Br. J. Pharmac. Chemother. (1967), **29**, 309–318.

CATION DEPENDENCE OF SYMPATHETIC TRANSMITTER RETENTION BY SLICES OF RAT VENTRICLE

BY

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(*Received October* 25, 1966)

It is now generally accepted that exogenous noradrenaline is removed from the external medium and stored in peripheral adrenergic nerve endings by a process that has two components: (a) the amine first crosses the nerve membrane by active transport—that is, uptake—and (b) is then bound in or to subcellular particles—that is, storage—(Iversen, 1965).

The concept of an active transport of noradrenaline into nerve endings is supported by several observations. Peripheral organs accumulate noradrenaline to levels several times those in the external medium (Whitby, Axelrod & Weil-Malherbe, 1961 ; Dengler, Michaelson, Spiegel & Titus, 1962). This accumulation represents net uptake and not simply exchange ; and the initial rate of influx obeys Michaelis–Menton kinetics (Iversen, 1965 ; Green & Miller, 1966a). The accumulation of amine is stereospecific, while noradrenaline and adrenaline compete for accumulation by a common mechanism (Iversen, 1965). Accumulation of the amine is reduced by ouabain and various inhibitors of metabolism (Dengler, 1965 ; Green & Miller, 1966a) and the process, in uterine slices, has a Q_{10} of 2.2 (Green & Miller, 1966a) and is saturable (Dengler *et al.*, 1962 ; Iversen, 1965).

The active transport of other non-electrolytes, such as amino acids and glucose, is dependent on the presence and concentrations of certain cations in the bathing medium (Csaky, 1965). The activity of the enzyme, ATPase, possibly implicated in the accumulation of noradrenaline (Titus & Dengler, 1966) is also modified by the nature of the cations to which it is exposed (Skou, 1965). It was, therefore, appropriate to investigate the basic cationic requirements for the retention of noradrenaline. In addition, the effect of experimentally induced anoxia on the process of retention was studied. This paper describes the effect of alterations in the cationic composition of the medium and of anoxia on the retention of (^{3}H)-noradrenaline by rat ventricular slices. A preliminary account of a portion of this work has been presented elsewhere (Gillis & Paton, 1966a).

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METHODS

Technique for incubation of slices

Adult male Sprague–Dawley rats were killed by a blow on the head, after which the hearts were excised rapidly, washed with ice-cold saline, and the atria and fat removed. Ventricular tissue slices, averaging 31 mg in weight (range 25–40 mg), were prepared and weighed. From two rat hearts, 16–20 slices were obtained and pooled. Preparation of the slices took 15–20 min.

Each slice was placed in 10 ml. of the medium to be studied in 25 ml. Erlenmeyer flasks, which were then set in a shaker at 37° C and gassed with 95% oxygen (O_2) and 5% carbon dioxide (CO_2). In one series of experiments, designed to investigate the effect of anoxia on retention of (^{3}H)-noradrenaline, equilibration was carried out with 95% nitrogen and 5% CO_2. After 30 min, pre-incubation, (^{3}H)-noradrenaline was added to the flasks so as to achieve a final concentration of 10 mμg of the base per ml. unless otherwise stated, and the incubation was continued for a further 30 min.

Measurement of (^{3}H)-noradrenaline retention

Slices were incubated with the amine for 30 min except when the kinetics of retention were investigated, the duration of incubation then being 8, 16 or 24 min. At the end of the incubation the slices were removed, rinsed briefly in ice-cold saline, blotted and homogenized in 2 ml. 0.4 M perchloric acid (PCA). After 45 to 60 min at 0° C the homogenates were centrifuged at 21,000$\times g$ for 20 min at 4° C. The pellet was re-suspended in 0.5 ml. of 0.4 M PCA and then centrifuged as above. A 0.1 ml. aliquot of the supernatant fluid was added to Bray's phosphor (Bray, 1960) and total (^{3}H) counted on a Packard tri-carb liquid scintillation counter that had a practical counting efficiency for tritium of 22–24%. In all cases duplicate portions were counted for 10 min each and corrected for quenching with ^3H_2O as an internal standard. In every case slices from the same two rats were incubated under control and test conditions. Two slices and their respective media were always pooled and their combined (^{3}H) measured. Duplicate 0.1 ml. portions of the medium were counted for (^{3}H) (as described for slices above).

When the amount of (^{3}H) specifically associated with noradrenaline was measured, the alumina column chromatographic methods of Schneider & Gillis (1965) and Schneider (1966) were used. In these instances, 1/10 volume of 4 M PCA and 1/50 volume of 5% sodium edetate (EDTA) were added to the media immediately after the removal of the slices and the media cooled rapidly. One tenth volume of 1% EDTA was added to the extracts of heart slices before alumina column chromatography.

Retention of (^{3}H)-noradrenaline was, unless otherwise stated, expressed as a ratio (R), calculated by dividing the (^{3}H) counts/min/g of heart slice by (^{3}H) counts/min/ml. of medium (see also Dengler *et al.*, 1962).

Measurement of (^{3}H)-noradrenaline efflux

Slices were incubated in Krebs solution with (^{3}H)-noradrenaline as described above. After 30 min incubation with the amine, the slices were removed, blotted, rinsed briefly in ice-cold saline and then returned to fresh amine-free medium (subsequently described as Medium II). Half the slices were placed in Krebs solution and served as controls. The other half were placed in solutions that had varying ionic compositions or contained a drug. After 30 min all slices were removed and the (^{3}H) content of media and slices determined as described previously.

Because of variation in retention of amine from slice to slice, even when obtained from the same animal, the amount of (^{3}H)-noradrenaline present in the slice before incubation in amine-free medium was taken to be equal to the sum of (^{3}H)-noradrenaline present in the slice at the completion of the experiment and (^{3}H)-noradrenaline present in medium II. Efflux of (^{3}H)-noradrenaline was expressed as a percentage of that originally present on the slice. Thus efflux=

$$\frac{(^3\text{H}) \text{ content of Medium II}}{\text{Original } (^3\text{H}) \text{ content of slice}} \times 100.$$

TABLE 1

COMPOSITION OF MEDIA USED

Media	Constituents (mM)											
	NaCl	$NaHCO_3$	NaH_2Po_4	KCl	$KHCo_3$	KH_2Po_4	$MgSo_4$	$CaCl_2$	Glucose	Sucrose	CaNaEDTA	Na_2EDTA
Krebs solution	119	25	—	4·81	—	1·19	1·19	2·54	11·10	—	0·03	—
High KCl solution (sodium free)	—	—	—	123·81	25	1·19	1·19	2·54	11·10	—	0·03	—
Modified KCl solution	—	25	—	123·81	—	1·19	1·19	2·54	11·10	—	0·03	—
Low Na^+ solution	—	25	—	4·81	—	1·19	1·19	2·54	11·10	238·0	0·03	—
K^+ free solution	119	25	1·19	—	—	—	1·19	2·54	11·10	9·62	0·03	—
Ca^{++} free solution	119	25	—	4·81	—	1·19	1·19	—	11·10	5·08	—	0·03
Mg^{++} free solution	119	25	—	4·81	—	1·19	—	2·54	11·10	2·38	—	0·03
Ca^{++} and Mg^{++} free solution	119	25	—	4·81	—	1·19	—	—	11·10	7·46	—	0·03

Media used

The ionic composition of the media used is shown in Table 1. When the ionic composition of the solution was altered, isotonicity was maintained by using appropriate amounts of a different ion or of sucrose.

Drugs

Chromatographically pure (±)-noradrenaline-7-(^{3}H)-hydrochloride (in 0.1 N-acetic acid) with a specific activity of 6.61 c/m-mole was obtained from the New England Nuclear Corporation. The solution of (^{3}H)-noradrenaline was diluted with 0.2 N hydrochloric acid (final concentration 0.001 N) and 10% sodium metabisulphite (final concentration 1%) and glass distilled water to give a stock solution containing 10 μg/ml. of the base, which was stored at 4° C.

Statistical analysis

When the significance of data was evaluated, Student's t test was the method used (Bernstein & Weatherall, 1952).

RESULTS

The effect on (^{3}H)-noradrenaline stability of incubation at 37° in various media was studied. Equilibration with 95% O_2/5% CO_2 produced similar degrees of amine destruction in all media tested for this effect (Table 2). However, equilibration with 95% N_2/5% CO_2 resulted in less amine destruction. In contrast, equilibration with room air produced more destruction probably because the pH of such media rose to 7.8 to 7.9, whereas after aeration with 95% O_2/5% CO_2, the pH of media was 7.3 to 7.4. For this reason, aeration in all subsequent work was carried out with 95% O_2 (or N_2 and 5% CO_2. The tritium associated specifically with noradrenaline was estimated for at least two pairs of slices after their incubation in the various media used (see below). After correction for a 76.1 ± 1.8% mean recovery of added (^{3}H)-noradrenaline, the amount of (^{3}H)-noradrenaline in all extracts was within 72–83% of the total tritium retained.

TABLE 2

EFFECT OF VARIOUS MEDIA ON THE STABILITY OF (^{3}H)-NORADRENALINE

(^{3}H)-Noradrenaline, 10 mμg/ml., was incubated in the absence of tissue for 30 min in the media and under the conditions indicated below. The amount of tritium specifically associated with noradrenaline was then determined by alumina column chromatography (see Methods). Values were corrected for a 70·0±2·6% recovery of (^{3}H)-noradrenaline added (n=6) to media immediately before the analyses

Medium	Aerated with	% of total ^{3}H associated with noradrenaline (mean±S.E.M. of 4 determinations)
Krebs solution	Room air	65·1±6·7
Krebs solution	95% O_2/5% CO_2	88·6±8·3
Krebs solution	95% N_2/5% CO_2	>100
KCl solution (Na^+ free)	95% O_2/5% CO_2	93·0±3·2
K^+ free solution	95% O_2/5% CO_2	91·9±8·1

Effect on (^{3}H)-noradrenaline retention of:

(a) *Amine concentration and duration of incubation*

In these experiments slices were incubated in Krebs solution containing (^{3}H)-noradrenaline 2.5, 6.25 and 10.0 mμg/ml. for 8, 16 and 24 min. Values for retention of (^{3}H)-noradrenaline were corrected for simple diffusion of the amine into slices as

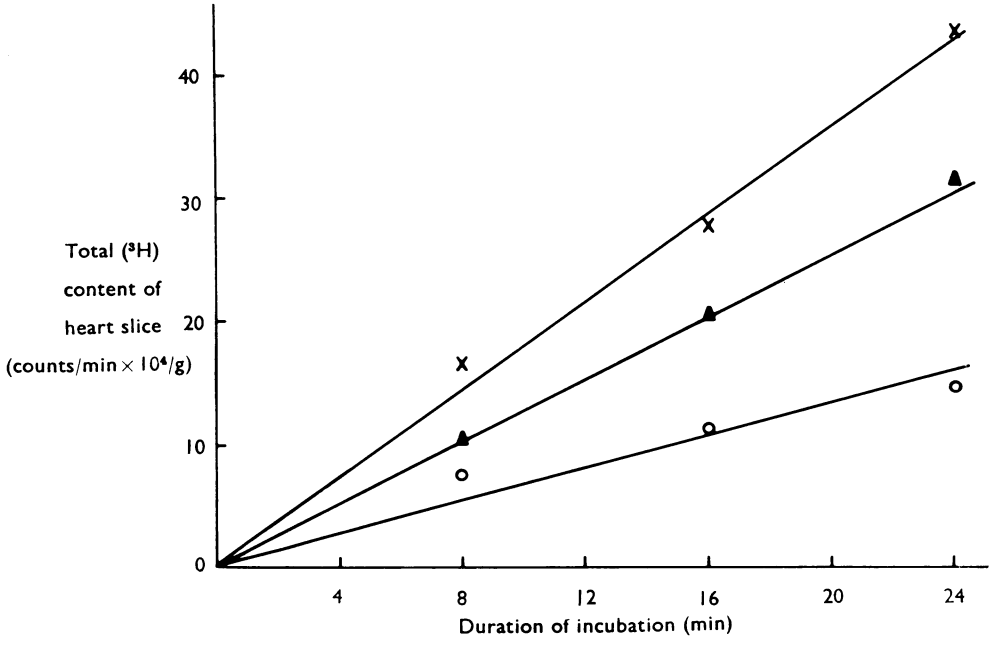

Fig. 1. The effect of (^{3}H)-noradrenaline concentration and the time of incubation on the retention of total tritium by slices of rat ventricle. ^{3}H-noradrenaline was used at concentrations of 2.5 (○——○), 6.25 (▲——▲ and 10 (×——×) mμg/ml. of incubation medium.

described by Green & Miller (1966a). Uptake was linear during the above incubations at all concentrations: retention of amine was most efficient at the lowest concentration of amine used (Fig. 1). From these data, the initial rates of retention of (^{3}H)-noradrenaline were determined. Parameters equivalent to the Michaelis Constant (Km and V_{max}) were then determined by plotting S against S/V (Iversen, 1965) and were found to be 0.8×10^{-7}M and 2.5 mμg/min/gm heart slice respectively.

(b) *Anoxia*

Equilibration of the Krebs solution used with 95% N_2/5% CO_2 resulted in a significant reduction in retention (Table 3). It should be noted than the total period of exposure to anoxia was 60 min—that is, 30 min pre-incubation and 30 min incubation with (^{3}H)-noradrenaline.

(c) *Alterations in cationic composition of the medium*

i. *Potassium.* It can be seen (Table 3) that complete elimination of K^+ (using the K^+ free solution) greatly reduced retention. However, the retention of amine was within normal limits when only one-half of the usual amount of K^+ (that is, 3 mM) was present in the medium (Table 4).

ii. *Sodium.* Complete elimination of Na^+ and its replacement with K^+ by means of KCl solution, greatly reduced retention (Table 3). As the Na^+ concentration was reduced progressively from its normal value of 144 mM and that of K^+ simultaneously raised from 6 mM, retention fell progressively (Table 4).

TABLE 3

EFFECT OF VARIOUS MEDIA ON RETENTION OF (^{3}H)-NORADRENALINE BY SLICES OF RAT VENTRICLE

In all cases control slices were incubated in Krebs medium that had been equilibrated with 95% O_2/5% CO_2

Medium	R value (mean±S.E.) Control	R value (mean±S.E.) Experimental	Pairs of slices (No.)
KCl (Na^+ free)	4·7±0·3	1·6±0·1	6
Low Na^+	3·3±0·2	1·9±0·1	8
Modified KCl	3·3±0·2	2·1±0·1	8
K^+ free	4·3±0·5	1·7±0·1	6
Ca^{++} free	4·0±0·3	2·3±0·1	5
Ca^{++} free (plus EDTA)	4·4±0·2	1·8±0·1	6
Ca^{++} and Mg^{++} free	4·1±0·2	1·4±0·1	7
Mg^{++} free	4·2±0·3	4·4±0·3	4
Krebs (equilibrated with 95% N_2/5% CO_2)	4·2±0·7	1·7±0·1	3

TABLE 4

THE INFLUENCE ON (^{3}H)-NORADRENALINE RETENTION OF SIMULTANEOUSLY INCREASING [K^+] WHILE LOWERING [Na^+]

Medium content (mM) Na^+	Medium content (mM) K^+	R values (average of 2 pairs of slices/experiment)
150	0	1·7
147	3	5·0
144	6	5·5
120	30	3·5
80	70	3·3
40	110	1·7
0	150	1·8

These results were investigated further as follows: slices were incubated in Krebs solution, modified KCl solution and low Na^+ solution (Table 1). Both the modified solutions reduced retention of the amine to a similar extent (Table 3). The use of the low Na^+ solution establishes that the increased K^+ present in the modified KCl solution was not responsible for the inhibition seen when the latter medium was used.

iii. *Calcium and magnesium.* The following procedure was followed: slices were incubated in Ca^{++} and/or Mg^{++} free solution for a total of 80 min before the addition of (^{3}H)-noradrenaline. During the pre-incubation period, slices were transferred to fresh solution every 20 min. The use of Ca^{++} free medium reduced retention (Table 3). More complete removal of Ca^{++} using disodium EDTA (5 mM for the first 20 min, 2.5 mM for the second 20 min) still further impaired retention. Mg^{++} free solution had no effect on retention; however, the absence of both Ca^{++} and Mg^{++} reduced retention to the greatest extent (Table 3).

Effects of various conditions on the efflux on (^{3}H)-noradrenaline

As can be seen in Table 5, the absence of either Na^+ or K^+ from the medium significantly increased the efflux of previously bound (^{3}H)-noradrenaline. Anoxia (aeration with N_2/CO_2, see Methods), however, failed to affect amine efflux (Table 5).

TABLE 5

EFFECT OF VARIOUS CONDITIONS ON THE EFFLUX OF TRITIUM FROM SLICES PREVIOUSLY INCUBATED WITH (^{3}H)-NORADRENALINE

Medium used during efflux experiment	% efflux of tritium (mean±S.E.)	Pairs of slices used (No.)
Krebs (control)	8·4±0·4	28
KCl solution (Na^+ free)	21·5±1·4*	12
K^+ free solution	22·6±2·7*	16
Krebs (control)	9·4±0·8	10
Krebs (aerated with 95% N_2/5% CO_2)	10·1±0·6	10

* $P<0·01$, when value compared with appropriate control.

DISCUSSION

Since the bulk (72–83%) of tritium found in slices, after incubation in the various media, was actually associated with noradrenaline, it is apparent that the measurement of total tritium accurately reflects the retention of the amine.

Values for "Km" and "V_{max}" were calculated to allow comparison of our data with similar information (Iversen, 1965), relating to the retention of noradrenaline by the perfused rat heart. It should be emphasized, however, that the use of these terms does not necessarily imply the existence, in heart slices, of a single enzymic process that is responsible for retention of the amine. Our values for these constants are from 10 to 100 times smaller than those reported by Iversen (1965). It seems very likely that the metabolism of heart slices proceeds at a much slower rate than in the intact, perfused heart (Bing, 1965). In addition, access of amine to the adrenergic nerve ending may be less adequate than that permitted by perfusion of the coronary system of the heart.

In considering the results presented here, it is important to note that a reduction in amine retention, produced by the various experimental procedures used, could reflect actions on one or both of the components of the total process; thus transport into the nerve, or its intraneuronal binding following transport, might be decreased.

The calcium ion has previously been shown to be necessary for optimal retention of noradrenaline by rabbit heart slices (Dengler, 1965) but is not essential for retention of the amine by rat uterus (Green & Miller, 1966a) or the perfused rat heart (Iversen & Kravitz, 1966). Lack of calcium causes membrane depolarization and increases the sodium conductance of the membrane (Marshall, 1965). In addition, perfusion of the rat isolated heart with calcium-free medium causes the liberation of noradrenaline (Taylor & Nash, 1966). In view of these diverse actions, which must reflect quite pronounced alteration in cellular function, the decreased amine retention in the absence of calcium (Table 3) is not unexpected.

High concentrations of potassium (16–66 mM) also depolarize cell membranes and recently have been shown to liberate noradrenaline from brain slices (Baldessarini & Kopin, 1966). However, low concentrations of potassium (3 mM) in the present investigation were sufficient to maintain essentially normal retention, although the complete absence of this ion markedly reduced the concentration ratios as did increasing its concentration above 6 mM (Table 4).

It is apparent from our results that the noradrenaline retention process is dependent on the presence of sodium, potassium, calcium and possibly magnesium in the external medium. Very recently, Iversen & Kravitz (1966) reported that the retention process in perfused hearts also requires sodium. Also, Bogdanski & Brodie (1966) demonstrated a decreased retention of tritium after exposure of rat heart slices to ^{3}H-noradrenaline in sodium-free medium. Such cation dependence is best explained by assuming that the electrolytes are required for the energy producing system on which the active transport depends and/or for the mechanism of the transport system itself.

In addition, however, the significantly elevated efflux of tritiated amine, caused by exposure of " loaded " heart slices to sodium or potassium-free media (Table 5) suggests an effect of these conditions also on binding of noradrenaline previously taken up. The absence of effect of anoxia on amine efflux supports this suggestion for the following reason. If the absence of sodium or potassium increased efflux by inhibiting the re-uptake of spontaneously liberated noradrenaline, then anoxia, which lowers the initial uptake of the amine (Table 3), would be expected to have the same effect on efflux; such was not the case (Table 5).

Thus our evidence indicates that the cation dependence of the noradrenaline retention process is linked both to the necessity for these ions for transport of the amine into the slice and its subsequent binding at intracellular sites.

Our observation that anoxia significantly lowered retention of (^{3}H)-noradrenaline is in contrast to previous findings in this laboratory (Gillis & Paton, 1966b), indicating that retention of the amine by cat perfused hearts was unaffected by anoxia. Also, oxygen lack has previously been shown not to influence noradrenaline retention by either isolated atria (Wakade & Furchgott, 1966) or rat uterus (Green & Miller, 1966a). The difference between the effect of anoxia in intact organs and in heart slices is likely to be related to the altered metabolism of the latter: the much lower Km and V_{max} calculated in our work supports this possibility. In addition, it is clear that, when intact or functioning tissues are used, very short periods of oxygen lack cause marked disruption of function and therefore (as in our previous study of anoxia, Gillis & Paton, 1966b) termination of the experiment. In the present study, the total duration of the anoxia to which the heart slices were exposed was considerably greater (60 min).

Anoxia is known to liberate catecholamines from peripheral tissues as well as the adrenal medulla (Shahab & Wollenberger, 1965). In contrast, this condition did not affect the release of previously bound amine from heart slices (Table 5). This difference is probably another reflection of differences in behaviour of intact organs and partially disrupted preparations of these organs.

The failure of anoxia in our work to change the rate of amine efflux might suggest that there is little uptake of spontaneously released amine, since anoxia very markedly depressed the initial uptake of the amine and could, therefore, be expected to act equally well to inhibit re-uptake, if it did in fact occur. However, it should be considered that the slice: medium ratio is high when " pre-loaded " slices are exposed to amine-free Krebs. Green & Miller (1966b) calculated a ratio of approximately 1,500: 1 under these circumstances. Amine efflux, resulting from a large concentration gradient might therefore obscure an opposing movement of amine that was caused by re-uptake.

SUMMARY

1. Rat ventricular slices were utilized to investigate the effect of various conditions on the retention and efflux of (^{3}H)-noradrenaline.

2. For (±)-noradrenaline, the Km was 0.8×10^{-7} M and V_{max} 2.5 mμg/min/g heart slice.

3. Retention of (^{3}H)-noradrenaline was reduced by anoxia and the absence of Na^+, K^+ or Ca^{++} from the incubation medium.

4. Efflux of (^{3}H)-noradrenaline from prelabelled slices was increased by incubation in KCl solution (Na^+ absent, high K^+ content) or by the absence of Na^+ or K^+, but not by anoxia.

5. It is concluded that the reduced retention of amine found when Na^+, K^+ and Ca^{++} are absent from the medium is due to a combination of (a) impairment of active transport into the cell and (b) interference with intracellular binding.

This investigation was supported by USPHS Grant No. H-7249 from the National Heart Institute and by Grant No. G-65-46 from the Life Insurance Medical Research Fund. C. N. G. is an established Investigator of the American Heart Association. One of us (D. M. P.) was the recipient of a Wellcome Research Travel Grant. The technical assistance of Mrs. J. Crockett and Miss C. Payne is gratefully acknowledged.

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