The Use of a Selective Acetylcholinesterase Inhibitor in the Estimation of Pseudocholinesterase Activity in Rat Brain

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The generally accepted method of estimating 'pseudo'- or butyryl-cholinesterase activity consists in measuring the rate of hydrolysis of one of the 'specific' substrates for this enzyme (Mendel, Mundell & Rudney, 1943), the most commonly used being benzoyl- and butyryl-choline. However, such specificity as the enzyme possesses is relative rather than absolute, for the two esters are hydrolysed also by acetylcholinesterase, though at much slower rates than acetylcholine and acetyl- β -methylcholine (Adams, 1949).

Where pseudocholinesterase is present in a tissue to an equal or greater extent than acetylcholinesterase, the contribution of the latter enzyme to the total hydrolysis of benzoyl- or butyryl-choline is negligible. Generally, however, acetylcholinesterase activity in a tissue is greater than pseudocholinesterase activity; this applies particularly to the central nervous system of the rat, where Ord & Thompson (1950) have found the ratio of activities to be about 20:1; in a subsequent paper (Ord & Thompson, 1952) they put forward evidence to suggest that as much as 50% of the apparent pseudocholinesterase activity may be due to acetylcholinesterase.

The present paper describes work undertaken to minimize this source of error as a preliminary to the study of the development of both cholinesterases in the central nervous system of the rat (Elkes & Todrick, 1955). The object was to depress the acetylcholinesterase activity without significantly affecting the pseudocholinesterase activity, by adding a highly selective inhibitor of acetylcholinesterase. Some of the results have been the subject of a preliminary communication (Bayliss & Todrick, 1953).

METHODS

Cholinesterase activity was estimated by a modification of Ammon's (1933) manometric method: the Warburg flask contained 0.5 ml. of enzyme preparation in bicarbonate saline (NaCl, 0.0115M; KCl, 0.0024M; CaCl₂, 0.0024M; NaHCO₃, 0.034M; total molarity 0.154M; equilibrated with CO₂ + N₂ (5:95) to pH 7.6 and 2.2 ml. of the inhibitor dissolved in the same medium; the side arm contained

0.3 ml. of a solution of the substrate (in distilled water, to minimize hydrolysis before mixing).

Enzyme preparations. The 'typical' acetyl- and pseudocholinesterase preparations that were used in selecting the most suitable inhibitors were respectively: (a) A 1:10 (w/v) suspension of rat brain in bicarbonate saline; this was prepared by pounding and grinding the whole of the rat brain in a glass mortar without the addition of saline until it appeared homogeneous, then adding the same volume of saline and continuing the grinding until homogeneity was retained; the bulk of the saline was then added and the grinding continued (Wajda, 1951; cf. also Long, 1952). (b) The intestinal mucosa from one rat, ground in a glass mortar in 20 ml. of the bicarbonate saline and diluted to a final volume of 60–400 ml., according to the activity of the preparation and the substrate used; the ground undiluted suspension could be centrifuged for 10–15 min. at 3000 r.p.m. to give a clear solution which retained 80% of the original activity.

In the experiments concerned with the pseudocholinesterase activity of the central nervous system, a more concentrated preparation was used, 1 g. of brain being homogenized in bicarbonate saline to a final volume of 4 or 5 ml.

Substrates. 0.03M Acetyl- β -methylcholine, 0.006M benzoylcholine and 0.01M butyrylcholine were used. They were dissolved in distilled water and brought to pH 5 with a trace of hydrochloric acid to prevent hydrolysis.

Calculation of results. The output of gas/flask/hr. was calculated by the method of Aldridge, Berry & Davies (1949); it was then corrected for non-enzymic hydrolysis and gas production by the enzyme preparation in the absence of substrate. The results have been quoted variously in terms of μ l. of CO₂/flask/hr., μ l. of CO₂/g. wet wt. of tissue/hr., or percentage inhibition based on a simultaneous estimation of uninhibited activity. The method chosen was that which most clearly emphasized the point at issue; the use of the wet-weight basis in all cases tends to give a misleading idea of the absolute output of gas being measured (e.g. Tables 3 and 4), but it is necessary when relating the activities of the two enzymes as in Table 5.

RESULTS

Comparison of the relative selectivity of acetylcholinesterase inhibitors

The first stage of the investigation consisted in choosing the most effective from a number of compounds which were known to be highly selective inhibitors of acetylcholinesterase. The ratio of the concentrations causing 50% inhibition of the

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pseudo- and acetyl-cholinesterases (I_{50} ratio) has been taken as a measure of the selectivity of an inhibitor (cf. Adams & Thompson, 1948; Todrick, 1954). In the present investigation, we were more concerned with the capacity of the inhibitors to cause very high or very low inhibitions; about this less is known, but theoretically it should be directly related to the I_{50} ratio in competitive reversible inhibitors, and the ranking of any series of inhibitors should be the same in the two cases. Therefore, as a working rule, no compound was examined which was not at least 500 times as effective against acetylcholinesterase as against pseudocholinesterase.

Of the four compounds available which satisfied this criterion, one, bis-(2-diethylaminoethyl) phthalate diethiodide, 'IS 337' (Bovet, Bovet-Nitti, Guarino, Longo & Marrotta, 1949), has not been tested because it is hydrolysed by pseudocholinesterase (Bovet-Nitti, 1949), which would involve uncertainty about its concentration during the estimation. The others are: the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide, 'Nu 1250' Roche (Hawkins

& Mendel, 1949); 1:5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide, '62C47' Wellcome (Burgen, 1948; Copp, 1953); and 1:5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one diiodide, '284C51' Wellcome (Austin & Berry, 1953; Copp, 1953).

Experiments were carried out to ascertain how completely rat-acetylcholinesterase activity could be depressed by these compounds in concentrations that would not interfere significantly with rat-pseudocholinesterase activity. In the first experiments, benzoylcholine was used as the substrate for pseudocholinesterase; the results are given in Table 1. It should, however, be remembered that the experimental error of the manometric method is generally taken as about 3 %.

It was concluded that Nu 1250 was insufficiently selective for the purpose in view and that, although 284C51 appeared to be slightly more effective than 62C47, it was doubtful whether the differences were real. These results thus tended to support the assumption that the effectiveness was directly related to the I_{50} ratio, of which the values are 500 for Nu 1250, 50 000 for 62C47 and 300 000 for

Table 1. *Inhibition of rat acetyl- and pseudo-cholinesterase by selective inhibitors**

Acetylcholinesterase, 1 g. of brain homogenized in 10 ml. of bicarbonate (0.034M) saline; pseudocholinesterase, intestinal mucosa from one rat ground in 20 ml. saline and diluted 4- to 6-fold according to activity; substrates, 0.01M acetyl- β -methylcholine and 0.014M benzoylcholine respectively: the figures are means of three estimations.

Inhibitor	Concn. (M)	Percentage inhibition	
		Acetyl- cholinesterase	Pseudo- cholinesterase
Selective acetylcholinesterase inhibitors			
Nu 1250	5×10^{-7}	81	1
	2×10^{-6}	98	8
62C47	5×10^{-6}	94	1
	2×10^{-5}	101	3
284C51	3×10^{-5}	100	2
	1×10^{-4}	102	5
Selective pseudocholinesterase inhibitors			
Ethopropazine methosulphate (Lysivane)	3×10^{-5}	- 1	90
	8×10^{-5}	9	95
Tetra <i>isopropyl</i> pyrophosphoramidate (<i>iso</i> -OMPA)	1×10^{-5}	0, 1	90
	3×10^{-5}	6, 11	92
	1×10^{-4}	24	—

Table 2. *Effect of selective anticholinesterases on the hydrolysis of butyrylcholine by pseudocholinesterase*

Pseudocholinesterase preparation, see Table 1; substrate, 0.01M butyrylcholine.

Inhibitor	Concn. (M)	Percentage inhibition of enzyme activity				
		Rat 1	Rat 2	Rat 3	Rat 4	Mean
A. 62C47	5×10^{-6}	- 1	+ 2	- 2	- 3	- 1
	2×10^{-5}	- 2	- 1	- 6	- 10	- 5
B. Lysivane		Rat 5	Rat 6	Rat 7	Mean	
	3×10^{-5}	99	99	102	100	
	8×10^{-5}	97	99	102	99	

284C51. The last two inhibitors were tested with butyrylcholine as substrate; the results for 62C47 are given in Table 2; the 5% activation observed at $2 \times 10^{-5} \text{M}$ may be real, as 284C51 at $3 \times 10^{-5} \text{M}$ did cause 10–20% activation. In subsequent work, 62C47 was used and the concentration was reduced to $7 \times 10^{-6} \text{M}$ to minimize the chances of slight activation. The stability of this compound in solution was investigated: a 10^{-4}M solution was compared after 6 months' storage in a refrigerator with a freshly made solution, both being equally diluted in order to cause inhibition of acetylcholinesterase of the order of 50%. Deterioration was hardly measurable and, considering the way in which the solution was used, appeared to be of little significance.

Comparison of the relative selectivity of pseudocholinesterase inhibitors

Selective inhibitors of pseudocholinesterase were tested in a similar manner. Only compounds at least 500 times as effective against pseudocholinesterase as against acetylcholinesterase were examined. Those tested were the irreversible inhibitor tetra-isopropylpyrophosphoramidate, 'iso-OMPA' (Aldridge, 1953), and the reversible inhibitor, ethopropazine methosulphate, 'Lysivane methosulphate' May and Baker (Todrick, 1954). The results of tests against acetylcholinesterase and pseudocholinesterase with benzoylcholine as substrate are given in Table 1; there was little difference between them, but the maximum inhibition of pseudocholinesterase caused by Lysivane methosulphate appeared to be slightly greater; in addition, this compound was preferred because it was a reversible

inhibitor, and the degree of inhibition caused by irreversible inhibitors varies markedly with time of contact (Mackworth & Webb, 1948). Tested against pseudocholinesterase, using butyrylcholine as substrate, Lysivane did achieve more complete inhibition (Table 2). In subsequent work it was used at a concentration of $5 \times 10^{-5} \text{M}$ with both substrates, though the results suggest that, with butyrylcholine, a concentration of $3 \times 10^{-5} \text{M}$ would be preferable.

Hydrolysis of benzoyl- and butyryl-choline by rat brain

Having selected an inhibitor which appeared to be capable of inhibiting 95% of acetylcholinesterase activity at a concentration that did not reduce pseudocholinesterase activity significantly, and another that was equally selective for pseudocholinesterase, we have used them to analyse the hydrolysis of butyryl- and benzoyl-choline by a homogenate of rat brain. Table 3 shows that the rate of hydrolysis is reduced by both compounds, but to a greater extent by the pseudocholinesterase inhibitor; the fact that the sum of the two inhibited activities is, within the limits of experimental error, equal to the uninhibited activity suggests that the two compounds are inhibiting different enzymes and not acting to different extents on a single enzyme. This is confirmed by experiments in which the inhibitors, used jointly, have reduced the rate of hydrolysis to an almost negligible level (Table 4).

In view of the known properties of the two inhibitors, the simplest explanation of the results is that acetylcholinesterase has contributed to the

Table 3. *Analysis of the choline-ester-hydrolysing activity of rat-brain homogenate, using selective inhibitors of acetyl- and pseudo-cholinesterase*

Homogenate, 1 g. of rat brain in 5 ml. of bicarbonate (0.034M) saline, 0.5 ml./flask; acetylcholinesterase inhibitor, 62C47; pseudocholinesterase inhibitor, Lysivane methosulphate; figures are mean of three estimations, corrected for substrate and tissue blanks.

Substrate	Rate of hydrolysis ($\mu\text{l. CO}_2/\text{hr.}$)				Difference between uninhibited activity and sum of two inhibited activities
	In presence of 10^{-5}M 62C47	In presence of $5 \times 10^{-5} \text{M}$ Lysivane	Sum of two inhibited activities	In absence of inhibitors	
Benzoylcholine 0.006M	35	16	51	48	-3
	35	16	51	52	+1
	32	15	47	49	+2
	39	16	56	59	+3
Mean	35	16	51	52	+1
Butyrylcholine 0.01M	107	11	118	116	-2
	118	11	129	128	-1
	89	7	96	98	+2
	108	7	115	118	+3
	100	13	113	119	+6
Mean	104	10	114	116	+2

Table 4. *Effect of addition of both an acetyl- and a pseudo-cholinesterase inhibitor on the hydrolysis of benzoyl- and butyryl-choline by rat-brain homogenate*

Experimental details as for Table 4.

Substrate	Inhibitors	Rate of hydrolysis of substrate ($\mu\text{l. CO}_2/\text{hr.}$)	
		In absence of inhibitors	In presence of inhibitors
0.006 M-Benzoylcholine	10^{-5} M 62C47 and 5×10^{-5} M Lysivane	30	0
		33*	4
		34*	1
		25	-7
		31	-4
	Mean	31	-1
0.01 M-Butyrylcholine	7×10^{-6} M 62C47 and 5×10^{-6} M Lysivane	109†	3
		116	1
		109†	-6
		101	3
	Mean	109	0

* Homogenate in these experiments contained 1 g. of brain in 3 and 4 ml. respectively; values have been scaled down to correspond with the others.

† These results were obtained in micro-flasks, using one-fifth of normal quantities; they have been multiplied by 5 so as to give figures comparable with the others.

Table 5. *Effect of the selective acetylcholinesterase inhibitor 62C47 on the hydrolysis of choline esters by rat brain and spinal cord*

Homogenates, brain, various strengths, and spinal cord, various strengths, 0.5 ml./flask; substrates, acetyl- β -methylcholine, 0.03 M, butyrylcholine, 0.01 M, benzoylcholine, 0.006 M; 62C47 concentrations, 7×10^{-6} M with butyrylcholine, 10^{-5} M with benzoylcholine; figures in column 5 give the difference between the uninhibited and the inhibited rates of butyrylcholine hydrolysis in terms of the rate of hydrolysis of acetyl- β -methylcholine [(col. 2 - col. 3)/col. 1]; this is interpreted as the ratio of the rates of hydrolysis of butyryl- and acetyl- β -methylcholine by acetylcholinesterase, see text; figures in column 6 give the ratio of the net rates of hydrolysis of butyryl- and benzoyl-choline by brain pseudocholinesterase.

		Rate of hydrolysis ($\mu\text{l. CO}_2/\text{g. wet wt./hr.}$)			Ratio: BuCh MCh hydrolysis for acetyl- cholinesterase	Ratio: BuCh BzCh hydrolysis for pseudo- cholinesterase
Tissue	Acetyl- β - methylcholine (MCh)	Butyrylcholine (BuCh)		Benzoyl- choline (BzCh) Inhibited		
		Uninhibited	Inhibited			
Brain	4180	1225	1120	183	0.025	6.1
	4900	1245	1210	205	0.007	5.9
	5450	1230	1135	217	0.017	5.2
	4580	1240	850	160	(0.085)	5.3
	4530	1170	1090	200	0.018	5.5
Spinal cord	2660	714	680	154	0.013	4.4
	2490	408	386	88	0.009	4.4
	2340	650	576	118	0.032	4.9
				Mean	0.017 $\pm$ 0.003*	5.2 $\pm$ 0.2

* Figure in brackets omitted.

total hydrolysis to the extent of 30 % with benzoylcholine and 9 % with butyrylcholine; alternatively, the estimate of pseudocholinesterase activity with benzoylcholine as substrate is 43 % high, and with butyrylcholine as substrate is 10 % high.

Relative rates of hydrolysis of choline esters by acetylcholinesterase

The figures given above are insufficient to permit the calculation of the ratios of the rates of hydrolysis of butyryl- and benzoyl-choline by acetyl-

cholinesterase to the rate of hydrolysis of acetyl- β -methylcholine. Since the earlier estimates of these ratios showed some disagreement (see Table 7), it was considered worth while to redetermine this ratio for butyrylcholine and acetyl- β -methylcholine by using a method involving a different principle, namely the employment of a selective inhibitor rather than a purified enzyme.

The depression by 62C47 of the rate of hydrolysis of butyrylcholine by rat-brain and spinal-cord homogenates was measured, together with the net

rate of hydrolysis of acetyl- β -methylcholine by the same homogenates; the ratio of these two values is taken to be the required ratio. The results are given in Table 5, the mean being 0.017 ± 0.003 (omitting one result). The ratio of the rate of hydrolysis of benzoylcholine by acetylcholinesterase to that of acetyl- β -methylcholine has not been determined, since it was confirmed that the rate of hydrolysis of benzoylcholine by pseudocholinesterase is only about one-fifth of that of butyrylcholine.

Effect of post-mortem perfusion on the estimation of brain-cholinesterase activity

The estimation of pseudocholinesterase activity was studied as a preliminary to the investigation of its development in the young rat and embryo, circumstances in which the post-mortem perfusion of the brain to remove the acetyl- and pseudocholinesterase in the blood might prove difficult. It was therefore decided to determine whether perfusion materially affected the estimates of enzyme activity. Estimations of both enzymes

were carried out on the brains of pairs of litter mates of the same sex aged about 30 days; one was effectively perfused, as judged by the alteration in colour of the eye; the other was superficially washed in saline. The results given in Table 6 do not demonstrate any material effect of perfusion on the pseudocholinesterase values.

DISCUSSION

The results presented in this paper confirm the view that acetylcholinesterase is partly responsible for the hydrolysis of butyryl- and benzoyl-choline by homogenates of rat brain and spinal cord (Ord & Thompson, 1952). The accepted method of estimating pseudocholinesterase therefore gives high results; the magnitude of the error will be proportional to the acetylcholinesterase/pseudocholinesterase quotient of the tissue.

The acetyl- and pseudo-cholinesterase activity has been measured in eight parts of the brain of the rat, and the ratio has been found to vary from 0.8

Table 6. *Effect of post-mortem perfusion on the estimates of cholinesterase activity in the rat brain*

Litter mates of same sex and age (approx. 30 days) killed by asphyxiation, one brain immediately perfused, the other removed and washed in saline; acetyl- and pseudo-cholinesterase activity measured by using 0.03M acetyl- β -methylcholine and 0.006M benzoylcholine as substrates.

Litter	Sex	Activity of perfused brain (μ l. CO ₂ /g./hr.)		Activity of washed brain (μ l. CO ₂ /g./hr.)	
		Acetylcholin- esterase	Pseudocholin- esterase	Acetylcholin- esterase	Pseudocholin- esterase
A	F.	5190	189	4970	183
A	M.	5330	189	5410	203
B	F.	4300	230	4500	266
C	F.	4440	242	4440	192
C	M.	3370	175	3590	214
Mean		4530	205	4530	212

Table 7. *Rate of hydrolysis of specific substrates of pseudocholinesterase by acetylcholinesterase as percentage of rate of hydrolysis of acetyl- or acetyl- β -methylcholine*

Source of enzyme	Pseudocholinesterase substrate	Acetylcholinesterase substrate	Ratio of rates of hydrolysis	Reference
Human erythrocyte	0.006M Benzoylcholine	0.02M Acetylcholine	0	Mendel <i>et al.</i> (1943)
Electric eel, purified	0.015M Butyrylcholine	0.015M Acetylcholine	2	Nachmansohn & Rothenberg (1945)
Human erythrocyte, partly purified	0.03M Butyrylcholine	0.03M Acetyl- β -methylcholine	5	Adams (1949)
Human erythrocyte, partly purified	0.03M Benzoylcholine	0.03M Acetyl- β -methylcholine	1.5	Adams (1949)
Horse erythrocyte, purified	0.006M Butyrylcholine	0.006M Acetyl- β -methylcholine	2.5	Mounter & Whittaker (1950)
Horse erythrocyte, purified	0.015M Butyrylcholine	0.015M Acetylcholine	2.3	Aldridge (1953)
Brain	0.03M Butyrylcholine	0.0138M Acetylcholine	2.3	Davison (1953)
All tissues (mean)	0.03M Butyrylcholine	0.0138M Acetylcholine	2.2	Davison (1953)
Brain and spinal cord	0.01M Butyrylcholine	0.03M Acetyl- β -methylcholine	1.7	This paper

to 19.1 (Elkes & Todrick, 1955). It can be calculated that the error involved in neglecting the acetylcholinesterase contribution ranges from 6 to 120% with benzoylcholine as substrate, and from 2 to 36% with butyrylcholine as substrate.

Another method of correction has been employed by Davison (1953). He has estimated the ratio of the rates of hydrolysis of butyrylcholine and acetylcholine and has corrected his estimated pseudocholinesterase activity by the estimated acetylcholinesterase activity multiplied by the previously determined ratio. This doubles the number of estimations to be carried out.

The experimental determination of a ratio of the order of 0.02 has its difficulties. However, setting aside earlier statements that acetylcholinesterase does not attack these substrates, the published values are not markedly discrepant (see Table 7), particularly when it is considered that the ratio will be modified by the use of different substrates and concentrations.

The emphasis which has been placed in this work on the estimation of pseudocholinesterase in the presence of a large excess of acetylcholinesterase has been dictated primarily by our interest in the central nervous system. However, there are also tissues in which pseudocholinesterase is present in considerable excess of acetylcholinesterase (Ord & Thompson, 1950); here, it should be possible to improve the accuracy of the estimation of acetylcholinesterase activity by adding Lysivane methosulphate to the reaction mixture. In choosing the optimum inhibitor concentration, and perhaps even the most suitable inhibitor, species differences will, however, have to be borne in mind.

SUMMARY

1. Determination of pseudocholinesterase by the use of the selective substrates butyrylcholine or benzoylcholine leads to high results when acetylcholinesterase is present in excess (as in rat brain).

2. The determination of pseudocholinesterase with butyrylcholine as substrate is improved by using, in addition, a selective inhibitor, '62C47', for acetylcholinesterase.

3. Similarly, the determination of acetylcholinesterase in the presence of excess of pseudocholin-

esterase could be improved by using a selective inhibitor, Lysivane methosulphate, for pseudocholinesterase.

4. The activities of acetyl- and pseudo-cholinesterases of rat brain are not significantly altered by post-mortem perfusion.

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