https://ntrs.nasa.gov/search.jsp?R=19660012894 2020-03-16T22:58:26+00:00Z

THE CHEMISTRY AND USE OF CELLULOSE DERIVATIVES FOR THE STUDY OF BIOLOGICAL SYSTEMS*

by

N. Weliky and H. H. Weetall

Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California

GPO PRICE $ _____

CFSTI PRICE(S) $ _____

Hard copy (HC) 3.00

Microfiche (MF) .50

ff 653 July 65

Suggested Running Title: Chemistry and use of cellulose derivatives

N66-22183 (ACCESSION NUMBER) (THRU)
57 (PAGES) 1 (CODE)
CR 63141 (NASA CR OR TMX OR AD NUMBER) 06 (CATEGORY)
FACILITY FORM 602

* This paper presents results of one phase of research carried out at the Jet Propulsion Laboratory, California Institute of Technology, under Contract No. NAS7-100, sponsored by the National Aeronautics and Space Administration.

[redacted]

TABLE OF CONTENTS

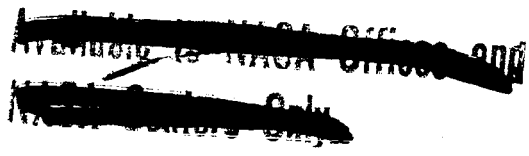


Table of Contents (cont.)

I. INTRODUCTION

The increasing number of publications dealing with the coupling of biologically important molecules or their substrates to soluble and particularly insoluble polymers reflects a growing acknowledgment of the usefulness of such systems for the isolation and synthesis of molecular species, the study of chemical and biological mechanisms, and the study of molecular interactions.

Examples of the uses to which such systems can be put include:

a. The coupling of antigens, antibodies, enzymes, enzyme inhibitors and nucleic acids (including fragments or monomers) in order to purify or isolate complementary substances with which they form specific or cross-reacting complexes. This includes the "sandwich technique" in which an antigen is coupled, antibody is complexed to the antigen, and advantage taken of the bivalence of antibody to detect or quantitate antigen by further exposure of the immunoadsorbent to solutions of that substance. Detection can be accomplished by radioactive labeling or by dissociation under appropriate conditions for the detection or recovery of the antigen.

b. Study of the bound substance to observe the effect of coupling at different molecular sites on reactivity. For example we have found that bovine serum albumin is as effective in removing antibody from immune serum, if coupled to cellulose through an azo link by reaction with a diazonium cellulose derivative, as by reaction with an acidic cellulose derivative to form an amide.

c. Study of the bound substance to observe the effect of coupling on chemical properties such as reactivity of enzymes, or physical properties such as fluorescence.

d. The study of the effects of reagents on the binding of complexed species with polymer bound molecules; e.g. acid dissociation. The separation of the components of the complex for quantitation requires no additional time consuming steps or the use of semi-permeable membranes with complicating physical interactions of their own.

e. Bound substances may, in favorable cases, be localized in particular environments where their biological effects can be observed without the possibility of physical transfer and the introduction of ambiguities caused by possible interactions at other locations. This technique is applicable to enzyme, hormone and antibody systems. Potentially, the possibility exists that deficiencies in organisms may be supplemented by biologically active molecules bound to insoluble or soluble polymers.

f. Model biological systems may be set up using bound enzymes, hormones and other biologically active molecules to study controlled sequential reactions.

g. The step wise synthesis of polymers in a controlled fashion may, in principle, be accomplished by binding a monomer and successively adding other components in high yield reactions. Some success has already been achieved in this area.

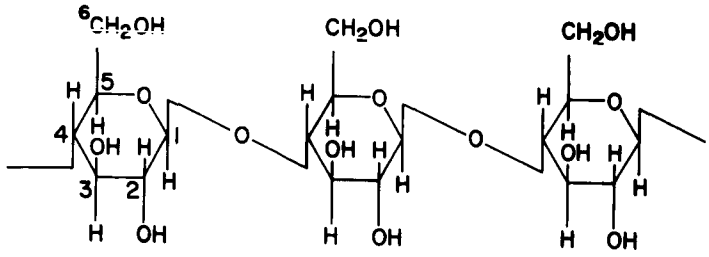
Polymers used as insoluble carriers for reactive molecules can be classified into those which bind by ostensibly physical adsorption - these include charcoal, clays and glass beads - and those which have groups that can be coupled covalently to the substance to be bound under conditions that are known to induce such chemical reactions. The latter class includes polyvinylbenzene derivatives (aryl amines, carboxylic acids and sulfonic acids), polyethylene derivatives (such as polyacrylic acids), cellulose and its derivatives, fibroin (silk), wool, synthetic polypeptides, precipitated proteins

and red blood cell stroma. A further class of which only few examples are published include proteins or other substances entrapped in an organic polymer matrix.

Reports of relatively high nonspecific adsorption of proteins by hydrocarbon polymers induced us to reexamine cellulose as a carrier for proteins, other polymers and simple organic molecules. The rest of this paper will be limited to a survey of the chemistry of cellulose and its derivatives as it applies to the preparation of systems useful for the study of biochemical substances and their properties. The usefulness of a particular method for synthesizing a particular cellulose derivative can be judged by its simplicity, availability of reagents and equipment, physical characteristics of the regenerated cellulose, stability of the cellulose derivative under the experimental conditions to be encountered, and lack of adsorption of the cellulose derivative for interfering substances, i.e. specificity for the substance which is to be complexed, under both adsorption and desorption conditions.

II. PROPERTIES OF CELLULOSE

Cellulose is a vegetable fiber composed of linear polymers of β-D-glucose. It is a polyhemiacetal of the cyclic pyranosyl form of β-D-glucose in which coupling is from the 4-hydroxy group of one glucose unit to the 1-hydroxy acetal group of the next. Cellulose swells in strongly alkaline solution but the fibrous form can be regenerated. Its insolubility is not appreciably affected by dilute acids, but it is hydrolyzed to small soluble units by boiling in strongly acidic solution.



CELLULOSE

Except for end groups, there are three reactive hydroxyl groups per glucose unit: two are secondary (positions 2 and 3) and one is primary (position 6).

Coupling of a particular molecular species to cellulose may be accomplished directly, to cellulose with partially blocked hydroxyl groups, or to a cellulose derivative with added groups available for further reaction. The hydroxyl groups of cellulose undergo the usual reactions of alcohols to form ethers, esters, urethanes, halides, amines, thio derivatives etc. It is desirable to break up the hydrogen bonded structure during modification reactions. However if the resultant substance is to be used as a column packing or has to be filtered, the cellulose must be regenerated in such a form that it can be made into a free flowing powder - not colloidal, gelatinous or gummy. The swelling agent, the solvent, the charge and other characteristics of modifying groups, the order in which operations are performed, and the temperature of the reaction and wash solutions all contribute to the physical form and properties of the regenerated cellulose derivatives.

Many reactions can be accomplished with high degrees of substitution using cellulose swollen in 30-50% sodium hydroxide or in pyridine. Swelling also occurs in glacial acetic acid. Ammoniacal solutions of copper, zinc or cadmium can be used to prepare clear solutions of cellulose.

Ethyl ethers of cellulose are soluble in water or organic solvents, depending on the degree of substitution. Highly substituted forms of ethyl

cellulose (the ethyl ether) are soluble in methanol, ethanol, pyridine, aromatic solvents and tetrahydrofuran. Cellulose acetates of low degree of substitution are soluble in acetone and other organic solvents. Highly substituted cellulose acetate is soluble in pyridine and chloroform-methanol, but not in acetone.

Cellulose and its derivatives will undergo many heterogeneous reactions, so that lack of solubility in a solvent does not rule out reaction in that solvent.

III. MODIFICATION OF CELLULOSE-PREPARATION OF REACTIVE INTERMEDIATES

Cellulose undergoes all the characteristic reactions of polyhydric alcohols: oxidation, esterification, ether formation, halogenation and reaction with reactive unsaturated substances such as acrylonitrile, isocyanates and ketene. Compared to low molecular weight alcohols, the reactivity of cellulose is modified by its secondary structure, which can be characterized as fibrous: made up of linear glucose chains interacting through hydrogen bonds. In the case of cellulose with substituted hydroxyl groups, chain interaction may also take place through dipole-dipole interaction, nonpolar van der Waal's forces (dispersion forces), charge interaction (electrostatic Coulomb forces), or combinations of the three depending on the nature of the substituents. Reactivity is thus dependent on the degree to which a solvent separates or penetrates the fibers, the properties of the solvent as an environmental factor in the reaction scheme, and the past history of the cellulose, i.e. if it has been dried at high temperature, exposed to solvents, and the method of regeneration if it has been in solution.

Properties of regenerated cellulose, such as flow resistance in columns and swelling in acid, base, aqueous salts and nonaqueous solutions are dependent on the treatments it has undergone, the order of events, and the

manner and sequence in which the cellulose was returned to a suspension in pure water. In addition, its physical properties are modified by changes in number of hydroxyl groups, changes in charge, and other characteristics of the modifying groups. If the cellulose has been swollen in nonaqueous solvents, the concentration of the cellulose in the nonaqueous solvent, the volume of water into which it may have been poured for regeneration, the rate at which it was poured, the manner and degree of agitation and the nature of the solvent itself are among the factors which determine the physical properties of the final product. If the cellulose has been swollen in a water immiscible solvent, in order to replace that solvent with water, solvent exchange with a water miscible solvent, or drying may be necessary.

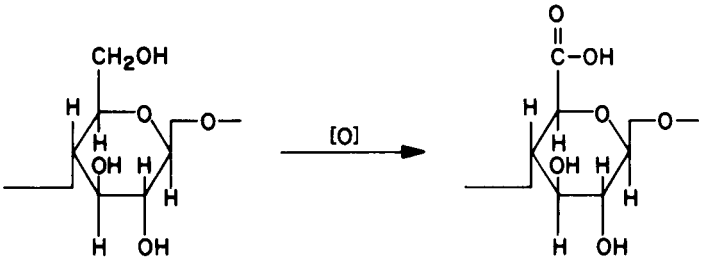
Some of the reactions which have been found useful for the preparation of cellulose derivatives with specific properties, or for the preparation of cellulose intermediates, will be outlined below.

A. Oxidation of Cellulose to Oxycelluloses

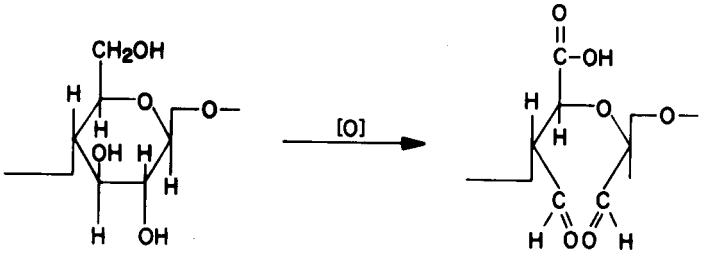
Cellulose can be oxidized to products with acidic properties.[1,2] At the same time, aldehydic (reducing) groups are formed to a greater or lesser extent.[1] Periodate, which oxidizes vicinal hydroxyl groups (glycols) to aldehydes or acids, and nitrogen dioxide which oxidizes mainly 6-hydroxy groups to form glucuronic acids, are both reported to attack the amorphous and crystalline regions of cellulose "without causing so much degradation that the material loses its fibrous form. With the commoner types of oxidants, the reaction is virtually confined to the amorphous regions and the surfaces of the crystallites, and the material falls to a powder at comparatively low degrees of oxidation".[1] Cellulose oxidized by nitrogen dioxide has not been reported used for coupling to proteins and other organic molecules, probably because of the availability of carboxymethylcellulose. Chromic acid also

oxidizes glucose units to uronic acids but reducing properties increase considerably.[1]

The methyl ester of oxycellulose, produced by alkaline permanganate oxidation of cuprammonium cellulose followed by treatment with diazomethane, has been coupled to proteins by the azide method.[2]



OXIDATION TO GLUCURONIC ACID



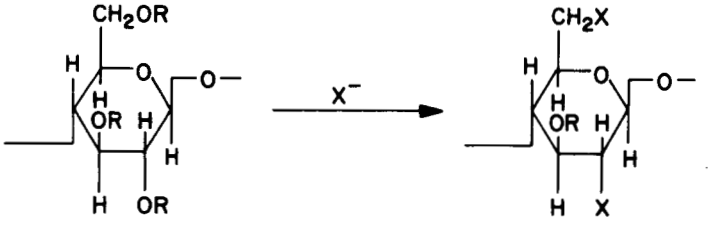
OXIDATION TO ACIDIC AND ALDEHYDIC GROUPS

Acidic oxycelluloses should couple to alcohols, amines and proteins to form esters or amide derivatives.

B. Halogenation of Cellulose

A number of methods are described for halogenating cellulose.[3,4] The most general technique is to form an ester of a strong acid such as p-toluenesulfonic acid (Fig. 1f), methanesulfonic acid (Fig. 1e) or nitric acid (Fig. 1h) and displace the acid by halide ion.[3] Pyridinium chloride in pyridine, and sodium iodide in acetone or in 2,5-hexadione (acetylacetone)

give the chloride and iodide respectively. Concentrated aqueous solutions of sodium halides halogenate methanesulfonic esters of cellulose if buffered to neutralize the acid formed. Some halogenation occurs if the temperature is too high while making p-toluenesulfonyl esters from p-toluenesulfonyl chloride. In general, because displacement reactions are used to make the halide, halogenation occurs mainly at primary hydroxyls.



Halogenation using phosphorus tribromide has also been reported[4] (see Section III. H).

Although few reports of such use seem to have been, cellulose halides, particularly bromides and iodides substituted on the 6-position, should couple readily to proteins, amines, alkoxides, mercaptans and salts of acids and phenols.[4,5]

Hydroxyethylcellulose, which is commercially available, should halogenate at the hydroxyethyl position by analogous procedures, and should be similarly susceptible to coupling to the same kinds of substances (Fig. 1 l).

C. Esterification of Cellulose

Some cellulose esters, such as acetates, are useful where free hydroxyl groups are objectionable, and prolonged basic or highly acidic conditions are not encountered. Others, such as p-toluenesulfonate (Fig. 1 f),

methanesulfonate (Fig. 1e) and nitrate esters (Fig. 1h) are useful as intermediates for the preparation of cellulose halides (see Section B) or amines (Fig. 1i).

1. Highly substituted cellulose acetates (Fig. 1a) and acetates of partially substituted cellulose derivatives such as carboxymethylcellulose can be made by treatment of cellulose with: (1) acetic anhydride - acetic acid with a small amount of sulfuric or perchloric acid[6], (2) acetic anhydride - acetic acid - toluene with a small amount of perchloric acid[6], (3) acetic anhydride - pyridine[7], (4) trifluoroacetic anhydride - acetic acid[8], (5) acetic anhydride with a small quantity of perchloric acid[9]. The physical form of the product is dependent on the method used for regeneration.

2. p-Toluenesulfonic esters of cellulose (Fig. 1f) can be obtained by[10]: (1) treating cuprammonium rayon, in pyridine, with a pyridine solution of p-toluenesulfonyl chloride (2) solvent exchanging cellulose, swelled in 17.5% sodium hydroxide, into pyridine and treating with a pyridine solution of p-toluenesulfonyl chloride (3) treating acetone soluble cellulose acetate with a pyridine solution of p-toluenesulfonyl chloride, which esterifies any unacetylated groups (free hydroxyl groups of partially ethylated cellulose should be esterified similarly). Toluenesulfonation with toluenesulfonyl chloride in pyridine at temperatures above about 20°C results in appreciable replacement of toluenesulfonyl groups by chlorine.[10]

3. Methanesulfonic esters (Fig. 1e) are made[10] by first swelling the cellulose in about 20% sodium hydroxide. The base is washed out and the cellulose either solvent exchanged through methanol into pyridine, or just washed with pyridine to remove excess water. Methanesulfonation is accomplished by treating the swelled cellulose with a solution of methanesulfonyl chloride in pyridine.

4. Bromoacetylcellulose (Fig. 1c) has been prepared by treating cellulose with bromoacetic acid and bromoacetyl chloride in dioxane.[11]

5. The p-aminobenzoyl ester of cellulose (Fig. 1d) has been made by reaction of cellulose with p-nitrobenzoyl chloride followed by reduction with Ti (III) or V (II).[12,13]

6. Other cellulose esters can also be made by the above methods, i.e. through anhydrides or acid chlorides. Alternative standard methods for esterification may be used if appropriate solvents and conditions are found. Procedures are described in many organic and biochemical texts and reviews.

Some proteins and organic molecules, including haptens, may be coupled to cellulose through ester bonds if they are to be used under conditions where hydrolysis is negligible, or not important.

D. Etherification of Cellulose

Cellulose can be etherified to block unwanted hydroxyl groups, to couple on a reactive group that may be used as in intermediate for coupling other groups, or for coupling desired molecules directly to cellulose. Ethers are among the most stable derivatives of cellulose and may be counted upon to remain intact under most conditions that will degrade or depolymerize the cellulose itself.

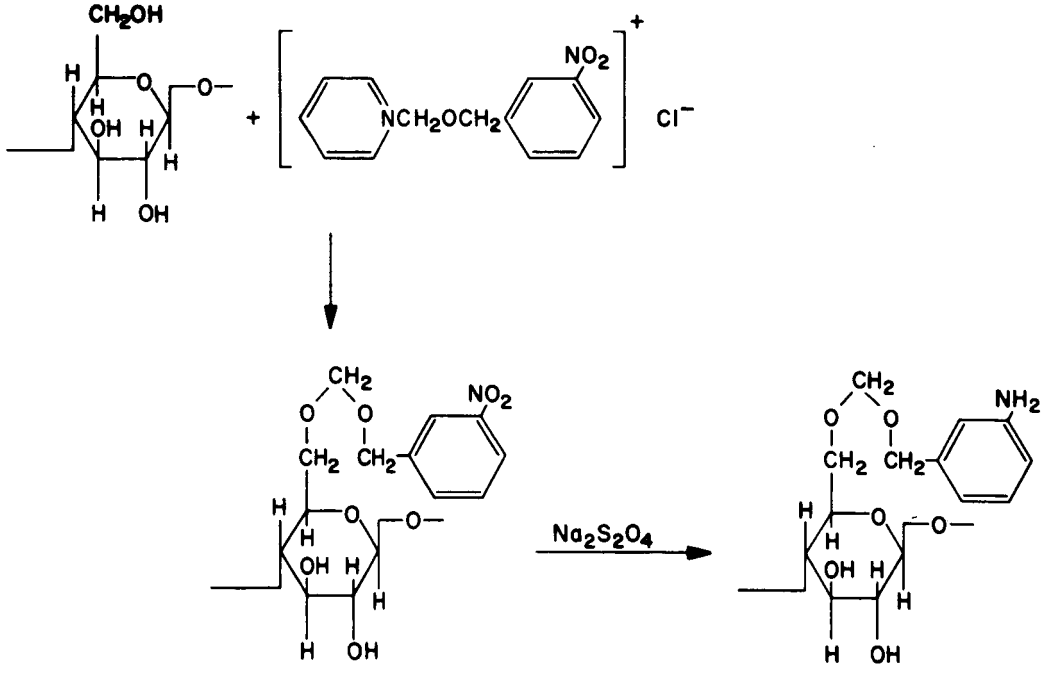
1. Water insoluble ethyl cellulose of different degrees of substitution is commercially available. Cellulose or derivatives such as carboxymethylcellulose with free uncoupled hydroxyl groups, can be highly ethylated by treating the cellulose previously swelled in 40% sodium hydroxide with ethyl chloride and additional sodium hydroxide for 8 hrs at 110°C in a stainless steel bomb tube[14] (Fig. 1k).

Varying degrees of substitution can be obtained. Some intermediate

degrees are soluble in alkaline solution. Hydroxyethylcellulose can be made by a similar procedure but using ethylene oxide (Fig. 1i). Substitution can also be achieved at atmospheric pressure[14] using ethylene oxide in a solvent.

2. Methyl cellulose is available commercially in a water soluble form. It can be made in the laboratory in a water insoluble form by treating cellulose with dimethyl sulfate[15] (Fig. 1j).

3. m-Aminobenzyloxymethyl ether of cellulose[13] (Fig. 1 m) can be made by wetting the cellulose with an acetate buffered solution of N-(m-nitrobenzyloxy)-methyl pyridinium chloride, drying the cellulose at 60-80°C and heating to 125°C for 40 min. The nitro groups are then reduced with an aqueous solution of sodium dithionite for 30 minutes. The choice of the 6-position for coupling is arbitrary in the following reaction scheme:

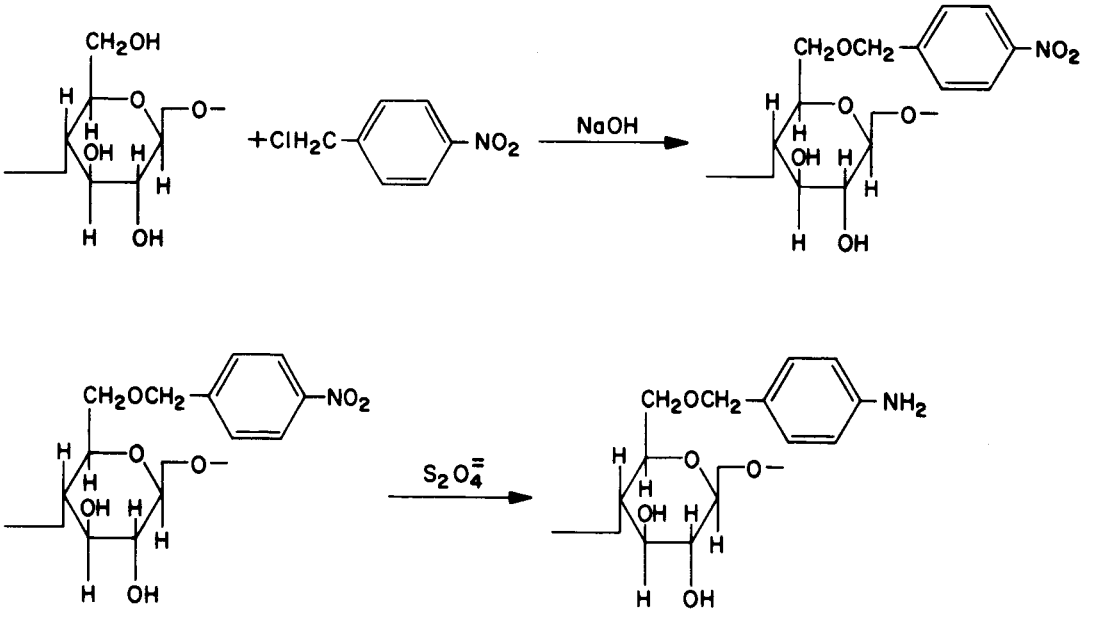


4. Both water insoluble and soluble carboxymethylcellulose (Fig. 1p) are commercially available. They can also be made by treating cellulose, swollen in 30-40% sodium hydroxide, with chloroacetic acid.[2] If substitution is low, the product is insoluble in water. Derivatives of soluble carboxy-

methylcellulose may or may not be soluble depending on the nature of the substituent, e.g. the methyl ester is insoluble[2] while we have found that the solubility of amides made with ε-DNP-lysine or 4-(p-aminophenylazo)-phenylarsonic acid depends on the degree of substitution.

5. Aminoethylcellulose is commercially available (Fig. 1o).

6. p-Aminobenzylcellulose (Fig. 1 n) is commercially available. Some samples have required very extensive washing. It can be made[16] by heating, for 4 hrs. at 95°C, a mixture of p-nitrobenzyl chloride and cellulose powder in 40% sodium hydroxide. Extensive washing with water, ethanol and acetone is required. The nitro group is then reduced to an amino group by suspending the p-nitrobenzylcellulose in boiling ethanol and adding a water solution of sodium dithionite. The light yellow product is ready for washing after thirty minutes.



The degree of substitution and the designation of coupling at positions C2 and C6 are arbitrary. In fact, it was found that 1.5 positions per glucose unit were substituted under these conditions.[16]

E. Amides of Acidic Cellulose Derivatives

This section will concern coupling of amines to carboxymethylcellulose having either free hydroxyl groups or with hydroxyl groups blocked by ethylation or acetylation. Although not reported, the methods should apply equally well to acidic oxycelluloses.

The most straightforward procedure of forming such amides is by reaction of a basic amine, amino acid or protein with carboxymethylcellulose in the presence of diimides such as N,N-dicyclohexylcarbodiimide or water soluble diimides. The reaction can take place in water, tetrahydrofuran, methanol, pyridine or other organic solvents. Some solvents work better than others for particular cases.

1. 4-(p-aminophenyl)-anilide of carboxymethylcellulose (carboxymethylcellulose half amide of benzidine)[17] (Fig. 2a) can be made by allowing benzidine to react with insoluble carboxymethylcellulose (0.7 meq/g) in water or methanol, in the presence of dicyclohexylcarbodiimide (DCCI) or other diimides to form the half amide, one amino group remaining free. This has been verified by determining the loss of titratable groups after treating the derivative with the amine blocking agent, benzenesulfonyl chloride.

If the reaction is attempted with soluble carboxymethylcellulose, gelation occurs because of cross linking.

2. Carboxymethylcellulose amide of tyramine[18] (Fig. 2b) has been made by coupling tyramine to carboxymethylcellulose in dimethylformamide using dicyclohexylcarbodiimide as the coupling agent.

3. 1-Hydroxy-5-naphthylamide of carboxymethylcellulose (Fig. 2c) can be made by coupling 1-hydroxy-5-naphthylamine to carboxymethylcellulose in water, in the presence of dicyclohexylcarbodiimide using the procedure previously described.[19]

F. 1-Hydroxy-5-naphthylazo-4-benzylcellulose

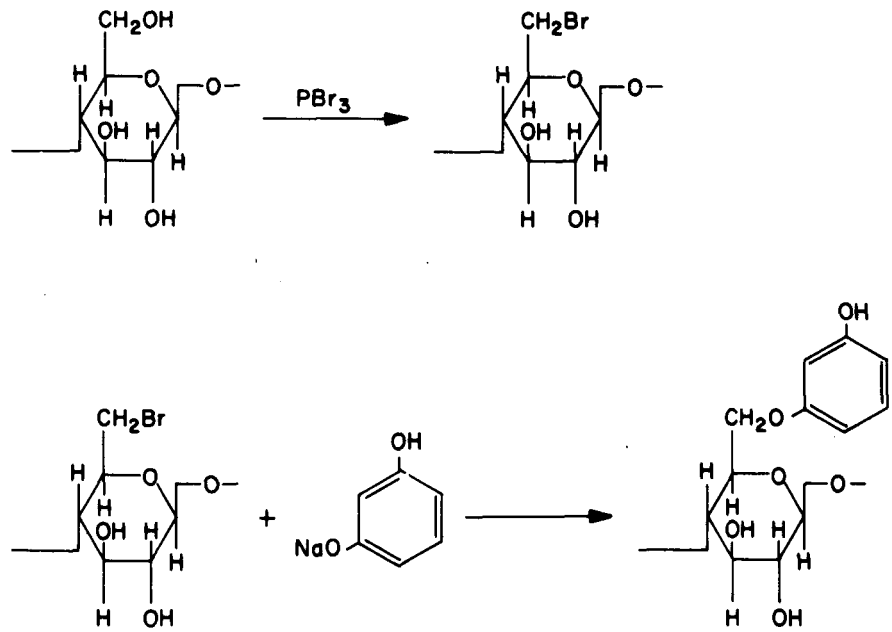
We have coupled[19] 1-hydroxy-5-naphthylamine to p-aminobenzylcellulose in slightly acid solution (Fig. 4f). It could be coupled in a similar manner to any other cellulose derivative with free aromatic amino groups.

G. Phosphocellulose

Phosphocellulose or cellulose phosphate (Fig. 1g) is available commercially.

H. Cellulose Monoresorcinol Ether

This was made[4,5] by refluxing a mixture of powdered cellulose and benzene, with phosphorus tribromide (2 g/100 g cellulose) (See section B) followed by refluxing of the cellulose bromide in alcohol with an excess of sodium m-hydroxyphenoxide (monosodium salt of resorcinol).



I. Cellulose Urethans

Organic isocyanates react with alcohols to form urethans. The

reaction of phenyl isocyanate with, e.g. acetylated cellulose, can be used to determine remaining free hydroxyl groups because it reacts equally well with primary and secondary hydroxyl groups (Fig. 1r).[6] This reaction might also be used for blocking free hydroxyl groups where the presence of aromatic groups would not interfere with the use of the product. Finally, although it has not been reported, reaction of cellulose with diisocyanates like toluene or xylene diisocyanates, by analogy with the coupling of benzidine to carboxymethylcellulose, should result at least in part in coupling at one end, leaving the other end free for coupling to proteins, organic amines, alcohols or phenols.

IV. COVALENT BINDING OF COMPLEXING AGENTS, BIOLOGICALLY ACTIVE MOLECULES AND OTHER REAGENTS TO CELLULOSE

Antigens, antibodies, haptens, enzymes, proteins in general, and organic reagents with selected structures, have been coupled to cellulose through covalent bonds for analytical purposes, for synthetic purposes or for the study of the properties of bound or complexed substances. Those derivatives we are aware of as having been reported and some we have made, but have not yet reported, will be discussed, together with reaction conditions, in this next section.

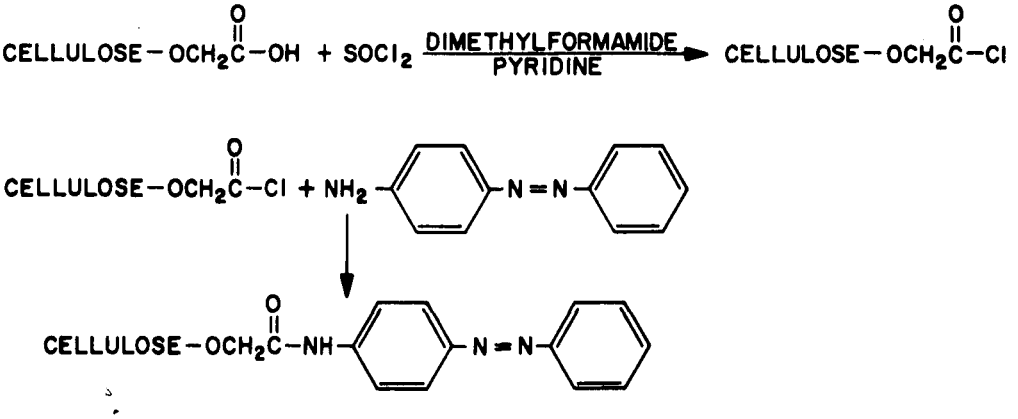
A. Direct Coupling to Cellulose and Ethyl Cellulose

Direct chemical coupling of functional molecules to cellulose has seldom been used for biologically active molecules or their substrates. Some ion exchange celluloses and reactive intermediates are described in another section. In principle, however, any molecule with a group that reacts with alcohols can be coupled to cellulose if the molecule is stable and soluble under reaction conditions.

B. Coupling to Cellulose Halides and Halogen Derivatives

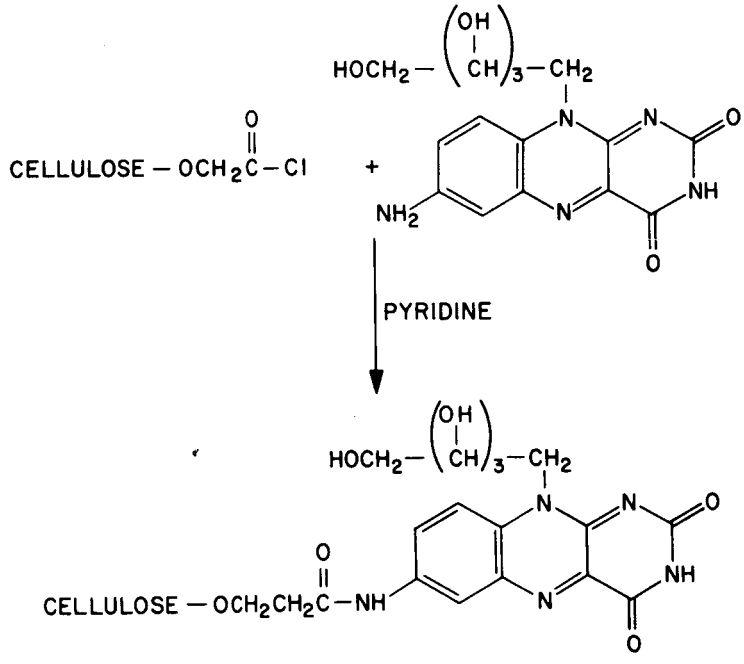
In principle, cellulose halides, with a halogen atom at the C6 position of glucose, should be susceptible to displacement reactions by amines, phenols, acids and mercaptans under basic conditions. Halogen is particularly likely to be present at the C6 position if cellulose halides are prepared by displacing toluenesulfonate or methanesulfonate groups, a reaction which is most likely to take place at a primary carbon. Few studies have been reported using derivatives made by displacement of halogen at the C6 position[4,5], and in the two cases cited the monoresorcinol ether formed was used as an intermediate.

1. p-Phenylazoanilide of carboxymethylcellulose. - The acid chloride of carboxymethylcellulose was made by the procedure of Bassard and coworkers.[20] To 2-3 ml of pyridine was added 100-200 mg carboxymethylcellulose (0.62 meq/g). About 1.5 ml of dimethylformamide was then added followed by the slow addition of an equal volume of thionyl chloride. After the initial reaction, the mixture was heated at 85°C for 3/4 hr. About 100 mg of p-phenylazoaniline was added and heating continued for another 3/4 hr. After cooling for 1 hr., the liquid was decanted and the solid washed with several portions of acetone followed by aqueous sodium carbonate. The product was a clear colored cellulose, yellow in acid, red in base.

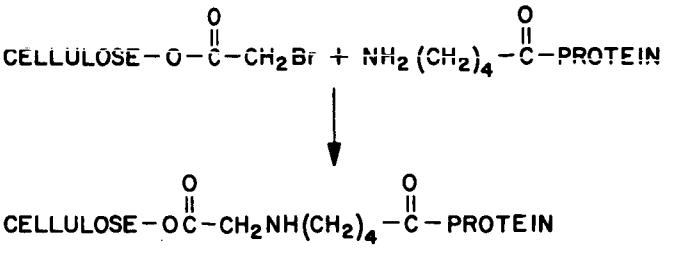


This synthesis was part of a study on the isolation of anti-azobenzene antibody.

2. Carboxymethylcellulose amides of flavins. - Arsenis and McCormick[21] made the acid chloride of carboxymethylcellulose using thionyl chloride in pyridine. To this they coupled 7-amino-6,9-dimethylisoalloxazine, in pyridine. The formation of the acid chloride is illustrated in the previous section. Coupling to the ribosyl derivative of a flavin is shown below. The primary amino group of the other flavin couples in an analogous manner.



3. Coupling to bromoacetylcellulose. - Jagendorf, Patchornik and Sela[11] report that they were able to couple active antibody to bromoacetyl-cellulose by violently stirring a mixture of the cellulose derivative with immune serum globulin in phosphate-citrate buffer at pH 4.6, in the presence of an anti-foaming agent, and then soaking the washed cellulose in dilute ethanolamine-bicarbonate solution adjusted to pH 8.7 with HCl. Coupling is represented below as through the ε-amino group of lysine, although coupling could as well have taken place through sulfhydryl, tyrosyl or guanidyl groups:

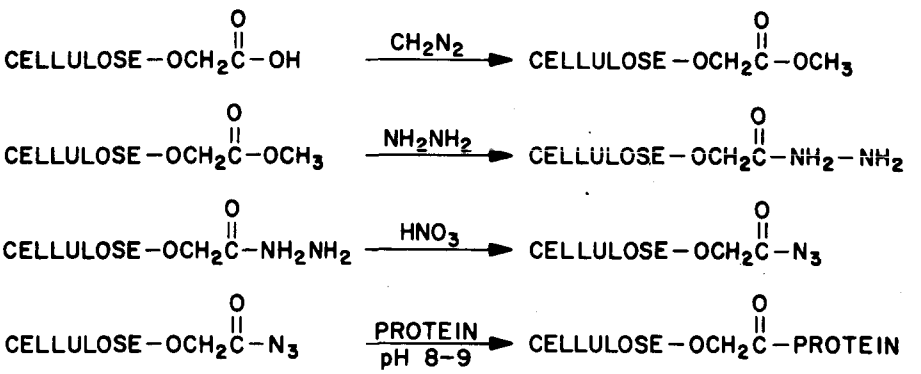


It is obvious, although not so reported, that other proteins, peptides, amines, phenols and mercaptans, such as those described in other sections, could be coupled in a similar manner.

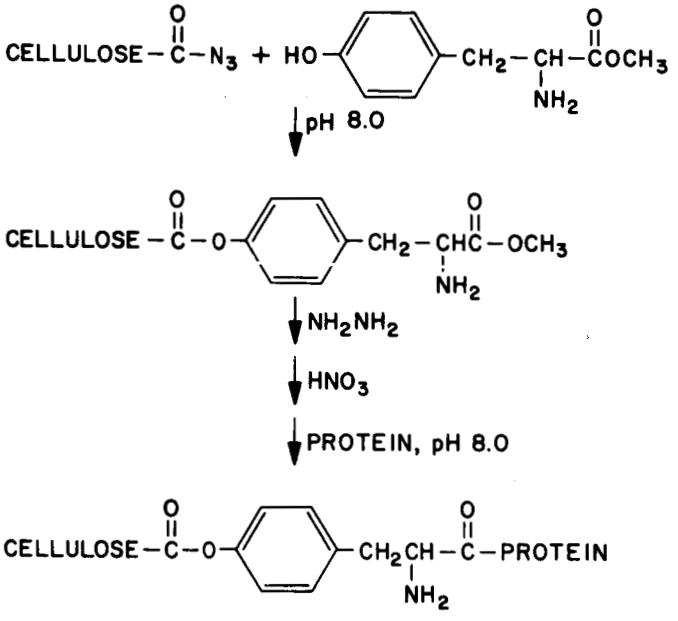
C. Coupling to Insoluble Carboxymethylcellulose and Acidic Oxycellulose Using the Azide Derivative

Stable derivatives of acidic celluloses are amides and esters, the former being more stable to acid and base than the latter. Proteins have been reported coupled to oxycellulose and carboxymethylcellulose by converting the acid to the azide.[2]

1. Coupling to protein via the azide.[2]

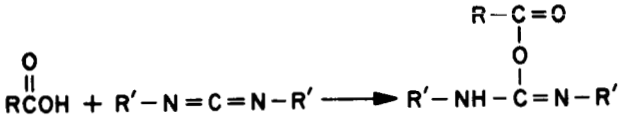


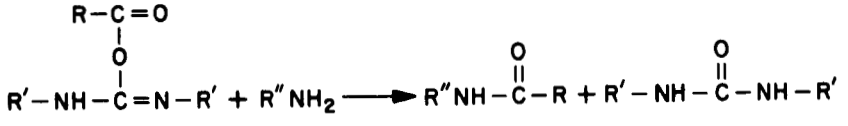
2. Coupling to protein via a tyrosyl azide (see 1. above).[2]



D. Coupling to Insoluble Carboxymethylcellulose Using the Diimide Reaction

Proteins and basic amines, including biologically active molecules and antigens, couple directly to carboxymethylcellulose in the presence of N,N-dicyclohexylcarbodiimide.[19] The mechanism of the diimide reaction is reported to involve acid activation:[22]





We found that the reaction goes to a greater extent in water at pH 3.5 - 5.0 than in tetrahydrofuran. Coupling has also been reported

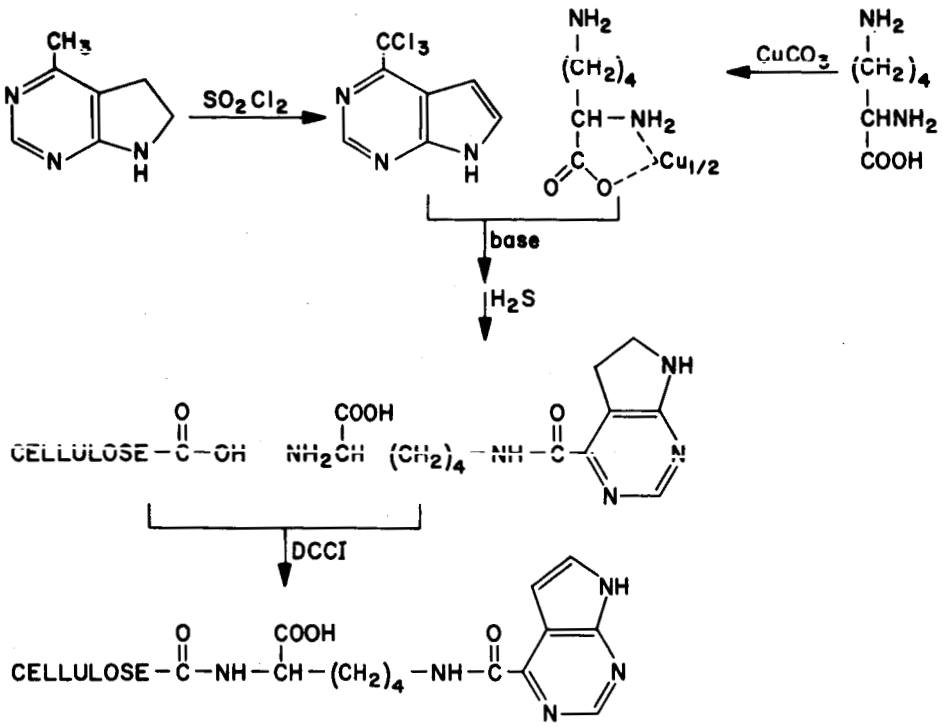
in dimethylformamide.[18] Proteins and many haptens and other organic molecules reacted after appropriate forms for coupling to carboxymethylcellulose were found or synthesized. The diimide reaction should also be applicable for coupling carboxymethylcellulose to amino derivatives of sugars and lipids.

1. Proteins - Proteins coupled[19] include bovine serum albumin, egg albumin, bovine gamma globulin, human gamma globulin, denatured hemoglobin, giant keyhole limpet hemocyanin, trypsin, chymotrypsin, pan protease, subtilisin, nagarse, ficin, β-glucuronidase, alkaline phosphatase, acid phosphatase, RNAase, DNAase, glucose oxidase and horseradish peroxidase (Fig. 2d). Activity was found in all the enzyme preparations. The solvent, water, was kept to a minimum. If dicyclohexylcarbodiimide was used, it was dissolved in a volume of tetrahydrofuran amounting to about 10 to 20 percent of the water used. Water soluble diimides were added directly. The quantity of diimide used was about one-third the weight of cellulose derivative. The pH was usually adjusted to 4.0.

2. Aminophenylazophenylarsonic acid can be coupled for use as an immunoadsorbent[19] (Fig. 2e) using the same procedure described above for coupling proteins.

3. ε-DNP-lysine can be coupled for use as an immunoadsorbent[19] (Fig. 2f) by the same procedure described above for coupling proteins.

4. Coupling of ε-N-(6-purinoyl)-lysine for use as a purine immunoadsorbent. - We prepared a purine hapten intermediate by oxidizing 6-methylpurine to 6-trichloromethyl purine.[61] This was coupled to the copper complex of lysine at the ε-amino group followed by reaction with carboxymethylcellulose in the presence of dicyclohexylcarbodiimide after removing the copper:



5. p-Aminophenylazobenzene (p-phenylazoaniline) can be coupled for use as an immunoadsorbent (Fig. 2g) using the procedure described for coupling proteins.[19]

6. Preparation of carboxymethylcellulose derivatives of protein-hapten conjugates. - We prepared such derivatives in either of two ways. One procedure was to use the diimide reaction to couple carboxymethylcellulose to a previously prepared protein-hapten conjugate. In the other procedure, carboxymethylcellulose was first coupled to the protein using the diimide reaction and the hapten coupled in a second step. Examples of the first method are the reaction of carboxymethylcellulose with the azophenylarsonate conjugate of bovine serum albumin, or dinitrophenylated egg albumin. An example of the second case included coupling of carboxymethylcellulose first to bovine serum albumin in the presence of dicyclohexylcarbodiimide followed by reaction of the product with diazotized arsanilic acid in carbonate solution.

Depending on the reaction, some hapten may couple directly to the cellulose using this technique. If this is not permissible, the second method must be used with care.

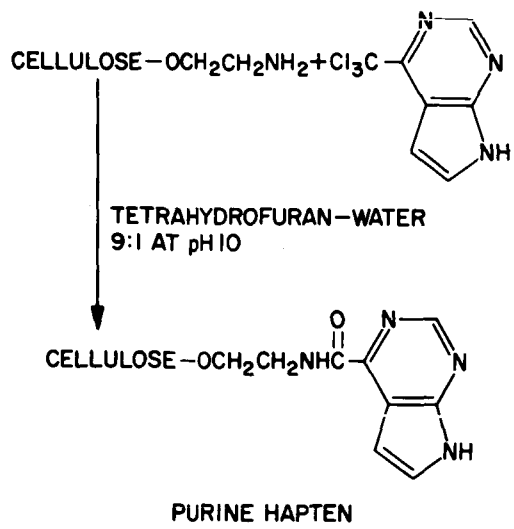
E. Coupling to Soluble Carboxymethylcellulose

We have made soluble amide derivatives of a commercially available low viscosity carboxymethylcellulose using aminophenylazophenylarsonic acid (Fig. 2e) or ε-DNP-lysine (Fig. 2f) and the diimide reaction described above.[19] The amines were mixed with a very viscous water solution of the carboxymethylcellulose, to which a solution of N,N'-dicyclohexylcarbodiimide in a small amount of tetrahydrofuran had been added. After precipitation and washing with acetone, the insoluble material was redissolved in water, centrifuged and extensively dialyzed against water, saline, carbonate or buffer. Enzymes have been coupled by the azide method.[23]

F. Coupling to Aminoethylcellulose

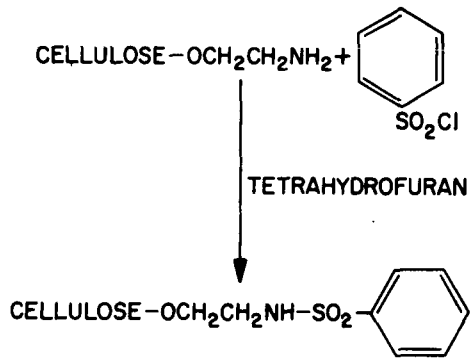
Aminoethylcellulose is a cellulose derivative with the particular properties of a basic aliphatic amine. It may thus be coupled directly to acid chlorides, alkyl or benzyl halides, activated aryl halides or it may be coupled to aliphatic and aromatic acids in the presence of activators such as diimides. We have made the following derivatives:

1. Coupling of 6-trichloromethylpurine.[24]



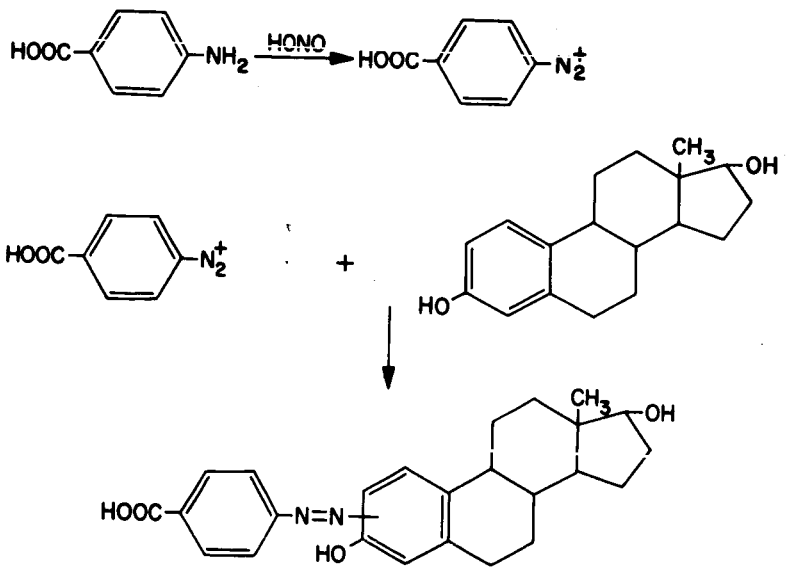
PURINE HAPTEN

2. Coupling of benzenesulfonyl chloride.

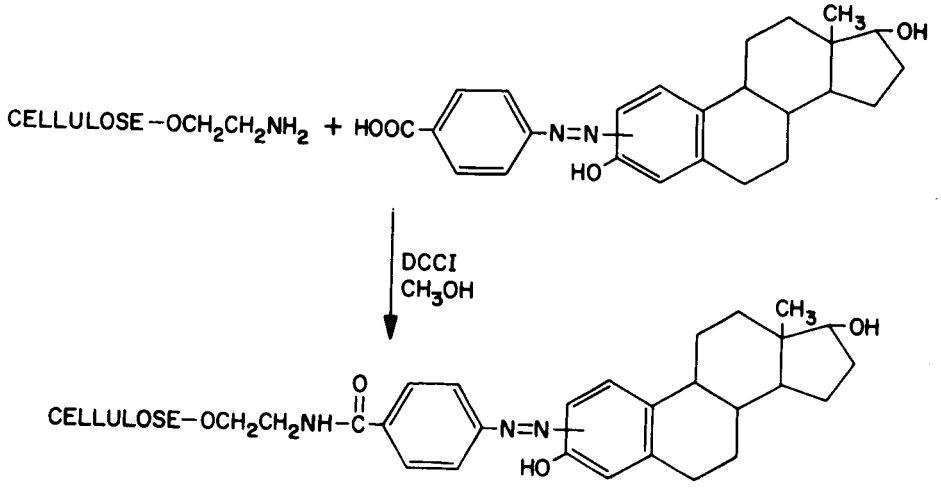


BENZENESULFONAMIDE HAPTEN

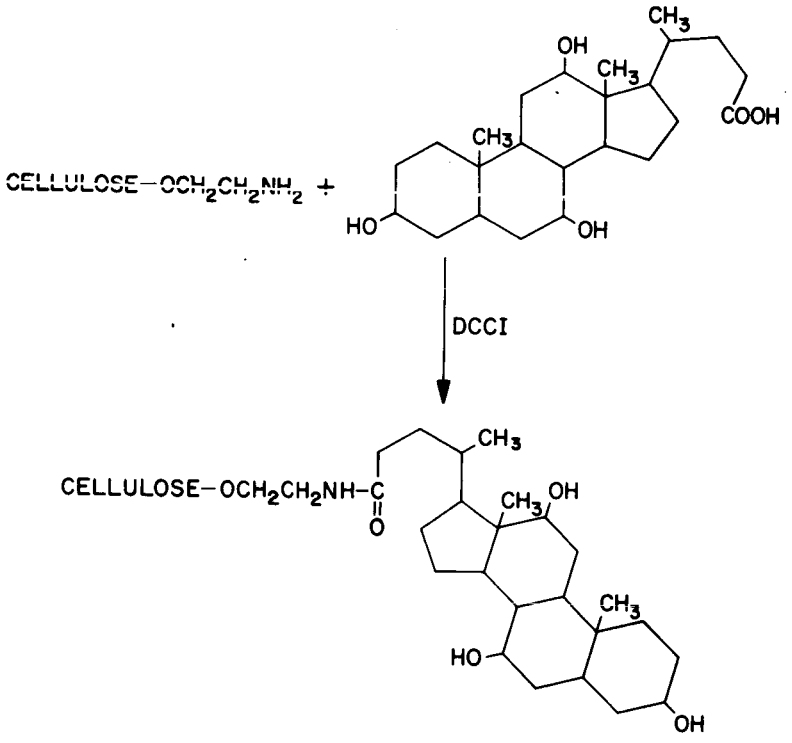
3. Coupling of 17-β-estradiol derivative. We first coupled 17-β-estradiol to diazotized p-aminobenzoic acid.



Coupling of the diazonium group could have taken place at the C2 or C4 position of the steroid, or at both. The azo derivative was then coupled to aminoethylcellulose in the presence of N-N'-dicyclohexylcarbodiimide (DCCI).



4. Coupling of cholic acid. - This derivative was made in methanol-water (90 to 10) using the diimide reaction by the method previously described.[19]



5. Coupling of proteins. - The reaction of aminoethylcellulose with proteins in the presence of diimides is no doubt general for proteins with acidic side chains, such as glutamyl and aspartyl. The reaction conditions are the same as for the reaction of carboxymethylcellulose with the basic groups on proteins.

G. Coupling to p-Aminobenzylcellulose

p-Aminobenzylcellulose is a weak base which, besides its use as an ion exchange material, has the properties of a polyaromatic polyamine which can be diazotized and coupled to many proteins, aromatic hydroxy compounds, aromatic amines and other substances which, under appropriate conditions,

couple to diazonium salts. In principle, it will form amides with acids and active acid intermediates by any of the several methods described.

1. Coupling to proteins. - Diazotization of p-aminobenzylcellulose (Fig. 3a) in an excess of nitrous acid followed by thorough washing and destruction of excess nitrous acid by urea or sulfamic acid, and subsequent coupling to protein in slightly alkaline solution has been used to make immunoadsorbents with bovine serum albumin,[16,17,25] human gamma globulin,[17,26,37] ragweed pollen extract[25] and timothy pollen extract[25] (Fig. 4a) as antigens.

2. Coupling to low molecular weight organic molecules. - Diazotized p-aminobenzylcellulose (Fig. 3a) has been coupled to phenol (Fig. 4c), p-toluol (Fig. 4d) and 4-(p-hydroxyphenylazo)-phenylarsonic acid (Fig. 4e) for use in an investigation of tyrosine inhibitors, and for the purification of tyrosinase.[4] Steroids with a hydroxyaromatic A ring will also couple (Fig. 4b). The phenol derivative has also been used for coupling to diazotized arsanilic acid and diazotized p-aminophenyl-N-trimethylammonium chloride for the purpose of isolating antibody to the latter two haptens.[27]

H. Coupling to m-Aminobenzyloxymethylcellulose and p-Aminobenzoylcellulose

m-Aminobenzyloxymethylcellulose, like p-aminobenzylcellulose and the 4-(p-aminophenyl)-anilide of carboxymethylcellulose, is effectively a polyaromatic amine which can be diazotized and coupled to aromatic amines and phenols, among other substances. The amine form should also couple to acids and acid derivatives to form amides. The diazonium salt derivative (Fig. 3b) has been reported coupled to human, cat, horse and rabbit gamma globulins, turkey and hen egg albumin, horse and human serum albumin, edestin, vicilin, glycinin, casein and water soluble proteins from liver and brain extracts.[13,28,29] Viruses of influenza A and B, ornithosis and poliomyelitis

have also been coupled.[28] These protein conjugates have been used as immunoadsorbents in the form of powders and paper discs (Fig. 4a).

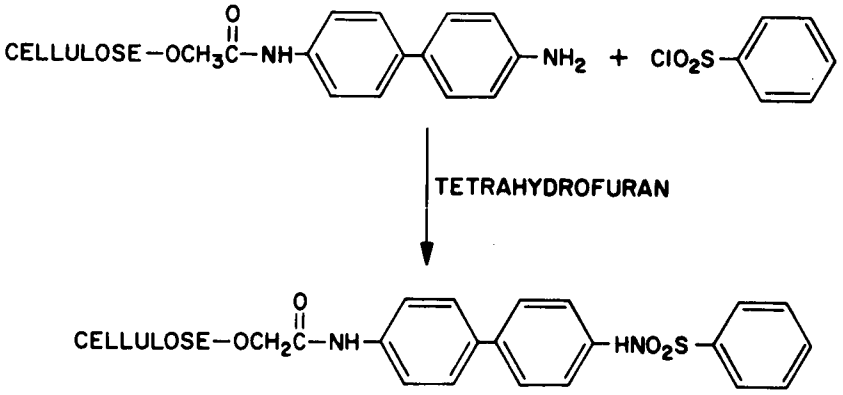
p-Aminobenzoylcellulose has been diazotized (Fig. 3c) and coupled to horse serum in an analogous manner[30] (Fig. 4a).

I. Coupling to 4-(p-aminophenyl)-anilide of Carboxymethylcellulose (Monobenzidine Amide of Carboxymethylcellulose)

This substance like p-aminobenzylcellulose and the m-aminobenzyloxymethyl ether of cellulose can be diazotized and coupled to proteins, aromatic amines and other appropriate substances, or it may be coupled directly to acids or acid derivatives in the presence of diimides.[17]

1. Coupling to proteins. - We have diazotized (Fig. 3d) and coupled this substance to bovine serum albumin,[17] peroxidase, and immune globulin (Fig. 4a).

2. Coupling to benzenesulfonyl chloride.[17]



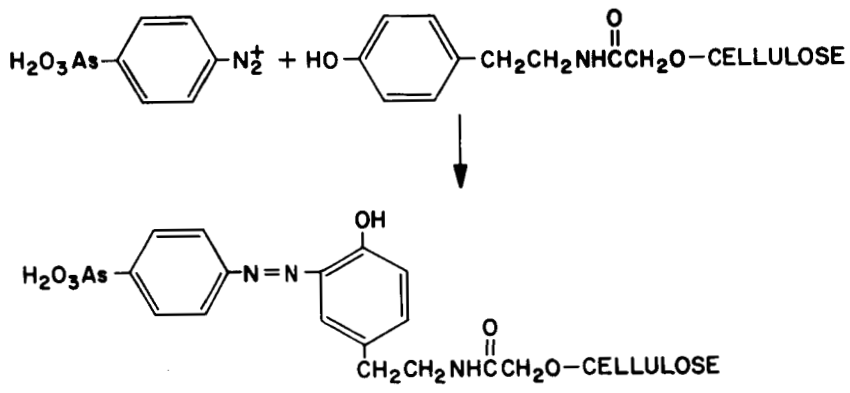
J. Coupling of Diazonium Salts to Hydroxyaromatic Derivatives of Cellulose

We have sometimes found it difficult to remove all soluble organic matter from products of this type of reaction.

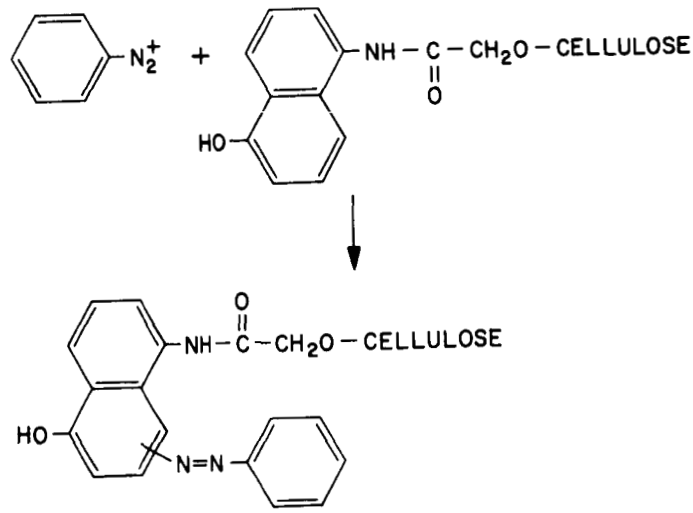
1. Coupling to the monoresorcinol ether of cellulose[4,5] (Fig. 1p).

Several aromatic azoresorcinol cellulose derivatives were made as test inhibitors or complexing agents for tyrosinase[4] (Fig. 5).

2. Coupling of diazotized arsanilic acid to the tyramine amide of carboxymethylcellulose[18] (Fig. 2b).

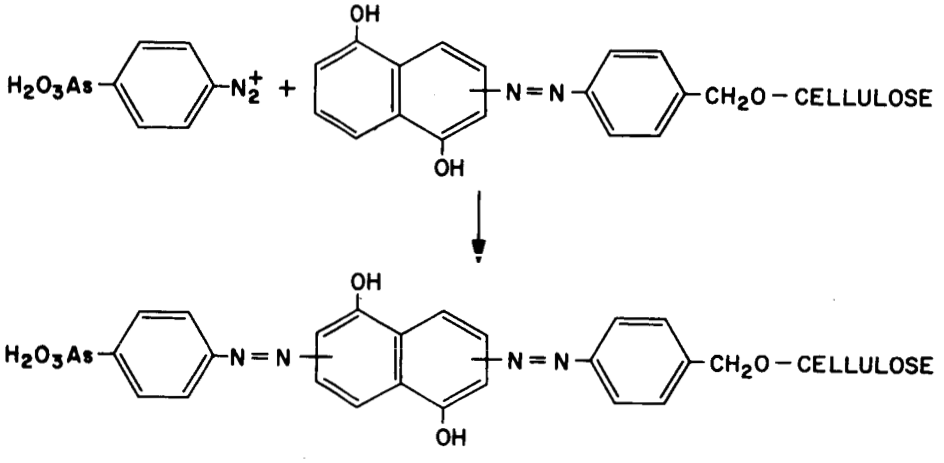


3. Coupling of diazotized arsanilic acid to the 1-hydroxy-5-naphthylamide of carboxymethylcellulose (Fig. 2c).

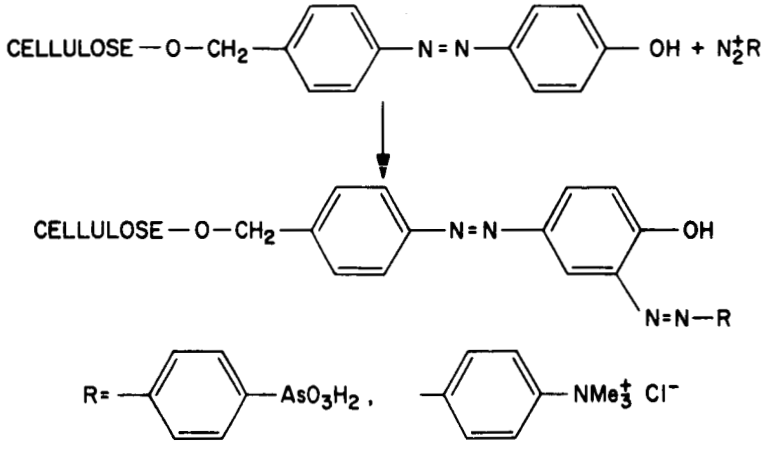


AZOPHENYL HAPTEN

4. Coupling of diazotized arsanilic acid to 1,5-dihydroxynaphthyl-azobenzylcellulose[19] (Fig. 4g).



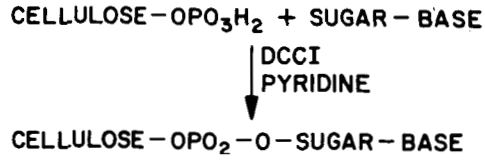
5. Coupling to the p-hydroxyphenyl derivative of diazotized p-aminobenzylcellulose. - Diazotized arsanilic acid and diazotized p-aminophenyl-N-trimethylammonium chloride have been coupled to the phenol derivative of diazotized p-aminobenzylcellulose (Figs. 3a, 4c) for use as hapten immunoadsorbents.[27]



K. Coupling to Phosphocellulose

Polynucleotides and DNA have been coupled to phosphocellulose using dicyclohexylcarbodiimide (DCCI) in dry boiling pyridine.[31,32,58,59] Coupling to DNA

apparently takes place either at chain ends or at reactive groups on the component bases of uncoiled polymer chains, because there are no free pentose hydroxyl groups except at chain ends.



L. Coupling to Cellulose with Blocked Hydroxyl Groups

Many of the reactions described are applicable to cellulose acetate or ethyl cellulose of which some hydroxyl groups are still unreacted, or substituted with other reactive groups. Where we have found interference by hydroxyl groups (e.g. in the range pH 3-4, protein is strongly nonspecifically adsorbed), partially acetylated or ethylated cellulose, or acetylated or ethylated carboxymethylcellulose have proven useful using coupling methods already described.[33]

V. CELLULOSE DERIVATIVES COUPLED TO ANTIGENS, AS IMMUNOADSORBENTS

The usefulness of cellulose immunoadsorbents for the purification, isolation, identification and study of the properties of antibodies, biological (and other) polymers and simple organic molecules, is determined by the degree of nonspecific interaction between these substances and the cellulose derivative (including coupling and blocking groups, if any) under the various conditions required. Also important are the particle size, porosity, the resistance to flow through columns (if required), the capacity of the cellulose for binding functional molecules, buffering capacity, the effect of dissociating reagents on the reactivity of bound groups, and other factors

too numerous to be enumerated.

Most of the systems reported for the isolation and study of antibody have been developed for a specific purpose or in order to overcome one or more difficulties discovered in attempting to apply an existing method. There are also factors involved in adsorption and elution techniques which are not well understood with respect to their effects on the extent or degree of what is commonly termed denaturation of antigenic sites or antibody binding sites. The variety of solvents used for eluting antibody from antigen bound polymers introduces further uncertainties into the ultimate evaluation of the effectiveness of a particular procedure. In brief, there remains much work to be done before final decisions can be made concerning the relative values of the different techniques available.

Comparing the use of cellulose derivatives to substituted polyvinylbenzene or polyacrylic polymers, the amount of nonspecific protein reported in discussions by Isliker,[34] Sehon,[35] and Moudgal and Porter[29] for antibody recovered from such adsorbents considerably exceeds that found for the case of cellulose derivatives. A small amount of work in our laboratories with cellulose coupled to various quantities of β-naphthol[17] tends to confirm this. Buckley, Whitney and Tanford[36] report as much as 28% nonspecific protein contaminating antibody isolated using a polyaminopolystyrene-antigen column. Webb and La Presle[37] found that the ratio of antibody adsorbed to antigen bound, was higher for a protein-cellulose derivative than for the corresponding protein-polystyrene derivative.[37]

Statements, by those who have used physical adsorption or entrapment to bind antigens to insoluble cellulose and other substances[38,39,40], that the integrity or properties of such bound molecules are less affected than where covalent coupling was used must be tested by experiment. Reference to the mechanism of adsorption and catalysis on solid surfaces should be sufficient

to remove any doubt that "physical adsorption" must cause extensive polarization in the bound region and probably extensive distortion in the rest of the polymer molecule. In fact, covalent binding at a specific side chain of a polymer could result in less overall distortion than that resulting from the polarization forces such a molecule is subjected to at a surface where it is adsorbed or in a solid solution of which it is a part. It is likely that neither really affects many protein antigens sufficiently that they cannot be used in these forms to complex with antibody, and any affect that does occur is minimized where random coupling has taken place.

Alteration of the native states of protein antigens and the polynucleotide DNA,[41] does not seem to reduce the effectiveness of these substances to precipitate antibody. In fact, denatured E. coli DNA precipitates with anti-polyuridine,[42] anti-purine and anti-nucleoside[43] antibody while undenatured DNA does not. Evidence exists that antigenic sites are present within a bovine serum albumin molecule.[44] Webb and La Presle isolated antibody to a fragment of human serum albumin.[37] Bovine serum albumin coupled either by amide bonds to carboxymethylcellulose[19] or by azo bonds to diazonium salts of aromatic amino derivatives of cellulose[16,17] can remove all the antibody specific to the immunizing antigen from rabbit antiserum. Jagendorf, Patchornik and Sela[11] report that antibody can be coupled to a cellulose halide by mixing them in a homogenizer with an antifoaming agent, and still retain activity. Antibody activity is retained after exposure to 0.1 N and even 1 N hydrochloric acid.[45] Serum antibodies have been complexed to antigen at pH 10.4 with retention of activity.[46] Antigens and antibodies thus appear to be rugged and can take considerable mistreatment without affecting binding capacity. Some characteristics, as evidenced by shifts in the antigen to antibody weight ratios at equivalence, are affected.[24]

There is some evidence that large numbers of azonaphthol groups increase nonspecific binding and release of protein at low pH.[17] This is consistent with observations that hydrocarbon polymers adsorb and release more nonspecific protein than the cellulose columns reported.

We have found that, particularly between pH 3-4, cellulose adsorbs serum proteins which are not released at pH 7.0 or 1.0[33] but can be removed with 0.1 N sodium hydroxide. Highly acetylated or ethylated carboxymethyl-celluloses adsorb globulin at pH 3.0 but may reversibly desorb the protein at pH 7.0. The implication is that the adsorption at pH 3-4 is by hydrogen bonding. If scattered hydrogen bonding takes place only between weak acid groups (on a cellulose with blocked hydroxyl groups) and protein at pH 3-4, the single bond is broken when the weak acid forms a salt at pH 7.0. If the hydroxyl groups are largely unblocked on the cellulose, or carboxy-methylcellulose, multiple site attachment may take place between the protein and cellulose with binding energy sufficient to prevent release until the correct kind of denaturation occurs to permit dissociation. This may explain why Talmage, Baker and Akeson[26] obtained only 25% dissociation of antibody at pH 3.2; some antibody may have been nonspecifically readsorbed on stirring during the batch elution. We have not found, in the cases of bovine serum albumin, human gamma globulin, anti-azophenylarsonate or anti-purine, that there are appreciable amounts of "tightly bound" antibody that cannot be dissociated by hydrochloric acid at pH 2.0. On those occasions where we have had reason to suspect this, the effect was finally attributed to nonspecific adsorption, aging or inactivation of the antibodies by buffers.

Another phenomenon reported to occur on cellulose immunoadsorbents is tighter binding of antigen and antibody on long standing, i.e. of the order of days.[16,47] This effect is commonly observed in the aging of colloids, such as the exudation of water from gelatin gels on standing, or in the

decrease in dissociation rate of antigen-antibody complexes on standing[48], particularly overnight at 4°C.[49] The conclusion to be drawn is that whereas in a precipitin reaction, where insolubility and minimum dissociation is desirable, long standing improves the results, in the case of immunoadsorbents, where dissociation is the objective, the shorter the time between binding and dissociation the better the yield.

On immunoadsorbents, stoichiometry between antigen and antibody need not be expected for many reasons, particularly steric.[19]

A last point concerning pitfalls in the use of immunoadsorbents is avoidance of unblocked ionic groups on the cellulose which may react with nonspecific serum proteins and desorb them under the conditions that antibody is dissociated. This can easily be detected by appropriate controls.

The concentration of antibody found on elution from an immunoadsorbent depends partly on whether batch or column elution was used. Batch elution results in more concentrated solutions, at least in the first extractions. Ease of elutions from columns is dependent on the buffering capacity of the cellulose: the higher the buffering capacity the slower the pH changes, particularly if unbuffered hydrochloric acid is used. Elution with solvents like 1 M acetic acid[50,70] or buffers such as glycine,[21,29,37] citric acid[26] or phosphate[18,19] may result in incomplete removal of antibody from the column or partial or total loss of antibody activity (see Tables 1 and 2). This along with release of antigen may explain some reports of univalent or nonprecipitable antibody eluted from cellulose antigen columns. We have observed no effect which could possibly be attributed to even as much as 10% of nonprecipitable antibody, and this was within estimates of the errors and losses expected. Antigens studied were bovine serum albumin,[17,19] human gamma globulin,[19] azophenylarsonate[19] and 6-amidocarboxypurine.[24] We have found that acidic phosphate buffers decrease the precipitability of anti-

protein and anti-phenylarsonate antibody from immunoadsorbents, the degree being proportional to the concentration of phosphate.[19]

It is difficult to rate the several methods for coupling antigens to cellulose. It is easier to point out the best features and drawbacks of each (see Tables 1 and 2).

The important factors to be considered are (a) overall yield, the completeness with which antibody is removed and recovered from serum (b) the purity or percent precipitability of the recovered antibody and the related nonspecific adsorption of the cellulose derivative (c) the capacity of the cellulose derivative for antibody (d) the availability of the cellulose derivative or the ease of its synthesis (e) the ease of coupling to the antigen (f) the versatility of the cellulose derivatives for coupling to different antigens.

The m-aminobenzyloxymethylcellulose[28,29,30] derivative shows the highest capacity if it has been reprecipitated in fine form from cuprammonium solution. Reported recoveries from the columns are about 50%. Precipitability is high. Nonspecific adsorption is low, particularly compared to the total quantity of antibody recovered.

p-Aminobenzylcellulose columns have low nonspecific adsorption.[19] Antibody recovery and precipitability are high according to Campbell, Luescher and Lerman[16] and Malley and Campbell[25] but not according to reports of Talmage, Baker and Akeson[26] and Sehon.[35] Webb and La Presle[37] coupled a fragment of enzymatically degraded bovine serum to diazotized p-aminobenzyl-cellulose and used this to extract antibody to anti-human serum albumin from rabbit serum. They report the use of glycine to block uncoupled diazonium groups instead of the usual 8-naphthol.

Columns made with the 4-(p-aminophenyl)-anilide of carboxymethyl-

cellulose behave in an analogous fashion to other cellulose derivatives of aromatic amines.[17] The simplicity of the synthesis and the absence of undesirable intermediates created during preparation makes it easier to clean up, and probably contributes to its improved properties with respect to recovery and yield. Like the other aromatic amines which are coupled through diazonium salts, blocking of excess groups has been achieved with β-naphthol which probably contributes to the nonspecific adsorption of all three. It is possible the excess diazonium groups may be blocked in another way to minimize this effect.

Carboxymethylcellulose - tyramine[18] derivatives follow the same pattern. Low antibody precipitability reported is probably due to the presence of phosphate in the elution solvent. Precipitability with phosphate elution alone (the antibody accounted for in the table was eluted with HCl after phosphate buffer was used) amounted to 45%.

The carboxymethylcellulose derivatives[19] are lowest in nonspecific protein released and high in overall yield and antibody precipitability. High overall yield, i.e. maximum extraction of antibody from serum and maximum recovery of antibody is important in view of the degree of heterogeneity of antibody observed. It does not require blocking groups for antibody isolation and so its low nonspecific protein per ml serum enables antibody to be isolated from lower titer serum in higher purity. Attempts have not been made to increase or test maximum capacity. Versatility is better than most of the other intermediates.

Other varieties of immunoadsorbents have not been adequately tested yet. Bromoacetylcellulose[11] shows promise. Cellulose derivatives with blocked hydroxyl groups will help in specific applications and may be generally more useful when further developed because we have found, for example, that anti-

azophenylarsonate antibody can be eluted at pH 3.0 from acetylated or ethylated carboxymethylcellulose coupled to aminophenylazophenylarsonic acid, instead of at pH 2.0 - 2.3. In fact, we have been able to do acid dissociation studies of bovine serum albumin and azophenylarsonate hapten complexes with their respective antibodies.[33] It is still difficult to make successful columns and we are continuing to look for improved methods for their synthesis.

Only acid dissociation has been discussed, because one of the most useful aspects of the methods using immunoadsorbents is the elimination of the requirement for hapten to dissociate antigen-antibody complexes and the subsequent minimizing of contamination of the antibody by antigen. In the case of anti-DNP antibodies, acid does not dissociate the complexes[19] and resort must be made to hapten elution or weaker binding cross reacting haptens.[51] Lerman used hapten elution to investigate heterogeneity of anti-azophenylarsonate antibody.[5]

Those techniques published in which antibody was not eluted have not been discussed. One in which BSA was coupled by physical adsorption to cellulose and antibody complexed to it is mentioned in another section.[38] Michael and Ewers[2] coupled gelatin and pseudoglobulin to acidic oxycellulose and carboxymethylcellulose but did not include any data on their effectiveness as immunoadsorbents. Brown used the anion exchange resin ECTEOLA cellulose to bind antigens of small size derived from adenovirus, influenza virus and typhus rickettsiae.[39] The resulting substances combined specifically with homologous antibody in sera from infected guinea pits or humans. Nonspecific adsorption of antibody was low.

In summary, it now appears practical, using cellulose immunoadsorbents, to isolate more than 90% of the antibody to a specific antigen from many immune sera, with precipitability of the isolated antibody exceeding 90%, in favorable cases.

VI. CELLULOSE DERIVATIVES COUPLED TO ANTIBODY

Several papers have appeared in which use has been made of cellulose derivatives coupled to antibody.

Stone and Williams complexed anti-subtilisin antibody to subtilisin physically adsorbed to cellulose.[52] On passing carboxypeptidase through the antigen-antibody column, all detectable subtilisin-like activity was removed and the carboxypeptidase was recovered almost quantitatively.

Jagendorf, Patchornik and Sela[11] coupled anti-ovalbumin antibody to bromoacetyl cellulose. On passing ovalbumin through the column, the albumin was adsorbed but could not be recovered. Nonspecific adsorption was very high for ribonuclease and relatively high for cytochrome c and BSA. For 50 mg of cellulose-antibody which adsorbed 70 μg ovalbumin, 2.49 mg out of 3.33 mg ribonuclease, 0.05 - 0.2 mg out of 3.33 mg of cytochrome c and 0.05 - 0.2 out of 3.33 mg of bovine serum albumin were picked up.

Gurvich and Drizlikh[53] recently used a similar technique in which 30 - 50 mg of human gamma globulin were coupled to 1 gm of the diazonium salt of m-aminobenzyloxymethylcellulose. Using a 1.5 - 3.0 mg column, 0.63 ml antiserum containing 0.54 mg antibody was passed through. This column was then able to pick up 710 out of 795 cpm I^{131} labeled human gamma globulin.

In a similar experiment we coupled the globulin fraction of rabbit anti-bovine human gamma globulin serum to the diazonium salt of the 4-(p-aminophenyl)-anilide of carboxymethylcellulose and were able to demonstrate that it could complex antigen at neutral pH and release it with 1% NaCl

acidified to pH 2.3 with HCl. The only other insoluble antibody derivatives reported to complex with and dissociate from specific antigen is a copolymer of globulin from immune sera, with tetrazotized benzidine[46] and an adsorbent made by coupling globulin from an immune serum to polyaminopolystyrene.[54]

VII. COUPLING OF CELLULOSE DERIVATIVES TO ENZYMES AND ENZYME INHIBITORS

Little has been published in this area. There is some work published on the coupling of enzymes to synthetic polypeptides[55,56] and other noncellulosic polymers. This is discussed by Manecke.[57]

Lerman[4] coupled a number of organic molecules to cellulose and found that three p-hydroxyphenyl derivatives acted as tyrosinase inhibitors and complexed with soluble tyrosinase from mushroom, while four other hydroxy derivatives where the hydroxy groups were not para or were sterically hindered did not do this. He was thus able to obtain tyrosinase solutions with 61 times the activity of the crude extract with a recovery of 50 - 60%, using columns of 0.2 gm of cellulose derivative or less.

Arsenis and McCormick[21] coupled flavins to the acid chloride of carboxymethylcellulose and used these flavin derivatives to concentrate flavokinase solutions and obtain specific activities greater than found possible by other methods.

Stone and Williams,[52] using the "sandwich technique," first complexed anti-subtilisin to subtilisin which had been physically adsorbed to cellulose and then used this material to remove subtilisin activity from carboxypeptidase solutions.

Mitz and Sumara,[23] using the azide method described earlier by Michael and Ewers,[2] coupled trypsin and chymotrypsin to soluble and insoluble carboxymethylcellulose. In the case of chmotrypsin on insoluble carboxymethylcellulose, 35 mg % coupled with an activity of 8.6 mg %. In the case of chymotrypsin on soluble carboxymethylcellulose, 55.8 mg % coupled with an activity of

107.1 mg %, i.e. the activity was higher coupled to soluble carboxymethylcellulose than in free solution. This observation warrants further investigation. If coupled to diazotized p-aminobenzylcellulose, 34 mg % of the chymotrypsin coupled with an activity of 1.3 mg %, considerably lower. Whether this was due to coupling with different parts of the chymotrypsin molecule, or because the surface environment around the molecule affected the reaction or affected the approach of substrate to the enzyme is problematical. Ribonuclease coupled to diazotized p-aminobenzylcellulose with a yield of 23 mg % and an activity of 0.6 mg %.

We have coupled a number of enzymes to carboxymethylcellulose in the presence of dicyclohexylcarbodiimide or soluble diimide.[17,19] All the enzymes displayed activity but data on efficiency or effectiveness is not yet available. Enzymes included chymotrypsin, trypsin, pan protease, subtilisin, nagarse, alkaline phosphatase, acid phosphatase, β-glucuronidase, DNAase, RNAase, glucose oxidase and horseradish peroxidase. We have coupled horseradish peroxidase to wool also, by the same method. The activity of horseradish peroxidase is affected by binding in that it oxidizes benzidine but not p-aminobenzoic or p-aminosulfonic acids.

VIII. COUPLING OF NUCLEIC ACIDS, NUCLEOTIDES, NUCLEOSIDES, PURINES AND PYRIMIDINES TO CELLULOSE DERIVATIVES

The coupling of purines and pyrimidines poses the same problems as for other simple organic molecules. The coupling of 6-trichloromethylpurine to aminoethylcellulose was described above.[24] Analogous derivatives can be made from other trichloromethyl compounds. Purines and pyrimidines, their derivatives and polymers may also be coupled if suitable functional groups are present or formed.

Several papers have appeared in which polynucleotides, RNA and DNA have been coupled to insoluble cellulose in order to extract, by complexation, specific complementary substances from solutions containing mixtures.

Bautz and Hall[31] labeled E. coli RNA with P^{32} (in vivo), and then incubated it with T4 bacteriophage and H^3 labeled uracil. A cellulose derivative was made by heating T4 phage DNA, in pyridine at 110°C, with phosphocellulose which had its free hydroxyl groups blocked by acetylation. Coupling presumably took place between the cellulose phosphate and the free hydroxyl groups of the hydroxymethylcytosine glucose units although reactions involving other reactive groups on the bases, terminal deoxyribose units or polyphosphate bonds are possible. On passing the isolated RNA from the culture through the DNA cellulose column at 55°C, adsorption of RNA occurred (but not below 40°C). One band containing a very low H^3/P^{32} ratio came off first. A second came off at 60°C and lower ionic strength, which had an H^3/P^{32} ratio 8 times that of the starting material, demonstrating a difference in binding for the two RNA types. This was related to base composition.

Adler and Rich[32], by the same synthetic method coupled poly A, poly C, poly I, poly U, bacteriophage virus RNA, E. coli transfer RNA, and ribosomal RNA to cellulose. Using the cellulose coupled to homopolymers, binding to the expected complementary homopolymers were found: A+U, A+I, C+T, A+A, I+I, U+U. Complexing was found to occur at 23°C in 0.5 M NaCl, 0.01 M tris buffer pH 7.4. Complexed material was released with 0.001 M tris buffer pH 7.4. Elution could also be accomplished by raising the temperature to above 40°C.

Gilham[58] and Gilham and Robinson[59] coupled nucleotides to cellulose by soaking the cellulose and nucleotide separately with N,N-dicyclohexylcarbodiimide in pyridine and then shaking them together. In this way thymidine

polynucleotide, deoxycytidine nucleotide and deoxyadenosine polynucleotide were coupled. A mixture of deoxyadenosine oligonucleotides (3 - 7 units) in 1 M sodium chloride - 0.01 M sodium phosphate pH 7 was fractionated using a thymidine polynculeotide cellulose column at 0°C. Elution was carried out by stepwise increases in temperature. A separation was also made of the larger polynucleotides from ribonuclease digestion of the ribonucleic acid of broomgrass mosaic virus. This technique is hoped to assist in sequence studies of nucleic acids.

IX. SYNTHESIS AND DEGRADATION

Although there is little information reported on the use of the synthesis of macromolecules starting with monomers bound to solid state supports, it is worthwhile mentioning the success of the synthesis of bradykinin[60] starting with t-butyloxy-carbonyl-nitro-L-arginine triethyl-ammonium salt coupled to chloromethylcopolystyrenedivinylbenzene to give an ester. The ease of purification of the desired coupled product, in each step, from excess reagents is potentially a powerful method which may make use of cellulose or other reactive polymers as carriers for the products.

There are no reports of techniques by which enzymes or other reagents coupled to polymers are used for synthetic or degradative processes. Such systems individually or in sequence also have potentialities as models for the study of organized processes in living organisms.

Acknowledgements. - We are grateful to Professor D. H. Campbell, of the Department of Chemistry, California Institute of Technology, for his encouragment, advice, confidence and foresight. We also wish to thank J. Podliska of the California Institute of Technology, Jet Propulsion Laboratory, Chemistry Section, for determining the hydroxyl content of ethyl cellulose.

REFERENCES

1. Nevell, T. P., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 164ff. Academic Press, New York (1963).
2. Michael, F. and Ewers, J., J. Makromol. Chem. 3, 200 (1949).
3. Pacsu, E., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 259ff. Academic Press, New York (1963).
4. Lerman, L. S., Proc. Nat. Acad. Sci., Wash. 39, 232 (1953).
5. Lerman, L. S., Nature 172, 635 (1953).
6. Tanghe, L. J., Gerung, L. B. and Mench, J. W., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 193ff, Academic Press, New York (1963).
7. Siggia, S., Quantitative Organic Analysis via Functional Groups, p. 4-7. John Wiley and Sons (1949).
8. Tedder, J. M., Chem. Rev. 55, 787 (1955).
9. Powers, J., California Corporation for Biochemical Research, personal communication.
10. Pacsu, E., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 251ff. Academic Press, New York (1963).
11. Jagendorf, A. T., Patchornik, A. and Sela, M., Biochim. Biophys. Acta 78 516 (1963).
12. Sun Tun, Derevitskaya, V. A. and Rogovin, Z. A., Visokomolekul Coyedinyeniya 12, 1768 (1960).
13. Gurvich, A. E., Kuzonlova, O. B., and Tamanova, A. E., Biokhimiya 26, 803 (1962).
14. Jullander, I. and Lagerstrom, O., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 303ff, Academic Press, New York (1963).

15. Croon, I. and Manley, R. St. J., Methods in Carbohydrate Chemistry (Edited by R. L. Whistler) Vol. III, p. 274ff, Academic Press, New York (1963).

16. Campbell, D. H., Luescher, E. and Lerman, L. S., Proc. Nat. Acad. Sci., Wash. 37, 575 (1951).

17. Weetall, H. H. and N. Weliky, Nature 204, 896 (1964).

18. Vannier, W. E., Bryan, W. P. and Campbell, D. H., Immunochemistry (in press).

19. Weliky, N., Weetall, H. H., Gilden, R. V. and Campbell, D. H., Immunochemistry 1, 219 (1964).

20. Bassard, H. H., Mory, R., Schmid, M. and Zellinger, H., Helv. Chim. Acta 42, 1653 (1959).

21. Arsenis, C. and McCormick, D. B., J. Biol. Chem. 239, 3093 (1964).

22. Greenstein, J. P. and Winitz, M., Chemistry of the Amino Acids, Vol. II, p. 1018. John Wiley and Sons, New York (1961).

23. Mitz, M. A. and Sumara, L. J., Nature 189, 576 (1961).

24. Weetall, H. H. and Weliky, N., Science (in press).

25. Malley, A. and Campbell, D. H., J. Chem. Soc. 85, 487 (1963).

26. Talmage, D. W., Baker, H. R. and Akeson, W., J. Infect. Dis. 94, 199 (1954).

27. Gold, E. F., Knight, K. L., Haurowitz, F., Biochem. Biophys. Res. Commun. 18, 76 (1965).

28. Gurvich, A. E., Immunological Methods (Edited by J. F. Ackroyd) p. 113ff. F. A. Davis Company, Philadelphia, Penna. (1964).

29. Moudgal, N. R. and Porter, R. R., Biochim. Biophys. Acta 71, 185 (1963).

30. Gurvich, A. E., Kapner, R. B. and Nezlin, R. S., Biokhimiya 24, 129 (1959).

31. Bautz, E. K. F. and Hall, B. D., Proc. Nat. Acad. Sci., Wash. 48, 400 (1962).

32. Adler, A. J. and Rich, A., J. Am. Chem. Soc. 84, 3977 (1962).

33. Weliky, N. and Weetall, H. H., Abstracts 148th Meeting Amer. Chem. Soc. (1964).

34. Isliker, H. C., Advances in Protein Chemistry, Vol. 12, p. 387-463. Academic Press, New York (1957).

35. Sehon, A. H., Brit. Med. Bull. 19, 183 (1963).

36. Buckley, C. E. III, Whitney, P. L. and Tanford, C., Proc. Nat. Acad. Sci., Wash. 50, 827 (1963).

37. Webb, T., and La Presle, C., Biochem. J. 91, 24 (1964).

38. Williams, R. R., and Stone, S. S., Arch. Biochem. Biophys. 71, 377 (1957).

39. Brown, N. K., Science 133, 331 (1961).

40. Bernfeld, P. and Wan, T., Science 142, 678 (1963).

41. Plescia, O. J., Braun, W. and Palczuk, N. C., Proc. Nat. Acad. Sci., Wash. 52, 285 (1964).

42. Sela, M., Ungar-Waron, H., Shechter, Y., Proc. Nat. Acad. Sci., Wash. 52, 285 (1964).

43. Beiser, S. M., Tanenbaum, S. W. and Erlanger, B. F, Nature 203, 1381 (1964).

44. Ishizaka, T., Campbell, D. H. and Ishizaka, K., Proc. Soc. Exptl. Biol. Med. 103, 5 (1960).

45. Haurowitz, F., Tekman, S. H., Bilen, M. and Schwerin, P., Biochem. J. 41, 304 (1947).

46. De Carvalho, S., Lewis, A. J., Rand, H. S. and Uhrick, J. R., Nature 204, 265 (1964).

47. Nezlin, R. S., Biokhimya 24, 486 (1960).

48. Talmage, D. W., J. Inf. Dis. 107, 115 (1960).

49. Farr, R. S., private communication.

50. Bennett, J. C. and Haber, E., J. Biol. Chem. 238, 1362 (1963).

51. Cheng, W. C. and Talmage, D. W., Abstracts 146th Meeting Amer. Chem. Soc. Jan. 19-24, 1964.

52. Stone, S. S. and Williams, R. R., Arch. Biochem. Biophys. 71, 386 (1957).

53. Gurvich, A. E. and Drizlikh, G. I., Nature 203, 648 (1964).

54. Sehon, A. H. and Richter, M., J. Immunol. 82, 190 (1959).

55. Cebra, J. J., Gival, D., Silman, H. I., Katchalski, E., J. Biol. Chem. 236, 1720 (1960).

56. Bar-Eli, A., Katchalski, E., Nature 188, 856 (1960).

57. Manecke, G., Pure Appl. Chem. 4, 501 (1962).

58. Gilham, P. T., J. Am. Chem. Soc., 86, 4982 (1964).

59. Gilham, P. T. and Robinson, W. E., J. Am. Chem. Soc. 86, 4985 (1964).

60. Merrifield, R. B., J. Am. Chem. Soc. 86, 304 (1964).

61. Cohen, S. C., Tom, E., and Bendich, A., J. Org. Chem. 29, 3545 (1962).

General References for the Properties of Cellulose and Some of Its Derivatives

Whistler, R. L., (Editor), Methods in Carbohydrate Chemistry, Vol. 3. Academic Press, New York (1963).

Ott, E., Spurlin, H. M. and Grafflin, M. W., Cellulose and Cellulose Derivatives, 2nd Ed. Interscience Publishers Inc., New York (1954).

Jones, D. M., Adv. Carbohydrate Chem. 19, 219 (1964).

Rogovin, Z. A., J. Polymer Sci. 48, 443 (1960).

Table 1.

Characteristics of Cellulose Immunoadsorbents

Cellulose Derivative	PABZ[30]	MABOM[13]	MABOM[30]	MABOM[29]	MABOM[29]	MABOM[29]	MABOM[29]	MABOM[29]	PAB[37]
Coupled Antigen	horse serum	horse serum	horse serum albumin	HSA	HGG	RGG	Hen EA	Turkey EA*	HSA fragment
Bound Antigen mg/gm Cellulose	117	200	--	125	271	450	450	320	5
Antibody Source	rabbit	rabbit	rabbit	rabbit	rabbit	horse	rabbit	rabbit	rabbit
Cellulose/g	2	--	2	0.05	0.05	0.05	0.05	0.05	6
Antibody + Complexed mg	104	330	--	125	103	150	100	60	--
% Antibody Complexed	--	--	--	60	50	40	10-25	55	--
Complexed Antibody Recovered (mg)	--	--	0.09-4.5	75	51.5	60	10-25	33	22-33
% Antibody Precipitable	--	--	44-48	90-100	90-100	90-100	90-100	90-100	--
Nonspecific Protein mg/ml serum	4.4	0.25	--	< 1	< 1	< 1	< 1	< 1	--
Elution Solvent	--	--	--	HCl pH 2	HCl pH 2	HCl pH 2	HCl pH 2	HCl pH 2	0.5 M glycine HCl pH 1.0

+ Serum was passed through till the capacity of the material for antibody was exceeded.

* The antibody isolated was from rabbit anti-hen egg albumin serum.

PABZ - p-aminobenzoylcellulose

MABOM - m-aminobenzyloxymethylcellulose

EA - egg albumin

RGG - rabbit gamma globulin

HSA - human serum albumin

HGG - human gamma globulin

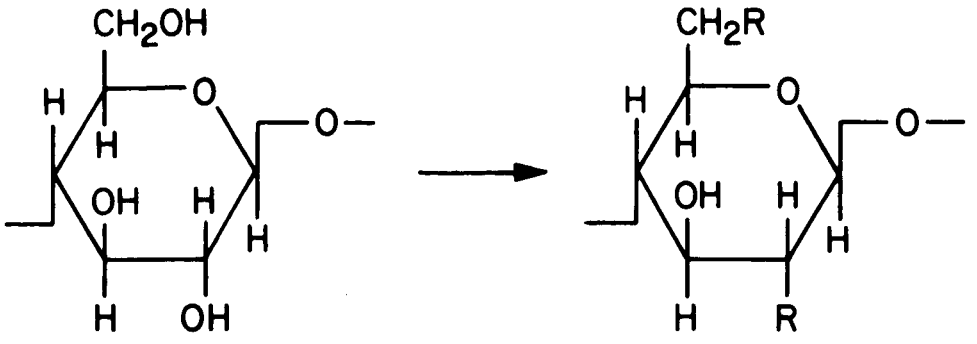
Table 2.

Characteristics of Cellulose Immunoadsorbents

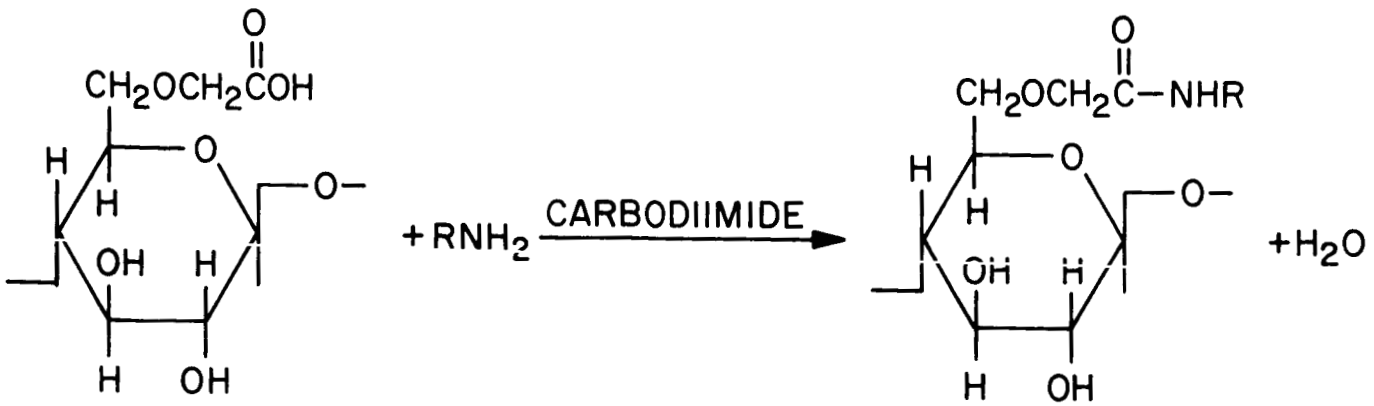
Cellulose Derivative	PAB[16]	PAB*[25]	PAB[26]	PAB[26]	PAB*[19]	CMCB[17]	AEC[24]	CMC[19]	CMCT[18]
Coupled Antigen	BSA	BSA	HGG	HGG	HGG, BSA, R'	BSA	Purine	BSA, HGG, R'	R'
Bound Antigen mg/gm Cellulose	15	--	33	33	--	--	47	up to 100	--
Antibody Source	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit
Antibody mg/ml	3.4	2.5	0.06	0.06	--	2.1	1-3	0.02-3	--
Antibody ml Applied	5	20	10	10	--	5	5	1-20	--
Antibody Applied mg	17	50	0.6	0.6	--	10.5	5-15	--	--
Cellulose/g	0.9	1.0	0.01	0.01	--	1.0	1.0	0.5-1.0	--
Antibody Complexed mg	17	--	--	--	--	--	5-15	1-20	--
% Complexed Antibody Recovered	86	46-70	25	67	--	100	83-91	71-100	75-80
% Antibody Precipitable	89	83	--	--	--	82	90	86-100	70
% Overall Yield	86	--	--	--	--	100	81-91	as high as 90-96	--
Nonspecific Protein mg/ml Serum	--	--	--	--	0.08-0.21	0.4	0.016	0.011	< 0.1
Elution Solvent	HCl pH 3.2	glycine HCl pH 3.0	HCl pH 3.2	0.2 M citric acid pH 3.2	HCl pH 2.3	HCl pH 2.3	HCl pH 2.3	HCl pH 2.3	HCl 0.1 N

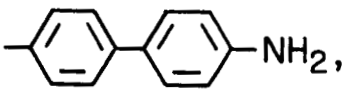
Footnotes:
HGG - human gamma globulin
BSA - bovine serum albumin
PAB - p-aminobenzylcellulose
PABX* - p-aminobenzylcellulose (commercial)
CMCB - 4-(p-aminophenyl)-anilide of carboxymethylcellulose
R' - aminophenylazoarsonate
HSA - human serum albumin
AEC - aminoethylcellulose
CMC - carboxymethylcellulose
CMCT - tyrosyl amide of carboxymethylcellulose

FIGURES

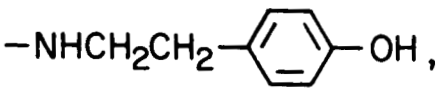


PROCESS	R
ESTERIFICATION	(a) $-OC(=O)-CH_3$, (b) $-O_2S-CH_3$, (c) $-OC(=O)CH_2Br$, (d) $-OC(=O)-C_6H_4-NH_2$, (e) $-O_2SCH_3$, (f) $-O_2S-C_6H_4-CH_3$, (g) $-OPO_3H$, (h) $-ONO_2$, (i) $-NH_2$
ETHERIFICATION	(j) $-OCH_3$, (k) $-OCH_2CH_3$, (l) $-OCH_2CH_2OH$, (m) $-OCH_2OCH_2-C_6H_4-NH_2$, (n) $-OCH_2-C_6H_4-NH_2$, (o) $-OCH_2CH_2NH_2$, (p) $-OCH_2C(=O)-OH$, (q) $-O-C_6H_4-OH$
URETHAN FORMATION	(r) $-OC(=O)NH-C_6H_5$

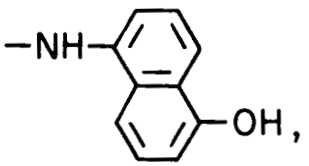


–NHR = 

(a)



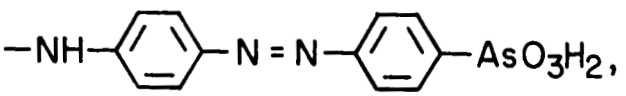
(b)



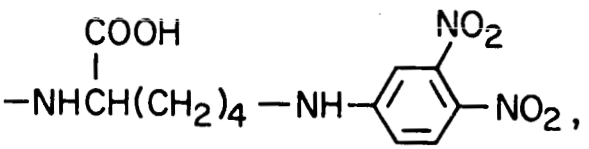
(c)

–PROTEIN
(THROUGH FREE AMINO GROUPS),

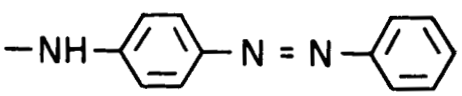
(d)



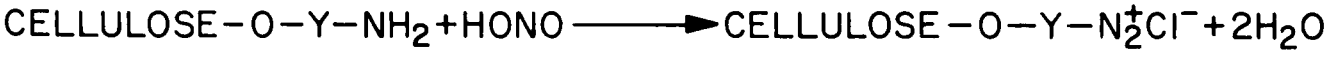
(e)

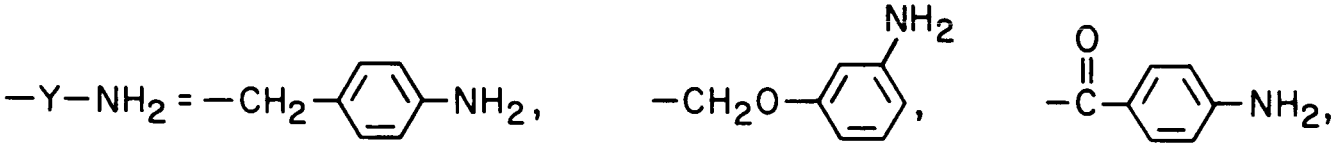


(f)

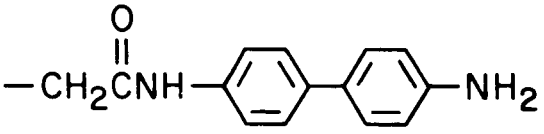


(g)

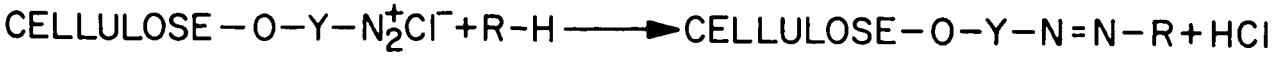




(a) (b) (c)

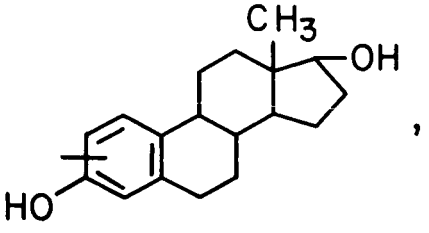


(d)

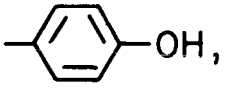


−R = −PROTEIN (THROUGH AROMATIC AND FREE AMINO GROUPS),

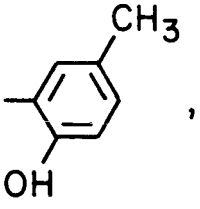
(a)



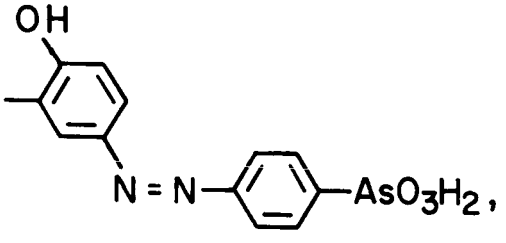
(b)



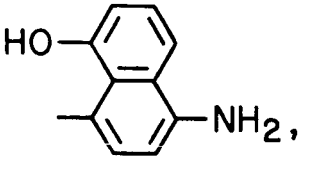
(c)



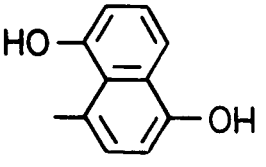
(d)



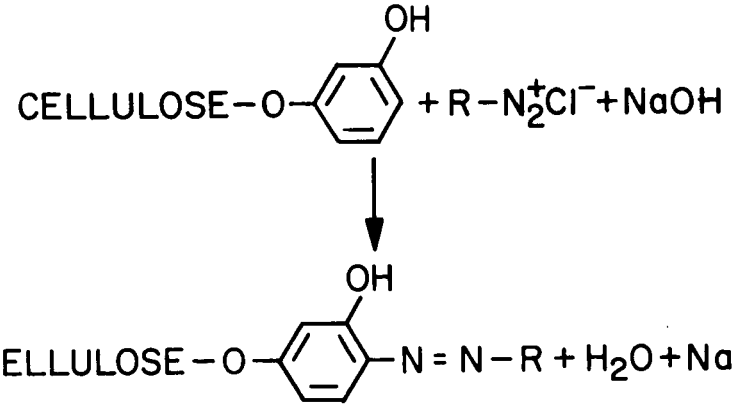
(e)

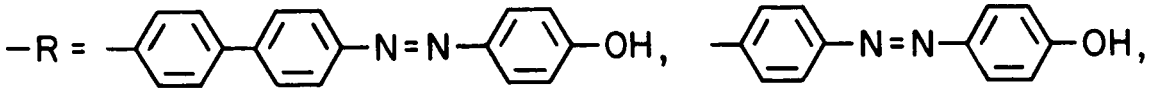


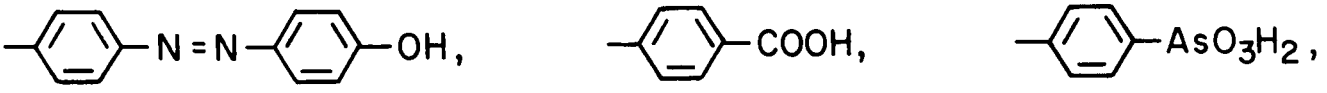
(f)

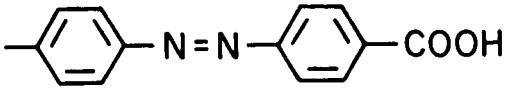


(g)


OH
CELLULOSE–O
+R–N$_2^+$Cl$^-$+NaOH
OH
CELLULOSE–O
N=N–R + H$_2$O + NaCl


–R =
N=N
OH,
N=N
OH,


N=N
OH,
COOH,
AsO$_3$H$_2$,


N=N
COOH