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Production of Antibodies to Bovine Adrenal Tyrosine Hydroxylase: Cross-reactivity Studies with Other Pterin-Dependent Hydroxylases

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SUMMARY

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A specific antiserum to purified bovine adrenal tyrosine hydroxylase was produced in sheep. Enzyme inhibition studies and agar double-immunodiffusion reactions revealed extensive cross-reactivity between the antiserum and tyrosine hydroxylases from bovine, rat, monkey, and rabbit adrenal glands, as well as from bovine caudate and human pheochromocytoma. The specificity of the antiserum was demonstrated by the lack of inhibition of phenylalanine hydroxylase, tryptophan hydroxylase, dopamine β-hydroxylase, and tyrosinase.

INTRODUCTION

There are many indications that the physiological control of catecholamine biosynthesis is exerted at the first, and presumably rate-limiting, step in the pathway, i.e., the reaction catalyzed by tyrosine hydroxylase (1–3). A wide variety of pharmacological and hormonal manipulations appear to alter the catalytic activity and perhaps the turnover rate of this enzyme (4–6).

Experience with other enzymes suggested that the availability of specific antibodies to tyrosine hydroxylase would be an invaluable tool in the further studies of the physiological regulation of the enzyme and the related question of its intracellular localization. With this end in mind, we have, by disc gel electrophoresis, prepared highly purified bovine adrenal tyrosine hydroxylase and used this preparation to induce the formation of specific antibodies to the hydroxylase.

This report describes the preparation of a specific antiserum to bovine adrenal tyrosine hydroxylase and its interaction with several other tyrosine hydroxylases, as well as with other pterin-dependent hydroxylases.

METHODS

Materials. Highly purified mouse melanoma tyrosinase, 12 units/ml (0.24 mg/ml), was a generous gift of Dr. Seymour Pomerantz, Department of Biochemistry, University of Maryland School of Medicine, Baltimore. Two samples of human pheochromocytoma tissue were kindly provided by Dr. Norman Weiner, Department of Pharmacology, University of Colorado School of Medicine, Denver. Frozen monkey and rabbit adrenal glands were purchased from Pel-Freez Biologicals, Inc.

[3,5-^{3}H]L-Tyrosine (specific activity, 30.6 Ci/mmole) was purchased from New England Nuclear Corporation and purified prior to use (7). Complete Freund's adjuvant was

purchased from Difco. Catalase and glucose 6-phosphate dehydrogenase were purchased from C. F. Boehringer und Soehne. All other chemicals were obtained from standard commercial sources.

Preparation of tissues and enzyme assays. Highly purified (85% pure) rat liver phenylalanine hydroxylase was prepared and assayed as previously described (8, 9). Tyrosine formation was measured either spectrophotometrically or fluorometrically by the nitrosonaphthol method (10).

The intracellular localization of bovine adrenal tyrosine hydroxylase has not been well established, since it is present in both the supernatant and particulate fractions of homogenates (4). Particulate bovine adrenal tyrosine hydroxylase can be solubilized by treatment with chymotrypsin (11), and both solubilized and soluble preparations were used in this study. Solubilized bovine adrenal tyrosine hydroxylase was prepared according to the method of Shiman *et al.* (11). Soluble bovine adrenal tyrosine hydroxylase was prepared according to the method of Nagatsu *et al.* (12). Bovine caudate tyrosine hydroxylase was purified 30-fold.[1] All other tyrosine hydroxylases were prepared at 0–4° as follows. All tissues were finely minced and then thoroughly homogenized with glass Duall homogenizers in 10 volumes 0.02 M potassium phosphate, pH 7.0. The homogenates were centrifuged at 40,000 × *g* for 30 min, and the supernatant fraction was decanted and made 80% saturated with respect to ammonium sulfate by the addition of cold saturated ammonium sulfate, pH 6.8. After equilibration, the mixtures were centrifuged at 15,000 × *g* for 15 min, the supernatant fractions were discarded, and the pellets were dissolved in 1–2 volumes (with respect to original tissue weight) of 0.02 M potassium phosphate, pH 7.0. This procedure effectively eliminated all tyrosine hydroxylase inhibitors, principally catechols, and preserved nearly all the enzymatic activity. Tyrosine hydroxylase activity was assayed by the tritium release method as used in this laboratory (7). All incubations were carried out at 37° for 15 min.

[1] T. Lloyd and S. Kaufman, procedure to be published.

Rabbit hind brain tryptophan hydroxylase was purified 10-fold and assayed as previously described (13). The tyrosine-hydroxylating capacity and the dopachrome formation (cresolase and catecholase functions) of mouse melanoma tyrosinase were measured according to the procedures described by Pomerantz and Li (14). Dopamine β-hydroxylase activity and catalase activity were assayed according to previously published procedures (15, 16).

Preparation of antigen and immunization schedule. Prior to use as an antigen, highly purified, solubilized bovine adrenal tyrosine hydroxylase (11) was further purified by gel electrophoresis essentially as described for the purification of rat liver phenylalanine hydroxylase (17). About 200 μg of the hydroxylase fraction (40–60% ammonium sulfate fraction after the substituted Sepharose step), specific activity, 22 nmoles of tritiated water (THO) formed per minute per milligram of protein, were routinely applied to each gel; tyrosine hydroxylase activity was localized on a sample gel in each run by cutting one gel into 2.5-mm slices and assaying for tyrosine hydroxylase activity, which was always found between relative mobilities of 0.62 and 0.67. The purity of the tyrosine hydroxylase was also established by staining one gel overnight with either Amido black or Coomassie brilliant blue and destaining, as previously described (11). The results of these studies always showed only one band with a relative mobility greater than 0.60 and only two very minor ones between 0.50 and 0.60. This relative isolation of the tyrosine hydroxylase band under these conditions facilitated removal of the antigen without contamination. For the initial and booster immunizations, 20–25 gels were run and the 2.5–5.0-mm section containing tyrosine hydroxylase was cut out of each gel. These sections (containing 0.5–1.0 mg of tyrosine hydroxylase protein in all) were pooled, homogenized in 2.0 ml of 0.02 M Tris-HCl, pH 7.0, in the cold, and emulsified with 2.0 ml of Freund's adjuvant prior to injection.

After control serum had been obtained, the emulsified gel preparation was injected intramuscularly at four sites into a sheep.

Five booster immunizations were prepared and administered at 4–6-week intervals in a fashion identical with the original immunization. Six days after the final injection, 500 ml of serum were obtained and stored in aliquots at −20°. Double-immunodiffusion studies were performed as previously described (17). Protein was determined by the procedure of Lowry *et al.* (18) with bovine serum albumin as the standard. Preliminary incubation of enzymes with serum was carried out essentially as previously described (17). The quantity of enzyme to be assayed was added to a series of test tubes, containing zero and increasing amounts of antiserum. Control serum was added so that all tubes would contain the same amount of serum. The tubes were incubated for 2–4 hr at 4°. Since the amount of precipitating complex formed in the activity—inhibition studies was too small to see after centrifugation, no attempts to remove it were made before the assay was begun.

RESULTS

Presence and specificity of antiserum. In the double-diffusion studies described below, serial dilutions were made of tyrosine hydroxylase-directed antiserum and of all the antigens examined for cross-reactivity. In double-diffusion reactions the control serum (Fig. 1A) and the antiserum (Fig. 1B) were tested against partially purified bovine adrenal tyrosine hydroxylase, an extract of bovine adrenal medulla, crude rat adrenal tyrosine hydroxylase, crude monkey adrenal tyrosine hydroxylase, highly purified mouse melanoma tyrosinase, and highly purified bovine adrenal dopamine β-hydroxylase. A single precipitin line was observed against the tyrosine hydroxylases, and none was observed against the other hydroxylases. Only a single precipitin line was observed toward an extract of bovine adrenal medulla. This finding indicates that the antiserum is highly specific for tyrosine hydroxylase and that there are no other cross-reacting proteins in this tissue. The control serum plate is included in Fig. 1 to demonstrate a slight cloudiness around the serum-containing center well, which apparently was due to nonspecific precipitation of serum proteins in the gel and was always observed with both the control and immune sera used in this study. Attempts to avoid this slight haze by dialysis of the sera, equilibration with the

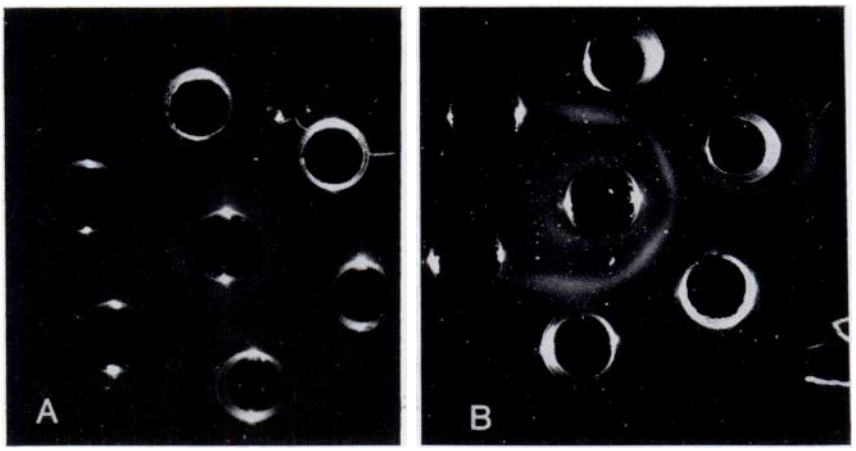

FIG. 1. *Agar gel double-immunodiffusion reactions*

The center well of Fig. 1A contained 10 μl of control serum and the center well of Fig. 1B contained 10 μl of antiserum. The peripheral wells of both plates were filled as follows: 12 o'clock, 10 μl of partially purified solubilized bovine adrenal tyrosine hydroxylase (0.04 mg); 2 o'clock, 10 μl of crude rat adrenal tyrosine hydroxylase (0.17 mg); 4 o'clock, 10 μl of crude monkey adrenal tyrosine hydroxylase (0.38 mg); 6 o'clock, 10 μl of bovine adrenal medulla extract (0.67 mg); 8 o'clock, 3 μl of highly purified bovine adrenal dopamine β-hydroxylase (0.02 mg) and 7 μl of 0.9% NaCl; 10 o'clock, 4 μl of highly purified mouse melanoma tyrosine (0.05 mg) and 6 μl of 0.9% NaCl. The plates were developed for 30 hr at 4°.

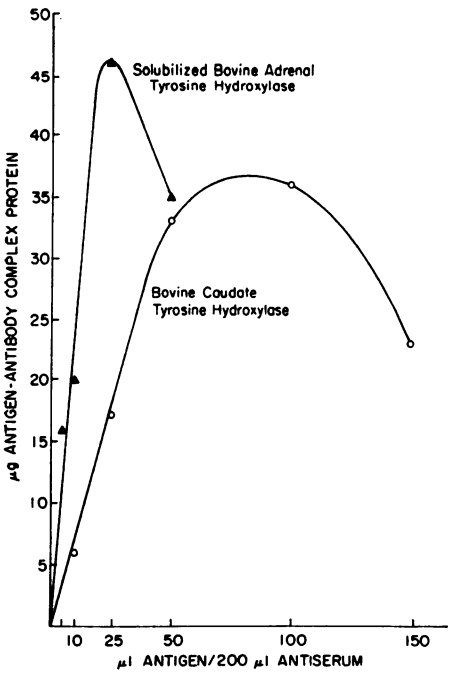

FIG. 2. *Quantitative precipitin curve*

Increasing amounts of either tyrosine hydroxylase preparation were added to 200 μl of antiserum, and the mixtures were incubated for 36 hr at 4°. The precipitates were recovered by centrifugation and washed three times with 2.0 ml of cold 0.9% NaCl. The antigen-antibody complex protein was determined by the method of Lowry *et al.* (18).

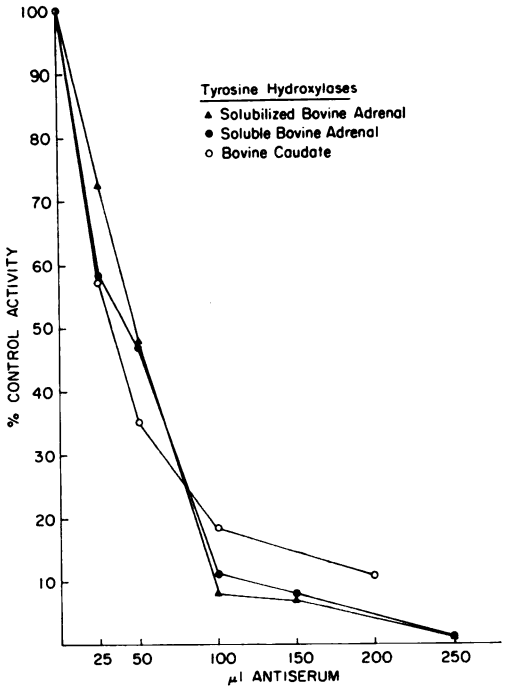

FIG. 3. *Effect of antiserum on activity of three tyrosine hydroxylases*

The following amounts of the three enzyme preparations used were incubated, as described in METHODS, with the indicated volumes of antiserum and then assayed for tyrosine hydroxylase activity (control activity, expressed as nanomoles of tritiated water (THO) released, is shown in parentheses): bovine caudate tyrosine hydroxylase, 0.28 mg (1.90); soluble bovine adrenal tyrosine hydroxylase, 0.10 mg (1.95); solubilized bovine adrenal tyrosine hydroxylase, 0.010 mg (2.04).

diffusion gel buffer, or fractionation of the γ-globulins were all unsuccessful, although the intensity of the haze varied somewhat from plate to plate. Further double-diffusion reactions against all other tyrosine hydroxylases studied also showed a single line of identity. In contrast, all the other enzymes which were found to have no demonstrable cross-reactivity by hydroxylase activity measurements also produced no precipitin lines against the antiserum. The nonreacting enzymes were always diluted to ensure that the antigen was not present in excess of the specific antibodies. The relatively light precipitin lines shown in Fig. 1B indicate that the anti-tyrosine hydroxylase titer of the antiserum is low.

Quantitative precipitin analysis of the antiserum was performed with partially purified, solubilized bovine adrenal tyrosine hydroxylase and with partially purified bovine caudate tyrosine hydroxylase. The results, shown in Fig. 2, demonstrate relatively sharp precipitin curves for both antigens and are consistent with the existence of specific antibodies to a pure antigen; the antibody content of the antiserum was calculated to be about 0.20 mg/ml.

Enzyme inhibition and cross-reactivity studies with antiserum. The dose-dependent inhibition of three bovine tyrosine hydroxylases by the antiserum is shown in Fig. 3. The amounts of soluble and solubilized bovine adrenal tyrosine hydroxylase used in this experiment were adjusted so that the tyrosine hydroxylase activities in the control tubes were very similar. The titration curves of enzyme activity with respect to antiserum

shown in Fig. 3 are nearly identical, suggestive of very similar antigenic sites on the two forms of bovine adrenal tyrosine hydroxylase. Although the molecular size of soluble bovine adrenal tyrosine hydroxylase has been reported to be about 4 times that of the tyrpsin-digested enzyme, interpretation of these data is complicated by the tendency of the soluble enzyme to form macromolecular aggregates, a property not shared by the trypsin-digested enzyme (19). It is possible, therefore, that the size of the subunits of the native enzyme is not significantly different from that of the solubilized enzyme.

Cross-reactivity between several other tyrosine hydroxylases and other enzymes involved in biologically active amine biosynthesis was studied by double-diffusion and enzymatic methods. The inhibition of enzymatic activity of seven tyrosine hydroxylases by the antiserum is presented in Table 1. In general, a typical dose-dependent extent of inhibition was seen, except in the case of the rat adrenal tyrosine hydroxylase, in which the lowest amounts of antiserum had already effected nearly complete inhibition.

In addition to the tyrosine hydroxylases, cross-reactivity between the antiserum and the following enzymes was studied: rat liver phenylalanine hydroxylase, bovine adrenal dopamine β-hydroxylase, mouse melanoma tyrosinase (both cresolase and catecholase activities), rabbit hind brain tryptophan hydroxylase, and catalase. Where cross-reactivity was thought, a priori, to be likely (phenylalanine hydroxylase, tryptophan hydroxylase, and tyrosinase), enzyme assays and double-diffusion studies were performed with a series of enzyme samples which were diluted over a 10-fold range against a series of antiserum samples which were diluted over a 100-fold range in an attempt to demonstrate cross-reactivity. No inhibition of enzymatic activity was observed, nor were any precipitin lines to any of the above enzymes detected with any amount of antiserum.

DISCUSSION

Through the use of gel electrophoresis we have prepared tyrosine hydroxylase of sufficient purity to elicit a moderate titer of tyrosine hydroxylase-directed antibody in sheep serum. This antiserum has been shown to be highly specific since it interacts, by both enzymatic and immunological criteria, with other tyrosine hydroxylases, but not with other enzymes involved in biologically

TABLE 1

Effect of antiserum on activity of a series of tyrosine hydroxylases

All assays and enzyme preparations were performed as described in METHODS. The activity of the tyrosine hydroxylases, when measured in the presence of either 200 or 250 μl of control serum, expressed as nanomoles of tritiated water (THO) released, is shown in parentheses.

Enzyme	Activity at various levels of antiserum						
	0 μl	25 μl	50 μl	100 μl	150 μl	200 μl	250 μl
				% *control*			
Soluble bovine adrenal tyrosine hydroxylase, 0.20 mg	(5.30) 100		95	73	50		29
Solubilized bovine adrenal tyrosine hydroxylase, 0.02 mg	(2.50) 100		67	32	8		0
Bovine caudate tyrosine hydroxylase, 0.28 mg	(1.90) 100	63	37	18		10	
Rat adrenal tyrosine hydroxylase, 0.24 mg	(1.54) 100		13	16		14	
Human pheochromocytoma tyrosine hydroxylase, 0.12 mg	(0.61) 100		30	25		21	
Rabbit adrenal tyrosine hydroxylase, 0.29 mg	(0.26) 100	33	31	24	15		
Monkey adrenal tyrosine hydroxylase, 0.94 mg	(1.95) 100	36	24	18			

active amine synthesis. Although an earlier report described the production of antiserum to a partially purified tyrosine hydroxylase (20), neither the specificity nor the purity of the antibodies thus produced was evaluated. The antiserum described in this report has been shown to react with soluble and particulate bovine adrenal tyrosine hydroxylases, as well as with tyrosine hydroxylases from bovine caudate and rat, rabbit, monkey, and human adrenals. This extensive interspecies and organ cross-reactivity and antigenic identity strongly suggest close structural similarity, if not identity, among the mammalian tyrosine hydroxylases.

The present finding that the chymotrypsin-solubilized and the soluble bovine adrenal tyrosine hydroxylases are immunologically indistinguishable demonstrates not only that the protease treatment does not significantly change the catalytic properties of the enzyme (11), but also that its antigenic determinants are unaltered.

Our previous study (17) demonstrated that the highly specific antiserum to pure rat liver phenylalanine hydroxylase did manifest some cross-reactivity toward tyrosine hydroxylases from both bovine adrenal medulla and rat brain. Although precipitating antigen-antibody complexes were not observed, cross-reactivity was detected by antibody inhibition of the hydroxylase activity. In contrast, our present study has shown that tyrosine hydroxylase-directed antibody has absolutely no cross-reactivity toward rat liver phenylalanine hydroxylase.

We included tyrosinase in our study of the specificity of the antiserum because it is known that the first step in the conversion of tyrosine to melanin catalyzed by tyrosinase is the hydroxylation of tyrosine to dopa; i.e., tyrosinase has a tyrosine hydroxylase-like activity. This partial overlap of catalytic activities between tyrosine hydroxylase and tyrosinase suggests that the two enzymes might have evolved from a common protein precursor. Our negative cross-reactivity results indicate that even if the two enzymes were once related, the mammalian enzymes are now structurally dissimilar.

Although a higher antibody titer is desirable, and can undoubtedly be raised by subsequent booster immunization, the presently described antiserum is sufficiently potent to carry out a number of further biochemical investigations. Preliminary investigations in this laboratory have shown that the antiserum can be used for purifying diverse tyrosine hydroxylases.

This specific antiserum should also prove valuable in carrying out both regional and intracellular localization studies of tyrosine hydroxylase, similar to those already reported for dopamine β-hydroxylase (21, 22). Finally, in view of its widely held role as the rate-limiting enzyme in the biosynthesis of norepinephrine, we believe that the careful use of a specific antiserum to tyrosine hydroxylase should provide the first truly quantitative method for studying the turnover time of this enzyme under normal conditions and under physiological and pharmacological manipulation.

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