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THE UPTAKE OF NORADRENALINE BY THE ISOLATED PERFUSED RAT HEART

BY

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The uptake of noradrenaline by the isolated perfused rat heart was studied after perfusion with a medium containing various concentrations of (±)-[^{3}H]-noradrenaline. Simultaneous measurement of the uptake of [^{3}H]-noradrenaline and of the net increase in the noradrenaline content of the heart showed that [^{3}H]-noradrenaline entering the heart both increased the tissue content and exchanged with endogenous noradrenaline. A large part (about 75%) of the endogenous noradrenaline pool, however, exchanged very slowly if at all with exogenous noradrenaline. The initial rates of noradrenaline uptake satisfied Michaelis-Menten kinetics with a Km for (±)-noradrenaline of 6.64×10^{-7} M. Further analysis of the uptake process indicated that noradrenaline entered into at least two intracellular pools at different rates. Measurement of the initial rates of noradrenaline uptake during perfusion with various concentrations of nonradioactive (+)- and (−)-noradrenaline showed that the uptake process exhibited stereochemical specificity. The Km values for (+)- and (−)-noradrenaline were 13.9×10^{-7} and 2.66×10^{-7} M respectively. Cocaine acted as a potent competitive inhibitor of noradrenaline uptake. This finding suggested that diffusion did not play any significant role in the entry of noradrenaline into the tissue.

The ability of tissues innervated by the sympathetic nervous system to accumulate and store [^{3}H]-noradenaline was demonstrated by *in vivo* studies on the fate of [^{3}H]-noradrenaline after intravenous infusion into the cat (Whitby, Axelrod & Weil-Malherbe, 1961). From a series of experiments on the uptake of [^{3}H]-noradrenaline by heart and brain slices and by pineal glands incubated *in vitro* it has been suggested that noradrenaline uptake obeys saturation kinetics of the Michaelis-Menten type (Dengler, Spiegel & Titus, 1961a ; Dengler, Wilson, Spiegel & Titus, 1962a ; Dengler, Michaelson, Spiegel & Titus, 1962b), and on the basis of this and other evidence these workers concluded that the uptake involved active membrane transport. In support of the hypothesis that noradrenaline uptake obeys saturation kinetics, previous studies from this laboratory have shown that the uptake of circulating [^{3}H]-noradrenaline by mouse tissues *in vivo* is a dose-dependent process which becomes saturated as the injected dose of noradrenaline is increased (Iversen & Whitby, 1962).

In all the experiments cited above the uptake of noradrenaline was measured by assaying only the uptake of [^{3}H]-noradrenaline ; in the absence of a simultaneous measurement of the total tissue content of noradrenaline it was impossible to determine whether this uptake of [^{3}H]-noradrenaline represented a net uptake of

noradrenaline or an exchange of [^{3}H]-noradrenaline with endogenous noradrenaline in the tissue stores. To distinguish between these two possibilities, and in an attempt to obtain more detailed information on the kinetics of the noradrenaline uptake, both total noradrenaline and [^{3}H]-noradrenaline uptake have been measured simultaneously in the present studies. The isolated perfused rat heart was chosen as a convenient *in vitro* preparation; it is able to accumulate [^{3}H]-noradrenaline from the perfusion medium (Axelrod, Gordon, Hertting, Kopin & Potter, 1962; Kopin, Hertting & Gordon, 1962), and a net uptake of exogenous noradrenaline in this preparation has also been demonstrated (Lindmar & Muscholl, 1962).

The present studies also included an investigation of the stereochemical specificity of noradrenaline uptake, and of the action of cocaine which has been reported to be a potent inhibitor of noradrenaline uptake (Whitby, Hertting & Axelrod, 1960; Dengler, Spiegel & Titus, 1961b; Muscholl, 1961).

METHODS

Drugs

(±)-β-[^{3}H]-Noradrenaline hydrochloride (9 mc/mg), obtained from the New England Nuclear Corporation, Boston, Mass., U.S.A., showed a single peak of radioactivity after paper chromatography using a *n*-butanol:acetic acid:water (4:1:1, v/v) mixture as developing solvent. This compound was diluted with a 1% solution of sodium metabisulphite in glass-distilled water to yield a stock solution containing 1 μg (free base)/ml. and 9 μc/ml. and was stored at −15° C. This stock solution was diluted with various amounts of nonradioactive (±)-noradrenaline and added to the perfusion medium to give a final concentration of 4.5 mμc[^{3}H]/ml. and from 10 to 1,000 ng/ml. of noradrenaline.

(±)-[^{3}H]-Normetanephrine (7.65 mc/mg) was used as a reference compound in ion-exchange chromatography; it was prepared enzymically from [^{3}H]-noradrenaline (Kopin, Axelrod & Gordon, 1961).

(±)-Noradrenaline bitartrate (Hopkins & Williams), (−)-noradrenaline bitartrate (L. Light & Co.) and (+)-noradrenaline bitartrate (Sterling-Winthrop, New York) were each prepared as stock solutions in 0.1 N-hydrochloric acid containing 1 mg (free base)/ml. These solutions were stored at 4° C.

Perfusions

The perfusion medium. This was Krebs-Henseleit solution in which the calcium content was reduced to 1.275 mM (Axelrod *et al.*, 1962). The medium was gassed with 5% carbon dioxide in oxygen and contained 1 g/l. of glucose and various concentrations of noradrenaline. The addition of ascorbic acid (20 mg/l.) and edetic acid (disodium salt 10 mg./l.) prevented the auto-oxidation of noradrenaline in the perfusate under these conditions. The amount of edetic acid added was insufficient to cause a significant decrease in the calcium or magnesium ion concentration of the perfusate, but enhanced the stabilizing effect of ascorbic acid.

Perfusion technique. Male and female albino Wistar rats (150 to 200 g) were injected intraperitoneally with sodium pentobarbitone (15 mg) and heparin (1,000 U). The hearts were removed 5 min later and perfused by the Langendorff technique in a gravity-feed apparatus at 37° C (Morgan, Henderson, Regen & Park, 1961). The perfusion rate was 8 to 10 ml./min at a pressure of 90 cm of water. The heart could be supplied by either of two gravity-feed systems connected to the perfusion cannula by a three-way tap. One system contained the noradrenaline perfusion medium and the other contained a noradrenaline-free medium. Hearts were perfused initially with the latter medium for 2 to 3 min to wash out blood and to allow rhythmic beating to be established. Perfusion was then continued with the noradrenaline medium for 1 to 60 min, and finally for a further 2 min with noradrenaline-

free medium to wash out extracellular noradrenaline. Previous studies with [^{14}C]-sorbitol in this preparation have shown that about 95% of the extracellular space is cleared by a wash-out perfusion of this duration (Morgan *et al.*, 1961).

Extraction and purification of noradrenaline and normetanephrine

Ion-exchange chromatography. In the experiments involving the use of (±)-[^{3}H]-noradrenaline it was essential to separate unchanged [^{3}H]-noradrenaline from any radioactive metabolites which might be present in the tissue extracts. The alumina adsorption method of Whitby *et al.* (1961) was found to give unsatisfactory results in the fluorimetric assay method, and could also be criticized on the grounds that it would not separate [^{3}H]-noradrenaline from other catechol metabolites, such as dihydroxymandelic acid, which might be expected to be present in the tissue extracts. The alternative ion-exchange isolation procedure of Bertler, Carlsson & Rosengren (1958), though satisfactory for the fluorimetric assay procedure, would not completely separate [^{3}H]-noradrenaline from other basic compounds such as normetanephrine. The following procedure, based on the methods described by Häggendal (1962a, b), was therefore used.

At the end of the perfusion the heart was blotted, weighed and then ground with 2 ml. of edetic acid (disodium salt, 1% w/v) and acid-washed sand in a mortar. The ground tissue was extracted with 8 ml. of 0.4 N-perchloric acid and, after standing for at least 30 min at 4° C, precipitated material was removed by centrifugation. The pellet was resuspended in a further 2 ml. of 0.4 N-perchloric acid and centrifuged again; the supernatant fluids were mixed and adjusted to *p*H 6.0–6.5 with 4 N-potassium hydroxide solution and the precipitated potassium perchlorate was removed by centrifugation. The neutralized extract was then passed through a 50 mm×6 mm column of a strong acid ion-exchange resin. The resin used was Amberlite CG-120 in the Na+ form, previously treated according to Häggendal (1962b). The column was washed with 25 ml. of glass-distilled water to remove acidic and neutral substances, and elution was performed by gravity with 7.5 ml. of N-hydrochloric acid (rejected) followed by a further portion of 15 ml. of N-hydrochloric acid (Fraction I, collected and used for noradrenaline assays); if required, elution was continued with 10 ml. of 2 N-hydrochloric acid (Fraction II, collected and used for normetanephrine assay). In a series of forty recovery experiments, [^{3}H]-noradrenaline added to heart minces and carried through the purification procedure was recovered in a yield of 84.3±4.8% (mean and standard deviation) in Fraction I and in an average yield of 4% in Fraction II. The data were corrected for an assumed recovery of 85% in Fraction I. In similar experiments [^{3}H]-normetanephrine added to heart minces and carried through the purification procedure was recovered almost entirely in Fraction II, and less than 2% of the added radioactivity appeared in Fraction I. A recovery correction was not applied to the normetanephrine results, but a correction was made for the presence of 4% of the total noradrenaline in Fraction II.

The specificity of this purification procedure was examined by paper chromatography of eluate Fractions I and II. An extract prepared from two hearts perfused for 20 min with (±)-[^{3}H]-noradrenaline (1 μg/ml., concentration of radioactivity raised to 45 mμc/ml.) was carried through the purification procedure. Eluate Fractions I and II were concentrated to small volumes in a rotary evaporator and applied to Whatman No. 1 filter paper. The chromatograms were developed with an *n*-butanol:acetic acid:water (4:1:1 v/v) mixture as a descending system, and the distribution of radioactivity on the papers was measured in a liquid scintillation spectrometer by the method of Wang & Jones (1959). Fig. 1*a* and *b* shows that Fraction I yielded a single peak of radioactivity corresponding in R_F to authentic noradrenaline, and Fraction II yielded a smaller peak corresponding in R_F to authentic normetanephrine together with a minor peak of noradrenaline.

Simplified ion-exchange procedure. In the experiments involving the use of nonradioactive (+)- and (−)-noradrenaline it was unnecessary to effect a complete separation of noradrenaline and normetanephrine since the latter compound did not interfere with the fluorimetric assay which was the only procedure used in these experiments. It was therefore possible to use a simplified ion-exchange purification procedure based on the method of

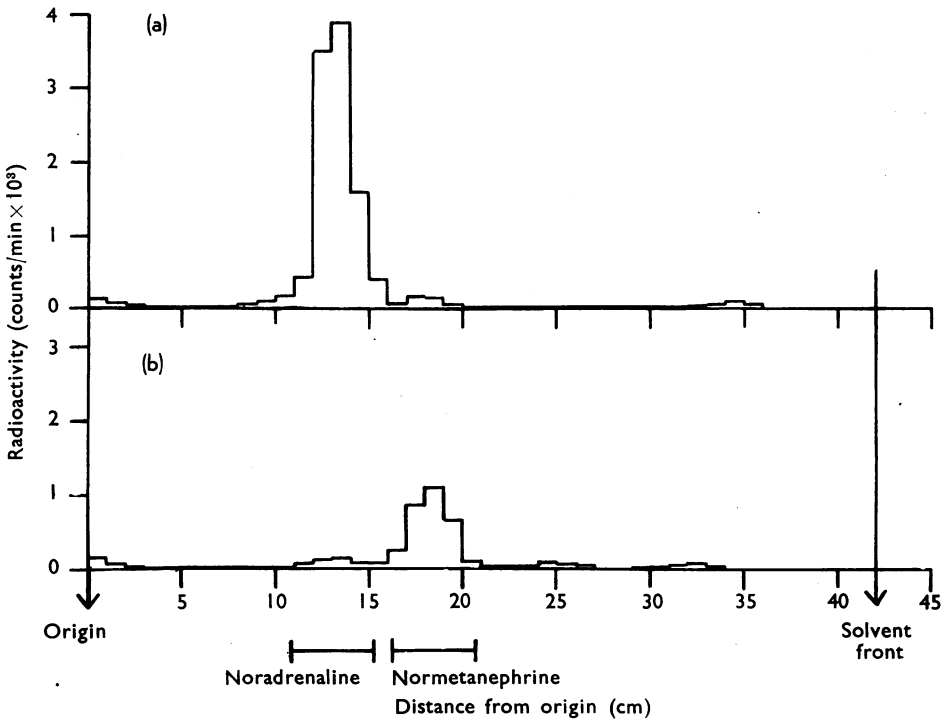

Fig. 1. Paper chromatography of radioactive substances isolated from a heart extract by ion-exchange chromatography. (*a*) Shows the distribution of radioactivity on a chromatogram of material present in Fraction I of the eluate and (*b*) shows a chromatogram of material present in Fraction II. The chromatograms were developed using an *n*-butanol : acetic acid : water (4 : 1 : 1 v/v) mixture as the solvent system. Nonradioactive noradrenaline and normetanephrine were used as marker materials (horizontal lines under (*b*)).

Bertler *et al.* (1958). This differed from the method described above only in that the length of the resin-bed was reduced from 50 to 20 mm, and in that elution was performed with a single portion of 10 ml. of N-hydrochloric acid. The recovery of [^{3}H]-noradrenaline added to heart minces and isolated by this method was practically the same as in the method described above, and the results were corrected for an assumed recovery of 85%.

Fluorimetric assay of noradrenaline

The total noradrenaline content of the purified eluates (Fraction I) was measured by a modification of the trihydroxyindole method (Euler & Lishajko, 1961) in an EIL Model 27A direct reading fluorimeter. The primary filter was Chance OX 1 (365 mμ) and the secondary was a combination of Chance OY 13 and Chance OY 6 (peak transmission above 480 mμ). Duplicate 2 ml. samples of each eluate were used for fluorimetric assay, and a third 2 ml. sample was used as a faded blank. Although adrenaline would also be measured by this method, the amount of endogenous adrenaline in the rat heart is so small that no correction was made for the presence of adrenaline in the heart extracts.

Assay of [^{3}H]-noradrenaline and [^{3}H]-normetanephrine

Duplicate 1.5 ml. samples of Fraction I (noradrenaline) or Fraction II (normetanephrine) were evaporated to dryness *in vacuo* in 15 ml. counting vials. The residue was taken up in

1 ml. of ethanol, and 10 ml. of phosphor (0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene) was added for counting. All samples were counted for two 10 min periods in a Packard TRI-CARB liquid scintillation spectrometer Model 314a. Internal standards were used to monitor quenching.

RESULTS

The uptake of (±)-[³H]-noradrenaline

The uptake of noradrenaline by the heart was measured after perfusion with several concentrations of (±)-[³H]-noradrenaline ranging from 10 to 1,000 ng/ml. Fig. 2 shows the curves for [³H]-noradrenaline and net noradrenaline uptake against

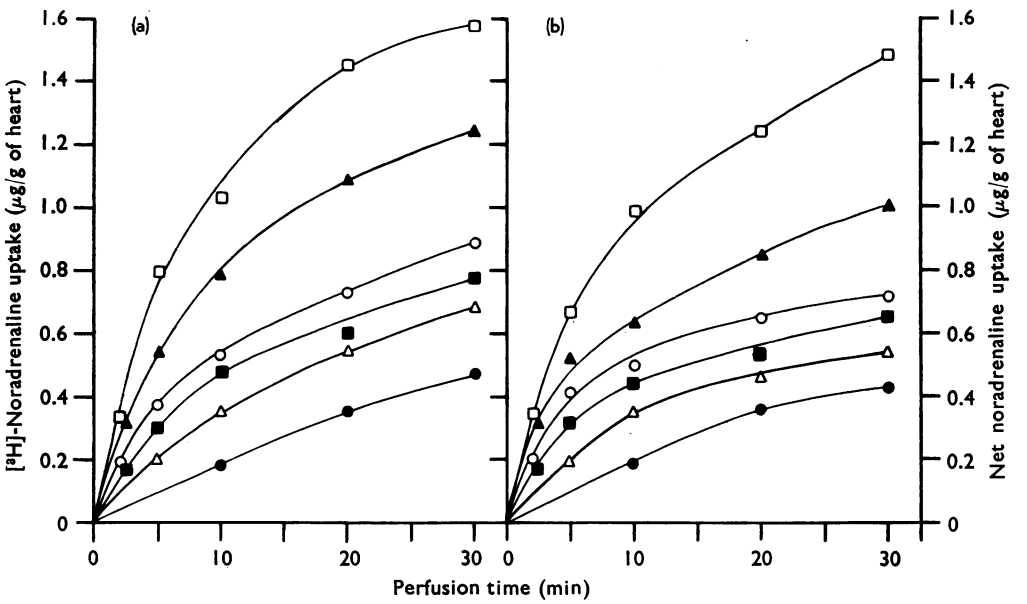

Fig. 2. (*a*) The uptake of [³H]-noradrenaline; (*b*) the net uptake of noradrenaline by the rat isolated heart during perfusion with various concentrations of (±)-[³H]-noradrenaline. ●, 10 ng/ml.; △, 20 ng/ml.; ■, 50 ng/ml.; ○, 100 ng/ml.; ▲, 500 ng/ml.; and □, 1 μg/ml. Each point represents the mean value of a group of six hearts.

time at various perfusion concentrations. Each point on these curves represents the mean value for a group of six hearts. Each group was perfused for 1 to 30 min with [³H]-noradrenaline and the tissue contents of [³H]-noradrenaline and of total noradrenaline were measured in each heart at the end of the perfusion. The uptake of [³H]-noradrenaline was expressed as μg/g of heart by dividing the mean [³H]-noradrenaline content of each experimental group (counts/min/g of heart) by the specific activity of the [³H]-noradrenaline in the medium. The net uptake of noradrenaline in μg/g of heart was expressed as the difference between the mean noradrenaline content of the experimental group and the mean noradrenaline content of a control group of hearts which had been perfused with noradrenaline-free medium. The mean of individual values within each experimental group had a

standard error of about ±5% for the fluorimetric determinations and about ±4% for the [^{3}H]-noradrenaline determinations. The mean noradrenaline contents of control groups which had been perfused for different times did not differ significantly and a control value of 0.97±0.05 μg/g of heart (mean and standard deviation), representing the overall mean for a total of seventy-six control hearts, was therefore used for all experiments.

The uptake curves for (±)-[^{3}H]-noradrenaline at a perfusion concentration of 200 ng/ml. were extended by a series of perfusions of up to 60 min duration (Fig. 3). From these results a plot of rate of [^{3}H]-noradrenaline uptake against time was

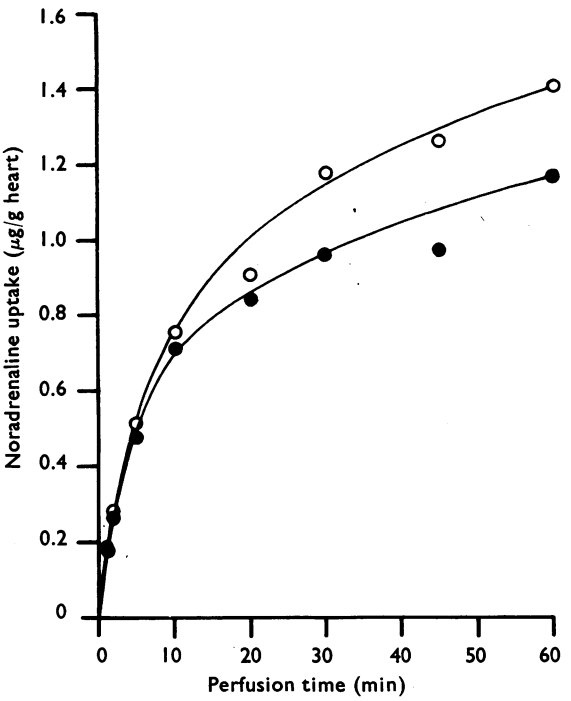

Fig. 3. The uptake of noradrenaline by the rat isolated heart perfused with (±)-[^{3}H]-noradrenaline (200 ng/ml.). Each point represents the mean value of a group of six hearts. ○, [^{3}H]-noradrenaline uptake; and ●, net noradrenaline uptake.

made on log paper (Fig. 4). This curve was resolved into two linear components (A) and (B), as shown in Fig. 4, by extrapolating the final linear portion of the curve to zero time to give component (B) and then subtracting this component from the initial part of the curve to give the second component (A). A plot of the rate of net uptake of noradrenaline against time at this perfusion concentration could be similarly resolved into two linear components in this way.

The uptake of (+)- and (−)-noradrenaline

Uptake curves for the net uptake of (+)- and (−)-noradrenaline were constructed in a similar manner by fluorimetric assay of the net increase in the noradrenaline

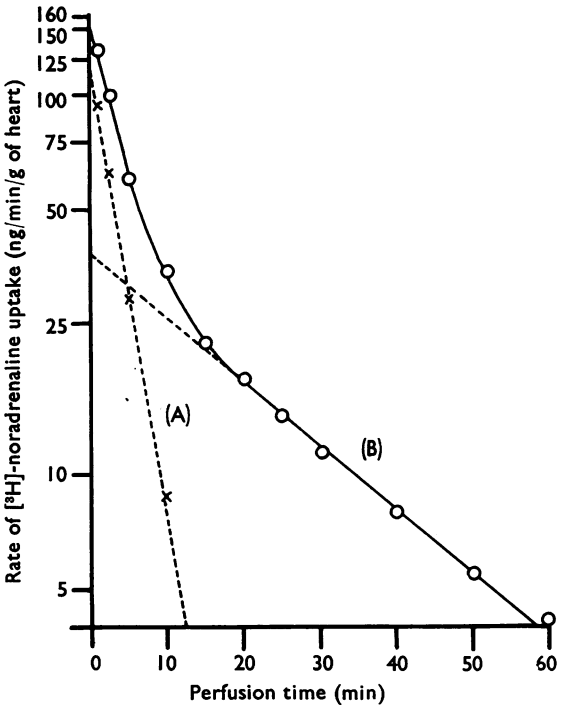

Fig. 4. The rate of [³H]-noradrenaline uptake plotted against time during perfusion of the rat isolated heart with (±)-[³H]-noradrenaline (200 ng/ml.). The curve was resolved into the linear components (A) and (B) as described in the text. The rate constants of these two components were 0·277 min^{-1} and 0.038 min^{-1} respectively. ○, values derived from the uptake curve for [³H]-noradrenaline at this perfusion concentration (Fig. 3); and ×, values derived by subtraction of (B).

content of hearts perfused for various times with nonradioactive (+)- or (−)-noradrenaline added to the perfusion medium. The concentrations tested were for (+)-noradrenaline 20, 100, 200 and 400 ng/ml. and, for (−)-noradrenaline, 10, 20, 50, 100 and 400 ng/ml. For each concentration three groups of six to eight hearts were perfused for periods of 1 to 10 min.

Measurement of initial rates of noradrenaline uptake

From the uptake curves obtained in this way (for example, Fig. 2) it was found that at low perfusion concentrations the uptake of noradrenaline was initially linear for 10 to 20 min, but at higher concentrations it was apparent that there was no true linear rate of uptake, but both [³H]-noradrenaline uptake and net noradrenaline uptake became progressively slower with time. Since there was no period of linear uptake at these higher perfusion concentrations it was impossible to measure the initial rates of noradrenaline uptake directly from the uptake curves. The initial rates at these concentrations were therefore found by plotting the rate of noradrenaline uptake against time on log paper and extrapolating to zero time (for

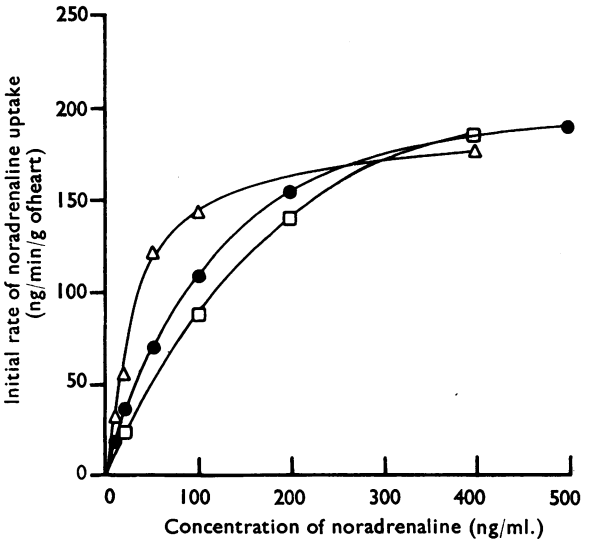

Fig. 5. Initial rates of noradrenaline uptake by the rat isolated heart perfused with various concentrations of noradrenaline. △, (−)-noradrenaline; □, (+)-noradrenaline; and ●, (±)-noradrenaline.

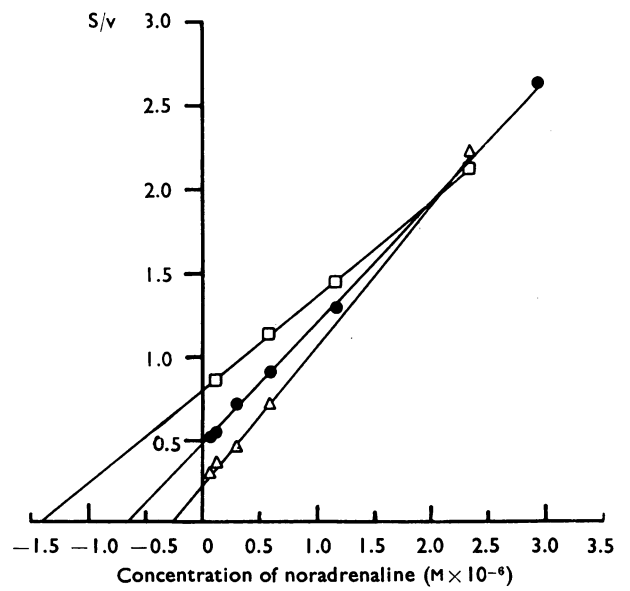

Fig. 6. Michaelis–Menten analysis of the results in Fig. 5 plotted as S/v (ordinate) against S (abscissa). S=perfusion concentration of noradrenaline; v=initial rate of noradrenaline uptake by the isolated heart. Kinetic constants were determined as follows: intercept on base-line=−Km; slope=1/Vmax; and intercept on vertical axis=Km/Vmax. The values of Km and Vmax determined in this way are summarized in Table 1. △, (−)-noradrenaline; □, (+)-noradrenaline; and ●, (±)-noradrenaline.

example, Fig. 4). In this way initial rates of [^{3}H]-noradrenaline uptake and initial rates of net uptake of (+)-, (−)- and (±)-noradrenaline were obtained. The initial rates of [^{3}H]-noradrenaline uptake and the initial rates of net noradrenaline uptake were identical at each perfusion concentration of (±)-[^{3}H]-noradrenaline tested. Fig. 5 shows the initial rates of noradrenaline uptake plotted against perfusion concentration for (+)-, (−)- and (±)-noradrenaline.

Kinetic analysis

In order to test the hypothesis that noradrenaline uptake obeys Michaelis-Menten kinetics the results obtained on the initial rates of noradrenaline uptake at various perfusion concentrations of noradrenaline were plotted in the form S/v against S (S = perfusion concentration of noradrenaline, v = initial rate of noradrenaline uptake). As shown in Fig. 6, the experimental values were found to lie on straight lines as predicted from the Michaelis-Menten equation (Dixon & Webb, 1958). From the slopes and intercepts of these graphs the kinetic constants for (+)-, (−)- and (±)-noradrenaline were determined and these values are summarized in Table 1. The results were plotted in the form S/v against S only

TABLE 1

KINETIC CONSTANTS FOR NORADRENALINE UPTAKE IN RAT HEART

The values were determined graphically from Fig. 6. Values for Vmax are means with standard errors

	Michaelis constant (Km) ($M \times 10^{-7}$)	Vmax (ng/min/g of heart)
(±)-Noradrenaline	6·64	234±5·1
(−)-Noradrenaline	2·66	196±20·2
(+)-Noradrenaline	13·90	295±8·4

because this gave a more satisfactory distribution of the experimental values for illustrative purposes than the more commonly used plot of 1/v against 1/S.

Exchange of [^{3}H]-noradrenaline with endogenous noradrenaline

Although the initial rates of [^{3}H]-noradrenaline and net noradrenaline uptake were found to be equal at each perfusion concentration of (±)-[^{3}H]-noradrenaline, the net uptake of noradrenaline declined more rapidly than the [^{3}H]-noradrenaline uptake (Figs. 2 and 3). For instance, at a perfusion concentration of 200 ng/ml., after a 30 min perfusion the average increment in total noradrenaline was 0.944 μg/g of heart, yet as indicated by radioactive measurement the tissue had taken up an average of 1.179 μg/g. The difference of 0.235 μg/g of heart can be accounted for by exchange of [^{3}H]-noradrenaline with the endogenous noradrenaline pool, which was thus 24.2% complete at this time. An alternative method is to calculate the exchange for individual hearts within the experimental groups and then to take the mean percentage exchange for the group. This latter method was used to calculate the extent of exchange of [^{3}H]-noradrenaline with endogenous noradrenaline after a 30 min perfusion at various perfusion concentrations of (±)-[^{3}H]-noradrenaline, as it allows some measure of the errors inherent in this type of calculation to be

TABLE 2

THE EXTENT OF EXCHANGE OF [^{3}H]-NORADRENALINE WITH ENDOGENOUS NORADRENALINE IN THE ISOLATED RAT HEART PERFUSED WITH VARIOUS CONCENTRATIONS OF (±)-[^{3}H]-NORADRENALINE

Values are means with standard errors of groups of six hearts perfused for 30 min. Percentage exchange is defined as:

$$\frac{\text{Heart content of [}^3\text{H]-noradrenaline} - \text{Net increase in heart content of noradrenaline } (\mu g/g)}{\text{Average endogenous heart content of noradrenaline } (\mu g/g)} \times 100$$

Perfusion concentration (ng of (±)-[^{3}H]-noradrenaline/ml. of medium)	Exchange (%) after 30 min perfusion
10	4·6±6·10
20	10·6±5·68
50	12·8±5·33
100	23·3±1·84
200	23·8±5·64
500	27·9±2·18
	after 60 min perfusion
200	29·1±6·02

made. Table 2 shows the exchange results calculated in this way together with the standard error of the mean for each group.

The accumulation of [^{3}H]-normetanephrine in heart tissue

[^{3}H]-Normetanephrine in the heart extracts prepared from hearts perfused with (±)-[^{3}H]-noradrenaline at concentrations of 20 ng/ml. and 1 μg/ml. was isolated

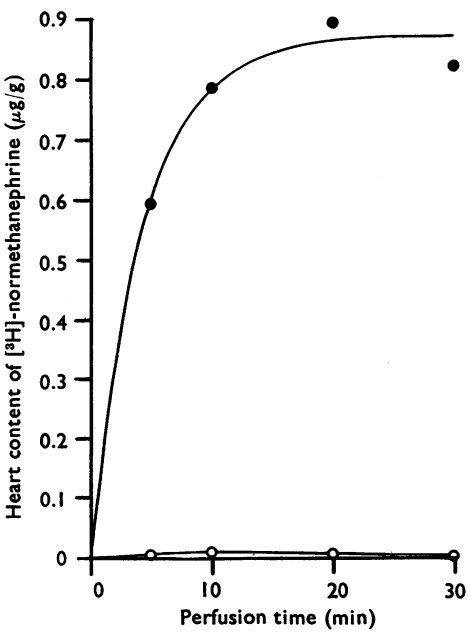

Fig. 7. The accumulation of [^{3}H]-normetanephrine in the rat isolated heart during perfusion with (±)-[^{3}H]-noradrenaline (20 ng/ml., ○; and 1 μg/ml., ●). Each point represents the mean value of a group of six hearts.

by ion-exchange chromatography and counted. The results shown in Fig. 7 indicate that at a perfusion concentration of 20 ng/ml. only negligible amounts of normetanephrine accumulated in heart tissue. However, at a perfusion concentration of 1 μg/ml. considerable amounts of [^{3}H]-normetanephrine were present in heart tissue, thus after a 10 min perfusion at this concentration the amount of [^{3}H]-normetanephrine present was equivalent to 80% of the [^{3}H]-noradrenaline content of the tissue at that time.

Inhibition of noradrenaline uptake by cocaine

The effects of cocaine were studied in a series of perfusions in which various concentrations of the drug were added to the noradrenaline perfusion medium so that the heart was exposed to cocaine and noradrenaline simultaneously. These experiments were carried out at two perfusion concentrations of (±)-[^{3}H]-nor-

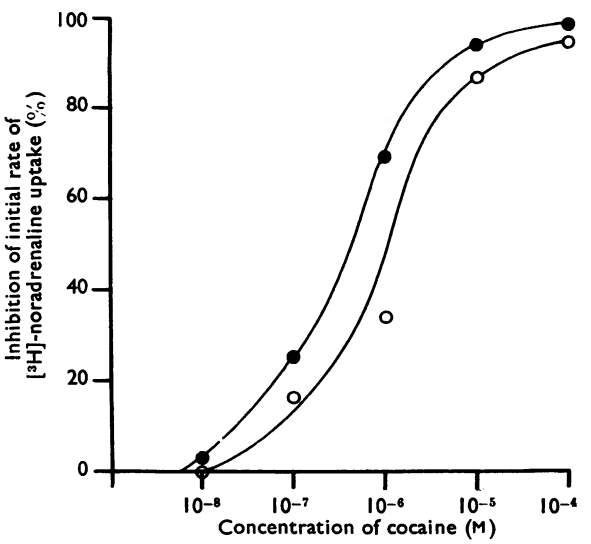

Fig. 8. Log dose/response curves for inhibition by cocaine of initial rates of uptake of [^{3}H]-noradrenaline by the rat isolated heart perfused with (±)-[^{3}H]-noradrenaline (20 ng/ml., ●; and 200 ng/ml., ○).

adrenaline, 20 and 200 ng/ml. The initial rate of [^{3}H]-noradrenaline uptake was measured at each drug concentration by assaying the [^{3}H]-noradrenaline content of groups of three hearts perfused for 5 and 10 min. Fig. 8 shows the dose/response curves for inhibition by cocaine of the initial rate of [^{3}H]-noradrenaline uptake at the two perfusion concentrations tested. A cocaine concentration of 10^{-4} M produced an almost complete inhibition of [^{3}H]-noradrenaline uptake ; the inhibition exceeded 99% at a perfusion concentration of 20 ng/ml. and 95% at a perfusion concentration of 200 ng/ml.

DISCUSSION

The present results confirm the previous findings of Lindmar & Muscholl (1962), Axelrod *et al.* (1962) and Kopin *et al.* (1962) that the isolated perfused rat heart can

accumulate exogenous noradrenaline supplied in the perfusing medium. A detailed study of the initial uptake rates of both total and radioactive (±)-noradrenaline confirms the suggestion of Dengler *et al.* (1961a, 1962a, b) that the system obeys kinetics of the Michaelis-Menten type, but yields the considerably larger value of 112 ng/ml. (6.64×10^{-7} M) for the Km as compared with about 25 ng/ml. (1.48×10^{-7} M) obtained by these workers. The difference lies in the method of calculating initial rates. Dengler *et al.* measured the uptake of [^{3}H]-noradrenaline against time at a concentration of 5 ng/ml. and found that the rate of uptake remained substantially constant for 20 to 30 min. They then made the assumption that this linearity would be apparent at *all* concentrations of noradrenaline, and therefore chose a fixed time of 20 min for measurement of uptake rates. The results presented in Fig. 2 show that this assumption is erroneous and that the method of Dengler *et al.* grossly underestimates the initial rate of noradrenaline uptake at higher noradrenaline concentrations and leads to an underestimate of the value of the Km.

Although the mathematical descriptions of the kinetics for binding to a fixed intracellular site and for binding to a carrier are formally similar, this similarity only applies to a consideration of overall rates of transfer and not to undirectional fluxes (Wilbrandt & Rosenberg, 1961). In the present studies initial rates of noradrenaline uptake were measured and it was found that the initial rates of [^{3}H]-noradrenaline uptake and net noradrenaline uptake were equal at each perfusion concentration tested. The initial rates of noradrenaline uptake therefore refer to unidirectional fluxes at zero time, when the presence of intracellular binding sites can have no influence on uptake kinetics. The uptake of noradrenaline in the rat isolated heart thus represents a membrane transport rather than binding to an intracellular site. This finding does not mean that intracellular binding may not be involved in the maintenance of a high intracellular concentration of noradrenaline, but merely that the accumulation of exogenous noradrenaline proceeds *initially* by a saturable process of membrane transport. The identity of initial rates of noradrenaline uptake measured by both radioactive uptake and net uptake incidentally provides the first evidence that isotopic noradrenaline, labelled with tritium in the β-position, and the normal compound have identical uptake kinetics.

The results obtained with (+)- and (−)-noradrenaline show that the uptake system exhibits stereochemical specificity in favour of the physiologically occurring (−)-isomer. The affinity of the uptake system for (−)-noradrenaline is extremely high, a finding which is consistent with the hypothesis that noradrenaline uptake has an important physiological role in the disposition of circulating and locally released noradrenaline. Although it is impossible to estimate accurately the local concentrations of noradrenaline likely to be encountered in heart tissue under physiological conditions, it seems probable that these would be considerably less than 100 ng/ml. and would thus lie in a range at which noradrenaline uptake would be functioning most effectively. The uptake system has a considerably lower affinity for (+)-noradrenaline, thus at very low concentrations the (+)-isomer is taken up at a lower rate than the (−)-isomer since under these conditions the rate of uptake is inversely proportional to Km. However, at high concentrations the rate

of uptake of the (+)-isomer was greater than that for the (−)-isomer at the same concentration (Fig. 5). This apparently anomalous finding is predicted on theoretical grounds (Wilbrandt & Rosenberg, 1961) since at high concentrations the rate of uptake of a transported material tends to become directly proportional to the Km. The significantly larger value of Vmax for (+)-noradrenaline as compared with (−)-noradrenaline found in the present studies was not expected, as it was anticipated that the maximal rates would be the same. It is clear that the relative proportions of (+)- and (−)-noradrenaline taken up by tissues from a racemic mixture will depend on its concentration; at low concentrations the (−)-isomer will be preferentially taken up, but this effect will not be evident at higher concentrations where the rate of uptake of the (+)-isomer may even exceed that of the (−)-isomer. These considerations may explain why Kopin & Bridgers (1963) failed to detect any significant difference in the initial uptakes of (+)- and (−)-noradrenaline from a racemic mixture by rat tissues *in vivo*. The present findings also suggest that many of the previous studies on the disposition of labelled (±)-noradrenaline may need careful re-examination in the light of the stereochemical specificity of the tissue uptake system. It seems possible that the results obtained with a racemic mixture may have underrated the importance of tissue uptake and storage as a physiological mechanism for the disposition of noradrenaline, and that this process may play an even more important role than hitherto supposed.

The results of the experiments with cocaine confirm previous reports that this drug is a potent inhibitor of noradrenaline uptake (Whitby *et al.*, 1960; Dengler *et al.*, 1961b). These results do not support the conclusion of Weiner & Trendelenburg (1962) that cocaine has no effect on the initial uptake of noradrenaline by tissues. The fact that the dose/response curve for inhibition by cocaine is shifted to the right when the noradrenaline concentration is raised lends support to the suggestion that cocaine acts competitively (Muscholl, 1961). It was possible to show that cocaine, at a concentration of 10^{-4} M, produced virtually complete inhibition of noradrenaline uptake even at the high perfusion concentration of 200 ng/ml. of (±)-[^{3}H]-noradrenaline. These results indicate that the entry of noradrenaline into intracellular space in heart tissue can be completely suppressed by an inhibitor, and therefore that the amounts of noradrenaline entering the tissue by simple diffusion must be negligible under these conditions. These results and the accuracy with which the kinetic values fitted the Michaelis-Menten equation suggest that if any diffusional component is present in the uptake process it is very small, even at the highest concentrations tested, and must be trivial at physiological concentrations. In this respect the present findings disagree with those of Dengler *et al.* (1961a, 1962a, b). These results also suggest that the criterion used by Dengler *et al.* (1961b) for evaluating drug inhibition of [^{3}H]-noradrenaline uptake by tissue slices incubated *in vitro* may not be valid. In those experiments 100% inhibition of noradrenaline uptake was defined as

$$\frac{\text{counts/min/g of control slice} - \text{counts/min/g of drug-treated slice}}{\text{counts/min/g of control slice} - \text{counts/min/ml. of incubation medium}} = 1$$

on the assumption that diffusion of [^{3}H]-noradrenaline into the tissue would give a ratio, $R = \frac{\text{counts/min/g of tissue}}{\text{counts/min/ml. of medium}} = 1$. The present findings show, however,

that [^{3}H]-noradrenaline does not enter the intracellular space of a tissue by diffusion. It seems probable, therefore, that under the conditions used by Dengler *et al.* (1961b) the maximum value of R which could have been expected from diffusion of [^{3}H]-noradrenaline into the extracellular space of the tissue would have been considerably less than 1.

A detailed study of the way in which [^{3}H]-noradrenaline uptake into the rat isolated heart falls off with time is presented in Fig. 4 for a perfusion concentration of 200 ng/ml. Under the conditions of these experiments where the external concentration of [^{3}H]-noradrenaline in the perfusion medium was maintained at a constant level throughout the experiment, the rate of [^{3}H]-noradrenaline uptake into heart tissue would be expected to decrease exponentially if the material were simply entering a single intracellular pool. If this were the case a plot of the rate of uptake of [^{3}H]-noradrenaline against time on log paper should be linear; the actual result (Fig. 4) was a curve which could be resolved into two linear components (A) and (B). This finding is interpreted as indicating that [^{3}H]-noradrenaline enters at least two intracellular pools in the tissue; furthermore the rate constants of the two components of Fig. 4 indicate that [^{3}H]-noradrenaline enters one pool at approximately seven-times the rate at which it enters the other pool.

The exchange of [^{3}H]-noradrenaline with endogenous noradrenaline (Table 2) was found to be limited to a maximum of 25 to 30% of the endogenous noradrenaline. This finding agrees with other reports that exogenous [^{3}H]-noradrenaline does not mix freely with endogenous noradrenaline (Dengler *et al.*, 1961a, 1962a, b; Chidsey & Harrison, 1963). This suggests that 70 to 75% of the endogenous noradrenaline in the rat heart is present in a third intracellular pool which exchanges at a negligible rate with exogenous noradrenaline or with either of the other two intracellular pools during the limited duration of these experiments. These findings are consistent with the growing evidence that the noradrenaline store in sympathetically innervated tissues cannot be considered to be a single homogenous entity (Trendelenburg, 1961, 1963; Campos & Shideman, 1962; Kopin *et al.*, 1962; Potter, Axelrod & Kopin, 1962; Iversen & Whitby, 1963), but it will require more experiments to delineate the characteristics of the various intracellular pools more precisely.

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