Production of Antibodies to Dopamine-β-hydroxylase of Bovine Adrenal Medulla

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SUMMARY

Purified dopamine-β-hydroxylase was prepared from bovine adrenal medulla and a potent and specific antibody to the enzyme was produced in rabbits. The antibody markedly inhibited dopamine-β-hydroxylase from bovine, dog, guinea pig adrenals, and human pheochromocytoma. Agar double diffusion studies also revealed an interaction between the antibody and enzyme from the various species. The specificity of the antibody was demonstrated by the lack of inhibition of tyrosine hydroxylase and DOPA decarboxylase, the other two enzymes in the pathway of catecholamine biosynthesis.

INTRODUCTION

One of the three enzymes involved in norepinephrine biosynthesis, dopamine-β-hydroxylase, has been obtained in highly purified form (1) permitting its use as an antigen. This report concerns the production of antibody to dopamine-β-hydroxylase of bovine adrenal medulla and an evaluation of its interaction with dopamine-β-hydroxylase from other species and with the other two enzymes involved in norepinephrine synthesis.

METHODS

Preparation of enzymes. Dopamine-β-hydroxylase to be used as antigen was isolated from bovine adrenal medulla and purified by a modification[1] of the method of Friedman and Kaufman (1). The preparations were assayed for specific activity and homogeneity (see below). Dopamine-β-hydroxylase from human pheochromocytoma, dog and guinea pig adrenal was extracted and purified to the first ammonium sulfate step according to the procedure of Friedman and Kaufman (1).

Aromatic L-amino acid decarboxylase was prepared from guinea pig kidney according to the procedure of Clark *et al.* (2). Tyrosine hydroxylase represented a 60-fold purification from a homogenate of bovine adrenal medulla according to the procedure of Nagatsu *et al.* (3).

Enzyme assays. Hydroxylation of dopamine was measured essentially according to the spectrophotometric assay of Pisano *et al.* (4). The reaction mixture contained (in micromoles): tyrosine 10; ascorbate 10; fumarate 10; potassium phosphate, pH 6.2, 100; and the enzyme to be assayed. The final volume was 1 ml. Incubation was for 10 min at 37°, and the reaction was stopped with 2 ml of 4 N NH_4OH, after which 0.30 ml of 2% $NaIO_4$ was added. The mixture was allowed to stand for 3 min at room temperature, and exactly 0.30 ml of 10% $NaHSO_3$ was then added. The *p*-hydroxybenzaldehyde that had been formed was measured by its absorption at 330 mμ.

Aromatic L-amino acid decarboxylase

[1] Details of the modified procedure will appear in a subsequent publication by L. A. Fahien, J. W. Gibb, and S. Udenfriend.

activity was assayed by following the conversion of 5-hydroxytryptophan to serotonin as described by Lovenberg *et al.* (5). Tyrosine hydroxylase was assayed by following the release of tritium from 3,5-tritiotyrosine as described by Nagatsu *et al.* (6).

Immunization of the rabbits. Purified bovine adrenal dopamine-β-hydroxylase (see below) in 0.15 M NaCl–0.01 M potassium phosphate pH 7.4, was emulsified with an equal volume of Freund's adjuvant which was composed of a 4:1 mineral oil–Arlacel A (v/v) mixture. The mineral oil contained dried, heat-killed *Mycobacterium butyricum* (10 mg/100 ml). Immunization was performed by injecting 1 mg of dopamine-β-hydroxylase in 1 ml of Freund's adjuvant into the foot pads of randomly bred albino rabbits (0.25 mg per foot pad). Following the primary injection of the antigen, subsequent booster injections (1 mg) were given every 3 weeks for a 2-month period. When the antibody reached suitable levels according to precipitin analysis (see below) animals were bled (by cardiac puncture or ear vein) at weekly intervals.

Precipitin analysis. The concentration of dopamine-β-hydroxylase antibodies in serum was measured by quantitative precipitin analysis using purified bovine adrenal dopamine-β-hydroxylase as the antigen. Antigen-antibody mixtures were incubated for 1 hr at 37° and then left for 18–24 hr at 4°. The amount of antibody in each washed precipitate, dissolved in 0.5% sodium dodecyl sulfate, was calculated from the total absorbance at 278 mμ minus the absorbance attributed to the antigen.

Antibody diffusion methods. Ouchterlony plates were prepared using 0.1% agar in 0.045 M barbital buffer, pH 8.5. The agar was made from Seakem agarose, which was obtained from Bausch and Lomb.

Disc gel electrophoresis, as described by Davis (7), was performed at 4° with 7.5% polyacrylamide. Samples of 0.2 mg of dopamine-β-hydroxylase were applied and run at a constant current of 6 mamp per tube at pH 9.5 for 3 hr.

RESULTS

Purity of the Antigen

The specific activity[2] of dopamine-β-hydroxylase (32 μmoles of octopamine per milligram of protein per 10 min) purified from bovine adrenal medulla compared favorably with the enzyme activity described by Friedman and Kaufman (1),

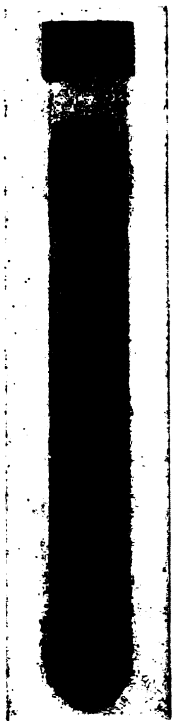

FIG. 1. *Electrophoretic pattern of purified bovine adrenal dopamine-β-hydroxylase on polyacrylamide gel*

0.2 mg of enzyme was applied to the gel.

whose preparations were found essentially homogeneous by ultracentrifugation and starch gel electrophoresis. The preparations obtained in the present studies when subjected to disc gel electrophoresis yielded essentially one protein band containing almost all the enzyme activity (Fig. 1). The minor protein band which appeared half

[2] Kaufman and Friedman measured norepinephrine formation from dopamine whereas the production of octopamine from tyramine was measured in this study. Tyramine and dopamine have comparable activity as substrates.

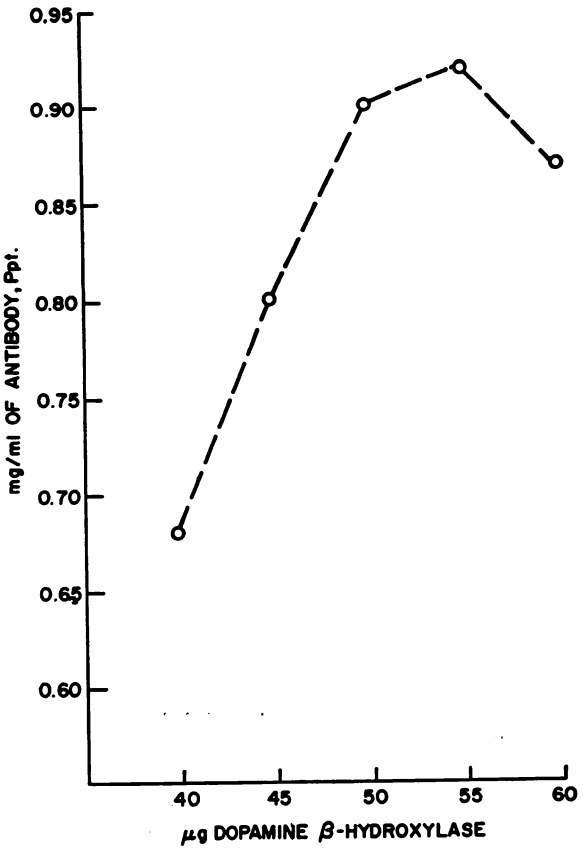

FIG. 2. *Quantitative precipitin curve*

Increasing amounts of dopamine-β-hydroxylase were added to a constant amount of antibody. Antigen mixtures were incubated for 1 hr at 37° and then left for 18–24 hr at 4°. The washed precipitate was dissolved in 0.5% sodium dodecyl sulfate, and the antibody present was calculated from the total absorbance at 278 mμ minus the absorbance attributed to the enzyme.

way along the gel column possessed dopamine-β-hydroxylase activity and may represent some altered form of the enzyme.

The dopamine-β-hydroxylase preparations from human, guinea pig, and dog adrenals were carried only to the first ammonium sulfate precipitation step (1). In spite of the crude nature of the preparations and the presence of endogenous inhibitor at this early stage of purification, activity was easily measured by the assay procedure.

Antibody Titer

The titer of pooled rabbit antiserum collected over a period of several months was estimated by quantitative precipitin analysis using the purified bovine adrenal enzyme. As shown in Fig. 2 the antibody content of the precipitated complex at the equivalence point was 0.92 mg per milliliter of antiserum.

Cross Reactivity

Interaction of antibody with dopamine-β-hydroxylase from other sources and with the other two enzymes involved in norepinephrine biosynthesis was investigated by immunologic and enzymic methods. Extensive cross reactivity of the antibody with preparations of dopamine-β-hydroxylase from dog adrenals and human pheochromocytoma was observed in precipitin reactions. In double diffusion reactions with the antibody, lines of precipitation were obtained with dopamine-β-hydroxylase

from human pheochromocytoma and dog and guinea pig adrenal. Ouchterlony plates of the first two preparations are shown in Fig. 3. A line of precipitation was also observed with the partially purified tyrosine hydroxylase from bovine adrenal medulla.

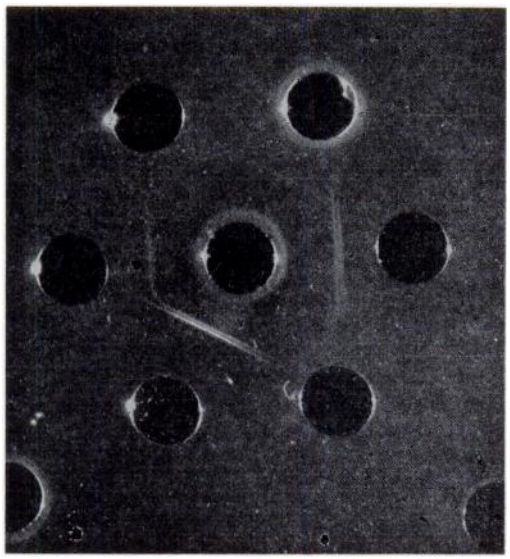

FIG. 3. *Agar gel double diffusion experiment*

Rabbit antiserum to bovine dopamine-β-hydroxylase was placed in the center well. The peripheral wells were filled as follows: 1 o'clock, human pheochromocytoma; 3 o'clock, bovine dopamine-β-hydroxylase; 5 o'clock, saline; 7 o'clock, dog dopamine-β-hydroxylase; 9 o'clock, bovine adrenal tyrosine hydroxylase; and 11 o'clock, saline.

No cross reactivity was observed between the dopamine-β-hydroxylase-directed antibody and aromatic L-amino acid decarboxylase from guinea pig kidney. Cross reactivity to dopamine-β-hydroxylase was also demonstrated by enzyme inhibition. As shown in Table 1 an amount of antiserum which inhibited the homologous antigen from 90 to 98% inhibited the enzyme from human pheochromocytoma 61–86% and the guinea pig adrenal enzyme 47–51%.

The antisera to dopamine-β-hydroxylase were not inhibitory to partially purified tyrosine hydroxylase obtained from the same organ, bovine adrenal medulla. This is shown in Table 2. No inhibition of tyrosine hydroxylase was observed even when the proportion of antibody to enzyme was raised to 30-fold that in the experiment shown in Table 2. Antisera to dopamine-β-hydroxylase had no appreciable inhibitory effect on the activity of

TABLE 1

Inhibition of dopamine-β-hydroxylase activity by antibody

In each experiment the enzyme was added to 0.02 ml of the antiserum from one of the immunized animals. The reaction was started by addition of substrate and incubated for 10 min. The percent inhibition is the dopamine-β-hydroxylase activity observed with the antiserum compared to the enzyme activity obtained when normal rabbit serum was added to the incubation mixture.

Rabbit No.	Inhibition (%)		
	Bovine	Human	Guinea Pig
1	94	79	51
2	90	66	47
3	98	61	51
4	91	86	47

TABLE 2

Tyrosine hydroxylase activity in the presence of antiserum to dopamine-β-hydroxylase

Serum, 0.01 or 0.02 ml, from normal or immunized rabbits was added to 0.03 ml of tyrosine hydroxylase (specific activity 35 mμmoles/mg protein/hr). The reaction was started by addition of a mixture of substrate and cofactor and was incubated for 10 min.

	Serum added (ml)	DOPA formed[a] (mμmoles/hr)
Control	–	74.5
	–	80.5
Normal serum	0.010	86.0
	0.020	81.5
Rabbit antiserum 1	0.010	80.5
	0.020	78.0
Rabbit antiserum 2	0.010	84.0
	0.020	78.0
Rabbit antiserum 3	0.010	78.5
	0.020	76.5
Rabbit antiserum 4	0.010	86.5
	0.020	78.0

[a] This was calculated from the tritium released into water.

TABLE 3

Effect of antiserum to dopamine-β-hydroxylase on aromatic L-amino acid decarboxylase

All flasks contained guinea pig kidney enzyme, 10^{-3} M iproniazid, 7×10^{-5} M pyridoxal 5-phosphate, 0.08 M Tris, pH 9.0, and 0.02 ml of serum, where added. After 10 min of prior incubation, the substrate, 5-hydroxytryptophan, was added to start the reaction. 5-Hydroxytryptamine (5HT) was assayed at 15 and 30 min.

	μg 5HT/15 min		μg 5HT/30 min	
	0.1 ml enzyme	0.2 ml enzyme	0.1 ml enzyme	0.2 ml enzyme
Control	54	118	118	232
Normal serum	64	—	132	—
Dopamine-β-hydroxylase antiserum	56	124	118	236

aromatic-L-amino acid decarboxylase from guinea pig kidney (Table 3).

DISCUSSION

Immunization of rabbits with purified bovine dopamine-β-hydroxylase yielded a highly potent antibody. Sage *et al.* (8) had previously noted that dopamine-β-hydroxylase, present as a contaminant in a preparation of chromaffin granule protein, was a potent antigen. The antibody produced in the present studies not only was directed against dopamine-β-hydroxylase from beef adrenals but also exhibited cross reactivity with the enzyme from dog, human, and guinea pig sources. This was shown by immunologic and enzymic methods.

The antibody to dopamine-β-hydroxylase does not appear to cross react with the other two enzymes involved in norepinephrine biosynthesis. The observance of a weak line of identity on double diffusion plates between the antibody and tyrosine hydroxylase is due to a slight amount of dopamine-β-hydroxylase which was found as a contaminant in the tyrosine hydroxylase preparation. This is not surprising since the latter is only 60-fold purified and was obtained from the same organ as the dopamine-β-hydroxylase antigen. Failure to observe tyrosine hydroxylase inhibition even with massive amounts of antibody suggests that the antibody does not react with tyrosine hydroxylase itself.

The cross reactivity between dopamine-β-hydroxylase from human, guinea pig, and dog with the antibody against the bovine enzyme indicates a structural similarity of this enzyme in these widely divergent species. It will be of interest to see whether the enzyme from even more distant species interacts with the antibody.

The ability of the antibody to inhibit dopamine-β-hydroxylase activity so markedly suggests that it may serve as a useful pharmacologic tool. The most obvious use of such a specific antibody is to help determine the localization of the norepinephrine synthesizing apparatus within the sympathetic nervous system. This can be done with immunochemical methods in conjunction with labeling for fluorescence and electron microscopy, which provide powerful tools for histochemical studies. Species cross-reactivity is fortunate since labeled antibody against the beef adrenal enzyme will be useful for experimental and diagnostic studies in tissues of species which are generally used in the laboratory.

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