THE SYNTHESIS AND CHROMATOGRAPHIC PROPERTIES OF CARBOXYL CELLULOSE*

DAVID D. GILBOE AND ROBERT M. BOCK
Departments of Surgery and Biochemistry, University of Wisconsin, Madison, Wisc. (U.S.A.)
(Received May 4th, 1964)

INTRODUCTION

The chromatographic separation of compounds with similar chemical characteristics has been one of the important advances in biochemistry, however, these techniques utilizing ion exchange resins were not universally successful in affecting separation of protein mixtures. Ion exchange resins usually form strong bonds with proteins making it difficult to adsorb and desorb the molecules without destroying the comparatively weak bonds which hold them in their native configuration. While the ion exchange resins have a relatively high number of adsorptive sites, the sites are not limited to the surface of the resin particle thus substantially reducing the capacity of the resins for large molecules like proteins.

Cellulose ion exchange materials[1–3] are derived by the controlled substitution of anionic or cationic groups onto the cellulose polymer. The reactions utilized in their synthesis have been known for some time, however, the prime factor in the synthesis is the restriction of substitution to control undesirable changes in physical properties of the cellulose such as formation of a gel or a soluble or granular product. The weak bonds formed between proteins and the ionizing groups on the cellulose may be severed by relatively mild changes of pH or ionic strength. Most cellulose ion exchange materials do not contain as many binding sites per gram as the ion exchange resins, but their charges are confined mostly to the surface making them more available for bond formation. Consequently, the substituted celluloses often bind nearly their own weight in protein.

The variety of binding sites and molecular structures that characterize protein molecules make it reasonable to assume that varying the configuration in which particular groups are placed on cellulose may change the characteristics as an ion exchange material for separation of protein mixtures.

Our purpose was: (1) to investigate the feasibility of converting one or both of the hydroxyl groups on carbon atoms 2 and 3 of the glucose sub-unit in the surface chains of cellulose to carboxyl groups while retaining the basic physical properties of the cellulose; (2) to determine whether the resultant material possessed the properties of a satisfactory ion exchange adsorbent.

* Supported in part by Grant No. 2185 of the National Institute of Neurological Diseases and Blindness.

EXPERIMENTAL

Preparation of dialdehyde cellulose

Cellulose with approximately 1 mequiv. carboxyl per gram is produced at room temperature by the initial reaction of 60 g of cellulose (SW-40-B Solkafloc) in 1 l of 0.0435 *M* potassium metaperiodate solution for 16 h[4]. The amount of aldehyde formed can be controlled by limiting exposure of the cellulose to potassium metaperiodate. With excess periodate present, a reaction time of 16 h or less prevents product loss and formation of granular material lacking the ideal flow quality of the natural cellulose. When limiting the reagent to form a specific number of aldehyde groups, 10 % more than the stoichiometric amount must be provided to compensate for the spontaneous decomposition of potassium metaperiodate in aqueous solutions at room temperature. After proper substitution has been achieved the remaining periodate and by-products are removed by repeated suspension of the product in distilled water followed by settling and decantation.

Preparation of carboxyl cellulose

Dialdehyde cellulose (60 g) is added to 2 l of 0.1 *M* acetic acid which is 0.11 *M* with respect to sodium chlorite[5,6]. The reaction is allowed to proceed with continuous mixing for 24 h at room temperature. Because of the noxious fumes generated, the reaction is best carried out in a hood. After oxidation, the supernatant fluid is decanted and the product is washed three times by resuspension in distilled water and filtered through a Buchner funnel. Finally, the product is suspended in 95 % ethanol and filtered. The filter cake is removed, broken into small pieces and allowed to dry in air or under an infrared lamp.

To summarize, each 1.1 mequiv. of potassium metaperiodate will form approximately 2.0 mequiv. of aldehyde which can subsequently be oxidized to 1.5 mequiv. of carboxyl groups. When limiting the reagent, one should provide 0.733 mequiv. of periodate for every mequiv. of carboxyl desired in the final product.

Aldehyde content

Aldehyde content was calculated from data on periodate consumption corrected for spontaneous breakdown under conditions of the reaction. Since each molecule of periodate is capable of producing two aldehyde groups on each glucose sub-

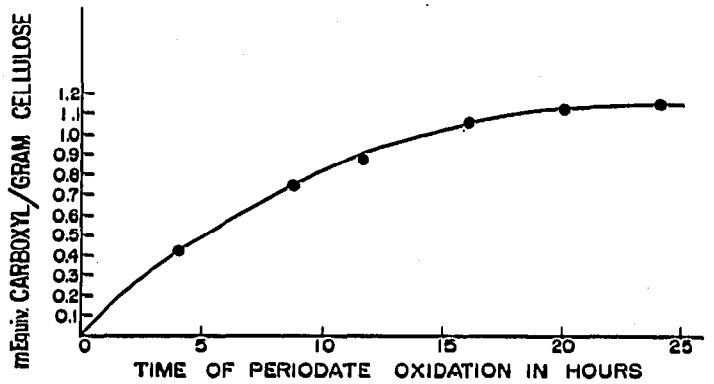


Fig. 1. Increase in titrable carboxyl groups after chlorite oxidation of cellulose samples exposed to periodate from 4–24 h.

unit of the cellulose, the number of mequiv. of aldehyde formed in the reaction is twice the mequiv. of periodate reacting with the cellulose.

The ultimate effect of increased aldehyde content is expressed best by the increase in carboxyl groups produced from cellulose samples treated with periodate for varying periods of time. Fig. 1 shows the increase in titrable acidity when 1 g of cellulose is exposed to 1.5 mequiv. of periodate for periods of 4–24 h with a subsequent 24 h chlorite oxidation. While the rate of carboxyl formation is not linear, it is reproducible when periodate, in excess, is permitted to react with cellulose at room temperature. The most rapid increase in carboxyl content occurs in samples exposed to periodate during the initial 8 h of the reaction. Samples exposed over 16 h tend to decrease in weight due to formation of soluble by-products.

Carboxyl contents

The carboxyl groups in the cellulose were determined by electrometric titration before and after oxidation. The rate at which dialdehyde cellulose is oxidized to carboxyl cellulose is shown in Fig. 2. Fifty percent of the total aldehyde groups present were oxidized within the first hour. This apparently indicates that one of the aldehyde groups is less easily oxidized than the other due to steric hindrance or the negative charge repulsion which would exist at the pH of the reaction. Only 73–75 % of the aldehyde present in the cellulose polymer can be oxidized even when the product was exposed to chlorous acid for several days. A slight decrease in carboxyl content has been observed when chlorous acid oxidation is continued for longer than 24 h.

Electrometric titration

Representative titration curves of carboxyl cellulose are shown in Fig. 3. The pK of 4.55 in water is higher than that reported for carboxymethyl cellulose and most substituted carboxyl groups in aqueous solution. In a 0.2 M sodium chloride solution,

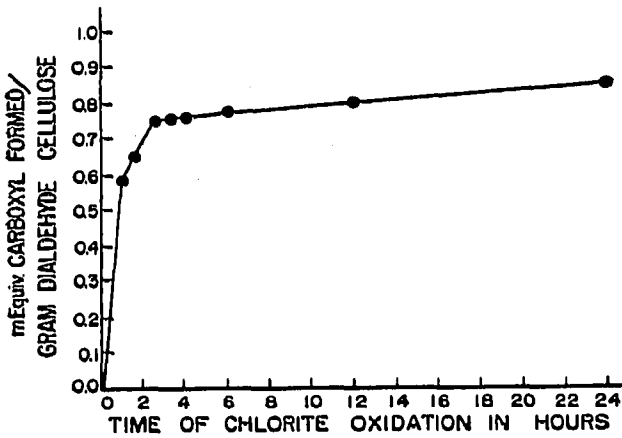


Fig. 2. Increase in titrable carboxyl groups in samples of dialdehyde cellulose exposed to chlorous acid for 0.5–24 h.

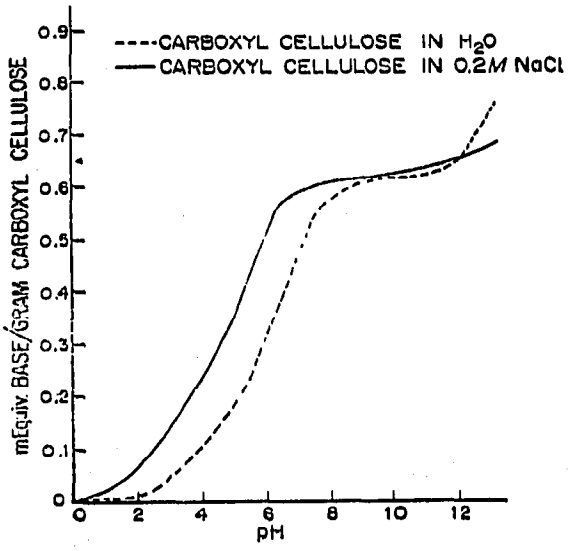


Fig. 3. Titration curves of carboxyl cellulose in distilled water and 0.2 M sodium chloride.

the pK shifts to 3.6, a value close to the one expected. It was not possible to determine from the titration curve whether the titrable groups were homogeneous with respect to pK.

pH stability

There is a measurable reduction of titrable carboxyl groups when carboxyl cellulose stands in solutions below pH 4 or above pH 11 for 24 h. The downward shift in pH noted after the addition of base during the titration of material exposed to pH values below 4 suggests that the loss of titrable groups may have been due to lactone formation. Dialdehyde cellulose was observed to lose weight in basic solutions. It would appear that cleavage of the pyranose ring decreases the stability of the cellulose polymer in alkaline solution.

Ultraviolet absorbing material

Ultraviolet absorbing material is bound on the cellulose after the chlorite oxidation step. This material cannot be completely removed from carboxyl cellulose which is prepared in large batches, consequently, some will appear in the first few ml of eluate. The interfering material can be eluted with about 1.5 bed volumes of the starting buffer.

Separation of serum albumin and lysozyme

Lysozyme is a very basic protein with an isoelectric point of pH 10.7 while the isoelectric point of bovine serum albumin is pH 4.7. Chromatographic separation of a mixture of the two was effected on a 1.2 × 25 cm carboxyl cellulose column using a 1 l linear gradient 0.01–0.50 M citrate buffer pH 5.0. There was no binding of the albumin which emerged in one bed volume while it took 350 ml of buffer to elute the lysozyme. Basic proteins are bound to the ion exchange material and may be eluted under relatively mild conditions.

Chromatography of cytochrome C

An easily obtainable mixture containing basic proteins suitable for chromatography on this ion exchange material is horse heart cytochrome C. Others have reported and we have confirmed the presence of 4 components[7,8] in cytochrome C prepared by the method of KEILIN AND HARTREE.

The chromatography of cytochrome C was performed on a 20 × 1.5 cm carboxyl cellulose column using a 0.2 M sodium phosphate buffer pH 7.04. The fractions observed were: a rapidly moving straw-colored band, reduced and oxidized cytochrome C appearing in a two-component band (the leading edge contained reduced cytochrome C and the remainder oxidized cytochrome C as demonstrated by their respective absorption spectra), and one component which remained at the origin. The straw-colored component may be eluted with distilled water and has been identified by others as myoglobin[7]. The remaining component has resisted all efforts to remove it including elution with 1 N acid or base. The material is thought to be cytochrome C which was denatured during isolation.

Iron determinations were made by the method of THEORELL AND PEDERSEN[9] on 2 mg samples of oxidized cytochrome C which had been dialysed and dried to constant weight. Analysis shows the oxidized cytochrome C to contain 0.43 % iron, a

value identical with that reported by MARGOLIASH[10] for oxidized cytochrome C purified at pH 7.0, but somewhat lower than the 0.46 % iron reported when chromatographic separation was done at pH 9.6.

Effects of pH and ionic strength on elution volume

Elution volume has been defined as the total volume of liquid which flows from the column from time of application of solute until the maximum solute concentration emerges from the column minus the volume of solvent held between the resin particles[8]. These studies were done using 4 g of carboxyl cellulose (0.48 mequiv./g) in a 1.5 cm diameter column, whose height varied with pH and ionic strength. Sodium phosphate buffers of appropriate ionic strength and pH were used to elute 2 mg samples of cytochrome C from the columns. Fig. 4 shows the effect of ionic strength at pH 7.04 on the elution volume of cytochrome C. Elution volumes increase sharply

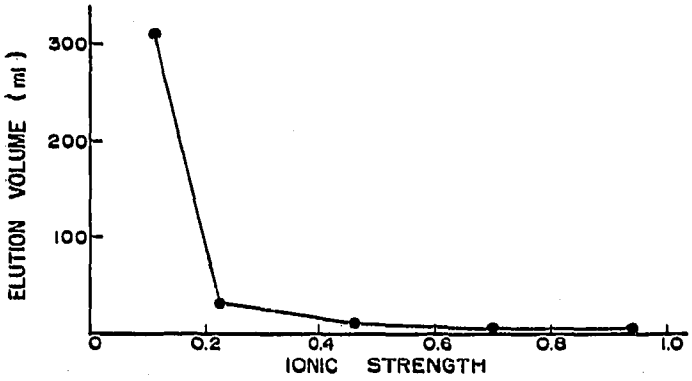


Fig. 4. Effect of ionic strength on the elution of cytochrome C at pH 7.04.

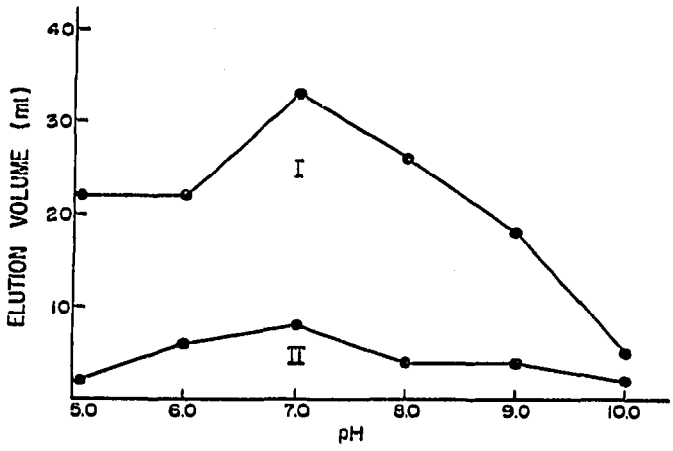
below an ionic strength of 0.2. Cytochrome C appears to remain stationary when the ionic strength is reduced below 0.02. The elution volume is maximal at pH 7.0 with ionic strength 0.22 (I) and 0.46 (II) (Fig. 5) and decreases both above and below this pH. The elution volume of cytochrome C increases below pH 7.0 with the carboxyl-containing resin IRC-50. The reduced elution volume of cytochrome C with carboxyl cellulose at lower pH values may reflect some lactone formation between the carboxyl and adjacent hydroxyl groups.

Adsorption capacity

The capacity of carboxyl cellulose was evaluated with two proteins, lysozyme (approx. mol. wt. 16,000) and cytochrome C (mol. wt. 12,100). At near zero ionic strength and room temperature with a pH of 5.4, 1 g of carboxyl cellulose (0.74 mequiv./g) adsorbs 270 mg of lysozyme and 280 mg of cytochrome C from 200 ml of a 0.1 % solution stirred continuously for 2 h. One gram of carboxyl cellulose (0.48 mequiv./g) adsorbs 183 mg of cytochrome C under similar conditions. It is difficult to compare the capacity of carboxymethyl cellulose with carboxyl cellulose since binding studies were not done with the same proteins. Taking into consideration the number of molecules of protein adsorbed per carboxyl, the capacities appear to be similar. It is considered probable that positions with adjacent carboxyl groups have no greater capacity than similar positions with a single carboxyl present.

Adsorption of lysine

Fig. 6 shows the effect of pH on the capacity of 1 g of carboxyl cellulose (0.48 mequiv./g) to adsorb lysine from 200 ml of a 0.0005 *M* solution. The solutions were adjusted to the specified pH using 6 *N* hydrochloric acid or sodium hydroxide.



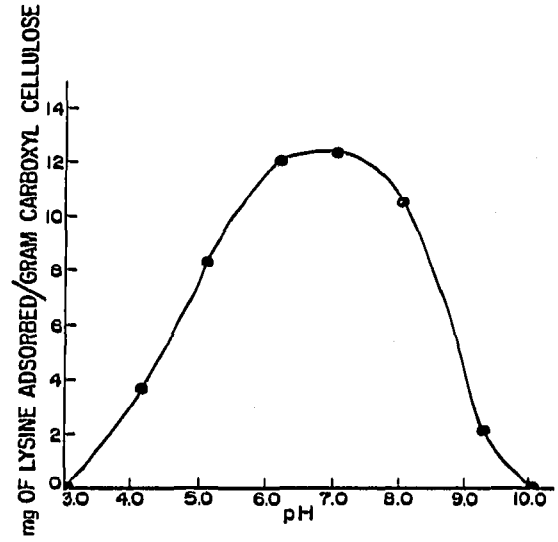


Fig. 5. Effect of pH on the elution of cytochrome C at ionic strength 0.22 (I) and 0.46 (II).

Fig. 6. Effect of pH on the adsorption of lysine by carboxyl cellulose.

The plot is for the average pH during the equilibration period and shows maximum adsorption at pH 7.0 with no binding when the carboxyl cellulose is not charged (pH 3.0) and no binding when the lysine is not charged (pH 10.0). Lysine was determined by the method of MOORE AND STEIN[11].

Peptides

An apparent difference in the ability of carboxyl cellulose and carboxymethyl cellulose to hold the tannin precipitable peptides responsible for formation of beer haze has been noted[12]. Carboxymethyl cellulose will adsorb all the peptides until it becomes overloaded, however, carboxyl cellulose allows 36 % of the tannin precipitables to come through. The phenomenon is not an effect of overloading and is thought to reveal a selectivity that reflects the difference in characteristics of the two materials as ion exchangers.

Other modifications

Periodate cleavage and chloride oxidation can be carried out on Whatman No. 1 filter paper to form ion exchange paper capable of binding cytochrome C and separation of its oxidized and reduced forms. A problem of keeping the paper intact and retaining dimensional stability is encountered due to the prolonged emersion of the paper in an aqueous medium. Placing the paper to be modified between two pieces of fiber glass screening gives sufficient support during the reaction and drying the paper on a photographic print dryer lends stability when the reaction is completed.

Sephadex, a high molecular weight dextran, may also be used as a base for

adding carboxyls. It does not react as readily under the conditions described as does cellulose and yields only 0.53 mequiv./g titrable carboxyl after 24 h exposure to periodate with subsequent chlorous acid oxidation. The resulting product is changed little in physical appearance by the reaction and it binds and permits chromatographic purification of cytochrome C by the system described for carboxyl cellulose.

Amino cellulose

Attempts to place amino groups on the dialdehyde cellulose have been made with limited success. About 10 % of the substitution predicted on the basis of aldehyde content is achieved when dialdehyde cellulose, in the presence of Raney nickel, is reacted with liquid ammonia and catalytically hydrogenated for 4 h under 750 lb. pressure. Further substitution may be possible if a method allowing the mixing of the dry reactants under high pressure were used.

DISCUSSION

Three common methods of placing carboxyl groups on the cellulose polymer are: (1) substitution of a carboxyl-containing prosthetic group via an ether linkage to one of the free hydroxyl groups on the glucose sub-unit[1]; (2) conversion of cellulose sub-units from glucose to glucuronic acid by nitrogen tetroxide oxidation[13]; (3) periodate cleavage and subsequent chlorite oxidation of the resulting carbonyl groups on carbon atoms 2 and 3 of the glucose sub-units. The latter method produces a carboxyl ion exchange material with many of the properties of those presently in use, plus some properties which are unique. Unlike the first two methods, the synthesis is simple and requires a minimum of attention. The ease of handling the product and its intermediate allows synthesis of larger batches in the laboratory. The amount of substitution is low and can be controlled within fairly narrow limits permitting the production of batches with similar degrees of substitution. The one expensive reagent, potassium metaperiodate, can be economically regenerated[14] when larger amounts of carboxyl cellulose are required. Considering reagent cost and personnel time in synthesis, it is an inexpensive ion exchange material.

The difference in retention of tannin precipitable peptides by carboxyl cellulose and carboxymethyl cellulose suggests the existence of subtle differences in their adsorption properties. While carboxyl ion exchangers may behave in a similar manner with some proteins, the supporting structure and spacial placement of the carboxyl groups on ion exchange adsorbants appear to effect their ability to bind proteins and peptides. The effect of having adjacent carboxyl groups in some positions and carboxyl and carbonyl groups adjacent in other positions is unclear; however, the adjacent carboxyl groups have been useful in the chromatography of nickel and iron[15]. It is probably fair to say that the presence of the unique groupings function to limit the bonding potential of the carboxyl group making it more selective.

SUMMARY

A carboxyl cellulose ion exchange adsorbent can be synthesized by controlled periodate cleavage of SW-40-B Solkafloc followed by chlorous acid oxidation. The material is capable of separating the components of a cytochrome C preparation in

much the same way as IRC-50. The properties of carboxyl cellulose and the synthesis of carboxyl cellulose paper and carboxyl Sephadex are discussed.

REFERENCES

1 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
2 J. D. GUTHRIE, *Ion Exchangers in Organic and Biochemistry*, Interscience, New York, N.Y., 1957, p. 554.
3 J. PORATH, *Arkiv Kemi*, 11 (1957) 97.
4 M. L. MALAPRADE, *Bull. Soc. Chim. France*, 43 (1928) 683.
5 A. JEANES AND H. S. ISBELL, *J. Res. Natl. Bur. Std. A.*, 27 (1941) 125.
6 G. F. DAVIDSON AND T. P. NEVELL, *J. Textile Inst., Trans.*, 46 (1955) 407.
7 E. MARGOLIASH, *Biochem. J.*, 56 (1954) 529.
8 N. K. BOARDMAN AND S. M. PARTRIDGE, *Biochem. J.*, 59 (1955) 543.
9 H. THEORELL AND K. O. PEDERSEN, *The Svedberg*, Almqvist and Wiksells Boktryckeri AB, Uppsala, 1944, p. 525.
10 E. MARGOLIASH, *Biochem. J.*, 56 (1964) 535.
11 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
12 H. REICH, personal communication.
13 E. C. YACKEL AND W. O. KENYON, *J. Am. Chem. Soc.*, 64 (1942) 121.
14 C. L. MEHLTRETTER, J. C. RANKIN AND P. R. WATSON, *Ind. Eng. Chem.*, 49 (1957) 350.
15 V. I. IVANOVA, N. YA. LENSHINA AND V. S. IVANOVA, *Izv. Akad. Nauk SSSR., Otd. Khim. Nauk*, (1957) 118; *C.A.*, 51 (1957) 7801d.