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THE TECHNIQUE OF FLUORESCENT ANTIBODIES AND ITS APPLICATION IN VIROLOGY*

[Following is a translation of an article by David Kirsh, M.D. of the Center of Contagious Diseases, Public Health, Service, Secretary of Health, Education and Welfare, Atlanta, Georgia, appearing in the Spanish-language periodical Boletin de la Oficina Sanitaria Panamericana (Bulletin of the Panamerican Sanitary Office) Vol 55, July-December 1963, pages 68-76.].

Landsteiner (1) was the first one to utilize "labelled" substances in immunology. This investigator and his collaborators used azo-proteins to study the serological specificity of proteins. Mainer (2) modified the antipneumococcic serum with diazoatoxyl and observed that the serum retained its specificity. Heidelberger (3) altered egg protein with a benzidine salt introducing a diazo-bond. All of these investigators and others used a drastic chemical treatment modifying the chemical structure of the antibody in a certain manner. A. Coons (4) and his collaborators deserve the merit of being the first ones who used fluorescein isocyanate to conjugate proteins without seriously damaging their specificity. These investigators conjugated antipneumococcic serum with isocyanate for the purpose of studying the fate and the localization of the pneumococcic polysaccharides in experimental animals. Organic solvents, dioxane

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* Work based on a series of lectures and laboratory demonstrations organized by the National Institute of Virology, Mexico, D.F. Mexico, in March 1962, under the sponsorship of the Panamerican Sanitary Office.

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and acetone, were used for protein conjugation. Riggs (5) reported that the fluorescein isothiocyanate salt was superior because the labelled product obtained with this reagent was more stable than the protein labelled with isocyanate. Besides, fluorescein isothiocyanate is more soluble than the corresponding isocyanate.

Other fluorochromes, particularly, lisaminerhodamine B 200 (6), have been used, but fluorescein isothiocyanate continues to be the preferred compound for labelling, due to the fact that this dye has been found to be appropiate for ultraviolet radiations because the eye is very sensitive to the fluorescent color emitted, and also because of the fact that the yellowish-green color emitted is very rarely found as autofluorescence in normal tissues.

Papers explaining how antibodies were labelled with Ferritin, an iron compound containing protein (7), have been published recently. This procedure has been used in electron microscopy to determine the localization of the antibody/antigen. Other materials of great electron density, which have been used, are mercury and lead salts.

However, for ordinary purposes, the fluorescein isothiocyanate method is considered to be the most adequate because the antibody remains physically and chemically unaltered. Also, by this method the reactions of antigen/antibody may be localized with great precision and in a relatively simple manner.

The Optical System

Microscopy using fluorescent light is more complicated than microscopy based on ordinary white light because the mechanical and optical alignments are harder to obtain when the image is relatively diffused.

1. Microscope

A simple microscope may be used. Achromatic objectives are suitable, although a clearer image may be obtained with apochromatic lenses or fluorite lenses. A dark field condenser is indispensable because fluorescence may be detected easier on a dark background. Besides, fluorescence is more intense with a dark field condenser. However, a translucent field condenser is useful in setting the field. The use of a monocular or binocular microscope is a question of personal preference. However, it should be remembered, that a binocular microscope loses almost 40 per cent of the light intensity.

2. Light Source and Filters

The use of a high-pressure mercury vapor lamp within a quartz chamber is preferred because it produces a high emission of energy in the ultraviolet and blue bands of the spectrum.

The brightness of fluorescence is low in comparison with the light of excitation. In order that fluorescence is not attenuated, the light of excitation must be eliminated. Therefore, a primary filter is placed between the lamp and the object, stopping any undesirable wavelength, thus allowing the passage of only those waves exciting fluorescence, that is, radiation of 360μ and 490μ (green-blue).

A secondary filter (a barrier filter) is placed between the object and the observer, to see only the wavelengths characteristic of fluorescein, and also to protect the sight of the observer. Many investigators utilize:

a) a blue-violet light of excitation between 400 and 450μ with a secondary filter distinctly yellow;
b) a combination of ultraviolet and blue-violet light of excitation between 350μ and 450μ and a secondary filter distinctly yellow;
or
c) an ultraviolet light of excitation between 350μ and 450μ, which is more frequently utilized with FA virus.

Fig. 1 is a representation of the optical system.*)

A critical factor for obtaining a satisfactory visibility of fluorescence is the proper alignment of the optical system and of the illumination system. When the Reichert system is used, it is convenient to proceed in the following manner:

a) Open the lamp chamber and put the mercury lamp in its place. To mount the Reichert apparatus, an Osram HBO 200, L1 is used. It is important to examine the cables of the transformer and to handle the mercury lamp with care, as not to leave traces of grease from finger prints because grease is burned and imbibes in the glass diminishing the efficiency of the lamp. Close the lamp chamber.
b) Place all the small handles of the filter holders in a vertical position and remove the manual screws from the upper part of the filter chamber. Raise the frame and move the handles, and mark them with 5940 (dark violet) or with 5970 (blue-violet).
c) Connect the three-pronged plug of the lamp to the transformer, which, in turn, has been connected to the laboratory circuit. Light the lamp and let pass 15 minutes to reach full intensity.

*) All figures appended at end of article.

When the lamp is shut off, allow 30 minutes before lighting it again.

d) Reduce the light intensity by closing the shutter of the lamp casing. Place a piece of white paper on the light aperture located on the base of the microscope. The image of the electrodes must appear on the white paper. Focus it clearly by means of the convergent lenses (the handle is on the front part of the lamp casing). Fuse the true image of the electrodes with the image of the same on the mirror, adjusting the screws centering the lamp in the upper part of the lamp casing. As soon as the two images coincide, tighten the screws in position. Then adjust the mirror so that the beam of light passes vertically through the center of the light aperture of the microscope base.

e) Place the centering device (a metal disk adjusted on the mirror) in position. This metal disk has a small hole in the center above the aperture of the exit of the light. Adjust the adapter of the microscope in such a manner that the light beam penetrates through the center at the base of the condenser.

f) Raise the condenser as much as possible. After fixing the centering device of the condenser, center the condenser by means of screws located at both sides. Place in position the low power objective (10x).

g) Place a drop of immersion oil ("Cargille" non-drying immersion oil, type A, of very low fluorescence) on the condenser, avoiding air bubbles. Place the microscope slide on the platform and center the specimen over the condenser lenses. Move the condenser up and down meanwhile the light shutter is opened and closed. A circular and very fluorescent area must appear in the center of the dark field.

h) Put a drop of oil on the specimen and change the low power objective to the oil immersion objective (40x). Focus and adjust the diaphragm until a good contrast is obtained.

i) Loosen the mirror clamp and adjust it with respect to the center of the circular fluorescent area. Adjust the condenser to obtain a clearer image and a maximum light intensity.

Labelling Procedure

Integral serum or precipitated globulins may be utilized. We prefer the latter for two reasons: first because the use of isothiocyanate is found to be more economical, and second, because it tends to decrease the non-specific spots.

To obtain good globulin preparations several methods may be utilized, such as the methanol-acetate precipitation of Dubert (6), the half saturation with ammonium sulfate or the fractionating method of D.E.A.E. (7). We utilize the ammonium sulfate method because it is simple and very satisfactory results are obtained. In short, this method consists of adding a volume of a cold saturated solution of ammonium sulfate to an equal volume of serum.

This addition is carried out slowly and with constant stirring of the mixture while it is in an ice bath. A magnetic stirrer may be used to obtain good results. Continue stirring the mixture for 30 minutes. Then the precipitate is placed into a Spinco centrifuge at 10,000 revolutions per second for 15 minutes. The supernatant liquid is removed. The precipitate 2X is washed with cold 50 per cent ammonium sulfate solution, and centrifuged in the Spinco centrifuge after each washing. Dissolve the precipitate in a minimum of cold, distilled water. Dialyze the dissolved globulin at 0° C with a 0.85 per cent NaCl solution exchanging frequently the saline solution. The dialysis is carried out until sulfate is no longer detected in the dialyzate by means of $BaCl_2$. The protein content is determined by the Biuret reaction. The globulin solution may be stored in the frozen state until the time when it will be used.

Labelling Globulin with Fluorescein Isothiocyanate

Fluorescein isothiocyanate is added, according to the protein content at a ratio of 1:20, or, 1 part of isothiocyanate per 20 parts of protein. It has been reported that the rabies antiglobulin can be satisfactorily conjugated in a ratio between 1:40 and 1:80. We do not know that this has been determined for other systems, although in experiments performed with anti-mice serum of rabbits, we conjugated the globulin in the ratio 1:40, and satisfactory results were obtained in the indirect experiment. Generally, the protein concentration, is adjusted to 2 per cent by diluting with 85 per cent NaCl solution, and adding 0.5 M carbonate-bicarbonate solution of pH 9 (10 per cent of the total volume) as stabilizer. An example of the calculations follows:

To label 5 milliliter of 3.4 per cent globulin solution
3.4 gram per cent = 34 milligram per milliter.

To obtain a 2 per cent protein solution, it is necessary to adjust the volume to 8.5 milliliter. However, because 10 per cent of the total volume must act as a carbonate-bicarbonate stabilizer, 0.425 milliliter of 0.5 M $NaHCO_3$ and 0.425 milliliter of 0.5 M Na_2CO_3 are added. Saline solution (2.8 milliliter) is added to the 5 milliliter of 3.4 per cent protein solution containing 0.85 milliliter of stabilizer. In this way, a total volume of 8.5 milliliter of 2 per cent protein solution at a pH 9 is obtained. To achieve an optimum of labelling, 8.5 milligram of fluorescein isotiocyanate is also added in a proportion of 1 milligram of isothiocyanate per each 20 milligram of protein. To calculate the amount of fluorescein isothiocyanate which must be added to a 2 per cent proteinic solution, the following equation can be used:

(total volume in milliliter)x(total milligram of protein)x0.05 = milligram of fluorescein isothiocyanate which must be used.

(In the above-mentioned case, this would amount to 8.5x20x0.05 = 8.5 milligram).

For this percentage of protein, with 0.05 milligram of isothiocyanate per milligram of protein, a simple rule of practice consists in adding 1 milligram of isothiocyanate per milliliter of a 2 per cent globulin solution at pH 9.0. It is convenient to adjust the pH of the solution to 9.0 since at this pH the isothiocyanate is more soluble and attaches itself more easily to the protein. Coons (10) states that fluorescein isothiocyanate reacts with the lysine residues of the protein molecule. The globulin and the isothiocyanate are mixed overnight in the regrigerator by a magnetic stirrer. Then, the mixture is dialyzed with frequent changes of PBS (saline solution at a 7.2 pH, stabilized with 0.01M phosphate solution) at low temperature, until the dialyzate is free of fluorescein. This may be observed by placing a sample under the microscope. If a greenish-yellow color is observed, the dialysis is continued until the solution stops emitting the characteristic fluorescence of fluorescein (greenish-yellow or green apple color). At this point, it is advisable to determine the ratio fluorescein/protein (11). The ideal conjugate has a ratio between 10×10^{-8} and 20×10^{-8}. However, it is wise to state here that a conjugate may still be satisfactory even if it does not fall between these limits. This can only be determined by testing the conjugate with homologous and heterologous antigens. The ratio fluorescein/protein indicates only the efficiency of the conjugation. One of the greatest problems in the technique of fluorescent antibodies, particularly in virology is the non-specific coloration of tissue cells. Many procedures have been described to eliminate this problem. The first one consists in the absorption of the conjugate with tissue powder dried in acetone. According to this method, for each milliliter of conjugate 100 milligram of tissue powder prepared from monkey liver or any suitable organ is used. The powder is introduced into a plastic tube with several milliliter of PBS, and is thoroughly mixed so that the powder becomes fairly moist. Then the mixture is centrifuged in the Spinco apparatus at 15,000 revolutions per minute, for 15 minutes. The supernatant liquid is carefully taken out and the conjugate is added to the sedimented tissue; this is completely mixed and placed in the refrigerator for 1 hour, shaking it frequently. Afterwards the mixture is centrifuged in the same manner as it was done before. The supernatant liquid represents the absorbed conjugate. Maybe it is necessary to repeat this operation. When tissue culture systems are used, such as those employed in the field of virology, it has been advantageous to add 0.5 milliliter moist

compact volume of non-infected tissue culture to absorb the conjugate. Another method consists in the usage of Dowex 2-X4 (in the chloride form, mesh 20-50). According to this method, an equal volume of Dowex and conjugate are mixed in a wide-mouth container and placed in the refrigerator for an hour, where the mixture is frequently stirred. The material is then centrifuged and the supernatant liquid is taken out. It can be dialyzed with P.B.S. or may be tested as it is.

A third method of elimination of non-specific spots consists in passing the conjugate through diethylaminoethyl cellulose (D.E.A.E.). The gradient effluents, from an increasing sodium chloride molarity, are collected and tested to determine the specificity (12). It may be possible to concentrate the effluents. The usage of Carbowax constitutes a satisfactory method of concentration.(13).

Perhaps it will be necessary to combine the described methods to eliminate the non-specific coloration. This can only be determined by testing the particular system under study.

Staining Methods

1. Direct Method

In this method, the antibody which has been first conjugated with fluorescein isothiocyanate is put in direct contact with the antigen, mixing them on the surface of the slide, or — as in viriology — by direct application to the cover glass with Leighton tissue which has been dried in the air and fixed in acetone. After an adequate incubation period, the slide and the cover glass are washed with P.B.S. and afterwards with distill water. The slide is dried in the air, mounted, and examined. Stabilized glycerine (90 per cent glycerine and 10 per cent P.B.S. at pH 7.2) or Elvanol (Elvanol 51-05, Dupont de Nemours, Electrochemistry Department) may be used as mounting media. The advantage of the Evanol method (14) is that it offers a semipermanent mounting. The storage and mailing of slides must not present difficulties. A representation of this reaction is shown in Fig. 2.

In the fluorescence microscope, this labelled antigen--antibody complex has the characteristic greenish-yellow fluorescence. Of course, in the case of tissue preparations, it can only be stated that this complex represents the virus antigen, but this does not imply that it is the virus particle itself. Besides, one must be sure that this labelled antigen-antibody complex is intracellular in the tissue system studied.

In the case that coloration already exists, it is indispensable to demonstrate its specificity; this may be attained by establishing the following controls:

a) Slide with non-infected tissue culture stained with the conjugate. This must not show any coloration. If in this control a non-specific coloration is obtained, it is necessary to reabsorb the conjugate.

b) Coloration inhibition. This is achieved, by first applying non-conjugated homologous serum, and, after an adequate incubation period and adequate washing applying the conjugated homologous serum. Once this is done, there must not be any coloration, or if there is one, it must have been attenuated.

2. Indirect Method

The direct method is simpler because it consists of the interaction of two reagents, the fluorescent antibody and the antigen, with the subsequent fluorescent antibody-antigen complex formation, which proceeds in one step. If one must examine, as it occurs in any serological survey, antibodies of numerous serums, the conjugation of each one of the serums, will be expensive with respect to time as well as to materials.

This difficulty has been overcome by using a third reagent: a specific antiglobulin labelled with fluorescein. In other words, by following a modified reaction of the Coombs type, in which the labelled antibody plays a double role: 1) it acts as antibody directed against the specific virus agent and 2) it acts as antigen of the antiglobulin.

To produce the antiglobulin, globulins of normal serum of the same species, provided by the immune serum, are precipitated. This globulin is subcutaneously injected into another species, in a coadjuvant, 80 milligram per injection. Four injections in intervals of 10 to 12 days with a total of 320 milligram of protein are administered. Fourteen days after the last injection blood is taken from the animal. This is done in portions instead of at one time. The coadjuvant we use consists of creamaline (aluminum hydroxide gel Winthrop Laboratories, New York Co, N.Y.) which has been washed in several volumes of saline solution and its original volume is restored with saline solution. We accustome ourselves to test the antiglobulin serum by double diffusion in gel, the Oudin type of diffusion, before precipitation and conjugation with fluorescein isothiocyanate.

This indirect method is performed in two steps. After fixing the tissue culture, the immune homologous serum is applied, incubated and washed with P.B.S. Then the antiglobulin, labelled

with fluorescein is applied and, after a second incubation period and washings, the slide is examined to determine the specific coloration. In this case, adequate controls must also be established as follows:

a) Slide with non-infected tissue culture, stained with immune serum and with labelled antiglobulin .
b) Slide with infected tissue culture, stained with normal serum and with labelled antiglobulin.
c) Slide with non-infected tissue culture, stained with only labelled antiglobulin.
d) Slide with infected tissue culture, stained with only labelled antiglobulin.

None of these controls must show specific flourescence.

A representation of the indirect coloration is shown in Fig 3.

Frequently, particularly in systems of tissue cultures, it is difficult to eliminate the non-specific coloration of the cells of tissue cultures by the common absorption procedures. In this case rhodamine may be applied as countercolorant. It is possible to obtain serum from a healthy calf, which has subsequently been conjugated with rhodamine. We have observed that a concentration of 1:20 of serum conjugated with rhodamine in our antiglobulin conjugated with fluorescein reduces considerably the non-specific coloration.

3. Staining of Complement (11)

The staining of the complement is similar to the indirect method with exception that the antiglobulin conjugate is directed against the species which provides the complement. In summary, if we examine human serum for antibodies against a specific agent of virus or rickettsias, we will apply the immune serum plus a drop, more or less, of serum of a healthy guinea pig at a ratio of 1:10. In order that the staining be satisfactory all the elements making up the complement must be present. After an adequate incubation period and the usual washing with P.B.S., a conjugated anti-guinea pig serum is applied. The final washing and final examination are made in the form described for the other methods. It is essential, in this modification, that the system antigen-antibody studied fixes the guinea pig complement. Besides the already mentioned controls carried out in relation with the indirect method, another specificity test must be performed, that is:

Omission of the complement of guinea pig at a ratio 1:10 in the staining procedure. This test must not show specific coloration. Of course, the guinea pig serum must be tested previously

to be sure that it does not contain antibodies homologous to the system under study. A representation of the staining of the complement is shown in Fig 4.

The procedure of the staining of the complement allows one to obtain a much brighter fluorescence than the one obtained by the usual procedures. A modification of this technique, to increase fluorescence, consists in adding a drop of serum of a healthy guinea pig 1:10 in the first step of the indirect procedure.

The Use of the Fluorescent Antibody Technique in the Identification and Localization of Virus

The fluorescent antibody techniques are attempts to solve the question of localization and the fate of the antigens. Numerous investigators have studied the infection kinetics at the cellular level. Watson, using fertile eggs, studied the course of mumps virus infection and influenza, whereas Liu, in his study on the influenza virus, used ferrets. The technique of fluorescent antibodies, using systems of tissue cultures, has been applied to the study of vaccinia (15); to epidemic hemorrhagic fever; influenza (17); to Newcastle's disease (18); to Sendai virus (19); and to poliomyelitis. This is only a representative list of the virus agents studied. In the works of Coons (10) and of Cherry and others (21) a wide study will be found, not only of the virus agents studied, but also of the fluorescent antibody technique.

The localization of the antigen of the virus is divided into three main categories:

1) The cytoplasmic localization, represented by the poliomyelitic virus (20); 2) the nuclear localization, presented by the simian "foamy agent" (22); and 3) the nuclear and citoplasmic localization, as the one observed in influenza (23).

The study of the kinetics of the virus infection on a cellular level reveals that the Newcastle disease virus (18) and the poliomyelitis virus (24) are propagated by the extracellular phase, whereas the virus of herpes simplex (25), chicken pox (24) and measles (26) are propagated from cell to cell without contaminating the extracellular medium.

The sequence of the development of virus antigens has been studied, using the fluorescent antibody technique. Thus, Loh, and Riggs (15) found that in cells infected by vaccinia, the thermolabile (L) and the thermostable (E) could be demonstrated four hours after infection, followed by the appearance of the nucleoprotein antigen (N) after six hours. The hemaglutinant antigen (HA), product derived from the virus-cell interaction, can be demonstrated

only after the appearance of mature virus. A variable in this type of study is the multiplicity of infection. The variations of quantity and quality of the influenza virus injected caused differences, not only in the virus production, but also in the histochemistry of the infection (23).

Finally, the application of the methods of fluorescent antibodies to the diagnosis in the field of virology may be considered, in the case of two virus: the influenza and the poliomyelitis viruses.

Liu (27) applied the FA (fluorescent antibody) technique to the nasal washings of patients with respiratory diseases. In a study of 20 patients, positive results were obtained in 12 cases, or, 71 per cent of the cases, which were diagnosed as influenza A cases by HI tests when convalescent serum could be obtained. At a later date, in another study of 21 cases, only 38 per cent was diagnosed as influenza B, from positive serological cases.

Hatch and others (28), in a study of 85 fecal specimens for the determination of the presence of poliomyelitic virus, identified 34 of them by the FA technique, in comparison with 38 determined by the usual identification methods (neutralization). The other 47 specimens were negative with the usual methods as well as with the FA technique. It is encouraging to observe the absence of false positive reactions.

Before the fluorescent antibody technique is applied in virology as a means of diagnosis (not counting the one of the rabies), a wide evaluation of the field is necessary.

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Figures

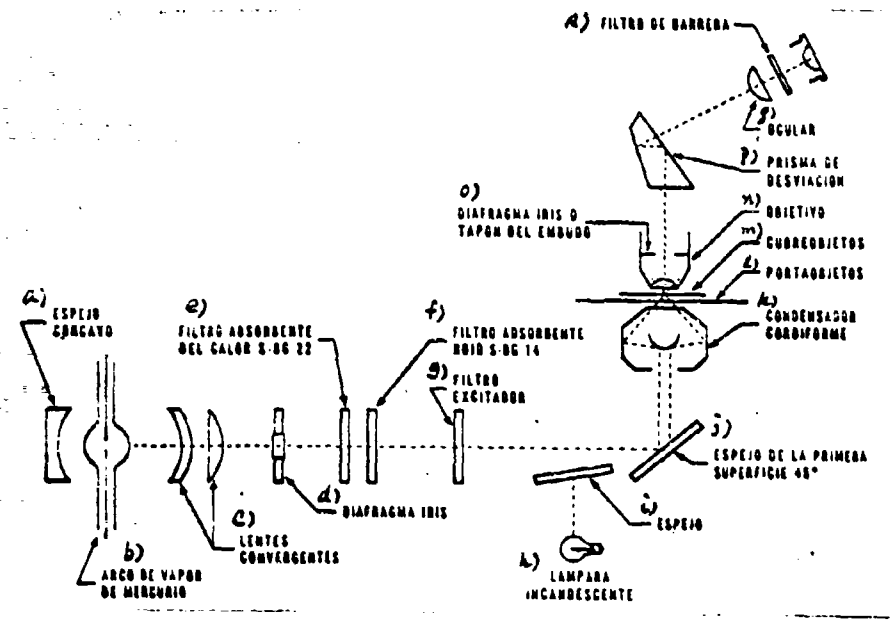

Figure 1. Schematic representation of the equipment for fluorescence microscopy.

[Legend:] a) concave mirror; b) mercury arc lamp; c) convergent lenses; d) iris diaphragm; e) filter absorbing heat S BG 22; f) filter absorbing red S BG 14; g) excitation filter; h) incandescent bulb; i) mirror; j) first surface mirror 45 ; k) "cardioid" condenser; l) slide; m) cover glass; n) objective; o) iris diaphragm or funnel stopper; p) deviation prism; q) ocular; r) barrier filter.

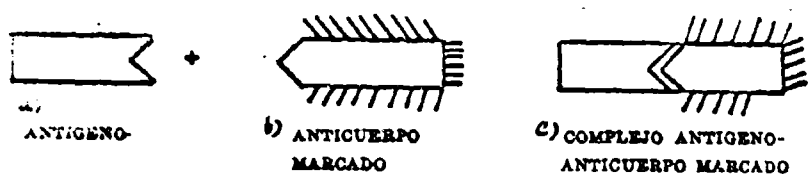

Figure 2 [Legend on following page]

[illegible] Fig. 2.[illegible]7 a) antigen; b) labelled antibody; c) labelled antigen-antibody complex.

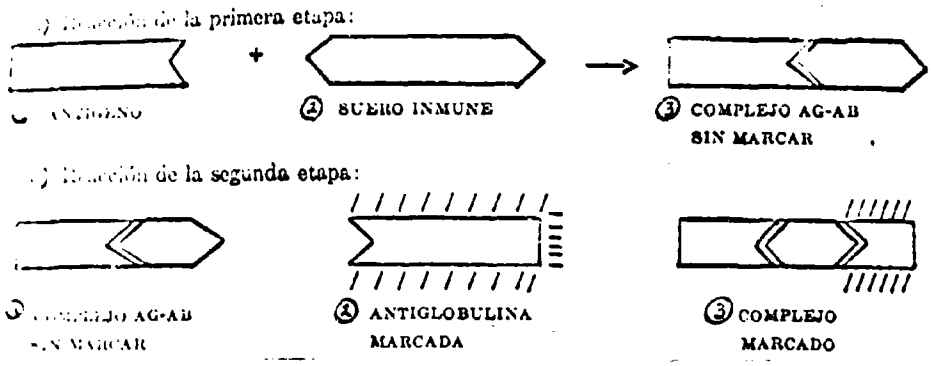

Figure 3

[illegible] Fig. 2.[illegible]7 a) First step reaction: 1) antigen; 2) immune serum; 3) AG-AB complex not labelled. b) Second step reaction: 1) AG-AB complex not labelled; 2) labelled antiglobulin; 3) labelled complex.

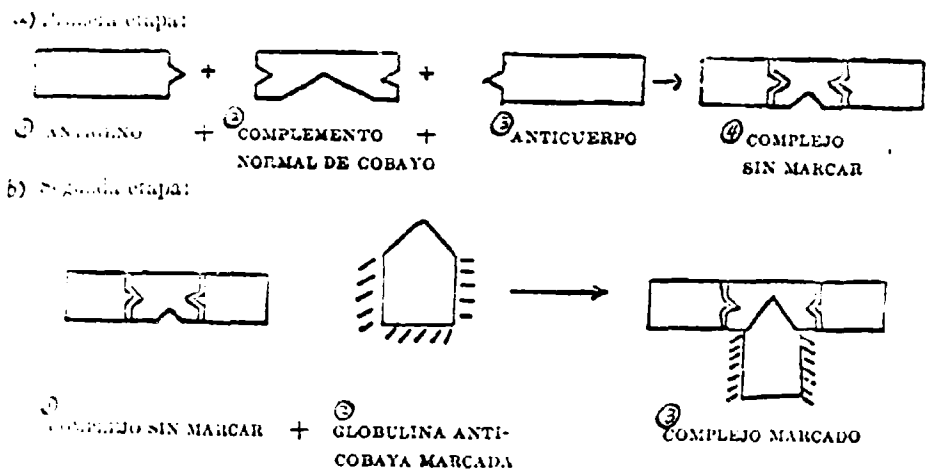

Figure 4

[illegible] Fig. 2.[illegible]7 a) First step: 1) antigen; 2) complement of a normal guinea pig; 3) antibody; 4) unlabelled complex. b) Second step: 1) unlabelled complex; 2) labelled anti-guinea globulin; 3) labelled complex.