A Subunit-Sized Butyrylcholinesterase Present in High Concentrations in Pooled Rabbit Serum

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A butyrylcholinesterase of mol.wt. approx. 83000 was observed in pooled rabbit serum. The enzyme was named monomeric butyrylcholinesterase to distinguish it from the larger oligomeric butyrylcholinesterase of horse and human serum whose subunits are the same size as the monomeric enzyme. The active-site concentration of monomeric butyrylcholinesterase in the pooled serum was $0.18\ \mu\text{M}$, which is five times the concentration of butyrylcholinesterase in pooled horse serum. This was surprising, since the horse serum is regarded as a rich source of butyrylcholinesterase, whereas rabbit serum is not generally thought to contain significant amounts of any butyrylcholinesterase. The explanation, in large part, was the relatively low k_{cat} of the monomeric enzyme, which was approx. 57 s^{-1} with butyrylthiocholine as substrate and is one-thirtieth of the comparable k_{cat} of horse butyrylcholinesterase. The substrate specificity of monomeric butyrylcholinesterase also differed significantly from that of horse and human butyrylcholinesterase. For example, with the monomeric enzyme, the hydrolysis of 1 mM-acetylthiocholine was only 4% the rate for 1 mM-butyrylthiocholine, whereas human and horse butyrylcholinesterases hydrolysed 1 mM-acetylthiocholine at 50% of the rate for 1 mM-butyrylthiocholine. Moreover, monomeric butyrylcholinesterase generally hydrolysed aromatic esters more rapidly than choline esters, whereas the reverse is true of the butyrylcholinesterases. To facilitate the study of monomeric butyrylcholinesterase, it was separated from the larger butyrylcholinesterase and acetylcholinesterase, also present in rabbit serum, and purified 89-fold by fractionation with $(\text{NH}_4)_2\text{SO}_4$ and ion-exchange chromatography.

We report a butyrylcholinesterase (EC 3.1.1.8) in pooled rabbit serum which was first located on polyacrylamide gels after disc electrophoresis as an unusually fast-moving band which stained preferentially with butyrylthiocholine substrate. The butyrylcholinesterase in this band was separated from other esterases and purified 89-fold by ion-exchange chromatography and fractionation with $(\text{NH}_4)_2\text{SO}_4$. The partially purified preparations were concentrated 50-fold over the serum activity to facilitate kinetic studies.

Perhaps the most intriguing property of this rapidly migrating butyrylcholinesterase was its relatively small molecular weight of about 83000. This is one-quarter the size of the predominant butyrylcholinesterases in horse and human serum, whose molecular weights have been estimated within the range 315000–360000 (Haupt *et al.*, 1966; Das & Liddell, 1970; Main *et al.*, 1972; Teng *et al.*, 1976). With the subunit structure of cholinesterase in mind, this relationship may be of particular interest, since rabbit serum also contains a large butyrylcholinesterase.

We suggest the name 'monomeric' butyrylcholin-

esterase for this smaller enzyme since it is about the same size as the subunits produced when the larger horse butyrylcholinesterase is subjected to sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis (Teng *et al.*, 1976; A. R. Main & S. C. McKnelly, unpublished work).

Rabbit serum is classed with those mammalian sera that have low cholinesterase activities (Augustinsson, 1959; Ecobichon & Comeau, 1973). The butyrylcholinesterase activities of horse and human sera, on the other hand, are among the highest reported. Surprisingly then, the active-site concentration of monomeric butyrylcholinesterase in pooled rabbit serum was five times that of the butyrylcholinesterase in horse serum. The low activity was explained primarily by the low k_{cat} of monomeric butyrylcholinesterase in reaction with butyrylthiocholine.

Similarities in the choline ester substrate specificities of a 'benzoylcholinesterase' reported by Ellis (1947) and Koelle (1953) in the sera of certain rabbits and of monomeric butyrylcholinesterase suggested that the two enzymes may be the same.

An acetylcholinesterase of the type found in erythrocytes (EC 3.1.1.7) is generally considered to be

the predominant cholinesterase in rabbit serum (e.g. Mendel & Rudney, 1945; Augustinsson, 1958), and this too was found in the pooled rabbit serum.

Experimental

Materials

Pooled rabbit serum (type 1 SQ) was from Pel-Freez Biologicals, Rogers, AR, U.S.A. The fresh blood serum and liver of a female New Zealand strain of rabbit were also used. Phenyl thioacetate and phenyl thiobutylate substrates were prepared as described by Booth & Metcalf (1970). *o*-Nitrophenyl butyrate was prepared as described by Main *et al.* (1961). DEAE-Sephadex A-50 and Sepharose 6-B were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Electrophoretically pure horse serum butyrylcholinesterase was prepared by the 23°C method of Main *et al.* (1974). Other chemicals and reagents were from the usual commercial sources.

Methods

Determination of cholinesterase activities. The method of Ellman *et al.* (1961) was used for most studies. In addition to the esters of the phenyl thiol acids, the substrates included the iodides of acetylthiocholine, propionylthiocholine and butyrylthiocholine. Activities were determined with u.v. spectrophotometers co-ordinated with a 10 in linear-log recorder, as previously described (Main *et al.*, 1974). Enzyme solution (5–50 μ l) was pipetted directly into 3 ml of substrate to start the reaction. Velocities were taken from the slopes of the linear progress curves generated on the recorder. Non-enzymic blanks were subtracted. These increased significantly when the enzyme samples contained $(\text{NH}_4)_2\text{SO}_4$ at the concentrations used for protein fractionation. Activities were determined at 25°C in 0.1 M-sodium phosphate buffer, pH 8.0. One unit of monomeric butyrylcholinesterase activity was the hydrolysis of 1 μ mol of 1 mM-butyrylthiocholine substrate/min under these conditions. In general, an activity unit refers to the hydrolysis of 1 μ mol of substrate/min at 25°C.

Measurement of protein concentrations. Protein was estimated as a routine by A_{280} with 1 cm-light-path cuvettes. With the relatively crude samples involved, an absorption of 1.0 for a concentration of 1 mg of protein/ml was a reasonable approximation.

Disc gel electrophoresis. Disc-gel-electrophoresis runs were made with a Savant Instruments apparatus by the standard method of Davis (1964). Resolving gels typically contained 6% (w/v) acrylamide with 3% cross-linking and were formed in 5 mm $\times$ 100 mm glass tubing at 23°C. Electrophoresis was at $20 \pm 0.5^\circ\text{C}$ at a constant 2.5 mA/gel. A Bromophenol Blue tracking dye was used. The run was allowed to continue

until the dye had travelled to within 5 mm of the end of the resolving gel. This required about 2 h on 6% (w/v) gels. The tracking dye band was marked by injecting the gel with black ink at its position, as shown, for example, in Fig. 1.

After electrophoresis, esterases were located on the gels by substrate-staining procedures adapted from histochemical techniques. To locate cholinesterases, the gels were incubated in the thiocholine ester-staining solutions recommended by Koelle (1963) with minor modifications. Polyvinylpyrrolidone (7½%, w/v) was substituted for Na_2SO_4 as suggested by Bernsohn *et al.* (1961). Acetylthiocholine, propionylthiocholine and butyrylthiocholine were used. Their concentration in the substrate stain was 6 mM. Some gels were incubated for about 18 h at 25°C rather than for 4 h at 37°C. The longer incubation was more effective in bringing out the position of monomeric butyrylcholinesterase. The insoluble white cupric thiocholine that was deposited on the gel in the region of the cholinesterases was converted into dark-brown CuS by treatment with $(\text{NH}_4)_2\text{S}$ for better detection.

Esterases were also located by incubation with indoxyl acetate substrate (Holt, 1952; Barnett & Seligman, 1951). According to Augustinsson (1959), indoxyl acetate is a general substrate and will react with all three classes of esterase in sera, namely cholinesterase, carboxylesterases, and arylesterases (Aldridge, 1953), but the sensitivity varied, depending on the esterase. Both cholinesterases and carboxylesterases reacted readily, but indoxyl acetate appeared to be a poor substrate for arylesterases.

The best results were obtained when 0.02–0.04 unit (μ mol/min) of each of the activities of interest was applied to a gel. Activity loadings of this size were not possible when serum was the sample because of the relatively high protein concentration. The loading of the larger butyrylcholinesterase activity in particular was then so low that its presence in serum samples was not always clearly revealed (see Fig. 1, gels 1–4).

To give an indication of the position of a band on the gel relative to other bands, the distance that each band had travelled down the resolving gel relative to the distance travelled by the Bromophenol Blue tracking dye was measured. This ratio was the relative mobility of the esterase in the band (Weber & Osborn, 1969). The relative mobilities of a given esterase agreed within 5% when measured on different gels run at different times under the same conditions. Consequently, relative mobilities were one of the criteria used to identify the esterase complement of various samples from the purification steps.

Another criterion was substrate specificity as revealed by the depth of staining of bands on different gels characterized by the same relative-mobility values but treated with different substrate stains.

Valid comparisons could be made when the same sample and loading had been applied to each of several gels, which were then treated identically except for the substrates in which they were incubated.

Results

Separation and partial purification of rabbit serum cholinesterases

Disc gel electrophoresis of pooled rabbit serum. A typical experiment involved applying 500 μg of serum protein in 0.1 ml of an appropriate solution to each of eight gels. After electrophoresis, two gels were stained with butyrylthiocholine substrate, two with propionylthiocholine, two with acetylthiocholine and two with indoxyl acetate. Examples of the gel-staining patterns obtained with rabbit serum and partially purified preparation of monomeric butyrylcholinesterase are illustrated in Fig. 1. Four different relative-mobility values, 0.12, 0.19, 0.67 and 0.73, characterized the bands on the different gels. The depth of staining by the various substrates indicated that the first band (relative mobility 0.12) was a butyrylcholinesterase, the second (relative mobility 0.19) was an acetylcholinesterase, the third (relative mobility 0.67) was a carboxylesterase, and the fourth (relative mobility 0.73) was the monomeric butyrylcholinesterase. No bands attributable to an arylesterase were observed.

Preparative separation and partial purification of rabbit serum cholinesterases. The nature of the cholinesterases in rabbit serum reflected by disc-gel-electrophoresis results were further investigated by separating them on a preparative scale. The procedure involved two stages: fractionation with $(\text{NH}_4)_2\text{SO}_4$ and ion-exchange chromatography. The steps were closely patterned after comparable steps used in the purification of horse serum butyrylcholinesterase as described by Main *et al.* (1974).

Two successive concentrations of $(\text{NH}_4)_2\text{SO}_4$ (33 and 60% satn.) were used at room temperature. The supernatant from the first and the precipitate from the second, containing 85% of the cholinesterase activity, were retained after centrifugation. The precipitate was dissolved in 50 mM-sodium phosphate, pH 8.0, and dialysed twice at 5°C, first against 6 litres of water and then against 6 litres of 40 mM-sodium phosphate buffer, pH 8.0.

After dialysis, the product, containing approx. 10 g of protein from 500 ml of starting rabbit serum, was applied to a column (490 ml bed vol.; 5 cm $\times$ 25 cm) of DEAE-Sephadex A-50. Rinsing and elution with a linear choline chloride gradient followed the procedure described previously (Main *et al.*, 1974). The eluate was collected in 20 ml fractions.

The elution profiles of activity with thiocholine ester substrates are shown in Fig. 2. They reveal three

Fig. 1. Disc-gel substrate-activity staining patterns of rabbit serum and of an 89-fold purified preparation of monomeric butyrylcholinesterase

Rabbit serum (0.01 ml) containing 500 μg of protein and 0.003 activity unit was applied to each of gels 1, 2, 3 and 4. Gel 1 was treated with butyrylthiocholine, gel 2 with acetylthiocholine, gel 3 with indoxyl butyrate and gel 4 with indoxyl acetate substrates. An 89-fold purified preparation of monomeric butyrylcholinesterase (0.1 ml) containing 75 μg of protein and 0.04 activity unit was applied to each of gels 5, 6, 7, 8 and 9. Gel 5 was treated with butyrylthiocholine, gel 6 with propionylthiocholine, gel 7 with acetylthiocholine, gel 8 with indoxyl butyrate and gel 9 with indoxyl acetate substrates. The position of the tracking dye is located by the black ink marks at the ends of the gel.

peaks, corresponding to three different cholinesterases. Comparison of the relative activity under the peaks toward each of the substrates indicated that the predominant cholinesterase under the first peak, which was eluted at 620 ml, was butyrylcholinesterase. Similarly, the activity under the second peak, at 820 ml, was an acetylcholinesterase, and that under the third peak, at 1020 ml, was monomeric butyrylcholinesterase. The contents of the tubes under each of the peaks were pooled in three fractions. Samples from each fraction were subjected to disc electrophoresis, followed by substrate staining. In addition, more extensive substrate-specificity studies were made.

Both the disc gels and the extended specificity studies confirmed the identity of the predominant

Fig. 2. Cholinesterase activity elution profiles from the first DEAE-Sephadex A-50 ion-exchange column to which an $(\text{NH}_4)_2\text{SO}_4$ fraction of rabbit serum had been applied. A 5 cm $\times$ 25 cm column (bed vol. 490 ml) was used. The sample was eluted from the column with a linear choline chloride gradient (----) in 50 mM-sodium phosphate buffer, pH 8.0. The eluate was collected in 20 ml fractions. Individual fractions were assayed with 1 mM-butyrylthiocholine ($\bullet$), 10 mM-butyrylthiocholine ($\circ$), 1 mM-propionylthiocholine (Δ) and 1 mM-acetylthiocholine ($\blacksquare$). The major component in each of the numbered peaks was: 1, oligomeric butyrylcholinesterase; 2, acetylcholinesterase; 3, monomeric butyrylcholinesterase.

cholinesterase in each of the peaks. In the present report attention is focused on the monomeric butyrylcholinesterase in the third fraction.

This monomeric enzyme in the third fraction was purified 14-fold and contained 2 g of proteins and 150 activity units. Although the disc gels indicated that monomeric butyrylcholinesterase was predominant, they also indicated the presence of small amounts of contaminating acetylcholinesterase and carboxylesterase. To separate these from the monomeric butyrylcholinesterase, the third fraction was chromatographed a second time on an ion-exchange column (44 ml bed vol.; 2.5 cm $\times$ 9 cm) of DEAE-Sephadex A-50. This second column was eluted with a linear NaCl gradient as described by Main *et al.* (1974). The eluate was collected in 5 ml fractions, and the fractions containing the monomeric butyrylcholinesterase were pooled. Their volume was 70 ml. Treatment by the second column removed the contaminating esterases as shown in Fig. 1, gels 5–9. In addition, a further 6-fold purification was effected, so that overall purification was 89-fold. To facilitate kinetic studies, the activity of the pool, which was

1.2 units/ml, was concentrated to 18.6 units/ml by precipitation with 70 %-satd. $(\text{NH}_4)_2\text{SO}_4$.

Characterization of monomeric butyrylcholinesterase

Inhibition by di-isopropyl phosphorofluoridate and paraoxon. Biomolecular rate constants, k_i , governing inhibition were determined from measurements of rates of inhibition in the presence of butyrylthiocholine substrate (Main & Dauterman, 1963). Values of ρ , the first-order rate constant, at a given constant value of i , the inhibitor concentration, were calculated from the inhibited progress curves by the Guggenheim method (Main, 1974). By obtaining ρ values at several values of i and s , the substrate concentration, both K_m , the Michaelis constant of the substrate reaction, and the k_i were obtained from the plot of i/ρ against s from the equation:

$$i/\rho = s/k_i K_m + 1/k_i \quad (1)$$

which assumes that $i \ll K_a$, where K_a is the initial binding constant for the reversible enzyme-inhibitor complex. The intercept on the i/ρ axis gave $1/k_i$, and that on the s axis gave $-K_m$.

With di-isopropyl phosphorofluoridate the Guggenheim plots were linear over the first 90% of inhibition, indicating that the reaction had followed first-order kinetics. Four values of s and i were used. The s values were 0.5, 1, 2.5 and 5 mM-butyrylthiocholine. The i values were 0.167, 0.33, 0.66 and 0.83 μM -di-isopropyl phosphorofluoridate. The i/ρ values deviated slightly from the linear expectations of eqn. (1) at 5 mM-butyrylthiocholine, but the plot gave values of K_m and k_i that appeared adequate for present purposes. The k_i value for the inhibition of monomeric butyrylcholinesterase by di-isopropyl phosphorofluoridate was 2.4 $\mu\text{M}^{-1} \cdot \text{min}^{-1}$ at 25°C, pH 8.0 in 0.1 M-sodium phosphate. The K_m value was 1.6 mM with butyrylthiocholine substrate.

This value of K_m was used to calculate the k_i for the inhibition of monomeric butyrylcholinesterase by paraoxon (diethyl 4-nitrophenyl phosphate). The Guggenheim plots used to calculate ρ were not linear, but curved because the paraoxon concentrations of 16.7 nM and 33 nM were not much greater than the estimated monomeric butyrylcholinesterase concentration of 13 nM. In these circumstances, second-order kinetics were used to calculate a k_i value of 36 $\mu\text{M}^{-1} \cdot \text{min}^{-1}$ for the inhibition of monomeric butyrylcholinesterase by paraoxon at 25°C, pH 8.0, in 0.1 M-sodium phosphate. The comparable k_i value for the inhibition of horse butyrylcholinesterase was 0.75 $\mu\text{M}^{-1} \cdot \text{min}^{-1}$, or 50-fold smaller.

Although determining k_i values in the presence of substrate involved a degree of uncertainty, determination by the conventional method, which involves incubating enzyme and inhibitor alone before adding substrate (Aldridge, 1950), could not be accomplished with butyrylthiocholine as substrate. The reason

Fig. 3. Concentration of monomeric butyrylcholinesterase by titration with paraoxon

An 89-fold purified preparation with an initial activity (1 mM-butyrylthiocholine) of 3.91 units/ml was allowed to react with paraoxon at the concentrations shown. The residual activities were assayed after incubation for 2 h (○) and 19 h (●) with 1 mM-butyrylthiocholine. The activity was also assayed with 1 mM-phenyl thiobutyrate (■). The lines were fitted by regression analysis. The intercepts on the paraoxon-concentration axis gave the concentration of monomeric butyryl cholinesterase. With 1 mM-butyrylthiocholine, the intercept gave a value of $3.04\text{ }\mu\text{M}$ -monomeric butyrylcholinesterase; with 1 mM-phenyl thiobutyrate, the value was $3.07\text{ }\mu\text{M}$ -monomeric butyrylcholinesterase. The average was $3.06\text{ }\mu\text{M}$ -monomeric butyrylcholinesterase.

was the low k_{cat} of the substrate reaction and the high k_i values for inhibition, particularly by paraoxon.

A preliminary study of inhibition by eserine was also made. Incubation of monomeric butyrylcholinesterase with $10\text{ }\mu\text{M}$ -eserine for 15 min at 25°C resulted in 21 % inhibition, and at $100\text{ }\mu\text{M}$ -eserine 55 % of the activity was inhibited. Monomeric butyrylcholinesterase was then relatively resistant to inhibition by eserine compared with horse butyrylcholinesterase. In this respect, the monomeric enzyme was like 'benzoyl cholinesterase' (Gunter, 1946).

Titration of monomeric butyrylcholinesterase with paraoxon. The active-site concentration of a product from a second ion-exchange-column treatment was determined by titration with eight final concentrations of paraoxon ranging from 0.167 to $4.00\text{ }\mu\text{M}$ and spaced as shown in Fig. 3. The final volume of each

of the inhibitor solutions was adjusted to 0.50 ml by mixing appropriate volumes of enzyme, paraoxon and buffer stock solutions. Inhibition occurred at 25°C in 50 mM -sodium phosphate buffer, $\text{pH } 8.0$. Samples (0.02 ml) were withdrawn from each of the eight inhibition solutions for determination of residual activities after incubation for 15 min, 2 h and 19 h. The 15 min and 19 h samples were assayed with butyrylthiocholine, and the 2 h samples were assayed with both butyrylthiocholine and phenyl thiobutyrate. Inhibition was practically complete after 15 min, since further inhibition of significance had not occurred after incubation for 2 h and 19 h.

The results after incubation for 2 h and 19 h are shown in Fig. 3. The points obtained with each substrate lay on straight lines between 5 and 90 % inhibition. Separate lines were fitted to the two sets of points by regression analysis. The line obtained with butyrylthiocholine gave an intercept on the paraoxon axis of $3.04\text{ }\mu\text{M}$, and that obtained with phenyl thiobutyrate gave an intercept of $3.07\text{ }\mu\text{M}$. These results indicated that the average active-site concentration of a monomeric butyrylcholinesterase solution with an activity of 3.91 units/ml was $3.06\text{ }\mu\text{M}$. The concentration of a horse butyrylcholinesterase solution of the same activity is about 50 nM , or 60 times less.

Since the butyrylthiocholine and phenyl thiobutyrate activities were on almost the same line, it was concluded that the preparation of monomeric butyrylcholinesterase used was free from significant amounts of other cholinesterases or carboxylesterase.

The possibility that the monomeric butyrylcholinesterase solution might have contained an arylesterase capable of hydrolysing paraoxon was also tested. Rabbit serum is a rich source of this type of arylesterase (Aldridge, 1953). The pooled rabbit serum hydrolysed $1.77\text{ }\mu\text{mol}$ of paraoxon/min per ml at 25°C . However, no enzymic paraoxon hydrolysis by the monomeric butyrylcholinesterase sample could be detected. Moreover $10\text{ }\mu\text{M}$ -di-isopropyl phosphorofluoridate completely inhibited all the activity toward phenyl thioacetate, suggesting that no other arylesterases were present.

Comparison of substrate-specificity patterns. The substrate specificity of rabbit serum and of the three cholinesterase fractions from rabbit serum are compared with patterns from pure horse butyrylcholinesterase and human serum in Table 1. The specificity of monomeric butyrylcholinesterase differed significantly from that of the horse and human serum butyrylcholinesterases in several respects. For example, hydrolysis by monomeric butyrylcholinesterase of 1 mM-acetylthiocholine was at only 4 % the rate of butyrylthiocholine hydrolysis. By comparison, horse and human butyrylcholinesterases hydrolyse acetylthiocholine at about 50 % the rate of

Table 1. *Esterase substrate specificities of rabbit serum, various rabbit serum fractions, human serum and pure horse butyrylcholinesterase*

To establish specificity patterns based on butyrylcholinesterases, the relative activities of each sample toward the various substrates are given as a percentage of a sample's activity toward 1 mM-butyrylthiocholine. An exception is the rabbit serum acetylcholinesterase sample, where the activities are given as a percentage of the activity with 1 mM-acetylthiocholine. The activities of the samples (in units/ml) with 1 mM-butyrylthiocholine were: rabbit serum, 0.31; monomeric butyrylcholinesterase second column fraction, 18.6; rabbit butyrylcholinesterase first column fraction 5.03; human serum, 3.63; pure horse butyrylcholinesterase, 12.1; acetylcholinesterase second column fraction, 0.14 (19.5 units/ml with 1 mM-acetylthiocholine). The specific activity of the rabbit serum was 0.0056 unit/mg of protein. The concentration of the substrates in mM is given by the number in parentheses under the abbreviation for the substrate.

Sample	BuSch*		PrSch	AcSch	PhSBu	PhSAc	<i>o</i> NpBu
	(1)	(10)	(1)	(1)	(1)	(1)	(1)
Rabbit serum	100	215	177	197	1060	12200	1000
Monomeric butyrylcholinesterase, second column fraction	100	255	41	4	287	148	804
Butyrylcholinesterase, first column fraction	100	124	88	84	97	83	93
Human serum	100	133	88	47	82	52	93
Pure horse butyrylcholinesterase	100	133	86	58	57	38	46
Acetylcholinesterase, second column fraction	<1	1.3	58	100	<1	38	8

* Abbreviations: BuSch, butyrylthiocholine; PrSch, propionylthiocholine; AcSch, acetylthiocholine; PhSBu, phenyl thiobutylate; PhSAc, phenyl thioacetate; *o*NpBu, *o*-nitrophenyl butylate

butyrylthiocholine. In addition, monomeric butyrylcholinesterase hydrolysed aromatic esters at much higher rates, relative to choline esters, than did the butyrylcholinesterases. The results also suggested that the large butyrylcholinesterase of rabbit serum, as distinct from the monomeric enzyme, was characterized by a substrate specificity similar to that of the butyrylcholinesterases in human and horse sera. Minor discrepancies with respect to acetate ester specificity could be accounted for by the presence of contaminating acetylcholinesterase in the sample studied.

Relationships of v to s of monomeric butyrylcholinesterase and thiocholine ester substrates. The monomeric butyrylcholinesterase preparations with an active-site concentration (E_0) of $3.06 \mu\text{M}$ had an activity, v , of 3.91 units/ml with 1 mM-butyrylthiocholine. The $v/(E_0)$ value of monomeric butyrylcholinesterase with 1 mM-butyrylthiocholine was then 21 s^{-1} . The comparable value for horse butyrylcholinesterase was 1350 s^{-1} , or 63 times greater. It was therefore decided to determine if this low $v/(E_0)$ value followed from a low k_{cat} for the reaction or from a relatively high K_m . In addition, the substrate-specificity patterns indicated that monomeric butyrylcholinesterase hydrolysed acetylthiocholine much less readily than butyrylthiocholine, giving rise to analogous questions about this relationship.

The hydrolysis of butyrylthiocholine by monomeric butyrylcholinesterase and horse butyrylcholine-

sterase did not follow the Michaelis equation, as shown by the curved plots of $v/(E_0)$ against $v/(E_0)s$ in Fig. 4(a). To compare the plots of monomeric butyrylcholinesterase with those for horse butyrylcholinesterase, the units on the $v/(E_0)s$ and $v/(E_0)$ axes of the latter plots were multiplied by factors of 25 and 200 respectively. Given that adjustment, the two plots ran nearly parallel, suggesting that the binding of butyrylthiocholine to horse butyrylcholinesterase was about 10 times that to monomeric butyrylcholinesterase.

Curving plots of the type shown in Fig. 4(a) may be caused by binding of a second molecule of substrate to the butyrylated active site, EA, to form an EAS complex. The EAS complex is then deacylated faster than the EA complex. The equation of such a system is of the form:

$$\frac{v}{(E_0)} = \frac{k_{\text{cat}}}{1 + \frac{K_m}{s} + \frac{s}{K_{ss}'}} + \frac{k'_{\text{cat}}}{1 + \frac{K_{ss}'}{s} + \frac{K_m K_{ss}'}{s^2}} \quad (2)$$

where K_{ss}' includes the constant governing formation of EAS and k'_{cat} is the constant governing its breakdown. The system includes a Michaelis enzyme-substrate complex in addition to the EA and EAS complexes mentioned. Then $k_{\text{cat}} = k_2 k_3 / (k_2 + k_3)$, where k_2 is the rate constant governing formation of EA from the enzyme-substrate complex and k_3 governs the rate at which EA gives products. At low

Fig. 4. Plots of v against s for monomeric butyrylcholinesterase with thiocholine esters, and comparison with pure horse butyrylcholinesterase

(a) Plot of $v/(E_0)$ against $v/(E_0)s$, where (E_0) is the concentration of monomeric butyrylcholinesterase (13.7 μM) or horse butyrylcholinesterase (0.23 μM). The substrates with monomeric butyrylcholinesterase were butyrylthiocholine (●), propionylthiocholine (■) and acetylthiocholine (▲). The substrate with horse butyrylcholinesterase was butyrylthiocholine (○). For comparison, the $v/(E_0)$ scale with the horse butyrylcholinesterase is $\times (4 \times 10^{-2})$ and the $v/(E_0)s$ scale is $\times (5 \times 10^{-3})$. The maximum $v(E_0)$ for horse butyrylcholinesterase is then, for example, 1733 s⁻¹. (b) Plot of v against s , where v is given as percentage of the maximum velocity measured, except when acetylthiocholine was the substrate. With acetylthiocholine, v is given as percentage of the calculated V_{max} . With monomeric butyrylcholinesterase, the substrates were butyrylthiocholine (●) and acetylthiocholine (▲). With horse butyrylcholinesterase, the substrates were butyrylthiocholine (○) and acetylthiocholine (△). (c) As (b), except that the s (mm) values were 10-fold greater to extend the range over which comparisons can be made.

Table 2. Comparison of K_m and k_{cat} values for the reaction of monomeric butyrylcholinesterase and horse butyrylcholinesterase with thiocholine ester substrates

The reactions of both butyrylcholinesterases with butyrylthiocholine and of horse butyrylcholinesterase with acetylthiocholine did not follow the Michaelis equation. Estimates of K_m and k_{cat} were then made by three approaches. One was to equate K_m with s values at $\frac{1}{2} V_{max}$ from plots of v against s . V_{max} and the maximum velocity, determined experimentally, were assumed to be equal. The second approach assumed that the reaction followed eqn. (2). K_m and k_{cat} were estimated by extrapolation of the linear regions, at low values of s , of v against v/s plots. The third approach involved irreversible inhibition in the presence of substrate according to eqn. (1) and gave only K_m . When the reaction did follow the Michaelis equation, K_m and k_{cat} were from plots of v versus v/s . Substrate abbreviations are defined in Table 1.

Enzyme	Substrate	K_m (mm)	k_{cat} (s ⁻¹)	Method
Monomeric butyrylcholinesterase	BuSch	3	65.8	$\frac{1}{2} V_{max}$
	BuSch	1.4	47.5	eqn. (2)
	BuSch	1.6	—	eqn. (1)
	PrSch	7	89.3	
	PrSch	63	52.3	
Horse butyrylcholinesterase	BuSch	0.35	1790	$\frac{1}{2} V_{max}$
	BuSch	0.15	1248	eqn. (2)
	AcSch	0.5	888	$\frac{1}{2} V_{max}$

values of s relative to $K_{ss'}$, eqn. (2) simplifies to the Michaelis equation, $v/(E_0) = k_{cat}/[1 + (K_m/s)]$, and the plots of $v/(E_0)$ against $v/(E_0)s$ become linear in this region. Estimates of K_m and k_{cat} were made by extrapolating the linear portion of the curves in Fig. 4(a) in the low-substrate regions. The results are

given in Table 2 and indicate again that the K_m of the monomeric enzyme is 10 times that of the horse butyrylcholinesterase.

The value obtained for the K_m of the monomeric butyrylcholinesterase (1.6 mm) is the same order as the K_m often attributed to horse butyrylcholinesterase,

whereas the K_m of the latter enzyme is about one-tenth the value usually reported (Augustinsson, 1963). To offer a more convincing demonstration that these relatively low values of K_m are valid, plots of v against s are shown in Fig. 4(b) and 4(c). The operational K_m values at $\frac{1}{2} V_{\max}$ were obtained from these plots. These values were about double those obtained by using eqn. (2) (Table 2), as might be expected from the curving plots of $v/(E_0)$ against $v/(E_0)s$. However, even these estimates of the K_m for horse butyrylcholinesterase remain much lower than those reported by Augustinsson (1963), but his values were usually obtained from velocities determined at relatively high concentrations and probably more nearly reflect K_{ss}' than K_m values.

The reactions of monomeric butyrylcholinesterase with acetylthiocholine and propionylthiocholine appeared to follow the Michaelis equation, judging from the linear plots (Fig. 4a). The K_m and k_{cat} values from these plots are given in Table 2. The k_{cat} values were of the same order for the three thiocholine ester substrates, indicating that the specificity of monomeric butyrylcholinesterase for the butyrate ester depended largely on K_m .

Both the k_{cat} and binding of butyrylthiocholine with monomeric butyrylcholinesterase were much lower than with horse butyrylcholinesterase, indicating that the relatively low activity of the monomeric enzyme results from both factors, although the difference in the k_{cat} values was the predominant factor.

Molecular weight of monomeric butyrylcholinesterase by gel filtration. Two Sepharose 6-B columns were used. The first was 82cm $\times$ 1.5cm (145ml) and was eluted at 6ml/h at 4°C. The second was 84cm $\times$ 1.5cm (148ml) and was eluted at 10ml/h at 25°C. The first column was calibrated with seven proteins of known molecular weight. Similarly, the second column was calibrated with eleven proteins. The concentrations of the calibration protein samples varied from 0.5 to 2mg/ml. Sample volumes (3ml) were applied and the columns run by upward flow. The peak elution volumes of each of the calibration proteins were determined by separate duplicate runs. The eluants were collected in fractions of approx. 3ml (90 drops) and were monitored by A_{280} . The peak elution volumes were determined by pooling the contents of all the tubes up to and including the peak tube in a graduated cylinder and estimating to the nearest 0.5ml.

When the samples were cholinesterases, the fractions were monitored by determining the activities by the method of Ellman *et al.* (1961) with the appropriate thiocholine ester substrate.

The calibration proteins, their molecular weights and the elution volumes at which they formed peaks are given in Table 3. The logarithms of the molecular weights of each calibration protein and the corres-

ponding peak elution volumes were related by a linear-regression analysis. The slopes and intercepts were used to calculate the molecular weights of monomeric butyrylcholinesterase and of rabbit serum acetylcholinesterase. The results are given at the end of Table 3.

The molecular weight of monomeric butyrylcholinesterase averaged 82900. Although both horse butyrylcholinesterase and eel acetylcholinesterase were used as calibration proteins, their molecular weights were also estimated from the regression line and are also given in Table 3. The values given by the regression calculations closely agreed with literature values, suggesting that the molecular weights calculated from the same regression for monomeric butyrylcholinesterase and rabbit serum acetylcholinesterase were reasonable.

There has been some disagreement as to the correct molecular weight of horse butyrylcholinesterase. Main *et al.* (1972) reported a value of 315000, but Lee & Harpst (1973) gave one of 440000. However, the latter group (Teng *et al.*, 1976) have repeated their work and have reported a new estimate of 317000 ± 12000 , which is in good agreement with value of 315000 reported by Main *et al.* (1972). The value of 260000 for eel acetylcholinesterase is from Leuzinger *et al.* (1969) and appears to be the best value of the preparations supplied by Worthington Biochemical Corp., Freehold, NJ, U.S.A., from whom we obtained this enzyme. The eel acetylcholinesterase preparation gave rise to two activity elution peaks. The smaller of these came out near the void volume and probably represents an 18S form (Massoulié & Rieger, 1969). The larger peak was accepted as the 11S form, of mol.wt. 260000. Worthington also supplied most of the other calibration proteins, and we accepted the molecular-weight values given in their catalogue.

Discussion

The most significant question concerning monomeric butyrylcholinesterase would appear to be its relationship to the larger oligomeric butyrylcholinesterase. Although the size of monomeric butyrylcholinesterase suggests that it could be a subunit of butyrylcholinesterase, we obtained no direct evidence that it was. For example, there was no indication that monomeric butyrylcholinesterase aggregated. On the other hand, the oligomeric butyrylcholinesterase of horse and human sera does not appear to dissociate spontaneously to give substantial amounts of active subunits that can be isolated and characterized. Current concepts of quaternary protein structure provide several models which could account for the failure of monomeric butyrylcholinesterase to aggregate or for the oligomeric butyrylcholinesterase to dissociate spontaneously. Consequently, the failure

Table 3. *Molecular weight of monomeric butyrylcholinesterase by gel filtration on Sepharose 6B*

The calibration proteins used, their molecular weights and peak elution volumes on each of two columns are listed, together with the peak elution volumes of monomeric butyrylcholinesterase and rabbit serum acetylcholinesterase. The calculated molecular weights of these two enzymes are also listed. They were calculated from a regression fit of the log (mol.wt.) of the calibration proteins and their peak elution volumes. Each peak elution volume is the average volume from two runs.

Calibration protein	$10^{-2} \times$ Mol.wt.	Peak elution vol. (ml)	
		Column 1	Column 2
Cytochrome <i>c</i>	130		161
α -Chymotrypsin	250	150.5	149
Ovalbumin	450	128.5	127
Bovine serum albumin	670	122	121
Alkaline phosphatase	800		118
Glutamate-oxaloacetate transaminase	1100		124
Aldolase	1530	119	118
Human γ -globulin	1600		118
Catalase	2500	116	114.5
Eel acetylcholinesterase	2600	101	
Horse butyrylcholinesterase	3150	97.5	99
Thyroglobulin	6600		93
Sample	Calculated mol.wt.		
Monomeric butyrylcholinesterase	756	126	
	902		124
Average	829		
Rabbit serum acetylcholinesterase	2545	102	
Internal calibration checks of interest			
Horse butyrylcholinesterase	3196	97.5	
	3633		99
Eel acetylcholinesterase			
First peak	2705	101	
Second peak	24813	57	

to aggregate does not necessarily indicate that monomeric butyrylcholinesterase is not a subunit of butyrylcholinesterase.

If monomeric butyrylcholinesterase were a subunit, then it would seem that the widely differing kinetic properties of the monomeric compared with the oligomeric forms could be related to the different states of aggregation.

The active-site concentration of monomeric butyrylcholinesterase in pooled rabbit serum was calculated from its activity in the serum and from the paraoxon titration, which indicated that an activity of 1 unit/ml was equivalent to a monomeric butyrylcholinesterase concentration of $0.78 \mu\text{M}$. The average activity toward 1 mM-butyrylthiocholine in pooled serum was 0.31 unit/ml. However, the yields and recoveries observed when butyrylcholinesterase was separated from monomeric butyrylcholinesterase indicated that only 75% of the total serum activity toward butyrylthiocholine, i.e. 0.23 unit/ml, could be attributed to monomeric butyrylcholinesterase.

Hence the concentration of monomeric butyrylcholinesterase in pooled rabbit serum was calculated to be 180 nM.

By comparison, the concentration of butyrylcholinesterase in horse serum is estimated at 30–40 nM. The concentration of monomeric butyrylcholinesterase in pooled rabbit serum is then five times that of butyrylcholinesterase in horse serum, and yet the activity in horse serum is 15 times that in rabbit serum when 1 mM-butyrylthiocholine is the substrate. It seems possible that monomeric butyrylcholinesterase and other dissociated forms of cholinesterase could be present in other sera and other tissues at significant concentrations and yet be overlooked because of their low activities with specific choline ester substrates. However, the distribution of monomeric butyrylcholinesterase in other sera and tissues remains to be studied extensively. Preliminary studies (A. R. Main & S. C. McKnelly, unpublished work) indicate that rabbit liver contains monomeric butyrylcholinesterase at 3–4 times the concentration in serum, and

disc gels indicate that human serum may contain a monomeric butyrylcholinesterase.

As mentioned above, monomeric butyrylcholinesterase and 'benzoylcholinesterase' (Koelle, 1953) are probably identical. They have a similar preference for butyrylcholine and react slowly with acetylcholine. Both are relatively resistant to inhibition by eserine. According to Ellis (1947) and Koelle (1953), benzoylcholinesterase activity varies widely between the sera of individual rabbits. The sera of about half the rabbits that they examined were rich in benzoylcholinesterase activity, but the remainder had activities which varied from 0 to about 20% the activity of the richer sera. The livers of both rabbit and guinea pig (Sawyer, 1945) contained benzoylcholinesterase, although again there were wide variations in the activities observed.

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