THE ALTERATION OF INTRACELLULAR ENZYMES

III. The Effect of Temperature on the Kinetics of Altered and Unaltered Yeast Catalase*

By M. J. FRASER‡ and J. GORDIN KAPLAN

(From the Department of Physiology, Dalhousie University, Halifax, Canada)

(Received for publication, August 4, 1954)

INTRODUCTION

As a result of certain similarities in behavior between surface-unfolded (3) and intracellular erythrocyte catalase (4), the hypothesis was advanced "that catalase within the cell exists partially, but reversibly, unfolded at some intracellular interface; upon cytolysis, the enzyme is desorbed from the interface and changed to the rolled-up (native), soluble condition, with consequent increase in activity . . .". The series of investigations of which this is part is designed to put this *interfacial hypothesis* to the test.

In the first of these papers ((1) hereafter referred to as Paper I), it was shown that the catalase of the intact yeast cell could be caused to undergo *in situ* an abrupt change in a wide variety of properties and thus made to imitate in almost every detail the behavior of catalase extracted from the cell into solution. The process by which this change was brought about was called *enzyme alteration*, and the existence of this phenomenon was taken to indicate that catalase could exist in no less than 2 distinct states, the rolled-up, highly active, soluble configuration (in which it is usually studied) which it possesses following alteration *in situ* or after extraction, and also the configuration, whatever it may be, which it possesses within the intact cell. Those properties of the intracellular catalase which underwent change were: (*a*) its specific activity,

* The authors are indebted to Dr. Charles Masson and Dr. Walter Trost of Halifax, and to Dr. Britton Chance of Philadelphia, for their very helpful comments. Plans for a kinetic study of surface-spread catalase were made jointly with Professor Teru Hayashi of Columbia University in 1949, and while such a study has not as yet been undertaken, this discussion was the seed from which the present experiments have grown. This work has been supported by grants from the National Cancer Institute of Canada, and the National Research Council, Ottawa.

‡ Recipient of National Research Council bursary, and at present 1851 Exhibition Scholar, Department of Colloid Science, University of Cambridge. The data to be presented in this paper have been abstracted from a thesis submitted by M. J. Fraser to the Faculty of Graduate Studies, Dalhousie University, in partial fulfillment of the requirements for the M.Sc. degree.

which increased by more than an order of magnitude (this is the Euler effect, named after its discoverer (5)); (*b*) its optimal substrate concentration, which was greatly reduced, owing to its much greater sensitivity to the destructive effect of H_2O_2; (*c*) its response to changes in the pH of the external medium (the activity of the altered intracellular enzyme was virtually abolished below pH 4 and above pH 10, whereas the unaltered enzyme was only slightly inhibited at the former pH and strongly activated at the latter); (*d*) its activity following pretreatment with heat which, at durations and intensities causing but little change in the unaltered catalase, caused total destruction of the altered enzyme. In Paper I, chloroform and ultraviolet radiation were the agents used to achieve the *in situ* enzyme alteration.

In the second paper ((2), hereafter referred to as Paper II), the ability of surface-active agents (alcohols, ketones, aldehydes) to cause enzyme alteration was shown to be closely correlated with their surface activity. For example, concentrations of these agents, varying over a 4,000-fold range, which caused equal activation of the catalase of a yeast suspension, depressed the surface tension of the treatment mixture to the same extent (to 42 ± 2 dynes, for doubled activity). It was also shown that the altering activity of these agents was not correlated with their ability to kill the yeast cells. The conclusion was drawn that altering agents studied in Paper II caused the alteration of catalase by adsorbing at some critical intracellular interface, and this was taken as support for, but not proof of, the interfacial hypothesis.

On the basis of the interfacial hypothesis, it was possible to make some interesting predictions concerning the changes in kinetic properties of the catalase which should accompany desorption of the enzyme from an interface and it has proven possible to confront these predictions with the testimony of experiment. We propose to show in this paper that the regular changes in enzyme kinetics which accompany enzyme alteration are of a nature and in a direction predicted by, or at least consistent with, the interfacial hypothesis.

THEORETICAL

A. Effect of Temperature on the Enzyme-Catalyzed Reaction.—It is well known that the rates of enzyme-catalyzed reactions generally follow the Arrhenius equation, one form of which is

$$\mu = 2.3\ R\ \frac{(\log k'_2 - \log k'_1)}{1/T_1 - 1/T_2} \qquad (1)$$

in which the specific rate constants k'_1 and k'_2 are determined at two absolute temperatures, T_1 and T_2. Hence a plot of log k' *versus* $1/T$ yields a straight line whose slope is $\mu/2.3\ R$, in which R is the gas constant, and μ the experimental energy of activation. The energy of activation is believed to be the energy required to reach a critical intermediate configuration, the activated complex, whose attainment is necessary before the reaction can proceed to

completion (6). It is at present agreed that catalysts in general, and enzymes in particular, accelerate the rate of a reaction by reducing its energy of activation; that is, the energy barrier over which the reactants must pass before the products of the reaction can be formed (6).

We should thus predict that the altered enzyme, shown in Papers I and II to be in a much more highly active state, and pictured according to our hypothesis as having been desorbed from the interface into a more specific configuration, should catalyze the decomposition of H_2O_2 with a lower energy of activation than that exhibited by the less specific, and less active, unaltered enzyme-substrate system.

From the experimental energy of activation, μ, determined by means of an Arrhenius plot, we may calculate the heat of activation $\Delta H\ddagger$, from the relation (see reference 6),

$$\Delta H\ddagger = \mu - RT \tag{2}$$

While, strictly, Equation 2 should have a term $(+p\Delta V\ddagger)$ for the volume change associated with the activation process, the magnitude of this term is so small at atmospheric pressure (we calculate 1.4 cal./mole, using the $\Delta V\ddagger$ of luciferin (7)) that it may safely be neglected. Knowing $\Delta H\ddagger$ and the specific second order velocity constants for the catalase-H_2O_2 system (about which more below), we may determine the entropy of activation, $\Delta S\ddagger$, by substituting these constants in the Eyring modification of the Arrhenius equation (6):

$$k'' = \kappa_t \frac{kT}{h} \cdot e^{-(\Delta H\ddagger)/RT} \cdot e^{(\Delta S\ddagger)/R} \tag{3}$$

Here k'' is the specific second order rate constant, κ_t, the so called transmission coefficient, assumed to be unity in these experiments (*i.e.*, ignoring the possibility that some of the activated complexes might be turned back to their initial state; we shall omit it in the treatment which follows), k is the Boltzmann constant, and h Planck's constant. The factor kT/h is a universal frequency factor, having the dimensions of reciprocal seconds, and being dependent only on temperature and not on the nature of the reaction.

Having determined $\Delta H\ddagger$, and $\Delta S\ddagger$, we may then determine the free energy of activation ($\Delta F\ddagger$) by applying to the activation process the thermodynamic equation,[1]

$$\Delta F\ddagger = \Delta H\ddagger - T\Delta S\ddagger \tag{4}$$

B. Effect of Temperature on the Enzyme Alone.—At higher temperatures, heat, in addition to accelerating the rate of the enzyme-catalyzed reaction,

[1] We shall refer to these quantities as thermodynamic constants, although they refer strictly to the (assumed) activation process, and are simply analogous to the same constants determined for the usual reversible reactions of classical thermodynamics.

will also irreversibly decrease the amount of active enzyme by converting part (or all) into a structurally modified, inactive ('denatured') form. The process of heat destruction of the enzyme may also be subjected to a kinetic analysis, and data relating the rate of catalase destruction in the absence of substrate to varying temperatures permit the calculation of the various thermodynamic constants of the activation of this process, exactly as described above for the activation of the enzyme-substrate systems. In this case, we substitute in the Eyring equation an experimentally measured first order specific reaction rate constant.

Our interpretation of the reason for which one may substitute 2 dimensionally different rate constants (*i.e.*, first and second order) in this equation, without modifying the dimensions of $\Delta F\ddagger$ or $\Delta S\ddagger$ calculated from it, stems from a consideration of another form of the Eyring equation,

$$k' = \frac{kT}{h} \cdot \mathrm{K}\ddagger \tag{5}$$

in which K‡ is the equilibrium constant for the formation of the activated complex ($X\ddagger$) through the reaction

$$A + B + \cdots \rightleftharpoons X\ddagger \tag{6}$$

In a monomolecular reaction of the type $A \rightleftharpoons A\ddagger \rightarrow$ products, it follows that

$$\mathrm{K}\ddagger = [A\ddagger]/[A] \tag{7}$$

hence K‡ is dimensionless and the right-hand side of Equation 5 has the proper dimensions, corresponding to a first order k', of sec.$^{-1}$, which, as we have seen above, is the unit of the frequency factor kT/h. In a bimolecular reaction of the type $A + B \rightleftharpoons X\ddagger \rightarrow$ products, it follows that

$$\mathrm{K}\ddagger = [X\ddagger]/[A][B] \tag{8}$$

hence K‡ has the units of liter·mole^{-1}, and the right-hand side of Equation 5 will thus have, as it ought, the dimensions of liter·mole^{-1}·sec.$^{-1}$, corresponding to a second order rate constant. If the van't Hoff equation is applied to an equilibrium between activated complex and reactants,

$$\mathrm{K}\ddagger = e^{-(\Delta F\ddagger)/RT} \tag{9}$$

and the right-hand side is substituted for K‡ in Equation 5, one obtains

$$k' = \frac{kT}{h} \cdot e^{-(\Delta F\ddagger)/RT}; \tag{10}$$

by substituting the right-hand side of Equation 4 for $\Delta F\ddagger$ in Equation 10, one obtains the relation shown in Equation 3, which is the form of the Eyring equation to be used in the calculation of our data. It is clear that by substituting the proper rate constant in Equations 10 and 3, the symbols $\Delta F\ddagger$,

$\Delta H\ddagger$ and $\Delta S\ddagger$ will represent the standard free energy, heat content, and entropy changes, respectively, occurring upon formation of the activated complex, either for the enzyme-catalyzed decomposition of H_2O_2, or for the heat-induced inactivation of the enzyme itself.

The interpretation which we shall make of differences (as between altered and unaltered enzymes) in $\Delta H\ddagger$ and $\Delta S\ddagger$ of the heat inactivation process is based on treatment by Glasstone, Laidler, and Eyring (6) of the pH dependence of these thermodynamic constants. It is known that $\Delta H\ddagger$ and $\Delta S\ddagger$ for the heat-induced inactivation of enzymes and for the "denaturation" of proteins are strongly pH-sensitive, possessing maximum values at the pH of optimal activity or maximum stability, falling off as the pH is varied in either direction. The explanation of this phenomenon (6) is based on the assumption (amply supported; see Mirsky and Pauling (8)) that the soluble protein is restrained to its highly specific configuration by many weak secondary valences which can be destroyed by acid, alkali, or heat; structural modification ("denaturation") of the protein causes rupture of these bonds and hence a very large increase in entropy. A large entropy of activation for the heat modification process indicates that attainment of the activated complex was accompanied by rupture of a high proportion of these bonds. Hence, if acid and alkali have spontaneously loosened the structure of the unheated protein by having destroyed certain of these secondary valences, the $\Delta S\ddagger$ accompanying the activated complex required for heat modification to occur will be small, since much of the loosening of structure has occurred prior to formation of the activated complex. Since the $\Delta F\ddagger$ of heat modification changes but little with pH, it follows from equation 4 that a reduction in $\Delta S\ddagger$ will be accompanied by a corresponding reduction in $\Delta H\ddagger$.

According to the interfacial hypothesis, the unaltered enzyme possesses lower activity because it is in a structurally less specific state owing to its being partially, but reversibly, unfolded at the interface in question, whereas the altered enzyme is in a more specific, and hence more active, state. It ought therefore to have been possible to predict that the rate of heat destruction of the altered enzyme would be more sensitive to change in temperature than that of the unaltered, leading to higher values of $\Delta H\ddagger$ and $\Delta S\ddagger$ for the former reaction than for the latter; this "prediction" (which we must admit was made retroactively, therefore being more of a postdiction) is based on the assumption that there will be a relatively small difference between the $\Delta F\ddagger$ values of the two reactions; i.e., compared to the difference in $\Delta H\ddagger$.

Glasstone, Laidler, and Eyring (reference 6, p. 443) also discuss the reason that heat modification of the structure of proteins occurs at moderate temperatures despite the unusually high energies of activation characteristic of this process; *e.g.*, in the case of ovalbumin, 132 kcal.). The large compensatory increases in entropy of activation which accompany the high $\Delta H\ddagger$ values en-

sure a comparatively low $\Delta F\ddagger$, from which they conclude that "according to the theory of absolute reaction rates, the reaction will be rapid at ordinary temperatures, in spite of the high heat of activation." We have reported in Paper I that at temperatures between 50 and 56°C. the rate of heat inactivation of the altered catalase is much greater than that of the unaltered enzyme. Hence it might be anticipated that this would be reflected in a significantly lower $\Delta F\ddagger$ for the heat inactivation of the altered catalase.

We must emphasize that we have undertaken this study in order to determine whether kinetic data can shed light on the essentially biological problem of the structure and organization of an enzyme within the cell. We should like to make it clear that we consider this study to be a contribution neither to the exact measurement of catalase rate constants or turnover numbers nor to the mechanism of catalase action, since the work of Chance (9) has made such a pre-occupation unnecessary. One assumption which we have made in our work is that the activated complexes for enzymatic decomposition of substrate and for heat destruction of the enzyme are the same for both altered and unaltered enzymes.

EXPERIMENTAL

A. General.—Methods of washing and handling yeast and of making extracts of yeast catalase have been described in Paper I. All yeast suspensions and enzyme and substrate solutions were made up in M/15 Sörensen's phosphate buffer at pH 7.1–7.2. In experiments on the effect of pH, phosphate buffer was also used; pH 4 was reached by adding a small amount of phosphoric acid, since it was felt that the disadvantage of reduced buffering capacity was outweighed by the advantage of not using a different buffer for this one point. Three methods of causing enzyme alteration were employed: chloroform and ultraviolet (UV) irradiation, described in Paper I, and *n*-propanol, described in Paper II. Activity measurements were made manometrically, unless otherwise specified, using apparatus and methods described in Paper I. In studies of the effect of temperature on the enzyme-catalyzed reaction, use was made of the American Instrument Company, Silver Spring, Maryland, refrigerated Warburg respirometer, not described in previous papers.

The crystalline beef liver catalase was obtained from the Worthington Laboratories, Freehold, New Jersey. Final substrate concentration in the Warburg vessels was 0.44 M, unless otherwise specified. In all our experiments, each manometric activity determination was done in duplicate.

B. Comparison of Manometric and Titrimetric Methods.—Since it had been reported that there existed large discrepancies between catalase activity constants determined manometrically and titrimetrically, (10), we decided to calculate turnover numbers from the rate constants determined by each of these two methods. Turnover numbers will be defined as the number of molecules of H_2O_2 decomposed by 1 molecule of catalase in 1 second.

Our titrimetric method was adapted from the standard manganimetric procedure of von Euler and Josephson (11), the principal differences being that 2 ml. aliquots of

reaction mixture were withdrawn at 1 minute intervals and that the reaction mixtures were incubated at temperatures from 20–30°C., using the sensitive (±0.01°C.) constant temperature bath and shaker described in Paper I. The purpose of these modifications was to permit comparison with an identical enzyme-substrate mixture run under closely similar conditions in the Warburg respirometer. In both cases, substrate was made up in M/20 phosphate buffer at pH 7.2.

With both methods, the reaction mixture contained 2.5×10^{-6} gm. crystalline catalase per ml., and was 0.044 M with respect to H_2O_2. Since 2 ml. of reaction mixture was analyzed in each case, the aliquots contained 5×10^{-6} gm. catalase, or, assuming a molecular weight of 225,000 for this protein,

$$\frac{5 \times 10^{-6}\ (\text{gm.}) \times 6.023 \times 10^{23}\ (\text{mols./mole})}{225{,}000\ (\text{gm./mole})} = 1.34 \times 10^{13}\ \text{mols. catalase} \tag{11}$$

Using the titrimetric method, turnover numbers were calculated by making use of the first order equation

$$v_0 = -\frac{d[H_2O_2]_0}{dt} = k'[H_2O_2]_0 \tag{12}$$

in which v_0 is the initial velocity of decomposition of substrate in moles H_2O_2/liter/sec., and k' is the velocity constant, measured in sec.$^{-1}$. Since the titrimetric first order velocity constants, K, were expressed as min.$^{-1}$ and $\log_{10}$, it was necessary to introduce the following conversion factor in order to use these constants:—

$$k' = \frac{\text{K}(\text{min.}^{-1}) \times 2.3}{60(\text{sec./min.})} \tag{13}$$

The initial velocity of substrate decomposition is thus

$$v_0 = \frac{2.3\text{K}}{60} \times 0.044 = 1.69 \times 10^{-3}\ \text{K moles } H_2O_2\text{/liter/sec.} \tag{14}$$

or, to convert to molecules of substrate,

$$= \frac{1.69\ \text{moles } H_2O_2 \times 2\ \text{ml.} \times 6.023 \times 10^{23}\ \text{mols./mole} \times \text{K}}{\text{Liter/sec.} \times 1000\ \text{ml./liter}} \tag{15}$$

$$= 2.03 \times 10^{18}\ \text{K mols. } H_2O_2\text{/sec.} \tag{16}$$

We then obtain the titrimetric turnover number (T.N.$_t$) by dividing this expression by Equation 11,

$$\text{T.N.}_t = \frac{2.03 \times 10^{18}\ \text{K mols. } H_2O_2\text{/sec.}}{1.34 \times 10^{13}\ \text{mols. catalase}} = 1.51 \times 10^{5}\ \text{K mols. } H_2O_2\text{/mol. catalase/sec.} \tag{17}$$

The pseudo-zero order rate constants obtained manometrically have dimensions of μl./min., so that these activity constants (A) must first be converted to moles O_2/sec., by the following expression:—

$$\frac{A(\mu\text{l. } O_2\text{/min.}) \times 10^{-6}\ (\text{liter}/\mu\text{l.})}{60(\text{sec./min.}) \times 22.4\ (\text{liter/mole})} = 7.44 \times 10^{-10}\ A\ \text{moles } O_2\text{/sec.} \tag{18}$$

From the stoichiometry of the decomposition of H_2O_2,

$$2H_2O_2 = 2H_2O + O_2, \tag{19}$$

it follows that expression 18 must be multiplied by 2 in order to convert to moles substrate broken down per second:—

$$\text{Rate of } H_2O_2 \text{ decomposition} = 1.49 \times 10^{-9} A \text{ moles } H_2O_2/\text{sec.} \tag{20}$$

Converting to molecules by multiplying by Avogadro's number, we obtain

$$\text{Rate of } H_2O_2 \text{ decomposition} = 8.97 \times 10^{14} A \text{ mols. } H_2O_2/\text{sec.} \tag{21}$$

and to obtain the corresponding turnover ($T.N._m$) we divide this expression by Equation 11,

$$T.N._m = \frac{8.97 \times 10^{14} A \text{ mols. } H_2O_2/\text{sec.}}{1.34 \times 10^{13} \text{ mols. catalase}} = 66.9 A \text{ mols. } H_2O_2/\text{mol. catalase/sec.} \tag{22}$$

Thus, by substituting the appropriate rate constants into Equations 17 and 22, we obtain directly the corresponding turnover numbers for both titrimetric and manometric methods.

C. Determination of Thermodynamic Constants of Enzyme-Substrate Systems.— 1 ml. aliquots of altered and unaltered yeast cells, of yeast catalase extract, or of a suitably diluted crystalline catalase preparation (exact concentration given in Results) were pipetted into the Warburg flasks, and run at temperatures from 5 to 40°C. at intervals of 5°C. Altered cells were generally run at a concentration of $\frac{1}{50}$th per cent (wet weight/volume) and unaltered at $1\frac{8}{50}$ths per cent; it will be shown below that the effect of temperature on the rate of H_2O_2 breakdown was independent of concentration of enzyme (or of substrate).

The linear portions of the curves relating O_2 produced to time permitted calculation of pseudo-zero order velocity constants (dimensions-microliters/minute), whose logarithms could then be plotted against the reciprocal of the corresponding absolute temperatures in the Arrhenius plot. The experimental energy of activation was determined from the slope of this curve, and the other thermodynamic constants calculated as described above.

It was found that catalase activity of yeast suspensions, both altered and unaltered, was directly proportional to the concentration of the yeast (or, up to a certain point, of H_2O_2) at temperatures at either end of the range employed in these experiments. This fact permitted development of what we call the short method of determining experimental energies of activation, calculated from the slopes of (or from pairs of points along) the straight lines relating activity to enzyme concentration at two different temperatures (5 and 25°C.). As will be seen below, values obtained by means of the short method were very close to those of the usual method, in which a standard yeast and substrate were run at 8 different temperatures.

D. Thermodynamic Constants of the Heat Inactivation Process.—50 ml. of yeast suspensions or of enzyme solutions contained in Erlenmeyer flasks were heated while being shaken at 60 C.P.M. in the constant temperature bath described above; 4 ml.

aliquots were withdrawn at intervals that varied with the temperature employed (from 15 minutes at lower temperatures to 1 minute at higher) and pipetted quickly into test tubes immersed in an ice bath. Usually 6 aliquots were withdrawn to determine a rate of inactivation at any temperature, and these rates were determined in any experiment at 8 temperatures at intervals of 1 or 2°C. Of course, preliminary experiments were necessary to establish for any given type of enzyme preparation the range within which the rate of inactivation could be measured with accuracy and convenience. Attainment of temperature equilibrium within the Erlenmeyers occurred (to within 0.1°C. of bath temperature) in approximately 3 minutes; this would introduce a slight error at the highest temperatures employed, when the final aliquots were withdrawn after from but 4 to 7 minutes' treatment. Unaltered cells were run at a concentration of $^{1}\!/_{50}$th per cent, and altered at $^{3}\!/_{50}$th per cent.

Heat inactivation of all catalases was found to follow the first order equation at all temperatures; hence a plot of log residual activity *versus* time gave a straight line whose slope was taken as the rate of inactivation. The log of these rates was then plotted against the reciprocal of the absolute temperature at which they were determined, and the Arrhenius equation was found to be closely followed for all types of preparation. From the Arrhenius plot, the other thermodynamic constants were calculated as described above. The lag period observed during heat pretreatment of unaltered catalase was not included in calculations of the rate of inactivation. A problem was posed by the fact that there was no overlap of temperature ranges for the inactivation of unaltered on the one hand, and of altered, extracted, or crystalline enzymes on the other. In order to compare the free energies of activation of the various preparations, it was necessary to calculate the $\Delta F\ddagger$ values at the same temperature for all, since this constant varies sufficiently with temperature to mask a small but significant difference between altered and unaltered enzymes. Consequently all $\Delta F\ddagger$ values were calculated using T = 60°C. (333°K.) in Equation 4, even though $\Delta H\ddagger$ and $\Delta S\ddagger$ may have been calculated at temperatures from 50 to 62°C. The reason for which this is justifiable is that $\Delta H\ddagger$ and $\Delta S\ddagger$ are relatively less temperature-sensitive; for example, $\Delta S\ddagger$ of the chloroform-altered cells at T = 50°C. is 340.6 entropy units (cal./deg./mole) and at T = 60° is 341.1 e.u. The $\Delta F\ddagger$ values could also be calculated at T = 60° by taking the observed first order rate constants for heat inactivation at another temperature, and correcting them to 60° by means of Equation 1. These corrected rate constants were then substituted in Equation 10, thus obtaining $\Delta F\ddagger$ directly, without first having determined the $\Delta S\ddagger$. The values of $\Delta F\ddagger$ obtained by this method were the same as those obtained with the quicker method discussed above (*i.e.*, substituting the uncorrected $\Delta S\ddagger$ and $\Delta H\ddagger$ values in Equation 4).

We should point out that the kinetic study of the heat inactivation process was a much more difficult affair than that of the activation of the enzyme-substrate system. For one thing, duplicate catalase assays of 6 aliquots at 8 temperatures involve an enormous amount of experimental work. Further, the first order rate constants for this process were more variable than for the enzyme-substrate system; consequently, the differences between the μ values of the unaltered and of the altered and extracted catalases were somewhat less clear cut than they were found to be for the enzyme-substrate system. The same difficulty did not obtain in the case of $\Delta S\ddagger$ and $\Delta F\ddagger$.

E. Statistical Treatment.—We have found it essential to treat our data statistically, to be certain of their significance. We have used the t test (see Fisher and Yates (12)); whenever means are presented, the ± symbol will be a standard deviation, and whenever differences between means, it will be the standard error of the difference between the means.

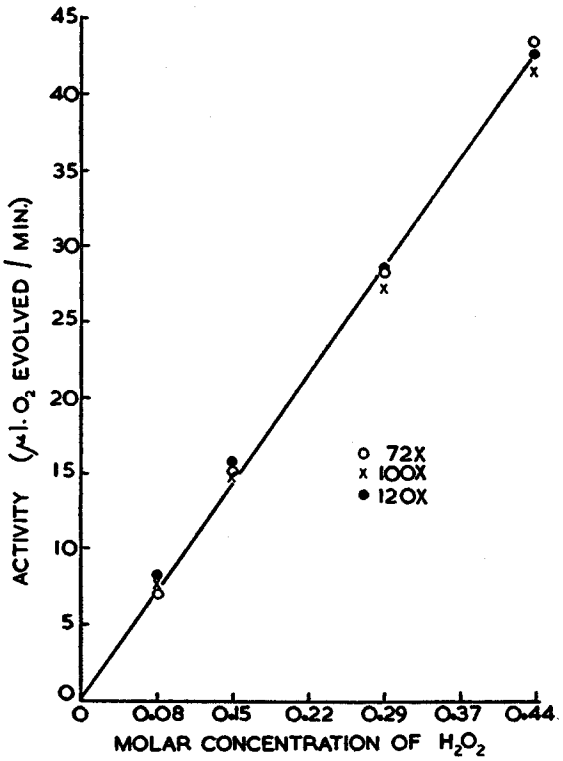

FIG. 1. Variation of activity with substrate concentration at three rates of agitation (72 ×, 100 ×, and 120 × per minute) of reaction mixture: 2 cc. unaltered intracellular yeast catalase (0.03 per cent yeast and 0–0.44 M H_2O_2). Each point represents the average of two measurements at 25.0°C.

RESULTS

A. Preliminary Experiments.—It was shown in Paper I that the critique of the manometric technique of catalase assay advanced by Beers and Sizer (13) was without either experimental or theoretical foundation. In order to demonstrate that the pseudo-zero order rate constants obtained by our methods are, contrary to the claims of these authors, related to the laws of mass action governing the catalase H_2O_2 reaction, we have again studied the effect of substrate and enzyme concentration on these constants, as well as the rate of shak-

ing of the reaction vessels. Fig. 1 shows that our velocity constants were directly proportional to substrate concentration, and, contrary to Beers and Sizer (13), independent of rate of shaking. Fig. 2 shows that our rate constants were directly proportional to enzyme concentration. The linearity, also reported in Paper I, of the curves relating manometric activity constants to enzyme and substrate concentrations shows that these constants, even if

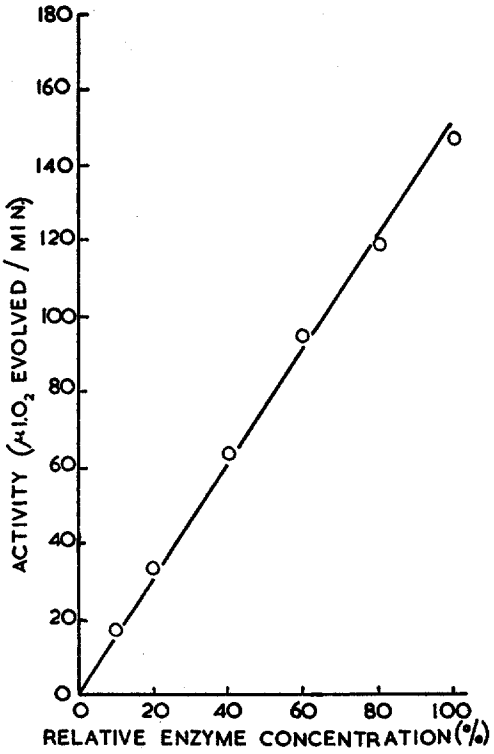

FIG. 2. Variation of activity with enzyme concentration at a rate of agitation of 120 × per minute. Reaction mixtures: 2 cc. of UV-altered intracellular yeast catalase (0–0.01 per cent yeast and 0.15 M H_2O_2) 25°C.

inexact, bear some definite relation to the mass action laws controlling the reaction between enzyme and substrate.

B. Comparison of Manometric and Titrimetric Methods.—Table I presents rate constants, turnover numbers and experimental energies of activation of the crystalline catalase-substrate system, determined manometrically and titrimetrically. It will be noted that there is only a 21 per cent average difference between turnover numbers obtained in these two ways, which is a very much smaller difference than that previously noted by Theorell and Ag-

ner (10). It will be noted also that the μ values are, within experimental error, the same by either method (standard deviations for this constant are given in Table III), since the per cent difference in turnover numbers ($T.N._M/T.N._t \times 100$) is unaffected by temperature within this range. However, the small temperature intervals in the two experiments (5 and 10°) would of course make the μ values quite inaccurate and would probably account for the 4 to 5 kcal. difference in the two experiments.

In view of the data of Table I, conclusions derived from use of manometric rate constants are not artifacts of the manometric technique. All data to be presented below were obtained manometrically, unless otherwise specified.

TABLE I

Comparison of Manometric and Titrimetric Techniques

Experiment No.	Temperature	A	$(T.N.)_m$	μ	K	K^* gm. enzyme/cc.	$(T.N.)_t$	μ	$\frac{(T.N.)_m}{(T.N.)_t} \times 100$
	°C.	*μl./min.*		*kcal./mole*	*min.*$^{-1}$			*kcal./mole*	
1	20	142	9.51×10^3	2.4	0.080	32,000	1.21×10^4	3.8	78.6
	30	163	1.09×10^4		0.099	39,600	1.49×10^4		73.2
2	25	165	1.11×10^4	7.4	0.088	35,200	1.33×10^4	8.3	83.5
	30	203	1.36×10^4		0.111	44,400	1.68×10^4		81.0
Average									79.1

* By analogy to "Kat f."

C. Thermodynamic Constants of Enzyme-Substrate Systems.—Variation of catalase activity with temperature of the reaction mixture of a standard suspension of unaltered yeast is demonstrated in Fig. 3. After a short lag period of unknown significance,[2] all curves became linear, thus permitting calculation of pseudo-zero order activity constants. When $\log_{10}$ activity is plotted against $1/T$°K. in the Arrhenius plot, we obtain the remarkably linear curves shown in Fig. 4. It will be immediately obvious, from curve A, that the activity of the unaltered yeast catalase was much more sensitive to changes in temperature than was that of either B, altered or C, extracted yeast, or of D, crystalline liver, catalase. This means that the μ value for the unaltered enzyme-substrate system was higher than that of the other systems studied; the magnitude of this difference is indicated on the figure.

In these Arrhenius plots, we have observed that the curve for the unaltered enzyme was linear through 40°C., whereas those for the altered intracellular,

[2] Dr. Britton Chance informs us that the lag period is unrelated to the degree of saturation of the reaction medium with O_2.

extracted, and crystalline catalases were linear through 30°, 25°, and 20°C., respectively, above which temperatures they fell off the curve in the direction of lower activity, as illustrated in Fig. 4, curve C. This appears to be due to the greater sensitivity of the altered, extracted, and crystalline enzymes, to inactivation by H_2O_2, demonstrated in Paper I; if this be so, the inactivation of catalase by its substrate, here demonstrated, must be itself a temperature-sensitive process, as has been found by Williams (14).

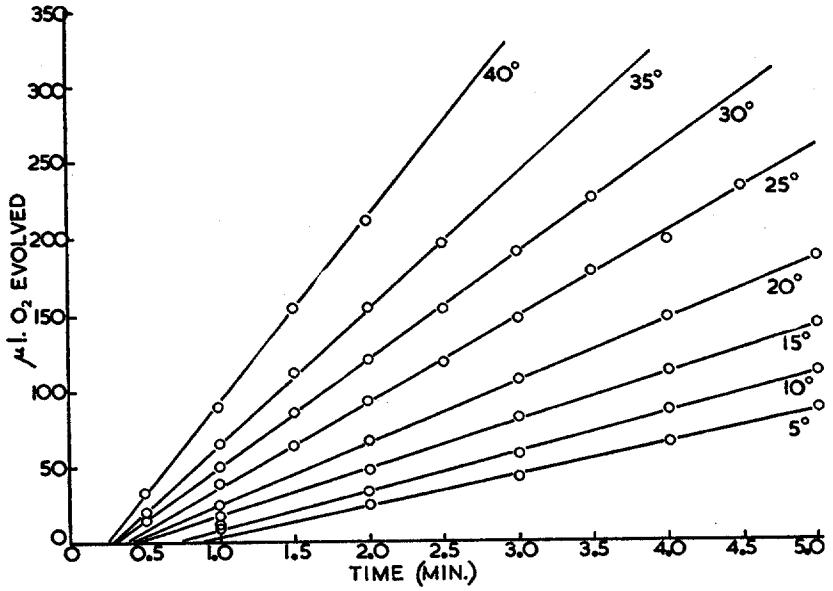

FIG. 3. O_2 evolution curves for unaltered intracellular yeast catalase (0.09 per cent yeast, with 0.44 M H_2O_2) from 5–40°C.

The μ values thus obtained may now be substituted in Equation 3, in order to obtain the $\Delta S\ddagger$, but a problem arises in the choice of the bimolecular rate constant, k''. Since, as will be shown below, the enzymatic properties of altered intracellular, extracted, and crystalline catalases do not differ significantly, we have adopted the same k'' value for all, namely, 3.5×10^7 liters $mole^{-1}sec.^{-1}$, a value which is the average of the k'' values of Chance (15) for erythrocyte and of Chance and Herbert (16) for bacterial catalases, corrected to 0°C. on the basis of the μ value of 1410 cal./mole reported by Chance and Herbert (16).

The activity of the unaltered cells bore, at constant temperature, a constant ratio to that of the altered cells, as shown in Table II; the activity of the latter at 30°C. is seen to be 17 times that of the former, and by extrapolation of the Arrhenius plots, to be 26 times at 0°C. Another way in which the ratio of ac-

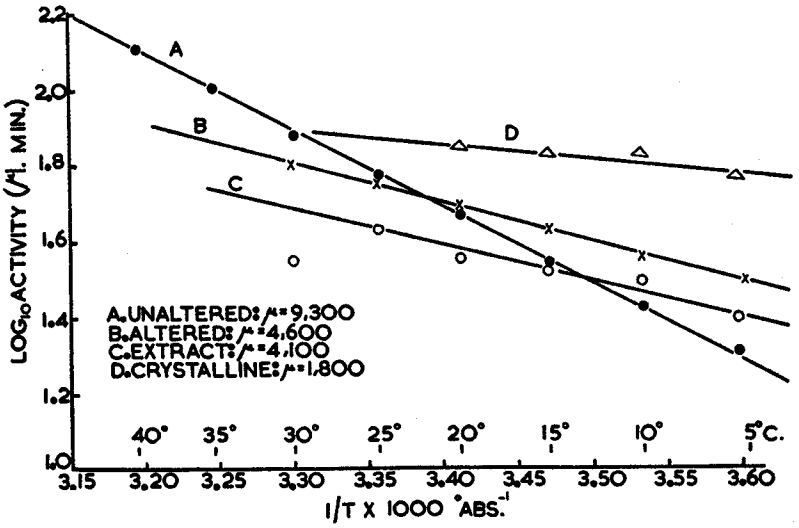

FIG. 4. Arrhenius curves of the enzyme-catalyzed decomposition of H_2O_2 for unaltered (A) and altered (B) intracellular yeast catalases, yeast catalase extract (C), and crystalline beef liver catalase (D).

TABLE II

Ratio of Activities of Unaltered and Altered Yeast Catalase at Standard Yeast Cell Concentration

Activity of altered catalase ÷ Activity of unaltered catalase	
30°C. (observed), n = 7	0°C. (calculated)
17.4 ± 3.2	26.0 ± 4.8

tivities at 0°C. may be calculated, is by making use of Equation 10. We may write, for the altered enzyme, A,

$$k''_A = \frac{kT}{h} \cdot e^{-\Delta F_A \ddagger / RT} \tag{23}$$

and for the unaltered enzyme, U,

$$k''_U = \frac{kT}{h} \cdot e^{-\Delta F_U \ddagger / RT} \tag{24}$$

At 30°C. (303°K.) we know, from Table II that

$$\frac{k''_A}{k''_U} = 17.4 = e^{(\Delta F_U \ddagger - \Delta F_A \ddagger)/1.98 \times 303} \tag{25}$$

from which it follows that

$$\Delta F_U \ddagger - \Delta F_A \ddagger = 1710 \text{ cal./mole.} \tag{26}$$

Hence, at 0°C., (273°K.), it follows that

$$\frac{k''_A}{k''_U} = e^{1710/1.98\times 273} = 24 \tag{27}$$

assuming that $\Delta F_U\ddagger - \Delta F_A\ddagger$ changes negligibly in this 30° range. This ratio agrees well with that determined graphically by extrapolation (Table II). Therefore, in order to obtain a value of k_U at 0°, we shall correct the bimolecular rate constant used for the altered and extracted enzymes by the factor 1/26 (Table II). That the extent of the Euler effect may also be determined by the physiological state of the yeast cell is suggested by the work of Yamahuzi *et al.* (24).

On the basis of the 26-fold difference of k'' for altered and unaltered enzymes, we may calculate the $\Delta S\ddagger$ and $\Delta F\ddagger$ values, as described above. The results are summarized in Table III. It will be remarked that the experimental energy, enthalpy, entropy, and free energy of activation of the unaltered enzyme-substrate system are all significantly higher (in all but one case at the 1 per cent level) than the same constants for the altered, extracted, or crystalline enzyme-substrate systems. It will be also remarked that these constants do not significantly differ (even at the 10 per cent level) among the latter three enzyme-substrate systems. Section B shows that all altering agents apparently alter the enzyme in the same way, at least as far as these thermodynamic constants are concerned. This confirms the evidence presented in Paper I, that crystalline liver catalase, and extracted yeast catalase behave in all respects like the intracellular enzyme altered by any of the agents used, and that all of these catalases differ significantly from the yeast enzyme *in situ* within the untreated cell. That these data are consistent with the interfacial hypothesis will be made clear in the Discussion.

We felt it necessary to recalculate $\Delta S\ddagger$ and $\Delta F\ddagger$ in order to see how they would vary if they were determined on the basis of a second order rate constant and μ value obtained from our own experiments, rather than from those of Chance. We substitute in Equation 14 a titrimetric value of K, 0.080 min.$^{-1}$, obtained from Table I (T = 20°C.), and a substrate concentration of 0.044 M. We solve for v_0 and obtain a value of 1.35×10^{-4} moles H_2O_2/liter/sec. The enzyme concentration used in this experiment was 2.5×10^{-6} gm./ml., or on the basis of a molecular weight of 225,000, 1.13×10^{-8} moles catalase/liter.

Since the initial velocity obviously depends also on the enzyme concentration, a factor which was assumed to be constant in Equation 14, it is clear that the first order rate constant, K, actually must include this term:—

$$\mathrm{K} = k''[E] \tag{28}$$

in which k'' is the specific second order rate constant, and (E) is the concentration of enzyme. We may substitute this for K in Equation 14, to obtain

$$v_0 = k''[E][S] \tag{29}$$

TABLE III

Thermodynamic Constants for Catalase H_2O_2 Reaction ($pH = 7.2$) Using Chance's $(k'')_{0^\circ} = 3.5 \times 10^7$ Liter Mole^{-1} Sec.$^{-1}$

Starred values obtained by short method.

μ	$\Delta H\ddagger$	$\Delta S\ddagger$	$\Delta F\ddagger$
kcal./mole	*kcal./mole*	*e.u.*	*kcal./mole*
A. Unaltered intracellular yeast catalase			
+12.1	+11.6	+12.3	+8.2
8.2	7.7	−2.0	8.2
9.3	8.8	+2.0	8.3
9.2	8.7	+1.7	8.2
9.4	8.9	+2.4	8.2
9.2*	8.7	+1.6	8.3
7.6*	7.1	−4.2	8.2
7.8*	7.3	−3.5	8.2
8.0*	7.5	−2.8	8.3
+9.0 ± 1.4	+8.5 ± 1.4	+0.8 ± 5.0	+8.2 ± 0.1
B. n-Propanol altered intracellular yeast catalase			
+4.6	+4.1	−8.7	+6.5
5.2	4.7	−6.5	6.5
7.2	6.7	+0.8	6.5
5.4	4.9	−5.8	6.5
4.6	4.1	−8.7	6.5
5.3*	4.8	−6.2	6.5
3.3*	2.8	−13.5	6.5
3.1*	2.6	−14.2	6.5
3.5*	3.0	−12.7	6.5
2.3*	1.8	−17.1	6.5
+4.5 ± 1.4	+4.0 ± 1.4	−9.3 ± 5.2	+6.5 ± 0.0
B′. Chloroform altered intracellular yeast catalase			
+3.4*	+2.9	−13.1	+6.5
B². Uv light–altered intracellular yeast catalase			
+3.7*	+3.2	−10.2	+6.5
C. Yeast catalase extract			
+4.5	+4.0	−10.6	+6.9
2.9	2.4	−17.3	7.1
4.1	3.6	−12.3	7.0
+3.8 ± 1.3	+3.3 ± 1.3	−13.4 ± 3.5	+7.0 ± 0.1

TABLE III—*Concluded*

	μ	$\Delta H\ddagger$	$\Delta S\ddagger$	$\Delta F\ddagger$
	kcal./mole	*kcal./mgle*	*e.u.*	*kcal./mole*
D. Beef liver catalase				
	+4.7	+4.2	−9.7	+6.8
	5.1	4.6	−8.0	6.8
	1.8	1.3	−22.0	7.3
	+3.9 ± 1.8	+3.4 ± 1.8	−13.2 + 7.6	+7.0 + 0.3
Differences between the means				
A–B	+4.5 ± 0.7 (P < 0.01)	+4.5 ± 0.7 (P < 0.01)	+10.1 ± 2.4 (P < 0.01)	+1.7 ± 0.02 (P < 0.01)
A–C	+5.2 ± 1.0 (P < 0.01)	+5.2 ± 1.0 (P < 0.01)	+14.2 ± 2.7 (P < 0.01)	+1.2 ± 0.06 (P < 0.01)
A–D	+5.1 ± 1.3 (P < 0.01)	+5.1 ± 1.3 (P < 0.01)	+14.0 ± 5.1 (P < 0.05)	+1.2 ± 0.2 (P < 0.01)
B–C	+0.7 ± 0.9 (P > 0.1)	+0.7 ± 0.9 (P > 0.1)	+4.1 ± 2.7 (P > 0.1)	−0.5 ± 0.3 (P > 0.1)
B–D	+0.6 ± 1.2 (P > 0.1)	+0.6 ± 1.2 (P > 0.1)	+3.9 ± 4.9 (P > 0.1)	−0.5 ± 0.3 (P > 0.1)

Rearranging, and solving for k'' by substituting the known values of v_0, (E) and (S), we obtain

$$k''_{20^\circ} = \frac{1.35 \times 10^{-14} \text{ moles } H_2O_2 \text{ liter}^{-1} \text{ sec.}^{-1}}{1.13 \times 10^{-8} \text{ moles catalase liter}^{-1} \times 0.044 \text{ moles } H_2O_2 \text{ liter}^{-1}} \tag{30}$$

$$= 2.7 \times 10^5 \text{ liter moles catalase}^{-1} \text{ sec.}^{-1}$$

This value of k'' may be corrected to 0°C., by substituting in Equation 1 a value of μ (3,900 cal./mole) found in Table III, section D, yielding $k''_0{}^\circ$ = 1.7×10^5 liter/mole enzyme/sec. The two thermodynamic constants recalculated substituting this value of k'' in the Eyring equation are presented in Table IV. Again, the unaltered enzyme is seen to be different from the altered, extracted, and crystalline forms; the $\Delta F\ddagger$ values for these enzyme-substrate systems are all approximately 3 kcal./mole higher and the $\Delta S\ddagger$ values about 10 e.u. lower, than those calculated using Chance's velocity constant.

It will be noted from Equations 25 and 26, that it is possible to calculate the difference in $\Delta F\ddagger$ between altered and unaltered enzyme-substrate systems, given only the ratio of their activity, determined manometrically, at constant temperature. The difference thus obtained is indistinguishable from that

obtained by substituting Chance's second order rate constant (and $\frac{1}{26}$th this value for unaltered enzyme) into the Eyring equation, then calculating the $\Delta F_U\ddagger$ and $\Delta F_A\ddagger$, and subtracting to obtain the difference; when done this way, $\Delta F_U\ddagger - \Delta F_A\ddagger = 1700$ cal./mole (Table III). From the Arrhenius plot, we have obtained the difference in μ between altered and unaltered systems, and we may thus calculate the difference in $\Delta H\ddagger$ ($\Delta_{H\ddagger}$) which, from Table III, is 4500 cal./mole. Given this, and the known difference in $\Delta F\ddagger$ ($\Delta_{\Delta F\ddagger}$), we may

TABLE IV

Thermodynamic Constants for Catalase H_2O_2 Reaction Calculated Using $(k'')_{0°} = 1.7 \times 10^5$ Liter mole^{-1} Sec.$^{-1}$

Catalase preparation	$\Delta S\ddagger$	$\Delta F\ddagger$
	e.u.	*kcal./mole*
A. Unaltered intracellular yeast catalase	-9.5 ± 5.6	$+11.1 \pm 0.2$
B. Altered intracellular yeast catalase	-21.4 ± 6.1	$+9.8 \pm 0.2$
C. Yeast catalase extract	-24.0 ± 3.5	$+9.9 \pm 0.1$
D. Crystalline beef liver catalase	-23.9 ± 9.6	$+10.0 \pm 0.2$
E. Crystalline beef liver catalase, Sizer* (18)	-22.5	$+9.8$

* Statistical treatment and sample size not given; value of $\Delta F\ddagger$ in table calculated from $\mu = 4.2$ kcal./mole and $\Delta H\ddagger = +3.7$ kcal./mole.

calculate directly the difference in entropy of activation of altered and unaltered enzyme-substrate systems ($\Delta_{\Delta S\ddagger}$) from the equation

$$\Delta_{\Delta F\ddagger} = \Delta_{\Delta H\ddagger} - T\Delta_{\Delta S\ddagger} \tag{31}$$

At 0°C.,

$$1710 \text{ cal./mole} = 4500 \text{ cal./mole} - 273°\text{K.}\ (\Delta_{\Delta S\ddagger}) \tag{32}$$

from which we may calculate $\Delta_{\Delta S\ddagger}$ directly as 10.2 cal. mole^{-1} deg.$^{-1}$. The difference obtained by actually subtracting the measured values of $\Delta_{S\ddagger A}$ from those of $\Delta_{S\ddagger U}$ was 10.1 cal. mole^{-1} deg.$^{-1}$ (Table III).

D. Effect of Enzyme and Substrate Concentrations on μ.—It was at this point necessary to demonstrate that the thermodynamic constants, as well as the differences between them, were valid generally, and not simply for the conditions of enzyme and substrate concentrations obtaining thus far in our experiments. It was shown in Figs. 1 and 2 that at constant temperature the relation between activity and enzyme or substrate concentrations is linear. If these relations remain linear at the other end of the temperature range used in these studies, it follows that μ would be independent of enzyme and

substrate concentrations, since the same μ value would be obtained by comparing points anywhere along two lines coming from the origin of a graph relating activity to enzyme (or substrate) concentration at two different temperatures.

Fig. 5 represents the variation in catalase activity of altered and unaltered cells as a function of enzyme concentration, at 5 and 25°C. The curves are linear at both temperatures, showing that the energies of activation are independent of the concentration of cells, at least within the range examined. As explained above, the slope of these curves may be used to calculate μ from

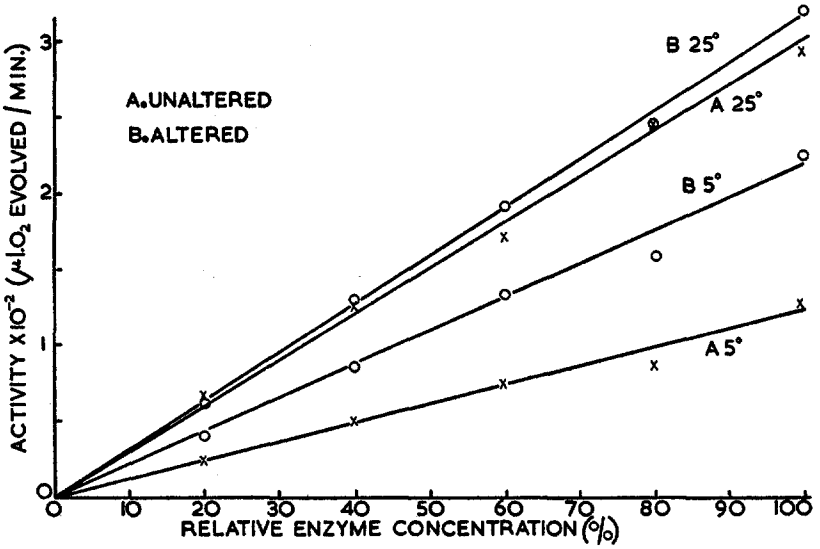

FIG. 5. Variation of activity with enzyme concentration at 5 and 25°C. A. Unaltered intracellular yeast catalase (0–0.18 per cent yeast, 0.44 M H_2O_2). B. Altered intracellular yeast catalase (0–0.01 yeast, 0.44 M H_2O_2). A curves give μ_A = 3,300 cal./mole, B curves give μ_{UN} = 7,600 cal./mole.

Equation 1. The μ values calculated by this short method have been presented, starred, in Table III, and are not statistically distinguishable from the values obtained by the longer method, in which a standard cell suspension is subjected to 6 or 8 different temperatures.

Fig. 6 shows the effect of substrate concentration on the activity of yeast suspensions at 5 and 25°C. From part A, it is noted that there is, for the unaltered enzyme, a linear relation between activity and substrate concentration throughout the range examined, at both temperatures, giving a μ value of 7,800 cal./mole, a value only slightly lower than those obtained by the long method (Table III). Part B, the propanol-altered catalase, shows a linear relation between activity and substrate concentration up to 0.13 M, above

which the curves fall off the line at both temperatures. Below 0.13 M, the activation energy for the altered enzyme-substrate system, calculated from the slopes, is 3.1 kcal.; above this concentration, the μ values calculated for 0.145 M, 0.295 M, and 0.44 M H_2O_2 are respectively 3.3, 4.2, and 3.5 kcal./mole. Since the mean value of μ in Table III (which does not include data from ex-

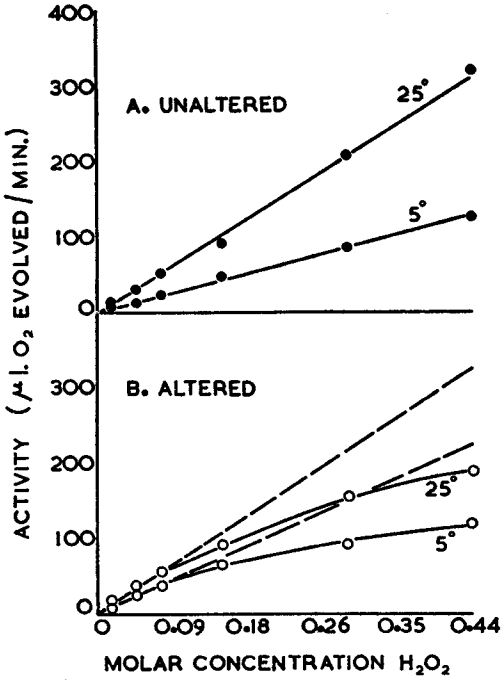

FIG. 6. A. Variation of activity with substrate concentration at 5 and 25°C. for unaltered intracellular yeast catalase (0.18 per cent yeast, 0–0.44 M H_2O_2). Curves give μ = 7,800 cal./mole. B. Variation of activity with substrate concentration at 5 and 25°C. for *n*-propanol-altered intracellular yeast catalase (0.01 per cent yeast). Linear portions of curves give μ = 3,100 cal./mole; points at 0.15, 0.29, and 0.44 M H_2O_2 give μ values of 3,300, 4,200, and 3,500 cal./mole respectively.

periments in which substrate concentration was varied) was 4.5 ± 1.4 kcal./mole, it appears that activation energies do not change with varying concentration of H_2O_2, even though the activity of these altered preparations deviates from linearity at higher substrate concentrations. As opposed to the temperature-sensitive substrate-induced inactivation of catalase demonstrated in section B above, the inactivation of catalase by H_2O_2 shown in Fig. 6 is *not* temperature-sensitive, since μ values are the same all along these curves. The evidence suggests, but does not establish, identification of these two distinct

types of H_2O_2-induced inactivation of catalase with the formation of the two inactive enzyme-substrate complexes (one reversible, one irreversible), in the reaction mechanism of Chance (9).

Fig. 6, B, shows that at 0.44 M, the concentration of substrate used in determination of thermodynamic constants, the activity of the altered enzyme has decreased by about one-third from the expected value (*i.e.*, if there were no inhibition by substrate); at this concentration, there was no inhibition of the unaltered enzyme. Hence, the observed difference in activity of altered and unaltered enzymes would have been larger had we worked at lower substrate concentrations, and this would have had the effect of exaggerating the differences between $\Delta S‡$ and $\Delta F‡$ values of altered and unaltered enzyme-substrate systems. For example, the $\Delta_{\Delta F‡}$ would have been approximately 2100 cal./mole, instead of the 1700 cal. reported in Table III.

E. Effect of pH on Thermodynamic Constants of Enzyme-Substrate Systems.— It was reported in Paper I that altered and unaltered enzymes respond differently to change in pH, and that cells, in which the catalase has been fully altered, have been killed by the alteration process.[3] Since the pH of the living yeast cell is given as 5.8 (Conway and O'Malley (17)), it seemed possible that some of the changes in enzyme properties following alteration might be due to an increase in the effective pH of the intracellular enzyme, as the dead cell comes into equilibrium with the medium, buffered at pH 7.2. This hypothesis was tested by performing four experiments in which activity of altered and unaltered suspensions, of different concentration, were determined at two temperatures and at varying pH; this permitted determination of μ values by the short method, and, from these, calculation of the other constants. The results of a typical experiment are presented in Table V.

Section A reveals that the activity and the thermodynamic constants of the unaltered catalase do not change within the pH range examined (5.3–8.0). On the other hand, the activity of the altered enzyme diminished appreciably at pH 6.0 and at this pH there is a rise in the $\Delta H‡$. There is also a compensatory rise in the $\Delta S‡$, with the result that, even at pH 4.0, where the $\Delta H‡$ has risen to within the usual range for the unaltered enzyme-substrate system, there has been only a slight change in the $\Delta F‡$. We may conclude that it is

[3] Although cells in which enzyme alteration is complete are dead cells (as determined by inability to grow on nutrient medium), cells killed by heat (Paper I) possess a catalase of unchanged kinetic properties, as do cells killed by a brief exposure to UV (unpublished data). Further, it was shown in Paper II that alteration and killing by *n*-butanol were unrelated processes. Hence the kinetic changes in catalase caused by altering agents do not follow simply from the death of the cell. Indeed, the *unaltered* cells in the heat inactivation studies (section F following) were probably dead, since 30 minutes at 56°C. *i.e.* 2°C. below the range used in the present studies, was sufficient to cause almost complete sterilization of a yeast population (Paper I).

possible that some, but not all, of the changes in kinetic properties of the catalase-substrate system which accompany enzyme alteration may be due to a rise in the intracellular pH. This point will be discussed below.

F. Thermodynamic Constants for the Heat Destruction Process.—Preheating the various enzyme preparations caused an irreversible loss in catalase activity when this was subsequently assayed at 30°C. Fig. 7 shows the effect of heating for 0 to 10 minutes an unaltered cell suspension while shaking at 63°C.; during this 10 minute period, there has been more than a 6-fold reduc-

TABLE V

Effect of pH on Thermodynamic Constants for the Enzyme Catalyzed Reaction

Activities of unaltered intracellular yeast catalase measured with 0.18 per cent yeast and 0.44 M H_2O_2, and of altered intracellular yeast catalase with 0.01 per cent yeast and 0.44 M H_2O_2, the latter being corrected for this 18-fold dilution of cells.

pH	Activity at 5°C.	μ	$\Delta H\ddagger$	$\Delta S\ddagger$	$\Delta F\ddagger$
	µl./min.	*kcal./mole*	*kcal./mole*	*e.u.*	*kcal./mole*
A. Unaltered intracellular yeast catalase					
5.3	111	+7.8	+7.3	−3.9	+8.4
6.0	115	+8.2	7.7	−2.0	8.2
7.1	106	+7.6	7.1	−4.2	8.2
8.0	103	+8.0	7.5	−2.8	8.2
B. n-Propanol altered intracellular yeast catalase					
4.0*	582	+9.8	+9.3	+7.8	+7.2
5.3	1197	7.1	6.6	+0.4	6.5
6.0	1782	5.0	4.5	−9.2	6.5
7.1	2070	3.3	2.8	−13.5	6.5
8.0	2133	3.5	3.0	−12.7	6.5

* k''_0° value corrected for lowered activity in calculation of $\Delta S\ddagger$ and $\Delta F\ddagger$ values. In other cases small variation makes this correction unnecessary.

tion in activity. Within the temperature range chosen for any given catalase preparation (intra- or extracellular) the inactivation takes place very slowly at the lower end of the range, and very rapidly 7 or 8° higher. When $\log_{10}$ residual activity is plotted against time, a series of straight lines is obtained, showing that the heat destruction is a first order process. Fig. 8 presents such a plot for the destruction of the unaltered, intracellular enzyme. In the case of the unaltered catalase, one invariably observes a marked lag phase, during which there is often remarked a slight *increase* in activity; the duration of the lag phase varies inversely with temperature. Following the lag, the diminution of activity is seen to follow a first order course. In the case of the altered intracellular enzyme, or of the extracted and crystalline catalases, such a lag period has never been observed by us; as shown in Fig. 9, the heat destruction process

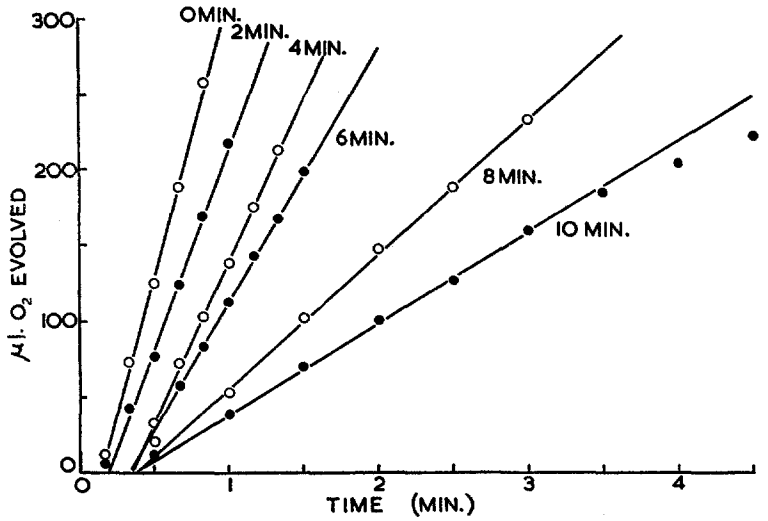

FIG. 7. O_2 evolution curves for unaltered intracellular yeast catalase (0.18 per cent yeast tested at 30°C. with 0.44 M H_2O_2) heated at 63°C. for 0, 2, 4, 6, 8, and 10 minutes, giving activities of 385, 277, 215, 167, 87 and 61 μl. O_2/minute.

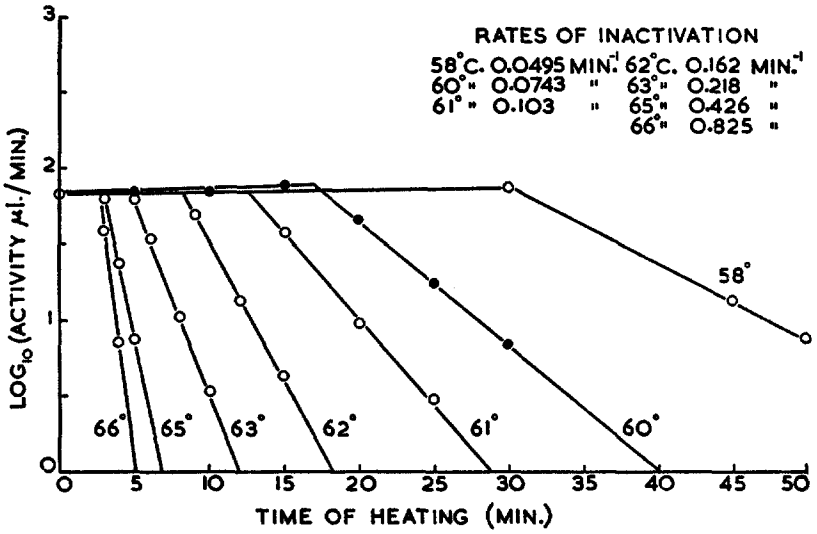

FIG. 8. Heat inactivation curves for unaltered, intracellular catalase (0.36 per cent yeast), heated in the range 58–66°C. The 60° curve is shown in greater detail to illustrate the lag phase. Each point is the average of two activity determinations.

follows a strict first order course from the beginning of the heating of the preparation.

The slopes of these first order curves are a measure of the rate of heat destruction at the temperature at which they were measured, and may be used for the Arrhenius treatment. Typical curves are plotted in Fig. 10: all curves are linear, showing that the rates of heat destruction of unaltered and altered intracellular, catalase, and of extracted yeast and crystalline liver catalases vary logarithmically with temperature according to the Arrhenius equation. The temperature range within which inactivation is conveniently measurable is strikingly different for the unaltered (58–66°C.) than for the other preparations (*ca.* 45–55°C.), confirming the evidence in Paper I. Experimental ac-

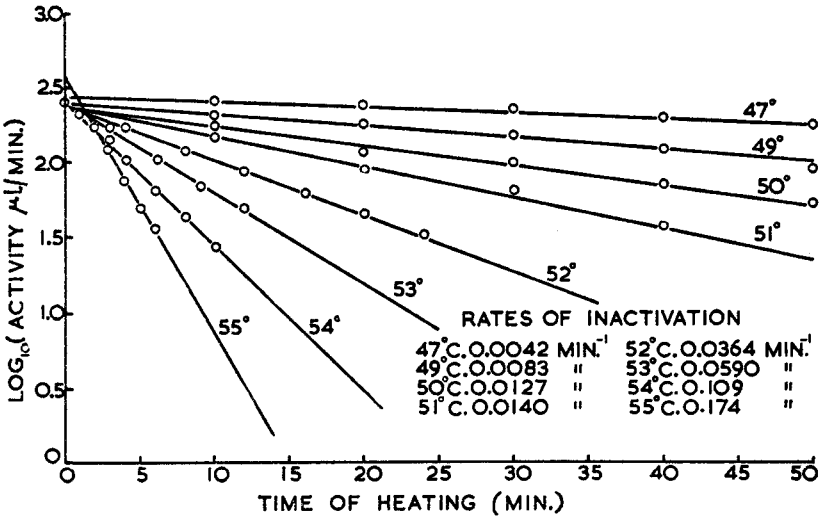

FIG. 9. Heat inactivation curves for *n*-propanol-altered, intracellular catalase (0.02 per cent yeast) heated in the range 47–55°C. Each point is the average of two activity determinations.

tivation energies were calculated from the slopes of these curves, and the other thermodynamic constants calculated from μ, using an experimentally determined value of k' ($\log_e$ and seconds^{-1}) to substitute in the Eyring equation.

These data have been collected in Table VI. The mean μ and $\Delta H\ddagger$ for the unaltered catalase are 20.6 kcal./mole lower than those for the altered, but the difference between the means is not quite significant at the 5 per cent level ($p = 0.1$). However, when the method of paired comparisons is used, in which unaltered and altered values were paired in chronological order, as presented in Table VI (including two altered-unaltered pairs, actually run as controls for each other), the average difference between the individual paired values of μ ($\mu_U - \mu_A$) is -22.2 ± 9.6, (in which 9.6 is the standard error of

the difference) which is significant at the 5 per cent level. The reason for which the difference in this constant for altered and unaltered catalases is less significant than that in the case of $\Delta S\ddagger$ and $\Delta F\ddagger$, is the large scatter in μ values of the unaltered enzyme.

Despite the fact that there is overlap in the $\Delta S\ddagger$, the 70 e.u. difference between the mean values for altered and unaltered is significant at the 1 per cent level: there is a greater entropy increase upon activating the heat destruction of the altered enzyme than occurs upon activating the destruction of the un-

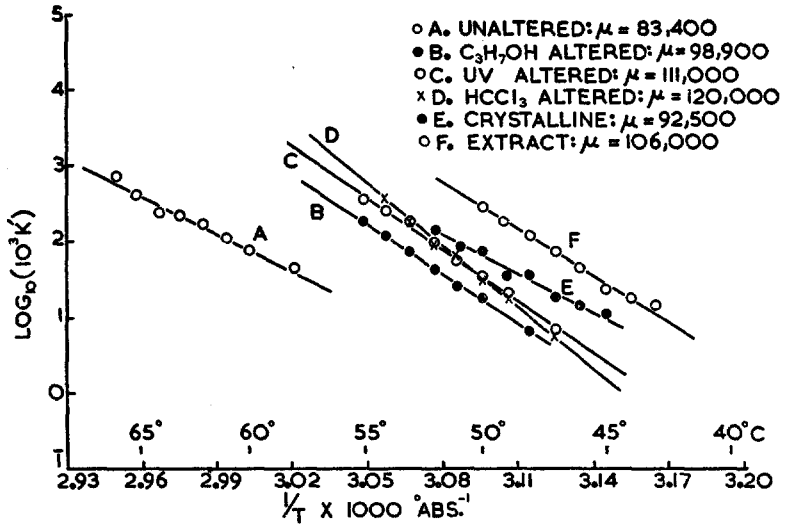

FIG. 10. Arrhenius curves for the heat inactivation of: A, unaltered, intracellular yeast catalase (58–66°C.); B, *n*-propanol-altered intracellular yeast catalase (47–55°C.); C, UV-altered intracellular yeast catalase (47–55°C.); D, chloroform-altered intracellular yeast catalase (47–55°C.); E, crystalline beef liver catalase (45–52°C.); F, extracted yeast catalase (43–51°C).

altered enzyme. On the other hand, there is no overlap in the $\Delta F\ddagger$ values, those for the unaltered catalase being 2.8 $\pm$ 0.3 kcal./mole *higher* than for the altered, intracellular enzyme; this difference is also significant at the 1 per cent level. All our $\Delta F\ddagger$ values for the various enzyme preparations, were between 21 and 24 kcal./mole, which are within the narrow range characteristic of the free energy of activation of the heat modification of protein structure and of the heat inactivation of enzymes (see reference 6).

We attempted to study the effect of pH on the thermodynamic constants of the heat inactivation process, but did only two complete experiments, owing to the enormous amount of experimental work involved. While the paucity of data precluded statistical treatment of the results and makes it impossible for

TABLE VI

Thermodynamic Constants for the Heat Inactivation of Catalases

Except when indicated $\Delta F\ddagger$ values are calculated at 60°C. $\Delta H\ddagger$ and $\Delta S\ddagger$ values of *A* calculated at 62°, of *B* at 52°, and of *C* and *D* at 50°C.

μ	$\Delta H\ddagger$	$\Delta S\ddagger$	$\Delta F\ddagger$
kcal./mole	*kcal./mole*	*e.u.*	*kcal./mole*
A. Unaltered intracellular yeast catalase			
+70.8	+70.1	+140	+23.5
64.4	63.7	122	23.1
75.9	75.2	156	23.3
103.0	102.3	236	23.7
121.9	121.2	293	23.6
120.5	119.8	288	23.9
111.3	110.7	261	23.8
+95.4 ± 24.5	+94.7 ± 24.5	+214 ± 73	+23.6 ± 0.3
B. Altered intracellular yeast catalase			
Alteration by n-propanol			
+98.9	+98.3	+231	+21.4
104.0	103.4	247	21.1
121.4	120.8	298	21.6
131.6	131.0	329	21.4
Alteration by chloroform			
134.0	133.4	341	19.8
120.0	119.4	298	20.2
Alteration by ultraviolet light			
111.0	110.4	270	20.5
107.0	106.4	259	20.2
+116.0 ± 12.8	+115.3 ± 12.8	+284 ± 39	+20.8 ± 0.7
C. Yeast catalase extract			
+99.4	+98.7	+236	20.1
106.0	105.3	254	20.7
120.0	119.3	296	20.7
D. Crystalline beef liver catalase			
+92.5	+91.8	+214	+20.5
88.3	87.6	201	20.7
E. Crystalline beef liver catalase (Sizer)			
Below 62°C. +55.0	54.3*	+90	+24.2*
Above 62°C. +255.0	254.3*	+290	+23.2*
F. Differences between the means			
A–B			
−20.6 ± 10.6 (P < 0.1)	−20.6 ± 10.6 (P < 0.1)	−70 ± 30 (P < 0.01)	2.8 ± 0.3 (P + 0.01)

* Calculated by us for $T = 62°C$.

us to regard these data as definitive, the results of the two experiments were in qualitative agreement, and one of them is presented in Table VII. It will be noted, from section A, that the constants for the unaltered enzyme appeared to be invariant in the range pH 5.3–8.0, whereas, from section B, there seemed to be at pH 5.3 a large decrease in μ and, of course, $\Delta H\ddagger$, which, since the $\Delta F\ddagger$ remained constant, caused a large compensatory decrease in $\Delta S\ddagger$. Thus, at low pH the $\Delta H\ddagger$ and $\Delta S\ddagger$ of the altered enzyme were not different from those of the unaltered, whereas the $\Delta F\ddagger$ continued to be below the range characteristic of the unaltered enzyme. The altered enzyme thus appeared to possess a maximum energy of activation for the heat destruction process at the pH of maximum activity (see Table V) and of minimum μ for the enzyme-substrate

TABLE VII

Effect of pH on Thermodynamic Constants for the Heat Inactivation of Catalases

All $\Delta F\ddagger$ values calculated at 60°C, $\Delta H\ddagger$ and $\Delta S\ddagger$ values calculated at 62° in *A*, at 52° in *B*.

pH	μ	$\Delta H\ddagger$	$\Delta S\ddagger$	$\Delta F\ddagger$
	kcal./mole	*kcal./mole*	*e.u.*	*kcal./mole*
A. Unaltered intracellular yeast catalase				
5.3	+98.9	+98.2	+226	+22.9
7.1	110.4	109.7	257	24.1
8.0	96.6	95.9	217	23.6
B. n-Propanol altered intracellular yeast catalase				
5.3	+94.3	93.6	+217	+21.3
7.1	131.6	131.0	329	21.4

system; this is exactly what one would expect on the basis of the treatment of Glasstone *et al.*, discussed above in the Theoretical section.

DISCUSSION

There can be no doubt that the phenomenon of enzyme alteration, demonstrated in Paper I, has been unequivocally confirmed in this paper, in which highly significant differences in the kinetics of intracellular catalase, before and after treatment of the cells with the altering agents $CHCl_3$, UV, and C_3H_7OH, have been observed. We must now see to what extent these differences are consistent with the interfacial hypothesis of enzyme alteration, which states that the enzyme within the intact, unaltered cell is in a structurally less specific, partially unfolded condition and that following desorption, it is converted to the more specific, more active, altered configuration. The basis of interpretation of these differences in thermodynamic constants has been presented in the Theoretical section; much of what follows will be speculation.

As a result of the lack of significant difference in any of the kinetic properties among altered intracellular, extracted yeast and crystalline liver catalases, our data have confirmed the evidence presented in Paper I showing that the extracted and crystalline enzymes have also undergone enzyme alteration, and are in a state identical with, or very similar to, that of the altered intracellular catalase. We shall therefore consider the term "altered" to embrace the extracted and the crystalline catalases, as well.

The essential facts, with respect to the enzyme-substrate systems will now be enumerated: (*a*) the activity of the altered enzyme is greater by more than an order of magnitude than that of the unaltered; (*b*) the energy of activation of the altered enzyme is lower; (*c*) the entropy of activation of the altered enzyme has a comparatively large negative value, by contrast to the unaltered; (*d*) the free energy of activation of the altered enzyme-substrate system is lower than that of the unaltered.

(*a*) The activity of an enzyme should be a measure of its structural specificity. The low activity of the unaltered enzyme is consistent with its existence within the cell in a partially unfolded condition.

(*b*) The more efficient the catalyst, the lower the energy of activation required to activate decomposition of its substrate, or to put it operationally, the less should the rate of its action vary with temperature. The μ, $\Delta H\ddagger$ values for the altered enzyme have been shown to fulfill this expectation.

(*c*) Sizer (18) has shown that the $\Delta S\ddagger$ of the crystalline catalase-substrate system is a negative quantity (with reference to the standard state used by him and by us); he has interpreted this finding, plausibly, as indicating an increase in order upon formation of the activated enzyme-substrate complex. We have found that the more active, altered enzyme reduces its entropy on formation of the activated complex by about 10 e.u. more than does the unaltered; it was possible to calculate this, knowing only the differences in activity and in μ value between altered and unaltered enzyme-substrate systems.

(*d*) At any given temperature, the more rapid the reaction, the lower the free energy of its activation. The significantly lower (approximately 1.5 kcal./mole) $\Delta F\ddagger$ of the altered enzyme-substrate system probably accounts for its much greater activity; *i.e.*, point (*a*).

The essential changes in the kinetics of heat destruction of catalase which follow alteration will also be enumerated: (*A*) the altered enzyme is rapidly destroyed by treatment at temperatures and durations to which the unaltered catalase is quite indifferent. (*B*) The μ and $\Delta H\ddagger$ for this process have been found to be higher for the altered enzyme. (*C*) The $\Delta S\ddagger$ was also considerably higher for the altered enzyme. (*D*) On the other hand, the $\Delta F\ddagger$ was lower after alteration.

A. The relative stability of the unaltered enzyme to heat, and the rather long lag periods before appreciable heat destruction commences, might be

imagined as being due to the many weak linkages which hold the catalase adsorbed to its intracellular interface. The altered, desorbed enzyme would not be protected in this way.

B. The interfacially bound protein is already partially opened up within the cell, whereas the soluble, altered enzyme possesses certain weak valence forces which confer upon it its high degree of specificity. Hence, the energy required to activate the destruction of the former by heat should be lower than that required for the latter, since these extra bonds must be destroyed. This is consistent with the observed 21 kcal./mole increase in μ and $\Delta H\ddagger$, following alteration.

C. If alteration causes only a relatively small change in $\Delta F\ddagger$ of heat destruction (which is what we should anticipate, given the very narrow range of $\Delta F\ddagger$ values for a wide variety of proteins (6)), the increase in $\Delta H\ddagger$ following alteration should be accompanied by a compensatory increase in $\Delta S\ddagger$. In terms of the interfacial hypothesis, the increase in entropy, which occurs upon formation of the activated complex for the unaltered catalase, is low since the protein, partially extended at the interface, is already in a relatively disordered, less specifiable state. Thus the 70 e.u. increase in the $\Delta S\ddagger$ of the altered enzyme is consistent with this hypothesis.

D. The rate of heat destruction at any temperature should be determined by the magnitude of the free energy of activation of this process. The relative instability to heat of the altered enzyme must be, at least partially, due to the 3 kcal. decrease in $\Delta F\ddagger$ of heat destruction, which accompanied alteration.

Certain of the changes in activity and thermodynamic constants which occur after alteration could be accounted for if the enzyme existed within the cell in equilibrium with a medium of very low pH (*e.g.*, pH 4.0). It was shown in Paper I that at this pH, the activity of altered catalase was but approximately $\frac{1}{15}$th that of the same enzyme at pH 7 (although from Table V it is seen that, in these experiments, activity at pH 4 was approximately $\frac{1}{4}$th that at pH 7). Similarly, we have shown above that energies and entropies of activation (of both processes studied) for the altered enzyme, change at low pH in a direction and magnitude so as to resemble the unaltered enzyme. According to the pH hypothesis, alteration consists in changing the effective pH of intracellular catalase from 4.0 to that of the external medium (in this case, pH 7.2), thus activating the enzyme and changing certain of its kinetic properties. It is apparent that many of the changes in properties and kinetics following alteration cannot be explained in this manner; for example, the $\Delta F\ddagger$ of both reactions studied increased only slightly at low pH, and further it is difficult to attribute the relative resistance to heat destruction of the unaltered enzyme to its being surrounded by a more acid medium. Yet, admitting that the pH hypothesis of alteration cannot explain a good part of our data, we might admit it tentatively as an explanation for part of them, provided that there

were a reasonable explanation of an effective pH of 4.0 in the neighborhod of catalase, while the lowest estimate of the pH of the intact yeast cell, that of Conway and O'Malley (17), is 5.8. If the catalase existed at an intracellular interface, a reasonable explanation would, in fact, exist, for Danielli has emphasized (19) that hydrogen ions tend to concentrate at interfaces, often producing a pH of as much as 2 units lower at the interface than obtains in the bulk phase. Thus, the pH hypothesis would extend and modify, but not replace, the interfacial hypothesis: part of the decreased structural and enzymatic specificity of the interfacially adsorbed catalase might then be due to the comparatively high local concentration of hydrogen ions at the interface. It might be mentioned that Agner and Theorell (20) found that catalase activity at pH 4 was 70 per cent optimal, whereas Chance (21) has found with his rapid flow methods that activity at pH 4 was undiminished.

Any explanation of the 200-fold difference in k'' between our values (at 0°C., 1.7×10^5 liter mole^{-1} sec.$^{-1}$, titrimetric; 1.4×10^5 liter mole^{-1} sec.$^{-1}$, manometric, calculated on the basis of the 20 per cent reduction in manometric turnover number (see Table I)) and that of Chance (3.5×10^7 liter mole^{-1} sec.$^{-1}$), is beyond the scope of this paper and the capacity of its authors. Suffice it to say that, as we have shown, our conclusions are valid no matter whose k'' we use, although, of course, the constants obtained by the fast flow spectrophotometric techniques of Chance have to be taken more seriously than ours, obtained by more workaday methods. Our values do agree with those calculated by Sizer (18), ($k'' = 3.4 \times 10^5$ liter mole^{-1} sec.$^{-1}$), from the "Kat.f" of Sumner (22). Surprisingly enough, despite the similarity of rate constants, our turnover numbers, corrected to 0°C. (7.5×10^3 mols. H_2O_2/mol. catalase/sec.) do not at all agree with Sizer's (3.4×10^5 mols. H_2O_2/mol. catalase/sec., which is numerically equal to his k'', quoted directly above); the reason for the disparity is that Sizer failed to correct for the substrate concentration used in Kat.f determinations (usually 0.01 M), whereas his value would be correct only if a substrate concentration of 1 M had been employed. When such a correction is introduced, the turnover number calculated from Sumner's data is only slightly lower than our own: 3.4×10^5 liter mole^{-1} catalase sec.$^{-1}$ $\times$ 0.01 M H_2O_2 = 3.4×10^3 mols. H_2O_2 mol.$^{-1}$ catalase sec.$^{-1}$.

We have been unable to confirm one other interesting finding of Sizer (18), that the energy of activation of the heat destruction process suddenly increases approximately 5-fold at 62–68°C. We have found that in this temperature range the inactivation of even our most heat-resistant preparations (unaltered intracellular) occurs too rapidly to permit accurate rate determinations, especially in view of the finite time required to bring the reaction mixture to the bath temperature.

A comparison of the kinetics of other enzymes inside and outside of the cell led Sizer to the general conclusion that "it thus appears that the activation

energy is not changed by the extraction of an enzyme from cells or tissues" (23). This conclusion is no longer tenable, in view of the results of the present paper.

While the next paper in this series will present a kinetic study of the process of enzyme alteration itself, we may permit ourselves to speculate that the energy of activation of alteration should be, on the basis of the present data, of the order of 21 kcal., if our interpretation of the meaning of the difference in μ values for heat destruction of altered and unaltered enzymes is correct. The fact that the unaltered enzyme requires 21 kcal./mole less energy to activate its destruction indicates that bonds totalling 21 kcal. bond strength (perhaps 4 hydrogen bonds?) must be formed during the process of enzyme alteration and this would therefore represent a minimum energy of activation for this process.

SUMMARY AND CONCLUSIONS

1. The very large increase in catalase activity (Euler effect) which follows treatment of yeast cells with $CHCl_3$, UV and *n*-propanol is accompanied by highly significant changes in kinetic properties. With respect to the enzymatic decomposition of H_2O_2, the thermodynamic constants of the activation process μ, $\Delta H\ddagger$, $\Delta S\ddagger$, $\Delta F\ddagger$, decrease, following treatment of the intracellular enzyme, by 4.5 kcal., 4.5 kcal., 10.1 e.u. and 1.7 kcal., respectively, all these differences being significant at the 1 per cent level.

2. Similar differences exist between the untreated, intracellular enzyme on the one hand, and the extracted yeast and crystalline beef liver catalases on the other. Significant differences in these thermodynamic constants do *not* exist among the treated intracellular, extracted yeast, and crystalline liver catalases.

3. These data provide unequivocal confirmation of the phenomenon of enzyme alteration reported previously, and confirm previous evidence that the extracted and crystalline enzymes have also undergone enzyme alteration and have properties which are identical with, or very similar to, those of the catalase altered *in situ*.

4. With respect to the process of heat destruction of catalase, the greatly diminished stability to heat of the altered enzymes, previously reported, has been confirmed. The thermodynamic constants of activation of this process have likewise changed following alteration, in the case of μ, $\Delta H\ddagger$, and $\Delta S\ddagger$ an increase of 20.6 kcal., 20.6 kcal., and 70 e.u., respectively, and of $\Delta F\ddagger$ a decrease of 2.8 kcal.

5. All these data have been shown to be consistent with, and in some cases predictable from, the interfacial hypothesis, which states that the unaltered catalase exists within the cell adsorbed to some interface, in a partially, but reversibly, unfolded configuration of relatively low specificity; enzyme altera-

tion consists, in the case of catalase, of desorbing the enzyme from the interface into its rolled-up, soluble, highly specific configuration. While the interfacial hypothesis has successfully withstood this experimental attack, the present data do not provide its unequivocal proof, since they are consistent with any hypothesis of alteration in which the unaltered, intracellular enzyme is in a relatively disordered state by comparison to the altered enzyme. While evidence of an interfacial process in enzyme alteration has been adduced previously, critical proof of the interfacial hypothesis awaits creation of a model system, in which most of the aspects of intracellular alteration can be reproduced.

6. Certain of the changes in kinetic properties following alteration of the intracellular enzyme, such as increased activity and the modified energies and entropies of activation of both enzyme-substrate system and heat destruction of the catalase itself, might be explained by a decrease (two orders of magnitude) in the effective hydrogen ion concentration, allowing the intracellular enzyme to be brought to the same pH as the extracellular medium. If such a pH change does, in fact, occur, it is necessary to invoke the interfacial hypothesis to explain why the unaltered, intracellular enzyme is in equilibrium with a medium whose pH is approximately 2 units lower than that of the cytoplasm itself.

7. It is concluded that kinetic data of this kind may be used to shed light on the structure of a soluble, cytoplasmic enzyme, not attached to any of the formed elements within the cell, yet organized within it in a condition of relatively low structural specificity; further, that information obtained exclusively from a study of the kinetics of the extracted or crystalline enzymes may not, in the case of this enzyme, at least, be extrapolated to the same enzyme within the intact cell.

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