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INACTIVATION OF ACETYLCHOLINESTERASE BY PROPANOL AND SODIUM DODECYL SULPHATE

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Summary

Departure from first-order kinetics was observed for the inactivation of bovine erythrocyte and electric eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by *n*-propanol. This was attributed to the presence of isoenzymes in the commercial preparations, although inactivation via a two-step process cannot be eliminated. The rate of inactivation of bovine erythrocyte enzyme by propanol or sodium dodecyl sulphate was decreased by the presence of added protein and increased by Triton X-100. The presence of a substrate (acetylthiocholine) decreased the rate of inactivation of bovine erythrocyte acetylcholinesterase by sodium dodecyl sulphate, but increased the rate of inactivation by propanol. Re-interpretation of earlier data on the inactivation and reversible inhibition of acetylcholinesterase by simple aliphatic alcohols indicated that they do not bind to hydrophobic regions of the active site.

Introduction

In a recent paper, Krupka [1] described some differential effects of denaturing agents on the reaction of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) with some active site-directed compounds. However there is little published data on the irreversible inactivation of acetylcholinesterase by such denaturing agents themselves. This paper presents some aspects of the inactivation of acetylcholinesterase by *n*-propanol and sodium dodecyl sulphate, further to the observations made by Dawson and Crone [2] and Heller and Hanahan [3] respectively. In addition the previous data on the inactivation of acetylcholinesterase by aliphatic alcohols [2] has been re-examined in the light of the proposal [4] that activities (computed for Raoults' reference system), rather than concentrations, of alcohols in aqueous systems are more relevant to the study of their effects on proteins.

Materials and Methods

Bovine erythrocyte acetylcholinesterase was obtained from Sigma Chemical Co., U.S.A. as a mixture of active protein, gelatin and buffer salts. Electric eel acetylcholinesterase was obtained from Worthington Biochemical Corp., U.S.A. Both enzyme preparations were used as received, without purification. Sodium dodecyl sulphate and Triton X-100 were obtained from BDH Chemicals Ltd. and H.B. Selby and Co., Australia, respectively.

Inactivation of acetylcholinesterase was initiated by adding a solution of *n*-propanol or sodium dodecyl sulphate in 0.05 M phosphate buffer, pH 7.0, to a solution of the enzyme in the same buffer solution at 25°C. At various times, an aliquot of the solution at 25°C was diluted (at least 6-fold) with a solution of 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M phosphate buffer, pH. 8.0, to terminate the inactivation process. Residual enzymic activity was determined after addition of acetylthiocholine iodide (final concentration, 1 mM) by observing the rate of change of absorbance at 412 nm.

To study the effect of acetylthiocholine on inactivation, a solution of 5,5′-dithiobis(2-nitrobenzoic acid) (0.3 mM), acetylthiocholine (1 mM) and denaturant in 0.05 M phosphate buffer pH 7.0 at 25°C was placed in a cell in the spectrophotometer and acetylcholinesterase added. The absorbance at 412 nm was read every 15 s to 6 min, during which time no more than 6% of substrate was consumed. The concentration of denaturant was chosen such that the half-life for the inactivation was 2.5—4 min. The experiment was repeated using water in place of acetylthiocholine, and aliquots were taken for later assay as described above.

Dialfiltration of solutions of acetylcholinesterase was performed at 5—8°C with a Diaflo PM10 membrane. The cell volume was maintained at 5—10 ml, nitrogen pressure was 140 kPa, and the reservoir buffer was 0.05 M phosphate buffer, pH 7.0.

Results

Inactivation of acetylcholinesterase by n-propanol

Previous work had shown that bovine erythrocyte acetylcholinesterase is approximately 50% inactivated in 40 min in the presence of 11% propanol (v/v) at pH 7.0, 25°C [2]. In the present work, the time-course of this inactivation was investigated, with an enzyme concentration of 70 μg/ml, and the results are illustrated as a semi-logarithmic plot in Fig. 1. The graph is not linear; and this indicates that the inactivation is not a simple first-order process. When the propanol concentration was raised to 12%, however, inactivation proceeded much more rapidly and first-order kinetics were apparently followed to 10% of initial enzymic activity (Fig. 1). For five other concentrations of propanol from 10.50 to 11.75% (v/v) graphs were linear within the range of enzymic activity, 100% to 60%. The apparent first-order rate constant was calculated for each graph from linear regression analysis, and these are listed in Table I. The table shows that the rate constant is not a simple function of alcohol concentration, and this is consistent with previous qualitative observations [2] and the manner in which other proteins are denatured by simple aliphatic alcohols [5]. Inactiva-

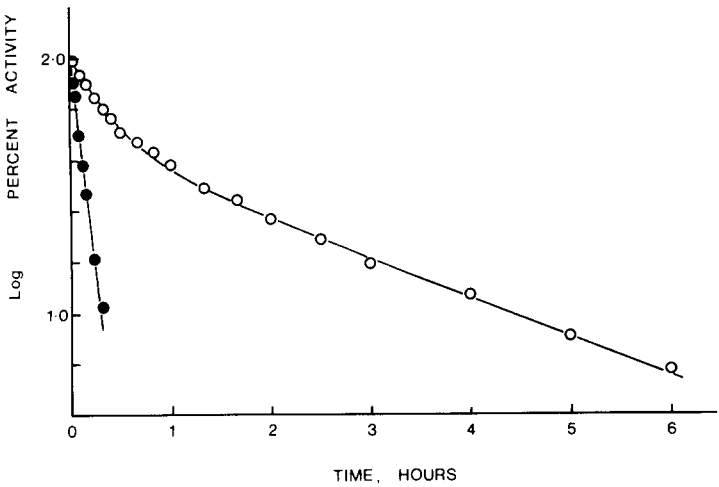

Fig. 1. Inactivation of bovine erythrocyte acetylcholinesterase by *n*-propanol at pH 7.0, 25°C in 0.05 M phosphate buffer. Enzymic activity was measured at various times as described in Materials and Methods. ○, 11% *n*-propanol (v/v); ●, 12% *n*-propanol (v/v).

tion of electric eel acetylcholinesterase (1.3 μg/ml) by 11% and 12% propanol gave similar log activity vs. time curves to those shown for bovine erythrocyte acetylcholinesterase in Fig. 1, although the deviation from first-order kinetics was not as pronounced.

The inactivation of bovine erythrocyte acetylcholinesterase (65 μg/ml) by propanol (11% or 11.6%, v/v) was then studied over the interval in which the initial inactivation is linear. Table II illustrates the effect of bovine serum albumin (650 μg/ml), Triton X-100 (65 μg/ml), substrate (acetylthiocholine, 1 mM) or a 10-fold increase in the concentration of enzyme on the first-order rate constant for inactivation. The rate constant was reduced by factors up to 4-fold by albumin or increasing the concentration of enzyme, and increased by a similar extent by Triton X-100 or substrate. No inactivation in the presence of Triton X-100 alone (65 μg/ml) was observed.

TABLE I

Apparent first-order rate constants (k) for the inactivation of bovine erythrocyte acetylcholinesterase by *n*-propanol, pH 7.0, 25°C.

Propanol concentration (vol.%)	$10^3 \cdot k$ (min^{-1})
10.50	1.5
10.75	3.6
11.00	8.9
11.25	22.7
11.50	44.7
11.75	70.7
12.00	123.2

TABLE II

Effect of varying reaction conditions on the apparent first-order rate constant (k) for the inactivation of bovine erythrocyte acetylcholinesterase by *n*-propanol, pH 7.0, 25°C. The value of k in the presence of acetylthiocholine (1 mM) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; 0.25 mM) is relative to the value of k in the presence of DTNB alone.

Concentration		Additive	Relative k
Propanol (vol.%)	Enzyme (μg/ml)		
11.0	65	None	1.0
11.0	650	None	0.43, 0.56, 0.69
11.0	65	Bovine serum albumin (650 μg/ml)	0.52, 0.24, 0.44
11.0	65	Triton X-100 (65 μg/ml)	2.6, 1.2, 3.6, 1.6
11.6	65	Acetylthiocholine + DTNB	1.8, 2.5, 3.9, 1.0, 2.1

Interrupted inactivation

Inactivation of diafiltered eel acetylcholinesterase (final concentration, 66 μg/ml) by 11.8% propanol was followed from 0 to 180 min. At 120 min, when the remaining free enzymic activity was 2.5%, a portion of the solution was diluted with cold phosphate buffer to stop further inactivation and immediately concentrated by ultrafiltration and diafiltered to remove propanol. No recovery of enzymic activity was observed on diafiltration, nor on incubation of some of the diafiltered solution at 25°C for 24 h. The inactivation is therefore irreversible or not readily reversible. The rest of the diafiltered enzyme solution was diluted and treated with propanol such that the final concentration of propanol and enzyme (active + inactive) was the same as that before interruption of the initial inactivation. First-order rate constants (k) for inactivation were derived by linear regression from linear sections of the log activity vs. time curves from both experiments and were as follows. The 95% confidence limits, and the number of points (n) for the regression are also listed:

	k (min^{-1})	n
Initial inactivation		
0—60 min	$k_1 = (3.46 \pm 0.22) \cdot 10^{-2}$	9
120—160 min	$k_2 = (2.02 \pm 0.23) \cdot 10^{-2}$	7
Inactivation of 97.5% inactivated enzyme		
0—40 min	$k_3 = (2.19 \pm 0.22) \cdot 10^{-2}$	8

It can be seen that $k_3 = k_2$, and $k_3 \neq k_1$. Inactivation of partially inactivated enzyme therefore proceeds at the same rate as if the inactivation had not been interrupted, rather than at the same rate as the fully active enzyme. Similar results were obtained when the experiment was repeated twice. The inactivation of bovine erythrocyte acetylcholinesterase was also shown to be irreversible, but the other experiments reported above could not be repeated on this enzyme due to its unreliable stability after partial inactivation and diafiltration.

Inactivation of acetylcholinesterase by sodium dodecyl sulphate

Inactivation of bovine erythrocyte acetylcholinesterase at pH 7.0, 25°C was studied for five concentrations of sodium dodecyl sulphate from 0.005% to

TABLE III

Apparent first-order rate constants (k) for the inactivation of bovine erythrocyte acetylcholinesterase by sodium dodecyl sulphate, pH 7.0, 25°C.

Concentration of sodium dodecyl sulphate (μg/ml)	$10^3 \cdot k$ (min^{-1})
50	1.0
55	4.6
60	14.2
65	27.2
70	45.9

0.007% (w/v). Plots of log (enzymic activity) versus time were linear for activities from 100% to 10%, i.e. in no case was a departure from first-order kinetics observed. The apparent first-order rate constants as a function of the concentration of sodium dodecyl sulphate are given in Table III. As in the case of propanol (above), the rate constant is not a simple function of detergent concentration.

Triton X-100 (32 μg/ml) increased the rate constant for inactivation of acetylcholinesterase (65 μg/ml) by 0.005% sodium dodecyl sulphate by factors of 9—15. On the other hand, the rate of inactivation by sodium dodecyl sulphate (0.0065% or higher concentration) was reduced by bovine serum albumin (650 μg/ml; 13—22-fold) or acetylthiocholine (1 mM; 2.4—4.4-fold) or by increasing the concentration of enzyme to 650 μg/ml (9—10-fold). The details are given in Table IV.

Re-examination of previous data

Dawson and Crone recently reported that the concentration at which several simple aliphatic alcohols inactivated bovine erythrocyte acetylcholinesterase was a function of their hydrophobicity, as reflected by their octanol-water

TABLE IV

Effect of varying reaction conditions on the apparent first-order rate constant (k) for the inactivation of bovine erythrocyte acetylcholinesterase by sodium dodecyl sulphate, pH 7.0, 25°C. Three sets of comparative figures are given. DTNB = 5,5′-dithiobis(2-nitrobenzoic acid).

Concentration		Additive	Relative k
Sodium dodecyl sulphate (μg/ml)	Enzyme (μg/ml)		
65	65	None	1.0
65	650	None	0.097, 0.110, 0.137
65	65	Bovine serum albumin (650 μg/ml)	0.069, 0.045, 0.076
50	65	None	1.0
50	65	Triton X-100 (32.5 μg/ml)	9.1, 14.8, 9.6
86	65	DTNB (0.25 mM)	1.0
86	65	Acetylthiocholine (1 mM) + DTNB (0.25 mM)	0.41, 0.23, 0.33, 0.25, 0.24

TABLE V

Effect of aliphatic alcohols on bovine erythrocyte acetylcholinesterase, pH 7.0, 25°C. D_{50} = concentration of alcohol required to cause 50% inactivation of acetylcholinesterase in 40 min [2]. pD_{50} = negative logarithm of D_{50}, expressed in mol/1. I_{50} = concentration of alcohol required to inhibit hydrolysis of 0.15 mM acetylcholine by acetylcholinesterase by 50% [2]. pI_{50} = negative logarithm of I_{50}, expressed in mol/1. log p_{oct} = logarithm of the octanol/water partition coefficient [2]. Activity = activity according to the calculations described by Schlusselberg and Paredes [4] and applying to the particular concentration of alcohol (D_{50} or I_{50}) shown.

Alcohol	D_{50} (vol.%)	pD_{50}	Activity (mole fraction)	I_{50} (vol.%)	pI_{50}	Activity (mole fraction)	Log P_{oct}
Methanol	37	—0.96	0.26	9.6	—0.37	0.066	—0.74
Ethanol	24	—0.61	0.24	4.4	0.12	0.045	—0.32
n-Propanol	12	—0.19	0.27	1.8	0.63	0.047	0.34
n-Butanol	5.5	0.22	0.39	1.4	0.83	0.113	0.88
n-Pentanol	2.1	0.71	0.53	0.9	1.09	0.240	1.40

partition coefficient [2]. Reversible inhibition of the enzyme by the alcohols also appeared to be a function of hydrophobicity. Since then Schlusselberg and Paredes have claimed that conversion of concentration into activity (using Raoults' reference system) gives a more realistic picture of this hydrophobic interaction of an alcohol with a protein [4]. The data of Dawson and Crone [2] for the five *n*-alcohols, and the relevant calculated activities of these alcohols [4] are given in Table V. When activities are considered, both inactivation and reversible inhibition of acetylcholinesterase are independent of the number of methylene groups (n) where n is between 1 and 3. According to Schlusselberg and Paredes, this conclusion indicates that methanol, ethanol and propanol are completely bound to hydrophobic regions of the enzyme, but for butanol and pentanol some methylene groups are not in contact with these hydrophobic sites and penetrate the solvent instead.

Discussion

The data for the inactivation of bovine erythrocyte acetylcholinesterase by 11% *n*-propanol is consistent with the following equation:

$$A = N_1 \exp(-k_1 t) + N_2 \exp(-k_2 t) \quad (1)$$

where A is the enzymic activity at time t and N_1, N_2, k_1 and k_2 are constants. This equation was found to hold for the thermal inactivation of mammalian erythrocyte acetylcholinesterase [6]. The curve drawn in Fig. 1 for 11% propanol was calculated from Eqn. 1 with:

$N_1 = 0.533$, $N_2 = 0.467$, $k_1 = 0.0444$ and $k_2 = 0.0059$.

The experimental points shown in Fig. 1 fit well to the calculated curve. Two possible explanations were offered to account for the observation that thermal inactivation of acetylcholinesterase follows Eqn. 1 above [6], and both could well apply to the present case. One is that active enzyme A_1 is transformed irreversibly into active enzyme A_2 (with rate constant k_a) and both A_1 and A_2 form inactive enzyme (with rate constants k_b and k_c respectively). This theory is consistent with the report that the denaturation of proteins by simple ali-

phatic alcohols is a two-step process [5]. Alternatively, isoenzymes or subunits of the commercial preparations of acetylcholinesterase studied above (and the mammalian erythrocyte enzyme of ref. 6) may each inactivate at different rates. The results for the interrupted inactivation of eel acetylcholinesterase (above) agree with both these explanations, but do not allow one to distinguish between them. I favour the latter proposal; kinetic inhomogeneity of both bovine erythrocyte acetylcholinesterase and electric eel acetylcholinesterase has been reported [7,8]. In the presence of sodium dodecyl sulphate, the isoenzymes and subunits of bovine erythrocyte acetylcholinesterase apparently inactivate at the same rate.

Sodium dodecyl sulphate and propanol are on the whole qualitatively similar in their mode of action on acetylcholinesterase. Both show a narrow concentration range separating comparative enzyme stability from rapid inactivation, and for both reagents the rate of inactivation decreases with increasing enzyme concentration, is retarded by bovine serum albumin, and increased by Triton X-100. Bovine serum albumin probably protects acetylcholinesterase against inactivation by reducing the concentration of free denaturant. Similarly, increasing the concentration of crude enzyme preparation increases the amount of protein material (other than acetylcholinesterase) present, and this protein may bind sodium dodecyl sulphate or propanol, thereby reducing slightly the free concentration of the denaturant and reducing the rate of inactivation. Triton X-100 is believed to remove lipids from integral membrane proteins such as acetylcholinesterase, possibly in its monomer form (in which form it probably wholly exists at the concentrations used in the present work) [9,10]. If the soluble acetylcholinesterase from bovine erythrocytes still contains some bound lipid material, the removal of this lipid material might expose fresh binding sites of the enzyme to sodium dodecyl sulphate or propanol, and hence increase its rate of inactivation.

The presence of a substrate (acetylthiocholine) during inactivation of acetylcholinesterase by propanol or sodium dodecyl sulphate establishes one point of contrast between the effects of the two denaturing agents. Acetylthiocholine protects the enzyme against inactivation by sodium dodecyl sulphate, and this effect can be ascribed to its stabilising effect on the conformation of the active site [1]. It may also sterically hinder binding of sodium dodecyl sulphate to hydrophobic areas of the enzyme adjacent to the active site [1]. By the same reasoning, acetylthiocholine should also provide some protection against inactivation by propanol. In practice, however, acetylthiocholine caused a small acceleration of the rate of inactivation. The reason for this is not clear. However the re-examination of previous data is pertinent in this regard; it suggests that the binding site(s) for simple aliphatic alcohols are apparently capable of accommodating only three methylene groups (see Results above), whereas hydrophobic regions near the active site of acetylcholinesterase are capable of binding 4 (esteratic site) or 6—8 (anionic site) methylene groups [11,12]. Propanol may therefore bind to sites remote from the active site, and this may be related to the lack of protection provided by a substrate against propanol inactivation. Whether or not such binding sites are the same as those which bind allosteric effectors of acetylcholinesterase [13] is an interesting problem for further study.

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