The Synthesis of Noradrenaline by Isolated Guinea-Pig Atria

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(Received 8 November 1965)

1. The initial rate of formation of noradrenaline by isolated atria from a physiological concentration of tyrosine was measured. 2. Study of the time-course of the formation led to the conclusion that newly formed noradrenaline enters a fraction of the total pool. Synthesis and turnover was maintained for at least 1.5 hr. In this time it was not possible to increase the concentration in the storage pool by employing high concentrations of precursor. 3. Preincubation of the tissue with noradrenaline resulted in an accumulation within the tissue and inhibited new formation. It is suggested that this indicates the existence of a physiological mechanism to limit the synthesis of noradrenaline, avoid saturation of storage sites and enable the 're-uptake' process to operate. 4. The amount of noradrenaline formed from 3-hydroxytyramine (dopamine) was considerably greater than from tyrosine. The kinetic constants of the reactions are in agreement with the view that hydroxylation of tyrosine to dihydroxyphenylalanine (dopa) is rate-limiting.

The biosynthesis of noradrenaline proceeds by means of the introduction of a phenolic hydroxyl group into tyrosine to form 3,4-dihydroxyphenylalanine (dopa) followed by decarboxylation to dopamine. Side-chain hydroxylation of dopamine by the enzyme dopamine β -oxidase results in noradrenaline, which exists in a bound form in adrenergic nerve fibres and serves as a transmitter substance. This synthetic route was first suggested by Blaschko (1939) and is now well established, although results based on the synthesis of noradrenaline from labelled tyrosine and other labelled precursors have been mostly qualitative (Musacchio & Goldstein, 1963; Goldstein & Musacchio, 1963). Quantitative measurements were attempted by Spector, Sjoerdsma, Zaltzman-Nirenberg, Levitt & Udenfriend (1963), employing guinea-pig hearts perfused by Tyrode solution containing tracer amounts of [^{14}C]tyrosine. The estimated rate of synthesis of noradrenaline of about $0.05 \mu\text{g./g./hr.}$ ($0.3 \mu\text{mole/kg./hr.}$) was recognized as being too low, first because of release of acidic metabolites of noradrenaline into the perfusion medium and secondly because tyrosine was employed at a concentration of 1–2% of that in normal plasma. More recently the same group of workers (Levitt, Spector, Sjoerdsma & Udenfriend, 1965) investigated the formation of noradrenaline from larger amounts of tyrosine and found a rate of synthesis comparable with that estimated from studies *in vivo*, 0.11 – $0.17 \mu\text{g./g./hr.}$ (0.65 – $1.0 \mu\text{mole/kg./hr.}$) (Udenfriend, Zaltzman-Nirenberg & Levitt, 1963).

Montanari, Costa, Beaven & Brodie (1963) employed a method based on the rate of disappearance of injected DL- $[\text{}^3\text{H}]$ noradrenaline from the hearts of intact animals, and estimated the rate of synthesis to be $0.17 \mu\text{g./g./hr.}$ ($1.0 \mu\text{mole/kg./hr.}$). This result, however, was somewhat obscured by the presence of the unnatural D-isomer and by the biphasic decline in radioactivity supposedly due to differing rates of release from two pools of noradrenaline: the 'readily available' pool and the 'reserve' pool. It was subsequently suggested (Crout, 1964) that the former merely represents storage granules lying near the axonal membrane.

It seemed likely therefore that a study based on synthesis rather than measurements of uptake and disappearance would give more valid information, but the isolated heart suffers from the disadvantage that relatively large amounts of radioactivity are required and progressive mechanical failure occurs during continuous reperfusion. However, isolated atria will continue to beat rhythmically for many hours when suspended in oxygenated Krebs-Henseleit solution. They also contain a considerably higher concentration of noradrenaline than the whole heart and thus might be expected to synthesize noradrenaline at a greater rate.

We have used this technique for studying the effects of drugs thought likely to interfere with the synthesis of noradrenaline. To understand the action of these drugs it was necessary to study the kinetics of the formation of noradrenaline from

tyrosine and dopamine and the present investigation deals mainly with this aspect.

MATERIALS

Radioactive materials. L-[U-¹⁴C]Tyrosine, specific activity 240 mc/m-mole, and DL-3-(3,4-dihydroxyphenyl)[2-¹⁴C]-alanine, ([¹⁴C]dopa), 31.2 mc/m-mole, were obtained from The Radiochemical Centre (Amersham, Bucks.).

2-(3,4-Dihydroxyphenyl)[2-¹⁴C]ethylamine, ([¹⁴C]dopamine), 0.79 mc/m-mole, was purchased from Tracerlab (Waltham, Mass., U.S.A.).

The purity of these compounds was checked by paper chromatography (Roberts, 1962) and by the column-chromatographic method described below.

Other materials. 3,4-Dihydroxyphenylacetic acid (dopac) was supplied as the dicyclohexylamine salt by Calbiochem (Los Angeles, Calif., U.S.A.).

L-3-(3,4-Dihydroxyphenyl)alanine (dopa), 3-hydroxytyramine (dopamine), normetanephrine and noradrenaline were bought from L. Light and Co. Ltd. (Colnbrook, Bucks.).

Inulin, obtained from British Drug Houses Ltd. (Poole, Dorset), was purified as described by Phelps (1965).

N-(*m*-Hydroxybenzyl)-*N*-methylhydrazine was a gift from Dr D. J. Drain (Smith and Nephew Research Ltd., Harlow, Essex).

METHODS

Preparation of tissue. Albino guinea pigs (Porton strain, 350–450 g.) were killed by a sharp blow on the head. The heart was rapidly removed and immersed in oxygenated saline at 0°. The atria were dissected and suspended in an organ bath containing 25 ml. of Krebs–Henseleit solution (Krebs & Henseleit, 1932). The left atrium was attached to a glass hook sealed to the bottom of the bath and the right atrium to a lever in order that contractions of the tissue could be observed. An organ bath of standard pattern was employed and maintained at 37° by means of a water jacket. The contents were oxygenated with O₂ + CO₂ (95:5) bubbled through a sintered-glass disk at the base of the bath. Under these conditions the atria continued to beat regularly and at the same amplitude for at least 6 hr.

On removal from the bath, the tissue was washed in three changes of Krebs–Henseleit solution, blotted and weighed. It was then ground in 5 ml. of 0.4 N-perchloric acid with a little sand. The homogenate was stored at –20° for at least 1 hr. or overnight. Catechols were subsequently separated from other tissue constituents by adsorption on alumina and elution with dilute acetic acid (Merrills, 1963). A portion of this eluate was employed for column chromatography as described below.

Column chromatography

Preparation of adsorbent for chromatography. Whatman P11 cellulose phosphate powder was washed by decantation with 0.5 N-HCl followed by aq. 0.5 N-NH₃ soln. until the supernatant fluid was free from fines and pigment. Excess of ammonia was removed by suspending the powder in water and adjusting the pH to about 6.0 with acetic acid. It was finally equilibrated with 0.05 M-ammonium acetate (adjusted to pH 6.0 by the addition of *n*-acetic acid) by washing repeatedly on a Buchner filter, and stored moist at 4°.

Chromatographic separation of reaction products. Because of the instability of the catechol metabolites of tyrosine, their isolation for radioassay presented some difficulty. Adsorption on alumina was an obvious choice for the initial separation from other tissue constituents since this adsorbent possesses considerable specificity for catechol compounds (Shaw, 1938). Unfortunately, the presence of aluminium salts in the acid eluate interfered with subsequent paper chromatography. For this reason a column-chromatographic procedure employing cellulose phosphate powder was developed. This had the advantage that the eluate from alumina could be used without further manipulation and also that the fractions from the column were suitable for direct chemical and radioactivity measurements.

Preparation of columns. A glass tube (0.5 cm. × 35 cm.) fitted with a sintered-glass support and a B10 ground-glass joint at the upper extremity was employed. The column was packed with a dilute (1 in 20) suspension of cellulose phosphate in the 0.05 M-ammonium acetate buffer solution. This was applied with an extension piece 45 cm. long. Buffer was subsequently pumped through the column at a rate of 0.7 ml./min. until no further reduction in the volume of the adsorbent occurred.

The sample (1 ml.) with the addition of carrier dopac, dopa, normetanephrine, dopamine and noradrenaline was added to the column and allowed to enter by gravity. The recovery of dopac, tyrosine, dopa, dopamine and noradrenaline was quantitative (95–100%) in amounts as low as 5 µg., although in practice a larger amount (100 µg.) was added as a carrier of radioactivity so that the substances could be detected spectrophotometrically.

Elution. The starting buffer (0.05 M-ammonium acetate, pH 6.0; 25 ml.) was placed in a beaker and pumped through the column at a nominal rate of 0.27 ml./min. 0.6 M-Ammonium acetate, pH 6.0, was added to the beaker at the same rate. Mixing was achieved by means of a magnetic stirrer. A proportioning pump (Technicon Ltd., Chertsey, Surrey) and the appropriate Tygon tubing was employed for the pumping operation. Fractions (approx. 1.5 ml.) were collected and the substances eluted by this procedure detected by spectrophotometric measurement at 280 mµ. The identity of the peaks was determined by paper chromatography (Roberts, 1962; Hagopian, Dorfman & Gut, 1961).

Assay procedures

Assay of radioactivity. After spectrophotometry the fractions were quantitatively transferred to counting vials with 10 ml. of Bray's solution (Bray, 1960). A Packard Tri-Carb Liquid Scintillation Spectrometer series 3000 (Packard Ltd., Wembley, Middlesex) was employed for measurement of the radioactivity. Because of the low count-rate of the samples the maximum available pre-set time (100 min.) was used.

Counting efficiency of about 55% was obtained with this scintillator; the actual efficiency for each sample was determined by the method of Baillie (1960), and the appropriate correction made to determine the absolute count-rate in disintegrations/min. based on the calibrated [¹⁴C]toluene supplied with the instrument.

Noradrenaline. This was estimated by a fluorimetric method after preliminary adsorption on alumina and elution with dilute acetic acid (Merrills, 1963).

Tyrosine. The concentration of tyrosine in blood

and tissue was determined by a fluorimetric method (Udenfriend, 1962). In experiments on the uptake of [^{14}C]-tyrosine by atria a 'reverse isotope dilution' procedure was employed: to the filtrate obtained after adsorption of the catechols on alumina (see above) 30 mg. of L-tyrosine was added and dissolved by heating to 100°. The amino acid was subsequently allowed to separate and recrystallized from hot water to constant specific activity. Radioactivity measurements were made by dissolving a known weight in 2 ml. of 0.01N-hydrochloric acid before the addition of scintillator solution.

Inulin space. This was measured by the procedure developed by Varon & McIlwain (1961).

RESULTS

Radioactivity in fractions derived from guinea-pig atria. Preliminary experiments were carried out by incubating isolated atria for 1.5 hr. in Krebs-Henseleit solution containing [^{14}C]tyrosine (45 μM , 5 μC). Column chromatography of an extract of the tissue demonstrated the presence of a number of radioactive substances. In a typical experiment 87% of the radioactivity recovered from a chromatogram coincided with carrier noradrenaline and 7.2% with dopamine (Fig. 1).

Three small radioactive peaks were also detected; the first coincided with dopac and appeared at the hold-up volume of the column. Since there was no resolution of acids on this type of ion-exchanger it probably represented a mixture of catechol acids and no attempt was made to identify them. A trace of activity corresponding to tyrosine was found to be due to incomplete elimination in the preliminary purification stage; this was understandable in view of the relatively enormous excess of this substance.

Fig. 1. Column chromatogram of an extract of guinea-pig atria incubated for 1.5 hr. in Krebs-Henseleit solution containing [^{14}C]tyrosine (4.5 μM , 5 μC). The order of elution of the substances is: dopac, tyrosine, dopa, normetanephrine, dopamine and noradrenaline. Normetanephrine was included as a marker only. ●, Extinction at 280 m μ . ○, ($10^{-2} \times$ Radioactivity disintegrations/min.).

Dopa was never observed except in experiments in which the decarboxylase inhibitor *N*-(*m*-hydroxybenzyl)-*N*-methylhydrazine was included in the incubation medium at a concentration of 0.1 mM.

Nagatsu, Levitt & Udenfriend (1964) reported the presence of labelled dopa as an impurity in samples of [^{14}C]tyrosine. Paper chromatography of the material used by us seemed to confirm this, but on cellulose phosphate the impurity in the tyrosine appeared as a peak adjacent to that of authentic L-dopa. It was presumed to be identical with the substance detected in the tissue extracts.

Uptake of tyrosine by guinea-pig atria. In previous experiments with perfused hearts tracer amounts of tyrosine were employed and release of unlabelled tyrosine from the heart resulted in dilution of the isotope, thus rendering the interpretation of results difficult (Spector *et al.* 1963). In the experiments reported here, tyrosine was usually employed at 45 μM . This approximated to the normal concentration in guinea-pig serum, which in four estimations was found to be 64 ± 7.9 (S.E.M.) μM . In order that calculation of the rate of noradrenaline synthesis should be meaningful it was necessary to show that the endogenous tyrosine equilibrated with reasonable rapidity with the label. Measurement of the specific activity of this amino acid in the tissue after incubation for various periods could not be carried out because available chemical methods were not sufficiently sensitive. However, it was possible to show by isotope-dilution analysis that the radioactivity in the tissue attributable to tyrosine reached a value within 30 min. which was not exceeded by more prolonged incubation. The concentration of the amino acid in one experiment in which four normal atria were pooled was found to be 16 $\mu\text{moles/kg}$. Thus the amount released from a single atrium was not sufficient to affect significantly the specific activity of the [^{14}C]tyrosine added to the organ bath.

Formation of noradrenaline from [^{14}C]tyrosine. When atria were incubated with [^{14}C]tyrosine (45 μM , specific activity 0.1 mC/m-mole), chromatographic analysis demonstrated that about 85% of the radioactivity present was due to noradrenaline; the remainder consisted of dopamine and traces of unidentified products as in Fig. 1.

In a series of ten experiments the mean production of [^{14}C]noradrenaline in 1.5 hr. was 2.25 ± 0.33 (S.E.M.) $\mu\text{moles/kg}$. The total concentration of noradrenaline was measured concurrently with the assay of radioactivity, and no correlation could be detected between the two measurements. From this it appeared that the synthetic ability of the tissue was not related to its normal storage capacity.

It was realized that the rate of synthesis could not be derived from measurements made at a single time-interval of 1.5 hr. unless the rate was constant

and unless the amine was stored without subsequent loss by diffusion or metabolism. In spite of the non-physiological condition of the tissue it continued to beat regularly for many hours, maintained its noradrenaline content and appeared to be metabolically intact. Thus there was some justification for assuming a constant turnover through the noradrenaline pool. The following experiments were designed to test this assumption.

Formation of noradrenaline as a function of time. The appearance of radioactive noradrenaline during incubation of atrial tissue for various periods is shown in Fig. 2. From the experimentally determined points a polynomial equation of best fit was calculated by the method of least squares. This was found to approximate the expanded form of the exponential: $n = 8.2(1 - e^{-0.24t})$ where n is concentration of [^{14}C]noradrenaline in $\mu\text{moles/kg.}$, and t is time in hours. By differentiating the equation with respect to time, giving $dn/dt = 1.72e^{-0.24t}$, the initial rate of synthesis was calculated as $1.72 \mu\text{moles/kg./hr.}$ This is somewhat higher, as expected, than that measured over a 1.5 hr. period.

The total noradrenaline concentration in the atria appeared to increase in experiments of long duration, but statistical evaluation (Student's t test) showed that only the accumulation observed after 6 hr. was significant ($P < 0.01$). This is shown in Table 1; it appears to indicate a failure of utilization or metabolism of the amine. When the synthesis of noradrenaline was increased to near maximal values by employing high concentrations of precursor for shorter (1.5 hr.) periods a net accumulation was not observed.

Time-lapse experiments. In these experiments the tissue was incubated with unlabelled tyrosine

for either 1.5 or 4 hr. before the addition of radioactivity. The formation of [^{14}C]noradrenaline plotted additively for the periods 1.5–4 and 4–6 hr. formed a curve, shown as a broken line in Fig. 2, which coincided with the curve obtained when [^{14}C]tyrosine was present for the whole of the periods. This clearly demonstrated that the initial rate of synthesis was not maintained, since if this were so the slope of the line between these points would have approximated the slope of the early part of the main curve.

Effect of unlabelled noradrenaline on the formation of [^{14}C]noradrenaline from tyrosine. Because noradrenaline has been found to inhibit tyrosine hydroxylase it has been suggested that this may signify the existence of a feedback control mechanism (Nagatsu *et al.* 1964). It was of interest to endeavour to increase the content of noradrenaline in atria by pretreatment with noradrenaline, which is known to be rapidly taken up by heart tissue (Iversen, 1963), and to measure the rate of synthesis of [^{14}C]noradrenaline from labelled tyrosine thereafter. Experiments were carried out in which atria were preincubated for various times with buffer containing noradrenaline and unlabelled tyrosine ($45 \mu\text{M}$). Ascorbic acid (0.1 mM) was also added (Iversen, 1963) to avoid oxidation of the catecholamine. The atria were subsequently washed in four changes of Krebs–Henseleit buffer, and the rate of synthesis was measured over a 1.5 hr. period according to the standard procedure.

The results demonstrated a considerable reduction in the rate of synthesis and the degree of this reduction was found to be related to the noradrenaline remaining in the tissue at the end of the experiment (Table 2).

Formation of noradrenaline as a function of tyrosine concentration. In these experiments tyrosine was added to the incubation fluid in concentrations varying from $22 \mu\text{M}$ to 0.55 mM . A standard

Fig. 2. ●, [^{14}C]Noradrenaline content of guinea-pig atria incubated for various periods in solutions containing radioactive tyrosine ($45 \mu\text{M}$). Each point is the mean of at least four estimations. The vertical lines represent the standard error of the mean. ○, As above, but after preincubation for either 1.5 or 4 hr. with unlabelled tyrosine at the same concentration.

Table 1. Content of noradrenaline in guinea-pig atria after incubation with [^{14}C]tyrosine ($45 \mu\text{M}$) for various times

The amount of [^{14}C]noradrenaline accumulated by the tissue is also shown for comparison. Results are given as mean of each group $\pm$ s.e.m. P , Probability of difference from control group (Student's t test).

Time (hr.)	Total content of noradrenaline ($\mu\text{moles/kg.}$)	[^{14}C]Noradrenaline ($\mu\text{moles/kg.}$)
0	20.3 ± 1.3 (10)	
0.5	19.7 ± 1.48 (4) $P 0.8$	1.02 ± 0.15
1.5	22.8 ± 2.01 (10) $P 0.3$	2.27 ± 0.11
3.0	25.0 ± 3.38 (4) $P 0.1$	3.78 ± 0.40
6.0	26.6 ± 1.24 (4) $P 0.01$	5.93 ± 1.10

Table 2. *Effect of preincubation with unlabelled noradrenaline on the synthesis from tyrosine*

Atria were incubated for various times in buffer containing unlabelled tyrosine and noradrenaline (0.12 mM). They were then washed and incubated for an additional 1.5 hr. with [^{14}C]tyrosine. The amount of [^{14}C]noradrenaline and the total noradrenaline present in the tissue at the end of the experiment were measured. Control values were obtained from tissue preincubated for 30 min. without the addition of noradrenaline. Results are the means of six estimations $\pm$ S.E.M.

Time of preincubation (min.)	[^{14}C]Noradrenaline ($\mu\text{moles/kg.}$)	Total noradrenaline ($\mu\text{moles/kg.}$)
0	2.25 $\pm$ 0.33	22.8 $\pm$ 2.0
5	0.62 $\pm$ 0.06	26.6 $\pm$ 1.2
15	0.36 $\pm$ 0.03	28.3 $\pm$ 1.3
30	0.15 $\pm$ 0.03	33.5 $\pm$ 3.4
Control	1.65 $\pm$ 0.34	23.0 $\pm$ 2.1

amount (1 μC) of [^{14}C]tyrosine was included. The formation of noradrenaline was assumed constant over a 1.5 hr. period. A double-reciprocal plot of product formation against the concentration of precursor showed that the overall reaction obeyed Michaelis-Menten kinetics (Fig. 3). The apparent K_m was estimated to be 45 μM . The maximum reaction velocity, 3.2 $\mu\text{moles/kg./hr.}$, is not incompatible with that obtained for a preparation of tyrosine hydroxylase from bovine adrenal medulla (Nagatsu *et al.* 1964).

Concentration of the intermediates dopa and dopamine. The formation of dopa from tyrosine was not detected in any of the experiments, except those in which a decarboxylase inhibitor [*N*-(*m*-hydroxybenzyl)-*N*-methylhydrazine] was added. In one experiment a high concentration of radioactive tyrosine (10 μC) was used and carrier dopa was added to the tissue during homogenization, to ensure that the failure to detect this substance was not caused by loss in processing the tissue. The absence of this intermediate is in accord with the known high activity of dopa decarboxylase (Hess, Connamacher, Ozaki & Udenfriend, 1961). From a consideration of the sensitivity of the method it was calculated that the concentration of dopa normally present in the atria is below 0.05 $\mu\text{mole/kg.}$

Carlsson (1959) failed to detect the presence of dopamine in heart tissue, but when a more sensitive fluorimetric assay was employed (Lavery & Sharman, 1964) it was demonstrated in some extracts of mouse heart in amounts of the order of 0.06 $\mu\text{mole/kg.}$ (Lavery, Sharman & Vogt, 1965). In the present experiments the production of [^{14}C]dopamine from tyrosine was of the order of 6% of the [^{14}C]noradrenaline formed in 1.5 hr.

Fig. 3. Lineweaver-Burk plot of noradrenaline formed by guinea-pig atria when incubated with various concentrations of tyrosine for 1.5 hr. Kinetic constants: K_m 45 μM ; V_{max} 3.2 $\mu\text{moles/kg./hr.}$

Since the endogenous content of dopamine is small it was concluded from the rate of synthesis of noradrenaline that the size of the dopamine pool must be 6% of the noradrenaline pool or about 0.12 $\mu\text{mole/kg.}$

Formation of noradrenaline from dopamine. In experiments with [^{14}C]dopamine, ascorbic acid (0.1 mM) was added to the Krebs-Henseleit solution. Failure to include this resulted in the appearance of a pink oxidation product. The formation of noradrenaline was more variable than when tyrosine was used as a precursor. Levitt *et al.* (1965), in investigations on the perfused heart, also noted considerable variation.

In an experiment in which atria were incubated for 1.5 hr., with concentrations of [^{14}C]dopamine varying from 0.26 to 10.4 μM , the amount of dopamine taken up by the tissue was found to be an almost constant fraction (about 80%) of the initial concentration (Fig. 4). This was a surprising observation, since it was expected that the amine would be rapidly degraded by monoamine oxidase present in the tissue, and perhaps also non-enzymically in the oxygenated incubation fluid. Analysis at the end of the experiment did indeed show that only 50% of the radioactivity in the bath fluid was then due to dopamine itself; the remainder was found in the column fraction corresponding to phenolic acids.

The uptake of dopamine shown in Fig. 4 was corrected for an extracellular (inulin) space of 15%. This was determined by parallel experiments in which 1% (w/v) of inulin was added to the incubation medium (Varon & McIlwain, 1961). In this correction it was assumed that dopamine contained within the extracellular space was in equilibrium with that in the exogenous fluid at the end of the

Fig. 4. Synthesis of noradrenaline by guinea-pig atria incubated for 1.5 hr. with various concentrations of [^{14}C]-dopamine. ●, [^{14}C]Noradrenaline accumulated by the tissue during this period. ○, Uptake of dopamine corrected for the extracellular (inulin) space. Each point is the mean of two observations.

experiment, i.e. 50% of the initial value. In the Figure the formation of noradrenaline is also given; this is considerably greater than that arising from a similar amount of tyrosine. Subsequent experiments demonstrated that the time chosen was needlessly long, and the formation of noradrenaline, uptake of dopamine and residual dopamine were therefore reinvestigated at shorter time-intervals at a fixed dopamine concentration ($5\text{ }\mu\text{M}$). This amount was chosen as the lowest compatible with adequate count-rates. The results are shown in Fig. 5 and demonstrate a rather slow uptake and a linear decrease in exogenous dopamine. As before, the radioactivity in the bath fluid remained virtually constant; the deficiency in dopamine was accounted for by the formation of acidic metabolites or oxidation products. At the longest incubation time employed in this experiment the intracellular concentration slightly exceeded the extracellular concentration. It can be seen from the Figure that the formation of noradrenaline proceeded rapidly; the initial rate was estimated graphically to be about $25\text{ }\mu\text{moles/kg./hr.}$

The time-course of the reaction was similar, but of different magnitude, from that found on the experiments with tyrosine; in spite of the eventual formation of labelled noradrenaline equal to half the content of normal tissue, a net accumulation was not observed, indicating a rapid rate of turnover.

An attempt was made to assess the kinetic

Fig. 5. ▲, Accumulation of [^{14}C]noradrenaline in guinea-pig atria incubated for various periods with [^{14}C]dopamine ($5\text{ }\mu\text{M}$). ■, Uptake of dopamine by the tissue, corrected for extracellular fluid (inulin) space. ●, Dopamine remaining in the incubation fluid. Each point is the mean of two results; at the shorter-time intervals (5 and 15 min.) atria from two animals were used for each determination.

Fig. 6. Lineweaver-Burk plot of noradrenaline formed from dopamine. Kinetic constants: $K_m\text{ }3\text{ }\mu\text{M}$; $V_{\text{max.}}\text{ }30\text{ }\mu\text{moles/kg./hr.}$ For details see the text.

constants of the conversion of dopamine into noradrenaline. To obtain sufficient count-rates at the substrate concentrations employed ($0.52\text{--}10.4\text{ }\mu\text{M}$) it was necessary to use two atria for each determination. An incubation time of 30 min. was chosen as the minimum practicable and under these conditions the total count-rate measured in the chromatographic fractions corresponding to noradrenaline was about 300 counts/min. From a double-reciprocal plot (Fig. 6) the apparent K_m for

the conversion of dopamine into noradrenaline was found to be $3\text{ }\mu\text{M}$.

DISCUSSION

The time-course of the formation of [^{14}C]noradrenaline by isolated guinea-pig atria shows that the initial rate of synthesis is $1.7\text{ }\mu\text{moles/kg./hr.}$, nearly twice that of the heart *in vivo* (Udenfriend *et al.* 1963; Nikodijevic, Creveling & Udenfriend, 1963; Montanari *et al.* 1963) and of the perfused heart (Levitt *et al.* 1965). The higher rate of synthesis in atria is commensurate with the greater degree of sympathetic innervation and higher noradrenaline content. In the present work the latter is found to be $22.8\text{ }\mu\text{moles/kg.}$ as opposed to $11.1\text{ }\mu\text{moles/kg.}$ for the whole heart (Montanari *et al.* 1963).

The rate of accumulation of radioactive noradrenaline decreases with the time of incubation so that after 6 hr. it is only 28% of the initial rate. This decrease can be explained either by reduction in the rate of formation, caused perhaps by loss of essential cofactors, or by turnover of the newly formed amine through the storage pool. The progress curve (Fig. 2) is of the exponential form expected from turnover-rate theory (Reiner, 1953), and the tissue shows no sign of fatigue during long periods of incubation and appears to be metabolically intact. Also, it is not possible to increase the total content of noradrenaline by employing a high concentration of tyrosine (or dopamine) for 1.5 hr. although the amount formed in this time is relatively high under these conditions. This clearly means that turnover of the noradrenaline is proceeding at a rapid rate. Calculation of the turnover rate requires a knowledge of the rate of increase in specific activity and the size of the pool. The specific activity can be obtained from the results given in Fig. 2. If it is assumed that the initial rate of synthesis is maintained the size of the pool into which the newly formed noradrenaline enters can be thus calculated to be $8.2\text{ }\mu\text{moles/kg.}$ or 36% of the total pool. There is considerable evidence that the store in sympathetically innervated tissue cannot be considered a single homogeneous entity (Trendelenburg, 1961, 1963; Campos & Shideman, 1962; Kopin, Hertting & Gordon, 1962; Potter, Axelrod & Kopin, 1962; Iversen & Whitby, 1963). This conclusion has been reached mainly from studies on the displacement of noradrenaline from heart tissue by tyramine and from the rate of uptake and subsequent release of [^3H]noradrenaline. A hypothetical model recently suggested (Neff, Tozer, Hammer & Brodie, 1965) is an open two-compartment system consisting of a 'readily available' compartment of free noradrenaline in dynamic equilibrium with a larger compartment of noradren-

aline complexed in granules. The 'readily available' pool comprises about 40% of the total (Montanari *et al.* 1963). This fraction is very similar to that obtained from the calculation mentioned above, and suggests that the amine initially enters the smaller pool. Experiments of longer duration (6 hr.) show that a statistically significant net accumulation is eventually obtained (Table 1) and, if the atria are preincubated with unlabelled tyrosine for 1.5 hr. or longer (Fig. 2) before the addition of radioactive precursor, the subsequent rate of formation of [^{14}C]noradrenaline is diminished relative to that in the experiments without preincubation. It is apparent therefore that, though turnover of the catecholamine must proceed initially, this function and the synthetic ability are ultimately lost.

It is of interest to speculate that the accumulation of noradrenaline in storage sites may inhibit synthesis. Because noradrenaline inhibits purified adrenal tyrosine hydroxylase it has been suggested (Nagatsu *et al.* 1964) that this may indicate a feedback control mechanism. However, the pretreatment of guinea pigs with monoamine-oxidase inhibitors to increase the noradrenaline content of the heart failed to decrease the rate of synthesis (Levitt *et al.* 1965). In guinea-pig atria the addition of noradrenaline to the organ bath results in a considerable reduction in the rate (Table 2). This effect may, of course, be caused by product inhibition of dopamine β -oxidase or simply decrease in binding capacity, rather than inhibition of tyrosine hydroxylase. But there appears to be a necessity for some control mechanism, since *in vivo* the storage sites are not fully saturated; this is an essential condition for the operation of the 're-uptake mechanism'. The latter (Paton, 1960; Kopin *et al.* 1962) is believed to play a major role in limiting the physiological action of noradrenaline released from sympathetic nerve endings (Kopin, 1964).

Investigations on the production of noradrenaline from dopamine are complicated by several factors. It is an effective substrate for monoamine oxidase and would be expected to be rapidly destroyed; it also acts both as a precursor and as a depleting agent (Harrison, Levitt & Udenfriend, 1963), presumably by displacing noradrenaline from storage granules. In isolated atria the uptake of dopamine is rather slow, but the amine finally achieves a concentration which is greater than that of the incubation fluid (Fig. 5), in spite of utilization in the formation of noradrenaline and possible destruction by monoamine oxidase. This appears to indicate the presence of some form of binding. Presumably this is necessary to protect the amine during transport to the sites of action of dopamine β -oxidase.

The apparent K_m for the conversion of extracellular dopamine into noradrenaline is $3\text{ }\mu\text{M}$ (Fig. 6).

Because of the rather slow uptake and decrease in substrate concentration during the duration of the experiment, it is clear that the kinetic measurements are somewhat artificial or inaccurate. It would perhaps be more logical to employ, as a point of reference, the mean intracellular substrate concentration. At present this is not feasible since the uptake of dopamine in the time (30 min.) is small and its measurement is subject to a large experimental error at the lower concentrations.

In guinea-pig hearts perfused with [^{14}C]dopamine, Levitt *et al.* (1965) found the production of noradrenaline to be very variable and were unable to approach a maximum rate even with concentrations as high as 0.2 mM. The reason for the discrepancy between this work and the results obtained with atria is not clear, but is perhaps related to the depleting action of dopamine, which, if more pronounced in the whole heart, would reduce the rate of accumulation of [^{14}C]noradrenaline and prevent a maximum being attained. After incubation of atria with [^{14}C]dopamine (10.4 μM for 1.5 hr.; Fig. 4) the amount of radioactive noradrenaline is nearly equal to the normal content of noradrenaline in atria. In spite of this, a net accumulation is not found, thus indicating a rapid rate of turnover.

The decarboxylation of dopa is a very fast reaction (Lovenberg, Weissbach & Udenfriend, 1962; Hess *et al.* 1961), as evinced by the virtual absence of this intermediate and by the rapid production of $^{14}\text{CO}_2$ from [*carboxy- ^{14}C*]labelled dopa in homogenates of guinea-pig atria (unpublished observation). It is clear from the V_{max} of the dopamine-noradrenaline conversion (30 $\mu\text{moles/kg./hr.}$) and from that of the overall reaction tyrosine-noradrenaline (3.2 $\mu\text{moles/kg./hr.}$) that the latter is controlled by the activity of tyrosine hydroxylase, in confirmation of the finding of Levitt *et al.* (1965) that this enzyme is rate-limiting.

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