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Immunofluorescent Localization of Dopamine β-Hydroxylase in Tissues

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SUMMARY

Dopamine β-hydroxylase from bovine adrenal medulla has been prepared in a form free from antigenic contaminants. The purified enzyme was used to induce the production of immunologically pure antienzyme in rabbits. The latter was utilized in the indirect immunofluorescent method for localization of dopamine β-hydroxylase in peripheral tissues and brain. The cross-reactivity of the antibody to the bovine enzyme permits its application to studies with tissues from laboratory animals.

INTRODUCTION

Of the three enzymes involved in the synthesis of norepinephrine from tyrosine, dopamine β-hydroxylase is the most difficult to localize in tissue extracts or homogenates. The adrenal medulla is the only organ in which meaningful measurements of dopamine β-hydroxylase activity can be made. In all other organs, only a small percentage of the activity which would be predicted from norepinephrine synthesis rates observed *in vivo* can actually be measured. This difficulty is apparently due to the presence of potent native enzyme inhibitors in tissue extracts (1–4). Antibodies to dopamine β-hydroxylase have been prepared (5), and they offer a possible solution to this problem. Since immunochemical methods of protein analysis operate independently of enzymatic activity, they permit localization of the enzyme in the presence of inhibitors. In addition, antibody-tracing methods can be applied to thin tissue sections in which the morphological relationships have not been disturbed by homogenization. Cellular localization of the synthetic enzymes involved in norepinephrine biosynthesis can also yield information which may not be obtainable by direct staining of the catecholamine itself (6).

This paper reports the preparation of a highly specific antibody to bovine adrenal dopamine β-hydroxylase and its application to immunofluorescent tracing of the enzyme in tissues.

MATERIALS AND METHODS

Enzyme Assay

The assay described by Friedman and Kaufman (7), using ^{3}H-tyramine as substrate and measuring formation of ^{3}H-octopamine, was modified in the following manner. To each 1 ml of incubation mixture were added 5–10 μmoles of *N*-ethylmaleimide, to inactivate sulfhydryl inhibitors (2), and 1.0 μmole of the monoamine oxidase inhibitor pargyline hydrochloride (Abbott

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Laboratories). Incubation time was 10 min at 30°.

Enzyme Purification

All steps were performed at 4°. Buffers were prepared by dilution of 1 M potassium phosphate, pH 6.2, to the desired concentration.

Preparation of bovine adrenal medullary particles. With the exception that the adrenal cortex was grossly separated from the medulla prior to homogenization, adrenal particles were prepared according to Friedman and Kaufman (7).

Solubilization of enzyme. Adrenal particles were suspended in an amount of 0.1% hexadecyltrimethylammonium bromide (Distillation Products Industries) 1.5 times the medullary weight and homogenized for 1 min in a Waring Blendor. The homogenate was then centrifuged at 17,000 × *g* for 20 min. The supernatant fluid, collected by filtration through a plug of glass wool, was brought to 80% saturation with solid ammonium sulfate and adjusted to pH 7. The precipitate was collected by centrifugation at 17,000 × *g* for 20 min and dissolved in a volume of 0.02 M buffer equivalent to 30% of the original medulla weight. This solution was brought to 70% saturation with solid ammonium sulfate, and the precipitate was again collected by centrifugation and redissolved in a volume of 0.02 M buffer equivalent to 25% of the original medulla weight. This solution was gently stirred for 10 min following the addition of acid-washed Norit (1 mg/ml). The Norit was removed by centrifugation, and the supernatant solution was dialyzed against 0.02 M buffer.

Ammonium sulfate fractionation. The following ammonium sulfate precipitates were prepared by adding saturated ammonium sulfate, pH 7.0: 10%, 30%, 40%, 50%, and 60%. Each of the precipitates was dissolved in 2 ml of 0.02 M buffer and assayed for enzymatic activity. The precipitates obtained at 40% and 50% saturation generally contained up to 80% of the total activity. These ammonium sulfate fractions were dialyzed against the starting buffer and subjected to DEAE-Sephadex (A-50) and Sephadex G-200 chromatography according to Friedman and Kaufman (7). Following the second chromatography on Sephadex G-200, the protein appeared as a single band on disc electrophoresis [method of Davis (8)], with a specific activity of 75–85 μmoles of octopamine formed per milligram in 10 min at 30°.

Preparation of Antibody

In the above manner, 8.0 mg of pure dopamine β-hydroxylase were prepared. As a final step in purification, the enzyme was subjected to disc gel electrophoresis on eight gels with 0.15 mg of protein in each. Immediately after electrophoresis each gel was removed from the tube, immersed for about 3 min in cold 0.003% anilinonaphthalenesulfonate, buffered with 0.05 M sodium phosphate (pH 6.4), and observed with a portable ultraviolet lamp. This procedure for localizing unfixed protein on acrylamide gels is described in detail elsewhere (9). When the enzyme band was visible it was excised. The small gel segments containing pure enzyme from each tube were combined and homogenized in a VirTis blender with 2 ml of 0.9% NaCl and 2 ml of complete Freund's adjuvant. Then 0.25-ml aliquots of the homogenates were injected subcutaneously in four places on the backs of each of four rabbits, each animal receiving 0.3 mg. The dose was repeated every 2 weeks for 6 weeks, at which time all the rabbits had specific precipitating antibody levels exceeding 2 mg/ml of serum by quantitative precipitin titration. The rabbits were given booster injections at 2-month intervals.

Specificity of Antibody

Immunoglobulin was precipitated from the serum at 50% saturation with ammonium sulfate (10). Double-diffusion agar plates were prepared to test the antibody for reaction against the following: (a) purified dopamine β-hydroxylase, (b) adrenal medulla particle (0.1% hexadecyltrimethylammonium bromide) extract concentrated by precipitation with 50% saturated ammonium sulfate, and (c) the protein eluted before and after the enzyme from the Sephadex G-200 chromatograms, pooled, and concentrated by precipitation in 70%

saturated ammonium sulfate. The last of these solutions (c) contained no enzyme activity and served as a control to detect any contaminants likely to have been injected during inoculation. As shown in Fig. 1, the first two protein solutions gave a single precipitin band, and the third, containing the most likely contaminating proteins, showed no reaction.

Immunoelectrophoresis was done on the purified enzyme and crude adrenal extract, and the antibody was compared with an antibody previously prepared to an enzyme carried through fewer steps of purification (5). Figure 2 shows that the antibody to the less purified preparation produced multiple precipitin lines to the crude adrenal extract, and two lines with the enzyme purified through the entire procedure except for the disc gel electrophoresis. The second component in the latter is a large molecular weight protein eluted from Sephadex G-200 chromatograms just before the dopamine β-hydroxylase. The purified fractions from the Sephadex G-200 chromatogram contained less than 3% of this contaminating protein. Because the contaminant migrated toward the negative electrode, it was completely separated from dopamine β-hydroxylase on disc gel electrophoresis. As shown in Fig. 2, the antibody produced from the enzyme further purified on disc gel electrophoresis showed only one line against the purified enzyme. Furthermore, only a single precipitin line was obtained between the antibody to the disc gel-purified enzyme and the crude adrenal particle extract. This last observation is of primary importance, since it indicates that the antibody was specific enough to distinguish the enzyme from other proteins which would be present in tissue sections during attempts to localize the enzyme by the application of immunofluorescent techniques.

Previous studies from this laboratory had shown that antibody to bovine dopamine β-hydroxylase cross-reacts with the enzyme derived from the adrenals of all other species investigated (5). Because of the cross-reactivity, reconfirmed in the present study, it was possible to use the antibody for fluorescent studies in tissues of other species.

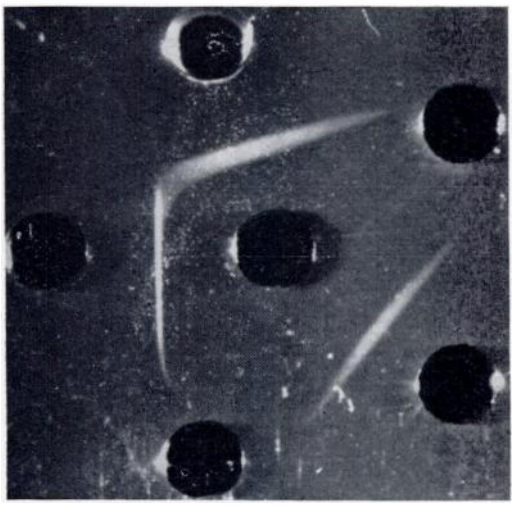

FIG. 1. *Ouchterlony plate, showing the specificity of anti-dopamine β-hydroxylase*

The center well contained anti-dopamine β-hydroxylase; peripheral wells: 11 o'clock, purified enzyme; 2 o'clock and 7 o'clock, enzyme-free contaminants from Sephadex G-200 chromatograms; 4 o'clock and 9 o'clock, bovine adrenal medulla particle extract. The plate was developed for 4 days at 4°.

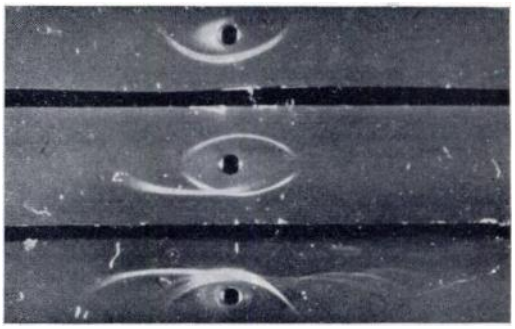

FIG. 2. *Immunoelectrophoretic demonstration of the specificity of antienzyme*

The top trough contained anti-dopamine β-hydroxylase induced with enzyme chromatographed by disc gel electrophoresis (see the text). The bottom trough contained antibody to enzyme purified only as far as the DEAE-cellulose chromatography step. The top and bottom wells contained crude adrenal particle extract, and the center well contained enzyme purified through the entire procedure except for disc gel electrophoresis. The positive terminal is at right; medium, 1% agarose-barbiturate buffer, pH 8.2, ionic strength 0.04; developed for 4 days at 4°.

TABLE 1

Comparison of fluorescence emitted by methanol-fixed bovine adrenal sections following addition of specific antienzyme of various control solutions in the first step of the indirect method

Tissue	Step I: nonfluorescent controls or antienzyme[a]	Observed fluorescence intensity
1. Adrenal medulla	None	None
2. Adrenal medulla	0.9% NaCl	None
3. Adrenal medulla	Normal rabbit globulin (2 mg/ml globulin)	Faint
4. Adrenal medulla	Antienzyme adsorbed with dopamine β-hydroxylase (2 mg/ml globulin)	Faint
5. Adrenal medulla	Specific antienzyme (2 mg/ml globulin)	Strong
6. Adrenal cortex	Specific antienzyme (2 mg/ml globulin)	Faint to none

[a] In step II of the indirect method, antibody was localized in the tissue section with fluorescein-conjugated anti-rabbit globulin (sheep origin), 0.5 mg/ml. See the text for details of the experimental procedure.

Fluorescent Staining Technique

Preparation of fluorescent anti-rabbit globulin. Concentrated anti-rabbit globulin (sheep origin) was obtained from Progressive Laboratories, Inc., Baltimore, lot 4312. This γ-globulin fraction was first chromatographed on DEAE-cellulose and then conjugated with fluorescein isothiocyanate according to Wood *et al.* (11). The conjugated protein was chromatographed on DEAE-cellulose, and fractions containing 2–4 moles of fluorescein per mole of γ-globulin [determined by the method of Wells *et al.* (12)] were used in the second step of the indirect (antiglobulin) method for localizing specific antibody (10).

Three-millimeter slices of fresh tissue were immediately frozen against the wall of a beaker immersed in Dry Ice–acetone and sectioned at 3–7 μ with a cryostat. The sections were then allowed to dry in air and fixed in methanol or chloroform-methanol (2:1 by volume) for 30 min at 0° prior to staining. Unfixed tissues in general gave adequate fluorescence but showed substantial loss of cytological detail. The following fixatives were tried but were found to inhibit the antigen-antibody reaction: ethanol, acetone, 10% formaldehyde, and 2% glutaraldehyde. Methanol gave excellent results in tissues other than brain, and chloroform-methanol (2:1) was found to be best for brain.

Method of staining. Fixed sections were covered with unconjugated antienzyme and various control solutions (see below). The slides were allowed to incubate at 30° for 1 hr in a 100% humidity chamber. They were then removed, washed vigorously by immersion and agitation in three changes of phosphate-buffered NaCl (0.16 M NaCl and 0.02 M sodium phosphate, pH 7.5), and immersed once in distilled water to remove the salt residue. The washed slides were covered with fluorescent anti-rabbit globulin, 0.5 mg/ml, and again incubated for 1 hr. After this, the slides were washed as before and mounted in a mixture of equal volumes of glycerol and 0.1 M phosphate buffer, pH 8.0. The sections were observed with a Leitz Orthomat fluorescence microscope equipped with an HBO 200 high-pressure mercury lamp and a blue excitation filter, No. BG 12. An amber barrier filter with a low cut-off wavelength, including a portion of the blue spectrum, was used.[3] Photographs were taken with Anscochrome 500 film.

Table 1 lists the various types of controls used and the results obtained in each case when slices of bovine adrenal gland were used. Similar controls were carried out with the other tissues studied, and similar results were obtained. The controls shown in the figures described under RESULTS were prepared with antienzyme adsorbed with dopamine β-hydroxylase purified to the second Sephadex G-200 step. Exposure times

[3] A more suitable barrier filter may be the K530 blue absorption filter. However, one must accept some decrease in sensitivity with this filter.

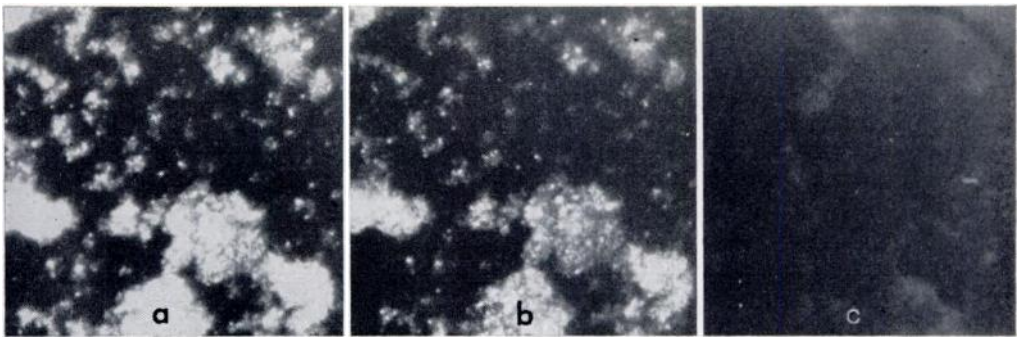

FIG. 3. *Demonstration of dopamine β-hydroxylase in purified bovine adrenal particles*
(a) Standard dark-field illumination of an immunofluorescent preparation of adrenal particles. (b) Specific immunofluorescence emitted from the same field as (a) when excited with ultraviolet light as described under MATERIALS AND METHODS. (c) Control for (b): adrenal particles were treated with anti-enzyme adsorbed with dopamine β-hydroxylase as described in Table 1. The particle smear was fixed with methanol and photographed at 400×.

varied from 1 to 3 min, depending upon the intensity of the individual slide. In each case the control photographs were exposed manually for the same time as that determined by the automatic light meter in order to obtain the best results with the specifically stained microscopic field.

RESULTS

Purified Washed Bovine Adrenal Particles

Figure 3a shows a microscopic field from a smear preparation of washed and stained adrenal particles as seen under standard dark-field illumination. Figure 3b shows the specific fluorescence emitted from the same field. It should be noted that virtually every particle emitted fluorescence. The control (Fig. 3c) shows essentially no fluorescence. The implication is that every medullary particle had enzyme associated with it and was capable of converting dopamine to norepinephrine.

Beef Adrenal

Frozen sections (3 μ) were prepared and fixed in methanol. In such sections, as in adrenal particles, specific staining of dopamine β-hydroxylase was easy to demonstrate. The stained adrenal medulla section and its control are shown in Fig. 4a and b. Medullary cells stand out as bright, greenish-yellow cytoplasmic fluorescence. Essentially all the medullary parenchymal cells showed a positive reaction. There was, however, a moderate amount of variation in intensity, suggesting heterogeneity in the number of particles per cell. The most intensely stained cells were generally found in the peripheral part of the medulla (Fig. 4a).

Rat Adrenal and Kidney

Figure 5a and b shows the adrenal cortical medullary junction and its control with brightly fluorescent (greenish-yellow) medullary parenchymal cells. In these sections the specific fluorescence appears granular, with the granules being more prominent near the cell membrane. The adrenal cortex shows bright blue autofluorescence, with almost complete absence of green fluorescence in both control and stained sections.

The kidney section and its control (Fig. 6a and b) show the specific fluorescence for dopamine β-hydroxylase as a fine network around renal tubules. These may represent sympathetic fibers. There is no fluorescence in the glomeruli. This field is from the distal cortex. The intensity of staining in the renal medulla (not shown) was much less than in the cortex.

Rat Brain

It has been possible to demonstrate specific immunofluorescence in brain following fixation of the tissue with chloroform-methanol (2:1). However, most areas of the

brain have shown considerable nonspecific binding of control globulins. For this reason, interpretation of immunofluorescent localization of dopamine β-hydroxylase in most areas of brain cannot yet be reported with certainty. Recent modifications of the staining procedure have solved many of the technical problems and should make it possible to apply the immunofluorescent technique successfully to brain.

The one area where there has been no difficulty in demonstrating the presence of dopamine β-hydroxylase, even without fixation, is the hypothalamus. Figure 7a and b shows specific staining in the hypothalamus; Fig. 7c serves as a control. These fields are from the area of the supraoptic nuclei, the area most easily recognized anatomically. Fluorescence was found both in cell bodies of neurons and in what appear to be neuronal processes.

Blood Vessels

One or two large, intensely staining areas were almost always observed adjacent to muscular arteries in the adrenal and kidney. Figure 8 shows examples of such observations. Comparable fluorescent areas have not been observed by the fluorescent histochemical stain for catecholamines (6). The large amounts of dopamine β-hydroxylase adjacent to blood vessels indicate that the norepinephrine found there is synthesized locally.

DISCUSSION

The purpose of this paper was to demonstrate the feasibility of using immunofluorescent techniques for the localization of the enzymes involved in catecholamine biosynthesis and metabolism. The major problem in each application is the preparation of antigenically pure enzyme. The procedures reported here for the preparation of immunologically specific anti-dopamine β-hydroxylase and its use for immunofluorescent localization in tissues can serve as a model for subsequent studies with the other enzymes involved in the biochemistry of the sympathetic nervous system. Some of the technical problems raised in this report have been solved by recent advances in staining procedure. Furthermore, by substituting an electron-dense label for fluorescein, it should be possible to increase the resolution of this procedure to the level of electron microscopy.

It is already evident from these initial studies with dopamine β-hydroxylase that immunofluorescent staining can be used as an independent method to verify observations made by other procedures and to yield information which is not obtainable by existing methods. For example, the method of Falk for demonstrating catecholamines (6) provided a great deal of valuable information concerning the localization of neurotransmitters in tissues. However, it is conceivable that some tissues

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FIG. 4. *Demonstration of dopamine β-hydroxylase in bovine adrenal medulla*

(a) Specific immunofluorescence observed in medullary cells. (b) Control section, similar field. Sections 3 μ thick were fixed in methanol and photographed at 400×. The blue emission in this and subsequent figures represents connective tissue or scattered light from artifacts present in some of the fields.

FIG. 5. *Rat adrenal cortical medullary junction*

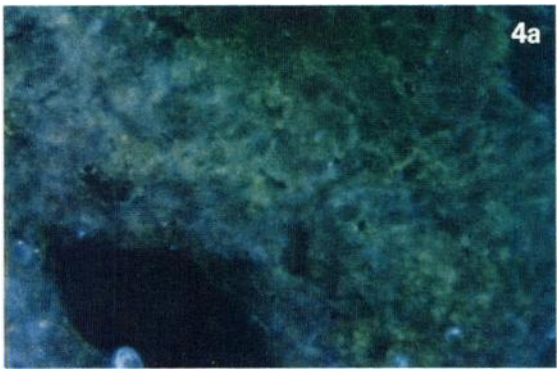
(a) Specific immunofluorescence in the medulla (*M*) and nonspecific blue emission in the cortex (*C*). (b) Control section, similar field. Sections 5 μ thick were fixed in methanol and photographed at 250×.

FIG. 6. *Dopamine β-hydroxylase in rat kidney*

(a) Specific immunofluorescence observed in the distal cortex: *T* = tubules; *G* = glomerulus. (b) Control for (a): the blue line transecting the artery is caused by light scattered from a surface scratch on the slide. Sections 5 μ thick were fixed in methanol and photographed at 250×.

FIG. 8. *Immunofluorescent localization of dopamine β-hydroxylase adjacent to blood vessels in rat kidney cortex*

(a) Small artery. (b) Medium-sized artery. Sections 5 μ thick were fixed in methanol and photographed at 250×. The control for these sections is Fig. 6b.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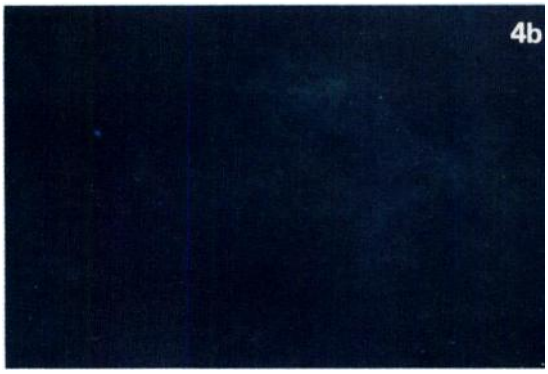
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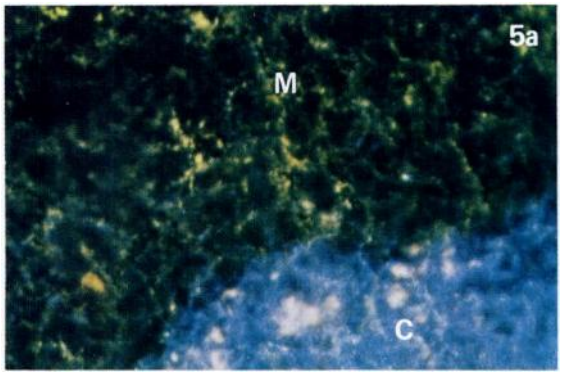
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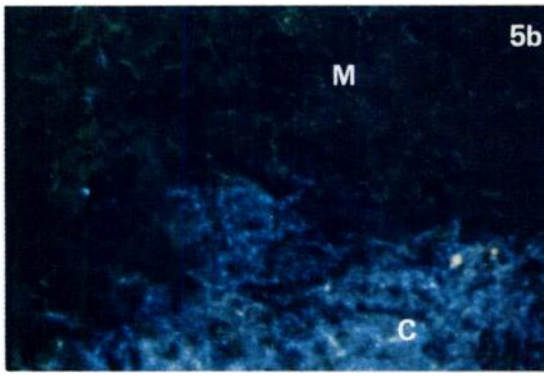
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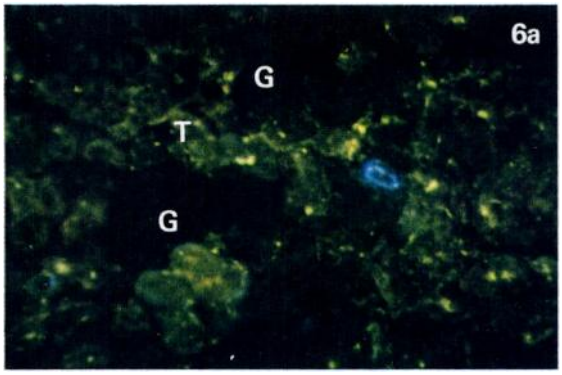
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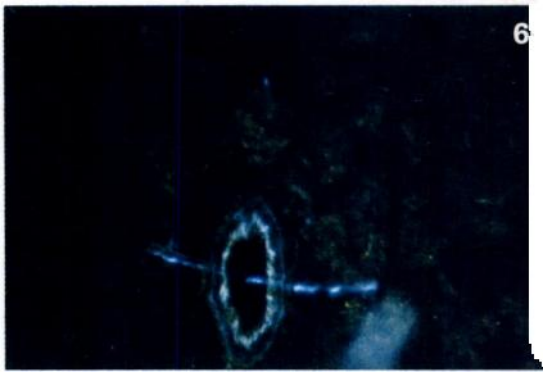
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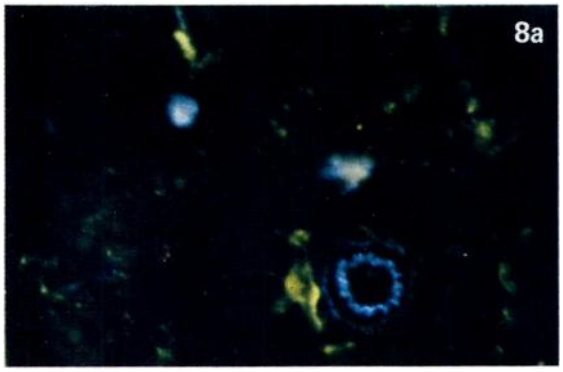
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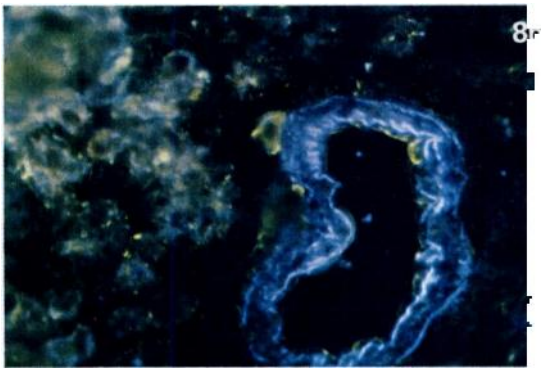
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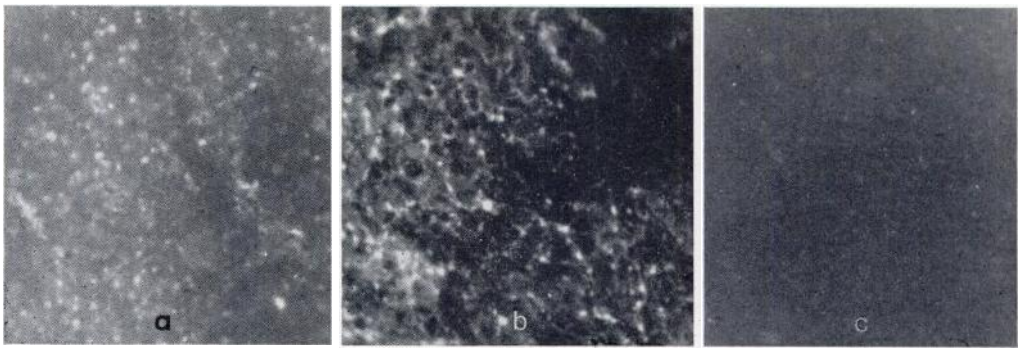

FIG. 7. *Demonstration of dopamine β-hydroxylase in rat brain from the area of the supraoptic nucleus of the hypothalamus*

(a) Specific immunofluorescence, photographed at 100×. (b) Specific immunofluorescence from another preparation, photographed at 250×. (c) Control for (a). Sections 5 μ thick were stained without the use of a fixative.

which do not show much staining for neurotransmitters by this method may contain large amounts of the enzymes responsible for their synthesis. This would be true in areas where the transmitter pool size is small but the turnover is very rapid. Such situations can be revealed readily by the immunofluorescent procedure. Furthermore, on theoretical grounds, histochemical localization of a macromolecule should be more reliable than the localization of a small molecular weight, diffusible substance such as norepinephrine.

Several of the observations made in this initial report are interesting enough to warrant further investigation. One of these observations relates to the catecholamine-containing adrenal particles, which have been widely investigated (13). Since all the particles were found to contain dopamine β-hydroxylase, there would appear to be none which specialize solely in storage of the transmitter or solely in the conversion of norepinephrine to epinephrine.

The finding of large accumulations of dopamine β-hydroxylase adjacent to blood vessel walls indicates a capacity for producing large amounts of norepinephrine. Since the total mass of blood vessels is very large, it is possible that blood vessels are the major producers of norepinephrine in the body. Similar suggestions concerning the blood vessels as a major site for norepinephrine production were made by Bigelow *et al.* (14) and by Spector and Berkowitz[4] on the basis of entirely different experimental procedures.

ADDENDUM

A preliminary communication concerning immunohistochemical evidence for the transport of dopamine β-hydroxylase in sympathetic nerves appeared while this manuscript was in preparation (15).

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