Denaturation and Reactivity of Invertase in Frozen Solutions*

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ABSTRACT: Comparisons of frozen and supercooled solutions of yeast invertase show that this enzyme by itself is stable toward freezing, but is denatured when frozen with some dilute solutes. At 0.01 M concentrations, dimethyl sulfoxide, urea, NH_4Cl, or NaCl had no effect after 3 hr at −5°, while the effects of other solutes at 0.01 M followed an order Li > Na, I > Br > Cl. In the absence of other solutes, HCl at pH 2 resulted in complete inactivation of the enzyme in frozen solutions but not in supercooled solutions. Both NH_4Cl and dimethyl sulfoxide at high concentrations (>0.3 M) completely protect the enzyme from the partial denaturating effects of dilute HCl at pH 3 and 4, but dimethyl sulfoxide is more effective than NH_4Cl, and small concentrations (*ca.* 0.025 M) of these solutes enhance the harmful effects of HCl. The pH dependence of the denaturation, the effects of ion and enzyme concentrations were generally consistent with denaturation due to high concentrations of solutes in liquid regions of the frozen system. Small increases, as well as the more commonly observed decreases, in rates of enzymatic reaction in frozen systems relative to otherwise identical supercooled systems, could be demonstrated with hydrolysis of sucrose by invertase. With dilute substrate (0.007 M) and enzyme (0.00015 wt %) in unbuffered solutions, sucrose hydrolysis at −4.0° proceeded 1.6 times faster in frozen solutions than in supercooled solutions. A kinetic treatment for enzymatic reactions in frozen solutions is consistent with these observations but complete quantitative evaluation of enzyme action in frozen systems will generally be difficult.

There exists an important technology and extensive literature concerned with frozen storage of biological substances. Although reported studies have frequently dealt with systems containing viruses, cells, tissues, and even higher organisms (Stowell, 1965; Meryman, 1966), there are few studies of the effects of freezing on individual enzymes (see Tappel, 1966; Chilson *et al.*, 1965). Some further understanding of enzymatic reactivity in frozen systems would seem to be of fundamental use in practical storage of still more complex materials, but in addition, a study of such systems has further theoretical interest. Thus, the intriguing suggestions of Szent-Gyorgyi (1957) regarding effects of freezing on some biochemicals and dyes have led to further investigations of reactions under frozen conditions and to suggestions of many possible effects. These include heterogeneous catalysis by the ice lattice (Grant and Alburn, 1965), fast proton or electron transfers through ice (Prusoff, 1963), changes in water structure at low temperatures (Grant and Alburn, 1966), special orientation of reactants in solid-state reactions (*cf.* Fuchtbauer and Mazur, 1966), as well as the concentration of reactants in liquid regions of the frozen system (*cf.* Butler and Bruice, 1964, 1965). Of these possibilities, the "concentration effect" is well established and data from at least simple "frozen state reactions" may be treated by a general kinetic equation (Pincock and Kiovsky, 1966a,b) to determine if observations of accelerations, changes in kinetic order, and effects of variations of temperature and concentrations can all be accounted for by the concentration effect alone.

The purpose of the work reported here was to see to what extent the concentration effect might account for an example of enzymatic denaturation and reactivity in frozen systems. Invertase, a readily available enzyme of well-characterized kinetic behavior (in normal nonfrozen solutions at least), was chosen for study. The denaturation of invertase when frozen in dilute acid solutions or with other solutes was studied first, followed by comparisons of enzymatic hydrolysis of sucrose in frozen and in supercooled solutions at the same temperature.

Experimental Section

Yeast invertase (Melibiase Free, analytical grade), sucrose (enzyme grade), and Tricine buffer (*N*-[(tris-hydroxylmethyl)methyl]glycine) were obtained from Nutritional Biochemical Corp., Cleveland, Ohio 44128. The activities of the enzyme solutions were determined polarimetrically (Bendix ETL-NPL Automatic Polarimeter, type 143A) at 25° after adding standard solutions of sucrose and Tricine to thawed samples. The change in optical activity during the sucrose hydrolysis was zero order at the concentrations used (5% or greater sucrose), and in a check on the method of

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analysis, the slopes (in degrees per minute) of the lines obtained were shown to be proportional to active enzyme concentration.

In order to separate out only the results of freezing, for every series of frozen samples, a nonfrozen, supercooled control solution was otherwise treated in a manner identical with that of the frozen solutions. Supercooled solutions often crystallized during a run, especially if they were jarred or otherwise subjected to sharp movements. This difficulty required the preparation of several identical control samples; usually one survived at −5° for the required time without freezing. The ratio of slopes in zero-order kinetic plots for the frozen and supercooled solutions then gave the fraction of enzyme activity left in the frozen sample. This control method obviated analytical difficulties due to changes in absolute activities caused by added solutes or to slow loss of activity in enzyme solutions at room temperature. Samples were equilibrated at −5° and those to be frozen were dipped quickly into a Dry-Ice–acetone bath and then returned to the −5° bath. After storage at −5° for 3 hr (unless otherwise noted), the samples were thawed under lukewarm tap water and equilibrated at 25° for 1 hr before analysis of activity.

The study of the protective action of various solutes on the HCl-promoted denaturation (*i.e.*, using a set of samples at identical pH and enzyme concentration but different dimethyl sulfoxide, NH_4Cl, or NaCl concentrations) required a number of sample analyses, each of which was itself a full kinetic run. It required too much time and was basically unnecessary to retain and analyze a supercooled sample for *each* frozen sample in a set. At a given pH value the control samples for a set of frozen samples (which gave a set of rate constants $k_{S,F}$) were of two types; one with no added solute but frozen (which gave rate constant $k_{SA,F}$), and one with no added solute but supercooled (giving rate constant $k_{SA,NF}$). On, say, a second day, samples at the same pH but with various concentrations of solutes were analyzed for enzyme activity directly without being frozen. These gave rate constant $k'_{SA,NF}$ together with a set of rate constants $k'_{S,NF}$. The denaturation due to freezing was then obtained by multiplying ratios of measured activities (*i.e.*, k values in degrees per minute) as follows; relative activity after being frozen 3 hr = $k_{(frozen)}/k_{(supercooled)} = (k_{S,F}/k_{SA,F})$. $(k_{SA,F}/k_{SA,NF})(k'_{SA,NF}/k'_{S,NF})$, where subscripts S, SA, F, and NF stand for solute present, solute absent, frozen sample, and nonfrozen sample, respectively. Thus, the rate constants in respective brackets were experimentally obtained in paired runs and the last bracket is a correction on the analysis at 25° due to the solute itself (this last ratio differed from unity only at higher concentrations). This method required a minimum of supercooled control samples (which were sometimes difficult to keep from freezing) and avoided variations due to small differences in activity of enzyme or of concentration of solutions prepared independently over a period of days.

Rather than obtaining a percentage activity after 3 hr several attempts were made to study the complete kinetics of HCl denaturation in frozen solutions. For example, 4-ml aliquots of a 50-ml solution containing 9 mg of enzyme at pH 4.46 (*i.e.*, HCl with no other solutes present) were frozen at −5°. At various times a sample was removed, quickly thawed, and its activity was determined as described above. Although the relative activities at various pH values after *ca.* 3 hr were quite consistent, the earlier samples of a run often showed nearly as much loss of activity and sufficiently reproducible and unscattered kinetic plots could not be obtained.

For enzymatic reaction itself in frozen solutions the rate of enzymatic (0.01 or 0.00015 wt %) hydrolysis of sucrose (1.5, 0.15, or 0.007 M) in buffered (0.01 M Tricine) and unbuffered frozen solutions was compared with hydrolysis in identical solutions supercooled to the same temperature or to identical solutions at room temperature. Enzymatic reaction was quenched in cold samples by addition of sufficient NaOH to denature the enzyme, followed by warming to room temperature, and polarimetric analysis of the sucrose.

Results

Invertase solutions in the absence of added solutes may be repeatedly frozen and thawed between −195° and room temperature without loss of activity (*cf.* Sizer and Josephson, 1942; Neumann and Lampen, 1967). However, direct comparison of frozen and supercooled solutions (0.018% enzyme and 0.01 M solute) after 3 hr at −5° showed that several solutes were effective denaturants in frozen systems. The ratio of observed activities is shown in Table I.

TABLE I: Effect of Solutes on Invertase Activity after 3 hr in Frozen Solutions at −5°.

Solute[a]	Act.[b]	Solute[a]	Act.[b]
Dimethyl sulfoxide	1.0[c]	$CaCl_2$	0.92
NH_4Cl	1.0	NaI	0.84
$MgBr_2$	1.0	LiBr	0.70
NaCl	1.0	LiI	0.42
Urea	0.99	Phenol	0.27
NaBr	0.97	HCl	0.0

[a] Solute concentration 0.01 M in initial unfrozen solutions; invertase at 0.018 wt %. [b] Activity = ratio of observed zero-order rate constants for 5% sucrose hydrolysis in frozen and supercooled solutions after returning to 25°. The sucrose and buffer (0.1 M Tricine) were added just before analysis and after the samples had been at −5° for 3 hr. [c] The maximum experimental variation in ratio of zero-order rates constants among several runs (say, runs with four different concentrations of NaCl from 0 to 0.07 M) was about 5%. The comparison of identical solutions, one frozen and one supercooled, eliminated many possible sources of variation that would appear in separate measurements of absolute rate constants.

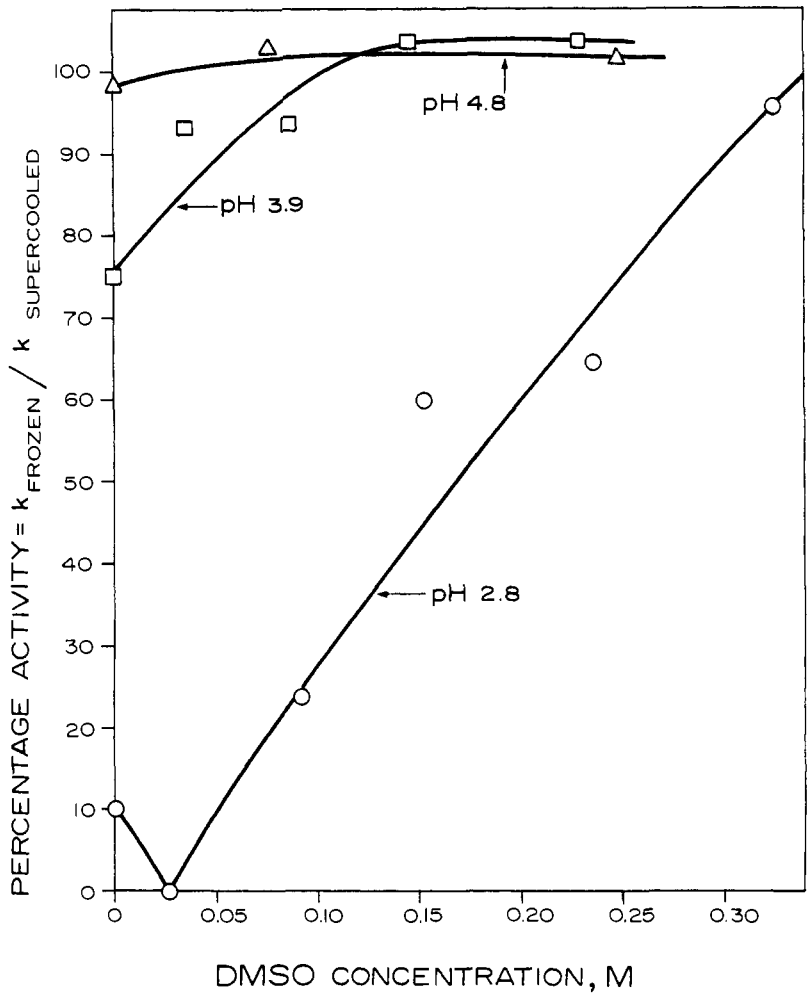

FIGURE 1: Percentage activity of invertase solutions, frozen 3 hr at −5°, as a function of initial pH and dimethyl sulfoxide concentrations.

TABLE II: Denaturation of Invertase by HCl in Frozen Solutions Relative to Supercooled Solutions at Various Initial pH.[a]

pH	Act.[b]	pH	Act.
1.95	0	4.06	0.73 ± 0.03[c]
2.53	0.05	4.46	0.85
2.97	0.18	4.8	1.0
3.01	0.33	5.0	1.0
3.68	0.57	6.5	1.0

[a] Conditions: enzyme at 0.016 wt %, all solutions held at −4.2 for 3 hr. [b] Activity = $k_{(frozen)}/k_{(supercooled)}$ where both are measured after returning to 25° and sucrose and buffer are added. [c] Variation on three separate determinations.

The highly destructive effect of HCl was studied with an enzyme concentration of 0.013% over a range of pH values in unbuffered solutions. These results are shown in Table II; the activity after 3 hr in frozen solutions at −5° varied with initial acid concentration such that above pH 4.8 no loss occurred while below pH 2.5 essentially all activity was lost in frozen solutions but not in supercooled solutions.

As NaCl, NH_4Cl, and dimethyl sulfoxide (frequently used as a "protective agent" in frozen systems) had no harmful effects by themselves (see Table I), their influence on the HCl denaturation was studied. Figures 1 and 2 show that while high concentrations of these solutes prevent loss of activity, lower concentrations actually result in total loss of activity, or at least a greater loss than would be caused by HCl alone (see Figure 2 at pH 4 for NH_4Cl solutions). Interestingly, dimethyl sulfoxide is a more effective protective agent than NH_4Cl (or NaCl, not shown) at the same concentration, *e.g.*, compare dimethyl sulfoxide at 0.1 M and pH 2.8 which is "protective" in Figure 1, with NH_4Cl at 0.1 M, pH 3.0, which is totally "destructive" in Figure 2; 25% activity would be present at pH 3 in the absence of NH_4Cl.

In order to study enzymatic action itself in frozen systems, aliquots of a solution of invertase, sucrose, and buffer were frozen, held at −5° for various times, and compared with otherwise identical, but nonfrozen samples. With 0.146 M sucrose, 0.01% enzyme, and 0.01 M Tricine buffer, paired sets of initial rates were obtained for runs at: A, 25° and frozen at −5°; B, 25° and supercooled at −5°. The ratio of initial rates for

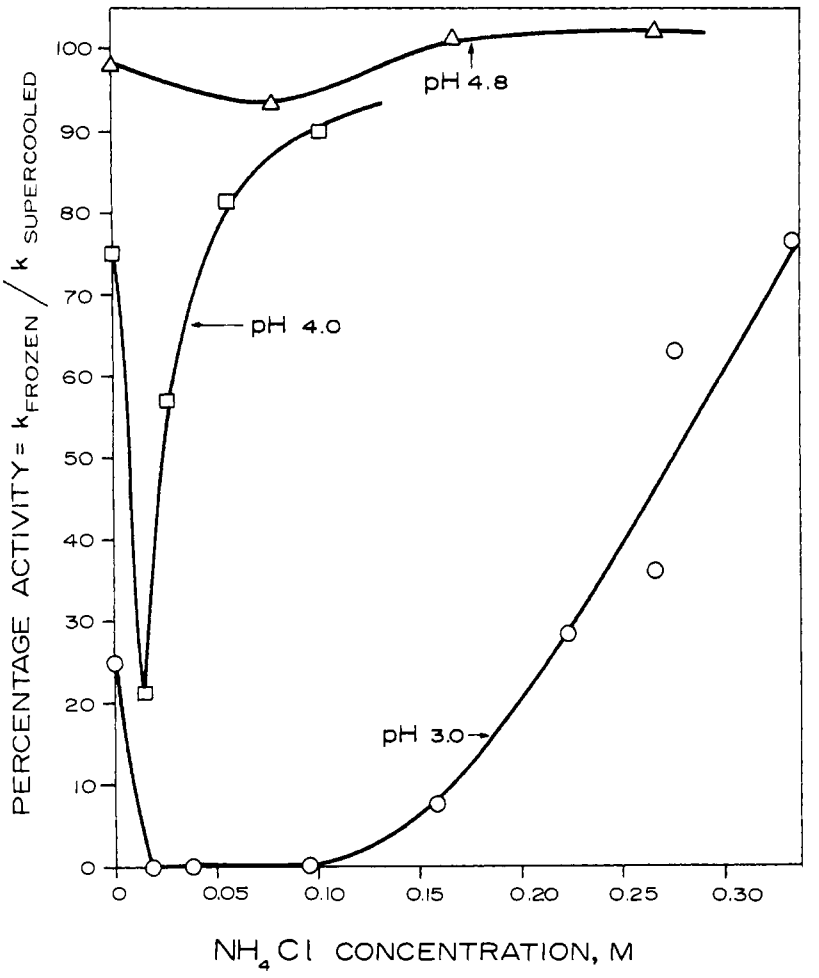

FIGURE 2: Percentage activity of invertase solutions, frozen 3 hr at −5°, as a function of initial pH and NH_4Cl concentrations.

pair A was 3.5 and for pair B was 3.0. As these frozen solutions contain liquid regions at very high concentrations (*ca.* 2.6 M at −5°), a further comparison of a frozen run with a nonfrozen run at as high a sucrose concentration as possible was carried out. The ratio of initial rates for a run at 25° containing 1.5 M sucrose to a frozen run at −5° at (initially) 0.146 M sucrose was 3.3. Comparison of this ratio with that of experiment A above suggests that a very high concentration of substrate somewhat slows the enzymatic hydrolysis (as suggested by Nelson and Vosburgh, 1917) and may account for the small difference in ratios obtained. In any case, at these initial concentrations, the rate of enzymatic hydrolysis of sucrose at −5° is not noticeably dependent upon the physical state (*i.e.*, frozen or nonfrozen) of the system.

The smaller the reactant concentration in an initial nonfrozen solution, the greater the relative change to the high concentrations present in liquid regions of a frozen solution. Any possible accelerations due to concentration changes can therefore be expected to show up most distinctly at low total solute concentrations (Pincock and Kiovsky, 1966a,b). To test this with the invertase–sucrose reaction, a pair of runs with the minimum feasible substrate and enzyme concentrations and with no buffer present were carried out. At 0.007 M sucrose and *ca.* 0.00015% enzyme, runs with frozen and supercooled samples at −4° gave fairly good plots of log (optical rotation) against time and the ratio of slopes was $k_{(frozen)}/k_{(supercooled)} = 1.6$. However, at 1.31 M sucrose with 0.005 wt % enzyme and no buffer the ratio of rates at −5° was reversed, $k_{(frozen)}/k_{(supercooled)} = 0.63$. In this way, changes, both weakly accelerative as well as decelerative, could be demonstrated for enzymatic hydrolysis of sucrose in frozen systems. Only in the case of very low initial concentrations were frozen samples slightly but consistently more reacted than supercooled samples; with a high sucrose concentration or with 0.01 M buffer present, the rate of reaction in frozen samples was less than in supercooled samples.

Discussion

There are frequent but often only qualitative reports of denaturation or other changes due to freezing of protein solutions (*cf.* Chilson *et al.*, 1965; Levitt, 1964; Schildkneckt, 1964). Invertase, in the absence of added solutes, is stable in frozen systems and is not affected by the freezing and thawing process. Although a protein surface might be covered with a mobile water layer even at very low temperatures (Bielski and Freed,

1965), it seems that the macroscopic change from water to ice structure around the enzyme does not permanently modify its activity. However, when frozen with certain solutes at temperatures where liquid regions at high concentrations still exist, invertase is denatured (see Table I). Acids are most effective (HCl, phenol) and the salts followed the order Li > Na and I > Br > Cl which has been found for denaturation proteins at high concentrations (*e.g.*, Putnam, 1953). There seems little doubt that the cause of such denaturation by solutes in frozen systems is a concentration effect, *i.e.*, the solidification of water leaves, at equilibrium, liquid regions in the frozen systems which contain high concentrations of solutes (at −5° the total concentration is *ca.* 2.6 M). Denaturation is greatly promoted by such high concentrations, although the initially dilute solutes (as in supercooled solutions) are relatively ineffective. Even though the molecular basis of this type of denaturation remains obscure (Jencks, 1965), many qualitative observations of protein denaturation by freezing, such as the greater effects of slow freezing and thawing and the protection by added solutes, are consistent with denaturation by concentration changes in frozen systems (*e.g.*, Chilson *et al.*, 1965). The concentration of solutes when dilute solutions are frozen could be a convenient way to measure the effects of high concentrations on proteins in cases where a continual presence of such high concentrations interferes with an analytical technique.

The variation of denaturation with pH of initial solutions shown in Table II is also consistent with a concentration explanation. At −4° the total concentration of solute in the liquid part of a frozen system reaches about 2.0 M, and if the initial HCl concentration is greater than the total of all other solutes, this is the acid concentration approached in the frozen system. Myrback and Willstaedt (1955) have reported that complete loss of invertase activity occurs by storage in 0.1 M HCl for 24 hr, so a *ca.* 1 M concentration can be expected to be highly effective at −4°. Melting point depression experiments on enzyme solutions (*e.g.*, 0.163 g of invertase in 25 ml gave $\Delta = 0.05°$) showed that total solute concentration arising from the enzyme preparation used in the HCl tests was about 10^{-4} M. The presence of these solutes will then keep the absolute concentration of HCl in liquid regions at −4° relatively low until the initial concentration of acid becomes greater than 10^{-4} (pH 4). Consistent with this, as shown in Table II, the denaturation by HCl becomes effective only at pH values less than *ca.* 4.5. Therefore, although the enzyme is quite stable at pH 2 in nonfrozen solutions, it is quickly denatured in otherwise identical frozen solutions at −4° when the actual acid concentration reaches *ca.* 1 M.

The protective action of NH_4Cl and dimethyl sulfoxide on the HCl denaturation also showed the general form expected for a concentration effect, *i.e.*, high concentrations of solutes completely protected the enzyme and at lower pH values a greater solute concentration was required to retain full activity after freezing (Figures 1 and 2). Not consistent with a simple concentration effect, however, are the observations that small concentrations of both NH_4Cl and dimethyl sulfoxide with HCl gave more effective denaturing conditions than HCl alone. Possibly these solutes act to keep in solution (and therefore subject to the action of HCl) some protein which would otherwise be precipitated or trapped into the ice by the freezing process, or, the solutes may modify the activity of HCl in the concentrated frozen systems. Also not consistent with a simple effect based on the colligative properties of solutes is the more effective protection by dimethyl sulfoxide than by NH_4Cl at comparable pH. Dimethyl sulfoxide may be an excellent general protective agent (Farrant, 1965) not only because of its physical properties (*i.e.*, the ability to penetrate tissues and cells), but also because it seems to have little chemical effect on enzymes at low temperatures.

The inactivation of proteins by freezing is also generally dependent upon their own concentration (Chilson *et al.*, 1965). In the case of invertase it was found that solutions containing greater enzyme concentrations were less susceptible to denaturation at a comparable pH value; *i.e.*, the solutes present in the enzyme preparation act as protective agents.

On enzymatic action itself at low temperatures, a majority of the few studies of enzyme kinetics in frozen systems have reported that rates are unaffected (Balls and Tucker, 1938) or decreased (Lineweaver, 1939; Kertesz, 1942; Sizer and Josephson, 1942) in the frozen state. But there are a few reports of accelerated enzymatic reactions (Kiermeier, 1948; Tappel, 1966; Grant and Alburn, 1966), and one suggestion of enzyme-like catalytic activity by active sites on ice surfaces in a nonenzymatic reaction (Grant and Alburn, 1965). In the latter case, inhibition by compounds of analogous structure gave rise to kinetic plots resembling linear Lineweaver–Burk plots for competive inhibition. However, any effect of concentration of solutes by freezing was not initially separated out, and as the generalized kinetic equation for the concentration effect on a bimolecular reaction (Pincock and Kiovsky, 1966) also gives the Lineweaver–Burk kinetic form for competitive inhibition[1] (*i.e.*, $1/v_s = (1/k_2C_h)\{1/[A_s] + 1/[B_s] + [I_s]/[A_s][B_s]\}$), the actual existence of any more unusual phenomena is unclear.

As enzymatic reactions are bimolecular, a concentration change brought about by freezing can, in principle, lead to accelerated reactions. Assuming normal Michaelis–Menten kinetics in the concentrated liquid regions of a frozen system, the observed velocity in thawed solutions will be[1] $v_s = v_h(V_h/V_s)$, where $v_h = k_3[E_h][S_h]/(K_a + [S_h])$. If all solutes present in

[1] Abbreviations used: k_2, second-order rate constant for bimolecular reaction of A and B; C_h, total constant concentration of solutes in liquid regions of a frozen solution; [A] and [B], concentrations of reactants in a bimolecular reaction; [I], concentration of any other added solutes (*i.e.*, "inhibitors"); v_h, actual reaction velocity in liquid regions of a frozen system; v_s, observed velocity in thawed solutions; V_h, total volume of liquid regions in a frozen system; C_s, total solute concentration in thawed solution; subscript s refers to thawed solutions; subscript h refers to concentrated liquid regions of frozen solutions.

thawed solutions were present in liquid regions of frozen solutions, then $C_hV_h = C_sV_s$, $[E_h]V_h = [E_s]V_s$, and $[S_h]V_h = [S_s]V_s$. The observed velocity in thawed solutions can then be put in the following form

$$v_s = \frac{k_3[E_s][S_s]}{K_a(C_s/C_h) + [S_s]} \quad (1)$$

This equation shows that at high substrate concentration the observed rate of reaction in frozen solutions (as measured in thawed solutions) is identical with that in supercooled solutions, *i.e.*, the enzyme is saturated with substrate in either case and $v_s = k_3[E_s]$. At low substrate concentrations, eq 1 predicts a relative acceleration, since $K_a(C_s/C_h)$ may not be neglected relative to $[S_s]$ and the total concentration in liquid regions of frozen solutions, C_h, is greater than the total concentration of thawed solutions, C_s. The observed velocity will then be $v_s = (C_h/C_s)(k_3[E_s][S_s]/K_a)$ which is greater than the velocity in a nonfrozen control sample by the factor C_h/C_s. In an extreme case of this effect, the enzyme–substrate complex would be at a maximum concentration in a frozen system but not in a supercooled system at the same temperature. This is probably the origin of the small increase in rate observed for invertase action at the lowest initial sucrose and enzyme concentrations, *i.e.*, under conditions where the ratio C_h/C_s is maximized. Such "concentration effects" may account for other accelerated enzyme reactions in frozen solutions. In principle, such cases of unusually fast enzyme reactions in frozen systems should be treated by eq 1 in order to see if effects other than a concentration effect are present.

On the other hand, the reported *decreases* in observed enzymatic rates in frozen systems relative to supercooled solutions cannot be even qualitatively accommodated by *direct* application of eq 1. In the absence of other factors, decreases in rate cannot occur simply by a concentration of reactants due to freezing. However, secondary effects of high concentrations of buffer, substrate, or salts can modify the values of k_3 and K_a, and pH shifts as well as partial denaturation or precipitation of the enzyme can all contribute to slower reactions in frozen systems. Such complications make a more quantitative evaluation of the kinetics of enzyme action in frozen systems very difficult, but these factors should be separated out before more exotic possibilities can be established.

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