amino acid portions of the *P*-Pxy-Tyr analogs. Also, the aldehyde group of pyridoxal-*P* displayed a special property, markedly increasing the binding of coenzyme to these antibodies but not to antibodies against the coenzyme alone.

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The Antibody–Enzyme Analogy. Comparison of Enzymes and Antibodies Specific for Phosphopyridoxyltyrosine†

Vic Raso‡ and B. D. Stollar*

ABSTRACT: Reduced Schiff base compounds of pyridoxal-*P* and tyrosine, which were used to induce specific antibodies described in the preceding article (V. Raso and B. D. Stollar, *Biochemistry,* 1975), caused active site-directed inhibition of tyrosine transaminase and tyrosine decarboxylase. The antibodies, studied as analogs of enzymes, were able to bind an unsaturated Schiff base catalytic intermediate, as shown by equilibrium dialysis and absorbance difference spectroscopy. Schiff base formation can proceed while the pyridoxal-*P* and tyrosine are within the antibody combining site, but the rate of this bimolecular condensation within the sites was not greater than the rate in free solution. Antibody did effect a small rate enhancement for the pyridoxal-*P*-catalyzed transamination of L-tyrosine. These results are discussed in light of current ideas in the mechanisms of enzyme catalysis.

The requirement for a good fit between protein and ligand in the formation of an enzyme–substrate complex provides adequate explanation for the specificity displayed in enzyme-catalyzed reactions. The extraordinarily high reaction velocities produced by enzymes are more difficult to account for and the problem of describing the mechanisms of these rate accelerations has remained an enigma for biochemists.

Fundamental similarities between enzymes and antibodies are manifest at the levels of binding specificity, strength of interaction, and kinetics of formation and disruption of ligand–protein complexes. It therefore appeared that an immunochemical approach to this problem might help to elucidate some aspects of the nature of enzyme catalysis. Following such an approach, we have obtained antibodies with combining site specificities similar to those of pyridoxal-*P* enzymes that act on the substrate tyrosine. A previous article presented the design, synthesis, and chemical and structural characterization of two compounds which resemble the Schiff base intermediate of pyridoxal phosphate dependent enzymes that act on the substrate tyrosine (Raso and Stollar, 1973). The preceding work (Raso and Stollar, 1975) described methods for attachment of these analogs to protein carriers and insoluble cellulose supports to provide both antigens for antibody induction and affinity adsorbants for isolation of the antibodies. The purified anti-hapten antibodies were characterized and compared with respect to their ligand binding properties.

The present article describes the interaction of the haptens, as inhibitors, with two tyrosine-utilizing pyridoxal-*P*-

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dependent enzymes. In addition, antibodies to the stable *P*-Pxy-Tyr[1] hapten were examined for their ability to interact with a pyridoxal-*P*-tyrosine Schiff base, and the kinetics of the bimolecular condensation that yields the Schiff base were measured in the presence of antibody. In further experiments, the transamination of L-tyrosine catalyzed by pyridoxal-*P* free in solution was compared with the same reaction occurring in the presence of the antihapten antibodies.

Materials and Methods

Sources for many of the materials have been cited in the preceding article (Raso and Stollar, 1975). L-Tyrosine (1-^{14}C-labeled) was purchased from New England Nuclear and L-tyrosine (side chain 2,3-^{3}H-labeled) from Amersham Searle.

Tyrosine Decarboxylase (EC 4.1.1.25). Streptococcus faecalis, grown on a vitamin B_6-deficient medium, was the source of tyrosine apodecarboxylase (Gunsalus and Smith, 1957). The enzyme preparation and assay mixture were as described by Maruyama and Coursin (1968). The crude apoenzyme alone had only 2.5% of the activity it showed in the presence of added coenzyme. The order of addition of reagents for assay was: buffer, enzyme, inhibitor, pyridoxal-*P,* and a final delivery of L-tyrosine-*1*-^{14}C to start the reaction. A filter paper soaked with hydroxide of hyamine was inserted into the top portion of the test tube (Klein and Sagers, 1966), the tube was sealed with parafilm, and the reaction was stopped with acid, and $^{14}CO_2$ that had adsorbed to the filter paper was determined as described by Klein and Sagers (1966).

Tyrosine Aminotransferase (EC 2.6.1.5). Rat liver tyrosine aminotransferase was induced and purified through the ammonium sulfate fractionation step as described by Hayashi *et al.* (1967). The crude enzyme was resolved of coenzyme by dialysis against 0.1 M potassium phosphate–0.005 M tyrosine (pH 6) (Hayashi *et al.,* 1967) and the resulting preparation alone had only 10% of the activity seen upon the addition of excess coenzyme. Reaction conditions described by these authors as assay III were used for determination of activity. The order of addition of reagents was: buffer, α-ketoglutarate, tyrosine, inhibitor, enzyme, and pyridoxal-*P* to begin the 15-min incubation period.

3-N-Acetyl-3-aminotyrosine. Ten millimoles (1.96 g) of 3-amino-L-tyrosine and 30 mmol (4.1 g) of sodium acetate · $3H_2O$ were added to about 50 ml of water and the pH was adjusted to 4.8 with 2 N HCl; 10 mmol (1.92 g) of water-soluble carbodiimide was then added with continuous stirring, and the pH was maintained with 2 N HCl. After 20 min the material was loaded on to a 98 × 3.7 cm column of XE-64 ion exchange resin. Elution with water was monitored by the absorbance at 285 nm; one major peak (700 ml) was obtained. This material was flash evaporated to a volume of about 20 ml, and the resulting precipitate was redissolved by heating the mixture on a steam bath; when it cooled, crystals were obtained. The crystals were washed gently with cold water. Mother liquor and washings could be further concentrated to obtain more crystals. The compound was dried at reduced pressure in a desiccator with P_2O_5. The yields were 75% or greater. The acetylated derivative moved as a single species on chromatography (Raso and Stollar, 1973); it was unreactive with aromatic amine reagents but was readily detectable with ninhydrin.

Antibodies. The previous article (Raso and Stollar, 1975) described the induction and purification of the anti-pyridoxal-*P,* anti-*P*-Pxy-Tyr(NH_2), and anti-*P*-Pxd<Tyr(NH_2) antibodies. Pool I preparations in each case were obtained between 1 and 2 months after the initial immunization. Pool II antibodies to *P*-Pxy-Tyr(NH_2) were obtained after reinjection between 7 and 9 months after primary immunization. Late antibody was obtained from a single rabbit in this same group from several weekly bleedings after reimmunization at 13 months.

A portion (100 mg) of the purified pool II antibody was treated with maleic anhydride in the presence of free hapten according to the method of Freedman *et al.* (1968), except that the exposure to hydroxylamine was omitted. The extent of maleylation was determined spectrophotometrically (Freedman *et al.,* 1968).

Equilibrium Dialysis and Competitive Binding. Dialysis experiments were carried out with radiolabeled *P*-Pxy-Tyr(NHAc) as previously described (Raso and Stollar, 1975). Association constants were determined by Scatchard analysis (1949). Equilibrium dialysis was also used to measure binding of Schiff base by purified pool I antibody. Glass dialysis chambers (Karush, 1956) were used for these experiments. The antibodies or normal rabbit γ-globulin were at a concentration of 3 mg/ml. The Tyr(NHAc) was added to both sides of a dialysis chamber at a concentration of 2.5×10^{-2} M. Varying amounts of pyridoxal-*P* were delivered to the protein-free side to provide final Schiff base concentrations ranging from 1×10^{-5} to 15×10^{-5} M. A standard plot of absorbance at 420 nm *vs.* the concentration of pyridoxal-*P* added to 2.5×10^{-2} M Tyr(NHAc) gave a straight line that did not change significantly on incubation under the conditions of the dialysis experiment (18°, 18 hr). The concentration of unbound Schiff base on the protein-free side was measured by absorbance at 420 nm, determined with a Cary 14 spectrophotometer on a 0–0.1 expanded scale, or with a Gilford spectrophotometer.

Difference Spectra. All solutions were dissolved in P_i/NaCl and adjusted to pH 8. Absorbance cells with a 1-cm path length were used with a Cary Model 14 spectrophotometer. The reference solution contained 1×10^{-4} M pyridoxal-*P* and 2.5×10^{-2} M Tyr(NHAc). The experimental cell contained either normal γ-globulin or antibody at 7.5 mg/ml (1×10^{-4} M sites) in addition to the coenzyme and Tyr(NHAc).

Rate of Schiff Base Formation. Stopped-flow measurements were made in the laboratory of D. Robinson, with an apparatus designed for use with a Gilford spectrophotometer and equipped with a storage oscilloscope. Experiments were performed at room temperature and all reactants were dissolved in P_i/NaCl, and the pH was adjusted to 8.0. Stock solutions were: 2×10^{-4} M pyridoxal-*P;* 2×10^{-2} M Tyr(NHAc); 15 mg/ml of antibody in 2×10^{-2} M Tyr(NHAc); and 15 mg/ml of normal γ-globulin in 2×10^{-2} M Tyr(NHAc). Equal volumes of pyridoxal-*P* and one of the solutions containing amino acid were injected simultaneously into the observation chamber and the reaction was monitored at 430 nm. The purified antibodies used in these experiments were late antibodies isolated from the serum of a single hyperimmunized rabbit.

[1] Abbreviations used are: Tyr(NHAc), 3-*N*-acetyl-3-aminotyrosine; *P*-Pxy-Tyr(NH_2), *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine; *P*-Pxd<Tyr(NH_2), cyclic *N*-(5-phosphopyridoxylidene)-3′-amino-L-tyrosine; *P*-Pxy-Tyr(NHAc), *N*-(5-phosphopyridoxyl)-3′-*N*-acetyl-3′-amino-L-tyrosine; *P*-Pxy-Tyr, *N*-(5-phosphopyridoxyl)-L-tyrosine; P_i/NaCl, 0.15 M NaCl–0.005 M phosphate buffer (pH 8).

The effect of maleylated pool II antibody on the rate of Schiff base formation was measured without the stopped-flow apparatus. Pyridoxal-*P* (3×10^{-7} mol) and 3×10^{-7} mol of antibody sites were mixed in 0.5 ml of P_i/NaCl in a 1-cm path spectrophotometer cuvet. After 10 min L-tyrosine (3×10^{-6} mol in 0.5 ml of the same buffer) was injected into the cuvet in a Gilford spectrophotometer equipped with a recorder, and mixed rapidly. The progress of Schiff base formation was followed by comparing the increase of absorbance at 430 nm with a blank containing 3×10^{-4} M pyridoxal-*P* alone. Controls were run with maleylated γ-globulin from nonimmune rabbits or with no protein added. The L-tyrosine could be dissolved at 6×10^{-3} M in P_i/NaCl with heating, and remained in solution for some time after it was slowly cooled to room temperature.

Nonenzymatic Transamination Reaction. Reaction mixtures contained: 0.1 ml of 1.2 M potassium acetate buffer (pH 6.2); 0.25 ml of either purified pool I antibody (24 mg/ml), normal γ-globulin (26 mg/ml) or water; 0.04 ml of 5×10^{-2} M tyrosine in 0.16 N HCl, 0.01 ml of 8×10^{-2} M pyridoxal-*P* in H_2O at pH 7, and, last, ^{3}H-labeled L-tyrosine was added in 0.02 ml (approximately 10^7 cpm). A final pH of 5.5 was obtained with this mixture, which was incubated in sealed tubes at 37°. Prior to use, the radioactive L-tyrosine (side chain 2,3-^{3}H-labeled) was lyophilized, redissolved in an equal volume of distilled water, and lyophilized once again. This procedure prevented high background from ^{3}H that had exchanged into water. The radioactive material was usually dissolved in water just before delivery into the reaction mixtures. A control with buffer, tyrosine, and water, but lacking pyridoxal-*P*, was run simultaneously. Tritium release from this mixture was slow, about 1/5 of that seen in the presence of coenzyme; this nonspecific exchange was subtracted from the experimental values. A modified micro distillation technique was used to measure the amount of tritium released into water (Fellman and Roth, 1971). A sample of 0.02–0.05 ml was removed from the reaction mixture and was delivered into a Thunberg tube that contained enough water to make a total volume of 1 ml. The changes made in the Fellman–Roth procedure included: evacuation of the tube with a Duo Seal Vacuum pump; distillation at 40°; condensation in a trough containing a Dry Ice–ethanol mixture; and the use of a different scintillation mixture (Maruyama and Coursin, 1968).

Commercially available [^{3}H]tyrosine is labeled in positions 2 and 3 of the side chain, with tritium introduced by the hydrogenation of an unsaturated intermediate, and it was assumed that the label was equally distributed at these two positions. Thus one half of the total radioactivity added was taken to equal all of the α (2 position) hydrogen in the original reaction and to have a concentration of 5×10^{-3} M; on this basis the radioactivity labilized was related to the number of moles converted in the reaction.

Results and Discussion

Reduced Schiff Base Interactions with Enzymes. The spontaneous condensation of amino acids with pyridoxal-*P* results in the formation of a Schiff base, a covalent reaction intermediate which is crucial for catalysis by pyridoxal-*P*-dependent enzymes (Hammes and Fasella, 1963; Malakhova and Torchinskii, 1965; Fasella, 1967; Morino and Snell, 1966). Chemical reduction of the Schiff base provides a stable link between the coenzyme and amino acids; these reduced compounds are not substrates but are effective inhibitors of corresponding enzymes, indicating that they conform to and fill both the coenzyme and specific amino acid binding sites (Dempsey and Snell, 1963; Fellman and Roth, 1971; Khomutov *et al.*, 1971).

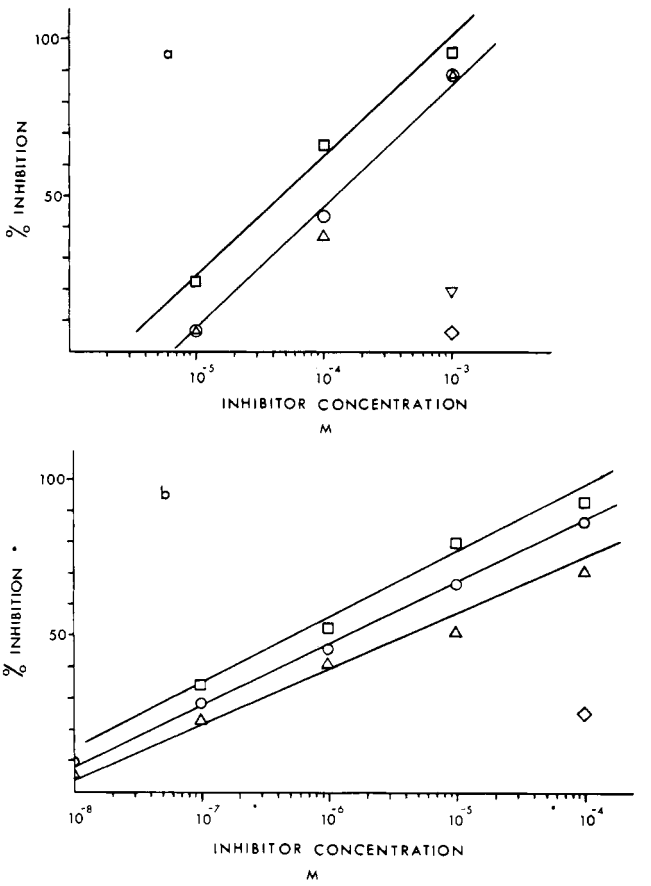

FIGURE 1: Inhibition of (a) tyrosine decarboxylase and (b) tyrosine transaminase apoenzymes. Inhibitors were: *P*-Pxy-Tyr (□); *P*-Pxy-Tyr(NH_2) (○); deoxypyridoxine-*P* (△); pyridoxamine-*P* (▽); and *P*-Pxd<Tyr(NH_2) (◇).

Tyrosine apodecarboxylase was one of two enzymes used to test the complementarity of our haptens with enzyme active sites. Inhibitors were tested at varying concentrations in the presence of a constant concentration of coenzyme (10^{-6} M) that was in excess of the amount needed for maximal activation of the apoenzyme. *P*-Pxy-Tyr was the most potent inhibitor, *P*-Pxy-Tyr(NH_2) was about 40% as efficient, and the cyclic *P*-Pxd<Tyr(NH_2) had only a slight effect even at 10^{-3} M (Figure 1a). The reduction of potency by the NH_2 substituent on the amino acid indicates that the tyrosyl portion of the inhibitor interacted with the enzyme in a specific way. When this region was conformationally distorted, as in the cyclic compounds, a drastic loss of effectiveness was noted. Pyridoxamine-*P* alone was a rather weak inhibitor, while deoxypyridoxine-*P* in which a hydrogen replaces the NH_2 function, was more efficient than pyridoxamine-*P* but still less effective than *P*-Pxy-Tyr or *P*-Pxy-Tyr(NH_2). Thus both coenzyme and tyrosine regions together were needed for most efficient inhibition.

Specificity of binding in the active site was further supported by competition studies. When the inhibitor was added, at a concentration of 10^{-4} M, to the apoenzyme before pyridoxal-*P* (10^{-6} M), 68% inhibition resulted; however, when apoenzyme and pyridoxal-*P* were mixed first, subsequent addition of *P*-Pxy-Tyr resulted in only 20% inhibition. When the inhibitor (10^{-4} M) was premixed with

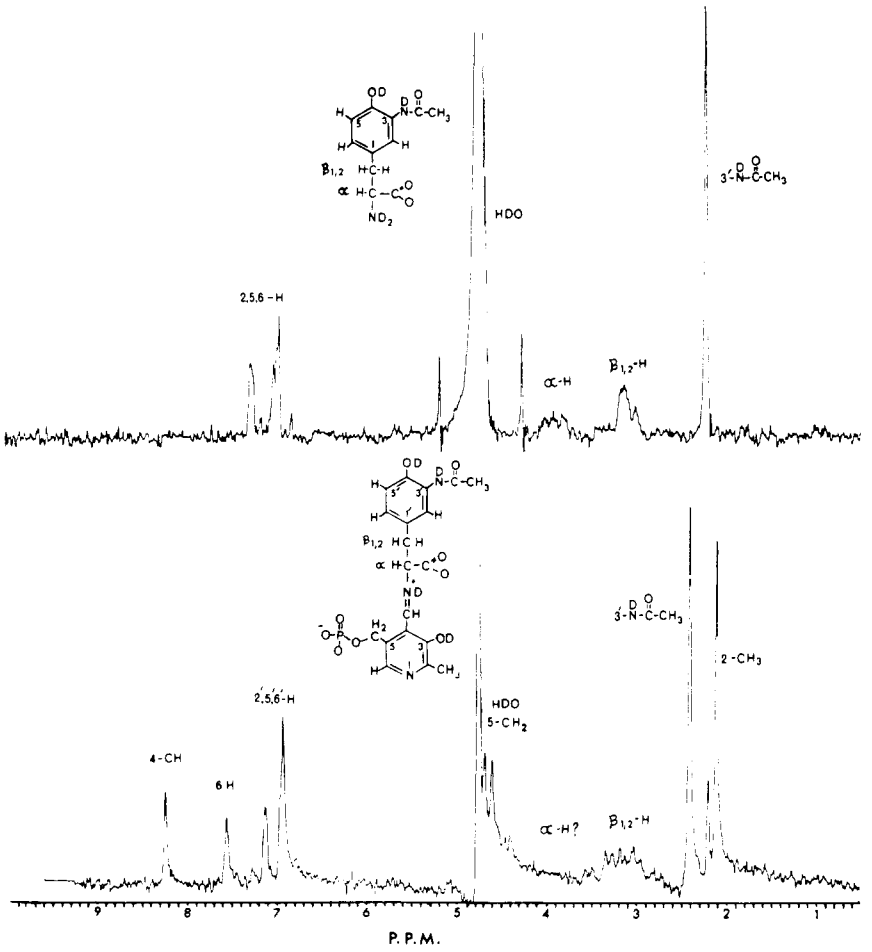

FIGURE 2: Nmr spectra of free Tyr(NHAc) (top) at approximately 0.05 M in D_2O pH 7.5–8.0 and a mixture of Tyr(NHAc) and pyridoxal-*P* (bottom), each at approximately 0.1 M in D_2O pH 7.5–8.0.

apoenzyme, a tenfold higher amount of coenzyme displaced it, as evidenced by complete recovery of decarboxylase activity. Thus the interaction of these inhibitors with tyrosine apodecarboxylase appeared to be competitive with respect to pyridoxal-*P*.

A similar pattern of inhibition was observed with rat liver tyrosine apotransaminase, for which the coenzyme concentration was kept in excess at 10^{-4} M (Figure 1b). Deoxypyridoxine-*P* showed 15% of the inhibitory strength of *P*-Pxy-Tyr again indicating the simultaneous interaction of the latter with both coenzyme and substrate binding regions of the enzyme. A correlation was noted between the potency of *P*-Pxy-Tyr and *P*-Pxy-Tyr(NH_2) as inhibitors and the relative effectiveness of tyrosine and 3-NH_2-tyrosine as substrates for the enzyme (Jacoby and La Du, 1964).

The mechanism of inhibition of tyrosine apotransaminase by these analogs appears to be complex, and depends on the order of addition of inhibitor and coenzyme. When 10^{-6} M *P*-Pxy-Tyr was added to the apoenzyme first, subsequent addition of pyridoxal-*P* up to a 1000-fold molar excess did not relieve the inhibition. When, however, 10^{-4} M pyridoxal-*P* was added first and the inhibitor delivered immediately afterward, no inhibition was detected. The ability of coenzyme to protect against inhibition again strengthens the conclusion that the analog was specific for the enzyme active site. Since the analog was not displaced by large amounts of coenzyme, it is possible that the interaction consisted of a reversible binding step followed by an irreversible covalent combination with enzyme (Khomutov *et al.*, 1969, 1971). Alternatively, the apparent tight binding of *P*-Pxy-Tyr may be explained in terms of transition state theory (Wolfenden, 1969; Lienhard, 1973). This analog contains the tetrahedral configuration of the carbinolamine transition state, an integral part of the Schiff base formation that must be catalyzed by tyrosine transaminase (Fasella, 1967).

Binding of the Schiff Base Intermediate. Antibody was induced against the phosphopyridoxyltyrosine haptens primarily because these molecules resemble the Schiff base intermediate. Thus it was important to determine whether the Schiff base itself as well as the reduced analogs could interact with these binding sites.

A problem in these studies arose from the reversible nature of Schiff base formation. When 10^{-4} M pyridoxal-*P* is mixed with 5×10^{-3} M tyrosine (the limit of its solubility) at room temperature in P_i/NaCl, only about 30% of the coenzyme exists in the Schiff base form once equilibrium is established. Acetylation of the aromatic amino group of 3-amino-L-tyrosine provided an analog which was soluble to the extent of 0.1 M at room temperature in P_i/NaCl. Since the acetylated analog closely resembled the hapten as it is attached to carrier protein (Raso and Stollar, 1975), the modification should not hinder its binding to antibody.

The mixture of pyridoxal-*P* and Tyr(NHAc) gave rise to a new λ_{max} at 420 nm, typical for Schiff base formation (Matsuo, 1957). Spectrophotometric titration at 420 nm showed that with pyridoxal-*P* at 1.06×10^{-4} M, the Schiff base intermediate was maximally formed with 10^{-2} M Tyr(NHAc), giving an extinction coefficient of 5400.

Table I: Intrinsic Average Association Constants for Ligand Binding to the Three Types of Pool I Antihapten Antibody ($\times 10^{-4}$).

	Anti-pyridoxal-*P*	Anti-*P*-Pxy-Tyr(NH_2)	Anti-*P*-Pxd<Tyr-(NH_2)
Pyridoxamine-*P*[b]	11.0	4.5	0.7
P-Pxy-Tyr(NH_2)[a]	20.0	45.0	4.0
P-Pxd<Tyr(NH_2)[b]	2.2	2.7	47.0
Schiff base[c]	5.6	9.0	5.2
Pyridoxal-*P*[b]	11.0	59.0	9.8

[a] Values obtained by equilibrium dialysis studies employing radio-labeled *P*-Pxy-Tyr(NHAc) (Raso and Stollar, 1975). [b] Constants calculated using the intrinsic association of *P*-Pxy-Tyr(NHAc) and the relative constants previously obtained by inhibition of precipitation studies (Raso and Stollar, 1975). [c] Values determined by equilibrium dialysis experiments as described in text.

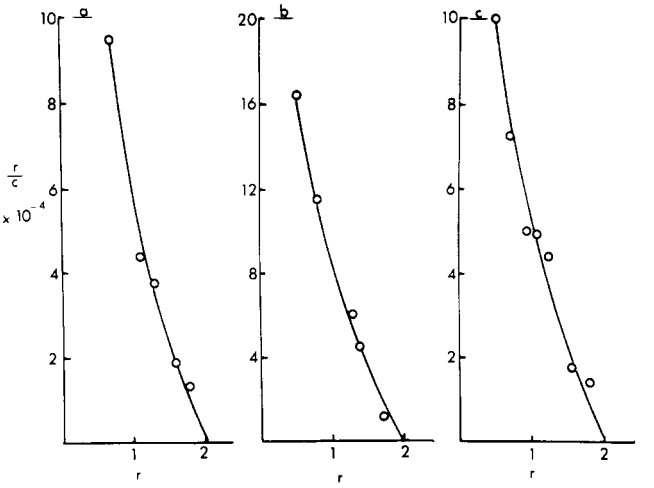

FIGURE 3: Scatchard plots of the binding data obtained from equilibrium dialysis experiments with the Schiff base of Pyridoxal-*P* and Tyr(NHAc) and antibodies to: (a) pyridoxal-*P*; (b) *P*-Pxy-Tyr(NH_2); and (c) <Tyr(NH_2).

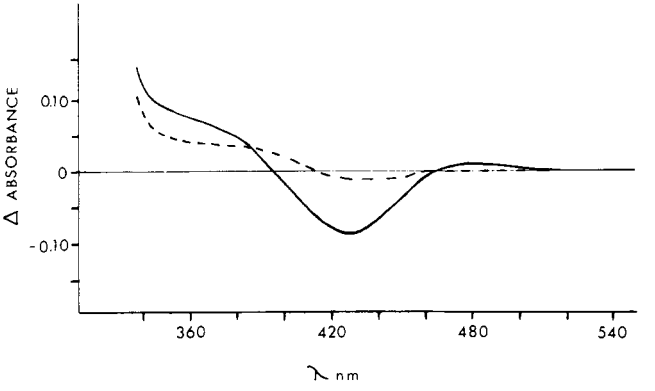

FIGURE 4: Difference spectra obtained with 1×10^{-4} M Schiff base in the reference cell and 1×10^{-4} M Schiff base plus either purified antibody to *P*-Pxy-Tyr(NH_2) (—) or normal γ-globulin (- - - - -) at 7.5 mg/ml (1×10^{-4} M antibody combining sites) in the second cell of a split beam spectrophotometer. All solutions were in P_i/NaCl.

Comparisons of nuclear magnetic resonance of a mixture of pyridoxal-*P* and Tyr(NHAc) and of free Tyr(NHAc) showed features which hallmark Schiff base formation (Figure 2), especially the downfield 4-CH hydrogen (Abbott and Martell, 1970). Interestingly, there appear to be some other subtle changes in Tyr(NHAc) absorbance when it combines with pyridoxal-*P* and its α hydrogen appears to have exchanged for a deuterium.

The equilibrium constant for Schiff base formation was determined from difference spectra of 10^{-4} M pyridoxal-*P* with varying concentrations of Tyr(NHAc) compared to 10^{-4} M coenzyme alone in a reference cell. A plot of these data according to the graphic method of Isenberg and Szent-Gyorgyi (1958) gave an apparent equilibrium constant, in P_i/NaCl, at 25° of 550 M^{-1}.

Scatchard plots of equilibrium dialysis data with Schiff base and purified pool I anti-pyridoxal-*P*, anti-*P*-Pxy-Tyr(NH_2), and anti-*P*-Pxd<Tyr(NH_2) antibodies (Figure 3) gave curves which extrapolated to a binding of 2 mol of ligand per antibody molecule, so that the Schiff base must be able to combine with all potential binding sites of each type of antibody. Anti-*P*-Pxy-Tyr(NH_2) antibody bound the intermediate with a K_a of 9×10^4, about two times higher than the K_a found with either of the other two types of antibodies (Table I). With the anti-pyridoxal-*P* antibodies, the Schiff base bound only half as tightly as the homologous hapten, pyridoxamine-*P*, and was distinct from the reduced *P*-Pxy-Tyr(NH_2) (Table I). While the saturated bond has an sp^4 tetrahedral configuration, the Schiff base is a planar sp^3 bond and the stereochemical consequences of this difference might adversely affect the position of the Schiff base within this site.

The anti-*P*-Pxy-Tyr(NH_2) antibodies showed a different picture, since the Schiff base bound more effectively than pyridoxamine-*P*. Thus the tyrosyl portion of the intermediate must have conformed to the site, though the Schiff base interaction with antibody was only one-fifth as strong as that of homologous reduced analog.

Antibody to the cyclic hapten interacted with the Schiff base with less than one-tenth the affinity that it showed for the homologous cyclic compound. This antibody saw little difference between the unsaturated intermediate and the reduced *P*-Pxy-Tyr(NH_2) molecule. Both of these were bound sevenfold more strongly than was pyridoxamine-*P*; perhaps both the Schiff base and its saturated analog can assume a quasicyclic configuration in the combining site of this antibody (Raso and Stollar, 1973).

Electronic interactions between the Schiff base and antibody binding sites were reflected in a substantial hypochromic effect in the 420–430-nm region (Figure 4). This occurred with each of three types of antibody, while no such effect was seen with normal γ-globulin (Figure 6). Since there was no concomitant increase in the absorbance at 388 nm, the hypochromicity in the 425-nm region could not be attributed to a simple antibody-dependent breakdown of Schiff base into free pyridoxal-*P* (λ_{max} 388 nm) and amino acid; it must have been due to electronic alterations of the phosphopyridoxyl chromophore portion of the Schiff base. Combination with antibody also effected a slight (5 nm) hypsochromic shift in the λ_{max} of the Schiff base absorbance.

Rate of Schiff Base Formation in the Presence and Absence of Anti-P-Pxy-Tyr(NH_2) Antibody. Decarboxylation, transamination, racemization, and other reactions catalyzed by pyridoxal-*P* enzymes involve many steps but one of the initial reactions that is accelerated is the condensation of coenzyme with amino acid to form a Schiff base (Cordes

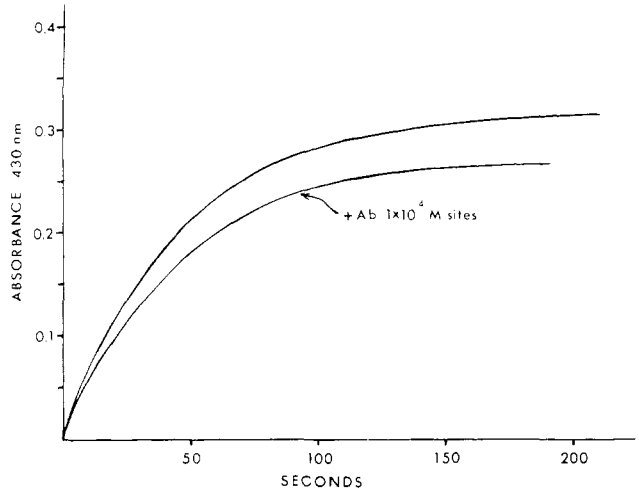

FIGURE 5: Schiff base condensation reaction of Tyr(NHAc) and pyridoxal-*P*. Curves were drawn from photographs of oscilloscope tracings that monitored absorbance at 430 nm. The upper curve shows the time course of reaction with no protein additions or with normal rabbit γ-globulin at 7.5 mg/ml. The lower curve represents the reaction in the presence of purified antibody to *P*-Pxy-Tyr(NH_2) at 7.5 mg/ml (1×10^{-4} M antibody combining sites). The initial absorbance of pyridoxal-*P* alone (about 0.2) was set as zero.

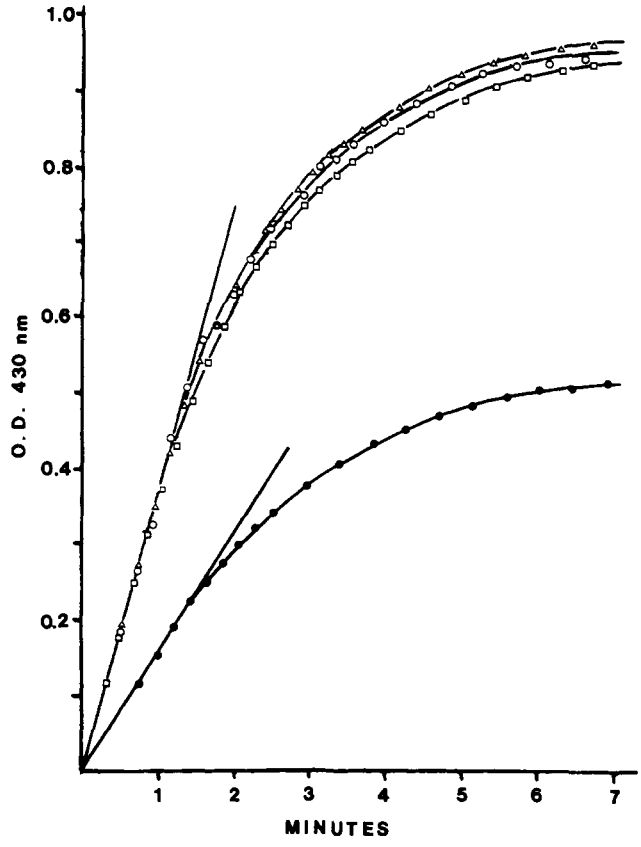

FIGURE 6: Schiff base formation with 3×10^{-3} M L-tyrosine and either 3×10^{-4} M pyridoxal-*P* (○) or 1.5×10^{-4} M pyridoxal-*P* (●) in Pi/NaCl (pH 8). The same reaction was carried out with 3×10^{-4} M pyridoxal-*P* in the presence of maleylated anti-*P*-Pxy-Tyr(NH_2) antibody, 22.5 mg/ml (3×10^{-4} sites) (□), or maleylated normal rabbit γ-globulin, 22.5 mg/ml (△).

and Jencks, 1962; Fasella and Hammes, 1967; Fasella, 1967). In solution the rate-limiting step for Schiff base formation is the attack of the amino group on the carbonyl group of the coenzyme (Auld and Bruice, 1967 a-c). When pyridoxal-*P* and tyrosine are mixed with antibody, the combining sites of antibodies to *P*-Pxy-Tyr(NH_2) would be expected to position the two reactants as they are arranged in the composite hapten molecule, so that the carbonyl region of the coenzyme is juxtaposed to the amino group of tyrosine. If these conditions are realized, the sites should be able to restrict the translational and rotational movements of the two molecules, orient their reactive groups, and prolong the lifetime of any activated complex that might form.

Purified antibodies from either a single hyperimmunized rabbit or from pool II sera were tested for their ability to enhance the rate of Schiff base formation. A stopped-flow apparatus was used to follow the reaction of pyridoxal-*P* (final concentration 10^{-4} M) with excess Tyr(NHAc) (final concentration 10^{-2} M). Antibody was introduced together with the Tyr(NHAc) so that the final antibody concentration was 7.5 mg/ml (10^{-4} M binding sites). Under these conditions, more than 60% of the pyridoxal-*P* is bound since the K_a for this ligand is 3.4×10^4 M^{-1} (Raso and Stollar, 1975). Assuming a K_a of 10^2 M^{-1} for the binding of Tyr(NHAc), it would fill 50% of the sites at the concentration used. Both in the absence and presence of purified late antibody, pseudo-first-order kinetics were followed, with a half-time of 32 sec in each case (Figure 5). Antibody did not affect the rate of Schiff base formation. The tracing of the reaction with antibody leveled off with a lower absorbance due to the aforementioned hypochromicity that occurs on combination of antibody and Schiff base.

The pseudo-first-order rate constant was 0.02 sec^{-1} under these conditions. Since the Tyr(NHAc) concentration was 10^{-2} M, the bimolecular rate constant, k_1, would be about 2 M^{-1} sec^{-1}; from the equilibrium constant of 550 M^{-1}, k_2 was calculated to be 0.0036 sec^{-1}.

It is known that the combination of antibody with hapten is a very fast reaction that occurs with a half-time in the range of milliseconds under the conditions of temperature jump experiments (Froese, 1968; Pecht *et al.*, 1972). The bimolecular rate constants for such combinations are typically 10^7 M^{-1} sec^{-1}. Thus the coenzyme and amino acid would be expected to have arranged themselves within the combining site well before the Schiff base condensation had proceeded to any extent. The most probable conclusion then is that the reaction did indeed occur at the antibody binding site but at a rate that was neither faster nor slower than the rate of the reaction occurring in an aqueous environment.

Schiff Base Formation in the Presence of Maleylated Antibody. To reduce the possible combination of pyridoxal-*P* with antibody lysyl groups that may have interfered with its combination with amino acid, a large excess of Tyr(NHAc) was used in the previous experiments, and pyridoxal-*P* was added to premixed antibody and Tyr(NHAc). To more directly eliminate the possible side reaction, to allow the use of lower relative concentrations of amino acid, and to allow the use of a preincubation of antibody with coenzyme, the antibody lysyl groups were blocked by maleylation; any lysine residues in the binding sites were protected by the presence of hapten during maleylation. The modified antibody bound *P*-Pxy-Tyr(NHAc) with a K_a of 5×10^5 M^{-1}, in comparison with a K_a of 17×10^5 M^{-1} for the untreated antibody; 82% of the potentially available sites could still interact with homologous hapten. From displacement experiments, a K_a of 1.9×10^4 M^{-1} was calculated for the binding of pyridoxal-*P*.

Pyridoxal-*P* and antibody were preincubated for 10 min, and L-tyrosine was then quickly added to make the final

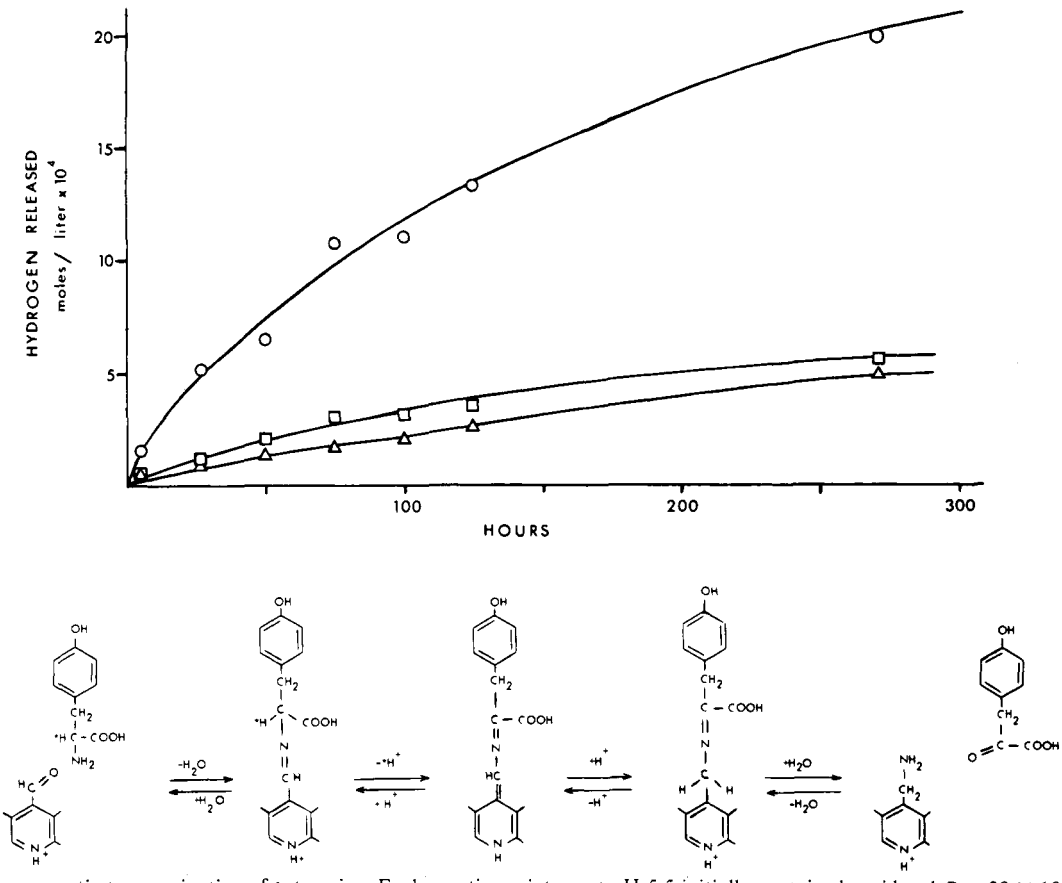

FIGURE 7: Nonenzymatic transamination of L-tyrosine. Each reaction mixture at pH 5.5 initially contained pyridoxal-*P* at 20 × 10^{-4} M and ^{3}H-labeled L-tyrosine at 50 × 10^{-4} M alone (Δ), with normal rabbit γ-globulin at 15 mg/ml (□) or with anti-*P*-Pxy-Tyr(NH_2) antibody at 15 mg/ml (2 × 10^{-4} M sites) (○).

concentration of coenzyme and sites 3 × 10^{-4} M, while the amino acid concentation was tenfold higher. The reaction between coenzyme and L-tyrosine proceeded at the same rate with either maleylated specific antibody or maleylated normal γ-globulin present during the condensation. Pseudo-first-order kinetics were followed in each case, and if the concentration of coenzyme were halved, the reaction rate with or without antibody was affected in direct proportion to this change (Figure 6).

From the K_a determined for the binding of pyridoxal-*P*, it was calculated that, at the concentrations used, ⅔ of the available sites would be combined with coenzyme, leaving only ⅓ of the pyridoxal-*P* (10^{-4} M) free in solution. The fact that coenzyme was bound in the expected amount was confirmed by equilibrium dialysis experiments with maleylated antibody. If only the unbound pyridoxal-*P* were able to condense with amino acid, there would have been a decreased rate of Schiff base formation (Figure 6); thus it must be concluded that the Schiff base formation did occur while the coenzyme was within the antibody site.

Nonenzymatic Transamination. Once Schiff base formation has occurred a number of rearrangement reactions must ensue before decarboxylation, transamination, or any one of the other possible pyridoxal-*P* mediated transformations is complete (Snell, 1962; Snell and Di Mari, 1970). Thus while the specific binding provided by the antibody combining sites did not affect the condensation of amino acid and coenzyme, there remained a possibility that this antibody would accelerate the rate of one or more of these intermediate steps. The transamination of tyrosine seemed like a good test of this hypothesis since the rate-limiting step in these reactions appears to be the tautomeric shifts which occur in the Schiff base (Banks *et al.*, 1961). Figure 7 shows the mechanism of transamination reactions catalyzed by pyridoxal-*P* as described by Snell (1958). When L-tyrosine, tritium labeled in the α position, is used in the reaction, the release of radioactivity into water can be used as a measure of the extent of transamination. The lowest curve in Figure 7 depicts the time course of hydrogen release from L-tyrosine (50 × 10^{-4} M) as catalyzed by 20 × 10^{-4} M pyridoxal-*P* at pH 5.5. Similar reaction mixtures containing either normal γ-globulin or pool I antibody to *P*-Pxy-Tyr(NH_2) at 15 mg/ml (2 × 10^{-4} M sites) were run simultaneously. There was only a slight difference between the rates of reaction of tyrosine with coenzyme alone or in the presence of normal γ-globulin, while the addition of purified antibody did provide a fivefold rate acceleration. Thin-layer chromatography of a sample taken from the reaction mixture after 100 hr showed the presence of a substantial amount of pyridoxamine-*P* and it was concluded that the reaction proceeded as shown in Figure 7. The antibody-containing mixture showed an elevated rate even after hydrogen release was tenfold higher than the number of sites present, indicating that the antibody acted in a catalytic way, displaying turnover. Condensation of the products, *p*-hydroxyphenylpyruvic acid and pyridoxamine-*P*, results in a Schiff base identical with that formed in the forward reaction. Thus it was expected that antibody should accelerate the reverse reaction and thereby continuously regenerate pyridoxal-*P*. The consequences of this dynamic equilibrium would be an eventual release of all of the tritium in the α position of the labeled tyrosine. At the end of this experi-

ment, the amount of hydrogen released was equal to the amount of coenzyme initially added and appeared to be still rising.

Conclusions

The antibody sites specific for the Schiff base analog used in these studies were designed not only to simulate the binding characteristics of pyridoxal-*P* enzymes but also to include some of the properties that are currently believed to mediate enzyme catalysis in general. The *N*-(5-phosphopyridoxyl)-L-tyrosine compound incorporates both the coenzyme and substrate portions into one molecule and in this respect resembles the monomolecular Schiff base intermediate. Because it is reduced, the carbon–nitrogen linkage is similar to the tetrahedral carbinolamine transition state that leads to Schiff base formation (Auld and Bruice, 1967a–c). Either of these features can explain the specific active site-directed enzyme inhibition caused by this analog.

The demonstration of Schiff base binding to the three types of antibodies extends the analogy between these combining sites and pyridoxal-*P* enzymes and, more importantly, sets the stage for assessing the effects of protein binding on chemical reactions. The fact that the extended *P*-Pxy-Tyr(NH_2) analog was a more effective enzyme inhibitor than was either pyridoxamine or the cyclic compound suggested that antibody to the extended form would be the most appropriate model for native enzyme sites. This antibody should be able to restrain the movements of both coenzyme and tyrosine and bring them into close proximity, with their reactive groups in a proper orientation for condensation. This antibody site may also be seen in terms of strain or distortion models that suggest that enzymes can produce rate accelerations by forcing substrate into a configuration that approaches that of the transition state for the reaction (Jencks, 1966). In this respect, antibody to *P*-Pxy-Tyr(NH_2) showed tightest binding for the saturated analog, which posseses sp^4 tetrahedral stereochemistry and thus resembles the carbinolamine transition state through which Schiff base formation proceeds.

Schiff base condensation was shown to occur while the reactants were protein bound and residing within the antibody combining site. The fact that this specific binding to antibody had no effect on the rate of Schiff base formation is contrary to what would be expected from approximation, orientation, and entropy trap models (Bruice and Pandit, 1960; Storm and Koshland, 1970; Page and Jencks, 1971; Reuben, 1971). In addition the potential strain effects were not realized in the form of catalysis. If the antibody model is a valid one, it implies that alternative factors are indispensable for catalysis.

The binding of tyrosine by the antibody is apparently not very strong, and this might account for the failure to observe any antibody-mediated rate increase in the bimolecular reaction of Schiff base formation. Interestingly, however, pyridoxal-*P* enzymes usually display weak binding for their amino acid substrate as well (Hayashi *et al.*, 1967).

It is clear that in nonenzymatic transamination Schiff base formation is not the rate-limiting step, as it occurs much more rapidly than the overall transamination. Thus the effect of antibody on the rate of transamination reflected modification of a later stage of the reaction. While the extent of rate acceleration was clearly not in the realm of that provided by enzymes, the mechanism may be analogous to an enzyme mechanism and of theoretical interest.

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Multiple Forms of Phosphodeoxyribomutase from *Escherichia coli.* Physical and Chemical Characterization†

Johan Christian Leer‡ and Karin Hammer-Jespersen*

ABSTRACT: Phosphodeoxyribomutase from *Escherichia coli* has been purified to homogeneity. Chromatography on DEAE-Sephadex revealed three peaks of enzyme activity, designated form I, form II, and form III. Form III could be further separated into form III-1 and form III-2 by polyacrylamide gel electrophoresis. The four different molecular forms of the enzyme thus isolated were shown not to be products of the column chromatography *per se.* The amino acid composition as well as the N-terminal amino acid were found to be identical for the different forms. Molecular weight determinations demonstrated that all four forms of the enzyme consist of a single polypeptide chain with a molecular weight of 45,000 ± 1000. Measurements of partial specific volumes, sedimentation coefficients, and absorption coefficients for form I and form II did not reveal any differences. It is concluded that the multiple forms of phosphodeoxyribomutase are caused by modifications of the polypeptide chain. Evidence is presented that form II is formed *in vitro* from form I by deamidation. It is probable that this deamidation occurs *in vivo* also. The different forms displayed only minor changes with respect to K_M for substrate and cofactor. Greater differences seem to exist among the four enzyme forms with respect to V_M and to cobalt binding.

Phosphodeoxyribomutase catalyzes the reversible transfer of a phosphate group between the C_1 and the C_5 carbon atoms of ribose and deoxyribose, respectively. This reaction has been demonstrated to occur both in bacteria and in mammal tissues and has in most cases been shown to be catalyzed by an enzyme different from the phosphoglucomutase. For further references see Kammen and Koo (1969), Hoffee and Robertson (1969), and Hammer-Jespersen and Munch-Petersen (1970).

The partial purification and some properties of phosphodeoxyribomutase from *E. coli* were reported earlier (Hammer-Jespersen and Munch-Petersen, 1970). This paper also reported the finding of three separate peaks of activity after hydroxylapatite chromatography.

The purpose of the present study was to investigate the polymorphism of the enzyme and to characterize the multiple forms of phosphodeoxyribomutase according to the subdivision of enzymes recommended by the IUPAC-IUB Commission on Biochemical Nomenclature ((1971), *Biochemistry 10,* 4825).

By use of genetic arguments some of the reasons for enzyme multiplicity could be excluded beforehand: mutants impaired in phosphodeoxyribomutase activity do arise with the same frequency as other mutations in single genes of *E. coli, e.g.,* mutations in *dra, tpp,* and *pup*[1] (K. Hammer-Jespersen, unpublished results). Thus the cause of the multiple forms of phosphodeoxyribomutase does not seem to be the existence of genetic independent proteins with phosphodeoxyribomutase activity.

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[1] Abbreviations used are: Dns dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; BAWP, 1-butanol–water–pyridine–glacial acetic acid (15:12:10:3); SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; dRib-P, deoxyribose 1-phosphate; Rib-P_2, ribose 1,5-diphosphate; TS 6.9 and TS 7.4, buffers containing 50 mM Tris, 50 mM succinic acid, and 1 mM EDTA, which are adjusted with solid sodium hydroxide to pH 6.9 or 7.4, respectively; MG-buffer, a 8.9 mM phosphate buffer adjusted to ionic strength 0.1 M with NaCl (pH 7.0) (Miller and Golder, 1950) (EDTA was added to a final concentration of 1 mM.); *dra, tpp, pup,* the genes coding for deoxyriboaldolase, thymidine phosphorylase, and purine nucleoside phosphorylase. Enzymes: Purine (deoxy)ribonucleoside phosphorylase or purine-nucleoside:orthophosphate (deoxy)ribosyltransferase (EC 2.4.2.1); thymidine phosphorylase or thymidine:orthophosphate deoxyribosyltransferase (EC 2.4.2.4); deoxyriboaldolase or 2-deoxy-D-ribose-5-phosphate acetaldehyde-lyase (EC 4.1.2.4); phosphoglucomutase or α-D-glucose-1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase (EC 2.7.5.1); phosphodeoxyribomutase or α-D-glucose-1,6-bisphosphate:deoxy-D-ribose-1-phosphate phosphotransferase (EC 2.7.5.6).