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The accumulation of [^{3}H]noradrenaline in the adrenergic nerve fibres of reserpine-treated mice

A. CARLSSON AND B. WALDECK

Mice, pretreated with reserpine, were given [^{3}H]noradrenaline (^{3}H-NA) intravenously and were killed 30 min later. Only small amounts of ^{3}H-NA were recovered from the hearts. The monoamine oxidase inhibitor nialamide in a dose of 10 mg/kg increased the ^{3}H-NA recovered from the hearts of reserpine-treated animals severalfold. In higher doses, however, this effect of nialamide was no longer observed. When ^{3}H-NA was replaced by [^{3}H]α-methylnoradrenaline, nialamide only reduced the amount of amine recovered from the hearts. The balance between the opposing actions of nialamide depended on dosage, time interval and temperature in a manner suggesting that the inhibitory influence was due to accumulation of endogenous amines in the adrenergic nerve fibres. Bretylium and harmaline also preserved ^{3}H-NA in reserpine-treated animals whereas iproniazid, pheniprazine, pargyline and tranylcypromine did not do this. Moreover, pargyline blocked the ability of nialamide to enhance ^{3}H-NA accumulation. The uptake of ^{3}H-NA observed after monoamine oxidase inhibition in the hearts of reserpine-treated mice was almost completely blocked by pretreatment with desipramine or protriptyline, potent blockers of amine uptake by adrenergic nerve fibres.

IN 1964, Hamberger, Malmfors, Norberg & Sachs, using a histochemical method, were able to demonstrate that the adrenergic neuron can take up and concentrate noradrenaline (NA) even after pretreatment with reserpine. Later this uptake mechanism was shown to be located at the level of the cell membrane, to be selectively blocked by cocaine, and to differ from the storage mechanism of the granules which is blocked by reserpine (Hillarp & Malmfors, 1964). These findings provided the first direct evidence for the dual amine uptake and storage mechanism of the adrenergic neuron postulated by Carlsson, Hillarp & Waldeck (1963). On the basis of experiments on the perfused rat heart, Muscholl (1963) suggested a reserpine-resistant uptake of NA into the cell. To achieve an accumulation of NA in the nerves of reserpine-treated animals it is necessary to block monoamine oxidase which otherwise will destroy the NA taken up, since reserpine excludes its protection by the amine-granules. This agrees with the findings of Kopin, Hertting & Gordon (1962) that monoamine oxidase plays a major role in the metabolism of NA in the hearts of reserpine-treated rats.

The reserpine-resistant uptake of NA has been further examined by Malmfors (1965), Carlsson & Waldeck (1966a) and, *in vitro*, by Hamberger & Masuoka (1965). At first when we tried to reproduce the histochemical findings biochemically we ran into difficulties, the uptake being relatively small (Andén, Carlsson & Waldeck, 1963). Later we found that the dose of the monoamine oxidase inhibitor was critical. Moreover, successful results were achieved only with certain monoamine oxidase inhibitors; others reduced the accumulation and antagonized the effect of inhibitors which increased accumulation.

It therefore seemed to be important to determine the optimal conditions under which NA can accumulate in the adrenergic nerves of reserpine-treated animals.

From the Department of Pharmacology, University of Göteborg, Sweden.

Experimental

MATERIALS

Commercially available (±)-[7-^{3}H]noradrenaline* (^{3}H-NA) with a specific activity of about 6 c/m-mole was used. α-Methyl[7,8-^{3}H]noradrenaline** (^{3}H-α-Me-NA) with an approximate specific activity of 30 mc/m-mole was prepared in co-operation with the Research Laboratories of Hässle Ltd (Hallhagen, G. & Waldeck, B., unpublished). Drugs used were: bretylium tosylate (Burroughs Wellcome & Co.); desipramine hydrochloride (Geigy); harmaline; iproniazid phosphate (Roche); nialamide (Swedish Pfizer); pargyline (Dr. G. M. Everett, Abbott Laboratories); pheniprazine hydrochloride (Draco); protriptyline hydrochloride (Dr. C. A. Stone, Merck Institute for Therapeutic Research); reserpine (Swedish Ciba); tranylcypromine sulphate (Smith, Kline & French).

METHODS

When not otherwise stated the experiments were made at room temperature (22–24°). Mice, divided at random into groups of six, were given reserpine (10 mg/kg i.p.) 6 hr, and a monoamine oxidase inhibitor in various doses and at different times, before the intravenous injection of 1 μg/kg ^{3}H-NA or 20 μg/kg ^{3}H-α-Me-NA. Thirty min later the animals were killed, and the hearts removed and extracted in 0·4N perchloric acid. The extracts were chromatographed on Dowex 50 W X4 ion-exchange resin. ^{3}H-NA and ^{3}H-α-Me-NA were determined in the eluate by liquid scintillation counting (for details see Carlsson & Waldeck, 1963; and unpublished).

Results

Nialamide in various doses was given to reserpine-treated mice 2 hr before ^{3}H-NA or ^{3}H-α-Me-NA. After 30 min the animals were killed and their hearts were analysed for ^{3}H-NA and ^{3}H-α-Me-NA, respectively. In the absence of a monoamine oxidase-inhibitor, reserpine caused a pronounced reduction of the amount of ^{3}H-NA recovered (Fig. 1). With increasing doses of nialamide the amount of ^{3}H-NA at first increased, reaching a maximum at about 10 mg/kg; it then decreased so that, at a dose of 100 mg/kg of nialamide, ^{3}H-NA was at about the same level as when reserpine had been given alone. The concentration of ^{3}H-NA observed after 10 mg/kg of nialamide was about 0·9 ng/g which is about one half of the value observed in normal animals at this time interval (compare Table 1). When the experiment was made at 30° the ^{3}H-NA levels were much lower. Even here a maximum was obtained after about 10 mg/kg of nialamide.

In reserpine-treated animals, 49 ng/g of ^{3}H-α-Me-NA was found in the heart 30 min after the administration. This is about the same amount as in animals not pretreated with reserpine (Carlsson, A. & Waldeck, B., unpublished). Nialamide had only an inhibitory influence on the accumulation

* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H] ethanol.
** 2-Amino-1-(3,4-dihydroxyphenyl)-[1,2-^{3}H] propanol.

of ^{3}H-α-Me-NA. With increasing doses of nialamide, the amount of ^{3}H-α-Me-NA decreased continuously so that at a dose of 100 mg/kg of nialamide only 12 ng/g could be detected. Between 10 and 100 mg/kg of nialamide, the ^{3}H-NA and ^{3}H-α-Me-NA curves (at 22–24°) took a similar course.

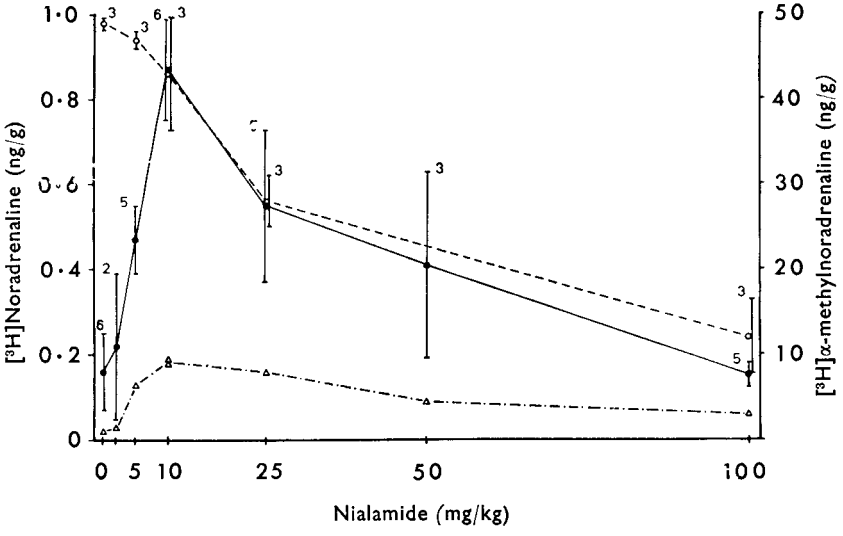

FIG. 1. Effect of nialamide on the accumulation of [^{3}H]noradrenaline and [^{3}H]α-methylnoradrenaline in the hearts of reserpine-treated mice. Reserpine (10 mg/kg) was given to mice i.p. 6 hr, and nialamide in various doses i.p. 2 hr, before intravenous [^{3}H]noradrenaline (1 μg/kg) or [^{3}H]α-methylnoradrenaline (20 μg/kg). The animals were killed 30 min later and the amine levels in the hearts were determined. Shown are the means ± s.e.m. with the number of experimental groups indicated at each point. However, the symbols of the curve obtained at an ambient temperature of 30° represent single values. Each experimental group consists of 6 animals. △----△, +30°, [^{3}H]noradrenaline. ●——●, +22–24°, [^{3}H]noradrenaline. ○----○, +22–24°, [^{3}H]α-methylnoradrenaline.

A number of monoamine oxidase inhibitors and bretylium were tested for their ability to preserve ^{3}H-NA taken up in the hearts of reserpine-treated mice (Table 1). Of these, only bretylium, harmaline and nialamide proved effective. When nialamide in a high dose (100 mg/kg) was given a short time (15 min) before ^{3}H-NA it appeared to be almost as efficient as 10 mg/kg given 2 hr before (compare Fig. 1).

In another experiment nialamide or pargyline, or a combination of both, was given to reserpine-treated mice before ^{3}H-NA. Pargyline proved inefficient in restoring ^{3}H-NA uptake in reserpine-treated animals, and moreover, blocked the positive effect of nialamide (Fig. 2). As the dose of pargyline used could be regarded as relatively high, a second experiment was designed using a smaller dose (10 mg/kg) given intravenously at various times before or after ^{3}H-NA (Fig. 3). Given 60 or 5 min before, pargyline inhibited the accumulation of ^{3}H-NA by about 2/3. When given 5 or 15 min after ^{3}H-NA, however, pargyline seemed to be less efficient in this respect.

Desipramine or protriptyline, efficient and specific blockers of the amine uptake at the level of the cell membrane of the adrenergic nerve fibre, were given a short time before ^{3}H-NA to mice pretreated with reserpine and nialamide. This resulted in a reduction of ^{3}H-NA uptake to the level observed in animals pretreated with reserpine only (Fig. 4).

TABLE 1. EFFECT OF SOME MONOAMINE OXIDASE INHIBITORS AND BRETYLIUM ON THE ACCUMULATION OF [^{3}H]NORADRENALINE (^{3}H-NA) IN THE HEARTS OF RESERPINE-TREATED MICE. Reserpine and a monoamine oxidase inhibitor were given at various times before i.v. injection of 1 μg/kg ^{3}H-NA. The animals were killed 30 min later and the levels of ^{3}H-NA in the hearts were estimated. Experiments made at an ambient temperature of 30° are indicated by an asterisk. The other experiments were made at 22–24°

Reserpine 10 mg/kg i.p. hr before	Inhibitor	Dose mg/kg	Hr before ^{3}H-NA	^{3}H-NA ng/g ± s.e.m.	n
None	None	—	—	1·90 ± 0·11	17
6	None	—	—	0·08 ± 0·01	11
6	None	—	—	0·03 ± 0·01*	2
6	Bretylium	10 i.v.	¼	0·25 ± 0·04	4
6	Harmaline	10 i.p.	½	0·53 ± 0·03	3
6	Harmaline	20 i.p.	½	0·66 ± 0·24	4
6	Iproniazid	10 i.p.	2	0·10	1
6	Iproniazid	100 i.p.	2	0·12	1
18	Iproniazid	100 i.p.	2	0·05	1
6	Nialamide	100 i.p.	¼	0·53 ± 0·11	4
18	Nialamide	100 i.p.	2	0·21 ± 0·15	2
6	Pargyline	10 i.p.	½	0·02*	1
6	Pargyline	100 i.p.	½	0·03*	1
6	Pargyline	10 i.p.	2	0·10 ± 0·01	2
6	Pargyline	10 i.p.	2	0·02*	1
6	Pargyline	100 i.p.	2	0·04*	1
18	Pargyline	100 i.p.	2	0·05	1
6	Pargyline	400 i.p.	2	0·05	1
6	Pheniprazine	10 i.p.	2	0·07	1
6	Tranylcypromine	10 i.v.	½	0·03	1
18	Tranylcypromine	10 i.v.	½	0·03	1

Discussion

The ability of the adrenergic neuron to retain NA depends largely on the amine-storing granules. If the granular storage mechanism is impaired by, for example, reserpine, NA will still be taken up by the amine transport mechanism at the level of the cell membrane (the "membrane pump") but as the monoamine oxidase is intact no accumulation of NA can occur. With a proper dose of a suitable monoamine oxidase inhibitor the NA taken up by the membrane pump will accumulate and stay in the neuron for some time.

We have shown that the conditions under which such an accumulation can occur are stringent. When nialamide was given 2 hr before the ^{3}H-NA, a relatively small dose (10 mg/kg) seemed to be most efficient in increasing the amount recovered. When the dose of nialamide was increased, this reduced the amount of ^{3}H-NA. This action could be even more clearly demonstrated when ^{3}H-NA was replaced by ^{3}H-α-Me-NA, an amine resistant to monoamine oxidase and thus being able to accumulate in the adrenergic neurons of reserpine-treated animals in the absence of a monoamine oxidase inhibitor. Nialamide at 100 mg/kg, a

dose which had little effect in preserving ^{3}H-NA when given 2 hr before it, seemed to have a good effect when given 15 min before ^{3}H-NA. This supports the view that the effect of nialamide in reducing the amount of amine recovered is a secondary phenomenon. It is possibly a consequence of monoamine oxidase inhibition; that is, blockade of the enzyme will result in accumulation of endogenous amines which may then compete with exogenous amines for uptake and binding mechanisms of the adrenergic nerve fibres. The ability of nialamide to preserve ^{3}H-NA in the hearts was less marked if hypothermia was prevented by keeping the reserpine-treated mice at an elevated ambient temperature.

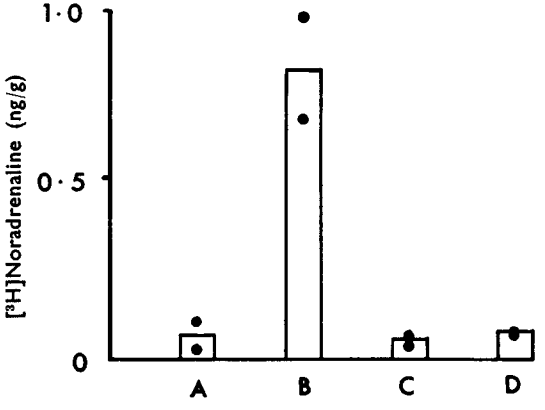

FIG. 2. Effect of pargyline on the accumulation of [^{3}H]noradrenaline in the hearts of mice. [^{3}H]Noradrenaline (1 μg/kg) was given i.v. to mice pretreated with reserpine, nialamide or pargyline as described below. The animals were killed 30 min later and the amine levels in the hearts were determined. Each point represents one experimental group consisting of six animals. A. Reserpine. B. Reserpine + nialamide. C. Reserpine + pargyline. D. Reserpine + nialamide + pargyline. Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [^{3}H]noradrenaline; nialamide 10 mg/kg, i.p. 2 hr before [^{3}H]noradrenaline; pargyline 100 mg/kg, i.p. 1 hr before [^{3}H]noradrenaline.

It has been observed (Carlsson, A. & Lindqvist, M., unpublished data, *see also* Carlsson, Dahlström, Fuxe & Lindquist, 1965) that the accumulation of monoamines in the brains of reserpine-treated mice brought about by nialamide or pargyline is much accelerated if hypothermia is prevented by keeping the animals at about 30°. If the inhibitory effect on accumulation by nialamide observed in the present experiments is due to accumulation of endogenous amines, it would be expected that this influence would be pronounced at an elevated temperature. Nialamide and pargyline have been shown previously to be capable of releasing [^{3}H]metaraminol from the hearts of mice not pretreated with reserpine (Carlsson & Waldeck, 1966b). Under these conditions it could be shown that inhibition of dopa decarboxylase blocked the releasing effect of the monoamine oxidase inhibitors (Carlsson, A., Lindqvist, M. & Waldeck, B., unpublished). The nature of the endogenous amines apparently involved here is obscure. It may be newly synthesized NA but other

amines such as octopamine cannot be excluded. Kakimoto & Armstrong (1962) have demonstrated the presence of octopamine in the rabbit heart after monoamine oxidase inhibition.

In view of the apparently subtle balance between the enhancing and antagonizing influences of monoamine oxidase inhibitors on ³H-NA accumulation in the heart it is not surprising that the scatter in some of the experiments was large.

FIG. 3. Effect of pargyline on the accumulation of [³H]noradrenaline in the hearts of mice pretreated with reserpine and nialamide. Indicated are the times before or after the i.v. administration of [³H]noradrenaline (1 μg/kg). The animals were killed 30 min later and the amine levels were determined. Each point represents one experimental group consisting of six animals. A. Control. B. Reserpine. C. Reserpine + nialamide. D. Reserpine + nialamide + pargyline at −60 min, at −5 min (E), at +5 min (F), and at +15 min (G). Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [³H]noradrenaline; nialamide 10 mg/kg, i.v. 2 hr before [³H]noradrenaline; pargyline 10 mg/kg, i.v.

In experiments on reserpine-resistant uptake mechanisms it is important that the dose and time interval be adequately chosen; recovery of the granular function sets in early (Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966). In the present experiments a large dose of reserpine (10 mg/kg) and a relatively short interval corresponding to the maximum action of the drug have been used.

In addition to the monoamine oxidase inhibitors nialamide and harmaline, bretylium preserved ³H-NA in hearts of reserpine-treated mice. Bretylium has been observed to behave as an inhibitor of the enzyme under various experimental conditions (Malmfors, 1965; Furchgott, 1966; Carlsson, A. & Waldeck, B., unpublished). In fact, Jonason, J. (unpublished), has observed an inhibitory action of bretylium (5×10^{-4}M) on a monoamine oxidase preparation *in vitro*.

In the present experiments iproniazid, pargyline, tranylcypromine and pheniprazine proved inefficient in preserving ³H-NA in the hearts of reserpine-treated mice. A full explanation of this failure is not yet possible. The amphetamine-like structure and activity of tranylcypromine and pheniprazine may be important (cf. Carlsson & Waldeck, 1966b).

This inability to preserve ^{3}H-NA by certain inhibitors of the enzyme does not accord with the observations of Malmfors (1965) who found all the inhibitors tested enhanced accumulation of NA. But his observations were made on the iris of the rat and the histochemical method he used is very sensitive so that variations in NA concentration above a certain level may not have been detected. Pargyline not only proved inefficient in preserving ^{3}H-NA but even after small doses actually blocked the enhancing action of nialamide. It is doubtful whether this action is the result of an inhibition of the enzyme.

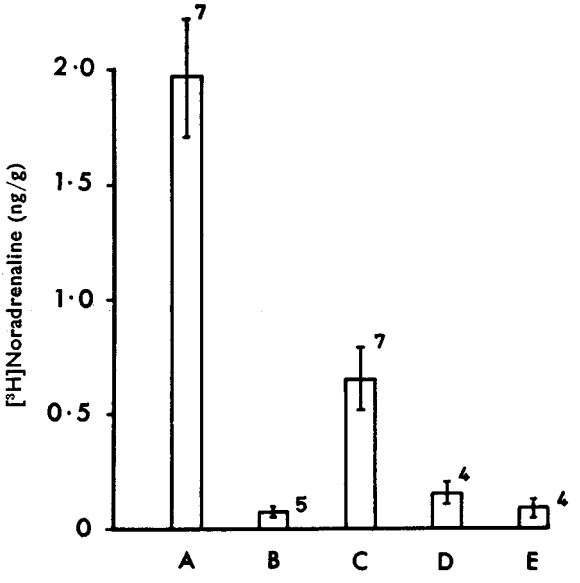

FIG. 4. Effect of desipramine and protriptyline on the accumulation of [^{3}H]noradrenaline in the hearts of mice pretreated with reserpine and nialamide. [^{3}H]Noradrenaline (1 μg/kg) was given i.v. to mice pretreated as described below. The animals were killed 30 min later and the level of [^{3}H]noradrenaline in the heart estimated. Shown are the means $\pm$ s.e.m. with the number of experimental groups indicated at each point. Each experimental group consists of six animals. A. Control. B. Reserpine. C. Reserpine + nialamide. D. Reserpine + nialamide + desipramine. E. Reserpine + nialamide + protriptyline. Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [^{3}N]noradrenaline; nialamide 10 mg/kg, i.p. 2 hr before [^{3}N]noradrenaline; disipramine 5 mg/kg, i.v. 5 min before noradrenaline; protriptyline 5 mg/kg, i.v. 5 min before [^{3}N]noradrenaline.

There is little doubt that the ^{3}H-NA determined is within the nerves. Histochemical work (Hamberger & others, 1964; Hillarp & Malmfors, 1964; Malmfors, 1965) clearly demonstrates that under conditions similar to those used by us exogenous NA accumulates in the adrenergic nerves. Further, potent and selective blockers of the "membrane pump" such as desipramine and protriptyline, when given before the test amine, effectively inhibit the accumulation of ^{3}H-NA in the hearts of reserpine-treated mice as well as the accumulation of NA in the adrenergic nerve fibres as observed histochemically. When the blocking agent is given after the

test amine, however, ^{3}H-NA already accumulated seems to be largely unaffected (Carlsson & Waldeck, 1966a).

The question arises whether the reserpine-resistant NA is free or particle bound. Recent work by Stitzel & Lundborg (cf. Lundborg & Waldeck, 1966) has shown that 30 min after the intravenous injection of ^{3}H-NA in mice pretreated with reserpine and nialamide as described here, as much as 15% of the ^{3}H-NA was found in the "particulate" fraction (calculated as per cent of the sum of particulate and supernatant fractions), that is, about one-third of the percentage observed in normal animals. These data indicate that at least part of the reserpine-resistant NA fraction is particle bound. Whether the particles in question are specific storage granules, microsomal particles, or fragments of the cell membrane is difficult to answer. It is interesting that, in the particulate fraction of the adrenal medulla, an amine uptake mechanism has been detected which does not require ATP and Mg^{2+} and is insensitive to reserpine (Lundborg 1966).

In the hearts of rats pretreated with reserpine 24 hr before the experiment, Iversen, Glowinski & Axelrod (1966) observed an uptake of ^{3}H-NA which occurred even when monoamine oxidase was intact and was not blocked by desipramine. Their observations were made only 5 min after the administration of the ^{3}H-NA. It seems probable that under such conditions most of the ^{3}H-NA is located outside the nerves. This would explain why the ^{3}H-NA uptake was unaffected by desipramine.

In a review, Costa, Boullin, Hammer & others (1966) have reached conclusions opposite to ours. They express the view that reserpine blocks amine uptake at the cell membrane level, whereas desipramine blocks uptake by amine granules. The data on which these conclusions are based, are only briefly reported in the article and do not seem to have been published.

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