cats. However, approximately 60% recovery from the depressor response occurred within 15–30 min. The response to bilateral carotid occlusion was blocked and the epinephrine, norepinephrine, and angiotensin effects potentiated. Transmission through sympathetic ganglia was inhibited but not blocked; and postganglionic transmission also appeared depressed, although not to the same degree as preganglionic transmission. At least part of the hypotensive effect produced by this compound appeared to be due to interference with sympathetic nervous system activity.

EX 4922, 1-hydrazinophthalazine 3,4-dihydro-6-nitro - 7 - sulfamoyl - 1,1,3 - trioxo - 2H - 1,2,4-benzothiadiazinate, produced hypotensive effects in rats, cats, and dogs, with the rat being the most sensitive of the species tested. The compound did not appreciably alter the response of the nictitating membrane to pre- and postganglionic stimulation but did markedly depress the pressor effect of exogenous epinephrine in the cat. EX 4922, 5 mg./Kg. i.v., to dogs attenuated the pressor effect of norepinephrine and reversed the pressor effect of epinephrine to a depressor action. A moderate direct vasodilatory effect was observed in dogs and cats. The data suggest that this compound produces depressor activity by blocking α adrenergic receptors and by a direct depressant action on vascular smooth muscle.

EX 4526, 2,2,6,6-tetramethylpiperidine 3,4-dihydro - 6 - nitro - 7 - sulfamoyl - 1,1,3 - trioxo - 2H-1,2,4-benzothiadiazinate, produced hypotensive effects in rats, cats, and dogs. The compound exerted a depressant action on the sympathetic nervous system at the level of the ganglia and at a more distal locus. The α adrenergic receptors were not inhibited when exogenous epinephrine was administered after EX 4526. The compound also produced a moderate direct vasodilatory effect and marked depression in cardiac output. The data suggest that the following factors may be involved in the hypotensive response elicited by EX 4526: (*a*) a reduction in cardiac output, (*b*) a moderate direct vasodilation, (*c*) an interruption of sympathetic function distal to the ganglia, and (*d*) possible ganglionic blockade.

SUMMARY

The hypotensive activities of four pempidine derivatives and four benzothiadiazine structures related to chlorothiazide and diazoxide are reported. The possible mechanisms of action of the more potent compounds are discussed.

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Single-Step Stability Studies

By S. P. ERIKSEN and H. STELMACH

By the use of a reciprocal heating machine, kinetic studies under nonisothermal conditions have been made. These single-step concentration-time-temperature studies permit a complete investigation of those parameters important for stability prediction to be made on one sample, in one run, regardless of whether previous screening studies have been made. The hydrolysis of two esters (ethyl acetate and *p*-nitrophenol acetate) has been followed to demonstrate the suitability of the theory and the method for determining both the energy of activation and the rate constant at any temperature. The potential of a single-step study method such as the one proposed, for investigations where large numbers of temperature sensitivity studies must be made, as for formula stability predictions, will be apparent, but other advantages over classical kinetic methods, such as number of samples and the volume of controlled temperature space required, increase its potential still further.

THE METHOD of exaggerating temperatures in order to accelerate degradation and thus mathematically to predict the shelf-life stability of formulations of pharmaceutical interest has been used considerably in the past 10 years. The literature is replete with examples of both its use and its usefulness (1). The basic procedure, that of determining the concentration independent rate constant at several temperatures, calculating the activation energy and then predicting the rate at shelf temperature, has been changed or improved remarkably little since the idea was first presented, either in the pharmaceutical or the chemical literature. A survey of the pertinent literature indicates that the improvements in the classical method have been limited to the introduction of differential thermal

Received March 15, 1965, from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication April 28, 1965.
Presented to the Scientific Section, A.PH.A., New York City meeting, August 1964.
This reasearch was supported in part with funds supplied by Smith Kline & French Laboratories, Philadelphia, Pa.

analysis (DTA) by Borchardt and Daniels in 1957 (2) and a spectrophotometric method in 1959 (3). The DTA method depends for its determinations on the detection of the net heat of reaction as the temperature is raised and uses both the area under the differential heat curve and its slope to determine the ΔH, the k_{obs}, and the E values for the reaction. The spectrophotometric method utilizes the slope of a continuous spectrophotometric curve plotted against time, obtained while the temperature of the sample cell is raised, to obtain the E and k_{obs} values. Both of these methods require an estimate of the slope of a nonlinear set of experimental points to be determined.

DISCUSSION

This report concerns a method utilizing a programmed temperature change during the reaction such that the analytical results obtained from samples removed during the run can be graphed directly, the energy of activation calculated from the slope of the straight line so obtained, and from a second calculation, the rate constant determined at any desired temperature.[1] No recourse is made to slopes of concentration-time plots.

While the classical kinetic method for determining the energies of activation is perhaps suitable for chemical kinetic studies, the large number of samples required, the controlled temperature storage space thus required, and the difficulty of maintaining assay precision constant over the prolonged storage periods of some of the samples make it difficult to apply completely to formulation stability studies. In addition, precise studies by the classical method require some preliminary estimate of the stability of the drug in the formula in order to time the sampling and assay periods for the best accuracy. The method presented here overcomes most of these problems. Only one run, requiring one sample, is used to determine all the desired constants and the run can theoretically be programmed to be complete within 1 working day. Precise results require repetition, of course, but each repetition is performed as an entity in itself independent of all other runs, and its speed adjusted to give reinforcement to the portion of the curve where it is desired without affecting the results. No preliminary studies or data on the formula are required, the sampling schedule being largely controlled by the mathematics and the assay convenience.

The theory involved is exact, with no approximations, so that the precision obtained is only a function of the effort expended on assay precision and numbers, provided the reaction follows some reaction order that can be classified as simple, and that the Van't Hoff-Arrhenius law holds. No initial rate or slope calculations are required.

The utility, method, and precision of the theory described are shown with studies of two ester hydrolyses, ethyl acetate and *p*-nitrophenol acetate.

[1] After the work for this report had been completed, the authors became aware of the recent paper of Rogers (4), in which he reported a similar but different derivation intended for the same purpose. The authors felt it necessary to append a comparison of the two methods to this paper.

THEORETICAL

A reaction having a nonfractional order can be described by some form of the general equation

$$f(C_a, C_b, \ldots) = -k_{obs}t + f(C_a^\circ, C_b^\circ, \ldots) \quad \text{(Eq. 1)}$$

where k_{obs} is the observed rate constant, t the time, and $f(C_a, C_b, \ldots)$ and $f(C_a^\circ, C_b^\circ, \ldots)$ are the concentration functions with which the reader is already familiar. (Table I.)

Equation 1 is obtained by integration from the general differential equations of the zero-, first-, and second-order reactions, when one assumes that k_{obs} is a constant. If temperature is allowed to vary with time however, k_{obs} must be integrated with respect to time as well, and the result obtained depends on the temperature-time function used. The effect of temperature on the k_{obs}

$$k_{obs} = Pe^{-E/RT} \quad \text{(Eq. 2)}$$

controls somewhat the choice of the temperature function to be used, in order that the resulting $k_{obs} = f(t)$ can be integrated at all, since the general function

$$k_{obs} = Pe^{(-E/R)[1/G(t)]} \quad \text{(Eq. 3)}$$

where T is any function $G(t)$ of time, is not integrable.

If one selects the temperature function,

$$1/T = 1/T_0 - at \quad \text{(Eq. 4)}$$

where a is a reciprocal heating constant, and T_0 and T are the temperatures at initial time and time t, respectively, the general differential equation including Eq. 3 (and therefore the differential equations related to Eq. 1) become exactly integrable, producing the general equation

$$f(C_a, C_b, \ldots) = \frac{Rk_{obs(T_0)}}{aE} e^{(aE/R)t} + f(C_a^\circ, C_b^\circ, \ldots) - \frac{Rk_{obs\,(T_0)}}{aE} \quad \text{(Eq. 5)}$$

where all terms are defined as before, and $k_{obs(T_0)}$ is the rate constant at the initial temperature. In its present form, Eq. 5 can be solved exactly only by the process of successive approximation (effective for synthesized data but difficult with actual assay results) or under certain special conditions. (See below and *Appendix*.) If Eq. 5 is rearranged and solved at two times, t and $t + \Delta t$

$$f(C_a^t, \ldots) - f(C_a^\circ, \ldots) = \frac{Rk_{obs(T_0)}}{aE} e^{aEt/R} - \frac{Rk_{obs(T_0)}}{aE} \quad \text{(Eq. 6)}$$

$$f(C_a^{t+\Delta t}, \ldots) - f(C_a^\circ, \ldots) = \frac{Rk_{obs(T_0)}}{aE} e^{[aE(t+\Delta t)]/R} - \frac{Rk_{obs(T_0)}}{aE} \quad \text{(Eq. 7)}$$

the difference between these two solutions yields an equation without the constant

$$\log [f(C_a^t, \ldots) - f(C_a^{t+\Delta t}, \ldots)] = \frac{aE}{2.303\,R} t + \log \left\{ [1 - e^{(aE/R)\Delta t}] \left[\frac{Rk_{obs(T_0)}}{aE} \right] \right\} \quad \text{(Eq. 8)}$$

TABLE I.—CONCENTRATION FUNCTIONS

Order	$f(C_a, C_b, \ldots)$	$f(C_a^\circ, C_b^\circ, \ldots)$
Zero	C_a	C_a°
First	$2.303 \log C_a$	$2.303 \log C_a^\circ$
Second	$1/C_a$	$1/C_a^\circ$
Second	$\frac{2.303}{(B-A)} \log \frac{(B-x)}{(A-x)}$	$\frac{2.303}{(B-A)} \log \frac{B}{A}$

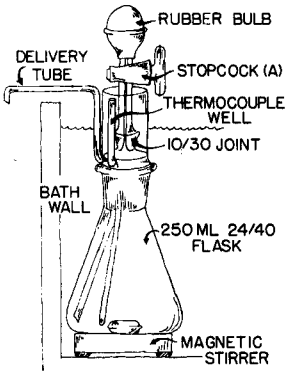

Fig. 1.—Submerged sample cell for use in nonisothermal kinetic studies.

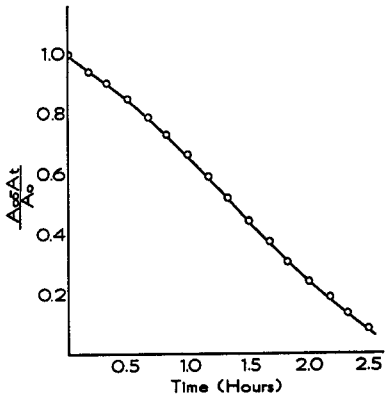

Fig. 2.—Crude concentration data plotted as a function of time, obtained during a nonisothermal kinetic run on *p*-nitrophenol acetate. Data are plotted as a function of the ultraviolet absorbance at 318 mμ.

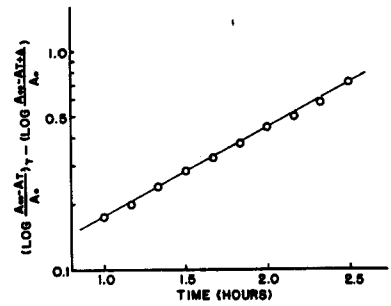

Fig. 3.—A plot of the Guggenheim solution of Eq. 5 (see text) for the data shown in Fig. 2. All samples were taken at 10-min. intervals, the time interval, Δt, used for the plot is 60 min.

Equation 8 represents a straight line for plots of the log [concentration function at $t - (t + \Delta t)$] *versus* time whose slope is $aE/2.3R$, provided a constant time interval (Δt) is maintained.[2]

The value of $k_{obs(T_0)}$ (the apparent rate constant at any starting temperature) can most easily be found by solving Eq. 6 for $k_{obs(T_0)}$

$$k_{obs(T_0)} = \frac{aE[f(C_a, \ldots) - f(C_a^\circ, \ldots)]}{R[e^{(aE/R)t} - 1]} \quad \text{(Eq. 9)}$$

at several times and taking the average. If T_0 is the desired shelf temperature, the above k is the desired one; if T_0 is not shelf temperature, the E and $k_{obs(T_0)}$ may be used to extrapolate.

The general Eq. 6 suggests that under the situation where $k_{obs(T_0)}$ is very small (usually the case for effective drug formulas), the term $Rk_{obs(T_0)}/aE$ may become negligible in comparison to log $[f(C_a, \ldots) - f(C_a^\circ, \ldots)]$ such that Eq. 8 would become

$$\log[f(C_a, \ldots) - f(C_a^\circ, \ldots)] = \frac{aE}{2.3R} t + \log \frac{Rk_{obs(T_0)}}{aE} \quad \text{(Eq. 10)}$$

and both E and $k_{obs(T_0)}$ could be found directly from a plot of this equation.

EXPERIMENTAL

Materials.—Mallinckrodt analytical reagent grade ethyl acetate was used as received. Original bottles were opened and small samples of the ester sealed in hard glass ampuls for storage in the refrigerator until used. The samples of *p*-nitrophenol acetate were prepared according to the method of Chataway (6) and stored in a desiccator until used.

Equipment.—The reaction vessel and stirrer are shown in Fig. 1. It was immersed in a water bath whose temperature was controlled by a variable speed programmer [described elsewhere (7)]. Temperatures inside the reaction vessel were monitored using an iron–constantan thermocouple having ice water as its reference temperature and recorded on a calibrated potentiometric recorder.

Procedure.—*A.*—The hydrolysis solvent was allowed to come to the bath starting temperature.

B.—The ester was injected with a 100-μl. syringe and the programmer and timer started (ethyl acetate was injected as such, *p*-nitrophenol acetate as a solution in dry acetonitrile).

C.—The initial sample was pumped from the vessel immediately by opening stopcock A and squeezing the bulb into a precooled (0°) test tube and analyzed immediately.

D.—Samples were then taken and assayed at regular intervals for about 4 hr.

E.—After the last sample had been collected, the programmer was stopped and the remainder of the solution left over night at the maximum temperature to obtain the infinite time assay.

Analysis.—Ethyl acetate was assayed by potentiometric titration of the residual base in a measured aliquot, with standard acid, to a pH of 7.0. *p*-Nitrophenol acetate was analyzed spectrophotometrically at 318 mμ by measuring the absorbance of the *p*-nitrophenol produced during the reaction. Concentrations were adjusted to

[2] This solution is completely analogous to the Guggenheim method for plotting kinetic data when the infinite time assay is not known (5).

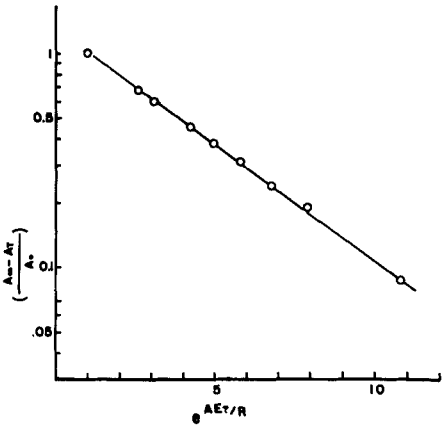

Fig. 4.—A plot of Eq. 5 using the value of E found from Fig. 3 to correct the abscissa.

yield absorbance readings of about 1 at infinite time.

RESULTS AND DISCUSSION

The data obtained from a representative run using *p*-nitrophenol acetate in 1 *N* HCl are shown in Figs. 2–4. Figure 2 shows the crude concentration data as a function of time of reaction, while temperature was being programmed according to Eq. 4. In Fig. 3, the plot of the data for the Guggenheim-like solution to Eq. 5 is shown using the time interval of 1 hr. [$(t + \Delta t) - t = 60$ min.] and indicates very well the linearity of the plots obtained from the equation. Figure 4 shows a plot of Eq. 5 directly, indicating the fit of the data to this general equation after the value of E has been found from the graph as shown in Fig. 2.

Table II contains the experimentally determined data for the two esters by this method. Isothermal data are included for comparison.

The above examples are intended not so much as *proof*, but more as demonstrations of the method. The linearity of the Guggenheim plot implies the validity of the equations used, while the variability of the E and the k_{T_0} values reflect the crude temperature program control obtained with the programmer used. The program rate (a here) is only correct to about 10%, and thus while the value of aE/R for any one run (slope of Eq. 8) is certainly more accurate than this, the E calculations from this slope and the k_{T_0} extrapolation from Eq. 9 reflect the variability in the heating rate, a.

For the accuracy demanded of stability measurements, the precision afforded even by this programmer seems adequate, though with a more accurate and reproducible temperature programmer the results could easily be made less variable. As the over-all precision of the mechanical system was low and method demonstration the aim, no great efforts were made to control the speed of quenching of the reaction after sampling, although this could be expected to have a *changing* effect on a system such as ours, while it is usually considered to have a *constant* effect in isothermal studies.

The errors of this system then can be gathered into three groups.

(a) Those errors inherent in the Guggenheim solution. These are minimized by increasing the time gap, Δt, and by decreasing the over-all variability of the analytical procedure.

(b) Inaccuracies in the infinite time assay. Because the reactions run by the programmed temperature method often cover a considerable range of degree of reaction, not infrequently the last few plotted values reflect not so much the true concentrations but rather the error in the infinite time assay. As in most kinetic studies, the data used should cover only the first 20–50% of the reaction for the best accuracy, although this depends on the assay and the calculation method used.

(c) Inaccuracies in the temperature program and measurement. These are essentially instrumental, but as discussed above are of concern.

The precision obtained by this procedure seems limited then only by the precision of the assay, the quality of the equipment, and the precision demanded by the study. The major source of error in this study appeared to be the imperfections in the heating program, though this limitation could easily be overcome by more sophisticated instrumentation.

MEANING OF THE E OBTAINED

The meaning of the E found by the method suggested in this report (and also of the E found by the more conventional treatment of k_{obs} values at several temperatures) is not as straightforward as it may seem at first glance, nor as it has been implied by other authors. Even for simple reactions such as those reported here, the observed rate constant is a multiple of at least three individual k values (8)

$$-d(E)/dt = k_{obs}(E) = k_H(H^+)(E) + k_{OH}(OH^-)(E) + k_w(H_2O)(E) \quad \text{(Eq. 11)}$$

or

$$k_{obs} = k_H(H^+) + k_{OH}(OH^-) + k_w(H_2O) \quad \text{(Eq. 12)}$$

each of which has its own E (or ΔH^* value), often quite different. The determination of an over-all E for the k_{obs} data by plotting log k_{obs} *versus* $1/T$ is graphically easy but represents quite a complex situation.

$$k_{obs} = Pe^{-E/RT} = P_H(H^+)e^{-E^1/RT} + P_{OH}(OH^-)e^{-E^2/RT} + p_w(H_2O)e^{-E^3/RT} \quad \text{(Eq. 13)}$$

Table II.—Rate Data for *p*-Nitrophenol Acetate and Ethyl Acetate Nonisothermal Runs

Compd.	E(Kcal./mole)	$k_{obs(25°)}$	$E_{(lit.)}$	$k_{(lit.)}$[a]
Ethyl acetate[b]	11 ± 1.0	.06 ± .02	11.7	0.11
PNPA[c]	21 ± 2	$5 \pm 1 \times 10^{-5}$	18[d]	5.3×10^{-5}[e]

[a] *Reference 10.* [b] Ethyl acetate runs in concentrations of 2.02×10^{-3} *M* base and 1.01×10^{-3} *M* ester; constants determined by standard second-order kinetics. [c] *p*-Nitrophenol acetate runs in concentrations of 10^{-4} *M* ester in 0.982 *N* HCl; constants determined by standard first-order kinetics. [d] Determined in our laboratories; Bruice suggests it lies at about 20 Kcal. (9). [e] *Reference 8.*

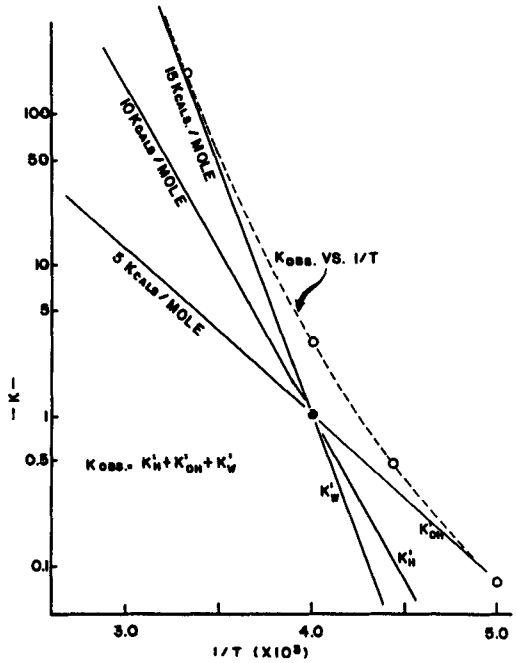

Fig. 5.—A hypothetical plot of log k_{obs} *vs.* $1/T$. The k_{obs} data have been synthesized from three individual k^* values, each having different E energies. An iso-rate point at 250°K has been chosen for all three reaction pathways, and the over-all k_{obs} representing the sum all three k^*'s has been plotted as a dashed line.

It should be apparent that a plot of log k_{obs} *versus* $1/T$ is a function of all these terms, although they are not all of equal importance in all cases. In the situation presented here for instance, in one case $(OH^-) = 0$ (for *p*-nitrophenol acetate) and in the other $(H^+) = 0$ (for ethyl acetate), and thus the appropriate terms of Eq. 13 will vanish leaving an equation of only two terms. In the example of the phenol acetate case

$$k_{obs} = (H^+)P_H e^{-E^1/RT} + (H_2O)P_w e^{-E^3/RT} \quad \text{(Eq. 14)}$$

but even with this simplification, unless the product $(H_2O)P_w = 0$, the assumption that the observed E value is a unique single value is still incorrect, unless E^1 and E^3 are the same. At other pH's, it may well be incorrect to presume that any term is equal to zero. An estimate of just what an E obtained for a plot of log k_{obs} *versus* $1/T$ really represents can be made if one considers the various situations possible for a reaction having a k_{obs} described by Eq. 13. For each individual k, the size of the exponential term controls the effect of the changing temperature, the P terms the absolute rate, and the relative contribution of each of the terms to the found E will depend on their absolute rate constants at the temperature in question. In other words, the E of the predominant reaction will control the E measured from the k_{obs}. The relationship between k_{obs} and E for several situations can be shown with Fig. 5 where lines representing reasonable but hypothetical rate-temperature data for a reaction having a three part k_{obs} are shown. A temperature of 250°K has been selected as the point where all the rate constants (k_{H^+}*) are equal to each other and to 1, the "iso-rate" point [in accordance with general stability practice $(H^+)P_H e^{-E^1/RT} = k_{H^+}$* as the rate constant has been used]. Appropriate lines representing E values of 5, 10, and 15 Kcal. were drawn through this point for each part of Eq. 13. The k^* for each portion of the k_{obs} was read from the line at 200, 225, 250, 300, and 350°K and the hypothetical k_{obs} calculated as the sum at each temperature. These sums are plotted as the dashed line on Fig. 5 (while it was assumed that all three k^* values had the same iso-rate point, an intersection of any two would have given the same general result).

The result of this type of analysis (the dashed line) indicates that one would not expect a log k_{obs} *versus* $1/T$ line to be straight unless the tested temperatures were far removed from any iso-rate point, or unless all the dominant reactions had the same E. In every other case, one may expect that the measured E will be different from any of the true ones, and thus neither indicative of the true temperature dependence nor useful for extrapolation and prediction purposes.

Iso-rate points can easily occur near room temperature, and in some cases are even sought for formulation purposes. Figure 6 contains three common types of pH-rate profiles drawn to show the contribution of each part of the k_{obs}. The over-all k_{obs} is shown in each case by the heavy line. The indicated points are the iso-rate points at 25° where the rate constants for two pathways are equal. An exaggerated temperature study of a solution with a pH in the proximity of any of these points may well produce a curved log k_{obs} *versus* $1/T$ plot quite irrespective of the other reasons for the line to be nonlinear (reaction mechanism changes, polymorphic transitions, ΔH^* inconstancy, etc.). Because iso-rate points are often associated with pH's that produce minima in the pH rate curve, these pH's are often sought for formulation, and thus these considerations become of concern.

The importance of recognizing the proximity of an iso-rate point lies in the fact that the E and the prediction line obtained for an exaggerated temperature study near such a point will always be in

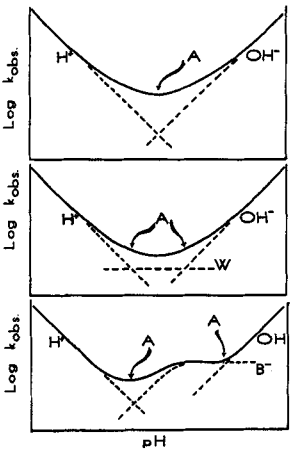

Fig. 6.—Three common pH-rate profiles showing the contribution of each part of the rate expression and indicating the iso-rate points in each (A).

error and normally on the *unsafe* side; the E will be too high and the predicted shelf rate therefore too low. An iso-rate point can only be detected by measuring the desired E and $k_{obs(T_0)}$ values at several pH's or by more sophisticated kinetic procedures designed to indicate the shape of the pH-rate curve (or to separate the actual k^* values at each pH). At the pH's where each constant predominates, the E's may be determined individually and the over-all Arrhenius curve properly drawn and interpreted.

SUMMARY

A nonisothermal kinetic procedure has been designed and tested. It will permit determination of both energies of activation and specific rate constants at any temperature as the direct result of linear plots of analytical data obtained during a temperature programmed run. The method produces both of these values as the result of a single run on one sample, and its accuracy is apparently limited only by the precision of the assay, the perfection of the equipment, the applicability of the Arrhenius equation, and the constancy of the activation energy.

In the case where the rate constant is made up of major contributions from several kinetic factors (*i.e.*, catalysts, etc.), each of which has a different E, the E obtained by the Arrhenius plot of k_{obs} values may not be used as a single entity and thus may not be used in stability prediction methods, but must be treated as a composite and proper consideration given to determining the contribution of its various constituent parts in order to extrapolate exaggerated temperature data properly for the purposes of shelf-life prediction.

APPENDIX

The equations of Rogers (4) utilize the heating program

$$1/T = 1T_0 - 2.303\, a \log (1 + t) \quad \text{(Eq. 1}a\text{)}$$

(his notation has been changed here to conform to that of this report for the purposes of comparison). With this temperature program, and again assuming that k_{obs} is an exponential function of the temperature

$$\log k_t = \log k_{obs(T_0)} + \frac{E}{2.303\, R}(1/T_0 - 1/T_t) \quad \text{(Eq. 2}a\text{)}$$

the specific rate constant at any time t, (k_t) becomes an integrable function of t

$$k_t = k_{obs(T_0)}\,(1 + t)^{(aE/R)} \quad \text{(Eq. 3}a\text{)}$$

so that his equivalent of the general Eq. 4 of this report is

$$\log[f(C_a, \ldots) - f(C_a^\circ, \ldots)] = \log k_{obs(T_0)} - \log\{[1 + (aE/R)] + [1 + (aE/R)]\,[\log(1 + t)]\} + \log\left\{1 - \left[\frac{k_{obs(T_0)}}{k_t}\right][1 + (R/aE)]\right\} \quad \text{(Eq. 4}a\text{)}$$

where k_t is the specific rate constant at time t, and all other terms have the same definitions as given in this report. Equation 4a suggests that a plot of log $[f(C_a, \ldots) - f(C_a^\circ, \ldots)]$ *versus* log $(1 + t)$ will produce a straight line of slope aE/R and an intercept that is a complex function of $k_{obs\,(T_0)}$ and E. The examples shown by Rogers (riboflavin and sucrose degradations in water) demonstrate the applicability of the method.

The proposal described here and that of Rogers can most conveniently be compared by considering Eq. 4a and Eqs. 7 and 8. For the situations given by Rogers where the $k_{obs(T_0)}$ approaches zero (actually 5×10^{-7} sec.$^{-1}$), either system, Eq. 10 of this report or Eq. 4a of the above, will produce the same result. When the reaction rate at the initial temperature is an appreciable value (as it is in this report, $= 10^{-5}$ sec.$^{-1}$), the essential nonlinearity of Eq. 4a at early time intervals will become apparent. The last term of Eq. 4a is *not* time independent, containing a k_t, and thus unless $k_{obs(T_0)} < k_t$, this term will contribute to curvature in the plotted line. Because curvature would lead one to suspect an incorrect order selection (indeed this is the only way to detect such an incorrect assumption), as well as an appreciable $k_{obs(T_0)}$, this condition could lead to ambiguity. The Guggenheim solution (Eq. 8) proposed in this report *is* completely linear, and thus a straight line will always be obtained for a correct order assumption and assay procedure.

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