THE DEVELOPMENT OF CURRENT CONCEPTS OF CATECHOLAMINE FORMATION

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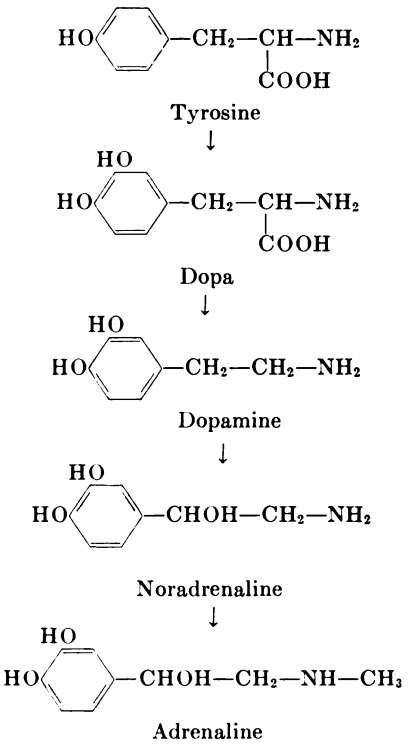
The chemical constitution of adrenaline and the other catecholamines has been known for over half a century, but it is only now that we can talk with some degree of confidence of the intermediate stages of catecholamine formation in the animal body. Speculations on the pathway of formation of adrenaline are almost as old as our knowledge of adrenaline itself; this has been pointed out in recent reviews on the subject (9, 38). Some of these old ideas, *e.g.*, on the stepwise formation of adrenaline (39) and on the importance of dopa as an intermediate (33), have been proved to be entirely correct.

1. *The pathway of formation.* The main stages of the formation of adrenaline, as they are known today, are shown below.



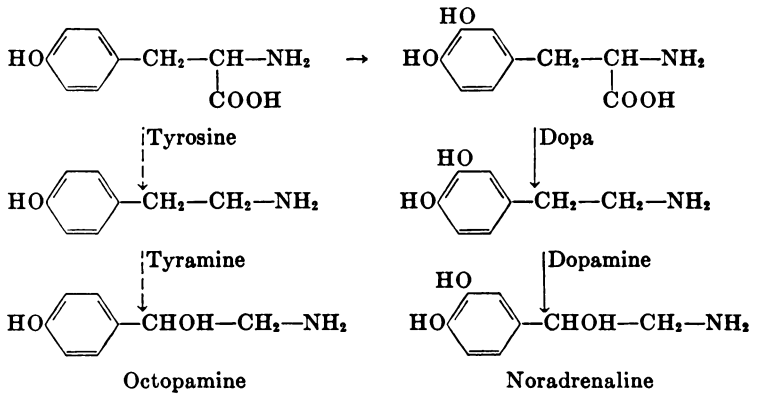
Main pathway of formation of catecholamines

According to this scheme, noradrenaline is the immediate precursor of adrenaline. Noradrenaline, therefore, has a dual role, not only as a hormone (and neurohormone), but also as an intermediate, *i.e.*, in adrenaline formation.

Our knowledge of noradrenaline as a neurohormone, securely based upon U. S. von Euler's work, began, as you all know, with the analysis of Cannon's "Sympathin E" in the nineteen thirties. That it was a precursor of adrenaline arose from the finding that, in contrast to dopa, N-methyl dopa was not decarboxylated by the mammalian decarboxylase. Thus it was recognized that a primary amine like noradrenaline must occur as a precursor in the biosynthesis of adrenaline (5, 6).

For dopamine, the third catecholamine, it is not yet clear whether it has a similar dual role. Its occurrence as a precursor of noradrenaline and adrenaline is now well established. In support of the idea that it has some functional role in the brain one might refer to its disappearance from this tissue after administration of reserpine (22). This may be due to the fact that the structural elements, in which it appears to be stored in this tissue (59), have undergone an alteration under the action of reserpine. The fact that in the brain as well as in adrenergic neurones (53) and in the lungs (31) large amounts of dopamine are found, makes it necessary to consider the possibility that the third catecholamine is not simply a precursor.

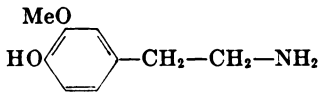
The sequence of reactions outlined above seems today fairly well established. The possibility has been discussed that hydroxylation in the side chain, with formation of dihydroxyphenylserine, may precede decarboxylation, but there is no convincing evidence that a phenylserine derivative appears as an intermediate compound in the formation of the catecholamines (see also 46). However, we ought to keep our minds open to the possibility of the existence of secondary pathways. A pathway of amine formation different from the main pathway outlined above occurs in octopods, but here too the occurrence of a serine derivative as an intermediate appears unlikely: the work of Henze (40) and Erspamer (29) suggests that in the posterior salivary gland of *Octopus* the sequence of decarboxylation preceding side-chain oxidation is exactly parallel to the stages of catecholamine formation in mammals, with the one difference, *i.e.*, the absence



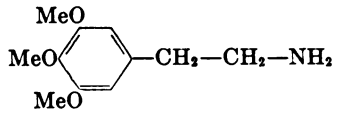
Probable pathway of formation of octopamine in Octopus macropus (dotted lines) and of noradrenaline in vertebrates (solid lines)

in *Octopus* of the reaction in which the second phenolic hydroxyl group is introduced.

In addition to alternative pathways, the possibility must be considered that there occurs a branching off from the main pathway. This possibility is particularly important now that we know that, like noradrenaline and adrenaline, dopamine may undergo O-methylation (1). We might therefore expect the normal occurrence in the tissues of a compound closely related to mescaline.

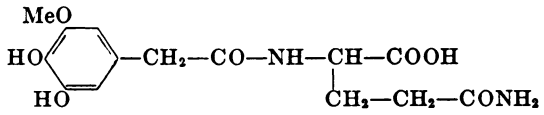


3-Methoxytyramine



Mescaline

It is also known that in the human body there occurs a partial demethylation in the metabolism of mescaline, with the formation of this compound (39a):



It looks, therefore, as if the pathways of metabolism of the catecholamines and of mescaline converge. We are interested in this, as we have recently studied at Oxford the substrate specificity of the amine oxidases of mammalian plasma and we have found that all the plasma oxidases will act on mescaline (10, 12).

The important outcome of the biochemical work on catecholamine formation is the recognition that the chromaffin tissue, as well as the other tissues that contain catecholamines, are the seat of certain specific chemical abilities (9). The demonstration of the occurrence of the enzyme dopa decarboxylase in the adrenal medulla (48) was really the first definite piece of evidence in support of the scheme outlined above. We shall, I hope, hear more about this enzyme from its discoverer, Dr. P. Holtz. Two puzzling facts still await interpretation. Firstly, there is the occurrence of large amounts of the enzyme in organs like liver and kidney, locations for which a high turnover of catecholamines has never been demonstrated. Some time ago, I discussed the possibility that precursors of adrenaline might reach other tissues by the way of the blood stream (7), and this idea has recently been revived in order to account for the high dopamine content of the lungs, a tissue from which dopa decarboxylase is absent (54).

There is, also, the unsolved problem of the relation between decarboxylation of dopa and of 5-hydroxytryptophan (5-HTP), activities found together in so many different locations; I remind you of the puzzling fact, reported by Dr. H. Langemann (49), that the human enterochromaffinoma decarboxylates dopa as well as 5-HTP. Similarly, extracts of mouse mast cell tumours act not only on 5-HTP but also on dopa; these cells contain 5-hydroxytryptamine (5-HT) but no catecholamines (37). If it is confirmed that dopa decarboxylase and 5-HTP

decarboxylase are in fact not two enzymes but one (60), it would seem that the relation between catecholamines and 5-HT is even closer than hitherto suspected: these amines would not only share amine oxidase as a catalyst taking part in their inactivation but also an enzyme that participates in their formation.

The introduction of the side-chain hydroxyl group was the last reaction to be demonstrated. The initial work, on preparations of chromaffin tissue, was carried out in the laboratory of Dr. A. D. Welch, at Yale (23, 38); it has since been confirmed and extended to nerve tissue (36) and to the intact animal (58). The intimate mechanism of this reaction is still little explored. I feel convinced that such a study would be most rewarding. I believe this is the rate-limiting step in noradrenaline formation (8). The reaction can be formulated as a dehydrogenation (9).

Fortunately, we have learned more in recent years about the biochemistry of the methylation of noradrenaline (18), and I hope Dr. Kirshner will tell us more about the part played by S-adenosylmethionine as a methyl donor in this reaction. I was interested to learn in a recent report (4) of the presence of free homocystine (or homocysteine) in the human adrenal gland, and particularly in a case of human pheochromocytoma. This indicates that the methylation of noradrenaline may occur at such a rate that the supplies of methyl donor are exhausted. A similar condition may occur where noradrenaline accumulates during active resynthesis of adrenaline, *e.g.*, after administration of insulin or acetylcholine (19, 44). I do not know of any other tissue for which the occurrence of free homocyst(e)ine has been reported.

The dual role of noradrenaline, as precursor and as hormone, is of interest in view of reports on the presence of different adrenaline—and noradrenaline—storing cells in the adrenal glands of many species (28). The chromaffin tissue of adrenal medulla may be a composite tissue, and only one of the two types of cell may contain the methylating mechanism; this may be absent in those cells in which noradrenaline normally accumulates as the end-product.

In homogenates of the bovine adrenal medulla in isotonic sucrose both noradrenaline and adrenaline are sedimented together, but some time ago my colleague, Dr. N. R. Eade (26, 27), noted a difference in the sedimentation of the two amines when suspensions of bovine adrenal medullary granules were centrifuged over hypertonic sucrose at high speeds. When such a suspension, in isotonic sucrose (0.3 M), was layered over 2.0 M sucrose and centrifuged, more of the noradrenaline was sedimented and more of the adrenaline was retained at the boundary of the isotonic and the hypertonic sucrose solutions. When Dr. H. J. Schümann joined us, he prepared a homogenate from the adrenal glands of the domestic fowl and carried out a density-gradient centrifugation. Here a separation of the two amines was more readily demonstrated: he was able to obtain a less dense fraction of granules from the upper part of the density-gradient tube that contained practically only adrenaline, and he also obtained a sediment that contained practically only noradrenaline and no adrenaline (51). In an experiment in which the glands were depleted of catecholamine by giving insulin, the less dense fraction, which normally contained only adrenaline and no noradrena-

line, contained both, adrenaline and noradrenaline. This is an indication that in the actively re-synthesizing gland the adrenaline-forming cells not only contain noradrenaline, but that they store it also, temporarily.

Dopamine does not normally accumulate in the adrenal gland of many species, but it has been detected in the adrenal medulla of the sheep (35) and ox (56). The method used by Eade (27) for the study of the distribution of dopamine in the bovine adrenal medulla was only semi-quantitative, but it was sufficiently accurate to show that the distribution of this amine was very similar to that of the two other catecholamines; most of the dopamine was recovered in the granules. This was unexpected: dopamine is, we believe, formed in the cytoplasmic sap (15). We must assume that it finds its way readily into the storage granules. In brain tissue, the location of the dopamine appears to be also in structural elements (59). On the other hand, in the splanchnic nerves, where almost one-half of the total catecholamine present is dopamine, it is present mainly in the supernatant fluid after centrifugation, whereas noradrenaline is held in granules, as in the chromaffin tissue (53; see also 30).

2. *Intracellular localization.* In the last few years the study of the sequence of chemical events culminating in the formation of the finished hormone (or neurohormone), has been supplemented by the study of the distribution of the amines in the cells that store them. We know now that they are not evenly distributed throughout the cell, but that they are held in specific cell organelles. This is true not only for the catecholamines, but also for the other biologically active amines. In tissue homogenates prepared and suspended in isotonic sucrose we find that the storage granules for amines are sedimented together with the mitochondria. However, it has now become possible to demonstrate that these storage granules are not identical with mitochondria.

Three years ago, Mrs. Hagen and I carried out some experiments on homogenates of dog liver in isotonic (0.3 M) sucrose. The so-called "mitochondrial" suspensions obtained, resuspended in isotonic sucrose, were centrifuged at high speed over 1.75 M sucrose. We found that after centrifugation in the ultracentrifuge 43% of the histamine was recovered in the sediments, but only 6% of the total protein nitrogen was recovered in these sediments. Clearly one had obtained a fraction of granules more dense than the mitochondria of the liver cells, and in these granules histamine was present in a very high concentration.

Recently, Mrs. Baker has carried out an analogous study for the 5-HT in the dog duodenal mucosa. She has found that in a homogenate of this tissue in isotonic sucrose the greater part of the 5-HT was sedimented by centrifugation (2), together with the mitochondrial enzymes, but more recently she has shown that in a density gradient tube the pattern of distribution of the mitochondrial enzymes, fumarase and succinic dehydrogenase, is entirely different from that of the 5-HT (3).

I mention these results, because they show that there is a fundamental similarity in the mechanism of intracellular storage of amines. As far as the catecholamines are concerned, in our first experiments, now six years ago, we found that in isotonic sucrose they were sedimented together with the mitochondrial en-

zymes. We pointed out, however, that this did not imply that the storage granules were mitochondria (16). The subsequent history owes much to help given by Dr. J. R. Baker, of Oxford. We had noted early in these studies that the granular sediments obtained by centrifugation of homogenates of bovine adrenal medulla were not homogeneous; they could be separated into a "top" and a "bottom" layer. We found that the bottom layer was richer in amines and the top layer richer in succinic oxidase (15). By the help of the "chondriocrit," a device that we found useful in the study of these sediments, the differences between "top" and "bottom" layers could be seen very clearly, and in one experiment carried out in April, 1953, we removed fairly clean samples of the layers from the chondriocrit and took them to Dr. Baker. He looked at them under the microscope, and he reported that the top layer was less homogeneous than the bottom layer. Both contained an abundance of minute granules, but in addition there were larger granules, and of these he saw more in the top layer and fewer in the bottom layer. In other words, the larger granules had sedimented less readily than the smaller granules.

It was this observation that led to the centrifugation over hypertonic sucrose solutions (11, 13, 14). If the smaller granules sedimented more readily, this might be due to their higher density, we thought. This hypothesis was confirmed when it proved possible, eventually, to obtain in a density gradient a fraction of granules essentially free of catecholamines, which contained the mitochondrial enzymes, fumarase and succinic dehydrogenase. This fraction was recovered in sucrose solutions of relatively low density, whereas the catecholamines were recovered in the lower parts of the density gradient tube. In their distribution in a density gradient, there is remarkably little difference between mitochondria from chromaffin tissue, from duodenal mucosa and from liver.

Our knowledge of the finer structure of the chromaffin cell does not rest on studies of homogenates only. It is possible now to correlate our findings to those obtained by means of the electron microscope. The presence of two entirely distinct types of granule has been described: typical mitochondria and a second type of much smaller, intensely osmiophilic particles (50, 57, 61). The latter must be the storage granules. There is even some indication of two types of cell in the chromaffin tissue (61), but it cannot be said whether these correspond to the adrenaline and the noradrenaline storing cells.

The recognition that the catecholamines are stored in a specific cell organelle, presents us with a number of new problems. There is the question as to the sites of the different chemical reactions in the chromaffin cell. All three catecholamines are stored in granules, but I have mentioned already that the dopamine-forming enzyme is probably not granule-bound. One could therefore think that the changes subsequent to dopamine formation all occur in the granules. According to Kirshner (46), the introduction of the side-chain hydroxyl group requires the presence of the granule fraction. Are the granules involved the chromaffin granules or the mitochondria? The methylating enzyme has been recovered from the supernatant fluid after centrifugation (47). We can, therefore, not yet exclude the possibility that all chemical reactions of catecholamine formation take place

outside the storage granules. However, a localization of noradrenaline formation inside the granule and of adrenaline formation (methylation) outside, might account for the fact that after a depletion of the adrenal medulla there is, during recovery, a prolonged presence of noradrenaline, whereas there is no evidence of accumulation of dopamine. The noradrenaline, in order to be converted to adrenaline, would have to leave the storage granules, to be picked up again by granules. Here are many new questions which arise out of recent discoveries.

The sequence of chemical reactions leading to noradrenaline and adrenaline does not constitute the final events in recovery. The final event is the uptake of amine by a granule.

3. *Mechanism of amine storage.* Early in the study of the chromaffin storage granules, when we began to measure the amine concentration (15), it became clear that there must exist a specific mechanism that held the amines in the storage position. We must assume the presence of a specific receptor for catecholamines at the site of storage. So here is another interesting problem: is there a similarity between the specific receptor at the storage site and the specific receptors for the catecholamines in the effector organ? It is in this connexion that the discovery, by Hillarp and his colleagues (32), of large amounts of adenosinetriphosphate (ATP) in the chromaffin tissue of the adrenal medulla is of great interest. We soon established that the ATP was also granule-bound (11), and that in the density gradient tube it is distributed over the different fractions of granules just as the catecholamines and in a manner entirely different from the distribution of the mitochondrial enzymes (13).

The presence of large amounts of ATP has recently also been established in a human pheochromocytoma (34). ATP is also present, together with noradrenaline, in the granules isolated from adrenergic neurones (55); thus, we can conclude that the mechanism of binding of the adrenergic mediator closely resembles that of the adrenal medullary hormones.

In the adrenal medulla, the ATP is characterized by its presence in stoichiometric, not in catalytic, amounts. We have, in our experiments, therefore often determined the molar ratio, catecholamine:ATP. A molar ratio of about 4 could be accounted for by the fact that, at neutrality, one molecule of ATP carries, approximately, four negative charges, as against the one positive charge of a molecule of catecholamine. In the bovine adrenal medulla, the molar ratios originally found at Oxford usually exceeded 4 (11); similarly Schümann and Grobecker (55) have calculated a molar ratio of 5 from the data of Falck, Hillarp and Högberg (32). More recently, we have reported molar ratios closer to 4 (25; see also 42). However, from unpublished experiments by my colleagues, Friedman and Hornykiewicz, it looks as if the true molar ratio *in vivo* may be even lower: they find in adrenal glands of rabbits killed by an intravenous injection of pentobarbital sodium (Nembutal) molar ratios near 3 or even a little lower. Possibly in the storage position one of the four negative charges of the ATP molecule is not used to neutralize a positive charge of a catecholamine, but it may be used for anchoring the ATP in the storage position, *e.g.*, to a storage receptor protein.

Ten years ago, Zeller (62) reported the presence of a very active ATP-ase in the adrenal medulla. This suggests the possibility that the hydrolysis of ATP, with an accompanying change in the charge configuration in the storage receptor, is responsible for the mobilization of the amine and its appearance, eventually, in the extracellular fluids. D'Iorio and Eade (25) did not find at first a significant decrease in ATP in the rabbit adrenal glands when the amines were mobilized by a dose of insulin; the molar ratio 2½ hours after the onset of convulsions had dropped from about 4 to 1.0–1.5. They discussed the possibility that in these experiments a resynthesis of ATP had occurred. A loss of ATP parallel to a loss of amine was clearly demonstrated in the experiments of Carlsson and Hillarp (20, 21), after insulin administration to sheep, cats and rats. Schümann (52) has recently shown that in the rat the resynthesis of ATP does in fact precede resynthesis of catecholamines, and this is supported by recent experiments carried out by Dr. O. Hornykiewicz (45), who has found a depletion in ATP in rabbit adrenals after administration of insulin, but a presence of normal amounts of ATP in animals in which the catecholamine content was still low.

The fate of the ATP lost during depletion of the adrenal gland is not yet known. D'Iorio (24) has found that sulphydryl reagents like *p*-chloromercuribenzoic and *p*-chloromercuriphenylsulphonic acids not only release catecholamines from the storage position, but also destroy the granule-bound ATP; at the same time two molecules of inorganic phosphate appear in the suspending fluid for each molecule of ATP lost. A formation of adenosinemonophosphate also occurs when granules are broken up in hypotonic media (41); evidence of the presence of an adenylate kinase in addition to the ATP-ase has also been given (41, 43).

It is of interest to compare the mechanism of storage of the catecholamines with that of the other biogenic amines. Born (17) has given good reasons for the belief that ATP is involved in the storage of 5-HT in the blood platelets, but the mechanism of binding of 5-HT in its principal site of storage, the intestinal mucous membrane, remains to be discovered. In the mast cell granules, histamine is stored in close association with heparin. The main difference is that the catecholamines are stored together with ATP, a labile substance of low molecular weight, and it seems likely that the storage complex contains also a protein, not yet characterized. Here is much room for further exploration.

Summary

We can today, with a fair degree of certainty, describe the main stages in the formation of the catecholamines. But many problems remain, *e.g.*, that of the intimate nature of some of the intermediate chemical reactions and also that of the spatial relationship of the various stages and their correlation in the living cell. It must remain for the future to discover the nature of the intracellular signalling system, by which the storage site is told that amine is to be released, and also to study what we might call the "adequate stimulus" for re-synthesis.

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