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Estimation of the stability of dry horse serum cholinesterase by means of an accelerated storage test

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The thermal inactivation of dry horse serum cholinesterase was found to obey first order kinetics over the temperature range 38–140°. Rogers' (1963) accelerated storage test was applied to this reaction and gave estimates of the rate coefficients and energy of activation of the inactivation reaction which agreed with the values determined in isothermal experiments. The stability of the enzyme preparation at 38° predicted by means of the accelerated storage data coincided with that found by isothermal storage of the enzyme at 38°.

MANY biological and pharmaceutical preparations are stored in the solid state. Hitherto the stabilities of these solid preparations have been estimated empirically by relating the rate of inactivation at some arbitrary temperature, higher than that of storage, to the established stabilities of similar preparations at the elevated and storage temperatures. This type of experiment can take several weeks or months to perform and a more rapid means of estimating the stability of solid preparations would be advantageous.

In a previous report (Cole & Leadbeater, 1966) a critical assessment of the accelerated storage test, described by Rogers (1963), was made and it was confirmed that the technique could be applied successfully to reactions in solution. The object of the present work was to establish that Rogers' technique can be used to estimate the stability of solid preparations of horse serum cholinesterase [EC 3.1.1.8].

Experimental

CHOLINESTERASE

Two preparations of partially purified horse serum cholinesterase were investigated. One was a preparation made in these laboratories by a method involving chromatography on diethylaminoethyl cellulose (DEAE-cholinesterase) which hydrolyzed 0·1 μmole acetylcholine/mg enzyme/min at 25°: the other, supplied by Organon Laboratories Ltd., was obtained by ammonium sulphate fractionation of horse serum (Organon-cholinesterase) and was about 40 times more active. The enzyme preparations were stored over phosphorus pentoxide.

TECHNIQUE

Cholinesterase (10 mg) was pressed onto the bottom of a tube of 10 mm internal diameter giving a layer of enzyme about 0·1 mm thick, assuming it to be uniformly distributed over the hemispherical surface of the base of the tube. The atmosphere above the sample was replaced with nitrogen. The tubes were fitted with guard-tubes containing silica gel

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and were heated in an oil bath. At appropriate times during the experiments tubes were withdrawn from the bath and cooled in an ice-water bath. The enzyme was dissolved in water, to a final activity of 0·2 units/ml, and the cholinesterase activity determined from the rate of hydrolysis of acetylcholine, using an automatic titrimeter (Cole & Leadbeater, 1966).

TEMPERATURE CONTROL

The temperature of the oil bath was controlled by means of an Ether Programme Temperature Controller, Type 994. For the accelerated storage experiments the temperature was within $\pm 0{\cdot}5°$ of that required by the programme and in the isothermal experiments the temperature control was better than $\pm 0{\cdot}1°$.

Results

ISOTHERMAL EXPERIMENTS

The thermal inactivation of both cholinesterase preparations followed first order kinetics. Typical curves are shown in Fig. 1, in which exponential curves for first order inactivation were fitted to the experimental data

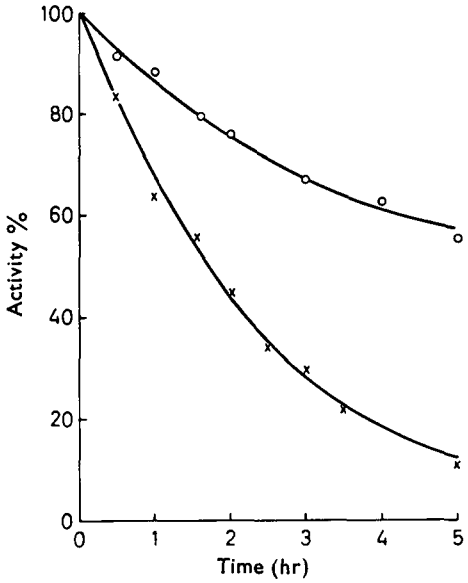

FIG. 1. The isothermal inactivation of dry cholinesterase. The points represent the experimental data for the inactivation of DEAE-cholinesterase at 120° (○) and Organon-cholinesterase at 140° (×) and the lines are the exponential curves fitted to the data.

by the method of least squares using a Ferranti Mercury computer. The exponential curves had the general form

$$\frac{a - x}{a} \cdot 100 = e^{-kt}$$

where a is the enzymic activity at zero time; x is the enzymic activity at time t; k is the rate coefficient. The average standard deviation of the points from the curves for all the isothermal data was $\pm$ 2·9.

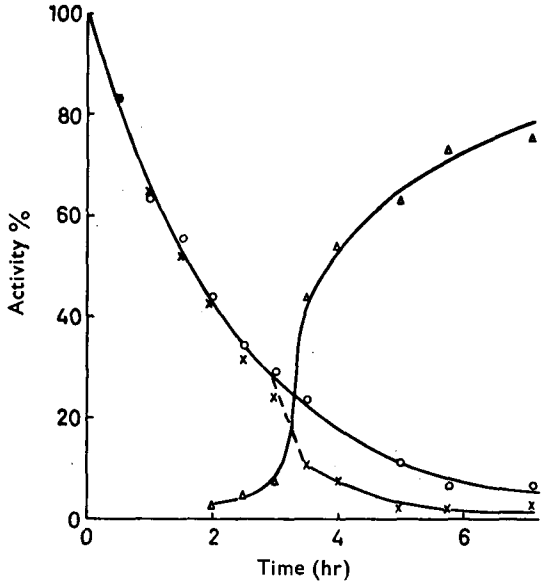

FIG. 2. The isothermal inactivation and denaturation of dry cholinesterase at 135°. The activities of the whole suspension of enzyme (○) and of the soluble fraction of the enzyme (×) are shown. The denaturation of the enzyme (△) was measured by the decrease in material absorbing at 280 mμ in the soluble fraction.

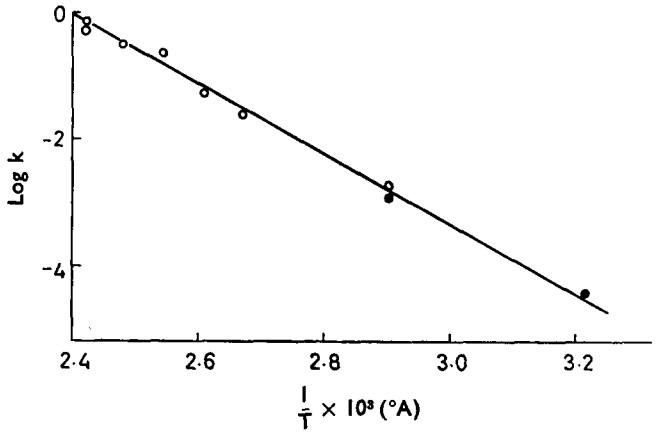

FIG. 3. The Arrhenius plot for the thermal inactivation of dry cholinesterase. The circles represent the rate coefficients determined isothermally and the line was calculated from the accelerated storage data. The solid circles represent data for the inactivation of the enzyme at 71° and 38° obtained by Mr. C. Stratford of this laboratory.

ESTIMATION OF DRY HORSE SERUM CHOLINESTERASE STABILITY

In most of the experiments the enzyme was readily soluble, but when it was more than 80% inactivated some of the protein would not dissolve. In these cases the suspension was homogenized for 10 sec using the microhead of a Silverson laboratory mixer (the enzyme was not inactivated under these conditions). The homogenate was used in the assay and smooth activity - time curves were obtained (Fig. 2). If the insoluble material was removed by centrifuging there was a break in the curve which corresponded with the point of rapid denaturation of the enzyme. Denaturation was measured by the decrease in the material absorbing at 280 mμ in the supernatent solution after centrifuging.

The effect of temperature on the rate of inactivation of DEAE-cholinesterase is shown in Fig. 3. The inactivation reaction obeyed the Arrhenius equation over the temperature range (140–71°) used in this series of experiments. Data obtained at lower temperatures (71 and 38°) by other workers in this laboratory were in excellent agreement with the present data (Fig. 3).

ACCELERATED STORAGE EXPERIMENTS

The enzyme was equilibrated at 110° and then the temperature was raised to 150° over a period of 6 hr according to the following program:

$$\frac{1}{T_o} - \frac{1}{T_t} = 2{\cdot}303.\ B.\log(1 + t)$$

Where $T_o = 383{\cdot}2°\ A$: $B = 2{\cdot}92 \times 10^{-4}$.
The Rogers' equation for the inactivation of cholinesterase, assuming first order kinetics, is shown below.

$$\log\left[2{\cdot}303.\log\left(\frac{a}{a-x}\right)\right] = \log k_{110} - \log\left(1 + \frac{EB}{R}\right) + \left(1 + \frac{EB}{R}\right)\log(1 + t) + \log\left[1 - \left(\frac{k_{110}}{k_t}\right)^{1 + \frac{R}{EB}}\right]$$

where E is the energy of activation; k_{110} is the rate coefficient at 110°; k_t is the rate coefficient at time t.

The data obtained by applying the accelerated storage technique to the thermal inactivation of both DEAE-cholinesterase and Organon-cholinesterase are shown in Table 1, together with the isothermal data for DEAE-cholinesterase. There was good agreement between the data for the two

TABLE 1. THE THERMAL STABILITY OF DRY CHOLINESTERASE

Preparation	Method of determination	No. expts.	E (kcal mole^{-1})	$k_{110} \times 10^5$ (sec^{-1})	Half-life at 38° C (years)
DEAE-cholinesterase	Accelerated storage	6	25·1 ± 2·6	1·77 ± 0·50	2·5 ± 0·7
DEAE-cholinesterase	Isothermal	1	25·9	1·42	2·2
Organon-cholinesterase ..	Accelerated storage	4	24·7 ± 1·4	1·28 ± 0·11	3·9 ± 0·3

preparations of cholinesterase, indicating that the different methods of isolation of the enzyme from horse serum yielded products with similar stabilities.

The agreement between the accelerated storage data and the isothermal data for the inactivation of DEAE-cholinesterase was very good (Table 1). This agreement is emphasized in Fig. 3 where the line was calculated from the accelerated storage data and the points represent the individual isothermal experiments.

Discussion

The data demonstrate that, over the range 38–140°, the thermal inactivation of cholinesterase follows first order kinetics. However, the data give no indication of the nature of the reaction involved in the inactivation and it is not possible to establish whether the inactivation process is in fact a true first order reaction or a complex change in the tertiary or secondary structure of the protein which yields apparent first order kinetics.

Inactivation of the enzyme preceded its denaturation since little or no insoluble protein was detected until the enzyme was about 80% inactivated. Inactivation probably involves a relatively minor change in the configuration of the peptide chain in the vicinity of the active centre of the enzyme whereas denaturation is the result of larger changes in the secondary or tertiary structures of the molecule rendering the protein insoluble. These experiments did not demonstrate whether the insoluble protein was enzymically active. The data in Fig. 2 show that the removal of the insoluble material from the enzyme suspension resulted in a loss of enzymic activity. This could have been due to the insoluble protein having enzymic activity or to molecules of the active native enzyme being carried out of solution with the insoluble material.

The present data clearly demonstrate that the accelerated storage test can be applied to the thermal inactivation of dry cholinesterase since the data obtained by this technique were reproducible (Table 1) and in good agreement with isothermal data (Table 1, Fig. 3). Moreover, the Rogers' experiments were completed within 8 hr whereas the isothermal studies at 71° and 38° were made over periods of eleven weeks and two years respectively. This technique eliminates the necessity of maintaining a standard method of assay (both chemicals and equipment must be standardized) for a long period. In addition to the great saving in time the accelerated storage procedure provides an estimate of the energy of activation of the inactivation process. This parameter enables the Arrhenius equation to be used to calculate the stability of the enzyme at any temperature in the range 38–140°, within which the inactivation was shown to follow first order kinetics. The energy of activation may also be used as a second parameter for comparing the stability characteristics of different samples of the same preparation.

Little work on the kinetics of the thermal inactivation of dry enzymes has been reported in the literature. In their review, Eyring & Stearn

(1939) quote papers by Tamman (1895) and Nicloux (1905) who showed respectively that the inactivation of dry preparations of emulsin and lipase followed first order kinetics. More recently, Mullaney (1966) demonstrated that the thermal inactivation of both trypsin and ribonuclease were first order processes. This work suggests that Rogers' accelerated storage test may have a general application in the estimation of the thermal stabilities of dry preparations of enzymes although, so far, it has only been applied to the inactivation of cholinesterase.

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