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UPTAKE OF BIOGENIC AMINES BY TWO DIFFERENT MECHANISMS PRESENT IN ADRENERGIC GRANULES

BY

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Recent work in our laboratory (Lundborg & Waldeck, 1966 ; Stitzel & Lundborg, 1967) has suggested the existence of two distinct mechanisms by which adrenergic nerve granules concentrate biogenic amines. One of these mechanisms has properties similar to those described by Carlsson, Hillarp & Waldeck (1962) and Kirshner (1962a, b) for granules of the adrenal medulla. These authors showed that adrenal medullary granules were able to take up and concentrate monoamines *in vitro* by a Mg^{++}-ATP-dependent storage mechanism and that this mechanism could be blocked by low concentrations of reserpine. The other incorporation mechanism is apparently not sensitive to reserpine and is virtually unaffected by elimination of ATP and Mg^{++} from the incubation medium. These two uptake processes apparently also differ in their ability to concentrate specific sympathomimetic amines since Lundborg & Waldeck (1966) have shown that metaraminol is preferentially taken up by the reserpine-resistant system. In the present investigation the specificity of the two mechanisms was examined in greater detail. The *in vivo* and *in vitro* interaction of adrenergic granules with three structurally related compounds, noradrenaline (NA), α-methyl noradrenaline (α-MeNA) and metaraminol, is reported.

METHODS

In vivo *experiments*

Mice, divided into groups of six, were given ^{3}H-NA, 1 μg/kg (6 c/mM) ; ^{3}H-α-MeNA, 100 μg/kg (30 mc/mM) or ^{3}H-metaraminol, 40 μg/kg (100 mc/mM) intravenously. Reserpine (10 mg/kg) and/or nialamide (10 mg/kg) were given intraperitoneally 6 and 2 hr, respectively, before the intravenous injection of the labelled amine. The animals were sacrificed at varying time intervals after amine administration. The hearts were removed and homogenization performed in an ice bath using a plastic pestle. The homogenization medium was 0.25 M sucrose containing 0.005 M phosphate buffer, pH 7.4 and 0.001 M $MgCl_2$. A coarse fraction was obtained by centrifugation of the homogenate in the cold at 2,000 *g* for 10 min. The supernatant obtained was then centrifuged at 100,000 *g* for 60 min in a Spinco Model L Ultracentrifuge, providing two more fractions, particulate (sediment) and high speed supernatant. After protein precipitation of the various fractions ^{3}H-NA or ^{3}H-α-MeNA were separated from their methylated metabolites on an ion exchange column (Dowex 50W X4) as described by Carlsson & Waldeck (1963). Further details of the analytical procedure have been previously described (Stitzel & Lundborg, 1967). Since metaraminol is not a substrate either for monoamine oxidase or catechol-O-methyl transferase, it could be analysed in a more direct manner. The particulate fraction was extracted with 5 ml. of 0.01 N HCl in absolute alcohol. One ml. of the acid alcohol was then added to 5 ml. of a

scintillation mixture (3 g 2,5-Diphenyloxazol and 0.3 g 1,4-Di-2-(5-phenyloxazolyl)-benzene in one litre of toluene), and taken for counting in a liquid scintillation system. Two ml. of the high speed supernatant fraction were added to 13 ml. of a dioxane scintillation mixture (120 g Naphtalene, 0.05 g 1,4-Di-2-(5-phenyloxazolyl)-benzene and 7 g 2,5-Diphenyloxazol in 1 l. of dioxane) and counted. The degree of quenching was corrected through the use of internal standards. Identical results were obtained in experiments where the metaraminol-containing subcellular fractions were first placed on an ion exchange column.

In a control experiment a quantity of ^{3}H-NA, ^{3}H-α-MeNA or ^{3}H-metaraminol was added to samples of cardiac tissue immediately before homogenization, and a subcellular distribution then was carried out. Virtually all radioactivity (about 97%) was found in the supernatant fraction. Thus in the present experiments any uptake of labelled amines above 3% by the particulate fraction must have occurred *in vivo*. All animals receiving reserpine were kept at 30° C. *In vivo* curves for NA (Stitzel & Lundborg, 1967) and metaraminol (Lundberg & Waldeck, 1966) are included for comparative purposes.

In vitro *experiments*

Amine granules from cow adrenal medulla were prepared essentially as described by Hillarp (1958). The medulla was homogenized with a loose-fitting plastic pestle in 0.3 M sucrose. Unbroken cells and nuclei were removed by centrifugation at 800 *g* for 5 min. The supernatant was decanted and centrifuged at 26,000 *g* for 20 min. The supernatant was decanted and the loose layer above the more tightly packed bottom sediment was removed by swirling with sucrose. The granules were then suspended in 0.3 M sucrose and stored at 0° C for use on the same or the next two days.

An aliquot (50 μl.) of the granule suspension, corresponding to about 125 μg catecholamines, was transferred to 1 ml. of an incubation mixture (at 0°) containing glycylglycine, ATP and $MgCl_2$. Amines were added in concentrations such that the total amount of labelled plus unlabelled drug was 0.30×10^{-3}M. Incubations were performed without shaking at 0° and 31° for 30 min. Further details can be found in the work of Lundborg (1966).

Substances used

^{3}H-metaraminol and ^{3}H-α-MeNA were prepared by the research laboratory of Hässle Ltd. in co-operation with this department (Carlsson & Waldeck, 1965; Hallhagen & Waldeck, unpublished). ^{3}H-NA was obtained from New England Nuclear Corp. Reserpine and nialamide were generously supplied by Swedish Ciba Ltd. and Swedish Pfizer Ltd., respectively.

RESULTS

Subcellular distribution of ^{3}H-NA, ^{3}H-α-MeNA and ^{3}H-metaraminol

After an intravenous injection all three titriated amines were taken up and retained by the particulate fraction derived from mouse heart homogenates (Table 1). One hr after administration of the labelled amine, NA was found to be present in a greater concentration (48%) than was either α-MeNA (37%) or metaraminol (19%). The

TABLE 1

SUBCELLULAR DISTRIBUTION OF ^{3}H-NORADRENALINE, ^{3}H-α-METHYL NORADRENALINE AND ^{3}H-METARAMINOL IN MOUSE HEART

All labelled compounds were given intravenously and the animals were sacrificed 1 hr later. The % retention in the particulate fraction is expressed as a % of ^{3}H-amine in the particulate + supernatant fractions. Each determination was performed on six pooled hearts

Drug(s)	Determinations (No.)	% in particulate fraction
^{3}H-Noradrenaline	6	48·02 ± 0·97
^{3}H-Noradrenaline + nialamide	6	47·60 ± 2·35
^{3}H-α-Methyl noradrenaline	6	36·9 ± 1·82
^{3}H-Metaraminol	8	18·8 ± 1·66

addition of a monoamine oxidase inhibitor, nialamide, did not alter the distribution of NA between the particulate and supernatant fractions. The other two compounds are not substrates for monoamine oxidase and, therefore, no experiments were performed using a combined regimen of either α-MeNA or metaraminol with nialamide.

In other experiments hearts were removed from mice at various time intervals after the intravenous injection of the labelled amine. NA, αMeNA and metaraminol show a continuous, although gradual, increase in the amount of compound appearing in the particulate fraction (Fig. 1). The amount of labelled amine in the particulate fraction is expressed as a percentage of that found in the particulate + supernatant fractions.

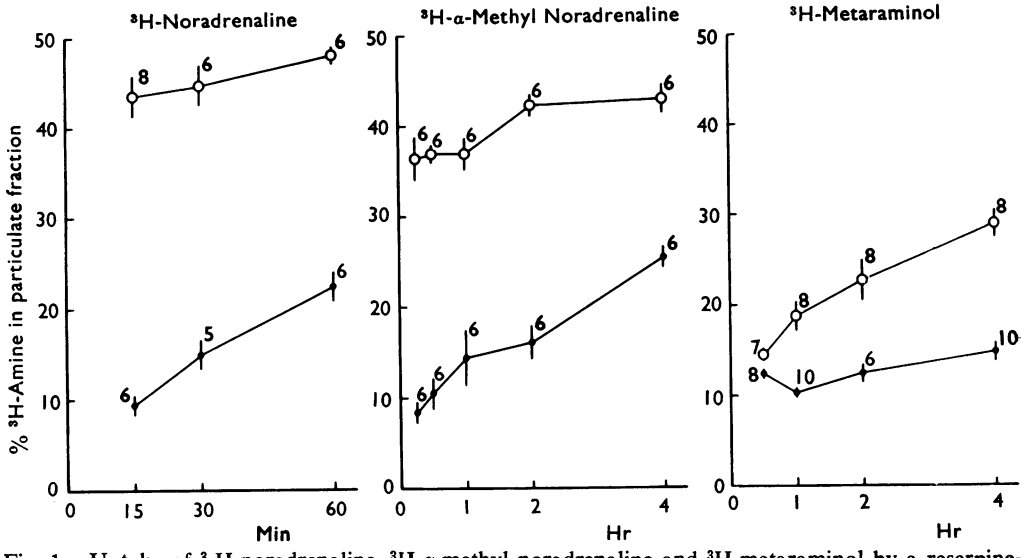

Fig. 1. Uptake of ³-H-noradrenaline, ³H-α-methyl noradrenaline and ³H-metaraminol by a reserpine-resistant mechanism in the particulate fraction of the mouse heart. The results are expressed as amounts of ³H-amine in the particulate fraction as a percentage of the ³H-amine in the particulate + supernatant fractions. Each determination was performed on six pooled hearts, and each point is the mean of 5–10 determinations. The vertical bars are standard errors of the mean. ○——○, control animals; ●——● mice treated with reserpine. Mice given reserpine and ³H-noradrenaline were pretreated with nialamide.

Effect of reserpine on the subcellular distribution of ³H-NA, ³H-α-MeNA and metaraminol

Mice given reserpine showed a greatly diminished uptake of all three amines in the particulate fraction (Fig. 1). This was accompanied by an appreciable accumulation in the supernatant fraction. The latter accumulation was particularly marked at the early intervals following the intravenous administration of the labelled amine. The accumulation of ³H-NA could be clearly demonstrated only in animals which had been pretreated with nialamide.

Influence of ATP and Mg^{++} and temperature on the in vitro *uptake of ³H-NA, ³H-α-MeNA and ³H-metaraminol*

Adrenergic granules derived from bovine adrenal medullae can take up and store NA, α-MeNA and metaraminol (Fig. 2). Incubation at 0° C markedly decreased the amount

of amines that was taken up at 31° *in vitro*. Apparently the uptake process involved in the concentration of all three amines is temperature dependent.

In some experiments ATP and Mg^{++} were not included in the incubation system. There was no significant decrease in the uptake of metaraminol if Mg^{++} and ATP were excluded. NA and α-MeNA (Fig. 2) uptake, however, were greatly depressed.

Influence of reserpine on the in vitro *uptake of ^{3}H-NA, ^{3}H-α-MeNA and ^{3}H-metaraminol*

Reserpine, even in relatively high concentrations, had only a slight influence on the accumulation of metaraminol by bovine medullary granules, while NA and α-MeNA uptake were impaired (Fig. 2). Increasing the concentration of reserpine in the incuba-

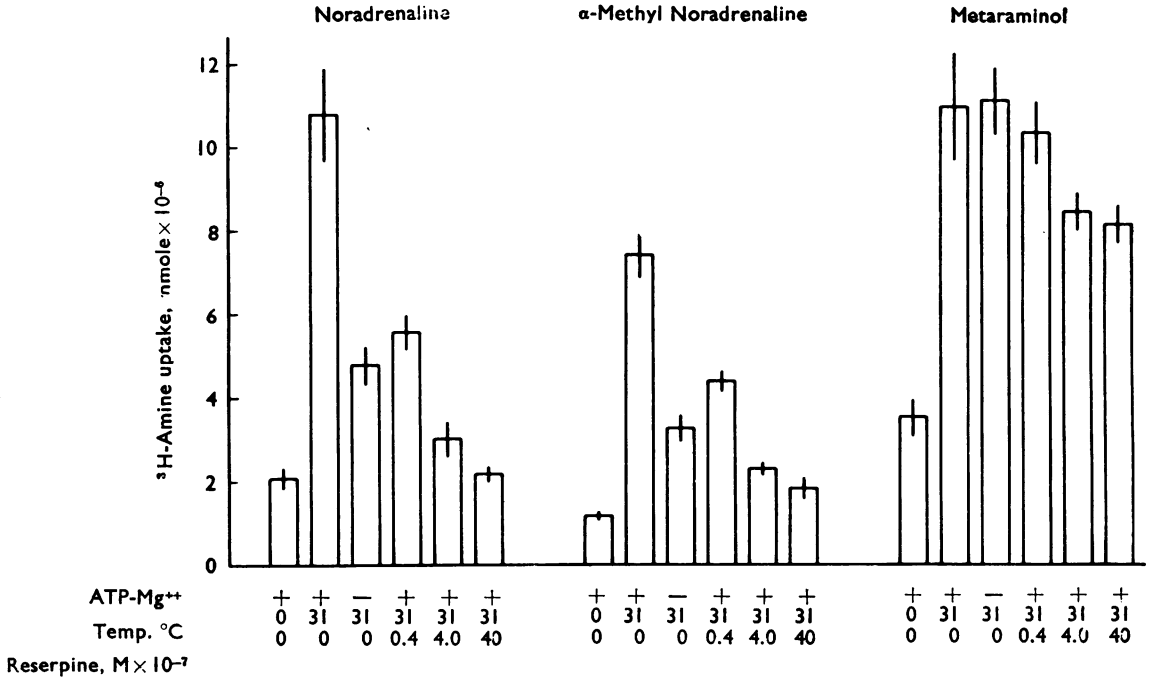

Fig. 2. Influence ot ATP-Mg^{++}, reserpine and temperature on the uptake of ^{3}H-metaraminol, ^{3}H-α-methyl noradrenaline and ^{3}H-noradrenaline by bovine adrenal medullary granules. The final concentrations of each amine, labelled plus unlabelled, was 3×10^{-4}M. The vertical bars represent means ± S.E.M. and are based upon 4–8 determinations at each point. Unlabelled amines added were levorotatory while the tritiated amines were a dl. mixture. If there is a varying stereospecificity of uptake for each amine then the absolute uptake of the three compounds cannot be compared directly.

tion medium resulted in an increase in the degree of inhibition of uptake of NA and α-MeNA. Reserpine in a concentration of 4×10^{-8}M reduced the accumulation of NA by 80% and reduced the incorporation of α-MeNA by about 50%, while metaraminol accumulation was decreased by only about 8%. Percentages were computed after subtracting the non-specific uptake that occurred at 0° C.

DISCUSSION

Enzymatic destruction has been considered an important factor in the inactivation of monoamines. Recently uptake by adrenergic neurones has also been shown to be of importance (Hertting, Axelrod & Patrick, 1961 ; Axelrod, Hertting & Potter, 1962). It has been postulated that the uptake mechanism consists of two major components, active transport through the nerve cell membrane and an incorporation into the storage granule complex (Carlsson, Hillarp & Waldeck, 1963). Both of these mechanisms can be selectively blocked by drugs. Thus protriptyline and desipramine were found to block the former and reserpine and prenylamine the latter mechanism (Carlsson & Waldeck, 1965).

The amine uptake and storage mechanism of the nerve granules appear to have a limited specificity, since compounds more or less closely related to NA, such as sympathomimetic amines of a simple structure (Musacchio, Kopin & Weise, 1965) and possibly even guanethidine (Chang, Costa & Brodie, 1965) can be taken up and retained. This limited specificity may, however, be due to the presence of more than one uptake mechanism in the amine-containing granules, each possessing its own characteristics and requirements.

It has been shown that the amine granules derived from the adrenal medulla (Carlsson *et al.*, 1962 ; Kirshner, 1962a, b) and from splenic nerve (Von Euler & Lishajko, 1963) are able to take up and concentrate NA *in vitro* by a Mg^{++}-ATP dependent storage mechanism. This mechanism is blocked by low concentrations of reserpine. Recently Lundborg (1966) has demonstrated that metaraminol is incorporated *in vitro* into amine granules by a mechanism which is only slightly, if at all, affected by Mg^{++} and ATP. This mechanism is temperature dependent and is not influenced by reserpine. The current investigation was undertaken to see if the above *in vitro* findings could be extended to the *in vivo* situation and also to study the relative specificity of the two systems both *in vivo* and *in vitro*.

All three drugs studied, NA, α-MeNA and metaraminol, were incorporated into the particulate fraction of the mouse heart after intravenous administration of the amines. A subcellular distribution of cardiac tissue showed that 48% of the NA was present in the particulate fraction 1 hr after its administration, while α-MeNA and metaraminol were present to the extent of 37% and 19% respectively. It has been argued that sympathomimetic amines are retained less efficiently by the granular fraction because they may lack one of the three hydroxyl groups of NA (Musacchio *et al.*, 1965). This cannot be the entire explanation since we have found that α-MeNA, which is identical with NA except that it has an additional methyl group, is also less efficiently retained. An alternative hypothesis might be considered. If there are at least two uptake mechanisms in adrenergic granules which differ slightly in their structural requirements for transport of biogenic amines, then the amount of amine incorporated might be expected to differ with each drug. In the present work the percentage retention in the particulate fraction was greatest for NA and least for metaraminol. Evidence is presented which shows that the principal incorporation of metaraminol and NA occurs by different mechanisms.

The *in vitro* studies show that the incorporation of NA into bovine granules is facilitated by ATP and Mg^{++} and can be blocked by reserpine, while metaraminol

incorporation is relatively independent of ATP and Mg^{++} and can occur even in the presence of concentrations of reserpine which completely inhibit NA uptake. α-MeNA, which has structural properties which are related to both NA and metaraminol, is able to utilize both uptake mechanisms. This is illustrated by the finding that either removal of ATP and Mg^{++} or the addition of reserpine to the incubation medium will only partially inhibit α-MeNA incorporation.

If the rates at which the reserpine-sensitive and reserpine-resistant mechanisms incorporate amines differ then the amount of amine retained will depend upon which mechanism is the predominant one for that particular compound. Amines with structures intermediate between NA and metaraminol may have some affinity for both mechanisms, but will probably utilize each mechanism to a degree dependent upon their structure. Musacchio *et al.* (1965) have found that different phenylethylamine derivatives are found in the supernatant fraction in proportions that are characteristic for each compound. This may be the result of the different rates and extent to which each compound utilizes the two granular uptake mechanisms.

The *in vitro* studies show that the incorporation of metaraminol into adrenergic granules is only slightly affected by reserpine ; however, the *in vivo* results demonstrate that the ability of the heart to retain metaraminol is significantly impaired by reserpine, although it is not completely blocked. A similar *in vivo* impairment of metaraminol retention by reserpine has been shown by Shore, Busfield & Alpers (1964) and Carlsson & Waldeck (1965). Presumably in the intact animal, metaraminol can also utilize the reserpine-sensitive mechanism. The *in vivo* incorporation of NA and α-MeNA is also impaired by pretreating the animals with reserpine, but these two compounds, like metaraminol, are still seen to be taken up. This uptake in reserpine-treated animals illustrates the *in vivo* functioning of the reserpine-resistant storage mechanism.

Apparently the rates at which these two mechanisms function for different compounds are quite different. Although metaraminol can use both mechanisms, the reserpine-sensitive process evidently is too slow to be observed *in vitro*. A similar conclusion was reached by Lundborg & Waldeck (1966). However, when sufficient time is allowed to elapse after administering metaraminol to the intact animal, metaraminol can be shown to be taken up at least partially by the reserpine-sensitive process. NA on the other hand appears to be incorporated *in vitro* only by the ATP-Mg^{++} dependent, reserpine-sensitive process. Evidently any utilization by NA of the reserpine-resistant mechanism is too slow to be observed *in vitro*. *In vivo*, however, NA is seen to be concentrated by both mechanisms, since, after blockade of the ATP-Mg^{++} dependent process by reserpine, NA is still shown to be taken up and stored. α-MeNA can be shown to utilize both the reserpine-sensitive and the reserpine-resistant systems *in vitro* as well as *in vivo*. *In vivo* studies do not rule out the possibility that the two mechanisms for amine concentration are not necessarily restricted to adrenergic granules. However, the similar results obtained from *in vitro* experiments make this possibility less likely.

The *in vivo* incorporation of NA, α-MeNA and metaraminol in reserpine-treated animals corroborates the *in vitro* data which show that metaraminol utilizes the reserpine-resistant mechanism to a greater extent than does either α-MeNA or NA. NA, on the other hand, utilizes the reserpine-sensitive system to a greater degree than does either α-MeNA or metaraminol.

SUMMARY

1. The *in vivo* and *in vitro* uptake of ^{3}H-NA, ^{3}H-α-MeNA and ^{3}H-metaraminol into subcellular fractions of cardiac and adrenal tissue was studied both in the presence and absence of reserpine.

2. After intravenous administration the three tritiated amines were retained by the particulate fraction derived from mouse heart homogenates. NA was retained to the greatest extent and metaraminol least.

3. Reserpine greatly impaired the *in vivo* uptake of all three amines into the particulate fraction. This was accompanied by an accumulation in the supernatant fraction. Even high doses of reserpine, however, did not completely prevent accumulation of the monoamines in the particulate fraction.

4. The *in vitro* incorporation of NA into granules from bovine adrenals was dependent upon Mg^{++} and ATP and could be prevented by reserpine. This was also true of α-MeNA, but to a lesser degree. Metaraminol incorporation was relatively independent of Mg^{++} and ATP and was only slightly affected by reserpine. The uptake of all three compounds was temperature dependent.

5. It is concluded that there are at least two uptake mechanisms in adrenergic granules for the incorporation of sympathomimetic amines, a reserpine-sensitive and a reserpine-resistant mechanism.

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