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The Antibody–Enzyme Analogy. Characterization of Antibodies to Phosphopyridoxyltyrosine Derivatives†

Vic Raso‡ and B. D. Stollar*

ABSTRACT: Stable analogs of the crucial Schiff base intermediate of enzymatic and nonenzymatic pyridoxal phosphate catalysis have been used as haptens for induction of specific antibodies. *N*-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine and its conformationally distinct cyclized derivative resemble the Schiff base formed upon mixing tyrosine with pyridoxal phosphate. These compounds were covalently coupled to a protein carrier *via* the 3′-amino group so as to confer a prescribed orientation, with the coenzyme region farthest removed from the carrier. A third antigen, with the phosphopyridoxyl group alone as the hapten, was prepared by linkage of pyridoxal phosphate directly to free amino groups on the carrier protein. Antibodies elicited for each determinant were purified by means of appropriate affinity columns. Antibody heterogeneity was observed in that different species could be separated from a given serum by sequential elution from the affinity columns with 1 M sodium phosphate buffers of pH 7.6, 5.2, 2.6, and 1.5. In assays of quantitative precipitation, inhibition of precipitation, equilibrium dialysis, and fluorescence quenching, antibodies to the phosphopyridoxyltyrosine haptens showed specificity for the phosphorylated form of the coenzyme and binding activity for both the coenzyme and tyrosine portions of the hapten. Antibodies to the phosphopyridoxyl groups alone did not display a similar reactivity toward the tyrosine portion of the complex haptens. The cyclic and noncyclic conformations of the hapten were serologically distinct, as antibody to each reacted preferentially with the homologous form.

The relationship between enzyme and antibody proteins has long been of formal interest, since both types of proteins show similar kinds of specificity in binding small molecules–substrates, coenzymes, or haptens. While no catalytic activity has been associated with the binding of haptens by antibodies, it remained possible that a suitably designed antibody, with simultaneous specificity for a pair of substances which undergo slow spontaneous reactions, might demonstrate catalysis by virtue of binding the reactants in close proximity and suitable orientation on the protein surface. Such an antibody would be of value as a model in studying factors that contribute to the catalytic activity of enzymes.

The nature of the combining region of an antibody is prescribed to fit the chemical and steric features of the hapten determinant of an antigen. To elicit antibodies with enzyme-like binding specificity, we synthesized *P*-Pxy-Tyr(NH_2)[1] and its cyclized derivative, which are stable analogs of coenzyme–substrate Schiff base complexes, and we immunized rabbits with protein conjugates of these haptens.

Experiments described in this article demonstrate that the combining sites of the resulting antibodies did encompass both the coenzyme and the tyrosine portions of the hapten. Further experiments, described in the following article, showed that these analogs, which fit the antibody sites as haptens, also inhibit the enzymes tyrosine decarboxylase and tyrosine transaminase, that the condensation reaction between pyridoxal-*P* and tyrosine can proceed at the antibody combining site, that the resulting Schiff base is reversibly bound, but that the antibodies did not greatly accelerate Schiff base formation or reactions which proceed *via* this intermediate. Another antigen was also constructed by attaching the pyridoxal-*P* directly to a carrier protein; the antibodies induced by this conjugate did not have a similar specificity for tyrosine.

Materials and Methods

The synthesis and characterization of *P*-Pxy-Tyr(NH_2) and the cyclic *P*-Pxd<Tyr(NH_2) were previously described (Raso and Stollar, 1973). Deoxypyridoxine-*P* was synthesized and purified by the method of Peterson and Sober (1954). Pyridoxal-*P* and pyridoxamine-*P* were purchased from Sigma Chemical Company; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride from the Ott Chemical Company (Muskegon, Mich.); carboxymethylcellulose from H. Reeve Angel, Inc.; aminoethylcellulose from Nutritional Biochemical Company; Amberlite XE-64 from Rohm and Haas Company; complete Freund's adjuvant

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[1] Abbreviations used are: *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_i/NaCl, 0.15 M NaCl–0.005 M phosphate buffer (pH 8).

from Difco, and Sephadex from Pharmacia. Pooled normal rabbit serum was obtained from Pel Freez. Amicon ultrafiltration membranes (PM-10 or PM-30) were employed for concentration steps. Tritium-labeled sodium acetate, 0.5 Ci/mmol, was a product of Schwarz/Mann. Ultraviolet absorption spectra were recorded with a Cary Model 14 spectrophotometer and fluorometry was performed with an Amino-Bowman spectrofluorometer and recorder.

Preparation of Normal Rabbit γ-Globulin. γ-Globulin was isolated from a 250-ml pool of normal rabbit serum by repeated ammonium sulfate fractionation (Stelos, 1967) and Sephadex G-200 gel filtration. An E_{280}(1%) of 14 was assumed for the use of this γ-globulin as a standard for colorimetric protein assay.

Phosphopyridoxyl-γ-globulin. The conjugation of pyridoxal-*P* to γ-globulin was similar to that described by Cordoba *et al.* (1966); 1 mmol (250 mg) of pyridoxal-*P* was mixed with 600 mg of γ-globulin in 40 ml of 0.15 M NaCl–0.05 M $NaHCO_3$, adjusted to pH 8.5, and kept in the dark for 1 hr. For reduction, 5 mmol (190 mg) of $NaBH_4$ was added. The colorless product was dialyzed against the saline–bicarbonate buffer. Its protein content was determined by three different methods (Gornall *et al.*, 1949; Lowry *et al.*, 1951; Itzhaki and Gill, 1964). The degree of substitution was determined by spectrophotometry and by phosphorus analysis of the digested conjugate (Bartlett, 1959).

Maleylated Rabbit γ-Globulin. The maleylated γ-globulin was prepared as described by Freedman *et al.* (1968a,b) but on a 500-fold greater scale. Treatment with hydroxylamine was omitted, and the amount of maleyl substitution was determined spectrophotometrically.

Protein Conjugates of Phosphopyridoxylaminotyrosine. The *P*-Pxy-Tyr(NH_2) and its cyclic analog were conjugated to maleylated γ-globulin in identical reactions. Finely powdered hapten (170 mg, 0.4 mmol) was added to 300 mg of protein in 10 ml of water and stirred until the solid was well suspended and the pH was steady at about 5. Solid carbodiimide (80 mg, 0.4 mmol) was added; the pH dropped to 4.7 and most of the hapten was solubilized. After 1 hr, the substituted protein was separated on a Sephadex G-200 column (4.2 × 92 cm) developed with P_i/NaCl. The degree of substitution, as determined from nitrogen (Lang, 1958) and phosphorus (Bartlett, 1959) analyses of water-dialyzed samples, was 50 mol of hapten/mol of protein for *P*-Pxy-Tyr(NH_2) and 80 mol/mol for *P*-Pxd<Tyr(NH_2).

Phosphopyridoxyl-Aminoethylcellulose. Two grams of AE-cellulose (0.85 mequiv/g) was washed with 0.5 N HCl, water, 0.5 N NaOH, and water, and suspended in 30 ml of 0.15 M NaCl–0.05 M $NaHCO_3$ (pH 8.8). Pyridoxal-*P* (3.5 g) was added, the pH was adjusted to 9 with 4 M NaOH, and the mixture was stirred in the dark for 3 hr. The mixture was then cooled on an ice bath, and 3 g of $NaBH_4$ was added. After an hour, the substituted cellulose was washed with 0.2 N NaOH, water, 0.2 N HCl, and water, and stored moist at 4°, shielded from light. A dried sample contained 0.8 mmol of phosphorous/g of cellulose; thus 95% of available amino groups had been substituted. The substituted resin reacted with Gibbs reagent to yield a blue color typical for the pyridoxyl group (Rodwell *et al.*, 1958).

Phosphopyridoxyltyrosine-Carboxymethylcellulose. Ten grams of CM-cellulose (0.6 mequiv/g) was washed with 0.5 N NaOH, water, 0.5 N HCl, and water, and suspended in water in a total volume of 150 ml. Then 1.28 g (3 mmol) of the *P*-Pxy-Tyr(NH_2) or *P*-Pxd<Tyr(NH_2) was added and the pH adjusted to 4.8 with 1 N NaOH. Carbodiimide (6 mmol, 1.15 g) was added and the pH was kept at 4.8 by dropwise addition of 2 N HCl. After 20 min the substituted resin was washed with water, 2% Na_2CO_3, water, 0.2 N HCl, and water, and stored moist at 4° shielded from light. A dried sample contained 0.12 mmol of P/g of cellulose in the case of *P*-Pxy-Tyr(NH_2) and 0.16 mmol of P/g in the case of the cyclic derivative; thus about 25% of the carboxyl groups of the resin were substituted. The modified cellulose gave a blue color with Gibbs reagent.

Acetylation of the 3′-Amino Group of the Phosphopyridoxylaminotyrosine Haptens. A mixture of 0.3 mmol of either amine and 0.9 mmol of sodium acetate in 4 ml of water was adjusted to pH 4.8 with 2 N HCl. Then 0.6 mmol of carbodiimide was added, the pH was maintained with acid, and after 15 min the mixture was applied to an Amberlite XE-64 column (2.5 × 67 cm). A major 325-nm absorbing peak was eluted with water, pooled, and flash evaporated. In each case the product moved as a single spot on thin-layer chromatography and gave appropriate chemical reactions for the acetylated compound (Raso and Stollar, 1973). *P*-Pxy-Tyr(NH_2) was also acetylated with ^{3}H-labeled sodium acetate in an identical but scaled down procedure. The product *P*-Pxy-Tyr(NHAc) had a specific activity of 19 Ci/mol, based on an ϵ_{325} 6500 at pH 7. On thin-layer chromatography the radioactive product moved as a single spot coincident with the blue fluorescence of the compound.

Immunization. Each of three separate groups of New Zealand White rabbits (6–10 per group) was immunized with the γ-globulin conjugate of pyridoxal-*P*, *P*-Pxy-Tyr(NH_2), or *P*-Pxd < Tyr(NH_2). Each rabbit received three weekly doses of 1 mg of antigen, emulsified with complete Freund's adjuvant, subcutaneously and intradermally, and an intravenous booster dose of 1 mg in buffered saline on the 4th week. Serum was obtained 7 days later and at weekly intervals afterward, with occasional intravenous boosters. Each initial pool (pool I) was from the first four bleedings of one group of animals. Serum was also collected from a single animal 13 months after the primary immunization (late antibodies).

Affinity Chromatography. Antibodies from each group of rabbits were purified separately with the appropriate affinity absorbant. A crude γ-globulin fraction was precipitated with 50% saturated ammonium sulfate, redissolved, and dialyzed against P_i/NaCl, giving a final volume of about one-half of the original serum sample. The concentrated globulin solution was clarified by centrifugation before chromatography.

The substituted cellulose was filtered to dryness, suspended in P_i/NaCl, and titrated to pH 8 with NaOH, packed into a glass column, and washed with more buffer. The globulin solution (30–50 ml) was allowed to pass through the column over a 3-hr period and was followed by P_i/NaCl. After the peak of unadsorbed protein emerged, the column was washed more rapidly with buffer until the Abs_{280} of the eluate was less than 0.05. Antibody was eluted in two steps with 1 M phosphate buffers (pH 2.6 and 1.5). Antibody-containing fractions (100–200 ml total volume) were pooled and adjusted to pH 5 with saturated Na_2HPO_4. The solutions were concentrated by ultrafiltration and dialyzed against three 4-l. portions of P_i/NaCl. A small amount of insoluble material was removed by centrifugation.

Serological Reactions. Quantitative precipitin tests were performed with 0.2–0.25 ml of serum or about 200 μg of purified antibody and varying amounts of antigen, brought

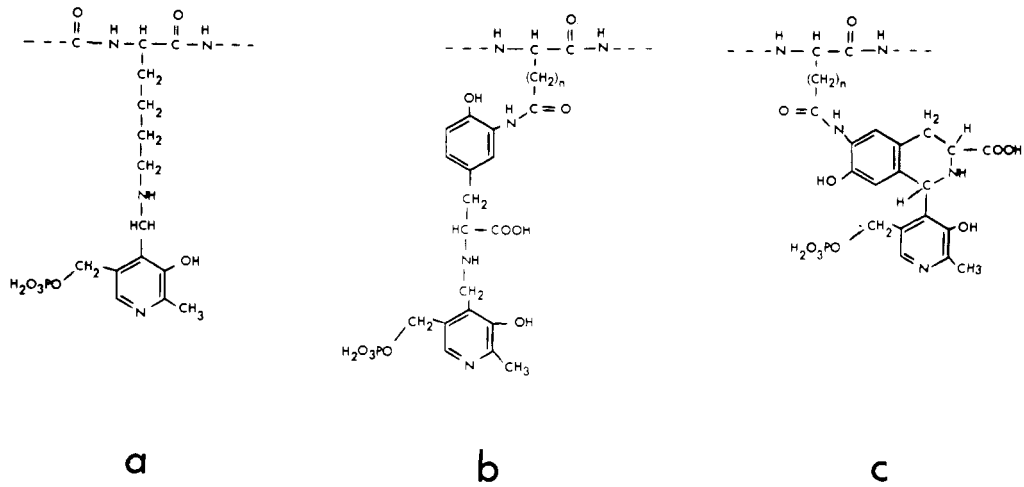

FIGURE 1: Structures of the hapten–protein conjugates used for immunization: (a) pyridoxal-*P* linked to lysine of γ-globulin; (b) *P*-Pxy-Tyr(NH_2) linked to maleylated γ-globulin; (c) *P*-Pxd<Tyr(NH_2) linked to maleylated γ-globulin.

to a final volume of 1 ml with P_i/NaCl. The reaction mixtures were incubated at 37° for 2 hr. Vigorous vortex mixing was then used to suspend the precipitate and the turbidity was immediately evaluated by optical absorbance at 500 nm, in comparison with readings for solutions of antibody or antigen alone. Analogous curves were obtained by determination of absolute antibody and antigen composition of the precipitate as described by Rubenstein and Little (1970).

For hapten inhibition experiments, amounts of antigen and antibody giving optimal precipitation were used: 516 μg of purified anti-pyridoxal-*P* antibody with 40 μg of homologous antigen, 214 μg of anti-*P*-Pxy-Tyr(NH_2) antibody with 80 μg of homologous antigen, and 200 μg of purified anti-*P*Pxd < Tyr(NH_2) antibody with 20 μg of homologous antigen. Inhibitors, buffered at pH 8, were preincubated with the antibody for 15 min at 37° before antigen was added. The complete reaction mixtures were then incubated and turbidity was determined as described above. If the higher concentrations of inhibitor had significant absorbance at 500 nm, the total absorbance was determined before and after centrifugation of the mixture, and the turbidity value was taken as the difference between the two readings.

Equilibrium dialysis experiments were carried out with plastic chambers having a 0.2-ml capacity on each side, as described by Karush (1956). Circular discs were cut from cellulose dialysis tubing and were boiled first in 0.01 M EDTA and then several times in distilled water. The sampling ports of each chamber were sealed with nylon screws since direct contact of dialysis samples with metal interfered with binding. Tritium-labeled *P*-Pxy-Tyr(NHAc) (19 Ci/mol) was used as the hapten for all three antibody preparations.

Purified anti-pyridoxal-*P* antibody was used at 240 μg/ml or 630 μg/ml, anti-*P*-Pxy-Tyr(NH_2) antibody at 400 μg/ml, and anti-*P*-Pxd<Tyr(NH_2) antibody at 320, 1200, and 2400 μg/ml. All solutions were in P_i/NaCl at pH 8 and equilibrium was established after 18 hr at 25°. A 0.1-ml sample was removed from each side of the membrane for determination of the radioactivity. Controls with hapten and normal rabbit γ-globulin or with hapten and buffer alone showed that nonspecific binding to protein and to the dialysis membrane did not occur.

For competitive binding studies, concentrations were such that 90% of the total available antibody sites would bind the radiolabeled hapten alone in a control chamber; increasing concentrations of unlabeled inhibitor were added to identical chambers and binding of the radiolabeled hapten was determined. For each analog, the per cent displacement of radioactive hapten as a function of inhibitor concentration was determined and compared with that caused by unlabeled *P*-Pxy-Tyr(NHAc). Since the average intrinsic K_a was determined directly for the binding of *P*-Pxy-Tyr(NHAc), a K_i for other analogs could be calculated from this comparison (Karush, 1956).

Fluorescence Quenching Titrations. Fluorescence quenching experiments were performed by adding 5–15-μl aliquots of the appropriate hapten to purified antibody at 150 μg/ml in phosphate-buffered saline (pH 8) (Eisen, 1964). Parallel determinations were made with normal rabbit γ-globulin.

Results and Discussion

Synthesis of Conjugates. The described mode of attachment of pyridoxal-*P* and of the coenzyme–tyrosine haptens to protein was unidirectional and provided each hapten with a defined orientation (Figure 1). To facilitate a high degree of substitution on the γ-globulin, we employed relatively high protein and pyridoxal-*P* concentrations and a slightly basic pH to favor Schiff base formation. Upon reduction, covalent substitution occurred at 30 of the 70 free amino groups of the protein. The conjugate showed absorbance peaks typical for pyridoxyl substitution at 250 and 325 nm.

Covalent linkage *via* the 3′-NH_2 group of either *P*-Pxy-Tyr(NH_2) or the cyclic *P*-Pxd<Tyr(NH_2) would place the coenzyme region in an immunodominant (most accessible) position and should also provide adequate exposure of the tyrosyl moiety. The 3′-amino function of the hapten was linked to the carboxyl groups of protein by carbodiimide-facilitated amide linkage. The possibility of condensing the hapten carboxyl to free amino groups of the protein was eliminated by preliminary blockage of all γ-globulin amino functions with maleyl groups. When an analog with no 3′-amino group was reacted under identical conditions, only one-tenth as much was coupled to protein; thus the main linkage of the phosphopyridoxylaminotyrosine forms did involve the amino group. With these two haptens, a substitution of 50–80 groups per IgG molecule was achieved.

Purification of Antibodies. Each rabbit produced between 0.7 and 1.0 mg/ml of antihapten antibody. For its

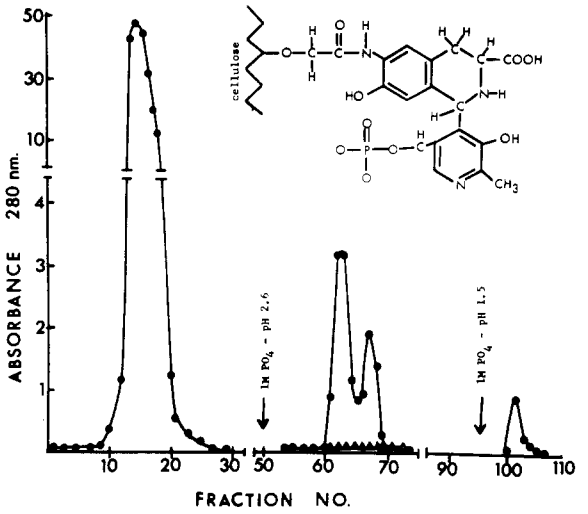

FIGURE 2: Affinity chromatography of the anti-*P*-Pxd<Tyr(NH_2) antibody. After extraneous proteins were washed off, hapten specific antibody was released with low pH phosphate buffers (arrows) (○). When normal rabbit γ-globulin was used in place of antibody, the phosphate buffers did not release protein (△) from the washed column.

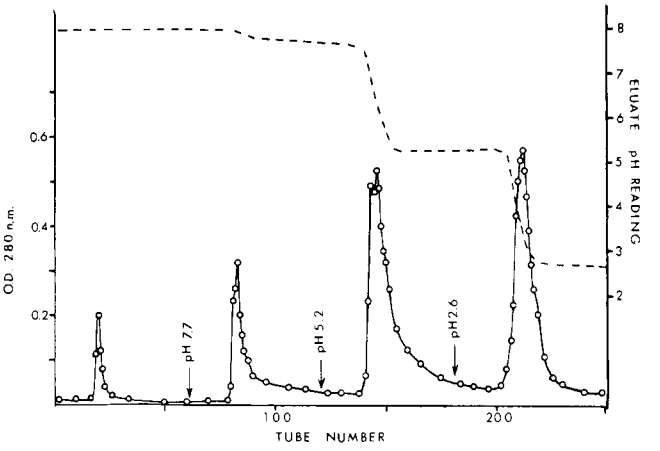

FIGURE 3: Purified anti-*P*-Pxd<Tyr(NH_2) antibody was rechromatographed on the homologous affinity column. The first peak is protein that was not retained. Antibody was released and eluted stepwise with 1 M phosphate buffers at the indicated pH values (arrows). The dashed line shows the pH profile of the eluate. The fraction size was 5 ml.

purification, crude γ-globulin was applied to an appropriate affinity absorbant column. With anti-*P*-Pxd < Tyr(NH_2) antibody on its affinity column for example, the extraneous proteins, free of antibody, were washed off with buffered saline and the hapten-specific antibody was released with 1 M phosphate buffers of pH 2.6 and 1.5 (Figure 2). When an identical procedure was followed with crude globulin from unimmunized rabbits, no protein was eluted with the acidic 1 M phosphate buffer (Figure 2). Antibodies to the other two haptens were similarly purified with their specific affinity columns.

Elution of the antibody shown in Figure 2 with pH 2.6 phosphate buffer produced two peaks of protein, and only a small amount was released on further elution at pH 1.5; each peak retained antihapten antibody activity. All the material eluted at pH 2.6 was combined and used for the characterization described below. When this material was applied to a fresh affinity column, over 95% was bound (Figure 3). After this second column was washed, elution was carried out consecutively with 1 M phosphate buffers of pH 7.7, 5.2, and 2.6. Three peaks of protein with antibody activity were obtained (Figure 3). A qualitatively similar pattern was observed with antipyridoxal-*P* antibody and its specific affinity column, though there were differences in the relative distribution of proteins in the various peaks. Thus, site heterogeneity was demonstrated, various antibody types requiring different pH phosphate buffers for elution. Differential elution at pH 7.7 and 5.2 may have been due to varying antibody specificity for the doubly or singly ionized form of the phosphate group on the phosphopyridoxyl portion of these haptens (Kitagawa *et al.*, 1967).

Quantitative Precipitation. Since there was no detectable anticarrier response even in the whole serum, and since the antibody had been purified with a completely different carrier as absorbant, the homologous antigens were used as the precipitating conjugates to measure hapten-specific antibody. Both the purified anti-*P*-Pxy-Tyr(NH_2) and anti-*P*-Pxd<Tyr(NH_2) antibodies were quantitatively precipitated by homologous antigen (Figure 4a,b); however, with purified anti-pyridoxal-*P* system, only ⅓ of the expected turbidity appeared when 250 μg of antibody was used (Figure 4c, lowest curve). The effectiveness of this precipitation was increased when the reaction was carried out in the presence of 0.25 ml of normal rabbit serum (Figure 4c, middle curve), but was still less than the expected level, which would have given a turbidimetric reading of 0.7. When 515 μg of purified antibody was used, 200 μg (36%) was precipitable (Figure 4c, upper curve). Still, the purified antibody was bound to the extent of 90% by the homologous affinity column and, on equilibrium dialysis, was found to have 2 binding sites per 150,000 molecular weight, characteristic of fully reactive immunoglobulin. The nature of the nonprecipitable antibody which still retained binding activity, and the enhancing effect of normal serum, have not been clarified.

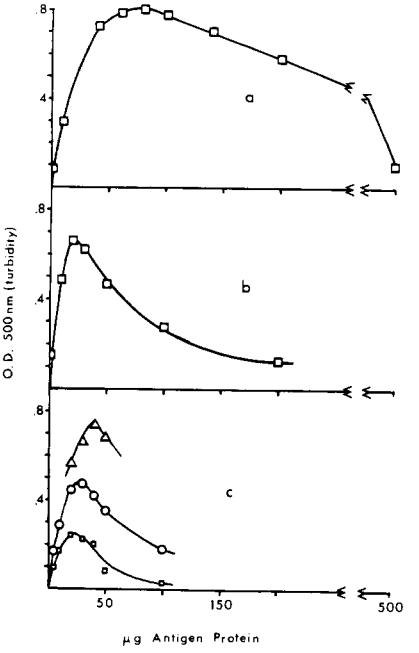

FIGURE 4: Quantitative precipitin curves using purified antibodies. (a) Purified anti-*P*-Pxy-Tyr(NH_2) (214 μg) with varying amounts of the maleylated γ-globulin conjugate of *P*-Pxy-Tyr(NH_2). (b) Purified anti-*P*-Pxd<Tyr(NH_2) (200 μg) with varying amounts of the maleylated γ-globulin conjugate of *P*-Pxd<Tyr(NH_2). (c) Purified anti-pyridoxal-*P* antibody with varying amounts of the globulin conjugate of pyridoxal-*P*; 250 μg of antibody (□), 250 μg of antibody plus 0.25 ml of normal rabbit serum (○), 516 μg of antibody (△).

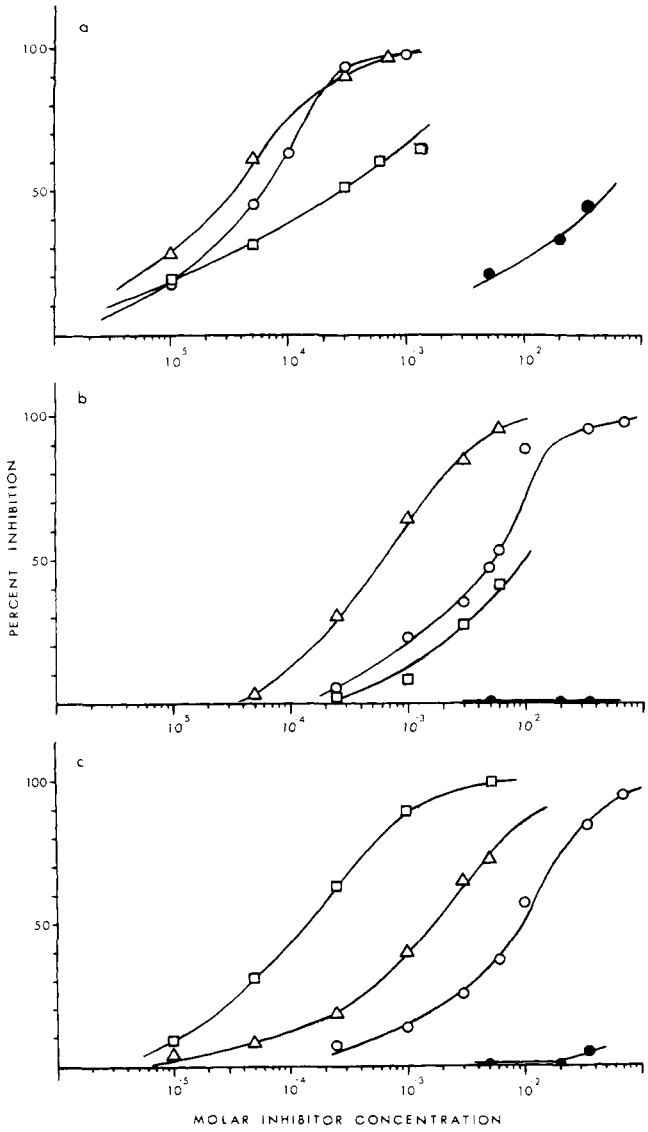

FIGURE 5: Inhibition of precipitation. The precipitation of each of the three purified antibody–antigen systems was competitively inhibited by prior addition of various hapten analogs: (a) antipyridoxal-*P*; (b) anti-*P*-Pxy-Tyr(NH_2); and (c) anti-*P*-Pxd < Tyr(NH_2). The inhibitors are: pyridoxamine (●); pyridoxamine-*P* (○); *P*-Pxy-Tyr(NH_2) (Δ); and *P*-Pxd<Tyr(NH_2) □).

Inhibition of Precipitation. ANTIBODIES TO PYRIDOXAL-P. In a particular system, relative association constants of various haptens which bind to a given antibody can be determined by comparing the hapten concentrations required to give 50% inhibition of the precipitin reaction of a standard amount of antigen and antibody (Pauling *et al.*, 1944).

With the purified anti-pyridoxal-*P* antibody system, the homologous hapten pyridoxamine-*P*, gave 50% inhibition of precipitation at a concentration of 6.2×10^{-5} M (Figure 5a). A 1000-fold higher concentration of nonphosphorylated pyridoxamine was required for comparable inhibition; thus there is a phosphate-specific region in the antibody site, as has been described previously for similar antibodies (Cordoba *et al.*, 1966). Cordoba *et al.* (1970) have also shown that, in comparison with pyridoxamine-*P* alone, its derivatives with a substituent resembling the aliphatic portion of a lysine side chain can bind 2- to 5-fold more effectively to this type of antibody. We similarly observed an almost twofold enhancement in the binding of *P*-Pxy-Tyr(NH_2) as compared to pyridoxamine-*P*, so that the amino acid portion of this flexible molecule may resemble the lysine carrier group. On the other hand, in the cyclic *P*-Pxd<Tyr(NH_2), the tyrosine ring is covalently locked in a bent position close to the phosphopyridoxyl group, a configuration which hinders binding, as this analog was fivefold less effective an inhibitor than pyridoxamine-*P*.

ANTIBODIES TO P-PXY-TYR(NH_2). Complete inhibition of the purified anti-*P*-Pxy-Tyr(NH_2) system (with pool I antibodies) could be achieved with high concentrations of pyridoxamine-*P* (Figure 5b), but the homologous hapten, including the aminotyrosine, was much more potent, binding 10 times as strongly as coenzyme alone. When the 3′-amino group of the complete hapten was absent or replaced by a nitro function, inhibition was unchanged. Thus, while the binding sites included reactivity with tyrosine, the antibody did not seem to interact with the most distal 3′ region of the extended compound. *P*-Pxd<Tyr(NH_2) inhibited 4 less effectively than pyridoxamine-*P* so that, once again, when the tyrosine was held close to and perpendicular to the pyridoxyl ring, it interfered with the binding effectiveness of the coenzyme portion of the molecule. Since this effect was not nearly as dramatic as that observed for the antibody to pyridoxal-*P*, the anti-*P*-Pxy-Tyr(NH_2) combining site appeared to be more accommodating. This antibody, like the former, was specific for the phosphorylated coenzyme.

ANTIBODIES TO P-PXD<TYR(NH_2). Antibody directed against the cyclic hapten had a markedly different pattern of binding specificity (Figure 5c). It bound the cyclic compound 65 times more effectively than free pyridoxamine-*P*, so that in this case the rigid configuration of the tyrosine portion enhanced rather than hindered binding. With the tyrosine portion pulled close to the pyridoxyl ring, the cyclic hapten presents itself as a compact structure (Raso and Stollar, 1973), and evidence for antibody interaction with the whole tyrosine ring was obtained. For example, with a compound in which the distal 3′-amino group of *P*-Pxd< Tyr(NH_2) was acetylated, binding was twice as strong as that of the free amine compound; acetylation creates a linkage similar to that formed when hapten was conjugated to glutamic acid and aspartic acid residues of the protein carrier and, unlike the case of the anti-*P*-Pxy-Tyr(NH_2) system, the linkage region was still within a range in which it affected binding.

The extended coenzyme–aminotyrosine compound was bound about sixfold more strongly than was pyridoxamine-*P* (Figure 5c). This indicated that the flexible tyrosine portion of this molecule can be used to increase binding above that of the phosphopyridoxyl region alone. The antibody may strain the tyrosine region of this derivative into a quasicyclic configuration. This effect would facilitate useful contact of the tyrosine ring with the combining sites even though it was not as effective as the rigid structure of the cyclic hapten. When the phosphopyridoxyltyrosine compound lacking a 3′-amino group was tested, its binding was only threefold greater than that of pyridoxamine-*P*. Thus, just as the 3′ region of the cyclic compound made contact, so did the same region of the flexible analog, again suggesting that this molecule may be folded within the antibody combining site. The lack of inhibition by pyridoxamine (Figure 5c) indicated that the anti-*P*-Pxd<Tyr(NH_2) antibody has the same phosphate specificity as the other two systems. The affinity for the nonphosphorylated derivative

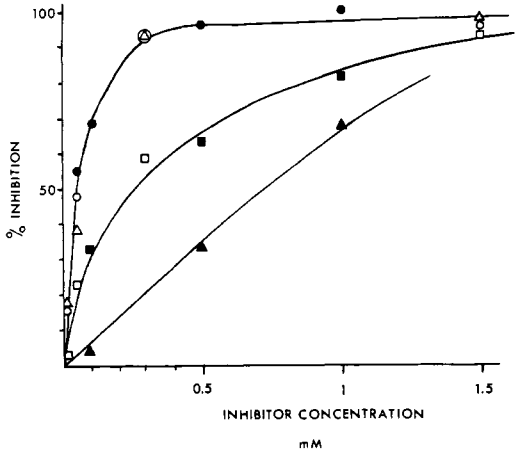

FIGURE 6: Inhibition by pyridoxal-*P* (▲, △), pyridoxamine-P (●, ○), and deoxypridoxine-*P* (■, □) of precipitation of the anti-pyridoxal-*P* antibody-antigen system using whole serum (closed symbols) or purified antibodies (open symbols).

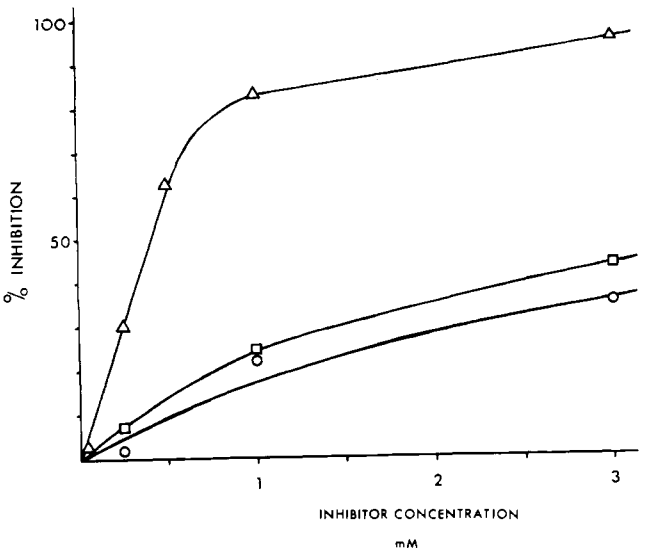

FIGURE 7: Inhibition of the purified anti-*P*-Pxy-Tyr(NH_2) antibody-antigen precipitation by pyridoxal-*P* (△) pyridoxamine-*P* (○), and deoxypyridoxine-*P* (□).

was, at most, $1/100$ that for phosphorylated form.

In control experiments, the effects of these inhibitors on the unrelated anti-dinitrophenyl-serum albumin system were tested. This antibody preparation, in the form of γ-globulin prepared by ammonium sulfate fractionation, was not inhibited by pyridoxamine-*P* or by *P*-Pxy-Tyr(NH_2) at 1×10^{-2} M, nor by the cyclic hapten at a 5×10^{-3} M concentration.

Inhibition by Pyridoxal-P. Some unexpected results appeared in comparison of the inhibitory effectiveness of pyridoxal-*P* and pyridoxamine-*P* in the three systems (Figures 6 and 7). As had been observed previously by Cordoba *et al.* (1966), when whole anti-pyridoxal-*P* serum was used, pyridoxamine-*P* was 15 times more effective an inhibitor than was pyridoxal-*P* (Figure 6). However, we found that when purified antibody was used for precipitation, the two inhibitors were equally effective, and the amount required for 50% inhibition was the same as the amount of pyridoxamine-*P* required with the whole serum; that is, the curve for pyridoxal-*P* inhibition shifted to lower concentration requirements. It is probable that the large amounts of albumin and other proteins in whole serum bound some of the pyridoxal-*P* and substantially reduced its effective concentration (Dempsey and Christensen, 1962). Deoxypyridoxine-*P*, which has a hydrogen atom instead of the amino or aldehyde function, gave the same inhibition curve with either whole antiserum or purified antibody (Figure 6). Since both pyridoxamine-*P* and pyridoxal-*P* were fivefold more effective than deoxypyridoxine-*P*, either the amino group or the aldehyde function can provide a contribution to their binding energy.

A still different picture was obtained with the purified antibodies to phosphopyridoxylaminotyrosine haptens (Figure 7). In both of these cases, pyridoxal-*P* inhibited 13 times more effectively than did pyridoxamine-*P*, while deoxypyridoxine-*P* and pyridoxamine-*P* caused similar inhibition; therefore, the higher efficiency of the pyridoxal-*P* must have been due to its aldehyde group. It is not clear why this occurred in the two systems which have amino acid specificity but not in the anti-pyridoxal-*P* antibody system.

In control experiments, pyridoxal-*P* inhibited the unrelated antidinitrophenyl-serum albumin system to the extent of 13 and 26% at 10^{-3} and 10^{-2} M, respectively.

Equilibrium Dialysis. From Scatchard (1949) analyses

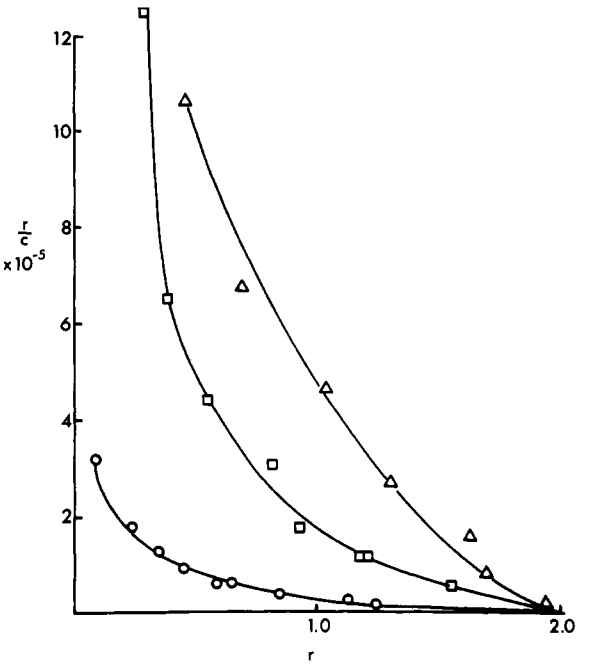

FIGURE 8: Equilibrium dialysis analysis of the interaction of *P*-Pxy-Tyr(NHAc) with anti-pyridoxal-*P* (□), with anti-*P*-Pxy-Tyr(NH_2) (△), and with anti-*P*-Pxd Tyr(NH_2) (○) antibodies.

of the reaction of *P*-Pxy-Tyr(NHAc) with each of the purified antibodies (Figure 8), average K_a's of 2.0×10^5, 4.5×10^5, and 0.40×10^5 M^{-1} obtained for pool I anti-pyridoxal-*P*, anti-*P*-Pxy-Tyr(NH_2), and anti-*P*-Pxd<Tyr(NH_2) antibodies, respectively.

The hapten employed for the dialysis was homologous for anti-*P*-Pxy-Tyr(NH_2) antibody. Since anti-pyridoxal-*P* antibody bound this hapten almost as tightly as it was bound by the homologous antibody, the anti-pyridoxal-*P* probably interacted with coenzyme region more strongly than did anti-*P*-Pxy-Tyr(NH_2) antibody, but the latter gained an advantage in being able to simultaneously interact with the tyrosyl moiety as well (Table I). The labeled analog was bound by antibody to the cyclic hapten, but with a relatively low K_a.

Fluorescence Quenching. The inherent protein fluores-

Table I: Relative Average Association Constants for Binding of Haptens by Different Antibodies.[a]

	Anti-pyridoxal-*P*	Anti-*P*-Pxy-Tyr (NH_2)	Anti-*P*-Pxd< Tyr (NH_2)
Pyridoxamine-*P*	1.0	1.0	1.0
P-Pxy-Tyr(NH_2)	1.8	10.0	5.5
P-Pxd< Tyr(NH_2)	0.2	0.6	64.0
Pyridoxal-*P*	1.0	13.0	13.5
Pyridoxamine	0.001	<0.01	<0.01

[a] For each antibody, the binding ability of pyridoxamine-*P*, the basic determinant recognized by all three antibodies, was assigned a K_a of 1.0, and a relative value for other haptens was determined from inhibition of precipitation data as described by Pauling *et al.* (1944).

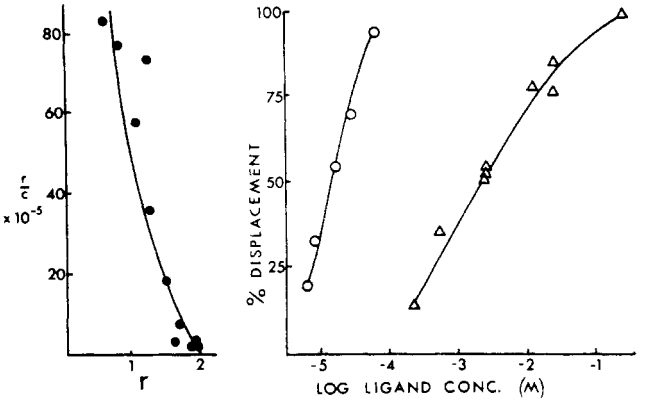

FIGURE 9: Equilibrium dialysis and inhibition of binding with purified late anti-*P*-Pxy-Tyr(NH_2) antibody from a hyperimmune rabbit. (a) Scatchard analysis for the interaction of radiolabeled *P*-Pxy-Tyr(NHAc) with antibody. (b) Binding competition with unlabeled *P*-Pxy-Tyr(NHAc) (○) or pyridoxal-*P* (△), displacing radioactive *P*-Pxy-Tyr(NHAc) from antibody.

cence of antibody to either phosphopyridoxyltyrosine hapten was quenched when hapten was bound. Quenching titrations were not suitable for determination of antibody-ligand association constants since they gave values five to ten times greater than those obtained by the definitive method of equilibrium dialysis (Eisen, 1964).

Both pyridoxamine-*P* and *P*-Pxy-Tyr(NH_2) are sufficiently fluorescent at pH 8 so that they could be used as site specific probes. When anti-pyridoxal-*P* antibody and pyridoxamine-*P* were mixed at an equimolar hapten and combining site concentration of 2×10^{-6} M, a 30% reduction in the fluorescence intensity of the coenzyme occurred. Since the antibody bound this hapten with a K_a of 1×10^5, less than half of the coenzyme was bound under these conditions; thus the actual reduction of fluorescence of the bound ligand was closer to 80%. The wavelength maxima of excitaton and emission were unchanged and a normal γ-globulin control gave no quenching of the coenzyme fluorescence. A similar phenomenon was demonstrated for *P*-Pxy-Tyr(NH_2) on its combination with homologous antibody. This analog is also fluorescent by virtue of its pyridoxyl ring (Raso and Stollar, 1973). Therefore, the coincidence in quenching effects indicates some basic resemblance between the coenzyme binding regions of both types of antibodies.

Characterization of Late Sera. The properties of late anti-*P*-Pxy-Tyr(NH_2) were compared with the early pool I antibodies described above. While the early antibody displayed a tenfold differential between the binding of pyridoxamine-*P* and *P*-Pxy-Tyr(NH_2), late antibody isolated from a single animal that was hyperimmunized by several courses of injections over 13 months showed a greater specificity for the tyrosyl portion of the complete hapten, which was 300 times more efficient than pyridoxamine-*P* in inhibition of precipitation. Pyridoxal-*P* was again more potent than pyridoxamine-*P*, but the difference was less dramatic than that found with the early pool I antibody. The acetylated analog and the nonacetylated hapten were indistinguishable. Thus, even though the antibody showed increased recognition of the tyrosyl moiety, interaction with the linkage portion of the hapten was still not demonstrable. An average intrinsic K_a of 53×10^5 M^{-1} was obtained from dialysis experiments with this late antibody and the acetylated hapten (Figure 9). Unlabeled *P*-Pxy-Tyr(NHAc) was 150 times more effective than pyridoxal-*P* in displacing the homologous ^{3}H-labeled hapten (Figure 9); these data corresponded well with the relative efficiency of the two molecules as inhibitors of immune precipitation. An average intrinsic K_a of 0.34×10^5 M^{-1} was calculated from these data for the antibody interaction with pyridoxal-*P*.

Conclusion

The antibodies elicited in response to these three hapten systems comprise a useful system for a comparative study of protein–ligand interactions relating to both antibodies and enzymes. The orientation of the three haptens on their carrier proteins has placed the coenzyme region of each in an immunodominant position. Thus, as anticipated, each of the resulting types of antibody showed strong phosphopyridoxyl specificity. The different hapten determinants begin to diverge from one another in the area of the 4-CH_2-R group of the coenzyme and it is here where differences in binding specificity began to appear. The anti-pyridoxal-*P* antibodies conformed to the amino group of pyridoxamine-*P* or to the aldehyde function of pyridoxal-*P*, since the replacement of these groups with a hydrogen caused a fivefold decrease in binding ability. In contrast, the combining sites of antibodies directed to *P*-Pxy-Tyr(NH_2) and *P*-Pxd<Tyr(NH_2) did not distinguish between the hydrogen and the amino group in this 4-CH_2-R position but each bound the aldehydic coenzyme, pyridoxal-*P*, 13-fold more strongly than the other two coenzymes. Further along the structure of the three determinants more obvious differences become evident, as reflected in the binding specificities of each type of site. Even though these regions are close to the carrier protein and are removed from the immunodominant portion of the hapten, each antibody recognizes and interacts best with the hapten determinant initially injected. The basis of the differential recognition of the extended and cyclic haptens by the antibodies must certainly reside in the configurational differences between the tyrosyl regions of the two molecules.

Comparison with the properties of pyridoxal-*P* enzymes show that certain formal correlations exist between the active sites of these enzymes and the combining sites of antibody against the phosphopyridoxyltyrosine determinants. Like pyridoxal-*P* enzymes these antibodies bind coenzymes and coenzyme analogs, display a strong preference for the phosphorylated form, and recognize both the coenzyme and

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†

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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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