J. Pharm. Pharmac., 1966, **18**, 101–111 Received July 21, 1965

A critical assessment of an accelerated storage test

B. R. COLE AND L. LEADBEATER

The accelerated storage test proposed by Rogers enables the rate constant (at a known temperature) and the energy of activation of a reaction occurring in solution to be calculated from a single experiment. This test has been applied to a number of first and second order reactions, including the heat inactivation of horse serum cholinesterase. The theoretical and practical limitations of the technique are discussed. It is concluded that the test has an important role in determining the stability of pharmaceutical and related compounds in solution and in comparing the stabilities of different batches of the same preparation.

AN accelerated storage test, which enables the rate constant of a reaction at a known temperature, and the energy of activation of a reaction, to be calculated from data obtained in a single experiment, has been described by Rogers (1963). The novelty of the technique is that the temperature is increased with time in the following way

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

Where T_o is the temperature (°K) at the start of the experiment, T_t is the temperature (°K) at time t, and B is a constant which determines the rate of rise of the temperature.

By substituting this equation into the Arrhenius equation and assuming an order of reaction it may be shown that

$$\log f(c) = \log k_o - \log\left(1 + \frac{E_A B}{R}\right) + \left(1 + \frac{E_A B}{R}\right) \log (1 + t) + \log\left[1 - \left(\frac{k_o}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$$

where f(c) is a function of the concentration of the reactants; k_o is the rate constant at temperature T_o; k_t is the rate constant at temperature T_t; E_A is the energy of activation; and R is the gas constant (1·987 cal. degree^{-1} mole^{-1}).

For first order reactions $f(c) = 2{\cdot}303 \log \frac{c_o}{c_t}$

for second order reactions $f(c) = \frac{1}{c_t} - \frac{1}{c_o}$

or $\frac{2{\cdot}303}{a_o - b_o} \log \frac{a_t}{b_t} + \frac{2{\cdot}303}{a_o - b_o} \log \frac{b_o}{a_o}$

where a_o and b_o are the concentrations of the reactants at the beginning of the experiment and a_t and b_t their concentrations at time t.

The value of the final term of the equation rapidly tends to zero as k_t becomes greater than k_o. Thus, a graph of log f(c) against log (1 + t) will

From the Ministry of Defence, Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wiltshire

be a straight line. From the slope of the line the energy of activation may be calculated and the rate constant (k_o) may then be calculated from the intercept when $\log (1 + t) = 0$.

Rogers suggested that this accelerated storage test has advantages over the conventional methods for determining stabilities of pharmaceutical preparations (Garrett, 1962). These were: (1) the data required to calculate the stability of the compound are obtained in a single experiment lasting one day rather than in a series of experiments which may last for several weeks; (2) no preliminary experiments are required to determine the optimum temperatures for the accelerated storage test; (3) the linearity of the plot of log f(c) against log (1 + t) confirms that the correct order of reaction for the decomposition has been assumed. Deviations from linearity may indicate that the wrong order of reaction has been chosen or that the mechanism of the reaction changes as the temperature is raised.

Since this accelerated storage test could be of great use in rapidly providing data for the stabilities of compounds in solution, the technique has been investigated both theoretically and experimentally.

Experimental

TEMPERATURE CONTROL

The temperature of a bath containing 17·5 litres of oil was raised, at the rate required by the programme, by the continuous manual adjustment of a variable transformer connected to a 2 kW heater. At any given instant the temperature of the bath was within less than 1° of that required by the programme. Typical programmes are shown in Fig. 1.

EXPERIMENTAL TECHNIQUE

The solution to be tested was divided amongst an appropriate number of tubes with ground glass stoppers. The tubes were placed in the bath and brought to the initial temperature before the temperature programme was commenced. At appropriate times during the experiments tubes were withdrawn and chilled in an ice-water bath.

METHODS OF ASSAY

Hydrolysis of sucrose. The reaction was followed polarimetrically as described by Rogers (1963).

*Solvolysis of methyl toluene-*p*-sulphonate.* A modification of the method of Robertson (1953) was used. The ester concentration was 10^{-2}M and the sulphonic acid produced was titrated with $7{\cdot}5 \times 10^{-3}$M sodium hydroxide using B.D.H. "4·5" indicator in the case of the 50% (v/v) ethanol-water solutions and lacmoid indicator for the 50% (w/w) dioxane-water solutions.

Hydrolysis of ethyl benzoate. The reaction was followed by a modification of the method described by Ingold & Nathan (1936). Equal volumes of 0·05M ethyl benzoate and 0·05M sodium hydroxide were mixed together and 10 ml samples pipetted into the stoppered tubes. Immediately after cooling the samples, excess hydrochloric acid (5 ml, 0·06M) was added.

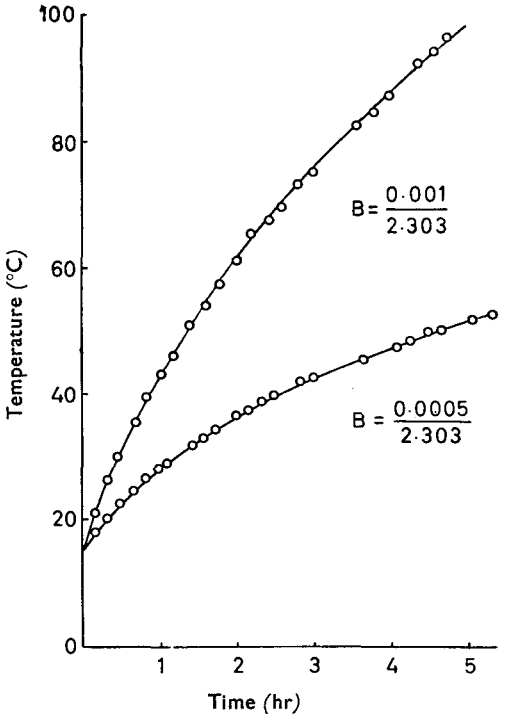

FIG. 1. Temperature programmes. The temperature was raised so that $\left(0{\cdot}00347 - \frac{1}{T_t}\right) = 2{\cdot}303.B.\log(1 + t)$. The points represent the temperature of the sample at the times indicated and the lines the required programme.

Carbon tetrachloride (1 ml) was added to remove the unhydrolysed ester from the aqueous phase and the solution titrated, under nitrogen, with 0·01M sodium hydroxide using bromothymol blue indicator.

Decomposition of N-*methyl pyridinium-2-aldoxime methane sulphonate* (*P2S*). The disappearance of the oxime group was determined by measuring the decrease in the extinction at 335 mμ of the P2S in alkaline solution (Creasey & Green, 1959).

Cholinesterase activity. 15 ml of 0·9% (w/v) saline and 1 ml of acetylcholine chloride (100 mg/ml) were pipetted into a titration cell. The pH was adjusted to 7·40 and the temperature was maintained at 25·00 $\pm$0·05°. 1 ml of the cholinesterase (from horse serum) solution was added and the acetic acid produced by the enzymic hydrolysis of the acetylcholine was titrated with 0·0125M sodium hydroxide, under nitrogen, at pH 7·40, using a Radiometer automatic titrimeter.

The standard deviations of the data are presented in Tables 1 and 2 to present an estimate of the reproducibility of the accelerated storage test.

Results

VALIDITY OF THE TECHNIQUE

Rate constants and energies of activation for the following reactions were determined by the accelerated storage technique and compared with data published in the literature: (*a*) the hydrolysis of sucrose, 40% (w/v), by 0·167N hydrochloric acid; (*b*) the solvolysis of methyl toluene-*p*-sulphonate (10^{-2}M) in 50% (v/v) ethanol-water and in 50% (w/w) dioxane-water; (*c*) the hydrolysis of ethyl benzoate ($2{\cdot}5 \times 10^{-2}$M) by sodium hydroxide ($2{\cdot}5 \times 10^{-2}$M) in 50% (v/v) and in 85% (w/w) ethanol-water.

The data are summarised in Table 1. The rate constants for the solvolysis of methyl toluene-*p*-sulphonate were determined at 40° and were calculated for 50° by means of the Arrhenius equation. Similarly the rate constants for the hydrolysis of ethyl benzoate were determined at 20° and calculated for 25°.

THE STABILITY OF P2S IN SOLUTION

The decomposition of P2S in dilute aqueous solution (3×10^{-5} M) was shown to follow first order kinetics, since log c_0/c_t varied linearly with time when the solution was maintained at 60° and 71° and the first order equation for Rogers' test gave a straight line (Fig. 2).

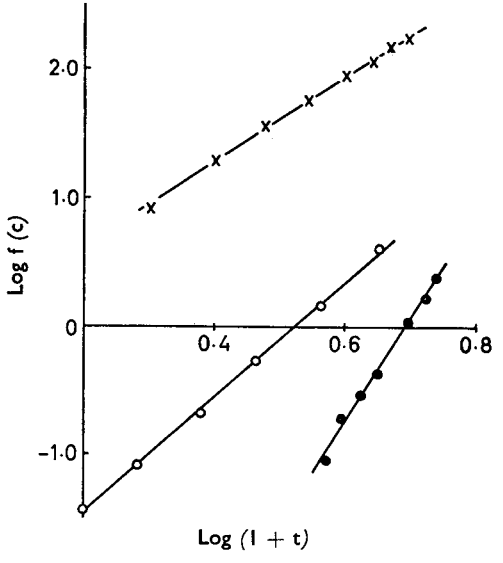

FIG. 2. Plots derived from Rogers' equation for the alkaline hydrolysis of ethyl benzoate (×), the decomposition of P2S in phosphate buffer (○) and the thermal inactivation of horse serum cholinesterase (●).

The stability of P2S was determined both in 0·0485M phosphate buffer, pH 7·38, and in unbuffered aqueous solution. The pH of the latter solution was 5·80 and was unchanged at the end of the reaction. The results are shown in Table 2.

TABLE 1. THE HYDROLYSIS OF SUCROSE, METHYL TOLUENE-*p*-SULPHONATE AND ETHYL BENZOATE

Reaction	No. Expts.	Rate constant	Energy of activation kcal mole^{-1}	Method of determination	Reference
Hydrolysis of sucrose in 0·167N HCl	3	$k_{20} = 6{\cdot}90 \pm 2{\cdot}82 \times 10^{-5}$	26·9 ± 1·6	Accelerated storage	—
Hydrolysis of sucrose in 0·167N HCl	1	$k_{20} = 3{\cdot}49 \times 10^{-5}$	25·8	Accelerated storage	Rogers (1963)
Hydrolysis of sucrose in 0·00167N HCl	1	$k_{20} = 3{\cdot}66 \times 10^{-5}$	27·6	Accelerated storage	Rogers (1963)
Hydrolysis of sucrose in 0·19N HCl	—	$k_{20} = 7{\cdot}11 \times 10^{-5}$ (litre mole^{-1} sec^{-1})	25·4	Isothermal storage	Moelwyn-Hughes (1934)
Solvolysis of methyl toluene-*p*-sulphonate in 50% (v/v) ethanol-water	4	$k_{50} = 4{\cdot}22 \pm 0{\cdot}21 \times 10^{-5}$	20·1 ± 1·6	Accelerated storage	—
	—	$k_{50} = 4{\cdot}41 \times 10^{-5}$ (sec^{-1})	—	Isothermal storage	Robertson (1953)
Solvolysis of methyl toluene-*p*-sulphonate in 50% (w/w) dioxane-water	3	$k_{50} = 1{\cdot}91 \pm 0{\cdot}12 \times 10^{-5}$	19·7 ± 0·2	Accelerated storage	—
	—	$k_{50} = 2{\cdot}29 \times 10^{-5}$ (sec^{-1})	22·1	Isothermal storage	Robertson (1953)
Alkaline hydrolysis of ethyl benzoate in 50% (v/v) ethanol-water	3	$k_{25} = 1{\cdot}27 \pm 0{\cdot}08 \times 10^{-3}$	14·8 ± 0·4	Accelerated storage	—
	—	$k_{25} = 1{\cdot}33 \times 10^{-3}$ (litre mole^{-1} sec^{-1})	—	Isothermal storage	Ingold & Nathan (1936)
Alkaline hydrolysis of ethyl benzoate in 85% (w/v) ethanol-water	3	$k_{25} = 5{\cdot}90 \pm 0{\cdot}66 \times 10^{-4}$	17·0 ± 1·1	Accelerated storage	—
	—	$k_{25} = 5{\cdot}5 \times 10^{-4}$ (litre mole^{-1} sec^{-1})	17·7	Isothermal storage	Ingold & Nathan (1936)

TABLE 2. THE STABILITY OF P2S IN DILUTE AQUEOUS SOLUTION

Solution		No. Expts.	k_{15} (sec^{-1})	Energy of activation (kcal mole^{-1})	Half-life at 15° (years)	Method
pH 7·38	0·0485M phosphate	4	$7{\cdot}55 \pm 2{\cdot}75 \times 10^{-9}$	34·15 ± 1·14	2·9 ± 0·8	Accelerated storage
		3	$9{\cdot}61 \pm 4{\cdot}07 \times 10^{-9}$	33·51 ± 2·35	2·3 ± 1·0	Isothermal storage at 60° and 71°
	0·01M phosphate	—	$6{\cdot}8 \times 10^{-9}$	32	3·2	Isothermal storage*
pH 5·8	Water unbuffered	4	$5{\cdot}28 \pm 2{\cdot}55 \times 10^{-11}$	43·34 ± 2·38	416 ± 163	Accelerated storage

* Data interpolated from the data of Fan, Nairn & Walker (1964).

THE THERMAL STABILITY OF CHOLINESTERASE

Horse serum cholinesterase which had been purified eighteenfold by chromatography on diethylaminoethylcellulose and by gel filtration through Sephadex was employed. The enzyme was dissolved either in carbon dioxide-free water, when the final pH was 7·35, or in 0·0056M veronal-acetate buffer, pH 7·40 (Michaelis, 1931) at a concentration of 2 mg protein per ml. The inactivation was assumed to be first order and good straight lines were obtained (Fig. 2). The data obtained from two preparations of cholinesterase are shown in Table 3.

TABLE 3. THERMAL STABILITY OF HORSE SERUM CHOLINESTERASE IN AQUEOUS SOLUTION

Preparation number	No. assays	Solution	E kcal mole^{-1}	k_{25} sec^{-1}
A	2	H_2O	84·4	$2·6 \times 10^{-10}$
X12	2	H_2O	66·9	$8·1 \times 10^{-9}$
X12	2	Veronal-acetate	75·8	$4·8 \times 10^{-9}$

The half-life of preparation X12, dissolved in water, was calculated for various temperatures, from the data obtained by Rogers' technique, and the results compared with direct observations made on the enzyme solution stored at those temperatures (Table 4).

TABLE 4. A COMPARISON OF THE STABILITY OF HORSE SERUM CHOLINESTERASE AT VARIOUS TEMPERATURES DETERMINED BY ISOTHERMAL STORAGE AND CALCULATED FROM DATA OBTAINED IN THE ROGERS ACCELERATED STORAGE TEST

Half life $t_{\frac{1}{2}}$	Temperature °C			
	30	50	60	71
Determined	>> 70 hr	3·5 hr	4·6 min	21 sec
Calculated	76 days	3·75 hr	9·8 min	19 sec

The half-life of the preparation was not determined at 30° since no special precautions were taken to ensure that the solution was aseptic. No loss of activity was detected over 70 hr but inactivation after that length of time could have been produced by bacterial invasion of the solution.

Discussion

LIMITATIONS OF THE ARRHENIUS EQUATION

The accelerated storage test is based on the Arrhenius equation which relates the rate constant of a reaction with temperature, thus:

$$\log k_t = \log k_o + \frac{E_A}{2·303\,R}\left(\frac{1}{T_o} - \frac{1}{T_t}\right)$$

This equation is an approximation but nevertheless it permits the calculation of rate constants, over a range of temperatures, which are in reasonable agreement with those determined experimentally (Moelwyn-Hughes,

1947) and it has been widely used as a basis for accelerated storage tests (Garrett, 1962).

In Rogers' equation the slope of the line obtained, which is equal to $(1 + E_A B/R)$, is not constant but changes as the energy of activation varies. However, this change in slope has not been detected in any of the experiments reported in this paper (see Figs 2 and 3), even though they were made over a temperature range of at least 40°. The value of the activation energy determined by this method is an average value for the temperature range employed.

There are a number of reactions to which the Arrhenius equation does not apply (Moelwyn-Hughes, 1947). Thus accelerated storage tests based on the equation, either the test devised by Rogers or the conventional method of a series of isothermal experiments, are not of universal application. However, in Rogers' method, deviations from the Arrhenius equation would be detected as a change in slope of the line.

The mechanism of a decomposition may change as the temperature is raised. The change can be detected by Rogers' technique provided it involves a change in the energy of activation or order of reaction. Even slight changes in the energy of activation could be detected since Rogers' equation gives good linear plots (see Figs 2 and 3).

LIMITATION OF ROGERS' EQUATION

In the accelerated storage test it must be assumed that the final term in Rogers' equation, namely,

$$\log\left[1 - \left(\frac{k_o}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$$

rapidly tends to zero and can be ignored. It is essential to establish the conditions when this assumption is valid.

The values of the expression have been calculated for a hypothetical first order reaction. The rate constant for the reaction at 15° was assumed to be 10^{-4} hr^{-1} and the energy of activation 20·0 kcal $mole^{-1}$. The temperature was raised from 15° according to the following programme:

$$\frac{1}{288{\cdot}2} - \frac{1}{T_t} = 0{\cdot}001 \log (1 + t).$$

The values of the expression are recorded in Table 5. It may be seen that the expression rapidly tends to zero and within a temperature rise of 10° it is less than 2% of the value of

$$\left[\log 2{\cdot}303 \log \frac{c_o}{c_t}\right]$$

Thus it is safe to neglect this term provided that data are not used which were obtained within 10° of the starting temperature. For reactions with activation energies less than 20 kcal $mole^{-1}$ the value of the factor would be slightly higher and a slightly larger temperature interval would be required before data could be used. This gap between the initial

TABLE 5. THE RATE OF DECREASE OF $\log \left[1 - \left(\frac{k_o}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$ WITH RISE IN TEMPERATURE

Equation	Temperature °C						
	17·5	20·0	22·5	25·0	30·0	40·0	60·0
$\log \left[1 - \left(\frac{k_o}{k_t}\right)^{1 + \frac{R}{E_A B}}\right]$	−0·509	−0·284	−0·177	−0·067	−0·055	−0·012	−0·001
$\log \left[2{\cdot}303.\ \log \frac{c_o}{c_t}\right]$	−4·570	−4·412	−4·257	−4·105	−3·808	−3·244	−2·212

temperature of the first observation which can be employed is normally unavoidable for relatively stable compounds where no detectable decomposition occurs until the temperature has been raised to 40–60°.

It is inherent in the method of Rogers that the error in the estimate of the rate constant (k_o) must be large since it is calculated from the following expression:

$$\log k_o - \log (\text{Slope}) = \text{Intercept}$$
$$[\log (1 + t) = 0]$$

Thus the integer of the intercept determines the characteristic of log k_o and all the error in the estimate of the intercept, together with the error in the estimate of the slope, appears in the mantissa of log k. For example, if the intercept is −5·80 and the slope is 2·00 and the error in the estimates of these values is ±1% then the error in the estimate of k_o is not ±1% but either ±15% ($k_o = 3{\cdot}17 \pm 0{\cdot}47 \times 10^{-6}$) or ±13% ($k_o = 3{\cdot}17 \pm 0{\cdot}41 \times 10^{-6}$), depending on whether or not the errors in the estimates of the slope and intercept are additive.

REQUIREMENTS FOR THE APPLICATION OF ROGERS' ACCELERATED STORAGE TECHNIQUE

(*a*) The reaction to be studied must occur in a homogeneous liquid or gaseous phase since the theory on which the method is based is only applicable to reactions occurring under these conditions.

(*b*) A method of assay specific either to the original compound or to one of its decomposition products must be available in order to follow the decomposition. In the latter instance the decomposition product must be stable under the conditions of the accelerated storage test.

(*c*) Before the energy of activation and the rate constant can be calculated by Rogers' equation the order of the reaction must be established. This may be found by substituting the experimental data into the zero, first, second, etc., forms of the equation. The equation which gives the best straight line allows the order of the reaction to be determined. This approach is illustrated in Fig. 3 where the data for the acid catalysed hydrolysis of sucrose are plotted as zero, first and second order reactions with respect to the sucrose concentration. If the decomposition is followed to completion, as in Fig. 3, there is no difficulty in deciding that

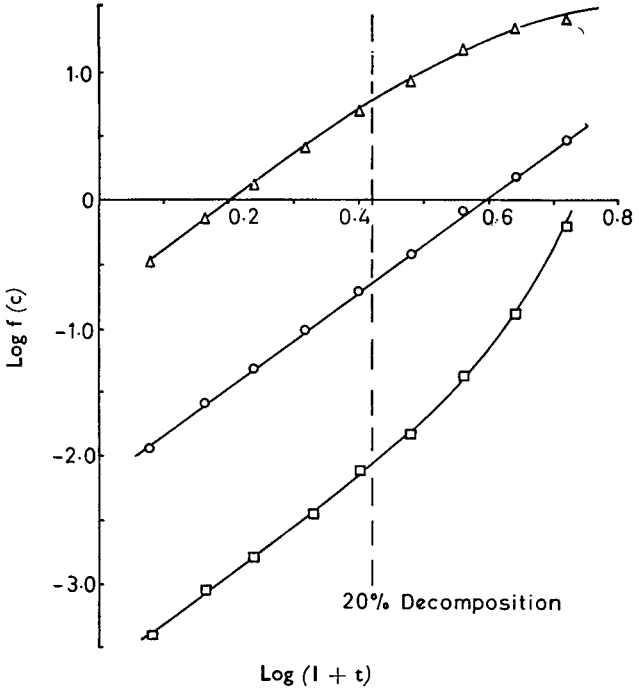

FIG. 3. The data for the acid hydrolysis of sucrose plotted in the zero (Δ), first (○) and second (□) order forms of Rogers' equation.

the hydrolysis of sucrose is a first order reaction with respect to sucrose, as was demonstrated by Moelwyn-Hughes (1934). However, if only the initial portion of the decomposition is followed, say 20%, then it is not possible to determine the order of the reaction (see Fig. 3). In studying the decomposition of more stable compounds it may not be possible to continue the experiment to 100% decomposition and in such cases the order of the reaction must be established by isothermal studies (Moelwyn-Hughes, 1947). This experiment can be performed easily in most instances and would not delay the production of data by Rogers' technique by more than one or two days.

EXPERIMENTAL ACCURACY

The values for the energies of activation and rate constants, determined by Rogers' technique, for the acid catalysed hydrolysis of sucrose, the solvolysis of methyl toluene-*p*-sulphonate and the hydrolysis of ethyl benzoate agreed very well with those reported in the literature (Table 1). Thus, in these cases, Rogers' test yielded accurate and reliable data.

As was suggested above, the data for the values of the rate constants were found to be more scattered than the data for the energies of activation; the mean of the standard deviations for the rate constant data

quoted in Table 1 was $\pm 14\%$ whereas that for the energy of activation data was $\pm 5\%$. While a large error in the value of the rate constant is an inherent feature of Rogers' technique some of the scatter in the data reported in Table 1 may be attributed to the relatively crude temperature control used for those experiments. While the temperature was within 1° of that required by the programme, the temperature programmes were not exactly reproducible in different runs—differences of up to 2° might be expected in two different runs. A more sophisticated method of temperature control which would give more reproducible temperature programmes would reduce the scatter in the data.

THE STABILITY OF P2S IN DILUTE AQUEOUS SOLUTION

The data obtained for the energy of activation and rate constant for the decomposition of P2S in dilute solution in phosphate buffer at pH 7·38 (Table 2) were in good agreement with the values reported by Fan, Nairn & Walker (1964). The study on P2S confirmed its greater stability in acid solution than in neutral or alkaline solution (Fan & others, 1964; Creasey & Green, 1959). There appear to be no other published data for the stability of P2S in dilute solution but Ellin, Carlese & Kondritzer (1962) have studied the stability of PAM (*N*-methyl pyridinium-2-aldoxime methiodide) which is likely to decompose in a similar way to P2S, *N*-methyl pyridinium-2-aldoxime methanesulphonate. Using the conventional method of storage at elevated temperatures they predicted that at 15° a solution of PAM in phosphate buffer at pH 7 would have a half-life of three years. This value is in good agreement with that found for P2S in phosphate buffer at pH 7·38 (Table 2, $2{\cdot}9 \pm 0{\cdot}8$ years).

THERMAL STABILITY OF CHOLINESTERASE

The data reported in Tables 4 and 5 demonstrate that Rogers' test may be used to determine the thermal inactivation of enzyme since the half-life of the activity of the enzyme solution at various temperatures, determined isothermally, agreed reasonably well with the values calculated from the Rogers' data. These experiments suggest an important role for this accelerated storage test in comparing the stabilities of different batches of the same preparations.

Rogers' test has thus been demonstrated to be extremely useful in determining the stabilities of compounds in the liquid phase. It may be used to determine the stability of any compound which can be investigated by isothermal studies and can produce data for the rate constant and energy of activation of the decomposition reaction very much more rapidly than the conventional methods. The results obtained by the technique are more scattered than those obtained isothermally. However, in many instances an estimate of the order of the stability is satisfactory. Thus the test may have its greatest use in preliminary studies of the storage stability of pharmaceutical and related compounds.

Acknowledgements. The samples of purified cholinesterase were generously supplied by Dr. J. J. Gordon of the Chemical Defence Experimental Establishment, Porton.

References

Creasey, N. H. & Green, A. L. (1959). *J. Pharm. Pharmac.*, **11**, 485–490.
Ellin, R. I., Carlese, J. S. & Kondritzer, A. A. (1962). *J. pharm. Sci.*, **51**, 141–146.
Fan, M. C., Nairn, J. G. & Walker, G. C. (1964). *J. Pharm. Pharmac.*, **16**, 493–496.
Garrett, E. R. (1962). *J. pharm. Sci.*, **51**, 811–833.
Ingold, C. K. & Nathan, W. S. (1936). *J. chem. Soc.*, 222–225.
Michaelis, L. (1931). *Biochem. Z.*, **234**, 139–141.
Moelwyn-Hughes, A. E. (1934). *Z. phys. Chem.*, **B26**, 281–287.
Moelwyn-Hughes, A. E. (1947). *The Kinetics of Reactions in Solution*, Chapters I and II. Oxford University Press.
Robertson, R. E. (1953). *Can. J. Chem.*, **31**, 589–601.
Rogers, A. R. (1963). *J. Pharm. Pharmac.*, **15**, 101*T*–105*T*.