Pyruvate Carboxylase. Reversible Inactivation by Cold*

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ABSTRACT: Pyruvate carboxylase from chicken liver mitochondria is rapidly inactivated by exposure to low temperatures and loss of catalytic activity is accompanied by an apparent dissociation of a tetrameric form of the enzyme into four protomers. Both inactivation and dissociation can be reversed almost completely under appropriate conditions by rewarming. The results of a more detailed study of these processes are consistent with a rapid and reversible dissociation reaction which involves the intermediate formation of an inactive tetramer. The results also suggest that on longer exposure to low temperatures, further changes occur in the protomer which prevent reactivation by rewarming although association to tetramers or aggregates may still occur. Inactivation and dissociation effects similar to those observed at low temperatures can occur at room temperature in the presence of low concentrations of urea and to some extent after adjustment to mildly alkaline pH ranges. Acetyl coenzyme A affords partial or complete protection against the inactivation and dissociation caused by cold, urea, or pH changes.

The concentration range, high degree of concentration dependence, and specificity of the protective effects of acyl coenzyme A are very similar to those observed for the activation of the catalytic reaction of this enzyme by this class of compounds. The enzyme is also protected against dissociation and to some extent against inactivation by adenosine triphosphate and several related substances. The presence of adenosine triphosphate considerably enhances the reactivation process in terms of recovery of catalytic activity and restoration of the original sedimentation pattern. The presence of a number of other substances including inorganic phosphate, methanol, several polyols, high concentrations of KCl, and oxalacetate, protect pyruvate carboxylase against inactivation by low temperatures.

Pyruvate carboxylase (EC 6.4.1.1) catalyzes reaction 1.

$$\text{pyruvate} + CO_2 + \text{ATP} \xrightleftharpoons{Mg^{2+},\ \text{acetyl-CoA}} \text{oxalacetate} + \text{ADP} + P_i \quad (1)$$

Previous papers from this laboratory (Keech and Utter, 1963; Scrutton and Utter, 1965, 1967; Scrutton *et al.*, 1965, 1966; Mildvan *et al.*, 1966; Valentine *et al.*, 1966; Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1968) have presented studies of the nature of the reaction, and of the physical, chemical, and catalytic properties of highly purified pyruvate carboxylase obtained from chicken liver mitochondria.

The enzyme has an approximate molecular weight of 660,000 and contains 3.6 moles of biotin/mole (Scrutton and Utter, 1965) and 3.4 g-atoms of Mn^{2+}/mole (Scrutton *et al.*, 1966). As in the case of other biotin-containing carboxylases (Knappe *et al.*, 1963; Lane and Lynen, 1963), the reaction mechanism has been shown to involve the ATP-dependent formation of an enzyme–biotin$\sim CO_2$ intermediate, followed by carboxylation of the acceptor. Acetyl-CoA is absolutely required for formation of the $CO_2\sim$ enzyme. Pyruvate carboxylase catalyzes the ATP–$^{32}P_i$ and pyruvate–[^{14}C]oxalacetate exchanges predicted by this mechanism (Scrutton *et al.*, 1965).

Pyruvate carboxylase purified from chicken liver is rapidly inactivated by cold, but can be partially reactivated by rewarming (Scrutton and Utter, 1965). The ATP–$^{32}P_i$ and pyruvate–[^{14}C]oxalacetate-exchange reactions undergo inactivation and reactivation (Scrutton *et al.*, 1965) parallel to the overall reaction during exposure to cold and rewarming. Exposure to low temperatures has also been reported to cause a change in the enzyme's ultracentrifugal sedimentation characteristics (Scrutton and Utter, 1965). Highly purified preparations exhibit a main component, $s^0_{20,w}$ = 14.8 S, which is enzymically active, and a much less abundant component, $s^0_{20,w}$ = 6.75 S, which appears to be inactive and which has a molecular weight approximately one-fourth that of the active enzyme. Incubation in the cold causes a shift of material from the faster moving to the slower moving component (Scrutton and Utter, 1965). Electron microscopic examinations of the enzyme have shown that this shift is accompanied by a loss of tetrameric structures which are believed to be the active forms of this enzyme (Valentine *et al.*, 1966).

The present studies are concerned with the various factors which influence the inactivation of pyruvate carboxylase during exposure to cold, urea, and alkaline pH, and the nature of the structural changes which accompany inactivation by these agents.

A preliminary account of some of these findings has appeared elsewhere (Irias, 1965).

Methods

Cold inactivation was carried out at 0° at pH 7.2 in potassium phosphate (10 mM), in 0.15–0.2 M KCl containing 1 mM EDTA unless otherwise indicated. The experiments were

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conducted with preparations of the enzyme which had been previously brought to the desired salt and buffer concentration by passage through a properly preequilibrated Sephadex G-50 column. Samples (usually 0.5 ml of the enzyme with 0.5 mg of protein/ml) in small tubes were placed in a cold bath and stirred manually. Larger and more concentrated samples used in ultracentrifugal studies were cooled as rapidly as possible with the aid of a small magnetic stirrer. Reactivation was achieved by placing the undiluted enzyme in a 23° bath for 20–30 min (with continued stirring of the larger samples) before aliquots were removed for assay.

Pyruvate carboxylase activity was measured spectrophotometrically by coupling with malate dehydrogenase as described previously (Keech and Utter, 1963) at a pH of 7.8, which is optimal for activity (Keech and Utter, 1963). Enzyme units are reported as micromoles of oxalacetate formed per minute and specific activities as units per milligram of protein.

Analytical. Protein was determined by the spectrophotometric method of Warburg and Christian (1941–1943). A Radiometer Model 22 meter with a type C electrode and a temperature compensator was used to determine pH values. Ultracentrifugal examinations were carried out in a Beckman-Spinco Model E analytical ultracentrifuge at 50,740 or 52,640 rpm using the AN-E rotor and a 30-mm aluminum centerpiece at temperatures as indicated.

Materials

Sigma Trizma Tris base was recrystallized from 1 mM EDTA in 85% ethanol as previously described (Sutherland and Wosilait, 1956). Analytical reagent grade salts and urea were also recrystallized once and ammonium sulfate twice from 1 mM EDTA.

Acetyl-CoA was prepared from coenzyme A (P-L Laboratories) and acetic anhydride as described by Stadtman (Stadtman, 1957); excess acetate was removed by four ether extractions at pH 2, and the pH was readjusted. Acetyl-CoA was estimated by citrate synthase (Ochoa, 1957).

Pyruvate carboxylase was purified as described elsewhere (Scrutton *et al.*, 1969). The enzyme was normally stored until used in the cold at high protein concentrations (15–20 mg/ml) in 1.5 M sucrose containing 0.1 M potassium phosphate (pH 7.2) or 0.1 M Tris-SO_4 (pH 6.9) and 0.06 M $(NH_4)_2SO_4$ (Scrutton and Utter, 1965).

Other enzymes and reagents were commercial products.

Results

General Features of Cold Inactivation and Reactivation. The overall character of the cold inactivation of pyruvate carboxylase is demonstrated by the typical experiment summarized in Figure 1. In this and other experiments small aliquots were removed for assay at 25°, using micropipets adjusted to the temperature of the sample. Reactivation does not occur under the conditions of the assay where low concentrations of protein are present.

The lower curve in Figure 1 shows the considerable cold inactivation which occurred under these conditions; at 90 min, the activity of the cold sample was only 23% that of the room temperature control. Further characteristics of the process became evident when samples of the enzyme inactivated at 0° for varying periods were rewarmed at 23° for 30 min before assay. These results are plotted on the middle curve, each point showing an activity obtained by assay at $(t + 30)$ min.[1] By this treatment of the data, cold inactivation can be dissected into reversible and irreversible phases. The reversible phase always proceeds to a maximum extent rapidly, in about 25–30 min in the instance shown. After that, the amount of reversibly inactivated enzyme, as indicated by the difference between the middle and lower curves, may remain nearly unchanged for a considerable period; eventually it begins to diminish slowly. In the experiment shown in Figure 1 this did not occur until 120–180 min and is not shown. The time course of irreversible inactivation due to cold in Figure 1 is shown by the difference between the upper and middle curves. There is some inactivation in 90 min in this experiment even in the sample held at 23° (top curve). This type of inactivation is variable with different preparations of the enzyme, and the relationship of the room temperature inactivation to that induced by cold is not known.

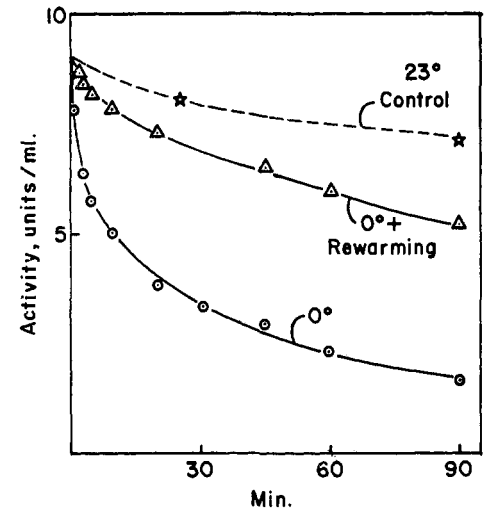


FIGURE 1: Cold inactivation of pyruvate carboxylase and reactivation by rewarming. Pyruvate carboxylase (0.5 mg/ml; specific activity 18.4) inactivated at 0° as described in Methods. The points on the middle curve indicate the activities of samples which have been held at 0° for the indicated time and then rewarmed for 30 min at 23° before assaying.

As previously reported (Scrutton and Utter, 1965; Valentine *et al.*, 1966; Irias, 1965) incubation of pyruvate carboxylase in the cold appears to cause dissociation of the enzyme. On examination in the ultracentrifuge material from the main 14.8S peak is found to have shifted to the 6.75S peak. Early attempts to demonstrate reassociation on rewarming were inconclusive and both dissociation and the reactivation on rewarming were incomplete. Rewarming tended to restore the ratio of 14.8S to 6.7S material toward that present before cooling, but the sum of the two components often diminished so that an absolute increase in the fast component could not be shown in convincing fashion. The occasional tendency of the enzyme to form visible precipitates during rewarming suggested that further aggregation to species larger than 14.8 S might occur to variable extents. This process is minimized, and the degree of

[1] These values were corrected for loss of activity in 30 min at 23°; the correction does not alter the qualitative relationship between the curves.

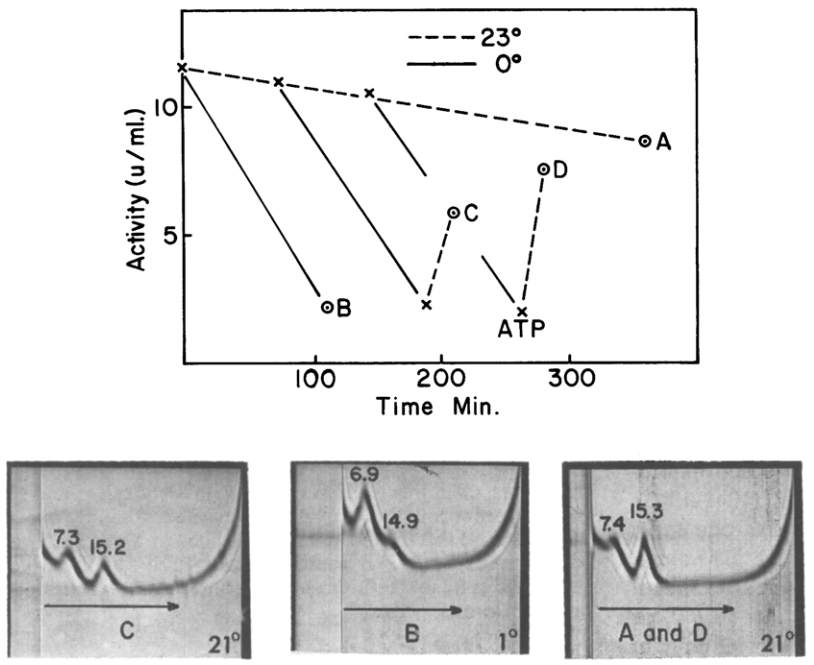


FIGURE 2: Effects of exposure to cold and rewarming in the presence and absence of ATP on the catalytic and sedimentation properties of pyruvate carboxylase. Pyruvate carboxylase (specific activity 11.6;[2] 1 mg of protein/ml) was treated as described below. Examination of the sedimentation pattern and catalytic activities were carried out as described in Methods with additional information supplied below. The letters A, B, C, and D on the graph show catalytic activities at times approximating those at which the accompanying sedimentation patterns were obtained. All photographs were taken at a bar angle of 45°. The direction and temperature of sedimentation and $s_{20,w}$ values are shown on the photographs. The various samples were treated as follows: (A) held at 23° for approximately 360 min; (B) held at 0° or 1° for approximately 100 min; 90 min after the start of the experiment sample C was placed at 0° for 115 min, then rewarmed for 48 min at 23°; and about 140 min after the start of the experiment sample D was placed at 0° for 115 min, then 5 mM ATP added and rewarmed for 48 min at 23°. Actual times at full speed in the centrifuge were: 16 min for A, C, and D and 32 min for B.

reactivation enhanced, by the addition of ATP before rewarming.

The experiments of Figure 2 show the effect of cold (B), simple rewarming (C), and rewarming with ATP (D) on the catalytic activity and the sedimentation patterns of pyruvate carboxylase. A sample not exposed to the cold represents the control (A). Return of material to the faster moving peak was shown quite clearly when ATP was used: the sedimentation patterns for A and D were identical. There was no apparent decrease in the sum of the two peaks in D in contrast to C. Figure 2 also shows the concomitant changes in catalytic activity. The cold-inactivated material (B) was approximately 80% inactivated at the time of examination.

In Figure 1, "irreversible inactivation" was defined as the fraction of activity not restored by rewarming. It is clear from Figure 2 that the extent of this process is influenced by the addition of ATP prior to reactivation. Although the presence of ATP increases the extent of reactivation at all points tested as shown in Figure 3, some irreversibly inactivated material remains (difference between curves A and B) which increases with time. These results show that the definition of "irreversible" inactivation is merely an operational one and that the process may involve two or more intermediates. Reactivation in the presence of ATP appears to restore the original sedimentation pattern (A and D, Figure 2). The temporary rise in activity sometimes observed in samples standing at room temperature is well illustrated in curve A of Figure 3.

Different batches of highly purified pyruvate carboxylase vary in their susceptibility to cold inactivation. Figure 4 shows the time course of the overall cold inactivation from two experiments using different enzyme preparations which illustrate the observable differences in cold lability. At the left, where per cent initial activity is plotted *vs.* time, curve B clearly shows a more rapid inactivation than A. The extent of the rapid phase of inactivation is also greater in B than in A. Experimental variables were comparable in the two experiments. Even after extensive investigation, the basis for differences in cold lability of different preparations of the enzyme is not known, nor do our data as yet permit conclusions about variability in reversible *vs.* irreversible inactivation, but the more rapidly inactivated preparations have generally shown more complete dissociation on ultracentrifugal examination, higher initial specific activities, and greater stability on storage.

The right-hand side of Figure 4 shows log plots of the data from curves A and B. Such plots may seem to suggest two successive first-order reactions, corresponding to early rapid (primarily reversible) and later slower (irreversible) inactivation. However, repeated experiments have failed to give a straightforward confirmation of this interpretation. Reversible inactivation is highly temperature dependent and very rapid, so that accurate analysis of its time course is difficult; measurements of half-times at varying protein concentrations have shown only that dilution favors the process. Irreversible inactivation is relatively slow, but activity decline curves show considerable scatter, preventing reliable kinetic analysis although the results most nearly conform to first-order kinetics. As previously mentioned, irreversible inactivation probably does not represent a single process.

[2] It should be noted that the variable specific activities of the different enzyme preparations used in these studies result mainly from activity losses during storage and handling. Preparations with rather low activities such as the one used in this experiment had a much higher specific activity immediately following purification. The slower moving peak contains inactive material but cannot account for all of the inactivated enzyme (*cf.* photograph A, Figure 2). Since sedimentation changes appear to be relatively independent of the specific activity existing at the time of cold treatment it would seem that enzymically inactive tetramers (15S material) can also undergo reversible dissociation.

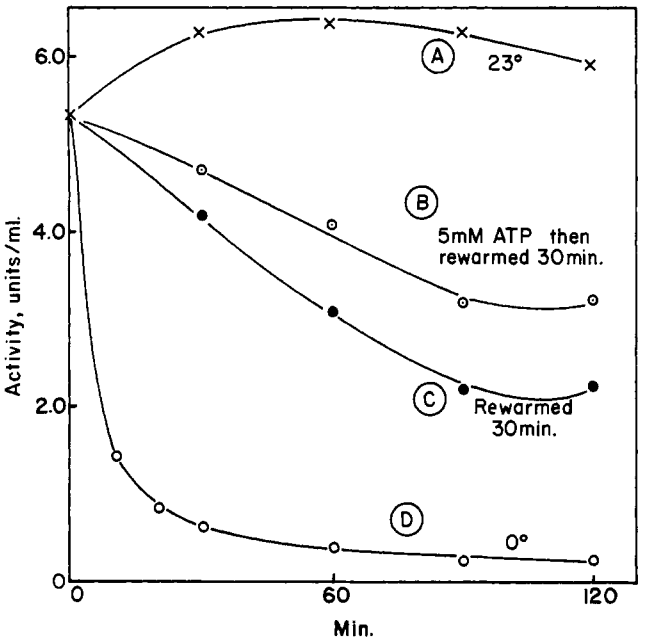


FIGURE 3: Extent of irreversible inactivation in the presence and absence of ATP during reactivation. Pyruvate carboxylase (specific activity 12) was inactivated under conditions described under Methods. Curves B and C show activities obtained after incubation at 0° for times indicated and reactivation at 23° for 30 min in the presence of 5 mM ATP (B) and absence of ATP (C).

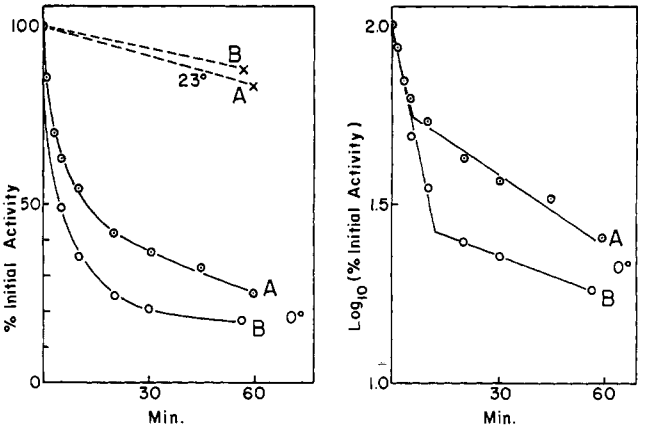


FIGURE 4: Variations in cold lability of different samples of pyruvate carboxylase. In both experiments, enzyme in a concentration of 0.5 mg/ml was incubated at 0 and 23° in 0.15 M KCl (A) and 0.2 M KCl (B), 1 mM EDTA, and 10 mM potassium phosphate buffer, pH 7.2 (A) and 7.25 (B). Specific activities were 18 (A) and 13 (B).

The kinetics of the reactivation process appear to be first order. For example, the half-time required for reactivation remained constant at different protein concentrations (Table I). These data also demonstrate that the reactivation times used in Figures 2 and 3 and in later experiments are adequate for achieving complete reactivation.

Figure 5 shows further evidence for first-order kinetics of reactivation. The time course described by the reactivation curve approaches the maximum approximated by the horizontal line (left part of Figure 5). The vertical distance from the curve to the line at a given time represents the concentration of reversibly inactivated enzyme. The instantaneous rates of reactivation at different times can be estimated from the slopes of secants to the curve. When log (reactivation rate) is plotted *vs.* log (reversibly inactivated enzyme) a straight line with a slope of approximately 1.0 is obtained (right part of Figure 5). Th s value has been consistent in repeated experiments and can be considered as good evidence for first-order kinetics. By contrast, application of this procedure to the inactivation data gave variable and unsatisfactory results.

If the hypothesis is correct that reactivation is accompanied by the formation of a tetramer from four protomers, the first-order kinetics of reactivation indicate that this process involves a rate-limiting step distinct from reassociation, such as a preliminary conformational change in the protomer, or the conversion of an initially formed inactive tetramer into an active one. Evidence consistent with the latter hypothesis will be presented later in this paper.

Experimental Variables Affecting Cold Inactivation. The effect of protein concentration on cold inactivation was previously reported (Scrutton and Utter, 1965); inactivation is more marked at lower protein concentrations. Further experi-

TABLE I: Effect of Protein Concentration on the Half-Time of Reactivation of Pyruvate Carboxylase after Exposure to Cold.[a]

Protein Concn (mg/ml)	$t_{1/2}$ (min)
0.5	3.0
1.0	3.2
1.5	2.5
2.0	2.7
	(av 2.9 ± 0.4)

[a] Pyruvate carboxylase (specific activity 17) was incubated at indicated concentrations of protein at 0° for 60–70 min and then rewarmed to 23° with activities measured at appropriate intervals. Activities were plotted against time with full reactivation usually obtained in 20–30 min. The $t_{1/2}$ values represent the times required to reach one-half of the maximal regained activity.

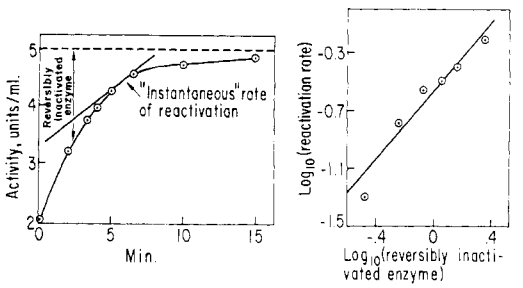


FIGURE 5: Kinetics of reactivation. Pyruvate carboxylase, specific activity 18.5, protein (0.5 mg/ml), in 0.15 M KCl, 1 mM EDTA, and 10 mM potassium phosphate buffer (pH 7.0) was incubated at 0° for 45 min, then transferred to a 24° bath at zero time and reactivated by rewarming. Activity was assayed at intervals during the reactivation.

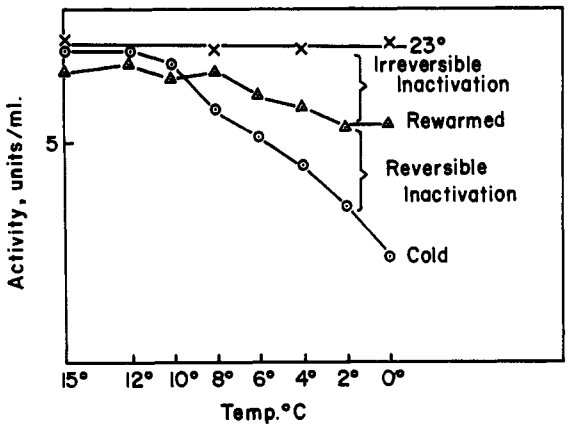


FIGURE 6: Temperature dependence of cold inactivation. Pyruvate carboxylase (specific activity 14). Aliquots of a sample held at 23° were removed at 2-min intervals, incubated for 20 min at the temperature shown, and assayed at 25°. These samples were then rewarmed for 20 min at 23° and reassayed at 25°.

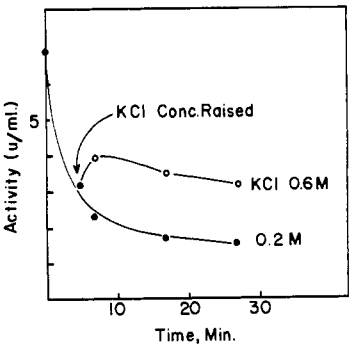


FIGURE 8: Effect of increasing KCl during cold inactivation. Pyruvate carboxylase (specific activity 18) was incubated at 0° in two aliquots. To one aliquot, 2 M KCl was added after 5 min to bring the KCl concentration to 0.6 M. Activity was assayed at 25° at intervals and corrected for dilution in the sample brought to higher salt concentration.

ments, which confirmed this finding, will not be presented here, but it may be mentioned that reversible inactivation is particularly enhanced at low protein concentrations.

Cold inactivation is more marked at lower temperatures as shown in Figure 6 where little inactivation was demonstrable above 10°. "Reversible" and "irreversible" inactivations could not be distinguished at the higher temperatures since the handling involved in rewarming simply caused slight additional inactivation. Below 10° there was increasing reversible and irreversible inactivation. Incubation at the lowest temperatures caused little additional change in the rate of irreversible inactivation. The greater temperature dependence of reversible inactivation has been a consistent finding. The maximum temperature at which cold inactivation may be observed, and the temperature at which irreversible inactivation reaches a maximum rate, are subject to some variation depending upon other experimental conditions.

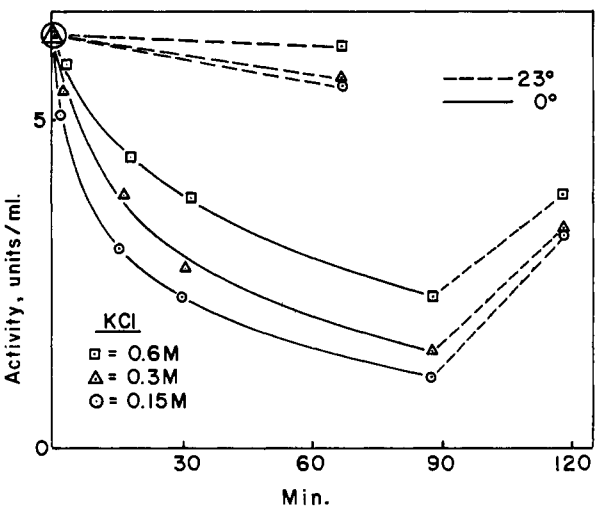


FIGURE 7: Effect of KCl concentration on cold inactivation. Pyruvate carboxylase (specific activity 12) was equilibrated with solutions of KCl having the concentrations shown, all containing 1 mM EDTA and 10 mM potassium phosphate buffer, pH 7.0, to give protein concentrations of 0.5 mg/ml. Reactivation was carried out in the absence of ATP.

Low temperatures increase the concentrations of O_2 and CO_2 in solution. The possibility that cold inactivation might in part involve oxidative reactions was tested by comparing cold inactivation in solutions exposed to air with that occurring under N_2. To obtain anaerobic conditions, enzyme samples were equilibrated with buffer using a Sephadex G-50 column in an anaerobic elution apparatus similar to that of Sakami (1962) which involved flushing with CO_2-free N_2. Samples were obtained containing less than 10% of the O_2 present in a sample exposed to air but there was no observable difference in inactivation between these experiments and control experiments conducted in air. It thus appears unlikely that increased O_2 or other gases in solution play any part in the cold inactivation of pyruvate carboxylase.

High ionic strength has been reported to increase the extent of cold inactivation of muscle glycogen phosphorylases *a* and *b* (Graves *et al.*, 1965) and to decrease that of glutamate decarboxylase from *E. coli* (Shukuya and Schwert, 1960). The experiments of Figure 7 demonstrate the effects of differing KCl concentrations on the progress of cold inactivation of pyruvate carboxylase. Loss of activity is considerably less at higher salt concentration but differences are less marked after reactivation. KCl protects mainly against reversible inactivation with a lesser effect on irreversible inactivation. Stability at room temperature is also favored at higher salt concentrations, as seen from the three upper lines. Reversible inactivation seems to reach a maximum within approximately the same period of time in each instance, but is less in extent at higher salt concentrations, as if the position of a near equilibrium had been shifted. Indeed, raising the concentration of KCl after initial cold inactivation results in partial reactivation in the cold, as shown in Figure 8. Very high concentrations of KCl, *e.g.*, 2 M, protect completely against both reversible and irreversible inactivation for several hours. Other salts, such as K_2SO_4 or $(NH_4)_2SO_4$ (not shown), give effects similar to those shown by KCl but the time course of inactivation depends upon the specific salt used. This factor and the marked instability of the enzyme at room temperature in the presence of certain salts (nitrates, bromides) prevent straightforward inter-

TABLE II: Effect of Methanol on the Irreversible and Reversible Inactivation of Pyruvate Carboxylase by Cold.[a]

Type of Inactivation	Extent of Inactivation (% of Original Act. Lost)		
	0 M MeOH	1.25 M MeOH	2.5 M MeOH
Reversible	54	10	0
Irreversible	21	6	0

[a] Pyruvate carboxylase (specific activity 14, 0.8 mg/ml) was incubated at 0° for 65 min in 0.15 M KCl, 1 mM EDTA, 10 mM potassium phosphate (pH 7.2), and methanol as indicated. Reactivation was carried out for 20 min at 23° without added ATP. "Reversible" inactivation refers to the difference between the inactivated and reactivated samples and "irreversible" inactivation to the difference between reactivated and control samples held at 23° throughout.

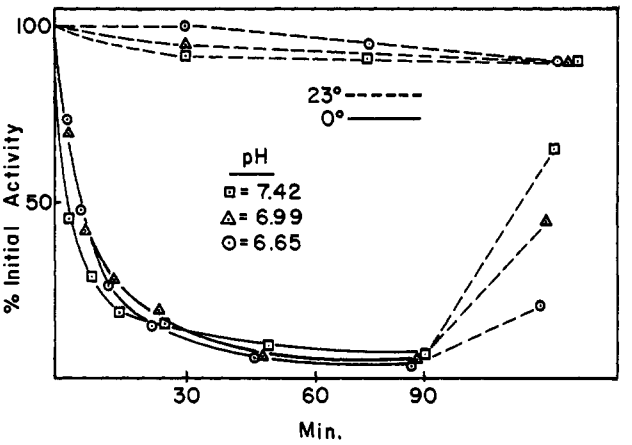


FIGURE 9: Effect of pH on the nature of inactivation by cold. Pyruvate carboxylase (specific activity 11) was equilibrated with 0.2 M KCl and 1 mM EDTA to give a protein concentration of 6 mg/ml; aliquots were diluted to 0.5 mg of protein/ml in 10 mM potassium phosphate of different pH values and containing 0.2 M KCl and 1 mM EDTA with final pH readings as shown. Reactivation was carried out without ATP. For each sample, the activity at the start of cold incubation is taken to be 100%. Prior activity losses during handling were 9, 13, and 35% for samples at pH 6.65, 6.99, and 7.42, respectively. All activities were assayed at pH 7.8.

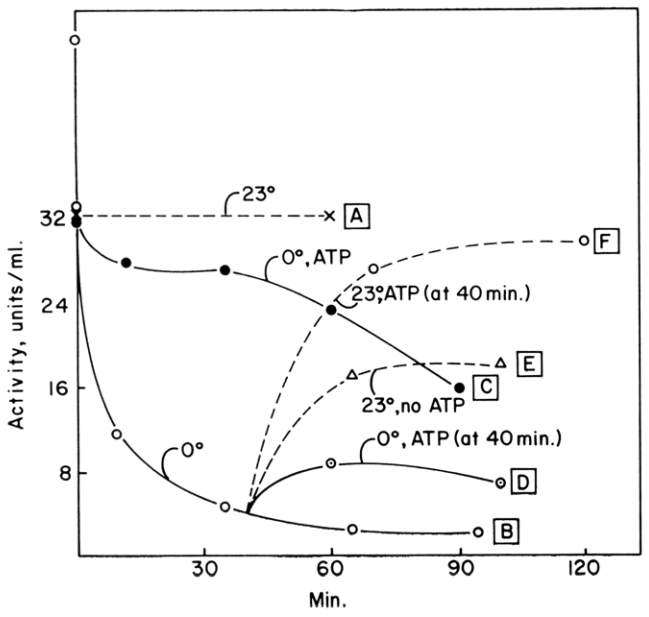
pretations of the salt effects in terms of ionic strength and type of salt.

It was previously reported (Utter *et al.*, 1964) that sucrose in high concentrations (1.5 M) protects pyruvate carboxylase completely against cold inactivation. Several other polyols have a similar effect, as do the simpler compounds methanol, ethanol, and acetone. Ethanol and acetone decrease the enzyme's stability at room temperature, but methanol does not have this effect. Table II shows the effect of methanol in two concentrations on cold inactivation: there is marked protection by 1.25 M methanol against both reversible and irreversible inactivation and complete protection by 2.5 M methanol. Addition of methanol after partial cold inactivation causes partial reactivation in the cold similar to that shown for KCl in Figure 8. When aliphatic alcohols of higher molecular weight than ethanol were tested, there was very marked denaturation of the enzyme immediately on mixing.

The marked effect of a variety of solutes in high concentrations on cold inactivation suggests that interaction of the protein with water may play a part in the process. Measurements of pH have been made before and after cooling unbuffered solutions of the enzyme in an attempt to detect possible net uptake or release of protons by the protein; no consistent change has been found. When cold inactivation takes place in buffers of different pH, the results are as seen in Figure 9. Overall cold inactivation is little affected by pH over the range tested, as shown by the fairly close clustering of the inactivation curves. However there is a striking difference in reactivation properties. Higher pH greatly favors reactivation, *i.e.*, there is proportionately more reversible inactivation; while lower pH values favor irreversible inactivation. Experiments not included in the figure have shown that cold inactivation at low pH followed by rewarming at higher pH does not increase reactivation. Thus pH appears to affect the relative amounts of reversible and irreversible cold inactivation rather than primarily affecting reactivation. Studies similar to those reported in the present paper have also been carried out in solutions buffered by Tris, instead of phosphate, with qualitatively similar results. The pH of such Tris solutions increases considerably as the temperature is lowered, thus complicating the interpretation of temperature studies. This problem is avoided by the use of phosphate buffer; at the phosphate and total salt concentrations used, the pH drops slightly (0.05–0.1 pH unit) on cooling from 23 to 0°.

Effects of Some Reaction Components and Related Compounds on Cold Inactivation. In addition to enhancing reactivation by rewarming as shown earlier (Figures 2 and 3) ATP protects pyruvate carboxylase against cold inactivation and dissociation when added before incubation of the enzyme in the cold. The protective effects of ATP are shown in Figure 10a,b. Figure 10a shows the catalytic activities of samples incubated at 23° (A) and at 0° without additions (B) and at 0° in the presence of ATP (C). The effect of adding ATP after inactivation without concomitant warming is shown in (D) and reactivation by warming with (F) or without (E) ATP are included for comparison. Ultracentrifugal examinations of the same samples after incubation are shown in the Schlieren patterns of Figure 10b which are designated in the same manner as the curves of catalytic activity. The protection by ATP against dissociation in a typical experiment is shown by comparison of patterns B and C. Pattern C is nearly the same as that of A, the room temperature control; protection against dissociation is nearly complete. By contrast, protection against inactivation is only partial as shown by curve C in the graph.

It should be noted that the sedimentation pattern for C in Figure 10b was obtained after about 75 min in the cold, at which time the sample had lost about 40% of its catalytic activity. Most of the inactivation shown in curve C was reversible by rewarming; this result was omitted from the figure for simplicity. These findings suggest that in the presence of ATP reversible inactivation can take place in the cold without accompanying dissociation, as would occur if an inactive tetramer was formed prior to dissociation. The existence of such a species is also consistent with the results obtained by adding ATP to an inactivated sample without subsequent re-



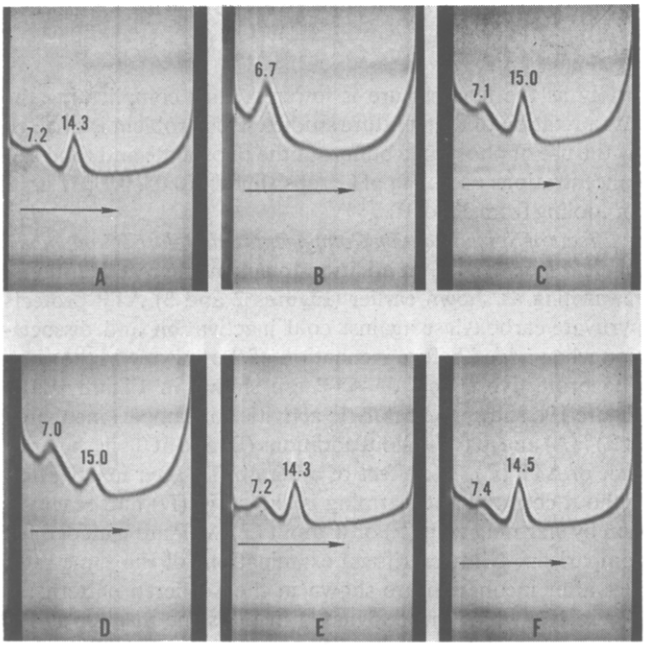


FIGURE 10: Effects of ATP. (a, top) On inactivation and reactivation of pyruvate carboxylase at low temperature. Pyruvate carboxylase (specific activity 21; 1.4 mg of protein/ml) in 10 mM potassium phosphate buffer (pH 7.25), 0.2 M KCl, and 1 mM EDTA was incubated at the temperature shown. ATP (4 mM) was present throughout in sample C, and added at 40 min to D and F. (b, bottom) On dissociation and reassociation of pyruvate carboxylase at low temperatures. The samples designated in part a as A–F were examined in the analytical ultracentrifuge. Individual samples were treated as follows, with photographs at times indicated. (A) Held at 23° for 60 min; (B) held at 0–1° for 75 min; (C) 4 mM ATP added, then held at 0–1° for 75 min; (D) held at 0° for 40 min, then 4 mM ATP added and held at 0–1° for an additional 60 min; (E) held at 0° for 40 min and then rewarmed to 23° for 60 min; and (F) held at 0° for 40 min, then 4 mM ATP added and rewarmed to 23° for 85 min. Actual times at full speed and temperatures in the centrifuge were 24 min at 23° for A, E, and F and 48 min at 1° for B, C, and D. Direction of sedimentation and approximate $s_{20,w}$ values are indicated on the individual photographs.

TABLE III: Specificity of Nucleoside Phosphate Protection against Cold Inactivation.[a]

Additions	% Original Act. Remaining after 15 min	30 min
None		23
ATP (5 mM)	102	92
ADP (5 mM)	104	81
5′-AMP (5 mM)	60	42
CTP (5 mM)		88

[a] Pyruvate carboxylase (specific activity 10) incubated at 0° with additions as indicated. K_m values for ATP and ADP in the catalytic reactions have been reported (Scrutton and Utter, 1965b) as 5.8 and 6.3 $\times$ 10^{-5} M, respectively, and K_I values against ATP and ADP, respectively, as 9.6 $\times$ 10^{-5} M for CTP and 9.3 $\times$ 10^{-3} M for 5′-AMP.

warming (D): reassociation is much more marked than reactivation.

Experiments E and F show the effects of rewarming alone and rewarming with ATP. As previously shown, ATP markedly enhanced reactivation by rewarming. In this experiment, over 90% of the activity of the control sample was obtained in the presence of ATP compared with less than 60% in its absence. Reassociation, on the other hand, was nearly as great on simple rewarming as on rewarming with ATP. This result contrasts with the enhancement of reassociation by ATP evident in Figure 2, but both types of results have been observed a number of times. Possible explanations for this combination of findings will be discussed later in connection with a proposed model of the dissociation–association phenomena.

The above described effects of ATP have been observed in the absence of added metal ions although Mg^{2+} or Mn^{2+} must be present for demonstration of catalytic activity. The unchelated form of ATP does bind to the enzyme, however, and produces marked changes in its properties; for example, unchelated ATP protects completely against inactivation of the enzyme by avidin (Scrutton and Utter, 1965). Other nucleoside triphosphates such as CTP which are inhibitors of the catalytic activity also protect the enzyme against inactivation by avidin in a manner very similar to that observed with ATP (Scrutton and Utter, 1965). ADP and AMP are also effective against avidin although very high concentrations of AMP are required. The experiments of Table III show that CTP and ADP protect against inactivation by cold about as well as does ATP and that AMP at 5 mM offers some protection. Thus, nucleoside phosphates protect against inactivation by cold or avidin and with about the same specificity. The K_m for ATP in the catalytic reaction is 60 μM, the K_d for ATP in the protection against avidin is 75 μM (Scrutton and Utter, 1965). Figure 11 shows the effects of ATP at varying concentrations on the early stages of cold inactivation. Since it has not been possible to establish the order of this rapid inactivation reaction with certainty, these data do not lend themselves to calculations of a dissociation constant for ATP. Nevertheless, it is apparent that 500 μM ATP affords complete protection during the ex-

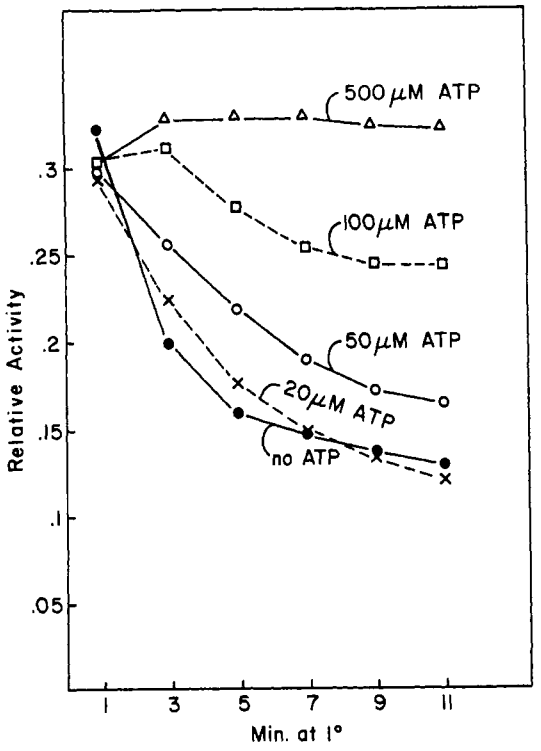


FIGURE 11: Effect of ATP concentration on protection of pyruvate carboxylase against cold inactivation. Pyruvate carboxylase (specific activity 21) incubated as described in Methods with addition of ATP as indicated.

perimental period and that 100 μM ATP affords considerable protection. The results suggest that the K_d may be between 50 and 100 μM which is in reasonable agreement with values obtained from catalytic experiments or with avidin inactivation. The latter experiments were carried out at 23°, which should be kept in mind when comparing such values with data from cold inactivation studies. In experiments not shown here, cold inactivation was shown to be markedly sensitive to CTP concentration and approximately half-protection was provided by 500 μM CTP.

Inorganic phosphate, also a reaction component, has been used as buffer in most of the studies reported here. However, in the concentration used (10 mM), phosphate protects only very slightly as compared with a sample of the enzyme buffered only with 1 mM EDTA. The latter situation was found to be inconvenient for routine use as the pH tends to drift downward. When higher concentrations are used, phosphate does provide considerable protection against cold inactivation. Figure 12 shows typical results; there was less reversible inactivation and almost no irreversible inactivation at the higher phosphate concentrations. Phosphate also conferred greater stability at room temperature. In other experiments arsenate has been shown to have effects very similar to those of phosphate.

Although most of the substrates, inhibitors, and activators of pyruvate carboxylase show protective effects against cold inactivation, pyruvate is a striking exception as shown in Figure 13. In the early stages of inactivation, pyruvate actually appears to accelerate the inactivation process as shown in this experiment. In other experiments not shown here it has

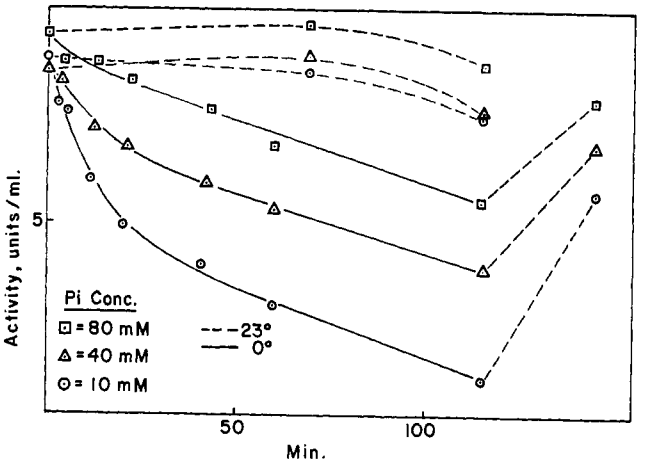


FIGURE 12: Cold inactivation in varying phosphate concentrations. Pyruvate carboxylase, specific activity 20, in 0.2 M KCl, 1 mM EDTA, and potassium phosphate buffer in the concentrations shown, final pH 7.2, diluted to protein concentration, 0.5 mg/ml. Reactivation was carried out without ATP.

been demonstrated that pyruvate has no deleterious effect on the stability of the enzyme held at room temperature nor is reactivation hindered by the presence of pyruvate. Earlier experiments with this enzyme strongly support the hypothesis

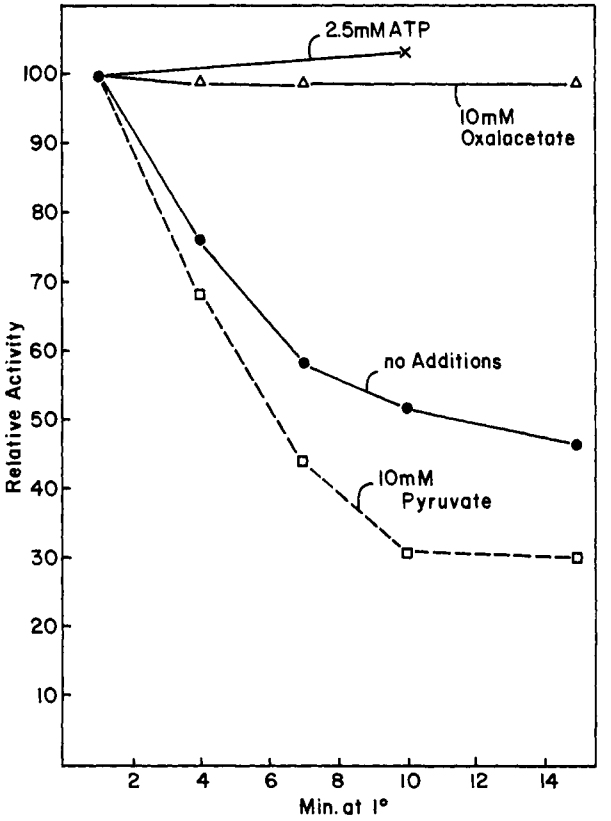


FIGURE 13: Effects of pyruvate and oxalacetate on cold inactivation of pyruvate carboxylase. Pyruvate carboxylase (0.5 mg/ml, specific activity 18) in 10 mM phosphate (pH 7.25), 0.2 M KCl, 1 mM EDTA, and other additions as indicated. Activities are expressed as percentages of values observed 1 min after cooling for each sample.

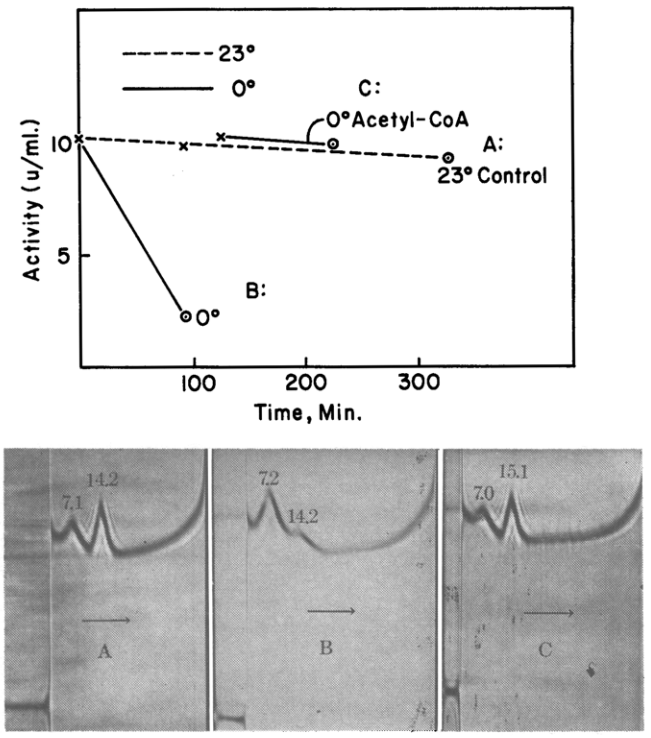


FIGURE 14: Acetyl-CoA protection against cold inactivation and dissociation. Pyruvate carboxylase (specific activity 10.6; 1 mg of protein/ml) was treated as described below. Direction of sedimentation and $s_{20,w}$ values are shown on the Schlieren photographs. Sedimentation patterns were photographed after the times indicated below with a bar angle of 40°. (A) After 320 min at 23°, (B) after 89 min at 0–1°, (C) after 90 min at 0–1° in the presence of 5×10^{-4} M acetyl-CoA. Actual times at full speed and temperatures in the ultracentrifuge were 16 min at 23° for A and 32 min at 1° for B and C.

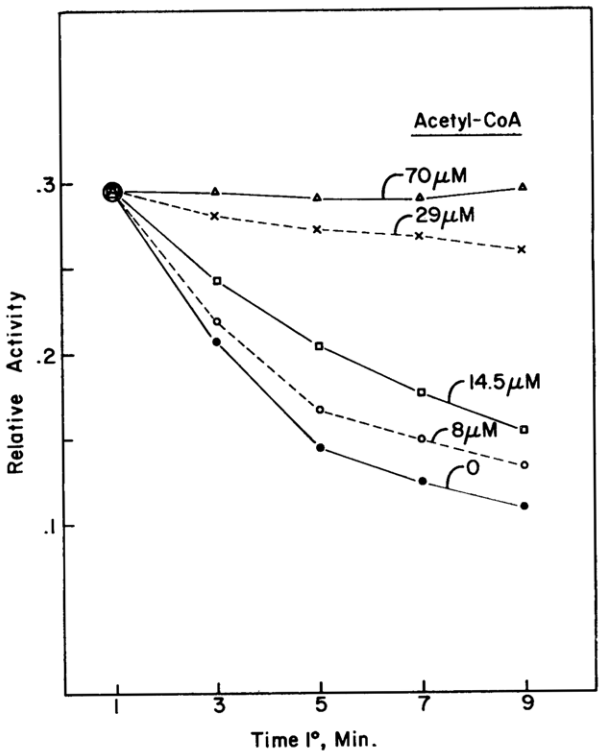


FIGURE 15: Effect of acetyl-CoA concentration on protection of pyruvate carboxylase against cold inactivation. The conditions are similar to those of Figure 13 with the exception of the addition of acetyl-CoA as indicated.

that pyruvate interacts with the tightly bound Mn ions present in pyruvate carboxylase (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967). Oxalacetate has also been shown to interact with the protein-bound Mn ions (Mildvan *et al.*, 1966) but as shown in Figure 13, oxalacetate is almost as effective as ATP in protecting the enzyme against cold inactivation. As discussed previously (Mildvan, *et al.*, 1966; Scrutton and Mildvan, 1968) the nature of the binding of pyruvate and oxalacetate to the bound metal of the enzyme is somewhat different and these differences may be responsible for the opposing effects of pyruvate and oxalacetate noted here.

An earlier paper in this series reported that acetyl-CoA, alone or in combination with ATP, Mg^{2+} and HCO_3^-, partially protected pyruvate carboxylase against cold inactivation; when the other reaction components were present the protection was essentially complete (Scrutton and Utter, 1965). Further experiments have shown that acetyl-CoA alone, added in very small volumes to minimize dilution of the enzyme, protects completely against cold inactivation. Figure 14 demonstrates that acetyl-CoA prevents both the loss of catalytic activity and the dissociation otherwise induced by cold. It has been suggested that the role of acetyl-CoA in the pyruvate carboxylase reaction (reaction 1) is one of altering the enzyme's conformation. Acetyl-CoA causes no significant changes in the sedimentation pattern of pyruvate carboxylase when examined at room temperature (Scrutton and Utter, 1967), but there may be conformational changes not readily detected by ultracentrifugation. The existence of such changes is strongly suggested by the greatly enhanced susceptibility of the biotin of the enzyme to inactivation by avidin in the presence of acetyl-CoA (Scrutton and Utter, 1967). Unlike ATP, the addition of acetyl-CoA after inactivation by cold has no appreciable effect on reactivation effected by rewarming.

The protective effects of acetyl-CoA against cold inactivation are highly concentration dependent as demonstrated in Figure 15. Because of the uncertain order of the inactivation reaction these data are not suitable for calculation of K_d values for acetyl-CoA. It is apparent, however, that a concentration of about 20 μM would afford approximately half protection. This value compares with values of 10–30 μM which have been reported (Keech and Utter, 1963; Scrutton and Utter, 1967) for the activation constant of acetyl-CoA in the catalytic reaction. Plots of acetyl-CoA concentration against initial velocity rates of the catalytic reaction show a marked sigmoidal character with a Hill coefficient of about 3 (Scrutton and Utter, 1967). The protection experiments shown in Figure 15 appear to show a similar cooperative effect since a fourfold change in concentration (8–29 μM) increases very slight protection to almost complete protection. The high degree of concentration dependence is quantitatively very similar to that observed in the catalytic reaction, and differs greatly from that predicted from a Michaelis–Menten relationship.

Pyruvate carboxylase from chicken liver is activated not only by acetyl-CoA but by several other acyl-CoA compounds notably propionyl-CoA (Scrutton and Utter, 1967). Experi-

TABLE IV: Specificity of Acylthiol Protection against Cold Inactivation by Pyruvate Carboxylase.[a]

Expt	Additions	% of Original Act. Remaining after 15 min at 1°	30 min at 1°
1	None	45	28
	Acetyl-CoA (0.1 mM)	96	93
	Propionyl-CoA (1 mM)	78	68
	Crotonyl-CoA (1 mM)	83	75
	CoA-SH (1 mM)	45	31
	Hydrolyzed acetyl-CoA	38	25
2	None	66	44
	Acetylpanthetheine (0.5 mM)	64	46
	Acetyl-CoA (0.5 mM)	108	108

[a] Pyruvate carboxylase (0.5 mg/ml, specific activity 17 in expt 1 and 21 in expt 2) incubated at 1° in 10 mM phosphate (pH 7.25), 0.2 M KCl, and 1 mM EDTA with additions as indicated. Acetyl-CoA was hydrolyzed by adjusting to pH 11 with KOH and reneutralizing after 90 min.

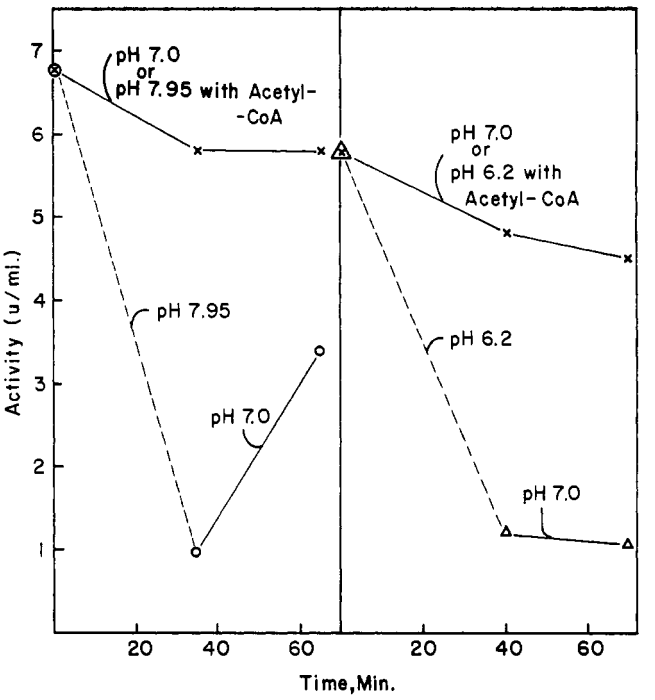


FIGURE 16: Effect of acetyl-CoA on the inactivation of pyruvate carboxylase by acid and base. Pyruvate carboxylase (specific activity 13.5, 0.5 mg of protein/ml) was equilibrated with 0.2 M KCl and 1 mM EDTA (pH 7.0) at 23°. Control aliquots and aliquots containing 0.2 mM acetyl-CoA were adjusted to the indicated pH values by addition with stirring of small (approximately 60 μl) volumes of 0.01 N KOH or HCl to 3-ml aliquots of the enzymes. All activities assayed at pH 7.8.

ment I in Table IV shows that these compounds also protect the enzyme against cold inactivation. The degree of protection appears to be slightly less than that afforded by acetyl-CoA. Experiment 1 in Table IV also shows that neither CoA-SH, nor a hydrolyzed sample of acetyl-CoA, protects the enzyme against cold inactivation.

Several acyl thioesters including acetylpantetheine have been shown to inhibit the catalytic activation of pyruvate carboxylase by acetyl-CoA in an apparently competitive fashion (Scrutton and Utter, 1967). Experiment 2 of Table IV shows that acetylpantetheine at a concentration of 0.5 mM offers no protection against cold inactivation.

Activation of the catalytic reaction of pyruvate carboxylase and protection against cold inactivation of the enzyme by acyl-CoA compounds show similarities in effective concentration ranges, high concentration dependence, and specificity. These observations suggest that the same changes caused in the molecule by acyl-CoA's which permit catalytic activity also confer stability against cold. The failure of acetylpantetheine, a competitive inhibitor of acetyl-CoA, to protect against inactivation also suggests that the conformational changes necessary for stability at low temperature may be quite specific.

Inactivation of Pyruvate Carboxylase by pH Alterations and Urea: Points of Similarity to Cold Inactivation. The earlier finding that pH affects cold inactivation (Figure 9) suggested that it might be possible to mimic some of the effects of cold by keeping the enzyme at room temperature and exposing it to changes in pH. This possibility was tested with results as shown in Figure 16. The enzyme was inactivated when the pH was raised to 7.95 or lowered to 6.2, and acetyl-CoA provided protection in each case. Inactivation at low pH was not reversed by neutralization. In contrast, inactivation at high pH was partly reversed by restoring the pH to 7.0. The demonstration of reversibility during certain types of pH changes supports the hypothesis that the mechanism may be related to that of cold inactivation and reactivation. On further examination of the effects of high pH on the enzyme, it was found that inactivation was generally rapid, with losses of as much as 65% of initial activity within 3 min after addition of base, and return to as much as 50% of initial activity by 10 min after neutralization. Quantitatively, the results varied somewhat in different experiments. Acid and base were always added with stirring in small volumes to nearly unbuffered solutions in order to avoid dilution of the enzyme; overshoot of pH could thus cause irreversible denaturation in a given region of the solution.

Inactivation at high pH may be said to mimic in part the effects of low temperature but an analogous dissociation has not been shown clearly. Ultracentrifugal examination has shown that high pH causes a decrease in the fast component (14.8 S) but the accompanying increase in the slow component (7 S) is not as large. Dissociation may be overshadowed by aggregation to species larger than 14.8 S. Acetyl-CoA prevents the changes in sedimentation pattern as well as those in catalytic activity brought about by high pH. Reneutralization does not result in a complete restoration of the original sedimentation pattern after exposure to high pH, owing perhaps to the aggregation factor as suggested above, but it should be emphasized that catalytic activity is also not restored to the original level.

Pyruvate carboxylase can also be inactivated at room temperature by low concentrations of urea (0.4 M). As shown in the lower curve (C) of Figure 17, an approximately 70% loss of the catalytic activity occurs over a 75-min period. In the

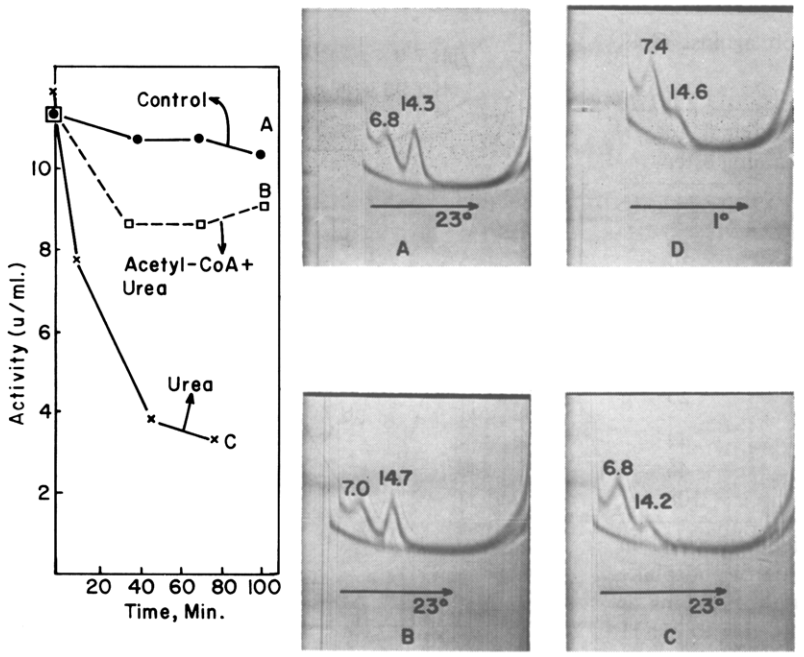


FIGURE 17: Effects of cold, 0.4 M urea, and acetyl-CoA on the activity and dissociation of pyruvate carboxylase. Pyruvate carboxylase (1.25 mg/ml for A and D and 1 mg/ml for B and C) was treated as described below: (A) held at 23° for 90 min; (B) incubated at 23° with 0.4 M urea and 5×10^{-4} M acetyl-CoA for 60 min; (C) incubated at 23° in 0.4 M urea without acetyl-CoA; (D) held at 0° for 60 min. Schlieren photographs were taken at approximately these times. Actual time at full speed in the centrifuge was 16 min for A, B, and C, and 32 min for D. Direction of sedimentation, temperature in the centrifuge, and $s_{20,w}$ values are shown on the photographs.

same length of time, the control (A) has lost only about 5% of its activity. The presence of acetyl-CoA (B) provides marked protection against inactivation by urea after an initial loss of about 20% of the activity. Ultracentrifugal examination of these samples (photographs accompanying Figure 17) shows that urea has produced a marked shift of material from the faster moving peak to the slower moving component. The latter has a sedimentation coefficient very similar to that found after cold inactivation as is shown by sedimentation pattern D of Figure 17 where the effect of exposure of the same sample of enzyme to cold is shown. Acetyl-CoA also prevents the shift in sedimentation pattern in both instances. These results support the view that low concentrations of urea and low temperatures cause the same dissociation reaction. Other studies not shown here indicate that the effects of urea and low temperature are at least partially additive. No attempts have been made as yet to reverse the dissociation caused by urea. High concentrations of urea (6 M) cause immediate inactivation and precipitation of the enzyme.

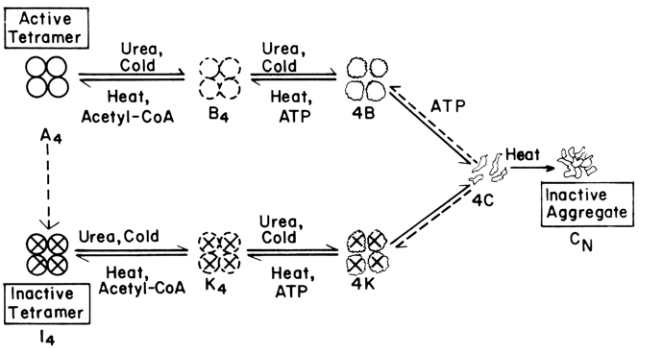


FIGURE 18: Proposed scheme representing interconversions between active and inactive forms of pyruvate carboxylase. See text for details.

Discussion

The results presented here suggest that the mechanism of the reversible cold inactivation and dissociation of pyruvate carboxylase is complex. For the purpose of discussing the various observations the following hypothesis is proposed (Figure 18) although it should be clear that this scheme is a very tentative one. In this scheme A_4 represents the active tetramer and is the only catalytically active species (or group of species) present. On exposure to cold A_4 is converted into an inactive tetramer (B_4) which then dissociates to four protomers (B). The need to postulate an inactive tetramer comes from the experiments of Figure 10a,b where the presence of ATP during exposure to cold preserves the initial sedimentation pattern although considerable loss of catalytic activity occurs. In the reactivation phase of this same experiment, the addition of ATP without concomitant rewarming causes a considerable shift toward aggregation with only a minor increase in activity. Both of these results are consistent with the intervention of an inactive tetramer between the active tetrameric species and the dissociated form of the enzyme. The first-order kinetics of reactivation (Figure 5 and Table I) are also consistent with a rate-limiting conversion of a catalytically inactive tetramer into an active one. Because the effects of cold on dissociation and reassociation appear to be essentially unaffected by the actual specific catalytic activity of the enzyme it is necessary to postulate a parallel series of reactions (perhaps with different rates) for an inactive form of the enzyme (I_4, K_4, and 4K). Possible slow conversion of A_4 into I_4 is also indicated, since the enzyme is slowly and irreversibly inactivated on storage without dramatic shifts in the sedimentation pattern.

Another species of protomers, here designated as C, is necessary to explain the observation of the slow irreversible inactivation. The species is also indicated as the link between the members of the two dissociation–association series, $A_4 \rightleftarrows 4B$ and $I_4 \rightleftarrows 4K$. The conversion of B or K into C is presumably slow and accounts for the gradual increase in the propor-

tion of irreversible inactivation. During rewarming, the extent of regain of active tetramer (A_4), inactive tetramer (I_4), and inactive aggregate (C_N) would be dependent upon the relative amounts of B, K, and C. The multiple routes available to the various protomeric forms for aggregation during rewarming could account for almost complete restoration of sedimentation pattern without accompanying catalytic activity (Figure 10a,b) or an incomplete restoration of the sedimentation pattern with apparent loss of protein through aggregation (Figure 2). The presence of ATP would favor the formation of B_4 and A_4 from the protomers and lead to greater restoration of catalytic activity on rewarming as well as to essential restoration of the original sedimentation pattern.

Acetyl-CoA is shown as favoring the A_4 or I_4 forms of the enzyme. ATP is also indicated to inhibit dissociation of B_4 or K_4 in accordance with its observed effects in Figure 10a,b.

One of the main features of the above scheme is the existence of a mainly reversible series of reactions with only slow irreversible reactions at A_4 to I_4 and B and K to C and a rapid irreversible aggregation (C to Cn) at room temperature after prior dissociation reactions. Many of the observations made here suggest the existence of equilibrium reactions during cold inactivation with the position of the equilibrium subject to alteration by many variables such as pH, phosphate concentration, salt concentration, presence of methanol, addition of ATP, and so on.

It is also possible to adapt the above scheme to the changes produced by low concentrations of urea and, with some assumptions concerning the relative rates of the reactions, to the effects of high pH.

Most reaction components exert their effects on cold inactivation at concentrations low enough to indicate that specific reactions of the compounds with the enzyme are involved. In general, the quantitative relationships of the enzyme's interaction with these components are very similar to those observed in catalytic studies.

The kinds of bonds involved directly or indirectly in the dissociation of pyruvate carboxylase and the physical-chemical details of the reaction are unknown. Hydrophobic bonds are certainly implicated but some findings suggest that bonds of more than one type may be involved, for example, the protection against cold inactivation by high salt concentrations and the ability of urea and high pH to mimic cold-induced dissociation at room temperature. Interaction between the protein and water probably plays a role; this would be consistent with the pronounced effect of sucrose and other solutes in high concentrations in protecting against cold inactivation. Also, Scrutton and Mildvan[3] have recently found that pyruvate carboxylase from chicken liver is markedly more stable in D_2O than in H_2O in the cold.

The dissociation and reassociation of pyruvate carboxylase exhibit some features inviting comparison with the reversible polymerization of tobacco mosaic virus protein. As reported by Stevens and Lauffer (1965) this reaction proceeds to an equilibrium in which an increased proportion of monomer is favored by low salt concentration, high pH, and low temperature. It has been reported that the protein releases bound water on polymerization, liberating the energy required by the process. One might speculate whether such a reaction could be involved in the case of pyruvate carboxylase as well.

[3] Private communication by M. C. Scrutton and A. S. Mildvan.

Cold lability has now been reported as a property of a number of enzymes (Scrutton and Utter, 1965; Graves *et al.*, 1965; Shukuya and Schwert, 1960; Hofstee, 1949; Fincham, 1957; Pullman *et al.*, 1960; Raijman and Grisolia, 1961; Kirkman and Hendrickson, 1962; Shuster and Doudoroff, 1962; Dua and Burris, 1963; Racker *et al.*, 1963; Numa and Ringelmann, 1965; Havir *et al.*, 1965; Penefsky and Warner, 1965; Jarabak *et al.*, 1966). Certain similarities are apparent on comparing these reports, although there is much variation among the enzymes described in reversibility of cold inactivation, rates of inactivation, concomitant physical changes, and effects of experimental variables. It is important to note that recent experiments indicate that cold lability is not a property of all species of pyruvate carboxylase. The enzyme as isolated from yeast[4] or calf liver[5] is not particularly susceptible to inactivation at low temperatures although the thermal properties of the enzyme from turkey liver[5] are very similar to those described here for the chicken liver enzyme. A preliminary report on the properties of pyruvate carboxylase of *Arthrobacter globiformis* (Bridgeland and Jones, 1967) also suggests that this species of the enzyme is cold labile.

Reactivation by simple rewarming has been reported for most of the cold-labile enzymes. In the case of a glutamate decarboxylase from *E. coli*, thermal reactivation was reported by Shukuya and Schwert (1960) to be essentially complete even after repeated cycles of cooling and rewarming;[6] thus in this instance there may be a true equilibrium between active and reversibly inactivated species, not appreciably affected by ongoing irreversible inactivation. For most of the other enzymes, the extent of reactivation is variable, and is generally less after prolonged incubation in the cold. The effect was very clearly shown by Penefsky and Warner (1965) for the mitochondrial ATPase of beef heart, and by Jarabak *et al.* (1966) for the 17β-hydroxy steroid dehydrogenase of human placenta. The latter enzyme especially resembles pyruvate carboxylase in seeming to undergo early rapid reversible cold inactivation and concurrent slower irreversible inactivation. A further similarity is the greater temperature dependence of the reversible phase compared with the irreversible.

Acetyl-CoA carboxylase from rat liver, like pyruvate carboxylase a biotin enzyme, has been reported by Numa and Ringelmann (1965) to exhibit a peculiar kind of cold lability: the portion of activity which is due to citrate activation is subject to inactivation by cold. Rewarming restores the original response to citrate unless the enzyme has been kept cold for prolonged periods.

As regards physical changes accompanying cold inactivation of the various enzymes, it is difficult to make generalizations. On examination in the analytical ultracentrifuge, or by sucrose density gradients, a number of enzymes have been reported to show appearance or increase of slowly sedimenting components after incubation in the cold. This effect, interpreted as a dissociation, has been described with the mitochondrial ATPase (Penefsky and Warner, 1965), with argininosuccinase (Havir *et al.*, 1965), with erythrocyte glucose 6-phosphate dehydrogenase (Kirkman and Hendrickson, 1962), and with

[4] Unpublished experiments by M. Young, B. Tolbert, and M. F. Utter.

[5] Unpublished experiments by J. Wallace and M. F. Utter.

[6] Unpublished observations indicate that pyruvate carboxylase can also be subjected to repeated cycles of cooling and rewarming with only gradual loss of activity.

citrate-activated acetyl-CoA carboxylase (Numa and Ringelmann, 1965), as well as with pyruvate carboxylase. Other kinds of evidence, such as findings of increased cold inactivation at low enzyme concentrations, allow for dissociation as at least a possible mechanism with the glutamate decarboxylase from *E. coli* (Shukuya and Schwert, 1960) and with the cold-labile nitrogen-fixing enzyme of Dua and Burris (1963). In contrast, glycogen phosphorylase b of muscle has been reported by Graves *et al.* (1965) to undergo reversible aggregation to a more rapidly sedimenting species. As in the case of pyruvate carboxylase, the cold inactivation of other enzymes may not be related in a simple fashion to dissociation or aggregation. For example, cold inactivation of 17β-hydroxy steroid dehydrogenase was accompanied by the appearance of high molecular weight components; separation by gel filtration, however, was reported to show that most or all reactivation on rewarming occurred in lower molecular weight components, so that reversible inactivation might be a conformational change distinct from aggregation (Jarabak *et al.*, 1966). A cold-inactivated enzyme may be predisposed to further structural changes; thus mitochondrial ATPase cold inactivated at high salt concentrations was reported to precipitate on rewarming (Penefsky and Warner, 1965).

As noted earlier with regard to pyruvate carboxylase, the physico-chemical basis of cold lability in enzymes in general remains unclear. Protective effects of glycerol, methanol, and related substances in high concentrations, as found for pyruvate carboxylase, have been reported in several instances (Graves *et al.*, 1965; Shukuya and Schwert, 1960; Penefsky and Warner, 1965; Jarabak *et al.*, 1966) and may imply that protein–water interactions are involved. Salt effects have been variable. Thus high salt concentrations were reported to enhance cold inactivation of phosphorylases *a* and *b* (Graves *et al.*, 1965) and of mitochondrial ATPase (Racker *et al.*, 1963); with the latter, KI exerted the largest and K_2SO_4 the smallest effects. In contrast, high salt concentrations were found to lessen cold inactivation of glutamate decarboxylase (Shukuya and Schwert, 1960) and argininosuccinase (Havir, *et al.*, 1965) as well as pyruvate carboxylase. Effects of both kinds were reported for 17β-hydroxy steroid dehydrogenase (Jarabak *et al.*, 1966), chloride and nitrate enhancing but phosphate and sulfate lessening cold inactivation. Reaction components have exerted several kinds of effects: protection against cold inactivation is commonly reported; there may also be partial reactivation in the cold, as reported for the glutamate decarboxylase with pyridoxal phosphate (Shukuya and Schwert, 1960), or enhancement of reactivation on rewarming, as found for the glucose 6-phosphate dehydrogenase with nicotinamide–adinine dinucleotide phosphate (Kirkman and Hendrickson, 1962), and for pyruvate carboxylase with ATP. These properties of cold inactivation in the several enzymes suggest that varied and complex processes may be involved. As further information becomes available about cold inactivation, better understanding may be provided of the relationship of cold-labile bonds and configurations to the structure and biological properties of enzymes and other proteins.

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