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The role of Uptake$_2$ in the extraneuronal metabolism of catecholamines in the isolated rat heart

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1. (±)-^{3}H-NA and labelled metabolites of NA were estimated in rat hearts after perfusion with various concentrations of NA in the range 0·01–50·0 μg/ml. Labelled metabolites of NA accounted for only a small proportion of the total uptake of radioactivity at low perfusion concentrations, but accounted for 50% of the total uptake at 1 μg NA/ml., thereafter declining to progressively smaller proportions at higher perfusion concentrations.

2. If the formation of labelled metabolites of ^{3}H-NA was blocked by a combination of monoamine oxidase and catechol-O-methyl transferase inhibitors, the accumulation of unchanged ^{3}H-NA was doubled when hearts were perfused with 1 μg NA/ml.

3. In hearts perfused with 0·5 μg NA/ml., an accumulation of unchanged ^{3}H-NA was demonstrated in the presence of a combination of metabolic inhibitors and metaraminol. This appeared to be due to Uptake$_2$, since the accumulation of NA under these conditions could be prevented by a low concentration of normetanephrine.

4. Phenoxybenzamine prevented extraneuronal uptake (Uptake$_2$) and metabolism of ^{3}H-NA with an estimated ID50 of 2·5 μM. The inhibition of Uptake$_2$ by phenoxybenzamine (2·0 μM) was diminished at very high NA concentrations, suggesting that the drug may act competitively with NA.

5. It was concluded that Uptake$_2$ operates at all catecholamine concentrations in the rat heart, but that in the lower range (less than 2·5 μg/ml. for NA and less than 0·75 μg/ml. for adrenaline) any catecholamine taken up by this process is rapidly metabolized. Thus the accumulation of unchanged amine is seen only at high perfusion concentrations.

6. The relevance of these results to an understanding of the possible physiological and pharmacological importance of Uptake$_2$ is discussed.

Previous studies of the uptake of catecholamines by the isolated perfused rat heart have demonstrated the existence of two mechanisms, Uptake$_1$ and Uptake$_2$ (Iversen, 1963, 1965). Uptake$_1$ represents the removal of exogenous catecholamine into the sympathetic innervation of the tissue, and is a high affinity system which becomes saturated at catecholamine concentrations above 0·5 μg/ml. Uptake$_2$ has

only been observed at high catecholamine concentrations (0·5 μg/ml. for adrenaline and 2·5 μg/ml. for noradrenaline, [NA]). Uptake$_2$ is mediated by a mechanism which has a different pattern of specificity and drug sensitivity from that of Uptake$_1$ (Iversen, 1965). Uptake$_2$ only becomes saturated at very high external catecholamine concentrations (20 μg/ml. for adrenaline and 100 μg/ml. for NA). Recent histochemical studies of the rat heart after perfusion with high concentrations of NA indicate that Uptake$_2$ represents an accumulation of unchanged catecholamine in cardiac muscle cells (Ehinger & Sporrong, 1968; Farnebo & Malmfors, 1969). Similar studies have demonstrated an uptake of exogenous catecholamine into smooth muscle cells of both the spleen and blood vessels after exposure to NA at 10 μg/ml. (Gillespie, Hamilton & Hosie, 1967; Avakian & Gillespie, 1968; Gillespie, 1968). In all cases the extraneuronal accumulation of catecholamines was not observed when tissues were exposed to catecholamine concentrations below 5 μg/ml.

One possible explanation of these findings is that the extraneuronal uptake of catecholamines (Uptake$_2$) is a threshold phenomenon which becomes activated at high catecholamine concentrations. Alternatively, a metabolic barrier might exist which prevents the extraneuronal accumulation of catecholamines under conditions in which the catecholamine metabolizing enzymes are not fully saturated. Thus, at low catecholamine concentrations, all the amine taken up into the extraneuronal sites would be metabolized. The latter hypothesis is supported by the findings of Eisenfeld, Axelrod & Krakoff (1966) and Eisenfeld, Landsberg & Axelrod (1967), who found that drugs which are known to inhibit Uptake$_2$ blocked the extraneuronal formation of ^{3}H-NA metabolites in rat hearts perfused with NA 5 ng/ml.

The present study provides evidence in favour of the view that Uptake$_2$ operates at all catecholamine concentrations in the rat heart, and that this process is intimately related to the subsequent extraneuronal metabolism of catecholamines.

Methods

Heart perfusion

Hearts from adult male hooded and albino Wistar rats were perfused by the Langendorff technique, as previously described (Iversen, 1963). The perfusion medium contained (±)-^{3}H-NA (Radiochemical Centre, Amersham, 4·7 c/m-mole) 50–100 nc/ml., diluted with various concentrations of non-radioactive (±)-NA. At the end of a 5 or 10 min perfusion, the hearts were removed and analysed. The results were corrected for the presence of ^{3}H-NA in the extracellular space of the tissue, assuming this to be 33% of the wet weight (Iversen, 1963).

Isolation of ^{3}H-NA and metabolites

^{3}H-NA was isolated from perchloric acid extracts by ion-exchange chromatography (Iversen, 1963) and the results were corrected for an average recovery of 68% for this procedure. 5·0 × 0·6 cm columns of Zeo-Karb 225,X8, >200 mesh, Na^+ form resin, was used instead of Amberlite CG-120 resin. An aliquot of each tissue extract was used to estimate the total radioactivity, and radioactive metabolites of ^{3}H-NA were determined as the difference between the total radioactivity and the corrected value for ^{3}H-NA, isolated by the ion-exchange procedure.

Inhibition of monoamine oxidase and catechol-O-methyl transferase

In order to obtain complete inhibition of monoamine oxidase (MAO), rats were pretreated with the compound Su-11,739 (Maître, 1968), 50 mg/kg given subcutaneously 12 hr before the perfusion experiment. No MAO activity could be detected in heart homogenates obtained from treated animals, by the radiochemical assay technique of McCaman, McCaman, Hunt & Smith (1965).

Catechol-O-methyl transferase (COMT) activity was inhibited by the addition of β-thujaplicin (4-isopropyl tropolone, Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire) to the perfusion medium at a concentration of 0·2 mM. No enzyme activity could be detected in heart homogenates in the presence of this concentration of β-thujaplicin, using the radiochemical assay procedure of McCaman (1965). β-thujaplicin was dissolved in a small volume of warm propylene glycol before dilution in saline solutions.

Results

Accumulation of radioactive metabolites in the heart during perfusion with various concentrations of (±)-^{3}H-NA

In the initial studies of Uptake$_2$, no measurements were made of the accumulation of catecholamine metabolites in the hearts perfused with high catecholamine concentrations. Such measurements have now been made in hearts perfused for 10 min with concentrations of ^{3}H-NA from 0·01 μg/ml. to 50·0 μg/ml. (Fig. 1). The results indicate the percentage of the total uptake of radioactivity which was accounted for by radioactive metabolites of ^{3}H-NA. At very low perfusion concentrations of NA (0·01 μg/ml.) radioactive metabolites accounted for less than 5% of the total radioactivity, which was almost entirely present as unchanged ^{3}H-NA. As the perfusion concentration was increased, metabolites accounted for an increasing proportion, reaching a maximum at a perfusion concentration of 1·0 μg/

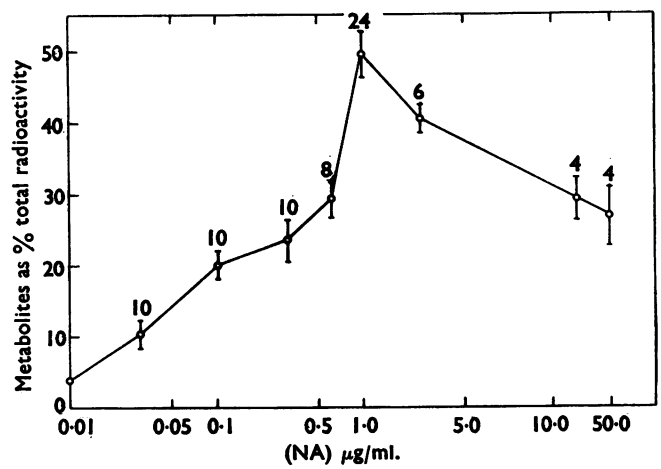

FIG. 1. Radioactive metabolites as a proportion of the total of metabolites + ^{3}H-NA in the rat heart after 10 min perfusions with various concentrations of NA. Number of observations at each concentration is indicated by the figure over each point. Results are mean values ± S.E. of mean.

ml., at which labelled metabolites accounted for about 50% of the total radioactivity. At higher concentrations there was a progressive decline in the proportion of the total uptake accounted for as labelled metabolites, suggesting that the catecholamine metabolizing systems become saturated at these concentrations. The formation of labelled metabolites of ^{3}H-NA was most important in the range 0·6–2·5 μg/ml. This is the range of concentrations in which previous results indicate that Uptake$_1$ is saturated, and Uptake$_2$ is not detectable (Iversen, 1965). Furthermore, the present results show that appreciable quantities of labelled metabolites are formed even at low perfusion concentrations of ^{3}H-NA.

Effects of inhibitors of catecholamine metabolism on the accumulation of unchanged NA

If the extraneuronal metabolism of catecholamines occurs after the uptake of exogenous amines by Uptake$_2$, then inhibition of the degradative enzymes would be expected to increase the accumulation of unchanged catecholamine in the tissue. This prediction was confirmed in experiments in which hearts were perfused with 1 μg ^{3}H-NA/ml. for 10 min in the presence of an inhibitor of MAO or COMT or a combination of both inhibitors (Fig. 2). When both enzymes were inhibited, the accumulation of ^{3}H-NA was doubled, this increase being stoichiometrically related to the amount of labelled metabolites present in the control hearts. The total accumulation of unchanged NA after enzyme inhibition was 2·72 μg/g ; this value

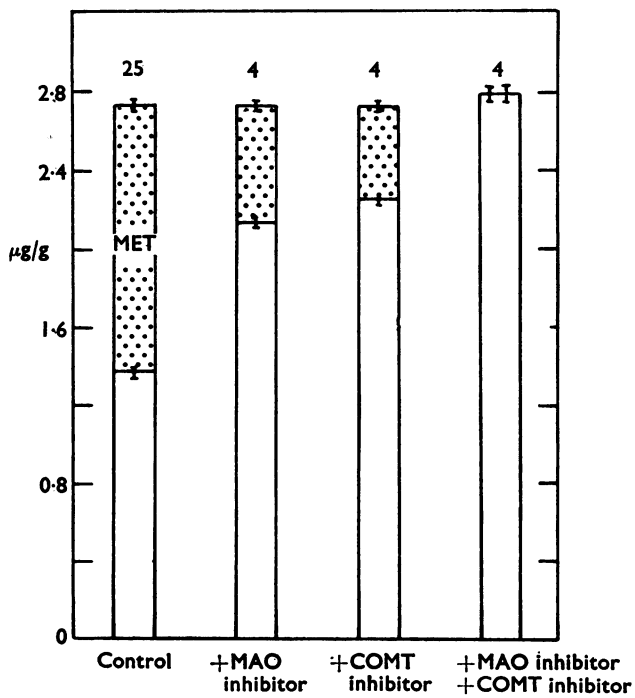

FIG. 2. ^{3}H-NA and labelled metabolites in the rat heart at the end of 10 min perfusions with NA 1 μg/ml., in the presence and absence of inhibitors of NA metabolism. Number of observations indicated by figure over each histogram ; results are mean values ± S.E. of mean. The shaded portion of the histograms represents the proportion of labelled metabolites.

is greater than could be accounted for by $Uptake_1$, which has an initial rate of NA uptake of only 0·2 μg/g per min at a NA concentration of 1·0 μg/ml. Most of the accumulation of labelled metabolites in hearts perfused with 1 μg NA/ml. probably derives from NA taken up into extraneuronal sites by $Uptake_2$. This continues to accumulate, in the form of unchanged NA, when both degradative enzymes are inhibited.

Similar experiments were performed with hearts perfused with ^{3}H-NA 30·0 μg/ml. for 10 min. In the presence of a combination of MAO and COMT inhibitors labelled metabolites were again found to be stoichiometrically replaced by an increase in the accumulation of unchanged ^{3}H-NA. In these experiments, as in those illustrated in Fig. 2, inhibition of either enzyme alone produced a significant effect, which was smaller than that produced by the inhibition of both enzymes, indicating that the metabolism of ^{3}H-NA involves both MAO and COMT.

Demonstration of $Uptake_2$ in hearts perfused with a low concentration of NA

The accumulation of appreciable amounts of labelled metabolites in hearts perfused with NA concentrations above 0·1 μg/ml. (Fig. 1) suggested that $Uptake_2$ might operate even at low concentrations of NA. If this were so, it should be possible to demonstrate an accumulation of unchanged NA in hearts perfused with a low concentration of NA, provided that the metabolism of the accumulated amine were prevented. This was investigated in a series of experiments in which hearts perfused with 0·5 μg ^{3}H-NA/ml. for 10 min were used. The neuronal uptake of NA was suppressed by the addition of 0·5 mM (−)-metaraminol, which is a potent inhibitor of $Uptake_1$, while having almost no inhibitory effect on $Uptake_2$ (Iversen, 1965). As expected, metaraminol reduced the uptake of unchanged NA, but had no significant effect on the accumulation of labelled metabolites (Fig. 3). When a medium containing metaraminol was perfused through hearts in which both MAO and COMT were inhibited, labelled metabolites were no longer detectable and there was a stoichiometric increase in the accumulation of unchanged NA (Fig. 3) as in the previous experiment (Fig. 2). This increase could be abolished by the $Uptake_2$ inhibitor normetanephrine at a concentration of 5·0 μM. At this low concentration normetanephrine has little inhibitory effect on $Uptake_1$ but produces a marked inhibition of $Uptake_2$ (Burgen & Iversen, 1965).

Effects of phenoxybenzamine on $Uptake_2$ and on the extraneuronal metabolism of NA

In a previous study it was shown that phenoxybenzamine is a potent competitive inhibitor of $Uptake_1$ in the rat heart, with a *Ki* of 0·75 μM. It was, furthermore, shown that the presence of 3·3 μM phenoxybenzamine in the perfusate almost completely blocked the formation of labelled metabolites of NA in hearts exposed to NA concentrations from 0·01–0·30 μg/ml. (Iversen & Langer, 1969). In the present study further investigations were made of the action of phenoxybenzamine on the uptake and extraneuronal metabolism of ^{3}H-NA.

Hearts were perfused with 0·5 μg ^{3}H-NA/ml. in the presence of 0·5 mM (−)-metaraminol, in order to inhibit the neuronal uptake of NA, as described above. When phenoxybenzamine was added to the perfusion medium at a concentration of

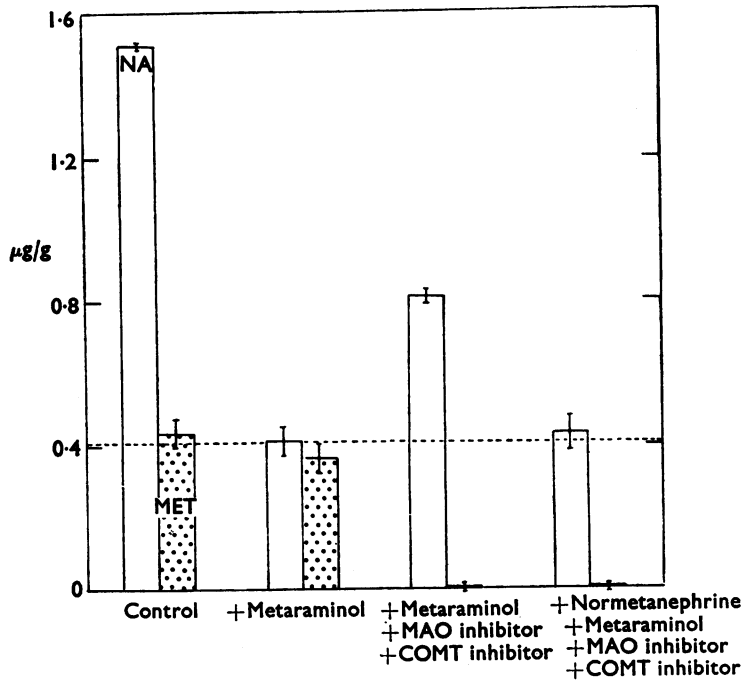

FIG. 3. ^{3}H-NA and labelled metabolites (shaded histograms) in the rat heart at the end of 10 min perfusions with NA 0·5 μg/ml., in the presence of metaraminol (0·5 mM), inhibitors of metabolism, and normetanephrine (5 μM). Each value is the mean of four to six observations, ±S.E. of mean. Dotted line indicates ^{3}H-NA content of metaraminol treated group.

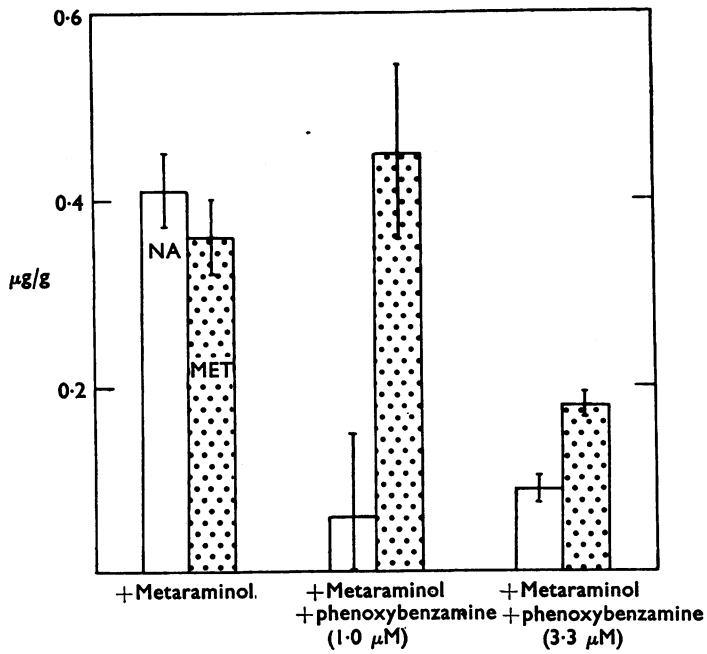

FIG. 4. Effect of phenoxybenzamine 1·0 μM and 3·3 μM on the metaraminol-resistant accumulation of ^{3}H-NA and labelled metabolites (shaded histograms) in the rat heart perfused for 10 min with NA 0·5 μg/ml. Each value is the mean of two to four observations, vertical lines indicate S.E. of mean or range.

1·0 μM, the accumulation of unchanged ^{3}H-NA was markedly reduced, but the level of radioactive metabolites was unchanged. This may be interpreted as an inhibition of residual Uptake$_1$ activity by low concentrations of phenoxybenzamine (Fig. 4). Higher concentrations of phenoxybenzamine (2·0–10·0 μM) led to a marked reduction in the accumulation of both unchanged NA and its labelled metabolites (Fig. 4). The concentration of phenoxybenzamine required to prevent the formation of 50% of the normal metabolites was between 2·0 and 3·0 μM. In further experiments, a concentration of 2·0 μM phenoxybenzamine was tested in hearts perfused for 5 min with concentrations of NA in the range 0·5–50·0 μg/ml. These experiments were also performed in the presence of 0·5 mM (−)-metaraminol to inhibit Uptake$_1$ activity. At a perfusion concentration of 1·0 μg NA/ml. phenoxybenzamine produced a 66% inhibition of the accumulation of unchanged NA. As the perfusion concentration of NA was increased, however, this effect was progressively diminished, so that at a perfusion concentration of 50·0 μg NA/ml. NA accumulation was about 40% reduced (Fig. 5). The inhibition of the accumulation of labelled metabolites was similar in all cases to that seen for unchanged NA. These results suggest that phenoxybenzamine produces a competitive inhibition of Uptake$_2$, and that the ID50 of phenoxybenzamine as an Uptake$_2$ inhibitor is approximately 2·5 μM.

In preliminary experiments, the effects of phenoxybenzamine (2 μM) on the accumulation of labelled metabolites have been found to be approximately doubled if hearts were perfused with the drug for 20 min before the addition of ^{3}H-NA. This suggests that the inhibitory effect of phenoxybenzamine is not immediate, but this is not necessarily inconsistent with a competitive action of the drug on Uptake$_2$ sites. In hearts from animals which had received phenoxybenzamine *in vivo* (9 mg/kg given intraperitoneally) 90–180 min before the experiment no consistent effects on the uptake or metabolism of ^{3}H-NA were found.

Discussion

The results obtained in the present study are consistent with the view that Uptake$_2$ in the rat heart is not a threshold phenomenon, but operates at all perfu-

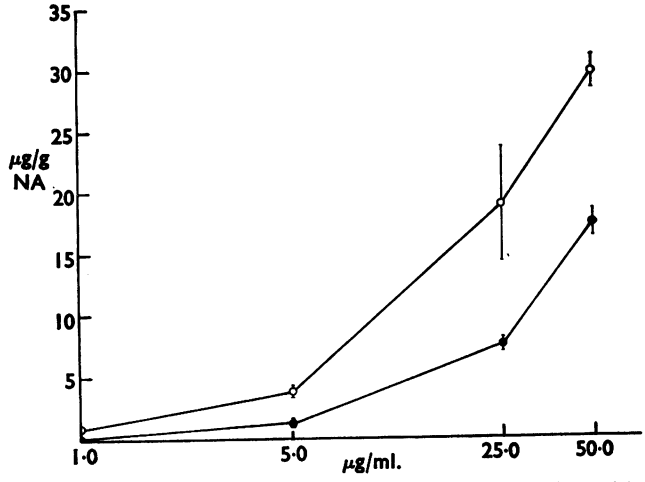

FIG. 5. Accumulation of ^{3}H-NA in the rat heart after a 5 min perfusion with various concentrations of NA in control hearts (○) and in the presence of phenoxybenzamine 2·0 μM (●). Each value is the mean of three observations, ± S.E. of mean.

sion concentrations of catecholamine. At concentrations of less than 2·5 μg NA/ml. or less than 0·75 μg adrenaline/ml., however, almost all of the catecholamine taken up by Uptake$_2$ is quickly metabolized. The accumulation of unchanged catecholamine by Uptake$_2$ would be expected to occur only when the rate of Uptake$_2$ exceeds the maximum rate of metabolism. Thus, if the metabolism of NA is blocked,

TABLE 1. *Kinetic constants for Uptake$_1$ and Uptake$_2$ in the rat heart*

Substrate	Uptake$_1$		Uptake$_2$	
	Km (μM)	V_{max} (μg/g per min)	*Km* (μM)	V_{max} (μg/g per min)
(−)-NA	0·27	0·20	252·0	17·0
(−)-Adrenaline	1·00	0·20	51·6	11·8

(From Iversen, 1965.)

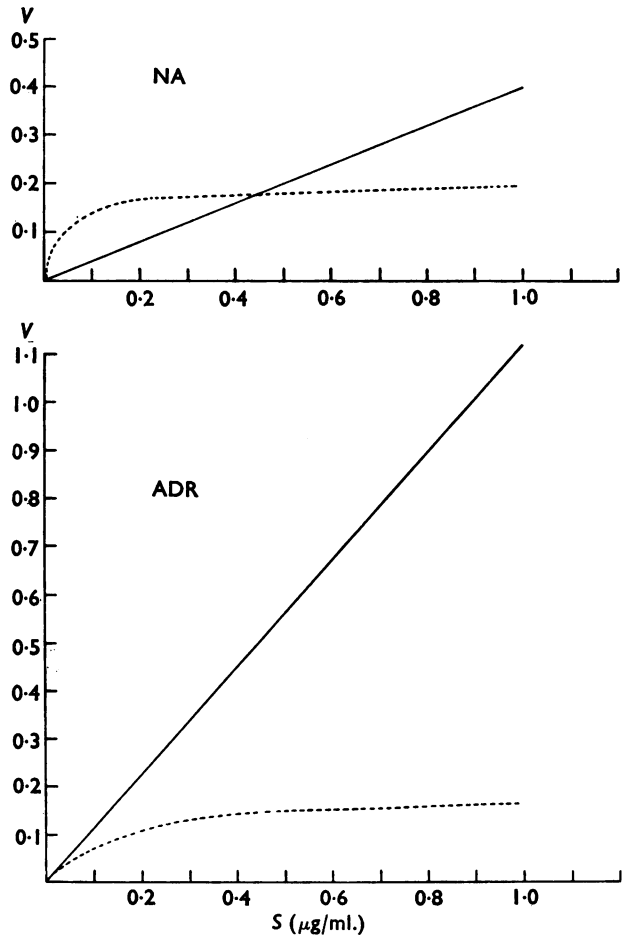

FIG. 6. Predicted values for the initial rates of uptake of (−)-NA and (−)-adrenaline (ADR) by Uptake$_1$ (dotted lines) and Uptake$_2$ (solid lines) in the rat heart at various catecholamine concentrations (*S*). These values were calculated from the Michaelis-Menten equation (rate of uptake$=V_{max}S/Km+S$), using the kinetic parameters given in Table 1.

Uptake$_2$ can be demonstrated even during perfusion with low concentrations of NA (0·5–1·0 μg/ml.). Uptake$_2$ is defined as an uptake of unchanged catecholamine which is sensitive to inhibition by a low concentration of normetanephrine but insensitive to inhibition by a high concentration of (−)-metaraminol (Iversen, 1965). It should be emphasized that the amount of ^{3}H-NA or labelled metabolite retained in the heart at the end of a 10 min perfusion probably reflects only a small proportion of the total amount taken up by Uptake$_2$, since neither NA nor metabolites are firmly retained in extraneuronal sites (Iversen, 1965).

It is possible to calculate the initial rates of uptake of NA and adrenaline by Uptake$_2$ predicted by the kinetic parameters previously determined for these substrates (Iversen, 1965) (Table 1). The results of these calculations are shown in Fig. 6. It can be seen that for NA Uptake$_2$ reaches a rate equal to that of Uptake$_1$ at an amine concentration of 0·5 μg/ml. and that the rates of Uptake$_2$ and Uptake$_1$ for adrenaline are similar even at very low catecholamine concentrations (0·01 μg/ml.). At a concentration of only 0·05 μg/ml. adrenaline is taken up by Uptake$_2$ at a predicted rate which is twice that of Uptake$_1$ (Fig. 6).

The threshold concentration at which Uptake$_2$ becomes apparent as an accumulation of unchanged catecholamine should correspond to the concentration at which Uptake$_2$ exceeds the maximum rate of metabolism of catecholamine. Previous studies have indicated that the threshold concentrations for Uptake$_2$ in the rat heart are 0·75 μg/ml. for adrenaline, and 2·5 μg/ml. for NA (Iversen, 1965). The calculated rates of Uptake$_2$ for these concentrations are 0·86 μg/g per min for adrenaline and 0·94 μg/g per min for NA. Because a higher concentration of NA is required to attain the critical rate of Uptake$_2$ at which metabolism becomes saturated, this would explain why the Uptake$_2$ threshold for NA is higher than that for adrenaline. Studies of the maximum rate of metabolism of catecholamines in rat heart homogenates indicated that the maximum rate of degradation of (−)-NA by MAO plus COMT is of the order of 1·7 μg/g per min (Crout, Creveling & Udenfriend, 1961). This estimate, which was made under optimal conditions for catecholamine metabolism, is not greatly in excess of the values predicted by the calculations outlined above.

The hypothesis proposed here is consistent with the results presented by Eisenfeld *et al.* (1966, 1967). These authors found that small amounts of ^{3}H-NA metabolites were formed in rat hearts perfused with a very low concentration of ^{3}H-NA (0·005 μg/ml.). These metabolites appeared to be extraneuronal, since their formation was unaffected by high concentrations of cocaine or by immunosympathectomy. The extraneuronal metabolism of ^{3}H-NA was blocked by various drugs, such as phenoxybenzamine, metanephrine, synephrine and methoxamine, which are known to be Uptake$_2$ inhibitors. The extraneuronal metabolism of ^{3}H-NA was not blocked by drugs which inhibited only Uptake$_1$, such as metaraminol.

Uptake$_2$ in the rat heart has been shown by histochemical studies to involve a massive accumulation of unchanged catecholamine in cardiac muscle cells (Ehinger & Sporrong, 1968; Farnebo & Malmfors, 1969). Other histochemical studies have shown that a similar normetanephrine-sensitive uptake of catecholamines occurs into various types of smooth muscle (Gillespie, 1968). Gillespie reported, however, that the uptake of NA into smooth muscle was not consistently observed in all species. Thus no fluorescence was seen in guinea-pig arterial smooth muscle after exposure to a high concentration of NA, whereas rabbit arterial and other smooth

muscle showed a marked accumulation of fluorescence. Our results suggest a possible explanation of such variations. Although Uptake$_2$ may be present in many effector tissues, it would only be expected to lead to a massive accumulation of catecholamine if the rate of uptake exceeded the maximum rate of metabolism. It is known that there are large differences in the activity of the enzymes MAO and COMT among different tissues and species (Jarrott, in preparation). In tissues in which the enzymes are highly active, Uptake$_2$ may not be demonstrable unless metabolism is inhibited.

The present studies also provide additional information about the actions of phenoxybenzamine on catecholamine uptake and metabolism. As shown in a previous paper, this drug is a potent inhibitor of the neuronal uptake of NA in the rat heart, with a *Ki* of 0·75 μM, similar to that of cocaine (Iversen & Langer, 1969). In the present study, it was confirmed that phenoxybenzamine also effectively blocks the extraneuronal uptake and metabolism of NA. Our results indicate that this effect develops only slowly on exposure to the drug, but it is not yet possible to determine whether the effect is irreversible. Our findings suggest that the inhibition of Uptake$_2$ produced by phenoxybenzamine may be competitive, since the effect was reduced at high concentrations of NA. The estimated ID50 of phenoxybenzamine as an Uptake$_2$ inhibitor was 2·5 μM, which suggests that this drug is the most potent inhibitor of Uptake$_2$ so far described (ID50 for (±)-metanephrine 2·9 μM, Burgen & Iversen (1965)). This finding is consistent with the suggestion of Langer (1968) that phenoxybenzamine blocks both the neuronal and the extraneuronal uptake of NA. Langer has, furthermore, provided evidence that the extraneuronal uptake and metabolism of NA accounts for a substantial proportion of the NA released at adrenergic synapses in the cat nictitating membrane. In general, however, it is not yet clear what role Uptake$_2$ and extraneuronal metabolism play in adrenergic transmission. The present results suggest that Uptake$_2$ operates at all catecholamine concentrations, although for (−)-NA the rate of Uptake$_2$ in the rat heart is less than that of Uptake$_1$, for NA concentrations of less than 0·50 μg/ml. Since the exact concentration of NA in the synaptic cleft is not known, it is impossible to estimate the relative importance of the two uptake mechanisms in the inactivation of released NA. However, in many tissues, in which the sympathetic innervation is only sparsely distributed in a large bulk of smooth muscle (for example, vascular smooth muscle), Uptake$_2$ may be the major mechanism for catecholamine inactivation. This would be consistent with the finding that Uptake$_1$ inhibitors do not markedly potentiate the effects of NA in such preparations (Maxwell, Wastila & Eckhardt, 1966), whereas the responses to NA and other agonists are potentiated by inhibitors of COMT and MAO (Kalsner & Nickerson, 1969).

In tissues with a dense sympathetic innervation, such as the cat nictitating membrane, there is good evidence that Uptake$_1$ plays an important role in the inactivation of various adrenoceptor agonists (Langer & Trendelenburg, 1969). However, certain agonists, such as adrenaline, which have a relatively low affinity for Uptake$_1$ and a high affinity for Uptake$_2$, may be inactivated mainly by the latter process. Thus, whereas the effects of (−)-NA are potentiated by about 40-fold in the nictitating membrane with cocaine, responses to (−)-adrenaline are potentiated by only 4-fold (Trendelenburg, Muskus, Fleming & Gomez, 1962). In the case of isoprenaline, which is an excellent substrate for Uptake$_2$ but is not taken up by Uptake$_1$

(Callingham & Burgen, 1966), Uptake$_2$ may constitute the only inactivation process. In this context, it is interesting to note that Foster (1967) found that the responses of bronchial smooth muscle to isoprenaline were potentiated by metanephrine and phenoxybenzamine, which are known to inhibit Uptake$_2$.

In conclusion, the present results suggest that Uptake$_2$ is a process which operates at all concentrations of catecholamine to transport the amine into non-neuronal tissues in which it is subsequently metabolized. This process may prove to have physiological importance in terminating the actions of NA after its release from adrenergic nerve terminals, and may have pharmacological significance in limiting the actions of certain other adrenoceptor agonists, such as adrenaline and isoprenaline. Uptake$_2$ may also play some role in the extremely rapid removal of circulating catecholamines which has been demonstrated in many perfused organs (Vane, 1969).

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