2. The general design and details of the essential parts of the osmometer are described. The osmometer requires less than 0·5 ml. of solution per measurement.

3. A new method for making thin collodion membranes has been developed, by which membranes of high permeability to the solvent but completely impermeable to lactoglobulin can be made.

4. The working procedure is described.

5. A new method is given for obtaining the surface tension and density corrections to be used in osmometry. The osmotic pressure, surface tension correction and refractive increment can all be measured on a single 0·5 ml. sample.

Part 2

1. The calculation of mole fractions from refractometric data, and the analysis of the data in terms of a two-component system are discussed.

2. The preparation of the β-lactoglobulin and solutions is described.

3. The refractometric procedure is outlined.

4. The results obtained by the methods described on unbuffered lactoglobulin solutions are presented and analysed. The solutions show a marked degree of non-ideality (up to 35% at a 10 g./100 ml. protein concentration). The methods of calculation of a molecular weight from such data are considered, and a value for this lactoglobulin of $37\,000 \pm 540$ is obtained, this being calculated from twenty-four measurements covering a range of concentrations from 1 to 10 g./100 ml. protein.

5. The results are compared with previous work, and it is concluded that the present method is considerably more precise (and capable of improvement by the use of buffered solutions) than the equilibrium methods with which it is compared. It is as precise as another dynamic method considered, but requires only one-tenth of the volume of solution.

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Studies on Cholinesterase

9. SPECIES VARIATION IN THE SPECIFICITY PATTERN OF THE PSEUDO CHOLINESTERASES

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As a result of their studies on the relative substrate specificities of various cholinesterase preparations, Mendel and co-workers concluded that vertebrate tissues contain two distinct types of enzymes capable of hydrolysing acetylcholine *in vitro*, both of which are sensitive to inhibition by low concentrations of eserine (Mendel & Rudney, 1943; Mendel, Mundell & Rudney, 1943). These two types were designated originally as true cholinesterase and pseudo cholinesterase (Mendel & Rudney, 1943).

Subsequent investigations have shown that true cholinesterases of human erythrocytes, horse erythrocytes and pigeon brain (Adams, 1949; Mounter & Whittaker, 1950; Whittaker, 1949) are most active towards those choline and non-choline esters whose structure approaches most closely

to that of acetylcholine. The pseudo cholinesterases of human serum and horse serum, on the other hand, exhibit their highest activity towards those esters which show a close structural resemblance to butyrylcholine (Adams & Whittaker, 1949*a*; Sturge & Whittaker, 1950). Consequently it has been proposed that the two types of cholinesterases should be classified on the basis of this difference in specificity patterns and designated as aceto-cholinesterases and butyro-cholinesterases respectively (Richter, cited by Sturge & Whittaker, 1950; cf. Whittaker, 1951).

These designations seemed to be particularly suitable from an enzymological point of view, whether or not they will prove to be related to the distinct physiological functions of the two types of cholinesterases. However, more recent investigations by Ord & Thompson (1951) have shown that rat heart ventricle contains a pseudo cholinesterase which acts most rapidly upon propionylcholine. These authors did not determine whether this difference was due to the species or to the tissue used, but it seems to justify the classification of this enzyme as a propiono-cholinesterase.

This finding suggested that a more extensive investigation of the substrate specificity patterns of pseudo cholinesterases from different species would be justified. It has been emphasized by Hawkins & Mendel (1946) and Mendel & Hawkins (1950) that the ratio of the maximal activities of a cholinesterase towards two different substrates may vary considerably from one species to another. Similar variations have been demonstrated by Augustinsson (1949), Sturge & Whittaker (1950) and Myers (1952*a*). The present investigation was designed, in part, to study these variations in specificity patterns in greater detail, and to determine whether the propiono-cholinesterase studied by Ord & Thompson (1951) was typical of the particular species used, or whether different enzymes are found in other tissues of the same animal.

A preliminary report on this work has been presented (Myers & Mendel, 1952).

METHODS

Estimation of esterase activity. As in previous investigations (Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1952*a*), the activity of the cholinesterases was measured manometrically by the Warburg method at pH 7·4 and 37·5° in a medium containing 0·025 M-$NaHCO_3$ and saturated with 5% CO_2 in 95% N_2 (v/v). The volume of enzyme preparation was selected so as to give an activity between 60 and 180 μl. CO_2 in 30 min. as far as was practicable. The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the enzyme inhibitor when used was added to the enzyme-bicarbonate mixture in the main compartment. After equilibration of the vessels at 37·5° for about 30 min., the substrate was tipped in from the side arm. Readings were started 2 min. later and repeated at appropriate intervals, usually every 3 or 5 min., for 30–40 min. Esterase activity was calculated from the initial constant rate of hydrolysis of substrate after corrections for spontaneous hydrolysis of the ester.

Substrates. Esters tested as substrates for the various pseudo cholinesterases were acetylcholine chloride and perchlorate (ACh), propionylcholine chloride and perchlorate (PrCh), butyrylcholine chloride and perchlorate (BuCh), acetyl-(±)-β-methylcholine chloride (MCh) and benzoylcholine chloride (BCh). Most of the data on relative rates of hydrolysis of propionylcholine and butyrylcholine were determined with the perchlorate salts of these esters. In confirmation of a communication from Prof. R. H. S. Thompson, no significant difference in rate of hydrolysis was noted when a perchlorate salt was used in place of chloride. Tributyrin was also tested as substrate in some experiments; it was suspended with 5% gum acacia and diluted 50-fold in the Warburg vessel to give a final concentration of 0·2% tributyrin with 0·1% gum acacia.

Routine estimations of pseudo cholinesterase and true cholinesterase activities were carried out with benzoylcholine and acetyl-β-methylcholine in final concentrations of 0·006 M and 0·03 M respectively (Mendel *et al.* 1943). Acetylcholine, propionylcholine and butyrylcholine were tested at a final concentration of 0·045 M, unless otherwise stated; the 0·3 M stock solutions of these esters were used since the perchlorate salts were not soluble in higher concentrations. The total volume of fluid in the Warburg vessel was limited to 2·0 ml. in these cases to keep the amount of ester necessary for the determinations to a minimum; when lower concentrations of substrate were used, the fluid volume was increased to 5·0 ml. (cf. Myers, 1952*b*). The relative activity of the pseudo cholinesterase towards these esters is expressed as a percentage of the activity towards acetylcholine (Ord & Thompson, 1950, 1951).

Inhibitors. The inhibitors used were physostigmine salicylate (eserine), the dimethylcarbamate of (2-hydroxy-5-phenyl)benzyltrimethylammonium bromide (Nu 683; Hawkins & Gunter, 1946), and di*iso*propyl phosphonofluoridate (di*iso*propyl fluorophosphonate, DFP).

Esterase preparations. Horse serum and human serum were dialysed and tested without any further treatment since these sera contain large amounts of pseudo cholinesterase and only insignificant amounts of true cholinesterase (Mendel *et al.* 1943). Unless the serum preparation is dialysed (Myers, 1950, 1952*b*), or the medium itself contains high concentrations of salts (Nachmansohn & Rothenberg, 1945; Augustinsson, 1949), the variations in salt concentration with different amounts of serum may cause appreciable errors in the measured ratio of activities towards two substrates.

Mouse and rat serum were obtained from mature female animals since these sera contain larger amounts of pseudo cholinesterase than the sera from male mice and male rats respectively. The sera were fractionated with $(NH_4)_2SO_4$ as follows. A saturated solution of $(NH_4)_2SO_4$ was added dropwise to the serum with continuous stirring until the mixture reached 0·45 saturation after 40 min. (cf. Hawkins & Gunter, 1946). After standing for a further 60 min. at room temperature with occasional stirring, the mixture was centrifuged and the pseudo cholinesterase precipitated from the supernatant by adding solid $(NH_4)_2SO_4$ to approximately 0·85 saturation. This precipitate was redissolved in

half the original volume of water and dialysed overnight. This treatment was usually sufficient to reduce the true cholinesterase activity to a small percentage of the total activity towards acetylcholine. If a significant amount of true cholinesterase activity still remained in the solution, the same procedure was repeated using 0·5 saturation with $(NH_4)_2SO_4$ to precipitate the true cholinesterase. The degree of purification of the pseudo cholinesterase from the original serum was not very large but was sufficient to eliminate the only other enzyme in the serum capable of hydrolysing choline esters.

Among the mammalian species studied was the Syrian hamster; the pseudo cholinesterase activity of the serum and tissues was extremely low. Thus it was not possible to obtain accurate results with this serum; the approximate results obtained are included because this enzyme also appeared to be a propiono-cholinesterase.

RESULTS

Enzymological characteristics of the serum pseudo cholinesterases

With each preparation of pseudo cholinesterase, the concentration of Nu 683 required to produce 50% inhibition of the enzyme activity was estimated in the manner described previously (Myers, 1952*a*). The measured values are summarized in Table 1; all of these enzymes are inhibited by very low concentrations of Nu 683, although the K_I values vary between 6×10^{-10} and 3×10^{-8}M. There seems to be a tendency for the K_I values to fall into three groups with values of about 7×10^{-10}, 3×10^{-9} and 2×10^{-8}M-Nu 683, but the series given is too small to allow an evaluation of this apparent tendency. An accurate estimation of the molar concentration of enzyme by the use of Nu 683 (cf. Myers, 1952*a*) can only be obtained with those pseudo cholinesterases in the first of the three groups.

All of these cholinesterase preparations exhibited their maximal activity towards acetylcholine at high concentrations of ester. With some of the preparations, an attempt was made to estimate the concentration of acetylcholine required to give 50% of the maximal activity; a plot of activity against the logarithm of the substrate concentration gave the sigmoid curves expected (cf. Goldstein, 1944; Myers, 1952*b*). The measured values of the Michaelis constant K_s were all of the same order of magnitude, varying from about 3×10^{-4} to $1{\cdot}6 \times 10^{-3}$M (Table 1). A substrate concentration of 0·045M was selected as the most convenient for further investigations; this concentration is similar to that recommended by Mendel *et al.* (1943) and, since the pseudo cholinesterase activity is about 98% of the theoretical maximum in this range of concentrations (cf. Goldstein, 1944), small alterations in substrate concentration have very little effect upon the activity.

Table 1 also shows the variation in the relative activities of the different serum pseudo cholinesterases towards 0·006M-benzoylcholine, the values given being expressed as a percentage of the activity towards 0·045M-acetylcholine and also as a percentage of the activity towards 0·045M-butyrylcholine. These results seem to confirm the hypothesis that the activity of the pseudo cholinesterases towards benzoylcholine is more closely related to the activity towards butyrylcholine than to the activity towards acetylcholine (cf. Adams & Whittaker, 1949*a*). As a rough approximation, the average pseudo cholinesterase activity towards butyrylcholine might be taken as seven times that towards benzoylcholine under the experimental conditions; the value for the pseudo cholinesterase of chicken serum is exceptional.

Table 1. *Enzymological characteristics of the pseudo cholinesterases from the sera of different species*

(The K_I values with Nu 683 for the pseudo cholinesterases from the sera of dog, man, horse, rat and mouse are taken from the data given previously (Myers, 1952*a*, 1952*c*). The relative activities towards 0·006M-benzoylcholine are expressed as percentages of the pseudo cholinesterase activities towards 0·045M-acetylcholine and towards 0·045M-butyrylcholine. Experimental methods are described in the text.)

	Michaelis constants		Activity ratios	
Species	K_s ($\times 10^{-3}$M-acetylcholine)	K_I ($\times 10^{-3}$M-Nu 683)	$\frac{BCh}{ACh} \times 100$ (%)	$\frac{BCh}{BuCh} \times 100$ (%)
Dog	0·5	3·2	62	23
Man	1·4	0·9	36	17·5
Horse	1·6	0·7	30	12
Cat	—	3·6	28	12·5
Ferret	—	3·6	28	17
Duck	—	4·5	26	16
Rat	0·5	3·1	24	18·5
Pig	0·9	22	18	14
Pigeon	0·3*	27	17	13
Squirrel	—	1·5	15	10
Mouse	1·4	0·6	11	14
Chicken	0·3*	25	7	8

* Approximate values.

The activity ratios given in Table 1 for the pseudo cholinesterases of dog, horse, cat and human serum can be compared with the values calculated from the corresponding data given by Mendel *et al.* (1943), Hawkins & Mendel (1947), Adams & Whittaker (1949*a*), Sturge & Whittaker (1950), and Myers (1952*a*). This shows a good agreement between the present results and those of other investigators; there can be little doubt that these values are highly characteristic of the particular pseudo cholinesterases concerned. Furthermore, no significant differences in the ratio of cholinesterase activities towards different substrates are observed upon purification of the cholinesterase concerned

(Mendel & Rudney, 1943; Adams & Whittaker, 1949*b*; Augustinsson, 1948, 1949; Ord & Thompson, 1951), nor were any differences observed in the specificity pattern of the pseudo cholinesterase of human serum from one individual to another.

Substrate specificity patterns

Comparative specificity patterns obtained with pseudo cholinesterases from the sera of thirteen different warm-blooded species are shown in Fig. 1. Eight of these hydrolysed butyrylcholine more rapidly than any of the other choline esters tested, while four hydrolysed propionylcholine most rapidly. The first eight enzymes might be classified as butyro-cholinesterases and the latter four as propiono-cholinesterases; however, there is no clear-cut distinction between these two groups. When the patterns are arranged into a series as in Fig. 1, a gradual transition from the one extreme to the other can be seen. We have as yet not found any pseudo cholinesterase which exhibits maximal activity towards acetylcholine, but the results suggest the possibility that a pseudo cholinesterase of this type may exist.

The substrate specificity pattern of the pseudo cholinesterase from rat serum is practically identical with that reported by Ord & Thompson (1951) for the cholinesterase from rat heart. There does not seem to be any special significance in the order of the species as given in Fig. 1. The pseudo cholinesterases from related species such as hamster, rat and mouse do show somewhat similar specificity patterns, but the pseudo cholinesterase of the red squirrel exhibits a dissimilar pattern.

The pseudo cholinesterase of pig serum

Levine & Suran (1950) have reported that pig serum contains a new type of cholinesterase which hydrolyses acetylcholine fairly rapidly but which does not exhibit any significant activity towards either acetyl-β-methylcholine or benzoylcholine; this enzyme, like the pseudo cholinesterases of other species, was found to be sensitive to inhibition by 10^{-4}M-pontocaine. In the present investigation it was found that dialysed pig serum does hydrolyse 0·006M-benzoylcholine at about 18% of the rate of hydrolysis of 0·045M-acetylcholine (Table 1). On plotting the activity towards acetylcholine against the logarithm of the molar concentration of acetylcholine, a typical S-shaped curve is obtained with a K_s value of the same order of magnitude as that found with other well known pseudo cholinesterases (Table 1). The activities towards acetylcholine and benzoylcholine were both inhibited by 10^{-5}M-eserine, and the serum exhibits moderately high activity towards butyrylcholine, an ester which may also be used as a 'specific' substrate for the pseudo cholinesterases (Cohen, Kalsbeek & Warringa, 1949).

This enzyme does exhibit an exceptional substrate specificity pattern; both acetylcholine and butyrylcholine are hydrolysed more rapidly than propionylcholine (Fig. 1). However, the activities towards all of these esters were inhibited to the same degree by various concentrations of DFP and Nu 683 (Table 2), and all were decreased to the same extent when the pseudo cholinesterase was partially inactivated by heating the serum at 55°

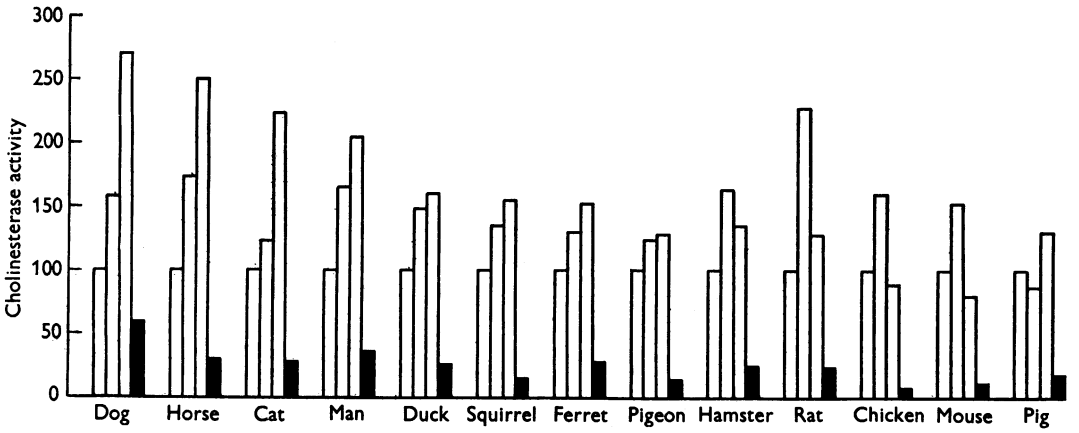

Fig. 1. Substrate specificity patterns of the pseudo cholinesterases from the sera of different species. In each case the first column indicates the relative rate of hydrolysis of 0·045M-ACh, given an arbitrary value of 100. The second column represents the relative activity of the pseudo cholinesterases towards 0·045M-PrCh, the third column that towards 0·045M-BuCh, while the fourth, in black, represents the relative activity towards 0·006M-BCh. Most of the pseudo cholinesterase preparations were obtained from their respective sera by fractional precipitation between 0·50 and 0·85 saturation with $(NH_4)_2SO_4$ as described in the text.

Table 2. *Inhibition by DFP and by Nu 683 of the hydrolysis of choline esters by pig serum*

(The pseudo cholinesterase preparation used was separated from pig serum as described in the text; this preparation showed an activity of 186 μl. CO_2/ml./20 min. towards 0·045 M-BuCl. The relative rates of hydrolysis of ACh, PrCh, BuCh and BCh are indicated in Fig. 1. The percentage inhibition of the activity towards each of these esters was measured after incubation of 1 ml. solution in a fluid volume of 5 ml. for 30 min. at 37·5° with various concentrations of DFP and Nu 682.)

Inhibition by DFP					Inhibition by Nu 683				
DFP concentration ($\times 10^{-7}$ M)	Percentage inhibition of the activity towards the following substrates				Nu 683 concentration ($\times 10^{-8}$ M)	Percentage inhibition of the activity towards the following substrates			
	ACh	PrCh	BuCh	BCh		ACh	PrCh	BuCh	BCh
0·8	15	—	—	10	0·8	20	—	—	25
1·3	26	22	25	19	1·3	32	33	38	35
2·0	36	36	42	37	2·0	44	48	45	46
3·0	53	56	53	54	3·0	61	59	58	59
5·0	71	72	75	69	5·0	73	—	—	70
					8·0	86	82	85	85

for 30 min. (cf. Myers & Mendel, 1949). No summation of the activities towards acetylcholine and butyrylcholine was observed. Fractional precipitation experiments were carried out with the technique described to separate successive fractions at 0·29, 0·40, 0·51, 0·65 and complete (1·0) saturation with ammonium sulphate. About 70% of the total pseudo cholinesterase activity was recovered from the fraction precipitated between 0·51 and 0·65 saturation with ammonium sulphate but the specificity pattern of the pseudo cholinesterase was not altered, despite the fact that nearly all of the small amount of true cholinesterase activity present in the original serum was removed. This evidence strongly suggests that the hydrolysis of all three choline esters is due to a single enzyme.

It might be noted that the pseudo cholinesterase of horse serum hydrolyses caproylcholine more slowly than valeryl- or heptoyl-choline (Glick, 1941), and that the pseudo cholinesterase of human serum hydrolyses *iso*amyl valerate more slowly than *iso*amyl butyrate or caproate (Adams & Whittaker, 1949*a*). In view of these unexplained peculiarities, the specificity pattern of the pig serum enzyme should not exclude its classification as a pseudo cholinesterase.

The hydrolysis of acetyl-β-methylcholine by avian pseudo cholinesterases

The results obtained by Mendel *et al.* (1943) indicate that highly purified preparations of the pseudo cholinesterases from dog pancreas and horse serum hydrolyse acetyl-β-methylcholine at slightly less than 1% of the rate at which they hydrolyse acetylcholine, while Adams & Whittaker (1949*a*) measured a corresponding value of 1·4% for the pseudo cholinesterase of human serum. All of the mammalian preparations which appear in Fig. 1 hydrolysed acetyl-β-methylcholine at 3% or less of the rate of hydrolysis of acetylcholine. It is probable that most of these preparations were still contaminated with a small amount of true cholinesterase; these contaminations would have very little influence on the specificity patterns given in Fig. 1, since it was found that in all cases a true cholinesterase preparation (washed erythrocytes or brain) from the same mammalian species hydrolysed 0·045 M-acetylcholine a little less rapidly than it hydrolysed 0·03 M-acetyl-β-methylcholine.

Chicken serum contains an enzyme which rapidly hydrolyses acetyl-β-methylcholine. Mendel *et al.* (1943) showed that chicken serum exhibits a much higher activity towards acetyl-β-methylcholine than towards benzoylcholine, but hydrolyses acetylcholine most rapidly at high concentration. Earl & Thompson (1952), using butyrylcholine as a specific substrate for pseudo cholinesterase, reported that the hydrolysis of acetyl-β-methylcholine and butyrylcholine was catalysed by the same enzyme in chicken serum. This report has been confirmed. Fractional precipitation with ammonium sulphate did not give any evidence for the presence of a considerable amount of a true cholinesterase in the serum. The activities towards acetylcholine, butyrylcholine and benzoylcholine were all inhibited to the same extent by various concentrations of Nu 683 and of DFP (cf. Earl & Thompson, 1952), indicating that the same enzyme was responsible for the hydrolysis of all three esters. The Michaelis constant K_s with acetylcholine as substrate had an unexpectedly low value (Table 1 and Fig. 2). However, this enzyme is capable of hydrolysing tributyrin (Table 3) and exhibited maximum activity towards high concentrations of the aliphatic choline esters (Fig. 2).

These results, combined with the sensitivity of the enzyme to inhibition by Nu 683, indicate that the enzyme concerned could be classified as a pseudo cholinesterase. To support this conclusion, a comparison was made between the characteristics of the cholinesterase of chicken brain and that of chicken serum. The results obtained (Fig. 2) show

that the cholinesterases found in the serum and in brain are different enzymes. The true cholinesterase of brain, in contrast to the pseudo cholinesterase of serum, exhibits the phenomenon of excess substrate inhibition with acetylcholine (Fig. 2) and does not appreciably hydrolyse either tributyrin or butyrylcholine (Table 3). Both enzymes hydrolyse acetyl-β-methylcholine, but the shape and position of the activity-substrate concentration curves are different. Both enzymes hydrolyse only the same half, presumably the (+)-form, of the racemic substrate acetyl-(±)-β-methylcholine (cf. Glick, 1938).

rat-brain homogenate are also included in Fig. 2. A possible explanation of the asymmetry in these curves has been suggested elsewhere (Myers, 1952*b*).

Despite the high activity of the true cholinesterase towards propionylcholine, this enzyme failed to act appreciably on butyrylcholine. A crude homogenate of chicken brain hydrolyses butyrylcholine slowly, but this activity must be due almost entirely to the pseudo cholinesterase present (cf. Ord & Thompson, 1952; Earl & Thompson, 1952) since it could be inhibited selectively by the addition

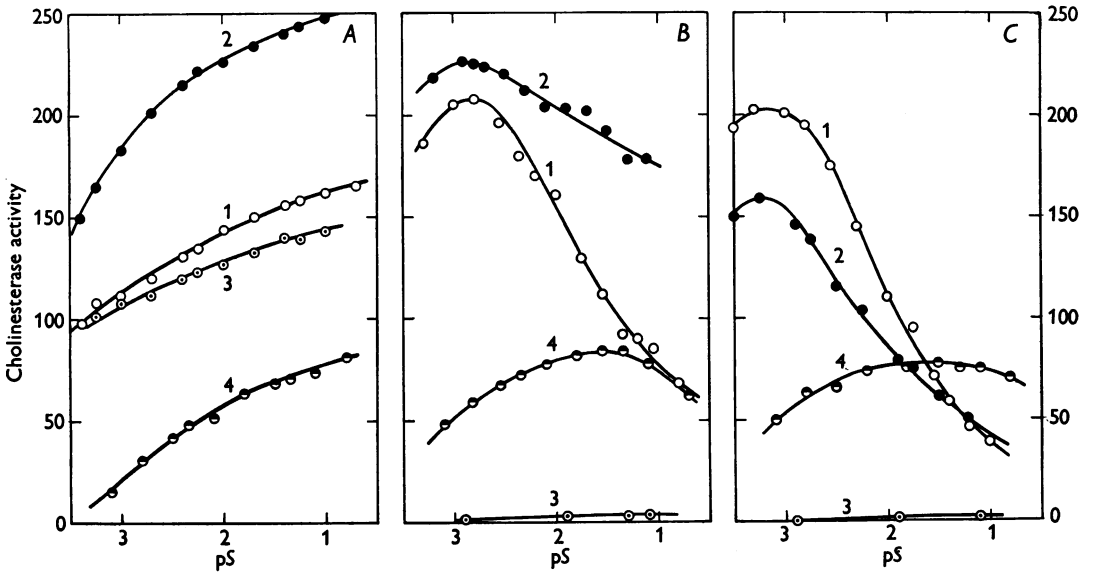

Fig. 2. Hydrolysis of ACh, PrCh, BuCh and MCh by (*A*) the pseudo cholinesterase of chicken serum, (*B*) the true cholinesterase of chicken brain, (*C*) the true cholinesterase of rat brain. The rates are plotted against −log (substrate concentration) (p*S*). ○—○, ACh; ●—●, PrCh; ⊙—⊙, BuCh; ◓—◓, MCh. The concentration of MCh is expressed as g.mol. acetyl-(±)-β-methylcholine/l. Rates are expressed as follows: pseudo cholinesterase of chicken serum as μl. CO_2/0·2 ml. solution/20 min.; true cholinesterase of chicken brain (measured in the presence of 4×10^{-9}M-DFP to inhibit any pseudo cholinesterase present) μl. CO_2/50 mg. fresh wt./20 min.; true cholinesterase of rat brain (measured in the presence of 10^{-8}M-DFP) μl. CO_2/75 mg. fresh wt./20 min.

Both the true cholinesterase and the pseudo cholinesterase of the chicken exhibit their maximal activity towards propionylcholine (Fig. 2). Certain of the true cholinesterases are known to exhibit approximately equal activities towards acetylcholine and propionylcholine (Augustinsson, 1949), but this appears to be the first example of a true cholinesterase which hydrolyses propionylcholine more rapidly than acetylcholine at optimal substrate concentrations. It is possible that this fact may be connected with the highly asymmetrical bell-shaped curves which are obtained on plotting the true cholinesterase activity against the logarithm of the substrate concentration (Fig. 2). For comparative purposes, the corresponding curves determined under similar experimental conditions with

of 4×10^{-9}M-DFP to the medium. The residual activity towards butyrylcholine in the presence of DFP was about 1% of the true cholinesterase activity towards acetylcholine (Table 3). Butyrylcholine is therefore a suitable specific substrate for determining the pseudo cholinesterase activity in the tissues of the chicken.

The pseudo cholinesterase preparations obtained from duck and pigeon sera also hydrolyse acetyl-β-methylcholine considerably more rapidly than the pseudo cholinesterases from mammalian sera (Table 3). This suggests that avian pseudo cholinesterases generally may exhibit appreciable activity towards this ester, although the pseudo cholinesterases of duck and pigeon hydrolyse acetyl-β-methylcholine much less rapidly than does the

chicken pseudo cholinesterase. The other substrate specificity characteristics of all of these pseudo cholinesterases are different from those of the true cholinesterases (Table 3).

Table 3. *Comparison of the rates of hydrolysis of MCh, tributyrin (TB) and BuCh by five pseudo cholinesterases and two true cholinesterases*

(The pseudo cholinesterase preparations were obtained from serum as described in the text. The true cholinesterase activities were determined with brain homogenate in the presence of a low concentration of DFP to inhibit any pseudo cholinesterase activity as described for Fig. 2. The activities towards MCh, TB and BuCh are expressed as a percentage of the cholinesterase activity towards optimal concentrations of acetylcholine, i.e. 0·045 M-ACh for the pseudo cholinesterases and approximately 0·001 M-ACh for the true cholinesterases (cf. Fig. 2). Since the hydrolysis of TB by the brain homogenates was not appreciably influenced by the addition of 10^{-6} M-eserine, the true cholinesterase activity towards TB must be less than 2% of the activity towards optimal concentrations of acetylcholine. These values and others given in brackets are approximations only since the activities are too low to allow accurate determinations.)

	Activity ratios		
Enzyme	$\frac{MCh}{ACh} \times 100$ (%)	$\frac{TB}{ACh} \times 100$ (%)	$\frac{BuCh}{ACh} \times 100$ (%)
Pseudo cholinesterase of			
Man	(1·2)	42	203
Pig	(0·7)	12	128
Duck	8	44	160
Pigeon	4	68	129
Chicken	42	58	89
True cholinesterase of			
Rat	37	(<2)	(0·5)
Chicken	42	(<2)	(1·0)

Specificity pattern of pseudo cholinesterases from different tissues of the rat

Various data have been published which indicate a variation in the specificity pattern of the pseudo cholinesterase from one tissue to another within the same animal as well as from one species to another. Calculations based on the data of Ord & Thompson (1950) show that the pseudo cholinesterases from intestinal mucosa, intestinal muscle and Harderian gland of the rat hydrolyse benzoylcholine at about 50% of the rate at which they hydrolyse acetylcholine, whereas the pseudo cholinesterases of rat heart and skin hydrolyse benzoylcholine at about 25% of the rate of acetylcholine. Similar variations were found by Sawyer & Everett (1947), although their data indicate that the pseudo cholinesterase of rat Harderian gland hydrolyses benzoylcholine at about 25% of the rate at which it hydrolyses acetylcholine. On the other hand, Goldstein (1951) found that the same type of pseudo cholinesterase is present in the serum, liver and gastric mucosa of the cat.

An attempt was made to confirm the results of Ord & Thompson (1950, 1951) by investigating the substrate specificity patterns of the pseudo cholinesterases from a series of rat tissues. To our surprise, the results did not give any indication of significant differences in the specificity pattern of the pseudo cholinesterases from different tissues of the rat. The results on comparative rates of hydrolysis of the various choline esters by rat heart ventricle agreed extremely well with those of Ord & Thomson (1951), and with those obtained on rat serum in the present investigation (Fig. 1). As judged by the close similarity of the specificity patterns obtained, the pseudo cholinesterases found in rat serum, heart ventricle, skin, pancreas, intestinal mucosa, intestinal muscle, uterus, multilocular adipose tissue (i.e. 'brown fat' or 'hibernating gland'), Harderian gland, parotid gland, lacrimal gland and mammary gland are all very similar if not identical. More limited investigations have been carried out on different tissues of the same animal in other species. The specificity patterns of the respective serum pseudo cholinesterases were found to be closely similar to those of the heart, Harderian gland and uterus of the hamster; the pancreas, heart and intestine of the ferret; the pancreas of the dog; the heart and liver of the mouse; and the liver and pancreas of the chicken. These results, in combination with those of Goldstein (1951), strongly suggest that the specificity pattern of the particular pseudo cholinesterase concerned is species specific but not organ specific.

Deviations from the usual pattern were observed with crude preparations of Harderian gland from the rat and hamster; in both cases it was found that the discrepancy was due to the presence of eserine-insensitive esterases capable of hydrolysing butyrylcholine but with very little activity towards either acetylcholine or benzoylcholine. Similar enzymes may be present in small amounts in the pancreas of the rat and intestine of the hamster.

Typical results obtained with a crude preparation of rat Harderian gland are given in Table 4; a portion of the total activity towards butyrylcholine is quite resistant to inhibition by any concentration of Nu 683 up to 10^{-3} M, whereas the pseudo cholinesterase activity is almost completely inhibited by 10^{-7}–10^{-6} M-Nu 683. The same portion of the activity towards butyrylcholine is not inhibited by a concentration (10^{-6} M) of eserine sufficient to inhibit the pseudo cholinesterase by about 90%. On the other hand, this esterase, like the pseudo cholinesterases, was sensitive to inhibition by low concentrations of DFP (10^{-7} M). Thus it appears to be similar to the enzymes found in the rabbit by

Table 4. *An esterase in rat Harderian gland which is capable of hydrolysing butyrylcholine but more resistant than the pseudo cholinesterase to inhibition by eserine and Nu* 683

(Harderian gland was obtained from adult female rats and homogenized with 9 vol. of water; the true cholinesterase activity towards 0·045 M-ACh was less than 2% of the total esterase activity. Native serum obtained from the same animals was also tested in the same way to allow comparison with an enzyme preparation containing only true cholinesterase and pseudo cholinesterase; the true cholinesterase was responsible for 6% of the total activity of the serum towards 0·045 M-ACh. The enzyme preparations were incubated at 37·5° for 30 min. with Nu 683 or eserine in the usual manner before measuring the residual esterase activity.)

	Nu 683 as inhibitor						Eserine as inhibitor	
	Esterase activity of serum towards the following substrates		Esterase activity of Harderian gland towards the following substrates				Esterase activity of Harderian gland towards the following substrates	
Concentration of inhibitor (M)	ACh (μl. CO_2/0·2 ml./20 min.)	BuCh	ACh	PrCh (μl. CO_2/20 mg. fresh wt./20 min.)	BuCh	BCh	ACh (μl. CO_2/20 mg. fresh wt./20 min.)	BuCh
0	121	156	106	266	171	24·8	107	175
10^{-8}	45·0	58·2	43	117	87	9·5	—	—
10^{-7}	10·5	12·5	14·6	47·9	48·2	2·5	61	135
10^{-6}	2·5	2·0	3·9	30·1	37·6	0·0	11·2	57·9
10^{-5}	0·0	0·0	2·0	28·0	36·2	0·0	3·1	38·5
10^{-4}	0·0	0·0	1·8	27·7	36·9	0·0	1·2	4·9
10^{-3}	—	—	—	—	32·6	—	—	—

Table 5. *Inhibition of the cholinesterases in the blood of mice injected with DFP*

(Female mice weighing 22–25 g. were injected intraperitoneally with solutions of DFP in saline; after observing the mice for 1 hr. subsequently to the injection, the animals were sacrificed and pooled samples of oxalated blood were collected from each group. The cholinesterase activities were determined in the usual manner using 0·03 M-acetyl-β-methylcholine as specific substrate for the true cholinesterase and 0·006 M-benzoylcholine for the pseudo cholinesterase.)

		True cholinesterase		Pseudo cholinesterase		
No. of mice	Amount of DFP injected (μg.)	Activity (μl. CO_2/ml. blood/20 min.)	Inhibition (%)	Activity (μl. CO_2/ml. blood/20 min.)	Inhibition (%)	Remarks
15	0	97·6	—	170·0	—	—
4	4	105·3	0	28·8	83	No symptoms
12	8	82·5	15	5·0	97	No symptoms
4	20	47·5	51	0·0	100	No symptoms
4	40	22·7	77	0·0	100	Listless
8	100	8·8	91	0·0	100	Severe fibrillation and diarrhoea

Koelle (1952); in view of their low activity towards benzoylcholine, these eserine-resistant esterases could hardly be designated as 'benzoyl-cholinesterases' (cf. Sawyer, 1945). Further studies on the substrate specificity and occurrence of these enzymes are being carried out; to avoid confusion with the true cholinesterases and pseudo cholinesterases, they will be referred to in the present publication simply as eserine-resistant enzymes which can hydrolyse butyrylcholine (cf. Koelle, 1952).

Inhibition of the pseudo cholinesterase in mice

The results given in Table 1 and Fig. 1 seem to justify a classification of all of these enzymes as pseudo cholinesterases, even though their specificity patterns show considerable variation from species to species. To substantiate the physiological classification of these enzymes as pseudo cholinesterases, it was demonstrated previously that selective inhibition of this enzyme in dog and man by Nu 683 (Hawkins & Gunter, 1946) and DFP (Hawkins & Mendel, 1947; cf. Mazur & Bodansky, 1946), respectively, does not cause any observable symptoms of acetylcholine poisoning. The pseudo cholinesterases both of dog and of man are, however, butyro-cholinesterases; since the enzyme found in mouse serum exhibits a quite different type of specificity pattern (Fig. 1), it was necessary to show that an enzyme such as this is also a pseudo cholinesterase physiologically, i.e. that it is not essential for the hydrolysis of the neurohumoral choline esters *in vivo*. Several groups of female mice were therefore injected with graded doses of DFP in aqueous solution; after observing the mice for 1 hr. subsequently, the animals were sacrificed and the

cholinesterase activities determined in pooled samples of the blood. The results of this experiment (Table 5) are almost identical with those obtained on dogs by Hawkins & Gunter (1946). No signs are observed when the pseudo cholinesterase activity is completely inhibited by a selective inhibitor. Signs of acetylcholine accumulation appear only when the true cholinesterase activity is inhibited more than 75–80%.

The ali-esterase (tributyrinase) of mouse serum proved to be even more sensitive than the pseudo cholinesterase to inhibition by DFP *in vivo*. This enzyme therefore appears to resemble the tributyrinase of rat serum, which behaves similarly *in vitro* (Myers, 1952c).

DISCUSSION

The physiological distinction between true cholinesterase and pseudo cholinesterase

Although the two types of cholinesterases were first distinguished by differences in enzymological characteristics, the original classification according to physiological function (Mendel & Rudney, 1943) has been supported by the results of subsequent investigations. The pseudo cholinesterase in the intact animal can be completely inhibited without causing any signs of acetylcholine accumulation (Hawkins & Gunter, 1946; Hawkins & Mendel, 1947; cf. Mazur & Bodansky, 1946). On the other hand, inhibition of the true cholinesterases by more than 70–80% leads to acute acetylcholine poisoning, while complete inhibition is invariably fatal (Gunter & Mendel, 1945; Hawkins & Gunter, 1946; Hawkins & Mendel, 1947, 1949; Nachmansohn & Feld, 1947). Experiments with various isolated organs usually show a close correlation between the inhibition of true cholinesterase and the pharmacological effects of the cholinesterase inhibitors (Blaschko, Bülbring & Chou, 1949; Kordik, Bülbring, & Burn, 1952; Kamijo & Koelle, 1952; cf. Koelle, Koelle & Friedenwald, 1950).

While no animal species devoid of true cholinesterase has been found, the sera and tissues of sheep and ox do not contain measurable amounts of pseudo cholinesterase (Mendel *et al.* 1943; Gunter, 1946), although they may contain eserine-resistant enzymes capable of hydrolysing choline esters. This has been confirmed by other workers; ox heart ventricle (Nachmansohn & Rothenberg, 1945) and adrenal gland (Langemann, 1951) show no significant activity towards benzoylcholine, in contrast to the heart (Ord & Thompson, 1950) and adrenal gland (Sawyer & Everett, 1947) of the rat.

The physiological function of the pseudo cholinesterases is still uncertain. Chance observations made in the present investigation suggest that it is unwise to draw any general conclusions regarding the function of the pseudo cholinesterases from a study of its distribution in different tissues of a given animal. For example, the pseudo cholinesterase activity of rat heart is very high (Ord & Thompson, 1950, 1951), but that of hamster heart was relatively low and there was almost no pseudo cholinesterase activity to be found in squirrel heart. A parallel observation is found in the results of Mendel *et al.* (1943), which show that the erythrocytes of avian species contain very little true cholinesterase activity despite the fact that mammalian erythrocytes often exhibit a high activity.

Recent results suggest that propionylcholine, as well as acetylcholine, might play a role as a neurohumoral transmitter in some cases (Banister, Whittaker & Wijesundra, 1951), but this would not alter the significance of the experimental observations on the pharmacological effects of the inhibition of true cholinesterase and pseudo cholinesterase. Whatever the physiological function of the pseudo cholinesterases may prove to be, the original distinction between them and true cholinesterases does seem to be justified by the primary physiological importance of the true cholinesterases for the rapid hydrolysis of acetylcholine in the intact animal.

Enzymological classification of the cholinesterases

A strict enzymological classification of the cholinesterases on the basis of their specificity patterns will often be confusing because the specificity pattern of the same type of enzyme varies from one species to another. The use of terms such as butyro-cholinesterase and propiono-cholinesterase may be useful to characterize the specificity pattern of the particular pseudo cholinesterase. However, the enzymological properties of an enzyme are typical of the species and vary within an apparently continuous range of specificity pattern; a particular enzyme must therefore be designated according to its source. It is also interesting that the pseudo cholinesterase of the pig does not show a marked preference for any one particular acyl group; the significance of this is not understood but it is obvious that such an enzyme would be difficult to classify on an enzymological basis.

A further complication is presented by the fact that the rate of enzymic hydrolysis of an ester will depend in part upon the physico-chemical properties of the ester, for example, the solubility, the surface activity and the inherent stability of the ester. For example, there may be a correlation between the rapid enzymic hydrolysis and the high rate of spontaneous hydrolysis of the haloacetate esters (cf. Adams & Whittaker, 1950). Thus a portion of what has been called specificity of an esterase may not be enzyme specificity at all but a function of the

lability of the bond that is exposed to the action of the enzyme (Smith, 1951).

A nomenclature based on specificity patterns is also disadvantageous when more than one type of enzyme exhibits similar specificity characteristics. In particular, this terminology fails to stress the characteristic differences between true cholinesterases, pseudo cholinesterases, and eserine-resistant enzymes which hydrolyse butyrylcholine. The limited data available on these eserine-resistant esterases indicate that some of these enzymes may also be butyro-cholinesterases, and the data given above (Fig. 1) point to the possibility that a pseudo cholinesterase with maximal activity towards acetylcholine may be found in species which have not yet been investigated. One example of a true cholinesterase which hydrolyses propionylcholine more rapidly than acetylcholine has been found (Fig. 2) and the true cholinesterases from certain invertebrates exhibit a greater activity towards acetyl-β-methylcholine than towards acetylcholine (Augustinsson, 1946; Metcalf & March, 1950).

Despite the fact that it is becoming more and more apparent that there are many types of cholinesterases from an enzymological viewpoint, it does seem justifiable and useful to retain the distinction between the two main groups of eserine-sensitive cholinesterases in vertebrate physiology. Apparent exceptions to this general classification can usually be explained on the basis of species variations in specificity patterns; the results with the pseudo cholinesterases of pig serum (Levine & Suran 1950), rat heart ventricle (Ord & Thompson, 1951) and chicken serum (Earl & Thompson, 1952) are of especial interest in this connexion and emphasize the importance of designating the particular pseudo cholinesterase or true cholinesterase according to the species from which it was obtained. The particular nomenclature which is used by various authors to distinguish these two groups of eserine-sensitive cholinesterases in mammalian tissues is not of great importance; however, the designation should not leave any room for ambiguity, and we believe that the evidence cited above still justifies a terminology which indicates a distinction according to physiological importance, e.g. true cholinesterase and pseudo cholinesterase.

The differentiation of true cholinesterase and pseudo cholinesterase activity

The main characteristics of a pseudo cholinesterase, as originally put forward by Mendel and co-workers, were that the enzyme should hydrolyse acetylcholine, benzoylcholine, tributyrin and methyl butyrate but not acetyl-β-methylcholine, and that the activity towards any ester should be inhibited by low concentrations of eserine. The true cholinesterases, on the other hand, should hydrolyse acetylcholine and acetyl-β-methylcholine but not benzoylcholine, tributyrin or methyl butyrate. Unlike the pseudo cholinesterases, the true cholinesterases exhibit maximal activity towards acetylcholine at low concentrations of this ester and are inhibited by excess of acetylcholine; like the pseudo cholinesterases, the true cholinesterases are inhibited by low concentrations of eserine. Tributyrin and methyl butyrate are also hydrolysed by the aliesterase, but these latter enzymes do not hydrolyse choline esters and are not inhibited by low concentrations of eserine.

The use of specific substrates. This was proposed by Mendel *et al.* (1943) and still appears to be the most convenient method for identification and quantitative measurement of the true cholinesterase and pseudo cholinesterase activity in various mammalian tissues (cf. Sawyer & Everett, 1947; Ord & Thompson, 1950), but it should always be supplemented by a test for sensitivity to inhibition by low concentrations of eserine and prostigmine or prostigmine analogues. Both benzoylcholine (Mendel *et al.* 1943) and butyrylcholine (Cohen *et al.* 1949; cf. Nachmansohn & Rothenberg, 1945) have been proposed as specific substrates for the measurement of pseudo cholinesterase activity, but other esterases which can hydrolyse one or both of these esters are known to exist (Sawyer, 1945; Koelle, 1952) and may be more widespread in occurrence than previously considered; these enzymes, unlike the true cholinesterases and pseudo cholinesterases, are not inhibited by 2×10^{-6}M-eserine (cf. Gunter, 1946; Hawkins & Gunter, 1946).

Since the specificity patterns of the pseudo cholinesterases differ greatly, the measured activity towards either butyrylcholine or benzoylcholine does not constitute an exact basis for comparison of the 'amount' of pseudo cholinesterase in the serum of different mammalian species, nor does it give an indication of the relative activity towards other esters. However, if the specificity pattern is constant from tissue to tissue within the same animal, we are justified in comparing the pseudo cholinesterase activities of these different tissues towards one of the specific substrates.

As pointed out by Adams & Whittaker (1949*a*), it may be unwise to attach too much significance to very small rates of hydrolysis of these substrates by enzyme preparations or to attempt to measure very low concentrations of the enzyme for which they are specific. The very small activity of the mammalian pseudo cholinesterases towards acetyl-β-methylcholine does not interfere greatly with the use of this ester as a specific substrate for determination of the true cholinesterase activities of mammalian tissues, but a complication is introduced by the fact that the pseudo cholinesterase of chicken serum also

hydrolyses acetyl-β-methylcholine (Earl & Thompson, 1952). Thus it is not possible to use this ester as a specific substrate for measurement for true cholinesterase activity in the chicken, and the same difficulty may be encountered in other non-mammalian species. However, since butyrylcholine is suitable as a specific substrate for the determination of pseudo cholinesterase activity in the chicken, an estimation can be made of the residual fraction of the total cholinesterase activity towards acetylcholine which must be due to true cholinesterase present.

The phenomenon of excess substrate inhibition. Excess substrate inhibition seems to characterize the activity of the true cholinesterases towards acetylcholine, whereas the pseudo cholinesterases do not show it. The concentration of acetylcholine at which inhibition may be observed varies considerably with the true cholinesterases from lower forms of life (Hawkins & Mendel, 1946; Augustinsson, 1946, 1948, 1949) and one true cholinesterase is known (in *Planaria*) which exhibits its maximal activity towards relatively high concentrations of acetylcholine (Hawkins & Mendel, 1946). Moreover, this characteristic, like the use of specificity patterns, is of very limited value for assaying mixtures of true cholinesterase and pseudo cholinesterase.

The ability of a cholinesterase to hydrolyse tributyrin. This can generally be used as additional evidence for its classification as a pseudo cholinesterase, but the lack of an appreciable eserine-sensitive activity towards tributyrin does not allow any conclusion as to the type of cholinesterase concerned. The pseudo cholinesterases of man, horse and dog all hydrolyse tributyrin and methylbutyrate at an appreciable rate (Mendel & Rudney, 1943; Adams & Whittaker, 1949*b*), while the true cholinesterases of man, horse, dog, sheep, ox and mouse show no significant activity towards these esters (Mendel & Rudney, 1943). However, the pseudo cholinesterase of rat heart is characterized by the absence of an appreciable activity towards tributyrin (Wright, 1946; Ord & Thompson, 1951).

The pseudo cholinesterases of all the rat tissues studied in the present investigation proved to have the same specificity characteristics as that of rat heart. Thus it is not surprising to find recorded in an early paper that the cholinesterase of rat skin does not hydrolyse tributyrin (Thompson & Whittaker, 1944), even though the major portion of the acetylcholine hydrolysis by rat skin is due to a pseudo cholinesterase (Ord & Thompson, 1950). It is also of interest that a cholinesterase preparation purified from mouse liver (Mendel & Mundell, 1939, unpublished) also failed to act appreciably on tributyrin; since this cholinesterase preparation hydrolysed acetylcholine most rapidly at high concentrations of this ester and was inhibited by low concentrations of eserine, we can conclude that this enzyme must be another example of a pseudo cholinesterase which does not hydrolyse tributyrin.

Selective inhibitors. Selective inhibitors have been used by many workers for differentiating the activities of various esterases. Under the experimental conditions used by Mendel and co-workers (cf. Mendel & Hawkins, 1950), a concentration of 10^{-7}–10^{-6}M-eserine is usually sufficient to inhibit the true cholinesterase and pseudo cholinesterase activities of mammalian tissues by 90–100%. The ali-esterases, on the other hand, are inhibited only by much higher concentrations of eserine (approx. 10^{-3}M). The Harderian gland of the rat contains an enzyme which hydrolyses butyrylcholine and which is not appreciably inhibited by 10^{-6}M-eserine but is almost completely inhibited by 10^{-4} M-eserine; thus there is not a very large margin between the concentrations of eserine necessary to inhibit completely the cholinesterases of mammalian tissues and the concentrations which partially inhibit other enzymes, but this margin is probably sufficient to serve as an experimental criterion for identifying the cholinesterase activities of mammalian tissues (cf. Gunter, 1946; Myers & Mendel, 1949). However, the true cholinesterases found in lower forms of life may be considerably less sensitive to inhibition by eserine than are the cholinesterases of mammalian tissues (Hawkins & Mendel, 1946) so that caution must be used in interpreting these results. Prostigmine and analogues have not been as extensively investigated but these inhibitors might give slightly better results with the cholinesterases in lower forms of life (Hawkins & Mendel, 1946), and the data given above (Table 4) show that Nu 683 is much more suitable than eserine for distinguishing pseudo cholinesterase activity from that due to other enzymes which hydrolyse butyrylcholine.

Other investigators have used the sensitivity to inhibition by DFP as a criterion for distinguishing true cholinesterase and pseudo cholinesterase activity (Adams & Thompson, 1948; Ord & Thompson, 1950). Considerable information concerning the occurrence of true cholinesterase and pseudo cholinesterase in different tissues has been obtained by this means (Ord & Thompson, 1950, 1952), and the same technique proved to be extremely useful for the present investigation. However, it has been found that DFP and other alkyl phosphate derivatives may inhibit a great many types of hydrolytic enzymes including the eserine-resistant esterases which hydrolyse butyrylcholine (Koelle, 1952) and certain ali-esterases (Myers & Mendel, 1949; Myers, 1952*c*). The method using DFP to differentiate true cholinesterase and pseudo cholinesterase activities should be supplemented, therefore, by a test with

eserine and prostigmine or prostigmine analogues to show that the esterase activity in question is not due to other types of hydrolytic enzymes.

SUMMARY

1. The pseudo cholinesterases from the sera of ten mammalian and three avian species have been studied in detail. It was found that eight of these pseudo cholinesterases can be classified as butyrocholinesterases and four as propiono-cholinesterases, while the pseudo cholinesterase of pig serum could not be classified readily on the basis of its substrate specificity pattern alone. However, there is no clear-cut distinction between the two groups of pseudo cholinesterases; the substrate specificity patterns can be arranged into a series which shows a gradual, progressive shift in the optimal acyl group.

2. Closely similar specificity patterns were found for the pseudo cholinesterase in twelve different rat tissues, indicating that the enzyme is probably the same in all tissues. This conclusion is substantiated by the results of more limited investigations in other species; thus the pseudo cholinesterases appear to be species specific but not organ specific.

3. The Harderian glands of the rat and hamster also contain an eserine-resistant esterase capable of hydrolysing butyrylcholine.

4. In contrast to the true cholinesterases previously investigated, the true cholinesterase of chicken brain hydrolyses propionylcholine more rapidly than acetylcholine. It resembles other true cholinesterases in its remaining enzymological properties, which differ greatly from those of the pseudo cholinesterases; nevertheless, a rigid classification of the cholinesterases on the basis of substrate specificity patterns would characterize both the true cholinesterase and the pseudo cholinesterase of the chicken as propiono-cholinesterases.

5. Acetyl-β-methylcholine cannot be used as a specific substrate for measurement of true cholinesterase activity in the chicken; this seems to apply, to some extent, to avian species generally. However, butyrylcholine appears to be suitable as a specific substrate for measurement of the pseudo cholinesterase activity in these species (cf. Earl & Thompson, 1952).

6. The proposed classification of cholinesterases on the basis of substrate specificity patterns has been criticized, and the importance of designating the pseudo cholinesterases according to their source has been stressed.

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An Apparatus for the Spectrokinetic Study of Rapid Reactions

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The kinetic study of any chemical reaction requires the creation of a homogeneous, reacting mixture accessible to analysis at any instant during the change. For rapid reactions, this technical problem was first solved in a direct and general manner by Hartridge & Roughton (1923). Their method was based on the constant flow principle: the reactants were driven at high speed through separate jets into a small chamber, where rapid mixing occurred. The reacting mixture flowed thence at constant rate down a tube of uniform bore, and could be analysed by optical or other physical means at any distance along the tube. Originally the analysis was made with a reversion spectroscope; later thermal and photoelectric methods were used (Roughton, 1930; Bateman & Roughton, 1935; Roughton & Millikan, 1936). The validity of the technique was established by various controls (Hartridge & Roughton, 1923; Roughton & Millikan, 1936).

Modifications of the method were devised by Millikan and by Chance, with rather different aims in view. In order to extend the work of Hartridge and Roughton on sheep haemoglobin to other oxygen-carrying pigments, Millikan (1936) adapted the constant-flow apparatus to the use of small quantities of material, by replacing the original gas-pressure drive with motor-driven syringes and using a sensitive differential photoelectric colorimeter to analyse the flowing mixture. The apparatus of Chance (1940, 1951) was designed for the kinetic study of enzyme reactions and embodied fundamental alterations in technique. The requisite extension of the time range and economy of material were secured with an accelerated flow method and a very sensitive reaction meter with a high speed of response: a manual syringe drive gave an initial impetus to the reactants; and the subsequent variations of flow velocity, and therefore of time interval, and the corresponding variations in the extent of reaction, measured photoelectrically at a fixed distance from the mixing chamber, were continuously recorded with a double-beam cathode-ray tube. The complete kinetic curve was obtained directly as a trace, which could be photographed.

A modification of the Hartridge-Roughton apparatus, constructed for kinetic studies of human haemoglobin with precision as the major objective of the design, is described in this paper. The apparatus consists essentially of a constant-flow apparatus of the gas-pressure type and a photoelectric spectrophotometer (Beckman quartz Model DU), and can be used to record the absorption spectra of transient intermediate compounds (Dalziel & O'Brien, 1951, 1952*a*, *b*).