On the solvent-extraction of acetylcholine receptor

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Summary. Chloroform/methanol rechromatography on Sephadex LH-20 of fractions isolated from organic solvent extracts of *E. electricus* electric organs exhibited alterations in elution point compared to the original chromatography. Additionally, [^{3}H]decamethonium bromide did not coelute with any of the observed fractions. It is concluded that as presently described the organic solvent isolation procedure for the cholinergic receptor exhibits undesirable characteristics.

Acetylcholine receptor protein (AChR) has been isolated from the electric organ of the electric eel upon extraction of tissue with either of 2 different solvent systems. One system utilizes the presence of various detergents, e.g., Triton X-100, Emulphogene, or sodium deoxycholate to solubilize the membrane-bound receptor[2,3]. The other employs a mixture of 2 organic solvents[4], i.e., chloroform/methanol (C/M) to effect extraction of the receptor on the basis that it behaves as a classical proteolipid[5]. Subsequent steps in the isolation of receptor are performed in the particular medium used for extraction. We report here the apparent alteration of receptor proteolipid while attempting to purify it after extraction with organic solvents.

Materials. Electric eels *(E. electricus)* were purchased from Paramount Research Supply Company (Ardsley, N.Y.). Sephadex LH-20 was supplied by Pharmacia and Fluram by Roche Diagnostics. Aquasol was obtained from New England Nuclear and [methyl-^{3}H] decamethonium bromide (DMB) and [acetyl[N-methyl-^{14}C] choline chloride from Amersham/Searle Corporation. All solvents were distilled prior to use.

Methods. Eels were sacrificed by decapitation, and the electric organs were immediately excised and stored frozen at −20 °C. Extraction of the frozen tissue with solvent and incubation of the extraction with radiolabeled ligands were performed as described by DeRobertis et al.[4] with the following modifications: a) prior to use, all samples were centrifuged at 1500×g for 5 min at room temperature, b) solvent extracts were evaporated down to a volume of 10 ml, and c) ligand binding incubations were routinely prepared in a final volume of 2 ml. All experiments were performed with freshly extracted tissue.

Columns of LH-20 were prepared essentially as described by Soto et al.[6]. Sephadex LH-20 was suspended in C/M, 1/1 (v/v) and swelled for at least 1 day prior to use. Columns (1.5×40 cm) were poured and packed at a flow rate of 35±5 ml/h. The surface of each column was covered with a 1 cm thick layer of solvent-washed sand. Columns were equilibrated with chloroform by first flushing with a gradient of decreasing polarity consisting of 150 ml each of C/M (4/1) and chloroform, and then with 250 ml of chloroform. Samples were routinely applied in 1.5–2.0 ml volumes, followed by 10 ml chloroform and then the column was developed with a gradient of increasing polarity consisting of 150 ml each of chloroform and C/M (4/1)[7]. Fractions were analyzed for: a) radioactivity by counting 200 µl aliquots dissolved in 10 ml of Aquasol on a Beckman LS-250, b) solvent composition by determining refractive index at 25 °C on an Abbé refractometer, and c) amino functions by reacting with Fluram[8].

Results and discussion. Chromatography of free ^{14}C-acetylcholine (ACh) on LH-20 is shown in figure 1. ^{14}C-ACh was incubated in solvent that was prepared by adding 15 ml chloroform to 30 ml C/M(2/1) and evaporating the mixture to 10 ml under vacuum at 23 °C. ^{14}C-ACh was eluted in 1 peak representing about 80% of applied radioactivity at a solvent composition of C/M (6.7/1) or 87.0% chloroform. This result was obtained with ^{14}C-ACh at concentrations of 0.58 µM or 14.4 µM.

Analysis of the crude proteolipid extract by chromatography on LH-20 revealed the profile in figure 2. The extract was generally recovered in 4 Fluram-detectable peaks. This qualitative result was obtained in 5 experiments. However, the relative amounts in these peaks varied somewhat from run to run. Only 3 of the peaks were eluted in 100% chloroform, whereas the 4th appeared in C/M (21.2/1) or 95.5% chloroform.

The results of chromatographing an incubation of proteolipid extract and ^{14}C-ACh on LH-20 is shown in figure 3. 4 Fluram-reactive peaks and 1 peak of radioactivity were

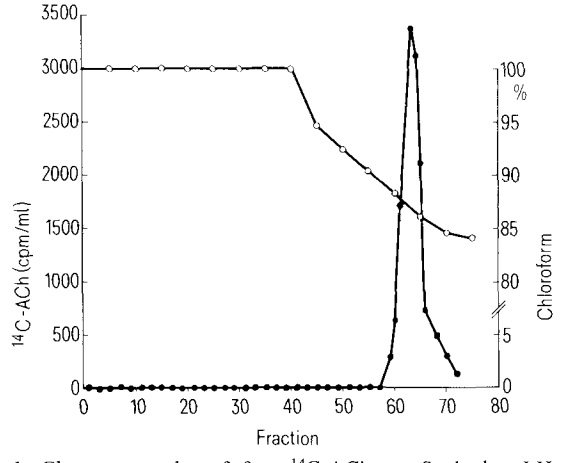

Fig. 1. Chromatography of free ^{14}C-ACh on Sephadex LH-20. Sample was prepared at a concentration of 0.58 µM as described in the text, and 1.5 ml was applied to the column. Eluate was collected in 2.9 ml fractions at a flow rate of 40 ml/h. Gradient, (○); radioactivity, (●).

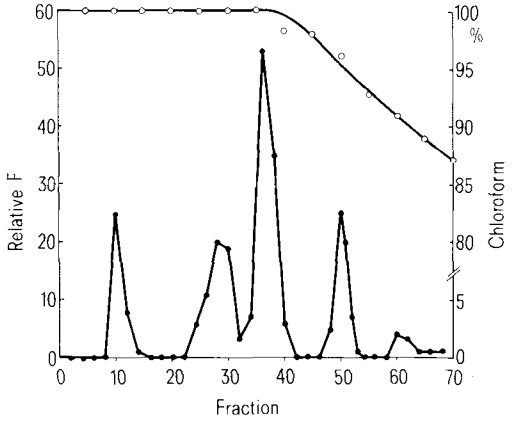

Fig. 2. Chromatography of crude proteolipid extract on Sephadex LH-20. Extract was prepared as described in Methods, and 1.7 ml was applied to the column. Eluate was collected in 2.9 ml fractions at a flow rate of 40 ml/h. Gradient, (○); fluorescence, (●).

obtained. It will be noted that the radiolabel was eluted at 93% chloroform and was not coincident with any of the Fluram-reactive peaks; but, it was significantly displaced from its elution value of 87% chloroform in the control run (see figure 1). This experiment was performed 4 separate times with the same result.

An attempt was made to isolate each peak of Fluram-reactive material and then to determine its ability to bind ^{14}C-ACh separately. Attempts to isolate AChR yielded the results shown in figure 4, A–D. All but 1 peak exhibited an altered elution profile upon rechromatography. Furthermore, 3 of the peaks which were initially resolved on LH-20 appeared on rechromatography to coelute. This result indicates that at least 2 of the peaks may have been altered simply by passage through an LH-20 column. This conclusion is consistent with the work of Heilbronn who observed that proteins similarly isolated from a C/M extract not only failed to bind α-neurotoxin but also were unable to react with antibodies raised to nicotinic AChR[9].

An additional chromatographic experiment involving an incubation of the crude extract with ^{3}H-labeled decamethonium bromide also resulted in no evidence for the presence of AChR, i.e., there were no coincident peaks of radioactivity and Fluram-reactive material. Finally, an attempt was made to show binding in the crude, solvent extract by incubating it with nitroxide, spin-labeled DMB and recording the resulting EPR-spectrum. No detectable binding was observed even though there should have been enough binding sites present based upon DeRobertis' value for the amount of receptor in electric organ tissue.

Considering our experimental results and the published objections[7] to the procedure for isolating AChR from C/M extracts, we consider the system too sensitive to variables beyond our control, and as such does not represent a reproducible technique for the isolation of acetylcholine receptor.

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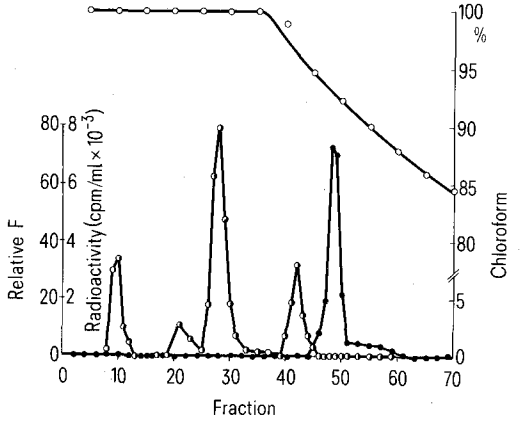

Fig. 3. Chromatography of an incubation of crude proteolipid extract and ^{14}C-ACh on Sephadex LH-20. Extract was incubated with 0.58 μM ^{14}C-ACh and 1.5 ml applied to the column. Eluate was collected in 2.0 ml fractions at 35 ml/h. Gradient, (○); radioactivity, (●); fluorescence, (◑).

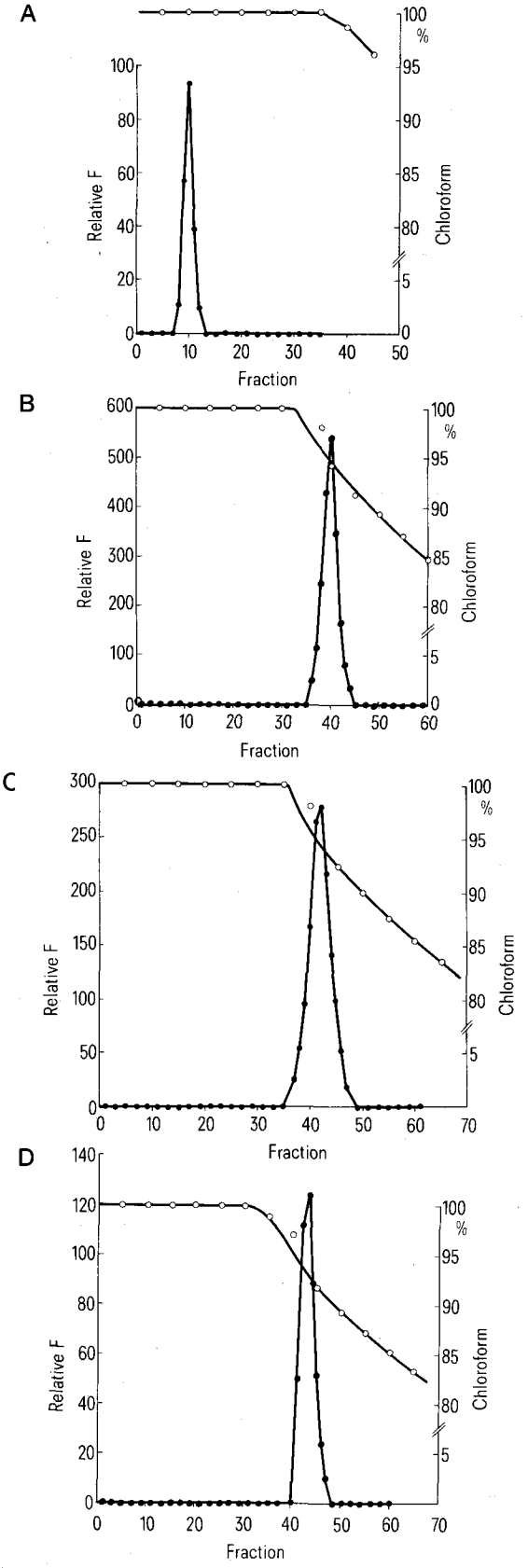

Fig. 4. Rechromatography on Sephadex LH-20 of peaks isolated from crude proteolipid extract. Isolated peaks were concentrated under vaccum and 1.5 ml applied to the column. Eluate was collected in 3 ml fractions at 40 ml/h. *A* peak I; *B* peak II; *C* peak III; *D* peak IV. Gradient, (○); fluorescence, (●).

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Induction of epidermal cell proliferation by a tumour promoter in vitamin B_6-deficient mice

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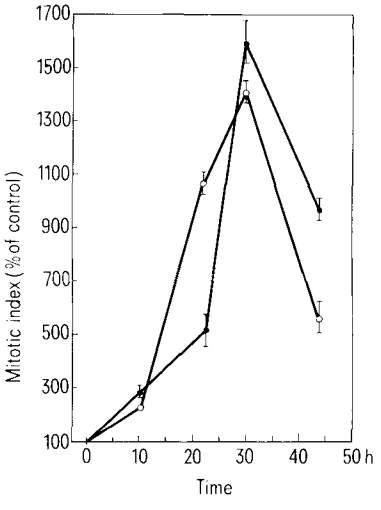
Summary. Topical application of the tumour promoter 12-0-tetradecanoyl-phorbol-13-acetate to skin caused a marked enhancement of mitotic activity both in mice maintained on a complete diet or on a vitamin B_6-deficient diet.

The enzyme ornithine decarboxylase catalyses the rate-limiting step in the biosynthesis of the polyamines in mammalian tissues[3]. The activity of the enzyme is enhanced by a wide range of physiological stimuli in a number of tissues. These stimuli are frequently associated with an increased rate of cellular proliferation, leading to the fairly general conclusion that induction of the decarboxylase is of significance in growth regulation[4-7]. The tumour promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) causes an early rise in mouse epidermal ornithine decarboxylase activity[8], followed by marked hyperplasia[9]. However, enhancement of epidermal cell proliferation by a range of stimuli is not invariably preceded by an increase in decarboxylase activity[8] and the 2 events may not be causally linked, at least in this tissue. Ornithine decarboxylase requires pyridoxal 5'-phosphate as a cofactor[10], and it has recently been shown that maintenance of mice on a vitamin B_6-deficient diet for 14 days greatly decreases the induction of decarboxylase in epidermis by TPA[11]. The purpose of the present study was to determine whether this decreased response modified the ability of TPA to enhance the proliferative activity of epidermal cells.



Mitotic indices were determined in epidermis at varying times after treatment with TPA. Animals were maintained on a complete diet (○) or a vitamin B_6-deficient diet (●) during the experiment and for 14 days prior to TPA treatment. Each point represents the mean ± SEM of results obtained with 3 (10-h point) or 4 (other time points) separate animals.

Materials and methods. Female Swiss albino mice were maintained for 14 days on either a complete or vitamin B_6-deficient diets as described before[11]. Animals were treated with TPA (17 nmoles in 0.2 ml acetone) or with acetone (0.2 ml). At varying times after treatment, groups of animals were injected i.p. with 0.1 mg colchicine (in 0.2 ml 0.9% NaCl) and were sacrificed after a further 2 h. Samples of skin were taken for histology as described before[12]; the mitotic index is expressed as the number of metaphase cells per 100 nucleated, interfollicular cells.

Results and discussion. It is clear from the results shown in the figure that TPA induced a marked proliferative response in the epidermis of mice maintained on both the complete and vitamin B_6-deficient diet. There was some suggestion of a more rapid early rise in mitotic activity in the control group, but in general the shape of the response curves was similar. The initial basal mitotic index in the B_6-deficient animals was 0.54 ± 0.06 and in the control animals 0.78 ± 0.09. These values were not significantly different at the 5% level. Mitotic indices determined in acetone-treated control animals (maintained on either a complete or B_6-deficient diet) at 22, 30 or 44 h did not differ from zero time control animals.

It was previously reported that the induction of epidermal ornithine decarboxylase by TPA in mice maintained on the deficient diet for 14 days was only about 5% of that in mice kept on a complete diet[11]. Consequently, the present data do not support a causal relationship between ornithine decarboxylase and induction of proliferative activity in mouse epidermis.

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