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CATION AND METABOLIC REQUIREMENTS FOR RETENTION OF METARAMINOL BY RAT UTERINE HORNS

BY

D. M. PATON

From the Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada
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It is now widely believed that noradrenaline is removed from the external bathing medium and stored in peripheral adrenergic nerve terminals as the result of the operation of a two-stage process. The first step in retention involves the passage of the amine across the nerve membrane and may involve active transport; this is known as uptake. The second stage is the subsequent binding of the amine in or to subcellular granules and is a storage phase. The evidence for this concept of retention has been recently reviewed by Iversen (1967).

The retention of exogenous noradrenaline is markedly influenced by cations (Iversen & Kravitz, 1966; Gillis & Paton, 1966, 1967) and energy supplies (Dengler, 1965; Green & Miller, 1966a). Whether cations and energy supplies are required for uptake or storage or for both has not, however, been clearly established. Certainly the efflux of [^{3}H]-noradrenaline from prelabelled tissues is increased in potassium-free (Gillis & Paton, 1967) and low sodium media (Keen, 1967) which suggests that these conditions may influence storage.

Metaraminol seems to be accumulated by organs in a manner essentially similar to noradrenaline; however, unlike noradrenaline, it is not a substrate for either catechol-*o*-methyl-transferase (COMT) or monoamine oxidase (MAO) and can thus be concentrated and retained *in vitro* by tissues pretreated with reserpine (Giachetti & Shore, 1966). Consequently Giachetti & Shore have proposed that reserpine prevents the intracellular binding of amines only without affecting their passage across the membrane; they proposed that any substance inhibiting the accumulation of metaraminol *in vitro* can be presumed to act on the membrane pump (Giachetti & Shore, 1966). The purpose of the investigations reported here was to utilize [^{3}H]-metaraminol to study the role of cations and metabolism in the retention of such amines; the immature rat uterus was chosen as the test organ because this preparation accumulates catecholamines very efficiently (Green & Miller, 1966a, b). A preliminary account of a portion of this work has been presented elsewhere (Paton, 1967).

METHODS

Technique for incubation of uterine horns

Immature female Wistar rats (35–55 g body weight) were killed by a blow on the head after which the uterine horns were excised rapidly, rinsed in Krebs-Ringer medium at room temperature

and dissected free of surrounding connective tissue. Each uterine horn was suspended on a separate metal hook, four to six horns being placed in 25 ml. of the medium at 37° C and gassed with 95% oxygen and 5% carbon dioxide. After varying durations of preincubation, described fully later, [^{3}H]-metaraminol was added to the containers so as to achieve a final concentration of 13.2 m μg of the base per ml. and the incubation continued for a further 45 min.

Measurement of [^{3}H]-metaraminol retention

At the end of the incubation, uterine horns were removed, blotted and homogenized in 1 ml. of 0.4 M perchloric acid (PCA). After 45–60 min at room temperature the homogenates were centrifuged in an International Clinical Centrifuge. A 0.2 ml. aliquot of the supernatant fluid was added to Bray's phosphor (Bray, 1960) and total ^{3}H counted in a Nuclear-Chicago liquid scintillation spectrometer that had a practical counting efficiency for tritium of 11.8%. In all cases one uterine horn served as a control while the other horn from the same rat served as the test organ. Duplicate 0.2 ml. portions of the medium were counted for [^{3}H] (as described for uterine horns above). In all cases portions were counted for at least 4 min each and corrected for quenching using ^3H_2O as an internal standard.

Retention of [^{3}H]-metaraminol was expressed as a ratio (R) calculated by dividing the [^{3}H] counts/min/g of uterine horn by the [^{3}H] counts/min/ml. of medium.

Technique for recording contractions of uterine horns

Isometric contractions of immature uterine horns at 37° C were recorded using Grass force displacement transducers connected to a Grass Model 5D Polygraph; the techniques employed have been described fully previously (Paton & Daniel, 1967).

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 sugar.

Drugs and chemicals

Chromatographically pure (±)-metaraminol-7-[^{3}H]-hydrochloride (in 0.01 N acetic acid) with a specific activity of 6.7 c/mmole was obtained from the New England Nuclear Corporation.

The following drugs and chemicals were used and obtained from the sources indicated: 2,4-dinitrophenol (DNP), rubidium chloride, lithium chloride, *d*-glucose, sucrose and *d*-fructose (Fisher Scientific Company); iodoacetic acid (IAA) (Eastman Organic Chemicals); *d*-galactose and 2-deoxy-*d*-glucose (Sigma Chemical Company); reserpine (Ciba Company); and acetylcholine chloride (British Drug Houses). The concentrations used throughout were: sugars, 49.2 mM; metabolic inhibitors, 1 mM.

Statistical analysis

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

RESULTS

The duration of incubation with [^{3}H]-metaraminol was chosen as 45 min following preliminary experiments which showed that retention had not reached equilibrium at that time.

Effects on [^{3}H]-metaraminol retention of:

(a) Alterations in cationic composition of the medium

i. *Potassium.* It can be seen (Table 2) that complete elimination of K^+ (using the K^+ free Krebs solution) greatly reduced retention. Rubidium was unable to substitute for potassium (Table 2).

TABLE 1

COMPOSITION OF MEDIA USED

Media	Constituents (mM)									
	NaCl	$NaHCO_3$	NaH_2PO_4	KCl	$MgSO_4$	$CaCl_2$	*d*-Glucose	Sucrose	LiCl	RbCl
Krebs solution	115·48	21·91	1·16	4·63	1·16	1·5	49·20	—	—	—
Low Na^+ Krebs solution	—	21·91	1·16	4·63	1·16	1·5	49·20	230·96	—	—
Lithium Krebs solution	—	21·91	1·16	4·63	1·16	1·5	49·20	—	115.48	—
K^+-free Krebs solution	115·48	21·91	1·16	—	1·16	1·5	49·20	—	—	—
Rb^+ Krebs solution	115·48	21·91	1·16	—	1·16	1·5	49·20	—	—	4·63
Ca^{++} free Krebs solution	115·48	21·91	1·16	4·63	1·16	—	49·20	—	—	—

ii. *Sodium.* Lowering of Na^+ to 23 mM (using the low Na^+ Krebs solution) greatly reduced retention. Lithium was unable to substitute completely for sodium (Table 2).

iii. *Calcium.* The following procedure was followed: horns were incubated in Ca^{++} and Mg^{++} free Krebs solution for a total of 80 min before the addition of [^{3}H]-metaraminol. During this pre-incubation period, horns were transferred to fresh solution every 20 min. In other experiments EDTA (1 mM) was present for the first 40 min of pre-incubation. During incubation with [^{3}H]-metaraminol, the Krebs solution contained Mg^{++} but no Ca^{++}. Retention of the amine was unaffected by either procedure, so all the values obtained have been pooled and are shown in Table 2.

TABLE 2

EFFECT OF CATIONS ON RETENTION OF [^{3}H]-METARAMINOL BY RAT UTERINE HORNS

In all cases control horns were incubated in Krebs solution that had been equilibrated with 95% oxygen and 5% carbon dioxide.

Medium	Duration of pre-incubation (min)	*R* value (mean ± S.E.) Control	Experimental	*t* test	Pairs of horns (No.)
Low Na^+ Krebs solution	30	8·7±0·4	2·8±0·1	$P<0.001$	8
Lithium Krebs solution	30	7·7±0·9	4·1±0·2	$P<0.01$	8
K^+-free Krebs solution	30	9·1±0·5	3·6±0·6	$P<0.001$	13
Rb^+ Krebs solution	30	8·1±1·0	3·9±0·6	$P<0.01$	8
Ca^{++}-free Krebs solution	80	8·4±0·5	7·6±0·4	N.S.	17

(*b*) *Inhibition of metabolism*

i. *Anoxia.* Equilibration of the Krebs solution used with 95% nitrogen and 5% carbon dioxide for 120 min (75 min pre-incubation and 45 min incubation with [^{3}H]-metaraminol) did not reduce retention (Table 3). These results suggested that the energy requirements, if any, for retention of metaraminol might be provided by anaerobic glycolysis. To test this possibility, retention was examined in horns incubated in glucose-free Krebs solution equilibrated with 95% nitrogen and 5% carbon dioxide; 120 min of such treatment did not reduce retention but, after 240 min, retention was somewhat reduced when compared with control horns incubated in Krebs solution containing glucose (49.2 mM) and equilibrated with 95% oxygen and 5% carbon dioxide (Table 3).

ii. *Dinitrophenol.* Retention was unchanged after 60, 120 and 240 min incubation with DNP 1 mM in the presence of *d*-glucose 49.2 mM (Table 3).

iii. *Iodoacetic acid.* Incubation with IAA 1 mM resulted in no reduction in retention after 60 min, a significant reduction after 120 min and a very marked reduction after 240 min (Table 3).

iv. *Dinitrophenol and iodoacetic acid.* Retention was greatly reduced after 60 min incubation with both DNP 1 mM and IAA 1 mM (Table 3).

v. *Dinitrophenol and various saccharides.* Studies had shown (see (*b*) ii above) that retention was not reduced by incubation in DNP 1 mM and *d*-glucose 49.2 mM. These results suggested that the energy requirements for retention of metaraminol, in the presence of DNP, were being supplied by glycolysis. To examine this possibility the following experimental design was adopted: all horns were incubated for 120 min (75 min pre-incubation and 45 min with [^{3}H]-metaraminol); one horn was incubated in Krebs solution containing DNP 1 mM and *d*-glucose 49.2 mM and served as a control; the other

TABLE 3

EFFECT OF METABOLIC INHIBITORS ON RETENTION OF [^{3}H]-METARAMINOL BY RAT UTERINE HORNS

In all cases control horns were incubated in Krebs solution equilibrated with 95% oxygen and 5% carbon dioxide.

Medium	Duration of pre-incubation (min)	*R* value (mean ± S.E.) Control	Experimental	*t* test	Pairs of horns No.
Krebs solution equilibrated with:					
95% N_2/5% CO_2	75	9·2±0·3	9·3±0·3	N.S.	4
95% N_2/5% CO_2 and glucose free	75	5·9±0·7	5·6±0·6	N.S.	11
95% N_2/5% CO_2 and glucose free	195	4·9±0·7	3·6±0·4	N.S.	6
Krebs solution containing:					
DNP (1 mM)	15	7·4±0·4	6·7±0·4	N.S.	7
	75	9·2±0·9	10·0±0·5	N.S.	7
	195	9·1±0·7	9·0±1·4	N.S.	4
IAA (1 mM)	15	7·3±0·7	6·9±1·0	N.S.	9
	75	9·0±0·7	4·5±0·5	$P<0·001$	8
	195	9·4±1·8	1·3±0·5	$P<0·01$	4
DNP (1 mM) and IAA (1 mM)	15	9·4±1·2	2·3±0·2	$P<0·001$	8

horn was incubated in Krebs solution containing DNP 1 mM and another saccharide, not *d*-glucose, 49.2 mM. It can be seen from Table 4 that only *d*-glucose and *d*-mannose were able to support normal retention in the presence of DNP; sucrose, *d*-galactose, *d*-fructose and 2-deoxy-*d*-glucose were unable to do this.

TABLE 4

EFFECT OF DINITROPHENOL AND VARIOUS SACCHARIDES ON THE RETENTION OF [^{3}H]-METARAMINOL BY RAT UTERINE HORNS

All horns were pre-incubated for 75 min in Krebs solution containing DNP 1 mM and equilibrated with 95% oxygen and 5% carbon dioxide. Control horns were pre-incubated in Krebs solution containing *d*-glucose and experimental horns in Krebs solution in which *d*-glucose had been replaced by another saccharide. The concentration of all sugars used was 49·2 mM.

	R value (mean ± S.E.) Control	Experimental	*t* test	Pairs of horns (No.)
DNP Krebs solution containing:				
Sucrose	6·7±1·3	2·6±0·1	$P<0·01$	6
d-Mannose	3·9±0·3	4·4±0·5	N.S.	8
d-Galactose	7·1±0·7	2·4±0·1	$P<0·001$	3
d-Fructose	6·2±0·4	3·0±0·3	$P<0·001$	4
2-deoxy-*d*-glucose	6·4±0·7	3·3±0·3	$P<0·01$	5

(*c*) *Saccharides*

It can be seen (Table 5) that incubation with any of the following saccharides, at a concentration of 49.2 mM, for 120 min in Krebs solution did not reduce retention: sucrose, *d*-galactose, *d*-fructose, 2-deoxy-*d*-glucose and *d*-mannose. The control horn in each case was incubated in Krebs solution containing d-glucose 49.2 mM.

TABLE 5

EFFECT OF VARIOUS SACCHARIDES ON RETENTION OF [^{3}H]-METARAMINOL BY RAT UTERINE HORNS

All horns were pre-incubated for 75 min in solutions that had been equilibrated with 95% oxygen and 5% carbon dioxide. The concentration of saccharide used was 49·2 mM throughout. Control horns were incubated in Krebs solution containing *d*-glucose and experimental horns in Krebs solution in which *d*-glucose had been replaced by another saccharide.

Saccharide	*R* value (mean ± S.E.) Control	Experimental	*t* test	Pairs of horns (No.)
Sucrose	8·0±0·7	7·4±0·8	N.S.	4
d-Mannose	5·7±0·7	5·7±0·6	N.S.	4
d-Galactose	6·9±0·3	6·7±0·4	N.S.	3
d-Fructose	8·8±0·9	9·1±1·3	N.S.	3
2-deoxy-*d*-glucose	9·6±0·9	8·8±0·5	N.S.	3

(*d*) *Reserpine pretreatment*

Rats were given reserpine, 5 mg/kg body weight, intraperitoneally, 18 hr before being killed. Horns from such animals were able to concentrate and retain [^{3}H]-metaraminol; this process was dependent on Na^+ and K^+, reduced by IAA and unaffected by DNP as in the case of horns obtained from normal animals (Table 6).

TABLE 6

EFFECT OF VARIOUS MEDIA ON THE RETENTION OF [^{3}H]-METARAMINOL BY RESERPINE PRE-TREATED RAT UTERINE HORNS

All horns were obtained from rats given reserpine (5 mg/kg intraperitoneally) 18 hr before. In all cases control horns were incubated in Krebs solution that had been equilibrated with 95% oxygen and 5% carbon dioxide.

Medium	Duration of pre-incubation (min)	*R* value (mean ± S.E.) Control	Experimental	*t* test	Pairs of horns (No.)
Low Na^+ Krebs solution	30	6·2±0·5	1·8±0·1	$P<0·001$	4
K^+ free Krebs solution	30	5·0±1·6	3·0±0·4	N.S.	4
Krebs solution containing:					
DNP (1 mM)	75	7·2±0·7	6·9±0·4	N.S.	8
IAA (1 mM)	75	6·6±0·4	2·8±0·1	$P<0·001$	8

Effects on contractility of metabolic inhibitors

Immature rat uterine horns were spontaneously active in Krebs solution at 37° C; this activity was rapidly abolished on exposure to either DNP 1 mM or IAA 1 mM. Contractions to supramaximal concentrations of acetylcholine were rapidly abolished by IAA 1 mM and this effect was irreversible. Contractions to acetylcholine continued for prolonged periods in DNP 1 mM when *d*-glucose 49.2 mM was present; however, in the presence of DNP and glucose-free Krebs solution, contractility rapidly disappeared. The effects of DNP were readily reversible. These features are shown in Fig. 1.

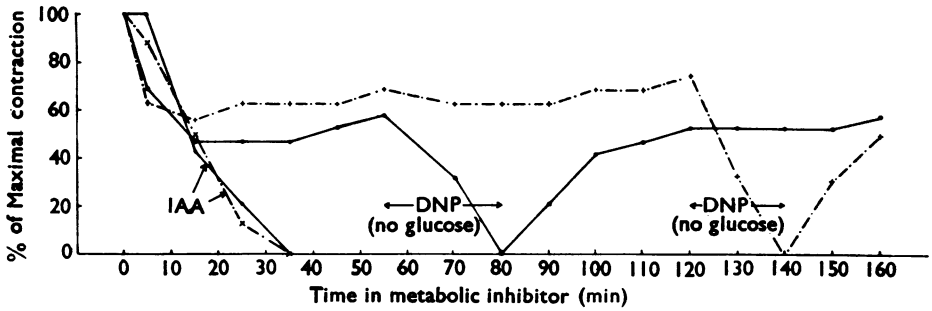

Fig. 1. Effect of metabolic inhibitors on contractility of immature rat uterine horns. Isometric contractions to a supramaximal concentration of acetylcholine (10 μg/ml.) were obtained in Krebs solution at 37° C (100% response). Two horns were then placed in Krebs solution containing DNP (1 mM) and are indicated: + — · — + ; ○——○. Two horns were placed in Krebs solution containing IAA (1 mM) and are indicated: ●——● ; × — · — ×. Responses to acetylcholine (10 μg/ml.) were elicited every 10 min and expressed as a percentage of the control response. The Krebs solution containing DNP had *d*-glucose (49.2 mM) present except as shown.

DISCUSSION

Immature rat uterine horns were utilized as a test system in the study reported here because the presence of two horns allows paired studies, the horns concentrate amines very efficiently, the ratio of cut to intact surfaces is small and diffusion should not be a great problem because the horns are very thin. A disadvantage is that the occasional preparation seems to be under oestrogen dominance because such horns are much heavier, larger and filled with liquid ; these were not used in the present studies because retention falls rapidly with increasing horn weight (Green & Miller, 1966a).

The subcellular localization and anatomical site for amine retention in rat uterine horns has not been clearly established. Wurtman, Axelrod & Potter (1964) reported that the retention of catecholamines by rat uteri was relatively unaffected by cocaine and tyramine and that the amines were predominantly in the supernatant fraction. Subsequently Green & Miller (1966a, b) showed that cocaine competitively inhibited the uptake of noradrenaline into rat uteri while the efflux of noradrenaline was increased by tyramine. Gutman and Weil-Malherbe (1967) were unable to confirm the previous report of the predominant localization of noradrenaline in the soluble fraction of the rat uterus.

The results presented in this paper provide additional evidence that metaraminol is taken up by peripheral tissues in a manner similar to that of noradrenaline. Thus in both cases retention is dependent on Na^+ and K^+ and energy supplies ; previous studies have shown that retention of both amines is dependent on an intact adrenergic innervation, results in concentration of the amine and is inhibited by ouabain, reduced temperatures and a number of compounds (Giachetti & Shore, 1966 ; Berti & Shore, 1967). As indicated earlier, however, a marked difference is seen when retention of the two amines is compared in tissues pretreated with reserpine ; retention of metaraminol is normal whereas that of noradrenaline is greatly reduced (Giachetti & Shore, 1966). The proposal

of Giachetti and Shore that any agent which impairs the retention of metaraminol must be acting by impairing the transport of the amine across the nerve membrane may not be entirely correct, in view of the demonstration that metaraminol can be bound by subcellular particles even in the presence of reserpine and that this process is not dependent on either ATP or Mg^{++} as is the case with the binding of noradrenaline to subcellular particles (Lundborg & Stitzel, 1967).

Catecholamine transport into peripheral tissues seems to be linked to cation transport. This is indicated by the observations that ouabain prevents amine retention (Dengler, 1965; Green & Miller, 1966a), that the action of various digitalis-like compounds on metaraminol retention seems to be proportional to inhibition of the Na^+-K^+ dependent adenosine triphosphatase (ATPase) (Berti & Shore, 1967), that omitting Na^+ or K^+ from the medium inhibits amine retention (Iversen & Kravitz, 1966; Gillis & Paton, 1966, 1967; Paton, 1967) and that an increase in K^+ concentration in the external medium overcomes the reduction in amine retention induced by ouabain (Titus & Dengler, 1966). The exact nature of the relationship between amine and cation transport is unknown. Possible mechanisms include: (*a*) dependence of amine retention on the maintenance of normal intracellular concentrations of Na^+ and K^+; (*b*) dependence of both amine retention and cation transport on the Na^+, K^+-activated ATPase of all membranes; and (c) dependence of amine retention upon Na^+ and/or K^+ movement across the membrane but not directly upon the activity of the ATPase. Of these possibilities, (*b*) and (*c*) seem to be most likely to affect the uptake stage of amine retention and (*a*) the storage or binding stage. The present results, in terms of the proposal of Giachetti & Shore (1966), would support a role of Na^+ and K^+ in the uptake phase of catecholamine retention.

Investigations into the role of Ca^{++} in retention of catecholamines have resulted in conflicting results. Thus Ca^{++} has been claimed to have no effect on the retention of amines by perfused rat heart (Iversen & Kravitz, 1966) perfused cat spleen (Kirpekar & Misu, 1967) and isolated rat uterine horns (Green & Miller, 1966a; present study) whereas Ca^{++} was needed for optimal retention of amines by heart slices (Dengler, 1965; Gillis & Paton, 1966, 1967) and guinea-pig uterus (Boullin, 1967). These differences may reflect variations in the amount of bound Ca^{++} remaining and/or in the degree of increased permeability produced by the absence of Ca^{++} (Hays, Singer & Malamed, 1965). Certainly the procedures adopted in the present study to eliminate Ca^{++} either abolish or very greatly reduce contractile responses of uterine horns to agonists (Paton & Daniel, 1967).

The following evidence was obtained that normal retention of [^{3}H]-metaraminol requires energy and that this can be provided by glycolysis: (*a*) retention was not reduced by DNP, provided that *d*-glucose was present; (*b*) retention was reduced by DNP where *d*-glucose was replaced by a non-metabolizable sugar (for example, sucrose, *d*-galactose, *d*-fructose, 2-deoxy-*d*-glucose); (*c*) retention was reduced by DNP in the presence of *d*-glucose if IAA was also present; (*d*) retention was reduced by IAA (but see below); and (*e*) anoxia did not reduce retention if *d*-glucose was present. Similar findings have been reported previously; retention of [^{3}H]-noradrenaline was abolished by DNP if *d*-glucose was absent (Dengler, 1965) while IAA had little (Dengler, 1965) or no effect (Green & Miller, 1966a) on the retention of [^{3}H]-noradrenaline.

If retention of metaraminol is in fact dependent on the energy supplied by glycolysis, then it is surprising that exposure of horns to IAA 1 mM for 1 hr had no effect; this concentration and exposure time would be expected to inhibit glycolysis (Webb, 1966). Webb has also pointed out that use of IAA 1 mM for more than 1 hr will certainly inhibit many enzymes apart from 3-phosphoglyceraldehyde dehydrogenase and thus cannot be regarded as a specific inhibitor of glycolysis in these circumstances. Iodoacetic acid 1 mM produces very marked effects on oestrogenized rat uterine horns; after 1 hr, marked downhill ion movements were present, adenosine triphosphate (ATP) levels had fallen by 90% and contractility was abolished (Daniel, Carroll, Robinson & Grahame, 1967). To exclude the possibility that immature horns were generally resistant to IAA, its effects on contractility were studied; this showed that contractility was as rapidly and completely abolished as in oestrogenized horns. The relative resistance of metaraminol retention to IAA could be the result of poor penetration of IAA into the adrenergic nerve ending and/or a large intraneuronal store of adenine nucleotides which falls slowly in the presence of IAA because of limited utilization of energy. The more rapid action of DNP in the absence of *d*-glucose or in the presence of IAA is explicable because DNP not only uncouples oxidative phosphorylation but also stimulates an ATPase (Slater, 1963); thus energy supplies in the presence of DNP would be more dependent on continuing glycolysis than on any pre-existing adenine nucleotide stores.

The retention of [^{3}H]-metaraminol could be dependent on glycolysis because: (*a*) the transport of the amine across the nerve membrane requires energy; (*b*) the storage (or binding) of the amine requires energy; or (*c*) transport of the amine is linked to energy dependent Na^+ and K^+ movements. Insufficient evidence is available to decide between these possibilties; however, the following observations appear pertinent. Retention of metaraminol by normal and reserpine pretreated horns (this study) and of noradrenaline by normal horns (Paton, unpublished observations) is dependent on glycolysis. The only stage or process in retention apparently common to these three situations seems to be the uptake or transport of the amine across the nerve membrane; the storage phase may not be dependent on glycolysis.

SUMMARY

1. Immature rat uterine horns were utilized to investigate the effect of various conditions on the retention of [^{3}H]-metaraminol.

2. Retention of [^{3}H]-metaraminol was reduced significantly by the reduction of Na^+ or absence of K^+ in the external bathing medium; Li^+ and Rb^+ were unable to substitute for Na^+ and K^+ respectively. Absence of Ca^{++} did not reduce retention.

3. Retention of [^{3}H]-metaraminol was reduced by iodoacetic acid after 2 hr but not by either dinitrophenol or anoxia when glucose was present.

4. Retention of [^{3}H]-metaraminol was normal in the presence of dinitrophenol if either *d*-glucose or *d*-mannose were present; *d*-galactose, *d*-fructose, 2-deoxy-*d*-glucose and sucrose were unable to maintain retention in the presence of dinitrophenol.

5. The above conditions influenced retention of [^{3}H]-metaraminol similarly in normal and reserpine pretreated uterine horns.

6. It was concluded that the uptake phase of retention of [^{3}H]-metaraminol requires Na^+ and K^+ in the external medium and an energy source; this can be provided by glycolysis. Glycolysis in adrenergic nerve endings seems to be relatively resistant to inhibition by iodoacetic acid.

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