THE PURIFICATION OF HYALURONIC ACID BY THE USE OF CHARCOAL*

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Numerous methods have been reported[1,2] for the purification of hyaluronic acid. Since most of the methods are either laborious or harsh, or yield contaminated products, a simple and mild method which provided hyaluronate of reasonable purity seemed desirable.

A method for purifying polysaccharides was suggested on the basis of observations that charcoal strongly adsorbs proteins[3–6], peptides[7,8], nucleic acid[9,10], and nucleotides[11–13], while being a weak adsorbent for highly charged molecules[5] and large molecular weight neutral polysaccharides. The development of such a method using Darco G-60 as adsorbent is described.

EXPERIMENTAL

Preparation of charcoal columns and pads

Equal weights of Darco G-60** and cellulose powder*** were mixed with water to make a smooth slurry for preparation of suitable columns. When larger volumes of extract were to be purified, pads prepared on Buchner funnels, 10–15 cm or more in diameter, were found more desirable. After covering the bottom of the funnel with a 0.5 cm layer of powdered cellulose, the Darco-cellulose slurry is added. A coarse filter paper is placed over the carbon-cellulose pad which is then washed with several bed volumes of water prior to use.

Isolation of hyaluronate from streptococcal extracts

A strain of Group A hemolytic *Streptococcus* (A111) was grown for 12–16 hours[14] in veal infusion broth with the pH being maintained at 7.0–7.2 by means of an automatic titrator. After centrifugation at 3000 r.p.m. for ½ hour, addition of two-tenths per cent *n*-octyl alcohol, the culture supernatant was concentrated to one-tenth volume in a flash concentrator or in a shallow pan set before a fan. One and one half volumes of 95% ethyl alcohol were then added with stirring to the concentrated culture fluid and the precipitate allowed to settle for ½ hour. After decanting most of the supernatant, which contains most of the pigments and much contaminating material, the residue was centrifuged for 5 minutes at 500 r.p.m., washed twice with equal volumes of 65% ethanol, and dissolved in a volume of water equivalent to one-fourth of that of the original media. Upon addition of one-tenth volume of

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** Obtained from Matheson, Coleman, and Bell, Inc., East Rutherford, New Jersey.

*** Whatman ashless powder, coarse grade, W. & R. Balston, Ltd., England.

References p. 400.

ethanol, the solution was passed over a Darco-cellulose pad prepared in a Buchner funnel. A 12.5 × 1 cm pad generally sufficed for the purification of material derived from 3–5 liters of culture. The purification was followed by testing the effluent at 280 mμ. An increase in absorbancy above a negligible value indicated approaching saturation of the carbon pad. The emergence of hyaluronate from the charcoal pad does not coincide with the liquid front, but requires the passage of 2 or 3 bed volumes of solution.

The presence of hyaluronate in the effluent was noted by a turbidity reaction upon the addition of a few drops of effluent to 10 volumes of acidified albumin reagent[15]. The hyaluronate solution was washed through the pad with a bed volume of water.

It was found that the hyaluronate effluents as obtained from the carbon columns contain various cations. These generally cause the effluent solution to develop a turbidity during concentration. Isolation of a precipitate by centrifugation shows the presence principally of magnesium, presumably arising from the Darco[16].

The effluent was dialyzed against tap water and concentrated, and if not cleared by the addition of a few drops of dilute acetic acid, was passed through a Seitz filter. After precipitation with four volumes of ethanol, the hyaluronic acid was washed with ethanol and ether and dried *in vacuo*. The yield was 300–400 mg per liter of culture medium. Preparations in the acid form were obtained either by precipitation with 3 volumes of acetic acid or by deionization with mixed bed resin, MB-2* and precipitation with ethanol.

A small amount of hyaluronate could be eluted from the carbon pad by the use of 40% ethanol containing 3% *n*-octyl alcohol, but this procedure was not routinely adopted because carbon generally emerged with the hyaluronate as a result of this treatment.

From umbilical cord extracts

One hundred grams of acetone-dried and ground human umbilical cords were mixed with 2 l of 0.15 *M* saline and 1–2 ml of toluene and shaken overnight. After centrifugation at 1000 r.p.m. for 10 minutes, the residue was washed with 500 ml of water and the wash liquids added to the extract. The residue was again extracted with 0.15 *M* saline. After adding one-tenth volume of ethanol the pooled extracts were passed over a Darco-cellulose pad (18.5 cm diameter) composed of 40 g of each constituent. With the umbilical cord extracts no advantage was found in precipitating the hyaluronate prior to carbon passage.

The effluent was dialyzed, clarified by means of Seitz filtration, and precipitated with 4 volumes of ethanol after concentration to small volume. The yield was 3.5–4%.

From vitreous humor

Vitreous humor was passed over a pad without previous treatment except for centrifugation. The isolation procedure followed that described for the other preparations. Yields are not recorded because experience in this type of preparation has been limited.

Purification with Dowex resin

Occasional preparations were not completely freed of materials absorbing in the ultraviolet. These were further purified by treatment with Dowex-1.

* A product of Rohm and Haas Co., Philadelphia, Pa.

References p. 400.

In a typical example 500 ml of streptococcal extract that had been passed over a Darco-cellulose pad and which retained material absorbing at 280 mμ was passed over a 22 × 40 mm column of Dowex-1, chloride (200–400 mesh, 2% cross linked). From the effluent was obtained 110 mg of material with 4.5% nitrogen and 17.5% uronic acid. Elution of the resin with 2 *M* saline yielded 800 mg of hyaluronate having 3.16% *N* and 39.7% uronic acid.

Viscosity measurements

These were done in an Ostwald viscometer at 25°. Umbilical cord hyaluronate was made up in 0.15 *M* saline while streptococcal and vitreous humor preparations were made up in 0.10 *M* phosphate buffer of pH 7.1. The hyaluronate concentration used for the viscosity determinations was 0.1%.

It is of some interest that occasionally an increase in viscosity of umbilical cord hyaluronate was noted after charcoal passage, the relative viscosity rising from 4 to 6 for equivalent concentrations. On other occasions the relative viscosities, both before and after carbon passage of umbilical cord extracts, remained the same on the basis of equal concentration of mucopolysaccharide. A similar result was noted for streptococcal preparations, the relative viscosity remaining unchanged both before and after carbon passage of the extract.

Chemical analyses

Uronic acid, hexosamine and nitrogen determinations were done as described earlier[14]. Ash was determined by combustion in a stream of oxygen at 500°.

RESULTS AND DISCUSSION

Utilization of carbon

The use of carbon was found to be simple and dependable, though certain precautions in its use for hyaluronate purification should be observed. Since carbon is a powerful adsorbent for numerous substances, while a weak adsorbent for highly charged ionic species[5] and neutral polysaccharides, it is not surprising that it has been found to be a practical agent for the purification of hyaluronate. Preliminary studies suggest its applicability for the purification of alkali-extracted chondroitinsulfuric acid as well as for glycogen. The charcoal method has also been found useful because of its rapidity and simplicity for the small scale purification of multiple samples of hyaluronate obtained from streptococcal cell extracts.

A certain, generally small, amount of hyaluronate is adsorbed onto carbon, the degree of adsorption increasing with the amount of carbon in excess of that required to adsorb the impurities present in the hyaluronate extracts. The use of a minimum amount of carbon in the purification procedures is, therefore, a consideration for maximum recovery of hyaluronate. The addition of 10–20% alcohol to the extracts tends to minimize adsorption of hyaluronate by carbon, and under proper conditions it was found that retention on the Darco pad of streptococcal hyaluronic acid is not more than 5–6%.

Darco G-60 appears to be more suitable in these methods than acid-treated carbons since the latter adsorb larger amounts of hyaluronate and are less efficient in separating hyaluronate and protein.

The results in Table I indicate the carbon method to be a mild one with little apparent degradative effect on hyaluronate. Similarly, the use of Dowex-1, chloride, for purification of hyaluronate appears to affect the viscosity only slightly. However, deionization of streptococcal hyaluronate with the mixed bed resin, MB-2, gave a product with a greatly lowered viscosity. Precipitation of streptococcal hyaluronate

References p. 400.

with glacial acetic acid yielded a product having a relative viscosity of 2.8, indicating that conversion to the acid form does not greatly affect the viscosity[17].

TABLE I

ANALYTICAL DATA FOR TYPICAL HYALURONATES PREPARED BY THE CHARCOAL METHOD

Hyaluronate source	*Nitrogen**	*Uronic acid*	*Hexosamine*	*Relative viscosity***	*Ash*
Group A *Streptococcus*	1.00	1.02	0.92	3.2	13.7
Group A *Streptococcus*, purified by Dowex-1, chloride	1.00	1.00	0.90	2.9	12.1
Streptococcus, deionized with MB-2 resin	1.00	1.00	0.94	1.2	1
Bovine vitreous humor	1.00	1.08	0.91	1.7	—
Umbilical cord, human	1.00	0.96	0.85	5.0	14.9
Umbilical cord, human	1.00	1.05	0.95	3.7	15.3

* Ratios are based on nitrogen values as 1.00.
** Not corrected for ash or moisture content.

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SUMMARY

A new method for the purification of hyaluronate requiring simply the passage of suitable extracts over a Darco-cellulose pad is described. Hyaluronate of reasonable purity has been obtained by this method from umbilical cords, streptococcal extracts and vitreous humor. The rapidity of the method renders it useful for the simultaneous purification of multiple hyaluronate samples, while the mildness of the method provides hyaluronate of minimum degradation.

REFERENCES

1 S. TOLKSDORF, in D. GLICK, *Methods of Biochemical Analysis*, Vol. I, Interscience Publishers, Inc., New York, 1954.
2 K. MEYER AND M. M. RAPPORT, *Advances in Enzymology*, 13 (1952) 199.
3 J. A. CIFONELLI, *Ph.D. Thesis*, University of Minnesota, 1952.
4 R. K. MORTON, *Biochem. J.*, 55 (1953) 795.
5 J. PORATH AND C. H. LI, *Biochim. Biophys. Acta*, 13 (1954) 268.
6 J. A. CIFONELLI AND F. SMITH, *J. Am. Chem. Soc.*, 77 (1955) 5682.
7 A. TISELIUS, *Advances in Protein Chem.*, 3 (1947) 81.
8 J. PORATH, *Arkiv Kemi*, 7 (1954) 535.
9 S. ZAMENHOF AND E. CHARGAFF, *Nature*, 168 (1951) 604.
10 S. ZAMENHOF, G. LEIDY, P. L. FITZGERALD, H. E. ALEXANDER AND E. CHARGAFF, *J. Biol. Chem.*, 203 (1953) 695.
11 S. F. VELICK, J. E. HAYES, JR., AND J. HARTING, *J. Biol. Chem.*, 203 (1953) 527.
12 R. K. CRANE AND F. LIPMANN, *J. Biol. Chem.*, 201 (1953) 235.
13 G. R. BARTLETT, *Federation Proc.*, 13 (1954) 179.
14 S. ROSEMAN, F. E. MOSES, J. LUDOWIEG AND A. DORFMAN, *J. Biol. Chem.*, 203 (1953) 213.
15 A. DORFMAN AND M. L. OTT, *J. Biol. Chem.*, 172 (1948) 367.
16 H. L. ROSENTHAL, *Arch. Biochem. Biophys.*, 54 (1955) 523.
17 C. E. JENSEN, *Acta Chem. Scand.*, 7 (1953) 603.

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