with thiamine treatment. Glyoxylate is presumably derived from glycine (Laing, 1963), but cannot be detected in normal blood (Buckle, 1963). Both complexes are thiamine-containing enzymes capable of reacting with glyoxylate, and both reactions represent potential pathways for the normal metabolism of glyoxylate. The ease with which thiamine pyrophosphate is removed from the pyruvate as compared with the α-ketoglutarate complex and the concomitant defect in glyoxylate metabolism during thiamine deficiency suggest a major role for the pyruvate reaction. However, in mammalian tissues, the reaction of glyoxylate with pyruvate is much slower than its reaction with α-ketoglutarate (unpublished), and it is not yet clear which reaction is more important in preventing a hyperoxaluria (Koch *et al.*, 1967).

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# Schiff Bases of Pyridoxal Phosphate with Active Center Lysines of Ribonuclease A†

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ABSTRACT: RNase A and pyridoxal phosphate react rapidly in aqueous solution, forming a Schiff base complex. Ultraviolet absorption spectra indicate that the formation of this aldimine species is extensive, even under conditions which render the model compound *N*-α-acetyllysine completely unreactive toward pyridoxal phosphate. The protein-bound cofactor is optically active, provided the tertiary structure is intact. The molar ellipticity at 408 nm as a function of pH is a bell-shaped curve and is in close agreement with a theoretical curve generated using the simple model $EPH_2 \leftrightarrows EPH \leftrightarrows EP$ where only EPH is optically active. A stable chemical modification is obtained by reduction of the complex with sodium borohydride. Modification with increasing amounts of coenzyme lowers the specific activity toward cytidine 2′,3′-cyclic phosphate to 20% at a fivefold ratio of coenzyme to enzyme, but the residual activity cannot be reduced further even with a 35-fold molar excess of pyridoxal phosphate. Amino acid analysis of the phosphorylpyridoxyl peptides obtained from tryptic–chymotryptic digests indicates that Lys-7 and Lys-41 are modified in a ratio of 2:3, when an equimolar complex is reduced with $NaBH_4$. This product distribution is constant as a function of temperature, pH, and borohydride concentration. Phosphate ions protect against modification at both sites by inhibiting the formation of the aldimine complex. The preferential binding of pyridoxal phosphate at the active site of RNase A is interpreted in terms of 3-dimensional structure of the enzyme.

The active center of an enzyme not only contains amino acid residues which function in catalysis but also those which form specific binding sites for substrates or cofactors. The specificity of both processes is highly dependent on local tertiary structure. Anomalous $pK_a$ values have often been found for such residues, and their reactivity has been observed

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to be chemically atypical compared to model compounds. This has led to the conclusion that active-center residues may in fact be poised for function in a local condition of high energy which has been described as an entatic state (Vallee and Williams, 1968).

Complex formation between pyridoxal-P and enzymes dependent on it for activity is an example of active-site-specific reactivity. As a general rule, the aldehyde group of the cofactor forms an aldimine linkage with one out of a large number of lysyl residues (Fasella, 1967). Presumably structural circumstances at the active site, such as the chemical effects exerted by neighboring amino acids (Ivanov and Karpeisky, 1969), enhance the affinity of the active-site lysyl residue for the coenzyme.

Active-site lysyl residues of enzymes not dependent on pyridoxal-P for activity might also be poised in a manner which could allow specific aldimine formation with pyridoxal-P, since a number of enzymes are inhibited in its presence (Rippa *et al.*, 1967; Shapiro *et al.*, 1968; Vallee and Riordan, 1969; Piszkiewicz *et al.*, 1970). Relatively few structural details are available for pyridoxal-P enzymes and other proteins which have been modified specifically with this cofactor. The chemical basis for the preferential reactivity of certain lysines in proteins with pyridoxal-P is therefore largely unknown.

As an approach to this problem we have studied the spectral and structural aspects of the interaction of pyridoxal-P with ribonuclease A (RNase A).[1] This enzyme does not require the cofactor for catalytic activity, but it does have a critical lysyl residue (41) at its active site which is unusually susceptible to chemical modification (Hirs and Kycia, 1965; Carty and Hirs, 1968a). The active center also has a high affinity for phosphate ions (Wyckoff *et al.*, 1967; Kartha *et al.*, 1967). The question thus arises whether or not the structural circumstances, which account for the anomalous chemical behavior of Lys-41, also make this residue preferentially reactive to pyridoxal-P. Our results indicate that pyridoxal-P does form an aldimine linkage preferentially with Lys-41, since reduction of the complex with $NaBH_4$ (Fischer *et al.*, 1958) permitted the isolation of a stable *N*-ε-phosphorylpyridoxyllysyl (41) peptide as a major product.

## Materials and Methods

RNase A was obtained from Worthington Biochemical Corp. (RAF-88B phosphate free). *N*-α-Acetyl-DL-lysine·$H_2O$, pyridoxal hydrochloride, and pyridoxal-P (Sigma Chemical Co.), guanidine hydrochloride (Alexander and Billings), and sodium borohydride (Fischer Reagent) were used without further purification.

Gel filtration chromatography was performed with Sephadex G-25 coarse (Pharmacia). Degradative proteolysis was carried out with *N*-tosyl-L-phenylalanyl chloromethyl ketone treated-trypsin (Worthington, lot 8113, 265 units/ml) and α-chymotrypsin (Worthington three times crystallized, CDI 6068-9). Dowex 50-X2 (Bio-Rad AG50 W-X2 200–400 mesh 4052) was treated as described by Schroeder (1967).

*Protein concentration* was measured by absorbance at 277.5 nm on a Zeiss PMQ II spectrophotometer assuming a molar absorptivity for native RNase A of 9800 (Sela and Anfinsen, 1957). Pyridoxal-P concentration was determined spectrophotometrically at 388 nm, using a molar extinction coefficient of 4900 at neutral pH (Peterson and Sober, 1954).

*Ultraviolet spectra* were obtained on a Cary 15 (MS) recording spectrophotometer between 500 and 280 nm in 1-cm cells at 25°. A spectrophotometric titration cell was used with the Cary 15 to measure changes in the ultraviolet absorption spectra as a function of pH (Auld and French, 1970). A scale-expanded Radiometer pH meter (Model pH 28) equipped with a Radiometer GK 2021 electrode was used to measure pH at 25°.

*Circular dichroic* spectra were recorded at 25° on a Cary 60 spectropolarimeter. The signal to noise ratio was maximized for protein and coenzyme concentrations on the order of $3 \times 10^{-4}$ M. Molar ellipticity, based on the amount of protein present in solution, was calculated as a function of wavelength from the observed spectrum corrected for the base line spectrum of the identical optical cell.

*Amino acid analysis* was carried out on a Beckman Model 120C automatic amino acid analyzer by the technique of Spackman *et al.* (1958). Acid hydrolysis was done in sealed evacuated tubes with 6 N (glass distilled) HCl at 110° for 20 hr.

Amino-terminal analysis, with 0.1 μmole of peptide, was achieved by Edman degradation (Konigsberg, 1967), and subtractive amino acid analysis. The residue obtained after evaporation of the trifluoroacetic acid was extracted for the thiazolinone amino acid with three washes of *n*-butyl chloride (2 ml each). The reagents and solvents for the procedure were prepared according to Edman and Begg (1967).

*Enzymatic activity* toward cytidine 2′,3′-cyclic phosphate, monosodium salt (Schwarz BioResearch 6082-1213-33), was determined at 25° from the change in the absorbance at 292 nm as a function of time, using a Gilford 2000 spectrophotometer (Herries *et al.*, 1962).

*N-α-Acetyl-N-ε-phosphorylpyridoxyllysine* was prepared by reducing 35 mg of *N*-α-acetyl-DL-lysine·$H_2O$ and 9.6 mg of pyridoxal-P dissolved in a solution of 0.4 ml of 95% ethanol and 0.15 ml of 1 M NaOH with 0.1 ml of 2% $NaBH_4$ (in 60% ethanol). Reduction was instantaneous and excess *N*-α-acetyl-DL-lysine was removed by preparative paper chromatography. The entire sample was applied in a 0.5 × 20 cm band to Whatman No. 3MM paper which was developed in the ascending direction for 6 hr, using propanol–acetic acid–$H_2O$ (5:1:1). The fluorescent band was cut out, eluted with water, and freeze-dried.

*N-ε-Pyridoxyllysine* was prepared from *N*-α-acetyl-*N*-ε-phosphorylpyridoxyllysine under conditions identical with those employed in amino acid analysis.

*Preparation of Phosphorylpyridoxylribonuclease.* Four milliliters of RNase A and pyridoxal-P, both $5 \times 10^{-4}$ M in 0.05 Mes buffer, pH 6.0, were equilibrated at 25° for 10 min. Addition of 50 μl of 0.1 M $NaBH_4$, freshly prepared in distilled $H_2O$, caused the yellow color of the aldimine chromophore to disappear instantly. The reduction product was applied directly to a 1.2 × 50 cm column of Sephadex G-25 coarse, previously equilibrated with 0.1 M acetic acid, and titrated to pH 5.1 with ammonia. Elution was carried out with the same buffer at room temperature with a flow rate of 60 ml/hr. Pyridoxine phosphate binds weakly to RNase A and was separated from the enzyme by the Sephadex chromatography. The covalently modified enzyme was detected by the absorption of the chromophoric cofactor at 325 nm. The protein peak was pooled, lyophilized, and stored frozen in the dark, since some phosphorylpyridoxyl peptides are photosensitive (Nolan *et al.*, 1964).

[1] Abbreviations used are: RNase A, bovine pancreatic ribonuclease A; Mes, *N*-morpholinoethane sulfonic acid; Bicine, *N,N*-bishydroxyethylglycine; CM-Sephadex, carboxymethyl Sephadex. The pH of Mes and Bicine is adjusted with KOH.

In the presence of 0.1 M mannitol this gel filtration method was employed to quantitate the amount of cofactor bound covalently to the enzyme by $NaBH_4$ reduction. Mannitol chelates the borate which otherwise binds reversibly to pyridoxine phosphate (Stock *et al.*, 1966) and significantly alters its absorption spectrum. The concentration of free pyridoxine phosphate could be determined spectrophotometrically, using a molar extinction coefficient of 7300 at 325 nm at neutral pH (Peterson and Sober, 1954). The extent of ribonuclease modification was calculated by subtraction of the amount of free pyridoxine phosphate present after reduction from the total amount of pyridoxal-P initially present in the system. Mannitol employed in the quantitative studies was removed by a second gel filtration.

*Performic Acid Oxidation.* The modified, desalted protein, prepared in the above manner, was oxidized with performic acid (Hirs, 1956). This procedure does not disrupt the model compound *N*-α-acetyl-*N*-ε-phosphorylpyridoxyllysine or destroy the chromophore on the modified enzyme. Performic acid was removed by lyophilization.

*Proteolysis.* The lyophilized protein was redissolved and digested with trypsin, followed by chrymotrypsin (Anfinsen *et al.*, 1959). Since no "core peptides" are formed in this degradation, the volatile buffer in which the proteolysis is carried out, is lyophilized away, leaving a white residue of soluble peptides.

*Isolation of Modified Peptides.* Ion-exchange chromatography of the modified peptides was performed under conditions similar to those described by Schroeder (1967). The peptide material representing 27.4 mg of reduced equimolar complex was dissolved in distilled water and acidified with dilute HCl to pH 2, the total volume being less than 2 ml. The sample was applied to a Dowex 50-X2 column (0.9 × 50 cm) equilibrated with 0.2 M pyridine acetate, pH 3.1, at 37°. Elution at 37° was performed with a pH gradient, consisting of 166 ml of pH 3.1 buffer in the mixing chamber and 332 ml of 2.0 M pyridine acetate, pH 5.0 in the reservoir. The flow rate was 20 ml/hr and fraction size 1.5 ml. Light was excluded from the column. Pyridoxyl peptides were located in the effluent by measuring absorbance at 325 nm.

The chromophoric peaks were pooled separately, lyophilized, and dissolved in 2 ml of 0.2 M $NH_4HCO_3$ (titrated to pH 8.0 with concentrated ammonia). Five microliters of a 5-mg/ml *Escherichia coli* alkaline phosphatase solution (Simpson and Vallee, 1968) was added. After a 2-hr incubation at room temperature, this reaction mixture was again lyophilized to dryness. The residue was redissolved in water, acidified with HCl, and rechromatographed over identical Dowex 50-X2 columns.

The elution of peptide material was followed by the alkaline ninhydrin method. A portion of each fraction, 50 or 100 μl, was sampled and hydrolyzed in 1 ml of 2.5 M KOH for 2 hr at 100°, followed by neutralization with 1 ml of 30% acetic acid. The amino group content of these samples was determined by reaction with ninhydrin using a Technicon Auto-Analyzer. The results are expressed as optical density at 570 nm corrected for base-line controls.

*Peptide Maps.* A sample of the unfractionated peptide mixture representing 1 mg of digested protein, dissolved in 50 μl of water, was applied to a sheet of Whatman No. 3MM paper, and developed by the method of Anfinsen *et al.* (1959). The pyridoxyl peptides were located by their light blue fluorescence. Interpretation of the fingerprints was further facilitated by employing a series of spray reagents on the same sheet, which permitted the sequential identification of arginine-, histidine-, and tyrosine-containing peptides (Easley *et al.*, 1969).

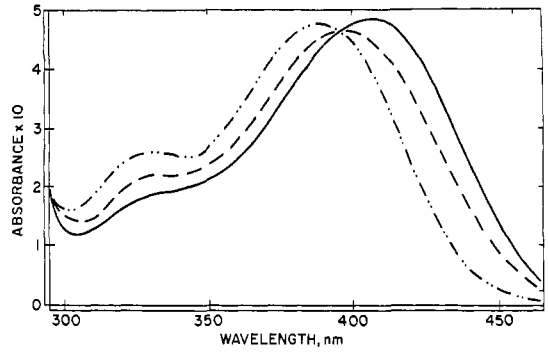


FIGURE 1: Absorption spectra of the equimolar pyridoxal-P–RNase A complex ($1 \times 10^{-4}$ M) in the absence of phosphate (—), in the presence of $4.1 \times 10^{-3}$ M (- -) and $8.1 \times 10^{-2}$ M phosphate (—· · —) in 0.05 M Mes, pH 6. The latter curve is almost identical with the spectrum of pyridoxal-P ($10^{-4}$ M) alone in the same buffer or in the presence of $10^{-3}$ M *N*-α-acetyl-DL-lysine.

*Separation of Two Modified Enzyme Species on CM-Sephadex.* Ribonuclease (27.4 mg) was derivatized with equimolar pyridoxal-P and $NaBH_4$ at pH 6 as described above. The product was applied to a 1 × 25 cm column of CM-Sephadex (G-50 medium), equilibrated with 0.01 M Tris-HCl, pH 8, at 4°, and was eluted with a 500 ml of linear salt gradient (0 to 0.1 M NaCl) in the same buffer. A flow rate of 10 ml/hr was maintained with an 80-cm hydrostatic head of pressure. Fractions of 1 ml were collected, which were analyzed for their optical density of 280 and 320 nm, as well as enzymatic activity.

Two major chromophoric peaks were observed which were desalted and degraded with trypsin and chymotrypsin. The site of modification in each case was determined by the peptide-mapping procedure described above.

## Results

*Reaction of the Coenzyme with Ribonuclease.* The visible ultraviolet absorption maximum of pyridoxal-P undergoes a red shift of 20 nm in the presence of equimolar RNase A (Figure 1), the resulting ultraviolet spectrum being consistent with formation of an aldimine complex (Heinert and Martell, 1963a). In contrast, $10^{-3}$ M *N*-α-acetyllysine does not perturb the spectrum of $10^{-4}$ M pyridoxal-P under similar conditions. This concentration of *N*-α-acetyllysine is equivalent to the lysyl ε-amino group content of RNase A under these conditions. The spectral changes associated with the enzyme–cofactor interaction can be reversed completely by adding increasing amounts of phosphate (Figure 1).

A characteristic aldimine spectrum is observed throughout the pH range of 6–9 (Figure 2). The only distinguishing feature of the ultraviolet spectrum at pH 6 is a shoulder near 325 nm, which disappears at pH 8. Above pH 11 the long-wavelength absorption maximum at 408 nm shifts back to 388 nm which is identical with that of the free cofactor in the aldehyde form (Peterson and Sober, 1954).

*Circular Dichroic Spectra of the Bound Cofactor.* Ribonuclease induces optical activity in the bound pyridoxal-P, generating a band coincident with the ultraviolet absorption maximum at 408 nm (Figure 3). The cofactor displays no optical activity when the protein is exposed to 6 M guanidine hydrochloride or is oxidized with performic acid prior to the addition of pyridoxal-P (Figure 3). Phosphate ions also di-

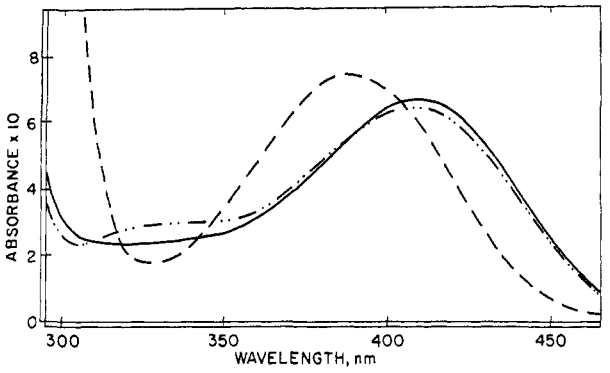


FIGURE 2: The absorption spectra of the equimolar pyridoxal-P–RNase A complex ($1.4 \times 10^{-4}$ M) in 0.02 M sodium acetate at pH 6.1 (—·—·—), pH 8.5 (—), and pH 11.7 (- -). Spectra and pH were recorded using a titration cell (Auld and French, 1970) placed inside a Cary 15 spectrophotometer. A number of spectra were also recorded at the intrevening pH values; titrant 0.5 M NaOH.

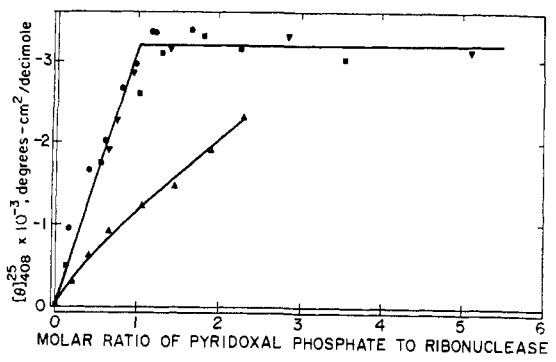


FIGURE 4: Circular dichroic titration of $3 \times 10^{-4}$ M (●) or $1.5 \times 10^{-4}$ M (■) RNase with pyridoxal-P in 0.05 M Bicine at pH 8.35. Same relationship is obtained for a constant pyridoxal-P concentration, $3 \times 10^{-4}$ M (▼) in presence of increasing enzyme concentration. Phosphate ions (0.05 M) have a marked effect on the titration of RNase ($3 \times 10^{-4}$ M) with pyridoxal-P (▲).

minish the extrinsic Cotton effect associated with the bound cofactor chomophore (Figures 3 and 4). Maximal optical activity is obtained at a 1 to 1 mole ratio of pyridoxal-P to enzyme (Figure 4).

In contrast to the ultraviolet spectra, the circular dichroic spectra of the aldimine has a marked pH dependence. The circular dichroism maximum, observed at 408 nm at pH 8.35 (Figure 3), is absent at pH 6 and 11. A plot of molar ellipticity at 408 nm as a function of pH is bell shaped (Figure 5). The same experimental curve is obtained when the pH of a titrated solution is lowered from pH 11 with HCl. The spectra are not noticeably altered either by buffers or the slight change in ionic strength during titration.

*Effects on Enzymatic Activity.* Reduction of the aldimine with $NaBH_4$ inactivates the enzyme to an extent dependent upon the level of pyridoxal-P present (Figure 6). The maximum inactivation is 80% under standard assay conditions employing the substrate cytidine 2′,3′-cyclic monophosphate, and is reached at a cofactor to enzyme ratio of five. In the absence of pyridoxal-P, the same levels of $NaBH_4$ cause no inactivation. Furthermore, pyridoxine phosphate at 20 times the molar amount of enzyme has no effect on catalytic activity. Thus the inactivation with $NaBH_4$ is almost certainly a result of the formation of a covalent bond between the phosphorylpyridoxyl moiety and the protein. Phosphate ions protect the enzyme from inactivation by this chemical modification, consistent with the ultraviolet spectra (Figure 1) which suggests that phosphate dissociates the complex. The phosphate moiety of the cofactor is clearly necessary for the modification of the enzyme, since a 35-fold excess of pyridoxal has no effect on RNase A activity.

*Extent of Irreversible Chemical Modification after $NaBH_4$ Reduction.* Pyridoxine phosphate does not bind tightly to ribonuclease and is readily separated from modified enzyme on Sephadex G-25. After $NaBH_4$ reduction of an equimolar mixture of the coenzyme and RNase A, only 15% of the starting pyridoxal-P is recovered as pyridoxine phosphate, indicating that 85% of the cofactor is bound irreversibly to the enzyme under these conditions. It was necessary to calculte the extent of modification by this indirect method because the extinction coefficient of the modified chromophoric enzyme was not known rigorously *a priori.*

*Phosphorylpyridoxyl Peptide Isolation over Dowex 50-X2.* The chromophoric peptides derived from the proteolytic digests of modified enzyme are purified initially by Dowex 50-X2 chromatography (Figure 7A). Ninhydrin-reactive material is separated effectively by this procedure, and two chromophoric peaks are observed which are sharp and symmetrical. The area ratio of peak I to peak II is approximately 3:2. Peak I elutes at pH 3.70, while peak II emerges at pH 4.05. Correction for the hydroxypyridine extinction coefficient which

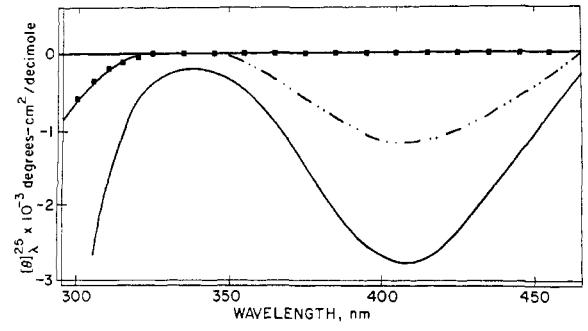


FIGURE 3: The circular dichroic spectra of the equimolar pyridoxal-P-RNase A complex ($3 \times 10^{-4}$ M) at pH 8.35, 0.05 M Bicine in absence (—) and presence of 0.05 M phosphate ions (—·—·—). The spectra in 6 M guanidine·HCl and performic acid oxidized RNase-pyridoxal-P (■—■) were essentially identical with the base line in the region of cofactor absorption.

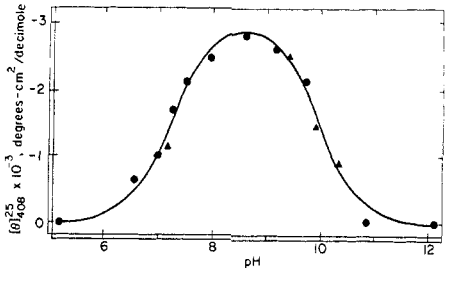


FIGURE 5: The optical activity of the equimolar RNase–pyridoxal-P complex ($3 \times 10^{-4}$ M) in 0.05 M sodium acetate, pH 5.0, titrated to pH 11 with 0.3 M NaOH (●). Concentrated base is introduced with micrometer syringe and does not markedly change the volume. Same curve obtained when complex back-titrated with 0.5 M HCl from pH 11 to 7 (▲). Theoretical curve (—) of best fit of data to eq 1 yields 11 out of 15 points within 5% of the experimental value.

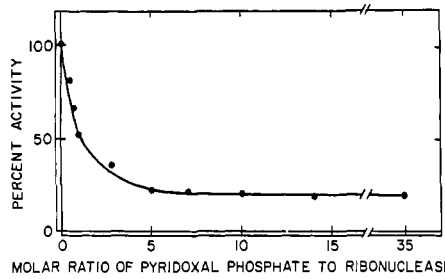


FIGURE 6: The inactivation of RNase A by covalently linking pyridoxal-P to the enzyme with $NaBH_4$. RNase ($10^{-4}$ M) and a variable molar ratio of pyridoxal-P (0- to 35-fold relative to enzyme), preincubated for 10 min at 25° in 0.05 M Mes, pH 6.0, is reduced with a 10-fold excess of $NaBH_4$ over enzyme. The modified enzyme solution is diluted 1:2 with water, and a 10-$\mu$l sample is assayed for hydrolysis of cytidine 2,'3'-cyclic monophosphate by addition to 1 ml of a $10^{-3}$ M substrate solution in 0.1 M sodium acetate, pH 5.0 (Herries *et al.*, 1962).

may vary as a function of pH (Peterson and Sober, 1954) is not necessary between pH 3.6 and 5 for these peptides.

Final purifications of both pyridoxyl peptides are achieved by separate rechromatography over identical Dowex 50-X2 columns (Figure 7B,C), after dephosphorylation of each peptide with alkaline phosphatase (Strausbauch and Fischer, 1970). Since removal of the phosphate alters the charge of the modified peptides significantly, they are retarded more strongly by the resin. All contaminants, however, are not affected in their elution properties. The sharp peak of absorption at 325 nm present very early in both rechromatographies (Figure 7B,C) is of unknown origin, but does not contain peptides. The recovery of chromophoric material from Dowex 50-X2 is 60%. All steps prior to Dowex chromatography give a quantitative recovery of 325-nm absorbing substance.

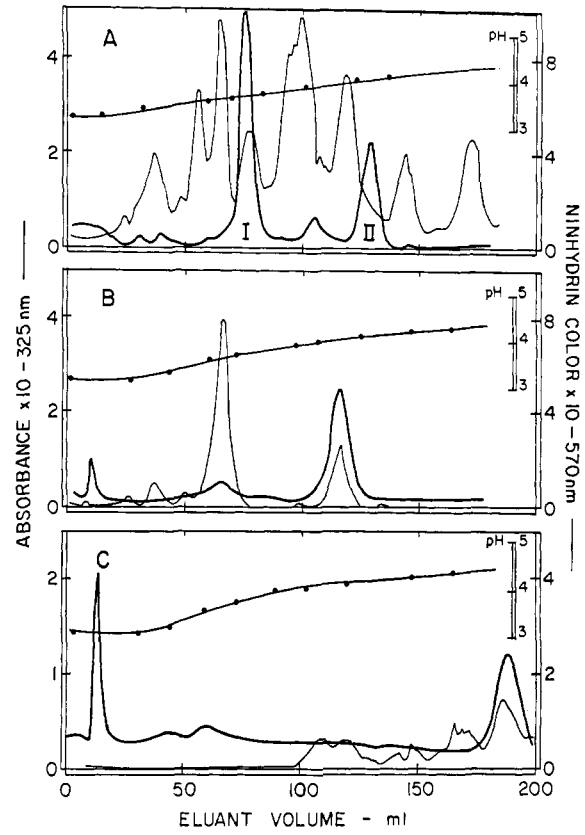


FIGURE 7: The purification of two chromophoric peptides obtained from tryptic-chymotryptic digest of the pyridoxal-P modified RNase. The peptides derived from 27.4 mg of modified RNase, were chromatographed on Dowex 50-X2 as described in the Experimental Section and measured by the ninhydrin color, the thin line. The two major optical density peaks at 325 nm indicated by the dark lines were pooled, treated with alkaline phosphatase, and rechromatographed. Figure 7B is the rechromatography of peptide I and 7C of peptide II.

TABLE I: The Amino Acid Composition of the Chromophoric Peptides Derived from Pyridoxal-P Modified RNase A.[a]

| Amino Acid | Peptide I[b] | Peptide II[b] | Peptide II[c] |
|---|---|---|---|
| Lys | 0.1 | 1.1 | 0.2 |
| PL-Lys[d] | 0.8 | 0.8 | 0.9 |
| Arg | 1.2 | | 0.1 |
| Asp | 2.0 | 0.1 | 0.1 |
| Thr | 1.0 | 1.0 | 1.0 |
| Glu | 0.1 | 1.1 | 1.0 |
| Pro | 1.1 | | |
| Ala | 0.1 | 3.0 | 3.0 |
| Val | 1.0 | | |
| Phe | 1.0 | 1.0 | 0.9 |
| Cysteic acid | 1.1 | | |

[a] Results are expressed as moles of amino acid per mole of peptide and are calculated on the basis of internal standards which are valine for peptide I and alanine for peptide II. [b] The composition of peptide I fits the sequence around Lys-41, which is Asp-Arg-Cys-$Lys_{41}$-Pro-Val-Asn-Thr-Phe. The composition of peptide II matches the sequence of the N-terminal region which is Lys-Glu-Thr-Ala-Ala-Ala-$Lys_7$-Phe. Other amino acids were not present at levels greater than 0.1 mole per mole of peptide. [c] Amino acid composition of peptide II after removal of the N-terminal amino acid. [d] PL-Lys refers to *N*-ε-pyridoxyllysine.

*The amino acid analysis of the two peptides* is presented in Table I. Proof that these chromophoric peptides actually contain *N*-ε-pyridoxyllysine was obtained by locating the elution position of an authentic sample of this compound on the short column of the Beckman amino acid analyzer. The standard was identical with a peak unique to the modified peptides, eluting 1.5 min before histidine on the Beckman short column. The modified peptides themselves contain no histidine.

The composition of peak I (Table I) identifies the modified residue as Lys-41 (Smyth *et al.*, 1963). Apparently in the Lys-41 modified protein the Arg-39–Cys-40 bond is not cleaved (Table I), whereas this bond is readily hydrolyzed in the native enzyme (Anfinsen *et al.*, 1959). Peptide II[2] is the N-terminal fragment of the protein, in which Lys-7 is conjugated with pyridoxal-P. Amino acid analysis subsequent to Edman degradation reveals that Lys-1 is unmodified, since pyridoxyllysine is not lost from the degraded peptide (Table I). The presence of phenylalanine in peptide II, likewise strongly suggests that Lys-7 is modified and that the usual tryptic cleavage does not occur. A chymotryptic split, how-

[2] This peptide contains two lysines since tryptic cleavage does not occur at N-terminal lysines.

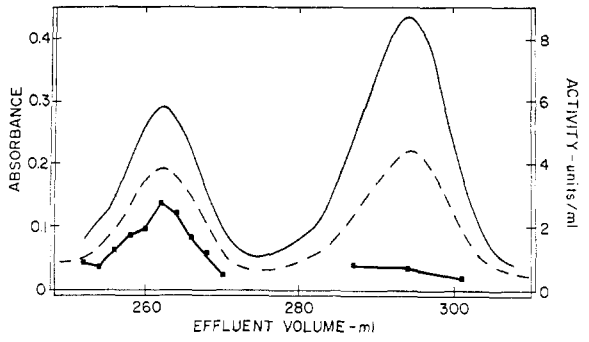


FIGURE 8: Separation of Lys-7 and Lys-41 phosphorylpyridoxyl-ribonucleases on CM-Sephadex. Chromophoric enzyme derivatives, containing the pyridoxyl moiety, were located by absorbance at 320 nm (- - -). The protein concentration determined by the method of Lowry *et al.* (1951) was proportional to the optical density at 280 nm (—). Units of enzymatic activity (expressed as μmoles of product per second) are calculated per ml of effluent (■—■). A small third peak, representing 5–10% of the material absorbing at 320 nm, eluted at an effluent volume of 200 ml.

ever, occurs after Phe-8, since Glu-9 and Arg-10 are not detected by amino acid analysis.

*Peptide mapping* of the unfractionated proteolytic digest of modified enzyme provides a rapid qualitative indication that only two phosphorylpyridoxyl peptides are present. The peptide corresponding to peak I (Figure 7A) migrates about 5 cm toward the anode under the conditions of electrophoresis employed, while peptide II migrates 2 cm toward the cathode. Both pyridoxyl peptides move with an $R_F$ of 0.3 during paper chromatography. In the final fingerprint (which was in close agreement with that observed by Anfinsen *et al.*, 1959) both peptides are resolved as new ninhydrin-positive spots, although peptide I is partially overlapped by a second arginine-containing peptide (probably peptide number 19 described by Anfinsen *et al.*, 1959). Tyrosine and histidine are not detected in either pyridoxyl peptide on paper, consistent with the amino acid analysis.

The product distribution of this chemical modification, as determined by peptide mapping, appears to be qualitatively independent of temperature, borohydride concentration, and pH. Thus if an equimolar complex is reduced between 0° and 40° or with a 1- to 50-fold excess of $NaBH_4$ at 25° (under conditions otherwise identical with those described under Methods), the same *N*-ε-phosphorylpyridoxyl peptides are obtained in similar relative proportions. The same fingerprint is also observed whether the complex is reduced in 0.05 M sodium acetate pH 5.0, in 0.05 M Mes pH 6.0, or in 0.05 M Bicine pH 8.25, although in the latter case a faint third fluorescent spot is present. If 0.1 M phosphate is included in the pH 6, 0.05 M Mes buffer, however, the modification of Lys-41 (peptide I) is blocked completely, and peptide II (Lys-7) is markedly diminished.

*Separation of the Lys-7 and Lys-41 Isomers on CM-Sephadex.* The two modified RNase A species, obtained by chromatography on CM-Sephadex with a shallow salt gradient (Figure 8), were analyzed by the peptide mapping procedure described above. The Lys-7 derivative, which emerges first, has about one-third the specific activity of the native enzyme, while the Lys-41 isomer is at least 95% inactivated. The residual activity associated with the Lys-41 derivative does not cochromatograph with the optical density at 280 nm (Figure 8) and might reflect the presence of low levels of an enzymatically active contaminant. The ratio of products obtained by chromatography on CM-Sephadex is in good agreement with that determined by Dowex chromatography of the peptides (Figure 7).

## Discussion

The interaction of pyridoxal-P with RNase A is highly active-center specific. The coenzyme reacts preferentially with Lys-41 and Lys-7, although each molecule of protein contains ten lysyl residues. The Lys-7 derivative has recently been identified in a tryptic digest of pyridoxal-P-modified RNase A (Means and Feeney, 1971) by isolation of a pyridoxyl peptide from a low molecular weight fraction obtained by chromatography on Sephadex G-10. In tryptic hydrolysates, however, Lys-41 is part of a much larger peptide than Lys-7. The results of the present study suggest Lys-41 to be modified to an even greater extent than Lys-7.

The structural factors which enhance the affinity of pyridoxal-P for the active center of RNase A may share some common features with the active centers of pyridoxal-P enzymes, since the active center lysines of RNase A can react with the cofactor under conditions which do not permit detectable aldimine formation with the model compound *N*-α-acetyl-DL-lysine (Figure 1).

It has been shown that a lowered amine $pK_a$ favors Schiff base formation between amino acids and 3-hydroxypyridine-4-carbaldehyde (French *et al.*, 1965; Auld and Bruice, 1967). A lowered $pK_a$ has also been postulated to account for the reactivity of Lys-41 of RNase A toward reagents such as dinitrofluorobenzene and 4-sulfonyloxy-2-nitrofluorobenzene (Murdock *et al.*, 1966; Carty and Hirs, 1968b) and may be of importance here. In addition, the proximity of these lysyl residues to the phosphate binding site of the enzyme may be critical in determining the specificity of aldimine formation, since phosphate ions compete with the coenzyme for binding to RNase A as judged by absorption and circular dichroic spectra (Figures 1 and 4). Furthermore, the cofactor analog pyridoxal, which lacks the phosphate group of the coenzyme, does not inactivate RNase A under these conditions. Regardless of which particular substituents on pyridoxal-P is most important in directing the specificity of this chemical modification, Lys-7 and Lys-41 are clearly poised in an environment which favors aldimine formation.

Since pyridoxal-P is a planar molecule, its absorption bands are not optically active unless it is bound stereospecifically by an asymmetry inducing center such as the active center of an enzyme (Fasella and Hammes, 1964; Ulmer and Vallee, 1965; Wilson and Meister, 1966). In the case of RNase A the bound aldimine is optically active at pH 8.5 (Figure 3). However, optical activity is not observed at pH 8.5 under conditions which disturb the native conformation of the enzyme, *i.e.*, if 6 M guanidine·HCl is present or if the disulfide bridges of the enzyme are oxidized with performic acid (Figure 3).

The molar ellipticity at 408 nm as a function of pH is a bell-shaped curve (Figure 5). A theoretical plot of ellipticity as a function of pH can be generated from the following simple model, which could serve to interpret the experimental data.

$$EPH_2 \overset{K_1}{\rightleftharpoons} EPH \overset{K_2}{\rightleftharpoons} EP \qquad (1)$$

The symbols "$EPH_2$," etc., represent the equimolar complex of cofactor and enzyme. The protons may belong either to pyridoxal-P or to the enzyme. Both $EPH_2$ and EP are optically

inactive,[3] but EPH has a finite molar ellipiticity ($3.15 \times 10^3$). The theoretical curve of "best fit" (Figure 5) was obtained when $pK_1$ and $pK_2$ equalled 7.22 and 9.91, respectively. The mean deviation of calculated and experimental values was 8.7%.

The $pK_a'$ of 9.91 is likely due to a lysyl or tyrosyl residue, or the chelate hydrogen bond of the aldimine. In this case the ribonuclease–coenzyme complex may actually dissociate since the absorption spectrum above 300 nm in the pH region near 11 is essentially identical with that of free pyridoxal-P (Peterson and Sober, 1954). Deprotonation of the aldimine nitrogen could also give rise to similar spectral changes, but is less likely since the absorption maximum of this species is generally below 380 nm (Metzler, 1957; Heinert and Martell, 1963b).

Although lowering of the pH to 6 also causes the circular dichroism band to disappear dependent on the dissociation of a group with a $pK_a'$ of 7.22 (Figure 5), the ultraviolet absorption spectra (Figure 2) in this case indicate that an aldimine complex is still present. Perhaps a subtle change in the mode of cofactor binding such as a slight change in the orientation of the chelate hydrogen bond of the aldimine might accompany this dissociation. Among the likely functional groups which may be reponsible for this ionization are His-119 or His-12, the pyridine nitrogen or the phosphate moiety of the cofactor.

A detailed structural analysis of these spectral phenomena in terms of the active-site environment should be feasible for the RNase A–pyridoxal-P complex. Recently nuclear magnetic resonance (nmr) and X-ray data have implicated His-119 and Lys-41 in the binding of the phosphate moiety of the inhibitors 2′- or 3′-cytidine monophosphate to RNase A (Meadows *et al.*, 1969). The histidyl $pK_a$ is shifted upwards by one or two pH units, as judged by the nmr spectrum of the $C_2$ proton, in direct proportion to the strength of inhibitor binding. Thus, the $pK_a'$ of His-119 is raised from 5.8 to 7.4 or over 8.0 for the 3′- and 2′-monophosphates, respectively. Since at least one binding mode of pyridoxal-P might similarly interact with this residue, the nmr spectrum of the RNase A–pyridoxal-P complex may also reveal a perturbation of the $pK_a'$ of His-119. This approach might allow the structural identification of the group with a $pK_a'$ of 7.2 associated with the appearance of the optical activity of the coenzyme chromophore.

An unusual feature in the mode of pyridoxal-P binding to RNase A is suggested by the circular dichroic titration shown in Figure 4, which displays a relatively sharp discontinuity at a 1:1 mole ratio of cofactor to enzyme. Although such data suggest that a single lysine of RNase A is capable of complexing the cofactor, peptide analysis shows that in fact, two lysines are modified. However, the interaction of cofactor with either lysine may exclude the interaction of the other. Indeed, chemical evidence strongly suggests a close steric promixity between Lys-7 and Lys-41, since these residues can be cross-linked with the bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene (Marfey *et al.*, 1965). Thus it is possible that aldimine formation at Lys-7 sterically hinders the same process at Lys-41 and *vice versa*. The phosphate moiety might also be noncovalently bonded to that amino group of the 7–41 pair not involved in the aldimine linkage or to His-12 and His-119 which are also part of the phosphate-binding region of the enzyme.

The characteristics of the individual modified RNase isomers are consistent with this hypothesis. The Lys-7 derivative retains considerable catalytic activity, while the Lys-41 isomer is largely inactive (Figure 8). Consequently the inability of pyridoxal-P to inactivate ribonuclease completely (Figure 6) suggests that modification of Lys-41 is not quantitative, no matter how great an excess of pyridoxal-P is employed, and supports the notion that the presence of a Schiff base with Lys-7 prevents Schiff base formation with Lys-41.

Specific chemical modifications of lysines with pyridoxal-P in enzymes not requiring the cofactor for catalysis suggests that these lysines are in a chemical environment, favorable to the binding of the coenzyme. Since most other enzymes that have been modified selectively with this cofactor thus far have organophosphates as natural substrates, the phosphate group is probably the major determinant in directing the specificity of the reaction, *e.g.*, aldolase (Shapiro *et al.*, 1968), phosphogluconate dehydrogenase (Rippa *et al.*, 1967), fructose 1,6-diphosphatase (Marcus and Hubert, 1968), and phosphoglucose isomerase (Schnackerz and Noltman, 1971). It is possible that many more enzymes will be found that interact specifically with pyridoxal-P, since organophosphates are extremely common in biological metabolism. In this regard it may be of interest to examine other coenzymes as chemical-modifying agents of active-center residues (Vallee and Riordan, 1969).

The reaction of pyridoxal-P with an "active" residue of an enzyme not requiring it may be an interesting model for enzymes which are dependent on it for activity. The accessibility of RNase–inhibitor complexes to nmr and X-ray crystallographic techniques should be a great asset in these endeavors. Such studies may also elucidate the basis for the chemically atypical nature of active-center lysines compared to similar lysines in catalytically inert regions of the protein (Vallee and Riordan, 1969).

## Acknowledgment

The authors are deeply indebted to Dr. Bert L. Vallee, who suggested pyridoxal-P as an active-site lysine reagent for ribonuclease.

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[3] The absorption spectrum in the pH region near 11 is more representative of unbound cofactor than of a bound complex. It is, therefore, possible for the EP to be optically active but present at very low concentrations because of dissociation of pyridoxal-P from the enzyme.

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# Models for Metalloenzymes. Zinc Ion Catalyzed Phosphorylation of 1,10-Phenanthroline-2-carbinol by Adenosine Triphosphate†

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ABSTRACT: Zinc ion catalyzes the phosphorylation of 1,10-phenanthroline-2-carbinol by ATP. The reaction is absolutely dependent on the metal ion and proceeds through the formation of a reactive ternary complex composed of 1,10-phenanthroline-2-carbinol, ATP, and zinc ion. In addition to serving as a template for the reaction of two dissimilar ligands, the metal ion serves the additional catalytic functions of enhancing the nucleophilicity of the carbinol group by lowering its $pK_a$ and of neutralizing the negative charge of the $\gamma$-phosphate to permit attack of an anionic nucleophile. The mechanistic function of the metal ion in this simple system corresponds to that postulated for the transition metal ion in terminal deoxynucleotidyl transferase from calf thymus.

Most enzymes which catalyze phosphoryl-transfer reactions require divalent metal ions (Mildvan, 1970). One approach to understanding the role of metal ions in these enzyme systems is to determine how metal ions can facilitate nonenzymic phosphoryl-transfer reactions.

An attractive hypothesis for the function of metal ions in pyrophosphate-bond cleavages stems from the highly reactive nature of unsymmetrical pyrophosphate diester dianions. For example, Miller (1969) has demonstrated that $P_1,P_1$-diethyl pyrophosphate dianion hydrolyzes with a half-life at 35° of 10 min most likely *via* a "metaphosphate" mechanism (Ia in eq 1). The suggested catalytic function of metal ions based on these studies would involve the metal ion binding to the penultimate, but not the terminal phosphate, and generating a species comparable in reactivity and structure by virtue of its neutralized charge on the penultimate phosphate (Ib in eq 1). Cooperman (1969) has made a direct

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‡ Supported by Public Health Service Training Grant GM 00364.