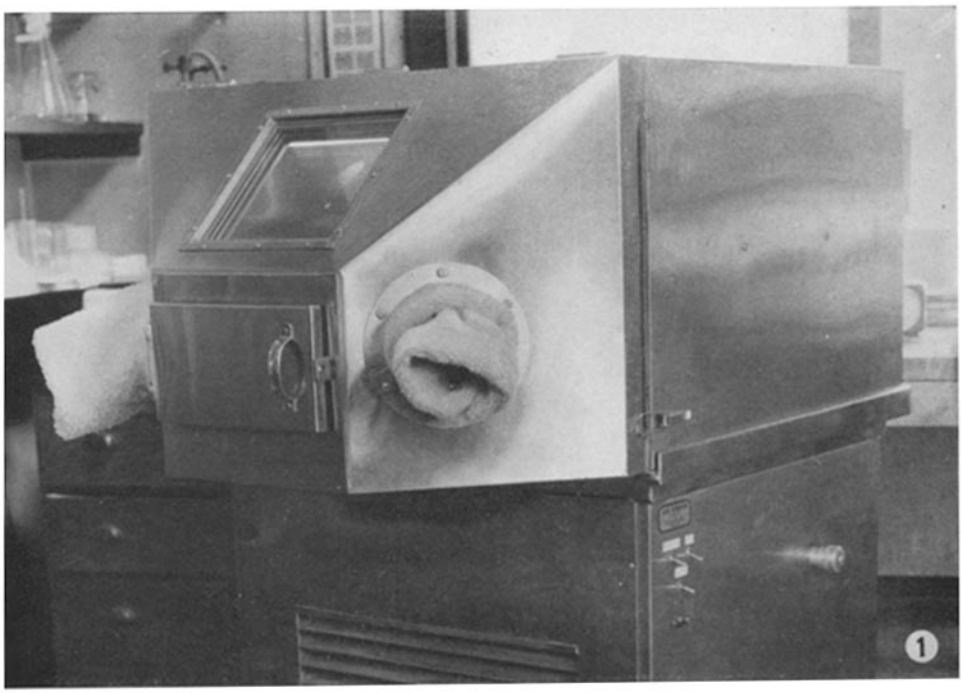
EXPLANATION OF PLATES

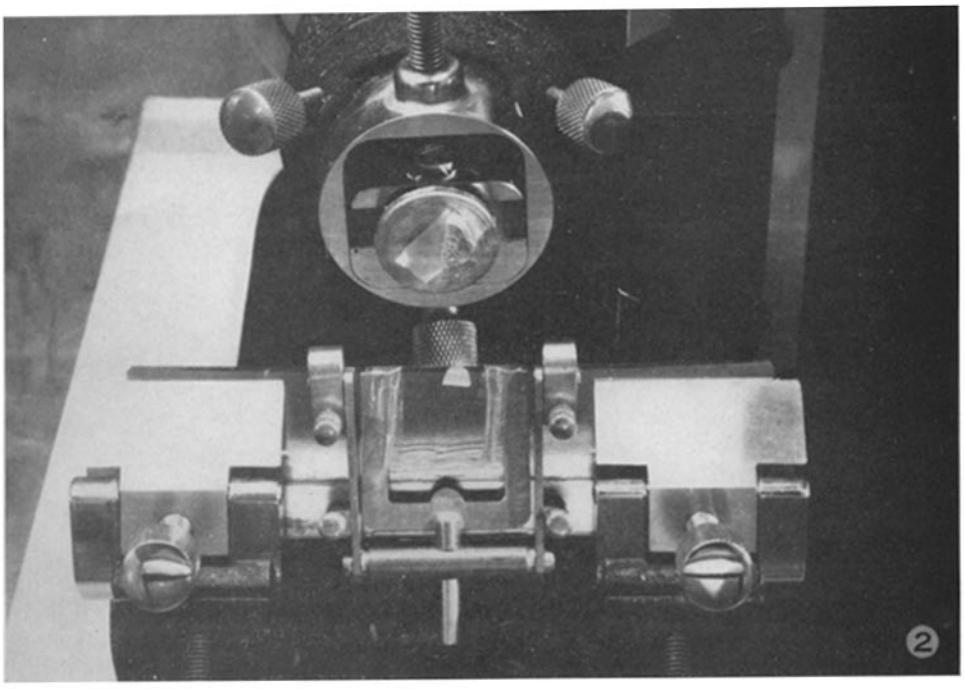
All photomicrographs are of organs from mice injected with human γ-globulin or crystalline egg albumin. Frozen sections were cut at 4 micra, and exposed to fluorescein-labelled homologous antibody. Except where noted, the lightest areas represent the bright yellow-green fluorescence of the fluorescent specific precipitate, as visualized under the fluorescence microscope. The histological topography is made visible by a faint bluish autofluoresecnce of the tissues. Fig. 8 was photographed under the light microscope.

PLATE 3

FIG. 1. Exterior of cryostat.

FIG. 2. Microtome in cryostat, with glass "window" in place. (The section beneath it is too wrinkled to use.)





(Coons *et al.*: Localization of antigen in tissue cells. VI)

PLATE 4

FIG. 3. Renal cortex (mouse 388, 10 mg. human γ-globulin; killed after 10 minutes). Antigen already concentrated in glomeruli and stroma around tubules. × 140.

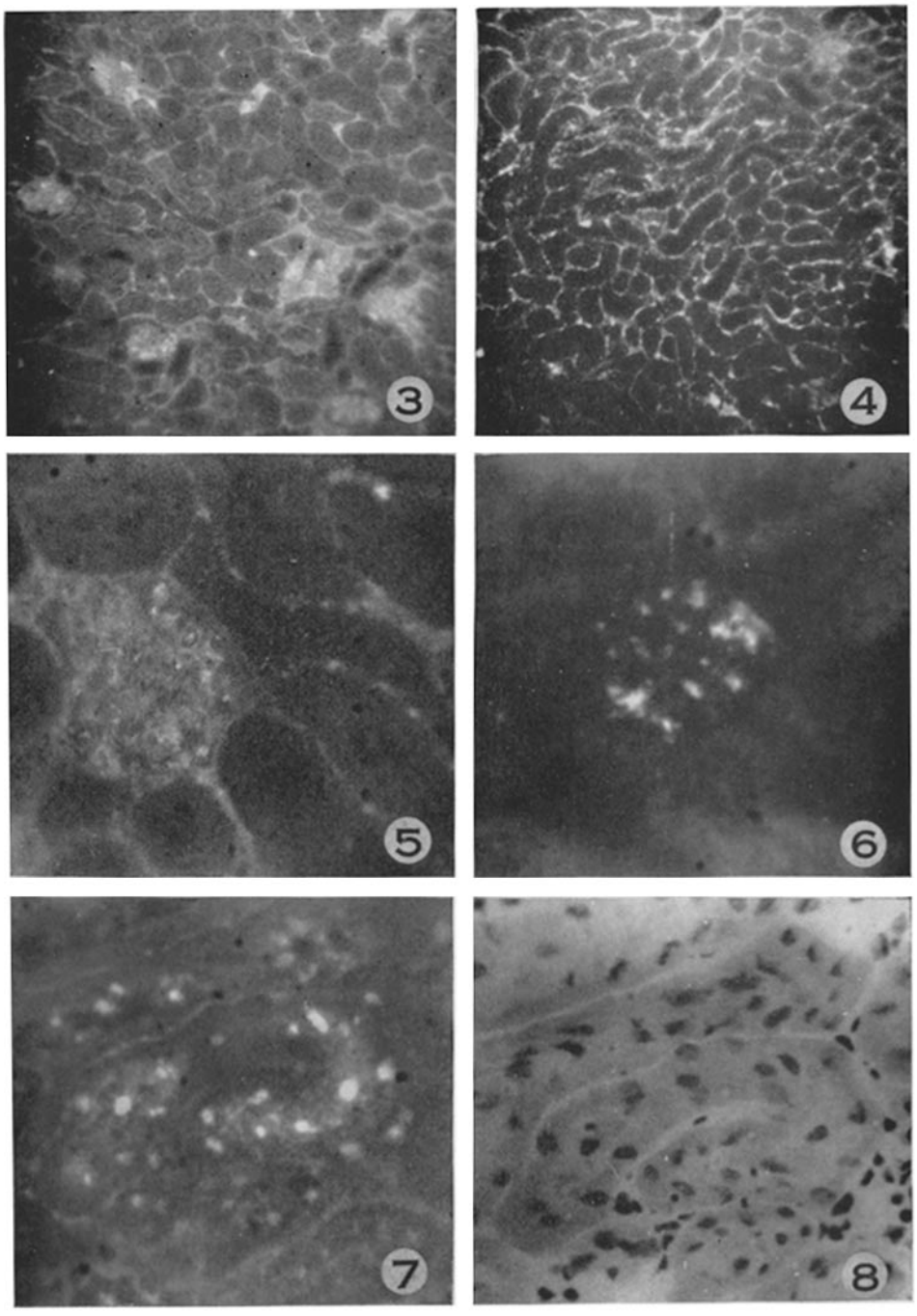
FIG. 4. Renal cortex (mouse 303; 4 mg. human γ-globulin; killed after 30 minutes). Bright fluorescence in glomerulus (upper right corner), stroma, and in nuclei of cells lining tubules. × 140.

FIG. 5. Renal cortex (mouse 328; 10 mg. human γ-globulin; killed after 24 hours). Antigen present in glomerulus and stroma. Diffuse green fluorescence in cytoplasm of cells around capillary loops outlines dark nuclear shadows. Within latter are points of brilliant fluorescence. × 560.

FIG. 6. Renal cortex (mouse 329; 10 mg. human γ-globulin, killed after 8 days). Antigen has disappeared from all elements of kidney except glomeruli. × 560.

FIG. 7. Convoluted tubule of kidney (mouse 377; 10 mg. egg albumin; killed after 30 minutes). Antigenic material is present in the cytoplasm as well as the nuclei of the renal cells; this localization is characteristic of egg albumin. × 560.

FIG. 8. Same field of same section as in Fig. 7 stained with hematoxylin and eosin. The foci of high antigen concentration (Fig. 7) correspond in position to the nuclei of the renal epithelium. Note that not all the nuclei contain antigen. × 560.



(Coons *et al.*: Localization of antigen in tissue cells. VI)

PLATE 5

FIG. 9. Liver (mouse 427; 10 mg. human γ-globulin; killed after 2 hours). Large amount of antigen concentrated in the hepatic nuclei. Shadows of the nucleoli may be seen against the fluorescent background of the nucleus. The cytoplasm contains no antigen. × 560.

FIG. 10. Liver (mouse 381, 10 mg. egg albumin; killed after 10 minutes). Antigen present in large amounts in both cytoplasm and nuclei of an isolated binucleate hepatic cell. × 560.

FIG. 11. White pulp of spleen (mouse 303; 4 mg. human γ-globulin; killed after 30 minutes). Diffuse fluorescence in cytoplasm of lymphocytes which outlines nuclear shadows and somewhat brighter fluorescent spots in the center of the nuclei. × 560.

FIG. 12. Duodenum (mouse 328; 10 mg. human γ-globulin; killed after 24 hours). High concentrations of antigen in connective tissue stroma and in cytoplasm and nuclei of Paneth cells in the glands at the base of the villi. × 560.

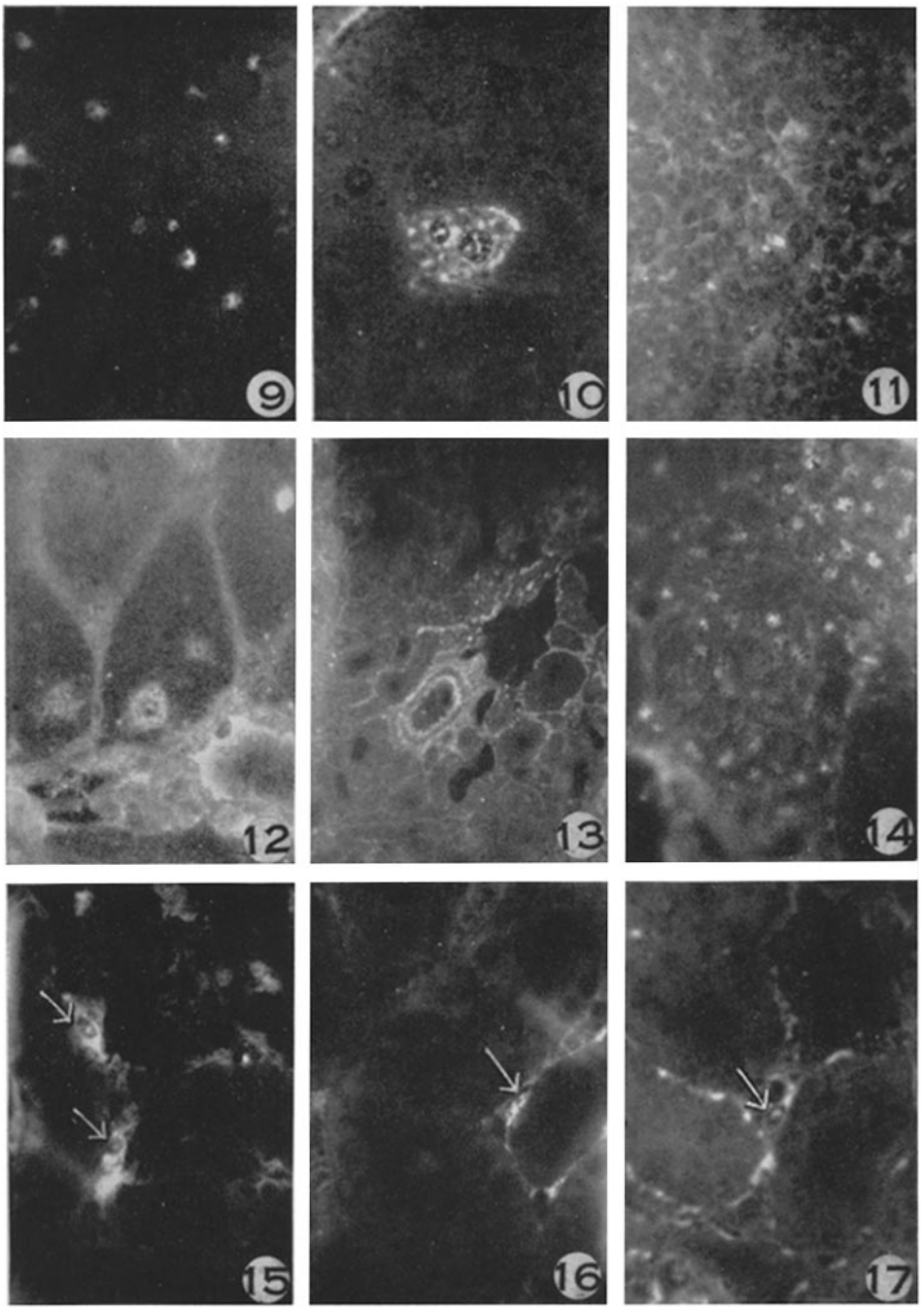
FIG. 13. Renal medulla (mouse 328; 10 mg. human γ-globulin; killed after 24 hours). A high concentration of antigen in the adventitia around an artery. The fluorescence of the elastic membrane is bright blue and does not indicate antigen. × 140.

FIG. 14. Glomerulus of kidney (mouse 427, 10 mg. human γ-globulin; killed after 2 hours). Nuclei contain irregular masses of antigenic material. Some of the nuclear membranes are also fluorescent. × 560.

FIG. 15. Stroma of kidney (mouse 91, 2 mg. Type II pneumococcal polysaccharide, killed after 4 days). The polysaccharide is present within the nuclear shadows of two fibroblasts (arrows). × 560.

FIG. 16. Stroma of kidney (mouse 267, 2 mg. Friedländer B polysaccharide; killed after 4 hours). Two rod-shaped accumulations of polysaccharide can be seen within the nucleus (arrow) of a connective tissue cell. × 560.

FIG. 17. Stroma of kidney (mouse 328; 10 mg. human γ-globulin, killed after 24 hours). Antigenic material is concentrated in the middle of a fibroblast nucleus (arrow). × 560.



(Coons *et al.*: Localization of antigen in tissue cells. VI)