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Physical and Catalytic Properties of Tryptophanase from *Bacillus alvei**

J. A. Hoch† and R. D. DeMoss

ABSTRACT: Tryptophanase from *Bacillus alvei* was resolved from its coenzyme, pyridoxal phosphate, by extended dialysis against Tris–EDTA buffer. Equilibrium dialysis of the enzyme against pyridoxal phosphate in potassium phosphate or Tris buffer indicated that 1 mole of coenzyme is bound/125,000 or 130,000 g of enzyme, in the respective buffers. Kinetic analysis of coenzyme binding suggests that two molecules of pyridoxal phosphate are required for full activity of each active site.

Dissociation constants calculated from the kinetic data for the two coenzyme molecules were 1.14 and 14.4 μM, respectively. A sedimentation constant of 10.8 S was calculated for the enzyme at infinite dilution in 0.01 M potassium phosphate, pH 7.0. The sedimentation constant decreases in buffers of higher ionic strengths. If resolved from coenzyme, the enzyme readily dissociates upon dilution in Tris–EDTA buffer, forming a particle with sedimentation constant about 5.4 S. The dissociated material can be fully reactivated by dialysis against potassium phosphate containing pyridoxal phosphate. The molecular weight of the enzyme in both the native and reconstituted form is approximately 220,000.

The authors have described the purification and some of the catalytic properties of tryptophanase from *Bacillus alvei* (Hoch *et al.*, 1966). The enzyme is constitutive and appears to participate in a regulatory role in the physiology of the organism (Hoch and DeMoss, 1965, 1966).

During additional sedimentation experiments, anomalous behavior of the protein was observed. In this report, we present data concerning the enzyme–coenzyme complex and some properties of the apoenzyme.

Materials and Methods

Tryptophanase. Tryptophanase was purified as previously described from extracts of *B. alvei* (Hoch *et al.*, 1966). The purified preparation was subjected to chromatography on Sephadex G-200 to remove a small amount of contaminating nucleic acid. The 280/260 mμ absorbancy ratio of the final preparation was 1.75. Tryptophanase activity was assayed as previously described (Hoch and DeMoss, 1965). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Pyridoxal Phosphate. Pyridoxal phosphate[1] was determined with phenylhydrazine as described by Wada and Snell (1961). Protein was precipitated from the contents of the dialysis bags by the addition of H_2SO_4 before determination of the PLP content (Dempsey and Snell, 1963).

Sedimentation. Sedimentation experiments were performed in a Spinco Model E ultracentrifuge. The density of all buffers was assumed to be the same as the density of water at the temperatures used. Sedimentation velocity experiments were conducted at 59,780 rpm in the standard 4° sector cell. Sedimentation equilibrium determinations were performed at 20° in a double-sector cell at 7257 rpm. Speed was determined from the

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[1] Abbreviation used: PLP, pyridoxal 5-phosphate.

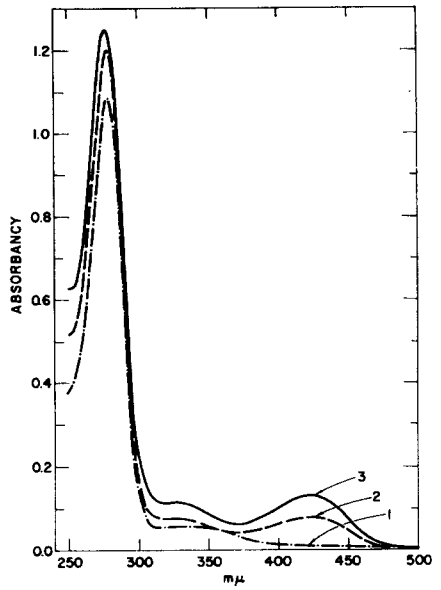

FIGURE 1: Spectra of holo- and apotryptophanase. Curve 1, tryptophanase resolved of pyridoxal phosphate by the method described in the text; protein concentration, 1.5 mg/ml. Curve 2, tryptophanase dialyzed 24 hr against 0.05 M Tris, pH 7.0, supplemented with 0.1 mM pyridoxal phosphate; protein concentration, 1.59 mg/ml. Curve 3, tryptophanase dialyzed 24 hr against 0.05 M potassium phosphate, pH 7.0, supplemented with 0.1 mM pyridoxal phosphate; protein concentration, 1.64 mg/ml.

odometer readings. C_0 for these determinations was calculated from a separate determination in a capillary-type synthetic boundary cell. The area under the peak in the latter experiment was determined by numerical integration using the trapezoidal rule. The peak areas in both the synthetic boundary and the approach to equilibrium determinations were evaluated from enlargments of the schlieren pictures traced on graph paper.

Results

Resolution of Tryptophanase. The enzyme was freed of PLP by dialysis at 5°, first for 48 hr against three changes of 1000 volumes each of 0.01 M Tris–Cl (pH 8.0), second for 24 hr against two changes of 1000 volumes each of 0.05 M Tris–Cl (pH 8.0) containing 0.5 mM EDTA, third for 12 hr against two changes of 1000 volumes each of 0.01 M Tris–Cl, 0.01 M potassium phosphate (pH 8.0), and finally for 12 hr against two changes of 1000 volumes each of 0.02 M potassium phosphate (pH 8.0). The visual estimation of residual yellow color suggested that PLP was removed mainly during dialysis against Tris supplemented with EDTA.

The resolved enzyme is completely active in potassium phosphate buffer, pH 8.0, supplemented with PLP without the addition of divalent cations.

TABLE I: Equilibrium Dialysis of Tryptophanase against Pyridoxal Phosphate.[a]

Buffer	Excess Pyridoxal Phosphate Concn inside Dialysis Bag (mμmoles/ml)	Protein (mg/ml)	g of Protein/Mole of Pyridoxal
Tris	12.23	1.594	130,335
Potassium phosphate	13.02	1.638	125,806

[a] Conditions: enzyme solution (3 ml) was dialyzed for 44 hr at 5° against 2000 ml of 0.05 M Tris–Cl, pH 8.0, or 0.05 M potassium phosphate, pH 8.0. Both buffers were supplemented with 0.1 mM pyridoxal phosphate.

Figure 1 shows the absorption spectra of the apoenzyme and of the enzyme dialyzed against Tris or potassium phosphate buffers containing PLP. The enzyme-bound PLP shows an absorption maximum in the 410–425-mμ region similar to the absorption displayed by tryptophanase of *Escherichia coli* (Newton *et al.*, 1965). Since the enzyme is inactive in Tris buffer without the addition of ammonium or potassium ions, it was of interest to determine if dialysis against Tris buffer containing PLP would alter the absorption spectrum of the bound PLP. The spectrum in Tris buffer exhibited no substantial shift of absorption maxima as compared to the spectrum observed in potassium phosphate buffer.

Pyridoxal Phosphate Content of the Holoenzyme. The amount of PLP bound to the enzyme was determined by equilibrium dialysis against PLP. The dialysis was performed both in Tris and potassium phosphate buffers to determine if the Tris buffer affected the amount of PLP bound to the enzyme. Table I shows the results of PLP analysis after 44-hr dialysis against the respective buffers. The PLP concentration excess within the dialysis bags corresponded to the binding of 1 mole of PLP/126,000 g of protein in potassium phosphate buffer and 1 mole of PLP/130,000 g of protein in Tris buffer.

Affinity of the Apoenzyme for Pyridoxal Phosphate. Tryptophanase resolved of PLP by the procedure described above was assayed in the presence of increasing concentrations of PLP to determine the affinity of the apoenzyme for the cofactor. The PLP saturation curve is shown in Figure 2A.

Sedimentation Coefficient of Tryptophanase. Purified tryptophanase displays a single, symmetrical boundary in sedimentation velocity experiments. A typical schlieren curve for the enzyme sedimenting in 0.01 M potassium phosphate, pH 7.0, is shown in Figure 3A–C. In this buffer the enzyme sediments at a higher velocity (10.8 S) than in buffers of higher ionic strength (9.75 S, Figure 4).

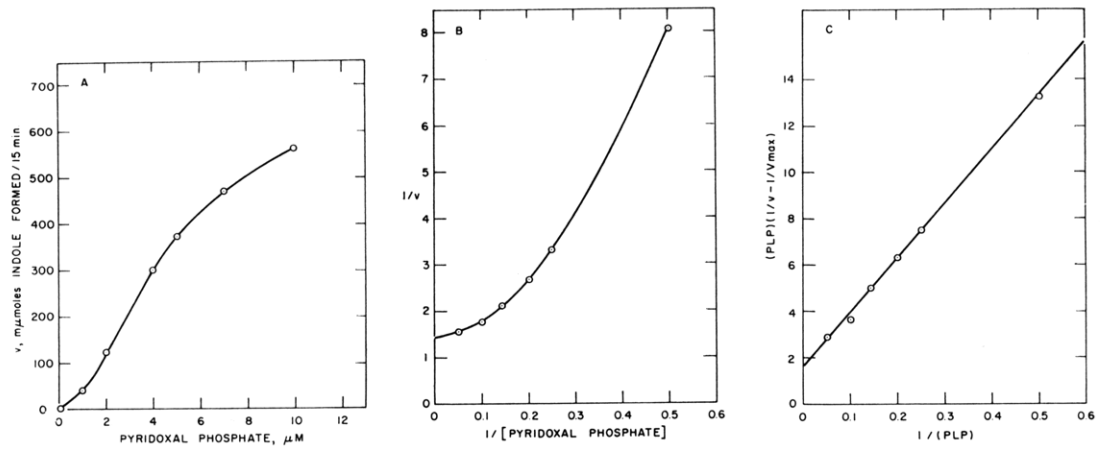

FIGURE 2: Pyridoxal phosphate saturation curve. Conditions: enzyme, 7 μg, resolved of pyridoxal phosphate as described in text, 200 μmoles of potassium phosphate, pH 8.0, 250 μg of bovine serum albumin, and various levels of pyridoxal 5-phosphate in a total volume of 1.5 ml were incubated for 10 min at 37°. After addition of 0.5 ml of 0.02 M L-tryptophan and 4 ml of toluene, the mixture was shaken gently at 37° for 15 min. The reaction was stopped by the addition of 0.1 ml of 2 N NaOH, and a sample of the toluene layer was analyzed for indole. (A) Plot of v, initial reaction velocity, as a function of pyridoxal phosphate concentration; (B) Lineweaver–Burk plot, $1/v$ as a function of 1/[pyridoxal phosphate]; (C) data are plotted on the assumption that two molecules of coenzyme are required for full enzyme activity (see text).

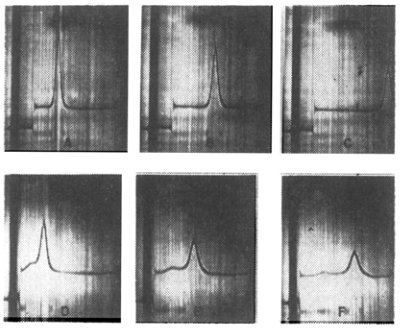

FIGURE 3: Sedimentation of tryptophanase from *B. alvei*. Tryptophanase in 0.01 M potassium phosphate, pH 7.0, containing 0.1 mM PLP; speed 59,780, 20°. Protein concentration was 7.3 mg/ml. Figures A–C were taken 16, 32, and 64 min after reaching speed. Tryptophanase was dialyzed against 0.05 M Tris–Cl, pH 7.5, containing 0.25 mM EDTA, for 24 hr; speed, 59,780 rpm, 20°. Protein concentration was 7.4 mg/ml. Figures D–F were taken 16, 32, and 48 min after reaching speed.

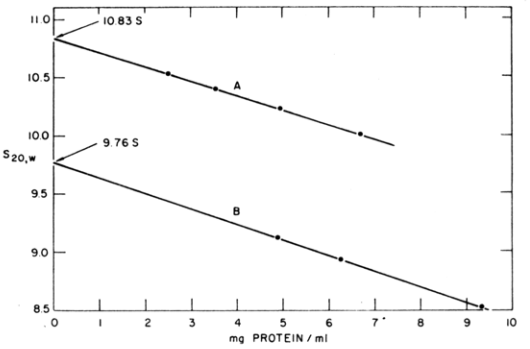

FIGURE 4: Sedimentation coefficient of tryptophanase. The sedimentation behavior of tryptophanase at various protein concentrations was determined in: (A) 0.01 M potassium phosphate, pH 7.0, containing 0.1 mM pyridoxal phosphate, at 18°, (B) 0.05 M Tris, pH 7.0, containing 0.1 mM pyridoxal phosphate, at 20°. In both cases, a nominal speed of 59,780 rpm was employed in the Spinco Model E analytical ultracentrifuge. The observed s values at 18° were corrected to $s_{20,w}$.

The purified enzyme was centrifuged in a sucrose density gradient as described by Martin and Ames (1961). The enzyme activity was distributed mostly in a single peak corresponding to 9.5 S (Figure 5) as measured by its mobility relative to that of catalase, the standard enzyme. In addition to the main peak, two other activity peaks sedimented at slower rates corresponding to 5.7 and 4.3 S.

Molecular Weight. The molecular weight of tryptophanase was determined by the approach to sedimentation equilibrium method as described by Schachman (1957). Tryptophanase (*ca.* 8 mg/ml) in 0.1 M potassium phosphate, pH 8.0, was centrifuged at 7257 rpm. Pictures were taken at intervals over a 2-hr period and the molecular weight calculated both at the menis-

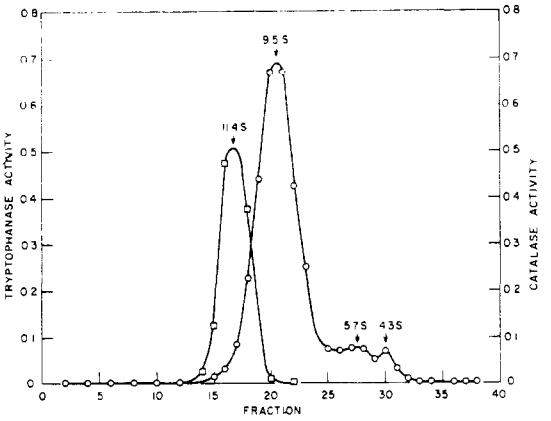

FIGURE 5: Sucrose density gradient centrifugation. Tryptophanase, 80 μg, and catalase, 39 μg, were layered, in 0.10 ml, on a 5–20% linear sucrose gradient, 4.6 ml, containing 0.1 M potassium phosphate, pH 7.0, and 0.04 mM pyridoxal phosphate. The gradient was centrifuged at 38,000 rpm, at about 4°, for 8.5 hr in the SW39 rotor of the Spinco Model L centrifuge.

cus and at the bottom of the cell for the various time intervals. The molecular weight calculated at the meniscus was 212,000 and at the bottom was 224,000. The molecular weight at both the meniscus and bottom remained constant for different sedimentation times (Table II). The partial specific volume of tryptophanase

TABLE II: Approach to Sedimentation Equilibrium.

Sedimentation Time (min)	Mole Wt Calcd at	
	Meniscus	Bottom
60	208,746	224,764
90	Not determined	226,202
110	217,289	224,341
140	208,735	220,536

was assumed to be 0.75 ml/g.

Dissociation of Tryptophanase by Tris–EDTA. Dialysis of tryptophanase against 0.05 M Tris, pH 7.5, containing 0.25 mM EDTA for 24 hr partially resolved the enzyme of PLP. The schlieren pattern for tryptophanase dialyzed in this manner is shown in Figure 3D–F. A peak with a slower sedimentation value appeared in this material. The majority of the enzyme, however, sedimented with a sedimentation constant corresponding to that of the native enzyme. If tryptophanase were completely resolved of PLP by dialysis, first against 0.05 M Tris, pH 7.5, containing 0.25 mM EDTA for 24 hr and then against 0.05 M Tris, pH 8.0, containing 0.5 mM EDTA for 24 hr, the enzyme showed a concentration-dependent dissociation to the slower sedimenting material. The *s* value for this material calculated at one concentration is 5.4 S. At high protein concentrations (*e.g.*, 1%) the preparation sedimented mainly as 9.5S material. Dialysis of the diluted material against 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM pyridoxal phosphate restored the enzyme completely to the 9.5S form.

Discussion

The tryptophanase of *E. coli* is easily resolved of PLP by crystallization (Newton *et al.*, 1965). The procedure followed for purification of tryptophanase from *B. alvei* does not result in coenzyme resolution. The exhaustive dialysis procedure of Dempsey and Snell (1963) for the resolution of pyridoxamine–pyruvate transaminase also was not effective in the removal of the coenzyme. A modification of their procedure, however, resulted in complete removal of the bound PLP as measured by lack of enzyme activity of the preparation in the absence of added PLP. The observation that dialysis in the absence of EDTA did not completely resolve the enzyme suggests that EDTA chelates a metal ion that either helps bind the PLP to the enzyme or helps bind protein subunits together, or more likely, EDTA may act as a protein "deforming agent" (Shaltiel *et al.*, 1966).

The values for bound PLP are not significantly different for the two buffers. Tryptophanase of *E. coli* has been reported (Newton *et al.*, 1965) to bind 1 mole of PLP/57,500 g of protein. This value corresponds to about twice as much PLP bound per gram of protein as that found for the *B. alvei* tryptophanase. If the number of PLP molecules bound is indicative of the number of active sites on the enzyme, the tryptophanase of *B. alvei* would possess half as many active sites. In keeping with this conclusion, the calculated turnover number for the tryptophanase of *E. coli* is twice that of the enzyme from *B. alvei* (Hoch *et al.*, 1966).

The kinetic behavior of the enzyme was unusual. The sigmoidal shape of the curve relating initial velocity to coenzyme concentration differs greatly from the hyperbolic saturation curves generally found for substrate or cofactor saturation. A Lineweaver–Burk plot of the data is nonlinear (Figure 2B). A nonlinear plot such as this is characteristic of enzymes catalyzing a reaction between two identical molecules and the reaction is second order with respect to the substrate (Dixon and Webb, 1958). An analogous situation can occur if the substrate acts as an activator of the enzyme (Dixon and Webb, 1958).

Neuhaus (1962) has derived equations to evaluate the Michaelis constants for D-alanyl-D-alanine synthetase which catalyzes dipeptide formation from two molecules of alanine. The reaction in this case is second order with respect to the amino acid and nonlinear Lineweaver–Burk plots are obtained. The present data for PLP binding have been treated in

a manner analogous to that for alanine binding in the above case. Since 2 moles of PLP is bound/mole of enzyme, the analysis assumes that both PLP molecules are required for full activity. The assumption does not specify the number of active sites. The following sequence of reactions is considered

$$E + (PLP) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(PLP) \quad (1)$$

$$E(PLP) + (PLP) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E(PLP)_2 \quad (2)$$

$$E(PLP)_2 + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E(PLP)_2S \quad (3)$$

$$E(PLP)_2S \xrightarrow{k_4} E(PLP)_2\text{–}S \quad (4)$$

where E is enzyme, (PLP) is pyridixal phosphate, E(PLP) and $E(PLP)_2$ are the binary and ternary complexes of the enzyme and pyridoxal phosphate, S is L-tryptophan, $E(PLP)_2S$ is the complex between enzyme, pyridoxal phosphate, and tryptophan, and $E(PLP)_2$–S is the enzyme–product complex. The rate constant k_4 reflects the limiting step in the conversion of $E(PLP)_2S$ to $E(PLP)_2$–S. If $k_4 \ll k_3 \ll k_1$ and k_2, the initial velocity of $E(PLP)_2$–S formation may be given approximately by

$$v = \frac{V_{max}}{1 + \frac{K_2^a}{(PLP)}\left[1 + \frac{K_1^a}{(PLP)}\right]} \quad (5)$$

in which K_1^a and K_2^a are the apparent dissociation constants for reactions 1 and 2, respectively. The reciprocal velocity equation rearranged to evaluate K_1^a and K_2^a is

$$(PLP)\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_2^a}{V_{max}} + \frac{K_1^a K_2^a}{V_{max}(PLP)} \quad (6)$$

The constants may be evaluated from the slope and intercept of a plot of $(PLP)(1/v - 1/V_{max})$ *vs.* 1/(PLP). A plot of this type is shown in Figure 2C. Using the data of Figure 2B, V_{max} was estimated to be 4.66×10^{-2} μmole/min. K_2^a was calculated from the intercept of Figure 2C as 1.14 μM. Calculation of K_1^a from the slope gave a value of 14.4 μM. If $k_3 \gg k_4$, then K_3^a should be the apparent dissociation constant for the enzyme–$(coenzyme)_2$–tryptophan complex, previously reported to be 270 μM (Hoch *et al.*, 1966).

A steady-state treatment of the reaction sequence for the general case of reactions 1–4 (Bloomfield, 1962) yielded expression 7.

In developing eq 7, the contributions of enzyme intermediates involving product complexes were ignored. The subscripts 1–3 correspond to Bloomfield's subscripts A–C, respectively. Unfortunately, the complex constants, *i.e.*, $K_{1,2,3}$, $K_{1,2}$, and $K_{2,3}$, cannot be evaluated from the data presently available. It is apparent, however, from the more extensive steady-state treatment, that K_2^a is a function of the substrate concentration, whereas K_1^a is a function of both substrate and coenzyme concentration.

$$(PLP)\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{1}{(PLP)}\left[\frac{K_{1,2,3}}{V_{max}}\left(\frac{1}{S} + \frac{1}{K_3} + \frac{(PLP)^2}{SK_{1,2}}\right)\right] + \frac{K_{1,2,3}}{V_{max}}\left[\frac{1}{SK_1} + \frac{1}{K_{1,3}} + \frac{1}{K_{2,3}}\right] \quad (7)$$

With the appropriate assumptions, expression 7 reduced to a form equivalent to eq 6. If $(PLP)^2/K_{1,2} \ll 1$ and $K_1/K_{2,3} \ll 1/S$, then $K_1^a \cong K_1$ and $K_2^a \cong K_{1,2,3}/K_1S + K_{1,2,3}/K_1K_3$. It is apparent that K_2^a may be evaluated more precisely from data obtained at varying substrate concentrations. Further experiments are planned to satisfy this point. Inspection of Figure 2C indicates, however, that even if K_2^a is markedly affected by high concentrations of substrate, the value of K_2^a will probably not exceed 1.7 μM and the corresponding value for K_1^a will probably remain larger than 9.3 μM.

The relative values calculated for K_1^a and K_2^a above are consistent with the sigmoidal shape of the curve (Figure 2A) and with the hypothesis that the first coenzyme molecule bound to the enzyme exerts an allosteric effect which results in increased binding of the second coenzyme molecule.

Burns and DeMoss (1962) reported a sedimentation coefficient of 9.0 S for tryptophanase from *E. coli*, when determined in 0.01 M potassium phosphate, pH 6.8. In the present experiments, tryptophanase from *B. alvei* exhibited a sedimentation constant of 10.8 S in the same buffer, pH 7.0. Sedimentation analysis in 0.1 M potassium phosphate or 0.05 M Tris results in lower values of the sedimentation coefficient. The sedimentation coefficient is 9.75 S at infinite dilution under these conditions (Figure 3).

Sedimentation analysis of the enzyme in a sucrose gradient revealed two minor peaks of enzyme activity which sedimented more slowly than the major portion of the protein. The sedimentation rates of the minor peaks corresponded to 5.7 and 4.3 S. In a similar experiment, crude extracts of *E. coli* showed activity maxima at about 6.8 and 9.5 S (Newton *et al.*, 1965; Feiss and DeMoss, 1965). The 6.8S material predominated in crude extracts whereas the purified enzyme sedimented at 9.5 S in the analytical ultracentrifuge. The small amount of tryptophanase in crude extracts of *B. alvei* precluded a direct comparison with the *E. coli* enzyme.

The molecular weight of tryptophanase from *B. alvei* is 220,000 as estimated from approach to sedimentation equilibrium analysis. The good agreement of the values for the meniscus and the bottom of the cell indicates a high degree of homogeneity of the preparation. The molecular weight of *E. coli* tryptophanase

calculated by this method is 281,000 (Newton *et al.*, 1965).

Acknowledgment

We are indebted to Dr. V. A. Bloomfield for helpful discussions concerning the considerations of kinetic analysis.

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Decomposition of a Phosphonylated Pyridinium Aldoxime in Aqueous Solution*

George M. Steinberg and Stanley Solomon

ABSTRACT: In aqueous solution, pH 7.6, the potent anticholinesterase *O*-(isopropylmethylphosphonyl)-4-formyl-1-methylpyridinium iodide oxime (4-PPAM) decomposes, with loss of anticholinesterase activity, to the corresponding nitrile, 4-cyano-1-methylpyridinium iodide, rather than to 4-formyl-1-methylpyridinium oxime iodide (4-PAM) as anticipated on the basis of its reaction with the enzyme. Nucleophilic attack at the phosphorus atom to give 4-PAM can be achieved with hexanohydroxamate anion, imidazole, and phosphate, nucleophilic reagents of known high reactivity with organophosphonates. Thus, 4-PPAM is subject to attack by bases *via* two competitive pathways: a general base reaction at the aldehydic hydrogen atom to give Beckman elimination and nucleophilic attack on phosphorus to yield oxime.

The product ratios are consistent with the effect of these nucleophiles on the kinetics of decomposition of 4-PPAM. With effective nucleophiles such as the enzyme the latter pathway predominates.

Pyridinium aldoximes are important in the treatment of organophosphonate poisoning (Hobbiger, 1963). They function principally by reactivating the inhibited enzyme acetylcholinesterase (inhibited by phosphonylation at the active site), although in the presence of free phosphonofluoridates they may also function by reacting with the latter compounds. In both cases, the first product of reaction is a phosphonylated oxime (Hackley *et al.*, 1959; Kewitz *et al.*, 1956). The phosphonylated oximes are themselves quite toxic and a study of the kinetics of decomposition of one member of the group, *O*-(isopropylmethylphosphonyl)-4-formyl-1-methylpyridinium iodide oxime, 4-PPAM,[1] in near neutral aqueous solution has been reported (Hackley *et al.*, 1959; Lamb *et al.*, 1965). Decomposition under these conditions is comparatively slow and can be speeded appreciably by the addition of a variety of nucleophiles. In this work we have studied the pathway of decomposition in aqueous solution and the effect of accelerating nucleophiles on reaction pathway through product identification.

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[1] Abbreviations used: 4-PPAM, *O*-(isopropylmethylphosphonyl)-4-formyl-1-methylpyridinium iodide oxime; 2-, 3-, or 4-PAM, 2-, 3-, or 4-formyl-1-methylpyridinium iodide oxime; 4-CY, 4-cyano-1-methylpyridinium iodide; GB, isopropyl methylphosphonofluoridate.