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ON THE PROBLEM OF ISOLATION OF THE SPECIFIC ACETYLCHOLINE RECEPTOR

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Abstract—A protein fraction derived from the electric organ of *Electrophorus electricus* has been examined for homogeneity and binding of *d*-tubocurarine. This fraction was previously considered homogeneous and identified by others as the acetylcholine receptor. It is shown in this paper that this fraction is readily resolved by chromatography on cellulose DEAE into numerous subfractions, no one of which represents a large portion of the total protein in the fraction. The binding of *d*-tubocurarine to several of these subfractions is compared to binding by other proteins isolated from the tissue. There is no indication that any of these subfractions binds *d*-tubocurarine more tightly than many other proteins obtained from the tissue. Mass-action plots of the binding curves of the various subfractions tested show departure from linearity which may be due to inhomogeneity, different classes of binding sites, or both. It is shown that the analysis which led to the conclusion that the specific receptor had been isolated is erroneous. A discussion is presented of the difficulties associated with the isolation and identification of this cellular constituent.

IT HAS been postulated that the depolarization of conducting membrane brought about by acetylcholine is due to its reaction with a specific membrane constituent—the acetylcholine receptor.[1, 2] Indeed, such a postulate is virtually self-evident. It is not, however, self-evident what the nature of such a receptor should be or how one should undertake its isolation and identification.

The acetylcholine receptor reacts with a wide variety of compounds, some of which bring about depolarization of the conducting membrane and block of electrical activity (receptor activators), while others block electrical conduction without depolarization (receptor inhibitors).[1, 3] The activators and inhibitors are competitive with one another, indicating their interaction with the same receptor;[4] block of electrical activity can be achieved with essentially unaltered acetylcholinesterase activity, indicating the distinction between the enzyme and the receptor.[5, 6]

The acetylcholine receptor exhibits striking specificity in its reactions with both activators and inhibitors, extremely small changes in chemical structure giving rise to great differences in response as measured *in vivo* with the monocellular electroplax preparation.[7, 8] If the receptor is a distinct macromolecular constituent rather than a structural unit comprising different kinds of molecules, then it is likely to be a

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protein in view of its great specificity of interaction with very similar small molecules and ions. Nachmansohn's well-known hypothesis describing the molecular events associated with electrical activity postulates that the receptor is, indeed, a protein, and that the change in membrane permeability which allows the inward rush of Na^+ ions is caused by an alteration in the receptor consequent to its interaction with acetylcholine.[1-3]

d-Tubocurarine is known to be a strong receptor inhibitor. At external concentrations of about 10^{-6} M it blocks electrical activity in the monocellular electroplax. Higman *et al.*[9] have estimated the dissociation constant *in vivo* of the receptor–tubocurarine complex to be about 10^{-7}. Several years ago Chagas and his associates[10, 11] used radioactive curare-like compounds in an attempt to isolate complexes with tissue components which interacted well with curare. They isolated an acidic polysaccharide. Curare, however, is bound well to many negatively charged constituents of the tissue, and binding alone cannot serve to identify the receptor.

After Nachmansohn's explicit proposal that the receptor is a protein, Ehrenpreis fractionated a soluble extract of the electric organ of the electric eel with ammonium sulfate.[12, 13] This tissue is rich in acetylcholinesterase, low in protein, and highly specialized to perform its function—the generation of bioelectric currents. To the 30% of saturation ammonium sulfate fraction, Ehrenpreis added *d*-tubocurarine and obtained a precipitate. On removal of tubocurarine by dialysis at pH 7·5, part of the precipitated material solubilized, the rest only after raising the pH to 9. The latter fraction was reported by Ehrenpreis to be homogeneous on the basis of electrophoresis and sedimentation.

The binding of a great many compounds to this fraction were studied by equilibrium dialysis. These compounds exhibited varying potencies in blocking electrical activity *in vivo*. Ehrenpreis reported parallelism between the effect *in vivo* of these neurotropic compounds and the extent of binding to this isolated protein, and identified the pH 9-solubilized fraction as the acetylcholine receptor.

There were, however, several deficiencies in this analysis. In the first place, the kind of comparison of binding curves being made was not explicitly stated. No association constants were presented, and many of the curves contained points at only two or three concentrations. Thus it was not possible to judge how the curves were being compared. Second, the fractionation procedure employed depended on the ability of tubocurarine to precipitate the protein, and the binding comparisons employed sometimes depended on the ability of different neurotropic compounds to precipitate the isolated protein at different concentrations. The relationship implied between solubility of the complex and tightness or extent of binding was not defended. Finally, the criteria of purity presented were not convincing.

In this communication, we report results of an examination of two of the fractions described by Ehrenpreis from the point of view of chromatographic behavior and the binding of tubocurarine to several of the subfractions obtained with simple chromatographic procedures. It will be shown that (1) the fraction solubilized at pH 9 and considered to be homogeneous is readily resolved into numerous subfractions, no one of which represents a very large portion of the total protein; and (2) there is no indication that any of these subfractions tested binds tubocurarine more tightly than subfractions obtained from the material which readily solubilizes at pH 7·5. If anything, the reverse is true.

EXPERIMENTAL

Procedures used to prepare the soluble extract from the electric organ and to obtain the fractions isolated by Ehrenpreis were those described by him.[12, 13] Subsequent treatment of the *d*-tubocurarine-precipitated material is described in Results and Discussion.

d-Tubocurarine was kindly supplied by Dr. Dutcher of Squibb. All other chemicals used were reagent grade.

Equilibrium dialysis measurements were performed according to the method of Klotz.[14] All cellophane strips were weighed before washing and a separate determination made of the binding of *d*-tubocurarine to the cellophane strips. The number of millimoles of *d*-tubocurarine bound per milligram cellophane was thus measured and plotted versus the final equilibrium concentration of *d*-tubocurarine. This curve was used in subsequent corrections for binding to the cellophane when protein was present. At low concentrations of *d*-tubocurarine the cellophane bag frequently bound as much tubocurarine as the contents of the bag, which generally contained protein at a concentration near 1%. At concentrations below 10^{-5} M *d*-tubocurarine, duplicates often differed by as much as 25%, largely because of the high blank correction.

RESULTS AND DISCUSSION

Preliminary experiments indicated that the distinction between material which solubilizes at pH 7·5 when the tubocurarine is removed by dialysis and that which requires elevated pH for solubilization breaks down when the attempt is made to repeat the selective solubilization. If material solubilized at pH 9 is restored to

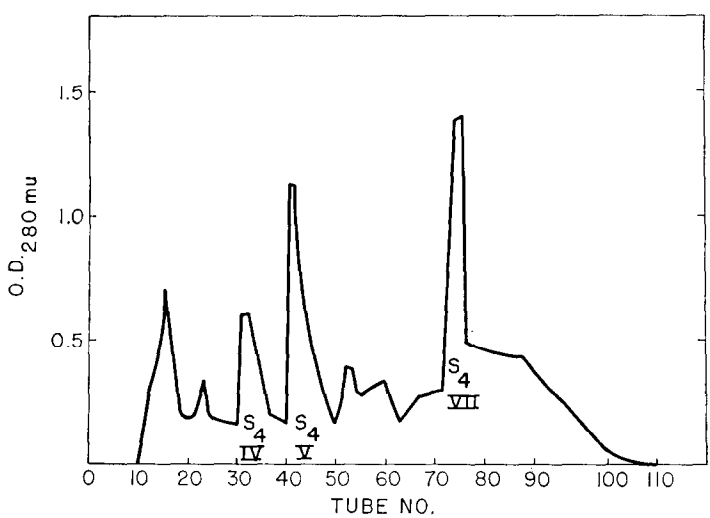

FIG. 1. Resolution on DEAE cellulose of the *d*-tubocurarine-precipitated fraction which was solubilized at pH 9. The fraction was applied in 0·1 ionic strength phosphate buffer at pH 7·5. At tube No. 15, the eluting buffer was increased to ionic strength 0·2 with NaCl. Thereafter, the ionic strength was increased by 0·1 at intervals of 10 tubes until tube No. 70. At tube No. 70, 1 M HCl was added, and at tube No. 80 buffer was reapplied.

pH 7·5, reprecipitated with tubocurarine, and set to dialyze again at pH 7·5, most of the precipitate quickly dissolves without requiring elevated pH. In fact, on first obtaining this precipitate and dialyzing against pH 7·5, it is only necessary to re-suspend what has not dissolved in the first day or two of dialysis in a fresh portion of buffer, and the remaining precipitate dissolves after another day of dialysis at pH 7·5.

Figure 1 shows the resolution by DEAE cellulose of the tubocurarine-precipitated material which was solubilized at pH 9. The fraction was applied in phosphate buffer at pH 7·5 to a cellulose DEAE column and eluted with increasing salt concentration as indicated. The fraction applied is thus hetereogeneous, comprising, as it does, several readily resolved subfractions. Inhomogeneity of this fraction has also been noted by Trams *et al.*[15]

Figure 2 shows the results of the same procedure applied to the portion of the tubocurarine precipitate that redissolves readily on removal of the tubocurarine by dialysis at pH 7·5. Eight peaks appear in addition to the smaller amounts in the various shoulders and that which is not absorbed by the cellulose DEAE under these conditions.

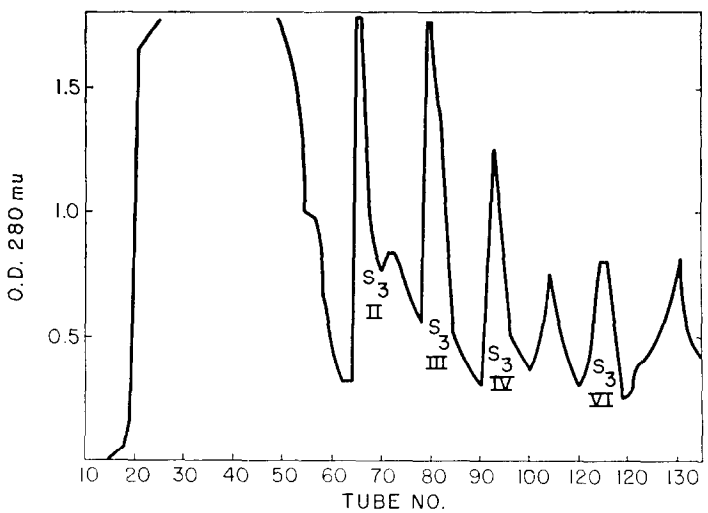

FIG. 2. Resolution on DEAE cellulose of the *d*-tubocurarine-precipitated fraction which was solubilized at pH 7·5. The fraction was applied in 0·1 ionic strength phosphate buffer. At tube No. 60 the ionic strength was increased to 0·20 with NaCl and thereafter increased by 0·1 at intervals of 20 tube numbers.

The contents of tubes collected at the centers of several of the peaks shown in Figs. 1 and 2 were dialyzed against phosphate buffer, pH 7·5, for several days, after which these sub-fractions were separately tested for binding of tubocurarine. As an illustration of the kind of results obtained, Fig. 3 shows two subfractions from the pH 9-solubilized material and two from the pH 7·5-solubilized material. The former clearly bind *more* tubocurarine per gram at high concentrations of tubocurarine, although saturation was not achieved for any of these fractions because precipitation occurred. However, when mass-action plots of these data are drawn[16] as in Fig. 4,

it is seen that both subfractions from the pH 9-solubilized material bind tubocurarine less tightly than either subfraction from the pH 7·5-solubilized fraction. Of five subfractions tested from the latter fraction, all showed steeper slopes in the initial linear parts of the curves than any of four which were tested from the pH 9-solubilized fraction.

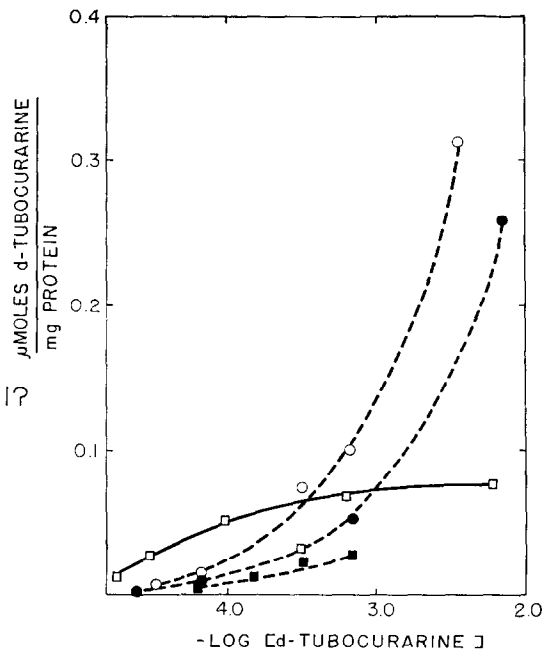

FIG. 3. Binding of *d*-tubocurarine by two subfractions from pH 9·0-solubilized material and two subfractions from pH 7·5-solubilized material. Binding measurements performed at pH 7·50 in phosphate buffer at ionic strength 0·10. Protein concentrations were determined by dry weight. S_3 II, ■; S_3 IV, □; S_4 IV, O; S_4 V ●.

These preliminary results merit little further analysis, because the critical requirements of homogeneity have not been satisfied, and it is pointless to examine impure materials for specific binding properties. The fraction earlier identified as acetylcholine receptor is grossly heterogeneous; indeed, the numerous subfractions obtained in the present work are probably still inhomogeneous. Thus the curvature obtained in the mass-action plots could equally well represent mixtures of proteins, different classes of binding sites, or both. It is important to view these results with the distinction in mind—long recognized in studies of multiple opportunities for the binding of a small molecule to different sites in a macromolecule—between the number of ligands bound and how tightly they are bound.[16, 17] Comparisons of the untreated binding data obscure this distinction and are deceptive. While this objection applies even when homogeneous proteins are examined, the views presented in earlier papers on this subject become all the more untenable since the protein fractions were inhomogeneous.

In earlier papers no suitable criterion was presented which allowed a decision that a given step in the attempted purification procedure increased or decreased the

concentration of the sought-after receptor. The concentration of *d*-tubocurarine required to precipitate a particular protein at a given pH gives no direct indication of affinity or capacity. It may be mentioned that proteins in other fractions which are not precipitated by *d*-tubocurarine and were not considered in the earlier studies were also found to bind *d*-tubocurarine.

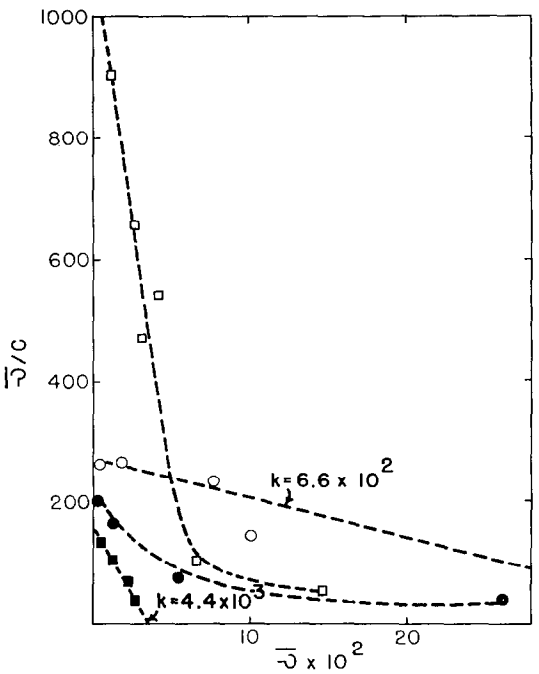

FIG. 4. Mass-action plots of data shown in Fig. 3. The form of the equation used is that suggested by Scatchard[16] $p/c = \nu(n - \bar{\nu})$; c as shown in the figure is the concentration of *d*-tubocurarine in moles per liter and $\bar{\nu}$ is the number of micromoles of *d*-tubocurarine bound per milligram of protein. S_3 II, ■; S_3 IV, □; S_4 IV, ○; S_4 V ●.

Ehrenpreis has himself questioned his earlier identification of the isolated fraction as the acetylcholine receptor.[18] His reasons for doubting the earlier identification, however, are not based on those presented in this report—that the comparison of the data for binding of various neurotropic compounds to the isolated fraction was made without a decision as to what was being compared and, most important, that the isolated fraction comprised many proteins. Other arguments raised by Ehrenpreis relating to the properties to be expected of the acetylcholine receptor have been discussed in detail elsewhere.[19]

Finally we may point out that the specificity to be expected of the receptor in its binding of inhibitors or activators will not extend to the numerous sites which will bind these substances nonspecifically. It is reasonable to expect that the receptor should bind at least one equivalent of, say, *d*-tubocurarine with an association constant of the order of 10^6 or greater. Many other sites in the same molecule may bind *d*-tubocurarine with far lower constants, nonspecifically. Comparison of the binding of various neurotropic compounds at these sites is without value in attempting to

gain information about the specific active site. Neither Ehrenpreis' work nor the work reported here sheds light on this specific interaction.

Further attempts at isolation of the specific acetylcholine receptor depend critically, for success, on knowledge of specific properties of the acetylcholine receptor which distinguish it from numerous other proteins and polyelectrolytes found in the tissue. Highly specific responses to various slightly different compounds, *in vivo*, probably provide the most promising approach to the underlying difficulty in the desired isolation, which is the absence, at present, of a suitable assay.

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