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Acceleration of noradrenaline biosynthesis in the guinea-pig vas deferens by potassium

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Summary

1. Increasing the concentration of KCl in Krebs-Henseleit bicarbonate solution enhanced the formation of ^{14}C-noradrenaline (^{14}C-NA) from ^{14}C-tyrosine in the guinea-pig vas deferens. In 52 mM KCl Krebs-Henseleit solution the specific activity of the newly formed ^{14}C-NA was double that of controls.

2. The rate of synthesis of ^{14}C-NA from ^{14}C-tyrosine was constant for up to 2 h in 52 mM KCl Krebs-Henseleit solution and for 4 h in unmodified Krebs-Henseleit solution.

3. There was no increase in NA formation in the presence of KCl rich Krebs-Henseleit solution if ^{14}C-DOPA was used as the starting substrate instead of ^{14}C-tyrosine.

4. The specific activity of ^{14}C-tyrosine in the high KCl treated vas deferens was 80% of that of control tissues. Thus the enhanced synthesis of ^{14}C-NA in high KCl Krebs-Henseleit solution did not arise from an increase in the specific activity of precursor.

5. The effect of K^+ on NA synthesis was not mimicked by ganglionic stimulants nor blocked by tetrodotoxin.

6. Removal of Ca^{2+} ions or increasing the concentration of Mg^{2+} ions abolished the increase in synthesis of NA seen in high KCl Krebs-Henseleit solution but left the basal rate of NA synthesis in unmodified Krebs-Henseleit solution unaltered.

7. The spontaneous release of newly synthesized catecholamines (^{14}C-labelled) or tritiated noradrenaline (^{3}H-NA) from vasa deferentia was increased in 52 mM KCl Krebs-Henseleit solution. Removal of Ca^{2+} ions reduced the increased efflux of newly synthesized amine in high KCl media to that seen in unmodified Krebs-Henseleit solution. The efflux of ^{3}H-NA was reduced to one-third of its former rate in the absence of Ca^{2+}.

8. High KCl Krebs-Henseleit solution caused a substantial contraction of the vas deferens which was not abolished by tetrodotoxin. Release of ^{3}H-NA paralleled the contractile response, and was likewise unaffected by tetrodotoxin.

9. No evidence was obtained for any alterations in the activity of tyrosine hydroxylase, the rate limiting enzyme in the formation of NA from tyrosine, in homogenates of vas deferens which had been treated with 52 mM KCl Krebs-Henseleit solution.

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10. These results support the hypothesis that acceleration of NA synthesis occurs when tyrosine hydroxylase is freed from end-product inhibition by the release of noradrenaline, brought about in this case, by high concentrations of KCl.

Introduction

It appears that the rate of noradrenaline (NA) biosynthesis in peripheral sympathetic tissues and also in the central nervous system (CNS) is determined by the level of nervous activity in the adrenergic neurones. Observations made on several sympathetically innervated tissues (the vas deferens, salivary gland and heart) indicate that the rate of formation of ^{14}C-NA from ^{14}C-tyrosine increases when the post-ganglionic sympathetic neurones are stimulated *in vitro* or *in situ* (Alousi & Weiner, 1966; Roth, Stjärne & von Euler, 1966, 1967; Austin, Levitt & Chubb, 1967a; Sedvall, 1969) or when sympathetic tone is increased *in vivo* (Oliverio & Stjärne, 1965; Gordon, Spector, Sjoerdsma & Udenfriend, 1966b; Gordon, Reid, Sjoerdsma & Udenfriend, 1966a; Spector, 1966; Dairman, Gordon, Spector, Sjoerdsma & Udenfriend, 1968; Dairman & Udenfriend, 1970). The ability to modulate NA synthesis according to the physiological demands made on the tissue appears to be a property which is unique to the nerve terminal region. In the axon portion of a pure sympathetic neurone, the splenic nerve, where no transmitter release occurs, synthesis of NA proceeds rapidly but is not increased in response to nerve stimulation (Roth *et al.*, 1967).

The investigation of factors modulating NA synthesis at sympathetic nerve endings has relied heavily on techniques involving electrical stimulation of the nerve supply to various tissues. Preliminary experiments in this laboratory indicated that K^+ ions also accelerate NA synthesis (Roth, Boadle & Hughes, 1970). We have now examined this further in the hope that this technique would help to simplify investigative procedures and elucidate the mechanism or mechanisms whereby NA synthesis is controlled.

Some of these results were communicated to the Federation of American Societies for Experimental Biology (Boadle-Biber & Hughes, 1970).

Methods

Studies on synthesis of NA

Vasa deferentia from guinea-pigs weighing 300–400 g were incubated at 37° C in 10 ml of oxygenated Krebs-Henseleit bicarbonate solution or modified Krebs-Henseleit bicarbonate solution containing purified ^{14}C-L-tyrosine (L-tyrosine-^{14}C-uniformly labelled, New England Nuclear Corp.), specific activty 10 mCi/mmol, in a final, saturating concentration of 5×10^{-5} M (Weiner & Rabadjija, 1968) (see below for purification procedure). In preliminary experiments the synthesis of ^{14}C-catecholamines from ^{14}C-tyrosine in the vas deferens was found to be linear for 4 h in control Krebs-Henseleit medium and for up to 2 h in 52 mM KCl Krebs-Henseleit medium, a modified Krebs-Henseleit medium used frequently in this study. Therefore, in the experiments that follow an incubation time of 1 h was employed. Since a saturating concentration of ^{14}C-tyrosine was present in the incubation medium the amount of ^{14}C-catecholamine formed in the vas deferens

during 1 h gave us an estimation of the overall rate of catecholamine synthesis. Using the starting specific activity of tyrosine (10 mCi/mmol) and the fact that one ninth of the radioactivity is lost in the conversion of ^{14}C-tyrosine to ^{14}C-dopamine (DA), the results were converted from d.p.m. to (nmol catecholamine/g fresh tissue)/h. Since the ^{14}C-tyrosine in the bath had not equilibrated completely with the tissues at the end of 1 h (the specific activity of the total tyrosine in the tissues was less than that of tyrosine in the bath) these results may give a conservative estimate of catecholamine synthesis. In one set of experiments the tyrosine was replaced by ^{14}C-DOPA (DL-3,4-dihydroxyphenylalanine-2-^{14}C, specific activity 3·94 mCi/mmol, New England Corp.) in a final concentration of $2{\cdot}54 \times 10^{-4}$M. At the end of the incubation period the tissues were removed from the medium, blotted, frozen on solid carbon dioxide, weighed and then homogenized in 4·0 ml 15% trichloracetic acid (TCA, analytical reagent). In the experiment in which ^{14}C-DOPA was used as starting substrate, homogenization of the tissues was carried out in 0·4 M perchloric acid (PCA). The following unlabelled compounds were added to the homogenates: L-tyrosine (50 μg); L-noradrenaline (NA, 50 μg); DA (50 μg), except when determinations were to be made of the total endogenous NA and tyrosine present in the tissues. Tissue extracts were stored at $-20°$ C. Precipitated protein was removed from the samples by centrifugation (10,000 rev/min for 10 min) and the supernatants were analysed for ^{14}C-labelled catecholamines by chromatography on alumina columns, or, in the case of experiments in which ^{14}C-DOPA was the starting substrate, by ion exchange chromatography on Amberlite CG-120 columns.

Studies on release of NA

The release of endogenous NA from the vas deferens was examined by using either ^{14}C-NA, formed from ^{14}C-tyrosine, or tritiated noradrenaline (^{3}H-NA) as markers for the endogenous NA. In the experiment utilizing ^{14}C-NA, vasa deferentia were first incubated for 1 h in ^{14}C-tyrosine and then placed individually in 5 ml of control or high KCl Krebs-Henseleit solution containing 10 μg/ml ascorbic acid in the presence or absence of $CaCl_2$. The time course of release of ^{14}C-NA was followed by transferring each vas deferens to fresh media at 5, 15, 30 and 60 minutes. The tissues and media were analysed for ^{14}C-catechol compounds by alumina chromatography. The ^{14}C-catechols released into the bathing medium were expressed as a percentage of the total ^{14}C-catechols found in the tissue and its bathing media at the end of the 1 h incubation. Two types of experiment were carried out with ^{3}H-NA. In the first type, vasa deferentia were opened longitudinally and then loaded with ^{3}H-NA by incubation for 1 h at 37° C in Krebs-Henseleit solution containing DL-7-^{3}H-NA (9 μCi/ml, 20 ng/ml) and ascorbic acid (20 μg/ml). At the end of this time the vasa deferentia were rinsed twice in normal Krebs-Henseleit solution and then left in 10 ml fresh Krebs-Hensleit solution for 15 min at 37° C. The amount of unchanged ^{3}H-NA and deaminated metabolites released into the medium was determined by analysis of the bathing medium by alumina chromatography and was expressed as a percentage of the ^{3}H-NA remaining in the tissue at the end of the experiment. In the second type of experiment a single vas deferens was loaded with ^{3}H-NA by incubating for 1 h at 37° C in Krebs-Henseleit solution containing DL-7-^{3}H-NA (5 μCi/ml, 20 ng/ml) and ascorbic acid (20 μg/ml). It was thoroughly rinsed with fresh Krebs-Henseleit solution and then suspended in a superfusion apparatus (Hughes & Roth, 1969) at a resting tension

of 2 g. Samples of the superfusion fluid which had passed over the vas deferens were collected for 30 s periods directly into scintillation vials. The mechanical response of the vas deferens to the high KCl Krebs-Henseleit solution was recorded semi-isometrically with an F-103 Grass Force displacement transducer. The vas deferens could be stimulated transmurally with two platinum electrodes on either side of the tissue.

Solutions

Krebs-Henseleit solution

The Krebs-Henseleit solution used in these experiments had the following composition: NaCl, 118·07 mM ; KCl, 4·75 mM ; $CaCl_2$, 2·54 mM ; KH_2PO_4, 1·19 mM ; $MgSO^4$, 1·19 mM ; $NaHCO_3$, 25·00 mM ; glucose, 11·10 mM ; EDTA, 0·027 mM.

Modified Krebs-Henseleit solution

Varying proportions of NaCl were replaced by equimolar amounts of KCl. Sucrose and choline chloride were also used as NaCl substitutes. For most of the experiments the modified Krebs-Henseleit solution had the following composition: NaCl, 70·84 mM ; KCl (4·75 + 47·23) mM ; $CaCl_2$, 2·54 mM ; KH_2PO_4, 1·19 mM ; $MgSO_4$, 1·19 mM ; $NaHCO_3$, 25·00 mM ; glucose, 11·10 mM ; EDTA, 0·027 mM. In some experiments the concentration of KCl was increased without altering the concentration of NaCl. In such cases controls were made hypertonic by the addition of sucrose in amounts osmotically equivalent to the excess KCl in the corresponding experimental medium. Other changes made to the Krebs-Henseleit and modified Krebs-Henseleit media are described in the text.

Purification of ^{14}C-L-tyrosine

A solution containing 0·1 mCi L-tyrosine (L-tyrosine-^{14}C-uniformly labelled, specific activity 352 mCi/mmol, New England Nuclear Corp.), 0·2 ml 1 M Tris buffer, pH 8·2, and 1·8 ml L-tyrosine (1 mg/ml) was adjusted to a pH of 8·4 with NaOH, and run twice through an alumina column (1·5 × 0·4 cm). The effluent from the column was collected and made up to 8·0 ml with distilled water washings from the column. A portion (0·4 ml) of this stock tyrosine was added to 9·6 ml Krebs-Henseleit incubation medium to give a final concentration of 5×10^{-5} M.

Column chromatography

Alumina chromatography

This procedure, which was modified from that of von Euler & Lishajko (1959) (see Roth & Stone, 1968), allows the separation of catecholamines which adhere to alumina at pH 8·4 from tyrosine and O-methylated catecholamine metabolites, which are covered quantitatively in the effluent. The alumina (British Drug Houses Ltd) was washed repeatedly to remove fines and then poured to give columns 1·5 × 0·4 cm in size. Tris buffer (0·2 ml, 1M, pH 8·2) and EDTA (to a final concentration of 10 mg/ml) were added to the tissue extracts, the pH of which was then adjusted

to between 8·3 and 8·5 with NaOH. The samples were passed through the columns immediately and recycled once. The effluent was collected and made up to 10 ml with washings of distilled water. Then the column was washed with 20–30 ml distilled water, to remove residual tyrosine, and blown dry. Catecholamines and catechol metabolites were eluted by suspending the alumina in 2·0 ml 0·2 M PCA, draining, and then washing with 2·0 ml 0·1 N PCA or 2·0 ml distilled water. The column was blown dry so as to give a total volume of eluate of 4·0 ml. One ml of each of the eluate and effluent were taken for scintillation spectrometry. The recovery of catechol compounds in the acid eluate from the alumina column was $83 \pm 6\%$ for NA ($n=34$); $81 \pm 4\%$ for dopamine ($n=74$) and $65 + 2$ for DOPA ($n=12$). Reported values are not corrected for recovery.

Ion exchange chromatography

Amberlite CG-120 columns were used to separate DA, NA, their deaminated metabolites and DOPA in the PCA eluates from the alumina columns and in the PCA tissue extracts obtained from the experiment in which ^{14}C-DOPA was used as catecholamine precursor instead of ^{14}C-tyrosine (Stjärne & Lishajko, 1966; Roth & Stone, 1968).

Scintillation counting

All samples were counted in a Packard Scintillation Counter for 10 min or to a standard deviation of less than 2·5%. Radioactivity in c.p.m. was converted to d.p.m. by means of the Packard Automatic External Standardization technique. The samples were counted in 10 ml of a scintillation fluid made up as follows: 1 litre Dioxane (Spectral quality); 1 litre toluene; 1 litre ethanol; 0·3 g dimethyl-POPOP (1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)-benzene, Scintillation Grade; Packard Instrument Co., Inc.); 15 g PPO (2,5-diphenyloxazole, Scintillation Grade, Packard Instrument Co., Inc.); 240 g naphthalene.

Determination of endogenous NA

The method used was based on that of von Euler & Lishajko (1961). 0·5 ml samples of the acid eluate from the alumina columns were adjusted to pH 6·5 with 0·3 of 0·5 N KH_2PO_4 buffer, pH 7·0. Potassium ferricyanide (0·05 ml of a 0·25% solution) was added to the reaction mixture which was agitated vigorously on a Vortex mixer and allowed to stand for exactly 3 minutes. Freshly prepared alkaline ascorbate (1·0 ml) was added and the fluorescence read on the Aminco Bowman fluoro microphotometer, using a primary filter, with a peak narrow band pass of 405 nm and a secondary filter (WRATTEN-65A) with a peak narrow band pass of 495 nm. The alkaline ascorbate contained 10 ml 2% (w/v) ascorbic acid in water and 2 ml 1,2-diaminoethane made up to 100 ml with 5 N NaOH. Blanks were prepared from 0·5 ml fractions of the same samples by adding the alkaline ascorbate before the potassium ferricyanide.

Determination of endogenous tyrosine

The method of Wong, O'Flynn & Inonye (1964) (and see Udenfriend, 1962) was used with slight modifications. The volume of the tyrosine sample was changed from 20 μl to 500 μl and 500 μl instead of 200 μl of the l-nitroso-2-naphthol-nitrate-nitrite reagent was added to the reaction mixture. Double volumes of water and ethylene dichloride were also used.

Assay for tyrosine hydroxylase

A 20% (w/v) homogenate of vasa deferentia from twelve animals was prepared in 0·1 M NaH_2PO_4 buffer, pH 7·0, using either an Ultra Turrax (Kebjo, Sweden) or a Kontes all glass homogenizer. Unbroken cells, nuclei and other debris were removed by centrifugation for 10 min at 10,000 *g* and 0·3–0·5 ml samples of the remaining supernatant fraction were taken for the tyrosine hydroxylase assay. For this the procedure of Nagatsu, Levitt & Udenfriend (1964) was employed with minor changes. The reagents present in 1 ml of reaction medium in the final concentrations were: ^{14}C-L-tyrosine, specific activity 10 mCi/mmol, in a final concentration of 5×10^{-5} M; $FeSO_4$, 10^{-5} M; the aromatic amino-acid decarboxylase inhibitor NSD-1055, 10^{-4} M; mercaptoethanol, 10^{-2} M; freshly prepared solution of tetrahydropteridine (2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride), 10^{-3} M; KH_2PO_4/CH_3COONa buffer, 0·1 M; pH 6·0 and up to 0·5 ml tissue homogenate. The reaction which was carried out at 37° C, was started by addition of the enzyme. It was found that the $FeSO_4$ could not be left standing in the presence of tetrahydropteridine for any length of time as a precipitate formed. Therefore, $FeSO_4$ was added immediately before the start of the experiment. The reaction was terminated after 5, 10, 15 or 20 min by addition of 2·0 ml of 0·4 M PCA. Non-radioactive carrier substances, L-DOPA (50 μg) and L-tyrosine (50 μg), were added after the acid precipitation. The samples were centrifuged for 10 min at 10,000 rev/min and the supernatants analysed for ^{14}C-DOPA by alumina chromatography as described above. In the elution step 4·0 ml 0·5 N HCl was used instead of PCA. The recovery of DOPA was $88 \pm 3\%$ ($n=8$).

Chemicals and drugs

The following chemicals and drugs were used in this study: McNeil's Compound, McN - A - 343, 4 - (3 - chlorophenylcarbamoyloxy) - 2 - butyryl trimethylammonium chloride, McNeil Laboratories, Inc., Fort Washington, Pennsylvania; nicotine, Eastman Organic Chemical Co.; tetrodotoxin, Calbiochem; tetrahydropteridine (2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride), Aldrich Chemical Co.; brocresine, NSD 1055; Cl 54,998 (4-bromo-3-hydroxybenzyl oxyamine), Lederle Division of American Cyanamid.

Results

Effects of changing the Na^+ and K^+ composition of the medium

Biosynthesis of ^{14}C-catecholamines from ^{14}C-tyrosine was studied in guinea-pig vasa deferentia incubated in a Krebs-Henseleit medium in which part of the NaCl was replaced with sucrose, choline chloride or KCl. An enhancement of synthesis of ^{14}C-catecholamine occurred when 50% of the NaCl was replaced with KCl (Table 1). Choline chloride and sucrose did not have this effect.

In the following experiment the NaCl in the medium was replaced by equimolar amounts of KCl and the amount and specific activity of NA formed in 1 h was determined (Fig. 1). A stimulation of catecholamine synthesis was seen when the concentration of KCl reached 28 mM (20% NaCl replaced with KCl) and it attained a maximum at 52 mM KCl (40% NaCl replaced with KCl). Thereafter, the acceleration in catecholamine synthesis fell off rapidly with increasing concentra-

tions of KCl, and when all the NaCl had been substituted by KCl there was no difference between rates of catecholamine synthesis in the control or high K^+ media. The acceleration of catecholamine synthesis seen in the presence of high concentrations of KCl is evident both from the increase in the total amount of labelled catecholamines present per gramme vas deferens after 1 h and from the increase in the specific activity of the NA isolated from this tissue (Fig. 1).

The diminished acceleration of catecholamine biosynthesis observed when the concentration of KCl in the incubation medium rose above 52 mM may have

TABLE 1. *Effect of changes in the Na^+ and K^+ composition of the Krebs-Henseleit incubation medium on the conversion of ^{14}C-tyrosine to ^{14}C-NA in guinea-pig vas deferens*

% NaCl replaced	Substituent	NA synthesis (nmol/g tissue)/h
0	—	2·09±0·14 (12)
50	Choline chloride	2·17±0·27 (4)
100	Choline chloride	2·74±0·29 (4)
50	Sucrose	2·03±0·15 (8)
50	KCl	3·61±0·14* (3)
100	KCl	2·05±0·28 (4)

Results expressed as mean±S.E. of mean. Figures in parenthesis give the number of individual vasa deferentia analysed. Comparison of tissues incubated in modified Krebs-Henseleit solution with those incubated in normal Krebs-Henseleit solution: $*P<0·05$.

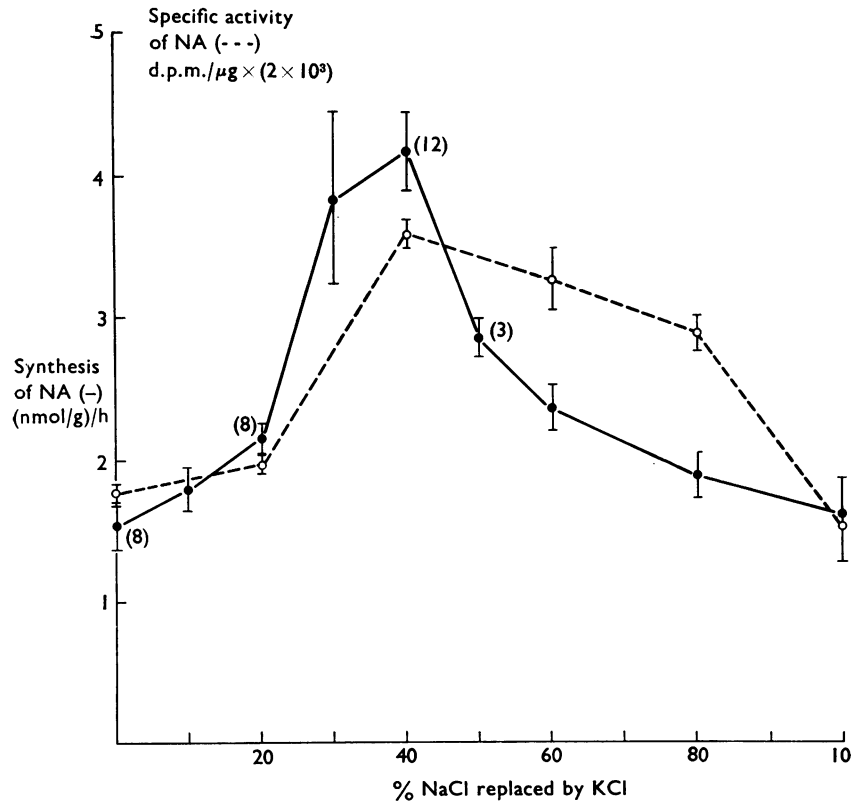

FIG. 1. Effect of increasing concentrations of KCl on the synthesis of ^{14}C-NA from ^{14}C-tyrosine (—) and on the specific activity of the newly formed NA (- - -) in guinea-pig vasa deferentia. The points are the mean of values from four individual vasa deferentia±S.E. of mean, except where indicated otherwise by figures in parenthesis.

resulted from the high KCl concentration or the lowered concentration of NaCl in the medium. To distinguish these two possibilities the rate of synthesis of ^{14}C-catecholamines in the vas deferens was studied in modified Krebs-Henseleit media containing increasing concentrations of KCl but normal concentrations of NaCl. In Fig. 2 the catecholamine formed is plotted against the concentration of KCl. There was an acceleration of CA biosynthesis in the presence of 52 mM KCl. At higher concentrations of KCl the rate of catecholamine synthesis levelled off instead of falling as in the earlier experiment when the concentration of NaCl dropped below 60% of normal (70·5 mM).

Analysis of KCl induced acceleration of catecholamine biosynthesis

Time course

The rate of catecholamine synthesis was constant for 2 h in 52 mM KCl Krebs-Henseleit solution but levelled off more quickly than in control Krebs-Henseleit solution (Fig. 3).

Effect of potassium on the uptake of ^{14}C-tyrosine

The ^{14}C-tyrosine concentrations in vas deferens incubated in high KCl Krebs-Henseleit solution (>60% NaCl replaced with KCl) were significantly lower than

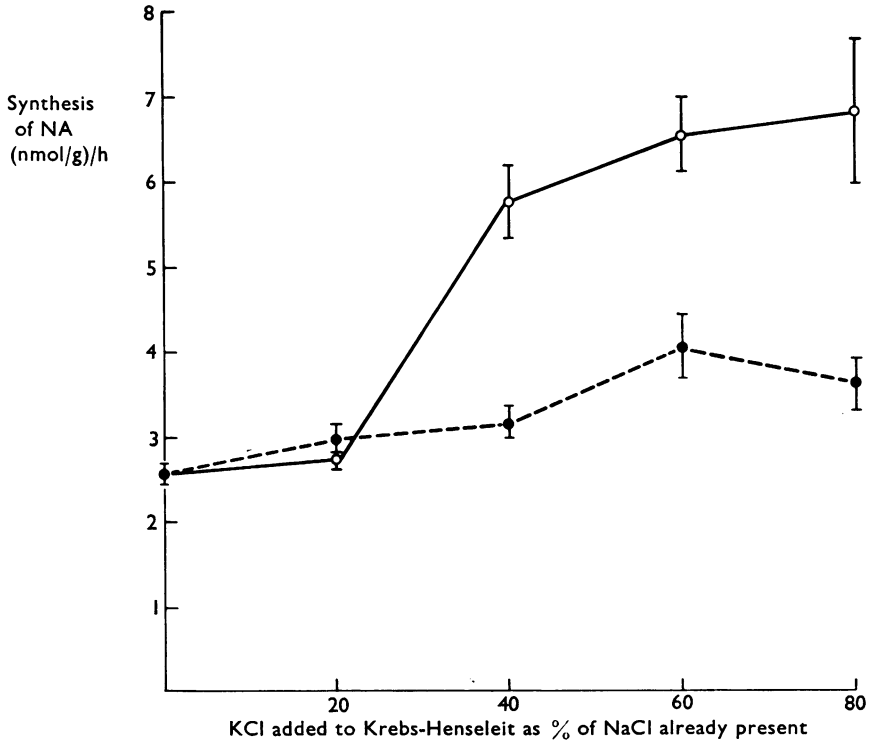

FIG. 2. Effect of increasing concentrations of KCl, in the presence of normal NaCl concentrations, on the synthesis of NA from ^{14}C-tyrosine in guinea-pig vasa deferentia (—). Controls (- - -) were made appropriately hypertonic by the addition of sucrose. Each point represents the mean of values from four individual vasa deferentia ± S.E. of mean.

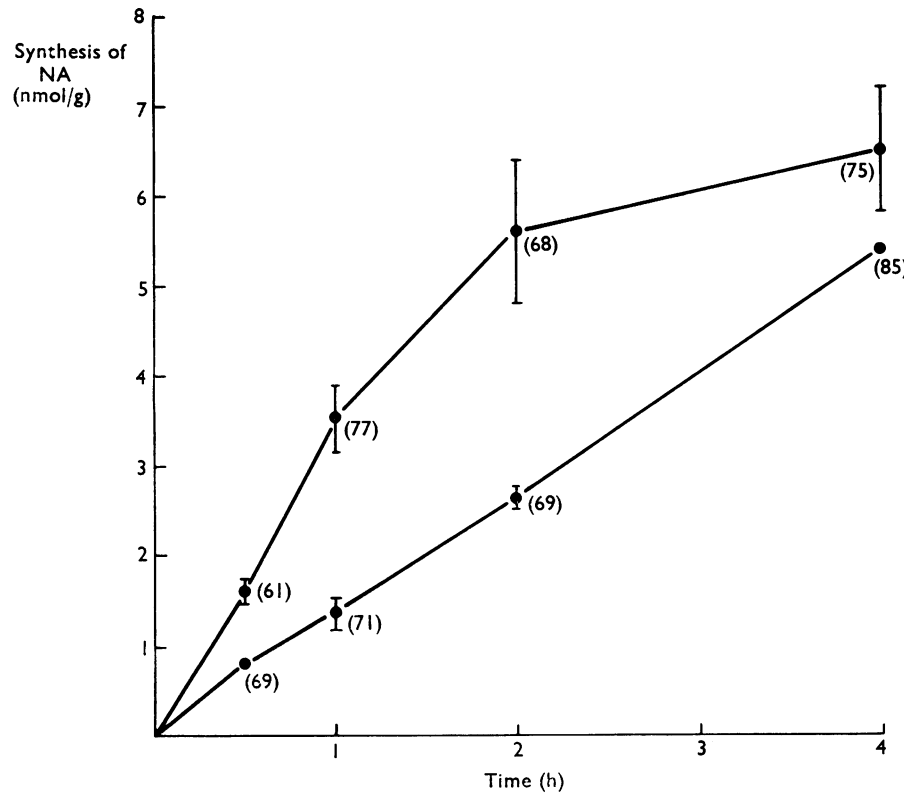

FIG. 3. Time course of synthesis of ^{14}C-catecholamines and ^{14}C-catechol compounds from ^{14}C-tyrosine in guinea-pig vasa deferentia. Lower curve: unmodified Krebs-Henseleit solution. Upper curve: Krebs-Henseleit solution in which 40% of the NaCl had been replaced with KCl giving a total KCl concentration of 52 mM. Each point on the curve is the mean ± S.E. of mean of determinations on four individual vasa deferentia, except for two points which are the mean of two observations only. Figures in parenthesis indicate the % of catechols present as NA in samples pooled from individual vasa deferentia at each time point.

TABLE 2. *^{14}C-tyrosine content of guinea-pig vas deferens at the end of 1 h incubation in Krebs-Henseleit solution containing increasing concentrations of KCl*

% NaCl replaced with KCl	Concentration of KCl (mM)	*n**	^{14}C-tyrosine content (%)§	*n**	Specific activity of tyrosine† (d.p.m./μg
0	4·8	8	100	18	52059 ± 3760
10	16·6	4	102 ± 14		
20	28·4	8	90 ± 7		
30	40·2	4	97 ± 10		
40	52·0	12	95 ± 7	17	41320 ± 2066‡
50	63·8	3	97 ± 4		
60	75·6	4	67 ± 3		
80	99·2	4	66 ± 4		
100	122·8	4	67 ± 8		

* *n*, Number of individual vasa deferentia analysed.
† Specific activity of tyrosine in the incubation medium. 121,500 d.p.m./μg.
‡ Significant at the 0·05 level when compared with controls.
§ ^{14}C-tyrosine content of vasa deferentia (d.p.m./g tissue) expressed as % of the control value (1·95 ± 0·016 × 10^6 d.p.m./g) ± S.E. of mean.

the concentrations in the controls (Table 2). However, the ^{14}C-tyrosine content of tissues exposed to high KCl concentrations in the presence of normal NaCl concentrations did not differ significantly from that of the controls. The specific activity of tyrosine isolated from vas deferens incubated for 1 h in 52 mM KCl Krebs was 80% of the control value.

Ganglionic mechanisms

Since high concentrations of KCl may excite the sympathetic neurones of the vas deferens by ganglionic stimulation we examined the possibility that ganglion stimulating agents might mimic the effects of potassium. Nicotine (10 μg/ml and 200 μg/ml) and McNeil's compound, McN-A-343 (1 μg/ml and 10 μg/ml) (Roszkowski, 1961) did not produce any significant alteration in the basal rate of catecholamine synthesis in the vas deferens.

As a further check on a possible ganglionic site of action for potassium, the ganglia were removed from the vas deferens by stripping off the outer serous coat (Birmingham, 1970). Under these conditions an acceleration of catecholamine biosynthesis was still observed in the presence of high KCl (Table 3).

Tetrodotoxin

Tetrodotoxin abolishes sympathetic nerve activity without affecting smooth muscle responsiveness (Toida & Osa, 1965; Bülbring & Tomita, 1966, 1967; Gershon, 1967); the effects of this toxin on catecholamine synthesis were studied in the vas deferens incubated with both normal and high KCl Krebs solutions. The tissues were preincubated in the Krebs-Henseleit solution containing 5×10^{-7} g/ml tetrodotoxin for 15 min at 37° C before incubation with the ^{14}C-tyrosine precursor. The contralateral paired vas deferens was run as an untreated control. Tetrodotoxin had no effect on the basal rate of catecholamine synthesis or on the stimulation of synthesis seen in the presence of 52 mM KCl.

Effect of high KCl on biosynthesis of catecholamines from dopa

The rate of formation of ^{14}C-catecholamines from dopa was found to be similar in both normal Krebs-Henseleit solution and 52 mM KCl Krebs-Henseleit solution. If anything, there was a slightly slower rate of synthesis in the high KCl medium. Thus the stimulation of synthesis by KCl must occur before the DOPA decarboxylase step.

TABLE 3. *Effect of removing the peripheral ganglia from guinea-pig vas deferens on the potassium induced acceleration of NA biosynthesis*

	NA synthesis (nmol/g tissue)/h	
Treatment	Control Krebs	52 mM KCl Krebs
Ganglia removed	1·78±0·22 (4)	3·61±0·33* (4)
Ganglia intact	2·04±0·07 (44)	4·24±0·24† (32)

Results expressed as mean±S.E. of mean. Figures in parenthesis give the number of vasa deferentia analysed. Comparison of tissues incubated in 52 mM KCl Krebs-Henseleit solution with controls: *$P<0·02$; †$P<0·001$.

Effect of Ca^{2+} and Mg^{2+} ions on NA synthesis

Omission of $CaCl_2$ from, or inclusion of, 14 mM $MgSO_4$ in the Krebs-Henseleit medium abolished the acceleration of catecholamine biosynthesis seen in the 52 mM KCl Krebs-Henseleit solution. These alterations left the basal rate of catecholamine synthesis unaffected. No changes were observed in the stimulation of catecholamine biosynthesis by KCl when the $CaCl_2$ was increased, or $MgSO_4$ was left out of the medium (Table 4).

Effect of exogenous NA on synthesis of NA

The presence of NA (1 μg/ml) in control or 52 mM KCl-Krebs-Henseleit solution depressed NA synthesis by about 60%. However, in spite of this inhibitory effect, synthesis of NA in vasa deferentia incubated in high KCl media still proceeded twice as fast as in controls (Table 5).

Effect of potassium on the release of NA

Endogenous NA

The endogenous NA remaining in the vasa deferentia at the end of a 1 h incubation in Krebs-Henseleit solution containing ^{14}C-tyrosine and different KCl concentrations was not significantly different from NA concentrations in control tissues incubated for the same time in normal Krebs-Henseleit solution.

TABLE 4. *Effect of alterations in the concentration of calcium and magnesium on the synthesis of ^{14}C-NA from ^{14}C-tyrosine in guinea-pig vas deferens*

	Synthesis of NA (nmol/g tissue)/h				Specific activity d.p.m./μg NA			
$CaCl_2$ mM	Control Krebs		52 mM KCl Krebs		Control Krebs		52 mM KCl Krebs	
0	1·86±0·06	(8)	1·99±0·22*	(8)	4464±829	(4)	4777±379*	(4)
2·54	1·73±0·08	(12)	3·98±0·34§	(12)	3415±365	(8)	8304±417§	(8)
5	2·04±0·18	(4)	4·22±0·27†	(8)	—		7706±609	(4)
10	—		3·64±0·68	(4)	—		10743±725	(4)
15	—		2·82±0·34	(4)	—		9646±643	(4)
20	—		3·00±0·15	(4)	—		9405±284	(4)
$MgSO_4$ mM								
0	1·94±0·21	(4)	4·73±0·38‡	(4)				
1·19	2·20±0·28	(4)	4·99±0·49†	(4)				
14	2·49±0·35	(4)	2·87±0·29*	(4)				

Results expressed as mean±S.E. of mean. Figures in parenthesis give the number of vasa deferentia analysed. Comparison of tissues incubated in 52 mM KCl Krebs-Henseleit solution with controls incubated in normal Krebs: * not significant at 0·1 level according to the t test; † $P<0·02$; ‡ $P<0·01$; § $P<0·001$.

TABLE 5. *Inhibition of NA biosynthesis in guinea-pig vas deferens by NA*

Additions to the incubation medium	NA synthesis (nmol/g tissue)/h	
	Normal Krebs	52 mM KCl Krebs
—	2·28±0·08	5·89±0·73*
NA (1 μg/ml)	0·80±0·03	2·21±0·16†

Each value is the mean of determinations made on four individual vasa deferentia±S.E. of mean. Comparison of tissues incubated in 52 mM KCl Krebs-Henseleit solution with controls: * $P<0·02$. † $P<0·01$.

Newly synthesized NA

After the initial 5 min, the release of newly synthesized catechols and catecholamines in 52 mM KCl Krebs-Henseleit solution was double that for controls. Removal of $CaCl_2$ from the unmodified Krebs-Henseleit solution did not alter the spontaneous efflux of newly synthesized amine but reduced the output of catecholamine in the high KCl medium to control values (Table 6).

^{3}H-NA

The release of ^{3}H-NA was studied in groups of four vasa deferentia incubated for 15 min with ^{3}H-NA in 10 ml of Krebs-Henseleit medium or in 10 ml of a modified Krebs-Henseleit medium (Table 7). In the presence of high KCl (52 mM) Krebs-Henseleit the output of ^{3}H-NA into the bath fluid was increased eight-fold over the control rate. If, however, the NaCl concentration was maintained at 118 mM in the high KCl Krebs-Henseleit solution, then the output of ^{3}H-NA exceeded that of the control by only 2·5 times. The release of ^{3}H-NA into the high KCl medium could also be reduced by making the medium hypertonic with sucrose. Finally, removal of $CaCl_2$ from the high KCl medium diminished the efflux of ^{3}H-NA from eight-fold to approximately 2·5 times that observed in normal Krebs-Henseleit solution.

^{3}H-NA from superfused vas deferens

When superfused with Krebs-Henseleit bicarbonate solution containing 52 mM KCl, the vas deferens exhibited a rapid initial contraction which declined quickly to a third of the maximal response (Fig. 4a). Reintroduction of normal Krebs-Henseleit solution led to a small further increase in contraction followed by a rapid

TABLE 6. *Release of newly synthesized ^{14}C-catecholamines and ^{14}C-catechols from guinea-pig vas deferens incubated in control and* 52 mM *KCl Krebs-Henseleit solution*

	% release of ^{14}C-catechol and ^{14}C-catecholamines into incubation medium*			
Time (min)	Control Krebs	52 mM KCl Krebs	Control Krebs Ca^{2+} free	52 mM KCl Krebs Ca^{2+} free
0–5	2·55±0·14	2·99±0·45	2·69±0·19	2·31 (2)
5–15	2·93±0·29	4·34±0·35	3·22±0·51	3·35 (2)
15–30	3·80±0·18	8·23±1·07	3·35±0·54	4·23 (2)
30–60	3·96 (2)	10·51±1·58	4·39±0·61	5·32 (2)
Total 0–60	11·92±1·30	26·08±3·28	13·65±1·47	15·23 (2)

* ^{14}C-catechols and ^{14}C-catecholamines released into the incubation medium for each time period were expressed as a % of the total ^{14}C-catechol radioactivity in the tissue at the end of the experiment plus that released into the bath during the experiment. Each value is the mean±S.E. of mean for three individual vasa deferentia, unless indicated otherwise by figures in parenthesis.

TABLE 7. *Effect of potassium on release of ^{3}H-NA from guinea-pig vas deferens*

	^{3}H release d.p.m./g	% release*	Alteration in release
Normal Krebs	53,585	0·26	—
52 mM KCl Krebs	446,875	2·04	8×
High osmol. Krebs†	32,660	0·21	—
52 mM KCl+normal Na	134,458	0·64	2·5×
52 mM KCl+sucrose†	216,873	0·98	3·8×
52 mM KCl—Ca^{2+} free	128,353	0·62	2·4×
Normal Krebs—Ca^{2+} free	37,001	0·21	—

* % release expressed as a fraction of the total catechol d.p.m. in the vas deferens at the end of the experiment. Each value is the mean of two experiments.
† High osmolarity Krebs-Henseleit solution contained 104 mM sucrose.

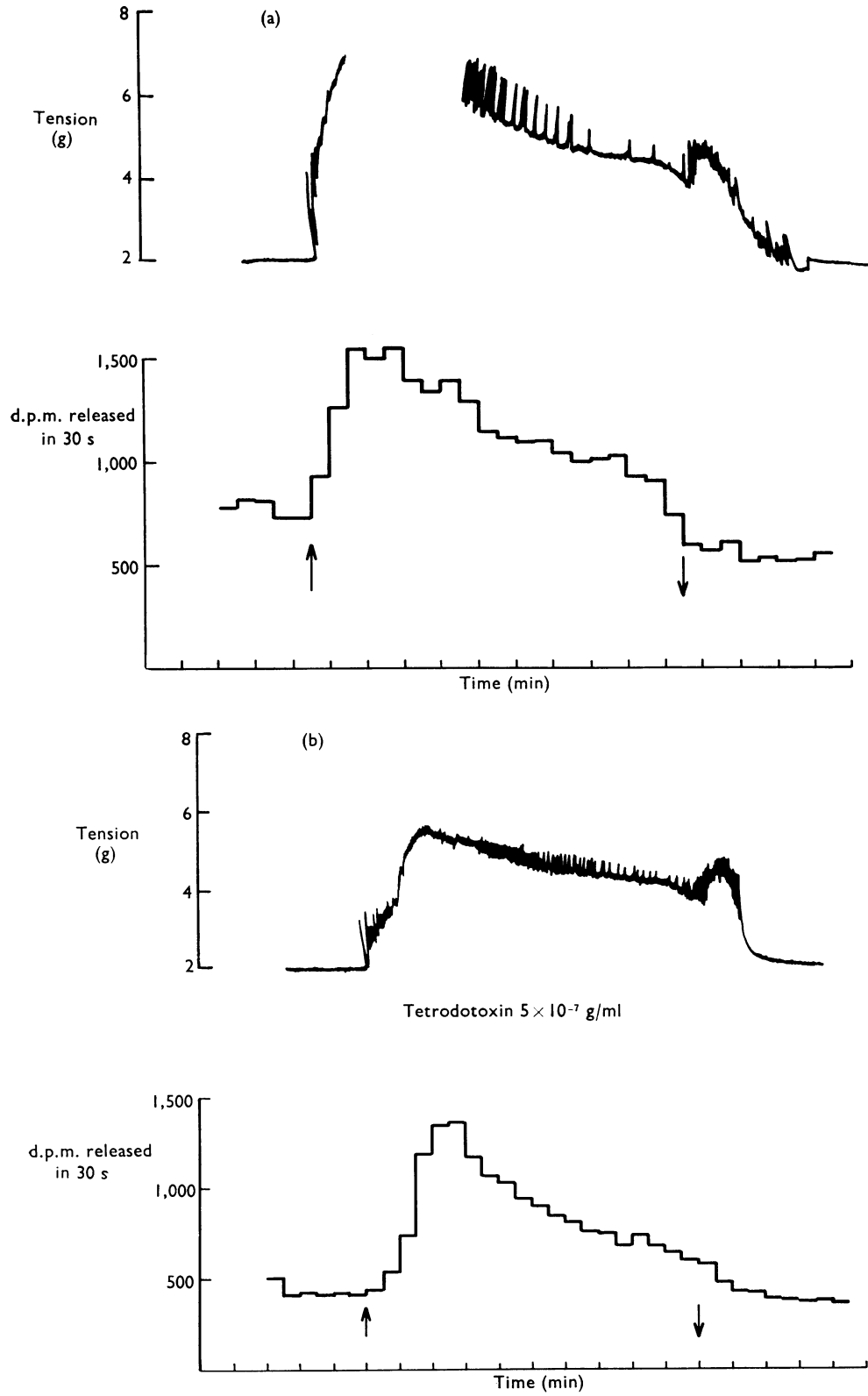

FIG. 4. a, Contraction of the superfused guinea-pig vas deferens (upper tracing) and efflux of ^{3}H-NA (lower tracing). The preparation was superfused initially with unmodified Krebs-Henseleit solution. Between the arrows the superfusion fluid was changed to Krebs-Henseleit solution in which 40% of the NaCl had been replaced with KCl giving a final concentration of 52 mM KCl. b, Identical with Fig. 4a except that the 52 mM KCl Krebs-Henseleit solution contained tetrodotoxin (5×10^{-7} g/ml).

return to the baseline. The ^3H efflux also increased quickly on the introduction of high KCl Krebs-Henseleit solution and declined in parallel with the contraction. No secondary rise in ^3H efflux was seen on reintroduction of normal Krebs-Henseleit solution. During exposure to high KCl Krebs-Henseleit solution the vas deferens did not respond to transmural electrical stimulation (5-50 Hz, 0·5 ms for 5 s).

Tetrodotoxin (5×10^{-7} g/ml) abolished the initial rapid contraction seen in high KCl Krebs-Henseleit solution and also abolished the contractions in response to transmural stimulation but did not reduce the slow contracture of the vas deferens seen in the high KCl medium. The ^3H efflux in the tetrodotoxin treated vas deferens was almost identical to that seen in the vas deferens treated only with high KCl Krebs-Henseleit solution (Fig. 4b).

In the presence of high KCl Krebs-Henseleit solution containing normal concentrations of NaCl a slow contracture of the vas deferens occurred which declined slowly after reaching a peak within 6 minutes. The increase in ^3H efflux was very small. Reintroduction of 52 mM KCl Krebs-Henseleit solution with only 70·5 mM NaCl caused a rapid contraction that was associated with an increase in the ^3H efflux that was at least 10 times as great as that seen with high KCl Krebs-Henseleit solution containing normal (118 mM) NaCl.

Studies on tyrosine hydroxylase in homogenates of guinea-pig vas deferens

Pretreatment of intact vas deferens with high potassium-Krebs-Henseleit medium

Vasa deferentia were left for 1 h at 37° C in either normal Krebs-Henseleit solution or Krebs-Henseleit solution containing 52 mM KCl and 70·5 mM NaCl. The tissues were then homogenized and assayed in triplicate for tyrosine hydroxylase activity by measuring the formation of ^{14}C-DOPA from ^{14}C-tyrosine at 5, 10 and 15 min. AV_{max} of (69 ± 8 nmol DOPA/g tissue)/h was found for controls and of (58 ± 3 nmol DOPA/g tissue)/h for K^+ pretreated tissues, indicating no significant difference in the activity of tyrosine hydroxylase in the two groups of tissues. However, the activity of tyrosine hyroxylase in these two fortified homogenates, in (nmol ^{14}C-DOPA/g tissue)/h, was found to be about twenty times greater than that measured in whole tissue in normal Krebs-Henseleit solution, expressed as (nmol catecholamine/g tissue)/h and about ten times that seen in whole tissue incubated in high KCl Krebs-Henseleit solution.

Tyrosine hydroxylase activity in buffers containing only potassium or sodium

Potassium may produce its effect on catecholamine biosynthesis by directly activating tyrosine hydroxylase; therefore the activity of this enzyme present in homogenates of guinea-pig vas deferens was compared in sodium phosphate buffer and potassium phosphate buffer at pH 6·5. Conversion of ^{14}C-tyrosine to DOPA was (72·1 nmol/g)/h in potassium phosphate buffer, compared with (68·7 nmol/g)/h in sodium phosphate.

Discussion

An increase in the potassium concentration of the Krebs-Henseleit bicarbonate medium to 52 mM has been shown to produce a large acceleration of synthesis of NA

in the guinea-pig vas deferens. In thirty-two vasa deferentia the mean rate of synthesis in 52 mM KCl Krebs-Henseleit was (4·24 ± 0·24 nmol/g tissue)/h showing an increase of more than 100% compared with the control value of (2·04 ± 0·07 nmol/g)/h (mean of forty-four observations). The decrease in the acceleration of NA synthesis seen in very high concentrations of KCl may have been due in part to the diminished uptake of ^{14}C-tyrosine resulting from the lowered sodium concentration (Guroff, King & Udenfriend, 1961). However, the fact that the fall in specific activity of the NA was not so large as the fall observed in the rate of NA synthesis suggests that release of amine into the bath may have contributed to the apparent reduction in synthetic rate. Bogdanski & Brodie (1969) found that reducing the sodium concentrations in the bathing medium to below 50 mM enhanced the release of ^{3}H-NA from slices of rat atria, and that this release of ^{3}H-NA became even more pronounced when high concentrations of KCl were also present.

The increases in NA synthesis reported for nerve stimulated preparations of the guinea-pig vas deferens are not as dramatic as that seen in 52 mM KCl (Roth, Stjärne & von Euler, 1966, 1967; Alousi & Weiner, 1966; Weiner & Rabadjija, 1968). Weiner & Rabadjija (1968) found a 44–65% increase in NA synthesis after 1 h of stimulation. However, these authors only stimulated their preparation for 5 s in every minute whereas in the present experiments the vasa deferentia were in continuous contact with K^+ for the duration of the incubation. In only one nerve stimulated preparation, the rat submaxillary gland, has an enhancement of NA synthesis been observed which was greater than that induced by high K^+ in the guinea-pig vas deferens (Sedvall & Kopin, 1967a; Sedvall, 1969). In this case the circulation to the submaxillary gland was intact which may well explain the more dramatic increase seen in NA synthesis.

In contrast to most sympathetically innervated tissues, the vas deferens of the guinea-pig receives the major part of its sympathetic innervation from peripheral ganglia which lie in and around the mesentery of the tissue (Sjöstrand, 1962, 1965 ; Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Ferry, 1967; Birmingham, 1970). There was a possibility, therefore, that K^+ might produce its effects on synthesis by depolarizing the ganglion cells and thereby stimulating the post-ganglionic sympathetic neurones. However, experiments in which ganglionic stimulants and tetrodotoxin (which blocks action potentials) were used and in which the ganglia were removed by stripping off the serosal membrane of the vas deferens (Birmingham, 1970) have indicated that this mechanism plays little or no part in the K^+ induced acceleration of NA synthesis.

In accord with the earlier observations reported for nerve stimulated preparations (Gordon, *et al.*, 1966a; Weiner & Rabadjija, 1968) we found that the K^+ induced acceleration of NA synthesis disappeared if DOPA was used as the starting substrate instead of tyrosine. This finding suggests that the acceleration occurs at the rate limiting step in the series of reactions leading from tyrosine to noradrenaline, that is tyrosine hydroxylation. In our experiments, where the stimulation was of a relatively short duration (1 h) the tyrosine hydroxylase activity did not change. This finding is in agreement with the results of Sedvall & Kopin (1967b) for the rat salivary gland, and the unpublished observations of Thoa, Kopin & Weiner (see Weiner, 1970) on the nerve stimulated guinea-pig vas deferens. However, under other conditions where prolonged activation of sympathetic neurones is involved—for example, reserpine

treatment—a significant increase in tyrosine hydroxylase has been seen (Mueller, Thoenen & Axelrod, 1969).

The tyrosine hydroxylase of the guinea-pig vas deferens, like that of the adrenal medulla, was not activated directly by K^+ (Ikeda, Fahien & Udenfriend, 1966). There was, however, a great increase in the activity of the enzyme when measured in tissue homogenates as compared with the activity in whole tissue which was estimated from the synthesis of NA. This can be attributed to a number of factors. One is the removal of the enzyme from end product inhibition on homogenization; a second is the greater ease of access of substrate to the enzyme in the homogenate; and a third is that the homogenate was fortified with pteridine co-factor. In the whole tissue the availability of reduced pterdine co-factor may well be determined by the activity of NADH dehydrogenase, the enzyme which regenerates the reduced pteridine (Musacchio, 1969). The fact that the activity of this enzyme may in turn be regulated by the physiological activity of the neurone has not been explored but should be kept in mind.

The increase in synthesis seen in the presence of K^+ did not appear to be due to an enhanced uptake of the radioactive substrate tyrosine into the K^+ treated tissues since the specific activity of the tyrosine in K^+ treated tissues was actually lower than that of controls. However, this observation does not exclude the possibility that K^+ may alter the uptake of tyrosine into the sympathetic neurone.

The most widely held view on the mechanism of the regulation of tyrosine hydroxylase activity which was recently reviewed by Weiner (1970) is that of end product feedback inhibition of the enzyme by NA. This appears to be a feasible mechanism in the guinea-pig vas deferens because synthesis of NA can be inhibited by exogenously applied NA (Alousi & Weiner, 1966; this paper). The proposal has been made that release of a small intraneuronal pool of NA during nerve stimulation leads to an increase in the activity of the tyrosine hydroxylase and an associated increase in the rate of NA synthesis (Alousi & Weiner, 1966; Roth, *et al.*, 1967; Weiner & Rabadjija, 1968; Weiner, 1970). The small size of the pool has been postulated to account for the observation that a large increase in the rate of NA synthesis takes place during nerve stimulation *in vitro* (Alousi & Weiner, 1966; Austin *et al.*, 1967a; Roth *et al.*, 1967; Sedvall & Kopin, 1967a; Weiner & Rabadjija, 1968; Sedvall, 1969) and *in vivo* (Oliverio & Stjärne, 1965; Gordon *et al.*, 1966b; Gordon *et al.*, 1966a; Spector, 1966; Dairman *et al.*, 1968; Dairman & Udenfriend, 1970) without any significant diminution of endogenous NA (Gordon *et al.*, 1966b; Roth *et al.*, 1967; Weiner & Rabadjija, 1968; Blakeley, Dearnaley & Harrison 1970) and is consistent with reports that tyrosine hydroxylase does not appear to be associated with the major NA pool in the neurone, the storage granules (Austin, Levitt & Chubb, 1967b; Stjärne & Lishajko, 1967). In this study also no evidence was obtained for a significant depletion of endogenous NA after K^+ treatment. We did find that 52 mM KCl doubles the spontaneous release of newly synthesized NA from the vas deferens as well as producing a 100% increase in NA synthesis. A similar study in rat brain slices, where a greater proportion of newly synthesized amine is released during K^+ depolarization, revealed a close correlation between the acceleration of synthesis seen in high K^+ media and release of newly synthesized catecholamine (Harris & Roth, 1970).

That the release of NA may be a triggering mechanism for acceleration of NA synthesis is further supported by the observation that removal of Ca^{2+} ions, which

are required for the normal output of sympathetic transmitter (Boullin, 1967), blocks the increase in synthesis normally seen in high K^+ but leaves the basal rate of synthesis unaffected. Under these conditions the release of newly synthesized NA seen in the high K^+ media is also reduced to the level of the spontaneous efflux seen in control Krebs-Henseleit media.

A much smaller proportion of the total ^{3}H-NA accumulated by the vas deferens was released by 52 mM KCl Krebs-Henseleit solution than the proportion of newly synthesized catecholamine released by this procedure. If one assumes that most of the radioactivity released is unchanged amine then these observations suggest that there is a preferential release of the newly synthesized amine. Clear-cut evidence for preferential release of newly synthesized amine has been obtained for the spleen of the cat (Kopin, Breese, Krauss & Weise, 1968) and for the portal vein of the rabbit (Hughes & Roth, unpublished observations).

In experiments on the vas deferens, the release of ^{3}H-NA was not as closely correlated with acceleration of NA synthesis as the release of the newly synthesized amine. For instance, in Ca^{2+} free 52 mM KCl Krebs-Henseleit solution, the efflux of ^{3}H-NA was reduced by 75% whereas the increase in synthesis was completely abolished under these conditions. Also the efflux of ^{3}H-NA in 52 mM KCl Krebs-Henseleit solution containing a normal Na^+ concentration was greatly reduced, whereas a corresponding reduction in synthesis was not observed under identical conditions.

Despite the good correlation observed between the release of newly synthesized catecholamine and the acceleration in synthesis of NA, the possibility cannot be ruled out that K^+ ions may increase synthesis by increasing Ca^{2+} influx into the neurone and perhaps inducing some secondary intracellular effects on synthesis not necessarily coupled to the release of NA. However, the simplest and most plausible hypothesis is that the release of NA from a small pool determines the alteration in synthesis rate and that the release from this pool requires Ca^{2+} ions. The possibility of a plurality of regulating factors should not, however, be ignored.

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