in an increase in the time needed for the onset of lysis. On the other hand, infection with a high multiplicity of T2r produced lysis, whereas a low multiplicity of infection resulted in the complete inhibition of lysis. In order to test the production of infective particles under conditions of multiple infection with T2r, samples of a logarithmically growing culture were infected at two different ratios of phage to bacteria, namely, 1:5 and 2:1. Table I indicates that the lysis caused by multiple infection with T2r in the presence of fluorouracil is not accompanied by the release of infective particles.

Since under these conditions the lysis of a cell does not release infective phage, a noticeable drop in turbidity is not to be expected when only a small proportion of the cell population is infected with T2r. If, on the other hand, the first cells to be infected do give rise to infective particles, these will infect the remainder of the population and cause visible lysis regardless of whether new phage particles are or are not synthesized in this second cycle of infection. In all subsequent experiments on lysis, infection was performed at a ratio of one phage per ten bacteria so that less than 10 % of the cells would be infected in the first cycle.

In order to test the effects of the duration of fluorouracil treatment, the substance was added (a) 10 min before the addition of phage, (b) together with the phage, or (c) 12 min after the addition of phage. Fig 2 indicates that the effect of fluorouracil on

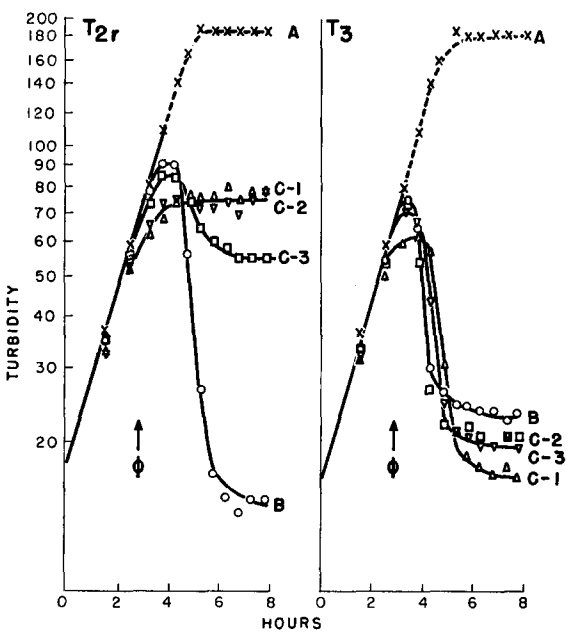

Fig. 2. Effects of duration of treatment with 5-fluorouracil. A, ×—×, control; B, ○—○, infected in absence of 5-fluorouracil; C, 50 μg/ml 5-fluorouracil added: △—△, 10 min before infection; ▽—▽, at time of infection; □—□, 12 min after infection.

lysis induced by T3 is only due to the depression of cell growth. In cultures infected with T2r—although fluorouracil completely inhibits lysis when added simultaneously with phage or before phage addition—some lysis does occur when the compound is added after the phage. This interval—12 min after infection—is about the time of appearance of the first intracellular phage particles[14,15]; and this may be taken to

compounds insofar as their effect on nerve and muscle is concerned, the interpretations being based on interactions with both the enzyme and receptor protein. Thus, the reaction of a number of tertiary and quaternary nitrogen compounds with cholinesterase and their effects on electrical activity of various cells have certain features in common[3,4]. As a further extension from the work on the enzyme, it was postulated that the apparent configurational changes of cholinesterase effected by acetylcholine during hydrolysis[5] might, if paralleled by similar changes in the receptor protein, provide a molecular mechanism for the altered ion permeability associated with nerve conduction[1].

While the notion of a receptor protein is a widely accepted one, direct experimental evidence for its existence in any sort of isolated form has been completely lacking, all data in this field having been obtained exclusively with living cells. A notable attempt to obtain the receptor in solution has recently been made by CHAGAS *et al.*[6,7]. Those workers injected radioactive triethiodide of gallamine—a curare-like substance—into electric eels, extracted material from the electric organ, a tissue known to be rich in receptor, and considered the retention of the radioactivity after exhaustive dialysis of the extract against distilled water as the basis for receptor activity. As has been found[8–10] and will be elaborated on below, a number of macromolecules, such as acidic polysaccharides and nucleic acids, bind curare—and presumably TRIEG—non-specifically, although such complexes behave in a manner similar to that described by CHAGAS *et al.*, *i.e.*, slow dialyzability of the curare in distilled water, rapid dialysis in dilute salt. Recognizing this fact, these investigators have now concluded that their findings may have uncovered nonspecific receptors for curare or TRIEG rather than the cellular component with which ACh reacts during electrical activity[11]. This interpretation has recently been stressed by CHAGAS at a recent symposium in Rio de Janeiro. In this report, evidence will be presented that a protein is present to a small extent in the extract of electric tissue and muscle which interacts much more strongly with curare. The high affinity of this substance for curare, differentiating it from other curare-binding components, gives rise to the intriguing possibility of a physiological role for this protein.

In pursuing the problem of attempting to isolate the receptor, CHAGAS' procedure has been modified in two important aspects: First, after extracting the electric tissue with dilute buffer, an ammonium sulfate fractionation procedure was undertaken in order to eliminate any non-protein components such as free acidic polysaccharides or nucleic acids present in the extract. Next, equilibrium dialysis has been used under controlled conditions of pH and ionic strength to study the interaction of various neurotropic compounds rather than exhaustive dialysis against distilled water which introduces complications due to DONNAN effect. As a check on the specificity of interaction, binding studies were conducted with bovine serum albumin and chondroitin sulfate. Whenever possible, results in solution were compared with those obtained from studies on single cells of the electric organ, although the difficulties of precise comparison are obvious.

METHODS AND MATERIALS

Preparation of tissue proteins for various studies

The method adopted for obtaining the proteins of the electric tissue in solution is as follows: The main organ of electric tissue was excised from the freshly killed

animal and freed as much as possible from muscle, connective tissue, blood, etc. If not used immediately, the tissue was stored at —15° at which temperature it can be kept for a long time with no evidence of decomposition. Extraction of the proteins was carried out as follows: Small portions of the frozen or fresh tissue were cut with scissors, placed in ice cold phosphate buffer at pH 7.5, ionic strength 0.1, containing a small amount of merthiolate and homogenized for about 5 min in a Waring blender. The volume of buffer was 1 to 1.5 ml/g of tissue. The mixture was shaken overnight to complete the extraction after which it was centrifuged at 4000 rev./min at 0° for about 1 h. Coagulated fat, which floats to the surface, was removed by aspiration. The residue was stored at —15° and was used without further treatment. The supernatant was concentrated to about one third of the original volume by pervaporation at 0°; after that it was dialyzed first against running tap water, then against dilute phosphate buffer followed by fractionation of the proteins with ammonium sulfate as described below.*

TABLE I

FRACTIONATION OF ELECTRIC TISSUE EXTRACT WITH AMMONIUM SULFATE

% saturation ammonium sulfate	*% of total protein precipitated*	
	Undiluted extract	*After 5 fold dilution*
20	4	0
30	27	20
40	44	38
50	55	44
60	60	44

Preliminary ammonium sulfate fractionation was carried out as follows: To the undiluted extract containing about 2 % protein and to a 5-fold dilution of the extract varying amounts of saturated ammonium sulfate were added to give 20, 30, 40, 50 and 60 % saturations. After standing overnight in the refrigerator, all solutions were centrifuged and the O.D. of the supernatants determined at 280 mμ. Table I shows (a) that the composition of each fraction depends quite markedly on the protein concentration and (b) that even at 60 % saturation a considerable amount of material remains in the supernatant with more being present at the lower protein concentration. Since a greater number of fractions were obtained at higher protein concentrations (about 2 %), all large scale fractionations were carried out with the tissue extract at about this protein concentration.

In this procedure the saturated ammonium sulfate was added dropwise to the ice cold extract to obtain 30 % saturation, the solution was placed in the refrigerator for about 1 h to complete precipitation after which it was centrifuged at 4000 rev./min for about 1 h. The precipitate was suspended in 30 % ammonium sulfate, recentrifuged and dialyzed first against running tap H_2O, then against phosphate buffer to remove the sulfate. Additional saturated ammonium sulfate was added to the supernatant to 40 % saturation, followed by the above treatment. This was repeated at intervals of 10 % saturation up to the 60 % level, except that with increasing saturation much longer periods of time (up to one day) were required to complete

* Pre-extraction of the tissue with toluene, used in the preparation of cholinesterase to remove mucin[12], did not improve the yield of extractable protein.

precipitation. 60 % saturation was considered the highest feasible ammonium sulfate concentration for obtaining a precipitate. In another procedure, the ammonium sulfate concentration was adjusted directly to either 30 % or 60 % saturation followed by centrifugation, washing etc. These precipitates are referred to as 30 % and 60 % fractions.

In order to determine whether precipitation was complete in the time allotted, an aliquot of each supernatant fraction was removed and placed in the refrigerator for several days. In no instance were additional precipitates observed.

In later procedures, especially for the preparation of 30 % fraction, solid ammonium sulfate was added to the ice cold protein solution with constant stirring to give the desired saturation.

Isolation of receptor protein by curare precipitation from the 30 % fraction

Although the procedure to be described is applicable to any fraction of the extract containing receptor protein, use of the 30 % fraction markedly reduces the amount of curare needed. All steps are done at 0°. The 30 % fraction was thoroughly dialyzed against phosphate, pH 7.5, $\mu = 0.1$ (hereafter referred to as the phosphate buffer), followed by centrifugation at 20,000 rev./min to remove insoluble material. To the clear solution obtained, a concentrated solution of *d*-tubocurarine chloride (50–100 mg/ml) in distilled water was added until precipitation was complete. The approximate amount of curare required was determined before-hand on 1-ml aliquot of the protein solution. A small excess of curare was added to the bulk of the solution to insure completeness of precipitation. The suspension was kept at about 0° overnight and centrifuged at 4000 rev./min for about 30 min. The resulting precipitate was suspended in the phosphate buffer and dialyzed for several days against the same buffer, after which the suspension was centrifuged as before and the precipitate washed with the buffer. The precipitate was resuspended in Tris buffer, $\mu = 0.3$, pH 9, and dialyzed against this buffer until solubilization was complete (about 3 days). Dialysis was then continued against the phosphate buffer to adjust conditions to those used for binding studies or against 0.02 ionic strength buffer for lyophilization purposes.

Source of compounds used in binding studies

d-Tubocurarine dichloride obtained from K and K Chemical Co., was purified according to DUTCHER's procedure[13]. Chloride analysis revealed that the samples used were about 99 % pure. Dimethyl *d*-tubocurarine diiodide was a gift from Dr. O. K. BEHRENS of Eli Lilly Company. Its u.v. absorption spectrum and extinction coefficients were virtually identical with the same compound synthesized from our *d*-tubocurarine by the Tracerlab Co. of Boston, Mass. Prostigmine bromide was a gift from Dr. J. A. AESCHLIMANN, Hoffmann LaRoche. Atropine sulfate, eserine salicylate (Merck), methyl pyridinium iodide (K & K), succinylcholine dichloride (Winthrop-Stearns), and the various other choline derivatives were the commercially available compounds used. PAM and other pyridinium derivatives were synthesized by Dr. S. GINSBURG of this Laboratory.

Analytical procedures

Protein concentration was determined from u.v. O.D. using extinction coefficients

which were based on dry weight. The u.v. absorption spectrum of the tissue extract is not that of a typical protein, *e.g.*, bovine serum albumin. As shown in Fig. 1 neither the 280 mμ maximum or 250 mμ minimum are exhibited by the extract; prolonged dialysis seemed to alter the spectrum somewhat. In dilute alkaline solution (pH 12) spectral changes were observed although once again different from those encountered

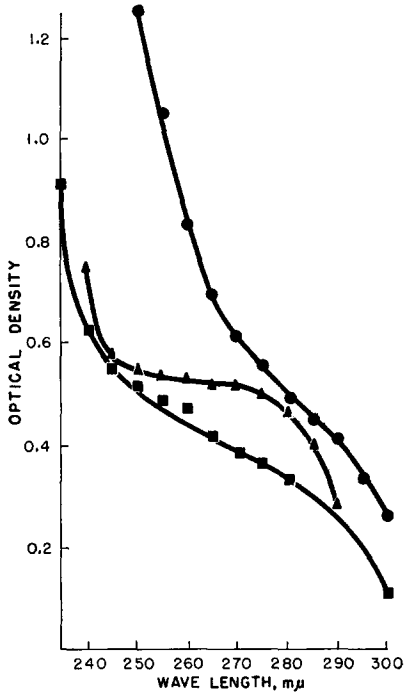

Fig. 1. U.v. absorption spectra of electric tissue extract, obtained from Electrophorus electricus. ■, undialyzed, diluent phosphate, pH 6.5; ▲, dialyzed, diluent phosphate, pH 6.5; ●, dialyzed, diluent 0.1 *M* NaOH.

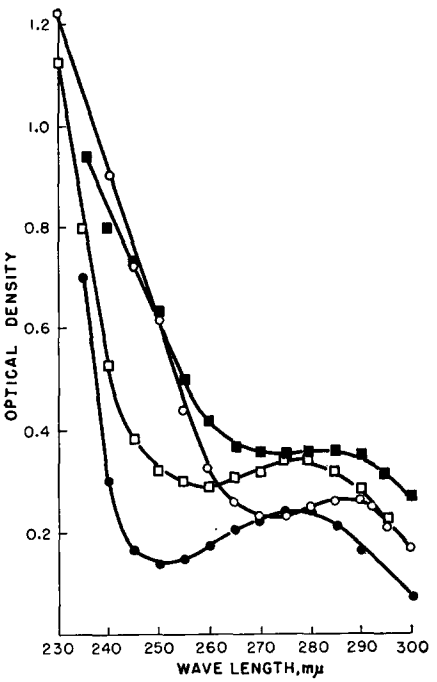

Fig. 2. U.v. spectra, 40% ammonium sulfate fraction and purified receptor. ●, 40% fraction, in 0.1 *N* HCl; □, receptor, 0.01 *N* HCl; ○, 40% fraction, in 0.1 *N* NaOH; ■, receptor, in 0.1 *N* NaOH.

TABLE II

U.V. ABSORPTION CHARACTERISTICS OF THE VARIOUS COMPOUNDS USED

Compound	*λ Max*	E_{mg}, *1 cm cell*
d-tubocurarine dichloride (curare)	280	11.21
Dimethyl *d*-tubocurarine diiodide (dimethyl curare)	280	8.0
Eserine salicylate	300	15.2
Prostigmine bromide	260	1.53
Atropine sulfate	260	0.43
Procaine hydrochloride	280	6.39
Pyridine 4 aldoxime methiodide	280	68.34
Pyridine 2 aldoxime dodeciodide	285	22.36
Hexamethylene bis-4 PAM	280	76.50
Decamethylene bis-4 PAM	280	70.14
Methyl pyridinium bromide	260	17.54
Cetyl pyridinium chloride	260	11.17
Hexamethylene bis-pyridinium	260	21.48
Decamethylene bis-pyridinium	260	19.94

with most proteins. However, the absorption spectrum of all the ammonium sulfate fractions as well as the purified receptor fraction were normal both at low and high pH (Fig. 2) although the shift occurring in alkaline solution was still not as pronounced as with bovine serum albumin. Whether this indicates a large proportion of H-bonded tyrosine residues, still only partially dissociated even at pH 12, has not been determined. A pH of 13 might be required to titrate all the tyrosyl groups as found by CRAMMER AND NEUBERGER for egg albumin[14].

U.v. analysis was also used to determine concentrations of many of the compounds studied. Table II lists the wave lengths used as well as the extinction coefficients. Analyses were performed in 0.01 *M* HCl to avoid spectral changes which may occur with variations in pH, especially in the case of curare.

Acetylcholine was determined by HESTRIN's colorimetric method[15]. Benzoylcholine was analyzed both by this method as well as by u.v. absorption. For the colorimetric determinations, calibration curves using known concentrations of these esters were run along with the unknowns.

Equilibrium dialysis

These experiments were performed essentially according to the method of KLOTZ *et al.*[16]. Cellophane bags prepared from 6 mm Visking dialysis tubing were filled with 1 ml of the protein solution and equilibrated with 1 or 3 ml of a solution containing the neurotropic compound. Most of the experiments to be reported were carried out in the phosphate buffer at 0°. Analysis of the outside solution consisted of diluting 0.1 or 0.2-ml aliquots with the appropriate volumes of either 0.01 *N* HCl or distilled water for u.v. or colorimetric determination respectively. These analyses were done on successive days after setting up the experiment. In many instances, the contents of the bags were analyzed at equilibrium to determine the inside concentration of diffusible compound, thus providing a direct measure for the extent of binding.

The following controls were set up for each experiment: the protein solution dialyzed against buffer, solution of neurotropic compound against buffer and buffer against buffer. The first control was necessary since u.v. absorbing and ninhydrin reacting material frequently appeared in these dialyzates. From control 2 it was established that no significant binding of the various compounds to the dialysis bag had occurred and that at equilibrium the concentration of diffusible compound inside and outside of the bag were identical within experimental error. On the other hand, it could be shown that in some instances, *i.e.*, at concentrations above 10^{-3} *M* the formation of micellar aggregates evidentally prevented equilibration across the membrane of both CPC and PAD. Control 3 was necessary to correct for the small but significant amount of u.v. absorbing material elaborated from the bags themselves.

Calculation of the amount of binding was based on the outside concentration of the compound when dialyzed against the protein as compared with the concentration in the buffer control. These values were in good agreement with those obtained from analyses of the inside solutions of the protein set ups at equilibrium.

Experiments involving the various choline derivatives were performed in the presence of 20 μg of TEPP in order to eliminate acetylcholinesterase activity. Although the amounts of enzyme are extremely small in absolute values, enough is present to interfere with experiments carried out over a period of many hours. Analysis by titration at constant pH after treatment with TEPP revealed the complete

absence of enzymic activity at the beginning of the experiment. However, about 5 days later a small fraction of the activity had returned. It is assumed that during the first 3 days of the experiment no activity was present since the concentrations of the choline compounds in dialyzates on two successive days remained constant.

Precipitation studies

To determine the combining ratio of curare with the receptor protein the following procedure was used: The protein was exhaustively precipitated with a concentrated solution of curare. After centrifuging and washing, the precipitate was dissolved with a known volume of dilute HCl and the O.D. at a series of wave lengths determined. From the extinction coefficients of curare and the protein the concentration of both could be determined in the mixture. Alternatively, protein concentration was determined by the ninhydrin method[17].

Many qualitative precipitation reactions were carried out with the receptor protein, chondroitin sulfate, bovine serum albumin, egg albumin and casein.

RESULTS

The primary aim of the investigations reported in this paper was to isolate the protein presumably reacting in the intact cell with acetylcholine and other neurotropic compounds and which may be responsible for the biological effects of acetylcholine action. This required two different lines of experiments: (a) testing the proteins of the tissue in terms of their ability to bind curare and other neurotropic compounds and (b) identification of the protein as the receptor. A few preliminary observations have been reported previously[18,19].

Curare interaction by equilibrium dialysis

The first studies using unfractionated tissue extract revealed that there was no

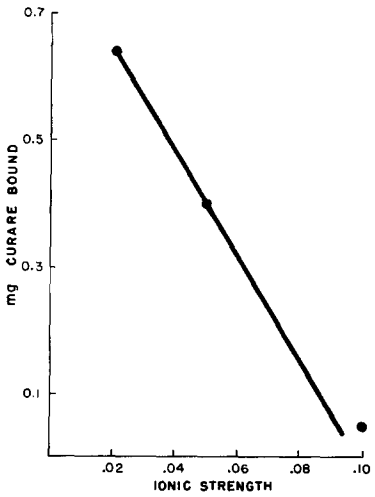

Fig. 3. Binding of curare to 60% fraction as a function of ionic strength at pH 7.5. Initial curare concn., 0.3 mg/ml. Phosphate, 0.02 μ with NaCl contributing the remaining ionic strength.

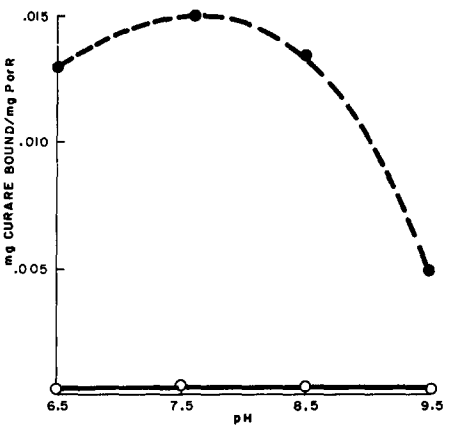

Fig. 4. ●, binding of curare to 60% fraction; ○, to tissue residue, as a function of pH at ionic strength 0.1. Initial curare concn., 0.3 mg/ml. Ordinate: mg curare bound/mg protein (P) or tissue residue (R).

significant binding of curare at an ionic strength of 0.1. This finding may be explained by the fact that the concentration of receptor present in the intact extract is too small to provide for measurable interaction. Complexing to the 60% fraction at 0.1 ionic strength could be readily demonstrated (Fig. 3), binding being greatly enhanced by decreasing ionic strength. Fig. 4 shows that binding to the 60% fraction is optimal at pH 7.5 as well as the fact that binding to the residue remaining after extraction was so negligible that this material was not used for further study.

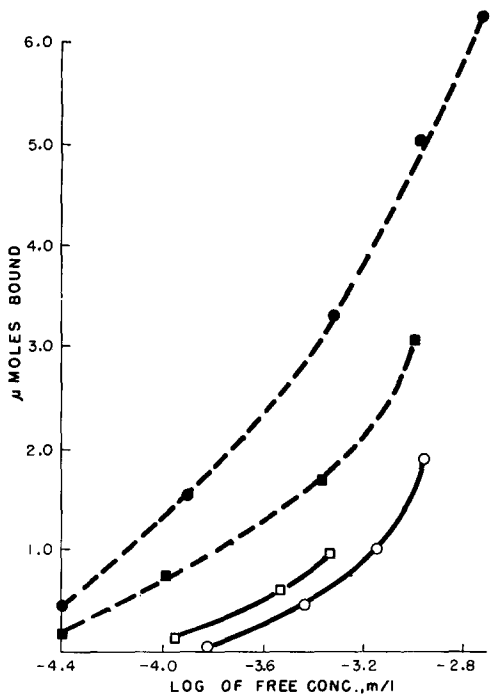

Fig. 5. Comparative binding of curare and dimethyl curare at pH 7.5 (phosphate) and pH 9.5 (borate); μ = 0.02. ●, curare, pH 7.5; ■, dimethyl curare, pH 7.5; ○, curare, pH 9; □, dimethyl curare, pH 9.

Fig. 5 gives a plot of data on the interaction of curare and dimethyl curare to the 60% fraction at pH 7.5 and 9.5. At pH 7.5 the affinity of the methylated derivative is less than that of curare; at pH 9.5 the situation is reversed, probably due to ionization of the phenolic hydroxyl groups of curare.

Curare interactions by precipitation

From equilibrium dialysis experiments in which binding to the 60% fraction as a function of curare concentration was determined, it was observed that use of high curare concentration gave rise to precipitates in the dialysis bag. Since precipitation, if specific enough, might be useful as a quick measure of receptor, it was decided to investigate this property of curare more extensively. In extending these studies it was found that curare not only precipitates material in the extract, but RNA, DNA and chondroitin sulfate A as well—the latter reaction having been studied rather extensively and reported elsewhere[8-10]. Precipitation of both chondroitin sulfate and

the component of the 60% fraction had the features in common that, at fairly low concentration of macromolecule, precipitation was surpressed by increasing μ (Table III). This was in complete agreement with the equilibrium dialysis data. However, the character of the precipitates once formed was found to be radically different insofar as lability to salt was concerned. Thus while the curare—chondroitin

TABLE III

EFFECT OF IONIC STRENGTH ON PRECIPITATION OF COMPONENT OF TISSUE EXTRACT AND CHONDROITIN SULFATE A BY CURARE

Final curare concn., 5 mg/ml, pH 7.5.

Ionic strength	*Unfractionated tissue extract*	*60% fraction*	*Chondroitin sulfate*
0.006	Dense precipitation	Precipitation	Precipitation
0.11	Precipitation	Turbid	Slight precipitation
0.16	Slight precipitation	Clear	Slight turbidity
0.20	Slight precipitation	Clear	Clear
0.26	Clear	Clear	Clear

sulfate precipitate was readily soluble in dilute salt and could be rapidly solubilized by dialysis against phosphate buffer at an ionic strength of 0.1, the tissue component—curare precipitate was quite stable to increases in ionic strength and failed to dissolve to any significant degree despite prolonged dialysis against the phosphate buffer. Both types of precipitate were readily soluble in dilute acid and base as well as in 1–2 *M* urea (Table IV), the latter suggesting that precipitation involves H-bonds. In accordance with these observations, dialysis of the curare—tissue component precipitate against buffer at pH 9, at which pH the phenolic

TABLE IV

EFFECT OF UREA ON CURARE PRECIPITATION

Urea Concn., M	*60% fraction*	*Chondroitin sulfate A*
0	Precipitation	Precipitation
1.0	Precipitation	Turbid
2.0	Turbid	Clear
3.0	Clear	Clear
4.0	Clear	Clear

hydroxyls of curare are ionized, resulted in complete solublization. Tris buffer is preferable, although solubilization in borate buffer at the same pH could also be achieved. However, it was later found that borate actually potentiated the interaction of curare with some proteins. Thus bovine serum albumin which ordinarily reacted weakly with curare (see below), precipitated with that substance in the presence of borate. The procedure used for isolating the receptor protein in solution depended on the lability of the precipitate in high pH buffer as previously described.

It is to be noted that curare precipitation of the receptor protein occurred at much lower concentrations of both reactants than with other extract fractions, indicating a high degree of purification (Table V).

At room temperature, curare at very high concentrations failed to precipitate

egg albumin, bovine serum albumin, pepsin, trypsin and lysozyme. Precipitation with isoelectric, unfractionated casein, as well as with the α and β fractions of that protein* were observed and will be discussed below. At 60°, curare caused a rapid rise in the viscosity of bovine serum albumin and subsequent precipitation of the protein.

As an indication of the usefulness of curare precipitation, the data of Table VI may be cited. This experiment shows quite clearly that the major portion of the curare precipitating component, later identified as the receptor protein, is present in the 30 % ammonium sulfate fraction. By contrast, the long chain quaternary CPC, which also reacts with the receptor, yielded precipitates in all fractions tested. Evidentally, precipitation by this compound is far too non-specific to be of use either in localizing or in isolating the receptor.

Precipitation studies using other quaternary compounds

Table VII summarizes the results of a great number of precipitation studies involving a large number of mono- and bis-quaternary compounds, all of which interact with the receptor as shown by studies on intact cells. These compounds seem to fall into three distinct types insofar as their precipitating ability is concerned: (a) Non-precipitating quaternary and bis-quaternary compounds. (b) Bis-quaternary compounds with a relatively high degree of specificity, *i.e.*, they do not precipitate egg albumin. These include curare, dimethyl curare, stilbamidine and decamethylene bis-PAM. (c) Mono-quaternary compounds which precipitate with a lesser degree of specificity. Examples include DPC, PAD, and nor-acetylcholine 12.

Binding studies with other compounds

All of these studies were carried out at pH 7.5, $\mu = 0.1$, using various ammonium sulfate fractions as well as the receptor protein. Results using several series of compounds are shown in Figs. 6–8.

DISCUSSION

The results presented in this paper show that it has been possible to isolate and to identify the acetylcholine receptor.

Several criteria for identification of the receptor have been used in the present investigations. The molecule must first of all have the ability to bind acetylcholine analogues and related compounds in a graded fashion consistent with that demonstrated by their affinity for the receptor in the intact electroplax. Based on studies in solution, it should be possible to make predictions about the effectiveness of previously untested compounds on the electrical activity of the electroplax. Effects of pH, ionic strength, specific ions, temperature etc., on binding in solution should yield results which agree at least in a qualitative way with observations on the living cell. The material must be obtained in quantities compatible with the information available about the amount in the cell.

Binding of various compounds in solution and the relation to their effects on electrical activity

The affinity of the various compounds to the proteins of the 30 % and 60 % ammonium sulfate fractions as well as to the curare precipitating protein has been

* Generously provided by Dr. G. PERLMANN of the Rockefeller Institute, New York City.

TABLE V

SENSITIVITY OF VARIOUS PREPARATIONS TO CURARE PRECIPITATION

pH 7.5, $\mu = 0.1$.

Preparation	*Protein concn. mg/ml*	*Curare concn. for first ppt. mg/ml*
Tissue extract	25	10.5
60% fraction	23	6
30% fraction	4	4.5
Receptor	0.08	2

TABLE VI

PRECIPITATION OF VARIOUS AMMONIUM SULFATE FRACTIONS BY CURARE AND BY CPC

$(NH_4)_2SO_4$ fraction	*Curare*	*CPC*
0–30% saturation	++++	++++
30–40% saturation	+	++++
40–50% saturation	—	++++
50–60% saturation	—	++++

TABLE VII

QUALITATIVE PRECIPITATION REACTIONS BETWEEN VARIOUS COMPOUNDS AND A NUMBER OF MACROMOLECULES

	Receptor protein	*Unfractionated Casein*	*BSA*	*Egg albumin*	*Chondroitin sulfate*
Curare	++++	++++	— (++ at 60°)	—	++++
Dimethyl curare	+	+	—	—	+
Prostigmine	Negative throughout				
Eserine	Negative throughout				
Atropine	Negative throughout				
Decamethonium	Negative throughout				
Acetylcholine	Negative throughout				
Carbamylcholine	Negative throughout				
Benzoylcholine	Negative throughout				
Butyrylcholine	Negative throughout				
Noracetylcholine 12	+++	+++	—	+++	+++
Norcholine 12	+++	+++	—	+++	+++
Tetrabutyl ammonium	—	—	—	—	—
Methyl pyridinium	—	—	—		—
Dodecyl pyridinium	+++	+++	—	++	+++
Cetyl pyridinium	++++	++++	—	+++	++++
Hexamethylene bis-pyridinium	—	—	—		—
Decamethylene bis-pyridinium	±	±	—		—
Hexamethylene bis-4-PAM	±	++	— (— at 60°)		—
Decamethylene bis-4-PAM	++	++	— (+ at 60°)		—
Stilbamidine	++	++	— (++ at 60°)		++

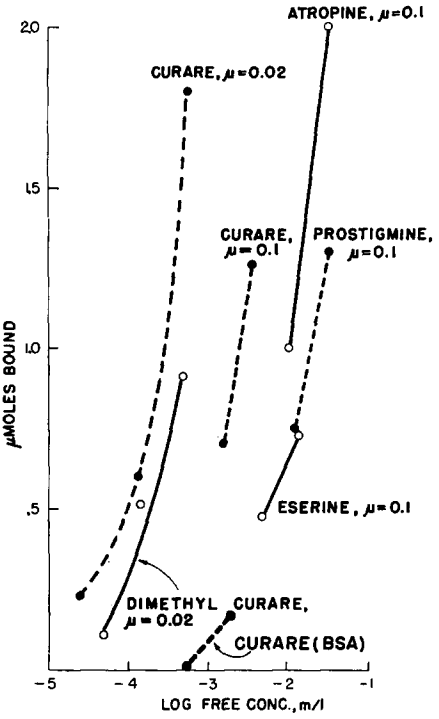

Fig. 6.

Fig. 6. Binding of curare and related nitrogen compounds to 60% fraction, pH 7.5, $\mu = 0.1$ (phosphate). Protein concn., 15 mg/ml. The various compounds are indicated in the figure. Binding of curare and dimethyl curare at $\mu = 0.02$ is shown for comparison. Also shown is the binding of curare by BSA, 10 mg/ml, under similar conditions.

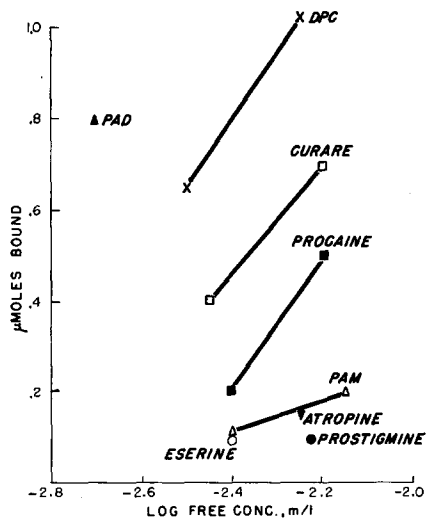

Fig. 7.

Fig. 7. Binding of curare and related compounds to 30% fraction, pH 7.5, $\mu = 0.1$ (phosphate) protein concn., 4.5 mg/ml.

Fig. 8. ●, binding of acetylcholine, ○, binding of benzoylcholine, to purified receptor. A single point (■) is shown for acetylcholine interaction with BSA.

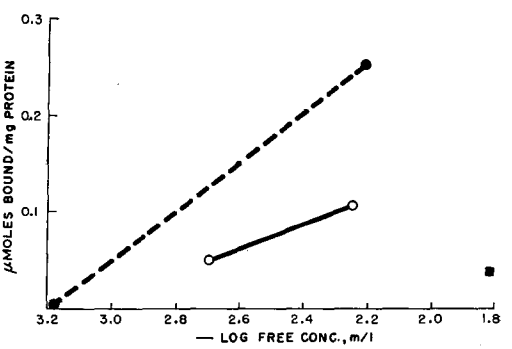

Fig. 8.

shown to follow in a qualitative way the ability of these compounds to interfere with the electrical activity of the intact cell. Thus the order of binding is: curare, procaine, atropine, eserine and Prostigmine. Dimethyl curare has been found in the present studies to be more weakly bound in solution than curare itself; this seemed to be in

contradiction to results repeatedly reported from whole animal experiments[13,21]. However, when tested on the intact electroplax, the methylated derivative indeed proved to be a weaker blocking agent than curare[22]. The stronger binding of curare to the receptor may be explained by the two phenolic hydroxy groups which contribute to the binding force by the formation of hydrogen bonds, a fact which was demonstrated in solution. The difference in behavior of these two compounds in the whole animal and on the single cell must be attributed to factors other than affinity to receptor. A possible explanation is offered by the finding that dimethyl curare is slightly chloroform soluble, apparently enabling it to penetrate to regions inaccessible to curare, a factor which may be important in effects on the animal.

Other comparisons among quaternary compounds of a given series revealed a parallelism insofar as binding in solution and effect on electrical activity were concerned. In both types of studies, PAD was superior to PAM, dodecyl pyridinium better than methyl pyridinium. Perhaps most striking was the finding that the order of affinities: decamethylene bis-PAM > hexamethylene bis-PAM > PAM and decamethylene bis-pyridinium > hexamethylene bis-pyridinium > methyl pyridinium as determined in solution (Fig. 9) was completely maintained in experiments of ROSENBERG AND HIGMAN on the electroplax[22]. The bis compounds of these series had not previously been tested on the cell. Another aspect of these findings will be discussed below.

As for the choline compounds, only the binding of acetylcholine and benzoylcholine to the receptor has been examined thusfar. Although the data are only preliminary, the order of binding in solution and effect on the cell yielded parallel results at least insofar as demonstrating the superiority of acetylcholine.

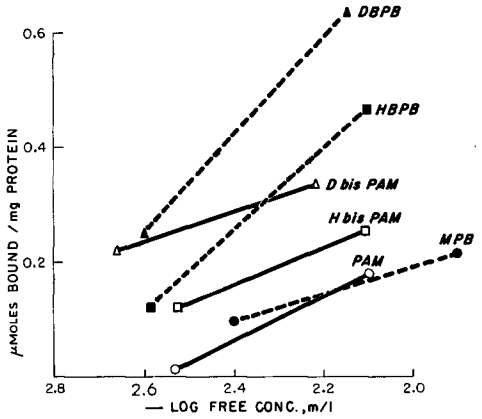

Fig. 9. Binding of bis-quaternary ammonium compounds to the receptor protein pH 7.5, $\mu = 0.1$ (phosphate). Protein concn., 10 mg/ml. Abbreviations: HBPB, hexamethylene bis-pyridinium; DBPB, decamethylene bis-pyridinium; H bis PAM, hexamethylene bis-PAM; D bis PAM: decamethylene bis-PAM.

Specificity of binding in comparison with other macromolecules

On the basis of studies using other macromolecules, it is apparent that binding in a graded fashion is by itself not a sufficiently specific criterion to identify a molecule as the receptor. For example, considering unpublished data on the binding to

chondroitin sulfate, it might be concluded that this substance also exhibits receptor-like activity inasmuch as the gradation in affinities of a number of compounds also may in some instances parallel that of the *in vivo* studies. Moreover, curare quantitively precipitates this polysaccharide and does so in a most interesting fashion: the curare molecule seems to have the exact dimensions to fit between the two negative charges of the chondroitin sulfate monomer[8–10]. It would therefore appear that a molecule of this sort possesses some of the structural requirements to act as a receptor for curare and other curare-like agents, most of which are di-quaternary compounds presumably having a 14–15 Å distance between cationic groups. However, on the basis of at least four different types of experimental evidence, this polysaccharide may be eliminated as the physiological receptor: (a) Although chondroitin sulfate does bind acetylcholine and other choline esters, the affinities in solution do not parallel the effectiveness of these compounds on the cell (unpublished experiments). (b) The configuration of the molecule is such that it apparently does not interact in a graded fashion with a variety of di-quaternary compounds. (c) The binding capacity for curare and related compounds on a mole to mole basis is far too great to be reconciled with WASER's data on binding to the intact tissue. (d) Binding which does occur is more or less non-specific *i.e.*, not relegated to specific regions in the molecule. The latter three considerations will be expanded on below.

The lack of versatility of chondroitin sulfate was clearly brought out when the interaction of methyl pyridinium was compared with that of hexamethylene and decamethylene bis-pyridinium or the corresponding PAM series. It was found that the polysaccharide could hardly distinguish the various bis-quaternary compounds from the mono-quaternary[9]. The binding of all the derivatives was almost identical and very weak. On the other hand, (Fig. 9) the receptor protein showed a distinct gradation in affinity towards these various compounds, in complete agreement with their effects on the intact cell.

While the distance between adjacent negative charges in the condroitin sulfate molecule appears to be suitable for accommodating two cationic groups in a given molecule, compounds with a rigid structure apparently combine more readily with two such sites. Thus of the many di-quaternary compounds examined (Table VI) only curare and stilbamidine precipitate chondroitin sulfate. Non-rigid molecules such as decamethonium or the decamethylene derivatives discussed apparently are not readily bound simultaneously to the two adjacent negative groups. Thus these compounds interact essentially like the mono-quaternary compounds. Conversely, a protein molecule could have many contributory side chains or secondary foldings which become involved in binding and in this way show a gradation in affinity for the great variety of compounds or neurotropic agents known to be bound to the receptor. This is particularly true insofar as curariform action is concerned: although the optimum distance between the two cationic nitrogens is 14–15 Å, pronounced effects may be elicited with molecules differing greatly with respect to this distance[23]. This fact alone suggests that the receptor may have some configurational adaptability, perhaps similar to that proposed by KARUSH for the bovine serum albumin molecule[24,25], a property evidentally absent in polysaccharides and nucleic acids. Alternatively, the receptor may also impose alterations in structure of the small molecule.

The other point of interest concerns the comparative curare-binding capacity of the curare-precipitating protein and chondroitin sulfate, *i.e.*, the number of curare

molecules bound at saturation. It may be expected, by analogy with enzymes, that the receptor may have a few binding sites and that perhaps the binding of one or two curare molecules would be sufficient to produce the physiological effects of the drug. From Fig. 10 it can be seen that, at saturation, approximately 0.1 mg curare is bound/mg receptor protein. This value is in good agreement with that obtained by precipitating the receptor and analyzing the precipitate for both components. This corresponds to about 10 curare molecules/molecule of protein, assuming a molecular weight of 100,000. On the other hand, chondroitin sulfate has a much higher combining capacity for curare[8,9], corresponding to about 150 curare molecules bound/100,000 molecular weight, or a complete neutralization of all the charges on the polysaccharide. This order of magnitude of binding is inconsistent with the results of WASER[20] who found by auto-radiographic techniques on intact rat diaphragm that a single receptor molecule most likely will combine with but a few curare molecules at saturation.

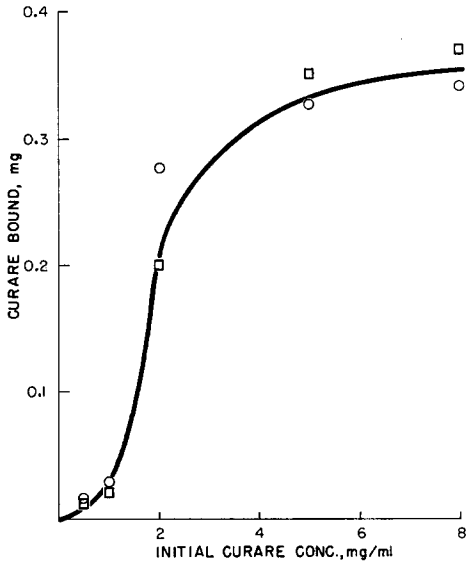

Fig. 10 Binding of curare to purified receptor protein prepared from 30% fraction □, and from tissue extract ○. pH 7.5 μ = 0.1 (phosphate). Protein concn. 3 mg/ml.

Effect of other variables on curare binding in solution and on its action on the intact cell

Reduction of ionic strength in terms of Na ion concentration greatly enhances the affinity of curare to its cellular receptor as well as to the tissue component in the extract. Urea may be employed for dissociating the complex in the test tube, presumably because of its hydrogen bond breaking ability. The effect of urea on curare action in the intact cell is under study, but it may be noted that guanidine, another compound known to break hydrogen bonds, has been reported to be antagonistic to curare[26,27].

Calcium is also effective in dissociating the curare–protein complex. Increasing the Ca concentration from 2 to 8 m*M* resulted in the abolition of the action of curare (2 μg/ml) on the intact electroplax The effect of Ca suggests that the curare binding site might contain one or more phosphate groups and indeed the receptor protein has been found to contain a significant amount of phosphate. Curare precipitates α-casein

more readily than β-casein; this observation also suggests that curare interaction with proteins may involve phosphate groups whereas carboxyls may play only a limited role. Proteins such as serum albumin and egg albumin, having many carboxyl groups but devoid of phosphate, failed to precipitate with curare (Table VI).

Binding of curare by the receptor protein in relation to the amount fixed to tissue

A rough estimate may be made of the number of receptors per cell as well as the number of curare molecules bound per synapse at saturation. From the weight of tissue used the approximate number of electroplax extracted may be estimated. The amount of receptor obtainable from the extract by curare precipitation has been determined on a number of occasions. The receptor per cell has been estimated to be about 1 μg. Assuming a molecular weight of 100,000, this gives a total of 10^{-11} moles receptor or $6 \cdot 10^{12}$ molecules/cell. There are approximately $3–10 \cdot 10^4$ synapses per electroplax[4], constituting approximately 3–6 % of the active membrane which may be considered to have similar receptors distributed throughout, all of which are extractible. The number of receptors per synapse is then $2 \cdot 10^6$ to $2 \cdot 10^7$. These admittedly crude estimates are in good agreement with WASER's estimate for rat diaphragm[20].

At saturation, 1 mg of receptor (in solution) binds 0.1 mg curare (approx. 10^{-7} moles). Since each synapse contains approximately $3 \cdot 10^{-11}$ to 10^{-12} g of receptor (calculated on the basis of 1 μg receptor/cell), the amount of curare expected to be bound at saturation would be $3 \cdot 10^{-14}$ to 10^{-13} g, corresponding to between 2.4 and $7 \cdot 10^7$ molecules, in reasonable agreement with WASER's figure of $8 \cdot 10^6$. This calculation is independent of the molecular weight of the receptor since the combining ratio in solution is in terms of mg of protein.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professor D. NACHMANSOHN for continuous encouragement, advice and stimulation throughout the course of this investigation. He wishes to acknowledge the able technical assistance of Mr. J. DE MAIOS. The help given in some of the experiments by Miss M. KELLOCK from the University of Glasgow, a visitor to this Laboratory on a Fulbright Scholarship, was invaluable.

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, and by the Medical Research and Development Board, Department of the Army, Office of the Surgeon General, Contract No. DA-49-007-MD 988.

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

THE INTERACTION OF QUATERNARY AMMONIUM COMPOUNDS WITH CHONDROITIN SULFATE

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(Received February 22nd, 1960)

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.