*Br. J. Pharmac.* (1972), **46**, 696–707.

# Effect of drugs on the synthesis of noradrenaline in guinea-pig vas deferens

MARGARET C. BOADLE-BIBER AND R. H. ROTH

*Departments of Pharmacology and Psychiatry, Yale University, School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, U.S.A.*

**Summary**

1. Reserpine *in vitro* ($10^{-5}$M) caused a profound inhibition (>85%) of the formation of both $^{14}$C-catecholamine ($^{14}$C-CA) and $^{14}$C-dihydroxyphenylalanine ($^{14}$C-DOPA) (in the presence of the amino acid decarboxylase inhibitor brocresine) from $^{14}$C-tyrosine in guinea-pig vas deferens. The magnitude of the inhibition was similar for both $^{14}$C-CA and $^{14}$C-DOPA suggesting that the inhibition occurred primarily at the tyrosine hydroxylase step.

2. One hour after *in vivo* treatment with reserpine (1 mg/kg) when tissue stores of noradrenaline (NA) were depleted by 50%, there was a significant inhibition of the formation of $^{14}$C-DOPA. Twenty-four hours after such treatment, when endogenous NA could no longer be detected, synthesis of $^{14}$C-DOPA was indistinguishable from untreated controls. However a 45% inhibition of $^{14}$C-DOPA synthesis from $^{14}$C-tyrosine could be produced in tissues which had been depleted of NA for 24 h or 48 h by the addition of reserpine, $10^{-5}$M, to the incubation medium.

3. Addition of pteridine cofactor, 2-amino-6,7,-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, to the incubation medium in a concentration of $5 \times 10^{-3}$M enhanced the formation of both $^{14}$C-CA and $^{14}$C-DOPA from $^{14}$C-tyrosine in guinea-pig vas deferens. In 52 mM KCl Krebs–Henseleit medium $^{14}$C-CA formation increased from $2{\cdot}58 \pm 0{\cdot}20$ (nmol/g)/h to $6{\cdot}35 \pm 0{\cdot}47$ (nmol/g)/h whilst $^{14}$C-DOPA formation increased from $5{\cdot}04 \pm 0{\cdot}88$ (nmol/g)/h to $11{\cdot}29 \pm 0{\cdot}59$ (nmol/g)/h.

4. Pteridine cofactor ($5 \times 10^{-3}$M) did not reverse the inhibition of $^{14}$C-DOPA formation seen with reserpine ($10^{-5}$M) in previously untreated tissues or in vasa deferentia from animals pretreated with reserpine 1 mg/kg for 24 hours. However, the inhibition did disappear in the presence of pteridine cofactor when treatment with reserpine was prolonged to 48 h and included two doses of reserpine of 2 mg/kg.

5. Tyramine ($5{\cdot}8 \times 10^{-5}$M) and bretylium ($10^{-5}$M) *in vitro* inhibited the formation of $^{14}$C-CA and $^{14}$C-DOPA from $^{14}$C-tyrosine to the same extent in guinea-pig vas deferens again indicating that their major site of action is on tyrosine hydroxylase. The inhibitory effects were reversed by pteridine cofactor.

6. Synthesis of $^{14}$C-NA from $^{14}$C-tyrosine in calf splenic nerve was not increased by incubating the tissue in 52 mM KCl-Krebs–Henseleit solution.

## Introduction

When sympathetically innervated tissues or brain slices are depolarized by electrical stimulation (Alousi & Weiner, 1966 ; Gordon, Reid, Sjoerdsma & Udenfriend, 1966 ; Roth, Stjärne & von Euler, 1966 ; 1967 ; Austin, Levitt & Chubb, 1967 ; Sedvall & Kopin, 1967 ; Weiner & Rabadjija, 1968 ; Sedvall, 1969) or by high concentrations of KCl (Roth, Boadle & Hughes, 1970 ; Boadle-Biber, Hughes & Roth, 1970 ; Harris & Roth, 1970, 1971 ; Harris, 1971) there is an acceleration in the formation of catecholamines (CA). This increase in synthesis of CA is apparently a result of an increase in the rate-limiting hydroxylation of tyrosine. It has been proposed that the biosynthesis of CA is accelerated when tyrosine hydroxylase is freed from end-product inhibition by the release or removal of catecholamine from a strategic site within the neurone.

We have examined the effects of various pharmacological agents on the enhanced biosynthesis of CA which is observed in the guinea-pig vas deferens preparation incubated with high concentrations of K. It was hoped that the use of some of these agents would lead to a better understanding of the localization of the pool of CA which seems to regulate the rate of CA biosynthesis. In addition we have looked to see whether the synthesis of CA in the axon region of calf splenic nerve is modified in the presence of high concentrations of potassium.

## Methods

Vasa deferentia from 300–400 g guinea-pigs were incubated for 1 h at 37° C in oxygenated Krebs–Henseleit bicarbonate solution or 52 mM KCl Krebs–Henseleit bicarbonate solution containing purified $^{14}$C-tyrosine (specific activity 10 mCi/mmol, final concentration $5 \times 10^{-5}$M) as described previously (Boadle-Biber *et al.*, 1970). In the initial experiments 4 vasa deferentia were incubated in 10 ml Krebs–Henseleit medium, but later the tissues were incubated singly or in pairs in 2 or 5 ml of medium. Wherever possible controls were matched with experimental tissues taken from the same animal and a paired analysis made. Drugs were made up in distilled water and added to the Krebs–Henseleit incubation medium in a volume not exceeding 0·1 ml per 10 ml incubation medium. Experimental tissues were preincubated with the test drug for 5 min in unmodified Krebs–Henseleit solution before transferring to the experimental media. During this time the paired control tissues remained in oxygenated Krebs–Henseleit solution. In some of the experiments the vasa deferentia were obtained from guinea-pigs that had been pretreated for various lengths of time with drugs (reserpine, 1 mg/kg or 2 mg/kg ; pargyline 75 mg/kg) which were administered intraperitoneally. In such cases unless specifically indicated no further drug was added to the incubation medium. A series of experiments were carried out with pteridine cofactor, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride ($DMPH_4$) added to the medium in a final concentration of $5 \times 10^{-3}$M. To prevent spontaneous oxidation of the pteridine cofactor, 2-mercaptoethanol ($5 \times 10^{-2}$M final concentration) was also included in the medium and controls were run in the presence of mercaptoethanol. There is a nonenzymic conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) in both the bath fluid and the tissues in the presence of $DMPH_4$ but this was corrected for by using an inhibitor of the enzymic formation of CA (see below). In all these experiments the formation of either $^{14}$C-labelled CA and catechol metabolites or of $^{14}$C-DOPA

was measured separately in bath fluids and tissues by alumina chromatography and liquid scintillation counting. To study formation of DOPA, incubations were carried out in the presence of the decarboxylase inhibitor, brocresine. In the presence of brocresine ($10^{-4}$M) more than 90% of the $^{14}$C-catechol compounds were identified as DOPA by Amberlite CG 120 cation exchange chromatography (Stjärne & Lishajko, 1967) (see **Results**). The procedures for the isolation of tissue CA and DOPA have been described earlier (Boadle-Biber *et al.*, 1970); those used for the analysis of the bath fluids were as follows: After the vasa deferentia had been removed from the incubation media, trichloracetic acid (TCA) (1·0 ml 100% w/v) was added to the bath fluids together with nonradioactive NA (50 $\mu$g), dopamine (50 $\mu$g), DOPA (50 $\mu$g), 3,4-dihydroxyphenylacetic acid (50 $\mu$g) and 3,4-dihydroxymandelic acid (50 $\mu$g) when CA formation was being measured or DOPA (50 $\mu$g) when the formation of DOPA only was determined. The procedure for isolating the CA, their deaminated metabolites and DOPA from the bath fluids by alumina chromatography resembled the one described earlier for the tissue extracts (Boadle-Biber *et al.*, 1970) except that potassium phosphate buffer (1 M pH 7·0) was added to the acidified medium (final concentration 0·1 M, McGeer, Gibson & McGeer, 1967) in addition to Tris buffer and disodium ethylene diamine tetra-acetic acid prior to bringing the pH to 8·4. The presence of potassium phosphate improves the recovery of tyrosine and non-catechol metabolites in the effluent from the column. CA was eluted with 2·0 ml 0·2 N perchloric acid (PCA) and 2 ml of water (recovery 83%). So little $^{14}$C-CA was present in the bath that it was ignored in computing total CA synthesis. In those experiments in which the formation of $^{14}$C-DOPA was measured, the DOPA present in the bath fluids which amounted to 40% of total $^{14}$C-DOPA formed, was eluted from the alumina column with 4·0 ml 0·5 N HCl. This gave a recovery of DOPA of $88{\cdot}4 \pm 0{\cdot}5\%$ ($n=12$). The $^{14}$C-DOPA isolated from the tissue extracts was always eluted with 4·0 ml 0·2 N PCA (recovery of $65 \pm 2\%$, $n=12$). In this way it was possible for determinations of endogenous NA concentrations also to be carried out on the alumina eluate. The non-enzymic conversion of $^{14}$C-tyrosine to $^{14}$C-DOPA in the bath fluid was determined by incubating vasa deferentia with $^{14}$C-tyrosine in the presence of $\alpha$-methyl-*p*-tyrosine (AMPT; $10^{-4}$M) or 3-iodotyrosine ($5 \times 10^{-3}$M) drugs which inhibit the formation of DOPA from tyrosine. The values obtained were similar in both cases. Bath and tissue blanks for the non-enzymic formation of $^{14}$C-catechol compounds from tyrosine in the presence and absence of $DMPH_4$ were determined by the use of 3-iodotyrosine ($5 \times 10^{-3}$M) to inhibit the synthesis of catechol compounds. In KCl-rich media the non-enzymic conversion of $^{14}$C-tyrosine to $^{14}$C-DOPA in the absence of $DMPH_4$ amounted to $1{,}680 \pm 47$ dpm ($n=8$) per 2 ml of medium, and in the presence of $DMPH_4$, $30{,}825 \pm 1{,}037$ dpm ($n=8$). These values are uncorrected for recovery from the alumina columns and were subtracted from the total counts of the incubation medium in experiments in which formation of $^{14}$C-DOPA was being measured so as to determine the amount of newly synthesized DOPA that had been released from the tissues. Values for the non enzymic formation of $^{14}$C-CA and $^{14}$C-DOPA in vas deferens were similar and when corrected for recovery from the alumina column amounted to $259 \pm 80$ (dpm/g)/h in the absence of $DMPH_4$ and $7{,}449 \pm 692$ (dmp/g)/h in the presence of $DMPH_4$.

Measurements were made of the specific activity of tyrosine in the tissues at the end of the incubation. The tyrosine-containing effluent from the alumina column was purified on DOWEX-50 columns by a slight modification of the procedure of

of Weiner & Rabadjija (1968) (Boadle-Biber & Roth, 1972) so as to remove any radiolabelled metabolites of tyrosine. The effluent from these columns was evaporated to dryness, taken up in 1·0 ml of water and the tyrosine content was determined by a minor modification of the procedure of Wong, O'Flynn & Inonye (1964) which has been described previously (Boadle-Biber *et al.*, 1970). No significant differences were observed between the specific activity of tyrosine in untreated and drug treated tissues.

Determinations of endogenous NA were made on the CA fraction isolated from the tissue extracts by alumina chromatography. For this the method of von Euler & Lishajko (1961a) in a modified form was used (Boadle-Biber *et al.*, 1970).

A number of experiments were carried out on splenic nerves obtained from freshly slaughtered calves. The nerves were kept on ice until they were dissected free from contaminating tissue. They were then desheathed and cut into lengths of approximately one centimetre. Samples of each individual nerve were distributed between all experimental and control groups. In this way it was possible to reduce the variability arising from the widely different biosynthetic capacities which are found in nerves from different animals.

Synthesis of NA in vasa deferentia or calf splenic nerve is expressed as (nmol NA/h)/g of fresh tissues ±S.E.M. Specific activities of the newly formed NA are given in dpm/$\mu$g endogenous NA ± S.E.M. In the calculation of the specific activity of NA a correction was made for the proportion of radiolabelled catechol compounds present as NA (71% for tissues incubated in control media and 77% for tissues treated with KCl-rich media, Boadle-Biber *et al.*, 1970). All values were corrected for recovery from the alumina columns.

### *Chemicals and drugs*

The following chemicals and drugs were used in this study: Brocresine (NSD 1055, CL 54998 (4-bromo-3-hydroxybenzyloxyamine)), Lederle division of American Cyanamid; cocaine hydrochloride, B.D.H.; tyramine hydrochloride (4-hydroxy-$\beta$-phenylethylamine hydrochloride), Calbiochem; (−)-noradrenaline bitartrate (NA), Calbiochem; $\alpha$-methyl-*p*-tyrosine (AMPT), Merck, Sharp & Dohme; 5-hydroxytryptamine creatinine sulphate (5-HT), Sigma; carbamylcholine chloride (Carbachol); pargyline (Eutonyl), Abbott; reserpine (Serpasil), Ciba; bretylium tosylate (Bretylate), Burroughs Wellcome; dibutyryl cyclic AMP, Schwartz; 2-mercaptoethanol, Eastman Organic Chemicals; 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride, Aldrich Chemical Co.; 3-iodotyrosine, Aldrich Chemical Co.

## Results

### *Effect of drugs on the formation of catecholamines in guinea-pig vas deferens*

The effects of some compounds on synthesis of NA from tyrosine in guinea-pig vas deferens incubated in unmodified and 52 mM KCl Krebs–Henseleit bicarbonate solution are summarized in Table 1. Table 2 shows the specific activity of NA isolated from the vasa deferentia in two of these experiments.

TABLE 1. *Effect of drugs on the synthesis and concentration of endogenous noradrenaline (NA) in guinea-pig vas deferens incubated in unmodified and 52 mM KCl Krebs–Henseleit medium*

| Drug treatment | Final drug concentration (M) | Synthesis of NA ((nmol/g fresh tissue)/h±S.E.M.) | | Endogenous NA (μg/g fresh tissue±S.E.M.) | |
|---|---|---|---|---|---|
| | | Control Krebs–Henseleit | 52 mM KCl Krebs–Henseleit | Control Krebs–Henseleit | 52 mM KCl Krebs–Henseleit |
| None | | 2·79±0·38 | 6·28±0·81 | 10·34±0·94 | 10·41±1·01 |
| Bretylium | $10^{-5}$ | 1·39±0·26 | 2·98±0·27 | 10·07±0·29 | 8·57±0·69 |
| | | $P<0·025$* | $P<0·005$* | | |
| None | | 2·20±0·25 | 4·32±0·15 | 12·87±0·95 | 13·69±0·63 |
| Tyramine | $5·8\times10^{-5}$ | 0·97±0·13 | 2·03±0·13 | 12·37±0·66 | 12·71±0·50 |
| | | $P<0·005$ | $P<0·001$ | | |
| None | | | 3·90±0·65 | | 14·00±0·83 |
| Reserpine | $10^{-5}$ | | 0·58±0·05 | | 11·11±0·72 |
| | | | $P<0·005$ | | $P<0·05$ |

Each result is the mean±S.E. of the mean of values obtained from four individual vasa deferentia.
* Comparison of data from drug-treated vasa deferentia with the corresponding untreated tissues using Student's *t* test.

*Bretylium*

In a final concentration of $10^{-5}$M bretylium caused a 50% inhibition of synthesis in both control and 52 mM KCl Krebs–Henseleit medium. There remained, however, the same percentage increase in synthesis in high KCl Krebs–Henseleit solution in the presence or absence of the drug. These changes were also reflected in the specific activities for NA.

TABLE 2. *Effect of drugs on the specific activity of newly synthesized noradrenaline (NA) in guinea-pig vas deferens*

| Drug treatment | Final drug concentration (M) | Specific activity of NA† (dpm/μg endogenous NA) Control Krebs–Henseleit | 52 mM KCl Krebs–Henseleit |
|---|---|---|---|
| None | | 3,718±271 | 9,033±370 |
| Bretylium | $10^{-5}$ | 1,793±251 | 5,258±265 |
| | | $P<0·005$* | $P<0·001$* |
| None | | 2,484±442 | 4,792±289 |
| Tyramine | $5·8\times10^{-5}$ | 1,098±158 | 2,422±196 |
| | | $P<0·05$ | $P<0·001$ |

* Comparison of data from drug-treated vasa deferentia with the corresponding untreated tissue using Student's *t* test.
† Each result is the mean±S.E. of the mean of values obtained from four individual vasa deferentia.

*Tyramine*

There was a 50% inhibition of NA synthesis in vasa deferentia incubated in both unmodified and 52 mM KCl Krebs–Henseleit solution in the presence of tyramine ($5·8\times10^{-5}$M).

*Reserpine*

With reserpine ($10^{-5}$M) in the high K incubation medium, an 85% inhibition of synthesis was seen.

*Other substances*

No alteration in the synthesis of NA from $^{14}$C-tyrosine was seen when vasa deferentia were incubated in unmodified Krebs–Henseleit medium in the presence

of 5-hydroxytryptamine ($5 \times 10^{-6}$M), carbachol ($5 \times 10^{-6}$M), dibutyryl cyclic AMP ($10^{-5}$; $10^{-4}$M), cocaine ($1{\cdot}5 \times 10^{-5}$M) or phenoxybenzamine ($2{\cdot}9 \times 10^{-5}$M).

### *Effect of drugs on the synthesis of DOPA*

The effect of drugs was studied on the rate-limiting step in the sequence of reactions leading from tyrosine to NA, that is to say the conversion of tyrosine to DOPA. The degree of inhibition of DOPA decarboxylase by brocresine was estimated by determining the fraction of total labelled catechol compounds present as DOPA, dopamine and NA. More than 90% of the $^{14}$C-catechols formed was DOPA. The results of the drug studies are summarized in Tables 3 and 4.

#### *Bretylium*

Synthesis of $^{14}$C-DOPA from $^{14}$C-tyrosine in KCl-rich Krebs–Henseleit medium was inhibited by about 40% in the presence of a bretylium concentration of $10^{-5}$M. At $10^{-6}$M no effect on synthesis of DOPA was observed. The drug produced no changes in endogenous concentrations of NA at either concentration.

TABLE 3. *Effect of drugs on the synthesis of $^{14}$C-DOPA from $^{14}$C-tyrosine in guinea-pig vas deferens incubated in 52 mM KCl Krebs–Henseleit solution in the presence of brocresine* ($10^{-4}$M)

| Drug treatment | Final drug concentration (M) | $n$* | Synthesis of $^{14}$C-DOPA ((nmol/g fresh tissue)/h ± S.E.M.) Tissue | Tissue and bath | $n$ | Endogenous NA ($\mu$g/g fresh tissue ± S.E.M.) |
|---|---|---|---|---|---|---|
| None | | 4 | 3·20±0·11 | 4·48 | 4 | 14·12±0·24 |
| Bretylium | $10^{-6}$ | 4 | 3·43±0·49 NS | 4·86 | 4 | 15·95±0·58 NS |
| | $10^{-5}$ | 4 | 1·75±0·09[d] | 2·51 | 4 | 14·75±0·90 NS |
| None | | 4 | 3·06±0·10 | 4·44 | | — |
| Tyramine | $5{\cdot}8 \times 10^{-5}$ | 4 | 1·82±0·18[c] | 2·39 | | — |
| None | | 3 | 4·10±0·33 | 5·79±0·59 | 6 | 14·60±2·24 |
| Pargyline† | | 3 | 1·92±0·24[b] | 2·85±0·44[a] | 6 | 19·21±1·79 NS |

* Number of individual vasa deferentia analysed. † Animals were treated with pargyline 75 mg/kg, 23 h beforehand. [a–d] Comparison of data from drug-treated vasa deferentia with that from the corresponding untreated tissues using Student's $t$ test. a=$P<0{\cdot}02$; b=$P<0{\cdot}01$; c=$P<0{\cdot}005$; d=$P<0{\cdot}001$. NS=not significant.

#### *Tyramine*

An inhibition in the formation of DOPA in vasa deferentia incubated in KCl-rich Krebs–Henseleit solution was observed in the presence of tyramine ($5{\cdot}8 \times 10^{-5}$M). The inhibition was of a similar magnitude to that seen for overall catecholamine formation.

#### *Pargyline*

Pretreatment with pargyline (75 mg/kg) given 23 h before the experiment resulted in an increase in the concentrations of endogenous NA and a large (>50%) inhibition of synthesis of $^{14}$C-DOPA in both control and KCl-rich Krebs-Henseleit solution.

#### *Reserpine*

Incubation of vasa deferentia in KCl-rich Krebs–Henseleit medium containing reserpine ($10^{-5}$M) produced a large inhibition (85%) of DOPA formation and a

concomitant reduction in the endogenous NA concentrations during the incubation period (Table 4). If, instead of adding the reserpine to the incubation medium, the animals were treated with the drug (1 mg/kg) 1 h before the incubation, an inhibition of $^{14}$C-DOPA formation was still seen, but this was not as large (only 40% compared with 85%). There was, however, a greater reduction (50%) in the concentration of endogenous NA compared with the untreated controls. If the reserpine was given 24 h before the experiment, then the synthesis of $^{14}$C-DOPA in the vasa deferentia incubated in KCl Krebs–Henseleit solution could not be

TABLE 4. *Effect of reserpine on synthesis of $^{14}$C-DOPA from $^{14}$C-tyrosine in guinea-pig vas deferens incubated in 52 mM KCl Krebs–Henseleit in the presence of brocresine* ($10^{-4}$ M)

| Time after treatment with reserpine (1 mg/kg) (h) | Concentration of reserpine added *in vitro* (M) | *n** | Synthesis of $^{14}$C-DOPA ((nmol/g fresh tissue)/h±S.E.M.) Tissue | Tissue and bath | *n** | Endogenous NA (μg/g±S.E.M.) |
|---|---|---|---|---|---|---|
| — | — | 6 | 3·62±0·39 | 5·91±0·72 | 6 | 12·57±0·86 |
| — | $10^{-5}$ | 4 | 0·88±0·08 | 1·02±0·13[d] | 6 | 9·50±0·84[a] |
| 1 | — | 4 | 1·87±0·18 | 2·99±0·22[b] | 6 | 6·95±1·2[c] |
| 3 | — | 4 | 2·33±0·07 | 4·14 | | — |
| 5·5 | — | 4 | 3·46±0·37 | 4·88 | | — |
| 24 | — | 4 | 4·78±0·34 | 7·23±0·34 NS | 6 | <0·4 μg/g |

* *n*=Number of individual vasa deferentia analysed. [a–d] Comparison of data from reserpine-treated vasa deferentia with that from the untreated tissues using Student's *t* test. a=$P<0·05$; b=$P<0·01$; c=$P<0·005$; d=$P<0·001$. NS=not significant, $P>0·05$.

distinguished from that observed in untreated tissues. At the same time the concentration of NA in the tissues treated with reserpine had fallen below the sensitivity of our fluorescence assay method (10 ng/ml or 40 ng per vas deferens). The question arises whether the inhibition seen with reserpine is related to the stores of endogenous amines remaining in the tissue. An experiment was therefore carried out to determine whether reserpine ($10^{-5}$M *in vitro*) would inhibit the formation of DOPA in vasa deferentia taken from guinea-pigs that had been treated with reserpine 24 h (1 mg/kg) or 48 h (2 doses, 2 mg/kg at 0 and 24 h) beforehand and in which endogenous NA could no longer be detected. The results in Table 5 indicate that there was an inhibition of DOPA synthesis of about 45% in both cases.

TABLE 5. *Effect of reserpine* ($10^{-5}$ M) *on synthesis of $^{14}$C-DOPA from $^{14}$C-tyrosine in guinea-pig vas deferens pretreated with reserpine and incubated in 52 mM KCl Krebs–Henseleit solution in the presence of brocresine* ($10^{-4}$ M)

| *In vivo* pretreatment duration and dose of reserpine | Concentration of reserpine added *in vitro* (M) | *n** | Synthesis of $^{14}$C-DOPA ((nmol/g fresh tissue)/h±S.E.M.) Tissue | Tissue and bath | Endogenous NA (μg/g±S.E.M.) | Synthesis of $^{14}$C-DOPA in presence of reserpine (% of control† ±S.E.M.) |
|---|---|---|---|---|---|---|
| — | — | 4 | 3·01±0·51 | 5·04±0·88 | 18·8±2·0 | |
| — | $10^{-5}$ | 4 | 0·64±0·12 | 1·20±0·36 | 15·3±1·1 | 22·5±3·0 |
| 24 h (1 mg/kg) | — | 6 | 6·88±0·64 | 11·56±1·01 | <0·4 μg/g | |
| 24 h (1 mg/kg) | $10^{-5}$ | 6 | 3·71±0·69 | 6·13±1·19 | <0·4 μg/g | 52·0±7·1[a] |
| 48 h (2 mg/kg twice) | — | 6 | 5·35±0·57 | 9·98±1·03 | <0·4 μg/g | |
| 48 h (2 mg/kg twice) | $10^{-5}$ | 6 | 3·67±0·52 | 5·55±0·90 | <0·4 μg/g | 55·0±5·9[a] |

* *n*=Number of individual vas deferens and bath fluids analysed for $^{14}$C-DOPA. † Synthesis of $^{14}$C-DOPA in vasa deferentia incubated in the presence of reserpine ($10^{-5}$ M) was expressed as a % of the synthesis for the contralateral control. [a] Comparison using Student's *t* test of the inhibitory effect of reserpine on the synthesis of $^{14}$C-DOPA in vasa deferentia pretreated for different periods with reserpine, with the inhibitory effect seen in control vasa deferentia which had received no pretreatment with the drug. $P<0·05$.

### *Experiments with pteridine cofactor*

Pteridine cofactor (2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, $DMPH_4$) accelerates the synthesis of $^{14}$C-CA from tyrosine in guinea-pig vasa deferentia. In control Krebs–Henseleit solution the synthesis of CA increased from $1{\cdot}34 \pm 0{\cdot}16$ (nmol NA/g fresh tissue)/h ($n=8$) to $4{\cdot}67 \pm 0{\cdot}47$ (nmol/g)/h ($n=8$) and exceeded that obtained for vasa deferentia incubated in KCl-rich Krebs solution ($2{\cdot}58 \pm 0{\cdot}20$ (nmol NA/g)/h, $n=8$). The synthesis of CA in KCl-rich Krebs fortified with cofactor was $6{\cdot}35 \pm 0{\cdot}47$ ($n=8$), a value which did not differ significantly from that obtained in the control Krebs solution in the presence of the cofactor. The rate of synthesis of CA was linear for at least 1 h in vasa deferentia incubated in the control and KCl-rich media fortified with pteridine.

When these experiments were repeated in the presence of the amino acid decarboxylase inhibitor brocresine it was found that the formation of DOPA was also increased by the pteridine cofactor from $5{\cdot}04 \pm 0{\cdot}88$ (nmol/g)/h to $11{\cdot}29 \pm 0{\cdot}59$ (nmol/g)/h in KCl-rich media.

An analysis of the $^{14}$C-catechol compounds formed from $^{14}$C-tyrosine in tissues incubated in KCl-rich Krebs–Henseleit both with and without added pteridine revealed that dopamine was the predominant catechol formed (Table 6). Practic-

TABLE 6. *Analysis* by Amberlite CG-120 chromatography of $^{14}$C-catechol compounds formed in guinea-pig vasa deferentia and the KCl-rich Krebs–Henseleit incubation medium in the presence and absence of pteridine cofactor ($DMPH_4$) during incubation† for 1 hour*

| | % of total $^{14}$C-catechol counts recovered from Amberlite column | | | |
|---|---|---|---|---|
| | | | $DMPH_4$ | |
| $^{14}$C-catechol metabolite | Tissue | Bath | Tissue | Bath |
| Deaminated metabolites | 9·8 | 24·9 | 7·6 | 9·0 |
| DOPA | 19·2 | 73·2 | 28·4 | 86·0 |
| Noradrenaline | 0 | 1·4 | 1·6 | 3·5 |
| Dopamine | 71·0 | 0·5 | 62·4 | 1·5 |

* $^{14}$C-catechol compounds were isolated by alumina chromatography and then identified by Amberlite CG-120 chromatography. † All tissues were incubated in the presence of mercaptoethanol ($5 \times 10^{-2}$ M).

ally no NA was found suggesting that the presence of 2-mercaptoethanol, a sulphydryl agent which was added to both media inhibits dopamine $\beta$-oxidase and thereby prevents the conversion of dopamine to NA. The predominant catechol compound isolated from the bath fluid was DOPA.

An attempt was made to see if the inhibition of CA or DOPA synthesis produced by drugs like bretylium, tyramine and reserpine could be reversed using the pteridine cofactor. In Table 7 the inhibitory effects of bretylium and tyramine on CA synthesis in vasa deferentia have almost completely disappeared in the presence

TABLE 7. *Effect of pteridine cofactor ($DMPH_4$) ($5 \times 10^{-3}$ M) on the inhibition of $^{14}$C-catecholamine (CA) synthesis produced by tyramine and bretylium in guinea-pig vas deferens incubated in 52 mM KCl Krebs–Henseleit medium*

| Drug | Concentration of drug (M) | $n$* | $^{14}$C-CA synthesis (% of control ± S.E.M.) | $+DMPH_4$ |
|---|---|---|---|---|
| Tyramine | $5{\cdot}8 \times 10^{-6}$ | 4 | $77{\cdot}6 \pm 2{\cdot}5$ | $88{\cdot}4 \pm 2{\cdot}8^{a}$ |
| Tyramine | $5{\cdot}8 \times 10^{-5}$ | 4 | $57{\cdot}7 \pm 6{\cdot}7$ | $84{\cdot}0 \pm 6{\cdot}0^{a}$ |
| Bretylium | $2 \times 10^{-5}$ | 4 | $61{\cdot}5 \pm 4{\cdot}7$ | $103{\cdot}7 \times 8{\cdot}2^{b}$ |

* Number of individual vasa deferentia analysed. [a–b] Significantly different from tissues not treated with $DMPH_4$. a = $P<0{\cdot}05$; b = $P<0{\cdot}002$.

of the cofactor. Table 8 summarizes the results obtained with $DMPH_4$ on the inhibition of synthesis of DOPA by reserpine ($10^{-5}$M). The cofactor did not reduce the inhibition of DOPA formation seen with reserpine ($10^{-5}$M) in previously untreated tissues or in vasa deferentia from animals pretreated with reserpine 1 mg/kg for 24 hours. However, the inhibition did disappear in the presence of $DMPH_4$ when pretreatment with reserpine was prolonged to 48 h and included two doses of reserpine of 2 mg/kg.

TABLE 8. *Effect of pteridine cofactor ($DMPH_4$) on the reserpine-induced inhibition of $^{14}$C-DOPA formation in guinea-pig vas deferens incubated in 52 mM KCl Krebs–Henseleit medium in the presence of brocresine ($10^{-4}$M)*

| *In vivo* pretreatment | Concentration of reserpine added *in vitro* (M) | *n** | Synthesis of $^{14}$C-DOPA† ((nmol/g fresh tissue)/h ± S.E.M.) Tissue | Tissue and bath | Endogenous NA (μg/g ± S.E.M.) | Synthesis of $^{14}$C-DOPA in presence of reserpine (% of control ± S.E.M.)‡ |
|---|---|---|---|---|---|---|
| — | — | 4 | 9·35±0·52 | 11·29±0·59 | 17·3 ±2·7 | |
| — | $10^{-5}$ | 4 | 3·15±0·39 | 3·15±0·39 | 14·2 ±1·3 | 28·4±4·4 |
| 24 h (1 mg/kg) | — | 3 | 7·30±0·41 | 12·03±0·82 | 0·79±0·17 | |
| 24 h (1 mg/kg) | $10^{-5}$ | 3 | 5·44±0·50 | 5·96±0·34 | 1·09±0·13 | 50·1±1·7[a] |
| 48 h (2 mg/kg twice) | — | 3 | 8·36±0·65 | 9·62±1·9 | <0·4 μg/g | |
| 48 h (2 mg/kg twice) | $10^{-5}$ | 3 | 8·24±0·83 | 9·07±1·4 | <0·4 μg/g | 96·0±8·3[b] |

* $n$ = Number of individual vasa deferentia and bath fluids analysed for $^{14}$C-DOPA. † The incubation medium contained $DMPH_4$ in a final concentration of $5 \times 10^{-3}$ M. ‡ Synthesis of $^{14}$C-DOPA in vasa deferentia incubated in the presence of reserpine ($10^{-5}$ M) is expressed as a % of the synthesis of the contralateral control tissue. [a–b] Comparison using Student's *t* test of the ability of $DMPH_4$ to reverse the inhibition of $^{14}$C-DOPA synthesis produced by reserpine ($10^{-5}$ M) in tissues pretreated for different periods with reserpine. a = $P<0.005$; b = $P<0.001$.

### *Effect of KCl-rich Krebs–Henseleit on the synthesis of catecholamines in calf splenic nerve*

Incubation of splenic nerve in 52 mM Krebs–Henseleit medium did not enhance the formation of catecholamines from $^{14}$C-tyrosine over that of control tissue. There was no significant difference between the overall amounts of CA isolated from the tissues incubated in the two media nor in the specific activity of the newly formed CA.

## Discussion

Experiments with splenic nerve demonstrated that nerve terminals and/or effector organs appear to be a prerequisite for the $K^+$-induced acceleration of CA synthesis. Thus incubation of portions of splenic nerve axons with high $K^+$ was observed to be without effect on the synthesis of CA from tyrosine. This observation was interpreted as being a result of the inability of $K^+$ to release a strategic pool of CA from the axon thereby relieving tyrosine hydroxylase from endproduct inhibition. Similar observations have also been reported for electrically stimulated splenic nerve (Roth *et al.*, 1967).

The observation that reserpine, *in vitro*, at a concentration of $10^{-5}$M produced a great inhibition of CA biosynthesis was not an unexpected finding since previous studies in heart (Rutledge & Weiner, 1967) and splenic nerve (Roth & Stone, 1968) have indicated that reserpine effectively blocks NA biosynthesis. However, in these earlier experiments it was suggested that the block in synthesis was occurring

primarily at the dopamine-$\beta$-hydroxylase step, whereas in the present study with the vas deferens, it appeared that the block in CA biosynthesis was taking place mainly at the initial tyrosine hydroxylase step. Thus when the conversion of DOPA to dopamine was prevented by including a decarboxylase inhibitor (brocresine) in the incubation medium, reserpine inhibited the formation of DOPA to the same extent as overall CA synthesis, that is about 85%. *In vivo* drug treatment also resulted in an inhibition of DOPA formation (and see Weiner, 1970). If guinea-pigs were pretreated with reserpine (1 mg/kg) 1 h beforehand, then the subsequent *in vitro* conversion of tyrosine to DOPA was reduced by 50% and there was an approximately 50% depletion of endogenous NA. If, on the other hand, the guinea-pigs were killed 24 h after being given reserpine, when the endogenous concentration of NA was depleted by more than 95%, no significant effect was observed on the conversion of tyrosine to DOPA. These experiments suggested that during the initial phase of the action of reserpine when NA is no longer accumulated in the storage granules and the extragranular concentration of NA is elevated (von Euler & Lishajko, 1961b, 1963 ; Kirshner, 1962 ; Kopin & Gordon, 1962) this extragranular pool of NA inhibits tyrosine hydroxylase. However, such a mechanism does not account for the observation that reserpine ($10^{-5}$M) produced a 45% inhibition of the formation of DOPA in tissues already depleted of endogenous NA unless one supposes that this high concentration of reserpine is able to mobilize residual NA present in the tissues in amounts that are not detected by our fluorescence assay (<40 ng per vas deferens or about 0·4 $\mu$g/g) but which are large enough to inhibit the formation of $^{14}$C-DOPA. *In vitro* experiments have indicated that catechol compounds can inhibit tyrosine hydroxylase by competing with the essential pteridine cofactor involved in the hydroxylation reaction (Ikeda, Fahien & Udenfriend, 1966). The observation that the addition of $DMPH_4$ relieves the inhibition produced by reserpine *in vitro* in vas deferens in which 95% or more of the endogenous NA had been depleted suggests that small amounts of extragranular CA may in fact play a significant role in the reserpine induced inhibition of DOPA formation. The fact that $DMPH_4$ is unable to reverse the inhibition seen with reserpine in tissues initially containing larger amounts of amine presumably reflects the fact that the CA compete effectively for both endogenous and exogenous cofactor under these conditions.

Both tyramine and bretylium inhibited the formation of DOPA in guinea-pig vas deferens to the same extent as they inhibited overall CA biosynthesis, again suggesting that the major site of the inhibition was the tyrosine hydroxylase step. Similar findings have been reported by Weiner for bretylium (Posiviata, Becker & Weiner, 1971) and tyramine (Weiner & Selvaratnum, 1968) on synthesis of $^3$H-CA from $^3$H-tyrosine in guinea-pig vas deferens hypogastric nerve preparations both in the resting state and when the nerve was stimulated. In view of the observation that neither tyramine (Weiner & Selvaratnum, 1968) nor bretylium (Posiviata *et al.*, 1971) have an effect on tyrosine hydroxylase activity in tissue homogenates, it seems likely that these drugs exert their inhibitory action indirectly. One possibility (Weiner, 1970) is that they mobilize a small pool of NA, and that this leads to an increase in the concentration of extragranular NA which then acts as a feedback inhibitor of tyrosine hydroxylase. That the inhibitory effects of both tyramine and bretylium on DOPA formation could be reversed in the presence of the enzyme cofactor, $DMPH_4$, a substance which interacts competitively with catechols, lends support to this proposal. Also consistent with this hypothesis is the observation that

tyramine can release NA directly from isolated storage granules (Schümann & Philippu, 1962; von Euler & Lishajko, 1968).

This work was supported in part by Grants from the USPHS, MH 14092 and NS 09389. We would like to thank Miss Ilona Mihaly and Mr. Karlis Rozitis for their excellent technical assistance. Gifts of Bretylate from Dr. Stanley T. Bloomfield, NSD 1055 from Dr. Robert Levine, α-methyl-*p*-tyrosine from Dr. Elmer Alpert and dibutyryl cyclic AMP from Dr. Paul Greengard, are gratefully acknowledged.

## REFERENCES

ALOUSI, A. & WEINER, N. (1966). The regulation of norepinephrine synthesis in sympathetic nerves: effect of nerve stimulation, cocaine and catecholamine releasing agents. *Proc. natl Acad. Sci., U.S.A.*, **56**, 1491–1496.

AUSTIN, L., LEVITT, B. G. & CHUBB, I. W. (1967). Increased synthesis and release of norepinephrine and dopamine during nerve stimulation. *Life Sci.*, **6**, 97–104.

BOADLE-BIBER, M. C., HUGHES, J. & ROTH, R. H. (1970). Acceleration of noradrenaline biosynthesis in the guinea-pig vas deferens by potassium. *Br. J. Pharmac.*, **40**, 702–720.

BOADLE-BIBER, M. C. & ROTH, R. H. (1972). Factors modifying the synthesis of dopamine from tyrosine in pedal ganglia of *Mercenaria mercenaria* (Mollusca). *General and Comparative Pharmac.*, **3**, 61–74.

GORDON, R., REID, J. V. D., SJOERDSMA, A. & UDENFRIEND, S. (1966). Increased synthesis of norepinephrine in the rat heart on electrical stimulation of the stellate ganglion. *Mol. Pharmac.*, **2**, 606–613.

HARRIS, J. E. (1971). Factors controlling the regulation of catecholamine biosynthesis in the brain. Ph.D. Thesis, Yale University.

HARRIS, J. E. & ROTH, R. H. (1970). Effect of potassium on catecholamine biosynthesis and release in rat brain cortical slices. *Fedn Proc.*, **29**, Abstract 941.

HARRIS, J. E. & ROTH, R. H. (1971). Potassium induced acceleration of catecholamine biosynthesis in brain slices: 1. A study on the mechanism of action. *Mol. Pharm.*, **7**, 593–604.

IKEDA, M., FAHIEN, L. A. & UDENFRIEND, S. (1966). A kinetic study of bovine adrenal tyrosine hydroxylase. *J. biol. Chem.*, **241**, 4452–4456.

KIRSHNER, N. J. (1962). Uptake of catecholamines by a particulate fraction of the adrenal medulla. *J. biol. Chem.*, **237**, 2311–2317.

KOPIN, I. J. & GORDON, E. K. (1962). Metabolism of $H^3$-norepinephrine released by tyramine and reserpine. *J. Pharm. exp. Ther.*, **138**, 351–359.

MCGEER, E. G., GIBSON, S. & MCGEER, P. L. (1967). Some characteristics of brain tyrosine hydroxylase. *Can. J. Biochem.*, **45**, 1557–1563.

POSIVIATA, M., BECKER, G. & WEINER, N. (1971). The effect of bretylium tosylate on catecholamine synthesis in the guinea pig isolated vas deferens preparation. *The Pharmacologist*, **13**, Abstract 344.

ROTH, R. H., BOADLE, M. & HUGHES, J. (1970). Acceleration of the rate-limiting step in norepinephrine biosynthesis by potassium. *Experientia*, **26**, 494–495.

ROTH, R. H., STJÄRNE, L. & VON EULER, U. S. (1966). Acceleration of noradrenaline biosynthesis by nerve stimulation. *Life Sci.*, **5**, 1071–1075.

ROTH, R. H., STJÄRNE, L. & VON EULER, U. S. (1967). Factors influencing the rate of norepinephrine biosynthesis in nerve tissue. *J. Pharmac. exp. Ther.*, **158**, 373–377.

ROTH, R. H. & STONE, E. A. (1968). The action of reserpine on noradrenaline biosynthesis in sympathetic nerve tissue. *Biochem. Pharmac.*, **17**, 1581–1590.

RUTLEDGE, C. O. & WEINER, N. (1967). The effect of reserpine upon the synthesis of norepinephrine in the isolated rabbit heart. *J. Pharmac. exp. Ther.*, **157**, 290–302.

SCHÜMANN, H. J. & PHILIPPU, A. (1962). The mechanism of catecholamine release by tyramine. *Int. J. Neuropharmacol.*, **1**, 179–182.

SEDVALL, G. C. (1969). Effect of nerve stimulation on accumulation and disappearance of catecholamines formed from radioactive precursors *in vivo*. In: *Metabolism of Amines in the Brain*, Proc. Symp. British and Scandinavian Pharmacological Societies, Edinburgh, July, 1968, ed. Hooper, G. London: Macmillan.

SEDVALL, G. C. & KOPIN, I. J. (1967). Acceleration of norepinephrine synthesis in the rat submaxillary gland *in vivo* during sympathetic nerve stimulation. *Life Sci.*, **6**, 45–51.

STJÄRNE, L. & LISHAJKO, F. (1967). Localisation of different steps in noradrenaline synthesis to different fractions of a bovine splenic nerve homogenate. *Biochem. Pharmac.*, **16**, 1719–1728.

VON EULER, U. S. & LISHAJKO, F. (1961a). Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. Scand.*, **51**, 349–356.

VON EULER, U. S. & LISHAJKO, F. (1961b). Effect of reserpine on the release of catecholamines from isolated nerve and chromaffin cell granules. *Acta physiol. Scand.*, **52**, 137–145.

VON EULER, U. S. & LISHAJKO, F. (1963). Effect of adenine nucleotides on catecholamine release and uptake in isolated adrenergic nerve granules. *Acta physiol. Scand.*, **59**, 454–461.

VON EULER, U. S. & LISHAJKO, F. (1968). Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules. *Acta physiol. Scand.*, **73**, 78–92.

WEINER, N. (1970). Regulation of norepinephrine biosynthesis. *Ann. Rev. Pharmac.*, **10**, 273–290.

WEINER, N. & RABADJIJA, M. (1968). The effect of nerve stimulation on the synthesis and metabolism of norepinephrine in the isolated guinea-pig hypogastric nerve vas deferens preparation. *J. Pharmac. exp. Ther.*, **160**, 61–71.

WEINER, N. & SELVARATNUM, I. (1968). The effect of tyramine on the synthesis of norepinephrine. *J. Pharm. exp. Ther.*, **161**, 21–33.

WONG, P. W. K., O'FLYNN, M. E. & INONYE, T. (1964). Micromethods for measuring phenylalanine and tyrosine in serum. *Clin. Chem.*, **10**, 1098–1104.

(*Received February* 22, 1972)