NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Technical Report No. 32-900

Immunoadsorbents: Preparation and Use of Cellulose Derivatives

Dan H. Campbell

Norman Weliky

JET PROPULSION LABORATORY

CALIFORNIA INSTITUTE OF TECHNOLOGY

PASADENA, CALIFORNIA

May 1, 1966

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Technical Report No. 32-900

Immunoadsorbents: Preparation and Use of Cellulose Derivatives

Dan H. Campbell
Norman Weliky

N. H. Horowitz, *Manager*
Bioscience Section

JET PROPULSION LABORATORY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

May 1, 1966

Prepared Under Contract No. NAS 7-100
National Aeronautics & Space Administration

CONTENTS

ABSTRACT

Chemicals, biochemicals, and biological materials that combine or complex specifically with insoluble substances can be isolated from mixtures by combination with these insoluble substances followed by dissociation under conditions that do not significantly alter the properties of interest of the desired materials. Antibodies (i.e., proteins of immunity that are synthesized by animals and humans and react specifically with foreign macromolecules) can be isolated from the blood by directly linking these foreign macromolecules (antigens), or small portions of them (haptens), by chemical bonds to insoluble cellulose derivatives and using these insoluble substances (immunoadsorbents) to complex with, and remove antibodies from, the other components of blood serum or plasma. Methods are described in detail for performing these operations, and precautions are discussed for avoiding difficulties in applying these methods to different adsorbents and different antigen–antibody systems. Many antibodies can be isolated in greater than 90% yield and purity.

A brief review is given of the history of the insoluble adsorbent technique and its effectiveness and applicability for the isolation of antibody, antigens, and the study of antigen–antibody interactions.

I. INTRODUCTION

An "immunoadsorbent"[a] may be defined as an insoluble material that has the property of combining with either a specific antibody or 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 also utilized other materials such as polystyrene (Refs. 11–13) and polyacrylic (Ref. 4) ion-exchange resins, silk fibroin (Ref. 14), organic polymer matrix (Ref. 15), antigen-coated glass beads (Ref. 16), and cross-linked proteins (Ref. 17). For general reviews dealing with the various types of adsorbents, including cellulose derivatives, reference should be made to Refs. 2, 4, and 18–23. See also Table 1.

One of the first investigations making use of a cellulose derivative for the isolation of antibody was described by Campbell *et al.* (Ref. 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 (Ref. 25) using a pollen antigen conjugated to cellulose. Webb and LaPresle (Ref. 26) isolated antibodies to fragments of digested protein antigens. Jagendorf *et al.* (Ref. 27) and Weetall and Weliky (Ref. 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 (Ref. 29). Similar studies have been reported by Gurvich *et al.* (Ref. 30) and by Weliky and Weetall (Ref. 31). The combining strength of a simple hapten with a complementary antibody combining site is many times greater when the hapten is attached to an insoluble matrix. Thus, when coupled to cellulose, it functions as a good specific adsorbent, but will still release antibody in the presence of soluble hapten (Ref. 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 that provide them with advantages as well as disadvantages.

[a] 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, e.g., bacteria [Ref. 1 (in Ref. 2)]; erythrocytes (Refs. 2–8) and recently protein antigens insolubilized by mild denaturation (Ref. 9) or polymerization of antibody with bifunctional coupling agents such as bisdiazotized benzidine (Ref. 10) for the isolation of antigens.

Table 1. Isolation of antibody using specific insoluble adsorbents

Carrier	Antigen	Elution solvent	Milligrams of antibody per gram of adsorbent	Overall yield, %	Recovery from adsorbent, %	Precipitable protein, %	Ref.
Charcoal	Protein azobenzoate	0.1 M acetic acid	1			30 to 60	44
Glass beads	Ovalbumin	Glycine-HCl, pH 3.0	5		90	80	16
RBC stroma	Azohaptens	Acetic acid, 0.01 M				50	45
RBC stroma	Azophenyl arsonate	Sodium arsanilate	10	30 to 65	30 to 65	50 to 67	46
RBC stroma	Azophenyl arsonate	HCl, pH 3.2	10	40 to 50	40 to 50	40 to 50	46
Silk (fibroin)	Azophenyl arsonate	HCl, pH 3.2	16	36	78	100	14
Tanned RBC stroma on IRA 410 resin	Blood group factor B	Sugars at 37°C			55 to 65	75 to 90[a]	4
Cross-linked protein	Azohaptens	Hapten	>1500	60 to 80	82 to 86	>90[b]	17
Cross-linked protein	Bovine serum albumin	Glycine-HCl, pH 2.3	400 to 700	Up to 90	90	88	17
Cross-linked protein	Insulin, human serum albumin	Glycine-HCl, pH 2.3	100 to 130			72 to 80[c]	17
Polyacrylic acid	Human serum albumin	pH 3.6		44	54	84	4
Polyaminopolystyrene	Bovine serum albumin	Citrate-phosphate, pH 3.1	5	27 to 45	30 to 45	52	11
Polyaminopolystyrene	Ovalbumin	Citrate-phosphate, pH 3.1	6	26	42	63	11
Polyaminopolystyrene	Human gamma globulin	HCl, pH 3.0	3		35	82	12
p-Aminobenzylcellulose	Bovine serum albumin	HCl, pH 3.2	15	86	86	89	24
p-Aminobenzylcellulose	Human gamma globulin	0.2 M citric acid, pH 3.2			25 to 67		35
p-Aminobenzylcellulose	Human gamma globulin	pH 3.2			25		35
m-Aminobenzyloxy-methylcellulose	Human serum albumin, rabbit gamma globulin, human gamma globulin	HCl, pH 3.2	100 to 150		40 to 60	90 to 100	38
Half benzidine amide of carboxymethylcellulose	Bovine serum albumin	HCl, pH 2.3	14	87 to 97	87 to 97	80 to 82	36
Aminoethylcellulose	6-Carboxypurine	HCl, pH 2.3	14	83 to 91	83 to 91	90	40
Carboxymethylcellulose	Bovine serum albumin, human gamma globulin, azophenyl arsonate	HCl, pH 2.0–2.3		71 to 96	78 to 100	86 to 100	14, 36
Tyramine amide of carboxymethylcellulose	Azophenyl arsonate	0.05 M phosphate at pH 3.0, and 0.1 M HCl			75 to 80	70	43

[a]Identified as specific antibody.

[b]By equilibrium dialysis.

[c]Method of Berson and Yalow.

II. GENERAL 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:

The selection and treatment of starting material will depend to some extent upon 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 a derivative such as carboxymethylcellulose, the material should be thoroughly washed with dilute acid (pH 1–2) and dilute alkali (pH 9–10); at times it is even desirable to wash with an organic solvent such as methanol. During the process of washing, it is advantageous to allow the suspension to stand for a while and decant 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.

The capacity of an adsorbent will depend upon the number of specific groups attached to the cellulose and also upon 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 7S gamma globulin, having a minor-to-major axial ratio of 300 Å/38 Å, would be expected to cover several hundred glucose units if oriented parallel to the long axis of the cellulose fiber. If 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 because of steric hindrance.

The adsorbent may be used either as in column chromatography or in a batch by mixing with the solution containing antibody. The former is usually preferable. No special precautions other than uniformity, suitable flow rate, and elimination of air are necessary in packing such columns. One can utilize a semi-batch 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 storage under concentrated salt solution.

When 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 that 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 for a small amount of antibody that 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 or hapten derivative which complex with the desired antibody are free and exposed and in the proper spatial relationship (relative to the cellulose surface) to complex effectively with the antibody combining site. In all instances, the final product must be thoroughly washed to remove the last traces of soluble reagents. This is particularly true of haptens, because 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.

The adsorption of antibody from serum is accomplished by merely exposing it to the adsorbent either by slow passage through a column or mixing in a flask. In order to eliminate adsorption of nonantibody protein as much as possible, serum should first be decomplemented with a heterologous antigen–antibody precipitate and then diluted with 1 to 2 volumes of 0.9% NaCl solution. If one is only interested in recovering pure precipitating antibody, and nonspecific adsorption of albumin is high, it is an advantage to adsorb a solution consisting only of the gamma globulin fraction, which can easily be prepared by salt precipitation (Ref. 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 extremely critical.

However, it is advisable to allow sufficient time for all of the adsorbent surface to have contact with the serum. The rate of passage through a 1- × 10-cm column should be approximately 5 ml/hr. It is important to ensure that all interspaces of the adsorbent are filled with liquid before the sample of serum is applied. After the antibody containing solution has passed through the column, the rate of washing may be increased. It is generally agreed that antigen–antibody complexes become increasingly stable with time so that it is advisable to start dissociation within 12 hr. The adsorbent is then washed with saline until completely free of protein, which is indicated by tests on the effluent, supernatant, or filtrate depending upon the procedure used.

Elution of antibody consists first of dissociation of antibody from its antigen template and then elution from the column or removal of the adsorbent by centrifugation. 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 involved in phosphate or acetic acid buffers) either inhibit dissociation or inactivate antibody under acid conditions, it is advisable to carefully adjust pH 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 (Refs. 14 and 33), but apparently will dissociate under alkaline conditions of approximately pH 12.[b] Optimum conditions for dissociation by means of pH changes may depend upon the ionizable character of the antigenic combining site. Lerman (Ref. 29) has reported the successful use of hapten to elute antiazophenylarsonate antibody from a cellulose adsorbent. A serious problem that may occur following dissociation of antigen and antibody is the nonspecific readsorption of antibody. In order to avoid such reactions, 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 reaction time for reversal of antigen–antibody combination is much greater than the forward reaction. Thus, more time must be allowed to give complete dissociation, and the flow through a column may have to be reduced; if a batch method is used, stirring should be continued for 1 to 2 hr. Dissociation is usually faster at room temperature than in the cold. Regardless of how the antibody is separated from the adsorbent, it should be returned to neutrality as soon as possible if pH changes have been made.

Purity, recovery, and yield of the final product should always be given careful consideration. Ordinarily such information is provided by quantitative precipitation on the basis of specific precipitable protein versus total protein recovered (purity), by the difference between antibody removed from the antiserum and the amount recovered from the adsorbent (recovery from adsorbent), and by the difference between total antibody in the serum and the amount recovered from the adsorbent (overall yield). In the case of a 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 (Ref. 16). Another method would be that described in Ref. 34, in which I^{131} tagged antigen is added to a solution containing antibody, the antibody then precipitated with salt, and subsequently estimated in terms of the antigen which would not have precipitated unless attached to antibody globulin. In many systems, both purity and yield may approach 100% by the use of immunoadsorbents. As stated previously, lack of purity or apparent lack of purity may be the result of failure to decomplement the antiserum, adsorption of nonspecific protein, inhibition by soluble antigen, denaturation during the final stages of isolation, or nonprecipitating forms of antibody.

Analytical procedures, in general, are relatively simple and utilize physical, chemical, and serological reactions. Detailed procedures will be included in other chapters of *Methods in Immunology and Immunochemistry*.[c] Proteins and haptens bound to cellulose may be determined best by the use of radioactive labeling and counting devices.

[b] Unpublished material by N. Weliky and H. H. Weetall, 1965.

[c] *Methods in Immunology and Immunochemistry*, Academic Press, New York (to be published).

III. SPECIFIC PREPARATIONS

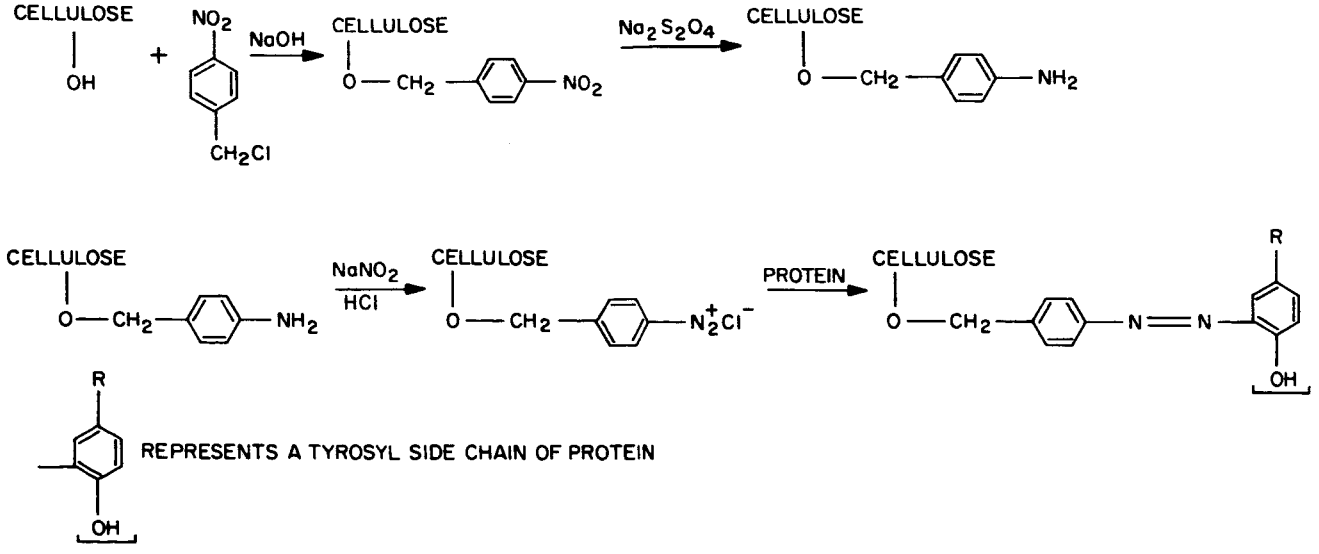
Only the preparation of adsorbents which have been demonstrated to be effective in extracting antibody from serum and from which antibody can be recovered in useful form will be described here. The adsorbents described are most conveniently classified by the functional groups of the cellulose derivatives used to react with antigen, i.e., carboxylic acid, alkylamino, aminoarylcellulose, 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 III-C) or the half benzidine amide of carboxymethylcellulose (Section III-A-2) are the preparations of choice. The protein derivative of m-benzyloxymethylcellulose (Section III-A-3) has the highest capacity for antibody; however, recovery of antibody from the adsorbent is lower than that of the preceding ones. It should be remembered that, in order to achieve maximum capacity, an excess of serum must usually be used; thus, overall yields are low, and the possibility of antibody fractionation great. Simple organic molecules (haptens) can usually be synthesized in forms which can be coupled to carboxymethylcellulose, aminoethylcellulose, a phenolic cellulose, the half benzidine amide of carboxymethylcellulose, or some other cellulose derivative. For the haptens which have been commonly used, those which form aryldiazonium salts, such as arsanilic acid, sulfanilic acid, and p-aminophenyltrimethylammonium, can be coupled easily to protein derivatives of cellulose (Sections III-A; III-C) phenolic cellulose derivatives (Section III-D), or, if available in the form of a p-azoaniline derivative (such as p-aminophenylazophenylarsonic acid), can be coupled directly to carboxymethylcellulose (Section III-C). ε-(2,4-dinitrophenyl)-lysine can be coupled directly to carboxymethylcellulose (Section III-C). Halides such as 6-trichloromethylpurine and benzenesulfonyl chloride may be coupled to aminoethylcellulose (Section III-B) or to aminoarylcellulose (Section III-A). For additional information, reference may be made to Ref. 23 or other reviews of the chemistry of cellulose and its derivatives.

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.

A. 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 III-C.)

SKETCH I



1. 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 has to be washed exhaustively with organic solvents before it can be used. A procedure for synthesis and coupling to proteins has been described in Ref. 24. Adsorbents made by this procedure have been used to isolate antibody with recoveries and precipitability in excess of 88%. Variability of results have 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 (Ref. 35),[d] but purity is uniformly high. Coupling to protein may also take place at tryptophan, histidyl, lysyl, or arginyl side chains. (See Sketch 1.)

The preparation described below is that of Campbell *et al.* (Ref. 24) with some modifications.[d]

a. Preparation of p-nitrobenzylcellulose. The reaction is done in a 1000-ml, three-neck flask, with a motor-driven stirrer in the center, a Liebig condenser on one side, and a thermometer on the other. The flask is kept in a water bath on a hot plate in order to maintain the temperature of 95°C needed throughout the reaction. Powdered cellulose is thoroughly washed with dilute acid, dilute base, and finally water; following this procedure, it is dried. A 20-g portion of this material is mixed with 60 g of p-nitrobenzyl chloride and 150 ml of 40% sodium hydroxide. The mixture is stirred vigorously at 95°C. (Cooling may be required during the first part of the exothermic reaction.) After 4 hr, the mixture is poured into approximately 4 liters of cold distilled water and filtered on a Buechner funnel. The residue is washed with water, ethanol, and finally with approximately 300 ml of acetone in a Soxhlet extractor. The extraction is continued for 3 to 4 days until no more color is extracted; then the cellulose derivative is pressed between pieces of filter paper and left to dry.

b. Preparation of p-aminobenzylcellulose. This reaction is done in a 500-ml, three-neck 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 g) is suspended in 125 ml of 95% ethanol and heated to near boiling. The mixture is then stirred vigorously and 12.5 g 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 approximately 30 min, the light yellow product is filtered off on a Buechner funnel and washed with cold water. The color of the final product varies from a light yellow to light brown. It is dried in a desiccator over calcium chloride.

c. Diazotization and coupling of p-aminobenzylcellulose to proteins. A 5-g portion 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 min as measured by starch-iodide paper. Stirring is continued for another 15 min; the material is then filtered on a Buechner funnel and washed with 1 liter of approximately 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 hr, and stored at 4°C for 5 to 6 days. The product is then filtered on a Buechner 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 min in approximately 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.

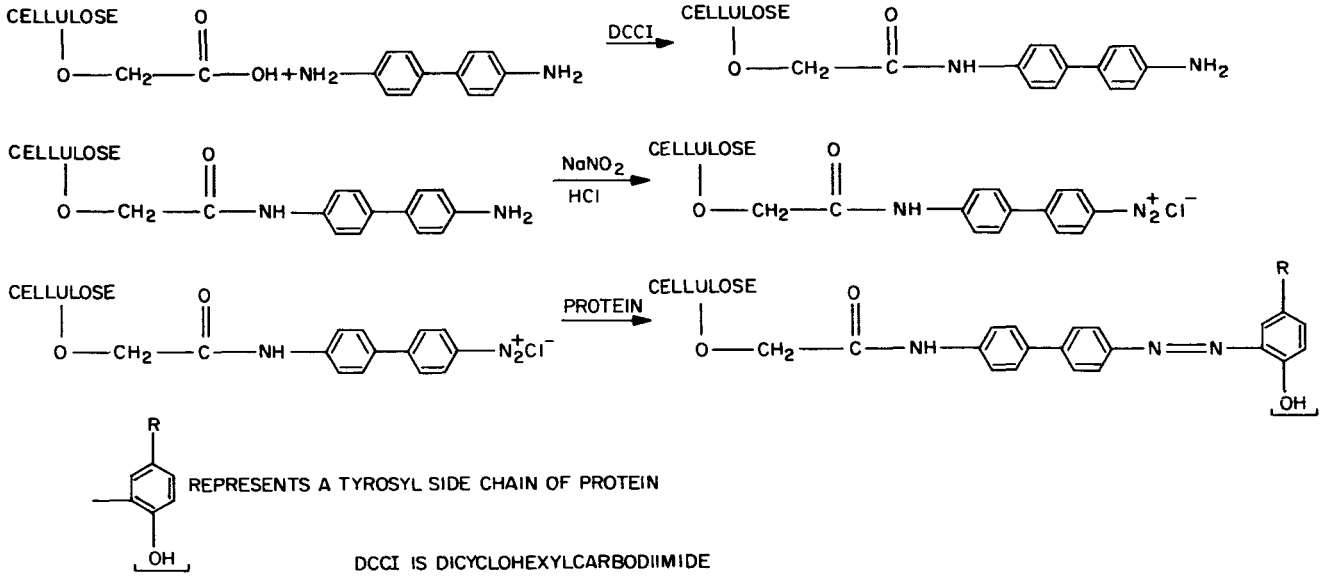
Recovery of antibody from the adsorbent varies from 15 to 40% with purity (precipitability with specific antigen) of 61 to 92%. The best results have been obtained by the column method with acid dissociation at pH 2.5.

2. Preparation of the Benzidine Derivative of Carboxymethylcellulose and Its Protein Derivatives

Proteins, phenols, aromatic amines, and other substances which couple to aryldiazonium salts can be coupled to the half benzidine amide of carboxymethylcellulose, i.e., the 4-(p-aminophenyl)-anilide of carboxymethylcellulose (Ref. 36). The procedure and precautions for coupling benzidine to carboxymethylcellulose are the same as those for coupling proteins and haptens to carboxymethylcellulose using dicyclohexylcarbodiimide (DCCI, discussed in Section III-C). Benzidine also reacts with carboxymethylcellulose in 90:10 water–methanol

[d] Weliky, B. G., and Campbell, D. H., 1965 (unpublished material).

SKETCH 2



and in methanol itself in the presence of carbodiimides. Coupling to protein may also take place at tryptophan, histidyl, lysyl, or arginyl side chains. (See Sketch 2.)

A 1-g portion of carboxymethylcellulose is stirred in a beaker or flask with 200 ml of 2 N hydrochloric acid for 1 hr, filtered on a Buechner funnel, stirred with 400 ml of water, filtered, resuspended in approximately 400 ml of water, and refiltered. Excess carboxymethylcellulose over that required for immediate use may be acid-washed, suspended in acetone, air-dried, and stored. Portions of 1 g of carboxymethylcellulose and water are added alternately to a suspension of 0.2 g of benzidine in 2 ml of water in a 25-ml Ehrlenmeyer flask, equipped with a magnetic stirrer, until about 80% of the cellulose is added and the mixture is semi-solid. A solution of 0.4 g 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 2 days if creamy, or it may be stirred for 2 days if the mixture is fluid. At the end of 2 days, the mixture must still be semi-solid 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 stirred well, and the contents 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 Buechner filter and washed well with water; the cellulose derivative is then resuspended in 200 to 400 ml of acetone. After stirring for 1 hr, 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 the precaution that quantities of water required are usually less than calculated.

A 1-g portion 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 of the mixture should be below 15°C.) An excess of sodium nitrite is then added (5 ml of 14% sodium nitrite solution). After stirring for 1 hr, or standing with occasional shaking, the mixture is filtered in a Buechner 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 g of protein in 20 to 30 ml of water at pH 7–9, adjusting the pH 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 conducted below 15°C and preferably

near 0°C. The preferred pH for coupling is usually 8 to 9, but may be as low as 6 or as high as 11 for some substances. After stirring for 1 hr, 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 gamma globulin, the adsorbent may be washed successively with water, bicarbonate, 0.1 N hydrochloric acid, and again water, resuspending the adsorbent frequently to ensure even washing. In order to remove fines, it is desirable to permit the derivative to settle for 30 min out of 300 to 500 ml of water and decant. If the adsorbent is to be used in column form, this 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 g) is dissolved in a small amount of 2.0 N sodium hydroxide, the solution diluted to 1 liter, and the pH adjusted to 8.0 with acetic acid. Any insoluble material should be filtered off. Add the protein–cellulose adsorbent and stir overnight. If the solution must be stirred in the cold, the β-naphthol solution may be filtered cold to avoid 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.

3. Preparation of m-Aminobenzyloxymethylcellulose and Its Protein Derivatives

Among the cellulose derivatives available, adsorbents made from m-aminobenzyloxymethylcellulose, which has been reprecipitated from cupra-ammonium solution, are reported to have the highest capacity for antiprotein antibody (Refs. 37 and 38). The adsorbent is made by coupling the quaternary pyridinium salt of m-nitrobenzyloxymethyl chloride (Ref. 39) to cellulose to form m-nitrobenzyloxymethylcellulose. The nitroaryl cellulose derivative then can 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 another section). 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 mg/g adsorbent specific for antibody against human serum albumin, human gamma globulin, many other proteins, and some viruses (Ref. 20). Recovery of protein from the adsorbent seems to be about 40 to 60% (Ref. 38) using hydrochloric acid at pH 2.0–3.0 for dissociation. Coupling may also take place with tryptophan, histidyl, lysyl, or arginyl side chains of proteins. (See Sketch 3.)

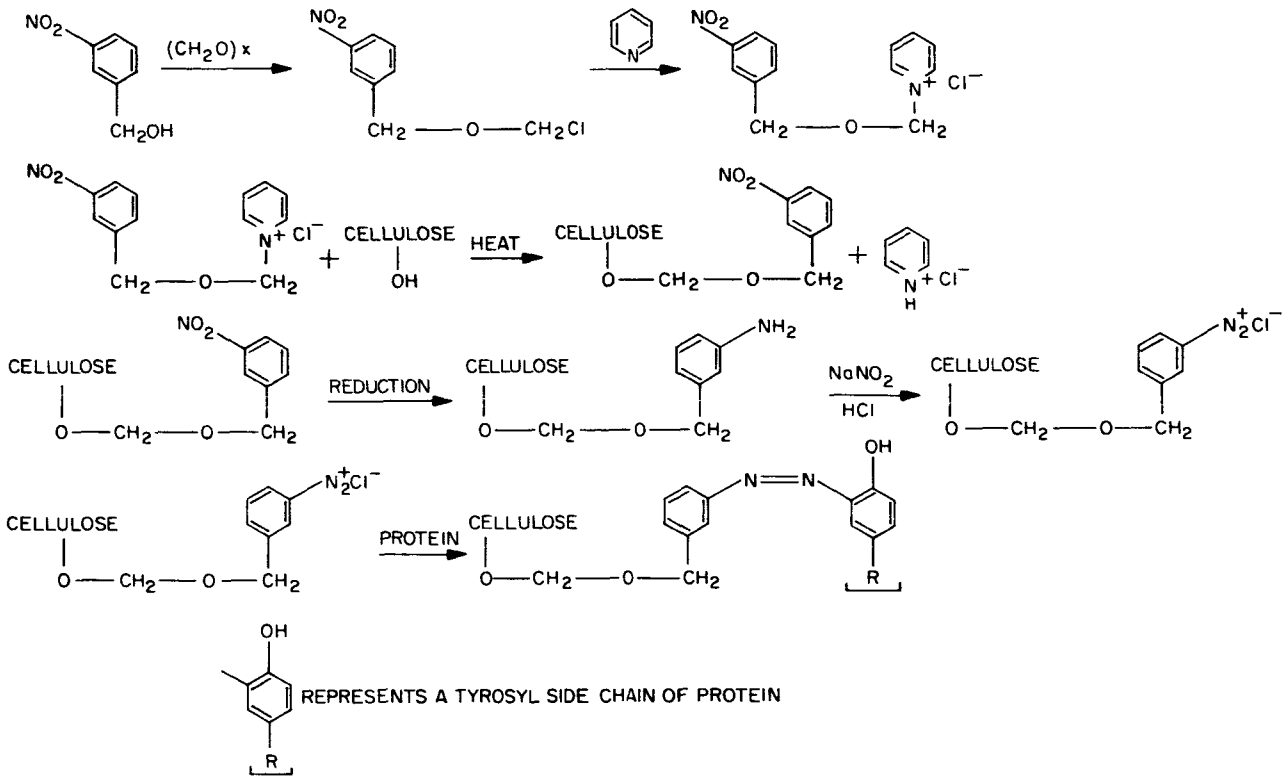
a. Preparation of N-(m-nitrobenzyloxymethyl)-pyridinium chloride **(Ref. 39).** Polyoxymethylene (4.8 g) is mixed with 6 g of m-nitrobenzyl alcohol in 35 cc of benzene and treated with dry hydrochloric acid for 2 hr with stirring. After standing overnight, the upper layer is separated and fractionated to yield 45.7% m-nitrobenzylchloromethyl ether ($b_{0.6}$ 147.5 to 148°C). The chloromethyl ether yields the desired pyridinium compound in 90.5% yield if treated with pyridine.

b. Preparation of m-nitrobenzyloxymethylcellulose **(Refs. 2, 30, and 38).** Cellulose powder (50 g) 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°C and heated at 125°C for 40 min. The product is washed thoroughly on a Buechner funnel with water and dried at 80°C. It is then washed with three 200-ml portions of benzene and dried on a Buechner funnel.

Alternatively, it is reported (Ref. 30) that time may be saved by dissolving the pyridinium salt and sodium acetate in 90% ethanol. Drying time of cellulose saturated with this solution is shortened; the resultant powder can be washed with benzene and then water immediately after heating for 40 min at 125°C.

c. Reduction and coupling to proteins. The procedure given in Ref. 37 follows: m-nitrobenzyloxymethylcellulose (3 g) is dissolved in 100 ml of an ammoniacal copper solution made by mixing 61 ml of ammonia, 4.5 g of cupric hydroxide [$Cu(OH)_2$], 1 g saccharose, and 39 ml of water. After adding an additional 50 ml of ammonia, tepid water is added until the cellulose precipitates. The supernatant liquid is decanted, the precipitate neutralized with 10% sulfuric acid, and washed several times with water. The washed precipitate is weighed and cold hydrochloric acid added so that its final concentration (calculated as HCl) is 5% in a total volume of 500 ml. Sodium nitrite (10 g) is added to the ice-cooled mixture; stirring is continued for 30 min. The suspension is then rapidly washed with ice water (2 liters) and once with cold borate buffer at pH 8.6. Borate buffer, 2% in human serum albumin (or other protein), is added to the washed precipitate to a total volume of 150 ml. The suspension is kept overnight under

SKETCH 3



refrigeration and the unbound protein washed off. The adsorbent is kept under refrigeration as an aqueous suspension.

The following procedure is that described in Ref. 38: A suspension of 1 g of m-nitrobenzyloxymethylcellulose is made in 33 ml of a solution consisting of 13 ml water, 20 ml of aqueous ammonia (specific gravity, 0.88), 1.5 g of cupric hydroxide [$Cu(OH)_2$], and 0.33 g of sucrose. A further 40 ml of ammonia solution is added, followed by 40 ml of warm water (70°C). Precipitation of cellulose begins as the water is added; it 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°C. (This procedure is a modified version of the procedure given in Ref. 37.)

42.5 ml of water and 7.5 ml of hydrochloric acid (36% w/v) are added to 25 ml of suspension containing 250 mg of aminoarylcellulose. The mixture is cooled in an ice bath; 1.9 g of sodium nitrite is added, and the mixture is stirred for 30 min. The cellulose derivative is centrifuged and washed twice with cold water; then it is washed 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 stirred at 2°C for 24 hr. 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.

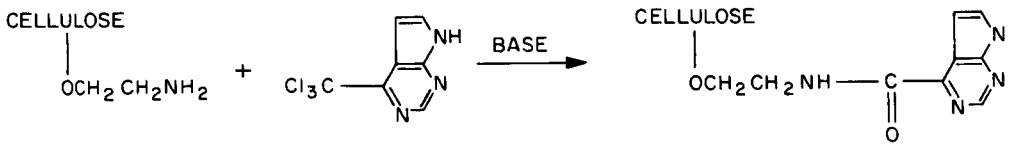
B. Aminoalkylcellulose Derivatives

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.

1. Preparation of an Adsorbent from Aminoethylcellulose and 6-Methylpurine, for Isolating Anti-Purine Antibody

Compounds with trichloromethyl groups will react with amines, including aminoethylcellulose (Ref. 40), to form derivatives which hydrolyze to amides in the presence of water. Trichloromethylpurine can be made from 6-methylpurine (Ref. 41) by several methods; the one resulting in the highest yield is described below. The preparation of other 6-trichloropurines and related compounds has also been published (Ref. 42). (See Sketch 4.)

SKETCH 4



Sulfuryl chloride (50 ml) is added to a solution of 6-methylpurine (13 g) in trifluoroacetic acid (50 ml). When the exothermic reaction has subsided, the solution is refluxed for 1 to 2 hr. The solution is brought to a syrupy consistency by evaporation under reduced pressure, and the clear residue 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. For further reaction, trichloromethylpurine is obtained in 73% yield (17 g) as a white crystalline material of sufficient purity.

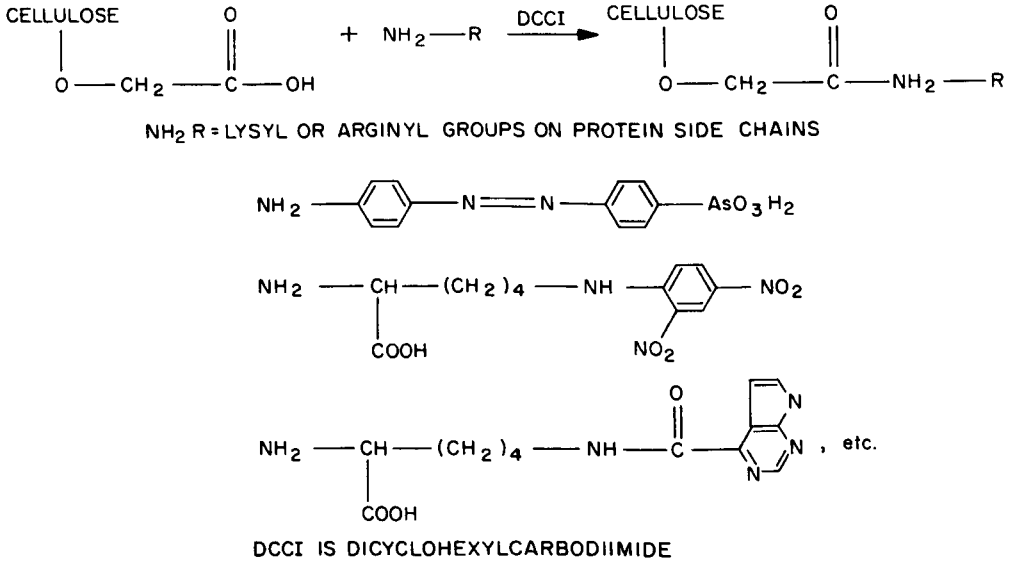
Aminoethylcellulose (5 g) is suspended in sufficient tetrahydrofuran–water (90:10) that it can be efficiently stirred. To this mixture is added 6-trichloromethylpurine (1 g) and 0.5 N NaOH in small portions so as to maintain the pH at 10.0 to 10.5. The pH should be checked occasionally over a period of about 6 hr and adjusted if necessary. After stirring overnight the mixture is filtered in a Buechner funnel, and the cellulose derivative repeatedly suspended in acetone and filtered until free of reagent. Before use, it should be suspended several times in sodium bicarbonate and then 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.

C. Carboxymethylcellulose Derivatives

Carboxymethylcellulose couples to basic amines in the presence of carbodiimides (Refs. 14 and 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/g of adsorbent per milliliter of serum, so that 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. Using hydrochloric acid of pH 2.0–2.3, antibodies can be isolated with recoveries of 80 to 100% and precipitability of 80 to 100%. (See Sketch 5.)

The capacity of commercial carboxymethylcellulose ranges from 0.2 to 0.8 meq/g. Preparations of the highest available capacity should be used, preferably above 0.6 meq/g.

SKETCH 5



1. Preparation of Protein and Hapten Derivatives of Carboxymethylcellulose by the Carbodiimide Reaction

Carboxymethylcellulose powder (0.6 to 0.8 meq/g) is stirred in 2 to 4 N hydrochloric acid for 30 min to 1 hr, filtered on a Buechner funnel, and washed extensively with water. If amounts are prepared larger than 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 g of protein (e.g., bovine serum albumin or human gamma globulin) or 0.1 to 0.4 g of a suspension or solution of hapten [e.g., 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 adjusted to 3.6 to 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 g) is added in portions with strong 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 g dicyclohexylcarbodiimide in 2.0 ml of tetrahyrofuran 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 semi-solid. If the mixture is fluid, it should be stirred for 2 days. Refrigeration should be used only if required for enzymes or other substances which 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 poured into approximately 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 which dissolves the antigen: water, acid, base, or for 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 washing the cellulose 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. Usually several times in each solvent is sufficient; however, ultimately, the effectiveness of washing must be determined by:

(1) Stirring it overnight and observing the color, ultraviolet absorbency, or some other characteristic of the wash solution.

(2) Setting up a column and observing column effluent for color, fluorescence, or absorption in the visible or ultraviolet spectrum.

(3) Using the cellulose derivative as a specific adsorbent and determining whether color appears or whether acid dissociated antibody is inhibited or deactivated by free antigen.

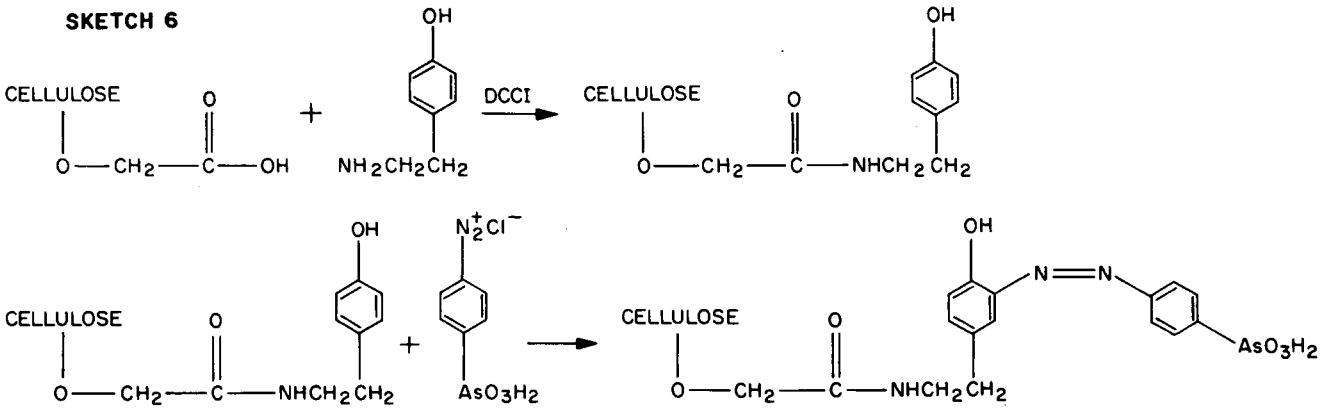
Hapten adsorbents, and perhaps some protein adsorbents, may be washed with acetone, air-dried, and stored.

D. Phenolic Derivatives of Cellulose Useful for Combination with Diazonium Salts

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 (Ref. 43). Some haptens may be coupled directly to carboxymethylcellulose by the carbodiimide reaction. (See Section III-C.) 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 using antiserum to azophenylarsonate hapten. Dissociation was by phosphate buffer at pH 3.0 followed by 0.1 N hydrochloric acid. (See Sketch 6.)

The following procedure is that given in Ref. 43: 35 g of carboxymethylcellulose (0.78 meq/g) are 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 g (40.3 mM) tyramine hydrochloride and 5.8 ml (41.8 mM) triethylamine. To this mixture,

SKETCH 6



DCCI IS DICYCLOHEXYLCARBODIIMIDE

11.0 g (53.3 mM) of N, N′-dicyclohexylcarbodiimide is added; stirring is continued at room temperature for 1 wk. During this time, two 6.5-g (31.5 mM) additions of N, N′-dicyclohexylcarbodiimide are made. The cellulose is washed five times with N, N-dimethylformamide and then copiously with water, 1% sodium chloride solution, and again with water.

For diazotization, 5.84 g of p-arsanilic acid (26.9 mM) in 90 ml of 1 N hydrochloric acid is cooled in an ice bath and a solution of 2.13 g (30.9 mM) of sodium nitrite in 20 ml of water added to the p-arsanilic acid solution. After 20 min 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 g of sodium carbonate in 700 ml of water). The pH is frequently adjusted to pH 8–9 over the course of the 1½ hr used for the addition of the diazonium salt. The mixture is allowed to remain 16 to 18 hr in the cold room (4°C) with constant stirring; it is then copiously washed with water, 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.

REFERENCES

1. Jenkins, C. E., "The Release of Antibody by Sensitized Antigens," *Brit. J. Exper. Path.*, Vol. 27, p. 111, 1946 (also in Ref. 2).
2. Kabat, E. A., and Meyers, M. M., *Experimental Immunochemistry*, 2nd Edition, Charles C. Thomas, Springfield, Illinois, 1961.
3. Landsteiner, K., and Miller, G. P., Jr., "Serological Studies on the Blood of the Primates. II. The Blood Groups in Anthropoid Apes," *J. Exp. Med.*, Vol. 42, p. 853, 1925 (also in Ref. 2).
4. Isliker, H. C., "Purification of Antibodies by Means of Antigens Linked to Ion Exchange Resins," *Ann. N.Y. Acad. Sci.*, Vol. 57, p. 225, 1953.
5. Weber, R., "Molecular Weight of Cold Hemagglutinins," Vox Sanguinis, Vol. 1, p. 37, 1956.
6. Kabat, E. A., *Blood Group Substances*, Academic Press, N.Y., 1956.
7. Fudenberg, H. A., and Kunkel, H. G., "Physical Properties of the Red Cell Agglutinins in Acquired Hemolytic Anemia," *J. Exptl. Med.*, Vol. 106, p. 689, 1957.
8. Goodman, H. S., "Chromatographic Fractionation and Characterization of Antibody Based on the Thermal Dissociation of the Specific Antigen-Complex," *Nature*, Vol. 193, p. 350, 1962. "Antigen- and Antibody-Combining Properties and Their Influence on the Immune Reaction of Red Cells," *Nature*, Vol. 194, p. 134.
9. McDuffie, N. M., and Sutherland, G. B., "Immunochemistry of Coagulated Antigen–Antibody Complex I, II," *J. Immunol.*, Vol. 90, pp. 438, 444, 1963.
10. DeCarvallo, S., Lewis, A. J., Rand, H. J., and Uhrick, J. R., "Immunochromatographic Partition of Soluble Antigens on Columns of Insoluble Diazo-Gamma-Globulins," *Nature*, Vol. 204, p. 265, 1964.
11. Yagi, Y., Engel K., and Pressman, D., "Quantitative Determinations of Small Amounts of Antibody by Use of Solid Adsorbents," *J. Immunol.*, Vol. 85, p. 375, 1960.
12. Gyenes, L., and Sehon, A. H., "Preparation and Evaluation of Polystyrene–Antigen Conjugates for the Isolation of Antibodies," *Can. J. Biochem. Physiol.*, Vol. 38, p. 1235, 1960.
13. Gyenes, L., Rose, B., and Sehon, A. H., "Isolation of Antibodies on Antigen–Polystyrene Conjugates," *Nature*, Vol. 181, p. 1465, 1958.
14. Weliky, N., Weetall, H. H., Gilden, R. V., and Campbell, D. H., "The Synthesis and Use of Some Insoluble Immunologically Specific Adsorbents," *Immunochem.*, Vol. 1, p. 219, 1964.
15. Bernfeld, P., and Wan, J., "Antigens and Enzymes Made Insoluble by Entrapping Them Into Lattices of Synthetic Polymers," *Science*, Vol. 142, p. 679, 1963.

REFERENCES (Cont'd)

16. Sutherland, G. B., and Campbell, D. H., "The Use of Antigen-Coated Glass as a Specific Adsorbent for Antibody," *J. Immunol.*, Vol. 80, p. 294, 1958.

17. Onouye, K., Yagi, Y., and Pressman, D., "Immunoadsorbents with High Capacity," *Immunochem.*, Vol. 2, p. 181, 1965.

18. Marrack, J. R., "The Chemistry of Antigens and Antibodies," Privy Council Medical Research Council, Special Report Series No. 230, His Majesty's Office, London, England, 1958.

19. Sehon, A. H., "Physicochemical and Immunochemical Methods for the Isolation and Characterization of Antibodies," *Brit. Med. Bull.*, Vol. 19, p. 183, 1963.

20. Gurvich, A. E., "The Use of Antigens on an Insoluble Support," *Immunological Methods* (Edited by J. F. Ackroyd), F. A. Davis Co., Philadelphia, 1964.

21. Manecke, G., "Über Serologisch Wirksame Proteinharze und Enzymharze," *Naturwissenschaften*, Vol. 51, p. 25, 1964.

22. Singer, S. J., "Structure and Function of Antigen and Antibody Proteins" in *The Proteins*, Vol. 3 (Edited by H. Neurath), Academic Press, New York, 1965.

23. Weliky, N., and Weetall, H. H., "The Chemistry and Use of Cellulose Derivatives for the Study of Biological Systems," *Immunochem.*, Vol. 2, p. 293, 1965.

24. Campbell, D. H., Luescher, E., and Lerman, L. S., "Immunologic Adsorbents. I. Isolation of Antibody by Means of a Cellulose–Protein Antigen," *Proc. Nat. Acad. Sci.*, U.S.A., Vol. 37, p. 575, 1951.

25. Malley, A., and Campbell, D. H., "Isolation of Antibody by Means of an Immunological Specific Adsorbent," *J. Am. Chem. Soc.*, Vol. 85, p. 487, 1963.

26. Webb, T., and LaPresle, C., "Isolation and Study of Rabbit Antibodies Specific for Certain of the Antigenic Groups of Human Serum Albumin," *Biochem. J.*, Vol. 91, p. 24, 1964.

27. Jagendorf, A. T., Patchornik, A., and Sela, M., "Use of Antibody Bound to Modified Cellulose as an Immunospecific Adsorbent of Antigens," *Biochim. Biophys. Acta.*, Vol. 78, p. 516, 1963.

28. Weetall, H. H., and Weliky, N., "The Coupling of Biologically Active Molecules to Insoluble Polymers: Antibody on Cellulose," *Biochim. Biophys. Acta.*, Vol. 107, p. 150, 1965.

29. Lerman, L. S., "Antibody Chromatography on an Immunologically Specific Adsorbent," *Nature*, Vol. 172, p. 635, 1953.

30. Gurvich, A. E., Kapner, R. B., and Nezlin, R. S., "Isolation and Examination of Pure Antibodies, Prepared by the Use of Antigens Fixed on Cellulose," *Biokhimiya*, Vol. 24, p. 144, 1959.

31. Weliky, N., and Weetall, H. H., "Carrier-Bound Proteins," Abstracts of 148th Meeting of the American Chemical Society, Chicago, Illinois, 1964.

32. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., *Methods in Immunology*, W. A. Benjamin, Inc., New York, 1963.

REFERENCES (Cont'd)

33. Velick, S. F., Parker, C. W., and Eisen, H. N., "Excitation Energy Transfer and the Quantitative Study of the Antibody Hapten Reaction," *Proc. Natl. Acad. Sci.*, U.S.A., Vol. 46, p. 1470, 1960.

34. Farr, R. S., "A Quantitative Immunochemical Measure of the Primary Interaction Between I* BSA and Antibody," *J. Infect. Dis.*, Vol. 103, p. 239, 1958.

35. Talmage, D. W., Baker, H. R., and Akeson, W., "The Separation and Analysis of Labelled Antibodies," *J. Infect. Dis.*, Vol. 94, p. 199, 1954.

36. Weetall, H. H., and Weliky, N., "New Cellulose Derivatives for the Isolation of Biologically Active Molecules," *Nature*, Vol. 204, p. 896, 1964.

37. Gurvich, A. E., Kuzovleva, O. E., and Tumanova, E. E., "Production of Protein–Cellulose Complexes (Immunosorbents) in the Form of Suspensions Able to Bind Great Amounts of Antibody," *Biokhimiya*, Vol. 26, p. 803, 1962.

38. Moudgal, N. R., and Porter, R. R., "The Use of Antigen–Cellulose Suspensions for the Isolation of Specific Antibody," *Biochim. Biophys. Acta.*, Vol. 71, p. 185, 1963.

39. Kursanov, D. N., and Solodkov, P. A., "A New Method for the Preparation of Colored Derivatives of Cellulose," *J. Appl. Chem.* (USSR), Vol. 16, p. 351, 1943; C. A., Vol. 38, p. 6551, 1944.

40. Weetall, H. H., and Weliky, N., "Immunoadsorbent for the Isolation of Purine-Specific Antibodies," *Science*, Vol. 148, p. 1235, 1965.

41. Cohen, S. C., Thorn, E., and Bendich, A., "The Preparation and Properties of 6-Halomethylpurines," *J. Org. Chem.*, Vol. 29, p. 3545, 1962.

42. Cohen, S. C., and Vincze, A., "6-Trichloromethylpurines and Related Compounds." *Israel J. Chem.*, Vol. 2, p. 1, 1964.

43. Vannier, W. E., Bryan, W. P., and Campbell, D. H., "The Preparation and Properties of a Hapten-Cellulose Antibody Adsorbent," *Immunochem.*, Vol. 2, p. 1, 1965.

44. Svensson, H., "Preliminary Attempts to Purify Antibodies by Specific Methods," *Ark. Kemi. Min. Geol.*, Vol. 17A, p. 1, 1943.

45. Landsteiner, K., and van der Scheer, J., "Of Cross Reactions of Immune Sera to Azoproteins," *J. Exptl. Med.*, Vol. 63, p. 325, 1936.

46. Froese, A., and Sehon, A. H., "Problems Involved in the Purification of Antihapten Antibodies by the Use of Stroma–Hapten Conjugates," *Am. J. Biochem. Physiol.*, Vol. 39, p. 1067, 1961.