# *Observations on the Properties of Cetyltrimethylammonium Salts of Some Acidic Polysaccharides.*

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The solubilities of the cetyltrimethylammonium salts of some mono- and poly-uronic acids and sulphated mono- and poly-saccharides are examined and compared with those of other amine (*e.g.*, 4-amino-4′-chlorodiphenyl) salts. An improved method has been developed, utilising the cetyltrimethylammonium salt as an intermediate, for the isolation, from bovine trachea, of inorganic salts of chondroitin hydrogen sulphate (suitable for the preparation of D-chondrosamine hydrochloride). The interconversion of salts of acidic polysaccharides may be achieved conveniently *via* the cetyltrimethylammonium salt. A method, based on cetyltrimethylammonium bromide (Cetavlon), for the detection of a wide range of acidic polysaccharides in paper chromatography and paper electrophoresis is described.

CETAVLON (cetyltrimethylammonium bromide) has been introduced recently as a precipitant for acidic (non-sulphated) polysaccharides (Jones, *Biochim. Biophys. Acta*, 1953, **10**, 607) and for sulphated polysaccharides (Stacey and Barker, in " Biochemistry of Nitrogen," Acad. Sci. Fennica, 1955, p. 262; Scott, *Chem. and Ind.*, 1955, 168) since it yields water-insoluble quaternary salts with these polysaccharides; neutral polysaccharides are not precipitated by this reagent (cf. Stacey and Barker, *loc. cit.*). We now report some observations on the properties of cetyltrimethylammonium salts of certain acidic polysaccharides.

The efficiency of Cetavlon as a precipitant for sulphated polysaccharides does not appear to depend critically on the molecular size of the carbohydrate since some simple sugar sulphates, *e.g.*, 1 : 2-5 : 6-di-*O*-*iso*propylidene-D-glucofuranose 3-sulphate, are precipitated. The general properties (cf. Jones, *loc. cit.*; Stacey and Baker, *loc. cit.*) of the cetyltrimethylammonium salts of mono- and poly-saccharide sulphates appear to be essentially similar. For acidic (non-sulphated) polysaccharides molecular weight is important since simple uronic acid derivatives, *e.g.*, sodium D-glucuronate, do not yield water-insoluble cetyltrimethylammonium salts.

A number of amines have been reported as precipitants for highly sulphated polysaccharides, especially for heparin, *e.g.*, benzidine (Charles and Scott, *Biochem. J.*, 1936, **30**, 1927), piperidine, *n*-pentylamine, *iso*pentylamine (Scott, Charles, and Fischer, *Trans. Roy. Soc. Canada*, 1942, V, **36**, 49), and decamethylenediamine (Lee and Berger, U.S.P. 2,561,384; *Chem. Abs.*, 1951, **45**, 10,515), but the applicability of these reagents as general precipitants for acidic polysaccharides appears not to have been studied. It is of interest that whilst the cetyltrimethylammonium salts of a range of acidic polysaccharides (Jones, *loc. cit.*; Stacey and Barker, *loc. cit.*; Scott, *loc. cit.*) have been found to be water-insoluble the corresponding salts derived from 4-amino-4′-chlorodiphenyl were found to be water-insoluble only in the case of highly sulphated polysaccharides (*e.g.*, heparin and dextran sulphates with 5—6 sulphate ester groups per tetrasaccharide unit). Polysaccharides which had less than 3 sulphate ester groups per tetrasaccharide unit [*e.g.*, de-*N*-sulphated heparin (Foster, Martlew, and Stacey, *Chem. and Ind.*, 1953, 825, 899), sodium chondroitin sulphate, and some polyuronic acids] did not give water-insoluble salts with 4-amino-4′-chlorodiphenyl (Foster and Martlew, unpublished data). It is possible that the combined use of the reagents Cetavlon and 4-amino-4′-chlorodiphenyl might be advantageous in certain cases. A further significant difference between these reagents is that the former yields a water-soluble sulphate whilst the latter may be used to precipitate sulphate ion quantitatively (Belcher, Nutten, and Stephen, *J.*, 1953, 1334). It has been pointed out (Stacey and Barker, *loc. cit.*) that separation into fractions of low and high sulphate content of a mixture of sulphated polysaccharides may be achieved by the use of sub-optimal amounts of Cetavlon.

Although the minimum number of acid functions per polysaccharide molecule necessary to give a water-insoluble cetyltrimethylammonium salt is not known precisely, it would appear to be small.

In order to assess the value of Cetavlon as a reagent for facilitating the isolation of salts of chondroitin hydrogen sulphate from bovine trachea a series of experiments were made in which fresh and " dried " trachea (see Experimental section) were subjected to various extraction procedures. The results are listed in Table 1. The general method employed

TABLE 1. *Analytical and other data on the various preparations of salts of chondroitin hydrogen sulphate.*

| Sample | Extractant | Yield (g.) | Moisture (solvent) (%) | N (%) [a] | S (%) [a] | *N*-Acetyl (%) [a] | $[\alpha]_D^{17-21}$ in $H_2O$ | Salt |
|---|---|---|---|---|---|---|---|---|
| | | | *From fresh trachea* | | | | | |
| I | NaOH | 3·05 [c] | 17·73 | — | 6·3 | 8·0 | −18·6° | Na |
| II [b] | NaOH | 3·20 [c] | 20·87 | 2·15 | 6·1 | 8·4 | −21·0 | Na |
| | | | *From dried trachea* | | | | | |
| III | NaOH | 3·45 | 18·07 | 2·1 | 5·6 | 7·45 | −17·4 | Na |
| IV | NaOH | 3·00 | 19·11 | 2·1 | 5·5 | 7·8 | −15·6 | Na |
| V | $CaCl_2$ | 0·45 | 17·39 | 3·4 | 4·2 | 7·0 | −17·6 | Na |
| VI | $CaCl_2$ | 1·28 | 17·37 | 2·9 | 5·0 | 7·0 | −21·8 | Na |
| VII | NaCNS | 0·70 | 17·42 | 3·9 | 4·3 | 6·1 | −29·4 | Na |
| VIII | NaCNS | 0·35 | 15·49 | 3·6 | 4·3 | 5·95 | −25·5 | Na |
| IX | $Ba(OH)_2$ | 2·10 | 17·69 | 2·0 | 5·45 | 7·8 | −21·4 | Ba |
| X | NaOH | — | 18·81 | 2·1 | 5·7 | 7·4 | −16·3 | Ba |

Col. 5—9 : data determined on moisture (solvent)-free salts. [a] Calc. for sodium chondroitin sulphate ($C_{14}H_{19}O_{14}NSNa_2$) : N, 2·8; S, 6·4; *N*-Ac, 8·55; for barium chondroitin sulphate ($C_{14}H_{19}O_{14}NSBa$) : N, 2·4; S, 5·4; *N*-Ac, 7·24%. [b] Sodium salt (II) was subjected to deproteinization. [c] Yields are calculated and approximate.

was to precipitate cetyltrimethylammonium chondroitin sulphate under suitable conditions after the extraction procedure, and then to convert the quaternary salt into other required salts.

In the case of " dried " trachea hot dilute aqueous sodium hydroxide (Bray, Gregory, and Stacey, *Biochem. J.*, 1944, **38**, 142) proved to be the most efficient extractant in that it afforded the highest yield of sodium chondroitin sulphate (Samples III and IV). The analytical data indicated that the salts (III) and (IV) contained some protein. Extraction of fresh trachea under similar conditions gave almost the same yield of sodium chondroitin sulphate (I) which appeared to have lower protein content than the salts (III) and (IV). Application of a standard deproteinisation procedure to the salt (I) reduced easily and substantially the traces of protein, to give the salt (II). No special precautions were taken to exclude oxygen in the hot alkaline extractions and it is not unlikely that the sodium salts (I)—(IV) had suffered some degradation (cf. Blix and Snellman, *Arkiv Kemi Mineralog. Geol.*, 1945, **19**, No. 32, 1; Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541). Such degradation is relatively unimportant if the mucopolysaccharide is to be further degraded, for example, to chondrosine (Levene and La Forge, *J. Biol. Chem.*, 1913, **15**, 69, 155; Levene, *ibid.*, 1941, **140**, 267) or D-chondrosamine hydrochloride (cf. *inter al.*, Levene and La Forge, *loc. cit.*; Stacey, *J.*, 1944, 272; Gardell, *Acta Chem. Scand.*, 1951, **5**, 195).

The use of Cetavlon in the above extraction procedure constitutes an improvement in the method of isolation, from bovine trachea, of salts of chondroitin hydrogen sulphate suitable for studies of degradation and for the preparation of D-chondrosamine hydrochloride. On the basis of the detailed study a simplified extraction procedure has been developed.

The sulphur percentages in Table 1 were obtained by the application of a modified method of ester-sulphate determination developed in these laboratories by Belcher and Fildes (unpublished work). Hydrolysis of the sulphate groups was effected under oxidising conditions (nitric acid) and thereafter the released sulphate ions were determined by a standard procedure. The method was found to be convenient and of particular value for the determination of sulphur in sulphated carbohydrates.

There has been no report of the nature and composition of the quaternary salts of acidic polysaccharides. The isolation of cetyltrimethylammonium and cetylpyridinium chrondroitin sulphates gave no difficulty and the quaternary salts were found to have low (<0·5%) ash contents. This observation, which indicates the almost complete replacement of other cations by the cetyltrimethylammonium or cetylpyridinium ion, supplements that of Scott (*loc. cit.*), who found that the titration of sulphate groups, in dextran sulphate, with Cetavlon corresponded to stoicheiometric salt formation. Scott (*loc. cit.*) reported a flocculation end point in these titrations, which we have noted also with salts of chondroitin sulphate. The low ash content of the quaternary salts also indicates that the interconversion of salts of an acidic polysaccharide (as, for example, in the conversion of sodium into barium chondroitin sulphate before preparation of D-chondrosamine hydrochloride) may be achieved conveniently *via* the cetyltrimethylammonium salt. Ammonium chondroitin sulphate prepared from the sodium salt in this manner had a very low (0·06%) ash content. By a similar procedure heparin (sodium salt) was converted *via* the benzidine salt into an ammonium salt with 0·7% ash content (Charles and Scott, *loc. cit.*; cf. Wolfrom, Weisblat, Karabinos, McNeely, and McLean, *J. Amer. Chem. Soc.*, 1943, **65**, 2077).

The yields of sodium chondroitin sulphate (V and VI) obtained on extraction of dried trachea with aqueous calcium chloride (cf. Meyer and Smyth, *J. Biol. Chem.*, 1937, **119**, 507) were much lower than in extraction with hot alkali, and the salts contained significant amounts of protein. It appears that appreciable contamination with protein did not increase the solubility of the cetyltrimethylammonium salt in water. Salts (VII and VIII) of essentially similar composition were obtained when aqueous sodium thiocyanate (cf. Snellman, Jensen, and Sylvén, *Nature*, 1948, **161**, 639) was used as extractant. The use of hot aqueous baryta as an extractant gave barium chondroitin sulphate (IX) with essentially the same elemental composition as, but in lower yield than, the corresponding salt (X) obtained after sodium hydroxide extraction.

Few reagents have been reported for the detection of polysaccharides in paper chromatography and ionophoresis. Aniline oxalate has been used to detect certain neutral polysaccharides (Preece and Hobkirk, *Chem. and Ind.*, 1955, 257) but a satisfactory general method for the detection of acidic polysaccharides has not been described. Sulphated polysaccharides may be located after paper electrophoresis by means of Toluidine-blue (Rienits, *Biochem. J.*, 1953, **53**, 79) and the same reagent and also Azure I have been used similarly in paper chromatography (Kerby, *Proc. Soc. Exp. Biol. Med.*, 1953, **83**, 263; Ricketts, Walton, and Saddington, *Biochem. J.*, 1954, **58**, 532; cf. Ball and Jackson, *Stain Technol.*, 1953, **28**, 33). The detection of acidic (non-sulphated) polysaccharides with Toluidine-blue is unsatisfactory. We have found that Cetavlon may be used to detect satisfactorily a wide range of acidic polysaccharides in paper chromatography and in ionophoresis in acetate (pH 5·0) and borate (pH 10·0; cf. Foster, *J.*, 1953, 982) buffer systems. After chromatography or ionophoresis the acidic polysaccharides on the paper were converted into the cetyltrimethylammonium salts by treatment with Cetavlon. After removal of excess of the detergent the insoluble cetyltrimethylammonium polysaccharide salts were detected with Bromocresol-purple. In this manner sodium alginate, luteic acid, *Azotobacter chroococcum* polysaccharide, gum arabic, gum tragacanth, hyaluronic acid, sodium chondroitin sulphate, heparin (sodium salt), and barium 1 : 2-5 : 6-di-*O*-*iso*-propylidene-D-glucofuranose 3-sulphate were found to be located readily.

The observation that cetyltrimethylammonium chondroitin sulphate was soluble in alcohols up to *n*-pentyl alcohol prompted an examination of the behaviour of the quaternary salt in paper chromatography. It was found that with the organic phase of acidic (butanol–water–acetic acid, 4 : 5 : 1), neutral (butanol–ethanol–water, 4 : 1 : 5) and basic (butanol–ethanol–water–ammonia, 40 : 10 : 49 : 1) solvent systems, dissociation of the quaternary salt occurred. The polysaccharide was retained near the origin and the cetyltrimethyl ammonium moiety ran just behind the solvent front.

## Experimental

*Preparation of "Dried" Trachea.*—Fresh bovine trachea (850 g.) were freed as completely as possible from adhering fat, flesh, and extraneous matter, minced, and defatted by exhaustive

extraction with acetone at room temperature. The material (200 g.) obtained after air-drying is subsequently referred to as " dried " trachea (moisture content, 15·94%).

*Extraction of " Dried " Trachea.*—(a) *With* N-*sodium hydroxide* (Bray, Gregory, and Stacey, *loc. cit.*). Dried trachea (30 g.) was extracted at 70° with N-sodium hydroxide (1 l.) for 30 min. The cooled, turbid solution was neutralised with acetic acid and dialysed against running tap-water for 2 days, and insoluble material then removed by centrifugation. Aqueous cetyltrimethylammonium bromide (Cetavlon; 2—20% w/v) was added to the centrifugate until precipitation of the quaternary salt was complete (flocculation end point). The precipitate was collected by centrifugation, washed twice with distilled water, and then dissolved in the minimum volume of aqueous sodium chloride (10% w/v). Sodium chondroitin sulphate was precipitated by the addition of ethanol (1—2 vols.). The polysaccharide was collected by centrifugation and dissolved in water, and the solution dialysed against running tap-water for 2 days; the dialysate was then passed down a column (25·5 × 2·5 cm.) of Zeocarb cation exchanger (sodium-salt form), and the sodium salt (III) isolated from the eluate by freeze-drying.

In a second extraction the above procedure was repeated with the omission of the first dialysis stage to give the sodium salt (IV).

(b) *With* 10% *calcium chloride* (Meyer and Smyth, *loc. cit.*). Dried trachea (30 g.) was extracted with aqueous calcium chloride (300 ml.; 10% w/v) by shaking at room temperature for 1 day. Two consecutive extracts were combined and after dialysis against running water for 2 days they were worked up as in (a), to give the sodium salt (V).

The residue remaining from the above extractions was further extracted with aqueous calcium chloride during 10 days and then worked up as above to give the sodium salt (VI).

(c) *With* 10% *sodium thiocyanate.* Dried trachea (30 g.) was extracted with aqueous sodium thiocyanate (300 ml.; 10% w/v) and the extracts were worked up as for the calcium chloride extraction, to give the sodium salts (VII) and (VIII) from two combined 24-hr. extractions and from a 10-day extraction respectively.

(d) *With* N-*barium hydroxide.* Dried trachea (30 g.) was extracted with N-barium hydroxide (500 ml.) at 70° for 30 min. The cooled solution was neutralised by the addition of solid carbon dioxide and clarified by centrifugation. The clear centrifugate was treated with aqueous Cetavlon (2—20% w/v), and the precipitated quaternary salt was collected and washed as in (a). Thereafter the quaternary salt was dissolved in aqueous barium chloride (10% w/v; 70 ml.), and barium chondroitin sulphate precipitated by the addition of alcohol (1—2 vols.). The barium salt was dissolved in water, converted into the insoluble quaternary salt, and reconverted into the barium salt as described above. An aqueous solution of the product was dialysed against running water for 2 days and the barium salt (IX) isolated from the dialysate by freeze-drying.

A sample of sodium chondroitin sulphate prepared from fresh trachea (see below) was converted into the cetyltrimethylammonium salt and thence into the barium salt (X) as described above.

*Extraction of Fresh Trachea with* N-*Sodium Hydroxide.*—Cleaned and minced bovine trachea (540 g.; equivalent to *ca.* 130 g. of " dried " trachea) was extracted as in (a). Thereafter the solution was neutralised with acetic acid and then treated with barium carbonate (100 g./l.) at 100° for 6 hr. Insoluble material was removed by centrifugation and washed with water, and the combined centrifugates were concentrated (1600 ml.). Aqueous Cetavlon (2—20% w/v) was added until precipitation of the quaternary salt was complete, the precipitate collected (centrifuge), and dissolved in the minimum volume of aqueous sodium chloride (10% w/v), and sodium chondroitin sulphate precipitated by the addition of alcohol (1—2 vols.). The precipitate (33 g.) was collected, washed with alcohol and then ether, and dried. Attempts to remove or decrease the protein content of this salt, (1) by passage of its aqueous solution through a Celite column (*ca.* 15 × 2 cm.) and (2) by repeated conversion into the cetyltrimethylammonium salt, thorough washing, and reconversion into the sodium salt, were unsuccessful. An aqueous solution of a portion (5·0 g.) of the product was dialysed against running water for 2 days and the sodium salt (I) treated and isolated as in (a). A similar amount was substantially freed from traces of protein by shaking seven times with chloroform–pentyl alcohol (Sevag, *Biochem. Z.*, 1934, **273**, 419), and the sodium salt (II) was isolated as described above.

The sodium salts (I) and (II) were found to be suitable for the preparation of D-chondrosamine hydrochloride after their conversion into the barium salts by the general method described below.

The yields and analytical data for the salts (I)—(X) are recorded in Table 1. *N*-Acetyl

determinations were carried out by using toluene-*p*-sulphonic acid and the digestion conditions described by Wolfrom *et al.* (*loc. cit.*), and the method for acetic acid determination described by Belcher and Godbert (" Semi-micro Quantitative Organic Analysis," Longmans, Green and Co., 1954, p. 161). Sulphur was determined according to the method of Belcher and Fildes (*loc. cit.*): A sample of the sulphated polysaccharide (30—50 mg.) was hydrolysed by boiling under reflux with 8N-nitric acid (5 ml.) for 1 hr. Thereafter the nitric acid was evaporated and the last traces removed by co-distillation with hydrochloric acid. The residue was dissolved in 0·05N-hydrochloric acid (50 ml.) and barium sulphate was precipitated, collected, and determined by the method of Belcher, Gibbons, and West (*Chem. and Ind.*, 1954, 127, 850).

*Simplified Procedure for the Extraction of Salts of Chondroitin Hydrogen Sulphate from Bovine Trachea.*—Fresh, cleaned and minced trachea was extracted with N-sodium hydroxide at 70° for 30 min. Thereafter the solution was neutralised with acetic acid and clarified by centrifugation. Aqueous Cetavlon (2—20% w/v) was added to the centrifugate until precipitation of the quaternary salt was complete. The precipitate was collected and washed twice with water (centrifuge). Conversion of the quaternary salt into other salts was effected by its dissolution in an aqueous solution of the appropriate chloride (*e.g.*, Na, Ba, Ca, $NH_4$) and precipitation by the addition of alcohol (1—2 vols.). The precipitate was then washed with aqueous ethanol (80%) until free from chloride ion. Barium chondroitin sulphate prepared in this manner contained a trace of proteinaceous material but was suitable for the preparation of D-chondrosamine hydrochloride.

*Isolation of the Cetyltrimethylammonium and Cetylpyridinium Salts of Chondroitin Hydrogen Sulphate.*—A solution of sodium chondroitin sulphate (5 g.) in water (100 ml.) was treated with aqueous Cetavlon (20% w/v) until precipitation of the cetyltrimethylammonium chondroitin sulphate was complete. Under these conditions the quaternary salt was precipitated as a flocculent solid but on addition of excess of Cetavlon it became syrupy. The precipitate was collected by centrifugation. A portion was washed twice with water, dissolved in ethanol, and precipitated as a wax-like solid (A) by the addition of ether. A second portion was washed thrice with a little Cetavlon solution and then treated as above. A wax-like solid (B) was again obtained. If, however, the initial precipitate was washed with acetone and then with ether, the quaternary salt was obtained as a fine white powder (C). Cetylpyridinium chondroitin sulphate (D) was also isolated in this manner as a fine white powder. Some analytical data on A—D are included in Table 2. The quaternary salts A—D were soluble in alcohols up to

TABLE 2.

| | A | B | C | D |
|---|---|---|---|---|
| Moisture (solvent) content (%) ..... | 16·35 | 13·51 | 7·2 | 9·1 |
| Ash (calc. on dry solid) (%) ..... | 0·41 | 0·39 | 0·42 | 0·41 |

*n*-pentanol but were insoluble in other common organic solvents, aqueous ammonia, and acetic acid.

*Detection of Acidic Polysaccharides after Chromatography and Electrophoresis on Paper.*—(a) *On the paper.* Chromatograms (Whatman No. 1 paper) were examined which had been irrigated with a propan-1-ol–water (1 : 1) solvent system. Ionophoretograms (Whatman No. 3 paper) were examined which had been run in acetate buffer (pH 5·00; 19·04 g. of sodium acetate trihydrate and 3·6 g. of acetic acid per l.) and borate buffer [pH 10·0; 19·77 g. of boric acid and 16·0 g. of sodium hydroxide per 4 l. (cf. Foster, *loc. cit.*)] systems. The method of detection was the same in each case. The dried (100—110°) paper was dipped in aqueous Cetavlon (1% w/v), excess of reagent removed by immediate and thorough washing with hot, running tap water for 5—10 min. (the minimum time depends on the thickness of the paper), and the paper dried at 100°. Thereafter the paper was sprayed with ethanolic bromocresol-purple (0·04% w/v) to which dilute aqueous sodium hydroxide had been added until the solution began to assume a reddish tinge. The locations of the polysaccharides were indicated by blue spots (which did not fade appreciably on storage of the paper) on a pale yellow background. This procedure was used for the substances listed on p. 3790.

Incomplete washing of the paper led to a final overall blue colour on spraying with bromocresol-purple, and the polysaccharide locations were then not easily discernible. In this case the paper was dipped in N-acetic acid, washed thoroughly, and dried. On heating to 100° green spots appeared on a pale yellow background. The spots tended to fade but could be regenerated by heat. The acetic acid treatment may be repeated if necessary.

(b) *After elution.* In paper chromatography of sodium chondroitin sulphate an alternative

method of detection was as follows: the chromatogram was dissected into strips (the width dependent on the distance migrated) at right angles to the direction of movement of the acidic polysaccharide, and the strips were separately eluted with boiling water (cf. Rienits, *loc. cit.*). The extracts were treated with aqueous Cetavlon (2% w/v), and the presence of acidic polysaccharide was indicated by the development of turbidity or the formation of a precipitate.

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