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MULTIPLE FORMS OF ACETYLCHOLINESTERASE FROM RAT ERYTHROCYTES

EFFECT OF FAT-FREE DIET

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Summary

Electrophoretic patterns of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from rat erythrocyte were studied. The enzyme was solubilized by the following treatments: a) Triton X-100, b) sodium deoxycholate, or c) ultrasonic irradiation. When the erythrocyte membrane was solubilized by Triton X-100 at concentrations higher than 0.3%, by 10 mM sodium deoxycholate, or by ultrasonic irradiation for more than 5 min, a single band of acetylcholinesterase activity appeared in the gel. Two bands of activity were stained in the gel when the membrane was solubilized by Triton X-100 at concentrations between 0.1—0.2%, or by ultrasound for 5 min.

Electrophoretic patterns of acetylcholinesterase from rats fed a fat-sufficient diet were similar to those for the enzyme from animals fed a fat-free diet. The recombination of lipids with the enzyme eluted from the gels confirmed the "phenotypic allosteric desensitization phenomenon".

Introduction

The allosteric behavior [1] (Hill plots) and temperature-dependent activity [2] (Arrhenius plots) of erythrocyte membrane-bound acetylcholinesterase from rats fed a fat-free diet are anomalous. In previous papers we have related this fact with the alteration of fatty acid composition in the membrane [1,2].

Indirect experiments suggested that the acetylcholinesterase present in the

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membrane erythrocytes of animals fed with or without a fat-supplement diet are identical [1]. However, additional studies of some molecular aspects of this enzyme were necessary to corroborate the mechanism involved with kinetic changes observed in the acetylcholinesterase from essential fatty acid-deficient rats. A variable multiple form of human erythrocyte membrane-bound acetylcholinesterase was reported [3—6]. We thought that studies of multiple forms with rat erythrocyte could be relevant to explain the kinetic changes of this enzyme in rats fed a fat-deficient diet. Electrophoretic characterization of the soluble enzyme obtained from rats fed a fat-sufficient and a fat-deficient diet is presented in this paper. Furthermore the role of lipids in changes of the kinetic constant is confirmed by an in vitro recombination experiment.

Materials and Methods

Animals, diets and acetylcholinesterase assay

Details concerning experimental animals, diets, ghost preparations, measurement of enzymatic activity and calculation of the kinetic parameters have been previously published [1].

Protein concentrations were estimated by the method of Lowry et al. [7]. When Triton X-100 was present in the assay system, sodium dodecyl sulfate was used to modify the method, according to Bonsall et al. [8], and prevent the formation of the water-immiscible Triton X-100 phosphomolybdate complex.

Solubilization and reconstitution of membrane-like material

A suspension of ghost erythrocytes in 20 mosM sodium phosphate buffer pH 7.4 containing 1 mM EDTA was used for membrane solubilization. The different procedures for membrane solubilization are described in the figures. The procedures of reconstitution of membrane-like material are described in Table II. The enzyme remaining in the supernatant after 100 000 × *g* for 1 h at 4°C centrifugation was considered soluble.

Polyacrylamide-gel electrophoresis procedures

Disc electrophoresis was carried out according to the method of Ornstein [9] and Davis [10] using bromophenol blue as tracking dye. Gels were normally run for 3 h at a constant current of 3 mA per tube at 4°C. The sample was mixed with 50% sucrose solution and applied to the top of the gel. When Triton X-100 was present in the sample, the detergent was incorporated into gels (0.1%) as suggested by Dulaney and Touster [11].

Acetylcholinesterase was visualized on the gel by the thiocholine method of Koelle [12] or the α-naphthyl acetate method of McManus and Mowry [13].

Proteins were stained by immersing the gel for 30 min in a fresh solution of 7% (v/v) acetic acid containing 1% (w/v) Amido Black. Electrolytic destaining was applied in 7% (v/v) acetic acid until the bands were visible.

The lipid-reacting material was stained by the following procedure: the gels were immersed in the stain solution (3.0 g Lipid Crimson in 1000 ml of 60% ethanol) for 2 h at 65°C and then left overnight at room temperature in

the same stain solution. The gels were destained for 24 h in 10% (v/v) acetic acid which was renewed four times.

Sepharose 4B column chromatography

Exclusion chromatography was performed on a beaded agarose (Sepharose 4B, product of Pharmacia, Uppsala). The manufacturer's recommendations on technique were followed and details are given in Results. The usual eluting buffer contained 5 mM sodium phosphate pH 7.0 and Triton X-100 at the concentration indicated for each experiment. The column was maintained at 4° C.

Results and Discussion

Multiple forms of Triton X-100 solubilized acetylcholinesterase from rat erythrocyte membranes

After solubilization of erythrocyte membrane from rats fed a fat-sufficient diet with Triton X-100, one or more bands of activity were found. Fig. 1 shows bands of acetylcholinesterase activity with the different mobilities obtained when the membrane solubilization was carried out with the same protein quantities but at three different Triton X-100 concentrations. With 0.12% Triton X-100 two bands of activity, (b) and (c), were obtained. When 0.04% or 0.4% Triton X-100 was used, either band (a) or band (d), with slow and fast

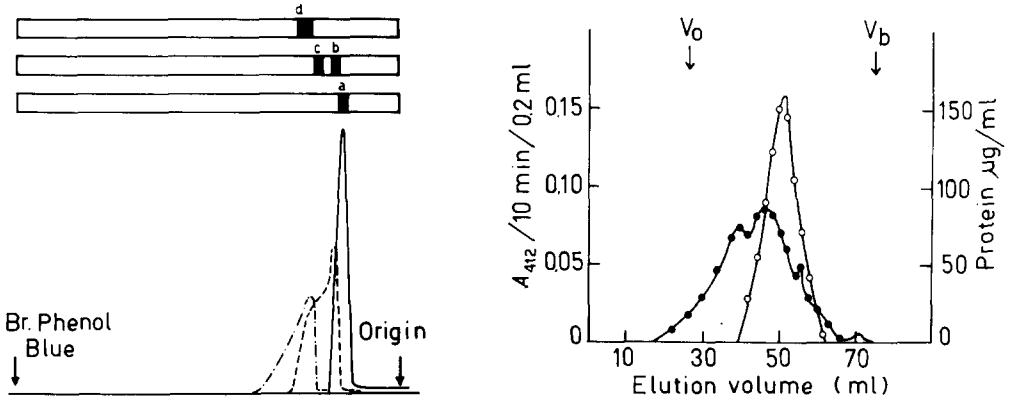

Fig. 1. Polyacrylamide-gel electrophoretograms of soluble erythrocyte acetylcholinesterase. The solubilization of red cell membrane from rats fed a fat-sufficient diet (0.50 mg protein/ml) was carried out by mixing 1 ml of membrane suspension with 3 ml of Triton X-100 to a final concentration of 0.04% (———), 0.12% (- - - - -) and 0.4% (— · — ·). After letting the preparations stand for 15 min at room temperature, they were centrifuged 100 000 × *g* for 1 h at 4° C and any pellet material was discarded. The supernatants containing 60—90% of enzyme activity were applied to the top of the gel. The gels were stained for acetylcholinesterase activity. Other experimental details are described in Materials and Methods.

Fig. 2. Elution of Triton-solubilized acetylcholinesterase from a column of Sepharose 4B. A sample (obtained by solubilization of erythrocyte membrane with 0.12% of Triton X-100) of 3.0 ml was applied to a 1.5 cm × 38 cm column, (bed volume 75 ml, void volume 26 ml). The elution rate was adjusted to 15 ml/h. Fractions of 2 ml were collected and assayed for acetylcholinesterase activity (○———○) and protein content (●———●). Recovery of enzymatic activity was about 76%. Other experimental details are described in Materials and Methods.

electrophoretic mobility respectively, were obtained. The same results were obtained when Triton X-100 was incorporated into the gel and buffer.

The difference in electrophoretic patterns obtained is due to the effect of Triton X-100 concentration and does not depend on the Triton X-100/membrane protein ratio, as demonstrated when identical electrophoretic patterns were obtained with several membrane suspensions containing different protein quantities, and solubilized with the same Triton X-100 concentration. Bands (a) and (d) can be transformed from one to the other. Band (a), obtained by solubilization with 0.04% Triton X-100, can be transformed into band (d) if the concentration of Triton X-100 is increased to 0.4%, and conversely band (d) can be transformed into (a) if the detergent is removed by Sephadex G-50 filtration.

Bands (a) and (d) were obtained simultaneously on the gel by mixing equal parts of supernatant containing (a) and (d) forms and immediate (15 min after mixing) electrophoresis. When the Triton X-100 extract containing band (a) was left two or more days at 0—4°C, band (d) was obtained. The total enzyme activity remained constant during the procedures.

The differences observed in the mobility of the enzyme obtained by solubilization with different Triton X-100 concentrations might be considered to be due to differences in surface charges and/or molecular weight. However, the latter assumption was ruled out since the enzyme preparations solubilized with 0.04%, 0.12% and 0.4% Triton X-100, showing multiple molecular forms (Fig. 1), presented a single and similar peak of acetylcholinesterase activity on Sepharose 4B. The gel column was equilibrated and eluted with buffers containing the same concentration of detergent used to obtain the soluble enzyme preparation. No activity was detected at the void volume. After column passage, the different solubilized preparations maintain their electrophoretic identity. Fig. 2 illustrates the elution profile of acetylcholinesterase solubilized by 0.12% Triton X-100 (sample that shows (b) and (c) electrophoretic bands, Fig. 1).

We could conclude that the multiple forms of acetylcholinesterase arise from differences in charge and not from differences in the apparent molecular weight. This observation is similar to that reported by Shafai et al. [3] for human erythrocytes.

Effect of Triton X-100 concentration on the electrophoretic pattern of proteins, lipids and acetylcholinesterase from rat erythrocyte membrane

Srinivasan et al. [14] reported that different Triton X-100 concentrations provoke changes in the electrophoretic pattern of whole rat brain acetylcholinesterase. These changes have been interpreted as due to the release of the catalytic protein in association with membrane fragments in various degrees of aggregation.

A gradual delipidization of the membrane protein from Semliki Forest virus [15] and red cell membrane [8] brought about by Triton X-100 was observed.

We can assume that the electrophoretic patterns of rat erythrocyte acetylcholinesterase, shown in Fig. 1, appear as a consequence of a similar process.

The effect of various concentrations of Triton X-100 on enzymatic activi-

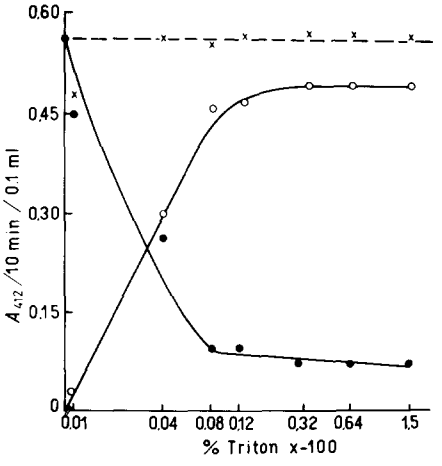

Fig. 3. Effect of Triton X-100 concentration on the acetylcholinesterase solubilization. The solubilization of membranes from fat-sufficient animals was carried out as indicated in Fig. 1. The membrane suspension used contained 1 mg protein/ml, and Triton X-100 concentrations are indicated in the logarithmic scale. After centrifugation 100 000 × *g* for 1 h at 4°C, the enzyme activity was measured in the supernatant (○) and in the pellet (●), the latter being resuspended in 4 ml of 100 mM sodium phosphate buffer pH 8.0. The total activity (X) was the sum of soluble (supernatant) and non-soluble (pellet) activity. Specific activity of the soluble enzyme was 1.8 μmol/h/mg protein at 0.01%. Triton X-100 concentration and in the range 8.5—9.5 μmol/h/mg protein for the other Triton X-100 concentrations.

ty of residue and 100 000 × *g* supernatant obtained from rats fed a fat-sufficient diet is shown in Fig. 3. The critical micellar concentration of this detergent is about 0.015% (Ref. 15). As can be seen, the acetylcholinesterase solubilization takes place over this concentration and 80—90% of the activity appears in the supernatant at Triton X-100 concentrations higher than 0.08%. It is interesting to note that the total activity remains virtually constant whatever the concentration of detergent tested.

The gel electrophoresis patterns developed for acetylcholinesterase activity and lipid materials solubilized at different concentration of Triton X-100 are shown in Fig. 4. Each gel was stained first for acetylcholinesterase activity and afterwards for lipid reacting materials, thus permitting the simultaneous observation of enzyme activity and lipid stain bands. As can be seen in Fig. 4 the major band of lipid-reacting material has identical mobility in every case but becomes broader and less intense as the concentration of Triton X-100 increases. At low Triton X-100 concentration, within the range 0.04—0.1%, soluble enzyme moves in the gel a short distance as a single band near the lipid material. Between Triton X-100 concentrations of 0.12—0.2%, two bands of activity appear and both of them move with the major lipid-stained material. At concentrations higher than 0.3%, only one band of enzyme activity appears again and its mobility increases with the Triton X-100 concentration, at the same time separating more and more from the lipid band. By increasing the time of electrophoresis from 3 to 6 h identical results were obtained.

The ranges of Triton X-100 concentration used did not modify the catalase and serum bovine albumin migration in the gel. In addition, the electrophoretic pattern of erythrocyte membrane protein solubilized with different

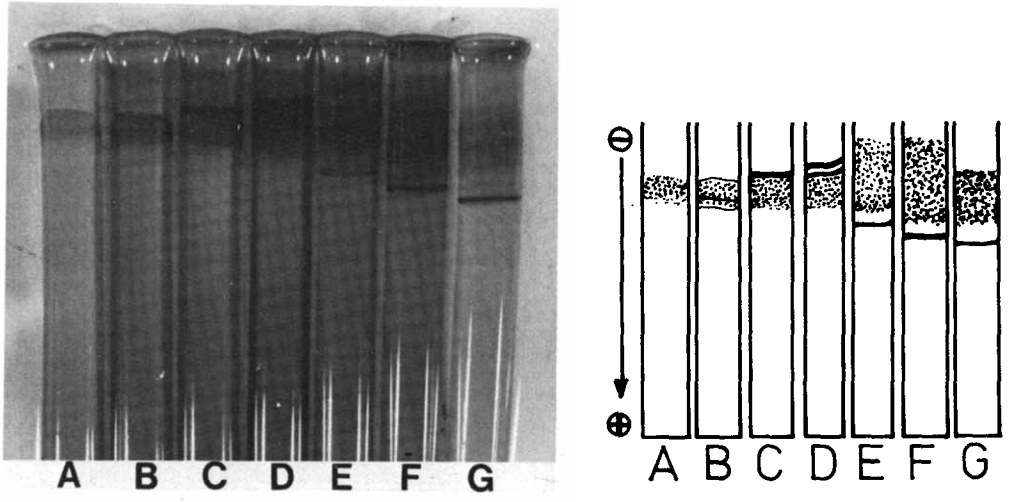

Fig. 4. Polyacrylamide-gel electrophoresis of soluble preparations obtained by Triton X-100 treatment. The different samples were prepared as indicated in Fig. 1. 1 ml of membrane suspension treated with Triton X-100 to final concentrations of: (A) 0.01%, (B) 0.04%, (C) 0.08%, (D) 0.12%, (E) 0.32%, (F) 0.64% and (G) 1.5% were layered on the top of the gels. The gels were stained for acetylcholinesterase activity (black bands) and lipid reacting materials (dotted areas). Migration was towards the anode (bottom of the gels). Other experimental details are described in Materials and Methods.

Triton X-100 concentrations shows different numbers of bands with different mobilities (not shown).

These facts suggest that the action of Triton X-100 on the enzymatic electrophoretic patterns could be due to the release of the catalytic protein from the membrane as a lipid protein complex, and to a gradual dissociation occurring when detergent concentration increases, just as it does when the charge of the particle is changed, and no changes in the molecular weight can be detected on Sepharose 4B column.

Electrophoretic patterns of acetylcholinesterase solubilized with sodium deoxycholate or ultrasonic irradiation

Several multiple enzymatic forms of acetylcholinesterase exist, the chemical nature of which are only partially understood. The number of forms reported varies widely and these variations appear to be dependent both upon species and methods of extraction [3—6,14,16].

Electrophoretic patterns of rat-liver membrane solubilized by sodium deoxycholate, sodium dodecyl sulphate or Triton X-100 were different for protein and for molecular species of phosphodiesterase, alkaline phosphatase and esterase [11]. These facts would indicate that the multiple enzymatic forms depend closely on the mechanism of membrane solubilization and therefore on the detergent used.

The presence of multiple acetylcholinesterase was investigated by solubilizing the erythrocyte membrane from rats fed a fat-sufficient diet with sodium deoxycholate or with ultrasound. A concentration of 10 mM sodium deoxycholate caused a dissociation of human red cell membrane into its component

TABLE I

R_F VALUES OF SOLUBLE ACETYLCHOLINESTERASE FROM RATS FED FAT-FREE AND FAT-SUPPLEMENTED DIETS

The solubilization procedure was described in the text and figures. R_F values were calculated by taking as reference the distance that the tracking dye (bromophenol blue) moved. The results are given as the mean ± S.E. The number of cases are in parentheses.

Solubilization treatment	Diet [a]	
	Fat-sufficient	Fat-deficient
Triton X-100 0.04% [b]	0.156 ± 0.011 (4)	0.147 ± 0.012 (3)
Triton X-100 0.12%	0.130 ± 0.009 (10) 0.190 ± 0.016	0.150 ± 0.010 (6) 0.220 ± 0.014
Triton X-100 0.4%	0.241 ± 0.009 (7)	0.275 ± 0.017 (3)
Sodium deoxycholate 10 mM	0.402 ± 0.009 (10)	0.411 ± 0.014 (12)
Ultrasound	0.21	0.20

[a] The R_F values from rats fed a fat-sufficient and a fat-deficient diet are not significantly different in any of the solubilization treatments.

[b] The R_F values for 0.04% and 0.4% Triton X-100 concentrations are significantly different ($P < 0.001$).

proteins and phospholipids [17]. When this detergent concentration was used to solubilize the membrane-bound acetylcholinesterase, a single band of activity with a high rate of migration (R_F 0.402 ± 0.009) moved into the gel (Table I). The recovery of enzyme activity in the supernatant was 63%.

Membrane solubilization can be brought about by ultrasonic irradiation

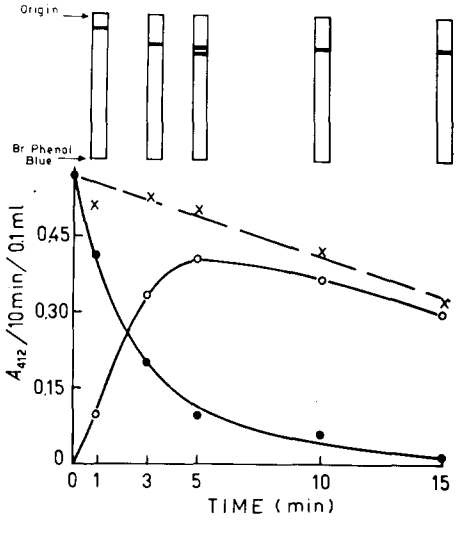

Fig. 5. Effect of different ultrasound irradiation durations on the acetylcholinesterase solubilization. 4 ml of membrane suspension from fat-sufficient animals (1 mg protein/ml) was irradiated with ultrasound at 4°C at progressively accumulated periods of time with a "Faetron" sonifier cell disrupter (Faeta S.A., Electronic Division, Argentina). After centrifuging at 100 000 × g for 1 h at 4°C, acetylcholinesterase activity was measured in soluble (○) and in pellet (●) fractions. The total activity (X) is the sum of the soluble and precipitated activity. Each polyacrylamide-gel electrophoresis of soluble enzyme is illustrated directly above its corresponding sonication duration.

[6,18]. Acetylcholinesterase activity plotted against sonication time is shown in Fig. 5. A sonication time of more than 5 min provoked a progressive inactivation of the soluble enzyme. Polyacrylamide-gel electrophoresis of the preparations treated with ultrasound for different periods of time are shown in the upper part of Fig. 5. With short periods of ultrasound irradiation (1 min), a single band of acetylcholinesterase activity was obtained. After treatments of 3 min, one band was again obtained, but this moved more rapidly than the other. Strikingly, the supernatant of erythrocyte membrane treated for 5 min presented two distinct bands in the gel. The R_F values obtained in five separate experiments were 0.24 ± 0.01 and 0.27 ± 0.01 respectively for the two bands. However, when 5% Triton X-100 was added to the ultrasound-treated solution containing two bands, and left for 3 h at 4°C, one single band was found on the gel. Only one band of acetylcholinesterase activity was found in preparations treated with ultrasound for more than 5 min.

These facts would confirm the presence of only one isoenzymatic form of acetylcholinesterase from rat erythrocyte membrane, and suggest that when different multiple forms appear, they could be due to the formation of different lipid-enzyme complexes.

Electrophoretic patterns of soluble acetylcholinesterase from rats fed a fat-sufficient and a fat-deficient diet

Several authors have pointed out the apparent lipoproteinic nature of acetylcholinesterase, although no evidence has been presented to define the lipids required for enzymatic activity. Interaction of lipid components with acetylcholinesterase from membrane erythrocyte [19,20], and electric organs [21], have also been studied. In addition, we have found that acetylcholinesterase solubilized from rat erythrocytes is inactivated by treatment with phospholipase A_1 (unpublished results). The above-mentioned reasons and our findings (Ref. 1) i.e. that the composition of red cell membrane fatty acids were different in rats fed fat-sufficient and fat-deficient diets, led us to compare the electrophoretic patterns of soluble acetylcholinesterase from both groups of animals.

Table I shows the R_F obtained for bands of acetylcholinesterase activity solubilized by different treatments, from rats fed fat-sufficient and fat-deficient diets respectively. The results are expressed as mean ± S.E. and no significant differences could be detected. These results would indicate that the phospholipid non-polar hydrocarbon chains have no important influence in the formation of the different net charge in the lipid-enzyme complex. These vary with the concentration of Triton X-100. It might seem more probable that the polar head of phospholipid plays a primary role in this phenomenon. However, as no difference in phospholipid composition has been found between fat-sufficient and fat-deficient rats [22,23], the electrophoretic patterns of acetylcholinesterase are identical in the two groups of animals.

Recombination of acetylcholinesterase with lipid extracted from fat-deficient animals: effect on the n values

In the previous papers, we have reported that the anomalous kinetic behavior of erythrocyte membrane-bound acetylcholinesterase from rats fed a

fat-free diet was normalized when the enzyme was released from the membrane by Triton X-100 solubilization [1,2]. Values of the Hill coefficient (n) for the inhibition by F^- of acetylcholinesterase from rats fed a fat-sufficient diet were found to be 1.6. Acetylcholinesterase from rats fed a fat-free diet gave n values close to 1.0. After disruption of this membrane by Triton X-100, the Hill coefficient shifted from 1.0 to 1.6, while the values of n remained unchanged when membrane from rats fed a fat-sufficient diet was subjected to similar treatment [1]. The acetylcholinesterase solubilized from both groups of animals by Triton X-100, sodium deoxycholate or sonication procedures presented n values close to 1.6.

The results of this paper show that a single band of activity in polyacrylamide-gel can be obtained under controlled and defined conditions and that this does not depend on the method employed (Triton X-100, deoxycholate and sonication). On the basis of this evidence, we made the following experiment that could provide useful information about the role of lipids in the allosteric changes that occur in the enzyme when it is bound to the fat-free membrane structure. After gel electrophoresis of solubilized acetylcholinesterase preparations from fat-sufficient animals, the gels were sectioned longitudinally: one half was stained for acetylcholinesterase activity and a 1-mm slice was sectioned from the other half at the place corresponding to the activity band, which was eluted with Triton X-100. The enzyme activity eluted from the gel was mixed with lipid extract of red cell membranes from rats fed a fat-free diet and diffused against the aqueous buffer. During diffusion the solution in the bag became opalescent and the turbidity increased with time.

TABLE II

RECOMBINATION OF ACETYLCHOLINESTERASE WITH LIPID MATERIAL EXTRACTED FROM ERYTHROCYTES OF RATS FED A FAT-FREE DIET

Eluted material: elution of activity bands was carried out after electrophoresis of the soluble enzyme. The enzyme solubilization with Triton X-100 and its electrophoretic pattern corresponded to case G described in Fig. 4. The gel was sectioned longitudinally; the activity was stained on one half of the gel and the zone corresponding to the enzymatic activity was eluted on the remainder with 0.08% Triton X-100 solution for 48 h at 0°C. Lipid extraction: 0.3 ml of erythrocyte membrane from rats fed a fat-free diet were extracted (according to the method of Folch et al. [24]) with chloroform-methanol 2 : 1, filtered through paper and partitioned with 0.2 vol. of water; the clear bottom layer was evaporated under a nitrogen stream. Reaggregation procedure: the lipid material (equivalent to 1.0 mg of membrane protein) was resuspended with 0.05 ml ethanol and 3.35 ml of 0.08% Triton X-100 solution and then 1.6 ml of eluted solution (containing 0.3 mg of protein) was added and shaken for 10—15 min at room temperature. The resulting solution was diffused for 36 h with 3—4 changes of 1000 ml of 5 mM sodium phosphate buffer, pH 6.5, containing 10 mM $MgCl_2$. The suspension was centrifuged at 100 000 × g for 15 min at 0°C. The pellet (reaggregated material) was resuspended in 100 mM sodium phosphate buffer, pH 8.0, containing 1 mM $MgCl_2$ and assayed for acetylcholinesterase activity. No activity was detected in the supernatant.

Enzyme preparation	Total activity (mol/h)	Total protein (mg)	Specific activity (mol/h per mg protein)	Yield %
Erythrocyte membrane	31.2	3.96	7.8	100
Supernatant after Triton X-100 treatment	24.6	2.96	8.2	78
Material eluted from polyacrylamide-gel	6.6	0.37	18.0	21
Material reaggregated by diffusion	3.9	0.37	10.5	12

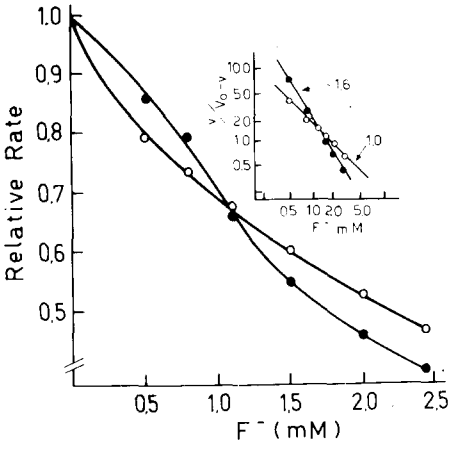

Fig. 6. Inhibition by F^- of acetylcholinesterase that is bound (○) and unbound (●) to lipid material. Both the lipid-unbound enzyme (corresponding to material eluted from polyacrylamide-gel) and the lipid-bound enzyme (corresponding to material reaggregated by diffusion) were obtained according to Table II. Inset: the same data were replotted according to Hill. The slope of each line corresponds to the Hill coefficient value (n), which is indicated in the figure.

After 36 h of diffusion, the pellet (reaggregated material) obtained by centrifugation showed 100% protein recovery; however, although the full acetylcholinesterase activity was present in the reaggregated material (no activity was found in the supernatant), only 50% of the specific activity of the material prior to lipid treatment was obtained (Table II). These results indicate that 50% of the enzyme activity was lost during the procedure (possibly as a consequence of the exposure to pH 6.5 for a long time). The reaggregated material in terms of allosteric kinetic constant closely resembles the original membrane from which the lipids were extracted. As can be seen in Fig. 6, sigmoidal (values of n 1.6) and non-sigmoidal (values of n 1.0) curves were obtained for unbound and bound enzymes respectively. These facts and the same electrophoretic patterns of the acetylcholinesterase from both groups of animals, confirm our previous hypothesis on the presence of the same enzyme in the "phenotypic allosteric desensitization phenomenon [1].

Acknowledgments

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