LOCALIZATION OF ANTIGEN IN TISSUE CELLS

II. Improvements in a Method for the Detection of Antigen by Means of Fluorescent Antibody*, ‡

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INTRODUCTION

A report from this laboratory published in 1942 presented a method for the detection of antigenic material in tissue cells in which antibody labelled with fluorescein was employed as a specific histochemical stain (1). It was based on previous observations (2–5) that the antibody molecule could be conjugated with simple chemical compounds without destroying its capacity to react specifically with its antigen. This method has since been further developed, applied to the demonstration of rickettsial and mumps virus antigens (6), and used in a study of the fate of pneumococcal capsular polysaccharide injected into the mouse (7). In the present paper improvements in the method will be described.

Attempts to follow the distribution of soluble antigens in the animal body began early in the history of immunology. Metchnikoff (8) was apparently the first; using toxicity as a tracer he studied the fixation of tetanus toxin in the tissues of the chicken. He pointed out that organs rich in blood could not be studied by this method because of circulating toxin. He found that the relatively bloodless ovary and testis of chickens previously injected with large doses of toxin produced tetanus in mice. In 1908, Wolff-Eisner (9) made a series of careful studies of washed organ minces at various intervals after the injection of tetanus toxin into rabbits. By assaying the washed cells in mice, he found toxin in the spleen, liver, kidney, lung, and bone marrow. Bieling and Gottschalk (10) performed the same type of experiment with both tetanus and diphtheria toxins in guinea pigs. The time interval studied was necessarily short (4 hours), and in all but one experiment the presence of blood (which contained a relatively large amount of toxin) compromised the results. In one experiment with washed cells, however, they found tetanus toxin in the spleen, none in kidney, muscle, liver, or brain.

* The first paper in this series was entitled "The Demonstration of Pneumococcal Antigen in Tissues by Means of Fluorescent Antibody" (1).

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Haurowitz and Breinl (11) injected antigens labelled chemically. They prepared horse serum diazotized with atoxyl, injected this into rabbits, and estimated the arsenic content of the organs. Six hours after injection most of the antigen had left the blood stream, and was found in the liver, spleen, bone marrow, kidney, and muscle, in descending order. They found no difference in the distribution of this antigen in normal animals and in those previously sensitized to horse serum. Later Haurowitz and Kraus (12) repeated these observations, and used in addition iodinated horse serum. They summarized their results by stating that they found the bulk of the antigen in the reticulo-endothelial organs, especially the liver.

The use of color for *in vivo* studies of antigenic distribution was introduced by Sabin (13) in her classical experiments with R-salt-azo-benzidene-azo-egg albumin. This dark red azoprotein was injected as an alum precipitate into rabbits, and could be found microscopically in the fixed tissue macrophages, the Kupffer cells, and the endothelial cells lining the lymphatic vessels. She described the "shedding" of cytoplasm by such dye-containing cells at about the time antibody was demonstrable in the serum. Polymorphonuclear leukocytes also took up this particulate antigen. These observations were confirmed in part and extended by Smetana (14) in 1947. He studied several such colored protein conjugates, and found that they were taken up by macrophages, and also by the proximal convoluted tubules of the kidney. He noted that traces of the material were still present 28 days after injection.

The use of radioisotopes as tracers in such studies was introduced in 1947 by Libby and Madison (15). They supplied tobacco plants infected with tobacco mosaic virus with P^{32} as $Na_2HP^*O_4$, found that the harvested virus was highly radioactive, and injected solutions of it into mice. Within the first 2 to 3 days, before most of the P^{32} had presumably been liberated from the virus, most of the radioactivity was found in the liver and spleen. A radioautograph confirmed the activity measurements. Pressman and Keighley (16), and Pressman, Hill, and Foote (17) labelled antikidney antibody with I^{131}, and found that after injection it was concentrated in the kidney, particularly in the glomeruli.

Warren and Dixon (18) produced anaphylactic shock in guinea pigs using antigen labelled with I^{131} for the shocking dose. Radioautographs of tissue sections from such animals showed a great part of the activity still in the blood stream (as was expected), and activity in the fibrous tissue and edema fluid around the bronchi. The liver also showed some activity.

The basic manipulations of the method to be described can be simply stated. An antibody solution of high titer is conjugated with fluorescein isocyanate. The resulting fluorescent antibody solution is employed as a specific histochemical stain on tissue sections. Wherever antigen-antibody precipitates form, the fluorescein-antibody is fixed; unreacted and indifferent fluorescent proteins can be washed away. The section is then examined under the fluorescence microscope; the brilliant yellow-green light from the deposited fluorescein-antibody reveals the presence and location of the homologous antigen.

The data to be presented relate to the synthesis of fluorescein isocyanate, the optimum conditions for carrying out conjugation, methods of purifying the

conjugates, methods for preparing tissue sections for examination without destroying antigenic activity, and the controls necessary to insure that the observed staining is immunologically specific.

1. Synthesis of Fluorescein Isocyanate

The synthesis was carried out by the methods of Bogert and Wright (19), and of Coons, Creech, Jones, and Berliner (1). The procedure to be described is an improvement of these in that the two isomers of nitrofluorescein theoretically possible from the fusion of 4-nitrophthalic acid and resorcinol have been separated and purified by fractional crystallization of their diacetates.

The course of the synthesis was as follows: 4-nitrophthalic acid was heated with two equivalents of resorcinol, with the production of nitrofluorescein. The crude product was refluxed with acetic anhydride, and the resulting nitrofluorescein diacetate subjected to fractional crystallization. Two isomeric diacetates were separated. Each was saponified and the pure nitrofluorescein isomer recovered. Catalytic hydrogenation produced the corresponding aminofluorescein, which was converted as needed to the isocyanate by treatment with phosgene. No attempt was made to isolate the isocyanates.

(*a*) *Preparation of Nitrofluorescein.*—100 gm. of 4-nitrophthalic acid[1] and 100 gm. of resorcinol[1] were intimately mixed in a beaker and heated on an oil bath at 195–200°C. until the mass was dry (12 hours). When cool, the melt was chipped from the beaker, ground in a mortar, and boiled with 1600 ml. 0.6 N HCl for 1 hour. After washing by decantation with three 300 ml. amounts of hot HCl solution, it was collected hot on a large Buchner funnel. The resulting brown paste was washed on the funnel with 5 liters of water, and dried in the oven at 110°C. Yield 176 gm. (98 per cent). *Crude nitrofluorescein.*

(*b*) *Preparation of Nitrofluorescein Diacetate and Separation of Isomers.*—100 gm. of this crude nitrofluorescein was refluxed with 400 gm. of acetic anhydride for 2 hours, and set aside to cool. Crystallization was induced by seeding from a small preliminary run. The whitish yellow crystals of diacetate were collected the next day on a Buchner funnel, and washed with two 10 ml. portions of acetic anhydride, and 20 ml. ethanol (excluded from the filtrate). Yield 21 gm. (17 per cent) (fraction 1).

The filtrate was concentrated by boiling to a volume of about 300 ml. and seeded as before. Yield 11.2 gm. (9.5 per cent) (fraction 2).

Fractions 1 and 2 were combined, dissolved with heat in about 110 ml. acetic anhydride, filtered hot, and set aside to crystallize. Yield 26 gm. (20 per cent). M.p. 213–219°C. After repeated recrystallizations from benzene and ethanol, m.p. 221–222.5°C.[2] Calculated for $C_{24}H_{15}O_9N$: C 62.47; H 3.28; N 3.04. Found: C 62.62; H 3.06; N 3.06.[3] *Nitrofluorescein diacetate I.*

The filtrate from fraction 2 was concentrated to a thick paste *in vacuo* on the boiling water bath. On cooling, the dark brown mass became a viscous gum. To it was added 90 ml. benzene; the solution warmed to about 60°C., and stirred. The gum slowly mixed with the benzene, and crystallization began at once. After several hours the yellow-white asbestos-like needles

[1] Eastman Kodak Company.

[2] All melting points are uncorrected.

[3] Micro analyses by Miss Shirley Katz.

were collected and washed quickly on the funnel with 30 ml. of cold benzene. Yield 33 gm. (26 per cent).

These needles were dissolved in 200 ml. benzene, filtered hot, and allowed to stand overnight. The resulting long white feathery needles were collected, washed quickly with about 40 ml. benzene, and dried in air. Yield 15 gm. (12 per cent). M.p. 189–190°C. After repeated recrystallization from benzene and ethanol, m.p. 215–216°C. Calculated for $C_{24}H_{15}O_9N$: C 62.47; H 3.28; N 3.04. Found: C 62.61; H 3.19; N 3.03. *Nitrofluorescein diacetate II.*

(*c*) *Recovery of Nitrofluorescein Isomers.*—5 gm. of diacetate I was added to 100 ml. hot filtered saturated alcoholic sodium hydroxide, warmed gently, and shaken for a few minutes. An immediate red color resulted. The solution was filtered and poured into 4 volumes of water, acidified with stirring with 2 ml. concentrated HCl, and allowed to stand for several hours. The nitrofluorescein isomer precipitated as a yellow powder which was separated with suction, washed with 500 ml. water, and dried. Yield 4.05 gm. (99 per cent). For analysis, a portion was crystallized from isopropanol. The orange crystals gradually darken on heating but do not melt up to 350°C. Calculated for $C_{20}H_{11}O_7N$: C 63.66; H 2.93; N 3.71. Found: C 63.47; H 3.04; N 3.59. *Nitrofluorescein I.*

To obtain the other nitrofluorescein isomer, 7 gm. of once recrystallized diacetate II was added to 100 ml. hot filtered saturated alcoholic NaOH with stirring. In this case, crystallization of the sodium salt began promptly, and after 2 hours 5 gm. of orange-red crystals were obtained (75 per cent). They were very soluble in water with a red color and faint green fluorescence. To obtain the nitrofluorescein itself, 3 gm. of these crystals was dissolved in 300 ml. water, and acidified with stirring by the addition of 2 ml. concentrated HCl. A prompt yellow precipitate formed which turned red on standing in its mother liquor overnight. Collected, washed, and dried. Yield: 2.1 gm. (74 per cent). A small portion was crystallized from isopropanol. It failed to melt up to 350°C. Calculated for $C_{20}H_{11}O_7N$: C 63.66; H 2.93; N 3.71. Found: C 63.41; H 3.16; N 3.43. *Nitrofluorescein II.*

(*d*) *Reduction of Nitro Compounds.*—2 gm. of nitrofluorescein I was suspended in 100 ml. absolute ethanol and shaken with about 1.5 gm. Raney nickel in an atmosphere of hydrogen at room temperature and pressure. The reaction began promptly and at the end of 90 minutes the theoretical amount of H_2 had been taken up. The nickel was removed by centrifugation, washed with 15 ml. of absolute alcohol, and the washings added to the main lot. The alcohol-amine solution was diluted with an equal amount (115 ml.) of water and allowed to stand. Colorless needles slowly formed which were separated by filtration after standing overnight. Yield: 580 mg. of colorless matted needles which turned red slowly in air and instantly in the presence of water. Kept in the desiccator they slowly darkened to yellow and then to brownish red. M.p. 215–220°C. (decomposed). (Addition of 50 ml. water to the mother liquor yielded another 510 mg.) Yield 61 per cent. *Amino-fluorescein I.* Micro analyses on two different samples of this compound gave low values for C, H, and N. Therefore, the hydrochloride was prepared for analysis. 400 mg. of this amine was dissolved with heat in 15 ml. 2 N HCl, filtered hot, and the dark red crystals collected and dried in the desiccator over H_2SO_4 and solid NaOH. Yield 330 mg. (81 per cent). This compound does not melt. Calculated for $C_{20}H_{14}O_5N \cdot HCl$: C 62.58; H 3.68; N 3.65; Cl 9.24. Found: C 62.71; H 3.66; N 3.61; Cl 9.26. *Amino-fluorescein HCl I.*

2 gm. of nitrofluorescein II was reduced and crystallized as above except that 4 volumes of water was added to the alcohol-amine solution. Canary yellow crystals which did not darken in air and which were stable on storage resulted. Yield: 1.12 gm. (67 per cent). M.p. 315–316°C. (decomposed) (put in bath at 285°). *Amino-fluorescein II.* Micro analyses on two different samples of this compound gave low values for C and N, a high value for H. For analysis, therefore, 60 mg. of amine II was dissolved in 1.5 ml. 2 N HCl with heat, filtered, redissolved by warming and the addition of a few drops of 2 N HCl, and allowed to stand. The red crystals were collected, dried in the desiccator with NaOH, and weighed. Yield:

50 mg. (82 per cent). This compound does not melt. Calculated for $C_{20}H_{14}O_5N \cdot HCl$: C 62.58; H 3.68; N 3.65; Cl 9.24. Found: C 62.66; H 3.85; N 3.67; Cl 9.56. *Amino-fluorescein HCl II.*

In common with fluorescein itself (20), each isomer of both nitro- and amino-fluorescein exists in a red and yellow form; the red form of amino-fluorescein II has been observed only on heating to about 150°C.

We have not attempted to determine which of the two possible positions in the molecule is occupied by the N atom in either of the two isomeric series described above.

(*e*) *Preparation of Fluorescein Isocyanate.*—The required amount of fluorescein amine (10 to 60 mg.) was added to 5 ml. of dry acetone,[4] and added dropwise from a dropping funnel to 15 ml. acetone saturated with phosgene, and through which phosgene was constantly bubbled.[5] As each drop of amine solution entered the reaction flask a yellow precipitate formed which rapidly dissolved. The solution in the flask became slightly warm. The reaction was allowed to continue for 30 minutes, by which time the flask had cooled again. The reaction flask was removed from the phosgene-train, three small anthracite chips added as an anti-bumping device, and the solution taken to dryness *in vacuo* over a water bath at 45°C. (10 to 15 minutes). This step served to remove the excess phosgene and acetone. The greenish brown gum was immediately dissolved in 2 volumes (1 to 2 ml.) of acetone and 1 volume of dioxane,[6] and this solution of fluorescein isocyanate added dropwise to the stirred chilled protein solution described below. Care must be taken to exclude water from the isocyanate solution until the moment of use, as it decomposes rapidly at room temperature in the presence of water.

2. Optimum Conditions for Conjugation of Isocyanate to Protein

Aromatic isocyanates were first conjugated to protein through the carbamido linkage by Hopkins and Wormall (21), who noted that 80 to 90 per cent of the free amino groups of caseinogen disappeared during its reaction with phenyl isocyanate in an aqueous medium at slightly alkaline pH. They concluded that the most likely reaction site was the ϵ-amino group of lysine. The general conditions for the conjugation of isocyanates of the higher aromatic hydrocarbons to protein were worked out by Creech and Jones (22, 23), and used by them in the conjugation of fluorescein isocyanate to antibody solutions (1). They agreed as to the site of the conjugation, although they recorded apparent slight conjugation with zein (a protein deficient in lysine). Miller and Stanley (24) have shown that under similar mild conditions phenolic hydroxyl groups in tobacco mosaic virus also participated in the reaction, though to a smaller extent.

Preliminary experiments with fluorescein isocyanate indicated that allowing conjugation to proceed for 18 hours increased the degree of fluorescent staining

[4] Dried over $CaSO_4$.

[5] This procedure was carried out in a hood with good forced draft. The reaction vessel formed part of a closed system under slight negative pressure. The phosgene was led from a tank through concentrated H_2SO_4, thence to the reaction flask, and thence through a trap to a solution of 20 per cent NaOH where the excess was destroyed.

[6] Purified by refluxing overnight with sodium hydroxide, then for 24 hours with sodium metal. It was then distilled and stored over sodium.

by the antibody-conjugates. The following experiment was carried out to determine the optimum amount of labelling material to use.

Materials and Methods

Antiserum.—The serum used was a refined and concentrated therapeutic antipneumococcal rabbit serum, Type III (Lederle).[7] It had a protein content of 11.3 per cent, and an agglutinin titer of 1/480.

Fluorescein isocyanate II as prepared above, was used as the labelling material.

Buffered saline, 0.15 M NaCl containing 0.01 M phosphate, pH 7.0.

Nitrogen was estimated by the method of Koch and McMeekin (25).

Fluorescein carbamido groups were estimated spectrophotometrically (490 mμ) using fluorescein purified by the method of Orndorff and Hemmer (20) as a reference standard.

Agglutinin titers were determined at the same time on solutions of the various conjugates and the starting serum diluted to the same protein concentration. The antigen was a fresh formalinized 6 hour culture of a highly virulent pneumococcus Type III. It was suspended in saline containing 0.3 per cent formalin to a density of 4 on the MacFarland scale.

Preparation of Conjugates.—A 50 ml. Erlenmeyer flask was fixed in an ice bath and provided with good mechanical stirring. Into it was put 10.5 ml. of saline (0.15 M NaCl), 3 ml. of carbonate-bicarbonate buffer (0.5 M, pH 9.0), 2.5 ml. of 1–4 dioxane, 0.5 ml. of acetone. When the temperature had fallen to 0–2°C. 3 ml. of serum (367 mg. protein) was added. The required amount of isocyanate (weighed as amine) was added dropwise in 1 ml. of acetone and 0.5 ml. of dioxane.[8] A fine yellow precipitate formed, which slowly, though not completely, dissolved during stirring for 18 hours in the cold (0–2°C.). The solution became progressively more fluorescent. At the end of 18 hours, the solution was transferred to a cellophane sac, and dialyzed against daily changes of buffered saline until the fluorescence of the solution outside the sac was less than that of a 1/20,000,000 solution of fluorescein (5 to 6 days). The precipitate was removed by centrifugation, 1/10,000 merthiolate[8] added, and the solution stored at 4°C.

The only variable in this series of conjugates was the amount of isocyanate added. The results are summarized in Table I.

It is apparent that the optimum amount of amine under these conditions was about 0.05 mg. per milligram of protein. Above that level the number of fluorescein-carbamido groups introduced stayed relatively constant, with progressively larger losses of protein. This loss of protein was indiscriminate, however, since the titer of antibody per milligram of remaining protein remained constant. It is possible that under different conditions the amount of conjugated fluorescein might be increased.

The general conditions in use at present for the preparation of conjugates are as follows:—

The protein concentration of the solution to be conjugated (usually a crude globulin fraction of antiserum) is determined, and a convenient amount of it selected for conjugation (for example 500 mg.). Some should be retained un-

[7] We are grateful to Dr. Rutledge W. Howard, and Dr. H. D. Piersma of the Lederle Laboratories, and to Dr. Geoffrey Edsall of the Massachusetts Department of Health Biologic Laboratories, for generous gifts of serum.

[8] Eli Lilly Company.

conjugated for control purposes. The protein concentration in the reaction mixture is fixed at 10 mg./ml.; the final mixture containing in addition 15 parts purified dioxane,[6] 15 parts carbonate-bicarbonate buffer (0.5 M, pH 9.0), 7 parts acetone, and enough saline to make 100 parts. Fluorescein amine, 0.05 mg. per mg. protein, is converted to isocyanate as described above, and the latter dissolved in 2 ml. acetone[5] and 1 ml. dioxane[6] (these quantities being subtracted from the calculated amounts of solvents initially added to the reaction vessel).

The mixture of solvents, buffer, and saline is chilled to 0-2°C. with good mechanical stirring, and then the chilled protein solution is added. Finally,

TABLE I

Preparation No.	Amine-protein ratio	Yield protein	Fluorescein protein ratio*	Agglutinin titer‡	Staining§ brightness (estimate)
		per cent	*μg./mg.*		
61	0.015	85	2.2	1/8	++
62	0.032	74	3.3	1/8	+++
63	0.046	65	5.1	1/8	++++
64	0.061	62	4.8	1/8	++++
65	0.091	53	4.7	1/8	+++
66	0.122	46	4.7	1/8	+++
Antipneumococcus serum Type III				1/16	

* These ratios are of only relative value since fluorescein is not a suitable reference compound for absolute measurements. Furthermore, there is evidence that these conjugates were not completely free from unconjugated material.

‡ Diluted to isoprotein concentration (see text).

§ Not adjusted to isoprotein concentration. Shaken twice with mouse liver powder (see text).

the isocyanate solution is added drop by drop, and stirring continued in the cold for 18 hours.

3. Choice of Isomer

The data presented in this and the accompanying papers were obtained by the use of conjugates prepared from fluorescein isocyanate II. However, two preparations were made with fluorescein isocyanate I. They stained tissues containing homologous antigen as effectively as their companion preparations prepared with fluorescein isocyanate II. The separation of the two isomers is not without advantage, however. As already pointed out, nitrofluorescein is difficult to crystallize, and contains gummy impurities. Preparation of the diacetates is the most effective way to remove these impurities. Reduction of the crude nitrofluorescein, and the recovery of the mixed amines by precipita-

tion with excess water, as described in (1), can be made a satisfactory procedure. In this case, a mixture of red (amine I) and yellow (amine II) crystals results.

4. Preparation of Tissue Sections

The two essentials in the preparation of tissue smears or sections for use with these conjugates are that the antigen retain its immunological activity, and that the architecture and cytology of the tissue be sufficiently well preserved. Clearly, methods involving tissue fixatives applicable to one antigen may not be suitable for another depending on the chemical characteristics of the antigen. For these reasons, the basic method in use in this laboratory for preparing tissue sections is that of Linderstrøm-Lang and Mogensen (26). This consists of cutting sections of quick-frozen unfixed tissue in a refrigerated cabinet. Such preparations show adequate cytological detail, and there is little likelihood of inactivating the antigen. The method has been modified by the substitution of mechanical refrigeration for the solid carbon dioxide as a cooling agent.

Pieces of tissue 4 to 5 mm. in their smallest dimensions are placed against the wall of a test tube near the bottom and plunged into a freezing mixture of dry ice and alcohol. They are then stored in the CO_2 ice box or in a freezing compartment at −20°C. until use.

The blocks can be taken from storage, put immediately into the refrigerated cabinet, frozen sections prepared, and the tissue returned to storage without thawing. Thawing and slow refreezing destroy the architecture of the tissue.

The sections, as cut, are thawed on the slide, dried in air 1 to 2 hours with the aid of a fan at room temperature, and used immediately or stored in the refrigerator. Storage of sections is complicated by the fact that the grey-blue autofluorescence of tissue proteins increases with time; for the first few days this is useful as it aids orientation during microscopy, but it gradually becomes so bright as to obscure the green fluorescence of the deposited antibody.

Antigens which are not inactivated by the procedure may be fixed, embedded in paraffin, and cut in the usual way. For use, they must be deparaffinized and hydrated (1, 7). Care must be taken not to wash diffusible antigens out of the sections before staining.

Both frozen and paraffin sections are mounted on clean glass slides which have previously been coated with formalinized gelatin as an adhesive (1).

5. Technique of Staining

The method of applying the conjugates to tissue sections is simple (1). A drop of conjugate is placed on the section, and evaporation prevented by covering with a small inverted dish containing a piece of moist filter paper. After 20 to 30 minutes, the conjugate is shaken off and the slide immersed in buffered saline in a Coplin jar. After a few seconds the saline is poured off,

the jar filled with fresh buffered saline, and the slide washed thus with very gentle agitation for 10 minutes only. It is then removed from the jar, and wiped dry except for the area of the section. A drop of reagent glycerol containing 1 part in 10 of buffered saline (commercial glycerol is slightly acid) is put over the section, and a clean coverslip dropped carefully over it. It is then ready for examination under the fluorescence microscope.

6. Fluorescence Microscope

A microscope similar to the one now in use for this work has already been described (1).

It consists of a carbon arc with automatic electromagnetic feed operated at 10 amperes; an aspheric glass condenser (6 cm. in diameter, 5 cm. focal length); as filters 3.2 cm. of $CuSO_4 \cdot 5H_2O$ (25 gm./100 ml.) in a pyrex cuvette, Corning filters No. 5840 ($\frac{1}{2}$ standard thickness—2.25 mm.), and No. 9863 (standard thickness—3.05 mm.); a quartz[9] condenser N.A. 1.25; a standard microscope (with non-fluorescing lenses); and a protecting filter (Wratten filter No. 2A[1]) in the ocular. This combination of filters admits a large amount of blue light to the optical system, but it is effectively removed by the filter in the ocular.

The most important elements are a high-intensity light source and a non-fluorescing optical system. The only satisfactory light sources in our hands have been the carbon arc described above, and a high-pressure water-cooled mercury vapor arc (AH-6[10]).

Much useful information is contained in monographs by Sjöstrand (27) and Glick (28).

7. Non-Specific Staining

Conjugates prepared and purified by dialysis as described above regularly stain many elements in normal tissue, due in part to unconjugated fluorescein derivatives which are still present. Additional purification by repeated precipitation at half saturation with ammonium sulfate, and then by precipitation with cold acetone (22, 23, 1) removes some but not all this staining. The nature of this "non-specific" staining is not clear at present, and there are perplexing aspects which have not as yet been systematically investigated. It was found empirically that this staining of normal tissue could be removed by shaking the dialyzed conjugates with a washed and dried suspension of ground liver. The animal species from which the liver is obtained has not so far been demonstrably important, although we have tended to use liver from the species whose tissues it was intended to stain. Conjugates prepared from rabbit, monkey, and horse serum have exhibited this staining.

Acetone-dried or lyophilized liver preparations are equally satisfactory. A measured amount of conjugate is shaken for 1 hour with 100 mg./ml. of such a powder, and the conjugate recovered by centrifugation. Two absorptions are usually adequate to abolish the non-specific staining of normal tissue. After the final absorption all particles, which are brightly fluorescent, must be removed, either by centrifugation or by filtration through a small Seitz EK pad.

[9] Most glass condensers fluoresce, filling the optical system with visible light.

[10] General Electric Company.

8. Proof of Specific Staining

The following controls are available to establish that any observed staining is immunologically specific:

1. Specific inhibition with unconjugated homologous antiserum, as previously described (1), and

2. Specific removal of antibody from an aliquot of the conjugate by precipitation with antigen.

Both these procedures abolish, or markedly diminish, specific staining.

TABLE II

An Example of Immunologically Specific Staining

Frozen sections of the parotid glands of monkeys stained with fluorescein-carbamido conjugate of convalescent mumps monkey serum (preparation No. 54).

Normal parotid	Mumps parotid	Mumps parotid	Mumps parotid
Treated with preparation No. 54 30 min.	Treated with preparation No. 54 30 min.	Treated with normal monkey serum 10 min.	Treated with anti-mumps monkey serum 10 min.
Washed in buffered saline with gentle motion. 10 min.		Rinsed in buffered saline 30 sec.	
Mounted in buffered glycerol and examined under the fluorescence microscope		Treated with preparation No. 54 10 min.	Treated with preparation No. 54 10 min.
Result		Washed, mounted, and examined	
No green fluorescence	++++ Green fluorescence in localized areas	*Result* ++ Green fluorescence in localized areas	Traces of green fluorescence in localized areas

3. In addition, it is essential to show that the conjugate does not stain normal tissue (unless, of course, normal tissue antigens are under investigation). It is also useful to determine that conjugates prepared from normal serum or heterologous antiserum and purified in the same way do not stain the tissue being studied, since normal tissue is not an ideal control for tissue in which pathological changes have occurred.

An example of two of these procedures is given in Table II. In the left-hand column the staining of a monkey parotid infected with mumps virus is compared with that of a normal gland, each exposed to conjugate for 30 minutes to achieve bright staining. In the right-hand column an inhibition experiment is outlined. It should be noted that in order to demonstrate clear cut inhibition long exposure to conjugate is undesirable, as replacement of unlabelled by

labelled antibody gradually takes place (1). With other antigen-antibody systems, and differing concentrations of antigen in the tissue, the time of staining or the concentration of conjugate may be altered so that inhibition is demonstrable. It is our experience that where it cannot be demonstrated, the staining is non-specific. Confusing combinations of specific and non-specific staining are encountered with inadequately absorbed conjugates.

DISCUSSION

As the accompanying papers indicate, this method of antigen localization has been successfully applied to pneumococcal polysaccharide, to rickettsiae, and to mumps virus. Experiments with other polysaccharides have been successful, and will be reported subsequently. It seems likely that polysaccharides which are or can be made antigenic so that antibodies can be produced against them can be traced by this means. Success with mumps virus indicates that the method is not restricted to polysaccharides. Preliminary experiments indicate that bovine albumin can also be traced. We have, however, had two failures: (1) with bovine gamma globulin early in the course of the work, and (2) with a neurotropic virus (SK) more recently. Both these cases will be restudied.

The possible use of other labelling materials has not been investigated. The original choice of fluorescein was based on the brilliance of its fluorescence, and the rarity of green-fluorescing materials in the tissues (*cf.* reference 27). It has the additional advantage that the wave-length of its emitted light (520 mμ-max., range 510 to 540 mμ) (29) is near the maximum sensitivity of the retina. Although radioisotopes show great promise in the study of immunity *in vivo* (15–18) particularly with regard to the fixation of antibody in specific locations (17), fluorescence has the advantages of good optical definition, and of the ease of using fluorescein-antibody conjugates once they have been prepared. (Methods of preparing tissues for examination, and the problem of establishing the specificity of the reaction probably would not be simplified by a change of labelling material.)

It should be pointed out that in each case studied so far, little or no circulating antibody was present. In the case of the rickettsiae (6), the cotton rats were moribund with infection; in the case of mumps (6), the monkeys were at the beginning of their disease; and in the case of the pneumococcal polysaccharides (7), the doses used are known to be far too large to confer protection. Whether excess antibody would mask the antigen being sought, and if so whether it could be displaced, is clearly a matter of importance if this method is to be applied to a study of immune reactions *in vivo*. Experiments on this point are planned.

The application of this method to the detection of viruses and rickettsiae (6) makes it an inviting one for use in the titration of such agents, and it may

prove to be of value for this purpose, since it is very sensitive (7). It is not, however, ideally suited to the examination of tissue suspensions, biological fluids, or other unorganized materials, unless the antigen being sought is particulate and its concentration relatively high. This limitation is inherent in a morphological method, depending as it does on differences in visual contrast. Tissue sections are favorable objects for study because they have an architecture enabling the observer to correlate any observed staining with its location. In the absence of a pattern, the appearance of a faint greenish coloration or a few green specks is difficult to evaluate.

These limitations would not, of course, apply to the measurement of fluorescence in solutions. Such conjugates could no doubt be fruitfully applied to the study of antigen-antibody reactions *in vitro*.

CONCLUSIONS

Improvements in a method for the specific microscopic localization of antigen in tissue cells are described. This method employs antibody labelled with fluorescein isocyanate as a histochemical stain, the specific antigen-antibody precipitate being made visible under the fluorescence microscope.

Two isomeric series derived from nitrofluorescein are described.

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