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Isolation of acetylcholine receptors by chloroform–methanol extraction: Artifacts arising in use of Sephadex LH-20 columns

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SUMMARY

Attempts were made to isolate acetylcholine receptors from the electric organ of *Electrophorus electricus* by means of chloroform–methanol extraction and subsequent purification on Sephadex LH-20 columns. Evidence is presented which indicates that the "receptor"–ligand binding observed may be artifactual.

De Robertis and co-workers have recently described a procedure for chloroform–methanol extraction of several tissues to yield drug receptors in at least a partially active form, *i.e.* able to bind ligands[1–6]. Their interest has centred especially on the isolation of acetylcholine receptors by this method[3,4,6]. Lipophilic Sephadex LH-20 columns have been used to separate from the tissue extracts a peak of protein which apparently has agonist and antagonist binding properties. Because of the simultaneous presence of lipid in this peak, the material has been designated as "receptor proteolipid". This report concerns attempts in our laboratory to repeat such extractions from electric organ, and discusses the results of some additional experiments designed to serve as controls for the purification system.

Extractions of both eel whole organ and electroplax membranes were performed according to the methods of La Torre *et al.*[3]. Briefly, the procedure was to extract either lyophilized whole tissue or lyophilized membranes, in each case representing 5–20 g of wet organ, in chloroform–methanol (2:1, v/v). The extract was filtered free of residue, and concentrated by evaporation as described in ref. 3 to a final volume of 5 ml. The concentrate was then allowed to equilibrate with radioactive ligands for 30 min. Preliminary studies utilized (+)-[^{14}C] tubocurarine, but [^{14}C] acetylcholine (with both acetyl and *N*-methyl labels, The Radiochemical Centre, Amersham) was used in later studies of

binding affinities. The range of acetylcholine concentrations used was 10^{-6}–10^{-5} M. After binding, the extract was fractionated on a Sephadex LH-20 column (Pharmacia Fine Chemicals) according to the methods of Soto *et al.*[7]; column dimensions were 2.1 cm x 18 cm, with a bed volume of about 40 ml eluant. In preliminary experiments the sample was eluted as described by La Torre *et al.*[3] (80 ml chloroform, followed successively by chloroform–methanol 15:1 (by vol.) (20 ml), 10:1 (20 ml), 6:1 (20 ml) and 4:1 (60 ml)), but after it became clear that intermediate proportions had little or no effect on the elution pattern, the elution was simplified to be 60 ml chloroform, followed by 10 ml 10:1 chloroform–methanol, and finally 60 ml 4:1 chloroform–methanol. The effluent was continuously monitored using a Uvicord spectrophotometer at 254 nm. This enabled solvent fronts and chloroform–methanol proportion changes to be detected, due to the greater absorbtivity of chloroform over methanol at this wavelength. Since proteins and lipids also absorb ultraviolet under these conditions, separate protein[8] and lipid phosphorus[9] assays were done on the effluent which was collected in a fraction collector. Radioactivity was assayed in the fractions using standard scintillation methods. All steps of the extraction and purification were done at room temperature.

A typical elution profile obtained with whole tissue is shown in Fig. 1; microsomes gave similar results. This profile agrees rather well with those obtained by previous investigators[3]. The main areas of interest in the profile are the coincident peaks of protein and radioactivity occurring approximately one column bed volume (40 ml) after sample application, and the smaller peaks of radioactivity, protein, and lipid occurring near the solvent front of the 4:1 eluant. The amount of radioactivity eluted in the first large peak represented about 95% of that applied, and was unaffected in 7 experiments by the concentration of acetylcholine used in labelling the extract or the amount of protein contained in the peak. Previous investigators found a concentration-dependent binding in these ranges of acetylcholine concentration, but also that the amount of radioactivity

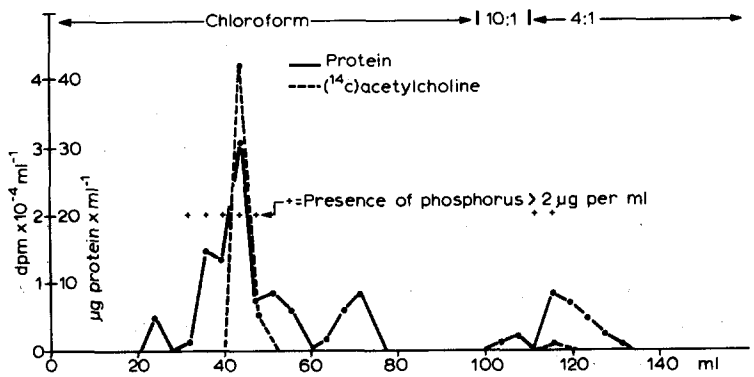

Fig. 1. Whole tissue extract of 1 g lyophilized organ. (1 of 7 experiments.) Acetylcholine concentration was $5 \cdot 10^{-6}$ M. Elution done according to a modified procedure of Soto *et al.* (see ref. 7 and text). Upper part of figure gives composition of effluent. Controls gave 95% retention of label.

contained in the peak was unchanged when the amount of protein varied; however, this radioactivity was only 60% of the total applied[6].

When a control bolus of [^{14}C] acetylcholine in 2:1 (v/v) chloroform–methanol is applied to the column and eluted as described, one observes retention of more than 95% of the label during the elution process. When LH-20 is equilibrated with solvents of low ionic strength or polarity (*i.e.* 100% chloroform) retention of compounds is common, apparently because of interactions between the Sephadex carboxyl groups and ionic groups in the retained compounds[10]. Raising the polarity or ionic strength of the eluting medium eliminates retention if the medium is able to compete with the retained compounds for the Sephadex carboxyl groups. As different compounds will have different affinities for the Sephadex, they will be eluted by solvents of different polarities or ionic strengths. Consideration of these facts in conjunction with the above results suggested that some component or components of the tissue extracts might be affecting the retentive properties of the LH-20 bed in a non-specific manner.

Further investigation revealed that the important peaks in Fig. 1 occurred in solvent fronts, where the polarity of the eluant abruptly increased. It thus seemed possible that the coincidence of the radioactivity and protein peaks in the solvent front was due to the simultaneous release of acetylcholine and protein from retention, rather than to a specific interaction between the two substances. Ligand, protein and lipid might all be released at different eluant polarities, but the stepwise elution procedure used would create solvent fronts which could simultaneously elute all substances that would otherwise be released separately at solvent polarities between those of the front and of the column bed solvent. For the large "binding" peak, ultraviolet chromatograms and the elution position one bed volume after sample application indicated that this was a solvent front caused by overloading the column with the 2:1 chloroform–methanol sample bolus, whose diffusion as it traversed the column was incomplete. Thus the effluent material was not 100% chloroform at this point, but some proportion intermediate between 100% chloroform and 2:1 chloroform–methanol. It was possible to eliminate this front by simply reducing the applied sample volume to 2 ml, as was confirmed by examining the elution patterns of [^{3}H] ouabain, which is normally retained in chloroform columns, but is released when the eluant polarity is higher than that found in 6:1 chloroform–methanol. When control boluses containing [^{3}H] ouabain in 2:1 chloroform–methanol were applied to the column in varying volumes and eluted as in Fig. 1, it was observed that significant quantities of radioactivity were eluted one bed volume after application (*i.e.* in a bolus solvent front) if the applied bolus volume was 3.5 ml or greater. Application of 6-ml boluses caused all of the applied radioactivity to be eluted in this position. Application of bolus volumes less than 3.5 ml caused all of the ouabain to be eluted much later in the solvent front of the 4:1 eluant. These results indicated that sample volumes of greater than 3.5 ml created solvent fronts which were eluted from the column at sufficient polarities to prevent the retention of the ouabain contained in the bolus. Ideally, one would like the sample bolus to equilibrate with the column bed chloroform so that one has control over retention of sample compounds. That 2-ml samples did equilibrate with the column could be inferred

from both the ouabain elution data and the lack of a solvent front appearing on the ultraviolet monitor traces. Elimination of the front could also be achieved by use of longer columns, which allowed for complete diffusion of the bolus during elution. In an attempt to separate the other coincident peaks in the 4:1 chloroform–methanol solvent fronts, the column was eluted with a continuous linear gradient of chloroform–methanol. 2 ml of sample were applied to the column, followed by 10 ml of chloroform to insure complete equilibration of the sample bolus with the column bed solvent. The column was then eluted with a 100-ml gradient, ranging from chloroform to 4:1 chloroform–methanol, using a standard gradient mixing chamber.

A typical result of eliminating solvent fronts by these methods is shown in Fig. 2, using electroplax microsomes as the receptor source. Use of whole tissue extracts gave similar results. The main peak of radioactivity was observed in 7:1 chloroform–methanol and considerably later than one bed volume after sample application. More important is the fact that protein was absent in significant quantities from this peak (6 experiments). However, in 3 out of 6 experiments lipid phosphorus was found in this position, as well as an unidentified residue observed on drying of the solvent. This peak contained virtually all the applied acetylcholine label, regardless of the amount of tissue protein applied or the concentration of acetylcholine used (in the range 10^{-6}–10^{-5} M). The 4:1 part of the gradient is not shown because it contained no protein, lipid, or radioactivity.

It thus appeared likely that the tissue extracts were interfering in a non-specific manner with the retention of acetylcholine. In order to show that the substances involved were not associated with acetylcholine binding *in vivo*, extracts were made of the electric organ of the catfish *Malapterurus*, which was likely to contain rather little acetylcholine receptor, because of the restricted area of its synaptic junctions[11]. Extracts were also made of purified ox brain phospholipids prepared according to Folch[12] that contained no protein.

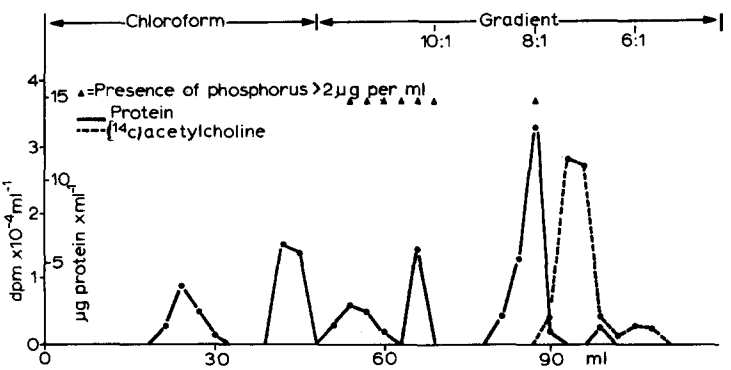

Fig. 2. Microsomal extract of electroplax (containing 10 mg protein before extraction). (1 of 6 experiments.) Acetylcholine concentration was $5 \cdot 10^{-6}$ M. Elution done by continuous linear gradient. Upper part of figure gives approximate composition of effluent as determined from ultraviolet traces of control runs. The parge peak contained 96% of the applied radioactivity, while controls gave 94% retention of the label.

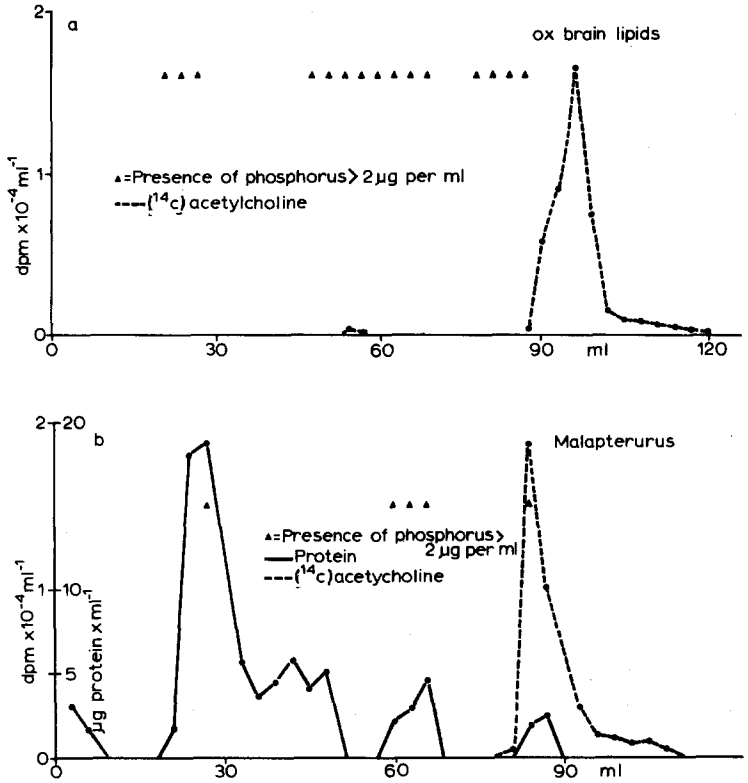

Fig. 3. (a) 2:1 chloroform–methanol solution of 50 mg ox brain phospholipids. Elution and controls as in Fig. 2. The large peak contained 98% of the applied radioactivity. (1 of 4 experiments.) (b) Extract of *Malapterurus* electric organ (1 g lyophilized tissue). Elution and controls as in Fig. 2. The large peak contained 98% of the applied radioactivity. (Single experiment.)

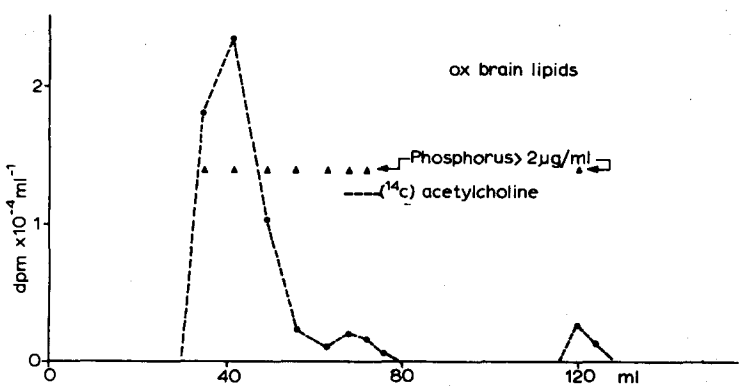

Fig. 4. 2:1 chloroform–methanol solution of 100 mg ox brain phospholipids. Elution and controls as in Fig. 1. Radioactivity in the large peak is 97% of that applied to the column. (1 of 2 experiments.)

Despite the lack of receptor in these extracts, they gave radioactivity elution patterns that closely mimicked those obtained with the eel extracts shown in Figs 1 or 2, depending on the elution procedure followed. Typical results using both elution procedures and non-receptor extracts are shown in Figs 3 and 4.

The effect of 2:1 chloroform–methanol treatment on the acetylcholine binding activity of eel microsome was assessed using equilibrium dialysis, as described by Eldefrawi *et al.*[13]. A 2:1 chloroform–methanol extract of microsomes was prepared as before, but then dried under nitrogen. Another batch of microsomes was also treated with 2:1 chloroform–methanol, but the solvents were not filtered free of residue before drying. This sample was prepared to determine if 2:1 chloroform–methanol treatment altered the binding activity of whole microsomes. Both dried samples were then reconstituted in a modified Ringer's solution[13]. Dialysis of these samples was done against $2 \cdot 10^{-4}$ M [^{14}C] acetylcholine in modified Ringer, and equilibrium binding was compared with both a Ringer control and reconstituted lyophilized microsomes. All samples contained 10^{-5} diisopropylfluorophosphate (DFP) to inhibit binding and hydrolysis due to acetylcholinesterase[14]. Under these conditions, no binding was observed in either of the samples treated with organic solvent. This experiment does not preclude the possibility that the receptors were denatured by the reconstitution process, but it would appear that 2:1 chloroform–methanol treatment eliminates the binding activity of whole microsomes and does not solubilize the receptor in an active form.

Our experiments have thus shown that acetylcholine binding peaks can be created using receptor-free and protein-free extracts, and suggest that the association of ligand with these peaks is non-specific. It would seem, therefore, that "receptor"–ligand interactions observed in Sephadex LH-20 purification may be artifactual. The precise nature of the interactions of column, ligand and extract compounds that affect the normal retention of acetylcholine is not yet clear. As the manufacturers warn in their pamphlet describing LH-20, "Factors other than molecular dimensions may also affect the elution pattern. These include specific interactions between the gel matrix and the substance to be fractionated. These effects are complicated and not completely elucidated" (ref. 10). In view of these effects when organic solvents are used as the binding medium, there would seem to be some doubt whether the receptor–ligand affinities previously described[7] do really represent the *in vivo* interactions observed in physiological solutions.

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