INTERACTION OF QUATERNARY AMMONIUM BASES WITH A PURIFIED ACID POLYSACCHARIDE AND OTHER MACROMOLECULES FROM THE ELECTRIC ORGAN OF ELECTRIC EEL

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SUMMARY

1. The interaction of quaternary ammonium bases with proteins and a purified acid polysaccharide from the electric organ of *E. electricus* has been studied. Extraction of these macromolecules was performed with water and with phosphate buffer (pH 7.5, μ 0.1). The water extract was fractionated under mild conditions by column chromatography and the saline extract through ammonium sulfate precipitation.

2. This interaction was measured by the differential-dialysis technique at increased electrolyte concentration which reduces binding strength. The observed effect is a function of the charge and the relative concentration of the cations present.

3. With the protein fraction no evidence was obtained of specific binding with TRIEG, a synthetic curare. Under the same conditions, the acid polysaccharide has a higher binding affinity that can be attributed to higher concentration of ionised carboxyl groups, which suggests an electrostatic interaction with the ionised quaternary bases.

4. The direct precipitation method was also tried with *d*-tubocurare. The ammonium sulfate fractions and the supernatant were precipitated with *d*-tubocurare, but not so easily by dimethyl-*d*-tubocurare. No precipitation occurred with TRIEG. These precipitates were soluble at pH 9.2 and pH 3. The acid polysaccharide also precipitates under these conditions.

Other proteins as insulin, pepsin and acetylcholinesterase are in the same way readily precipitated with *d*-tubocurare, less easily with the dimethyl derivative, but not with TRIEG.

INTRODUCTION

During the last five years[1–6] evidence has accumulated in this laboratory that quaternary ammonium bases with curarizing and depolarising activity can interact with

Abbreviations: TRIEG, Gallamine triethiiodide; SCh, succinylcholine chloride; ACh, acetylcholine chloride; DMTC, dimethyl-*d*-tubocurarine iodide; DTC, *d*-tubocurarine chloride; FAT, total electric organ aqueous extract; ETS, total saline extract; STCA, trichloroacetic acid supernatant from FAT or ETS; FY, alcohol precipitate from STCA; Sf_1, neutral polysaccharide from FY; Sf_3, acid polysaccharide from FY; AChE, acetylcholinesterase; CPC, cetylpyridinium.

several macromolecules to a variable extent depending on their electric charge and the ionic entourage in the media where this interaction is measured. To support these findings, it has been demonstrated by BRAUN CANTILO *et al.*[6] in this laboratory that after the injection of ^{14}C labelled quaternary bases to dogs, they are widely distributed in the body. He found no evidence of an increased specific binding in muscle but a high accumulation in cartilages. CHAGAS *et al.*[7], working with the electric eel, after injection of physiological curarizing doses of [^{14}C]TRIEG, isolated the electroplax from connective tissue, and found the curare distributed between both tissues.

In another line of work we have studied the binding of [^{14}C]TRIEG to extracts of the electric organ following the original findings of CHAGAS *et al.*[1] that the labelled base could be extracted together with non dialysable macromolecules from the electric organ.

Some of the results thus obtained have demonstrated that, from aqueous extracts (FAT) of the electric organ, an acid polysaccharide (Sf_3) was obtained which could bind [^{14}C]TRIEG, [^{14}C]SCh and ACh[4,5] with higher specific activity (expressed as counts/min/mg of dry residue) than the other macromolecules from FAT.

The extraction and purification of Sf_3 has been already described[4]. By chemical analysis and by electrophoretic and chromatographic methods, it has been determined that it is constituted of equivalent amounts of glucuronic acid and N-acetylglucosamine with no sulfate groups[5].

During the course of these studies, EHRENPREIS AND NACHMANSOHN published a series of papers[8–11] in which some objections were presented to our results. On the basis of *in vitro* pharmacological studies, these authors described the purification of a protein supposed to be the specific receptor for acetylcholine and other quaternary ammonium bases. It should be pointed out that as far as we are concerned, the acid mucopolysaccharide Sf_3 has never been described as the specific receptor for acetylcholine, though evidence was presented that it does bind the quaternary bases under our experimental conditions, established as a consequence of the previous *in vivo* experiments[3–5].

More recently, EHRENPREIS[11] has been confirming our finding[4] that chondroitin sulfate also binds curare and curare-like compounds[2–4]. Chondroitin sulfuric acid is an acid polysaccharide containing $2n$ acidic sites per n-disaccharide repeating unit while the acid polysaccharide from the electric organ, Sf_3, contains n acidic sites per n-disaccharide repeating units as indicated in Fig. 1. As that same author points out, the fact that these molecules bind quaternary bases of the CPC type has been recognized since a long time. DORFMAN (personal communication) working with the rat skin extract, was able to precipitate with CPC, hyaluronic acid, chondroitin sulfuric acids and heparin. These mucopolysaccharides were separated by gradient elution of the precipitate with 0.4 M–2.1 M NaCl. Heparin was eluted at 2.1 M concentration. This would also give an indication that heparin binds the quaternary base more strongly than the other products.

Other physico-chemical studies on the binding of cations and their influence in the molecular configuration of chondroitin sulfuric acid were developed by MATHEWS[12,13] at DORFMAN'S laboratory and, more recently, by GILBERT AND MYERS[14]. These authors have described potentiometric titration and viscosity measurements at different ionic strengths and in the presence of different cations (Ca^{2+}, H^+, Ir^{3+} and La^{3+}) which are apt to be bound by the carboxyl groups of chondroitin sulfate. The

sulfate groups have small influence upon the observed binding. The concentration of counter ions in the media under analysis is also effective in modifying the cations–polyacid interactions.

The finding that the acid polysaccharide isolated from the electric organ extracts was able to bind the curarizing ammonium bases *in vitro*, directed our attention to the study of their interaction.

In this paper, evidence will be presented that a complex is formed with TRIEG and the purified acid polysaccharide, Sf_3, and also with the FY fraction, at ionic strengths 0.01, 0.05 and 0.10. The binding extent is a function of the ionic strength and of the concentration of the base in the dialysis media. The effect of pH and ionic strength in the interaction of FAT, FY and Sf_3 fractions with SCh, ACh and DMTC will be also described. Binding was measured using the differential dialysis technique, and radioactivity determinations and colorimetric analysis under various conditions. No precipitated material has ever been found inside the dialysis bag even when quantitative data demonstrated that high interaction existed.

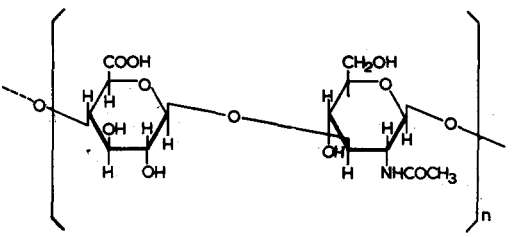

Fig. 1. Disaccharide repeating unit of Sf_3.

Results will be presented of fractionation of FAT and ETS by column chromatography and with ammonium sulfate in order to obtain purified proteins from the electric tissue and study their interaction with the bases. No direct evidence of specific binding of TRIEG to a single protein fraction was obtained. Paper electrophoresis of these fractions will show the extent of protein purification obtained by the two methods.

Direct precipitation of a specific protein[9] was tried with the ammonium sulfate fractions and TRIEG, DMTC and DTC. TRIEG even at a final concentration of $5 \cdot 10^{-2}$ *M* failed to precipitate any protein. DMTC formed precipitates with all the fractions, at $0.7 \cdot 10^{-2}$ *M* final concentration and DTC at $0.5 \cdot 10^{-2}$ *M*. The precipitates were insoluble at pH 8.0, $\mu = 0.1$. DTC proved to be a highly effective reagent for the precipitation of many proteins, as purified beef insulin at very low concentrations, pepsin and a highly active acetylcholinesterase preparation.

MATERIAL AND METHODS

The quaternary ammonium bases utilized were ACh, SCh, DTC, DMTC and TRIEG. [^{14}C]SCh and [^{14}C]DMTC were labelled in two methyl groups and [^{14}C]TRIEG in three ethyl groups as indicated in Fig. 2. The five bases were chromatographically pure in two different acid solvents, and their structure is also presented in Fig. 2.

TRIEG has been more extensively studied as it is the labelled ^{14}C synthetic

curare-like substance which has been used by CHAGAS *et al.* in the first *in vivo* experiments with the electric eel (*E. electricus*). The labelled bases were utilized for binding experiments, in solution with their inactive carriers.

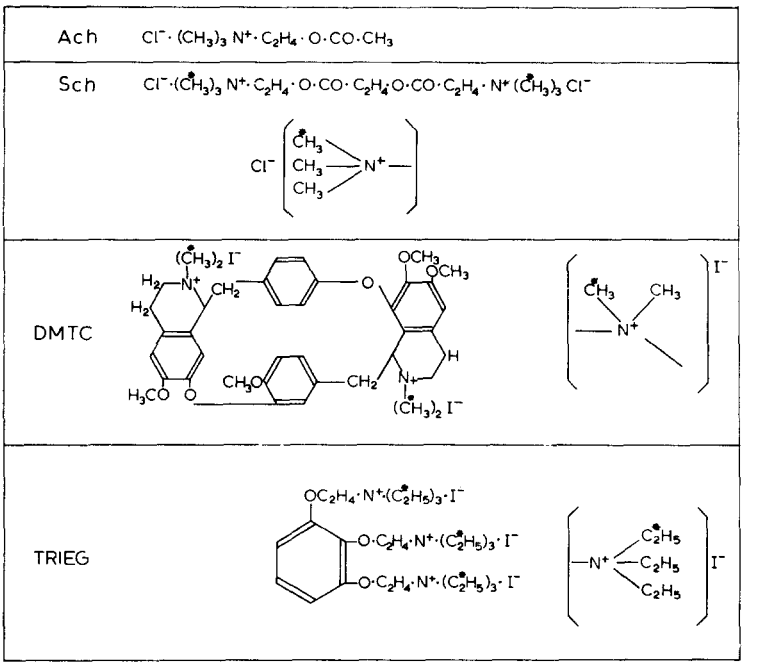

Fig. 2. Chemical structures of the quaternary ammonium bases studied: Labelled carbon (C*) has its position in the formula indicated under brackets.

Dialysis method

Binding measurements were made by the differential dialysis technique described by HUGHES AND KLOTZ[15] utilizing cellophane bags ("Visking" sausage casings type). 4 ml of solution containing the macromolecules under study were dialysed with mechanical shaking against 4 ml of the quaternary bases solution inside covered bottles. Before binding experiments, the macromolecules were dialysed against the same buffer solution where the bases would be dissolved.

As previously[5], through this paper, the word "receptor" will express the molecule with which the bases interact under the described conditions avoiding any ambiguity with pharmacological meanings.

Mechanical shaking was performed in a rotary multi-dialyzer where 16 simultaneous experiments can be performed under the same conditions. After 24 h, equilibrium was attained and it was checked by running also a blank containing no "receptor" molecule inside the bag. Dialysis experiments were carried out at + 5° in a cold room. Most of the experiments were conducted at pH 7.0 with phosphate buffer of 0.01, 0.05 and 0.1 ionic strengths (μ) regulated by NaCl.

When binding measurements were done at lower ionic strengths, 5 ml of the receptor molecules solution were dialysed against 1 l of base solution, with a magnetic stirrer until constant concentration of the base was measured in the outside solution.

pH's were measured with a Leeds and Northrop miniature glass electrode and they were measured in the internal and external solution of dialysis after equilibrium. If necessary, corrections for Donnan effect were calculated.

Analytical methods for binding extent

Binding analysis with the ^{14}C labelled compounds were made by radioactivity determinations with aliquots of the inside and outside solutions of the cellophane bags in an automatic and continuous gas flow counter (Nuclear Chicago) and corrections were made for ^{14}C "self absorption". The binding results are obtained as counts/min/mg of dry residue and then calculated as molar concentrations from known standards measured under the same conditions. Under our conditions, no significant losses of radioactivity were found on the cellophane bags. ACh binding was measured by the colorimetric procedure with hydroxamate and ferric chloride described by LIPMANN AND TUTTLE[16]. In a mixture of ACh and [^{14}C]SCh the radioactivity results were subtracted from the colorimetric results to determine ACh concentration.

Fractionation of electric organ extracts

Non dialysable molecules from electric organ homogenates were obtained from aqueous (FAT) and saline (ETS) extracts. The minced organ was homogenised in a Waring-blendor with water or sodium phosphate buffer, pH 7.5 μ 0.1, 1:1 (w/v) proportion, at $+5°$ in the cold room. The suspension was stirred for at least 3 h, centrifuged in a refrigerated centrifuge, the residues reextracted with the same extracting solution and the suspensions recentrifuged.

The supernatant solutions were concentrated by perevaporation in the cold room to an approximate protein concentration of 2% (w/v). These solutions were dialysed overnight against physiological saline. Any insoluble material was centrifuged off in a Servall angle centrifuge and the supernatants (FAT) and (ETS) kept in the refrigerator under a layer of toluene as preservative of deterioration. Before binding experiments, FAT or ETS were dialysed against the medium where interaction would be measured.

Column chromatography of FAT

FAT was fractionated in a DEAE-cellulose column with the procedure of PETERSON AND SOBER[17], utilizing an automatic fraction collector (Technicon). A Liebig condenser was adapted for column chromatography and water, refrigerated to $+5° \pm 1°$, was pumped through it. FAT was first dialysed against sodium phosphate buffer, pH 6, $\mu = 0.05$, and 10 ml (200 mg protein) was fractionated in 4 g DEAE-cellulose. Elution was performed with a pH gradient of phosphate and Tris buffers which was followed by a gradient of NaCl concentration in the buffer solutions as described in Fig. 3. Following the protein distribution[18], fractions were separated in the following way: fraction 1, containing eluates from tube 10–30; fraction 2, tubes 35–50; fraction 3, tubes 51–56; fraction 4, tubes 57–68; fraction 5, tubes 69–79 and fraction 6, tubes 80–94.

Aliquots from the contents of each even numbered tube were analysed for uronic acid[19] content and cholinesterase activity[20]. With both analyses, two peaks were obtained in the same set of tubes which composed fraction 6. Also in this fraction was eluted a yellow component of FAT which appeared as a distinct yellow band moving in the column when elution was performed with Tris – HCl buffer,

0.05 *M*, pH 8.6 containing 0.4 *M* NaCl. This was the final step of the fractionation procedure. The chemical composition of this yellow component is now under investigation. Total protein recovery after column fractionation was 96 %.

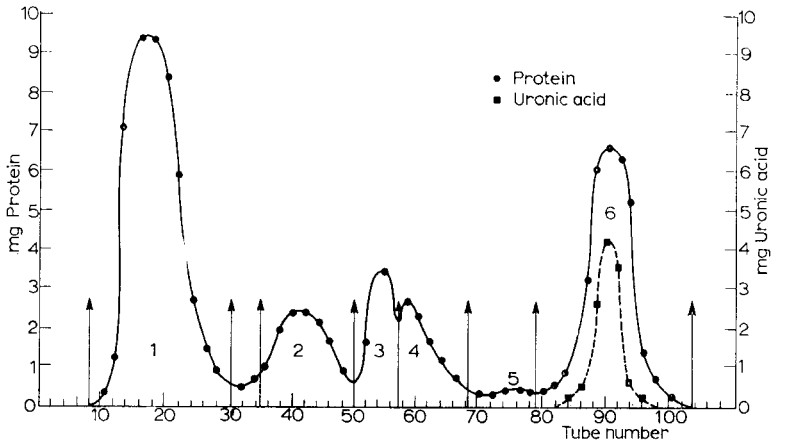

Fig. 3. Fractionation of FAT (200 mg protein/10 ml) in DEAE-cellulose column, at + 5°. Elution started with sodium phosphate-buffer, pH 6.0, μ = 0.05, followed by: sodium phosphate pH 7.6, μ = 0,05; the same buffer containing 0.1 *M* NaCl; 0.05 *M* Tris – HCl, pH 8.6, containing 0.1 *M* NaCl; the same buffer containing 0.3 *M* and 0.4 *M* NaCl. Fractionation performed with an automatic fraction collector (4 ml/tube). The ordinate to the left refers to total protein, ●—●, measured spectrophotometrically, and the ordinate to the right to total uronic acid; ■—■ measured by DISCHE's carbazol method. Arrows indicate tube numbers corresponding to assembled fractions.

Paper electrophoresis at pH 8.6 of the fractions collected is shown in Fig. 4 and they were stained for proteins with bromphenol blue reagent.

FY fraction was also prepared from FAT by protein precipitation with trichloroacetic acid, removal of the acid by dialysis, perevaporation to approx. 10 mg/ml of dry residue and followed by ethanol precipitation as described previously[5]. A dry white powder was obtained which was a mixture of Sf_1 and Sf_3.

Ammonium sulfate fractionation of ETS

Sixty millilitres of ETS (7.7 mg protein/ml) in phosphate buffer, pH 6.4, μ = 0.05 were fractionated with solid, finely powdered ammonium sulfate at 0°. The salt was added slowly with mechanical stirring over a period of 30 min, and stirring was continued each time over a period of 1 h prior to centrifugation at top speed in a Servall angle centrifuge. The solution was just brought to 0.30 saturation, the precipitate was centrifuged and redissolved in 5 ml Tris buffer 0.05 *M*, pH 8.7 and dialysed against two changes of the same buffer for 24 h.

The supernatant fluid was brought to 0.40 saturation with ammonium sulfate and the precipitate was centrifuged, dissolved and dialysed as before. The supernatant was brought to 0.60 saturation with ammonium sulfate and the same steps were followed. After the removal of ammonium sulfate by dialysis, the 30%, 40%, 60% precipitates and the last supernatant, which was concentrated by perevaporation, were dialysed against sodium phosphate buffer, pH 7.0, μ = 0.10 regulated with NaCl. Paper electrophoresis at pH 8.6 of these fractions is presented in Fig. 5 showing protein distribution as obtained after staining with bromphenol blue.

From ETS, the STCA and FY fractions were also prepared. Chemical analysis of all the fractions obtained from ETS will be presented later in Table IV together with binding measurements of TRIEG.

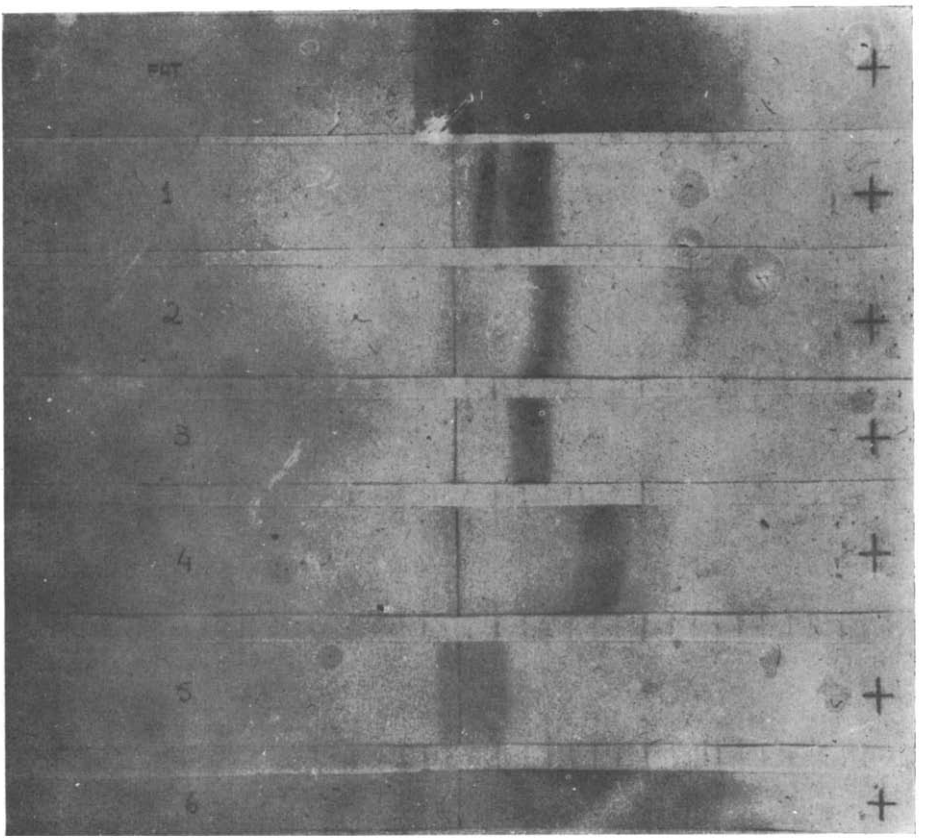

Fig. 4. Paper electrophoresis of FAT fractions from DEAE-cellulose column. Sodium barbiturate buffer, pH 8.6, μ = 0.05. Whatman No. 1 paper. 0.6 mA/cm for 6 h. Strips stained with bromphenol blue. Numbers on the strips correspond to fractions numbers.

Purification of Sf_3

The acid polysaccharide Sf_3 is obtained from FY by column chromatography fractionation, as previously described[4,5], in a DEAE-cellulose column developed at the beginning with sodium acetate buffer, pH 5.6, 0.1 *M*. The neutral polysaccharide Sf_1, which is a glycogen-like substance, is collected in the solution that passes through the column; this is washed with 0.1 *M* NaCl and the acid Sf_3 is eluted with 0.4 *M* NaCl. The relative proportions of Sf_1 and Sf_3 in FY vary with the preparation and are determined by chemical analysis of FY (uronic acid determined with DISCHE's carbazol[19] method and glucose with the anthrone reagent[19]) and by ultracentrifugation analysis in the Spinco analytical ultracentrifuge (model E)). In a 1% FY solution in 0.1 *N* sodium acetate buffer, Sf_1 has a sedimentation constant of the order of 66 Svedberg units and Sf_3, under the same conditions, has a sedimentation constant of 1.2 Svedberg units. Sf_1 and Sf_3 are precipitated, from the dialysed and concentrate

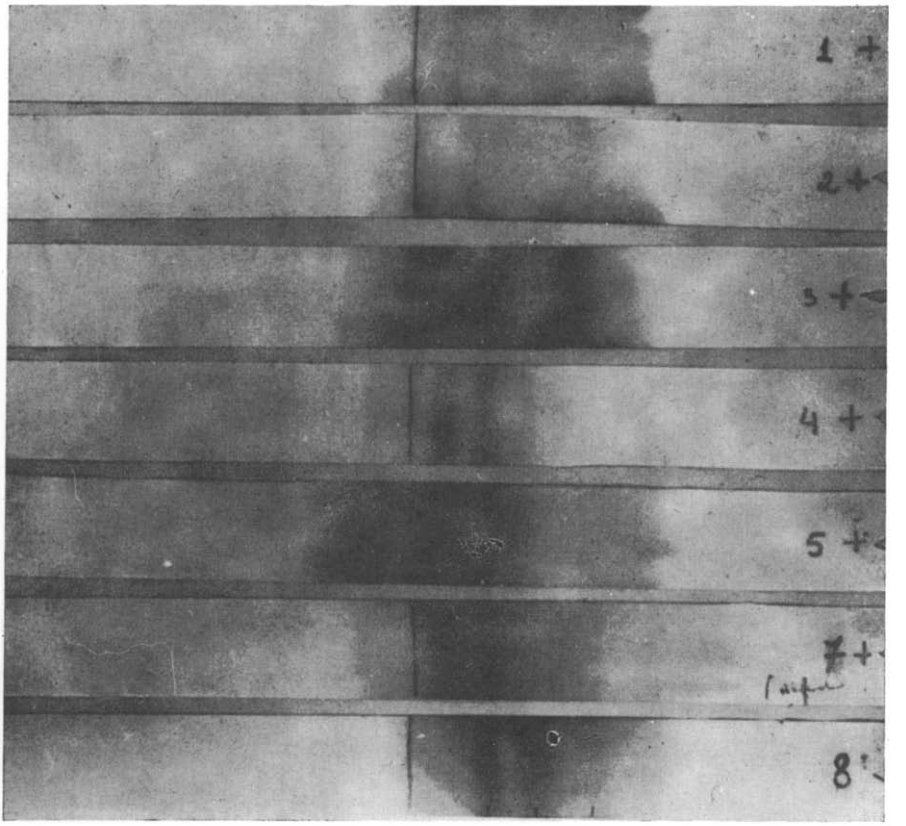

Fig. 5. Paper electrophoresis of ammonium sulfate fractions of ETS. Sodium barbiturate buffer, pH 8.6, $\mu = 0.05$. Whatman No. 1 paper. 0.125 mA/cm for 16 h. Strip No. 1, 30% precipitate (0.07 ml); No. 2, 40% precipitate (0.08 ml); No. 3, 60% precipitate (0.07 ml); No. 4, supernatant (0.07 ml); No. 5, same supernatant concentrated to 2.4 mg protein/ml; No. 7, ETS diluted 1:1 (0.10 ml); No. 8, ETS, 7.7 mg protein/ml (0.10 ml). Strips stained with bromphenol blue.

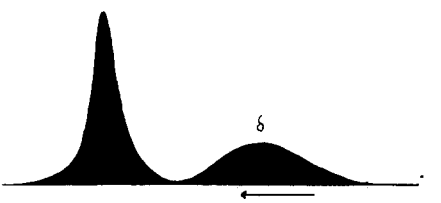

Fig. 6. Moving boundary electrophoretic pattern of purified Sf_3 fraction. 0.6% solution in sodium phosphate buffer, pH 6.0, $\mu = 0.1$. Potential gradient of 2.8 V/cm for 4800 sec.

eluates, with acid alcohol, centrifuged, washed with alcohol and ether, and both dried as white amorphous powder. Fig. 6 presents the free electrophoresis pattern of Sf_3 in phosphate buffer, pH 6.0, $\mu = 0.1$, in the Tiselius moving boundary apparatus at 1°. Fig. 7 shows paper electrophoresis of FY, Sf_1 and Sf_3 in ammonium acetate, 0.05 *M* pH 5.3, stained with Alcian Blue reagent for acid polysaccharides, as described by HEREMANS *et al.*[21]. Results of the chemical constitution of Sf_1 and Sf_3 determined by paper chromatography were presented at the Symposium on Comparative Bioelectrogenesis held in Rio de Janeiro[5]. These indicated that Sf_3 is similar to hyaluronic

acid. More recent physico-chemical studies (sedimentation in the ultracentrifuge and diffusion analysis) of Sf_3 gave evidence that, as extracted, it is not so highly polymerised as the hyaluronic acids described in the literature[22,23], its most probable molecular weight being near 10000.

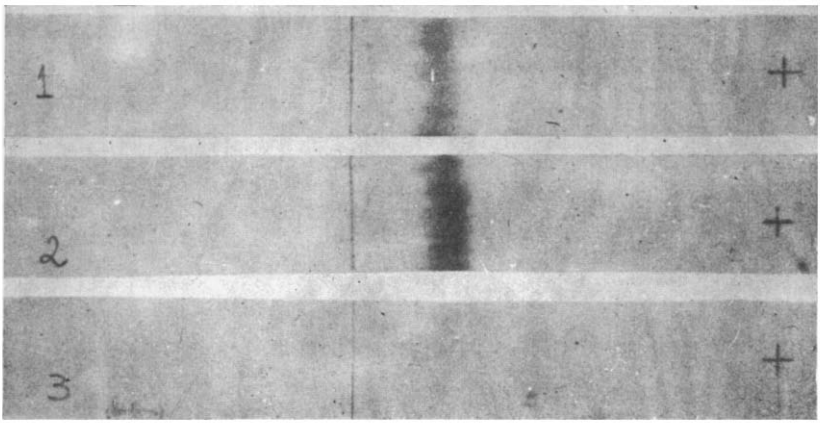

Fig. 7. Paper electrophoresis of FY, and its purified fractions Sf_1 and Sf_3 in ammonium acetate, 0.05 *M*, pH 5.3, 0.5 mA/cm for 195 min. Whatman No. 1 paper stained with Alcian blue reagent[21] for acid mucopolysaccharides. Relative concentration of Sf_3 is shown: in FY, 1% solution, (1); after Sf_3 purification, 1% solution, (2), and its absence in Sf_1 (3).

As Sf_3 does not contain sulfate groups, its acidic character is only related to the number of carboxyl groups. By potentiometric titration data[24], the number of reactive carboxyl groups per milligram of Sf_3 was found to decrease with increased ionic strength. It was calculated as $12 \cdot 10^{-7}$ moles/mg of Sf_3, the maximum of H^+ ions that would be bound, when the molecule is half dissociated, at lower ionic strength.

RESULTS

The effect of pH and ionic strength

The extent of TRIEG binding by FAT and its fractions was firstly measured at low ionic strength. It was then recognized that a non protein component had a higher affinity, measured as specific activity for the curare. This is put in evidence at Table I, where it can be seen that binding is affected by pH changes and increases in the absence of electrolytes. In the bottom of Table I it is also indicated that higher interaction is observed with the pure Sf_3 fraction. Sf_1, the glycogen-like polysaccharide, shows a negligible binding of TRIEG. SCh and ACh were also studied for interaction with FAT and FY as presented in Table II and the same effects of pH and of the absence of electrolytes were observed. These interactions were studied at low bases concentration in order to verify the analogy with the physiological doses though electrolytes concentrations are not physiological. Under the described conditions, it is apparent that the base with highest affinity for Sf_3 is succinylcholine and this is observed in Fig. 8 where the binding of SCh and TRIEG to an FY preparation containing 25% Sf_3 is plotted against the logarithm of free base concentration (Bjerrum's formation function[25]). These results were corrected for Donnan effect.

For DMTC binding by FAT the influence of ionic strength is also important and in Fig. 9 we present the interactions measured at different ionic strengths (0.01, 0.05, 0.10 and 0.20) and constant pH with the differential dialysis technique which is also named equilibrium dialysis.

TABLE I

TRIEG BINDING BY FAT AND FY IN $2 \cdot 10^{-3}$ *M* Tris – HCl, pH 7.4; $2 \cdot 10^{-3}$ *M* SODIUM ACETATE, pH 4, AND WATER, AT 4°

"Receptor"	*TRIEG concentration*	*Dialysis media-2 l*	*Moles TRIEG bound/mg*
FAT (52 mg/5 ml)	$1.12 \cdot 10^{-6}$ *M*	Tris – HCl, $2 \cdot 10^{-3}$ *M*, pH 7.4	$0.23 \cdot 10^{-7}$
FY (18 mg/5 ml)	$1.10 \cdot 10^{-6}$ *M*	Tris – HCl, $2 \cdot 10^{-3}$ *M*, pH 7.4	$0.63 \cdot 10^{-7}$
FAT (52 mg/5 ml)	$1.00 \cdot 10^{-6}$ *M*	Na acetate; $2 \cdot 10^{-3}$ *M*, pH 4	$0.01 \cdot 10^{-7}$
FY (18 mg/5 ml)	$1.00 \cdot 10^{-6}$ *M*	Na acetate; $2 \cdot 10^{-3}$ *M*, pH 4	$0.31 \cdot 10^{-7}$
FAT (52 mg/5 ml)	$1.12 \cdot 10^{-6}$ *M*	Water	$0.30 \cdot 10^{-7}$
FY (18 mg/5 ml)	$2.60 \cdot 10^{-6}$ *M*	Water	$1.40 \cdot 10^{-7}$
FY (20 mg/5 ml)	$22.6 \cdot 10^{-6}$ *M*	Water — 1 l	$2.60 \cdot 10^{-7}$
Sf_1 (20 mg/5 ml)	$22.6 \cdot 10^{-6}$ *M*	Water — 1 l	$0.02 \cdot 10^{-7}$
Sf_3 (20 mg/5 ml)	$22.6 \cdot 10^{-6}$ *M*	Water — 1 l	$6.60 \cdot 10^{-7}$

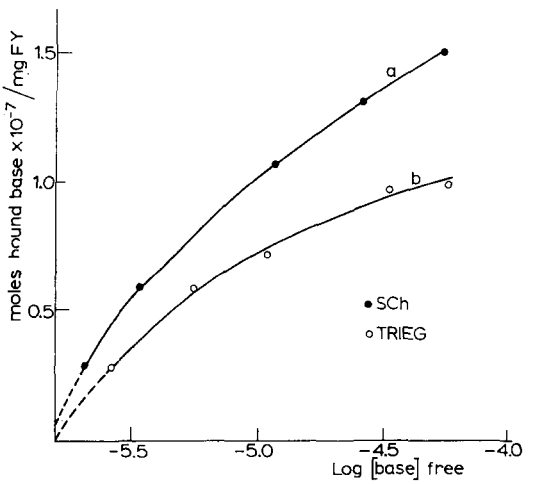

Fig. 8. Comparative binding of [^{14}C]SCh (curve a) and [^{14}C]TRIEG (curve b) by FY containing 25% Sf_3 and 75% Sf_1. 10 mg of FY/5 ml dialysed against base solution in Tris–HCl pH 7.2, 0.001 *M*, 1000 ml. Full line represents range of experimental values.

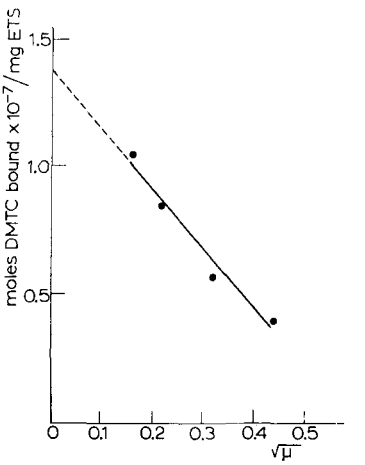

Fig. 9. Influence of ionic strength ($\sqrt{\mu}$) on binding of DMTC by ETS (8 mg/ml). DMTC initial concentration $5 \cdot 10^{-3}$ *M* in sodium phosphate buffer, pH 7.0, ionic strength regulated with NaCl. Solid line represents range of experital measurements and dotted line is extrapolation-to infinite dilution.

With another FY preparation which contained 45% Sf_3 and 55% Sf_1, the binding of TRIEG was also measured at different ionic strengths (0.01, 0.05 and 0.10) and utilizing different TRIEG concentrations as shown in Fig. 10. In this case, increase in ionic strength is a limiting factor only to a certain extent, the values obtained from 0.05 to 0.10 ionic strength reaching a constant level.

TABLE II

BINDING OF CHOLINE ESTERS (SCh AND ACh) BY FAT AND FY AT 4° IN $2 \cdot 10^{-3}$ *M* Tris – HCl, pH 7.4; $2 \cdot 10^{-3}$ *M* SODIUM ACETATE, pH 4.0 AND WATER.

neostigmine employed as ache inhibitor for FAT

"Receptor"	*Choline esters concentration*		*Dialysis media – 2 l*	*AChE**	*Neostigmine concentration/l*	*SCh moles bound/mg (radioact. determination)*	*ACh moles bound/mg (colorimetric determination)*
	[^{14}C]SCh	*ACh*					
			Tris – HCl				
FAT (52 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 7.4	0	$1.36 \cdot 10^{-6}$ *M*	$0.15 \cdot 10^{-7}$	—
FAT (52 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 7.4	96	0	$0.07 \cdot 10^{-7}$	—
FY (18 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 7.4	0	0	$0.27 \cdot 10^{-7}$	—
			Sodium acetate				
FAT (52 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 4	0	$2.6 \cdot 10^{-6}$ *M*	$0.02 \cdot 10^{-7}$	—
FAT (52 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 4	82	0	$0.0014 \cdot 10^{-7}$	—
FY (18 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	$2 \cdot 10^{-3}$ *M*, pH 4	0	0	$0.07 \cdot 10^{-7}$	—
FAT (52 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	Water	0	$1.77 \cdot 10^{-6}$ *M*	$0.38 \cdot 10^{-7}$	—
FY (18 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	—	Water	0	0	$2.23 \cdot 10^{-7}$	—
FY (18 mg/5 ml)	$6.25 \cdot 10^{-6}$ *M*	$27.5 \cdot 10^{-6}$ *M*	Water	0	0	$2.60 \cdot 10^{-7}$	$0.03 \cdot 10^{-7}$
FY (18 mg/5 ml)	—	$27.5 \cdot 10^{-6}$ *M*	Water	0	0	—	$2.47 \cdot 10^{-7}$

* AChE—specific activity in μmoles ACh/h/mg protein.

By the potentiometric titration of FY in different NaCl concentrations (0.01 M, 0.05 M and 0.1 M) and in water[24], we were able to confirm this statement as the electrostatic factor determined from the plot of pH and the calculated degree of association of H^+ has a constant value in 0.05 M and 0.1 M NaCl.

At 0.1 ionic strength, increased TRIEG concentration must be effective for higher binding until saturation of binding sites is reached. This can be observed in Fig. 11

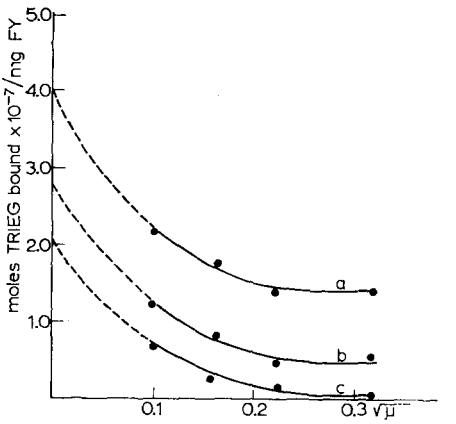

Fig. 10. Binding of TRIEG by FY (55% Sf_1 + 45% Sf_3) at different ionic strengths ($\sqrt{\mu}$) in sodium phosphate, pH 7.0. Ionic strengths regulated with NaCl. 15 mg FY/4 ml dialysed against 4 ml of TRIEG solution. Values obtained are for three concentrations of TRIEG: curve a, $6 \cdot 10^{-3}$ M; curve b, $2 \cdot 10^{-3}$ M and curve c, $0.6 \cdot 10^{-3}$ M. ———, The range of experimental measurements; ·····, represents extrapolation to infinite dilution.

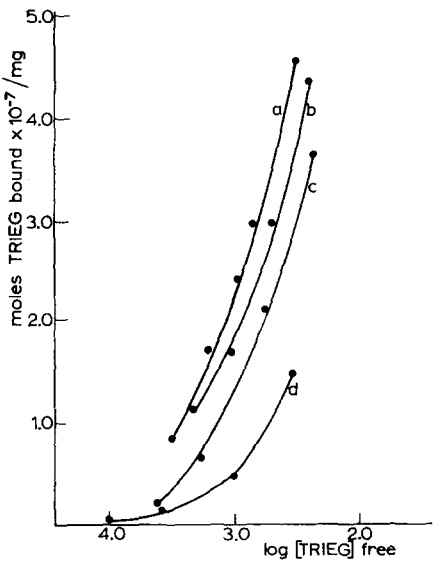

Fig. 11. Comparative binding of TRIEG by purified Sf_3 fraction (curve a) and by FY preparations containing decreasing amounts of Sf_3: 78%, curve b; 67%, curve c and 45%, curve d; sodium phosphate buffer, pH 7.0 and 0.1 ionic strength.

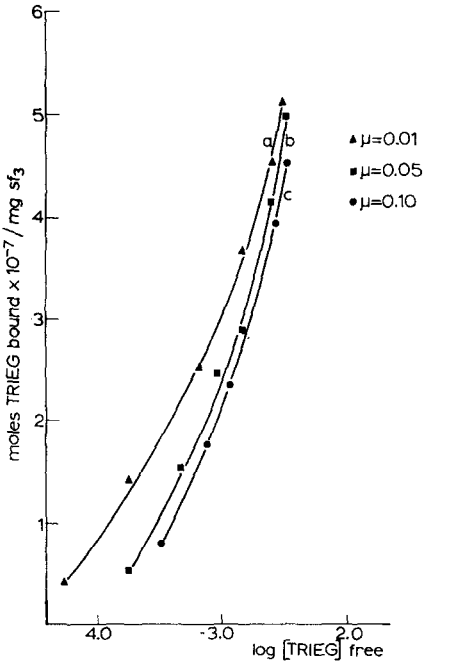

Fig. 12. Binding of TRIEG by Sf_3 in phosphate buffer at pH 7.0 and three ionic strengths (regulated with NaCl): μ = 0.01 (curve a); μ = 0.05 (curve b); μ = 0.10 (curve c). 8 mg Sf_3/4 ml dialysed against 4 ml of TRIEG solution.

where binding extent is plotted against logarithm of free concentration of TRIEG, at constant ionic strength and pH, for several FY preparations, where Sf_3 is present in different concentrations, as indicated, and also with the pure Sf_3 preparation.

The effect of ionic strength on the binding of TRIEG by purified Sf_3, which was also measured with the equilibrium dialysis method, is shown in Fig. 12, where the increased interaction at $\mu = 0.01$ is observed. It can be assumed, for the acid polysaccharide, that the shielding effect of the counter ions in the buffer solution hinders the binding of TRIEG molecules in higher ionic strengths.

Interaction with DEAE fractions

Interaction with DEAE fractions from FAT was measured in function of protein content which was determined spectrophotometrically with a Beckman DU model, by measurement of the light absorption at wave lengths 280 and 260 mμ with the nucleic acid content corrected[18]. The elution diagram and paper electrophoresis (Figs. 3 and 4) of the collected fractions give some idea of their degree of purification.

TABLE III

BINDING OF TRIEG TO FAT FRACTIONS FROM DEAE COLUMN

TRIEG solution: $4 \cdot 10^{-3}$ *M* in sodium phosphate buffer, pH 7.0, $\mu = 0.05$.

Fraction number	*Moles of TRIEG bound/mg protein*
1	$0.62 \cdot 10^{-7}$
2	$2.70 \cdot 10^{-7}$
3	$1.80 \cdot 10^{-7}$
4	$3.10 \cdot 10^{-7}$
5	$2.70 \cdot 10^{-7}$
6	$2.00 \cdot 10^{-7}$
FAT	$0.61 \cdot 10^{-7}$

Carbohydrate analysis indicated also that Sf_1 is in fraction 1 and that Sf_3 was eluted together with AChE and the yellow compound in fraction 6. The binding of TRIEG to these fractions was measured by equilibrium dialysis. They were previously concentrated by perevaporation to a minimum protein concentration of 2 mg/ml and then dialysed against sodium phosphate buffer, pH 7.0, $\mu = 0.05$. 4 ml of each fraction was dialysed against 4 ml of $4 \cdot 10^{-3}$ *M* [^{14}C]TRIEG in the same buffer. Binding results are given in Table III. Fraction 4 has the highest specific activity but fractions 2, 5 and 6, which have at least 3 components, as described, also show significant binding of the base. Paper electrophoresis shows that fractions 2 and 4 are composed of a single protein, and this would mean that higher binding should be expected should one of them be a specific receptor. In no case, precipitation was observed inside the dialysis bag.

Interaction with ammonium sulfate fractions of ETS

Chemical analysis and paper electrophoresis of ammonium sulfate fractions (as shown in Table IV and Fig. 5) did not give evidence of carbohydrates suppression, and neither did they provide higher protein purification in any fraction. Binding of [^{14}C]TRIEG was measured by equilibrium dialysis in sodium phosphate buffer, pH

7.0, $\mu = 0.10$. Results obtained suggest that the supernatant has a higher specific activity per milligram of dry residue and per milligram protein content where the existing proteins have mobilities between β and γ serum globulins. FY prepared from the same ETS solution has an increased specific activity which will be still higher if it is calculated only on the basis of Sf_3 content (45%) in FY. No precipitated material was found inside the bags after equilibrium was attained.

TABLE IV

BINDING OF TRIEG BY ETS; AMMONIUM SULFATE FRACTIONS AND FY

4 ml inside solution dialysed against 4 ml outside TRIEG solution $4 \cdot 10^{-3}$ *M* in sodium phosphate buffer, pH 7.0, $\mu = 0.10$.

"Receptor" mg/4 ml	*Uronic acid* g/100 ml*	*Hexoses** g/100 ml*	*Protein*** g/100 ml*	*ChE activity§ μmoles ACh/h*	*Binding of TRIEG*	
					moles/mg dry residue	*moles/mg protein*
precipitate 30% (25 mg)	0.011	0.068	0.550	0	$0.264 \cdot 10^{-7}$	$0.300 \cdot 10^{-7}$
precipitate 40% (26 mg)	0.008	0.078	0.580	210	$0.277 \cdot 10^{-7}$	$0.313 \cdot 10^{-7}$
precipitate 60% (48 mg)	0.020	0.323	0.890	117	$0.144 \cdot 10^{-7}$	$0.194 \cdot 10^{-7}$
Supernatant (12 mg)	0.021	0.072	0.240	0	$1.00 \cdot 10^{-7}$	$1.25 \cdot 10^{-7}$
ETS– (20.8 mg)	0.0175	0.136	0.385	100	$0.65 \cdot 10^{-7}$	$0.875 \cdot 10^{-7}$
ETS– (41.6 mg)	0.035	0.272	0.770	100	$0.65 \cdot 10^{-7}$	$0.875 \cdot 10^{-7}$
FY§§ (10 mg)	0.050	0.120	0	0	$1.62 \cdot 10^{-7}$ ($3.6 \cdot 10^{-7}$/mg Sf_3)	—

* Uronic acid measured by DISCHE's carbazol method[19].
** Hexoses measured with anthrone reagent[19].
*** Total protein by GORNALL's biuret method[31].
§ Cholinesterase activity by HARGREAVES' titimetric method[20].
§§ FY containing 0.45 mg Sf_3 /mg.

EHRENPREIS described the direct precipitation of the acetylcholine receptor protein[26] with the addition of DTC and DMTC. We have tried the same procedure with the ammonium sulfate fractions at pH 7.5, $\mu = 0.05$, $1.40 \cdot 10^{-2}$ *M* solution of DMTC (1 ml added to 1 ml of the fraction solution) was utilized. Precipitates were obtained at 0° with all fractions and ETS. They were centrifuged, resuspended in sodium barbiturate buffer, pH 8.0, $\mu = 0.1$ and dialysed against 2 changes of the buffer for 24 h and no solubilization was observed. The ionic strength of the buffer was increased to 0.2 with NaCl. Dialysis was continued for another 24 h with 2 changes of buffer and still the precipitates were not dissolved. The interaction was irreversible under these conditions but upon careful addition of 0.2 *N* NaOH to pH 9.0 in an ice bath, they were solublized to a clear solution. The same procedure was repeated with DTC and precipitation of the fractions was obtained upon addition of a 10^{-2} *M* solution. The precipitates which were formed at pH 7.5, $\mu = 0.05$ (sodium phosphate buffer) were also solubilized at pH 9.0 with careful addition of 0.2 *N* NaOH, in an ice bath; with a $5 \cdot 10^{-2}$ *M* solution of TRIEG no precipitate was formed. The curare action, towards the electric eel, with these three drugs is in the proportion of 5 (TRIEG):2 (DTC):1 (DMTC) mg/kg what does not coincide with precipitating ability towards the described extracts.

Precipitation of purified proteins and Sf_8

As the precipitation with *d*-tubocurare of a receptor protein has been described[26] as a specific reaction, on the basis of the precipitation results with FAT and ETS proteins, it seemed interesting to determine whether other proteins, commercially available, would also precipitate. Under the described conditions, *i.e.*, at pH 7.5 and $\mu = 0.05$, assays were performed with 2 ml of 0.2% protein solutions which were first dialysed against sodium phosphate buffer. Crystallized beef insulin (Armour laboratory), crystallized trypsin (Bios. laboratories), crystallized pepsin (Bios. laboratories), bovine serum albumin (Armour laboratory) and a purified preparation of acetylcholinesterase from the electric organ were assayed.

The same solutions of DTC, DMTC and TRIEG used with the ammonium sulfate fractions were employed.

Results concerning intensity of precipitate formation are described in Table V utilizing EHRENPREIS notation[26]. All the formed precipitates were not dissolved until the pH was brought to 9.0–9.2 with alkali. From the qualitative results concerning precipitation ability, insulin is more active than the other proteins. Insulin activity towards the two bis-quaternary compounds parallels that of the described specific receptor[26] in view of the amount of precipitate formed at low protein concentration (0.1 mg insulin/ml can precipitate with 2 mg of DTC) and influence of pH in the extent of precipitation.

TABLE V

QUALITATIVE PRECIPITATION OF PROTEINS WITH THE CURARIZING BASES

Protein solutions 0.2%, in phosphate buffer, pH 7.5, $\mu = 0.05$. To 1 ml of protein solution was added 1 ml of the bases solution.

Preparation	*DTC* $1 \cdot 10^{-2} M$	*DMTC* $1.4 \cdot 10^{-2} M$	*TRIEG* $5 \cdot 10^{-2} M$
Insulin	+++++	+++	—
Pepsin	+++	+	—
Acetylcholinesterase	+++	+	—
Trypsin	—	—	—
Serum albumin	—	—	—

Pepsin too is precipitated with DTC although the same author did not confirm it. The most interesting result is that highly purified acetylcholinesterase (prepared at Dr. A. B. HARGREAVE'S laboratory through phosphate gel adsorption and further magnesium sulfate fractionation) also precipitates with DTC which is not recognized as an inhibitor of the enzyme. TRIEG which is also a potent tris quaternary curare failed to precipitate these proteins. Trypsin and serum albumin did not precipitate with these quaternary bases.

The fact that insulin, which is devoid of phosphate groups, interacts so actively with the curares does not confirm the assumption[26] that phosphate groups are involved. It seems that the relative proportion of free carboxyl groups to the free cationic groups in the protein is an important factor as the precipitation results agree with the distribution of such groups in proteins[27].

Sf_8 also is precipitated with DTC, at pH 7.5, phosphate buffer, $\mu = 0.05$. To 5 mg Sf_8/ml were added 5 mg of DTC (0,72 ml of $1 \cdot 10^{-2} M$ solution). Turbidity was

first observed and, after standing, at 0°, a flocculent precipitate was formed. Under the same conditions, interaction with DMTC formed a smaller precipitate.

DISCUSSION

The data presented here suggest that the *in vitro* interaction of quaternary ammonium bases is not confined to a determined macromolecular configuration Electrostatic forces seem to act principally in the interactions of these bases with the described polyacid, Sf_3, hyaluronic acid and chondroitin sulfuric acids. These interactions are also observed with other kind of polyelectrolytes and some polyampholytes, bearing a negative charge, to which belong some kinds of proteins and the nucleic acids.

On the basis of our results no doubt exists that the purified acidic polysaccharide has the ability of binding a greater number of molecules of the ammonium bases in solution.

The observed interaction also exists at higher ionic strengths and can be compared with the results previously presented at zero ionic strength. It has been demonstrated that binding is a function of the ionic strength, the pH of the solution and of the base concentration. In view of the molecular characteristics of the polyacid which changes with chemical factors and its well known property of coiling-up as the ionic strength increases and expanding to a stretched condition in the absence of electrolytes, the higher interaction under the latter conditions can be explained. In water solutions, for instance, there is no counter-ions effect and all the carboxyl groups will be charged increasing the intramolecular repulsion, the molecule reaching its fully extended form, thus more binding sites being be available.

Much information can be gathered from the work of KATCHALSKY[28] and MATHEWS[13] about the behaviour in solution of polyelectrolytes.

Although no specific biological function has been attributed to such polyelectrolytes like hyaluronic acid, the knowledge of their interaction in solution with the ammonium bases may contribute to the knowledge of the action of these substances. The non specificity would not invalidate the hypothesis that owing to their regular structure and number of acid groups, these molecules can at least control cation exchanges between the body vessels and the cells. Many other physical aspects of their role in biological function could be reminded here, as for instance, in water retention and in the diffusion and exchange of small molecules. In the *in vivo* studies of neuromuscular transmission in the electric organ its distribution should be kept in mind as the acid polysaccharide is regularly present in this tissue[29] and may compete with any other receptor for the quaternary bases.

The objections presented by NACHMANSOHN[10] and EHRENPREIS[8] to the finding that Sf_3 and other acid polysaccharides could bind TRIEG, SCh and ACh, as it was described[4,5], were that complexes obtained at low ionic strenghts, were due to a Donnan effect and to Van der Waals forces. That this is not the case was demonstrated by the data presented above in which binding was measured at higher ionic strengths, under controlled pH conditions. The physical properties of the polyelectrolytes described give a satisfactory explanation for the results obtained by CHAGAS *et al.* with dialysis of [^{14}C]TRIEG complex extracted from the electric organ after *in vivo* experiments. The same dependence of binding extent on ionic strength was obtained in the potentiometric titration of Sf_3 which will be the subject of a

separate publication[24]. The pK of the polyacid changes with the ionic strength of the NaCl solution in which titration was performed. At $\mu = 0.1$ a major number of Na^+ is bound, decreasing the intramolecular Coulombic repulsion, and only 50% of the carboxyl groups are titrated.

In the absence of electrolytes all the carboxyl groups are titrated and $12 \cdot 10^{-7}$ moles of H^+ are bound to 1 mg of Sf_3. At $\mu = 0.1$, only $4.5 \cdot 10^{-7}$ moles of TRIEG are bound by 1 mg when the free base concentration is $2.3 \cdot 10^{-3}$ M. This would mean that 37.5% of the binding sites are available for the tris-quaternary derivative, or that 1 mole of it interacts with more than one anionic site.

From the work of UNNA[30] with a TRIEG analogue containing two quaternary ammoniums and a phenone group substituting the middle chain it was concluded that the curare activity of TRIEG is due to two quaternary nitrogen atoms; the third one, in the middle position, is not essential for curare-like action.

The presence of the base, as expected, contributes also for a change in the molecular configuration of the polyacid in accordance with results of potentiometric titration[24].

The results reported for protein fractionation with ammonium sulfate demonstrate that wide distribution of components is obtained in all fractions and a subsequent fractionation must be necessary.

The precipitation technique[26] for isolating a specific receptor is not the ideal method. Many types of proteins can precipitate with curare, as in the case of insulin and of acetylcholinesterase for which the electric organ is a rich source. Probably proteins containing phosphate groups will be more difficult to solubilize after precipitation with curare but the necessary presence of the prosthetic group for stronger interaction has to be proved. Curarization *in vivo* is a reversible phenomenon, thus it must not be necessarily related to a difficultly soluble precipitate; dilute acid and base and concentrated urea solution (3 M urea) have been described[26] as solvents for the so-called "specific receptor"–curare precipitated complex. Mild conditions for protein fractionation should instead be employed and evidence of purification by the currently used biochemical methods should be given.

High specificity of interaction should be significantly greater than the one obtained for Sf_3 and the other macromolecules described in the present paper, if one wishes to admit its participation as a specific receptor in the biological sense.

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