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Production of Antibodies to Rat Liver Phenylalanine Hydroxylase Cross-reactivity with Other Pterin-Dependent Hydroxylases

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SUMMARY

A potent, specific antiserum to rat liver phenylalanine hydroxylase was produced in sheep. Agar double-immunodiffusion reactions and enzyme inhibition studies revealed extensive cross-reactivity between the antiserum and phenylalanine hydroxylases from guinea pig, mouse, monkey, and human liver, as well as from rat kidney. No cross-reactivity with *Pseudomonas* phenylalanine hydroxylase could be demonstrated. Further studies suggested cross-reactivity with bovine adrenal and rat brain tyrosine hydroxylases, but not with rat brain tryptophan hydroxylase, rat kidney aromatic L-amino acid decarboxylase, or bovine adrenal dopamine β-hydroxylase.

INTRODUCTION

The pterin-dependent aromatic amino acid hydroxylases—phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase—show striking similarities in many of their kinetic and regulatory properties (1–6). Only rat liver phenylalanine hydroxylase has been purified extensively enough for detailed physical characterization (7). Since it was of interest to know if these hydroxylases manifest physical similarities, we have compared them immunochemically. In the present paper we describe the preparation of antiserum to highly purified rat liver phenylalanine hydroxylase and its interaction with several other phenylalanine hydroxylases, as well as with the other two pterin-dependent aromatic amino acid hydroxylases.

METHODS

Materials. Complete Freund's adjuvant was purchased from Difco. [4-^{3}H]L-Phenylalanine (specific activity, 17 Ci/mmole) was purchased from Schwarz-Mann. [3,5-^{3}H]L-Tyrosine (specific activity, 43.6 Ci/mmole) was purchased from New England Nuclear Corporation. *N*-Methyl-*N*-3-hydroxyphenylhydrazine (NSD-1034; an aromatic L-amino acid decarboxylase inhibitor) was a generous gift of Smith and Nephew, Ltd., Harlow, Essex, Great Britain. All other materials were obtained as previously described (4, 6–8).

Preparation of tissues and enzymes. All steps were performed at 2–4°. Osborne-Mendel male rats and Hartley guinea pigs were obtained from the National Institutes of Health animal supply section. A frozen African green monkey liver was purchased from Pel-Freez Biologicals, Inc., Rogers, Ark., and stored at −80°. Adult human liver tissue was kindly supplied by Dr. Elwood LaBrosse. The liver was removed at autopsy performed within 30 min of death; it was quick-frozen immediately and stored at −80°.

Extracts for phenylalanine hydroxylase assays and for immunodiffusion were prepared by manual homogenization, in all-glass

Duall homogenizers, of the minced livers or kidneys with the volume of 0.15 M KCl indicated for each experiment below. When human or monkey liver was to be assayed, a frozen portion of tissue was treated as previously described (9). The homogenates were centrifuged at 30,000 × *g* for 30 min, and the supernatant fluids were used immediately.

Rat brain tyrosine hydroxylase was prepared by homogenization of fresh hindbrain with 25 volumes of 0.0016 M KCl in a VirTis homogenizer, followed by centrifugation of the homogenate for 60 min at 100,000 × *g*. The resultant extract was brought to 80% of saturation with ammonium sulfate; the pellet obtained after centrifugation was dissolved in 3 volumes of 0.0016 M KCl. The preparation was used immediately for assays.

Rat hindbrain tryptophan hydroxylase was prepared by manual homogenization, in an all-glass homogenizer, of minced hindbrain in 2 volumes 0.05 M Tris-acetate, pH 7.5. The homogenate was centrifuged at 30,000 × *g* for 45 min. The supernatant fluid was made 2 mM with respect to dithiothreitol and used immediately for assays.

Highly purified phenylalanine hydroxylase (85% pure) from rat liver, purified through the Sephadex G-200 step (7), was used in all experiments unless stated otherwise. Phenylalanine hydroxylase from rat kidney was purified by the same procedure (7), except that the tissue was homogenized in 0.15 M KCl. *Pseudomonas* phenylalanine hydroxylase was a generous gift of Dr. Gordon Guroff, and was purified by the method of Guroff and Rhoads (10). Bovine adrenal tyrosine hydroxylase was purified through the first ammonium sulfate step (4). Dopamine β-hydroxylase was purified according to the method of Friedman and Kaufman (8). The first ammonium sulfate fraction of rat kidney aromatic L-amino acid decarboxylase, prepared according to Clark *et al.* (11), and dihydropteridine reductase from sheep liver, purified through the calcium phosphate gel step (12), were used.

Enzyme assays. Mammalian phenylalanine hydroxylase was assayed according to published procedures (12, 13). Bacterial phenylalanine hydroxylase was assayed according to the method of Guroff and Ito (14). Tyrosine formation was measured either spectrophotometrically or fluorometrically by the nitrosonaphthol method (15, 16).

Tyrosine hydroxylase was assayed by the tritium release method as used in this laboratory (4, 17). In one instance the hydroxylation of phenylalanine by tyrosine hydroxylase was measured; the tritium release assay described by Guroff and Abramowitz (18) was used. Tryptophan hydroxylase (6) and dopamine β-hydroxylase (8) were assayed by fluorometric methods. Aromatic L-amino acid decarboxylase was assayed by the method of Lovenberg *et al.* (19); 5-hydroxytryptophan was the substrate. Dihydropteridine reductase was assayed independently of hydroxylase activity by a spectrophotometric assay (20). Glucose 6-phosphate dehydrogenase and catalase were assayed according to published procedures (21, 22).

Preparation of antigen. Rat liver phenylalanine hydroxylase [Sephadex G-200 peak fractions (7)] was purified further as follows. Electrophoresis in polyacrylamide gel (7%) was carried out according to Ornstein (23) and Davis (24), except that 0.0005% riboflavin replaced persulfate as the catalyst. About 150 μg of protein were subjected to electrophoresis per gel slice (0.5 cm in diameter × 5 cm in length). After electrophoresis, the bands on one gel were visualized by staining with anilinonapthalenesulfonate as has been described (25); two protein bands [isozymes of mol wt 110,000 (7)] with relative mobilities of 0.37 and 0.41 in this gel system were identified. Using the stained gel for reference, a single piece of gel, which included only the two hydroxylase bands, was cut from each of the remaining unstained gels. The pieces (eight or nine) were placed in an all-glass homogenizer to which 1.5 ml of 0.01 M Tris-HCl with 0.12 M KCl had been added, and the gel was homogenized. The resulting homogenate was transferred to a 5-ml syringe to which 1.5 ml of complete Freund's adjuvant had been added. This mixture was kept at 0° and emulsified prior to injection.

Immunization schedule. After control serum had been obtained, enough of the emulsion described above was injected

intramuscularly at four sites into a sheep to deliver about 1 mg of phenylalanine hydroxylase. After 14 days the sheep received (at two sites) another 0.5 mg of hydroxylase in an identical emulsion. Seven days later serum was obtained. Serum was stored in aliquots at −20°.

Preliminary incubation of enzymes with serum. When the effect of the antiserum on enzyme activity was to be determined, zero and increasing volumes of antiserum were added to individual test tubes, each of which contained an equivalent amount of the enzyme to be assayed. So that each sample would contain the same amount of serum, control serum was added to bring them all to the same volume. The tubes were incubated for 4 hr at 4°. After centrifugation at 5000 × *g* for 10 min at 4° to reduce to a pellet any precipitating antibody-antigen complexes, aliquots of the supernatant fractions were transferred to individual enzyme assay tubes, and the assays were begun.

Immunodiffusion. Double-immunodiffusion reactions were performed with the use of Ouchterlony plates containing 1.8% Agarose, 0.3 M glycine, and 0.02 M potassium phosphate, pH 7.0.

FIG. 1. *Agar gel double-diffusion experiment*
Highly purified rat liver phenylalanine hydroxylase (0.6 mg/ml) was placed in the center well. The peripheral wells were filled as follows: 2 o'clock, undiluted sheep serum after immunization; 10 o'clock, undiluted sheep serum before immunization. The volume per well was 0.007 ml; plates were developed for 26 hr at 4°.

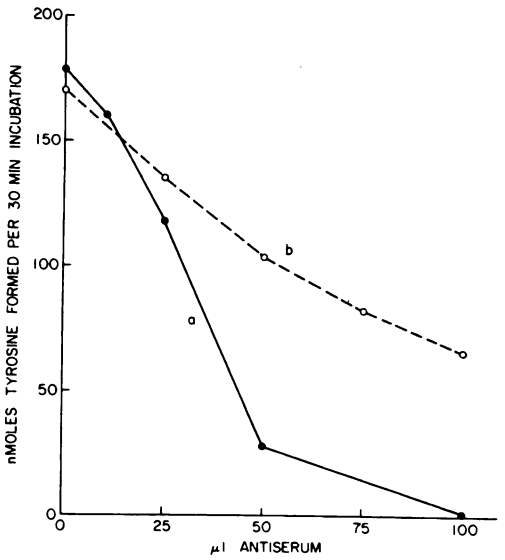

FIG. 2. *Effect of sheep serum on activity of purified rat liver phenylalanine hydroxylase*
Purified rat liver phenylalanine hydroxylase (0.015 mg) was first incubated, as described in METHODS, with the indicated volumes of antiserum in a total volume of 0.13 ml. Subsequently 0.1-ml aliquots were taken either after centrifugation (*a*) or after homogeneous resuspension of the precipitate (*b*), and transferred to assay tubes. The reaction was initiated with the addition of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine at a final concentration of 0.2 mM. The assay was performed according to Fisher and Kaufman (13). The final volume was 0.5 ml, and incubation was conducted for 30 min at 25°.

Protein was determined by the microbiuret procedure, with the use of bovine serum albumin as a standard (26).

RESULTS

Existence of an antiserum. In double-diffusion reactions (Fig. 1), purified rat liver phenylalanine hydroxylase formed a single precipitin line with serum obtained from the sheep after treatment by the immunization schedule described above, but not with the control serum. A precipitin line was observed with phenylalanine hydroxylase at concentrations of the enzyme as low as 0.025–0.050 mg/ml.

The effect of this serum on the activity of purified rat liver phenylalanine hydroxylase was examined (Fig. 2). Assay of aliquots of the supernatant fractions after preliminary incubation as described in METHODS showed a progressive loss of soluble enzymatic activity with increasing levels of antiserum (Fig. 2, curve *a*). If the assays were carried out on aliquots of the uncentrifuged preliminary incubation samples, substantially less inhibition was observed at each of the antiserum levels tested (Fig. 2, curve *b*). These results indicate that, over the range of antiserum studied, phenylalanine hydroxylase that had precipitated was not completely inactivated.

Since phenylalanine hydroxylase was assayed in the presence of catalase, glucose 6-phosphate dehydrogenase, and dihydropteridine reductase, it was necessary to eliminate the possibility that the antiserum inhibited the hydroxylation reaction by inhibition of one of these enzymes. Each enzyme, at the concentrations present in the phenylalanine hydroxylase assay, was first incubated with different amounts of antiserum exactly as described in Fig. 2. After centrifugation, aliquots were taken, and the enzymes were assayed by the methods referred to above (20–22). The antiserum had no demonstrable effect on the activity of these enzymes.

On the basis of these results, we concluded that antibodies to rat liver phenylalanine hydroxylase had been produced. Quantitative precipitin analysis of the antiserum was performed with highly purified rat liver

FIG. 3. *Cross-reactivity study*
Undiluted antiserum was placed in the center well. The peripheral wells were filled as follows: 12 o'clock, purified rat liver phenylalanine hydroxylase (0.6 mg/ml); 2 o'clock, rat liver extract (28 mg/ml); 4 o'clock, mouse liver extract (12.5 mg/ml); 6 o'clock, purified *Pseudomonas* phenylalanine hydroxylase (3.1 mg/ml); 8 o'clock, guinea pig liver extract (34.5 mg/ml); 10 o'clock, rat kidney extract (40 mg/ml). Extracts were obtained from homogenates made with the volume of 0.16 M KCl indicated (per wet weight of tissue): rat liver, 4; mouse liver, 2; guinea pig liver, 2; rat kidney, 1. The volume per well was 0.007 ml; plates were developed for 16 hr at 4°.

FIG. 4. *Cross-reactivity study*
Undiluted antiserum was placed in the center well. The peripheral wells were filled as follows: 12 o'clock, purified rat liver phenylalanine hydroxylase (0.6 mg/ml); 2 o'clock, human liver extract (44 mg/ml); 4 o'clock, rat hindbrain extract (12.2 mg/ml); 6 o'clock, 0.9% NaCl; 8'oclock, purified bovine adrenal tyrosine hydroxylase 20 mg/ml); 10 o'clock, monkey liver extract (52 mg/ml). Extracts were obtained from homogenates made with the volume of 0.16 M KCl indicated (per wet weight of tissue): human liver, 1; rat brain, 1; monkey liver, 1. The volume per well was 0.007 ml; plates were developed for 16 hr at 4°.

TABLE 1

Effect of antiserum on activity of a series of phenylalanine hydroxylases

The enzymes were first incubated, as described in METHODS, with the indicated volumes of antiserum in a total volume of 0.13 ml. After centrifugation, 0.1-ml aliquots of the supernatant fractions were transferred to assay tubes, and the assay was begun with the addition of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. The mammalian hydroxylases were assayed according to Fisher and Kaufman (13); the concentration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine was 0.2 mM. The bacterial enzyme was assayed according to Guroff and Ito (14); the concentration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine was 2 mM. The final volume was 0.5 ml, and incubation was carried out for 30 min at 25°. The number of nanomoles of tyrosine formed during 30 min with 50 µl of control serum is shown in parentheses. All extracts were derived from homogenates of tissues with 3 volumes of 0.15 M KCl.

Enzyme	Activity		
	No antiserum	25 µl of antiserum	50 µl of antiserum
		% control	
Purified rat liver phenylalanine hydroxylase, 0.01 mg	100 (178)	65	16
Rat liver extract, 1.14 mg	100 (165)	63	35
Purified rat kidney phenylalanine hydroxylase, 0.06 mg	100 (100)	69	6
Mouse liver extract, 0.75 mg	100 (218)	40	17
Guinea pig liver extract, 1.38 mg	100 (191)	81	28
Human liver extract, 2.64 mg	100 (191)	64	43
Pseudomonas phenylalanine hydroxylase, 0.06 mg	100 (30)	101	102

phenylalanine hydroxylase; the antibody content of the precipitated complex at the equivalence point was found to be about 1.3 mg/ml of antiserum. Despite this level of antibody, relatively high levels of antiserum were needed to remove active enzyme from the supernatant fraction in the enzyme inhibition experiments (Fig. 2, curve *a*). It should be noted, however, that the turnover number for phenylalanine hydroxylase is low compared with those of many other enzymes. Since we utilized protein-protein interaction to remove the enzyme from the supernatant fraction, and since relatively large numbers of enzyme molecules must be present to measure activity spectrophotometrically, it was not surprising that these high levels of antiserum were required.

Cross-reactivity with other phenylalanine hydroxylases. Interaction of antibody with phenylalanine hydroxylase from other sources was investigated by immunological and enzymatic methods. Figures 3 and 4 show double-diffusion reactions of the antiserum with a variety of other phenylalanine hydroxylases. Single precipitin lines of identity (with highly purified rat liver phenylalanine hydroxylase) were observed with extracts from rat, mouse, guinea pig, monkey, and human adult liver,[1] as well as with purified rat kidney phenylalanine hydroxylase. A precipitin line of identity was also obtained with an extract from human fetal liver. Phenylalanine hydroxylase activity has been reported in mouse pancrease (27); we have obtained a precipitin line of identity to an extract of mouse pancreas. By contrast, the phenylalanine hydroxylase from *Pseudomonas* showed no precipitin line (Fig. 3).

Cross-reactivity could also be demonstrated in enzyme assays. For all the mammalian phenylalanine hydroxylase prepara-

[1] Incubating the Ouchterlony plates for 30–36 hr led to the formation of "spurs" on the precipitin line formed with the purified rat liver phenylalanine hydroxylase at its intersections with the lines formed with human and monkey liver extracts. Thus, while primate phenylalanine hydroxylases share common antigenic sites with rat liver phenylalanine hydroxylase, they are probably not immunologically identical with it.

TABLE 2

Effect of antiserum on other pterin-dependent hydroxylases

In experiment 1, 0.25 ml of a rat hindbrain extract, prepared as described in METHODS, was first incubated with the indicated volume of antiserum in a total volume of 0.35 ml. Then the following components (in micromoles) were added to make a final volume of 0.5 ml: L-tryptophan, 0.5; TPNH, 0.05; glucose 6-phosphate, 0.5; NSD-1034, 0.7; catalase, glucose 6-phosphate dehydrogenase, and dihydropteridine reductase in excess; and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.1 (added last). After 1 hr of incubation at 37°, the reaction was terminated by the addition of 0.05 ml of 70% perchloric acid. After centrifugation, an aliquot of the supernatant was made 3 N HCl, and the quantity of 5-hydroxytryptophan formed was determined fluorometrically (6). In experiment 2, 0.10 mg of bovine adrenal medulla tyrosine hydroxylase was first incubated with the indicated volumes of antiserum in a total volume of 0.15 ml. Subsequently the samples were assayed by tritium release, according to the published procedure (17), in a final volume of 0.50 ml. Incubation was carried out for 15 min at 37°. Experiment 3 was performed in the same way as experiment 2, except that the antiserum was first treated with an excess of purified rat liver phenylalanine hydroxylase; the resulting precipitate was discarded, and the remaining serum was then used for the preliminary incubation. In experiment 4, 0.10 mg of bovine adrenal medulla tyrosine hydroxylase was first incubated with the indicated volumes of antiserum in a total volume of 0.10 ml. Subsequently the samples were assayed as in experiment 2, except that 50 nmoles of [4-^{3}H]L-phenylalanine (specific activity, 0.3 Ci/mmole) replaced the tyrosine and 50 nmoles of tetrahydrobiopterin replaced the 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. Tyrosine formation was determined from tritium release as described previously (18). Experiment 5 was the same as experiment 2, except that 0.17 mg of rat hindbrain tyrosine hydroxylase, prepared as described in METHODS, was used.

Enzyme	Activity				
	No antiserum	25 μl of antiserum	50 μl of antiserum	75 μl of antiserum	100 μl of antiserum
			% control		
1. Tryptophan hydroxylase, 2 mg[a]	100 (1.2)[b]	98	101	98	108
2. Adrenal tyrosine hydroxylase, 0.10 mg	100 (6.8)	92	88	75	66
3. Adrenal tyrosine hydroxylase, 0.10 mg	100 (6.8)		105		97
4. Adrenal tyrosine hydroxylase, 0.10 mg	100 (1.3)	92	84	65	50
5. Brain tyrosine hydroxylase, 0.17 mg	100 (0.95)	76	73	69	57

[a] The amount of total protein contributed to the assay by each enzyme preparation is indicated.

[b] The actual number of nanomoles of product formed in each experiment is indicated in parentheses.

tions tested (Table 1), the enzyme activity remaining in the supernatant fractions after preliminary incubation and removal of the precipitate by centrifugation decreased as the amount of antiserum present during the preliminary incubation period was increased. Neither a precipitate nor inhibition of enzyme activity was observed with *Pseudomonas* phenylalanine hydroxylase.

Thus extensive cross-reactivity of antiserum to rat liver phenylalanine hydroxylase was observed with all the mammalian phenylalanine hydroxylases tested, including those from kidney and pancreas. No cross-reactivity was observed with the bacterial enzyme. Conversely, antibody to the bacterial enzyme showed no cross-reactivity with rat liver phenylalanine hydroxylase.[2]

Cross-reactivity with other pterin-dependent hydroxylases. Interaction of antibody with bovine adrenal and rat brain tyrosine hydroxylases, and with rat hindbrain tryptophan hydroxylase, was studied in a similar manner. Figure 4 shows that no precipitin lines were formed with purified bovine adrenal tyrosine hydroxylase or with

[2] G. Guroff, personal communication.

TABLE 3

Preliminary treatment of antiserum with adrenal tyrosine hydroxylase

The initial incubation was carried out at 4° for 2 hr in a total volume of 0.1 ml. The total serum volume was 0.07 ml, of which 0.04 ml was antiserum when it was used. After 2 hr either buffer or purified rat liver phenylalanine hydroxylase in a volume of 0.01 ml was added, and a second incubation (0° for 3 hr) was carried out. The samples were then centrifuged, and 0.075-ml aliquots of the resulting supernatant fractions were transferred to assay tubes. The assay was begun with the addition of 0.1 nmole of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. The assay was performed as described by Fisher and Kaufman (13). The final volume was 0.5 ml, and incubation was carried out for 30 min at 25°.

Initial conditions	Addition after 2 hr	Tyrosine formed
		nmoles/30 min
Tyrosine hydroxylase (0.6 mg) + control serum	Buffer	0
Tyrosine hydroxylase (0.6 mg) + antiserum	Buffer	0
Buffer and control serum	Phenylalanine hydroxylase, 0.04 mg	222
Tyrosine hydroxylase (0.6 mg) + control serum	Phenylalanine hydroxylase, 0.04 mg	220
Buffer + antiserum	Phenylalanine hydroxylase, 0.04 mg	98
Tyrosine hydroxylase (0.6 mg) + antiserum	Phenylalanine hydroxylase, 0.04 mg	22

an extract of rat hindbrain. In this and other experiments (e.g., with the purified *Pseudomonas* phenylalanine hydroxylase) in which no precipitin lines were observed, serial dilutions of both antigen and antibody were made, and no combination of these dilutions gave visible precipitin lines. While these results might indicate a lack of cross-reactivity, they are consistent both with the presence of cross-reacting but intrinsically nonprecipitating antibodies and with the presence of precipitating antibodies which cross-react with the enzyme being studied; however, in the preparations used, the enzyme was present at concentrations too low to yield a visible precipitin line when antibody and enzyme were near the equivalence point. For the brain extract this second possibility is not unreasonable, but it seems more remote for the purified bovine adrenal tyrosine hydroxylase (and for the *Pseudomonas* phenylalanine hydroxylase), since the amount of protein contributed by these enzymes should have been enough to yield visible precipitin lines if significent levels of the precipitating antibodies had cross-reacted.

The possibility that antibodies might be present that could cross-react with the other pterin-dependent hydroxylases but not yield visible precipitin lines in the double-diffusion reactions was examined. Table 2 shows the effect on enzyme activity of preliminary incubation of the enzymes with increasing amounts of antiserum. There was no effect on the activity of rat hindbrain tryptophan hydroxylase.[3] With both the bovine adrenal and rat brain tyrosine hydroxylases, progressive inhibition of enzyme activity occurred with increasing levels of antiserum. The phenylalanine-hydroxylating activity of bovine adrenal tyrosine hydroxylase was also sensitive to the antiserum (Table 2, experiment 2). As shown, inhibition of tyrosine hydroxylation was abolished if the anti-

[3] It should be mentioned that 0.1 ml of the control serum inhibited the activity of the enzyme 70% as compared to enzyme that had first been incubated in an equal volume of 0.05 M Tris-acetate, pH 7.5. However, as shown, the immune serum had no further inhibitory effect. The reason for the inhibition by control serum is unclear. Tryptophan hydroxylase incubated in an identical fashion was assayed simultaneously by the radioisotopic method of Lovenberg *et al.* (28); results identical with those presented in Table 2 were obtained.

serum had been treated previously with an excess of highly purified rat liver phenylalanine hydroxylase (Table 2, experiment 3). This preparation of phenylalanine hydroxylase (85% pure by disc gel electrophoresis) exhibited neither tyrosine hydroxylase activity nor protein bands on disc gel electrophoretic patterns that corresponded to those of purified bovine adrenal tyrosine hydroxylase. In addition, since the antiserum was elicited from two discrete phenylalanine hydroxylase bands cut from disc gels, it is highly unlikely either that the sheep had been immunized with contaminating tyrosine hydroxylase or that contaminating tyrosine hydroxylase was responsible for the reversal of inhibition observed with the previously treated serum. This information, coupled with the fact that "immune" serum from a sheep subjected to an identical immunization schedule (except that no phenylalanine hydroxylase was included in the injections) did not inhibit tyrosine hydroxylase, suggests that the antiserum contained antibodies to rat liver phenylalanine hydroxylase which cross-reacted with tyrosine hydroxylase.

Table 3 illustrates the effect of antiserum that had been incubated with bovine adrenal tyrosine hydroxylase on the activity of phenylalanine hydroxylase. After the serum had been incubated with the tyrosine hydroxylase, an aliquot of phenylalanine hydroxylase was added, and another preliminary incubation was carried out. The experiment was designed to produce a 3-fold excess of tyrosine hydroxylase molecules compared to the concentration of phenylalanine hydroxylase monomers (7). After the second preliminary incubation and centrifugation, the phenylalanine hydroxylase activity remaining in the supernatant fraction was assayed. Less activity in the supernatant fraction was observed when the antiserum had been treated with tyrosine hydroxylase than when it had not. These results can be explained if one makes the following assumptions: (a) in addition to precipitating antibodies, the antiserum also contained nonprecipitating antibodies to phenylalanine hydroxylase, which either do not inhibit, or inhibit only partially, the enzymatic activity of phenylalanine hydroxylase; (b) these nonprecipitating antibodies might have interfered with the interaction of the enzyme with the precipitating antibodies; (c) tyrosine hydroxylase could have combined with the nonprecipitating antibodies. It would follow that removal of some of the competing nonprecipitating antibodies by tyrosine hydroxylase could have led to a greater degree of precipitation of phenylalanine hydroxylase by the remaining precipitating antibodies and thereby resulted in the greater loss of phenylalanine hydroxylase activity that we observed.

Cross-reactivity of other related enzymes. Two other enzymes, aromatic L-amino acid decarboxylase and dopamine β-hydroxylase, were not found to cross-react with the antiserum by either double-diffusion or enzyme assay techniques.

DISCUSSION

The antiserum to rat liver phenylalanine hydroxylase that we have produced possesses sufficient potency and specificity to make possible the cross-reactivity studies reported. Several other applications for the antiserum are apparent. With the fluorescent antibody technique, it might be possible to determine whether the hydroxylase is localized within the hepatic architecture or is generally distributed; its localization in kidney and pancreas might also be determined.

The existence of a cross-reacting protein in the livers of phenylketonuric individuals [who have no measurable hepatic phenylalanine hydroxylase activity (29–32)] should be detectable by utilizing the appropriate antiserum. One approach would involve purification of human liver phenylalanine hydroxylase and subsequent production of antiserum to it. Such a project, however, would require an ample supply of fresh, active necropsy liver. Alternatively, a comparison in double-diffusion reactions of precipitin lines formed between the antiserum to rat liver phenylalanine hydroxylase and extracts of biopsy samples from normal or phenylketonuric livers could be made. In double-diffusion reactions and enzyme inhibition studies our antiserum showed cross-reactivity with human liver phenylalanine hydroxylase (see Fig. 4 and Table 1). Such a

comparative study is currently in progress in this laboratory.

It has recently been suggested that the phenylalanine hydroxylase from rat kidney is a different form of the enzyme from that in rat liver (33). This suggestion was based on differences in heat stability of the enzymes in extracts from the respective organs, as well as on differences in stability during storage (33). It was felt that different forms of the enzyme in liver and kidney might explain the variants of phenylketonuria observed in humans (34). Our experiments show that these two enzymes yielded precipitin lines of complete identity with the antiserum. Thus the phenylalanine hydroxylase from rat kidney (and most likely from pancreas also) seems structurally very similar to, and probably is identical with, the enzyme from rat liver. Based on this argument and the assumption that phenylalanine hydroxylase exists in normal human kidney and pancreas, it can be predicted that the enzymatic defect previously established in the livers of phenylketonuric individuals, i.e., lack of a functional phenylalanine hydroxylase (29–32), will also extend to their kidneys and pancreas.

The absence of cross-reactivity of the antiserum with *Pseudomonas* phenylalanine hydroxylase is not surprising; it is one further example of a mammalian enzyme and a bacterial enzyme that perform identical functions in the two organisms but are structurally so dissimilar as to be immunologically distinct.

Our data are highly suggestive of cross-reactivity of the antiserum with both adrenal and brain tyrosine hydroxylases; a structural similarity between tyrosine hydroxylase and phenylalanine hydroxylase may exist. Both the tyrosine hydroxylases can hydroxylate phenylalalanine (4, 35).[4] Further clarification of this potential cross-reactivity awaits more detailed immunological investigation. While it is conceivable that the brain tryptophan hydroxylase interacts with the antiserum but is neither precipitated nor inhibited by the antibodies, it seems more likely that the enzyme is immunologically distinct from phenylalanine hydroxylase, despite the fact that hepatic phenylalanine hydroxylase has been shown to hydroxylate tryptophan (36).

[4] T. Lloyd and S. Kaufman, unpublished observations (1971).

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