On the Role of Pyridoxal 5′-Phosphate in Phosphorylase. I. Absence of Classical Vitamin B_6–dependent Enzymatic Activities in Muscle Glycogen Phosphorylase*

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ABSTRACT: Phosphorylases *b* and *a* were purified and crystallized according to various procedures and examined for transaminase and other enzymatic activities characteristic of B_6-dependent enzymes. Aspartate-α-ketoglutarate and alanine-α-ketoglutarate transaminase activities were determined by coupled spectrophotometric techniques. Transamination and decarboxylation of other amino acids were determined by paper electrophoresis. In addition, the enzyme preparations were examined for their ability to catalyze specific β- and γ-elimination reactions. Contrary to the findings of others, phosphorylase *b* or *a* was found to be absolutely free of enzymatic activities associated with "typical" pyridoxal phosphate–containing enzymes. No evidence could be found in nonenzymatic model systems for the participation of vitamin B_6 and several B_6 derivatives in the glycogen-dependent release of phosphate from glucose-1-phosphate.

The role of pyridoxal-5′-phosphate in glycogen phosphorylase (E.C. 2.4.1.1, α-1,4-glucan:orthophosphate glucosyltransferase) has remained a mystery ever since its discovery in the enzyme (Baranowski *et al.*, 1957; Cori and Illingworth, 1957; Kent *et al.*, 1958). On the one hand, Illingworth *et al.* (1958) reported that removal of the cofactor resulted in loss of enzymatic activity; the inactive material could be partially reactivated by addition of pyridoxal phosphate. On the other hand, it was shown that treatment of the enzyme with sodium borohydride during which pyridoxal phosphate is irreversibly "fixed" to the protein yielded an enzymatically active product (Fischer *et al.*, 1958a). Since the reduction involved the carbonyl group of pyridoxal phosphate, this finding indicated that the cofactor had to function in a manner different from that of all other pyridoxal phosphate–catalyzed reactions for which a potential aldehyde group is required.

In view of the fact that all well-characterized pyridoxal phosphate–containing enzymes, with the exception of phosphorylase, are involved in amino acid metabolism, the possibility existed that the latter enzyme possessed a secondary catalytic activity in which pyridoxal phosphate was directly involved. The purpose of this work was therefore to examine phosphorylase for reactions normally catalyzed by B_6-containing enzymes. Furthermore, since it was shown (for review see Snell, 1963) that most enzymatic reactions involving pyridoxal enzymes can be catalyzed nonenzymatically by pyridoxal phosphate, model systems were set up in the hope of demonstrating a glycogen-dependent hydrolysis of glucose-1-phosphate in the presence of vitamin B_6 and a number of B_6 derivatives.

Subsequent to a preliminary account of this work (Fischer *et al.*, 1963) and contrary to it, Waksman and Roberts (1963) reported that phosphorylase exhibited transaminase activity. It therefore appeared necessary to thoroughly reexamine the possibility that purified phosphorylase could also catalyze reactions normally expected from B_6 enzymes. For this purpose phosphorylase *b* and *a* were purified and crystallized according to various procedures, and the distribution of phosphorylase and transaminase activities was followed throughout the purification. In addition, recrystallized phosphorylase was subjected to a number of tests designed to detect enzymatic activities normally associated with pyridoxal-containing enzymes.

Materials and Methods

Preparation and Assay of Glycogen Phosphorylase. Phosphorylase *b* was prepared according to the method of Fischer *et al.* (1958b). Phosphorylase *a* was prepared by the conversion of crystalline phosphorylase *b* to phosphorylase *a* (Fischer *et al.*, 1958b) using purified phosphorylase kinase (Krebs *et al.*, 1964), and directly from crude muscle extracts, using the original method of Illingworth and Cori (1953). Three-times-recrystallized phosphorylase *b* was routinely used for the enzymatic tests and for conversion to phosphorylase *a*. Phosphorylase *a* produced from phosphorylase *b* was recrystallized twice after the *b*-to-*a* conversion, and twice when prepared directly from the muscle extract.

The phosphorylase assay (Illingworth and Cori, 1953) was modified in order to measure enzymatic activities under zero-order kinetics. To 0.2 ml of phosphorylase (1–6 μg) diluted in 0.04 M mercapto-

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ethanol and 0.1 M maleate, and containing 1 mg/ml crystalline bovine serum albumin (Sigma), *p*H 6.5, was added 0.2 ml of substrate containing 0.15 M glucose-1-phosphate, 2% glycogen (Krishell), 0.002 M AMP,[1] and 0.1 M maleate, *p*H 6.5. The glycogen was further purified by the method of Somogyi (1957), or by passage through charcoal. Final concentrations were 0.075 M glucose-1-phosphate, 1% glycogen, 0.001 M AMP, 0.02 M mercaptoethanol, 0.5 mg/ml albumin, and 0.1 M maleate. After 5 minutes at 30°, the reaction was stopped by addition of acidified ammonium molybdate solution and inorganic phosphate released was determined by the method of Fiske and Subbarow (1925), following the usual procedure of Illingworth and Cori (1953). Under these conditions, one unit of activity is defined as the amount of enzyme causing the release of 1 μmole inorganic phosphate from glucose-1-phosphate per minute; specific activities for both phosphorylase *b* and *a* in the presence of AMP are 80 units/mg, as compared to 1600 and 2200 units/mg, respectively, obtained in the original assay system of Illingworth and Cori (1953). In the absence of AMP, phosphorylase *b* has a specific activity of <0.8 unit/mg, and phosphorylase *a* of 54 units/mg. The range of precision of this method is ±5%. The protein concentration was determined by the biuret method described by Layne (1957), or by optical density using the absorbancy index $A_{278}^{1\%} = 11.9$ (Appleman *et al.*, 1963).

Amino acids and polylysine (DP-30 and DP-100) were purchased from Mann Research Laboratories. Pyridoxine, pyridoxal, pyridoxamine, analogs of vitamin B_6, and their phosphorylated derivatives were purchased from Calbiochem (Los Angeles, Calif.) and DPNH from Pabst Laboratories. ε-*N*-Pyridoxyllysine and poly-ε-*N*-pyridoxylpolylysine were prepared by Dr. Arden Forrey (1963). Pyruvate, α-ketoglutarate, and lactic and malic dehydrogenase were obtained from Calbiochem, and glutamic dehydrogenase from Sigma.

Transaminase Activities. L-Aspartate-α-ketoglutarate (E.C. 2.6.1.1) and L-alanine-α-ketoglutarate (E.C. 2.6.1.2) transaminase activities were determined spectrophotometrically. In the first instance, the oxalacetate produced was measured by coupling the reaction with malic dehydrogenase, while in the second the pyruvate formed was determined by the use of lactic dehydrogenase. Both reactions were followed by the disappearance of DPNH at 340 mμ. The reaction mixture contained 9×10^{-3} M α-ketoglutarate, 9×10^{-3} M alanine, or aspartic acid, 0.07 mg/ml (1.05×10^{-4} M) DPNH, 0.02 mg malic or 0.1 mg lactic dehydrogenase, 0.07 M sodium phosphate buffer, *p*H 7.4, in a total of 3 ml solution. The reaction was run at room temperature (23°) and the reaction followed for 3–10 minutes. The reaction was linear for approximately 90% of DPNH disappearance. The sensitivity of the method was such that 2.4×10^{-4} unit of transaminase activity could have been easily determined; this is equivalent to 0.031 μg of pure transaminase, assuming a specific activity of 75 units/mg at 23° for pure L-aspartate-α-ketoglutarate transaminase. A unit of transaminase activity was defined as that amount of enzyme catalyzing the conversion of 1 μmole substrate per minute; the specific activity was expressed in units transaminase per mg protein.

Results

Separation of Transaminase from Phosphorylase Activity during Purification of Phosphorylase b and a. If transaminase activity were an intrinsic attribute of phosphorylase, one would expect that the transaminase-to-phosphorylase activity ratio would reach a constant value during purification of the enzyme. Since the method of Fischer *et al.* (1958b) for the purification of phosphorylase *b* differed from the original method of Illingworth and Cori (1953) for the purification of phosphorylase *a*, it was felt necessary to follow both procedures; it could be argued that transaminase activity could be specifically destroyed in one of these. This could be the case, for instance, during the 1-hour treatment at 37°, *p*H 8.7, introduced in the purification of phosphorylase *b* to rid the enzyme of phosphorylase *b* kinase, phosphorylase phosphatase, and other contaminating enzymes.

Table I reports transaminase (aspartate-α-ketoglutarate and alanine-α-ketoglutarate) and phosphorylase activities, expressed in identical units at various steps during the purification of phosphorylase *a*. Table II reports similar data obtained during purification of phosphorylase *b*. It can be seen that both transaminase activities are rapidly removed during the purification of the enzymes.

It is also apparent that the two transaminase activities are associated with different proteins since they behave differently during the purification. The almost doubling of aspartate-α-ketoglutarate activity in the acid supernatant fractions obtained in both procedures has been consistently observed; it has not been further investigated. The transaminase/phosphorylase ratio falls to zero by the third crystallization step for both transaminases.

In the final assays involving crystalline phosphorylase *b* and *a*, up to 5 mg of enzyme was added to the reaction mixture. The sensitivity of the assay was such (see under Methods) that the presence of 0.0016% of transaminase would have been readily detected. In assuming that transaminase activity was somehow masked in the purified enzyme, but could be revealed by the presence of certain substrates, transaminase activity was measured under yet another set of conditions. In this instance, 2.5 mg phosphorylase *b* was incubated with glutamate (0.015 M) and pyruvate (0.01 M) at *p*H 7.5; the α-ketoglutarate produced in this reaction was coupled with DPNH, ammonia, and glutamic dehydrogenase and the decrease in absorbancy at 340 mμ was measured according to the general procedure of Hopper and Segal (1962). No formation of α-ketoglutarate could be detected over a period of 5 minutes.

[1] Abbreviations used in this work: AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; DPNH, reduced diphosphopyridine nucleotide.

TABLE I: Comparison of Phosphorylase and Transaminase Activities during Purification of Phosphorylase *a*.

Fraction	Phosphorylase *a*			Alanine-α-ketoglutarate Transaminase			Aspartate-α-ketoglutarate Transaminase		
	Total Activity[a] (units)	Total Activity[b] (units)	Specific Activity[b] (units/mg)	Total Activity (units)	Specific Activity (units/mg)	Transaminase/ Phosphorylase	Total Activity (units)	Specific Activity (units/mg)	Transaminase/ Phosphorylase[b]
Extract	170,000	308,000	7.9	375	9.5×10^{-3}	1.2×10^{-3}	1800	4.6×10^{-2}	5.8×10^{-3}
Acid supernatant	138,000	248,000	8.5	356	1.2×10^{-2}	1.4×10^{-3}	3360	1.2×10^{-1}	1.4×10^{-2}
Acid pellet	580	5,760	1.7	0	0	0	1	2.9×10^{-4}	1.7×10^{-4}
Dialyzed ammonium sulfate (precipitate)	11,700	168,000	47	31	8.5×10^{-3}	1.8×10^{-4}	20	5.6×10^{-3}	1.2×10^{-4}
Dialyzed ammonium sulfate (supernatant)	226	70,500		30		4.3×10^{-4}	21		3.0×10^{-4}
First crystals	16,000	68,500	75	3.7	4.0×10^{-3}	5.4×10^{-5}	0.47	5.2×10^{-4}	6.9×10^{-6}
First crystals (supernatant)	3,900	31,700		3.4		1.1×10^{-4}	0.08		2.5×10^{-6}
Second crystals	10,500	19,500	79	0.3	1.2×10^{-3}	1.5×10^{-5}	0	0	0
Second crystals (supernatant)	836	2,040		0.3		1.5×10^{-4}	0	0	0
Third crystals	7,700	16,500	80	0	0	0	0	0	0

[a] Measured in the absence of AMP. [b] Measured in the presence of 10^{-3} M AMP.

TABLE II: Comparison of Phosphorylase and Transaminase Activities during Purification of Phosphorylase *b*.

Fraction	Phosphorylase *b*		Alanine-α-ketoglutarate Transaminase			Aspartate-α-ketoglutarate Transaminase		
	Total Activity[a] (units)	Specific Activity (units/mg)	Total Activity (units)	Specific Activity (units/mg)	Transaminase/ Phosphorylase	Total Activity (units)	Specific Activity (units/mg)	Transaminase/ Phosphorylase
Extract	642,000	8.1	770	9.7×10^{-3}	1.2×10^{-3}	8,000	9.7×10^{-2}	1.2×10^{-2}
Acid supernatant	500,000	8.7	760	1.3×10^{-2}	1.5×10^{-3}	13,300	2.4×10^{-1}	2.7×10^{-2}
Dialyzed ammonium sulfate (precipitate)	440,000	56	335	4.3×10^{-2}	7.6×10^{-4}	127	1.6×10^{-2}	2.9×10^{-4}
First crystals	300,000	72	15	3.6×10^{-3}	5.0×10^{-5}	72	1.7×10^{-2}	2.4×10^{-4}
Second crystals	300,000	75	2.0	5.0×10^{-4}	6.7×10^{-6}	0	0	0
Third crystals	280,000	80	0.4	1.1×10^{-5}	1.4×10^{-6}	0	0	0
Fourth crystals	320,000	80	0	0	0	0	0	0

[a] Measured in the presence of 10^{-3} M AMP.

If there were a lag period before which transaminase activity appeared, short incubations as used above would not be appropriate. Therefore, incubations in the presence of the substrates were carried out for up to 40 minutes before coupling with the glutamic dehydrogenase reaction. Again, no traces of transaminase activity could be detected. These results were confirmed by measurements of pyruvate concentration at the beginning and end of the reaction using lactic dehydrogenase; no disappearance of pyruvate occurred during the incubation. Contamination of phosphorylase by transaminase in the order of one part per million would have been easily detected.

In the improbable event that the enzyme could undergo partial resolution during purification, it appeared advisable to test certain fractions in the presence of added pyridoxal-5′-phosphate. Therefore, the crude muscle extract, the supernatant solution obtained after heat treatment, and all crystalline fractions were also assayed in the presence of 10^{-4} M pyridoxal-5′-phosphate. No increase in transaminase activity could be observed; on the contrary, the enzymes appeared to be slightly inhibited if anything. Finally, pyridoxal-5′-phosphate was also added to a few other purified proteins to check for a possible potentiation of the catalytic activity of the free coenzyme. In assuming that interaction of certain groups on the protein with the coenzyme could be slow and rate limiting, pyridoxal-5′-phosphate (10^{-4} M) was incubated overnight at 0° with the proteins, then 40 minutes at 30° prior to the assay. No transaminase activity could be observed in the presence of 2 mg each of albumin (Pentex), muscle hexokinase (Pabst), and ribonuclease (Worthington).

Absence of Other B_6-dependent Enzymatic Activities in Crystalline Phosphorylase. In spite of the negative results afforded by the coupled spectrophotometric assays for transaminase activity reported here, it was felt necessary to check for the presence of other catalytic activities characteristic of pyridoxal phosphate–containing enzymes in purified phosphorylase *b* or *a*. To this effect, phosphorylase was incubated with all usual amino acids and the products of the reaction were examined by paper electrophoresis. This reaction mixture consisted of 0.01 M amino acid, 0.01 M α-ketoglutarate, and up to 2.5 mg/ml phosphorylase in a final volume of 2 ml. The reaction was run for 1 hour at 30° with or without 10^{-3} M AMP at *p*H 6.8 (0.05 M phosphate buffer) or *p*H 8.5 (0.367 M Tris-Cl^-). After incubation, the reaction mixture was deproteinized with $HClO_4$ and the protein precipitate was removed by centrifugation. The supernatant solution was adjusted to *p*H 6.5 by KOH and the insoluble $KClO_4$ was removed by a second spin. A 50-μl portion of this clear solution was spotted on Whatman 3 MM paper and subjected to high-voltage electrophoresis (60 minutes at 2000 v) in pyridine-acetate buffer, *p*H 6.5. The paper was sprayed with ninhydrin to detect glutamic acid produced by amino transfer to α-ketoglutarate, or the formation of amines if decarboxylation of the added amino acids occurred. The method was sensitive enough to enable detection of 0.01 μmole of any new ninhydrin-positive material produced in the reaction; this amount would correspond to 1% substrate converted.

The following amino acids were used as substrates: L-glutamine, L-aspartic acid, L-asparagine, L-histidine, L-lysine, L-arginine, glycine, L-alanine, L-isoleucine, L-leucine, L-valine, L-threonine, L-serine, L-phenylalanine, L-tyrosine, L-tryptophan, L-cysteine, and L-methionine. L-Glutamic acid was also tested for possible β- and γ-decarboxylation. In spite of the fact that the amount of phosphorylase added to these reaction mixtures was approximately 200-fold greater than that used in measuring phosphorolytic activity, and the time of incubation twelve times longer, neither the formation of glutamic acid nor that of any new ninhydrin-positive spot could be detected under the experimental conditions described here.

Attempts to find *cysteine desulfhydrase* activity (E.C. 4.4.1.1) in crystalline phosphorylase were made by using the lactic dehydrogenase–coupled system in the presence of 0.013 M L-cysteine (and leaving out α-keto acids). The results were negative.

Serine and *threonine dehydrase* activities (E.C. 4.2.1.13 and 4.2.1.16) were run by Dr. Helen Whiteley using the spectrophotometric method of Whiteley and Hayaishi (1964). Using 1 mg of phosphorylases *a* and *b* in the presence or absence of added pyridoxal-5′-phosphate (0.1 mg/ml), AMP, or ADP (2.5×10^{-3} M) at 37°, *p*H 9.5, and incubations up to 9 hours, no significant dehydrase activity could be detected.

Nonenzymatic Model Studies on Glycogen-dependent Hydrolysis of Glucose-1-phosphate Catalyzed by B_6 Derivatives. Snell (1945) and Metzler and Snell (1952) have shown that various B_6 derivatives including pyridoxal or pyridoxamine phosphate can catalyze nonenzymatic transaminations when heated in the presence of various α-amino acids (see also Snell, 1963). The reaction was greatly accelerated by catalytic amounts of multivalent metal ions. It appeared therefore advisable to check if pyridoxal-5′-phosphate or various other B_6 derivatives could catalyze a nonenzymatic reaction between glucose-1-phosphate and glycogen.

The model reaction mixture contained 5×10^{-3} M α-glucose-1-phosphate, 0.4% glycogen, and 5×10^{-5} M of the pyridoxal derivatives listed in Table III in a total of 5 ml. The reaction was run at three different *p*H values, namely, *p*H 4.7 (0.1 M acetate buffer), *p*H 7.0 (no buffer added), and *p*H 9.6 (0.02 M glycine), for 60 minutes at 100°. Inorganic phosphate released was determined according to Fiske and Subbarow (1925); this method could easily detect 0.1% conversion of the added glucose-1-phosphate.

The results are given in Table III. As can be seen, no significant difference could be detected in the amount of inorganic phosphate released between controls where no pyridoxal derivative was added and the mixtures where they were present. Similar results were obtained when the reactions were run in the presence of 10^{-3} M Al^{3+} ions.

TABLE III: Release of Inorganic Phosphate from α-Glucose-1-phosphate in the Presence of Various B_6 Derivatives.[a]

B_6 Derivatives	Inorganic Phosphate Released[b] *p*H 4.7 (μmoles)	*p*H 7.0 (μmoles)	*p*H 9.6 (μmoles)
Control	1.98	0.50	0.41
+ pyridoxine	2.04	0.50	0.41
Control	1.96	0.54	0.42
+ pyridoxamine	1.89	0.47	0.42
Control	2.18	0.47	0.39
+ pyridoxal	1.97	0.47	0.40
Control	2.38	0.98	0.92
+ pyridoxine phosphate	2.38	0.99	0.81
Control	2.17	0.72	0.61
+ pyridoxamine phosphate	2.05	0.63	0.56
Control	2.04	0.68	0.61
+ pyridoxal phosphate	1.93	0.66	0.58
Control	2.45	0.46	0.55
+ ε-pyridoxyllysine	2.49	0.47	0.56
Control	2.51	0.50	0.56
+ poly-ε-pyridoxyl polylysine	2.47	0.56	0.60

[a] Conditions as indicated in the text. [b] Total amount of glucose-1-phosphate present = 25 μmoles.

Discussion

Multiple enzyme activity has been reported for a large variety of enzymes. It is well known, for instance, that many proteolytic enzymes possess peptidase and esterase activity, that triose phosphate dehydrogenase can act as a dehydrogenase, a phosphotransacetylase, a transacetylase, or a hydrolase, that pyruvic decarboxylase can yield a number of products, and so on. However, in all these cases multiple enzymatic activities result from the fact that the enzyme acts as a group carrier forming an intermediate with part of the substrate that can then be transferred to a number of acceptors. The bond-breaking reaction catalyzed by the enzyme in forming the reactive intermediate is always the same.

Certain pyridoxal phosphate–containing enzymes are known to catalyze several simultaneous reactions, but these usually also proceed from the decomposition of a common intermediate in which, for instance, the α-carbon of an α-amino acid is activated by interacting with pyridoxal-5′-phosphate according to the classical formulation by Snell and Braunstein (for review, see Snell, 1958, and Braunstein, 1960). Labilization of the bonds surrounding the α-carbon will allow racemization, decarboxylation, transamination, deamination, or elimination reactions to take place. In yet another instance, β-aspartic decarboxylase (E.C. 4.1.1.12) was shown to catalyze both transamination and β-decarboxylation, perhaps owing to an error on the part of the enzyme in selecting a single chemical route or possibly as a means of controlling enzyme activity, as pointed out by Novogrodsky and Meister (1964a).

If phosphorylase were shown to catalyze both a phosphorolytic (or glycosyl transferase) reaction *and* a transamination reaction (or another reaction characteristic of B_6-dependent enzymes), the situation would be quite different. The substrates for glycogen phosphorylase are nonnitrogeneous and therefore do not contain an amino group capable of interacting with the aldehyde group of pyridoxal phosphate. Furthermore, $NaBH_4$ reduction of the carbonyl group of pyridoxal phosphate, when linked to phosphorylase by a Schiff base, decreases only slightly the activity of the enzyme (Fischer *et al.*, 1958a). Even if another group on the pyridoxyl nucleus were involved in phosphorolysis, this reaction would have to proceed by a different mechanism than that postulated for transamination. Therefore, the likelihood that the two reactions would occur on the same catalytic site would be extremely tenuous: to catalyze both phosphorolysis and transamination, the enzyme would have to be a real "double-headed" enzyme.

There are several other arguments against the probability that phosphorylase would act as a transaminase as well as a glucosyltransferase. All indications are that at neutral *p*H pyridoxal phosphate in phosphorylase is unavailable for chemical interaction, as if it were "buried": (a) No reduction of the carbonyl group occurs with $NaBH_4$ under these conditions, which led to the postulate that the coenzyme is bound to the protein in the form of a substituted aldamine (Kent *et al.*, 1958); (b) it does not react with any of the weak or strong carbonyl reagents that have been tested unless the protein molecule is distorted by the appropriate agent;[2] and (c) release of the pyridoxyl peptide (after fixation of pyridoxal phosphate on the protein by borohydride reduction) occurs only after prolonged proteolytic attack (Kent, 1959; Forrey, 1963).

As shown in this study, no typical enzymatic activity characteristic of pyridoxal phosphate could be demonstrated in phosphorylase under a whole host of conditions. This is in direct contrast to a report by Waksman and Roberts (1963). However, if one calculates the turnover rate of phosphorylase *b* for the phosphorolysis of glycogen (20,000 moles/minute per mole of enzyme at 30°) and compares it to the turnover rate of transamination for this enzyme as reported by Waksman and Roberts (2.5×10^{-4} mole/minute per mole of enzyme for the alanine-α-ketoglutarate transaminase reaction at 37°), it can be seen that phosphorolysis is at least 10^8 times greater than transamination. This enormous difference in rate between the two types of activities is further emphasized if one considers the situation as it exists in crude muscle extract. If one assumes that the transaminase activity found by Waksman and Roberts in muscle phosphorylase is an intrinsic attribute of the

[2] J. L. Hedrick, S. Shaltiel, and E. H. Fischer, unpublished results.

enzyme, and not mere contamination, the contribution made by phosphorylase to the total transaminase activity of the muscle extract would only be 0.0008%, even though phosphorylase constitutes 10% of the total proteins in this extract. Under these conditions, it would seem exceedingly unlikely that the transaminase activity attributed to phosphorylase could have any kind of physiological significance. But even then, as shown here, these low levels of transaminase activities must be ascribed to contamination, since they could be entirely eliminated by repeated crystallization.

The above-mentioned findings once more raise the question as to the role of pyridoxal phosphate in phosphorylase. At the present time, the most obvious conclusion is that the coenzyme is involved solely in maintaining the tertiary structure of the enzyme in the proper conformation for enzymatic activity. There are, of course, numerous examples of enzymes stabilized by their coenzymes, cofactors, or substrates. Novogrodsky and Meister (1964b) have shown that aspartate β-decarboxylase is stabilized by 4′-deoxypyridoxine-5′-phosphate. DeMoss and Bonner (1959) have reported that in a mutant of *Neurospora crassa* the aldolytic activity of tryptophan synthetase (E.C. 4.2.1.20), i.e., the reversible splitting of indole-3-glycerophosphate into indole and D-glyceraldehyde-3-phosphate, was stimulated by pyridoxal phosphate even though this coenzyme would not be expected to act catalytically in this reaction. Carsiotis and Suskind (1964) have shown that the aldehyde group of pyridoxal phosphate was not involved in this stimulation, since pyridoxine phosphate and 4-deoxypyridoxine phosphate, as well as the hydroxylamine and semicarbazide derivative of pyridoxal phosphate, were also effective. However, in this particular instance, the reaction in point is intimately related to two reactions in which pyridoxal phosphate is directly required for catalysis. No such close relationship in the case of phosphorylase can be visualized at the present time.

Although no transamination was found to be catalyzed by phosphorylase, the possibility remained that pyridoxal-5′-phosphate in the enzyme could itself undergo changes in form (such as, for instance, conversion to pyridoxamine-5′-phosphate), resulting in changes or loss of enzymatic activity. This, then, could be visualized as a possible means of control for the enzyme, as postulated by Novogrodsky and Meister (1964a) for aspartate-β-decarboxylase. This possibility appears extremely remote not only because of the reasons discussed here but also because no other form of vitamin B_6 other than pyridoxal-5′-phosphate has been isolated from this enzyme. Further work on the role of pyridoxal-5′-phosphate in phosphorylase is in progress.

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Addendum

Since submitting this work for publication, a report by Waksman and Roberts (*Arch. Biochem. Biophys. 109*, 522 [1965]) has appeared which essentially retracts their original conclusions regarding the transaminase activity of glycogen phosphorylase.

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Electrolytic Oxidation of Uric Acid: Products and Mechanism*

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ABSTRACT: Alloxan is the dominant product of the chemical oxidation of uric acid under strongly acid conditions; allantoin is the corresponding product for less acidic to alkaline conditions; separate reaction paths have generally been postulated to account for this difference. Investigation of the electrolytic oxidation of uric acid under moderately acidic conditions indicates the presence of a common path, which eventually diverges to produce both alloxan and allantoin in comparable amounts. To this extent, the mechanism of electrolytic oxidation bears a resemblance to the mechanism proposed for the enzymatic oxidation of uric acid. Uric acid gives a well-defined anodic voltammetric wave at a graphite electrode. When uric acid is electrolytically oxidized in dilute acetic acid at large graphite electrodes, 2.2 faradays are passed, and 0.25 mole CO_2, 0.25 mole of a precursor of allantoin, 0.75 mole urea, 0.3 mole parabanic acid, and 0.3 mole alloxan simultaneously appear per mole of uric acid oxidized. At any stage during electrolysis, the sum of the moles of allantoin precursor and urea equals the moles of uric acid oxidized. This material balance and the stability of the allantoin precursor indicate that the production of urea is associated with the pathway(s) that produce alloxan and parabanic acid.

These and other facts indicate a mechanism whereby uric acid is oxidized in a 2*e* process to a primary short-lived dicarbonium ion intermediate, which undergoes three simultaneous transformations: (1) hydrolysis to the allantoin precursor, (2) hydrolysis to alloxan and urea, and (3) further oxidation and hydrolysis leading to parabanic acid and urea. The nonintegral number of electrons involved are accounted for by the formation of parabanic acid. The primary oxidation intermediate ultimately produces both allantoin and alloxan, suggesting that this intermediate may be common to all uric acid oxidations and that the ultimate product heretofore considered to be typified by either allantoin or alloxan (but not both) is most likely controlled by experimental conditions. A major conclusion is that the electrochemical oxidation more nearly resembles the enzymatic oxidation than the chemical oxidation.

Uric acid gives a well-defined anodic voltammetric wave at the graphite electrode (Smith, 1962). Macroscale electrolysis is accompanied by complete disappearance of the characteristic ultraviolet absorption bands, indicating that oxidation must have occurred at the 4,5 double bond (see subsequent discussion).

In virtually all earlier chemical investigations of uric acid[1] oxidation, two fundamentally separate and distinct mechanisms are implied, one occurring under strongly acid conditions and the other under weakly acid, neutral, and alkaline conditions (Biltz and Schauder, 1923). The most characteristic product of the latter conditions is allantoin, but many other products have been isolated (Brandenberger, 1956); however, there is no report of alloxan ever having been identified. Similarly, strongly acid conditions produce alloxan as the most characteristic oxidation product with no apparent involvement of species that could lead to products encountered when allantoin is the characteristic product. The present investigation indicates a possible common primary oxidation intermediate that can subsequently produce either allantoin or alloxan, analogous to the mechanism suggested (Canellakis and Cohen, 1955; Paul and Avi-Dor,

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[1] To facilitate reading of the text, structural formulas of the principal compounds discussed are given in Figures 1 and 5. The formulas used are commonly accepted ones; allowance must be made for alteration owing to keto-enol and acid-base equilibria.