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THE ACCUMULATION AND METABOLISM OF [^{14}C]HYPOXANTHINE BY SLICES OF RABBIT RENAL MEDULLA

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SUMMARY

The uptake of hypoxanthine by rabbit renal medulla has been studied with *in vitro* conditions. Unlike the uptake by renal cortex slices reported earlier, no evidence was found for involvement of an organic cation transport system. Medullary accumulation of the ^{14}C-labeled material occurred in the absence of O_2 if glucose was present as substrate. Uptake of the ^{14}C label was not supported by other sugars or metabolic intermediates, however. Metabolic inhibitors reduced both aerobic and anaerobic uptake. Tissue extracts were subjected to high-voltage electrophoresis and gel filtration for identification of possible metabolites. These studies indicated that most of the uptake of ^{14}C-labeled material was accounted for by its conversion to inosine-like and/or inosinic acid-like compounds. That is, when experimental conditions were designed to retard slice metabolism of hypoxanthine, tissue to medium ratios for ^{14}C approximated 1.0. A third metabolite was found occasionally, but remains unidentified.

INTRODUCTION

A number of studies from this laboratory and others have described the *in vitro* handling of a variety of oxypurines by renal cortical tissues[1-6]. At least two oxypurines, xanthine and uric acid, are not transported by renal medullary tissue of the rat or rabbit. That is not to say, however, that renal medullary tissue is devoid of metabolic or transport activities.

Kean and collaborators[7,8] demonstrated that renal medullary tissue possessed marked glycolytic activity while only modest respiratory activity. Although there has been some debate about the relative importance of these metabolic pathways[9,10], most workers agree that these pathways exist and probably are important to conservation and production of energy in renal tissue.

In addition to this, Kean *et al.*[11] showed that leached medullary slices accumulated K^+. Although from a quantitative point of view this process was a modest one, it was temperature sensitive and could be affected by some metabolic inhibitors. Most interesting was the observation that uptake of K^+ in a N_2 atmosphere was as marked as the uptake in the presence of O_2. Neither procedure, however, permitted accumulation of K^+ to control levels.

The present study describes another medullary accumulation process. In this case uptake of an organic compound, hypoxanthine, can be demonstrated in medullary slices but not by means of a specific, active system. The accumulation of labeled hypoxanthine appears to be related to its metabolism to other compounds.

METHODS

Rabbits were killed by a blow to the base of the skull. The kidneys were removed immediately and placed in ice-cold, balanced salt solution. Free-hand slices of medullary tissue were prepared either before or after separation of the cortex. Papillary and medullary slices were pooled for these studies. As much as possible red medullary tissue was excluded from the tissue pools. In some experiments free-hand cortical slices were also made. In order to obtain enough medullary tissue for most experiments, it was necessary to pool the tissues from two animals.

The incubation solution in most experiments was a modified Krebs–Ringer phosphate solution ($[Ca^{2+}] = 1.0$ mM, $[K^+] = 5$ mM). In most experiments 8-[^{14}C]-hypoxanthine was added to a concentration of 0.1 μCi/ml, and enough unlabeled hypoxanthine to give a total chemical concentration of about 10 μM. The incubations were performed in a Dubnoff shaker at 25 °C with either 100% O_2 or 100% N_2 in the gas phase. Media osmolalities of from 285 to 295 mosM (as determined by freezing point depression) were used throughout this study. At the end of the experimental period, the tissues were blotted, weighed, and homogenized in cold, distilled water. Enough trichloroacetic acid was added to both the tissue homogenates and the incubation media to give a final concentration of 5 %. After 20 min at room temperature the samples were centrifuged and aliquots of the supernatants were added to Bray's solution prior to counting in a Packard Tri-Carb spectrometer. Appropriate quench corrections were employed and the radioactivity uptake data were expressed as the slice/medium ratio, *i.e.* the ^{14}C concentration in the tissue divided by that in the bathing solution. Total recovery of radioactivity (tissue label *plus* medium label) was about 97 % complete.

To determine the chemical nature of the label in the tissues and media after incubation, the protein-free supernatants were subjected to high-voltage electrophoresis and gel filtration. The electrophoresis was performed on Whatman 3MM paper with a standard flatplate system. A borate buffer (pH 9.5) was used and the voltage (1500 V, about 30 V/cm) was imposed for 3 h. After drying the papers at room temperature, the radioactivity on each strip was determined with an automatic gas-flow (Packard Radiochromatogram scanner). The gel filtration was performed with either Sephadex G-10 or G-15 in a 2.5 cm × 100 cm column. The column was eluted with 0.05 M phosphate buffer (pH 7.4) and the samples collected in an automatic fraction collector. The gel filtration separations were performed either at room temperature or at 3 °C and the radioactivity of the fractions determined by liquid scintillation counting.

When appropriate, statistical analyses were performed using Students' t test or a paired comparison.

RESULTS

Earlier studies in this laboratory showed that hypoxanthine uptake by rabbit

renal cortex was marked. In Table I accumulation by medullary tissue is compared with that for cortex. Although higher slice/medium ratios were found with cortical tissue, the rabbit renal medulla accumulated significant amounts of ^{14}C.

Effect of substrates and metabolic inhibitors

Acetate failed to enhance [^{14}C]hypoxanthine uptake by medullary slices. Both pyruvate and glucose, however, promoted a significant increase in label accumulation. These data are presented in Table I.

TABLE I

COMPARISON OF CORTICAL AND MEDULLARY UPTAKE AND THE EFFECT OF SUBSTRATES ON [^{14}C]-HYPOXANTHINE UPTAKE BY RENAL MEDULLARY SLICES

Incubations were for 3 h in 100% O_2 at 25 °C.

	n	*^{14}C slice/medium ratio ± S.E.*	*P*
Cortex *	4	14.9 ± 3.0	—
Medulla			
Control	12	5.6 ± 0.55	—
Glucose	7	9.7 ± 1.27	<0.01
Acetate	4	5.3 ± 0.70	—
Pyruvate	2	8.0	—

* Data published previously[3].

TABLE II

EFFECT OF METABOLIC INHIBITORS ON [^{14}C]HYPOXANTHINE UPTAKE BY RENAL MEDULLARY SLICES

Incubations were for 3 h at 25 °C. The gas phase was 100% O_2 and 10 mM glucose was present as substrate.

	Concn (mM)	*n*	*^{14}C slice/medium ratio ± S.E.*	*P*
Control		7	9.7 ± 1.27	—
2,4-Dinitrophenol	0.1	3	1.5 ± 0.06	<0.01
	0.01	5	2.5 ± 0.77	<0.01
Iodoacetamide	0.1	3	3.6 ± 0.52	<0.01

In Table II the effects of two metabolic inhibitors are summarized. Both 2,4-dinitrophenol and iodoacetamide depressed the ^{14}C uptake, although the greater effect was noted with dinitrophenol. In these experiments, glucose was used as a substrate, but similar effects were found in the absence of substrate.

Effect of potential competitors

Hypoxanthine uptake by renal cortex occurred partly by the classical renal cation transport process[8,18]. This conclusion was reached mainly on the basis of competition studies, *i.e.* the reduction of hypoxanthine uptake by compounds known to be transported by the organic base system, *e.g.* quinine. To test this possibility as a mechanism for medullary uptake, two specific and efficacious inhibitors were

TABLE III

EFFECT OF VARIOUS POTENTIAL INHIBITORS ON [^{14}C]HYPOXANTHINE UPTAKE BY RENAL MEDULLARY SLICES

Incubations were for 3 h at 25 °C in the presence of 100% O_2.

	Concn (mM)	*n*	*Slice/medium ratio ± S.E.*	*P*
Control		4	3.8 ± 0.93	—
Cyanine No. 863	0.038	4	1.9 ± 0.35	<0.05
Control		6	2.8 ± 0.21	—
Phenoxybenzamine	0.1	6	2.4 ± 0.19	>0.2
Control		7	5.6 ± 0.47	—
6-Mercaptopurine	0.1	4	2.5 ± 0.49	<0.01
	1	2	0.92	—
Allopurinol	0.1	4	6.2 ± 0.94	N.S.
Adenine	0.01	2	2.4	—
	0.1	2	1.2	—
Guanine	0.01	2	3.0	—
	0.1	2	1.6	—

tested (cyanine No. 863 (ref. 13) and phenoxybenzamine[14]), and the data from these experiments are presented in Table III. Phenoxybenzamine failed to alter label accumulation while cyanine No. 863 reduced it significantly.

With renal cortical tissue, a specific purine uptake process was observed[3] in addition to the classical organic base system. To test for the existence of such a process in medullary tissue several purines or related substances were tested as potential inhibitors of hypoxanthine uptake. These data are given in Table III. All three of the purines tested, 6-mercaptopurine, guanine, and adenine, reduced the uptake of labeled material significantly. As with hypoxanthine uptake by cortical slices[3], allopurinol failed to alter label accumulation.

Effects of incubations in a N_2 atmosphere

The data from experiments in which medullary slices were incubated in N_2

TABLE IV

COMPARATIVE EFFECT OF O_2 AND N_2 AND THE EFFECTS OF VARIOUS METABOLIC SUBSTRATES ON [^{14}C]HYPOXANTHINE UPTAKE BY RENAL MEDULLA IN PRESENCE OF 100% N_2

Incubations were for 4 h at 25 °C. All substrates were present in a concentration of 10 mM.

	n	*^{14}C slice/medium ratio ± S.E.*	*P*
O_2	12	5.6 ± 0.55	—
N_2			
Control	13	1.2 ± 0.06	—
Glucose	10	2.9 ± 0.43	<0.01
Fructose	5	1.6 ± 0.29	>0.05
Ribose	5	1.4 ± 0.20	—
Acetate	5	1.3 ± 0.12	—
Pyruvate	2	1.2	—
α-Ketoglutarate	1	1.1	—

are compared in Table IV with those presented earlier. In the absence of substrate, N_2 caused a significant reduction in the uptake of label.

The effects of various substrates on label accumulation in nitrogen were also examined. These data are presented in Table IV. Only glucose increased significantly

TABLE V

EFFECT OF METABOLIC SUBSTRATES ON [^{14}C]HYPOXANTHINE UPTAKE BY RENAL CORTEX IN PRESENCE OF N_2

Incubations were for 4 h at 25 °C. Substrates were employed in a concentration of 10 mM.

	n	*^{14}C slice/medium ratio ± S.E.*	*P*
Control	7	1.04 ± 0.02	—
Glucose	7	1.18 ± 0.04	<0.05
Fructose	4	1.02 ± 0.04	—
Ribose	4	1.03 ± 0.03	—
Acetate	4	1.0 ± 0.03	—

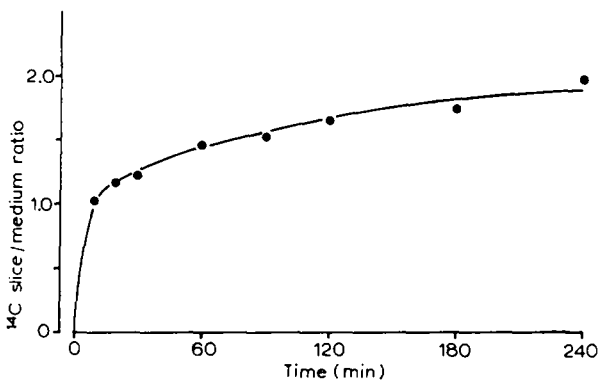

Fig. 1. The time course of [^{14}C]hypoxanthine uptake by rat medullary slices. These data are from a typical experiment in which the incubations were performed in a N_2 atmosphere with glucose present.

TABLE VI

EFFECTS OF INHIBITORS ON MEDULLARY UPTAKE OF [^{14}C]HYPOXANTHINE IN PRESENCE OF 100% N_2

Incubations were for 4 h at 25 °C with glucose (10 mM) present as substrate.

	Concn (mM)	*n*	*^{14}C slice/medium ratio ± S.E.*	*P*
Control		4	2.4 ± 0.13	—
2,4-Dinitrophenol	0.01	3	2.3 ± 0.25	—
	0.1	4	1.7 ± 0.05	<0.01
Iodoacetamide	0.1	4	1.4 ± 0.05	<0.01
6-Mercaptopurine	0.01	4	2.6 ± 0.15	—
	0.1	4	1.8 ± 0.08	<0.01

the uptake of label under anaerobic conditions. The steady state slice/medium ratio developed obtained by these tissues was not as great as that seen in the presence of O_2, but was nearly three times that in the absence of substrate. None of the other substrates had any effect.

A qualitatively similar, but quantitatively different response was obtained with renal cortex slices (Table V). Cortical tissue incubated in N_2 showed no net accumulation of [^{14}C]hypoxanthine. Several substrates were tested and only glucose produced a significant increase in label uptake. However, the magnitude of the stimulation (about 15 %) did not approach that noted with medullary tissue (about 300 %).

The time course of glucose-stimulated label accumulation by medullary slices incubated in nitrogen is presented for a typical experiment in Fig. 1. After a relatively rapid initial uptake, the process proceeds at a modest rate, tending towards a steady-state value only after 4 h.

Three inhibitors were tested on the anaerobic, glucose-stimulated label uptake by medullary slices, and the data are presented in Table VI. Although dinitrophenol significantly depressed label accumulation, the magnitude of this effect was less than that seen for iodoacetamide. This situation is the reverse of that seen when the incubations were performed in O_2 (Table II), where dinitrophenol produced the greater inhibition. 6-Mercaptopurine produced a relatively small, but statistically significant, decrease in the glucose-stimulated label accumulation by medullary slices incubated in N_2.

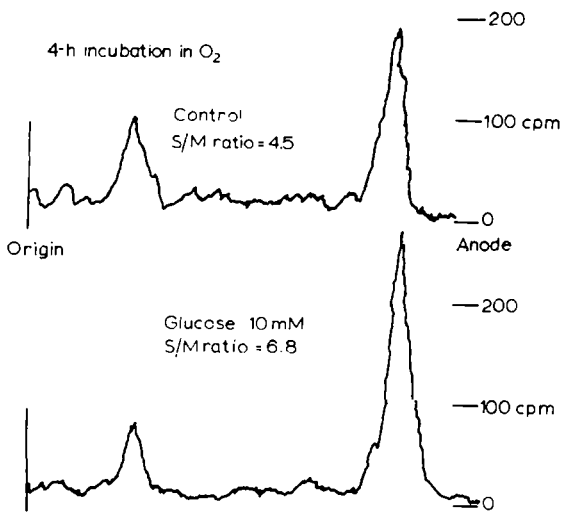

Fig. 2. Electrophoretogram from a medullary tissue extract. Medullary slices were incubated in O_2 with and without glucose prior to extraction of the label. S/M = slice/medium.

Slice metabolism of [^{14}C]*hypoxanthine*

Earlier studies on cortical slices were interpreted to mean that the tissue from most rabbits did not metabolize hypoxanthine. However, because some animals did show this capability the question of *in vitro* metabolism of [^{14}C]hypoxanthine was also examined with the medullary tissue.

In Fig. 2 is presented an electrophoretogram of medullary extracts from tissues

incubated in O_2 with and without glucose. The radioactivity peak near the origin corresponded to authentic hypoxanthine, (as determined by the use of standards) while the second radioactive peak was a metabolite of hypoxanthine. The second peak was increased in magnitude by performing the incubation in the presence of glucose. Some difficulty was found in obtaining reproducible results with these experimental conditions. The existence of one or two metabolites was found most often, although occasionally additional radioactive peaks were noted. The appearance of these was unpredictable and no identification of the peaks was attempted.

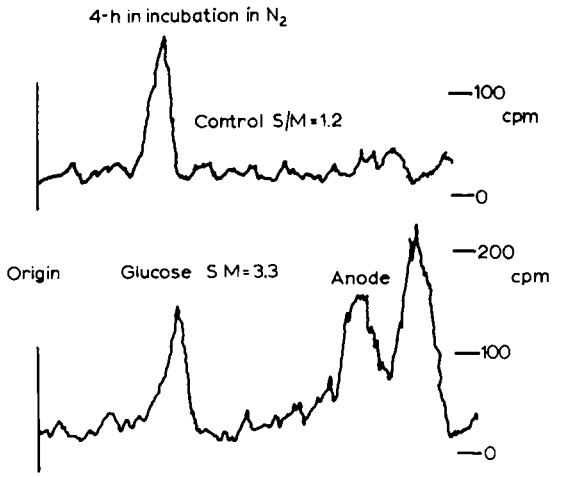

Fig. 3. Electrophoretogram from a medullary tissue extract. Medullary slices were incubated in N_2 with and without glucose prior to extraction of the label. S/M = slice/medium.

In Fig. 3 are presented similar data for tissue extracts from tissues incubated with and without glucose in a N_2 atmosphere. The radioactivity near the origin corresponds to hypoxanthine and is the only significant radioactive peak noted in the glucose-free incubation. These tissues showed no net label uptake, *i.e.* the slice/medium ratio was about 1.0. When glucose was present two additional radioactive peaks were noted, presumably representing metabolites of the original hypoxanthine. Similar data were obtained with gel filtration. In Fig. 4 are presented representative data with this technic. In the control, the radioactive peak corresponded to authentic hypoxanthine. When glucose was added two additional radioactive peaks were noted. The effluent volumes for these two peaks corresponded to those for known samples of inosine (small peak) and inosinic acid (large peak).

Similarly, for the electrophoresis studies the metabolite peaks noted in the tissue extracts had mobilities similar to those for inosine and inosinic acid standards. A comparison of these mobilities is given in Table VII. In some experiments a total of two rather than three peaks was found in the tissue extracts. When this was true, the metabolite peak had usually migrated about 12 cm from the origin.

DISCUSSION

The data presented here indicate clearly that isolated medullary tissue is capable of metabolizing [^{14}C]hypoxanthine. Also when metabolism occurs with

cortical tissue[8], the metabolites are the same as those found with medullary slices. In general two metabolites were discovered. When compared to standards these metabolites migrated in an electrical field and were handled on gel filtration in a manner similar to inosine and inosinic acid. In occasional experiments a third metabolite was observed. This substance was seen most readily as a peak in the gel filtration

TABLE VII

ELECTROPHORETIC MOBILITIES

Values are means of triplicate analyses from each of three experiments. In each experiment, tissues were incubated in N_2, at 25 °C, for 4 h in presence of 0.01 M glucose. 1500 V, 3 h, 0.05 M borate buffer (pH 9.5).

	Number of peaks	*Distance from origin (cm)*
Standard hypoxanthine	1	5.5
Standard inosine	1	9.0
Standard inosinic acid	1	12.0–13.5
Media	1 *	5.3
Tissue extracts	3	5.1
		9.5
		12.6–14.0

* Occasionally more than one peak was seen in the media.

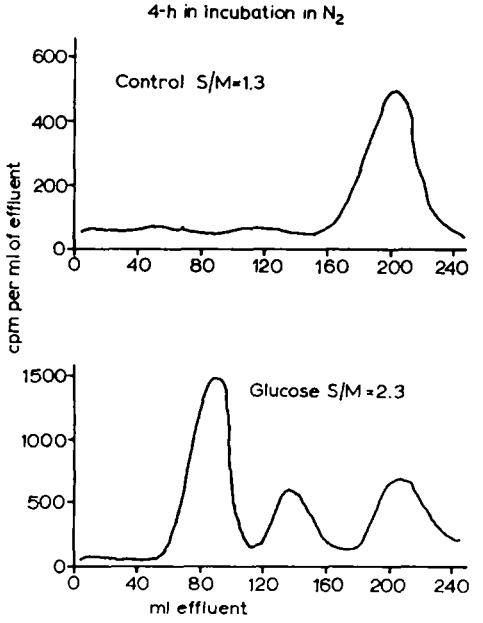

Fig. 4. Patterns of radioactivity obtained in the effluent from a Sephadex G-15 column after addition of medullary tissue extracts. Medullary slices were incubated with and without glucose in a N_2 atmosphere prior to extraction of the label. S/M = slice/medium.

effluent curve between the first (inosinic acid) and second (inosine) peaks when Sephadex G-10 was used rather than Sephadex G-15. In those experiments where this radioactive peak was prominent, the second peak was minimal. The third metabolite has not been identified. Very rarely additional radioactive peaks were seen with electrophoresis, but these data were not readily reproducible.

Most of the uptake of ^{14}C-labeled material was attributable to metabolite accumulation. In the tissue extract electrophoretograms, for example, from tissues incubated in N_2 with no substrate, no evidence of any metabolite was noted. In every case, the slice/medium ratios for the label was either 1.0 or only slightly greater than 1.0 (about 1.2 or 1.3). This slight accumulation might be interpreted as active uptake, albeit, of a modest degree. Medullary tissue has a somewhat higher extracellular fluid space (*i.e.* inulin space) than cortical tissue[15]. In this laboratory a value for total tissue of 88 % was found with an inulin space of about 45 % of the total water. If the slice/medium ratio of 1.2 is corrected for this inulin space an intracellular/extracellular distribution ratio of nearer 2.0 is found. A ratio of this magnitude is usually considered to represent active transport.

The only other data in this study which deals with the active transport question are those with metabolic inhibitors. In general, these data discredit the idea of the hypoxanthine accumulation being active. For example, neither the presence of high concentrations of 2,4-dinitrophenol (Table II) under aerobic conditions nor iodoacetamide (Table VI) under anaerobic conditions reduced the slice/medium ratio below 1.3. Given that the tissue stores of label are present as unaltered hypoxanthine, then this represents a sizable distribution ratio which is apparently uninfluenced by the usual metabolic relationships. The only inhibitor that reduced the slice/medium ratio below 1.0 was 6-mercaptopurine.

Whether the ^{14}C-labeled material was accumulated by a hypoxanthine active transport process or entirely by metabolite formation or both, it is clear that this activity is unlike anything seen in cortical tissue[3]. For example, evidence of hypoxanthine uptake by the renal organic cation transport process was obtained with cortical tissue. Both quinine and tetraethylammonium reduced hypoxanthine accumulation by cortical tissue. In this study two inhibitors that were presumed to be specific for the cortical base transport system were tested (Table III) and although one inhibited ^{14}C uptake, the other did not. Since no conclusive evidence for active transport of hypoxanthine as such was found (see below), it is likely that the cyanine No. 863 acted on the synthetic pathway. This is in keeping with other observations[15] which failed to show the classical organic base transport system in medullary tissue.

The most striking difference between these two tissues relates to the effects of N_2 on hypoxanthine uptake. Under aerobic conditions, cortical tissue accumulated more ^{14}C-labeled material than medullary slices, while the reverse was true under anaerobic conditions. With medulla, whether in the presence of O_2 or in its absence, only glucose was found to increase significantly the uptake of ^{14}C-labeled material. Further, on the basis of metabolite studies, the entire increase in label uptake was attributable to enhanced metabolite formation. With anaerobiosis cortex slices showed no net uptake of [^{14}C]hypoxanthine. The addition of glucose to the bathing solution produced a statistically significant increase in ^{14}C uptake. However, the magnitude of this enhanced uptake is small and its biological significance is not obvious.

The finding of an anerobic process in renal medullary tissue is not entirely new. For example, others have reported K^+ accumulation[11] and amino acid uptake[15] by rat renal medullary tissue in the absence of O_2. In particular the amino acid studies are noteworthy. Lowenstein *et al.*[16] reported distribution ratios of 4–5 for the non-metabolizable amino acid, α-aminoisobutyric acid, under anaerobic conditions. The findings in the present study re-emphasize the anaerobic capabilities of medullary tissue. Whether or not active accumulation of hypoxanthine occurs, its conversion to inosine-like and inosinic acid-like compounds is unequivocal under anaerobic conditions if an energy source is present.

The role of this process in renal medullary economy is not clear. Probably, however, this is not indicative of a special metabolic process, but rather just additional evidence that medullary tissue can carry on normal activities under anaerobic conditions. This would be in keeping with all the earlier demonstrations that medullary tissue contains the energy-making, metabolic machinery for anaerobic activity[7,8,10,17,18]. Furthermore, the importance of glucose as a medullary energy source is emphasized by the recent work of Abodeely and Lee[19].

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