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THE ACTION OF RESERPINE ON NORADRENALINE BIOSYNTHESIS IN SYMPATHETIC NERVE TISSUE

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Abstract—In postganglionic sympathetic nerve tissue, reserpine produced a marked inhibitory effect on the synthesis of noradrenaline from tyrosine. This block in synthesis was localized at the dopamine-β-hydroxylase step. In contrast to similar studies in nerve homogenates, dopamine was not observed to accumulate in the intact nerves exposed to reserpine except when monoamine oxidase (MAO) was inhibited by pargyline. However, a larger increase in the acid metabolites of dopamine (notably, dihydroxyphenylacetic acid) was found in the nerves and medium after reserpine treatment. This indicates that in nerve tissue MAO plays an important role in the degradation of any free dopamine which cannot be protected by being taken up into the granular vesicles and subsequently converted to noradrenaline. Inhibition with an MAO inhibitor partially reversed the inhibitory action of reserpine on noradrenaline biosynthesis. These studies indicate that in intact postganglionic sympathetic nerves, reserpine inhibits the synthesis of noradrenaline by inhibiting the uptake of the precursor dopamine into the granular vesicles which contain the enzyme necessary for its ultimate conversion to noradrenaline.

THE ADMINISTRATION of reserpine is known to cause an extensive depletion of tissue catecholamines.[1–3] This depletion can be produced with small doses of reserpine, develops slowly, is usually quite prolonged and is not accompanied by marked sympathomimetic effects. Although a great deal of research effort has been devoted to the elucidation of the mechanism of this depletion, it is as yet poorly understood despite the fact that many modes of action have been proposed for this alkaloid.

It has been suggested by many investigators that the primary action of reserpine is to prevent the normal storage of NA* in storage granules by disrupting the uptake or binding mechanism for this amine.[4–7] However, it has also been proposed that reserpine may, in addition, cause a lowering of NA levels by interfering with the biosynthesis of this neurohormone through a blockade of the uptake of DA into its synthesizing site within the granule.[8–10] In this case, part of the depletion would be a result of the lack of newly synthesized NA available to replace that lost during functional activity. This latter alternative has received little attention, although it appears possible that this might be a mechanism by which reserpine could deplete a functional pool of NA. Experiments *in vitro* have already demonstrated that the uptake of DA and NA into chromaffin granules[9] and into splenic nerve granules is

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Abbreviations used in this paper: NA, noradrenaline; DA, dopamine; MAO monoamine oxidase; PCA, perchloric acid; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, dihydroxyphenylacetic acid; DOMA, dihydroxymandelic acid; NM, normetanephrine; DHPG, dihydroxyphenyl-glycol; DHPE, dihydroxyphenyl-ethanol.

blocked by reserpine.[6] In intact tissue, a block in DA uptake would be expected to expose newly synthesized DA to intraneuronal MAO, resulting in both an increase in deaminated metabolites and a reduction in the synthesis of NA. Recent evidence *in vitro* has in fact demonstrated that reserpine does block the formation of NA from tyrosine in homogenates of nerve tissue.[11] This block in synthesis occurs selectively at the DA to NA conversion.[12] Moreover, inhibition of the conversion of DA to NA was shown to be of a competitive nature, since addition of progressively more DA to the medium overcame the inhibition exerted by reserpine. This finding suggests that the block in NA synthesis produced by reserpine is not an irreversible phenomenon. However, whether this mechanism is functional in intact nerve tissue remains to be clarified. Therefore, the purpose of this investigation is 2-fold: (1) to determine if reserpine has any effect on NA synthesis from tyrosine in intact bovine splenic nerve; (2) if any effect is apparent, to localize it to one or more steps in the biosynthetic pathway for NA formation.

METHODS

Bovine splenic nerves were obtained from the slaughterhouse 20–40 min post-mortem and immediately chilled on ice. The nerves were then carefully dissected free from contaminating tissue and desheathed. The nerves (about 0·5 g) were weighed and transferred to 10-ml Erlenmeyer flasks containing 5 ml of Tyrode's solution, 20 μg/ml ascorbic acid, L-tyrosine (2×10^{-5} M) and ^{14}C-L-tyrosine ($7{\cdot}1 \times 10^5$ cpm/ml, ^{14}C-L-Tyrosine, New England Nuclear Corp., Boston, Mass.; sp. act. 367 mc/m-mole, uniformly labeled and purified on alumina prior to use). The nerves were preincubated at 0° with or without 10^{-5}M reserpine (reserpine phosphate, lyophilized, Ciba). In some cases, pieces (about 1 g) of nerve were incubated in Tyrode's solution and ascorbic acid with 50 μc H^3-NA (New England Nuclear Corp., Boston, Mass.; DL-Norepinephrine, 8·8 c/m-mole). Incubations were carried out in a Dubnoff metabolic shaker at 37° for 30 min and in all but one experimental group aerated with 97% O_2 + 3% CO_2. This increased the oxygen concentration of the media to about 5 times that found when the media were saturated with air. At the end of the incubation the samples were placed in an ice bath, the nerves were removed, blotted and homogenized by means of an Ultra-Turrax apparatus in 5 ml of 10% TCA. The incubation media were acidified with 0·5 ml of 50% TCA. The samples were left to extract at 0° for 30 min and the precipitated protein was then removed by centrifugation at 30,000 *g* for 10 min; 100 μg of cold carrier tyrosine, 0·2 ml of 1 M Tris buffer and 1 ml of 10% EDTA were added and the samples brought to a final volume of 10 ml. Each sample was neutralized to pH 8·3 to 8·5 with NaOH and passed twice over a $1 \times 1{\cdot}5$ cm column of aluminum oxide in order to retain selectively the catechol compounds. The effluent and the first 10 ml of water wash containing the noncatechols and *O*-methylated compounds were saved for subsequent analysis by ion-exchange column chromatography and the columns were washed with an additional 30–50 ml of distilled water. The catechol compounds were eluted with 6 ml PCA (3 ml of 0·2 M + 3 ml of 0·1 M). A portion of the eluate was oxidized according to a modification of the method of von Euler and Lishajko.[13] Fifty μg carrier NA, DA and DOPA and 100 μg DOPAC and DOMA were added to each of the remaining eluates. The pH of each sample was adjusted to 4 and the samples were frozen and retained for chromatography on Amberlite CG-120 according to a modification of the procedure

of Stjärne and Lishajko.[11] The effluent and water wash from the Amberlite column, which contains catechol acid and glycol metabolites, was in some cases initially extracted at pH 7·0 with 5 vol. of ethyl ether to remove the glycols, which turned out to be only a small percentage of the metabolites (*ca.* 20 per cent). The pH of the aqueous phase was then adjusted to pH 1 and the aqueous phase was extracted again with ether to remove the acids. (By means of this procedure, 80 per cent or more of the radioactivity was extractable into the ether phase.) The ether phase was blown off with nitrogen and the acids were taken up in a small volume of ethanol and subjected to thin-layer chromatography. Thin-layer chromatography (TLC) was carried out largely according to the procedure of de Potter *et al.*[14] Thin-layer plates were coated with a 250-μ layer of MN cellulose 300. An aliquot (50 μl) of the ethanol extract as well as standards of DOPAC and DOMA were applied to the plate and the plates were developed in a solvent of n-butanol saturated with 3 N HCl for about 3 hr or until the solvent had moved about 15 cm. After development, the plates were dried and the separated substances detected by spraying the plate with ethylenediamine mixed with an equal volume of distilled water. Fluorescence was observed with the aid of a portable Mineralight; and the fluorescent areas were marked, removed with a spatula and placed directly in a scintillation vial containing 10 ml of scintillation mixture (2:1, toluene:absolute ethanol containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4 *bis*-2-(5-phenyloxazolyl)benzene per liter of toluene + 4% Cab-O-Sil). Blanks consisted of equal areas of cellulose similarly removed from the thin-layer plates in the position of standards of DOPAC and DOMA. The R_f values for DOPAC and DOMA were 0·84 and 0·72 respectively. In some cases the partially purified acid and glycol fractions (consisting largely of DOPAC, Fig. 4) obtained after alumina and Amberlite column chromatography were pooled, counted and again passed over the alumina column to evaluate the overall recovery of the acidic and neutral metabolites isolated from the nerves and media by this procedure. The average recovery of the acids and glycols isolated from the nerves was 36 per cent, while the recovery of the metabolites isolated from the bath was 24 per cent. Fig. 1 illustrates the details of the separation procedure.

RESULTS

In a preliminary study we were not able to obtain consistently high and reproducible recoveries of dopamine by the alumina column adsorption method.[15] Kindwall and Weiner reported that higher recoveries could be achieved if adsorption was carried out at pH 9·6 at 4°.[16] However, at this pH we found DA recoveries by our column procedure at room temperature to be quite variable. When samples are passed over alumina according to the method of von Euler and Lishajko, the pH of the effluent always exceeds 8·4, the pH to which the extract was initially adjusted. Therefore, we passed the extracts through the alumina column twice (see Methods) and by this means achieved excellent recoveries of both NA and DA. This procedure provided an NA recovery of 83·9 $\pm$ 4 per cent and a DA recovery of 84·9 $\pm$ 3·4 per cent (mean $\pm$ S.D.). Therefore this technique was employed throughout the following experiments. The results reported are corrected for recovery of individual metaboltes on the alumina column.

Recent experiments by Roth *et al.*[17] have indicated that the synthesis of NA from tyrosine in bovine splenic nerve trunk is very rapid and linear for at least 1 hr. There-

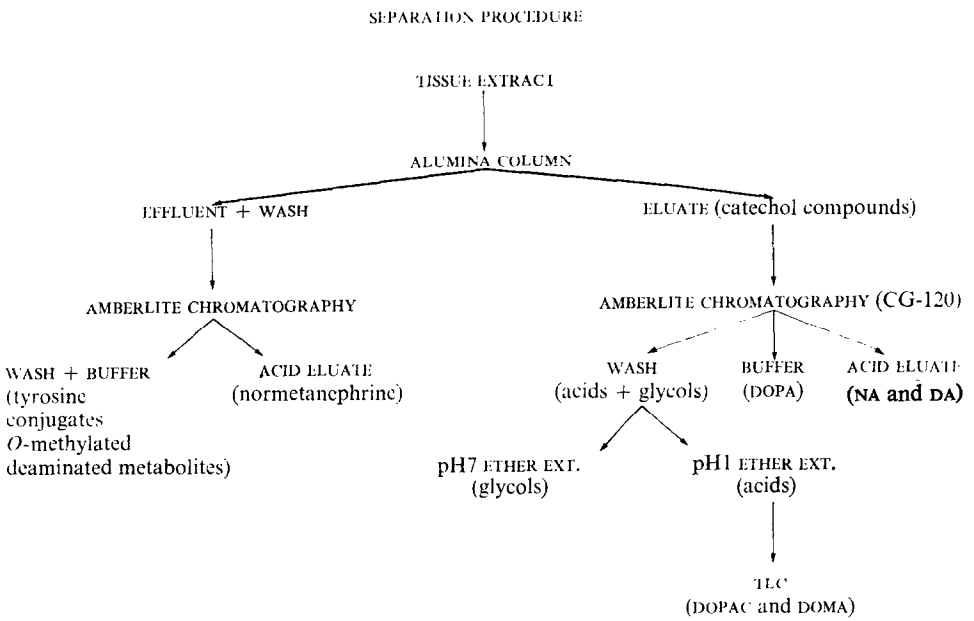

FIG. 1. Schematic diagram of the procedure used to separate the radioactive metabolites of tyrosine.

fore, in this investigation a time interval of 30 min was selected for all the synthesis studies. Initial experiments on the synthesis of NA from ^{14}C-tyrosine demonstrated that oxygenated media were necessary for the maintenance of an optimal synthesis of NA as well as for total catechol synthesis (see Table 1). The inhibition seen in the absence of oxygen bubbling is apparent at both steps in the biosynthetic pathway known to be dependent upon atmospheric oxygen.[18] Therefore, in all other experiments the incubation medium was oxygenated by constant bubbling with 97% O_2 + 3%

TABLE 1. SYNTHESIS OF CATECHOLAMINES IN BOVINE SPLENIC NERVE INCUBATED WITH 14C-TYROSINE: EFFECTS OF GASSING WITH O_2 OR INCUBATION WITH RESERPINE (10^{-5}M)

	Synthesis‡ (n-mole/g/hr)	% NA	% DA	% DOPA	% Acids +glycols	Recovery*
Nerves						
Nonoxygenated (n = 4)	11·14 ± 0·94	41·8 ± 4·7	35·8 ± 4·2	3·1 ± 0·3	5·2 ± 0·7	86·6
Oxygenated (n = 3)	15·16 ± 0·40	68·6 ± 4·6	12·0 ± 0·8	2·5 ± 0·3	7·9 ± 2·3	91·0
Reserpine (n = 4)	11·22 ± 0·95	19·3 ± 1·6	9·4 ± 1·8	5·9 ± 1·0	51·1 ± 1·6	85·7
Incubation medium						
Nonoxygenated (n = 4)	7·49 ± 0·47	5·2 ± 0·4	12·0 ± 1·3	32·4 ± 5·7	35·9 ± 4·2	85·5
Oxygenated (n = 3)	8·43 ± 0·69	6·5 ± 2·3	9·1 ± 1·6	33·8 ± 7·1	45·8 ± 8·3	95·2
Reserpine (n = 4)	11·15 ± 0·49	3·5 ± 0·6	6·4 ± 0·3	28·8 ± 9·7 (n = 2)	53·5 ± 11·5 (n = 2)	92·2

* Percentage of the radioactivity recovered from the alumina column which was identified as NA, DA, DOPA or acids and glycols.
† Results are expressed as the mean ± S.E.M.
‡ Total catechol synthesis corrected for recovery of each individual metabolite isolated by the alumina column procedure.

CO_2 throughout the incubation period. Since we were concerned with all the catechol metabolites, it was of initial interest to determine if any significant portion of the catecholamines formed *in situ* from ^{14}C-tyrosine were *O*-methylated. *O*-methylated catechols do not adsorb on alumina and therefore the effluent + water wash from the alumina column procedure was taken for chromatography on Amberlite CG-120 after addition of carrier NM. A chromatogram from a typical synthesis experiment is illustrated in Fig. 2. No labeled normetanephrine was ever detected in the nerves or in

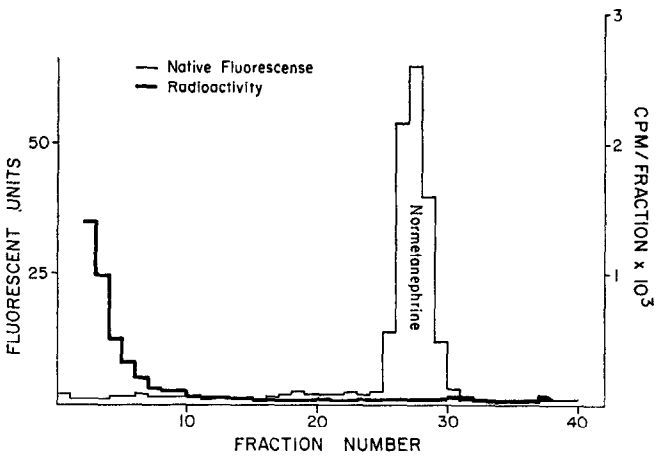

FIG. 2. Typical ion-exchange chromatogram of alumina column effluents obtained from splenic nerves incubated with ^{14}C-tyrosine.

the medium even when the nerves were incubated with reserpine and pargyline. However, in order to be certain that the splenic nerves did not contain substantial *O*-methylating capacity that might have been overlooked due to subsequent deamination (in experiments lacking an MAO inhibitor), larger amounts of nerve (*ca.* 1 g) were incubated in Tyrode's solution containing ascorbic acid and 50 μg of ^{3}H-NA with or without pargyline (10^{-4}M). In addition, to avoid the possibility that the normetanephrine formed was immediately released into the medium, both the incubation medium and the nerve were extracted together, passed over alumina to remove the majority of intact NA and a portion of the effluent + water wash was subjected to Amberlite chromatography after addition of carrier NA and NM. Fig. 3 shows a typical chromatogram obtained from such an experiment. Only a trace of the NA added to the medium was converted to NM even in the presence of an MAO inhibitor (*cf.* Table 2).

In view of the absence of substantial *O*-methylating capacity of the splenic nerve tissue under the conditions of these experiments, an analysis of the effect of drugs on the biosynthesis of catecholamines in this tissue becomes less complicated. Reserpine at a concentration of 10^{-5}M has a profound effect on the synthesis of catechol compounds from ^{14}C-tyrosine. Total synthesis is blocked only about 5 per cent while the apparent synthesis of NA is blocked about 86 per cent (*cf.* Table 1). Reserpine also changed the distribution of the products formed from ^{14}C-tyrosine. As has been previously demonstrated in splenic nerve homogenates, reserpine decreased the proportion of newly synthesized NA, but in contrast to those experiments, we did not observe any concurrent accumulation of DA in either the nerve tissue or the

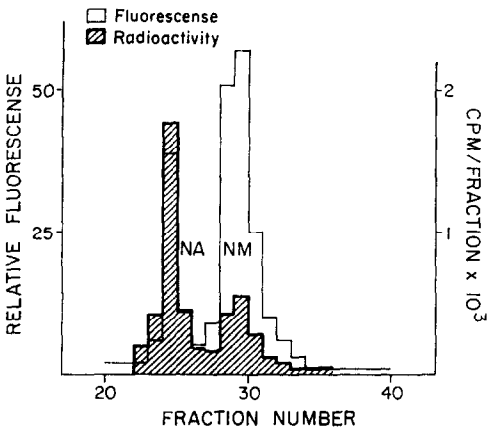

FIG. 3. Typical ion-exchange chromatogram of alumina column effluents obtained from splenic nerves incubated with 50 μc of ^{3}H-noradrenaline. Carrier added:noradrenaline (NA) = 50 μg, normetanephrine (NM) = 100 μg.

TABLE 2. *O*-METHYLATION OF ^{3}H-NORADRENALINE BY INTACT SPLENIC NERVES*

	% NA *O*-methylated	Normetanephrine formed (n-mole/g)
Control (n = 4)	0·145 ± 0·022	0·0094 ± 0·0013
Pargyline (n = 4)	0·148 ± 0·039	0·0096 ± 0·0027

* Results are expressed as mean ± S.E.M.

medium. Rather, reserpine produced a large increase in acid and glycol metabolites in the intact nerve. Reserpine in this short time did not, however, produce any significant depletion of endogenous NA in the nerve trunk. Also very little intact ^{3}H-NA was released into the incubation medium in the presence of the alkaloid (*cf.* Table 1). Incubation of the reserpinized nerves with pargyline was found to partially reverse the action of reserpine as evidenced by the production of a large increase in DA which partially overcame the inhibition of NA biosynthesis. Thus pargyline caused about a 16-fold increase in the DA in the nerve and only about a 3-fold increase in NA (Table 3).

TABLE 3. REVERSAL OF RESERPINE-INDUCED INHIBITION OF CATECHOLAMINE SYNTHESIS BY MAO INHIBITION

	Synthesis* (n-mole/g/hr)	NA	DA	DOPA	Acids + glycols
Control (n = 2)	12·54	7·29	1·28	0·21	3·76
Reserpine (n = 2)	3·15	0·48	0·20	0·10	2·37
Pargyline (n = 2) + reserpine	5·45	1·38	1·38	0·12	0·79

* Total catechol synthesis corrected for recovery of each individual metabolite isolated by the alumina column procedure.
Mean synthesis in n-mole/g/hr corrected for recovery on alumina and Amberlite.

Since in nerve homogenate studies it has been demonstrated previously that reserpine inhibits the conversion of DA to NA with a concurrent build-up of DA, whereas in the present experiments reserpine did not produce a marked accumulation of DA except after MAO inhibition, it was of interest to determine the origin of the large amounts of acids or glycols or both in the presence of reserpine (i.e. whether derived from DA or NA). Initial experiments demonstrated that less than 30 per cent of the radioactivity in the acid + glycol fraction was extractable at pH 7, suggesting that the majority of the metabolites in this fraction were acids since 80 per cent or more was extractable into ether at pH 1. TLC of the ether-extractable acids demonstrated that the majority of the radioactivity in both the control nerves and in those treated with reserpine had the same R_f as authentic dihydroxyphenylacetic acid. Only trace amounts of labeled dihydrocymandelic acid were found (Fig. 4).

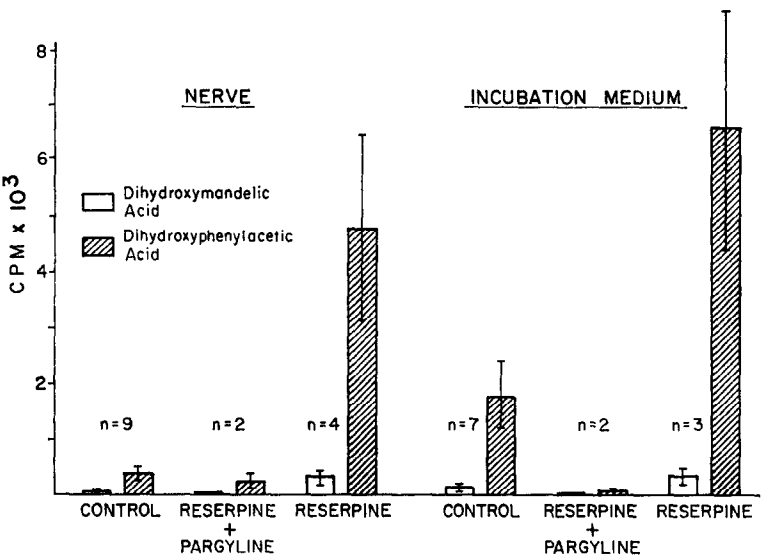

FIG. 4. Radioactive acid metabolites isolated from splenic nerve by TLC after incubation with ^{14}C-tyrosine. n = Number of experiments. Results are illustrated as the mean ± S.E.M.

DISCUSSION

The results obtained in these experiments are in many ways similar to those observed in nerve tissue homogenates treated with reserpine, which indicate that in intact nerve tissue as well as in homogenates reserpine has a profound effect on the synthesis of NA from tyrosine.[11, 12] However, in contrast to the homogenate studies, DA was not observed to accumulate except when MAO was inhibited by pargyline. Previous experiments in both homogenates and intact nerve have demonstrated that reserpine produces a strong inhibition in the uptake of NA into the storage granules.[6, 19] In the intact tissue it appears as if this uptake mechanism is important for protecting newly formed DA from attack by MAO. When this uptake and binding mechanism is interfered with, the newly formed DA is deaminated very rapidly by MAO, thus explaining the large increase in acid metabolites in the nerve and medium after

reserpine treatement. This may indicate that in nerve tissue in general MAO plays an important role in the degradation of any "free" DA which cannot be taken up into the granules and subsequently converted to NA. Therefore, MAO may also serve to exert some influence on the biosynthetic pathway for NA formation by regulating the supply of DA reaching the β-hydroxylating enzyme as well as by playing a role in the degradation of intact NA released intraneuronally.

Since reserpine produces a major shift ih the metabolism of newly synthesised DA to deaminated products this leads to a lower overall efficiency in the recovery of catechol metabolites on alumina chromatography. If the recovery of individual metabolites is not corrected for, reserpine could appear to cause an inhibition of catechol biosynthesis at the tyrosine hydroxylase step. In addition, by facilitating the deamination of DA, reserpine causes an increase in the newly synthesised metabolites released into the incubation medium. Therefore, if the media or perfusion fluid are not analysed simultaneously for metabolites reserpine would appear to cause a net reduction in catecholamine biosynthesis. However, when these factors are taken into account reserpine appears to affect catechol biosynthesis primarily at the site whese DA is converted to NA causing a decrease in NA formation and an increase in DA metabolites.

Recent experiments with inhibitors of NA biosynthesis have demonstrated that some behavioral parameters can be correlated more closely with NA synthesis inhibition than with total brain concentrations of NA.[20] Thus, alterations in behavior are observed after treatment with alpha-methyl-*p*-tyrosine at a time when amine levels are at about 80 per cent of the normal concentration. These findings may suggest that newly synthesized NA feeds directly into the functional pool of NA without initially mixing with the endogenous store. Likewise, NA biosynthesis has been shown to be essential for the central action of some drugs like amphetamine.[21]

The demonstration that reserpine can block NA synthesis in intact sympathetic neurons, coupled with the homogenate studies *in vitro* indicating a difference in the sensitivity between the inhibitory effects of reserpine on NA binding and synthesis,[12] could indicate that shortly after reserpine administration when tissue levels of reserpine are high, synthesis as well as binding is effectively blocked. However, in the face of a waning drug concentration, synthesis may be restored while binding is still effectively inhibited. This may explain the finding of Haggendal and Linqvist[22] and Haggendal *et al.*[23] who, on chronic administration of reserpine to rabbits, were able to disclose a small, labile pool of NA which appeared to be essential for function. This could represent the newly synthesized pool of NA that is restored only when the extra-granular tissue concentration of DA reaches a level at which it overcomes the inhibition exerted by reserpine at the level of the uptake of DA into the synthesizing granules. It is quite conceivable that the restoration of normal synthesis may be more closely related to the return of function than the restoration of the tissue uptake of circulating NA or the total tissue levels of amine, since only about 10 per cent of the NA present in tissue appears to be necessary for intact adrenergic function.

Recently, Glowinski *et al.*[24] have demonstrated that in rat brain reserpine did not have a marked effect on the conversion of DA to NA. However, in their experiments they used an MAO inhibitor which might be expected to allow an abnormal accumulation of DA in the brain and thereby reverse the inhibitory effect of reserpine on NA biosynthesis. The fact that MAO inhibitors not only reverse the behavioral

effects produced by reserpine but also partially restore tissue levels of NA make this possibility even more feasible.

Fig. 5 depicts a conceptual model of the sympathetic nerve ending and illustrates the postulated sites of action of reserpine. Initially tyrosine is taken up into the neuron

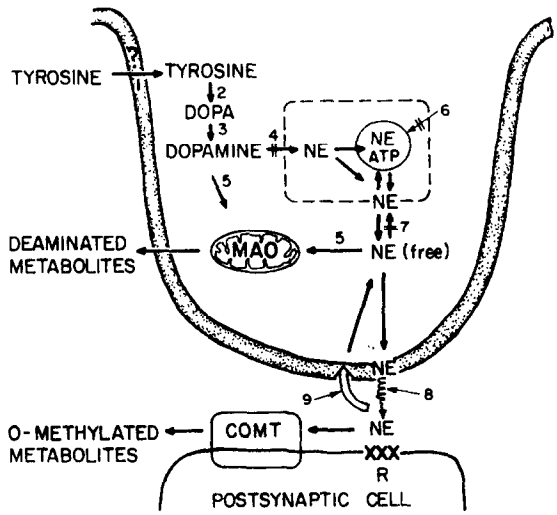

FIG. 5. Schematic representation of an adrenergic nerve ending. 1. Tyrosine uptake into the neuron. 2. Conversion of tyrosine to DOPA by means of the enzyme tyrosine hydroxylase. 3. Conversion of DOPA to DA by dopa decarboxylase. 4. Uptake of DA into the granular vesicle and subsequent conversion to NA by means of the enzyme DA-β-hydroxylase. Reserpine blocks this step by interfering with the uptake of DA into the β-hydroxylation sites on or within the granule membrane. 5. Destruction of intraneuronal free DA or NA by MAO giving rise to dihydroxyphenylacetic acid and dihydroxyphenyl-ethanol (DA metabolites) and dihydroxymandelic acid and dihydroxyphenyl-glycol (NA metabolites). 6. Binding of NA within the granular vesicle, presumably to ATP and protein, to form a stable complex. Reserpine in some manner may disrupt this stable binding of NA leading to release of bound NA. 7. Uptake of NA into the granular vesicle. Reserpine interferes with both the uptake and release of NA from the storage granules. 8. Nerve stimulation brings about the release of intra neuronal NA. The majority of NA released, after exerting its action is "recaptured" and taken back up into the neuron.

and is subsequently converted to dopa via the enzyme tyrosine hydroxylase. The dopa formed is then converted to DA by means of the cytoplasmic enzyme, dopa decarboxylase. The DA is either taken up into the NA storage vesicles where it is subsequently converted to NA or metabolized intraneuronally by MAO giving rise to either DOPAC or DHPE. The newly formed NA can either be stored in the storage granules or mobilized to the "free" pool of NA. This unbound NA, which is in dynamic equilibrium with the granule-bound NA, is also exposed to attack by MAO. However, NA is not nearly as good a substrate for MAO as is DA,[25, 26] perhaps explaining why more labeled DA metabolites as compared to NA metabolites are found even in control nerves. Reserpine appears to have three possible actions in this model system: (1) its well known effect on the binding and storage of NA; (2) its action on the uptake and release of NA from the nerve granules; (3) a block in the uptake of DA into the site where it is ultimately converted to NA (i.e. in or on the granule membrane) resulting

in an effective block of NA biosynthesis. At the present time it is not known whether the inhibition of storage of endogenous NA or the inhibition in the formation of NA is more important for the development of the functional sympathectomy resulting after treatment with reserpine. However, it is conceivable that the maintenance of complete amine depletion is dependent on effective synthesis inhibition as well as on a disruption of the storage capacity of the tissue. The observation that certain organs remain functional despite a depletion of the amine content to only 10–20 per cent of the control level suggests that a small fraction of the tissue content of amine may be related to function. Chronic treatment with reserpine abolishes this small pool for a time interval well correlated with the absence of function.[22, 23] The possibility remains that part of reserpine's action may be exerted by the depletion of a small functionally essential pool of NA by interfering with the biosynthesis of this neurohormone by blocking the uptake of DA into the site where it is converted to NA.*

* While this manuscript was in preparation, C. O. Rutledge and N. Weiner published a paper (*J. Pharmac.* **157**, 290, 1967) which strongly documented the inhibitory action of reserpine upon the synthesis of NA in the rabbit heart. This inhibitory action of reserpine on NA biosynthesis was demonstrated to be the result of an inhibition of DA uptake into the site where it is subsequently converted to NA.

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