Porter, D. H., & Cardenas, J. M. (1980a) *Arch. Biochem. Biophys. 202*, 54–62.
Porter, D. H., & Cardenas, J. M. (1980b) *Biochemistry 19*, 3447–3452.
Steinmetz, M. A., & Deal, W. C., Jr. (1966) *Biochemistry 5*, 1399–1405.
Strandholm, J. J., Dyson, R. D., & Cardenas, J. M. (1976) *Arch. Biochem. Biophys. 173*, 125–131.

Effects of 1,2-Dimethoxyethane on the Catalytic and Coenzyme Properties of Glycogen Phosphorylase†

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ABSTRACT: Dimethoxyethane, a good activator of phosphorylase *b*, has been used to study mechanisms of phosphorylase activation and the catalytic reaction. Activation can be explained best by an alteration of the allosteric equilibrium in favor of the active R conformation. Lesser effects are seen with phosphorylase *a*, and activation does not alter appreciably the equilibrium between the dimeric and tetrameric forms. With 20% 1,2-dimethoxyethane, the V_m value of phosphorylase *b* is 74% of that obtained in the presence of adenosine monophosphate. In the presence of 10% 1,2-dimethoxyethane, the K_i value for glucose inhibition is increased 3-fold, but inhibition by 1,5-gluconolactone is increased. The allosteric activation of glycogen phosphorylase results in a change in pK_1 for the pH–activity profile. The formation of the dianionic form of the phosphoryl group of the coenzyme, pyridoxal phosphate, may account for this change. By analogy to the effects of anions and a change in dielectric on the acid hydrolysis of glucose 1-phosphate, it is suggested that the dianion of the coenzyme could stabilize the developing positive charge of an oxonium ion intermediate. Dimethoxyethane also affects the interaction of pyridoxal phosphate with phosphorylase. It influences the rates of both resolution and reconstitution. Good preparations of apophosphorylase *a* can be made by using 1,2-dimethoxyethane in the resolution medium.

The activity of glycogen phosphorylase is controlled by an equilibrium between inactive and active conformations. The equilibrium is shifted toward an active form by substrates, phosphorylation, and the allosteric activator AMP (Madsen et al., 1978; Kastenschmidt et al., 1968; Helmreich et al., 1967). Specific conformational changes in the protein result in activation (Madsen et al., 1978; Weber et al., 1978), and changes include the active site, which contains the coenzyme pyridoxal phosphate (Parrish et al., 1977; Sygusch et al., 1977). Although there is considerable information about the phosphorylase molecule and the activation process, there is some uncertainty about how these changes in protein structure cause activation of the catalytic reaction. Kinetic studies of the reaction suggest that α-glucan phosphorylases from skeletal muscle (Engers et al., 1969), liver (Maddaiah & Madsen, 1966), and *E. coli* (Chao et al., 1969) proceed via a rapid equilibrium random Bi–Bi mechanism. 1,5-Gluconolactone is a potent inhibitor of glycogen phosphorylase (Tu et al., 1971; Gold et al., 1971). Because of its inhibitory action and the fact that this compound possesses a structure similar to the half-chair conformation of an oxonium ion (Hackert & Jacobsen, 1969), it has been suggested that the transition state involves formation of an enzyme–glycosyl complex in which the glucosyl residue is in the half-chair conformation (Tu et al., 1971). Similar suggestions about the reaction were made by Gold et al. (1971).

Recently it has been shown that organic solvents cause activation of glycogen phosphorylase (Dreyfus et al., 1978; Uhing et al., 1979). This report characterizes the properties of muscle phosphorylase in the presence of an organic solvent, 1,2-dimethoxyethane, in order to further investigate the process of activation and the nature of the catalytic process. The cofactor of glycogen phosphorylase, pyridoxal phosphate, has been determined to be an indispensable component of the enzyme because its removal results in the loss of catalytic activity (Hedrick et al., 1966). Both structural (Feldmann et al., 1976; Shimomura & Fukui, 1977, 1978) and catalytic (Feldmann & Hull, 1977; Palm et al., 1979) roles for the coenzyme have been suggested. The effects of 1,2-dimethoxyethane on the coenzyme site in phosphorylase and its uses in the resolution of rabbit skeletal muscle phosphorylase *a* are described in this report.

Experimental Procedures

Preparation of Enzymes. Rabbit skeletal muscle glycogen phosphorylase *b* was prepared according to the method of Fischer & Krebs (1962) and recrystallized at least 3 times before use. Residual AMP was removed by treatment with Norit A. Muscle phosphorylase *a* and liver phosphorylase *a* were prepared from the respective phosphorylase *b* forms by phosphorylation with rabbit skeletal muscle phosphorylase kinase (Krebs, 1966). Apophosphorylase *b* was prepared according to Shaltiel et al. (1966) and passed over a Sephadex G-25 column that had been equilibrated with 0.2 M imidazole and 0.05 M L-cysteine at pH 6.0.

Apophosphorylase *a* was prepared from phosphorylase *a* by modifying the procedure of Shaltiel et al. (1966). Phosphorylase *a* (5 mg/mL) is incubated for approximately 12 h at room temperature (22–23 °C) in the presence of 0.4 M imidazolium citrate–0.1 M L-cysteine, pH 6.0, which also contains 10% (v/v) 1,2-dimethoxyethane (the solvent is added to the deforming buffer immediately before use to minimize the precipitation of salts). After the incubation period, an equal

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amount of neutralized saturated ammonium sulfate is added and the suspension centrifuged. The precipitate is dissolved in 0.2 M imidazolium citrate–0.05 M L-cysteine at pH 6 and desalted over Sephadex G-25. The protein-containing fractions are combined, made 50% saturated with respect to ammonium sulfate, and centrifuged. The precipitate is dissolved in 0.04 M β-glycerophosphate–0.03 M 2-mercaptoethanol at pH 6.8 and dialyzed against the same buffer. The protein crystallizes when dialyzed in the cold. The resulting apoenzyme preparation is greater than 99% resolved on the basis of activity and can be reconstituted at 5 mg/mL phosphorylase with a 5-fold molar excess of pyridoxal 5′-phosphate to the same specific activity as the phosphorylase *a* from which it is prepared. The reconstituted enzyme has the same properties as native phosphorylase *a* as determined by sedimentation characteristics, by enzymatic activities, and as a substrate for phosphoprotein phosphatase. The yield of apophosphorylase is routinely greater than 75%. Higher incubation temperatures during resolution result in lower yields even at shorter incubation times. Higher temperatures also result in a lower specific activity for the reconstituted enzyme. Lower incubation temperatures greatly decrease the rate of resolution.

Enzymatic Assays. Phosphorylase activity in the direction of glycogen synthesis was measured either by the liberation of inorganic phosphate from glucose-1-P as described by Illingworth & Cori (1953) or by the incorporation of [^{14}C]-glucose into glycogen from [^{14}C]glucose-1-P by utilizing the filter paper assay of Thomas et al. (1968). When the radioactive assay was used, absolute ethanol was substituted for acetone in the final wash. Unless otherwise specified, the assay system contained 1% glycogen, 16 mM glucose-1-P, 20 mM β-glycerophosphate, and 15 mM 2-mercaptoethanol at pH 6.8 and 30 °C. AMP (1 mM) also was included for the measurement of phosphorylase *b* activity. Activity is expressed as IU/mg where 1 IU is 1 μmol of inorganic phosphate liberated or 1 μmol of [U-^{14}C]glucose incorporated per min.

Phosphorylase concentrations were measured spectrophotometrically by using an extinction coefficient, $\epsilon^{1\%}_{10\,mm}$ at 280 nm, of 13.2 (Kastenschmidt et al., 1968). The monomeric molecular weight of phosphorylase was taken to be 97 420 (Titani et al., 1975). Sedimentation velocity experiments were performed with a Beckman Spinco Model E ultracentrifuge. The movement of protein boundaries was followed by Schlieren optics, and the Schlieren patterns were analyzed with a microcomparator. $s_{20,w}$ values were calculated by a linear regression program of an HP-25 calculator. pK_a values were determined by the protocol of Alberty & Massey (1954).

Materials. AMP, glucose-1-P, L-cysteine, pyridoxal phosphate, Tris, and β-glycerophosphate were obtained from Sigma Chemical Co. and used without further purification. Grade I imidazole from Sigma was used after 3 times recrystallization from reagent grade acetone. Shellfish glycogen from Sigma was further purified according to the method of Anderson & Graves (1973). 2-Mercaptoehtanol was obtained from Aldrich and [U-^{14}C]glucose-1-P from Amersham. 1,2-Dimethoxyethane was obtained from Eastman Kodak.

Results

Normally it is considered that phosphorylase *b* exhibits a requirement for a nucleotide for catalytic activity, but in the presence of organic solvents, phosphorylase *b* is active even in the absence of nucleotide (Dreyfus et al. 1978; Uhing et al. 1979). The extent to which this activation can be achieved by using 1,2-dimethoxyethane is shown in Figure 1. Whereas AMP activation is characterized by a decrease in the K_m for the substrate, glucose-1-P, and an increase in the V_m value

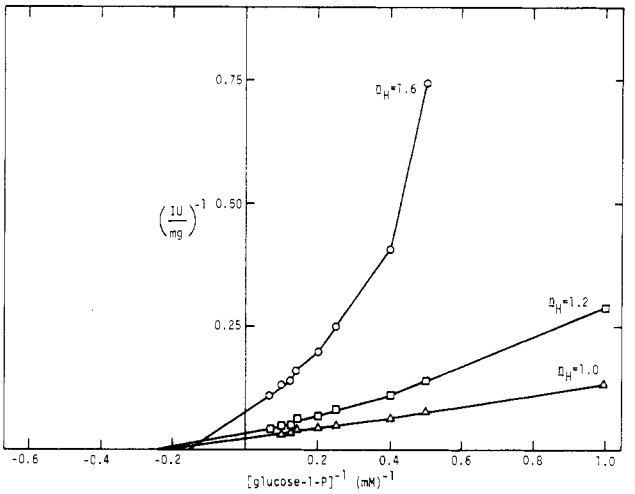

FIGURE 1: 1,2-Dimethoxyethane activation of phosphorylase *b* in the absence of nucleotides. Activity was measured according to Illingworth & Cori (1953) in the presence of 1% glycogen, 20 μg/mL phosphorylase *b*, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 10% (○), 15% (□), or 20% (△) (v/v) 1,2-dimethoxyethane, and the indicated amounts of glucose-1-P at pH 6.8 and 30 °C.

Table I: Effect of 1,2-Dimethoxyethane on Kinetic Constants for the Different Forms of Rabbit Muscle Glycogen Phosphorylase

1,2-dimethoxyethane (%, v/v)	K_m(glucose-1-P[a]) (mM)		V_{max}[b] (IU/mg)	
	0	10	0	10
phosphorylase *b* (−AMP)	~50	6.4	2	14
phosphorylase *b* (+AMP)	5.4	3.2	66	75
phosphorylase *a* (−AMP)	3.8	3.2	53	60
phosphorylase *a* (+AMP)	2.7	2.7	73	68

[a] Measured in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 4 μg/mL enzyme, and glucose-1-P concentrations between 2 and 10 mM at pH 6.8 and 30 °C whether in the presence or absence of 1 mM AMP as indicated for phosphorylase *b* (+AMP), phosphorylase *a* (−AMP), and phosphorylase *a* (+AMP). For phosphorylase *b* (−AMP), the K_m was measured in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 67 μg/mL enzyme, and glucose-1-P concentrations between 10 and 160 mM at pH 6.8 and 30 °C in the absence of 1,2-dimethoxyethane. In the presence of 1,2-dimethoxyethane, the same enzyme form was assayed in the presence of 1% glycogen, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 20 μg/mL enzyme, and glucose-1-P concentrations between 1 and 15 mM at pH 6.8 and 30 °C. [b] Extrapolated from plots of (velocity)$^{-1}$ vs. (glucose-1-P)$^{-1}$ from *a*.

(Madsen, 1964), activation by 1,2-dimethoxyethane in the absence of nucleotide, throughout the solvent concentration range utilized here, seems to be due to an increase in the maximal velocity with only a slight change in the affinity for the substrate. Dreyfus et al. (1978) also have recently investigated the action of organic solvents on phosphorylase *b*. These authors reported that at lower concentrations of organic solvent there was an increase in substrate binding, the K_m being lowered from 11 to 2.3 mM as the *tert*-butyl alcohol concentration was raised from 5 to 10%. Some change in the K_m for glucose-1-P would be expected at lower concentrations of 1,2-dimethoxyethane also because the substrate affinity of phosphorylase *b* in the absence of AMP is much higher than that seen in the presence of 10% (v/v) 1,2-dimethoxyethane (Table I). The maximal velocity obtained at the highest solvent concentration used (20%) is 46 IU/mg or 75% of that obtained in the presence of saturating AMP. No activity measurements were performed at concentrations of 1,2-dimethoxyethane higher than 25% because at these concentrations glycogen was precipitated. Another property of nu-

cleotide activators that also was found to be characteristic of organic solvent activation was the reduction of homotropic cooperativity for the substrate. The Hill coefficient decreases from 1.6 to 1.0 as the concentration of 1,2-dimethoxyethane increases from 10 to 20%. A plot of the reciprocal of the maximal velocity vs. the reciprocal of the 1,2-dimethoxyethane concentration exhibits significant curvature similar to the cooperativity seen for AMP activation. Inasmuch as higher concentrations of 1,2-dimethoxyethane could not be used, neither the K_a for solvent activation nor the maximal velocity obtainable for saturating solvent effect could be determined.

Glucose, caffeine, and AMP alter the properties of phosphorylase by binding, respectively, to the active site, nucleoside inhibitor site, and nucleotide activator site. It is known that glucose and caffeine stabilize the inactive T state of phosphorylase *b* whereas AMP promotes formation of the active R state (Fletterick & Madsen, 1980). Earlier, Uhing et al. (1979) showed that dimethoxyethane decreases the K_a value for AMP but increases the K_i value for caffeine. The presence of 1,2-dimethoxyethane lowers inhibition by glucose by elevating the K_i value from 6 to 21 mM. The Hill coefficient for glucose-1-P at the K_i value in the absence of solvent is 1.4, but it is lowered to 1.2 in its presence. These effects and the change in K_a and the Hill coefficient for AMP suggest that dimethoxyethane activates phosphorylase *b* by altering the allosteric equilibrium in the direction of the active R conformational state.

For further investigation of the effect of 1,2-dimethoxyethane on the allosteric equilibrium, its effects on the different forms of phosphorylase were investigated because phosphorylase *a* has a lower allosteric constant, i.e., a larger relative amount of the active R conformer (Kastenschmidt et al. 1968; Helmreich et al. 1967). The influence of 10% solvent on the K_m and V_m values for different forms of glycogen phosphorylase is presented in Table I. The largest effect is on phosphorylase *b*. Without AMP or solvent, phosphorylase *b* has a low V_m value and a high K_m value for glucose-1-P. 1,2-Dimethoxyethane increases the V_m value from 2 to 14 IU/mg and lowers the K_m to 6.4 mM. In the presence of AMP, this solvent increases the V_m for phosphorylase *b* about 13% and causes a reduction in the K_m value. Phosphorylase *a* in the presence of AMP shows a decrease in the maximal velocity in the presence of 10% (v/v) 1,2-dimethoxyethane. This enzyme form also shows no effect of the solvent on glucose-1-P binding. Some stimulation of phosphorylase *a* activity is seen when AMP is omitted from the reaction mixture.

The effect of organic solvents on the dissociation–association properties of phosphorylase has been further examined by sedimentation velocity experimentation to investigate whether the effects of these solvents could be explained by an alteration of this equilibrium. Phosphorylase exists as either a dimer or a tetramer, depending on the state of phosphorylase and the presence of various effectors of the enzyme. Under the buffer conditions utilized in these experiments (36 mM β-glycerophosphate, 27 mM 2-mercaptoethanol, pH 6.8), phosphorylase *b* sediments as a dimer ($s_{20,w}$ = 8.4 S), and phosphorylase *a* as a tetramer, in the presence or absence of 1,2-dimethoxyethane. In the presence of AMP, phosphorylase *b* sediments primarily as a tetramer at 10 °C, although a significant amount of dimer is still present. With the inclusion of solvent, this profile is shifted totally toward the tetramer, in agreement with the results of Dreyfus et al. (1978).

When phosphorylase *a* is used, the effect of 1,2-dimethoxyethane on the specific activities of both the dimeric and tetrameric forms of this enzyme was examined, as well as the

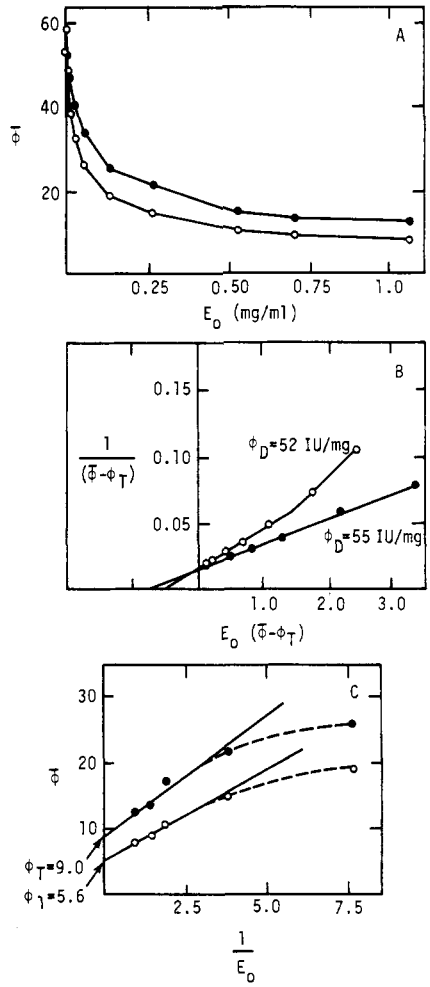

FIGURE 2: Effect of 1,2-dimethoxyethane on the specific activity of phosphorylase. (A) Activity was assayed as described uncer Experimental Procedures in the presence of 1% glycogen, 18 mM β-glycerophosphate, 0.9 mM EDTA, 0.5% KCl, and the indicated amounts of phosphorylase *a* at pH 6.8 and 24 °C in the absence (○) or presence (●) of 10% (v/v) 1,2-dimethoxyethane. For enzyme concentrations below 0.5 mg/mL, 0.5 mg/mL bovine serum albumin was also included in the assay. (B) Data from (A) are plotted as $\bar{\phi} - \phi_T)^{-1}$ vs. $[E_0(\bar{\phi} - \phi_T)]$ in order to obtain ϕ_D and K_d. (C) Data from (A) are plotted as $\bar{\phi}$ vs. $(E_0)^{-1}$ in order to obtain ϕ_T.

effect of the solvent on the dimer–tetramer equilibrium under the normal assay conditions. Huang & Graves (1970) have shown that both the dissociation constant for this equilibrium and the specific activities of the two forms can be determined from the activity of phosphorylase *a* as a function of protein concentration. As can be seen, the curves differ slightly due to the presence of 1,2-dimethoxyethane (Figure 2a). Figures 2B and 2C show extrapolations to obtain the specific activities of the dimer and tetramer, respectively. The plot for Figure 2B is from

$$\frac{1}{\phi - \phi_T} = \frac{1}{K_d(\phi - \phi_T)^2}[E_0(\phi - \phi_T)] + \frac{1}{\phi_D - \phi_T}$$

where ϕ = measure specific activity, $\phi_T = [(1/E_0) \rightarrow 0]\bar{0}$, E_0 = total enzyme concentration, and ϕ_D = specific activity of the dimer. In Figure 2C, by the law of mass action, the specific activity of the tetramer is that at infinite protein concentration. As in seen at lower protein concentrations, there is significant curvature due to the presence of dimeric phosphorylase *a*. Figure 2B shows that there is a slight difference for the specific activity of the dimer due to the presence of the solvent. The solvent increases the specific activity of the phosphorylase *a* dimer about 6% under these conditions. There is little change

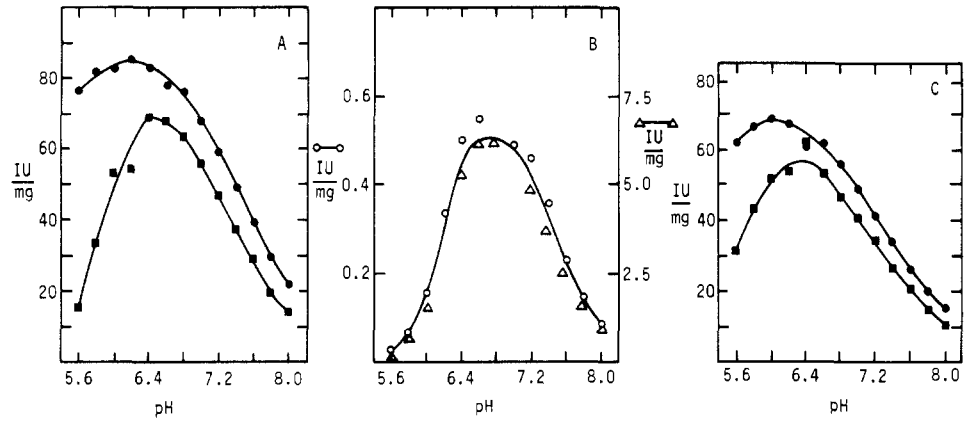

FIGURE 3: Effect of pH on the stimulation of phosphorylase *b* activity by 1,2-dimethoxyethane. Enzymatic activity was measured according to Illingworth & Cori (1953) in the presence of 1% glycogen and 50 mM glucose-1-P at the indicated pH values and 30 °C. The presence of other substances was as indicated. Both substrates and buffers were adjusted to the indicated pH values. The addition of 10% solvent did not alter the pH value. (A) Phosphorylase *b* (16 μg/mL) was assayed as described above in the presence of 50 mM Tris–maleate: 10 mM AMP for the pH values 5.6–6.2 and 3 mM AMP for the pH values 6.4–8.0, in the absence (■) or presence (●) of 10% (v/v) 1,2-dimethoxyethane. (B) Phosphorylase *b* (49 μg/mL) in the presence of 10% (v/v) 1,2-dimethoxyethane (△) or 3 mg/mL phosphorylase *b* in the absence of 1,2-dimethoxyethane (○) was assayed as described above in the presence of 50 mM Tris–maleate. (C) Assay conditions were as described in (A) with the substitution of 50 mM Tris–50 mM imidazole for the Tris–maleate buffer.

in the dissociation constant for the dimer–tetramer equilibrium, the K_d being increased from 7.8×10^{-3} g/L to 9.1×10^{-3} g/L due to the inclusion of 10% (v/v) 1,2-dimethoxyethane. The tetramer, which under the conditions used here has about 10% of the activity of the dimer in the absence of solvent, shows a much larger stimulation due to the solvent. As can be determined from Figure 2C, this stimulation is on the order of 60%. Because 1,2-dimethoxyethane affects the specific activities of both the dimeric and tetrameric forms of phosphorylase *a* while doing little to alter the dissociation constant, it seems unlikely that the changes seen in the activity properties of the enzyme could be explained by large changes in the enzyme's aggregation state.

The effect of 1,2-dimethoxyethane on the pH profile of glycogen phosphorylase *b* was undertaken to further investigate the mechanism by which the enzyme is activated by the solvent. As can be determined from Figure 3a, there is little effect of the solvent on the alkaline limb of the pH profile. Kasvinsky & Meyer (1977) have suggested that this ionization is that of a histidyl group on the basis of observed p*Ka* and the heat of ionization. This assumption also is consistent with the fact that the pK_a is not perturbed by an environmental change induced by the presence of the solvent. The acidic limb of the pH profile exhibits a pronounced dependency on the environment. The pK_1 is shifted due either to the presence of the solvent itself or to a conformational change induced by the solvent. The change in the pH profile for the AMP-activated phosphorylase *b* in the presence of 1,2-dimethoxyethane cannot be explained by a change in the pK_a of the substrate because, in the absence of AMP, the 1,2-dimethoxyethane-induced profile appears to have a pK_1 similar to nonactivated phosphorylase *b* (Figure 3B). The differences in the profiles also are not due to a perturbation of the buffer pH by the solvent because, as is seen in Figure 3C, the same shift in the acidic limb is seen in Tris–imidazole buffer. Clearly, the changes seen here are due to a perturbation of some group on the enzyme that is important for the catalytic reaction rather than the binding of the substrates or activators because all effectors used here should be saturating according to the results reported by Kasvinsky & Meyer (1977) and Hollo et al. (1966).

Figure 4A shows the effect of 1,2-dimethoxyethane on the inhibition by 1,5-gluconolactone. This compound has been suggested to be a transition-state analogue for the phosphorylase reaction (Tu et al., 1971; Gold et al., 1971). Interestingly, the solvent increased the inhibitory action of this compound, but as mentioned earlier, caused less inhibition by glucose. The increased inhibition by 1,5-gluconolactone in the presence of the solvent cannot be explained by a change in the rate of hydrolysis of the compound because, as is seen in Figure 4B, the inclusion of 1,2-dimethoxyethane actually accelerates this process. The increased inhibition by this compound is suggestive of a more stabilized transition state under the conditions in which the maximal velocity is increased. Bunton et al. (1958) and Bunton & Humeres (1968) have provided evidence that the acid hydrolysis of glucose-1-P proceeds through an S_N1 mechanism via an oxonium intermediate through the pH range 2–4, thus providing an analogy with the phosphorylase reaction. Below this range, there is an increase in rate, which is not correlatable with an increase in hydrogen ion concentration (Bunton & Humeres, 1968). Tu et al. (1971) have shown an isotope effect, k_H/k_D, of 1.13 for the hydrolysis of glucose-1-P in 1.1 N HCl. This also is suggestive of an S_N1 mechanism. Figure 5 shows the rate of hydrolysis of glucose-1-P in 1.2 N HCl as a function of chloride concentration. The rate shows a linear dependency on the salt concentration as well as a further stimulation by the organic

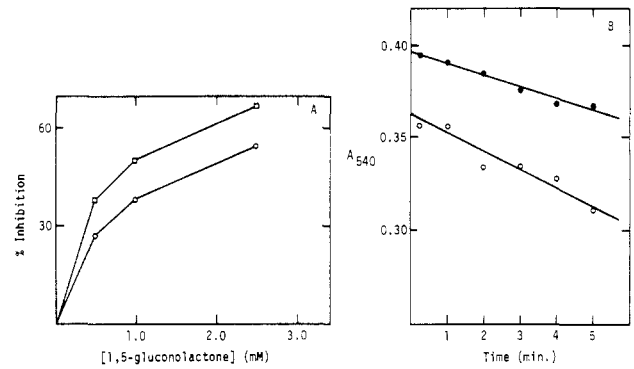

FIGURE 4: Effect of 1,2-dimethoxyethane on the inhibition of phosphorylase *b* activity by 1,5-gluconolactone. (A) Assay conditions were as described under Experimental Procedures in the presence of 13 μg/mL phosphorylase *b*, no (○) or 10% (□) (v/v) 1,2-dimethoxyethane, and the indicated amounts of 1,5-gluconolactone. (B) Effect of 1,2-dimethoxyethane on the hydrolysis of 1,5-gluconolactone. Gluconolactone (3 mM) was incubated in the presence of 40 mM β-glycerophosphate and no (●) or 10% (○) (v/v) 1,2-dimethoxyethane at pH 6.8. At the indicated times, aliquots were removed and hydrolysis was determined as in Tu et al. (1971).

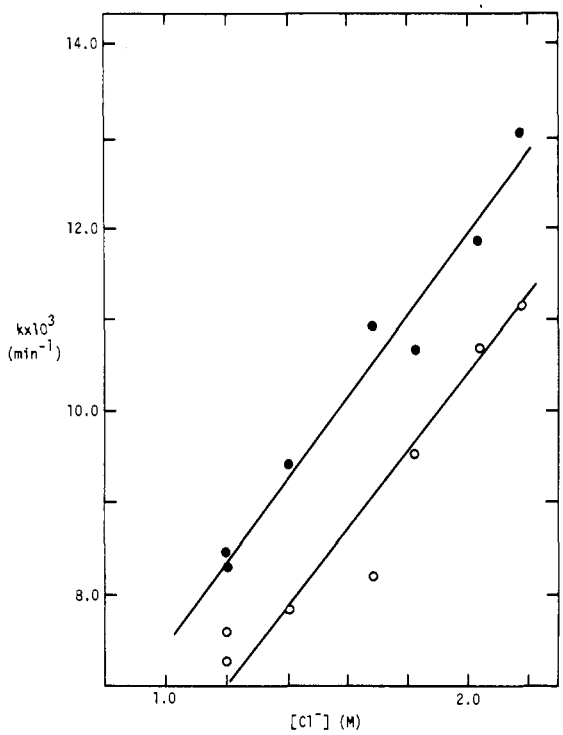

FIGURE 5: Effect of ionic strength and dielectric constant on the acid hydrolysis of glucose-1-P. Glucose-1-P (200 mM) was incubated in 1.2 N HCl with the concentration of chloride anion adjusted to the indicated levels by addition of KCl. The reaction was carried out in the absence (○) or presence (●) of 10% (v/v) 1,2-dimethoxyethane. The dielectric constant of pure 1,2-dimethoxyethane is 6.7 (Vincenza & Zampetti, 1973) at 30 °C. At intervals between 5 and 45 min, aliquots were removed and phosphate was determined in a manner analogous to that utilized for the phosphorylase reaction (Illingworth & Cori, 1953).

solvent, 1,2-dimethoxyethane. Bunton & Humeres (1968) and co-workers previously have reported a salt effect on the hydrolysis of glucose-1-P and from their work on electrolyte effects on acid-catalyzed ester hydrolysis (Bunton et al., 1968) have suggested that the carbonium intermediate is stabilized by an increase in water activity as well as by a specific salt interaction. Although the effects of salts have been shown previously, the data in Figure 5 are presented because the conditions used are similar to those showing the kinetic isotope effect and also to present the effect of a lower dielectric on the process.

Shaltiel et al. (1966) found that pyridoxal phosphate could be resolved from phosphorylase *b* under mild conditions by using a deforming buffer, imidazolium citrate, and a stereospecific aldehyde trapping agent, L-cysteine. These authors further reported that phosphorylase *a* could not be resolved under these conditions. Other, more drastic, conditions have been utilized in the resolution of the cofactor from the *a* form of the enzyme (Shaltiel et al., 1969). However, these conditions result in an apophosphorylase *a* that can be reconstituted only to about half of the original activity.

It was found that inclusion of 10% (v/v) 1,2-dimethoxyethane in the resolution medium accelerates the rate of resolution of phosphorylase *b* and *a*. The faster rate can be attributed to the increased accessibility of the pyridoxal phosphate (Figure 6A,B). In the presence of 0.4 M imidazolium citrate, but in the absence of L-cysteine, there is a gradual appearance of the 415-nm band of the bound pyridoxal phosphate. This band has been attributed to the exposed protonated imine (Shaltiel & Cortijo, 1970). The presence of 10% (v/v) 1,2-dimethoxyethane results in a large acceleration in the rate of appearance of this band. The process

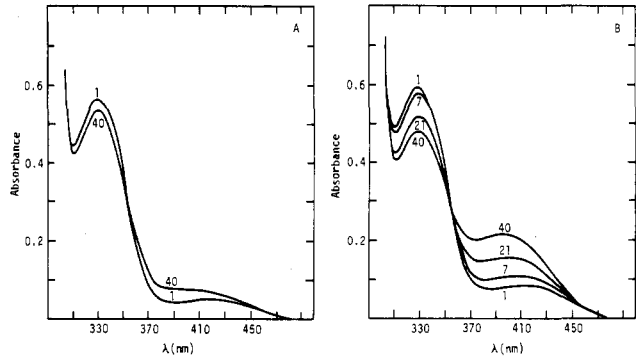

FIGURE 6: Effect of the resolution conditions on the spectral properties of pyridoxal phosphate bound to phosphorylase *b*. Phosphorylase *b* (9 mg/mL) was incubated in the presence of 0.4 M imidazolium citrate, 42 mM β-glycerophosphate, and 9 mM 2-mercaptoethanol at pH 6.0 in the absence (A) or presence (B) of 10% (v/v) 1,2-dimethoxyethane. At the times indicated by the numbers on the curves, spectra were recorded on a Cary 15 recording spectrophotometer.

observed is not due to resolution of the cofactor since it was found that no loss of enzymatic activity occurs under these conditions. Shaltiel et al. (1966) suggested, on the basis of exchange experiments, that the effect of the deforming buffer was to expose the coenzyme, but no data have been presented previously that correlate this hypothesis with spectral evidence.

Because inclusion of the solvent resulted in an increased rate of resolution of the *b* form of phosphorylase, the possibility of its use in the resolution of phosphorylase *a* was investigated. From Figure 7, it can be seen that no resolution of phosphorylase *a* is seen at 30 °C under the resolution conditions described by Shaltiel et al. (1966). Inclusion of 10% (v/v) 1,2-dimethoxyethane results in a rapid loss of activity which has been found to be due to resolution of the coenzyme because incubation of pyridoxal phosphate, upon removal of the resolution buffer, results in a return of the enzymatic activity. Shaltiel et al. (1966) have suggested that formation of monomers may be a prerequisite for resolution of the coenzyme on the basis of the finding that phosphorylase *b* formed monomers in the presence of the deforming buffer. As is seen in Figure 7 (inset), the results obtained with phosphorylase *a* also are in agreement with this suggestion. In the presence of the deforming buffer alone, phosphorylase *a* sediments predominantly as the dimer and is not capable of being resolved. When the deforming buffer contains 10% (v/v) 1,2-dimethoxyethane, conditions under which the enzyme would be resolved if L-cysteine were included, the enzyme sediments as a monomer. Good preparations of apophosphorylase are obtainable in the presence of 1,2-dimethoxyethane with the procedure outlined under Experimental Procedures.

Figure 8 demonstrates the effect of 1,2-dimethoxyethane on the reconstitution of apophosphorylase *b*. Again, in the presence of the solvent acceleration occurs. The two effects of 1,2-dimethoxyethane on resolution and reconstitution are not contradictory and are due to different experimental conditions that influence the aggregation state of the enzyme. The inset of Figure 8 shows that apophosphorylase *b* exhibits an increased predominance of a species with a lower *s* value in the presence of solvent. The $s_{20,w}$ of the major form is 8.5 S, characteristic of the dimeric form of the enzyme.

Discussion

Rabbit skeletal muscle glycogen phosphorylase *b*, as initially purified, was found to require AMP for activity, yet this nucleotide was not utilized during the catalytic process (Cori et al., 1938), thus presenting an early recognition of the allosteric

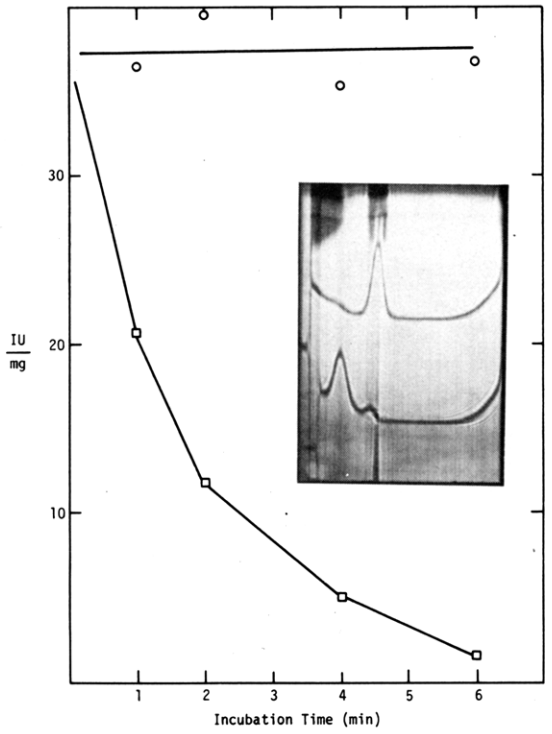

FIGURE 7: Effect of 1,2-dimethoxyethane on the resolution of phosphorylase *a*. Phosphorylase *a* (1.3 mg/mL) was incubated in the presence of 0.4 M imidazolium–citrate and 0.1 M L-cysteine at pH 6.0 and 30 °C in the absence (○) or presence (□) of 10% (v/v) 1,2-dimethoxyethane. At the indicated times aliquots were removed and assayed as described under Experimental Procedures. (Inset) Effect of the resolution buffer on the aggregation state of phosphorylase *a*. Sedimentation velocity experimentation was performed as described under Experimental Procedures at 52000 rpm. The sample contained 3.5 mg/mL phosphorylase *a* and 0.4 M imidazolium citrate at pH 6.0 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 23 °C. $s_{20,w}$ values are 9.5 (top) and 4.2 S (bottom).

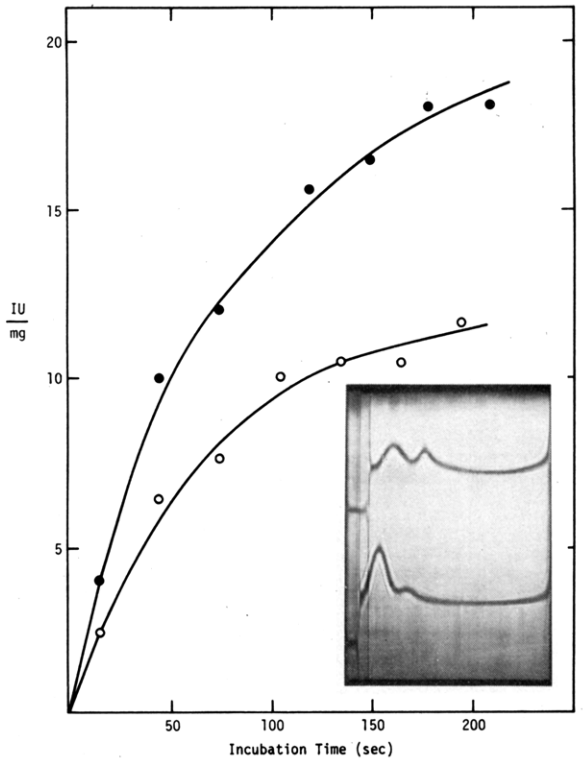

FIGURE 8: Effect of 1,2-dimethoxyethane on the rate of reconstitution of phosphorylase *b* with pyridoxal phosphate. Apophosphorylase *b* (5 mg/mL) was incubated in the presence of 50 μM pyridoxal phosphate, 30 mM β-glycerophosphate, and 23 mM 2-mercaptoethanol, at pH 6.8 and 19 °C in the absence (○) or presence (●) of 10% (v/v) 1,2-dimethoxyethane. At the indicated times aliquots were removed and assayed as described under Experimental Procedures. (Inset) Effect of 1,2-dimethoxyethane on the aggregation state of apophosphorylase *b*. Sedimentation velocity experiments were performed as described under Experimental Procedures at 52000 rpm. The sample contained 2.5 mg/mL apophosphorylase *b* in the 36 mM β-glycerophosphate and 27 mM 2-mercaptoethanol at pH 6.8 in the absence (top) or presence (bottom) of 10% (v/v) 1,2-dimethoxyethane. The run was performed at 20 °C. $s_{20,w}$ values are 8.2 and 18.0 S (top) and 8.5 S (bottom).

control of this enzyme. Further allosteric controls on phosphorylase activity subsequently have been recognized. These include the phosphorylation of the enzyme to the *a* form and the presence of a second nucleotide site where binding is inhibitory. In 1965, Monod et al. provided a model for enzymatic allosteric transitions assuming an inactive T state and an active R state. The existence of these two conformations has been confirmed for the phosphorylase molecule by various methods, most notably through recent X-ray crystallographic studies, which have delineated the sections of the molecule that undergo major conformational changes during the T → R transition (Madsen et al., 1978). The effect of 1,2-dimethoxyethane on the different forms of phosphorylase found here presents evidence that this solvent activates by shifting the allosteric equilibrium.

The data presented here suggest that the activation properties of the solvent cannot be explained by a difference in the aggregation state of the enzyme. Inclusion of the solvent facilitates the AMP-induced tetramerization of phosphorylase *b*. This again is probably due to the ability of the solvent to stabilize the R form since glucose-1-P, which facilitates this transition in the presence of AMP (Kastenschmidt et al., 1968), also increases the amount of tetramer in the presence of the nucleotide (Black & Wang, 1968). Helmreich and co-workers have invoked an additional conformational state for the tetramer to explain its allosteric properties (Kastenschmidt et al., 1968). The lower activity of the tetramer and the difference in the solvent stimulation for this form also are consistent with this suggestion.

The results obtained with 1,2-dimethoxyethane in the absence of nucleotides show that, in the presence of substrates, the allosteric equilibrium can be shifted to a large extent to the active form by this solvent alone. The activity obtained cannot be explained by contaminating AMP in the enzyme preparation or substrates because further increases in the solvent concentration result in an increased maximal velocity with little change in the K_m for glucose-1-P. AMP activation is characterized by a change in the K_m for substrates at limiting concentrations of this nucleotide (Helmreich & Cori, 1964; Madsen, 1964). Further evidence that organic solvent activation is not attributable to endogenous nucleotides has been presented by Dreyfus et al. (1980). These authors reported that butanedione modification of an essential arginine residue eliminated the ability of phosphorylase *b* to be activated by AMP while there was no effect on *tert*-butyl alcohol activation. Still further evidence for differences in solvent activation, and that of AMP, is provided by the differences in the acidic limb of the pH profiles.

The effects of pH on glycogen phosphorylase activity obtained here suggest that the pK exhibits a wide variance dependent upon the activity state of the enzyme. Kasvinsky & Meyer (1977) have reported that phosphorylase *a* exhibits a pK_1 at least one-half pH lower than that of phosphorylase *b* in the presence of AMP. The results obtained here show that 1,2-dimethoxyethane activation of phosphorylase *b* in the

presence of AMP correlates with a substantial lowering of the pK_1, again suggesting the presence of a phosphorylase *a* type molecule under these condtions. This change in the pH profile can be attributed to the catalytic reaction because the concentrations of effectors used here should be saturating according to the results of Kasvinsky & Meyer (1977) utilizing P_i and the consideration of the lower pK_2 for glucose-1-P which was utilized in these experiments. Phosphorylase *b* in the absence of activators and in the presence of 10% (v/v) 1,2-dimethoxyethane as the activator exhibits a pK_1 approximately 0.2 unit higher than that of AMP-activated phosphorylase *b*.

Various authors have invoked an ionization of the phosphoryl portion of pyridoxal phosphate to explain the acidic limb of the pH profile (Kasvinsky & Meyer, 1977; Chao & Graves, 1970). Although deprotonation of a carboxyl group could also be suggested to account for this ionization, Feldmann & Hull (1977), using ^{31}P NMR and phosphorylase *b*, found that the presence of AMP and a substrate analogue resulted in a deprotonation of the phosphoryl portion of pyridoxal phosphate. These authors further presented evidence that the resonance for phosphorylase *b* in the absence of effectors was insensitive to pH between 5.8 and 8.5. Addition of the activator AMPS and the substrate analogue arsenate together, or arsenate alone, resulted in a titration of the resonance below pH 6.4. These results, together with the X-ray crystallographic results, which locate the coenzyme at the active site (Weber et al., 1978; Sygusch et al., 1977), make it attractive to suggest that the acidic ionization of the pH–activity curve is due to the deprotonation of the phosphoryl portion of the coenzyme.

The results obtained on ionic strength activation of the acid hydrolysis of glucose-1-P suggest the importance of ions on this process while evidence supports an S_N1 mechanism under these conditions (Tu et al., 1971; Bunton et al., 1958). From their work on ester hydrolysis, Bunton et al. (1968) have presented evidence that this stimulation is attributable to the anion and that the rate enhancement is due to stabilization of the developing carbonium, both via an increase in the activity coefficient of the medium and through specific interactions. Further, these authors found that the catalytic order of strong acids and salts suggested that the lower the charge density of the anion the more it stabilizes transition states with carbonium ion character (Bunton et al., 1968).

The effect of low charge density reagents is clearly seen in micellar catalysis (Bunton, 1977). The ability of the cationic surfactant, hexadecyltrimethylammonium bromide, to catalyze unimolecular decarboxylations and dephosphorylations is greatest when a high charge density anion in the initial state is converted with a low charge density transition state. The anion in the transition state is stabilized by the low charge density cationic, trimethylammonium, head of the surfactant much more readily than the initial high charge density anion.

The anion stimulation and the further stimulation by a lower dielectric suggest that a similar situation may be important in the action of glycogen phosphorylase. The most likely candidates for such a function in phosphorylase would be the dianionic phosphoryl group of pyridoxal or a carboxylate anion. The increased inhibition by the transition-state analogue 1,5-gluconolactone in the presence of 1,2-dimethoxyethane suggests that the transition state is stabilized under conditions in which the maximal velocity also is increased. This oxonium intermediate can be considered to be a low charge density cation because of the delocalization of charge between C-1 and the ring oxygen. Feldmann & Hull (1977) have shown that activation results in a deprotonation of the phosphoryl group of pyridoxal phosphate. The results presented here also suggest the importance of the activity state of the enzyme on the pK_1 of the pH profile. The lesser effects of 1,2-dimethoxyethane on phosphorylase *a* in comparison with phosphorylase *b* is consistent with a more highly formed active site in phosphorylase *a* in which the coenzyme is in a dianionic form (Feldmann & Hull, 1977; Helmreich & Klein, 1980). By analogy with the results on the acid hydrolysis of glucose-1-P, the activation process may be such as to allow the dianion of pyridoxal to stabilize the developing oxonium transition state. X-ray crystallographic studies on the T form of the enzyme, however, also show the presence of two carboxyl groups near the catalytic site (Fletterick & Madsen, 1980). A role for a carboxylate group was suggested by the experiments of Ariki & Fukui (1978) utilizing water-soluble carbodiimide modification. The theoretical studies of Warshel and Levitt on lysozyme (1976) suggest that electrostatic stabilization of the carbonium may be considerable. These authors have suggested that the presence of Asp-52 lowers the energy of the carbonium ion relative to the ground state by 9 kcal mol^{-1}.

An involvement of the phosphoryl dianion, as suggested here, would allow for nucleophilic assistance by this group as well as electrostatic stabilization of the developing transition state. Such a mechanism would be considered to have both S_N1 and S_N2 characteristics, thus offering a possible explanation for the absence of a secondary isotope effect reported by Firsov et al. (1974) under conditions where no covalent intermediate is found. Recently, Johnson et al. (1980) have suggested, on the basis of crystallographic studies of the binding of glucose-1-P to phosphorylase *b*, a role for the dianionic form of the coenzyme as a nucleophile in the stabilization of an oxonium ion intermediate.

The results reported here show that good preparations of apophosphorylase *a* can be made by using 1,2-dimethoxyethane in the resolution medium. The effect of this solvent on the resolution does not appear to be related to its effect on the allosteric equilibrium since both AMP and caffeine have been reported to inhibit the resolution of phosphorylase *b* (Hedrick et al. 1969). Good preparations of apophosphorylase *a* allow the effects of different pyridoxal phosphate analogues on the properties of phosphorylase *a* to be determined. Yan et al. (1979) have recently reported that the structure of the coenzyme is important for the regulation by glucose and caffeine of the dephosphorylation by phosphatase and their regulations of the dimer–tetramer equilibrium. These authors have presented evidence for the importance of the dianionic form of the phosphoryl portion of the coenzyme in this regulation. The importance of the dianionic form in the expression of activity of the *b* form of the enzyme has been previously suggested by Parrish et al. (1977).

References

Alberty, R. A., & Massey, V. (1954) *Biochim. Biophys. Acta 13*, 347.

Anderson, R. A., & Graves, D. J. (1973) *Biochemistry 12*, 1895.

Ariki, M., & Fukui, T. (1978) *J. Biochem. (Tokyo) 83*, 183.

Black, W. J., & Wang, J. H. (1968) *J. Biol. Chem. 243*, 5892.

Bunton, C. A. (1977) *Pure Appl. Chem. 49*, 969.

Bunton, C. A., & Humeres, E. (1968) *J. Org. Chem. 34*, 572.

Bunton, C. A., Llewellyn, D. R., Oldham, K. G., & Vernon, C. A. (1958) *J. Chem. Soc.*, 3588.

Bunton, C. A., Crabtree, J. H., & Robinson, L. (1968) *J. Am. Chem. Soc. 90*, 1258.

Chao, J., & Graves, D. J. (1970) *Biochem. Biophys. Res. Commun. 40*, 1398.

Chao, J., Johnson, G. F., & Graves, D. J. (1969) *Biochemistry 8*, 1459.
Cori, G. T., Colowick, S. P., & Cori, C. F. (1938) *J. Biol. Chem. 123*, 381.
Dreyfus, M., Vanderbunder, B., & Buc, H. (1978) *FEBS Lett. 95*, 185.
Dreyfus, M., Vanderbunder, B., & Buc, H. (1980) *Biochemistry 19*, 3634.
Engers, H. D., Bridger, W. A., & Madsen, N. B. (1969) *J. Biol. Chem. 244*, 5936.
Feldmann, K., & Hull, W. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 856.
Feldman, K., Zeisel, H., & Helmreich, E. J. M. (1976) *Eur. J. Biochem. 65*, 285.
Firsov, L. M., Bogacheva, T. I., & Bresler, S. E. (1974) *Eur. J. Biochem. 42*, 285.
Fischer, E. H., & Krebs, E. G. (1962) *Methods Enzymol. 5*, 369.
Fletterick, R. J., & Madsen, N. B. (1980) *Annu. Rev. Biochem. 49*, 31.
Gold, A. M., Legrand, E., & Sanchez, G. (1971) *J. Biol. Chem. 246*, 5700.
Hackert, M. L., & Jacobsen, R. A. (1969) *Chem. Commun.*, 1179.
Hedrick, J. L., Shaltiel, S., & Fischer, E. H. (1966) *Biochemistry 5*, 2117.
Hedrick, J. L., Shaltiel, S., & Fischer, E. H. (1969) *Biochemistry 8*, 2422.
Helmreich, E., & Cori, C. F. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 131.
Helmreich, E., & Klein, H. W. (1980) *Angew. Chem. 19*, 441.
Helmreich, E., Michaelides, M. C., & Cori, C. F. (1967) *Biochemistry 6*, 3695.
Hollo, J., Laszlo, E., & Hoschke, A. (1966) *Staerke 18*, 337.
Huang, C. Y., & Graves, D. J. (1970) *Biochemistry 9*, 660.
Illingworth, B., & Cori, G. T. (1953) *Biochem. Prep. 3*, 1.
Johnson, L. N., Jenkins, J. A., Wilson, K. S., Stura, E. A., & Zanotti, G. (1980) *J. Mol. Biol. 140*, 565.
Kastenschmidt, L. L., Kastenschmidt, J., & Helmreich, E. (1968) *Biochemistry 7*, 4543.
Kasvinsky, P. J., & Meyer, W. L. (1977) *Arch. Biochem. Biophys. 181*, 616.
Krebs, E. G. (1966) *Methods Enzymol. 8*, 543.
Maddaiah, V. T., & Madsen, N. B. (1966) *J. Biol. Chem. 241*, 3873.
Madsen, N. B. (1964) *Biochem. Biophys. Res. Commun. 15*, 390.
Madsen, N. B., Kasvinsky, P. J., & Fletterick, R. J. (1978) *J. Biol. Chem. 253*, 9097.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88.
Palm, D., Schächtele, K., Feldmann, K., & Helmreich, E. J. M. (1979) *FEBS Lett. 101*, 4031.
Parrish, R. F., Uhing, R. J., & Graves, D. J. (1977) *Biochemistry 16*, 4824.
Shaltiel, S., & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun. 41*, 594.
Shaltiel, S., Hedrick, J. L., & Fischer, E. H. (1966) *Biochemistry 5*, 2108.
Shaltiel, S., Hedrick, J. L., Pocker, A., & Fischer, E. H. (1969) *Biochemistry 8*, 5189.
Shimomura, S., & Fukui, T. (1977) *J. Biochem. (Tokyo) 81*, 1781.
Shimomura, S., & Fukui, T. (1978) *Biochemistry 17*, 5359.
Sygusch, J., Madsen, N. B., Kasvinsky, P. J., & Fletterick, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4757.
Thomas, J. A., Schlender, K. K., & Larner, J. (1968) *Anal. Biochem. 25*, 486.
Titani, K., Cohen, P., Walsh, K. A., & Neurath, H. (1975) *FEBS Lett. 55*, 120.
Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsh, K. A., Neurath, H., & Fischer, E. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4762.
Tu, J.-I., Jacobsen, G. R., & Graves, D. J. (1971) *Biochemistry 10*, 1229.
Uhing, R. J., Janski, A. M., & Graves, D. J. (1979) *J. Biol. Chem. 254*, 3166.
Vincenza, V., & Zampetti, P. (1973) *Chem. Phys. 2*, 233.
Warsehl, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227.
Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G., Wild, D. L., & Jenkins, J. A. (1978) *Nature (London) 274*, 433.
Yan, S. C. B., Uhing, R. J., Parrish, R. F., Metzler, D. E., & Graves, D. J. (1979) *J. Biol. Chem. 254*, 8263.