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# IMMUNOFLUORESCENCE, AN ANNOTATED BIBLIOGRAPHY

## VI. TECHNICAL PROCEDURES

Warren R. Sanborn

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## VI. TECHNICAL PROCEDURES

Warren R. Sanborn

December 1965

Pathology Division
DIRECTORATE OF MEDICAL RESEARCH

## FOREWORD

The use of immunofluorescence, or fluorescent antibodies, was initiated by Dr. Albert H. Coons and his co-workers in 1942. Dr. Coons has modestly stated that making antibodies fluorescent was "simply another variation of their use as reagents for the identification of specific antigen. . . ." However, this "variation" has proved to be one of immense significance to modern immunology. Its importance lies in the wedding of the two broad areas of investigation, morphology and immunology, thus allowing the detection of immunologic reactions at the cellular level.

The expanding volume of literature covering uses of immunofluorescence bears witness to the value of the technique. Through 1954 only about 40 articles had been published. In the next two years 58 articles were published. In 1957 and 1958 there were 83 and 96, respectively. By 1961 this figure had risen to more than 260 in that one year alone. Apparently more than 400 articles per year can be expected for 1964 and 1965.

It would be virtually impossible to cite every article that refers to the use of immunofluorescence, but an attempt has been made in this six-volume annotated bibliography. Fifteen languages are represented, and more than 150 journals have been searched. Six abstracting journals have been included in the search. Translations were provided by several co-workers, government translating services, and the compiler. The earliest entry is dated 1905; significant publications through 1962 are included. Subsequent entries are being compiled and will be incorporated into revisions of this bibliography. The additions will, no doubt, increase considerably the bulk of these volumes.

The bibliography is intended to aid investigators in following the expanding mass of literature on the technique and to improve their skill in its use. The entire publication, Miscellaneous Publication 3, carries the title: "Immunofluorescence, an Annotated Bibliography." The subtitles for the six volumes are: Volume I, "Bacterial Studies"; Volume II, "Viral Studies"; Volume III, "Studies of Fungi, Metazoa, Protozoa, and Rickettsiae"; Volume IV, "Studies of Animal Physiology"; Volume V, "Diagnostic Applications and Review Articles"; and Volume VI, "Technical Procedures." Each of the volumes is subdivided into subject categories that should, hopefully, aid the reader in finding pertinent information in his field of interest without his spending undue time in scanning superfluous citations. Articles within subject categories are arranged alphabetically by senior author.

Accession numbers in each volume were assigned to articles by tens to allow room for expansion in subsequent editions. Accession numbers within each volume are consecutive throughout that volume, so the volume number must accompany the accession number to identify an entry unmistakably. Entries applicable to more than one subject category appear more than once, and these will have an accession number for each placement in the volumes.

A complete author index is included in each volume; the author's name is listed with the accession numbers of the entries with which he is associated. The asterisk designates those for which he is senior author.

The second parts of Volumes V and VI contain only references to articles printed in the other four volumes. As in the other volumes, the references are placed in subject categories, and are arranged alphabetically by senior author within categories. The authors, the year of publication, and the volume and accession number are shown to indicate where the complete entry can be found.

For brevity, certain abbreviations in common usage in this field have been used rather than the more ponderous longer form. For unmistakable identification, they are listed below.

| | |
|---|---|
| DANS | a. 1-dimethylaminonaphthalene-5-sulfonic acid<br>b. 5-dimethylamino-1-naphthalene sulfonic acid or its sulfonyl chloride form. |
| FIC | fluorescein isocyanate |
| FITC | fluorescein isothiocyanate |
| FTA | fluorescent treponemal antibody |
| FTA-200 | a modification of the above based on serum dilution. |
| PAP | primary atypical pneumonia |
| PAS | para-aminosalicylic acid |
| PBS | phosphate-buffered saline |
| RB 200 | a. lissamine rhodamine RB 200<br>b. lissamine rhodamine B 200<br>c. lissamine rhodamine B<br>d. sulphorhodamine B<br>e. acid rhodamine B |
| TPFA | *Treponema pallidum* fluorescent antibody |
| TPI | *Treponema pallidum* immobilization |

Generally, the citations follow the format prescribed by the second edition of "Style Manual for Biological Journals," American Institute of Biological Sciences, 2000 P Street, N.W., Washington, D.C., 20036. Abbreviations follow "American Standard for Periodical Title Abbreviations, Z39.5-1963, American Standards Association Incorporated, New York.

The compiler started collecting this information in 1959 while he was stationed at the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland. Since his transfer to the Naval Medical Research Institute, Bethesda, Maryland, in 1963, he has continued this work with the encouragement and support of both installations.

The information in these volumes was originally recorded on coded Keysort cards. With the compilation of this publication, the citations and annotations have been transcribed on punched tape for conversion to automatic data processing and for use in updating later editions. Each entry is coded for recall by authors, date, title, and source publication to allow compilation of more selective listings.

Readers are invited to report errors or suggest added entries to the compiler or to Editorial Branch, Technical Information Division, U.S. Army Biological Laboratories, Frederick, Maryland, 21701, for improvement of the subsequent editions.

## ACKNOWLEDGMENTS

This compilation would have been much more difficult if not impossible without the guidance, help, and encouragement of:

Dr. Harold W. Batchelor, who introduced the compiler to card-sorting systems;

The staff of Naval Medical Research Institute, who provided both support and personnel for assistance in this work;

My colleagues at the U.S. Army Biological Laboratories and the Walter Reed Army Unit at Fort Detrick and at the Naval Medical Research Institute in Bethesda, who volunteered their technical competence and supplied moral support.

Obviously, outstanding cooperation and assistance of librarians was required for this work. The staff of the Technical Library, Fort Detrick, under the direction of Mr. Charles N. Bebee, was continually patient, understanding, and essential for the entire period of compilation.

Another essential in the chain to final publication: Mrs. Madeline D. Warnock and her staff in the Editorial Branch at Fort Detrick have served as editors, of course, but also as confessors, encouragers, consciences, and the required driving force, all of which have brought this to its publication. The compiler is most grateful to Mrs. Warnock and her people.

## ABSTRACT

This volume is one of a series of six annotated bibliographies on various aspects of immunofluorescence and its use. Citations cover the period 1905 through 1962. Volume VI is divided into two major sections. The first section contains 169 annotated citations to general procedures in the use of immunofluorescence, arranged by subject areas. The second section contains 226 cross-references to citations in the other volumes that describe specific applications of this technique; these are arranged to correspond with the subject areas of the other volumes. A complete author index for these 425 citations is included.

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# I. GENERAL PROCEDURES

## A. ANTISERUM PRODUCTION

10

Mueller, A.P.; Wolfe, H.R. 1961. Precipitin production following massive injection of BSA in adult chickens. Intern. Arch. Allergy 19:321-330.

Adult Arbor Acre White Rock chickens, 45 and 69 weeks of age, were given 2.61 gm BSA-KBW or roughly 65 times the immunizing dose usually administered in this laboratory, 40 mg. Nine of the chickens given this dose failed to produce precipitins following the elimination of antigen. Subsequent injection of 40 mg BSA-KBW at 59, 84, 96, 103, and 123 weeks of age showed eight of these birds to be unresponsive to BSA. Failure to produce precipitins after the challenging injection was accompanied by a lengthened antigen clearance period. Antigen was found in the circulation of the unresponsive chickens as late as 29 days following the injection. The induced unresponsiveness to BSA was specific. Injection of HGG demonstrated that chickens unresponsive to BSA produced anti-HGG in amounts comparable to control animals.

20

Pernis, B.; Paronetto, F. 1962. Adjuvant effect of silica as tridymite on antibody production. Proc. Soc. Exp. Biol. Med. 110:390-392.

Crystalline silica particles 1 to 3 microns in diameter were injected intravenously into rabbits and rats. At various intervals from 0 to 90 days the animals were immunized with ovalbumin or horse serum. Antibody production was greatly enhanced in silica-treated animals. The antibody titer was higher when the interval between silica and antigen injection was longer. Many more antibody-containing cells were observed in silica-treated animals. It is suggested that the adjuvant effect of silica is related to the proliferation of immunologically competent cells in the RES. This might play a role in the pathogenesis of silicosis.

30

Proom, H. 1942. The preparation of precipitating sera for the identification of animal species. J. Pathol. Bacteriol. 55:419-426.

Specific precipitating sera of high potency have been regularly prepared in rabbits by intramuscular injection of alum-precipitated antigen; in a group of 165 rabbits 84 per cent responded satisfactorily to one or two injections. This method has the advantage of reducing the number of injections to a minimum and so favors the production of specific sera. Attempts to prepare specific sera against heated proteins, which would be suitable for detecting the nature of cooked meats, were unsuccessful. It was possible to identify the nature of lightly cooked meats using precipitating sera prepared with unheated material.

B. CONJUGATE PREPARATION

40

Bien, T.K. 1962. A rapid method of labeling proteins with fluorescein isothiocyanate using dry Sephadex G-25. Rev. Franc. Etude Clin. Biol. 7:638-639. In French.

The use of Sephadex G-25 in dry powder form saves much time and work. Removal of excess dye by column chromatography and further concentration of the eluate are avoided.

50

Bronfenbrenner, J. 1930. A useful modification in the preparation of therapeutic sera. Proc. Soc. Exp. Biol. Med. 27:734-735.

The alteration of therapeutic sera to deprive them of their species specificity is discussed in relation to the problem of the use of such sera, particularly horse sera, in repeated treatment of individuals. Azotization, acetylation, halogenation, or coupling with carbohydrates or lipoids are methods successfully used to destroy species specificity and impart new specificities to the sera. By selecting chemical procedures of a milder nature, therapeutic value of such sera was maintained, but anaphylactic-producing properties were destroyed.

60

Bronfenbrenner, J.; Hetler, D.M.; Eagle, I.O. 1931. Modification of therapeutic sera with a view of avoiding complications of allergic nature. Science 73:455-457.

Methods to minimize allergic reaction to sera in patients are presented. By coupling immune sera with diazonium salts of para-toluidin, para-anisidine, atoxyl, sulphanilic acid, anthranilic acid, naphthionic acid, and amino R salt, a much safer immunization program was established.

70

Chase, M.W.; Slizys, I.S.; Dukes, C.D. 1959. Studies of fluorescent antibody in a particular form of immunologic sandwich. Bacteriol. Proc. M104:89.

To visualize the sites of retention of chemical groupings of allergenic chemicals in animal tissues, in vitro studies have been made on picryl groupings attached to erythrocyte stromata, picryl-specific rabbit antibody, and fluorescent antirabbit-globulin sheep antibody. Labeled antisera were tested by double diffusion precipitation to detect antibody activity loss and for ability to complete the immunologic sandwich. Coupling globulins in the cold at pH 10.5 for short periods with 3 to 6 mg of fluorescein derivatives per gram of protein, and precipitating the product by pH and salt variations, was found superior to the conditions usually employed to yield minimal antibody loss and maximal fluorescence. The relative efficiency of coupled antibody preparations was tested by using standard amounts of picrylated guinea pig stromata coated with rabbit antipicryl antibody, reading the washed reaction products macroscopically for brightness under a tubulary UV emitter, and confirming by fluorescence microscopy. Specific inhibition of the first

antibody coupling was demonstrated with picryl glycine and of the second antibody coupling with normal rabbit globulin. Relationships were demonstrable between the amounts of the two antibodies required to complete the sandwich.

80

Cohn, E.J.; Gurd, F.R.N.; Surgenor, D.M.; Barnes, B.A.; Brown, R.K.; Derouaux, G.; Gillespie, J.M.; Kahnt, F.W.; Lever, W.F.; Liu, C.H.; Mittelman, D.; Mouton, R.F.; Schmid, K.; Uroma, E. 1950. A system for the separation of the components of human blood: Quantitative procedures for the separation of the protein components of human plasma. J. Amer. Chem. Soc. 72:465-474.

The fractionation of plasma into its various components through the use of relative solubilities in inorganic salt solutions, organic solvents, and various combinations thereof is described in detail. A protein-protein and protein-metal interaction system for separation and analysis of plasma or other tissue components is proposed. Apparently, very precise separation of components with a minimum of denaturation is possible. Solubilities and other characteristics of plasma proteins are discussed in detail.

90

Coons, A.H.; Kaplan, M.H. 1950. Localization of antigen in tissue cells: II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91:1-13.

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

100

Creech, H.J.; Jones, R.N. 1940. The conjugation of horse serum albumin with 1,2-benzanthryl isocyanates. J. Amer. Chem. Soc. 62:1970-1975.

Conjugated proteins containing the 1,2-benzanthryl radical as the prosthetic group have been prepared in a condition suitable for studies of their possible carcinogenic and serological properties. Coupling was effected by interaction of the protein with the hydrocarbon isocyanate in an aqueous dioxane medium. By ultraviolet spectrophotometry, it has been possible to establish the fact that true conjugation has occurred and to determine the degree of conjugation. Purified samples of 1,2-benzanthryl-10-carbamido horse serum albumin contain approximately 12 hydrocarbon residues per molecule; the best samples of 1,2-benzanthryl-3-carbamido horse serum albumin contain 18 hydrocarbon prosthetic groups per molecule.

110

Creech, H.J.; Jones, R.N. 1941. The conjugation of horse serum albumin with isocyanates of certain polynuclear aromatic hydrocarbons. J. Amer. Chem. Soc. 63:1661-1669.

A number of conjugated proteins having various polynuclear aromatic hydrocarbons as prosthetic groups have been prepared for studies of their possible immunological and carcinogenic properties. Coupling was effected in an aqueous dioxane medium, the conjugates were purified by ammonium sulfate and acetone precipitations, and the degree of coupling was determined by ultraviolet spectrophotometry. Marked differences in the extent of conjugation with protein were observed with the various isocyanates under a given set of experimental conditions: those that were more soluble in dioxane underwent reaction with the serum albumin to the extent of introducing about 16 groups per molecule of protein, the less soluble formed conjugates containing nine to four groups per molecule. By increasing the dioxane concentration of the reaction medium, 30 and 19 hydrocarbon groups per molecule were obtained. Fractionation experiments indicated that the major portion of the conjugate contains close to the average number of prosthetic groups.

120

Creech, H.J.; Jones, R.N. 1941. Conjugates synthesized from various proteins and the isocyanates of certain aromatic polynuclear hydrocarbons. J. Amer. Chem. Soc. 63:1670-1673.

Conjugates prepared by the interaction of isocyanates of 1,2-benzanthracene and 3,4-benzpyrene with bovine serum albumin were found to possess the same hydrocarbon content as horse serum albumin conjugates prepared under identical conditions. Only a small number of hydrocarbon groups could be introduced into egg albumin and bovine and horse serum pseudoglobulins without causing marked denaturation of the protein component. Beta-anthryl isocyanate was coupled with an antipneumococcus serum to give a new type of labeled antibody. No significant degree of coupling was observed with zein.

130

Deutsch, H.F. 1952. Separation of antibody-active proteins from various animal sera by ethanol fractionation techniques, p. 284-300. In A.C. Corcoran, ed. Methods in medical research, Vol. 5. Year Book Medical Publishers, Inc., Chicago.

This chapter deals with the principles and methodology for the precipitation of globulin fractions from sera with ethanol. Detailed instructions are given for the general technique, and the peculiarities of various animal sera are pointed out.

140

Dubert, J.M.; Slizewicz, P.; Rebeyrotte, P. Macheboeuf, M. 1953. New methods of separating proteins of serum with methanol. Ann. Inst. Pasteur 84:370-375.

By the successive action of a 30 per cent concentration of methanol and of a controlled acidification, working at a temperature below 0 C, the method that we propose permits obtaining separately, from 1 or 2 ml of rabbit or horse serum, first the gamma globulins contaminated only by a little beta-2-globulin; second, the gamma globulins; and third,

the albumins containing no more than a trace of globulins. The entire operation requires less than 3 hours. Paper electrophoresis has guided us constantly in the work, and permits simple control of the results of all the fractions obtained. Results parallel those obtained by classic electrophoresis.

150

Eagle, H.; Smith, D.E.; Vickers, P. 1936. The effect of combination with diazo compounds on the immunological reactivity of antibodies. J. Exp. Med. 63:617-643.

It was possible to conjugate five diazo compounds to antisera, but continued coupling eventually destroyed the reactivity of the antisera. This inactivation occurred in stages as more diazo molecules were added. It was possible to prepare partially active antisera by halting the conjugation reaction. The various activities successively lost in various antigen-antibody systems are described. The groups in the protein that probably participate in this conjugation probably include the aliphatic amines, the imidazole ring of histidine, the indole group of tryptophane, the NH of proline, and the phenyl group of tyrosine.

160

Fieser, L.F.; Creech, H.J. 1939. The conjugation of amino acids with isocyanates of the anthracene and 1,2-benzanthracene series. J. Amer. Chem. Soc. 61:3502-3506.

Beta-anthryl-, 1,2-benzanthryl-3-, and 1,2-benzanthryl-10-isocyanate have been prepared and characterized by conversion to various carbamates and substituted ureas. The isocyanates were coupled with glycine and with aminocaproic acid to give alkali-soluble substances, some of which are being tested for carcinogenic activity. The method of conjugation in aqueous dioxane was found suitable for application to proteins.

170

Frommhagen, L.H.; Spendlove, R.S. 1962. The staining properties of human serum proteins conjugated with purified fluorescein isothiocyanate. J. Immunol. 89:124-131.

Electrophoretic analysis has shown that six preparations of fluorescein isothiocyanate, FITC, from one source contain degradation products that exhibit essentially similar fluorescence properties and a similar capacity to a couple with serum protein, but that confer upon conjugate the propensity for nonspecific staining. It is recommended that only undegraded FITC, e.g. BBL, which manifests superior specificity and higher fluorescence intensity, be used for conjugation for specific immune gamma globulins, and that the pH of the conjugation system not exceed 8.8. Purified FITC is now commercially available or may be easily obtained by continuous-flow electrophoresis. It is emphasized, however, that the degradation products of FITC constitute only one of a number of factors contributing to nonspecific fluorescent staining. Two other such factors, relating to the overlabeling of the gamma globulin and to the serum proteins, are discussed.

180

Goldwasser, R.A.; Shepard, C.C. 1958. Staining of complement and modifications of fluorescent antibody procedures. J. Immunol. 80:122-131.

A modification is described for the procedure for conjugating antisera with fluorescein, involving use of fluorescein isocyanate-impregnated filter disks. Labeling is carried out without use of organic solvents. Quality of the conjugate is determined by spectrophotometric determination of the fluorescein and protein contents. A ratio of fluorescein to protein of $3.8 \times 10^{-3}$ to $5.5 \times 10^{-3}$ indicates satisfactory conjugation, but lower ratios give less satisfactory staining. In a variation of the indirect staining procedure, guinea pig complement can be used in the staining of several antigens in combination with various antisera. Only one conjugate is needed to visualize CF antigens. The role of the various components of complement in the staining reaction was investigated and no evidence was found for the fixation of component 3. Components 1, 2, and 4 all had to be present simultaneously for full staining to occur.

190

Gornall, A.G.; Bardawill, C.J.; David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-776.

Techniques are described for performing the biuret reaction. These are simple, precise, and require a minimum amount of sample.

200

Griffin, C.W.; Carski, T.R.; Warner, G.S. 1961. Labeling procedures employing crystalline fluorescein isothiocyanate. J. Bacteriol. 82:534-537.

The effect of altering the ratio of dye to protein in the labeling of three immune globulins with crystalline fluorescein isothiocyanate was studied. Direct staining of group A streptococci and rabies-infected mouse brain, and indirect staining of Treponema pallidum, Nichols strain, were the three immunological systems selected for investigation. Maximal specific staining and minimal nonspecific background staining were demonstrated by the use of low dye-to-protein labeling ratios.

210

Heidelberger, M.; Kendall, F.E.; Soo Hoo, C.M. 1933. Quantitative studies on the precipitin reaction: Antibody production in rabbits injected with an azo protein. J. Exp. Med. 58:137-152.

A red protein azo dye preparation is described. By precipitin test the dye protein conjugate was demonstrated to be quantitatively specific.

220

Hess, R.; Pearse, A.G.E. 1959. Labeling of proteins with cellulose-reactive dyes. Nature 183:260-261.

Study of the labeling of proteins with reactive dyes was reported. Fluorescent dichlorotriazinyl dye was used. Labeling of antibodies and demonstration of antigen-antibody reactions in tissue were successful. The workers noted negligible cross-linking using dyes containing one dichlorotriazinyl group.

230

Hopkins, S.J.; Wormall, A. 1933. XCVII. Phenyl isocyanate protein compounds and their immunological properties. Biochem. J. 27:pt.1:740-753.

The preparation of phenyl isocyanate protein conjugates and their properties are described. The isocyanate compounds reacted with the free amino groups of the lysine molecules in the protein. When conjugates of one species globulin were injected into rabbits, precipitins resulted that would react with similar conjugates of other species serum proteins. The significance of the findings of loss of original species specificity is discussed. The precipitin reaction between phenylureido-proteins and their antisera is specifically inhibited by phenylureido-amino-acid compounds such as glycine, alanine, and lysine. Since inhibition is complete with lysine and not with the others, the view is supported that the characteristic conjugate grouping is phenylureido-lysine.

240

Kaufman, L.; Cherry, W.B. 1961. Technical factors affecting the preparation of fluorescent antibody reagents. J. Immunol. 87:72-79.

Interference by ammonium sulfate contamination of globulin, resulting from the serum fractionation procedure, was eliminated by dialyzing 5- to 10-ml samples of the antiglobulin in dialysis tubing 0.0625 inch in diameter for 4 hours against two liters of 0.85 per cent sodium chloride. Contamination of Brucella abortus antiglobulin by 0.08 M or greater concentrations of ammonium sulfate caused interference with the biuret estimation of the protein and its subsequent conjugation to fluorescein isothiocyanate. Conjugates prepared in the presence of graded concentrations of the salt showed proportionally greater fluorescence and greater light absorption values prior to dialysis than did conjugates prepared in the absence of this salt. The results suggested that ammonium sulfate reacted with free fluorescein isothiocyanate to form an unstable dialyzable complex that was more brilliant than the conjugate alone. Ammonium sulfate-contaminated conjugates from which salt and free dye were removed by dialysis fluoresced weakly and showed poorly staining titers compared with salt-free controls. Solutions of fluorescein isothiocyanate stored in the dark at 4 C for 70 days showed a marked reduction in fluorescence intensity. On the other hand, Brucella melitensis antiglobulin labeled with fluorescein isothiocyanate and similarly stored did not.

250

Levy, H.B.; Sober, H.A. 1960. A simple chromatographic method for preparation of gamma globulin. Proc. Soc. Exp. Biol. Med. 103:250-252.

A relatively simple method for separation of gamma globulin from serum has been presented. The procedure has been applied to a number of antisera, and has been shown, at least with them, to give reasonably good localization of antibody in the gamma globulin fraction.

260

Marshall, J.D.; Smith, C.W.; Eveland, W.C. 1958. An evaluation of various antisera conjugated with fluorescein by four methods. Bacteriol. Proc. PH15:136-137.

Two fluorescein compounds were evaluated by two separate techniques of conjugation. Antisera representing various bacterial strains, virus and fungal agents, and antiglobulin fractions of human and rabbit origin were divided into four portions. These were conjugated by the standard method of Coons and the filter paper technique of Goldman, using fluorescein isocyanate and fluorescein isothiocyanate. In all instances the fluorescein isothiocyanate-conjugated antisera yielded the more intensely stained preparation when examined by ultraviolet microscopy. Upon serial dilution of the various sera, the point of extinction of specific fluorescence was reached at lower dilution with the fluorescein isocyanate-conjugated sera. Agglutination studies, where practicable, demonstrated the least diminution of titer by the filter paper technique. The method of choice for conjugation of antisera, as indicated by this study, is the fluorescein isothiocyanate filter paper method, which eliminates the necessity of maintaining a supply of highly purified organic solvents and specialized equipment. This method of preparation might be adapted to the small laboratory.

270

Putnam, F.W. 1960. The plasma proteins: Vol. I. Isolation, characterization, and functions. Academic Press, New York. 420 p.

This is a basic reference to technical portions of the fluorescent antibody technique involved in serum fractionation. Chapters of particular importance are on precipitation methods, chromatography, gamma globulin, and antibodies.

280

Reiner, L. 1930. On the chemical alteration of purified antibody proteins. Science 72: 483-484.

Diazonium salts of well-defined chemical compounds coupled to proteins have been used in the study of the relation of biological specificity to chemical constitution. The coupled compound does not fully determine the specificity of the protein as an antigen because the original biological specificity of the protein also remains. This method presents the possibility of altering chemically with the same methods such substances as display a specific biological activity without altering the latter. The antibodies have a specific affinity toward their antigens, but they usually do not destroy themselves.

290

Rinderknecht, H. 1960. A new technique for the fluorescent labeling of proteins. Experientia 16:430-431.

A new technique for labeling proteins with fluorescent dyes is presented. The method involves use of specially dried diatomaceous earth. Conjugation time is approximately 30 minutes. Dyes used successfully were 5-dimethylaminonaphthalene sulfonylchloride, lissamine rhodamine RB 200, and fluorescein isothiocyanate.

300

Rinderknecht, H. 1962. Ultra-rapid fluorescent labeling of protein. Nature 193:167-168.

A method for labeling proteins with fluorescein isothiocyanate, lissamine rhodamine RB 200 chloride, or dimethylaminonaphthalene sulphonylchloride is described. The dyes are mixed with celite and shaken with the serum specimen, and the conjugate is purified on Sephadex. The method permits rapid labeling, removal of excess dye, and the establishment of pH, all within 30 minutes.

310

Singer, S.J.; Fothergill, J.E.; Shainoff, J.R. 1960. A general method for the isolation of antibodies. Amer. J. Chem. Soc. 82:565-571.

A simple and general method is described for the isolation of pure antibodies, particularly those directed against protein antigens. The essential features of the method are the use of a thiolated antigen to precipitate the specific antibody, and the subsequent removal of the thiolated antigen by cross-linking with a bifunctional organic mercurial. The details of the method have been thoroughly studied. Antibodies to three protein antigens, bovine serum albumin, ovalbumin, and ribonuclease, have been isolated and shown to be at least 98 per cent pure and active.

320

Smith, M.L.; Carski, T.R.; Griffin, C.W. 1962. Modification of fluorescent antibody procedures employing crystalline tetramethylrhodamine isothiocyanate. J. Bacteriol. 83: 1358-1359.

Optimal results were obtained with conjugates labeled with TMRITC at a dye-to-protein ratio of 1:40 in a staining period of 4 hours in two different fluorescent antibody systems. The optimal labeling ratio and staining time for rhodamine-labeled antibody may vary with other immunological systems.

330

Sokol, F.; Hulka, A.; Albrecht, P. 1962. Fluorescent antibody method: Conjugation of fluorescein isothiocyanate with immune gamma globulin. Folia Microbiol. 7:155-161.

The influence of several factors, such as pH, dye-to-protein ratio, concentraion of components, presence of organic solvents in the reaction mixture, temperature, and reaction

time on the efficiency of conjugation of gamma globulin with fluorescein isothiocyanate was studied. The amount of dye bound by gamma globulin increased markedly with increasing pH of the reaction mixture in the range of 7.0 to 10.0; other factors did not affect the labeling of the protein within the range examined. The specific fluorescence of the bound dye decreased appreciably with increasing labeling of the protein; thus, it seems most reasonable to use conjugates with a protein-to-dye weight ratio of about 60 in the fluorescent antibody method.

340

Vodrazka, Z. 1960. Protein interactions: XIX. Interaction of serum albumin with fluorescein dyes. Collection Czech. Chem. Commun. 25:410-419. In German.

Quinoid forms of fluorescein dyes were studied for their formation of complexes with albumin. Complex stability increased with addition of halogen to the dye. Amino groups were involved in the bond formation.

350

Weber, G. 1952. Polarization of the fluorescence of macromolecules: II. Fluorescence conjugates of ovalbumin and bovine serum albumin. Biochem. J. 51:155-167.

The preparation and characterization of 1-dimethylaminonaphthalene-5-sulphonylchloride and several sulfonamido derivatives are detailed. The preparation of conjugates between ovalbumin or bovine serum albumin and the dye is outlined. Extensive studies involving polarization, depolarization, and relaxation of rotation of the conjugates are described in relation to pH, denaturation, heat, and other factors.

360

Wolfson, W.Q.; Cohn, C.; Calvary, E.; Ichiba, F. 1948. Studies on serum proteins: V. A rapid procedure for the estimation of total protein, true albumin, total globulin, alpha globulin, beta globulin, and gamma globulin in 1.0 ml of serum. Amer. J. Clin. Pathol. 18:723-730.

Simple procedures have been described for estimating the serum proteins indicated in the title. The method is rapid and permits handling of many samples.

C. SPECIMEN PREPARATION

370

Oystese, B. 1962. Critique of the immunofluorescence method in histology. Verhandl. Deut. Ges. Pathol. 46:120-124. In German.

The author has investigated some sources of error in fluorescent antibody histochemistry, including those concerned with nonspecific staining, handling the specimen, and fixation. The best specificity of staining was obtained by the use of antisera fractionated on the DEAE-cellulose column. Cryostat sectioning, while generally very useful, may cause dislocations of the substance in question and should be supplemented by freeze-drying for control. The problem of fixation is particularly intriguing when nonbound proteins are to be demonstrated. Investigations on the secretion of albumin and gamma globulin by the human stomach reveal that the best fixation so far obtained for these substances are absolute alcohol followed by acetone for 30 minutes each at room temperature.

380

Rodriguez, J.; Deinhardt, F. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12:316-317.

A semipermanent mounting medium contains Elvanol 51-05 in phosphate-buffered saline plus glycerol. It is reported to set to a gel containing no bubbles at pH 6 to 7 and to be nonfluorescent. Fluorescence was maintained in virus specimens for longer than 9 months.

390

Zubzhitsky, Y.N. 1957. A substitute for immersion oil for fluorescent microscopy. Microbiology USSR, English Trans. 26:383.

Anisol meets the practical requirements of fluorescent microscopy and is offered as a possible substitute of the nonfluorescent immersion oil.

## D. NONSPECIFIC FLUORESCENCE

400

Allen, S.J.; Donaldson, P. 1961. A comparison of methods to eliminate nonspecific fluorescent antibody staining. Texas Rep. Biol. Med. 19:30.

Portions of fluorescein-conjugated rabbit antihuman serum globulin were treated by absorption with liver powder, by addition of 0.05 part of rhodamine-conjugated normal calf serum, by fractionation with a diethylaminoethyl-cellulose chromatographic column, and by the addition of the rhodamine-conjugated calf serum to the selected chromatographic fraction. Frozen sections were cut off the liver of a mouse sacrificed during anaphylactic shock involving human serum as antigen. These were stored 7 weeks at both 0 and 25 C, both over Drierite and in slide boxes, and in a sealed container at -20 C. These sections stained with the fluorescent antiserum portions were observed by ultraviolet illumination for specific staining of the antigen-containing Kupffer cells and nonspecific staining of the parenchyma. Sections stored at 0 C over Drierite showed minimal nonspecific staining of the parenchyma and good specific fluorescence of antigen in the Kupffer cells, especially with the chromatographic fraction alone and with this fraction combined with rhodamine-coupled calf serum. There was little specific or nonspecific staining of sections stored at 25 C. Storage at -20 C produced results similar to those of 0 C storage.

410

Beck, J.S. 1962. Investigations into the affinity of some preparations of human serum albumin for cell nuclei. Exp. Cell Res. 26:296-303.

Weak solutions of human serum albumin stored for long periods are altered so that they have an affinity for interphase nuclei and in particular for the surface of deoxyribonucleoprotein strands. The phenomenon has been reproduced by oxidation under fairly critical conditions. It is considered that the nuclear staining produced by this method indicates the presence of deoxyribonucleoprotein as a naturally occurring conjugated protein in the intact interphase nucleus.

420

Chadwick, C.S.; Nairn, R.C. 1960. Fluorescent protein tracers: The unreacted fluorescent material in fluorescein conjugates and studies of conjugates with other green fluorochromes. Immunology 3:363-370.

Protein conjugates with fluorescein isocyanate and isothiocyanate contain unreacted fluorescent material, UFM, that contributes to specific immunological staining but is troublesome because it also stains nonspecifically. Attempts have been made to find an alternative to fluorescein isocyanate and isothiocyanate conjugates, with similar green fluorescence but free from UFM and more easily prepared. Conjugates were made with fluorescein and certain derivatives of fluorescein. None proved satisfactory. At the present time, green fluorescent protein tracers are probably best prepared from fluorescein isothiocyanate. UFM accounts for nearly half of fluorescence after full dialysis and two extractions with tissue powder. Extraction with powdered, activated charcoal removes the UFM almost completely, but the staining intensity of the conjugate

is so reduced that the procedure cannot be recommended. This property of charcoal provides a means of testing fluorescein conjugations. A sample from a satisfactory conjugate should retain substantial fluorescence after such extraction.

430

deRepentigny, J.; Sonea, S. 1960. Presentation in evidence of some properties of the primary fluorescence of microorganisms and discussion of their interference in the diagnosis made by the aid of fluorescent antibodies. Can. J. Pub. Health 51:36.

Having detected a primary yellow-green fluorescence in many bacteria, we observed that this primary fluorescence could sometimes equal the fluorescence given by fluorescent antibodies. In quantitive study of the intensity of the primary fluorescence of the microorganisms we have observed a reddish fluorescence in certain species. To our knowledge this observation has not yet been reported. Such a reddish primary fluorescence could lend confusion in the case of coloration of microorganisms with antibodies coupled to a reddish fluorochrome. We have tried to measure the intensity and the colors of primary fluorescence, which could limit the value of diagnoses made with fluorescent antibodies.

440

Frommhagen, L.H.; Spendlove, R.S. 1962. The staining properties of human serum proteins conjugated with purified fluorescein isothiocyanate. J. Immunology. 89:124-131.

Electrophoretic analysis has shown that six preparations of fluorescein isothiocyanate, FITC, from one source contain degradation products that exhibit essentially similar fluorescence properties and a similar capacity to couple with serum protein, but that confer upon the conjugate the propensity for nonspecific staining. It is recommended that only undegraded FITC, e.g. BBL, which manifests superior specificity and higher fluorescence intensity, be used to conjugate specific immune gamma globulins, and that the pH of the conjugation system not exceed 8.8. Purified FITC is now commercially available or may be easily obtained by continuous-flow electrophoresis. It is emphasized, however, that the degradation products of FITC constitute only one of a number of factors contributing to nonspecific fluorescent staining. Two other such factors, relating to the overlabeling of the gamma globulin and to the serum proteins, are discussed.

450

Goldstein, G.; Slizys, I.S.; Chase, M.W. 1960. Nonspecific fluorescence of tissue treated with fluorescent globulins. Bacteriol. Proc. M166:139.

Studies were made with normal sheep globulin and sheep antirabbit globulin coupled at various ratios with fluorescein isothiocyanate. Serum at 2.5 per cent protein concentration was fractionated with ammonium sulfate, 40 per cent saturation at 4 C. Couplings were made with 7 to 50 mg per gram of protein. Certain couplings were made stepwise, with the coupling product isolated from the low-molecular-weight compounds by one-step passage for 2 to 4 hours through a Sephadex G-50 column and reconcentration to the desired endpoint by pressure dialysis under nitrogen. Substrates were imprints or tissue sections of normal guinea pigs, for nonspecific fluorescence, and of

specifically restimulated rabbits and normal rabbits. Fluorescent globulin preparations were applied to substrates both with and without tissue powder absorption and other adsorbants to determine the amount that could be applied without inducing nonspecific fluorescence and the amount capable of producing specific fluorescence. The margin of difference between the two amounts is often little when one is searching for small concentrations of antigen in the section, perhaps twofold to fourfold, and may be lessened by choice of tissue sections of different thickness.

460

Goldstein, G.; Slizys, I.S.; Chase, M.W. 1961. Studies on fluorescent antibody staining: I. Nonspecific fluorescence with fluorescein-coupled sheep antirabbit globulins. J. Exp. Med. 114:89-110.

In tissue imprints made with the spleens of antigen-stimulated animals, no morphological distinction was evident between areas showing nonspecific fluorescence and specific fluorescence. Elimination of nonspecific fluorescence was not achieved by any one, or any combination of, the following: conjugating only gamma globulin with isothiocyanate; removal of dialyzable fluorescent products on Sephadex, followed by concentration by use of pressure dialysis; and use of crystalline preparations of fluorescein isothiocyanate. The coupling ratio of 50 mg fluorescein isothiocyanate per gm of protein, as commonly advocated, cannot be recommended for the precise localization of antibody globulin in tissues because of the capacity of the coupled products to give nonspecific fluorescent staining. When crystalline preparations of fluorescein isothiocyanate are used instead of the amorphous product at 50 mg per gm of protein, far too high a nonspecific fluorescence results. A fraction with bright specific fluorescence and negligible nonspecific fluorescence was obtained from each fluorescent antibody that was prepared by using 6 to 8 mg of crystalline fluorescein isothiocyanate per gram of globulin. The fraction was subjected to DEAE-cellulose chromatography and gradient elution to eliminate the most highly coupled molecules.

470

Hall, C.T.; Hansen, P.A. 1962. Chelated azo dyes used as counterstains in the fluorescent antibody tracing technic. Zentralbl. Bakteriol. Parasitenk. Abt. I. Orig. 184:1:548-554.

A counterstaining procedure for use in conjunction with fluorescein-conjugated antisera has been developed that uses aluminum chelates of dihydroxyazo dyes. Successful counterstaining of formalin-fixed tissues and antigenically heterologous bacteria has been achieved with no apparent deleterious effects upon the immune reaction.

480

Maisel, J.C. 1962. Nucleolar reaction with normal serum shown by nonimmune fluorescent staining. J. Lab. Clin. Med. 60:357-364.

The normal sera of a wide variety of animal species react in vitro with nucleoli of the cells of homologous and heterologous animal species. Autologous cell-serum combinations also produce the nucleolar reaction. The substance in normal serum that binds to nucleoli is an alpha globulin glycoprotein that supports logarithmic

proliferation of primary monkey kidney cells in the absence of other serum proteins. The nucleolus-absorbed protein can be demonstrated, if calcium ions are present, by staining fixed preparations with fluorescein-labeled serum globulins. The latter are not specific antibodies to the nucleolus-absorbed glycoprotein. They are apparently globulins that, after fluorescein labeling and in the presence of calcium ions, have a nonspecific affinity for the nucleolus-bound serum glycoproteins.

490

Maisel, J.C.; Lytle, R.I.; Marston, J. 1962. Nucleolar reaction with normal serum shown by nonimmune fluorescent staining. Bacteriol. Proc. V52:142.

A wide species variety of normal animal sera react in vitro with cell nucleoli of homologous and heterologous animal species. Autologous cell-serum combinations also exhibit nucleolus-serum interaction. Unfixed cell culture monolayers are exposed first to normal serum, washed once, fixed in acetone, then incubated in the presence of calcium ions with fluorescein-conjugated normal serum, and again washed. The cell-serum interaction is indicated under ultraviolet illumination by nucleolar fluorescence in every cell of the monolayer. Exposure of fixed cells to serum prior to attempted fluorescent staining also produces nucleolar fluorescence. Cells not incubated with serum before or after fixation do not afford nucleolar fluorescence after incubation with fluorescein-conjugated normal serum. Neither the nucleolus-serum interaction nor adherence of conjugated normal serum to serum-treated nucleoli appears to be a specific antigen-antibody reaction. The nucleolar-reacting serum substance has the electrophoretic mobility of an alpha globulin and the chemical properties of a glycoprotein. Since this serum protein fraction is capable of supporting logarithmic growth of primary rhesus renal epithelial cells diluted in Hanks BSS in the absence of other serum protein, it appears that nucleolar interaction with this serum component may have a nutritional significance for cells cultured in vivo.

500

Mayersbach, H. 1959. Unspecific interactions between serum and tissue sections in the fluorescent antibody technic for tracing antigens in tissues. J. Histochem. Cytochem. 7:427.

The problem of nonspecific reactions encountered when using fluorescent antibody methods in tissue sections is discussed. Absorptions with organ powders to eliminate these reactions were not successful. The potency of sera subjected to repeated absorptions was lowered. The theory that common antigens and antibodies yield nonspecific fluorescence is attacked. Results indicate that nonspecific reactions are the result of serum-tissue electrostatic absorptions. Methods of reducing this electrostatic absorption without resorting to absorptions of the serum that may damage its immunologic properties are suggested.

510

Mayersbach, H.; Schubert, G. 1960. Immunohistochemical methods: III. The unspecific reaction between labeled serum and tissues in the immunohistological technique. Acta Histochem. 10:1:44-82. In German.

Thorough investigations were made into the unspecific attachment of fluorescence-marked sera to native and fixed frozen sections in the technique of immunohistology. It is not a question of immunologic serum reaction, but rather of electrostatic affinities of the marked sera to frozen section material that is similar to a staining reaction with the customary histological dyestuffs. Both the occurrence and conditions of incidence, and the possibilities of prevention of these unspecific reactions, are dealt with at length.

520

Smith, C.W.; Marshall, J.D., Jr.; Eveland, W.C. 1959. The use of contrasting fluorescent dye as counterstain in fixed tissue preparations. Proc. Soc. Exp. Biol. Med. 102: 179-181.

A counterstaining method is described that gives a contrasting reddish-orange background when used with fluorescein-labeled antibody systems. It curtails nonspecific fluorescence in tissues and tissue cultures. The possibility of a nonspecific protein reaction is discussed. This reaction apparently plays no part in the serological system to which it has been added.

530

Thivolet, J.; Cherby-Grospiron, D. 1961. Studies on nonspecificity factors in immunofluorescence reactions. Ann. Inst. Pasteur 101:869-875. In French.

The authors studied certain nonspecificity factors in the immunofluorescence reaction applied to the diagnosis of syphilis. They demonstrated the part played by a testicular extract and antitesticular serum, obtaining the elimination of the serum factor by adsorption on testicular powder. They suggest a routine technique that apparently yields specific results.

E. LABELING COMPOUND

540

Bogert, M.T.; Wright, R.G. 1905. Some experiments on the nitro derivatives of fluorescein. J. Amer. Chem. Soc. 27:1310-1316.

The nitro and amino derivatives of fluorescein were investigated. The influence of substituent groups on fluorescence weakening is usually directly proportional to the molecular weight of the group. The directions for preparing the various nitrofluoresceins are given. Reductions were attempted on the following nitrofluoresceins: 4-nitro-; 3-nitro-; dinitro-; penta- or tetra-nitro. Directions are given and also comments on difficulty and product purity.

550

Borek, F.; Silverstein, A.M. 1960. A new fluorescent label for antibody proteins. Arch. Biochem. Biophys. 87:293-297.

A new orange-fluorescent dye, aminorosamine B, was prepared by condensation of two moles of m-diethylaminophenol with one mole of p-nitrobenzaldehyde, followed by catalytic reduction of the nitro group. This dye was found to be useful as a label for antibody proteins, with which it may be conjugated as an isocyanate, isothiocyanate, or diazonium derivative. The optimal dye-protein ratio was determined corresponding to a maximum intensity of staining with a minimum decrease in antibody activity. A diazorosamine B antibody conjugate was found useful in staining mixed bacterial smears, in combination with a fluorescein antibody conjugate.

560

Chadwick, C.S.; McEntegart, M.G.; Nairn, R.C. 1958. Fluorescent protein tracers: A trial of new fluorochromes and the development of an alternative to fluorescein. Immunology 1:315-327.

The following fluorochromes were examined as possible alternatives to fluorescein: aminoeosin, aminorhodamine B, 3-phenyl-7-isocyanatocumarin, 5-carboxyethylaminoacridine, R 4388, fluolite C, lissamine flavine FFS, lissamine rhodamine GS, and lissamine rhodamine RB 200. Only the last was satisfactory. RB 200 is a successful alternative to fluorescein. Conjugation of dye to protein by a sulphonamido linkage is quick, simple, and does not materially affect the physico-chemical or biological properties of the protein. Conjugates are stable, have a brilliant orange fluorescence in UV light, and good contrast with tissue autofluorescence. Contrast permits use in microscopy of ultraviolet plus blue light with a yellow filter above the object to ensure a black background. Conjugates injected intravenously are distributed in the tissues and eliminated from plasma in much the same way as conjugates of fluorescein or radioactive isotopes. Antibody conjugated with RB 200 retains immunological specificity. Use has been made of RB 200 conjugates as plasma tracers and as specific immunological stains, alone and in combination with fluorescein conjugates in double-tracing experiments.

570

Chadwick, C.S.; McEntegart, M.G.; Nairn, R.C. 1958. Fluorescent protein tracers: A simple alternative to fluorescein. Lancet 1:412-414.

Serum protein conjugates with RB 200 are as useful as fluorescein conjugates for direct and immunological tracing. They have the advantage that their preparation is simple, requiring no special laboratory facilities, and their color contrasts well with tissue autofluorescence. RB 200 conjugate is sufficiently different from the apple-green of fluorescein conjugates for them to be used together in double-tracer experiments; it may be applied either as simple plasma labels or as immunological stains.

580

Colobert, L.; Demont, G.; Domanski, B. 1959. Preparation of fluorescein isocyanate for fluorescent microscopy. Compt. Rend. Soc. Biol. 153:1029-1031. In French.

Modifications on the Coons method are presented.

590

Coons, A.H.; Kaplan, M.H. 1950. Localization of antigen in tissue cells: II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91:1-13.

Improvements in a method for the specific microscopic localizatio of antigen in tissue cells are described. This method employs antibody labeled with fluorescein isocyanate as a histochemical stain; the specific antigen-antibody precipitate is made visible under the fluorescence microscope. Two isomeric series derived from nitrofluorescein are described.

600

Corey, H.S., Jr.; McKinney, R.M. 1962. Chromatography of nitrofluoresceins, aminofluoresceins, and fluorescein isothiocyanates. Anal. Biochem. 4:57-68.

Paper chromatography was used in a study of FITC isomers I and II. The amine and isomer I gave superior fluorescence per microgram. The chromatographic methods developed could lead to more uniform commercial products. Most commercial samples contained multiple components.

610

deRepentigny, J.; James, A.T. 1954. A chromatographic separation of the aminofluorescein isomers. Nature 174:927-928.

In the preparation of aminofluorescein by the condensation of 4-nitrophthalic acid and resorcinol followed by reduction, two isomers are formed at the condensation stage, and these must be separated if unequivocal results are to be obtained in the subsequent reaction of the derived fluorescein isocyanate with proteins to give fluorescent protein conjugates. The conventional method of separation of the two isomers, consisting

of fractional crystallization of the nitrofluorescein diacetates, is laborious and wasteful; to obviate this we have devised a chromatographic separation of the two isomers.

620

Dowdle, W.R.; Hansen, P.A. 1959. Labeling of antibodies with fluorescent azo dyes. J. Bacteriol. 77:669-670.

The labels available for the fluorescent antibody technique have so far been limited to the isocyanates of fluorescein and the related compounds rhodamine B and tetramethylrhodamine or to some of the corresponding isothiocyanates. Chelates of the azo dyes were used successfully and produced brilliant fluorescence.

630

Emmart, E.W. 1958. Observations on the absorption spectra of fluorescein, fluorescein derivatives, and conjugates. Arch. Biochem. Biophys. 73:1-8.

Rapid loss in the characteristic maximum at 490 mu occurs in solutions of fluorescein below pH 7.0, with elimination of fluorescence at pH 2.0. A new absorption band appears in these solutions below pH 5.0 with a maximum at 430 mu at pH 2.0. On the basis of samples of fluorescein amine II prepared in different laboratories, but having a melting point of 313 C, infrared absorption spectra have been obtained that serve as a standard for the preparation of this amine. The ultraviolet absorption spectrum has been shown of fluorescein amine isomer II in dioxane-acetone solution of the change in spectrum that occurs after conversion by phosgene to fluorescein isocyanate. The ultraviolet absorption spectrum of the fluorescein nucleus in aqueous-buffered solution, pH 7.6, showing a maximal band at 490 mu and two minor bands at 280 and 325 mu, has been obtained. The changes in the intensity of these bands after conjugation to rabbit globulin have been shown. The emission spectra solutions of fluorescein and fluorescein globulin conjugates have a longer wavelength than their absorption spectra, with an optimum emission band at 550 [illegible].

640

Felton, L.C.; McMillion, C.R. 1961. Chromatographically pure fluorescein and tetramethylrhodamine isothiocyanates. Anal. Biochem. 2:178-180.

Paper chromatography is a significant criterion of purity for the isothiocyanates studied, demonstrating isomeric separation, decomposition, and byproducts. These compounds should be protected from moisture to prolong shelf life.

650

Frommhagen, L.H.; Spendlove, R.S. 1962. The staining properties of human serum proteins conjugated with purified fluorescein isothiocyanate. J. Immunol. 89:124-131.

Electrophoretic analysis has shown that six preparations of fluorescein isothiocyanate, FITC, from one source contain degradation products that exhibit essentially similar fluorescence properties and a similar capacity to couple with serum protein, but that

confer upon the conjugate the propensity for nonspecific staining. It is recommended that only undegraded FITC, e.g. BBL, which manifests superior specificity and higher fluorescence intensity, be used to conjugate specific immune gamma globulins, and that the pH of the conjugation system not exceed 8.8. Purified FITC is now commercially available or may be easily obtained by continuous-flow electrophoresis. It is emphasized, however, that the degradation products of FITC constitute only one of a number of factors contributing to nonspecific fluorescent staining. Two other such factors, relating to the overlabeling of the gamma globulin and to the serum proteins, are discussed.

660

Goldman, M.; Carver, R.K. 1957. Preserving fluorescein isocyanate for simplified preparation of fluorescent antibody. Science 126:839-840.

The advantages to be obtained from the use of dried isocyanate in the manner described above are that the isocyanate can be prepared centrally in either commercial or noncommercial laboratories and can be sent out to smaller research or diagnostic laboratories for actual use. Also, an antiserum can be made to stain more intensely by relabeling, without danger of loss of protein content as a result of denaturation.

670

Hamashima, Y. 1955. Synthesis of aminofluorescein. Acta Sch. Med. Univ. Kioto 32:112-122.

The best method for the synthesis of 4-nitrophthalic acid is as follows: Phthalic anhydride to phthalimide to 4-nitrophthalimide to 4-nitrophthalic acid by saponification. Optimum pH for the conjugation of fluorescein to protein was 7.8. Complete exclusion of water was necessary for conversion of amino compound to isocyanate.

680

Hess, R.; Pearse, A.G.E. 1959. Labelling of proteins with cellulose-reactive dyes. Nature 183:260-261.

Study of the labeling of proteins with reactive dyes was reported. Fluorescent dichlorotriazinyl dye was used. Labeling of antibodies and demonstration antigen-antibody reactions in tissue were successful. The workers noted negligible cross-linking with dyes containing one dichlorotriazinyl group.

690

Hiramoto, R.; Engle, K.; Pressman, D. 1958. Tetramethylrhodamine as immunohistochemical fluorescent label in the study of chronic thyroiditis. Proc. Soc. Exp. Biol. Med. 97:611-615.

A new fluorescent label, tetramethylrhodamine isocyanate, is described and its use demonstrated in the immunohistochemical fluorescence technique. The label fluoresces orange and is of particular value for staining tissues that show a green autofluorescence similar to fluorescein, even when unstained. Serum from a patient with chronic

thyroiditis combined with normal human serum showed no such staining. Thyroid from a patient with chronic thyroiditis, infiltrated with lymphocytic cells, was stained by rabbit antibody to human globulin in the region occupied by the infiltrating cells. Normal lymph nodes showed no such staining. This indicates that the infiltrating cells contained human globulin and may have been important in the destruction of the thyroid during the course of the disease.

700

Marshall, J.D.; Eveland, W.C.; Smith, C.W. 1958. Superiority of fluorescein isothiocyanate for fluorescent antibody technic with a modification of its application. Proc. Soc. Exp. Biol. Med. 98:898-900.

Globulin fractions of antisera representing bacterial, viral, and mycotic agents and antiglobulin fractions were labelled with two derivatives of fluorescein amine by three methods. Fluorescein isothiocyanate was superior in stability, ease of conjugation, and degree of fluorescence. This direct method of adding the dye to a dilute, buffered antiserum eliminates the need for organic reagents that may denature protein.

710

Marshall, J.D.; Smith, C.W.; Eveland, W.C. 1958. An evaluation of various antisera conjugated with fluorescein by four methods. Bacteriol. Proc. PH15:136-137.

Two fluorescein compounds were evaluated by two separate techniques of conjugation. Antisera representing various bacterial strains, virus and fungal agents, and antiglobulin fractions of human and rabbit origin were divided into four portions. These were conjugated by the standard method of Coons and the filter paper technique of Goldman, using fluorescein isocyanate and fluorescein isothiocyanate. In all instances the fluorescein isothiocyanate-conjugated antisera yielded the more intensely stained preparation when examined by ultraviolet microscopy. Upon serial dilution of the various sera, the point of extinction of specific fluorescence was reached at lower dilution with the fluorescein isocyanate-conjugated sera. Agglutination studies, where practicable, demonstrated the least diminution of titer by the filter paper technique. The method of choice for conjugation of antisera, as indicated by this study, is the fluorescein isothiocyanate filter paper method, which eliminates the necessity of maintaining a supply of highly purified organic solvents and specialized equipment. This method of preparation might be adapted to the small laboratory.

720

Mayersbach, H. 1958. Immunohistological methods: II. Further particulars on the dye: Dimethyl-1-aminonaphthalene-5-sulfonyl chloride. Acta Histochem. 5:5-8:351-368. In German.

An investigation was made of 1-dimethylaminonaphthalene-5-sulfonic acid, the sulfonyl chloride of which can be conjugated to protein by a sulfonamidic linkage. Immune conjugates of this dye are satisfactory replacements for conjugates of fluorescein isocyanate for immunohistological investigations. These conjugates are also suitable for injection tests. Dye preparation, protein conjugation, and conjugate properties are discussed at length.

730

Orndorff, W.R.; Hemmer, A.J. 1927. Fluorescein and some of its derivatives. J. Amer. Chem. Soc. 49:1272-1280.

Purified fluorescein has been prepared in two isomeric forms, a red quinoid form and a yellow lactoid form. No other forms were obtained. Different yellow hydrochlorides may be prepared from the two forms. Derivatives of the lactoid form are diacetate, bisphenyl carbonate, hydrazide, eosin, and dibromofluorescein dibenzoate. Derivatives of the quinoid form are mono-ammonium suet, methylester, and acetate.

740

Peck, R.M.; Creech, H.J. 1952. Isocyanates of dimethylaminostilbenes and acetylaminofluorene. J. Amer. Chem. Soc. 74:468-470.

The synthesis of isocyanates of certain systemic carcinogens represents the initial step in an extension of a research program involving immunochemical studies of carcinogen-protein conjugates. 4-Dimethylaminostilbenyl-4-isocyanate was prepared in 61 per cent yield by the interaction of phosgene with 4-dimethylamino-4-aminostilbene. 4-Dimethylamino-4-amino-2-methylstilbene, which had been prepared in 36 per cent yield by a two-step process from 4-nitro-o-xylene and p-dimethylaminobenzaldehyde, was converted into the corresponding isocyanate in 79 per cent yield. 2-Acetylaminofluorenyl-7-isocyanate was obtained from the amine in 63 per cent yield. The isocyanates were characterized by conversion into their ethyl urethanes.

750

Redetzki, H.M. 1958. Labeling of antibodies by 5-dimethylamino-1-naphthalene sulfonyl chloride, its effect on antigen-antibody reactions. Proc. Soc. Exp. Biol. Med. 98:120-122.

Properties of the fluorescent dye DNS for labeling proteins have been described. Investigations of conditions of DNS binding to serum proteins revealed ratios of 3 to 4 moles of DNS per mole of gamma globulin and about 7 moles of DNS per mole of albumin. Dealing with the use of DNS in immunochemistry, experiments have been carried out with the ADH-rabbit antiserum system, measuring enzyme inhibition by the antibody. Labeling of antibody with DNS did not affect rate of formation of antigen-antibody complex and had no influence on quantity of formed precipitate. The reported data suggest that because DNS does not affect the antigen-antibody reaction, it can be successfully applied in histochemical studies.

760

Riggs, J.L. 1957. Synthesis of fluorescent compounds and their use for labelling antibody. Master's Thesis, Univ. Kansas, Lawrence, Kansas.

Complete thesis.

770

Seiwald, R.J.; Riggs, J.L.; Burckhalter, J.H.; Downs, C.M.; Metcalf, T.G. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Bacteriol. Proc. PH14:136.

The instability of fluorescein isocyanate and the difficulty of production led to efforts to produce a more stable product to be used in the labeling procedure. This report deals with the synthesis of stable, solid isothiocyanates of fluorescein and rhodamine B, and the use of these two fluorescent compounds in labeling antibodies for the specific staining of antigens. The dyes have been used for both the direct and indirect methods of staining as given by Coons, and have shown satisfactory fluorescence when labeled antibody is applied to bacterial smears, bacteria in tissues, and to bacteria, rickettsiae, and viruses in cultured cells.

780

Sokol, F.; Hana, L.; Albrecht, P. 1961. Fluorescent antibody method: Quantitative determination of 1-dimethylaminonaphthalene-5-sulphonic acid and protein in labeled gamma globulin. Folia Microbiol. 6:145-150.

A spectrophotometric method for the quantitative determination of protein and 1-dimethylaminonaphthalene-5-sulphonic acid in fluorescent antibodies was elaborated. After exhaustive dialysis the conjugates do not contain free dye that would disturb the analysis. According to the conditions used during conjugation, the average weight ratio of protein to fluorescent dye in the labeled gamma globulin was 76 to 245, corresponding to 3 to 9 molecules of dye bound to one molecule of globulin.

790

Uehleke, H. 1958. The labeling of proteins with fluorescent stains. Naturwissenschaften 45:4:87. In German.

The preparation and use of sulfonated fluorescent dyes for conjugation of antibodies as substitutes for the unstable isocyanate are discussed. Methods for determining the stability of the conjugates by electrophoresis and problems regarding autofluorescence of tissues are included in the paper.

800

Uehleke, H. 1958. New techniques for preparing fluorescent-labeled proteins. Z. Naturforsch 13b:11:722-724. In German.

Various sulfo group dyes were used to label protein, and the products were studied electrophoretically and spectrophotometrically. Detailed instructions are presented for labeling. Conjugate comparisons were made.

810

Uehleke, H. 1959. Investigations with fluorescent antibody: IV. Labelling of antibodies with sulfonyl chlorides of fluorescent dyes. Schweiz. Z. Allg. Pathol. Bakteriol. 22:5:724-729. In German.

An extensive review of fluorescent dyes and their mechanisms of protein conjugation is given, with particular emphasis on the sulfochloride forms. Problems involved in synthesis, conjugation with proteins, and nonspecific staining are included. The two dyes recommended for use are 1-dimethylnaphthalene-5-sulfonic acid and sulforhodamine B.

820

Vasser, P.S.; Culling, C.F. 1959. Fluorescent stains, with special reference to amyloid and connective tissues. AMA Arch. Pathol. 68:487-498.

This paper presents an account of various experiments to assess the value of simple fluorescent staining techniques, using a small ultraviolet light source. The characteristics of various fluorochromes are presented.

830

Weber, G. 1952. Polarization of the fluorescence of macromolecules: II. Fluorescence conjugates of ovalbumin and bovine serum albumin. Biochem. J. 51:155-167.

Details in the preparation and characterization of 1-dimethylaminonaphthalene-5-sulphonyl chloride and several sulfonamido derivatives are given. The preparation of conjugates between ovalbumin or bovine serum albumin and the dye are outlined. Extensive studies involving polarization, depolarization, and relaxation of rotation of the conjugates are described in relation to pH, denaturation, heat, and other factors.

F. STAINING PROCEDURES

840

Coons, A.H.; Kaplan, M.H. 1950. Localization of antigen in tissue cells: II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91:1-13.

Improvements in a method for the specific microscopic localization of antigen in tissue cells are described. This method employs antibody labeled with fluorescein isocyanate as a histochemical stain; the specific antigen-antibody precipitate is made visible under the fluorescence microscope. Two isomeric series derived from nitrofluorescein are described.

850

Hall, C.T.; Hansen, P.A. 1962. Chelated azo dyes used as counterstains in the fluorescent antibody tracing technic. Zentralbl. Bakteriol. Parasitenk. Abt. I. Orig. 184:1: 548-554.

A counterstaining procedure for use in conjunction with fluorescein-conjugated antisera has been developed using aluminum chelates of dihydroxyazo dyes. Successful counterstaining of formalin-fixed tissues and antigenically heterologous bacteria has been achieved with no apparent deleterious effects upon the immune reaction.

860

Hirschberg, N. 1962. Simplified method for staining smears for fluorescent antibody. J. Bacteriol. 84:1126.

In many laboratories, the staining of large numbers of slides for fluorescent antibody studies has become a time-consuming procedure. The use of staining jars, regardless of size, involves picking up and transferring the slides several times, and, in the indirect procedure, doubles the time for the performance of the test. The use of aluminum slide trays like those used to store histological sections eliminates the transfer of the slides. The entire fixing and staining procedure can be carried out in the same tray. Multiple holes can be punched in the back of the trays to allow the liquids held by capillary action under the slides to be removed by blotting on absorbent paper or towel. The slides should be set or fastened on applicator sticks or wire laid vertically in the tray. The trays are covered with an oblong aluminum cooking pan with a wet filter-paper insert, for incubation. Slightly larger pans are used for fixing and washing. The method requires the use of somewhat more solution than does the use of staining jars. This is relatively inexpensive because the cost is more than offset by the saving of time. When the slides are ready for mounting, the bottoms must be wiped on a gauze or cloth pad. Slides stained by this method have been shown to be well washed and free of residue.

870

Ishida, N.; Hinuma, Y.; Miyamoto, T. 1962. Investigations of FA technique: II. Emphasis on the complement staining method. Jap. J. Clin. Pathol. 10:60-66. In Japanese.

The FA complement staining method is completely explained.

880

Marshall, J.D.; Eveland, W.C.; Smith, C.W. 1958. Superiority of fluorescein isothiocyanate for fluorescent antibody technic with a modification of its application. Proc. Soc. Exp. Biol. Med. 98:898-900.

Globulin fractions of antisera representing bacterial, viral, and mycotic agents and antiglobulin fractions were labeled with two derivatives of fluorescein amine by three methods. Fluorescein isothiocyanate was superior in stability, ease of conjugation, and degree of fluorescence. This direct method of adding the dye to a dilute buffered antiserum eliminates the need for organic reagents that may denature protein.

890

Mayersbach, H. 1959. Unspecific interactions between serum and tissue sections in the fluorescent antibody technic for tracing antigens in tissues. J. Histochem. Cytochem. 7:427.

The problem of nonspecific reactions encountered when using fluorescent antibody methods in tissue sections is discussed. Absorptions with organ powders to eliminate these reactions were not successful. The potency of sera subjected to repeated absorptions was lowered. The theory that common antigens and antibodies yield nonspecific fluorescence is attacked. Results indicate that nonspecific reactions are the result of serum-tissue electrostatic absorptions. Methods of reducing this electrostatic absorption without resorting to absorptions of the serum that may damage its immunologic properties are suggested.

900

Silverstein, A.M. 1957. Contrasting fluorescent labels for two antibodies. J. Histochem. Cytochem. 5:94-95.

The development by Coons of techniques employing antibodies labeled with fluorescein led to significant advances in the histological tracing of antigenic substances. The technique has been extended to studies on the site and mode of production of antibodies. Fluorescent labeling may serve as a specific histochemical stain for antigen or for antibody. A second fluorescent label may be useful. Requirements are indicated.

910

Silverstein, A.M.; Berrigan, P.J. 1957. Applications of contrasting fluorescent labels for two antibodies. Federation Proc. 16:1860:433.

A second fluorescent label for antibodies is needed for use in conjunction with the yellow-green fluorescein label. This label should have a contrasting color, so that each label can be visualized separately when the two are used in conjunction in the same field of view, or in the same cell. A method for specific staining of two antigenic components simultaneously might have several applications: It would simplify the establishment of a schema for the rapid identification of microorganisms, and would allow a study of the distribution and effects of two antigenic components in vivo, and of the antibody response evoked by the several antigens. Preliminary studies have indicated that an amino derivative of rhodamine B might satisfy these requirements. This fluorochrome emits a bright orange fluorescence in ultraviolet light, in marked contrast to the yellow-green of fluorescein. It is conjugated to antibody in a manner analogous to that of fluorescein.

920

Smith, C.W.; Marshall, J.D., Jr.; Eveland, W.C. 1959. Use of contrasting fluorescent dye as counterstain in fixed tissue preparations. Proc. Soc. Exp. Biol. Med. 102:179-181.

A counterstaining method is described that gives a contrasting reddish-orange background when used with fluorescein-labeled antibody systems. It curtails nonspecific fluorescence in tissues and tissue cultures. The possibility of a nonspecific protein reaction is discussed. This reaction apparently plays no part in the serological system to which it has been added.

G. TISSUE CULTURE

930

Chen, H.Y. 1962. Studies on the staining reaction of the fluorescent conjugate of globulin. Shih Yen Sheng Wu Hseuh Pao 7:283-298. In Chinese.

A review of past work is first presented. Then staining of sections of various tissues and tissue cultures are studied with respect to staining intensity under varying conditions such as pH, ionic strength of diluents, fixation methods, and effects of normal versus tumorous tissues. An interesting point was the use of pH 12.5, which gave very intense staining and good differentiation between the nucleus and the cytoplasm of cells. The dyes DANS and RB 200 were used for conjugation.

940

Hiramoto, R.; Goldstein, M.N.; Pressman, D. 1958. Reactions of antisera prepared against HeLa cells and normal fetal liver cells with adult human tissues. Cancer Res. 18:668-669.

The reactions of antibodies prepared in rabbits against tissue culture cells of human origin, i.e., HeLa and fetal liver, with human thyroid, liver, kidney, and adrenal were studied by the immunohistochemical staining technique involving fluorescein as a label. Both sera acted similarly and stained stromal elements, i.e., reticular tissues, basement membranes, and sinusoidal linings. Thus, the tissue-cultured cells of both lines contained components in common with or closely related to those of stromal elements of normal tissue.

950

Hiramoto, R.; Goldstein, M.N.; Pressman, D. 1960. Limited fixation of antibody by viable cells. J. Nat. Cancer Inst. 24:255-265.

The indirect fluorescent antibody procedure was used to study the reaction of antibody, active against human connective tissue, with living and dead HeLa cells. These antibodies were fixed only at the cell surface of viable HeLa cells and were able to react with intracellular cytoplasmic components only in killed cells. Antisera to human fibrinogen and human globulin did not give this type of fixation. Components taken up by the cell from the culture medium were demonstrable in the cytoplasm of killed cells.

960

Hiramoto, R.; Jurandowski, J.; Bernecky, J.; Pressman, D. 1961. Immunohistochemical identification of tissue culture cells. Proc. Soc. Exp. Biol. Med. 108:347-353.

Cell lines of different species can be differentiated by immunohistochemical techniques using properly absorbed antisera. In the work reported here the antisera were prepared against human and mouse tissues and reacted strongly with the connective tissue of the corresponding species in sections of adult human or adult mouse organs, respectively. These antisera also reacted individually with tissue culture cells of human

and mouse organs. In all cases the use of the proper tissue in sufficient amounts to absorb the antisera plays a crucial part in being able to demonstrate the specific staining of cells. Differentiation is possible even within the cell lines of a single species when unique components are present in one of them. The use of anti-myosin serum to detect muscle cells is an example. The technique of using paired labeled antiserum quickly and effectively demonstrates that very slight contamination can be recognized. Such a small contamination would be difficult to detect by other tests such as chromosome morphology, in which the cell must be in the metaphase before it can be observed and chromosomes of each cell must be counted.

970

Holter, H.; Holtzer, H. 1959. Pinocytotic uptake of fluorescein-labeled proteins by various tissue cells. Exp. Cell Res. 18:421-423.

Various tissue slices and tissue culture cells were exposed to different fluorescein-labeled proteins. The cells were inspected after incubation by phase and fluorescence microscopy for pinocytotic vacuoles. The authors speculate on the criteria for various cells being capable of pinocytosis.

980

Korngold, L. 1962. Antigens as genetic markers. Univ. Mich. Med. Bull. 28:337-346.

Immunologic methods for study of mammalian cells in tissue culture may become valuable tools. FA will probably be a major tool. A prime problem will be suitable antisera and a clearing house to provide reference antisera. A number of specific problems that need resolution are discussed.

990

Louis, C.J.; White, J. 1960. Fluorescein globulin staining of cells in tissue cultures. Lab. Invest. 9:273-282.

Fluorescein globulin stain has been applied to cells in tissue culture. To begin with, normal cells stain, but cells of malignant tumors fail to stain. In this regard they conform with observations made on tissues in the body. As normal tissues grow, the cells gradually lose their capacity for staining, so that after a few days no cell stains. The significance of this observation is uncertain, but it appears to conform with the view that in tissue culture, cells derived from normal tissues gradually adopt a status comparable with that of the cells derived from neoplasms.

1000

Reusser, F.; Smith, C.G.; Smith, C.L. 1962. Investigations on somatotropin production of human anterior pituitary cells in tissue culture. Proc. Soc. Exp. Biol. Med. 109:375-378.

In vitro cell lines freshly isolated from human anterior pituitary glands showed fibroblastic growth. Some cells did not retain eosinophilic and basophilic characteristics. No human growth hormone was detected within the cells with fluorescein-labeled

antibody specific for human growth hormone. Also, no growth hormone was present in the supernatant medium. These findings suggest that human pituitary cells grown in tissue culture do not retain their functional properties for growth hormone production under the described culture conditions.

1010

Rosenau, W.; Moon, H.D.; McIvor, B.C. 1962. Localization of antibody to tissue culture cells by fluorescent antibody technique. Lab. Invest. 2:199-203.

The fluorescent antibody technique was used to demonstrate that cytolic antibodies to tissue culture cells localized specifically on the cell membranes. Cytoplasmic or nuclear binding of antibody could not be demonstrated. There was no localization of antibody by cell lines not affected by the antisera. It is suggested that the toxic effect on cells produced by antisera is due to binding of antibody to the cell membrane.

1020

Simpson, W.F.; Stulberg, C.S.; Peterson, W.D. 1962. Further studies of species specificity of cultured animal cells by immunofluorescence. Federation Proc. 21:159.

Antibodies were tested against a variety of strains of diverse species origin: human Det.-6, -504 Fb-1, HeLa, KB, AV-3, and LLC-Hel; monkey LLC-MK2; mouse tumors S-180 and CA-775; and Chinese hamster 15F-FAF-28. They stained cells of homologous but not of heterologous species. These also exhibited species specificity for additional cell strains: human Minn. EE, Det.-32, -52, -98, and -508 Fb-1; monkey LLC-Mel; normal mouse CCRF 228; and dog MDCK. The labeled antisera detected cells of varied origin in artificially prepared mixtures. Within a given species, antibodies against cells with different characteristics or tissue origins, e.g., ploidy, malignancy, embryonic, etc., could not be distinguished antigenically. Preliminary results indicate that antibodies against established strains are specific for primary cultures of the same species, but antibodies against primary kidney cultures do not show the same degree of specificity.

1030

Spear, G.S. 1962. Forssman antigen in tissue culture. Bull. Johns Hopkins Hospital 111:307-314.

Forssman antigen was demonstrated in tissue culture of trypsinized kidney cells from normal guinea pigs. Specific FA fluorescence was particulate, cytoplasmic, and possibly related to pinocytosis. Antigen was not present in fresh maintenance medium, suggesting synthesis by the cells. Antigen was demonstrable 53 days after primary cell establishment. Cell borders were not specifically stained.

1040

Spendlove, R.S.; Lennette, E.H. 1962. Viral antigen and infectious virus development in reovirus-infected HeLa cells. Bacteriol. Proc. V14:132.

Cell-associated, CA, and extracellular, EC, virus determinations were made at intervals postinfection. Viral antigen synthesis was followed by FA staining. Synthesis of CA virus was relatively slow. About 5 per cent of the maximal amount was present by 8 hours, 30 to 35 per cent by 24 hours, and the maximal concentration of about 400 plaque-forming units per cell was not reached until after 48 hours. This concentration remained fairly constant until 96 hours, but dropped to about 35 per cent by 7 days. There appeared to be little release of virus from infected cells. At 24 hours and earlier, there was less than 1 PFU of EC virus per cell. At 48 hours and later, there were about 30 PFU of EC virus per cell. Viral antigen was present throughout the cytoplasm by 8 hours as fine particulates, which increased in size, became globular, and coalesced to form a reticulum that was evident by 14 hours. This network of antigen tended to concentrate in a perinuclear position, where it became more dense and in most cases completely surrounded the nucleus. The antigen subsequently lost much of its reticular structure and spread confluently throughout the cytoplasm. A final stage involved a streaming of the cytoplasm, and processes containing viral antigen often became constricted and pinched off from the cell. The possible relationship of these results to some of the unusual characteristics of reovirus is discussed.

1050

Stulberg, C.S.; Simpson, W.F.; Berman, L. 1961. Species-related antigens of mammalian cell strains as determined by immunofluorescence. Proc. Soc. Exp. Biol. Med. 108:434-439.

A procedure has been described whereby species-associated antigens were directly identified in cultured mammalian cells by fluorescent antibody. Guinea pig anti-cellular globulin fractions were conjugated with fluorescein isothiocyanate. Dispersed cells were prepared from monolayer cultures of frozen cell suspensions and the resulting wet cell suspensions were treated with the labeled antibodies. Staining specificity was recognized by presence or absence of peripheral fluorescence of the treated cells. Four cell strains derived from human tissues, three from mouse, and one from Chinese hamster reacted specifically with homologous but not with heterologous labeled antibodies. Antibodies to monkey- and human-derived strains exhibited reciprocal cross-reactions that were eliminated by absorption.

1060

Twarog, J.M.; Larson, B.L. 1962. Cellular identification of beta-lactoglobulin synthesis in bovine mammary cell cultures. Exp. Cell Res. 28:350-359.

Approximately 50 per cent of the cells present in a collagenase dispersion of bovine mammary tissue were found to survive and grow when placed in in vitro culture. These cultures are composed of predominantly epithelial cells. A net synthesis of beta-lactoglobulin could be demonstrated in the cultures by a quantitative immunological procedure until 20 days after their establishment. The use of the fluorescent antibody technique indicated that the epithelial cells were those responsible for the

synthesis of the beta-lactoglobulin. Since previous studies have indicated that other specialized functions, i.e., lactose synthesis and UDP Gal-4-epimerase activity, are lost in a nonparallel manner with the ability to synthesize beta-lactoglobulin, these results indicate that the secretory cells survive and proliferate in culture but with time lose their ability to perform specialized functions because of a dedifferentiation or modulation in function.

1070

Twarog, J.M.; Larson, B.L. 1962. Cellular identification of beta-lactoglobulin in synthesis in bovine mammary cell cultures. Federation Proc. 21:160.

Approximately 50 per cent of the cells present in a collagenase dispersion of bovine mammary tissue near parturition survived and grew in culture in a defined medium supplemented with autologous serum. A net synthesis of the unique milk protein beta-lactoglobulin could be demonstrated by a quantitative immunological procedure in these cultures for 20 days after their establishment. The synthesis rate decreased from an initial 0- to 6-hour level of 58 uug per cell per day to 0.3 uug per cell per day at 20 days. These cultures were composed predominately of proliferating epithelial cells. The epithelial cells were shown to be those responsible for the synthesis of beta-lactoglobulin by the fluorescent antibody procedure. These observations complement previous studies that have demonstrated that other specialized functions such as lactose synthesis and UDP Gal-4-epimerase activity are lost in a nonparallel manner with the ability to synthesize beta-lactoglobulin. The results indicate that a high proportion of the secretory cells of bovine mammary tissue survive and proliferate in culture but lose their ability with time to perform specialized functions because of dedifferentiation or modulation in function.

## H. CONJUGATE PURIFICATION

1080

Chadwick, C.S.; Nairn, R.C. 1960. Fluorescent protein tracers: The unreacted fluorescent material in fluorescein conjugates and studies of conjugates with other green fluorochromes. Immunology 3:363-370.

Protein conjugates with fluorescein isocyanate and isothiocyanate contain unreacted fluorescent material, UFM, that contributes to specific immunological staining but is troublesome because it also stains nonspecifically. Attempts have been made to find an alternative to fluorescein isocyanate and isothiocyanate conjugates, with similar green fluorescence but free from UFM and more easily prepared. Conjugates were made with fluorescein and certain derivatives of fluorescein, but none proved satisfactory. At the present time, green fluorescent protein tracers are probably best prepared from fluorescein isothiocyanate. UFM accounts for nearly half of fluorescence after full dialysis and two extractions with tissue powder. Extraction with powdered activated charcoal removes the UFM almost completely, but the staining intensity of the conjugate is so reduced that the procedure cannot be recommended. This property of charcoal provides a means of testing fluorescein conjugations. A sample from a satisfactory conjugate should retain substantial fluorescence after such extraction.

1090

Coleman, R.M.; Dutt, G.B. 1960. Conjugation of an anti-Hymenolepis rabbit globulin with lissamine rhodamine RB 200. J. Parasitol. 46:sect. 2:12.

Antigens were prepared from Hymenolepis nana var. fraterna. Following a protein determination the saline antigenic extract was mixed with an equal volume of aluminum hydroxide gel. It was inoculated into rabbits either subcutaneously or intramuscularly and titered 10 days following the last inoculation. Serum from exsanguinated rabbits was subjected to ethodin fractionation. The protein content of immune globulins was determined. In preparing the fluorescent dye, 500 mg of rhodamine was ground in a mortar with one gram of phosphorus pentachloride for five minutes. Fifteen ml of dry acetone was then added, allowed to stand for 15 minutes, filtered, and brought back to volume with acetone. The optical density was determined at 610 millimicrons. The acetone solution of the sulphonyl chloride of RB 200 was used the same day. Conjugation and subsequent steps were carried out at 0 to 2 C. An equal volume of carbonate-bicarbonate buffer, 0.5 M and pH 9, was added to the globulin solution. A solution of 0.05 to 0.01 ml of dye per mg of protein was conjugated.

1100

Curtain, C.C. 1961. The chromatographic purification of fluorescein antibody. J. Histochem. Cytochem. 9:484-486.

Crude fluorescein antibody globulin conjugates may be freed of soluble fluorescein derivatives and other low-molecular-weight byproducts by filtration through a Sephadex G-25 column. Nonspecifically staining conjugate may be removed by filtration of the fluorescein globulin through a column of N, N-diethyaminoethyl cellulose in 0.02 M phosphate buffer, pH 6.0. The method may be used for either purifying fluorescent antibody or for isolating the nonspecifically staining components of the crude conjugates.

1110

Dineen, J.K.; Ada, G.L. 1957. Rapid extraction with ethyl acetate of free fluorescein isocyanate globulin conjugates. Nature 180:1284.

Extraction of fluorescein-conjugated antisera with ethyl acetate at neutrality was found to eliminate from the reaction mixture the free fluorescein derivatives, the acetone, and the dioxane. The extraction procedure is described.

1120

Fothergill, J.E.; Nairn, R.C. 1961. Purification of fluorescent protein conjugates: Comparison of charcoal and Sephadex. Nature 192:1073-1074.

The Sephadex method, although time-consuming and less simple, is as efficient as charcoal extraction and may be preferred for fluorescein isothiocyanate conjugates of any weak sera that cannot be handled by sandwich staining.

1130

George, W.H; Walton, K.W. 1961. Purification and concentration of dye protein conjugates by gel filtration. Nature 192:1188-1189.

The method of use of Sephadex G-25 for purifying fluorescein and rhodamine isothiocyanates is described. Its use for conjugate concentration is also indicated.

1140

Goldstein, G.; Slizys, I.S.; Chase, M.W. 1960. Nonspecific fluorescence of tissue treated with fluorescent globulins. Bacteriol. Proc. M166:139.

Normal sheep globulin and sheep antirabbit globulin coupled at various ratios with fluorescein isothiocyanate, FITC. Serum at 2.5 per cent protein concentration was fractionated with ammonium sulfate, 40 per cent saturation at 4 C. Couplings were made with 7 to 50 mg per gram of protein. Certain couplings were made stepwise, with the coupling product isolated from the low-molecular-weight compounds by one-step passage of 2 to 4 hours through a Sephadex G-50 column and reconcentration to the desired endpoint by pressure dialysis under nitrogen. Substrates were imprints or tissue sections of normal guinea pigs for nonspecific fluorescence and of specifically restimulated and normal rabbits. Fluorescent globulin preparations were applied to substrates both with and without tissue powder adsorption and other adsorbants to determine the amount that could be applied without inducing nonspecific fluorescence and the amount capable of producing specific fluorescence. The margin of difference between the two amounts is often little when one is searching for small concentrations of antigen in the section, perhaps twofold to fourfold, and may be lessened by choice of tissue sections of different thickness.

1150

Goldstein, G.; Spalding, B.H.; Hunt, W.B. 1962. Studies on fluorescent antibody staining: II. Inhibition by suboptimally conjugated antibody globulins. Proc. Soc. Exp. Biol. Med. 111:416-421.

Three rabbit immune globulin preparations were coupled at 1 or 2 per cent protein concentration with 8 or 10 mg of crystalline FITC per gram of protein. Each preparation was heterogeneous with respect to the number of fluorescent groupings. In these preparations, 35 to 80 per cent of the conjugated globulins contained less than an optimal number of fluorescent groupings. Maximum brightness of specific staining was obtained after each preparation was fractionated on DEAE-cellulose to separate the optimally coupled from the under-coupled and the over-coupled globulins.

1160

Gordon, M.A.; Edwards, M.R.; Tompkins, V.N. 1962. Refinement of fluorescent antibody by gel filtration. Proc. Soc. Exp. Biol. Med. 109:96-99.

Gel filtration through a Sephadex column permits a significantly simpler and more rapid purification of fluorescent conjugates than either anion exchange or electrophoresis. Dialysis was eliminated and unconjugated dye as well as other low-molecular-weight fractions were separated with minimum loss of protein or titer. Detailed data on purification of some antifungal conjugates are presented. Ethodin extraction of serum antibody preparatory to conjugation and gel filtration further expedites the process.

1170

Hospelhorn, V.D. 1961. A convenient technique for dialysis. Anal. Biochem. 2:180-182.

A simple, rapid method for dialysis suitable for removing ammonium ion from solutions is presented. This may be applicable to some FA reagent preparation procedures.

1180

Hudson, B. 1961. Column adsorption of fluorescein isothiocyanate-labeled antibodies. Bull. WHO 24:291-292.

A chromatographic technique is described for purifying an anti-P. pestis conjugate on columns composed of a tissue powder and diatomite filter aid. Saline elutions from such a column were superior to conjugates adsorbed 2 or 3 times by standard techniques, and the method represented a substantial time saving.

1190

Johnson, G.D. 1961. Simplified procedure for removing nonspecific staining components from fluorescein-labeled conjugates. Nature 191:70-71.

Nonspecific staining material in conjugates prepared from fluorescein isothiocyanate can all be removed by serial treatment with liver suspension alone, thus eliminating the long dialysis procedure.

1200

Killander, J.; Ponten, J.; Roden, L. 1961. Rapid preparation of fluorescent antibodies using gel filtration. Nature 192:182-183.

The use of gel filtration in place of dialysis in the preparation of fluorescent antibodies is described.

1210

Lipp, W. 1961. Use of gel filtration and polyethylene glycol in the preparation of fluorochrome-labeled proteins. J. Histochem. Cytochem. 9:458-459.

Equipment and steps in the subject procedures are described.

1220

Oystese, B. 1962. Critique of the immunofluorescence method in histology. Verhandl. Deut. Ges. Pathol. 46:120-124. In German.

The author has investigated some sources of error in fluorescent antibody histochemistry, including those concerned with nonspecific staining, handling the specimen, and fixation. The best specificity of staining was obtained with antisera fractionated on the DEAE-cellulose column. Cryostat sectioning, while generally very useful, may cause dislocations of the substance in question and should be supplemented by freeze-drying for control. The problem of fixation is particularly intriguing when nonbound proteins are to be demonstrated. Investigations on the secretion of albumin and globulin by the human stomach reveal that the best fixative so far obtained for these substances is absolute alcohol followed by acetone for 30 minutes each at room temperature.

1230

Peters, J.H.; McDevitt, H.O.; Pollard, L.W.; Harter, J.G.; Coons, A.H. 1961. Purification of fluorescent conjugates by column chromatography. Federation Proc. 20:17.

Rabbit antisera against bovine serum albumin were conjugated with fluorescein by the method of Marshall et al. After extensive dialysis against 0.005 M phosphate buffer, pH 8.1, each conjugate was adsorbed to a DEAE-cellulose column and eluted by stepwise increases in phosphate molarity at pH 6.3. Fraction I was eluted at 0.05 M phosphate, II at 0.10 M, and III with 1 N NaCl, pH 7.0. Each fraction was concentrated to the original volume with polyvinyl pyrrolidone. Fraction I contained 40 to 60 per cent of the original antibody nitrogen. Specific precipitate from it had a molar fluorescein to antibody protein ratio of 1.06, determined by adsorption at 490 mμ and by biuret reaction. It gave specific staining and was free of nonspecific staining of frozen sections of rabbit lymph node. Fraction III gave moderate specific and marked nonspecific staining, and had a molar fluorescein to antibody protein ratio of 3.92. The original conjugates and their fractions were found to fluoresce with 14 to 21 per cent of the intensity of purified, unconjugated sodium fluorescein. It should now be possible to prepare conjugates free of nonspecific reactivity, with known fluorescence intensity and antibody content.

1240

Porath, J.; Flodin, P. 1959. Gel filtration: A method for desalting and group separation. Nature 183:1657-1659.

Gel filtration in columns is discussed with particular reference to Sephadex, a dextran gel. The possibilities of use for purification of fluorescent protein conjugates are excellent with this technique.

1250

Tokumaru, T. 1962. Kinetic study on the labeling of serum globulin with fluorescein isothiocyanate by means of the gel filtration technique. J. Immunol. 89:195-203.

Sephadex gel filtration was used for the quantitive analysis of reactants by rapid separation. Using simultaneous gel filtration of samples in a number of sister columns, association constants and the number of sites were obtained for the kinetics of the labeling process of globulins with fluorescein. Reagents for protein precipitation were used to remove superficially adsorbed dye from fluorescent antibody. The results of the precipitation showed that the gel-filtered material contained smaller amounts of adsorbed dye than dialyzed material. The patterns of DEAE-cellulose and DEAE-Sephadex column chromatography were compared for fluorescent antisera. The complex of papain and fluorescent antibody could be separated from free papain satisfactorily on a Sephadex G-75 column.

1260

Wagner, M. 1962. Use of Sephadex for rapid purification of fluorescence-labeled antibody solutions. Zentralbl. Bakteriol. Parasitenk. Abt. I. Orig. 185:1:124-128. In German.

Quantitative separation of the unbound fluorescence dye from antibody solutions labeled with fluorescein is achieved by gel filtration with Sephadex G-50. As compared with the previously used dialysis, the purification is terminated in a few minutes and therefore can be done at room temperature. Because of the good possibility of optical control it permits regaining the serum conjugate with only negligible decrease of concentration.

1270

Zwaan, J.; van Dam, A.F. 1961. Rapid separation of fluorescent antisera and unconjugated dye. Acta Histochem. 11:306-308.

Sephadex, a cross-linked dextran, was found very useful for rapid removal of free dye from fluorescent antisera.

## I. HISTOLOGIC TECHNIQUES

1280

Allen, J.C. 1962. Identification of antigens in solution by the fluorescent antibody technique. Federation Proc. 21:15.

During studies on microbiological antigens it became desirable to extend the use of the fluorescent antibody staining technique to the identification of antigens in aqueous solution. Antigen solutions are mixed with melted agar at 40 to 50 C, allowed to gel, and quick-frozen. Sections are made at 32 mu in a cryostat at -15 C. Human albumin, HSA, human gamma globulin, HGG, and diphtheria toxin, DT, have been studied. Sections of agar stained by methylene blue and examined under light reveal a mesh of agar strands. FA staining of these sections reveals antigen localized within the strands of HSA and DT or present as aggregates on the surface of or between the strands of HGG. HSA has been detected in concentrations as low as 0.5 milligram per ml; HGG, at 0.25 milligram per ml; and DT, at 0.1 plus unit per ml. All procedures usable in FA staining of tissue-localized antigens are applicable to this technique with the possible exception of counterstaining. Agar presumably holds these antigens by a mechanical entrapment of antigen molecules within or around agar micelles. The specificity and sensitivity of the systems are, as in classical FA procedures, functions of the antibody used. The technique is rapid and allows the use of nonprecipitating antibodies.

1290

Balfour, B.M. 1961. Immunological studies on a freeze substitution method of preparing tissue for fluorescent antibody staining. Immunology 4:206-218.

A freeze-substitution, wax-embedding method for preparing tissue for fluorescent antibody studies is described. Solutions of ovalbumin and gamma globulins were subjected to the whole embedding procedure and their solubility and immunological reactivity were estimated quantitatively. More than 60 per cent of these proteins remained soluble after embedding. The precipitating power of the soluble fraction of ovalbumin and antibody globulin was found to be reduced by half. The insoluble fraction of antibody globulin bound more antigen than would be precipitated at equivalence.

1300

Burkholder, P.M.; Littell, A.H.; Klein, P.G. 1961. Sectioning at room temperature of unfixed tissues, frozen in a gelatin matrix, for immunohistologic procedures. Stain Technol. 36:89-91.

Frozen sections of unfixed tissues are usually cut for immunohistological procedures with a microtome mounted within a cryostat. The cost, tedious maintenance, and use of such apparatus have been obviated in this laboratory by employing a simpler method that permits cutting frozen sections as thin as 2.5 microns at room temperature. Unfixed tissues are enclosed in a gelatin matrix, stored in a freezer, and sectioned at room temperature while frozen.

1310

George, W.H.; Walton, K.W. 1962. Preservation of tissue sections in Coons fluorescent antibody technique. Nature 194:693.

Any material used to preserve tissue sections for immunological analysis must fulfill certain criteria: It must not interfere with the antigen-antibody reaction by denaturation of protein, it must itself be nonreactive in the antigen-antibody system, and it must be easy to apply. The polyethylene glycol Carbowax, from Carbide and Carbon Chemical Co., has a molecular weight of 285 to 315 and a freezing range of -15 to 8 C. It fulfilled the criteria for use as a preserving medium.

1320

Louis, C.J. 1957. Cutting unfixed frozen sections for fluorescent antibody studies. Stain Technol. 32:279-282.

A simplified method of section cutting that dispenses with guide attachments on the microtome blade and is suitable for serial sections of unfixed frozen tissue of 4 microns is described. Essential features are maintenance of low temperature, -13 C, critical angle of knife, and moistening slides in cold alcohol or isopentane. This method has been found suitable for fluorescence antibody studies where maintenance of low temperatures is necessary.

1330

Marcato, D. 1962. Immunohistochemical aspects of the human palatine tonsil: Preliminary report on the immunofluorescence method. Arch. Ital. Otol. Rinol. Laringol. 78:6:895-900. In Italian.

The FA method for demonstrating immunohistochemical phenomena in the tonsils by microfluoroscopy is described.

1340

Rahman, A.N.; Luttrell, C.N. 1962. Albumin embedding method for frozen sectioning of fresh tissues for histological, fluorescent antibody, and histochemical studies. Bull. Johns Hopkins Hosp. 110:66-72.

A simple method is described whereby crude egg albumin is utilized for frozen embedding and sectioning of fresh tissues in the cryostat. The greatest advantage of frozen albumin sectioning lies in the ease with which it is possible to obtain quality sections of varying thicknesses from different organs. The albumin method can be used for ordinary histology, as well as histochemical, autoradiographic, and fluorescent antibody studies. One technician can freeze, section, mount, and stain an average of 40 to 50 specimens a day. The albumin blocks can be stored for months in a deepfreeze for future sectioning if needed.

1350

Sainte-Marie, G. 1962. A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem. 10:250-256.

A method is described for the fixation of blocks of tissue for use in studies employing immunofluorescence. This method consists of fixing thin blocks in 95 per cent ethanol and carrying out the subsequent dehydration and clearing at refrigeration temperatures of 4 C. Thereafter, embedding in paraffin and sectioning by the standard microtome is easy. This method results in preparations that are histologically more precise in the localization of antigen or antibody than preparations of frozen tissues; in addition, with rabbit antibody and bovine serum albumin, the sensitivity of detection is enhanced. Bovine serum albumin can be found for longer periods after injection than is possible with frozen sections. Other antigens for which this procedure has proved satisfactory are bovine gamma globulin, horse ferritin, influenza virus A, and diphtheria and tetanus toxoids, but hen ovalbumin deteriorated.

1360

Steedman, H.F. 1960. Polyester wax, p. 52-55 and 121. In H.F. Steedman, ed. Section cutting in microscopy. Blackwell Scientific Publications, Oxford.

Polyester wax block preparation of tissues for FA and other histology studies is described. Sections may be made at 17 to 22 C.

1370

Tobie, J.E. 1958. Certain technical aspects of fluorescence microscopy and the Coons fluorescent antibody technique. J. Histochem. Cytochem. 6:271-277.

A fluorescence microscope using the 1000-watt A-H6 lamp is described with special reference to its application in the fluorescent antibody technique and tissue localization of tetracyclines. The spectral characteristics of the light source and the filter combinations are discussed. In fluorescent antibody studies with fluorescein isocyanate a wavelength of 390 to 440 mu seems satisfactory. A method of quick-freezing fresh tissues in petroleum ether at approximately -65 C has been found satisfactory for preserving the cellular architecture of tissues. The operation of a rotary microtome in a cryostat at subfreezing temperatures has been improved by special lubrication, so that sections can be cut at a microtome setting of 3 microns. With thin sections, it is possible to observe minute cytological details and localize fluorescent substances accurately.

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J. MICROSCOPY EQUIPMENT

1380

Brumberg, Ye.M.; Krylova, T.N. 1953. The use of interference separatory reflectors in fluorescent microscopy. J. Gen. Biol. 14:6:461-464. In Russian.

The use of an interference separatory reflector for incident lighting in fluorescent microscopy to increase the efficiency of the exciting light is presented. The theory is discussed, and a system is described. A light utilization of 90 per cent is claimed to exceed that of a standard opaque illuminator by 4 or 5 times. The brightness of the object increases with increase in strength of objective, in contrast to the usually encountered reverse ratio. Other advantages of this method of illumination in fluorescent microscopy claimed are elimination of need for nonfluorescent immersion oil, and high-powered light sources, less intense exposure of objects to UV light, and the possibility of use of weaker light filters.

1390

Butslov, M.M.; Korn, M.Ya.; Muromtsev, S.N. 1961. A brightness amplifier applied to light and luminescence microscopy. Dokl. Akad. Nauk SSSR 139:568-570.

An apparatus described as an electron optical transducer, or brightness amplifier, is presented that is a multistage amplifier for image brightness having a high conversion factor and resolving power. The image projected from the microscope is played on the photocathode of the amplifier placed above the microscope. The bright image then appears on a fluorescent screen, where it may be photographed. The apparatus was tested with acridine orange and immunofluorescent systems. In FA preparations of anthrax and Brucella, exposures without the brightness amplifier for 12 minutes were underexposed, but satisfactory photographs were made with the brightness amplifier at exposures of one-fifth and one-tenth second.

1400

Kunz, C.; Gabler, F.; Herzog, F. 1961. Contrast fluorescence, a new fluorescence microscopy method. Mikroskopie 16:1:1-7. In German.

Fluorescence equipment is described that permits observations of fluorescence and simultaneously of positive- or negative-phase contrast, a so-called simultaneous contrast fluorescence. Various globulin solutions were labeled with fluorescein isothiocyanate, and the advantage of this contrast fluorescence equipment for the fluorescent antibody technique was demonstrated by fluorescence microscopic examinations of yeast, virus-infected cells, and histological tissue sections. The superiority of this equipment over other fluorescent equipment mainly lies in the fact that an exact localization of antigens in tissue cultures or in histological tissue sections is greatly facilitated.

1410

Kunz, C.; Klaushofer, H. 1961. Purity control of the production of baker's yeast employing a fluorescent antiserum. Appl. Microbiol. 9:469-471.

A simple staining procedure for the rapid detection of wild yeasts contaminating baker yeast during the course of industrial production is described. Fluorescein-labeled specific antiserum against Saccharomyces cerevisiae is applied to smears of baker's yeast, which are then examined by ultraviolet microscopy. Optimal results are obtained with the combined phase contrast and fluorescence that makes the S. cerevisiae appear green, whereas cells of wild yeasts are visible in bright red counterstain. With this method, wild yeasts could be identified in baker's yeast at a dilution of 1:10,000.

1420

Tobie, J.E. 1958. Certain technical aspects of fluorescence microscopy and the Coons fluorescent antibody technique. J. Histochem. Cytochem. 6:271-277.

A fluorescence microscope using the 1000-watt A-H6 lamp is described with special reference to its application in the fluorescent antibody technique and tissue localization of tetracyclines. The spectral characteristics of the light source and the filter combinations are discussed. In fluorescent antibody studies with fluorescein isocyanate a wavelength of 390 to 440 mu seems satisfactory. A method of quick-freezing fresh tissues in petroleum ether at approximately -65 C has been found satisfactory for preserving the cellular architecture of tissues. The operation of a rotary microtome in a cryostat at subfreezing temperatures has been improved by special lubrication, so that sections can be cut at a microtome setting of 3 microns. With thin sections, it is possible to observe minute cytological details and localize fluorescent substances accurately.

K. QUANTIFICATION

1430

Chadwick, C.S.; Johnson, P. 1961. Depolarization of the fluorescence of proteins labeled with various fluorescent dyes. Biochim. Biophys. Acta 53:482-489.

The behavior of a series of fluorescent dyestuffs conjugated to protein molecules has been examined. Assuming the relaxation time for bovine serum albumin, the excited lifetimes of the dyestuffs vary from $0.25 \times 10^{-8}$ to $9 \times 10^{-8}$ second. Depolarization measurements have been reported for conjugates with proteins whose molecular weights vary from 15,000 to 650,000. A pyrene derivative with a particularly long excited lifetime such as the greater time cited above shows anomalous behavior when conjugated with bovine serum albumin under mild conditions, and the need for caution in interpreting depolarization measurements is stressed.

1440

Chase, M.W.; Slizys, I.S.; Dukes, C.D. 1959. Studies of fluorescent antibody in a particular form of immunologic sandwich. Bacteriol. Proc. M104:89.

To visualize the sites of retention of chemical groupings of allergenic chemicals in animal tissues, in vitro studies have been made on picryl groupings attached to erythrocyte stromata, picryl-specific rabbit antibody, and fluorescent antirabbit-globulin sheep antibody. Labeled antisera were tested by double diffusion precipitation to detect antibody activity loss and for ability to complete the immunologic sandwich. Coupling globulins in the cold at pH 10.5 for short periods with 3 to 6 mg of fluorescein derivatives per gram of protein, and precipitating the product by pH and salt variations, was found superior to the conditions usually employed to yield minimal antibody loss and maximal fluorescence. The relative efficiency of coupled antibody preparations was tested by using standard amounts of picrylated guinea pig stromata coated with rabbit antipicryl antibody, reading the washed reaction products macroscopically for brightness under a tubulary UV emitter, and confirming by fluorescence microscopy. Specific inhibition of the first antibody coupling was demonstrated with picryl glycine and of the second antibody coupling with normal rabbit globulin. Relationships were demonstrable between the amounts of the two antibodies required to complete the sandwich.

1450

Dandliker, W.B.; Feigen, G.A. 1961. Quantification of the antigen antibody reaction by the polarization of fluorescence. Biochem. Biophys. Res. Commun. 5:299-304.

We have concluded that it is possible to determine by measurements of fluorescence polarization two important parameters, the equilibrium constant and the combining capacity characterizing the antigen-antibody reaction. The combining capacity is proportional to the number of antibody sites in a preparation and the equilibrium constant, together perhaps with certain kinetic quantities, constitutes a quantitative measure of avidity. Further results will show to what extent the presence of the fluorescent label interferes in the reaction, but, in any case, it appears that the effect is relatively small.

1460

Dandliker, W. B.; Feigen, G.A. 1961. Detection of the antigen-antibody reaction by fluorescence polarization. Federation Proc. 20:11.

The use of fluorescence polarization in connection with the antigen-antibody or antibody-haptene reaction follows implicitly from the work of Weber and of Steiner. This polarization is of special interest in reactions of univalent or nonprecipitating antibody, but it can also be used to quantify the reaction between precipitating antibody and the antigen or haptene. Rabbits were immunized to ovalbumin coupled to fluorescein and the gamma globulin fraction was prepared. Measurements of polarization and intensity, taken after adding normal globulin to the fluorescein with or without ovalbumin, were in all cases nearly the same as when buffered saline was added. Easily measured increases in polarization and decreases in intensity resulted when immune globulin was used. Low antibody concentrations were usually necessary in the region of equivalence to postpone the development of turbidity. Quantitative treatments based upon either polarization or intensity data gave concordant results. Scratchard plots showed pronounced curvature, possibly indicating a wide spectrum of association constants and or interaction between binding sites. Our results demonstrate directly the specific combination of antibody and antigen in solution without precipitate formation and also the union between antibody and haptene.

1470

Haber, E.; Bennett, J.C. 1962. Polarization of fluorescence as a measure of antigen-antibody interaction. Proc. Nat. Acad. Sci. 48:1935-1942.

Polarization of fluorescence is a sensitive and direct method for studying antigen-antibody interactions. Examples are given of titrations of the B chain of insulin, coupled to PAH and fluorescein against antipara-amino-hippurate, and fluoresceinated ribonuclease and bovine serum albumin against their antibodies. The utility of the method in measuring interaction between univalent antibody fragment and antigen is likewise demonstrated. Rotational relaxation times for antigen-antibody complexes in various proportions are determined.

1480

Lange, K.; McPherson, S.; Wenk, E.J. 1959. The intravenous injection of fluorescein-labeled antibody as a means to localize and quantitate antigen location. AMA J. Dis. Child. 98:615.

The gamma globulin fraction of duck serum against rabbit kidney labeled with fluorescein isothiocyanate was injected intravenously into rabbits and its localization was observed after intervals from one hour to several months. Animals killed after one hour showed an intense crisp fluorescence of the basement membrane of all glomeruli but no staining of any other part of the kidney or any other organ except the spleen, where it appeared irregularly in droplet form in the red pulp and in the lymph nodes. The labeled fraction of unmodified duck serum does not localize in the kidney but in the spleen. There is no nonspecific background staining with this method. The labeled gamma globulin is nontoxic and produces disease as well as nonconjugated duck serum against rabbit kidney. This method gives a more nearly quantitative impression of

antibody localization than the original Coons method. Since fluorescein-conjugated antibody to whole rabbit kidney localized exclusively in the basement membrane, the basement membrane is the main antigenic factor in nephrotoxic sera. Under normal conditions the endothelium of the glomerulus is freely permeable to proteins the size of gamma globulin.

1490

Lange, K.; McPherson, S.; Wenk, E.J. 1959. The intravenous injection of fluorescein-labeled antibody as a means to localize and quantitate antigen location. Federation Proc. 18:1920:487.

The gamma globulin fraction of duck serum against rabbit kidney labeled with fluorescein isothiocyanate was injected intravenously into rabbits and its localization observed after intervals from one hour to several months. Animals sacrificed after one hour showed an intense fluorescence of the basement membrane of all glomeruli but no staining of any other part of the kidney or other organ except for the spleen, where the substance appeared irregularly in droplet form in the red pulp. Gamma globulin fraction of unmodified duck serum does not localize in the kidney, but it can be found in the spleen. There is no nonspecific background staining with this method. The labeled gamma globulin appears to be nontoxic, to give a more quantitative impression of the antibody location.

1500

Miquel, J.; Horvath, B.; Klatz, I. 1960. A chromatographic technique for the quantitative study of the precipitin reaction. J. Immunol. 84:545-550.

A chromatographic technique for quantitative estimation of precipitin reactions is presented. The main advantages of the technique are the small amount of serum required, 0.01 ml, the high sensitivity, 1 gamma of nitrogen, and the simplicity of procedure. The ratio of antigen to antibody in the precipitate can be determined by fluorescent antibody. The abbreviated version of the technique can be used for qualitative precipitin reactions or for testing the reactivity of fluorescent antibodies. The minimal concentration of precipitated nitrogen required for quantitative determinations is 100 gammas for one ml of antiserum.

L. PROPERTIES: FLUOROCHROMES, CONJUGATES

1510

Alper, T. 1946. Fluorescence fatigue. Nature 158:451.

The phenomenon of fluorescent fading or fatigue under light excitation has been neglected in the biological literature, and at the same time the intensity of fluorescence is suggested as a criterion in certain identification procedures. Results with a number of fluorescent dyes including fluorescein are discussed and were generally parallel, displaying a constant logarithmic rate of intensity decay over a time period. An equilibrium state reported by some workers was not observed. Results of intensity measurements should be considered in the light of these findings and of previous exposure history of the sample.

1520

Curtain, C.C. 1958. Electrophoresis of fluorescent antibody. Nature 182:1305-1306.

The problem of nonspecific staining by fluorescein-conjugated sera was studied from an electrophoretic standpoint. Human gamma-2 globulin was absorbed with acetone-dried rat liver powder. Comparative electrophoresis demonstrated a reduction of the area of the leading peak. In gamma-2 globulin against ovalbumin the diminution was accompanied by a 25 per cent loss of antibody activity. Nonspecific fluorescence was nearly eliminated. Starch block and powdered cellulose electrophoretic separation techniques were disappointing; however, fractionation by electrophoretic convection was possible. Human serum albumin and a myeloma globulin increased in mobility but only slightly in heterogeneity on conjugation with fluorescein isocyanate.

1530

Pringsheim, P. 1949. Fluorescence and phosphorescence. Interscience Publications, John Wiley and Sons, Inc., New York. 794 p.

Basic reference information is presented on the physical and chemical nature of the fluorescent phenomenon. Data on absorption and emission for many dyes and other chemicals are available. General techniques and types of equipment are presented.

1540

Schiller, A.A.; Schayer, R.W.; Hess, E.L. 1953. Fluorescein-conjugated bovine albumin: Physical and biological properties. J. Gen. Physiol. 36:489-505.

Conjugates of fluorescein and bovine albumin have been prepared and found not to differ appreciably in size, shape, and homogeneity from the precursor, bovine serum albumin. Fluorescein has also been conjugated to rat plasma proteins. Their disappearance rates from the circulation of rats correspond with those obtained from the use of isotope labeling. Fluorescein conjugates have been used to visualize the transcapillary passage of circulating proteins in the mesenteric circulation of frogs and rats by direct microscopic observation and found to diffuse slowly in the manner predicted for plasma proteins. The process of labeling does not render guinea pig albumin antigenic,

although slight antigenicity results from labeling whole-plasma protein. It is believed that sufficient biological evidence is presented to support the conclusion that fluorescein-conjugated plasma proteins, particularly albumin, behave in vivo like their native precursors.

M. SERLOGIC TECHNIQUES

1550

Alexander, W.R.M. 1958. The application of the fluorescent antibody technique to hemagglutinating systems. Immunology 1:217-223.

The Coons technique has been modified for the detection of antibodies on erythrocytes agglutinated by fluorescein-conjugated antisera. The results of experiments with several hemagglutinating systems suggested that specific fluorescence was found only on erythrocytes that had been agglutinated by species-specific antibodies. The significance of these findings is discussed.

1560

Allen, J.C. 1962. Identification of antigens in solution by the fluorescent antibody technique. Federation Proc. 21:15.

During studies on microbiological antigens it became desirable to extend the use of the fluorescent antibody staining technique to the identification of antigens in aqueous solution. Antigen solutions are mixed with melted agar at 40 to 50 C, allowed to gel, and quick-frozen. Sections are made at 32 mu in a cryostat at -15 C. Human albumin, HSA, human gamma globulin, HGG, and diphtheria toxin, DT, have been studied. Sections of agar stained by methylene blue and examined under light reveal a mesh of agar strands. FA staining of these sections reveals antigen localized within the strands of HSA and DT or present as aggregates on the surface of or between the strands of HGG. HSA has been detected in concentrations as low as 0.5 milligram per ml; HGG, at 0.25 milligram per ml; and DT, at 0.1 plus unit per ml. All procedures usable in FA staining of tissue-localized antigens are applicable to this technique with the possible exception of counterstaining. Agar presumably holds these antigens by a mechanical entrapment of antigen molecules within or around agar micelles. The specificity and sensitivity of the systems are, as in classical FA procedures, functions of the antibody used. The technique is rapid and allows the use of nonprecipitating antibodies.

1570

Chase, M.W.; Slizys, I.S.; Dukes, C.D. 1959. Studies of fluorescent antibody in a particular form of immunologic sandwich. Bacteriol. Proc. M104:89.

To visualize the sites of retention of chemical groupings of allergenic chemicals in animal tissues, in vitro studies have been made on picryl groupings attached to erythrocyte stromata, picryl-specific rabbit antibody, and fluorescent antirabbit-globulin sheep antibody. Labeled antisera were tested by double diffusion precipitation to detect antibody activity loss and for ability to complete the immunologic sandwich. Coupling globulins in the cold at pH 10.5 for short periods with 3 to 6 mg of fluorescein derivatives per gram of protein, and precipitating the product by pH and salt variations was found superior to the conditions usually employed to yield minimal antibody loss and maximal fluorescence. The relative efficiency of coupled antibody preparations was tested by using standard amounts of picrylated guinea pig stromata coated with rabbit antipicryl antibody, reading the washed reaction products

macroscopically for brightness under a tubulary UV emitter, and confirming by fluorescence microscopy. Specific inhibition of the first antibody coupling was demonstrated with picryl glycine and of the second antibody coupling with normal rabbit globulin. Relationships were demonstrable between the amounts of the two antibodies required to complete the sandwich.

1580

Coleman, R.M.; Dutt, G.B. 1960. Conjugation of an anti-Hymenolepis rabbit globulin with lissamine rhodamine RB 200. J. Parasitol. 46:sect. 2:12.

Antigens were prepared from Hymenolepis nana var. fraterna. Following a protein determination the saline antigenic extract was mixed with an equal volume of aluminum hydroxide gel. It was inoculated into rabbits either subcutaneously or intramuscularly and titered 10 days following the last inoculation. Serum from exsanguinated rabbits was subjected to ethodin fractionation. The protein content of immune globulins was determined. In preparing the fluorescent dye, 500 mg of rhodamine was ground in a mortar with one gram of phosphorus pentachloride for five minutes. Fifteen ml of dry acetone was then added, allowed to stand for 15 minutes, filtered, and brought back to volume with acetone. The optical density was determined at 610 millimicrons. The acetone solution of the sulphonyl chloride of RB 200 was used the same day. Conjugation and subsequent steps were carried out at 0 to 2 C. An equal volume of carbonate-bicarbonate buffer, 0.5 M and pH 9, was added to the globulin solution. A solution of 0.05 to 0.01 ml of dye per mg of protein was conjugated.

1590

deLong, R. 1961. Use of agar diffusion and fluorescent antibody. Nature 190:1126.

The agar diffusion technique is very useful and has made a significant contribution in the analysis of antigenic materials. However, the difficulty encountered when trying to observe some of the precipitation zones is known to anyone who has used this method. Successful photography of the zones can be especially difficult. By using the fluorescent antibody in the agar diffusion procedure, the precipitation zones when viewed or photographed in the near-ultraviolet range appear much clearer than with nonfluorescent antibody. Photographs are presented for comparison.

1600

Klein, P.G.; Burkholder, P.M. 1959. The demonstration of fixed complement with labeled anti-complement. Schweiz. Z. Allg. Pathol. Bakteriol. 22:6:729-731. In German.

The specific nature of the CF test was demonstrated with alexinized sheep cells and with labeled anti-guinea pig globulin. Using marked anti-guinea pig complement and suitable controls on the Masugi kidney, it was shown that the antigen-antibody complexes did absorb complement.

1610

Klein, P.G.; Burkholder, P.M. 1960. The demonstration of complement-fixation by fluorescence microscopy. Ger. Med. Mon. 5:37-40.

A method is described for demonstrating fixed complement by the fluorescent antibody technique. The basis of the method is the production of specific antibodies against fixed complement and their serological demonstration. These antibodies or anti-complement, are directed against two serologically distinct components of fixed complement. When labeled with fluorescein they produce specific staining of complement fixed on sensitized red cells. When unfixed sections of a Masugi kidney from a rat are treated with guinea pig serum they fix complement from it. This can then be made visible by specific staining with labeled antibody. Complement fixation takes place in the glomerulus. By means of labeled antirat complement it can also be shown that the glomeruli of the Masugi kidney are coated with endogenous complement from the animal itself. Kidney sections from control animals fix neither their own complement nor that from other species.

1620

Klein, P.G.; Burkholder, P.M. 1960. Studies on the antigenic properties of complement I. Demonstration of agglutinating antibodies against guinea pig complement fixed on sensitized sheep erythrocytes. J. Exp. Med. 111:93-106.

Evidence is presented to show that guinea pig complement fixed on sensitized sheep red cells acts as a specific agglutinogen. Agglutinating antibodies that react with cell-fixed complement can be produced by immunizing rabbits with a complex of stromata, amboceptor and complement or with guinea pig serum globulin. These agglutinins can be removed by precipitation with guinea pig serum. They are, therefore, distinct from immunoconglutinins.

1630

Lachman, P.J.; Mueller-Eberhard, H.J.; Kunkel, H.G.; Paronetto, F. 1962. The localization of in vivo bound complement in tissue sections. J. Exp. Med. 115:63-82.

A technique has been described for demonstrating a human complement component by an immunofluorescent method. The component detected is beta 1-C-globulin, a moiety of the third complement component in pure form, to which a specific antiserum has been prepared. The binding of beta 1-C-globulin as shown by immunofluorescence is strictly equivalent to complement fixation as assessed by standard serological methods. In vivo complement binding occurs in the lesions of several human diseases, but not elsewhere in the same tissues. In vivo complement binding was found particularly in systemic lupus erythematosus, various nephritides, and amyloidosis, as well as in single cases of some other diseases. The spectrum of in vivo complement binding has been compared with that of gamma globulin binding, 7S and 19S types, and with the demonstration of in vitro complement fixation and rheumatoid factor fixation. It was distinct from each of these. Rheumatoid factor fixation, detected by anti-19S antiserum, showed promise as a method for the detection of antigen-antibody complexes and aggregated gamma globulin in tissue sections.

1640

Mikhailov, I.F.; Dashkevich, I.O. 1961. Detection of fixed complement by the fluorescent antibody technique. J. Microbiol. Epidemiol. Immunobiol. 32:1284-1289.

Complement fixation demonstration by fluorescent antibody is specific for bacterial antigens. Modifications indicated can be used for research as well as for investigations in practice.

1650

Miquel, J.; Horvath, B.; Klatz, I. 1960. A chromatographic technique for the quantitative study of the precipitin reaction. J. Immunol. 84:545-550.

A chromatographic technique for quantitative estimation of precipitin reactions is presented. The main advantages of the technique are the small amount of serum required, 0.01 ml, the high sensitivity, 1 gamma of nitrogen, and the simplicity of procedure. The ratio of antigen to antibody in the precipitate can be determined with fluorescent antibody. The abbreviated version of the technique can be used for qualitative precipitin reactions or for testing the reactivity of fluorescent antibodies. The minimal concentration of precipitated nitrogen required for quantitative determinations is 100 gamma for each ml of antiserum.

1660

Mueller, F.; Giese, G.; Ricken, D. 1961. Investigations on the specificity of staining bound complement with fluorescent-labeled anticomplement. Z. Hyg. Infektionskrankh. 147:5:434-446. In German.

Anticomplement was prepared by immunizing rabbits with complexes of complement, antigen and antibody. Egg albumin and Proteus OX-19 were the antigens used. The fixation reaction was specific. In virus-tissue culture systems showing CPE, there was nonspecific deposition of complement-independent components of guinea pig serum on nuclear and plasma constituents of tissue cells. The reasons for this were discussed, along with techniques for the elimination of these nonspecific reactions.

1670

Rice, C.E. 1962. The effect of unheated normal bovine serum on the complement-fixing activity of heat-inactivated bovine antiserum with homologous antigen: II. Aggregative properties. Can. J. Comp. Med. Vet. Sci. 26:107-115.

To determine whether the greater fixation of complement observed in modified complement-fixation tests is related to an increased aggregation of the antigen-antibody complexes, parallel tests by the two methods have been made with two different particulate bacterial antigens and corresponding bovine antisera. After fixation, the mixtures were centrifuged and washed. Smears of each sediment were examined by immunofluorescence using fluorescein-labeled rabbit anti-guinea pig complement. A greater degree of aggregation of the antigen-antibody complexes was observed in sediments from tests with modified complement. Aggregates from mixtures showing increased fixation of complement appeared more brightly fluorescent. The reaction was specific.

1680

Sacchi, R.; Costanzi, G.; Mancini, A.M. 1962. Active complement in guinea pig sert conjugated with fluorescein isothiocyanate. Boll. Soc. Ital. Biol. Sperim. 38:19:928-932. In Italian.

The role of complement and subfractions of complement was demonstrated with FA.

1690

Taschini, P.; Rappaport, B.Z. 1960. Use of optical density of fluorescent conjugate for analysis of co-precipitating antibody. Proc. Soc. Exp. Biol. Med. 105:73-76.

The optical density of fluorescein isothiocyanate permits determination of the quantit of protein derived from a conjugated precipitin serum when this is added to an unconju gated precipitin serum. This permits one to distinguish in a specific precipitate the antibodies of the two sera. This finding could have practical application in co-preci itating techniques.

## II. REFERENCES TO PROCEDURES WITH SPECIFIC APPLICATIONS

### A. BACTERIA

#### 1. Actinomycetales

1700
Ehrlich, R.; Ehrmantraut, H.C. 1955. See MP 3, Vol. I, No. 20.

#### 2. Bacillaceae

1710
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1720
Cherry, W.B.; Freeman, E.M. 1959. See MP 3, Vol. I, No. 150.

1730
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1740
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1750
Kuzmin, N.A. 1962. See MP 3, Vol. I, No. 230.

1760
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1770
Riggs, J.L.; Seiwald, R.J.; Burckhalter, J.H.; Downs, C.M.; Metcalf, T.G. 1958. See MP 3, Vol. I, No. 280.

#### 3. Bedsonia (Miyagawanella)

1780
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1790
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1810
Starr, T.J.; Pollard, M.; Tanami, Y. 1960. See MP 3, Vol. I, No. 350.

1820
Starr, T.J.; Pollard, M.; Tanami, Y.; Moore, R.W. 1960. See MP 3, Vol. I, No. 360.

4. Brucellaceae

1830
Chadwick, P.; Slade, J.H.R. 1960. See MP 3, Vol. I, No. 460.

1840
Eldering, G.; Eveland, W.C.; Kendrick, P.L. 1961. See MP 3, Vol. I, No. 520.

1850
Eldering, G.; Eveland, W.C.; Kendrick, P.L. 1962. See MP 3, Vol. I, No. 530.

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Jaeger, R.F.; Spertzel, R.O.; Kuehne, R.W. 1961. See MP 3, Vol. I, No. 580.

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Janney, G.C.; Berman, D.T. 1962. See MP 3, Vol. I, No. 590.

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Riggs, J.L.; Seiwald, R.J.; Burckhalter, J.H.; Downs, C.M.; Metcalf, T.G. 1958. See MP 3, Vol. I, No. 800.

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1990
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2000
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5. Corynebacteriaceae

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2020
Marshall, J.D.; Eveland, W.C.; Smith, C.W. 1959. See MP 3, Vol. I, No. 1010.

2030
Smith, C.W.; Marshall, J.D.; Eveland, W.C. 1960. See MP 3, Vol. I, No. 1050.

6. Enterobacteriaceae

2040
Dashkevich, I.O.; Diakov, S.I. 1959. See MP 3, Vol. I, No. 1160.

2050
Dashkevich, I.O.; Diakov, S.I.; Ermakov, N.V.; Ivanova, M.T.; Maiboroda, G.M. 1959. See MP 3, Vol. I, No. 1170.

2060
Dashkevich, I.O.; Mikhailov, I.F. 1957. See MP 3, Vol. I, No. 1190.

2070
Ehrlich, R.; Ehrmantraut, H.C. 1955. See MP 3, Vol. I, No. 1220.

2080
Goldman, M.; Carver, R.K. 1961. See MP 3, Vol. I, No. 1260.

2090
Marrack, J. 1934. See MP 3, Vol. I, No. 1400.

2100
Martineau, B. 1962. See MP 3, Vol. I, No. 1410.

2110
Petuely, F.; Lindner, G. 1959. See MP 3, Vol. I, No. 1520.

2120
Poetschke, G.; Uehleke, H.; Killisch, L. 1959. See MP 3, Vol. I, No. 1540

2130
Riggs, J.L.; Loh, P.C.; Eveland, W.C. 1960. See MP 3, Vol. I, No. 1560.

2140
Riggs, J.L.; Seiwald, R.J.; Burckhalter, J.H.; Downs, C.M.; Metcalf, T.G. 1959. See MP 3, Vol. I, No. 1570.

2150
Silverstein, A.M.; Eveland, W.C.; Marshall, J.D. 1957. See MP 3, Vol. I, No. 1620.

2160
Tanaka, N.; Yamaguchi, H.; Nishimura, T.; Yoshiyuki, T. 1960. See MP 3, Vol. I, No. 1670.

2170
Thomason, B.M.; Cherry, W.B.; Davis, B.R.; Pomales-Lebron, A. 1961. See MP 3, Vol. I, No. 1700.

7. Lactobacillaceae

2180
Fox, E.N. 1962. See MP 3, Vol. I, No. 1890.

2190
Moody, M.D.; Ellis, E.C.; Updyke, E.L. 1958. See MP 3, Vol. I, No. 2070.

2200
Pittman, B.; Moody, M.D. 1960. See MP 3, Vol. I, No. 2120.

2210
Redys, J.J.; Ross, M.R.; Borman, E.K. 1960. See MP 3, Vol. I, No. 2140.

2220
Sercarz, E.; Coons, A.H. 1959. See MP 3, Vol. I, No. 2180.

8. Micrococcaceae

2230
Cohen, J.O.; Cowart, G.S.; Cherry, W.B. 1961. See MP 3, Vol. I, No. 2300.

2240
deRepentigny, J.; Sonea, S. 1959. See MP 3, Vol. I, No. 2330.

9. Mycoplasma (PPLO)

2250
Barile, M.F.; Malizia, W.F.; Riggs, D.B. 1962. See MP 3, Vol. I, No. 2350.

2260
Carski, T.R.; Shepard, C.C. 1961. See MP 3, Vol. I, No. 2370.

2270
Clyde, W.A. 1961. See MP 3, Vol. I, No. 2410.

2280
Clyde, W.A. Denny, F.W. 1962. See MP 3, Vol. I, No. 2420.

2290
Eaton, M.D.; Farnham, A.E.; Levinthal, J.D.; Scala, A.R. 1962. See MP 3, Vol. I, No. 2470.

10. Neisseriaceae

2300
Deacon, W.E. 1961. See MP 3, Vol. I, No. 2570.

2310
Harris, A.; Deacon, W.E.; Smith, C.A. 1957. See MP 3, Vol. I, No. 2610.

11. Pseudomonadales

2320
deRepentigny, J.; Sonea, S. 1959. See MP 3, Vol. I, No. 2670.

12. Spirillaceae

2330
Marrack, J. 1934. See MP 3, Vol. I, No. 2760.

2340
Tabakov, P.K.; Chibrikova, E.V.; Shurkina, I.I.; Velner, E.I. 1962. See MP 3, Vol. I, No. 2790.

13. Spirochaetales

2350
Deacon, W.E.; Freeman, E.M. 1960. See MP 3, Vol. I, No. 2900.

2360
Deacon, W.E.; Freeman, E.M.; Harris, A. 1960. See MP 3, Vol. I, No. 2910.

2370
Harris, A.; Deacon, W.E.; Smith, C.A. 1957. See MP 3, Vol. I, No. 3000.

2380
Vanini, G.C.; Mandras, A.; Ciarlini, E. 1962. See MP 3, Vol. I, No. 3180.

14. Other Bacteria

2390
Dashkevich, I.O.; Diakov, S.I.; Nikitin, V.M.; Osipova, I.V. 1962. See MP 3, Vol. I, No. 3270.

2400
deRepentigny, J.; Sonea, S. 1962. See MP 3, Vol. I, No. 3280.

2410
Lipkin, M.E.; Veselov, V.A.; Pushkova, K.T. 1961. See MP 3, Vol. I, No. 3330.

2420
Sonea, S.; deRepentigny, J. 1961. See MP 3, Vol. I, No. 3360.

B. VIRUSES

1. Adenovirus

2430
Boyer, G.S.; Denny, F.W.; Ginsberg, H.S. 1959. See MP 3, Vol. No. 1070.

2440
Boyer, G.S.; Denny, F.W.; Ginsberg, H.S. 1959. See MP 3, Vol. II, No. 1080.

2450
Moulton, J.E.; Frazier, L.M. 1961. See MP 3, Vol. II, No. 1110.

2460
Philipson, L. 1961. See MP 3, Vol. II, No. 1130.

2470
Riggs, J.L.; Seiwald, R.J.; Burckhalter, J.H.; Downs, C.M.; Metcalf, T.G. 1958. See MP 3, Vol. II, No. 1140.

2. Arbovirus

2480
De Groot, C.J.; Metzger, J.F.; Smith, C.W.; Hoggan, M.D. 1960. See MP 3, Vol. II, No. 30.

2490
Gaidamovich, S.Ya.; Lvova, A.I.; Klimenko, S.M. 1961. See MP 3, Vol. II, No. 40.

2500
Ivanovsky, D.I. 1[illegible]. See MP 3, Vol. II, No. 50.

2510
Lvova, A.I.; Tutova, N.G. 1962. See MP 3, Vol. II, No. 70.

2520
Mettler, N.; Buckley, S.M.; Casals, J. 1961. See MP 3, Vol. II, No. 80.

2530
Metzger, J.F.; Banks, I.S.; Smith, C.W.; Hoggan, M.D. 1961. See MP 3, Vol. II, No. 90.

2540
Noyes, W.F. 1955. See MP 3, Vol. II, No. 110.

2550
Rapp, F.; Buckley, S.M. 1962. See MP 3, Vol. II, No. 120.

2560
Wiebenga, N.H. 1960. See MP 3, Vol. II, No. 140.

2570
Wiebenga, N.H. 1961. See MP 3, Vol. II, No. 150.

2580
Wiebenga, N.H. 1961. See MP 3, Vol. II, No. 160.

3. Herpesvirus

2590
Enders, J.F. 1956. See MP 3, Vol. II, No. 910.

2600
Enders, J.F. 1956. See MP 3, Vol. II, No. 920.

2610
Hoggan, M.D.; Roizman, B.; Roane, P.R., Jr. 1960. See MP 3, Vol. II, No. 930.

2620
Hoggan, M.D.; Roizman, B.; Roane, P.R., Jr. 1961. See MP 3, Vol. II, No. 940.

2630
Lebrun, J. 1956. See MP 3, Vol. II, No. 960.

2640
Lebrun, J. 1956. See MP 3, Vol. II, No. 970.

2650
O'Dea, J.F.; Dineen, J.K. 1957. See MP 3, Vol. II, No. 990.

2660
Roizman, B. 1961. See MP 3, Vol. II, No. 1000.

2670
Ross, R.W.; Orlans, E. 1958. See MP 3, Vol. II, No. 1010.

2680
Toriumi, J. 1962. See MP 3, Vol. II, No. 1020.

2690
Weller, T.H.; Coons, A.H. 1954. See MP 3, Vol. II, No. 1050.

4. Myxovirus, including Influenza

2700
Baker, R.F.; Rapp, F.; Grogan, E.A.; Gordon, I. 1957. See MP 3, Vol. II, No. 1550.

2710
Bennett, C.R.; Hamre, D. 1961. See MP 3, Vol. II, No. 1560.

2720
Bennett, C.R.; Hamre, D. 1962. See MP 3, Vol. II, No. 1570.

2730
Breitenfeld, P.M.; Schafer, W. 1957. See MP 3, Vol. II, No. 1580.

2740
Bukrinskaya, A.G. 1962. See MP 3, Vol. II, No. 1590.

2750
Bukrinskaya, A.G.; Zhdanov, V.M. 1961. See MP 3, Vol. II, No. 1600.

2760
Cantell, K.; Skurska, Z.; Paucker, K.; Henle, W. 1962. See MP 3, Vol. II, No. 1610.

2770
Cohen, S.M.; Gordon, I.; Rapp, F.; Macaulay, J.C.; Buckley, S.M. 1955. See MP 3, Vol. II, No. 1650.

2780
Deibel, R.; Hotchin, J.E. 1959. See MP 3, Vol. II, No. 2110.

2790
Duemler, L.P. 1958. See MP 3, Vol. II, No. 1670.

2800
Enders, J.F. 1956. See MP 3, Vol. II, No. 2120.

2810
Franklin, R.M. 1958. See MP 3, Vol. II, No. 1680.

2820
Franklin, R.M.; Breitenfeld, P.M. 1959. See MP 3, Vol. II, No. 1690.

2830
Frickey, P.H.; Stewart, R.B. 1962. See MP 3, Vol. II, No. 2150.

2840
Gilfillan, R.F. 1961. See MP 3, Vol. II, No. 2160.

2850
Hillis, W.D.; Moffat, M.; Holtermann, O.A. 1960. See MP 3, Vol. II, No. 2190.

2860
Hinuma, Y.; Sekino, K.; Kashiwazaki, H. 1962. See MP 3, Vol. II, No. 2210.

2870
Holtermann, O.A.; Hillis, W.D.; Moffat, M. 1960. See MP 3, Vol. II, No. 2220.

2880
Huang, J.S.; Bang, F.B. 1961. See MP 3, Vol. II, No. 2230.

2890
Ishida, N. 1962. See MP 3, Vol. II, No. 2260.

2900
Kisch, A.L.; Johnson, K.M.; Chanock, R.M. 1962. See MP 3, Vol. II, No. 1700.

2910
Liu, C.; Chu, I.S.; Sharp, E.; Detert, A. 1961. See MP 3, Vol. II, No. 1720.

2920
Loffler, H. 1962. See MP 3, Vol. II, No. 2350.

2930
Loffler, H.; Henle, G.; Henle, W. 1962. See MP 3, Vol. II, No. 2360.

2940
Maassab, H.F.; Loh, P.C. 1962. See MP 3, Vol. II, No. 1750.

2950
Mason, R.J.; Titus, D.J. 1962. See MP 3, Vol. II, No. 1760.

2960
Moffat, M.; Holtermann, O.A.; Hillis, W.D. 1960. See MP 3, Vol. II, No. 2380.

2970
Oosato, T.; Ishida, N. 1961. See MP 3, Vol. II, No. 1790.

2980
Paucker, K.; Cantell, K.; Henle, W. 1962. See MP 3, Vol. II, No. 1800.

2990
Paucker, K.; Skurska, Z.; Henle, W. 1962. See MP 3, Vol. II, No. 2390.

3000
Prince, A.M.; Ginsberg, H.S. 1957. See MP 3, Vol. II, No. 1810.

3010
Rapp, F.; Gordon, I. 1958. See MP 3, Vol. II, No. 1820.

3020
Rapp, F.; Gordon, I.; Baker, R.F. 1960. See MP 3, Vol. II, No. 1830.

3030
Rapp, F.; Seligman, S.J.; Gordon, I. 1959. See MP 3, Vol. II, No. 1840.

3040
Rapp, F.; Seligman, S.J.; Jaross, L.B.; Gordon, I. 1959. See MP 3, Vol. II, No. 1850.

3050
Riggs, J.L.; Loh, P.C.; Eveland, W.C. 1960. See MP 3, Vol. II, No. 1860.

3060
Roizman, B.; Schluederberg, A.E. 1961. See MP 3, Vol. II, No. 1870.

3070
Roizman, B.; Schluederberg, A.E. 1962. See MP 3, Vol. II, No. 1880.

3080
Rustigian, R. 1962. See MP 3, Vol. II, No. 1890.

3090
Sugiura, A.; Enomoto, C.; Ayai, M. 1962. See MP 3, Vol. II, No. 2410.

3100
Tanaka, N.; Yamaguchi, H.; Kobavashi, M. 1959. See MP 3, Vol. II, No. 1910.

3110
Toyoshima, K.; Hata, S.; Takahashi, M.; Miki, T.; Okuno, Y. 1960. See MP 3, Vol. II, No. 1920.

3120
Toyoshima, K.; Takahashi, M.; Hata, S.; Kunita, N.; Okuno, Y. 1959. See MP 3, Vol. II, No. 1930.

3130
Traver, M.I.; Northrop, R.L.; Walker, D.L. 1960. See MP 3, Vol. II, No. 2450.

3140
Tyrell, D.A.J.; Klemperer, H.G. 1959. See MP 3, Vol. II, No. 2460.

3150
Walker, D.L.; Hinze, H.C. 1962. See MP 3, Vol. II, No. 1940.

3160
Watson, B.K. 1952. See MP 3, Vol. II, No. 1950.

3170
Wheelock, E.F. 1960. See MP 3, Vol. II, No. 2500.

3180
Wheelock, E.F.; Tamm, I. 1959. See MP 3, Vol. II, No. 2510.

3190
Wheelock, E.F.; Tamm, I. 1961. See MP 3, Vol. II, No. 1970.

3200
Wheelock, E.F.; Tamm, I. 1961. See MP 3, Vol. II, No. 1980.

3210
Zhdanov, V.M.; Azadova, N.B.; Bukrinskaya, A.G. 1962. See MP 3, Vol. II, No. 1990.

3220
Zhdanov, V.M.; Bukrinskaya, A.G. 1962. See MP 3, Vol. II, No. 2000.

3230
Zhdanov, V.M.; Bukrinskaya, A.G.; Azadova, N.B. 1961. See MP 3, Vol. II, No. 2010.

5. Picornavirus

3240
Buckley, S.M. 1956. See MP 3, Vol. II, No. 1240.

3250
Buckley, S.M. 1957. See MP 3, Vol. II, No. 1250.

3260
Crowther, D.; Melnick, J.L. 1961. See MP 3, Vol. II, No. 1260.

3270
Hatch, M.H. 1962. See MP 3, Vol. II, No. 1290.

3280
Hatch, M.H.; Kalter, S.S.; Ajello, G.W. 1961. See MP 3, Vol. II, No. 1300.

3290
Henry, C.; Franklin, R.M. 1959. See MP 3, Vol. II, No. 1310.

3300
Hinuma, Y.; Hummeler, K. 1961. See MP 3, Vol. II, No. 1320.

3310
Hinuma, Y.; Sekino, K.; Kashiwazaki, H. 1962. See MP 3, Vol. II, No. 133(

3320
Kalter, S.S.; Hatch, M.H.; Ajello, G.W. 1959. See MP 3, Vol. II, No. 134

3330
Kirillova, F.M. 1961. See MP 3, Vol. II, No. 1350.

3340
Larson, V.M. 1962. See MP 3, Vol. II, No. 1360.

3350
Lebrun, J. 1957. See MP 3, Vol. II, No. 1370.

3360
Maksimovich, N.A.; Golub, N.F.; Borodai, V.M. 1962. See MP 3, Vol. II, No. 1380.

3370
Mattern, C.F.T.; Chi, L.L. 1962. See MP 3, Vol. II, No. 1390.

3380
Mayor, H.D. 1961. See MP 3, Vol. II, No. 1400.

3390
Mayor, H.D.; Jordan, L.E. 1962. See MP 3, Vol. II, No. 1410.

3400
Mueller, F.; Klein, P. 1959. See MP 3, Vol. II, No. 1440.

3410
Mussgay, M. 1958. See MP 3, Vol. II, No. 1450.

3420
Nuggolo, L.; Ravetta, M.; Vellucci, A. 1962. See MP 3, Vol. II, No. 1460

3430
Page, R.H.; Stulberg, C.S. 1962. See MP 3, Vol. II, No. 1470.

3440
Pauluzzi, S.; Felici, A. 1961. See MP 3, Vol. II, No. 1480.

3450
Riggs, J.L.; Brown, G.C. 1960. See MP 3, Vol. II, No. 1490.

3460
Riggs, J.L.; Brown, G.C. 1961. See MP 3, Vol. II, No. 1500.

3470
Riggs, J.L.; Brown, G.C. 1962. See MP 3, Vol. II, No. 1520.

3480
Riggs, J.L.; Loh, P.C.; Eveland, W.C. 1960. See MP 3, Vol. II, No. 1530.

3490
Toriumi, J. 1962. See MP 3, Vol. II, No. 1540.

6. Poxvirus

3500
Avakian, A.A.; Altshtein, A.D.; Kirillova, F.M.; Bykovski, A.F. 1961. See MP 3, Vol. II, No. 170.

3510
Cairns, J. 1960. See MP 3, Vol. II, No. 180.

3520
Easterbrook, K.B. 1961. See MP 3, Vol. II, No. 190.

3530
Easterbrook, K.B. 1961. See MP 3, Vol. II, No. 200.

3540
Enders, J.F. 1956. See MP 3, Vol. II, No. 210.

3550
Enders, J.F. 1956. See MP 3, Vol. II, No. 220.

3560
Holden, M.; Adams, L.B. 1962. See MP 3, Vol. II, No. 230.

3570
Joklik, W.K.; Rodrick, J.McN. 1959. See MP 3, Vol. II, No. 240.

3580
Loh, P.C.; Riggs, J.L. 1961. See MP 3, Vol. II, No. 290.

3590
Loh, P.C.; Riggs, J.L. 1961. See MP 3, Vol. II, No. 300.

3600
Mastyukova, Yu.N.; Kabanova, Ye.A. 1961. See MP 3, Vol. II, No. 310.

3610
Noyes, W.F.; Watson, B.K. 1955. See MP 3, Vol. II, No. 350.

3620
Spendlove, R.S.; Lennette, E.H. 1962. See MP 3, Vol. II, No. 380.

3630
Weller, T.H.; Coons, A.H. 1954. See MP 3, Vol. II, No. 420.

7. Rabiesvirus

3640
Etchebarne, M.; Bernal, P.G.; Leyton, G.R. 1960. See MP 3, Vol. II, No. 1150.

3650
Goldwasser, R.A.; Kissling, R.E. 1958. See MP 3, Vol. II, No. 1160.

3660
Kaplan, M.M.; Forsek, Z.; Koprowski, H. 1960. See MP 3, Vol. II, No. 11

3670
McQueen, J.L.; Lewis, A.L.; Schneider, N.J. 1960. See MP 3, Vol. II, No. 1210.

8. Reovirus

3680
Rhim, J.S.; Jordan, L.E.; Meyor, H.D. 1962. See MP 3, Vol. II, No. 2050.

3690
Spendlove, R.S.; Lennette, E.H. 1962. See MP 3, Vol. II, No. 2070.

9. Tumorvirus

3700
Fraser, K.B.; Gharpure, M. 1962. See MP 3, Vol. II, No. 450.

3710
Girardi, A.J.; Sweet, B.H.; Slotnick, V.B.; Hilleman, M.R. 1962. See MP Vol. II, No. 490.

3720
Hampton, E.G.; Eidinoff, M.L. 1962. See MP 3, Vol. II, No. 500.

3730
Henle, G.; Deinhardt, F.; Rodriguez, J. 1959. See MP 3, Vol. II, No. 510

3740
Ito, M. 1960. See MP 3, Vol. II, No. 520.

3750
Ito, M.; Nishioka, K. 1959. See MP 3, Vol. II, No. 530.

3760
Malmgren, R.A.; Rabotti, G. 1960. See MP 3, Vol. II, No. 580.

3770
Noyes, W.F. 1960. See MP 3, Vol. II, No. 670.

3780
Padgett, B.L.; Walker, D.L. 1962. See MP 3, Vol. II, No. 690.

3790
Rubin, H.; Vogt, P.K. 1962. See MP 3, Vol. II, No. 720.

3800
Shein, H.M.; Enders, J.F.; Levinthal, J.D. 1962. See MP 3, Vol. II, No. 750.

3810
Shein, H.M.; Levinthal, J.D. 1962. See MP 3, Vol. II, No. 760.

3820
Takahashi, M.; Kato, S.; Kameyama, S.; Kamahora, J. 1959. See MP 3, Vol. II, No. 820.

3830
Vogt, M.; Dulbecco, R. 1962. See MP 3, Vol. II, No. 840.

3840
Vogt, P.K.; Rubin, H. 1961. See MP 3, Vol. II, No. 850.

10. Other Viruses

3850
Carski, T.R. 1959. See MP 3, Vol. II, No. 2530.

3860
Carski, T.R. 1960. See MP 3, Vol. II, No. 2540.

3870
Dowdle, W.R.; Hansen, P.A. 1961. See MP 3, Vol. II, No. 2620.

3880
Hillis, W.D. 1961. See MP 3, Vol. II, No. 2630.

3890
Hopkins, S.R.; Janney, G.C. 1962. See MP 3, Vol. II, No. 2650.

3900
Livingston, C.W.; Moore, R.W. 1962. See MP 3, Vol. II, No. 2680.

3910
Rapp, F.; Friend, C. 1962. See MP 3, Vol. II, No. 2720.

3920
Shurin, S.F.; Yavorovskaya, B.Ye.; Lozovoi, V.P. 1961. See MP 3, Vol. II, No. 2760.

## C. FUNGI, METAZOA, PROTOZOA, RICKETTSIAE

### 1. Fungi

3930
Al-Doory, Y.; Gordon, M.A. 1962. See MP 3, Vol. III, No. 10.

3940
deRepentigny, J.; Sonea, S. 1959. See MP 3, Vol. III, No. 40.

3950
Kase, A.; Marshall, J.D. 1960. See MP 3, Vol. III, No. 130.

3960
Kaufman, L.; Kaplan, W. 1961. See MP 3, Vol. III, No. 150.

### 2. Metazoa

3970
Anderson, R.I.; Sadun, E.H.; Williams, J.S. 1961. See MP 3, Vol. III, No. 370.

3980
Coleman, R.M.; Dutt, G.B. 1960. See MP 3, Vol. III, No. 430.

### 3. Protozoa

3990
Beale, G.H.; Mott, M.R. 1962. See MP 3, Vol. III, No. 610.

4000
Carver, R.K.; Goldman, M. 1959. See MP 3, Vol. III, No. 630.

4010
Fife, E.H.; Muschel, L.H. 1959. See MP 3, Vol. III, No. 660.

4020
Goldman, M. 1957. See MP 3, Vol. III, No. 690.

4030
Goldman, M. 1959. See MP 3, Vol. III, No. 710.

4040
Goldman, M. 1960. See MP 3, Vol. III, No. 720.

4050
Goldman, M.; Carver, R.K.; Gleason, N.N. 1960. See MP 3, Vol. III, No. 740.

4060
Tobie, J.E.; Burgdorfer, W.; Larson, C.L.; Evans, C.B.; Robinson, C.E. 1961. See MP 3, Vol. III, No. 960.

4. Rickettsiae

4070
Lipkin, M.E.; Veselov, V.A.; Pushkova, K.T. 1961. See MP 3, Vol. III, No. 1120.

4080
Roberts, A.N.; Downs, C.M. 1959. See MP 3, Vol. III, No. 1140.

4090
Tobie, J.E.; Burgdorfer, W.; Larson, C.L.; Evans, C.B.; Robinson, C.E. 1961. See MP 3, Vol. III, No. 1170.

D. PHYSIOLOGICAL APPLICATION

1. Collagen Diseases

4100
Beutner, E.H.; Nagy, C.F. 1962. See MP 3, Vol. IV, No. 120.

4110
Crawford, H.J.; Wood, R.M.; Lessef, M.H. 1959. See MP 3, Vol. IV, No. 210.

4120
Friou, G.J. 1958. See MP 3, Vol. IV, No. 260.

4130
Friou, G.J. 1962. See MP 3, Vol. IV, No. 300.

4140
Mandema, E.; Pollack, V.E.; Kark, R.M.; Rezaian, J. 1961. See MP 3, Vol. IV, No. 530.

4150
Shurin, S.F.; Yavorovskaya, B.Ye.; Lozovoi, V.F. 1961. See MP 3, Vol. IV, No. 740.

4160
Widelock, D.; Gilbert, G.; Siegel, M.; Lee, S. 1961. See MP 3, Vol. IV, No. 870.

2. Miscellaneous Applications

4170
Cher, H.Y. 1962. See MP 3, Vol. IV, No. 3720.

4180
Clayton, R.M. 1960. See MP 3, Vol. IV, No. 3730.

4190
Cormack, D.E.; Easty, G.C.; Ambrose, E.J. 1961. See MP 3, Vol. IV, No. 3760.

4200
Goldstein, M.N.; Hiramoto, R.; Pressman, D. 1959. See MP 3, Vol. IV, No. 4040.

4210
Hasebe, H. 1962. See MP 3, Vol. IV, No. 1400.

4220
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13. ABSTRACT

This volume is one of a series of six annotated bibliographies on various aspects of immunofluorescence and its use. Citations cover the period 1905 through 1962. Volume VI is divided into two major sections. The first section contains 169 annotated citations to general procedures in the use of immunofluorescence, arranged by subject areas. The second section contains 226 cross-references to citations in the other volumes that describe specific applications of this technique; these are arranged to correspond with the subject areas of the other volumes. A complete author index for these 425 citations is included.

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