PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

Printed in U.S.A.

SECOND SYMPOSIUM ON CATECHOLAMINES

OPENING ADDRESS

TWENTY YEARS OF NORADRENALINE

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EARLIER OBSERVATIONS

At the Ciba Symposium on Adrenergic Mechanisms in London, in 1960, the historical survey was centered around the early findings before 1945 in the excellent review by Sir Henry Dale (14a), that is to say, before the noradrenaline era. I shall therefore start at this point and only touch upon some earlier observations.

Noradrenaline (norepinephrine, NE) is by no means a new substance, having been synthesized by Stölz in 1904 (60). However, it received little attention for a long period of time. The first hints as to its relationships with the sympathetic nervous system were perhaps when Barger and Dale in 1910 (4) studied the biological effects of various sympathomimetic drugs. They made the interesting observations that epinephrine (E) did not mimic all the actions of sympathetic nerve stimulation as one might expect if it were the transmitter, and that in fact certain amines of the ethanolamine series showed closer similarity in this respect. Then in 1934 Bacq (3), who had worked in Cannon's laboratory, suggested that "sympathin E" was NE while sympathin I should correspond to E. This view was discussed in Cannon and Rosenblueth's classical book on sympathetic neuroeffector systems (11). Bacq's idea was taken up again in a careful study by Greer *et al.* in 1938 (36), who gave what seemed to be good evidence that at least some sympathetic nerve effects fitted in well with the assumption that the transmitter was NE. Only shortly afterwards, however, Cannon and Lissák (10) concluded from their studies on sympathomimetic activity in extracts of nerves and organs that the transmitter was identical with E. It is of interest to note that in the thirties both Stehle and Ellsworth (58) in Canada and Tiffeneau (62) in France commented upon the quirk of nature which made use of a compound like E when the nonmethylated compound seemed to serve the purpose better and was chemically simpler. The first suggestions based on chemical considerations that the sympathetic neurotransmitter might not be E originated from Raab (54), who in 1943 found that extracts from spleen contained a catechol compound with properties different from those of E.

From these scattered data one might believe that the ground was well prepared to accept our findings that extracts of sympathetic nerves and organs innervated by such nerves acted differently from E and closely mimicked NE and also α-methyl-NE, which we used for comparison in the early experiments. Such was not the case, however, and it took many years to convince the scientific community, and particularly writers of textbooks, who may be somewhat more

conservative. At about the same time, and independently, Holtz *et al.* (44) discovered NE in cat adrenals and in normal urine. The paper describing this finding was sent in for publication in 1944 and published in 1947. We had missed the NE in the adrenals for the simple reason that we used adrenals from the rabbit, which contain only small amounts of NE. In this context it may also be mentioned that Loewi (49) was convinced that the sympathetic transmitter which he had discovered in the frog heart was E, and this also turned out to be correct. In this case again, the frog is an exception, containing only E in its adrenergic nervous system. No wonder that NE had some difficulties in breaking through. It is perhaps of some historical interest that Schild (55) came close to finding the NE in adrenal extracts in 1933 when, by virtue of careful biologic assay, he observed that extracts made from cat adrenals raised blood pressure more than could be accounted for by their E content.

The next problem was to find a method to determine NE and E in a mixture, since both compounds are present in adrenal extracts. This became possible by using a pair of biological test systems such as the cat's blood pressure and the rectal caecum of the chicken, which responded much more strongly to E than to NE. Differential estimation of the two amines by means of the iodine oxidation method and analysis after separation by paper chromatography and ion exchange resins also proved useful for convenient and accurate estimation of NE and E. A study of adrenals from a large number of species showed that the relative proportions of the two amines varied from almost exclusively E in rabbit adrenals to almost exclusively NE in the adrenals of the whale. In the cat the two amines occurred in about equal amounts. The functional significance of the varying proportions of NE and E in different animals, even among mammals, has naturally been the subject of some speculation. Dr. Goodall (34), who worked in our laboratory around 1950, even bought a plane and flew to Africa to collect adrenals in order to test his theory that aggressive animals had much NE in their adrenals while their prey, prepared to flee, had much E.

The presence of two hormones in the adrenals also raised the question as to whether they occurred in different cells. This was shown to be the case by Hillarp and Hökfelt (40). Later experiments also showed that they could be activated separately by direct hypothalamic stimulation (29), by hypoglycemia (16), or by psychic stress (21). Goodall (33) was also the first to show that dopamine was a natural component of the adrenals. The dualistic concept of adrenal catecholamine secretion has been much debated (see 52), and although a differential secretion has been demonstrated, it appears that the most common secretion types are either of E alone or a mixture of NE and E.

Of paramount importance was the almost simultaneous discovery by Blaschko and Welch (6) and of Hillarp *et al.* (41) that the adrenal amines were harbored in subcellular granules. It is true that Cramer in 1919 (14) had observed a particulate distribution of the amines in osmium-stained sections of adrenals, but this observation was never followed up. By a relatively simple technique it became possible to separate the granules after homogenization of the chromaffin cells, and the properties of these granules were elucidated in a brilliant series of

studies by the authors mentioned. It was even shown that the NE-containing granules could be separated from the original mixture, at least to some extent. Particularly interesting was the finding by Hillarp *et al.* (39) that the adrenal granules contained ATP in a fixed relation to the amines, with a corresponding number of negative and positive charges.

ADRENERGIC NERVES

It was not difficult to verify the fact that extracts of various organs and sympathetic nerves had sympathomimetic activity. Comparative tests with E on a series of biological preparations soon indicated that a parallellism between the active principle and E did not exist. After many trials and doubts and guesses it became plausible that the active compound was NE. Later we found that most organs contained small amounts of E in addition to NE. It soon emerged that although the amount of NE per gram of organ varied greatly in different organs it had a characteristic value for any one organ. Its occurrence in adrenergic nerves and disappearance from organs after nerve degeneration indicated that it was bound to nerves (34). Accordingly placenta lacked the amine. These studies may seem somewhat trivial, but the important implications were that the neurotransmitter seemed to occur in a stoichiometric relationship, as it were, to the number of neurons, a relation that indicated a rather orderly way of binding. Particular interest was attached to the finding of Vogt (64) that NE occurred in relatively high amounts in certain parts of the brain, such as the hypothalamus. The problem then arose where and how is the transmitter bound in the adrenergic neurons. A comparison of the amounts of NE in the splenic nerve and the spleen indicated that the transmitter must be accumulated in the nerve terminals (20). We assumed that hardly more than 1 % of the spleen could consist of intrasplenic nerves and 0.1 % of true nerve terminals. Consequently the NE concentration in them would be close to 3 mg per g, which is of the same order of magnitude as in chromaffin tissue. This estimate later proved to be not so far from the truth. Moreover we found less NE in the sympathetic ganglia than in the nerve trunk. Our conclusion was that this rather uneven distribution of NE in the neuron was hardly compatible with the idea that NE should occur in freely diffusible form. This was unlikely also for other reasons. It then seemed not too long a shot to assume that the transmitter was bound to certain structures and since granules had been shown to occur in chromaffin cells they might perhaps also occur in their homologues, the adrenergic neurons. Hillarp kindly consented to homogenize adrenergic nerves and some organs, centrifuge the homogenate in the same way as he had done for adrenals and send us the preparations (22). This was in 1955. The first tests showed what we had hoped for, namely that the high-speed sediment contained NE in high concentration, but biologically inactive prior to treatment with acid or heat. We thus had evidence for structurally bound NE. This observation was commented upon by Otto Loewi (50), who put it in relation to his own findings that extraction of acetylcholine from organs was more efficient with acid than with physiological media. Our first electron microscopic pictures with Bahr in 1956 and Bloom in 1957

showed that the high-speed sediment from homogenized splenic nerves was rich in osmiophilic granules of sizes about 500 to 1000 Å in diameter. I shall return to them a little later, and in the meantime touch on a few other problems.

METHODOLOGICAL PROBLEMS

No studies of the catecholamines would of course be possible without methods for purification and quantitative and sensitive methods for estimation. The biological tests served well during the first years but were time-consuming. The basic discoveries for purification and chemical estimation were the adsorption of catechol compounds on alumina (57), and the trihydroxyindol fluorimetric method as developed by Ehrlén (17) and by Lund (51). Subsequent refinements of these methods make it now easy to determine amounts even in the nanogram range accurately. The ethylenediamine method used by Weil-Malherbe and Bone (63) has in many instances proved useful. These methods also allow estimation of NE and E in a mixture without prior separation. With chromatographic separation on strong cation resins by a technique worked out in detail by Häggendal (37) it is now possible to separate a number of phenolic amines and related compounds.

PROBLEMS OF PHYSIOLOGICAL RELEASE AND ACTION

For the physiologist, the pharmacologist, and the clinician it became a question of interest to know in which ways the actions of NE differed from those of E. The careful studies by Goldenberg *et al.* in 1948 (32) largely cleared up the circulatory actions of NE and E in man. On the other hand the actions of sympathetic nerve stimulation were relatively well known and the identification of the transmitter did not change the situation very much in the beginning. When it became possible to measure sympathetic nerve activity, however, the physiological problems gained new interest. Estimation of NE and E in urine proved to be an important aid in obtaining new knowledge in this field. Such studies made it clear that most of the E in urine was derived from the adrenals while almost all of the NE originated from the adrenergic nerves. Perhaps one of the most striking findings was that simple change of posture from reclining to standing greatly increased the excretion of NE in urine—a sign of homeostatically induced increased activity of the vasoconstrictor nerves to the legs (26). Each time we stand up NE is poured out from nerve terminals in increased amounts, and this is a prerequisite for the privilege of remaining for some length of time in a vertical position. During heavy muscular work NE is also released and excreted in increased amounts. On the other hand, insulin hypoglycemia triggers a selective release of E which can be measured in urine (25). Stress can be quantitatively estimated by measuring the output of catecholamines in urine, mostly E, and this method has found wide use.

It first appeared that E was predominantly a metabolic hormone while NE was chiefly concerned with circulatory homeostasis. Through the brilliant work of Sutherland and his associates (61) we know now that even NE can activate phosphorylase by stimulating the formation of cyclic 3′,5′-AMP, and the finding

by Carlson and his co-workers (46) that NE enhances metabolism of rats exposed to cold has given new aspects to this problem, further elucidated by the work initiated by Dole (15) and others showing that both amines enhance the release of free fatty acids in plasma. Equally exciting are the findings originating from Brody and his group (7) about the importance of amines for psychic activity. This discovery has opened up an entirely new field of research with the widest implications. This area deserves a historical survey of its own.

Even a brief survey of the catecholamine field should include a mention of the finding of Holton (42) that phaeochrome cell tumours may consist of almost only NE-producing cells. This finding is the basis for the best present method to diagnose such tumours by catecholamine analysis of urine (18). In this connection it may be recalled that NE has been found useful as a therapeutic agent in many cases of circulatory failure.

BIOSYNTHESIS AND INACTIVATION

The history of our concepts of catecholamine biosynthesis is a winding path which was straightened out in the late thirties by the discovery by Holtz (43) of dopadecarboxylase and by Blaschko's (5) bold and correct biosynthetic scheme, starting from tyrosine. Nevertheless, it took several years before the conversion of dopamine to NE became established, and we have recently witnessed the conquering of the most difficult step, from tyrosine to dopa by Udenfriend and his associates (53).

Equally important physiologically is the problem of inactivation, and the unsatisfactory situation of allotting the inactivation only to monoamine-oxidase (MAO) obtained its solution when Axelrod (1) discovered the enzyme responsible for the O-methylation and found the corresponding catecholamine derivatives. The uptake of excessively released transmitter by appropriate binding sites in the neuron is another kind of inactivation or, as Axelrod terms it, of termination of the action.

MORPHOLOGY OF SYMPATHETIC NERVE TERMINALS

Since 1945 many attempts have been made to apply fluorescence techniques to adrenergic nerves to clarify a number of problems involved in this system. Through the ingenuity and skill of the late professor Hillarp and his colleague Falck (28) it finally became possible to demonstrate the finest terminals by means of their content of NE by applying a fluorescence technique based on interaction with formaldehyde, a procedure previously studied and developed by Eränkö (19). A large amount of work with this technique has already been done and important results are accumulating at a great rate. I shall not go into details but only mention that the demonstration of the terminals with their varicosities confirm Hillarp's often contested concept first reported in 1946 (38). The recent disclosure of monoaminergic nerve paths within the central nervous system, greatly extended through the work of Fuxe (30) and others, has opened up new and fruitful fields of the highest significance.

The application of electron microscopy to the study of finer structures of the

adrenergic nervous system has similarly contributed much to our understanding of the transmission mechanism. It is surprising that the first convincing pictures of a section through a varicosity with its granules were not published until 1961 (48). Even the uptake of amines into such granules has been demonstrated with autoradiography (65).

SPECIFIC GRANULES

I now return to the nerve granules, which I think deserve a special brief survey since they represent the minute manufacturing units which presumably ultimately govern the whole transmission process. As mentioned above, homogenization or squeezing of bovine splenic nerves and subsequent centrifugation at high speed yields granules of a uniform type but varying in size between 300 and 1000 Å, with occasional larger ones. Subsequently published electron micrographs of sections through tissues show the same kind of granules in adrenergic terminals.

To recall some of the basic properties of these granules it may suffice to mention that they retain their NE firmly at low temperatures, but release it rapidly at 37° (24). In this respect as in several others they differ from adrenal medullary granules, which release their hormones slowly even at 37°. This difference can be regarded as being of great physiological significance as it presumably determines the maximal rate of release. In the nerve terminals it may be considered as essential that the release occurs rapidly and efficiently while a similar almost explosive rate of release from the suprarenal medulla could lead to unwanted effects. We know, however, that even intense stimulation of the adrenal medulla releases the hormones at only a moderate rate. This is of the order of a few percent of the total amount in the gland per minute and roughly corresponds to the release rate at 37° from isolated medullary granules. The large stores in the adrenals, therefore, can serve to produce a prolonged and well controlled intravenous infusion of catecholamines. In contrast to the adrenal medullary granules, the nerve granules, probably for the same chemical and biological reasons, are able to take up amines much more rapidly and efficiently. This has become the basis for extensive studies both *in vivo* and *in vitro*. These studies have been stimulated by the introduction and use of radioactive NE, principally by Axelrod and his associates (2), particularly after it had been shown that the radioactive compound was bound mainly to the microsomal fraction, which contains the granules.

Another observation was that the rate of release of NE from the granules was lowered when NE was added to the suspension medium (24). Addition of radioactive NE showed that the release was compensated for by an uptake of exogenous NE (27). Similarly, on incubation with E a certain amount of endogenous NE is replaced by E, as space becomes available by the release of NE. Even high concentrations of the amines do not raise the NE (or E) content of the granules much above the original level, and this suggests that they have a limited binding capacity. This also affords a good explanation for the early finding of fixed and characteristic NE stores in organs under physiological conditions.

The metabolic activity of isolated nerve granules is being currently investigated by Giacobini (31), a problem which appears of great interest since it has been shown that amine uptake is greatly enhanced by ATP and ADP, and the release strongly enhanced by dinitrophenol.

The occurrence of specific particles in large numbers at the site of physiologic release naturally raises the point whether they synthesize the transmitter as well as harbor it. This has been proved for adrenal medullary granules (35), but only recently (59) has it been shown that nerve granules, washed and resuspended, are able to carry on the synthesis from dopa to NE, thereby partially confirming Udenfriend's hypothesis expressed at the Stockholm Symposium (*cf.* Stjärne, Section IV E). The properties of the adrenergic nerve granules—synthesis, uptake, storage, and release at a controlled rate—obviously place them in a central position in the physiology of adrenergic nerve transmission.

Sometimes there is a tendency to blur the distinctions between adrenal medullary granules and nerve granules, not unlike the situation some 15 years ago between E and NE. This is a temporary phenomenon not without historical interest. Just to show how careful one has to be in this respect I will mention briefly a new finding. Lishajko and I some 5 years ago decided to check the catecholamines in the vesicular gland of the bull. We found some 5 to 10 μg NE per g, which is far in excess of the NE content of any organ except the adrenals. Recent studies of the behaviour of the granules in the vesicular gland of the bull showed that their release rate was neither that of bovine splenic nerves nor of bovine adrenal medulla but something in between. What seemed to be ordinary adrenergic neurons in the organ, as studied with the histochemical fluorescence method, thus turned out to be structures containing granules with a release rate which placed them in a group of their own. Other properties, such as the behaviour towards reserpine and other drugs, also distinguished them from ordinary nerve or organ granules as well as from chromaffin cell granules. In other words, by studying the nerve granules of a tissue we have a way of characterizing the system from a kinetic and functional point of view. From a historical point of view it might perhaps be of interest to add that J. B. Collip in 1929 (13) had prepared highly active extracts from the so-called prostate gland of the bull. He noted that the active principle was similar to E in many respects, except that it had less hyperglycemic effect and that its effect on the blood pressure was not reversed by ergotamine; the latter is typical for NE.

ACTION OF DRUGS

Again, this field seems to merit a survey of its own, but brief mention should be made of some discoveries which have had a strong impact on our views and concepts. I am referring to the discovery of Brodie and his group (7) that reserpine exerts an amine-depleting action, later shown by Carlsson and Hillarp (12) and by Holzbauer and Vogt (45) to be true also for catecholamines. This drug has been followed by others, and by now there is an array of compounds, even including rocket fuels, having a more or less pronounced ability to deplete the NE stores. Our knowledge of the mechanism of action of reserpine is still far from complete, but two actions seem to merit special consideration. One is the

peculiar blocking of release observed in isolated nerve granules which can be demonstrated even in concentrations of 10^{-8} M (23). The other is the finding by Kirshner (47) that reserpine prevents the uptake and conversion of dopamine to NE in adrenal medullary granules. In accordance with this we have found (59) that reserpine blocks the synthesis of NE from dopamine also in isolated nerve granules.

The other drug which I would like to mention is tyramine, which has been known to have relations to the adrenergic nervous system for some 35 years, since Burn and Tainter (9) made the basic discoveries that adrenergic nerves were a prerequisite for its action and that its action was blocked by cocaine. When the NE stores are depleted, tyramine is no longer active, as shown by Carlsson, and it is generally believed to act by release of NE, as suggested by Burn and Rand (8). As shown by Schümann (56), this also occurs in isolated granules. I believe Dr. Stjärne is going to challenge this concept and I shall leave this subject. Before doing so I will mention that it is possible to fill a NE-depleted rabbit heart with NE simply by injecting 0.15 mg tyramine per kg intravenously; this finding suggests that tyramine may serve efficiently as a precursor of NE under certain circumstances.

A recent finding which seems to be significant is that all the adrenergic blockers which we have tested inhibit the release of NE from isolated nerve granules. Unless this is a peculiar coincidence, it might give some clue to the mechanism of binding not only in the granules but also to the receptors on the effector side

Inhibition of NE synthesis is a new field offering possibilities with far-reaching implications and some success has already been registered.

ADRENERGIC NERVE TRANSMISSION

Catecholamine studies have in recent years attracted the interest of many prominent research workers in widely divergent fields, as we can witness today. For the physiologist there are two main problems which still await elucidation; *viz.*, mechanism of action, that is, triggering the effector cell, and mechanism of release. Progress is noted in both these areas but much essential knowledge is still lacking. As to the second problem I believe we are closer to the solution. Cannon and Loewi first established the basic facts, and we know now at least something about release from the granular store. Opinions differ as to whether the transmitter is directly transferred to the receptor from the granules or whether it has an intermediate station from which it can be delivered. For the time being this postulated store may be referred to as the extragranular pool, from which the release may even occur in several steps.

This is not the right moment for discussing the various possibilities in the transmitter release process, but we may safely assume that depolarization of the axon terminal during nerve excitation alters its properties in such a way that the transmitter passes the synaptic cleft, perhaps aided by ionic currents. The resulting local deficit can further be assumed to disturb the equilibrium between the granular and the extragranular pool, causing a transfer of NE from the granules and a refilling of the partly emptied extragranular sites. This again in some

way triggers resynthesis, which must be a rapid process since we hardly notice any deficit even at high nerve activity with a large demand for the transmitter. Uptake from the outside fits in well with this concept. As to extragranular binding, we know virtually nothing except that it is loose. A possible candidate for holding the NE before it is discharged might be phospholipids, which we know to occur in considerable amounts in adrenergic nerves and which can bind catecholamines reversibly, a binding which is immediately broken up by small concentrations of polyvalent anions. This possibility was also considered by Loewi (50).

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