THE INTERACTION OF QUATERNARY AMMONIUM COMPOUNDS WITH HYALURONIC ACID

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(Received May 16th, 1960)

SUMMARY

The interaction of *d*-tubocurarine (curare) and stilbamidine with hyaluronic acid has been investigated at pH 7.5, ionic strength 0.1. Curare fails to bind to the polysaccharide while strong binding of stilbamidine is observed. This observation, in conjunction with others, support the view that this acidic polysaccharide is not essential for the pharmacological effects of curare on nerve impulse conduction. Considerable binding of curare and other quaternary ammonium compounds is observed in distilled water and is evidently due to the Donnan effect.

Lack of binding of curare to hyaluronic acid may be due to the fact that unlike stilbamidine the inter-quaternary distance is not complementary to the inter-anionic distance of the polysaccharide. Since stilbamidine and curare have been considered to have the same interquaternary distance—14 Å—the present findings raise the question whether current views on the configuration of the curare molecule may have to be revised.

INTRODUCTION

During the past few years, attempts have been made to isolate the specific receptor substance of nerve and muscle which combines with acetylcholine and related quaternary ammonium compounds when they produce their biological effects. A protein which forms strong complexes with *d*-tubocurarine (curare) has been obtained in solution from the electric organ of the electric eel[1-3]. This protein interacts in a graded way with a large number of compounds demonstrated by NACHMANSOHN *et al.* to combine with the acetylcholine system[4]. Considerable evidence has accumulated which supports the assumption[3] that this protein may be identical with the physiological acetylcholine receptor.

In a series of studies, CHAGAS *et al.* have reported the isolation of a different component from electric tissue which combines with a curare-like compound TRIEG when it is injected *in vivo*. The TRIEG remains associated with the component when extracts of the electric tissue are made and dialyzed against distilled water[5-8]. The component has recently been identified as a polysaccharide similar to hyaluronic acid[8].

Abbreviations: TRIEG, triethiodide of gallamine; PAM, pyridine-2-aldoxime methiodide.

In view of the highly anionic character of this macromolecule, it appeared interesting to examine the nature of its binding properties more closely under conditions other than distilled water. Accordingly, the interaction of curare and a number of other quaternary compounds with hyaluronic acid has been investigated in salt solution at neutral pH. Using equilibrium dialysis, it was found that hyaluronic acid fails to interact with curare at pH 7.5, ionic strength 0.1. On the other hand, considerable binding of curare and other quaternary compounds is observed in water; this may be attributed primarily to the Donnan effect.

These studies, in conjunction with others previously reported[9,10] have led to interesting problems about the configuration of the curare molecule. Some speculations along these lines will be presented in this paper.

MATERIALS AND METHODS

Hyaluronic acid, obtained from the Mann Research Laboratories, was about 50% pure on the basis of the Dische carbazole reaction. *d*-Tubocurarine, acetylcholine and benzoylcholine were obtained from the K & K Chemical Company. Stilbamidine was a product of Merck Chemical Co. PAM, hexamethylene bis-PAM and decamethylene bis-PAM were synthesized by Dr. SARA GINSBURG of this laboratory.

The concentration of acetylcholine was determined by HESTRIN's method[11], that of the other compounds spectrophotometrically. Some of their u.v. characteristics have been presented elsewhere[3,10].

Equilibrium dialysis experiments were carried out as previously described[2,3,10]. It was necessary to use tris buffer since stilbamadine is quite insoluble in phosphate.

Dialysis experiments in distilled water were carried out in a manner similar to that described by CHAGAS *et al.* The hyaluronic acid was dialyzed first against dilute NaCl then against distilled water. The polysaccharide was mixed with the various quaternary compounds at concentrations about 10 times greater than required to neutralize all the negative charges in the polysaccharide. The mixtures were dialyzed against several changes of distilled water until the dialyzates were essentially free of u.v. absorbing material. The concentration of quaternary compound and hyaluronic acid within the bags was then determined.

RESULTS AND DISCUSSION

The binding of curare and stilbamidine to hyaluronic acid at pH 7.5, ionic strength 0.1 is shown in Fig. 1. It is apparent that under these conditions curare failed to interact with the polysaccharide. Strong binding of stilbamidine was observed.

The fact that hyaluronic acid does not combine with curare in dilute salt solution at pH 7.5 makes it likely that this macromolecule does not play an important role as a physiological receptor for curare and related compounds in the sense defined above. The behavior of this polysaccharide may be contrasted with that exhibited by the receptor protein isolated from the electric organ which combines very strongly with curare under these conditions[1-3]. The present result clearly shows that binding of curare to the receptor protein is indeed unique and not merely the result of non-specific coulombic interaction. Nevertheless, the complete absence of binding of a diquaternary compound like curare to the highly anionic polysaccharide was some-

what unexpected and may have considerable bearing on the configuration of the curare molecule.

The strong binding of stilbamidine is in contrast to that of curare (Fig. 1). Moreover, stilbamidine quantitatively precipitates hyaluronic acid[12] with an equivalence ratio of one stilbamidine for two negative charges. It has been shown[10,12] that this kind of interaction of a rigid diquaternary compound such as stilbamidine may be used as the basis for measuring the inter-anionic distance of the repeating unit of a macromolecule, *e.g.*, chondroitin sulfate. Thus it would appear that the inter-anionic distance in hyaluronic acid is fairly close to 14 Å, the inter-quaternary distance in stilbamidine. The distance between positive charges in curare has also been considered to be about 14 Å. However, the fact that curare fails to interact with hyaluronic acid

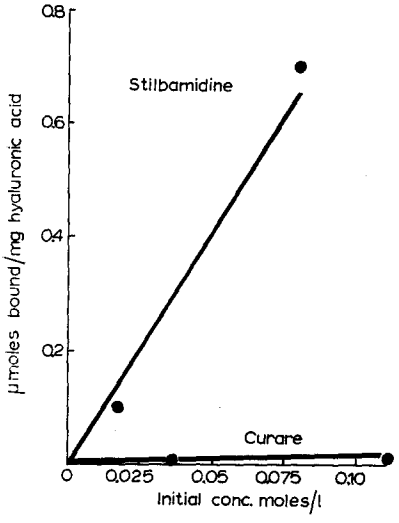

TABLE I

RETENTION OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS BY HYALURONIC ACID IN DISTILLED WATER

Compound	*moles "bound" per mole hexuronic acid*
Curare	0.50, 0.43
Stilbamidine	0.31, 0.23, 0.35
Acetylcholine	0.37
Benzoylcholine	0.50
Hexamethylene bis-PAM	0.43
Decamethylene bis-PAM	0.43
PAM	0.36

Fig. 1. Comparison of binding of stilbamidine and curare to hyaluronic acid. pH 7.5, $\mu = 0.1$ (Tris), 0°.

as well as the marked differences in the combination of curare and stilbamidine with a variety of other macromolecules[12] lead to the speculation that the inter-quaternary distance in curare may be significantly less than 14 Å. Such a conclusion is particularly interesting in view of the report by LOEWE AND HARVEY[13] that a model of curare may be made in which the cationic groups are separated by only 6.5 Å. If this premise is correct, the lack of binding of curare to hyaluronic acid would be explained by the fact that the anionic groups are at a distance which is far too great to accommodate the curare molecule; conversely, this finding supports the notion that the inter-quaternary distance may be considerable less than 14 Å. For more definitive evidence, it is clear that other types of experiments, such as X-ray crystallography of the curare molecule are required.

It is apparent that in distilled water the interaction of curare and other quaternary compounds observed (Table I) is non-specific, *i.e.*, no distinction in binding is made between a variety of cationic substances. Certainly the degree of binding in no way paralleled the affinity of these compounds for the physiological receptor as determined by experiments on the intact cell[14,15]. Retention of these compounds most likely results from the Donnan membrane effect rather than from true complex formation. However,

it is to be noted that in no instance was the amount of quaternary compound retained sufficient to completely neutralize all the charges on the hyaluronic acid. This is partly explained by the fact that the final pH inside the dialysis bags was fairly low and thus some of the carboxyl groups were in the unionized state. Even if this were the case, the interesting point is that the amount of di-quaternary and mono-quaternary retained was almost the same. This shows that in distilled water, where repulsion of similar charges of a flexible molecule should be at a maximum, the fully extended form did not seem to predominate. Thus in decamethylene bis-PAM the maximum distance between cationic groups is approx. 14 Å; if the fully extended configuration occurred to a significant extent the compound could combine simultaneously with two negative charges in hyaluronic acid as is the case with stilbamidine. It would be expected that the number of moles of this compound bound would be significantly less than the mono-quaternary, PAM; this was not found. Similar results were obtained with chondroitin sulfate (unpublished experiments), indicating that even the shorter distance separating negative groups of this polysaccharide is not preferred either by the hexa- or decamethylene bis-quaternary compounds.

These results appear to provide some justification for LOEWE AND HARVEY'S objections[13] to the equidistance concept of curariform action which postulates that strong curare action results when a compound has two quaternary groups separated by 14 Å. This situation pertains only when the molecule is rigid. It is evident that decamethylene bis-PAM, a flexible molecule having very potent curarizing properties, fails to attain the 14-Å distance to any significant extent.

ACKNOWLEDGEMENTS

This work was supported in part by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant No. B-400, U.S. Public Health Service, by the National Science Foundation, Grant No. G-4331.

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