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Conference Paper

The Use of Affinity Gels for the Study of the Ligand Binding Properties of Mammalian Acetylcholinesterase

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An affinity adsorption technique for the analysis of the binding sites of acetylcholinesterase for small ligands is presented. By studying the interaction of decamethonium and edrophonium the second anionic binding site of decamethonium is characterized as having affinity for trimetyl-(*p*-aminophenyl)ammonium-CH- Sepharose 4B but not for trimethyl-(*m*-aminophenyl)ammonium--CH-Sepharose 4B. The directional localization of a hydrophobic binding area in the vicinity of the anionic subsite of the catalytic site is tentatively defined.

The affinity chromatographic method is now established as an efficient method for protein purification. As pointed out by O'Carra[1] the development of the method has however in some respects not been sufficiently rigorous. This has had the consequence that some as it seems widely accepted generalizations are incorrect or at least misleading. In fact, from several studies the conclusion has been drawn that adsorption and desorption to the gel are not simple equilibrium processes and such concepts as »progressively perpetuating effectiveness« and considerable time dependence of the desorption process compared to the adsorption process has been advanced in order to explain various anomalies encountered. There are now, however, reasons to believe that these anomalies are due to non-specific adsorption mainly to the spacer arm and that if this complication can be mastered the results obtained by the method lend themselves to quantitative analysis according to the relations derived from the law of mass action. From this it follows that the affinity chromatographic method, at least in principle, could be used for studies of the binding of small ligands to macromolecules. We have now started an investigation of the applicability of the method for the study of the interaction of established reversible inhibitors of the acetylcholinesterase and this paper presents the method and some of our results, which are mainly of a preliminary character.

The method used is not really chromatographic, but a batch procedure. To a test tube are added enzyme, gel and medium to a final volume of one milliliter. After equilibration the ligand or ligands are added and their concentrations are then step-wise increased by successive additions. The volumes withdrawn for the quantitation of the eluted enzyme by the acetylthiocholine method of Ellman are in the microliter range. The sample is thus diluted up to 3 000 times in the measuring cuvette. There is thus in most instances no need of running controls in order to correct for inhibition of the enzyme

activity during the measurement. The enzyme used was a not extensively purified preparation from the caudate nucleus of the calf brain[2]. The specific activity was 625 μmol Ac—S—Ch per mg protein and per hour at 27 °C; based on the highest specific activity for the brain enzyme obtained by us (575 mmol mg^{-1} h^{-1}) the enzyme concentration used varied between 10^{-8} M and 3×10^{-8} M in the various experiments. The main characteristics of this preparation are its low molecular weight, about 80 000, and its non-aggregating properties. It is possible that these properties underlie the fact that the enzyme is not at all unspecifically adsorbed to the spacer arms including such of highly hydrophobic character.

In spite of the fact that the spacer arm does not by itself has any noticeable affinity for our enzyme the arm seems to contribute to the binding by the immobilized ligand under certain conditions. Such a case, is illustrated by some experiments using trimethyl-(m-aminophenyl)ammonium and its para-analogue as the immobilized ligands. The dissociation constants of the

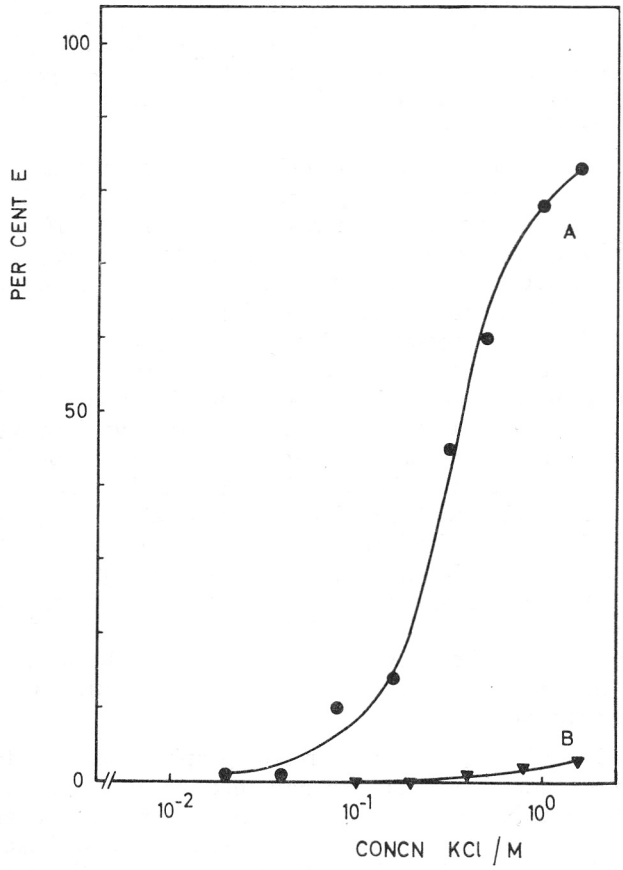

Fig. 1. Effect of ionic strength on the adsorption of AChE to (A) *meta*- and (B) *para*-analogue gels.

100 mg trimethyl-(*m*-aminophenyl)ammonium-CH-Sepharose 4B and trimethyl-(*p*-aminophenyl)ammonium-CH-Sepharose 4B suspended in 0.025 M *tris*-HCl buffer pH = 8.0. Final immobilized ligand concentration was 3.2×10^{-4} M and 6×10^{-4} M.

two ligand-enzyme complexes are about the same, 5×10^{-5} M and 7×10^{-5} M, indicating only a small contribution of the amino-group to the binding. This conclusion is further supported by the finding that the dissociation constant of the immobilized para-ligand is 6×10^{-5} M, a value that is not materially different from that of the free ligand. The dissociation constant for the immobilized meta-compound, however, is 6×10^{-6} M, ten times lower than that of its free counterpart. The difference indicates that the hydrophobic arm in this case is involved in the binding. This conclusion is corroborated by the finding that the enzyme can not be eluted by 1.6 M KCl from the meta-gel while the elution by KCl is easily brought about by relatively low concentrations of the salt from the para-gel (Fig. 1). It seems that these experiments define a hydrophobic binding site on the enzyme situated in such a position in relation to the anionic site that it can interact with the hydrophobic spacer

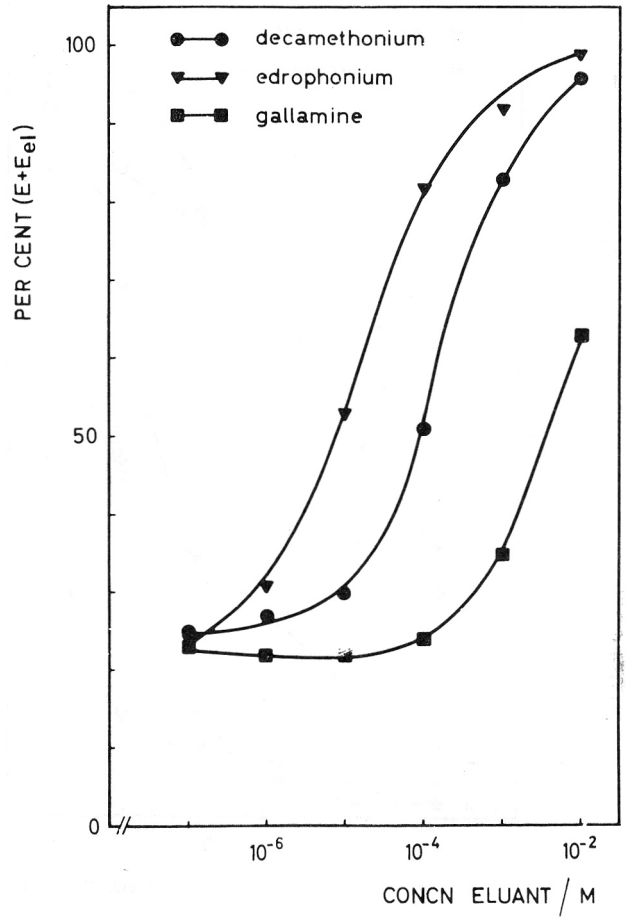

Fig. 2. Desorption of AChE by edrophonium, decamethonium and gallamine from meta--analogue gel.

10 mg trimethyl-(*m*-aminophenyl)ammonium gel suspended in 0.017 M *tris*-HCl buffer pH = 8.0. Final immobilized ligand concentration was 3.2×10^{-5} M.

arm only when the arm is in meta position to the positive charge of the immobilized ligand.

Fig. 2 shows that edrophonium, decamethonium as well as gallamine effectively elute the enzyme from the meta-gel and thus have definite affinities for the site of the enzyme involved in the binding to the immobilized ligand.

The elution pattern from the para-gel is somewhat more complex. Decamethonium behaves, it is true, as expected but the anomalous curve for the elution by edrophonium (Figs. 3 and 4) indicates two binding sites of the enzyme, site A and site B (Fig. 5) and a low ratio between site B affinity to the second molecule of edrophonium and site B affinity to the gel. The failure of decamethonium to reveal the B-site would be due to a high ratio between site B affinity to the second molecule of decamethonium

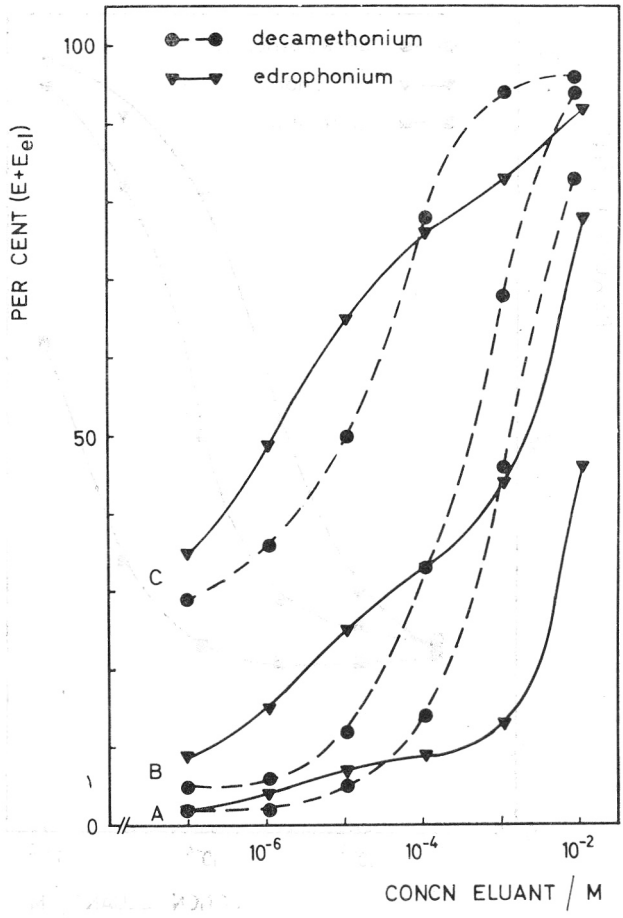

Fig. 3. Desorption of AChE by edrophonium from para-analogue gel.

40 mg (A), 25 mg (B) and 15 mg (C) trimethyl-(*p*-aminophenyl)ammonium gel suspended in 0.017 M *tris*-HCl buffer pH = 8,0. Final immobilized ligand concentration 2.4×10^{-4} M, 1.5×10^{-4} M and 9×10^{-5} respectively.

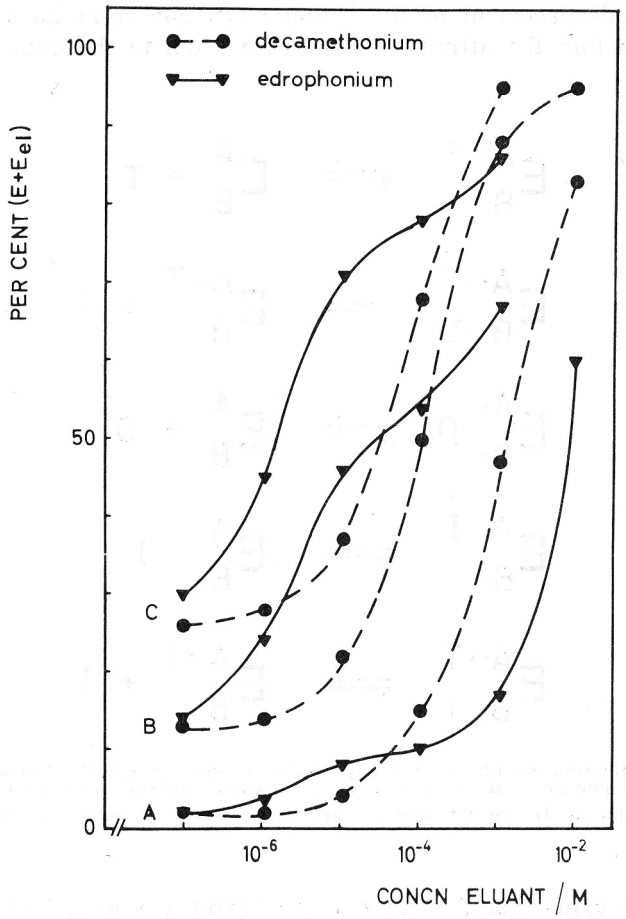

Fig. 4. Desorption of AChE by edrophonium from para-analogue gel. Effect of KCl.

40 mg trimethyl-(*p*-aminophenyl)ammonium gel suspended in 0.017 M *tris*-HCl buffer pH = 8.0. A = 0 KCl, B = 0.08 M KCl and C = 0.15 KCl. Final immobilized ligand concentration 2.4×10^{-4} M.

and site B affinity to the gel. Such a model requires that the effects of edrophonium and decamethonium as eluants should be additive. This however is not the case. On the contrary the elution by decamethonium is decreased by edrophonium (Figs. 6 and 7). The minimum of the curve appears at a concentration of 10^{-4} M to 10^{-3} M edrophonium and the elution at this minimum is about the same as attained by 10^{-3} M edrophonium alone. These results suggest that it is the same decamethonium molecule which binds to the two sites. By the displacement of the cationic function from its binding to site A by edrophonium the affinity of decamethonium to the enzyme is reduced

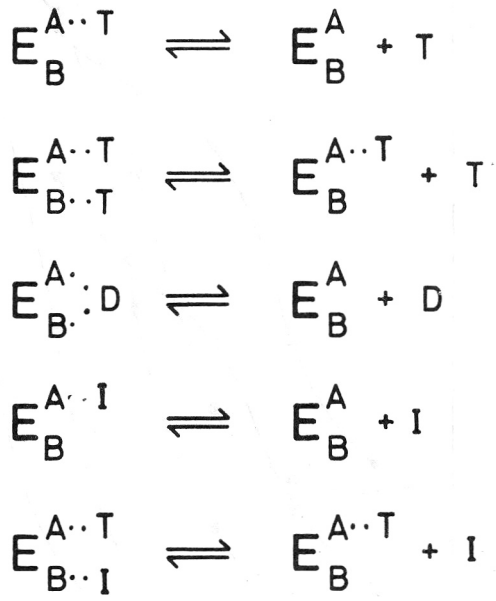

Fig. 5. The dominating equilibria in the proposed model for AChE adsorption to the para analogue gel and its desorption by edrophonium and decamethonium.
T = edrophonium, D = decamethonium and I = immobilized para analogue.

to such a low value that the immobilized ligand now effectively can compete with the second cationic function for the B site of the enzyme. This explanation thus takes in account the chelate character of the decamethonium binding to acetylcholinesterase. If our data really conform with the proposed model remains to be proved by a computer analysis using the experimental values for the dominating equilibrium constants.

At least one further anionic site on the enzyme has been postulated for the binding of d-tubocurarine, gallamine and similar compounds. This possibility is now under investigation in our laboratory with the presented method using d-tubocurarine and procaine as the immobilized ligands.

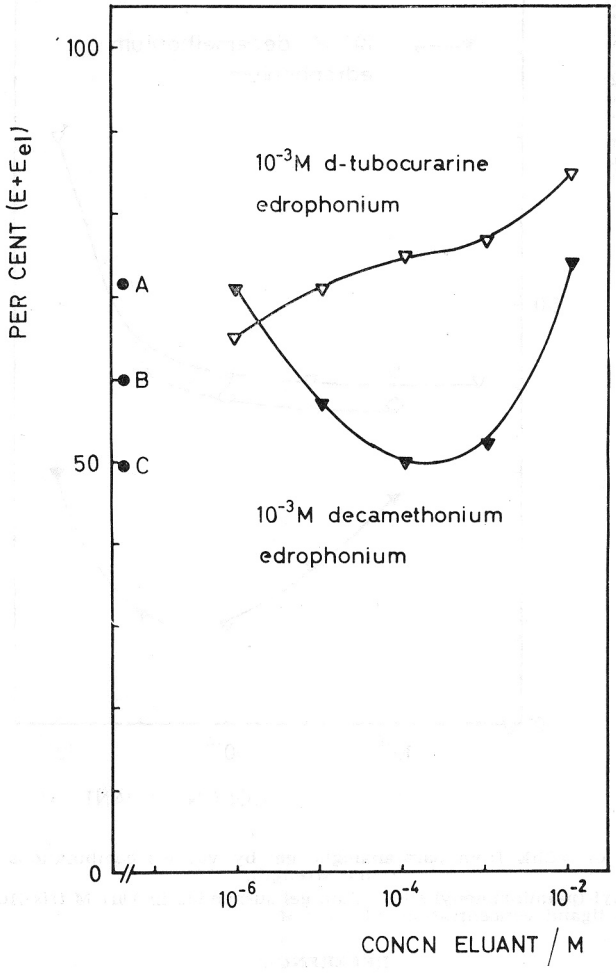

Fig. 6. Desorption of AChE from para-analogue gel by various combinations of ligands. High ionic strength.

100 mg trimethyl-(*p*-aminophenyl)ammonium gel suspended in 0.017 M *tris*-HCl buffer pH 8.0 + 0.15 M KCl. A = decamethonium 10^{-3} M, B = *d*-tubocurarine 10^{-3} M and C = edrophonium 10^{-3} M. Final immobilized ligand concentration 6×10^{-4} M.

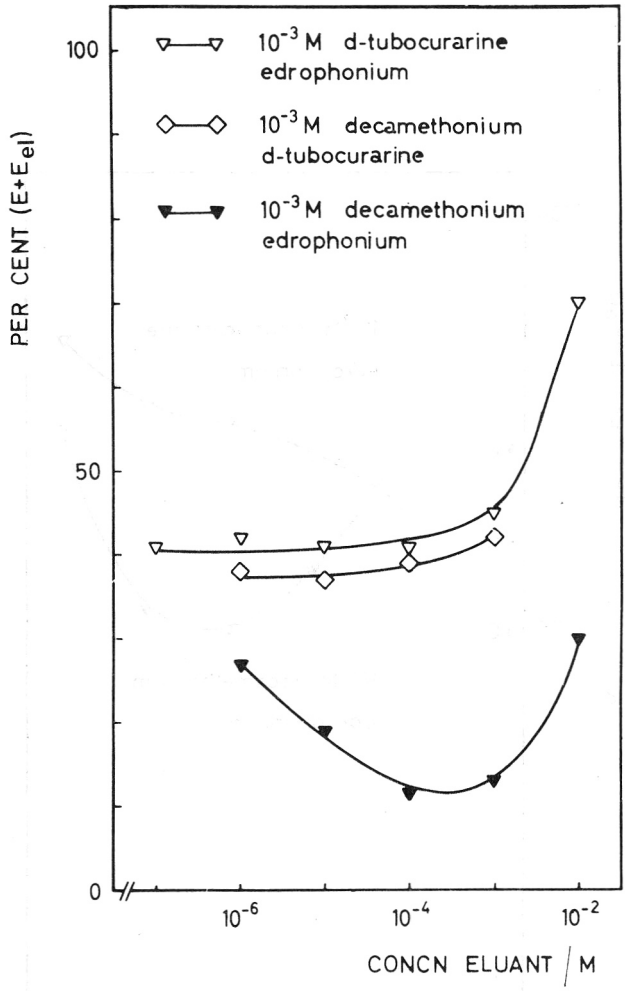

Fig. 7. Desorption of AChE from para-analogue gel by various combinations of ligands. Low ionic strength.

40 mg trimethyl-(*p*-aminophenyl)ammonium gel suspended in 0.017 M *tris*-HCl buffer pH=8.0 Final immobilized ligand concentration 2.4×10^{-4} M.

REFERENCES

1. P. O'Carra, *Affinity Chromatography of Enzymes*, in: E. D. Spencer (Ed.), *Proceedings of the FEBS special meeting in Dublin*, April 1974, Industrial Aspects of Biochemistry, North Holland, Amsterdam. 1974. pp. 107—133.

2. E. G. Hollunger and B. H. Niklasson, *J. Neurochem.* **20** (1973) 821.

DISCUSSION

P. J. Jewess:

(a) How much ligand do you have bound per millilitre of gel? (b) Have you tried more polar spacer arms? (c) Does the spacer arm alone bind acetylcholinesterase?

G. Hollunger:

(a) The ligand concentration in the meta-gel was 6×10^{-3} M and in the para-gel 3.2×10^{-3} M. The highest final concentration used in the experiments with specific eluants was 6×10^{-5} M and 3.2×10^{-4} M respectively. (b) It is our experience from a few experiments that somewhat more hydrophylic arms do not change the dissociation constant to any great extent. (c) The spacer arm does not bind the enzyme to a measurable extent. Even after eliminating the negative charge from the carboxylic group of the arm by acetylation, no binding occurs. Furthermore, the anilin gel has a very low affinity, if any, to the enzyme.

U. Brodbeck:

What is the composition of your eluting solution, i. e. what is the total ionic strength of the eluting medium? Could an increase in ionic strength *per se* explain some of your data?

G. Hollunger:

In all experiments the basic ionic strength was 0.017 (tris buffer) and in some experiments the ionic strength was increased by adding KCl. The effect of increasing the ionic strength seems to be the same as decreasing the concentration of the immobilized ligand.

SAŽETAK

Primjena afinitetne adsorpcije na gelu u istraživanju svojstava acetilkolinesteraze sisavaca za vezanje liganada

G. Hollunger i B. Niklasson

Opisana je tehnika afinitetne adsorpcije za analizu mjesta vezanja malih liganada na acetilkolinesterazu. Istraživanjem učinaka dekametonija i edrofonijuma nađeno je da sekundarno anionsko mjesto vezanja dekametonijuma pokazuje afinitet za trimetil-(*p*-aminofenil)amonij-CH-sefarozu 4B, ali ne i za trimetil-(*m*-aminofenil)amonij-CH-sefarozu 4B. Postulirano je hidrofobno područje vezanja koje bi se nalazilo u blizini anionske strane katalitičkog mjesta.

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