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Correlation of the recovery of the granular uptake-storage mechanism and the nerve impulse induced release of [^{3}H]noradrenaline after reserpine

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Rats were treated intraperitoneally with 10 mg/kg reserpine. After various time intervals 1 μg/kg $\pm$ [^{3}H]noradrenaline (^{3}H-NA) was administered intravenously, and the uptake into subcellular fractions of the submaxillary gland was measured or, in some of the rats, the sympathetic chain of the neck was electrically stimulated with 10 impulses/s for 30 min. The release of ^{3}H-NA and the contraction response of the lower eye-lid were measured. A striking parallel was observed between the recovery of the ^{3}H-NA uptake into the amine storage particles and the nerve impulse-induced release of ^{3}H-NA, and also the recovery of the functional response. The somewhat earlier recovery of the ^{3}H-NA uptake into the coarse fraction might reflect the existence of another type of amine storage granule or might represent granules present near the nerve cell membrane. A possible increase in the turnover of the adrenergic transmitter during the period of recovery after reserpine is discussed.

Treating animals with reserpine depletes the stores of noradrenaline in the tissues and also reduces the response of various organs to sympathetic nerve stimulation (Bertler, Carlsson & Rosengren, 1956; Muscholl & Vogt, 1958).

After a single dose of reserpine the nerve function recovers centrally (Häggendal & Lindqvist, 1963; 1964) and peripherally (Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966) within 2 or 3 days, while the monoamine concentrations are still low. At the time of functional recovery there is a sudden rise in the ability of the amine granules to take up and retain amines; in the adrenal medulla the uptake of monoamines increases to almost normal values during this time (Lundborg, 1963; Carlsson, Jonason & Rosengren, 1963) but there is only partial recovery in the sympathetic nerves (Lundborg & Stitzel, 1968). It would, however, be of value to relate the recovery of the ability of the neuron to take up and to retain noradrenaline to the release of the amine from the neuron after electrical stimulation of the nerve.

In the present investigation we have compared in the same organ (salivary gland of the rat) the uptake of tritiated noradrenaline (^{3}H-NA) into the catecholamine-containing granules and the release of ^{3}H-NA elicited from the gland by sympathetic nerve stimulation. These studies were conducted from 4 to 72 h after reserpine administration. The effect of nerve stimulation on the tension of the lower eye lid was also investigated.

MATERIALS AND METHODS

Male Sprague-Dawley rats, 175–225 g, were used.

In one series of experiments, rats were given ^{3}H-NA, 1 μg/kg (7·45 Ci/mmol), intravenously into a tail vein. Atropine (1 mg/kg) was given 30 min before the

labelled amine to reduce the variations in ^{3}H-NA uptake into the salivary glands (Almgren, 1970).

In some experiments reserpine (10 mg/kg) was given intraperitoneally at various intervals before the ^{3}H-NA.

The animals were killed 30 min after the administration of ^{3}H-NA. The submaxillary plus sublingual glands were removed, weighed and homogenized in an ice bath with a Teflon pestle in 0·25M sucrose containing 0·005M phosphate buffer, pH 7·4 and 0·001M $MgCl_2$. The coarse particles were removed by centrifuging the homogenate at 4° at 2000 *g* for 10 min. The resulting supernatant was then centrifuged at 100 000 *g* for 60 min in a Spinco Model L ultracentrifuge at 4° to give the particulate fraction and the high speed supernatant used. After protein precipitation, extracts of the various fractions were passed through cation-exchange resin columns. The catecholamines were eluted with hydrochloric acid and the tritium content of the eluate was determined by liquid scintillation counting (Stitzel & Lundborg, 1967).

In other experiments rats were given reserpine, atropine and ^{3}H-NA as described above. The rats were anaesthetized with urethane (1 g/kg) intraperitoneally and the right cervical sympathetic trunk was dissected free for stimulation (Almgren, Lundborg & Stitzel, 1969). The sympathetic trunk of the contralateral side was cut proximal to the superior cervical ganglion. Thirty min after the injection of ^{3}H-NA, the sympathetic chain was stimulated preganglionically with a Grass S4 Stimulator giving supramaximal pulses (3–5 V, 2 ms) at a frequency of 10 impulses/s. The contraction of the lower eye-lid was recorded according to Obianwu (1967). The stimulation was continued for 30 min. Immediately after the stimulation period the submaxillary plus sublingual glands from each side were removed, weighed and homogenized in ice-cold 0·4N perchloric acid. The ^{3}H-NA content was determined as described above.

Substances used: (±)-Noradrenaline-1-^{3}H (New England Nuclear Chemicals). Commercially available atropine sulphate was used. Reserpine (Serpasil) was generously supplied by the Swedish Ciba, Ltd, Vällingby.

RESULTS

Effect of reserpine on the uptake of ^{3}H-NA into subcellular fractions of salivary glands

The absolute values are given in Table 1, and in Fig. 1 the recovery of the coarse and particulate fractions as well as the total ^{3}H-NA content of the glands are expressed as a percentage of controls.

Six and 12 h after pretreatment with reserpine, there was an almost complete blockade of the uptake-retention of ^{3}H-NA into the salivary glands (Fig. 1), the value obtained being about 6% of the control values. A slight recovery was observed 24 h after the injection of reserpine, reflecting an increased amount of ^{3}H-NA recovered from the coarse and supernatant fractions at this time. The particulate fraction was unchanged. Thirty-six h after reserpine, there was an increase in ^{3}H-NA content of the particulate fraction to about 10% of the control values, which had increased to about 40% at 48 h, while in the coarse and supernatant fractions about 65% of the noradrenaline content, as compared to the control values, had been recovered.

Effect of reserpine on the release of ^{3}H-NA from rat salivary glands by nerve stimulation

In untreated animals, stimulation of the cervical sympathetic chain for 30 min caused about a 56% reduction in the ^{3}H-NA content of the stimulated gland (Fig. 2a).

Table 1. *Uptake and retention of ^{3}H-NA in subcellular fractions of rat salivary glands at various time intervals after reserpine.* The animals except the controls received an i.p. injection of 10 mg/kg reserpine. At various time intervals after the reserpine injection 1 mg/kg atropine was administered i.p. and 30 min thereafter 1 μg/kg ($\pm$) ^{3}H-NA. 30 min after the administration of the labelled amine the rats were killed and the submaxillary and sublingual glands were removed. The analytical procedure is given in the text. The values are in ng/g and represent mean $\pm$ s.e. C = coarse fraction, P = particulate fraction, S = supernatant fraction.

Time interval after reserpine (h)	No. of exp.	^{3}H-NA content ng/g		
		C	P	S
Controls	14	0·81 ± 0·07	0·24 ± 0·02	0·33 ± 0·03
6	4	0·03 ± 0·00	0·01 ± 0·00	0·05 ± 0·01
12	6	0·03 ± 0·00	0·01 ± 0·00	0·05 ± 0·00
24	10	0·15 ± 0·05	0·01 ± 0·00	0·11 ± 0·03
36	12	0·30 ± 0·04	0·03 ± 0·00	0·11 ± 0·01
48	6	0·54 ± 0·05	0·10 ± 0·00	0·23 ± 0·02

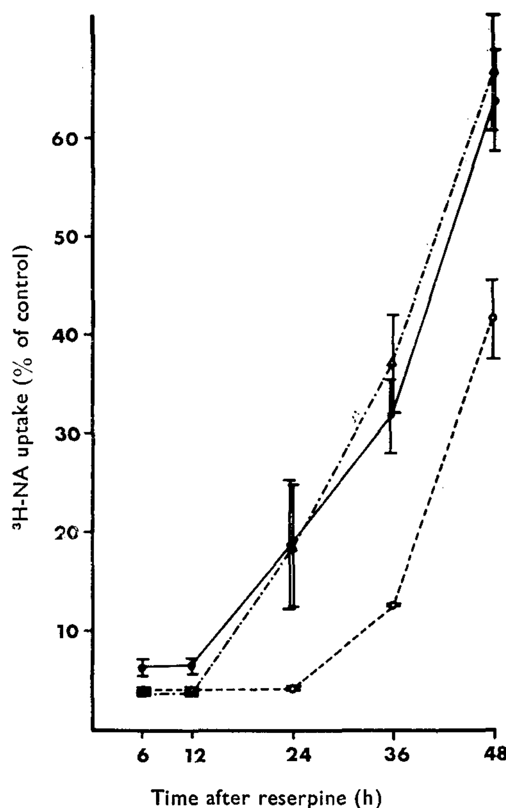

Fig. 1. Effect of reserpine on the uptake of ^{3}H-NA into subcellular fractions of the rat salivary glands. The total uptake is the sum of the coarse, particulate and supernatant fractions. The ^{3}H-NA values are given as a percentage of controls (n = 14). The absolute values, corresponding to 100% are given in Table 1. Vertical lines indicate standard errors of the means, and the number of experiments were 4, 6, 10, 12 and 6 at 6, 12, 24, 36 and 48 h respectively.

Four and 24 h after reserpine, nerve stimulation caused no further reduction in the already low ^{3}H-NA content of the gland. At 36 and 48 h after reserpine the ^{3}H-NA content of the stimulated gland was reduced by 53 and 59% respectively.

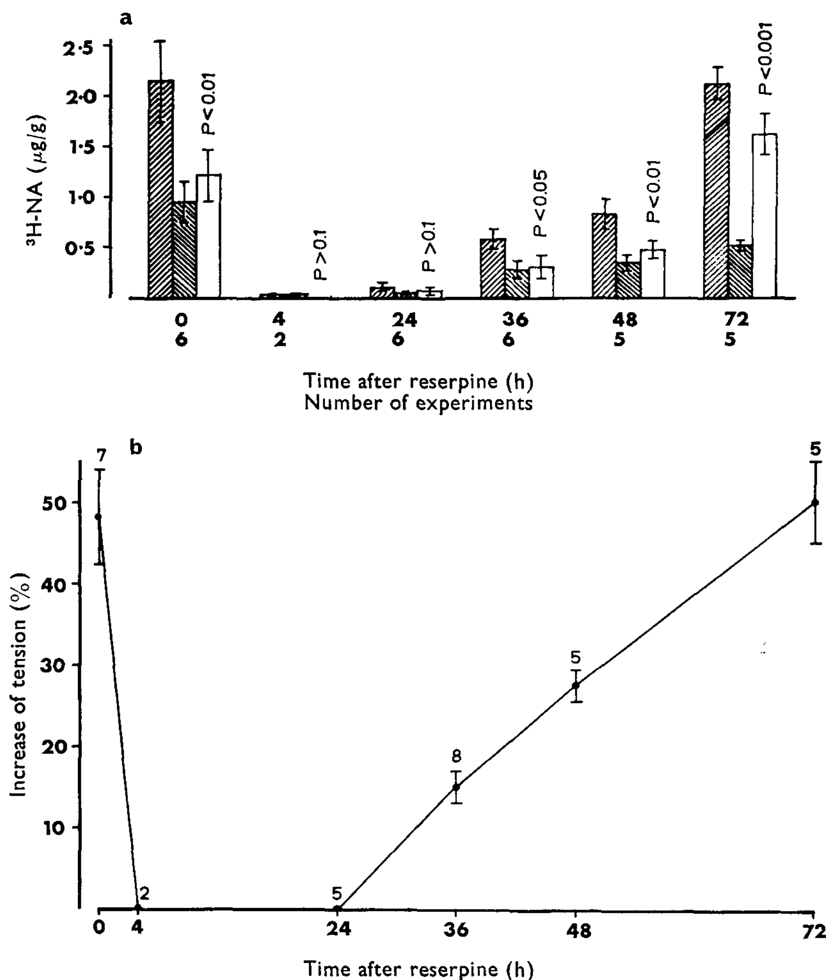

FIG. 2. a. Release of ^{3}H-NA from rat salivary glands by sympathetic nerve stimulation. The experiments were made at various time intervals after the i.p. injection of 10 mg/kg of reserpine (RES). Thirty min after the i.v. injection of 1 μg/kg of ^{3}H-NA, the right sympathetic chain was stimulated with supramaximal pulses at a frequency of 10/s for 30 min. The left (control) side was decentralized. The vertical bars indicate the standard errors of the means. The P values given were calculated by a t-test on the difference between matched pairs.

b. The contraction response of the lower eye-lid of rats, induced by sympathetic nerve stimulation at various intervals after the administration of 10 mg/kg reserpine. The eye-lid was given a base tension at rest of 0·5 g. The increase of tension was recorded on a Grass polygraph, and is given in the figure as per cent of the base tension. Supramaximal pulses at a frequency of 10/s were given over 30 min. Vertical bars indicate the standard errors of the means and the figures the number of experiments.

Seventy-two h after reserpine the uptake of ^{3}H-NA into the unstimulated (decentralized) glands had returned to normal. Nerve stimulation reduced the ^{3}H-NA content by about 75% (which was a significantly greater reduction than in the controls ($P < 0{\cdot}05$).

Effect of reserpine on the response of the lower eye-lid of the rat to nerve stimulation

In untreated rats stimulation of the sympathetic chain resulted in an increase in the tension of the lower eye-lid by about 0·25 g (Fig. 2b). Four and 24 h after reserpine the response of the lower eye-lid to stimulation was completely abolished. Thereafter a gradual recovery in response was observed until at 72 h it was normal.

DISCUSSION

Considerable evidence indicates that reserpine acts on particle-bound stores.

In vitro, reserpine blocks the ATP-Mg $^{+}$-dependent uptake of catecholamines into isolated adrenal medullary (Carlsson, Hillarp & Waldeck, 1962; Kirshner, 1962) and nerve (Euler and Lishajko, 1963) granules. Also, after *in vivo* administration of reserpine the *in vitro* uptake of catecholamines is blocked (Lundborg, 1963).

Histochemical studies have shown that after pretreatment with reserpine and an inhibitor of the monoamine oxidase, exogenous noradrenaline can accumulate in the sympathetic nerves (cf. Malmfors, 1965) thus indicating that reserpine is not interfering with the uptake at the level of the nerve cell membrane.

^{3}H-NA given to reserpine-treated animals is not accumulated in sympathetic nerves. Such an accumulation can however be demonstrated with [^{3}H]α-methylnoradrenaline, an amine resistant to monoamine oxidase, which is accumulated in the nerves after reserpine treatment. However, there is a pronounced blockade of the uptake of this amine into the particle-bound stores (particulate fraction) with its subsequent extra-particular accumulation (Lundborg & Stitzel, 1967).

Thus much evidence has accumulated in support of the hypothesis that reserpine exerts its effect by interfering with the particle-bound stores. The immediate conclusion could be drawn that the data obtained in the present study are not in favour of this concept. However, accepting the evidence that reserpine acts on particle-bound stores, factors influencing the recovery of noradrenaline uptake-retention after reserpine and the isolation of amine-containing particles will be discussed.

A clearcut difference was revealed between the coarse and the particulate fractions for recovery of ^{3}H-NA uptake-retention after reserpine. Already at 24 h there was a slight but significant increase in ^{3}H-NA content of the coarse fraction while the particulate fraction was still unchanged. This discrepancy between the two fractions remained at 48 h. The earlier recovery in the ^{3}H-NA content of the coarse fraction might indicate that that fraction contains not only "pinched off nerve-endings" but also amine storage particles which differ in uptake properties from those of the particulate fraction.

The noradrenaline content of the supernatant fraction, although not reduced by reserpine to the same extremely low values as the coarse and particulate fractions, may in part be an artifact representing amines released during the homogenization procedure (Lundborg, 1967). Therefore, in the present study, this fraction appeared to be less important in providing information on the recovery of noradrenaline uptake-retention after reserpine.

The possible existence of two different types of granules has been pointed out. By a continuous gradient centrifugation technique Roth, Stjärne & others (1969) have been able to differentiate between "light" and "heavy" granules. In electron microscopical studies, dense-cored vesicles of different sizes have been observed in adrenergic neurons (for ref. see Hökfelt, 1968). The small type (around 50 nm) is very

common in the nerve terminals. The large type is also found in the nerve terminals, but there are more in the cell bodies and in the axons (Hökfelt, 1969). Häggendal & Dahlström (1970, 1971) have suggested the possibility that the large dense-cored vesicles correspond to the young amine granules and that with the gradual loss of chromogranin (see Geffen, Livett & Rusk, 1969), the large vesicles shrink and develop into the smaller type. The small type would then possibly represent the old amine granules. According to the suggestion discussed above, it is possible, that in the present subcellular distribution studies, the young large vesicles have properties causing them to be "trapped" and spun down with the low speed sediment (coarse fraction). The suggested (see above) dominance of young granules in the recovery phase after reserpine could thus account for the early recovery in ^{3}H-NA content of the coarse fraction. It is also possible that, during the first period of recovery, all functioning granules will be localized close to the nerve cell membrane. During the fractionation procedure these granules might stick to membrane fragments and be spun down with the low speed sediment.

In experiments in which the cervical sympathetic chain was stimulated, observations were run up to 72 h after injection of reserpine. At this time the ^{3}H-NA content of the unstimulated glands was back to normal, a result that would appear to be inconsistent with previous findings (e.g. Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966) since in these related studies the ^{3}H-NA content of various organs at 72 h was respectively 40 and 25%. A possible explanation for the discrepancy is that in the present study the glands were preganglionically denervated before stimulation was started (see Methods). Thus the ^{3}H-NA content of these glands was not influenced by the physiological impulse flow during a period of 45 out of 60 min. It might be speculated that, during the recovery period after reserpine, there is an increased turnover rate of the transmitter (Iggo & Vogt, 1960). The ^{3}H-NA should then be released at a higher rate from intact (not decentralized) sympathetic nerves. In the studies of Andén & others quoted above, the content of ^{3}H-NA of various organs was measured 30 min after the administration of the labelled amine and, in the reserpine-treated animals, more of the ^{3}H-NA initially taken up might have been released by them than in untreated animals. The recovery of this granular uptake function might thus have been partly masked by an increased turnover and release of ^{3}H-NA. By using decentralized organs, this experimental error can be circumvented. The observation (see Fig. 2a) that about 75% of the ^{3}H-NA content was released by nerve stimulation 72 h after reserpine than in the untreated animals (about 55%) could be taken as support for such a view.

In the present study a striking correlation in time was observed between the reappearance in (i) granular uptake-storage function, (ii) the ability of nerve stimulation to release ^{3}H-NA and (iii) functional response, thus demonstrating the importance of functioning storage granules for normal neuronal function such as release of transmitter onto specific receptor sites.

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