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WITH  
THE HELP OF FLUORESCENT ANTIBODIES

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DETECTION OF ANTIGENS WITH THE HELP OF FLUORESCENT ANTIBODIES

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## Detection of Antigens with the Help of Fluorescent Antibodies

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### Introduction

In recent years a new method of detecting antigens with the help of fluorescent antibodies has been receiving more and more dissemination. It is the method which was proposed and developed by Coons and coauthors. The foundation for this was the work which indicated that it was possible to introduce simple chemical radicals into a molecule of an antibody without the loss of its specificity.

The essence of the method is that the antibodies of immune serum are combined with fluorescein-4-isocyanate. A section of tissue, in which the antigen being traced is present, is treated with a solution of a labeled antibody. On the surface of the tissue section, over the sites where the antigen is located, there takes place a microprecipitation of the antibody which is labeled with fluorescein isocyanate. Upon studying this section under a fluorescence microscope under strong illumination, the antibody is detected, thus indicating the location of the antigen.

At first a method was developed for detecting a foreign antigen which was introduced into an organism, because the fate of antigens in an organism has interested investigators from the moment immunology originated right up to the present day. However, subsequent experiences have shown that we can adapt this method for solving the most diversified problems.

In this article fluorescent antibody technique will be described, its practical value for solving various problems of experimental biology will be pointed out and an evaluation will be given of it.

### Fluorescent Antibody Technique

Direct method. In 1941 a report appeared by Coons, Creech and Jones (1941). It dealt with the fact that, based on the method of Creech and Jones (1941a, b), they succeeded in obtaining a conjugation of rabbit anti-pneumococcal serum with anthracene and successfully used this labeled serum for the demonstration of type III pneumococci in an agglutinate after anti-serum action on them and on slides in smears from cultures of pneumococci. But the weak bluish fluorescence of serum labeled in this way didn't permit the tracing of microorganisms in mammal tissues, which in ultraviolet rays also possess a bluish autofluorescence.

Therefore, Coons, Creech, Jones and Berliner (1942) resorted to a conjugation of rabbit antipneumococcal serum with fluorescein isocyanate. Serum labeled in this manner gave off a bright fluorescence of a greenish hue in ultraviolet rays which was easily distinguished from the weak bluish autofluorescence of the tissue. The procedure for obtaining fluorescein isocyanate consisted of the following. Upon heating, 4-nitrophthalate acid entered into a reaction with resorcinol and formed 4-nitrofluorescein. The 4-nitrofluorescein was reduced in an atmosphere of hydrogen in the presence of a catalyzer, with the formation of 4-aminofluorescein, which following its treatment with phosgene, was converted to fluorescein isocyanate.

For the conjugation of fluorescein isocyanate with antibodies, an antiserum solution was prepared by mixing immune rabbit serum with saline and with an 0.5N carbonate-bicarbonate buffer at pH 9.0. During this reaction, the temperature was maintained at from 0 to 2 degrees C. Fluorescein-4-isocyanate in dioxane and acetone was added to the antiserum solution and stirred with it for 30 minutes. This resulted in the formation of a greenish fluorescent solution which is dialyzed for 20 hours at 5 degrees C against several changes of saline. A globulin fraction was separated from the antiserum which is conjugated with fluorescein isocyanate. It was purified by repeated reprecipitation with cooled ammonium sulfate or acetone with intermediate dialysis initially against tap water and then against saline. The process of purifying the preparation took 5-7 days. After such processing the agglutinin titer didn't change and equaled 1:800, though the fluorescence of the agglutinate was observed at no more than 1:400 in the dilution of the labeled antiserum.

Experimental test mice were administered an intravenous 0.5 ml suspension of live pneumococcal cells (with a concentration of  $10^{10}$  microbial bodies in 1 ml) and following this were slaughtered following 30 minutes, 1 hour, and 4 hours. Their tissues were fixed with formaline and embedded in paraffin. Sections were prepared from them which prior to conjunction with labeled antibodies were deparaffinated and conducted by an alcohol battery into an aqueous medium. The prepared sections were washed in water for the same period of time. Then they were rapidly washed by a short rinse in saline, rinsed in distilled water, mounted in buffered glycerin on slides made from ordinary glass which are covered with a thin layer of gelatin, and covered with cover glasses that are also made of ordinary glass. The finished preparations were studied in a fluorescence microscope immediately after preparation. After being stored for 2-3 days in cold and heat they were still completely suitable. Later in this period the intensity of the specific fluorescence in the preparations started to decrease and the tissue autofluorescence of nonfixed sections increased.

The authors used an ordinary research microscope, having adapted it for fluorescence microscopy. A carbon arc was used as the source of light. Its light rays passed through a quartz fixing lens and a series of filters. For eliminating the infrared rays they used cuvettes with a 1.5% solution of copper sulfate, for absorbing the violet rays - cuvettes with 0.002% solution of R-nitroso-dimethyl-aniline, and for eliminating the rays of the central part of the visible spectrum - a Corning 986 filter. A pencil of ultraviolet rays was directed onto the stage of the microscope with the help of a quartz prism and a quartz condenser. A protective gelatin filter was put on the ocular.

Pneumococcal antigen was detected in all the organs that were studied in the experimental mice, mainly in the reticuloendothelial system. There was a particularly large amount in the liver and spleen. For verifying the specificity of the reaction, control sections from the same mice were treated initially with homologous antiserum, which wasn't conjugated with fluorescein isocyanate, in a 1:50 dilution for 20 minutes, and then after washing in a salt solution for 3 minutes they were treated with homologous labeled antiserum in 1:50 dilution for 10 minutes. This didn't cause the tissue to fluoresce. With a greater concentration of labeled antisera (1:10, 1:5) and a longer period for its reaction, tissue sometimes stained which had been preliminarily exposed to reaction with homologous unlabeled antiserum. This staining rarely reached such an intensity as staining which was observed following preliminary reaction with heterologous antibodies or normal rabbit serum and was always manifested in areas containing the largest amount of antigen. Apparently this staining took place owing to the gradual dissociation of the antigen-antibody complex and the formation of a chemical equilibrium between the precipitated unlabeled antibodies in the section of tissue and the fluorescent antibodies located in the solution.

Subsequently Coons and Kaplan (1950) improved certain phases of the fluorescent antibody technique. Below we will present these changes and other variations worked out by other authors.

During the process of synthesizing fluorescein isocyanate, Coons and Kaplan isolated and purified two isomers of nitrofluorescein. From the two isomers of nitrofluorescein, two isocyanates of fluorescein were obtained; I and II. The authors did the biggest part of their work with isomer II though preparations of antiserum prepared with fluorescein isocyanate I stained the tissue with the same intensity as those prepared with isomer II. Subsequently, Marshall (1951), in order to decrease the secondary reactions during the synthesis of fluorescein isocyanate, applied Hardy's method (1934) which was proposed for obtaining stable arylcarbimides. Apparently this enabled him to obtain preparations of fluorescein isocyanate that were more stable and had a large yield.

Coons and Kaplan made small changes in the methods of combining antiserum with fluorescein isocyanate. Thus the entire serum wasn't used in the reaction but only the globulin fraction. Marshall (1951, 1954) combined the gamma 2-globulins of antiserum with fluorescein isocyanate. The duration of the reaction of combining the serum with the dye was increased up to 18 hours by Coons and Kaplan. This was accompanied by an ~~increase~~ in the fluorescence of the antigen being stained. The authors consider that the optimum amount for conjunction with 1 mg of antiserum protein is 0.05 mg of fluorescein amine. During the storage of fluorescein isocyanate it is necessary to avoid actions of moisture, light and high temperature. Marshall (1951, 1954), and Vasquez and Dixon (1956), for the more rapid and complete removal of unconjugated fluorescein isocyanate, conducted three reprecipitations of the conjugated protein with ethanol and a subsequent dialysis for 24 hours.

A conjugation of fluorescein isocyanate with antiserum, purified by dialysis, as a rule would stain many elements of normal tissue. The nature of this "nonspecific staining" wasn't quite clear to Coons and Kaplan. Additional purification of the conjugation by repeated precipitation with half-saturated ammonium sulfate and an ensuing precipitation with cooled acetone eliminated only part of the nonspecific staining. Experimentally it was found that adsorption of the conjugation of antiserum and fluorescein isocyanate by dried

liver powder eliminated the nonspecific staining of the tissue. Usually liver was taken from the species of animal which was used in the experiment, though this didn't have a great significance. Two adsorptions, calculated at 100 mg of powder to 1 ml of conjugate, were sufficient. In a number of cases (Sheldon, 1953; Coons, Leduc and Connolly, 1955; and others), it required an additional adsorption of the antiserum by powder from rabbit bone marrow in order to eliminate the nonspecific staining of leucocytes.

Coons and Kaplan found better methods of preparing and processing the sections. The usual procedures for preparing the tissue sections were changed in a way that would preserve not only the architecture of the tissue but also the immunological activity of the nonstable antigens being studied. For this, fragments of the nonfixed tissue being investigated were placed in a test tube and were quickly frozen by submersion in a mixture of alcohol or acetone with carbonic acid. Prior to use they were stored at -20 degrees C. The nonfixed tissue was cut in a frozen condition in a portable box that was specially constructed for this. It had a refrigeration of from -15 degrees C up to -18 degrees C (for details, see Coons, Leduc and Kaplan, 1951). In order to keep the section of tissue from rolling up, a cover glass was set up parallel to the flat of the blade and at a distance of 50 microns from it. After extracting the section the glass was removed and the frozen section of tissue was transferred to a slide. After cutting, the piece of tissue continued to be stored at -20 degrees C to prevent it from thawing. The sections obtained were deposited on a gelatinized slide, thawed, dried in the air at room temperature for 1-2 hours, and used directly or else stored in a refrigerator for several days.

If the antigen is not inactivated, then the tissue can be fixed, embedded in paraffin and sections prepared from it. These can be deparaffinated and then subjected to the action of labeled antibodies.

A drop from a solution of labeled antiserum was placed on a tissue section on a slide. For the prevention of evaporation, the preparation was covered by a Petri dish containing a piece of moist filter paper or batting. After 30-45 minutes the antiserum is shaken off and the slide is rinsed, first for several seconds in buffered saline and then for 10 minutes in a fresh batch of the same solution. After this the slide is wiped dry, with the exception of the spot where the tissue section was located. A drop of buffered glycerin (1 part of glycerin to 10 parts of buffer solution with an ultimate pH 7) was placed on the section and a clean cover glass was sealed with paraffin. Following this the preparation is ready for investigation using a fluorescence microscope.

According to Marshall (1951), preparations which were hermetically sealed with polyethylene glycol along the edges of the coverslip could be preserved for several months with only a small decrease in the intensity of fluorescence.

Similarly, smears were prepared on slides and preparations of tissue cultures growing on coverslips (Liu Chien, 1956; Lebrun, 1956 and others).

In the first investigations conducted by a method of fluorescent antibodies (Hill, Deane, and Coons, 1950; Leduc, Coons and Kaplan, 1951), it was discovered that when a solution of labeled antiserum is deposited on a nonfixed tissue section, part of the antigen is washed out of the section, combines with the fluorescent antibodies and then haphazardly settles in the preparation. For decreas-

ing the solubility of the antigens in aqueous solutions without destroying their immunological activity, they used several fixatives with which the prepared section was already processed before staining. It was primarily acetone (Weller and Coons, 1954; Watson, 1952, and others), less often methyl or ethyl alcohol (Coons, Leduc and Kaplan, 1951; Gitlin, Landing and Whipple, 1953), sometimes alcohol in combination with acetone (Gitlin, Craig and Jane-way, 1957, and others). The preparations are usually placed in acetone for 10-15 minutes at room temperature, and in 95% ethyl alcohol for 20-30 minutes at room temperature, and in 95% ethyl alcohol for 20-30 minutes at a temperature of 37 degrees. For complete removal of the fixative, the slide is slightly dried in an incubator for 20-30 minutes at plus 37 degrees C. While studying bacterial polysaccharides, Hill, Deane, and Coons (1950), and Kaplan, Coons, and Deane (1950) fixed the tissue in a chilled mixture, consisting of picric acid, alcohol and formalin, which is used in histochemistry for the study of glycogen.

It should be kept in mind that at the present time it is impossible to give a method of fixation for all the possible cases on account of the great individual peculiarities of antigens. In each concrete case, the fixative, if it is still not known, should be carefully selected, taking into consideration its concentration, duration and temperature of the action.

Coons and Kaplan used an ordinary standard microscope with a collection of filters similar to those described before and which passed the ultraviolet and violet part of the spectrum. They considered that the most important elements of the set up were the nonfluorescent optical system of the microscope and a source of light with a high intensity (carbon arc or high pressure water cooled mercury lamp with a power no lower than 150-250 vt). Many authors used a dark field condenser (Buckley, Whitney, and Rapp, 1955; Weiter, 1956, and others), Marshall (1951) used a cardioid dark field condenser.

In the preparation under study, a weak autofluorescence of a bluish color was seen which permitted good orientation in the structure of the tissue. In this background of autofluorescence, the specific fluorescence was a rich yellow-bluish color. For a more accurate determination of the type of cells and the character of the microscopic changes, the following methods are available.

1. One part of a tissue was prepared for investigation in a fluorescence microscope and the other part was fixed and stained by the usual histochemical method (Mellors and Ortega, 1956; Ortega and Mellors, 1956 and others).
2. One of two adjacent sections of tissue was stained with fluorescent antibodies and the other with the usual histochemical dyes (Marshall, 1951; Coons, Leduc and Connolly, 1955, a, b, and others).
3. After study in the fluorescence microscope, the coverslips of the preparation were removed, the section of tissue was washed off, in case of necessity it was additionally fixed, stained with histochemical dyes, and the very same fields of vision were looked over in visible light (Watson, 1952; Moulton, 1956, and others).

4. They examined the preparation in ultraviolet light and then without moving the slide, they studied the same fields of vision in visible light with the help of a phase-contrast hook-up (Mellors, Siegel and Pressman, 1955).

For checking the specific reaction, Coons and Kaplan suggested the following tests:

- 1) a preliminary specific suppression of the tissue antigen with unlabeled homologous antiserum;
- 2) specific removal of labeled antibodies by means of precipitation with a homologous antigen;
- 3) the inability of labeled antibodies to stain normal or heterogenous tissue;
- 4) the inability of a labeled normal or heterogenous antiserum to stain a test tissue.

In addition, Vasquez and Dixon (1956) consider it expedient to investigate:

- 1) nonstained sections of the test tissue for the presence of autofluorescence;
- 2) sections of tissue with the antigen being studied, treated with a 40% solution of formalin for 30 minutes prior to specific staining, with a view to changing the specific properties of the antigen.

In summarizing the aforementioned statements it can be stated that work with fluorescent antibodies at the present time includes primarily the following stages:

- 1) synthesis of fluorescein isocyanate;
- 2) conjunction of fluorescein isocyanate with antibodies;
- 3) chemical purification of this conjugation from the unbound dye;
- 4) preventing the nonspecific staining of the tissue by adsorption of the labeled antiserum;
- 5) preparation of tissue sections, smears or preparations of tissue culture on coverslips;
- 6) fixation of preparations;
- 7) treating tissue with labeled antiserum;
- 8) studying the preparations in the ultraviolet or violet spectrum of illumination;
- 9) determining the type of cells and the histological changes present in the preparation;

10) determining the specificity of the fluorescence being observed.

Indirect method. In 1954, Weller and Coons (1954) described the so called indirect method of detecting antigens, which apparently gives a more specific reaction. In the direct method the gamma globulin of the antiserum, prepared directly against the antigen, is mixed with fluorescein isocyanate, while in the indirect method, the fluorescein isocyanate is bound with an antiserum prepared in a different species of animal, to the initial immune gamma globulin. Let us suppose that some sort of Virus A is detected in cultures of human tissue. A rabbit is immunized with a suspension of chorio-allantoic membrane which is infected with the virus. From it, gamma globulin antiserum is obtained (we will designate it as antibody 1). A hen is then immunized with antibody 1.

The gamma globulin of the hen antiserum (we will designate it as antibody 2) is conjugated with fluorescein isocyanate. For detecting the antigen in the experiment they initially apply antibody 1 to the section of tissue and then fluorescent antibody 2.

The majority of work which has been published recently has been carried out with the help of the indirect method.

In spite of the apparent allurements, there are a number of difficulties concealed in the method of fluorescent antibodies. First of all it is necessary to note the intricacy of synthesizing fluorescein isocyanate, which takes place under conditions of phosgene formation, i.e., it is possible only in specially equipped laboratories or plants. Therefore the necessity arises of searching for new fluorochromes which are simpler to obtain and handle, and more stable and capable of combining with the molecule of the antibody without the loss of its specificity. It is also necessary that there be available several fluorochromes of the same type which are different in their coloration for the purposes of bacteriological or virological diagnostics, during which it is expedient to simultaneously use several variously stained antisera. Attempts at searching for new fluorochromes capable of combining with an antibody molecule are already going on (Clayton, 1954), and it can be surmised that this problem will be solved in the near future.

Adaptation of the ordinary microscope for purposes of luminescence microscopy may be found in the works of V. M. Bergolts (1953) and M. N. Meysel (1955). The domestic illuminator, type OI-18 can be a satisfactory source of light. The ML-1 microscope, which will be produced in the near future is suitable for work.

An intricate portable refrigerated box is used primarily for cutting the tissue. It seems to us that it is necessary to propose simpler methods for cutting nonfixed frozen tissue at room temperature (Adamstone and Taylor, 1948; Bush and Hewitt, 1962), which in essence are modifications of a method of cutting tissue with a knife that is thoroughly cooled (see Romeys, 1954). It cannot be excluded that with time, conditions for fixation will be found which allow the tissue being investigated to be cut with an ordinary chilled microtome. Very much in perspective is the method of lyophilizing tissues which are being subjected to histological investigation, which is already being used in work with fluorescent antibodies (Marshall, 1955; Weller, 1956). Pirs (1956) and Gersh and Stefenson (1956) also describe methods of lyophilization.

The use of the method under consideration becomes more practicable if the antisera, combined with fluorescein isocyanate or with any other dye, would be prepared centrally and sent out to all the laboratories that need them. For preserving labeled antiserum a method of lyophilization may be proposed which is already being used with success (Marshall, 1951). It is especially important that with the use of the indirect method, for the overwhelming majority of work it will be sufficient to have 1-2 standard fluorescent antisera, in as much as it is always possible to prepare the appropriate "immunological bridge".

#### Detection of Foreign Soluble Antigens Introduced into an Organism

Tissue antigens. Coons with coworkers (Coons, Leduc and Kaplan, 1951; Coons, 1953) studied the distribution of egg albumin, albumin of bovine serum and the gamma globulin of man in the organism of a mouse. The antigens appeared rapidly and in a large concentration in connective tissue and in the reticuloendothelial system of all the investigated organs. Even though the observations were much the same for the distribution of all three antigens, there appeared noticeable differences in the duration that they could be detected in the organism. It can be roughly said that the speed of disappearance of all three proteins from the nuclear substance and from the cytoplasm of the cells was in reverse proportional dependence on their molecular weight. Similar data has been obtained by Schiller, Schayer and Hess (1953).

Waksman and Bocking (1953) injected egg albumin and bovine gamma globulin intracutaneously in normal and sensitized rabbits. Antigens were found in the area of their introduction intercellularly and intracellularly in the histiocytes and in the lymphocytes of the regional lymph nodes. In the sensitized animals, a slowing down in the disappearance of antigens was noted both from the skin and from the lymph nodes.

Bacterial polysaccharides. Bacterial polysaccharides, introduced into the organism of a mouse, were preserved considerably longer (Hill, Deane and Coons, 1950; Kaplan, Coons and Deane, 1950; Coons, 1953). Polysaccharides of Friedlander's bacillus and type II and III pneumococci were detected in the cells of the reticuloendothelial system of the majority of investigated organs during a period of 5-6 months.

Schmidt (1952), on the contrary, reports of the rapid revelation of streptococcal polysaccharide from the sanguiferous canal through the kidneys after intravenous administration in mice.

#### Studying Native Tissue Antigens of an Organism

Clayton (1954) used fluorescent antisera for studying the antigens of a mouse's eye. During the work, three fluorescent dyes were used:

- 1) isocyanate-4-fluorescein - green fluorescence;
- 2) 1-dimethyl-amino-5-sulfonil-chloride-napthalene - yellow fluorescence;
- 3) benzaldehyde-6-nitro-2-sodium-diazotate - red fluorescence.

The antiserum was prepared against the crystalline lens and muscle of a mouse. Eye sections of a one-day animal were treated with a mixture of two labeled antisera. If the crystalline lens antiserum was labeled with a red

fluorescence and the muscle with a green then the following picture was observed: The crystalline lens was a rich red color; the ciliary process, retina and epithelium of the cornea - pale-rose colored; external muscles and supporting connections of the eye - green; and the choroid - a pale green color.

Gitlin, Landing and Whipple (1953) in extensive investigations of human tissues with antibodies combined with fluorescein isocyanate against human gamma globulin, albumin, beta-lipoprotein, a combination of beta-globulin with metal, and fibrinogen, regularly detected all these substances, with the exception of fibrinogen, in the nuclei of many cellular types.

Fink, Holtzer and Marshall (1956) with the help of this method describe the distribution of myosin in the transversostriated muscular fibers.

In another work, Marshall (1951) studied the localization of adrenocorticotrophic hormone in a pig. The fluorescent antibody selectively stained the cytoplasm of basophil cells of the hypophysis of a swine and did not stain the cells of the hypophysis of a sheep and cow nor the kidney cells of swine.

This same author (Marshall, 1954) reports the distribution of chymotrypsinogen, procarboxypeptidase, desoxyribonuclease and ribonuclease in the cells of the pancreatic gland of a bull.

Hill and Cruickshank (1953a), on the basis of studying the antigenic components of a rat's kidney, conclude that it contains, to a minor degree, two antigens: One in the basal membranes and the other in the cytoplasm of the canalicular epithelium. They report (Hill and Cruickshank, 1953b) that the basal membranes and reticular tissue of many organs of a rat have common antigen.

#### Clinical and Experimental Study of Several Human Illnesses

Glomerulonephritis of man and experimental glomerulonephritis. Mellors and coworkers, with the help of the method of fluorescent antibodies, studied experimental glomerulonephritis, glomerulonephritis of man, and several other diseases. They determined, by direct and indirect methods, the localization of heterogenic proteins or nephrotoxic antibodies administered in animals in order to induce glomerulonephritis. Simultaneously the distribution of gamma globulins was studied in the tissues of an experimental animal. The considerable increase in their content at the sites of affection (determined and compared photometrically) during the corresponding controls allowed the authors to consider that, at any rate, part of these gamma globulins were introduced autogenously by the antibodies that were formed.

Mellors, Siegel and Pressman (1955), Ortega and Mellors (1956a, b) and Mellors (1955) found that during experimental glomerulonephritis of rats, caused by the administration of nephrotoxic serum, foreign antibodies are localized in the glomeruli of the kidney (in the basal membranes). In sites that are histologically close, if not identical, there is also a localization of autogenously formed antibodies which were possibly produced in opposition to a foreign antigen - rabbit protein.

Upon administration of a purified foreign protein to rabbits (Mellors, Siegel and Pressman, 1955; Mellors, 1955; Mellors, Aria-Stella, Siegel and Pressman, 1955) and with the advent of expressed glomerulonephritis, auto-genously formed antibodies were observed in the renal glomeruli and in affected sectors of myocardium, endocardium and blood vessels.

Amyloidosis. In man, Mellors and Ortega (1956) observed autogenously formed antibodies in the sites of active affections during glomerulonephritis, lipoid nephrosis, periarteritis nodosa and amyloidosis of the kidneys, in the walls of capillaries during the period preceeding the deposition of amyloid.

In amyloidai depositions in man and during experimental amyloidosis, Vasquez and Dixon (1956a) found a considerable amount of gamma globulin, which could have been the result of the antigen-antibody reaction. "Collagen diseases." Gitlin, Craig and Janeway (1957) found that fibrin is contained in fibrinoid formations during "collagen diseases" (rheumatoid arthritis, lupus erythematosus, dermatomyositis, etc.). They also reported that the method of determining fibrin by means of fluorescent antibodies is considerably more sensitive than the common histochemical reactions to fibrin.

Vasquez and Dixon (1956b) found an increased content of gamma globulin in fibrinoid depositions during "collagen diseases" in comparison with normal tissues.

Tumors of animals. Weiler (1955) reported that in rats, during the process of the malignant degeneration of liver cells, the hepatic antigen, which is detected with the help of fluorescent antibody, disappears. Weiler (1956) didn't find a specific hepatic antigen in the cells of hamster kidneys with cancer induced by stilbestrol.

Noyes and Rapport (1956) report of their detection of a common antigen in the lymphosarcoma cells of a rat and the epithelium, thymus and cutis of the same animal.

#### The Application of Fluorescent Antibody Technique for Studying the Sites of Antibody Formation in an Organism

Coons, Levine and Connolly (1955a), during investigation of rabbits given numerous immunizations with human gamma globulin and chick ovalbumin, found that the antibodies are present in groups of plasmatic cells in the red pulp of the spleen, in the substantia medullaris of the lymph nodes, in the sub-mucous of the small intestine, and in periportal connective tissue.

These same authors - Leduc, Coons and Connolly (1955), report about changes taking place in the popliteal lymph node of a rabbit after a single or dual administration of antigenic material. They found that after the initial antigenic stimulation, antibodies are detected in the cytoplasm and in the nuclei of the large immature cells of the cerebral regions of the lymph nodes which drain the site of administration of the antigen. These cells, as part of their development, increase the amount of antibody that they synthesize, and gradually become typical plasmatic cells. The number of these cells is considerably increased after the second antigenic stimulation. The authors expressed the thought that maybe for the synthesis of the antibody, the cell, in all cases, should come in contact with the antigen twice. The first such

encounter only prepares the cell to a certain degree for the subsequent synthesis of the antibody which takes place only after the second antigenic stimulation.

White, Coons and Connolly (1955a), during study of the immunization of rabbits with ovalbumin and diphtherial toxoid adsorbed in ammonium phosphate, discovered that the cells which develop antibodies appeared initially in the regional lymph node and then in the local granulation formed around the site of administration of the antigen.

The authors explain the stimulating effects of the ammonium phosphate on the development of the antibodies, first by the slowed down absorption of the antigen from the site of its administration, and secondly by the formation of a local granuloma which contains the plasmatic cells which form antibodies.

White, Coons and Connolly (1956b) also studied the role of the wax fraction of Mycobacterium tuberculosis in Freund's adjuvant during antibody formation. The high titer of serum antibodies, in their opinion, is explained by the widely distributed proliferation of plasmatic cells in the lymph nodes which are distant from the site of antigen injection, in the liver and in the spleen.

In other reports by Coons, Leduc and Connolly (1956b,v), and Witmer (1955a,b), the role of plasmatic cells in the generation of antibodies is confirmed.

#### Detection of Several Viruses by Fluorescent Antibody Technique

Virus of epidemic parotitis. Coons, Snyder, Cheever and Murray (1950) studied, by means of labeled antibodies, the virus of epidemic parotitis and the rickettsiae of exanthematous typhus and Rocky Mountain fever. The first mentioned agent was localized microscopically in the parotid salivary gland of an experimentally infected monkey, in the cytoplasm of acinous cells.

They found rickettsial antigen in various tissues, mainly intracellular, in infected cotton rats; they also detected it in cells of exudations from the serous cavities of these rats and in the organisms of infected lice.

Chu, Cheever, Coons and Daniels (1951) also studied the distribution of the virus of epidemic parotitis in one experimentally infected monkey 96 hours after it was infected (that is, prior to the appearance of the first clinical signs of illness). The virus was found in the brain and spinal column and in both parotid salivary glands of the animal. It follows that in this work the serum from a monkey convalescing from epidemic parotitis served as an immune serum. The work of Watson (1952a) is also devoted to the detection of this same virus in a tissue culture. In this work, convalescent monkey serum which is bound with fluorescein-isocyanate also serves as an immune serum. Distribution of the virus was limited by the cytoplasm of the cells of the chick embryo which came in direct contact with the inoculation substance regardless of the type of tissue used.

Influenza virus. The occurrence of the epidemic parotitis virus (Watson, 1952b; Watson and Coons, 1954) and the influenza virus (Watson and Coons, 1954) were also studied in a developing chick embryo.

Many general and distinguishing characteristics of behavior of these agents were found. Following inoculation of a 7-11 day embryo in the amniotic sac, in both cases the virus antigen was found in the cells lining the amnion and in the cells of the epidermal and pharyngeal epithelium, that is, directly in contact with the introduced antigen. The virus wasn't detected in other tissues of the embryo. If the influenza virus is injected into a 12 day embryo by the same route, then besides this, there appear large viral inclusions in the cells lining the respiratory tract. The virus of epidemic parotitis doesn't possess these properties. Cytologically, the influenza antigen appears first in the nucleus and later in the cytoplasm of the cells. The antigen of the epidemic parotitis virus was detected only in the cytoplasm.

With the help of fluorescent antibody technique, Watson (1956) studied the dual infection in a developing chick embryo with the viruses of epidemic parotitis and influenza. Depending on the conditions of the experiments, the multiplication of one virus or the other predominated. In a number of cases there was observed a simultaneous presence of both agents in one and the same cells of the embryo.

Liu (1955a), with the help of staining with a fluorescein-bound antibody, detected the virus of type A influenza in the cytoplasm and in the nuclei of ciliary epithelial cells, covering the nasal cartilage of infected ferrets. In the beginning of the febrile period, when, with the help of the usual cytological methods, it was impossible to detect any kind of pathological changes in the mucous, a large portion of the epithelium fluoresced. During this period, in smears made from secretions of the nasal passages, there were individual desquamated cells of epithelium and macrophage present which contained the viral antigen. The author suggests the use of this finding for the early and simple diagnosis of influenza. However, preliminary testing of this method in practice didn't give the expected results (Liu, 1956).

In another work conducted on the very same pattern, Liu (1955b) reports that nuclear fluorescence takes place at the expense of the soluble antigen (S), common for the three strains of influenza type A under study (Fm<sub>1</sub>, Farrington and PR8 strains), and cytoplasmic fluorescence - at the expense of the naturally viral antigen (antigen V).

Vaccinia virus. Noyes and Watson (1955) identified the Vaccinia virus in in vitro cultured cells of human epidermoid carcinoma. At first, the fluorescence of the cell cytoplasm bore a granulated character, but as part of the development of the infection and an increase in the number of antigens, it gradually acquired a mixed diffused appearance. In late stages of the infection there was sometimes noted also a diffused nuclear staining.

Virus of canine distemper. Moulton and Brown (1954) studied the virus of canine distemper in naturally diseased dogs. In the cytoplasm of the epithelial cells of the urinary bladder they found a viral antigen which by its localization fitted the inclusion corpuscles.

Moulton (1956) also studied the demyelination of neural tissue during the same disease of dogs. He found a viral antigen only in the nuclei of neuroglia, mainly in astrocytes. A direct connection between the presence of the virus and the process of demyelination has not been established.

Liu and Coffin (1956) studied the virus of canine distemper during the intranasal infection of ferrets. The authors were able to establish a rapid diagnosis of the disease by staining smears of the animal's blood with a specific antibody. In the natural diseases of canines, the establishment of a diagnosis was based on the staining with antibodies of smears from mucous conjunctiva.

Poliomyelitis virus. Buckley (1956a,b), with the help of the indirect method of fluorescent antibodies, detected antigens of the poliomyelitis virus, types I, II and III in monkey kidney tissue cultures. Only 5 hours after inoculation the antigen appeared in the cytoplasm, and in later stages, even in the nuclei.

Other viruses. Cohen, Gordon, Rapp, Macauley and Buckley (1955) isolated, from three persons who had measles, an agent (obviously a virus) in monkey kidney tissue cultures and demonstrated it with the help of the indirect method. The agent specifically reacted with serum from persons convalescing from measles.

Noyes (1955) studied the Egypt 101 virus (close to the virus of Western Nile fever) in tissue cultures of human epidermoid carcinoma and in neurons from the brain and spinal cord of infected mice. The method of fluorescent antibodies turned out to be 10 times more sensitive than the method of direct isolation of this virus during the intracerebral infection of mice.

Liu and Eaton (1955), with the help of the indirect method, detected the virus of primary atypical pneumonia after its inoculation in a developing chick embryo. By the same indirect method they isolated and demonstrated the virus of primary atypical pneumonia from the sputum of sick persons.

The report of Liu, Eaton and Heyl (1956) is devoted to the isolation of the primary atypical pneumonia virus in chick embryos and the successful detection of it with the help of the indirect method. The authors were also able to detect antibodies in convalescents, beginning with the 3rd week after the onset of the disease, utilizing convalescent serum conjugated with fluorescein isocyanate and sections of chick embryo lung tissue, infected with the primary atypical pneumonia virus.

Buckley, Whitney and Rapp (1955) report about their investigation, with the help of the indirect method of staining, of the development forms of the psittacosis virus in the tissue cultures from the livers of mice embryos. The results of this work allow the authors to consider that the psittacosis virus begins to multiply in the cells immediately after it penetrates them, because the first traces of fluorescence were noted in several cells only 1 hour after the introduction of the virus into the culture.

Coffin, Coons and Cabasso (1953) described the successive stages of appearance of the canine hepatitis virus in the nuclei of infected canine cells. The antigen of the virus first appeared in the nuclear membrane, from where it spread into the depth of the nucleus with a gradual forming of large fluorescent intranuclear granules homologous to inclusion corpuscles.

Lebrun (1956) described the appearance and expansion of the herpes zoster virus in the cells of the chorio-allantoic membrane of a chick embryo,

the brain of a mouse and in cultures of human epidermoid carcinoma, with the help of the indirect method of staining. The antigen of the virus initially appeared in the nucleus where it reached considerable numbers. At this time, they didn't find the typical intranuclear inclusion corpuscles. Then the antigen of the virus appeared in the cytoplasm where its quantity gradually increased along with a simultaneous decrease in the nucleus. By the time of almost complete disappearance of the antigen from the nucleus, the typical inclusion bodies were formed in it. The author analyzed the inclusion bodies as an "intranuclear scar" of infection.

Weller and Coons (1954), with the help of the indirect method suggested by them, confirmed the fact of the multiplication of isolated herpes zoster and varicella viruses in human tissue cultures.

#### Application of Fluorescent Antibody Technique in Microbiology

The initial works in this area have already been carried out (Coons, Greech and Jones, 1941; Coons, Greech, Jones and Berliner, 1942).

Moody, Goldman and Thomason (1956) prepared a fluorescent antiserum against Malleomyces pseudomallei and used it for staining fixed and nonfixed smears on a slide from pure cultures of M. pseudomallei, M. mallei, and other species of bacteria. Specific staining was positive with 33 strains of M. pseudomallei and with 3 strains of M. mallei, and negative with 19 strains of other species of microorganisms. In another work, Thomason, Moody and Goldman (1956) accurately detected M. pseudomallei, not only in pure cultures, but even in the tissues of infected animals, in soil samples, from the surface of tree leaves, and from suspensions containing massive concentrations of other bacteria, even if there were only 220 cells per ml. of M. pseudomallei in a ratio of 1:100,000 with other microorganisms.

The authors consider that by using the fluorescent antibody method, the process of identifying unknown bacteria may be accomplished in the matter of a few hours regardless of whether or not the bacteria are viable and without the necessity of complicated cultural, biochemical and serological procedures.

Sheldon (1953) writes about detecting with the help of fluorescent antibody of the leptospiral antigen in muscular lesions during leptospirosis.

Goldman (1953, 1954) prepared an antiserum conjugated with fluorescein isocyanate which specifically reacted with Entamoeba histolytica and Entamoeba coli. With the help of labeled antisera these protozoa were detected with great accuracy both in smears from pure cultures as well as from mixtures with other species of amoebae.

#### Conclusions

The method of fluorescent antibodies is a new method of investigation. Several details concerning its use are still unknown. For example, the minimum concentration of antigen in tissue which can be detected by method isn't known. It isn't known to what degree the "fragments" of an antigen administered in tissues preserves the ability to precipitate itself into an antibody. It isn't clear to what degree an antigen can be traced when it is blocked by the antibody formed to it.

Sometimes it is difficult to avoid nonspecific staining of tissue. There is no doubt, however, that the new method is very effective for studying both foreign as well as natural antigens of an organism. It is advantageously different from many other experimental methods in its feasibility to determine the whole protein molecule or its large fragments which have maintained immunological specificity. It is very important when using fluorescent antibodies that the proteins being studied are not subjected to any preliminary influences which could change their properties.

The new method considerably increases the bounds of histo and cytochemical determination of proteins, polysaccharides, ferments and even hormones. It can be boldly stated that with its help, one can investigate the fate of any antigen that is introduced or exists in an organism and even when this substance was not in a pure form. Reducing the strength of the labeled antiserum destroys secondary reactions.

The use of this method is very probable in conjunction with fractionation when investigating the antigenic structure of tissues. It is possible to investigate simultaneously the distribution of several antigens in one section.

The visual detection of specific tumoral antigens appears practicable and also the investigation of the antitumoral antibodies.

Fluorescent antibodies can be used in virology with great success. By using them it is possible to process single layer cultures of tissue inoculated with a virus for the precise determination of the number of patches of destructive cells that have developed (Dalbek method). This method may yield great help in the early diagnosis of viral infections both during the study of smears and prints and during the study of material introduced in tissue cultures. Without a doubt, with the help of fluorescein antibody technique, it is possible to study in detail the pathogenesis of viral infections and the effect of interference and immunity to viruses.

The use of labeled antiserum is very important for the rapid identification of unknown microorganisms and for investigating their antigenic structure.