Biochemical Pharmacology, Vol. 16, pp. 1729–1739. Pergamon Press Ltd. 1967. Printed in Great Britain

# NORADRENALINE FORMATION FROM DOPAMINE IN ISOLATED SUBCELLULAR PARTICLES FROM BOVINE SPLENIC NERVE

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(*Received* 14 *December* 1966; *accepted* 20 *March* 1967)

**Abstract**—The process of β-hydroxylation of dopamine (DA) and the fate of the newly formed noradrenaline (NA) were studied in high-speed sedimentable particles from bovine splenic nerves, after resuspension in potassium phosphate. The NA formation was found to be a linear function of the DA concentration in the medium. However, only a fixed amount of the newly formed NA was retained in the particulate fraction; the remainder appeared in the supernatant. Addition of NA to the medium reduced NA formation from DA, but hardly affected binding of the newly formed NA, while severely depressing DA retention. On the other hand, DA was shown to have about the same affinity as NA for the amine uptake mechanism of the particles. Reserpine added to the medium inhibited the formation of NA from DA, as well as the binding of NA and of DA, to an extent dependent on the drug/DA concentration ratio. The inhibition of NA formation was much less pronounced than that of firm amine binding. Although the results suggest that the membranes of the particles are permeable to NA and to DA, they do not rule out a certain inhibitory effect of reserpine on amine passage across the membranes. Washing was found to release loosely bound amines or amine derivatives from the particles. Loose binding appeared to be blocked by reserpine. The results indicate that deamination proceeds in the high-speed sedimentable particles. Inhibition of monoamine oxidase did not affect the NA formation from DA.

IN A STUDY of the localization of the different steps in the biosynthesis of noradrenaline (NA) from tyrosine in fractions from the bovine splenic nerve homogenate, only the dopamine (DA) β-hydroxylation step was found to require the presence of the high-speed particulate fraction containing the specific NA-storing vesicles.[1]

In the present investigation the properties of the DA β-hydroxylating mechanism and the fate of newly formed NA were studied in the above mentioned high-speed sedimentable particulate fraction resuspended in potassium phosphate. The results provide some information on the capacity of the DA β-hydroxylating enzyme, the storage compartment for newly formed NA, and the influence of certain drugs on the processes of synthesis and storage.

## MATERIAL AND METHODS

The bovine splenic nerve homogenate was prepared as previously described,[1] by means of an Ultra-Turrax apparatus. After removing coarse tissue particles by centrifugation at 9000*g* for 10 min the supernatant was centrifuged at 50,000*g* for

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30 min, and the sediment (derived from about 1*g* of nerve tissue) was resuspended in 8 ml of 0·13 M potassium phosphate (pH 7·5). Resuspended high-speed (HS) particles, which contain about 60 per cent of the total NA of splenic nerve tissue,[2] are not homogeneous, but consist of specific NA-storing vesicles contaminated to a certain (as yet undefined) extent with other particles that have similar sedimentation properties (work in progress). Since preliminary experiments indicated that the formation of NA from DA in this preparation was linear for 60 min at 20° the incubations were carried out for this time and at this temperature in the presence of ATP (3 mM), $MgSO_4$ (3 mM), tritium-labeled DA (New England Nuclear Corp. 3,4-dihydroxyphenylethyl-1-$^3$H-amine-HBr, sp. act. 50 mc/m-mole; about 1 $\mu$c/ml) and various amounts of unlabeled DA or NA. In control experiments with boiled HS particles, no NA was formed from DA. In experiments intended to study the affinity for the amine uptake mechanisms of the NA storage vesicles, tracer amounts of tritium-labeled DL-NA (New England Nuclear Corp., DL-NA-7-H$^3$ hydrochloride, sp. act. 7 c/m-mole, chromatographically purified prior to use; 1 $\mu$c/ml), were added together with various amounts of unlabeled DA or L-NA. In some experiments the preparation was preincubated at 0–4° for 30 min with the monoamine oxidase (MAO) inhibitor pargyline (Eutonyl, Abbott Laboratories, $10^{-4}$M) or with reserpine (reserpine phosphate lyophilized, Ciba, $10^{-5}$M).

After the incubation period the tubes were chilled in an ice bath and centrifuged at 50,000*g* for 30 min. The supernatant fractions were decanted, the walls of the tubes were carefully wiped dry and the sediments were resuspended in 9 ml potassium phosphate and again centrifuged at 50,000*g* for 30 min. The second supernatant fractions (wash) were decanted, the walls of the tubes were again wiped dry and the sediments were extracted with 0·5 ml 0·4 M perchloric acid. The two supernatants were extracted with 0·8 ml 4 M perchloric acid. The extracts were adjusted to pH 5, the perchlorate precipitate was removed by centrifugation, and the supernatants were kept at −30° until analyzed by cation-exchange column chromatography as previously described.[1]

## RESULTS AND DISCUSSION

### *NA formation in the presence of increasing concentration of DA*

In accordance with observations in the perfused guinea pig heart,[3] the formation of NA from DA in the resuspended HS particles from a bovine splenic nerve homogenate was found to increase with the DA concentration in the medium (Fig.1). When the DA concentration was increased 13-fold, there was a 5–6-fold rise in the amount of newly formed NA found in the tubes at the end of the incubation period.

### *Fate of newly formed NA*

*Incubation with increasing concentrations of DA.* While NA formation increased with the DA concentration in the medium, the amount of newly formed NA recovered from the washed high-speed pellet remained essentially constant, at about 1 nanomole per pellet, throughout the DA concentration range. The remainder of the newly formed NA was recovered from the incubation medium. The amount of chromatographically identified exogenous DA in the washed pellet also remained constant, at 4–6 nanomole per pellet, and the total amount of exogenous DA derivatives increased only

moderately, from about 7 nanomole in the low DA range to about 10 nanomole in the high DA range (Fig. 2).

The HS particles were thus found to bind constant amounts of newly formed NA and of DA irrespective of large changes in the concentration of newly formed NA or of DA in the medium. Occasional fluorimetric determination did not reveal any change in the total NA content of the pellet due to the increased DA concentrations in the medium.

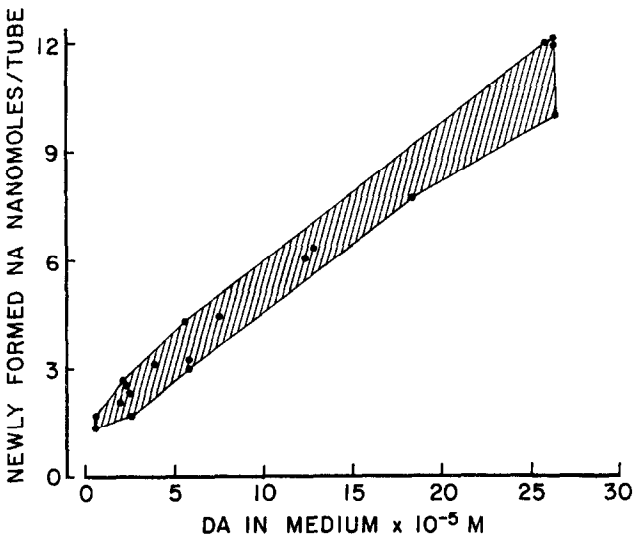


FIG. 1. NA synthesis as a function of the DA concentration in the medium.

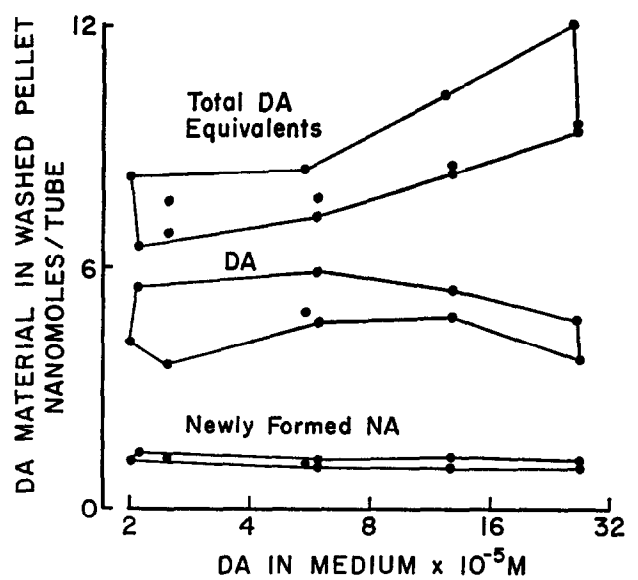


FIG. 2. Retention in the washed pellet of chromatographically identified DA, newly formed NA, and total exogenous amine material (calculated from the sp. act. of the DA in the medium), as a function of the DA concentration.

*Incubation with constant amounts of DA in the presence of increasing concentrations of NA.* In order to study the properties of the β-hydroxylating mechanism, the present preparation was incubated with constant amounts of DA in the presence of increasing concentrations of NA. Raising the NA concentrations in the medium from 0 to 15·6 × $10^{-5}$ M, which changed the molar NA/DA ratio from virtually 0 to 8/1, was found to reduce the NA formation from DA by 26 per cent. The percentage of the newly formed NA found in the washed pellet decreased from 50 to 40 per cent, while the chtomatographically identified exogenous DA in the pellet was reduced by 87·5 per cent (Fig. 3).

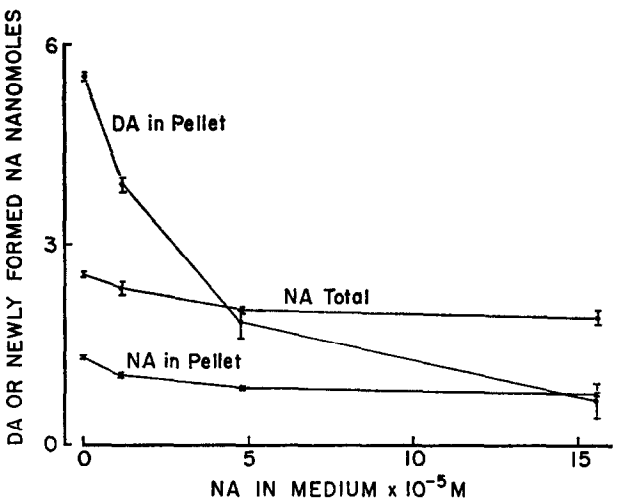


FIG. 3. NA formation, and retention in the washed particles of newly formed NA and chromatographically identified DA, as a function of the NA concentration in the medium.

These results indicate that NA in the medium to some extent inhibits the β-hydroxylation of DA, presumably by competing with DA for uptake into the β-hydroxylation sites. This interpretation is supported by similar results recently obtained with the bovine splenic nerve preparation in a study of the inhibitory effects of exogenous NA on the synthesis of NA from tyrosine.[4]

Although NA in the medium was found to compete with DA for storage in the HS particles, it did not appreciably reduce the percentage of the newly formed NA recovered firmly bound to the washed pellet at the end of the experiment. Since an increase in the ratio of exogenous NA/newly formed NA from virtually 0 to 680/1 caused a decrease in firmly bound newly formed NA only from 50 to 40 per cent, it must be concluded that the newly formed NA found in the washed pellet was stored immediately after it was formed from DA, in the same particles where it was formed, without previous passage into the medium and subsequent competition with NA in the medium for uptake into storage sites.

*Incubation with tracer amounts of $^3$H-DL-NA in the presence of large amounts of L-NA or DA.* In order to study the relative affinity of DA and NA for the NA uptake mechanism of the specific NA storage vesicles, the present preparation was incubated with tracer amounts of $^3$H-DL-NA in the presence of unlabeled L-NA or DA 0–15·6

$\times 10^{-5}$M. Both catecholamines were found to strongly inhibit the uptake or retention or both of the labeled NA in the washed pellet to about the same extent (Fig. 4).

On the basis of the findings described above, it may be concluded that NA is formed from DA in HS particles and up to a limit is stored in the particles where it was formed; the remainder appears in the incubation medium. The limitation of the

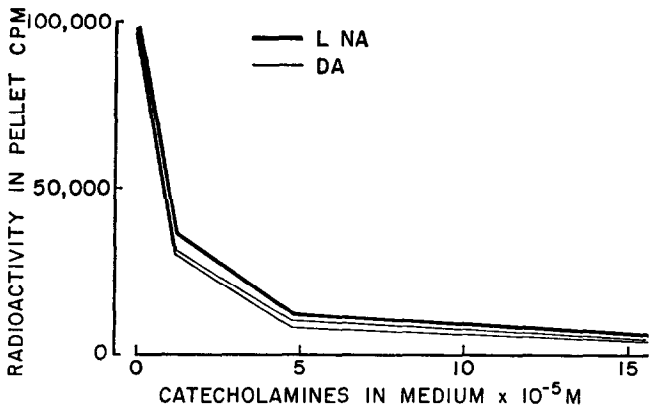


FIG. 4 Inhibition of uptake and retention of tracer amounts of $^{3}$H-DL-NA by L-NA and DA in the medium.

amount of newly formed NA bound in the washed pellet (5–10 per cent of the total amount of endogenous NA in nonincubated HS particles) makes it unlikely that newly formed NA is stored in the main endogenous NA store, and rather suggests the existence of a separate compartment for storage of newly formed NA, possibly with a rapid turnover, inside the NA storage vesicles or in separate synthesis vesicles.

The present experiments also show that DA in the incubation medium competes with NA for uptake into HS particles. The relatively constant amount of exogenous DA found in the washed HS particles (a level corresponding to 25–50 per cent of the total endogenous NA content of nonincubated HS particles), in the presence of a 13-fold increase in the DA concentration of the medium, suggests that exogenous DA can accumulate in some storage compartment that normally contains NA, as long as there are free binding sites available, and can thus fill the compartment to maximum capacity. It appears likely that this compartment is identical with the main NA store in the specific NA storage vesicles. In view of the finding that DA had at least as high an affinity as L-NA for the amine uptake mechanism of the HS particles, judged from its efficiency in inhibiting $^{3}$H-DL-NA uptake and retention, it was surprising that high concentrations of L-NA in the medium so strongly decreased the amount of DA found in the washed pellets, but only moderately decreased the conversion of DA to NA or the retention of newly formed NA. This suggests that DA, lacking the $\beta$-hydroxyl group, may be less firmly bound in the storage vesicles than NA.[5] The implication is that the amount of any exogenous amine found in NA storage vesicles represents the net result of competition with (endogenous or exogenous) NA, both for uptake and for retention. High affinity for uptake may occur in a compound which is subsequently less firmly bound.

*Effects of drugs on the β-hydroxylation of DA*

*Reserpine.* In previous experiments it was demonstrated that reserpine can completely inhibit the last step in NA synthesis from tyrosine without appreciably affecting the formation of DA.[6] In the present experiments preincubation with reserpine ($10^{-5}$M) was found to inhibit NA synthesis from DA almost completely when the DA concentration in the medium was so low that the molar reserpine/DA ratio approached unity. At higher DA concentrations the inhibitory effect on the β-hydroxylation of DA was decreased, and at molar ratios below 1/10 the presence of reserpine did not affect NA formation. However, at all DA concentrations the retention in the washed pellet of newly formed NA or of DA was strongly inhibited (Fig. 5).

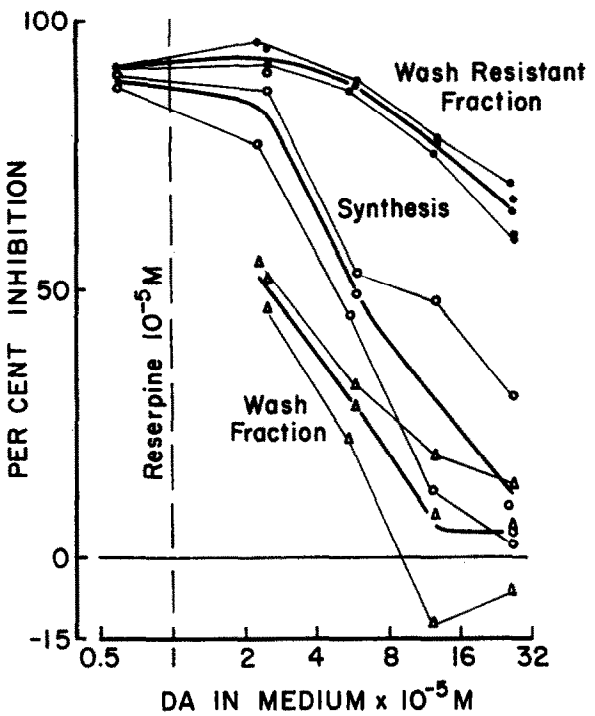


FIG. 5. Inhibition by reserpine $10^{-5}$ M of NA synthesis, and of uptake and retention in the wash-resistant and wash fractions, respectively.

These results indicate that reserpine interferes with the amine transport or binding mechanisms or with both in a complex manner, even in the present system *in vitro*. A 50 per cent inhibition of NA synthesis required a reserpine/DA ratio of about 1/5. However, extrapolation from the data in Fig. 5 suggests that the same degree of inhibition of wash resistant binding in the HS particles occurs even if the DA concentration is raised to reduce the reserpine/DA ratio to 1/100. This latter value is in agreement with that found in a previous study of the inhibitory effect of reserpine on NA uptake and retention in bovine splenic nerve storage particles.[7]

While the high amine concentration required to overcome the inhibitory effect of reserpine on firm binding indicates that the drug in this case acts at some 'bottle-neck'

in the specific amine transport in these particles, the observation that synthesis inhibition was virtually complete only at a drug/amine ratio approaching unity suggests that the reserpine molecules in this latter case actually competed with DA for binding to some sites to which DA must be bound in order to get access to the β-hydroxylating enzyme.

The present findings may provide a basis for explanation of the apparently conflicting evidence obtained in studies of the effects of reserpine on the synthesis of NA in intact tissues. The report that reserpine caused inhibition of the last, but not of the two first, steps in NA synthesis in slices from the bovine adrenal medulla or in the perfused rabbit heart[8] is in excellent agreement with the present results. On the other hand, the finding[9] that the synthesis of NA from tyrosine or DA in the rat brain 24 hr after administration of reserpine was subnormal, but could be restored by inhibition of MAO to levels higher than those found in untreated control animals (but lower than in controls treated with MAO inhibitor) is not incompatible with the present results. In fact it appears quite likely that the waning reserpine concentration 24 hr after administration would cause, at most, incomplete inhibition of NA synthesis, while still strongly inhibiting amine binding. Raising the intracellular DA concentration by inhibiting MAO would then reduce the reserpine/amine concentration ratio and overcome the inhibition of synthesis more or less completely. Whether reserpine inhibits the synthesis of NA would thus depend on the drug dose, the time interval after administration of the drug, and the various factors that influence the concentration of DA in the neurons.

*Pargyline.* Inhibition of MAO with pargyline ($10^{-4}$M) did not appreciably affect the NA formation from DA. This is in accordance with the recent observation that MAO inhibition did not change the synthesis of NA from tyrosine or DA in the guinea pig heart.[3] The finding that MAO inhibition in the present experiments did not affect synthesis appears surprising in view of the high MAO activity of the preparation.[10] However, only 0·5–1·5 per cent of the DA added to the medium was actually transformed to NA. Thus, obviously the supply of DA was sufficient to maintain NA synthesis in spite of substantial deamination of DA.

*Localization in the HS particles of the β-hydroxylation mechanism*

Since, according to the above results, only 10–50 per cent of the newly formed NA could be recovered from the washed HS particles, the two additional fractions, the wash and the supernatant, were also studied.

*The wash fraction.* Washing the high-speed sediment pellet, obtained on centrifugation of the HS particles at the end of the incubation period, resulted in removal from the pellet of a considerable amount of material derived from the DA added to the incubation medium. On chromatographic analysis, this material was found, in the absence of MAO inhibition, to consist predominantly of acids, but also of DA and of small amounts of newly formed NA. When MAO was inhibited no acids were found and all the material was recovered as intact DA or newly formed NA (Fig. 6). This DA material in the wash, which consisted of intact DA, newly formed NA, and acid metabolities, is hereafter referred to as the wash fraction.

The wash fraction was found to be less saturable than the wash-resistant fraction (material contained in the washed high-speed sediment pellet). With increasing concentrations of DA in the medium, the wash fraction contained an increasing

proportion of the total amount of DA derivatives recovered from the high-speed pellet after the first centrifugation. However, the wash fraction did not show unlimited capacity. Thus it was possible to demonstrate a decrease in the amount of radioactive DA material present in this fraction by addition of large amounts of unlabeled amines to the medium or by preincubation with reserpine (Fig. 5, 7, 8).

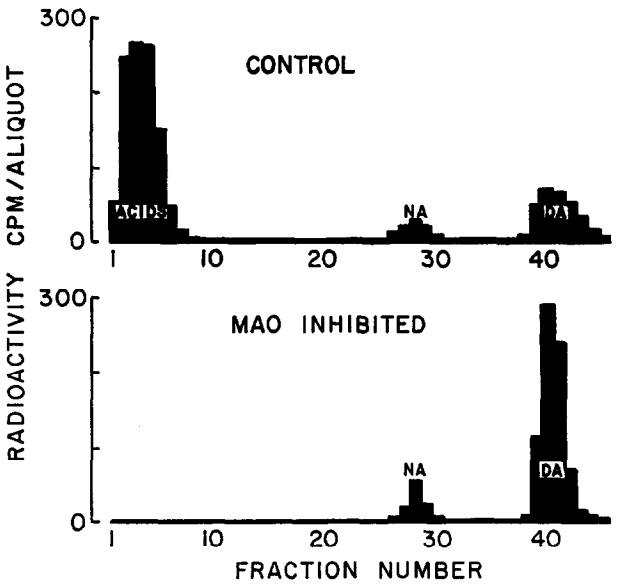


FIG. 6 Effect of MAO inhibition on the nature of the wash fraction. Ion-exchange chromatograms of the wash fraction after incubation with DA $2{\cdot}5 \times 10^{-5}$M, with or without preincubation with pargyline $10^{-4}$M.

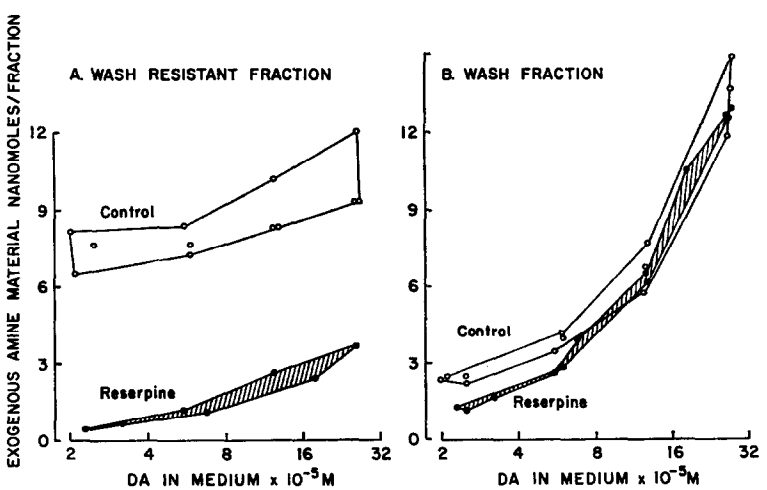


FIG. 7. Exogenous amine material in the (A) wash-resistant and (B) wash fractions in controls and after preincubation with reserpine $10^{-5}$M.

*Permeability of the membranes of HS particles.* In order to determine the significance of the wash fraction results, efforts were made to calculate the catecholamine diffusion space in the pellet, i.e. the total space in the high-speed pellet in which catecholamines from the medium would distribute by simple diffusion. An approximation of this space was obtained by the following formula: (catecholamine radioactivity in wash/ total catecholamine radioactivity) × volume of tube. With this procedure, the actual

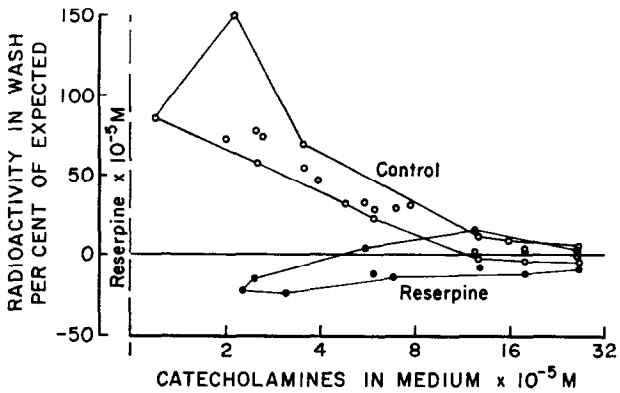


FIG. 8. Total radioactivity found in wash fractions, in controls and after preincubation with reserpine $10^{-5}$M in relation to the activity expected if the membranes of the particles allow free diffusion of DA and NA at all amine concentrations used. Amine diffusion space was calculated as described in text.

figures for the amine diffusion space were found to decrease with increasing catecholamine concentrations in the medium and reached a steady level at the highest exogenous amine concentrations used in the experiments, at a mean value of 0·059 ml (range, 0·045–0·073 ml; N = 9). Determination of the tritiated water space in several experiments, calculated in a similar way, gave a mean value of 0·054 ml.

The close agreement of the steady state values for the amine diffusion space with the similarly calculated tritiated water space strongly suggests that the catecholamine material found in the wash fraction, when the exogenous amine concentration in the medium was high, was essentially dissolved in the pellet water and was equally distributed on both sides of the membranes of the HS particles. If this assumption is correct, it follows that the excess of catecholamine material in the wash fraction, when the exogenous amine concentration in the medium was low (see Fig. 8), represents material originally bound in the HS particles but subsequently removed by a single wash. Such loose binding in the HS particles was evidently inhibited by large amounts of catecholamines in the medium. There is no evidence of competition for passage across the membranes into the intravesicular portion of the pellet water.[11] Preincubation with reserpine appears to abolish or strongly reduce loose binding, and possibly, to interfere to some extent with passage of amines across the membranes of the HS particles (Fig. 8).

*Implications of loose binding.* Although the amount of amine material washed out of the original high-speed sediment pellet, at least in the low exogenous amine range, consistently was found to be higher than could be accounted for by amines dissolved in the pellet water and although this amine material after MAO inhibition was found

to consist of intact amines, the true significance of this loosely bound amine pool remains obscure. The data do not allow a sharp distinction between firmly and loosely bound amine pools. It is quite possible that there is a gradual transition from relatively loose to increasingly firm amine binding. The apparent differences in the degree of blocking effect of reserpine on firm and loose binding, as seen in Fig. 5, may well be artifactual since amines dissolved in the pellet water were not subtracted from total amines in the wash. It is thus possible that reserpine interferes with amine binding at a single site and by a single mechanism and that the apparent block of loose binding, reflected in decreased amine material in the wash fraction, could be explained as less leakage of firmly bound amines during the washing procedure when the total amount of firmly bound material was strongly decreased by reserpine.

However, the present results clearly show that there is a distinct difference in the completeness of the inhibitory effects of reserpine on firm binding and on synthesis. It may be significant that the reserpine-induced block of synthesis resembles inhibition of binding in the wash fraction much more than in the wash-resistant fraction. Thus the possibility must be considered that the binding by which DA must be attached to the HS particles before it can be β-hydroxylated may be of the loose type.

It is also conceivable that loose binding is not only involved in the last step in NA synthesis, but that loosely bound amines represent a separate catecholamine pool in the HS particles, distinct from both the firmly bound pool and from the pools of catecholamines dissolved in the water phase on either side of the membranes of the HS particles. The possibility remains that the loosely bound pool of catecholamines observed in the present study may represent part of, or be identical with, the postulated 'extragranularly bound pool of NA'.[12]

*Overall distribution of DA, newly formed NA, and acid metabolites*

Chromatographic analysis of the washed pellet, wash, and supernatant fractions showed that about 90 per cent of the DA added to the incubation medium remained intact in the supernatant, while about 10 per cent was converted to acid metabolites and about 1 per cent to NA. The supernatant also contained the major part of the newly formed NA not firmly bound in the HS particles and most of the acid metabolites formed (Table 1).

TABLE 1. DISTRIBUTION OF EXOGENOUS DA AND ITS DERIVATIVES, NEWLY FORMED NA AND ACID METABOLITES, IN WASHED PELLET, WASH, AND SUPERNATANT FRACTIONS (ONE EXPERIMENT, DA IN MEDIUM $2{\cdot}5 \times 10^{-5}$M)

| | *Pellet* | | *Wash* | | *Supernatant* | |
|---|---|---|---|---|---|---|
| | (nanomole) | (% of total) | (nanomole) | (% of total) | (nanomole) | (% of total) |
| NA | 1·30 | 61 | 0·03 | 1·5 | 0·8 | 37·5 |
| DA | 3·60 | 1·8 | 0·17 | 0·1 | 180 | 98 |
| Acids | 0·32 | 1·5 | 0·71 | 3·2 | 20·9 | 95·3 |
| Total $DA_{Eq}$ | 5·22 | | 0·91 | | 201·7 | |
| | NA × 100/Total $DA_{Eq}$ | | DA × 100/Total $DA_{Eq}$ | | Acids × 100/Total $DA_{Eq}$ | |
| Pellet | 25 | | 69 | | 6·1 | |
| Wash | 3·3 | | 18·7 | | 78 | |
| Supernatant | 0·4 | | 90 | | 10·4 | |

The highest concentration of newly formed NA/total DA equivalents was found in the washed pellet. The relative concentration of newly formed NA in the wash fraction was about 10 times as high as that in the supernatant. This appears to fit in with the concept that the β-hydroxylation takes place after DA has become loosely bound in the wash fraction, and that the newly formed NA is immediately firmly bound until the specific compartment is saturated and the surplus passes into the supernatant.

The highest acid metabolite/total DA equivalent ratio was found in the wash fraction, which indicates that deamination takes place in this fraction. This brings up the question of the subcellular localization of MAO. Recent results in this laboratory indicate that the major part of the total MAO in the bovine splenic nerve homogenate cannot be sedimented in isotonic potassium phosphate by centrifugation at 9000*g* for 10 min. About half of the MAO in the low-speed supernatant, and thus about a third of the total MAO in the crude homogenate, was found in the HS particles.[10] Electron micrographs of this fraction* suggest that it is relatively free from contamination with mitochondria. Thus the possibility must be considered that part of the MAO in bovine splenic nerves is located in the NA storage vesicles themselves.[11] This problem is currently being investigated in this laboratory by biochemical and morphological analysis of different fractions obtained from the bovine splenic nerve homogenate by continuous gradient centrifugation.

* U. S. von Euler, F. Lishajko and G. Swanbeck, personal communication.

*Acknowledgements*—This investigation was supported by grants from the Swedish Medical Research Council, projects No. 14 X-97-02 (Professor U. S. von Euler) and 14 X-625-01 (L.S.), as well as by a Public Health Service postdoctoral fellowship, 1-F2-GM-30, 064-01 (R.R.), which are hereby gratefully acknowledged.

## REFERENCES

1. L. Stjärne and F. Lishajko, *Biochem. Pharmac.*, in press.
2. U. S. von Euler, *Acta physiol. scand.* **67**, 430 (1966).
3. M. Levitt, S. Spector, A. Sjoerdsma and S. Udenfriend, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
4. L. Stjärne, F. Lishajko and R. H. Roth, *Nature, Lond.* in press.
5. J. M. Musacchio, I. J. Kopin and S. Snyder, *Life Sci.* **3**, 769 (1964).
6. L. Stjärne and F. Lishajko, *Br. J. Pharmac.* **27**, 398 (1966).
7. L. Stjärne, *Acta physiol. scand.* **62**, *suppl.* 228 (1964).
8. N. Weiner and C. O. Rutledge, in *Mechanisms of Release of Biogenic Amines* (Eds. U. S. von Euler, S. Rosell and B. Uvnäs), pp. 307–318. Pergamon Press, Oxford (1966).
9. J. Glowinski, L. L. Iverson and J. Axelrod, *J. Pharmac. exp. Ther.* **151**, 385 (1966).
10. R. H. Roth and L. Stjärne, *Acta physiol. scand.* **68**, 342 (1966).
11. L. Stjärne, *Acta physiol. scand.* **67**, 441 (1966).
12. General Discussion, in *Mechanisms of Release of Biogenic Amines* (Eds. U. S. von Euler, S. Rosell and B. Uvnäs), pp. 471–474. Pergamon Press, Oxford (1966).