[7] C. CHAGAS, E. PENNA-FRANCA, K. NISHIE AND E. J. GARCIA, *Arch. Biochem. Biophys.*, 75 (1958) 251.
[8] S. EHRENPREIS AND M. M. FISHMAN, *Abstracts 135th Meeting Am. Chem. Soc., Boston, April 1959*, p. 7D.
[9] S. EHRENPREIS AND M. M. FISHMAN, *Biochim. Biophys. Acta*, 44 (1960) 577.
[10] S. EHRENPREIS, in preparation.
[11] C. CHAGAS, *Ann. N.Y. Acad. Sci.*, 81 (1959) 345.
[12] M. A. ROTHENBERG AND D. NACHMANSOHN, *J. Biol. Chem.*, 168 (1947) 223.
[13] J. D. DUTCHER, *J. Am. Chem. Soc.*, 68 (1946) 419.
[14] J. L. CRAMMER AND A. NEUBERGER, *Biochem. J.*, 37 (1943) 302.
[15] S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
[16] I. M. KLOTZ, F. M. WALKER AND R. B. PIVAN, *J. Am. Chem. Soc.*, 68 (1946) 1486.
[17] V. J. HARDING AND R. M. MACLEAN, *J. Biol. Chem.*, 24 (1916) 503.
[18] S. EHRENPREIS, *Science*, 129 (1959) 1613.
[19] S. EHRENPREIS, *Federation Proc.*, 18 (1959) 220.
[20] P. WASER, in D. BOVET, F. BOVET-NITTI AND G. B. MARINI-BETTOLO, *Curare and Curare-like Agents*, Elsevier Publishing Co., 1959, p. 219.
[21] O. WINTERSTEINER AND J. D. DUTCHER, *Science*, 97 (1943) 467.
[22] P. ROSENBERG AND H. HIGMAN, *Biochim. Biophys. Acta*, to be submitted.
[23] D. BOVET, in D. BOVET, F. BOVET-NITTI AND G. B. MARINI-BETTOLO, *Curare and Curare-like Agents*, Elsevier Publishing Co., 1959, p. 219.
[24] F. KARUSH, *J. Am. Chem. Soc.*, 72 (1950) 2705.
[25] F. KARUSH, *J. Am. Chem. Soc.*, 76 (1954) 5536.
[26] H. FUHNER, *Arch. exptl. Path. Pharmakol. Naunyn-Schmiedeberg's*, 58 (1908) 1.
[27] T. P. FENG, *Chinese J. Physiol.*, 13 (1938) 119.

Biochim. Biophys. Acta, 44 (1960) 561–577

THE INTERACTION OF QUATERNARY AMMONIUM COMPOUNDS WITH CHONDROITIN SULFATE

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SUMMARY

The interaction of a series of mono- and di-quaternary ammonium compounds with chondroitin sulfate A has been studied. Quantitative precipitation of the polysaccharide occurs with cetyl pyridinium chloride and *d*-tubocurarine. The ratio, moles quaternary bound/mole repeating unit of polysaccharide, is 2 for CPC and 1 for curare. Evidence is presented to show that the rigid curare molecule precipitates by combining simultaneously with both the carboxyl and sulfate groups of the repeating unit of ChSA, rather than causing inter-molecular aggregation. Di-quaternary compounds which do not have a rigid structure fail to precipitate chondroitin sulfate and show only weak interactions in solution.

Abbreviations: CPC, cetyl pyridinium chloride; MPB, methylpyridinium bromide; ChSA, chondroitin sulfate A; DPC, dodecyl pyridinium chloride; MPB, methyl pyridinium bromide; PAM, pyridine 4 aldoxime methiodide; HBPB, hexamethylene bis-pyridinium; DBPB, decamethylene bis-pyridinium.

INTRODUCTION

Numerous investigations have been carried out on the interaction of long-chain mono-quaternary ammonium compounds with both acidic and neutral polysaccharides[1–4]. As a result of such studies, methods for the isolation of polysaccharides from various tissues have been developed, based on precipitation with these quaternary compounds[5–7]. On the other hand, interactions with di-quaternary ammonium compounds have, in general, been neglected. Such reactions may be of particular interest for at least two reasons: (a) The repeating units of many acidic polysaccharides are bi-functionally charged; thus, studies of this kind may provide information about the configuration of these macromolecules in solution which in turn may lead to an understanding of how such polysaccharides are built into ground substance of connective tissue. (b) Diquaternary ammonium derivatives have powerful pharmacological action and find increasing use in medicine. One such compound, the nerve poison *d*-tubocurarine (curare) has recently been used for the selective precipitation and isolation of the specific acetylcholine receptor protein[8,9]. Since curare also forms a precipitate with chondroitin sulfate A (see ref. 10), it would be of some interest to evaluate the possible role which acidic polysaccharides play in cellular reactions of curare and other bis-quaternary compounds. This is particularly pertinent since CHAGAS has attributed a function to acidic polysaccharides in terms of binding quaternary ammonium ions, and has ascribed receptor activity to some polysaccharides[11,12].

MATERIALS AND METHODS

Quaternary ammonium compounds

The quaternary compounds used, together with some of their u.v. absorption characteristics, are given in Table I. Structures of most of these compounds are shown in Fig. 1. The compounds were dissolved in phosphate buffer, pH 7.5, $\mu = 0.02$, immediately before use. This is particularly important with regard to curare which turns yellow on standing even at 0°. The final concentration of the compounds used in the various reactions was such that their contribution to the ionic strength did not appear to affect the results.

TABLE I

U.V. ABSORPTION CHARACTERISTICS OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS

Compound	*λ max*	*Emg, 1 cm cell*
d-tubocurarine dichloride (curare)	280	11.4
Dimethyl *d*-tubocurarine diiodide (dimethyl curare)	280	8.0
Cetyl pyridinium chloride	260	11.2
Dodecyl pyridinium chloride	260	14.7
Methyl pyridinium bromide	260	20.0
Hexamethylene bis-pyridinium	260	21.5
Decamethylene bis-pyridinium	260	19.9
Pyridine 4 aldoxime methiodide	280	68.3
Hexamethylene bis-4 PAM	280	76.5
Hexamethylene-4-PAM	280	65.0
Decamethylene bis-4 PAM	280	70.1
Na chondroitin sulfate	260	0.3
	280	0.25

The CPC, MPB and curare were obtained from the K and K Chemical Company of New York. The dimethyl curare was a gift from Dr. O. K. BEHRENS of Eli Lilly and Co. The other compounds were synthesized by Dr. S. GINSBERG of this laboratory. All compounds were used without further purification.

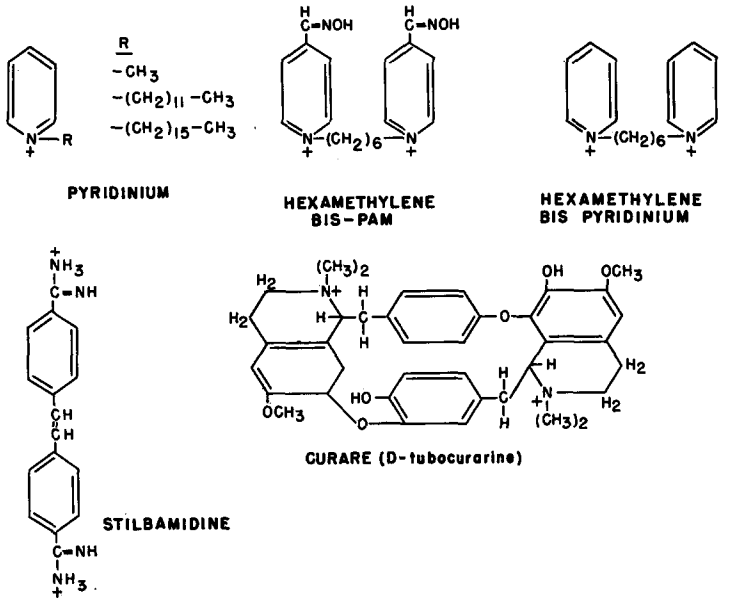

Fig. 1. Chemical structure of some of the quaternary ammonium ions used in this study.

Chondroitin sulfate A

The ChSA used in this investigation was obtained from various commercial sources and was used either as received or was purified following the method of EINBINDER AND SCHUBERT[13]. Prior to use, the polysaccharide was dissolved in the phosphate buffer at pH 7.5, $\mu = 0.02$, and dialyzed overnight at 0°.

A highly purified sample of ChSA was also supplied by Prof. K. MEYER, Department of Medicine, Columbia University. The uronic acid content of our purified preparations compared favorably with this sample, both by orcinol and carbazole analyses. On this basis, these ChSA preparations were about 90 % pure.

The structure of the ChSA repeating unit[14] is shown in Fig. 2.

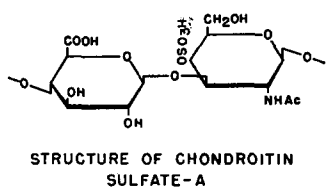

Fig. 2. Chemical structure of the repeating unit of chondroitin sulfate A.

Spectrophotometry

All reaction mixtures were analyzed with a model DU Beckman spectrophotometer. This method was particularly applicable to these experiments inasmuch as

the quaternary compounds have relatively high u.v. absorption spectra whereas ChSA shows only negligible absorption under these conditions (see Table I). Thus it was possible to check many of the results based on indirect measurements of precipitation and equilibrium dialysis experiments by direct analysis, *i.e.*, by determining the absorption of dissolved precipitates or solutions inside the dialysis bags (see below).

Equilibrium dialysis

These experiments were carried out essentially by the method of KLOTZ *et al.*[15]. The conditions were pH 7.5, $\mu = 0.1$ (phosphate), 0°. In each case, 1 ml of ChSA, 4 mg/ml, was equilibrated against 3 ml of the appropriate quaternary ammonium compound whose initial concentration was varied from 0.01 to 0.003 *M*. Controls were run by dialyzing these compounds against the buffer. In no instance was adsorption by the dialysis bag observed.

Calculation of the amount of quaternary ammonium compound bound at equilibrium was based on the difference between the dialyzate concentrations of polysaccharide and control. These results were in reasonably good agreement with those obtained by analyzing the contents of the bags containing polysaccharide and ammonium compound.

Precipitation studies

Stock solutions of ChSA (5 or 10 mg/ml), CPC (10 mg/ml), and curare (20 mg/ml), were prepared. Reaction mixtures of quaternary ammonium compound and polysaccharide were set up in duplicate at room temperature (about 23°) using 0.1 or 0.2 ml ChSA. 0.2 to 1.8 ml of buffer was added to bring the total volume to 2 ml. After precipitation had occurred, the solutions were allowed to stand for at least 15 min before centrifuging at 4000 rev./min, also for 15 min. Aliquots of the supernatants were removed for analysis as described above; u.v. absorption was used for the quaternary ammonium compound, orcinol for the ChSA.

It had been observed that some mixtures gave turbid supernatants, especially at the phase of precipitation prior to the equivalence point (see below). In order to avoid turbidities in the solutions read with the spectrophotometer, dilutions of supernatants were made with 3 *M* NaCl for the CPC analysis and 1 *M* NaCl for curare. During the course of the initial studies it was found that passage of u.v. light at 260 mμ through the NaCl solution gave rise to a progressive appearance of some substance having a distinct u.v. absorption maximum at this same wave length. Recrystallization of the NaCl or use of doubly-distilled water failed to suppress this effect. Passage of light of 280 mμ wave length through the same solution had no effect on the NaCl. The increased absorption at 260 mμ interfered with the CPC analysis and therefore distilled water was used in the reference cuvette, the O.D. of the CPC solutions being determined as rapidly as possible.

RESULTS

The precipitation of ChSA by both CPC and curare is shown in Fig. 3. μmoles of the quaternary compounds bound are plotted as a function of their initial concentration. The following phases of the reaction may be distinguished: (a) A phase in which soluble complexes have been formed but no precipitation has taken place; this was dem-

onstrated by equilibrium dialysis. (b) There is precipitation as well as a turbid supernatant but equivalence has not been reached. (c) An apparent equivalence in which the supernatant is clear but precipitation is nervertheless incomplete. (d) A true equivalence point. (e) A post-equivalence phase in which the quaternary compound

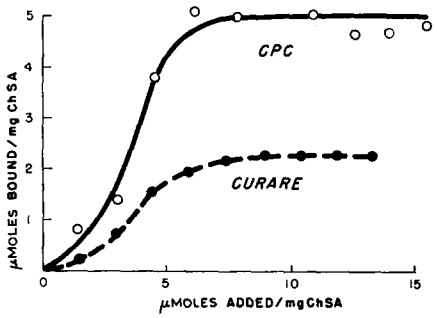

Fig. 3. Combining ratios of CPC and curare to chondroitin sulfate A, expressed as μmoles bound/mg polysaccharide. pH 7.5, ionic strength 0.02, room temperature.

is now present in excess but the amount bound remains constant. No solubilization of the precipitated complex could be detected even in the presence of a large excess of quaternary compound. This behavior is in marked contrast to the situation encountered with proteins where excess long-chain quaternary may solubilize the precipitate[16].

It is to be noted that no ChSA could be found in the supernatants either at the equivalence point or in the post-equivalence zone, indicating complete precipitation of the polysaccharide material.

Perhaps the most interesting feature of these precipitation reactions concerns the molar ratios of CPC and curare to ChSA, bound at the equivalence point and in the post-equivalence phase. It is evident (Fig. 3) that the ratio of CPC to chondroitin sulfate monomer is approximately 2:1 at equivalence, whereas that of curare is 1:1. In other words, at equivalence, the number of moles of CPC bound is twice that of curare. These ratios held regardless of the concentration of ChSA, which was varied from 1 to 4 mg/ml. Moreover, this value was independent of the purity of ChSA used. Data from solubilized precipitates were in good agreement with those obtained from analysis of the supernatant. It is also to be noted that the maximum binding of curare, as determined by equilibrium dialysis, was in excellent agreement with that determined by precipitation. Equilibrium dialysis could not be run with CPC above about 10^{-3} M due to micelle formation which prevented equilibration across the dialysis bag.

Additional differences in the behavior of the two types of complexes studied were indicated by observations on the properties of the precipitates (Table II). It is evident that the CPC–ChSA precipitate is quite stable to low concentration of salt, to heat, urea, acid, and alkali. In contradistinction, the curare complex is labile to each of these agents. A distinct difference in solubility in organic solvents is also to be noted. The CPC precipitate was soluble in 95% alcohol as well as in acetone, whereas the curare precipitate was almost insoluble in both of these solvents.

Equilibrium dialysis data on the binding of various other pyridinium compounds are shown in Fig. 4. It is evident that the order of binding is CPC > DPC > MPB. In this range of concentration, the binding of CPC is stronger than curare.

The binding of bis-quaternary compounds of two series—hexamethylene-bis-PAM, decamethylene-bis-PAM and the corresponding bis-pyridiniums (see Table I)—was compared with that of the corresponding methylated mono-quaternary-PAM and MPB. Initial molar concentrations of each compound were identical in order to provide direct comparison of the degree of binding. The affinity of all six compounds to ChSA was approximately the same (see Fig. 5). Certainly the polysaccharide has a markedly lower affinity for these bis-quaternary compounds than for curare.

TABLE II

SOLUBILITY CHARACTERISTICS OF CHONDROITIN SULFATE PRECIPITATES WITH CURARE AND CETYLPYRIDINIUM

	CPC	*Curare*
NaCl	Soluble in 1 *M*	Soluble in 0.1 *M*
Temperature (70°)	Stable	Soluble
Urea	Stable in 6 *M*	Soluble in 1 *M*
pH	Stable pH 3.5–9.5	(a) Slightly soluble, pH 3.5 (b) Completely soluble, pH 2 (c) Completely soluble, pH 12
Ethyl alcohol, acetone	Soluble	Insoluble

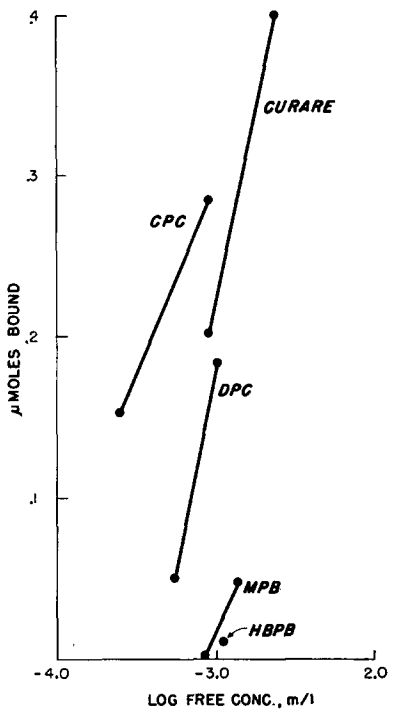

Fig. 4. Binding of various pyridinium derivatives by chondroitin sulfate A as determined by equilibrium dialysis at pH 7.5, ionic strength 0.1, 0°. The binding of curare is shown for comparison with that of HBPB. Binding of the latter is similar to that of MPB, a finding confirmed by the more extensive data of Fig. 5.

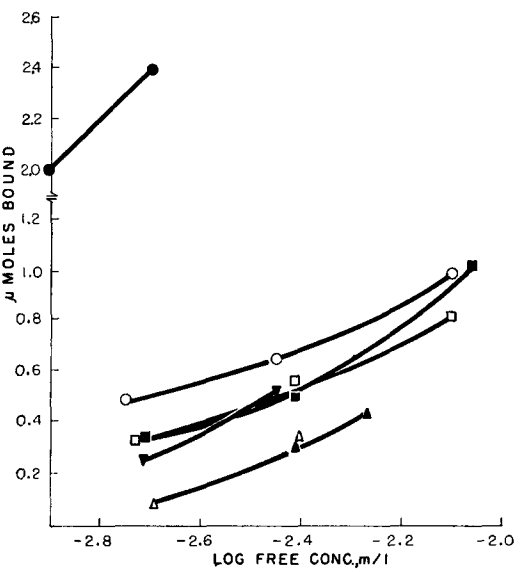

Fig. 5. Binding of various bis-quaternary compounds to ChSA compared with the corresponding mono-quaternary compounds, pH 7.5, ionic strength 0.1, 0°. Data for curare binding under comparable conditions are also shown. ●, curare; ○, HBPB; □, DBPB; △, MPB; ■, curve for both H bis PAM and D bis PAM; ▲, PAM; ▼, hexamethylene PAM.

DISCUSSION

The data based on precipitation and equilibrium dialysis studies indicate that there may be stoichiometric binding between some quaternary ammonium compounds and chondroitin sulfate. Two molecules of CPC, each with a single functional group, react with both functional groups of the repeating unit of ChSA. However, only one molecule of the bifunctional curare is required, suggesting that the inter-quaternary distance in this compound is such as to permit it to attach simultaneously to the two negative groups of the repeating unit. Regardless of the mode of interaction, it is safe to assume that the principal binding force is electrostatic. Thus we find that the precipitated complexes may be dispersed by increasing concentrations of salt. However, this cannot be the only factor involved as indicated by the marked difference in the stability of these complexes to salt, pH, urea, or heat. Thus curare–ChSA precipitates are readily soluble in 0.1 *M* NaCl, whereas CPC–ChSA precipitates are completely soluble only when the concentration of NaCl is increased to 1 *M*. It would appear that at low concentrations of electrolyte, mutual repulsion of the negatively charged groups on ChSA occurs, this apparently providing the proper spatial configuration for the two-point attachment of curare. However, as the ionic strength increases, the configuration of the ChSA may change[17,18], the two-point attachment of the relatively rigid curare molecule may be broken and there is either a decrease in the amount of curare bound or solubilization. On the other hand, the CPC–ChSA complex would be expected to be less salt sensitive because only single charges are involved. Moreover, there is the additional stabilizing factor due to the VAN DER WAALS' forces exerted through the hydrophobic portion of the long chain compound.

Changes in pH down to pH 3.5 produce effects which are somewhat comparable to changes in electrolyte concentration. As the pH is lowered, ionized carboxyl groups on the ChSA are converted to COOH. Under these conditions, the ChSA would be expected to undergo configurational changes together with a decrease in the number of binding sites. Consequently, the binding of curare is very much reduced whereas the CPC–ChSA complexes are quite stable over a wider range of pH since binding occurs with individual carboxyl and sulfate groups.

The effect of either changes in temperature or the addition of urea point to the contribution of hydrogen bonding toward the stabilization of curare complexes. Thus curare–ChSA precipitates (Table II) are dispersed by warming to 70° or by the addition of urea to a final concentration of 1 *M*. Under similar conditions, CPC precipitates are quite stable. The influence of hydrogen bonding is further substantiated by a comparison of curare with its dimethyl derivative. Both compounds form precipitates with ChSA which are salt-labile, but the affinity of curare for ChSA is much greater than that of the dimethyl derivative; much higher concentrations of the latter are required for precipitation. Similar differences in their binding properties were obtained by equilibrium dialysis experiments. Thus, the phenolic hydroxyl groups of curare, which are present as O-methyl ethers in the methylated derivative, are free to form hydrogen bonds and thereby would be expected to contribute to the binding force. Interestingly, the dimethyl curare–ChSA complex is also dispersed by urea, indicating the possibility that still other hydrogen bonds may be involved.

Many investigators have reported that an increase in the chain length of a quaternary compound enhances its binding to polymeric materials. Our results on

the influence of chain length, based on equilibrium dialysis, which appear in Fig. 4, confirm this general conclusion. MPB gives soluble complexes whereas both DPC and CPC produce precipitates; PAM complexes are soluble whereas those formed with its dodecyl derivative are insoluble. With an increase in the chain length of the quaternary compound, the complex becomes more hydrophobic and less water soluble. Preliminary experiments indicate that the six carbon side chain, *e.g.*, on H-PAM, is not sufficiently long to result in the precipitation of ChSA or even to significantly enhance the affinity of the compound over that of PAM itself (Fig. 5). In addition, the longer chains are more likely to aggregate into micelles through VAN DER WAALS' forces, thereby imparting a reduced water solubility. Indeed, it is apparently this tendency of the nonpolar end of the complex to associate through secondary valence forces which accounts for the solubility of the CPC–ChSA complex in solvents such as alcohol or acetone. This is evidently not the case with either the curare or dimethyl curare–ChSA complexes, both of which are insoluble in these solvents.

From our results, it was also evident that a number of di-quaternary compounds, viz., bis-4-PAM, bis-pyridinium, not only failed to give precipitates but did not bind nearly as well as curare. In fact, the binding of these compounds to ChSA was only slightly greater than that of the monoquaternary compound (MPB or PAM). It is to be noted that in solution none of these compounds have the structural rigidity of a molecule such as curare, in which the distance between the positive charges is fixed. This factor is apparently necessary for the formation of a firmly-bound complex. This possibility is supported by observations with stilbamidine, a compound of considerable lower molecular weight and of lesser complexity than curare, but one which also has a rigid structure in solution (Fig. 1). It, like curare, forms insoluble precipitates with ChSA. The nature of the interaction of this compound with ChSA is under investigation.

We may now consider some of the general conclusions which have emerged from these studies regarding the structure of ChSA in solution. The fact that there is a fairly exact equivalence of 1 curare for each 2 negative charges suggests that the negative groups are regularly spaced and that the inter-anionic distance is almost exactly the same as the inter-quaternary distance in curare. The question which may be raised is whether a molecule like ChSA, generally considered to be a random coil in dilute salt solution [17,18], would be expected to have the regular spacing of its negative groups which is required for the type of interaction observed. However, the possibility still exists that the curare molecule itself induces changes in the ChSA configuration such that the negative groups approach the inter-quaternary distance. Two pieces of evidence rule out such a mechanism: (a) Curare failed to alter the viscosity of ChSA and (b) curare failed to precipitate chondroitin sulfate C (CHSC). In the latter polysaccharide, the sulfate group is removed by a few Ångström units from its position in ChSA. Failure of ChS-C to precipitate with curare further substantiates the hypothesis that curare attaches itself to two negative groups and that small differences in the inter-anionic distance results in much reduced interaction. The experiment with ChS-C would appear to provide the best evidence that curare precipitation is not due to inter-molecular binding.

From the evidence presented in this paper, it is apparent that even a very highly anionic polysaccharide such as chondroitin sulfate may play only a minor role *in vivo* insofar as complexing with quaternary ammonium ions is concerned. Of

the many such compounds examined, only curare and stilbamidine have an affinity which is sufficiently high to permit interaction with cellular chondroitin sulfate. Other compounds having profound effects when applied to the neuromuscular junction—such as the decamethonium type—interact only weakly. Thus CHAGAS' suggestion[11,12] that acidic polysaccharides may function as "receptors" for various quaternary compounds in general should be modified so as to include only those diquaternary compounds which fit the inter-anionic distance of the polysaccharide repeating unit.

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REFERENCES

1 J. E. SCOTT, *Chem. and Ind.*, (1955) 168.
2 J. E. SCOTT, *Biochim. Biophys. Acta*, 18 (1955) 428.
3 M. M. FISHMAN AND R. S. MILLER, *J. Colloid Sci.*, in the press.
4 S. A. BARKER, M. STACEY AND G. ZWEIFEL, *Chem. and Ind.*, (1957) 330.
5 I. DANISHEFSKY, E. T. OPPENHEIMER, M. WILLHITE, A. P. STOUT AND M. M. FISHMAN, *Cancer Research*, 19 (1959) 1234.
6 E. D. KORN, *J. Biol. Chem.*, 234 (1959) 1325.
7 H. G. B. SLACK, *Biochem. J.*, 69 (1958) 125.
8 S. EHRENPREIS, *Science*, 129 (1959) 1613.
9 S. EHRENPREIS, *Biochim. Biophys. Acta*, 44 (1960) 561.
10 S. EHRENPREIS AND M. M. FISHMAN, *Abstracts 135th Meeting, Am. Chem. Soc., April, 1959.*
11 C. CHAGAS, E. PENNA-FRANCA, K. NISHIE AND E. J. GARCIA, *Arch. Bioch. Biophys.*, 75 (1958) 251.
12 A. HASSON AND C. CHAGAS, *Biochim. Biophys. Acta*, 36 (1959) 301.
13 J. EINBINDER AND M. SCHUBERT, *J. Biol. Chem.*, 191 (1951) 591.
14 E. A. DAVIDSON AND K. MEYER, *J. Am. Chem. Soc.*, 77 (1955) 4796.
15 I. M. KLOTZ, F. M. WALKER AND R. B. PIVAN, *J. Am. Chem. Soc.*, 68 (1946) 1486.
16 F. W. PUTNAM, *Advances in Protein Chem.*, Vol. 4, Academic Press, Inc. New York, 1948, p. 80.
17 M. B. MATHEWS AND A. DORFMAN, *Arch. Biochem. Biophys.*, 42 (1953) 41.
18 M. B. MATHEWS, *Arch. Biochem. Biophys.*, 43 (1953) 181.