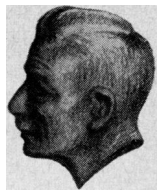
The R. E. Dyer Lecture



IMMUNOFLUORESCENCE

ALBERT H. COONS, M.D.

IT IS a high honor to be invited to deliver the R. E. Dyer Lecture. Dr. Dyer was actively studying rickettsiae when I was still a college student. Tonight he sits among us in the midst of this galaxy of institutes of which he was the first director and the initial guiding force. We are all here to do him honor and I know we are all joined in wishing him many more happy anniversaries.

Such an occasion is both a challenge and a responsibility to the lecturer, and I feel humble under its weight. Up to the present, my activities have been increasingly specialized, so that there is little choice in the subjects of which I can claim sufficient expert knowledge to justify your coming to listen. I am a musician with a single tune; when I am called upon to sing, I must hope that the audience is new because, alas, the song is not. However, the subject of immunofluorescence is not out of place in this environment. Indeed both the cellular aspects of immune reactions and the specific identification of pathogens in smears are matters not only of obvious interest but of active advance within the Public Health Service. The fact is that, although my colleagues and I had a hand in introducing fluorescent antibodies as useful immunological reagents, the largest and most active group now working with them is at the Communicable Disease Center of the Public Health Service at Atlanta, Ga.

Fluorescent antibodies, whatever their scientific merits, are very attractive under the microscope. They shine in the dark, a brilliant greenish-yellow glow. Like pebbles in the moonlight, they weave a pattern in the forest which leads the weary children home.

In the space of an evening it is not possible to describe in detail the multiplying examples of the application of these labeled antibody molecules to the many special problems of infectious disease. Rather, I propose to describe briefly what fluorescent antibodies are and then to single out examples of their use: the specific identification of a virus and a bacterium in a diagnostic situation; the study of tissue cells infected with a virus; and the synthesis of antibody in cells.

Antibody molecules are proteins synthesized by cells apparently specialized for that process, and then secreted into the circulation, where they persist for a few weeks in gradually diminishing amounts. Their half-life in man is about 13 days, and in the animal most favored by immunologists, the rabbit, about 5 days (*1*). The special property which makes them objects of intense current interest is their possession, as structural features, of two specific reactive areas, apparently concave. They are complementary to and fit more or less snugly around molecular configurations projecting from the antigen molecule which stimulated their synthesis in the first place (*2,3*). These comple-

Dr. Coons, a career investigator of the American Heart Association and visiting professor of bacteriology and immunology at Harvard Medical School, delivered this lecture December 1, 1959, at the National Institutes of Health, Public Health Service.

mentary patches account for the specific interactions between the antigen and the antibody. For reasons not understood, the patches on a given antibody molecule apparently react with the same antigen molecule, and perhaps always with the same configuration, not with a different one. Since such antibodies appear in any appreciable amount only after exposure of the cells to the antigen, the mechanism of their synthesis is a fascinating and unsolved problem. I will come back to this later.

Meantime, regardless of the details of the method by which antibodies are synthesized in such exquisite complementary form, their specificity can be harnessed as a tool. The history of immunology is largely that of the utilization of antibodies, produced by systematic injection of an antigen into animals, for various specific purposes. Making them fluorescent is simply another variation of their use as reagents for the identification of specific antigen, whether it be vaccinia virus or hen's ovalbumin.

Fluorescent Antibodies

Let me describe briefly how one can make an antibody fluorescent. Under suitably alkaline conditions (pH 9.0) aromatic isocyanates will react with the free amino groups of protein molecules to form a urea-like linkage, a reaction introduced into immunology by Hopkins and Wormall in 1933 (*4*) and later used by Creech and Jones (*5*) to couple carcinogenic hydrocarbons to carrier proteins. After an initial demonstration that a reaction between anthracene isocyanate and rabbit antibody could be carried out without serious damage to the specificity of the antibody, Creech, Jones, Berliner, and I (*6*) demonstrated that antigen could be visualized in the phagocytic cells of the mouse by means of specific antibody labeled with fluorescein.

The choice of fluorescein has proved a happy one. Fluorescein was originally chosen because of the virtual absence of green fluorescing materials from mammalian tissue, and because of the brilliance of the greenish-yellow light which fluorescein emits. In fact, the quantum efficiency of fluorescein is reported to be about 75 percent (*7*). Moreover, although we did not think of it at the time, its emission wave length of 5,200 Å is very close to the maximum of retinal sensitivity (*8*). Recently, several new compounds have been successfully tried as labeling materials in order to provide more than one color for purposes of identification: rhodamine, orange-red (*9*) and dimethylaminonaphthyl sulfonic acid, yellow (*10*, *11*). The most recent advance in labeling was the introduction by Riggs and co-workers (*12*) of the isothiocyanate instead of the isocyanate (*13*). The isothiocyanates of fluorescein and of rhodamine are stable solids which can be added to buffered dilute antibody solutions and will react without need of organic solvents. They are commercially available and have put the labeling procedure into the hands of everyone.

Such labeled antibody solutions are the simple means of merging immunology and morphology; they bridge the gap between the world of the microscope and the world of immunological specificity. As such they give the specificity of antigen-antibody reactions to the cytologist, and add the microscope to the weapons of the serologist. Under favorable circumstances an enzyme can be precisely localized to a secretion granule (*14*) and a few bacterial cells identified in a large, mixed flora (*15*). The immunologist can study some of the reactions of antigen and antibody in vivo, for example, and look to see where injected antigen is concentrated and where the resulting antibody makes its first appearance.

The actual specificity of the reactions depends of course on the quality of the antibody solution. If it contains an unknown mixture of antibodies, the observations made with it will be uncertain. What one sees of such a specific reaction depends on the fact that antibody molecules, once reacted with specific antigen, cannot be dislodged by the saline used to rinse off the excess, unreacted molecules. The reliability of observations depends on the care with which control observations are carried out, and upon the appropriateness of their selection. Although this is not the place to describe or to mourn the technical details involved in the successful use of labeled antibody for tissue localization of a chosen antigen, it would be unfair not to mention that there are difficulties, sometimes almost insurmountable, due to interactions between fluorescent protein molecules in the

antibody solution and components in the tissue section.

Identification of Virus and Bacteria

As an example of the result of a search with specific labeled antiserum for cells infected with a virus, I refer to a portion of a microscopic preparation photographed by my former colleague, Dr. Chien Liu (*16*, fig. 1). The photograph shows 10 or so cells lining the nasal cavity of a ferret. These are typical columnar epithelial cells, with one end along the basement membrane, and the other ciliated end forming the wall of the nasal cavity. The four blue-gray cells at the top show the normal fluorescence of tissue cells in frozen section; those below show yellow-green fluorescent patches where labeled antibody has reacted with antigen in the cells. In this case, the antiserum was prepared in rabbits against the A strain of influenza virus, and aggregations of viral antigen are revealed both in the cytoplasm and the nucleus of infected cells. At the time this ferret was killed, on the third day of an experimental infection, the infection in this particular spot was demonstrable in a group of cells, but not in the contiguous ones. Moreover, there is a considerable amount of cytological detail visible; heavy amounts of antigen along outer cell walls, patches in the cytoplasm, and surprising amounts of what Liu proved to be S antigen in the nuclei. Other examples of antibody deposited over collections of antigen in infected cells are mumps virus in the acinar cells of the parotid of an infected monkey (*17*), vaccinia virus in F–J cells (*18*), and herpes simplex virus in its early stages in F–J cells (*19*).

To mention two more examples, Liu (*20*) has been able to make accurate diagnoses of influenza A in three-quarters of the cases in a small series by the examination of smears from a single nasal washing each, although the results with an outbreak of influenza B were less accurate, and Goldwasser and Kissling (*21*) have demonstrated rabies virus in Negri bodies and in brains known to contain virus in which no Negri bodies could be found. They have also found rabies antigen in the salivary glands of infected dogs, foxes, and other species. There seems little question that for some virus diseases the use of labeled antibody will become the diagnostic method of choice.

An illustrative example of the diagnosis of a bacterial disease is provided by the data of Moody and Winter (*22*) for a case of experimental *Pasteurella pestis* infection in mice, where organisms were specifically identifiable in impression smears of the spleen for a few hours after infection. Many other organisms have been investigated in a preliminary way, and it is already clear that in the cases where the serologic relationships have previously been established, specific diagnoses can be made in material containing only a few organisms. However, unexpected situations arise. For example, Thomason, Cherry, and Edwards (*23*) found among Enterobacteriaceae in the intestinal tract of man and animals many organisms having serologic relationships to *Salmonella* serotypes. Smears of feces contained many reacting organisms which were not the *Salmonella typhi* sought. Moreover, Thomason and her co-workers found that normal rabbit serum often contained nonagglutinating antibodies reactive with *Escherichia coli* and with *Proteus*, thereby producing false positive reactions when the animals were subsequently used in the preparation of a specific antiserum.

Clearly, marked antibodies are of great potential interest in the diagnosis of viral (Liu), protozoal (Goldman, *24*), and bacterial (Moody) diseases because they offer the possibility of specific identification without waiting for pure cultures or large numbers of the organisms. It is, I think, equally clear that a considerable amount of developmental work will be necessary before they are useful in the practical diagnosis of a specific infection. In the course of such developmental work it is almost certain that new information concerning the distribution of antigenic determinants and the surface structure of bacteria will be uncovered, and those who are engaged in these tasks should be alert for these dividends. Moreover, it is likely that viruses masked as completely as were the adenoviruses which we carry in our tonsils will come to light as tissue is exposed to interaction with serum from various sources. The history of immunology is largely bound up with the exploitation of such circular situations, where convalescent serum reacts with the organism

isolated earlier from the patient. (For a summary of the diagnostic uses of fluorescent antibody, see Coons, reference 25).

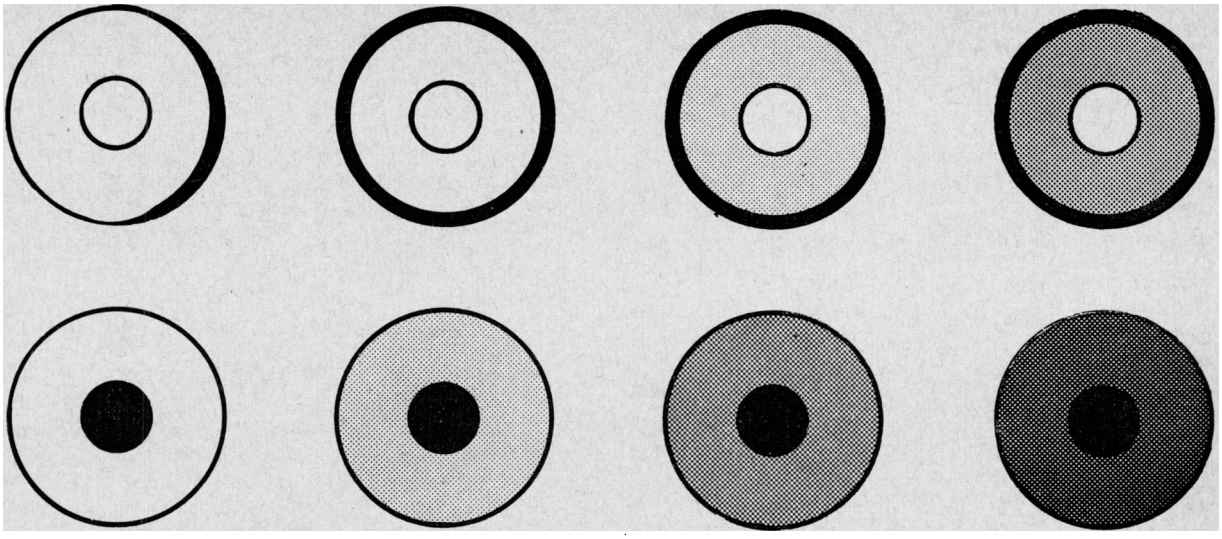
Virus-Infected Tissue

I come now to another subject because, fascinating though the problems of diagnosis are to the physician, the diagnostic bacteriologist, and the public health official, it would not be right to fill our evening with them when there are other matters of interest and concern. Also, if a speaker is unwise enough to set as his subject a method of procedure, he must be ready to follow more than one trail.

Some years ago, Dr. Barbara Watson and I (*26*) published an account of the infection of the chick embryo with the PR8 strain of influenza virus. We had examined the tissues of the embryo for viral antigen at various moments during the progression of the infection. At that time it was noticed that the first evidences of viral antigen appeared, under the conditions of the experiments, in the nuclei of the cells lining the amniotic sac into which the viral inoculum had been introduced. Later, antigenic material made its appearance throughout the infected cell. During the next year, my colleague, Dr. Liu (*16*) was able to show that the antigen demonstrable in the nuclei of epithelial cells in the infected ferret was the so-called soluble or S antigen of Hoyle (*27*). This material, which can be extracted from virus particles by ether and which contains the ribonucleoprotein of the virus has recently been found to be a rodlike structure of varying length and about 16 mμ in diameter. Antigenically, this material is known as the S (for "soluble"), or as the complement-fixing, antigen. It was shown by Hoyle to appear in infected cells before other antigenic components were detectable. The antigen is common to all strains of influenza A, which of course differ in other antigenic properties revealed by other methods of testing (hemagglutinin-inhibition). A further fact is that the S antigen of influenza A and the g or "gebundenes" antigen of fowl plague cross react powerfully, but their hemagglutinins, composed of protein and carbohydrate, do not.

Breitenfeld and Schäfer (*28*) set themselves to study the chain of events leading to the formation of completed virus particles. They employed tissue cultures of chick embryo fibroblasts infected with about 100 particles per cell of fowl plague virus. After various periods of growth, they examined the cells by means of fluorescent antibody and the yield of virus and of various antigenic components by appropriate tests. They found that the g antigen appeared in the cells before any other component was detectable, and that it was first seen 3 hours after the start of the infection. It was visible in the nucleus of the infected cells and later could also be detected in the cytoplasm. Beginning at about the fourth hour, hemagglutinin was detectable both by staining and by assay of the tissue culture cells; it was first seen in a small concentrated area near the cell nucleus, but not in it. Later, the whole cell filled with hemagglutinin, which obscures the g antigen; and filaments could be seen at the cell wall.

Watson has studied the problem of influenzal replication in the infected amniotic membrane of the chick embryo and has kindly consented to let me present a summary of her main findings. These data have not been published. Watson carried out an extensive series of experiments employing doses varying from 1,000 virus particles per cell to 1,000 cells per particle. She found that the dose has a profound effect on the appearance of viral antigens detectable inside the infected cells. When sections of the infected amnion were stained for viral antigens, it was immediately obvious that the infection was not uniformly distributed among the cells. When there was only an occasional virus particle available, antigen in the infected cells was first detectable late in the course of the infection and then usually only in and near that part of the cell membrane which faced the amniotic cavity. Only gradually, if at all, and after the appearance of infectious viral particles in the fluid, was antigenic material found deeper in the cells, and here it was usually limited to the cytoplasm. Hence, if the genetic material of the virus must enter the nucleus, as many believe, in order to initiate virus infection, the quantity is not large enough to be detectable. This condition of only an occasional virus particle per cell is of course the one found by Von Magnus to favor the production of infectious virus.



Gradual development of influenza A antigens in infected cells. Progression from left to right. Top row, infection with less than one particle per cell. Bottom row, infection with many particles per cell. (From B. K. Watson, unpublished data.)

An extreme example of the condition favoring the production of incomplete or noninfectious virus as described by Von Magnus (*29*) is that in which 1,000 particles are available per cell. In this situation Watson found that antigen, shown by absorption to be S antigen, was first visible in the nucleus and only subsequently did viral antigens spread to the cytoplasm and to a lesser extent to the cell wall. During the period when most of the cells contained antigen in the cytoplasm, noninfectious hemagglutinin appeared in the amniotic fluid.

These findings are illustrated in the diagram, which shows from left to right in the top row the usual situation when infection is initiated by small doses. The blackened areas represent viral antigen. It might be said parenthetically that only in or near the cell membrane of infected cells can identifiable virus particles be found by electron microscopy. The antigenic material deeper in the interior revealed by fluorescent antibody is evidently too close to the size of other particles normally present in the cell nucleus and the cell cytoplasm to be distinguishable. In the bottom row the reverse situation, representing infection initiated by high multiplicities of virus, is diagramed.

There is unfortunately no time to illustrate the impressive beginnings which have been made by experimental pathologists in the use of fluorescent antibody in the study of serum disease, or in the analysis of fibrin and globulin deposits in histological lesions. One curious finding has turned up in the investigation of disseminated lupus erythematosis, where the so-called L.E. factor in the serum of sufferers is found to interact with the cell nuclei of cells from many species, including fish (*30*).

Antibody Synthesis

Studies of antibody formation (*31*) have localized the site of synthesis somewhat more precisely than was possible before and have shown that the synthetic machinery is gradually established during the orderly differentiation of a specialized family of cells, plasma cells. The impression is strong that this family of cells is a specialized response to antigenic stimulation. Moreover, the marked difference in the behavior of the lymph node population during a second exposure from its relatively inapparent reaction to a first exposure is indicative of a profound change in the responsiveness of the cell population. In order to determine whether this change was progressive or whether it became stabilized, Dr. Fecsik and I (*32*) investigated the effect of prolonging the interval between the two antigenic injections on the maximum height of the secondary response in a large series of mice. We found that the responsiveness increased for 3 or 4 weeks and that thereafter it stayed at a high but fixed level for as long as half a year.

A great stimulus to investigation of the antibody response has come in the last 2 years from the introduction by Burnet (*33*), Talmage (*34*), and Lederberg (*35*) of the notion that the effect of an antigen injection is not to instruct responsive cells in the elaboration of an antibody specific to the antigen, but rather to select spontaneously appearing cells to multiply and synthesize a specific protein which by chance they are genetically capable of doing. Although some of the pictures my colleagues and I have published look like clones which might have arisen from a single cell, we have seen them spring from areas where there were at least several precursors when antibody became first distinguishable. These precursors were not at that time distributed in a clump. These observations do not rule out the possibility that such clusters are clones, nor is it necessary to postulate that antibody-forming cells spring from a single cell. There could be a number of specific mutants available. However, the appearance of these clusters must not mislead us into supposing that they must be clones.

This lecture has summarized some of the uses to which visible antibody molecules have already been put. As I predicted, the discussion has been somewhat rambling because the element uniting all these diverse findings has been a way of looking at the world rather than a unifying idea. For this reason, too, it has not been feasible to take any one of these subjects all the way to its present frontier. However, I think you will agree that immunology married to morphology has a usefulness in many areas of biology. It only remains to remember that the quality of the observations will depend on the careful analysis of the antibody solution employed as much as on the morphological knowledge which one brings to his microscope.

REFERENCES

(*1*) Dixon, F. J., Talmage, D. W., Maurer, P. H., and Deichmiller, M.: The half-life of homologous gamma globulin (antibody) in several species. J. Exper. Med. 96: 313–318 (1952).

(*2*) Pressman, D., Siegel, M., and Hall, L. A. R.: Closeness of fit of antibenzoate antibodies about haptens and the orientation of the haptens in combination. J. Am. Chem. Soc. 76: 6336–6341 (1954).

(*3*) Kabat, E. A.: Heterogeneity in extent of the combining regions of human antidextran. J. Immunol. 77: 377–385 (1956).

(*4*) Hopkins, S. J., and Wormall, A.: Phenyl isocyanate protein compounds and their immunological reactions. Biochem. J. 27: 740–753 (1933).

(*5*) Creech, H. J., and Jones, R. N.: The conjugation of horse serum albumin with 1,2–benzanthryl isocyanates. J. Am. Chem. Soc. 62: 1970–1975 (1940).

(*6*) Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E.: The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 45: 159–170 (1942).

(*7*) Umberger, J. Q., and La Mer, V. K.: The kinetics of diffusion-controlled molecular and ionic reactions in solution as determined by measurement of the quenching of fluorescence. J. Am. Chem. Soc. 67: 1099–1109 (1945).

(*8*) Granit, R.: The colour receptors of the mammalian retina. J. Neurophysiol. 8: 195–210 (1945).

(*9*) Silverstein, A. M.: Contrasting fluorescent labels for two antibodies. J. Histochem. 5: 94–95 (1957).

(*10*) Clayton, R. M.: Detection of embryonic antigens by antisera labelled with fluorescent dyes. Nature 174: 1059 (1955).

(*11*) Mayersbach, H.: Immunohistologische Methoden. II. Ein weiterer Markierungfarbstoff: Dimethyl–1–Napthyl-aminosulfonsäure–5. Acta Histochem. 5: 351–368 (1958).

(*12*) Riggs, J. C., Seiwald, R. J., Burckhalter, J., Downs, C. M., and Metcalf, T. G.: Isothiocyanate compounds as fluorescent labelling agents for immune serum. Am. J. Path. 34: 1081–1097 (1958).

(*13*) Marshall, J. D., Eveland, W. C., and Smith, C. W.: Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technic with a modification of its application. Proc. Soc. Exper. & Biol. Med. 98: 898–900 (1958).

(*14*) Marshall, J. D.: Distributions of chymotrypsinogen, procarbooxypeptidase, desoxyribonuclease, and ribonuclease in bovine pancreas. Exper. Cell Res. 6: 240–242 (1954).

(*15*) Hobson, P. M., and Mann, S. O.: Some studies on the identification of rumen bacteria with fluorescent antibodies. J. Gen. Microbiol. 16: 463–471 (1957).

(*16*) Liu, C.: Studies of influenza infection in ferrets by means of fluorescein labelled antibody. I. The pathogenesis and diagnosis of the disease. J. Exper. Med. 101: 665–676 (1955).

(*17*) Coons, A. H., Snyder, F. C., Cheever, F. S., and Murray, E. S.: Localization of antigen in

tissue cells. IV. Antigens of rickettsiae and mumps virus. J. Exper. Med. 91: 31–38 (1950).

(*18*) Noyes, W. F., and Watson, B. K.: Studies on the increase of vaccine virus in cultured human cells by means of the fluorescent antibody technique. J. Exper. Med. 102: 237–242 (1955).

(*19*) Lebrun, J.: Cellular localization of herpes simplex virus by means of fluorescent antibody. Virology 2: 496–510 (1956).

(*20*) Liu, C.: Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein labeled antibody. Proc. Soc. Exper. Biol. & Med. 91: 460–464 (1956).

(*21*) Goldwasser, R. A., and Kissling, R. E.: Fluorescent antibody staining of street and fixed rabies virus antigens. Proc. Soc. Exper. Biol. & Med. 98: 219–223 (1958).

(*22*) Moody, M. D., and Winter, C. C.: Rapid identification of *Pasteurella pestis* with fluorescent antibody. III. Staining *Pasteurella pestis* in tissue impression smears. J. Infect. Dis. 104: 288–294 (1959).

(*23*) Thomason, B. M., Cherry, W. B., and Edwards, P. R.: Staining bacterial smears with fluorescent antibody. VI. Identification of *Salmonellae* in fecal specimens. J. Bacteriol. 77: 478–486 (1959).

(*24*) Carver, R. K., and Goldman, M.: Staining *Toxoplasma gondii* with fluorescein-labeled antibody. III. The reaction in frozen and paraffin sections. Am. J. Clin. Path. 32: 159–164 (1959).

(*25*) Coons, A. H.: The diagnostic application of fluorescent antibodies. Schweiz. Ztchr. allg. Path. 22: 700–723 (1959).

(*26*) Watson, B. K., and Coons, A. H.: Studies of influenza virus infection in the chick embryo using fluorescent antibody. J. Exper. Med. 99: 419–428 (1954).

(*27*) Hoyle, L.: The structure of the influenza virus. The relation between biological activity and chemical structure of viral fractions. J. Hyg. 50: 229–245 (1952).

(*28*) Breitenfeld, P. M., and Schäfer, W.: The formation of fowl plague virus antigens in infected cells, as studied with fluorescent antibodies. Virology 4: 328–345 (1957).

(*29*) Von Magnus, P.: Incomplete forms of influenza virus. Advances in Virus Research 2: 59–79 (1954).

(*30*) Friou, G. J., Finch, S. C., and Detre, K. D.: Interaction of nuclei and globulin with lupus erythematosus serum demonstrated with fluorescent antibody. J. Immunol. 80: 324–329 (1958).

(*31*) Coons, A. H.: Some reactions of lymphoid tissues to stimulation by antigens. The Harvey Lectures, 1957–1958. New York, Academic Press, 1959.

(*32*) Fecsik, A., and Coons, A. H.: Effect of the interval between two stimuli on the height of the secondary antibody response. Science 130: 1427 (1959).

(*33*) Burnet, M.: The clonal selection theory of acquired immunity. Nashville, Tenn., Vanderbilt University Press, 1959.

(*34*) Talmage, D. W.: Immunological specificity. Science 129: 1643–1648, June 1959.

(*35*) Lederberg, J.: Genes and antibodies. Science 129: 1649–1653 (1959).

Driver Health and Traffic Mishaps Studied

A pioneer project to explore the relationship between the physical and psychological status of drivers and traffic accidents was launched in May 1960 by the Connecticut Departments of Health and of Motor Vehicles and the Public Health Service. Using a mobile health examination unit, the study is scheduled to screen between 15,000 and 25,000 selected drivers during a period of 18 months to 2 years. These include chronic violators, drivers in personal injury accidents, and some whose licenses have been suspended or revoked. Selected drivers with good records serve as controls.

Examinations are made of vision and hearing, height and weight, hemoglobin, blood pressure, and the heart and chest. Also carried out are tests for anemia, diabetes, and glaucoma.

The Connecticut State Medical Society is actively participating in followup; screening test findings are evaluated by private physicians of the examinees. The findings may also be of help in shaping legislation for physical examinations of drivers.