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GUANETHIDINE UPTAKE AND NORADRENALINE DEPLETION IN NORADRENALINE STORAGE PARTICLES OF THE RAT HEART*

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Abstract—Rats received an intravenous injection of [^{3}H]noradrenaline or of [^{3}H]guanethidine. The noradrenaline storage particles of the heart were isolated by sucrose density gradient centrifugation. Their analysis confirmed that both agents are taken up into subcellular granules by a reserpine-sensitive process and showed that they have a similar pattern of subcellular distribution into two types of gradients. The amounts of [^{3}H]guanethidine found in the granules 30 min and 5 hr after its intravenous injection were compared with the loss of noradrenaline from these granules after intravenous injection of the same dose of unlabelled guanethidine. After 30 min an appreciable amount of guanethidine was found, whereas no significant loss of noradrenaline could be detected. Between 30 min and 5 hr, the granules lost an amount of noradrenaline which was greater than the supplementary amount of guanethidine taken up. Since the rate of neuronal uptake of guanethidine is not correlated with the rate of noradrenaline loss, it is concluded that the noradrenaline depletion caused by guanethidine in the rat heart is not due to a simple displacement mechanism.

Chromatographic experiments carried out in two solvent systems failed to demonstrate the presence of metabolites in the granule fraction, whereas at least one metabolite was detected in the coarser particles. This suggests that the guanethidine metabolites play at best a minor rôle in the mechanism of action of guanethidine.

SEVERAL studies have indicated that guanethidine causes a long lasting depletion of peripheral stores of noradrenaline (see e.g. review articles Refs. 1, 2). It was also shown that guanethidine is taken up and concentrated in various tissues in the rat.[3–6] Considerable evidence suggests that it may be taken up and retained in sympathetic nerves by a process involving such a high specific affinity of the drug for noradrenaline storage sites[7–9] that it can be released by sympathetic nerve stimulation[10] and can affect both the sympathetic nerve cell membrane pump and the intracellular catecholamine-concentrating mechanism.[11,12]

Obianwu *et al.*[13] have found that [^{3}H]guanethidine injected intravenously into mice is preferentially retained in the high speed particulate fraction of the heart and Chang *et al.*[8] have reported a direct relationship between the amount of guanethidine retained in the rat heart and the degree of noradrenaline depletion. The latter results were obtained by calculating the differences of guanethidine uptake and noradrenaline depletion in the whole heart of normal rats and of rats pretreated with various doses of amphetamine. It seemed of interest, therefore, to study the exact distribution of

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[^{3}H]guanethidine in subcellular particles of the rat heart, to compare it with that of noradrenaline and to study the relationship between the amount of [^{3}H]guanethidine found in these particles and the degree of their noradrenaline loss.

MATERIALS AND METHODS

DL-7-[^{3}H]noradrenaline (6·6 c/mM) was obtained from New England Nuclear, Boston, Mass., U.S.A. and [^{3}H]guanethidine sulphate (0·13 μc/μg) from Dr. D. F. Elliott, CIBA Horsham, England.

Isolated bovine splenic nerve granules

Bovine splenic nerve granules were isolated by differential centrifugation with some modifications[14] of the method described by von Euler[15] and by Schümann.[16] The incubation mixture contained: nerve granules corresponding to 0·5 g of nerve tissue, 0·3 M sucrose, 0·009 sodium phosphate buffer pH 6·8, 0·003 M ATP, 0·003 M $MgCl_2$ and 0·1 mM [^{3}H]noradrenaline (0·12 μc/μg). In some experiments [^{3}H]noradrenaline was replaced by [^{3}H]guanethidine (0·13 μc/μg). After an incubation at 37° for 20 min aliquots of the suspension were removed and the granules collected by passage through a HAWP 025-Millipore filter. The filters were washed with ice-cold 0·25 M sucrose, dried and counted by liquid scintillation spectrometry after addition of 10 ml of a 0·6% butyl-PBD (Scintillator CIBA) solution in toluene. Granules kept in an ice bath during incubation time were used as controls.

Uptake and retention of [^{3}H]noradrenaline and [^{3}H]guanethidine in subcellular particles of the rat heart in vivo

(a) *Treatment.* Male albino rats (190–210 g body weight) were injected intravenously with either 100 μc (2·56 μg) [^{3}H]noradrenaline or 100 μc (760 μg) [^{3}H]guanethidine. Thirty min or 5 hr later the rats were killed by decapitation.

(b) *Density gradients.* The hearts were removed quickly and homogenized in 0·25 M sucrose containing 0·001 $MgCl_2$ and 0·005 M phosphate buffer pH 7·4 (5 ml per heart) using a Polytron PT 20 OD homogenizer. The homogenate was centrifuged at 750 *g* for 5 min. The supernatant fraction was centrifuged again at 105,000 *g* for 5 min (including speed-up time) and the new supernatant fraction was recentrifuged at the same speed for 1 hr, according to the method of Michaelson *et al.*[17] The microsomal pellet was resuspended in 0·5 ml of the sucrose solution used for homogenization and layered on linear sucrose density gradients. The two gradient types A and B described by Roth *et al.*[18] were used. Type A consisted of 0·25–2·0 M sucrose, type B of 0·5–1 M sucrose with 0·32 ml 2·0 M sucrose in the bottom. The tubes were centrifuged in the SW 65 L Spinco rotor at 169,000 *g* for 1 hr, and they were then emptied by constant-rate infusion of 2·5 M sucrose through a perforation in the bottom of the tube.[18] Radioactivity was determined in fractions of four drops after addition of 10 ml of methanol and 10 ml of the scintillator solution mentioned above.

(c) *Chromatography.* Ascending paper (Whatman I) chromatography of 2 N HCl extracts of the pellet from each centrifugation step was carried out in two solvent systems (a) *n*-butanol–ethanol–10% ammonia (240:120:78) and (b) isopropanol–conc. ammonia–water (400:40:60). The chromatograms were developed for about 18 hr, dried and cut into 1-cm wide strips. The strips were counted after addition of 10 ml of the scintillator solution.

Determination of noradrenaline

The content of endogenous noradrenaline was estimated fluorometrically[19] in fractions of 10 drops obtained from the gradient type B mentioned above, after adsorption into alumina at pH 8·6 and elution with 0·25 N HCl.

Renal hypertensive rats

Production of renal hypertension (Goldblatt method) and plethysmographic measurements of blood pressure under light ether anaesthesia were carried out as described previously.[20]

RESULTS

1. *Isolated bovine splenic nerve granules*

Guanethidine is known to cause a profound depletion of endogenous noradrenaline stores. Since this property is shared by substances which inhibit the transmitter uptake into isolated amine storing granules, e.g. reserpine or prenylamine, the ability of guanethidine to influence noradrenaline uptake into bovine splenic nerve granules was investigated and compared with that of reserpine itself. The results presented in Fig. 1 show that guanethidine at concentrations as high as 10^{-3} M was unable to counteract [^{3}H]noradrenaline uptake, while reserpine inhibited it by 50 per cent already at concentrations of 10^{-7} M.

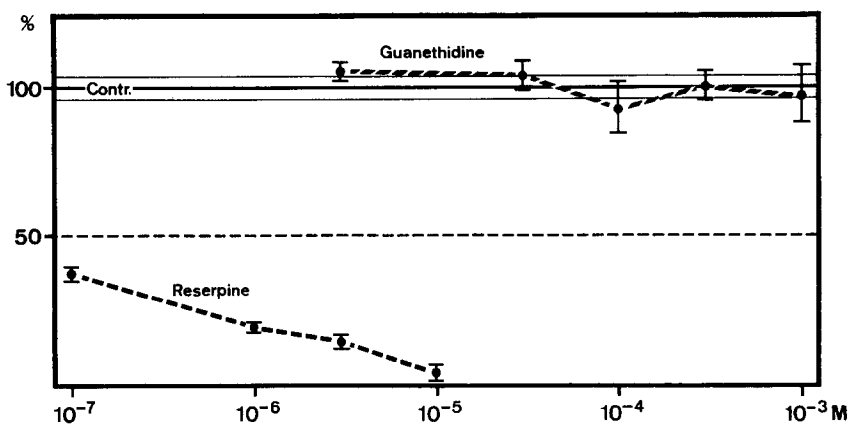

FIG. 1. Effects of guanethidine and reserpine on the uptake of [^{3}H]noradrenaline in isolated bovine splenic nerve granules. Granules suspensions were incubated at 37° for 20 min with [^{3}H]noradrenaline as described under Material and Methods in the presence of various concentrations of reserpine phosphate or guanethidine sulphate. The symbols represent mean values ± S.E. of three to six experiments. Twelve control determinations were carried out.

The same system was used in a second series of experiments to compare the ability of the granules to take up [^{3}H]noradrenaline or [^{3}H]guanethidine, respectively. Whereas [^{3}H]noradrenaline was effectively taken up, no measurable uptake of [^{3}H]guanethidine could be noted (Table 1).

Since *in vivo* experiments clearly indicate that guanethidine interacts with the subcellular storage mechanisms and displays a strong affinity to the adrenergic nerve

TABLE 1. UPTAKE OF [^{3}H]NORADRENALINE AND OF [^{3}H]GUANETHIDINE IN ISOLATED BOVINE SPLENIC NERVE GRANULES

Granules incubated with	Counts/min/0·2 ml granules suspension 0°*	37°
[^{3}H]noradrenaline	704 ± 30	1550 ± 17†
[^{3}H]guanethidine	745 ± 50	756 ± 34 N.S.

Aliquots of the same granule suspension were incubated for 20 min with either [^{3}H]noradrenaline (2 μc/16·9 μg/ml) or [^{3}H]guanethidine (2 μc/15·2 μg/ml).

The uptake was determined as described under Material and Methods.

* 0° means that granules were prepared for incubation, but kept in an ice bath during the incubation period.

† $P < 0{\cdot}001$.

N.S. = not significant.

endings,[7–9] the system of the isolated splenic nerve granules seemed to be inadequate to get a better insight into the mechanism by which guanethidine may be stored in the nerve endings and lower their noradrenaline content.

We studied therefore the uptake and exact subcellular distribution of [^{3}H]guanethidine *in vivo* and compared the amounts of the drug present in the noradrenaline granules of the heart with the amounts of endogenous noradrenaline in the same subcellular fractions.

2. *Subcellular distribution of* [^{3}*H*]*guanethidine and of* [^{3}*H*]*noradrenaline in the rat heart. Effect of pretreatment with reserpine*

The subcellular distribution of intravenously injected [^{3}H]guanethidine was compared with that of [^{3}H]noradrenaline. The microsomal pellet obtained from a heart homogenate was subjected to different types of density gradient centrifugation. A large portion of labelled noradrenaline eluted in a sharp band indicated its presence in a fairly homogenous kind of particles. A considerable portion of the [^{3}H]noradrenaline was also present in a broader region of particles sedimenting more slowly. It is not known whether these represent a separate class of particles or whether they are the result of partial damage of the amine storage granules. The fact that the proportion of the two species varies from experiment to experiment seems rather to indicate that the latter is the case.

These results show also that in both types of density gradient most of the [^{3}H]guanethidine is present in the same narrow band of particles containing the [^{3}H]noradrenaline (Fig. 2, a, b). It is therefore very likely that guanethidine is taken up into the same granules which store noradrenaline.

To study the effect of reserpine on the uptake of [^{3}H]guanethidine, rats were treated as described above but in addition, groups of rats were also pretreated with reserpine (1 mg/kg s.c.) 17 hr prior to the injection of the labelled compound. Figure 3 shows that the uptake of [^{3}H]noradrenaline is greatly reduced in reserpine-treated rats. The peak fractions contain only 50 counts/min in contrast to over 1000 counts/min in control experiments. The uptake of [^{3}H]guanethidine is also greatly reduced after pretreatment with reserpine in the main region as well as in the region containing smaller particles.

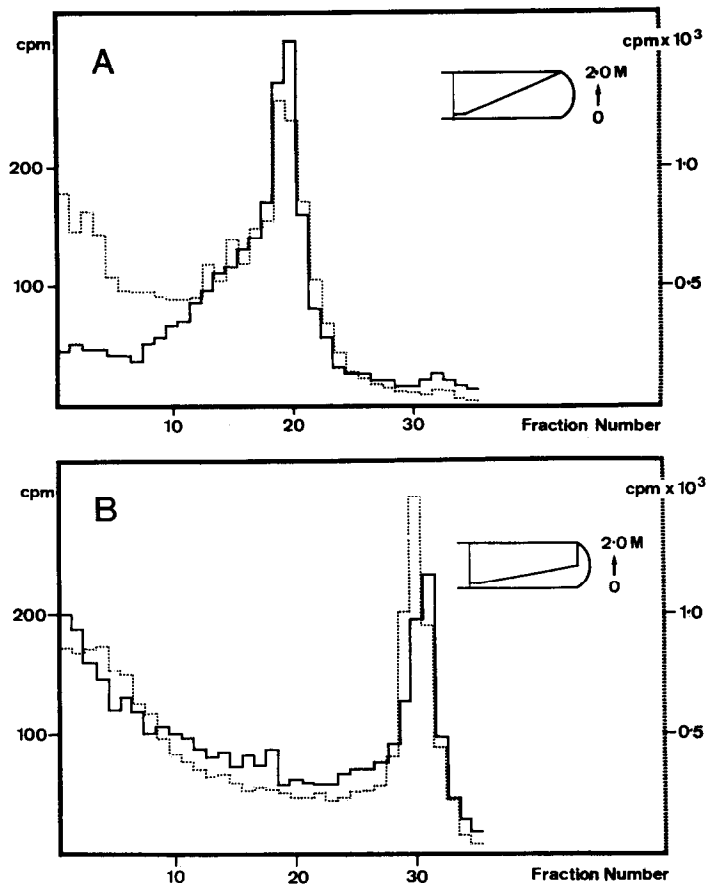

FIG. 2. Subcellular distribution of [^{3}H]guanethidine and [^{3}H]noradrenaline in resuspended high-speed sediment from rat heart. [^{3}H]noradrenaline (100 μc/rat) or [^{3}H]guanethidine (100 μc/rat) were administered 30 min before removing the hearts. Preparation of the granule fraction and density gradient centrifugation were carried out as described under Material and Methods. (a) Gradient type A (0·25–2 M sucrose) (b) Gradient type B (0·5–1 M sucrose with a layer of 2 M sucrose in the bottom). These gradients are schematically shown in the figure. Solid line: guanethidine. Dotted line: noradrenaline.

3. *Comparison of guanethidine content and noradrenaline depletion in the subcellular fraction of amine storage granules*

In order to analyse the possible relationship between uptake of guanethidine and disappearance of noradrenaline measurements of both amines were carried out 30 min and 5 hr after guanethidine injection. This time schedule was chosen because preliminary experiments with this i.v. dose of guanethidine have shown that the noradrenaline content in the rat heart strongly diminishes between 30 min and 5 hr.

The [^{3}H]guanethidine content increased significantly between 30 min and 5 hr, as shown in Fig. 4. The mean guanethidine concentration found in six experiments were 101 $\pm$ 4 pM/heart and 165 $\pm$ 18 pM/heart after 30 min and 5 hr respectively. The noradrenaline concentrations were determined in the corresponding subcellular fractions of hearts from rats treated similarly but with unlabelled guanethidine.

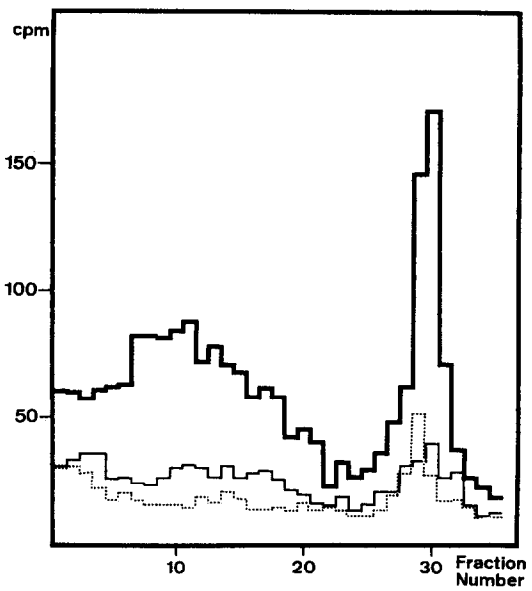

FIG. 3. Reserpine-induced inhibition of uptake of [^{3}H]noradrenaline and [^{3}H]guanethidine in noradrenaline storage granules of the rat heart. [^{3}H]noradrenaline (100 μc/rat) or [^{3}H]guanethidine (100 μc/rat) were injected intravenously in reserpine treated rats 30 min before removing the hearts. Control rats were similarly injected with [^{3}H]guanethidine. Preparation of granule fractions and density gradient centrifugation as described in Material and Methods with a sucrose density gradient of type B (0·5–1 M sucrose with a layer of 2 M sucrose in the bottom). Reserpinization (1 mg/kg, subcutaneously) was performed 17 hr before injection of the [^{3}H] substances.

——— [^{3}H]guanethidine injected to untreated rats.

——— [^{3}H]guanethidine injected to reserpinized rats.

- - - - - [^{3}H]noradrenaline injected to reserpinized rats.

Thirty min after drug injection the granule fraction had lost 53 ± 5 pM/heart of their endogenous noradrenaline which correspond to 20–25 per cent of the control values (220 ± 19 pM/heart). After 5 hr the mean noradrenaline depletion was 136 ± 9 pM/heart.

Thus, a measurable amount of guanethidine has been taken up into the amine storage granules at a time (30 min) when the loss of noradrenaline amounted to only about half the guanethidine taken up. Between 30 min and 5 hr the granules lost an amount of noradrenaline (83 pM) which exceeded the amount of guanethidine they took up during this time (64 pM).

Several metabolites of guanethidine have been described.[4,6,21,22] At least one of them was found in the heart of rats treated with guanethidine.[4,6] For that reason a chromatographic analysis of all fractions of rat heart homogenates was carried out. The hearts were removed 5 hr after [^{3}H]guanethidine treatment and prepared in a similar manner as for density gradient centrifugation. The acidic extracts of all fractions were submitted to paper chromatography as described in Materials and Methods.

The results of a typical experiment are shown in Fig. 5. All fractions contained a main peak corresponding to the R_f value of authentic guanethidine. The pellet used for

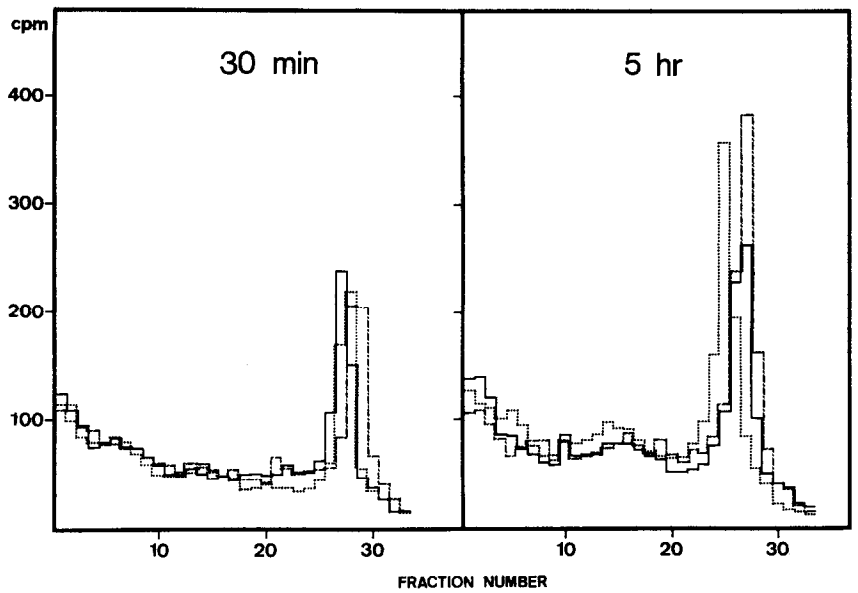

FIG. 4. Comparison of the contents of [^{3}H]guanethidine in the noradrenaline storage granules of the rat heart 30 min and 5 hr after injection of [^{3}H]guanethidine. Rats were injected with [^{3}H]guanethidine (100 μc/rat, intravenously) and the hearts were removed 30 min or 5 hr later, respectively. Experimental details as in Fig. 3. The curves represent the results of three different experiments.

density gradient centrifugation showed only a single peak which was indistinguishable from guanethidine, whereas a second peak was present on the chromatograms of the two coarse fractions. This second peak corresponded to the R_f of all three identified guanethidine metabolites[22] which could not be separated from another in the two solvent systems used. This indicates that the amine storage granules of the rat heart most probably store only unchanged drug. The result obtained after chromatography in the second solvent system used (isopropanol–conc. ammonia–water, 400:40:60) were essentially the same as those shown in Fig. 4.

4. *Antihypertensive effects*

It seems clear that guanethidine can produce its adrenergic neurone blocking action before depleting the stores of noradrenaline in sympathetic nerves.[23] A further series of experiments was undertaken to determine whether the postganglionic blockade caused by guanethidine could be correlated with its selective accumulation in noradrenaline granules. The antihypertensive effect of guanethidine was taken as a measure of the blockade of the postganglionic neurones.

Renal hypertensive rats were injected intravenously with unlabelled guanethidine at a dose (3·75 mg/kg) corresponding to that of [^{3}H]guanethidine injected in the normotensive rats. The arterial blood pressure was recorded 30 min and 5 hr later, i.e. at times when [^{3}H]guanethidine contents in the amine storage granules of the heart were measured. The results are shown in Table 2.

Guanethidine produced a decrease of arterial blood pressure which was already highly significant after 30 min and still more pronounced after 5 hr.

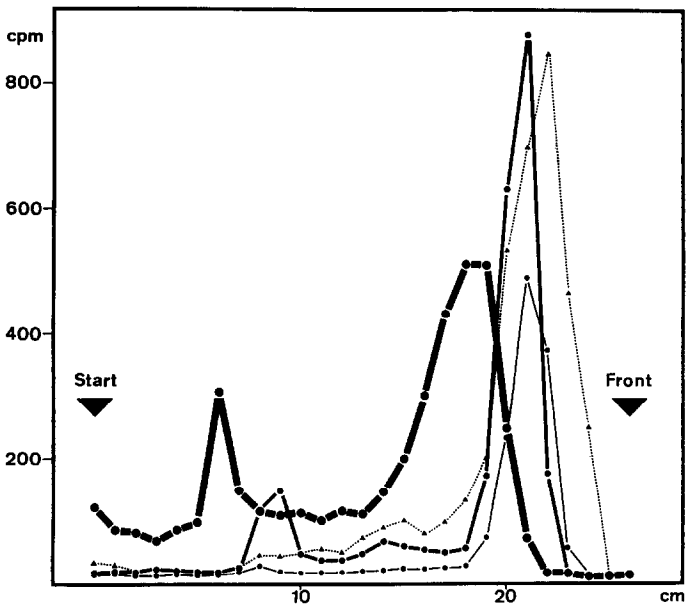

FIG. 5. Distribution of radioactivity on paper chromatograms of different subcellular fractions of the rat heart. [^{3}H]guanethidine (100 μc/rat) was injected intravenously 5 hr before removing the hearts. Fractions were prepared by differential centrifugation. The pellets were extracted with 2 N HCl and the extracts chromatographed in *n*-butanol–ethanol–ammonia (see Material and Methods).

750 *g* sediment.

105,000 *g* sediment (5 min).

105,000 *g* sediment (60 min).

[^{3}H]guanethidine used for treatment.

TABLE 2. EFFECT OF GUANETHIDINE (3·75 mg/kg, i.v.) ON THE ARTERIAL BLOOD PRESSURE OF THE RENAL HYPERTENSIVE RAT

Time after guanethidine	Decrease of arterial blood pressure (mm Hg)
30 min	−54 ± 4*
5 hr	−86 ± 6†

Seven renal hypertensive rats were used. The initial blood pressure measured 2 hr before the injection of guanethidine was 212 ± 4 mm Hg.

* $P < 0.001$ as compared with initial values.

† $0.001 < P < 0.01$ as compared with 30 min values.

DISCUSSION

The results described in this paper present direct evidence that guanethidine is taken up through a reserpine sensitive process into the same subcellular particles which store noradrenaline. The extent of the uptake of the two agents cannot be compared from these experiments since the absolute amount of [^{3}H]guanethidine injected was much higher than that of [^{3}H]noradrenaline. It seems justifiable, however, to conclude from these experiments that the uptake of guanethidine into storage granules *in vivo* is quite considerable and may be of importance for the mechanism of action of guanethidine. The finding that the amount of guanethidine in the granular fraction increases between 30 min and 5 hr contrasts with several observations reported earlier that the amount of guanethidine in the *whole heart* decreases during this time.[4,8] This means that the drug is bound much more firmly by the noradrenaline storage sites than by the other cardiac tissue components and is in agreement with the view that guanethidine is retained in sympathetically innervated tissues by two types of processes: an unspecific adsorption as well as a specific retention at sites concerned with the storage of noradrenaline.[5,8]

Most interesting studies in recent years[7–10] have led to the general view that guanethidine displaces noradrenaline by its incorporation into sympathetic nerve endings. However, the presence of measurable amounts of guanethidine in the noradrenaline storage particles of the rat heart 30 min after drug treatment is not in accord with a simple displacement process. Nor is the observation that the subsequent strong noradrenaline decrease is associated with a relatively small supplementary uptake of guanethidine. A displacement of noradrenaline by guanethidine at the granular level cannot be ruled out, however, but if so, it would consist of at least two distinct phases. In the first one guanethidine would be taken up into the granules without influencing noradrenaline stores. The second phase would represent a displacement process. Such an assumption implicates a kind of migration of guanethidine from uptake sites to release sites within the granules. In fact, as early as 1961, Cass and Spriggs[23] have shown that adrenergic blockade by guanethidine is induced more rapidly than is amine depletion, and even that a complete block of sympathetic nerve stimulation could be produced in isolated tissues without diminution of catecholamine levels in these tissues. This would suggest that the occupancy of the uptake sites by the drug represents the primary mechanism of the blocking action, whereas noradrenaline depletion would have only a minor rôle, if any. In this context, it is of interest to compare the levels of [^{3}H]guanethidine in the rat heart after 30 min and 5 hr with the antihypertensive effects induced by the same treatment after these times. The magnitude of the amounts of [^{3}H]guanethidine found remarkably parallels that of the blood pressure decreases. Of course, a larger time course of both parameters is needed to admit the idea of a close relationship between the concentration of the blocking drug in the noradrenaline storage particles and the block itself.

The reason why no guanethidine was taken up into isolated bovine splenic nerve granules is not clear and might indicate that not guanethidine itself but a metabolite enters the granule and would be responsible therefore for the biological activity of the drug. Three metabolites of guanethidine have yet been identified.[22] They did not inhibit the uptake of [^{3}H]noradrenaline into isolated bovine splenic nerve granules at a concentration of 10^{-4} M and caused at best a very small inhibition at 10^{-3} M.[24] On the other hand only guanethidine-*N*-oxide among them lowered arterial blood pressure

and cardiac noradrenaline stores in renal hypertensive rats, but it was much less active than guanethidine itself.[24]

Finally the results obtained from chromatography of all subcellular fractions of the heart of rats treated with guanethidine accord well with the view that most probably only guanethidine itself and not its metabolites enters the specific sites of noradrenaline storage, thus producing depletion of the endogenous transmitter.

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