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THE ACCUMULATION OF ^{14}C-HYPOXANTHINE BY SLICES OF RABBIT KIDNEY CORTEX*

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Abstract—The accumulation of C^{14}-hypoxanthine by slices of rabbit kidney cortex has been examined. The label was taken up over a long time course and reached steady state levels only after 4 hr. No evidence was found of metabolic transformation of the labeled material indicating that the slow accumulation is a characteristic of the transport process. The uptake of ^{14}C-hypoxanthine was depressed by metabolic inhibitors (dinitrophenol, cyanide, iodoacetamide). Of the substrates tested, only acetate stimulated uptake, while succinate, α-ketoglutarate, pyruvate, and citrate depressed uptake. Substances which affect the organic acid transport system (bromcresol green and probenecid) were without effect. Tetraethylammonium chloride and quinine, both of which are transported by the organic base system, significantly depressed ^{14}C-hypoxanthine uptake.

ALLOPURINOL‡ (4-hydroxypyrazolo (3,4-d) pyrimidine) is an inhibitor of xanthine oxidase.[1] Although initially developed to prolong the biological half-life of 6-mercaptopurine,[1, 2] it was soon recognized that allopurinol also reduced the conversion of hypoxanthine and xanthine to uric acid.[3, 4] Administration of this substance to subjects with elevated blood uric acid levels resulted in prompt reduction of the hyperuricemia.[4] Allopurinol will readily effect a reduction in blood urate levels not only in gouty patients but also in those individuals undergoing treatment for malignancies in whom the high urate levels are due to the sudden destruction of large tissue masses.

In patients treated with this drug there occurs, along with the reduction in blood urate levels, a concomitant elevation of the blood hypoxanthine and xanthine levels. This is reflected in an increased renal excretion of these oxypurines without increased excretion of other purine bases. Some difficulties have been reported in the clinical use of allopurinol, wherein acute attacks of gout have been noted at a time when blood urate levels were normal. The possibility of this being a 'xanthine gout' has been suggested, although it seems unlikely for several reasons.[4]

The renal mechanisms of hypoxanthine and xanthine excretion have not been extensively examined. Some studies *in vivo* indicate that tubular secretion of oxypurines is a possibility, although ordinarily only reabsorption was noted. A notable finding,

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however, was that uricosuric agents reduced the renal excretion of these compounds.[5, 6] In an attempt to better understand the nature of these renal transport mechanisms, the present study *in vitro* was undertaken. A preliminary report of this material has been presented.[7]

MATERIALS AND METHODS

Rabbits were stunned by a blow to the base of the skull and the kidneys were removed immediately and placed in cold Krebs–Ringer phosphate solution. The kidney cortex slices were prepared freehand and stored in cold, balanced salt solution until used.

The incubations were performed in a Dubnoff metabolic shaker or in a new Brunswick reciprocal-model Metabolyte. Shaking speed was between 90 and 100 opm and unless otherwise specified, the gas phase was 100% oxygen and the temperature 25°. Tissue slices weighing from 100–300 mg were incubated in a modified Krebs–Ringer phosphate solution. This solution was similar to that used in earlier studies,[8] except that it contained 5 mM potassium and 10 mM sodium acetate. The pH was maintained at 7·3 with a sodium phosphate buffer.

For most of these studies, $1{\cdot}4 \times 10^{-6}$ M hypoxanthine was incorporated into the bathing solutions. Measurement of uptake of this material was possible through the use of ^{14}C-8-hypoxanthine obtained from several commercial sources, to which was added an appropriate amount of unlabeled material. The amount of isotope present was usually 0·01 μc/ml. Total recoveries of radioactivity were calculated routinely and were found to range from 90–100 per cent.

After incubations, the tissues were blotted, weighed, and homogenized in 5% TCA After appropriate dilution and centrifugation the supernatants were neutralized. Aliquots of the neutralized supernatant solutions were plated on aluminum planchets prior to gas-flow counting. The incubation solutions were treated similarly. In a few experiments the tissue TCA precipitates were also analyzed for radioactivity. For this purpose the precipitates were washed twice with cold TCA, alcohol, and acetone. After drying to a constant weight they were dissolved with gentle heating in 1 N NaOH, after which the solutions were neutralized and appropriately diluted before plating for gas-flow counting.

The authenticity of the labeled hypoxanthine in the tissue extracts was verified by ascending paper chromatography. The solvents used were water and methanol : formic acid : water (160 : 30 : 10). The radioactive spots from the tissue extracts were analyzed by cutting the paper strips into several small segments, placing these in liquid scintillation vials, and counting the ^{14}C activity. These chromatograms were compared to hypoxanthine standards run simultaneously and prepared from commercial ^{14}C-hypoxanthine. In addition, unlabeled material was spotted on top of the tissue extracts and the standards to verify that the labeled material behaved the same as the unlabeled chemical.

Most statistical analyses as well as some of the routine calculations were performed on a digital computer. Statistical methods employed were Student's *t*-test, both for paired and unpaired data, and the completely randomized analyses of variance. Much of the data is presented as the slice : medium ratio (S/M ratio), i.e. the concentration of the hypoxanthine in the tissue divided by its concentration in the bathing solution.

RESULTS

Studies were undertaken to determine whether or not the radioactive material that entered the tissue slice remained as hypoxanthine, since some degree of exogenous or catabolically produced hypoxanthine can be incorporated into nucleic acids. In Fig. 1 is shown the analysis of a typical chromatogram. For this experiment, kidney cortex slices were incubated for various times with ^{14}C-hypoxanthine, the tissues were

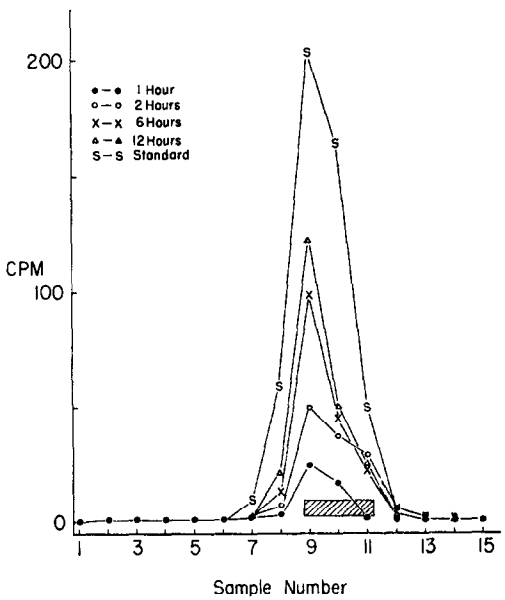

FIG. 1. Radiochromatograms of a ^{14}C-hypoxanthine standard (S-S) and tissue samples incubated for various times with ^{14}C-hypoxanthine (●—●). The hatched area indicates the location of unlabeled hypoxanthine in the same chromatograms.

extracted, and aliquots of the extracts were spotted for chromatography. Another spot was made with a ^{14}C-hypoxanthine standard and on top of this standard a sample of authentic, unlabeled hypoxanthine was spotted. The standard and unlabeled hypoxanthine appeared in the same region of the chromatogram. The radioactivity extracted from the tissue samples at the various time periods occurred at the same place on the chromatogram, and these peaks coincided exactly with the occurrence of the standard radioactivity. Furthermore, at no time period was more than one peak noted, indicating that the labeled material was not metabolized. The chromatograms depicted in Fig. 1 were developed with water as the solvent phase.

The above data indicated that the labeled material extracted from the supernatant was authentic hypoxanthine. The recoveries of total radioactivity from the supernatants and incubation media were 85–90 per cent of that added initially The tissue plugs (i.e. trichloracetic acid precipitates) that remained after extraction were examined therefore to test the extent of labeling of this material. The accumulation into the TCA precipitates (data not presented here) indicated that these were labeled to an amount equal to 5–15 per cent of the total ^{14}C-labeled material. Therefore, the amount of labeling of the TCA precipitate plus that in the tissue extracts and the incubation

media accounted for all of the radioactive substance added at the beginning of the incubation.

The rate of ^{14}C-hypoxanthine uptake was examined at three temperatures and the data are presented in Fig. 2. The incubations performed at 35° were not conducted

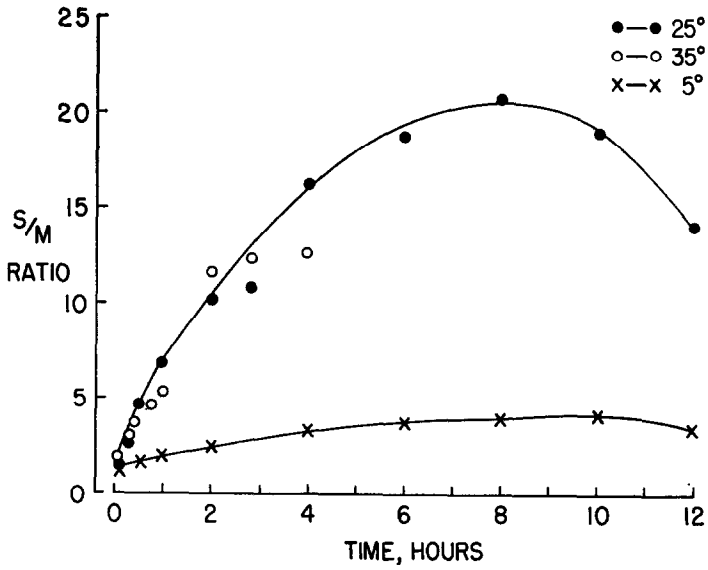

FIG. 2. Accumulation of ^{14}C-hypoxanthine by cortex slices at three temperatures. Each point is the mean of 4–6 experiments. The S/M ratio is the concentration of hypoxanthine in the tissue divided by its concentration in the bathing solution.

beyond 4 hr, since by that time there was gross evidence of tissue disintegration. At 5° the rate of uptake and the steady state values were both reduced. At 25° the steady state was reached in 4 hr, i.e. the 4, 6, 8, and 10 hr values were not significantly different from one another. In all of the subsequent experiments an incubation time of 3–4 hr was used.

The effect of hypoxanthine concentration on the 4-hr uptake of the label is presented in Fig. 3. The high S/M ratios found at low hypoxanthine concentrations were depressed as the hypoxanthine concentration was increased. For all subsequent experiments the hypoxanthine concentration employed was $1{\cdot}4 \times 10^{-6}$ M.

The effects of several metabolic inhibitors were tested and the data are presented in Table 1. All the agents tested were effective in significantly reducing the 3-hr S/M ratios. Dinitrophenol (DNP) was the most effective agent over the concentration range tested.

In Fig. 4 are presented the effects of two substrates on ^{14}C-hypoxanthine accumulation. The incubations were performed for 4 hr. Sodium acetate was found to produce a significant enhancement of uptake, while succinate significantly reduced accumulation. Because this response pattern was similar to that for *p*-aminohippurate (PAH) uptake, other substances known to modify PAH uptake were also tested. The data presented in Fig. 5 are from 4-hr incubations. Glucose failed to alter the uptake process while each of the three organic acids, α-ketoglutarate, citrate, and pyruvate,

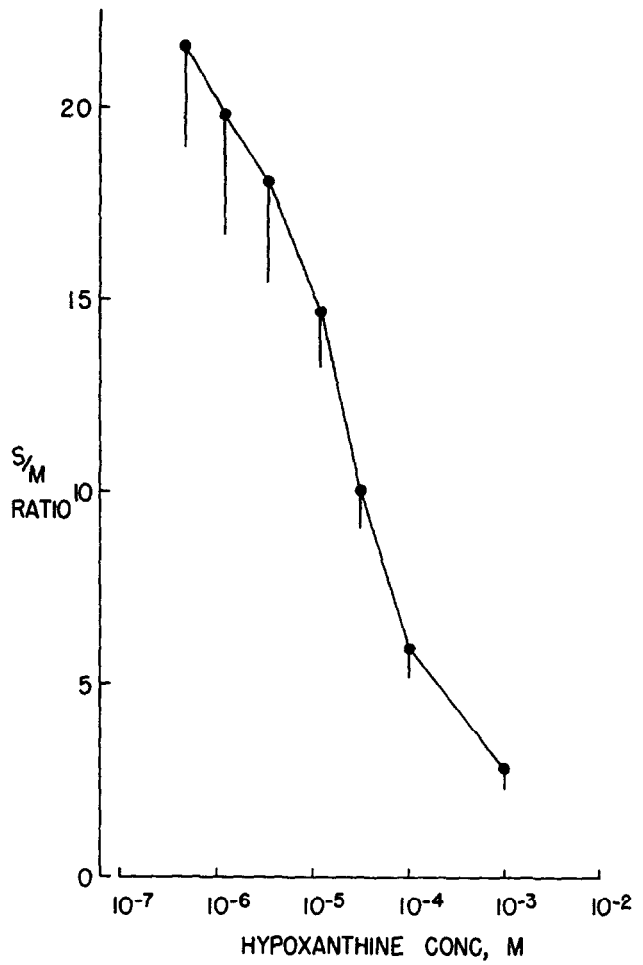

FIG. 3. The influence of hypoxanthine concentration in the bathing solution on the 4-hr ^{14}C-hypoxanthine uptake. The mean values and their S.E. are from 6–10 experiments.

TABLE 1. EFFECT OF THREE METABOLIC INHIBITORS ON ^{14}C-HYPOXANTHINE ACCUMULATION AFTER 3 HR OF INCUBATION

Inhibitor	S/M ratio ± S.E.	N	P*
Control	16·10 ± 1·56	15	
Dinitrophenol			
10^{-4} M	2·59 ± 0·164	9	<0·01
5×10^{-4} M	1·35 ± 0·109	6	<0·01
Sodium cyanide			
10^{-3} M	7·93 ± 1·45	5	<0·02
Iodoacetamide			
10^{-4} M	10·53 ± 1.01	13	<0·01
5×10^{-4} M	1·86 ± 0·12	3	<0·01

* The probability values were determined in relation to the control situation.

significantly reduced the accumulation. Tenfold lower concentrations of several of these materials were tested also, and were found to be ineffective in altering the uptake process.

In Table 2 are summarized the data for several substances that might be expected to alter the accumulation of ^{14}C-hypoxanthine. Allopurinol was tested here not as a

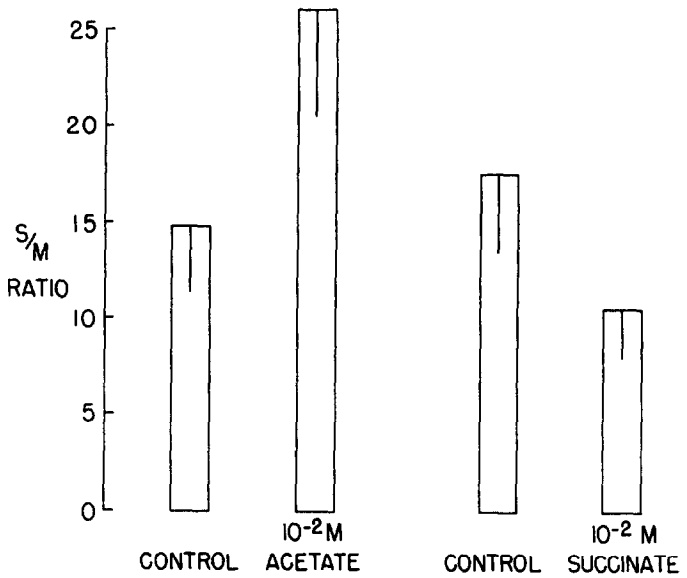

FIG. 4. The influence of acetate and succinate on the 4-hr ^{14}C-hypoxanthine accumulation. The acetate values are from 5 experiments and the succinate values from 4 experiments. Statistical analyses were by means of a paired comparison, and in each case the effect was significant ($P < 0{\cdot}05$).

xanthine oxidase inhibitor, but because its chemical structure is closely related to that for hypoxanthine, and therefore might be expected to act as a competitive antagonist. This did not prove to be true, however, since concentrations as high as 10^{-3} M failed to alter ^{14}C-uptake. Incidental to these studies, paper chromatograms of the tissue extracts from allopurinol-treated tissues were also examined. No effect on the radiochromatographic pattern was detected.

TABLE 2. EFFECT OF VARIOUS SUBSTANCES ON THE 3-HR. ^{14}C-HYPOXANTHINE UPTAKE

	Concn. (M)	S/M ratio ± S. E.	N	P*
Control		12·84 ± 3·64	4	
Allopurinol	10^{-4}	9·58 ± 2·03	5	>0·6
	10^{-3}	10·92 ± 2·85	5	>0·6
Probenecid	10^{-5}	11·29 ± 2·88	5	>0·6
	10^{-4}	10·52 ± 2·98	5	>0·6
	3×10^{-4}	8·00 ± 1·79	5	>0·2
Bromcresol green	10^{-5}	9·82 ± 2·95	4	>0·5
	10^{-4}	10·38 ± 2·55	5	>0·5
	3×10^{-5}	8·93 ± 1·84	5	>0·3

* The probability values were determined relative to the control situation.

Both probenecid and bromcresol green are known to block organic acid transport[9, 10] by renal tissue both *in vivo* and *in vitro*. Neither of these substances produced significant reductions in the steady state accumulation of label, even in the highest concentrations tested.

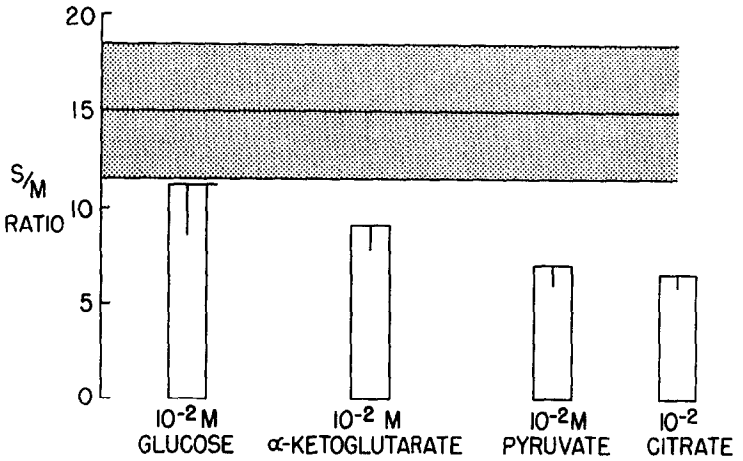

FIG. 5. Effect of several metabolic substrates on the 4-hr ^{14}C-hypoxanthine uptake. The horizontal line represents the mean control value and the stippled area one S.E. All mean S/M ratios are from 5 experiments. Statistically significant effects were noted with α-ketoglutarate ($P < 0.05$), pyruvate ($P < 0.05$), and citrate ($P < 0.02$).

In Table 3 are presented the effects of four purine substances on ^{14}C-hypoxanthine uptake. Over the concentration ranges studied two compounds, adenine and guanine, markedly reduced the ^{14}C accumulation, whereas uric acid had only a modest effect and xanthine none at all. The methylated purines, theophylline, theobromine, and caffeine, had no significant effect on hypoxanthine accumulation (data not presented).

TABLE 3. EFFECTS OF VARIOUS PURINES ON THE 4-HR ^{14}C-HYPOXANTHINE ACCUMULATION

Purine	Concn. (M)	N	S/M ratio ± S. E.	P*
Control		6	15·6 ± 1·86	
Xanthine	10^{-6}	6	15·2 ± 1·65	>0·8
	10^{-5}	6	13·2 ± 1·33	>0·3
	10^{-4}	6	12·4 ± 1·09	>0·1
Uric acid	10^{-6}	6	13·2 ± 1·24	>0·3
	10^{-5}	6	12·6 ± 1·63	>0·1
	10^{-4}	6	10·4 ± 1·43	=0·05
Adenine	10^{-6}	6	13·8 ± 0·72	>0·3
	10^{-5}	6	10·8 ± 0·65	<0·05
	10^{-4}	6	5·1 ± 0·02	<0·01
Guanine	10^{-6}	6	16·1 ± 1·06	>0·8
	10^{-5}	6	12·5 ± 1·13	>0·1
	10^{-4}	6	2·5 ± 0·02	<0·01

* P values were determined relative to control situation where no potential inhibitor was employed.

The effect of potassium on the accumulation of ^{14}C-hypoxanthine was not significant at any of the levels tested (see Table 4). Despite the apparent trend toward higher S/M ratios at higher potassium values, analysis of variance at each time period indicated no significant effects. Although there was no increase in label uptake, it is worth noting that very high levels of extracellular potassium did not inhibit the uptake process.

TABLE 4. EFFECT OF POTASSIUM ON THE ACCUMULATION OF ^{14}C-HYPOXANTHINE AT TWO TIME PERIODS

Time* (hr)	K^+ concentration (mM)				
	0	10	20†	40†	80†
1·5	10·9± 1·69‡	11·0± 1·14	13·8± 1·37	15·4± 2·08	16·4± 3·55
3	12·8± 1·49	16·8± 2·34	20·2± 2·57	16·8± 3·01	17·5± 3·64

* Use of analysis of variance indicated no significance at either time period. For 90 min, $F = 1{\cdot}065$ on 4 and 19 degrees of freedom and at 180 min, $F = 1{\cdot}561$ on 4 and 19 degrees of freedom.

† At the three higher concentrations of potassium, the sodium concentration of the bathing solution was reduced so that $[Na^+] + [K^+] =$ constant.

‡ Values are given as mean S/M ratios of ^{14}C-hypoxanthine ± S.E.

Finally, the effects of several organic bases were tested on ^{14}C-hypoxanthine uptake (Table 5). All of the materials have been shown to be transported by the renal organic base transport system.[11–15] A statistically significant depression in accumulation was noted with tetraethylammonium chloride (TEAC) and quinine sulfate, although in both cases very high concentrations of the inhibitors were required. No effect was noted with choline chloride in any concentration tested.

TABLE 5. EFFECTS OF VARIOUS ORGANIC BASES ON ^{14}C-HYPOXANTHINE ACCUMULATION

Organic base	Concn. (M)	N	S/M ratio ± S. E.	P*
Control		5	19·51 ± 2·37	
Choline chloride	10^{-5}	5	16·46 ± 1·30	>0·05
	10^{-4}	5	21·06 ± 2·99	>0·6
	10^{-3}	5	19·22 ± 2·70	>0·8
	10^{-2}	5	16·97 ± 0·89	>0·3
Tetraethyl-ammonium chloride	10^{-5}	5	16·81 ± 1·23	>0·2
	10^{-4}	5	17·16 ± 2·33	>0·2
	10^{-3}	5	15·52 ± 1·89	<0·02
	10^{-2}	5	12·67 ± 1·59	<0·01
Quinine sulfate	10^{-5}	5	17·54 ± 2·39	>0·5
	10^{-4}	5	13·23 ± 1·36	<0·05

* The probability values were determined relative to the control.

DISCUSSION

No evidence was found in the present study to indicate a significant degree of metabolism of the labeled hypoxanthine. Other attempts to verify this were made by using a spectrophotometric analysis. For these experiments, however, the concentration of hypoxanthine used in the bathing solution was elevated to 10^{-4} M, so the data may not be strictly comparable to those obtained at the lower concentration. After chromatography of tissue extracts, the visible spots were cut out, shaken with dilute NaOH, and after appropriate pH adjustment, analyzed in a recording spectrophotometer. The spectra of tissue samples were compared with simultaneously run hypoxanthine standards.

It was possible in some of these experiments to detect two spots with the u.v. lamp. The major spot corresponded to the majority of the ^{14}C label (an estimated 90 per cent) and was chromatographically identical to the ^{14}C-hypoxanthine standard. Furthermore, upon elution this material showed a u.v. absorption spectrum similar to that for hypoxanthine standard. The minor spot was not identifiable spectrophotometrically and contained only a small part of the ^{14}C label. The minor spot was never noted when the lower hypoxanthine concentration was employed in the bathing solution (see Methods). In terms of R_f values, however, the position of this minor spot corresponded to that of inosinic acid and was readily separated from hypoxanthine with water as the solvent. Average R_f values obtained for this system were: hypoxanthine, 0·62; xanthine, 0·76; uric acid, 0·79; inosinic acid, 0·92.

Alexander *et al.*[16] have reported metabolism of ^{14}C-hypoxanthine by isolated rat kidney tissue. Their data indicated that the rat kidney mince metabolized a sizable proportion of the extracted label to xanthine and uric acid. The extent of this metabolism was altered by administration of allopurinol. Allopurinol has been tested *in vitro* in the present study (Table 2 and unpublished observations), but failed to alter the steady state accumulation of hypoxanthine, the rate of label uptake at 25°, or the radiochromatographic patterns obtained with tissue extracts. The differences noted between these data and those of Alexander *et al.* may be due to species variation or differences in experimental design.

The time required in these experiments to reach a steady state of hypoxanthine uptake was surprising. The uptake curves for urate, for example, indicate attainment of a steady state by 60 min on incubation.[9] This is also true for many foreign substances[8] accumulated by kidney tissue. Since the slow accumulation of hypoxanthine does not reflect metabolic transformation, it seems likely that it is simply a characteristic of the transport process. Reduction of the temperature to 5° did alter the accumulation process. Indeed both the rate of uptake and the steady state values were reduced by lowering the temperature. Radiochromatograms of tissue extracts incubated at 5° were in no way different from those derived from tissues incubated at 25°.

The reduction in ^{14}C-hypoxanthine uptake by lowering the temperature probably reflects a generalized decrease in available metabolic energy thereby interfering with the transport process which is apparently energy-dependent. All of the metabolic inhibitors tested produced marked reductions in label uptake. Indeed, iodoacetamide (which inhibits glycolysis) was just as effective as dinitrophenol (which acts by uncoupling oxidative phosophorylation) in reducing the uptake. Although it is possible that dinitrophenol might itself be transported as an organic acid and as such might inhibit hypoxanthine uptake, this seems unlikely since certain other organic acid inhibitors do not have such an effect (see below).

All of the data considered thus far indicate that hypoxanthine accumulation is energy-dependent and carrier-mediated. At least the decreasing S/M ratio noted with increasing hypoxanthine concentrations is consistent with a carrier-mediated process, although admittedly this does not prove the existence of such a mechanism. These data, however, do not give any information about the specificity of the transport process. Therefore the other experiments reported here were undertaken to define the specificity of the hypoxanthine uptake process and to compare it with the transport for other organic substances by kidney tissue.

The effects of various metabolic substrates were tested and the results are similar to those found for urate uptake by kidney tissue,[9] and in part like those for *p*-aminohippurate (PAH) uptake.[17] For example, acetate is known to stimulate uptake and succinate to depress uptake of both of these organic acids just as was the case for hypoxanthine accumulation. Also, inhibition of organic acid accumulation by α-ketoglutarate and citrate has been reported. Lactate and pyruvate, on the other hand, which produce an enhancement of transport in the case of PAH,[17] have been reported to have no effect on urate transport.[9] Lactate was not employed in the present study, but pyruvate produced a significant reduction in hypoxanthine uptake. The mechanism of these inhibitory effects was not examined but, since such metabolic intermediates are known to stimulate tissue respiration, it is likely that their effects are due to an interference with the specific transport site or carrier.

The transport of both urate[9, 18] and PAH[19] shows a requirement for potassium. With these acids, very low S/M ratios were found in the absence of potassium relative to those found in the presence of 5 mM potassium or more. The failure to detect a significant potassium effect in the present study indicated further that the requirements of hypoxanthine transport are not the same as those for uric acid or PAH.[11]

Also the failure of probenecid and bromcresol green to alter hypoxanthine uptake is in agreement with this idea. It was a bit surprising, however, that probenecid did not produce any effect in view of the fact that it has been shown to alter oxypurine excretion in man.[6] The effect in man was the opposite of that on uric acid excretion, i.e. decreased oxypurine excretion compared to an enhanced urate excretion.

The data presented in Tables 4 and 5 do indicate something about this transport process, however. Apparently, the purine structure can interact in this transport process. The nature of the inhibition may be competitive, but that can not be established from the data presented here. All of the basic substances tested, tetraethylammonium chloride, choline chloride and quinine sulfate, have been shown by several investigators to be handled by a 'specific' renal transport sytem, namely, that for organic bases.[11–13] That is, each substance can interfere with the transport of another and the transport of all three is blocked by the inhibitor cyanine No. 863. Two of these substances were capable of depressing hypoxanthine uptake, at least when present in high concentrations. These effects might be taken as evidence that hypoxanthine was carried by the organic base transport process.

Although the substrate effects mentioned earlier appear to be more or less like those expected for transport of an organic acid, it is possible that instead these effects reflect an action on organic base transport. Lesher and Shideman[14] found results somewhat similar to those reported here for mepiperphenidol (Darstine). For example, these authors found inhibitory effects of succinate and α-ketoglutarate, a stimulatory effect of acetate, and no effect with glucose. These data were interpreted to mean that either

the transport of this substance showed peculiar characteristics relative to other organic bases or that a third transport system existed. This contention was supported by the fact that neither PAH, carinamide, nor *N*-methylnicotinamide interfered with mepiperphenidol transport.

The quantitative aspects of this inhibitory phenomenon are unusual, however. No significant effect was noted with less than about a 100-fold excess of inhibitor over substrate. Although in cases in which organic bases interact in renal transport processes inhibitor concentrations of this magnitude have been reported, significant effects are seen often with much lower concentrations.[14, 20]

In view of the data presented in this report it might be suggested that hypoxanthine accumulation by isolated kidney tissue displays the following characteristics: (a) the kidney cortex from most rabbits (all of those reported here) do not show a significant degree of metabolism of the ^{14}C-hypoxanthine; (b) the transport is dependent on metabolic energy; (c) it is a carrier-mediated process; (d) the accumulation, at least in part, is by means of the organic base transport system known to exist in renal tissue; and (e) the accumulation, at least in part, may be by means of a purine transport system.

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