

Cold Inactivation of Glycogen Phosphorylase*

Donald J. Graves, Robert W. Sealock,[†] and Jerry H. Wang

ABSTRACT: Glycogen phosphorylase *b* has been found more sensitive to storage at 0° than at 20° in glycerophosphate-cysteine buffer at pH 6.0. After storage at cold temperatures, enzymic activity is lost, inhomogeneous material can be detected in the ultracentrifuge, and a shift and increase of absorbance of enzyme-bound pyridoxal phosphate occur to shorter wavelengths. Inactivation may be reversed upon rewarming. While the initial rate of cold inactivation of phosphorylase *b* has been shown to follow first-order kinetics at all protein concentrations, the entire course of inactivation can be described by the first-order law only at low protein concentrations. Inactivation is effectively slowed by glycogen, pyridoxal phosphate, AMP,

ATP, organic solvents, and buffer at pH 6.8, and is accelerated at pH 6.0 in the presence of cysteine and NaCl. An Arrhenius plot of phosphorylase *b* activities at pH 6.0 shows a marked discontinuity at approximately 13°, with activation energies of 17,000 and 46,000 calories for the upper and lower limbs, respectively. NaBH₄-reduced phosphorylase *b* is also cold sensitive. Phosphorylase *a*, in contrast to phosphorylase *b*, is sensitive to cold only in solutions of high ionic strength. The present data suggest that the inactivation of phosphorylase results from a conformational change of enzyme structure induced by cold temperatures and is not caused by a loss of enzyme-bound pyridoxal phosphate.

The sensitivity of the secondary and tertiary structure of catalytic proteins in solution to high temperatures has been extensively investigated and well documented. More recently it has been shown that exposure of protein molecules to cold temperatures can also disrupt their native conformations (Penefsky and Warner, 1963; Scrutton and Utter, 1964; Rosenberg and Lumry, 1964); such exposure results in the inactivation of glutamic acid decarboxylase (Skukuya and Schwert, 1960), pyruvate carboxylase (Scrutton and Utter, 1964), and mitochondrial ATPase (Pullman *et al.*, 1960). The instability of glycogen phosphorylase *a* in high ionic strength solutions below pH 7.0 during dialysis at 3–4° (Wang and Graves, 1963) prompted an investigation of the effect of cold temperature on the catalytic and physical properties of glycogen phosphorylase.

Experimental Procedures

Materials. Crystalline phosphorylase *b* was isolated by the procedure of Fischer and Krebs (1958). Phosphorylase *a* was prepared with the use of phosphorylase kinase (Fischer and Krebs, 1962) and crystalline phosphorylase *b*.

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[†] National Science Foundation undergraduate research participant.

Reduced phosphorylase *b* was prepared by first adding solid NaCl to enzyme (40 mg/ml) in 0.08 M glycerophosphate, 10⁻³ M EDTA, pH 6.8, to a final concentration of 1.5 M. After the material was chilled for several minutes in a cold-temperature bath at -5°, 0.2 mg of NaBH₄/ml of solution was added. Addition of NaBH₄ was repeated after 0.5 hour and again after 1 hour of incubation. The reaction mixture was then diluted 10-fold in 0.08 M glycerophosphate-10⁻³ M EDTA, pH 6.8, to which an equal volume of neutral saturated ammonium sulfate was added. The precipitate was collected after centrifugation, dissolved-dialyzed against 0.04 M glycerophosphate-0.03 M cysteine, pH 6.8, and crystallized according to Fischer and Krebs (1958). Per cent reduction of bound pyridoxal phosphate was measured by the analysis of free pyridoxal phosphate in deproteinized supernatants according to Kent *et al.* (1958). A reduction of 93% was obtained with an enzyme specific activity of 1020 units/mg.

AMP and ATP were purchased from Pabst Laboratories, Milwaukee, Wis. Bovine plasma albumin was supplied by California Corp. for Biochemical Research. Cysteine-HCl, sodium glycerophosphate, potassium glucose-1-phosphate, and shellfish glycogen were obtained from Sigma Chemical Co., St. Louis, Mo.

Methods. Phosphorylase *a* activities were measured according to the procedure of Krebs *et al.* (1964); phosphorylase *b* activities were similarly measured in the presence of 10⁻³ M AMP. Enzyme concentration was determined spectrophotometrically with the use of an absorbancy index of 11.7 for a 1% solution of protein (Velick and Wicks, 1951). Ultracentrifugation and measurement of sedimentation coefficients were as previously described (Wang and Graves, 1963).

Ultraviolet and visible spectroscopy were carried out in a Cary Model 14 double-beam recording spectrophotometer. For low temperature spectroscopy, the temperature of the cell in the sample beam was controlled by circulating water at 2° through the cell housing while the reference cell was maintained at room temperature.

Results

Cold Inactivation of Phosphorylase *b*. The effect of preincubation of phosphorylase *b* at cold temperatures upon the catalytic activity of 30° is illustrated in Figure 1. The data clearly show that storage of enzyme (5 mg/ml) in a glycerophosphate-cysteine buffer with 0.2 M NaCl at pH 6.0 at 0° results in a greater extent of inactivation than in samples incubated at 20°. This differential inactivation by temperature will subsequently be referred to as "cold inactivation."

To test whether cold inactivation was reversible upon rewarming, enzyme stored at 0° for 4 hours was rewarmed and incubated at 20° prior to the dilution and activity measurements. As shown in Table I, although only 14% of the original activity remained after storage at 0°, rewarming enzyme (5 mg/ml) to 20° for 2 hours reactivated enzyme to 75% of its original value; rewarming enzyme in the presence of pyridoxal phosphate, a prosthetic group of glycogen phosphorylase, restored the enzyme to full catalytic activity. The return of enzymic activity upon rewarming was dependent upon protein concentration; at 0.1 mg/ml, where 65% of enzymic activity was lost after 1 hour at 0°, no reactivation could be demonstrated after 2 hours at 20° in the absence of added pyridoxal phosphate. In the

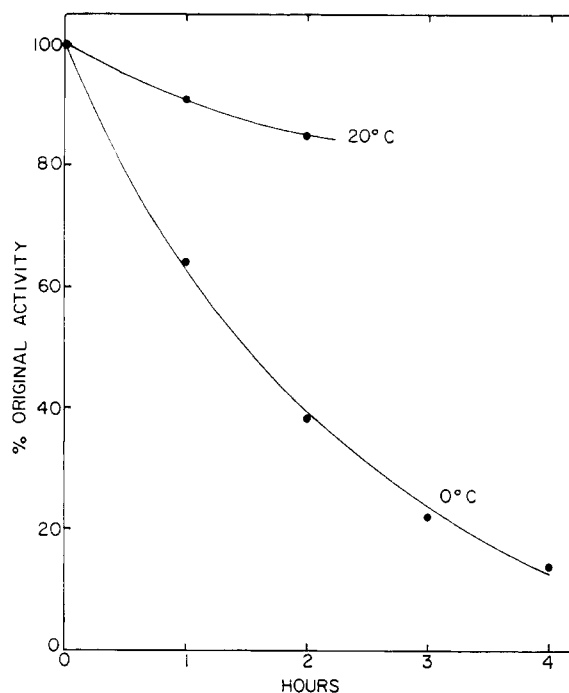


FIGURE 1: Effect of preincubation at cold temperatures on the activity of phosphorylase *b* at 30°. Phosphorylase *b* (5 mg/ml) was incubated in 0.2 M NaCl containing 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, for various intervals prior to a 125-fold dilution in the same buffer at 30°. After a 2-minute incubation at 30° enzyme activities were measured.

presence of 10^{-4} M pyridoxal phosphate enzymic activity was returned to 87% of its initial value.

The effect of protein concentration on the rate of cold inactivation was studied to define more precisely the characteristics of this reaction. Phosphorylase *b* was incubated at different concentrations at 0° in 0.2 M NaCl, pH 6.0, diluted in a glycerophosphate-cysteine buffer to a concentration less than 0.1 mg/ml, a protein concentration where reversal of inactivation did not occur upon rewarming, and assayed for enzymic activity at 30°. The initial rates of cold inactivation are plotted in Figure 2 according to the differential method of Van't Hoff (1884), which may be derived from the relationship, $V = kc^n$, where V is velocity, k is a rate constant, c is protein concentration, and n is the kinetic order of the reaction. The data of Figure 2 show that the order of the reaction does not change with protein concentration, and the line with a slope of 0.93 ± 0.04 , calculated from the experimental points, indicates that cold inactivation of phosphorylase *b* is a first-order reaction. Cold inactivation does not appear, however, to be a simple unimolecular decay of native to inactive enzyme, since the kinetic order for the course of the inactivation varied with protein concentration. At 0.08 mg/ml, the reaction followed first-order kinetics through 90% inactivation, whereas at 10 mg/ml the half-life was increased, and the reaction departed from its initial first-order kinetics after about

TABLE I: Reversal of Cold Inactivation.^a

Time after Rewarming of Enzyme from 0° to 20° (min)	Per Cent of Original Activity	
	No Additions	+PLP
0 ^b	14	
30	51	81
120	76	101

^a Phosphorylase *b* (5 mg/ml) was incubated in 0.2 M NaCl containing 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, for 4 hours at 0°. At this time the reaction mixture was divided into two portions and placed in a 20° water bath. To one portion, pyridoxal phosphate was added to a final concentration of 10^{-4} M; no addition was made to the second aliquot. After various intervals aliquots were removed, diluted 125-fold in 0.2 M NaCl containing 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, and assayed for enzymic activity at 30°. ^b Zero time, in this case, refers to the activity of enzyme after 4 hours of storage at 0° as determined by dilution and assay as in Figure 1.

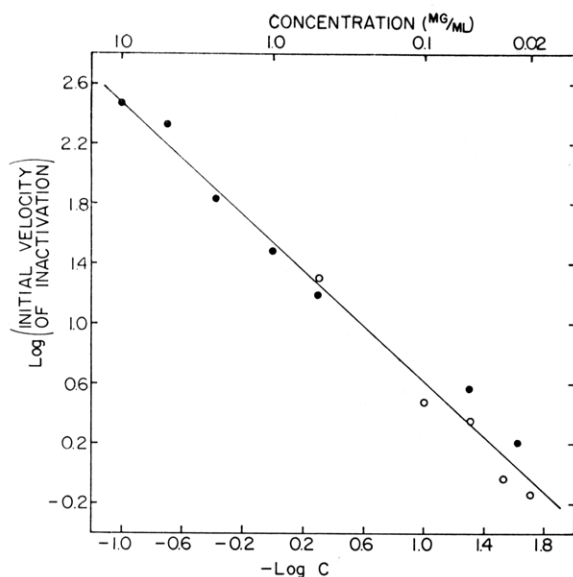


FIGURE 2: Kinetics of the inactivation of phosphorylase *b* by cold temperatures. Phosphorylase *b* was incubated at 0° at different protein concentrations in 0.2 M NaCl containing 0.04 M glycerophosphate–0.03 M cysteine, pH 6.0. At various intervals, aliquots were removed, diluted in buffer at 30°, and assayed for enzymic activity as in Figure 1. Initial slopes of the inactivation curves were measured by determination of the tangent at t_0 with the use of a tangentimeter. The line drawn through the experimental points was calculated by the method of least squares. Closed and open circles, independent experiments.

23% inactivation. It appears that the product, cold inactivated phosphorylase, has no influence on the half-life since identical rates of inactivation of phosphorylase *b* (1 mg/ml) were obtained in 0.2 M NaCl and in 0.2 M NaCl containing totally inactivated enzyme.

Since pyridoxal phosphate has been shown to be essential for enzymic activity of phosphorylase (Cori and Illingworth, 1957) and important for maintenance of its native conformation (Illingworth *et al.*, 1958), the involvement of this prosthetic group in cold inactivation was investigated. Table II illustrates results obtained with NaBH_4 -reduced phosphorylase *b*, a form of enzyme in which the prosthetic group is attached as a stable pyridoxylamine derivative (Fischer *et al.*, 1958). The data show that this form of phosphorylase is also more sensitive to storage at 0° than at 20°. As pyridoxal phosphate would not be expected to be released from the protein under these conditions, this experiment suggests that loss of enzymic activity may be more directly related to alteration of the protein structure than to a loss of pyridoxal phosphate.

In an attempt to delineate the relationship between loss of enzymic activity and alteration of the physical structure of glycogen phosphorylase, ultracentrifugal analyses were carried out under conditions of cold in-

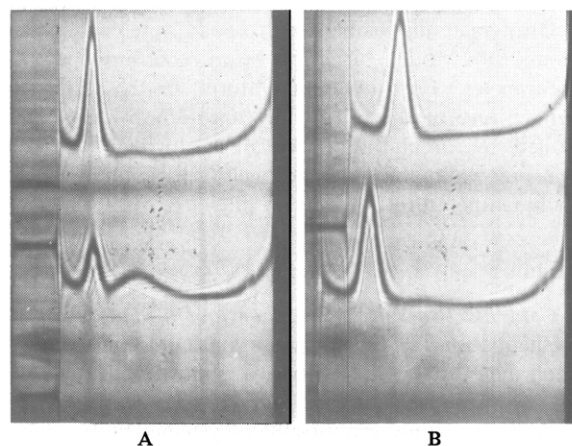


FIGURE 3: Ultracentrifugal patterns of cold-inactivated phosphorylase *b*. Phosphorylase *b* (5 mg/ml) in 0.2 M NaCl containing 0.04 M glycerophosphate–0.03 M cysteine, pH 6.0. Upper curves: enzyme incubated for 2 hours at 20°. Lower curves: enzyme incubated for 2 hours at 0°. (A) Rotor temperature maintained at 7°. (B) Rotor at 20°. Pictures were taken 16 minutes after the centrifuge reached a speed of 59,780 rpm. Sedimentation direction is from left to right.

TABLE II: Cold Inactivation of Reduced Phosphorylase *b*.^a

Time of Incubation (hr)	Per Cent of Original Activity	
	0°	20°
1	46	83
2	31	71

^a Enzyme (2 mg/ml) was incubated in 0.2 M NaCl with 0.04 M glycerophosphate–0.03 M cysteine, pH 6.0, at 0 and 20°. Aliquots were removed, diluted in the same buffer at 30°, and assayed for enzymic activity as in Figure 1.

activation. If enzyme (5 mg/ml) is stored for 2 hours at 0° and centrifuged at 7°, two boundaries can be detected in the ultracentrifuge with $s_{20,w}$ of 8.3 S and 20.2 S (Figure 3A, lower curve). When ultracentrifugation is carried out at 7° on enzyme which has not been exposed to 0°, only one main sedimenting component can be identified with an $s_{20,w}$ of 8.4 S (A, upper curve). The effect on sedimentation characteristics of rewarming to 20° enzyme which had been stored for 2 hours at 0° is illustrated in Figure 3B. The rewarmed enzyme clearly shows one major component in the ultracentrifuge with an $s_{20,w}$ of 8.7 S and nearly complete deletion of heavy sedimenting matter (B, lower curve). As native phosphorylase *b*, not treated at 0°, shows a component with an $s_{20,w}$ also of 8.7 S (B, upper curve), the ultracentrifugal data show that physical alteration

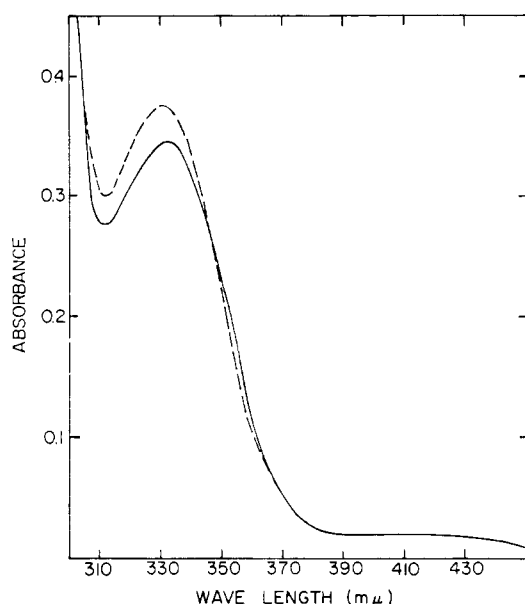


FIGURE 4: Effect of ionic strength and temperature on the spectral properties of phosphorylase *b*. Phosphorylase *b* (6.5 mg/ml) in 0.2 M NaCl containing 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, at 20°, solid line; 22 minutes at 7.5°, broken line.

of enzyme structure by cold essentially may be reversed upon rewarming.

The nature of cold inactivation was further investigated by examining the effect of temperature on the spectral properties of phosphorylase *b*. The data of Figure 4 show that cooling a solution of phosphorylase *b* (6.5 mg/ml) in 0.2 M NaCl to 7.5° causes a significant increase in absorbance and a shift in the absorption maximum of the prosthetic group, pyridoxal phosphate, to shorter wavelengths. Under these conditions (22 minutes at 7.5°), 12% inactivation occurs, whereas with storage at 20° no activity loss or spectral alterations could be demonstrated. NaBH₄-treated phosphorylase *b* showed similar alterations of the absorption spectrum upon cooling. As imine formation (absorption maximum, 415 mμ) is believed to precede the loss of pyridoxal phosphate (Kent *et al.*, 1958), the cold inactivation demonstrated in 0.2 M NaCl, where no 415-mμ component is formed, further supports the view that inactivation of phosphorylase *b* by cold temperature is not necessarily associated with the loss of pyridoxal phosphate.

The influence of various reagents on the cold inactivation of phosphorylase *b* was tested. The data illustrated in Table III show that differential inactivation of enzyme at 0 and 20° is extensive in glycerophosphate-cysteine buffer, pH 6.0, but more pronounced in buffer containing NaCl. The inactivation is not affected by inorganic phosphate or bovine plasma albumin but is effectively blocked in the presence of glycogen, AMP, pyridoxal phosphate, methanol, propylene glycol, dimethyl sulfoxide, or in buffer at pH

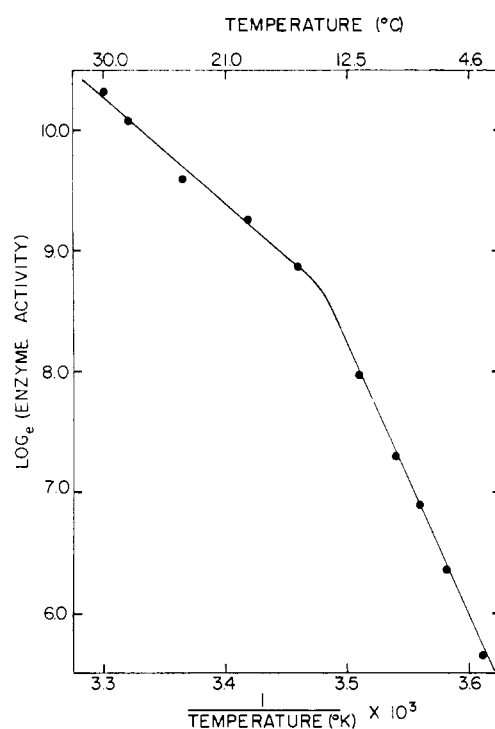


FIGURE 5: Effect of temperature on phosphorylase *b* activity. Enzyme was diluted in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, preincubated for 10 minutes at the temperature used for the assay, and then tested for activity. The assay time was 5 minutes for all temperatures, and the protein concentration varied from 0.02 to 0.2 mg/ml as the temperature was changed from 30 to 2°.

6.8, and is markedly reduced in the presence of ATP or in buffer without cysteine.

The mechanism of action of these reagents is not understood. It does appear, however, that the role of cysteine is not to remove enzyme-bound pyridoxal phosphate as visualized by Appleman (1962) for urea denaturation of phosphorylase *b*, as the cold inactivation of NaBH₄-reduced enzyme is accelerated approximately 3-fold in the presence of cysteine. The action of methanol against cold inactivation may be demonstrated on the physical structure of phosphorylase *b*. Ultracentrifugation at 5° of enzyme (5 mg/ml) stored for 2 hours in glycerophosphate-cysteine, pH 6.0, with 10% methanol showed only a symmetrical component with an $s_{20,w}$ of 8.9 S.

The determination of the activation energy of the enzyme-catalyzed reaction at pH 6.0 further demonstrates the effect of cold temperatures on phosphorylase *b*. The Arrhenius plot illustrated in Figure 5 shows a discontinuity at 13° with activation energies for the upper and lower limbs of 17,000 and 46,000 calories, respectively. It is not clear whether the activation energy for the lower limb is a result of a mixture of native and inactivated enzyme, as suggested by Kistiakowsky and Lumry (1949) for urease, or of enzyme of a dif-

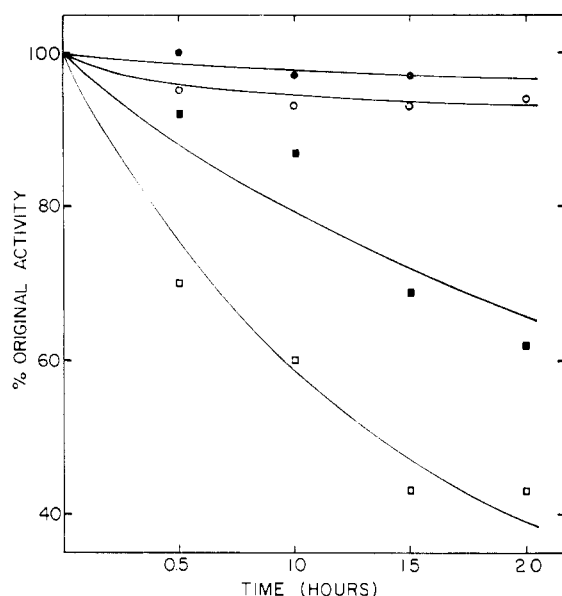


FIGURE 6: Cold inactivation of phosphorylase *a*. Enzyme was diluted to 2 mg/ml in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, at 0°, solid circles; in buffer with 0.2 M NaCl, open circles; with 1.2 M NaCl, open squares; with 1.2 M NaCl at 20°, solid squares. At various intervals aliquots were removed, diluted 60-fold in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, and assayed for enzymatic activity at 30°.

ferent conformation with lower catalytic activity as proposed by Levy *et al.* (1962) for myosin ATPase.

Cold Inactivation of Phosphorylase *a*. The high sensitivity of phosphorylase *b*, a dimer, to cold temperatures prompted a study of the effect of temperature on phosphorylase *a*, a tetramer. Figure 6 shows, in contrast to results obtained with phosphorylase *b*, that little cold inactivation could be demonstrated with phosphorylase *a* in glycerophosphate-cysteine buffer or in buffer with 0.2 M NaCl. In 1.2 M NaCl inactivation was apparent at 20 and 0°, although the data clearly show that phosphorylase *a* is inactivated at 0° to a greater extent.

Ultracentrifugation of phosphorylase *a* was carried out to relate cold inactivation to dissociation of enzyme in NaCl. The results illustrated in Figure 7, upper curve, show that in 0.2 M NaCl one molecular species predominates at 20° with an $s_{20,w}$ of 14.9 S, whereas in 1.2 M NaCl two components are evident with $s_{20,w}$ of 14.9 S and 8.9 S. As cold inactivation occurs in 1.2 M NaCl, the ultracentrifugal data suggest that the component with an $s_{20,w}$ of 8.9 S is cold sensitive. Previous studies with phosphorylase *a* in NaCl suggest that this slow-sedimenting component is a dimeric species (Wang and Graves, 1963).

Discussion

The sensitivity of the secondary and tertiary structure of protein molecules to various agents depends primarily

TABLE III: Effect of Various Reagents on the Cold Inactivation of Phosphorylase *b*.^a

Additions or Deletions	Per Cent of Original Activity	
	20°	0°
None	90	31
— Cysteine	100	85
+ 0.1 M NaCl	100	25
+ 0.2 M NaCl	59	5
+ 0.3 M NaCl	42	5
+ 1% Glycogen	88	88
+ 0.01 M Inorganic phosphate	99	38
+ 10 ⁻³ M AMP	100	93
+ 10 ⁻³ M ATP	92	65
+ 2 × 10 ⁻⁴ M Pyridoxal phosphate	100	90
Buffer at pH 6.8	100	100
+ Plasma albumin (5 mg/ml)	100	26
+ 10% Methanol ^b	100	95
+ 10% Propylene glycol ^b	100	100
+ 10% Dimethyl sulfoxide ^b	100	100

^a Enzyme (1 mg/ml) was incubated for 4 hours at 20° and 0° prior to a 25-fold dilution in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, for activity measurements at 30°. Buffer used for incubation was 0.05 M glycerophosphate-0.038 M cysteine, pH 6.0. ^b For experiments with organic solvents the incubation buffer was 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0.

upon (1) the nature of the agent, (2) the type of forces involved in the stabilization of these structures, and (3) the extent to which these individual forces are involved in maintenance of structure. Since hydrophobic interactions were found in model studies and theoretical treatments by Kauzmann (1959), Nemethy and Scheraga (1962), Schneider *et al.* (1964), and Scheraga *et al.* (1964) to be sensitive to cold temperatures, the inactivation of phosphorylase *b* at pH 6.0 by cold temperatures may perhaps be best explained by disturbance of hydrophobic bonds that are essential for maintenance of the active conformation of the enzyme. The lack of sensitivity of phosphorylase *b* to cold at pH 6.8 suggests that, under these experimental conditions, other forces may be as important or more important for stabilization of the active conformation of phosphorylase *b*.

Organic solvents which might be expected to weaken hydrophobic interactions (Nemethy and Scheraga, 1962) did not inactivate phosphorylase but provided stabilization. The mechanism of this stabilization is uncertain. The protection provided by a substrate, glycogen, an activator, AMP, and an inhibitor, ATP suggests that binding of these molecules induces structural changes which render the enzyme insensitive to cold.

The present kinetic experiments suggest to us the

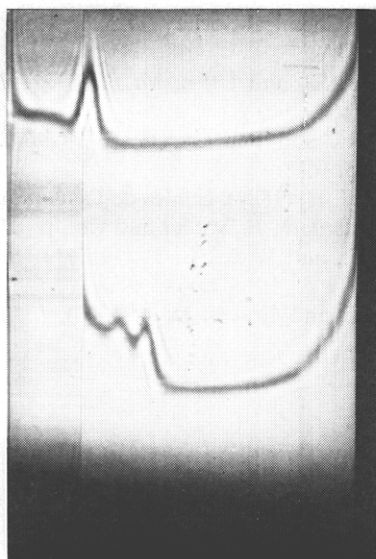
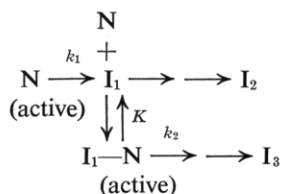


FIGURE 7: Effect of ionic strength on ultracentrifugal properties of phosphorylase *a*. Phosphorylase *a* was diluted to 2 mg/ml in 0.04 M glycerophosphate-0.03 M cysteine, pH 6.0, at 20°. Upper curve, with 0.2 M NaCl; lower curve, with 1.2 M NaCl. Pictures were taken 15 minutes after the speed of the centrifuge reached 59,780 rpm. Sedimentation direction is from left to right.

following reaction scheme for cold inactivation:



where N is native enzyme, I₁, I₂, and I₃ are inactive forms, I₁—N is a complex of unknown description in which the native enzyme is less sensitive to cold, *k*₁ and *k*₂ are rate constants, and *K* is a dissociation constant. The loss of enzymic activity may be described by the differential equation

$$\frac{-d(A)}{dt} = k_1(N) + k_2 \frac{(N)(I_1)}{K}$$

where

$$K = [(N)(I_1)]/(I_1\text{—N}).$$

This mechanism predicts that inactivation, as measured by initial rates, depends only on the first term of the above equation and therefore would be a first-order reaction at all protein concentrations. The amount of the complex, I₁—N, formed during the course of cold inactivation determines the significance of the second

term. The fact that the course of cold inactivation did follow first-order kinetics at low protein concentrations but deviated at high protein concentrations suggests that significant amounts of the complex are formed at high concentrations as inactivation proceeds. As 100% inactivated enzyme did not influence cold inactivation, it has been assumed that only intermediates (inactive forms with incomplete structural alterations) can interact with native enzyme.

Although the exact mechanism of cold inactivation is uncertain, the reversibility of inactivation, the lack of imine formation under conditions where inactivation occurs, and the instability of a pyridoxalimino derivative of phosphorylase to cold suggest that cold inactivation is not caused by the loss of the prosthetic group, pyridoxal phosphate. The effect of pyridoxal phosphate on reactivation and stabilization does suggest, however, that some pyridoxal phosphate may be lost as a result of cold inactivation. The increase in the absorption maximum and blue shift of the pyridoxal phosphate moiety in 0.2 M NaCl at cold temperatures is similar to results obtained by Dempsey and Christensen (1962) with a bovine plasma albumin-pyridoxal phosphate complex in urea and with 3-hydroxypyridines in solutions of increasing dielectric constant (Metzler and Snell, 1955). These data suggest that loss of enzymic activity by cold temperatures is a result of a conformational change that results in the exposure of the prosthetic group to a more polar environment.

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Intracellular Distribution and Characterization of the Lipids of *Streptococcus faecalis* (ATCC 9790)*

Marie L. Vorbeck† and G. V. Marinetti

ABSTRACT: The lipids of exponential phase *Streptococcus faecalis* cells have been studied with respect to their intracellular distribution and nature. Subcellular fractions were prepared by enzymatic disintegration with muramidase followed by differential centrifugation of the released cell constituents. The membrane fraction contained 94% of the total cell lipid with the remainder in the protoplasm fraction. Lipids were not detected in the cell wall fraction. No differences were observed in the chemical and chromatographic properties of lipids extracted from either the membrane or protoplasm.

The major components obtained by silicic acid column chromatography were identified as phosphatidyl glycerol, amino acid esters (lysine, glycine, and alanine) of phosphatidyl glycerol, monoglucosyl diglyceride, and a glycosyl diglyceride containing both glucose and galactose. Phosphatidic acid and diphosphatidyl glycerol (cardiolipin) were minor components. These lipids were identified by determination of chromatographic behavior, staining characteristics with specific reagents, ester/sugar ratios, and by the analysis of the water-soluble products obtained by partial hydrolysis.

Recent studies in this laboratory on the lipids of *Streptococcus faecalis* (ATCC 9790), *Lactobacillus plantarum* (Vorbeck and Marinetti, 1964, 1965), and other lactic acid bacteria¹ revealed considerable amounts of carbohydrate-containing lipids to be present. The chromatographic and staining properties of these lipids were similar to those of the galactolipids (mono-galactosyl and digalactosyl diglycerides) observed in photosynthetic tissues (Benson *et al.*, 1958; Zill and

Harmon, 1962), wheat flour lipids (Carter *et al.*, 1956), leaf lipids (Kates and Eberhardt, 1957; Kates, 1960), and grasses (Weenink, 1961). Kolb *et al.* (1963) found the phosphatides of *S. faecalis* localized in the cell membrane; however, little is known about either the nature of the individual phosphatides or the distribution and nature of glycosyl diglycerides within the bacterial cell.

In the present study with *S. faecalis*, both phosphatides and nonphosphatides have been investigated. Data are presented on the intracellular distribution and characterization of the major lipid components. Fractionation of the cell constituents preceded extraction and characterization of the lipids. Subcellular fractions were prepared by enzymatic disintegration with muramidase (E. C. 3.2.1.17, formerly called lysozyme) and subsequent fractionation of the released cell constituents by differential centrifugation. The lipid components were identified by chromatographic behavior, staining properties with specific reagents, radioisotopic data, and by examination of the water-soluble products formed on hydrolysis.

* From the Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y. Received September 2, 1964. This work was supported by a grant (H-2063) from the National Heart Institute, National Institutes of Health.

† Postdoctoral trainee under a training grant (TI-00137) of the National Institutes of Health. Present address: Temple University Medical School, Department of Microbiology, Philadelphia, Pa.

¹ N. L. Vorbeck and G. V. Marinetti, unpublished observations.