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STABILITY STUDIES ON CRUDE AND PURIFIED HORSE SERUM CHOLINESTERASE

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Abstract—The stability characteristics of purified and serum preparations of horse and human pseudocholinesterase were examined. Light, oxygen, magnesium and removal of heavy metal ions was shown to have negligible effect on the activity of a purified pseudocholinesterase preparation, but stabilisation of all preparations was achieved by addition of gelatin. Two commercial preparations were found to be unsatisfactory.

IT HAS been shown by Goldstein and Doherty[1] that dilute solutions of purified pseudocholinesterase (PCE) (acylcholine acylhydrolase EC 3.1.1.8) are unstable but that low temperatures (0–4°), isotonic sodium chloride and a pH of 6 all improve stability. An increase in enzyme concentration or the addition of albumin also increases the stability of the enzyme, and these workers suggest that the loss of stability in dilute solution arises from the dissociation of a protein–protein interaction which albumin restores. It has also been reported that the addition of albumin[1] or gelatin[2] increases the hydrolytic activity of PCE but this potentiation phenomenon has not been observed by other workers, although the stabilising effect of "inert" protein is well known.[3–5]

The stability characteristics of several PCE preparations were therefore studied and factors which might influence stability examined.

METHODS AND RESULTS

Enzyme preparations

The enzyme characteristics of purified PCE prepared in this laboratory (E1) were compared with commercially available samples (E2) and native serum (E3).

E1—Two samples (a) and (b) were prepared from fresh horse blood by the method of Strelitz[5] (the last two stages being omitted), and stored as freeze-dried powders at 0°. The product isolated was that fraction precipitated between 0·54 and 0·66 per cent saturation by ammonium sulphate.

E2—Commercially available preparations were obtained from (a) Koch-Lights and (b) Serva-Entwicklungslabor v.Grothe & Co., Heidelburg.

E2a—(Batch No. 13058) was stated to be the fraction of horse serum that precipitates between 0·49 and 0·75 per cent saturation with ammonium sulphate, and had a specification of 5 units/mg. E2b—(Batch No. not stated) had a specification of 6 units/mg.

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E3—Native serum (a) horse, obtained from Wellcome Research Laboratories (Batch No. 96918) and (b) human, from blood (C.L.V.) centrifuged at 2500 r.p.m. for 10 min.

Enzyme studies

The hydrolysis and inhibitor studies were carried out at 37° using the standard Warburg manometric technique as described elsewhere.[6] Enzyme solutions were prepared such that 1·5 ml in a total volume of 3·0 ml hydrolysed approximately 6 μmole of butyrylcholine iodine (BuCh) ([S] = 3×10^{-2}M) in 30 min. Concentrated solutions (0·6 mg/ml) were prepared from each purified preparation (Solution I) and were stored at 0° until required. Solution I was diluted to give Solution II as follows:—E1a, 1:10; E1b, 1:40; E2a, 3:100; E2b, 1:25; The sera were diluted—E3a, 3:100; and E3b, 1:40.

Each preparation was characterised by using specific substrates (BuCh), acetylmethylcholine[7] and tributyrin[8]) and specific inhibitors (Nu1250* at 10^{-7}M inhibiting horse erythrocyte and serum cholinesterase 100 and 70 per cent respectively,[9] and Nu 683* at 10^{-8}M inhibiting horse serum cholinesterase completely[10]). All the preparations studied, hydrolysed only BuCh; the percentage inhibitions of this hydrolysis by Nu683 and Nu1250 were respectively:—E1a (0·06 mg/ml), 100 and 85 per cent; E1b (0·015 mg/ml), 100 and 68 per cent; E2a (0·018 mg/ml), 98 and 78 per cent. E2b was so unstable that control values upon which to base inhibitor studies could not be obtained.

The K_m values using BuCh (3×10^{-2}M) were determined from 1/V against 1/[S] Lineweaver–Burk plots, and were as follows:—E1a, $0{\cdot}8 \times 10^{-3}$M; E1b $1{\cdot}52 \times 10^{-3}$M; E2a, $3{\cdot}32 \times 10^{-3}$M; E3a, $0{\cdot}77 \times 10^{-3}$M and E3b, $0{\cdot}91 \times 10^{-3}$M. Preparation E2a exhibited the phenomenon of excess substrate inhibition ($[S]_{opt} = 0{\cdot}8 \times 10^{-2}$M) and was therefore not a typical PCE.

The data obtained indicated that the preparations studied, with the exception of E2a, contained PCE as the only choline ester hydrolysing enzyme.

Stability characteristics of the purified PCE preparations

The hydrolytic activity of Solution I was determined by diluting to the concentration of Solution II immediately before use. The rates of hydrolysis of BuCh ([S] = 3×10^{-2}M) by both Solution I and II were determined at intervals over a period of several days, and the results obtained are presented in Table 1. In each case, Solution I (concentrated) was more stable than Solution II (dilute).

A concentrated solution of E1a was stable over a long period but the dilute solution was unstable. However, the addition of gelatin (0·1%), *vide infra*, to Solution II produced a preparation as stable as Solution I. Solution I for E1b was stable when initially prepared, and the deterioration of Solution II was linear over the first 26 hr. The stability characteristics of E1b were reassessed after an interval of six months; it was found that (i) the absolute activity of both Solutions I and II had decreased by half and that (ii) Solution I retained its original stability characteristics whilst Solution II deteriorated rapidly and irregularly.

All solutions of E2a and E2b were unstable. In particular, solutions of E1b were

* Nn1250 and Nu683 were supplied by Hofmann La Roche.

so unstable that it was only possible to obtain reproducible results when gelatin was included in the system.

The horse serum (E3a) was completely stable over the period investigated and when diluted (Solution II) it deteriorated by only 4·5 per cent in 70 hr. Serum E3b was completely stable even when diluted.

TABLE 1. THE DECREASE IN HYDROLYTIC ACTIVITY OF CONCENTRATED (I) AND DILUTE (II) PCE SOLUTIONS WHEN STORED AT 0°

	Hydrolytic activity (μmole/30 min) using enzyme preparations							
Time	E1a		E1b		E1b*		E2a	
(hr)	I	II	I	II	I	II	I	II
0	5·84	5·7	6·51	6·51	3·16	3·16	6·91	6·91
3						2·50		
6				6·36				
8						2·41		
24	5·84	4·50	6·45	5·98		2·00	6·25	5·35
45	5·71	4·15				1·60		
70							5·89	3·48
75			6·31	5·40	3·08	1·51		
94	5·62	3·79						
100							5·40	2·00
121						1·20		
168	5·26	3·25	6·04	4·88	2·94	0·93	5·18	1·74
			% Activity remaining					
24	100	78	99	92	99	63	90	77
168	90	56	93	75	93	30	75	25

* Values determined six months after the initial determinations.

Investigation of possible factors affecting stability of PCE in solution

The following factors were investigated using preparation E1b with BuCh ([S] = 3×10^{-2}M) as substrate:—

(1) *Light.* A freshly prepared Solution II was divided into three parts. The hydrolytic activity of one sample (a) was determined immediately and of the other two samples (b) and (c) after a period of 3 days under a 150 watt tungsten lamp; sample (c) was contained in a blackened flask to prevent exposure to the light. The percentage activity remaining after 70 hr was 35 and 46 per cent for (b) and (c) respectively. Solutions of PCE would therefore appear to be less stable in the presence of light. However, the decrease in activity reported here corresponds to approximately 0·15 per cent per hr, and this would not constitute a significant source of error during a normal enzyme study.

(2) *Oxygen.* All solutions were saturated with and stored under CO_2 (5%) in N_2, the oxygen originally in solution being removed in the CO_2–N_2 gas stream. The hydrolytic activities of Solution I and II were determined at intervals and the activity remaining after 120 hr was found to be 94 and 50 per cent respectively. The deterioration of the dilute solution was very erratic, results being obtained which were similar to those reported in Table 1. It was concluded that the presence of oxygen does not materially effect the stability of PCE in solution.

(3) *Absence of magnesium.* Buffer solutions were prepared from which magnesium chloride was omitted. The hydrolytic activity of Solution II still deteriorated in a rapid and erratic manner.

(4) "*Heavy metals*". Complete removal of trace heavy metal impurities from aqueous solutions can be achieved by treatment with 8-hydroxyquinoline (oxine),[11, 12] and a modification of the method of Waring[11] was used in the present study. Chloroform (5ml) containing oxine (250μg) was added to the buffer solution (100 ml). The mixture was shaken vigorously and the aqueous layer extracted with chloroform (3 $\times$ 30 ml) to remove any dissolved oxine. The addition and extraction of oxine was repeated five times and finally the aqueous layer was extracted with chloroform (6 $\times$ 40 ml). The absence of oxine from the aqueous layer was checked spectrophotometrically—oxine has an absorption band at 240 mμ. Traces of chloroform were removed by gassing the buffer solution for 1 hr with CO_2 (5%) in N_2.

The hydrolytic activity of E1b was determined using solutions prepared in (a) standard, and (b) oxine treated buffer; the corresponding buffer being used for all solutions required in each determination. The initial activity of PCE in oxine treated buffer was 42 per cent higher than when prepared in untreated buffer, and the activity remaining after 80 hr was 67 and 43 per cent for preparations (a) and (b) respectively. The initial potentiation produced by using an oxine treated buffer was not standard and the use of such a buffer decreased rather than increased the stability of the enzyme solution.

(5) "*Inert*" *protein.* Gelatin (0·1%) was added to a freshly prepared Solution II of each PCE preparation studied, to give Solution III (in the case of E1b, a solution was also prepared containing 0·1% albumin). The hydrolytic activity of solution III was determined after 0, 24 and 168 hr and the results are shown in Table 2. The

TABLE 2. THE EFFECT OF THE ADDITION OF GELATIN (0·1%) ON THE ACTIVITY AND STABILITY OF DILUTE PCE SOLUTIONS (SOLUTION III)

Enzyme preparation	Initial activity (μmole/30 min)	Potentiation (in %)	% activity remaining 24 hr	168 hr
E1a	5·93	0	99	91
E1b	7·18	10	99	93
	5·00‡	58	98	86
	6·78*	114	96	78
	7·81†	146	90	70
E2a	9·15	32	96	91
E2b	9·68	—	99	97
E3a	6·83	0	100	99
E3b	6·92	0	100	100

* Results obtained using a different batch of gelatin.
† Results obtained using albumin instead of gelatin.
‡ Results obtained six months after the initial determination.

percentage potentiation was calculated from the activity of the corresponding Solution II, and for comparative purposes the percentage activity remaining in Solution I after 24 and 168 hr is included. The deterioration of enzymic activity in the presence of added protein was in all cases similar to that observed for the concentrated solution.

DISCUSSION

Loss of stability on dilution is typical of many proteins including PCE.[1, 5] Light appeared to increase slightly the rate at which hydrolytic activity of a dilute PCE solution decreases but activity was apparently uninfluenced by the presence of oxygen or magnesium. Many heavy metals are non-specific enzyme poisons[13] and trace contamination with heavy metals can cause a reduction in enzymic activity.[14, 15] Since many proteins, including gelatin, are capable of binding metal ions,[16, 17] the stabilising and potentiating effects of added inert protein could be the result of the removal of such ions from the system. The treatment with oxine which is a metal complexing agent, was designed to test this possibility; there was no significant improvement in enzyme stability in systems from which trace heavy metal impurities had been removed, but initial activation was observed. The stabilising effect of gelatin cannot therefore, be due to its ability to absorb metal ions but must be a result of a protein interaction. The potentiation and stabilisation produced by addition of gelatin to dilute PCE solutions appeared to be occurring as independent phenomena since a quantitative relationship could not be established between them. Both the sera and the concentrated PCE solutions studied were of excellent stability.

All the preparations studied differed in both reaction and stability characteristics. It has been shown[18] that, in order to produce enzyme preparations having reproducible characteristics, rigid controls must be enforced at each stage of the preparative procedure. The present study has shown that it is essential to determine both kinetic and stability characteristics of a PCE preparation before an experimental investigation is undertaken.

REFERENCES

1. A. Goldstein and M. E. Doherty, *Arch. Biochem. Biophys.* **33**, 22 (1951).
2. L. H. Easson and E. Stedman, *Proc. R. Soc. (B)*, **121**, 142 (1936).
3. P. J. Moloney and E. M. Taylor, *Trans. R. Soc. Canada*, **25**, 149 (1931).
4. A. A. Scott and J. R. Mendive, *J. biol. Chem.* **139**, 661 (1961).
5. F. Strelitz, *Biochem. J.* **38**, 86 (1944).
6. A. H. Beckett, N. J. Harper and J. W. Clitherow, *J. Pharm. Pharmacol.* **15**, 362 (1963).
7. G. A. Alles and R. C. Hawes, *J. biol. Chem.* **133**, 375 (1940).
8. H. Blashcko and P. Holton, *Br. J. Pharmacol.* **4**, 181 (1949).
9. R. D. Hawkins and J. M. Gunter, *Biochem. J.* **40**, 192 (1946).
10. R. D. Hawkins and B. Mendel, *Biochem. J.* **44**, 260 (1949).
11. W. S. Waring and C. H. Werkmann, *Arch. Biochem.* **1**, 303 (1962).
12. A. A. Vahora, Ph.D.Thesis, Univ. of London (1957).
13. J. L. Webb, in *Enzymes and Metabolic Inhibitors*, Academic Press, New York (1963).
14. E. S. G. Barron and S. Levine, *Arch. Biochem. Biophys.* **41**, 175 (1952).
15. F. J. Hoch, R. G. Martin, W. E. C. Wacker and B. L. Valles, *Arch. Biochem. Biophys.* **91**, 166 (1960).
16. W. Grossman and D. Kusch, Hoppe-Seyl, *Z. Physiol. Chem.* **290**, 216 (1952).
17. G. H. Wolff and S. Fallab, *Helv. Chim. Acta.* **39**, 837 (1956).
18. E. J. Cohn, L. E. Strong, W. L. Hughes, D. J. Melford, J. N. Ashworth, M. Melin and H. L. Taylor, *J. Am. Chem. Soc.* **68**, 459 (1946).