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HEN'S EGG YOLK CHOLINESTERASE

PURIFICATION, CHARACTERIZATION AND COMPARISON WITH HEN'S LIVER AND BLOOD PLASMA CHOLINESTERASE

AHMAD SAEED *, STEFAAN DE BOECK, IGNACE DEBRUYNE, JAN WOUTERS and JAN STOCKX **

Laboratorium voor Fysiologische Scheikunde, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000 Gent (Belgium)

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Summary

The cholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) of chicken egg yolk was partly purified and characterized. It was compared to homologous enzymes of liver and blood plasma of laying hens.

During gel filtration, yolk and liver cholinesterase were resolved into two fractions. Blood plasma cholinesterase showed one form only, identical with yolk and liver cholinesterase 1 *** (EC 3.1.1.8). This form has an M_r of 440 000 and may be a tetramer of a cholinesterase form present in yolk and liver (M_r 104 000). Substrate specificity, pH optima, K_m values, the influence of effectors (ammonium derivatives, choline, eserine, fluoride), gel filtration, gel electrophoresis, isoelectric focusing and affinity chromatography, all point to a very close similarity, if not identity, of the corresponding forms.

Introduction

Cholinesterases catalyse the hydrolysis of choline esters. In this paper we will use acetylcholinesterase in the sense of acetylcholine acetylhydrolase (EC 3.1.1.7) and cholinesterase in the sense of acylcholine acylhydrolase (EC 3.1.1.8). Information on acetylcholinesterases has been gathered mainly from

* Present address: Gomal University, Department of Chemistry, Dera Ismail Khan, Pakistan.
** To whom correspondence should be addressed.
*** As used in this paper, cholinesterase 1 and cholinesterase 2 refer to higher and lower molecular weight species of EC 3.1.1.8; this nomenclature has no connection with the terms cholinesterase I and cholinesterase II sometimes employed.

the study of the *Electrophorus electricus* and *Torpedo marmorata* enzymes [1,2].

We found cholinesterase activities in the eggs of duck, goose, hen, peacock and pheasant [3]. For the hen's egg we used the enzyme as a marker in the fractionation of components, as it was one of the most active enzymes [4]. Its relation to the cholinesterases of the laying hen's liver and blood plasma was also investigated as it is generally accepted that yolk proteins are synthesized in the liver, under oestrogen control, and transported to the oocytes via the blood plasma.

Materials and Methods

Materials. We used fresh unfertilized eggs from white leghorns. Fresh blood plasma and livers were obtained from laying hens. The chemicals used were of the purest grade available. Concanavalin A-Sepharose 4B was obtained from Pharmacia Fine Chemicals AB.

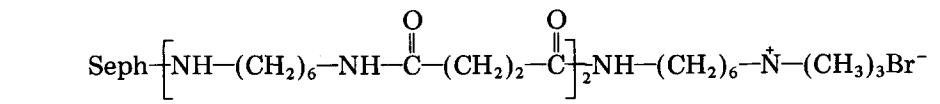
Protein assay. Protein was determined at 260—280 nm according to Layne [5], or by the method of Lowry et al. [6], using bovine serum albumin as standard.

Enzyme assays. Cholinesterase was determined with acetylthiocholine or butyrylthiocholine according to Ellman et al. [7]: 0.9 mM substrate in 0.05 M phosphate pH 7.2 at 37 °C, and measured at 405 nm. Where mentioned, the method of Hestrin [8] was used: 0.9 mM acetylcholine in 0.05 M phosphate, pH 7.2, at 37 °C, and measured at 490 nm.

Cholinesterase activities were checked with relation to time and enzyme concentration. They were perfectly linear in the conditions used.

Acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), phosphodiesterases (EC 3.1.4.1), ribonuclease (EC 3.1.27.5) and α-D-mannosidase (EC 3.2.1.24) were determined as described by Willems et al. [4]. Carboxylesterases (EC 3.1.1.1) and (EC 3.1.1.3) were determined titrimetrically.

Affinity adsorbents. Sepharose 2B-1-(*N,N,N*-trimethylammonium)-6-hexylamine was synthesized according to Hopff et al. [9,10]:



Isoelectric focusing. Isoelectric focusing was done with a 110 ml electrofocusing column (LKB) with 1% LKB ampholytes [11]. The protein was dissolved in the low-density solution. 2 ml fractions were collected and analysed for pH and enzyme activities.

Gel electrophoresis. Gel electrophoresis was done on 5% acrylamide gels in a Tris-glycine buffer, pH 8.5 [12]. Gels were loaded after 30 min pre-electrophoresis at 10 V/cm, and were run during 210 min at 10 V/cm with bovine hemoglobin as internal marker. Gels were then incubated as a whole and scanned at 385—420 nm (Kipp densitometer DD2). Duplicate gels were frozen, cut into slices of 1 mm, and incubated in the standard solution.

Preliminary purification. (1) Whole yolks were rinsed with distilled water and the contents drained through gauze. 0.02% NaN_3 was added to the yolk

and to all further preparations. The supernatant obtained after centrifugation of diluted yolk (yolk/water, 1 : 1 (v/v)) was delipidated (supernatant/diethyl-ether/ethanol, 1 : 1 : 0.1 (v/v/v)). Ether and ethanol were removed by evaporation at 35°C in vacuo and this sample was dialysed against 0.01 M Tris-HCl, pH 7.5. Centrifugation for 10 min at 27 000 × g yielded a clear supernatant. (2) Blood plasma was obtained by centrifugation of 4 vols. blood of laying hens + 1 vol. 0.35% NaCl and 3.5% trisodium citrate at 3000 × g for 10 min. The resulting plasma was delipidated and treated further as yolk samples. (3) Liver was minced, rinsed with 0.9% NaCl until A_{417} (hemoglobin) was zero, homog-

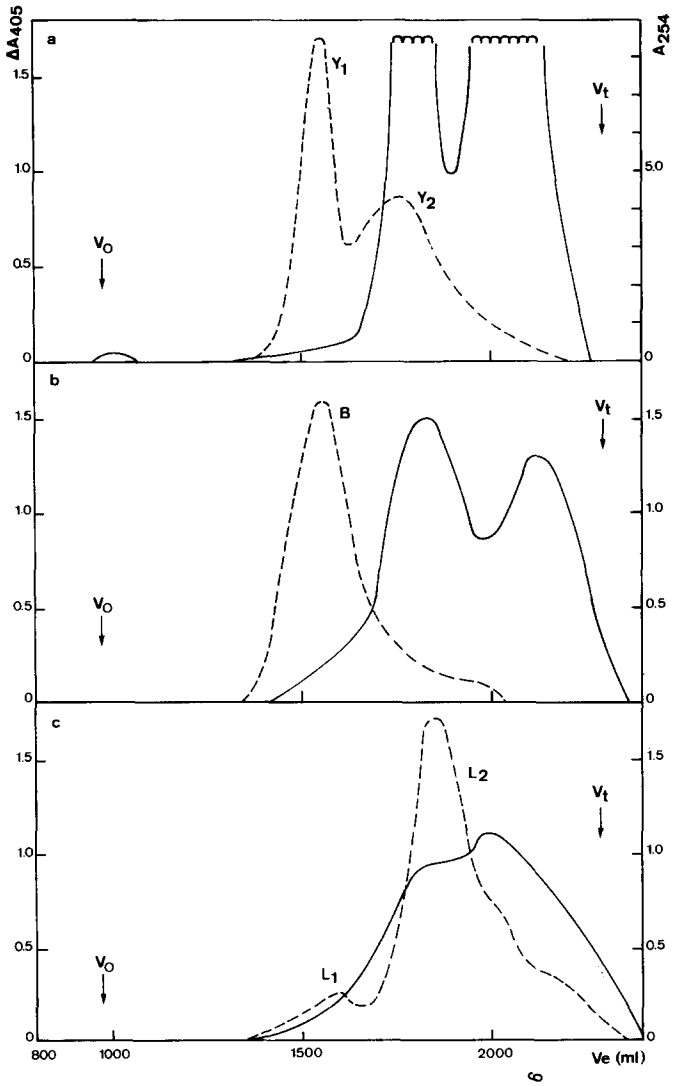


Fig. 1. Gel filtration of yolk, blood plasma and liver cholinesterase on Sepharose 6B. Column: 6 × 85 cm; eluent: 75 mM NaCl; sample: 150 ml after DEAE-cellulose chromatography; 150 ml/h; room temperature. (a) yolk; (b) blood plasm; (c) liver. (———) A_{254}; (- - - - -) ΔA_{405} cholinesterase activity with acetylthiocholine as substrate.

enized with a Virtis homogenizer (2—3 min at 30 000 rev./min; 1 g liver/ml 0.9% NaCl), and centrifuged at 48 000 × *g* for 30 min at 2°C. The supernatant was delipidated and treated further as were the yolk samples (1).

Ion-exchange chromatography. DEAE-cellulose chromatography was performed in 0.01 M Tris-HCl pH 7.5. A linear gradient (0—0.2 M NaCl) in the same buffer was applied. Cholinesterase always eluted between 25 and 75 mM NaCl. A 2-fold purification was obtained, without separation between the activities against acetyl- and butyrylthiocholine.

Gel filtration. Gel filtration on Sepharose 6B of the concentrated active fractions from the DEAE-cellulose was done in 75 mM NaCl (Fig. 1).

Blood plasma yields one peak. Liver yields two peaks: peak 1 (liver cholinesterase 1) accounting for 6% and peak 2 (liver cholinesterase 2) for 94% of the activity. Yolk also yields two peaks: peak 1 (yolk cholinesterase 1) comprising 70% of the activity and peak 2 (yolk cholinesterase 2) that accounts for 30% of it.

Concanavalin A-Sepharose 4B. The active fractions after gel filtration on Sepharose 6B were dialysed against distilled water, lyophilized, dissolved in 0.02 M Tris-HCl pH 7.0, and applied on concanavalin A-Sepharose 4B (Fig. 2).

Sepharose 2B-1-(N,N,N-trimethylammonium)-6-hexylamine [9,10]. Preparations purified on DEAE-cellulose were dialysed against 0.02 M Tris-HCl, pH 7.0. Chromatography was performed on 25 ml adsorbent, equilibrated in the same buffer. Cholinesterase was retained quantitatively and could be recovered with 22 mM NaCl, 33 mM trimethylammonium chloride, 13 mM tri-*n*-butylammonium chloride, 17 mM tetramethylammonium chloride, 13 mM tetra-

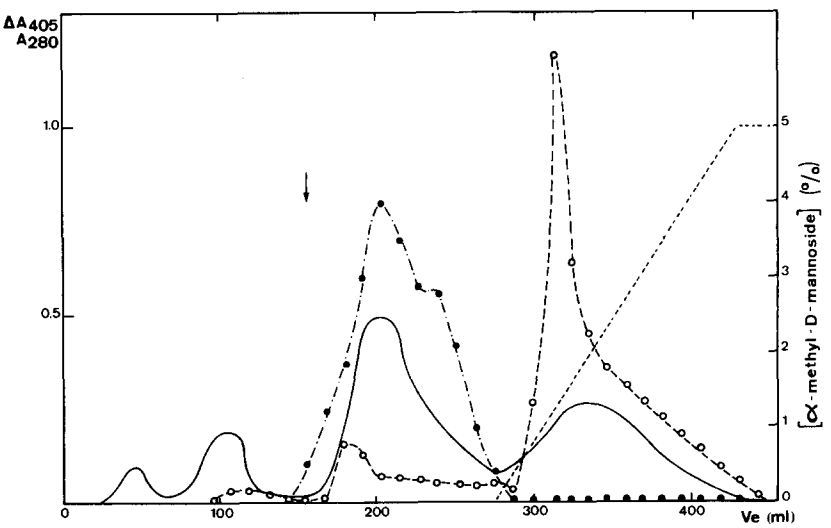


Fig. 2. Concanavalin A-Sepharose. Column: 1.6 × 50 cm; eluent; 0.02 M Tris-HCl pH 7.0; sample: 100 ml yolk cholinesterase 2 in the same buffer, after Sepharose 6B; room temperature. Arrow: buffer + 0.5% α-methyl-D-glucoside; gradient (------) α-methyl-D-mannoside in 0.02 M Tris-HCl pH 7.0. (———) A_{280}; (○———○) ΔA_{405}: cholinesterase activity with acetylthiocholine; (●———●) ΔA_{405}: 0.9 mM *p*-nitrophenylacetate as substrate.

n-butylammonium chloride, 5 mM *N,N,N',N'*-tetramethylethylenediamine or 5 mM *N,N,N',N'*-tetramethyl-1,6-hexane diamine, thus showing the nonspecific character of the adsorption.

In the final purification procedure, cholinesterase preparations after concanavalin A-Sepharose were chromatographed on Sepharose 2B-1-(*N,N,N*-trimethylammonium)-6-hexylamine, equilibrated with 0.02 M Tris-HCl, pH 7.0, and eluted with a gradient from 0 to 0.1 M tetramethylammonium chloride in the same buffer. Shortening the spacer only reduced the capacity. An adsorbent, having only a spacer without affinity ligand, partly bound the enzyme and elution was possible with 1 M NaCl.

Results

Purification procedure. The final purification is summarized in Table I. Due to difficulties in protein determination after concanavalin A-Sepharose, purification factors are given for the two chromatographic steps (concanavalin A-Sepharose and affinity chromatography) together. Affinity chromatography of yolk samples after gel filtration, without chromatography on concanavalin A, gives purification factors of 20. The presence of a few contaminating enzymes was investigated in the purified samples. The rate of hydrolysis of *p*-nitrophenylacetate, a substrate used for the determination of enzymes as divergent as carboxylesterases, chymotrypsin, trypsin and carbonic anhydrase,

TABLE I

PURIFICATION OF HEN'S EGG YOLK, BLOOD PLASM AND LIVER CHOLINESTERASE

After affinity chromatography refers to activities and purification factors after concanavalin A-Sepharose and affinity chromatography on Sepharose 2B-1-(*N,N,N*-trimethylammonium)-6-hexylamine. n.d., not determined.

		Vol (ml)	mg protein/ml	mU/mg protein	Purification factor	Percent recovery
(1)	Yolk 1:1 H_2O	2300	92.0	1	1	100
	Delipidated, dialysed	850	24.5	7.75	7.5	75
	DEAE-cellulose	600	10.6	27.7	27	80
	Sepharose 6B peak 1	440	3.5	71.4	70	51
	peak 2	275	10.0	5.6	6	7
	After affinity chromatography					
	peak 1	n.d.	0.015	12 500	12 100	n.d.
	peak 2	n.d.	0.036	240	230	n.d.
(2)	Blood plasm	160	40.5	9.41	1	100
	DEAE-cellulose	1250	1.40	17.1	1.8	50
	Sepharose 6B	280	0.27	238.0	25	30
	After affinity chromatography	n.d.	0.120	4 625	490	n.d.
(3)	Liver supernatant	600	58.0	3.00	1	100
	Delipidated, dialysed	425	15.0	12.54	4.2	76
	DEAE-cellulose	1545	0.500	116.7	39	86
	Sepharose 6B peak 1	272	0.070	201	67	3
	peak 2	810	0.290	169	56	52
	After affinity chromatography					
	peak 1	n.d.	0.080	584	185	n.d.
	peak 2	n.d.	0.105	4 170	1 390	n.d.

TABLE II

ISOELECTRIC POINTS OF THE EGG YOLK, BLOOD PLASMA AND LIVER CHOLINESTERASE

In brackets: minor peaks or shoulders. Substrates: acetyl- and butyrylthiocholine.

Enzyme	Isoelectric points				
Yolk cholinesterase 1	4.80	(4.95)			
Yolk cholinesterase 2	4.80				
Blood plasma cholinesterase	4.80				
Liver cholinesterase 1	(4.80)		5.30	(5.80)	6.80
Liver cholinesterase 2	(4.90)		5.30	5.80	6.80

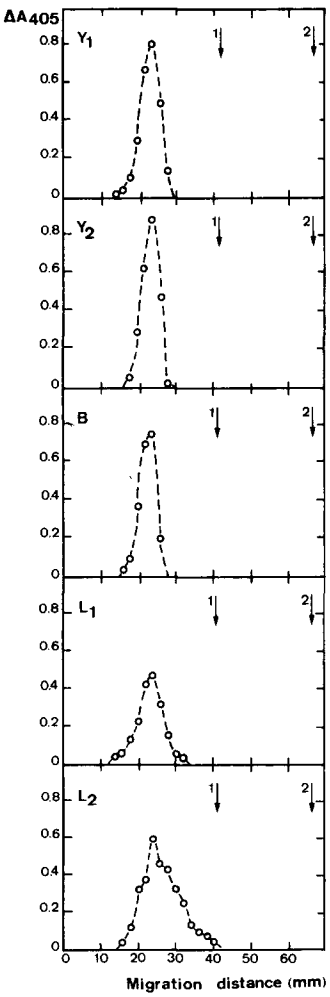


Fig. 3. Polyacrylamide gel electrophoresis. 5% acrylamide gels; Tris-glycine buffer pH 8.5; sample after affinity chromatography: 5 μg dissolved in 60 μl. Arrow 1: hemoglobin; arrow 2: bromophenolblue. Y1 yolk cholinesterase 1; Y2: yolk cholinesterase 2; B; blood plasma cholinesterase; L1: liver cholinesterase 1; L2: liver cholinesterase 2. Activities with acetyl- and butyrylthiocholine are completely coincident.

was only 1.0—1.5% compared to that with acetylthiocholine. Only trace amounts of acid and alkaline phosphatases and diesterases could be detected (rate of hydrolysis: 2% or less as compared to acetylthiocholine). Mannosidase and ribonuclease were absent. Lipase (substrate: olive oil or glycerintributyrate) and carboxylesterase (methyl-, ethyl-, butyl-, isoamylacetate as substrates) were present in insignificant amounts.

Enzyme location. The egg cholinesterase is located almost exclusively in the yolk. White is 40-times less active, whereas ovovitelline membrane is practically devoid of it. The specific activities were determined in different maturation stages of the oocytes, defined according to Gilbert [13], only relatively small changes occur between the 5th and the 9th stage.

Enzyme stability. As lyophilized powder, cholinesterases retain full activity for more than six months at −16 °C. Dialysis against distilled water at 4 °C and repeated freezing, thawing and lyophilization have no influence. The stability at 25 °C is excellent between pH 6—9. All activity is destroyed after 10 min at 70 °C (pH 7.2).

Isoelectric focusing. The enzyme p*I* values determined by isoelectric focusing are summarized in Table II.

Gel electrophoresis. Blood plasma, yolk cholinesterases 1 and 2 and liver cholinesterase 1 give one band. Liver cholinesterase 2 shows a somewhat more complex pattern (Fig. 3).

pH optimum. pH optima were measured with acetylcholine chloride according to Hestrin [8] (Fig. 4). No significant differences are seen in the profiles between pH 6—9. The pH optima are all near pH 8.0.

K_m values. The K_m values are shown in Table III. Substitution of acetylthiocholine by the butyryl derivative yields the same K_m for yolk cholinesterase 1.

Molecular weights. Molecular weights were determined by gel filtration on a calibrated Sephadex G-200 and Sepharose 6B column in 0.01 M Tris-HCl, pH 7.0. We found the following values: yolk and liver cholinesterase 1 and blood plasma cholinesterase, 440 000 ± 30 000 (Sepharose 6B); yolk and liver cholinesterase 2, 104 000 ± 9000 (Sephadex G-200). Within experimental error the M_r values are the same in each group.

Substrate specificity. Different choline and thiocholine esters were tested. Results are summarized in Table IV. Some non-choline esters withstood hydrolysis; among them *p*-nitrophenylsulfate, *O*-acetyl-hydroxyproline, *N*-acetyl-5-hydroxytryptamine and *O*-acetyl-L-serine. Acetyl-β-methylcholine chloride is only a poor substrate.

Effect of ammonium compounds. The kind of influence of tetramethylammonium chloride and choline chloride is dependent on their respective concentrations. All enzymes show identical responses. Choline chloride stimulates enzyme activity between 0 and 0.02 M. Activity decreases at higher concentrations (Fig. 5).

The inhibition by eserine is of special importance in the study of cholinesterases, since this alkaloid is a specific cholinesterase inhibitor with an exceptional potency [1,14]. We assayed it in the concentration range $1.43 \cdot 10^{-8}$ M to $2.86 \cdot 10^{-5}$ M. All enzyme preparations showed similar sigmoid curves: $I_{50} = (4.6 \pm 0.6) \cdot 10^{-7}$ M (S.E.).

The inhibitory action of ammonium compounds on yolk cholinesterases was

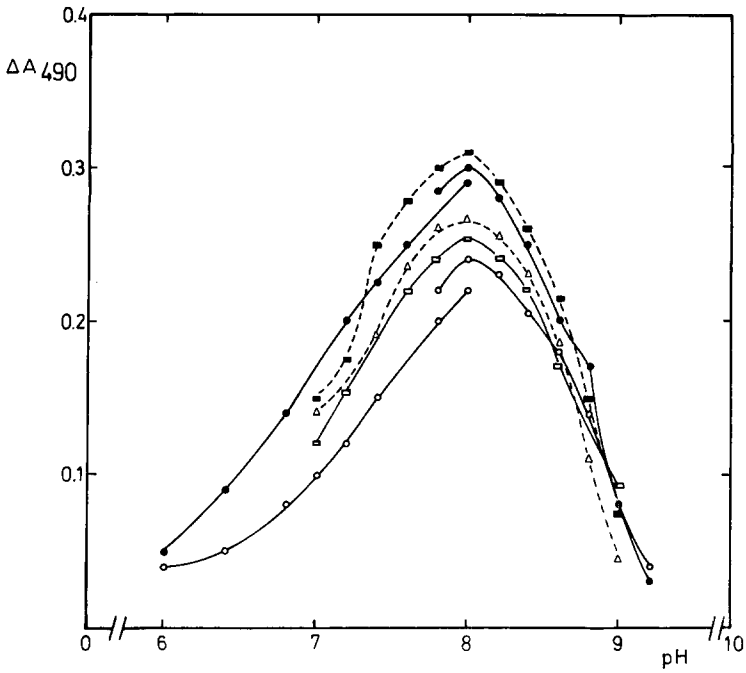


Fig. 4. pH optima. Enzymes after Sepharose 6B, determined according to Hestrin [8]. pH 6.0—8.0: phosphate; pH 7.8—9.2. Tris-HCl (ionic strength 0.05). (●———●) Yolk cholinesterase 1; (○———○) yolk cholinesterase 2; (△- - - - -△) blood plasma cholinesterase: (■- - - - - -■) liver cholinesterase 1; (□———□) liver cholinesterase 2.

predominantly either of the competitive or uncompetitive type. Cetavlon inhibits competitively: K_i = 0.15 ± 0.02 mM (S.D.). Tetrabutylammonium inhibition is predominantly competitive (K_i = 0.98 ± 0.04 mM with acetylthiocholine and 0.93 ± 0.05 mM with butyrylthiocholine). Inhibition produced by *N,N,N',N'*-tetramethylethylene diamine (K_i = 7.5 ± 0.6 mM against acetyl- and 4.0 ± 0.7 mM against butyrylthiocholine) and by *N,N,N',N'*-tetramethyl-1,6-hexane diamine (K_i = 2.2 ± 0.2 mM against acetyl- and 2.5 ± 0.3 mM against butyrylthiocholine) are of the uncompetitive type.

Effects of cations and anions. Cations (20 mM) had comparable effects on all enzyme preparations with either substrate (chlorides and acetates were used).

TABLE III

K_m values of the cholinesterases. Standard incubation mixture: substrate:acetylthiocholine iodide; enzyme:purified samples after affinity chromatography. K_m values ± S.D. are given.

Enzyme	K_m (10^{-5} M)
Yolk cholinesterase 1	2.4 ± 0.1
Yolk cholinesterase 2	2.3 ± 0.2
Blood plasma cholinesterase	2.6 ± 0.2
Liver cholinesterase 1	3.2 ± 0.2
Liver cholinesterase 2	3.0 ± 0.4

TABLE IV

SUBSTRATE SPECIFICITY

Activities are expressed as percentage compared to acetylcholine chloride. Y1, yolk cholinesterase 1. Y2, yolk cholinesterase 2. L1, liver cholinesterase 1. L2, liver cholinesterase 2. B, blood plasma cholinesterase.

Substrate	Enzyme fraction				
	Y1	Y2	L1	L2	B
Acetylcholine chloride *	100	100	100	100	100
Propionylcholine chloride *	114	117	98	102	100
Butyrylcholine chloride *	107	104	94	98	96
Succinyl dicholine dichloride *	6	8	0	0	6
Benzoylcholine chloride *	12	17	12	15	16
Acetyl-β-methylcholine chloride *	14	16	8	14	15
Acetylthiocholine iodide **	132	133	130	131	132
Propionylthiocholine iodide **	178	164	170	200	208
Butyrylthiocholine iodide **	149	145	130	136	142

* Determined according to Hestrin [8].

** Determined according to Ellman et al. [7].

Whereas Mg^{2+} and Ba^{2+} were more or less activating (up to 130% activity), Mn^{2+}, Ca^{2+} and Sr^{2+} were without any effect and Co^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+} were inhibiting (80—35% residual activity, in this order).

Cl^-, Br^-, I^-, NO_3^-, SO_4^{2-} and acetate did not affect the activities. Citrate was the strongest activator (140% activity for 20 mM). The strongest inhibition was shown by fluoride: I_{50} is 20 ± 1 mM (S.E. of the mean) in 0.05 M phosphate pH 7.2 and 58 ± 4 mM (S.E. of the mean) in 0.1 M Tris-HCl pH 7.2 for all enzymes. EDTA was without any effect. All anions had Na^+ as common cation.

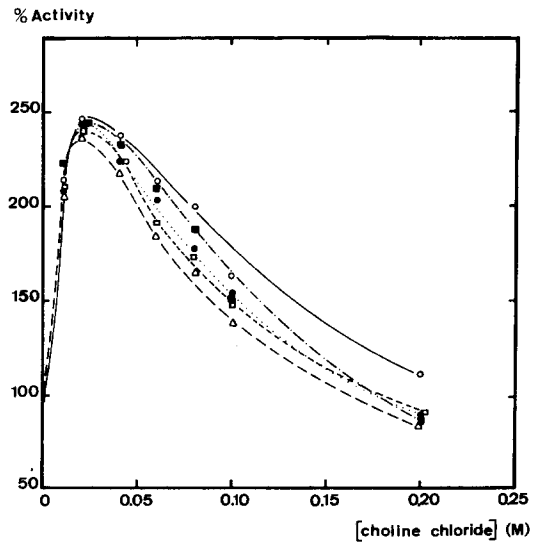


Fig. 5. Effect of choline chloride. Standard incubation mixture. (●———●) Yolk cholinesterase 1; (○———○) yolk cholinesterase 2; (△———△) blood plasma cholinesterase; (■———■) liver cholinesterase 1; (□———□) liver cholinesterase 2.

Discussion

Two cholinesterase fractions can be isolated from egg yolk and hen's liver while hen's blood plasma shows only one form. All these enzymes could be appreciably purified using ion-exchange chromatography, gel filtration and affinity chromatography. In the different purification steps no distinction could be made between activities against acetyl- and butyrylthiocholine. All five enzymes hydrolyse both substrates equally.

The absorption on concanavalin A-Sepharose 4B points to the glycoprotein nature of the five enzyme varieties. Most cholinesterases have indeed been characterized as glycoproteins [15,16].

Although affinity chromatography is a well documented technique for purifying acetylcholinesterases, it is not frequently used for cholinesterases. Our results and a previously published purification of human serum cholinesterase [17] indicate that adsorbents designed for acetylcholinesterase purification, are equally suited for cholinesterases.

1-(*N*,*N*,*N*-Trimethylammonium)-6-hexylamine is not a good inhibibor, but affinity chromatography with this compound as ligand yields excellent purification factors.

Sepharose 2B-1-(*N*,*N*,*N*-trimethylammonium)-6-hexylamine is not a pure affinity adsorbent for our enzymes, since these partly adsorb to the spacer. This may be due to hydrophobic interactions. Massoulié and Bon [18] also emphasized the role of hydrophobic interactions in affinity chromatography of cholinesterases. The molecular weights for yolk and liver cholinesterases 1 and for blood plasma cholinesterase amount to 440 000 and to 104 000 for yolk and liver cholinesterase 2. A tetramer state seems plausible for the first group. Analogous situations, where active subunits and higher molecular weight forms are found together, are quite common for cholinesterases and acetylcholinesterases [2,19—21].

The pH optimum, 8.0, is the same for all enzymes studied, K_m values against acetylthiocholine are also very alike (Table III). The fact that no substrate inhibition is found once more points to cholinesterases (EC 3.1.1.8). With regard to substrate specificity, no differences could be detected between the different enzyme forms (Table IV). The comparable hydrolysis rates for acetyl and butyryl derivatives and the very low activity against acetyl-β-methylcholine provide further evidence for a classification under EC 3.1.1.8. The yolk cholinesterase thus seems different from that found in chicken embryo leg muscle (EC 3.1.1.7) [22]. Simple carboxylesters and lipids are not hydrolysed to any significant degree. This, together with the strong inhibitory action of eserine on the five enzyme forms, rules out interferences with carboxylesterases (EC 3.1.1.1) and lipase (EC 3.1.1.3).

No effector seems able to discriminate between the five enzyme forms. This is particularly well illustrated by choline (Fig. 5). Cations and anions act in the same way against the different forms. Fluoride, a known inhibitor of cholinesterases [23] displays the same effect on the five cholinesterases.

Isoelectric focusing shows an identical enzyme profile for yolk cholinesterase 1 and blood plasma cholinesterase. Yolk cholinesterase 2 yields a slightly different distribution (Table II). The liver enzymes, however, are more

diversified and the main p*I* values are higher. But here, too, we find minor peaks with p*I* values close to those of blood plasm and yolk cholinesterases. Charge differences seem to exist between the liver enzymes and the blood and yolk enzymes.

Gel electrophoresis likewise points to very similar enzymes. Here, all cholinesterases move with the same speed (Fig. 3). Bands for the liver enzymes are somewhat broader and different enzyme forms may be present.

All results confirm the very close similarity of a probable tetrameric form in liver, blood plasm and yolk. They also reveal the presence of a form with a lower molecular weight (maybe the monomer) that is absent in blood plasma. We may therefore conclude tentatively that the yolk cholinesterase is synthesized in the liver and transported via the blood to the oocytes. Similar transport systems were described for egg yolk adenosine deaminase [24], alkaline phosphatase [25], and other proteins [26].

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