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EFFECT OF RESERPINE AND MONOAMINE OXIDASE INHIBITION ON THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF ^{3}H-NORADRENALINE

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Evidence is available which indicates that the monoamine depletion induced by reserpine is, in all probability, due to a blockade of amine uptake by the storage particles (Bertler, Hillarp & Rosengren, 1961; Carlsson, Hillarp & Waldeck, 1962; Kirshner, 1962a, b). Most of the evidence is of an indirect nature, however, being derived from studies *in vitro*. Incorporation *in vivo* of noradrenaline (NA) into subcellular fractions derived from reserpine-treated animals has been examined in the adrenal medulla (Bertler *et al.*, 1961), but few studies are available relating to adrenergic nerves. In order to demonstrate an amine accumulation in adrenergic nerves in reserpine-treated animals it is necessary to pretreat the animals with a potent monamine oxidase (MAO) inhibitor (Andén, Carlsson & Waldeck, 1963) or, alternatively, to administer an amine resistant to this enzyme—for example, α-methyl NA or metaraminol. Recent histochemical studies (Hillarp & Malmfors, 1964; Hamberger, Malmfors, Norberg & Sachs, 1964; Malmfors, 1965) indicate that in reserpine-treated animals exogenous NA or α-methyl NA accumulate in adrenergic nerves under these conditions, and that they are located extragranularly. Similarly, biochemical evidence indicates that metaraminol accumulates in adrenergic nerves after reserpine treatment (Carlsson & Waldeck, 1966) but is not incorporated in the granular fraction as readily as in normal animals (Lundborg & Waldeck, 1966). Uptake of ^{3}H-NA by adrenergic nerves after MAO inhibition has also been demonstrated in animals pretreated with reserpine (Carlsson & Waldeck, 1966). In the present study the subcellular distribution of ^{3}H-NA in adrenergic nerves, both in the presence and absence of reserpine, was investigated.

METHODS

Mice, divided into groups of six, were given ^{3}H-NA, 1 μg/kg (approximately 6 c/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 either 15, 30 or 60 min after ^{3}H-NA 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 removing the protein the ^{3}H-NA was separated from ^{3}H-normetanephrine (NM) on an ion exchange column (Dowex 50W X4) as described by Carlsson & Waldeck (1963). The eluates were evaporated to dryness under reduced pressure in a rotating evaporator at 42°, and the residues taken up in 1 ml. of 99.5% ethanol containing 1% concentrated HCl. After adding 5 ml. of scintillation mixture (3 g 2,5-Diphenyloxazol and 0.3 g 1,4-Di-2-(5-phenyloxazolyl)-benzene in 1 l. of toluene), 5 ml. of the sample were transferred to a counting vial. The tritium content was determined in a liquid scintillation counter. In a control experiment a quantity of ^{3}H-NA was added to samples of cardiac tissue immediately before homogenization, and a subcellular distribution was then carried out. Virtually all radioactivity (about 97%) was found in the supernatant fraction. Thus any uptake of ^{3}H-NA above 3% by the particulate fraction in the present experiments must have occurred *in vivo*. All animals receiving reserpine were kept at 30° C.

RESULTS

In the untreated animals ^{3}H-NA was about equally distributed between the particulate and the supernatant fractions at all time intervals studied (Table 1). Treatment with nialamide alone did not significantly influence the results, while treatment with reserpine resulted in a greatly diminished uptake of ^{3}H-NA both in the particulate and in the supernatant fractions. Animals given both reserpine and nialamide still showed low concentrations of ^{3}H-NA in the particulate fraction, but there was an appreciable accumulation in the supernatant fraction. This latter accumulation was particularly marked at the 15 min time interval.

TABLE 1

BLOCKADE OF UPTAKE OF ^{3}H-NORADRENALINE INTO THE PARTICULATE FRACTION BY RESERPINE

Values in the table are expressed as ng/g of fresh tissue. Each determination was performed on six pooled hearts and each value represents the mean of six determinations. The time intervals indicate the time of sacrifice after injection of ^{3}H-noradrenaline. MAOI=monoamine oxidase inhibition

	15 min		30 min		60 min	
	Particulate	Supernatant	Particulate	Supernatant	Particulate	Supernatant
Control	0·484±0·046	0·629±0·032	0·619±0·059	0·765±0·066	0·455±0·027	0·490±0·023
MAOI	0·565±0·027	0·726±0·034	0·584±0·024	0·755±0·045	0·440±0·056	0·471±0·041
Reserpine	0·005±0·001	0·062±0·018	0·004±0·001	0·021±0·003	0·002±0·003	0·015±0·003
Reserpine + MAOI	0·041±0·006	0·397±0·026	0·025±0·003	0·143±0·005	0·013±0·002	0·045±0·003

After reserpine and/or nialamide there was a marked increase in the appearance of the O-methylated metabolite of NA, normetanephrine (NM) (Table 2). This metabolite was restricted exclusively to the supernatant fraction. A combination of reserpine and MAO inhibition produced the largest increases in NM concentration. The NM data has been corrected for a small (about 5%) overlap in the elution patterns of NA and NM.

Even after a large dose of reserpine, which should completely impair the storage capacity of the amine-concentrating granules, a small but significant amount of ^{3}H-NA can be recovered from the particulate fraction. Figure 1 shows the amount of ^{3}H-NA taken up into the particulate fraction in untreated and in reserpine and nialamide pretreated mice. The amount of ^{3}H-NA in the particulate fraction is expressed as a percentage of ^{3}H-NA in the particulate + supernatant fractions.

TABLE 2

SUBCELLULAR DISTRIBUTION OF ^{3}H-NORMETANEPHRINE FORMED FROM EXOGENOUSLY ADMINISTERED ^{3}H-NORADRENALINE UNDER VARIOUS EXPERIMENTAL CONDITIONS

Values in the table are expressed as ng/g of fresh tissue and are corrected for an approximately 5% overlap in elution patterns of noradrenaline and normetanephrine. Each determination was performed on six pooled hearts and each value represents the mean of six determinations. The time intervals indicate the time of sacrifice after injection of ^{3}H-noradrenaline. MAOI = monoamine oxidase inhibition

	15 min		30 min		60 min	
	Particulate	Supernatant	Particulate	Supernatant	Particulate	Supernatant
Control	0·011±0·005	0·143±0·016	0·000±0·000	0·064±0·003	0·008±0·004	0·045±0·004
MAOI	0·024±0·010	0·214±0·016	0·003±0·002	0·143±0·013	0·005±0·003	0·058±0·015
Reserpine	0·009±0·001	0·351±0·047	0·001±0·001	0·111±0·015	0·001±0·001	0·073±0·010
Reserpine + MAOI	0·015±0·001	0·533±0·021	0·003±0·001	0·307±0·074	0·002±0·001	0·107±0·004

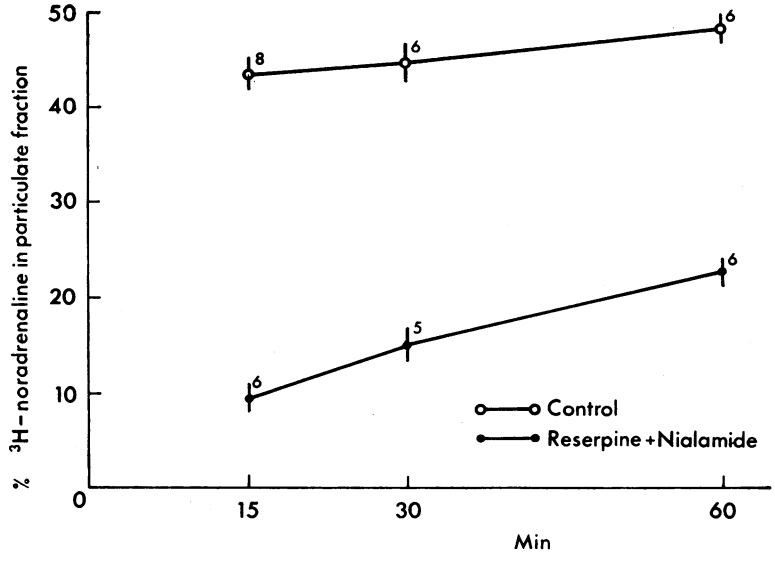

Fig. 1. Uptake of ^{3}H-noradrenaline by a reserpine-resistant mechanism in the particulate fraction of the mouse heart. The results are expressed as amount of ^{3}H-noradrenaline in the particulate fraction as a percentage of the ^{3}H-noradrenaline in the particulate + supernatant fractions. Each determination was performed on six pooled hearts, and each point is the mean of five to eight determinations. The vertical bars are standard errors of the mean.

DISCUSSION

The uptake of ^{3}H-NA by sympathetically innervated tissues is reduced to very low levels following sympathectomy (Hertting, Axelrod, Kopin & Whitby, 1961), immunosympathectomy (Klingman, 1965; Iversen, Glowinski & Axelrod, 1965a) or treatment with selective and potent blockers of amine uptake by the adrenergic nerve fibres such as desipramine and protriptyline (Carlsson & Waldeck, 1965). All these findings point to an intraneuronal binding of exogenously administered NA. Therefore there seems to be little doubt that under the present experimental conditions the ^{3}H-NA recovered from the heart was essentially localized within the adrenergic nerves. The histochemical observations quoted above (see introduction) support this view.

Approximately equal amounts of ^{3}H-NA were recovered from the supernatant and particulate fractions of the normal mouse heart. This distribution may, in part, be due to the release of ^{3}H-NA from the particulate fraction during homogenization. This is supported by the observation that the supernatant ^{3}H-NA content of the normal heart did not show much decrease with time, suggesting that *in vivo* it was bound in some manner and therefore protected from intraneuronal MAO. The view that intraneuronal NA is largely particle-bound now seems to be generally accepted (Euler, 1966).

In the reserpine-treated animals very little ^{3}H-NA was found in either the particulate or supernatant fractions. The low levels of NA found in the supernatant fraction could be due to a rapid destruction by MAO. In support of this view it was found that following administration of reserpine and a MAO inhibitor the supernatant fraction contained appreciable amounts of ^{3}H-NA. Thus NA, which would normally tend to accumulate in the cytoplasm as a consequence of the blockade of uptake by reserpine, preferentially undergoes oxidative deamination. If MAO is inhibited, however, an extragranular accumulation of ^{3}H-NA can clearly be demonstrated. This provides direct biochemical evidence for the hypothesis that reserpine blocks the incorporation of circulating NA into adrenergic nerve granules, but not the entry of the amines into the cell. Carlsson & Waldeck (1965) also have evidence that reserpine has little or no effect on the cell membrane transport mechanism. Iversen, Glowinski & Axelrod (1965b) have also studied the uptake of ^{3}H-NA in reserpine-treated animals. It is not possible to correlate their findings with those reported in the present investigation since their experimental conditions were quite different. They sacrificed their animals only 2 min after giving ^{3}H-NA, and thus it is probable that most of their ^{3}H-NA was extraneuronal. This is made even more likely by their observation that desmethylimipramine, a potent blocker of catecholamine transport at the cell membrane, did not block the uptake of ^{3}H-NA.

There appears to be a rapid disappearance of ^{3}H-NA in animals pretreated with reserpine, even when the amine is protected from enzymatic destruction by nialamide. All of the extragranular ^{3}H-NA which had been accumulated is lost within 60 min. This is in contrast with the findings of Lundborg & Waldeck (1966) who found that ^{3}H-metaraminol was still present in the extragranular fraction 4 hr after its administration to reserpine-treated mice. In their experiments MAO was not inhibited since metaraminol is not a substrate for this enzyme. It is possible that in the present experiments a complete inhibition of MAO was not achieved, but this cannot be the complete explanation since Carlsson & Waldeck (in preparation) have shown 10 mg/kg of nialamide 2 hr before ^{3}H-NA administration to be optimal conditions for measurement of accumulation of injected NA. Therefore, some additional factor(s) must be involved. Nialamide may have some releasing action of its own or in the presence of MAO inhibition another amine may be accumulated which displaces the ^{3}H-NA from its extragranular site (Carlsson & Waldeck, personal communication).

Pretreatment of the animals with reserpine causes a pronounced increase in the amount of labelled NM recovered from tissues. This is a true increase in NM since it can be blocked by prior administration of H 22/54, a potent catechol-O-methyl transferase (COMT) inhibitor (Carlsson, Corrodi & Waldeck, 1963; Stitzel & Lundborg, unpublished results). This increase may be due to a decreased capacity of the adrenergic nerves to bind NA, thus exposing it to enzymatic destruction, although other explanations

cannot be ruled out. Our data indicate that the adrenergic granules were incapable of incorporating the NM either in control or in reserpine treated animals. Similar observations have been reported by Potter & Axelrod (1963). The rapid disappearance of ^{3}H-NM in reserpine-treated animals is probably due to lack of binding and to destruction by MAO. A rapid tissue disappearance of NM has also been reported by Carlsson & Waldeck (1963).

Although most of the ^{3}H-NA uptake seen in reserpine-treated animals was restricted to the supernatant fraction, a small but significant portion (about 10%) did appear in the fraction containing cardiac adrenergic granules. Apparently there is an uptake of NA in the particulate fraction even after a large dose of reserpine. This is consistent with the recent findings of Lundborg & Waldeck (1966) and Lundborg (1966), who demonstrated two distinct mechanisms for the incorporation of ^{3}H-metaraminol into the particulate fraction. Their data and ours suggest the possible existence of at least two different mechanisms for the incorporation of amines into the particulate fraction: (a) a reserpine-sensitive mechanism, probably identical with the ATP-Mg^{++} dependent uptake observed by Carlsson *et al.* (1962) and Kirshner (1962a, b); and (b) a reserpine-resistant uptake.

SUMMARY

1. The uptake *in vivo* of ^{3}H-NA into subcellular fractions of the mouse heart was studied both in the presence and absence of reserpine.

2. Reserpine, when administered alone, was found to decrease the uptake of ^{3}H-NA into both the particulate and high speed supernatant fractions. If MAO was inhibited, however, the blockade of uptake could be shown to be restricted to the particulate fractions since appreciable amounts of ^{3}H-NA now accumulated in the high speed supernatant.

3. Reserpine appears to prevent the accumulation of ^{3}H-NA into the particulate fraction but not into the adrenergic neuron.

4. ^{3}H-normetanephrine apparently is not bound to the particulate fraction and rapidly disappears from heart fractions.

5. There appears to be an uptake of ^{3}H-NA in the particulate fraction even after a large dose of reserpine. This suggests the possible existence of at least two different mechanisms for the incorporation of amines into the particulate fraction.

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