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Methods in IMMUNOLOGY and IMMUNOCHEMISTRY

Edited by

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Volume I

Preparation of Antigens and Antibodies

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Preface

The rapid growth of research in immunochemistry and immunology warrants the initiation of an open-end treatise dealing with methodology. The increasing number of applications of immunological methodology to problems in other areas of biology dictates an organization, content, and style which will be helpful to the nonspecialist and specialist alike. Our aim, therefore, has been to open our colleagues' notebooks to bring together detailed procedures that are hard to retrieve from original literature. But the presentation and discussion of reliable methods are intended to provide confidence and guidance, not rigidity. The solution of research problems often demands inventive modifications and sometimes the development of new and specialized approaches. Accordingly, contributors were asked to include not only the details of procedures they had found most satisfactory in their own laboratory, but also critical remarks about common pitfalls and interpretation of results, references to alternative methods, and mention of applications to other problems. While not all topics are easily suited to this format, we feel that insofar as our general objectives are achieved, these volumes represent high potential energy.

Other publications have appeared with similar titles. Some are intended primarily for teaching purposes, others have appeared as reports of symposia. Many are excellent aids to workers in laboratories. None we have seen to date, however, encompasses the scope of the present volumes. Volume I is concerned with typical preparative methods employed in handling antigens, antibodies, and laboratory animals. Volume II presents general chemical and physicochemical methods of great usefulness for immunological research. Volume III is devoted to techniques for the analysis of the antigen-antibody reaction, both *in vitro* and *in vivo*. Volume IV includes methods and interpretations of approaches to the study of the immune response. Unavoidably, some important general topics as well as many specific methods had to be postponed for subsequent volumes, which will treat hypersensitivity, transplantation, immunogenetics, immunity to parasites, and histochemistry, in addition to updating material already presented and introducing new fields of interest.

It would clearly be impossible to compile high quality material of this scope without the enthusiastic support and creative advice of the advisory editors. Their contributed sections, their help in suggesting topics and authors, and in some cases their assistance with the editing is not only greatly appreciated by us but, we are certain, will be appreciated by the users of these volumes.

CURTIS A. WILLIAMS
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Contents of Other Volumes

VOLUME II

VOLUME III

CHAPTER 1

Antigens

No anthology of manageable size and scope could compile detailed instructions for the preparation of all the materials that have been employed for immunizing animals or for analysis by immunochemical methods. These preparations do fall into general categories, however, and certain ones have proved particularly important in the history and development of the related disciplines of immunology and immunochemistry.

In this first chapter of *Methods in Immunology and Immunochemistry,* the authors have attempted, in ways suitable to their particular topic; to relate the specifics of their experience to the general problem of obtaining satisfactory antigen preparations. If the reader-investigator finds in this chapter the precise procedure he is looking for, he will be more than fortunate. That is not to be expected—but what is certain to be found, among the details of the illustrative preparations, is an approach and an understanding of the special considerations necessary for the satisfactory handling of material of that category.

Certain interesting functional categories of antigens or antigen preparations have been omitted. It was felt either that the time was not ripe for definitive exposition on these topics, or that the special problems accompanying their preparation were due not to their physical and chemical nature, where known, but rather to the means available for assessing the purity or relevance of the final product. Among such substances would be the so-called histocompatibility antigens and various structural proteins and complexes of intracellular origin, etc. There are others, certainly, and some perhaps should have been covered, but they must await the compilation of a future volume.

A. Protein Antigens

FACTORS IN THE PREPARATION OF SOME COMMON PROTEIN ANTIGENS*

a. Introduction

The extensive application of immunological tools in recent years to many areas of research has resulted in the employment of a wide variety

* Section 1,A was contributed by Harold F. Deutsch.

of antigens. The more classical immunological studies utilized only a few specific proteins. The methods for the preparation of proteins such as crystalline ovalbumin and serum albumins have been adequately presented in the past,[1] and their presentation once again would serve no significant purpose. Immunoglobulin preparations are given in Chap. 3. This section will deal with modern fractionation techniques applicable to the preparation of many protein antigens. To illustrate them and the various factors in their successful use, we will discuss a few preparations with which we have personal experience.

One of the marked changes in immunological research in recent years is that the investigator seldom prepares his own antigens. Whatever the source, there may be no assurance of the quality of the preparation even when the method has followed a published procedure. Workers should become familiar with the physical-chemical characterization methods which permit some evaluation of protein homogeneity or lack thereof.

It is to be stressed that the purification effort must be related to the particular protein and to the goal faced by the investigator. While commercial preparations may be adequate for certain types of experiments, some further fractionation may often be desirable.

The isolation of protein antigens presents various problems which are related to their source and their physical-chemical properties. If unwanted, associated proteins possess quite divergent chemical and physical properties with respect to the antigen, their removal may prove relatively easy. However, this is usually not the case, and one often obtains an antigen containing small amounts of impurities.

A second consideration is the relative response of the animal immunized to an antigen containing impurities. Considerable amounts of contaminants, if they are weakly antigenic, may elicit insignificant amounts of antibody. Alternatively, traces of very antigenic impurities may produce a disproportionately high amount of antibody. Also, the antibody responses of individual animals and of different species to a given antigen often show wide variations. Routes of injections and immunization routines are important but variable factors. The final evaluation of the purity of any antigen must be in terms of the antibody or antibodies formed against it.

One difficulty in determining whether a considerable antibody response to a minor antigenic component has resulted should be pointed out. The immune serum should be reacted with a crude fraction containing relatively large amounts of the component in question so that enough

[1] E. A. Kabat, Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

of this antigen is present to give a visible specific precipitate. The purified antigen may fail to provide a sufficient amount of the minor component to realize an adequate evaluation.

When a combination of different techniques is used, a high degree of purification can be effected for most antigens. Many of the currently available techniques for separating proteins from complex biological mixtures are described in Chaps. 6 through 9, Vol. II. The methods employed for the separation of a series of protein antigens will be given. The particular examples presented were selected to indicate the variety of techniques generally employed and at the same time to show some of the limitations of a single method. It should be pointed out that the physical-chemical methods used in the purification of proteins are constantly being improved and extended.

b. Crystallization

The concept that a crystalline protein represents a highly purified antigen finds wide acceptance. Various proteins such as chicken egg white lysozyme,[2] ovalbumin[3,4] and iron-conalbumin,[4,5] and bovine β-lactoglobulin[6] may be readily crystallized from solutions containing considerable amounts of impurities. The ovalbumins,[7] the conalbumins,[7] and β-lactoglobulin[8,9] show evidence of several components when subjected to various physical-chemical studies. Crystalline β-lactoglobulin prepared by two different methods may show marked differences in its antigenic behavior.[10] Antisera to highly purified egg white proteins have been found to contain antibodies to other proteins of the system.[11-13] Thus, crystallization is not in itself an index of antigenic purity.

The preparation of lysozyme by direct crystallization[2] is an example of some inherent limitations of crystallization procedures. Whole chicken egg white is homogenized gently without foaming in a Waring blendor to break up the mucin stands. Solid sodium chloride is added to a concentration of 5%, and the mixture is adjusted to a pH of about

[2] G. Alderton and H. L. Fevold, *J. Biol. Chem.* **164**, 1 (1946).
[3] R. A. Kekwick and R. K. Cannan, *Biochem. J.* **30**, 227 (1936).
[4] R. C. Woodworth and A. L. Schade, *Arch. Biochem. Biophys.* **82**, 78 (1959).
[5] R. C. Warner and I. Weber, *J. Biol. Chem.* **191**, 173 (1951).
[6] R. Cecil and A. G. Ogston, *Biochem. J.* **44**, 33 (1949).
[7] M. B. Rhodes, P. R. Azari, and R. E. Feeney, *J. Biol. Chem.* **230**, 399 (1959).
[8] C. H. Li, *J. Am. Chem. Soc.* **68**, 2746 (1946).
[9] R. Aschaffenburg and J. Drewry, *Nature* **176**, 218 (1955).
[10] H. F. Deutsch, *J. Biol. Chem.* **185**, 377 (1950).
[11] J. H. Vaughan and E. A. Kabat, *J. Allergy* **25**, 387 (1954).
[12] J. H. Vaughan and E. A. Kabat, *J. Immunol.* **73**, 205 (1954).
[13] M. Kaminski, *J. Immunol.* **75**, 367 (1955).

9. The solution is stored in the cold room (0° to 4°) overnight; then a small amount of seed crystals is added. Crystallization proceeds slowly and is allowed to continue for 4 to 7 days. The crystals are then removed by centrifugation and dissolved in 0.05 *M* acetate buffer at pH 4 to 5. The insoluble mucins are removed by centrifugation, and the solution is then returned to the original pH 9 crystallization conditions. After several days the crystals which have formed are removed. Recrystallization may be repeated as described above until it is considered that the lysozyme is antigenically pure.

The number of crystallizations necessary to effect purification must be considered. Egg white contains nearly 11% protein, 3.7% of this being lysozyme.[14,15] Thus, a liter of egg white contains about 4.1 gm of this protein. Let us assume that 80% of it may be isolated in any crystallization step and that the crystals packed by centrifugation are 30% protein. The first crystals from a liter of egg white will then contain 3.3 gm of lysozyme, and within the crystals will be about 7.7 gm of

TABLE I
APPROXIMATE VALUES FOR ONE LITER OF CHICKEN EGG WHITE

Number of crystallizations	Lysozyme (mg)	Other proteins (mg)	Purity of lysozyme (%)
1	3.3	850	80
2	2.6	31	98.8
3	2.1	1.2	99.95

what is really supernatant liquid containing 11% egg white protein, or about 0.85 gm of contaminant egg white proteins within the 3.3 gm of lysozyme crystals. Lysozyme, once-crystallized and not washed, is thus about 80% pure. The need for washing out the occluded soluble material within the crystal mass is therefore obvious, and it can be accomplished by washing several times at approximately 0° with 5% sodium chloride at pH 9. Let us suppose that washing is not practiced. Recrystallization will gradually remove the contaminating proteins as shown in Table I, if we assume that lysozyme is recrystallized from 2% solution.

Crystallization effects a rather rapid removal of soluble impurities but will never remove them entirely. The same considerations apply to reprecipitation of any protein.

Although three-times-crystallized lysozyme would be expected to con-

[14] L. G. Longsworth, R. K. Cannan, and D. A. MacInnes, *J. Am. Chem. Soc.* **62**, 2580 (1940).

[15] L. R. Wetter and H. F. Deutsch, *J. Biol. Chem.* **192**, 237 (1951).

tain only 0.05% of impurities due to liquid occluded between the centrifuged crystals, other considerations suggest that this is not a realistic figure. Lysozyme with an isoelectric point near 11[15] would be expected to form electrostatic complexes with some of the more acidic proteins of egg white when it is crystallized at pH near 9. Lysozyme is also a relatively weak antigen, and the contaminant proteins even though in low concentration might stimulate a considerable production of antibody. Seven-times-crystallized lysozyme, however, has been found to be a satisfactory antigen.[15]

Even though an extreme case is presented here in that lysozyme has a very high isoelectric point and is readily crystallized in the presence of a high concentration of other proteins, the same considerations apply to other antigens as regards purification by crystallization or reprecipitation. The immunological effect of impurities in a crystalline antigen is exemplified by the immunization of a horse with three-times-crystallized ovalbumin.[16] More antibody was formed against a conalbumin impurity than against the ovalbumin.[17] Thus, crystallization techniques, while useful, should be supplemented with other isolation procedures.

In the preparation of lysozyme antigen the trace impurities in several-times-crystallized protein can readily be removed by passage of a solution of approximately 0.05 ionic strength over a DEAE-cellulose or DEAE-Sephadex column at pH 7 to 8. Under these conditions lysozyme passes through, and the more acidic components are held on the column. If a commercial preparation of crystalline lysozyme is employed, it may be subjected to the DEAE-cellulose chromatography. This step, coupled with a laboratory procedure which includes washing of the crystals, can provide an even more favorable result and a better preparation of this enzyme.

c. Chromatographic and Electrophoretic Separations

These methods offer some of the best possibilities for the preparation of purified antigens. Both procedures depend on variations in the net charge of the proteins with pH and with the ionic strength of the medium. Some limitations of these methods are mentioned.

The separation of the Fab and Fc fragments[18] of 7 S γG-immunoglobulins formed on digestion with papain[19] (see Chap. 5) illustrates the use

[16] A. M. Pappenheimer, Jr., *J. Exptl. Med.* **71**, 263 (1940).
[17] M. Cohn, L. R. Wetter, and H. F. Deutsch, *J. Immunol.* **61**, 283 (1949).
[18] Nomenclature for Human Immunoglobulins, *Bull. World Health. Organ.* **30**, 447 (1964).
[19] G. M. Edelman, J. F. Heremans, M.-Th. Heremans, and H. G. Kunkel, *J. Exptl. Med.* **112**, 203 (1960).

of several ion exchange cellulose derivatives. The Fc fragments have isoelectric points near pH 6.5, while those of the Fab fragments, separated from various γG-immunoglobulins, range from pH 7 to 9. The moving-boundary electrophoretic diagram of Fig. 1 shows the charge relationships of a γG-myeloma globulin and its papain digestion products, of which the latter are to be isolated for use as antigens.

Choice of the cellulose derivative will depend on which of the papain digest fractions it is desired to isolate in antigenically pure form. Only the first component eluting from the chromatographic column will be free of the second component. Since elution of a given component is

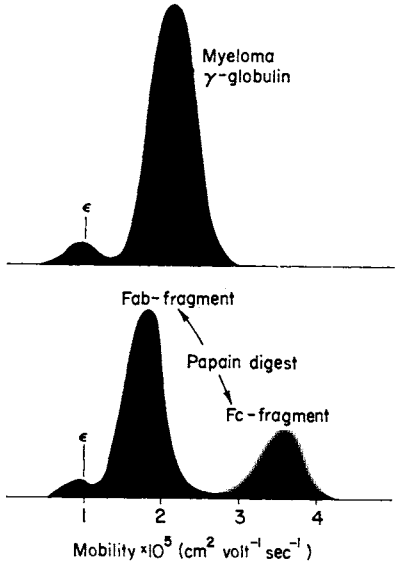


FIG. 1. Moving-boundary electrophoretic patterns of a γG-myeloma protein and its papain digestion products.

never quantitative, second components, which are usually eluted after establishment of a salt or a pH gradient or both, are always contaminated with the first component. In practice it is found that antiserum produced against the first-eluting component is satisfactory but that antibody prepared against one or more components eluting later will also contain antibody against the components that were eluted first.

If the Fc fragment is desired, the papain digest is chromatographed on a column of CM-cellulose. Conditions as exemplified in Fig. 2A allow it to elute following the holdup or first column volume. The Fab fragment is then eluted as a second fraction after either the ionic strength or the pH of the eluting media is raised. It is always contaminated with residual Fc fragment.

The Fab fragment of the immunoglobulin may be separated on DEAE-cellulose at approximately pH 7.0 in a form suitable for use as an antigen. Under the conditions indicated in Fig. 2B, it elutes shortly after the holdup volume of the column, free of the Fc fragment. The latter component elutes only after the ionic strength is increased or the pH is lowered, and it is contaminated with Fab fragment.

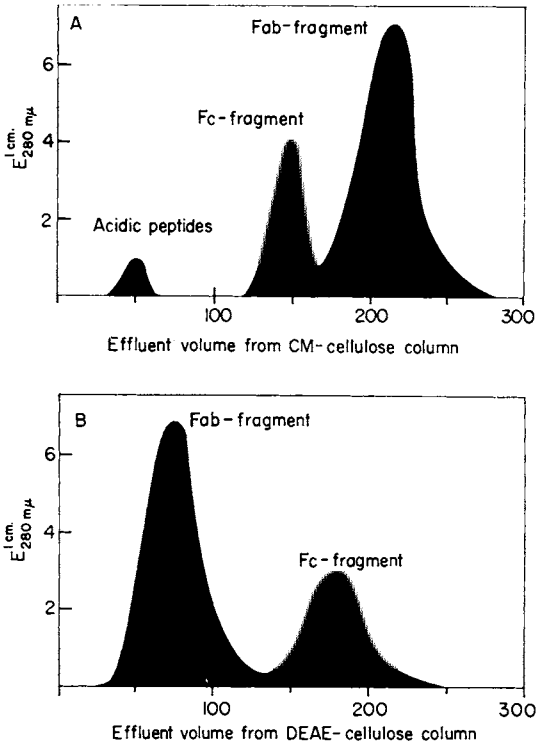


FIG. 2. A, Chromatography on CM-cellulose of undialyzed papain digest of a myeloma globulin. A 2 × 30-cm column was employed for 150 mg of digest. A constant-gradient elution technique employed pH 5.5 sodium acetate buffer, from 0.05 to 0.3 *M*. B, Chromatography of a similar papain digest on a 2 × 30-cm column of DEAE-cellulose. A constant-gradient elution technique employed pH 7 sodium acetate, from 0.05 to 0.3 *M*.

The same considerations apply to electrophoretic separations of these proteins. Figure 3 illustrates the electrophoretic separation of a similar papain digest by the column electrophoresis method[20] (see Chap. 6,D,2, Vol. II) at pH 6. Here only the Fab fragment, which is the fastest migrating at this pH and also the first to be eluted, may be considered suitable

[20] J. Porath, *Acta Chem. Scand.* 8, 1813 (1956).

as an antigen. If the Fc fragment is to be separated by this technique, a buffer in the range pH 8 to 8.5 would be employed. This would permit its rapid anodic electrophoretic migration and thus permit separation free of the Fab fragment.

While only the Fc fragment or the Fab fragment of a papain digest will be separated in a form suitable as an antigen by the application of one separation technique, the second eluting fragment may be subjected to an additional separation under conditions where it elutes as the first component. In this manner both of these fragments from a single papain digestion may be isolated in antigenic purity.

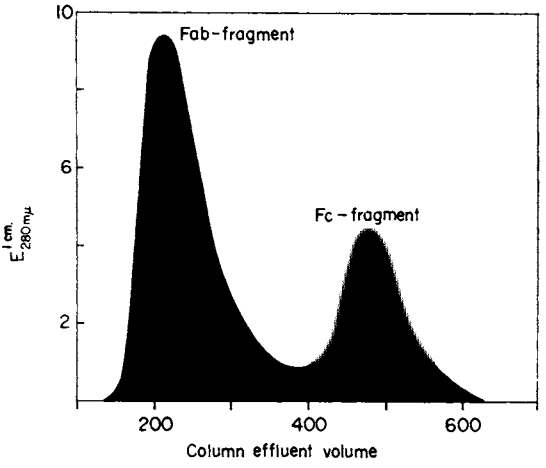


FIG. 3. Elution pattern of papain-digested myeloma globulin after electrophoresis on a cellulose column at pH 6.0.

In either chromatographic or electrophoretic separations for obtaining the Fc fragment, one must consider the presence of an acidic peptide fraction (A-fraction) in the papain digestion mixture of the immunoglobulins.[21] It would not be a contaminant of the Fab fragment under conditions where this material was eluted as the first component. It is not known whether this peptide material is antigenic or if it plays a role in the immunochemical reactions of the Fc fragment. In the absence of knowledge of its possible antigenic role, it is desirable to remove it from any Fc fragment being employed as antigen. It is possible to remove this peptide material by exhaustive dialysis before separation of the papain digest or by passage of the isolated Fc fragment fraction over Sephadex G-25 or G-50 columns.

[21] H. F. Deutsch, E. R. Stiehm, and J. I. Morton, *J. Biol. Chem.* **236**, 2216 (1961).

Electrophoretic separations on supporting media such as starch grains[22] (see Chap. 6,D,1, Vol. II) also yield proteins that are contaminated by materials that have previously migrated through a given area. Only the fastest migrating fraction may be considered adequate as an antigen. The same consideration applies to components separated by gel filtration on columns. Only the highest-molecular-weight component of a mixture of proteins which elutes as the first component may be considered suitable as an antigen. Thus, in the separation of the γ-globulins of different molecular weight—that is, the 18 to 19 S macroglobulins (γM) and the 7 S γ-globulins (γG and/or γA)—only the γM-globulins would be considered suitable for use as an antigen. Small amounts of the γM-globulin elute behind the main peak into the region where the other components elute. This situation obtains even when one uses a column sufficiently long to permit separation of components by a considerable volume difference.

Continuous-flow electrophoretic methods such as paper curtain electrophoresis[23] and the free-flow method of Hannig[24] should permit the separation of a series of antigens in any given experiment, provided the components to be separated possess adequate mobility differences. Thus, it would be expected that both the Fab and Fc fragments of the papain digest of an immunoglobulin could be prepared in a single experiment by such methods. Data are not yet available as to whether several components separated in a single experiment of this type are suitable as antigens.

d. Use of Antibodies to Remove Highly Antigenic Impurities

Occasionally a purified protein that one wishes to use as an antigen is contaminated with small amounts of a highly antigenic impurity. An example of this is crystalline ceruloplasmin. This protein is prepared from fraction IV-1 of human plasma[25] which is available from various pharmaceutical houses.

The fractionation is carried out as follows[26]:

1. One hundred grams of fraction IV-1 is suspended in 500 ml of 0.05 *M* sodium acetate with a Waring blendor and stirred for 30 to 60 minutes.

[22] H. G. Kunkel, *in Methods of Biochemical Analysis* (D. Glick, ed.), Vol. 1, p. 141. Interscience, New York, 1954.

[23] W. Grassmann and K. Hannig, *Z. Physiol. Chem.* **292**, 32 (1953).

[24] K. Hannig, *Z. Anal. Chem.* **181**, 244 (1961).

[25] E. J. Cohen, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor, *J. Am. Chem. Soc.* **68**, 459 (1946).

[26] H. F. Deutsch, C. B. Kasper, and D. A. Walsh, *Arch. Biochem. Biophys.* **99**, 132 (1962).

The blendor is controlled by an external voltage regulator and is operated at low speed, since foaming must be avoided. In practice, a large test tube is held down into the liquid or suspension in the blendor to prevent any vortex from forming and drawing air into the solution being stirred.

2. Insoluble proteins are removed by centrifugation at a temperature of about 0°. Re-extraction of the sediment with 200 ml of 0.05 *M* sodium acetate provides an additional 10 to 15% yield of ceruloplasmin if wanted.

3. Fifteen to eighteen grams of DEAE-cellulose (about 0.8 meq. of amino group per gram) previously equilibrated against pH 5.5, 0.05 *M* sodium acetate buffer is added as a thick slurry to the above supernatant, and the suspension is stirred for 30 to 60 minutes. The suspension is then allowed to settle, the supernatant is decanted, and the DEAE-cellulose is poured into a column containing a bed of approximately 3 inches of fresh DEAE-cellulose at the bottom of the column. The latter serves to detect ceruloplasmin eluting from the DEAE-cellulose.

4. The DEAE-cellulose column is washed with pH 5.5, 0.05 *M* sodium acetate containing 0.05 *M* sodium chloride until the $E^{1cm}_{280m\mu}$ value of the effluent is below 0.200.

5. The ceruloplasmin is then eluted from the column with the pH 5.5, 0.05 *M* sodium acetate buffer containing 0.5 *M* sodium chloride.

6. The ceruloplasmin is equilibrated by dialysis against pH 5.5, 0.05 *M* sodium acetate buffer (overnight in the cold) and then chromatographed on a DEAE-cellulose column equilibrated against this buffer. A constant-volume, continuous-gradient elution technique is employed in which the higher salt concentration buffer is the above-mentioned pH 5.5, 0.05 *M* sodium acetate buffer containing 0.3 *M* sodium chloride. Fractions having $E_{280/610m\mu}$ ratios of less than 40 are pooled, adjusted to pH 7.4 (± 0.2), and dialyzed against 0.05 *M* sodium acetate (overnight in the cold).

7. The ceruloplasmin is chromatographed on a DEAE-cellulose column which has been adjusted to pH 7.4 and then equilibrated against 0.05 *M* sodium acetate. A constant-volume, continuous-gradient elution technique is employed in which the higher salt concentration solution is 0.05 *M* sodium acetate containing 0.3 *M* sodium chloride. Fractions having $E_{280/610m\mu}$ ratios of less than 30 are pooled.

8. The ceruloplasmin is precipitated at 40% saturation with ammonium sulfate, and the precipitate is taken up in sufficient water to give a ceruloplasmin concentration of 3 to 5% ($E^{1\%.1cm}_{610m\mu}$ is 0.68 for pure ceruloplasmin). The sample is dialyzed against pH 5.25 (± 0.05), 0.025 *M* sodium acetate buffer for 2 to 3 days. Crystallization is usually effected at the end of 24 hours; higher yields are obtained when the crystallization is allowed to proceed for several additional days.

9. The crystals are removed by centrifugation in the cold and then washed by centrifugation two or three times at 0° with pH 5.25, 0.025 *M*

sodium acetate buffer. This washing procedure appears to be more effective in removing impurities occluded within the crystals than recrystallization and in addition may be carried out much more readily. The washed crystalline ceruloplasmin is then reconstituted into the desired working solution.

Although the isolated crystalline ceruloplasmin appears pure by various physical-chemical evaluations,[27] antibody produced against it in rabbits, goats, and guinea pigs shows the presence of relatively large amounts of antibodies to serum γM-globulins and little against ceruloplasmin in the early stages of immunization.[28] Such an antibody preparation is useful to remove the small amount of contaminating macroglobulins and thus to yield a suitably pure ceruloplasmin antigen. The usual crystalline ceruloplasmin[26] is used to immunize a goat or a series of rabbits. When antiserum giving good precipitin reactions with whole normal human serum is formed, it is found to give a minimal precipitin reaction with crystalline ceruloplasmin. This is apparent from the lack of blue color in the specific precipitates or from Ouchterlony-type experiments (see Chap. 14, Vol. III). This early antiserum is collected, and the γ-globulin fraction is separated by ethanol fractionation[29] or by chromatographic procedures.[30] The isolated γ-globulins are then allowed to react with crystalline ceruloplasmin, and the mixture is incubated at about 0° for 1 to 2 days. Any precipitate forming is removed by centrifugation. The supernatant is dialyzed against 0.05 *M* sodium acetate at pH 7 ± 0.2. This solution is then chromatographed on DEAE-cellulose and the pH 7, 0.05 *M* sodium acetate is used as the initial eluting fluid.

The γ-globulins elute with the first column volume. Next, sodium chloride is added to the eluting fluid to 0.05 *M* concentration. A small blue-colored fraction is then usually eluted before the ceruloplasmin peak. This is apparently a soluble complex of ceruloplasmin with specific antibody in antigen excess. Following this, the sodium chloride concentration of the eluting fluid is raised to 0.5 *M*, and the ceruloplasmin is eluted. Figure 4 illustrates the type of chromatogram obtained.

The ceruloplasmin component is concentrated and used to immunize the same species of animal from which antibody to the impurity was obtained. Although the ceruloplasmin is isolated as the third component in this chromatographic system and is contaminated with small amounts of rabbit γ-globulins, these latter proteins are homologous to the animals

[27] C. B. Kasper and H. F. Deutsch, *J. Biol. Chem.* **238**, 2325 (1963).
[28] C. B. Kasper and H. F. Deutsch, *J. Biol. Chem.* **238**, 2343 (1963).
[29] J. C. Nichol and H. F. Deutsch, *J. Am. Chem. Soc.* **70**, 80 (1948).
[30] H. B. Levy and H. A. Sober, *Proc. Soc. Exptl. Biol. Med.* **103**, 250 (1960).

being immunized. The γM-globulin contaminant has been removed, and antibody produced against this ceruloplasmin is highly specific. It is to be stressed that, while the removal of an impurity in a ceruloplasmin preparation by the use of an antibody is an important step in the preparation of a pure antigen, it is the combination of chromatographic and crystallization methods in conjunction with an antigen absorption step that gives the desired result. Any single purification method has its

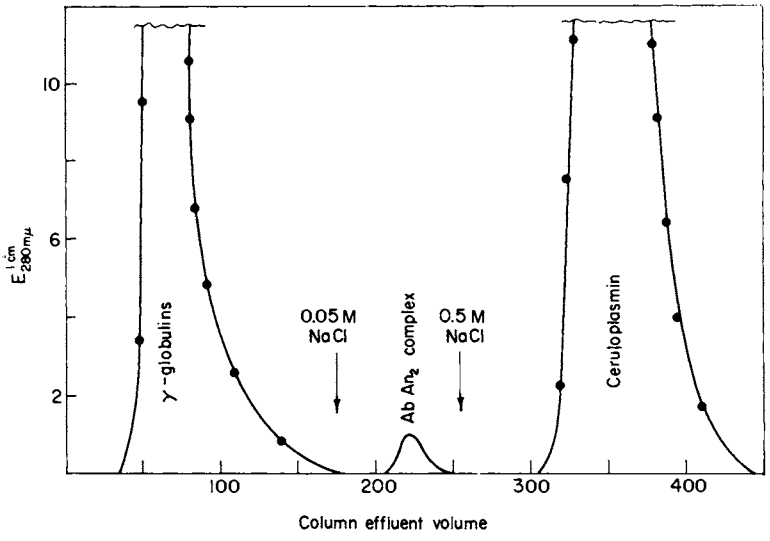


FIG. 4. Elution pattern of a mixture of 500 mg of crystallized human ceruloplasmin and a rabbit γG-globulin antibody preparation on a 2 × 40-cm column of DEAE-cellulose. Sodium chloride was added to the pH 7, 0.05 *M* sodium acetate eluting fluid at the elution volumes marked by the arrows (see text).

limitations, and these often can be circumvented by use of a combination of methods.

e. STABILIZATION OF ANTIGEN

Following isolation, certain antigens may undergo physical-chemical changes which result in a modification of their antigenic properties and thus introduce new molecular species which may act as antigenic impurities in the preparation. The stabilization of protein antigens therefore often becomes necessary during their isolation. For example, when ceruloplasmin loses a portion of its copper, it undergoes marked changes in its immunological behavior.[28,31] Thus, consideration must be given to conditions of preparation and storage that minimize losses of copper from this protein.

[31] H. F. Deutsch and G. B. Fisher, *J. Biol. Chem.* **239**, 3325 (1964).

Another example is provided by the serum albumins, which have been used as antigen in many immunological investigations. Albumin has a marked tendency to form dimers as the result of disulfide bond formation. Levine and Brown[32] have shown that the monomer and dimer forms of serum albumin may be distinguished by the quantitative precipitin reaction. Certain immunochemical investigations may require preparations in which the monomer has been separated from the dimer and in which the sulfhydryl group of the purified monomer has been alkylated to prevent it from undergoing further dimer formation. In practice, a 1% solution of a serum albumin is alkylated with 0.005 *M* iodoacetamide at pH 8. The mixture of protein and salts is then passed over a column of Sephadex G-100. Most of the dimer may be resolved from the monomer by such treatment. Starch gel electrophoretic analyses of monomer and dimer preparations indicate that they are well separated. The very small amount of dimer that is expected to elute with the secondarily eluted monomer component does not introduce a serious problem in most immunochemical investigations because of the close antigenic similarity of the two forms of the serum albumin.

Generally one is never completely satisfied with the methods employed to store protein antigens or with their purity. However, it is necessary to reorganize the problems entailed and to know the methodologies currently available to circumvent them. These procedures permit one to prepare protein antigens that are in general superior to those used earlier.

[32] L. Levine and R. K. Brown, *Biochim. Biophys. Acta* **25**, 329 (1957).

B. Bacterial Antigens

1. DISRUPTION OF BACTERIA AND PREPARATION OF CELL WALLS*

a. Methods for Disruption of Bacteria

i. Introduction

Ideally, one would choose from the great variety available that method for disruption of bacteria which was best suited to each special purpose; but disruption for preparation of antigens is not necessarily different from disruption for any of several other purposes. Neither is disruption of bacteria unlike that of other microorganisms such as rickettsiae or certain fungi and large viruses. This discussion proceeds, therefore, from general principles.

*** Section 1,B,1 was contributed by Edgar Ribi and Kelsey C. Milner.**

Disruption of cells may be an end in itself, as when it is desired only to increase the availability of enzymes or toxins. Often, however, some separation is to be made, and in discussing such cases it will be useful to employ the terminology of de Duve,[1] even though he was referring to fractionation of animal tissue. In following this convention, we shall distinguish between two classes of cellular entities: the morphological *components* or organelles, by which we mean such things as cell walls, cell membranes, and cytoplasmic reticula; and the biochemical *constituents*, such as proteins, lipopolysaccharides, and enzymes. Frequent reference will also be made to *protoplasm*, which will arbitrarily be classed as a component, and which will mean simply the dispersed material from fragmented cells remaining in the supernatant fluid after the cell walls and any unbroken cells have been sedimented by centrifugation. In addition to cytoplasm and nuclear material, it may include fine fragments and soluble constituents of the cell wall or cell envelope and, according to the method of disruption, will or will not include the solubilized cell (cytoplasmic) membrane.

Neither chronological treatment nor completeness of coverage of all the methods that have been devised will be attempted. Reviews in English have extensively covered methods aimed at recovery of active enzymes[2] and methods applied especially to the isolation of bacterial cell walls.[3] The thesis of Edebo[4] contains more general treatment of disruption methods in addition to complete details of the author's own device, the X-press. An evaluation of selected methods for disruption of bacteria was undertaken at the International Symposium on Biotechnical Developments in Bacterial Vaccine Production, Stockholm, 1965.[5] Methods that can be described as useful, in one circumstance or another, for preparation of antigens may be classified under the following heads: lysis (autolysis, enzymatic, osmotic), grinding, sonic and ultrasonic vibrations, freeze-thawing, shaking, decompression, and high-pressure extrusion. These are characterized and a selection from the most useful devices and methods is described below.

ii. Lysis

Autolysis, although it has been employed with some apparent success,[6,7] cannot be recommended for disintegration of cells with a view

[1] C. de Duve, *J. Theoret. Biol.* **6, 33** (1964).
[2] W. B. Hugo, *Bacteriol. Rev.* **18, 87** (1954).
[3] M. R. J. Salton, "The Bacterial Cell Wall." Elsevier, Amsterdam, 1964.
[4] L. Edebo, "Disintegration of Microorganisms." Almqvist and Wiksell, Uppsala, 1961.
[5] "Symposia Series in Immunobiological Standardization," Vol. III, Karger, Basel, 1967.
[6] W. Weidel, *Z. Naturforsch.* **6b, 251** (1951).
[7] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **16, 184** (1957).

to isolating either components or constituents except in those cases where autolysis proceeds rapidly and the desired product is stable to the enzymes that may be released. Tetanal toxin, for example, is thought to be produced in the cytoplasm, but the aging organisms lyse so early and regularly that it is satisfactory to begin isolation of this constituent from well-grown fluid cultures that have been clarified by centrifugation or filtration.

The most feasible procedure for isolating components reasonably identifiable with the cell membrane of bacteria is by osmotic shock of protoplasts.[8] Protoplasts or spheroplasts may be prepared from certain organisms by treatment with lysozyme as originally described by Weibull,[9] by cultivation in the presence of low concentrations of penicillin,[10] and with other inducing agents or combinations. Sato *et al.*[11] have also found that lysis of mycobacterial (BCG) cells could be produced—not necessarily via spheroplast induction, however—by cultivating the organisms in Dubos medium containing 1.5% glycine, 0.1% lithium chloride, and 0.01% lysozyme.

Most normal bacteria are exceptionally resistant to simple osmotic lysis; nevertheless, some disintegration may often be obtained, and osmotic lysis is the easiest and most inexpensive method. Gillchriest and Bock[12] grew cells of *Azotobacter vinelandii* in the presence of 2 *M* glycerol, collected them by low-speed centrifugation, and produced a useful degree of rupture by discharging the pellet into 8 volumes of a low-ionic-strength "RNP" buffer (1.6×10^{-3} *M* K_2HPO_4, 0.4×10^{-3} *M* KH_2PO_4, and 5×10^{-3} *M* $MgSO_4$). From the resulting suspension they isolated protoplasmic granules of nucleoprotein of a uniform size (86 S) that could not have passed the unbroken cell wall.

iii. Grinding

Only limited notice will be taken of grinding for disruption of bacteria, although it is one of the time-honored, widely used methods. Today, grinding is employed mainly for fractionation of animal tissues, and its use with bacteria is restricted to studies of biochemical constituents. Better methods have been devised for isolation of antigens and morphological components.

Disruption of microorganisms by grinding may be accomplished with

[8] S. Brenner, F. A. Dark, P. Gerhardt, M. H. Jeynes, O. Kandler, E. Kellenberger, E. Klieneberger-Nobel, K. McQuillen, M. Rubio-Huertos, M. R. J. Salton, R. E. Strange, T. Tomcsik, and C. Weibull, *Nature* **181**, 1713 (1958).

[9] C. Weibull, *J. Bacteriol.* **66**, 688 (1953).

[10] J. Lederberg, *Proc. Natl. Acad. Sci. U.S.* **42**, 574 (1956).

[11] H. Sato, B. B. Diena, and L. Greenberg, *Can. J. Microbiol.* **12**, 255 (1966).

[12] W. C. Gillchriest and R. M. Bock, *in* "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), p. 1. Pergamon, New York, 1958.

no more complicated apparatus than hand-operated mortar and pestle with an abrasive such as quartz sand, ground glass, glass beads, alumina, or Carborundum in one of the finer grades. To increase the speed and efficiency of the operation, numerous mills have been built for use on wet or dry material, with and without abrasives or control of temperature. Relatively few of these mills, whether commercially or privately constructed, are adaptable for use with small quantities of material. As is the case with nearly all methods for mechanical disintegration, when grinding is sufficiently prolonged to rupture most of the cells, continued fragmentation of components of the cells that ruptured earlier has also taken place, with resultant multiplication of the difficulties of making separations and purifications. In addition, grinding is slower than most other techniques, and during the process heat is developed that must be dissipated if the product is not to be injured. Not only are heat-labile antigens destroyed, but proteins, as they denature, coat and adhere to other constituents, rendering clean separations virtually impossible.

In wet grinding with abrasives, the relative proportions of cell paste, abrasive, and diluent are often critical for a high percentage of disintegration and quality of product.[13] These must be determined for each application.

Phillips *et al.*[14] have described a method for "grinding" microorganisms, including bacterial spores, by the action of a peristaltic pump on a slurry of organisms and minute glass beads in rubber tubing. Its utility for separation of antigens and whole cell walls will have to be assessed by longer experience, but the method would appear to have more in common with the shaking methods described below and to be correspondingly more useful than the other types of grinding. Temperature control is said to be effective, and the quantities of material that can be processed are flexible over wide limits.

iv. Sonic and Ultrasonic Vibrations

Agitation of sufficient energy to fragment bacterial cells in aqueous suspension can be imparted to fluids by suitable transducers vibrating at frequencies through most of the range of audible sound and on up to at least several hundred kilocycles per second. The disintegration achieved is largely independent of the frequency but is controlled to some extent by the intensity and amplitude of the waves.[15,16] Devices

[13] G. Kalnitsky, M. F. Utter, and C. H. Werkman, *J. Bacteriol.* **49**, 595 (1945).
[14] J. W. Phillips, C. Lamanna, and M. F. Mallette, *Appl. Microbiol.* **13**, 460 (1965).
[15] P. K. Stumpf, D. E. Green, and F. W. Smith Jr., *J. Bacteriol.* **51**, 487 (1946).
[16] D. E. Hughes and W. L. Nyborg, *Science* **138**, 108 (1962).

of two kinds have been extensively employed and are offered commercially: (1) *magnetostrictive* oscillators transmit the contractions of a metal (nickel laminate) rod in an alternating magnetic field; (2) *electrostrictive* oscillators make use of the expansion and contraction of certain crystals or ceramics when an alternating potential difference is applied. The latter are also known as piezoelectrical devices although the principle involved is the reverse of the usual piezoelectric effect.

The mechanism by which sonic and ultrasonic vibrations bring about shear forces sufficient to disrupt bacteria has been the subject of much study and speculation. The phenomenon of gaseous cavitation and collapse, in which minute bubbles undergo a cycle of several thousandfold expansion and violent collapse to less than the original size in less time than one cycle of the sound, is known to generate the required energy in terms of heat and pressure. Hughes and Nyborg[16] have shown, however, that bacteria can be ruptured by ultrasonic vibrations at amplitudes and pressures below the threshold for cavitation-collapse under circumstances that implicate eddying and related motions imparted by vibrating bubbles as the sources of shearing action. These findings could have an important bearing on the design of new instruments, since most available devices have been aimed at producing the collapse phenomenon. Because the threshold for free radical formation is the same as that for cavitation-collapse,[16] and because free radicals are definitely harmful to enzymes[17] and probably to some antigens, an instrument that would effectively disrupt cells at lower amplitudes would have a distinct advantage.

At present, easily operated instruments of both the magnetostrictive and the electrostrictive types are available in commercial models. They readily disrupt most bacteria and other cells. The oxidizing effect of free radicals can be diminished by addition of cysteine,[17] and other oxidations can be countered by disruption in an atmosphere of hydrogen.[18] For extraction of certain antigenic constituents such methods may be entirely suitable, but for isolation of morphological components—particularly of whole cell walls—these instruments cannot be recommended. The difficulty is similar to that with grinding; if the process is carried on long enough to disrupt a majority of cells, a further comminution of many of the organelles also takes place so that, for example, instead of finding a high proportion of large cell-wall sheets in the product, one finds a spectrum of different-sized pieces with a high proportion of very fine fragments that are not easily separated by sedimentation. There is also a tendency from the beginning to produce

[17] D. E. Hughes, *J. Biochem. Microbiol. Technol. Eng.* **4**, 405 (1962).
[18] A. G. Marr and E. H. Cota-Robles, *J. Bacteriol.* **74**, 79 (1957).

a greater shattering of cell walls than with other methods. This explanation, derived empirically from attempts to prepare cell walls, finds experimental verification in studies of the kinetics of disruption with sonic vibration by Marr and Cota-Robles.[18] Their data, based on turbidity and rates of change in direct counts of whole cells correlated with phase contrast photomicrographs, indicate that, with *Azotobacter vinelandii*, the first "hit" on a cell disintegrates an average 40% of the cell wall into submicroscopic units, killing the cell and releasing the contents from the envelope. The remaining portion of envelope or "hull" (consisting of cell wall and possibly cell membrane) then disintegrates progressively with succeeding "hits."

v. Freeze-Thawing

Disruption of bacteria by alternate freezing and thawing of aqueous suspensions has been practiced for about as long as disruption by autolysis or grinding. It has the merit of simplicity and avoids excessively harsh treatments. It is also a tedious and time-consuming method when performed without specialized apparatus, and it does not always yield the desired product. In 1959, Billaudelle and Brunér[19] described "an automatic apparatus for extraction of cell suspensions by repeated freezing and thawing," which is adaptable to large-scale preparations. If offered commercially, this will probably be one of the most complex and expensive machines yet devised for the disruption of cells. At the recommended setting, it subjects cell suspensions to repeated cycles of rapid freezing at −56° and thawing at 37°. Even with this machine, the time required to achieve a moderate percentage of disintegration is probably greater than with other methods. In the case of *Salmonella typhi*, virtually no intact cells remain after 36 hours of treatment.[20] Possibly because of the cost factor, the device has not been extensively employed.

vi. Shaking

Shaking with abrasive particles has developed as an alternative to grinding in mortars or mills for disruption of cells. The introduction of small (about 0.1 to 0.2 mm in diameter) smooth glass beads in place of rough abrasives[21] and use of a device (Mickle tissue disintegrator) that shakes the mixture rapidly at moderate amplitudes[22] led to the

[19] H. Billaudelle and P. O. Brunér, *J. Biochem. Microbiol. Technol. Eng.* **1**, 229 (1959).

[20] H. Billaudelle, H. Lundbeck, and M. O. Tirunarayanan, *Z. Immunitäts-Allergieforsch.* **127**, 164 (1964).

[21] H. R. Curran and F. R. Evans, *J. Bacteriol.* **43**, 125 (1942).

[22] H. Mickle, *J. Roy. Microscop. Soc.* **68**, 10 (1948).

first really good preparations of clean cell walls from both gram-positive and gram-negative bacteria.[23-26] The potentialities of the method have been notably exploited by Salton[3] and colleagues and other investigators too numerous to mention. The Mickle device, used in conjunction with glass beads, remains one of the most successful and inexpensive tools for disruption of small quantities of bacteria, especially for production of cell walls or protoplasm when numerous different experimental products are desired. The operation of the machine is discussed later in connection with methods for preparation of cell walls.

Other instruments for disrupting cells by shaking with glass beads have been fabricated to increase capacity, shorten time of agitation, and provide better control of temperature. Nossal[27] described a considerably more vigorous shaker capable of completely disrupting 0.5 gm of Proteus or Lactobacilli in about 2 minutes when the bacteria were mixed with 10 gm of glass beads and 10 ml of 0.9% KCl. By chilling the suspension in the stainless-steel capsule to near 1°, but not freezing, and by operating the shaker in a room maintained at —1°, the temperature of the suspension remained under 10° after 30 seconds of agitation and under 20° after 90 seconds. Without special cooling, however, suspensions in Nossal-type shakers heat at the rate of about 1 degree per second.[28] A Nossal machine with a cooling system is now commercially available in the United States (McDonald Engineering Company, Cleveland, Ohio).

The Braun shaker (The Braun Company, Melsungen, Germany), built to the specifications of Merkenschlager *et al.*,[29] has greater capacity and uses standard glass reagent bottles in place of metal capsules. When loaded with 36 ml of cell suspension (about 42 mg dry weight per milliliter) and 44 gm of glass beads, it disrupted about 1.5 gm of staphylococci in 5 minutes of total shaking over a 15- to 20-minute period.[28] During this treatment, the temperature never rose above 5°. Another convenient shaking device is the adaptation by Shockman *et al.*[30] of a standard centrifuge shaker head. By operating it in a refrigerated centrifuge the temperature of suspensions could be maintained near 0° throughout continuous operation, and up to 6 gm of dry bacteria

[23] I. M. Dawson, *Soc. Gen. Microbiol. Symp.* **1**, 119 (1949).
[24] M. R. J. Salton and R. W. Horne, *Biochim. Biophys. Acta* **7**, 177 (1951).
[25] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **5**, 981 (1951).
[26] D. H. Northcote and R. W. Horne, *Biochem. J.* **51**, 232 (1952).
[27] P. M. Nossal, *Australian J. Exptl. Biol. Med. Sci.* **31**, 583 (1953).
[28] E. Huff, H. Oxley, and C. S. Silverman, *J. Bacteriol.* **88**, 1155 (1964).
[29] M. Merkenschlager, J. Schlossman, and W. Kurz, *Biochem. Z.* **329**, 332 (1957).
[30] G. D. Shockman, J. J. Kolb, and G. Toennies, *Biochim. Biophys. Acta* **24**, 203 (1957).

could be disrupted in a single, short operation. In still another development of this principle, Ross[31] has described a continuous-flow shaker capable of completely disrupting heavy suspensions of bacteria at rates ranging from about 1 to 4.5 liters/hour. Plastic rather than glass beads were employed. Although an exit temperature of 18° to 21° for an organism with holdup time of 35 seconds appears to be moderately satisfactory, no data were given on temperature rise during the longer holdup times required for certain common bacteria (for example, 96 seconds for *Aerobacter aerogenes*).

vii. Decompression

Fraser[32] demonstrated that bacteria in aqueous suspension could be disrupted by mixing them with a gas under pressure and then rapidly returning them to normal atmospheric pressure. The method was crude, and a substantial portion of the product was liberated into the air. Foster *et al.*[33] designed an instrument for the same purpose which permitted almost instantaneous decompression and safe handling of pathogens. The capacity was rather small and many of the cells remained intact, but a moderate yield of apparently clean cell walls was achieved. Future utility of the method should depend largely on whether or not the product can be shown to have any distinctive advantages over that of other methods.

viii. High-Pressure Extrusion

In 1950 Milner *et al.*[34] described a method for dispersing components and constituents of cells without abrasives by forcing them through a constricted orifice at high pressure. This original "pressure cell" displayed a limited efficiency in disrupting cells of yeasts and *Escherichia coli* when operated at the maximum pressure of 20,000 psi. After progressive modification,[35] its direct descendant, now manufactured commercially (Aminco French Pressure Cell, American Instrument Company, Silver Spring, Maryland), is equipped with O-ring seals and a built-in, side-opening needle valve, but is still designed for maximum pressures of about 20,000 psi. In operation, the cylinder, with valve closed, is loaded with the suspension (for example, 20 to 40 mg/ml) of cells to be disrupted, and the fitted piston is introduced. The cell is inverted, the

[31] J. W. Ross, *Appl. Microbiol.* **11**, 33 (1963).
[32] D. Fraser, *Nature* **167**, 33 (1951).
[33] J. W. Foster, R. M. Cowan, and T. A. Maag, *J. Bacteriol.* **83**, 330 (1962).
[34] H. W. Milner, N. S. Lawrence, and C. S. French, *Science* **111**, 633 (1950).
[35] C. S. French and H. W. Milner, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 64. Academic Press, New York, 1955.

valve opened, and the piston depressed manually to expel air; then with valve closed, the cylinder is placed upright in a hydraulic press. When operating pressure is reached, the valve is opened slightly to deliver liquid at the rate of a few milliliters per minute. The desired pressure is maintained by pumping until the entire charge has been expelled. Extrusion through the narrow orifice in conjunction with sudden release of pressure creates the shear forces that bring about disruption.

Although the Aminco cell and a hand-operated press make a satisfactory and inexpensive apparatus for certain purposes, many bacteria and nearly all spores rupture only at higher pressures. With higher pressures, generation of heat increases rapidly with consequent denaturation of materials at the orifice, and hand-pumping becomes quite exhausting; stronger cells, better seals, automatic pressure control, and more sensitive valves are demanded. Work with pathogens is impossible without some elaborate type of shielding. Indeed, the engineering problems encountered in meeting the needs for higher pressures and greater versatility are formidable. Following the direction of Ribi *et al.*,[36,37] either the Aminco cell or a more ruggedly designed cell has been adapted to special needs in many laboratories where the necessary engineering skills and milling facilities are available. An automatic apparatus of large capacity, great convenience, and versatility is now on the market (Sorvall-Ribi Refrigerated Cell Fractionator, Model RF-1. Ivan Sorvall, Inc., Norwalk, Connecticut; Operating Manual, 1964). Among many distinctive features offered is a newly designed valve, provided with efficient cooling of the orifice, which permits sensitive fine adjustment for positive control of flow rate over a broad range of pressures.

Another means by which microorganisms may be disrupted through application of pressure was supplied by Hughes.[38] He found that when a mixture of bacteria and abrasive particles in a cylinder was compressed slowly with a piston driven by a hydraulic press most of the cells remained intact, but when pressure was delivered by sudden, powerful blows the bacteria were ruptured. The technical problems of making this principle useful were solved by constructing the Hughes press in two halves which were bolted together. The paste of bacteria to be disrupted was then driven across the interface of the closely fitted blocks into a collecting channel milled into one of them. By chilling the press to —20° or below, the use of abrasives could be dispensed with, since,

[36] E. Ribi, T. Perrine, R. List, W. Brown, and G. Goode, *Proc. Soc. Exptl. Biol. Med.* **100**, 647 (1959).

[37] T. D. Perrine, E. Ribi, W. Maki, B. Miller, and E. Oertli, *Appl. Microbiol.* **10**, 93 (1962).

[38] D. E. Hughes, *Brit. J. Exptl. Pathol.* **32**, 97 (1951).

presumably, ice crystals performed the function of abrasives. The drop hammer which is supplied with commercial models (Colab Laboratories, Inc., Chicago Heights, Illinois) is said to deliver a force of the order of 12 to 15 tons/in^2.

The X-press described by Edebo[39] appears to combine features of the Milner-French (Aminco) cell and the Hughes press. The prototype model was later modified to render it more suitable for work with pathogenic microorganisms.[40] For disruption in any version of the X-press, packed sediments of cells may be first molded to shape by freezing them in plastic tubes. The tubes are then cut away, and the cells inserted into a chilled cylinder with a small orifice of fixed size at the bottom. Alternatively, provision may be made for freezing cell pastes directly in the cylinder, without preliminary molding in tubes. A piston driven by a hydraulic press then forces the frozen cell mass through the orifice into the collecting flask. For *Salmonella typhimurium*, and probably for many other organisms, one pressing serves to disrupt about 95% of the cells, and pressing twice more brings the proportion to over 99%. Repeated pressings, however, result in further comminution of the already-broken cells.[40]

Both the Hughes press and the X-press have the advantages of speed of operation on cells that remain cold throughout the processing, and both have excellent capacity because they admit solidly packed cells instead of suspensions. Both can be operated without abrasives. Disintegration of pathogens in the Hughes press is inadvisable, and hand operation of the drop hammer may become somewhat arduous with organisms that resist breaking. Although repeated processing to achieve complete disruption is readily handled in the X-press, the recovery of cell walls may be rendered progressively more difficult because of excessive fragmentation of the walls. For this purpose single pressings may be preferable.

b. Preparation of Whole Cell Walls

i. General Considerations

In the literature on methods for disruption of bacteria, one finds a major emphasis on achieving the highest possible proportion of breakage. This is a legitimate concern when the objective is recovery of cell-free enzymes or other soluble constituents, but it can be a misplaced effort for isolation of cell walls. With most methods and most organisms, the excessive force required to break nearly every cell produces a degree

[39] L. Edebo, *J. Biochem. Microbiol. Technol. Eng.* **2**, 453 (1960).
[40] L. Edebo and T. Holme, *Acta Pathol. Microbiol. Scand.* **51**, 173 (1961).

of fragmentation in many of the walls that is incompatible with recovery by available means. The obverse situation also should not be overlooked: the noncell-wall material that we call protoplasm may be the desired product, in which case contamination with cell-wall fragments should be held to the minimum. In certain of our studies we have sought to make the cleanest possible separation between cell walls and protoplasm in order to assess the biological activities associated with each. For such purposes it is often better to settle for a small percentage of rupture than to risk excessive fragmentation. It has always been possible to devise methods for separating cell walls from unbroken cells, but the procedure must be adjusted to the individual case. With shaking methods we have usually found it desirable to employ the minimum agitation that will just "crack" about 50% of the cells.[41] A substantially higher proportion of rupture without excessive fragmentation is possible in the modern refrigerated pressure cell. There are also exceptions such as *Staphylococcus aureus*, which is among the most difficult bacteria to rupture and whose cell walls will resist much additional shaking without further fragmentation. Cell walls of such bacteria may be best prepared in the more vigorous shaking machines, so long as adequate temperature control is maintained.

Both for selection of conditions necessary to satisfactory disruption and for control of purification of cell walls, the use of electron microscopy is indispensable. Examination of a few fields quickly shows the proportion of disrupted cells and the degree of fragmentation of the walls with an accuracy that is not approximated by sedimentation behavior, electrophoretic mobility, or chemical analysis. The characteristic electron-transparency of clean cell walls is quickly recognized and can be only slightly supplemented by more objective but fallible measurements of density and absorption of utraviolet wavelengths. When a given substance (for example, rhamnose) is known to be associated only with the cell wall, assays for this substance may be employed as an index to the proportion of wall material that has escaped into the soluble fraction.[18,42] Light-scattering patterns in cesium chloride gradients are also useful in identifying fractions containing cell walls or unbroken cells.[28]

Cells to be disrupted are first washed well and then resuspended in water or dilute buffer, usually with a high-speed mixer, to a concentration that is best determined by a series of trials for each organism followed by observation of the result with the electron microscope. Much better results have been obtained with freshly grown and harvested cells

[41] E. Ribi, K. C. Milner, and T. D. Perrine, *J. Immunol.* **82**, 75 (1959).
[42] H. D. Slade and J. K. Vetter, *J. Bacteriol.* **71**, 236 (1956).

than with cells killed by heat or chemicals or even with lyophilized or frozen and stored cells. With only rare exceptions, the use of killed or stored cells has resulted in greatly increased difficulty of removing adherent protoplasmic constituents from the ruptured cell walls. When working with pathogens one must, of course, take adequate precautions to shield operators from aerosols and other dangerous exposure.

It is obviously impractical to give explicit instructions here for preparation of cell walls from a large number of species after disruption in each type of machine that might be suitable. More detailed descriptions are offered, however, for isolation of walls from two very different species disrupted in two of the devices that have been found most generally useful.

ii. Disruption of Salmonella enteritidis in the Mickle Tissue Disintegrator

A harvest of early stationary-phase cells grown, preferably, in liquid synthetic medium (because it is virtually impossible to free polysaccharide constituents of the bacteria from agar polysaccharide in the case of agar-grown cells) is drawn through a double thickness of filter paper in a Büchner funnel and washed three times in water or dilute buffer by centrifugation at 2000 *g* for 45 minutes. The combination of filtration and washing removes all flagella that can be detected with the electron microscope, although some flagellar substance undoubtedly remains with the cells. An appropriate concentration for disruption is conveniently obtained by adjusting turbidity to a scale reading of 770 in a Klett-Summerson colorimeter with filter No. 520. Five milliliters of suspension is then mixed with 5 gm of glass beads 0.1 to 0.2 mm in diameter (glow beads, paving beads, or Ballotini) and vibrated in a smooth cup on the Mickle machine. Under these conditions, a 3-minute shaking period usually produces the desired 50% disruption. All of these procedures, as well as those for recovery of fractions, are carried out at 4°. Since this is not a concentrated suspension, and since the volumes employed are small, the method is not suitable for large-scale production, but the quality of cell walls that may be recovered is excellent. With the pressure cell discussed below, quantities of Enterobacteriaceae similar to those described for Mycobacteria may be disrupted efficiently.

The quality and treatment of the glass beads may be of importance. Kolb[43] has reported that all but one of eleven brands tested imparted significant alkalinity to fluids during shaking and that this could not be eliminated by washing. He identified one brand (Prismo Safety Corporation, Huntingdon, Pennsylvania) that did not release alkali. He

[43] J. J. Kolb, *Biochim. Biophys. Acta* **38**, 373 (1960).

also recommended against treatment with strong acid or drying with excessive heat on the ground that these injured the surfaces and increased the dissolution of materials from the glass. After treatment with mild acid and thorough washing, the beads employed by the authors (Cataphote No. 12 glass beads, Cataphote Corporation, Toledo, Ohio) appear to be satisfactory, possibly because of the short periods of shaking and the buffering capacity of the disrupted cells.

iii. Disruption of Mycobacterium Tuberculosis (BCG) in the Sorvall Cell Fractionator

Rigorous pretreatment, as with potassium hydroxide–ethanol mixture,[44] by which some of the problems of disrupting Mycobacteria have been solved, is hardly desirable for immunological studies. Although cell walls of acid-fast organisms have been prepared by shaking methods,[45] the process is arduous and the yields are small. Such cell walls are readily prepared in the automatic pressure cell,[46] and the capacity of the machine is almost without limit. Suspensions containing 200 mg (wet weight) of BCG per milliliter are processed at the rate of 130 ml per stroke, and as much as 500 gm of bacteria may be prepared, disrupted, and washed in one day. Ninety-five per cent or more of the cells are broken, and electron micrographs give the impression that most have been simply torn in half, spilling the contents.

A satisfactory procedure is to grow BCG (or other Mycobacteria) as a heavy pellicle on Sauton's medium[47] (about 10 days of incubation at 37° for BCG) and harvest by filtration onto sterile gauze supported by a stainless-steel screen.[48] After being washed with water through the same filter, the cell mass is weighed and suspended in water to a maximum concentration of 200 to 250 mg/ml with the aid of a high-speed mixer. Even dispersion is of no particular importance for this method except that the aggregates should not be large enough to settle out during a single stroke of the piston. Pressure is maintained at 40,000 psi, and temperature at the orifice is held at 5° to 10° by playing a jet of nitrogen gas, automatically precooled to —50°, onto the orifice as the effluent emerges. The cell itself can be maintained at any desired temperature by a special cooling jacket. When the preset pressure is reached, the valve is opened slightly until fluid begins to emerge. The speed of the

[44] C. S. Cummins and H. Harris, *J. Gen. Microbiol.* **14**, 583 (1956).
[45] E. Ribi, C. L. Larson, R. List, and W. Wicht, *Proc. Soc. Exptl. Biol. Med.* **98**, 263 (1958).
[46] C. L. Larson, E. Ribi, W. C. Wicht, R. H. List, and G. Goode, *Nature* **198**, 1214 (1963).
[47] B. Sauton, *Compt. Rend.* **155**, 860 (1912).
[48] E. Ribi, C. L. Larson, W. Wicht, R. List, and G. Goode, *J. Bacteriol.* **91**, 975 (1966).

stroke is then governed chiefly by maintenance of the desired temperature and pressure. Although the apparatus is enclosed for protection of the operator, and exhaust air is incinerated when one is working with dangerous organisms, additional safety measures under development would further diminish the hazard from aerosols by providing another method for cooling the orifice.

iv. Recovery and Refinement of Cell Walls

Isolation of the walls is essentially the same after either method of disruption; the subject has been well discussed by Salton.[3] Glass beads have first to be removed from the Mickle product by settling or by passage of the material through a course (grade 2) sintered-glass filter. A short, low-speed centrifugation (3000 to 4000 *g* for 10 minutes) may remove most of the unbroken cells when the proportion of these is substantial. At an early stage of processing it is well to take some measure to prevent degradation by autolytic enzymes that may be activated by disruption. Heat has been employed for the purpose,[49,50] but this may hinder the cleaning-up process and degrade or release antigens. For many purposes, including the two described here, we have employed an early wash in water containing 0.1% formalin.

A scheme of differential centrifugation designed to fit the individual case may then be applied. Centrifugal force as low as 2500 *g* for 70 minutes has been found to sediment practically all wall material from *Salmonella enteritidis* disintegrated in the Mickle apparatus as described here.[41] Usually, however, forces in the range of 15,000 to 30,000 g have been applied for 15 minutes to 2 hours.[3,51] Billaudelle *et al.*[52] found it desirable to centrifuge at forces up to 100,000 *g* for 60 to 180 minutes in order to sediment the washed cell walls of *Bordetella pertussis* from the disruption product of the X-press. Unbroken cells are then found in an opaque bottom layer, and cell walls in a more translucent upper layer which is ordinarily removed by gentle washing. Repeated centrifugations complete the separation. On occasion difficulties are presented by the tendency of cell walls to aggregate during the process of purification. In such cases the best solution is to turn directly to some form of gradient centrifugation. Some bacteria yield additional layers of sedimentable particles which must be dealt with as found. Bits of cell membrane may be found clinging to Mickle-prepared cell walls (R. L.

[49] R. E. Strange and F. A. Dark, *J. Gen. Microbiol.* **16**, 236 (1957).
[50] J. Mandelstam and H. J. Rogers, *Biochem. J.* **72**, 654 (1959).
[51] K. Fukushi, R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner, and E. Ribi, *J. Bacteriol.* **87**, 391 (1964).
[52] H. Billaudelle, L. Edebo, E. Hammarsten, C.-G. Hedén, B. Malmgren, and H. Palmstierna, *Acta Pathol. Microbiol. Scand.* **50**, 208 (1960).

Anacker, unpublished observation), and other fragments may lie in a top layer of sediment.[3] The location of membrane fragments may be traced through examination for succinic dehydrogenase activity, which is known to be associated with these structures.[53a–53d] The pressure cell appears to lyse the membrane, because resolvable fragments are not often found in the sediments, and nearly all the succinic dehydrogenase may be recovered from the soluble fraction (protoplasm).[53e] The freeing of the walls from other constituents may include stages of treatment with trypsin[44,54–56] and washing with 1 *M* NaCl. Trypsin is not known to attack the wall proper, but the possibility should be checked in untried cases. Since 1 *M* NaCl is a rather good extractant for endotoxin,[57] there may be some loss of this constituent in the washing process.

Centrifugation of disrupted bacteria in density gradients of sucrose,[58] glycerol,[59] and CsCl[28] has been employed with good results in the isolation of cell walls. Separation in two-phase polymer systems according to Albertsson[60,61] has also been employed. These methods may be superseded by the development of zonal centrifugation,[62] which provides high resolution, large capacity, and quantitative collection of fractions without mixing (see Chap. 7,E, Vol. II). Indications from recent experience (E. Ribi and C. Price, unpublished observations) are that 2 gm or more of whole-cell/cell-wall mixture may be sedimented through a sucrose gradient in the Anderson A-IX low-speed rotor to yield pure, discrete cell walls in a single step. The principal sedimentation bands are clearly visible through the transparent end plates, and the patterns of continuous nephelometer readings may be recorded during collection of fractions.

[53a] P. Mitchell and J. Moyle, *Biochem. J.* **64,** 19P (1956).
[53b] R. Storck and J. T. Wachsman, *J. Bacteriol.* **73,** 784 (1957).
[53c] C. Weibull, H. Beckman, and L. Bergström, *J. Gen. Microbiol.* **20,** 519 (1959).
[53d] E. Vanderwinkel and R. G. E. Murray, *J. Ultrastruct. Res.* **7,** 185 (1962).
[53e] C. Weibull, W. D. Bickel, W. T. Haskins, K. C. Milner, and E. Ribi, *J. Bacteriol.* **93,** 1143 (1967).
[54] M. McCarty, *J. Exptl. Med.* **96,** 569 (1952).
[55] M. R. J. Salton, *J. Gen. Microbiol.* **9,** 512 (1953).
[56] R. L. Anacker, R. A. Finkelstein, W. T. Haskins, M. Landy, K. C. Milner, E. Ribi, and P. W. Stashak, *J. Bacteriol.* **88,** 1705 (1964).
[57] M. Digeon, M. Raynaud, and A. Turpin, *Ann. Inst. Pasteur* **82,** 206 (1952).
[58] B. S. Roberson and J. H. Schwab, *Biochim. Biophys. Acta* **44,** 436 (1960).
[59] J. W. Foster and E. Ribi, *J. Bacteriol.* **84,** 258 (1962).
[60] P. Å. Albertsson, *Biochim. Biophys. Acta* **27,** 378 (1958).
[61] P. Å. Albertsson, "Partition of Cell Particles and Macromolecules." Wiley, New York, 1960.
[62] N. G. Anderson, H. P. Barringer, N. Cho, C. E. Nunley, E. F. Babelay, R. E. Canning, and C. T. Rankin, Jr., "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis: The Development of Low-Speed "A" Series Zonal Rotors," Monograph 21. J. Natl. Cancer Inst. Series (1966).

v. Concluding Observations

Although cell walls may be prepared from bacteria disrupted in almost any of the ways described, methods employing shaking with glass beads or high-pressure extrusion appear to have special advantages. Bacteria and spores having tough walls that resist fragmentation may be best ruptured in quantity in the large-capacity shaking devices. The Mickle apparatus has proved its value over the years as an inexpensive and efficient disintegrator for work with small batches, and one which is highly adaptable to different needs. With the various forms of control that may be exerted by simple means, it may be used to prepare high-quality walls from a great variety of cells. The Hughes press will disrupt substantial quantities of cells while giving excellent protection to constituents and components by maintaining freezing temperatures. The X-press has the same advantages plus greater capacity, ease of operation, and safety for the operator. For those who need and can afford it, the modified, automatic pressure cell described here offers the greatest versatility as regards controls, capacity, variety of cells it can disrupt, and quality of product.

2. PREPARATION OF CELL WALL ANTIGENS FROM GRAM-NEGATIVE BACTERIA*

a. General Procedures and Properties

Many reagents have been used for extractions of cellular antigens (O antigens) from gram-negative bacteria. These have been reviewed by Burger[1] and more recently by Davies.[2] They include such substances as pyridine, ethylene glycol, and urea. Since the latter review appeared, ether has been used with success by Ribi and his group (see Section D,3,b).

This section describes four techniques currently used to extract (1) the whole antigen with trichloroacetic acid (TCA), (2) the lipopolysaccharide with hot phenol, (3) the polysaccharide with acetic acid (AP), and (4) the polysaccharide by alkaline hydrolysis (KP).

The properties of the different extraction products differ widely. Only the TCA-extracted material is a good antigen in the rabbit; the lipopolysaccharide is a poor antigen in this species, whereas the two polysaccharide preparations are not in themselves antigenic. However, it is possi-

* **Section 1,B,2 was contributed by A. M. Staub.**

[1] M. Burger, "Bacterial Polysaccharides." Thomas, Springfield, Illinois, 1950.
[2] D. A. L. Davies, *Advan. Carbohydrate Chem.* **15**, 271 (1960).

ble to obtain antipolysaccharide antibodies by coupling the hydrolyzed alkaline polysaccharide to stromata[3] or the acid polysaccharide to protein.[4,5] While polysaccharide preparations are completely devoid of the biological properties associated with the more complex preparations (toxicity, pyrogenicity, enchancement of hormonal and enzymatic activities, etc.[6,7]), they possess part if not all of the specificity of the whole 0 antigen complex.[3]

The two polysaccharide preparations differ in the following three respects: (1) The acetic acid-degraded polysaccharide (AP) is a much smaller molecule than the polysaccharide obtained by alkaline hydrolysis (KP). (2) AP precipitates fewer antibodies from horse antisera than KP. (3) Antibody complexes of AP do not fix complement and AP cannot be fixed onto erythrocytes, whereas KP possesses both properties.

b. Trichloroacetic Acid Extraction of the Whole Antigen Complex

This method, introduced by Boivin and Mesrobeanu,[8] extracts only a part of the antigen present in the bacteria. Preliminary digestion by trypsin should permit a total extraction,[9] but in our hands this technique yielded a product containing much more nitrogen than was obtained by direct extraction.[10] Dry or wet bacteria can be extracted, but products containing less nitrogen are obtainable when wet bacteria are used.

The gram-negative bacteria are grown for 18 hours on nutrient agar in Roux bottles and harvested in 0.9% sodium chloride solution (about 20 ml of saline per Roux bottle). After two washings in 0.9% saline in a weighed centrifuge tube, the packed cells are suspended in five times their weight of water at 0°. An equal volume of 0.5*N* trichloroacetic acid is added, and the temperature of the mixture is maintained at 4° for 3 hours.

The mixture is then warmed to room temperature and centrifuged.*

[3] A. M. Staub, *in* "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), p. 38 Rutgers Univ. Press, New Brunswick, New Jersey, 1964.

[4] P. Grabar and J. Oudin, *Ann. Inst. Pasteur* **73**, 627 (1942).

[5] U. Hämmerling, "Immunchemische Untersuchungen zur Überführung von bakteriellen Polysaccharid—Haptenen in Vollantigene," Thesis Universität Freiburg, Freiburg, Germany, 1965.

[6] O. Westphal, *Ann. Inst. Pasteur* **98**, 789 (1960).

[7] M. Landy and W. Braun (eds.), "Bacterial Endotoxins." Rutgers Univ. Press, New Brunswick, New Jersey, 1964.

[8] A. Boivin and L. Mesrobeanu, *Rev. Immunol.* **1**, 553 (1935).

[9] A. Boivin, L. Mesrobeanu, and I. Mesrobeanu, *Compt. Rend. Soc. Biol.* **117**, 271 (1934).

[10] A. M. Staub and R. Combes, *Ann. Inst. Pasteur* **80**, 21 (1951).

* It has been observed that centrifugation at 4° results in a very opalescent product which contains much more nitrogen and is much less toxic.

The supernatant is neutralized to pH 6.5 with concentrated and then dilute sodium hydroxide and cooled to 0°. The complex is precipitated by 2 volumes of ethanol at —4° by pouring the 0° solution into ethanol cooled to —10° to —15°. The precipitate which sediments overnight at —4° is centrifuged at this temperature. It is then dissolved in distilled water at one-tenth of the starting volume, neutralized if necessary, and dialyzed for 2 days against tap water followed by 2 days against distilled water. The final product is centrifuged at 27,000 *g* to eliminate microbial debris.

It is possible to freeze or lyophilize the solution, but some denaturation may occur, and it seems preferable to keep the solution in the cold with a preservative such as merthiolate at a concentration of 1/5000. Such solutions keep their immunological properties for a long time, although biological activity may diminish somewhat during storage.

Such preparations are never immunologically or chemically identical from one batch to another; nitrogen content may vary from 2 to 4% with any given species. A good preparation from *Salmonella typhi* contains about 2.5% nitrogen and gives a final yield of 3 to 5% of the dry microbial weight.

The purity of this material can be improved by ultracentrifugation[11] or by filtration on Sephadex G-200. With either of these methods it is possible to remove additional protein, nucleic acid, and degraded polysaccharides which together represent about 20% of the weight of the whole antigen.

c. Phenol–Water Extraction of the Lipopolysaccharide

Phenol was first introduced by Palmer and Gerlough[12] to extract O antigens from bacteria. These authors used successive extractions by phenol followed by water. Westphal *et al.*[13] showed later that this procedure could be simplified by shaking bacteria directly in an emulsion of equal volumes of liquid phenol and water for a few minutes at low temperature (5° to 10°). However, lipopolysaccharides extracted from bacteria with *cold* emulsions of phenol-water still contain varying amounts of firmly bound protein. An improvement of the technique was to treat the cells with a solution of equal volumes of phenol and water at 65° to 68°.[13]

Such preparations contain mixtures of lipopolysaccharide, nucleic acid, and more degraded (lipo)polysaccharide. Purification of the large molecules of lipopolysaccharide can be achieved by ultracentrifugation at 105,000 *g* which leaves in solution most of the nucleic acid and degraded

[11] L. Chedid, R. C. Skarnes, and M. Parant, *J. Exptl. Med.* **117**, 561 (1963).
[12] J. W. Palmer and T. D. Gerlough, *Science* **92**, 155 (1940).
[13] O. Westphal, O. Lüderitz, and F. Bister, *Z. Naturforsch.* **7b**, 148 (1952).

product. The remaining nucleic acid can be eliminated by precipitation with Cetavlon (cetyl trimethylammonium bromide), and the small quantities of degraded antigen eliminated either by further ultracentrifugation or by filtration on Sephadex.[14]

i. Desiccation of the Bacteria

Bacteria grown on agar are harvested in 0.9% sodium chloride solution and washed twice with saline solution and three times with acetone (about 500 ml per 15 gm of dry cells). After the last washing, the liquid paste must be dispersed in a thin film along the wall of the centrifuge tube and stirred in order to obtain a fine powder of dry cells which is sieved through a double thickness of fine gauze. This latter step eliminates most of the agar particles.

ii. Phenol–Water Extraction

Twenty grams (dry weight) of bacteria are suspended in 350 ml of water maintained at 65° to 68°; 350 ml of 90% phenol, preheated to 65° to 68°, is then added with vigorous stirring, and the mixture is kept for 10 to 15 minutes at 65°. The mixture is cooled to about 10° in an ice bath, and the emulsion is centrifuged at 3000 rpm for 30 to 45 minutes, resulting in the formation of three layers: water, phenol, and an insoluble fraction. The insoluble residue sometimes forms a layer at the phenol–water interface. The water phase is taken off by suction, and the phenol layer and the insoluble residue are extracted with another 350 ml of hot water as described above. The combined water extracts are dialyzed for 3 to 4 days against distilled water to remove phenol and small amounts of low-molecular-weight bacterial substances. The slightly opalescent solution containing the lipopolysaccharide and ribonucleic acid (RNA) is concentrated at 35° to 40° under reduced pressure to a volume of about 100 ml. After low-speed centrifugation to remove traces of insoluble material, the supernatant solution is freeze-dried to give a fluffy, almost white powder.

iii. Removal of Nucleic Acid

The lyophilized crude extract is dissolved in water to give a 3% solution which is then centrifuged for 6 to 8 hours at 80,000 *g*. The sediment is resuspended in water and centrifuged for 3 hours at 105,000 *g*. This latter step is repeated one or two more times, after which the final sediment is taken up in a minimum amount of water and freeze-dried. It contains 3% of nucleic acid.

One gram of crude lipopolysaccharide, containing 2 to 5% of RNA,

[14] O. Westphal and K. Jann, *in* "Methods in Carbohydrate Chemistry" (R. C. Whistler, ed.), Vol. V, p. 83. Academic Press, New York and London, 1965.

is dissolved in 150 ml of water; 15 ml of a 2% aqueous Cetavlon solution is added, and the mixture is stirred for about 15 minutes at room temperature. The turbid mixture is then centrifuged for 20 minutes at 3000 rpm to remove the precipitated RNA. The opalescent supernatant is lyophilized, and the fluffy residue is dissolved in 50 to 60 ml of 0.5 *M* sodium chloride. The solution is poured into a tenfold volume of ethanol to precipitate the lipopolysaccharide while leaving the excess Cetavlon in solution. After standing for 1 to 2 hours at 0° to 4°, the precipitate is centrifuged and redissolved in water.

The final yield after these two purifications is about 2% of the dry weight of bacteria. Another possibility is to precipitate the RNA directly from the crude extract by Cetavlon according to a technique described by Westphal and Jann.[14]

d. Somatic Degraded Polysaccharide Obtained by Acetic Acid Hydrolysis of the Bacteria

This procedure was first used by White[15] and later refined by Freeman,[16] who isolated the polysaccharide in a high state of purity. The advantage of this technique is that it permits a quantitative extraction of the polysaccharides from the bacteria, enabling one to titrate the polysaccharide in a culture of bacteria.[17]

i. Extraction and Purification of the Polysaccharides

Bacteria are prepared as described above (Section B,2,c,i). Fifty to sixty grams of acetone-dried bacteria are suspended slowly and evenly in 900 ml of water. The suspension is heated in a boiling water bath, and 100 ml of hot acetic acid is added when the temperature has reached 90°. The mixture is heated for 1.5 hours under reflux in the boiling water bath. After cooling, the mixture is centrifuged, and the cells are hydrolyzed once more under the same conditions. The two supernatants are desiccated separately under reduced pressure at 55°, and the dry residues are suspended in water (100 ml for the first residue, 50 ml for the second). The insoluble portions are washed with water.

ii. Purification of Polysaccharides

The crude polysaccharides are precipitated from the extracted supernatant (including the washings) by adding 1 volume of ethanol and

[15] B. White, *J. Pathol. Bacteriol.* **34,** 325 (1931).
[16] G. G. Freeman, *Biochem. J.* **36,** 340 (1942).
[17] A. M. Staub and R. Combes, *Ann. Inst. Pasteur* **83,** 528 (1952); A. M. Staub and J. Wiart, *ibid.* **107,** 791 (1964).

allowing the solution to stand overnight at 4°. The precipitate is taken up in a small volume of water in which it should not dissolve. If a part of the material dissolves, it must be added back to the supernatant and precipitated with 6 volumes of ethanol.* The procedure is repeated, with smaller volumes each time until no more precipitate forms after addition of 1 volume of ethanol or until this precipitate becomes completely soluble in water. Generally, five to six precipitations are required, and the last precipitation is done in 20 ml of water. It is usually necessary to add some sodium acetate to facilitate the precipitation.

The second step of the purification uses 94%, 96%, or 98% acetic acid as the precipitant, depending on the bacterial species. With some bacteria, this process may be repeated three times, as indicated by Freeman for *S. typhi* and *S. typhimurium*.[16] However, significant losses in yield may occur after more than one precipitation so that it is often better to omit further precipitations. In some cases, it seems that very few impurities remain after the alcoholic precipitation, and thus the acetic acid step may be completely eliminated.

Final purifications are performed with alternate precipitations using 1 and then 6 volumes of ethanol until no precipitation occurs after the addition of 1 volume or until the precipitate can be completely solubilized in water. One precipitation is often sufficient. Finally, the solution is dialyzed against distilled water and freeze-dried.

Such polysaccharides are always mixtures of molecules which consist of variously degraded fractions of the polysaccharide with an average molecular weight of 20,000 to 40,000. The yield varies between 1 and 2% of the dry weight.

Such preparations are immunologically identical from one batch to another. However, their nitrogen content varies between 0.4 and 0.8%,† owing to a small amount of remaining D-glucosamine and of residual amino acids. Also, the phosphorus content varies between 0.4 and 0.9%, probably owing largely to the phosphate present in the core of the polysaccharide.[18]

e. Polysaccharide Obtained by Alkaline Hydrolysis

The degraded polysaccharide prepared by acetic acid hydrolysis cannot be fixed onto red cells, and its complexes with antibodies do not fix complement; it is therefore useful to prepare a less degraded polysaccharide which possess such properties. One means is to cleave the lipopoly-

* Some polysaccharides (such as *S. paratyphi* A) required 9 volumes of alcohol to precipitate.

† It may be higher if the polysaccharides contain hexosamines.

[18] O. Lüderitz, A. M. Staub and O. Westphal, *Bacteriol. Rev.* **30**, 193 (1966).

saccharides by alkaline hydrolysis. According to Neter *et al.*,[19] heating for 1 hour in 0.25 *N* NaOH at 56° produces a polysaccharide with a molecular weight of about 200,000, which is devoid of toxicity but which can be fixed onto red cells. Another possibility is to extract the polysaccharide directly from the cells by alkaline hydrolysis according to a procedure described by Thomas and Mennie[20] and refined by Staub and Davarpanah.[21]

i. Extraction of Polysaccharides

Bacteria are prepared as indicated above (Section B,2,c,i). Twenty-five grams of acetone-dried bacteria are hydrolyzed with 500 ml of 0.25 *N* NaOH during 5 hours at 56°.

ii. Partial Purification of Polysaccharides

The soluble extract is precipitated by 2.5 volumes of alcohol overnight at 4°. The precipitate is suspended in 0.25 *N* NaOH during 24 hours at 4°, and the soluble part is precipitated again by alcohol. The precipitate is acetone-dried. The resulting powder is suspended in water (0.5 gm for 40 ml), and the soluble fraction is precipitated by 0.25 *N* CCl_3COOH at 4°. The supernatant is neutralized and precipitated by 3 volumes of alcohol overnight at 4°. The precipitate is acetone-dried and extracted by 90% phenol twice during 24 hours at 37°; after three washings by acetone, the precipitate is dried. The yield is about 5%. The final product contains 0.5 to 1% nitrogen in the case of *S. typhi*. The product is heterogeneous, and various preparations are not immunologically identical. Moreover, further treatment does not improve its purity. Filtration on Sephadex was not available when this technique was elaborated. Such methods might be useful as means to obtain more homogeneous products.

[19] E. Neter, O. Westphal, O. Lüderitz, E. A. Gorzynski and E. Eichenberger, *J. Immunol.* **76**, 377 (1956).
[20] J. C. Thomas and A. T. Mennie, *Lancet* **259**, 745 (1950).
[21] A. M. Staub and C. Davarpanah, *Ann. Inst. Pasteur* **91**, 338 (1956).

3. PREPARATION OF CELL WALL ANTIGENS FROM GRAM-POSITIVE BACTERIA*

a. Introduction

As a result of the isolation and chemical characterization of bacterial cell walls, it has become apparent that many bacterial somatic antigens are wall constituents. The extraction and subsequent purification of cell

* Section 1,B,3 was contributed by Richard M. Krause.

wall antigens have been facilitated in large measure by the development of techniques for the preparation of cell walls from bulk lots of disrupted bacteria. It is usually possible by differential centrifugation to obtain cell wall preparations essentially devoid of the other cellular elements. If, because of incomplete separation, the cell walls contain elements of the capsule and the protoplast membrane, the final antigenic product may contain other polysaccharides or proteins. Figure 1 is a schematic diagram depicting the relationships of the surface structures of group A streptococci. In the case of gram-positive bacteria the cell wall antigens are either polysaccharides or proteins.

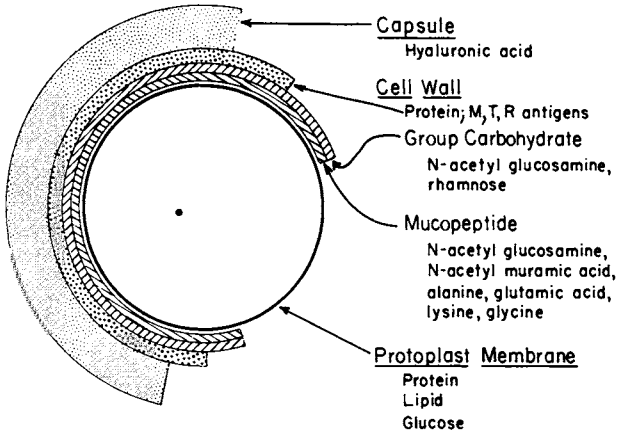


FIG. 1. Diagrammatic representation of the subcellular components of hemolytic streptococci. The monosaccharides listed here for the carbohydrate are those of group A streptococci. From R. M. Krause, *Bact. Rev.* **27**, 369, 1963.

A major class of polysaccharides, termed teichoic acids,[1] has been detected in the cell walls of many gram-positive bacteria. The teichoic acids are discussed and their preparation is described in Section B,4.

A new class of heteropolymers, termed mucopeptides[2] or glycosaminopeptides,[3] has been found in the walls of all bacteria so far examined. The substances contain *N*-acetylglucosamine, *N*-acetylmuramic acid, and a peptide of a limited number of amino acids, most notably alanine, glycine, glutamic acid, and lysine. Although the relative concentrations of these substances in the heteropolymer vary among the bacterial species, extensive structural studies indicate a backbone of repeating units

[1] J. J. Armstrong, J. Baddiley, J. G. Buchanan, B. Carss, and G. R. Greenberg, *J. Chem. Soc.* 4344 (1958).

[2] J. Mandelstam, and H. J. Rogers, *Biochem. J.* **72**, 654 (1959).

[3] M. R. J. Salton, "The Bacterial Cell Walls." Elsevier, New York, 1964.

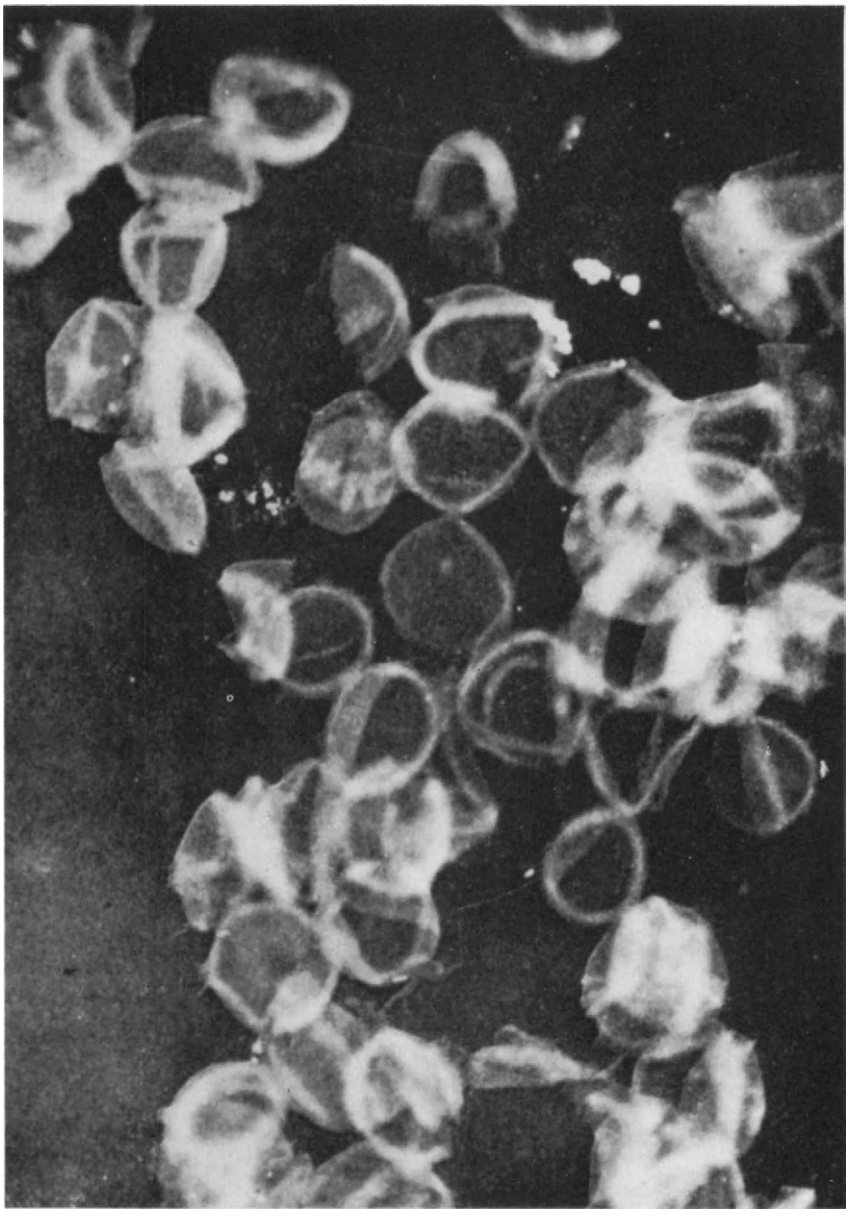


FIG. 2. Electron micrograph of hot formamide extraction of group A cell walls. These discrete disc structures are composed of mucopeptide. Magnification approximately ×15,000. From R. M. Krause and M. McCarty, *J. Exptl.* **114**, 127 (1961).

of the two amino sugars. There is a considerable body of evidence to suggest that this family of heteropolymers is a major chemical component which is the rigid structural element or matrix of the cell wall. For example, after treatment of streptococcal cell walls with proteolytic enzymes and hot formamide, glycosaminopeptide is the major component of the residual cell wall framework (Fig. 2). The antigenic properties of the glycosaminopeptide have not been examined. It is conceivable, however, that such studies in conjunction with chemical analysis will provide additional information on the differences in the glycosaminopeptide(s) among the bacterial species.

Hydrolysis with acid or alkali, and other reagents such as urea, formamide, and phenol, has been employed to extract bacterial antigens from cell walls. Antigens have also been isolated from the digest of cell walls hydrolyzed with enzymes that disrupt the mucopeptide and thus bring about solubilization of the cell wall components. It is not feasible in this discussion to describe the methods of isolating the antigens in all the gram-positive bacteria so far examined. The reader is referred to several recent reviews for additional information.[3-5] Special mention will be made of streptococci and pneumococci.

b. Hemolytic Streptococci

The two major antigens of the wall which have received particular attention are the M protein and the group-specific carbohydrate. Both of these antigens are extracted from whole bacteria or cell walls with pH 2 HCl at 100° for 10 minutes, the method initially employed by Lancefield.[6]

i. M Protein

Following neutralization, the residue is removed by centrifugation, and the M protein is precipitated with 2 volumes of absolute alcohol. After several reprecipitations the final product is devoid of the carbohydrate antigen. The carbohydrate is readily precipitated from the alcoholic solution by the addition of 5 volumes of acetone. As an alternative method, the M protein has been collected from the neutralized acid extract by precipitation between 0.33 and 0.6 saturation with $(NH_4)_2SO_4$ at pH 6 or 8.[7] After several reprecipitations the final product gave posi-

[4] H. J. Rogers, *in* "The Structure and Function of the Membranes and Surfaces of Cells" (D. J. Bell and J. K. Grant, eds.), p. 55. Cambridge Univ. Press, London, 1963.

[5] M. McCarty and S. I. Morse, *Advan. Immunol.* **4**, 249 (1964).

[6] R. C. Lancefield, *J. Exptl. Med.* **47**, 91 (1928).

[7] R. C. Lancefield and G. E. Perlman, *J. Exptl. Med.* **96**, 71 (1952).

tive reactions at a final concentration as low as 5 μg/ml with absorbed-type antiserum. The preparation and properties of M protein have been reviewed by Lancefield.[8]

ii. Group Carbohydrate Antigen

Several procedures have proved valuable for the extraction of the group carbohydrate antigen. Among them are those employing enzymes that digest cell walls. One such enzyme has been isolated from culture filtrates of *Streptomyces albus*,[9] and another is produced by group C streptococci undergoing virulent bacteriophage lysis.[10] The polysaccharide purified from the cell wall digests obtained with either enzyme, although composed primarily of group-specific carbohydrate, contains some mucopeptide material.

TABLE I
COMPOSITION OF GROUP A CELL WALLS AND OF THE SOLUBLE CARBOHYDRATE (CHO) AND THE INSOLUBLE RESIDUE AFTER HOT, FORMAMIDE EXTRACTION

		Formamide treatment		
			Formamide residue	
Constituent	Cell walls (%)	Extracted group A CHO (%)	Amount (%)	Mole ratio
Rhamnose	34.0	60.0	1.2	—
Glucosamine	18.8	30.0	11.0	1.6
Muramic acid	6.4	*a*	9.4	(1)
Alanine	17.4	*a*	31.0	9.3
Glutamic acid	7.9	*a*	17.0	3.1
Lysine	8.0	*a*	14.0	2.6
Glycine	1.0	*a*	0.9	0.3

[a] Less than 1%.

The carbohydrate extracted with formamide at 180° has proved particularly useful for immunochemical purposes.[11] In this procedure 5 ml of formamide is added to each 100 mg of lyophilized cell walls, and extraction is carried out with continuous stirring at 180°, maintained by means of an oil bath. After extraction, removal of the insoluble material by centrifugation is facilitated by the addition of 2 volumes of acid alcohol (95 volumes of alcohol and 5 volumes of 1 *N* HCl). The

[8] R. C. Lancefield, *J. Immunol.* **89**, 307 (1962).
[9] M. McCarty, *J. Exptl. Med.* **96**, 555 (1952).
[10] R. M. Krause, *J. Exptl. Med.* **108**, 803 (1958).
[11] R. M. Krause and M. McCarty, *J. Exptl. Med.* **114**, 127 (1961).

carbohydrate is collected from the alcoholic supernatant by the addition of 5 volumes of acetone. Although the bulk of the carbohydrate is extracted from the cell walls by the first extraction, the residue is virtually devoid of carbohydrate after a second treatment with formamide and is composed of mucopeptide. The acetone-precipitable carbohydrate obtained from the initial extraction is dissolved in water and after dialysis against water, is passed through Dowex-2X10 200- to 400-mesh, medium-porosity anion exchange resin, and Dowex-50X8 200- to 400-mesh, medium-porosity cation exchange resin.

The chemical analysis of a typical formamide extraction of group A cell walls is outlined in Table I. The group carbohydrate is composed almost exclusively of glucosamine and rhamnose.

c. Pneumococci

Cell walls of pneumococci, as in the case of group A hemolytic streptococci, contain an M protein and a C carbohydrate antigen. Pneumococci of a particular capsular type commonly contain an M protein characteristic of that type. These M proteins have chemical properties similar to those of the group A streptococci but are immunologically distinct.[12]

The C carbohydrate is of special interest because it forms a precipitate when mixed with the C-reactive protein of acute phase sera obtained from patients with a variety of inflammatory diseases. Isolation and purification of the C carbohydrate are complicated by the fact that large batches of cell walls are difficult to prepare. It is thus necessary to purify the carbohydrate from the whole bacteria. The pneumococci are lysed with deoxycholate, and the carbohydrate is isolated from the complex mixture by a somewhat lengthy procedure which cannot be included here. A detailed description of a method is given by Liu and Gotschlich.[13]

d. Other Bacteria

The principal antigenic substances which have been extracted from the cell walls of staphylococci are the teichoic acids (see Section B,4). Indeed, the group-specific polysaccharides initially described by Julianelle and Wieghard,[14] for *Staphylococcus aureus* and *Staphylococcus albus* were probably the teichoic acids. Teichoic acids have been isolated from the cell walls of other bacteria including *Bacillus subtilis* and the lactobacilli.

[12] R. Austrian and C. M. MacLeod, *J. Exptl. Med.* **89**, 439 (1949).
[13] T. Liu and E. C. Gotschlich, *J. Biol. Chem.* **238**, 1928 (1963).
[14] L. A. Julianelle and C. W. Wieghard, *J. Exptl. Med.* **62**, 11 (1935).

A teichuronic acid, identified in the walls of *Micrococcus lysodeikticus*, is a polymer containing equal molar amounts of glucose and an amino uronic acid (probably 2-amino-2-deoxymannuronic acid).[15] The several recent reviews should be consulted for additional details of these and other bacteria.[3-5]

[15] H. R. Perkins, *Biochem. J.* **86**, 475 (1963).

4. TEICHOIC ACIDS*

a. Introduction

The teichoic acids are a class of microbial carbohydrates characterized by the presence of either glycerol or ribitol linked together by phosphodiester bonds to form polymers. Substituent sugar groups and D-alanine residues in labile ester linkage are frequently attached to the polyolphosphate chain. Originally thought to be present only in bacterial walls (*teichos* = wall), teichoic acids have been found in other loci (reviewed by Baddiley).[1] These are termed intracellular teichoic acids, although their exact location has not been established. Many organisms contain both cell wall and intracellular teichoic acids, but these are generally of different composition. Thus far, ribitol teichoic acids have been found only in walls, but glycerol teichoic acids have been demonstrated both in walls and as nonwall components. Teichoic acids have been identified with certainty only in gram-positive microorganisms, although related material has been described in *Escherichia coli*.[2]

i. Composition and Structure

Identification of teichoic acids is readily achieved by examination of the products of acid hydrolysis. Acid hydrolysis of ribitol teichoic acids yields a complex mixture including alanine and any substituent sugars, inorganic phosphate, ribitol, and its monophosphates and diphosphates. A major product is 1,4-anhydroribitol.[3] The conversion of ribitol phosphate to anhydroribitol under acid conditions proceeds much more rapidly than the conversion of ribitol to anhydroribitol. However, sufficient anhydroribitol for chromatographic standards can be obtained by heating ribitol (adonitol) in 2 *N* HCl at 100° for 18 to 24 hours. The major product of hydrolysis of glycerol teichoic acids is glycerol monophosphate, in addition to any substituent moieties.

*** Section 1,B,4 was contributed by Stephen I. Morse.**

[1] J. Baddiley, *Federation Proc.* **21**, 1084 (1962).
[2] P. H. Clark and M. D. Lilly, *Nature* **195**, 516 (1962).
[3] J. Baddiley, J. G. Buchanan, and B. Carss, *J. Chem. Soc.* 4058, (1957).

Structural studies of various teichoic acids indicate that in the case of the ribitol teichoic acids the ribitol units are connected 1,5 by phosphodiester bridges. Substituted sugars are usually in glycosidic linkage at the 4 position of the D-ribitol residues, occasionally at both the 3 and 4 positions, and D-alanine is in ester linkage with the hydroxyl groups at either C-2 or C-3. The glycerol teichoic acids are 1,3-glycerol phosphate polymers, and D-alanine or other substituted groups are attached at the 2 position. An exception is the intracellular group antigen of group D streptococci in which D-alanine is linked to the compound via the hydroxyl groups of substituent glucosyl units.[4]

ii. Antigenicity

Teichoic acids are now known to be important antigenic constituents of many gram-positive organisms (reviewed by McCarty and Morse[5]), particularly of the staphylococci and streptococci. Many isolated teichoic acids have thus far not been tested for serological reactivity. Depending on the compound examined, immunological specificity has been shown to be conferred by substituted sugar groups, by D-alanine, or, in the case of streptococcal polyglycerophosphate, by glycerophosphate. Ribitol phosphate has not as yet been shown to bear immunological specificity.

b. PREPARATION

Preparation of teichoic acids from microbial cell walls generally presents few problems, whereas isolation of intracellular teichoic acids is attended by great difficulty. The following procedure for the isolation of serologically active ribitol teichoic acid from *Staphylococcus aureus* is essentially the same procedure as that described by Armstrong *et al.*[6] in their initial studies on teichoic acids and is generally applicable for the isolation of cell wall glycerol or ribitol teichoic acids.

i. Preparation of Cell Walls

Cell walls are prepared by mechanical disruption of the intact bacterial cells in the Mickle disintegrator or other high-speed shaking device in the presence of glass beads. Optimal conditions vary considerably, depending on the organism used. A generally effective method for *Staph. aureus* is to suspend 40 mg (dry weight) of cells per milliliter of distilled water, saline, or phosphate buffer at pH 7.0. Five milliliters of the cell suspension, 4 ml of No. 12 Ballotini beads, and one drop of the anti-

[4] A. J. Wicken, S. D. Elliott, and J. Baddiley, *J. Gen. Microbiol.* **31**, 231 (1963).
[5] M. McCarty and S. I. Morse, *Advan. Immunol.* **4**, 249 (1964).
[6] J. J. Armstrong, J. Baddiley, J. G. Buchanan, B. Carss, and G. R. Greenberg, *J. Chem. Soc.* 4344 (1958).

foaming agent tri-*n*-butyl phosphate are added to the cups of the Mickle device. The system is vibrated at maximum frequency and amplitude in the cold for 15 to 20 minutes.

The resulting suspension is then passed through a coarse sintered-glass filter, and the beads are washed with a small amount of buffer. The cell walls are sedimented by centrifugation at 13,000 *g* for 20 minutes at 4°. If significant numbers of unbroken cells remain, the cell wall layer can usually be removed from the underlying intact cells with a spatula. The preparation is examined for homogeneity by phase or electron microscopy and by the inability of the cell walls to retain crystal violet in the Gram stain. Small amounts of ribonucleic acid and protein often remain attached to the cell walls. The cell walls can be further purified by washing them with 10% NaCl, or by digesting a suspension of walls at a concentration equivalent to 100 mg/ml of starting material with ribonuclease (100 μg/ml) and trypsin (200 μg/ml). It is extremely important to avoid exposure of the cell walls to extremes of pH. In particular, alkaline conditions are hazardous because even quite dilute alkali will split the labile ester linkage of D-alanine to teichoic acid. This will occur even when the teichoic acid is still bound to the cell wall.

ii. Extraction of Teichoic Acid

The cell walls are suspended at a concentration of approximately 4% (w/v) in cold 10% trichloroacetic acid (TCA). The suspension is stirred at 4° for 48 to 72 hours, and the insoluble residue is removed by centrifugation and washed with a small volume of cold 10% TCA. The antigen is then precipitated from the combined supernatant fluid and washings by the addition of 1 to 2 volumes of acetone or 2 volumes of ethanol. After standing at 4° for 18 hours, the glassy-appearing precipitate is harvested by centrifugation, washed with absolute ethanol or acetone, and dried with ether.

c. Strain and Species Differences

The *Staph. aureus* wall teichoic acid contains *N*-acetylglucosamine in addition to ribitol phosphate and D-alanine.[7,8] Preparations are usually devoid of contaminating mucopeptide elements. The ratio phosphorus–amino sugar–alanine is 1:1:0.66 for the ribitol teichoic acid of *Staph. aureus* strain H,[7] and 1:1:0.5 for the antigen from strain Copenhagen.[8] A preparation from strain NYH-6 contained very little alanine, presum-

[7] J. Baddiley, J. G. Buchanan, F. E. Hardy, R. O. Martin, U. L. RajBhandary, and A. R. Sanderson, *Biochim. Biophys. Acta* **52**, 406 (1961).

[8] J. Baddiley, J. G. Buchanan, R. O. Martin, and U. L. RajBhandary, *Biochem. J.* **85**, 49 (1962).

ably because of exposure to alkaline conditions during isolation, but was nevertheless fully active serologically.[9] On the basis of analyses after treatment of the compounds with periodate and phosphomonoesterase, the average chain lengths of preparations are between eight and fifteen.[7,10]

The cell wall ribitol teichoic acid of *Staph. aureus* is the species antigen of these organisms, and the immunological determinant is the *N*-acetylglucosamine component. Both α- and β-linked acetylglucosamine are found, and either or both anomers may confer immunological specificity.[11-15]

The wall glycerol teichoic acid of *Staph. epidermidis* is also a species antigen; specificity is determined by glucosyl residues.[16,17] In this case serologically active material can also be extracted from walls by boiling a cell wall suspension at pH 2 for 20 minutes. This procedure, however, splits the alanine ester linkage.

As was noted above, purification of intracellular teichoic acids often presents major difficulties because of the amount of contaminating cytoplasmic material present in the usual extracts of whole bacterial cells. McCarty[18] isolated unsubstituted polyglycerophosphate from intact group A streptococci after acid hydrolysis or disruption in the Mickle disintegrator. The compound reacted with various anti-streptococcal antisera, and cross-reacting antigens were found to be released from a variety of other gram-positive organisms. The immunological determinant is glycerol phosphate. Using milder methods, McCarty[19] also isolated alaninated polyglycerophosphate from group A streptococci. The preparation was serologically reactive but did not cross-react with anti-polyglycerophosphate antisera. It was found that the immunological determinant of the alaninated product was the ester-linked D-alanine. When the alanine was removed by exposing the antigen to dilute alkali, it

[9] S. I. Morse, *J. Exptl. Med.* **116**, 229 (1962).
[10] A. R. Sanderson, J. L. Strominger, and S. G. Nathenson, *J. Biol. Chem.* **237**, 3603 (1962).
[11] G. Haukenes, D. C. Ellwood, J. Baddiley, and P. Oeding, *Biochim. Biophys. Acta* **53**, 425 (1961).
[12] A. R. Sanderson, W. G. Juergens, J. L. Strominger, *Biochem. Biophys. Res. Commun.* **5**, 472 (1961).
[13] W. G. Juergens, A. R. Sanderson, and J. L. Strominger, *J. Exptl. Med.* **117**, 925 (1960).
[14] M. E. Torii, E. A. Kabat, and A. E. Bezer, *J. Exptl. Med.* **120**, 13 (1964).
[15] S. I. Morse, *Ann. N. Y. Acad. Sci.* **128**, 191 (1965).
[16] S. I. Morse, *J. Exptl. Med.* **117**, 19 (1963).
[17] A. L. Davison and J. Baddiley, *Nature* **202**, 873 (1964).
[18] M. McCarty, *J. Exptl. Med.* **109**, 361 (1959).
[19] M. McCarty, *Proc. Natl. Acad. Sci. US.* **52**, 259 (1964).

then fully cross-reacted with antipolyglycerophosphate antisera. It is now clear that these compounds are at least partially responsible for the reaction originally described by Rantz *et al.*,[20] who showed, by passive hemagglutination techniques, the presence of a common heterophile antigen in many species.

The group antigens of group D[21] and group N streptococci[22] are also intracellular glycerol teichoic acids. They have been isolated after extraction of organisms with dilute alkali or after disruption of the cells in the Mickle disintegrator. In these instances serological specificity appears to be determined by substituted sugar units—kojibiose and kojitriose in the group D antigen,[23] and galactose phosphate in the group *N* antigen.[22]

[20] L. A. Rantz, E. Randall, and A. J. Zuckerman, *J. Infect. Diseases* **98**, 211 (1956).
[21] S. D. Elliott, *Nature* **193**, 1105 (1962).
[22] S. D. Elliott, *Nature* **200**, 1184 (1963).
[23] A. J. Wicken and J. Baddiley, *Biochem. J.* **87**, 54 (1963).

5. FLAGELLAR ANTIGENS*

a. General Considerations and Principles

Bacterial flagella are organelles of locomotion consisting of three distinct morphological regions.[1,2a,2b,2c,2d] The *basal region* is closely associated with the cytoplasmic membrane; its structural and functional nature is not fully understood. A bent region, the *hook*, is connected by a constriction to the basal region. The hook can be differentiated from the main spiral filamentous portion of the organelle on the basis of ultrastructure and solubility characteristics.

This *spiral filament* is a tube the wall of which is constructed of coiled protein fibers, six in the case of the flagellum of *Bacillus pumilus*[3,4a,4b] (for other proposed structures, see the literature[5–9]). Each fiber represents

*** Section 1,B,5 was contributed by Henry Koffler.**

[1] D. Abram, H. Koffler, and A. E. Vatter, *J. Bacteriol.* **90**, 1337 (1965).
[2a] D. Abram, A. E. Vatter, and H. Koffler, *J. Bacteriol.* **91**, 2045 (1966).
[2b] D. Abram, H. Koffler, and A. E. Vatter, *J. Gen. Microbiol.* **44**, v (1966).
[2c] D. Abram, H. Koffler, and A. E. Vatter, *J. Bacteriol.* in press.
[2d] D. Abram, H. Koffler, J. R. Mitchen, and A. E. Vatter, *Bacteriol. Proc.*, 39 (1967).
[3] D. Abram, A. E. Vatter, and H. Koffler, *Abstr. Biophys. Soc.* WC4 (1964).
[4a] D. Abram and H. Koffler, *J. Mol. Biol.* **9**, 168 (1964).
[4b] D. Abram, H. Koffler, and A. E. Vatter, *Abstr. 2nd Intern. Biophys. Congr., September 1966, Vienna*, Abstr. No. 45 (1966).
[5] R. E. Burge, *Proc. Roy. Soc.* **A260**, 558 (1961).
[6] D. Kerridge, R. W. Horne, and A. M. Glauert, *J. Mol. Biol.* **4**, 227 (1962).
[7] J. Lowy and J. Hanson, *Nature* **202**, 538 (1964).
[9] J. Lowy and J. Hanson, *J. Mol. Biol.* **11**, 293 (1965).
[8] G. Swanbeck and B. Forslind, *Biochim. Biophys. Acta* **88**, 422 (1964).

a strand of globular subunits consisting of the protein flagellin,[4a,6,10-23b] In a few cases, the surface of the filament shows additional morphological features, perhaps due to the fraying of the constituent fibers or the existence of external materials superimposed upon the filament.[2a] The possibility of antigenic "contamination," especially by cytoplasmic membrane or cell wall materials, must be kept in mind if this is relevant to a given experiment.

Before isolation of flagella is attempted, it may be advisable to permit the passage of organisms through semisolid media contained in U-tubes (inoculation at one end; isolation at the other). It is thought that more heavily flagellated strains can be selected in this manner, although without additional study it is not always obvious whether these actually are more flagellated or more actively motile members of the population.

Usually flagella are isolated from cells grown on suitable solid media. The degree of flagellation varies with the phase of growth from which the cells are harvested; therefore, before a specific procedure becomes established it is advisable to check microscopically the number and length of flagella per cell. For certain strains, cells from the late logarithmic or the early stationary phase of growth are more heavily flagellated than those isolated at other times. Similarly, the extent to which all the morphological components of the flagellum are represented in a given isolate depends on the organism and the conditions of growth. For example, many, perhaps all, so-called pure strains of *Bacillus* are infected by specific bacteriophages. Cells from such cultures tend to

[10] C. Weibull and A. Tiselius, *Arkiv Kemi Mineral. Geol.* **20B**, No. 3 (1945).
[11] C. Weibull, *Biochim. Biophys. Acta* **2**, 351 (1948).
[12] C. Weibull, *Biochim. Biophys. Acta* **3**, 378 (1949).
[13] C. Weibull, *Acta Chem. Scand.* **4**, 268 (1950).
[14] C. Weibull, *Discussions Faraday Soc.* **11**, 195 (1951).
[15] C. Weibull, *Acta Chem. Scand.* **5**, 529 (1951).
[16] C. Weibull, *Acta Chem. Scand.* **7**, 335 (1953).
[17] H. Koffler, *Bacteriol. Rev.* **21**, 227 (1957).
[18] T. Kobayashi, J. N. Rinker, and H. Koffler, *Arch. Biochem. Biophys.* **84**, 342 (1959).
[19] R. P. Ambler and M. W. Rees, *Nature* **184**, 56 (1959).
[20] C. Weibull, *in* "The Bacteria" (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. 1, p. 153. Academic Press, New York, 1960.
[21a] D. Abram and H. Koffler, *Abstr. 8th Intern. Congr. Microbiol., August 1962, Montreal,* p. 21 (1962).
[21b] H. E. Abron, M.S. Thesis, Purdue University, Lafayette, Indiana, 1966.
[22] G. L. Ada, G. J. V. Nossal, J. Pye, and A. Abbot, *Australian J. Exptl. Biol. Med. Sci.* **42**, 267 (1964).
[23a] M. W. McDonough, *J. Mol. Biol.* **12**, 342 (1965).
[23b] M. N. Farquhar, M.S. Thesis, Purdue University, Lafayette, Indiana, 1966.

lyse, especially during the later stages, and at least some of the flagella isolated from such cells possess all three morphological regions. Preparations of flagella isolated under such circumstances are likely to contain also membrane fragments, wall materials, and/or proteolytic enzymes.[23b] To accomplish the isolation of antigenically more homogeneous material one may need to use different organisms or cells from younger cultures. Ordinarily, one can obtain the filamentous portion of the flagellum, free from basal materials, by vigorous shaking of intact cells and subsequent fractional centrifugation; apparently, flagellar filaments break off at or near the constriction between the basal region and the hook. It is likely, therefore, that most isolated flagella contain at least some hooks, the antigenic properties of which are not yet known. The occurrence and relative abundance of hooks should be established in each case by electron microscopy if this information is critical to given experimentation.

Fortunately, the hook is more stable to acid and/or alcohol than the filamentous portion of the flagellum, and it is thus possible to isolate purified flagellin by fractional solubilization.[2b,2c] In general, the filaments can be disintegrated into individual flagellin molecules or small multiples thereof by treatment with a variety of agents, such as acid,[11,12,17,18,22,23a,25,26,26a] alkali,[11,17,25,27] urea,[6,17,24-26] guanidine,[17,24,27] acetamide,[17,24,25] sodium dodecyl sulfate,[6,17,25] cetyl pyridinium chloride,[27] various alcohols,[27,30] dioxane,[26,30] heat,[6,17,18,24,26] and sonication.[6,24] Preparations of purified flagellar filaments may contain only one or several proteins[28] when examined by disc electrophoresis on urea gels.[28,29] Water in the standard gel formula is replaced by 6 *M* urea (see Chap. 6,C,4, Vol. II). It is not clear whether a given cell is capable of making more than one flagellin, contained in one or more types of flagellar filaments, or whether the observed molecular heterogeneity is due to different flagellins, each synthesized by a different cell type of the population. In any case, individual flagellins can be isolated by disc electrophoresis[28,29] and probably also by preparative zone electrophoresis (LKB-3340c apparatus; ethanolyzed cellulose column; see Chap. 6,D,2, Vol. II).

Flagellin is a unique protein, with a molecular weight varying from 20,000 to 50,000, depending on the species.[6,17-20,21a,21b,22,23a,25,30] Cysteine-

[24] H. Koffler, G. E. Mallett, and J. Adye, *Proc. Natl. Acad. Sci. U.S.* **43**, 464 (1957).
[25] S. Erlander, H. Koffler, and J. F. Foster, *Arch. Biochem. Biophys.* **90**, 134 (1960).
[26] J. Stenesh and H. Koffler, *Federation Proc.* **21**, 406 (1962).
[26a] A. Vegotsky, F. Lim, J. F. Foster, and H. Koffler, *Arch. Biochem. Biophys.* **111**, 296 (1965).
[27] G. E. Mallett, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1956.
[28] F. Gaertner, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1966.
[29] L. Ornstein, *Ann. New York Acad. Sci.* **121**, 321 (1964).
[30] J. Stenesh and H. Koffler, unpublished results, 1962.

cystine, histidine, proline, tryptophan, and tyrosine are either absent in a given flagellin or present in only small amounts.[17-23a] Some flagellins apparently are glycoproteins.[31,32] The flagellins are at least partially helical when they exist in the flagellum, as judged by X-ray diffraction methods[33,34] and their optical rotatory dispersion properties.[26,35a,35b,36] After disaggregation the individual flagellin molecules are capable of assuming a variety of conformations, depending on the ionic and molecular environment in which they find themselves. For example, at pH 4 most flagellins from mesophilic cells have a helix content up to 34%, but undergo a configurational transition as the pH is lowered to 2, a pH at which they exist as coils; the helical regions of flagellin molecules from thermophiles do not undergo such transitions. In brief, at least certain flagellins are capable of existing in several conformational states, each probably with its own characteristic immunogenic and antibody-binding behavior.[37,38] For example, flagellar filaments and flagellin from *Salmonella adelaide* differ in immunogenicity; flagellin from cells of *Proteus vulgaris* in the disaggregated form (acid-treated or heat-treated flagellar filaments[37]) binds only 15 to 20% of the antibodies produced in response to injections of flagellar filaments, while the intact filaments are capable of binding all the antiflagellin antibodies in solution. It is not astonishing, however, that the conformation and antigenic properties of flagellin vary with the ionic and molecular environment. When flagellin exists in the flagellum, its characteristics are strongly influenced by flagellin–flagellin interactions, many of which probably are hydrophobic in nature, whereas in solution, especially in low concentrations, each flagellin molecule is surrounded by water molecules and also is more accessible to various ions and other molecules. Conformational variations express themselves also in differences in stability; flagellar filaments are considerably more stable to heat and urea than are monomeric flagellin molecules. These conformational possibili-

[31] R. J. Martinez, *Biochem. Biophys. Res. Commun.* **12**, 180 (1963).
[32] D. Abram and H. Koffler, *J. Cell Biol.* **19**, 3A (1963).
[33] W. T. Astbury, E. Beighton, and C. Weibull, *Symp. Soc. Exptl. Biol.* **9**, 282 (1955).
[34] E. Beighton, A. M. Porter, and B. A. D. Stocker, *Biochim. Biophys. Acta* **29**, 8 (1958).
[35a] M. Yaguchi, J. F. Foster, and H. Koffler, *Abstr. 6th Intern. Congr. Biochem., July 1964, New York*, p. 189 (1964).
[35b] H. Koffler, F. H. Gaertner, J. F. Foster, and D. Klein, *Abstr. 2nd Intern. Biophys. Congr., September 1966, Vienna*, Abstr. No. 44 (1966).
[36] D. Klein, J. F. Foster, and H. Koffler, *Abstr. Biophys. Soc. 1967*, 28 (1967).
[37] K. S. Read, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1957.
[38] G. J. V. Nossal, G. L. Ada, and C. M. Austin, *Australian J. Exptl. Biol. Med. Sci.* **42**, 283.

ties need to be kept in mind when one considers the ability of flagellin, in solution or in polymeric form, i.e., as filaments, either to stimulate the synthesis of antibodies or to react with antibodies. Furthermore, one needs to remember that the actual state of flagellin after injection in the animal is unknown, regardless of the condition of the antigen before injection.

Under certain conditions, flagellin molecules can be made to reassemble to form filaments[4a,22,39a,39b,40] that in morphology and fine structure resemble flagellar filaments, except that they are longer. However, such preparations may not be antigenically identical to native flagellar filaments.[38] Nevertheless, repeated disintegration of flagella at pH below 4 and subsequent reconstitution of flagella-like filaments may be a useful practice in the removal of nonflagellin antigens, especially if soluble flagellin rather than flagellar filaments is to be used as the eventual experimental material.

b. Isolation of Flagella

Cells in the desired phases of growth, usually the late logarithmic phase, are washed with water from a suitable solid medium, and then sedimented by centrifugation at 3300 to 6000 *g* for 30 to 60 minutes. To remove their flagella, cells resuspended in water at a concentration of 30 to 60 gm/liter (wet weight) are shaken for 10 minutes at room temperature on a shaking machine having about six hundred 1½-inch strokes/min. Extensive fragmentation must be avoided if fractional centrifugation is to be used as the basis for further purification, but this is not a serious problem in the method developed by Martinez.[41] The deflagellated cells are sedimented by centrifugation at 3300 *g* for 30 minutes, and the remaining cells and debris are removed from the supernatant liquid by centrifugation at 16,000 *g* for 15 to 20 minutes. Flagella are brought down in a Spinco model L preparative centrifuge at 40,000 *g* for 2 to 3 hours, and the pellets are resuspended in water and kept at 5°.

Further purification is effected by centrifugation of flagellar suspensions at concentrations of 0.3 to 0.7 mg/ml in a Spinco model L centrifuge. Pellets are obtained after centrifugation in rotor No. 30 at 12,500 *g* for 20 to 30 minutes with a subsequent increase to 78,000 *g* for 90

[39a] A. Asakura, G. Eguchi, and T. Iino, *J. Mol. Biol.* **10**, 42 (1964).
[39b] S. Asakura, G. Eguchi, and T. Iino, *J. Mol. Biol.* **16**, 302 (1966).
[40] J. Lowy and M. W. McDonough, *Nature* **204**, 125 (1964).
[41] R. J. Martinez, *J. Gen. Microbiol.* **33**, 115 (1963).

minutes, or in rotor No. 40 at 15,000 *g* for 15 to 20 minutes, then at 100,000 *g* for 60 minutes. Such pellets have the consistency of a firm gel and are water-clear, sometimes with a faintly blue tinge. One can remove colored or white opaque materials, well packed and centered at the bottom portion of the pellets, by cutting through the cellulose nitrate tubes with a razor blade. A portion of this material, which contains also flagellar filaments, and the clear portion of the pellets are resuspended separately in water. This step is repeated, usually one to four times, until clear pellets are obtained. To avoid unnecessary fragmentation, flagella are treated as gently as possible; one can obtain uniform suspensions without stirring by allowing the pellets to stand in water for at least 5 hours at 5°. All centrifugations are performed at 2° to 5°; deionized water is used. Suspensions of purified flagella at concentrations of 2 to 5 mg/ml are kept at 2° and used within a few days.

A suitable column method for purifying more highly fragmented flagella was developed by Martinez.[41]

c. Preparation of Flagellin

Flagellar filaments usually disintegrate at pH below 4. Since the basal material and the hook remain relatively stable under such conditions, purified flagellin can be obtained by fractional solubilization. Therefore, if a preparation of flagella, as demonstrated by electron microscopy, contains basal structures and hooks, the fractional solubilization should be performed at pH as close to 4 as possible. During this process suspensions of flagella lose their viscosity and take up hydrogen ions[26a]; thus, the conversion of flagella to soluble flagellin can be regarded as completed if viscosity and pH remain unchanged over a period of time. Viscosity can be conveniently determined with a Cannon-Manning semi-micro viscometer, size 75, and 2-ml samples of aqueous suspensions. Samples containing about 0.5 mg/ml give relative viscosities of 1.4 to 1.7 at 26°. Most preparations can be completely disintegrated within 80 hours at pH 3.4, or within 20 minutes at pH 2.5.[26a]

The following is a sample procedure: Aqueous suspensions of purified flagella at concentrations of 1 to 2.5 mg/ml are acidified with 0.1 *N* HCl to give a final concentration of 0.05 to 0.005 *N* HCl. After 1 hour at 26°, the suspension is centrifuged at 100,000 *g* in a Spinco model L centrifuge for 60 minutes at 5°. The acid-insoluble sediments contain the bulk of nonflagellin materials present in the preparation of flagella and are discarded; the clear supernatant liquid contains one or more

flagellins. If desirable, the acid can be removed by dialysis against cold distilled or deionized water; solutions must be dilute (<0.2 mg/ml) to avoid reconstitution of flagellar filaments.

d. Preparation of Reconstituted Flagellar Filaments

Under certain conditions, which probably differ from organism to organism, flagellin molecules in solution reassemble to form flagella-like filaments,[4a,22,39a,39b,40] Concentration of flagellin, hydrogen ion, salt, and temperature are the most critical variables. In some cases, primer may be necessary.[39a,39b] In general, solutions as concentrated as possible (about 5 mg/ml) should be used. To reconstitute flagellar filaments from flagellin synthesized by *Bacillus pumilus*, the following procedure is used[4a]: The pH of the flagellin solution in 0.005 *N* HCl (the acid-insoluble material had been removed) is adjusted to 3.9 with 1 *N* KOH while the solution is being stirred. After no more than 30 minutes at 26°, the solution is mixed with 0.5 *M* KH_2PO_4–K_2HPO_4 buffer, pH 5.4, to give a final pH of 5.3 to 5.5. Under these conditions reconstitution is practically instantaneous at a concentration of 5 mg/ml and is completed within 4 hours at 2 mg/ml.

Repeated "crystallization" of flagellin (solubilization of flagellar filaments and reconstitution) should be a useful procedure for obtaining antigenically highly purified flagellin; however, experience regarding this has been limited.

e. Homogeneity

Usually, preparations of flagella devoid of acid-insoluble residue are essentially free from nonflagellin components. Thus, the amount of acid-insoluble material is the most meaningful single criterion for the relative purity of preparations of flagella.[17,22] The number of flagellins in acid- or urea-disintegrated preparations of flagella can be determined by analytical ultracentrifugation,[16,17,20,22,25,26a,30] by column chromatography,[22,28,41] or by disc electrophoresis[29] on urea gel.[28] Antigenic homogeneity can be gauged by immunodiffusion[22,42,43] and immunoelectrophoresis,[22] with sera against flagella, soluble flagellin, reconstituted flagella, and acid-insoluble material as specific reagents. Other useful criteria, when applicable, are quantitative measurements of amino acids absent in the flagellins; N-terminal amino acids; carbohydrate and lipids (before and after acid hydrolysis); nucleic acids; and ash.

[42] D. Weinstein, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1959.
[43] D. S. Nasser, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1964.

6. PREPARATION OF CAPSULAR POLYSACCHARIDES*

a. Definition and Demonstration of Capsular Polysaccharides

Several criteria have been suggested to distinguish between bacterial cell wall and bacterial capsule.[1] The capsule is a gelatinous, extracellular layer (thickness $\lesssim$ 200 mμ) composed of either protein or polysaccharide, which can be resolved by the light microscope. It is not essential for cell viability and is chemically and immunologically different from the cell wall. In some cases the extracellular, gelatinous material may be present as a loose slime which can often be removed from the cells by repeated washings.

Capsular polysaccharides can be demonstrated by a variety of staining techniques.[2] The periodate–Schiff stain[3] and the Alcian blue stain[4,5] have been suggested for the specific demonstration of capsular polysaccharides, the latter dye staining the capsule without preliminary mordanting. Capsules are best demonstrated by techniques in which the capsule is outlined while in a wet (unshrunken) state. Thus, in the wet-film Indian ink method, which Duguid considers the most reliable, informative, and generally applicable method, the size and shape of the capsular material are outlined by particles of Indian ink unable to penetrate the gelatinous capsule. In addition, the method can be used to demonstrate extracapsular slime.

i. Wet-Film Indian Ink Method

Duguid[2] recommends shaking coarse Indian ink with 0.25 volume of grade 12 (0.2 mm) Ballotini in a Mickle tissue disintegrator to obtain a suitably dense, homogeneous ink which may be further improved by concentration. A large loopful of undiluted ink is mixed on a clean slide with either a small portion of the bacterial colony or a small loopful of the centrifuged deposit of a liquid culture. The ink should be diluted as little as possible during this process. A clean cover glass placed on the drop is pressed down under a pad of filter paper. It may be necessary to repeat the whole process to obtain a film of satisfactory thickness. The application of too much pressure may result in capsules that are

* Section 1,B,6 was contributed by Mervyn J. How.

[1] J. F. Wilkinson, *Bacteriol. Rev.* **22,** 46 (1958).
[2] J. P. Duguid, *J. Pathol. Bacteriol.* **63,** 673 (1951).
[3] R. D. Hotchkiss, *Arch. Biochem.* **16,** 131 (1948).
[4] R. E. McKinney, *J. Bacteriol.* **66,** 453 (1953).
[5] A. Novelli, *Experientia* **9,** 34 (1953).

flattened, distorted, or even disintegrated to simulate loose slime. The capsules should appear as clear, light zones between the cell outline and the dark background of ink. In order to demonstrate extracapsular slime, the film must be prepared from a surface culture on a solid medium.

ii. Periodate–Schiff Stain[3]

In this reaction some polysaccharides are oxidized by periodic acid to polyaldehydes which give colored compounds with Schiff's reagent, fuchsin sulfite. A negative reaction will be obtained with capsular polysaccharides which resist oxidation by periodic acid, and a lack of staining should not be considered to demonstrate beyond doubt the absence of appreciable amounts of polysaccharide.

iii. Alcian Blue Stain[4,5]

The dye (Alcian blue 8GN 150, produced by Imperial Chemical Industries Ltd., London), used as a 1% solution in ethanol, stains bacterial capsules blue and bacteria red and shows a great affinity both for capsules and for the isolated polysaccharide.

iv. Phase Contrast Microscopy

Structural details in unstained living cells may be observed by phase contrast microscopy, which saves time and damage to the cells, and has been used to demonstrate capsular polysaccharides in pneumococcus.

b. Preparation and Purification of Capsular Polysaccharides

The isolation and subsequent purification of capsular polysaccharides are illustrated with examples of alkali-labile, acidic and neutral, capsular specific polysaccharides of pneumococcus.* Although it is not anticipated that the methods described will be appropriate for the isolation and purification of capsular polysaccharides from all capsulated bacterial species, it is hoped that the examples described will suggest practical solutions to a wide range of such problems.

* Pneumococcal types and capsular specific polysaccharides are designated by Pn and S, respectively, followed by the appropriate Roman numeral. The chemistry and immunochemistry of pneumococcal capsular polysaccharides have been reviewed.[6,7]

[6] M. Heidelberger, *Fortschr. Chem. Org. Naturstoffe* **18**, 503 (1960).

[7] M. J. How, J. S. Brimacombe, and M. Stacey, *Advan. Carbohydrate Chem.* **19**, 303 (1964).

i. Growth Requirements of Pneumococcus and Preparation of Culture Medium

The use of a completely dialyzable medium avoids contamination of the capsular polysaccharide with polysaccharides from the medium. A partially defined, completely dialyzable medium which supports the growth of most pneumococcal types and permits both heavy growth from a reasonably small inoculum and unlimited subculture has been devised by Hoeprich (see Table I). Pneumococcus metabolizes glucose

TABLE I

MODIFIED MEDIUM FOR THE CULTURE OF *Diplococcus pneumoniae*[a]

Solution I—for 100 ml of medium:			
Acid hydrolyzate of casein	2.0	gm	
L-Cystine	0.015	gm	(0.15 ml 10% solution in *N* HCl)
L-Tryptophan	0.002	gm	(0.20 ml 1% solution in *N* HCl)
L-Tyrosine	0.020	gm	(0.20 ml 10% solution in *N* HCl)
$K_2HPO_4 \cdot 3H_2O$	0.65	gm	
0.2% phenolsulfonphthalein	0.25	ml	
Distilled water, q. s. ad.	50.0	ml	
The pH was adjusted to 7.8 with NaOH. Using glass tops, 5-ml portions in 15 × 125-mm tubes were autoclaved at 10 pounds steam pressure for 10 minutes.			
Solution II—for 100 ml of medium:			
D-Glucose	1.25	gm	
Mineral solution	0.10	ml	
Distilled water, q. s. ad.	37.0	ml	
3.7-ml amounts in 15 × 125-mm tubes were autoclaved at 10 pounds steam pressure for 10 minutes. The mineral solution contained:			
$MgSO_4 \cdot 7H_2O$	50.0	gm	
$FeSO_4 \cdot 7H_2O$	0.50	gm	
$ZnSO_4 \cdot 7H_2O$	0.080	gm	(8.0 ml 1.0% solution)
$MnSO_4 \cdot 4H_2O$	0.036	gm	(3.0 ml 1.2% solution)
Concentrated HCl	15.0	ml	
Distilled water, q. s. ad.	100.0	ml	
Solution III—accessory growth substances for 2.0 ml of medium:			
Biotin	0.0075	mg	
Nicotinic acid	5.00	mg	
Pyridoxine	5.00	mg	
Riboflavin	5.00	mg	
Thiamine	5.00	mg	
Calcium pantothenate	25.00	mg	
Adenine sulfate	50.00	mg	
Uracil	50.00	mg	
Choline chloride	50.00	mg	
Asparagine	500.00	mg	
Distilled water, q s. ad	100.00	ml	
After sterilization by filtration, solution stored in refrigerator.			

TABLE I (*Continued*)
MODIFIED MEDIUM FOR THE CULTURE OF *Diplococcus pneumoniae*[a]

Solution IV—2.5 ml sufficient for 100 ml of medium:

L-Glutamine	2.5%

After being sterilized by filtration, the solution was stored in the refrigerator. Since glutamine is unstable, the solution was not used 2 weeks after preparation.

Solution V—for 100 ml of medium:

0.10-gm portions of dry, solid $NaHCO_3$, in 15 × 125-mm tubes, were sterilized by autoclaving for 10 minutes at 10 pounds steam pressure. Before use, the sterile salt was dissolved in 5.0 ml of sterile distilled water, then mixed with 0.10 ml of sterile 10% thioglycolic acid; 10% thioglycolic acid was sterilized by heating in a boiling water bath for 10 minutes. The bicarbonate–thioglycolate mixture is unstable and must be prepared fresh each time cultures are set up; it was added to the medium just before inoculation.

Solutions I and II were mixed as soon as they were cool enough to permit handling. When this mixture was at room temperature, solutions III and IV were added. Solution V was not prepared and added unless the inoculum was at hand.

[a] [From P. D. Hoeprich, *J. Bacteriol.* **69,** 682 (1955) **74,** 587 (1957)]. Improvement in the medium of M. H. Adams and A. S. Roe [*J. Bacteriol.* **49,** 401 (1945)] resulted from omission of copper and calcium; addition of L-tyrosine; increase in concentration of iron, potassium, phosphate, glucose, glutamine, asparagine, uracil, adenine, choline, pantothenate, pyridoxine, riboflavin, thiamine, nicotinic acid, and biotin; decrease in concentration of zinc and manganese.

with the production of acids which lower the pH of the medium below the optimum (7.2 to 7.8) required for growth of pneumococcus, and growth ultimately stops. Massive growth of the organism can be maintained by intermittent addition of alkali to the medium, but this is permissible only when organisms possess alkali-stable capsular polysaccharides. The alkali lability of certain pneumococcal capsular polysaccharides, notably S-I and S-V, prompted the isolation of such polysaccharides under as mild conditions as possible from cultures that were not neutralized.[8] The presence in some capsular polysaccharides of immunologically significant acetyl groups and other relatively labile linkages emphasizes that the use of heat, strong acid, or alkali must be avoided in the isolation of such biologically active materials.

ii. Growth and Isolation of Pneumococcal Capsular Polysaccharides

In the preparation of capsular polysaccharides, all operations should be carried out in the cold (0 to 5°) and within a period not exceeding 2 to 3 weeks.

[8] M. Heidelberger, C. M. MacLeod, H. Markowitz, and A. S. Roe, *J. Exptl. Med.* **91,** 341 (1950).

(*a*) *Alkali-Stable Capsular Polysaccharides.*[9] A highly virulent strain of pneumococcus is obtained by serial passage through three mice and isolation from the heart's blood. The organisms are cultured for a further 12 hours in the modified medium (see Table I), centrifuged, washed with sterile normal saline, and suspended in sufficient sterile water so that 1 ml of inoculum, sufficient for 100 ml of medium, contains approximately 0.5 mg of total bacterial nitrogen. Large batches (about 10 liters) of modified medium in sterile flasks are inoculated and incubated at 37°, with the intermittent addition of 1 *N* sodium hydroxide to neutralize acidic metabolic products. For maximum production of polysaccharide S-II, the medium should be freshly prepared before inoculation and the concentration of glucose increased to 2.5% when the culture reaches its logarithmic phase of growth. Such conditions lead to heavy growth followed by almost complete autolysis (72 to 96 hours), after which phenol is added to 1% and the culture is allowed to stand overnight at room temperature. Individual flasks are tested for sterility and examined for contamination by direct microscopy. Any contaminated culture is discarded. After centrifugation of the culture in a Sharples centrifuge, the effluent is concentrated ten- to twenty-fold by ultrafiltration under sterile conditions,[9,10] which also removes much nitrogenous material. A 4.5% solution of nitrocellulose (Parlodion, available from Mallinckrodt) in glacial acetic acid is used to coat an alundum cylinder support (No. 5154C, available from Arthur H. Thomas). The ultrafilter will retain type-specific pneumococcal capsular polysaccharides, although polysaccharides of lower molecular weight, such as the species-specific pneumococcal C substance, may not be retained. Prior to ultrafiltration the solution should be centrifuged to remove turbidity or treated with Celite and either filtered through paper or passed through an uncoated alundum candle. Such techniques will minimize clogging of the ultrafilter. The ultrafiltrate should be tested for the presence of type-specific capsular polysaccharide with homologous antipneumococcal serum which has been absorbed with C substance, and any solution that gives a positive precipitin reaction should be refiltered through an intact membrane. After passage of all the culture medium, saline containing 1% phenol is passed through the ultrafilter until the effluent is nearly colorless.

The ultrafiltered concentrate is centrifuged (2500 rpm, 0°, several hours) to remove as much turbidity as possible, and the precipitate is washed several times with water, until the washings no longer react with specific antiserum. The concentrate is brought to approximately

[9] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

[10] R. Brown, *J. Immunol.* **37**, 445 (1939).

5% with respect to sodium acetate by addition of saturated sodium acetate solution adjusted to pH 6.05 with glacial acetic acid, and the crude polysaccharide is precipitated with 95% ethanol (1 to 1.5 volumes) at 0°. In all precipitation procedures, the chilled precipitant is added slowly to the chilled polysaccharide solution with constant stirring in a thick-walled beaker. Occasionally the polysaccharide may be precipitated in a more tractable form by the addition of the polysaccharide solution to the precipitant. The optimal ratio of precipitant to polysaccharide solution may be determined from preliminary small-scale experiments. Although slightly larger volumes of ethanol may be required to precipitate certain pneumococcal capsular polysaccharides, a large excess (3 to 5 volumes) should be avoided, since the species-specific pneumococcal C polysaccharide precipitates under these conditions. Most of the supernatant solution can be decanted off, and the crude polysaccharide centrifuged, drained, and purified (see Section B,6b,iii).

(*b*) *Alkali-Labile Pneumococcal Capsular Polysaccharides.*[8] Heavy growth of gram-positive forms of pneumococcus type I is obtained with minimum autolysis after inoculation of batches (7.5 liters) of medium (see Table I) with an 8-hour culture of pneumococcus in the same medium (75 ml) for 7 to 8 hours at 37°. No alkali is added to the culture (final pH, 6.2 to 6.7). Subsequently the culture is chilled in an ice–salt mixture, and an equal volume of chilled (0° to 4°) 95% ethanol is added to prevent autolysis. The agglomerated organisms are centrifuged after settling in the cold for a few hours, and a negative test for S-I should be obtained with the supernatant solution. The capsular polysaccharide S-I is removed by grinding the pneumococci in acetate buffer, pH 6.05, with stainless-steel balls (2 mm in diameter) at 0° for 6 hours. After centrifugation (2500 rpm, 0°) crude S-I is precipitated from the supernatant at 0° with cold isopropanol (0.5 volume). The cell debris is further extracted with acetate buffer, and a second fraction of S-I is obtained by precipitation with isopropanol. Other alkali-labile capsular polysaccharides of pneumococcus (for example, S-V) may be precipitated only by larger volumes of ethanol or isopropanol.

The crude, precipitated, capsular polysaccharide is centrifuged (2500 rpm, 1 hour, 0°), drained free from as much excess alcohol as possible, and purified (see Section B,6b,iii).

iii. Procedures for Purification

(*a*) *Techniques of General Application.* Traces of cell debris may be removed by centrifugation from an aqueous solution of the crude polysaccharide (5% or higher, depending on the viscosity of the solution)

to which is added an equal volume of ethanol, but no electrolyte. Centrifugation is at high speed (15,000 to 20,000 rpm) at about 4° for 1 hour. The gelatinous pellet is washed with a small volume of water until the washings give a negative precipitin reaction with homologous antiserum, and the supernatant solutions are combined.

The chilled polysaccharide solution is agitated in a Waring blendor with 0.5 volume of a chilled mixture of chloroform (5 parts) and *n*-butanol (1 part) in a cold room to remove denaturable protein. To prevent excessive heating, the mixture is agitated for 5 minutes and allowed to cool for at least 5 minutes; the process is repeated twice during 30 minutes. Centrifugation at 2000 rpm for 1 hour in the cold separates the mixture into a clear supernatant solution and a thick, white emulsion. The supernatant, which contains the polysaccharide, is separated with a siphon or a pipet, and the treatment with mixed solvents is repeated, as described, until no emulsion (interfacial layer) forms. At each stage the emulsion layer is washed with water in a Waring blendor and centrifuged, and the combined supernatant solutions are added to the bulk of the polysaccharide solution. Trichlorotrifluoroethane may be used to denature protein by an essentially similar technique.[11,12]

The "deproteinized" solution is brought to 5% with respect to sodium acetate (pH 6.05) by addition of saturated sodium acetate solution (pH 6.05), and the polysaccharide is precipitated with chilled ethanol. After centrifugation, the precipitate is drained free from alcohol, and more alcohol is removed by centrifugation after the tightly packed precipitate has been broken up. After several reprecipitations with ethanol or isopropanol, the polysaccharide may be further purified by methods outlined below.

Cationic detergents such as Cetavlon (cetyl trimethylammonium bromide) precipitate polysaccharides containing uronic acids and sulfated polysaccharides. These two types of acidic polysaccharide may be separated by precipitation with Cetavlon at pH 1.5, at which the ionization of the carboxylic acid group is suppressed. Cetavlon can also be used to precipitate the complexes of neutral polysaccharides with borate, and various fractions can be obtained from the mixture according to the pH (7 to 10) of the borate buffer. Although Cetavlon precipitates both nucleic acids and acidic polysaccharides at neutral pH, the Cetavlon complexes of the acidic polysaccharides are soluble in 0.25 *M* sodium chloride. This method has been used to separate polysaccharide S-V from nucleic acid. For certain fractionations the free quaternary base

[11] A. E. Gessler, C. E. Bender, and M. C. Parkinson, *Trans. N.Y. Acad. Sci.* **18**, 701 (1956).

[12] A. S. Markowitz and J. R. Henderson, *Nature* **181**, 771 (1958).

may be used to advantage.[13] The use of detergents in fractionation has been comprehensively reviewed.[14]

Fractionation of macromolecules according to molecular size can be achieved by gel filtration on molecular sieves, such as Sephadex or Bio-Gel P. Ion exchangers derived from Sephadex, such as the anion exchanger diethylaminoethyl(DEAE)-Sephadex, the weak cation exchanger carboxymethyl(CM)-Sephadex, and the strong cation exchanger sulfethyl(SE)-Sephadex, available in different porosities, can be used to separate acidic, basic, and neutral polysaccharides. (Chromatographic procedures are described in Chap. 9, Vol. II.) Solutions of substances of high molecular weight can be concentrated by the addition of dry Bio-Gel P-10 or Sephadex G-25 beads to form a thick suspension, leaving the high-molecular-weight substance as the external solution which may be recovered by filtration or by centrifugation (preferably with a swinging-bucket centrifuge).

The gels, which are chemically stable, can be used repeatedly; it is recommended that Sephadex gels in the wet state be stored after the addition of formaldehyde (5%) or chloroform to the buffer.

(*b*) *Special Techniques.* (*i*) *Purification of Acidic Pneumococcal Capsular Polysaccharides.* Polysaccharide S-I, which contains alkali-labile *O*-acetyl groups and approximately 60% of anhydrohexuronic acid residues, has been purified by fractionation with isopropanol. Crude S-I and fractions were analyzed by moving-boundary electrophoresis and serology.[15] Polysaccharide S-I, free from nucleic acid, is precipitated in the cold from solution in 3% sodium acetate (pH 7) by isopropanol (25%). A fraction which contains species-specific pneumococcal C polysaccharide, nucleic acid, and only a small amount of S-I is precipitated from the 25% supernatant by addition of isopropanol to 73%.

The acid form of S-I is precipitated from aqueous solution with glacial acetic acid and ethanol; it should be washed free of excess acid with ethanol. Further purification can be achieved by precipitation of S-I from solution in 5% barium chloride with isopropanol (15 to 20%). Pneumococcal C substance remains in solution under these conditions. Polysaccharide S-I is precipitated as its copper complex by addition of 0.1 volume of saturated copper acetate solution which has been neutralized until the copper hydroxide first formed begins to dissolve with difficulty. Copper is removed from the precipitated polysaccharide by repeated precipitation with ethanol from 10% sodium acetate solution.

[13] H. O. Bouveng and B. Lindberg, *Acta Chem. Scand.* **12**, 1977 (1958).

[14] J. E. Scott, *in* "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 8, p. 145. Interscience, New York, 1960.

[15] R. A. Alberty and M. Heidelberger, *J. Am. Chem. Soc.* **70**, 211 (1948).

Recent work on the purification of the acidic capsular polysaccharides of pneumococcus types I, II, and V has shown[16,17] that traces of pneumococcal C polysaccharide, nucleic acid, polyglutamate, and other components cannot be separated from the capsular polysaccharides by fractional precipitation with alcohol or detergents. However, the respective capsular polysaccharides have been purified by chromatography on DEAE-Sephadex.

Polysaccharide S-I, obtained by successive precipitations by ethanol or isopropanol from solution in acetic acid, neutral sodium acetate, or barium chloride solutions, showed three components by moving-boundary electrophoresis in acetate buffer, pH 7, and liberated, among other products, up to 10% of glutamic acid on hydrolysis with acid.[17] Analysis by gel diffusion showed the presence of S-I and pneumococcal C polysaccharide. A solution of S-I in 0.02 *M* sodium acetate solution, pH 7, was fractionated on DEAE-Sephadex A-50 by gradient elution with a solution of sodium chloride ($0 \rightarrow 1\ M$) in the same buffer. Three electrophoretically homogeneous components were eluted by $0 \rightarrow 0.2\ M$, 0.4 *M*, and 0.75 *M* sodium chloride, and were shown, by chemical and immunological analyses, to be C polysaccharide, S-I, and protein, respectively. The protein fraction contained a high proportion of glutamic acid.

Polysaccharide S-V, which could not be further fractionated by precipitation with alcohol or with the detergent cetyl trimethylammonium bromide, was separated into four components by chromatography on DEAE-Sephadex equilibrated with 0.05 *M* phosphate buffer, pH 6, by elution with a $0 \rightarrow 1.0\ M$ sodium chloride gradient.[16] The first component was largely protein in nature, the second and third contained *C* substance, and the fourth was S-V. The persistent impurity of nucleic acid present in a preparation of S-II was removed similarly by chromatography on DEAE-Sephadex. Polysaccharide S-II, a complex of polysaccharide and nucleic acid, and nucleic acid were eluted from the ion exchanger by increasing the concentrations of sodium chloride; further dissociation of the complex was obtained by repeated fractionation on DEAE-Sephadex.

The acidic polysaccharide S-VIII, a linear chain polymer of high molecular weight with the repeating unit *O*-β-D-glucuronosyl-(1 → 4)-*O*-β-D-glucosyl-(1 → 4)-*O*-α-D-glucosyl-(1 → 4)-α-D-galactose, is purified by precipitation from Fehling solution A (alkaline tartrate) by dropwise addition of Fehling solution B (copper sulfate). The complex is

[16] S. A. Barker, Susan M. Bick, J. S. Brimacombe, and P. J. Somers, *Carbohydrate Res.* **1**, 393 (1966).

[17] R. C. E. Guy, M. Heidelberger, M. J. How, and M. Stacey, unpublished results, 1965.

filtered on a glass sinter and washed well with water; copper is removed by the addition of *N* hydrochloric acid (60 ml) to a suspension of the complex in water (1 liter) at 0°. After centrifugation, the supernatant solution is poured with vigorous stirring into 4 volumes of ethanol to precipitate polysaccharide S-VIII. It has not been possible to purify the preparation further either by precipitation as the cetyl trimethylammonium salt or by electrophoresis on a glass fiber support with an electrolyte of borax and sodium hydroxide.

Polysaccharide S-III, a polymer of the disaccharide cellobiouronic acid (4-*O*-β-D-glucuronosyl-D-glucose), is obtained, free from nitrogen and phosphorus, by repeated precipitation with ethanol and isopropanol, and by precipitation as the water-insoluble barium salt with barium acetate at pH 6.6.

(*ii*) *Purification of Other Pneumococcal Capsular Polysaccharides.* Polysaccharide S-VI, a linear polymer which contains phosphodiester linkages, can be purified from a 7% solution in 5% calcium acetate, pH 5, by four precipitations with ethanol. A small precipitate of high nitrogen content is removed by centrifugation after the addition of 0.75 volume of ethanol, and the main fraction is precipitated by addition of a further 0.15 volume of ethanol to the supernatant. The nitrogen content of the polysaccharide can be reduced to below 0.1% by subsequent fractionation with glacial acetic acid in the presence of calcium acetate.

c. Criteria of Purity and Homogeneity of Pneumococcal Capsular Polysaccharides

It is important to establish the physical and immunochemical homogeneity of preparations of capsular polysaccharides by as many techniques as possible before making chemical studies.

i. Immunological Criteria

The analysis of mixtures containing immunologically reactive substances, such as the pneumococcal capsular polysaccharides, by the quantitative precipitin method (Chap. 13,A, Vol. III) in the region of antibody excess offers great advantages over the usual chemical methods. In particular, the method is highly specific and requires very small amounts of material for analysis. Analysis of the nitrogen content of washed, specific immune precipitates is extremely useful for comparing different polysaccharide preparations. In such analyses homologous rabbit antipneumococcal serum is much more sensitive to degradation of the pneumococcal capsular polysaccharide than is horse antiserum.

Qualitative immunochemical analysis of mixtures of immunologically reactive substances is very useful, particularly in assessing the purity of polysaccharide fractions obtained in later stages of fractionation. Gel diffusion techniques with whole antiserum will give a minimal estimate of the various antibody–antigen systems. Good resolution of the components may be obtained by use of immunoelectrophoresis. A single line of precipitation obtained in such tests indicates an adequately purified antigen, provided a range of dilutions of antigen has been tested, in order not to overlook any impurity (see Chap. 14,E, Vol. III). To test for homogeneity of antiserum against the purified antigen, the antiserum should give a single line in gel diffusion when it is allowed to react with the *crude* antigen.[18] These tests are minimal evidence for immunological homogeneity.

The recovery of a capsular polysaccharide from its immune specific precipitate with partially purified homologous antibody and its subsequent analysis are very important criteria of purity in such polysaccharides. The immune specific precipitate obtained from the S-XVIII anti-Pn-XVIII system is kept in the cold for several days, centrifuged, and washed three times with cold saline. The precipitate is dissolved in water, with the aid of a few drops of *N* sodium hydroxide (as little as possible alkali should be used), and the solution is treated rapidly with an equal volume of chilled 10% aqueous solution of trichloroacetic acid. After centrifugation, the precipitate is washed twice with small volumes of 5% trichloroacetic acid, the combined supernatants are cautiously neutralized to pH 5.8 with sodium hydroxide solution, and the solution is thoroughly dialyzed against water in the cold. The contents of the dialysis bag are lyophilized and dried to constant weight prior to analysis.

ii. Electrophoresis

Electrophoresis of charged polysaccharides, or of the complexes of neutral polysaccharides with borate ion, on filter paper or glass fiber can be used for both analytical and preparative purposes. A solution of polysaccharide is applied to thick filter paper (for example, Whatman No. 3) which is subsequently impregnated with a suitable buffer (acetate, pH 4; borate, pH 11), and clamped between two sheets of thick glass placed on a metal plate through which cold water circulates.[19] After electrophoresis, polysaccharides may be detected by using aniline hydrogen oxalate reagent. Glass fiber has distinct advantages as a supporting

[18] D. A. L. Davies, *Advan. Carbohydrate Chem.* **15**, 271 (1960).
[19] A. B. Foster, *Chem. Ind. (London)* 1050 (1952).

medium for electrophoresis of polysaccharides. In particular, more drastic reagents (0.5% $KMnO_4$ in N NaOH, or 5% 1-naphthol in 10 N H_2SO_4) may be used to detect polysaccharides. The sample is applied to the smooth side of a strip of glass fiber, held between two insulating sheets of polyethylene, and placed on a sheet of foam rubber (0.5 inch thick). The "sandwich" is clamped between two sheets of plate glass (0.5 inch thick). Several preparations of plant gums were shown to be heterogeneous by electrophoresis on glass fiber with 2 N sodium hydroxide as electrolyte.[20]

A preparative method for the separation of polysaccharides has been described in which the supporting medium was a column of glass powder, obtained by grinding glass wool in a ball mill.[21] Approximately 40 to 100 mg of a mixture of polysaccharides (for example, yeast mannan and yeast glycogen) could be separated by electrophoresis (450 volts, 28 to 30 ma, 24 hours) in 0.05 M borate buffer, pH 9.2. Bromcresol green was used as a reference point on the column. After electrophoresis, the polysaccharides were eluted in 5-ml fractions with the same buffer and detected by the anthrone reagent.

iii. Column Chromatography

Ion exchange resins have low capacities for polysaccharides and can irreversibly absorb part of the material.[22] The cationic derivatives of cellulose (for example, DEAE-cellulose) give better results.[23] The development of a range of gels (Sephadex and Bio-Gel P) for molecular sieve chromatography enables the homogeneity of polysaccharides of differing molecular weights to be investigated. The range and use of such gels, including ion exchangers derived from Sephadex, are described in Chap 9, Vol. II.

iv. Analytical Data

Additional analytical techniques, some of which may provide useful criteria by which to assess the efficacy of purification procedures, should be considered. For such methods, a standard solution of the polysaccharide is prepared from a sample dried to constant weight (±0.02 mg) over phosphorus pentoxide in high vacuum. On addition of water to the polysaccharide, transparent gels form, and complete solution may

[20] B. A. Lewis and F. Smith, *J. Am. Chem. Soc.* **79**, 3929 (1957).
[21] B. J. Hocevar and D. H. Northcote, *Nature* **179**, 488 (1957).
[22] K. Steiner, H. Neukom, and H. Deuel, *Chimia (Aarau)* **12**, 150 (1958). [C.A. **52**, 1823f (1958).]
[23] H. Neukom, H. Deuel, W. J. Heri, and W. Kundig, *Helv. Chim. Acta* **43**, 64 (1960).

take several days. Care should be taken to ensure that the solution is homogeneous.

Infrared spectroscopy. Infrared spectroscopic analysis of milligram quantities of polysaccharides, either as a mull in liquid paraffin or Chlorofluorolube, in a potassium bromide disk, or as a solid film, can give valuable information as to the presence or absence of, for example, carboxylic acid and *N*-acetyl and *O*-acetyl groups. In certain cases information may also be obtained about the stereochemistry of polysaccharides. The application of infrared spectroscopy in the determination of the structure of carbohydrates has been reviewed by Barker *et al.*[24]

Specific optical rotation $[\alpha]_D^t$

Nitrogen by the micro-Kjeldahl method or the Markham modification (for 10 to 100 μg of nitrogen) of the micro-Kjeldahl method (see Chap. 12,A Vol. II).

Relative viscosity in 0.9% sodium chloride solution. Depolymerization of a polysaccharide by drastic treatment will be reflected in a lower viscosity.

Neutral equivalent.[9]

Acetyl. Total acetyl content of a polysaccharide is best determined by acid hydrolysis of the sample to liberate acetic acid which is distilled off and titrated. Any other volatile acids will be titrated. Details of various methods have been reviewed.[9,25] Colorimetric methods have been developed for the determination of *O*-acetyl[26] and total acetyl.[27]

Reducing sugars liberated on hydrolysis. A comprehensive review of reagents for the determination of reducing sugars is given by Hodge and Hofreiter.[28] It is first necessary to establish conditions of hydrolysis that give maximum liberation of reducing sugars from unknown polysaccharides.

Phosphorus. Phosphorus (0.2 to 10 μg) can be determined colorimetrically by using *N*-phenyl-*p*-phenylenediamine monohydrochloride.[29]

Ash. Flame photometry is recommended for the microdetermination of ash. *All analytical data should be corrected to account for the ash content of the polysaccharide.*

[24] S. A. Barker, E. J. Bourne, and D. H. Whiffen, *in* "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 3, p. 213. Interscience, New York, 1956.

[25] R. Belcher and A. L. Godbert, "Semi-micro Quantitative Organic Analysis." Longmans, Green, New York, 1954.

[26] S. Hestrin, *J. Biol. Chem.* **180**, 249 (1949).

[27] J. Ludowieg and A. Dorfman, *Biochim. Biophys. Acta* **38**, 212 (1960).

[28] J. E. Hodge and B. T. Hofreiter, *in* "Methods in Carbohydrate Chemistry" (R. L. Whistler and M. L. Wolfrom, eds.), Vol. I, pp. 380–394. Academic Press, New York, 1962.

[29] R. L. Dryer, A. R. Tammes, and J. I. Routh, *J. Biol. Chem.* **225**, 177 (1957).

Uronic anhydride. Uronic anhydride (4 to 40 μg) can be determined by a modification of the Dische carbazole method.[30] Colorimetric methods for the determination of uronic anhydride do not give absolute values and are subject to interferences, the effects of which are difficult to predict and difficult to correct.[31] Analytical methods based on acidic decarboxylation are preferable when appropriate quantities of polysaccharide are available.[32]

[30] T. Bitter and H. M. Muir, *Anal. Biochem.* **4**, 330 (1962).
[31] D. M. W. Anderson and S. Garbutt, *Anal. Chim. Acta* **29**, 31 (1963).
[32] D. M. W. Anderson, S. Garbutt, and S. S. H. Zaidi, *Anal. Chim. Acta* **29**, 39 (1963).

7. EXTRACELLULAR BACTERIAL PRODUCTS AND TOXINS*

a. General Methodology

The preparation of extracellular antigens and toxins from bacterial sources is complicated by the fact that these products are generally recovered in the supernatant fluid in low concentration. Concentration from tens or even hundreds of liters of culture supernatant may be required to obtain relatively small amounts of a purified material. Moreover, some bacteria need complex media for adequate growth and yield of extracellular products, and many fabricate not a single extracellular product but a *potpourri*[1-3] of products with both widely varying and similar biological, enzymatic, and antigenic properties. Separation from ill-defined constituents of culture media and often equally ill-defined bacterial materials is frequently necessary. Some of these difficulties can be minimized by careful choice of strain and judicious selection of growth medium and conditions.

i. Choice of Strain

Simple screening of a large number of strains of a bacterial species often reveals strains that are particularly good producers of the antigen or toxin of interest. Occasionally on selective media or on media designed to detect a particular biological activity, mutants can be found that will be especially favorable.[4] In special circumstances, choice of a strain

* Section 1,B,7 was contributed by Lewis W. Wannamaker.

[1] C. G. Pope, M. F. Stevens, E. A. Caspary, and E. L. Fenton, *Brit. J. Exptl. Pathol.* **32**, 246 (1951).
[2] S. P. Halbert and S. L. Keatinge, *J. Exptl. Med.* **113**, 1013 (1961).
[3] J. MacLennan, *Bacteriol. Rev.* **26**, 177 (1962).
[4] P. H. A. Sneath, *J. Gen. Microbiol.* **13**, 561 (1955).

that fails to yield a contaminating product difficult to separate from the antigen or toxin of interest may be advantageous. Selected strains that grow and yield the desired product on relatively simple media may be useful.[5]

*ii. Selection of Media**

Ideally, to exclude contamination with protein and other macromolecular constituents of complex media, bacteria should be grown in liquid media of simple defined composition.[7] For some products of certain species—for example, diphtheria toxin and clostridial toxins[8-10]—this is feasible. Unfortunately, many species or strains either do not grow readily or do not produce good yields of the wanted antigen or toxin in such media. A practical compromise for more fastidious species with less simple or less well-defined needs is to use a medium that is freed of large molecular substances by dialysis.[11,12] Use of a dialyzate or simple defined medium is not always essential; in the isolation of certain proteases, such as streptococcal protease,[13] it is possible to rid the preparation of extraneous protein by simple activation of the protease at a later stage of preparation. Extracellular products may also be extracted from solid media, on which bacteria have been grown, by freezing and thawing with consequent separation of the agar from the fluid components,[14] but it is difficult to obtain large volumes by this method.

Certain organisms have specific requirements for growth or for toxin production—for example, low iron for production of diphtheria toxin.[8]

iii. Culture Conditions

It is advantageous to know or to determine optimal conditions for cellular growth and for toxin or enzyme production. Many extracellular

[5] A. W. Bernheimer, W. Gillman, G. A. Hottle, and A. M. Pappenheimer, Jr., *J. Bacteriol.* **43**, 495 (1942).

* Although technically somewhat difficult, recovery of extracellular antigens is also possible from *in vivo* environments.[6]

[6] D. W. Watson, *in* "Streptococcal Infections" (M. McCarty, ed.), p. 92. Columbia Univ. Press, New York, 1954.

[7] A. M. Pappenheimer, Jr., *Advan. Protein Chem.* **4**, 123 (1948).

[8] J. H. Mueller and P. A. Miller, *J. Immunol.* **40**, 21 (1941).

[9] J. H. Mueller and P. A. Miller, *J. Immunol.* **47**, 15 (1943).

[10] A. Abrams, G. Kegeles, and G. Hottle, *J. Biol. Chem.* **164**, 63 (1946).

[11] V. P. Dole, *Proc. Soc. Exptl. Biol. Med.* **63**, 122 (1946).

[12] L. W. Wannamaker, *J. Exptl. Med.* **107**, 783 (1958).

[13] S. D. Elliott, *J. Exptl. Med.* **92**, 201 (1950).

[14] S. D. Elek, *J. Hyg.* **51**, 125 (1953).

antigens appear in the supernatant fluid during the logarithmic phase of growth; and some are produced in parallel with cellular growth.[15,16] It is therefore a common custom to continue growth to the completion of the logarithmic phase. In general, unusually prolonged incubation should be avoided, since extracellular toxins or enzymes may be labile at incubator temperatures, and bacterial cells may lyse, releasing proteins, nucleic acids, and other macromolecular substances which may complicate purification procedures. It may be questioned whether toxins obtained under such circumstances can be considered extracellular.[16] However, with some organisms and some products, such as streptokinase, increased yields can be obtained by prolonging growth with frequent additions of glucose and alkali.[17] Continuous cultures have also been used for the preparation of certain extracellular enzymes and toxins.[18,19]

The possibility that some enzymes may be released as a precursor or in an inactive state should be considered.[13,20] Such precursors or enzymatically inactive forms may be antigenically similar to or different from the active enzyme.

Optimal pH, temperature, and gaseous environment should be considered. With some enzymes or their precursors, release does not ordinarily occur until the pH has dropped to a certain level.[13] Carbon dioxide is required for maximal production of some staphylococcal products[21,22] and aeration by vigorous shaking is required for others.[23,24]

The addition of antifoam agents may be helpful in reducing surface denaturation due to excessive foaming under some cultural conditions.[25] The incorporation of bacterial proteinase inhibitors—for example, chelating agents such as potassium citrate[25]—in the growth medium has proved useful in special situations.

Specific inducers may be useful in the preparation of adaptive enzymes. For example, the addition of hyaluronate to the medium significantly increases the yield of streptococcal hyaluronidase, and the addi-

[15] M. McCarty, *J. Exptl. Med.* **88,** 181 (1948).
[16] M. R. Pollock, *in* "The Bacteria" (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. 4, p. 121. Academic Press, New York, 1962.
[17] L. R. Christensen, *J. Gen. Physiol.* **28,** 363 (1945).
[18] M. Raynaud, A. Turpin, R. Mangalo, and B. Bizzini, *Ann. Inst. Pasteur* **87,** 599 (1954).
[19] M. R. Pollock, A. Torriani, and E. J. Tridgell, *Biochem. J.* **62,** 387 (1956).
[20] E. M. Ayoub and L. W. Wannamaker, *J. Immunol.* **90,** 793 (1963).
[21] G. F. Leonard and A. Holm, *J. Immunol.* **29,** 209 (1935).
[22] S. Kumar and R. K. Lindorfer, *J. Exptl. Med.* **115,** 1095 (1962).
[23] E. S. Duthie and G. Haughton, *Biochem. J.* **70,** 125 (1958).
[24] B. G. Weckman and B. W. Catlin, *J. Bacteriol.* **73,** 747 (1957).
[25] M. Kogut, M. R. Pollock, and E. J. Tridgell, *Biochem. J.* **62,** 391 (1956).

tion of penicillin markedly improves the production of staphylococcal penicillinase.[16,26]

iv. Separation and Sterilization of Culture Supernatant

Relatively small volumes (4 to 12 liters) of supernatant fluid can be removed by batch centrifugation in one to three runs with a swinging-bucket or angle-type head holding large cups. The enormous volumes frequently required for preparation of extracellular products can be more readily and efficiently handled by some type of continuous-flow centrifugation. For this purpose a Sharples centrifuge or a continuous-flow attachment for a large conventional centrifuge may be used. It is important to determine whether the foaming frequently accompanying such procedures results in significant surface denaturation of the required product. A slower rate of flow may obviate this problem but may also remove the advantage which this type of centrifugation has over batch operation. From organisms that sediment firmly, supernatant fluid can sometimes be removed by simple decantation followed by filtration. However, if significant amounts of bacterial cells remain, these may clog filters.

It is not possible to centrifuge large volumes of culture supernatant under sterile conditions. Therefore, centrifugation is accomplished with clean rather than sterile technique. Cooling of the supernatant prior to and during centrifugation inhibits growth of extraneous bacterial contaminants and possible loss due to instability of the bacterial product of interest. With reasonable care, significant contamination of the atmosphere with bacteria from the culture supernatant can usually be avoided, although this may be a difficult problem with continuous-flow centrifugation. Some of the more recently introduced attachments for continuous-flow centrifugation (International) are said to be aerosol-free and therefore more suitable for centrifuging infectious material. In some instances it may be acceptable and desirable to sterilize the bacterial culture prior to filtration—for example, by the addition of chloroform or ethylene oxide (see below).

With a few bacterial products which are unusually heat resistant (for example, staphylococcal deoxyribonuclease), sterilization of the culture supernatant can be accomplished by heating. In other instances chloroform (0.7%), merthiolate (1:10,000), or ethylene oxide (1%) may sterilize or inhibit growth.[12,21,27,28] Filtration (Coors, Seitz, Berkefeld, Selas, sintered glass, or Millipore) may be satisfactory, provided volumes

[26] H. J. Rogers, *Biochem. J.* **39**, 435 (1945).
[27] A. T. Wilson and P. Bruno, *J. Exptl. Med.* **91**, 449 (1950).
[28] P. G. Quie and L. W. Wannamaker, *J. Bacteriol.* **82**, 770 (1961).

are not too small or too large. With many enzymes and toxins significant amounts of the active product may adsorb to the filter during the early stages of filtration (that is, until saturation has occurred), which is a particular problem with small volumes. Loss by adsorption can often be minimized by pretreatment of the filter with a solution of another protein (for example, albumin) or by terminal elution with a high concentration of buffer or salt solution.[25] With large volumes, filtration is time-consuming unless multiple filters are used, in which case the problems of adsorption are increased. Filtration is preferably performed in the cold, particularly when it is carried out over a long period of time.

v. Preliminary Concentration

Because the amounts of product available in culture supernatants are usually small, preliminary concentration by one or more of a variety of methods is almost always necessary prior to purification. Alcohol precipitation in the cold has been used in some of the classical methods of toxin preparation.[29] Certain bacterial nucleases can be safely and conveniently concentrated and desalted by extraction with phenol.[30] Despite their usefulness in some circumstances and certain special advantages which they may have (such as simultaneous sterilization and possible avoidance of the necessity for dialysis), these methods have generally been replaced by milder procedures.

Precipitation by salting out is widely used as a method of both preliminary concentration and preliminary purification. Ammonium sulfate is commonly employed. Slow addition of the solid salt to 0.8 saturation (560 gm/liter) will result in precipitation of all protein from supernatant culture fluid. It is important to allow several hours to achieve equilibrium. Overnight refrigeration appears to result in increased precipitation, perhaps owing to temperature effects on salt saturation. The precipitate can be conveniently collected by suction filtration on a thin layer or pad of washed diatomaceous earth (Celite or Filter-Cel) or washed potato starch. The precipitated extracellular products can be redissolved by washing the pad with appropriate buffer solutions. Ammonium sulfate precipitation is a generally mild method of concentration, although some problems may be encountered with pH control, particularly if technical-grade reagents are used, and extensive dialysis or gel filtration is necessary to remove residual salt. A consideration of some of the theoretical

[29] L. Pillemer and K. C. Robbins, *Ann. Rev. Microbiol.* **3**, 265 (1949).

[30] G. W. Rushizky, A. E. Greco, R. W. Hartley, Jr., and H. A. Sober, *Biochem. Biophys. Res. Commun.* **10**, 311 (1963).

and practical problems which may be encountered in salting out proteins is available in a useful review.[31]

Pervaporation is also a convenient method of concentration of culture supernatants. Water and other volatile components are removed by placing supernatant fluid in seamless cellulose casings of the type commonly used for dialysis and exposing them to an air current. Refrigeration is advisable, especially during the later stages of this process when less cooling from pervaporation may be expected. This method results in the retention of small as well as large molecular components in the fluid concentrate. Because of salt concentration, preparations commonly show precipitation, perhaps containing the product of interest, and dialysis to remove salt and other small molecular components is generally necessary. Despite the possible risk of denaturation due to drying at the receding fluid edge, concentration by pervaporation appears to give the highest yields for certain extracellular products.

Distillation under reduced pressure has proved satisfactory for preliminary concentration of some extracellular enzymes.[19,32]

Osmotic dialysis with high-molecular-weight substances such as dextran, polyvinylpyrrolidone (PVP), or polyethylene glycol, sold under a variety of trade names (Carbowax 20-M, Cellugel Super-3000, Aquacide), is an effective and easy method of concentration. To avoid membrane rupture from osmotic pressure, the dry absorbent should be placed on the outside of dialysis casings containing the solution to be concentrated. Low-molecular-weight substances present in some commercial preparations will cause contamination of the sac contents.

Concentration by ultrafiltration can be achieved in several different ways (see Chap. 8, Vol. II).[33,34] A convenient method,[12] is to apply suction to a solution of extracellular products placed in a collodion bag (Schleicher and Schuell Co.). If the bag is attached to an appropriate holder, additional material can be added as concentration proceeds. This method is most useful as a secondary or final method of concentration. It has the advantage that dialysis can be performed simultaneously with concentration. Concentration may require several days and should be performed in a cold room (at 4°).

A useful method which provides some preliminary concentration of extracellular macromolecular products and, simultaneously, exclusion of macromolecular constituents of the medium is the cellophane bag technique. Bacteria are grown in a bag of Visking dialysis tubing immersed

[31] M. Dixon and E. Webb, *Advan. Protein Chem.* **16**, 197 (1961).
[32] L. L. Campbell, Jr., *J. Am. Chem. Soc.* **76**, 5256 (1954).
[33] A. Wadsworth and J. J. Quigley, *Am. J. Hyg.* **20**, 225 (1934).
[34] H. Mies, *Klin. Wochschr.* **31**, 159 (1953).

in suitable media. Several kinds of apparatus convenient for this purpose have been described.[18,35,36]

vi. Purification Procedures

Fractional precipitation with salt or with organic solvents has been used in most of the classical methods of toxin purification.[29] With salt fractionation, it is important to be aware that the limits of precipitation vary with the concentration of enzyme.[31] Careful attention must be paid to the pH, to the effect of temperature on the percentage of salt saturation, and to the significant changes in volume which may occur following the addition of some salts—for example, ammonium sulfate.[31,37] Some investigators prefer phosphates to ammonium sulfate.[31] Low temperatures, precisely controlled, are essential when fractional precipitation is attempted with acetone or alcohol.[32,38] Methanol is considered superior to ethanol for purification of bacterial toxins.[29] Simple lowering of the pH will result in precipitation of certain extracellular products with removal of considerable acid-soluble materials.[17,39] Adsorption with a variety of materials (magnesium hydroxide, kaolin, glass powder, starch, charcoal, ion exchange resins) has also been used for purification.[25,40,41] Protamine has been useful in some instances for removing nucleic acids and other unwanted nitrogenous materials.[42,43]

The above procedures may still be highly useful for preliminary purification, but final purification is best achieved by a combination of one or more of the more modern methods of protein separation—zone electrophoresis (Chap. 6,D, Vol. II), ultracentrifugation (Chap. 7, Vol. II), column chromatography (Chap. 9, Vol. II), or gel filtration (Chap. 9,B, Vol. II). Detailed discussion of these and other methods may be found elsewhere.[44]

Several extracellular products of bacteria have been obtained in crys-

[35] M. Sterne and L. M. Wentzel, *J. Immunol.* **65**, 175 (1950).
[36] W. Koch and D. Kaplan, *J. Immunol.* **70**, 1 (1953).
[37] M. Dixon, *Biochem. J.* **54**, 458 (1953).
[38] L. Pillemer, R. G. Whittler, J. I. Burrell, and D. B. Grossberg, *J. Exptl. Med.* **88**, 205 (1948).
[39] C. Lamanna, H. W. Eklund, and O. E. McElroy, *J. Bacteriol.* **52**, 1 (1946).
[40] C. G. Pope and M. F. Stevens, *Brit. J. Exptl. Pathol.* **39**, 139 (1958).
[41] B. Hagihara. H. Matsubara, M. Nakai, and L. Okunuki, *J. Biochem. (Tokyo)* **45**, 185 (1958).
[42] L. R. Christensen, *J. Gen. Physiol.* **30**, 465 (1947).
[43] L. R. Christensen, *J. Clin. Invest.* **28**, 163 (1949).
[44] H. A. Sober, R. W. Hartley, Jr., W. R. Carroll, and E. A. Peterson, *in* "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. III, p. 1. Academic Press, New York, 1965.

talline form by subjecting purified and concentrated solutions to appropriate manipulations of pH, salt concentration, and temperature, sometimes with the addition of alcohol.[10,13,19,38-40] To assist in crystallization with alcohol, a sealed bath at 2° which allows very gradual equilibration of the volatile precipitant between bath and solution has sometimes proved useful.[19,45] It is important to realize that crystalline preparations are not necessarily homogeneous.

vii. Methods of Monitoring

During successive concentration and purification steps, careful monitoring will reveal gains in concentration and losses in total product. Depending on the nature of the product desired, it may be convenient to assay for biological (for example, hemolytic, lethal), enzymatic (proteolytic), or immunological (precipitation or neutralization with specific antiserum) activity. At certain stages some apparent increase in total activity may occasionally be observed, apparently owing to removal of inhibitors which may be present in certain culture media.[15]

viii. Tests for Purity

In addition to the usual tests of homogeneity by physical characteristics, it is advisable to use immunological tests, such as double diffusion in gel (Chap. 14, Vol. III),[44,46] and to examine the final product for specific biological or enzymatic activity of likely contaminants.[13,47] It is important to recall that crystalline preparations are frequently impure and that one organism can produce several different toxins or enzymes with similar biological or enzymatic activities.[48] Conversely, with some toxins, such as staphylococcal Panton-Valentine leucocidin, biological activity requires the presence of two or more immunologically distinct components.[49] The possibility that diphtheria and anthrax toxins are also mixtures or complexes of several molecular species is discussed in a recent review.[50]

ix. Storing and Stabilizing Preparations

Since many extracellular products are highly labile, it is generally advisable to undertake preparative procedures and store the preparations

[45] B. W. Low and F. M. Richards, *J. Am. Chem. Soc.* **76**, 2511 (1954).
[46] P. Grabar, *Advan. Protein Chem.* **13**, 1 (1958).
[47] C. G. Pope, *Brit. J. Exptl. Pathol.* **38**, 207 (1957).
[48] L. W. Wannamaker, *J. Exptl. Med.* **107**, 797 (1958).
[49] A. M. Woodin, *Biochem. J.* **75**, 185 (1960).
[50] W. E. van Heyningen and S. N. Arseculeratne, *Ann. Rev. Microbiol.* **18**, 195 (1964).

at 4°, and to avoid excessive foaming, drying, dialysis, or dilution, or exposure to strong acids, alkalis, or heavy metals during preparation and purification. Some toxins may adsorb to glass surfaces.[51] Many bacterial enzymes and toxins can be easily and safely stored as a suspension in 0.8 saturated ammonium sulfate solution.[17,31] Bacterial growth is prevented by the high salt concentration, eliminating the need for addition of chloroform or methiolate. Freezing and lyophilizing are convenient methods of storing but may result in some initial or delayed loss of activity. Lyophilized preparations are usually brought to constant weight over a drying agent and stored *in vacuo* at 4° to minimize further possible loss in activity and deliquescence. Various proteins (for example, albumin, gelatin), peptides (peptone solution) or amino acids (glycine) have been used as stabilizers for storing bacterial extracellular products in the liquid or dried state.[12,19,48] Calcium ions may be useful stabilizers of certain bacterial enzymes, apparently by protection against proteolytic inactivation.[16,52,53] On the other hand, some bacterial enzymes may be more stable in the presence of chelating agents such as citrate or ethylenediaminetetraacetic acid (EDTA).[16,25] Diisopropylphosphorofluoridate (DFP) may also be useful for its protective effect against proteolytic inactivation.[54] Substrates or their digestive products are also sometimes protective.[52]

It is of interest and of some practical importance that loss of antigenicity does not necessarily occur in parallel with loss of enzymatic activity or toxicity.

b. Specific Preparations

i. Examples of Extracellular Antigens

Preparation of the following selected extracellular antigens has been described: α-Hemolysin (*Staphylococcus aureus*)[22,55-57]; botulinum toxin, type A (*Clostridium botulinum*)[10,39]; coagulase (*Staphylococcus*

[51] L. Pillemer, *J. Immunol.* **53**, 237 (1946).
[52] B. Hagihara, T. Nakayama, H. Matsubara, and K. Okunuki, *J. Biochem.* **43**, 469 (1956).
[53] E. A. Stein and E. H. Fischer, *J. Biol. Chem.* **232**, 867 (1958).
[54] J. M. Junge, E. A. Stein, H. Neurath, and E. H. Fischer, *J. Biol. Chem.* **234**, 556 (1959).
[55] S. Kumar, K. I. Loken, A. J. Kenyon, and R. K. Lindorfer, *J. Exptl. Med.* **115**, 1107 (1962).
[56] A. W. Bernheimer and L. L. Schwartz, *J. Gen. Microbiol.* **30**, 455 (1963).
[57] I. Lominski, J. P. Arbuthnott, and J. B. Spence, *J. Pathol. Bacteriol.* **86**, 258 (1963).

aureus)[23,58,59]; diphtheria toxin (*Corynebacterium diphtheriae*)[40,60]; penicillinase (*Bacillus cereus*)[19,25]; proteinase (group A streptococcus)[13,61]; and tetanus toxin (*Clostridium tetani*).[38,62,63]

ii. Preparation of Streptococcal Deoxyribonuclease B (modified from Wannamaker et al.)[12,48,64]

The preparation and purification of a bacterial extracellular product from a species which generally requires complex media and releases a conglomerate of such products is presented as a typical example. The Dematteo strain of group A streptococci was grown in 10 liters of a dialyzate medium, consisting of combined dialyzates of beef heart infusion, casamino acids, peptone (Pfanstiehl, R.I.), plus dextrose, sodium bicarbonate, and buffer salts. A sample of the completed medium was brought to 0.8 saturation by the addition of ammonium sulfate (0.56 gm/ml). Suitable preparations, from previously screened lots of peptone (Pfanstiehl, R.I.) gave no precipitate at this salt concentration.

The cells from a 40-ml 18-hour culture in dialyzate broth were washed twice in sterile physiological saline and inoculated into 250 ml of dialyzate broth. After 6 hours at 37°, this culture was inoculated in 50-ml amounts into flasks containing 1.5 liters of dialyzate broth. The inoculated flasks were incubated at 37° for 16 hours. The growth in each flask was checked for contaminants by Gram's stain and by subculture on blood agar. Cultures were immediately chilled in an ice bath, and all further preparative procedures were done in a cold room at 4°. The bulk of the bacterial cells was removed by decanting of the supernatant fluid after centrifugation at 2000 rpm for 1 hour in an International SB2 centrifuge. A swinging-bucket head fitted with 6 cups of 600-ml capacity each or 4 cups of 1-liter capacity each is useful for this purpose. For larger volumes, centrifugation was performed in a continuous-flow centrifuge (Servall KSB) at 12,000 g. Chloroform (0.7%) was added, and, after mixing, the excess chloroform was removed by decanting. This procedure usually, but not always, resulted in disinfection.

The supernatant fluid was brought to 0.8 saturation by the addition

[58] M. C. Drummond and M. Tager, *J. Bacteriol.* **78**, 413 (1959).
[59] M. C. Drummond and M. Tager, *J. Bacteriol.* **83**, 432 (1962).
[60] A. M. Pappenheimer, Jr., *J. Biol. Chem.* **120**, 543 (1937).
[61] T. Liu, N. P. Neumann, S. D. Elliott, S. Moore, and W. H. Stein, *J. Biol. Chem.* **238**, 251 (1963).
[62] A. Turpin and M. Raynaud, *Ann. Inst. Pasteur* **97**, 718 (1959).
[63] M. C. Hardegree, *Proc. Soc. Exptl. Biol. Med.* **119**, 405 (1965).
[64] H. C. Dillon, Jr., and L. W. Wannamaker, *J. Exptl. Med.* **121**, 351 (1965).

of 560 gm of ammonium sulfate per liter. After standing for 1 to 15 hours, the resulting precipitate was removed by filtration *in vacuo* through a thin pad of washed potato starch on filter paper. Better yields were obtained with this material than with Filter-Cel. Care was taken not to disrupt the starch pad during filtration. To prevent denaturation by drying, suction was discontinued while the collected precipitate on the starch pad was still moist.

The precipitate was redissolved by washing the starch pad three times with glycine buffer, pH 9, $\mu = 0.01$, in a total volume equal to approximately one hundredth of the original culture supernatant. The solution of extracellular products was placed in several collodion bags (Schleicher and Schuell Co.) and dialyzed under vacuum (50 cm Hg) against glycine buffer, pH 9, $\mu = 0.01$. Further concentration was achieved by vacuum filtration through the collodion bags, with pooling of partially concentrated preparations by quantitative transfer with buffer into a single bag and final concentration to a volume of approximately 1 ml.

The concentrated preparation either was immediately submitted to further purification by zone electrophoresis or column chromatography or was frozen and lyophilized. Fluid preparations concentrated approximately 1000-fold lost almost 50% of their deoxyribonuclease activity after 7 days at 4°. (Recent experience in this laboratory indicates that calcium and magnesium ions may effectively stabilize such preparations.) Lyophilization resulted in approximately 20% loss of activity. About 50% of the original deoxyribonuclease activity in the culture supernatant was present in the crude lyophilized preparation. Activity was measured by an adaptation[12] of the viscosimetric assay of McCarty.[65]

Concentrates were prepared for zone electrophoresis or column chromatography by dialysis against the buffer to be used. Thin-wall dialysis casings, $^{2}\%_{30}$ inch inflated diameter (The Visking Corporation), were soaked in sodium Versenate (EDTA), pH 7.6, to remove heavy metals and rinsed in distilled water and buffer. Preparations were dialyzed on a mechanical rocker or rotator for 6 hours at 4°. Insoluble residue was removed by centrifugation at 2500 rpm for 30 minutes at 4°.

Zone electrophoresis with starch as a supporting medium was done by the method of Kunkel and Slater (see Chap. 6,D,1, Vol. II).[66] The starch was washed twice in distilled water and once in buffer prior to pouring the 45-cm block. Glycine buffer, $\mu = 0.1$, pH 9, was used. A direct current with a potential difference of 400 volts was applied for 16 hours at 2° to 4°. The protein was eluted from 1-cm sections of the starch block by washing with 2 ml of glycine buffer. Quantitative re-

[65] M. McCarty, *J. Gen. Physiol.* **29**, 123 (1946).

[66] H. G. Kunkel and R. J. Slater, *Proc. Soc. Exptl. Biol. Med.* **80**, 42 (1952).

covery was facilitated by suction and rinsing through a sintered-glass filter.

Column chromatography was performed with diethylaminoethyl-(DEAE)-cellulose as the ion exchange material (Selectacel, Schleicher and Schuell Co., capacity 0.87 meq/gm). Cellulose was prepared by the methods described by Peterson and Sober.[67] Glycine buffer, $\mu = 0.01$, pH 9.0, was used as the final wash and suspending buffer. A column with a 1.0-cm inside diameter, packed to 15 cm, was used. Elution was carried out with a linear gradient of $0 \rightarrow 0.5$ *M* NaCl in glycine buffer with a total volume of 600 ml.

Fractions from the starch block or column were screened for deoxyribonuclease activity by a modification[12] of the alcohol precipitation test of McCarty.[68] Protein was measured by the modified Folin-Ciocalteu phenol reaction.[69]

By both zone electrophoresis and column chromatography, deoxyribonuclease B separated cleanly from the other streptococcal nucleases. This enzyme could be distinguished by its physical and immunological properties. Preparations of deoxyribonuclease B often contained small amounts of streptococcal nicotinamide adenine dinucleotidase (NADase) and sometimes streptolysin O if strains that produce these biologically active products were used. Preparations of streptococcal deoxyribonuclease B regularly showed ribonuclease activity, and evidence has been obtained[70] suggesting that these two activities may reside in a single protein. As a general rule, more homogeneous preparations of streptococcal deoxyribonuclease B were obtained by column chromatography. Pooled block or column eluates containing the active enzyme were stored in the frozen state or lyophilized and preserved at —10°.

[67] E. A. Peterson and H. A. Sober, *in* "Methods in Enzymology" (S. P. Colowich and N. O. Kaplan, eds.), Vol. V, p. 3. Academic Press, New York, 1962.
[68] M. McCarty, *J. Exptl. Med.* **90**, 543 (1949).
[69] H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.* **35**, 89 (1951).
[70] L. W. Wannamaker, *in* "The Streptococcus, Rheumatic Fever and Glomerulonephritis," p. 140. Williams & Wilkins, Baltimore, Maryland, 1964.

C. Blood Group Antigens

1. SOLUBLE BLOOD GROUP SPECIFIC SUBSTANCES*

a. Introduction

The blood group specific substances that occur in secretions of human origin are glycoproteins. They are most readily obtained from lyophilized

* Section 1,C,1 was contributed by W. T. J. Morgan.

secretions and body fluids by extraction with liquid phenol at room temperature.[1] Good sources of the specific substances are saliva, gastric juice, meconium (the first stool of the newborn),[2] tissue digests, and the fluid contents of some ovarian cysts. Such cyst fluids frequently contain large amounts of specific substance, and in certain instances they reach a volume of many liters and contain several grams of group specific substance.[3] The specific substances are usually insoluble in liquid phenol, whereas extraneous protein and fatty materials are readily soluble. The specific substances are somewhat soluble in phenol after they have been liberated from tissues by peptic digestion; the active substance is then recovered by the addition of relatively low concentrations of ethanol to the phenol solution.[4,5] The group specific substances isolated by these procedures are largely soluble in water and can be recovered from solution after dialysis, centrifugation to remove water-insoluble material, and precipitation by the addition of ethanol. Further purification can sometimes be achieved by saturating a solution of the substances with ammonium sulfate and heating at 55°.[6] In most instances, but not all, the active materials are separated into two molecular species; one is insoluble under these conditions, the other is soluble. After removal of the ammonium sulfate by dialysis, the two substances are recovered by precipitation from aqueous solution by ethanol, or by drying from the frozen state. Sometimes, but not always, further purification can be obtained by fractionation on a column of DEAE-cellulose.[7]

Specific substances obtained by these procedures are free from contamination with unspecific protein and fatty substances. They are composed of a family of macromolecules, ranging in size up to several millions, which are closely similar, but not identical, in composition. The largest molecules are sparingly soluble in water and tend to be eliminated during purification. The specific serological activity of a preparation is measured by the hemagglutination inhibition or precipitation test.[8,9]

It is to be emphasized that there is extreme diversity in the contents of cyst fluids, which vary from clear, colorless, nonviscous fluids containing largely protein with little carbohydrate, through intermediate types

[1] W. T. J. Morgan and H. K. King, *Biochem. J.* **37**, 640 (1943).
[2] D. J. Buchanan and S. Rapoport, *J. Biol. Chem.* **192**, 251 (1951).
[3] W. T. J. Morgan and R. van Heyningen, *Brit. J. Exptl. Pathol.* **25**, 5 (1944).
[4] E. A. Kabat and A. E. Bezer, *J. Exptl. Med.* **82**, 207 (1945).
[5] C. Howe and E. A. Kabat, *Arch. Biochem. Biophys.* **60**, 244 (1955).
[6] A. Pusztai and W. T. J. Morgan, *Biochem. J.* **80**, 107 (1961).
[7] A. Pusztai and W. T. J. Morgan, *Biochem. J.* **93**, 363 (1964).
[8] E. A. Kabat, "Blood Group Substances." Academic Press, New York, 1956.
[9] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

consisting of viscoelastic, ropy slimes that are rich in carbohydrate and possess varying amounts of protein, to water-insoluble "trembling jelly" or a paramucinous type of content that contains a carbohydrate–protein complex and much free protein. This variability in composition and physical character makes it impossible to describe in advance an isolation procedure that will be completely successful in every instance. The following description is based on a wide experience and in most instances can be used for the isolation of blood group specific substances irrespective of their serological character within the ABO and Lewis systems.[10]

b. PROCEDURE

i Extraction

The cyst fluid, immediately after removal from the patient, is shaken with a few milliliters of toluene, cooled to 1° to 3°, and held at this temperature until it can be dried from the frozen state. The weight of dry material obtained varies from a few grams up to a kilogram; but, because many cyst fluids contain high concentrations of serum proteins, the final weight of dry substance obtained bears no direct relationship to the amount of specific substance in the material.

A convenient amount of dried material that can be extracted with liquid phenol in the first step in the isolation procedure is 50 or 100 gm. This amount is ground with ten times its weight of liquid phenol (90 or 95%, w/w) in a large mortar and allowed to stand for 2 or 3 hours to give time for the solid to swell and for the protein to dissolve. The resulting viscous solution or semisolid sludge is then centrifuged, the dark-colored phenol extract is set aside, and the clear yellow gelatinous mass is again extracted by grinding with half the original volume of phenol. The viscous suspension is centrifuged, the phenol solution is added to that recovered from the first extraction, and the deposit of clear yellow jelly is extracted a third time with a quarter of the original volume of liquid phenol. The final gelatinous deposit is kept in the centrifuge bottle and repeatedly extracted with ethanol until it is free from phenol. The deposit is then suspended in 100 to 200 ml of water, stirred or shaken until it is thoroughly dispersed, poured into suitable lengths of Visking dialysis tubing, and dialyzed with gentle agitation for several days at 1° to 3° against several changes of distilled water. The dialyzed material is then collected and centrifuged to remove any water-insoluble gel, and the gelatinous deposit is repeatedly extracted

[10] R. R. Race and R. Sanger, "Blood Groups in Man," 4th ed. Blackwell, Oxford, 1962.

with water until no further material passes into solution. The amount of water-insoluble gel finally obtained varies considerably from preparation to preparation. The combined aqueous extracts are concentrated to yield a 1 to 2% (w/v) solution, centrifuged at 30000 *g* for 30 minutes, and the clear supernatant solution, which contains the specific substance, is dried from the frozen state. Most cyst fluids give only 1% or so of their dry weight as active substance; a strongly active cyst fluid, however, will yield as much as 10% of its dry weight of specific substance. The isolation procedure described above is repeated until the whole of the crude cyst contents have been extracted.

ii. Purification

A 1 or 2% (w/v) solution of the crude specific substance is saturated with ammonium sulfate and heated in the presence of an excess of the solid salt to 55° to 60°, stirred, and held at this temperature for about 10 minutes. Most preparations give a precipitate. This is collected after the solution has cooled, either by skimming the sticky, matted precipitate from the surface of the saturated ammonium sulfate solution, or by centrifugation, or by simple filtration through paper. A combination of all these procedures may be necessary according to the physical nature of the precipitate. The material is washed with saturated ammonium sulfate, redissolved in water to give a 1 to 2% solution, reprecipitated in the presence of excess ammonium sulfate at 55° to 60°, and collected as described above. The material finally obtained is dissolved in water, dialyzed until free from salt, and dried from the frozen state.

The combined ammonium sulfate solutions obtained after removal of the material precipitated at 55° to 60° are dialyzed to remove the salt, concentrated under reduced pressure, and dried.

The amount of specific substance found to be insoluble or soluble in saturated ammonium sulfate varies considerably from one cyst fluid to the next; whereas in some instances almost the whole of the specific substance is insoluble, with most preparations it is found that the component insoluble in saturated ammonium sulfate makes up rather more than half the total amount of material recovered. (The specific substances obtained to date from six B-active cyst fluids and a few fluids with other specificities have been completely soluble in hot saturated ammonium sulfate.)

The specific substances insoluble or soluble in saturated ammonium sulfate at 55° to 60° and obtained from the same cyst fluid have the same qualitative composition, but differ in certain physical and chemical properties. The substances insoluble in ammonium sulfate have a greater content of amino acids, are more viscous, have higher molecular weights,

and are serologically more active than are those substances soluble in hot ammonium sulfate.

Each substance, soluble or insoluble in hot saturated ammonium sulfate, is finally fractionated with ethanol from a 1 to 2% (w/v) solution in water containing 1% potassium acetate. Usually the largest amount and the most active and viscous substance is precipitated first. This occurs mostly at ethanol concentrations between 45 and 50% (v/v). Smaller amounts of less active and less viscous material separate at higher concentrations of ethanol. The fractional precipitation is repeated two or three times on each material within the same ethanol concentrations, and finally the precipitates are dissolved in water, dialyzed, and centrifuged at 30,000 *g* for 30 minutes. The clear solution is dried from the frozen state. The main fractions are blood group specific substances in a high state of purity, suitable for most immunochemical studies.

Should a preparation be required that is less heterogeneous in its physical and chemical properties than are the materials described above, this can be obtained by fractionating the substance on a column of DEAE-cellulose with a continuous gradient of sodium chloride (0.01 to 1 *N* NaCl) as eluant.[7] This procedure detects and separates substances with different amounts of sialic acid and acidic amino acids.

Other methods of isolation of the blood group specific substances from tissue fluids and secretions have involved the formation of borate complexes[11] and the use of electrodecantation,[12] but these appear to be less satisfactory procedures than those described above.

c. Chemical Properties

An examination of many specimens of each of the four specific (A, B, H, and Le^a) substances has shown them to be macromolecules with molecular weights ranging from 200,000 to several millions, and to have similar chemical patterns. Each substance contains 80 to 90% of carbohydrate, the remaining part of the molecule consisting of amino acids. The substances contain D-galactose, L-fucose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine. *N*-Acetylneuraminic acid is also frequently a component, varying in amount in different preparations from 1% or less to about 20%. The analytical figures obtained vary within a relatively wide range from preparation to preparation and also depend to a considerable extent on the particular fraction of the preparation taken for analysis. Typical analytical figures for the specific A, B, and H substances fall within the ranges: N, 4 to 6%, fucose, 16 to 22%;

[11] C. A. Zittle, L. De Spain Smith, and L. E. Krejci, *Arch. Biochem.* **19**, 9 (1948).
[12] G. Holzman and C. Niemann, *J. Am. Chem. Soc.* **72**, 2044 (1950).

hexosamines (as base), 20 to 30%; and reducing sugars (as glucose), 48 to 54%, after hydrolysis with 0.5 *N* HCl for 18 hours at 100°. The following methods are suitable for chemical analysis: nitrogen—Kjeldahl determination[13]; fucose—Gibbons[14]; amino sugars—Rondle and Morgan[15]; reducing sugars—Nelson.[16] The Le^a substances usually have a fucose content between 8 and 14%. It is to be emphasized that the analytical figures sometimes fall outside the ranges given, although the materials have passed through all the purification steps described above.

The analytical procedures available for the determination of the amount of each component sugar in the blood group substances are not entirely satisfactory; nevertheless it is evident that the variation in composition found far outweighs the errors due to the methods employed. It is probable that the variation in the amounts of individual sugars from one preparation to the next is real and is a direct reflection of the degree of reproducibility and extent of completeness of the biosynthesis of the specific macromolecules, the result of which depends on several gene-controlled steps.

The composition of the amino acid-containing moiety is noteworthy in that the hydroxyamino acids, threonine and serine, make up almost half the total amino acids, and the aromatic and sulfur-containing amino acids are poorly represented. The total content of amino acids can vary considerably (7 to 26%) from one preparation to the next. The amino acid composition, however, is remarkably constant in all highly purified preparations of the specific substances.

d. Serological Properties

The purified specific substances are not strongly antigenic in rabbits but can be made so by complexing with the conjugated protein component of the O somatic antigens of a number of gram-negative bacteria.[17,18] This method has been used for many years at the Lister Institute to produce potent A, B, and H rabbit antibody for use as blood-grouping reagents and in specific precipitation and hemagglutination inhibition tests. Specific anti-A and anti-B sera have been produced in man by immunization with blood group A and B substances of human origin.[8,9]

[13] R. Markham, *Biochem. J.* **36**, 790 (1942).
[14] M. Gibbons, *Analyst* **80**, 268 (1955).
[15] C. J. M. Rondle and W. T. J. Morgan, *Biochem. J.* **61**, 586 (1955).
[16] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944).
[17] W. T. J. Morgan, *Brit. J. Exptl. Pathol.* **24**, 41 (1943).
[18] S. G. Rainsford and W. T. J. Morgan, *Lancet* **154** (1946).

i. Serological Testing

The soluble blood group specific glycoproteins neutralize the corresponding antibody, and agglutination does not occur on the addition of the appropriate red cells to the system. This reaction forms the basis of the hemagglutination inhibition test. Serial dilutions of the specific substance are made by transferring 0.1 ml of 1.0% solution in 0.15 *M* NaCl to the first of a series of tubes (1 × 5 cm), each containing 0.1 ml of 0.15 *M* NaCl. The contents of the first tube are mixed; 0.1 ml is transferred to the next tube and mixed, and the operation is repeated until the necessary serial dilutions have been made. A suitable dilution of the test serum (0.1 ml), equivalent to three to four completely agglutinating doses, is then added to each tube, the contents are mixed by gentle shaking, and the tubes are allowed to stand at room temperature for 15 to 30 minutes. A 1% suspension (in 0.15 *M* NaCl) of freshly collected red cells of the appropriate group is added (0.1 ml) to each tube, the contents are shaken, and the tubes are put aside for 1 to 2 hours. The contents of the tubes are examined microscopically for agglutination. The end point of the titration, the inhibition titer, is taken as the reciprocal of the dilution of the substance in the tube in which agglutination just appears.

2. ISOLATION OF ABO ANTIGENS FROM RED CELLS*

a. Introduction

This section describes the isolation from human erythrocytes of blood group active materials which are glycolipid in nature. These substances contain residues of sphingosine, fatty acids, glucose, galactose, glucosamine, galactosamine, sialic acid, and fucose.[1-3] The carbohydrate moiety comprises about 40 to 50% of the total weight of glycolipids. The amount of fucose is low, usually in the range of 1 to 2.5%. Sialic acid is invariably present in the preparations obtained from blood group A erythrocytes. Preparations of blood group B erythrocytes were found to contain also a specific glycolipid containing only a little sialic acid.[1,4] Glycolipids similar in chemical composition to those present in erythro-

* Section 1,C,2 was contributed by J. Kościelak.

[1] S. Handa, *Japan. J. Exptl. Med.* **33**, 347 (1963).
[2] J. Kościelak, *Biochim. Biophys. Acta* **78**, 313 (1963).
[3] T. Yamakawa, R. Irie, and M. Iwanaga, *J. Biochem.* (*Tokyo*) **48**, 490 (1960).
[4] E. Grochowska, and J. Kościelak, *I Kongr. Biochem. Lodz, 1963* p. 150 (1963).

cytes of blood groups A and B are present in erythrocytes of blood group O; these glycolipids do not display any appreciable O or H activity.[5,6]

Purified blood group specific glycolipids are soluble both in water and in some organic solvents (methanol, pyridine, mixtures of methanol and chloroform). In aqueous solution the glycolipids tend to form large molecular aggregates or micelles. The degree of physical dispersion of the glycolipids greatly affects their ability to inhibit the agglutination of erythrocytes by specific antibodies. It was found that only highly aggregated preparations exhibited substantial blood group activity in hemagglutination inhibition tests.[2] Since the degree of aggregation of the glycolipids depends to a large extent on the presence of other lipids, sudden changes of serological activity occur at certain steps of the purification procedure (see below). Therefore, the blood group activity of purified glycolipids in hemagglutination inhibition tests may be properly evaluated only when the glycolipids are combined into a so-called carrier lipid—that is, are transformed into a highly aggregated state (see Section C,2,c).

For the sake of convenience, the method of isolation of A and B antigens is divided into two parts; the first deals with the isolation of a crude substance, the second with the preparation of antigens free of gross contamination. Large-scale fractionation is necessary to obtain reasonable quantities of purified products; it may therefore, be advantageous to isolate and work with the crude substance which, although less active than the purified product, nonetheless exhibits the same order of blood group specificity.

b. Preparation

i. Crude A and B Substances

(*a*) *Preparation of Stroma.* Packed erythrocytes (1 liter) are hemolyzed with 7 to 10 liters of distilled water. Tap water may be used when one is working with a large volume of erythrocytes. The hemolyzate is acidified to pH 5.6 to 5.7 by adding pieces of solid CO_2; capryl alcohol is added to prevent foaming. After 1 hour the hemolyzate is centrifuged, and the stroma paste is collected. With large volumes of hemolyzate it is advisable to use a high-speed continuous-flow centrifuge (Sharples). The hemolyzate is centrifuged at 22,000 to 30,000 rpm with a flow rate

[5] J. Kościelak, and K. Zakrzewski, *Proc. Intern. Symp. Biol. Active Mucoids, Warsaw. 1959* p. 21.

[6] J. Kościelak, *Proc. 10th Congr. Intern. Soc. Blood Transfusion, Stockholm, 1964* p. 453 (1965).

of about 10 liters/hour; the rotor of the Sharples is emptied of the sedimented stroma every 30 to 45 minutes. The pH of the hemolyzate is maintained throughout the procedure by adding solid CO_2 if necessary. The effluent from the Sharples should be completely clear, and a small sample of the effluent should not leave any sediment after centrifugation in an ordinary centrifuge for 10 minutes at 3000 rpm.

(*b*) *Ethanol Extraction of Stroma* The stroma paste is suspended in approximately 2 volumes of ethanol and stirred vigorously for 5 minutes. Further ethanol is added dropwise until the specific gravity of the supernatant reaches 0.855 (this is conveniently checked by means of a hydrometer). The suspension is stirred for 1 hour, transferred to a stoppered container, and left for 2 days at room temperature, with occasional shaking. The material is filtered through a Büchner funnel with suction. The residue is washed on a filter once with a small volume of 83% ethanol. The washing is added to the original filtrate, and the residue is discarded.

(*c*) *Precipitation at Low Temperature.* The clear yellow ethanolic filtrate is transferred to a narrow-bottom flask and cooled to about —10°. The white precipitate which separates is allowed to settle for 2 to 3 days at this temperature. The clear supernatant is decanted and discarded. The lower layer which contains the precipitate is centrifuged at —10°. The deposit is washed once with 2 volumes of cold 83% ethanol and then twice with acetone at room temperature. The white sediment, consisting of crude blood group substance, is dried *in vacuo*. The yield is 250 to 300 mg/liter of packed red cells. Lower yields indicate incomplete separation of stroma from the hemolyzate.

The material does not exhibit Le^a, Rh_0, M, N, H, or P activity and fails to inhibit the hemagglutination brought about by influenza virus. The A or B blood group activity, expressed according to Morgan and King[7] as hemagglutination inhibition activity (HIA), is 2 to 5 μg.* The content of reducing sugar is 12 to 16%; of hexosamine, 2.5 to 5.0%.

ii. Purification of A and B Substances

(*a*) *Extraction with Organic Solvent.* (*i*) *Extraction with Acetone, Ethyl Ether, and Petroleum Ether.* The crude preparations from individual runs are pooled; the total weight should be at least 2.0 to 2.5

[7] W. T. J. Morgan, and H. K. King, *Biochem. J.* 37, 640 (1943).

* This is the minimum amount of substance which completely inhibits the agglutination of A or B erythrocytes by four agglutinating units of the appropriate antiserum. Under the conditions, highly purified A and B active glycoproteins (kindly donated by Dr. W. T. J. Morgan) displayed hemagglutination inhibition activity equal to 0.1 μg.

gm. The material is extracted for 4 hours with 150 to 200 ml of acetone in a Soxhlet extractor. The acetone extract is discarded, and the extraction is continued for 2 hours with 150 to 200 ml of diethyl ether. The insoluble residue is transferred to a centrifuge tube of 100-ml capacity; the residue is washed twice with 50-ml portions of petroleum ether (b.p. 40° to 60°), and the sediment after centrifugation is dried *in vacuo* (yield, 1.5 to 1.7 gm; reducing sugar, 16 to 20%; hexosamine, 3.5 to 5.5%).

(*ii*) *Extraction with Methanol.* The residue insoluble in acetone, ether, and petroleum ether is suspended in methanol (30 ml of methanol per gram of the substance), and the suspension is refluxed for 3 to 4 minutes in a boiling water bath. It is filtered while hot, and the residue is discarded. The filtrate is transferred to a centrifuge tube and left at 4° for 24 hours. The precipitated material is recovered by centrifugation at 4°, washed once with acetone, and dried *in vacuo* (yield, 1.0 to 1.3 gm; HIA, 0.5 to 1.0 μg; reducing sugar, 20 to 25%; hexosamine, 6 to 6.5%).

(*b*) *Chromatography.* (*i*) *Cellulose Column.* Cellulose powder (125 gm, Whatman) is suspended in a homogenizer in 500 to 600 ml of the lower layer of a chloroform–ethanol–water (16:4:1 by volume) mixture. A 3.5 × 5.0-cm all-glass column is filled with the cellulose suspension and allowed to settle for a day. One gram of the residue, recovered from cold methanol, is applied to the column as a 10% solution in the chloroform–ethanol–water solvent mixture. The solvent is then run through the column until a sample of the effluent gives no trace of opalescence after addition of 5 volumes of acetone. The effluent, which contains acetone-precipitable material, is concentrated to a small volume in a flash evaporator at temperatures between 30° and 40°, precipitated with 5 volumes of acetone, and dried *in vacuo* (fraction C-I; yield, 700 mg; HIA, more than 100 μg; reducing sugar, 10 to 16%; hexosamine, 2 to 3%). This "carrier lipid" fraction will subsequently be used for the activation of purified blood group active glycolipids.

The column is then eluted with a chloroform–methanol–water solvent mixture (5:15:1 by volume); the effluent containing material precipitable by acetone is evaporated to dryness in a flash evaporator and is then dried over $CaCl_2$ (fraction C-II; yield about 200 mg). The HIA of this material is only 10 to 20 μg—that is, 5% of the original activity of the material applied to the cellulose column. The activity of this fraction (C-II) may be restored by combination with the "carrier lipid" (see Section C,2,c). Reducing sugar is 40 to 50%; hexosamine, 10 to 15%.

(*ii*) *Silicic Acid Column.* Silicic acid (Mallinckrodt) is activated by heating at 110° for 1 hour. Twenty grams of the adsorbent is suspended

in chloroform and poured into a 2.2 × 18-cm all-glass column fitted at the top with a bulb of 300-ml capacity. A 200-mg portion of fraction C-II is dissolved in the minimum volume of 20% methanol in chloroform (with the aid of heating) and applied to the top of the column. The column is eluted with 200 ml of chloroform followed by 200 ml of 20% methanol in chloroform. These effluents are discarded. The column is then eluted with 40% methanol in chloroform. Fractions of 10 ml each are collected. The progress of fractionation is followed by evaporating suitable aliquots of each fraction (0.05 to 0.3 ml) to dryness at 60° to 80°, dissolving the residues obtained in 1 ml of water, and analyzing each for hexose by the phenol-H_2SO_4 test[8] or any similar method.

A sharp glycolipid peak (110 mg) which is eluted from the column represents a "globoside"[9] and is completely devoid of blood group activity. The elution of the column with 40% methanol in chloroform is continued until the concentration of sugar in the effluent remains steady at the level of 10 to 20 μg/ml in three to four consecutive 10-ml fractions. Then the solvent mixture is changed to 60% methanol in chloroform, and the above procedure is repeated. Under these conditions the first blood group active glycolipid peak (S-IV) is eluted from the column. Portions of the effluent comprising a clearly defined glycolipid peak (which, however, exhibits some trailing) are pooled and dried *in vacuo* (yield, 30 to 50 mg; HIA, 10 to 20 μg; HIA after combination with the carrier, 0.2 to 0.4 μg; reducing sugar, 40 to 45%; hexosamine, 10 to 15%). The column is finally eluted with 80% methanol in chloroform, which yields an additional amount of the blood group active material (S-V; yield 8 to 12 mg; HIA, 2 to 4 μg; HIA after combination with the carrier, 0.2 to 0.4 μg; reducing sugar, 35 to 40%; hexosamine, 8.5 to 13%). These preparations may yield varying amounts of precipitates with immune antiserums.

iii. Thin-Layer Chromatography

Fractions S-IV and S-V are tested for the presence of impurities by thin-layer chromatography on silicic acid. Plates (20 × 20 cm) with the layer of adsorbent 0.3 mm thick are prepared in a usual way by using a suitable applicator. Silicic acid (Merck G) was found to give satisfactory separation of specific glycolipids. The thin-layer plates are activated for 1 hour at 120° and then stored in a desiccator over $CaCl_2$.

Fractions S-IV and S-V (5 to 10 μl of 2 to 3% solutions) are applied to a starting line, and the chromatogram is developed in chloroform–

[8] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.* **28**, 350 (1956).

[9] T. Yamakawa, S. Yokohama, and N. Handa, *J. Biochem.* (*Tokyo*) **53**, 28 (1963).

methanol–water solvent (60:35:8 by volume). Chromatograms are sprayed with 0.5% orcinol in ethanol containing 2 *N* H_2SO_4 and then heated for 10 minutes at 105°. Both fractions should migrate as single spots with only a trace of impurities. If appreciable amounts of impurities are still present, the fractions may be purified by elution from thin-layer chromatograms. For preparative purposes, thin-layer plates are prepared with silicic acid which preferably does not contain any binder. Single 20 × 20-cm plates will allow separation of up to 10 mg of the material. The materials are applied to the plates as streaks. After development, the plates are dried and then sprayed with water. The fractions show up as white bands on a translucent background. The location of bands is marked, and the plates are allowed to dry. The portion of the adsorbent which contains the main fraction is eluted three times with 20 ml of 20% chloroform in methanol. The pooled extracts are separated from the adsorbent by centrifugation and evaporated to dryness in a flash evaporator. The residue is dissolved in water and is exhaustively dialyzed against distilled water; finally it is freeze-dried. The blood group activity and analytical values for hexosamine and reducing sugar of the materials do not change appreciably after separation on thin-layer chromatograms. The recovery from thin-layer chromatograms varies from 30 to 50%.

c. Determination of Serological Activity

The estimation of the blood group activity, as performed by hemagglutination inhibition tests or the estimation of Forssman activity by inhibition of hemolysis tests, should always be performed in the presence of the carrier lipid. The precipitation with immune sera is less affected by physical dispersion of the glycolipids, and the tests may be performed by standard techniques. The carrier blood group active glycolipid complex is prepared by dissolving 3 parts of carrier (by weight) with 1 part of the active glycolipid in chloroform–methanol (1:1 by volume) and then evaporating the solution with a stream of nitrogen or a flash evaporator. The residue is then dissolved in water or in saline by heating in a boiling water bath for 30 to 60 seconds. The carrier lipid, as obtained by the present procedure, does not display any blood group activity at the concentration of 1 mg/ml; therefore, the minimum amount of the substance which inhibits the hemagglutination is expressed as the amount of pure blood group active glycolipid present in the complex. In quantitative studies, however, it is better to use the carrier lipid isolated from erythrocytes of a different blood group; blood group A active glycolipids should be activated with the carrier lipid isolated from erythrocytes of blood group B, and vice versa. A highly aggregated blood group A antigen preparation may be obtained from fraction S-IV

by methanol fractionation.[2] This preparation displays a high blood group activity even in the absence of the carrier, but the activity may diminish during storage.

D. Viruses

1. THE PREPARATION OF ANIMAL VIRUSES FOR USE AS ANTIGENS*

a. Introduction

The fact that animal viruses must be propagated in living host systems—laboratory animals, embryonated hens' eggs, or *in vitro* cell cultures—creates certain problems in the preparation of both immunizing and serologic antigens. First, it is necessary to utilize infected tissues containing high concentrations of virus in relation to the large amounts of associated host material; in some instances concentration procedures must be employed to obtain sufficient quantities of virus. Second, for certain purposes it is essential to free viral antigens from contaminating host antigens; virus propagated in animals or embryonated eggs is associated with species-specific host antigens, and virus propagated in tissue culture systems retains not only antigens of the host cells, but also antigens of the serum employed for both growth and maintenance of the cell cultures. The intended use of the antigen determines the need for concentration and/or purity. It is important in the use of concentration and purification procedures to avoid chemical or physical treatments that denature the viral protein, producing changes in antigenic properties.

Many viruses possess several antigens with different serologic properties; some are associated with the intact, infectious viral particle, some are comprised of "coreless" viral particles lacking nucleic acid, and others are so-called "soluble" antigens, smaller in size than the viral particle and separable by simple high-speed centrifugation. Special cultural and preparative procedures are involved in the production of each of these kinds of antigens, and different immunization procedures may be required for production of antibody to the various antigens.

b. Viral Immunizing Antigens

In the preparation of viral antisera a primary consideration, aside from obtaining suitably high antibody levels, is the avoidance of treatments that might serve to distort or destroy the antigenicity of the virus employed for immunization. Immunizing antigens are usually not inactivated, unless the virus is one that produces fatal infections in

* Section 1,D,1 was contributed by Nathalie J. Schmidt and Edwin H. Lennette.

the animal species to be immunized. Formalin, phenol, ultraviolet irradiation, and heat inactivation may all distort the antigenicity of viruses to some extent. Inactivation of viral infectivity with β-propiolactone (BPL) has been shown to be more rapid and less detrimental to antigenicity than many other chemical or physical treatments.[1]

BPL is stored at —20° in flame-sealed glass ampules. For use, a 10% solution is first prepared in distilled water at 4° to 5°; the diluent and the BPL are kept in an ice bath. (BPL should form globules on addition to the water; deterioration is evidenced by crystallization and cloudiness on addition to the cold water.) A 0.4% solution of the BPL is then prepared by mixing appropriate volumes of the 10% aqueous BPL solution and cold saline–bicarbonate solution (1.68 gm of $NaHCO_3$, 0.85 gm of NaCl, and 1.0 mg of phenol red in 100 ml of distilled water). Equal volumes of the 0.4% BPL solution and a viral suspension (for example, a 20% suspension of infected mouse brain) are mixed and incubated at 37° for 2 hours. The pH is adjusted to 7.2 to 7.4 with 1 *M* Na_2HPO_4 if necessary, and the treated material is stored at —20°.

For the preparation of immune sera to certain lethal viruses—for example, rabies virus or certain arboviruses—the animals may be immunized initially with BPL-inactivated virus, followed by inoculation with uninactivated virus.

Viral antibody production may be stimulated through the use of hyperimmunization, concentrated antigens, and/or adjuvants. A drawback to the use of hyperimmunization is the fact that it tends to broaden the reactivity of the antiserum produced; this is particularly marked among members of certain viral groups such as the arboviruses. Hence, for the preparation of sharply type-specific immune serum a single injection of the virus is given, but for the preparation of group-reactive antiserum repeated injections are employed. Further, hyperimmunization with unpurified antigens builds up increasing amounts of antibody to associated host proteins.

If viral antigens are to be concentrated for the preparation of immune serum, the method employed should be a gentle one such as high-speed centrifugation [Section D,1,d,i,(b),(v)] or liquid partition [Section D,1,d,iii(d)].

An adjuvant widely employed for preparation of viral immune sera consists of 9 parts of light mineral oil (Marcol 52* or Standard C.T. 70†) and 1 part of dispersing agent (Arlacel A‡). Equal parts of the

[1] G. A. LoGrippo and F. W. Hartman, *J. Immunol.* **75**, 123 (1955).

* Esso, Standard Oil Company, New Jersey. This N.F. grade of oil replaces the former Bayol F.

† Standard Oil Company of California, Richmond, California.

‡ Atlas Powder Company, Wilmington, Delaware.

adjuvant and a viral suspension are emulsified by vigorous shaking in a vaccine vial containing glass beads. The virus–adjuvant mixture is inoculated by the intramuscular route. Also, viruses may be precipitated with aluminum salts[2] or adsorbed onto cholesterol,[3] and the complex inoculated directly into animals for the preparation of immune serum.

Viral antisera employed for certain serologic procedures should be free from antibodies directed against the host tissues or cells employed for preparation of the serologic antigen. This is particularly important in the case of sera to be utilized in complement fixation (CF) tests, fluorescent antibody staining, and precipitation tests, and antihost antibodies may even interfere in tissue culture neutralization tests by inhibiting viral cytopathic effects[4–6] or by exerting a toxic effect on the cell cultures.

It is extremely difficult to free viral immunizing antigens from host proteins that elicit the formation of antibodies. Even fluorocarbon treatment, which satisfactorily removes reactive host material from viral complement-fixing antigens, is not entirely effective in the removal of host material from immunizing antigens.[7]

Viral antisera free from host antibodies may be prepared by immunizing with virus propagated in a homologous host system—for example, by immunizing mice with coxsackievirus propagated in mouse muscle or mouse brain, or by immunizing monkeys with enteroviruses propagated in monkey kidney cell cultures (*grown* and *maintained* on media containing monkey serum or no serum).

Alternatively, the adverse effects of antihost antibodies may be circumvented by employing different host systems for preparation of immunizing antigens and serologic antigens. Thus, the antihost antibodies in the immune serum do not react with heterologous host material in the serologic antigens. For example, immune serum to herpes simplex virus prepared by immunization of hamsters with infected mouse brain may be used in complement fixation tests with viral antigens derived from infected eggs or from chick embryo tissue culture materials. Since tissue culture-derived virus is associated not only with host cell antigens but also with antigens of the serum employed for growth and mainte-

[2] J. L. Melnick, H. A. Wenner, and L. Rosen, *In* "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.), 3rd ed., p. 194; American Public Health Association, New York, 1964.

[3] J. S. Younger and H. Noll, *Virology* **6**, 157 (1958).

[4] K. Habel, J. W. Hornibrook, N. C. Gregg, R. J. Silverberg, and K. K. Takemoto, *Virology* **5**, 7 (1958).

[5] L. Quersin-Thiry, *J. Immunol.* **81**, 253 (1958).

[6] M. C. Timbury, *Virology* **20**, 629 (1963).

[7] P. Halonen and R. J. Huebner, *Proc. Soc. Exptl. Biol. Med.* **105**, 46 (1960).

nance of the cell cultures,[8] it is important to use heterologous sera for growth and maintenance of cell cultures employed for production of the immunizing antigen and for production of the serologic antigen. For example, enterovirus immune sera for use in CF tests may be prepared by immunizing monkeys with virus propagated in monkey kidney cells grown and maintained on bovine serum, and the resultant immune sera may be employed in CF tests with antigens propagated in either monkey kidney or human cell cultures grown and maintained on sera other than bovine, such as horse or monkey.

If viral antiserum can be produced by infection of an animal species through oral or intranasal inoculation of the virus—for example, intranasal infection of ferrets with influenza virus—the resultant immune serum is usually free from host antibodies.

For detailed information on suitable host animals and immunization schedules for preparation of immune sera to specific viral agents, the reader is referred to the third edition of "Diagnostic Procedures for Viral and Rickettsial Diseases."[9]

c. Viral Serologic Antigens

The type of serologic test for which a viral antigen is intended determines the particular qualities of potency and/or purity which the antigen must possess.

i. Neutralization Tests

Viral neutralization tests conducted in animals, embryonated eggs, or *in vitro* cell cultures are based on the ability of serum antibodies to render the virus noninfectious—that is, to neutralize its pathologic or cytopathic effects. Antigens for use in these tests need not be purified, but they should contain a high proportion of infectious virus as compared to the content of noninfectious virus, which is also capable of binding antibody. Titers should be high enough to permit adequate dilution to eliminate much of the host material and noninfectious virus. Virus intended for use in neutralization tests should be cultivated under conditions yielding maximum quantities of infectious virus, and should be handled and stored in such a manner as to prevent loss of infectivity.

ii. Hemagglutination Tests

Viral hemagglutination (HA) tests are based on the ability of certain viruses to combine with receptor sites on erythrocytes and produce visible

[8] R. N. Hamburger, D. A. Pious, and S. E. Mills, *Immunology* **6**, 439 (1963).

[9] E. H. Lennette and N. J. Schmidt (eds.), "Diagnostic Procedures for Viral and Rickettsial Diseases," 3rd ed. American Public Health Association, New York, 1964.

agglutination; hemagglutination inhibition (HI) tests are based on the ability of specific viral antibody to inhibit the combination of virus with erythrocytes, and thus to prevent hemagglutination. Hemagglutinating antigens must possess sufficiently high titers to permit their use at dilutions containing four or eight antigenic units. In some cases it is necessary to free the viral preparation of host material masking hemagglutinins in order to obtain sufficient HA activity. Myxovirus HA antigens may be freed from considerable amounts of host material by adsorption to and elution from erythrocytes [Section D,1,d,iii(a)]; arbovirus HA antigens are prepared by extraction of infected tissue with acetone and ether, or sucrose and acetone,[10] and in some cases enterovirus hemagglutinins in infected tissue culture fluids may be "unmasked" by treatment with fluorocarbon.[11,12] The HA titers of certain viral antigens may be increased by alternate cycles of freezing and thawing or by sonication, and HA titers of measles virus antigens have been increased by treatment with Tween 80 and ether.[13] An important consideration in the preparation of HA antigens in tissue culture systems is the avoidance of serum in the maintenance medium which might possess agglutinins for the erythrocytes to be employed in HA and HI tests.

iii. Complement Fixation Tests

Primary considerations in the preparation of viral complement-fixing (CF) antigens are potency, freedom from host materials that might fix complement with test sera, and freedom from anticomplementary activity. Antigenic potency may be increased simply by harvesting and processing only the tissues or organs of infected animals or eggs that are richest in viral antigen[14–16] or by employing tissue culture techniques for viral cultivation which give rise to large yields of viral antigen.[17–19]

[10] D. H. Clarke and J. Casals, *Am. J. Trop. Med. Hyg.* **7**, 561 (1958).
[11] N. J. Schmidt, V. L. Fox, and E. H. Lennette, *J. Immunol.* **89**, 672 (1962).
[12] N. J. Schmidt, J. Dennis, M. N. Hoffman, and E. H. Lennette, *J. Immunol.* **93**, 377 (1964).
[13] E. Norrby, *Proc. Soc. Exptl. Biol. Med.* **111**, 814 (1962).
[14] J. E. Smadel, R. D. Baird, and M. J. Wall, *Proc. Soc. Exptl. Biol. Med.* **40**, 71 (1939).
[15] J. Sosa-Martinez and E. H. Lennette, *J. Bacteriol.* **70**, 205 (1955).
[16] S. Fazekas de St. Groth, D. M. Graham, and I. Jack, *J. Lab. Clin. Med.* **51**, 883 (1958).
[17] N. J. Schmidt, E. H. Lennette, J. H. Doleman, and S. J. Hagens, *Am. J. Hyg.* **66**, 1 (1957).
[18] G. Churcher, F. W. Sheffield, and W. Smith, *Brit. J. Exptl. Pathol.* **40**, 87 (1959).
[19] J. C. N. Westwood, G. Appleyard, D. Taylor-Robinson, and H. T. Zwartouw, *Brit. J. Exptl. Pathol.* **41**, 105 (1960).

Also, the physical and chemical concentration procedures described below may be utilized for concentrating viral CF antigens. Host material may be reduced by extraction of the antigens with lipid solvents or fluorocarbon, and combinations of some of the physicochemical procedures described below may virtually eliminate host material. Fluorocarbon treatment [Section D,1,d,ii(d)] has also been recommended for removal of anticomplementary activity from viral antigens,[20,21] as has treatment with heated guinea pig complement.[22] For the latter procedure guinea pig serum, inactivated by heating at 56° for 30 minutes, is added to tissue culture antigens to give a final concentration of 5%; the mixture is then incubated at 37° for 30 to 60 minutes. Treated antigens may be stored at —20°.

iv. Flocculation and Precipitation Tests

Viral antigens for use in flocculation or precipitation tests must be highly concentrated, in some instances several hundredfold, in order to form visible aggregates with specific antibody. Such concentration may be effected by high-speed centrifugation or by certain combinations of physicochemical concentration procedures. Freedom from host material which might interfere with precipitation or give a cloudy appearance to the antigen is important in the case of antigens employed for tests in an aqueous medium, but to a lesser extent for antigens utilized in gel double-diffusion systems, as in the latter instance antigens and host materials tend to separate on the basis of differences in diffusion through the agar medium.

v. Precautionary Techniques

Performance of *in vitro* serologic tests with infectious viral antigens constitutes a hazard, and good precautionary techniques must be observed. With some agents it is possible to use inactivated antigens, but with others, particularly the enteroviruses, inactivation by physical or chemical methods distorts the reactivity of the antigen. Ultraviolet irradiation has been employed for preparation of certain myxovirus antigens[23] and for arborvirus antigens.[24] Adenovirus CF antigen may be heated at 56° without distorting its reactivity, and group CF antigens for members of the psittacosis–lymphogranuloma venereum group can also be heat-inactivated. β-Propiolactone has been recommended for in-

[20] K. Hummeler and V. Hamparian, *Science* **125**, 547 (1957).
[21] V. Hamparian, F. Muller, and K. Hummeler, *J. Immunol.* **80**, 468 (1958).
[22] G. von Zeipel, *Arch. Virusforsch.* **8**, 246 (1958).
[23] G. Henle, S. Harris, and W. Henle, *J. Exptl. Med.* **88**, 133 (1948).
[24] J. Casals, *Proc. Soc. Exptl. Biol. Med.* **49**, 501 (1942).

activation of serologic antigens for certain of the arborviruses.[25] The use of attenuated viral strains for preparation of serologic antigens would appear advantageous from the standpoint of reducing the hazard of handling; attenuated poliovirus strains have been found to give rise to CF antigens comparable in potency and sensitivity to those derived from unattenuated viral strains.[26,27]

d. Concentration and Purification of Viral Antigens

Concentration procedures utilized for the preparation of viral antigens may merely reduce the volume of fluid in which the virus is suspended without resulting in appreciable removal of host material, or techniques may be employed that both concentrate the virus and separate it from host material. Also, purification of viral preparations may or may not involve concentration of the antigenic material. It might appear that procedures resulting in a high degree of concentration and purification are usually preferable, but several factors must be taken into consideration. These include (1) the laboratory facilities and personnel time available for preparation of antigens, (2) the purpose of the antigen (is there an actual need for a highly purified or concentrated reagent?), and (3) the possible denaturing and inactivating effect of complex concentration and purification procedures on the viral antigen.

i. Simple Viral Concentration Procedures

The techniques described below are simple in principle and, with the exception of ultracentrifugation, do not result in appreciable purification of the virus.

(a) Cultural Procedures. Some degree of viral concentration can be achieved through certain manipulations of tissue culture systems in which virus is propagated. One may infect a large number of host cells in a small volume of maintenance medium; the virus produced in the cells is concentrated by virtue of release into a small volume of suspending medium.[17-19] Also, viral antigens may be concentrated by harvesting and utilizing only the cellular phase of the infected cultures—before the virus is released into the fluid phase.[28,29] The infected cells are resus-

[25] G. R. French and R. W. McKinney, *J. Immunol.* **92**, 772 (1964).
[26] N. J. Schmidt, E. H. Lennette, S. J. Hagens, and J. Dennis, *J. Immunol.* **84**, 160 (1960).
[27] N. J. Schmidt, E. H. Lennette, J. Dennis, and S. J. Hagens, *J. Immunol.* **85**, 67 (1960).
[28] F. Artzet and M. L. Ryhiner, *Ann. Inst. Pasteur* **99**, 852 (1960).
[29] N. J. Schmidt, E. H. Lennette, C. W. Shon, and T. T. Shinomoto, *Proc. Soc. Exptl. Biol. Med.* **116**, 144 (1964).

pended in a small volume of fluid, and the virus is released by alternate cycles of freezing and thawing or by sonic oscillation.

(*b*) *Physical Procedures.* Several simple physical methods may be employed for concentration of virus; for most of these the viral preparation is first clarified of larger cellular debris by horizontal centrifugation at 2000 rpm for 20 minutes.

(*i*) *Pervaporation.*[30,31] Clarified viral suspensions are dialyzed against approximately 100 volumes of distilled water for 18 to 24 hours at 4°, after which the dialysis bag is placed approximately 1½ feet in front of a household fan which is allowed to play on the bag until the volume of the fluid is reduced to the desired fraction of the original. Pervaporation is conducted at 4° to minimize viral inactivation. After the volume of the suspending fluid is reduced to the desired level, the concentrates are dialyzed for 18 to 24 hours against 100 volumes of physiological saline or buffered saline.

(*ii*) *Dialysis against Hydrophilic Compounds.*[32,33] The volume of suspending medium of a viral preparation may be reduced by dialysis against such hydrophilic compounds as polyvinylpyrrolidone or polyethylene glycol (Carbowax 20-M*). In the use of Carbowax, clarified viral suspensions are simply placed in dialysis tubing which is covered with Carbowax chips and held at 4° until the volume of fluid is reduced to the desired degree. The concentrate is then dialyzed against 100 volumes of physiological saline or phosphate-buffered saline for 18 to 24 hours at 4°.

(*iii*) *Ultrafiltration.*[34,35] Viral preparations may be concentrated on filters which retain the viral antigens, allowing the suspending medium to pass through; the virus is then resuspended in a smaller volume of fluid. Filters are prepared by impregnating alundum candles with a 7% solution of collodion in glacial acetic acid. (Ultrafilters are also commercially available.†) The filter candle is placed in the clarified viral suspension, and fluid is removed by vacuum. Viral particles are retained

[30] A. Polson and J. W. F. Hampton, *J. Hyg.* **55**, 344 (1957).

[31] N. J. Schmidt and E. H. Lennette, *Am. J. Hyg.* **70**, 51 (1959).

[32] M. A. Bucca, H. L. Casey, and J. F. Winn, *Proc. Soc. Exptl. Biol. Med.* **104**, 247 (1960).

[33] N. J. Schmidt and E. H. Lennette, *J. Immunol.* **89**, 85 (1962).

* Carbide and Carbon Chemicals Company, Union Carbide and Carbon Corporation, New York.

[34] A. Svedmyr, J. F. Enders, and A. Holloway, *Proc. Soc. Exptl. Biol. Med.* **79**, 296 (1952).

[35] T. H. Weller and H. M. Witton, *J. Exptl. Med.* **108**, 869 (1958).

† LKB Instruments, Inc., 4840 Rugby Avenue, Washington, D.C.

in the residue on the surface of the filter and are washed by passing Veronal buffer or physiological saline through the filter. The washed virus is then resuspended in a small volume of fluid.

(*iv*) *Lyophilization.* Certain viral antigens, such as those of the varicella-zoster virus,[36] may be concentrated by drying the viral preparation in the frozen state and reconstituting the desiccated material in a small volume of fluid. This procedure is not suitable, however, for use with certain viruses, notably the enteroviruses.

(*v*) *Ultracentrifugation.* The simple physical concentration procedures mentioned above result in the concentration of host material as well as viral antigens. However, greater amounts of host material may be removed from viral preparations by ultracentrifugation. The following procedure[37] may be employed for concentration of enteroviruses or rhinoviruses for use as immunizing antigens or for serologic antigens in complement fixation or precipitation tests. Infected tissue culture fluids are clarified, first by horizontal centrifugation at 1500 rpm for 15 minutes, followed by high-speed centrifugation in an angle centrifuge at 15,000 rpm for 45 minutes. The resultant clarified fluids are then centrifuged at 30,000 rpm for 5 hours to sediment the virus. The supernatant fluids are discarded, and the pelleted virus is resuspended in a small volume (one-tenth to one-hundredth or less of the original) of physiological saline or balanced salt solution. In concentrating the larger viruses preliminary centrifugation at 15,000 rpm is eliminated, as this would tend to sediment the virus; also, the larger viruses may be pelleted at 20,000 rpm for 2 hours. Baron[38] has demonstrated the importance of including protein in the suspending medium for effective pelleting of the small enteroviruses; 2% calf serum or 0.06% gelatin is recommended.

ii. Viral Purification Procedures

(*a*) *Extraction with Lipid Solvents.* Viral preparations containing large quantities of host material—for example, infected mouse brains or muscles—are extracted with lipid solvents for use as antigens in *in vitro* serologic tests. Benzene[39] or acetone–ether extraction[40] is employed for preparation of arbovirus CF antigens, and acetone–ether or sucrose–ether extraction is utilized for the production of arbovirus HA antigens.[10] Detailed descriptions of these techniques are given in the references

[36] A. E. Caunt, C. J. M. Rondle, and A. W. Downie, *J. Hyg.* **59**, 249 (1961).
[37] W. Smith, F. W. Sheffield, L. H. Lee, and G. Churcher, *Lancet* **I**, 710 (1956).
[38] S. Baron, *Proc. Soc. Exptl. Biol. Med.* **95**, 760 (1957).
[39] C. Espana and W. McD. Hammon, *J. Immunol.* **59**, 31 (1948).
[40] J. Casals, *Proc. Soc. Exptl. Biol. Med.* **70**, 339 (1949).

cited as well as in the third edition of "Diagnostic Procedures for Viral and Rickettsial Diseases."[9]

(*b*) *Protamine Sulfate Precipitation.*[10,41] The addition of protamine sulfate to certain viral suspensions precipitates much of the host material, leaving the virus in the supernatant fluid. However, some viruses are precipitated along with the host material. To each milliliter of chilled viral suspension is added 5.0 mg of protamine. The mixture is agitated and held at 4° for 30 minutes, after which the precipitate is sedimented by centrifugation at 3000 rpm for 15 minutes. The supernatant fluid containing the virus is withdrawn, and excess protamine is precipitated by the addition of 3.8 mg of heparin per milliliter of the fluid. Protamine sulfate precipitation is effective for the partial purification of certain arboviruses and enteroviruses, but not for the viruses of herpes simplex, vaccinia, rabies, lymphocytic choriomeningitis, or murine encephalomyelitis.

(*c*) *Bentonite Adsorption.*[42] Adsorption of viral suspensions with a 1% concentration of Bentonite (a clay mineral with high cation exchange and adsorbing properties) has been employed for removal of host material from coxsackievirus preparations. With certain viruses, however, Bentonite treatment results in a loss of viral activity.

(*d*) *Fluorocarbon Treatment.* Emulsification of viral suspensions with certain fluorocarbons such as Genetron 113* results in the precipitation of host proteins in the fluorocarbon phase, leaving the virus in the aqueous phase.[43] Fluorocarbon treatment has been utilized for ridding viral CF antigens of anticomplementary activity[20,21] and of host proteins reacting "nonspecifically" with animal immune sera;[21,44] it has also been employed for "unmasking" viral hemagglutinins in tissue culture preparations.[11,12,45] One volume of Genetron 113 is added to 2 volumes of the viral suspension, and the mixture is stirred in a Virtis homogenizer at 20,000 rpm for 5 minutes, after which the homogenate is centrifuged at 2000 rpm for 10 minutes. The aqueous phase containing the virus is collected and employed as antigen. In some instances two fluorocarbon extractions may be required to free CF antigens of reactive host material.[44] It should be pointed out, however, that viral antigens differ in

[41] J. Warren, M. L. Weil, S. B. Russ, and H. Jeffries, *Proc. Soc. Exptl. Biol. Med.* **72**, 662 (1949).

[42] N. Oker-Blom and E. Nikkilä, *Ann. Med. Exptl. Fenn.* **33**, 190 (1955).

* Allied Chemical and Dye Corporation, New York.

[43] A. E. Gessler, C. E. Bender, and M. C. Parkinson, *Trans. N.Y. Acad. Sci.* **18**, 701 (1956).

[44] P. Halonen, R. J. Huebner, and H. C. Turner, *Proc. Soc. Exptl. Biol. Med.* **97**, 530 (1958).

[45] A. J. Girardi, *Virology* **9**, 488 (1959).

their susceptibility to fluorocarbon; enterovirus CF antigens can withstand several treatments without loss of titer, but certain myxovirus antigens may be precipitated after two treatments.[21] Fluorocarbon treatment is frequently performed as a preliminary step to other viral purification procedures—for example, density gradient centrifugation.

iii. Viral Concentration–Purification Procedures

The following techniques may be employed for both concentration and purification of virus; they may be used in combination with other procedures for the preparation of highly purified and concentrated viral suspensions.

(*a*) *Adsorption and Elution from Erythrocytes.* Viruses possessing hemagglutinins may be concentrated and partially purified by adsorbing them onto erythrocytes and subsequently eluting them into a small volume of fluid. This method is most effective with the myxoviruses. Influenza virus from infected allantoic fluids is adsorbed onto chicken erythrocytes at 0° for 1 hour; the erythrocytes are then sedimented by centrifugation in the cold and washed once with ice-cold 0.01 *M* phosphate-buffered saline solution, pH 7.0. The washed erythrocyte–virus complex is then resuspended in a small volume of the buffered saline solution containing 1% RDE (receptor-destroying enzyme of *Vibrio cholerae*), and virus is eluted by incubation at 37° for 2½ hours. The erythrocytes are sedimented by centrifugation, and the supernatant fluid constituting the viral concentrate is collected.

(*b*) *Precipitation Methods.* Virus may be precipitated from suspension by treatment with ammonium sulfate, methanol, or heavy metals, or by adjusting the pH to that of the isoelectric point of the virus. Concentration is effected by resuspending the virus in a smaller volume of fluid. Considerable quantities of host protein may be precipitated along with the virus; if a more purified suspension is desired, the concentrate may be subjected to ultracentrifugation, column chromatography, or density gradient centrifugation. For examples of the use of precipitation as a step in the preparation of highly concentrated and purified poliovirus, the reader is referred to the literature.[46,47] Also, a simple method for concentrating poliovirus by precipitation with cobalt chloride has been described.[48]

Polson and Selzer[49] described the use of isoelectric precipitation for

[46] C. E. Schwerdt and F. L. Schaffer, *Virology* **2**, 665 (1956).

[47] J. Charney, R. Machlowitz, A. A. Tytell, J. F. Sagin, and D. S. Spicer, *Virology* **15**, 269 (1961).

[48] N. Grossowicz, A. Mercado, and N. Goldblum, *Proc. Soc. Exptl. Biol. Med.* **103**, 872 (1960).

[49] A. Polson and G. Selzer, *Biochim. Biophys. Acta* **14**, 67 (1954).

concentration and partial purification of polioviruses; the following modification of their method has been employed in this laboratory for the preparation of poliovirus flocculating antigens.[31] Infected HeLa cell culture fluids are dialyzed (without preliminary centrifugation) against approximately 60 volumes of a 1:5 dilution of McIlvaine's buffer, pH 4.3, for 48 hours at 4°. The resultant precipitate is sedimented by horizontal centrifugation (in the cold) at 2000 rpm for 20 minutes. After removal of the supernatant fluid the virus is eluted from the precipitate by incubation for 2 hours at room temperature in a small volume of a 1:5 dilution of McIlvaine's buffer, pH 8.0. The eluate is clarified by centrifugation at 2000 rpm for 20 minutes. Isoelectric precipitation has also been described for concentration of adenovirus antigens.[50,51]

Other methods for purification and concentration of viruses by precipitation are to be found in reports dealing with the use of zinc compounds for the precipitation of certain viruses.[52-54]

(*c*) *Adsorption Procedures.* Virus may be concentrated and purified by adsorption with certain gels or ion exchangers. In some systems the virus is adsorbed, leaving host proteins in the suspension, and in others the host proteins are adsorbed from the viral suspension.

Fantes[55] has described a simple method for the concentration and partial purification of poliovirus in which the virus is adsorbed onto aluminum phosphate at pH 5.0 and subsequently eluted at pH 7.0 to 8.0. Aluminum phosphate is added to a poliovirus suspension to give a final concentration of 0.1 to 0.2 mg/ml. The pH of the mixture is then adjusted to 5.0 by the addition of *N* HCl or 10% acetic acid, and the mixture is agitated at room temperature for 2 hours. After centrifugation at 1000 to 1500 *g* for 10 minutes, the supernatant fluid is discarded, and the virus is eluted in one-hundredth of the original volume of a mixture of 9 parts of tissue culture Medium 199 and 1 part of 10% $NaHCO_3$. After being shaken for 30 minutes at room temperature, the suspension is centrifuged at 1000 to 1500 *g* for 10 minutes, and the supernatant fluid containing the concentrated, partially purified virus is withdrawn.

Column chromatography with cellulose ion exchangers (DEAE-SF or ECTEOLA-SF) has been employed for purification of a variety of ani-

[50] H. G. Steinman and P. A. Murtaugh, *Virology* **7**, 291 (1959).
[51] C. D. Brandt, A. L. Neal, R. E. Owens, and K. E. Jensen, *Proc. Soc. Exptl. Biol. Med.* **113**, 281 (1963).
[52] T. G. Metcalf, *J. Infectious Dis.* **101**, 40 (1957).
[53] N. Newton and R. E. Bevis, *Virology* **8**, 344 (1959).
[54] W. J. Hausler, Jr., and E. C. Dick, *J. Infect. Diseases* **107**, 189 (1960).
[55] K. H. Fantes, *J. Hyg.* **60**, 123 (1962).

mal viruses. The polioviruses and coxsackievirus type A9 are not adsorbed onto the columns, but chromatography of suspensions containing these viruses results in a high degree of purification, host material being retained on the columns. On the other hand, echovirus type 13 and Colorado tick fever virus are adsorbed onto the columns and may then be washed, and finally eluted into the appropriate buffer solution.[56]

Highly purified preparations of poliovirus may be obtained in the following manner.[56,57] Virus is first concentrated by ultracentrifugation, and the pellet is washed by resuspension in 0.02 *M* phosphate buffer, pH 7.1, and centrifugation at 100,000 *g* for 2 hours. The washed virus is then resuspended in phosphate buffer, pH 7.1, and passed through a column of ECTEOLA-SF cellulose (height 15 cm, diameter 1 cm). The virus is eluted with 2-ml volumes of 0.02 *M* phosphate buffer, pH 7.1, and sedimented at 100,000 *g* for 2 hours. The viral pellets are then resuspended in the desired volume of suspending medium.

Viruses with lipophilic properties, such as myxoviruses, vaccinia, and certain bacteriophages, may be adsorbed onto cholesterol columns,[3] the host proteins passing into the effluent. The virus–cholesterol complexes may be suspended and employed directly for immunization purposes.

(*d*) *Liquid Partition Systems.* The concentration and partial purification of viral antigens by distribution in liquid–liquid two-phase systems represents a highly effective procedure which does not require complicated apparatus. For further purification the concentrates obtained in the two-phase system may be subjected to column chromatography or density gradient centrifugation.

In a liquid partition system, particles distribute according to their size and surface properties. Concentration of virus can be achieved through the use of a system in which most of the virus is transferred to a phase with a small volume; purification is effected if the host materials distribute in the other phase. The use of systems consisting of aqueous polymers provides a mild treatment which does not denature viruses, in contrast to organic solvent systems which are deleterious to certain viruses with essential lipids.

Phase separation is performed in a separatory funnel which should be constructed with a narrow, graduated tube at the bottom into which the complete bottom phase settles and can be collected. Thus, the surface of the phase boundary does not decrease during collection, which would cause material attached to the phase boundary to drag down into the bottom phase.

[56] B. H. Hoyer, E. T. Bolton, R. A. Ormsbee, G. LeBouvier, D. B. Ritter, and C. Larson, *Science* **127**, 859 (1958).

[57] L. Levintow and J. E. Darnell, Jr., *J. Biol. Chem.* **235**, 70 (1960).

As an example of the use of a liquid partition system for the concentration and purification of viruses the following method[58] for the concentration of polioviruses is given. The two-phase polymer system consists of sodium dextran sulfate* (limiting viscosity number of 70 ml/gm) and polyethylene glycol (Carbowax 6000†). Infected tissue culture fluids are first clarified by centrifugation at 2000 rpm for 10 minutes, after which the polymers are added to give a final concentration of 0.2% (w/w) of sodium dextran sulfate and 6.45% (w/w) of polyethylene glycol. A 5 *M* solution of NaCl is then added until the salinity of the mixture is 0.3 *M*. The system is carefully mixed in a separatory funnel and allowed to separate for 24 to 48 hours at 4°. The bottom phase represents about one-hundredth of the total volume of the system and contains most of the virus, which thus becomes concentrated ninety to one hundred times. Dextran sulfate may be removed from the viral concentrate by precipitation with 0.69 ml of 3 *M* KCl or 0.3 ml of 1 *M* $BaCl_2$ per gram of the concentrate.

The virus in the bottom phase can be further concentrated by the addition of 5 *M* NaCl to give a final concentration of 1 *M*; this change in molarity produces a new phase system in which the virus distributes in the top phase. Since the volume of the top phase is about one-fifth of the total volume of the secondary system, a further concentration of virus is obtained. The small amount of dextran sulfate in the top phase can be eliminated by precipitation with KCl or $BaCl_2$.

This same polymer system can also be employed for concentration of certain echoviruses, but different systems may be required for concentration of other viruses. For detailed information on the principles and applications of liquid partition systems for viral concentration and purification, the reader is referred to the excellent monograph by Albertsson.[59]

Organic solvent partition systems may be used for purification and concentration of certain viruses which do not contain an essential lipid—for example, the enteroviruses.[60] Virus-infected tissue culture fluids are first clarified by centrifugation at 2500 rpm for 10 minutes. One volume of the clarified tissue culture fluid is then mixed with 1 volume of phosphate solution (2.5 *M* K_2HPO_4, adjusted to pH 7.5 with 33.3% H_3PO_4)

[58] E. Norrby and P. A. Albertsson, *Nature* **188**, 1047 (1960).

* Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, New Market, New Jersey.

† Carbide and Carbon Chemicals Company, Union Carbide and Carbon Corporation, New York.

[59] P. A. Albertsson, "Partition of Cell Particles and Macromolecules." Wiley, New York, 1960.

[60] T. Kitano, I. Haruna, and I. Watanabe, *Virology* **15**, 503 (1961).

and 0.8 volume of the organic solvent mixture (2 volumes of 2-ethoxyethanol and 1 volume of 2-butoxyethanol). The mixture is gently agitated twenty times and then centrifuged at 3000 rpm for 5 minutes. The top layer contains most of the organic solvent, and the bottom layer most of the aqueous solution; almost all the virus is recoverable at the interface, while the host material is dispersed in the top and bottom layers.

(*e*) *Density Gradient Centrifugation.* Centrifugation of particles in a column (tube) with a gradient of densities separates them on the basis of differences in sedimentation rates or density, depending on the system employed. Gradients of sucrose or cesium chloride have been the most widely used for the separation of viral antigens. Not only is virus freed from host material, but viral particles with different physicochemical and serologic properties are separable by this technique. Density gradient centrifugation usually constitutes a final step in the production of highly purified viral antigens; virus is first concentrated by ultracentrifugation or other appropriate methods.

For the preparation of purified poliovirus antigens,[46] sucrose density gradients are prepared in 5-ml centrifuge tubes by successively layering 0.7-ml volumes of 45%, 37%, 29%, 21%, and 11% sucrose (by weight) solutions containing 0.14 *M* NaCl. The gradients are permitted to become continuous by diffusion at 4° for 12 hours. Viral concentrates are layered over the gradients, which are then centrifuged at 30,000 rpm for 2 hours in a Spinco SW-39 swinging-bucket rotor. Centrifugation is stopped after 2 hours—after the various components are separated, but before equilibrium is attained. This is called "zone centrifugation," and various components are separated on the basis of differences in sedimentation rates. Two poliovirus antigens are separable in this manner, the so-called D antigen, contained in a dense band at the bottom of the gradient and consisting of viral particles containing nucleic acid and possessing infectivity, and the so-called C antigen, less dense than the D antigen and lacking nucleic acid and infectivity. These two antigens possess different serologic properties in CF and precipitation tests.

Two antigens with different serologic properties are separable from coxsackievirus preparations by centrifugation in a cesium chloride density gradient.[61] In this system centrifugation is continued until each particle has reached a point where the suspending medium has a density equal to that of the particle; this is called "isopycnic gradient centrifugation," or equilibrium centrifugation, and particle separation is based solely on differences in density. The viral suspension is first clarified

[61] N. J. Schmidt, J. Dennis, L. H. Frommhagen, and E. H. Lennette, *J. Immunol.* **90**, 654 (1963).

by centrifugation at 1500 rpm for 15 minutes, followed by centrifugation in an angle centrifuge at 15,000 rpm for 45 minutes, and the virus is then concentrated 200-fold by high-speed centrifugation at 30,000 rpm for 5 hours. The concentrates are centrifuged in cesium chloride of initial density 1.34 for 22 hours at 35,000 rpm in a Spinco swinging-bucket rotor SW-39. Coxsackievirus antigens with high infectivity titers and type-specific activity in precipitation and CF tests position in a band in the center of the gradient, while less dense antigens with low infectivity titers and group reactivity in precipitation and CF tests position at the top of the gradient.

Density gradients of potassium tartrate[62] have been employed for purification of certain of the larger animal viruses such as herpes simplex and vaccinia.

[62] J. F. McCrea, R. S. Epstein, and W. H. Barry, *Nature* **189**, 220 (1961).

2. THE PREPARATION OF PLANT VIRUSES FOR USE AS ANTIGENS*

a. Introduction

In this chapter, it is not intended to devise a single scheme which would be expected to give satisfactory results in the purification of all plant viruses. The aim of this article is to outline some techniques which the author has found successful and which can be included, with perhaps a few modifications, in the purification of many plant viruses when a high degree of purity is required, as in immunological studies. For the various purification procedures successfully developed for many plant viruses, the reader is referred to a review by Steere.[1]

In the purification of plant viruses for use as antigens, the procedures considered successful are those that effect separation of the virus, in intact form, from all normal plant tissue antigens. Some of the techniques described may appear to be excessively time-consuming, but the need for a very high degree of purity in virus preparations to be used as antigens justifies any additional effort which the techniques demand.

Improved knowledge of the nature of viruses now allows us to consider each as a distinct macromolecular particle having well-defined physical and chemical properties. Use has been made of these properties in devising methods of purification. For example, in differential and "rate" zonal density gradient centrifugation, separation of viruses from some normal

* Section 1,D,2 was contributed by Roderick MacLeod.

[1] R. L. Steere, *Virus Res.* **6**, 3 (1959).

tissue constituents is effected by differences in their sedimentation characteristics. In "equilibrium" zonal density gradient centrifugation, differences in density bring about the separation. In zonal density gradient electrophoresis, the total surface charge of the particles is used to separate the viruses from normal host constituents.

The first essential is a good source of the virus. The success of any procedure is largely dependent on the selection of a suitable host and optimal conditions for the vigorous growth of the host and rapid invasion of the host by the virus. In many cases, analytical centrifugation will give a good indication of the quantity of virus that a particular host plant will yield. A small volume of juice extracted from the infected tissue can be examined directly in an analytical centrifuge.[2] The lower limit of virus concentration which can be detected is 0.1 mg of virus per milliliter of the infected sap. Tissues showing this concentration would be expected to yield sufficient virus for a successful purification, provided the virus can withstand the purification procedure. This method may be used to compare the virus content of different hosts and of certain tissues in the same host, and to determine the peak of virus concentration in the infected tissue.

b. Methods

i. Purification by Differential Centrifugation

The procedure described is that used by the author for the purification of the turnip crinkle virus. All manipulations are carried out at 4°.

Procedure 1

1. Wash the infected tissues in 0.001 *M* potassium cyanide.
2. Macerate the tissues in a meat grinder.
3. Wrap the pulp in bandage gauze, and extract the juice by means of a press.
4. Centrifuge the juice at 15,000 *g* for 15 minutes.
5. Discard the pellets, and centrifuge the supernatant fluid at 100,000 *g* for 60 minutes.
6. Discard the supernatant fluid, cover the pellets with distilled water, and allow them to stand for several hours.
7. Resuspend the virus from the softened pellets by gentle agitation of the tubes.
8. Repeat alternate low-speed and high-speed centrifugation as in steps 4 and 5.

[2] R. Markham, *Advan. Virus Res* **9**, 241 (1962).

Although it is recommended that as few chemicals as possible be added during the stages of purification, EDTA (ethylenediaminetetraacetic acid), adjusted to pH 7.5, should be added to the extracted juice to a final concentration of 0.02 *M*, providing it has been ascertained that it does not affect the virus adversely. This treatment greatly facilitates removal of the ribosomes, especially when one is attempting to purify viruses having sedimentation rates close to those of the ribosomes.

Treatments that coagulate normal host proteins should be avoided. Apparently, aggregates of plant proteins may be produced by some treatments,[2] and these proteins may have sedimentation rates similar to those of some viruses. These aggregates may persist through differential centrifugation cycles. If the pellets are soaked for several hours, gentle agitation of the tubes will usually resuspend the virus, leaving most of the normal host constituents compacted. Subsequent low-speed centrifugation will remove these large aggregates. Differential centrifugation, such as that described, has been used successfully to purify many of the stable plant viruses.

ii. Zonal Density Gradient Centrifugation

Zonal density gradient centrifugation was first applied to the purification of plant viruses by Brakke.[3] For a detailed description of the theory of this technique, the reader is referred to Brakke[4] and to Chap. 7, Vol. II.

Two types of sucrose gradients are used in the purification of plant viruses by zonal density gradient centrifugation. The first, the "rate" gradient, is a column of relatively low density with movement of particles through it being dependent mainly on their sedimentation rates. The second, the "equilibrium" gradient, has a density which approaches that of the virus particles, and the density of the particles is the main factor determining their movement through this gradient. (It should be noted that this is "quasi" equilibrium.) If both "rate" and "equilibrium" gradients are included in a purification scheme, a more effective separation of viral particles from normal host constituents occurs through differences in sedimentation rates and, in addition, through differences in densities.

A gradient-making device may be used to prepare sucrose gradients, or it may be more efficient to layer sucrose solutions when several gradient columns are to be prepared. In this laboratory, satisfactory gradients are prepared in centrifuge tubes by using funnels with capillary stems.[5]

[2] M. K. Brakke, *J. Am. Chem. Soc.* 73, 1847 (1951).
[4] M. K. Brakke, *Advan. Virus Res.* 7, 193 (1960).
[5] M. K. Brakke, *Arch. Biochem. Biophys.* 45, 275 (1953).

The volumes of the different sucrose solutions used to prepare gradients in Beckman Spinco S.W. 25.1 and S.W. 25.2 tubes are listed in Table I. The figures are for the preparation of gradients for the purification of wound tumor virus and potato yellow dwarf virus. Buffer and the range of sucrose concentrations may be varied to suit the particular

TABLE I
VOLUMES OF SUCROSE SOLUTIONS USED TO PREPARE GRADIENTS IN SPINCO CENTRIFUGE TUBES

Tube S.W. 25.1 (volume, 33 ml)		Tube S.W. 25.2 (volume, 60 ml)	
Sucrose concentration	Volumes per layer	Sucrose concentration	Volumes per layer
	Rate Gradients		
0%	3.2 ml	0%	3.2 ml
10%	6.4 ml	5%	3.2 ml
20%	6.4 ml	10%	6.4 ml
30%	3.2 ml	15%	6.4 ml
		20%	6.4 ml
		25%	6.4 ml
		30%	6.4 ml
	Equilibrium Gradients		
30%	3.2 ml	25%	3.2 ml
40%	3.2 ml	30%	3.2 ml
50%	3.2 ml	35%	3.2 ml
60%	3.2 ml	40%	3.2 ml
		45%	3.2 ml
		50%	3.2 ml
		55%	3.2 ml
		60%	3.2 ml

virus being prepared. The layered solutions are allowed to stand overnight at 4° so that a gradient will form by diffusion.

Procedure 2

1. Extract the juice, and clarify as in procedure 1, steps 1 to 4. If the virus content of the juice is less than 0.25 mg/ml, the virus should be concentrated by pelleting once, as in procedure 1.
2. Carefully layer the virus suspension on each of the rate gradients (8 ml on the S.W. 25.1 tubes; 16 ml on the S.W. 25.2 tubes).
3. Centrifuge in a swinging-bucket rotor at about 60,000 *g* for ap-

proximately the length of time required to pellet the virus from an aqueous solution with use of the same force.

4. Remove the virus zones with a hypodermic syringe fitted with a 20-gauge needle that has a right-angle bend both at the base and at 1 cm from the tip. (The virus zone should be identified by infectivity tests.)
5. Pool the samples, and layer on each of the equilibrium gradients (8 ml or 16 ml, according to the tube size).
6. Carefully layer buffer on the zones to 0.5 cm from the tops of the tubes. The tubes are thus prevented from collapsing during centrifugation.
7. Centrifuge at 60,000 *g* for about ten times as long as the time determined in step 3 above.
8. Remove the virus zones, as in step 4.
9. Pool the samples, and dilute with an equal volume of buffer.
10. Layer the virus on a second set of equilibrium gradients as in steps 5 and 6.
11. Centrifuge as in step 7. The virus, which is the only clearly visible zone at this stage, can be removed as described previously.

iii. Zonal Density Gradient Electrophoresis

Zonal density gradient electrophoresis was first applied to the purification of plant viruses by Brakke.[6]

It is best to apply zonal density gradient electrophoresis as a final purification step, immediately following the second equilibrium centrifugation. The virus samples collected from the equilibrium gradients are in a solution containing approximately 45% sucrose and can be introduced as a zone in the high-density (lower) region of the sucrose gradient in the electrophoresis apparatus.

An L.K.B. column electrophoresis apparatus is used in this laboratory for zonal density gradient electrophoresis. All sucrose solutions are made up in an appropriate buffer with readjustment of the pH when necessary (a 0.02 *M* potassium phosphate buffer, pH 7.7, is used for electrophoresis of the potato yellow dwarf virus). The density of the sucrose gradient ranges from 40% to 5%.

The apparatus is diagramed in Fig. 1. Modifications for its use to suit virus purification are mentioned in the detailed procedure which follows. Ascending electrophoresis is used with the positions of the electrodes interchanged. Thus the anode vessel of the apparatus houses the cathode. This arrangement can be used successfully if the period of electrophoresis is of relatively short duration.

[6] M. K. Brakke, *Arch. Biochem. Biophys.* **55**, 175 (1955).

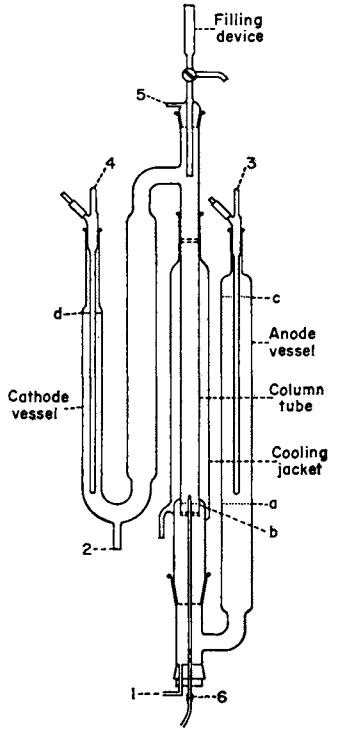


FIG. 1. A diagrammatic illustration of the L. K. B. type 3340 column electrophoresis apparatus as used for zonal density gradient electrophoresis of the virus preparations.

Procedure 3

1. Start the circulation of cold water through the cooling jacket of the column.
2. Allow a 50% sucrose solution to flow into the apparatus from a Mariotte flask through inlet 1 until it reaches level *a* in the anode vessel and *b* in the inner column tube.
3. Remove the electrode from the anode vessel, and close all other outlets.
4. Layer buffer on the sucrose solution in the anode vessel to level *c*.
5. Replace the electrode, and close the outlet to the anode vessel.
6. Remove the filling device.
7. Mount a gradient-making device at a level higher than the top of the apparatus; charge the lower vessel of the gradient-maker with a 50% sucrose solution and the upper vessel with buffer.
8. Attach a length of fine-bore rubber tubing to the outlet of the

gradient-maker, and lower the tube into the column until its outlet is about 1 inch above the surface of the sucrose solution at level *b*.

9. Start the gradient-maker, and allow the gradient to flow down the wall of the column at a rate that will not cause excessive mixing at the interface (*b*).
10. Fill the column with the gradient, raising the tube periodically to maintain the outlet 1 inch above the surface of the solution in the column.
11. When the level of the gradient has reached the junction of the column and the cathode vessel, remove the gradient-maker, and replace the filling device.
12. Allow buffer to flow through inlet 2 until it reaches level *d* in the cathode vessel.
13. Close outlet 4 of the cathode vessel, and continue filling the apparatus slowly until buffer flows from outlet 5 of the filling device.
14. With outlet 5 open and all other outlets closed, collect three successive 3-ml samples through the capillary tube (6). Mix each thoroughly.
15. Adjust the sucrose concentration of the virus suspension so that a drop of the sample will float on gradient sample 1, suspend in sample 2, and sink in sample 3. Alternatively, the virus can be pelleted and resuspended in sample 2.
16. Collect the virus sample in a 5-ml hypodermic syringe, fitted with a 22-gauge needle, and expel all air bubbles.
17. Insert the needle into the rubber tubing attached to the capillary tube (immediately behind the lower end of the capillary), and clamp the tube behind the point of insertion of the needle.
18. Expel the sample from the syringe by applying a low, steady pressure to the piston. The sample will enter the gradient at the top of the capillary and form a zone on the shelf produced in the gradient in step 14.
19. Close outlet 5 of the filling device, and carefully open outlets 3 and 4 of the electrode vessels. (There may be slight movement of the liquid in the column when outlets 3 and 4 are opened. The level of the liquid in the anode and cathode vessels at hydrostatic equilibrium will be found to differ slightly, owing to the differences in the densities of the liquids in the apparatus. When hydrostatic equilibrium has been attained, these levels should be marked and prearranged each time the apparatus is filled.)
20. Raise the position of the zone in the column by allowing a small volume of buffer to flow from outlet 2 of the cathode vessel with outlet 3 open and all other outlets closed.

21. The capillary can now be flushed with a small volume of liquid from the gradient (the tip of the capillary is now about 1 cm below the virus zone).
22. Make sure that both anode and cathode outlets (3) and (4) are open, and start the electric current. Allow electrophoresis to proceed for 12 hours. (The potato yellow dwarf virus migrates a distance of 20 cm in 12 hours when 450 volts and 35 ma are applied through the above-mentioned buffer.)
23. Discontinue electrophoresis, close outlets 3 and 4, and open outlet 5 of the filling device.
24. Unload the gradient through the capillary, and collect the virus zone.
25. Pellet the virus, and resuspend the pellets in buffer.

When a total of about 5 mg of the potato yellow dwarf virus is loaded in the column, the virus zone is clearly visible after electrophoresis. Faintly opalescent zones can be seen above and below the virus zone, indicating separation of small amounts of contaminants. (When this concentration of virus was used in the initial zone, droplet sedimentation was not observed.) The virus may be subjected to a second electrophoresis if incomplete removal of contaminants is suspected.

Although this treatment has been found to be highly successful, it will not effect separation of contaminants which may be adsorbed to the surface of the virus particles. In some preparations of the potato yellow dwarf virus, there is evidence of the adsorption of green pigment onto the surface of the virus particles. This is indicated by a green pigmentation of the virus zone in the equilibrium gradients. It is suspected that the age of the infected tissue at harvesting may be related to the degree of adsorption of this pigment to the virus particles. Clearly, this is also a function of properties of the virus itself. The high degree of adsorption of pigment sometimes found in preparations of the potato yellow dwarf virus has never been found by the author in preparations of several other viruses extracted from the same host by similar procedures. It has been shown that antisera prepared against potato yellow dwarf virus with a heavy green pigmentation usually reacted with normal tissue constituents, even after the virus was subjected to electrophoresis.[7] In this instance the green pigment is a good indicator of the presence of normal host antigens on the surface of the virus particles.

Experiments carried out on the virus preparations showing this green pigmentation suggest that the electrophoretic properties of the virus are changed by the adsorption of contaminants. Complete separation of un-

[7] S. Wolcyz and L. M. Black, personal communication, Department of Botany, University of Illinois, Urbana, Illinois, 1963.

coated particles from contaminant-coated particles was not achieved by zonal density gradient electrophoresis. The virus zone widened considerably during electrophoresis, suggesting a distribution of particles with a range of electrophoretic mobilities. This fact indicates strong complexing of varying amounts of contaminants with the virus.

iv. Celite Filtration

A successful means for the prevention of adsorption of normal host antigens onto the surface of the potato yellow dwarf virus was found in a method of R. L. Steere (personal communication).

Procedure 4

1. Prepare a 1-inch pad of Celite (grade 535) on Whatman No. 1 filter paper in a Büchner funnel with the aid of a vacuum pump. The Celite should be made to settle into a well-compacted pad by pounding the sides of the funnel.
2. Wash the Celite pad twice with distilled water. (Care should be taken not to let the pad dry out and crack.)
3. Extract the juice as in procedure 1, steps 1 through 3.
4. Add 5% (w/v) of dry Celite to the extracted juice.
5. Adjust the juice to pH 8 by adding NaOH dropwise, with continuous stirring.
6. Pour the juice onto the Celite pad, and apply vacuum.
7. Collect the effluent which shows a brown coloration.
8. Flush the pad with distilled water until all the brown effluent has been collected. (A section cut through the pad will show the green pigment trapped in the upper half-inch.)
9. Centrifuge the brown effluent as in procedure 1, step 5.
10. Resuspend the virus in buffer, continue the purification from procedure 2, step 2, and subject the purified virus to zonal density gradient electrophoresis as described previously.

Preparations of the potato yellow dwarf virus subjected to Celite filtration, followed by zonal density gradient centrifugation and electrophoresis, showed no pigmentation in the gradient zones or in the final virus pellets.

v. Partial Fractionation on a Granulated Agar Gel Column

This technique was first used by Polson[8] for the fractionation of protein mixtures. It has been applied to the purification of plant viruses.[9,10]

[8] A. Polson, *Biochim. Biophys. Acta.* **50**, 565 (1961).
[9] M. H. V. van Regenmortel, *Virology* **17**, 601 (1962).
[10] R. L. Steere and G. K. Ackers, *Nature* **196**, 475 (1962).

Procedure 5

1. Select a gel concentration that will give a pore diameter slightly smaller than that of the virus. [For example, a 2% (dry wt/vol) ion agar No. 2 gel excludes the potato yellow dwarf virus, which has a diameter of approximately 150 mμ.]
2. Prepare the selected concentration of agar in distilled water, and allow the gel to solidify.
3. Homogenize the gel in 2 volumes of water in a Waring Blendor to a particle size that will pass through a 40-mesh (U.S. standard) sieve and be retained by a 60-mesh sieve.
4. Pour a suspension of the granulated agar in water into a chromatographic column, and allow the gel granules to settle to give a column of packed granules filling the chromatographic tube to three-fourths of capacity. (The column *must not* be allowed to dry at any time during the procedure.)
5. Place a piece of filter paper on the surface of the settled granules.
6. Wash the column with 3 volumes of distilled water, and saturate with 3 to 4 volumes of an appropriate buffer.
7. Filter the extracted juice through a Celite pad as in procedure 4, steps 1 through 9.
8. Allow the buffer to drain from the column until it is just at the surface of the filter paper on the gel.
9. Layer the brown extract on the filter paper as a zone to a depth approximately equal to the width of the column. The rate of flow of the column should be regulated for optimal separation of the fractions.
10. Carefully layer buffer on the filter paper when the extract has just passed into the gel. (Always keep a head of buffer on the column.)
11. Collect fractions when the effluent appears opalescent, and test for virus. (This usually occurs when the brown pigment is about half-way through the column.)
12. Pool all samples containing virus. Centrifuge to pellet the virus, and continue purification from procedure 2, step 2.

When potato yellow dwarf virus is prepared by this procedure, distinct opalescence can be seen when the virus is present in the effluent, owing to the high degree of light scattering caused by the virus particles. When the virus concentration in the infected juice is too low to give opalescence, other methods for detection of the virus in the effluent should be adopted. This technique permits rapid separation of the virus from many of the lower-molecular-weight tissue constituents by the principle

of molecular sieving. Inclusion of this step in virus purification is recommended when it is desirable to effect rapid removal of virus from the plant juice. In some cases, this apparently may be essential to maintain the virus particles intact. Removal of the C-terminal threonine from the peptide chains of the subunits of the tobacco mosaic virus by the action of carboxypeptidase has been shown to depress the titer of this virus against antiserum to the unaltered form.[11] This indicates that the serological properties of some viruses may change noticeably as a result of very minor changes in the surface of the virus protein. The identification of carboxypeptidase in sap extracted from bean leaves suggests the possibility that significant changes in the serological properties of some virus may result from their exposure to certain plant juices. When plant viruses are being purified for use as antigens, it is a good practice to effect the rapid removal of the virus from the plant sap. Not only can this reduce the likelihood of enzymatic digestion of the protein, but it can also significantly reduce the degree of complexing of normal tissue constituents with the virus.

c. Tests of Purity and Intactness of the Purified Virus

i. Analytical Centrifugation

Although analytical centrifugation should be a routine test applied to the virus preparation, it cannot be relied upon for the detection of small amounts of impurities. If, for example, a contaminant is a homogeneous plant protein, the lowest concentration detectable would be in the region of 0.1 to 0.2 mg/ml. Plant ribosomes, however, can be detected in concentrations as low as 0.01 mg/ml by use of the ultraviolet optical system. If the contaminant is polydisperse, much larger quantities may go undetected.[2] Depending on the nature of the contaminant, much lower quantities than those detectable by analytical centrifugation may stimulate antibody production.

ii. Electron Microscopy

Electron microscopy is not a satisfactory test of purity. It can be of use, however, to determine whether or not the virus has survived the purification procedure in an intact form.

Electron microscopy of thin sections of tissues infected with potato yellow dwarf virus and of negatively stained preparations of the extracted virus[12] has contributed greatly to our understanding of the serological behavior of this virus.

[11] J. I. Harris and C. A. Knight, *J. Biol. Chem.* **214**, 215 (1955).
[12] S. Brenner and R. W. Horne, *Biochim. Biophys. Acta* **34**, 103 (1959).

Thin sections of infected tissues have shown[13] that potato yellow dwarf virus particles acquire a portion of the nuclear membrane of the host cell as an envelope. This fact has an important bearing on our interpretation of cross-reaction tests carried out between this virus and several other plant and animal viruses which have been shown to have a similar ontogeny. The presence of this envelope immediately suggests the possibility that the virus may carry antigens analogous to the heterophile antigens, and positive cross reactions may well involve these and not be a true reflection of phylogenetic relationship.

In this regard also, electron microscopic examination of extracts has shown potato yellow dwarf virus to be extremely susceptible to loss of native morphology immediately following removal from its intracellular environment. Experiments have been carried out to investigate the effect of numerous treatments on the morphology of this virus and observations have led to the inclusion of an additional preliminary step in the purification schedule: the infected leaves are quartered and submerged in a suitable buffer (0.1 *M* glycine:0.01 *M* $MgCl_2$, pH 7.0 for potato yellow dwarf virus). The air spaces in the tissue are evacuated with the aid of a vacuum pump. Release of the vacuum results in an impregnation of the tissues with the buffer and they are allowed to remain for 2 hours at 4° in this buffer before grinding. The resulting virus preparation is noticeably less pigmented even at the initial clarification step and electron microscopy shows that the virus is intact, having the same internal components and complement of membranes as seen in thin sections of the virus in the tissues.

There is good reason to suspect that potato yellow dwarf virus is not the only plant virus that acquires cellular membranes, and the detection of such phenomena may have significance in the interpretation of serological reactions involving these viruses.

iii. Immunological Methods

(*a*) *Reaction of Antisera with Normal Tissue Constituents.* In investigating cross-relationships among plant viruses, it is necessary to determine whether observed positive reactions indicate the presence of common antigenic sites on different viruses or the presence of serologically related contaminating antigens. One such antigen, the F_1 protein, was reported[14] to be common to many plant species. It is essential to test for the presence of antibody to this protein in antisera to be used for the investigation of cross-reactions between any plant viruses. Antisera should be prepared against normal tissue constituents and used to test

[13] R. MacLeod, L. M. Black, and F. H. Moyer, *Virology* **29**, 540 (1966).
[14] M. H. V. van Regenmortel, *South African J. Lab. Clin. Med.* **8**, 164 (1962).

for the presence of F_1 protein or any other impurities which may contaminate the virus preparation. It is important that the tests carried out for the detection of contaminants be of the same sensitivity as those used to test for cross-reactions between the viruses.

(*b*) *Bacterial and Fungal Contamination of Purified Virus Preparations.* Highly purified virus preparations stored in aqueous suspension at 4° are susceptible to bacterial and fungal contamination. Although low-speed centrifugation of the antigen prior to injection may remove most of the bacterial or fungal growth, the supernatant can contain metabolic products of the microorganisms, and the possibility of alteration of the viral structure by microbial enzymes must be kept in mind. There is a strong possibility of obtaining spurious cross-reactions with antisera prepared against virus samples thus contaminated. The virus to be used for each injection in an immunization schedule should be freshly prepared just prior to injection. Viruses to be used as antigens should not be prepared in bulk and stored under conditions that would allow contamination to develop.

Prevention of contamination of the growing plants by other viruses or by other strains of the same virus is of extreme importance. The accumulation of contaminants on the apparatus used in the purification schedule can be avoided by careful cleaning and, wherever possible, sterilization of all equipment.

(*c*) *Immunoelectrophoresis.* Immunoelectrophoresis is a useful test for the detection of unbound contaminants, but it will not detect contaminants that are strongly complexed with the virus particles.

d. Discussion

It is maintained by some that differential centrifugation will not yield virus preparations of sufficiently high purity for use as antigens. Preparations of several of the stable "spherical" plant viruses, purified by this method in investigations by the author,[15] did not react with antisera prepared against normal plant antigens. Several groups of viruses purified in this manner were subjected to precipitin tests, cross-absorption tests, immunoelectrophoresis, and indirect hemagglutination tests when similarities in the physical and chemical properties of these viruses suggested the possibility of serological cross-reactions. Not all tests for cross-reactions were positive, as one would expect if each immunizing antigen contained F_1 protein—not even the highly sensitive indirect hemagglutination test in which cells sensitized with purified antibody consistently gave end-point titers in the homologous system at an antigen dilution of 0.1 μg/ml. False positive reactions due to the presence of F_1 protein and its antibody would be expected in this sensitive test if the purification

[15] R. MacLeod, Ph.D. Thesis, Cambridge University, 1963.

procedures had not completely removed this protein from the preparations.

The zonal density gradient centrifugation procedure is recommended for viruses that will not survive repeated differential centrifugation and for the large plant viruses that will sediment in the normal clarification procedure (procedure 1, step 4).

It is suggested that zonal density gradient electrophoresis should be included as the final treatment of preparations, but, as mentioned above, it is not the answer to all problems. Tests for the presence of antibody to contaminants must be carried out on all antisera, even if the immunizing antigen has been repeatedly subjected to zonal density gradient electrophoresis.

We may sum up with the view expressed by Kabat[16]: "In the present state of our knowledge about impurities, it is inexcusable to immunize with an antigen, no matter how pure it is thought to be, and then take for granted that the animal has produced only antibody to this antigen. All antisera must be examined for the presence of antibodies to impurities by as many and as sensitive techniques as possible before use."

[16] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed., p. 394. Thomas, Springfield, Illinois, 1961.

3. THE PREPARATION OF ANTIGENS OF BACTERIAL VIRUSES*

a. Production of Bacteriophage

Bacterial viruses are potent antigens; the smallest quantity of bacteriophage with the capacity to elicit formation of detectable antibody in rabbits has been found to be 10^7 particles per animal.[1] Since the lysates of phage-infected bacterial cultures contain between $10^9$and 10^{11} phage particles/ml, they can be used for immunization without further concentration. The preparation of such lysates and the techniques of their assay have been described by Adams[2] and are presented in Chap. 17, Vol. III.

The crude lysates are contaminated, however, with large amounts of bacterial constituents, some of which are antigenic and toxic as well. When it is desired to obtain potent antiphage sera relatively free of antibacterial antibodies, it is necessary to use purified viral vaccines. The methods of preparation of such vaccines depend on the size and

* Section 1,D,3 was contributed by M. A. Jesaitis and Norton D. Zinder.

[1] E. M. Miller and W. F. Goebel, *J. Exptl. Med.* **100**, 525 (1954).

[2] M. H. Adams, *Bacteriophages*, pp. 443–522. Interscience, New York, 1959.

shape of the virus. The larger phages can be sedimented by passage of lysates through a continuous-flow centrifuge such as a Sharples. The smaller phages must be precipitated prior to the collection in the centrifuge. For further purification of the virus, filtration and differential centrifugation are used. These methods take advantage of differences between the size of phage and that of bacterial constituents. Finally, when it is necessary to remove bacterial components having the same sedimentation constant as the phage, density gradient centrifugation and chromatographic methods have to be used.

The following protocols illustrate the procedures for the purification of the large T-even phages (particle weight 3×10^8) and the small f2 phage (particle weight 3×10^6).

i. Preparation of T2 Phage

A 2-liter culture of *Escherichia coli* B is grown in glucose–phosphate medium[3] at 37° until it contains 5×10^8 cells/ml. The culture is then supplemented with 20 ml of 10% nutrient broth and infected with 10^{10} phage particles. A few drops of tributylphosphate are added to control foaming. The culture is then aerated for 5 to 6 hours and left overnight to lyse. After filtration of the lysate through a layer of Hyflo Super-Cel, the phage is collected by spinning the filtrate in a refrigerated Sharples ultra-centrifuge equipped with a self-sealing rotor. The sedimented virus is then suspended in the medium remaining in the rotor, and the suspension is spun in an angle centrifuge for 10 minutes at 5000 *g*. The supernatant containing the phage is decanted, adjusted to pH 7, and digested with deoxyribonuclease (1 μg/ml) for 30 minutes at 37°. The solution is filtered through a porcelain candle (Coors P2 or P3), the filtrate is centrifuged for 45 minutes at 12,000 *g*, and the phage pellets are resuspended in 10 ml of saline. The suspension is purified further by one cycle of centrifugation at low and at high speed, and the final phage pellet is resuspended in 5 ml of saline. The phage suspension thus obtained contains about 10^{13} T2 particles/ml.[3,4] Other techniques for preparation and purification of the T-even phages have been reviewed by Adams.[2]

ii. Preparation of f2 Phage

Host bacterium *E. coli* K 12 Hfr is grown in 40-liter batches in a Biogen (American Sterilizer Co.) until the culture contains about 2×10^8 cells/ml. Phage is added to give a multiplicity of 0.1 virus per bacterium, and the culture is incubated further for 2½ hours. Under these

[3] M. A. Jesaitis and W. F. Goebel, *J. Exptl. Med.* **102**, 733 (1955).
[4] M. A. Jesaitis, *J. Gen. Physiol.* **44**, 585 (1961).

conditions many bacteria do not release the virus. Phage and bacteria are then precipitated by adding 300 gm of ammonium sulfate per liter of culture. After storage of the culture overnight at room temperature, the precipitate is collected in a Sharples ultracentrifuge by passing the fluid at a rate of 6 liters/hour at 50,000 rpm, and the sediment is resuspended in 400 ml of water. To complete the lysis, EDTA is added to give 0.002 *M*, and lysozyme is added to a concentration of 100 μg/ml. Since the solution becomes very viscous, it is digested with deoxyribonuclease (1 μg/ml) in the presence of 0.01 *M* $MgCl_2$. When the viscosity decreases, the bacterial debris is removed by centrifugation at 12,000 *g* for 1 hour. The phage is then precipitated from clear supernatant with 2 *M* ammonium sulfate, the precipitate is collected by centrifugation at 10,000 *g* for 1 hour, and the pellet is resuspended in 100 ml of 0.1 *M* phosphate buffer at pH 7.5.

To purify the phage further, the suspension is centrifuged in an ultracentrifuge at 100,000 *g* for 1 hour, and the resulting phage pellets are resuspended in 60 ml of water. On addition of 36 gm of CsCl, the phage suspension is centrifuged at 35,000 rpm for 20 hours in a Spinco 40 rotor. The phage bands are collected from the middle of the tubes. These are then diluted with 5 volumes of water, and the dilutions are centrifuged at 100,000 *g* for 2 hours. The final phage pellets are suspended in 0.1 *M* Tris buffer at pH 7.4. Thus, some 500 mg of phage is obtained of which 5 to 15% is viable.[5]

b. Preparation of the Components of T-Even Phages

The particles of T-even phages are composed of a polyhedral head and a rigid tail. The head consists of a protein membrane containing viral nucleic acid, internal protein, and polyamines. The tail consists of an outer contractile sheath, an inner hollow core, and several tail fibers attached to a terminal plate.[6] Four of the viral protein components—the head membrane, the tail sheaths, the tail fibers, and the internal protein—are antigenic.[7-9] The serological properties of the tail cores and plates have not yet been investigated. The nucleic acids of these phages were also found to be immunologically active.[10]

When a virus grows in a bacterial cell, each of its constituents is

[5] T. Loeb and N. D. Zinder, *Proc. Natl. Acad. Sci. U.S.* **47**, 282 (1961).
[6] S. Brenner, G. Streisinger, R. W. Horne, S. P. Champe, L. Barnett, S. Benzer, and M. W. Rees, *J. Mol. Biol.* **1**, 281 (1959).
[7] F. Lanni and Y. T. Lanni, *Cold Spring Harbor Symp. Quant. Biol.* **18**, 159 (1953).
[8] N. C. Franklin, *Virology* **14**, 417 (1961).
[9] L. Levine, J. L. Barlow, and H. Van Vunakis, *Virology* **6**, 702 (1958).
[10] L. Levine, W. T. Murakami, H. Van Vunakis, and L. Grossman, *Proc. Natl. Acad. Sci. U.S.* **46**, 1038 (1960).

synthesized separately. During maturation, these components are assembled to form a complete viral particle. Some of the constituents escape the assembly process, however, and they are released into the culture medium, when the bacterial cell lyses. Electron microscopical examination of the lysates of bacteria infected with T2 or T4 phage has revealed that they contain about twenty-three head membranes and ten tail rods for each one hundred complete phage particles.[11]

Lysates containing large amount of components of the T-even phages can be prepared by inhibiting the formation of complete particles in bacteria with an acridine dye, proflavine.[12] For this purpose a bacterial culture containing about 5×10^8 cells/ml is infected with 2 to 5 phages per bacterium, supplemented 1 to 3 minutes later with 1 to 5 μg of proflavine per milliliter, and incubated until it lyses.[13] The lysis can be accelerated by adding chloroform to the culture after incubating it for 1 or 2 hours.[8,11] The lysates obtained in this manner contain less infectious phage than the lysate of an untreated culture by a factor of about 1000, yet the quantity of phage antigens in treated and untreated lysates is essentially the same.[13,14] Electron microscopy has revealed that in the proflavine lysates there are about six viral head membranes and two tail rods for each intact viral particle.[11]

The various phage components can be isolated from the lysates by centrifugation through buffered sucrose gradient. First the bacterial debris is removed by spinning the lysate at 8000 *g* for 10 minutes, and the complete phage particles are sedimented at 12,000 *g* for 1 hour. Then a gradient consisting of 1-ml portions of 30%, 40%, 50%, and 60% sucrose solutions is formed in a centrifuge tube and overlayered with an aliquot of purified lysate. The tubes are spun at 70,000 *g* for 5 hours in a Spinco swinging-bucket rotor; 70% of the tail fibers are found in the top sucrose layer, 90% of the cores are present in the 30 to 40% of sucrose, and 80% of the head membranes are found in the layers containing 50 to 60% of sucrose.[8]

Since the head membranes are sedimented more rapidly than other phage components, they can also be prepared by centrifuging debris-free proflavine lysates at 20,000 *g* for 2 hours. The sediment consists predominantly of tailless viral membranes, called "doughnuts," which are accompanied by 1% of tailed viral particles.[13] The latter can be removed by absorbing the doughnut suspension with bacterial cells. Sera of rabbits

[11] E. Kellenberger and J. Sechaud, *Virology* **3**, 256 (1957).
[12] R. A. C. Foster, *J. Bacteriol.* **56**, 795–809 (1948).
[13] R. I. De Mars, S. E. Luria, H. Fisher, and C. Levinthal, *Ann. Inst. Pasteur* **84**, 113 (1953).
[14] R. I. De Mars, *Virology* **1**, 83 (1955).

immunized with such membrane suspension contain a considerable quantity of antibody to viral head proteins, and only a small amount of antibody reacting with viral tail constituents.[15]

Viral head membrane consists of a multitude of protein subunits. To prepare the latter, phage is disrupted by osmotic shock, and the released nucleic acid is digested with DNase. The protein coats of the virus, called "phage ghosts," are sedimented by centrifugation at 100,000 *g* for 1 hour and resuspended in saline.[16] The phage ghosts are then treated either with an alkaline saline solution containing 0.1 to 1.0% Dupanol at pH 10.2,[17] or with 66% acetic acid containing 0.3 *M* NaCl.[18] Under these conditions, viral head membranes dissociate into subunits. After dialysis of the solution against Tris buffer at pH 10.5, the undegraded viral components are removed by centrifugation at 120,000 *g* for 2½ hours, while the membrane subunits remain in the supernatant. Physicochemical measurements have revealed that the subunits formed on treatment with acetic acid have a molecular weight of 42,000, and that those obtained by alkaline dissociation have a molecular weight between 86,000 to 100,000. The latter may be regarded as dimers of the smaller particles.[17,18] The subunits obtained by treatment with alkali still are capable of binding complement in the presence of an antiserum to phage ghosts. Their complement fixation curves differ, however, from those given by the ghosts, a fact suggesting some denaturation of the subunits.[17]

Viral tail sheaths and fibers can be obtained from T2L phage.[6] This is achieved by denaturing the virus with glycine–hydrochloric acid buffer at pH 2.0 and degrading viral nucleic acids and head membranes with DNase, trypsin, and chymotrypsin. The tail sheaths are sedimented from the digested phage solution by centrifugation at 100,000 *g* for 1 hour and resuspended in 1% ammonium acetate at pH 6.5. The nonsheath material is then removed by centrifuging the suspension at 10,000 *g* for 10 minutes, while the sheaths are collected by recentrifuging the supernatant at 100,000 *g*. The fibers are isolated from the viral digest, from which the sheaths have been removed, by absorption on an hydroxyapatite column and elution with 0.08 to 0.12 *M* phosphate buffer at pH 6.8. This procedure fails with other T-even phages, such as T2H, T4B, and T6, as they are not denatured by the glycine–HCl buffer. However, the tail sheaths of these phages may be prepared by using 0.5 *N* sulfuric acid in the presence of 0.15 *M* sodium chloride as the denaturing agent.[6]

[15] M. A. Jesaitis, *J. Exptl. Med.* **121**, 133 (1965).
[16] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.* **40**, 809 (1957).
[17] H. Van Vunakis, W. H. Baker, and R. K. Brown, *Virology* **5**, 327 (1958).
[18] D. J. Cummings, *Biochim. Biophys. Acta* **68**, 472 (1963).

The tail sheaths and fibers of T2L phage were found to be serologically active. Absorption of T2 antiserum with tail fibers reduced its neutralization constant to 10% of its original value (see Chap. 17, Vol. III). Subsequent absorption of the serum with tail sheaths further reduced its K value to 0.3%.[8] The serological properties of tail sheaths prepared by degradation with sulfuric acid have not been described.

The internal protein of T2 and T4 can be isolated by rupturing the particles by repeated freezing and thawing or by osmotic shock, followed by digestion of the nucleic acid with DNase.[9,19] The phage components having high molecular weights are removed by centrifugation at 93,000 g for 5 hours. The internal protein remains in the supernatant.[19] It is detected by complement fixation using antisera to ruptured phage absorbed with intact homologous virus.[9]

The immunologically active nucleic acids of the T-even phages can be obtained by extracting the proteins from phage suspension with phenol and precipitating the nucleic acid from the aqueous phase with two volumes of ethanol.[20] An alternative method is to rupture the phage by repeated freezing and thawing, remove protein from the homogenate with chloroform–octyl alcohol in the presence of 1 M NaCl, and recover the nucleic acids from the solution by alcohol precipitations.[21] The nucleic acids thus prepared are not immunogenic, but they bind complement in the presence of antisera containing specific antibodies. Such antisera can be obtained by prolonged immunization of rabbits with large quantities of ruptured virus[10] or with a mixture of viral nucleic acid and methylated bovine serum albumen.[22]

[19] T. Minagawa, *Virology* **13**, 515 (1961).
[20] J. D. Mandell and A. D. Hershey, *Anal. Biochem.* **1**, 66 (1960).
[21] M. A. Jesaitis, *J. Exptl. Med.* **106**, 233 (1957).
[22] O. J. Plescia, W. Braun, and N. C. Palczuk, *Proc. Natl. Acad. Sci. U.S.* **52**, 279 (1964).

E. Conjugated and Synthetic Antigens

1. COUPLING OF DIAZONIUM COMPOUNDS TO PROTEINS*

a. General Procedure and Properties

Because of the simplicity of the method and the availability of a great variety of suitable substances, the coupling of diazonium salts of aromatic compounds has been extensively used as a means of introducing small molecules ("haptens") into proteins. Immunization with

* Section 1,E,1 was contributed by Alfred Nisonoff.

such protein–hapten conjugates will frequently elicit antibodies with specificity directed toward the hapten group. Evidence for antihapten specificity may include: reaction of the antiserum with a conjugate consisting of the same hapten coupled to a protein other than that employed for immunization; the inhibition of such precipitation by free (uncoupled) hapten or by molecules closely related structurally to the hapten (see Chap. 18,E, Vol. III); a direct demonstration of binding by a method involving only the antibody and hapten, such as equilibrium dialysis,[1] as discussed in Chap. 18,B. Vol. III; or quenching of the fluorescence of antibody,[2] as discussed in Vol. II, Chap. 18,C, Vol. III. Classical immunological studies carried out with haptens coupled to proteins through azo linkages are exemplified by the work of Landsteiner[3] and Pauling *et al.*[4]

Most aromatic amines react with nitrous acid to form a diazonium salt. An example is the diazotization of aniline hydrochloride. The prod-

uct has the characteristics of an ionic salt, is soluble in water and insoluble in ether. Since the dry diazonium chloride may explode if subjected to shock or heat, it should be handled as an aqueous solution. Many diazonium fluoroborates, however, are stable if dried rapidly and protected from sunlight; such compounds have been used by Singer and his associates for specific labeling of antibody combining sites.[5]

At slightly alkaline pH a diazonium salt will couple to side chains of a protein molecule. If the number of moles of diazonium salt used is comparable to the number of moles of tyrosine, histidine, and lysine residues in the protein, the reaction yields mainly monosubstituted tyrosine and histidine, and disubstituted lysine groups; coupling occurs through azo linkages in each case. Some reaction with arginine and tryptophan may also take place.[6–8] The reaction with tyrosyl side chains

[1] J. R. Marrack and F. C. Smith, *J. Exptl. Pathol.* **13**, 394 (1932).

[2] S. F. Velick, C. W. Parker, and H. N. Eisen, *Proc. Natl. Acad. Sci. U.S.* **46**, 1470 (1960).

[3] K. Landsteiner, "The Specificity of Serological Reactions," rev. ed. Harvard Univ. Press, Cambridge, Massachusetts, 1945.

[4] L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, *J. Am. Chem. Soc.* **64**, 2994 (1942).

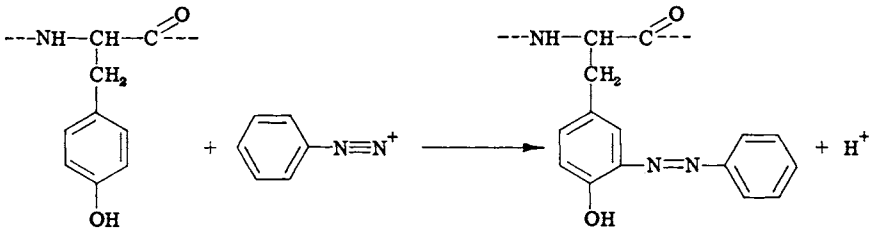
[5] L. Wofsy, H. Metzger, and S. J. Singer, *Biochemistry* **1**, 1031 (1963); H. Metzger, L. Wofsy, and S. J. Singer, *ibid.* **2**, 979 (1963).

[6] E. W. Gelewitz, W. L. Riedeman, and I. M. Klotz, *Arch. Biochem. Biophys.* **53**, 411 (1954).

[7] A. N. Howard and F. Wild, *Biochem. J.* **65**, 651 (1957).

[8] M. Tabachnick and H. Sobotka, *J. Biol. Chem.* **235**, 1051 (1960).

proceeds as shown. When relatively large amounts of the diazonium salt are employed, disubstitution of tyrosine and histidine and more extensive reaction with arginine and tryptophan residues may occur.[7,8] Reaction mechanisms are discussed in detail by Howard and Wild.[7]



Reactivities of various diazonium derivatives in the coupling reaction are described by Migrdichian.[9]

b. Procedure

The procedure used for conjugation will be illustrated by the method used in this laboratory to couple diazotized *p*-aminobenzoic acid to bovine γG-globulin. The product is used for immunization of rabbits.

i. Preparation of Diazonium Salt

Forty milligrams of *p*-aminobenzoic acid is dissolved in 2 ml of 1 *N* HCl and cooled by immersion in an ice bath. A cold solution of sodium nitrite in water (14 mg/ml) is added dropwise. After each addition the mixture is stirred magnetically for about half a minute. A droplet is then transferred on the end of a thin stirring rod to starch–iodide paper. The presence of excess nitrous acid (end point) is indicated by the *immediate* appearance of a blue color. The reaction between the p-aminobenzoic acid and sodium nitrite is stoichiometric, and 1.45 ml of nitrite solution is therefore required.

ii. Coupling Procedure

One gram of bovine γG-globulin (6.5 S γ-globulin) is dissolved in 20 ml of a buffer consisting of 0.13 *M* NaCl, 0.16 *M* boric acid, and sufficient sodium hydroxide to raise the pH to 9. The beaker containing the solution of γ-globulin is surrounded by an ice bath contained in a large crystallizing dish with a flat bottom; this permits magnetic stirring of the contents of the beaker.

The solution of diazonium salt is added dropwise, with rapid stirring, to the cold protein solution. After addition of each drop the pH is re-

[9] V. Migrdichian, "Organic Synthesis," Vol. 2, p. 1511. Reinhold, New York, 1957.

adjusted to 9.0 to 9.5 with 0.5 *N* NaOH. When all the solution has been added, the reaction is allowed to continue with slow stirring for at least an hour, with further additions of sodium hydroxide solution as necessary to maintain the pH in the range 9.0 to 9.5.

The solution is then dialyzed exhaustively against a number of changes of a cold, neutral buffer solution, over a period of a week. The final dialysis should be against an isotonic buffer suitable for use in immunization. The solution is then diluted with buffer to give a protein concentration of 10 mg/ml.

In place of dialysis, unreacted small molecules may be removed from the protein by passage through a column of Sephadex G-25 in the cold room, with an isotonic salt solution as the eluting buffer. The protein solution may be stored in a number of small containers in the frozen state; portions are then thawed prior to use. Alternatively, as a preservative, phenol may be added to a final concentration of 0.1%, and the solution stored in the refrigerator.

c. Notes on the Procedure

i. In the example given above, the reaction with nitrite proceeds very rapidly. Some aromatic amines, however, react more slowly, and it is necessary to wait for an appreciable period of time after each addition of nitrite before testing with starch–iodide paper. The stoichiometry of the reaction between nitrite and the aromatic amine offers a direct test for completeness of the diazotization.

ii. Some aromatic amines are sparingly soluble in dilute hydrochloric acid. Nevertheless, the reaction may proceed smoothly, owing to continued solubilization as the products are formed.

iii. If it is desired to stop the reaction prior to dialysis, resorcinol may be added as an aqueous solution in an amount equal to a hundredfold molar excess over the amine.

iv. From the earlier literature, one obtains the impression that coupling of limited amounts of diazonium salt occurs mainly to tyrosine and histidine side chains. However, recent work of Tabachnick and Sobotka clearly indicates that the reaction with lysine may be comparably fast.[8]

v. Detectable amounts of dialyzable colored material may be slowly released from a conjugated protein (over a period of weeks or months). This may have no deleterious effect on an antigen to be used for immunization. However, it can seriously interfere with an antigen used to determine the presence of precipitating antibodies in the serum of an immunized animal ("test antigen"); soluble hapten molecules compete with the conjugated protein for antibody combining sites. Such antigens should

therefore be dialyzed periodically or stored in the frozen state. Acetone extraction, in addition to dialysis, has been used for purification.[10]

vi. The number of hapten groups coupled to the protein has been determined by use of a radioactive aromatic amine[11-13]; by analysis for arsenic after coupling diazotized arsanilic acid[14]; or by analysis for iodine content after coupling diazotized iodoaniline.[8] By the use of radiolabeled *p*-aminobenzoic acid we have found that the coupling efficiency in the reaction described above (40 mg of diazotized *p*-aminobenzoic acid reacting with 1 gm of bovine γ-globulin) is about 35%; approximately 15 hapten groups are incorporated per molecule of protein. Table I lists some data on incorporation obtained with other systems.

vii. Since the azoproteins formed in these reactions are colored, one would expect that an estimate of the degree of coupling might be obtained from the absorption spectrum of the product. Actually, this is difficult because of the variety of side chains that undergo reaction and the fact that both mono- and bis-substitution of tyrosine and histidine are possible. The absorbancy at various wavelengths will depend on the proportion of each type of side chain that has undergone reaction. In addition, it is necessary to obtain spectral data with suitable model compounds. For example, mono-(*p*-azobenzenearsonic acid)-*N*-chloroacetyl-tyrosine has been used as a model for predicting the extinction coefficient at various wavelengths of the correspondingly substituted tyrosine side chain in a protein.[8] Spectral data on the azobenzenearsonic, azobenzenesulfonic, and azobenzoic acid derivatives of *N*-acetylhistidine and *N*-chloroacetyltyrosine are also given by Tabachnick and Sobotka.[15] These authors provide evidence for the validity of applying extinction coefficients of such compounds to conjugated proteins. As indicated above, they found that the coupling of limited amounts of arsanilic acid to bovine serum albumin at pH 9 involves almost exclusively tyrosine, histidine, and lysine side chains. After blocking the ε-amino groups of lysine by acetylation, they attempted to use the extinction coefficients of model compounds to estimate the extent of conjugation to tyrosine and histidine; the method was checked by arsenic analysis. In nonacetylated samples the spectral data were not useful for estimation of the extent of reaction with lysine residues, although this was shown by an

[10] A. Nisonoff and D. Pressman, *J. Immunol.* **80**, 417 (1958).
[11] C. F. Crampton, H. H. Reller, and F. Haurowitz, *Proc. Soc. Exptl. Biol. Med.* **80**, 448 (1952).
[12] J. S. Garvey and D. H. Campbell, *J. Exptl. Med.* **105**, 361 (1957).
[13] J. S. Ingraham, *J. Infect. Diseases* **89**, 109 (1951).
[14] W. C. Boyd and S. B. Hooker, *J. Biol. Chem.* **104**, 329 (1934).
[15] M. Tabachnick and H. Sobotka, *J. Biol. Chem.* **234**, 1726 (1959).

TABLE I

EFFICIENCY OF COUPLING OF DIAZONIUM COMPOUNDS TO PROTEINS

Aromatic amine	Protein	Molar ratio (amine/protein) in reaction mixture	pH of reaction	Efficiency of coupling (%)[a]	Analytical method[b]	Reference
p-Arsanilic acid	Ovalbumin	16	7	6	A	*c*
p-Arsanilic acid	Ovalbumin	16	8	20	A	*c*
p-Arsanilic acid	Ovalbumin	16	9	25	A	*c*
p-Arsanilic acid	Ovalbumin	16	10	25	A	*c*
p-Arsanilic acid	Pepsin (denatured)	18	7	20	A	*c*
p-Arsanilic acid	Pepsin (denatured)	18	8	43	A	*c*
p-Arsanilic acid	Pepsin (denatured)	18	9	45	A	*c*
p-Arsanilic acid	Pepsin (denatured)	18	10	56	A	*c*
p-Arsanilic acid	Bovine serum albumin	37	9	18	A	*c*
p-Arsanilic acid	Bovine serum albumin	185	9	16	A	*c*
o-Aminobenzoic acid-7-C^{14}	Ovalbumin	33	~10	36	B	*d*
p-Aminobenzene-sulfonic acid-S^{35}	Bovine γ-glubulin	120	8	31–50	B	*e*
p-Iodoaniline	Bovine serum albumin	10	Not specified	51	C	*f*
p-Iodoaniline	Bovine serum albumin	40	Not specified	52	C	*f*
p-Iodoaniline	Bovine serum albumin	125	Not specified	~45	C	*f*

[a] Percentage of aromatic amine used that was covalently bound to protein.

[b] Analytical methods: A, analysis for arsenic; B, measurement of radioactivity; C, analysis for iodine.

[c] M. Tabachnick and H. Sobotka, *J. Biol. Chem.* **235,** 1051 (1960).

[d] C. F. Crampton, H. H. Reller, and F. Haurowitz, *Proc. Soc. Exptl. Biol. Med.* **80,** 448 (1952).

[e] J. S. Ingraham, *J. Infect. Diseases,* **89,** 109 (1951).

[f] E. W. Gelewitz, W. L. Riedeman, and I. M. Klotz, *Arch. Biochem. Biophys.* **53,** 411 (1954).

indirect method to have occurred to a significant extent. When the coupling reaction was carried out at pH 8, they could account for only 50% of the bound arsenic in terms of substitution on tyrosine and histidine, even when lysine groups had been blocked by acetylation. Coupling to arginine and tryptophan at pH 8 was therefore postulated. Their data illustrate the difficulties inherent in accurate estimation of the total extent of coupling from spectral data, but also indicate that it is sometimes possible to estimate the degree of substitution of particular side chains, if suitable model compounds are available.

2. HAPTEN CONJUGATION OF PROTEINS THIOLATED WITH *S*-ACETYLMERCAPTOSUCCINIC ANHYDRIDE*

a. Introduction

Most reactions for the conjugation of haptens to proteins lack specificity in that the hapten couples to more than one species of amino acid residue. In immunochemical studies it is often desirable to have a conjugated protein preparation in which the hapten is reacted exclusively with a single amino acid. A useful reagent for this purpose is an acetylated mercaptan derivative of an acid anhydride which is reacted with the ϵ-amino groups of lysine side chains. This reagent *S*-acetylmercaptosuccinic anhydride (SAMSA), described by Klotz and Elfbaum,[1] is preferable to the earlier method using the thiolactone reagent,[2] since the mercapto group can readily be regenerated by the use of hydroxylamine. The Ag^+ and thiourea, necessary for the thiolactone reaction, are not required in the SAMSA reaction, thus eliminating problems of stability in the use of disulfide-containing proteins.

b. Method of Preparation

A typical protocol for the preparation of thiolated human serum albumin (HSA) is as follows: One gram of HSA (2% aqueous solution) is stirred in an anaerobic reaction vessel in the presence of a slow stream of prepurified nitrogen. After 1 hour the pH is adjusted to 7.5 with 1.0 *N* NaOH, and 0.155 gm of SAMSA is added. The pH is maintained at 7.5 with 1.0 *N* NaOH by a pH-stat. The reaction mixture is stirred for 1 hour, consuming approximately 1.8 ml of 1.0 *N* NaOH. The mixture

* Section 1,E,2 was contributed by Robert Marks.

[1] I. M. Klotz and S. G. Elfbaum, *Arch. Biochem. Biophys.* **96**, 605 (1964).
[2] R. Benesch and R. E. Benesch, *Biochim. Biophys. Acta* **63**, 166 (1962).

is then adjusted to pH 3.0 with approximately 0.4 ml of 12 *N* HCl. No precipitation should occur.

c. Isolation

Isolation of thiolated HSA is effected by passing the reaction mixture through a Sephadex G-25 column (4 × 34 cm) equilibrated with 0.1 *N* acetic acid. The elution is carried out with 0.1 *N* acetic acid, and the thiolated HSA is collected in 75 ml after the void volume of 60 ml has been discarded.

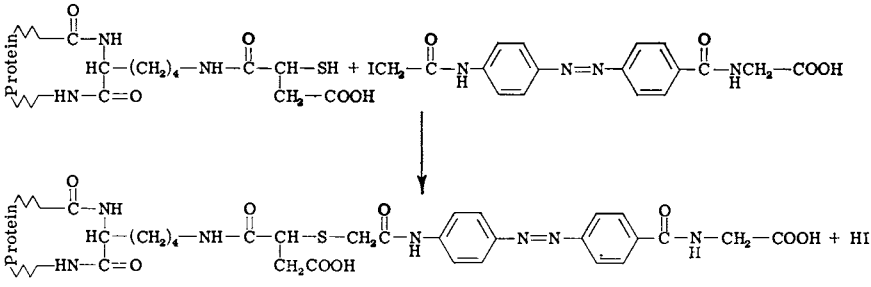
The use of SAMSA as the thiolation agent eliminates the necessity of introducing silver ions to protect the thiol groups. The *S*-acetyl linkage is stable at pH up to 9.5.[3] The regeneration of the sulfhydryl groups for subsequent reaction is readily achieved by exposure to pH 11.5, or by reaction with 0.01 *M* hydroxylamine at pH 7.2 to 7.8.[1]

Twenty sulfhydryl groups per mole of HSA are obtained when a 1:1 mole ratio of SAMSA to amino groups is used.[4]

d. Coupling Reaction with Thiolated Protein

i. Reaction

The thiolated protein can now be coupled with components capable of reacting with sulfhydryl groups. For example, thiolated HSA will react with iodo compounds such as the iodinated hapten, *p*-(*p*-iodoacetylaminobenzeneazo)hippuric acid. The resultant protein derivative is used as an immunizing or precipitating antigen.[5] The reaction is illustrated as follows:



ii. Procedure

The thiolated HSA (1 gm) which was isolated by the Sephadex G-25 column is placed in an anaerobic reaction vessel in the presence of a

[3] I. M. Klotz and R. E. Heiney, *J. Am. Chem. Soc.* **81**, 3802 (1959).
[4] K. Saha, Ph.D. Thesis, University of Pennsylvania, 1963.
[5] K. Saha, F. Karush, and R. Marks, *Immunochem.* **3**, 279 (1966).

stream of nitrogen throughout the course of the reaction. After 1 hour the pH is adjusted to 6.0 with 1.0 *N* NaOH. The iodo compound (0.18 gm) and hydroxylamine hydrochloride (0.75 ml, 1 *M*) are added to the reaction vessel, and the pH is raised to 7.5 with 1.0 *N* NaOH and maintained with a pH-stat. The SH groups are regenerated (deacetylated) at pH 7.5 by the hydroxylamine and become available for coupling with the iodo compound with the elimination of hydrogen iodide. The reaction is continued at pH 7.5 with a pH-stat overnight. The long reaction time is needed because of the low solubility of the iodo compound. The next morning iodoacetate (4 ml, 0.1 *M*, pH 7.5) is added to react with free -SH groups, thus preventing the formation of intra- or intermolecular disulfide bonds. The reaction mixture is centrifuged at 2500 rpm for 30 minutes to sediment unreacted insoluble iodo compound. The clear supernatant is passed through a Sephadex G-25 column (4 × 34 cm) equilibrated with 0.02 *M* phosphate buffer, pH 7.4. The protein derivative is collected in 75 ml after the 60-ml void volume has been discarded. The preparation is then suitable for use as an antigen.

3. PREPARATION OF IMMUNOGENIC 2,4-DINITROPHENYL AND 2,4,6-TRINITROPHENYL PROTEINS*

a. Principle

Proteins with covalently attached DNP or TNP groups are effective immunogens in many mammalian species.† The antibodies formed after immunization with proteins carrying many DNP or TNP substituents per molecule seem to be predominantly specific for the substituent group and the amino acid side chains to which they are attached.

DNP and TNP groups are attached to proteins by nucleophilic substitution. The ortho and para nitro groups of DNP and TNP facilitate nucleophilic attack on carbon-1 (C-1) by electron-donating groups; but the susceptibility of C-1 to such attack is greatly enhanced if an addi-

* Section 1,E,3 was contributed by J. Russell Little and Herman N. Eisen.

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Abbreviations are as follows: DNP, 2,4-dinitrophenyl group; TNP, 2,4,6-trinitrophenyl group; BGG, bovine γ-globulin.

tional electron-withdrawing substituent is attached to this ring carbon atom. Thus, with 2,4-dinitrochlorobenzene or 2,4,6-trinitrochlorobenzene the combined electron-withdrawing properties of the nitro groups and the halogen atom confer a positive-charge character on C-1 and render it highly susceptible to attack by electron-donating groups. In proteins the most effective electron-donating groups are the NH_2, OH, and SH groups of lysine, tyrosine, and cysteine residues, respectively. Accordingly, 2,4-dinitrobenzene and 2,4,6-trinitrobenzene with halogen atoms or sulfonic acid groups on C-1 are effective dinitrophenylating and trinitrophenylating reagents. The order of reactivity of these reagents is directly related to the electrophilic character of the C-1 substituent, since bond-making, not bond-breaking, is the rate-limiting step: the greater the positivity of C-1, the more rapid will be its reaction with an electron donor (a nucleophile). Thus, 2,4-dinitrofluorobenzene is more reactive than 2,4-dinitrochlorobenzene, which is more reactive than 2,4-dinitrobenzenesulfonic acid.

Although less reactive, the sulfonic acid derivatives have some advantages over the C-1 halogen-substituted nitrobenzenes. First, under rather mild and easily controlled conditions the polynitrobenzenesulfonates yield conjugated proteins in which essentially only ϵ-NH_2 groups of lysine residues are substituted. Tyrosine OH groups are not substituted; if α-NH_2 groups of N-terminal residues are free, they are likely to be substituted, but they are few in number relative to the lysine residues in most proteins. The SH groups of cysteine residues may also be substituted, if they are made accessible by reaction conditions, but S-DNP-cysteine residues are unstable at the alkaline pH at which the dinitrophenylation reaction is carried out, and they give rise to dehydroalanyl residues plus free 2,4-dinitrothiophenylate.[1] The analogous reaction probably occurs with 2,4,6-trinitrobenzenesulfonate. A second advantage of the nitrobenzenesulfonates is their water solubility, due to their ionization at pH values above 1.0. They are, therefore, easily introduced in aqueous reactions at varying concentrations, and the unreacted reagent may be readily removed from the derivatized protein product.

Nitrophenyl substituents can also be introduced into proteins by the classical diazonium reaction—for example, with diazotized 2,4-dinitroaniline. The azo substituents, however, are much less stable than the DNP and TNP groups introduced by desulfonation. In fact, the DNP-NH bond in proteins is even more stable than the peptide bond; and this stability accounts in part for the continued popularity of the DNP end-

[1] M. Sokolovsky, T. Sadeh, and A. Patchornik, *J. Am. Chem. Soc.* **86**, 1212 (1964).

group analysis method, following its introduction in the classical studies of Sanger.[2]

b. Crystallization of 2,4-Dinitrobenzenesulfonic Acid

The sodium salt of technical-grade 2,4-dinitrobenzenesulfonate is available commercially (Distillation Products Industries, Rochester, New York) and is readily purified by crystallization.[3] Fifteen grams of the salt is dissolved in about 1 liter of 95% ethyl alcohol with heating to 70°. Powdered charcoal (Norit) is added, and the mixture is stirred vigorously and then filtered through two to three thicknesses of Whatman No. 3 paper on a Büchner funnel. Crystals appear on slow cooling and are harvested for recrystallization two or three times. The air-dried crystals have a melting point above 300°. The molar absorbancy in water at 360 mμ is about 280. After heating in 0.02 *M* NaOH at 90° for 1 hour, the dinitrobenzenesulfonate is quantitatively converted to 2,4-dinitrophenol (E_M = 14,000 at λ_{max}, 360 mμ).

c. Dinitrophenylation Reaction

The following procedure is based on previous descriptions.[3,4] Equal weights of protein and potassium carbonate are dissolved in sufficient water to bring the concentration of each to about 20 mg/ml. An equal weight of crystalline sodium dinitrobenzenesulfonate is then added, and the solution is stirred at room temperature or at 37° until the desired extent of substitution is achieved. During the reaction the vessel should be shielded from light by wrapping it with aluminum foil. About 24 hours of reaction with bovine γ-globulin (BGG) at 37° achieves maximal substitution, close to 70 DNP residues per BGG molecule. Somewhat longer reaction time is required for maximal substitution of human serum albumin.

At the end of the reaction period the DNP protein is separated from unreacted dinitrobenzenesulfonate and from 2,4-dinitrophenol (formed as a by-product by hydrolysis of dinitrobenzenesulfonate) by one of the following procedures, used alone or in combination: (1) Dialysis against water for several days. (2) Passage through Sephadex G-25. (3) Passage through an anion exchange resin column such as Dowex 1 × 8, 200 to 400 mesh, in the Cl^- form. (Alternatively, IRA-400, 50 mesh, may be used.) Both dinitrobenzenesulfonate and 2,4-dinitrophenol are anionic at neutral pH and are bound firmly to either resin. (4) Alternat-

[2] F. Sanger, *Biochem. J.* **40**, 261 (1946).

[3] H. N. Eisen, *in* "Methods in Medical Research" (H. N. Eisen, ed.), Vol. 10, pp. 94–102. Year Book Medical Publishers, Chicago (1964).

[4] H. N. Eisen, S. Belman and M. E. Carsten, *J. Am. Chem. Soc.* **75**, 4583 (1953).

ing cycles of acid precipitation and dissolution. Highly substituted DNP proteins can be quantitatively precipitated below pH 4 and are highly soluble above pH 8.

The dinitrophenylated protein is stored in water, either frozen or at 4°. It is stable indefinitely but should be kept in the dark. Most preparations are readily precipitated at high ionic strength. In 0.15 *M* NaCl some substituted protein will precipitate from highly concentrated solutions. At the concentrations used for precipitating antibodies from antisera (less than 1 mg/ml), the preparations are soluble and stable in buffered saline (0.15 *M* NaCl–0.01 *M* phosphate pH 7.4). Solutions up to about 100 mg/ml DNP-BGG (pH 8, in water) can be prepared for immunization purposes.

d. Characterization of DNP-Proteins

The number of DNP groups per molecule of conjugated protein is calculated from the absorbancy at 360 mμ and from an estimation of total protein concentration by Kjeldahl nitrogen analysis or by determining the dry weight of the sample *in vacuo*. With the usual conditions for Kjeldahl digestion, the nitro groups do not contribute measurable ammonia. Dry weight is determined on samples after extensive dialysis against water or volatile salts, such as ammonium acetate. The samples are heated at 105° in a vacuum oven to constant weight, usually over a period of 1 to 3 days. For purposes of calculation we assume that: (1) the molar absorbancy of DNP-lysyl residues at 360 mμ is the same as that of ε-DNP-lysine, 17,530 at pH 7.4 in dilute salt; and (2) the molecular weight of the conjugate is the same as the unsubstituted protein. In a representative preparation of DNP-BGG a dry weight of 16.8 mg/ml and an absorbancy at 360 mμ of 87.5 corresponded to 50 dinitrophenyl groups per molecule of BGG.[3]

e. Preparation and Characterization of TNP-Proteins

2,4,6-Trinitrobenzensulfonic acid is available commercially (Nutritional Biochemicals Corp., Cleveland, Ohio). It is recrystallized from 1 *N* HCl, and the air-dried, nearly colorless crystals have a melting point of 168° to 169°. Elemental analysis* for $C_6H_3O_9N_3S_1 \cdot 4H_2O$ is as follows: Calculated: C, 19.73; H, 3.04; N, 11.50; S, 8.78; found: C, 20.00, H, 2.96; N, 11.32; S, 9.43. After vigorous dehydration a higher melting point (180°) has been reported.[5]

The reaction of 2,4,6-trinitrobenzenesulfonate with protein at elevated

* Elemental analysis performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

[5] T. Okuyama and K. Satake, *J. Biochem.* (*Tokyo*) **47**, 454 (1960).

pH is more rapid than the comparable reaction with 2,4-dinitrobenzenesulfonate. In 0.2 *M* borate at pH 9.2 the reaction with primary amino groups proceeds promptly at room temperature without undesirable side reactions.[6] After 24 hours of reaction with BGG maximal substitution is achieved (approximately 60 TNP groups per BGG molecule). The lysine residues are converted to the ϵ-TNP derivative and no other amino acid residues are substituted.[6]

The unreacted trinitrobenzenesulfonate and the trinitrophenol by-product are removed by passage through an anion exchange resin column—for example, Dowex 1, Cl^- form, 200 to 400 mesh. The conjugated protein is collected at the void volume and dialyzed against water before characterization. TNP-proteins may be stored frozen in the dark for many months. Thawing often results in significant precipitation, but completely clear solutions are once again obtained after the pH is adjusted to 7.0 to 7.4 with dilute buffer. Most preparations are partly precipitated at high ionic strength if the protein concentration is high (greater than 5 mg/ml).

The TNP-proteins are preferably characterized by relating their absorbancy at 348 mμ (λ_{max}) to a dry-weight measurement. The entire absorbance at 348 mμ is attributed to the TNP–amino acid conjugates, and ϵ-TNP-lysine is the predominant species. If the assumption is made that the molar extinction coefficient is the same for the peptide-bound ϵ-TNP-lysyl residue as for ϵ-TNP-L-lysine at pH 7.4 (15,400 at λ_{max} 348 mμ), then the molar concentration of TNP groups can be related to the molar concentration of protein determined by dry weight.

Satake and co-workers have studied the trinitrophenylation reaction with a variety of conjugated amino acids, peptides, and proteins in detail.[5,7,8] Their data provide evidence for a high degree of specificity in the reaction between trinitrobenzenesulfonic acid and primary amine groups.

f. Concluding Comment

With the techniques outlined above, the following DNP- and TNP-proteins have been found to evoke the production of anti-DNP or anti-TNP antibodies: DNP-BGG, DNP-*Limulus polyphemus* hemocyanin, DNP-human serum albumin, DNP-rabbit serum albumin, and TNP-BGG. ϵ-41-Mono-DNP-ribonuclease (bovine pancreatic ribonuclease with one DNP group per molecule, substituted in the ϵ-NH_2 of the

[6] J. R. Little and H. N. Eisen, *Biochemistry* **5**, 3385 (1966).
[7] K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem.* (*Tokyo*) **47**, 654 (1960).
[8] T. Shinoda and K. Satake, *J. Biochem.* (*Tokyo*) **50**, 293 (1961).

lysine residue in position 41), prepared with 2,4-dinitrofluorobenzene according to Hirs *et al.*,[9] also induces the formation of anti-DNP antibodies in rabbit and guinea pigs.[10] Likewise ε-DNP-insulin, with the DNP group attached to the one lysyl residue of bovine insulin,[11] induces the formation of anti-DNP antibodies.[12]

[9] C. W. H. Hirs, *Brookhaven Symp. Biol.* **15**, 154 (1962).
[10] H. N. Eisen, E. S. Simms, J. R. Little, and L. A. Steiner, *Federation Proc.* **23**, 559 (1964).
[11] C. H. Li, *Nature* **178**, 1402 (1956).
[12] J. R. Little and R. B. Counts, in preparation. See also R. B. Counts and J. R. Little, *Federation Proc.* **25**, 677 (1966).

4. CONJUGATION OF PENICILLIN AND ITS DERIVATIVES*

a. General Considerations

It is believed that organic molecules of the size of penicillin cause sensitization only if they can form a stable bond with endogenous protein. In accord with this concept the antigenic determinants which have been definitely or tentatively identified in penicillin hypersensitivity[1-8] all are penicillin derivatives capable of being bound covalently to functional groups on protein (Fig. 1, IVa, IVb, V, VII, IX, X, XI, XII). Penicillin itself (I), which binds reversibly to serum protein but does not form a covalent linkage, apparently does not cause antibody formation. The predominant reactions leading to protein substitution and antibody formation are acylation of protein amino groups (Fig. 1, pathways 2, 1b, 1c, 3a, 3b) and mixed disulfide formation with protein cysteine and half-cystine residues (Fig. 1, pathways 1a, 5b, and the reaction of IX and SH groups, not shown).

One of the major problems in the preparation of conjugates is the elimination of contaminating groups. Consider, for example, the reaction of penicillenate (III), which readily forms from penicillin in neutral aqueous solution, with a protein. As seen in Fig. 1, these reactions can

*** Section 1,E,4 was contributed by Charles W. Parker.**

[1] M. A. Budd, C. W. Parker, and C. W. Norden, *J. Am. Med. Assoc.* **190**, 203 (1964).
[2] B. B. Levine and Z. Ovary, *J. Exptl. Med.* **114**, 875 (1961).
[3] B. B. Levine and V. Price, *Immunology* **7**, 542 (1964).
[4] B. B. Levine, *Immunology* **7**, 527 (1964).
[5] B. B. Levine, *Federation Proc.* **24**, 45 (1965).
[6] C. W. Parker, J. Shapiro, M. Kern, and H. N. Eisen, *J. Exptl. Med.* **115**, 821 (1962).
[7] C. W. Parker, *Federation Proc.* **24**, 51 (1965).
[8] J. A. Thiel, S. Mitchell, and C. W. Parker, *J. Allergy* **35**, 399 (1964).

result in protein substitution with at least several different groups (IVb, V, XII). By a proper choice of reaction conditions or the use of chemically modified protein, reagents that are largely monospecific can be prepared. While the possibility that a minor substituent is contributing significantly in an immunological assay must be considered, this can

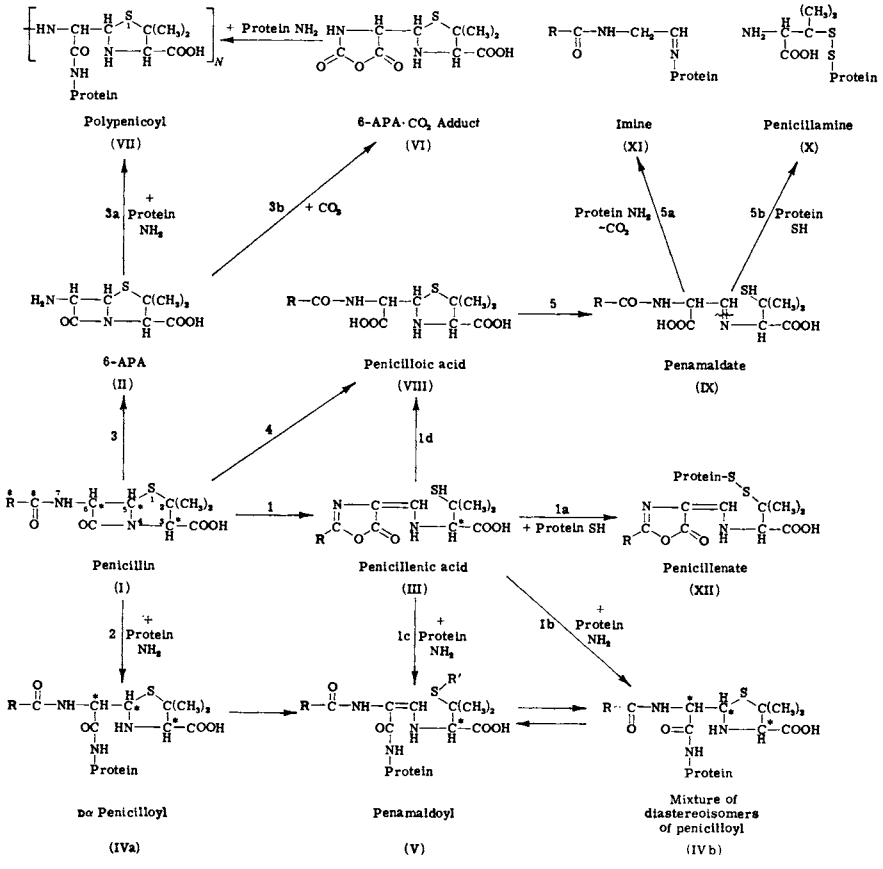

FIG. 1. Proposed pathways for formation of antigen in penicillin allergy (APA, aminopenicillanic acid).

be excluded if the univalent hapten corresponding to the major substituent produces complete inhibition. In other words, a reaction to a penicilloyl–protein can be fully accepted as penicilloyl-specific if, and only if, ϵ-*N*-penicilloyl–aminocaproate, which corresponds closely in structure to a penicilloyl–lysyl group on a protein, inhibits the reaction.[2,6] The demonstration of hapten inhibition should be possible in immediate hy-

persensitivity responses as well as *in vitro* precipitation, hemagglutination, and complement fixation reactions.

b. Penicilloyl Conjugates

We refer, unless otherwise indicated, to benzylpenicillin and its derivatives. The coupling procedures described are generally applicable to penicillins with other R groups (for further discussion, see the literature.[9-11] On the basis of present evidence, the most important antigenic determinant in penicillin allergy is the penicilloyl group. It can form from penicillin by two pathways (Fig. 1, pathway 2, and a combination of pathways 1 and 1b). It seems likely that both mechanisms contribute to formation of antigen *in vivo*. In each instance the reaction takes place with uncharged amino groups. Where the penicilloyl group is formed from penicillenate, racemization takes place. Therefore, with purified penicillenic acid as the coupling agent, a conjugate containing a mixture of penicilloyl diastereoisomers (IVb) is prepared. Where the penicillin reacts directly with protein amino groups (pathway 2), there is retention of the optical configuration of the original penicillin. A conjugate of this type (IVb) is prepared by reacting penicillin with protein or polylysine amino groups at pH above 9.0, where formation of penicillenate does not take place. The products differ not only in the optical configuration of the penicilloyl groups but also in the extent to which contaminating groups are present.[10]

The use of protein conjugates in the study of penicillin allergy in man entails the risk of stimulation of antibody formation. To minimize this possibility, cutaneous testing in man can be done with conjugates of penicilloyl with polylysine.[6] The reaction here is analogous to the formation of protein-bound penicilloyl (pathways 2 and 1b). Unsubstituted polylysines are toxic in human skin,[12] and a high degree of substitution of the polymer with negatively charged penicilloyl groups is desirable. On the basis of studies in the guinea pig, maximal substitution has a second advantage in that polylysines with at least 50% of ϵ-amino groups substituted are nonimmunogenic.[13] As a consequence, a large excess of penicillin or penicillenate is used in the conjugation reaction. This compensates for the fact that many molecules of coupling agent are hydrolyzed to free penicilloic acid and other products. The use of

[9] B. B. Levine, *in* "Methods in Medical Research" (H. N. Eisen, ed.), Vol. X, pp. 184–191. Year Book Publishers, Chicago, 1964.
[10] C. W. Parker and J. A. Thiel, *J. Lab. Clin. Med.* **62**, 482 (1963).
[11] C. W. Parker, *in* "Methods in Medical Research" (H. N. Eisen, ed.), Vol. X, pp. 192–196. Year Book Publishers, Chicago.
[12] C. W. Parker, M. Kern, and H. Eisen, *J. Exptl. Med.* **115**, 789 (1962).
[13] C. W. Parker, J. A. Thiel, and S. Mitchell. *J. Immunol.* **94**, 289 (1965).

a polymer with about 20 lysyl residues per mole gives a product of relatively low molecular weight which is highly effective as an elicitor of immediate skin reactions. The procedures outlined below are applicable to both penicilloyl–poly-L- and penicilloyl–poly-D-lysines. (For further discussion of the L- and D-polylysines, see the literature.[13,14])

i. Penicilloyl-Polylysines Prepared from Penicillenic Acid

(*a*) *Preparation of Penicillenic Acid.** The procedure described is based on the method of Carpenter *et al.*[15] as modified by Levine.[9] Mercuric chloride (26 gm) is dissolved in 1500 ml of water by heating. After the solution has cooled to room temperature, 30 gm of penicillin dissolved in 500 ml of water is added. The mixture is allowed to stand at room temperature for 3 to 5 hours. The insoluble mercury mercaptide of penicillenic acid is collected by filtration and washed three times with 200 ml of cold water and four times with 75 ml of cold ether. All subsequent operations are carried out as rapidly as possible with precooled glassware and solutions. The object is to keep the temperature of the two-phase system as low as possible (approximately 6° to 8°) without freezing the benzene phase. The precipitate is suspended in 300 ml of water and 1400 ml of benzene in a 4-liter beaker immersed in ice. Hydrogen sulfide is bubbled in for 15 minutes while the two-phase system is stirred vigorously (overhead stirrer). The precipitated mercuric sulfide is removed by filtration through a fresh bed of Celite, wet with water. The aqueous portion of the filtrate is removed in a separatory funnel, and the organic layer is washed three times with 100 ml of cold water. The benzene solution is dried over anhydrous sodium sulfate (20 minutes), distributed among three 2-liter round-bottomed flasks, and lyophilized. Penicillenic acid is obtained as an amorphous white powder which is 90 to 95% pure based on a molar extinction of 26,600 at 322 mμ in 95% ethanol for the pure compound. The yield averages about 25%. Known contaminants are penicillin (about 1%) and penicillenic acid disulfide. The material is stored under vacuum at —20° where it is stable for at least 6 months. Flasks should be allowed to equilibrate at ambient temperature before they are opened. Because of the relative instability of penicillenic acid in solution, only fresh solutions should be used in conjugation.

(*b*) *Conjugation.* One hundred and fifty milligrams of polylysine·HCl (an average of 20 lysyl residues per molecule) is dissolved in 60 ml

[14] B. B. Levine, *Nature* **202**, 1008 (1964).

* Penicillenic acid and penicilloyl-polylysines are now available commercially (Sigma Chemical Company, St. Louis, Missouri).

[15] F. H. Carpenter, R. H. Turner, and V. Du Vigneaud, *J. Biol. Chem.* **176**, 893 (1948).

of 0.1 *M* phosphate–0.002 *M* Versene (EDTA), pH 8.0. A freshly prepared solution of 7 gm of benzylpenicillenic acid in 20 ml of 95% ethanol is added dropwise with magnetic stirring over a 20- to 30-minute period.[10,11,16] During the addition, pH is maintained between 7.2 and 8.2 (pH meter) by dropwise addition of 0.5 *N* NaOH. After the final addition, incubation is continued for 1 hour at room temperature.

Purification and analysis are carried out as described in subsequent sections with the following modifications. Because a portion of the product is precipitated from the reaction mixture at relatively high salt and ethanol concentrations, the entire reaction mixture is transferred as a suspension to the dialysis bag. After 5 to 6 hours of dialysis against dilute buffer, any remaining insoluble material is discarded. To minimize losses of the relatively low-molecular-weight polymer during dialysis, dialysis is carried out in $1\frac{8}{32}$-inch Visking dialysis casing which has a relatively low permeability[16] (see Chap 8, Vol. II).

The product contains about 10 penicilloyl (IVb), 3 penamaldate (V and IX), and 2 penicillenate groups (XII) for each 20 residue polylysine molecules. The penicillenate presumably exists in mixed disulfide linkage with penamaldate (Fig. 1, derivative V, where R′ is penicillenate) and can be readily removed by incubation of the conjugate in 0.2 *M* 2-mercaptoethanol at pH 8.0 (0.1 *M* $NaHCO_3$) for several hours.[16]

ii. Penicilloyl–Polylysines Prepared from Penicillin at High pH

The reaction is initiated at pH 9.6 because the solubility of the polylysine is greatly decreased in more alkaline solution. Once substitution of a portion of the amino groups by negatively charged penicilloyl groups has taken place, the pH may be raised to 11 or more without precipitation.[10,11]

One hundred milligrams of polylysine · HCl (an average of 20 lysyl residues per molecule) is dissolved in a minimal volume of water (about 0.4 ml), and the solution is just neutralized with 5 *M* NaOH. One milliliter of 1 *M* carbonate, pH 10.4, is added, followed by 1.2 gm of penicillin with mixing. The pH is adjusted to 9.6 (pH meter) with 5 *N* NaOH, and, if necessary, additional water is added to dissolve the penicillin completely. Additional 600-mg amounts of penicillin are added at 16 and 24 hours. After each addition the pH is raised to 11.0. Six or more hours after the final addition the solution is purified by dialysis and characterized (see preceding and subsequent sections).

The product contains about 12 to 14 penicilloyl groups (IVa) per 20 residue polylysine molecules. The penicilloyl groups can be shown

[16] C. W. Parker, A. de Weck, M. Kern, and H. N. Eisen, *J. Exptl. Med.* **115**, 803 (1962).

to be in the D-α-configuration by optical rotation measurements. The penamaldate and penicillenate contents are less than 0.1 and 0.02 group per polylysine molecule, respectively.[10]

iii. Penicilloyl–Protein Conjugates

The reaction of penicillin and penicillenic acid with proteins leads primarily to the formation of penicilloyl–lysyl substituents.[2,9,16] If penicillenic acid or penicillin is coupled at or below neutrality, penamaldate and penicillenate groups also can be found coupled. Coupling conditions are substantially the same as with polylysine. On an equal-weight basis the reactive amino groups are much fewer in proteins than in polylysines, and the quantity of acylating agent can be proportionately reduced. In reactions employing penicillenate it is desirable to restrict the total volume of 95% ethanol added to no more than 15 to 20% of the total reaction volume to avoid precipitation of protein.

For details of coupling conditions, see the polylysine section. The following reaction of penicillenate with bovine γ-globulin (BGG) will serve as an example of the efficiency of a protein conjugation reaction using this reagent. One millimole of penicillenic acid in 4 ml of 95% ethanol was reacted with 160 mg of BGG (6×10^{-2} mmole, ϵ-NH_2), dissolved in 25 ml of 0.1 *M* phosphate, pH 7.5. The product was found to contain 50 penicilloyl (IVb), 8 penamaldate (V and IX), and 7 penicillenate groups (XII) per molecule of protein. The penicillenate groups could be readily cleaved by reaction with a mercaptan (see polylysine section). The efficiency of the coupling reaction of penicillin with protein varies widely, depending on pH and protein concentration. At pH 11.0, with a 30- to 50-fold molar excess of penicillin with respect to free amino groups, nearly quantitative substitution of protein lysyl residues can be achieved.

Penicilloyl groups are susceptible to destruction by a variety of oxidizing agents. In the preparation of I^{125}- or I^{131}-labeled penicilloyl–proteins, it is desirable to label the protein with radioactive iodine before penicilloyl groups are introduced.[8]

c. Polypenicoyl Conjugates

The reaction of 6-aminopenicillanic acid (6-APA, II) with polylysine and protein can be carried out in a similar manner to the reaction of penicillin with polylysine at high pH (Fig. 1, pathway 3a).[3]

d. The Preparation of Penicillenate–Protein Conjugates

To prepare protein conjugates largely specific for penicillenate, it is necessary to block protein amino groups, preventing reaction 1b. This

can be done by using *N*-acetyl homocysteine thiolactone[17] or other reagents, such as acetic anhydride, which acylate protein.[4]

In the acylation reaction the thiolactone rearranges to homocysteine, and the protein is greatly enriched with SH groups. Mixed disulfide formation between protein and penicillenate (Fig. 1, pathway 1a) is carried out in urea in neutral or weakly alkaline solution in the presence of an oxidizing agent. If hydrogen peroxide is used for oxidation, any penicilloyl groups which may have formed owing to failure to obtain quantitative substitution of amino groups will be oxidized to various forms of penamaldate.[16] Thus, products essentially free of penicilloyl can be obtained. The *N*-acetyl-DL-homocysteinyl residues are capable of contributing an additional antigenic specificity to the conjugate.[16]

i. The Preparation of Thiolated Protein

To a 5% solution of protein in 0.15 *M* NaCl–0.01 *M* phosphate, pH 7.4, is added solid *N*-acetyl-DL-homocysteine–thiolactone in 12-fold molar excess with respect to lysyl residues of protein. When the thiolactone has dissolved, the pH is raised by addition of 2 volumes of 1.0 *M* sodium borate, pH 11.3. After 2 hours at room temperature, the solution is chilled, rapidly acidified to pH 4 to 5 with ice-cold 6 *N* HCl (~0.1 volume), and ⅑ volume of ice-cold 50% trichloroacetic acid (TCA) is added. The precipitate is washed three times with ice-cold 5% TCA by centrifugation and dissolved by the careful addition of 0.1 *M* K_2HPO_4. The solution is adjusted to pH 6.5 to 6.8 with 1 *M* NaOH. Protein recoveries are regularly 60 to 80% based on absorbance at 280 mμ and on biuret analysis.[16] The degree of thiolation as estimated by titration with bis(mercurimethyl)dioxane[18] is generally at or close to the theoretical maximum—in essence, the number of lysyl residues of the protein. While the thiolated protein is relatively stable at 0° in a deoxygenated solution of pH 6.5, it is desirable that conjugation be carried out within 1 to 2 hours.

ii. Protein Conjugation

A suspension is prepared which contains the following: 23 gm of urea; 10 ml of 1 *M* phosphate–0.02 *M* Versene, pH 7.2; 40 ml of saturated sodium borate–0.01 *M* Versene (EDTA), pH 9.1; and 1 ml of 30% hydrogen peroxide. The thiolated protein solution (100 mg of thiolated human serum albumin, HSA, in 10 ml of 0.1 *M* K_2HPO_4, pH 6.6) and penicillenic acid in ethanol (5 mmole in 16 ml of 95% EtOH)

[17] A. DeWeck and H. N. Eisen, *J. Exptl. Med.* **112**, 1227 (1960).
[18] S. J. Singer, J. E. Fothergill, and J. R. Shainoff, *J. Am. Chem. Soc.* **82**, 565 (1960).

are added simultaneously in a dropwise fashion over a 20-minute period with stirring. At 5 and at 10 minutes an additional 0.5 ml of 30% hydrogen peroxide and 5 ml of H_2O are added. Incubation is continued for an additional hour before purification.

The product of the above reaction should contain about 50 penicillenate and 5 to 10 penamaldate groups per molecule of HSA.

e. Penicillamine–Protein Conjugates

Penicillamine may be coupled to thiolated proteins (Fig. 1, X), as described above for penicillenate–protein conjugates. In a simpler procedure the protein is reduced by penicillamine in a solution containing urea or a detergent.[4] An oxidizing agent is then added. The sulfhydryl groups of the reduced protein combine with the penicillamine to form a mixed disulfide bond. The degree of substitution of the protein with penicillamine obtained with the second procedure is usually in the range of 12 to 18 groups per molecule of protein, which is substantially less than with proteins thiolated with *N*-acetylhomocysteine thiolactone.

Human serum albumin (100 mg) and D-penicillamine (150 mg) are dissolved in 10 ml of 0.1 *M* K_2HPO_4–7 *M* urea. Incubation is carried out at 37° for 2 hours; 1.2 ml of a 1 *M* solution of potassium ferricyanide in water (freshly prepared) is then added rapidly with good mixing. After 30 minutes at room temperature, the reaction mixture is purified by dialysis as described below.

f. Purification, Recovery, and Stability

Purification of conjugates is carried out primarily by dialysis against 0.01 *M* phosphate, pH 8.0, at 4°. The time of dialysis may be shortened by preliminary purification on Sephadex G-25. Nearly all the noncoupled low-molecular-weight products have at least one negative charge, and the use of an anion exchange resin in the outside solution for the first several days of dialysis will aid in their removal. Dialysis for 6 to 7 days against 50 to 100 volumes of buffer which is changed four to five times a day usually will give a conjugate free of uncoupled products. Reanalysis of the conjugate after an additional 24 to 48 hours of dialysis will help to verify the absence of uncoupled materials. The final dialysis should be for at least 24 hours against the same dilute solution of buffer to assure equilibration for subsequent analyses.

Recovery of penicilloyl–, penicoyl–, and penicillamine–proteins is high, in the range of 90 to 100%. Recovery of penicillenate–proteins may be as low as 70 to 80% because of the formation of some insoluble protein. When a polymer having an average degree of polymerization

of about 20 residues per chain is used, penicilloyl–polylysine gives an average recovery of about 50%.

Penicilloyl, penicillenate, and penicillamine conjugates may be stored as lyophilized powders or in neutral aqueous solution in the frozen state or at 4°. A slow fall in number of substituent groups (of the order of 5%) may be observed over a 4- to 6-month period under the latter condition.

g. Analysis

After the final 24 hours of dialysis, aliquots of the inside and outside solutions are transferred to tared planchets, taken to dryness at 90°, and cooled in a desiccator. Once constant weights are obtained, the concentration of conjugate is determined by difference. Results obtained with this procedure correlate well with those derived from Kjeldahl nitrogen analysis. By relating the concentration of various substituents to a given weight of conjugate, the degree of substitution and the molecular weight of the product can be calculated.

i. Penicilloyl and Penicoyl Analysis

A stock 1.5×10^{-2} *M* solution of *p*-hydroxymercuribenzoate (POHMB) is prepared by dissolving the organic mercurial in a minimal volume of 0.1 *M* NaOH, diluting to volume with 0.1 *M* carbonate, pH 9.2, and centrifuging. The exact concentration is determined by the absorbance at 232 mμ, pH 7.0 ($\epsilon = 1.69 \times 10^4$). In a dark, tightly stoppered bottle this solution is stable for several months at room temperature.[9]

On the day of titration a 2×10^{-3} *M* solution of POHMB is prepared by dilution into 0.05 *M* carbonate, pH 9.2; 0.1 ml of this solution is added directly to a 2.0-ml cuvette containing exactly 1.0 ml of a solution of penicilloyl–protein or penicilloyl–polylysine in 0.05 *M* carbonate, pH 9.2, at an approximate penicilloyl concentration of 2 to 4×10^{-5} *M*. The solution is rapidly mixed with a capillary, and a reading is made on a spectrophotometer at 285 mμ after 5 to 10 minutes. The difference in the 285-mμ readings before and after the mercurial is due to the complexing of POHMB with penicilloyl to form a penamaldate (Fig. 1, V, where R′ is POHMB) ($\epsilon = 2.38 \times 10^4$).[9] Corrections must be made for dilution (the final protein concentration is 91% of the original) and for absorbance by uncombined POHMB at 285 mμ (absorbancy 0.038 at a final concentration of 1.82×10^{-4} *M*).

This assay also is applicable to substituents of the penicoyl or polypenicoyl type (Fig. 1, VII).[1]

ii. Penamaldate and Penicillenate Analyses

The penamaldate and penicillenate concentrations are determined by the absorbances at 282 mμ and 322 mμ, respectively, in the absence of mercurial ($\epsilon \simeq 23{,}500$ at 282 mμ for penamaldate; $\epsilon \simeq 26{,}600$ at 322 mμ for penicillenate). For protein conjugates a correction must be made for absorbance contributions by the protein at the above wavelengths. No correction is necessary for polylysine conjugates. The absorbances of penamaldate at 322 mμ and of penicillenate at 282 mμ are about 10% of their respective values at 282 mμ and 322 mμ.

iii. Penicillamine Analysis

When penicillamine–proteins are oxidized by aqueous bromine, penicillamine–cysteine mixed disulfide bonds are cleaved. Penicillamine is liberated in the form of dimethylcysteic acid (penicillamine sulfonic acid) which can be isolated and quantitated.[4] A freshly prepared, saturated solution of bromine in water is added in small increments to the concentrated protein solution at 0° until the bromine color persists. Aliquots of the oxidized solution are applied to Whatman No. 1 chromatography paper. Additional spots containing known amounts of authentic dimethylcysteic acid also are applied. The chromatograph is developed with phenol–water, and spots corresponding to dimethylcysteic acid are detected with ninhydrin ($R_f = 0.20$). Quantitation of dimethylcysteine in the oxidized protein sample is on the basis of the intensity of its ninhydrin color, as compared with the standard. Where available, the amino acid analyzer affords a convenient and accurate means of quantitation.

h. Red Blood Cell Conjugates

The incubation of human and animal erythrocytes with penicillin sensitizes the cells so that they are agglutinated by appropriate antipenicillin antisera.[19] Thiel, Mitchell, and Parker demonstrated that sensitization is markedly favored at alkaline pH.[8] The influence of alkalinity on sensitization is consistent with the direct reaction of penicillin (I) with uncharged amino groups on the surface of the erythrocyte, forming erythrocyte-bound D-α-penicilloyl (Fig. 1, pathway 2). In the evaluation of hemagglutination reactions produced by antisera from human beings with penicillin hypersensitivity, hemagglutination inhibition studies with univalent haptens indicate that antipenicilloyl antibodies (Fig. 1,IVa and IVb) are primarily responsible for hemagglutination. Occasionally, however, entirely different hemagglutination in-

[19] A. B. Ley, J. P. Harris, M. Brinley, B. Liles, J. A. Jack, and A. Cahan, *Science* **127**, 1118 (1958).

hibition patterns are obtained indicating that antibodies of other specificities also can be involved (Fig. 1,V,VII,XI,XII).[8] Since red cell–bound penicilloyl would not be expected to react with all the different kinds of antibody that participate in hemagglutination it seems likely that penicillin derivatives other than penicilloyl couple to the erythrocyte membrane during sensitization. The routine followed in this laboratory for sensitization of erythrocytes with penicillin is described below.[8] It involves the incubation of erythrocytes with penicillin in barbital buffer at pH 8.5–9.0. In a recent modification of this method it was reported that incubation of erythrocytes with penicillin in a trimethylamine buffer at pH 10.0 provides a somewhat more sensitive cell preparation (a one to four tube increase in titer in a serial dilution assay).[20] However, no evidence was presented to indicate that non-penicilloyl antibodies can be detected with cells prepared by the latter method. Until such evidence is obtained the cells prepared by the barbital technique appear to be preferable for routine hemagglutination studies.

i. Preparation of Human Cells with Penicillin

Freshly drawn blood (group O, Rh negative donor) is mixed with an equal volume of Alsever's solution and the cells are washed three or four times with 0.145 *M* NaCl–0.01 *M* phosphate, pH 7.4 (phosphate-saline). One volume of a 16% suspension of cells in phosphate-saline is then mixed with 2.5 volumes of barbital buffer 0.14 *M*, pH 8.6. To 3.5 volumes of this mixture is added 0.5 volume of a 400 mg per milliliter solution of benzylpenicillin in phosphate-saline. For the control red cells, 0.5 volume of phosphate-saline is substituted for the solution of penicillin in phosphate-saline. The penicillin solution is freshly prepared, and the addition is made with constant mixing. Cells are incubated with and without penicillin for 1 hour at 37° and for 16–40 hours at 4°. They are then washed with phosphate-saline, the amount of washing varying with the extent of hemolysis. At least three or four washes are made after the first wash that rendered the supernatant solution grossly free of hemoglobin. Cells are then stored at 4° in phosphate-saline and can be used for as long as 1 week thereafter. Cells are always washed at least several times more on the day of use.

ii. Preparation of Rabbit Cells with Penicillin

Rabbit cells are similarly prepared, with the exception that 0.5 volume of a 20 mg per milliliter solution of benzylpenicillin is used rather than the equivalent volume of a 50 ml per milliliter solution as described above. Use of the lower concentration of benzylpenicillin is necessary to avoid extensive hemolysis.

[20] B. B. Levine, M. J. Fellner, and V. Levytska, *J. Immunol.* **96**, 707 (1966).

5. THE PREPARATION OF STEROID–PROTEIN CONJUGATES TO ELICIT ANTIHORMONAL ANTIBODIES*

a. General Procedure

Antihormonal agents capable of counteracting the physiological effects of endogenous hormones could serve as important biochemical and clinical tools in many phases of endocrinology. It was with the purpose of obtaining antihormonal antibodies that the synthesis of steroid–protein conjugates was undertaken. Prior to the experiments described below, two attempts to prepare such antigenic conjugates were reported: an unsuccessful one by Mooser and Grilichess,[1] and one by Sprunt *et al.*[2] described in insufficient detail to permit evaluation of the results. More recently, Sehon and his collaborators reported the successful conjugation of an estrone derivative to proteins and the stimulation of specific antibodies.[3]

The conjugates described below may be prepared by linking appropriate steroid derivatives via an amide bond to the ϵ-amino groups of bovine serum albumin (BSA). Other protein carriers can serve as well. Figure 1 shows six steroid derivatives that have been linked to BSA. All except testosterone 17-chlorocarbonate (I) have carboxyl groups which were caused to react with the amino groups of the protein by means of a mixed anhydride intermediate according to the method of Vaughan.[4] In the case of testosterone 17-chlorocarbonate,[5] the Schotten-Baumann reaction was used.

b. Preparation of the Steroid Haptens

i. Testosterone 3-(O-Carboxymethyl)oxime (II)

A solution of 0.98 gm (3.4 mmoles) of testosterone and 1.07 gm (8.4 mmoles) of (*O*-carboxymethyl)hydroxylamine[6] in 200 ml of ethanol was made alkaline by the addition of 20 ml of 5% NaOH and heated at

* Section 1,E,5 was contributed by Bernard F. Erlanger, Sam M. Beiser, Felix Borek, Frances Edel, and Seymour Lieberman.

[1] H. Mooser and R. K. Grilichess, *Schweiz. Z. Allgem. Pathol. Bakteriol.* 4, 375 (1941).
[2] D. H. Sprunt, A. D. Dulaney, and R. Conger, *Cancer Res.* 11, 282 (1951).
[3] L. Goodfriend and A. H. Sehon, *Can. J. Biochem.* 39, 961 (1961).
[4] J. R. Vaughan, Jr., *J. Am. Chem. Soc.* 73, 3547 (1951).
[5] K. Miescher, H. Kagi, C. Scholz, A. Wettstein, and E. Tschopp, *Biochem. Z.* 294, 39 (1937).
[6] E. Borek and H. T. Clarke, *J. Am. Chem. Soc.* 58, 2020 (1936).

a reflux for 1.5 hours. The solution was reduced to a small volume, diluted with water, and extracted with ether. The alkaline aqueous phase was acidified with concentrated hydrochloric acid. The resulting precipitate was extracted into ether, and the ether extract was washed with water, dried over sodium sulfate, and evaporated to dryness. The crude product weighed 0.84 gm (68%), melting point 167° to 185°. Three recrystallizations from benzene–ligroin yielded 0.27 gm, melting point 179° to 181°, $[\alpha]_D^{25} + 143.8° \pm 2°$ (19.5 mg in 1.5 ml of ethanol; $+140° \pm 2°$ (26.2 mg in 5.0 ml of 0.05 *M* Tris buffer, pH 8.5). The

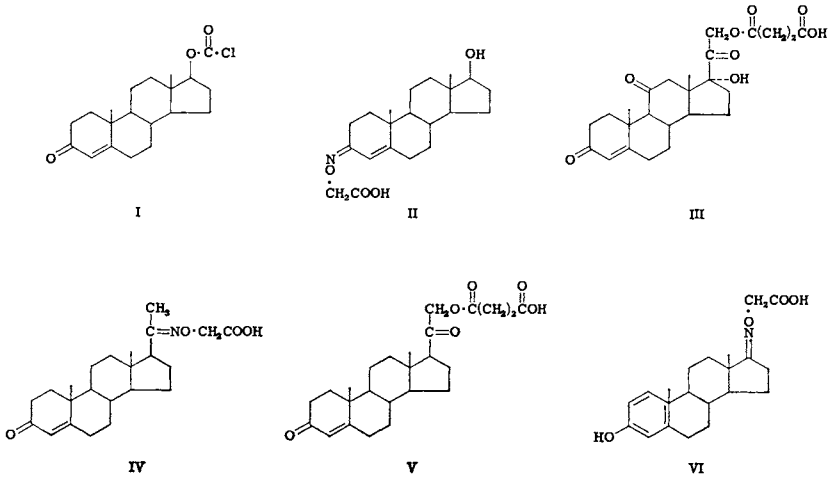

FIG. 1. Steroid derivatives linked to BSA: I, testosterone 17-chlorocarbonate; II, testosterone 3-(*O*-carboxymethyl)oxime; III, cortisone 21-hemisuccinate; IV, progesterone 20-(*O*-carboxymethyl)oxime; V, deoxycorticosterone 21-hemisuccinate; VI, estrone 17-(*O*-carboxymethyl)oxime. Reproduced with permission, from S. Lieberman, B. F. Erlanger, S. M. Beiser, and F. J. Agate, Jr., *Recent Progr. Hormone Res.* **15**, 169 (1959).

ultraviolet spectrum of the solution in 0.05 *M* Tris buffer (pH 8.5) showed a maximum at 251 to 252 mμ (ϵ = 15,360). The infrared spectrum (Nujol) showed a band at 1730 cm^{-1} (conjugated C = O of carboxyl) and no absorption in the region of 1670 to 1640 cm^{-1} (conjugated C = O). Calculated ($C_{21}H_{31}O_4N$): neutral equivalent, 361; C, 69.81; H, 8.59; N, 3.87 found: netural equivalent, 368, C, 69.72; H, 8.68; N, 3.92.

ii. Pregnenolone 20-(O-Carboxymethyl)oxime

A solution of 10.45 gm (0.033 mole) of pregnenolone and 10.45 gm (0.082 mole) of (*O*-carboxymethyl)hydroxylamine hydrochloride[6] in 517

ml of ethanol containing 46.5 ml of 2 *N* KOH was refluxed for 3 hours. The reaction mixture was reduced to a small volume in a vacuum, water was added (about 150 ml), and the pH was adjusted to 10 to 10.5 with 2 *N* KOH. After being extracted with ethyl acetate twice, the aqueous phase was acidified with concentrated hydrochloric acid to pH 2 and placed in the refrigerator for 24 hours. The precipitate was collected by filtration and washed with water; yield 13 gm (92%), melting point 196° to 197° with decomposition. It was recrystallized from ethyl acetate; yield 12 gm (85%), melting point 204° to 205° with decomposition, $[\alpha]_D^{25}$ 0° ± 3° (2% in methanol). The infrared spectrum (Nujol) showed a band at 1730 cm^{-1} (C = O of carboxyl) and no absorption at 1685 cm^{-1} (20-keto C = O). Calculated ($C_{23}H_{35}O_4N$): neutral equivalent, 389; C, 70.92; H, 9.05; N, 3.60; found: neutral equivalent, 398; C, 70.96; H, 9.04; N, 3.80.

iii. Pregnenolone 20-(O-Carboxymethyl)oxime Methyl Ester

A solution of 0.68 gm (1.8 mmoles) of pregnenolone 20-(O-carboxymethyl) oxime in 50 ml of ethyl acetate was treated with a solution of diazomethane in 10 ml of ether prepared from 1.2 gm (10.0 mmoles) of *N*-nitroso-*N*-methylurea. Gas evolution was apparent. The reaction mixture was allowed to stand overnight at room temperature. Excess diazomethane was decomposed by adding a small amount of glacial acetic acid. The reaction mixture was evaporated to dryness under reduced pressure, yielding 0.60 gm (85%) of crystalline product, melting point 191° to 192°. Recrystallization from methanol yielded material of unchanged melting point, $[\alpha]_D^{25}$ 0° ± 2° (17.0 mg in 2.0 ml of ethanol). The infrared spectrum showed a band at 1750 cm^{-1} (C = O of an ester). Calculated ($C_{24}H_{37}O_4N$): C, 71.43; H, 9.24; found: C, 71.14; H, 9.18.

iv. Progesterone 20-(O-carboxymethyl)oxime (by Oppenauer Oxidation) (IV)

A solution of 2.57 gm (6.4 mmoles) of pregnenolone 20-(*O*-carbomethyl)oxime methyl ester in 95 ml of dry acetone and 25 ml of dry benzene was heated to boiling and treated with 4.73 gm (19.2 mmoles) of purified aluminum isopropoxide dissolved in 100 ml of dry benzene. The reaction mixture became cloudy and, after heating under reflux for 8 hours, was transferred to a separatory funnel with an additional 25 ml of benzene and washed with 40 ml of 50% Rochelle salt solution. The two-phase mixture was freed of the suspended solid by filtration through Celite.

The benzene layer was separated, washed three times with water, dried

over sodium sulfate, and evaporated to dryness. The semisolid residue weighing 1.62 gm was dissolved in 10 ml of benzene and purified by passing through a Florisil column. Recrystallization of the purified material from acetone–Skellysolve B yielded 1.43 gm (56%), melting point 122° to 136°. Repeated recrystallization gave a crystalline product, melting point, 138° to 140°, $[\alpha]_D^{22}$ 123° ± 2° (20.0 mg in 2.0 ml of ethanol). The infrared spectrum (Nujol) showed a band at 1670 cm^{-1} (conjugated C = O).

A solution of 2.18 gm (5.2 mmoles) of progesterone 20-(*O*-carboxymethyl)oxime methyl ester in 78.5 ml of methanol was treated with 8.7 ml of *N* NaOH (final normality of base, 0.1 *N*) and allowed to stand at room temperature. Aliquots of 0.4 ml were drawn at 1-hour intervals, diluted with water, and examined for ester content with a quantitative hydroxamic acid–ferric perchlorate method.[7] After 3 hours, about 97% of the ester was saponified. The reaction mixture was evaporated to a smaller volume, diluted with water, and extracted with two 50-ml portions of ether. The ether extract was found to contain about 0.04 gm of progesterone. The aqueous layer was brought to pH 2 with 8.7 ml of *N* HCl. The resulting precipitate, after standing in the cold overnight, was collected by filtration, washed with water, and dried for 2 hours in a vacuum at 90° to 95°. The crude product weighed 1.91 gm (90%). Two recrystallizations from aqueous methanol yielded 1.35 gm, melting point 165° to 167° decomposed), $[\alpha]_D^{25}$ + 122° ± 2° (12.9 mg in 2.0 ml of ethanol). The ultraviolet spectrum of the solution in 0.05 *M* Tris buffer (pH 8.5) showed a maximum at 249 mμ (ϵ = 15,890). The infrared spectrum (Nujol) showed bands at 1725 cm^{-1} (C = O of carboxyl) and at 1635 cm^{-1} (conjugated C = O). Calculated ($C_{23}H_{35}O_4N$): neutral equivalent, 387; C, 71.27; H, 8.58; N, 3.61; found: neutral equivalent, 393; C, 71.23; H, 8.65; N, 3.68.

v. Progesterone 20-(O-carboxymethyl)oxime Methyl Ester (by Chromic Acid Oxidation)

Pregnenolone 20-(*O*-carboxymethyl)oxime methyl ester (2.33 gm, 5.55 mmoles), was dissolved in 350 ml of acetone (previously distilled over chromic acid), and the solution was cooled to 10°. While a current of nitrogen was passed through this solution, Jones' chromic acid oxidant[8] was added dropwise by buret, the temperature of the reaction mixture being kept at about 15°. Addition of the oxidant was terminated on the appearance of an olive-green color accompanied by precipitation.

[7] R. R. Goddu, N. F. Leblanc, and C. M. Wright, *Anal. Chem.* **27**, 1251 (1955).
[8] C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.* **21**, 1547 (1956).

A solution of 20 ml of methanol in 2 liters of water was added to complete the precipitation of the product. It was then collected by filtration, washed with water, and air-dried; yield 2.28 gm, melting point 113° to 125°. To complete isomerization the product was dissolved in 150 ml of methanol, to which was then added 5 drops of a 10% solution of KOH (yellow color). After standing overnight in the refrigerator, the solution was acidified to pH 2 with hydrochloric acid, and then enough water was added to precipitate the product. The latter was extracted into ethyl acetate; the ethyl acetate solution was washed with water, dried over sodium sulfate, and evaporated to dryness in a vacuum. Recrystallization of the residue from acetone–petroleum ether yielded 1.9 gm of ketone melting at 138° to 139°.

vi. Deoxycorticosterone 21-Hemisuccinate (V)

The method is the same as that used to prepare cortisone 21-hemisuccinate (III).

A solution of 1.0 gm (3.0 mmoles) of deoxycorticosterone and 1.0 gm (10.0 mmoles) of succinic anhydride in 10 ml of dry pyridine was refluxed for 4.5 hours. The reaction mixture was evaporated to dryness under reduced pressure, and the semisolid residue was dissolved in chloroform, washed three times with water, dried over sodium sulfate, and evaporated to dryness. The residue was recrystallized twice from acetone, yielding 1.12 gm (86%) of product, melting point 200° to 202°. Two further recrystallizations, one from benzene–hexane and one from acetone, gave pure compound, melting point 204° to 206°, $[\alpha]_D^{22}$ + 158° ± 2° (13.0 mg in 2 ml of ethanol). The ultraviolet spectrum of the solution in 0.05 *M* Tris buffer (pH 8.5) showed a maximum at 249 mμ (ϵ = 16,610). Calculated ($C_{25}H_{34}O_6$): neutral equivalent, 430; C, 69.82; H, 7.96; found: neutral equivalent, 430; C, 69.65; H, 7.74.

vii. Estrone 17-(O-carboxymethyl)oxime (VI)

This compound was prepared by the method of Huffman *et al.*[9] and also by the following method, which was found to be more satisfactory for the preparation of moderate quantities of material. A solution of 5 gm (18.5 mmoles) of estrone, 5 gm (39.2 mmoles) of (*O*-carboxymethyl)hydroxylamine,[6] 22.5 ml of 2 *N* KOH, and 250 ml of ethanol was refluxed for 3 hours. The alcohol was removed in a vacuum, 500 ml of water was added, the pH was adjusted to 8.5, and the aqueous solution was then extracted twice with volumes of 100 ml of ethyl ace-

[9] M. N. Huffman, D. W. MacCorquodale, S. A. Thayer, E. A. Doisy, G. V. Smith, and O. W. Smith, *J. Biol. Chem.* **134**, 591 (1940).

tate. Acidification of the aqueous layer yielded a precipitate which was collected by filtration; yield 6.5 gm (97%). Recrystallization from ethyl alcohol yielded 6.4 gm (95%), melting point 188° to 190° (decomposed) (reported melting point 188°[9]).

c. Preparation of the Conjugates by the Mixed Anhydride Technique

This procedure can be used to prepare conjugates prepared from all steroid derivatives having free carboxyl groups.

i. Preparation of Estrone 17-Albumin Conjugate

Estrone 17-(*O*-carboxymethyl)oxime (2.06 gm, 6.0 mmoles) and 2.86 ml (2.28 gm, 12.0 mmoles) of tri-*n*-butylamine were dissolved in 90 ml of dioxane. The solution was cooled and treated with 0.63 ml (0.70 gm, 6.0 mmoles) of isobutylchlorocarbonate. Precipitation sometimes occurs after addition of tri-*n*-butylamine. This precipitate redissolves on addition of isobutylchlorocarbonate. The reaction was allowed to proceed in the cold for 30 minutes, and then the mixture was added in one portion to a stirred, cooled solution of 7.0 gm (0.10 mmole) of BSA in 183.5 ml of water, 123.5 ml of dioxane, and 7.0 ml of *N* NaOH. Stirring and cooling were continued for 4 hours, the pH remaining at 8 throughout the reaction. The solution was dialyzed against running water for 17 hours and brought to pH 4.6 with *N* HCl. The resulting precipitate was allowed to stand in the cold overnight and then was collected by centrifugation. The product was suspended in 200 ml of water, dissolved by bringing the pH to 7.0 with a small amount of *N* NaOH, and reprecipitated by the addition of 300 ml of cold acetone followed by adjustment of the pH to 4.5 with *N* HCl. The precipitate was collected by centrifugation, and the acetone treatment was repeated twice. The third acetone extract was found to contain no detectable amount of estrone. The conjugate was taken up in 300 ml of water, and the pH was adjusted with *N* NaOH to 7.8. A small amount of solid which remained undissolved was removed by centrifugation and discarded. The supernatant liquid was dialyzed against running water for 8 hours and lyophilized, yielding 6.8 gm of conjugate, $[\alpha]_D^{25}$ $-40.8° \pm 2°$ (24.5 mg in 5.0 ml of 0.05 *M* Tris buffer, pH 11.3). Assumed molecular weight 76,500; 20 of 60 NH_2 groups substituted. Calculated: NH_2—N, 0.72; total N, 14.95; ratio of NH_2—N to total N, 0.048. Found: NH_2—N, 0.69; total N, 13.80; ratio of NH_2—N to total N, 0.050; moisture, 10.2.

Generally 20 to 30 steroid haptens could be linked to the protein

as determined spectrophotometrically, by amino nitrogen assay, and by using fluordinitrobenzene. Details of the characterization of the conjugate as well as their immunological and biological properties have been published.[10-14]

[10] B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.* **228**, 713 (1958).
[11] B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.* **234**, 1090 (1959).
[12] S. Lieberman, B. F. Erlanger, S. M. Beiser, and F. J. Agate, Jr., *Recent Progr. Hormone Res.* **15**, 165 (1959).
[13] S. M. Beiser, B. F. Erlanger, F. J. Agate, Jr., and S. Lieberman, *Science* **129**, 564 (1959).
[14] R. O. Neri, S. Tolksdorf, S. M. Beiser, B. F. Erlanger, F. J. Agate, Jr., and S. Lieberman, *Endocrinology* **74**, 593 (1964).

6. PROTEIN-PROTEIN CONJUGATION*,†

a. General Considerations

Protein–protein conjugates are by definition covalently bonded multimolecular protein complexes. Antigens may be coupled to erythrocytes or stromata for use in agglutination tests or for immunization of animals. Smaller proteins—for example, cytochrome c[1] and insulin[2]—or peptides—for example, bradikynin,[3] angiotensin,[4,5] and ACTH[3]—can be coupled to larger carrier proteins, thus increasing the probability of enhancing the immunogenicity of these "poor" antigens. Ferritin–antibody or ferritin–antigen conjugates may be used as specific electron-dense reagents for the detection and localization of antigens or antibodies in tissue by electron microscopy (see Chap. 28, Vol. IV).

Reactions of proteins in the presence of conjugating or crosslinking agents can give rise to different products, depending on several factors,

* Section 1,E,6 was contributed by Vinay Likhite and Alec Sehon.

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[1] M. Reichlin, S. Fogel, A. Nisonoff, and E. Margoliash, *J. Biol. Chem.* **241**, 251 (1966.)
[2] P. Mark and A. H. Sehon, "Abstracts of 48th Annual Canadian Institute of Chemistry Meeting, Montreal" (1965); P. Mark, Ph.D. thesis, Department of Chemistry, McGill University, to be submitted.
[3] T. L. Goodfriend, L. Levine, and G. D. Fasman, *Science* **144**, 1344 (1964).
[4] T. L. Goodfriend, G. Fasman, D. Kemp, and L. Levine, *Immunochemistry* **3**, 223 (1966).
[5] D. S. Kemp and R. B. Woodward, *Tetrahedron* **21**, 3019 (1965).

such as the particular conjugating agent, the reaction mechanism, the particular proteins being conjugated, and the relative concentrations of the conjugating agent and of the proteins. Thus, one may obtain (1) intramolecularly crosslinked products, which are due to coupling of the different groups within the same protein molecule; the formation of this type of compound will be favored at low concentration of the protein; (2) intermolecular conjugates, which are due to coupling covalently (*a*)

TABLE I
REAGENTS FOR PROTEIN–PROTEIN CONJUGATION

Conjugating reagents	Reactive groups on protein molecules
Diisocyanates	Amino,[a,b,c] sulfhydryl[d]
Carbodiimides	Carboxyl (with amino)[e,f,g]
Dihalogenated dinitrobenzenes	Amino,[h] phenolic,[h] histidyl[h]
Mercurials	Sulfhydryl[i]
Tris(1-(2-methyl)aziridinyl)phosphine oxide (MAPO)	Sulfhydryl[j]
Ethylene maleic anhydride copolymer (EMA)	Amino[k]
Diazonium compounds	Tyrosyl,[l,m] histidyl,[l,m] amino,[l,m] carboxyl,[l,m] phenolic[l,n]
N-Ethyl benzisoxazolium fluoborate (EBIZ)	Amino,[o] carboxyl[o]

[a] J. Givas and A. H. Sehon, unpublished observations, 1966.

[b] P. Mark and A. H. Sehon, "Abstracts of 48th Annual Meeting, Canadian Institute of Chemistry, Montreal" (1965).

[c] L. Gyenes and A. H. Sehon, *Immunochemistry* **1,** 43 (1965).

[d] F. W. Putnam, in "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. IB, p. 893, Academic Press, New York, 1953.

[e] T. L. Goodfriend, L. Levine, and G. D. Fasman, *Science* **144,** 1344 (1964).

[f] H. G. Khorana, *Chem. Rev.* **53,** 145 (1953).

[g] H. M. Johnson, K. Brenner, and H. E. Hall, *Federation Proc. Abstr.* **25,** 248 (1966); H. M. Johnson, K. Brenner, and H. E. Hall, *J. Immunol.* **97,** 791 (1966).

[h] H. Zahn, *Proc. Intern. Wool Textile Res. Conf.* **1955C,** 425 (1955); H. Zahn and A. Wung, *Biochem. Z.* **325,** 182 (1954).

[i] S. J. Singer, J. E. Fothergill, and J. R. Shainoff, *J. Am. Chem. Soc.* **82,** 565 (1960).

[j] K. Onoue, Y. Yagi, and D. Pressman, *Immunochemistry* **2,** 181 (1965).

[k] E. R. Centeno and A. H. Sehon, *Federation Proc. Abstr.* **25,** 729 (3044) (1966).

[l] J. Gordon, B. Rose, and A. H. Sehon, *J. Exptl. Med.* **108,** 37 (1958).

[m] L. O. Frick, L. Gyenes, and A. H. Sehon, *J. Allergy* **31,** 216 (1960); P. Gold, J. Jonson, and S. O. Freedman, *ibid.* **37,** 311 (1966).

[n] A. B. Stavitsky and E. A. Arquilla, *Intern. Arch. Allergy Appl. Immunol.* **13,** 1 (1958).

[o] T. L. Goodfriend, G. Fasman, D. Kemp, and L. Levine, *Immunochemistry* **3,** 223 (1966); D. S. Kemp and R. B. Woodward, *Tetrahedron* **21,** 3019 (1965).

two or more identical protein moelcules resulting in the formation of homopolymers, or (*b*) two or more different protein molecules leading to the formation of heteropolymers; and (3) a mixture of these types of conjugates.

Protein–protein conjugation can be achieved with the aid of bifunctional (or polyfunctional) reagents, which either become incorporated into the conjugate, or activate certain groupings of the reacting protein molecules for the subsequent formation of the corresponding crosslinkages, such as peptide, disulfide, or thioether bonds. The reactive functional groups of the protein molecules and some of the more common reagents used for conjugation are illustrated in Table I; the reactions involved are discussed in the following pages.

b. Diisocyanates

i. Reactions

Diisocyanates react with the free $-NH_2$ groups of proteins and can thus be used for the preparation of protein–protein conjugates. Among

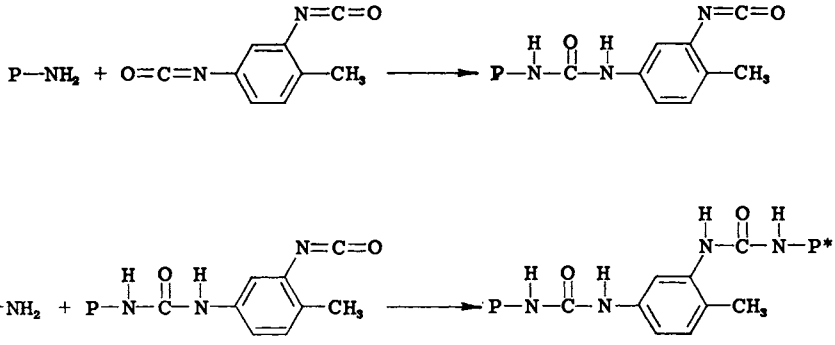


Fig. 1. Reactions involved in conjugation with TDIC.

the more commonly used diisocyanates are m-xylylene diisocyanate and tolylene-2,4-diisocyanate (TDIC); of these two reagents, TDIC is preferable because the isocyanate group in position 4 is more reactive than the one in position 2. Hence, stepwise reactions with each of the two conjugating proteins can be controlled; that is, it is possible to conjugate the diisocyanate first with one type of protein molecule, and the resulting product, in the absence of any free diisocyanate, may be coupled to the second protein. The conjugation procedure is illustrated in Fig. 1. Thus, TDIC is first added at 0° to the protein of

the first solution, represented here as P–NH_2. At this temperature the sterically less hindered isocyanate group in position 4 reacts readily with the free amino group. The TDIC–protein conjugate is then added to the second protein (P*–NH_2) solution at 37°, when the second isocyanate group in position 2 reacts with the amino groups present on the second protein molecule. It is obvious from this outline that to minimize destruction or blocking of the functional groups of the protein moiety responsible for the immunological activity—that is, the antibody sites or antigenic determinants—it is advantageous to react the antibody or antigen preparation in the second step.

The ferritin–antibody conjugate was prepared in this manner without significant loss of the antibody activity[6] by using the xylylene diisocyanate, as described in Chap. 4,C. Similarly, TDIC was used to conjugate insulin[2] and myoglobin[7] to bovine serum albumin, and a variety of other antigens to erythrocytes for use in passive hemagglutination reactions.[8]

ii. Conjugation of Ferritin to Antibody Molecules with TDIC[6]

Step 1. Ferritin (75 mg) is dissolved in 5 ml of phosphate buffer at pH 7.5, $\Gamma/2 = 0.1$ (0.947 gm of $NaH_2PO_4 \cdot 2H_2O$ and 4.41 gm of anhydrous Na_2HPO_4 in 1 liter of water). The solution is cooled to 0°, and 0.1 ml of TDIC is then added to it. The reaction mixture is stirred vigorously for 25 minutes at 0° and centrifuged to sediment the unreacted diisocyanate.† The supernatant is carefully removed by a syringe to avoid contamination with the diisocyanate in the film at the meniscus. It is then allowed to stand for an additional hour at 0° to permit any dissolved TDIC to react.

Step 2. The ferritin solution from step 1 is added to 5 ml of antibody solution containing 75 mg of globulin in borate buffer at pH 9.5, $\Gamma/2 = 0.1$ (9.27 gm of H_3BO_3 and 4.0 gm of NaOH in 1 liter of water), at 37°. The resulting mixture should be at pH 9.3. It is stirred for 1 hour at 37°, dialyzed against 0.1 *M* ammonium carbonate to destroy any unreacted isocyanate groups on the conjugate, and finally against a neutral buffer (precipitation of ferritin should not occur at any stage).

The conjugated globulins can be removed from the dialyzed preparation by centrifugation at 35,000 rpm in the preparative ultracentrifuge for 2 hours. The supernatant contains the unconjugated γ-globulins, and the pellet contains both the conjugated and the unconjugated ferritin.

[6] S. J. Singer and A. F. Schick, *J. Biophys. Biochem. Cytol.* **9**, 519 (1961).
[7] P. Mark, Ph.D. Thesis, McGill University (1967).
[8] L. Gyenes and A. H. Sehon, *Immunochemistry* **1**, 43 (1965).
† TDIC is insoluble in aqueous solutions and precipitates as a white solid at 0°.

iii. Conjugation of Insulin to Bovine Serum Albumin with TDIC[7]

Step 1. To 5 ml of a 1% solution of insulin in the form of the zinc salt, in borate buffer at pH 9.5, $\Gamma/2 = 0.1$, is added 0.10 ml of TDIC at 0°. The reaction mixture is stirred vigorously at 0° for 30 minutes and then centrifuged in a preparative ultracentrifuge. The supernatant solution is decanted into a tightly stoppered tube, and the reaction is allowed to proceed for an additional hour.

Step 2. The solution from step 1 is added to an equal volume of a 1% solution of bovine serum albumin (BSA) in borate buffer at pH 9.5, $\Gamma/2 = 0.1$, at 37°, and the reaction is allowed to proceed for 1 hour. The reaction mixture is dialyzed against 0.1 *M* ammonium carbonate, pH 8.8, to destroy any unreacted isocyanate groups on the insulin–BSA conjugate. It is then exhaustively dialyzed against the borate buffer.

Step 3. Purification. The insulin–BSA conjugate may be separated from the unreacted insulin and BSA by powder block electrophoresis (see Chap. 6,D,1, Vol. II); the conjugate is found to move with a mobility between that of the modified insulin and the BSA. The insulin–BSA conjugate is then eluted from the appropriate section of the block, exhaustively dialyzed against distilled water, and lyophilized.

*iv. Conjugation of Protein to Erythrocytes with TDIC**,[8]

A solution containing 0.021 gm of TDIC in 15 ml of *p*-dioxane is prepared. A volume of 0.1 ml of a 50% suspension of washed rabbit red blood cells (rbc) in saline is dispersed in 3ml of an antigen solution, such as BSA, at 20 mg/ml. To this solution is added 0.2 ml of the TDIC solution, and the reaction is allowed to proceed for 20 to 25 minutes at room temperature. The rbc–BSA conjugate is removed from the reaction mixture by centrifugation.

* As a general rule for passive hemagglutination procedures, the optimal proportion of the reagents used for the sensitization of red cells must be established. For this purpose, the ratio of the crosslinking agent to antigen, for a constant amount of red cells, is critical. This ratio should be established for each batch of the crosslinking agent (1) by varying the concentration of the crosslinking agent and keeping the antigen concentration constant, and (2) by varying the antigen concentration and keeping the concentration of the crosslinking agent constant. Each batch of sensitized cells is tested with an immune serum and with normal serum. The concentrations of crosslinking agent and antigen adopted are the ones that give the highest sensitivity—that is, the highest titer with the immune serum and no reaction with the normal serum.

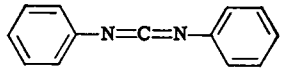
In a similar manner, 3 ml of a solution containing 0.4 mg of water-soluble ragweed pollen (WSR), 0.1 ml of rbc suspension, and 0.1 ml of the TDIC solution have been reacted to obtain the rbc–WSR conjugate.

c. Carbodiimides

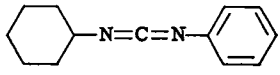
i. Reactions

Carbodiimides* can react with carboxyl groups of proteins[3,9,10] to form intermediates that can (1) undergo rearrangement to form the corresponding acyl urea, or (2) react with a second carboxyl group to form an acid anhydride and a disubstituted urea. The acid anhydride can then react with an amino group of another protein molecule to form the protein–protein conjugate by the creation of a peptide bond. The reaction can be illustrated by the flow chart given in Fig. 2.

* The general formula of carbodiimdes is R—N=C=N—R′, where R and R′ are alkyl or aryl groups. There are symmetrical carbodiimides (R = R′) and unsymmetrical carbodiimides (R ≠ R′). An example of a symmetrical aliphatic carbodiimide is diethylcarbodiimide, C_2H_5—N=C=N—C_2H_5; a symmetrical aromatic carbodiimide, diphenylcarbodiimide,



an unsymmetrical carbodiimide, ethylpropylcarbodiimide, C_2H_5—N=C=N—C_3H_7, and cyclohexylphenyl-carbodiimide,



Carbodiimides employed for the reactions described in this section tend to be less simple than those outlined in the general scheme, and not all authors use the same nomenclature for them. As an example, one may cite CH_3CH_2N=C=N—$CH_2CH_2CH(NHCH_3)_2$, which is variously designated as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, the preferred nomenclature being abbreviated as ECDI, or 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide.

[9] H. G. Khorana, *Chem. Rev.* **53**, 145 (1953).

[10] H. M. Johnson, K. Brenner, and H. E. Hall, *Federation Proc. Abstr.* **25**, 248 (1966).

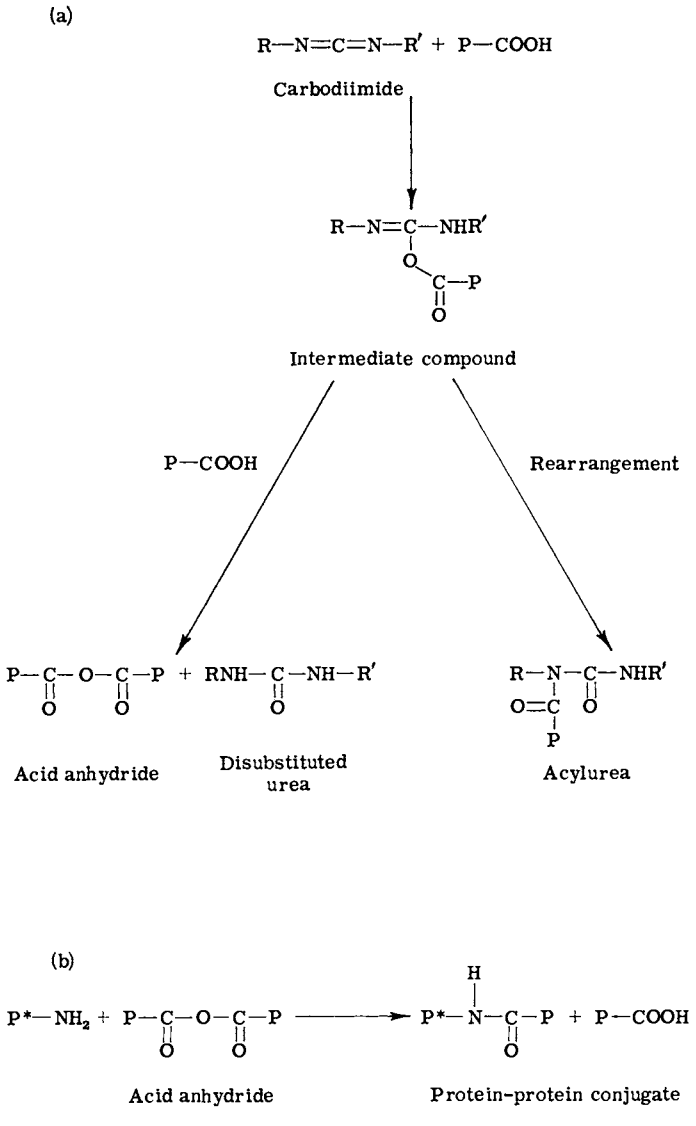


FIG. 2. Reactions involved in conjugation with carbodiimides.

The carbodiimides found to be particularly useful for conjugation of proteins are 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (ECDI) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide methyl-*p*-toluene sulfonate (morpho-CDI). ECDI has been used to conjugate bradykinin[3] and angiotensin[4,5] to rabbit serum albumin (RSA) and cytochrome c to acetylated bovine γ-globulin.[1] The resulting conjugates were shown to produce antibodies to bradykinin, angiotensin, and cytochrome c in rabbits. Recently ECDI was used to couple proteins to red blood cells for use in the passive hemagglutination test.[10,11]

ii. Bradykinin–RSA Conjugation with ECDI[3]

To a solution containing 10 mg of RSA and 20 mg of bradykinin in 0.5 ml of water, 100 mg of freshly dissolved ECDI in 0.25 ml of water is added. The reaction is allowed to proceed for 30 minutes at room temperature. The reaction mixture is then dialyzed against distilled water for 24 hours and lyophilized. Occasionally a colloidal suspension is formed in the dialysis bag. This is removed by centrifugation, and the clear supernatant is lyophilized.

iii. Angiotensin–RSA Conjugation with ECDI[4,5]

A reaction mixture containing 10 mg of [*l*-asparagine, 5-valine]-angiotensin, 5 mg of RSA, and 150 mg of ECDI* in 0.2 ml of water is allowed to stand at room temperature for 1 to 12 hours. The appearance of a cloudy or colloidal precipitate is taken as the end point of the reaction. (With some of the conjugations the precipitation does not occur, and overnight incubation was arbitrarily chosen as the length of the reaction.) On appearance of a precipitate, or at the end of incubation, the reaction mixture is dialyzed against water for 48 hours.

iv. Cytochrome c–BGG Conjugation with ECDI[1]

Five milligrams of cytochrome c is conjugated to 10 mg of acetylated BGG† by reacting the mixture with 200 mg of ECDI. The reaction mixture is then dialyzed and lyophilized.

[11] H. M. Johnson, K. Brenner, and H. E. Hall, *J. Immunol.* **97**, 791 (1966).

* Angiotensin-RSA conjugates are also prepared by using morpho-CDI.

† Acetylation of BGG results in the blocking of at least 85% of the $-NH_2$ groups of this carrier protein, which might otherwise react with the "activated" carboxyl groups of the BGG molecules, leading to intramolecular peptide bonds and to loss of efficiency of crosslinking with the cytochrome.

v. Protein–Erythrocyte Conjugation with ECDI[10]

To a mixture containing 0.1 ml of 50% rbc suspension in phosphate-buffered saline (PBS), pH 7.2, and 10 mg of BSA in 3 ml of PBS, is added 100 mg of ECDI in 0.5 ml of PBS. After standing for 60 minutes at room temperature, the BSA–rbc conjugate is washed with a solution of 2% EDTA in normal rabbit serum (NRS) diluted 100-fold with 1% NRS in PBS. It is then suspended to a 2% concentration in 1% NRS

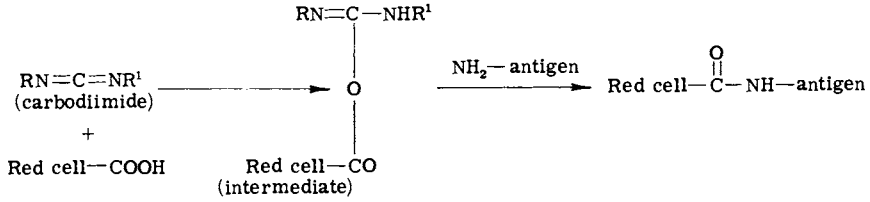


FIG. 3. Mechanism of coupling of protein antigens to red cells by carbodiimides.

and used in the passive hemagglutination test. The mechanism involved in this reaction is illustrated in Fig. 3.

d. DIHALOGENATED DINITROBENZENES

i. Reactions

Halogenated 2,4-dinitrobenzenes can bring about the crosslinking of proteins.[12] The conjugation of proteins by 1,5-difluoro-2,4-dinitrobenzene (DFDNB)[13] and by p,p′-difluoro-*m*,*m*′-dinitrodiphenyl sulfone (FNPS)[14] has been extensively studied. The reaction schemes proposed for the crosslinking of proteins by DFDNB involve the (1) amino, (2) histidyl, and (3) tyrosyl groups as depicted in Fig. 4. FNPS has been used for the formation of a homopolymer of rabbit serum albumin[14] and for the coupling of ferritin to antibody.[15] DFDNB has been used for the conjugation of protein antigens to red blood cells.[16]

ii. Human γ-Globulin (HGG)–Erythrocyte Conjugation with DFDNB[16]

Human group O Rh-negative red cells are washed four times in saline at 2400 rpm, and a 10% saline suspension is prepared. DFDNB reagent

[12] P. Alexander, M. Fox, K. A. Stacey, and L. F. Smith, *Biochem. J.* **52**, 177 (1952).
[13] H. Zahn, *Proc. Intern. Wool Textile Res. Conf.* **1955C**, 425 (1955); H. Zahn, and A. Wung. *Biochem. Z.* **325**, 182 (1954).
[14] F. Wold, *J. Biol. Chem.* **236**, 106 (1961).
[15] S. S. Tawde, and Ram. J. Sri, *Arch. Biochem. Biophys.* **97**, 429 (1962).
[16] N. R. Ling, *Immunology* **4**, 49 (1961).

(L. Light and Co.) (0.015 ml of 2% solution in acetone) is introduced into a 75 × 13-mm centrifuge tube containing 1 ml of EDTA buffer,* pH 8.4, and 0.5 ml of the cell suspension is added. The tube is placed in a 37° bath for 30 minutes, then lightly centrifuged, and the supernatant is discarded.

The cells are resuspended in 1 ml of buffer, pH 8.4, and 0.25 ml

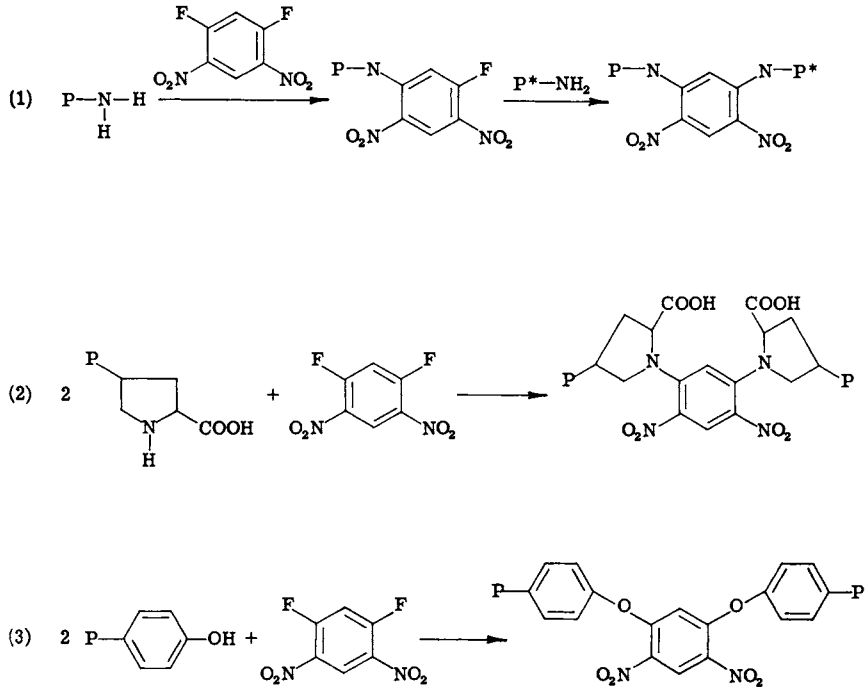


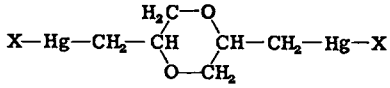
FIG. 4. Mechanism of the DFDNB reaction (in sodium bicarbonate or slightly alkaline conditions). Obviously, in view of the many different groups with which DFDNB can react, one would expect to obtain mixtures of conjugates in which different functional groups had been crosslinked.

(5 mg) of heated γ-globulin is added. The contents of the tube are mixed and incubated at 37° for 60 minutes. The tube is again lightly centrifuged, and the supernatant is discarded. The cells are washed three times with EDTA buffer, pH 7.5, and finally resuspended in 20 ml of the same buffer.

* Ethylenediaminetetraacetate (EDTA) buffers: (1) pH 8.4 and (2) pH 7.5; ethylenediamine tetraacetic acid disodium salt dihydrate (17 gm) is dissolved in water, the pH brought to 8.4 or 7.5, respectively, by the addition of 2 *N* sodium hydroxide, and the volume made up to 1 liter with distilled water.

e. MERCURIALS

Compounds of mercury (mercurials) can be used as protein–protein conjugating agents by virtue of their reaction with the –SH groups of proteins. More useful bifunctional organic mercurials of the type



where $X = Cl^-$, CH_3COO^-, or NO_3^-, ions for the coupling of proteins have also been developed.[17]

These reagents have been used in a general method for the isolation of antibodies[18] (see Chap. 3,B,3). For this purpose the protein antigen is reacted with N-acetyl homocysteine thiolactone (AHT), which results in the introduction of a number of –SH groups into the protein antigen molecule without affecting its ability to react with the antibody. The thiolated protein antigen (T-Ag) is then reacted with the antibody.* The resulting precipitate is freed from nonspecific proteins by centrifugation and washing and then dissolved in a glycine–sulfuric acid buffer at pH 2.4, which leads to the dissociation of antigen–antibody complexes. The appropriate amount of the bifunctional organic mercurial is added for the intramolecular conjugation of the T-Ag through the formation of Ag–S–Hg–R–Hg–S–Ag bonds. The crosslinked antigen molecules are insoluble and are removed from the solution by centrifugation, leaving the free antibodies in solution.

f. TRIS[(1-(2-METHYL)AZIRIDINYL)PHOSPHINE OXIDE] (MAPO)

i. Reactions

Recently, MAPO (Interchemical Corporation, New York) was used to crosslink proteins to form insoluble polymers which were employed in the preparation of immunosorbents.[19] The insoluble protein framework was synthesized by the series of reactions illustrated in Fig. 5.

In Step I, *S*-acetylmercaptosuccinic anhydride (SAMSA) is reacted with the amino groups of rabbit albumin or γ-globulin fractions at pH 8. (SAMSA can also be reacted with free hydroxyl groups of proteins

[17] J. T. Edsall, R. H. Maybury R. S. Simpson, and R. Straessle, *J. Am. Chem. Soc.* **76**, 3131 (1954).

[18] S. J. Singer, J. E. Fothergill, and J. R. Shainoff, *J. Am. Chem. Soc.* **82**, 565 (1960).

* For introduction of —SH groups one may use also *S*-acetylmercaptosuccinic anhydride (SAMSA) as described in the next section.

[19] K. Onoue, Y. Yagi, and D. Pressman, *Immunochemistry* **2**, 181 (1965).

and other macromolecules such as polysaccharides and nucleic acids.[20]) The resulting modified proteins are insoluble at pH 4.0 to 4.5 and can be solubilized at neutral or higher pH. Step II consists in the removal of the acetyl group of the mercaptosuccinyl residue by reaction with sodium hydroxide at pH 11.5 under nitrogen. In Step III the resulting

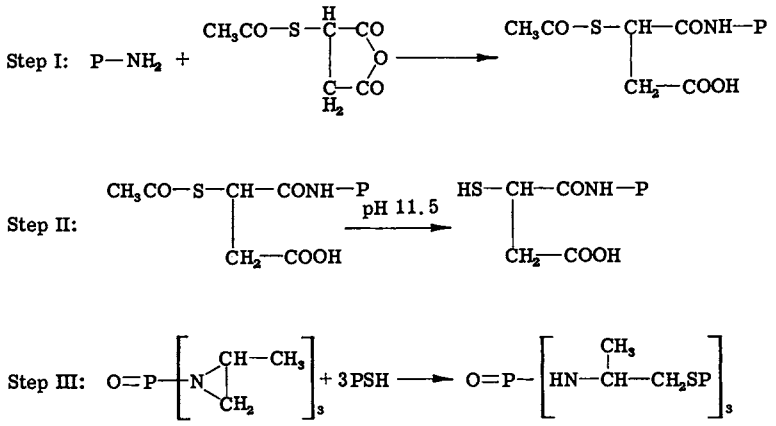


FIG. 5. Reactions for crosslinking proteins with MAPO.

modified proteins, rich in sulfhydryl groups, are crosslinked and converted to an insoluble polymer by reaction with MAPO.

ii. SAMSA–RSA Conjugation[19]

Three grams of rabbit serum albumin (RSA) is dissolved in 50 ml of borate buffer, pH 8.0. The pH of the solution is carefully adjusted to 8.0 with 1.0 *N* NaOH; it is then diluted to 75 ml with the buffer, and 885 mg of SAMSA is added in small amounts, over a period of 1 hour, with constant stirring under nitrogen. The resulting mixture is dialyzed for 24 hours at 0° against 0.15 *M* saline, buffered with 0.02 *M* borate buffer at pH 8.0. The acetyl groups of the mercaptosuccinyl residues are removed by keeping the modified RSA at a pH of 11.5 (with 1.0 *N* NaOH) for 1 to 5 hours under nitrogen.

iii. Reaction of Modified RSA with MAPO

To a solution of modified RSA, 0.92 ml of MAPO is added with stirring, and the pH is adjusted to 4.0 with 1.0 *N* HCl. The reaction mixture is stirred for 3 hours at room temperature and overnight in the cold. As the conjugation progresses, the homopolymer of the modified RSA

[20] I. M. Klotz and R. E. Heiney, *Arch. Biochem. Biophys.* **96**, 605 (1962).

becomes insoluble over the wide range of pH of 2 to 9. The modified protein is converted to the insoluble polymer after 3 hours at room temperature. The suspension is diluted three to four times with borate saline, pH 8.0, and the pH is adjusted to 8.5 with 1 *N* NaOH. The polymer of RSA is sedimented by centrifugation at 2500 rpm for 10 minutes. It is washed with borate–saline at pH 8.0 until $O.D._{280}^{1cm}$ of the washing is less than 0.05, and is then ready to be used as an immunosorbent.

g. Etyhlene Maleic Anhydride Copolymer (*EMA*)

i. Reactions

A simple and rapid procedure, originally developed for coupling enzymes to insoluble polymers,[20a] was recently adapted as a general method for the preparation of high-capacity immunosorbents with protein antigens.[21] Some of the general features and results of this method are given below: Protein antigens, such as BSA, human serum fractions, and aqueous soluble ragweed pollen constituents (WSR), were coupled to commercial samples of ethylene maleic anhydride (EMA) by the reaction schemes shown in Fig. 6; in this figure, for the sake of simplicity, EMA is shown as a regularly alternating array of ethylene and maleic anhydride residues.

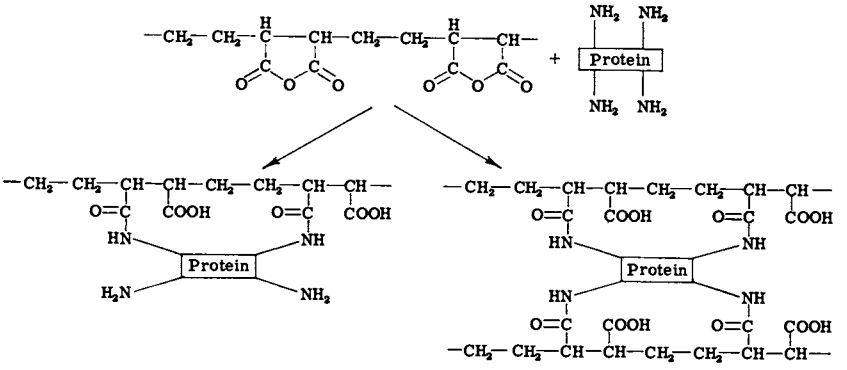
ii. Protein–Protein Conjugation with EMA[21]

Scheme I. The copolymer is dissolved in anhydrous dioxane or acetone to a concentration of about 0.6%. This solution is added dropwise to the solution containing the protein antigen at 0°, at the optimum pH, which has to be established for each antigen. For example, for the coupling of bovine serum albumin (BSA) or rabbit globulins, the optimum pH is found to be within the range of 5.8 to 6.1.

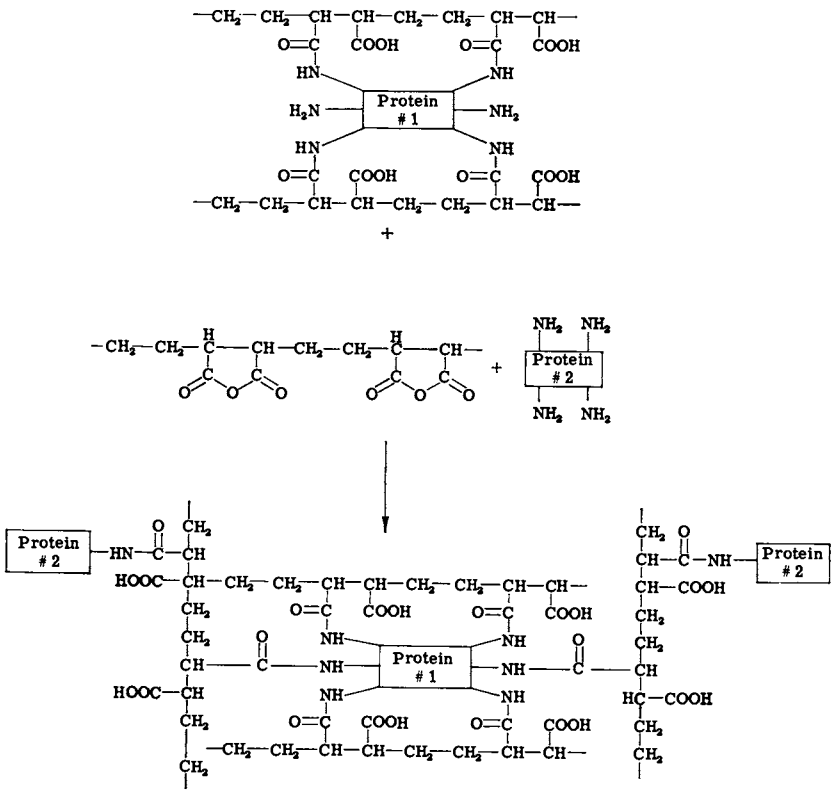
In general, the antigen solution consists of about 200 mg of the antigen in 50 ml of saline. The resulting EMA–protein conjugate separates out in the form of flakes, which are isolated by centrifugation. The supernatant is discarded, and the precipitate is washed three times with saline and then resuspended in buffered saline at pH 7.5 to 8.0. This suspension is stirred overnight in the cold room in order to remove any occluded protein from the immunosorbent. The immunosorbent is then separated by centrifugation and washed extensively with saline, then with glycine–HCl buffer (pH 3.0), and again with saline so as to eliminate any free protein.

Scheme II. Physically stable and insoluble immunosorbents can be prepared also with small antigen molecules, such as are found in the

[20a] I. H. Shilman and Katchalski, *Ann. Rev. Biochem.* **35**, 873 (1966).
[21] E. R. Centeno, and A. H. Sehon, *Federation Proc. Abstr.* **25**, 729 (3044) (1966).



Reaction scheme I



Reaction scheme II

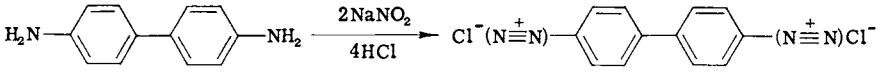
FIG. 6. Reaction scheme I represents crosslinking of a protein with EMA; reaction scheme II illustrates a graft polymerization process.

aqueous extract of pollens, or even with small haptens, by a "graft polymerization" procedure. This method consists in preparing first a crosslinked insoluble EMA–protein matrix in the form of a fine suspension by the reaction of EMA with a large-molecular-weight heterologous protein (for example, BSA), without blocking all its NH_2 groups. Onto this framework is then "grafted" the low-molecular-weight peptide or hapten by the addition of the corresponding peptide or hapten in the presence of a second portion of EMA. Haptens with appropriate groups can be also grafted directly onto the EMA protein matrix by reactions with the protein.

h. Diazonium Compounds

i. Reactions

The diazonium functional group reacts readily with the phenolic (tyrosyl), histidyl, amino, and carboxyl groups of proteins.[10,22,23] The simple organic chemical benzedine, on treatment with nitrous acid ($NaNO_2$ and HCl), is converted to the bifunctional bis-diazotized benzedine (BDB), which can react with the appropriate groups in the different protein molecule(s).



This reaction has been used widely for attaching the antigen to rbc in the BDB passive hemagglutination reaction employed for the detection of antibodies in immune sera produced in experimental animals or in sera of allergic individuals.[7,22,24,25] (See also Chap. 15,B,3, Vol. III.) Bis-diazotized-3,3′-dianisidine (BDD) has also been used for the conjugation of ferritin to its antibody.[26]

ii. Conjugation of Ferritin to Antibody with BDD[26]

A solution of 160 mg of ferritin and 80 mg of rabbit globulin in 7 ml of 0.1 *M* citrate buffer, pH 5.0, is treated at 4° with a 1-ml aliquot of a solution of 6.7 mg of dianisidine and 3.7 mg of sodium nitrite in 10 ml of 0.017 *N* hydrochloric acid. The solution is stirred in the

[22] J. Gordon, B. Rose, and A. H. Sehon, *J. Exptl. Med.* **108**, 37 (1958).
[23] F. W. Putnam, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. IB, p. 893. Academic Press, New York, 1953.
[24] L. O. Frick, L. Gyenes, and A. H. Sehon, *J. Allergy* **31**, 216 (1960); P. Gold, J. Jonson, and S. O. Freedman, *ibid.* **37**, 311 (1966).
[25] J. E. Moore and W. H. Ward, *J. Am. Chem. Soc.* **78**, 2414 (1956).
[26] F. Borek, *Nature* **191**, 1293 (1961).

cold for 2 hours, and then dialyzed against 0.08 *M* borate, pH 9.4, and finally against neutral saline. The ferritin–antibody conjugate can be separated from the unreacted proteins by electrophoresis.

*iii. Protein–Erythrocyte Conjugate with BDB**[,22,24]

Step 1. Preparation of BDB. BDB is prepared by dissolving 0.23 gm of benzedine in 45 ml of 0.2 *N* HCl, and adding 0.175 gm of $NaNO_2$ in 5 ml of distilled water to this solution at 0°. The reaction is allowed to proceed for 30 minutes with intermittent stirring. Aliquots of the solution are then placed into 2-ml vials, quick-frozen at —78° in a dry ice–acetone bath, and stored at —20° until required. (Some batches have been used over periods as long as 5 months without detectable deterioration.) For each experiment the content of a vial, immediately after thawing, is diluted fifteenfold with 0.15 *M* phosphate buffer (215 ml of 0.15 *M* Na_2HPO_4 and 49 ml of 0.15 *M* KH_2PO_4) at pH 7.3 and is used for coupling the antigen to the red cells.

Step 2. Sensitization of Erythrocytes. Rabbit red blood cells are washed three times with cold physiological saline, and the packed cells are resuspended in an equal volume of saline. The optimal quantity of the antigen is then placed into a 15-ml centrifuge tube and mixed with 0.1 ml of a 50% red cell suspension. Finally, an optimal amount of freshly prepared BDB–phosphate (1 ml of BDB and 15 ml of cold phosphate buffer at pH 7.3) is added, and the mixture is inverted several times to ensure even mixing. The reaction is allowed to proceed at room temperature (22° to 26°) for 15 minutes with occasional stirring. The cells are separated by centrifugation, and the brownish supernatant is discarded. The cells are washed with 3.5 ml of a solution consisting of heat-inactivated normal rabbit serum, diluted 100-fold with phosphate buffer. Finally, the cells are resuspended in the diluted serum to a final volume of 2.5 ml. With some antigens, higher hemagglutination titers have been obtained by a slight modification of the procedure, consisting in the addition of the BDB–phosphate solution to the red cells prior to reaction with the antigens in question.[24]

i. N-Ethyl Benzisoxazolium Fluoborate (EBIZ)[4,5]

i. Principle

EBIZ rearranges in the presence of base to the keto-ketinimine form. The latter compound gives readily an adduct with nucleophilic reagents such as carboxylates, which then rearrange to form activated acyl deriva-

* Compare procedure detailed in Chap. 15,B,3, Vol. III.

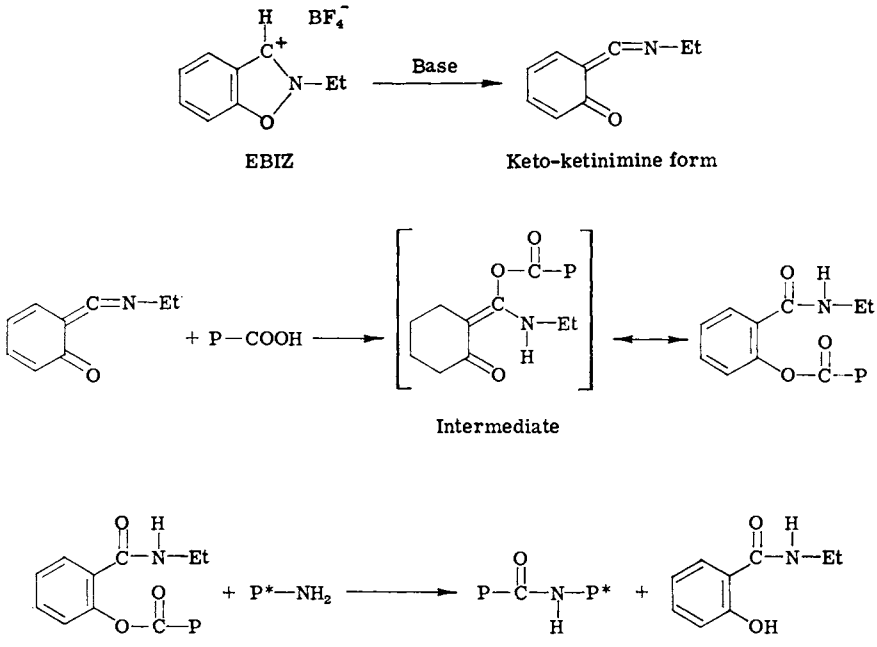


FIG. 7. Reactions involving EBIZ.

tives. These, in turn, react with amino groups to form the amide bond. The reaction mechanism is the principle for the preparation of Woodward's reagent K and is illustrated in Fig. 7.

ii. Conjugation of Angiotensin to Polylysine with EBIZ[4,5]

Ten milligrams of angiotensin is dissolved in 0.1 ml of water at pH 5.0. To this solution is added 20 mg of EBIZ, and the mixture is warmed slightly to dissolve the reagent. Immediately thereafter, 5 mg of polylysine and 0.15 ml of dimethylformamide are added, and the pH is adjusted to 8.5 with concentrated sodium hydroxide. The mixture is allowed to stand at room temperature for 2 days; then 1.0 ml of water is added, and the pH is brought to 2.0 with concentrated hydrochloric acid. Benzene (2 ml) is added, and the mixture is shaken to extract the side products of the EBIZ reagent. The aqueous layer is dialyzed to purify the antigen. The yield of the soluble conjugate is 5 mg.

j. CONCLUDING REMARKS

In addition to the procedures described in this section, other potentially useful reactions for the preparation of protein–protein conjugates

for immunological purposes may be noted. Among these are reactions employing nitrogen mustards,[23] formaldehyde,[23] disulfonyl chlorides,[23] and maleimides.[25] For example, bovine serum albumin was dimerized with *N,N'*-(1,3-phenylene)bismaleimide,[25] and formaldehyde has been used as a crosslinking agent in the tanning industry.[23] Recently, dimers and higher aggregates of ribonuclease have been prepared using the diimido ester, dimethyl adipimidate, as the crosslinking agent.[27]

Clearly, as in all other chemical reactions, the specific conditions, (pH, temperature, time of reaction, and appropriate solvents) for optimal yields of protein–protein conjugates must be established for each system.

It is obvious from the foregoing treatment that the great versatility and ingenuity of the organic and protein chemist have as yet not been fully exploited in this area. In principle, any bifunctional or polyfunctional compound capable of forming covalent bonds with the reactive groups of protein molecules could serve as a protein–protein conjugating agent.

[27] F. C. Hartman and F. F. Wold, *J. Am. Chem. Soc.* **88**, 3890 (1966).

7. PREPARATION OF SYNTHETIC POLYPEPTIDES AS ANTIGENS*

a. Introduction

It has been shown that synthetic polypeptides may elicit the formation of narrowly specific antibodies in experimental animals. Several reviews have summarized recent studies concerning the preparation and the immunological properties of linear and multichain polypeptides.[1-4] Such polypeptides serve as useful models in the elucidation of the minimal chemical requirements necessary for antigenicity. With the chemistry of these compounds known, it is possible, through a systematic study of copolymers showing only limited variations in their chemical formulas, to arrive at conclusions concerning the role of various structural features in their antigenic function.

*** Section 1,E,7 was contributed by Michael Sela and Sara Fuchs.**

[1] E. Katchalski, M. Sela, H. I. Silman, and A. Berger, *in* "The Proteins" (H. Neurath, ed.), Vol. 2, p. 405. Academic Press, New York, 1964.
[2] P. H. Maurer, *Prog. Allergy* **8**, 1 (1964).
[3] M. Sela, *in* "New Perspectives in Biology" (M. Sela, ed.), p. 225. Elsevier, Amsterdam, 1964.
[4] M. Sela, *Advan. Immunol.* **5**, 29 (1966).

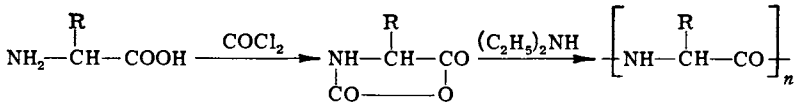
Polypeptidyl proteins are also of interest in immunological studies, as peptidylation of proteins gives rise to antigens with new specificity. Furthermore, peptidylation of proteins which are either poor antigens or not antigenic at all (because they are tested in homologous animals or in animals made experimentally unresponsive to them[5]) serves as an appropriate tool in the elucidation of the chemical basis of immunogenicity.

Besides synthetic antigens composed exclusively of amino acids, synthetic immunogens were reported[4] which elicit antibodies with sugar, nucleoside,[6] or coenzyme specificity. Such antigens may be of interest both because of the information they may yield concerning the molecular aspects of antigenicity, and because of their potential use in the chemistry and biology of nucleic acids, vitamins, etc. The procedure for the preparation of a uridine-synthetic polypeptide antigen is given in Section E,8,d.

b. Linear Polypeptide Antigens

i. General Procedure

Linear polypeptide antigens are prepared by random copolymerization of *N*-carboxy-α-amino acid anhydrides.[1] These are obtained from the respective amino acids and phosgene. The polymerization scheme is given here.



When *N*-carboxyanhydrides of several α-amino acids are polymerized together, R in the above scheme stands for the different side chains of the component amino acids. The polymerization is carried out in an organic solvent (usually dioxane, benzene, or dimethylformamide) in the presence of an initiator (usually an organic amine[7] or sodium methoxide[8]). Reactive groups, whenever present in the side chains of amino acids, are protected before polymerization with reagents which may be easily removed after the completion of the reaction.[7]

[5] I. Schechter, S. Bauminger, M. Sela, D. Nachtigal, and M. Feldman, *Immunochemistry* **1**, 249 (1964).

[6] M. Sela, H. Ungar-Waron, and Y. Shechter, *Proc. Natl. Acad. Sci. U.S.* **52**, 285 (1964).

[7] E. Katchalski, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 540. Academic Press, New York, 1957.

[8] E. Friedman, T. J. Gill III, and P. Doty, *J. Am. Chem. Soc.* **83**, 4050 (1961).

ii. Preparation and Characterization of a Copolymer of L-*Glutamic Acid,* L-*Lysine, and* L-*Tyrosine, in a Residue Molar Ratio of 55:39:6 (According to Nomenclature Suggested by Gill*[9]*: polyGlu*^{55}*Lys*^{39}*Tyr*^{6})

In a 1-liter flask equipped with a calcium chloride drying tube and a magnetic stirring bar, 7.23 gm (27.5 mmoles) of γ-benzyl-*N*-carboxy-L-glutamate anhydride, 6 gm (19.5 mmoles) of ε,*N*-carbobenzoxy-α,*N*-carboxy-L-lysine anhydride, and 0.63 gm (3 mmoles) of *N*-carboxy-L-tyrosine anhydride* are dissolved in 280 ml of anhydrous dioxane.† Triethylamine (0.15 ml) is added immediately, and the reaction mixture is left at room temperature for 3 days. After this period the polymer is precipitated with water (2 liters), filtered, washed several times with water, and dried—first over concentrated sulfuric acid and then over phosphorus pentoxide—in a vacuum desiccator.

To remove the protecting groups, the polymer is treated in a glass-stoppered vessel with 110 ml of a 33% solution of hydrogen bromide in glacial acetic acid for 72 hours at 2°.‡ After that time precipitation of the copolymer is completed with anhydrous ether (600 ml). The precipitate is centrifuged, the supernatant liquid discarded, and the polymer suspended and washed several times with anhydrous ether and finally dried in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide.

iii. Analysis

The copolymer obtained is characterized by chemical and physicochemical analysis. The copolymer is hydrolyzed with 6 *N* hydrochloric acid for 24 hours in a sealed evacuated tube at 105°. The amino acid

[9] T. J. Gill III, *Biopolymers* **2**, 283 (1964).

* γ-Benzyl-*N*-carboxy-L-glutamate anhydride is prepared from γ-benzyl-L-glutamate and phosgene, according to Katchalski and Berger.[10] *N*-Carboxy-L-tyrosine anhydride[11] and ε,*N*-carbobenzoxy-α,*N*-carboxy-L-lysine anhydride are prepared from phosgene and, respectively, L-tyrosine and ε,*N*-carbobenzoxy-L-lysine, in the same way.[10] ε,*N*-Carbobenzoxy-L-lysine is prepared by coupling an equimolar amount of the sodium salt of L-lysine with benzyl chloroformate in cold chloroform solution.

[10] E. Katchalski and A. Berger, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 546. Academic Press, New York, 1957.

[11] A. Berger, J. Kurtz, T. Sadeh, A. Yaron, R. Arnon, and Y. Lapidoth, *Bull. Res. Council Israel* 7A, 98 (1958).

† Each anhydride is dissolved separately in dioxane (1 gm per 20 ml) (filtered if necessary), and the three solutions are mixed together in the flask. *N*-Carboxy-L-tyrosine anhydride is dissolved by heating the dioxane solution to about 70°.

‡ These conditions are essential for removing the benzyl groups. To remove the carbobenzoxy groups, 30 minutes at room temperature are sufficient.

composition of the copolymer is determined by chromatography on an amino acid analyzer (or by quantitative paper chromatography in *n*-butanol–acetic acid–water, 50:12:50 v/v, followed by ninhydrin colorimetry). The content of tyrosine is calculated from the spectral absorption of a solution of the polymer at pH 13 at 293.5 mμ, with $\epsilon = 2330$ for the molar extinction of the phenolate ion at this wavelength. For polymers with degrees of polymerization lower than 200, the number average degree of polymerization may be determined from amino nitrogen (Van Slyke) determination.

For the determination of the molecular weight by physicochemical criteria, the copolymer is subjected to sedimentation and diffusion in the ultracentrifuge.[12] The experiments are performed on solutions of the copolymer in 0.1 *M* phosphate buffer, pH 7.0, which were dialyzed against that buffer. For the calculation of the average molecular weight, the partial specific volume of the copolymer is computed from the partial specific volumes of the amino acid residues and their proportion in the polymer.[8,12] The partial specific volume of tyrosine residue is taken as 0.71, that of lysine residue as 0.72, and that of sodium glutamate residue as 0.57. In a typical preparation, the polymer obtained had an average molecular weight of 61,000, and a molar residue ratio of Glu–Lys–Tyr, 59:35:6.

iv. Dissolution and Storage

For use as an antigen, 1 gm of the copolymer is dissolved in 100 ml of distilled water, and 1 *N* sodium hydroxide is added in drops until the material is completely dissolved (the pH of the solution should not exceed 8.5). The solution is then dialyzed against one change of 0.05 *M* sodium bicarbonate and three changes of distilled water. The contents of the dialysis bag are freeze-dried and stored at 2°.*

c. Multichain Polypeptide Antigens

i. General Procedure

Multichain polyamino acids are prepared by polymerization of the desired *N*-carboxy-α-amino acid anhydrides by using a multifunctional amine.[12,13] That initiator, for example poly-L-lysine hydrobromide, is dissolved in a neutral buffer solution and treated at 2° with the desired

[12] M. Sela, S. Fuchs, and R. Arnon, *Biochem. J.* **85**, 223 (1962).

* To remove fractions of relatively low molecular weight, which are not removed by dialysis, samples of the copolymer may be filtered through a Sephadex G-100 column, and only the first fraction collected.

[13] M. Sela, E. Katchalski, and M. Gehatia, *J. Am. Chem. Soc.* **78**, 746 (1956).

anhydrides dissolved in dioxane. Under these conditions each amino group of the initiator may start a polymeric chain, and the multichain polymer obtained is composed of peptidic side chains attached to the multifunctional core.

ii. Preparation and Characterization of Multi [*copoly*(L-*glutamyl*, L-*tyrosyl*)-*poly*-DL-*alanyl*]-*poly*-L-*lysine*

A schematic presentation of this polymer, denoted p(Glu,Tyr) -pDLAla- -pLys,[12] is given in Fig. 1.

(*a*) *Synthesis of N-Carboxy*-DL-*alanine* DL-Alanine (10 gm) and anhydrous dioxane (200 ml) are placed in a two-necked, 1-liter, round-bottomed flask, equipped with a reflux condenser, a gas inlet tube extending below the surface of the reaction mixture, and a magnetic stirring

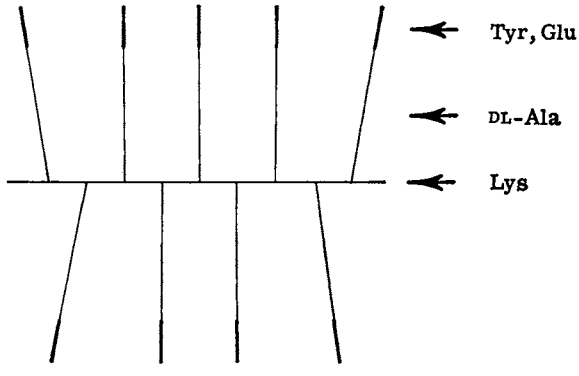


FIG. 1. Schematic presentation of the multichain copolymer p(Glu,Tyr)-pDLAla--pLys, in which L-glutamic acid and L-tyrosine residues are attached to multi-poly-DL-alanyl-poly-L-lysine.

bar. The mixture is kept at 40° in a water bath, and a stream of phosgene* is passed through the gas inlet tube for 3 hours, whereupon all the solid goes into solution. The hydrogen chloride formed and the excess of phosgene are passed through the top of the condenser into a wash bottle containing aqueous ammonia. After 3 hours the supply of phosgene is stopped, and a stream of dry nitrogen is blown through the solution for 2 hours, to remove excess phosgene. The solution is filtered through glass wool, and the clear solution is concentrated *in vacuo* at 40°. The oily residue is dissolved in anhydrous ethyl acetate (100 ml), which

* Phosgene is withdrawn from a cylinder and dried by bubbling through concentrated sulfuric acid.

is removed by distilling *in vacuo*. This procedure is repeated once, and the residue is dissolved in about 20 ml of anhydrous ethyl acetate, reprecipitated with anhydrous petroleum ether, and stored in the deep-freeze until it crystallizes. If the oil does not crystallize, the solvent is removed by distillation *in vacuo*, and the treatment with ethyl acetate is repeated. The crystals are collected, washed with petroleum ether, and dried *in vacuo* over phosphorus pentoxide. The yield of anhydride, melting at 70° (decomposed), is 7 gm.

(*b*) *Multichain Poly-*DL*-alanine* (pDLAla pLys) A solution of 1 gm of poly-L-lysine hydrobromide[7] in 300 ml of 0.05 *M* phosphate buffer, pH 7,* is introduced into a 1-liter flask. The flask is cooled with ice to about 2° and a solution of 14 gm of *N*-carboxy-DL-alanine anhydride in 200 ml of anhydrous dioxane is added slowly with vigorous shaking. At this stage there is a strong evolution of carbon dioxide. The reaction mixture is left with a magnetic stirring in the cold room overnight, dialyzed against several changes of distilled water at 2° for 5 days, and then freeze-dried† and stored at 2°.

(*c*) *p(Glu,Tyr)–pDLAla pLys* A solution of 6 gm of multichain poly-DL-alanine in 500 ml of 0.05 *M* phosphate buffer, pH 7, is introduced into a 2-liter flask. The flask is cooled with ice to about 2°, and a solution of 3.9 gm (15 mmoles) of γ-benzyl-*N*-carboxy-L-glutamate anhydride and 2.1 gm (10 mmoles) of *N*-carboxy-L-tyrosine anhydride in 120 ml of anhydrous dioxane‡ is added in several portions with vigorous shaking. There is an evolution of carbon dioxide, and a precipitate is formed. The reaction mixture is left under stirring in the cold room overnight. The precipitation of the reaction product is completed by adding 3 volumes of cold acetone.§ The mixture is allowed to stand for several hours in the cold room, the supernatant fluid is decanted, and the precipitate is filtered with suction or centrifuged, washed with acetone and water, and dried over sulfuric acid and phosphorus pentoxide in a vacuum desiccator. The protecting benzyl groups are removed as

* It is preferable to dissolve the polylysine hydrobromide in 150 ml of distilled water and then to add the same volume of 0.1 *M* phosphate buffer, pH 7.0.

† The reaction product may be concentrated to about half the volume before freeze-drying by evaporation or ultrafiltration. If the multichain poly-DL-alanine is prepared for use as a multifunctional initiator, the freeze-drying step may be omitted, and the solution may be brought directly to the desired concentration of the buffer by adding the buffer components.

‡ Each anhydride is dissolved separately in dioxane (1 gm per 20 ml) (filtered if necessary), and the three solutions are mixed together in the flask. *N*-Carboxy-L-tyrosine anhydride is dissolved by heating the dioxane solution to about 70°.

§ It is desirable to determine on a small scale how many volumes of acetone give an easily filtrable precipitate.

described above for the linear copolymer. The final product is dissolved in water, dialyzed for 3 days against several changes of distilled water, freeze-dried, and stored at 2°.

iii. Analysis

The multichain copolymer is characterized by chemical and physicochemical analysis. Determinations of the amino acid composition and of the molecular weight are done as described above for a linear copolymer.* Terminal amino groups are quantitated by dinitrophenylation, thus giving the number of polymeric side chains in the polymer.

d. Polypeptidyl Proteins

i. General Procedure

Proteins contain numerous free amino groups and may be used, similarly to synthetic polypeptides containing ϵ-amino groups, as multifunc-

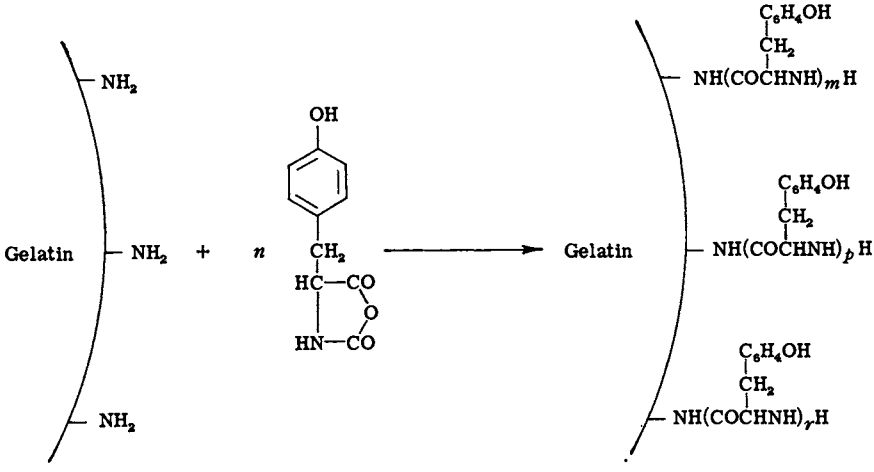


Fig. 2. Synthesis of polytyrosyl gelatin from gelatin and *N*-carboxytyrosine anhydride.

tional initiators. The polymerization of *N*-carboxy-α-amino acid anhydrides with proteins as multifunctional initiators[14,15] (Fig. 2) proceeds under mild conditions (aqueous media, low temperature, neutral pH)

* The partial specific volume of alanine residue[12] is taken as 0.72.

[14] R. R. Becker and M. A. Stahmann, *J. Biol. Chem.* **204**, 745 (1953).

[15] M. Sela and R. Arnon, *Biochem. J.* **75**, 91 (1960).

in which most proteins remain native. From a statistical analysis of the molecular weight distribution of multichain polyamino acids,[16] it may be predicted that the attachment of the peptide side chains to the protein will not greatly affect its molecular weight homogeneity.

When the *N*-carboxy-α-amino acid anhydrides contain blocking groups, these groups should be removed after the polymerization under mild conditions, to prevent the denaturation of the protein carrier. Such conditions are available for preparing polylysyl proteins (via poly-ϵ,*N*-trifluoroacetyllysyl proteins[17]).

*ii. Preparation of Poly-*L*-tyrosyl Gelatin*

A solution of 2 gm of gelatin in 100 ml of 0.05 *M* phosphate buffer, pH 7, is introduced into a 250-ml flask, cooled with ice to 2°, and a solution of 0.62 gm (3 mmoles) of *N*-carboxy-L-tyrosine anhydride* in 20 ml of anhydrous dioxane is added in several portions with vigorous shaking. The reaction is carried out with stirring for 24 hours at 2°, followed by an additional 24 hours at room temperature. The reaction mixture is dialyzed against several changes of distilled water at 2° for 3 days. The reaction product is centrifuged to remove any precipitate that has formed, and the solution is freeze-dried and stored at 2°.

*iii. Preparation of Poly-*L*-lysyl Rabbit Serum Albumin*

A solution of 1 gm of rabbit serum albumin in 50 ml of 0.05 *M* phosphate buffer, pH 7, is introduced into a 250-ml flask, cooled with ice to 2°, and a solution of 1.2 gm (4.5 mmoles) of ϵ,*N*-carboxy-L-lysine anhydride[17] in 17 ml of anhydrous dioxane is added. After 24 hours at 2° the reaction mixture is dialyzed against distilled water for 3 days at 2°, and the insoluble material formed is separated by centrifugation and lyophilized.

To remove the trifluoroacetyl groups, 1 gm of poly-ϵ,*N*-trifluoroacetyllysyl rabbit serum albumin is suspended in 70 ml of 1 *M* aqueous piperidine. After 1 hour a clear solution is obtained.† After an addi-

[16] E. Katchalski, M. Gehatia, and M. Sela, *J. Am. Chem. Soc.* **77**, 6175 (1955).

[17] M. Sela, R. Arnon, and I. Jacobson, *Biopolymers* **1**, 517 (1963).

* γ-Benzyl-*N*-carboxy-L-glutamate anhydride is prepared from γ-benzyl-L-glutamate and phosgene, according to Katchalski and Berger.[10] *N*-Carboxy-L-tyrosine anhydride[11] and ϵ,*N*-carbobenzoxy-α,*N*-carboxy-L-lysine anhydride are prepared from phosgene and, respectively, L-tyrosine and ϵ,*N*-carbobenzoxy-L-lysine, in the same way.[10] ϵ,*N*-Carbobenzoxy-L-lysine is prepared by coupling an equimolar amount of the sodium salt of L-lysine with benzyl chloroformate in cold chloroform solution.

† At this stage the protein derivative still contains about half of the original trifluoroacetyl groups.

tional 20 hours, the solution is neutralized with cold 0.5 *M* acetic acid dialysed for 3 days against distilled water at 2°, and freeze-dried.*

iv. Analysis

Determinations of the amino acid content and the molecular weight of polypeptidyl proteins are carried out as described above for a linear copolymer. Terminal amino groups are quantitated by dinitrophenylation or by deamination.[18] From the amino acid analysis of the modified and unmodified protein, it is possible to determine the enrichment of the protein with the amino acid which was polymerized on it. From the dinitrophenylation or deamination tests, it is possible to determine the number of the polypeptide side chains and thus to calculate their average length. (The number of lysines obtained in the amino acid analysis after deamination and total hydrolysis of the peptidylated protein gives the number of side chains, except for polylysyl proteins.)

e. Techniques of Immunization and Detection of Immune Response

Immunization with synthetic antigens is usually done in a complete Freund's adjuvant, while polypeptidyl proteins are injected either in adjuvant or in saline solutions. The first dose, in adjuvant, is usually 8 to 20 mg of antigen in rabbits, 10 to 1000 μg in guinea pigs, and 10 to 100 μg in mice.

The methods used to detect an immune response toward synthetic polypeptide antigens included immunospecific precipitin reactions and their inhibition, immunodiffusion, immunoelectrophoresis, complement fixation and hemagglutinin reactions, measurements of antigen-binding capacity, rate of elimination, delayed and immediate hypersensitivity reactions, and systemic anaphylaxis as well as passive cutaneous and systemic anaphylaxis reactions.[4] Most of these methods are described in Vol. II.

* The final material may contain up to 0.7% fluorine.

[18] C. B. Anfinsen, M. Sela, and J. P. Cooke, *J. Biol. Chem.* **237**, 1825 (1962).

8. PREPARATION OF ANTIGENS FOR ELICITING ANTIBODY WITH NUCLEOSIDE AND NUCLEIC ACID SPECIFICITY

a. Introduction†

The results of early attempts to produce antibodies against nucleic acids were encouraging but indecisive. Immunization of rabbits with

† Sections 1,E,8,a and 1,E,8,b were contributed by Otto J. Plescia.

a DNA-rich fraction of *Brucella abortus* elicited antibody, which reacted with DNA-associated antigens. These antigen complexes lost their reactivity upon DNase treatment, but the DNA fractions freed of protein and carbohydrate were unreactive by either precipitation or complement fixation tests. There was no evidence for immunological specificity for any of the nucleosides of DNA.[1]

Some human subjects with lupus erythematosus (LE) appear to produce DNA-specific antibody as do rabbits injected with a lysate to T4 bacteriophage.[2-5] Antibodies in LE sera that react with DNA are not species-specific, however, so it is assumed that DNA would not be recognized as foreign in any host, and thus not immunogenic. The key to immunogenicity has proved to be suitable methods for conjugating DNA, nucleosides, purines, or pyrimidines to immunogenic foreign proteins in stable complexes.

b. Complexes of Nucleic Acids and Oligonucleotides with Methylated Bovine Serum Albumin

Because DNA is an acidic polymer it interacts with basic proteins under physiological conditions to form stable complexes. Methylated bovine serum albumin (MBSA), a basic protein, is readily prepared by reacting bovine serum albumin with methanol in hydrochloric acid.

PREPARATION OF THE IMMUNOGEN

i. Preparation of MBSA

The essential steps are described in detail by Sueoka and Cheng.[6] One gram of bovine serum albumin is dissolved in 100 ml of absolute methanol, and 0.84 ml of 12 *N* HCl is added. The preparation can be on a smaller or larger scale in the same proportions. The protein

[1] O. J. Plescia, W. Braun, and N. C. Palczuk, *in* "Conceptual Advances in Immunology and Oncology," pp. 238–256. Harper and Row, New York, 1963.

[2] H. R. G. Deicher, H. R. Holman, and H. G. Kunkel, *J. Exptl. Med.* **109**, 97 (1959).

[3] D. Stoller, L. Levine, H. I. Lehrer, and H. Van Vunakis, *Proc. Natl. Acad. Sci. U.S.* **48**, 874 (1962).

[4] L. Levine, W. T. Murakami, and H. Van Vunakis, *Proc. Natl. Acad. Sci. U.S.* **46**, 1038 (1960).

[5] W. T. Murakami, H. Van Vunakis, L. Grossman, and L. Levine, *Virology* **14**, 190 (1961).

[6] M. Sueoka and T. Cheng, *J. Mol. Biol.* **4**, 161 (1962).

is readily soluble at first, but it precipitates as it becomes methylated. The reaction mixture is allowed to stand, with occasional mixing, at room temperature in the dark for at least 3 days. The mixture is centrifuged, and the precipitate is separated and washed twice, first with methanol and next with anhydrous ether. Residual ether is removed from the precipitate by evaporation in air. Residual acid is removed by drying *in vacuo* over KOH. Failure to remove traces of acid reduces the basicity of MBSA. The dried MBSA is commonly stored as a powder in a desiccator over KOH. An alternate procedure is given in Chap. 2,A,2,f.

ii. DNA–MBSA Complex Formation

The stability of complexes formed between DNA and MBSA depends on the concentration and type of salt in the medium and the pH. Dissociation occurs at either high or low pH and at salt concentrations greater than 0.15 *M*. Thus, a physiological diluent, 0.15 *M* NaCl at pH 7, has been selected as standard.

The concentration of DNA is arbitrary. Primary considerations are its solubility and the amount to be injected into the animal. A concentration of 0.5 mg of DNA per milliliter has proved effective and is therefore recommended.

Since MBSA is more easily dissolved in water than in 0.15 *M* NaCl, it is added in a 1% aqueous solution to the DNA solution. A small volume is required because of the relatively high concentration of MBSA, and the resultant concentration of salt in the final solution is still physiological.

The optimal ratio of DNA to MBSA in the complexes for antibody formation has not yet been investigated. This should be a factor, but, since the ratio may be varied over a wide range, it does not appear to be a critical one. Suitably immunogenic protein conjugates are formed by mixing equal amounts of DNA and MBSA by weight of solute. The reaction is carried out at room temperature with gentle mixing.

IMMUNIZATION AND TESTING OF ANTISERUM

i. Immunogen

The use of complete Freund's adjuvant in immunization influences greatly the immune response (see Chap. 2). If the antigen is a poor or a "nonforeign" one, adjuvant may be indispensable. Since nucleic acids may be regarded as "nonforeign," as discussed above, complete

Freund's adjuvant is routinely incorporated in the immunizing preparation by emulsifying the complexes of MBSA–hapten in an equal volume of adjuvant.

ii. Host

Experience to date has been limited to the rabbit, but there is reason to believe that conjugates of MBSA with nucleic acid or oligonucleotides should be immunogenic in any host capable of producing antibodies to MBSA.

iii. Immunization Schedule

Rabbits were injected weekly for 3 weeks with freshly prepared antigen containing 0.25 mg of hapten per milliliter. Each week 0.4 ml was given in the foot pad and 1.0 intramuscularly so that a single rabbit received a total of about 1 mg of hapten in the course of three injections. Serum was separated from blood obtained by cardiac puncture 7 to 10 days following the last injection.

iv. Methods of Analysis

Immune sera may be analyzed for antibodies by the quantitative precipitin method (see Chap. 13, Vol. III) or by complement (C′) fixation (see Chap. 16,A, Vol. III). Experience to date indicates that a serum will have both C′ fixing and precipitating antibody or none at all. Therefore either method may be used. The method of C′ fixation may be preferred because it is more sensitive than the precipitin test and hence requires much less antiserum.

Whenever DNA or synthetic polydeoxyribonucleotide is tested as reactive antigen, it is first heat-denatured to separate its strands of polynucleotides. Soluble RNA (sRNA) or synthetic polyribonucleotides may be used without prior denaturing.

v. Specificity of Antibodies

The immune response is typical of that induced by a protein–hapten conjugate. Some antibodies are specific for the MBSA or nucleic acid alone, and some are specific for the conjugate.

The specificity of antibodies to nucleic acids is determined by the hapten inhibition method (see Chap. 18,E, Vol. III), with mononucleotides and digests of nucleic acids as inhibitors. From such studies it has been concluded that antibodies induced by DNA and sRNA are specific for each of the common nucleotides, probably in sequence or groups. A

notable exception is T4 phage DNA, which contains glucosylated 5-hydroxymethylcytosine instead of cytosine. The antibodies formed are highly specific for glucosylated 5-hydroxymethylcytosine and cross-react only with DNA's having glucosylated bases. The specificity of the antibodies produced by the synthetic polynucleotides appear to be restricted to those nucleotides contained in the polynucleotides.

SPECIFIC APPLICATIONS

i. Natural DNA

Molecules of DNA in their native state are double-stranded helices held together by hydrogen bonds formed between complementary base pairs. In this state, the bases are not exposed and hence are not reactive. Thus, DNA must first be separated into single strands before the addition of MBSA to form complexes if it is desired to produce antibodies specific for the bases and not the deoxyribose phosphate backbone alone. Denaturation of DNA by heating at 100° for 10 minutes results in the formation of single strands, and, if the solution is cooled rapidly, strand separation is virtually preserved.[7] Examples of DNA tested as haptens are those from calf thymus and T4 bacteriophage.[8]

ii. Synthetic Polydeoxyribonucleotide

A synthetic copolymer consisting of repeating units of deoxyadenylate-thymidylate (d-AT) has been tested and found to be haptenic when complexed with MBSA. Like DNA, d-AT copolymer has a high degree of secondary structure owing to the formation of intra- and intermolecular hydrogen bonds at low temperatures. Therefore, d-AT copolymer is heat-denatured at 100° for 10 minutes and cooled rapidly immediately before MBSA is added.

iii. Oligodeoxyribonucleotides

Mixtures of oligodeoxyribonucleotides are prepared either by the digestion of calf thymus DNA with pancreatic deoxyribonuclease or by its depurination by the procedure of Burton and Petersen.[9] The resulting oligonucleotides are too small to have secondary structure, so it is un-

[7] P. Doty, J. Marmur, J. Eigner, and C. Schildkraut, *Proc. Natl. Acad. Sci. U.S.* **46**, 461 (1960).

[8] O. J. Plescia, W. Braun, and N. C. Palczuk, *Proc. Natl. Acad. Sci. U.S.* **52**, 279 (1964).

[9] K. Burton and G. B. Petersen, *Biochem. J.* **75**, 18 (1960).

necessary to denature them; the MBSA may be added directly in an amount equal to the weight of starting DNA.[10]

iv. Soluble RNA (sRNA)

Soluble RNA's from *Escherichia coli* and yeast have been used as haptens. Although a large portion of the sRNA molecule is hydrogen-bonded, denaturation was deliberately avoided in order to preserve its functional state as an amino acid-specific transfer RNA in the hope that antibodies produced might be specific for nucleotides in that portion of the molecule responsible for transfer activity. It has been shown that sRNA in this state functions as a hapten when linked to MBSA.[11]

v. Synthetic Polyribonucleotide

As an example of a synthetic polyribonucleotide, polycytidylate has been tested as a hapten. Like sRNA, it was complexed to MBSA without first being denatured by heating. Antibodies specific for polycytidylate were produced.

c. Preparation of Purine- and Pyrimidine-Protein Conjugates*

Methods for covalently linking purines and pyrimidines and their derivatives to proteins[12-15] have provided antigens capable of eliciting antibodies specific for the introduced grouping. These antibodies cross-reacted with denatured DNA, and there is also evidence for their reaction with RNA.[15,16]

All the conjugates that were effective in eliciting purine-, pyrimidine-, nucleoside-, and nucleotide-specific antibodies were prepared by coupling the hapten to free amino groups (presumably the ϵ-amino of lysine) of the protein.

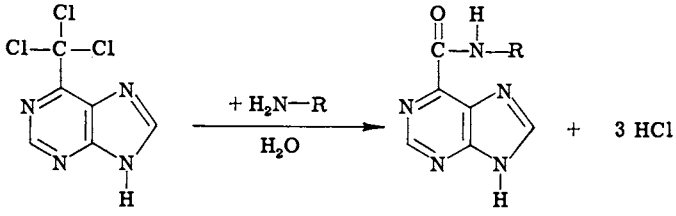
*** Section 1,E,8,c was contributed by Sam M. Beiser, Bernard F. Erlanger, and Stuart W. Tanenbaum.**

[10] O. J. Plescia, N. C. Palczuk, W. Braun, and E. Cora-Figueroa, *Science* **148**, 1102 (1965).
[11] O. J. Plescia, N. C. Palczuk, E. Cora-Figueroa, A. Mukherjee, and W. Braun, *Proc. Natl. Acad. Sci. U.S.* **54**, 1281 (1965).
[12] V. P. Butler, Jr., S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, *Proc. Natl. Acad. Sci. U.S.* **48**, 1597 (1962).
[13] S. W. Tanenbaum and S. M. Beiser, *Proc. Natl. Acad. Sci. U.S.* **49**, 662 (1963).
[14] B. F. Erlanger and S. M. Beiser, *Proc. Natl. Acad. Sci. U.S.* **52**, 68 (1964).
[15] M. Sela, H. Ungar-Waron, and Y. Shechter, *Proc. Natl. Acad. Sci. U.S.* **52**, 285 (1964).
[16] S. M. Beiser, unpublished observations, 1965.

PREPARATION OF ANTIGENS

i. Purin-6-oyl–Protein Conjugates[12]

A chilled solution containing 750 mg of bovine serum albumin (BSA) and 170 mg of 6-trichloromethylpurine[17,*] in 70 ml of 4% tetrahydrofuran–water is stirred at room temperature for 3 hours. It is necessary to add 0.1 *N* NaOH to maintain the pH between 10 and 10.5. The solution is dialyzed against running tap water overnight, and the conjugated protein is precipitated by adjusting the pH to 4.5 with 0.1 *N* HCl. The precipitate is dissolved in 25 ml of 0.15 *M* $NaHCO_3$, dialyzed as above, and lyophilized. The reaction is shown as follows:



R = Amino acid or protein residue

ii. 5-Acetyluracil–1-Protein Conjugates[13]

"Ethoxyacrylamide" (750 mg, 3.6 mmoles)[18a,18b] is added in small portions to a cold solution of 2.3 gm (0.03 mmole) of BSA in 50 ml of water plus 20 ml of 0.1 *N* NaOH, pH 11.2. After agitation for 5 minutes, 10 ml of tetrahydrofuran and another 20 ml of 0.1 *N* NaOH are added. The mixture is stirred for 1 hour at 37°, the pH is adjusted to 10.3, and then the mixture is stirred for 18 hours at 37°. Following dialysis for 24 hours against running tap water, 0.1 *N* HCl is added dropwise to bring the pH to 2.2. After 10 minutes the conjugate is precipitated by adding 10% Na_2CO_3 to pH 5.0. The precipitate is separated by centrifugation at 4° and is then dissolved in 25 ml of 0.15 *M* $NaHCO_3$. The conjugate is reprecipitated by adding 0.15 *M* HCl to pH 4.9, and the precipitate is dissolved in 30 ml of 0.1 *M* phosphate buffer, pH 7.0. After dialysis for 24 hours against running tap water, the solution may be lyophilized.

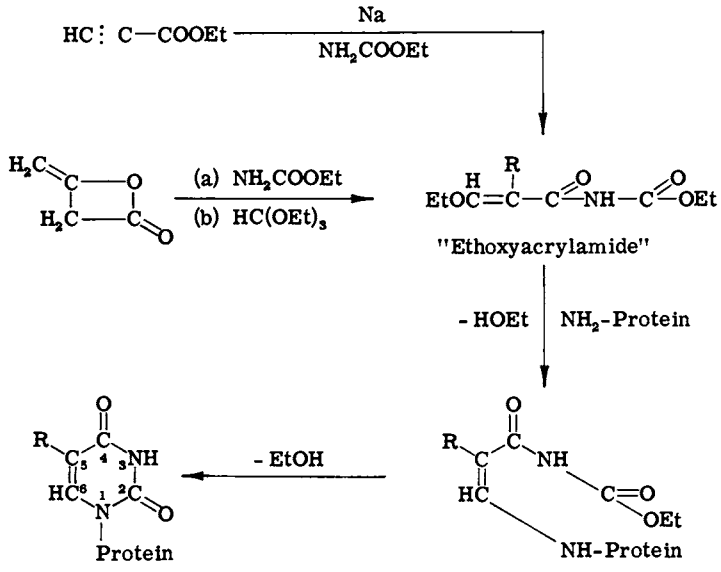
This reaction is depicted as shown on the following page, where R = H, polyvalent uracil–1-protein; R = CH_3CO, polyvalent 5-acetyl-uracil–1-protein.

[17] S. Cohen, E. Thom, and A. Bendich, *J. Org. Chem.* **27**, 3545 (1962).

* Commercially available from Cyclo Chemical Corporation, Los Angeles, California.

[18a] R. K. Ralph, G. Shaw, and R. N. Naylor, *J. Chem. Soc.* 1169 (1959).

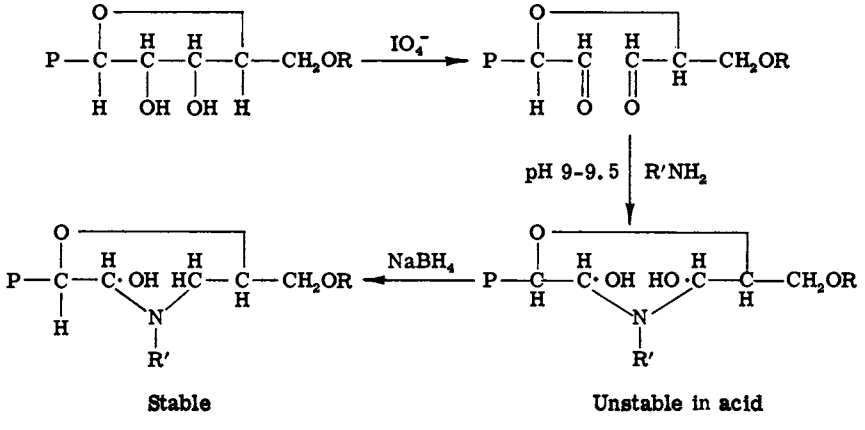
[18b] J. H. Dewar and G. Shaw, *J. Chem. Soc.* 3254 (1961).



iii. Coupling of Ribosides and Ribotides to Proteins[14]

The procedure utilized to couple purine and pyrimidine ribonucleosides and ribonucleotides to proteins is a general one requiring only the presence of vicinal hydroxyl groups on the sugar moiety. The method of coupling ribosides or ribotides to protein, illustrated below is based on the method used by Khym[18c] to determine terminal ribonucleotides. It has been used successfully for the preparation of protein conjugates of ribonucleosides of the five common purine and pyrimidine bases as well as for a number of di-, tri-, and tetra-ribonucleotides.

[18c] J. X. Khym, *Biochemistry* **2**, 344 (1963).



P = Purine or pyrimidine

R = H or 

R′ = Protein backbone; i.e. the N is supplied by the lysine residues

This method will be illustrated with AMP (adenylic acid). AMP dihydrate (100 mg) is dissolved in 5 ml of 0.1 *M* $NaIO_4$. After 20 minutes at room temperature, excess $NaIO_4$ is decomposed by adding 0.3 ml of *M* ethylene glycol. After another 5 minutes at room temperature, the reaction mixture is added with stirring to a solution of 280 mg of BSA dissolved in 10 ml of water adjusted to pH 9 to 9.5 with 5% K_2CO_3. Stirring is continued for 45 minutes, the pH being maintained with 5% K_2CO_3. A solution of 150 mg of $NaBH_4$ in 10 ml of water is added, and the mixture is kept in the refrigerator for 18 hours. Five milliliters of *M* HCOOH is added, and, 1 hour later, the pH is adjusted to 8.5 with *M* NH_4OH. The solution is dialyzed against running tap water for 36 hours and lyophilized. About 25 groups are incorporated per mole of BSA (molecular weight 70,000) as determined by ultraviolet spectrophotometry.

In some cases, changes in the above procedure will be necessary. In preparing the guanosine conjugate, periodate oxidation is carried out in 10 ml of 0.05 *M* $NaIO_4$ to prevent gelation of the oxidized product.

For the preparation of di-, tri-, and tetra-nucleotide conjugates, acidification with HCOOH is omitted subsequent to reduction with $NaBH_4$ because in some cases it has been found that HCOOH causes the formation of a precipitate that does not redissolve on addition of NH_4OH.

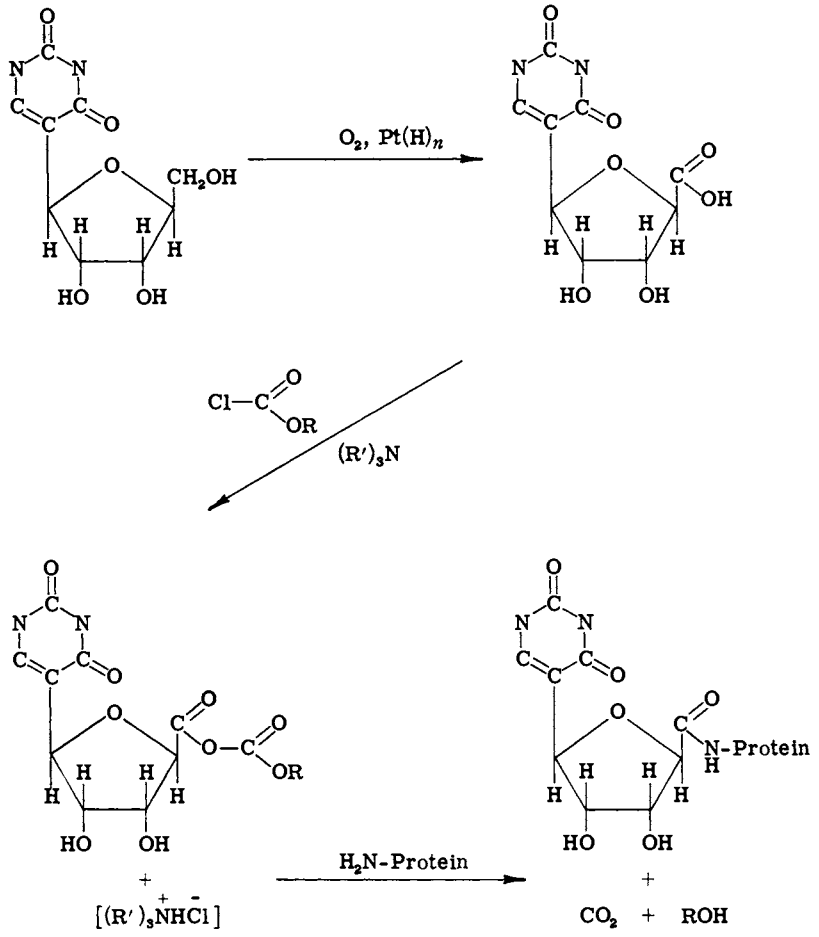
iv. Nucleoside-5′oyl Conjugates

Any nucleoside or nucleotide whose terminal hydroxymethyl can be selectively oxidized to a carboxyl group can be linked covalently to protein by using Boissonnas' mixed anhydride method.[19] With this technique, uridine, ψ-uridine, and N^6-methylaminopurine riboside conjugates have been made.[20] The synthesis is illustrated here with the preparation of the ψ-uridine-BSA conjugate.*

[19] R. A. Boissonnas, *Helv. Chim. Acta* **34**, 874 (1951).

[20] M. H. Karol and S. W. Tanenbaum, *Bacteriol. Proc.* (*Am. Soc. Microbiol.*) 59 (1965); also unpublished observations, 1965.

* An alternative method of acylation of amino groups by the nucleoside-5′-COOH has been used by Sela *et al.* See Section E,8,d.



ψ-Uridine-5′-carboxylic acid can be synthesized by using the catalytic oxidation method of Moss *et al.*[21] One hundred milligrams of ψ-uridine is dissolved in 14 ml of water containing 0.4 mmole of $NaHCO_3$. Na_2CO_3 is added to bring the pH to 8.8. After addition of 2.16 gm of reduced platinum oxide,[22] the suspension is maintained at 80°, and oxygen is bubbled through the rapidly stirred solution for 27 hours.* After removal

[21] G. P. Moss, C. B. Reese, K. Schofield, R. Shapiro, and A. R. Todd, *J. Chem. Soc.* 1149 (1963).

[22] K. Heyns and M. Beck, *Chem. Ber.* **90**, 2443 (1957).

* The time course of oxidation may be conveniently followed by paper chromatography of samples withdrawn from the oxidation mixture by using an isopropanol–ammonia–water (7:1:2) solvent. (R_f Ψ-uridine = 0.48; R_f ψ-uridine-5′-carboxylic acid = 0.24.) The reaction was terminated when about 90% oxidation was indicated on the chromatograms.

of catalyst by filtration of the oxidation mixture through celite and washing with water, the combined filtrate is reduced *in vacuo* to 20 ml. Treatment with Amberlite IR-120 (H^+), decantation, and evaporation gives a yellow oil which crystallizes rapidly (yield, 80%). Pseudouridine-5′-carboxylic acid is recrystallized from methanol to give an analytically pure sample, melting point 234.5° (decomposed) (λ_{max}^{tris} at pH 7.4, 263 mμ; $\epsilon = 7440$).

Pseudouridine-5-carboxylic acid is dried thoroughly *in vacuo* over H_2SO_4. A solution of 0.32 mmole in 0.9 ml of *N,N′*-dimethylformamide is chilled, and, to this, 0.32 mmole (0.077 ml) of tri-*n*-butylamine and 0.32 mmole (0.042 ml) of isobutylchloro-carbonate are added. Moisture is excluded with a drying tube, and the mixture is kept in an ice bath. After 15 minutes, the mixed anhydride is added to a stirred cold solution of 0.0057 mmole (400 mg) of BSA dissolved in a mixture of 7 ml of water and 6 ml of *N,N′*-dimethylformamide. The pH is adjusted to 10.5 with 0.1 *N* NaOH, and the reaction is stirred for 1 hour in the cold followed by at least 3 hours at room temperature. At the termination of the reaction, the pH should be in the range 8.2 to 8.6.

Dialysis against running tap water overnight, then against physiological saline, and finally against water again may be followed by lyophilization. Spectrophotometric analysis should indicate that the ψ-uridine BSA thus prepared contains approximately 10 groups per mole. The corresponding rabbit serum albumin conjugate contains 11 groups per mole (molecular weight, 70,000).

d. Preparation of Nucleoside Conjugates of Synthetic Polypeptides*

The catalytic oxidation of nucleosides yields nucleoside-5′-carboxylic acids,[21] which may be conjugated by means of *N,N′*-dicyclohexylcarbodiimide to the terminal amino groups of synthetic multichain polypeptides.[15] This kind of conjugation keeps intact the positions 2′ and 3′ of the sugar moieties. The attachment of uridine-5′-carboxylic acid to the nonantigenic multichain poly-DL-alanine (see Section E,7) yielded a completely synthetic antigen (Fig. 1), eliciting in rabbits antibodies with specificity toward uridine.[15] The antibodies formed reacted with polyuridylic acid, heat-denatured RNA, and DNA, but not with polyadenylic acid, native *Escherichia coli* RNA, or double-stranded calf thymus DNA. Ribose and deoxyribose contribute to the specificity of anti-nucleoside antibodies prepared by this technique. Thus, antibodies to

* Section 1,E,8,d was contributed by Michael Sela and Sara Fuchs.

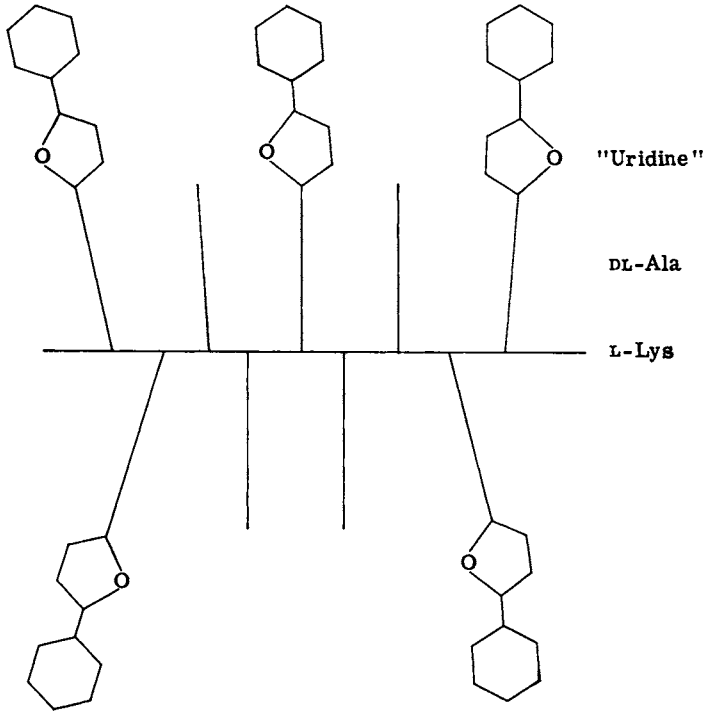


FIG. 1. Schematic presentation of the multichain nucleoside–polypeptide conjugate uridine–pDLAla–-pLys. From M. Sela, H. Ungar-Waron, and Y. Shechter, *Proc. Natl. Acad. Sci. U.S.* **52**, 285 (1964).

thymidine did not react with uridine, even though antibodies to uridine cross-reacted with thymidine.

i. Preparation of Uridine-5'-carboxylic Acid[21]

Uridine (0.794 gm, 3.25 mmoles) is dissolved in 110 ml of sodium bicarbonate buffer, pH 8.8 (0.275 gm of sodium hydrogen carbonate and 0.12 gm of sodium carbonate decahydrate). Platinum oxide (0.57 gm) reduced by hydrogenation in glacial acetic acid is added, and oxygen is bubbled into the rapidly stirred suspension, maintained at 80°. The reaction is virtually complete after 22 hours. The catalyst is collected by filtration, and the filtrate is concentrated to 25 ml under reduced pressure, treated with Amberlite IR-120 (H^+ form), and further evaporated to a pale yellow oil which crystallizes (after recrystallization from methanol–acetone, melting point 237° to 239°).

*ii. Preparation of the Uridine Conjugate of Multichain Poly-*DL*-alanine*[15]

Multichain poly-DL-alanine (pAla--pLys) is prepared by the method described in Section E,7. This product (1.5 gm) is dissolved in 10 ml of water and mixed with a solution of 0.77 g m(3 mmoles) of uridine-5'-carboxylic acid in 180 ml of dimethylformamide (the content of water in the final reaction mixture should not exceed 5%). *N,N'*-Dicyclohexylcarbodiimide (0.62 gm, 3 mmoles) is then added, and the reaction mixture is left at room temperature. After 18 hours the reaction product is dialyzed against several changes of distilled water for 3 days, the precipitate formed is removed by filtration and discarded, and the filtrate is dialyzed again, freeze-dried, and stored at 2°.

The conjugate (U–pAla--pLys) is represented schematically in Fig. 1. The uridine-CO content is determined from the extinction at 260 mμ (about 9% for the above polymer). The molecular weight and the amino acid analysis are determined as described in Section E,7.

F. Preparation and Testing of Lipids for Immunological Study*

1. INTRODUCTION

The immunological activity of lipids has been associated traditionally with such substances as the Wassermann and Forssman haptens because of the utility of the former in the diagnosis of syphilis and the seemingly irrational distribution of the latter in different biological species. Two others among the classical problems involving immunological activity of lipids concerned the nature of the heat-stable substance accounting for the organ specificity of antibrain sera, and the substance or substances responsible for the large differences in reactivity between tumor tissue lipids and normal tissue lipids when these were studied with antisera to tumors.

The chemical nature of animal tissue lipids that display immunological activity has been elucidated in several instances. On the basis of studies related to brain lipid specificity and tumor lipid specificity, it is possible to make a broad generalization of the relation of immunological activity to chemical structure: all neutral glycosphingolipids, and possibly all glycosphingolipids, may be expected to act as antigenic determinants and to display haptenic properties. The simplest molecule of this type

* Section 1,F was contributed by Maurice M. Rapport and Liselotte Graf.

is galactocerebroside (ceramide galactoside), and the immunological specificity is determined primarily by the carbohydrate residue. Among other lipids, only cardiolipin, a complex phosphatide whose structure may be regarded as *diphosphatidyl glycerol,* has been established unequivocally as having immunological activity.

The immunological method of principal value in the study of lipids is complement fixation. There are three essential reasons: First, it can be used for insoluble antigens; second, it can be used to establish the *quantity* of antigen; and third, it is sensitive enough to be usable even with the small quantities of antibody that are generally obtained by injecting tissue fractions into rabbits. The loss of any of these three capacities is a serious handicap (with precipitation methods, the first and third may be lost, and with hemagglutination methods, the second is lost).

Investigators who wish to measure the immunological activity of lipids must recognize three features that are unique or at least of special importance in this area. First, pure lipid haptens do not react well with antibody in the absence of auxiliary lipids; second, the fixation of complement to the immune complex is completely dependent on the presence of auxiliary lipids; and third, some lipids (as well as proteins and polysaccharides) are anticomplementary and interfere with measurements based on complement fixation. The emphasis placed here stems from the fact that, at the present time, major interest lies in studying the nature of lipid antigens and their specificity, rather than the reactivity of antibody.

2. IMMUNIZING ANTIGENS FROM TISSUES

These antigens are of two types: (1) a particulate fraction prepared by high-speed centrifugation and (2) a total lipid fraction extracted with chloroform–methanol.

a. Particulate Fraction

All operations are conducted at 5°. A homogenate is prepared in a Waring blendor at low speed from 30 gm of tissue and 270 ml of 25% sucrose containing 0.002 *M* $CaCl_2$. The homogenate is filtered through gauze, layered over 30% sucrose, and centrifuged at 800 *g* for 10 minutes. The sediment and 30% sucrose layer are discarded. The supernatant solution is centrifuged at 20,000 *g* for 1 hour. The sediment is suspended in 30% sucrose with a glass homogenizer using 60 ml (2 ml/gm of original tissue). Merthiolate is added (1:10,000), and the suspension is frozen for storage.

b. Lipid Fraction[1,2]

All operations are conducted at 5° with reagent-grade solvents that have been redistilled. Ten grams of tissue is cut into pieces and homogenized in a Waring blendor with 200 ml of a mixture of chloroform–methanol (2–1, v/v). The insoluble protein is removed by gravity filtration. Water-soluble contaminants are eliminated as follows: An Erlenmeyer flask is filled to within ½ inch of the top with filtrate and is then carefully submerged in 10 to 20 volumes of water. After 24 to 48 hours, the flask is removed, the upper phase is aspirated off, and the lower (chloroform) phase and interfacial fluff are placed in a freezer at —20°. When the interfacial (aqueous) layer has frozen, it is filtered by gravity (in the freezer). The solvent is stripped from the clear filtrate in a rotary evaporator at a bath temperature under 40°. The lipid residue is taken up in chloroform–methanol (2–1), made up to 10 ml, and stored in a brown bottle at 5°. Lipids that partition in the water phase (ganglioside, sulfatide, etc.) may be retained by using a dialysis bag to remove the methanol.[3,4]

3. IMMUNIZATION

With cell particulates, rabbits are given, over a 3-week period, nine intravenous injections of a quantity of fraction containing 5 to 10 mg of protein (albumin equivalent using the Lowry method) per injection. With lipids, a course of six intravenous injections is given, each containing 20 mg of lipid suspended in 1 ml of swine serum diluted 1–5 with saline. The rabbits are exsanguinated by cardiac puncture 4 or 5 days after the last injection. The sera are stored at —20° with merthiolate.

4. TEST ANTIGENS

Antisera containing antibody directed against lipids are selected on the basis of their capacity to react with total lipid extracts of the tissue. The reactivity of these sera with pure lipid haptens is then determined.

a. Total Lipid Extracts

A portion of chloroform–methanol solution containing 2 mg of crude lipid (the weight is determined on a separate portion which is dried *in vacuo* over P_2O_5 for at least 4 hours, and this sample is not used

[1] J. Folch, I. Ascoli, M. Lees, J. A. Meath, and F. N. LeBaron, *J. Biol. Chem.* **191**, 833 (1951).
[2] M. M. Rapport and L. Graf, *Cancer* **8**, 538 (1955).
[3] S. I. Hakomori and R. W. Jeanloz, *J. Biol. Chem.* **239**, PC3606 (1964).
[4] J. Folch, S. Arsove, and J. A. Meath, *J. Biol. Chem.* **191**, 819 (1951).

for tests) is evaporated just to the point of dryness in a stream of nitrogen at a water bath temperature of 40°. To the residue, 0.1 ml of absolute ethanol is added, the mixture is heated in a bath at 50° to 55° for 10 seconds, and then 1.9 ml of saline is rapidly added. The mixture is agitated well. In some cases flocculation occurs, but this rarely affects the final result if sampling errors are avoided during dilution.

b. Pure Lipids

In studies of pure lipids, auxiliary lipids must be added and the mixture made up in organic solvents before solution or suspension in the aqueous phase. The auxiliary lipid for use with cytolipin H is a mixture of lecithin and cholesterol (1:1, w/w).

i. Cytolipin H[5]

A stock solution having 10 μg/ml in chloroform–methanol is stored at 5°. A portion containing 2μg is mixed with 100 μg of egg lecithin and 100 μg of cholesterol (in ethanol or chloroform), and the organic solvents are removed in a stream of nitrogen as in Section F,4,a. The residue is treated with ethanol and dissolved in saline as in Section F,4,a.

ii. Cytolipin K, Galactocerebroside, and Other Glycosphingolipids

The procedure is essentially the same as for cytolipin H except that the proportions of lecithin and cholesterol may have to be varied, and the quantity of auxiliary lipid that is added may be different.

iii. Cardiolipin

A test antigen formulated with lecithin and cholesterol is available from the Sylvana Chemical Co. It contains 175 μg of cardiolipin, 875 μg of lecithin, and 3000 μg of cholesterol per milliliter of ethanol. For testing, 0.1 ml of alcoholic solution is diluted with 1.9 ml of saline. The opalescent solution does not flocculate.

iv. Forssman Substance

Since pure Forssman substance is not available, the presence in antisera of antibody to this substance is most conveniently estimated by the following hemolytic test. To 0.1 ml of antiserum and 0.1 ml of diluted guinea pig serum (representing 0.0075 ml of undiluted serum), 0.1 ml of saline and 0.2 ml of a 2.5% suspension of unsensitized sheep red cells are added. The mixture is incubated at 37° for 30 minutes in a shaking water bath, and the percentage of hemolysis is determined as

[5] M. M. Rapport, L. Graf, V. P. Skipski, and N. F. Alonzo, *Cancer* **12**, 438 (1959).

in the complement fixation test. The antiserum is studied in twofold serial dilutions, and activity is recorded as the number of "units" per milliliter of undiluted antiserum, where the "unit" is defined for 50% hemolysis. Since a good antiserum to sheep red cells possesses activity of the order of 240,000 units/ml, Forssman activity below 240 units/ml is insignificant.

c. Partially Purified Lipid Fractions

Lipid fractions that have been partially purified present a special problem, since the role of contaminating lipids cannot be assessed: the enhancement of activity by lipids other than lecithin and cholesterol has not been studied; the presence of excess lipid can be markedly inhibitory; and a number of lipids have exceptionally strong anticomplementary properties (sulfatide, cardiolipin). The procedures to be followed in such cases are empirical; in our hands, however, the following method has been found to be most consistent. Lipids fractionated solely with solvents are tested directly. Antigen titrations are begun with 100 μg of lipid at a sensitivity level of about 0.0075 ml of guinea pig serum (six 50% hemolytic units of complement in our system). Anticomplementary activity is detected by using 0.0025 ml of complement (two 50% units). Fractions obtained by column chromatography are tested directly (as above) and also in the presence of lecithin. Antigen titrations are begun with 10 μg of lipid combined with 100 μg of lecithin. These fractions are prepared in the same way as total lipid or pure lipids, with ethanol and saline. It is important to test the fractions recombined in the proportions in which they are obtained from the column, and also to test a judicious selection of groups of fractions that are free of anticomplementary interference. Fractions are tested with the largest quantity of antiserum (antibody excess) consistent with the available supply and its degree of freedom from anticomplementary properties. In general, we use 0.05 ml of a 1:5 or 1:10 dilution of rabbit antiserum for this purpose.

5. COMPLEMENT FIXATION TESTS

Since complement fixation is an indirect method of limited range (10% and 90% hemolysis occur with 0.6 and 1.6 units, respectively), and the sensitivity can be adjusted at will, a special effort is required to compare results obtained by introducing variations in technique. Three features of the method we use[6] are different from the method currently employed in most other laboratories studying antigenic activity based

[6] M. M. Rapport and L. Graf, *Ann. N.Y. Acad. Sci.* **69**, 608 (1957).

on complement fixation. First, the sensitivity is set at 6 units of complement with an end point of 50% hemolysis (0.2 ml of a 2.5% suspension of sheep red cells sensitized in a particular way is used[7]). Second, the interference of anticomplementary activity of antiserum and test antigens is tested at a level of 2 rather than 6 complement units. Third, the data are presented as isofixation curves, which relate the quantity of antiserum in milliliters to the quantity of antigen in micrograms (*on linear axes*) needed to give the same end point of 50% hemolysis with a given quantity of complement.[6]

The advantages of these features are described briefly below. The number of units per se is much less important in establishing sensitivity than the actual quantity of guinea pig serum used. The units are defined here in the absence of added calcium and magnesium, and six 50% units thus correspond to about 0.0075 ml of guinea pig serum. In the presence of added calcium and magnesium, six units would correspond to about 0.0040 ml of guinea pig serum,[6] and the test would therefore be almost twice as sensitive. (Calcium and magnesium are not used because they enhance the anticomplementary activity of some lipids and thereby increase the level of interference, particularly at intermediate stages of purification.) The tests for anticomplementary interference are set at a much greater level of sensitivity because of the limited range of the immune hemolysis reaction serving as indicator. Thus, if a given antigen displayed anticomplementary interference capable of eliminating 4 units in a 6-unit test, this would not be detected in a 6-unit control: the 2 units of activity remaining would be sufficient to produce complete hemolysis. Therefore, the difference between the sensitivity level used to detect interference and the sensitivity level used for measurement of immune complex formation is directly related to the reliability of the test. The constraint that prevents us from making this difference much larger is the limited availability of antibody. Most antisera do not contain a sufficiently high level of antibody against lipid to form enough immune complex to fix 12 units of complement (0.0150 ml of guinea pig serum).

Lastly, the use of isofixation curves to determine and compare the characteristics of antigen–antibody interaction of different immune systems and to communicate the results of complement fixation experiments is one of the most valuable features. Inasmuch as the unit of complement activity is variable and depends on many factors (the number and quality of the sheep red cells, the method of sensitization, the conditions

[7] A. B. Wadsworth, "Standards Methods of the Division of Laboratories and Research of the New York State Department of Health," 3rd ed., p. 379. Williams Wilkins, Baltimore, Maryland, 1947.

employed for immune hemolysis, and the presence of such cations as Ca^{++} and Mg^{++}), the information that is of greatest value for purposes of comparison and communication is the quantity of antigen and the quantity of antiserum that characterize the reaction in the zones of antibody excess and antigen excess. The isofixation curve is simply a line curve that represents the information obtained in a box titration,[6] in which several antigen titrations are carried out with different quantities of antiserum, and several antiserum titrations are carried out with different quantities of antigen. The end-point dilutions are then converted to quantities by taking their reciprocals, and the values are plotted

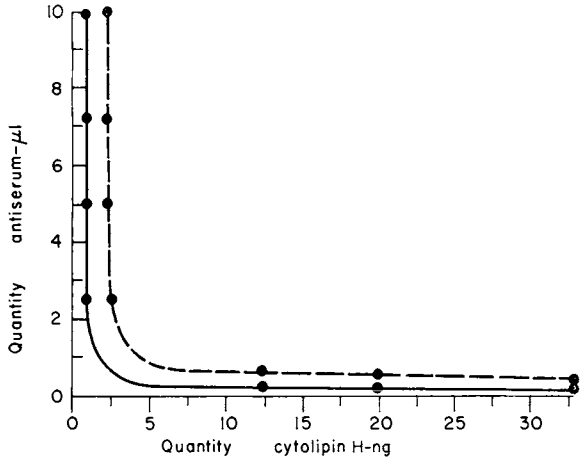


FIG. 1. Isofixation curves showing reactivity at different levels of sensitivity of cytolipin H with rabbit antiserum prepared to human reticulum cell sarcoma: solid line, with 3 units of complement (3H); broken line, with 6 units (6H).

on linear axes (Fig. 1). It is this type of information that has proved to be so useful in studies based on the direct method of precipitation. With immune systems that are operating properly, the isofixation curves show little slope in either antibody excess or antigen excess. In general the presence of marked slope in antibody excess indicates insufficient antibody[6] or anticomplementary property of the antiserum. Marked downward slope in antigen excess indicates anticomplementary property of the antigen or the presence of multiple immune systems.

Primary reaction. To 0.05 ml of appropriately diluted antiserum is added 0.1 ml of guinea pig serum diluted to contain sixty 50% hemolytic units/ml. This is followed by 0.1 ml of antigen dilution in saline. The volume is brought to 0.3 ml by adding 0.05 ml of saline (this final

addition permits small variations to be introduced, if necessary, in the volumes of the first three reagents). Six to ten tubes representing twofold serial dilutions are used for antigen titrations, and six tubes based on an arithmetic progression are used for antiserum titrations. The incubation is carried out for 2 hours in a water bath at 20°.

Indicator reaction. To each tube, 0.2 ml of a standardized 2.5% suspension of sensitized sheep cells is added, and hemolysis is allowed to proceed

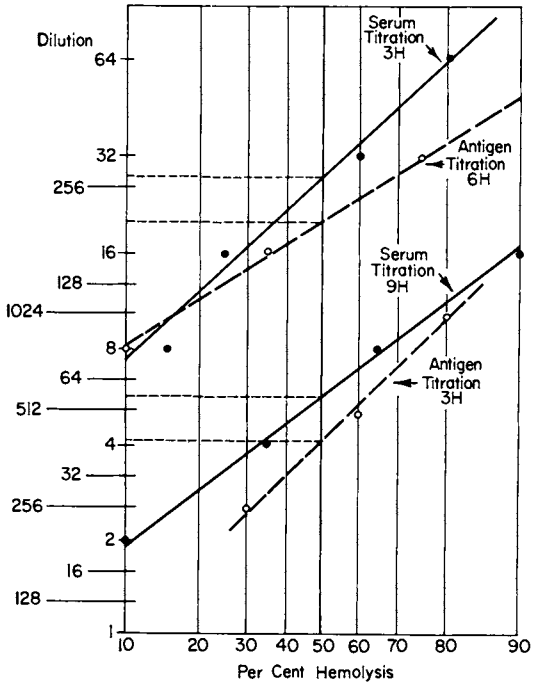


FIG. 2. Estimation of dilution titers for 50% hemolysis by graphic interpolation from a plot of log dilution (ordinate) versus logit hemolysis (abscissa). Straight lines usually are obtained when inhibition zones are absent and when the quantities of antigen and antibody are not close to the zone of equivalence.

at 37° for 30 minutes with mechanical shaking. The tubes are then chilled, and 1 ml of ice-cold saline is added. After mixing, the cells are centrifuged and the percentage of hemolysis is determined by reading the optical density at 545 mμ in a Coleman Junior spectrophotometer. The sheep cells are standardized by adjusting a 5% suspension until the absorbance is 0.58 in a 10-mm cuvette for a sample lysed with 14 volumes of distilled water. The adjusted suspension is then sensitized

with an equal volume of rabbit hemolysin, the concentration of the latter being the lowest that will produce maximal sensitization of the cells.[7]

Evaluation of reactions. The indicator reaction is regarded exactly as one would a chemical indicator. However, it is rare that a given tube in a titration will show exactly 50% hemolysis, and therefore the end-point value must be obtained by interpolation. This is done most conveniently on a log-logit plot (Fig. 2), since straight lines are usually obtained in zones removed from equivalence (the curved portion of the isofixation curve, and one of little importance in most studies of this type). As an example of the calculation of a point on the isofixation curve, suppose that, with a 1:10 dilution of an antiserum, it is found by interpolation that 50% hemolysis occurs at an antigen dilution of 4.1, the original concentration of antigen being 1 mg/ml (Fig. 2, lowest line). Then the quantity of antiserum for this point is 0.05 ml of a 1:10 dilution, or 0.005 ml. The quantity of antigen, if it had not required dilution, would have been 0.1 ml of 1 mg/ml, or 100 μg. Since the end-point dilution is 4.1, the quantity of antigen is 100 divided by 4.1, or 24.5 μg. These values, 0.005 ml of antiserum and 24.5 μg of antigen, determine one point on the curve. The complete curve may be obtained with five or six points—three in antibody excess and two or three in antigen excess. The quantities of antigen used for antiserum titration in the zone of antigen excess are about five to ten times the limiting quantity of antigen determined in the zone of antibody excess.

6. ABSORPTION STUDIES

Absorption studies with lipids have been very few.[8,9] Auxiliary lipids are important for absorption, and the requirements differ from those used to prepare the test antigens in complement fixation. In general, lecithin enhances interaction with antibody, whereas cholesterol has little effect. The optimal result is secured with only a small quantity of lecithin. Antigens are prepared in the same way as test antigens for complement fixation. The antiserum is then added, and, after incubation for an appropriate interval, the reaction mixture is centrifuged for at least 1 hour at 20,000 *g*. This is necessary to remove immune complexes from the supernatant, so that anticomplementary interference will be kept minimal. Residual antibody in the entire supernatant is then determined by complement fixation.

[8] M. M. Rapport, N. F. Alonzo, L. Graf, and V. P. Skipski, *Cancer* **11**, 1125 (1958).

[9] M. M. Rapport, L. Graf, L. A. Autilio, and W. T. Norton, *J. Neurochem.* **11**, 855 (1964).

7. PURIFICATION OF LIPIDS

It is not possible to present details of lipid purification, collection, and storage here. Almost all purification methods rely on chromatographic separations on columns. Of the principal adsorbents (silicic acid, Florisil, alumina, and DEAE-cellulose), silicic acid is the most useful for primary separations,[10,11] and most readily permits recovery of all lipids. Chromatographic fractionation should be applied only to lipids from which water-soluble impurities have been removed. It is best to conduct all such chromatographic procedures in the cold, and to protect the separated fractions from light and oxygen. Oxidized lipids become markedly anticomplementary. The lipids should be stored in solution (toluene, chloroform, chloroform–methanol) in the cold, under nitrogen and in brown bottles. It is interesting that many different haptens are found in a fraction eluted from silicic acid by increasing the solvent polarity from hexane–ethanol (70:30 v/v) to ethanol, and thus they are eluted between the ethanolamine phosphatide fraction and the choline phosphatides. These haptens include cytolipin K,[11] cytolipin G,[10] cytolipin R (from rat lymphosarcoma),[13] and Forssman substance from horse kidney.[12]

[10] L. Graf, M. M. Rapport, and R. Brandt, *Cancer Res.* **21**, 1532 (1961).
[11] M. M. Rapport, L. Graf, and H. Schneider, *Arch. Biochem. Biophys.* **105**, 431 (1964).
[12] M. M. Rapport, N. F. Alonzo, and L. Graf, unpublished studies (1961).
[13] M. M. Rapport, H. Schneider, and L. Graf, *Biochim. Biophys. Acta* **137**, 409 (1967).

CHAPTER 2

Production of Antiserum

A. Preparation of Immunogens*

1. INTRODUCTION

Materials to be used as antigens fall into several classes: soluble and insoluble proteins, viruses and subcellular particulates, and entire complex cells such as blood cells and bacteria. The numerous possibilities can be discussed only in principle.

Materials to be injected should be kept sterile, in order to assure that the immunogen has not been degraded prior to injection and that bacterial endotoxins have not accumulated. Soluble materials should be passed through sterilizing filters at the time of preparation. It is not always possible to make sterile preparations of particulates, but this goal can be approached closely if reasonable surgical care is taken. When applicable to an insoluble immunogen, lyophilization under sterile conditions can reduce markedly the population of viable microbes, particularly when toluol has been added before freezing. Materials that can withstand drying and defatting can be sterilized practically by being suspended in clean ether for several hours; then the ether is poured off, and the remainder is allowed to evaporate through a sterile cotton plug, the tube being laid flat on its side. Preservatives that do not alter antigenicity, such as 0.5% phenol or merthiolate (1:10,000 or 1:30,000), can be added at the time of preparation, but such materials are bacteriostatic in character and do not assure death of bacteria or molds even over many weeks. Incorporated into Freund's adjuvants (see Section A,2,g) contaminated antigens will be dispersed as water droplets and remain at 37° for hours or days before their liberation by cellular attack on the continuous phase of liquid hydrocarbon. Accordingly, unless solutions are sterile it can be advantageous to add an antiseptic in the aqueous phase that will retard microbial growth yet not affect stability of the emulsion or have adverse effects on the animal.

2. SOLUBLE PROTEINS OR PROTEIN–HAPTEN CONJUGATES

These materials range from purified proteins to complex mixtures such as serum or plasma or extracts of ragweed pollen. They may be injected

* Section 2,A was contributed by Merrill W. Chase.

in the fluid state, adsorbed to insoluble particles such as alumina, or incorporated in matrix materials such as agar, calcium alginate, or Freund's adjuvants ("complete" or "incomplete," depending on whether mycobacteria are incorporated).

a. Fluid Solutions

Sterile solutions, diluted suitably and divided into portions applicable to the injection schedule, may be kept fluid or held frozen (—14° or lower) conveniently in sterile screw-capped tubes (150 × 16 mm, or 100 × 16 mm). It is best not to clarify by very high or sustained centrifugal speeds, as aggregated serum globulins and albumins with dimers and higher polymers present are more immunogenic. Indeed, slight artificial aggregation of human globulin by heating at 63° for 10 minutes in 1% solution (in saline or in barbital buffer pH 8.6, $\Gamma/2 = 0.1$) has been found to be a useful practice.[1]

b. Adsorption of Soluble Proteins to Insoluble Colloidal Carriers

The effectiveness of alum precipitates appears to have been demonstrated first in 1926 on injection of horses with diphtheria toxoid.[2] Alumina cream or aluminum phosphate, prepared beforehand, is mixed with antigen. Alternatively, alumina can be formed in the presence of the antigen (Section A,2,b,ii), a method that we prefer in general.

The amount of a given soluble protein that can be bound by the adsorbent, especially at pH 7.2 and 0.15 *M* NaCl, should be known before preparation of the immunogen. This end can be determined readily by mixing several concentrations of antigen with small, constant volumes of adsorbent (either preformed or formed in the presence of the antigen as in Section A,2,b,ii), clarifying after 20 minutes, and testing portions of supernatant with equal volumes of 10% aqueous trichloroacetic acid.

The speed of desorption from such complexes can be appreciable: with alumina, the amount of BGG remaining bound after 2 days is perhaps one-fifth (data from Talmage and Dixon,[3] revised in accord with the amount that could have been bound initially), yet there is indubitable enhancement of antigenicity (Section B,1,c, Table I).

i. Preformed Absorbents

(*a*) *Willstaetter's Alumina, Type Cγ* ($Al_2O_3 \cdot 3H_2O$).[4] Dissolve 34 gm of aluminum ammonium sulfate in 50 ml of hot water at about 75°

[1] H. G. Kunkel, personal communication.

[2] A. J. Glenny, C. G. Pope, H. Waddington, and U. Wallace, *J. Pathol. Bacteriol.* **29**, 38 (1926).

[3] D. W. Talmage and F. J. Dixon, *J. Infect. Diseases* **93**, 176 (1953).

[4] E. Bauer, *in* "Die Methoden der Fermentforschung" (E. Bamann and K. Myrbäck, eds.), Vol. 2, p. 1426. Academic Press, New York, 1945.

(*a*). Heat 10 gm of $(NH_4)_2SO_4$ in 300 ml of water to 60°, and add 21.5 ml of 20% ammonia (*b*). Pour (*a*) into (*b*), and maintain at 60°; stir well for 15 minutes at least, the precipitate gradually becoming flocculent. Dilute to 2 liters; allow to settle, and remove supernatant by aspiration with aid of a hollow glass "cane." Fig. 1. Transfer to four 250-ml centrifuge bottles. Sediment, strip, and wash once with water at low speed (1100 rpm for 5 minutes). For the next washing, add 2 ml of 20% ammonia to destroy any residual basic aluminum sulfate. Continue washing until the supernatant fluid remains slightly turbid, and wash twice more* Bring the volume to 350 ml with water, dispense

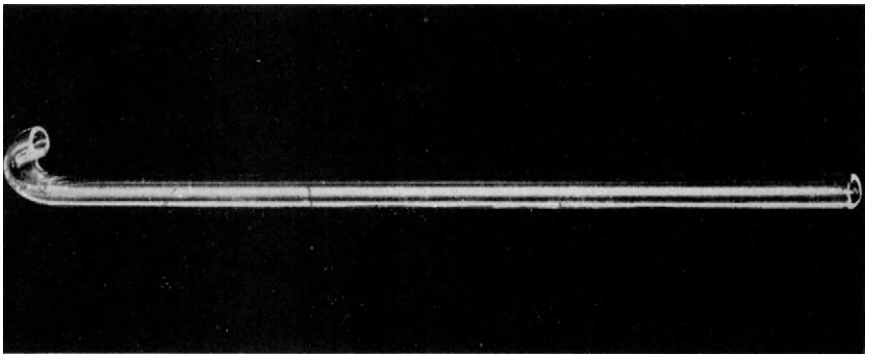


FIG. 1. Glass "cane" to remove supernatant solution from lightly sedimented precipitates by a suction device. It is drawn from 7-mm O.D. tubing, about 20 cm high. The tip is flattened to an oval 3×5 mm (not well shown) before bending.

as 50-ml portions in screw-capped "milk dilution" bottles (Pyrex No. 1372, Kimble No. 14925), and autoclave with caps loose. Allow to cool, and tighten caps. Ascertain the dry weight on 4 ml of the product (about 12 mg/ml expected), evaporating and drying finally over P_2O_5 for several days.

One milliliter of this product at 9.9 mg of dry weight has absorbed 1.25 mg of diphtheria toxoid (0.2 mg of toxoid-N).[4]

(*b*) *Simple Alumina, Gelatinous.* The Willstaetter product is superior for storage as a permanent, autoclaved material. A simpler product can

* For complete conversion to the γ-modification, the product is allowed to stand for several weeks in the refrigerator. It is used usually after ripening for 3 months, but the process of ripening appears of questionable value for adsorption of protein antigens.

be prepared as needed in the following way, with sterile technique. Apart from sterile glassware, one sterile reagent is needed: 10% solution of aluminum potassium sulfate (potassium alum, $AlK(SO_4)_2 \cdot 12H_2O$, N.F. XI or Mallinckrodt's "photopurified" granular product). The alum is dissolved in water, sterilized by passage through a Millipore or similar membrane filter, and dispensed sterilely in 150 × 16-mm screw-capped tubes.

To 10 ml of 10% aqueous potassium alum in a 50-ml screw-capped centrifuge tube, add slowly, with mixing, 22.8 ml of 0.25 *N* NaOH. After 10 minutes, centrifuge at low speed for a few minutes (do not pack hard), strip off the supernatant containing K_2SO_4, and wash once with sterile distilled water at the same total volume. Resuspend to 10 ml for use (about 10 mg of dry-weight alumina per milliliter). Adsorptions with this nitrogen-free product allow ready determination of nonadsorbed protein. The same type of product can be made also with ammonium alum.

(*c*) *Aluminum Phosphate.*[5] Dissolve 7 gm of $AlCl_3 \cdot 6H_2O$ in 70 ml of water, and dilute further to 420 ml. Add slowly with constant stirring 70 ± 0.6 ml of 15.75% (w/v) $Na_3PO_4 \cdot 12\ H_2O$; the actual amount is determined by a trial run with small amounts of the same solutions. Let the precipitate form for 5 minutes, and check the reaction of the supernatant as pH 5.0. The solution is designed to be approximately isotonic (NaCl) and to be sterilized by autoclaving at this point (about 13 mg/ml, dry weight).

With sodium acetate added for pH adjustment and merthiolate for bacteriostasis, $AlPO_4$ has absorbed diphtheria toxoid to the extent of 86 mg of toxoid/gm when the toxoid was present at a concentration of 54–70% of theoretical purity. The product is injected without need for washing.

(*d*) *Blood Charcoal.*[6] Charcoal made from blood possesses especial adsorptive properties. With a few materials of very low antigenicity (Section B,1, Table I, part 1), it has proved particularly useful in inciting production of antibody when injected into rabbits intraperitoneally on a weekly schedule. The charcoal is washed several times with large volumes of 0.1 *N* HCl, sedimented by centrifugation in 250-ml centrifuge bottles and stripped with a glass cane (Fig. 1). It is then washed free of acid with excess water, and pressed to a cake between sheets of hardened filter paper. The cake is conveniently dried in an oven. The rather fluffy powder is sterilized by dry heat in an oven.

[5] L. B. Holt, *Developments in Diphtheria Prophylaxis.* Heinemann, London, 1950.
[6] K. Landsteiner, *J. Exptl. Med.* **75**, 269 (1942).

ii. Adsorption onto Nascent Alumina

Adsorption occurs very well indeed when alumina forms in the presence of protein. In the writer's laboratory this procedure has been used most satisfactorily to prepare total volumes as small as 2.5 ml for injection. Besides sterile 10% potassium alum (see Section A,2,b,i(b)), sterile stirring rods (3 mm in diameter), capillary pipets, and tubes are used. Prepare 1.0 *N* NaOH and 0.25 *N* NaOH, and make a trial floc (2 ml of 10% alum, 2 ml of water, 1 drop of 0.02% aqueous phenol red, 1.0 ml of 1.0 *N* NaOH, plus 0.25 *N* NaOH to a bare trace of pink) as a guide to the amounts that will be used (1.00-ml pipets are adequate). (Theoretically, 10 ml of 10% potassium alum requires 6.3 ml of 1.0 *N* NaOH.) Also, if a particular protein has not been used previously, the requisite amount of floc to adsorb fully should be determined; for example, repeat the trial floc with 0.2 ml of 10% alum + 0.2 ml of water in five small tubes, adding 7, 6, 5, 4, and 3 mg of protein, respectively. Neutralize approximately with 0.45 ml of 0.25 *N* NaOH in the presence of phenol red, mix, let stand for 20 to 30 minutes, and centrifuge; test portions of the supernatants with equal volumes of 10% aqueous trichloroacetic acid.

The actual adsorption is done sterilely, and alkali is added to neutralize immediately after the protein is added to the alum, but with care being taken to mix well on each addition to avoid high local concentrations of alkali. The pH is checked by removing a droplet with a sterile rod to a porcelain depression plate, and adding 2 drops of distilled water and 1 drop of 0.02% aqueous phenol red indicator.

After standing for 30 minutes, the floc is diluted with sterile water or saline and is centrifuged at low speed to avoid hard packing. The supernatant is stripped with a sterile capillary pipet, and the floc is suspended in saline to the calculated concentration. The single washing is sufficient to reduce the potassium concentration adequately to allow even intravenous injection. Amounts adsorbed readily per gram of potassium alum have been 200 mg of bovine γ-globulin (BGG), 250 to 300 mg of bovine serum albumin, 325 mg of ovalbumin, 240 mg of picrylated BGG, 145 mg of picrylated guinea pig serum protein, and not less than 10 mg of unheated tuberculoprotein or the tuberculin preparation PPD. Stevens and Riley[7] found different figures for maximal absorption per gram of potassium alum: 690 mg of BGG and 200 mg of human serum albumin. Others have assumed that as much as 1200 mg of BGG or 2700 mg of ovalbumin would be adsorbed per gram of alum.[3] (The actual

[7] K. M. Stevens and P. A. Riley, Jr., *J. Immunol.* **76**, 181 (1956).

absorbent, alumina cream, has only about one-tenth the weight of alum used.)

A typical preparation, not approaching the maximal adsorptive capacity, might be: 5 mg of BGG in 3.0 ml of saline, 1.6 ml of 10% potassium alum, 0.7 ml of 1.0 *N* NaOH, 0.45 ml of 0.4 *N* NaOH, and 0.2 ml of saline to establish a 5.0-ml volume for washing and resuspension.

c. Calcium Alginate as Repository

Sodium alginate of controlled polymer length has adjuvant properties, which are increased markedly by conversion *in situ* to calcium alginate.[8a,*] Calcium chloride, used as a sterile 3.75% aqueous solution, will be needed. Two syringes are prepared, one holding a mixture of one part of 4% sodium alginate and one part of protein or of bacterial suspension contained in a calcium-deficient medium, the other an equal volume of $CaCl_2$. After injecting the antigen mixture into a thigh muscle or subcutaneously, grasp the hub of the needle with forceps and substitute the $CaCl_2$ syringe. Inject a volume equal to the 2% alginate–antigen suspension. The injection elicits no evident pain. Calcium alginate will form *in situ* as an essentially non-irritating, slowly absorbed white mass. Usually 1 ml is injected from each syringe.

Another method consists in chelating calcium to EDTA (disodium ethylenediaminetetraacetic acid) and incorporating it, in three times the theoretical amount, in the alginate–antigen mixture, allowing dissociation from EDTA *in vivo*. (The speed of dissociation is not described in ref. 8a.) Dissolve 6.7 gm of EDTA in 20 ml of water, then 1.5 gm of $CaCl_2$; back-titrate from pH 11.0 to pH 7.2 or 7.0 with concentrated HCl. Bring the volume to 33 ml with water, and sterilize. Mix one volume of 4% sodium alginate with one-sixth volume of antigen and one-third volume of chelated calcium; inject 1.5 ml.

d. Polyacrylamide Gel as Repository

The difficulty of eluting protein bands quantitatively from selected gel strips following electrophoresis suggested injection of the polyacrylamide gel intramuscularly into animals following homogenization of the gel in a chilled apparatus.[8b] The method proved to be quite efficient for inciting antibody-production to macroglobulin with amounts as small as 0.35 gm of gel containing 30 μg macroglobulin. Entrapping antigen within the matrix of synthetic polymers is described also.[8b] A preparation for

[8a] C. R. Amies, *J. Pathol. Bacteriol.* **77**, 435 (1959).

* Sodium alginate is available from Colab Laboratories, Inc., Chicago Heights, Illinois, as 10-ml vials of autoclaved 4% solution.

[8b] M. Weintraub and S. Raymond, *Science* **142**, 1677 (1963).

injection is conveniently made sterilely in 10 ml amounts in a 13 × 100 mm tube.* Dissolve the antigen (e.g., 50 mg) in 4.30 ml of 2% saline solution. Add 1.25 ml aqueous 28% acrylamide, 2.5 ml aqueous 2% solution of *N,N'* methylenebisacrylamide, and 1.25 ml containing 3 μliters of tetramethylenediamine (add 0.23 ml to 100 ml saline). Mix thoroughly, add 0.7 ml of freshly prepared 1% ammonium persulfate in water, mix at once and cap the filled tube to exclude air. Gelation occurs in 90 minutes at 37°; do not agitate. Break the polymer mechanically with 2 volumes of saline, as by use of an Omnimixer chamber, wash it once with saline, and suspend to 15 ml for intramuscular injection. A volume of 5–10 ml per rabbit should be sufficient divided among 2 to 4 sites.

e. Bentonite Used as Adjuvant

Bentonite has been employed successfully as adjuvant in mice for stimulating synthesis of antibovine γ-globulin[9] and antibovine serum albumin. (Details of the technique follow information generously contributed by Dr. H. N. Claman.) A stock bentonite solution, prepared as described below, adsorbs part of the protein, and the unadsorbed protein is removed by centrifugation. Mice responded very favorably when 1.9 mg of BGG was injected intraperitoneally in the form of bentonite-adsorbed material,[9] and 2 mg of bentonite-BSA produced to two to five times as much antibody in 14 to 28 days as did soluble BSA. For intravenous injection, care must be taken to resuspend thoroughly and to eliminate bentonite clumps. No comparison has been undertaken to equate efficiency with alum-adsorbed mixtures; it gives detectable antibody sooner than when Freund's adjuvant is used. The ultimate concentration of antibody secured by the several methods is not given. The preparations are injected into mice in nonsterile form and are prepared fresh each week.

i. Preparation of Stock Bentonite[10a]

In glassware washed free of detergents, suspend 0.5 gm of Wyoming bentonite (BC micron or No. 200 standard Volclay) in 100 ml of distilled water, and let run in a Waring blendor for 1 minute; repeat for 1 minute after waiting for 5 minutes. Transfer to a glass-stoppered graduate, and bring to 500 ml with distilled water. Shake, and let settle for 1 hour. Decant the supernatant in six 100-ml Pyrex centrifuge tubes (heavy-

* Huminori Kawata, unpublished data, 1967. The gel is superior to that described in ref. 8b.

[9] H. N. Claman, *J. Immunol.* **91**, 833 (1963).

[10a] K. J. Bloch and J. J. Bunim, *J. Am. Med. Assoc.* **169**, 79/307 (1959).

duty), and centrifuge for 15 minutes at exactly 1300 rpm in a No. 240 head International centrifuge, size 2. From the supernatant, recover the particle size that sediments on further centrifugation for 15 minutes at 1600 rpm. Discard the second supernatant. Suspend entire sediments in 100 ml of distilled water, and let run in the Waring blendor for 1 minute. This stock suspension has been observed to retain its adsorptive properties for 6 months. Claman keeps this stock in 0.15 *M* NaCl adjusted to an optical density of 0.7 at 655 mμ (Coleman colorimeter with 8-215 filter) and preserves it under refrigeration for several weeks.

ii. Adsorption of Protein

Mix in proportions of 2 ml of saline containing 40 to 50 mg of BGG with 1 ml of standardized bentonite, and stir magnetically at below 20° for 20 to 30 minutes. Centrifuge at 3000 *g* for 5 minutes, and resuspend the bentonite particles to the original volume. Radiotracer methods have indicated that about 20% of BGG binds to the bentonite in this procedure. (Only one-sixth less protein was bound when the protein concentration was reduced to 20 to 25 mg per 2 ml.) In other experiments, 40 to 60% was found to adsorb. The amount bound to bentonite appears to be fixed in a rather stable manner.

Several quite simple methods are available to determine the amount of protein remaining in the supernatant after the bentonite is sedimented by centrifugation; it is possible that clarification by passage of the supernatant through a nonsterile Millipore membrane would be helpful. Claman chooses to add a tracer amount of the same protein labeled with I^{131} or I^{125} and to compare the relative radioactivity of supernatant with that of the precipitate resuspended to the volume of the supernatant, with use of a well-type γ-ray scintillation counter.

f. Methylated Bovine Serum Albumin as Adjuvant

A method that has proved very efficient for complexing negatively charged materials (DNA, polynucleotides, and so on) and producing antibodies is to mix the antigen with methylated bovine serum albumin, which has a high positive charge, and to use the resulting precipitate for injection.[10b]

Methylated bovine serum albumin,[10c] described in Chap. 1,E,8,b,(i), is usually recovered by washing twice with excess methanol to reduce

[10b] O. J. Plescia, W. Braun, and N. C. Palczuk, *Proc. Natl. Acad. Sci. US.* **52**, 279 (1964); O. J. Plescia, N. C. Palezuk, and W. Braun, *Science* **148**, 1102 (1965).
[10c] J. D. Mandell and A. D. Hershey, *Anal. Biochem.* **1**, 66 (1960).

the acid promptly and then with ether before drying and pulverizing; it dissolves with difficulty. A readily soluble final product can be secured by dissolving the well-washed (methanol) precipitate in water, neutralizing, and drying at —16° by lyophilization. MBSA can be stored as a sterile neutral solution in 0.15 *M* NaCl, scanning in 0.25 *N* acetic acid at 277 mμ like unmodified BSA.

The complexing is made by mixing a solution of negatively charged antigen with sufficient 1 % solution of methylated BSA to provide equal weights of both. The resulting precipitate is suspended to an antigen concentration of 0.25 mg/ml and emulsified in an equal volume of Freund's complete adjuvant (Chap. 1,E,8,b).[10b]

g. Freund's Adjuvant as Repository[11]

The dispersion of water microdroplets in paraffin oil was introduced by Freund, who used several types of water-in-oil (W/O) stabilizers, from lanolin, to lanolin derivatives (Aquaphor, Falba, Protegin-X), to mannide monoöleate, Arlacel A.[11] The latter emulsifier* is used chiefly at present, partly because it can be injected in man. Some evidence exists, however, that enzymes *in vivo* can serve to break it down. For animal work the writer prefers Aquaphor† and a heavier paraffin oil (Saybolt viscosity 175 to 180 seconds at 100°F).

Freund's "complete" adjuvant contains mycobacteria (0.5 mg/ml); adjuvant without mycobacteria is called "incomplete." Evidence for local retention of antigen at the site of footpad injection is convincing, elimination occurring with an antigen "half-life" of 14 days.[8] The choice of "complete" versus "incomplete" emulsions rests on special considerations. A prolonged immunological stimulus is experienced when incomplete adjuvant is used, but it may be somewhat greater with complete adjuvant.[11] Mycobacteria can add a new quality: thus, guinea pigs respond by synthesizing larger amounts of precipitating antibody now termed 7 S γ_2 whereas without mycobacteria the synthesis is largely 7 S γ_1.[12,13]

Freund devised his "complete" emulsion for intramuscular injection.

[11] J. Freund, K. J. Thomson, H. B. Hough, H. E. Sommer, and T. M. Pisani, *J. Immunol.* **60**, 383 (1948).

* A special product, partly purified, is available from the Atlas Powder Company, Wilmington, Delaware.

† Available from Duke Laboratories, South Norwalk, Connecticut.

[12] R. G. White, G. C. Jenkins, and P. C. Wilkinson, *Intern. Arch. Allergy Appl. Immunol.* **22**, 156 (1963).

[13] B. Benacerraf, Z. Ovary, K. J. Bloch, and E. C. Franklin, *J. Exptl. Med.* **117**, 939 (1963).

With the increasing popularity of injection into footpads, the original Freund formula unfortunately has been retained. Furthermore, repeat injections of the same "complete" emulsion are often made thoughtlessly into the same feet, with resultant breakdown of tissue in species that develop hypersensitivity to the mycobacteria (starting usually within 10 days). Later injections can be made to advantage with the amount of mycobacteria greatly reduced or absent.

i. Commercial Adjuvant and Preparation of Emulsions

Freund's adjuvant is available commercially* in 10-ml ampules containing sterile light mineral oil and Arlacel A ("incomplete"), and 10 ml ampules of the same product containing heat-killed *Mycobacterium butyricum* (0.5 mg dry weight per milliliter), termed "complete." Also, desiccated killed mycobacteria are available in 100-mg amounts.* Freund's adjuvant is designed for emulsification with an equal volume of protein solution or lyophilic suspensoids such as bacterial vaccines at pH 7.2 to 7.4. "Complete" and "incomplete" adjuvants can be blended before use to vary the content of mycobacteria.

The "mixing" is largely a shearing effect, owing to the unequal viscosities of water and paraffin oil. Blending should be done under sterile conditions, and air should be excluded, else its compression and decompression during injection will continue to eject emulsion from the needle. Blending is done neatly when syringes are used in pairs, each having twice the capacity of the total volume being mixed. The syringes are connected by "double-hub" needles,† or, more flexibly, by connections of polyethylene tubing size PE 200.‡ For sterilization, 65-mm lengths of tubing are kept immersed in benzalkonium chloride (Zephiran chloride, Winthrop), 1:750. These are removed with sterile forceps as needed and rinsed with sterile water, and the midportion is grasped by the fingers. Each end is passed through the female end of a sterile metal "plastic tubing adapter"§ and caused to flare by being rotated while held

* Difco Laboratories, Detroit, Michigan. Packages of six ampules of 10 ml are available as follows: complete adjuvant containing *Mycobacterium butyricum* (No. 0638-60) or *M. tuberculosis* H37 Ra (No. 3113-60); incomplete adjuvant (No. 0639-60). Dried mycobacteria are sold separately in 100-mg amounts: *M. butyricum* (No. 0640-33), *M. tuberculosis* H37 Ra (No. 3114-33).

† Available from Eisele and Co., Nashville, Tennessee, as item 3000, gauges, 17, 18, 19, 20, 21, 22 in lengths (inches) of 1, 1.25, 1.5, or 1.75; or from Becton Dickinson, Rutherford, New Jersey, as #18—1¾ inches (¼-inch shank) or #20—2⅞ inches (1½-inch shank).

‡ Clay-Adam's "Intramedic" tubing, medical formulation PHF, ID = 0.055 inch.

§ Clay-Adam's No. A-1025, size C, sold singly. Two of the plastic tubing adaptors are required for each "gang."

close to the flame of a microburner: the flared tip is then secured when the male end of the fitting is screwed in place. Such "gangs" can be made in advance, and sterility secured by inserting sterile male plugs.*

Whatever the device, it is best to coat both (dry) syringes by drawing up some paraffin oil, rotating the barrel, and then ejecting the oil. One syringe then receives the adjuvant, the other the aqueous phase. The syringe tips are wiped free of oil by sterile dry squares of Kleenex. The connecting device is attached to one, the air expelled, and the second syringe attached. At this point, it is often useful to cool the connected syringes in the refrigerator for 30 minutes. By alternate rapid expulsions of syringe contents, the phases blend and the water disperses. Increasingly greater pressure will be required. Forty or fifty strokes are minimal. If free fluid is seen on releasing the pressure for a few minutes, the syringes should be chilled before the procedure is continued. The final test of droplet size can be made by transferring a minute droplet of emulsion to a glass slide, dropping on a cover glass (No. 1, ¾-inch square), and tapping gently with the point of a pencil to flatten. Microscopic examination at 450× will serve to reveal unevenly dispersed water and to detect the process of coalescence of water droplets. Emulsions for injection should be stable. There is no difference in preparation whether "complete" or "incomplete" adjuvant is used.

We prefer all-glass syringes for injecting emulsions. The high pressures needed are not easily applied through plastic syringes; and rubber-tipped plungers in the glass–plastic combination are not compatible with paraffin oil. The chosen syringes, dry, are loaded directly from one end of the "gang," the emulsion being expelled into the syringe, driving the plunger downward. After a small portion has entered, it is best to detach the syringe temporarily, coat the inside of the syringe, expel the air, and re-attach for complete filling. The tips of the syringes are wiped firmly with bits of sterile Kleenex, and needles are then attached with a firm twisting motion. When injection is to be delayed, the tips can be capped with sterile metal caps*; avoid contamination of the exposed syringe barrels.

Another method of blending is the "micro-Waring blendor"[3]; supposedly, the vortex can be eliminated by controlling the motor speed and having a tube, sterile externally, fixed at the vortex position. Cleaning such an apparatus will be a chore, unless it is reserved, for example, for making "complete" adjuvant regularly.

For intramuscular injection, we use 2-ml Yale all-glass syringes and

* Becton, Dickinson and Company's plugs for female Luer slip, item No. 426A. Syringes are closed similarly with caps for male Luer, item 425A.

No. 20 1-inch needles, preferably metal-hubbed disposable needles. For footpad injection, No. 23 gauge needles are used.* For footpad injection in guinea pigs, 0.25-ml tuberculin syringes are most convenient, with disposable No. 23 gauge 1-inch needles.†

ii. Other Adjuvant Mixtures

The commercial adjuvants described above can be altered by blending complete with incomplete adjuvant, or by increasing the amount of mycobacteria (*M. butyricum* or *M. tuberculosis*). For greatest flexibility in varying the adjuvant, the separate ingredients can be held and blended as wanted: (a) paraffin oil, (b) emulsifier such as the special Arlacel A‡ or Aquaphor,§ (c) killed mycobacteria suspended finely in sterile paraffin oil. Materials (a) and (b) are autoclaved in 50 ml Erlenmeyer flasks at 15 pounds pressure for 40 minutes or longer. The paraffin oil used by Freund was a light oil, Bayol F,|| equivalent to Drakeol; our choice is an oil of medium viscosity (Section i above), with the aim of enhancing prolongation of the depot. The mycobacteria (killed virulent human strains of *M. tuberculosis* or the special strains H37Rv or H37Ra or *M. butyricum*) are weighed sterilely and gently rubbed in a small mortar¶ then the sterile paraffin oil is added dropwise as mortarizing is continued to give a stock suspension of 10 mg/ml. The grinding must not be extreme: it is designed to secure a fine suspension free of clumps but not to crush the organisms and so destroy their acid-fastness. The suspension is held at 4° and used for 2 months or so.

For intramuscular injection, we prefer to blend 7 ml of aqueous phase at pH 7.1–7.2 with 7.5 ml of oil phase containing 35% Aquaphor and 1.46 mg *M. tuberculosis* per milliliter. The oil phase for a unit batch of 14.5 ml of emulsion is prepared by blending 3.8 ml of paraffin oil at about 50° with 2.6 ml of molten Aquaphor by means of a sterile 4 mm glass rod, after which 1.1 ml of stock suspension (10 mg/ml) of mycobacteria in paraffin oil is added. The blending can be done with "ganged" syringes

* Individually packaged sterile needles such as Monoject 200 (20 gauge) or 250 (23 gauge), 1 inch in length.

† For cleaning, excess emulsion is removed with cotton-tipped throat applicators, followed by removal of hydrocarbon with normal heptane, rinsing with 95% alcohol, soaking in 2 *N* NaOH, then washing as usual.

‡ A special product, partly purified, is available from the Atlas Powder Company, Wilmington, Delaware.

§ Available from Duke Laboratories, South Norwalk, Connecticut.

|| This former light oil (Saybolt viscosity 53 seconds) is presently replaced by Marcol 52 (N.F.) with viscosity of 51 seconds.

¶ Mortars of internal diameter of 45 mm and depth of 25 mm are available from W. Haldenwanger Porzellanwerke, Berlin, West Germany, as No. 55-00.

(Section i above) or by shearing the water phase dropwise into the oil phase actively stirred by a motor-driven spiral blade of nickel.[14] Shearing is continued until microscopic tests show that a finely divided, stable emulsion is attained. The device employed should not create a vortex and incorporate air into the emulsion. Guinea pigs are injected intramuscularly with 1 ml of emulsion, divided among 5 sites in the nuchal muscles, and rabbits similarly with 2 ml divided among 6 to 10 sites: the dose for rabbits contains 1.5 ml of mycobacteria and 2 to 20 mg of antigen.

For footpad injection, we prefer doses of 0.03 ml injected into each footpad of the guinea pig, with 3 μg of mycobacteria per 0.03 ml of depot. (This amount of mycobacteria is adequate for full effect, and the feet of the guinea pigs do not swell progressively or ulcerate.) The small amounts required have led to slightly changed ratios. A stock "adjuvant mixture" is blended warm, as above, consisting of 4.4 ml of paraffin oil, 2.5 ml of Aquaphor, and 1.4 ml of paraffin oil containing stock suspension diluted in paraffin oil to 1 mg/ml. By means of ganged syringes (Section i above), this stock adjuvant mixture is emulsified with saline phase in the ratio 1.7:1.2, such that 0.12 ml contains 12 μg of mycobacteria and the desired amount of antigen. (A 1% protein solution provides about 0.5 mg in a total injected volume of 0.12 ml.) Tuberculin syringes of 0.25-ml capacity with disposable, metal-hubbed No. 23 gauge 1-inch needles serve for injection of two guinea pigs. Doubling the dose should be appropriate to rabbits, but relatively more antigen should be provided.

[14] M. W. Chase, *Intern. Arch. Allergy Appl. Immunol.* **5**, 163 (1954).

B. Immunization Procedures*,†

1. IMMUNIZATION OF MAMMALS OTHER THAN MAN

a. Introduction

This section describes practical procedures that usually lead to high concentrations of serum immunoglobulins. Experiences with rabbits, so frequently used in laboratories, show clearly pronounced differences apparently genetically determined between individual rabbits in capacity for response to antigens. For example, a partially inbred strain of rabbits, Family III, developed at the former Bussy Institute, proved in our tests to be greatly superior to other rabbits in response to human group A

* Section 2,B,1 was contributed by Merrill W. Chase.

† Immunization with the primary objective of producing tissue damage—nephritis, allergic encephalitis, and so on—will be discussed in a later volume.

cells (production of hemolysin for sheep cells) and in response to two other common protein antigens (precipitins) as well. Offspring of English Blue by Lilac parents, although small in stature, have proved rather better than random stocks in response to immunization.

In practice, then, it is well to immunize groups of about five rabbits and to select those antisera that have the desired characteristics. Individual rabbits that make a better antibody response than others of the group are commonly revealed by means of trial bleedings early in the immunization process. Usually these are the individuals that eventually give high titers. Special reactors may warrant handling in individual rather than in group scheduling.

While many workers desire to obtain sera with immunoglobulins in high concentrations, others are chiefly interested in investigating the primary response and the early anamnestic response of animals, with regard to appearance of classes of immunoglobulins and to heterogeneity within the classes. Pappenheimer, for example, discovered that certain early bleedings of a horse under immunization, devoid of precipitating antibodies, actually contained immature "univalent" antibodies specifically capable of *increasing* the amount of precipitate (by coprecipitation) when mixed with later bleedings and tested with antigens. And Cann *et al.*,[1] on fractionating a nonprecipitating serum, found both a frank precipitin and an inhibitor of precipitation; a mixture of the two fractions failed to react. Talmage* once remarked that the early bleedings discarded by most investigators were probably those of greatest interest from the viewpoint of immunogenesis.

It is recommended that, at least when titers are first observable in the course of immunization, trial bleedings be preserved for subsequent direct comparison with later antibody output.

As to methods used to stimulate and restimulate rabbits, very few theoretical studies exist in which the efficiencies of different methods have been compared directly and adequately. General distinctions are recognized between the usefulness of some adjuvant method versus a nonadjuvant method.

The complexity of the immunization process is emphasized by recent findings that bovine serum albumin (BSA) and bovine γ-globulin (BGG), when freed of dimers and higher complexes, produce tolerance instead of antibody synthesis. Whether mixtures of monomer and polymer forms of an antigen cause individual rabbits to respond differently remains unresolved.

[1] J. R. Cann, D. H. Campbell, R. A. Brown, and J. G. Kirkwood, *J. Am. Chem. Soc.* **73, 4611 (1951).**

* D. W. Talmage, personal communication.

This chapter cannot deal with experimental design. The choice of species for injection can determine the degree of cross-reactivity. As Campbell *et al.*[2] have well said: "At times it is necessary to select for the production of antiserum an individual closely related to the one that supplied the antigen. For example, unique minor antigenic differences in cells may not be detected when injected into a species of animal different from that of the cell donor; when used to immunize a closely related animal, however, the unique antigen may become a major foreign antigenic structure. Thus, in order to obtain antiserum that will distinguish between Rh-positive and Rh-negative human red blood cells, Rh-negative human volunteers are immunized with Rh-positive cells. If rabbits are used, the antigens common to all human erythrocytes predominate and the small amount of Rh antigen is not effective."

b. Comments on Principles of Immunization

To suggest guidelines regarding the practice of immunization is hazardous when the basis consists in random observations. Final evaluation must rest on orderly accumulation of data. We offer tentatively the following conclusions:

1. The first injections of antigen serve to "load" the new animal so that—until the animal can clear its load by antibody production or by normal catabolism—further injections may well be an embarrassment. If one chooses to inject a new rabbit intravenously with, say, 5 mg of antigen daily for 6 days, are the injections of days 4 to 6 helpful, or should they be omitted? We have been surprisingly successful with single injections of material absorbed to alumina followed by a lengthy period of rest and one fluid reinjection (see Table I, part 1).

2. While antibody production is being sustained actively even though diminishingly, reinjection of antigen will not give a useful anamestic response. This fact was clearly evident in work done in our laboratory when the primary stimulus consisted of diphtheria toxoid adsorbed to Willstaetter's alumina: in this instance antitoxin was determined with precision to within 5%.

3. When antigen is administered in still longer-lasting form, as when dispersed in water droplets within paraffin oil (Freund's adjuvant), enough antigen seems to persist to provide anamnestic recall over a long period of time, with a sustained production of antibody. As in (2) above, it proves difficult to increase the titer by injection of such animals. Accordingly, it may be expected that higher titers will be found

[2] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology: A Laboratory Text for Instruction and Research," pp. 96–112, W. A. Benjamin, New York, 1963.

by giving a larger initial dose of antigen in a first injection of Freund's adjuvant than by planning to reinject. In this connection it should be recalled that dispersal of water-soluble antigens within paraffin oil, attended by slow release, allows administration of increased amounts of toxic materials: for example, 100 MLD of tetanus toxin is tolerated in mice.[3]

When reinjection is to be practiced with use of incomplete adjuvant [see (4) below], several months should elapse. One instance of reinjection of a soluble protein that boosted the antibody level was noted 18 months after the initial injection, although Freund did not experience such a rise with typhoid organisms as antigen. In some instances, however, by earlier reinjections we have apparently been able to secure a larger amount of hapten-specific antibody.

4. In species that develop high sensitization to tuberculin, particularly guinea pigs and rabbits, reinjection of antigen in complete Freund's adjuvant is followed by a violent inflammatory response to the mycobacterial component. The pathology of this response, associated with slough of tissue (including the antigenic depot when this is close to the skin), cannot be conducive to antigenic restimulation. Secondary injections intended to be given in Freund's adjuvant should be reduced in mycobacterial content to lessen the extent of allergic reaction incurred against the continuous phase of the emulsion. Such reduction is quite practicable by blending commercially available "complete" and "incomplete" antigens in a ratio, say, of 1:8 to 1:50.

5. Other methods than the use of Freund's adjuvants should receive serious consideration. In several instances we have been surprised to find unexpectedly good titers with use of antigen adsorbed to alumina (Table I), and with minimal animal handling. The capacity to "boost" by spaced reinjections can be a real asset.

6. The production of hapten-specific antibody by injection of artifically conjugated proteins is difficult in rabbits. Antibodies appear first to the whole complex and predominate throughout the course of immunization; hapten-specific antibodies accumulate rather late, and in relatively few individuals are these antibodies present in reasonably high concentration. A greater percentage of useful sera was secured in Landsteiner's laboratory by immunizing with insoluble complexes (couplings made to horse erythrocyte stromata) than with soluble mammalian proteins as carrier (Table I, part 2). Perhaps carriers such as synthetic polypeptides may offer still greater advantage. Groups of six to ten rabbits should be employed, with the hope that one or two will yield a good hapten-specific serum. Smaller groupings such as dinitrophenyl–

[3] J. Freund, *Ann. Rev. Microbiol.* **1**, 291 (1947).

and trinitrophenyl–protein complexes offer somewhat less of a problem than substances connected to protein by azo linkages.

c. Rabbits

The rabbit is an excellent animal for obtaining precipitating serum. Various procedures that have yielded satisfactory sera with a variety of antigens are listed in Table I. Such information, which is not at all complete, still should provide some general guidance in preparing sera. Entries in the table usually do not indicate the number of responding rabbits. At least five rabbits should be immunized unless availability of the intended antigen imposes limitations. The immune sera listed in Table I, part 1, should titer in the range of 0.2 to 1.2 mg of antibody-nitrogen (Ab-N) per milliliter.

In recent years, the injection of Freund's adjuvants into the footpads has become common practice, about 0.25 ml per pad. Freund's complete adjuvant was designed originally for deep intramuscular injection, and about 2 to 4 ml was injected per rabbit; the dose was divided among several depots. In using the usual commercial preparation of complete adjuvant, each pad would receive 60 μg of mycobacteria in 0.25 ml of emulsion. Hence it is important, particularly when rabbits are raised on elevated metal grids, to reduce the amount of mycobacteria and spare their feet from overly severe tuberculin reactions with a slough of tissue.

Attention is directed particularly to the adjuvant effect of endotoxin discovered by Johnson *et al.*[4a] illustrated by hen egg albumin (Ea) and diphtheria toxoid in Table I, part 1; BSA gave a similar response (0.16 mg of Ab-N per milliliter), no greater than that secured by the adjuvant effect of alumina on BSA.

d. Horses, Goats, Sheep

The larger domestic animals are immunized by methods similar to those used for rabbits (Section B,1,c).

Horses are likely to be under immunization for long periods of time. For example, the very excellent lots of horse antihuman serum that are produced and sold by the Pasteur Institute owe their broad spectrum to long immunization. Similarly, standard diphtheria horse antitoxin titering at 600 to 700 antitoxic units (AU) per milliliter is the result of rather long and vigorous immunization: one procedure calls for three subcutaneous injections per week, ascending over 48 days from an initial dose of 20 MLD of phenolized toxin to 1,000,000 MLD, and continuing at 4-day intervals with the latter dosage for several months more.

Immunization of goats and sheep is frequently carried out by labora-

[4a] H. G. Johnson, S. Gaines, and M. Landy, *J. Exptl. Med.* **103**, 225 (1956).

TABLE I

VARIOUS METHODS OF IMMUNIZING RABBITS

Antigen	Dose per injection	Injections	Comment	Ref.
		Part 1. Soluble Protein as Antigens		
Whole serum, for multivalent antiserum				
Used (a) in Freund's complete adjuvant, then (b) with secondary stimuli	2 ml 1:3 serum in 2 ml complete adjuvant i.m. divided among 5 to 6 sites	One initial i.m. injection; later injections as stated, testing by immunoelectrophoresis (IEP)	After 4 weeks, repeat in incomplete adjuvant or give i.v. After 4 weeks, inject serum (1:3) i.v. on 3 successive days (0.5, 1 ml, 2 ml) of each week for every other week up to 8 months. Some animals are allowed long rest periods. Producers of multivalent serum are kept in long production, taking 25 ml from ear twice per week.	Chap. 14,E, Vol. III
Adsorbed to alumina, with secondary stimuli.	0.5 ml guinea pig serum adsorbed on alumina from 500 mg potassium alum	One i.m. (in 2 thighs); 2 ml 1:3 serum i.v. on day 40 and day 70	Trial bleeding 7 days after each restimulation, tested by immunoelectrophoresis.	*a*
Native proteins[b]				
In saline	5 mg in 2 ml saline, or 2 ml 1:10 whole serum	6 injections i.v. per week, rest on alternate weeks	Trial bleeding 1 week after 3rd series. After 4th series, rest 1–3 months, give 2 further series.	*c*
Serum proteins or whole serum				
Hemocyanin (6,000,000 mol. wt.)	20 mg in 2 ml saline	6–9 injections made on alternate days	Trial bleeding 7 days later. Inject further only if initial bleeding shows antibody response.	*d*
BSA				
Incorporated in Freund's complete adjuvant	50 mg BSA + 2.5 mg mycobacteria in 5 × 0.2 ml sites s.c.	Once only	Trial bleeding at 8 weeks. Reinjection not suggested in Campbell *et al.*[d]	*d*
Used with endotoxin as adjuvant	2 mg Ea + 5 μg typhoid endotoxin i.v.	3 injections at intervals of 3 days	Bleedings on day 7 yield 0.26, 0.25, 0.24, and 0.13 mg Ab-N per milliliter.	*e*

Adsorbed to alumina	2.5 mg BSA adsorbed on nascent alumina (from 100 mg potassium alum)	One i.m. (in 2 thighs)	Rested 10 weeks; injected 5 mg BSA in saline i.v.; antibody found regularly on day 7.	*a*
Ragweed antigens				
Adsorbed to alumina	0.18 mg Ragweed Antigen E on nascent alumina (from 25.8 mg potassium alum)	One i.m. (thigh)	Rested 3 months; injected 0.1 mg Antigen E saline i.v.; antibody found regularly on day 7 (average titer 0.3 mg Ab-N per milliliter.	*a,f*
Adsorbed to alumina	200 mg extractives of defatted ragweed pollen on nascent alumina (from 60 mg potassium alum)	One i.m. (thigh)	Rested 33 weeks; injected 2.5 ml whole "7%" extract i.v.; antibody found on day 7.	*g*
Diphtheria toxoid				
Adsorbed to alumina (Jerne[k]; Freund and Bonanto[i])	Diphtheric toxoid 24 Lf adsorbed to Willstaetter alumina	2 injections 4 weeks apart	Titers maximal 10 days after reinjection on day 108—50–80 AU/ml.	*a*
Used with endotoxin as adjuvant	5 Lf + 5 μg typhoid endotoxin i.v.	3 injections at intervals of 3 days; 4th injection at 29 days	Bleeding 7 days later contained 32.0 AU/ml. (Without endotoxin, titer was 1.0 AU/ml.)	*e*
Tetanus toxoid				
Adsorbed to $AlPO_4$	5 Lf adsorbed to 5 mg $AlPO_4$ s.c.	2 injections at 6-week interval	Trial bleeding 1 week later. Found, 35 AU/ml.	*j*
Fluid toxin injected	5 Lf without $AlPO_4$ s.c.	2 injections at 6-week interval	Found, 13 AU/ml.	*j*
Silk fibroin				
Adsorbed to blood charcoal	25 mg fibroin + 275 mg charcoal in 15 ml i.p.	6 injections, one every 6–7 days	Trial bleedings 6 days after 4th injection, and thereafter.*	*l,m*
Heteroproteoses[n]				
Adsorbed to blood charcoal	25 mg proteose + 275 mg charcoal in 15 ml i.p.	Up to 9 weekly injections i.p.	Trial bleedings after 6 injections, and thereafter.	*a,o*

TABLE I (*Continued*)

Antigen	Dose per injection	Injections	Comment	Ref.
		Part 2. Hapten-Specific Antibodies		
Arsanilate azohemocyanin (20 groups/mol)	10 mg in 2 ml saline	18 injections made regularly 3 times per week	Trial bleeding 3 to 5 days after last injection.	*d*
Arsanilate azo-horse stromata[p]	1.5 mg complex in 1 ml	6 injections i.v. per week; rest on alternate weeks	Trial bleeding 7 days after 3rd series, likewise after each further series.	*c*
m-Aminobenzene sulfonic-azo-horse globulin	30 mg in 3 ml in saline	6 injections i.v. per week; rest on alternate weeks	Trial bleeding 7 days after 3rd series, likewise after each further series.	*c*
"Arsanilate azo-horse serum"	10 mg in 2 ml in saline	6 injections i.v. per week; rest on alternate weeks	Trial bleeding 7 days after 3rd series, likewise after each further series.	*c*
2,4-Dinitrophenyl-BGG in Freund's complete adjuvant, 38 groups per mole	0.5 to 5.0 mg + 40 μg mycobacteria in 2 ml of emulsion	10 simultaneous sites i.m. and footpads	Trial bleeding at 39 days. Reinjected i.m. in same way, with mycobacteria reduced to 8 μg, on days 40, 55, and 98. Hapten-specific antibody was satisfactory 8 days after 4th injection.	*q*
2,4-Dinitrophenyl-hemocyanin in Freund's complete adjuvant	1.0 mg, footpad route	2 further injections after 3 and 7 months	Bleedings pooled from days 10, 11, 16, and 18 days after the 3rd injection.	Chap. 3,B,5
		Part 3. Production of Hemolysin for Sheep Erythrocytes		
Sheep erythrocytes (heterophile plus isophile antigens)	1 ml 10% rbc in saline/kg i.v.	One injection can be adequate; or 10 i.v. injections in 16 days (1, 2, 3, 4, 6, 8, 10, 13, 15, 17)	High titers 13 days after 1st injection averaging 15,500 50% hemolysin units/ml, because heterophile response is of anamnestic character. Longer series of injections may be useful.	*r*
Sheep or horse erythrocytes (boiled to inactivate isophile antigen)	"Rbc stromata" heated at 100° for 1 hour, equivalent to 10 ml blood	3 injections i.p. at 8-day intervals	Bleeding 7–10 days after last injection.	*s*

"Forssman effect"[t] of human group A erythrocytes (washed cells brought to 10-fold original blood volume)	2 ml i.v.	6 injections per week; rest on alternate weeks	Test for sheep rbc hemolysin 4–7 days after 2nd course (add fresh guinea pig serum). Continue up to 6 series of injections.	*c*
"Forssman bacteria"				
Neisseria catarrhalis E heated 60° 1 hour	Increasing doses, $\frac{1}{20}$ up to $\frac{1}{2}$ agar slant growth	Inject on alternate days i.v. for 4–7 times	Test for sheep rbc hemolysin 3 days after the 4th injection, and 3 days after later injections (complement needed).	*a,u*
Pasteurella lepiseptica washed from broth, formalin-killed	3 ml "broth-equivalent density"	4 s.c. injections on successive days; 3 i.v. injections on successive days	Trial bleeding 4 days after 7th injection; rest 4 days, give 3 i.v. injections on successive days. Bleed on day 4.	*c*
		Part 4. Antibodies to Bacteria		
Agglutinating diagnostic sera[v,w] (gram-negative, nonsporing aerobes)	Increasing doses of killed broth cultures (Table II), 1 ml 1:2, 1, 2, 3 ml, i.v.	Days 0, 4, 8, 12	Trial bleeding on day 18. Rest 3 days, reinject on days 21, 26.[x] Bleed on day 9.	*d,v* Chap. 15,D, Vol. III
Bacterial spores (see Table II)	30 I.U. density	8 weekly injections, first 2 s.c., others i.v.[y]		Chap. 15,D, Vol. III
Staphylococci (see Table II)	Increasing doses i.v. 0.1, 0.2, 0.4, 0.4, 0.6, 0.8, 0.8, 1.0, 1.0 ml	9 injections		Chap. 15,D, Vol. III
Mycobacterial agglutinating sera	1–2 ml i.v. at 3.5 I.U. density, heated 80° for 30 minutes	8 injections 4 days apart		*z*
Salmonella typhosa agglutinins, in Freund's adjuvants and other menstrua with paraffin oil	3×10^8 bacteria in 1.5 ml emulsions, i.m. (0.1 mg mycobacteria, when used)	One injection adequate; second injection at 11 weeks ineffectual except with organisms in saline	Titers at 7 weeks between 1:1500 and 1:5000.	*i,aa*

TABLE I (*Continued*)

Antigen	Dose per injection	Injections	Comment	Ref.
Pneumococcus strains, encapsulated, for type diagnosis (see Table II)	Increasing doses of 2.5 × 10^9 cells/ml during 5 injections (0.1, 0.2, 0.5, 0.5, 1.0 ml)	Days 0, 1, 2; 7, 8, 9; 14, 15, 16; 21, 22, 23	Trial bleeding on day 30. Continue injections on days 31, 32, 33; 38, 39, 40.	*d*
Part 5. Rabbit Antibodies to Rickettsiae and Viruses				
Bacteriophage				
Coliphages T_2, T_4, T_6 in filtered lysate	Phage particles in lysate i.v., increasing 10^9, 2 × 10^9 . . . 5 × 10^9 . . . 10^{11}	2 to 3 injections per week for 4 weeks	Trial bleeding at 7 days. "Titer" (Neutralization Constant/min) is usually 500 — 1000. With repeated courses, "titer" of 5000–10,000 may result.	Chap. 17,E, Vol. III
Coliphage T_2	Immune precipitate of phage and rabbit antiphage mixed with alumina	1 injection, i.m., restimulation after 3 weeks by injection i.v. of 10^{10} phage particles on successive days	Bleeding 10–12 days later.	*bb*
Rickettsiae, in Freund's adjuvant	—	—	Titer 15- to 30-fold higher than when injected in saline.	Quoted in Freund[cc]
Animal viruses				
Rauscher and Maloney murine leukemia viruses	Virus pellet from leukemic mouse plasma resuspended in buffered saline to 1/10 volume of plasma; incorporated in equal volume complete Freund's adjuvant	0.5 ml into each footpad and 1 ml into each thigh intramuscularly as primary injection; 1 ml i.v. 4 weeks later then 1 ml i.p. every 2 weeks for 3 injections	Rest for 4 weeks; inject 0.5 ml i.p. each month. Make trial bleedings on day 7 after each injection. Take serum when precipitins for mouse plasma (contaminant) are readily demonstrable; absorb precipitins for normal plasma proteins with normal mouse plasma before making neutralization tests. Producing rabbits are maintained for 2 years on the monthly injection schedule.	*dd*

[a] M. W. Chase, unpublished experiments.

[b] Specific proteins are cited in the second column but each procedure is adaptable to other soluble proteins as well. Ea, egg albumin; BSA, bovine serum albumin.

[c] K. Landsteiner, unpublished experiments.

[d] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology: A Laboratory Text for Instruction and Research," pp. 96–112, W. A. Benjamin, New York, 1963.

[e] H. G. Johnson, S. Gaines, and M. Landy, *J. Exptl. Med.* **103,** 225 (1956).

[f] T. P. King, P. S. Norman, and J. T. Connell, *Biochemistry* **3,** 458 (1964).

[g] J. J. Cebra, *J. Immunol.* **79,** 118 (1957).

[h] N. K. Jerne, *Acta Pathol. Microbiol. Scand. Suppl.* **87,** 1 (1951).

[i] J. Freund and M. V. Bonanto, *J. Immunol.* **40,** 437 (1941); **45,** 71 (1942).

[j] J. C. Suri and S. D. Rubbo, *J. Hyg.* **59,** 29 (1961).

[k] Neither fluid injections nor injections in Freund's adjuvant produced Ab.

[l] K. Landsteiner, *J. Exptl. Med.* **75,** 269 (1942).

[m] J. J. Cebra, *J. Immunol.* **86,** 190 (1961).

[n] Fraction isolated from peptic digestion of coagulated beef albumin, sheep euglobulin, etc.

[o] J. L. Jacobs, *J. Exptl. Med.* **59,** 479 (1934).

[p] Applicable as well to *o*- and *p*-aminobenzoic, succinanilic, and suberic substituents, etc.

[q] N. T. Macris and M. W. Chase, unpublished experiments, 1965.

[r] W. H. Taliaferro and L. G. Taliaferro, *J. Infect. Diseases* **95,** 117 (1954).

[s] R. R. Hyde, *Am. J. Hyg.* **8,** 205 (1928), see K. Landsteiner and E. Prašek, *Z. Immunitaetsforsch.* **13,** 403 (1912).

[t] Special rabbits containing low-grade natural hemolysin must be selected for immunization.

[u] M. S. Shorb and G. H. Bailey, *Am. J. Hyg.* **19,** 148 (1934).

[v] P. R. Edwards and W. H. Ewing, "Identification of Enterobacteriaceae," 2nd ed. Burgess, Minneapolis, 1962.

[w] The production of diagnostic antisera has been studied extensively (see ref. *v* above). Thursday is most convenient to begin the sequence shown.

[x] Reinject with 3.0 and 4.0 ml only those animals demonstrating antibody on day 18. Discard others, for prolongation of immunization broadens the reactivity of the antiserum.

[y] For pathogenic spores, cover the first four injections by administration of 150,000 units of penicillin.

[z] W. B. Schaefer, *Am. J. Respirat. Diseases* **92,** No. 6, Part II, 85 (1965).

[aa] J. Freund, K. J. Thomson, H. B. Hough, H. E. Sommer, and T. M. Pisani, *J. Immunol.* **60,** 383 (1948).

[bb] H. Jaquet, B. Bloom, and J. J. Cebra, *J. Immunol.* **92,** 991 (1964).

[cc] J. Freund, *Ann. Rev. Microbiol.* **1,** 291 (1947).

[dd] K. Yoshida, K. L. Smith, and D. Pinkel, *Proc. Soc. Exptl. Biol. Med.* **121,** 72 (1966), slightly modified (D. Pinkel, personal communication).

TABLE II

NOTES ON PREPARATION OF BACTERIAL VACCINES FOR IMMUNIZATION

Organism	Preparation	Ref.
Salmonella H (flagellar) antigens	A highly motile (selected) strain is grown in broth for 15–18 hours at 37°. Add $\frac{1}{20}$ volume formalin to undiluted culture. Titers averaging 1:8000 should be secured by procedure described.	*a*
Salmonella O (somatic) antigens	Method a: growth in infusion broth for 6–8 hours; 100° for 2.5 hours, discard if autoagglutinable. Add $\frac{1}{20}$ volume formalin. Note that the heated bacteria are washed and resuspended in method of Campbell *et al.*[b] Titers averaging 1:400 should be secured.	*a*
	Method b: Harvest 24-hour infusion agar growth as dense suspension; 100° for 2 hours. Centrifuge, and suspend sediment in 95% ethanol for 4 hours at 37°. Wash twice with acetone. Grind dry to powder. Suspend to "broth density" for injection.	*c*
Shigella	Growth for 6–8 hours in meat infusion broth at pH 7.4; 100° for 2 hours. Resuspend growth in saline containing 0.5% formalin. Absorb alpha agglutinins if present in resulting antiserum.[d]	*a*
Staphylococci	Growth on agar for 18 hours, 37°, is harvested and killed with formalin. Adjust density to 5×10^9/ml.	Chap. 15,D, Vol. III
Pneumococci (capsular polysaccharide antigens plus somatic antigens)	Freshly mouse-passaged pneumococci shown to be fully encapsulated are seeded heavily in brain heart infusion broth. After 8 hours kill bacteria with 1.2% formalin. Harvest, resuspend to 10-fold concentration in buffered saline, heat at 70° for 20 minutes, formalinize at 0.1%, standardize for injection at about 2.7×10^9 cells/ml.	*e*

[a] P. R. Edwards and W. H. Ewing, "Identification of Enterobacteriaceae," 2nd ed. Burgess, Minneapolis, 1962.

[b] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology: A Laboratory Text for Instruction and Research," pp. 96–112. W. A. Benjamin, New York, 1963.

[c] R. Roschka, *Klin. Med. (Vienna)* **5**, 2, 88 (1950).

[d] L. Stamp and D. M. Stone, *J. Hyg.* **43**, 266 (1944).

tory workers, who use a variety of methods—intravenous injections, alumina adsorption, or Freund's complete adjuvant. The doses are scaled up from those used with rabbits roughly in ratio to body weights. Cebra *et al.*[4b] used an initial intramuscular injection of rabbit "light chain" immunoglobulin (17 mg) in Freund's complete adjuvant and five weeks later injected intramuscularly 15 mg, precipitated with alum.

[4b] J. J. Cebra and G. Goldstein, *J. Immunol.* **95**, 230 (1965).

When the schedule calls for reinjection of soluble antigen, several small, gradually increasing doses, as 0.1 mg, 0.5 mg, 1.5 mg, should be given subcutaneously at hourly intervals, and the effect noted carefully; if no pronounced reaction is seen, the remainder of the intended dose is injected several hours later.

e. Guinea Pigs

Under stimulation by soluble proteins, the guinea pig makes chiefly a cytophilic type of antibody (termed "$7S\gamma_1$" by Benacerraf *et al.*[5]), measurable by anaphylactic responses. The antibody can be quantitated in a reasonable way by the procedure of passive cutaneous anaphylaxis (PCA). Yet it had long been known that guinea pigs produce large amounts of diphtheria and tetanus antitoxins when these antigens are presented on adsorbents. Diphtheria toxoid adsorbed to Willstaetter's alumina, for example, leads to the same type of results seen in rabbits (Table I, part 1) with titers of 30 to 45 AU/ml; the half-life of guinea pig diphtheria antitoxin so produced is between 5.5 and 6.5 days. The same holds for tetanus toxoid[6]: two doses of 10 Lf adsorbed on 10 mg of aluminum phosphate (made as in Section A,2,b,i(c), given with an interval of 4 weeks between doses, yielded 30 AU/ml 2 weeks later. Again, BGG and picrylated BGG adsorbed to nascent alumina (Section A,2,b,ii) and injected into guinea pigs are found to stimulate precipitins readily.[7]

Shortly after Freund's adjuvant became available, it was found to cause marked precipitin formation in guinea pigs, and a distinction between guinea pig antibodies that were able to precipitate and those that caused passive anaphylaxis became more obvious. Finally, the difference became clear when the response to ovalbumin was compared in incomplete Freund's adjuvant and in complete adjuvant[8]: the PCA type of antibody belongs to the subclass of IgG later named γ_1, and the precipitin type is γ_2 G. These two antibodies have been clearly isolated and studied in detail by Benacerraf *et al.*[5] The use of Freund's complete adjuvant not only causes synthesis of $7S\gamma_1$ in marked amounts but increases the synthesis of $7S\gamma_2$ at the same time.

The preparation of antigens is the same as for rabbits. Repeated intravenous injections are hardly practicable, although possible. Injections

[5] B. Benacerraf, Z. Ovary, K. J. Bloch, and E. C. Franklin, *J. Exptl. Med.* **117**, 937 (1963).
[6] J. C. Suri, and S. D. Rubbo, *J. Hyg.* **59**, 29 (1961).
[7] J. R. Battisto, and M. W. Chase, *J. Exptl. Med.* **121**, 591 (1965).
[8] R. G. White, G. C. Jenkins, and P. C. Wilkinson, *Intern. Arch. Allergy* **22**, 156 (1963).

of Freund's adjuvants for the purpose of antibody formation is made intramuscularly (1 ml divided among five sites deep into the nuchal muscles) or into the footpad (0.03 ml per footpad has given good results in our hands). Hair should be removed locally, and the skin sponged with alcohol. When the footpad route is chosen, the amount of mycobacteria should be reduced to the point where allergic reactions owing to tuberculin sensitivity will not cause the feet to crack open. Footpad injection is described in Section D,3,i. We find 12 to 15 μg of mycobacteria in our total footpad dosage of 0.12 ml to be sufficient for good adjuvant effect and tolerable to the animals. Bleedings can be made between 3 to 8 weeks later.

f. MICE

Although mice are rendered anaphylactic by one or two injections of BSA without showing much if any antibody in the serum, Freund's adjuvant can produce respectably high amounts of antibody to soluble proteins (see below). BGG adsorbed to bentonite,[9] or BSA handled similarly, produces a marked stimulus, an effect not yet really explored. As with rabbits (Section B,1,c), bacterial endotoxin serves as adjuvant when injected with protein into mice. For example, injection of 1 Lf of diphtheria toxoid with 10 to 100 μg of typhoid endotoxin twice at a 14-day interval, followed by 2 weeks of rest, incited production of antitoxin measuring between 1 and 3 AU/ml, whereas without endotoxin the titer was below 0.001 AU/ml.[4]

The production of hemolysin on intraperitoneal injection of sheep erythrocytes, pointed out clearly by Newsom and Darrach,[9a] has been studied with regard to individual cells by Jerne (Chap. 26,D, Vol. IV); also agglutinins are formed to foreign erythrocytes.

Collection of adequate volumes of mouse serum remains a problem unless a particular investigation can be conducted with small amounts of serum. This handicap has been overcome in large measure by the finding of Munoz[10] that Freund's adjuvant introduced intraperitoneally will stimulate a proportion of the mice to secrete ascites fluid that can be rich in globulin. By this technique, not only antibody to soluble proteins but also agglutinins for certain bacteria (staphylococci, *Salmonella enteritidis*) can be secured, although the number of mice responding and the time of apperance of ascites cannot be selected in advance. Also, the apperance of fluid does not necessarily signify a useful antibody content. One must be prepared to make collections at appropriate times, preserve, test individually, and eventually pool. But the long period of time over which antibody is synthesized when Freund's adjuvant

[9] H. N. Claman, *J. Immunol.* **91**, 833 (1963).
[9a] S. E. Newsom and M. Darrach, *Canad. J. Biochem. Physiol.* **32**, 373 (1954).
[10] J. Munoz, *Proc. Soc. Exptl. Biol. Med.* **95**, 757 (1957).

is used overcomes the problem of the 2-day half-life of mouse antibody. By this procedure, large amount of immunoglobulin can be attained in satisfactory concentration (some anti-Ea pools showed 1.0 to 2.0 mg of Ab-N per milliliter; see Anaker and Munoz[11]).

With an expectation of securing satisfactory volumes of antibody-rich fluids in less than one-half of the animals, immunize a sufficiently large group of mice (50) with antigen in Freund's complete adjuvant. Inject 0.25 ml containing 0.5 mg of mycobacteria, intraperitoneally, and repeat 2 weeks later. Allow ascites to develop over 3 weeks.[10] When an abdomen shows marked distension, code the mouse for future identification (ear punch or dye markers) and tap. Wet the abdominal hair with 70% alcohol lightly, and with the fingers insert an 18-gauge needle. The fluid will drip out. To allow repeated collections, do not attempt to "strip." Yields from producing mice within a group of 50 have shown about 150 ml over a period of a month.

The collecting tubes are centrifuged so that oil from the cavity rises. Remove the free fluid by expelling most of the air from a Pasteur pipet fitted with a rubber teat, and expel the residual air just as it is plunged along the side of the tube and through the oil layer. A simple preliminary test for antibody is the Lancefield capillary tube test (Chap. 13, Vol. III); use one part of clarified ascites fluid and one part 1:50 of 5% concentration of the antigen, taking one after the other into the capillary. The column of partially mixed fluids is positioned about 1 cm from the end of the capillary bore by tipping it downward with finger closing the lower end; then invert the tube and plunge it downward into plasticine to support the fluid column on trapped air. Precipitation should be seen almost at once, if the antibody content is high.

Lieberman *et al.*[12] produced staphylococcal agglutinins by this method; nearly every mouse produced ascites. These workers gave larger doses (0.4 to 0.5 ml) two or three times at intervals of 5 to 7 days. Part of the regularity of response seemed to be caused by a soluble staphylococcal product present in the culture broth.

g. Other Mammals

Rats make precipitins when they are stimulated with soluble protein in Freund's adjuvants,[13] although weakly; the species responds to a single injection of bacterial vaccines or foreign erythrocytes. Campbell *et al.* (reference 2, p. 113) have tabulated the titers to cellular antigens secured by different routes of administration.

[11] R. L. Anacker and J. Munoz, *J. Immunol.* **87**, 426 (1961).
[12] R. Lieberman, J. A. O. Douglas, and N. Mantel, *J. Immunol.* **84**, 514 (1960).
[13] M. M. Lipton, S. H. Stone, and J. Freund, *J. Immunol.* **77**, 453 (1956).

Monkeys have shown good serum titers to poliomyelitis virus, when the first injection was given in Freund's complete adjuvant and virus alone was reinjected 5 and 11 weeks later.[14]

[14] R. Ward, D. Rader, M. M. Lipton, and J. Freund, *Proc. Soc. Exptl. Biol. Med.* **74**, 536 (1950).

2. PRODUCTION OF ANTIBODIES IN HUMAN SUBJECTS*

a. General Considerations

Before the use of human beings for experimental studies is undertaken, one must always be concerned with the ethical, moral, and legal considerations. The code laid down by the Nuremberg Military Tribunals is now the guiding principle.[1-4] Its opening sentence reads: *"The voluntary consent of the human subject is absolutely essential."* Many of the antigens employed for the experimental production of antibodies in human subjects are those in general use in medicine for routine immunization or as plasma volume expanders or in the production of antisera for blood grouping. They are produced commercially according to standards laid down by Federal agencies and involve minimal risk. Other materials such as the type-specific polysaccharides of pneumococci were at one time licensed for human immunization; purified substances are frequently isolated from microorganisms with which extensive data in man have been obtained by using the intact bacteria as a vaccine, so that the potential hazards may be evaluated. Even with such materials, one must always be alert to the possibilities of allergic reactions. Accordingly, human immunizations are best carried out by or in association with physicians, and in hospitals, clinics, or medical schools, in which facilities for dealing with allergic reactions are available. Many institutions have faculty committees which must evaluate such studies and assent to their being carried out. With substances which are prepared for injection in the laboratory, precautions to exclude pyrogens, toxicity, and to ensure sterility of the preparation are essential. It should be remembered that most antigenic materials may cause local reactions at the site of injection in some subjects, and attention should be given to the quantity of mate-

* **Section 2,B,2 was prepared by Elvin A. Kabat.**

[1] I. Ladimer, *J. Public Law* **3**, 467 (1954).
[2] I. Ladimer, *New Engl. J. Med.* **257**, 18 (1957).
[3] P. B. Beeson, P. K. Bondy, R. C. Donnelly, and J. E. Smith, *Yale J. Biol. Med.* **36**, 455 (1964).
[4] British Medical Research Council, *Science* **145**, 1024 (1964).

rial to be injected. Lipopolysaccharides from gram-negative microorganisms may be pyrogenic in extremely minute doses. The injection of products containing or derived from human blood from one human being to another, except for the standard licensed preparations of human albumin and human γ-globulin, involves a considerable risk of producing serum hepatitis. However, many individuals may have substantial levels of antibody to antigens with which they have come in contact, and good precipitating antisera may be obtained without deliberate immunization. Notable examples are antibody to the group-specific "C" polysaccharide of pneumococcus[5] and antibody to the teichoic acid of *Staphylococcus aureus*.[6]

Another guiding principle in studies in human beings, especially with polysaccharide antigens, is to use well-established injection schedules and select those individuals who show a satisfactory antibody response rather than to attempt prolonged immunization in subjects who fail to respond or who respond poorly; this reduces the risk of allergic reactions on repeated injection.

b. Human Antitoxins

Human diphtheria antitoxin has been widely used in immunochemical studies,[7-10] human tetanus antitoxin is produced commercially for passive protection,[11,12] and human antibody to purified botulinum toxoid has been prepared.[13,14]

The commercial preparations of toxoids usually include recommended injection schedules which may be followed. With alum-precipitated tetanus toxoid, an initial injection is given, followed four to six weeks later by a second injection, each of 0.5 ml. Blood samples may be taken two weeks to several months following the second injection. Booster

[5] M. Heidelberger, C. M. MacLeod, S. J. Kaiser, and B. Robinson, *J. Exptl. Med.* **83**, 303 (1946).

[6] M. Torii, E. A. Kabat, and A. E. Bezer, *J. Exptl. Med.* **120**, 13 (1964); J. C. Allen, H. G. Kunkel, and E. A. Kabat, *J. Exptl. Med.* **119**, 453 (1964); G. M. Edelman and E. A. Kabat, *J. Exptl. Med.* **119**, 443 (1964).

[7] W. J. Kuhns and A. M. Pappenheimer, Jr., *J. Exptl. Med.* **95**, 363 (1952).

[8] W. J. Kuhns and W. Dukstein, *J. Immunol.* **79**, 154 (1957).

[9] I. Finger and E. A. Kabat, *J. Exptl. Med.* **108**, 453 (1958).

[10] E. Henocq, E. H. Relyveld, and M. Raynaud, *Intern. Arch. Allergy Appl. Immunol.* **20**, 262 (1962).

[11] E. A. Kabat, *New Engl. J. Med.* **269**, 247 (1963).

[12] J. Ipsen, Jr., *J. Immunol.* **70**, 426 (1953); L. Levine, J. Ipsen, Jr., and J. A. McComb, *Am. J. Hyg.* **73**, 20 (1961).

[13] A. R. Prévot, and E. R. Brygoo, *Ann. Inst. Pasteur* **79**, 1 (1950).

[14] M. A. Fiock, M. A. Cardella, and N. F. Gearinger, *J. Immunol.* **90**, 697 (1963).

injections may be given at intervals. It is generally advisable to obtain a small sample of serum which is assayed for antitoxin. If the response is suitable, a large sample of antiserum may be obtained a few days later. If facilities for plasmapheresis are available, several liters of antiserum may be obtained within a few weeks while the antitoxin level is maximal.

Although local and systemic reactions to tetanus toxoid are rare, a considerable proportion of adults have acquired delayed-type hypersensitivity to constituents of the diphtheria bacillus and less frequently even to the toxoid itself. Accordingly, production of human diphtheria antitoxin is limited to anamnestic stimulation of Schick-negative individuals who do not show delayed hypersensitivity to the Schick test dose of toxin or to diphtheria toxoid.[7-9]

Procedure. A Schick test is performed by intradermal injection of 0.1 ml (1/50 MLD) of a commercial preparation of Schick toxin on the forearm. At the same time 0.1 ml (0.008 L_f) of a control preparation of diphtheria toxoid is injected at a second site. The sites are examined at 15 to 30 minutes, at 48 hours, and at 4 days. Individuals who show no reactions to either the toxin or the toxoid receive 40 L_f) of purified diphtheria toxoid subcutaneously in the upper arm. Samples of serum are tested for antibody 7 to 10 days after the injection, and, if levels of antitoxin are suitable, large samples of serum are obtained. It should be noted that antisera obtained do not contain antibody to diphtheria toxin alone, but an anamnestic response to many other proteins of the diphtheria bacillus generally occurs, and antisera may show as many as five or six lines in agar diffusion.[8,9] The antibody formed by these procedures is largely 7 S or γG-immunoglobulin. Although recent studies with many antigens indicate that 19 S or γM antibodies are generally formed early in the course of immunization and may often disappear on continued immunization, procedures for the production of a 19 S antitoxin in man have not yet been developed.

c. Human Antipolysaccharide Antibodies

Extensive data have been obtained in man on the production of precipitating antibodies to the pneumococcal type-specific polysaccharides,[5,15] dextrans,[15-17] and blood group A and B substances,[18] and more limited studies have been carried out on levan,[19] the Vi antigen of *E.*

[15] S. Leskowitz and F. C. Lowell, *J. Allergy* **32**, 152 (1961).
[16] E. A. Kabat and D. Berg, *J. Immunol.* **70**, 513 (1953).
[17] P. H. Maurer, *Proc. Soc. Exptl. Biol. Med.* **83**, 879 (1953).
[18] E. A. Kabat, "Blood Group Substances." Academic Press, New York, 1956.
[19] P. Z. Allen and E. A. Kabat, *J. Exptl. Med.* **105**, 383 (1957).

coli,[20,21] and the teichoic acids of *S. aureus*.[6] For additional references see Kabat.[18,22]

The antibody response to polysaccharide antigens in persons who respond well reaches a maximum 10 days to 6 weeks after stimulation and remains elevated for many years so that serum may be collected without further immunization. A satisfactory antibody response to pneumococcal polysaccharides and dextrans generally is accompanied by development of wheal and erythema skin sensitivity.

Commercially available* preparations of blood group substances may be used to produce high-titer precipitating anti-A or anti-B; a rise in anti-A or anti-B is obtained in a fair proportion of individuals who receive one or two subcutaneous injections of 0.5 ml to 1 ml of these preparations a day apart.

i. Preparation of Immunogen

The procedure employed for producing antipolysaccharide in man when commercial products are not obtainable is as follows: A weighed quantity of polysaccharide is dissolved in a volume of sterile saline to give a solution of 2 to 5 mg/ml. (All glassware to be used is sterilized in an oven at 180° for 2 hours.) Such solutions may be kept in the refrigerator indefinitely in tightly stoppered vessels in the presence of a drop or two of chloroform, a small droplet at the bottom of the tube indicating that the solution is saturated. When the tube of solution is opened for use, the odor of chloroform can be detected and a fresh drop of chloroform added if needed. To prepare the solution for injection, a volume containing about twice the quantity of antigen needed for the number of individuals to be injected is measured out. Five per cent phenol in saline is added such that the final phenol concentration will be 0.25%; the solution is made up to the final volume, mixed thoroughly, and distributed into 5-ml or 10-ml sterile bottles with self-sealing rubber stoppers. Dextrans and blood group substances are generally made up to 1 mg/ml, while the pneumococcal polysaccharides are made up to 50 μg/ml; for the Vi antigen 40 μg was used.[21] The bottles are allowed to remain at room temperature for 48 hours for the phenol to act. For skin testing studies it is desirable to prepare a control solution of phenolized saline without antigen in the same manner.

[20] M. Landy, *Am. J. Hyg.* **60**, 52 (1954).
[21] N. Huang, *Am. J. Hyg.* **70**, 275 (1959).
[22] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

* Merck, Sharp & Dohme, Rahway, New Jersey, and Pfizer Diagnostic, Division of Chas. Pfizer and Co., Inc., New York.

Sterility. For materials stable to autoclaving, such as dextrans and blood group substances, this method of sterilization may be used on the final solution. Solutions in 5-ml or 10-ml bottles are tested for sterility by streaking 0.1 ml on a blood agar plate and inoculating two tubes containing 20 ml of thioglycolate broth with 0.1 and 0.2 ml of the solution. Tubes and agar plates are incubated at 37° for 10 days and examined for growth daily.

Toxicity. Two-tenths ml and 0.5 ml of the solution are injected intraperitoneally into two mice. The mice are observed for 7 days and should show no ill effects.

One-tenth ml of solution is injected intradermally into a rabbit. The injection site should show no local reaction during 7 days.

ii. Immunization Procedure

A sample of blood is drawn prior to immunization, and an intradermal test is carried out with 0.05 ml of the solution to be used for immunization and with phenolized saline as a control. Wheal and erythema reactions are looked for after 15 to 20 minutes. If a negative or slight reaction is obtained, 0.5 ml of the polysaccharide antigen is injected subcutaneously in the upper arm. A second subcutaneous 0.5-ml injection is given on the following day. Ten days to 3 weeks later a second blood sample is taken; and a second set of intradermal 0.05-ml injections is carried out, and any development or increase in wheal and erythema sensitivity noted. The pre- and postimmunization samples are examined for antibody by the quantitative precipitin method[22-24] (see also Chap. 13, Vol. III) or by other methods. About 10 to 25% of the subjects produce antisera with 10 to 80 μg of antibody N per milliliter to the various antigens.

d. Miscellaneous Human Antibodies

Studies in human subjects have been carried out with streptococcal M protein[25,26] and with streptococcal cell walls.[26] With synthetic polypeptides[27] severe delayed skin reactions were encountered, and further studies with these materials would seem not to be indicated, especially since their antigenicity can readily be tested in animals. Antibody

[23] E. A. Kabat and G. Schiffman, *J. Immunol.* **88**, 782 (1962).
[24] G. Schiffman, E. A. Kabat, and W. Thompson, *Biochemistry* **3**, 113 (1964).
[25] W. C. Schmidt, *J. Infect. Diseases*, **106**, 250 (1960).
[26] C. K. Wolfe, Jr., J. A. Hayashi, G. Walsh, and S. S. Barkulis, *J. Lab. Clin. Med.* **61**, 459 (1963).
[27] P. H. Maurer, B. F. Gerulat, and P. Pinchuk, *J. Exptl. Med.* **116**, 521 (1962).

specific for Gm(a), Gm(b), and Gm(x) has been found in 17 of 24 children with thalassemia who had received multiple transfusions of human serum.[28]

[28] J. C. Allen and H. G. Kunkel, *Science* **139**, 418 (1963).

3. PRODUCTION AND PURIFICATION OF CHICKEN IMMUNOGLOBULINS*,†

a. Introduction

Wolfe and his associates[1-5] found that chickens respond to injection of heterologous serum proteins by synthesizing high levels of precipitating antibodies, and they also recorded that fowl antisera differ in a number of ways from typical mammalian antisera. The nature of the complexes composed of chicken antibody and antigen is perplexing. Precipitation of chicken antibody by antigen is greater at high salt concentrations (1.5 *M* NaCl) than at the usual physiological concentrations of salt (0.15 *M* NaCl). Moreover, aging of chicken sera at 4° or —20° results in a decrease in the quantity of precipitate formed with antigen in 1.5 *M* NaCl but results in an increase in the quantity of precipitate formed with antigen in 0.15 *M* NaCl. Normal serum macroglobulin was reported to coprecipitate with antigen–antibody precipitates formed in 1.5 *M* NaCl, regardless of the age of the antiserum; however, coprecipitation occurred in 0.15 *M* NaCl with aged antisera but not with fresh antisera.[6] More recently Orlans *et al.*[7-9] found that larger amounts of the macroglobulin coprecipitated in 0.15 *M* NaCl than in 1.5 *M* NaCl, and, furthermore, that small amounts of fowl serum albumin were present in immune precipitates formed in high salt concentrations.[10] In addition to the fact that normal serum components are included in immune com-

*** Section 2,B,3 was contributed by Albert A. Benedict.**

† The author's experimental work quoted was supported by a USPHS Training Grant (1 Tl AI 243-03) and a USPHS Research Grant (AI05660-02).

[1] H. R. Wolfe, *J. Immunol.* **44**, 135 (1942).
[2] M. Goodman, H. R. Wolfe, and S. Norton, *J. Immunol.* **66**, 225, (1951).
[3] N. Gengozian and H. R. Wolfe, *J. Immunol.* **78**, 401 (1957).
[4] J. Banovitz, S. J. Singer, and H. R. Wolfe, *J. Immunol.* **82**, 481 (1959).
[5] J. Banovitz and H. R. Wolfe, *J. Immunol.* **82**, 489 (1959).
[6] T. Makinodan, N. Gengozian, and R. E. Canning, *J. Immunol.* **85**, 439 (1960).
[7] E. Orlans, M. E. Rose, and J. R. Marrack, *Immunology* **4**, 262 (1961).
[8] E. Orlans, *Immunology* **5**, 306 (1962).
[9] E. Orlans, M. E. Rose, and K. H. Clapp, *Immunology* **5**, 656 (1962).
[10] E. Orlans, C. B. Richard, and M. E. Rose, *Immunochemistry* **1**, 317 (1964).

plexes, the antibodies are heterogeneous. Early primary-response antiprotein sera contain both γG and γM antibodies.[11-12] When it is separated from the macroglobulin fraction, the γG antibody in primary antisera is precipitated by antigen only in high salt.[12] Whether these and other peculiarities of chicken antisera militate against the use of chickens for production of antibody depends largely on the purpose of the studies an investigator wishes to conduct.

b. Immunization Procedures

There are no fixed schedules for immunization of chickens with the variety of antigens available; however, most studies done with the chicken have involved the use of either primary-response sera or antisera obtained after two injections of antigen. The procedures described here have been used by most investigators, and, except for minor modifications, particularly in regard to production of antisera for study of the classes of immunoglobulins with antibody activity, these procedures are essentially those introduced by Wolfe and his colleagues.

Most of the common breeds of chickens, and either males or females, may be used for antibody production. Although serological maturity is approached at about 9 weeks of age, older chickens are preferred for maximum production of antibodies. For most studies, antigen is administered either in the medial wing vein on the underside of a wing or in the jugular vein.

i. Protein Antigens

High concentrations of antibodies are obtained within a week following a single injection of mammalian serum proteins or of hemocyanin. Saline solutions of the serum protein are given in amounts of 40 mg/kg of body weight; of hemocyanin, 5 mg/kg is used. There is no advantage in giving these amounts in a series of injections. The chickens are bled 7 to 9 days following injection. The animals are not fed for at least 24 hours prior to bleeding, and then are bled either by cardiac puncture or from the jugular vein. As much as 0.5 mg of total N per milliliter of antiserum may be precipitated with antigen. Somewhat higher antibody concentrations are obtained by giving a second intravenous injection of 40 mg of protein per kilogram of body weight 2 to 3 weeks following the primary injection; again, bleedings are made 7 to 9 days following injection.

Antisera free of γM antibodies, as evidenced by autoradiography of immunoelectrophoretic patterns (see Chap. 14, Vol. III) after treatment

[11] A. A. Benedict, R. J. Brown, and R. T. Hersh, *J. Immunol.* **90**, 399 (1963).
[12] A. A. Benedict, R. T. Hersh, and C. Larson, *J. Immunol.* **91**, 795 (1963).

with radioactive antigen,[13] and by examination of macroglobulin fractions by passive hemagglutination[14] (see Chap. 15,B, Vol. III), are obtained by giving an intravenous injection of 20 mg of protein per kilogram of body weight 2 weeks following primary injection. Seven days later a series of four intramuscular injections of antigen in Freund's complete adjuvant is given at 4-day intervals. Bleedings are made 9 to 12 days following the last injection.

Hemoglobin is a poorer antigen than are the serum proteins, but satisfactory concentrations of precipitating antibody are obtained by giving an initial intravenous injection of 40 mg/kg of body weight followed by three to four intramuscular injections of the same amount on alternate days.[8] Bleedings are made 7 to 9 days later.

ii. Haptens

By passive hemagglutination[15] and by autoradiography of immunoelectrophoretic patterns,[16] antibodies to haptens, such as, *p*-aminobenzoic acid and arsanilic acid, are demonstrated in antisera obtained 6 to 9 days following intravenous injection of 70 mg of the azoprotein per kilogram of body weight. Gold *et al.*[17] have obtained precipitating antibody to haptens by giving a total of 40 mg of the azoprotein according to the following schedule: two intramuscular injections in Freund's complete adjuvant about 10 days apart, followed by weekly subcutaneous injections, without adjuvant, until precipitating antibody appears in sufficient concentration.

iii. Characteristics of Antibody Response

Recent studies by the author show that three to four days following immunization with bovine serum albumin (BSA) or keyhole limpet hemocyanin many sera contain only γM antibodies. No antibody activity is detected in the γG fractions of these sera. Four to five days following injection, the γG antibody may predominate, and sera collected 7 to 9 days following injection may contain only small amounts of γM antibody persisting for several days. The period of time in which the γM is the only antibody present is short (about 1 day). The time of conversion of production from γM to γG antiprotein antibodies does not appear to be dependent on the dose of antigen. The smallest immunogenic dose

[13] Y. Yagi, P. Maier, and D. Pressman, *J. Immunol.* **89**, 736 (1962).
[14] A. B. Stavitsky, *J. Immunol.* **72**, 360 (1954).
[15] E. F. Gold and A. A. Benedict *J. Immunol.* **89**, 234 (1962).
[16] G. Dreesman, C. Larson, R. N. Pinckard, R. M. Groyon, and A. A. Benedict, *Proc. Soc. Exptl. Biol. Med.* **118**, 292 (1965).
[17] E. F. Gold, K. Knight, H. Stine, M. Roelofs, and F. Haurowitz, *Bacteriol. Proc.* **M106**, 63 (1964).

of BSA (1 mg) induces a γM response of the same duration as do larger doses of antigen, and, as with larger doses of antigen, the conversion to γG antibody occurs rapidly. Assay for antibody by hemagglutination indicates that 6-day primary anti-BSA sera contain mainly γM hemagglutinins, and the synthesis of γG hemagglutinins follows later,[11,18] apparently in conflict with the above observation. Hemagglutination is extremely sensitive, however, for detecting chicken γM antibodies.[19] In fact, the γG antibodies in early primary sera often are not demonstrated by hemagglutination, although they may be detected by the precipitin reaction, immunoelectrophoresis, or the Farr method[16] (see Chap. 13,C, Vol. III).

The nature of the antigen plays some role in the synthesis of different chicken immunoglobulins. Chickens given a single intravenous inoculum of a hapten–protein conjugate produce antihapten γG antibody and an antihapten antibody tentatively identified as γA.[16] Recently, we have identified antiarsanilic γM antibodies. The antibodies to the protein moiety are mainly γG and γM antibodies. In contrast to the predominance of the γG antibody in primary anti-BSA sera, primary chicken neutralizing antibodies against bacteriophage are mainly γM.[20] R. M. Groyon in the author's laboratory found that primary chicken antibacteriophage precipitins are mainly γM-immunoglobulins; high concentrations of the 19 S antibodies persisted following a second injection with phage.

Ultracentrifugal examination of soluble antigen–antibody complexes, prepared from washed precipitates of chicken antibovine γ-globulin (pseudoglobulin fraction) and bovine γ-globulin, yields two peaks with sedimentation coefficients of 10.3 S and 14.9 S.[10] The formation of these two peaks is attributed to the presence of univalent (AbAg) and bivalent ($AbAg_2$) antibodies. In contrast, Williams and Donermeyer[21a] observed no intermediate complex (AbAg), and reported that the mole ratio of antigen to antibody approached the limiting value of 2 as the relative amount of antigen was increased. Possibly these studies should be repeated, using purified γG preparations.

The γG anti-BSA antibody in 6- to 7-day primary sera, when separated from the other serum proteins, precipitates with antigen only in high salt solutions, and the γM antibody precipitates optimally in low salt.[12] The role of other serum components causing precipitation of γG

[18] A. A. Benedict, C. Larson, and H. Nik-Khah, *Science* **139**, 1302 (1963).
[19] A. A. Benedict, *Nature* **206**, 1368 (1965).
[20] J. W. Uhr, M. S. Finkelstein, and E. C. Franklin, *Proc. Soc. Exptl. Biol. Med.* **111**, 13 (1962).
[21a] J. W. Williams, and D. D. Donermeyer, *J. Biol. Chem.* **237**, 2123 (1962).

by antigen in unfractionated serum in low salt is not understood. Purified γG antibody obtained from booster sera forms precipitins in high and low salt solutions, indicating that further changes in the γG antibody are a function of the duration of immunization.

Even precipitates formed of highly purified γG antibody and antigen may contain normal γG-immunoglobulins. Recent studies done in collaboration with R. T. Hersh[21b] show that in a solution of 1.5 *M* NaCl purified chicken γG forms a polymer with an extrapolated sedimentation coefficient of approximately 14 S. On the basis of molecular weight determinations, the polymer consists of either three or four 7 S units. In high salt the bivalent and univalent antibodies which have been postulated to exist[4,10] might polymerize with normal γG. The extent to which aggregation influences the amount of precipitate formed with specific antigen is not known. However, aggregation of chicken γG could complicate quantitative determination of the nature of the immune complexes formed in high salt solutions, and also could influence chromatographic and gel filtration separations which employ solvents containing high concentrations of salt.

In view of the complexities of the precipitin reaction, even with those that employ isotopic-labeled antigen, other methods of antibody assay might be advisable. The ammonium sulfate salting-out procedure of Farr[22] (see Chap. 13,C, Vol. III) avoids the disadvantages of the precipitin reaction, although it does not detect the γM antibody in primary sera taken very early in the immune response.

Chicken antiserum fails to fix guinea pig complement (C′) in the presence of homologous antigen, and is ineffective for eliciting passive cutaneous anaphylaxis (PCA) in guinea pigs. R. Kubo in our laboratory found PCA in chickens unreliable for quantitative assay of chicken antibody.

c. Purification of Chicken Immunoglobulins

The procedures for concentration and purification of chicken immunoglobulins are similar to those used for the immunoglobulins of other animal species (see Chap. 3,A). However, attention should be given to these aspects of chicken serum: the high serum lipid content; the tendency for the γG- and γM-immunoglobulins to aggregate during fractionation and to become denatured; the polymerization of γG in high salt concentrations; and the precipitation of γG during dialysis against buffers commonly used for chromatographic separation of the serum proteins.

[21b] R. T. Hersh, and A. A. Benedict, *Biochim. Biophys. Acta* **115**, 242 (1966).
[22] R. S. Farr, *J. Infect. Diseases* **103**, 239 (1958).

i. Salt Precipitation

Except for modification of salt concentrations, chicken globulins are precipitated with Na_2SO_4 according to the method of Kekwick.[23] All operations are performed at room temperature. The serum is cleared by centrifugation at 3000 rpm for 30 minutes. Sera heavily contaminated with lipids should be discarded. The globulins are precipitated by the slow addition of Na_2SO_4 to a final concentration of 0.18 gm/ml. After standing for 30 minutes, the precipitate is recovered by centrifugation and is dissolved to one-half the original serum volume in borate buffered saline, pH 8.2, $\Gamma/2 = 0.16$.* Two subsequent precipitations are made, each with Na_2SO_4 to a final concentration of 0.14 gm/ml. After the final precipitation, the globulins are dialyzed against the borate buffer for 48 hours at 4°. Following dialysis, the preparation is cleared by centrifugation at 3000 rpm for 15 minutes. Such preparations usually contain four sedimenting species of protein in the ultracentrifuge, having sedimentation coefficients of 7 S, 9 S, 15 S, and 19 S.

ii. Gel Filtration

Gel filtration, according to the method described by Flodin and Killander,[24] appears to be better suited than is diethylaminoethyl (DEAE)-cellulose chromatography for purification of chicken γG- and γM-immunoglobulins (see Chap. 9,B, Vol. II). The salt-precipitated immunoglobulins are separated by filtration on Sephadex G-200 with borate-buffered saline, pH 8.2, $\Gamma/2 = 0.16$, in a column 4.5 × 50 cm in size. Between 250 and 300 mg of protein is placed on the column, and filtration takes place at room temperature. Ten-milliliter fractions are collected, and the protein concentrations are determined by ultraviolet absorption at 280 mμ. The macroglobulins pass through with the void volume, and the 7 S globulins are delayed in their passage through the column.

The peak protein γG fractions are pooled and dialyzed against distilled water at 4° for at least 72 hours and finally lyophilized. A small amount of material which remains insoluble on redissolving the lyophilized preparation in either phosphate or borate buffers is eliminated by cen-

[23] R. A. Kekwick, *Biochem. J.* **34**, 1248 (1940).

* Borate buffer, pH 8.2, $\Gamma/2 = 0.16$

Salt	gm/liter H_2O
H_3BO_3	10.3
NaOH	1.1
NaCl	7.85

[24] P. Flodin, and J. Killander, *Biochim. Biophys. Acta* **63**, 403 (1962).

trifugation. Examination of the γG preparation by immunoelectrophoresis often reveals a β-globulin contaminant. The contaminant is eliminated by dissolving the lyophilized γG preparation in 0.1 *M* phosphate buffer, pH 6.4, and passing the mixture through DEAE-cellulose with the same buffer (see Section 3,C). The γG is eluted with the void volume from DEAE-cellulose. The γG so prepared may be separated into the euglobulin and pseudoglobulin fractions by dialysis against distilled water at 4° for 72 hours. Although antibody activity is found in both these fractions, they have not been studied in detail.

For purification of γM, a pool is made of the contents in the peak protein tubes from the first Sephadex fraction. Examination by immunoelectrophoresis using rabbit anti-chicken serum antiserum often reveals γG as a contaminant. The γM fraction is dialyzed against distilled water for at least 72 hours and precipitated as a euglobulin. It may be recovered by centrifugation, or the preparation may be lyophilized. To eliminate γG, the γM fraction is dissolved in 0.1 *M* phosphate buffer, pH 6.4, and chromatographed on DEAE-cellulose. Chromatographic fractions C and D (Section B,3,c,iii) contain γM free of contaminants detectable by immunoelectrophoresis.

A second method for purification of larger quantities of γM has been adopted recently by the author. After dialysis of the salt-precipitated globulins (Section B,3,c,i), Na_2SO_4 is added slowly to a final concentration of 0.09 gm/ml. The precipitate contains some γG, small amounts of γM, and all of the α_2-macroglobulin. After removal of the precipitate by centrifugation, Na_2SO_4 is added slowly to the supernatant to a final concentration of 0.14 gm/ml. The precipitate is recovered by centrifugation and is dissolved in borate-buffered saline. After dialysis against borate buffer, the preparation is filtered through Sephadex. A pool is made of the contents in the peak protein tubes from the first fraction, and the pool is concentrated by ultrafiltration. The concentrated preparation is recycled through Sephadex, and the γM fraction is concentrated by ultrafiltration. The preparation does not contain α2-macroglobulin, and it is usually free of γG as detected by gel diffusion.

iii. Chromatography

With chicken sera, unlike most mammalian sera, most of the γG- and γM-immunoglobulins precipitate when equilibrated with dilute buffers usually employed for chromatographic separation of the serum proteins. (See Chap. 9,A, Vol. II for general procedures.) Thus, buffers of higher ionic strength are required; however, poor separation of the serum proteins results. The use of DEAE-cellulose chromatography for large-scale fractionation of the immunoglobulins has been discontinued in the author's laboratory. It is useful, however, as a final step in eliminating

contaminants from γG and γM fractions prepared by gel filtration. Stepwise elution, employing a starting buffer of 0.1 *M* sodium phosphate, pH 6.4, has been used for following the synthesis of γG and γM hemagglutinating antibodies.[11]

The salt-precipitated globulins are dialyzed against the starting buffer at 4° for 48 hours. The small amount of precipitate which forms is removed by centrifugation. The globulins are eluted stepwise with the following buffers: (A) pH 6.4, 0.1 *M* sodium phosphate, (B) pH 5.8, 0.2 *M* sodium phosphate, (C) pH 5.4, 0.3 *M* sodium phosphate, (D) pH 4.7, 0.4 *M* sodium phosphate, (E) pH 4.4, 2 *M* NaCl in 0.4 *M* sodium phosphate. Fractions A and B together account for about 95% of the γG. The physical-chemical differences between the A and B fractions of γG have not been determined. Most of the γM immunoglobulins are eluted in fractions C and D. The protein in fraction E has not been identified. Globulin with a sedimentation coefficient of 15 S is eluted in fractions B and C.

d. Physicochemical Properties

Fewer chemical and physical studies have been made than biological studies. Limited studies on structure reveal that fowl immunoglobulins may be exceedingly interesting molecules for further investigation.

i. γG-Immunoglobulin

The isolated γG-immunoglobulin gives a single line by double diffusion studies and a single long arc by immunoelectrophoresis. On starch block electrophoresis about 60% of the protein migrates as γ_2-globulin, and 40% as γ_1-globulin. Banovitz *et al.*[4] reported an electrophoretic mobility of 2.55×10^{-5} cm²/volt/sec at pH 8.6, $\Gamma/2 = 0.3$. The $S_{20,w}$ is 6.9 to 7.1,[25] and from calculations which are dependent on a diffusion coefficient determined in agar gel Orlans *et al.*[7] estimated the molecular weight to be approximately 180,000. Light-scattering measurements have given a molecular weight value of 175,000.[26] The carbohydrate content is 2.5%.[26]

Like γG from other animal species, chicken γG hydrolyzed with papain and reducing agent is split into 3.4 S products.[27,28] Approximately 30 to 50% of the products are dialyzable peptides. The Fab and Fc fragments are electrophoretically slow (S) and fast (F), respectively.

[25] G. Dreesman and A. A. Benedict, *Proc. Natl. Sci. U.S.* **54**, 822 (1965).

[26] G. Dreesman, Chicken antibodies: Class of immunoglobulins and enzymatic and reductive dissociation of IgG. Ph.D. thesis, University of Hawaii, 1965.

[27] M. C. Michaelides, R. Sherman, and E. Helmreich, *J. Biol. Chem.* **239**, 4171 (1964).

[28] G. Dreesman and A. A. Benedict, *J. Immunol.* **95**, 855 (1965).

Dreesman and Benedict[25] have studied the heavy and light chains. The relative yields of heavy and light chains, and the antigenic relationships between isolated chains and papain-produced subunits, fit the four-chain structure proposed by Porter[29] for mammalian γG. Unlike most mammalian γG, reduced and alkylated chicken γG without a dispersing agent partially dissociates to yield heavy and light chains at pH 7.2 to 8.2. Apparently the noncovalent bonds holding chicken γG heavy and light chains are weak. In urea–starch gel electrophoresis at pH 8.8 reduced normal γG has ten distinct light chain bands.

ii. γM-Immunoglobulin

In immunoelectrophoresis γM-immunoglobulin forms a "Gull"-shaped arc extending out from the well. In starch block electrophoresis γM anti-BSA antibodies migrate as γ_2- and γ_1-globulins.[18]

Purified by recycle through Sephadex (Section B,3,c,ii), γM preparations have two peaks in the ultracentrifuge with $S_{20,w}$ values of 16.6 and 26 to 28, respectively. Williams and Donermeyer[21a] reported that a 25 S macroglobulin had a very concentration-dependent sedimentation coefficient.

According to our recent studies, the 16.6 S and 26 to 28 S proteins are reduced by 0.1 *M* mercaptoethanol to 7 S, 5 S, and 3.6 S subunits. Similar to chicken γG,[25] reduced γM partially dissociates to yield heavy and light chains in neutral buffer in the absence of a dispersing agent. In urea-starch gel electrophoresis, the γG and γM heavy chains migrate differently, and some of the γG and γM light chains appear to have different mobilities. Nevertheless, the γG- and γM-immunoglobulins are distinguished antigenically by their heavy chains.

There are no reports on the isolation and characterization of a γA-immunoglobulin. Attempts by the author to isolate a γA-immunoglobulin by the $ZnSO_4$ precipitation method of Heremans *et al.*[30] have been unsuccessful.

[29] R. R. Porter, *in* "Basic Problems in Neoplastic Disease" (A. Gellhorn and E. Hirschberg, eds.), pp. 177–194. Columbia University Press, New York, 1962.

[30] J. F. Heremans, M.-T. Heremans, and H. E. Schultze, *Clin. Chim. Acta* **4**, 96 (1959).

C. Collection and Handling of Serum*

Blood commonly is allowed to clot, the serum expressed on clot retraction then being clarified and preserved. All efforts should be taken to pre-

* Section 2,C was contributed by Merrill W. Chase.

vent hemolysis; if hemolysis is sufficiently strong, degradation of immunoglobulin can occur by enzyme action. As a rule of thumb, recovery of serum should be about one-half of the blood volume. The greater the bulk of a clot or its firmness, the less serum will be rendered. In special circumstances, as described below, clots are not allowed to form until cellular elements have been removed. None of the various methods of rendering serum quickly by defibrinating blood and centrifuging—inducing fibrin to form on glass beads or on metal whisks—can be recommended.

1. EXAMINATION OF TRIAL BLEEDINGS

A decision whether to bleed or to extend the immunization period of animals rests upon examination of trial bleedings of individual animals. The "half-life" of IgG and IgA in rabbits, guinea pigs, and most laboratory animals allows 1 or 2 days for obtaining a rough estimate of the concentration and characteristics of the immunoglobulins present if the trial bleeding is made 5 or 6 days after a restimulating injection of antigen; because of the shorter half-life of IgM, trial bleedings are made 3 days after restimulation.

It is possible to assay an antiserum quickly by precipitation, agglutination, or complement-fixation. Under some circumstances, even antiviral antibodies can be determined in a short time by coating virus-infected cultured tissue cells with antibody and determining the event of coating by fluorescent anti-γ-globulin.[1]

When multiple antibodies are wanted rather than a mono-specific serum, it is best to ascertain first that the total antibody content is satisfactorily high, then to proceed at once with further appropriate tests, such as micro-Ouchterlony tests (in which the agar field between antigen well and antibody well is reduced to 5 to 6 mm) or the Preer test. The Preer test,[2] requiring more skill to run, is started only after the trial bleeding becomes available; it is the most efficient one to determine the presence of antibodies that exist in only low concentration.[3] Plates required for micro-Ouchterlony or slides for immunoelectrophoresis are prepared before the trial bleeding is taken.

A simple method for evaluating trial bleedings applicable to soluble antigens is described.* Often, a decision whether to undertake major bleedings can be reached within 30 minutes, actual quantitative evaluation being made later. Three small tubes (70 × 8 mm) are used for

[1] S. M. Buckley, *Arch. Gesamt. Virusforsch.* **6**, 388 (1955).
[2] J. R. Preer, Jr., *J. Immunol.* **77**, 52 (1956).
[3] I. Finger and E. A. Kabat, *J. Exptl. Med.* **108**, 453 (1958).
* See also Chap. 13, A,3,a, Vol. III. The method given here is elaborated from one used by Karl Landsteiner.

each serum to be tested. The first tube should contain 6 μg in 0.2 ml, the second 20 μg, and the third 100 μg. To secure the highest concentration wanted (1:2000), it is simple to dilute 0.05 ml (50 lambda) of stock antigen with saline to a volume (in milliliters) that corresponds to the *percentage* concentration of stock antigen; that is, if the concentration of antigen is 28 mg/ml, dilute 0.05 ml to a volume of 2.8 ml. The second dilution is 1:5 of this, and the highest dilution is 1:3 of the second dilution. Add single drops of the trial bleeding to each tube, dropping them from a standardized capillary pipet (0.038 to 0.04 ml per drop) held at a 45° angle. When antibody is being prepared against native proteins of molecular weight 40,000 to 200,000, 1 drop of serum should be used; for hapten-specific antibody tested with a non-cross-reacting carrier protein, 2 drops will be needed. The tubes, positioned in a single row in narrow racks, are shaken well and observed against a dark background, illuminated by oblique light* directed from above, at about a 30° angle from horizontal. Readings are made within 5 minutes, and at intervals up to 60 minutes. A 6× or 7× optical loupe such as the Bausch and Lomb Hastings Triplet 7× is useful, held close to the eye and about 34 mm from the tubes. (If precipitation is not satisfactory within 10 minutes, a second drop of serum can be added to all tubes to reveal animals that possess antibody in only low concentration.)

We consider "one-plus" to be the first appearance of tiny discrete particles observable with a 6× or 7× loupe against a critically illuminated black background, and "four-plus" to be complete precipitation of antibody. Only seldom will a reading of "one-plus" as the maximum observed reaction at 10 minutes be meaningful in terms of a useful precipitating antiserum. An estimate of the antibody content depends on the relative turbidities observed within the three-tube test. If the maximum turbidity appears initially in, and the floc develops best in, the first tube (6 μg), the antibody-N will be below 0.1 mg/ml, since the formation of a similar amount of precipitate in tubes 2 and 3 is inhibited by antigen excess. As the immunoglobulin increases in concentration in the sera, the position of maximal precipitation "shifts to the right," to tube 2 and then to tube 3. Maximal precipitation (++ to +++) in tube 3, with use of 1 drop of antiserum, connotes a very good serum, of the order of 1.25 mg Ab-N per milliliter. A serum

* The total incident light is best directed downward by adjustable parallel wooden slats, painted black, the bottom one serving as the specified dark background. A more elaborate apparatus is described in D. H. Campbell, J. B. Garvey, N. E. Cremer, and D. H. Sussdorf, *Methods in Immunology. A Laboratory Text for Instruction and Research*, Benjamin, New York, 1963.

may be regarded as satisfactory when visual flocculation is essentially equal in tubes 2 and 3, or perhaps slightly greater in tube 2 than in tube 3 (about 0.6 mg Ab-N per milliliter).* Individual rabbits showing precipitation best in tube 2 can often be stimulated, by appropriately spaced reinjections of antigen, to make two- or threefold higher concentrations of immunoglobulins.

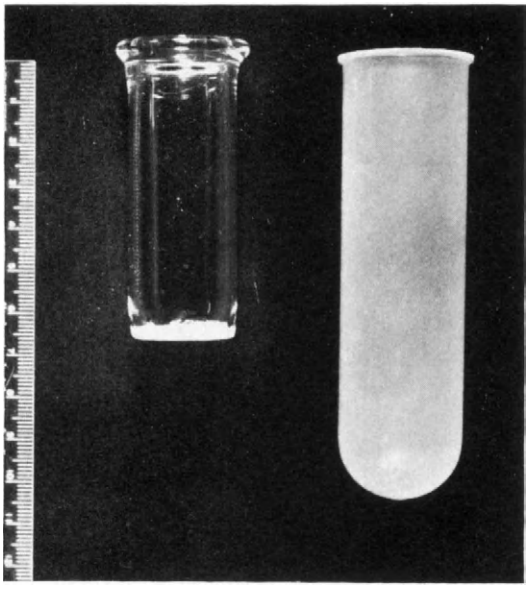


FIG. 1. Apparatus for small-scale concentration of proteins. A Pyrex glass tube (1 inch O.D.) is fused at one end to a 21-mm disk of coarse fritted Pyrex glass; the open end is provided with a lip ($1\frac{3}{16}$ inches O.D.). The total length is 68–70 mm. The lip rests above a 50-ml polycarbonate centrifuge tube, allowing collection of 15 ml of effluent.

In special cases, the presence of antibody capable of reacting with only a single antigen will be of greater concern than the absolute concentration of immunoglobulin, since further stimulation may excite the production of antibodies to minor contaminants. In these cases, a first, quick appraisal by the three-tube precipitin test mentioned above can be run with use of 2 drops (0.08 ml) of antiserum; within 2 hours the antibody can be concentrated four- to sixfold and given a final appraisal for monospecificity by appropriate methods. To effect rapid

* The figures were obtained by nitrogen value determined by the ninhydrin-hydrindantin method on the washed precipitate from tubes showing no excess of Ag or Ab in the supernatant; that is, $(N - AgN) \times 26.1$—the dropping pipet delivering 0.038 ml per drop.

concentration, mix 5 ml of antiserum, 5 ml of water, and 10 ml of saturated (25°) ammonium sulfate; after 20 minutes, centrifuge at 5000 rpm for 30 minutes; discard the supernatant, and wipe the walls of the tube with cotton; dissolve the precipitate with a *minimal* volume of water. The solution is desalted by passage through a column of Sephadex G-25 equilibrated with 0.85% NaCl*; concentrate the column effluent by mixing the effluent with Sephadex G-25, coarse grade, contained in an apparatus of the type shown in Fig. 1. Use 2 gm of Sephadex for each milliliter of unwanted fluid, since equilibrium will not be attained in the short time allowed. Centrifuge at once in a 50-ml (105 × 29-mm) wide-mouth polycarbonate centrifuge tube, such as Nalgene NI7-413. It is simple to calculate the concentration by weighing the centrifuge tube before and after receiving the concentrate. (Alternatively, one can introduce the ammonium sulfate precipitate as a thick white slurry into Visking tubing, tie the sac off, and dialyze against changes of saline for at least 24 hours.) Stabilization of the globulin concentrate can be effected by adding 0.1% of serum albumin, either a commercial preparation of albumin of the same species, or the crude ammonium sulfate supernatant. When the latter is used, the requisite amount determined by absorbancy at 2800 Å is added prior to the desalting procedure.

2. COLLECTION OF SERUM, SMALL ANIMALS

For *trial bleedings* intended to determine whether there is sufficient antibody to warrant larger collections, dry narrow tubes (100 × 10 mm O.D.) can be used to advantage for collecting blood, say from the marginal vein of the rabbit's ear. The clot forms quickly, owing to the large amount of glass surface; it is then separated from the walls and compressed by centrifugation 15 minutes later. With reasonable care, serum can be ready for testing within 90 minutes. Trial bleedings are made 5 or 6 days after a restimulating injection of fluid antigen, and heavy bleedings are made 1 or 2 days later. When IgM immunoglobulin is the antibody sought, rabbits are test-bled 3 days after a restimulating injection.

When the antibody titer is satisfactory and a rabbit is to be sacrificed, a moderately heavy bleeding (about 35 ml) is often taken from the ear on the fifth or sixth day after the last injection (Section D,2,e,i), and fluid balance is restored by injecting the equivalent volume of saline intraperitoneally; exsanguination is practiced 2 to 4 days later. The several serum collections are worked up separately and can be pooled.

* Test for freedom from sulfate on a few drops of final solution by adding 2 drops of 1% barium acetate solution and 1 drop of concentrated HCl.

The successive bleedings secure a greater recovery of immunoglobulin; the serum of the major bleeding will show some dilution in total proteins.

Techniques of taking blood from various species are given in Section D. With very small animals such as the mouse, bleedings are often pooled into one receiving vessel to reduce mechanical loss. Besides serum, ascites fluid rich in immunoglobulin can sometimes be induced to form. Repeated intraperitoneal injection of antigen in Freund's adjuvants can cause 0.5 to 14 ml of ascites fluid to accumulate. The procedure for handling such exudates is given in Section B,i,f.

a. Separation of Serum from Clotted Blood

For *heavy bleedings* of rabbits via heart puncture or via the carotid artery, care to avoid hemolysis is a prime goal. Food, but not water, should be removed from animals the night before the intended bleedings to obtain clear serum and to avoid subsequent, persisting separations of fatty materials. Occasionally, a quite milky serum specimen will be found, or blood specimens which clot only very slowly, allowing erythrocytes to settle out partially before clotting is completed. These conditions reflect physiological disturbances as a consequence of natural diseases or unusual experimental procedures.

Vaselined collection tubes and clot weights are prepared as follows. We prefer round-bottom 100-ml heavy-duty centrifuge tubes, 25.5 mm I.D. (not constricted at the neck),* or culture tubes, 16×150 mm. For small bleedings, culture tubes or the smaller Wassermann tubes, 13×100 mm, are quite suitable. The collection tubes, sterilized with cotton plugs, are then coated with a *very thin* layer of sterile Vaseline to allow spontaneous separation of clot from the vessel walls. Molten sterile Vaseline is introduced or sterilized within the tube. The tube is grasped in a test tube holder, and the walls are warmed sufficiently to melt the Vaseline; the excess is poured out onto paper towels, the plug replaced, and the tube held inverted in a rack so that the small remainder will drip into the cotton plug.

Weights cut from stainless-steel solid rodding, 22.2 mm in diameter $\times$ 17 mm high ($\frac{7}{8} \times \frac{11}{16}$ inch), weighing about 52 gm, are useful for compressing clots within 25.5-mm I.D. tubes. For other tubes, clot weights of appropriate diameter can be prepared.

The collection tubes are filled with blood to two-thirds of capacity. The enzymatic process of clotting is assisted by maintaining the tubes at 37° for 1 hour before transfer to the cold room for clot contraction. Two hours later, any clots that have not retracted from the walls are *gently* freed by careful "rimming" with a sterile rod: we use anodized

* Similar to Pyrex 8260, 32×152 mm, or Kimax 45206, 160 mm, but with rim instead of pour-out.

aluminum knitting needles, No. 2 (2.5 mm in diameter × 25 cm), sterilized in special glass tubes. Within 30 minutes, free serum can be decanted sterilely into 50-ml screw-capped glass centrifuge tubes. Later collections are added to the original portions. When the drained clot about equals the bulk of the serum collected from it, two or three clot weights are added; since clots move freely within the tubes, they can be "pooled" up to one-half the capacity of a tube before weights are added. The tubes are left overnight in the cold for a final collection of serum, which is decanted temporarily into separate tubes. (By this method, centrifugation is not needed to compress the clots.) Blood cells are removed by centrifugation (for example, 1500 rpm for 20 minutes), and serum is removed by means of a sterile pipet. A "sucker tube" of flexible rubber tubing (Scimatco, 3/16" bore by 1/32" wall, Fisher 14-150-5) ending in a glass mouthpiece assists considerably in stripping serum from sedimented cells at eye level. Straw-colored, limpid serum should result. Portions are pooled, or are held aside if later collections are tinged with hemoglobin. Preservation of serum is discussed below.

b. Separation of Serum from Clarified Plasma

In special circumstances, clotting is best prevented until erythrocytes have been removed by settling or centrifugation. The volume of serum rendered can be greater and hemolysis is prevented. Further, erythrocytes and peripheral white cells become available; sterile techniques must be used when the cells are wanted. Citrate or oxalates or EDTA can be added to prevent clotting; later, sufficient calcium is added to the clarified plasma to cause fibrin to form. Such clots, however, do not retract; clots are cut up or teased apart, and serum is extruded by applying rather high compression. The final concentration of serum proteins is usually about 85%, owing to volume increases during the manipulations. This technique is particularly applicable to the blood of mature chickens, which yields a firm, hardly contracting clot; alternatively, the clot is held for 4 hours at 40° or spun at 15,000 *g*.

Syringes are wetted with the chosen anticoagulant (isotonic 3.8% aqueous sodium citrate·$2H_2O$ or a mixture containing 1.2% ammonium oxalate and 0.8% potassium oxalate, both monohydrated, in saline, or 2% disodium EDTA in saline). An assistant has ready a series of 125-ml Erlenmeyer flasks and 5-ml pipets. As blood is drawn, approximately one-tenth its volume of the anticoagulant is placed in an Erlenmeyer flask, the blood added, and swirling rotation started at once and maintained for 1 minute. The total volume of anticoagulant used should be recorded. The specimens are pooled and strained through stainless-steel strainers of 40 mesh (40 wires per inch). Centrifuge bottles should not be overloaded; introduce not more than 60 ml of fluid blood per

250-ml centrifuge bottle to yield a packed erythrocyte volume of not more than 25 ml. Centrifugation should be made at about 2500 rpm for about 20 minutes to avoid hemolysis.

Fully clarified plasma should be clotted in a single vessel with walls lightly Vaselined. For each 10 ml of 3.8% sodium citrate present, add 2.0 ml 10% anhydrous calcium chloride; or for each 10 ml of 2% EDTA add 1.6 ml 2% calcium chloride; or for each 10 ml of mixed oxalates add 1.25 ml of 10% calcium chloride. Stir with a stout glass rod during addition of the calcium chloride. The plasma will clot shortly as a clear solid that, unless the vessel is Vaselined, will adhere firmly and nearly invisibly to glass and result in mechanical loss. After some hours, break the clot apart mechanically and squeeze within fine-quality bandage gauze to express the serum. Following recovery of serum, there will be present approximately 100 mg of calcium citrate per 100 ml, 110 mg of calcium EDTA per 100 ml, or only bare traces of calcium oxalate.

3. COLLECTION OF SERUM, LARGE ANIMALS

Horses, goats, and sheep are bled from the external jugular vein (Section D,6). Small bleedings are handled as in Section D,2,e. Large bleedings are usually drawn into rather large containers to preserve sterility, so that extrusion of serum from a massive solid clot requires the use of heavy clot weights and special techniques. Freund[4a] showed definitely that much more serum (110 to 195% of the amount secured from ordinary clots) could be obtained by use of anticoagulant, removal of cells, and preparation of serum from clarified plasma.

For processing bleedings of 1.5 to 3 liters taken from domestic animals, 3-liter portions are drawn into 40 ml of 25% sodium citrate sterilized in a $22\frac{1}{4} \times 6$-inch (55×15-cm) vessels; the blood is well mixed *at once*, and the vessel is left at room temperature overnight to allow erythrocytes to settle. Plasma is then drawn off by sterile siphon into an $8\frac{3}{4} \times 7$-inch (22×18 cm) straight-sided vessel containing 20 ml of 25% calcium chloride, the clot forming in 30 to 40 minutes. After 3 or 4 hours, the clot is cut in four or five places and a 15-pound lead weight, precisely formed in a special manner[4] to fit the straight-sided vessel, is added. Serum is withdrawn on the following day.

4. PRESERVATION AND STORAGE

a. Liquid Storage

Sterile immune serum of good initial titer can be held safely at 4° for long periods of time; precipitin titers have been maintained for more

[4a] J. Freund, *J. Infect. Diseases* **33**, 328 (1923).

than 35 years, and guinea pig antisera (PCA-type antibody) have proved stable for over 20 years. When serum is intended for subsequent testing *in vivo,* the absence of a preservative is highly advantageous. It is the practice in this laboratory to filter serum through cellulose acetate filters (Millipore Type GS, 0.22 microns average pore size) as described in Section C,5.

Sterile serum is stored optimally in thin-walled glass ampules sealed by torch, but it is more practical to dispense it sterilely in 4- to 8-ml amounts in 100 × 16-mm screw-capped tubes stoppered with Demuth one-piece pressure-expanding polypropylene closures.* The caps are forced tight and wiped with 70% alcohol; parafilm seals are then placed to keep the shoulders of the tubes clean and to check gaseous exchange.† Azide is added only to single tubes as desired for facility in making gel tests. Other types of containers include narrow-necked "serum vials" available in 1-, 2-, 5-, and 10-ml sizes (such as A. H. Thomas Co. No. 2319) and wide-necked "allergists' vials" (such as T. C. Wheaton Glass Company's item No. 5850), both of which are closed with rubber stoppers having a turn-down skirt covering the neck of the vial. Air must be introduced into closed vials equal to the amount of serum to be withdrawn, the top of the rubber cap has to be sterilized chemically, and inverting of the vial becomes necessary for withdrawal even after precipitates have settled to the bottom.

Preservatives can be employed, such as 1:10,000 merthiolate (thimerosal) ("1% of 1% stock" solution), or 0.1% sodium azide ("1% of 10% stock"), or 1:10,000 8-hydroxquinoline sulfate ("8-quinolinol sulfate"). Some workers add preservatives in pairs, such as merthiolate 1:10,000 plus phenol 0.25%, or 0.05% sodium azide plus 0.01% acriflavine. Preservative added to serum can impose limitations as to use. An injection of serum containing merthiolate into guinea pig skin, for example, will produce tuberculin-like toxic reactions. Sodium azide, an excellent preservative when reactions in gel are intended, is toxic for animals. It must be kept in mind that some preservatives, although effecting primary sterilization, will bind to certain proteins as to albumin and may not be free to thwart a contaminant introduced later, and also that some preservatives are removed with time by dissolving in rubber stoppers or caps. It is indeed disconcerting to find subsequent growth of yeasts or bacteria or molds in "preserved" sera.

* Available from Brockway Glass Company, Inc., Parkersburg, West Virginia. The soft glass type is suitable for storage.

† The double barrier is highly effective in retarding entry of CO_2 and H_2S. Ordinary screw-capped tubes with liners of cemented rubber or Teflon allow easy access of these gases.

Sera intended for subsequent high dilution can be preserved well by the addition of an equal volume of high-quality glycerine, especially agglutinating sera and rabbit anti-sheep cell hemolysin ("amboceptor"). Fifty percent glycerine is an excellent solvent for proteins. It is strongly advised to avoid exposing such mixtures to sunlight. The glycerine is not deliberately removed but is diluted out when the serum is used.

b. PRESERVATION BY FREEZING

Storage of frozen sera at about —16° is practiced often, with reliance placed on continuing maintenance of the frozen state. It is to be noted that chicken serum should not be frozen.

It is preferable to dispense serum in volumes sufficiently small to allow sampling without subjecting large volumes to repeated freezings and thawings. The containers should not allow escapement of water vapor or gaseous exchange. Again, we prefer screw-capped tubes with Demuth one-piece caps and overseals of parafilm. During frozen storage, water is constantly subliming and refreezing as pure ice at the top of the frozen plug, resulting in gradual condensation of protein in the bottom of the tube. Long-stored samples may show gelation of proteins, necessitating considerable effort in reconstituting the serum after melting. Storage of frozen samples, in our opinion, is applicable chiefly to experiments that are to be completed within a reasonably short period of time. It should be remembered that some makes of rubber stoppers, often used to close test tubes for frozen storage, lose resilience and contract away from the glass at —16° or below.

c. LYOPHILIZATION

Following lyophilization, on reconstitution some specimens of serum tend to precipitate nonspecifically[4b] and show turbidities which are difficult to clear. It is recommended that small samples first be tested by lyophilization and immediate reconstitution. Lyophilized specimens should be sealed in glass and stored at 4° with labels stating the volume to which sterile water should be added in reconstituting. Passage through a pre-wetted filter (Section 2,C,5,b) may be needed.

5. STERILE FILTRATION

With properly designed laboratory routine, no difficulty should be experienced in maintaining sterile conditions during filtration and dispensing of antiserum or other fluids. Filtration of small volumes through thin and disposable cellulose acetate membranes or their equivalent has practically supplanted older methods (Chamberland, Selas, Berkefeld,

[4b] M. Heidelberger and M. M. Di Lapi, *J. Immunol.* **61**, 153 (1949).

or Mandler "candles" of the rigid type), including full-thickness or split-thickness Seitz asbestos pads which have high adsorptive capacity. (For "production runs," other decisions regarding equipment may be useful.)

Positive-pressure devices are much to be preferred over those employing negative pressure, to avoid sudden foaming of protein solutions with the hazard of surface denaturation of proteins and the need for bleeding air into the system periodically. In a laboratory in which volumes of 4 ml to 300 to 400 ml will be filtered in single batches, only two types of positive filtering devices need be employed (Figs. 2 and 3). Large volumes, such as 1500 ml of serum or 6000 ml of buffer, may require larger apparatus—such as those holding 142-mm or even 293-mm membranes, available from the Millipore Corporation and Gelman Instrument Company. Very small volumes, 0.5 to 2 ml, are best passed by syringe pressure through 13-mm membranes held in a Swinney-type filter holder. The small metal Swinney filters, machined to hold asbestos pads, require special Teflon gaskets to seize membrane filters properly; very tight closures are required to avoid leaking. Special tightening tools are available from the Millipore Corporation. Newly introduced assembled and presterilized disposable plastic filters offer considerable facility when samples have been fully clarified. Both the 13-mm- and the 25-mm-diameter Swinnex filters are available with membranes of various average pore sizes—from 1.2 microns down to 0.22 micron. For sterile filtration, only the 0.45-micron and 0.22-micron porosities are needed. Prefiltration can be done nonsterilely in these plastic filters or in a metal device such as the one pictured in Fig. 2. Prefilters and filters of 0.8-micron, 0.45-micron, and 0.22-micron average pore sizes should be kept on hand. Membranes of different pore sizes may be used singly or "stacked."

The presence of detergents in membrane filters (2 to 3% of dry weight) has been pointed out by Cahn[5]; the detergent can be largely removed by a preliminary pass of very hot water (90° to 100°) through a filter, cooling, and conducting subsequent filtration in the cold.

Various devices have come on the market intended to utilize centrifugal force to push fluids through suspended membranes. These are judged unsuccessful in securing a sterile effluent for several reasons. (1) Since air does not escape through a membrane once it is wetted, pressure considerations dictate that the receiving chamber should not be of one-piece construction unless it is large in size relative to the effluent to be received; (2) devices for sealing *and venting* the receiving chamber appear not to have been designed adequately to ensure both sterility and subsequent removal of sterile filtrate; one device, the Hemmings'

[5] R. D. Cahn, *Science* **155**, 195 (1967).

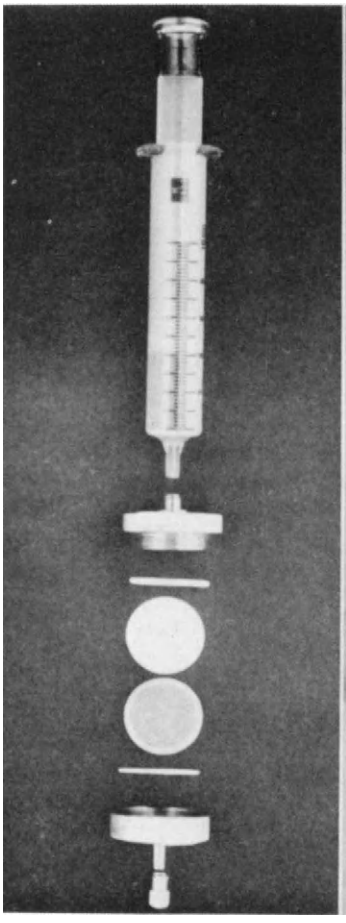


FIG. 2

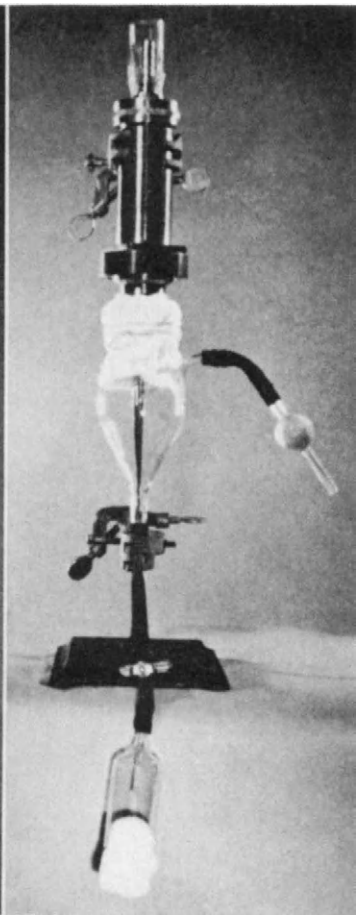


FIG. 3

FIG. 2. A useful apparatus for sterile filtration of serum (about 4 to 30 ml). The white membrane filter disc (25 mm or 1 inch in diameter) rests upon a perforated support screen. Below the support is seen a circular flat Teflon gasket that rests in the terminal stainless-steel element carrying the discharge Luer nozzle (left). The filter itself is pressed to the support screen by a circular Teflon O-ring fitting inside the stainless-steel upper element which bears a female Luer fitting. The dry assembly is screwed together somewhat loosely. A cap for a male Luer fitting (Becton-Dickenson, No. 425A), shown at the left, covers the discharge nozzle. The assembly is wrapped in a steam-pervious 7-inch square of Patapar vegetable parchment No. 27-2T (Paterson Parchment Paper Co., Bristol, Pennsylvania) and covered with Kraft paper which is sealed with a narrow strip of telltale autoclave tape (for ex-

ample, No. 1222 of Minnesota Mining and Manufacturing Company). The assemblies are sterilized for 20 minutes at a true internal autoclave temperature not exceeding 121° (250°F) (see text), allowing steam pressure to rise slowly and to fall slowly.

At the time of use, the filter is unwrapped and tightened with special wrenches provided, while the capped Luer nozzle is held inside the Patapar. A sterile disposable needle is then attached by sterile forceps to the Luer nozzle, the protective needle jacket being left in place. The fluid for filtration, clarified and passed previously through a nonsterile cellulose acetate membrane of 0.45-mμ pore size in a similar apparatus, is taken up in a syringe (10 or 20 ml) large enough to include 3 to 6 ml of air. Filtration is made directly into one or more sterile tubes held near the updraught of incombustible gases near a lighted Bunsen burner. The compressible head of air allows all fluid above the filter to pass the membrane. A slight amount of fluid (about 0.3 ml) is lost within the small space *below* the membrane, although it can be recovered and treated with preservative. Each assembly is unscrewed and inspected at once for integrity and positioning of the membrane as shown by the circular indentation caused by the upper pressure gasket. The particular apparatus shown is obtainable from the Millipore Filter Corp., Bedford, Massachusetts, as Micro-Syringe Filter Holder, Cat. No. XX3002500. A similar apparatus, although made of autoclavable plastic (see text), is sold by the Gelman Instrument Company, Ann Arbor, Michigan, as Model 4320.

FIG. 3. An apparatus suitable for sterile filtration and dispensing of serum or buffered solutions (about 20 to 300 ml). The filter unit shown consists of a 100-ml stainless-steel pressure filter holder (Millipore Cat. No. XX4004700, similar to Gelman Pressure Filtration Funnel Cat. No. 4240), entering the pear-shaped Pyrex collecting bulb through a one-hole rubber stopper, with joint covered by cotton packing. The collecting bulb has a pressure-venting side arm connected with a Pyrex bulb tightly packed with absorbent cotton. The tapered dispensing tip (35 mm long) below the collecting bulb is integral with a glass bell of extended lip (total height 68 mm), and sufficient width (34 mm I.D.) to receive necks up to the size of 250-ml Erlenmeyer flasks or "Milk Dilution Bottles" (Pyrex No. 1372, Kimble No. 14925). Rubber tubing is heat-resistant (A. H. Thomas Co., No. 8836, ¼-inch bore × 5/64-inch wall). The unit is clamped to a ringstand cut down to fit within the autoclave. During autoclaving, the gauze-covered cotton plug in the dispensing tip is protected with a wrapping of Kraft paper and is elevated by cord to avoid contact with the base plate.

The filter unit is essentially a larger model of Fig. 2, but holds 47-mm-diameter membranes which provide 37 mm of filter area. In the Millipore model, the Teflon pressure rings of Fig. 2 are integral, and a flat Teflon gasket is used on top of the flange at the bottom of the chamber. During autoclaving, the loosely tightened assembly is handled as in the legend for Fig. 2. Just before use, the lower thread assembly is tightened by special wrenches supplied with the device; the newest model of wrenches can be used without removal from the ringstand. Preclarified serum is then poured into the barrel after removal of the top element (which is tightened only by hand). Air pressure (about 4 psi) is applied to the top orifice. Filtration may be interrupted temporarily and the contents dispensed from the lower bowl whenever the amount to be filtered exceeds the 100-ml capacity of the receiving bowl. Similarly, the metal barrel can be loaded several times in succession.

filter, supplies a split rubber washer between filter element and receiver; another has an open plastic vent judged difficult to keep sterile.

Despite the thinness of membrane filters, it should not be assumed that adsorptive characteristics can be ignored. Common Millipore and Gelman membranes have both removed a surprisingly large amount of pepsin from solution in 0.0025 *N* HCl. In special cases—but probably this is not applicable to whole serum—one should reject the first 5 ml when a dilute protein reagent is being filtered; the principle has long been known with filters of the candle type and with asbestos filters, the amount to be rejected varying with the mass of the filter. The presence of detergent in membrane filters has been mentioned above; this is usually not an obstacle unless the filtrate is intended for purposes such as tissue culture.[5]

a. Filtration Assemblies

With the devices shown in Figs. 2 and 3, the membranes are autoclaved assembled in the instrument. Three precautions are to be observed: (1) The filter elements must be fully dry at the time of assembly, and membranes must be handled carefully to avoid mechanical damage. (2) Pressure differentials should not exist between the top of the membrane and the effluent side during autoclaving; else stretching and "doming" of the filter can occur. (3) The tolerated amount and length of heating should not be exceeded. Requirement 2 is met by avoidance of packings that will retard uniform rise and fall of steam pressure on both sides of the membrane during autoclaving. Requirement 3 depends on the particular membrane employed. Cellulose acetate filters are heat-sensitive at temperatures above 121° and shrink below the needed diameters; the temperature actually attained at "pressure" or "temperature" settings on the gauges should be determined for each autoclave with a maximum-registering thermometer (Taylor Instrument Co., Cat. No. 21464). Newer types of membranes are being introduced. Thus a membrane made of fluorocarbon (Gelman Metricel VF-6) is stated to be stable up to 148° (300°F).

The pore diameter of the membrane used for sterile filtration is important: Membrane filters of 0.45-mμ average pore diameter correspond to Berkefeld N ("normal") filters; those of 0.22 mμ are equivalent to the desirable Berkefeld W ("wenige") filters of bacteria-excluding size.

b. Preliminary Clarification

Premature clogging of a filter can occur on filtering a serum sample that "looked" clear. Samples of serum that, examined by transillumina-

tion, show a slightly "smoky" appearance are not ready for one-pass sterile filtration, and only seldom will the use of a "microfiber glass" prefilter placed over a sterilizing membrane permit a single "sterilizing pass."

If tiny particles are seen when the serum is viewed critically in thin layer by a hand loupe, a preliminary centrifugation in Lusteroid tubes at 13,000 rpm for 30 minutes should clear the serum considerably and usually deposit a small amount of sediment. If centrifugal force has caused particles to float owing to excess of fat, most of these can be excluded by nonsterile pressure-passage through a membrane filter (as 0.8-mμ mean pore size) that has been wetted with saline *after assembly*.

Ostensibly clear serum should be passed through a nonsterile membrane filter, 0.45 mμ in pore size, passage being rapid with only negligible losses in volume. The serum is then ready for the final, sterilizing pass. The use of fiberglass prefilters proves mostly to be unnecessary.

c. Filtration and Dispensing

Apparatus of the type shown in Figs. 2 and 3 is recommended. For a laboratory making three to twelve or more filtrations per week, seven units of the 1-inch type would be appropriate, one being held for nonsterile preliminary filtrations (or disposable presterilized 1-inch Swinnex filters can be used). Two sterile units of the 100-ml pressure-bowl type would be adequate, with a third bowl held for nonsterile preliminary filtrations.

When the 1-inch unit is used, an all-glass syringe is helpful, owing to its accurately ground Luer tip, simply tightened into the filter with a clockwise motion. A head of air should be maintained in the syringe during filtration. Sterility of the nozzle end is maintained, and the broad flat surface may be rested during filtration on the flamed end of the receiving tube. The operator holds each screw-cap between the palm and little finger, with the opening pointed downward. An assistant loosens caps of other tubes that are to be filled and flames the tops of the tubes. When a second portion of serum is to be passed through the same filter, syringes are detached without exerting pull on the plunger, to avoid raising the membrane from its supporting disk. Should clogging occur unexpectedly, stop the operation, turn the apparatus with needle upward, and remove the syringe. The contents are recovered by withdrawal above and below the filter by means of a sterile capillary and a rubber teat (see Fig. 4).

The caps of the tubes are screwed on tightly, wiped with 70% alcohol, and 2.5-inch squares of parafilm as sold in laboratory rolls (use the surface next to the protective paper) are placed over the cap and pressed

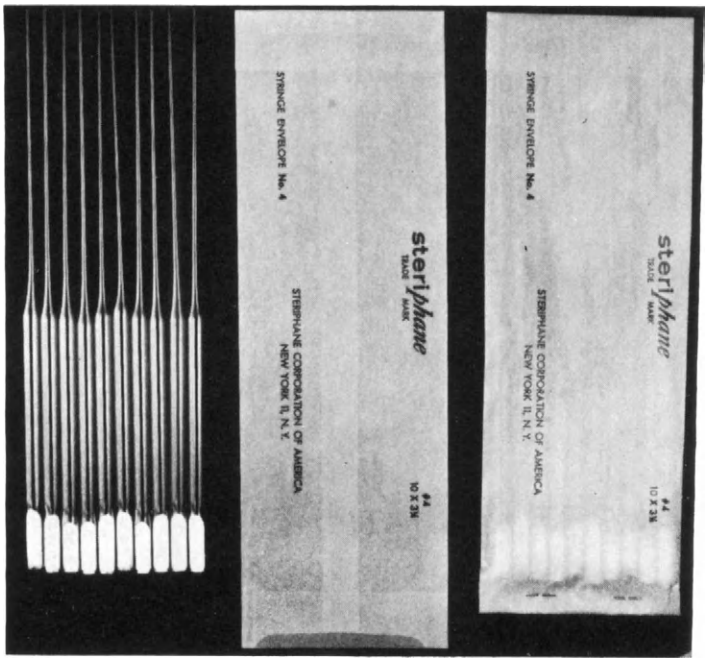


FIG. 4. A convenient sterile packaging of disposable Pasteur capillary pipets (9 inches long with 4½-inch capillary, ending in tips of 0.75 ± 0.25 mm I.D.) is shown. The pipets are cleaned on the steambath (at 70° or above for 20 minutes) with commercial sulfuric acid to which solid KNO_3 has been added (about 0.5 gm per 250 ml of acid). The pipets are rinsed thoroughly, shown to be free of acid, dried, and plugged with absorbent cotton; projecting cellulose fibers are removed by flaming (left view). Ten such pipets are introduced (center) into No. 4 steam-permeable 10 × 3¼-inch Steriphane syringe envelopes (Steriphane Corp. of America, New York, New York). The free end is closed by stapling, and the packages—rigid enough to avoid broken capillary tips—are sterilized by autoclaving (right). For use, a sharp cut of one lower corner is made with scissors. By compression of the envelope, pipets may be withdrawn singly without touching the envelope. A final pass through a Bunsen flame provides a pipet sterile both internally and externally.

close to the walls*; the warmth of the fingers will cause the film to adhere.

d. HANDLING OF STERILE SERUM

When a portion of sterile serum is to be withdrawn from a screw-capped tube, sterile pipets should be used, sterile outside as well as

* The double barrier is highly effective in retarding entry of CO_2 and H_2S. Ordinary screw-capped tubes with liners of cemented rubber or Teflon allow easy access of these gases.

inside. If capillary pipets are not individually wrapped and sterilized, the outside wall is thoroughly flamed and allowed to cool within a sterile culture tube 150 × 17 mm. We find it especially convenient to employ capillary pipets of the type shown in Fig. 4 whenever the volume wanted is not greater than 1.5 ml. Serum withdrawn and remaining unused is treated with sodium azide and added to the sample held with preservative.

e. Sterility Testing

Obviously it will not be possible to detect only one organism in an entire filtrate, particularly by culturing a single drop or even 0.5 ml of filtrate. Material that has been prefiltered cleanly will not place a large burden on the final membrane. Absence of external leakage and confirmation of proper seating of the membrane as seen upon unscrewing the apparatus usually indicate that sterile filtration has been accomplished. Owing to the slight chance that one or two organisms have passed into a filtrate, further safety is assured when filtrate is dispensed in amounts of 4 to 7 ml.

To check for gross contamination and laboratory error, simple sterility tests can be made in the following ways. Add 3 capillary drops of a filtrate to each of the following culture media, which should be stocked routinely. The media will be useful for a year if kept refrigerated in screw-capped tubes covered with parafilm caps. (1) Nutrient broth (0.3% beef extract and 0.5% peptone, pH 7.2) or meat infusion broth with peptone; (2) nutrient agar slants (nutrient broth with 0.5% sodium chloride and 1.5% agar); (3) Sabouraud's agar for fungi (1% high quality bacteriological peptone, 4% dextrose, 1.5% agar, pH 5.6).

The inoculated tubes are incubated for 10 days at room temperature in a dark cabinet, since the presence of pathogens is hardly to be suspected in the filtrate, and 37° may be too warm for commensal organisms; or two sets of media may be prepared for incubation at each temperature. A more critical test consists in using 15 ml of fluid thioglycolate medium with 0.075% agar in 150-mm culture tubes, which would be inoculated with the same capillary pipet used for media 1, 2, and 3, by plunging straight down to the base of the tube and allowing a vertical column of filtrate to flow out as the capillary tip is drawn upward. This medium is required for biologicals in commerce, since anaerobes and microaerophilic organisms are detected in addition to aerobic organisms, but the medium is not stable for more than a few days, owing to penetration of oxygen by diffusion. Laboratories wishing to use it are advised to purchase dehydrated fluid thioglycolate medium such as Difco's B-256 or B.B.L.'s No. 01-140 and to dissolve small amounts and autoclave a few tubes as wanted.

When the issue is very critical, the used filtration apparatus, without disassembly, can be employed to filter a culture of a bacterium and the entire bacterial filtrate collected and incubated. (This is essentially the procedure used by the manufacturers to test their batches of membrane material; thus Millipore Type GS (0.22 mμ) filters retain *Pseudomonas* in tenfold-concentrated tissue culture medium 199, the entire filtrate being incubated at 37° for 15 days.) Note that 0.45-mμ filters are not expected to pass the *Pseudomonas* test. If growth appears in the incubated tubes, the entire serum batch can be refiltered if it has been kept close to 0° during the period of sterility testing.

D. Animal Handling*

1. GENERAL CONSIDERATIONS

In recent years, standards have been formulated for housing animals, new types of cages have been introduced, and pelleted foods have been marketed. The manner of raising animals by commercial breeders has been much improved, so that investigators may expect to receive healthy and rather clean stock shipments. Many highly inbred and genetically described mouse lines are available, and several inbred strains of guinea pigs and rats are being propagated. Rabbits of known allotypes are being produced in certain laboratories. For special purposes, "gnotobiotes" can be used—animals raised under sterile conditions, derived initially by separation from maternal uteri in late term and free of many of the usual viruses and pathogenic bacteria; several commercial dealers now deliver gnotobiotic mice to the laboratory in sterile plastic equipment. Larger research institutions often have trained professional persons as supervisors.

Apart from these special considerations, the former problems persist: training the investigator to know and handle animals; providing clean water, food, and bedding on schedule; avoiding infection and cross-infection; and exercising vigilance over the continuing health and well-being of animals in their artificial social environments. The best assurance comes when the investigator visits the animal quarters periodically, to observe and to learn. Holiday and Sunday routines in animal quarters may offer less than standard care.

Particularly when questions of procedure or health arise, the investigator should turn at once to the supervisor of the animal facilities.

* Section 2D was contributed by Merrill W. Chase.

Books of general usefulness can be consulted,[1-2c] and excellently indexed annotations of publications appearing through 1961 are available, covering anatomy, physiology, diseases, and other aspects of animal care.[2d]

It is recommended that all persons who handle animals be immunized with tetanus toxoid under medical supervision by the standard procedure (usually toxoid adsorbed to alumina is given in spaced injections), with subsequent annual "booster shots." An established medical record will relieve persons who suffer skin lacerations—as from sharp edges of cages or occasional bites—from submitting to administration of tetanus antitoxin.

Physical handling of animals and their caging have been described well in other volumes and other years.[3a-3c] Repetition is needed chiefly because books age and become forgotten. There are, however, some new and excellent manuals[2b,2c,4,5] and a few useful newer techniques which will be listed. The discussion offered here is limited to the most commonly used laboratory animals.

Recent legislation has established certain standards in procuring dogs and cats, and in housing dogs, cats, guinea pigs, hamsters, rabbits, and nonhuman primates. Public Law 89-544 of August 1966, implemented by standards set by the Secretary of Agriculture, became effective in 1967, see *Federal Register* **32**, Part II, 3270 (1967). Sections of the law as these affect immunologists (who use chiefly rabbits and guinea pigs)

[1] "The Merck Veterinary Manual," 2nd ed. Merck & Company, Rahway, New Jersey, 1961.

[2a] R. W. Kirk, "Current Veterinary Therapy. Small Animal Practice, 1966/67." Saunders, Philadelphia, 1966.

[2b] W. Lane-Petter, "Animals for Research." Academic Press, New York, 1963.

[2c] W. I. Gay, "Methods of Animal Experimentation," Vol. I. Academic Press, New York, 1965.

[2d] J. S. Cass, I. R. Campbell, and L. Lange, "A Guide to Production, Care and Use of Laboratory Animals, An Annotated Bibliography," *Federation Proc.* Suppl. No. 6, December 1960; *ibid*, Suppl. A, *Federation Proc.* Suppl. No. 13, March/April 1963.

[3a] J. A. Kolmer, "Infection, Immunity and Biologic Therapy," pp. 32–52. Saunders, Philadelphia, 1925.

[3b] W. Smith and J. McIntosh, *in* "A System of Bacteriology in Relation to Medicine," Vol. 9, pp. 236–267. His Majesty's Stationary Office, London, 1931.

[3c] A. B. Wadsworth, "Standard Methods of the Division of Laboratories and Research of the New York State Department of Health." Chapters 3, 4, 5, and 70. Williams & Wilkins, Baltimore, 1947.

[4] A. N. Worden (ed.), "The UFAW Handbook on the Care and Management of Laboratory Animals." Baillière, Tindall and Cox, London, 1947.

[5] D. J. Short and D. P. Woodnott, (eds.), "The A(nimal) T(echnicians) A(ssociation) Manual of Laboratory Animal Practice and Techniques." Thomas, Springfield, 1963. See especially Chapter 5, "Animal Handling," by D. J. Short.

TABLE I
SPACE SUGGESTED FOR THE ROUTINE HOUSING OF COMMON LABORATORY ANIMALS[a]

		Housing			
Species	Weight	Desired area per animal (square feet)	Unit	Unit size width × depth × height (inches)	Floor space of unit (square feet)
Rabbits	2–4 kg	3.0	Cage for 1–2	18 × 24 × 16	2.9
Guinea Pigs	350 gm	0.5	Cage for 2–4	14 × 20 × 8	2.0
		0.7	Cage for 1	8 × 12 × 8	0.67
Rats	250 gm	0.2–0.5	Cage for 4–10	14 × 20 × 8	2.0
		0.2–0.7	Cage for 1	8 × 12 × 8	0.67
Mice	20 gm		Cage for 2	5 × 9 × 5	0.31
		0.05–0.1	Cage for 10–20	12 × 18 × 5	0.31
		0.1–0.7	Cage for 5–10	8 × 12 × 5	0.67
Chickens	3 kg	1.5	Cage for 2–4	36 × 24 × 24	6.0
		3.0	Cage for 1	18 × 18 × 21	2.25

[a] The table shows housing guidelines recommended for animals during experimentation, data being derived from Table 1 of "Guide for Laboratory Animal Facilities and Care," Public Health Service Publication No. 1024, Revised 1965. Prepared by the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council, Washington, D.C. or from Dr. Vernon Riley. The exact dimensions shown are idealized and are not generally available. Animals of greater weight, such as guinea pigs heavier than 350 gm, should have 90-square-inch floor area per animal, i.e., not over 3 animals in a cage measuring 14 × 20 × 8.

Production units to house larger numbers of animals are described in individual "Standards" for the breeding, care and management of each species, available from the Institute of Laboratory Animal Resources of the National Academy of Sciences–National Research Council: for *rabbits; rats* (of which 6 weight categories are recognized); *laboratory mice* (of which 4 weight categories are recognized); *Syrian hamsters; laboratory primates;* White Leghorn *chickens;* and so on.

Minimum standards for housing the following species are detailed in the *Federal Register* for February 24, 1967, exclusive of space for food and water.

Rabbits: Space per individual animal: 3–5 lb, 1 sq ft; 6–8 lb, 2 sq ft; over 9 lb, 3 sq ft; nursing female with litter, ascending according to weight from 4.7 sq ft (3–5 lb doe) to 7.5 sq ft (12 lb doe), with nesting box.

Guinea Pigs: Enclosure height must be at least 6½ inches. Space requirements *per animal:* weaning to 350 gm, 60 sq inches; 350 gm or more, 90 sq inches, cage limited to 10; breeders, 180 sq inches; nursing female with litter, 225 sq inches.

Hamsters: Enclosure height must be at least 5½ inches. Space requirements *per individual:* weaning to 5 weeks, 10 sq inches, cage limited to 20; 5–10 weeks, 12.5 sq inches, cage limited to 16; 10 weeks or more, 15 sq inches, cage limited to 13; nursing female, 121 sq inches, cage singly. Approximately two-thirds of the areas shown are appropriate to dwarf strains.

are given in Addendum 2 and should be understood. Listed below Table I are the promulgated minimum standards for housing. It is recommended that laboratories, at least as regards animals under experimentation, follow recommendations for housing listed in Table I.[6]

2. RABBITS*

a. Maintenance and Handling

Rabbits are commonly housed in cages containing an elevated grid of perforated metal, with a dropping tray beneath, which contains some absorbing material. Recommended cage dimensions are given in Table I. Food consists of pelleted rabbit chow and water. It is necessary to check the specifications of commercially available cages for the absence of sharp corners, proper discharge of urine and feces into the droppings pan, proper size of grid openings,† firm anchorage of water containers such that the rabbits cannot dislodge them, and adequate food hoppers of a design that prevents soiling of food, as well as overall sturdiness. A rather unique design is a device, fitted to cages by a suspending wire, that doubles as hopper and water fount (see Fig. 1, *l*, *q*). The water vessel of about 2-quart capacity is a relatively narrow-necked bottle (about ⅞ inch I.D.) inverted in the hopper to fill it partially and then to constitute an ample supply based on the "inverted ink well" principle. Room temperature should be maintained between 60° and 70°F, at a relative humidity of 40–60% for rabbits under immunization.

Proper handling is necessary, particularly when one is removing rabbits from their cages. Rabbits must not be picked up by the ears. The most suitable way is to pick up a broad fold of skin along the back with one hand (Fig. 1, *b*). The rabbit can then be slung with its head under the arm and the full weight of the body comfortably supported on the forearm (Fig. 1, *a*). For restraint of bucking and kicking, a grasp

[6] "Guide for Laboratory Animal Facilities and Care." Public Health Service Publication No. 1024, Revised 1965. Prepared by the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council, Washington, D.C.

* Physiological and biological data are given in Addendum 1.

† Grids are of various types, the important feature being to support the rabbit comfortably yet leave as little metal as possible: ½-inch squares of metal often are removed, to leave ¼-inch metal strips; or expanded metal can be used, with 3/16-inch metal supports around diamond cutouts of 1½ × ½ inches. Cage bottoms made of parallel round wires should be rejected as offering as inadequate support for comfort, as should bottoms made of woven ¼-inch flat metal.

can be secured on the lower back so that the thighs go into extension (Fig. 1, *c*). Rabbits should be placed or held only on surfaces that afford a sense of support; slippery surfaces cause a feeling of terror.

Initial body weight should be recorded as a base line to judge the state of health and any deleterious effect of injection procedures. Coat color should be glossy, the musculature across the lower back firm to the touch, the eyes bright, and the muzzle dry. Loss of weight is easily apparent by feeling the bulk and tonus of muscles across the lower back.

A principal disease of rabbits is "snuffles," due to *Pasteurella multocida* (formerly known as *lepiseptica*) infection. This disease spreads rapidly, but usually becomes chronic. Such animals are never quite well, and they should be discarded. Another problem is crusting within the ear as a consequence of infestation with burrowing ear mites which feed on the lymphatic vessels. Ears should be inspected frequently and prompt steps taken to eradicate infestations.*

The chief parasitic infestation is coccidiosis. Five species of parasites are known, distinguished by the oöcysts.[7,8] One species, *Eimeria stiedae,* causes hepatic coccidiosis, death losses peaking at 4 to 6 weeks after infection; terminally, yellowing of the eyeballs can usually be seen. Other species, causing intestinal coccidiosis, can be disclosed by fecal examination. Treatment consists in adding 0.03% sulfaquinoxaline to the drinking water.[9]

It is worth pointing out that hair growth is hormonally mediated and cyclic, and that rabbits often remain for one or two months in a "resting hair stage," especially in winter months. This stage is particularly favorable for study of skin reactions, since the skin is smooth and clean after the hair has been clipped away. Irritation of the skin, as by treatment, may start local regrowth of hair as elevated "hair islands."

b. Identification

Identifying numbers tattooed permanently on ear or hind footpad are particularly useful for long-term genetic studies. For short-term experiments a simple aluminum ear tag bearing three digits and provided

* Crusts are softened for a few hours by oil application and then scraped forward and out of the ear with a wire loop; a commercial preparation of rotenone in a complex solvent (Canex, Pitman-Moore Company), diluted with an equal volume of mineral oil to 0.06% rotenone, is applied and repeated at 3-day intervals for a total of four applications.

[7] J. F. Kessel and H. A. Jankiewicz, *Am. J. Hyg.* **14**, 304 (1931).

[8] E. A. Benbrook and M. W. Sloss, "Veterinary Clinical Parasitology." Iowa State Univ. Press, Ames, Iowa, 1961.

[9] K. W. Hagan, Jr., *J. Am. Vet. Med. Assoc.* **138**, 99 (1961).

with two long, sharp points is sufficient.* The points are bent carefully at right angles to the numbered disk, checked for sharpness, cleaned, and thrust through the cleaned outer surface of the right ear one-third the distance from the root of the ear to the tip so as to straddle the central vein. The protruding points are carefully bent inward to overlap, but not sharply, lest the ear tissue be constricted or crushed. A properly placed tag will not be lost for several years. It is useful to distinguish the sex of cage occupants by employing differently colored cage tags, such as yellow for females and manila for males. The cage tag should show the identification number, age, and any distinguishing colors of the hair coat.

c. INJECTION

i. Intraperitoneal Injection

For most procedures, rabbits can be handled by two workers. Intraperitoneal injection, for example, is best performed by suspending the rabbit in a head-down position so that the intestines sink forward, and the injection is made by a second operator who advances the needle through a raised fold of skin (clipped free of hair and rubbed with 70% alcohol) (Fig. 1, *j*). As the needle is pressed forward at a 30-degree angle, penetration of the thick abdominal muscles and peritoneum occurs suddenly and can be felt. The needle is raised parallel to the belly wall for the actual injection.

ii. Intravenous Injection

Injections into an ear vein are made most expeditiously by two workers as in Fig. 1, *d*, *e*. One person sits on the bench with the rabbit alongside his thigh. The left arm is passed back to allow the fingers to pull the rump of the rabbit forward. The right forearm presses the rabbit against the thigh; the fingers of the right hand hold the head at the base of the ears, while thumb and forefinger are readied to close off the venous return through the marginal vein. The course of the vein is dry-shaved with a single-bladed safety razor, and the skin is rubbed lightly with alcohol, without inducing chilling. If the room is warm and the assistant closes off the venous return as above, the vein should fill and allow injection. Heat supplied by a gooseneck lamp with a 60-watt bulb may be needed. Xylol will not be necessary with the temporarily closed venous return.

For injection, 1-inch or 1¼-inch needles, No. 25 or No. 23 gauge,

* N. Stafford Co., 120 Fulton Street, New York: 13-mm, numbered flat aluminum disks with two points.

are recommended. Penetration is made alongside the vein, and the point is then carried sideways into the vein. The operator soon learns the "feel." As soon as penetration is effected, the operator clamps the needle shaft against the ear by thumb and forefinger to maintain the position, holding the ear flat at the same time. Pressure on the vein at the base of the ear is relaxed by the assistant, and injection is made slowly. If any whiteness is seen in the tissue over the vein, the needle should be removed at once, and a new entry effected. Transillumination of the ear becomes useful only after considerable scarring has occurred by previous injections, and 30-gauge needles may become necessary. Various illuminators are offered, with light passing through flashed opal glass. Many of the laboratory illuminators that employ tiny fluorescent lamps can be used, or even a pen-type flashlight clamped to a small ringstand.

It is accepted practice for right-handed laboratory workers to reserve the left ear for injection and the right ear for bleeding. A good worker can make many injections in the marginal vein of one ear. One should remember that the needle should not penetrate far (valves of the veins are injured), that first injections are made close to the sensitive tip of the ear, and that succeeding injections are made as distal as possible, ascending gradually the venous pathway toward the base of the ear. The chief reasons for venous obstruction and capillary bypass of the marginal vein are: (1) mechanical trauma to the venous wall, either internally, or externally by infiltrative pressure from depositing antigen outside the vein; (2) improper use of xylol (used without removal of hair, or not removed fully by 95% alcohol); (3) sores arising under clotted blood that has been allowed to remain on the ear over the vein.

It is recommended that the volume of the solution containing antigen be at least 2 ml per injection, both because of possible mechanical loss and because any concentrated antigen that is deposited outside the venous wall constitutes foreign antigenic material that can cause inflammatory alterations as antibody develops.

When no assistant is available, mechanical restraint becomes necessary. For this purpose, a rabbit box is most suitable (Fig. 1, *f*). With such a device, however, the operator will usually not have the benefit of constriction of the vein below the ear and must rely on dilatation of the vein by dry shaving near the tip of the ear, by gentle massage, and by heat. The temptation to use xylol to cause dilatation often seems irresistible, yet its use decreases markedly the possible number of succeeding injections. Dilatation is under nervous control, and patience is required of the operator. When the rabbit loses its nervousness, dilatation occurs.

iii. Intramuscular Injection

The thigh muscles and the large nuchal muscles running parallel to the spine are used chiefly. As with human beings, slight retraction of the syringe plunger is practiced to assure that the needle has not penetrated a vessel. The thigh is clipped free of hair by an electric clipper (No. 000 blade followed by No. 0000 blade), and the leg is extended. The needle is inserted in the upper third of the thigh muscle and advanced distally a short distance into the thigh muscle without touching the femur. When the nuchal muscles are used, do not advance the needle more than ½ inch before making the injection, since penetration of the parietal pleura may accidentally occur.

iv. Methods of Mechanical Restrain during Injection

Many types of rabbit boxes have been suggested, from open-sided stalls with some sort of head stock[10,11] to box enclosures. The stalls lack a cross-member against which the rabbit could brace his forelegs and buck. Box enclosures suggested in the literature or offered commercially seldom have proper dimensions to prevent the rabbit from lateral movement or even from breaking its back by forcible thrusts of the hind legs. Proper inside dimensions of a box enclosure will serve to confine the rabbit properly, inhibiting sudden thrusts with shoulders or legs. The box of Fig. 1, *f*, with a cross section of only $4\frac{3}{4} \times 4\frac{1}{2}$ inches, is appropriate for all rabbits. The length is adjustable from 14 to 16 inches, but 15 inches is sufficient. Once the animal is introduced, the back of the rabbit is grasped, and the other hand passes under the belly and pushes the hind legs into extension. As the back is pressed down gently, the hind legs will assume the normal crouching posture of rabbits. If the animal is too short for the length of the box, one or more cloth towels are inserted at the back. The lid is eased forward while the back is held down. The neck slot comes fully forward, and the top plate is secured in position by a knurled screw. The particular box shown is made of sheet metal. Wooden boxes of the same internal spacing are equally suitable. This type of box is suitable not only for intravenous injection by one operator, but also, and principally, for taking ear bleedings (Fig. 1, *g, i*).

For special purposes, as in making intradermal injections under stan-

[10] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology, A Laboratory Text for Instruction and Research." W. A. Benjamin, New York, 1963.

[11] J. R. Leahy and P. Barrow, "Restraint of Animals," 2nd ed. Cornell Campus Store, Ithaca, New York, 1953.

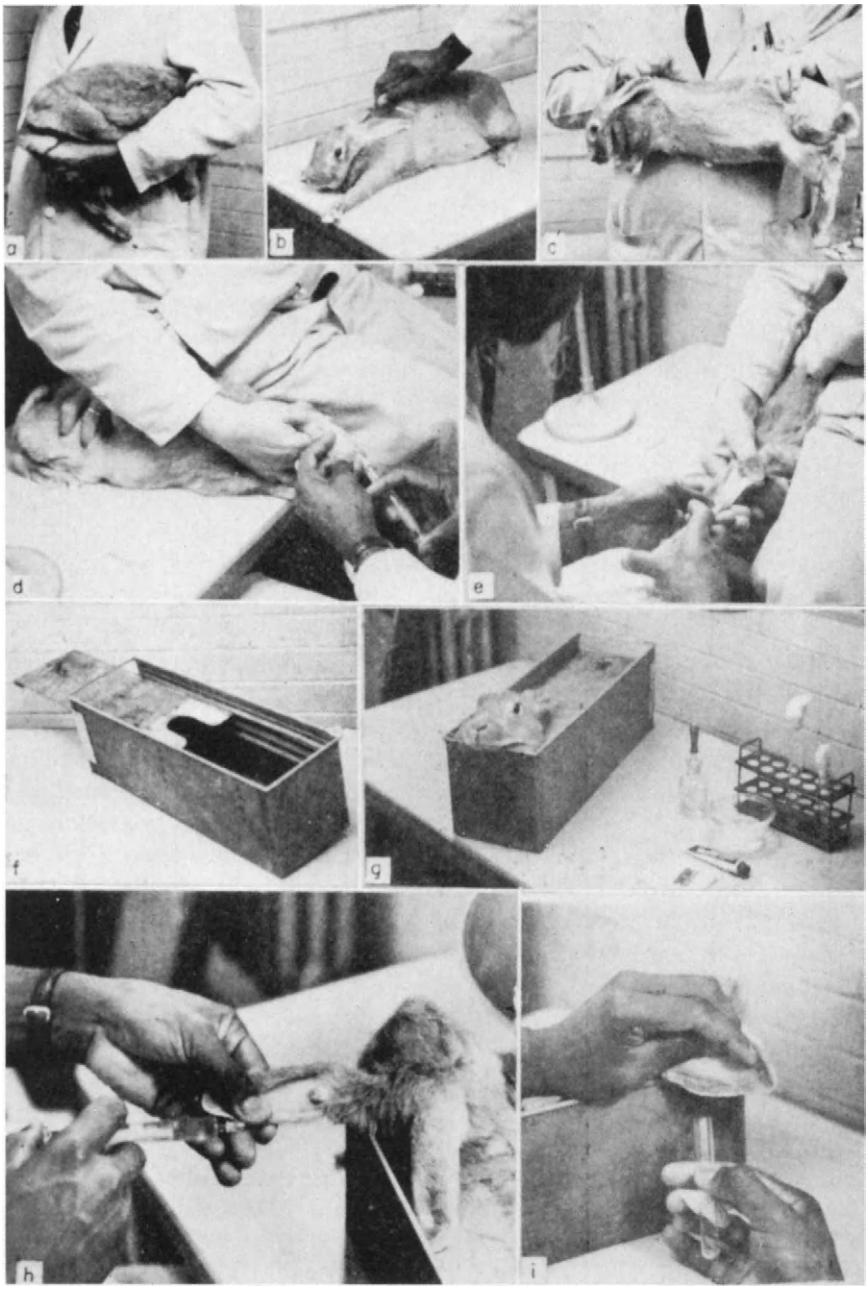


FIG. 1. Views *a–c*, carrying and holding rabbits: *c*, rear legs are kept in extension by position of operator's left hand.

Views *d*, *e*, rabbit held by assistant for intravenous injection of left ear: *e*,

dardized tension, scarification for viral studies, or a sequence of injections over a period of time, the animal board described below (Fig. 1, (*n*) will be helpful.

d. Gastric Intubation

A simple spindle of plastic, provided with a lateral "stop" and bearing holes in the spindle, is described by Di Pasquale and Campbell[12] for gastric intubation. While the plastic gag is in the animal's mouth, gastric tubes are easily passed into the stomach through one of the holes.

e. Bleeding

i. Ear Bleedings

Small bleedings are best made from the marginal vein while the animal is restrained in a box (Fig. 1, *i*). The room should be warm. The animal is bled from the right ear. (The left ear is used for injections as long as possible, to extend the total time of patency of the marginal veins.) The ear is dry-shaved to the lateral margin of the ear, cleaned with 70% alcohol, coated with Vaseline, and wiped free of excess Vaseline with clean gauze. When the marginal vein dilates, make a longitudinal incision (2 mm) into the vein with the corner of a sharp razor blade, avoiding lateral ear tissue. Compress the venous return proximal to the cut. Blood rises on the Vaselined surface and is directed into a tube. Trial bleedings of 4 to 8 ml are often taken, but amounts of 40 ml can be secured in this way. (For bleedings intended for storage, food should be removed the previous evening to reduce serum lipids.) If blood flow ceases prematurely, remove the clot by dry wiping across the cut. Heat can be applied locally by means of an 8-ounce polyethylene bottle filled with moderately warm water and stoppered tightly. Hold the bottle in the palm of the hand against the lower surface of the ear.

Bleeding usually stops when pressure is applied for 3 or 4 minutes through a wisp of cotton covered by a dry gauze sponge; watch for

[12] G. Di Pasquale and W. A. Campbell, *Lab. Animal Care* **16**, 294 (1966).

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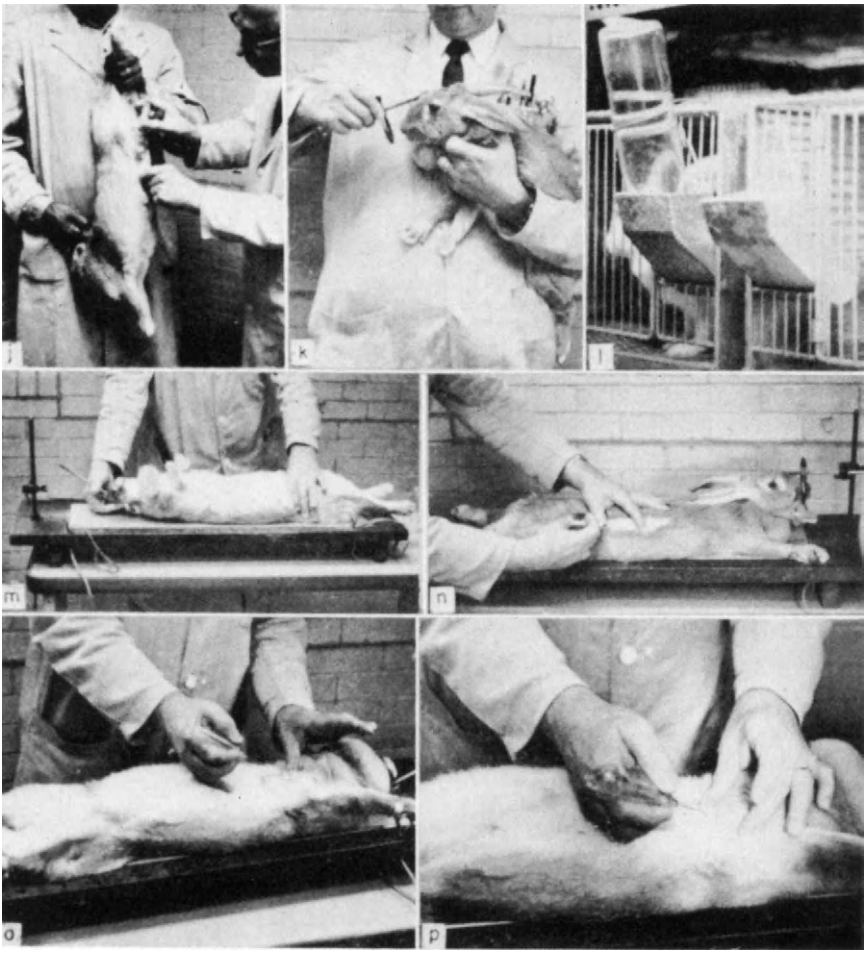
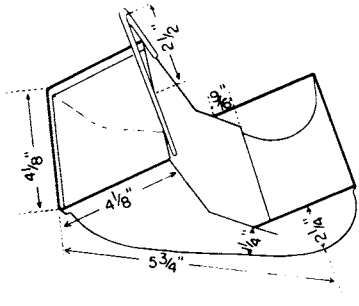
note position of assistant's right hand in holding head and clamping off the venous return with thumb and forefinger.

Views *f–i*, use of rabbit box for injection and bleeding: *f*, box has internal dimensions of 4½ inches wide, 4¾ inches high, and a variable length, set usually at 15 inches; *g*, accessories include razor blade, gauze, Vaseline, dish of alcohol with cotton pledgets, dry cotton, and tubes; *h*, injection into dry-shaved left ear; *i*, position for bleeding from right ear, the marginal vein dry-shaved and coated very lightly with Vaseline.





q

FIG. 1. (*Continued.*) See opposite page for legend.

resumption of bleeding after the rabbit is released. If bleeding persists, place a dry gauze sponge over the ear, and secure it by means of a 37-mm arterial clamp or paper clip. The ear should be cleaned thoroughly of Vaseline and external clotted blood within the next hour or so.

Bleeding from the central artery can be accomplished with a very sharp 1-inch gauge 23 needle attached to an all-glass syringe wetted beforehand with saline. The shaved artery is dilated by brisk rubbing with the finger; this failing, a xylene-moistened cotton ball will speed dilatation. The ear is transilluminated by placing a gooseneck lamp (60 watts) some inches beneath the ear. The tip of the ear is toward the operator. The needle is brought forward very slowly, directly over the artery against the direction of blood flow, and advanced slowly into the artery. If entry is correct, blood will flow downward when ear and syringe are lowered, with pressure sufficient to push backward the plunger of the syringe. Up to 40 ml of blood can be taken; do not pull on the barrel of the syringe. If xylene is used, the ear must be cleaned with 95% alcohol; xylene that has spread through hair is difficult to remove completely.

Another method for bleeding from the central artery employs a device that utilizes negative pressure.[13] A special Lucite cap is machined bearing a needle tubulature (to carry a 1-inch needle, gauge 22), a vacuum port, a bypass port to control vacuum by fingertip, and Lucite ears to attach to bleeding tubes. One size is made to fit 40-ml tubes, another to fit 12-ml collection tubes. The artery is dilated as above by xylene.

[13] R. A. Hammerstrom, *J. Lab. Clin. Med.* **61**, 352 (1963).

View *j*, assistant holding rabbit downward for intraperitoneal injection, and operator raising fold of abdominal skin in left lower quadrant to make the initial subcutaneous injection (see text).

Views *k*, *m*, *n*, use of special animal board with rabbits: *k*, attachment of headgear, lower jaw positioned upward, body supported and restrained by left arm after lifting animal as in view *b*; *m*, animal is restrained from moving head after headgear is positioned while rear legs are kept in extension; after headgear is attached, operator attaches ankles to board with thongs without releasing grip of left hand; forelegs are secured last; removal from board is in reverse order, always keeping hind legs in extension; *n*, use of board for intradermal injections for special purposes.

Views *o*, *p*, positioning for heart bleeding by right-handed operator: *o*, heart position outlined, heart displaced toward left by pressure with left thumb: median line drawn from top of rib cage to xiphisterum is bisected at right angles; heart lies below the intersection of the lines; alcohol-cleaned left forefinger palpates heart and determines position of entry; *p*, position of entry of needle, with syringe lowered about 20 degrees for purposes of photography.

Views *l*, *q*: combination food hopper-water reservoir device as used at Institut Pasteur, Annexe de Garches; two hoppers are fixed to each cage; the ends of two adjacent cages are shown.

ii. Heart Puncture

(*a*) *Methods of Restraint.* Various specialized devices are used to restrain rabbits while blood is being withdrawn from the heart. V-neck, or especially designed inclined boards to hold the head far backward,* or fabric sleeves[14] have been recommended. The animal board shown in Fig. 1, *k*, *m*, *n*, is far more versatile in providing restraint (in supine, prone, or flank-upward positions) for a variety of procedures apart from making bleedings from the heart (see Fig. 2 for general construction).

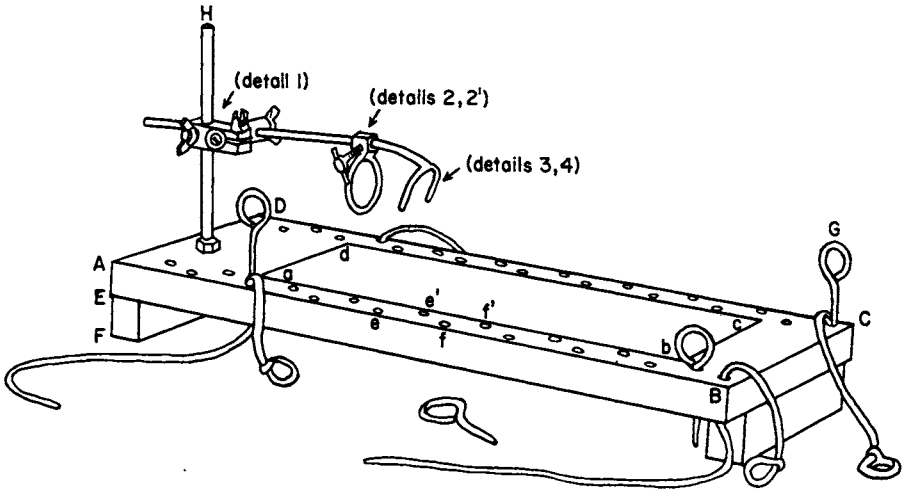


FIG. 2. Scheme of animal board with headgear. Details in Fig. 3 and Table II.

The supine and prone postures are based on mild triangulated tension exerted by the skull holder and the two hind legs; the forelegs are tied rather loosely. Dimensions for the rabbit holder are given in Table II. The skull yoke and nose ring are shown in Fig. 3; the yoke carrier rod is bent as shown in detail drawing 4*a* to pass over the skull.

Many sorts of tension cords are possible. For rabbits, thongs of rawhide work well. These are 22 inches long, cut to a width of ¼ inch except for a terminal double-width bulge which is slit to allow formation of a slipknot. The free end is wedged firmly in a hole of the board with a brass pin bearing a hand hold (Fig. 2) and a tapered pin 1¾ inches long (3/16-inch stock tapered to 5/32 inch). For exposing the flank, both

* See ref. 10, Fig. 1·A-1·1.

[14] R. M. Appleman, *Lab. Animal Care* **16**, 300 (1966).

TABLE II

SPECIFICATIONS FOR ANIMAL HOLDER (FIG. 2)

Parts	Rabbit holder	Guinea pig holder		
Animal board dimensions				
ABCD: oak frame				
AB	37 inches	18 inches		
BC	10½ inches	7 inches		
AE	1¼ inches	1 inch		
EF	2⅜ inches	1 inch		
abcd: metal insert panel				
ab	29 inches	12 inches		
bc	7 inches	4½ inches		
ef, e'f' (two rows, staggered)	2¼-inch spacing × ¼-inch diameter	2 inch spacing × 3/16-inch diameter		
Aa	5⅛ inches	4 inches		
Post (*H*)				
⅜-inch iron rodding, threaded for 2 inches	8 inches	7½ inches		
Headgear clamp[a]	As shown	As shown		
Wedge pin (*G*)	See text	See text		
		Small size, up to 300 gm	Medium size, 300–600 gm	Large size, 600 gm or heavier
Brass nose ring[b]				
3/16-inch stock		1 inch I.D.	1⅛ inches I.D.	1¼ inches I.D.
9/32-inch stock	1 9/16 inches[c]			
Skull yoke[d]				
3/16-inch stock:				
AB	1 11/16 inches	1 5/16 inch	1 1/16 inches	1 1/16 inches
CD	1 9/16 inches	⅞ inch	1 inch	1 3/16 inches
EF	1 9/16 inches	1 inch	1 inch	1 3/16 inches
Yoke carrier rod[e]				
¼-inch stock	10 inches	10 inches		

[a] Detail drawing 1, Fig. 3.

[b] Detailed drawings 2, 2', Fig. 3.

[c] Ring is filed flat across the ring on each side to lighten the weight.

[d] Detailed drawing 3, Fig. 3.

[e] Bent as in drawings 4*a* (rabbit size) and, for guinea pigs, 4*d*, 4*c*, and 4*b*, Fig. 3.

hind legs are bound by a single cord, and a second cord passes from this to the opposite side of the board to exert triangular tension in the fashion of Fig. 4, *j*.

(*b*) *Technique of Heart Puncture.* In advance, lay out syringes, an extra needle, stout forceps, and labeled tubes to receive the blood. In

first learning the art, choose an all-glass syringe of no greater capacity than 30 ml, or a lightweight all-plastic syringe of 20-ml capacity such as the excellent B-D Plastipak syringe. Use a regular rather than a disposable 19-gauge needle, no shorter than 2 inches, and be sure that

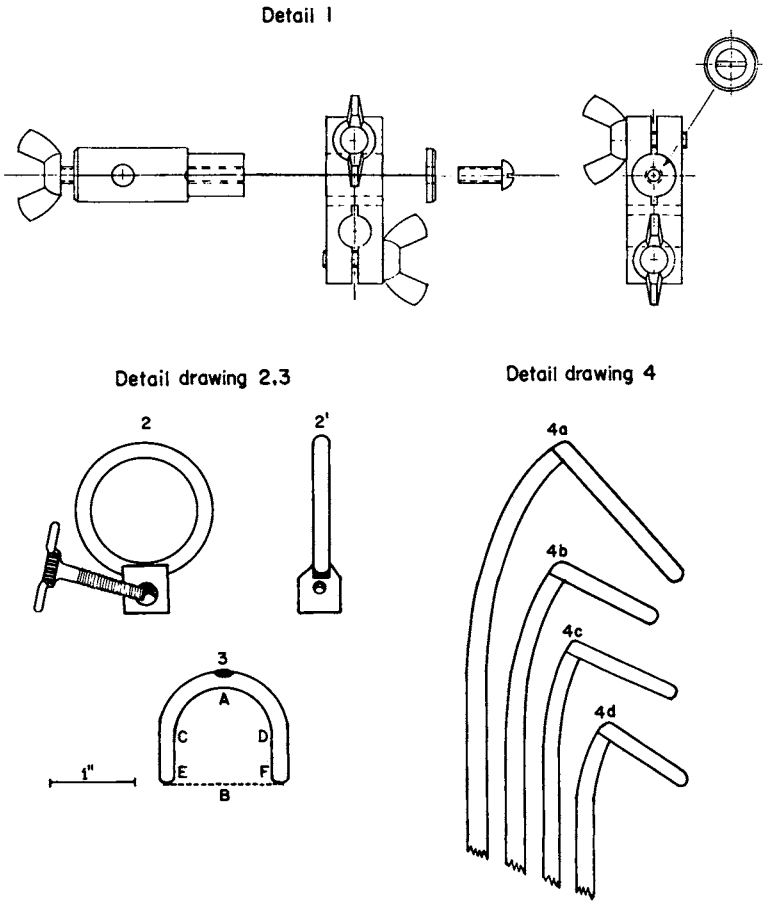


FIG. 3. Constructional details of headgear for animal board shown in Fig. 2. See Table II for dimensions of various yokes and muzzle rings.

it is sharp and free of burrs. Needles should be checked with a hand loupe.

The manner of restraint that one selects will determine the anatomical aspects of the approach to the heart. In using a mechanical restraining board, several points should be noted. The head should be extended

until the thongs attached to the hind legs are quite taut but do not cause discomfort. The headgear should position the skull without sideways twist, and the neck should rest at a natural angle to the body. The hind legs should be secured by the equal tension of short lengths of thong (2 or 3 inches) between ankle and point of insertion, made in corresponding holes on the two sides of the board. Avoid flattening the rib cage by use of too great tension on the forelegs; the animal should present a "barrel chest."

Apart from considerations of restraint equipment, there are three or four methods of approach. As in all animal work, one should learn a single method thoroughly and not shift from one procedure to another.

The hair should be clipped carefully from the chest area, care being taken to avoid excoriation of skin or abrasion of the thoracic nipples. One point of entry is the angle formed by the last sternal rib on the left side of the midline and the xiphoid process. The angle is easily located, since the finger will be able to depress the area deeply. Tactile sensation is acute when the needle is inserted prior to attachment of the syringe. Insert the needle, bevel up, in this angle, penetrate downward for a few millimeters, then slant the needle forward at an angle of approximately 30 degrees to the chest wall. Advance the needle until it transmits the heart beat; attach the syringe, and advance the needle into the ventricle. The left hand, holding the syringe and needle, can be steadied on the chest of the rabbit. Other details are given below.

The procedure described here (Fig. 1, *o*, *p*) utilizes a different approach. If approach to the heart is made from the left (Fig. 1, *o*, *p*), the hand that holds the syringe barrel and establishes the depth of penetration can be steadied by resting it on the rigid metal headgear, and the right elbow can be supported on the near edge of the animal board. (A rigid posture also allows changing of syringes during the bleeding, with the hub of the needle held fixed by forceps.) Changes of syringes are made readily with use of Luer-lok-tipped syringes and regular (as opposed to disposable) needles. Disposable needles have aluminum or plastic hubs which tend to bind to the syringe.

The thoracic area is sponged with 70% alcohol, the excess being removed with sterile gauze. The rib cage is palpated by thumb and middle finger, and the alcohol-wetted forefinger is used to palpate the heartbeat as in Fig. 1, *o*. The left thumb is transferred to the right side of the rib cage and pressed firmly against it. The position of entry is shown in Fig. 1, *p*, although the proper angle of entry of the needle is somewhat higher than depicted. The aim is to enter the left ventricle; the hazard consists in tearing the heart muscle so that bleeding into the pericardial sac occurs, causing death by clot pressure outside the heart.

Penetrate until the heart pulsation can be felt. (Note that use of large-capacity, heavy glass syringes dulls tactile sensitivity.) Advance the needle sharply about 3 mm, and apply slight negative pressure. If blood does not flow at once, first pull the needle back slightly, rather than advancing it deeper.

The needle position can require fine adjustment, making a rigid posture of great advantage to the operator. If blood is not obtained, withdraw the needle completely, palpate the chest area again, and re-enter the chest wall. The handling of blood in Vaselined tubes is discussed in Section C,2,a. Remove the needle before ejecting blood from the syringe. For ease of subsequent cleaning, rinse the needle and syringe at once, finally flushing out the needle hub separately.

iii. Terminal Bleeding

More blood can be withdrawn from an artery than most workers will be able to secure by cardiac puncture. As the blood pressure falls, it is difficult to retain the needle in the ventricle or to reinsert the needle into the heart when proper positioning is lost; and holding the needle *in situ* when syringes are changed during the bleeding presents an especial hazard (see Section e,ii(b) above). The carotid artery is an excellent choice, although the abdominal aorta has been recommended; the femoral artery collapses too quickly. For arterial bleedings, some surgical experience must be gained through dissection to ensure efficient moves during the actual procedure. Two persons should work regularly as a team.

Set out bone forceps, a scalpel (Bard-Parker No. 20 blade), scissors, two pairs of 4½-inch forceps for blunt dissection (free of palpable rough edges when the blades are squeezed together), two 4½-inch hemostat forceps, one 50-mm small artery clamp (serrefine), a pair of fine scissors, one pair of forceps with ultrafine tips that remain in contact even under pressure, and a 16-inch length of three-cord linen thread No. 40. Other accessories are saline, 3-inch square gauze, 70% alcohol, cotton balls, a laboratory towel to diaper the animal, two Vaselined 100-ml straight-walled centrifuge tubes (Section C,2,a), and one or two Vaselined culture tubes. In addition, if one wishes to cannulate, proper thin-walled cannulating needles, plastic tubing* and suitable ties should be at hand.

Prepare a syringe containing a sufficient amount of sodium pentobarbi-

* Tubing such as Becton, Dickinson, and Company's polyvinyl tubing is suitable; the 0.039-inch diameter (No. 442T) passes through 1½ and 2½-inch 18-gauge, thin-walled needles of series T462LNR (as does the more flexible Clay-Adams' PE 20 polyethylene tubing of somewhat smaller internal diameter); the 0.065-inch diameter tubing (No. 444T) is inserted through similar 14-gauge, 2-inch thin-wall needles. Cannulation is rather difficult; it is not needed.

tal (Nembutal) to deliver 30 mg/kg body weight. With the aid of an assistant, inject as in Fig. 1, *e*, but first deliver only about three-quarters of this dose; the assistant will detect relaxation of body muscles. More drug may not be necessary.

Attach the anesthetized animal to the board as in Fig. 1, *o*, but draw the forelimbs caudally to present the neck. Adjust the head clamp to present a straight and slightly rising throat. Clip away the hairs with a No. 000 blade, stretching the skin flat before the clipper is advanced. The female throat "ruff' requires much care in stretching flat as one proceeds. Complete the clipping with a No. 0000 blade. Sponge the area with alcohol.

The carotid runs close to the trachea and medial to the longitudinal throat muscles. It is round and pink in color, with a white thick nerve running closely alongside.

Stretch the skin of the left side of the throat sideways, and make an incision to the left of the trachea, commencing at the upper third of the throat and carrying the incision caudally for about 6.5 cm. Dissect the skin back bluntly and attach the hemostat forceps, letting their weight hold the incision open. Cut carefully through the fascia. With blunt dissection, only, separate the intact muscles and visualize the carotid. Continue the blunt dissection until nearly 35 to 40 mm of carotid is exposed. Separate the nerve and any fat. Sponge the field with saline-wetted cotton.

Raise the carotid on the open tips of one forceps, and pass a loop of cotton thread underneath, avoiding any rubbing of thread on the artery (local contracture can occur). Snip the thread to constitute two ties. Work one thread cephalad as far as possible, and make a firm tie. The other thread is laid in a loose tie for precaution and is carried caudad as far as possible. Close the cardiac supply by applying the 50-mm serrefine to the caudal end. The contained carotid artery should be about 28 to 32 mm in length. Raise the carotid, and, just below the cephalad tie, cut it completely across at a 45-degree angle with fine scissors. The very tip of the severed carotid is grasped with the fine-tipped forceps and carried obliquely to the right. The neck of the Vaselined tube is placed on the longitudinal muscles by the assistant. The carotid is entered into the mouth of the tube. When the serrefine is removed, blood will issue in a steady stream. During the bleeding, the right forearm of the operator presses firmly on the abdomen, and terminally the fingers of the right hand compress the rib cage. With falling pressure, the blood comes in spurts, with some pauses. Finally, the end is allowed to go free; some further blood may flow into the collection tube. The animal is then killed by advancing the bone forceps

through the incision and under the trachea, and the spinal column is severed.

The method of taking blood from the abdominal aorta[15] also requires anesthetization (sodium pentobarbital or ether). A midline incision is made, the organs are displaced to expose the kidneys, and fatty structures are teased away with gauze sponges. Entry is made into the aorta a few centimeters below the left kidney. A hemostat is kept ready as an emergency measure to allow the aorta to be clamped off above the point of entry. The apparatus suggested consists of a 100-ml syringe connected to a short-bevel, gauge 15 or 16 needle that has been bent upward at 45 degrees while the needle is held bevel up. A flexible connection between the bent needle and the syringe, made with wide-bore polyethylene tubing in the fashion described for another purpose in Section A,2,g,i, might be an improvement, with the syringe locked rigidly to a stand. Blood is easily withdrawn. If difficulty should arise, clamp cephalad to the point of entry, and make a fresh entry.

3. GUINEA PIGS*

a. Maintenance and Handling

Guinea pigs are housed for experimental purposes in groups of 3 or 4 of one sex; acceptable cage sizes are given in Table I. The hind feet require more support than is offered by grid-bottom cages†; heavy calluses develop in such cages, and serious sores can follow when animals injected in the footpads, as is often practiced, are so housed. Wood shavings or other absorptive material placed liberally on a flat floor serves well, with cages cleaned sufficiently often to maintain clean, dry bedding. Room temperature should be maintained at 70–75°F, with relative humidity of 40–60%.

Food containers should be attached firmly and designed so that soiling cannot occur; nipples delivering water should be rigid, with overflow of water directed outside the cage. The established "social order" of male cagemates must be respected, for males cannot be recaged randomly. Males attacking others must be segregated.

Special pelleted diets are made for guinea pigs. The high requirement

[15] A. F. Moreland (ed.), *Lab. Animal Digest* **1**, 7 (1963).

* Physiological and biological data are given in Addendum 1.

† The raising of guinea pigs from infancy on wire-floored grid bottoms having large openings (for example, ¾-inch squares or openings of ½ × 3 inches constructed of welded metal strips) is advocated by Lane-Petter.[2b] The large openings lessen the danger of the feet passing through, catching, and being broken. The writer, however, has seen only callused feet on such animals and believes that experimental ends, at least, are not served by such cages.

for vitamin C is often met by adding it during preparation of the pellets in at least threefold excess; the 50-pound sacks bear an "expiration date" indicating the expected time when the ascorbic acid will have become oxidized by air and natural moisture below the minimal necessary daily intake. In the writer's experience, hay and supplemental feedings of cabbage or other green fodder are needed in addition to pelleted food, to provide adequate roughage for a "full cecum" and additional supplies of vitamins.

A chief difficulty encountered in guinea pigs raised commercially in the United States is the prevalence of carriers of the natural guinea pig pathogen, group C streptococci, producing chronic lymphadenitis. In such infected stocks, frank buboes can be palpated in some individuals in the neck or groin. Intercurrent deaths are encountered; hence the numbers of animals purchased are usually increased by 20% or more to allow for losses during experimentation. The young of infected stocks are apt to show in only dull fashion reactions of passive cutaneous anaphylaxis.

Some "closed colonies" of guinea pigs are maintained free of group C streptococci (Rockefeller University, National Institutes of Health, etc.). Colonies of this sort can be set up by the procedure used by Moen,[16] by skin-testing and discarding stock showing delayed-type hypersensitivity to concentrated aqueous extract of group C streptococci.* The streptococcus-free animals live for 3 or 4 years, and deaths from other intercurrent infections such as *Pasteurella* are usually minimal.

Two strains of guinea pigs in the United States are nearly isologous with regard to intrastrain acceptance of skin grafts—Sewell Wright's Family II and Family XIII.[17a,17b] Narrowing of the gene pattern by selective breeding has resulted in special characteristics, both physical and immunological, for each strain.

The gestation period is around 68 days, the estrus cycle around 16 to 18 days (cf. Reid[17c]). Litters vary from 2 to 5 individuals. Young are commonly weaned at 3 to 4 weeks (200 to 270 gm of body weight). In institutions that raise their own pen-inbred guinea pigs, it is worth

[16] J. K. Moen, *J. Exptl. Med.* **64**, 553 (1936).

* Testing extracts are not available commercially. They are prepared by disrupting thick suspensions of streptococci with Ballotini beads in a Mickle shaker and discarding bacillary debris by centrifugation. Only one testing can be performed on an animal, owing to induced sensitization (chiefly of Arthus type).

[17a] S. Wright, *U.S. Dept. Agr. Bull.* **No. 1090**, Professional Paper (1922); **No. 1121** (1922).

[17b] S. Wright, *J. Cell. Comp. Physiol.* **56**, 123 (1960).

[17c] M. E. Reid, "The Guinea Pig in Research: Biology, Nutrition, Physiology." Publication No. 557, Human Factors Research Bureau, Washington 1958.

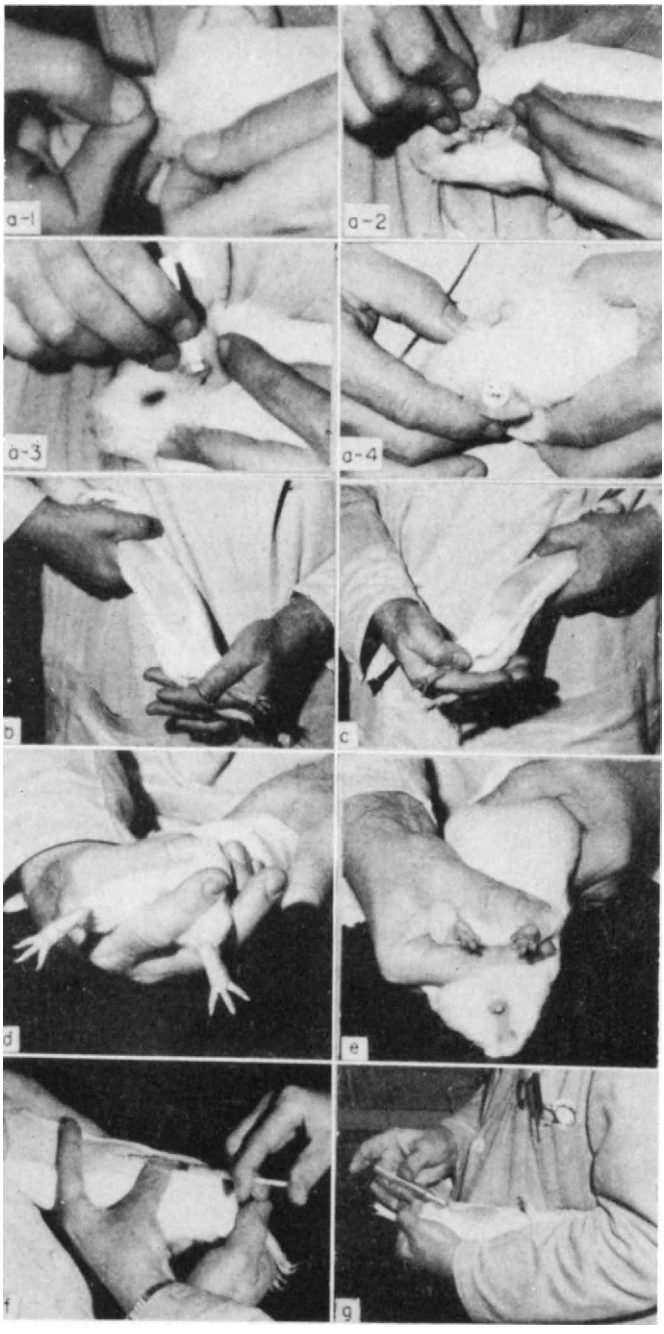


FIG. 4. Views, *a-1* to *a-4*, placing tag in left ear of guinea pig: *a-1*, margins of ear held flat while staple is positioned to straddle central vessels; *a-2*, staple thrust

remembering that siblings of like sex are apt to be transferred at weaning into the same holding cage; accordingly, nonrandom distribution must be taken into account in experiments in which genetic factors play a role.

b. IDENTIFICATION

Ear tags offer the most convenient means of identification*; fine-line tattooing is possible on the pads of the hind legs proximal to soles and toes. Ear tags, properly applied, are lost at a yearly rate of only about 5%. In multiple occupancy cages, tags should be checked about once in 4 to 6 weeks. Replacement numbers can be impressed readily on blank tags; new tags are attached to the second ear.

A correctly placed ear tag (Fig. 4, *a–4*) will rest high on the ear, the staple straddling the central artery and vein; it will not be "loose" or rise from the ear during head movements, yet the ear tissue will not be compressed or crushed by the folded staple. The attachment of an ear tag is shown in Fig. 4, a–*1* through *a–4*. It is done by one person, the guinea pig receiving support from the left arm as the animal is pressed against the operator's laboratory coat. The tag with staple inserted is held by thumb and forefinger of the right hand. The ear is flattened by the thumb and forefinger of the left hand, and—somewhat unnaturally—by the middle and fourth fingers of the right hand. The staple is inserted while the ear is stretched (Fig. 4, *a–2*). To avoid crush-

* Animal tags and accessories are available from Arthur H. Thomas Co., Philadelphia, Pennsylvania. Tags listed as item No. 1128 are not convenient; tags with black fill in the indented numbers are available on special order, as are four-place numbers; staples are supplied with free spacing bar as item No. 1129, and blank tags with staple holes as item No. 1128-A. Blank tags can be marked with machine-made steel figures (Millers Falls Co., Greenfield, Massachusetts, item No. 1550, ⅛ inch high), by applying a light hammer blow while the flat tag rests on a solid support.

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through the ear; *a-3*, closing of staple points over a tapered aluminum bar to avoid crushing ear tissues; *a-4*, a correctly positioned tag high on the ear.

Views *b*, *c*, *f*, holding for intradermal injection by a right-handed operator: *b*, right flank presented, chin pressed to assistant's body, rib cage not compressed, hind feet held between thumb and forefinger and between forefinger and second finger, respectively; *c*, left flank presented; *f*, operator's left hand seen between assistant's hands and body positioned as in *c*, skin held taut by thumb and forefinger, with operator's middle finger grasping the right flank from below.

Views *d*, *e*, holding for footpad injections.

View *g*, intraperitoneal injection by one operator: pectoral girdle held between operator's forearm and body, hind feet held as in view *b*. Operator's left wrist is extended to tense abdominal skin during subcutaneous entry of needle. Wrist is elevated to relax abdomen slightly during penetration of the peritoneum.

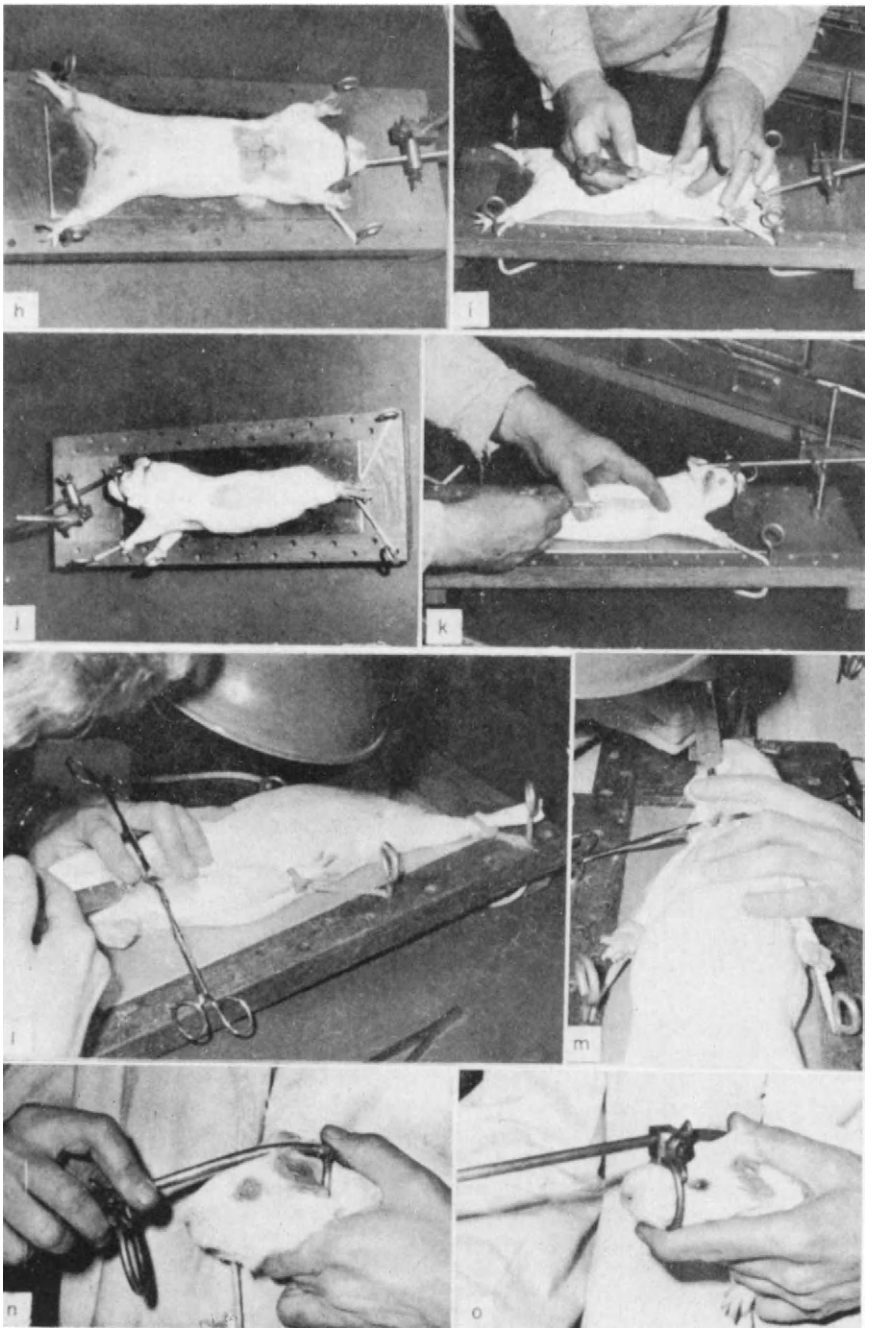

FIG. 4. (*Continued.*) See opposite page for legend.

ing the ear tissue when the points of the rigid Monel staple are closed over one another, a tapered, thin aluminum bar is held temporarily between the points (Fig. 4, *a–3*), the flat portion of the staple being held firmly against the face of the tag by means of the right thumb. Packets of staples with dull or hooked points should be returned to the dealer.

Cage tags should indicate sex of the occupants, either by shape or by color, such as yellow tags for females and manila for males. Red tags with special instructions to the attendant can be added as needed. In breeding work, special tags on cages indicating pregnant and lactating females have proved useful.

c. Methods of Restraint

For many types of work, a trained assistant will suffice for restraint (Fig. 4), but for heart puncture and intrajugular injections, a firm manner of restraint becomes necessary, including controlled positioning of the skull. A mechanical device (Fig. 2), having dimensions as given in Table II, offers great flexibility. It is highly useful also in positioning animals for deposition of serum samples for comparative passive cutaneous anaphylaxis (Fig. 4, *m*) or for operations involving the flank (Fig. 4, *l*), or for intraperitoneal injection of large volumes of paraffin oil. Three sizes of head harnesses are used, according to weight of the animals, as indicated in Table II. The respective yoke carrier rods are bent as in Fig. 3, detail drawings 4*b*, 4*c*, and 4*d*. The restraining ties are made from Venetian blind sash cord, which is cut at a sharp angle and bent to form a tight loop. The end of the loop is sewn to the cord

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Views *h*, *i*, heart puncture: *h*, guinea pig stretched on board, tensioned by headgear and hindfeet, with forefeet held forward loosely (see views *n*, *o*). Line drawn between xiphisternum and top of rib cage is bisected by cross line, with position of heart indicated by outline; *i*, operator's left thumb pressed against the ribcage to push heart toward left side, operator's forefinger (cleaned with alcohol) used to palpate heart beat; point of the needle will enter at position shown, with angle of syringe raised about 20 degrees higher than pictured.

View *j*, use of board to secure guinea pigs for flank operations.

View *k*, use of board to tension skin evenly for comparative PCA (passive cutaneous anaphylaxis) sites. Row on right back is made first, with hands in position of view *k*; row on left flank is made second, to avoid pressure on sites previously made. Sequence is reversed for left-handed operators.

Views *l*, *m*, injection of guinea pig into external jugular vein (see text). In the views shown, a retractor was not needed.

Views *n*, *o*, attaching headgear of animal board (see Fig. 2) to guinea pig: *n*, holding yoke over head while lower jaw is raised with forefinger; *o*, nose ring is slipped forward with the right hand, passed over nose and mandible, and tightened. The guinea pig must be restrained until the headgear is fixed in clamp (Fig. 2, detail 1), and the hind legs are attached to the board as in view *h*.

to fashion a tight slipknot, and the free end of the 18-inch cord is bound to prevent fraying.

d. INJECTION

i. Intradermal Injection

Restraint is offered manually in making injections (Fig. 4, *b, c, f*) except when a series of like injections is to be made under controlled tension of the skin (Fig. 4, *k*). Restraint when the footpads are to be injected is shown in Fig. 4, *d, e*. Footpads are cleansed with a solution of tincture of green (potassium) soap in water, applied with cotton pledgets; the soap is removed by sponging with 95% alcohol. Proper injection, made into the central pad from a lateral approach, should not give rise to bleeding.

ii. Intraperitoneal Injection

A technique is shown in Fig. 4, *g*, in which one operator performs the injection. (A 1-inch 20- to 23-gauge needle is satisfactory.) There are three steps: (1) holding the abdominal skin taut by wrist position while penetration is effected subcutaneously in the left lower quadrant of the abdomen, about 15 mm below the level of the umbilicus; (2) raising the wrist position to relax the abdominal muscles as the tip of the needle is lowered onto the muscles and penetrates the peritoneum and (3) elevating of the needle shaft to lift the peritoneum while the injection is made. When animals are to be held by a trained assistant the same three steps should be carried out.

iii. Intramuscular Injection

Intramuscular injections are made in the manner described for rabbits (Section 2,c,iii above). When nuchal muscles are to receive the deposit the head is bent forward by the assistant as the hind quarters are lowered so that the nuchal area is presented to the operator in a nearly horizontal plane. The position of the highly vascularized fat pad just below the skull is palpated; penetration is made in the 2- to 3-cm area just caudad by means of a 1-inch needle. (Do not enter more than 10 mm.) For aqueous solutions or nonviscous suspensions, a 1-inch 23-gauge needle is best; for emulsions with Freund's adjuvant, a 20-gauge needle should be used.

iv. Intravenous Injections

(*a*) *Superficial Vessels.* Considerable practice is needed to acquire skills that permit precise delivery of given volumes into a small vein. A large

vein passes over the arch of the hind foot between the lateral and middle *toes*. Clip the hair with a No. 0000 blade to expose the area, and sponge lightly with alcohol. The animal is held by a skilled assistant, who interrupts the venous return by pressure at the ankle; the operator himself curls the toes down slightly to flatten the area. On males that are not too young, the two lateral *penile veins* can be distended by pressing the penis into erection, allowing injection into these veins; anesthetization is recommended. The lateral vein on the *ear* can be entered by a No. 30 needle after dry-shaving, preferably during anesthetization and transillumination. It is helpful to use an animal board having a headgear (Fig. 2); if ether is used, the headgear is omitted. Most superficial veins require cutting down as described in Section D,5. Remove hair by clipping, and sponge down with 70% alcohol; never cut down directly over a vein. Close with one or two ties of linen thread or with a Michel skin clip (11-mm size). The *femoral vein* is approached as in the rat (Section D,5). A rather deep vein at the outer aspect of the *ankle* requires cutting down through the dermis for exposure. The *vein* on the lateral aspect of the forelimb is easily found, but it is delicate. Occasionally other prominent superficial vessels can be found and entered directly.

(*b*) *Intracardial Injection.* This method of approach cannot be recommended. Mortality is apt to be high, and there is uncertainty in complete intravenous or intra-arterial delivery. The technique follows that given in Section e,i below, the loaded syringe, completely free of air bubbles, being inserted intracardially. Blood is pulled into the syringe to confirm position, and injection is made slowly. Rapid distension is harmful.

(*c*) *Intrajugular Injection.* This method of approach, once mastered, allows precise delivery of volumes as large as 5.0 ml in a guinea pig of 450 gm in body weight. Only very shallow dissection is required. Set out clean dissecting equipment (Section D,2,e,iii), a small retractor,* and threaded needles as described below, as well as the filled syringe. Attach a very sharp, ⅞-inch hypodermic needle, gauge 26,† provided with a short bevel.

The guinea pig is anesthetized lightly and fixed on a board as in Fig. 4, *h*, with the differences that the hind limbs are fixed at the end of the board to provide sufficient working area between neck and post (Fig. 2,H), and forelimbs are drawn caudally to expose the full neck area. Hair is removed by clipping from the upper portion of the thorax to the angle of the jaw. When the animal is in proper position, the

* An excellent retractor is Aloe and Co.'s item No. B 1318, closed length 70 mm; the adjustable arms are provided with four quarter-circle blunt hooks.

† Available from Eisele and Company, Nashville, Tennessee, as Cat. No. 2001. Specify 26G, ⅞-inch short bevel.

skin of the neck will be held flat and reasonably taut, and the head will be centered in the headgear.

The head of the guinea pig and the rod carrying the yoke point toward the operator (Fig. 4, *m*). Clean the area with alcohol, and blot off any excess. Using the left thumb and forefinger, stretch the skin taut from midline to the side of the throat. Make a longitudinal incision about 28 mm long through the full thickness of the skin, parallel to and 1 cm distant from the trachea, by means of a clean Bard-Parker No. 20 blade attached to a No. 4 handle. Attach hemostat forceps as in Section D,2,e,iii, pull slightly to spread the skin, and then let these hang down as retractors.

Lift one hemostat forcep to tense the panniculus muscle. Next, by means of a pair of 4½-inch forceps, which is held closed and has been ascertained to present no rough edges, make a shallow blunt dissection through the muscle to expose the external jugular for a distance of 8 or 9 mm. (A collateral branch vein is present at times; avoid tearing it.) Spread the muscles with the small retractor, so that the anterior approach is free. At times, the retractor should be lifted slightly or pulled by an assistant during injection. The jugular vein is approached at about a 10 degree angle. The low angle of approach necessitates use of a ⅞-inch needle shaft, although penetration is made only to the extent of a few millimeters. The left middle finger is pressed against the skin just caudad to the incision to cause the vein to distend. On entry of the needle, the left forefinger exerts light pressure upon, and steadies the needle shaft (Fig. 4, *l*, *m*). After injection, the needle is withdrawn while pressure is exerted by dry gauze. No bleeding should occur. Before closure, make sure that the entire field is scrupulously clean and free of hairs. One or two ties of linen thread (three-cord, No. 40) are used to close the skin, by means of a cervix or fistula needle.* The stitches are tied but not drawn firmly: allowance is made for ensuing edema around the stitches so that the thread will not cut through the skin.

The entire procedure is carried out rapidly, in 5 minutes or less from the time the animal is made ready on the board. Postoperative attention is given. There will be a temporary edema of the throat region. If blunt dissection is minimal, the same vein can be entered again within the

* For operative closures of skin, a No. 5 fistula needle (half-circle, cutting edge) is used, such as Torrington style 725 or Anchor Brand style 1832, or Kelly's cervix needle No. 4, W. H. Welsh and Company's item 435. For operative closures of soft tissue (as parietal peritoneum together with abdominal muscles) a noncutting needle is used, such as a No. 6 Mayo intestinal needle (half-circle, taper point) available as Torrington style 705 or Anchor Brand style 1823, or Kelly's intestinal needle No. 17, W. H. Welsh and Company's item No. 115.

week through an adajacent slit in the skin (allow full healing of the original incision).

Another procedure involves tying off the external jugular vein, a permissible procedure owing to the circulatory loop in the head area. The external jugular is dissected out over a length of 23 mm, and a loop of linen thread is passed beneath as the vein is raised; two ties are established in the manner of Section D,2,e,iii. The cephalad end is tied off completely, and the ends of the tie are grasped by the operator. An assistant presses backward along the distal end of the vein, so that blood enters and distends it. A 50-mm serrefine is used to clamp off the caudal end, and the second tie is laid there as a precaution but is not closed. Just as the operator inserts the needle in this isolated segment, steadying it by pulling lightly on the ends of the tie, the assistant carefully opens the serrefine (but holds it open and poised) during the injection. At the end of the injection, the caudal tie is secured, the serrefine removed, and the skin approximated with linen ties as above. The greater amount of blunt dissection used in this method results in considerably more scar tissue during postoperative repair.

e. BLEEDING

i. Heart Puncture

Heart puncture is made as for the rabbit (consult Section D,2,e,ii(b)). The animal is affixed to the board as in Fig. 4, *h*, *n*, *o*, forefeet extended forward. The precautions noted in Section D,2,e,ii(b) should be observed in using the animal board. The position of the heart is outlined in Fig. 4, *h*, and the point of approach, determined by palpation, is shown in Fig. 4, *i*, but the angle of the syringe is raised to about 35 degrees on the longitudinal axis and pointed slightly inward toward the midline. Blood should be encountered before the shaft of the needle penetrates more than 18 mm (¾ inch). The needle position will require fine depth adjustment. For bleedings limited to volumes up to 8 ml, 1-inch gauge 23 needles are preferable. For exsanguination by heart puncture, 1-inch 20-gauge regular hypodermic needles can be used. As was noted before, the changing of syringes during bleeding is accomplished readily with Luer-lok-tipped syringes while the hub of the needle is gripped firmly.

The alternative approach, in the angle formed by the last sternal rib on the left side with the xiphoid process, is also possible, but 1¼-inch needles should be used.

Terminal bleedings are usually made by heart puncture. The rigid position afforded by the position of the operator in Fig. 4, *i*, allows holding the hub of the needle and changing syringes. For this purpose, alumi-

num-hubbed or plastic-hubbed disposable needles are not desirable; regular hypodermic needles are easily held by forceps and allow easy interchange of syringes. Simple all-glass syringes, wetted beforehand with saline, are recommended, and vaselined culture tubes (150 × 16 mm) for receiving the blood. The volume of blood that can be withdrawn in this way is about one-thirtieth of body weight.

ii. Bleeding from the Orbital Venous Plexus

Orbital bleedings can be made with a special pipet, as described for the rat in Section D,4,d,vi, penetration being about 12 mm for guinea pigs.[18] The blood is taken from the corner of the eye. The pipet is provided with a mechanically strong tip tapered to 3 mm O.D., cut at 90 degrees, and fire-polished. Since volumes of 0.5 to 1.0 mm are desired, several of the 0.2-ml commercial pipets that fill by capillarity can be used in succession (Section D,4,d,vi), marked at attained blood level, and discharged. If the volume contained per 5 cm of pipet length is known, the blood volume can be determined from the height of the column of blood. Workers often modify 2-ml heavy-wall glass pipets, at full or half length, to bear the proper tip. Here, mouth suction by means of a "sucker tubing" (Section C,2,a) is required to raise the blood into the pipet. The weight of such a pipet serves to dull tactile sensitivity during placement.

iii. Ear Bleeding

Amounts of 0.6 to 1.2 ml of blood may be taken from an ear vein with entire safety; owing to the time needed, the method is recommended only for serial bleedings of valuable animals. The animal is restrained as in Fig. 4, *k*, but the abdominal wall is raised on a cylindrical can covered with a laboratory towel, the head is lowered by repositioning the headgear, and the rear end of the board is elevated. A goose-neck lamp with a 60-watt bulb is used to warm the ear. After dry shaving and application of a thin layer of Vaseline to the ear, a tiny incision is made with the corner of a very sharp razor blade, poising the blade over the course of the vein and entering it with a quick jabbing thrust. The objective is to open the vein with minimal release of tissue-clotting factors. A small drop of blood appears at once and should well up sufficiently to be collected in a tube (75 × 8 mm). The large drops can be collected while the mouth of the tube is passed across the ear in a stroking motion, a procedure that stimulates flow. It is necessary to remove clots by dry wiping and to maintain the Vaseline layer. Owing

[18] A. Pettit, *Compt. Rend. Soc. Biol.* **74**, 11 (1913).

to the slow collection of drops, clotting and contracture are efficient, so the volume of serum will be somewhat greater than one-half the blood volume.

f. Anesthetization

For short-term anesthetization, U.S.P. anesthetic ether should be chosen and used in a room without flames or lighted cigarettes (see Section D,9). An initial etherization can be given in a glass battery jar with elevated wire-grid bottom, in which a common paper towel is used as wicking: part of it lies crumpled on the bottom of the jar, the remainder rising for a height of about 5 inches on one side of the jar. Ether poured in the bottom is carried upward by capillarity and vaporized high in the jar, the heavy vapors falling downward. Such a large volume of ether–air mixture brings the animal quickly to stage 3 anesthetization; the animal is removed, and anesthetization is continued by means of an ether cone as described in Section D,9. The animal is attached to a board, but the muzzle ring of the headgear is not used. Note that vaporization of ether chills the ambient air: water droplets collect in the ether cone and impede further vaporization. Several cones must be used in succession on humid days, being dried out later.

For very short-term etherization, the jar described above can be replaced with a small narrow glass jar about 4 cm in internal diameter (baby food jars are excellent). The ether–air mixture is created within the closed jar, and the muzzle of the animal is inserted. The air is shortly exhausted; hence the air–ether mixture should be adjusted at intervals by withdrawing the jar and swirling it around before replacing it.

Ethyl chloride U.S.P. is often used in England for very short-term anesthetization because induction proceeds so smoothly and rapidly; but prolonged exposure should be avoided. Avertin is used likewise (Section D,9,e).

4. MICE*

a. Raising and Maintaining

Mice are usually maintained in boxes with pine shavings, which are cleaned twice a week. Recommended cage sizes are given in Table I. Pelleted chow (free of antibiotics) should be provided in an overhead wire mesh hopper, commonly built into the top of the cage. (If the animals lack vigor, a few pellets are placed on the shavings.) Water

* Physiological and biological data are given in Addendum 1.

bottles are attached, with airtight rubber closures fitted with stainless-steel or glass tubing (slightly constricted at the tip) to serve as nipples. Although elaborate water supply systems can be installed with tongue-activated valves at each cage "station," individual water bottles offer flexibility in using various additives, such as antibiotics, during experimental work. Instead of plain drinking water, acidulated water has been introduced[19] to reduce bacterial growth and cross-contamination in the drinking water. This type of fluid intake has been used in breeding many successive generations of mice, and it is to be recommended: 3 ml of *N* HCl is added per liter of water. For details of anatomy, handling, breeding, and common infections of mice, see Green.[20]

Sudden, jerky motions are to be avoided in handling mice, as is true with all animals. One may pick up mice directly by the tail, or, if preferred, forceps may be used, care being taken to exert only the minimal amount of pressure needed.

Immunologists who purchase adult mice and conduct relatively short-term experiments may not encounter certain problems that are well known to breeders, to microbiologists, to geneticists, and to other persons making long-term observations. Those who raise special strains of mice sporadically encounter infantile diarrhea; this disease affects only suckling young, and it can be avoided by allowing pregnant females to deliver and raise entire litters to weanling age within special filter boxes; among the devices, a relatively cheap but successful box is described by Schneider and Collins.[21] An increasing array of pathological changes in the organs occurs during long-term experiments, even when the mice seem to be fully healthy and originate from specific pathogen-free (SPF) stock. Many strains of mice harbor *Corynebacterium kutscheri* in the form of a latent infection.[22] Overt diseases can break out, usually salmonellosis and at times corynebacterial pseudotuberculosis. The successful maintenance of mice without cross-infection has been solved by housing standard cages on "bookshelves" built along a wall, provided with sliding glass doors, each shelf separately vented by a gentle airstream drawn through a simple filter unit at one end, and pulled across the shelf through an adjustable damper by applying negative pressure at the other end of the shelf. (The device described here was designed by Dr. Vernon Riley.) Such devices remove all odor and avoid the spread of dander with the chance of acquiring hypersensitivity.

[19] R. W. Schaedler and R. J. Dubos, *J. Exptl. Med.* **115**, 1149 (1962).
[20] E. L. Green (ed.), "Biology of the Laboratory Mouse, 2nd ed., McGraw-Hill, New York, 1966.
[21] H. A. Schneider and G. R. Collins, *Lab. Animal Care* **16**, 60 (1966).
[22] R. M. Fauve, C. H. Pierce-Chase, and R. J. Dubos, *J. Exptl. Med.* **120**, 283 (1964).

Painless disposal can be effected in a closed chamber by CO_2 gas admitted from a cylinder, the heavy gas flowing downward.

b. Identification

After receiving an injection, every mouse should be marked by dye to distinguish it from normal mice. Group identification may prove to be adequate. Individual identification becomes necessary not only for genetic work (a standard system of ear notchings representing digits is used; see Green[20]) but also when serial bleedings are required. Color coding is adequate for white mice; color must be renewed every three weeks or so. The writer recommends simple laboratory dyes such as carbol–fuchsin, gentian violet, or picric acid: 1 volume of aqueous solution is diluted with 3 volumes of alcohol and 4 volumes of ethyl ether to provide a quick-drying dye. Cotton-tipped applicator sticks lightly moistened in dye are thrust below the hair and rolled upward with a twisting motion to color the lower hairs also. With three dyes and three positions—base of skull, back, base of tail—nine digits can be represented; 10 is represented by placing the 1 position on the left side of the skull; in this way, numbers up to 99 are easily coded and deciphered. The use of one dye will be adequate in many situations.

c. Injection

i. Subcutaneous Route

A simple method is shown in Fig. 5, *b*, in which the mouse, held by the tail, strains forward across a wire grid. Flank skin near the base of the tail is wetted lightly with 70% alcohol. Using a syringe with a long needle (at least 1 inch), gauge 22, introduce the needle beneath the skin slightly on either side of the tail in the dorsal area; advance the hypodermic, held horizontally as in Fig. 5, *b*, directing the needle away from the spinal column of the mouse in the event of a sudden jerk by the animal. Penetrate the subcutaneous tissue at least ¾ inch before expelling the contents of the syringe. Not more than 0.5 ml should be introduced at one site.

Very little force is required to inject the material, and an immediate bleb can be observed when the inoculum has been deposited correctly. If, instead, one finds force to be required for injection, the underlying tissue has been penetrated.

Occasionally, particularly when animals undergo repeated injections, mice will need to be restrained. This is readily accomplished by holding the tail, allowing the animal to retreat within the box, and sliding the

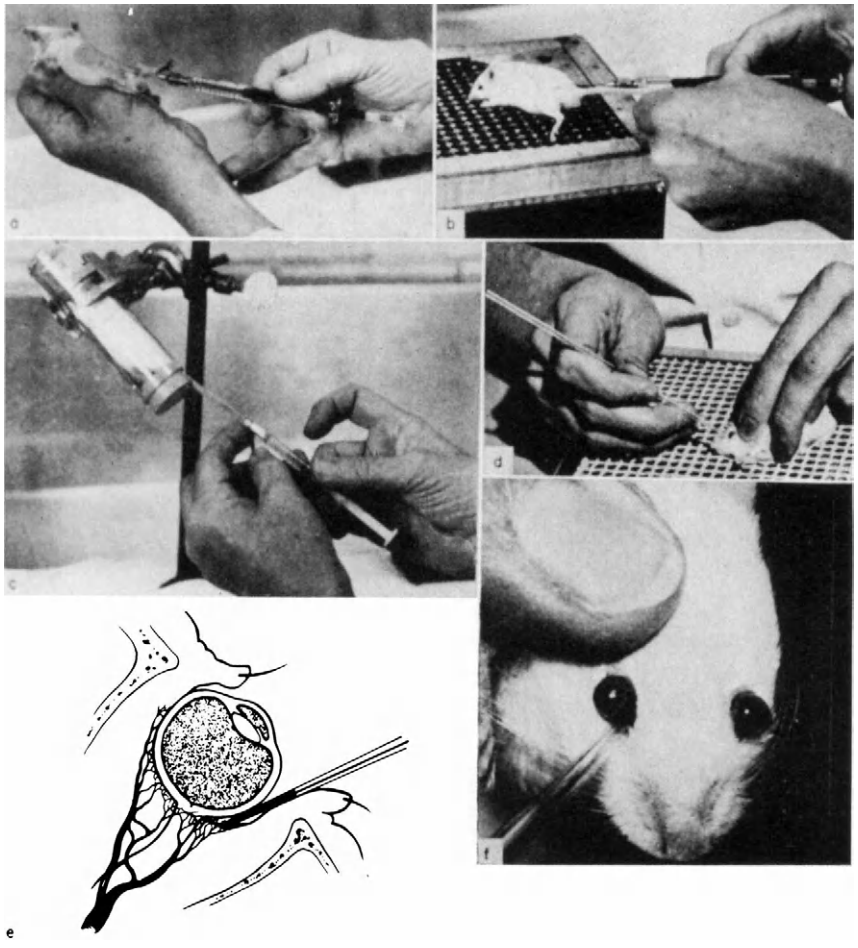

FIG. 5. Views *a–c*, injection of mice by the intraperitoneal (*a*), subcutaneous (*b*), and intravenous routes (*c*).

Views *d–f*, technique of obtaining mouse blood by rupture of venous capillaries of the ophthalmic venous plexus, after V. Riley, *Proc. Soc. Exptl. Biol. Med.* **104**, 751 (1960): *d*, positioning of mouse on wire cage top during bleeding, after fixation of scalp as described in text; *e*, anatomical detail of the reservoir formed by the orbital cavity from which blood is drawn by capillarity; *f*, entering position of pipet. Illustrations *d*, *e*, and *f* courtesy of Dr. Vernon Riley. View *e* is reproduced from Riley, *op. cit.*, by courtesy of author and publisher.

lid to within ¼ to ½ inch of the edge of the box before proceeding with the injection.

ii. Intraperitoneal Route

With the mouse in the position of Fig. 5, *b*, the operator grasps the back of the neck and inverts the mouse. As much of the dorsal skin is seized as possible, and the tail is anchored between palm and little finger; the unsupported hind leg can be anchored between fourth and little fingers if desired. A cotton pledget moistened with alcohol is used to swab the abdomen, and injection is made as in Fig. 5, *a*, with a 1¼-inch gauge 22 needle. Entry is made subcutaneously in the skin of the left lower quadrant, caudal to the umbilicus. Thrust forward subcutaneously about 1 cm, elevate the syringe slightly, and press downward with the needle. With the abdominal wall kept taut, the abdominal muscles and peritoneum will be seen to be depressed by the needle point. Thrust downward only a few millimeters, to penetrate; then lower the syringe and elevate the peritoneal wall by means of the needle shaft before injecting. Injection of liver and nicking of the gut are thus avoided.

iii. Intravenous Route

A useful and simple means of restraining mice for intravenous injection is composed of a Berkefeld filter mantle, 6 inches × 1 inch, the hole for attaching the filter candle serving as an air inlet when used as below. The open end is fitted with a No. 6 rubber stopper from which a 7-mm butt of rubber has been removed across the broader end and from which a wedge-shaped lateral segment has been removed so as to leave a space approximating the largest diameter of a mouse's tail. The glass is clamped to a ring stand (Fig. 5, *c*). The mouse is swung forward by the tail and induced to enter head first into the receptacle while the tail is held; the rubber stopper is then pushed securely into the open end.

Before the mouse is so positioned, the inoculum is introduced into a 1-ml tuberculin syringe with 27-gauge long-bevel needle; the volume to be injected per mouse is 0.2 to 0.3 ml. Disposable needles ensure sharpness, a most necessary condition for success. Just before injection, the tail is drawn taut and wiped with 70% alcohol; blood flow is stimulated by a series of sharp snapping motions with the tip of the operator's fingernail. Within seconds one can usually observe dilatation of the caudal vessels. (Some prefer to use xylene to secure dilatation; if so, xylene must be removed carefully by means of 95% alcohol at the end

of the procedure.) Other workers stimulate blood flow by working in the immediate vicinity of heat from a lamp. As a rule, however, the last two methods are unnecessary.

Only the lateral veins should be used as injection sites. When the veins become readily apparent, the only necessary precaution is to introduce the material slowly so that it does not infiltrate the tissues surrounding the vessel wall. If one must explore blindly, it is important to begin as closely as possible to the distal end; intravenous injection is obtained if the material is delivered from the syringe without force and if a small drop of blood appears at the site on withdrawal of the needle. If resistance is encountered, the needle should be withdrawn and another attempt made at a less distal site. Difficulties are sometimes encountered in mice having a dark skin or in older animals in which the skin is thickened. However, as with the case of venipuncture in human beings when the vein cannot be visualized, the experienced technician (operator) can tell by "feel" when the needle has been introduced into the vein.

If the volume injected is greater than that recommended, 0.2 to 0.3 ml, there is more of a tendency toward back leakage or collapsing of veins.

iv. Intradermal Injection

The common method for intradermal injection is altered somewhat in view of the thin skin of the mouse. With a 30-gauge needle attached to a ¼-ml tuberculin syringe, one can penetrate the dermis and deposit approximately 0.03 ml volume intradermally, with usually a small amount of leakage from the bleb so produced. A better procedure is to penetrate the skin fully and raise the needle tip up into the dermis from below. The position of the tip can be seen precisely. The sites of choice are the lateral dorsal areas.

Another technique is that of intradermal inoculation into the metatarsal pads of the foot.[23] With the mouse held by an assistant as in Fig. 5, *a*, the ventral surface is presented. The operator grasps the toes of a hind foot with thumb and forefingers, thus tensing the plantar surface. The footpad is turned slightly laterally and swabbed with 70% alcohol. The tip of a short-bevel 26-gauge needle attached to a ¼-ml tuberculin syringe is then introduced into the distal aspect of one of the pads. With slight pressure on the plunger, 0.03 ml is expelled, causing

[23] David F. Gray and P. A. Jennings, *Am. Rev. Tuberc. Pulmonary Diseases* **72**, 171 (1955).

distention of about 3 mm in the diameter of the sole of the foot surrounding the pad. If a subcutaneous injection is made accidentally, there is an immediate subcutaneous spread extending along the entire plantar surface to the tarsus. When intradermal tests are made at weekly intervals, alternate hind feet should be injected. Results can be read in units of 0.05 mm of anteroposterior thickening of the foot as determined by micrometer measurements.*,[24]

v. Intracerebral Inoculation

This route is used occasionally. A description is given by Standfast.[25] Mice are anesthetized lightly with ether or ether–chloroform, and a volume of 0.03 ml is injected intracerebrally from a 0.25-ml syringe fitted with a $\frac{3}{8}$-inch gauge 27 needle.

vi. Intranasal Inoculation

For virus research and special purposes, intranasal instillation can be used. An inoculum of 0.03 ml is deposited onto each nostril of mice anesthetized with a mixture of 2 parts of ether to 1 part of chloroform.[26] Inhibition of cough, sneeze, and swallowing reflexes allows the inoculum to be inhaled evenly into the lungs. The inoculum is best delivered from a calibrated Pasteur dropping pipet (33 drops per milliliter).

d. Bleeding Methods, Mice

i. General Information

When bleedings of individual mice are kept separate, precipitin tubes are used. Other receptacles are chosen if pooled blood is to be collected. To obtain nonhemolyzed serum, the same care must be taken as with separation of serum from blood samples of other species. One may expect to obtain as serum approximately half the total volume of blood withdrawn. When a serum is to be obtained quickly to measure antibody response, the blood may be discharged along the side of a tilted petri dish, and allowed to clot and retract; serum sufficient for a test can

* Measurements were made with the "Schnelltaster" measuring instrument (Type A·02T, available from H. C. Kröplin, Schlütern, Hessen, Germany), designed to determine thread diameter by closing the legs of the instrument against the thread. When the instrument is used with soft tissues, the end point for closure is partly subjective. Truly objective measurement would require a modification of the instrument to permit making readings at a standard pressure.

[24] David F. Gray, Heather Graham-Smith, and J. L. Noble, *J. Hyg.* **58**, 215 (1960).

[25] A. F. B. Standfast, *Immunology* **1**, 135 (1958).

[26] F. M. Burnet and C. Timmins, *Brit. J. Exptl. Pathol.* **18**, 83 (1937).

be collected within 30 minutes. Precautions are taken to avoid evaporation.

For full and rapid exsanguination designed to drain blood prior to the collection of organs such as the spleen, a special "mouse guillotine" is available commercially, which severs the head in one stroke.

ii. Cardiac Puncture and Sacrifice

To obtain the maximal volume of blood, chloroform is preferred for anesthesia, since it causes dilatation, first of the left atrium and later of the entire heart. The mouse is put into a beaker with a wire-grid bottom under which a pledget of chloroform-moistened cotton is present, the top closed by a petri dish. At a deep level of anesthesia, the animal is removed and is pinned by the feet, ventral side up and taut, onto a piece of heavy pressed cork. The chest area is washed with 70% alcohol. Blood may be obtained from the heart by either of the following two methods, using aseptic technique.

(*a*) *Withdrawal from Closed Chest by Syringe and Needle.* The skin is deflected from the chest wall, and a 27-gauge (short-bevel) needle attached to a 2-ml syringe is inserted between the left intercostal spaces at an angle designed to penetrate the left ventricle of the heart. When blood appears in the barrel of the syringe, the piston should be withdrawn very slowly, since too great negative pressure tends to collapse the heart wall. With experience one may eventually expect to obtain in this manner approximately 1.5 to 2 ml of blood from a 20- to 25-gm mouse. An inexperienced operator should secure at least 0.75 ml of blood. The operator must be able to extract the blood expeditiously to avoid clotting, and care must be taken in expressing the blood into the receiving vessel to avoid hemolysis.

(*b*) *Removal from Open Chest by Capillary Pipet.* This method is technically easier, although it does not allow exact measurement of blood volume. Commercial disposable Pasteur pipets (6 inches long) are plugged with cotton and dry sterilized. Immediately before use the capillary tip is flamed in a Bunsen burner and pulled out finely so that the wall is rather rigid and the bore approximates a 20- to 22-gauge needle. It is broken off, preferably leaving a jagged end more easily able to penetrate the heart wall. A series of pipets may be laid out on a rack in an area adjacent to a Bunsen burner where the conditions can be considered aseptic. Just before use, each pipet is fitted with a rubber teat. The mouse is anesthetized with chloroform and prepared as in Section (*a*) just cited, and the chest wall is opened. While the heart still beats, the pipet is introduced into the wall of the left ventricle, and blood is withdrawn slowly. As with the closed-chest method, practice

is essential to obtain the maximum amount of blood (1.5 to 2 ml) without clotting or hemolysis.

iii. Use of an Axillary "Pocket"

Serum obtained by this method must be sterilized by filtration for full assurance of sterility. Ether, which increases cardiac output during light surgical anesthesia by release of epinephrine, is the anesthetic of choice for blood drawn from peripheral areas. The mouse is anesthetized in a beaker with elevated wire-grid bottom, except that ether instead of chloroform is used on the cotton pledget. The stems of capillary pipets are broken off to provide stubby tips. The anesthesized animal is pinned upon the board and washed down with 70% alcohol; excess disinfectant is removed with cotton. The skin in the axillary area is deflected in such a manner that a small pocket is fashioned and slight dissection of the muscles will expose the subclavian artery. With one stroke of a sharp scalpel, the artery is nicked or severed, and the blood issuing forth is immediately drawn up into the capillary pipet before it overflows into the surrounding skin area. The procedure must be carried out with the minimum amount of trauma to prevent an undue amount of tissue juices, which serve both to dilute the blood and to activate rapid clotting.

iv. Jugular Vein Penetration

Micro quantities of blood may be withdrawn from the jugular vein of weanling mice without sacrifice, and repeated samples can be obtained in this manner from older mice.[27] The loose dorsal skin of the mouse is held firmly between the thumb and index finger. Hyperextension of the head to expose neck and upper thorax is accomplished by slipping several threads of a 2×2 inch gauze sponge over the upper central incisors, and the gauze, pulled backward, is grasped between the second and third fingers. Depilation of the skin from chin to midthorax exposes both external jugular veins, which are usually distended. The syringe with 26-gauge needle is wetted with a solution of anticoagulant, and, after sponging of the area with 70% alcohol, the needle is inserted subcutaneously 1 to 2 mm below the sternoclavicular junction so that the vessel may be approached in a cephalad direction. Blood must be withdrawn slowly.

v. Blood from the Tail

The tail is wiped vigorously with 70% alcohol, massaged gently or exposed to heat to dilate the vessels, and with sharp scissors approximately 1 cm of the distal end is cut off. Several drops of blood may be obtained

[27] R. Kassel and S. Levitan, *Science* **118**, 563 (1953).

freely in this manner. Further manipulation of the tail—that is, attempting to squeeze the veins manually to obtain more blood—should be avoided, since this dilutes the blood with tissue juices.

Another technique, described by Dr. Labe Scheinberg, removes 0.5 to 1.0 ml of blood by application of negative pressure to a cut made on the tail of an anesthetized mouse. A tail bleeding apparatus is used, consisting of a tube and a two-hole rubber stopper—one hole to fit the maximal diameter of the tail, the other connected to a source of negative pressure which is measured by a mercury manometer. After the mouse loses consciousness, the tail and hind quarters are warmed by a 100-watt bulb placed 4 or 5 inches above. The tail is wetted in a tube of heparin solution (2 mg of heparin in 10 ml of isotonic KCl), led through the stopper of the bleeding apparatus, and nicked with a scalpel about 1½ inches from the tip. Suction is applied, equal to 9 cm of mercury.

*vi. Orbital Bleeding Technique for Mice, Rats, Hamsters**

A procedure for securing blood from the orbital socket of a variety of rodents was first described by Pettit in 1913.[18] Although put to use by later workers, an expanded account was first presented fully by Riley.[28] Proper lightweight pipets (heparinized) are now available commerically,† in which volumes of 0.2 ml of blood rise by capillarity. It requires 1 minute to bleed a mouse; for exsanguination, five pipets can be used in succession. Usually, the method is employed for serial bleedings; due allowance should be made for loss of about 10% in blood volume of the mouse at each bleeding. Sedimentation rates and hematocrit values can be obtained on the blood within the loaded pipet, the plasma then being removed for antibody studies.

The mouse is grasped by picking up the hair of the scalp with thumb and forefinger as far forward as possible and then shifting the grasp to thumb and third finger. The freed forefinger is used to exert traction on the skin close to the eye, to make the eye bulge, and to constrict the superficial venous return (Fig. 5, *d*, *f*). With the mouse held against a wire-grid base as in Fig. 5, *d*, the tip of the pipet is inserted in the inner angle of the eye as in Fig. 5, *f*, and slid under the eyeball and over the bony socket. The tip of the pipet is held at about a 45-degree

* Material in this section, including information on rats and hamsters, is based on data generously offered by Dr. Vernon Riley.

[28] V. Riley, *Proc. Soc. Exptl. Biol. Med.* **104**, 751 (1960).

† Scientific Products, Division of American Hospital Supply Corp., micro blood collecting tubes, 150 mm long, 3 mm O.D., 1.5-mm bore, coated with ammonium heparinate, Cat. No. B 3095-2.

angle as it passes along the bone, directed at the optic nerve, where it ruptures the fragile venous capillaries of the ophthalmic venous plexus (Fig. 5, *e*). The passage is about 5 mm in the mouse; the tip of the pipet is then retracted slightly, and the blood collecting in the orbital cavity will rise into the pipet. The pipet, which has a fire-polished tip, is rotated slightly during the bleeding.

Relaxation of pressure by the forefinger after removal of the pipet returns the eyeball to the socket, exerting pressure on the ruptured venous network and ending extravasation. A tiny droplet of residual blood around the eyeball is removed by a cotton-tipped applicator.

Rats can be bled in the same way; an assistant is useful. The animals are grasped behind the neck with thumb and index finger of the left hand and positioned on a wire-mesh cage top under the palm and forearm. The eye is caused to bulge by placing the index finger above the eye and the thumb below it. The capillary pipet is used as in Fig. 5, *f*, and then given a sharp thrust to force the pipet about 10 mm toward the back of the orbit; the pipet is then withdrawn; the blood flows freely from the corner of the eye and is collected in the capillary tube.

Hamsters may be picked up easily by attaching hemostat forceps to the skin of the throat, the instrument being kept attached during bleeding.

The operator should not wear gloves. Light anesthesia can be employed when it is not disadvantageous for the experiment.

Blood-filled tubes are laid flat during the collecting operation. Closure is effected finally by pressing the top of each pipet into a disk of plasticene, after which the closed tip is secured in the bore of a rubber serum ampoule stopper,* This type of stopper has a rubber skirt which will support the capillary tube during centrifugation in a 13 × 100 mm glass tube (2000 rpm for 20 minutes, in bucket-type trunnion cups). Hematocrit values are read as percentage of the packed erythrocyte volume within total contained volume, for which a special scale has been designed.[29] To secure the plasma, the tubes are nicked with a file just above the erythrocyte column, and the upper part of the tube is snapped off. The plasma yield can be calculated from the height of the column, since the bores of the pipets are essentially uniform. For serological purposes, the plasma can be blown out into a suitable amount of diluent designed to yield a known primary dilution such as 1:5 to 1:20.

* The more expensive Critocap-J tube closures can be used (also sold as micro tube closures, Scientific Industries, item No. M-375).

[29] V. Riley and W. C. Valles, *Proc. Soc. Exptl. Biol. Med.* **91**, 341 (1956).

5. RATS*

Techniques for handling rats follow closely those used with mice. There is one very important difference in handling. The dermis of the tail cannot support the weight of the body; hence rats should not be taken from the cage by grasping the tail, or lifted even when anesthetized.

It is important to work daily with rats to train the animals to recognize their handlers, to avoid making sudden motions, and to position the holding cages so that the rats can look around the room. Various aspects of handling rats are discussed by Farris and Griffith.[30] Room temperature should be maintained between 75 and 80°F, with relative humidity at 40–70%, optimally 50–55%.

A gentle rat is grasped by placing the palm of the right hand over the back, using thumb and forefinger to encircle the upper part of the thorax; but do not squeeze the ribcage. The forelegs are pressed up under the chin by the thumb so as to prevent biting.[11] Unruly rats can be grasped with locking tongs,† seizing the skin at the base of the tail, and lifting the rat to a wire mesh surface. The middle third of the tail is grasped, and the hind quarters are lifted free of the surface. As the rat strains forward on the mesh, the skin at the nape of the neck is seized by the operator's right hand between thumb and forefinger.

Various restraints are pictured in Leahy and Barrow.[11] One useful device for restraint during femoral vein injections can be made from a box such as those in which large glass syringes are sold. A space for the tail is made by U-shaped grooves in box and cover at one end; a rectangular port for the hind legs is cut out 1¾ inches long in proper position, and a corresponding 2-inch section is cut from the cover. When the rat is introduced, the hind legs are passed out of the ports, the tail is placed in the tail slot, and the cover is put in place.

Caged wild rats are either anesthetized or driven out into a wire cone protruding from a sliding panel in the cage door. Blowing the breath on the animal will cause it to run into the cone, after which a wire "comb" of parallel wires soldered to a rod is run down behind the animal.[11] When anesthetization is used to permit handling, the cage is placed in a room without flames. Anesthetic ether can be vaporized close to the face by directing a stream from a syringe fitted with a fine-gauge hypodermic needle. Elaborate devices are available for administering tranquilizing drugs.

* Physiological and biological data are given in Addendum 1.

[30] E. J. Farris and J. Q. Griffith, Jr., "The Rat in Laboratory Investigation," 2nd ed. Hafner, New York, 1949.

† Foerster sponge holding forceps, straight, 7 inches with serrated jaws, and 9½ inches with smooth jaws.

Intravenous injection by tail vein demands greater skill than with mice, and injection through the femoral vein is recommended. After anesthetization with ether in a jar, the rat is placed in the supine position and secured on a board.* Wipe the skin of the right thigh with 70% alcohol, and cut down with a sterile Bard-Parker disposable blade No. 15 for a distance of 12 to 15 mm parallel to, but not over, the femoral vein. Do not cut the fascia of the leg muscle. Extend the right hind leg to straighten the course of the vein, displace the skin to expose the femoral vein, and inject through a ½-inch 30-gauge needle. After the needle is withdrawn, compress the vein by a pledget of cotton pressed firmly over the area for about a minute. When bleeding has stopped, close the incision with a tie of sterilized cotton thread or Michel skin clips, 11-mm size.

Rats are bled from the orbital sinus as in Section D,4,d,vi or from the heart as in Section D,3,e,i. The lightly anesthetized animal can be secured by its limbs to a board, and the head held by rubber bands, one being placed across the nose and another beneath the lower incisors.[11]

6. LARGE QUADRUPEDS

Horses are restrained by ropework employing twitches, hopples, rope halters, or the like.[11] For intravenous injection and bleedings, the head is tilted upward, an overhead beam being used for attaching a rope leading from the halter.

Sheep and *goats* are handled similarly for injection and bleeding, although their temperaments are different. With goats, it is best to maintain two together, since solitude is disquieting; and it should be remembered that one's back should never be turned to a goat. Animals of these species can be backed into a corner (Fig. 6, c, *d*), and the head can then be raised and held by an assistant astride the animal, exposing the area of the external jugular vein. The operator clips the wool or hair from the site, sponges the skin with alcohol, and distends the vein by blocking the venous return with his left thumb (Fig. 6, *d*). Injection, or bleeding by means of a needle and plastic tubing,† is then easily possible. Intramuscular injection in the thigh can often be made with

* A useful device (Campbell *et al.*[10]) consists of a metal cylindrical sleeve through which a heavy rubber band is passed; one end of the metal sleeve is then squeezed down flat to provide friction. The leg is grasped in one rubber loop, and the intact end of the sleeve is slid down to the limb. The opposite rubber loop is tensioned and fixed to the board by a thumbtack.

† A useful presterilized set is Abbott Laboratories' Stock No. 4700, blood collecting set, 24-inch tubing with siliconed needles. The vein is entered with the attached 1½-inch 17-gauge needle, and the needle to enter the collecting bottle is 15 gauge.

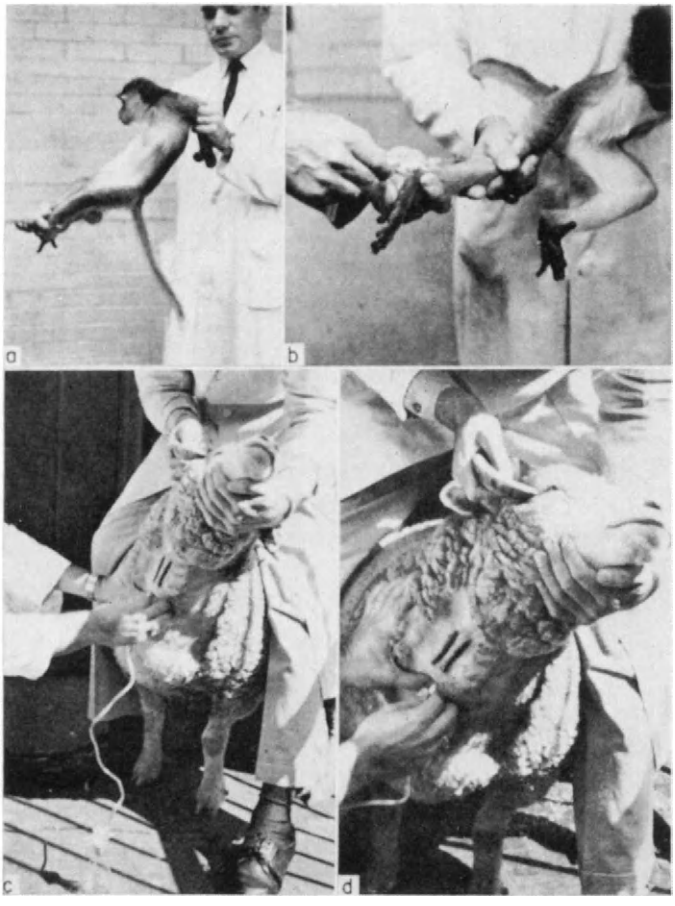

FIG. 6. Restraint of monkey (*a*) and position for intravenous injection (*b*) are shown.

Sheep and goats are backed into a corner and straddled by an assistant, who raises the lower jaw and turns the head away from the side of the neck on which the external jugular will be approached (*c*). The operator closes off the venous return with his free thumb and inserts the 2-inch 17-gauge needle into the distended vein (*c*, *d*).

this hold, or the animal can be thrown and the hooves tied together. Small samples of blood can be taken from the marginal vein of the ear as described for rabbits (Section D,2,e,i).

7. PRIMATES

Arrangements for the care, feeding, and housing of primates under rather constant temperature (between 70–78°F and relative humidity

40–60%) are complex and call for cooperation with veterinaries. *Monkeys** are commonly identified by numbered brass plates attached by chain around the neck. The caretaker should spend time in developing a reputation for gentleness but firmness in handling. Monkeys that cannot be trained by voluntary handling require special restraints, such as light chains attached to collar and cage door so that the monkey can be drawn to the cage door and seized by the upper arms when the door is opened, or other specialized devices.[31a] Figure 6, *a*, shows the handling of a trained monkey by an experienced handler, and Fig. 6, *b*, shows an intravenous injection made through the skin into the superficial femoral vein; the vein is readily apparent when the hair is clipped away, and the assistant temporarily blocks the venous return by pressure applied close to the knee. Persons working with monkeys should be prepared for sudden discharge of unformed fecal matter. Papers dealing with care and diseases of monkeys are provided by Sauer[31b] and Cass *et al.*[2d]

A few monkeys are carriers of B-virus, of which seven handlers bitten by monkeys have died. Immunization of those persons who already carry anti-herpes virus antibodies is apparently possible with a B-virus preparation, as judged by development of specific anti-B-virus antibodies; but the requirement for "proof of efficacy" as a prophylactic measure presently prohibits injection of man.

8. AVIAN SPECIES

a. Handling and Restraint

It will be assumed that the birds are in cages and are being raised on normal diets. Identification is usually made by color coding with plastic spiral leg bands. Literature on biological characters and physiology is given by Sturkie,[31c] Levi,[31d] and Cass *et al.*[2d]

*Pigeons** or birds of similar size are grasped by the assistant around the base of the tail, and the wingtips also are caught within the same hand; the legs are extended fully and replaced in the same hand, with the index finger inserted between the legs to spare the hocks. The opera-

[31a] F. G. Fielder and C. J. Casmer, *Lab. Animal Care* **16**, 395 (1966).

[31b] R. M. Sauer (ed.), "Care and Diseases of the Research Monkey." N. Y. Acad. Sci. **85**, 735 (1960).

[31c] P. D. Sturkie, "Avian Physiology," 2nd ed. Comstock Publishing Associates, Ithaca, New York, 1965.

[31d] W. M. Levi, "The Pigeon." Levi Publishing Co., Sumter, South Carolina, 1957.

* Physiological and biological data are given in Addendum 1.

tor has access to the head and neck or to a wing, which can be raised separately.

*Chickens** can be raised and supported on the left forearm by facing the bird and passing the forefinger caudally between the legs, encircling them with thumb and middle finger. The right hand is placed across the back to keep the wings from flapping. Held in this position, the chicken can be readily turned over and placed on its back. The assistant can then hold the legs with one hand and present and control the opposite wing. (If the head is lowered toward the ground, regurgitation may occur from the crop.)

Full mechanical restraint is possible by preparing a "chicken board" which holds head and legs and has spring-tension clamps to hold the wings extended, by clamping them below their bony portions (the radius, ulna, and carpometacarpus), as described in reference 10, p. 14.

Chickens can be "hypnotized" by tucking and holding the head under the left wing, grasping the body with legs flexed toward the bird's abdomen (the roosting position), and swinging the chicken through a vertical plane for ten to fifteen complete circles. The chicken will remain relaxed for some minutes and allow many manipulations. The procedure is harmless.

b. INJECTION

Intramuscular injection is made into the heavy pectoral muscles, with pressure sustained to prevent back-leakage.

Subcutaneous injections are done in the loose skin of the axillary area.

Intravenous injections are most conveniently carried out by injecting the alar vein, which lies just beneath the humerus on the underside of the wing. It is exposed by plucking feathers from the axillary area. After injection through a 23-gauge needle, a compress is held over the site for some minutes, since clotting will occur only slowly. For anaphylaxis in pigeons, the vein running on the outer aspect of the leg can be entered readily; again, cotton must be pressed firmly as with a rubber band while the pigeon is set free for observation of symptoms.

Other available vessels are the *jugular* vein, exposed by dissection near the ramus of the mandible, the *saphenous vein*, approached from the posterolateral aspect of the thigh by means of blunt dissection, and the *femoral vein*, in the area of the femoral triangle, exposed by minor dissection.

* Physiological and biological data are given in Addendum 1.

c. Bleeding

i. Alar (Brachial) Vein

As described under intravenous injections, the plucking of feathers from the axillary area reveals the priminent alar vein lying just beneath the humerus. The vein can constrict quickly if the animal feels pain. When proper insertion is made with a 19- or 20-gauge needle, it is possible to withdraw 20 ml of blood from a chicken, or half as much from birds of pigeon size. The point of entry must be compressed firmly for at least 3 minutes after the needle is withdrawn to avoid hematoma.

ii. Cardiac Puncture

With the bird on his back, the feathers are plucked from the crop area, and the V-shaped furcula (wishbone) formed by fusion of the clavicles is palpated. This V points toward the tail. Position the bird's head toward the operator, who places his left thumb in the V depression. Using a syringe fitted with a 2-inch 18- to 20-gauge needle, insert the needle just below the left thumb in a flat trajectory, about a 7-degree angle from the horizontal. Advance the needle at this angle along the midline, using slight negative pressure on the plunger, nearly as far as its full length for a 4-pound (2-kg) bird. If the heart is not met, withdraw completely and make a new approach. When blood flows into the syringe, retract the plunger very slowly; up to 50 ml may be withdrawn safely, or the bird can be so exsanguinated if desired.

Alternatively, a lateral approach to the heart is possible, the bird being held on its side. The maximum beat of the heart is palpated. The needle is inserted between the ribs just dorsal to the strong pectoral muscles, and the needle is advanced until the pulsation of the heart is felt. Penetration is then effected as in other species.

iii. Arterial Cannulation

Cannulation of the carotid artery or alar (brachial) artery is possible with Clay-Adams polyethylene PE 20 tubing or Becton-Dickinson No. 442T polyvinyl tubing, both of which pass through thin wall No. 18 gauge needles. The carotid is approached by dissection near the ramus of the mandible, the brachial by minimal dissection along the humerus close to the alar vein.

The segment to be cannulated is closed off; the end further from the heart is tied first, and then the end nearer the heart is clamped by means of a 50-mm serrefine (see Section D,2,e,iii). The thin wall No.

18-gauge needle is inserted into the segment, then the cannula is inserted and tied in place with silk thread passed around the artery. After the needle is withdrawn, the serrefine is removed from the heart end, and blood is allowed to flow.

iv. Terminal Bleeding

As terminal procedures, cardiac puncture, arterial cannulation, or decapitation may be practiced. When decapitation is chosen, precautions should be taken not to contaminate the blood with crop contents or feathers.

It is best to take chicken blood with citrate or oxalate and to separate the erythrocytes before the plasma is clotted (Section C,2,b), since the blood of mature chickens clots without contracting.

9. ANESTHESIA

a. General Remarks[2a,32-34]

A wide variety of sedatives and anesthetics is available for many specialized and prolonged operative procedures. For the immunochemist, short-term anesthetization usually suffices. His concern is with the small laboratory animals, and his needs are met by simple inhalant anesthetics or by pentobarbital sodium without pre-anesthetic medication; such pre-medication is recommended, however, by Gay.[2c] Any required anesthetization of primates and larger domestic animals should be carried out by a veterinary doctor.

Diethyl ether and chloroform are the most appropriate inhalant anesthetics for small animals. (Note that none of the species considered here can readily receive inhalant–air mixtures by endotracheal tube.) Almost always, ether will be the anesthetic of choice.

Liquid chloroform or ether should never touch the body or muzzle of the animal: in jars or boxes, an elevated grid bottom is needed. With use of cones, the use of a bland ophthalmic ointment is recommended to protect the eyes.*

It is well to note that animals receiving inhalant anesthetics should not be tilted head downward, since the lung volume decreases markedly.

[32] W. V. Lumb, "Small Animal Anesthesia." Lea and Febiger, Philadelphia, 1963.

[33] P. G. Croft, "An Introduction to the Anaesthesia of Laboratory Animals." The Universities Federation for Animal Welfare, London, 1958.

[34] C. D. Barnes and L. G. Eltherington, "Drug Dosage in Laboratory Animals: A Handbook." Univ. California Press, 1964.

* Sterilized Vaseline can be used, or special products used as eye drops, such as Tear-i-sol, Crookes-Barnes Laboratory (methyl cellulose base with boric acid and a preservative).

b. Diethyl Ether

Ether must be administered mixed with air. Induction is relatively slow, but the margin of safety is wide. Under controlled conditions, it has been observed that deep anesthesia is secured by ether–air mixtures of 3.5 to 4.5% (v/v); increasing the concentration to 6.7 to 8.0% brings on respiratory arrest.

The effort is to provide a high concentration at the outset, bringing the animal rapidly through stage 1 (inception of unconsciousness) and stage 2 (phase of excitability), up to plane 2 of stage 3 (full relaxation with slow and regular respiration, no eyeball or eyelid reflex, no reflex when toes are pinched, and normal color of mucous membranes). For the initial high concentration, a chamber with high wicking, as described in Section D,3,f, is appropriate, the heavy vapors being released high in the chamber. Once reached, stage 3 is best maintained with use of a cone* by an assistant functioning solely as anesthetist, who may drop ether onto an absorptive surface lining the pathway of air intake (Section D,3,f) or use air blown over the surface of ether contained in a bottle. When an animal exhibits the deeper planes of stage 3 anesthesia (pale mucous membranes, delayed thoracic respirations, or even chiefly abdominal respiration) or stage 4 (toxic stage, shallow and unpredictable respiration), administration of ether must be stopped at once, and the animal allowed to ventilate and become more lightly anesthetized.

For birds, ether can be employed to narcotize (in the veterinarian's sense—to sedate and make oblivious to pain), but the distribution of ether in the air sacs is too uncertain to suggest administering enough to permit operative work on head and neck. When ether is used, it is best to give it slowly, with long pauses during which the bird breathes air.[2c]

It is to be stressed that diethyl ether presents a high degree of fire hazard. During its use, avoid all open flames, lighted cigarettes, and the use of electrocautery. The vapors falling downward can function as a fire "trail" to carry flame rapidly to the major supply. Only U.S.P. anesthetic ether sold and maintained in copper-lined cans should be used, not the C.P. anhydrous grade, which contains peroxides. It is best

* Ether cones can be constructed of ¼-inch galvanized wire cloth, 19 gauge (hardware cloth). They are made 9 cm long, tapered from 1.5 cm at the tip to 5.0 cm at the base. The interior can be lined with an absorptive material such as Multilith pads or cotton within gauze, sewn to the frame, and sharp edges covered with an ether-impermeable material. Ether is dropped from a capillary pipet fitted with a nipple onto the inner absorptive material at the narrow tip of the cone.

to buy anesthetic ether in ¼-pound cans, since peroxides can form by oxidation after the can is opened. The temperature of storage should not exceed 25°C (77°F). Opened cans must be kept tightly stoppered and never placed on an upper shelf; refrigerators can be used for storage only when they possess a special sparkproof electric system or are brine-operated. Emptied cans should be filled completely with cold water to displace ether vapor before being emptied and discarded.

c. CHLOROFORM

Chloroform is reserved usually for terminal procedures, as for bleeding mice from the heart (Section D,4,d,ii), for which its dilating effect is useful, or for preparing guinea pigs for exsanguination by severance of major head vessels owing to its more rapid action and the absence of fire hazard. It has a narrow margin of safety, renders the respiratory center progressively less sensitive to CO_2 stimulation, and reduces cardiac output. Chloroform possesses pronounced toxicity for the heart, liver, and kidneys.[32] When chloroform is chosen, a high initial concentration secured by the wicking method of Section D,3,f would be inappropriate owing to chloroform's high toxicity and early arrest of respiration. In particular, chloroform is to be avoided with rabbits, death often occurring during the induction phase chiefly owing to ventricular fibrillation.

d. PENTOBARBITAL SODIUM

Pentobarbital sodium is given at the average intravenous dosage of 29 mg/kg of body weight for most species, somewhat more for mice (35 mg/kg) and less for monkeys (25 mg/kg) and, according to Sapirstein and Hartman,[35] for chickens (15 to 25 mg/kg) but not for pigeons.[36] Great variability is encountered from one individual to another. A deep anesthesia of 1 to 3 hours' duration ensues. For safety in laparotomy of rabbits, the dosage can be reduced to 18 to 20 mg/kg, and supplemental anesthetization given with ether.

Rabbits and birds are injected intravenously, the dose being delivered slowly. Narcotization ensues almost at once; the proper dosage is judged while the needle is still in the vein. About 75% of the average dose is injected first, and more only when it seems to be necessary. A pinch on the heel will tell whether the depth of anesthesia is sufficient.

Guinea pigs and rats can be handled likewise, but intravenous injection is often abandoned for the simpler intraperitoneal injection of larger doses, a procedure slowing the action of the drug and leaving the needed

[35] L. A. Sapirstein and L. A. Hartman, *Am. J. Physiol.* **196**, 751 (1959).
[36] T. B. Clarkson, R. W. Pritchard, H. B. Lofland, and H. O. Goodman, *Lab. Animal Care* **13**, 768 (1963).

dosage uncertain. Average recommended doses for intraperitoneal injection are, in milligrams per kilogram, guinea pig, 35; rat, 50; mouse, 48 to 60.

e. TRIBROMOETHANOL (AVERTIN)

For very short-term work with small animals (surgical anesthesia of around 15 minutes), tribromoethanol is recommended (reference 4, p. 129). It is purchased as a concentrated solution in amylene hydrate (1 gm/ml). It is diluted freshly on each occasion with 39 parts of saline at 60° to constitute a 2.5% (25 mg/ml) working solution, which is kept in the dark. Recommended doses per kilogram of body weight are: guinea pigs, 8 mg for 250- to 400-gm animals and 10 mg for larger ones; mice of 14 to 22 gm, 5 mg; rats weighing between 30 and 60 gm, 6 mg; up to 100 gm, 7mg; and over 100 gm, 8 mg.

10. EUTHANASIA AND DISPOSAL OF LABORATORY ANIMALS

a. EUTHANASIA

The immunochemist is usually concerned only with relatively small numbers of animals; the suggested disposal methods are directed to his problems. Euthanasia of dogs and cats is considered in detail by D. C. Smith in reference 2c.

To assure death of unconscious animals which have just been "exsanguinated" for serum, some simple life-terminating procedure should be used, such as severing the spine when carotid bleedings have been made (Section D,2,e,iii), establishing pneumothorax by entering the chest cavity, or making an incision in the left ventricle. In the case of heart puncture, when the exsanguinating needle is known to be positioned properly in a ventricle, air or a few milliliters of ether (via an all-glass syringe) can be injected intracardially.

Unwanted small animals can be killed by placing them in a preformed dense atmosphere of chloroform vapor, with use of the wicking device mentioned in connection with first-step anesthetization with ether (Section D,3,f), or placing them in a jar or gastight box in which an atmosphere containing at least 60% CO_2 can be established quickly from a CO_2 gas cylinder. The CO_2 method stuns painlessly but does not assure an early death; animals should be held in the CO_2 atmosphere for at least 20 minutes. Included would be mice, rats, guinea pigs, and hamsters. Alternate procedures with mice are snapping of the spinal column at the neck, crushing of the skull, or—when tissues are wanted as free from blood as possible—decapitation by a mouse guillotine.

Rabbits have long been killed by hunters by suspending the animals by the hind feet and striking the back of their necks smartly and powerfully with the side of the hand. Properly done, the neck is broken at once, but this method should not be used by the inexperienced. Air administered by intravenous injection can not be considered a satisfactory manner of killing. Rabbits succomb rapidly in a dense chloroform-air atmosphere. Narcotization by administering pentobarbital sodium (diabutal, nembutal) intravenously (see Section D,9,d), followed by opening the chest cavity and establishing pneumothorax, is entirely satisfactory. If injection via the marginal vein is difficult owing to scarring, an intrathoracic injection of isotonic pentobarbital can be made, the effect being more rapid than by intraperitoneal injection. The rabbit is raised from the bench top by an assistant, who lifts the chin and forelegs. Entry of the pentobarbital directly into lung tissue is desirable and secures most rapid absorption.

Sheep and goats can be dispatched conveniently by injecting an excess of pentobarbital sodium via the jugular vein.

b. Disposal

i. General

Dead animals should be held in a refrigerator and incinerated within 24 to 48 hours excepting when the tissues are radioactive (see Chap. 11A, Vol. II). Large animals such as sheep and goats should be dissected to allow incineration in association with waste bedding from the animal house.

ii. Infected Stock

Stock possessing transmissible infective agents should be killed in containers that can be sterilized or discarded (small cardboard boxes are applicable at times), and the attendant should place the corpse in a leakproof bag to avoid handling by others prior to incineration. The area chosen should have facilities for the attendant to decontaminate his person immediately. In some instances, full-length surgeons' gowns or disposable gloves should be worn and placed inside a paper bag to allow subsequent sterilization or incineration.

iii. Animals Containing Radioactive Compounds[2c]

Regulations of the Atomic Energy Commission *prohibit disposal by incineration* of radioactive materials, except as specifically approved by the AEC. Local laws are also in force in some areas. A full discussion is given in Chap. 11A, Vol. II.

ADDENDUM 1
Common Physiological Data on Laboratory Vertebrates[a]

Animal (species)	Gestation (days)	Weaning age	Rectal temp.[b] (°C)	Heart rate	Respiration (breaths/ minute)	Tidal volume (ml/breath)	Food consumption (gm/day)	Water consumption (ml/day)	Estrous cycle (days)
Rabbit, *Oryctolagus cuniculus*	28–36 (30)	6–8 weeks	37.5–39.9 (38.8)	123–304 (205)	36–56 (46–53)	19–25 (19)	25–49/kg	♂66–142/kg ♀61–138/kg	No cycle (ovulation postcoitus)
Guinea pig, *Cavia porcellus*	62–72 (68)	20–21 days	37.6–39.4 (38.4)	260–400 (280)	69–104 (90)	1–3.9 (1.8)	30/750 gm	84 (With 30 gm green feed)	16–18 (16)
Mouse, *Mus musculus*	18–21 (19)	18–28 days	35.7–37.9 (36.5)	328–780 (600)	84–230 (163)	0.09–0.23 (0.15)	3–4/20 gm	4.2–6.9	2–9 (4)
Rat, *Rattus norvegicus* (var, *albinus*)	21–23 (22)	18–28 days	37.5–38.1 (35.1)	261–600 (328)	66–114 (85.5)	0.6–1.35 (0.86)	10/100 gm 20/400 gm	13–31 (24–31)	4–6 (4–5)
Hamster, *Mesocricetus auratus*	15–19 (16)	20–22 days	36.1–38.1 (36.0)	300–600 (380–412)	33–127 (74)	0.42–1.2 (0.8)	up to 14 gm	8	4–5 (4)
Monkey, *Macaca mulatta*	146–180 (164–165)	4–6 months	37.2–40.0 (38.3–38.7)	165–240 (192)	31–52 (40)	10–29 (21)	52–210 (116) per 3–8.5 kg; 300 per 9.6 kg♂	171–710 per 3–8.5 kg; 811 per 9.6 kg♂	23–33 (28)
Chicken, *Gallus gallus*	Hatching time, 21 days		40.9–41.9 (41.2)	178–458 (312)	♂12–21 (17) ♀20–37 (28)	(45)	80–100 gm	150–200	
Pigeon, *Columba livia*	Hatching time, 17–19 days		39.9–41.9 (41.1)	141–244 (170)	25–30 (27.5)	4.5–5.2 (4.8)	30 gm	50–60	

[a] Observed normal ranges are shown, average values being given in parentheses; data from "Handbook of Biological Data," (W. S. Spector, ed.) Tables 105, 248, 311. W. B. Saunders, Philadelphia, 1956; "Handbook of Respiration," (D. S. Dittmar and R. M. Grebe, eds.) Table 41, W. B. Saunders, Philadelphia, 1958; references 17b, 30a, 30b, 31; A. Shaffer *in* "Methods of Animal Experimentation," (W. I. Gay, ed.) Vol. 1. Academic Press, New York, 1965; food consumption from Hoeltge Brothers, Inc., Cincinnati, Ohio.

[b] For Fahrenheit temperatures, construct a Centigrade-Fahrenheit graph, connecting by straight line the fixed points 35°C = 95°F and 40°C = 104°F.

ADDENDUM 2

Certain sections of Public Health Law 89-544 of August, 1966, are quoted here from the point of view of research institutions and laboratory workers; certain interpolations for clarity have been added within brackets. The primary objective deals with the procurement of cats and dogs. The law compels the user to procure *cats* and *dogs* from licensed dealers*; individual identification of animals of these species is required, and records of acquisition and previous ownership must be maintained see *Federal Register* **31**, No. 242 (1966); **32**, Part II, No. 37 (1967).

Section 13. The Secretary [of Agriculture] shall establish and promulgate standards to govern the humane handling, care, treatment, and transportation of animals [primates, dogs, cats, rabbits, guinea pigs, hamsters, but not mice] by dealers and research facilities. Such standards shall include minimum requirements with respect to the housing, feeding, watering, sanitation, ventilation, shelter from extremes of weather and temperature, separation by species, and adequate veterinary care. The foregoing shall not be construed as authorizing the Secretary to prescribe standards for the handling, care, or treatment of animals during actual research or experimentation by a research facility as determined by such research facility.

Section 16 (in part only). . . . The Secretary shall promulgate such rules and regulations as he deems necessary to permit inspectors [U.S. Department of Agriculture employees] to confiscate or destroy in a humane manner any animals found to be suffering as a result of a failure to comply with any provision of this Act of any regulation issued thereunder if . . . (2) such animals are held by a research facility and are no longer required by such research facility to carry out the research, test, or experiment for which such animals have been utilized.

Section 18. Nothing in this Act shall be construed as authorizing the Secretary to promulgate rules, regulations, or orders for the handling, care, treatment, or inspection of animals during actual research or experimentation by a research facility as determined by such research facility.

Section 6. Every research facility shall register with the Secretary [of Agriculture] in accordance with such rules and regulations as he may prescribe.

Section 9 (in part). . . . the act, omission, or failure of any individual acting for or employed by a research facility . . . shall be deemed the act, omission, or failure of such research facility . . . as well as of such individual.

* Technically speaking, direct purchases are permissible from unlicensed persons who breed cats or dogs as a sideline, as are acquisitions from municipal dog and cat pounds.

CHAPTER 3

Purification of Antibody

A. Preparation of Immunoglobin

1. NOMENCLATURE OF THE IMMUNOGLOBULINS*

Scientific advances often tend first to complicate and then to simplify a particular field. The immunoglobulin field is now in a self-accelerating complication phase and has recently been especially confusing because of conflicting and ever-changing terminologies which have hindered precise communication. The following summary should be studied with the realization that the immunoglobulins fall into several classes, each of which is functionally and structurally heterogeneous. In addition, owing largely to advances in structural and antigenic analysis, there is at the moment no hope in sight for much simplification.

The terminology outlined and discussed below adheres to the guides established by a committee convened by the World Health Organization (W.H.O.) in 1963 and published by that body in 1964.[1] It is strongly urged that all workers in the field consult this reference as well as their colleagues before introducing new terms or systems of terminology. It is especially to be hoped that investigators concerned with the genetic control of immunoglobulin structure and the mode of inheritance of antigenic variants will devise symbols which are not inconsistent with the W.H.O. guidelines. More difficult perhaps, but equally important to our future understanding, will be systems capable of imparting some comprehension of the similarities of genetic control which must exist among the different species of animals under investigation.

a. Classes of Immunoglobulins

The immunoglobulins, as currently defined, consist of at least four distinct classes of proteins with known antibody activity. Each of these four classes, in turn, consists of an indeterminate number of proteins which share certain structural, physicochemical, and antigenic properties but which also have individually specific properties presumably related

* Section 3,A,1 was contributed by H. Hugh Fudenberg.

[1] *Bull. World Health Organ.* **30**, 447 (1964).

to their functional (antibody), structural (antigenic), and genetic (allotypic) differences.

Much of the present knowledge of these characteristics has emerged from the study of monoclonal gammopathies—for example, multiple myeloma, in which the cells that normally produce immunoglobulins elaborate large quantities of proteins related to one of the four normal immunoglobulins in chemical structure and antigenic specificity. Current definitions of the immunoglobulins accordingly include within their scope the paraproteins of multiple myeloma and Waldenström's macroglobulinemia, although they are apparently devoid of any known antibody activity.

TABLE I
W.H.O. NOMENCLATURE FOR IMMUNOGLOBULIN CLASSES

Recommended standard usage[a]	Previous usage	Normal serum concentration[b] (mg/ml)	Sedimentation rate
γG or IgG	7 S γ, γ_2	12 ± 3.0	6.5–7 S
γA or IgA	β_2A, γ_1A	2 ± 0.6	7–17 S[c]
γM or IgM	γ_1M, β_2M, 19 S γ	1 ± 0.25	17–20 S
γD or IgD		0.1–0.3	7 S

[a] The Greek letter notations should not be employed by themselves but as modifiers for "immunoglobulin"—γG-immunoglobulin or γA-immunoglobulin.

[b] Levels vary according to methods used and according to laboratory. The values listed here were obtained by the radial diffusion method with monospecific antiserums (R. Stiehm and H. H. Fudenberg, *Pediatrics* **37**, 715 (1966).

[c] J. P. Vaerman *et al.*, *Immunochemistry* **2**, 263 (1965).

The recognition of these immunoglobulin classes and recent advances in the biological and chemical investigations of their properties led to the adoption of a standard terminology based in part on the identification of the polypeptide chains that make up the antibody molecules. The investigations of Edelman and Poulik[2] and of Porter *et al.*[3] strongly suggest that the γG-immunoglobulin of various species (molecular weight 150,000 to 160,000) consists of two pairs of polypeptide chains held together by disulfide bonds. Mercaptoethanol reduction of γG-immunoglobulin in neutral aqueous solution, followed either by dialysis against propionic acid or by treatment with urea or guanidine, dissociates the two types of chains; these can be separated and recovered on Sephadex columns. The methods employed for such structural analy-

[2] G. M. Edelman and M. D. Poulik, *J. Exptl. Med.* **113**, 861 (1961).

[3] J. B. Fleischman, R. H. Pain, and R. R. Porter, *Arch. Biochem. Biophys. Suppl.* **1**, 174 (1962).

ses are described in Chap. 5, and the figure on p. 407 will assist in an understanding of the notations suggested by the World Health Organization Committee on Nomenclature of Human Immunoglobulins.[1] These recommended designations, along with previous usage, are given in Tables I and II.

TABLE II
NOMENCLATURE FOR POLYPEPTIDES OF IMMUNOGLOBULINS

Polypeptide chain	Structural type		Antigenic type		Molecular weight
	Recommended usage	Previous usage	Recommended usage	Previous usage	
Light chains	κ	I	K	I,B	22,000
	λ	II	L	II,A	22,000
Heavy chains from:					
γG-immunoglobulin	γ	$H^{\gamma G}$		See Table IV	55,000
γA-immunoglobulin	α	$H^{\gamma A}$			?
γM-immunoglobulin	μ	$H^{\gamma M}$			70,000
γD-immunoglobulin	δ	$H^{\gamma H}$			?

Two types of light chains, κ and λ, have been identified in human immunoglobulins. These have corresponding antigenic types designated K and L. Both types of light chains occur in all immunoglobulins, and, insofar as can be ascertained with currently available methods, they are generically similar, if not identical, in all four classes of immunoglobulins. In contrast, the heavy chains of each of the four classes differ considerably in their biochemical and antigenic structure. Accordingly, different symbols have been applied to the different heavy chains unique to each class. In the W.H.O. terminology the Greek letters γ, α, μ, and δ (Table II), corresponding to the Roman capital letters G, A, M, and D identifying the immunoglobulin classes, designate the respective heavy chains of the different proteins.

A few intact myeloma proteins (usually γA-immunoglobulin) give no precipitate in diffusion reactions in agar with certain anti-K or anti-L antisera. Separated from the heavy chains, however, some or all of the light chains of these unusual proteins can be typed as K or L. Nevertheless, there is a possibility that additional antigenic types will be discovered. In the interest of a consistent nomenclature, a new light chain must obviously not be called a μ chain (in the κ, λ, μ sequence) with the antigenic type M, in view of previous designation of μ for the heavy chains of γM-immunoglobulin.

b. Proteolytic Fragments

Papain hydrolysis cleaves some peptide bonds of the heavy chains, and subsequent reduction of a single disulfide bond[4] between the residual heavy chains results in the formation of three fragments from each molecule of γG-immunoglobulin.[5] Two of these fragments contain one each of the *a*ntigen-*b*inding sites of the parent molecule and are called the Fab fragments. The third fragment, called Fc, is *c*rystallizable but lacks antigen-binding activity. The Fc fragment, however, is responsible for certain biological properties of the parent molecule (for example, complement fixation, fixation to skin tissue, and ability to cross the placenta and other membranes). Each Fab fragment consists of one light chain and the N-terminal portion of a heavy chain. Reduction and alkylation of the Fab fragment separates the light-chain from the heavy-chain fragment. The latter is termed the Fd fragment. Table III lists current

TABLE III
Nomenclature for Fragments Produced by Digestion with Papain

Recommended standard usage	Previous usage[a]
Fab fragment (antigen-binding)	A, C, S (I, II)
Fc fragment (crystallizable)	B, F (III)
Fd fragment[b]	A piece

[a] The terms in parentheses are used in referring to fragments of rabbit γG-globulin.

[b] See text for explanation.

and earlier terminologies, and Fig. 1 in Chap. 5 illustrates the formation of these fragments.

Peptic digestion of the γG-immunoglobulin at pH 4.5 to 5.0, followed by mild reduction, produces a fragment with bivalent antibody activity and a sedimentation rate of 5 S.[6] No official name has been applied to this fragment, but it can be cleaved by the reduction of a single disulfide bond into two fragments, closely resembling the Fab fragment. On further reduction these fragments also yield one light chain and the N-terminal portion of a heavy chain similar to the Fd fragment. It has been suggested that the fragments derived from the 5 S peptic hydrolysis product be termed Fab′ and Fd′, but there is as yet no official designation.

[4] A. Nisonoff and D. J. Dixon, *Biochemistry* **3**, 1338 (1964).
[5] R. R. Porter, *Biochem. J.* **73**, 119 (1959).
[6] A. Nisonoff, F. C. Wissler, and L. N. Lipman, *Science* **132**, 1770 (1960).

As additional fragments of conceptual importance are produced, suitably consistent designations will become necessary. For example, mild reduction of γG-immunoglobulin followed by acidification yields 4.5 S "half-molecules" with univalent antibody activity which consist of one light chain and one heavy chain.[7] Another interesting fragment, an intermediate of mild papain hydrolysis, is composed of one Fab fragment and one Fc fragment, presumably held together by one uncleaved heavy chain.[8]

It is hoped that authors, in describing the products of their research into the structure and activity of the immunoglobulins, will not feel constrained to defend too vigorously their working appellations when and if they are modified to reflect a reasonable system of nomenclature.

c. Intraclass Heterogeneity

i. Antigenic Heterogeneity of Heavy Chains

Human γG-immunoglobulin molecules can be divided into subclasses on the basis of antigenic differences located on the heavy chain. This is made possible by monkey antiserum to γG-immunoglobulin or rabbit antisera to selected myeloma proteins. These subclasses differ both in genetic determinants and in biological properties. Two systems of terminology have thus far been used in designating these variants,[9,10] and a third system is under consideration.[11] Similarly, at least two subclasses of γG-immunoglobulins identifiable by antigenic differences among heavy chains exist in the guinea pig[12] and in the mouse[13]; these are generally termed γG_1 and γG_2-immunoglobulins, but other terminologies are also used. Three antigenically distinct γG-antibodies were recently identified in preparations of purified horse antihapten antibody.[14]

There is an obvious need for a rational nomenclature for antigenic subgrouping of heavy-chain variants, even if it cannot be uniform in every case for the immunoglobulins of different species. It may also become necessary that such a system include a symbol for the animal producing the typing serum. Table IV lists the four known subclasses

[7] J. L. Palmer, A. Nisonoff, and K. E. Van Holde, *Proc. Natl. Acad. Sci. U.S.* **50**, 314 (1963).
[8] C. A. Nelson, *J. Biol. Chem.* **239**, 3727 (1964).
[9] W. D. Terry and J. L. Fahey, *Science* **146**, 400 (1964).
[10] H. M. Grey and H. G. Kunkel, *J. Exptl. Med.* **120**, 253 (1964).
[11] R. Ceppellini *et al.*, *Bull. World Health Organ.* **33**, 721 (1965).
[12] B. Benacerraf, Z. Ovary, K. J. Bloch, and E. C. Franklin, *J. Exptl. Med.* **117**, 937 (1963).
[13] J. L. Fahey, J. Wunderlich, and R. Mishell, *J. Exptl. Med.* **120**, 243 (1964).
[14] J. H. Rockey, N. R. Klinman, and F. Karush, *J. Exptl. Med.* **120**, 589 (1964).

TABLE IV

CURRENT TERMINOLOGY FOR ANTIGENIC VARIANTS OF THE HEAVY CHAINS OF HUMAN γG-IMMUNOGLOBULINS

Subclasses of γG-immunoglobulin[a]	Associated Gm factors identified[b]	Percent incidence among γG-myeloma proteins[c]
γ_{2a} or Ne	None	11
γ_{2b} or We	a, f, x	77
γ_{2c} or Vi	b^1, b^3, b^4, c, g	9
γ_{2d} or Ge	None	3

[a] Although subclasses were originally established by antigenic differences, it is now clear that there are certain biological and chemical differences among the different subclasses. See W. D. Terry and J. L. Fahey, *Science* **146**, 400 (1964); H. M. Grey and H. G. Kunkel, *J. Exptl. Med.* **120**, 253 (1964).

[b] Although the Gm factors listed appear specific to a given subclass, the Gm designations are not synonymous with the subclass designations. Also, individual immunoglobulins in a given subclass may be positive or negative for one or more Gm factors. See L. Mårtensson and H. G. Kunkel, *J. Exptl. Med.* **122**, 799 (1965); W. D. Terry, J. L. Fahey, and A. G. Steinberg, *ibid.* **122**, 1087 (1965).

[c] Data supplied by Drs. W. D. Terry and J. L. Fahey from study of 274 myeloma proteins. Precise data on the relative amounts of the different subclasses of γG-immunoglobulins in normal individuals are not available, but according to Dr. H G. Kunkel they approximate the incidence distribution among myeloma proteins.

of antigenic variants identified by two different systems in current use. Genetic variants among these, the Gm factors,[15,16] are also listed.

It should be emphasized that normal serum contains a mixture of immunoglobulin molecules of different antigenic types. It appears that each antibody molecule contains two light chains of the same antigenic type and two heavy chains of the same antigenic subclass. However, two light chains of one antigenic type may combine with two heavy chains of any single subclass *in vitro,* and appear to do so *in vivo;* hence, both K and L light chains are represented in normal serum in combination with all classes and subclasses of heavy chains. The paraproteins of multiple myeloma and Waldenström's macroglobulinemia, on the other hand, are antigencially homogeneous; each such paraprotein contains light chains of only one antigenic type and heavy chains of only one antigenic subclass.[17] Similarly, normal urine contains both K and L light chains in trace amounts. In contrast, a urinary Bence-Jones protein of any given patient with multiple myeloma contains either K or L light chains, but not both.

[15] L. Mårtensson and H. G. Kunkel, *J. Exptl. Med.* **122**, 799 (1965).

[16] W. D. Terry, J. L. Fahey, and A. G. Steinberg, *J. Exptl. Med.* **122**, 1087 (1965).

[17] H. H. Fudenberg, *Ann. Rev. Microbiol.* **19**, 285 (1965).

ii. Notations for the Factors of the Gm and Inv Systems of Human Immunoglobulin

The genetically determined serological factors (allotypic specificities) on immunoglobulin molecules have been given a variety of names. At the present time, accumulated information is insufficient to permit precise correlation of the genetic loci for the genes responsible for elaboration of these serological factors with the structures they influence. Consequently, at least with human immunoglobulins, it seems advisable at this time to retain the present symbols, Gm and Inv, to designate at least two distinct genetic loci governing serological factors on the heavy chains and light chains, respectively. The serological factors produced by genes at these loci are complex, and population studies are disclosing an ever-increasing complexity.

To avoid these complexities and the implication of possibly false relationships in the identification of the serological factors, a flexible numerical notation, similar to that suggested for the *Salmonella* antigens, Rh blood groups, and histocompatibility antigens in the mouse, has recently been recommended.[11] The numbers assigned to each of the already known Gm and Inv factors (Table V) have been based in part on the order

TABLE V
NOTATIONS FOR THE GM FACTORS

Original	Suggested[a]
a	1
x	2
b^2	3
f	4
b and b^1	5
c	6
r	7
e	8
p	9
b^α	10
b^β	11
b^γ	12
b^3	13
b^4	14
NOTATIONS FOR THE INV FACTORS	
1	1
a	2
b	3

[a] R. Ceppellini *et al.*, *Bull. World Health Organ.* **33**, 721 (1965).

of their discovery and in part on the known relationships of the Gm factors to the other subgroups of the heavy chains of γG-immunoglobulin listed in Table IV.

For recording the phenotype of an individual, it is recommended that results obtained with each typing system—for example, Gm (1, —2, 3, 4, —5), Inv (1, —2, 3)—be recorded. Alternatively, only the positive results may be listed—Gm (1, 3, 4), Inv (1, 3). In this case, however, negative results cannot be ascertained from the phenotype recorded without a list of typing systems utilized.*

iii. Allotypic Determinants in Other Species

For allotypic specificities of rabbit immunoglobulin, the "shorthand" nomenclature listed in Table VI has been generally employed in recent

TABLE VI
RABBIT ALLOTYPES

Official designation	"Shorthand" designation	Polypeptide chain
Aa1	a1	Heavy chain, Fd fragment
Aa2	a2	
Aa3	a3	
Ab4	b4	Light chain
Ab5	b5	
Ab6	b6	
Aa7	a7	Heavy chain, Fc fragment
Aa8	a8	

years, particularly in oral presentations. The two loci, officially designated Aa and Ab, determine allotypic specificities on the heavy and light chains, respectively, and each controls the elaboration of at least three allotypic determinants. This terminology has been satisfactory to date, but recent findings by Hamers, Hamers-Casterman, and Lagnaux[18] might complicate the terminology. The a1, a2, and a3 specificities are present in the Fd fragment of the heavy chains, but two new allotypic specificities, a7 and a8, found by Hamers, are associated with the Fc fragment of the heavy chains. The genes determining a7 and a8, if not at

* It should be noted that, in keeping with the standard genetic convention, symbols for alleles or genotypes are underlined when typed, and italicized when printed (*Gm*[1], *Gm*[4], etc.)

[18] R. Hamers, C. Hamers-Casterman, and S. Lagnaux, *Immunology* **10**, 399 (1966).

the Aa locus, appear closely linked genetically to the genes determining a1, a2, and a3.

Allotypic specificities for the heavy chains of mouse γG_1-, mouse γG_2-, and mouse γA-immunoglobulin have also been delineated in the past few years. However, the nomenclature of the mouse allotypes is especially difficult at present because of the rapid proliferation of many numbers and symbols for identical loci (for example, Asa, Ig-1, Mu A). Since it appears that international agreement on a uniform nomenclature for mouse immunoglobulin allotypes may soon be reached, listing of current notations at this time seems unwarranted.

2. PREPARATION OF IMMUNOGLOBULIN CONCENTRATES*

a. Introduction

Proteins with the physiochemical and serological properties of immunoglobulins have been studied extensively in recent years. They have proved to consist of a rather heterogeneous group of molecules as regards charge, mass, and biological activities. Their separation into discrete physico-chemical entities poses rather demanding problems in purification, currently under active investigation in various laboratories.

According to the interests of the investigator, emphasis may be directed to the immunoglobulins present in nonimmune or so-called "normal" serum, or to globulins from the sera of immunized animals, or to immunoglobulin-type molecules present in high concentrations in the sera of human beings exhibiting certain pathological conditions, notably multiple myeloma and Waldenström's macroglobulinemia. The immunoglobulins of different species may show quite divergent physicochemical properties, and thus the source of the material must enter into consideration. The conditions pertinent to the separation of globulin concentrates are discussed with reference to those animals that are most often used in immunological investigations.

Prior to the successful separation of any specific immunoglobulin, a preliminary fractionation procedure is usually made to prepare it in high yield and concentration.

The immunoglobulins represent the most basic globulins of the serum. Their solubilities, coupled with their relatively high isoelectric points, form the basis for most separations. Investigators who are concerned only with the separation of serum immunoglobulins may not wish to use the rather complicated and extensive fractionation conditions devel-

* Section 3,A,2 was contributed by Harold F. Deutsch.

oped by Cohn *et al.*[1] for the isolation of a series of plasma proteins, including γ-globulins. The present discussion will be directed only to the separation in good yield of immunoglobulin concentrates by relatively rapid and simple procedures. Three general methods may be employed: ethanol fractionation, precipitation by fairly high concentration of neutral salts, and the formation of insoluble protein complexes. They will be illustrated for animal species for which they are particularly suited.

b. Ethanol Fractionation Techniques

Concentrates of γG-immunoglobulins and of mixtures of γA- and γM-immunoglobulins* may be prepared as follows: Serum is diluted with 3 volumes of distilled water, and the pH is adjusted to 7.7 (±0.1). This solution is cooled to 0°, and sufficient precooled 50% ethanol (approximately −20°) is slowly added with vigorous stirring to give a final concentration of 20%. As the ethanol is added, the temperature is lowered and kept near the freezing point of the aqueous-ethanol mixture until −5° to −6° is reached. The insoluble proteins to be designated as precipitate A are removed by centrifugation at this temperature. This precipitation step serves to remove the major portions of the immunoglobulins of most animal species along with small amounts of other serum proteins.

Precipitate A is suspended in cold 0.015 to 0.02 *M* NaCl (containing ice), and the pH is lowered to about 5.1 by slow addition of cold 0.05 *M* acetic acid. The temperature should be kept slightly below 0°. The precise conditions vary for different animal sera, but the object is to leave the major portions of the γG-immunoglobulins in the supernatant while precipitating the γA- and γM-immunoglobulins (precipitate B). Table I lists conditions suitable for a variety of species.

The supernatant of precipitate B is raised slowly to about pH 7.4 by the addition of a cold 0.5 *M* Na_2HPO_4 or 0.5 *M* $NaHCO_3$–Na_2CO_3 in equimolar mixture. Precooled 50% (−20°) or 95% (−20° to −30°) ethanol is added to a concentration of 25%, the temperature being maintained near the freezing point of the mixture until −5° to −6° is attained. The 95% ethanol is generally used for those supernatants already containing 10 to 20% ethanol. The precipitated γG-immunoglobulins (precipitate C) are then removed by centrifugation at −6° and either

[1] E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor, *J. Am. Chem. Soc.* **68**, 459 (1946).

* The γG-immunoglobulins formerly were designated as γ_2-globulins. Mixtures of γA- and γM-immunoglobulins were formerly termed γ_1- or β_2-globulins, the 6 to 7 S portion of which was designated as γ_1A- or β_2A-globulins, and the 18 to 19 S portion as γ_1M or β_2M-globulins. See Section, A,1.

TABLE I

CONDITIONS FOR SEPARATING THE MAJOR PORTIONS OF γG- FROM γA- AND γM-IMMUNO GLOBULINS IN PRECIPITATE A FOR VARIOUS SPECIES

Animal	Precipitation condition			Yields,[a] γG-globulin	Ref.
	pH	Ethanol (%)	Ionic strength		
Man	5.1	15	0.01	65	(*b*, *c*)
Goat	5.2	0	0.01	65	(*c*)
Rabbit	5.2	10	0.01	70	(*c*)
Rat	5.0	10	0.01	50	(*c*)
Dog	5.2	10	0.005	55	(*c*)
Guinea pig	5.1	15	0.01	70	(*c*)
Cow	5.8	10	0.01	35	(*d*, *e*)
Swine	5.6	15	0.005	35	(*f*)
Horse	5.8	20	0.005	20	(*g*)
Chicken	5.1	15	0.01	30	(*h*)

[a] Yields of γG-immunoglobulins based on the original serum content. The relatively low yields for cow, swine, horse, and chicken are due to their immunoglobulins' being an electrophoretically heterogenous group of 7 S molecules. These have been arbitrarily separated into a slower (formerly called γ_2-globulins) and a faster (formerly called γ_1-globulins) migrating fraction.

[b] H. F. Deutsch, L. J. Gosting, R. A. Alberty, and J. W. Williams, *J. Biol. Chem.* **164**, 109 (1946).

[c] J. C. Nichol and H. F. Deutsch, *J. Am. Chem. Soc.* **70**, 80 (1948).

[d] E. L. Hess and H. F. Deutsch, *J. Am. Chem. Soc.* **70**, 84 (1948).

[e] E. L. Hess and H. F. Deutsch, *J. Am. Chem. Soc.* **71**, 1376 (1949).

[f] P. S. Cammarata and H. F. Deutsch, *Arch. Biochem.* **25**, 354 (1950).

[g] H. F. Deutsch and J. C. Nichol, *J. Biol. Chem.* **176**, 797 (1948).

[h] H. F. Deutsch, J. C. Nichol, and M. Cohn, *J. Immunol.* **63**, 195 (1949).

reconstituted into suitable solution or recovered by lyophilization. Whichever method is employed, low temperatures must be maintained until the ethanol has been removed. The globulin concentrate isolated usually consists of 90 to 98% γG-immunoglobulins and may be further purified by various chromatographic procedures (see Section A,5).

Precipitate B contains the major portions of the γA- and γM-immunoglobulins as well as certain nonimmunoglobulin proteins, notably β-lipoproteins. The recovery of the immunoglobulins from this precipitate is carried out as follows: The precipitate is suspended in distilled water at 0°, and the temperature is maintained. The pH is adjusted to 5.1, and the insoluble proteins (precipitate B-A) are removed by centrifugation and discarded. The pH of the supernatant is adjusted to 5.5, and the ionic strength to 0.01 to 0.0075. Precooled 50% ethanol is added to a concentration of 10%, the temperature being lowered to —2° to

—3° during the addition. The insoluble protein (precipitate B-B) contains the major portions of the serum γA- and γM-immunoglobulins. The 7 S and 19 S immunoglobulins in this precipitate may be separated by the application of a combination of techniques, the details of which are given in Section A,5. Moving-boundary electrophoretic patterns of

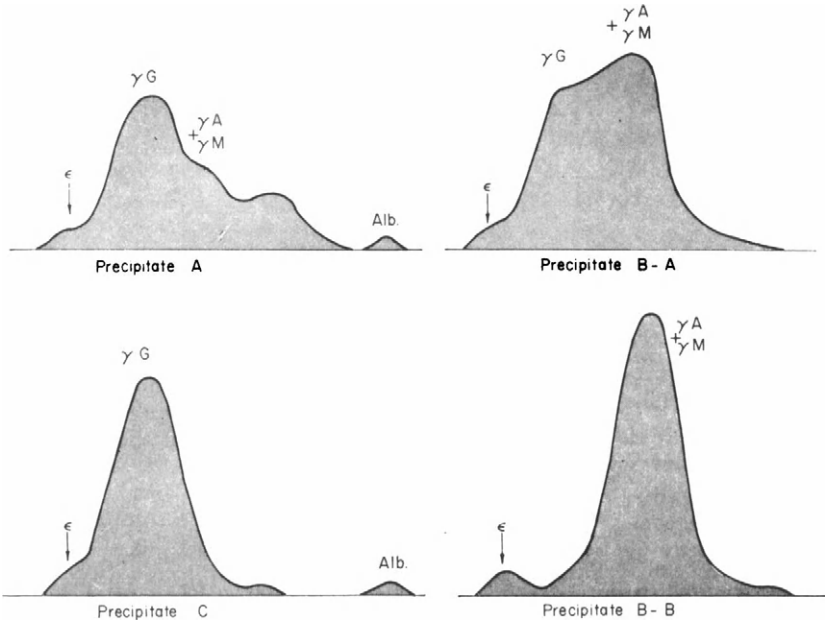

FIG. 1. Electrophoretic patterns of some crude immunoglobulin fractions of human serum separated by ethanol fractionation.

the γ-globulin concentrates separated from human serum by these methods are shown in Fig. 1.

c. FRACTIONATION WITH NEUTRAL SALTS

i. General Considerations

Salting-out procedures have certain advantages over ethanol fractionation for preliminary concentration and partial purification of immunoglobulins. Among these are simplicity and relatively slight danger of denaturation. In addition, high concentrations of salt in reconstituted precipitates may be rapidly removed by gel filtration techniques (see Chap. 9,B, Vol. II), whereas prolonged dialysis was formerly required. For the fractionation of serum from some species, notably chicken serum, precipitation with neutral salt is superior to ethanol purification on the basis of yield and purity. The procedures outlined below are type ex-

amples which may require some modification for serum of species other than that for which the method was designed.

In general it is preferable to work with proteins at cold-room temperatures and with preservatives to inhibit enzyme activity and the growth of microorganisms, some of which fluorish at remarkably high concentrations of salt. Whatever the temperature of the reaction, however, the salt concentration and the protein concentration determine the speed and completeness of the precipitation. It has in the past been customary to cite ammonium sulfate concentrations in percentage of saturation of the solution, but since this indication will vary with temperature it would seem preferable to cite molar concentrations. Saturated $(NH_4)_2SO_4$ at 0° is about 5.35 *M* and at room temperature about 5.75 *M*, or 700 gm/liter and 760 gm/liter, respectively. Concentrations of Na_2SO_4 in salting-out procedures have usually been given in percent w/v; thus confusion as to temperature–concentration effects does not come about in the same manner. At 5°, however, 18% Na_2SO_4 is a supersaturated solution and exceedingly unstable, tending to crystallize on the slightest jarring. It is therefore advisable to work at room temperature with this salt, and to add a drop of toluene to the batch to inhibite microbial growth, particularly if it is to stand for a long period. The latter decision is one of convenience, since the precipitate will settle, permitting removal of a large portion of the supernatant liquor and thus economizing on the subsequent centrifugation.

ii. $(NH_4)_2SO_4$ Precipitation of Human γG-Immunoglobulin[2]

This method is generally applicable to the sera of other animal species. Fifty milliliters of saturated $(NH_4)_2SO_4$ solution is added to 100 ml of serum. The precipitated proteins are removed by centrifugation and dissolved in 50 ml of water. To this solution is added 25 ml of saturated $(NH_4)_2SO_4$, and the precipitate is removed by centrifugation. This second precipitation step is repeated four or five times. The final precipitate is taken up in the desired buffer, and the residual ammonium sulfate is removed either by dialysis or by passage over a column of Sephadex G-25, G-50, or G-100. This concentrate can also be further purified by chromatography on DEAE-cellulose.

iii. Na_2SO_4 Precipitation of Human γG-Immunoglobulins[3]

To each 100 ml of serum that has dialyzed against 0.1 ionic strength phosphate buffer, pH 8, is added 18 gm of Na_2SO_4. (The serum may also be diluted with an equal volume of 0.2 ionic strength buffer prior

[2] F. E. Kendall, *Cold Spring Harbor Symp. Quant. Biol.* **6**, 376 (1938).
[3] R. A. Kekwick, *Biochem. J.* **34**, 1248 (1940).

to addition of Na_2SO_4.) The precipitate is dissolved and made up to 40% of the original serum volume in the pH 8 phosphate buffer, the solution is clarified by centrifugation, and 12 gm of Na_2SO_4 is added to each 100 ml of this solution. The precipitate formed is dissolved and made up of 20% of the original serum volume in the pH 8 phosphate buffer, the solution is again clarified by centrifugation, and 12 gm of Na_2SO_4 is added per each 100 ml of supernatant. The precipitated proteins are fairly pure γG-immunoglobulins which can be further purified by chromatography on DEAE-cellulose. The above method may be used to separate γG-immunoglobulins from other mammalian sera.

iv. Na_2SO_4 Precipitation of Chicken γ-Globulins

Chicken γ-globulins differ considerably from those of mammals in their physicochemical properties.[4] Their separation by ethanol fractionation conditions is quite involved and gives poor yields, whereas with Na_2SO_4 good results are obtained.[5] To 100 ml of chicken serum is added 100 ml of 34% Na_2SO_4 (w/v). The precipitate is suspended and washed twice with 100 ml of 17% (w/v) of Na_2SO_4 solution. The precipitate is dissolved in *M*/15 Tris–HCl buffer, pH 8, and reprecipitated by adding 15 gm of Na_2SO_4 per 100 ml of solution. The resultant precipitate is again twice washed by suspension and centrifugation in 100 ml of 15% (w/v) of Na_2SO_4 solution. The precipitate is then dissolved in the pH 8 Tris buffer and precipitated at a concentration of 15% Na_2SO_4 (w/v); the precipitate is washed twice with 15% Na_2SO_4 solution and then reconstituted into the desired solution. The precipitate is largely γ-globulins, with purity, yield of product, and ease of preparation much more favorable than with ethanol fractionation. Various chromatographic and/or gel filtration procedures may be employed to effect further purification.

d. Use of Complexing Precipitants

The γG-immunoglobulins have relatively high isoelectric points. It is possible, therefore, to form insoluble cation complexes with most of the other serum proteins at pH levels where the γG-immunoglobulins do not form such complexes. When plasma or serum is mixed with 3 volumes of 0.4% Rivanol (2-ethoxy-6,9-diaminoacridine lactate) and the pH is adjusted to 7.6 to 7.8, a precipitate forms which contains most of the non-γG proteins.[6] The supernatant to the precipitate is clarified by various means (Norit SX 30, Supercel, etc.), and the supernatant immunoglobulins may be precipitated by the addition of ethanol to 25%

[4] H. F. Deutsch, J. C. Nichol, and M. Cohn, *J. Immunol.* **63**, 195 (1949).
[5] E. Orlans, M. E. Rose, and J. Marrack, *Immunology* 4, 262 (1961).
[6] J. Horejsi and R. Smetana, *Acta Med. Scand.* **155**, 65 (1956).

concentration (temperature to —6°) or by the addition of 20 gm of Na_2SO_4 per 100 ml of solution. The concentrate, which consists of 80 to 98% γ-globulins, may be further purified by DEAE-cellulose chromatography.

Other procedures employing aluminum and zinc[7] salts and polyphosphates[8] have been used to obtain concentrates of γG-immunoglobulins. These methods depend in large measure on the formation of insoluble metal salts of either the γ-globulins or the other plasma proteins. An example of the latter is based on the work of Lewin and Steinbuch[7]:

To citrated plasma adjusted to pH 7.2 and 0°, an equal volume of 0.1 *M* $AlCl_3$ solution is added with thorough mixing. The mixture is adjusted to pH 4.7, and after 3 to 4 hours of stirring the insoluble proteins are removed by centrifugation. The γG-immunoglobulins in the supernatant fluid are recovered by precipitation at a concentration of 25% ethanol, temperature —6°. From 75 to 90% of the γG-immunoglobulins may be obtained in purity approaching 95%. This concentrate can be further purified by chromatographic methods.

The methods described or alluded to above generally do not give highly purified preparations of immunoglobulins. They do, however, lend themselves more readily to the fractionation of relatively larger volumes of serum than the chromatographic ones and also provide concentrates of γ-globulins that serve better than whole serum for purification by chromatography (Section A,3) gel filtration (Chap. 9, Vol. II), gradient centrifugation (Chap. 7, Vol. II) or electrophoresis (Chap. 6,D, Vol. II).

[7] J. Lewin and M. Steinbuch, *in* "Les Gamma Globulines et Le Medicine des Enfants," p. 87. Masson, Paris 1955.

[8] R. B. Pennell, in "The Plasma Proteins" (F. W. Putnam, ed.), Vol. 1, Academic Press, New York, London, 1960.

3. CHROMATOGRAPHIC SEPARATION OF IMMUNOGLOBULINS*

a. Introduction

i. Principle

Ion exchange chromatography, introduced in 1956 by Peterson and Sober[1] and by Sober *et al.*,[2] has proved useful for fractionation of anti-

* Section 3,A,3 was contributed by John L. Fahey.

[1] E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.* 78, 751 (1956).

[2] H. A. Sober, F. J. Gutter, M. M. Wyckoff, and E. A. Peterson, *J. Am. Chem. Soc.* 78, 756 (1956).

bodies and purification of immunoglobulins. For the purpose of ion exchange chromatography of protein mixtures such as serum, the insoluble adsorbent is commonly packed into a column, and buffer conditions are adjusted so that adsorbent and soluble proteins have opposite charges. The proteins become fixed to the adsorbent through electrostatic bonds. Proteins may be eluted sequentially from the adsorbent by changing the buffer environment—that is, by raising the ionic strength (salt content) or/and by changing the pH (hydrogen ion concentration) toward the isoelectric point of the protein.

The distribution of proteins in a chromatogram effluent is determined, in part, by the same factor that determines electrophoretic mobility—net charge density. The size of a molecule also plays a part by helping to determine the number of bonds between protein and adsorbent.

ii. Adsorbents

Adsorbents may be either cation exchangers or anion exchangers. Many ion exchangers are available. Those linked to insoluble cellulose fibers or to particles of cross-linked dextran (Sephadex) seem to be best suited to protein separation. Most studies have utilized diethylaminoethyl-(DEAE)-cellulose, an anion exchanger; therefore, techniques utilizing this adsorbent will be described first.

iii. Serum Immunoglobulins

Human immunoglobulins exist in four discrete classes, discussed in Section A,1: γG-, γA-, γM-, and γD-immunoglobulins. These classes differ on the basis of properties of the heavy polypeptide chains in the molecules and may be identified by specific antigenic determinants. The relative distribution of these immunoglobulin classes on DEAE-cellulose chromatography of normal human serum is seen in Fig. 1.

b. DEAE-Cellulose Chromatography

i. Technique

A description of procedures for carrying out substituted cellulose chromatography of proteins is presented in Chap. 9,C, Vol. II and elsewhere by Peterson and Sober.[3] The reader is referred to these publications for information concerning equipment, buffers, washing of adsorbent, packing of columns, preparation and application of sample, and procedures for eluting protein and for collecting, concentrating, and measuring protein distribution in the effluent. Two fractionation procedures are

[3] E. A. Peterson and H. A. Sober, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. V, pp. 3–27. Academic Press, New York, 1962.

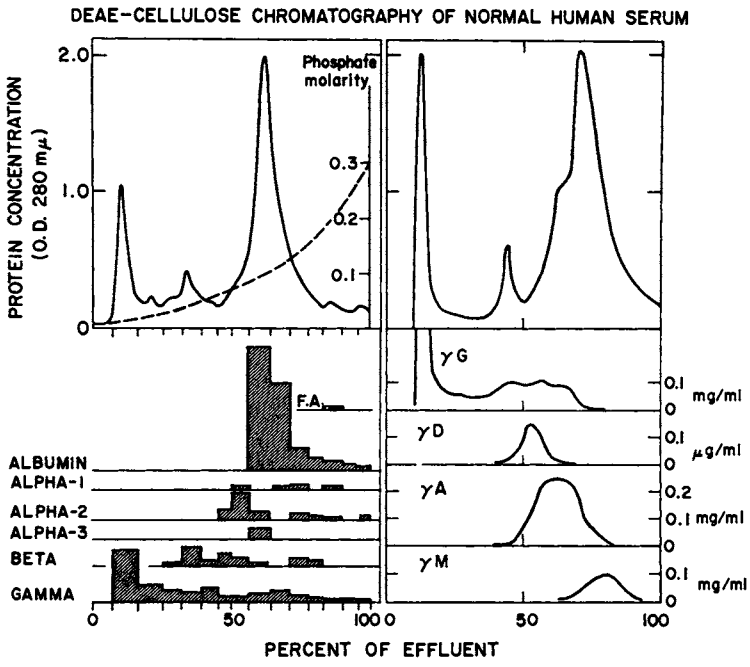

FIG. 1. DEAE-cellulose chromatography of normal human serum. On the left, the fractionation of 5 ml of serum is illustrated. Eight grams of DEAE-cellulose was packed into a 2.2 × 18-cm column. A gradient elution system was used with initial buffer in chambers 1, 2, 3, 5, and 7, final buffer in chambers 6, 8, and 9, and a 9:1 mixture (initial–final buffer) in the fourth chamber. Sixty-milliliter amounts were present in each chamber, giving a total effluent volume of about 550 ml which was collected in 6-ml fractions. The initial buffer was 0.02 *M* potassium phosphate, pH 8.0, and the final buffer was 0.30 *M* potassium phosphate, pH 8.0. The distribution of protein in the chromatogram effluent, determined by measuring the optical density at 280 mμ, is shown as a solid line in the figures. The phosphate gradient is indicated by the dashed line. The effluent was pooled and concentrated by ultrafiltration and examined by paper electrophoresis to determine the distribution of serum proteins (left). The first peak of the β-globulins is transferrin. On the right, 5 ml of serum was fractionated by a similar gradient employing an initial buffer of 0.015 *M* phosphate, pH 8, and a final buffer of 0.30 *M*. The chromatographic distribution of individual classes of immunoglobulins was determined by quantitative techniques. From D. Rowe and J. L. Fahey, *J. Exptl. Med.* **121**, 185 (1965); J. L. Fahey and G. McKelvey, *J. Immunol.* **94**, 84 (1965).

described below: one for preparation of γG-immunoglobulin alone, and the other for fractionation of whole serum.

(*a*) *Preparation of γG-Immunoglobulin.* From 50 to 100 mg of pure γG-immunoglobulin may be prepared from 10 ml of normal serum. A starting buffer of 0.01 *M* phosphate (potassium phosphate), pH 8, is

used. A 2.2 × 18-cm (or longer) column is packed with about 8 gm (dry weight) of DEAE-cellulose which has been thoroughly washed and then equilibrated with the starting buffer. Ten milliliters of serum is dialyzed against 100 volumes of starting buffer, usually for 18 hours.

The dialyzed serum is clarified by centrifugation, applied to the top of the DEAE-cellulose column, and washed in with several small volumes of buffer. Several milliliters of starting buffer is placed above the top of the DEAE-cellulose. A reservoir containing the starting buffer is connected by flexible tubing to the top of the glass column, and a flow rate of 1 to 2 ml/min is established. The flow rate may be controlled by hydrostatic pressure or by a pump. Fractions of 5- to 10-ml volume are collected, and the protein distribution is determined by measuring the optical density of the effluent fraction at 280 mμ.

Protein will begin to appear in the effluent after collection of about 32 to 38 ml of buffer. About 80% of the serum γG-immunoglobulin will be eluted in the next 20 to 26 ml of buffer. This fraction should be free of other serum protein. The effluent tubes containing γG-immunoglobulin may be pooled and concentrated by ultrafiltration or other techniques. After the first protein peak has been obtained and the optical density of the effluent has fallen toward the base line, the remaining serum proteins may be eluted by passing 200 ml of 0.5 *M* NaCl through the column.

Another useful procedure for DEAE-cellulose chromatographic preparation of γG-immunoglobulin has been described by Levy and Sober.[4] These authors recommend using 0.017 *M* phosphate buffer, pH 6.3. The use of such buffers, however, requires an excellent preparation of DEAE-cellulose; otherwise the γG fraction will be contaminated with other serum proteins (see below).

(*b*) *Whole Serum Fractionation.* DEAE-cellulose chromatography may be usefully employed for the fractionation of all the serum proteins including the immunoglobulins. The first part of the procedure described above for preparation of γG-immunoglobulin may be followed—starting buffer of 0.01 *M* phosphate, pH 8, a 2.2 × 18-cm column, and 10 ml of serum. After the serum has been applied to the top of the DEAE-cellulose column and covered with several milliliters of starting buffer, a flexible tubing filled with starting buffer is used to connect the top of the column to a device which will introduce a buffer gradient onto the column. Two buffers are needed—a starting buffer as noted above, and a final buffer, such as 0.3 *M* phosphate (potassium phosphate) buffer, pH 8.

Gradient elution may be produced with a varigrad[5] having nine cham-

[4] H. B. Levy and H. A. Sober, *Proc. Soc. Exptl. Biol. Med.* **103**, 250 (1960).
[5] E. A. Peterson and H. A. Sober, *Anal. Chem.* **31**, 857 (1959).

bers (starting buffer in chambers 1, 2, 3, 5, and 7, and final buffer in chambers 6, 8, and 9, with a 9:1 ratio of starting buffer to final buffer in chamber 4). A three-chamber device with starting buffer in chambers 1 and 2 and final buffer in chamber 3 may be used. Gradient elution also may be provided by using two vessels, such as a 600-ml beaker and a 250-ml Erlenmeyer flask. The two vessels are connected by a siphon arrangement filled with initial buffer or by direct connection between the bases of each vessel. Approximately 500 ml of starting buffer is placed in the beaker, which is used as a mixing chamber, and 250 ml of the final buffer is placed in the Erlenmeyer flask. A smaller elution volume may be achieved with a 250-ml beaker and a 125-ml Erlenmeyer flask. A similar arrangement is illustrated by Fahey *et al.*[6] The bottom level of the two vessels should be the same, and the level of fluid in both should reach the same height. The contents of the beaker is continuously mixed by a plastic-coated magnetic bar inside the beaker and a magnetic stirrer outside (usually beneath the beaker and supporting it).

Effluent fractions are collected, and the protein distribution is determined by measuring the optical density as noted above. The chromatogram is graphically plotted by relating the optical density of the effluent to the fraction number or elution volume (Fig. 1). Subsequently the effluent fractions can be pooled, concentrated, and tested for immunoglobulins.

A technique for DEAE-cellulose chromatography may be scaled down for fractionation of small (1-ml) quantities of serum by using smaller amounts of adsorbent and less volume of buffers.[7] A technique based on stepwise changes in buffer, described by Kochwa *et al.*,[8] has the advantage of requiring less buffer and producing more concentrated fractions. DEAE-cellulose chromatography can be used with even smaller amounts of adsorbent and protein, but stepwise elution systems are best when small amounts of protein are involved. Conditions for fractionation of large quantities of globulin, prepared from 100 ml or more of serum, are described by Strauss *et al.*[9]

ii. Distribution of Specific Immunoglobulins

(*a*) *γG-Immunoglobulin.* About 80% of γG proteins is eluted in the first protein peak from the column (Fig. 1). The γG molecules which are

[6] J. L. Fahey, P. F. McCoy, and M. Goulian, *J. Clin. Invest.* **37**, 272 (1958).
[7] J. L. Fahey, *J. Biol. Chem.* **237**, 440 (1962).
[8] S. Kochwa, R. E. Rosenfield, L. Talal, and L. R. Wasserman, *J. Clin. Invest.* **40**, 874 (1961).
[9] A. J. L. Strauss, K. G. Pierson, Jr., W. E. Vannier, and H. C. Goodman, *J. Immunol.* **93**, 24 (1964).

most cathodal on zone electrophoresis will be in this peak. The faster-migrating (more anodal) γG proteins will be retained on the column initially by interaction between the negative charge of these γG molecules and the positive charge of the adsorbent. They will be eluted as the buffer increases in ionic strength. Because the γG-immunoglobulin population is heterogeneous in electrical charge, these molecules are eluted through about 80% of the chromatogram effluent (Fig. 1).

(*b*) *γA-Immunoglobulin.* The γA proteins initially are retained on the column. With a gradient increase in ionic strength, the γA proteins are eluted in the fractions between 45 and 80% of the elution volume (Fig. 1). The chromatogram fractions containing γA-immunoglobulin also have other serum proteins, both immunoglobulins and nonimmunoglobulins. The earlier chromatogram fractions with γA-immunoglobulin may also contain γD molecules, and the later fractions may include γM molecules.

DEAE-cellulose chromatography by itself does not provide purified γA molecules, but it may be helpful in distinguishing the antibody activity of γA molecules from γG- or γM-immunoglobulins.[10]

(*c*) *γM-Immunoglobulin.* The γM proteins are eluted in the fractions between 60 and 90% of the elution volume. These fractions also contain some γG and γA molecules and many other serum proteins (Fig. 1).

The distribution of γM molecules on DEAE-cellulose chromatography is different from that of other classes of immunoglobulins. This difference may be used to advantage in distinguishing antibody activity of γM from antibody of γG molecules.[11,12] The relative amounts of antibody activity in each of these fractions can be determined after DEAE-cellulose chromatographic separation.

(*d*) *γD-Immunoglobulin.* This newly described member of the immunoglobulin family is present only in small amounts in normal serum (0.03 mg/ml).[13] The γD molecules are eluted after the γG-immunoglobulin peak and slightly before most of the γA molecules (Fig. 1). Other serum proteins will also be present in these chromatographic fractions of whole serum.

iii. Special Considerations

(*a*) *Variability of DEAE-Cellulose.* Commercial preparations of DEAE-cellulose are not uniform products. Many such preparations have fractionation characteristics different from those of the DEAE-cellulose

[10] P. Fireman, W. E. Vannier, and H. C. Goodman, *J. Exptl. Med.* **117**, 603 (1963).
[11] J. L. Fahey, *Science* **131**, 500 (1960).
[12] E. V. Barnett, J. J. Condemi, J. P. Leddy, and J. H. Vaughan, *J. Clin. Invest.* **43**, 1104 (1964).
[13] D. Rowe and J. L. Fahey, *J. Exptl. Med.* **121**, 185 (1965).

preparations described by Peterson and Sober[1] or those used for the procedures described here. The most common deficiency is a low protein-binding capacity. To correct for this defect it may be necessary to use more DEAE-cellulose per milliliter of serum, or to use a lower ionic strength (molarity) in the initial buffer, or to try another DEAE-cellulose preparation.

Preparations of DEAE-Sephadex may bind serum proteins rather more tightly than DEAE-cellulose adsorbent. Recovery of some immunoglobulin molecules may be less when DEAE-Sephadex preparations are used.

(*b*) *Protein Overloading.* The capacity of a DEAE-cellulose column to adsorb protein may be exceeded if the binding capacity of the adsorbent is poor (see above) or if the serum contains unusually large amounts of protein (myeloma or macroglobulinemic serums). When this occurs, protein components will be eluted sooner than anticipated and in a large (rather than restricted) volume of effluent. The possibility of overloading is one of the reasons why analytic testing of representative fractions is an important element in successful DEAE-cellulose chromatography.

(*c*) *Euglobulins.* Dialysis of serum against buffers of low ionic strength will precipitate a part of the serum globulins. Some nonimmunoglobulins and some of the normal immunoglobulins will be included in the precipitate. Many Waldenström macroglobulins, some myeloma proteins, and part of the increased immunoglobulin seen in disease may precipitate in 0.005 *M* phosphate buffers. The tendency to precipitate is reduced at pH levels above 7 and at higher salt concentration. For this reason, pH 8 buffers with 0.02 *M* phosphate have been used for DEAE-cellulose chromatography of immunoglobulins.[14] Many DEAE-cellulose preparations in 0.02 *M* buffers, however, will not completely adsorb transferrin and other serum proteins. In such circumstances, the salt content of the starting buffer will have to be lowered for good serum adsorption, but this brings the added risk of euglobulin losses. Sometimes a careful titration is necessary to find an initial buffer concentration (and pH) in which the euglobulin remains in solution and is fully taken up by the adsorbent.

(*d*) *Myeloma Proteins and Waldenström Macroglobulins.* G-myeloma protein (γG), A-myeloma protein (γA), and M (Waldenström)-macroglobulins (γM), may be partially purified by DEAE-cellulose chromatography (Fig. 2). These proteins are eluted in discrete peaks which are as distinctive as the peaks seen on zone electrophoresis.

Conditions for chromatography may have to be adjusted to achieve the best results. When one is fractionating serum containing G-myeloma

[14] J. L. Fahey and A. P. Horbett, *J. Biol. Chem.* **234**, 2645 (1959).

protein, it usually is desirable to have the G-myeloma protein adhere to the DEAE-cellulose column so that the bulk of normal γG-immunoglobulin may be eluted in the first peak and the G-myeloma protein in a subsequent fraction (see serum B.B. in Fig. 2). To obtain good adherence of the myeloma protein, the molarity of a starting buffer may have to be lowered. Such a change can precipitate euglobulins, as noted above, so preliminary testing of chromatography conditions

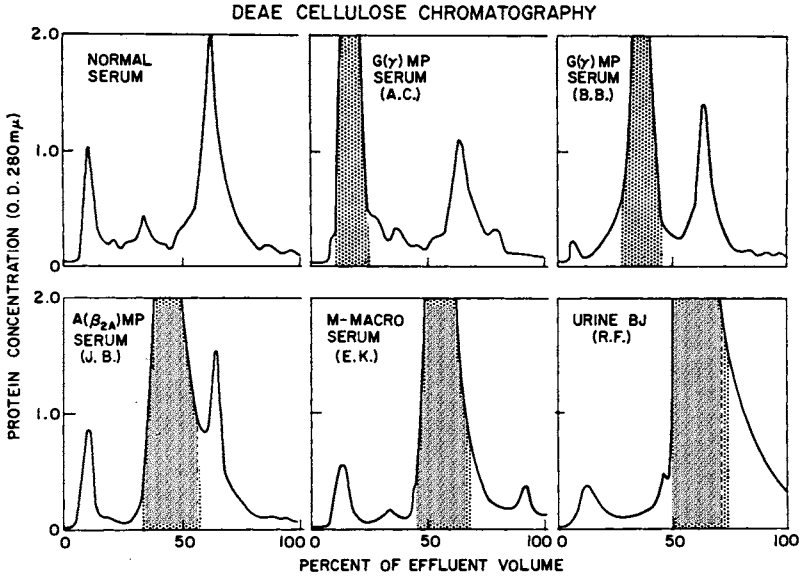

FIG. 2. DEAE-cellulose chromatography of myeloma and macroglobulinemic serums and a urine with Bence-Jones protein. Chromatography was carried out with 1 ml of serum (except with the last sample) and a gradient elution using potassium phosphate buffers, pH 8. [J. L. Fahey, *J. Biol. Chem.* **237**, 440 (1962).] The initial buffer was 0.02 *M* phosphate, and the final buffer was 0.30 *M* phosphate. The location of the anomalous protein (myeloma protein, etc.) is indicated by the stippled area on each chromatogram.

may be required to determine the best conditions for purification of each myeloma protein.

All chromatographically prepared anomalous protein fractions will contain some normal immunoglobulin contaminant. The amount of normal contaminant may be reduced by pooling only the effluent fractions from the peak region of protein elution—that is, the effluent with the greatest amounts of anomalous protein and the (relatively) least normal protein.

(*e*) *Bence-Jones Proteins.* The Bence-Jones proteins, present in the urine of many patients with multiple myeloma, may be concentrated by ammonium sulfate precipitation[15] or by ultrafiltration. The concentrate is then dialyzed against the starting buffer, and chromatography is carried out in the usual way (Fig. 2).

Urine normally contains about 100 mg of protein per 24 hours. Part of this is serum protein. In multiple myeloma with Bence-Jones proteinuria, renal damage may increase the amount of serum protein present in the urine, and urinary tract infection may increase the amount of other proteins. Thus, fractionation of the urine is required for purification of Bence-Jones protein. DEAE-cellulose chromatography is useful for purification of Bence-Jones protein because of the high capacity of DEAE-cellulose for protein adsorption and because of the high degree of resolution which can be achieved with proteins which are largely homogeneous on electrophoresis.

(*f*) *Purification of Normal γA- and γM-Immunoglobulins.* Although DEAE-cellulose chromatography provides a partial separation of γA- and γM-immunoglobulins from other serum immunoglobulins, this technique has not proved as valuable as other methods for purification of these proteins from normal serum. Zone electrophoresis and gel filtration,[16] ultracentrifugation,[17] and zinc precipitation techniques[18] have proved more useful in preparing γA or γM proteins from normal serum.

iv. Quantitation of γG-Immunoglobulin

The γG-immunoglobulin in serum may be measured by determining the protein content of the first effluent peak obtained on DEAE-cellulose chromatography.[7] The level of this γG fraction in normal serum can be determined and the changes in disease measured when standardized chromatographic conditions and constant volumes of serum are used.

This technique is particularly useful when a large amount of myeloma protein is present in the serum. If the anomalous protein can be made to adsorb onto the DEAE-cellulose, the first effluent peak will represent a significant part of the γG-immunoglobulin population. The levels of normal γG-immunoglobulin may be significantly reduced in myeloma and macroglobulinemia, as shown in Fig. 2. The amounts of anomalous protein in a serum may also be estimated from the size of the anomalous protein peak on chromatography (Fig. 2). Other analytical techniques

[15] E. C. Franklin, *J. Clin. Invest.* **38**, 2159 (1959).
[16] J. L. Fahey and C. L. McLaughlin, *J. Immunol.* **91**, 484 (1963).
[17] H. G. Kunkel, *in* "The Plasma Proteins" (F. W. Putnam, ed.), Vol. 1, pp. 279–307. Academic Press, New York, 1960.
[18] J. P. Vaerman, J. F. Heremans, and C. Vaerman, *J. Immunol.* **91**, 7 (1963).

such as zone electrophoresis and immunochemical techniques are easier to apply to large numbers of samples, so that the use of DEAE-cellulose chromatography for quantitative measurements is helpful only under special circumstances.

v. Species Differences

Purified γG-immunoglobulins from many species may be prepared by DEAE-cellulose chromatography of serum in a similar manner to that outlined for man. In the mouse, however, DEAE-cellulose chromatography may not be useful for preparing purified γG-immunoglobulin. We have routinely used zone (block) electrophoresis for purification of this component from mouse serum.[19] On chromatography much of the γG-immunoglobulin adheres to the adsorbent and is eluted with other serum proteins, notably transferrin. In Fig. 3 the distribution of mouse serum proteins on DEAE-cellulose chromatography is recorded. The first major elution peak (between 20 to 30% of the elution volume) is composed largely of transferrin. The γG-immunoglobulins (both 7 S γ_2- and 7 S γ_1-globulins) have a wide spectrum of electrophoretic heterogeneity and are eluted throughout much of the elution volume (Fig. 3). The γA and γM proteins are eluted in a more restricted portion of the chromatogram. In the mouse, as in man, the serum chromatogram fractions containing γA- and γM-immunoglobulins also include many other serum proteins (Fig. 3). An immunoelectrophoretic evaluation of a DEAE-cellulose chromatogram of mouse serum has been published by Talal and associates.[20]

Two categories of 7 S immunoglobulin (7 S γ_2-globulins and 7 S γ_1-globulins) have been identified in the guinea pig[21] and in the mouse.[19,22] The 7 S γ_2-globulins and 7 S γ_1-globulins in the guinea pig may be separated by DEAE-cellulose chromatography,[23] but the two forms of 7 S immunoglobulins in the mouse are only partially separated on DEAE-cellulose columns (Fig. 3).

c. Carboxymethyl (CM)-Cellulose Chromatography

Carboxymethylcellulose is a weakly acidic cation exchanger with functional groups titrating at pH between 3 and 6.[1] CM-cellulose chromatog-

[19] J. L. Fahey, J. Wunderlich, and R. Mishell, *J. Exptl. Med.* **120**, 223 (1964).
[20] N. Talal, G. Hermann, C. de Vaux St. Cyr, and P. Grabar, *J. Immunol.* **90**, 246 (1963).
[21] B. Benacerraf, Z. Ovary, K. J. Bloch, and E. C. Franklin, *J. Exptl. Med.* **117**, 937 (1963).
[22] R. S. Nussenzweig, C. Merryman, and B. Benacerraf, *J. Exptl. Med.* **120**, 315 (1964).
[23] Y. Yagi, P. Maier, and D. Pressman, *J. Immunol.* **89**, 442 (1962).

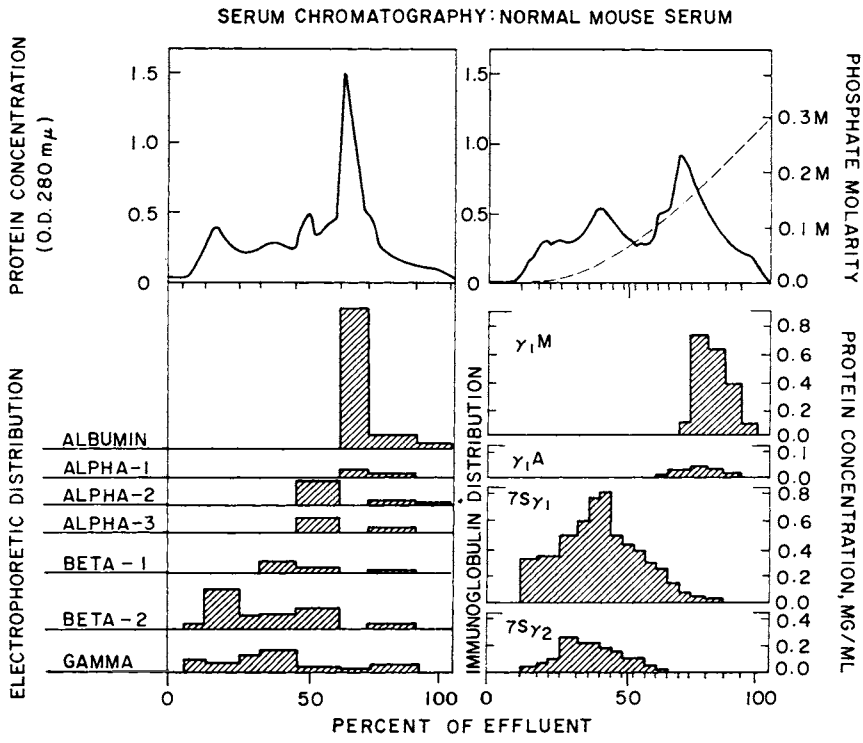

FIG. 3. DEAE-cellulose chromatography of mouse serum. Fractionations of 1 ml of serum are shown. Two grams of DEAE-cellulose was packed into a 1 × 22-cm column. A gradient elution system was used with an initial buffer of 0.015 *M* phosphate, pH 8, and a final buffer of 0.30 *M* phosphate, pH 8. A three-chamber variable gradient device was used with the initial buffer in chambers 1 and 2, and the final buffer in chamber 3. The effluent (total volume 180 ml) was collected in 3-ml fractions, and the protein concentration was determined from optical density measurements at 280 mμ. The protein distribution in the chromatogram is represented by the solid line, the phosphate molarity by the dashed line. *Left:* The distribution of serum protein components in normal mouse serum was determined by paper electrophoresis of pooled, concentrated effluent fractions. The first chromatogram peak of β-globulin electrophoretic mobility is due to transferrin. *Right:* The distribution of immunoglobulin components after DEAE chromatography of hyperimmune mouse serum was determined by quantitative immunochemical tests.

raphy is especially suitable for fractionation of immunoglobulins that are not adsorbed on the anion exchangers such as DEAE-cellulose.

Immunoglobulins of hyperimmune serums have been fractionated on CM-cellulose columns.[1,3] The γ-globulin prepared from 20 ml of pooled serum by ammonium sulfate precipitation was applied to a 19-gm CM-cellulose column equilibrated with 0.015 *M* sodium phosphate buffer,

pH 6.6. An elution gradient was used, with the final buffer containing 0.1 *M* NaCl and 0.2 *M* phosphate, pH 7.6. The γ-globulins were eluted in many chromatogram fractions, and differences in the distribution of specific antibodies were noted.

CM-cellulose chromatography was used by Porter[24] to separate the antibody fragments Fab (I, II) and Fc (III) obtained by papain digestion of rabbit γ-globulin. Sodium acetate buffers of pH 5.5 were used. The initial buffer was 0.1 *M* for acetate, and a gradient to 0.9 *M* acetate was used to elute the protein from the CM-cellulose column.

[24] R. R. Porter, *Biochem. J.* 73, 119 (1959).

4. ELECTROPHORETIC SEPARATION ON SOLID SUPPORTING MEDIA*

One of the more satisfactory means of separating the crude immunoglobulins from other serum proteins is powder block zone electrophoresis. The method is described in Chap. 6,D,1, Vol. II. If larger samples must be fractionated, electrophoresis columns may prove more practical. Such columns are discussed in Chap. 6,D,2, Vol. II.

Preparative electrophoresis by continuous elution from agar gel slabs is illustrated in Chap. 6,D,3, Vol. II, and recovery of electrophoretic fractions from polyacrylamide gels is described in Chap. 6,C,5, Vol. II. The latter technique is particularly useful when very small samples must be employed or when greater resolution is required.

The advantages and disadvantages of the several methods will depend largely on starting material and the purposes to which they are to be applied. These are presented in general terms by the authors of the respective sections. It must be remembered, however, that most electrophoretic fractions containing immunoglobulin will contain all classes of immunoglobulin, and that the fraction of higher mobility will contain significant amounts of other plasma proteins as well.

* Section 3,A,4 was contributed by the Editors.

5. PREPARATION OF IMMUNOGLOBULINS BY ELECTROPHORESIS-CONVECTION*†

Although γ-globulin migrates as a single boundary in an electric field over a wide range of pH and ionic strength, the reversible boundary-

* Section 3,A,5 was contributed by John R. Cann.

† Contribution No. 241 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver, Colorado.

spreading experiments of Alberty[1] indicated that it is nevertheless electrophoretically heterogeneous and possesses a continuous distribution of mobilities. This situation was confirmed by fractionation of γ-globulins in the course of one of the first applications of electrophoresis-convection to immunology. (The general method is described in Chap. 6,D,4, Vol. II.) Thus, bovine γ-globulin (fraction II of bovine plasma) was separated at pH 8.7 in barbital buffer, ionic strength 0.1, into eight fractions with mean mobilities ranging between -1.25×10^{-5} and -2.25×10^{-5} cm^2 sec^{-1} $volt^{-1}$ and mean isoelectric points in cacodylate-chloride buffer ranging from 7.31 down to 5.74.[2,3] The various fractions could be distinguished from one another by at least two electrophoretic properties. Furthermore, the mobility distribution of the unfractionated γ-globulin could be reconstructed by summation of the weighted distributions of the individual fractions. Human γ-globulin, prepared by ethanol fractionation from the plasma of individuals hyperimmunized to *Hemophilus pertussis* organisms, was also fractionated.[4] The four electrophoretically unique fractions possessed mean mobilities ranging between -0.88×10^{-5} and -1.60×10^{-5} and mean isoelectric points between 7.86 and 6.96. Immunological studies revealed a broad distribution of antibodies among the fractions with some separation of immune activities. All the fractions gave nearly the same agglutinin titer against two different antigen strains. Although all fractions showed protective activity, the greatest activity was associated with the fraction of lowest mobility. This was confirmed by the fractionation of a second sample of immune γ-globulin and subsequent subfractionation of the low-mobility material.

Following the demonstration that γ-globulin is electrophoretically heterogeneous, the method of electrophoresis-convection was used to fractionate a number of different types of antisera. (For routine separation of γ-globulin and other fractions from serum, however, see other sections of this chapter.) The studies with electrophoresis-convection showed that there is no simple pattern in the distribution of antibodies among the serum proteins. Thus, although most antibodies are found in the γ-globulins, in some cases they are broadly distributed throughout the serum globulins,[5] and in others they are associated chiefly with β-globulin frac-

[1] R. A. Alberty, *J. Am. Chem. Soc.* **70**, 1675 (1948).
[2] J. R. Cann, R. A. Brown, and J. G. Kirkwood, *J. Biol. Chem.* **181**, 161 (1949).
[3] J. R. Cann, R. A. Brown, and J. G. Kirkwood, *J. Am. Chem. Soc.* **71**, 2687 (1949).
[4] J. R. Cann, R. A. Brown, J. G. Kirkwood, and J. H. Hinks, Jr., *J. Biol. Chem.* **185**, 663 (1950).
[5] J. R. Cann, R. A. Brown, J. G. Kirkwood, P. Sturgeon, and D. W. Clarke, *J. Immunol.* **68**, 243 (1952).

tions.[6,7] Furthermore, a given type of antibody may be present in a single sharp fraction, or it may have a broad, and at times bimodal, distribution.[5,8] In the case of Rh antibodies,[5,9,10] different types of such antibodies were present in different components of serum, but the distributions were so broad that no effective separation could be obtained. However, partial separation of antibodies with different specificities (antiphenylarsonate and antibovine γ-globulin) and reactive capacities (precipitability) was obtained in the case of rabbit antiphenylarsonic azobovine γ-globulin antibodies,[11] and considerable separation and purification of human antibodies against two strains of poliomyelitis virus have been achieved.[7] The antipolio serum was from a single donor with naturally acquired immunity and showed high titers against all three principal types of poliovirus. Neutralizing antibodies against two strains were concentrated in different fractions: anti-type II in the slowest-migrating γ-globulin fraction, and anti-type I in a fraction consisting principally of γ-globulin of intermediate mobility. In contrast, the neutralizing activity of a pooled monkey hyperimmune serum to a type II attenuated virus was concentrated in a slow-moving β-globulin fraction. This, in turn, is in contrast to a monkey antipneumococcus body which appears to be associated with γ-globulin.[12] In the case of rabbit antisera to bacteriophage T2r$^+$, all the globulin fractions showed virus-neutralizing activity, but correlation of electrophoretic composition and neutralizing activity indicated that the virus-neutralizing antibodies are associated principally with γ-globulin.[13] Differences in the kinetic behavior of the virus-neutralizing antibody of the various serum fraction with respect to changes in the salt concentration of the antigen–antibody reaction mixture at pH 7 showed that the antibody molecules in the different fractions were different physical entities. In passing, it is worth noting that these particular experiments illustrate the value of using globulin fractions in preference to unfractionated antiserum for studying the mechanisms of the specific antigen–antibody reaction.

Electrophoresis-convection has also been utilized in an extensive investigation of the distribution of allergic antibodies among human serum

[6] J. R. Cann and M. H. Loveless, *J. Immunol.* **72**, 270 (1954).
[7] S. N. Timasheff, A. W. Moyer, R. A. Brown, and J. G. Kirkwood, *Proc. Natl. Acad. Sci. U.S.* **42**, 228 (1956).
[8] M. H. Loveless and J. R. Cann, *Science* **117**, 105 (1953).
[9] J. R. Cann, R. A. Brown, D. C. Gajdusek, J. G. Kirkwood, and P. Sturgeon, *J. Immunol.* **66**, 137 (1951).
[10] P. Sturgeon and R. A. Brown, *J. Immunol.* **68**, 287 (1952).
[11] J. R. Cann, D. H. Campbell, R. A. Brown, and J. G. Kirkwood, *J. Am. Chem. Soc.* **73**, 4611 (1951).
[12] A. Tiselius and E. A. Kabat, *J. Exptl. Med.* **69**, 119 (1939).
[13] J. R. Cann and E. W. Clark, *J. Am. Chem. Soc.* **78**, 3627 (1956).

proteins.[6,8,14,15] The sera contained reaginic and/or blocking antibodies against crystalline insulin, ragweed allergen, or a highly purified cottonseed allergen. From these investigations has emerged the fundamental fact that reagin and blocking antibody are distinct physical entities. Whereas reagin appears to be associated chiefly with β-globulin, the blocking antibody migrates electrophoretically with the γ_2-globulins.

[14] M. H. Loveless and J. R. Cann, *J. Immunol.* **74**, 329 (1955).

[15] J. R. Cann and M. H. Loveless, *J. Allergy* **28**, 379 (1957).

6. ULTRACENTRIFUGAL SEPARATION OF γG- AND γM-IMMUNOGLOBULINS*

It is frequently desirable to determine the relative amounts of antibody present in the 6.5 to 7 S and 19 S immunoglobulin fractions of an immune serum. This may be achieved by analysis of fractions separated by ultracentrifugation on sucrose or salt gradients. Ultracentrifugation methods are described in Chap. 7, Vol. II and the procedures applied to immunoglobulins are outlined in Section C,5 of that chapter.

Whole serum may be used if it is of interest only to test for antibody activity. If method is to be used as a preparative step, it is advisable to use immunoglobulin concentrates such as those described in Section 3,A,2 or electrophoretic fractions from powder blocks as described in Chap. 6,D,1, Vol. II.

Alternative methods for separating γG- from γM-immunoglobulins may be more convenient. Gel filtration is a simple procedure (see Chap. 9, Vol. II), and gels of larger porosities are now commercially available. DEAE-cellulose chromatography may also be employed for the separation of immunoglobulin classes (see Section A,3).

* Section 3,A,6 was contributed by the Editors.

B. Preparation of Specific Antibody

1. ANTIPOLYSACCHARIDE ANTIBODY PURIFIED FROM SPECIFIC COMPLEXES*

Many of the methods used for the purification of antibody from specific complexes, are also applicable to antipolysaccharide antibodies.[1,2]

* Section 3,B,1 was contributed by Elvin A. Kabat.

[1] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

[2] A. H. Sehon, *Brit. Med. Bull.* **19**, 183 (1963).

These include elution with hapten,[3,4] acid,[5] or alkali[1] from insoluble specific precipitates or from complexes with insoluble specific adsorbents. In addition, two methods have some degree of uniqueness for anti-polysaccharide antibodies—extraction with 15% NaCl from specific precipitates[6] or agglutinates,[7] and digestion of the antigen in the precipitate with specific enzymes such as lysozyme,[8] dextranase,[9,10] and salivary amylase[11] to release the antibody. This method has also been applied to a protein antigen; for example, collagenase has been used to digest gelatin–antigelatin precipitates.[12]

Extraction with 15% Salt. This method takes advantage of the finding that a given quantity of pneumococcal polysaccharide binds less antibody in 15% NaCl than it does in 0.9% NaCl.[13] This differential binding varies from antiserum to antiserum, but generally between 15 and 30% will go into solution in 15% NaCl from specific precipitates formed and washed in 0.9% NaCl. No such dissociation is observed with protein–antiprotein systems.

The procedure given in Kabat[1] is as follows: "The antibody from 100 ml. of type-specific antipneumococcus serum is precipitated at 0°C by addition of an amount of homologous polysaccharide calculated to bring the system to the beginning of the equivalence zone or preferably to leave a small excess of antibody. After the precipitate flocculates, the mixture is centrifuged in the cold, the supernatant is decanted and the precipitate evenly suspended in chilled saline and washed repeatedly until the amount of heat coagulable or sulfosalicylic acid precipitable protein is at a minimum, four to seven washings usually being sufficient. The washed specific precipitate is then extracted at 37°C with about 30 ml. of 15% sodium chloride solution for one hour in the presence of a drop of toluene. After centrifugation the precipitate is again extracted with an additional 15 ml. of the 15% salt solution. The 15% NaCl extracts are dialyzed in the cold under negative pressure against

[3] S. F. Schlossman and E. A. Kabat, *J. Exptl. Med.* **116**, 535 (1962).
[4] J. Gelzer and E. A. Kabat, *J. Exptl. Med.* **119**, 983 (1964); *Immunochemistry* **1**, 303 (1964).
[5] H. G. Kunkel, M. Mannik, and R. C. Williams, *Science* **140**, 1218 (1963).
[6] M. Heidelberger and F. E. Kendall, *J. Exptl. Med.* **64**, 161 (1936).
[7] M. Heidelberger and E. A. Kabat, *J. Exptl. Med.* **67**, 181 (1938).
[8] R. R. Feiner, K. Meyer, and A. Steinberg, *J. Bacteriol.* **52**, 375 (1946).
[9] E. A. Kabat, *Science* **120**, 782 (1954).
[10] O. Swineford, R. Hoene, S. Quelch, and D. Samsell, *J. Allergy* **30**, 433 (1959).
[11] M. Heidelberger, H. Jahrmärker, B. Björklund, and J. Adams, *J. Immunal.* **78**, 419 (1957).
[12] R. Arnon and M. Sela, *Science* **132**, 86 (1960).
[13] M. Heidelberger, F. E. Kendall, and T. Teorell, *J. Exptl. Med.* **63**, 819 (1936).

repeated changes of 0.9% saline in the presence of toluene until the desired volume has been reached. The precipitate usually formed during the dialysis is removed from the supernatant which constitutes the purified antibody solution. The degree of purity of the antibody solution is determined by the ratio of the antibody nitrogen content as measured with the quantitative precipitin or agglutinin methods, to the toal nitrogen content of the solution. The yield may be calculated from the amount of purified antibody recovered and the amount present in the volume of serum used."

This method has been used with several species of antipneumococcal sera of various types but is probably applicable to many polysaccharide–antipolysaccharide systems. Yields are small with rabbit antipneumococcal sera, but with horse antisera yields up to 30% may be obtained. The recovered antibody is generally 80 to 100% precipitable by antigen, but with some antisera it may be only 40 to 60%.[1]

Instead of soluble polysaccharide, a suspension of pneumococci killed by formalin and heat may be used to absorb the antibody. The agglutinated bacteria are washed, and the procedure is carried out as above. (The residual specific precipitates or agglutinates have been washed with 0.9% NaCl and used to reabsorb a portion of fresh antiserum, and the procedure repeated.) Alternatively, a portion of the residual antibody may be recovered by extraction with $Ba(OH)_2$ and $BaCl_2$ in the cold.[1]

Barbu *et al.*[14] have prepared rabbit and horse antisera to ribosomes which react with RNA and with polynucleotides such as polyadenylic acid (poly-A). The washed poly-A–antibody complex is extracted at 4° with 1.0 *M* $MgCl_2$; the antibody goes into solution, while the poly-A remains insoluble and is centrifuged off. A similar procedure was used to recover the antibody precipitable by polyinosinic acid (poly-I); poly-I also gave some precipitation with normal serum.

Digestion of Specific Complexes with Enzymes.[1,9] The dextranase of *Penicillium funiculosum*[15] will digest dextrans with high proportions of α-1,6 linkages such as the B512 strain which contains 96% α-1,6 and 4% α-1,3 linkages and will also digest Sephadex G-75 almost completely; only 32 mg of 120 mg of Sephadex G-25 was digested (Kabat, unpublished observations, 1962). It has been used at pH 5.1 and at pH 6.1. A washed specific precipitate of dextran–antidextran is homogenized in a Ten Broeck grinder with saline buffered with acetate at pH 5.1 or 6.1, and a small quantity of dextranase (about 0.2 mg) is added.

[14] E. Barbu, G. Quash, and J. P. Dandeu, *Ann. Inst. Pasteur* **105**, 849 (1963).

[15] H. M. Tsuchiya, A. Jeanes, H. M. Bricker, and C. A. Wilham, *J. Bacteriol.* **64**, 513 (1952).

The suspension is placed at 37° with occasional mixing; after several hours the precipitate goes almost completely into solution. Any trace of insoluble residue is centrifuged off, and the soluble oligosaccharides are removed by ultrafiltration through a Schleicher and Schuell membrane filter[4] and washing with saline or by dialysis against saline.

The antibody in type II[10] and in type XXII (Goodman and Kabat, unpublished observations, 1959) antipneumococcal serum cross-reacting with dextran has been recovered in a similar manner. The method is applicable to specific precipitates with antipneumococcal sera and glycogen when salivary amylase is used.[11]

The recovered antidextran was precipitable by dextran to the extent of 80 to 90%, and the yield was 90 to 95% of the nitrogen in the specific precipitate. The solutions contain residual amounts of active dextranase which complicate quantitative precipitin and inhibition assays. For quantitative precipitin assays this may be circumvented by using a more highly branched dextran which is not susceptible to the action of the enzyme, but in inhibition assays isomaltohexaose was found to be split extensively into isomaltose, isomaltriose, and isomaltotetraose during the time required for analysis (Kabat, unpublished observations, 1960) so that valid inhibition assays could not be obtained.

Elution of Antibody from Sephadex–Antidextran Precipitates. The use of Sephadex as an insoluble dextran offers substantial advantages in the purification of antidextran, since the antibody may be extracted without any of the antigen going into solution.[3,4] With human antidextran, Sephadex G-75 has been found most suitable. Absorption of the antidextran is carried out batchwise by adding an appropriate quantity of Sephadex G-75 which has been washed with saline. The antiserum and the Sephadex in 50-ml centrifuge tubes are rotated slowly at about 6 rpm on a clock motor for periods up to a week; samples of the supernatant are analyzed to check on the removal of the antibody. It is advisable to rotate the tubes along the long axis rather than end to end, since the latter causes nonspecific precipitation from serum. The antibody–Sephadex precipitate is centrifuged off and washed repeatedly in the cold with saline until no material absorbing at 2800 A is present in the washings. The antidextran may be eluted by extraction in the cold with acetate buffer at pH 3.5[5] and dialysis of the extract rapidly against phosphate buffer, pH 7.5. Elution may also be carried out by using as haptens the oligosaccharides of the isomaltose series. This procedure has been most extensively applied in the study of the heterogeneity of human antidextran from single individuals. Sequential extraction with smaller oligosaccharides such as isomaltose and isomaltotriose removed portions of antibody with higher affinity for smaller oligosaccha-

rides, leaving for subsequent extraction by larger oligosaccharides, such as isomaltohexaose, antibody which had relatively little affinity for the smaller oligosaccharides but which reacted strongly with the larger oligosaccharides[3,4]; assays were carried out by inhibition of precipitation and by inhibition of complement fixation. Eluates have been reabsorbed on Sephadex and re-eluted with oligosaccharide. About 50 mg of Sephadex G-75 has been used to absorb 1 mg of antidextran nitrogen. Recoveries of total nitrogen were about 60% for a single absorption and sequential elution with two to four haptens, and the precipitability of the recovered antibody ranged from 80 to 100%. Human antilevan has also been absorbed on a Sephadex type of material prepared from levan (Kabat and Ingelman, unpublished observations, 1963), and the antilevan eluted at pH 3.5.

The procedure of Smith *et al.*[16,17] of separating individual antigen–antibody bands from large-scale agar diffusion runs of mixtures of antigens and antibodies could provide a means for the purification of antipolysaccharide antibodies.

[16] H. Smith, B. T. Tozer, R. C. Gallop, and F. S. Scanes, *Biochem. J.* **84**, 74 (1962).
[17] H. Smith, R. C. Gallop, and B. T. Tozer, *Immunology* **7**, 111 (1964).

2. PURIFICATION OF ANTIBODY FROM ERYTHROCYTE–ANTIBODY COMPLEXES*†

a. Introduction

Multiple immune responses to differing erythrocytic antigenic determinants are observed both in homologous and in heterologous systems. Accordingly, there is a need, for both qualitative and quantitative studies, to isolate antibodies having single serological specificities. Purified preparations of antibody are necessary also for immunochemical characterization of immune responses, kinetic studies of antigen–antibody reactions, and evaluation of the genetically controlled expression of erythrocytic antigens. The selection of red cell antigens and the most efficient method of dissociating antigen–antibody complexes are of primary importance in antibody isolation.

* Section 3,B,2 was contributed by Richard E. Rosenfield and Shaul Kochwa.

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b. Factors Affecting Antibody Recovery

The efficiency of antibody recovery from serum is related to three factors: (1) the amount of antibody that can be bound initially to erythrocytes; (2) the degree of spontaneous dissociation of weakly bound antibody during washing of complexes; and (3) the degree of final dissociation that can be achieved and maintained during the separation of antibody from erythrocytes. When these conditions are readily controlled, as they can be with cold agglutinins, excellent recoveries are achieved.[1,2] In general, however, the amount of antibody recovered in eluates is inversely proportional to the strength of the erythrocyte–antibody bonds that must be dissociated. Thus, eluates from type A_2 cells contain more anti-A than similar preparations from type A_1 cells which bind more anti-A and are more intensely agglutinated by these antibodies. This phenomenon is also apparent in the Rh system where cell-bound Rh antibodies may be recovered more readily from weak than from strong Rh-positive cells. However, in the case of acid eluates from stroma,[3,4] the best antibody yields are obtained from cells that have the strongest Rh antigenic expression. At low pH values the antibody-binding capacity of Rh sites on stroma is quickly and completely lost,[5] ensuring the recovery of all cell-bound antibodies. Antibody losses in this method are, therefore, determined solely by the uncontrollable dissociation of bound antibody during lysis and washing.[4,6] Kell sites on stroma are also sensitive to low pH, but the antigens of blood group systems ABO, MNSs, Duffy, Kidd, and Lutheran are insensitive,[5,7] thereby limiting the efficient applicability of acid elution. Red cells and stroma can be used repeatedly for the binding of cold agglutinins. Elution of bound antibodies at 37° is the only method that has no detectable effect on the binding capacity of cellular antigenic sites.

i. Efficiency of Dissociation

A comparison of several methods of recovering cell-bound I^{131}-labeled Rh antibodies[8] showed 80% dissociation of fully active antibodies by

[1] D. Stats and L. R. Wasserman, *Medicine* **22**, 363 (1943).
[2] R. S. Gordon, J. Immunol. **71**, 220 (1953).
[3] P. Kidd, *J. Clin. Pathol.* **2**, 103 (1949).
[4] R. E. Rosenfield and S. Kochwa, *J. Immunol.* **92**, 693 (1964).
[5] S. Kochwa and R. E. Rosenfield, *J. Immunol.* **92**, 682 (1964).
[6] R. E. Rosenfield, I. O. Szymanski, and S. Kochwa, *Cold Spring Harbor Symp. Quant. Biol.* **29**, 427 (1964).
[7] P. Levine, F. J. Falkowsky, and M. Celano, personal communication, 1965. To be published.
[8] N. C. Hughes-Jones, B. Gardner, and R. Telford, *Biochem. J.* **88**, 435 (1963).

acid (pH 3.5),[3] 35 to 40% by heat,[9] and 40 to 70% by a nonpolar solvent.[10,11] Alkaline eluates (pH 11.3) contained 87% dissociated antibodies, but over 40% of their binding capacity was lost. At pH 3.0, in contrast to pH 3.5, approximately full dissociation of active antibodies has been achieved.[5]

ii. Effect of Complement

Harboe[12] found 99.95% of I^{131}-labeled cold agglutinins, previously bound to red cells at 4° in the presence of added fresh serum, to be dissociated at 37°. Both β_1E (C'4)- and β_1C (C'3a)-globulins were retained after antibody elution because the cells were still antiglobulin-positive in tests with specific serums. Evans *et al.*[13] also reported that complement had little effect on the uptake and retention of cold agglutinins.

On the other hand, Leddy *et al.*[14] reported that some acid eluates[3] prepared from red cells of patients with hemolytic anemia lacked hemolytic complement but contained complement protein apparently complexed with γG autoantibodies. In the absence of fresh complement, normal cells exposed to these eluates became antiglobulin-positive for complement as well as for γG-globulins.

c. Isolation of Specific Antibody Activity from Intact Erythrocytes

Absorption on and dissociation from intact red cells of appropriate type permit a selection of antibodies on the basis of specificity. Such eluates contain nonantibody protein including hemoglobin, but this has little or no effect on qualitative and semiquantitative agglutination tests. Furthermore, the procedures are short and do not require special equipment.

i. Heat

Landsteiner and Miller[9,15] applied 25 volumes of serum to 1 volume of washed packed red cells, but lesser amounts of stronger serums are needed for the equilibrium conditions that ensure maximal antibody binding to erythrocytes. The amount of antibodies to be applied to

[9] K. Landsteiner and C. P. Miller, Jr., *J. Exptl. Med.* **42**, 853 (1925).
[10] W. Weiner, *Brit. J. Haematol.* **3**, 276 (1957).
[11] H. Rubin, *J. Clin. Pathol.* **16**, 70 (1963).
[12] M. Harboe, *Brit. J. Haematol.* **10**, 339 (1964).
[13] R. S. Evans, E. Turner, and M. Bingham, *Am. J. Med.* **38**, 378 (1965).
[14] J. P. Leddy, R. F. Bakemeier, and J. H. Vaughan, *J. Clin. Invest.* **44**, 1066 (1965).
[15] K. Landsteiner, *Münch. Med. Wochschr.* **49**, 1905 (1902).

erythrocytes should, if possible, be sufficient so that not more than 50% of applied antibody activity will be absorbed. After an incubation period of 1 or 2 hours at an appropriate temperature, the antibody-sensitized erythrocytes are washed at least three times, each time with at least 10 volumes of 0.9% NaCl, then suspended in 3 volumes of saline, and heated to 56° for 5 minutes. The supernatant eluate, hemoglobin-tinged, should be removed immediately after centrifugation at 56°. These conditions can be achieved by preheating an entire angle rotor in which the cups are enclosed or by placing a small centrifuge in a heated box where even a hair dryer can serve as the heater. Unless antigen is destroyed at 56°, and few are, significant antigen–antibody recombination can occur if adequate heat is not maintained until the eluate is removed. Recovery of antibody activity is sometimes improved by heating for 20 minutes, or by using higher temperatures for dissociation, in which case saline containing 5% bovine albumin protects dissociated antibodies. At 65° significant loss of antibody activity occurs.

Goodman[16a] used formalin-stabilized red cells in polyurethan for controlled thermal elution from a column. Unfortunately the antibody-binding capacity of formalin-treated erythrocytes is very low.

Hughes-Jones *et al.*[16b] applied to packed cells almost sufficient I^{131}-labeled anti-Rh4 (hr′ or c) to achieve equilibrium concentrations. The mixture was incubated at 37° for 30 minutes, and the sensitized cells, sedimented at 2000 *g*, were washed eight times with saline chilled to 0°, a temperature at which antibody dissociation is minimal. When these antibody-sensitized cells were suspended in 20 to 40 volumes of saline at 37° for 30 minutes, significant dissociation of antibody occurred, and, in ten sequential applications of warm saline, 80% of antibodies were recovered in dilute eluates which required concentration by ultrafiltration. The process is not suitable for most antibodies because they will not dissociate at 37° as readily as do many examples of anti-Rh4.

ii. Nonpolar Solvent

A mixture of equal volumes of diethyl ether and a 50% saline suspension of Rh antibody-coated washed cells is inverted repeatedly for 1 minute and then centrifuged at 1000 *g* for 10 minutes to obtain three layers.[11] The top (ether) and middle (denatured stroma) layers are discarded. Residual ether present in the bottom layer of hemoglobin-containing eluate should be evaporated by warming to 37° for 15 minutes. Additional centrifugation helps to clarify the eluate. The 40% recovery

[16a] H. S. Goodman, *Nature* **139**, 350 (1962).
[16b] N. C. Hughes-Jones, B. Gardner, and R. Telford, *Biochem. J.* **85**, 466 (1962).

of cell-bound Rh antibodies by this method is increased to 70% by incubation of the ether mixture for 30 minutes at 37°.[8]

d. Isolation of Antibodies from Stroma

i. Preparation of Stroma

To purify antibody protein it is advisable to apply antiserum to intact cells rather than to stroma because the latter tend to trap significant amounts of nonantibody serum proteins. Intact red cells have many serum proteins on their surface, but these may be removed during lysis and washing. An efficient method of obtaining intact stroma that are free of serum proteins was described by Kochwa and Rosenfield.[5] Digitonin (0.5 ml of a 5-mg/ml suspension in 0.9% NaCl), when added to 10 ml of a chilled 10% red cell suspension, causes lysis in less than 1 minute, and aggregation of stroma occurs. The latter event, in the presence of a high concentration of hemoglobin, assists sedimentation at 34,000 *g* for 30 minutes in the cold, while in subsequent saline washings the digitonin is removed and the same stroma, now devoid of hemoglobin, sediment readily and disperse freely. In contrast, losses of stroma are considerable in some other methods of preparation, particularly where lesser centrifugal forces are used. Blood group antigen activity is unaffected by the digitonin method of preparing stroma.

ii. Dissociation of Antibodies from Stroma

Many methods of dissociating antibodies from stroma have been described. Heat,[8,17,18] the low pH principle of Landsteiner and van der Scheer,[3,5,19] and combinations of heat and low pH[20-22] have been employed. Evans (cited by Komninos and Rosenthal[23]) suggested use of 8% NaCl. Others have used ether[24] or a combination of ether and heat.[10,25] Undesired dissociation of antibody during washing of cells and stroma can be minimized by adjustment of pH to 6.5 to 7.5 and reduction of temperature to 0° to 4°.[8]

[17] S. P. Masouredis, *J. Clin. Invest.* **38**, 279 (1959); **39**, 1450 (1960).
[18] T. J. Greenwalt, *J. Lab. Clin. Med.* **48**, 634 (1956).
[19] K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **63**, 325 (1936).
[20] J. G. Selwyn, M. D. Thesis, Cambridge, cited by J. V. Dacie, in "The Haemolytic Anaemias: Congenital and Acquired," p. 494, Churchill, London, 1954, and by Rubin.[11]
[21] J. C. Mitchell, University thesis, Aberdeen, 1956, cited by Rubin.[11]
[22] N. C. Hughes-Jones, B. Gardner, and R. Telford, *Vox Sanguinis* **8**, 531 (1963).
[23] Z. D. Komninos and M. C. Rosenthal, *J. Lab. Clin. Med.* **41**, 887 (1953).
[24] G. H. Vos and G. A. Kelsall, *Brit. J. Haematol.* **2**, 342 (1956).
[25] K. G. Jensen, *Vox Sanguinis* **4**, 230 (1959).

e. Purification of Antibody Protein

i. Purification of Cold Agglutinins

Cold agglutinins are readily dissociated from stroma at 37°; their γM properties have been described by many investigators.[1,2,26-32] In view of the cryoglobulin nature of some cold agglutinins,[33] their peculiar immunochemical properties,[30,34,35] and their occurrence in some cases of Waldenström macroglobulinemia,[36] the assumed antierythrocytic specificities[37-40] have still to be proved, especially in view of the unique cold agglutinin activity of the γM-globulin antibody response of rabbits to *Listeria monocytogenes*.[41]

ii. Purification of Rh Antibodies

As was mentioned above (Section B,2,d,i), Kochwa and Rosenfield[5] used intact red cells at 37° to absorb Rh antibodies, washed these cells in the cold, and lysed them with digitonin. The antibodies, bound to chilled and washed stroma, were then dissociated at 0° to 2° in 0.1 *M* glycine buffer, pH 3.0, and the cell-free eluates were dialyzed against phosphate buffer, pH 7.3. These preparations were found to be free of all serum proteins other than eluted antibodies, but 85 to 95% of their nondialyzable nitrogen consisted of 2 S and 4 S components. These were probably derived from stroma because they were present in similar concentration even when antibodies had not been applied to red cells. Gel filtration

[26] R. Weber, *Vox Sanguinis* **1**, 37 (1956).
[27] H. H. Fudenberg and H. G. Kunkel, *J. Exptl. Med.* **106**, 689 (1957).
[28] W. N. Christenson, J. V. Dacie, and B. E. E. Croucher, with P. A. Charlwood, *Brit. J. Haematol.* **3**, 262 (1957).
[29] H. G. Kunkel, *in* "The Plasma Proteins" (F. W. Putnam, ed.), Vol. I, pp. 279–307. Academic Press, New York, 1960.
[30] T. N. Mehrotra, *Immunology* **3**, 265 (1960).
[31] T. N. Mehrotra and P. A. Charlwood, *Immunology*, **3**, 254 (1960).
[32] H. Olesen, B. Mansa, and K. Lind, *Scand. J. Haematol.* **1**, 257 (1964).
[33] W. N. Christenson and J. V. Dacie, *Brit. J. Haematol.* **3**, 153 (1957).
[34] E. C. Franklin and H. H. Fudenberg, *Arch. Biochem. Biophys.* **104**, 433 (1964).
[35] M. Harboe and J. Deverill, *Scand. J. Haematol.* **1**, 223 (1964).
[36] M. Harboe, *Ser. Haematol. Suppl. Scand. J. Haematol.* **4**, 65 (1965).
[37] A. S. Wiener, L. J. Unger, L. Cohen, and J. Feldman, *Ann. Internal Med.* **44**, 221 (1956).
[38] W. L. Marsh and W. J. Jenkins, *Nature* **188**, 753 (1960).
[39] D. M. Marcus, E. A. Kabat, and R. E. Rosenfield, *J. Exptl. Med.* **118**, 175 (1963).
[40] R. E. Rosenfield, R. Schroeder, R. Ballard, M. van der Hart, M. Moes, and J. J. van Loghem, *Vox Sanguinis* **9**, 415 (1964).
[41] N. Costea, V. Yakulis, and P. Heller, *Federation Proc.* **24**, 630 (1965).

on Sephadex G-200 permitted further purification because the initial effluent contained most of the antibody activity and γG-globulins. On ultracentrifugation these fractions were composed of equal parts of 7 S and 4 S components.[42] The γG-globulins in these eluates were measurable by specific quantitative precipitin tests and represented selection of Rh antibodies on the basis of their retentiveness because, when reabsorbed, they could be fully recovered in second eluates.[5] Immunoelectrophoresis reveals an uneven but long γ-line that is characteristic of electrophoretically heterogeneous γG-globulins.[5,43]

Isolated Rh antibodies from single serums occur as both K (type I) and L(type II) molecules.[43,44]

iii. Purification of Anti-A

Anti-A has been recovered at pH 3.8 from specific precipitates,[45] but more efficient recoveries are possible with insoluble polyleucyl derivatives containing A substance from which bound anti-A can be released with either *N*-acetyl galactosamine or acetate buffer, pH 3.6.[46] Eluates so prepared represented 11 to 34% of absorbed antibody and were 65 to 72% precipitable with A substance. If desired, stroma could be substituted in this method for insolubilized A substance, providing that substances of stroma origin in the eluate were recognized.

f. Recommended Methods

i. Purification of Human Blood Group Antibodies

(*a*) *Rh and Kell: Method of Kochwa and Rosenfield.*[5] Antibodies are applied to intact red cells at 37° and under equilibrium conditions of antibody excess (see Section B,2,c,i). Chilled sensitized cells are washed and then lysed in the cold with digitonin. These stroma, sedimented at 35,000 *g* for 30 minutes, are washed repeatedly in the cold until free of hemoglobin (see Section B,2,d,i). Bound antibodies are then dissociated with the application in the cold of 2 volumes of 0.1 *M* glycine buffer, pH 3.0, for each volume of starting intact packed red cells. The Rh and Kell sites of stroma are destroyed by this treatment so that essentially all bound antibody is recovered. The eluate, dialyzed against phosphate buffer, pH 7.3, can be concentrated by ultrafiltration.

[42] I. O. Szymanski, S. Kochwa, and R. E. Rosenfield, *Federation Proc.* **24**, 630 (1965).
[43] J. P. Leddy and R. F. Bakemeier, *J. Exptl. Med.* **121**, 1 (1965).
[44] M. Mannik and H. G. Kunkel, *J. Exptl. Med.* **118**, 817 (1963).
[45] H. G. Kunkel, M. Mannik, and R. C. Williams, *Science* **140**, 1218 (1963).
[46] M. E. Kaplan and E. A. Kabat, *J. Exp. Med.* **123**, 1061 (1966).

Additional purification can be achieved by gel filtration on Sephadex G-200.

The method of Rubin[11] is useful (see Section B,2,c,ii) for the rapid recovery of antibodies to be used only in serological tests.

(*b*) *Other Warm Reacting Blood Group Antibodies: Method of Landsteiner*[15] *and Landsteiner and Miller.*[9] See Section B,2,c,i.

(*c*) *Cold Agglutinins.* Cold agglutinins are absorbed at 0° to 1° either by intact cells or stroma and are readily released at 37° to 42°. Rarely, 46° is needed. Eluates made from intact cells will contain hemoglobin because of the intensity of cold agglutination. Some cold agglutinins will precipitate spontaneously in the cold.

(*d*) *Leukocyte Agglutinins.* Leukocyte agglutinins have been purified by absorption and elution.[47] For some antigens, bound antibody has been effectively recovered at pH 3.0, as described for stroma-bound antibody.[5] With other leukocyte antigens only heat[9] is effective.

ii. Purification of Nonhuman Antibodies

(*a*) *Mouse Antibodies.* In the H-2 system,[48] anti-D, anti-E, and anti-K have been recovered in acid eluates.[5] Thermal elution has also been employed.[49,50]

(*b*) *Dog Antibodies.* Rubinstein *et al.*[51] have purified antibodies to the genetically independent 1, 2, 3, 4, 5, 6, 7, and 8 antigens of dogs by the acid elution method of Kochwa and Rosenfield.[5]

[47] P. Lalezari, *Transfusion* **5**, 135 (1965).
[48] G. Hoecker and P. Rubinstein, personal communication (1965).
[49] H. M. Cann and J. Herzenberg, *J. Exptl. Med.* **117**, 259 (1963).
[50] R. A. Spencer, *Transplantation* **3**, 563 (1965).
[51] P. Rubenstein, N. Mollen, and J. W. Ferrebee, personal communication (1965).

3. ISOLATION OF ANTIPROTEIN ANTIBODIES BY THE THIOLATED ANTIGEN METHOD*

In this general method,[1] a number of SH groups are attached chemically to the surface of a protein antigen molecule to give a thiolated antigen (T-Ag). The T-Ag is then used to precipitate the antibodies prepared against the unmodified antigen. On dissociation of the specific precipitate in acid solution, the addition of a bifunctional mercurial compound, 3,6-bis(acetoxymercurimethyl)dioxane (MMD), cross-links and precipitates the T-Ag. The antibodies largely remain in solution.

*** Section 3,B,3 was contributed by S. J. Singer.**

[1] S. J. Singer, J. E. Fothergill, and J. R. Shainoff, *J. Am. Chem. Soc.* **82**, 565 (1960).

The thiolation of the protein antigens is carried out essentially by the method of Benesch and Benesch,[2] utilizing the reaction of *N*-acetylhomocysteine thiolactone (AHT) with the ϵ-NH_2 groups of lysine residues. About 3 to 4 SH groups per mole of protein of 20,000 molecular weight can be attached to a protein antigen surface without appreciably affecting the capacity of the antigen to combine with its antibodies. Care must be taken to prevent the oxidation of the SH groups on the T-Ag and to find conditions for the antibody precipitation under which the T-Ag is not itself molecularly aggregated. For the antigens bovine serum albumin (BSA), ovalbumin (OA), and bovine pancreatic ribonuclease (RNase), the following procedure (illustrated for OA) gives satisfactory yields of their respective pure rabbit antibodies.

To 4.5 ml of an 8.8% solution of OA in water are added 3.0 ml of carbonate buffer, pH 10.7 (170 gm of anhydrous K_2CO_3 and 15 gm of anhydrous $NaHCO_3$ per liter) and 0.30 gm of AHT in 1.5 ml of water. All three solutions are brought to 0° before being mixed. The reaction is stopped after 2 hours at 0°, by dilution with 30 ml of phosphate buffer, pH 6.8, ionic strength 0.4 (any concentrated neutral buffer will do), and the mixture is then dialyzed against phosphate buffer, pH 7.0, ionic strength 0.05, until essentially all the hydrolyzed and unhydrolyzed AHT is removed. (Passage of the mixture through Sephadex G-25 could probably replace this dialysis step.) In this latter buffer, all three T-Ag's are monodisperse and fairly stable, particularly if stored under nitrogen.

The γ-globulin fractions of the rabbit antisera are first prepared by precipitation with 40% saturated $(NH_4)_2SO_4$. Precipitin titrations are carried out with the specific T-Ag to determine the equivalence point. The specific precipitate is then made at the equivalence point in the phosphate buffer, pH 7.0, ionic strength 0.05. The mixture is kept at 37° for about 15 minutes and then at 4° for another 2 hours. (This short period is used to minimize the oxidation of SH groups and to permit maximal dissociation of the precipitate in the following step.) The precipitate is centrifuged and washed thoroughly with the phosphate buffer. It is then rapidly dissolved at 4° in a glycine–H_2SO_4 buffer, pH 2.4, ionic strength 0.35 (32 gm of glycine and 230 ml of 1 *N* H_2SO_4 per liter of solution). To this solution is added a 10^{-3} *M* solution of MMD in water in an amount about 1.5 times that required to titrate the SH groups on the T-Ag present in the solution. (As long as the MMD is in excess, the degree of excess does not appear to be critical.) A precipitate of the cross-linked T-Ag appears almost immediately and is allowed to form for an hour at 4° before it is centrifuged. The super-

[2] R. Benesch and R. E. Benesch, *J. Am. Chem. Soc.* **78**, 1597 (1956).

natant is adjusted to about neutral pH with a phosphate buffer, pH 7.2, ionic strength 1.0. After it has been allowed to stand overnight, the small amount of precipitate that usually forms is removed by centrifugation. The supernatant contains the pure antibodies. The yields obtained were about 45% for anti-OA, 75% for anti-BSA, and 25% for anti-RNase antibodies.

A number of different SH titration procedures might be equally useful. A modified nitroprusside method is quite satisfactory. A rough titration is first carried out on a 1.0-ml sample of about 1% T-Ag solutions by adding an equal volume of carbonate buffer, pH 10.6, ionic strength 0.4, one drop of a 10% sodium nitroferricyanide solution, and a sufficient measured volume of the MMD to discharge the pink color of the indicator. A more accurate titration is then made by adding about 90% of the previously determined volume of titrant to the T-Ag solution before the addition of the carbonate buffer and indicator, and then completing the titration as before. This procedure is required because of the rapid oxidation of SH groups at high pH.

MMD is easily made as follows. One hundred grams of mercuric acetate is added directly and with vigorous agitation to 42.5 ml of allyl alcohol and 0.5 ml of glacial acetic acid over a period of about 2 minutes. The reaction mixture is maintained at a temperature below 70° by several immersions in an ice bath. On subsequent cooling, a white crystalline product is obtained, which is recrystallized twice from hot water. Occasionally, supersaturated solutions form which require seeding to promote crystallization.

4. DISSOCIATION OF ANTIGEN–ANTIBODY COMPLEXES IN A SALT-FREE MEDIUM WITH CARBON DIOXIDE*

a. Introduction

Treatment with salt-free saturated aqueous carbon dioxide at pH 5 is a general method for dissociating antigen–antibody complexes.[1] The extent of the dissociation depends on the nature of the antigen and the course of immunization used to produce the antibody. It varies between complete dissociation of antigen from antibody (for example, for a hemoglobin complex) to the liberation of a small amount of antibody

* Section 3,B,4 was contributed by B. T. Tozer.

[1] B. T. Tozer, K. A. Cammack, and H. Smith, *Biochem. J.* **84**, 80 (1962).

from a residual insoluble complex (for example, for a complex of a pneumococcal polysaccharide).

b. Preparation of Specific Precipitates for Dissociation by Carbon Dioxide

Antiserum (3 volumes, heated at 56° for 30 minutes to inactivate complement) or concentrated immune γ-globulin solution (1 volume) is diluted to 10 volumes with phosphate-buffered saline (pH 7.0; 1.10 gm of $NaH_2PO_4 \cdot 2H_2O$, 4.53 gm of $Na_2HPO_4 \cdot 12H_2O$, and 7.31 gm of NaCl per liter). The calculated amount of antigen (0.1% in phosphate-buffered saline) is added, and the mixture is diluted to 15 volumes. The mixture is kept for 1 hour at 20° and for 16 hours at 2° to 3°; the precipitate is then centrifuged, washed twice with phosphate-buffered saline (2×15 volumes at 1100 *g* for 20 to 30 minutes), and three times with deionized water (2×15 volumes and 1×4 volume, at 15,000 *g* for 20 minutes). The final sludge contains approximately 1 gm of specific precipitate per 7 gm of water.

i. Treatment with Carbon Dioxide

The salt-free precipitate, prepared as described above, is mixed with aqueous CO_2 (sufficient to provide an approximately 1 to 2% w/v solution of precipitate) and transferred to the apparatus described below for saturating the solution with CO_2. A vertical stoppered tube (8 or 27 ml in total capacity to take volumes up to 4 and 20 ml, respectively), with a side arm for entry of water-washed CO_2 above the liquid level and an escape hole (1 mm in diameter) in the stopper, is fixed at the center of a hinged horizontal platform. The front edge of the platform is raised by a double-lift cam driven by a fractional-horsepower geared motor. The dimensions of cam and platform are such that the tube undergoes a vertical lift of 4 mm and an angular displacement of 5 degrees at speeds up to 110 cpm. This gentle agitation dissolves CO_2 and avoids surface denaturation.

The gas (50 to 100 ml/min) is passed for 1 to 2 hours to effect maximum solution of protein, which usually dissolves completely.

ii. Subsequent Treatment

Operations with CO_2 solutions (for example, the filling of centrifuge tubes or ultracentrifuge cells) are performed in an atmosphere of at least 95% CO_2 which can be maintained in a glove box, or more simply in a flat-bottomed glass cylinder (6 inches in diameter, 6 inches high), by the passage of CO_2 (2 liters/min).

The dissolved complex can be examined analytically in the ultracentrifuge to indicate the extent of the dissociation, and the immunoglobulin isolated by one of the following methods:

(a) Sparingly soluble complexes ($\leq 0.1\%$) are extracted with aqueous CO_2, and the immunoglobulin-containing extract is separated by centrifugation.

(b) When solubility of the complex exceeds 0.1% and the differential in sedimentation coefficient between residual complex and immunoglobulin is adequate, the latter may be isolated by preparative ultracentrifugation of the complex in aqueous CO_2 solution.

(c) When dissociation is complete and method b is not applicable, separation of immunoglobulin from antigen may be achieved by column chromatography.

iii. Typical Yields

Typical yields are given in Table I. Any residual complex in the separated immunoglobulin solution is precipitated by addition of sodium

TABLE I
METHOD OF ISOLATION AND YIELD OF IMMUNOGLOBULINS

Source	Antigen	Yield (%)	Method
Rabbit	*Pasteurella pestis*, antigen 3	12	(a)
Rabbit	Pneumococcus polysaccharide S111	30	(a)
Horse	Pneumococcus polysaccharide S1	48	(a)
Rabbit	Horse hemoglobin	15–25	(c) with $(Ca)_3(PO_4)_2$ or Sephadex G-50
Rabbit	Horse hemoglobin	40–50	(c) with carboxymethylcellulose chromatography
Rabbit	Ovalbumin	15–25	(b)[a]
Rabbit	Bovine serum albumin	15–25	(b)[a]

[a] Analytical ultracentrifugation suggests that method b could have been applied with equal success to diphtheria toxin–horse antitoxin complex and to rabbit immunoglobulin complexes of the following antigens: human serum albumin, horse serum albumin, sperm whale myoglobin, lysozyme, ribonuclease, and the polysaccharides (degraded and undegraded) of *Shigella dysenteriae*.

chloride (0.9%) and by removal of the CO_2 (1 to 2 hours at 10 cm Hg pressure over potassium hydroxide). Further details of these methods have been described.[1]

5. PURIFICATION OF ANTIBODIES TO THE 2,4-DINITROPHENYL (DNP) GROUP AND TO THE 2,4,6-TRINITROPHENYL (TNP) GROUP*†

a. General Considerations

It seems to be generally expected that methods for the purification of antibodies ought to be rigorously standardized, as are, for example, those used for the purification of enzymes. This expectation is unrealistic, since populations of antibody molecules of any particular specificity are heterogeneous in physicochemical properties and in their affinity for the corresponding antigenic determinant. Variations in these properties in different sera markedly influence the yield of purified antibodies.

The two procedures described below (Sections B,5,b and B,5,c) for isolating anti-DNP‡ antibodies from serum are both modifications of the procedure described earlier by Farah *et al.*[1] In addition, some procedures[2] for isolating anti-TNP antibodies will be considered under Section B,5,d. The various methods will be compared with respect to yield, purity, and affinity of the antibodies obtained as the final product.

By *yield* we mean the amount of antibody finally isolated, relative to what is initially precipitable from serum with the immunogen (usually DNP-BγG, or TNP-BγG, or another di- or trinitrophenylated protein similarly substituted in its lysine ϵ-NH_2 groups). By *purity* we refer to the extent to which the isolated antibody is specifically precipitated by the corresponding DNP- or TNP-protein. *Affinity* refers to the average intrinsic association constant for the reversible binding of a univalent ligand by the isolated antibody molecules.

i. Principles

The following methods apply particularly to antisera prepared against dinitrophenylated bovine γ-globulin (DNP-BγG) in which almost all

*** Section 3,B,5 was contributed by Herman N. Eisen, Walter Gray, J. Russell Little, and Ernest S. Simms**

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‡ Abbreviations used: DNP = 2,4-dinitrophenyl; TNP = 2,4,6-trinitrophenyl; BγG = bovine γ-globulin; HSA = human serum albumin.

[1] F. S. Farah, M. Kern, and H. N. Eisen, *J. Exptl. Med.* **112**, 1195 (1960).

[2] J. R. Little and H. N. Eisen, *Biochemistry* **5**, 3385 (1966).

lysine residues are substituted (see Chap. 1,E,3 for the preparation of DNP- and TNP-proteins). From these sera about 90% of the antibodies precipitated by the immunogen is usually precipitated by dinitrophenylated human serum albumin (DNP-HSA) in which, again, almost all lysine residues are substituted. These antibodies may thus be regarded as specific for DNP-lysyl, probably the only immunogenic group common to both dinitrophenylated proteins. We shall not consider antisera to lightly dinitrophenylated proteins, in which a substantial proportion of the antibodies is directed to the carrier protein.[3]

The main features of the present purification methods are best outlined in relation to the prototype procedure described previously.[1] Briefly stated, the older procedure is as follows:

1. Small aliquots of the antiserum are tested by the quantitative precipitin reaction with DNP-HSA to determine the concentration of precipitable anti-DNP antibody, and to establish the equivalent amount of DNP-HSA (the quantity needed per milliliter of serum for maximal precipitation).

2. Antibody is then precipitated from the desired volume of serum with the equivalent quantity of DNP-HSA.

3. The washed specific precipitate is suspended in buffered saline containing a high concentration of 2,4-dinitrophenol and streptomycin. The dinitrophenol extracts the antibodies specifically in the form of soluble complexes, and the streptomycin maintains the antigen, which is a polyanion, in insoluble form.

4. The soluble antibody–hapten complexes are then passed over a column of Dowex 1, which binds the dinitrophenolate ion tightly and allows the purified antibody to emerge from the column.

Yields with the foregoing procedure vary with the nature of the antiserum. Consider two types of sera: (1) Those populated by anti-DNP molecules of low affinity for the DNP-lysyl group; these are designated low-affinity sera, and they are obtained early after immunization. (2) Those populated by anti-DNP molecules of high affinity for the DNP-lysyl group; these are designated high-affinity sera, and they are usually obtained several months after immunization, especially after booster injections.

With low-affinity sera the yield with the method outlined above varies from 20 to 40%,[1] but with high-affinity sera the yield is often less than 10%.

The yield can be improved by extracting antibodies from precipitates with ϵ-DNP-lysine or ϵ-DNP-aminocaproate. These haptens, bound

[3] H. N. Eisen, M. E. Carsten, and S. Belman, *J. Immunol.* **73**, 296 (1954).

much more strongly than dinitrophenol by anti-DNP molecules,[4] solubilize virtually all precipitated anti-DNP antibodies. However, it is correspondingly difficult to separate these haptens from the isolated antibodies. Separation has been achieved through the use of large Sephadex columns (G-25) saturated with 2,4-dinitrophenol to exchange the strongly bound haptens for 2,4-dinitrophenol; the latter could then be easily removed on columns of Dowex 1.[5] Though cumbersome, this procedure gave considerably improved yields—about 60%—even with sera obtained 2 months after immunization.

ii. Current Methods

In the past few years three modifications of the foregoing methods have been used in this laboratory.

1. For high-affinity anti-DNP sera, trinitrophenylated human serum albumin (TNP-HSA) is used to precipitate the anti-DNP antibodies from serum. This heterologous antigen, also with most of its lysine residues substituted, is useful only with late bleedings: it is bound sufficiently well by high-affinity anti-DNP antibodies to precipitate them efficiently; but it does not compete effectively with 2,4-dinitrophenol, which can then solubilize almost all of the precipitated anti-DNP molecules, including those of very high affinity.

2. In another procedure, useful especially with low-affinity and moderately high-affinity sera, DNP-HSA is used to precipitate anti-DNP molecules, but DNP-glycine is used instead of 2,4-dinitrophenol to extract the precipitated antibodies. DNP-glycine is bound more strongly than dinitrophenol[4] and competes more efficiently with DNP-HSA for precipitated antibody molecules. Hence it dissolves completely almost all specific precipitates. The DNP-glycine is readily removed from low-affinity antibody molecules by passage through Dowex 1; but this hapten is bound too strongly by high-affinity antibodies to be removed in this fashion, and it is necessary to exchange it with dinitrophenol, using the dinitrophenol-saturated Sephadex G-25 columns.[5] Following exchange, the dinitrophenol is readily removed by Dowex 1 columns, even from high-affinity anti-DNP molecules.

3. We no longer rely on streptomycin to keep DNP- or TNP-proteins insoluble during the extraction of specific precipitates. Instead, the specifically dissolved precipitates—containing soluble antibody–hapten complexes, free hapten, and free antigen—are passed initially through a DEAE-cellulose column; the substituted proteins, which are highly

[4] H. N. Eisen and G. W. Siskind, *Biochemistry* 3, 996 (1964).

[5] H. N. Eisen, *in* "Methods in Medical Research" (H. N. Eisen, ed.), Vol. 10, p. 94. Year Book Publishers, Chicago, 1964.

anionic, are retained, but the soluble antibody–hapten complexes are not. The latter are then passed through Dowex 1, which binds DNP-glycine or dinitrophenol. As is shown in Fig. 1, a double-layered column is convenient: DEAE-cellulose on top of Dowex 1.

The effectiveness of the heterologous antigen, TNP-HSA, depends on the extent to which it precipitates anti-DNP molecules.

In sera obtained in 1 to 2 weeks after immunization, the proportion of anti-DNP antibodies precipitated by TNP-HSA is so low that it is necessary to use dinitrophenylated protein as the precipitating agent; we then use 0.1 *M* DNP-glycine to elute the precipitated antibodies.

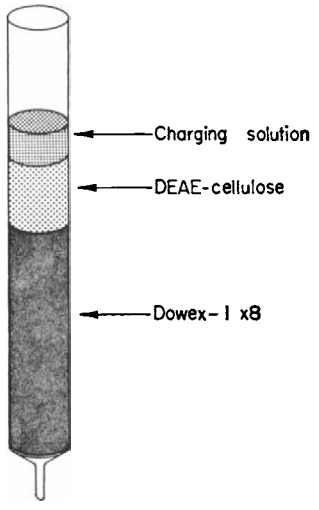


FIG. 1. Relative volumes of packed resin beds and "charging" solution in a double-layered column.

In sera obtained 1 to 2 months after immunization, TNP-HSA usually precipitates about 50 to 70% of the anti-DNP molecules. Though the heterologous antigen may be used, we generally prefer with these sera to use DNP-HSA to precipitate and DNP-glycine to elute antibodies.

In sera obtained 3 months or more after immunization, and especially after booster injections of the immunogen, TNP-HSA usually precipitates 85 to 95% of the antibodies precipitated by DNP-HSA. For these sera we select TNP-HSA for precipitation and 2,4-dinitrophenol for extraction.

The choice of reagents depends primarily, therefore, on establishing with each serum the precipitating effectiveness of TNP-HSA and DNP-HSA.

b. Use of TNP-HSA and Dinitrophenol to Isolate Anti-DNP Antibodies of High Affinity

i. Procedure

Five hundred milliliters of a serum pool* containing 3.3 mg and 3.2 mg of anti-DNP antibody per milliliter, as estimated by precipitin reactions with DNP-HSA and TNP-HSA, respectively, were cleared by centrifugation in the cold for several hours at about 10,000 *g* and then mixed with 90 mg of TNP-HSA. After incubation at 37° for 1 hour and at 4° overnight, the precipitate was collected by centrifugation and washed by alternate cycles of centrifugation and resuspension of the precipitate in cold 0.15 *M* NaCl. Washing was continued until the last two supernatants remained free of turbidity in 5% trichloroacetic acid.

The washed precipitate was then suspended in 110 ml of 0.1 *M* 2,4-dinitrophenol in buffered saline (0.15 *M* NaCl–0.01 *M* K-phosphate, pH 7.4 to 7.7) for 1 hour at 37°. (The volume is so chosen that the extracted antibody will be at a concentration of 5 to 15 mg/ml.)

After centrifugation at room temperature the supernatant (referred to below as the "charge") was passed through the following double-layered ion exchange column. Lower layer: Dowex 1 (×8) 200 to 400 mesh, 4.5 × 23.2 cm, equilibrated with buffered saline; upper layer: DEAE-cellulose, 4.5 × 8.1 cm, equilibrated with buffered saline. For 1 volume of "charge" we generally use 1 volume of packed DEAE-cellulose and 3 volumes of packed Dowex 1; the latter could doubtless be reduced safely to 1.5 volumes, since Dowex 1 binds dinitrophenolate very effectively. The column was developed with buffered saline at a flow rate of 3 ml/min. The column and solution were maintained at 37°, as we have the impression that recovery of antibody from the column is greater at elevated temperature (37° to 45°).

Usually, as a precautionary measure, a small volume of 0.1 *M* dinitrophenol, just enough to color the DEAE-cellulose layer, is added before the column is charged with the antibody–hapten solution; an equal volume is added afterwards. Thereafter the column is perfused with buffered saline. This precaution is taken to minimize the possibility that the free hapten concentration might drop in the DEAE-cellulose portion of the column and lead to recombination of some proportion of the antibody with the antigen bound to the DEAE groups. The column effluent is monitored for protein by testing occasional drops with 7%

* Rabbits were injected with 1.0 mg of DNP-*Limulus polyphemus* hemocyanin (in complete Freund's adjuvant, in footpads). The injections were repeated 3 and 7 months later. Bleedings were taken 10, 11, 16, and 18 days after the third injection and pooled.

perchloric acid. (The ion exchange resins are not used more than once because of difficulties in regenerating them.)

Effluent tubes containing protein were pooled, and the protein was concentrated by adding solid ammonium sulfate (0.3 gm/ml) slowly, with stirring. After standing overnight at 4° the precipitate was collected by centrifugation, dissolved in 125 ml of buffered saline, and dialyzed at 4°, first for 24 hours against several liters of buffered saline, and then (after a small amount of precipitated material had been removed) for about 20 hours against several liters of 0.001 *M* PO_4, pH 7.6. The protein that precipitated during the final dialysis was removed. The remaining soluble protein, representing the final product, was lyophilized.

Antibody isolated in 0.001 *M* PO_4 buffer may be stored for several days at 4° in this solvent, or it may be lyophilized. If it is to be frozen, however, the ionic strength should first be increased—for example, by adjusting to 0.15 *M* NaCl–0.01 *M* PO_4, pH 7.4 to 8.0; otherwise considerable amounts of precipitated protein will appear on thawing. When lyophilized, or frozen in buffered saline, the purified antibody remains active for prolonged periods (over a year).

ii. Analysis of Product

Yield. The yield after dialysis against buffered saline was 1.46 gm (88%); after the "euglobulin" precipitate that formed on dialysis against 0.001 *M* PO_4 had been removed the final yield was 0.846 gm (51%). Subsequent experience has shown that the precipitation of "euglobulin" antibody can be reduced by lowering the protein concentration, before the final dialysis, to 1 to 2 mg/ml.

Our usual experience in isolating anti-DNP antibodies from high-affinity sera by the method outlined above is as follows: 85 to 95% of the total antibody (that is, that precipitated by the immunogen) is precipitated by the cross-reacting antigen (TNP-HSA); about 85% of the precipitated antibody is extracted by elution with 2,4-dinitrophenol; about 15% of the extracted antibody is lost on the DEAE-cellulose–Dowex 1 column; and approximately 10% of the antibody eluted from the column is precipitated during the final dialysis. The overall yield is usually 50 to 60% (51% in the example given above). This is about ten times greater than is achieved with similar sera when the initial precipitation is made with DNP-HSA instead of TNP-HSA, and the rest of the steps are the same.

Purity. The final product was over 96% precipitable by DNP-HSA. As a rule the isolated antibodies are at least 90% precipitable with DNP-HSA; they also sediment as a single symmetrical boundary of 6.5 S (uncorrected) and form a single line of "γG" mobility by immuno-

electrophoresic analysis developed with polyvalent goat antiserum to rabbit immunoglobulin. (See *Note Added in Proof.*)

Affinity. The antibody isolated by the representative procedure given above bound ϵ-DNP-lysine and 2,4-dinitroaniline too strongly to permit measurements of association constants by fluorescence quenching ($K_0 \geq 1 \times 10^9\ M^{-1}$).

iii. Comment

The following two observations indicate that small losses of antibody on the DEAE-cellulose–Dowex 1 column are probably due to specific binding of antibody by the antigen or the hapten, both of which are tightly bound to ion exchange residues. First, rabbit γG-immunoglobulin (not anti-DNP) was recovered from these columns in virtually 100% yield. Second, when a sample of purified anti-DNP antibody was mixed, for control purposes, with 2,4-dinitrophenol and with TNP-HSA and then passed through a representative column, only 80% of the antibody was recovered. The loss was not evidently selective, since the antibody emerging from the column was not distinguishable from the starting antibody with respect to affinity for 2,4-dinitrotoluene and 2,4,6-trinitrotoluene, measured by fluorescence quenching.

Cheng and Talmage[6] have recently described a purification method in which anti-DNP antibodies are first isolated from serum by precipitation with a triply substituted protein (T-protein) containing *o*- and *p*-mononitrophenyl and 2,4,6-trinitrophenyl groups. Their procedure and that described above are otherwise essentially the same. The T-protein apparently precipitated virtually all the anti-DNP antibody from their sera, whereas TNP-protein precipitated only 60%.

Anti-DNP antibodies bind *o*- and *p*-mononitrobenzenes very weakly[4]; hence TNP- and T-proteins should be functionally almost identical. The superior precipitating effectiveness of T-proteins might not arise from a specific binding contribution of mononitrophenyl groups; it could be due to gross conformational changes in the T-protein (perhaps from substitution of mononitrophenyl groups in tyrosine and other residues) that increase nonspecifically the protein's effectiveness as a precipitating antigen. In any event we anticipate that T-proteins, as TNP-proteins, would also not be useful with low-affinity anti-DNP sera. With high-affinity sera they would seem not to offer a real advantage over TNP-proteins, which are much simpler to prepare and to characterize; but with sera of intermediate affinity they may indeed offer a significant advantage, as suggested by the results of Cheng and Talmage.[6]

[6] W. C. Cheng and D. W. Talmage, *J. Biol. Chem.* **240**, 3530 (1965).

c. Use of DNP-HSA and DNP-Glycine to Isolate Anti-DNP Antibodies of Low or High Affinity

There are two main differences between this procedure and that described in Section B,5,b,i. First, antibodies are precipitated from serum with DNP-HSA. Second, the washed specific precipitates are extracted with 0.1 *M* DNP-glycine in buffered saline, pH 8.5.

For the antibody recovered from the DEAE-cellulose–Dowex 1 column (see Fig. 1), as in Section B,5,b,i, the ratio of A_{278}/A_{360} indicates the extent to which DNP-glycine has remained associated with antibody. We assume that the A_{360} reading is due entirely to DNP-glycine and subtract from the gross A_{278} the contribution made by this hapten

$$[A_{278} - (0.36)(A_{360}) = \text{net } A_{278}].$$

The net A_{278} is used to estimate the antibody concentration

$$(E^{1\%}_{1cm} = 15.5),$$

and the A_{360} the DNP-glycine concentration (ϵ_M = 15,890 at 360 mμ). These estimates may be slightly in error because the absorption spectra of bound and free DNP-glycine are probably slightly different.[4] (See Appendix 1, Vol. II for extinctions of immunoglobulins and haptens.)

With antibody preparations of low affinity, DNP-glycine in the column effluent commonly amounts to about 5 to 10 moles % of the total antibody concentration. Usually no further attempt is made to remove the residual bound hapten from these preparations. The antibody solution is then treated as in Section b,i—dialysis against buffered saline, removal of any precipitate that forms, dialysis against 0.001 *M* PO_4, pH 7.6, etc.

With antibody preparations of high affinity, however, the tenaciously bound DNP-glycine may amount to 80 moles % of the antibody concentration. Under these circumstances the antibody obtained from the DEAE-cellulose–Dowex 1 column is brought to 0.1 *M* 2,4-dinitrophenol and passed through a column of Sephadex G-25, prepared and developed with 0.1 *M* 2,4-dinitrophenol in 0.1 *M* Tris-Cl, pH 8.0.[5] During passage through this second column the bound DNP-glycine is exchanged with 2,4-dinitrophenol. It is desirable to use large columns, about 0.5 gm of Sephadex G-25 per milligram of antibody. (See Chap. 9, Vol. II for general procedures of gel filtration.) Occasionally aliquots of the effluent are monitored for protein by qualitative spot tests with acetone: 1 volume of effluent plus 2 volumes of acetone precipitates the protein. The tubes containing protein are pooled and passed through Dowex 1. The dinitrophenolate is selectively retained. In the effluent the persisting A_{360}

readings correspond to persistent DNP-glycine (or dinitrophenol), often at a level of about 15 moles % (versus about 80 moles % before exchange on Sephadex). The remaining steps—concentration of antibody with ammonium sulfate, dialysis against buffered saline, removal of any precipitate that forms, and final dialysis against 0.001 M PO_4, pH 7.6—are carried out as in Section B,5,b,i.

Yields of about 60% and 70% have been obtained with high-affinity and low-affinity sera, respectively, and affinities for ε-DNP-L-lysine have ranged from values that are too high for accurate measurement by fluorescence quenching ($>1 \times 10^9$ M^{-1}) to 1×10^5 M^{-1}, in accord with the type of antiserum used as starting material.

d. Purification of Antibodies Specific for the 2,4,6,-Trinitrophenyl Group

The principles involved in the isolation of anti-TNP antibodies are analogous to those described above for anti-DNP.[2]

Rabbit anti-TNP antibodies are usually purified by precipitation from antisera with DNP-HSA, which precipitates 50 to 90% of the total antibody present, depending on the average affinity of the antibody population in the serum; and by eluting the precipitates with 0.01 M 2,4,6-trinitrophenol at pH 7.4 to 7.6. The pH is critical, since picric acid is a potent protein denaturant at acid pH. The eluted antibody is applied to a DEAE-cellulose–Dowex 1 column equilibrated with buffered saline as in Section B,5,b,i, and the protein effluent is dialyzed against buffered saline, and then against 0.001 M PO_4, pH 7.4.

The final yield from high-affinity sera is approximately 40 to 50% when DNP-HSA is used for precipitation and trinitrophenol for elution. But the yield is only 10 to 20% from low-affinity sera when either DNP-HSA or TNP-HSA is used to form the initial specific precipitate, and trinitrophenol is used for elution; with DNP-HSA a substantial proportion of the antibody is not precipitated, and with TNP-HSA a substantial proportion is not eluted. The combination of TNP-HSA and a TNP-amino acid should improve the yield from low-affinity sera.

Purified rabbit anti-TNP antibody is also found to be approximately 95% precipitable with the immunogen. It forms a single line by immunoelectrophoresic analysis with polyvalent antiserum and has a sedimentation constant of 6.5 S (uncorrected). Average intrinsic association constants of anti-TNP antibodies for TNP ligands can be measured by fluorescence quenching and are found to be in the same range encountered with anti-DNP antibody binding of DNP ligands.[2] Similar degrees of heterogeneity with respect to binding constants are also observed.

Table I lists sample yields from serum pools derived from rabbits

TABLE I

VARIATIONS IN YIELD OF PURIFIED ANTI-TNP ANTIBODIES WITH TIME AFTER IMMUNIZATION

Rabbit antiserum pool	Time antiserum obtained after immunization	Reagents used to purify	Yield (%)
TN-8	16 days after first injection of TNP-BγG	TNP-HSA to precipitate TNP-OH to elute[a]	14.1
		DNP-HSA to precipitate TNP-OH to elute	12.0
TN-9	1 year after first injection, 14 days after second injection of TNP-BγG	TNP-HSA to precipitate TNP-OH to elute	7.0
		DNP-HSA to precipitate TNP-OH to elute	44.5

[a] TNP-OH is 0.01 *M* 2,4,6-trinitrophenol. [J. R. Little and H. N. Eisen, *Biochemistry*, 5, 3385 (1966).]

immunized with TNP-BγG in complete Freund's adjuvant and bled either early (low-affinity antisera) or late (high-affinity antisera) after immunization.

Note Added in Proof: Recent studies in this laboratory by E. W. Voss, Jr., have shown that anti-DNP antibodies of the γM-immunoglobulin class are not precipitated with DNP-HSA from the anti-DNP sera obtained early after immunization with dinitrophenylated protein. However, the γM anti-DNP antibodies can be isolated (usually along with much larger amounts of γG anti-DNP antibodies) by the use of a specific adsorbent in which DNP-HSA is linked to cellulose through acetyl bridges, prepared and used according to J. B. Robbins, J. Haimovich, and M. Sela, who kindly provided us with a manuscript of their work in advance of publication.

6. PURIFICATION OF ANTIBODIES TO CARBOHYDRATE HAPTENS*

a. INTRODUCTION

The haptenic group *p*-azophenyl-β-lactoside is a very useful hapten for the study of the specificity and thermodynamics of the antibody–antigen reaction. Since its lactose moiety is responsible for much of the specificity and combining energy of the interaction of this haptenic group

* Section 3,B,6 was contributed by Norman R. Klinman.

with antibody directed against it,[1] this hapten has proved useful also as a source of information relative to the specific interaction of naturally occurring carbohydrate antigens and their specific antibodies.

The intermediate *p*-aminophenyl-β-lactoside is synthesized by the method of Babers and Goebel,[2] and the compound can be diazotized to carrier proteins with relative ease.[3] These protein–hapten complexes may then be used for immunization and for precipitation of the anti-hapten antibody for immune sera. (See also Chap. 1,E,1.)

Such a precipitation is carried out as the first step in the purification of the antihapten antibody. After the precipitate is washed free of serum, the purification is completed by the separation of the anti-Lac antibody from the antigen. This separation is carried out by displacing the antigen from the antibody combining site by treating the complex sequentially with high concentrations of two compounds with decreasing affinity for the combining site and finally with buffer. Thus, in the first stage of purification, the specific precipitate is extracted with a large amount of highly concentrated lactose. By the law of mass action, the lactose is able to replace the higher affinity haptenic group in the combining site, and the complex is solubilized. The antibody is separated from the antigen by utilizing the differential properties of the two proteins. The lactose is then removed from the antibody by dialysis against galactose in high concentrations, and the relatively weakly bound galactose is removed by dialysis against sodium phosphate buffer.

Prior to specific precipitation, coprecipitating factors are removed from the immune serum by precipitation with a nonrelated antigen–antibody system. To avoid bacterial contamination, all procedures are carried out at 4°, and the serum is stored frozen.

b. Purification Procedure

i. Specific Precipitation

Specific precipitation of the anti-Lac antibody is carried out by the addition of antigen to the immune serum. This antigen should preferably be a hapten–protein complex not used for immunization; however, the immunizing antigen may be used if the serum is previously treated with the carrier protein to remove all the antiprotein antibody.[4] Lac-human serum albumin (Lac-HSA) is usually used in our laboratory as a precipitating antigen and not as an immunizing antigen. A quantitative

[1] F. Karush, *J. Am. Chem. Soc.* **79**, 3380 (1957).
[2] F. H. Babers and W. F. Goebel, *J. Biol. Chem.* **105**, 473 (1943).
[3] F. Karush, *J. Am. Chem. Soc.* **78**, 5519 (1956).
[4] N. R. Klinman, J. H. Rockey, and F. Karush, *Immunochemistry* **2**, 51 (1965).

precipitin test is carried out to determine the amount of Lac-HSA to be added[5] (see also Chap. 13, Vol. III). A concentration of Lac-HSA is selected which corresponds to the amount used to obtain maximum precipitation in the precipitin curve. An example of an equine serum with a fairly high anti-Lac antibody level showed a maximum precipitation of 2.14 mg of antibody with the addition of 40 μg N of Lac-HSA per milliliter of serum. For the specific precipitation of this serum, therefore, 40 mg N of Lac-HSA was used per liter of serum.

After the addition of Lac-HSA, the serum is mixed thoroughly and incubated for 1 hour at 37° and for 5 days at 4° with occasional mixing. The serum is then centrifuged at 1000 *g* for 1 hour at 4°, and the supernatant is decanted. The precipitate is washed five times in 0.02 *M* sodium phosphate buffer with 0.15 *M* sodium chloride at pH 7.2, followed by two washes with 0.02 *M* sodium phosphate alone. The buffer is at 4°, and the volume of each wash is equal to the original serum volume. Care is taken to suspend thoroughly the precipitate in the buffer, and the suspension is allowed to mix gently for at least 1 hour prior to centrifugation.

ii. Solubilization of the Antibody

After the precipitate has been washed it is extracted with 0.5 *M* lactose in 0.02 *M* sodium phosphate at pH 7.2. This extraction is carried out at 37° in a volume of lactose equal to one-twentieth to one-tenth the original volume of serum. The exact volume of lactose solution necessary and the duration of extraction vary with the amount of precipitate to be solubilized. The end point of solubilization is reached when either all the precipitate is dissolved or a constant amount of precipitate remains on re-extractions with lactose. Such residual precipitate rarely exceeds 10% of the total protein in the original precipitate, and usually all the precipitate is soluble in the lactose within 3 hours.

iii. Removal of Antigens

The solution containing lactose, solubilized anti-Lac antibody, and Lac-HSA is then placed on a DEAE-cellulose column.[6] The column should contain at least 5 gm of DEAE (1 meq/gm) per 100 mg of protein. For a solution of 50 ml containing approximately 1 gm of antibody–antigen mixture, a column containing 100 gm of DEAE-cellulose is used, measuring 5 cm by 60 cm. The column is equilibrated with a solution containing 0.5 *M* lactose and 0.02 *M* sodium phosphate, pH 7.2, and the protein is eluted with the same buffer. Since Lac-HSA is

[5] E. A. Kabat, "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.

[6] S. Utsumi and F. Karush, *Biochemistry* **3**, 1329 (1964).

a relatively negatively charged material, it remains close to the top of the column. The antibody elutes just after the void volume as a large peak containing approximately one third of the total antibody and a long tail containing another third of the protein. After 1 liter of the initial solvent has passed through, the eluent fractions contain little or no protein. To elute the remaining antibody protein, a linear gradient of sodium chloride is initiated in the same solvent running from 0 to 1.0 *M* in 1 liter. This elutes a second peak of antibody followed closely by a fraction containing an antigen–antibody mixture. If it is desired to separate the antigen and antibody in this mixture, the material may be fractionated by starch block electrophoresis in a barbital buffer solution of ionic strength 0.05, pH 8.6, in the presence of 0.5 *M* lactose, collecting the slower migrating fractions which contain antibody freed from antigens.[7]

An alternative method of precipitation of anti-Lac antibody eliminates the necessity of separating the antigen from the antibody by column chromatography. This method utilizes coprecipitation of the anti-Lac antibody and is especially useful for sera with large amounts of nonprecipitable antibody.[8] In this instance, the antigen used for immunization is employed without prior absorption of the serum with the carrier protein. The serum antibody specific for this carrier protein is first assayed by a quantitative precipitin test, and an amount of antigen is selected which, although in a region of antibody excess, causes a large amount of precipitation. Thus, in a serum where the precipitate at maximum contains 5 mg of protein per milliliter of serum including 2 mg of the carrier protein, 0.5 mg of the antigen, Lac-hemocyanin, is added to the serum for precipitation of the antiprotein antibody as well as the anti-Lac antibody. Incubation is carried out as with Lac-HSA, and the precipitate is washed in a solution containing 0.15 *M* sodium chloride and 0.02 *M* sodium phosphate, pH 7.2, until the supernatant shows a minimal constant level of protein. Lactose extraction is carried out as above, but at least three such extractions are made, using as an end point a minimal protein concentration in the supernatant. Since in this case only the antihapten antibody is solubilized, while the Lac-hemocyanin remains precipitated, further separation of antibody from the antigen is not necessary.

iv. Removal of Lactose

The antibody solution containing lactose is dialyzed against 0.002 *M* sodium phosphate, pH 7.2, to remove all the free lactose. It is then

[7] J. H. Rockey, N. R. Klinman, and F. Karush, *J. Exptl. Med.* **120**, 589 (1964).
[8] N. R. Klinman, J. H. Rockey, and F. Karush, *Science* **146**, 401 (1964).

advisable to concentrate the protein solution. This may be done by lyophilization, ultrafiltration, or pervaporation. The protein, at a concentration of 10 to 20 mg/ml, is then dialyzed against 0.1 *M* galactose in 0.02 *M* sodium phosphate at pH 7.2 to remove the bound lactose and replace it with galactose. Two changes of the galactose solution in a volume fifty to one hundred times the volume of the protein solution is sufficient. The antibody solution is then dialyzed against large volumes of 0.02 *M* sodium phosphate at pH 7.85 to remove the galactose.

c. Recovery and Purity of Antibody

i. Yields

The specific precipitate of anti-Lac antibody and Lac-HSA should be 90 to 100% solubilized by treatment with lactose. DEAE-cellulose chromatography at pH 7.2 in 0.02 *M* sodium phosphate and 0.5 *M* lactose yields 50 to 60% of the equine anti-Lac antibody placed on the column. A sodium chloride gradient up to 0.5 *M* yields another 30 to 40% of the antibody protein; however, much of this contains antigen which can subsequently be separated by starch block electrophoresis.

During removal of the lactose and galactose small amounts of precipitate form and are removed by centrifugation. If care is taken in the removal of Lac-HSA from gradient fractions, as much as 70% of the original antibody protein is recoverable in purified form, as determined by quantitative precipitation. The method of coprecipitation with an immunizing antigen has yielded, in sera assayed by quantitative precipitation, as much as 60% of the measurable antibody in purified form.

ii. Assay for Purity and Homogeneity

The purity of the antibody preparation may be tested in several ways. The presence of antigen is detectable by measuring the optical density of the preparation at 365 mμ, the wavelength of maximum absorption of the Lac haptenic group. It should be virtually absent. Immunoelectrophoresis against antiserum directed against horse serum components may be used both to detect nonimmunoglobulin contaminants as well as to assay for the heterogeneity of the immunoglobulins contained in the specific anti-Lac antibody population. While as many as six antigenically distinct immunoglobulins have been found to have anti-Lac activity from a single horse serum,[7] no nonimmunoglobulin components should be present.

The purity of the antibody may also be determined by its precipitability with Lac-HSA and its ability to bind Lac-dye. While the precipitability may vary with the antibody preparation, 7 S anti-Lac antibody

should bind 2 moles of Lac dye per 150,000 gm of protein.[1] Other tests such as ultracentrifugation and gel filtration may also serve as assays of the purity of the antibody preparation.

7. IMMUNOADSORBENTS: PREPARATION AND USE OF CELLULOSE DERIVATIVES*†

a. Introduction

An "immunoadsorbent" may be defined as an insoluble material which has the property of combining either with a specific antibody or with a specific antigen.‡ Within recent years, the term has been used to designate compounds consisting of an insoluble matrix to which either antigen or antibody has been irreversibly bonded without loss of specific combining capacity. In principle, such adsorbents can be used for the isolation and purification of antibodies and antigens, as an analytical tool for the detection and estimation of antibody and antigen, or for the study of the physical properties of antigen–antibody interactions. Although powdered cellulose (solely considered here) has been commonly used as the insoluble supporting matrix, investigators have utilized other materials, such as polystyrene[11–13] and polyacrylic[3] ion exchange resins,

* Section 3,B,7 was contributed by Dan H. Campbell and Norman Weliky.

† Contribution No. 3383 from the California Institute of Technology.

‡ Although the present chapter is concerned with the synthesis and use of artificial adsorbents, certain natural adsorbents have been investigated for the isolation of antibody—for example, bacteria,[1] erythrocytes,[2–8] and recently protein antigens insolubilized by mild denaturation[9] or polymerization of antibody with bifunctional coupling agents such as bisdiazotized benzidine[10] for the isolation of antigens.

[1] C. E. Jenkins, *Brit. J. Exptl. Pathol.* **27**, 111 (1946).
[2] K. Landsteiner and G. P. Miller, Jr., *J. Exptl. Med.* **42**, 853 (1925) (in Kabat[7]).
[3] H. C. Isliker, *Ann. N.Y. Acad. Sci.* **57**, 225 (1953).
[4] R. Weber, *Vox Sanguinis* **1**, 37 (1956).
[5] E. A. Kabat, "Blood Group Substances." Academic Press, New York, 1956.
[6] H. H. Fudenberg and H. G. Kunkel, *J. Exptl. Med.* **106**, 689 (1957).
[7] E. A. Kabat, "Kabat and Mayers' Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.
[8] H. S. Goodman, *Nature* **193**, 350 (1962); **194**, 134 (1962).
[9] N. M. McDuffie and G. B. Sutherland, *J. Immunol.* **90**, 438, 444 (1963).
[10] S. DeCarvallo, A. J. Lewis, H. J. Rand, and J. R. Uhrick, *Nature* **204**, 265 (1964).
[11] L. Gyenes, B. Rose, and A. H. Sehon, *Nature* **181**, 1465 (1958).
[12] L. Gyenes and A. H. Sehon, *Can. J. Biochem. Physiol.* **38**, 1235 (1960).
[13] Y. Yagi, K. Engel, and D. Pressman, *J. Immunol.* **85**, 375 (1960).

silk fibroin,[14] organic polymer matrix,[15] antigen-coated glass beads,[16] and cross-linked proteins.[17] For general reviews dealing with the various types of adsorbents including cellulose derivatives, reference should be made to the following: Isliker,[3] Marrack,[18] Kabat,[7] Sehon,[19] Gurvich,[20] Manecke,[21] Singer,[22] and Weliky and Weetall[23]; see also Table I.

One of the first investigations making use of a cellulose derivative for the isolation of antibody was described by Campbell *et al.*,[24] who coupled bovine serum albumin (BSA) to the diazonium salt of *p*-aminobenzylcellulose powder. Antibody in good yield and purity was obtained from serum of rabbits immunized with BSA. Such adsorbents can also be used for the isolation of nonprecipitating antibodies such as reagins from serum of allergic patients as shown by Malley and Campbell[25] using a pollen antigen conjugated to cellulose. Webb and LaPresle[26] isolated antibodies to fragments of digested protein antigens, and Jagendorf *et al.*[27] and Weetall and Weliky[28] have recently described the preparation of cellulose–antibody adsorbents for the isolation of specific antigens. The use of a cellulose immunoadsorbent for the study of the heterogeneity of antibody interaction with antigen was first reported by Lerman.[29] Similar studies have been reported by Gurvich *et al.*[30] and by Weliky and Weetall.[31] The combining strength of a simple hapten with a complementary antibody combining site is increased many times when the hapten is attached to an insoluble matrix. When coupled to cellulose,

[14] N. Weliky, H. H. Weetall, R. V. Gilden, and D. H. Campbell, *Immunochemistry* **1**, 219 (1964).
[15] P. Bernfeld and J. Wan, *Science* **142**, 679 (1963).
[16] G. B. Sutherland and D. H. Campbell, *J. Immunol.* **80**, 294 (1958).
[17] K. Onoue, Y. Yagi, and D. Pressman, *Immunochemistry* **2**, 181 (1965).
[18] J. R. Marrack, *Med. Res. Council, Spec. Rep. Ser.* **230** (1958).
[19] A. H. Sehon, *Brit. Med. Bull.* **19**, 183 (1963).
[20] A. E. Gurvich, *in* "Immunological Methods" (J. F. Ackroyd, ed.). Davis, Philadelphia, 1964.
[21] G. Manecke, *Naturwissenschaften* **51**, 25 (1964).
[22] S. J. Singer, *in* "The Proteins" (H. Neurath, ed.), Vol. 3, Academic Press, New York, 1965.
[23] N. Weliky and H. H. Weetall, *Immunochemistry* **2**, 293 (1965).
[24] D. H. Campbell, E. Luescher, and L. S. Lerman, *Proc. Natl. Acad. Sci. U.S.* **37**, 575 (1951).
[25] A. Malley and D. H. Campbell, *J. Am. Chem. Soc.* **85**, 487 (1963).
[26] T. Webb and C. LaPresle, *Biochem. J.* **91**, 24 (1964).
[27] A. T. Jagendorf, A. Patchornik, and M. Sela, *Biochim. Biophys. Acta* **78**, 516 (1963).
[28] H. H. Weetall and N. Weliky, *Biochim. Biophys. Acta* **107**, 150 (1965).
[29] L. S. Lerman, *Nature* **172**, 635 (1953).
[30] A. E. Gurvich, R. B. Kapner, and R. S. Nezlin, *Biokhimiya* **24**, 144 (1959).
[31] N. Weliky and H. H. Weetall, *Abst. 148th Meeting, Am. Chem. Soc.* (1964).

therefore, the haptenic group functions as a good specific adsorbent but will still release antibody in the presence of soluble hapten (see Lerman[29]).

Most of the technical problems associated with the use of cellulose adsorbents will also be involved with other types of insoluble supporting materials, but each will have one or more unique properties which provide them with advantages as well as disadvantages.

b. General Procedures and Technical Considerations

Although the preparation and use of immunoadsorbents are relatively simple in principle, many factors must be taken into consideration. Some of the more important are as follows:

i. Selection and Treatment of Starting Material

Selection of material will depend to some extent on what is the most convenient or procurable, and the chemical nature of the immunological material to be coupled to the insoluble matrix. Regardless of whether one starts with plain purified cellulose or with a derivative such as carboxymethylcellulose, the material should be thoroughly washed with dilute acid (pH 1 to 2) and dilute alkali (pH 9 to 10). At times it is even desirable to wash with an organic solvent such as methanol. At some point during the washing procedures it is desirable to allow the suspension to settle for 15 minutes to one-half hour and to decant or siphon off the supernatant in order to remove the "fines." One must also keep in mind that different batches of commercial products may vary in their properties—both physical and chemical.

ii. Capacity of an Adsorbent

This property depends on the number of specific groups attached to the cellulose and also on the molecular size of both the specific unit of adsorbent (protein, polysaccharide, or simple hapten) and the material to be adsorbed. Since the latter is usually antibody, one can calculate roughly, on the basis of glucose units, an equivalent of a single antibody molecule. Thus, a 7 S γ-globulin, having a major to minor axial ratio of 300 A/38 A, would be expected to cover several hundred glucose units if oriented parallel to the long axis of the cellulose fiber. When a simple haptenic group such as arsanilic acid is attached to cellulose, one must consider that some will probably be attached within the fibrous structure of cellulose and not be available for reaction with large molecules owing to steric hindrance.

The adsorbent may be used either as in column chromatography or

TABLE I
ISOLATION OF ANTIBODY BY THE USE OF SPECIFIC INSOLUBLE ADSORBENTS

Carrier	Antigen	Elution solvent	Antibody (mg/gm adsorbent)	Overall yield (%)	Recovery from adsorbent (%)	Precipitable protein (%)	Ref.
Charcoal	Protein azobenzoate	0.1 *M* acetic acid	1	—	—	30–60	(*d*)
Glass beads	Ovalbumin	Glycine–HCl, pH 3.0	5	—	90	80	(*e*)
RBC stroma	Azohaptens	Acetic acid, 0.01 *M*	—	—	—	50	(*f*)
RBC stroma	Azophenyl arsonate	Sodium arsanilate	10	30–65	30–65	50–67	(*g*)
RBC stroma	Azophenyl arsonate	HCl, pH 3.2	10	40–50	40–50	40–50	(*g*)
Silk (fibroin)	Azophenyl arsonate	HCl, pH 3.2	16	36	78	100	(*h*)
Tanned RBC stroma on IRA 410 resin	Blood group factor B	Sugars at 37°	—	—	55–65	75–90[a]	(*i*)
Cross-linked protein	Azohaptens	Hapten	>1500	60–80	82–86	>90[b]	(*j*)
Cross-linked protein	Bovine serum albumin	Glycine–HCl, pH 2.3	400–700	up to 90	90	88	(*j*)
Cross-linked protein	Insulin, human serum albumin	Glycine–HCl, pH 2.3	100–130	—	—	72–80[c]	(*j*)
Polyacrylic acid	Human serum albumin	pH 3.6	—	44	54	84	(*i*)
Polyaminopolystyrene	Bovine serum albumin	Citrate–phosphate, pH 3.1	5	27–45	30–45	52	(*k*)
Polyaminopolystyrene	Ovalbumin	Citrate–phosphate, pH 3.1	6	26	42	63	(*k*)
Polyaminopolystyrene	Human γ-globulin	HCl, pH 3.0	3	—	35	82	(*l*)
p-Amino-benzylcellulose	Bovine serum albumin	HCl, pH 3.2	15	86	86	89	(*m*)
p-Amino-benzylcellulose	Human γ-globulin	0.2 *M* citric acid, pH 3.2	—	—	25–67	—	(*n*)
p-Amino-benzylcellulose	Human γ-globulin	pH 3.2	—	—	25	—	(*n*)

m-Aminobenzyloxymethylcellulose	Human serum albumin, rabbit γ-globulin, human γ-globulin	HCl, pH 3.2	100–150	—	40–60	90–100	(*o*)
Half benzidine amide of carboxymethylcellulose	Bovine serum albumin	HCl, pH 2.3	14	87–97	87–97	80–82	(*p*)
Aminoethylcellulose	6-Carboxypurine	HCl, pH 2.3	14	83–91	83–91	90	(*q*)
Carboxymethyl cellulose	Bovine serum albumin, human γ-globulin, azophenyl arsonate	HCl, pH 2.0–2.3	—	71–96	78–100	86–100	(*h*, *p*)
Tyramine amide of carboxymethylcellulose	Azophenyl arsonate	0.05 *M* phosphate at pH 3.0, and 0.1 *M* HCl	—	—	75–80	70	(*r*)

[a] Identified as specific antibody.
[b] By equilibrium dialysis.
[c] Method of Berson and Yalow.
[d] H. Svensson, *Arkiv Kemi Mineral. Geol.* **17A,** 1 (1943).
[e] G. B. Sutherland and D. H. Campbell, *J. Immunol.* **80,** 294 (1958).
[f] K. Landsteiner and J. van der Scheer, *J. Exptl. Med.* **63,** 325 (1936).
[g] A. Froese and A. H. Sehon, *Am. J. Biochem. Physiol.* **39,** 1067 (1961).
[h] N. Weliky, H H. Weetall, R. V. Gilden, and D. H. Campbell, *Immunochemistry* **1,** 219 (1964).
[i] H. C. Isliker, *Ann. N.Y. Acad. Sci.* **57,** 225 (1953).
[j] K. Onoue, Y. Yagi, and D. Pressman, *Immunochemistry* **2,** 181 (1965).
[k] Y. Yagi, K. Engel, and D. Pressman, *J. Immunol.* **85,** 375 (1960).
[l] L. Gyenes and A. H. Sehon, *Can. J. Biochem. Physiol.* **38,** 1235 (1960).
[m] D. H. Campbell, E. Luescher, and L. S. Lerman, *Proc. Natl. Acad. Sci. U.S.* **37,** 575 (1951).
[n] D. W. Talmage, H. R. Baker, and W. Akeson, *J. Infect. Diseases* **94,** 199 (1954).
[o] N. R. Moudgal and R. R. Porter, *Biochim. Biophys Acta* **71,** 185 (1963).
[p] H. H. Weetall and N. Weliky, *Nature* **204,** 896 (1964).
[q] H. H. Weetall and N. Weliky, *Science* **148,** 1235 (1965).
[r] W. E. Vannier, W. P. Bryan, and D. H. Campbell, *Immunochemistry* **2,** 1 (1965).

in a batch, by mixing it with the solution containing antibody. The former is usually preferable. No special precautions are necessary in packing such columns except the usual ones providing uniformity, suitable flow rate, and elimination of air. One can utilize a semibatch method by removing the column material in sections or *in toto* after antibody has been adsorbed and freeing the adsorbent of soluble nonspecific substances by washing it with 0.9% NaCl solution or a suitable neutral buffer. The extruded adsorbent can then be treated (see below) in suspension for the dissociation of antibody, and the insoluble adsorbent removed by filtration or by centrifugation. If one maintains the column throughout the entire procedure, it is advisable to use a fraction collector and an automatic detecting device such as an ultraviolet analyzer, a fluorimeter, or, if the adsorbed material has been labeled, a counter. Fresh or used adsorbent should be preserved in some manner when not in use, especially if one is dealing with some type of protein adsorbent. This can be accomplished by freezing or drying under mild conditions for most materials, or by storage under concentrated salt solution.

iii. Coupling Proteins or Simple Haptens to an Insoluble Matrix

Consideration should be given to possible denaturation of proteins and the destruction or masking of structural components which are involved in the specific combining sites of either antigen or antibody. In the case of protein antigen, loss of antigenic sites can be detected by testing the effluent antiserum for a small amount of unadsorbed antibody which persists after passage through a new unused sample of adsorbent. In the case of haptens, an attempt should be made to couple the hapten to the cellulose so that the portions of the hapten which complex with the desired antibody are free and exposed and in the proper spatial relationship, relative to the cellulose surface.

In all instances the final adsorbent product must be thoroughly washed to remove the last traces of soluble reagents. This is particularly important when haptens are used, since such materials often become trapped in the interstices of the cellulose and are released very slowly. Such washings may require several days. Before use, stored adsorbents should again be washed thoroughly under all the conditions to be encountered during the isolation procedure.

iv. Adsorption of Antibody

This step is accomplished by merely exposing the antiserum to the adsorbent either by slow passage through a column or by mixing them in a flask. To eliminate adsorption of nonantibody protein as much as possible, serum should first be decomplemented with a heterologous anti-

gen–antibody precipitate and then diluted with 1 to 2 volumes of 0.9% NaCl solution. If one is interested only in recovering pure precipitating antibody and if nonspecific adsorption of albumin is high, it is an advantage to adsorb a solution consisting only of the γ-globulin fraction, which can easily be prepared by salt precipitation (see Section A,2 or Campbell *et al.*[32]). In general, this problem is not encountered with cellulose adsorbents. Since the primary reaction of antigen–antibody combination occurs very rapidly, time is not highly critical. It is advisable, however, to allow sufficient time for all the adsorbent surface to have contact with the antiserum. The rate of passage through a 1 × 10-cm column should be about 5 ml/hour; one should make sure that all interspaces of the adsorbent are filled with liquid before the serum is applied. After the antibody-containing solution has passed through the column, the rate of washing may be increased. The adsorbent is then washed with saline until it is completely free of protein, which can be detected by tests on the effluent, supernatant, or filtrate, depending on the procedure used.

v. Elution of Antibody

It is generally agreed that antigen–antibody complexes become increasingly stable with time; therefore it is advisable to start dissociation of antibody within 12 hours. Antibody is dissociated from its antigen template and may be recovered by elution from the column or by removal of the adsorbent by centrifugation if the batch method is used.

For most antigens of interest, dissociation can be accomplished by adjusting the pH to 2.0 to 2.5 with either dilute hydrochloric acid or an acid buffer such as glycine–HCl. Since some molecules and ions, such as those in phosphate or acetic acid buffers, either inhibit dissociation or inactivate antibody under acid conditions, it is advisable to adjust the pH carefully with dilute HCl. Although acidification usually reverses antigen–antibody combination, this may not be true of all systems. For example, it has recently been found that 2,4-dinitrophenyl hapten and its specific rabbit antibody do not dissociate in dilute acid[14,33] but apparently will dissociate under alkaline conditions of around pH 12 (Weliky and Weetall, unpublished results, 1965). Optimum conditions for dissociation by means of pH changes may thus depend on the ionizable character of the antigenic combining site. Other dissociation methods may also be employed. Lerman[29] has reported the successful use of

[32] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology." Benjamin, New York, 1963.

[33] S. F. Velick, C. W. Parker, and H. N. Eisen, *Proc. Natl. Acad. Sci. U.S.* **46**, 1470 (1960).

hapten to elute anti-azophenylarsonate antibody from a cellulose adsorbent.

The reaction time for reversal of antigen–antibody combination is much greater than the forward reaction; hence more time must be allowed to give complete dissociation. Thus the flow through a column may have to be reduced, and if a batch method is used, stirring should be continued for 1 to 2 hours. Dissociation is usually faster at room temperature than in the cold.

Regardless of what pH changes have been made to separate antibody from the adsorbent, the recovered material should be returned to neutrality as soon as possible.

A serious problem which may occur following dissociation of antigen and antibody is the nonspecific readsorption of antibody. To avoid such difficulties, the particular adsorbent to be used should first be tested for nonspecific adsorption of serum globulin at the pH to be used for dissociation. The final product should always be carefully assayed. Information on purity and yield ordinarily is provided (1) by quantitative precipitation on the basis of specific precipitable protein versus total protein recovered (purity); (2) by the difference between antibody removed from the antiserum by contact with the adsorbent and the amount recovered from the adsorbent (recovery from adsorbent); and (3) by the difference between total antibody in the original antiserum and the amount recovered from the adsorbent (overall yield).

In the case of nonprecipitating antibody, estimation must be made by such methods as free-boundary electrophoresis in which nonprecipitating antibody is determined by the amount of soluble antigen–antibody complex.[16] Another suitable method would be that described by Farr (see Chap. 13,C, Vol. III and Farr[34]) in which I^{131}-labeled antigen is added to a solution containing antibody. The immunoglobin is then precipitated with ammonium sulfate and subsequently estimated in terms of the coprecipitated radioactive antigen.

In many systems both purity and yield may approach 100% by the use of immunoadsorbents. As stated previously, lack of purity and/or low yield may result from failure to decomplement the antiserum, from adsorption of nonspecific protein, from inhibition by soluble antigen contaminating the adsorbent, from denaturation during the final stages of isolation, or from nonprecipitating forms of antibody.

vi. Analytical Procedures

Analytical procedures are in general relatively simple and utilize physical, chemical, and serological reactions. Detailed procedures will be in-

[34] R. S. Farr, *J. Infect. Diseases* **103**, 239 (1958).

cluded in other chapters of these volumes. Proteins and haptens bound to cellulose may best be determined by the use of radioactive labeling.

c. Specific Preparations

i. General Considerations

Only the preparation of adsorbents which have been demonstrated to be effective in extracting antibody from serum and from which antibody can be recovered in useful form will be described here. The adsorbents described below are most conveniently classified by the functional groups of the cellulose derivatives used to react with antigen—carboxylic acid, alkylamino, aryldiazonium, and phenolic. These groups determine the kind or form of antigen which may be coupled.

Most protein antigens can be coupled to diazotized arylaminocellulose or, in the presence of carbodiimides, to carboxymethylcellulose. For maximum yield and recovery of antibody from the adsorbent, protein derivatives of carboxymethylcellulose (Section B,7,c,iv) or the half benzidine amide of carboxymethylcellulose [Section B,7,c,ii (b)] are the preparations of choice. The protein derivative of *m*-benzyloxymethylcellulose [Section B,7,c,ii(c)] has the highest capacity for antibody, but recovery of antibody from the adsorbent is lower than that of the preceding ones. It should be borne in mind that, to achieve maximum capacity, an excess of serum must usually be used; thus overall yields are low, and the possibility of antibody subfractionation is great.

Simple organic molecules (haptens) can usually be synthesized in forms that can be coupled to carboxymethylcellulose, aminoethylcellulose, a phenolic cellulose, the half benzidine amide of carboxymethylcellulose, or some other cellulose derivative. For the haptens commonly used, those that form aryldiazonium salts, such as arsanilic acid, sulfanilic acid, and *p*-aminophenyltrimethylammonium, can be coupled easily to protein derivatives of cellulose (Sections B,7,c,ii and B,7,c,iv) or phenolic cellulose derivatives (Section B,7,c,v); or, if available in the form of a *p*-azoaniline derivative (such as *p*-aminophenylazophenylarsonic acid), they can be coupled directly to carboxymethylcellulose (Section B,7,c,iv). ϵ-(2,4-Dinitrophenyl)-lysine can be coupled directly to carboxymethylcellulose (Section B,7,c,ii). Halides such as 6-trichloromethylpurine and benzenesulfonyl chloride may be coupled to aminoethylcellulose (Section B,7,c,iii) or to aminoarylcellulose (Section B,7, c,ii). Reference may be made to Weliky and Weetall[23] or to other reviews of the chemistry of cellulose and its derivatives for additional information.

The cellulose or cellulose derivatives used as starting materials may

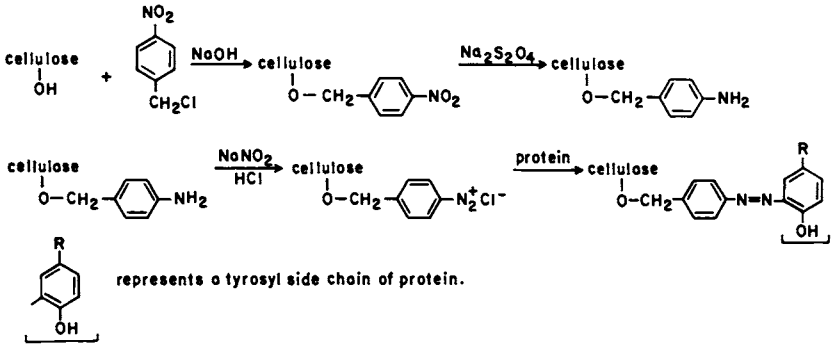
differ in physical form, water absorption, reactivity, or degree of substitution, depending on the batch or the manufacturer. Some modifications of procedure or use of alternative sources may be necessary to achieve optimum results.

ii. Aminoaryl Derivatives

Aminoaryl derivatives may be diazotized and coupled to proteins or phenolic haptens, or they may be coupled to proteins and basic amines by the carbodiimide reaction (see Section B,7,c,iv).

(*a*) *Preparation of p-Aminobenzylcellulose and Its Protein Derivatives.* *p*-Aminobenzylcellulose can be made by the reaction of *p*-nitrobenzyl chloride and cellulose followed by reduction of the nitro group. The resulting aminoarylcellulose can be diazotized and coupled to proteins and to haptens that react with such diazonium salts. Commercially available *p*-aminobenzylcellulose must be washed exhaustively with organic solvents before it can be used. A procedure for synthesis and coupling to proteins has been described by Campbell *et al.*[24] Adsorbents made by this procedure have been used to isolate antibody with recoveries and precipitability in excess of 88%. Variability of results has been observed, however, which may be attributed to sensitivity of the procedure to variations in the properties of the cellulose used. Recoveries as low as 30% have been found[35a] but purity is uniformly high. The general reactions are as shown. Coupling to protein may also take place at tryptophan, histidyl, lysyl, or arginyl side chains.

The preparation described below is that of Campbell *et al.*,[24] with some modifications.[35b]



(*i*) *Preparation of p-Nitrobenzylcellulose.* The reaction is performed in a 1000-ml three-necked flask, with a motor stirrer in the center, a

[35a] D. W. Talmage, H. R. Baker, and W. Akeson, *J. Infect. Diseases* 94, 199 (1954).
[35b] B. G. Weliky and D. H. Campbell, unpublished results, 1965.

Liebig condenser on one side, and a thermometer on the other. The flask is kept in a water bath on a hot plate to maintain the temperature of 95° needed throughout the reaction. Powdered cellulose is thoroughly washed with dilute acid, dilute base, then water, and dried. Twenty grams of this material is mixed with 60 gm of *p*-nitrobenzyl chloride and 150 ml of 40% sodium hydroxide and stirred vigorously at 95° (the reacting mixture may have to be cooled during the first part of the exothermic reaction). After 4 hours, the mixture is poured into approximately 4 liters of cold distilled water and filtered on a Büchner funnel. The residue is washed with water, with ethanol, and finally with about 300 ml of acetone in a Soxhlet extractor. The extraction is continued for about 3 to 4 days, until no more color is extracted. The cellulose is pressed between pieces of filter paper and left to dry.

(*ii*) *Preparation of p-Aminobenzylcellulose.* This reaction is performed in a 500-ml three-necked flask with a motor-driven stirrer in the center and a condenser on one side. The other side is used to introduce the sodium dithionite.

p-Nitrobenzylcellulose (12.5 gm) is suspended in 125 ml of 95% ethanol and heated to near boiling. The mixture is then stirred vigorously, and 12.5 gm of sodium dithionite ($Na_2S_2O_4 \cdot 2H_2O$) is dissolved in the minimum amount of water and added in small portions. After continued heating of the mixture for about 30 minutes, the light-yellow product is filtered off on a Büchner funnel and washed with cold water. The color of the final product varies from a light yellow to a light brown. It is dried in a desiccator over calcium chloride.

(*iii*) *Diazotization and Coupling of p-Aminobenzylcellulose to Proteins.* Five grams of the *p*-aminobenzylcellulose is suspended in 10 ml of 2 *N* hydrochloric acid, then mixed with 20 ml of water and chilled in an ice bath. With constant stirring, 0.5 to 1.0 ml of 0.5 *M* sodium nitrite solution is slowly added until sufficient nitrite is present to maintain an excess for 15 minutes as measured by starch–iodide paper. Stirring is continued for another 15 minutes, after which the material is filtered on a Büchner funnel and washed with 1 liter of about 0.3% sulfamic acid.

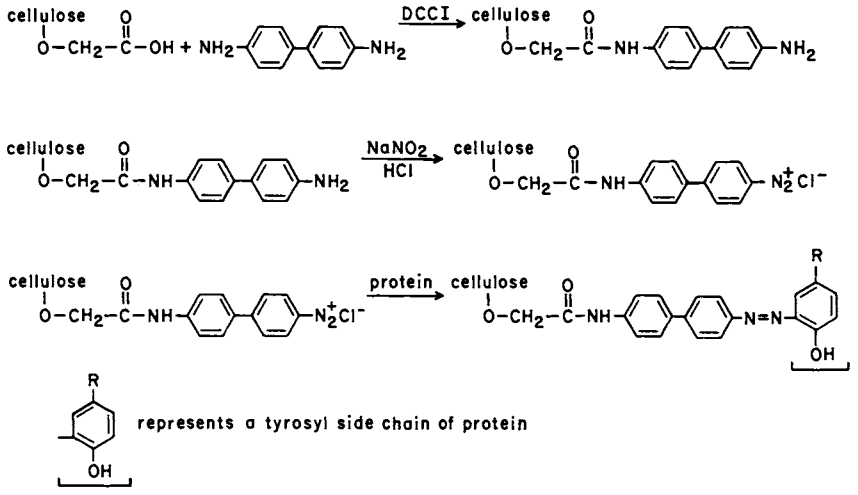
The diazotized cellulose derivative is added to an ice-cooled solution of bovine albumin, or other protein, in 50 ml of 0.15 *M* borate buffer at pH 8.5. The coupling mixture is stirred at low temperature for 2 hours and stored at 4° for 5 to 6 days. The product is then filtered on a Büchner funnel and washed with 1 to 2 liters of 0.15 *M* borate buffer, pH 8.5.

To block unreacted diazonium groups, the cellulose–protein conjugate is then stirred for 30 minutes in about 250 ml of a saturated ice-cooled

solution of β-naphthol in borate buffer at pH 8.75, followed by renewed washing on the filter with buffer and water.

Recovery of antibody from the adsorbent varies from 15 to 40%, with purity (precipitability with specific antigen) of 60 to 92%. The best results have been obtained by the column method with acid dissociation at pH 2.5.

(*b*) *Preparation of the Benzidine Derivative of Carboxymethylcellulose and Its Protein Derivatives.* Proteins, phenols, aromatic amines, and other substances that couple to aryldiazonium salts can be coupled to the half benzidine amide of carboxymethylcellulose—the 4-(p-aminophenyl)-anilide of carboxymethylcellulose.[36] The procedure and precautions for coupling benzidine to carboxymethylcellulose are the same as those for coupling proteins and haptens to carboxymethylcellulose by using dicyclohexylcarbodiimide (DCCI), discussed in Section B,7,c,iv. Benzidine also reacts with carboxymethylcellulose in 90:10 water–methanol and in methanol itself in the presence of carbodiimides. The general reactions



are as shown. Coupling to protein may also take place at tryptophan, histidyl, lysyl, or arginyl side chains.

One gram of carboxymethylcellulose is stirred in a beaker or flask with 200 ml of 2 *N* hydrochloric acid for 1 hour, filtered on a Büchner funnel, stirred with 400 ml of water, filtered, resuspended in about 400 ml of water, and refiltered. Carboxymethylcellulose in excess of that

[36] H. H. Weetall and N. Weliky, *Nature* **204**, 896 (1964).

required for immediate use may be acid-washed, suspended in acetone, air-dried, and stored. To a suspension of 0.2 gm of benzidine in 2 ml of water in a 25-ml Ehrlenmeyer flask equipped with a magnetic stirrer are added portions of 1 gm of carboxymethylcellulose and water, alternately, until about 80% of the cellulose is added and the mixture is semisolid. A solution of 0.4 gm of dicyclohexylcarbodiimide in 1 ml of tetrahydrofuran is added, followed by 1 ml of water. The remaining carboxymethylcellulose is now added, with additional water if necessary. At this point the mixture should be permitted to stand for 2 days if it is creamy, or it may be stirred for 2 days if it is fluid. At the end of 2 days, the mixture must still be semisolid or fluid. (If it has a dry appearance or does not flow if tapped gently, too little water was used and the material should be discarded.) Water is then added to the flask until it is almost filled. The mixture is stirred well, and the contents are poured into 400 to 500 ml of dilute sodium bicarbonate solution (the mixture should be alkaline) in a 600-ml flask and stirred overnight. It is then filtered in a Büchner filter and washed well with water, and the cellulose derivative is resuspended in 200 to 400 ml of acetone. After being stirred for 1 hour, the mixture is permitted to stand, and the supernatant liquid is decanted or filtered off. The cellulose derivative is then resuspended in acetone and washed in this manner at least twice more. It is then washed several times with dilute sodium bicarbonate, several times with 0.1 *N* hydrochloric acid, and finally several times with water. The completely washed half benzidine amide of carboxymethylcellulose may be suspended in acetone, filtered, air-dried, and weighed. Quantities specified may be scaled up, with only the precaution that quantities of water required are usually less than calculated.

One gram of the half benzidine amide of carboxymethylcellulose is suspended in 25 ml of 2 *N* hydrochloric acid in a 125-ml Ehrlenmeyer flask cooled in an ice bath (the temperature should be below 15°). An excess of sodium nitrite is then added (5 ml of 14% sodium nitrite solution). After being stirred for 1 hour, or standing with occasional shaking, the mixture is filtered in a Büchner funnel and washed successively with water, a dilute solution of sulfamic acid (about 1%), and finally water again.

The diazotized and washed cellulose derivative is then added to a solution of 0.1 gm of protein in 20 to 30 ml of water at pH 7 to 9, the pH being adjusted. if necessary, with sodium hydroxide solution, sodium carbonate, or sodium bicarbonate. The procedure is the same if simple organic molecules (haptens) are coupled. It is desirable to use a considerable excess of antigen if it is available. All operations should be performed at temperatures below 15° and preferably near

0°. The preferred pH for coupling is usually 8 to 9, but it may be as low as 6 or as high as 11 for some substances. After being stirred for 1 hour, the mixture is filtered and washed with water or other solvents in which excess antigen is soluble. For protein antigens such as bovine serum albumin or human γ-globulin, the adsorbent may be washed successively with water, bicarbonate, 0.1 *N* hydrochloric acid, and again water, the adsorbent being resuspended frequently to ensure even washing. To remove fines it is desirable to permit the derivative to settle for ½ hour out of 300 to 500 ml of water and decant. If the adsorbent is to be used in column form, this step should be repeated until the supernatant liquid is clear.

If proteins or other macromolecular antigens are coupled, some diazonium groups cannot be reached by the macromolecules, so that masking of excess groups with a reagent such as β-naphthol is desirable. An excess of β-naphthol reagent (0.25 to 1.0 gm) is dissolved in a small amount of 2.0 *N* sodium hydroxide, the solution is diluted to 1 liter, and the pH is adjusted to 8.0 with acetic acid. Any insoluble material should be filtered off. The protein–cellulose adsorbent is added, and the mixture is stirred overnight. If the solution must be stirred in the cold, the β-naphthol solution may be filtered cold to avoid subsequent precipitation of β-naphthol. When the mixture is filtered, care should be taken that the β-naphthol is completely removed by washing with sodium bicarbonate or sodium carbonate, by warming, or by a combination of the two.

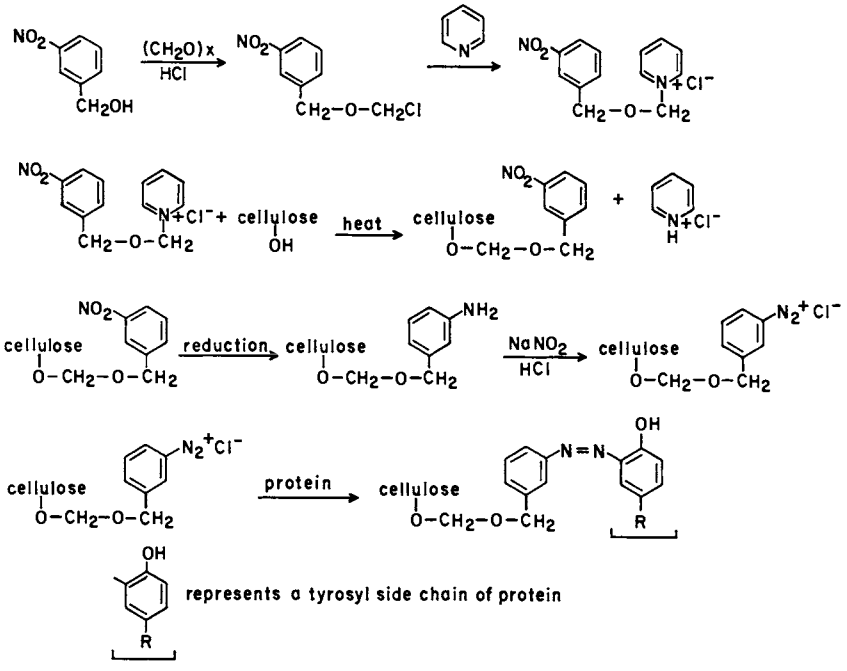
(*c*) *Preparation of m-Aminobenzyloxymethylcellulose and Its Protein Derivatives.* Among the cellulose derivatives available, adsorbents made from *m*-aminobenzyloxymethylcellulose which has been reprecipitated from cuprammonium solution are reported to have the highest capacity for antiprotein antibody.[37,38] The adsorbent is made by coupling the quaternary pyridinium salt of *m*-nitrobenzyloxymethyl chloride[39] to cellulose to form *m*-nitrobenzyloxymethylcellulose. The nitroarylcellulose derivative can then be reduced to an aminoaryl derivative which in turn can be diazotized and coupled to protein antigens in a manner analogous to the procedure for *p*-nitrobenzylcellulose, described in Section B,7,c,ii (a). If the *m*-nitrobenzyloxymethylcellulose is dissolved and reduced in an ammoniacal copper solution containing saccharose, it is reprecipitated in a fine form which has a high capacity for antibody: 100 to 150

[37] N. R. Moudgal and R. R. Porter, *Biochim. Biophys. Acta* **71**, 185 (1963).

[38] A. E. Gurvich, O. E. Kuzovleva, and E. E. Tumanova, *Biokhimiya* **26**, 803 (1962).

[39] D. N. Kursanov and P. A. Solodkov, *J. Appl. Chem.* (*U.S.S.R.*) **16**, 351 (1943) [*C.A.* **38**, 6551 (1944)].

mg/gm of adsorbent specific for antibody against human serum albumin, human γ-globulin, many other proteins, and some viruses.[20] Recovery of protein from the adsorbent seems to be about 40 to 60%, if one uses hydrochloric acid at pH 2.0 to 3.0 for dissociation.[37] The general



reactions are as shown. Coupling may also take place with tryptophan, histidyl, lysyl, or arginyl side chain of proteins.

(*i*) *Preparation of N-(m-Nitrobenzyloxymethyl)-pyridinium Chloride.*[39] Polyoxymethylene (4.8 gm) is mixed with 6 gm of *m*-nitrobenzyl alcohol in 35 ml of benzene and treated with dry hydrochloric acid for 2 hours with stirring. After standing overnight, the upper layer is separated and fractionated to yield 45.7% *m*-nitrobenzylchloromethyl ether ($b_{0.6}$ 147.5° to 148°). The chloromethyl ether yields the desired pyridinium compound in 90% yield if treated with pyridine.

(*ii*) *Preparation of m-Nitrobenzyloxymethylcellulose.*[7,30,37] Cellulose powder (50 gm) is mixed with 100 ml of a 6% solution of the pyridinium salt in 2.5% sodium acetate solution (2% and 7% solutions of the pyridinium salt and 0.7% solutions of sodium acetate have also been used). The mixture is dried at 60° to 80° and heated at 125° for 40 minutes. The product is washed thoroughly on a Büchner funnel with water

and dried at 80°. It is then washed with three 200-ml portions of benzene and dried on a Büchner funnel.

Alternatively, it is reported that time may be saved by dissolving the pyridinium salt and sodium acetate in 90% ethanol.[30] The drying time of cellulose saturated with this solution is shortened, and the resultant powder can be washed with benzene and then water immediately after heating for 40 minutes at 125°.

(*iii*) *Reduction and Coupling to Proteins.* (*a*) The procedure of Gurvich *et al.*[38] follows: The *m*-nitrobenzyloxymethylcellulose (3 gm) is dissolved in 100 ml of an ammoniacal copper solution made by mixing 61 ml of ammonia, 4.5 gm of cupric hydroxide ($Cu(OH)_2$), 1 gm of saccharose, and 39 ml of water. After addition of a further 50 ml of ammonia, tepid water is added until the cellulose precipitates. The supernatant liquid is decanted, and the precipitate is neutralized with 10% sulfuric acid and washed several times with water. The washed precipitate is weighed, and cold hydrochloric acid is added to give a final concentration of 25 gm of HCl in a volume of 500 ml. The mixture is cooled in ice. Sodium nitrite (10 gm) is added, and stirring is continued for 30 minutes. The suspension settles and is rapidly washed with 2 liters of ice water and once with cold borate buffer at pH 8.6. Human serum albumin (or other protein) at a concentration of 2% in borate buffer is used to suspend the washed precipitate in a total volume of 150 ml. The suspension is kept overnight under refrigeration, and the unbound protein is washed off. The adsorbent is kept under refrigeration as an aqueous suspension.

(*b*) The following procedure is that described by Moudgal and Porter[37] and is a modified version of the procedure of Gurvich *et al.*[38] A suspension of 1 gm of *m*-nitrobenzyloxymethylcellulose is made in 33 ml of a solution consisting of 13 ml of water, 20 ml of aqueous ammonia (specific gravity 0.88), 1.5 gm of cupric hydroxide ($Cu(OH)_2$), and 0.33 gm of sucrose. A further 40 ml of ammonia solution is added, followed by 40 ml of warm water (70°). Precipitation of cellulose begins as the water is added and is completed by cautiously adding 10% sulfuric acid solution until the solution just loses its blue color. The cellulose is centrifuged, washed six times with water, and stored as a well-dispersed suspension at 2°.

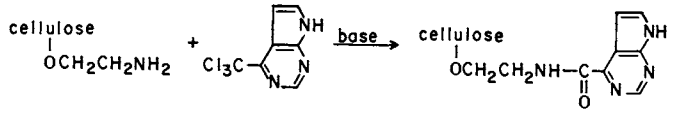
To 25 ml of suspension containing 250 mg of aminoarylcellulose are added 42.5 ml of water and 7.5 ml of hydrochloric acid (36% w/v). The mixture is cooled in an ice bath, 1.9 gm of sodium nitrite is added, and the mixture is stirred for 30 minutes. The cellulose derivative is centrifuged and washed twice with cold water and then twice with cold 0.2 *M* borate buffer (pH 8.7). The protein antigen (250 mg) dissolved

in 25 ml of cold 0.2 *M* borate buffer (pH 8.7) is added to the cellulose derivative, and the mixture is stirred at 2° for 24 hours. The cellulose antigen is then washed with cold 1% sodium chloride, once at neutral pH, three times at pH 3.2 (if lower pH is to be used for antibody dissociation, use that acidity), and again three times at neutral pH.

iii. Aminoalkylcellulose Derivatives

(*a*) *General Properties.* Aminoethylcellulose will react with halides such as trichloromethylpurine or benzenesulfonyl chloride. It will also react with proteins and organic acids in the presence of carbodiimides such as dicyclohexylcarbodiimide.

(*b*) *Preparation of an Adsorbent from Aminoethylcellulose and 6-Methylpurine, for Isolating Antipurine Antibody.* Compounds with trichloromethyl groups will react with amines, including aminoethylcellulose,[40] to form derivatives which hydrolyze to amides in the presence of water. Trichloromethylpurine can be made from the 6-methylpurine by several methods,[41] of which the one resulting in the highest yield is described below. The preparation of other 6-trichloropurines and related compounds has also been published.[42] The general reaction is as shown.



Sulfuryl chloride (50 ml) is added to a solution of 13 gm of 6-methylpurine in 50 ml of trifluoroacetic acid. When the exothermic reaction has subsided, the solution is refluxed for 1 to 2 hours. The solution is brought to a syrupy consistency by evaporation under reduced pressure, and the clear residue is freed from acid by the addition of methanol and evaporation. The product is taken up in water (100 ml) and shaken vigorously to ensure thorough mixing. Trichloromethylpurine is obtained in 73% yield (17 gm) as a white crystalline material of sufficient purity for further reaction.

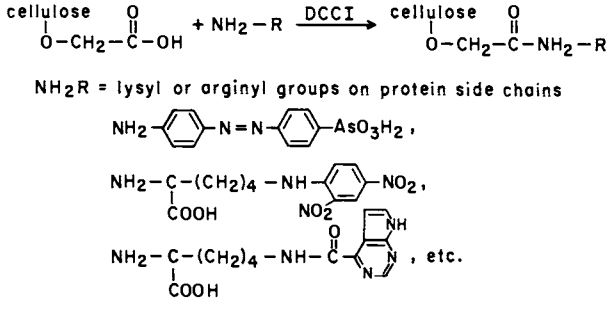
Aminoethylcellulose (5 gm) is suspended in sufficient tetrahydrofuran–water (90:10) that it can be efficiently stirred. To this mixture is added 1 gm of 6-trichloromethylpurine and 0.5 *N* NaOH in small portions maintaining the pH at 10.0 to 10.5. The pH should be checked occasionally over a period of about 6 hours and adjusted if necessary. After being stirred overnight the mixture is filtered in a Büchner funnel,

[40] H. H. Weetall and N. Weliky, *Science* **148**, 1235 (1965).
[41] S. C. Cohen, E. Thorn, and A. Bendich, *J. Org. Chem.* **29**, 3545 (1962).
[42] S. C. Cohen and A. Vincze, *Israel J. Chem.* **2**, 1 (1964).

and the cellulose derivative is repeatedly suspended in acetone and filtered until free of reagent. Before use, it should be suspended several times in sodium bicarbonate and then in 0.1 *N* hydrochloric acid until no more color is extracted and until no more impurity is extracted that can be detected by absorption in the ultraviolet region.

iv. Carboxymethylcellulose Derivatives

(*a*) *General Properties.* Carboxymethylcellulose couples to basic amines in the presence of carbodiimides.[14,36] Thus, specific adsorbents can be made for isolating antibody to most proteins and to haptens which have a basic amino group available for coupling such as aminophenylazophenylarsonic acid, aminophenylazobenzene, ε-(6-purinoyl)-lysine, and ε-DNP-lysine. Carboxymethylcellulose is a versatile support. Adsorption of nonspecific protein is low, about 0.010 mg/gm of adsorbent per milliliter of serum; therefore adsorbents made with this substance may be used for the isolation of antibody from sera with antibody concentrations of the order of tenths of milligrams per milliliter. With hydrochloric acid at pH 2.0 to 2.3, antibody can be isolated with recoveries of 80 to 100% and precipitability of 80 to 100%. The general reaction is as shown.



(*b*) *Preparation of Protein and Hapten Derivatives of Carboxymethylcellulose by the Carbodiimide Reaction.* Carboxymethylcellulose powder (0.6 to 0.8 meq/gm) is stirred in 2 to 4 *N* hydrochloric acid for ½ to 1 hour, filtered on a Büchner funnel, and washed extensively with water. If amounts are prepared in excess of that required for immediate use, the cellulose may be washed on the filter with acetone, suspended in acetone, refiltered, air-dried, and stored.

A solution of 0.4 gm of protein (for example, bovine serum albumin or human γ-globulin) or 0.1 to 0.4 gm of a suspension or solution of

hapten [such as aminophenylazophenylarsonic acid or ε-(2,4-dinitrophenyl)-lysine] in 19 ml of water is stirred with a magnetic stirrer in a 125-ml Ehrlenmeyer flask. The pH should be maintained between 3.6 and 4.5 if the antigen is stable in this range. If not, the pH may be adjusted to 4.5 to 6.0, although reaction may be slower or less complete. Carboxymethylcellulose (2.4 gm) is added in portions with vigorous agitation until the mixture becomes thick. Water and carboxymethylcellulose are then added alternately until about 80% of the cellulose has been added, keeping the suspension thick. The pH should be readjusted if necessary. A solution of 0.8 gm of dicyclohexylcarbodiimide in 2.0 ml of tetrahydrofuran is added, followed by the rest of the carboxymethylcellulose and water, if necessary, to keep the consistency such that the mixture flows if the flask is tilted. It is better to add too much water than too little. The mixture is permitted to stand for 2 days if it is semisolid. If the mixture is fluid, it should be stirred for 2 days. Refrigeration should be used only if required for enzymes or other substances that deteriorate at room temperature. For some haptens, up to 90% methanol or 90% tetrahydrofuran may be used for a solvent. Reaction is slow if tetrahydrofuran alone is used, probably because the cellulose does not swell in that solvent.

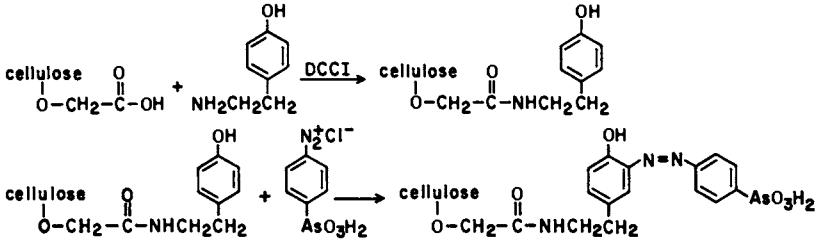
After 2 days the mixture should still have a wet appearance and flow if the flask is tilted and tapped gently. (If the preparation is dry, not enough water was used and it should be discarded.) Water is added to almost fill the flask, the mixture is stirred, and the contents of the flask washed into about 500 ml of water. The mixture may be stirred overnight to hydrolyze unreacted carbodiimide. After filtering, the cellulose derivative should be washed extensively on the filter with a solvent that dissolves the antigen: water, acid, base, or, in the case of some haptens, acetone, methanol, tetrahydrofuran, or other organic solvents. The latter solvents will also dissolve and remove excess carbodiimide and some of the dicyclohexylurea which results from hydrolysis of the carbodiimide. After the cellulose has been washed on the filter, it should be suspended in sodium bicarbonate solution several times and freed from solvent by decantation or filtration, then washed in a similar manner with 0.1 *N* hydrochloric acid, and finally with water. Several washes in each solvent are usually sufficient, but ultimately the effectiveness of washing must be determined (1) by stirring the solution overnight and observing the color, ultraviolet absorbance, or some other characteristic of the wash solution; (2) by setting up a column and observing the column effluent for color, fluorescence, or absorption in the visible or ultraviolet spectrum; or (3) by using the cellulose derivative as specific adsorbent and determining whether color appears and

whether acid-dissociated antibody is inhibited or deactivated by free antigen.

Hapten adsorbents, and perhaps some protein adsorbents, may be washed with acetone, air-dried, and stored.

v. Phenolic Derivatives of Cellulose Useful for Combination with Diazonium Salts

(*a*) *General Properties.* Carboxymethylcellulose will react with tyramine in the presence of dicyclohexylcarbodiimide to form an amide derivative. The resulting polyphenolic cellulose will react with aryldiazonium salts, such as the diazonium salt of *p*-arsanilic acid, to form azo derivatives which will complex, in this instance, to antibody directed against azophenylarsonate hapten.[43] Some haptens may be coupled directly to carboxymethylcellulose by the carbodiimide reaction (see Section B,7,c,iv). Adsorbents made in this manner may be used to isolate antibody in high purity, particularly if 1% sodium chloride adjusted to pH 2.0 to 2.3 is used to dissociate antibody from the adsorbent. Recoveries of 75 to 80% from the adsorbent and purity of 70% have been obtained by using antiserum to azophenylarsonate hapten. Dissociation was by phosphate buffer at pH 3.0 followed by 0.1 *N* hydrochloric acid. The general reactions are as shown.



DCCI is dicyclohexylcorbodiimide.

(*b*) *Preparation.* The following procedure is that of Vannier *et al.*[43] Carboxymethylcellulose (35 gm) is washed thoroughly with 0.1 *N* hydrochloric acid and water, then dried in a vacuum desiccator over calcium chloride. The dried cellulose is suspended in 998 ml of *N,N*-dimethylformamide (dried over anhydrous calcium sulfate) containing 7.0 gm of tyramine hydrochloride and 5.8 ml of triethylamine. To this mixture 11.0 gm of *N,N'*-dicyclohexylcarbodiimide is added, and stirring is continued at room temperature for 1 week. During this time two 6.5-gm additions

[43] W. E. Vannier, W. P. Bryan, and D. H. Campbell, *Immunochemistry* **2**, 1 (1965).

of *N,N'*-dicyclohexylcarbodiimide are made. The cellulose is washed five times with *N,N*-dimethylformamide and then copiously with water, with 1% sodium chloride solution, and again with water.

For diazotization, 5.84 gm of *p*-arsanilic acid in 90 ml of 1 *N* hydrochloric acid is cooled in an ice bath, and a solution of 2.13 gm of sodium nitrite in 20 ml of water is added to the *p*-arsanilic acid solution. After 20 minutes for diazotization, the solution is added dropwise with constant stirring to the tyramine amide of carboxymethylcellulose suspended in a precooled aqueous sodium carbonate solution (4.3 gm of sodium carbonate in 700 ml of water). The pH is maintained at 8 to 9 over the course of the 1½ hours used for the addition of the diazonium salt. The mixture is allowed to remain for 16 to 18 hours in the cold room (4°) with constant stirring, then copiously washed with water, with 0.1 *M* sodium carbonate, and finally again with water. The product may be stored in the cold room in water containing a few drops of toluene.

CHAPTER 4

Labeling of Antigens and Antibodies

A. Radioisotopes

1. INTERNAL (BIOSYNTHETIC) LABELING OF PROTEINS WITH C^{14}, S^{35}, AND H^3*

a. Introduction

Internal labeling with C^{14}, S^{35}, or H^3 may be used to study the synthesis and metabolic fate of antigens and immunoglobulins. This is in contrast to the external label with radioactive iodine (Section A,2), which can measure and trace labeled proteins only after they are formed and for so long as they remain intact (see also Section A,3).

Internal labeling of proteins with radioactive tracers is limited to the three isotopes listed above. The stable isotope N^{15} has been used in similar experiments, but it requires a mass spectrometer. Both C^{14} and S^{35} are usually counted in a thin-window gas-flow counter, but they may also be measured in a liquid scintillation counter. The latter instrument is required for H^3 counting, but many models can count both H^3 and C^{14} or H^3 and S^{35} simultaneously.

Internal labeling of proteins with S^{35} and H^3 can be accomplished only by the injection of a labeled amino acid. However, a wide variety of C^{14}-labeled compounds can be used, such as acetate, formate, or glucose, as well as a C^{14}-amino acid.

Internal labeling of protein with an S^{35}-amino acid has the advantage of combining moderate expense, ease of counting, and a high efficiency of incorporation. Losses of S^{35}-amino acids through metabolism and excretion are minimal. Yeast hydrolysate containing both S^{35}-cystine and S^{35}-methionine is probably the most economical source of an internal label. A method for preparing S^{35}-labeled yeast hydrolysate is given below.

One important source of misinterpretation following the use of S^{35}-cysteine is the capacity of this compound to combine with protein by

* Section 4,A,1 was contributed by David W. Talmage and Jevrosima Radovich.

the formation of disulfide bonds. A strong reducing agent such as sodium bisulfite will dissociate disulfide-bound radioactivity without affecting that which is incorporated through peptide bonds. To determine whether significant disulfide-bound radioactivity is present, the protein is precipitated with trichloroacetic acid (final concentration, 10%) with and without the addition of sodium bisulfite.

The amount of labeled compound injected will depend on the type and purpose of the experiment. To demonstrate synthesis, 50 μCi/kg of body weight is usually adequate. If the labeled protein is intended for administration to a second animal with a view to determining its fate, the production of protein of higher specific activity is required. For this purpose, administration of several millicuries per kilogram is usually adequate. For cell culture experiments 1 μCi or less per milliliter will be adequate.

An increased rate of incorporation can sometimes be obtained by starving an animal or culture of cells with respect to the compound to be injected. This is particularly true in cell culture experiments, and also when the labeled compound injected is an essential amino acid. Media or diets containing all the other essential amino acids are prepared. Cells are incubated in such media for 30 minutes before addition of the labeled amino acid. Animals must be fed the deficient diet for several days before an accelerated incorporation of the injected label will be obtained.

Additional general discussion of internal labeling may be found in Chap. 11,A, Vol. II.

b. Niklas method of preparing S^{35}-labeled yeast hydrolysate[1]

The following solutions are prepared in advance: *Solution I:* 1% glucose (w/v), distilled H_2O. *Solution II:* 10 gm of $(NH_4)H_2PO_4$, 10 gm of NH_4Cl, 2 gm of KCl, 0.05 gm of $CaCl_2$, and 0.05 gm of $MgCl_2$ in distilled water to make 100 ml. *Solution III:* 1% $MgSO_4$ (w/v) in distilled H_2O.

Step 1. Suspend 50 mg of yeast (wet weight of *Torula utilis* or *Saccharomyces cerevisiae*) in a mixture of 100 ml of solution I, 2 ml of solution II, and 1 ml of solution III. Add indicator solution for the range pH 5 to 7. Incubate for 5 to 7 hours at 37° with continuous aeration. Maintain the pH at approximately 7 by adding 0.25% NH_4OH dropwise as needed. Then centrifuge the yeast at 2500 rpm and wash it once with 100 ml of solution I.

[1] Annemarie Niklas, *Z. Physiol. Chem.* **301**, 194 (1955).

Step 2. Suspend approximately one-third of the yeast from step 1 in a mixture of 100 ml of solution I and 2 ml of solution II. Incubate at 37° for approximately 5 hours or until growth has ceased, as indicated by cessation of acid production. Centrifuge.

Step 3. Suspend approximately one-third of the sediment from step 2 in a mixture of 100 ml of solution I and 2 ml of solution II. Add 5 to 50 mCi of carrier-free inorganic S^{35} as sulfate. Incubate for 4 to 6 hours at 37°. Centrifuge the yeast, and count an aliquot of supernatant. The total supernatant should contain less than 1% of the radioactivity added. Wash the yeast cells twice with 1% Mg SO_4 to remove nonincorporated radioactivity. Centrifuge the yeast, and dry under vacuum or desiccator.

Hydrolysis. Draw out under oxygen flame the middle portion of a 20-ml Pyrex tube so that the internal diameter of the narrow portion will still admit a capillary pipet. Suspend S^{35} yeast cells in 5 ml of 5 *N* HCl. Transfer the suspension to the Pyrex tube, and seal it under vacuum, while the suspension is kept frozen in dry ice–alcohol mixture. Heat at 100° for 8 hours in oven. Evaporate the HCl solution under vacuum in a flash evaporator at 37°, and collect the water and HCl in a condenser. Dissolve the residue in H_2O, and filter it through a small filter. More than 50% of the S^{35} in the filtrate should be present as S^{35}-methionine. Smaller amounts should be present as cystine or cysteic acid.

2. EXTERNAL LABELING OF PROTEINS WITH I^{131}, I^{125}, AND I^{130}*

a. Introduction

Radioactive isotopes of iodine have been the most widely used radioactive tracers of antigens and antibodies. Their chief advantages are ease of preparation, accurate counting, stability *in vitro* and *in vivo,* diversity of half-life, and lack of reincorporation into new protein. The last-mentioned advantage derives from the fact that iodine is not normally found in protein (except for thyroglobulin). On the other hand, it is important to limit the amount of iodine introduced into protein if it is desired that the protein remain unchanged with respect to its immunological properties and its distribution and metabolism *in vivo.*

* Section 4,A,2 was contributed by David W. Talmage and Henry N. Claman.

Two methods for labeling proteins with radioactive iodine are given. The choice of isotope will depend on the proposed use of the labeled protein, the type of counting equipment available, and the necessity for a long half-life. Iodine-131 is least expensive and can be counted with only a well counter and scaler. Iodine-125 has a longer half-life (60 days, versus 8 for I^{131}), gives more definition in autoradiographs, and requires less shielding because of the lower energy of its γ-rays. However, I^{125} requires a spectrometer for accurate counting. Iodine-130 has a very short half-life (12.5 hours) and has been used only where it is desired to label three different proteins with different isotopes and count them simultaneously.

The choice of method will depend on the protein to be labeled and its proposed use. Method A was developed for milligram quantities of serum proteins, and method B for microgram quantities of growth hormone. Method B should be particularly valuable where small quantities of protein with high specific activity are desired or where proteins are easily denatured. However, this method also gives excellent results with serum proteins. With both methods it is essential to employ carrier-free isotope which does not contain a reducing agent such as cysteine or bisulfite, as these may markedly reduce the efficiency of iodination. It is also important that highly purified proteins be used, because minor impurities may be selectivity labeled.

b. Method A[1]

To 1 to 10 mCi of I^{131} in 0.5-ml volume or less, add the following: (1) 0.05 ml of a 0.01 *N* solution of KI, freshly diluted from stock 1*N* solution. (2) 1 drop of 0.5 *N* HCl. Check pH with paper—it should be below pH 5. If not, add another drop of HCl and recheck pH. (3) 1 drop of 0.1 $NaNO_2$. Solution should turn yellow, indicating presence of iodine (I°). (4) 1 drop of 0.1 *M* urea. (5) 0.5 ml of 0.15 *M* NaCl solution containing 5 to 10 mg of protein. Color should remain yellow. Mix with a quick shake, and follow with step 6 within a few seconds. (6) 0.5 ml of 0.2 M carbonate buffer, pH 10. Yellow color should disappear. Test pH with paper to make sure it is >9.5. If not, add more buffer. (7) 0.1 ml of 1 *N* KI.

With this technique, 30 to 35% of the radioactivity will be bound to proteins. This corresponds to an average of 2.5 atoms of total iodine per molecule of protein if 10 mg of globulin are used. Iodine-125 may be used instead of I^{131} without affecting the degree of iodination.

[1] Method A is a modification of a procedure reported by D. W. Talmage, H. R. Baker, and W. Akeson [*J. Infect. Diseases* **94**, 199 (1954)].

c. Method B (for Preparing Iodinated Proteins of High Specific Activity)[2]

Protein (5 μg to 5 mg) is dissolved in 0.025 to 4 ml of 0.05 *M* phosphate buffer, pH 7. With the larger quantities of protein and larger volumes, the protein solution is placed in a 30-ml beaker containing a 2.5-cm polyethylene-coated magnetic stirring bar. The beaker is placed in a crystallizing dish containing cracked ice to keep the reactants cold. The crystallizing dish is then placed on a magnetic stirrer, the appropriate quantity of carrier-free radioactive iodine is added, and the mixture is well stirred. While the protein–iodine mixture is being stirred, 40 to 100 μg of chloramine-T dissolved in 0.1 ml of phosphate buffer is added dropwise or injected by means of a 22-gauge hypodermic needle to ensure prompt mixing. One to ten minutes after the addition of chloramine-T, the same weight of sodium metabisulfite is added to neutralize any remaining oxidizing agent and stop the reaction. The chloramine-T and sodium metabisulfite should be made up fresh for each labeling procedure.

With 5 μg of growth hormone and 2 to 4 mCi of I^{131} this method should yield labeled hormone with a specific activity of 200 to 600 μCi/μg. The preparation with 600 μCi/μg would have 1.2 atoms of total iodine per molecule. If I^{125} is used, it is necessary to increase the amount of protein added by a factor of 7.5 to obtain the same degree of iodination. Thus, the specific activity obtainable with I^{125} is less by a factor of 7.5.

d. Removal of Nonprotein Iodine

To remove the nonprotein-bound iodine, one of the following methods may be used:

1. Pass the solution through a 15 $\times$ 150-mm column made by mixing equal parts of Amberlite IR-4B and AG-1-X8 (previously saturated with an unrelated protein solution, such as rabbit serum). This is rapid, but some labeled protein is lost on the column.

2. Dialyze against saline (globulins) or water (albumins). This is very effective, and, if I^{125} is used, the longer half-life of 60 days permits extensive dialysis without loss of significant amounts of radioactivity.

3. Pass the solution through a column of Sephadex G-50 (1 gm) which has been equilibrated with bovine serum albumin and washed with 20 ml of buffer. This is the method of choice for microgram quantities of growth hormone.

[2] Method B is a modification by P. J. McConahey and F. J. Dixon [*Intern. Arch. Allergy Appl. Immunol.* **29**, 185 (1966)] of a method reported by W. M. Hunter and F. C. Greenwood [*Nature* **194**, 495 (1962)].

3. EXTERNAL LABELING OF ANTIGENS BY DIAZOTIZATION-COUPLING WITH S^{35}-SULFANILIC ACID*†

a. Introduction

Certain radioactive haptens may be employed in the diazotization-coupling reaction described in Chap. 1,E,1, for labeling proteins of immunological interest. The use of S^{35}-labeled sulfanilic acid is rather a unique instance of hapten labeling in which very high specific radioactivity can be obtained as well as a stable linkage between hapten and protein. *In vivo* evidence for the stability of the label has been demonstrated in studies of antigen retention in the tissues of immunized animals,[1] and this demonstration of a reliable *in vivo* label has led to detailed studies of antigen metabolism.[2,3]

The isotope S^{35} has rather ideal properties of half-life (89 days) and radiation (soft β) for ease in counting measurements as well as for detection by autoradiography (see Chap. 11, Vol. II).

b. Preparation of Sulfanilic Acid Labeled with Sulfur-35

The synthesis of 1 mmole of sulfanilic acid is modified[4] from the commercial "baking" process[5,6] with particular attention to (1) efficient use of $H_2S^{35}O_4$ in the sulfonation of aniline and (2) observance of safety precautions to avoid radioactive contamination and also unnecessary transfer steps that may cause loss of material.

Approximately 100 mg‡ (1.1 mmoles) of aniline is weighed into a

*** Section 4,A,3 was contributed by Justine S. Garvey.**

† Contribution No. 3450. From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California.

[1] J. S. Garvey and D. H. Campbell, *Arch. Biochem. Biophys.* **73**, 507 (1958).

[2] D. H. Campbell and J. S. Garvey, *Advan. Immunol.* **3**, 261 (1963).

[3] A. Saha, J. S. Garvey, and D. H. Campbell, *Arch. Biochem. Biophys.* **105**, 179 (1964).

[4] D. H. Campbell, J. S. Garvey, N. E. Cremer, and D. H. Sussdorf, *in* "Methods in Immunology," pp. 83–88. Benjamin, New York, 1963.

[5] P. H. Groggins, "Unit Processes in Organic Synthesis," p. 287. McGraw-Hill, New York, 1938.

[6] J. J. Jacobs, D. F. Othmer, and A. Hokanson, *Ind. Eng. Chem. Anal. Ed.* **35**, 312 (1943).

‡ Aniline is redistilled and distributed in 1- to 2-ml volumes in vials which are tightly stoppered and refrigerated until used. The aniline is used only once after a vial has been opened. Aniline is weighed in slight excess of a stoichimetric amount with sulfuric acid, the excess being present for efficient utilization of the radioactive $H_2S^{35}O_4$ in the synthesis.

vial of known weight. To the aniline is added with stirring a solution of carrier-free $H_2S^{35}O_4$ (usually about 10 to 100 mCi), then 2.0 ml of 1.00 *N* H_2SO_4.* On addition of the nonradioactive H_2SO_4, a white precipitate of aniline sulfate develops. The vial is placed under a heat lamp, and most of the water is allowed to evaporate. While liquid is still present, the vial is removed from the heat and drying is continued for several hours over P_2O_5 in a desiccator. When the contents of the vial appear to be almost dry, the vial is placed into the drying chamber of an Abderhalden drying apparatus which contains P_2O_5 in the desiccant chamber and *m*-cresol in the boiling flask. The drying apparatus is assembled with a Glas-Col heating mantle and rheostat together with a vacuum pump equipped with a trap of desiccant and a manometer. Refluxing of the *m*-cresol is allowed to occur for 8 to 10 hours; during this period of time, it is important that the drying temperature be maintained at 190° (easily determined if a thermometer is placed inside the drying chamber and close to the contents of the vial) and that the system be under a partial vacuum of 8 to 10 mm Hg.

At the end of the 8- to 10-hour period allowed for drying, the pump and heat are turned off. When the apparatus is cooled partially, the vial is removed for cooling in a desiccator. The product is weighed, dissolved in hot distilled water, and transferred quantitatively to an Erlenmeyer flask for titration with 0.25 *N* NaOH.† If colored material is present, an intermediate step involving decolorization with charcoal is necessary. This involves mixing charcoal with the solution, heating the mixture until it boils, and filtering it while hot, using suction and a hardened retentive filter paper. The yield of product is greater than 90% of the theoretical yield. Diazotization of the sodium sulfanilate is accomplished with a stoichiometric amount of nitrous acid according to the procedure described above. When 1 mmole of diazonium salt is used in the coupling reaction with 1 gm of either bovine serum albumin or keyhole limpet hemocyanin, the percentage coupled is 35 to 40%.

* $H_2S^{35}O_4$ is obtained from several commercial suppliers in carrier-free form. If 1 mCi of S^{35}-labeled sulfuric acid (10^{14} molecules) is used in the synthesis of 1 mmole of sulfanilic acid (10^{20} molecules), the ratio of radioactive to nonradioactive molecules is about $10^{2}:10^{6}$. A ten- to hundredfold increase in labeled molecules is feasible and is recommended for some problems, particularly *in vivo* applications. To calculate the requirements for labeling and detection under different conditions, use can be made of the figure of 10^{9} molecules of sulfanilic acid giving a count rate of about 4000 cpm in a liquid scintillation system with about 90% efficiency.

† It is preferable to postpone the steps of dissolving and titrating if the material is not to be used immediately in diazotization and coupling, since greater stability of the sulfanilic acid is achieved in the dry state.

4. IDENTIFICATION OF PEPTIDES FROM THE COMBINING SITES OF ANTIBODIES BY THE PAIRED-LABEL TECHNIQUE*[1,2]

a. General Method

If a reagent causes an antibody to lose activity, and if this loss of activity can be prevented by the presence of the specific hapten during the chemical alteration, then the loss of activity is most simply interpreted as being due to a reaction in the site. Peptides can be identified as coming from the antibody site by treating the antibody with such a reagent in the paired-label procedure. Here one portion of the antibody is caused to react in the *absence* of hapten with a reagent containing a radioactive isotope, and another portion is carried through a parallel reaction in the *presence* of the hapten with the same reagent, but containing a different radioactive isotope.

In the case of sites containing tyrosine residues, radioactive iodines are used in the paired-label technique. Here one portion of antibody is iodinated with I^{131}-labeled iodine in the presence of hapten ("protected"), and a second portion is iodinated to the *same extent* in the absence of hapten ("unprotected") with I^{125}-labeled iodine. The preparations are mixed and digested with pepsin, and the resulting peptides are separated. Iodinated peptides showing isotope ratios different from that of the original mixture are derived from that portion of the molecule which iodinated differently in the antibody-hapten complex than in the free antibody. This portion of the molecule is most probably the binding site, but it can conceivably be due to conformational change elsewhere in the molecule as a result of hapten–antibody combination. Obviously, a hapten that reacts with iodine cannot be used.

b. Procedure

i. Iodination

The procedure given here is suitable for iodinating 5 mg of antibody in 1.5 ml of borate buffer (unprotected) or in 1.5 ml of borate buffer containing 0.018 *M* hapten (protected). The two solutions should be prepared from a common stock of the antibody; they are placed in

* Section 4,A,4 was contributed by Oliver A. Roholt and David Pressman.

[1] D. Pressman and O. Roholt, *Proc. Natl. Acad. Sci. U.S.* **47**, 1606 (1961).
[2] O. A. Roholt, G. Radzimski, and D. Pressman, *Sci.* **141**, 726 (1963).

covered 5-ml beakers containing stirring bars for magnetic stirring and brought to ice-bath temperature before the subsequent steps are begun.

Labeled I_2 of high specific activity in CCl_4 is prepared, and this is used to prepare hypoiodite of known specific activity. Volumes of I^{125} and I^{131} solutions, each containing about 2.5×10^8 cpm, are added to individual 12-ml centrifuge tubes containing 2 ml of 0.002 *N* NaOH. To each tube is added 0.5 ml of 12 *N* HCl, 0.2 ml of 4×10^{-4} *M* iodine monochloride (ICl) in 2 *N* HCl, and 0.2 ml of 4×10^{-4} *M* KI, with mixing after each addition. The resulting I_2 (less than 0.08 μmole) is extracted into about 5 ml of CCl_4. The tubes are centrifuged to clear the layers, and the aqueous layer of each is drawn off. The CCl_4 layer of each is transferred to an individual fresh centrifuge tube, care being taken not to carry over any of the residual aqueous layer. The CCl_4 is extracted with 1.5 ml of pH 9 glycine buffer (7.5 gm of glycine, 16 ml of 1 *N* NaOH, in a volume of 100 ml) and then centrifuged. As much as possible of the aqueous layer is transferred to a test tube in an ice bath. A portion of each of these radioisotope-containing glycine solutions should be counted to determine the approximate concentration of radioactivity.

For each iodination, a volume of glycine buffer containing about 10^8 cpm of the radioiodine is pipetted into an ice-cold test tube containing sufficient ice-cold glycine buffer to make a total volume of 1.5 ml, and then 0.15 ml of 0.01 *M* ICl (in 0.01 *N* HCl, 1.0 *M* NaCl) is added.

To each protein solution, 1.25 ml of the proper iodinating solution is added with stirring. The iodine incorporation is usually about 75% efficient, and this amount of iodinating solution incorporates an average of about 25 iodine atoms into each antibody molecule. The reaction mixtures are then covered and allowed to stand in the cold for an hour. Suitable dilutions of portions of the unused iodinating solutions are made in KI and thiosulfate in buffer solution for standards of specific activity. The protein solutions are then dialyzed overnight against 3 liters of 1:10 borate buffer containing 8 gm of NaCl per liter. The outer solution is changed, and 0.1 gm of KI is added to this fresh outer solution; dialysis is continued for several hours. Several dialyses against 3 liters of 5×10^{-3} *M* HCOOH at room temperature are then carried out, and the solutions are removed from the bags. The radioactivity and protein concentration of each are determined.

ii. Digestion

Volumes of each protein solution containing 2 mg of protein are mixed in 10-ml Erlenmeyer flasks (in duplicate) and frozen as a layer in the

bottom of the flask (not shell-frozen). The flasks are placed in a desiccator over KOH pellets, and the desiccator is evacuated to a pressure of 50 microns or less. The desiccator is then sealed off and allowed to stand overnight. The contents of the flasks should go to dryness, and nothing should have blown out of the flasks.

To each flask is then added 0.4 ml of 0.5 *M* HCOOH containing 0.133 mg of crystalline pepsin, and the contents are dissolved or suspended. The flasks are tightly stoppered, and digestion is allowed to proceed at 37° for 15 to 20 hours.

iii. Chromatography, HVPE, and Autoradiography

A volume of digest equivalent to 1 mg of protein is placed in several portions at the origin of an 18 × 22-inch sheet of Whatman 3 MM paper. Descending chromatography with *n*-butanol–acetic acid–water (4:1:5), followed by high-voltage paper electrophoresis (HVPE) in 1 *M* HCOOH, is employed to separate the peptides.

The dried paper is placed in contact with two pieces of 17 × 21-inch KK X-ray film (one on each side). Sheets of this size can be made by butting together one and one-half sheets of 17 × 14-inch film. One is developed after 8 hours, and the other after about 24 hours.

Areas on the chromatogram corresponding to the spots on the film are cut out, transferred to suitable test tubes, and counted in a γ-ray spectrometer. The I^{125}/I^{131} ratio for each spot is calculated and compared with that of a sample of the unfractionated digest also counted on a piece of filter paper. Spots with ratios different from those of the unfractionated digest are the peptides of interest.

iv. Control

A complete check of the whole experimental procedure for confidence in the results is easily made by making a second unprotected iodination with I^{131} and pairing this with the product from the unprotected iodination with I^{125}. The peptides should all have the same ratio; otherwise some technical detail is at fault.

5. LABELING OF ERYTHROCYTES WITH CHROMIUM-51

The labeling procedure and tracing of the fate of transferred labeled cells is developed in detail in Chap. 25,B, Vol. IV.

B. Ferritin-Labeled Antigens and Antibodies*

1. GENERAL METHOD

Ferritin is an iron-containing protein found in spleen, liver, and other tissues of animals.[1] It has a shell of apoferritin, the protein moiety, about 105 to 120 A in diameter, enveloping an inner core of ferric hydroxide micelles arranged at the apices of a roughly tetrahedral lattice that measures about 55 A across.[2] The molecular weight of apoferritin is approximately 460,000. Despite the low atomic number of iron, as compared with mercury[3] or uranium,[4] which have also been used to label antibody, the aggregate of 2000 to 3000 atoms of iron in a molecule of ferritin (about 23% by weight) confers high electron density.[5] Singer first reported the preparation of ferritin–antibody conjugates, using xylylene *m*-diisocyanate (XMDIC) as a coupling agent.[6] Singer and Schick used toluene-2,4-diisocyanate for the conjugation[7]; Borek and Silverstein used dianisidine[8]; and Ram his associates used *p,p'*-difluoro-*m,m'*-dinitrodiphenylsulfone.[9] XMDIC has consistently proved an effective coupling agent when it is stored under conditions that ensure minimal contact with air and moisture.[10]

The synthesis of the binary protein, ferritin–antibody or ferritin–antigen, is accomplished in a two-step reaction as illustrated in Fig. 1. In the first step, XMDIC, the coupling agent, is linked to ferritin by the formation of a ureido linkage through one of the two -NCO groups of the agent with ϵ-amino groups in the protein envelope of ferritin. Subsequently the ferritin intermediate is conjugated with antibody or antigen through a similar linkage of its unreacted -NCO group with ϵ-amino groups of the second protein. The yield of ferritin conjugate

* Section 4,B was contributed by Konrad C. Hsu.

[1] S. Granick, *Chem. Rev.* **38**, 379 (1946).
[2] J. L. Farrant, *Biochim. Biophys. Acta* **13**, 569 (1954).
[3] F. A. Pepe, *J. Biophys. Biochem. Cytol.* **11**, 515 (1961).
[4] L. A. Sternberger, E. J. Donati, and C. E. Wilson, *J. Histochem. Cytochem.* **11**, 48 (1963).
[5] S. J. Singer and J. D. McLean, *Lab. Invest.* **12**, 1002 (1963).
[6] S. J. Singer, *Nature* **183**, 1523 (1959).
[7] S. J. Singer and A. F. Schick, *J. Biophys. Biochem. Cytol.* **9**, 519 (1961).
[8] F. Borek and A. M. Silverstein, *J. Immunol.* **87**, 555 (1961).
[9] J. D. Ram, S. S. Tawde, Jr., G. B. Pierce, Jr., and A. R. Midgley, Jr., *J. Cell Biol.* **17**, 673 (1963).
[10] R. A. Rifkind, K. C. Hsu, and C. Morgan, *J. Histochem. Cytochem.* **12**, 131 (1964).

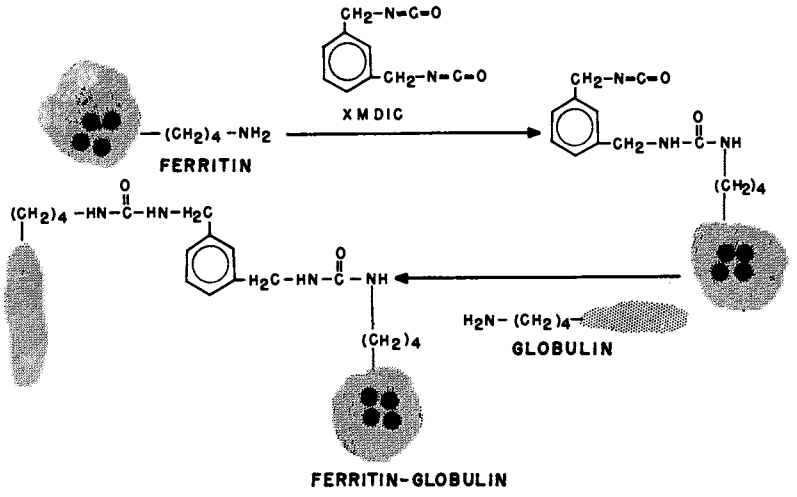


FIG. 1. Diagrammatic representation of the two-step reaction in the conjugation of ferritin with globulin, with xylylene *m*-diisocyanate (XMDIC) as a coupling agent.

depends on the presence of an unreacted -NCO group in the ferritin intermediate and its subsequent reaction with the second protein. A portion of the antibody or antigen in the reaction mixture nevertheless remains unlabeled.

2. PROCEDURE

a. PURIFICATION OF FERRITIN

Commercial horse spleen ferritin can be obtained from many sources, but further purification is essential for optimal experimental results in ferritin-labeled immunological reactants.[10]

i. Recrystallization

Commercial or crude ferritin is diluted to a 1 to 2% solution with 2% ammonium sulfate, pH 5.85 (critical). Ferritin is recrystallized repeatedly from this dilute solution in 5% cadmium sulfate until microscopic examination of a random sample shows only typical orange-brown ferritin crystals, as in Fig. 2.

ii. Reprecipitation

The purified crystalline product is redissolved in 2% ammonium sulfate, and amorphous ferritin is precipitated three times with ammonium sulfate at 50% saturation. After addition of a small amount of water to the final precipitate, the concentrated suspension is dialyzed first

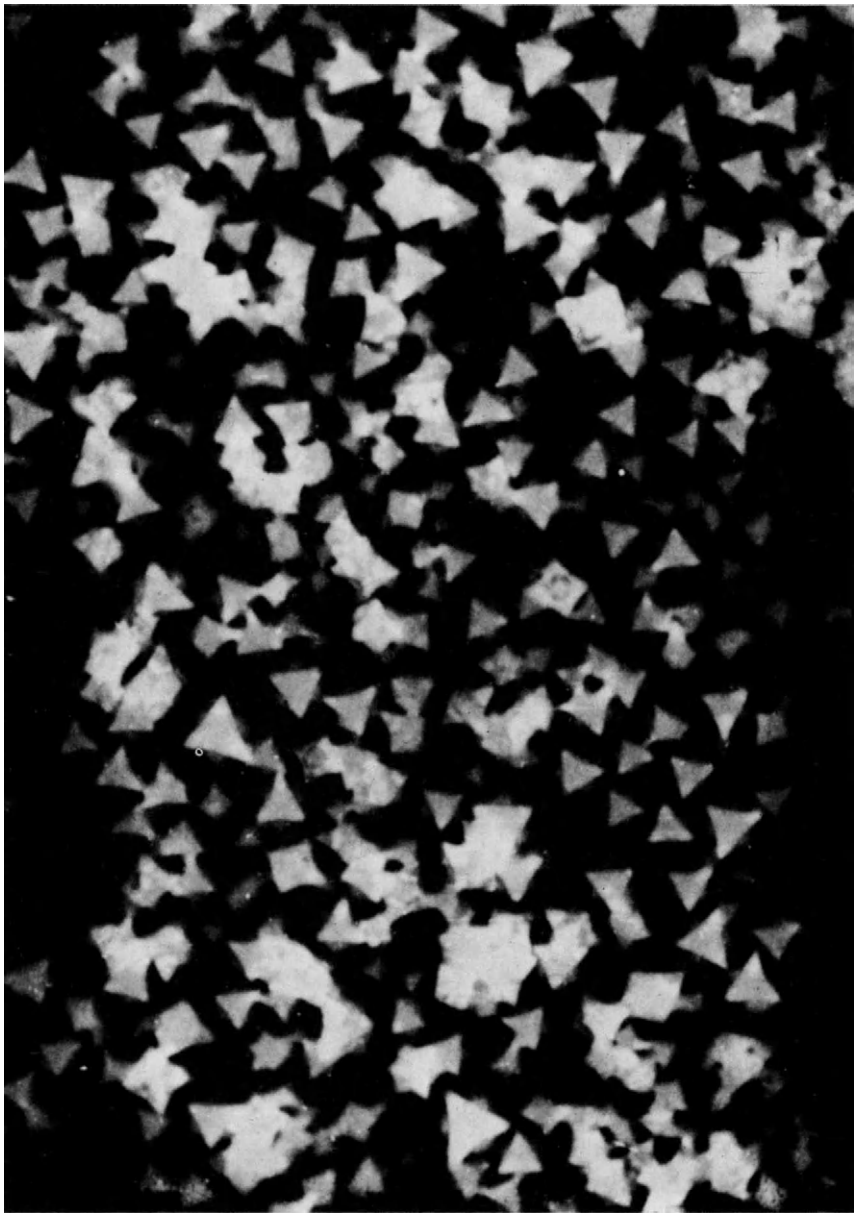


FIG. 2. Horse spleen ferritin recrystallized 5 times. Note the typical star-shaped crystals. Magnification ×400.

against cold running water until saltfree, and then against 0.05 *M* phosphate buffer, pH 7.5, overnight. The purified ferritin solution is sterilized by passage through a Seitz filter and is then stored at 4°.

b. Preparation of Antibody and Antigen

Specific antisera of high titer are essential, since only part of the antibody becomes labeled. Also, absorption of unwanted antibodies is often important. Immunoglobulin of both γG (7 S) or γM (19 S) types is used for conjugation.[11] Any protein with available ε-amino groups that remains in solution at pH 9.5 at 4° can be conjugated with ferritin. Many workers find it advisable to employ relatively pure globulin preparations, such as are described in Chap. 3.

c. Preservation of Coupling Agent

XMDIC can be obtained from Polysciences Inc., Bethayres, Pennsylvania. When the container is first opened the XMDIC should be transferred at once in 0.1-ml amounts to melting-point capillary tubes which are sealed and stored at 4° to minimize polymerization.

d. Conjugation

i. Step 1

The required amount of sterile ferritin is subjected to ultracentrifugation at 100,000 *g* for 2 hours. The upper three-fourths of the fluid in the centrifuge tubes, which is usually clear or very slightly yellow in color, is discarded. The remaining concentrated, purified, amorphous ferritin is then mixed in an ice bath with 0.05 *M* phosphate buffer, pH 7.5, and 0.3 *M* borate buffer, pH 9.5, in volumes calculated to achieve a final concentration of 20 to 25 mg of ferritin per milliliter in 0.1 *M* borate buffer. XMDIC is then added in the proportion of 0.1 ml per 100 mg of ferritin. The mixture is stirred vigorously in an ice bath on a magnetic stirrer for 45 minutes and then centrifuged at 4° at 1500 *g* for 30 minutes. The clear supernatant fluid is pipetted off and placed in an ice bath for one hour to complete the reaction.

ii. Step 2

The antibody or antigen is added to the reacted ferritin solution (ferritin–xylylene–NCO) in the proportion of 1 part of antibody or antigen

[11] A. J. L. Strauss, B. C. Seegal, K. C. Hsu, P. M. Burkholder, W. L. Nastuk, and K. E. Osserman, *Proc. Soc. Exptl. Biol. Med.* **105**, 184 (1960).

to 4 parts of ferritin by weight. Fresh borate buffer is added to maintain 0.1 molarity and pH 9.5. The mixture is stirred gently (to avoid foaming) for 48 hours at 4°. Then it is dialyzed in the cold, first against 0.1 *M* ammonium carbonate overnight, and next against 0.05 phosphate buffer, pH 7.5, for 2 to 3 hours.

e. Ultracentrifugation

The mixture of ferritin-conjugated protein, unconjugated protein, and unconjugated ferritin is centrifuged sequentially three times at 100,000 *g* for 4.5 hours. After each run the heavier ferritin-conjugated protein and unconjungated ferritin, which form a pellet, are resuspended in 0.05 *M* phosphate buffer at pH 7.5. After the final run a small quantity of phosphate buffer is added to the pellet, which is held at 4° overnight. After suspension, the solution is passed through a bacterial Millipore filter and stored at 4° in sterile tubes with sterile caps. Satisfactory results have been obtained with conjugates thus stored for over 1 year. This conjugate still contains unconjugated ferritin, which can be eliminated by starch block or continuous-flow electrophoresis.[8] In most experiments the unconjugated ferritin apparently has not interfered with the specificity of staining.

f. Testing of Conjugates for Success of Labeling Prior to Use for Electron Microscope Studies

Two relatively simple methods have been used as aids in selecting optimal conjugates for immunoferritin electron microscopy.

i. Fluorescent Antibody Technique

Antibodies or antigens may be sequentially labeled with fluorescein and ferritin. These doubly labeled conjugates retain their immunological specificity.[12] Figure 3 shows cells of group A type 4 streptococcus treated with fluorescein–ferritin doubly labeled antibody specific for the cell wall. The inset is a picture of the organisms viewed by ultraviolet microscopy and delineated by the fluorescent labeled antibody. The main photograph is an electron micrograph of the streptococci displaying ferritin granules in the bacterial cell wall which indicate the sites where the antigen molecules in the cell wall bind with molecules of the doubly labeled antibody. This technique permits the use of the relatively simple method of immunofluorescence for screening the conjugates prior to electron microscope studies.

[12] K. C. Hsu, R. A. Rifkind, and J. B. Zabriskie, *Science* **142**, 1471 (1963).

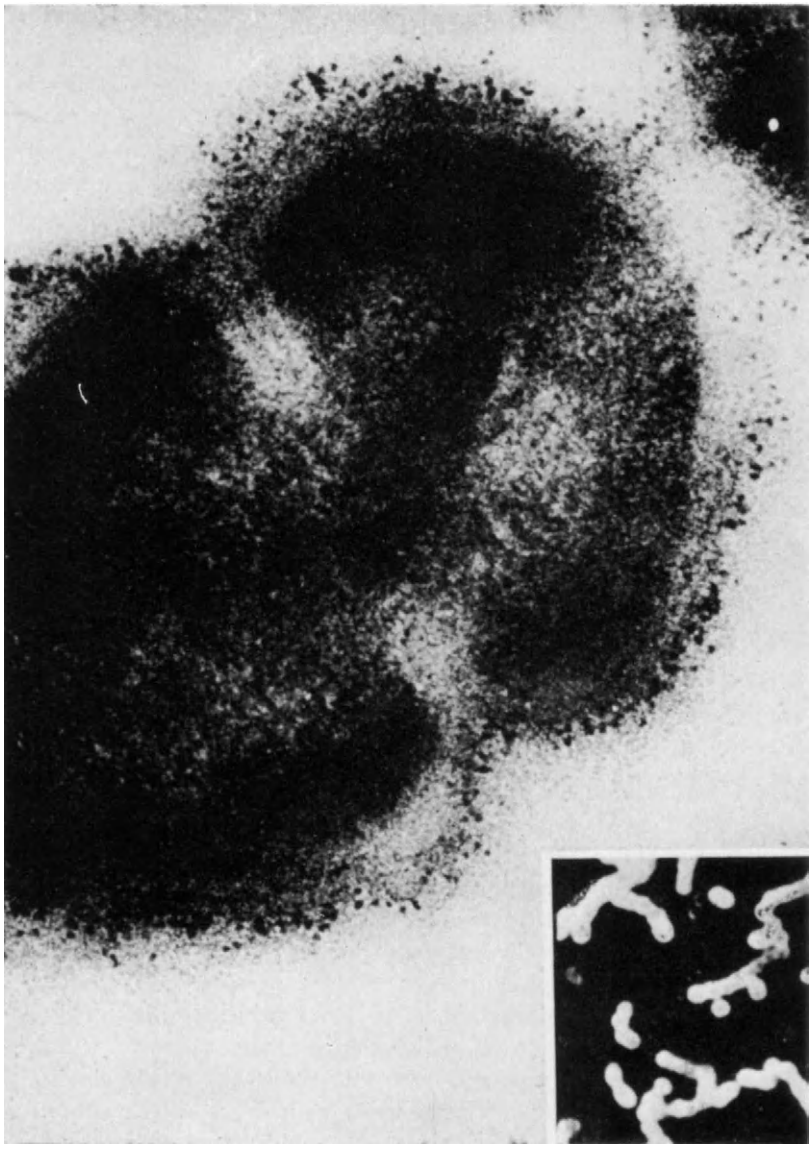


FIG. 3. Electron micrograph of cells of group A type 4 streptococci treated with fluorescein-ferritin doubly labeled antibody specific for the cell wall. *Ferritin* granules in the bacterial cell wall indicate the sites where the antigens of the cell wall bind with the doubly labeled specific antibody. Magnification ×54,000. Inset: organisms viewed by ultraviolet microscopy revealing the *fluorescent* label. Magnification ×400.

ii. Immunoelectrophoretic Analyses

The electrophoretic mobility of ferritin-labeled globulin is more anodal than that of γ-globulin.[8,10,12] By subjecting the labeled and unlabeled globulin to immuno electrophoretic analysis (see Chap. 14,E, Vol. III) it can be determined whether the globulin is conjugated to ferritin. Figure 4 is a photograph of immunoelectrophoretic patterns of ferritin-labeled hamster globulin and the same globulin unlabeled. The top and bottom wells were filled with different pools of hamster globulin, and the middle

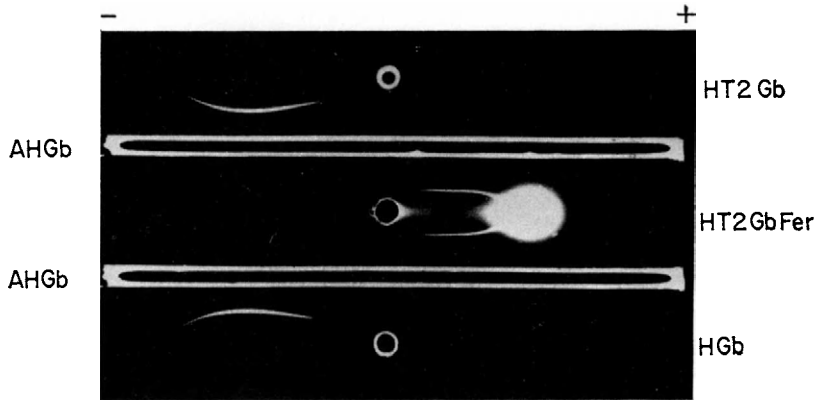


FIG. 4. The immunoelectrophoretic pattern of ferritin- labeled hamster globulin and unlabeled hamster globulin. Top well: pooled hamster globulin (HT2Gb). Middle well: hamster globulin of the same pool labeled with ferritin (HT2GbFER). Bottom well: hamster globulin from a different pool (HGb). The troughs were filled with rabbit antibody against hamster globulin. Precipitin arcs of ferritin-labeled globulin appeared in the β range, and those of the unlabeled globulins in the γ region. Note absence of unlabeled globulin in the central well. The white spot anodal to the central well is unconjugated ferritin.

well with ferritin-labeled hamster globulin. After electrophoresis on 2 × 3-inch slides at 11 ma per slide for 2.25 hours, the troughs were filled with antibody to hamster globulin. After 18 hours the precipitin arcs of the conjugated globulin appeared in the β range, whereas those of the unconjugated globulin were in the γ region.

3. APPLICATIONS

The application of ferritin-labeled antibody or antigen in immunological studies involves technical procedures which will be discussed in the section on electron microscopy (Chap. 29, Vol. IV). Suffice it to mention here that ferritin-labeled antibody has proved valuable in ultrastructural

studies concerning the identification of localization of microbial,[12] viral,[13–15] and tissue antigens,[16,17] as well as the pathogenesis of diseases due to antigen-antibody reactions.[18,19] The technique constitutes a strategic tool for the immunologist as well as for the virologist and experimental pathologist.

[13] S. Dales, P. J. Gomatos, and K. C. Hsu, *Virology* **25**, 193 (1965).
[14] C. Morgan, R. A. Rifkind, K. C. Hsu, M. Holden, B. C. Seegal, and H. M. Rose, *Virology* **14**, 292 (1961).
[15] R. A. Rifkind, K. C. Hsu, C. Morgan, B. C. Seegal, A. W. Knox, and H. M. Rose, *Nature* **187**, 1094 (1960).
[16] G. B. Pierce, Jr., J. S. Ram, and A. R. Midgley, Jr., *Intern. Rev. Exptl. Pathol.* **3**, 1 (1965).
[17] R. A. Rifkind, E. F. Osserman, K. C. Hsu, and C. Morgan, *J. Exptl. Med.* **116**, 423 (1962).
[18] G. A. Andres, C. Morgan, K. C. Hsu, R. A. Rifkind, and B. C. Seegal, *J. Exptl. Med.* **115**, 929 (1962).
[19] B. C. Seegal, G. A. Andres, K. C. Hsu, and J. B. Zabriskie, *Federation Proc. Symp.* **24**, 100 (1965).

C. Labeling of Antibody with Fluorescent Dyes

The method for preparing fluorescent antibodies for histological or immunochemical reactions is given in Chap. 27, Vol. IV, where their use as histochemical reagents is described.

CHAPTER 5

Methods Used in Studies of the Structure of Immunoglobulins*,†

A. Background Information

1. INTRODUCTORY REMARKS

Interest in the structure of antibodies and immunoglobulins stems largely from the desire to understand the molecular bases of immunological specificity. The purpose of the present review is to summarize some of the experimental techniques employed in this area of study. Inasmuch as highly specialized methods for the study of the amino acid sequence of antibodies are just beginning to be employed, they will not be considered here.

From the viewpoint of the experimental chemist, the structural analysis of molecules as large as the immunoglobulins must begin with efforts to isolate smaller portions of the molecules for study. If possible, these portions should retain biological activity or the capacity to regain activity. It is also highly desirable that they represent natural subunits of the molecule. Two approaches have been employed to realize some or all of these objectives: chemical degradation into natural subunits, and limited hydrolysis with proteolytic enzymes. Each major approach will be discussed separately. To simplify these discussions, consideration must first be given to terminology and to current models of immunoglobulin molecules.

2. CONVENTIONS, TERMINOLOGY, AND MODELS OF THE IMMUNOGLOBULINS

Different designations have been used for the various classes of immunoglobulins, their polypeptide chains, and the fragments resulting from proteolytic degradation. A summary of various nomenclatures is given in Table I. No further explanation of nomenclature will be given

* Chapter 5 was contributed by Gerald M. Edelman and John J. Marchalonis.

† Original research reported in this chapter was supported in part by grants from the National Institutes of Health and the National Science Foundation.

here; a more detailed discussion is given in Chap. 3,A,1, and reference may be made to the conference on nomenclature sponsored by the World Health Organization.[1]

The nomenclature is embodied in a model of the γG-immunoglobulin molecule shown in Fig. 1.[2,3] The model is presented in order to clarify presently accepted relationships between the polypeptide chains and the fragments produced by treatment with various enzymes. We shall assume that γA and γM immunoglobulins are similar to γG-immunoglobulins except perhaps for the number of associated subunits in the molecule.

TABLE I
NOMENCLATURE FOR IMMUNOGLOBULINS, THEIR POLYPEPTIDE CHAINS AND FRAGMENTS

	Previous usage	Proposed usage[a]
Classes of immunoglobulins	γ, 7Sγ, 6.6Sγ, γ_2, γ_{ss}	γG-immunoglobulin
	β_2A, γ_1A	γA-immunoglobulin
	β_2M, γ_1M, 19Sγ, γ-macroglobulin	γM-immunoglobulin
Polypeptide chains		
Heavy chains of class γG	Hγ, A	γ chain
Heavy chains of class γA	Hγ_{1A}	α chain
Heavy chains of class γM	Hγ_{1M}	μ chain
Light chains of type I (now antigenic type K)	L_I, B	κ chain
Light chains of type II (now antigenic type L)	L_{II}, B	λ chain
Fragments	A, C, S, I, II	Fab fragment
	B, F, III	Fc fragment
	A piece	Fd fragment

[a] *Bull. World Health Organ.* **30**, 447 (1964).

For example, from the symbols in Table I the molecular formula of a γM-immunoglobulin could be $(\mu_2\ \kappa_2)_n$, where n is most probably 5.

As suggested by its molecular formula, the γG-immunoglobulin molecule is composed of two light chains and two heavy chains. They are arranged in the model as two light–heavy pairs about a rotational symmetry axis, and it is currently believed that the two pairs are linked via the heavy chains. Each chain is linked to its neighbor by weak

[1] *Bull. World Health Organ.* **30**, 447 (1964).
[2] G. M. Edelman and J. A. Gally, *Proc. Natl. Acad. Sci. U.S.* **51**, 846 (1964).
[3] M. Fougereau and G. M. Edelman, *J. Exptl. Med.* **121**, 373 (1965).

interactions (hydrophobic bonds, hydrogen bonds) and a single interchain disulfide bond. Both the interchain disulfide bonds and the weak interactions must be disrupted in order to separate the chains. If the disulfide bonds are not cleaved, the chains will not dissociate even in solvents that perturb weak interactions ("dissociating solvents"). After

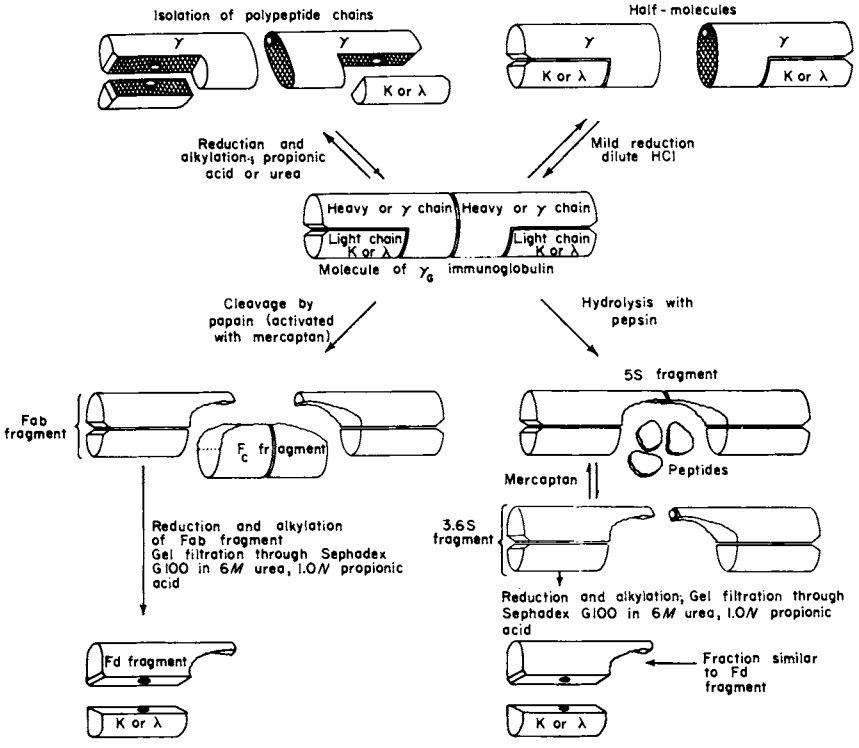


FIG. 1. Hypothetical model of γG-immunoglobulin molecule and sequence of steps of degradation to chains and fragments. Disulfide bonds and half-cysteine residues are represented as small discs between the chains. From M. Fougereau and G. M. Edelman, *J. Exptl. Med.* **121**, 373 (1965).

cleavage of the disulfide bonds, the γG-immunoglobulin molecule remains intact and dissociates only when placed in solvents such as 6 *M* urea, 6 *M* guanidine hydrochloride, or certain acids. Dissociation of the chains is thus a two-stage process.[4]

Also depicted in Fig. 1 are the fragments produced by treatment with papain and pepsin. The effects of these enzymes have been extensively

[4] G. M. Edelman and M. D. Poulik, *J. Exptl. Med.* **113**, 861 (1961).

studied by Porter[5-7] and by Nisonoff.[8-16] The Fab ("antigen-binding") fragments are composed of a light chain and the Fd fragment, a portion of the heavy chain. It has been suggested[8,9,17] that the disulfide bond between the heavy chains is cleaved during proteolysis as a result of the presence of reducing agents used to activate the papain. Another possibility is that papain hydrolyzes the molecule in such a way as to leave this bond in the Fc fragment which is comprised of the remaining portions of the heavy chains (Fig. 1). Treatment with pepsin does not disrupt this disulfide bond but converts portions of the heavy chains corresponding to Fc fragments into small peptides.

B. Isolation of Polypeptide Chains of the Immunoglobulins

The dissociation of immunoglobulins into their constituent polypeptide chains was reported by Edelman[18] in 1959. Treatment of human γG- and γM-immunoglobulins with mercaptans in urea, by sulfitolysis in urea, or by performic acid oxidation resulted in a large decrease in molecular weight. Subsequently, a number of reports[4,19-21] extended these observations, and new methods were devised for separation of the dissociated polypeptide chains. These methods will be described in detail after a brief consideration of the chemical methods for cleaving disulfide bonds and stabilizing the products.

[5] R. R. Porter, *Biochem. J.* **46,** 479 (1950).
[6] R. R. Porter, *Nature* **182,** 670 (1958).
[7] R. R. Porter, *Biochem. J.* **73,** 119 (1959).
[8] A. Nisonoff, F. C. Wissler, L. N. Lipman, and D. L. Woernley, *Arch. Biochem. Biophys.* **89,** 230 (1960).
[9] A. Nisonoff, G. Markus, and F. C. Wissler, *Nature* **189,** 293 (1961).
[10] J. L. Palmer, A. Nisonoff, and K. E. Van Holde, *Proc. Natl. Acad. Sci. U.S.* **50,** 314 (1963).
[11] S. R. Stein, J. L. Palmer, and A. Nisonoff, *J. Biol. Chem.* **239,** 2872 (1964).
[12] A. Nisonoff, F. C. Wissler, and D. L. Woernley, *Biochem. Biophys. Res. Commun.* **1,** 318 (1959).
[13] A. Nisonoff, F. C. Wissler, and L. N. Lipman, *Science* **132,** 1770 (1960).
[14] A. Nisonoff, *Biochem. Biophys. Res. Commun.* **3,** 466 (1960).
[15] W. J. Mandy, M. M. Rivers, and A. Nisonoff, *J. Biol. Chem.* **236,** 3221 (1961).
[16] A. Nisonoff, and M. M. Rivers, *Arch. Biochem. Biophys.* **93,** 460 (1961).
[17] J. J. Cebra, D. Givol, H. I. Silman, and E. Katchalski, *J. Biol. Chem.* **236,** 1720 (1961).
[18] G. M. Edelman, *J. Am. Chem. Soc.* **81,** 3155 (1959).
[19] F. Franěk, *Biochem. Biophys. Res. Commun.* **4,** 28 (1961).
[20] R. A. Phelps, K. A. Neet, L. T. Lynn, and F. W. Putnam, *J. Biol. Chem.* **236,** 96 (1961).
[21] J. B. Fleischman, R. H. Pain, and R. R. Porter, *Arch. Biochem. Biophys. Suppl.* **1,** 174 (1962).

1. CLEAVAGE OF DISULFIDE BONDS

An extensive review will not be given here; excellent descriptions of the theoretical and experimental approaches have been given by Boyer[22] and Cecil.[23]

Disulfide bonds in proteins may be cleaved by a number of methods. Most work on the immunoglobulins has utilized one of three reactions: reduction by mercaptans, sulfitolysis, or oxidation with performic acid.

a. Reduction

Reaction of protein disulfide bonds with mercaptide ions proceeds as follows:

$$RS^- + R'SSR' \rightleftarrows RSSR' + R'S^-$$

$$RS^- + RSSR' \rightleftarrows RSSR + R'S^-$$

R is the group present on the mercaptide ion, and R′ represents the protein. 2-Mercaptoethanol has been the most frequently used reagent for the reduction of disulfide bonds in immunoglobulins.

At neutral and alkaline pH values, the $R'S^-$ groups are reactive. If it is desired to stabilize the products, alkylation with iodoacetamide or iodoacetic acid may be resorted to:

$$R'S^- + ICH_2CONH_2 \rightarrow R'SCH_2CONH_2 + I^-$$

The S-carboxamidomethylated derivative is stable over a wide pH range at room temperature.

b. Sulfitolysis

In the presence of sulfite and cupric ions, disulfides are converted to S-sulfonates:

$$R'SSR' + 2Cu^{++} + 2SO_3^{--} \rightleftarrows 2R'SSO_3^- + 2Cu^+$$

This reaction has been used with proteins.[24] The modification of this reaction, described by Pechère *et al.*,[25] was employed on immunoglobulins

[22] P. D. Boyer, *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. I, p. 511. Academic Press, New York, 1959.

[23] R. Cecil, *in* "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. I, p. 379. Academic Press, New York, 1963.

[24] J. M. Swan, *Nature* **180**, 643 (1957).

[25] J. F. Pechère, G. H. Dixon, R. H. Maybury, and H. Neurath, *J. Biol. Chem.* **233**, 1364 (1958).

by Edelman[18] and has received extensive application in the hands of Franěk.[19,26,27]

c. Oxidation

Organic peracids such as performic acid may be used to cleave disulfide bonds,[28] yielding two molecules of sulfonic acid for each disulfide:

$$R'SSR' + 6[O] \rightleftarrows 2R'SO_3H$$

The cystine residues are converted to cysteic acid.

2. DETAILED PROCEDURES FOR DISSOCIATION OF IMMUNOGLOBULINS AND FRACTIONATION OF THE POLYPEPTIDE CHAINS

a. Chemical Treatment of Immunoglobulins

Depending on the purposes for which the final products are intended, reduction may be carried out in the presence or absence of denaturing solvents. The modified procedure of Edelman and Poulik[4] is as follows: 100 mg of immunoglobulin is added to 5 ml of a solution that is 8 *M* in urea and 0.1 *M* in 2-mercaptoethanol brought to an apparent pH of 8.0 by addition of small amounts of 1 *M* tris (hydroxymethyl)aminomethane (Tris). The reaction is allowed to proceed for 4 hours at room temperature, after which the solution is made 0.2 *M* in iodoacetamide and the pH is kept at 8.0 by addition of small amounts of 1 *M* Tris. After 10 minutes at room temperature, the preparation is dialyzed extensively against distilled water at 4° and lyophilized. This procedure leads to extensively reduced chains which are insoluble in aqueous buffers. The insolubility probably results from cleavage of intrachain S-S bonds. Extensively reduced preparations are useful for examination by starch gel electrophoresis in urea, and for chemical analyses requiring that most or all of the disulfide bonds of the molecule be cleaved.

To obtain soluble preparations of chains, Edelman and Poulik[4] substituted 0.15 *M* NaCl for urea in the above procedure. The final solution is dialyzed against 0.15 *M* saline, H_2O, or Tris buffer, depending on the purposes for which it will be used. Reduction in the absence of denaturing solvents presumably cleaves mainly interchain disulfide bonds. The same approach was used by Fleischman *et al.*,[21] whose procedure is as follows: A 2% (w/v) solution of immunoglobulin in 0.55

[26] F. Franěk and R. S. Nezlin, *Folia Microbiol. (Prague)* **8**, 128 (1963).
[27] F. Franěk and J. Zikan, *Collect. Czech. Chem. Commun.* **29**, 1401 (1964).
[28] C. H. W. Hirs, *J. Biol. Chem.* **219**, 611 (1956).

M Tris, pH 8.2, is degassed by suction on a water pump, and nitrogen is slowly bubbled through. 2-Mercaptoethanol is added to a final concentration of 0.75 *M*. After 1 hour at room temperature the solution is cooled in ice water. An equal volume of 0.75 *M* iodoacetamide at 0° (previously degassed) is added, and trimethylamine is added cautiously to maintain the pH at about 8 as judged by indicators. After 1 hour the solution is dialyzed against 100 volumes of cold 0.15 *M* saline. A subsequent report by Fleischman *et al.*[29] indicated that degassing is not necessary.

The procedure of S-sulfonation has been extensively applied to dissociation of immunoglobulins by Franěk.[19,26,27] It is based on methods of Swan[24] and Pechère *et al.*[25] According to Franěk and Zikan,[27] a useful method is to dissolve 500 mg of protein in 70 ml of 0.5 *M* ammonia buffer, pH 8.6 (0.5 *M* NH_4Cl adjusted to pH 8.6 with concentrated ammonia); 5.0 ml of 0.1 *M* $CuSO_4$ and 0.1 ml of concentrated ammonia are then added. The pH is adjusted to 8.6, and the volume is made up to 80 ml with buffer. To this solution is added 20 ml of the ammonia buffer in which sodium sulfite has been freshly dissolved to a final concentration of 1.25 *M*. The reaction mixture is stirred at room temperature in a closed vessel. After 24 hours, the solution is passed over a column (4 × 35 cm) containing Sephadex G-25* in 0.25 *M* ammonium carbonate, and the effluent containing the protein is freeze-dried.

Procedures employing oxidation have been less generally used but may be helpful in detailed chemical studies. Edelman[4,18] used the method of Hirs[28] on human γG-immunoglobulin: to 100 mg of protein in 9.0 ml of 88% formic acid, 1 ml of 30% H_2O_2 is added. After 20 minutes at room temperature (or preferably at +4°) the reaction is stopped by adding 200 ml of H_2O. The sample is then lyophilized.

b. Methods for Fractionation and Isolation of the Polypeptide Chains

Three main methods have been developed for separating or isolating polypeptide chains. They are starch gel electrophoresis in urea (see Chap. 6,C,3, Vol. II), ion exchange chromatography in urea (see Chap. 9,B, Vol. II), and gel filtration on Sephadex (see Chap. 9,A, Vol. II).

i. Starch Gel Electrophoresis. Starch gel electrophoresis in urea at acid pH[4,30] has the highest resolving power of any known method for the fractionation of polypeptide chains of immunoglobulins. It is not at present useful for isolation procedures but is extremely powerful in

[29] J. B. Fleischman, R. R. Porter, and E. M. Press, *Biochem. J.* **88**, 220 (1963).

* Pharmacia, Uppsala, Sweden.

[30] O. Smithies, *Biochem. J.* **71**, 585 (1959).

analysis and comparison of the chains of different classes of immunoglobulins. For example, it has played a dominant role in the characterization of the chains of myeloma proteins and Bence-Jones proteins.[31]

The apparatus for vertical starch gel electrophoresis and the detailed procedures according to Edelman and Poulik[4] are essentially as described in Chap. 6,C,3,c, Vol. II. Electrophoresis in urea-starch gel with formate buffer is carried out at a voltage gradient of 6 volts/cm for 16 hours. After electrophoresis has been carried out, the gel is sliced and is stained for 30 minutes to 1 hour with the following solution: 2 gm of Amido-Schwarz 10B is dissolved in 1 liter of a solution containing (by volume) 8 parts of water, 1 part of methyl alcohol, and 1 part of acetic acid. The stained gel is washed with 10% acetic acid in a recirculating gel washer.* The destained gel may be stored if desired in a solution of methanol–water–acetic acid (50–50–10, v/v). A typical pattern is shown in Fig. 2.

It is sometimes convenient to compare a number of immunoglobulins that are available only in small amounts. One milligram of protein is dissolved in 0.1 ml of solvent and is then loaded directly into the origin of the gel. Comparisons of the polypeptide chains of immunoglobulins may be made by dissolving the samples in a neutral buffer (0.05 *M* Tris, pH 7.0) or 8 *M* urea that has been made 0.1 *N* in 2-mercaptoethanol. After 1 hour at room temperature, solid iodoacetamide is added to a final concentration of 0.2 *M*, and the sample is loaded directly onto the gel. If the sample is reduced in urea, it is not strictly necessary to alkylate the reduced immunoglobulin preparations, since the -SH groups present in the sample are not ionized at pH 3.0, the approximate pH at which electrophoresis is carried out. In practice, however, slightly sharper patterns are obtained if iodoacetamide is added.

ii. Ion Exchange Chromatography. The polypeptide chains of immunoglobulins may also be fractionated and isolated by using ion exchange chromatography in urea.[4] For general methods and technical considerations, see Chap. 9,C, Vol. II.

The following technique is particularly useful for separation of the chains of pure immunoglobulin such as myeloma globulin.[31] Reduced alkylated immunoglobulin (50 to 100 mg) is loaded on 25 × 0.9-cm columns of carboxymethylcellulose (0.71 meq/gm),† using buffers made with 6 *M* urea. The urea solution is prepared at this concentration and is filtered through mixed-bed ion exchange resin (Amberlite MB1, column size 50 × 2 cm). The first three column volumes are discarded before

[31] G. M. Edelman, and J. A. Gally, *J. Exptl. Med.* **116**, 207 (1962).

* O. Heller, Madison, Wisconsin, or E-C Apparatus Co., Philadelphia, Pennsylvania.

† Schleicher and Schuell, Keene, New Hampshire.

the urea solution is collected. This purified urea solution is then used to make buffers with sodium acetate. The carboxymethylcellulose column is equilibrated initially with 0.01 *M* sodium acetate buffer made with urea, and gradient elution is performed to 1.0 *M* sodium acetate made with urea. Since 6 *M* urea is used as solvent to prepare the buffers, the urea concentration diminishes slightly in the course of elution. In

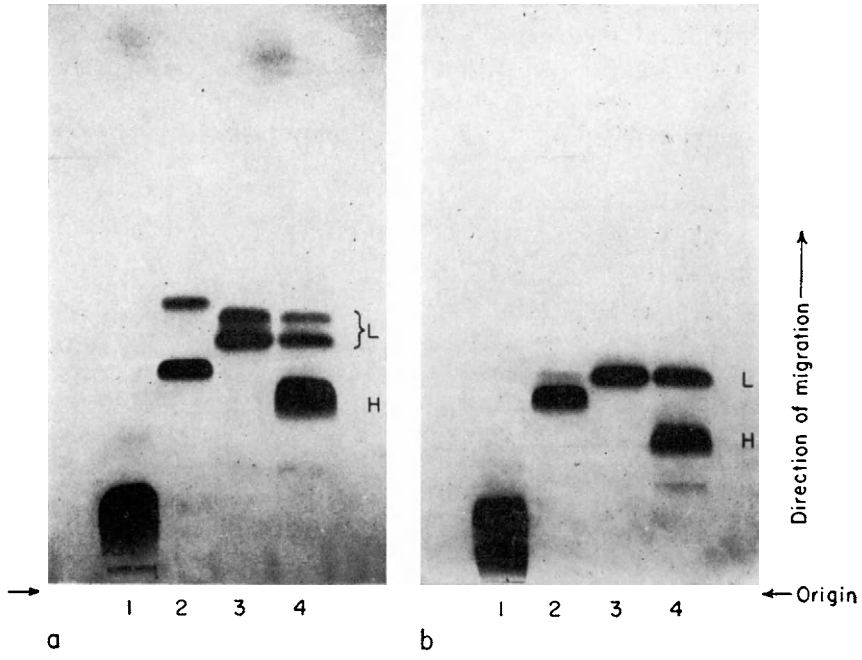


FIG. 2. Starch gel electrophoretic comparison of Bence-Jones proteins and myeloma proteins before and after reduction and alkylation in 8 *M* urea. (a) patient Haw; (b) patient S: (1) myeloma protein; (2) Bence-Jones protein; (3) Bence-Jones protein, reduced and alkylated; (4) myeloma protein, reduced and alkylated; L—light polypeptide chains, H—heavy polypeptide chains. From G. M. Edelman and J. A. Gally, *J. Exptl. Med.* **116**, 207 (1962).

practice, this has not been found to alter the results. It is essential that the chromatography be performed with fresh urea–buffer solutions, at 4°. The best apparent pH for the column must be determined in each case. A useful apparent pH for the sodium acetate–urea solutions is 6.0; the solutions may be adjusted with small amounts of NaOH or HCl if they deviate from this value.

The chromatographic method can be used for immunoglobulin solutions that are reduced in the presence or absence of urea. In the latter case,

soluble preparations of chains may be obtained as described by Edelman and Poulik.[4]

iii. Gel Filtration. A number of methods for the separation of immunoglobulin chains by gel filtration in dissociating solvents have been reported. They have the advantage of ease and convenience and are at present the methods of choice. Gel filtration methods are described in Chap. 9,B, Vol. II.

Although the method described here has been devised primarily for the fractionation of mammalian γG-immunoglobulins, it is applicable to other mammalian immunoglobulin classes as well as to immunoglobulins from lower vertebrates. The separation procedure of Fleischman *et al.*,[21] employing Sephadex G-75, first reported for partially reduced

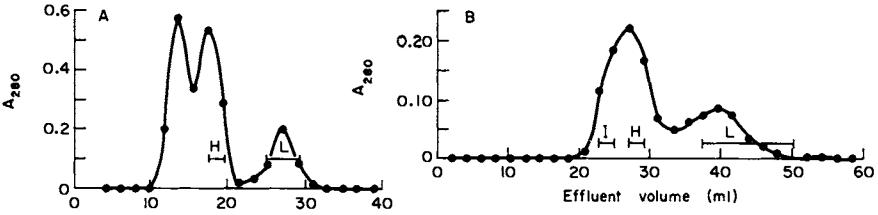


FIG. 3. Separation of heavy-chain and light-chain fractions from reduced alkylated antibodies by gel filtration on Sephadex G-100 in 0.5 *N* propionic acid. (*A*) Reduced alkylated antibodies to f1 bacteriophage; (*B*) reduced alkylated antibodies to f2 bacteriophage; A_{280} = absorbancy at 280 mμ. H—heavy-chain fraction; L—light-chain fraction; I—first fraction (containing aggregates of heavy chains). From G. M. Edelman, D. E. Olins, J. A. Gally, and N. D. Zinder, *Proc. Natl. Acad. Sci. US.* **50**, 753 (1963).

immunoglobulin, has been used to obtain soluble subunits from rabbit and human γM-macroglobulins.[32-34] Marchalonis and Edelman[35] used a 1 *M* propionate–6 *M* urea solvent system[3] to resolve extensively reduced light and heavy polypeptide chains from elasmobranch immunoglobulins.

Mention should be made of the separation of fully reduced immunoglobulin by gel filtration on Sephadex G-200 in 5 *M* guanidinium chloride by Small *et al.*[36] In addition, sodium decyl sulfate[37] and 6 *M* urea–formate buffer[26] have been used as solvents.

The method of Fleischman *et al.*[21] is as follows: A column of Sephadex

[32] S. Cohen, *Biochem. J.* **89**, 334 (1963).
[33] H. Chaplin, S. Cohen, and E. M. Press, *Biochem. J.* **95**, 256 (1965).
[34] F. Miller and H. Metzger, *J. Biol. Chem.* **240**, 3325 (1965).
[35] J. Marchalonis and G. M. Edelman, *J. Exptl. Med.* **122**, 601 (1965).
[36] P. A. Small, Jr., J. E. Kehn, and M. E. Lamm, *Science* **142**, 393 (1963).
[37] S. Utsumi and F. Karush, *Biochemistry* **3**, 1329 (1964).

G-75 (3 × 65 cm) is equilibrated with 1 *N* propionic acid at 4°. Immunoglobulin (100 to 200 mg), reduced in the absence of urea, is placed on the column, and fractions of 15 to 20 ml are collected. Two peaks are obtained. The first consists mainly of heavy chains, and the second of light chains. The amount of protein in the fractions is measured by determining absorbancy at 280 mμ. Several modifications of this method have been proposed. In a solvent of 0.5 *N* propionic acid on 1 × 100-cm columns of Sephadex G-100, collecting 2-ml fractions, three peaks are observed.[38] The first peak contains a polymer of heavy chains, the second contains heavy chains, and the third contains the light chains (Fig. 3A).

3. RECONSTITUTION OF IMMUNOGLOBULINS FROM POLYPEPTIDE CHAINS AND HALF-MOLECULES

Molecules resembling γG-immunoglobulins have been reconstituted both from polypeptide chains[39] and from half-molecules.[10,11] The methods employed will be reviewed briefly.

a. Reconstitution from Reduced Alkylated Chains

Fractions of heavy and light chains are obtained from immunoglobulin reduced and alkylated in the absence of urea. The chains are fractionated by means of gel filtration on Sephadex G-100 in 0.5 *N* propionic acid on a column measuring 1 × 100 cm, and 1-ml fractions are collected (see above, Section A,2,b,iii). While still in propionic acid, the light-chain and heavy-chain fractions are mixed, and the mixtures are dialyzed for 48 to 72 hours at 4° against 2 liters of 0.05 *M* Tris buffer, pH 7.2, 0.15 *M* NaCl.

The reconstituted products may be obtained from the dialyzed mixture by density gradient ultracentrifugation in a linear gradient of sucrose[39] or by gel filtration on Sephadex.[40] The latter is more convenient as a preparative method. The dialyzed mixtures are concentrated by ultrafiltration (see Chap. 8, Vol. II) and are centrifuged in a clinical centrifuge at 500 *g* for 15 minutes to remove a small amount of precipitate. The supernatant, containing from 2 to 10 mg of protein per milliliter, is loaded on a column (1 × 50 cm) of Sephadex G-200 in 0.05 *M* Tris–HCl, pH 8.0, 0.15 *M* NaCl. The flow rate is 2.0 ml/hour, and 1-ml fractions are collected. The patterns obtained are illustrated in Fig. 4.

If it is desired to follow the composition of reconstituted molecules,

[38] G. M. Edelman, D. E. Olins, J. A. Gally, and N. D. Zinder, *Proc. Natl. Acad. Sci. U.S.* **50**, 753 (1963).

[39] D. E. Olins and G. M. Edelman, *J. Exptl. Med.* **119**, 789 (1964).

[40] M. Fougereau and G. M. Edelman, *Biochemistry* **3**, 1120 (1964).

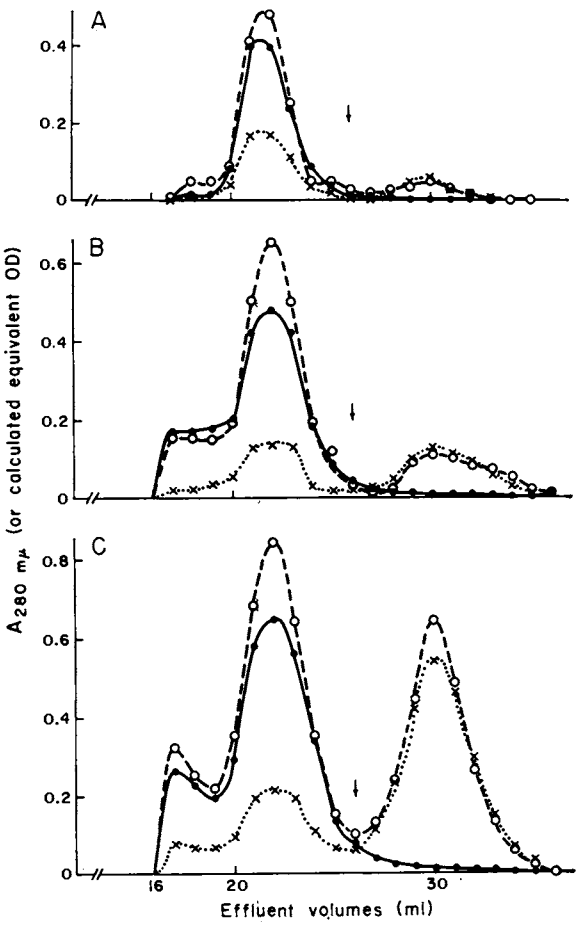


FIG. 4. Sephadex G-200 separation of reconstituted molecules prepared from reduced and alkylated heavy and light chains mixed at three different mass ratios. (*A*) Mass ratio of heavy chain to light chain = 3:1 in the original mixture; (*B*) mass ratio of heavy chain to light chain = 2:1 in the original mixture; (*C*) mass ratio of heavy chain to light chain = 1:1 in the original mixture. -○- - -○-, A_{280} mμ (absorbancy at 280 mμ); -●———●-, amount of ^{125}I-labeled heavy-chain fraction; and · × · · · · × ·, amount of ^{131}I-labeled light-chain fraction estimated by assay of radio-activity and converted into equivalent absorbancy values by the use of the known specific activities of the chains. The arrow indicates the peak of activity of alkaline phosphatase used as a marker. From M. Fougereau and G. M. Edelman, *Biochemistry* **3**, 1120 (1964).

the light chains used in the original mixture may be obtained from immunoglobulin labeled with I^{131}. The heavy chains may be obtained from immunoglobulin labeled with I^{125}. The methods used have been described elsewhere.[39,40]

Reconstituted immunoglobulins have been shown to recover antibody activity[26,38,41] and to have the same molecular weight and gross arrangement of chains as native immunoglobulins.[39,40] Hybrid molecules may be formed by mixing complementary chains of different origins within or across animal species lines.[42]

b. Reconstitution from Reduced Chains with Formation of Interchain Disulfide Bonds

Evidence has been obtained that interchain disulfide bonds may be re-formed in reconstituted molecules.[39] The procedures are exactly the same as those employed for reconstitution from reduced alkylated chains except that alkylation is omitted. Immunoglobulin is reduced as described above (Section B,2,b,iii), and the entire reaction mixture is placed on the Sephadex G-100 column in 0.5 *N* propionic acid. The chain fractions are mixed and dialyzed at 4° against a solution of 0.1 *M* 2-mercaptoethanol in 0.05 *M* Tris buffer, pH 7.2. The dialyzed mixtures are diluted with the same solvent to protein concentrations of 0.1% or less and are dialyzed against multiple changes of 0.05 *M* Tris, pH 7.2, in the absence of mercaptan. The products may be fractionated by gel filtration on Sephadex G-200 as described above.

c. Reconstitution from Half-Molecules

Nisonoff and co-workers[10,11,43,44] have shown that rabbit γG-immunoglobulin dissociates into half-molecules after reduction with 0.1 *M* mercaptoethylamine, addition of excess *p*-chloromercuribenzoate (PCMB) to prevent reoxidation, and acidification to pH 2.5 in aqueous salt solution. A single labile disulfide bond is cleaved in the process. If the pH is raised to neutrality, whole molecules are re-formed, and if the -SH groups are made available, the interchain disulfide bond may be re-formed.

In the procedure described by Nisonoff and Dixon,[44] immunoglobulin is reduced with 0.05 *M* 2-mercaptoethylamine hydrochloride under nitrogen for 75 minutes at 37° in 0.1 *M* sodium acetate buffer, pH 5.0. The

[41] O. Roholt, K. Onoue, and D. Pressman, *Proc. Natl. Acad. Sci. U.S.* **51**, 173 (1964).
[42] M. Fougereau, D. E. Olins, and G. M. Edelman, *J. Exptl. Med.* **120**, 349 (1964).
[43] J. L. Palmer and A. Nisonoff, *Biochemistry* **3**, 863 (1964).
[44] A. Nisonoff and D. J. Dixon, *Biochemistry* **3**, 1338 (1964).

mixture is passed through a column of IR-120 ion exchange resin in the same buffer. Excess PCMB is added. The protein is dialyzed against two 4-liter portions of cold 0.025 *M* NaCl, and the pH is lowered to 2.4 with 1 *N* HCl. This leads to dissociation into half-molecules. Readjustment of the pH to 8.0 leads to re-formation of 7 S molecules.

The interchain disulfide bond between the heavy chains may be reformed if the PCMB is first removed. The PCMB-treated protein is reduced with 0.1 *M* mercaptoethylamine for 3 hours at 37° in 0.1 *M* acetate buffer, pH 4.5. The mixture is passed over a column of Sephadex G-25 in 0.1 *M* sodium acetate buffer, pH 4.5. Adjustment of the pH to 8.0 with 1 *N* NaOH and allowing the solution to stand for 3 hours leads to re-formation both of the 7 S molecule and of the interchain disulfide bond. Reoxidation may also be achieved by overnight dialysis against cold 0.1 *M* sodium acetate adjusted to pH 7.5.[11]

The re-formation of immunoglobulins from half-molecules may be used to prepare hybrid molecules. For example, it has been shown[44] that half-molecules from specifically purified antiovalbumin are capable of combining with half-molecules of normal immunoglobulin to form hybrid molecules with active combining sites.

C. Preparation and Isolation of Fragments Produced by Enzymatic Hydrolysis of Immunoglobulins

The use of proteolytic enzymes to degrade antibodies has been prompted by practical objectives as well as by basic interests in antibody structure. One of the major practical uses of enzymes was to "despeciate" antitoxins of animal origin so that they would be less antigenic and safer for use in therapy of human disease. The use of proteolytic enzymes has increased our knowledge of the structure of antibodies; some of the main techniques will be reviewed below. Of the variety of enzymes that have been employed, papain and pepsin have so far proved most useful.

1. HYDROLYSIS WITH PAPAIN

In 1942, Petermann[45] showed that papain split bovine γG-immunoglobulin into fragments having sedimentation coefficients of 5.3 S and 3.7 S, the latter having a molecular weight of 45,000. She observed that the sedimentation coefficient of the larger fragment was similar to that obtained after digestion with pepsin.[46] Similar early studies on human

[45] M. L. Petermann, *J. Biol. Chem.* **144**, 607 (1942).
[46] M. L. Petermann, *J. Phys. Chem.* **46**, 183 (1942).

γG-immunoglobulin treated with papain yielded fragments having molecular weights of 47,000, some of which retained antibody activity.[47]

Although Petermann used both crude and crystalline papain in her original studies, the crystalline enzyme was not readily available. More recently, Porter[5-7] has shown that hydrolysis of rabbit γG-immunoglobulin with crystalline papain when combined with refined techniques of separation yielded two types of fragments. In one type ("fractions I and II," or Fab fragments), the antibody activity was retained; the other type of fragment was crystallizable and had no activity ("fraction III," or Fc fragments). The observations of Porter stimulated a number of studies on enzymatic hydrolysis of immunoglobulin.

a. Cleavage with Crystalline Papain[6,7]

γG-Immunoglobulin (150 mg) and mercuripapain (1.5 mg) are dissolved in 10 ml of buffer 0.1 *M* sodium phosphate, pH 7.0, 0.01 *M* cysteine, 0.002 *M* sodium ethylenediaminetetraacetate, EDTA). The solution is incubated for 16 hours at 37°. It is then dialyzed against water with vigorous stirring to inactivate the enzyme. The products are subsequently dialyzed against 0.01 *M* acetate buffer, pH 5.5, for chromatography on carboxymethylcellulose (0.71 meq/gm). The column dimensions are 2.4 × 35 cm, and the mixing chamber volume for gradient elution is 1200 ml. The buffers are 0.01 *M* sodium acetate, pH 5.5, with gradient to 0.9 *M* sodium acetate, pH 5.5. The fractionation obtained is shown in Fig. 5.

Subsequent studies of the fragments have indicated that they are not homogeneous chemical species or true subunits but are artifically produced by treatment with an enzyme capable of rather general attack on peptide bonds. The so-called fractions I and II are composed of similar Fab fragments but are derived from different immunoglobulin molecules.[48]

A number of modifications of the hydrolysis procedure have been proposed. The hydrolysis may be stopped by addition of *N*-ethylmaleimide,[7] *p*-chloromercuribenzoate,[49] or iodoacetamide.[50] Conditions for optimal cleavage were described by Putnam *et al.*[49] The pH optimum was in the range pH 5 to 6, and 66% of rabbit γ-globulin was cleaved in 15 minutes at 37°.

[47] M. L. Petermann, *J. Am. Chem. Soc.* **68**, 106 (1946).
[48] J. L. Palmer, W. J. Mandy, and A. Nisonoff, *Proc. Natl. Acad. Sci. U.S.* **48**, 49 (1962).
[49] F. W. Putnam, M. Tan, L. T. Lynn, C. W. Easley, and S. Migita, *J. Biol. Chem.* **237**, 717 (1962).
[50] E. Marler, C. A. Nelson, and C. Tanford, *Biochemistry* **3**, 279 (1964).

The rates of cleavage are slowed considerably if mercuripapain is used in the absence of sulfhydryl-containing activators and EDTA.[51,52] In the presence of 0.01 *M* cysteine, 80% of human and rabbit immunoglobulin molecules are cleaved within 10 minutes at pH 6.0 and 37°.[52] The rates of hydrolysis differ for immunoglobulins of different animal origin in the following order: rabbit > human > bovine > equine > porcine.[51]

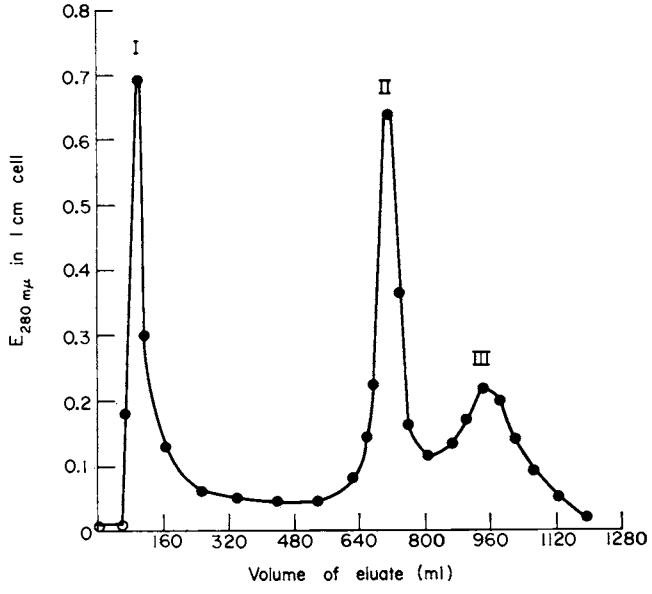


FIG. 5. Chromatography of papain digest of rabbit γG-immunoglobulin on carboxymethylcellulose. Weight of digest 150 mg. Column 2.4 × 30 cm. Volume of mixing chamber 1200 ml. Gradient from 0.01 *M* sodium acetate, pH 5.5, to 0.9 *M* sodium acetate, pH 5.5, commencing at 200-ml eluate volume. From R. R. Porter, *Biochem. J.* **73**, 119 (1959).

Chromatography of papain hydrolyzates of human γG-immunoglobulin on carboxymethylcellulose under the same conditions used for rabbit γG-immunoglobulin fails to resolve Fab and Fc fragments. Separation may be achieved on diethylaminoethylcellulose (0.9 meq/gm) under the following conditions[53]: column size 2 × 60 cm, mixing chamber 500 ml, buffers for gradient elution, 0.005 to 0.5 *M* sodium phosphate, pH 7.8. The fragments from human γG-immunoglobulin may also be fractionated

[51] S. H. Hsiao, and F. W. Putnam, *J. Biol. Chem.* **236**, 122 (1961).
[52] H. F. Deutsch, E. R. Stiehm, and J. I. Morton, *J. Biol. Chem.* **236**, 2216 (1961).
[53] G. M. Edelman, J. F. Heremans, M.-Th. Heremans, and H. G. Kunkel, *J. Exptl. Med.* **112**, 203 (1960).

(with less resolution) by starch zone electrophoresis in 0.05 *M* barbital buffer, pH 8.6.[54] Franklin has described additional chromatographic methods for fractionating papain fragments of human γG immunoglobulin.[54a]

b. Hydrolysis with Insoluble Papain

This method has the advantage of minimizing nonspecific and irrelevant proteolysis in the production of fragments of immunoglobulins. It may prove to be particularly useful in refined chemical studies of the fragments. Cebra *et al.*[17] have directly demonstrated that the cleavage of rabbit γG-immunoglobulin is a two-stage process consisting in proteolysis followed by breakage of disulfide bonds. After 5 minutes of treatment with water-insoluble papain, an average of five peptide bonds per molecule were cleaved with no change in molecular weight. Subsequent treatment with cysteine led to the production of 3.5 S fragments.

i. Preparation of Enzyme

A copolymer of L-leucine and *p*-amino-DL-phenylalanine hydrobromide (molar residue ratio 2.5–1) is prepared by the method of Bar-Eli and Katchalski.[55] The copolymer (250 mg) is dissolved in 3 ml of 50% aqueous acetic acid, and the solution is mixed with 3 ml of 2 *N* HCl. Diazotization is effected by dropwise addition of 1.5 ml of 0.5 *M* $NaNO_2$ to the ice-cold polymer solution. After 2 hours at 4°, the polydiazonium salt is precipitated by adding 2 *N* NaOH to a final pH of 8.0. The salt is washed twice with 12 ml of 10% sodium acetate followed by 30 ml of 0.1 *M* sodium phosphate buffer, pH 7.6. Eighty milligrams of crystalline papain (Worthington, twice crystallized) suspended in 2 ml of 0.03 *M* cysteine is added to a suspension of the diazotized copolymer made in 40 ml of buffer at pH 7.6 (0.075 *M* phosphate, 0.005 *M* cysteine, and 0.002 *M* EDTA). The mixture is flushed with nitrogen and is stirred for 20 hours at 4° in a closed vessel. The water-insoluble papain-conjugated copolymer is centrifuged and washed several times with phosphate buffer, pH 6.0, containing 0.005 *M* cysteine and 0.002 *M* EDTA. The product is stored in this buffer after homogenization in a Potter homogenizer with a Teflon piston.

ii. Cleavage of Immunoglobulin

Two hundred milligrams of γG-immunoglobulin in 0.9% NaCl (adjusted to pH 8.5 and containing 0.002 *M* EDTA) is mixed with 10 mg of

[54] D. E. Olins and G. M. Edelman, *J. Exptl. Med.* **116**, 635 (1962).
[54a] Franklin, E. C., *J. Clin. Invest.*, **39**, 1933 (1960).
[55] A. Bar-Eli and E. Katchalski, *Nature* **188**, 856 (1960).

insoluble enzyme. Before addition, the enzyme is washed in 5 volumes of 0.9% NaCl. The enzyme is kept suspended by stirring, and proteolysis may be followed by titration with 0.1 *N* NaOH in a pH-stat at pH 8.5 and 25°. The water-insoluble enzyme is removed rapidly at the desired time by filtration through Whatman No. 1 filter paper. Reduction of the hydrolyzates is then carried out in 0.9% NaCl, 0.01 *M* in cysteine at pH 8.0 and 37° for 12 hours.

2. HYDROLYSIS WITH PEPSIN

A number of studies have been reported indicating that pepsin reduces the molecular weight of immunoglobulins without destroying activity. Hydrolysis by pepsin has been studied extensively by Nisonoff and his colleagues.[8-16,56] Their work indicates that treatment of rabbit γG-immunoglobulin with pepsin yields a group of fragments with sedimentation coefficients of 5 S and molecular weights of approximately 106,000. These fragments lack that portion of heavy chains corresponding to the Fc fragment (see Fig. 1). They are divalent and are capable of precipitating with antigens. Mild reduction of the 5 S fragments with mercaptans results in cleavage of a single labile disulfide bond and the production of 3.5 S fragments (see Fig. 1)[13] with molecular weights of about 56,000. These fragments resemble Fab fragments. There is evidence that the disulfide bond linking the two 3.5 S fragments is the bond between the heavy chains. If this bond is reduced and the γG-immunoglobulin is then treated with pepsin, 3.5 S fragments are obtained directly. Reoxidation of reduced 3.5 S fragments leads to re-formation of 5 S fragments, and heteroligating 5 S fragments may be formed by reoxidizing mixtures of 3.5 S fragments from antibodies of different specificities.[44]

a. Formation of 5 S Fragments

Two grams of rabbit γG-immunoglobulin is treated with 20 mg of crystallized pepsin in 76 ml of 0.1 *M* sodium acetate buffer, pH 4.5. After 20 hours at 37°, a small amount of precipitate is removed by centrifugation, the pH is raised to 8 with 1 *N* NaOH, and Na_2SO_4 (25 gm per 100 ml) is added dropwise at room temperature, with stirring, to a final concentration of 18 gm per 100 ml. The heavy precipitate which forms is separated by centrifugation, dissolved in water, and dialyzed against two 4-liter volumes of cold 0.1 *M* sodium acetate. The 5 S fragments may also be fractionated by gel filtration and dialysis.[57,58]

[56] A. Nisonoff, *in* "Conceptual Advances in Immunology and Oncology," p. 273. Hoeber-Harper, New York, 1962.
[57] J. W. Goodman, *Biochemistry* **3**, 857 (1964).
[58] S. Utsumi and F. Karush, *Biochemistry* **4**, 1766 (1965).

b. Reduction and Reoxidation of 5 S Fragments

The labile disulfide bond holding the 3.5 S fragments together may be cleaved[15,43] by treating a solution of 5 S fragments containing 50 to 100 mg of protein per milliliter with 0.01 *M* 2-mercaptoethylamine hydrochloride at pH 5 for 75 minutes at 37°. The pH of the solution is lowered to 4.5 by the addition of 1 *N* acetic acid, and the reducing agent is removed[13,59] by passage of the solution through a column (8 × 280 mm) of IR-120 cation exchange resin (in the sodium cycle) at 4°. The protein is washed through with 0.1 *M* sodium acetate buffer, pH 4.5. The pH of the eluate is adjusted to pH 8.0 with 2 *N* NaOH. Reoxidation is then achieved by passing a stream of oxygen over the solution in an open beaker for 2 hours at room temperature, followed by dialysis overnight at 4° against 4 liters of 0.1 *M* sodium acetate adjusted to pH 7.5.

3. REDUCTION OF Fab FRAGMENTS AND RECOVERY OF THE Fd FRAGMENT

If Fab fragments are reduced in the absence of urea and placed in dissociating solvents, the light-chain moiety dissociates from the portion of the heavy chain corresponding to the Fd fragments,[29] as illustrated in Fig. 1.[3,29]

Fab fragments may be reduced by dissolving them in 0.05 *M* Tris buffer (pH 8.0) made 0.1 *N* in 2-mercaptoethanol. After 1 hour, the solution is made 0.2 *M* in iodoacetamide. To obtain Fd fragments of rabbit γG-immunoglobulin, the reduced alkylated Fab fragments are passed over a Sephadex G-100 column in 1 *N* propionic acid.[29] To fractionate Fd fragments of human γG-immunoglobulin, a column of Sephadex G-100 in 1 *N* propionic acid and 6 *M* urea has been found effective.[3] Two peaks are obtained in both cases: the first peak contains the Fd fragments, and the second peak contains light-chain moieties (occasionally contaminated with Fd fragments).

4. TREATMENT OF IMMUNOGLOBULINS WITH ENZYMES OTHER THAN PAPAIN AND PEPSIN

Various enzymes have been used in attempts to fragment immunoglobulins.[5,60] They include: (1) sulfhydryl-activated proteases (ficin, chymopapain, bromelin); (2) proteases and peptidases of the digestive tract; and (3) miscellaneous enzymes (lysozyme, neuraminidase).[60] The

[59] W. J. Mandy and A. Nisonoff, *J. Biol. Chem.* **238**, 206 (1963).

[60] F. W. Putnam, C. W. Easley, and L. T. Lynn, *Biochim. Biophys. Acta* **58**, 279 (1962).

sulfhydryl-activated proteases yield products similar to those obtained after papain treatment.[60] Exposure of the γG-immunoglobulins to carboxypeptidase, leucine aminopeptidase, crude hog peptidase, enterokinase, urease, and lysozyme had no apparent effect.[60]

Treatment with trypsin[5,61,62] yields fragments resembling those of papain-treated γG-immunoglobulin. The presence of cysteine increases the yield of 3.5 S fragments. The conditions used by Schrohenloher[61] for tryptic hydrolysis are: enzyme–substrate ratio 1–100, 0.1 *M* sodium phosphate buffer, pH 7.6, 37°, 72 hours.

D. Some Remarks on the Application of Structural Methods

Some final strictures on the application of the methods described above may be useful. In precise chemical studies of structure, it is usually necessary to work with fully reduced polypeptide chains and to assure the purity of these chains. On the other hand, for activity studies, the partially reduced chains must be employed. Here, too, the problem of purity is primary. Although pure light-chain preparations may easily be obtained by present methods, more care must be exercised to obtain pure heavy chains. Moreover, heavy-chain preparations tend to aggregate and occasionally become insoluble.

It has often been assumed that the fragments produced by enzymatic treatment of the immunoglobulins are homogeneous chemical entities or natural subunits of the molecule. This assumption is incorrect, and its adoption may lead to fruitless experimentation. The fragments of a particular type share many gross properties (such as antigenic structure); in general, however, they represent heterogeneous collections of polypeptides.[52,63,64] Although the exact sites of cleavage of the polypeptide chains of immunoglobulins are not known, it is fairly certain[3] that the proteolytic enzymes attack peptide bonds within the fragments as well as peptide bonds in stretches of the chains linking those regions that eventually comprise the fragments.

[61] R. E. Schrohenloher, *Arch. Biochem. Biophys.* **101**, 456 (1963).
[63] L. A. Hanson and B. G. Johansson, *Nature* **187**, 599 (1960).
[63] E. R. Stiehm, J. I. Morton, and H. F. Deutsch, *J. Immunol.* **85**, 337 (1960).
[64] J. L. Fahey and B. A. Askonas, *J. Exptl. Med.* **115**, 623 (1962).

Author Index

Numbers in parentheses are reference numbers and indicate that an author's work is referred to although his name is not mentioned in the text.

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