Analytical Cytology

ANALYTICAL CYTOLOGY

Methods for Studying

Cellular Form and Function

Edited by

ROBERT C. MELLORS, M.D., PH.D.

Pathologist, Director of Laboratories, and Associate Director of Research, Hospital for Special Surgery, Philip D. Wilson Research Foundation, an Affiliate of the New York Hospital-Cornell Medical Center, New York. Formerly at the Sloan-Kettering Institute for Cancer Research and the Memorial Center for Cancer and Allied Diseases

Second Edition

The Blakiston Division

McGRAW-HILL BOOK COMPANY, INC.

NEW YORK TORONTO LONDON

1959

Preface

The purpose of this work is to present a discussion of the theory and the practical applications of some of the physical and chemical methods presently used in the analysis of cellular structure and function. This field of scientific investigation, which encompasses biophysical and biochemical cytology, has been appropriately designated *analytical cytology* by Professor Francis Schmitt. The preparation of this book was undertaken with the collaboration of a panel of authors who are identified with original work in the various fields under discussion. It is my privilege and foremost obligation in this preface to thank these authors for their contributions. *Analytical Cytology* is intended to serve as a reference book for research workers, teachers, and graduate and advanced undergraduate students in the biological and the medical sciences. The need for a work of this type and the measure of its success were shown by the favorable reception afforded the first edition. As in that edition, the general format of each chapter includes an introduction, theoretical and practical aspects, applications, illustrations, interpretations, and references.

The first chapter, an entirely new one, is devoted to the technique and the applications of the fluorescent-antibody method. The scope of the work already undertaken with, or envisioned for, this new procedure appears ample to establish it as one of the major advances in microscopic methodology. The unique attribute of the fluorescent-antibody method is that it provides the microscopist with a staining procedure which has the inherent specificity of immunochemical reactions. The second chapter, dealing with the intracellular localization of chemical constituents, is a new contribution and presents the integrative synthesis of biomorphology and biochemistry which constitutes a principal objective and a major achievement of analytical cytology. Phase, interference, and polarizing microscopy are *in-situ* (nondestructive) physical methods which are, among other things, applicable to the study of living cells. The theory and the practice of these optical methods and their use in quantitative cytology, for example, in the measurement of the mass (dry weight) and the mass concentration of cellular constituents, are discussed in Chapter 3, which has been substantially revised. Contemporary knowledge of cellular structure and function is being refined and expanded at an enormous rate by electron microscopy as a consequence of the high

resolution of ultrastructure which this analytical method affords. The techniques and applications of electron microscopy in cytology are presented in Chapter 4, which has been materially changed to keep abreast of the rapid advances in this field. X-ray microscopy may be used to determine the mass (dry weight) and the thickness of cytological objects, and under specified conditions, as discussed in revised Chapter 5, certain elementary chemical analyses of microscopic structures may be performed. The utilization of radioactive tracers and the procedure of autoradiography affords an opportunity (shared by few techniques) of studying the in-vivo cellular localization of labeled constituents. The use of autoradiography in cytology is presented in Chapter 6, which is but little altered since the first edition. The last chapter, also revised, deals with the photometric chemical analysis of cells, a procedure which, dating from its original inception to the present day, has contributed immeasurably to the development of cytology and other fields of biology.

Acknowledgement is made throughout the text to individuals, societies, and publishers for their generous permission to reproduce illustrations (some of which have been kindly supplied in the original) and tables from journals and books.

ROBERT C. MELLORS

Contents

CHAPTER 1 *Fluorescent-antibody Method*

ROBERT C. MELLORS, M.D., PH.D.

The Hospital for Special Surgery, New York Hospital—Cornell Medical Center; formerly at Sloan-Kettering Institute for Cancer Research, Memorial Center for Cancer and Allied Diseases: New York

Introduction

The underlying principle of the fluorescent-antibody method developed by A. H. Coons and his associates (9–17) is based upon the observation (57) that antibody molecules can be chemically linked with simple compounds and dyes without destroying the specific reactivity of the antibody with its antigen. The first report on the detection of antigenic material by means of fluorescein-labeled antibody appeared in 1941 (11), and improvements in the method were presented in 1950 (13). Since then numerous papers dealing with biologic and medical applications of the fluorescent-antibody method have appeared. An essentially complete list of these publications is found at the conclusion of this chapter. The scope of the work already undertaken with, or envisioned for, this technique appears ample to establish this procedure as one of the major developments in microscopic methodology. Suitably controlled, the fluorescent-antibody method provides the microscopist with a staining procedure that has the inherent specificity of immunochemical reactions.

The method has been used for the microscopic identification of infectious agents (viruses, bacteria, rickettsiae, fungi, and protozoa); injected foreign antigens (proteins and polysaccharides); intrinsic antigens, proteins, enzymes, and hormones; and specific antibodies. Some of the additional applications include the study of glomerulonephritis and related renal diseases; rheumatic and related connective-tissue diseases; hypersensitivity; lupus erythematosus cell reaction; hemolytic anemia; thrombocytopenic purpura; cancer; and tissue-transplantation tolerance and immunity. In this chapter the discussion of the technique and the applications of the fluorescent-antibody method is concerned principally, although not solely, with fluorescein conjugates. Preliminary reports on the use of three other labeling compounds for antibodies have appeared in the literature: 1, 5-dimethylamino-5, 1-naphthalene sulfonyl chloride, producing a yellow fluorescence (6);

nuclear fast red, a red fluorescence (6); and rhodamine B, an orange-red fluorescence (86). The latter two dyes, like fluorescein, are chemically converted to the isocyanate for conjugation with protein.

Technique

When layered over a histologic section or cytologic preparation, fluorescent antibody is deposited from solution, as in a microprecipitin reaction, at sites of specific combination with antigen. The reaction

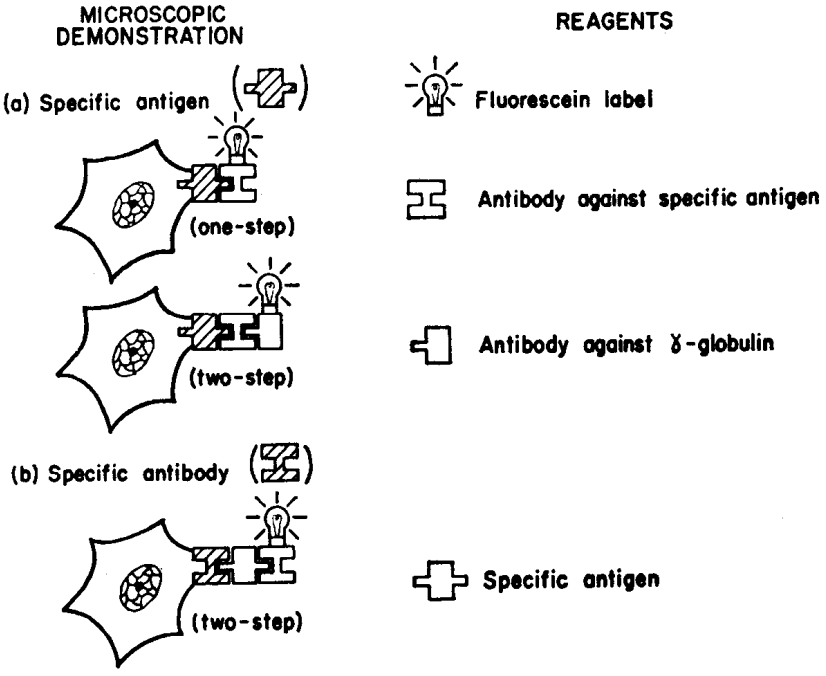

FIG. 1·1 Symbolic representation of the two principal uses of the fluorescent-antibody method for the microscopic detection of (*a*) specific antigen and (*b*) specific antibody.

sites are perceived in characteristic color in the fluorescence microscope, notably, with fluorescein-labeled antibody, in yellow-green (apple green), a color readily distinguished from, and rarely if ever shown by, the intrinsic fluorescence of tissue sections (35).

In theory and practice, as symbolized in Fig. 1·1, the two principal uses of the fluorescent-antibody method are for the microscopic detection of (*a*) specific antigen and (*b*) specific antibody. Specific antigen can be identified in the one-step, one-layer, or *direct* procedure, originally described by Coons and Kaplan (13), by staining directly with fluorescein-labeled antibody against the specific antigen. Spe-

cific antigen can also be detected by a two-step, two-layer, or *indirect* procedure (101, 53). In the first step of the procedure unlabeled specific antibody is deposited over the antigen. In the second step, fluorescein-labeled antibody against the deposited gamma globulin is used to form a second and an indirectly, identifying layer at the reaction site.

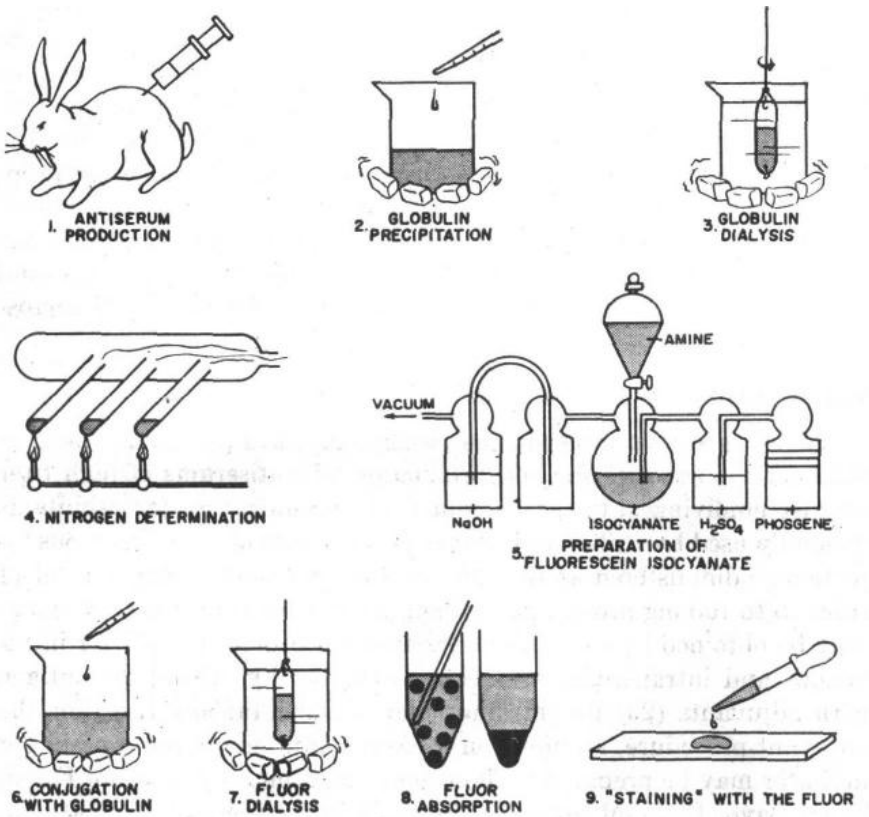

FIG. 1·2 Symbolic representation of some of the technical procedures in the fluorescent-antibody method.

Specific antibody in tissue sections is identified by a two-step procedure described by Coons, Leduc, and Connolly (15). In the first step of the procedure, the section is exposed to a solution of the specific unlabeled antigen which combines with free reactive sites, if present, on the antibody, and the section is subsequently washed in buffered saline solution to remove unbound antigen. In the second step, the binding of antigen in vitro is disclosed by staining with fluorescent antibody against the specific antigen. A companion slide, in which treatment in vitro with specific antigen is omitted, is similarly stained to serve as one of the control procedures. By these composite means the presence of specific antibody is indirectly detected.

Some of the technical aspects of the fluorescent-antibody method are symbolized in Fig. 1·2. They include (1) preparation of antiserum; (2) concentration of antibodies by precipitation of the crude gamma globulin fraction; (3) dialysis of the reconstituted globulin solution; (4) microkjeldahl determination of the nitrogen (and the protein) content of the globulin solution; (5) conversion of fluorescein amine to fluorescein isocyanate by dropwise addition of the amine to acetone saturated with phosgene gas; (6) addition of fluorescein isocyanate to the globulin and formation of the fluorescein conjugate of the globulin, i.e., the fluorescent antibody, called simply the fluor; (7) dialysis of the fluor; (8) absorption of the fluor with tissue powder to reduce the property of nonspecific staining; and (9) staining a tissue section with fluorescent antibody. In the use of the fluorescent-antibody method one must have an understanding of certain procedures in immunology (and immunochemistry), histology (and cytology), and fluorescence microscopy.

PREPARATION OF ANTISERUMS

There is great variation in the largely empirical procedures used in different laboratories for the production of antiserums of high titer against nonliving antigens. A course of immunization for rabbits as generally used by Kabat and Mayer (43) consists of 16 intravenous injections, administered at the rate of four per week, using a total of from 10 to 100 mg protein per animal. An enhanced antibody response may be obtained by using alum-precipitated protein (31,38) for intravenous and intraperitoneal injections (43) or by mixing the antigen with adjuvants (25) for intramuscular or subcutaneous use. For the adjuvant procedure, an injection mixture containing 5 mg protein per milliliter may be prepared by homogenizing a mixture of 10 ml Falba, 25 ml Bayol F, 25 ml isotonic saline solution of the protein (300 mg), and 25 mg heat-killed tubercle bacilli. Information relevant to the production of antiserums against viruses and other infectious agents is presented where pertinent in the section dealing with particular applications of the fluorescent-antibody method. Antiserums may be stored in the frozen state in the deep freezer, in the lyophilized state in the refrigerator, or in the sterile state in the refrigerator with or without the addition of preservative (Merthiolate, 1 part by volume in 10,000).

Antiserums obtained from man, rabbit, chicken, goat, monkey, dog, horse, and cow have been used with success in the fluorescent-antibody method. Serums containing high titers of antibodies are required because the layer thickness and the rapidity of deposition, and possibly the closeness of packing, of the antibody on the antigen are enhanced by high concentrations of antibody in simpler systems (1) and presum-

ably also in the fluorescent-antibody method (10). There is a reduction in the titer of antibody incident to the manipulations and the dilutions which occur in the fluorescein-coupling procedure.

PREPARATION OF CRUDE GAMMA-GLOBULIN FRACTION

The antibody-active proteins are separated from the antiserum by preparing the so-called gamma-globulin fraction. This procedure is generally required to reduce the relative concentration of the immunologically indifferent proteins which after conjugation with fluorescein contribute to undesirable nonspecific staining by the fluor. Three preparatory methods are available: (*a*) aqueous ethanol fractionation (8*a*); (*b*) ammonium sulfate fractionation (45); and (*c*) dissociation of specific precipitates.

The initial precipitate obtained in the ethanol-fractionation procedure as modified by Deutsch (23) contains practically all the antibody-active protein (gamma globulins) and lesser amounts of other serum proteins, notably beta globulin. The identical initial precipitation step may be carried out on a variety of animal serums (23). We regularly prepare the initial precipitate and reconstitute the crude gamma-globulin fraction in accordance with the following steps:

1. Dilute serum with 3 vol water.
2. Adjust pH to 7.6 to 7.8 with $NaHCO_3$ or dilute acetic acid.
3. Cool to 0°C.
4. Add cold (−10 to −20°C) 50 per cent ethanol to a concentration of 18 to 25 per cent by volume.
5. Stir for 20 min.
6. Centrifuge at −5°C for 10 min at 14,000 rpm.
7. Decant and reconstitute precipitate to one-third of original serum volume in buffered saline solution (0.8 per cent *w/v* sodium chloride containing 0.01 *M* phosphate at pH 7.0).
8. Dialyze against buffered saline solution overnight in cold room to remove ethanol.

The ammonium sulfate fractionation procedure is as follows:

1. Dilute serum with 1 vol buffered saline solution.
2. To this add equal volume of cold (4°C) saturated ammonium sulfate.
3. Centrifuge.
4. Wash precipitate in cold one-half saturated ammonium sulfate.
5. Centrifuge.
6. Reconstitute precipitate to one-third of original serum volume, using buffered saline solution.
7. Dialyze against buffered saline solution until dialysate is free of ammonium ion by Nessler's test.

PREPARATION OF FLUORESCEIN AMINE (AMINOFLUORESCEIN)

There are presently three commercial sources of fluorescein amine and of fluorescein isocyanate in absolute acetone (Sylvania Chemical Corp., Orange, N.J.; General Biochemicals, Inc., Chagrin Falls, Ohio; and Delta Chemical Works, Inc., New York, N.Y.).

The course of the synthesis of the two isomers of fluorescein amine is described by Coons and Kaplan (13) as follows. Nitrofluorescein is prepared by the fusion of 4-nitrophthalic acid with two equivalents of resorcinol, two isomeric nitrofluoresceins thus resulting. This crude product is refluxed with acetic anhydride to form two isomers of nitrofluorescein diacetate which are separated by fractional crystallization. Each isomer is saponified, and the corresponding nitrofluorescein is recovered. The nitro compounds are reduced by catalytic hydrogenation to the corresponding amino isomers, aminofluorescein I (red crystals) and aminofluorescein II (yellow crystals). Each isomer can be used in the fluorescent-antibody method (13), but in practically all the published work isomer II has apparently been utilized. An alternative synthesis (12) can be used to prepare a mixture of the isomeric aminofluoresceins. After preparing the crude nitrofluorescein, the amines are formed by reduction and are precipitated with excess water (12). The two isomers of aminofluorescein can be separated by chromatography (22).

PREPARATION OF FLUORESCEIN ISOCYANATE AND FLUORESCEIN ISOTHIOCYANATE

Fluorescein amine by treatment with phosgene (13) is converted to fluorescein isocyanate, which is unstable (unless stored in the absence of moisture, heat, and light) and generally used for coupling immediately after preparation. A closed system, Fig. 1·10, consisting of a phosgene-gas train and traps and a reaction vessel with dropping funnel is used under a chemical hood with adequate forced draft. Since phosgene is a toxic gas, it is best to keep a chemical warfare mask nearby for use in the event of untoward emergency. The phosgene is led from the tank, passes by a vacuum manometer, through concentrated H_2SO_4 for drying, thence to the reaction flask, and finally through two traps, the last of which contains 20 per cent NaOH, which destroys the unused phosgene.

The computed amount of fluorescein amine (10 to 50 mg) is dissolved in 5 ml anhydrous acetone (which has been dried over $CaSO_4$ for 12 to 24 hr) and added dropwise from a dropping funnel to 15 ml anhydrous acetone that is saturated with phosgene and through which phosgene is constantly bubbled. With the addition of each drop of amine solution a

yellow precipitate forms and rapidly disappears, and the reaction flask becomes slightly warmer. The reaction is allowed to continue for 30 min after addition of the amine, by which time the flask has returned to room temperature. The reaction flask is removed from the phosgene-gas train, three small anthracite chips are added to diminish bumping, and the excess phosgene and acetone are evaporated *in vacuo* over a water bath at 45°C, leaving a green-brown, gummy residue. This residue is dissolved in 3 ml anhydrous acetone (or in 2 ml anhydrous acetone plus 1 ml anhydrous dioxane), and the resulting solution of fluorescein isocyanate is immediately conjugated with protein, or placed in sealed dry ampuls over $CaSO_4$ desiccant (74) or under nitrogen gas, as is the commercial practice, and may be stored (in the dark) in the deep freezer for periods up to 1 month and on occasion longer. Ampuls containing 25 mg fluorescein isocyanate in 2.5 ml dry acetone are available from the commercial sources cited previously for fluorescein amine.

One of the most important achievements in the field is the synthesis by Riggs *et al.* (81*a*) of fluorescein isothiocyanate, which is prepared from fluorescein amine by the use of thiophosgene instead of phosgene gas. Fluorescein isothiocyanate, an orange-yellow powder, is stable when dry, and is now commercially available in 25-mg lots or multiples thereof from two sources (Sylvania Chemical Corp., Orange, N.J., and Baltimore Biological Laboratory, Baltimore, Md.); it has been used successfully in the preparation of fluorescent antibodies by numerous investigators.

PREPARATION AND PROPERTIES OF FLUORESCENT ANTIBODIES

Fluorescein Conjugates. The optimal conditions for conjugating fluorescein isocyanate to proteins, as specified by Coons and Kaplan (13), introduce about two groups of fluorescein per globulin molecule, through the carbamido linkage, most likely by coupling with the ϵ-amino groups of lysine. The optimum amount of isocyanate is 0.05 mg (calculated as fluorescein amine) per milligram of protein; and the protein concentration in the final reaction mixture is adjusted to 10 mg per ml. It is necessary first to determine by the microkjeldahl procedure the protein concentration in the crude gamma-globulin solution (which should contain at least 20 mg protein per milliliter) and to select a volumetric aliquot, usually one containing 500 mg protein (dry weight). This amount (500 mg) of protein will be adjusted to a concentration of 10 mg per ml in the final mixture, the total volume of reactants then being 50 ml and distributed in the following proportions:

3 ml acetone containing isocyanate equivalent to 25 mg fluorescein amine

7.5 ml purified dioxane
7.5 ml pH 9.0, 0.5 *M* carbonate buffer
500 mg aliquot of protein
isotonic saline solution to make total volume of 50 ml

Lesser or greater quantities (250 to 1,000 mg) of protein may be coupled, using a proportional adjustment in the amount of fluorescein amine (12.5 to 50 mg) and in the total volume of the reaction mixture (25 to 100 ml) while maintaining dioxane and carbonate buffer each at a volumetric proportion of 15 parts per 100 and acetone at 6 to 7 parts.

The necessary reagents are prepared as follows. Dioxane is purified by refluxing for 24 hr with chips of sodium metal and then by distillation at 101°C and storage under sodium. The carbonate buffer is prepared by mixing 0.5 *M* Na_2CO_3 and 0.5 *M* $NaHCO_3$ in the approximate ratio of 4:1 and adjusting the pH to 9.0 with HCl or NaOH.

The procedure for mixing the reactants is as follows. A 250-ml Erlenmeyer flask is placed in an ice bath on a magnetic stirrer. The required amounts of dioxane, buffer, and saline solution are added and chilled to 0–2°C. The chilled protein solution is next added. Finally, the solution of fluorescein isocyanate in acetone is added, drop by drop, and the stirring is continued in the cold for 18 hr.

After 18 hr, the solution is transferred to a cellophane sack and dialyzed for 5 to 6 days against buffered saline solution, changed once or twice daily. The solution is centrifuged, the precipitate is discarded, Merthiolate, 1 part in 10,000, is added to the supernate, and this, the fluorescent-antibody solution, is stored at 4°C, to be purified further as needed for staining. The buffered saline solution used for dialysis (and elsewhere in the fluorescent-antibody procedure) is 0.8 per cent sodium chloride containing 0.01 *M* phosphate buffer at pH 7.0.

If commercial preparations of fluorescein isocyanate are used, the same steps and conditions are employed in the conjugation procedure. For example, an ampul containing 25 mg fluorescein isocyanate in 2.5 ml dry acetone provides sufficient material for the conjugation of 500 mg protein (1 mg fluorescein isocyanate per 20 mg protein) in a final reaction volume of 50 ml (protein concentration, 10 mg per ml). The ampul of fluorescein isocyanate is opened, and the required volume is diluted with one-half volume of purified dioxane; these volumes of acetone (2.5 ml per ampul) and dioxane (1.25 ml per ampul) are subtracted from the calculated amounts of organic solvents added initially to the reaction vessel.

A modification of the conjugation procedure with fluorescein isocyanate has been introduced (34*a*). Five-tenths-milliliter quantities of a 1 per cent (*w*/*v*) fluorescein isocyanate solution (in dioxane-acetone

2:1) are pipetted onto ½-in. Seitz filter disks and allowed to dry *in vacuo* over $CaSO_4$. The disks are stored in a desiccator in the dark until used. To 10 ml of the globulin fraction containing 2.5 per cent protein in a 25-ml Erlenmeyer flask is added 1 ml 0.5 *M* $Na_2CO_3/NaHCO_3$ (pH 9.0). The solution is chilled in an ice bath, three impregnated disks are added, and the contents of the flask are stirred slowly on a magnetic mixer overnight at 4°C. The fluorescent-antibody solution is then centrifuged to remove filter disk material and dialyzed against buffered saline solution until the dialysate shows no trace of fluorescence. A variation of this method is used for labeling small amounts of protein. One impregnated filter pad is placed in a Swinny filter and attached to 2-ml hypodermic syringe containing 2 ml chilled globulin solution to which has been added 0.1 ml pH 9.0 0.5 *M* $Na_2CO_3/NaHCO_3$. The syringe piston is then advanced to force 0.1 ml through the impregnated pad every 5 min. The resulting conjugate is dialyzed as described above. Using these methods, approximately 95 per cent of the proteins originally present in the globulin solution were recovered in the conjugate. The fluorescein/protein ratios of the conjugates were determined by absorption spectrophotometry at 480 mμ (for fluorescein) and at 560 mμ (using the biuret reaction for protein). Ratios of (3.8 to 5.5) $\times$ 10^{-3} indicated satisfactory conjugation.

The procedure for the conjugation of protein with fluorescein isothiocyanate is extremely simple (81*a*). The chemical constituents and amounts used for 500 mg protein are as follows:

3 ml pH 9.0, 0.5 *M* carbonate buffer
2 ml dry acetone (reagent grade)
isotonic saline solution and 500 mg aliquot of protein to make a total volume of 25 ml (inclusive of buffer and acetone)
25 mg fluorescein isothiocyanate (0.05 mg per mg protein) dissolved in 1.5 ml acetone or used as dry powder

It is to be noted that the protein concentration is higher (20 mg per ml) and the volume yield of conjugate correspondingly less in this procedure. The carbonate buffer, acetone, and isotonic saline solution are mixed and chilled to 0°C, the chilled protein solution is added, and finally fluorescein isothiocyanate, either dissolved in acetone just before use or employed as a dry powder, is added directly to the reaction mixture. Stirring or horizontal shaking is continued in the cold for 6 to 18 hr. The solution is then transferred to a cellophane sack and dialyzed in the cold against buffered saline solution (0.15 *M* NaCl containing 0.01 *M* phosphate, pH 7.0), using daily changes for 5 to 6 days. Any precipitate which may form is removed by centrifugation, Merthiolate is added to a concentration of 1 part in 10,000, and the solution is

stored at 4°C, or frozen. There are two variations in the conjugation procedure which may be used in the event that the fluorescent antibody displays little staining activity. The conjugation procedure may be repeated once or more often, observing the same conditions described previously. The fluorescent antibody may be precipitated by cold ethanol or by ammonium sulfate (as described under preparation of crude gamma-globulin fraction) dialyzed free of the precipitant, and may be reconstituted in a minimum volume of buffered saline solution.

Other Labeling Compounds. Nuclear fast red, with red fluorescence (6), rhodamine B, with orange-red fluorescence (86), and tetramethylrhodamine, with orange fluorescence (40*a*), have after conversion to the isocyanates been conjugated with antibodies. 1, 5-Dimethylamino-5, 1-naphthalene sulfonyl chloride, with red fluorescence, has been coupled directly through—SO_2NH—linkage with various proteins (36, 97) and with antibodies (6). Rhodamine B isothiocyanate has been synthesized (81*a*); and lissamine rhodamine (Chadwick, *Immunol.*, **1**: 315, 1958).

Properties of Fluorescein-carbamido-proteins. A detailed study of the physical and biologic properties of fluorescein-labeled bovine serum albumin has been made by Schiller and his associates (83). The electrophoretic pattern of the protein-fluorescein conjugate was substantially the same as that of the unconjugated protein. The isoelectric point of the conjugate was decreased by 0.13 pH unit, corresponding to the introduction of 1.6 molecules of fluorescein per molecule of albumin. The visible absorption spectrum of the conjugate at pH 7.2 had a maximum at 490 to 495 mμ, an absorption peak lacking in bovine albumin but present in sodium fluorescein (see Fig. 1·8 and also discussion under Fluorescence Microscopy, further on in this chapter). The concentrations of fluorescein-labeled proteins were estimated from measurements of optical density at 490 mμ and by fluorophotometry, although the results by the two methods were not consistently the same. The process of conjugating guinea pig serum albumin did not render the protein antigenic for the guinea pig, although slight antigenicity resulted from labeling whole plasma protein. Fluorescein-labeled plasma proteins, particularly albumin, behaved in vivo like their native precursors. The conjugation of fluorescein with antibody does not alter its immunologic specificity (13).

PREPARATION OF TISSUE SECTIONS AND CYTOLOGIC MATERIALS

The requirements in the preparation of tissue sections for study by the fluorescent-antibody method are the preservation of both the specific activity of the antigen and the unaltered microscopic structure of the tissue. Some stable antigens, notably bacterial polysaccharides,

have been studied in tissue sections prepared after formalin and other fixation and paraffin embedding (40, 44*b*). The major work, however, has been done with unfixed tissues, utilizing the procedures of freezing and sectioning in the frozen state (13, 16) or of freezing-drying, paraffin embedding, and sectioning (58). The technique of freezing, substitution with ethanol, and clearing at low temperature, followed by paraffin embedding and sectioning, is worthy of evaluation for use under certain circumstances (72).

Freezing Tissues. Much that has been learned about the mechanics of freezing in living cells and tissues (2, 37, 69, 87) appears to be applicable to freezing for microtomy. The single most important aspect of the freezing process is the removal of pure water from solution and its transformation into ice crystals (69). The crystal size is approximately inversely proportional to the rate of freezing (69). Ice-crystal growth does not take place below approximately −130°C. Above this temperature recrystallization occurs in frozen biologic materials, i.e., there is a preferential growth of large ice crystals at the expense of smaller ones. Crystallization occurs in extracellular, intracellular, and intranuclear spaces and produces an artefactual distortion of the tissue section if the crystals are large. The coolants used in histologic freezing are baths of isopentane at −160°C in liquid nitrogen for rapid freezing, and dry ice–ethanol mixtures (−70°C) and dry ice itself for an intermediate rate of freezing. To minimize the effects of recrystallization, storage must be maintained at least at dry-ice temperature for material that has been rapidly frozen and at deep-freezer temperature (−20°C) for material frozen at an intermediate rate. The deleterious effects of storage are attributed principally to the process of denaturation by dehydration (69). Since denaturation and crystal growth both increase exponentially with temperature, the thawing procedure should be as rapid as possible (56). A second freezing of thawed tissue leads to the growth of large destructive crystals. The addition to tissues of hydrogen binders, such as glycerin (55) in isotonic saline solution, reduces the amount of water available for crystallization and minimizes denaturation by dehydration. Small amounts of certain alcohols, glycols, and sugars reduce the crystallization rate very substantially, for example, by a factor of 1,000 for ethanol at a concentration of 30 per cent (55). The geometry of the specimen is one major factor influencing rate of freezing (69).

A simple but not ideal procedure for freezing, utilized in our laboratories successfully in applications of the fluorescent-antibody technique, is as follows. Fresh tissue blocks are cut with an area and thickness (1 to 3 mm) suitable for microtomy. The blocks are transferred to microscope slides (or cardboard strips) and frozen by placing the slides on dry ice, and by laying a sheet of wax paper over the tissues

and covering the whole with powdered dry ice. After freezing, the slides are inserted into large-mouthed, flattened pyrex test tubes which are then sealed with tightly fitting rubber stoppers and stored at −20°C. A more accelerated and preferred rate of freezing is achieved by using a bath of dry ice and ethanol. The tissue blocks are placed against the inner planar surface of special flat test tubes, which are then partially immersed in the cooling bath, and, after freezing, are stoppered and stored at −20°C. When needed for sectioning, the tissue blocks are readily freed from their glass supports with a scalpel.

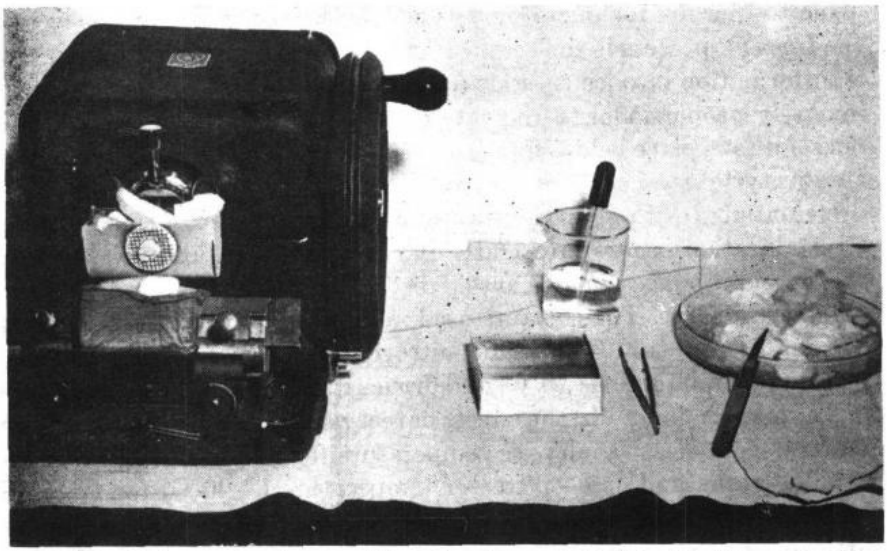

FIG. 1·3 Rotary microtome, with a dry ice–cooled object holder and knife, for the preparation of frozen sections. (*Courtesy of Dr. W. F. Noyes.*)

Tissues may be quickly frozen by immersion in a small beaker containing petroleum ether which has been chilled in a Dewar flask to about −65°C with a dry ice–alcohol mixture (88*a*). The highly commendable procedure of rapid freezing with liquid nitrogen is used in conjunction with freezing-drying and paraffin embedding, which is discussed subsequently.

Frozen Sections. These may be cut at −10 to −5°C using a microtome with a dry ice–cooled knife (66, 77), Fig. 1·3, or at a lower temperature (generally −25 to −20°C) utilizing a microtome and a cryostat (16), Fig. 1·4. The first method is simpler and readily put into operation in any histology laboratory, while the second method yields sections of better quality. The frozen tissue blocks are attached to microtome object holders which are precooled with dry ice and then fro-