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The Application of Immunological Techniques to the Study of Enzymes Regulating Catecholamine Synthesis and Degradation

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THE techniques of immunology are rapidly developing into major tools for the biochemist. Various immunological techniques may be used 1) for quantification of the amount of enzyme protein present, 2) to localize the enzyme in microscopic tissue sections where anatomical relationships are preserved, and 3) to aid in estimation of the rate of enzyme synthesis and degradation. Furthermore, because of the relative stability of the antigenicity compared to enzyme activity, it is possible to carry out these studies after conditions or treatment which would make the enzymes inactive.

In addition to the above applications, immunochemical techniques have proved useful in the study of protein structure. Antigen cross-reactivity between related proteins has been shown to be a good indicator of structural similarities or differences. It has thus been possible to examine the relationship between enzymes from different species, different organs of the same species, as well as different forms of an enzyme in the same organ.

This paper will discuss several studies which make use of antibodies directed toward dopamine-β-hydroxylase (DBH) and monoamine oxidase (MAO).

Enzyme Purification and Antibody Preparation

The most important factor in determining the success or failure of most of the immunological techniques is starting with an immunologically pure, antigenically specific, high titer antiserum. This in turn requires an enzyme with a greater degree of purity than is required for almost any other purpose. It has been necessary to use the resolving power of acrylamide electrophoresis as a final purification step. A rapid staining technique for localization of proteins on acrylamide gels without acid fixation has been previously described (10). The segment of the gel containing the enzyme is then cut from the remainder of the gel and homogenized with Freund's adjuvant. A suitable animal is then inoculated. After the antiserum is obtained, it is important to determine its purity and specificity, regardless of the apparent purity of the antigen. The demonstration of a single immunoprecipitin line on immunoelectrophoresis against

purified enzyme is not sufficient evidence for purity and specificity. Two criteria for purity and specificity should be met: (a) The antiserum should produce a single immunoprecipitin line when run against a crude tissue extract where the relative concentration of enzyme and other proteins is more consistent with that found in the systems to be investigated; and (b) no immunoprecipitin lines should form when antiserum is tested against the protein fractions eluted from chromatographs before and after the enzyme during purification. These fractions will contain the proteins most likely to contaminate the inoculating dose. An example of the application of these tests to our antibody to DBH has been previously described (11).

Immunoassay. In general, the sensitivity of immunoassays can be made to compete with some of the most sensitive enzyme assays. The method applied here to DBH is complement fixation. The complement fixation assay does not require the use of purified antigen and can be applied to proteins which are membrane-bound. These features make it especially useful for the assay of DBH. The microquantitative complement fixation procedure described by Perez and Moore (16) was used in this study.

With this procedure, it was possible to measure beef adrenal DBH at the level of 0.5 ng enzyme protein. Intact adrenal catecholamine storage vesicles were prepared from fresh beef adrenal medulla (4). The purified vesicles were washed three times in isotonic sucrose and resuspended in isotonic sucrose. This preparation retained 32% of the total adrenal DBH activity and will be referred to as "intact vesicles." One aliquot of intact vesicles was diluted 1 to 5 with distilled water and homogenized in a Virtis blender. This preparation will be referred to as "water lysed vesicles." A second aliquot was diluted 1 to 5 with 0.1% Triton X-100 and homogenized. This preparation will be referred to as "Triton X-100 lysed vesicles." These three preparations were then assayed for DBH activity as previously described (11) modified by decreasing the volume to 300 μl, increasing the specific activity of tyramine to 1 μc/μm and with Cu^{++} to block inhibitors. These preparations were also assayed for DBH by microcomplement fixation with anti-DBH. In order to compare the results, both assay procedures were standardized against a highly purified preparation of bovine adrenal DBH. Results are expressed in terms of enzyme protein per unit of adrenal vesicles. One unit of vesicles contained the quantity of vesicles purified from 1 mg wet weight of adrenal medulla. For the enzyme activity assay, 5 units were used, and for complement fixation only 5×10^{-3} to 10×10^{-3} units were used per assay. The results of this experiment are shown in table 1.

Both assays gave maximum results on Triton X-100 lysed vesicles, and after this form of treatment the quantity of enzyme as measured by both methods was essentially identical. In the case of the complement fixation assay, intact vesicles showed only 12% of the activity measured after Triton X-100 treatment. These results can most easily be explained by postulating that the DBH is located on the inside of the storage vesicles, where it is inaccessible to the antibody and complement. After water lyses, 78% of the complement-fixing

TABLE 1

Comparison of results obtained by enzymatic assay and microcomplement fixation assay† for dopamine-β-hydroxylase in vesicles purified from bovine adrenal medulla*

	DBH Activity			Complement Fixation	
	nm Octopamine formed/unit‡ ± S.E.§	Apparent enzyme/ unit‡	Percent maximum	Apparent enzyme/ unit ± SE	Percent maximum
		μg	%	μg	%
Intact vesicles	3.24 ± 0.22	0.45	88	0.058 ± 0.03	12
Water lysed vesicles	3.31 ± 0.15	0.46	90	0.38 ± 0.08	78
Triton-X-100 lysed vesicles	3.67 ± 0.12	0.51	100	0.49 ± 0.05	100

* DBH activity with tyramine (10^{-3} M) as substrates, pH 6.2, 20 min at 37°C; copper and catalase at optimal concentrations (5 units of vesicles/assay).

† Complement fixation assay was done on 5 to 10 × 10^{-3} units of vesicles/assay.

‡ One unit of vesicles consisted of the particles purified from 1 mg wet weight of adrenal medulla. The purified vesicle fraction contained 32% of the total DBH activity.

§ ±S.E.M. of three determinations.

DBH activity is now measurable. This is not only the result of the release of soluble DBH, because 48% of this activity could still be measured on membranes precipitated at 25,000 g for 1 hr (data not shown in table 1). Thus, once the vesicles are ruptured, even the membrane-bound DBH becomes accessible to antibody and complement. The maximum complement-fixing activity is not achieved, however, until the enzyme has been solubilized by Triton X-100. In the use of the enzyme activity assay, only a relatively small increase in activity is noted after lysis. This is presumably because the small substrate molecules can more easily gain access to the inside of the vesicle than antibody and complement. Thus, by combining standard enzyme techniques with immunological techniques in this relatively simple experiment, it has been possible to localize both the membrane-bound and the soluble DBH to the inside of the storage vesicle.

Several other aspects of the complement fixation assay may be noted. Although it is very sensitive, requiring only minute amounts of tissue, its precision is considerably less than that of the enzyme activity assay. In addition, the range of enzyme levels which can be read from the standard curve is relatively small (0.5 ng to 8 ng enzyme protein), so that it is often necessary to assay more than one dilution of an unknown.

Immunofluorescent Localization of DBH in the Central Nervous System

In brain, even more than in other tissues, the understanding of basic mechanisms requires the preservation of anatomical and physiological relationships between the various cell types. Much is known concerning the anatomical connections between various parts of the brain. Similarly, neurophysiological studies have yielded information about the functional importance of various regions of the central nervous system (CNS). Relative to the known neuro-

anatomical and neurophysiological data, however, the knowledge of the biochemical organization of the brain is quite primitive. One major exception to this generalization has been the study of the neuronal systems containing the biogenic amines. The development by Falck and Hillarp of the technique for histochemical localization of catecholamines and serotonin by formaldehyde induced fluorescence, with its improvement and application by many others, has yielded a great deal of information concerning this biochemical system.

One obvious use of antibodies to enzymes in the catecholamine pathway would be the application of antibody tracing techniques to the localization of catecholamine pathways. In the first attempts to localize DBH using the fluorescent antibody technique the resolution and sensitivity was not sufficient to clearly visualize terminals or neuronal fiber tracts (6, 11). Since then, several methodological improvements have permitted the visualization of the entire noradrenergic system, with DBH as a marker. The methodological details and the complete results of the localization and mapping of the central noradrenergic system based on DBH is presented elsewhere (13). The main aim here will be to outline some of our major findings, especially with regard to their possible implications concerning the sympathetic nervous system.

The antiglobulin sandwich technique has been used exclusively in this work. One major improvement has been the use of purified antiglobulin for conjugation with fluorescein isothiocyanate (FITC) (1). The antiglobulin was purified by affinity chromatography, with sepharose conjugated with purified rabbit IgG by cyanogen bromide linkage (2). Frozen sections (10 μ) of entire cross-sections of rat brain were fixed in chloroform methanol (2:1) and incubated with anti-DBH at 37°C. The fact that a large molecular weight protein is being localized rather than the small, diffusable catecholamines eliminates the necessity for freeze drying. The lack of cracking artifacts and the general ease of preparing tissue are major advantages of this technique. After thorough washing, the sections were incubated with purified FITC-rabbit antiglobulin (from goat). The sections were again washed and mounted for examination with a fluorescence microscope.

Cell bodies. The neuronal cell bodies containing DBH correspond precisely to those described by Dahlstrom and Fuxe (3) as containing norepinephrine (NE) by histochemical fluorescence. Most neurons are medium sized with multiple axons (fig. 1). The largest accumulation of cell bodies is in the locus ceruleus. No cell bodies were found in areas thought to contain cell bodies of dopamine neurons (3).

Nerve fibers and terminals. In contrast to DBH-containing cell bodies which are seen only in discrete areas of the medulla and pons, DBH-containing nerve fibers and terminals are observed in widespread areas of the brain. At a distance from cell bodies, the specific fluorescence for DBH is observed in several different patterns (figs. 2–7).

1. Random dots or clumps of fluorescence may represent either nerve fibers or terminals cut in cross-section (fig. 7). In a single brain section it may be impossible to distinguish these two structures. If in serial sections the random

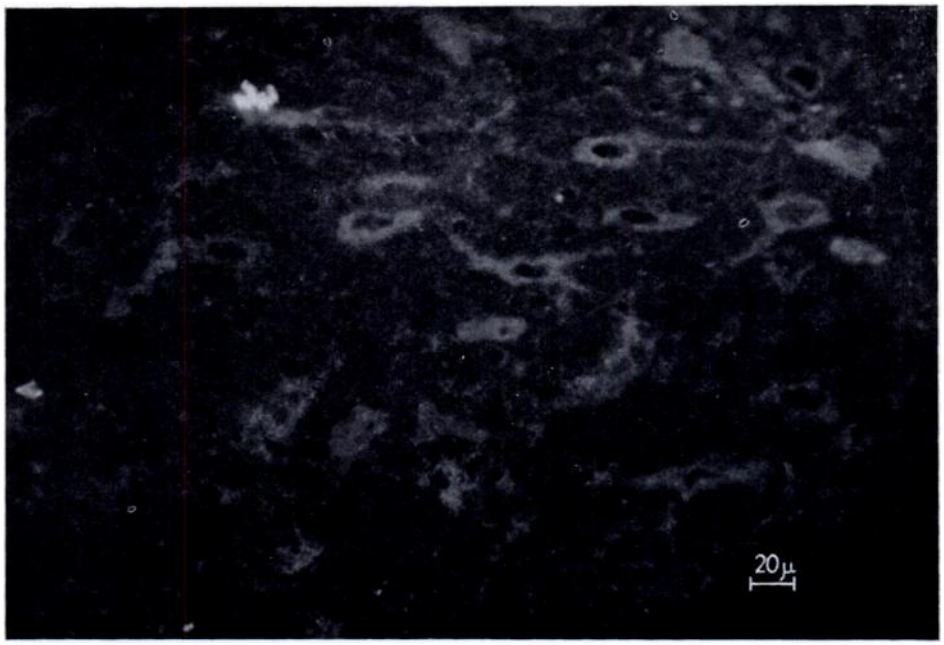

FIG. 1. Typical DBH-containing cell bodies showing multiple processes. These cells represent a ventral lateral extension of the rostral locus ceruleus.

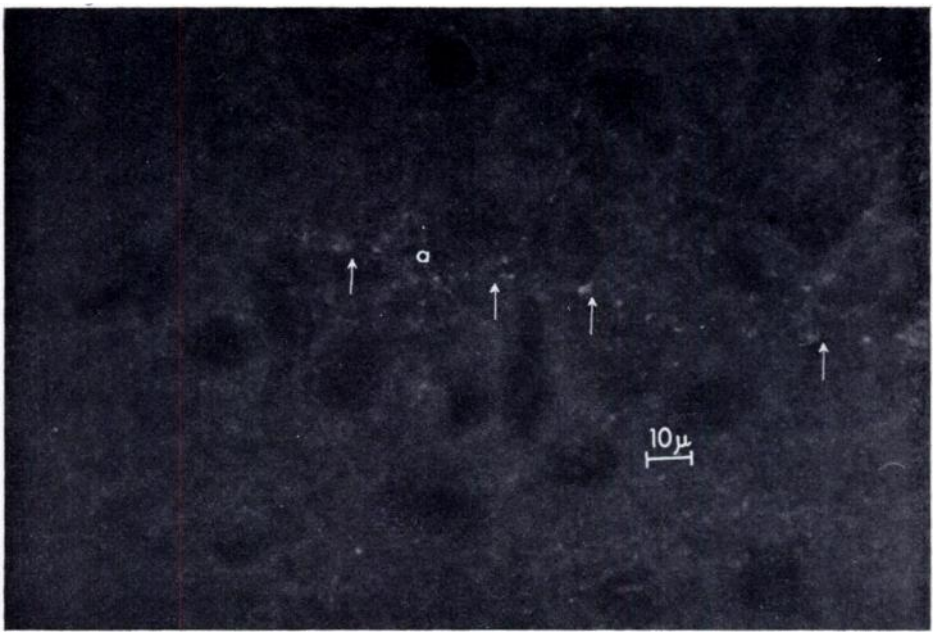

FIG. 2. Fine nerve fiber which after coming into contact with a small artery (a) gives off several branches (↑).

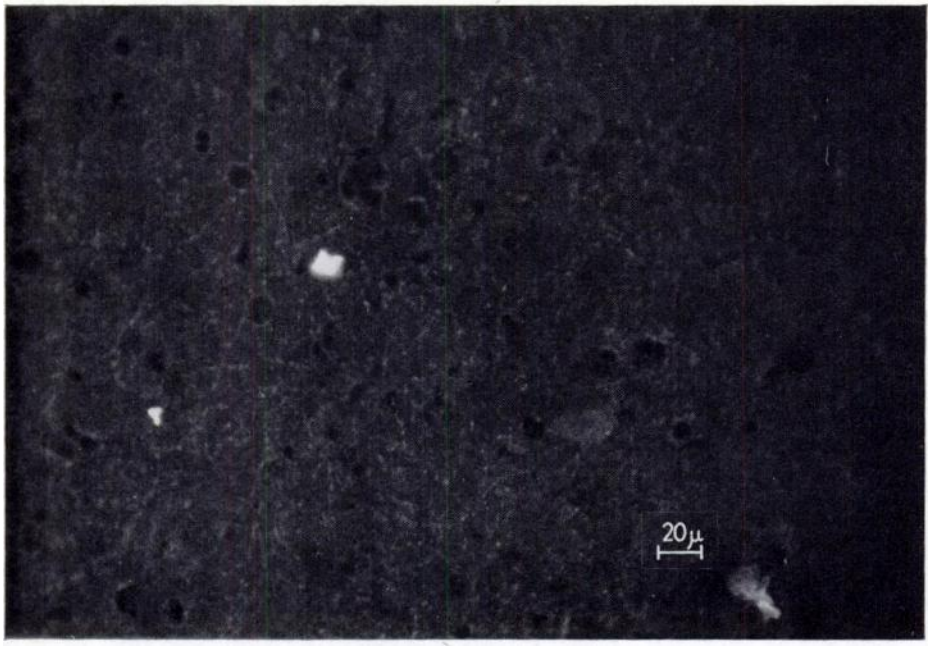

FIG. 3. Fine terminal fiber network in anterior ventral nucleus of the thalamus. Varicosities are approximately 1 μ in diameter.

dot pattern is maintained over distance it is assumed to represent a fiber pathway. The presence of such fiber pathways may be confirmed by making longitudinal sections through the same area.

2. Closely packed dots or clumps of fluorescence which form a line, but in which the dots of fluorescence do not have a regular distance between them (periodicity), are presently interpreted to represent noradrenergic nerve fibers containing many axons (fig. 5). The length of the line segment observed is related to how nearly the plane of tissue section approximates the longitudinal direction of the fiber. The thickness of these fibers is variable but their distinguishing characteristic is the lack of a regular periodicity between fluorescent dots.

3. Fluorescent dots which have a regular periodicity are interpreted as terminal fibers. The dots represent terminals (varicosities). The varicosities are seen in sizes ranging from 0.5 to 5 μ. (Example of a small terminal fiber is seen in fig. 8 and of a large terminal fiber in fig. 4.) The size range of the terminals is very similar to that observed for noradrenergic terminals using the histochemical fluorescence for NE (5).

In most sections a combination of the various patterns described is observed. The dotted or stippled appearance is characteristic of the specific staining for DBH. Smooth staining fluorescent nerve fibers have never been observed. Fibrous astrocytic processes are smooth and stain nonspecifically, but with lower intensity, and are easily recognized.

FIG. 4. High power view of a thick terminal fiber (↑). Field is from the paraventricular nucleus of the hypothalamus.

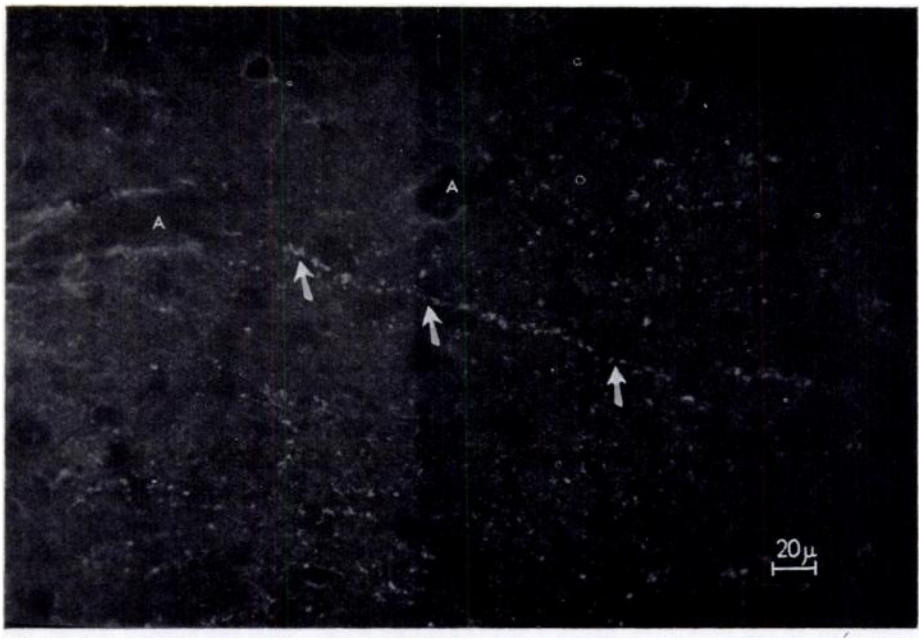

FIG. 5. Thick nerve fiber in the anterior hypothalamus cut longitudinally (↑) to its direction. This fiber appears to become associated with a muscular artery (A) which briefly dips out of the plane of section.

FIG. 6. Area of intense DBH fluorescence resulting from thick fibers and terminals in the ventral part of the interstitial nucleus of the stria terminalis.

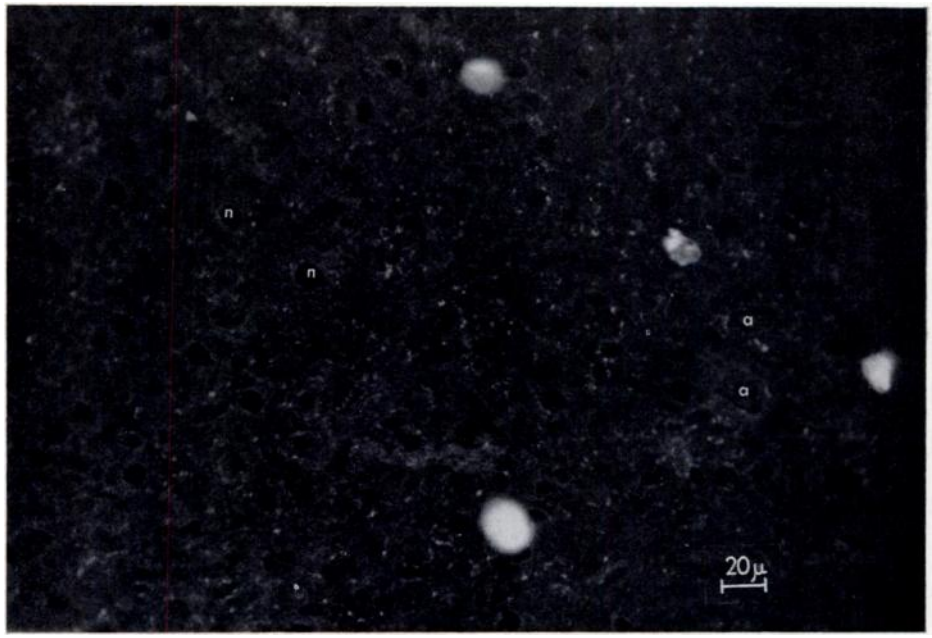

FIG. 7. Cross-section at right angles to dorsal medial fiber tract as it courses through the thalamus. Both fine fibers and thick fibers are seen, predominately as fluorescent dots of different diameters. Fibers are seen in close association with small arterioles (a) and neurons (n).

In contrast to staining observed for NE, in areas with a very high density of terminals (fig. 6) the dotted appearance is resolved and in contrast to staining for NE, no areas of diffuse fluorescence are observed. Also in contrast to the staining of NE non-terminal fibers are clearly visible in normal animals. When staining for catecholamines, some method, such as drug treatment or the creation of lesions, must be used to enhance the concentration of catecholamines before non-terminating axons can be visualized (18). This is perhaps one of the greatest advantages of the localization of DBH rather than NE for the mapping of fiber pathways in the CNS. It must be kept in mind that with the antibody technique, a protein is being localized. Once the vesicle leaves the cell body, where the enzyme is synthesized, the concentration of enzyme per vesicle is at its maximum. There is apparently enough DBH on vesicles in transit along axons to allow visualization. At the terminals the intensity of fluorescence observed is indeed increased, but this is presumably due simply to the additive effect of greater numbers of vesicles rather than increased DBH per vesicle. With localization of catecholamines the situation is different. The greater part of synthesis and accumulation of catecholamines occurs at the terminals, and synthesis in cell bodies and in transit along axons is small relative to that occurring at terminals.

With regard to the localization of noradrenergic nerve fiber pathways, the major differences observed using DBH as a marker as opposed to NE involve

the course of the dorsal fiber pathways as described in the most recent review by Ungerstedt (18). With the immunofluorescent technique, three major branches of the dorsal pathway have been observed as it transverses the mesencephalon and diencephalon. This is in contrast to a single dorsal pathway according to NE mapping. One portion of the dorsal bundle observed in the mesencephalon drifts ventrally to join the ventral pathway in the medial forebrain bundle essentially as described using staining for NE (18). Another branch (dorsal-medial pathway) drifts dorsally with the fasciculus retroflexus to a position immediately inferior to the habenula. It follows this course rosterally, giving terminal branches to the midline thalamic nuclei (fig. 7). The third dorsal pathway follows a complex diffuse route, as it spreads laterally and dorsally through the midbrain and thalamus. This pathway consists of multiple lateral branches splitting from the main trunk of the dorsal bundle. It appears as a layer of fibers spreading laterally just dorsal to the medial lemniscus. Its fibers are oriented in a rostral, lateral and dorsal direction. This pathway is responsible for fibers which send terminal branches, first into the medial and lateral geniculate bodies, next into the thalamic radiations to be distributed to the cerebral cortex, and next to the terminal areas in the anterior thalamic nuclei (fig. 3). Both of these dorsal pathways eventually appear to send fibers ventrally into hypothalamic nuclei. DBH mapping of the ventral ascending pathway coincides with that outlined by Ungerstedt (18).

With regard to the dopaminergic pathways, we have not seen any cell bodies or fiber tracts in those areas thought to be exclusively dopaminergic. These include the substantia nigra, and the caudate-putamen. This offers important confirmational data for the results of histochemical fluorescent localization of dopamine, a procedure requiring the observation of a spectral shift upon heating. Furthermore, the fact that DBH is specific for the NE pathway has important theoretical implications. One possibility was that DBH was indeed present in the dopaminergic system, but inhibited. Thus, if the inhibition were released, NE could have proceeded at an extremely rapid rate, because of the high concentration of substrate. The absence of DBH in these areas essentially rules out this possible mechanism. Another attractive hypothesis would be that all the genetic information for production of the catecholamine enzymes are located in a single cistron. If this is correct, some mechanism would need to be present to turn off the synthesis of DBH in dopaminergic neurons. The recent report of DBH activity in the caudate nucleus (17) can probably best be explained by the inclusion, during dissection, of parts of adjacent nuclei high in DBH content. For example, the very large interstitial nucleus of the stria terminalis (fig. 6) has the highest concentration of DBH terminals of any nucleus in the brain, and is very near to the caudate-putamen in some areas.

The terminals of the DBH neurons are found in the same anatomical regions as NE. Fibers appear to terminate on neuronal cell bodies (figs. 7 and 8) but a greater number seem to be in areas between neurons, where they may effect the dendrites of many neurons simultaneously. In addition, terminals and fibers very frequently are associated with blood vessels, mainly arteries and arterioles.

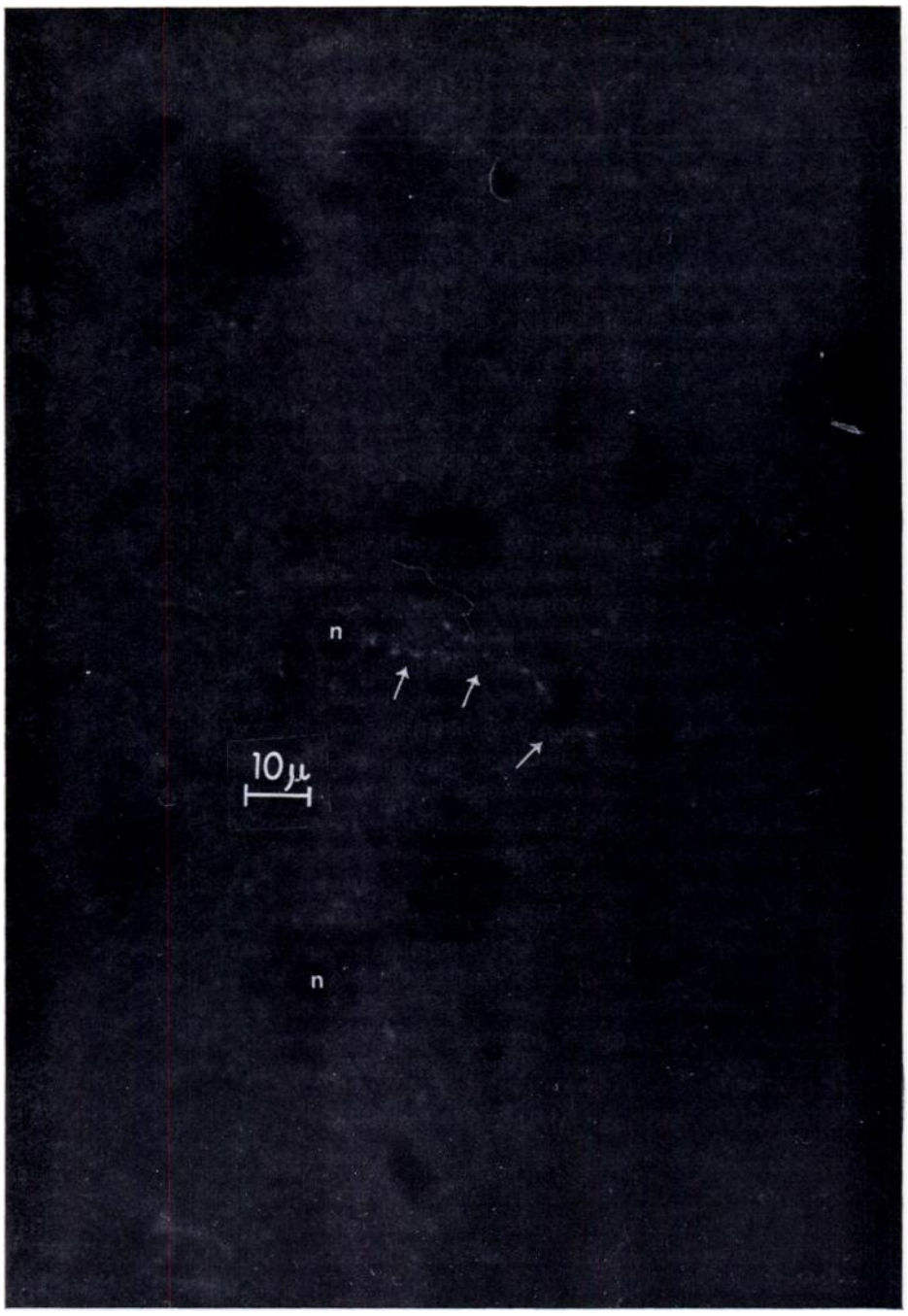

FIG. 8. DBH-containing terminal fiber giving off a small branch which makes close contact with the cell body of a neuron (n).

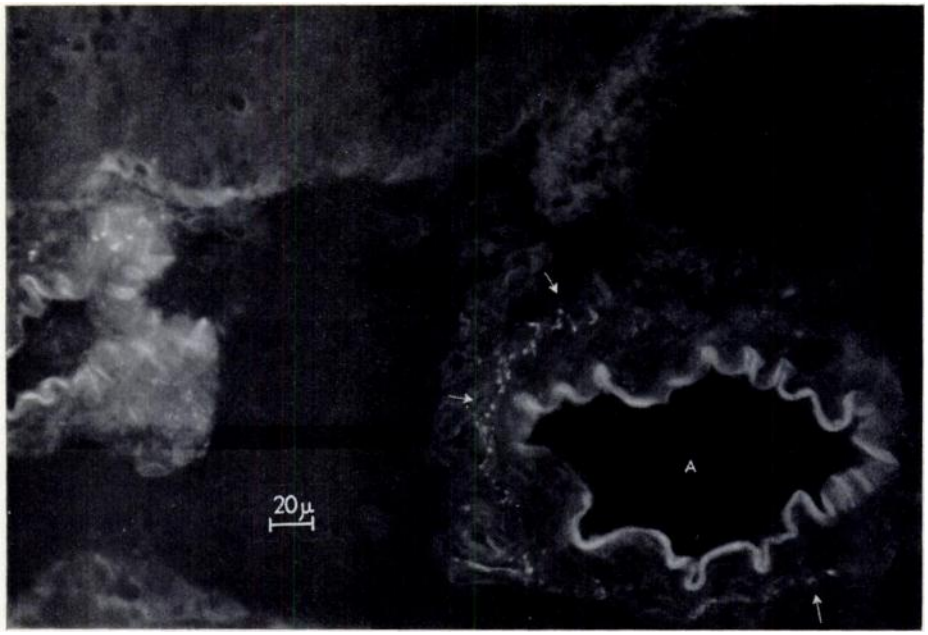

FIG. 9. Cross-section of a muscular artery (A) with a DBH fiber (↑) coursing through the outer muscular layer. The elastic tissue in the convoluted surface of the lumen is autofluorescent. These vessels are located on the ventral surface of the brain near the optic chiasm.

This is true not only near the surface of the brain (fig. 9), but deep within the parenchyma itself (figs. 2, 5, and 7). This observation has not been emphasized by those localizing NE alone. We have also traced fibers from cell bodies in the locus ceruleus to nearby blood vessels. Furthermore, fibers have been observed approaching small arteries at right angles, rather than coursing parallel to them, as one would expect if the axons were derived from the peripheral sympathetic nervous system. Near the brain surface, however, it is impossible to ascertain from anatomical data alone, whether the DBH-containing fibers originate from inside or outside of the brain. The point we wish to emphasize, is that we are confident that many CNS blood vessels derive their noradrenergic innervation from cell bodies located inside the brain. To our knowledge, this is the first description of connections between central noradrenergic neurons and the brain's vascular system. So that at least one function of this system is almost certainly involved with the regulation of cerebral blood flow.

Apart from the differences in interpretation of the route of several pathways, there is excellent agreement between both methods as to the major areas receiving terminals. Moreover, the general organization of the central noradrenergic system is identical, regardless of the method used to examine it. The system is derived in its entirety from a relatively few number of neurons located exclusively in the pons and medulla. We have estimated that there are no more than several thousand cell bodies in the entire CNS. These neurons give rise

to a large number of highly branched fibers, which are distributed to widely dispersed areas of the brain. The nuclei and regions associated with the limbic system receive the greatest representation. The nerve processes terminate not only on cell bodies and dendrites but also on blood vessels. Another system of neurons with essentially identical properties, including sharing the same neurotransmitter substance is the peripheral noradrenergic or sympathetic nervous system.

Speculations concerning the derivation and function of the central noradrenergic system. The cells of the embryonic neural crest are known to give rise to the DBH and NE-producing cell bodies of the sympathetic nervous system. Groups of these cells migrate to form a chain of ganglia along the spinal column, where they send numerous branching neuronal processes to diverse structures and areas of the body, including blood vessels. Another group of cells migrates all the way to a position near the kidney, to become the adrenal medulla. We postulate that still another group, destined to be the central noradrenergic neurons, migrates to a position spreading longitudinally along the fourth ventricle from the medulla through the pons. This group sends its long branching fibers throughout the brain, including the vascular system.

It is further proposed that the central noradrenergic system may also perform a role in the CNS, analogous to the function of the sympathetic nervous system peripherally. During normal conditions this system could operate as a regulator in response to local needs. There is no reason to assume that the only way by which NE can be released is by stimulation at the cell bodies. The terminals themselves may have the capacity to release NE in response to local stimulation. Many drugs and hormones have been shown to cause release from NE terminals in the peripheral nervous system, without affecting the sympathetic ganglia, (*e.g.*, angiotensin and amphetamines). We assume this may hold true in the CNS as well. The localized vasoconstriction which frequently is seen during cerebral angiography may be a clinical example of such local responses. This is not to imply that during a crisis, central stimulation of the entire system *via* the cell bodies might not occur, much as in the case of the peripheral sympathetic response. We would assume that the overall function of this system would again be analogous to the peripheral system (*i.e.*, to prepare the brain to cope with a crisis situation by a combination of stimulatory and inhibitory effects depending on the receptor specificity of the neurons or blood vessels innervated). The anatomy and distribution of the central noradrenergic system make it a logical candidate for this function. It has some representation in essentially all areas of the brain, but is heavily weighted toward the reticular activating system and the limbic system, two areas that have long been implicated by neurophysiological experiments to be involved in anger, rage, or other "emotionally charged" responses.

Rate of Synthesis of Dopamine-β-hydroxylase (DBH)

When reserpine is administered to rats in high doses over several days duration, an increase in adrenal DBH enzyme activity is observed (15). Several mechanisms could account for such an observation. Firstly, the same amount of

enzyme could be stimulated or released from inhibition. Secondly, the amount of enzyme could be increased, either by an increase in the rate of synthesis or by a decrease in the rate of degradation. In such cases it is important to obtain information about the rates of synthesis or turnover of the enzyme. The most frequently used method of examining this problem is to administer inhibitors of protein synthesis. The rate of disappearance of enzyme activity is then assumed to correlate with the rate of turnover of the enzyme. The action of protein synthesis inhibitors is of course not specific for the enzyme being measured. In addition, these inhibitors alter the parameter which is being measured. The result is that the system may be changed so drastically after administration of the inhibitors that accurate interpretation of results may be difficult.

The ideal method consists of administering radioactively-tagged amino acids (leucine is usually chosen because it almost exclusively enters the protein synthetic pathway) and following either their rate of incorporation into proteins or, once they have been incorporated, following their rate of disappearance as the proteins are degraded. The major drawback of this approach is that the tagged amino acids are incorporated into all proteins, so that, unless one is interested in total protein synthesis, it is necessary to separate the protein of interest from all the other labeled proteins. It is at this stage that immunochemical techniques have proved valuable. The data presented here is from a preliminary study done in collaboration with Perry Molinoff and was previously presented in abstract form (9). This work is at present not completed and these preliminary results are given mainly to show an example of the technique.

Reserpine was administered (3 mg/kg) intraperitoneally to rats for 3 consecutive days. On the 4th day both six controls and six reserpinized rats were dosed intravenously with 250 μc of ^{3}H-leucine 24 hr prior to sacrifice. The adrenals were removed, homogenized in buffered 0.1% Triton X-100 and centrifuged at 10,000 $\times$ *g* for 10 min. A small aliquot of supernatant was removed for assay of DBH activity and for counting of ^{3}H-leucine incorporation into total protein. The remaining supernatant was incubated with 0.1 ml of sepharose conjugated with specific anti-DBH (cyanogen bromide linkage) (2). This immunoadsorbant has the property of specifically binding DBH. In preliminary experiments, the adsorbant was shown to adsorb >95% of the DBH activity from similarly prepared rat adrenal homogenates. Furthermore, when ^{3}H-leucine-labeled rat adrenal DBH was eluted from the sepharose, approximately 75% of the radioactivity in the eluant migrated with authentic purified DBH on disc gel electrophoresis. After incubation of the adrenal homogenates, the sepharose was removed by centrifugation, exhaustively washed, and the *in vivo*-labeled DBH eluted from the anti-DBH sepharose with 1% sodium lauryl sulfate. The radioactivity in the eluant was counted by liquid scintillation spectrophotometry. Blanks were evaluated in two ways, each giving essentially identical results: (a) counting the eluant from the samples after a preliminary incubation with normal rabbit IgG sepharose, or (b) repeating the incubation of the supernatant from the first antibody adsorption, with an additional aliquot of anti-DBH sepharose. The results of this experiment are shown in table 2. It should be

TABLE 2

Effect of reserpine treatment on ^{3}H-leucine incorporation and DBH enzyme activity

	Time on Reserpine (3 mg/kg/day)		Increase
	Controls	4 Days	
			%
^{3}H-leucine in DBH (cpm/pair of adrenals)	778 ± 109	2726 ± 320	250
Enzyme activity μg/20 min/pair of adrenals	2.59 ± 0.18	6.29 ± 0.35	140
^{3}H-leucine in total protein cpm/pair of adrenals	38,500 ± 2100	74,200 ± 3800	93

noted that the DBH activity increased 140% during the reserpine treatment. The rate of incorporation of ^{3}H-leucine into total adrenal protein also increased 93% as a result of this treatment. However, the rate of incorporation of ^{3}H-leucine into DBH increased to an even greater extent (250%). The main objective of this section was to demonstrate the power of immunoadsorption as a method of purification. This is best illustrated by comparing the number of counts on DBH with the radioactivity incorporated into total protein. In the control adrenals, the immunoadsorbant was able to separate in one step the 778 cpm in DBH from the 38,000 cpm in total protein while in the reserpine treated rats 2726 cpm were separated from the 74,200 cpm in total protein.

In spite of the preliminary nature of this experiment (specific activity of free ^{3}H-leucine was not determined and the 24-hr time lapse before sacrifice probably resulted in some degree of metabolism of DBH before the counts were evaluated), the increase in ^{3}H-leucine incorporation into DBH is of sufficient magnitude to support the hypothesis that at least part of the increase in DBH activity observed after chronic high doses of reserpine is the result of an increase in the rate of synthesis of this enzyme.

Immunological Studies of Different Forms of Monoamine Oxidase (MAO)

In several protein systems the antigenic properties of proteins have been shown to correlate with structural differences or similarities. Thus, by studying antigen-antibody cross-reactivity, it has been possible to examine the degree of structural relatedness of forms of MAO derived from liver and brain. The application of this approach has resulted in the following conclusions: 1) The various separable forms of liver mitochondrial MAO as described by Gomes *et al.* (7), are antigenically identical. The differences in electrophoretic mobility of the forms observed on acrylamide gels can probably be best accounted for by aggregation or polymerization of the same protein unit. The presence or absence of detergent may also affect electrophoretic mobility. 2) The bulk (80%) of brain mitochondrial MAO is antigenically identical to liver MAO, and it seems likely that, at least in part, the multiple forms reported in brain

extracts may be due to modifications similar to those in liver. 3) The remaining 20% of MAO activity derived from the crude mitochondrial fraction of brain does not cross-react with the antibody to liver MAO, and probably represents a structurally unrelated enzyme. This MAO has been separated from the "liver type MAO," and has been shown to differ from other types of MAO, not only in antigenic properties, but also with regard to several enzymatic parameters. These conclusions are based on three studies which have been reported in detail elsewhere (8, 12, 14).

Gomes *et al.* (7) were able to separate three forms of MAO from beef liver mitochondria. Two were pure proteins, component 2 (C2) with a molecular weight of 1,200,000 and with 12 moles of flavin adenine dinucleotide (FAD)/mole, and component 1 (C1) with a molecular weight of 400,000 and with 4 moles FAD/mole. These two forms were separated by using hydroxylapatite chromatography. The protein fraction which did not adsorb to hydroxylapatite also contained MAO activity, but was contaminated with other proteins and with residual Triton X-100 detergent. This fraction was designated fraction A. Antibodies were prepared to chromatographically pure C2. Component 2 was chosen for antibody induction because it is the largest of the forms of MAO and presumably would contain the most antigenic sites and possibly different sites were not also found on C1 or fraction A.

Immunoprecipitin lines of antigenic identity formed between each of the forms of MAO in double diffusion experiments comparing C2, C1 and fraction A for

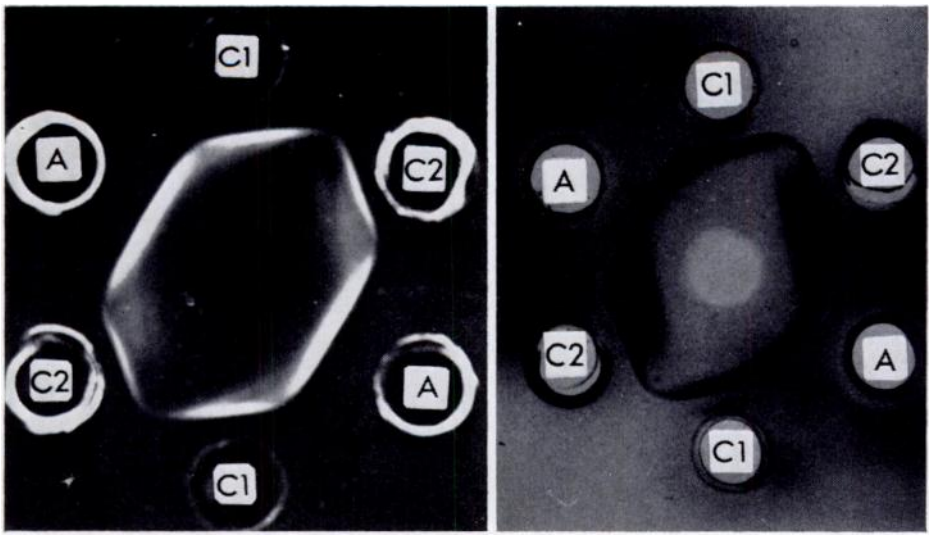

FIG. 10. Double immunodiffusion in agar gel of the separable forms of liver mitochondrial MAO against anti-component 2 (C2). Left: Immunoprecipitin lines formed after 6 days incubation at 4°C, the center well contained anti-C2. Photographed under indirect light. Right: The same plate after staining for MAO activity (nitrotetrozolium blue method, tryptamine as substrate). [From Hartman *et al.* (12).]

cross-reactivity with antibody to C2 (anti-C2) (fig. 10). This indicated that there were no antigenic sites present on C2 that were not also present on C1 and the MAO in fraction A.

Immunoprecipitin titrations were done by incubating constant quantities of antigen (MAO) and increasing amounts of anti-C2. Immunoprecipitates were removed by centrifugation. As would be predicted from the cross-reactivity shown by the double diffusion experiment and the fact that C2 and C1 are essentially pure proteins, it was possible to precipitate 100% of the MAO activity immunochemically. At equivalence, no MAO antibodies were detectable in the supernatants. Immunoprecipitin titration of fraction A proved more complex because of formation of soluble antigen antibody complexes in the presence of residual detergent. This problem was overcome by the addition of a sufficient amount of antiglobulin (anti-rabbit IgG prepared in goat) to quantitatively precipitate the antibody added. The addition of the antiglobulin step resulted in the quantitative removal of the MAO activity contained in fraction A from the supernatant. Thus there was no additional form of MAO present in fraction A which did not cross-react with anti-C2. It was thus concluded that within the sensitivity of the method, the separable forms of MAO are antigenically identical with regard to antigenic sites recognized by anti-C2.

This evidence strongly supports the hypothesis that in spite of apparent

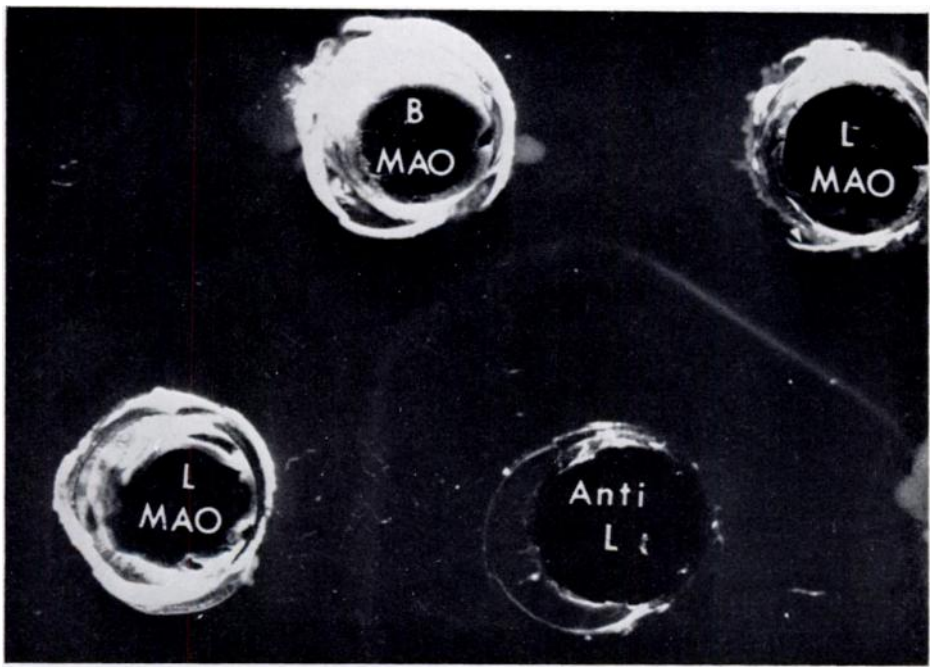

FIG. 11. Double immunodiffusion in agar comparing liver mitochondrial MAO (L-MAO) and brain mitochondrial MAO (B-MAO) against anti-liver MAO (anti-L). (From Hidaka *et al.* (14).]

heterogeneity in substrate and inhibitor specificity or the ability to separate forms of MAO with different electrophoretic mobility, the MAO solubilized from bovine liver mitochondria is composed of the same basic enzyme unit.

The cross-reactivity of anti-liver MAO toward MAO derived from bovine brain mitochondria was investigated next. Figure 11 shows the results of a double immunodiffusion experiment in agar comparing a partially purified preparation of liver mitochondrial MAO and brain mitochondrial MAO with anti-liver MAO. Immunoprecipitin lines of identity formed between the enzymes derived from the two organs. The absence of spurs indicates that there are no antigenic sites on the liver enzyme not also present on brain MAO.

Immunoprecipitation titration showed, however, that it was not possible to quantitatively precipitate the MAO activity, even when using an antiglobulin to precipitate any soluble antigen antibody complexes. It was possible to precipitate a maximum of 80% of the brain MAO activity (fig. 12). The remaining 20% of the MAO activity did not appear to cross-react with the anti-liver MAO. Thus the immunochemical evidence indicated that at least the bulk (approximately 80%) of the MAO activity derived from brain mitochondria is structurally very similar if not identical to liver MAO.

The fact that it was not possible to immunochemically precipitate 20% of the brain MAO with anti-liver MAO suggested that there might be a different

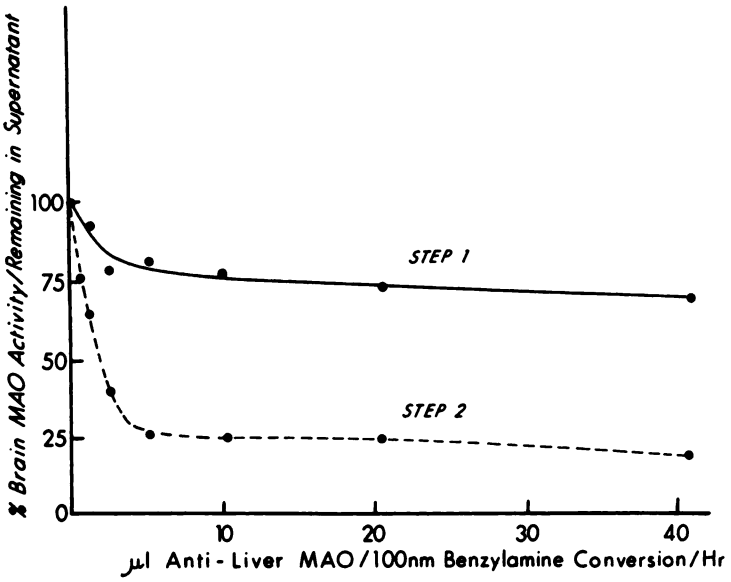

FIG. 12. Immunoprecipitin titration of constant quantity of brain mitochondrial MAO with increasing amounts of anti-liver MAO. Step 1 represents MAO activity remaining in supernatant after incubation with anti-liver MAO alone and removal of immunoprecipitate by centrifugation. Step 2 represents activity remaining in supernatant after precipitating excess antibody and soluble enzyme antibody complexes with antiglobulin. [From Hartman (8).]

MAO present in brain which is not found in liver. To test this hypothesis, it was necessary to make a preparation of the non-cross-reacting form of brain MAO which was free of the "liver type" MAO. This preparation could then be retested for cross-reactivity with anti-liver MAO to insure that the non-affinity was not an artifact of enzyme concentration or partial cross-reactivity.

Beef brain stem mitochondria were prepared by differential centrifugation and homogenized in buffered Triton X-100 to solubilize the MAO. The soluble MAO was then passed over Sephadex G-50 to reduce the concentration of detergent. The column eluate was concentrated by pressure dialysis and contained 70% of the total brain mitochondrial MAO activity. Subsequently this fraction will be referred to as total brain MAO. An excess of anti-liver MAO was first incubated with the solubilized total brain MAO. The immunoprecipitate formed was removed by centrifugation. Sufficient antiglobulin was then added to precipitate all excess antibody and soluble enzyme antibody complexes. This immunoprecipitate was also removed by centrifugation. The supernatant which now contained approximately 20% of the original MAO activity was passed over Sephadex G-200. The MAO activity was collected, and concentrated by pressure dialysis. This preparation of brain MAO was again subjected to a two-step immunoprecipitation first with anti-liver MAO and second with antiglobulin. In this case, 100% of the MAO activity remained in the supernatant (*i.e.*, no MAO activity was precipitated by anti-liver MAO), indicating that this fraction of brain MAO had no affinity to the anti-liver MAO and probably represents a different enzyme with an unrelated protein structure. This new MAO comprised approximately 20% of the total brain MAO activity and was not present in detectable amounts in liver mitochondrial preparations. In this regard, this form of the enzyme is brain "specific." Experiments have also been done which demonstrate differences between the non-immunoadsorbable brain MAO and total brain MAO, liver MAO and serum amine oxidase with regard to substrate and inhibitor specificity (8).

These studies demonstrate how immunochemical methods have aided in the understanding of the relationships between various forms of an enzyme as well as allowing the identification and isolation of a new antigenically unrelated enzyme.

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