SUMMARY

1. Insulin can be separated from protamine by paper chromatography developing with the upper phase of a mixture of *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). A dye such as bromocresol green is used for locating the zones.

2. The concentration of protamine and of various proteins in a solution can be estimated on filter paper by retention analysis with a suitable dye, e.g. erythrosine. The area of the unstained wedge formed above the protein spot is proportional to the amount of protein present. The method can be used to estimate the amount of protamine in protamine zinc insulin after removal of insulin by paper chromatography.

3. The concentration of insulin in protamine zinc insulin can be estimated, after separating the insulin from protamine by paper chromatography, by staining with bromocresol green solution, eluting the insulin-dye complex and comparing the colour of the eluate with that given by a standard solution of insulin treated in the same way.

4. Data are recorded on the composition of a number of suspensions of protamine zinc insulin and on the distribution of the components between the phases of the suspension.

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Studies on Cholinesterase

7. DETERMINATION OF THE MOLAR CONCENTRATION OF PSEUDO-CHOLINESTERASE IN SERUM

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The classical theory of reversible enzyme-substrate and enzyme-inhibitor reactions postulates that enzyme and substrate, or enzyme and inhibitor, form a reversible combination according to mass law principles (Michaelis & Menten, 1913). The mechanism of enzyme-inhibitor reactions has been analysed from this theoretical standpoint by Straus & Goldstein (1943) and Goldstein (1944), with special consideration being given to the concentration of free enzyme and free inhibitor. They pointed out that the degree of inhibition of enzyme activity effected by a certain concentration of inhibitor will be dependent in part upon the enzyme concentration when the so-called 'dissociation' or Michaelis constant of the enzyme-inhibitor system is of the same order of magnitude or much smaller than the molar enzyme concentration employed.

The theoretical treatments given by Goldstein (1944) were illustrated with experimental data obtained, using a pseudo-cholinesterase preparation

as the source of enzyme and eserine as the enzyme inhibitor. In this system it is possible to obtain appreciable inhibition of the cholinesterase activity with eserine concentrations as low as 10^{-8} M; nevertheless, the degree of inhibition is practically independent of enzyme concentration under the experimental conditions. Thus it was not possible to confirm the theoretical formulations completely at that time, or to arrive at any definite value for enzyme concentration in the cholinesterase system.

Since that time, other investigators, notably Bain (1949), have attempted to determine cholinesterase concentration by the use of irreversible alkylphosphate inhibitors such as diisopropyl fluorophosphonate (DFP) and tetraethyl pyrophosphate (TEPP). The lack of theoretical criteria which could be applied to the results with these inhibitors makes this a rather unreliable method for determining cholinesterase concentration. Moreover, the results obtained do not correspond with those expected of

an irreversible inhibitor which combines specifically and only with the active centre of the cholinesterase on an equimolar basis.

However, it has been observed that the reversible inhibition of pseudo-cholinesterase by the very potent inhibitor, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Nu683), is dependent upon the cholinesterase concentration (Myers, 1950) and that the experimental results obtained correspond with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) for a competitive reversible inhibitor of high potency. Evidently the concentrations of Nu 683 which produce appreciable inhibition of pseudo-cholinesterase activity are of the same order of magnitude as the concentrations of pseudo-cholinesterase. By applying the theoretical formulations given by Goldstein, pseudo-cholinesterase concentration in serum has been calculated by a method which appears to be completely justified theoretically.

METHODS

The activity of the cholinesterases was measured manometrically by a method similar to those described previously (Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1950). The medium used was a 0.025M-bicarbonate solution saturated with 5% CO₂ + 95% N₂ (v/v), pH 7.4 at 37.5°. Dialysed human, horse, dog or rat serum was used as source of pseudo-cholinesterase, usually with 0.006M-benzoylcholine as substrate (Mendel, Mundell & Rudney, 1943). Esterase activity for a wide range of cholinesterase concentrations was kept within accurately measurable range by varying the total volume of solution between 2.0 and 5.0 ml. and by the use of 0.06M-acetylcholine at very low enzyme concentrations with horse and human serum. The cholinesterase activity was, however, always calculated out to the value it would have had if 5.0 ml. of the same solution had been used and if benzoylcholine had been the substrate.

The pseudo-cholinesterase inhibitor in most of these experiments was Nu683, described by Hawkins & Gunter (1946). This inhibitor is still not sufficiently potent to allow the calculation of enzyme concentration from inhibition in a system where enzyme, inhibitor and substrate are all equilibrated, as has been recommended by Goldstein (1944). Therefore, the measurements must of necessity be done with a system in which only enzyme and inhibitor are in equilibrium. For a competitive reversible enzyme-inhibitor system of this kind, the relationship between inhibitor concentration I and fractional activity a is expressed as

$$I = K_I \frac{(1-a)}{a} + (1-a)E \quad (\text{Goldstein, 1944}).$$

The concentration of inhibitor required to produce 50% inhibition of the enzyme activity can be designated as I_{50} . When the ratio $E/K_I < 0.1$, the concentration I_{50} will be approximately equal to K_I and independent of the enzyme concentration E (zone A). When $E/K_I > 100$, the concentration I_{50} will be directly proportional to E and independent of the Michaelis constant K_I (zone C). For intermediate values of E/K_I , I_{50} will depend upon the values of both

E and K_I (zone B) (Straus & Goldstein, 1943; Goldstein, 1944). As will be shown below, the pseudo-cholinesterase-esterine system used by Goldstein (1944) must have been in zone A. The pseudo-cholinesterase-Nu683 system used in the present investigation usually lies in the lower part of zone B so that the values of both E and K_I can be accurately determined.

The equation above can be applied only when sufficient time has been allowed to ensure complete equilibration of the enzyme-inhibitor system. The time of incubation should not be too long, however, or errors may be introduced by the slow enzymic hydrolysis of the carbamic acid ester linkage in the combined inhibitor. Another factor is the progressive displacement of combined inhibitor and consequent increase in the fractional activity caused by the addition of substrate to the equilibrated enzyme-inhibitor system. The importance of these three factors has been stressed by Goldstein (1944), but, as will be described below, the possible errors which they would introduce are relatively small in the case of the enzyme-inhibitor system with Nu683 and the pseudo-cholinesterase of human or horse serum. During the course of these investigations, preliminary experiments were done with the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu1250) (Hawkins & Mendel, 1949) as inhibitor of the true cholinesterase activity of purified human erythrocytes (Mendel & Rudney, 1943). In this case the rate of displacement of combined inhibitor by added substrate proved to be appreciable under the experimental conditions. In addition, the ratio E/K_I was still quite low so that the shift in I_{50} was relatively small. Both of these factors increase the possibility of error in the estimations of enzyme concentration and consequently this enzyme-inhibitor system did not seem suitable for an accurate determination of cholinesterase concentration.

For purposes of greater accuracy with Nu683, the value of I_{50} at each enzyme concentration was estimated from a series of determinations with different inhibitor concentrations covering the range from 10 to 90% inhibition of the pseudo-cholinesterase activity. The precise value of I_{50} was obtained by plotting the fractional activity a against pI ($-\log I$) for each set of results, as recommended by Goldstein (1944). The theoretical equation relating inhibitor to enzyme concentration then becomes $I_{50} = K_I + 0.5E$. If I_{50} is determined for a series of different enzyme concentrations, a plot of I_{50} against the relative concentration of enzyme should give a straight line with intercept equal to K_I and a slope equal to one-half the molar concentration of enzyme.

RESULTS

The most convenient system for accurate determination of pseudo-cholinesterase concentration was that with Nu683 as inhibitor and the pseudo-cholinesterase of dialysed human serum as enzyme. It had already been shown (Myers, 1950) that this enzyme-inhibitor system exhibits the predicted characteristics (Goldstein, 1944) of a competitive reversible system for which the ratio of E/K_I has a value appreciably greater than 0.1 and in which the inhibitor combines specifically with the enzyme active centre on an equimolar basis ($n=1$). This is further illustrated by Fig. 1, in which typical

experimental points are compared with the theoretical curves expected for an enzyme-inhibitor system in zone B ($0.1 < E/K_I < 100$) (see a later section for theoretical treatment).

Fig. 1. Comparison of the theoretical curves (—) expected for a competitive reversible inhibitor in zone B with the experimental curves (-----) obtained using Nu683 as inhibitor of the pseudo-cholinesterase activity of dialysed human serum. Theoretical curves calculated for $K_I = 8.8 \times 10^{-10}$ M-Nu683 and $E = 2.3 \times 10^{-9}$ M-pseudo-cholinesterase/100 μ l. CO_2 activity. Experimental points of curve A obtained after 50 min. incubation of the Nu683 with a 2% (v/v) serum solution giving 70 μ l. CO_2 /5 ml. solution/20 min.; those of curve B with a 12% (v/v) serum solution giving 448 μ l. CO_2 /5 ml. solution/20 min. pI represents the negative log. of the molar concentration of Nu683 and a the fractional activity in the presence of this inhibitor.

Table 1. Effect of added benzoylcholine in displacing Nu683 combined with the pseudo-cholinesterase of human serum

(5 ml. of 6% (v/v) dialysed human serum in 0.025 M-bicarbonate was incubated with 6×10^{-9} M-Nu683 for 50 min. at 37.5° before adding 0.006 M-benzoylcholine at time = 0.)

Time after addition of benzoylcholine (min.)	Activity, for time stated, of control serum (μ l. CO_2)	Activity, for time stated, of serum incubated with Nu683 (μ l. CO_2)	Fractional inhibition by Nu683 ($1 - a$)
2-7	49.5	12.0	0.758
7-12	55.5	13.0	0.766
12-17	52.5	14.5	0.724
17-22	55.5	15.0	0.730
Average 2-22 min.			0.745
22-32	112.0	32.5	0.710
32-42	114.5	36.5	0.681
42-52	110.5	38.5	0.651
52-62	112.0	43.0	0.616
62-72	111.5	47.5	0.574
72-82	112.5	50.0	0.556
82-92	114.0	55.0	0.517

The error introduced by the displacement of combined inhibitor after addition of substrate was

relatively small when the activity was measured over the period 2-22 min. after the addition of benzoylcholine (Table 1). However, the fact that Nu683 combined with pseudo-cholinesterase was displaced by added benzoylcholine does indicate that Nu683 is a reversible competitive inhibitor of pseudo-cholinesterase.

It was also necessary, as noted previously, to determine an optimal time of incubation of the enzyme with inhibitor, that is, a time which would be long enough to ensure relatively complete equilibration of the enzyme-inhibitor, but not so long as to allow any appreciable destruction of inhibitor to take place. To this end, a series of determinations of pI_{50} ($-\log I_{50}$) after various times of incubation was carried out using 2 and 12% serum concentrations. The results obtained are given in Table 2; from this data, a standard time of 50 min. incubation was arbitrarily selected for use in subsequent determinations.

Table 2. Changes in pI_{50} after incubation of 2 and 12% (v/v) solutions of human serum with Nu683 for various times at 37.5°

Time of incubation (min.)	pI_{50}	
	2% serum	12% serum
30	8.83	8.18
50	8.77	8.21
75	8.79	8.07
160	8.64	7.92
270	8.53	7.62

A series of experimental a -pI curves, similar to those shown in Fig. 1, was determined for a total of fifteen different enzyme concentrations with several samples of serum. From each of these curves a value of I_{50} was calculated. The enzyme activity was directly proportional to the serum concentration with any one sample of serum (Fig. 2), but the activity per unit volume varied from one sample to another, as would be expected. Therefore the relative enzyme concentration was expressed on the basis of enzyme activity. A plot of I_{50} against relative enzyme concentration gave the experimental points of Fig. 3. The best straight line, fitted to these points by the method of least squares, proved to be that represented by the equation $I_{50} \times 10^9 = 0.880 + 1.15$ (per 100 μ l. activity).

From the theoretical equation $I_{50} = K_I + 0.5E$, it follows that $K_I = 8.8 \times 10^{-10}$ M-Nu683 and

$$E = 2.3 \times 10^{-9} \text{ M-pseudo-cholinesterase}$$

in a solution of which 5 ml. gave an activity of 100 μ l. CO_2 /20 min. with 0.006 M-benzoylcholine as substrate, when measured under the standard conditions of 37.5° , pH 7.4 and 0.025 M-sodium bicarbonate medium. This same concentration of the pseudo-cholinesterase of human serum would

give an activity of $292 \mu\text{l. CO}_2/20 \text{ min.}$ under the same conditions when using 0.06 M -acetylcholine as substrate.

An enzyme activity of this order of magnitude is usually obtained with a concentration of about 3% human serum. For a full serum which gives an activity of $700 \mu\text{l. CO}_2/\text{ml. serum}/20 \text{ min.}$ with benzoylcholine, the concentration of pseudo-cholinesterase would be precisely 35 times as high as the figure given above. That is to say, the concentration of pseudo-cholinesterase in full human serum would be about $8 \times 10^{-8} \text{ M}$ on the average.

Fig. 2. Linear relationship between the pseudo-cholinesterase activity and the concentration of serum used for two different samples of dialysed human serum. The original activity of the full serum corresponding to line A was $820 \mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$; this was diluted to 1.12 times the original volume in the process of dialysis. The full serum corresponding to the dialysed sample of line B had an activity of $685 \mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$ and was diluted to 1.38 times the original volume in the process of dialysis. The activity was determined using 0.006 M -benzoylcholine as substrate, a fluid volume of 5 ml. below 10% (v/v) dialysed serum and a fluid volume of 2.5 ml. above the 10% serum concentration, a rate of shaking of $150 \times 5 \text{ cm. strokes/min.}$, and the other conditions as described in the method.

A similar type of determination on dialysed dog serum gave an equation (from 5 points) $I_{50} \times 10^9 = 3.2 + 1.35$ (per 100 $\mu\text{l.}$ activity), from which $K_I = 3.2 \times 10^{-9} \text{ M}$ -Nu 683 and $E = 2.7 \times 10^{-9} \text{ M}$ -pseudo-cholinesterase in a solution of which 5 ml. gave an activity of $100 \mu\text{l. CO}_2/20 \text{ min.}$ with 0.006 M -benzoylcholine under the standard experimental conditions. In this case the ratio E/K_I would be approximately 0.8 for the pseudo-cholinesterase

concentration in a solution which gives the particular esterase activity selected as standard, whereas in the case of human serum previously the two values obtained would give a ratio $E/K_I = 2.6$. From the theoretical treatments of Straus & Goldstein (1943) and Goldstein (1944), it would be expected that the enzyme-inhibitor system is much

Fig. 3. Relationship between the relative concentration of pseudo-cholinesterase and the molar concentration of Nu 683 which would cause 50% inhibition of the pseudo-cholinesterase activity of dialysed human serum towards 0.006 M -benzoylcholine.

Fig. 4. Comparison of the theoretical (—) and experimental (-----) curves obtained using Nu 683 as inhibitor of the pseudo-cholinesterase activity of dialysed dog serum. (cf. Fig. 1). Experimental points of curve A obtained after 50 min. incubation of the Nu 683 with a 2% (v/v) serum solution giving $35.4 \mu\text{l. CO}_2/5 \text{ ml. solution}/20 \text{ min.}$; those of curve B with a 12% (v/v) serum solution giving $206 \mu\text{l. CO}_2/5 \text{ ml. solution}/20 \text{ min.}$

closer to zone A with dog serum than with human serum under the conditions used. In agreement with this fact, the a - pI curves obtained were somewhat flatter and less widely spread than those obtained with human serum. Also the experimental points agree relatively well with the theoretical curves expected from the values of E and K_I obtained (Fig. 4). It is a striking fact that these two sets of curves (Figs. 1 and 4) give similar values

for the pseudo-cholinesterase concentration per unit of activity despite their differences in shape and relative position.

The same concentration of dog serum gave an activity of 186 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.06M-acetylcholine as substrate. From this it can be calculated that the concentration of pseudo-cholinesterase in the 4.54% dog serum used by Goldstein (63.1 $\mu\text{l. CO}_2/20 \text{ min.}$ with a volume of 2.2 ml. and with 0.08M-acetylcholine as substrate) was probably $2.07 \times 10^{-8}\text{M}$, as compared with the value $E < 1.8 \times 10^{-8}\text{M}$ used by Goldstein (1944). This would mean that the ratio E/K_i with eserine as inhibitor ($K_i = 3.11 \times 10^{-8}\text{M}$) was of the order of 0.067 in Goldstein's investigations, and justifies the application of zone A equations to the experimental data obtained by Goldstein (1944) with eserine as the pseudo-cholinesterase inhibitor.

pseudo-cholinesterase in the serum. In human serum or horse serum, however, the amounts of true cholinesterase present are so small that no appreciable error will be introduced by the use of acetylcholine as substrate for the pseudo-cholinesterase.

Similar determinations of the values of E and K_i were also carried out with the pseudo-cholinesterases of horse, rat and mouse sera. The relative activities of the pseudo-cholinesterases of rat and mouse serum towards acetylcholine were determined by the use of Nu 1250 and half-saturated ammonium sulphate as previously described for dog serum. The results obtained are summarized in Table 3.

Difficulties were encountered with rat serum in that the relatively low pseudo-cholinesterase activity of the full serum does not permit such a wide range of enzyme concentrations to be used in

Table 3. Comparison of the values of K_i and E as determined with Nu 683 for the pseudo-cholinesterases from the sera of different species

Source of serum	Activity of full serum towards benzoylcholine ($\mu\text{l. CO}_2/\text{ml.}/20 \text{ min.}$)	No. of determinations of I_{50}	K_i ($\times 10^{-9}\text{M}$) Nu 683	E_{BCh}^* ($\times 10^{-9}\text{M}$)	ACh/BCh†	E_{ACh}^* ($\times 10^{-10}\text{M}$)
Mouse	355	7	0.56	3.27	8.2	3.98
Rat	180	8	3.3	2.05	4.25	4.82
Horse	790	7	0.72	2.24	3.35	6.70
Man	685-820	15	0.88	2.30	2.92	7.89
Dog	340	5	3.2	2.70	1.62	16.7

* E_{BCh} represents the molar concentration of pseudo-cholinesterase in a solution of which 5 ml. give an activity of 100 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.006M-benzoylcholine as substrate under the standard experimental conditions used. E_{ACh} defined similarly but with 0.06M-acetylcholine as substrate.

† ACh/BCh is the ratio of pseudo-cholinesterase activity towards 0.06M-acetylcholine over that towards 0.006M-benzoylcholine.

However, it should be noted that dog serum usually contains appreciable amounts of true cholinesterase as well as the pseudo-cholinesterase, and that both of these enzymes are capable of hydrolysing acetylcholine even at high concentrations of this substrate (Mendel & Rudney, 1943; Mendel *et al.* 1943).

To determine the relative pseudo-cholinesterase activity towards acetylcholine and benzoylcholine, it was first necessary to eliminate all true cholinesterase activity towards acetylcholine. This was done by two methods, by selectively inhibiting the true cholinesterase activity with 10^{-7}M -Nu 1250 (Hawkins & Mendel, 1949), and by precipitating the true cholinesterase from the serum by addition of an equal volume of saturated ammonium sulphate to the serum (Hawkins & Mendel, 1949). The residual pseudo-cholinesterase activity towards acetylcholine and benzoylcholine was subsequently determined in both cases. With the above example of dog serum, it appeared that only 162 of the 186 $\mu\text{l. CO}_2/20 \text{ min.}$ with 0.06M-acetylcholine as substrate were actually due to the activity of the

these determinations as in the other cases. It is known that female rat serum contains much larger amounts of pseudo-cholinesterase than the serum of male rats (Mundell, 1944); the same is also true to a lesser extent for the sera of male and female mice. Therefore, pooled samples of female rat serum and of female mouse serum were employed for these determinations. The pseudo-cholinesterase activity of the female rat serum was 180 $\mu\text{l. CO}_2/\text{ml. serum}/20 \text{ min.}$ with benzoylcholine as substrate; this is relatively high for rat serum, but even so the activity is still somewhat lower than that of the other types of sera used. In addition, the ratio E/K_i was relatively low so that the values of E and K_i could not be determined as accurately as with human serum or horse serum.

DISCUSSION

The absolute activity of pseudo-cholinesterases

The results given in Table 3 show that the pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-

cholinesterase activity towards 0.06 M-acetylcholine over that towards 0.006 M-benzoylcholine becomes progressively smaller. The values range from 8.2 to 1.6, a difference of 5.1-fold. In this same series, the pseudo-cholinesterase concentration which gives one unit of activity towards acetylcholine becomes progressively larger, that is to say, the absolute activities of the pseudo-cholinesterases in this series becomes progressively smaller. The values obtained cover a range of 4.2-fold. This is a surprising finding, but closer examination of the results show that there is not a close correlation between the figures in these two series. Moreover, the absolute activity of the pseudo-cholinesterases towards benzoylcholine do not form a parallel series. Thus it seems doubtful that there might be some theoretical significance for the rough correlation in this series of pseudo-cholinesterases between the absolute activity towards acetylcholine and the ratio of activity towards acetylcholine over that towards benzoylcholine.

The Michaelis constant for Nu683 also varies considerably from one pseudo-cholinesterase to the other, but varies independently of the other characteristics determined. Measurements of the Michaelis constant for acetylcholine show a similar lack of correlation with any of the other results.

From Table 3 it is apparent that the absolute activity of the pseudo-cholinesterases towards benzoylcholine does not vary as much from one type of serum to another as does the absolute activity towards acetylcholine. The former varies by only 1.50 times for the five preparations of pseudo-cholinesterase investigated, the latter by 4.20 times.

The absolute activity, however, is inversely proportional to the pseudo-cholinesterase concentration which gives one unit of activity, regardless of the particular units used. Easson & Stedman (1936) have defined the term absolute activity in terms of a turnover number, i.e. the number of molecules of substrate hydrolysed by one molecule of pseudo-cholinesterase per unit of time. Unfortunately the values given by these authors are not strictly comparable with the above. However, for purposes of comparison with results on other enzymes, turnover numbers can be calculated from the above results on the basis that

$$\begin{aligned} 100 \mu\text{l. CO}_2/5 \text{ ml. soln./20 min.} \\ &= 1 \mu\text{l. CO}_2/\text{ml. soln./min.} \\ &= 1 \text{ ml. CO}_2/\text{l. soln./min.} \\ &= 4.46 \times 10^{-5} \text{ moles/l. soln./min.} \end{aligned}$$

If this latter figure is divided by any one of the enzyme concentrations given in Table 3 the corresponding turnover number will be obtained, expressed in terms of min.^{-1} . The enzyme concentrations are expressed simply in moles of enzyme per litre, but inherent in this method of determining enzyme concentration is the assumption that these

concentrations are equivalent to the molar concentration of enzyme active centres. For the present it can only be assumed, in most cases, that each active centre corresponds to one molecule of the enzyme protein.

Thus, for example, each active centre of the pseudo-cholinesterase of horse serum will hydrolyse 6.87×10^4 mol. of acetylcholine or 1.99×10^4 mol. of benzoylcholine per min. under the experimental conditions used. (Compare the value 9.35×10^4 mol. of acetylcholine per min. reported by Berry (1951) after the completion of this paper.) With propionylcholine or butyrylcholine as substrate, this value would be considerably higher. As judged from the results of Augustinsson & Nachmansohn (1949) and Sturge & Whittaker (1950), this turnover number might approach a value of 1.4×10^5 mol. of butyrylcholine per min. A turnover number expressed in this way will be identical with the destruction constant k_d of Goldstein (1944) when the appropriate small correction is made for the effect of substrate concentration.

Another point of some interest is the order of magnitude of the activity of pure pseudo-cholinesterase. The exact molecular weight of pseudo-cholinesterase is unknown, but for a serum albumin we might assume a molecular weight of 70 000 (Kekwick, 1938). Further, assuming that one active centre corresponds to 1 mol. of pseudo-cholinesterase protein, it could be calculated that pure pseudo-cholinesterase from horse serum would have a $Q_{\text{acetylcholine}}$ value of 1300 000 $\mu\text{l. CO}_2/\text{mg. dry wt./hr.}$ This is of a similar order of magnitude to the $Q_{\text{acetylcholine}}$ values of 400 000 and 600 000 for the purest preparations of pseudo-cholinesterase obtained by Strelitz (1944) and Mendel & Mundell (1943) respectively.

The data presented in Table 3 show that the pseudo-cholinesterases from the sera of different species of animal may have considerably different affinities for the same inhibitor. Moreover, the absolute rate of hydrolysis (per mole of enzyme) of the same substrate may also differ from one preparation of pseudo-cholinesterase to another. The magnitude of these variations in the absolute rate of hydrolysis by various pseudo-cholinesterase preparations also depends upon the substrate concerned. This fact has already been indicated by the results of other investigators, which show that the pseudo-cholinesterase from horse serum differs from the pseudo-cholinesterase of human serum in the relative rate of hydrolysis of non-choline esters (Blaschko & Holton, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950).

It has been pointed out by Mendel and co-workers (Mendel & Rudney, 1943; Mendel *et al.* 1943; Mendel & Mundell, 1943; Strelitz, 1944; Hawkins & Gunter, 1946; Hawkins & Mendel, 1949) that pseudo-

cholinesterase is a distinct enzyme, with the same general and characteristic specificity towards certain substrates and certain inhibitors in all cases investigated, regardless of the source of the enzyme. The results of later investigations emphasize the fact that preparations of pseudo-cholinesterase from different sources often show minor differences in the relative specificity for various substrates and inhibitors. Consequently, it has been proposed that the pseudo-cholinesterases from various sources should be regarded as a special group or 'family' of related enzymes rather than as a special enzyme, and that the true cholinesterases, similarly, should be regarded as another special group of closely related enzymes (Bodansky, 1946; Augustinsson, 1948). The results of the present investigation would appear to justify such a concept—to the same extent as it would be justified to consider the haemoglobins, for example, as a group of closely related haem proteins.

In both cases it can be considered that the active centre or active grouping will determine the general characteristic specificity common to all different preparations of the same enzyme, while the minor variations in relative specificity will be determined mainly by the influence of the various species-specific (and possibly organ-specific) protein carriers. Thus it will depend upon the purposes of the investigation in hand whether the pseudo-cholinesterase (or the true cholinesterase, or the haemoglobin) from different sources is to be regarded as a special enzyme (from the viewpoint of physiological function) or as a special group of enzymes (from the viewpoint of enzymic or immunological investigations). The pseudo-cholinesterases from different sources would appear to have the same general characteristics in all cases, but it must be emphasized that the Michaelis constants, reaction velocity constants and enzyme concentration will not be identical from one preparation of pseudo-cholinesterase to another.

The use of inhibitors in the study of pseudo-cholinesterases

It has been found previously that the inhibition of pseudo-cholinesterase by Nu 683 is reversed only very slowly by dialysis or dilution of the enzyme-inhibitor system (Hawkins & Gunter, 1946). Nevertheless, Nu 683 must be a reversible competitive inhibitor of pseudo-cholinesterase since the combined inhibitor is displaced by added substrate. The previous findings are probably to be explained by the very high affinity of Nu 683 for pseudo-cholinesterase and by the fact that the enzyme-inhibitor system lies in zone *B* under the experimental conditions.

The experimental results with this inhibitor show sufficiently good agreement with the theoretical

predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the theoretical treatments of these authors, in so far as the concept of three zones of inhibitor action is concerned. And conversely, it would also indicate that Nu 683 combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. Thus the results with this inhibitor should give an accurate determination of pseudo-cholinesterase concentration with the mathematical methods used.

On the other hand, the use of irreversible inhibitors such as DFP or TEPP can lead to erroneous values for enzyme concentration. In the first place it might be noted that the data of Bain (1949) indicate apparent enzyme concentrations which differ by 250 times when these two compounds were used as 'specific' irreversible inhibitors of the true cholinesterase activity of rat brain. Evidently, at least one of the two cannot be a specific cholinesterase inhibitor, but there is no theoretical criterion which could be applied to such results to indicate when an irreversible inhibitor does combine specifically with the cholinesterase active centre on an equimolar basis.

The discrepancies observed in the results with these alkyl-phosphate inhibitors are further illustrated by the data obtained using DFP as an inhibitor of true cholinesterase activity (Fig. 5). The results obtained in this case are more typical of a reversible competitive inhibitor in zone *B* than of a specific irreversible inhibitor, despite the fact that for most practical purposes the inhibition of cholinesterase activity by DFP is completely irreversible under these experimental conditions. But while the experimental results with DFP would indicate an apparent enzyme concentration of 2.9×10^{-8} moles/100 μ l. activity with 0.03M-acetyl- β -methylcholine under standard conditions, the results obtained with Nu 1250 showed that the true enzyme concentration was no greater than 6.3×10^{-10} M in the same solution. The reactions of cholinesterases with DFP and similar inhibitors will be dealt with in more detail in a subsequent publication. For the present purposes it is sufficient to note that the explanation of these results with DFP is still uncertain, and that the use of this inhibitor will not give a reliable value for the concentration of cholinesterase under the above conditions.

At least three other groups of investigators (Easson & Stedman, 1936; Ellis, Plachte & Straus, 1943; Straus & Goldstein, 1943) have attempted to measure the pseudo-cholinesterase concentration in horse serum by the use of eserine or prostigmine as inhibitors. Their results have been criticized on theoretical and practical grounds by Goldstein (1944), who himself did not attempt to give anything more than a maximum value for the cholinesterase concentration. The results obtained in the present

investigation with Nu 683 as inhibitor do indicate considerably different concentrations of pseudo-cholinesterase in horse serum from those which have been previously proposed, as would be expected in view of Goldstein's criticism.

The difference between the method used in the present investigation for determination of pseudo-cholinesterase concentration and previous determinations with competitive reversible inhibitors

against pI to give a sigmoid curve. Easson & Stedman (1936), on the other hand, used an equation of the form

$$\frac{I}{(1-a)} = \frac{K_I}{a} + E,$$

and plotted $I/(1-a)$ against $1/a$ on a linear basis. The equation itself is theoretically correct, of course, and this mathematical method of analysing the results would give correct values for E and K_I if the experimental results correspond exactly with the theoretical expectations. But when the data are subject to some experimental error, the use of this equation can give results which are very misleading. Goldstein (1944) has pointed out that this latter method gives a vastly disproportionate weight to the experimental results with very small values of a , and that it is just these results more than any others which are most subject to large experimental errors. The method of plotting a against pI would weight all points to the same extent so that a theoretical curve can be obtained which will give a minimal standard deviation from all of the experimental points (Goldstein, 1944). Thus this method will give a more accurate representation of the experimental data, and the shift in I_{50} concentration with changes in enzyme concentration will allow the calculation of a reliable value for the molar concentration of enzyme. The a - pI plot has a further advantage in that the experimental results can be compared with the theoretical predictions for a competitive reversible inhibitor which combines specifically with the enzyme active centre on an equimolar basis. The combination of all of these factors seems to make this a reliable method for the accurate determination of pseudo-cholinesterase concentration in serum.

Fig. 5. Relationship between the relative concentration of true cholinesterase and the molar concentrations of Nu1250 or of DFP which would cause 50% inhibition of the true cholinesterase activity of human erythrocytes towards 0.03M-acetyl-β-methylcholine. The degree of inhibition effected by various concentrations of Nu1250 was determined after incubation with the erythrocyte preparation for 80 min., that effected by DFP after incubation for 120 min.

lie in three major points. In the first place, Nu 683 is a much more potent inhibitor of pseudo-cholinesterase than either eserine or prostigmine; consequently the degree of inhibition obtained is more dependent on the enzyme concentration. Secondly, the enzyme concentration and Michaelis constant are determined from the results at several different enzyme concentrations rather than from one set of results as in the generally accepted method of Easson & Stedman (1936). Thirdly, the experimental results are plotted as fractional activity a

SUMMARY

1. The experimental results show that Nu 683 (the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide) is a reversible competitive inhibitor of pseudo-cholinesterase which combines specifically with the pseudo-cholinesterase active centre on an equimolar basis. The ratio of E/K_I has a value appreciably greater than 0.1; this has made it possible to determine pseudo-cholinesterase concentration in serum accurately by a method which appears to be completely justified theoretically. The advantages of this method over those which have been used previously have been discussed.

2. The experimental results obtained with this inhibitor show sufficiently good agreement with the theoretical predictions of Straus & Goldstein (1943) and Goldstein (1944) to provide experimental confirmation of the concept of three zones of inhibitor action.

3. The pseudo-cholinesterase concentrations have been expressed on the basis of enzyme activity under standard conditions. The concentration of pseudo-cholinesterase in the particular samples of full serum used were as follows: horse serum 8.8×10^{-8} M; human serum, 7.9×10^{-8} M; female mouse serum, 5.8×10^{-8} M; dog serum, 4.6×10^{-8} M; female rat serum, 1.7×10^{-8} M.

4. The pseudo-cholinesterases from mouse, rat, horse, human and dog serum form a series in which the ratio of pseudo-cholinesterase activity towards 0.06 M-acetylcholine over that towards 0.006 M-benzoylcholine diminishes progressively from 8.2 to 1.6. In this same series, the absolute activity of the pseudo-cholinesterases towards acetylcholine becomes progressively smaller. Other characteristics

of the pseudo-cholinesterases also vary considerably from one type of pseudo-cholinesterase to another, but do not show any correlation to the above series.

5. The significance of these results has been discussed with respect to the concept of the pseudo-cholinesterases as a special group of closely related enzymes.

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The Chemical Estimation of Adrenaline-like Substances in Blood

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Of the numerous chemical methods which have been devised for the estimation of adrenaline the fluorimetric techniques seem most likely to reach a degree of sensitivity sufficient for the measurement of concentrations as low as those occurring in peripheral venous blood. Adrenaline, as is well known, forms an unstable fluorescent oxidation product in alkaline solution. Lund (1949b) recently described a method in which the fluorescent derivative is stabilized by the addition of ascorbic acid and which is capable of estimating concentrations of not less than $10 \mu\text{g./l.}$ Recovery of added adrenaline amounted to 80% and a factor was employed to correct for the loss. Lund did not find measurable

amounts of adrenaline in venous blood and concluded that adrenaline, if present at all, occurred in concentrations of less than $10 \mu\text{g./l.}$

Although the sensitivity of Lund's method could perhaps be increased by suitable modifications, the procedure seems to have other disadvantages. It is stated that adrenaline is quantitatively oxidized to adrenochrome by manganese dioxide without adrenochrome itself being further oxidized. The need for a rapid and complete removal of manganese dioxide is, however, emphasized and variations of such factors as temperature or the activity of the manganese dioxide preparation might, unless carefully controlled, entail the risk of a loss of adreno-