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ON THE METABOLISM OF ALLOPURINOL

FORMATION OF ALLOPURINOL-1-RIBOSIDE IN PURINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCY

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Abstract—Allopurinol-1-riboside, a major metabolite of allopurinol, is commonly thought to be directly synthesized by purine nucleoside phosphorylase (PNP) *in vivo*. As this enzyme is otherwise believed to function *in vivo* primarily in the direction of nucleoside breakdown, we have determined by high performance liquid chromatography and a conventional chromatographic method the urinary metabolites of allopurinol in a child deficient of PNP. In this patient approximately 40% of urinary allopurinol metabolites consisted of allopurinol-1-riboside, thus proving the possibility of indirect formation of allopurinol-1-riboside via allopurinol-1-ribotide *in vivo*, catalysed by hypoxanthine guanine phosphoribosyltransferase (HGPRT) and a phosphatase.

Oral doses of allopurinol are recovered to about 80% in the urine, mostly as oxipurinol (60–70% of total metabolites), unchanged allopurinol (6–12%), allopurinol-1-riboside (6–12%) and in some cases oxipurinol-7-riboside [1–3]. Allopurinol-1-riboside can be formed in two different ways (Fig. 8): either directly by the action of purine nucleoside phosphorylase (PNP, EC 2.4.2.1) or indirectly as the degradation product of allopurinol-1-ribotide, which itself may be synthesized by hypoxanthine guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) [3–5]. From *in vitro* kinetic data and the low tissue levels of allopurinol-1-ribotide following intravenous administration of [8-^{14}C]allopurinol in rats [6], it has been generally assumed that the direct phosphorylase pathway is more likely to occur *in vivo* than the indirect route [7, 8]. On the other hand, PNP is considered to function in animal tissues primarily in the direction of nucleoside breakdown, as in most tissues the concentration of inorganic phosphate is considerably greater than the concentrations of ribose-1-phosphate or purine bases [9], thus favouring theoretically the indirect route of allopurinol-1-riboside formation *in vivo*. To investigate these possibilities we studied the urinary metabolites of allopurinol in a patient deficient in PNP who received oral allopurinol and guanine hydrochloride in a therapeutic attempt to reconstitute lowered guanine nucleotide pools [10, 11].

MATERIALS AND METHODS

Patient S. B., a male infant aged 18 months [11], received on 3 days (3–5 November 1981) 120 mg (0.88 mmole) allopurinol (10 mg/kg) additional to 175 mg (0.96 mmole) guanine hydrochloride/day. Allopurinol was subsequently given alone for a 2-week period at the same dose (May 1982). Twenty-four hour urines were collected using toluene as preservative. Urinary metabolites of allopurinol, as well as urinary purine bases and nucleosides, were determined by different modifications of the reversed phase high performance liquid chromatography (HPLC) method of Webster *et al.* [12], and by the method of Simmonds [13] using conventional anion exchange column chromatography followed by two-dimensional high voltage thin-layer electrophoresis and UV spectrometry of the isolated compounds at pH 2.0 and 10.0.

Two different HPLC systems were used: (1) a Waters system consisting of two 6000 pumps, a 660 solvent programmer, a 710B injector and a 440 dual wavelength detector (254 and 280 nm, 0.05 AUFS) or (2) a Hewlett–Packard 1084B apparatus with a variable wavelength detector (attenuation 2^5).

The Waters system was used with either (1a) a Hichrome Spherisorb 5-ODS column (0.4 × 25 cm) or (1b) a Jones Super Apex ODS 3μ type B column (0.4 × 25 cm), and a Whatman Co-pell ODS pre-column. Urine samples were injected directly after appropriate dilution with water.

(1a) The Hichrome column was eluted at a flow rate of 1 ml/min either isocratically with phosphate buffer (1 g/l KH_2PO_4, BDH Aristar grade, pH 2.2) (method 1) or with a concave gradient (gradient 8) starting from 100% phosphate buffer (as above) and leading to 75% phosphate buffer and 25% methanol (Rathburn Chemicals HPLC grade) in 25 min (method 2).

(1b) Separations on the Jones column were performed with a linear gradient (gradient 6) starting from 100% phosphate buffer (1 g/l KH_2PO_4, pH 3.95) and leading to 90% phosphate buffer and

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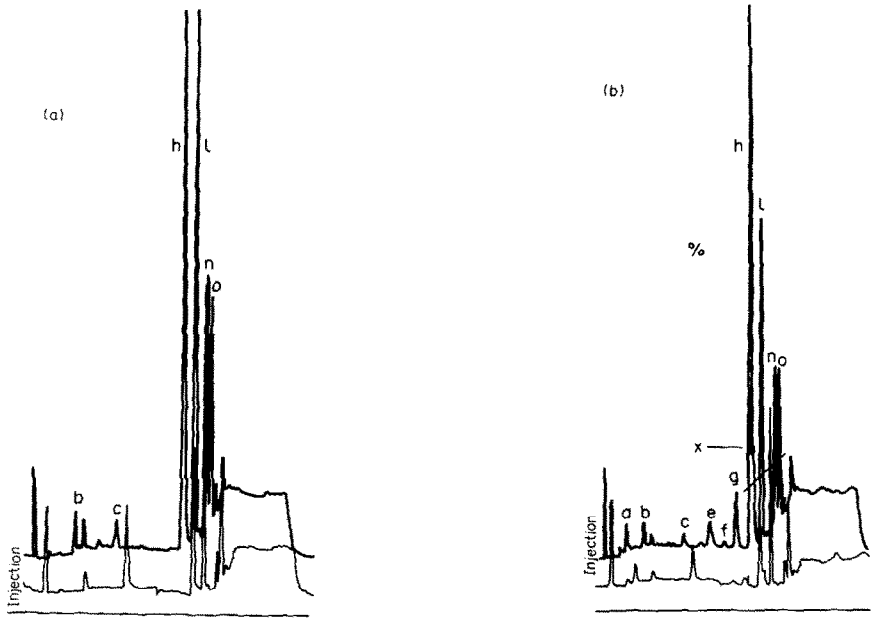

Fig. 1. HPLC chromatograms of urines from our PNP-deficient patient: (a) 8 February 1982, no treatment; (b) 4 November 1981, 120 mg (0.88 mmole) allopurinol and 175 mg (0.96 mmole) guanine hydrochloride/day. Injection volume 0.003 ml of a 1:10 dilution of urine with water. Method 2 (gradient 8). ———, Absorbance at 254 nm; – – –, absorbance at 280 nm. For peak identification, see Table 1.

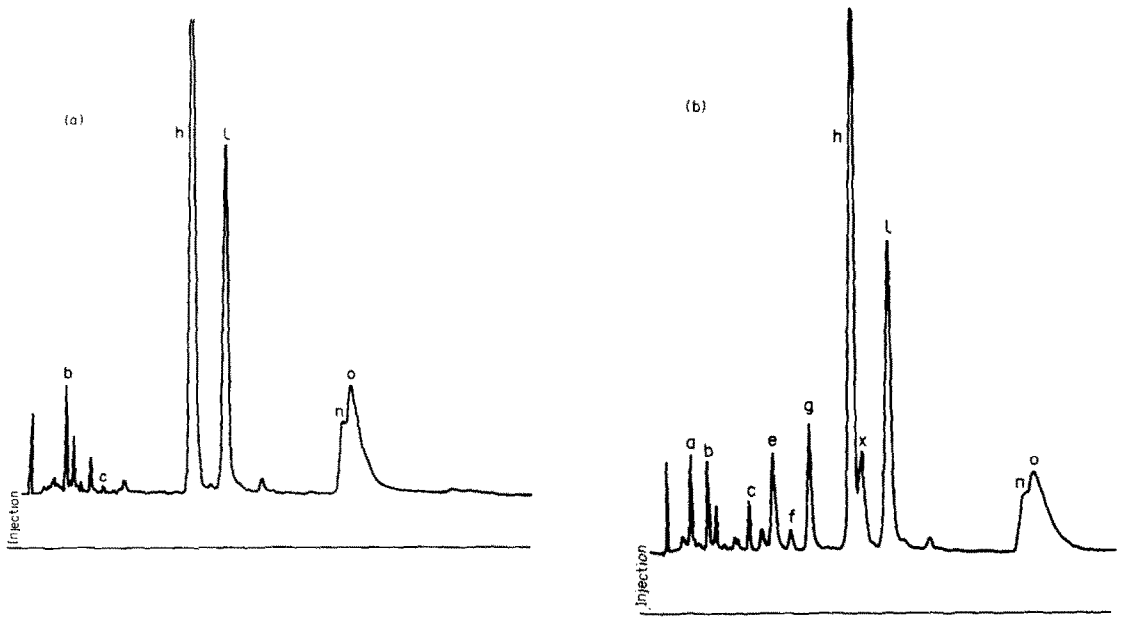

Fig. 2. (a) The same urine as in Fig. 1a (8 February 1982) chromatographed under isocratic conditions (method 1). Injection volume 0.01 ml of the 1:10 dilution with water. Absorbance at 254 nm. For peak identification, see Table 1. (b) The same urine as in Fig. 1b [4 November 1981], 120 mg (0.88 mmole) allopurinol and 175 mg (0.96 mmole) guanine hydrochloride/day] chromatographed under isocratic conditions (method 1). Injection volume 0.01 ml of the 1:10 dilution with water. Absorbance at 254 nm. For peak identification, see Table 1.

10% methanol in 40 min, the flow rate being 0.8 ml/min (method 3).

The Hewlett–Packard apparatus was used with a Chrompack RP-8 column (0.4 × 25 cm) which was eluted isocratically by phosphate buffer (1 g/l. KH_2PO_4, pH 2.3). The flow rate was 1 ml/min (method 4). Urine samples were pre-separated on small anion exchange columns (Bio-Rad AG 1X8, acetate form): 0.4 ml of urine, brought to pH 10.0 with 1 M NaOH, was applied to the column packed in a Pasteur pipette stoppered with glass wool; after a washing with 2 ml of water, purines including

Table 1. Standard retention times of identified purines and pyrimidines in the HPLC methods used

Symbol	Compound	Retention time in method 1 (mm)	2 (mm)	3 (min)	4 (min)
a	Orotidine	7	7		3.56
b	Pseudouridine	13	13		4.58
c	Uric acid	27	27		5.81
d	Guanine	32.5			6.09
e	Hypoxanthine	35	35		6.62
f	Xanthine	41	41		8.1
g	Oxipurinol	47	45	18.56	9.37
h	Inosine	61.5	50	27.43	12.51
x	Allopurinol-1-riboside	65	50.5	28.1	15.48
k	Allopurinol	69.5		20.4	11.68
l	Guanosine	73	53	29.5	12.51
m	Oxipurinol-7-riboside	111			22.39
n	Deoxyinosine	119	58	31.8	17.66
o	Deoxyguanosine	122	59	34.86	16.75

allopurinol and its metabolites were eluted with 6 ml of salt solution (15 g/l. NaCl in 0.04 M HCl).

Compounds were identified by their retention time, co-chromatography with standards, the ratio of absorbance at 280–254 nm and stop-flow UV spectra of single peaks.

To confirm the results obtained, urine samples were subjected to: (a) enzymatic degradation; (b) boronate affinity gel chromatography; and (c) acid hydrolysis.

(a) Enzymatic degradation studies were performed by incubating 0.1 ml of urine with 0.1 U of milk xanthine oxidase (Boehringer), 3.75 U of uricase (Loewens) and 0.025 ml of dialysed normal red blood cell lysate as PNP source in a final volume of 1 ml of 50 mM phosphate buffer, pH 7.4; after an incubation period of 10 hr at 37° the reaction mixture was deproteinized by heating for 3 min and chromatographed using HPLC method 1.

(b) For fractionation of nucleosides from urine by boronate affinity gel chromatography, the method of Pfadenhauer and Sun-de Tong [14] was used: 0.25 g Affi-Gel 601 (Bio-Rad) was allowed to swell in 0.25 M ammonium acetate, pH 8.8 and was then packed in a Pasteur pipette stoppered with glass wool; to 1 ml of urine 0.1 ml of 2.5 M ammonium acetate, pH 8.8, was added and 1 ml of this mixture was applied to the boronate column. After two washings with 4 and 5 ml of 0.25 M ammonium acetate, pH 8.8, nucleosides were eluted with 5 ml of 0.1 M formic acid. The eluates were chromatographed using HPLC method 4.

(c) Acid hydrolysis of nucleosides was carried out by the method of Krenitsky *et al.* [3]: 0.2 ml of urine was diluted 1:5 with 1 M HCl and heated for 20 min at 95°. Before chromatography with HPLC method 4, the sample was neutralized with 30% KOH.

Allopurinol-1-riboside and oxipurinol-7-riboside as reference substances were gifts of the Dr. G. Henning GmbH West Berlin.

RESULTS

An aliquot of a 24 hr urine sample of our PNP-deficient patient receiving no therapy (8 February 1982) separated by the gradient HPLC method 2 is shown in Fig. 1a. The chromatogram demonstrates the characteristic pattern of urinary purine metabolites in PNP deficiency: the major excretion products are inosine (7.77 mM), guanosine (2.74 mM), deoxyinosine (1.94 mM) and deoxyguanosine (1.46 mM) whereas uric acid (0.16 mM) represents only about 1% of total purine excretion.

The administration of allopurinol and guanine led to the appearance of additional peaks: orotidine, hypoxanthine, xanthine, oxipurinol and a small shoulder peak of the inosine peak (Fig. 1b); orotic acid was not detected.

To achieve complete resolution of the unknown shoulder peak (x) of inosine, the two urines were chromatographed under the isocratic conditions of method 1 (Fig. 2). This system separates allopurinol and its metabolites clearly from each other and from the excreted purine nucleosides (Table 1); it also separates well the unknown shoulder peak (x) from inosine (Fig. 2b). From the retention time and the 280:254 nm absorbance ratio of standard compounds chromatographed in the same system (Table 1, Fig. 3), this peak was identified as allopurinol-1-riboside. At the retention time of allopurinol and oxipurinol-7-riboside standards, no peaks were detectable in the chromatogram of our patient's urine (Fig. 2b).

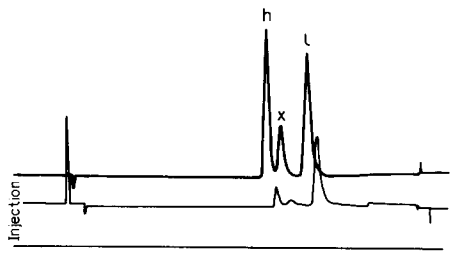

Fig. 3. Separation of a standard mixture consisting of inosine (h), allopurinol-1-riboside (x) and guanosine (l) using the isocratic HPLC method 1. ———, Absorbance at 254 nm; ———, absorption at 280 nm.

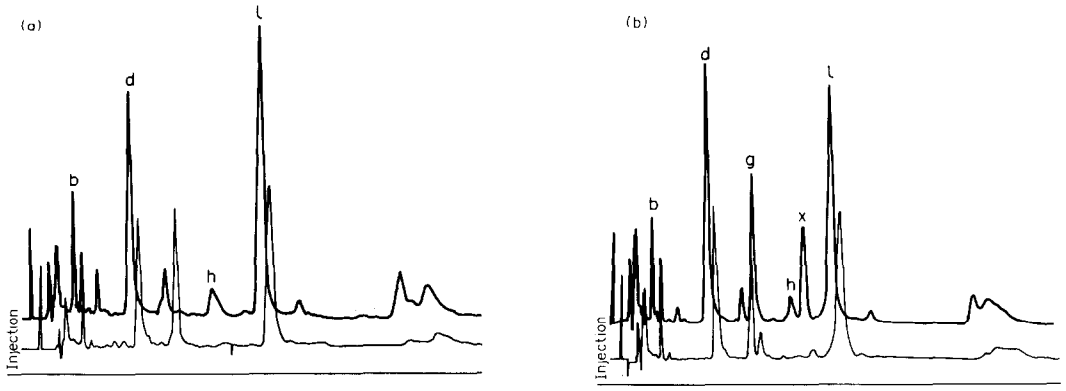

Fig. 4. (a) The same urine as in Figs. 1a and 2a (8 February 1982, no treatment) chromatographed under isocratic conditions (method 1) after incubation with PNP (lysed red blood cells), xanthine oxidase and uricase (see Materials and Methods for details). No uric acid and nearly no inosine (h) was left whereas guanosine (l) was only partly degraded due to the accumulation of guanine (d). Injection volume 0.01 ml, absorbance at 254 nm (———) and 280 nm (———). (b) The same urine as in Figs. 1b and 2b [4 November 1981, 120 mg (0.88 mmolc) allopurinol and 175 mg (0.96 mmolc) guanine hydrochloride/day] chromatographed under isocratic conditions (method 1) after incubation with PNP (lysed red blood cells), xanthine oxidase and uricase (see Materials and Methods for details). Oxipurinol (g) and allopurinol-1-riboside (x) were not degraded by the added enzymes thus confirming their identification and facilitating the measurement of allopurinol-1-riboside excretion. Injection volume 0.01 ml, absorbance at 254 nm (———) and 280 nm (———).

To confirm this finding and to quantify the excreted amount of allopurinol-1-riboside in the urine of our PNP-deficient patient, urines were subjected to enzymatic degradation. Allopurinol-1-riboside is not cleaved by PNP [15] and not oxidized by xanthine oxidase [16]. Therefore the urines were incubated with dialysed erythrocyte lysate as PNP source, xanthine oxidase and uricase in order to degrade the inosine peak which interferes with a proper measurement of allopurinol-1-riboside. The result is shown in Fig. 4. Inosine was degraded almost completely to allantoin as are hypoxanthine, xanthine and uric acid.

Oxipurinol and allopurinol-1-riboside are unchanged thus confirming their identification (Fig. 4b). The addition of xanthine oxidase (and uricase) was necessary due to the equilibrium of the PNP reaction under the chosen incubation conditions. Only about 50% of nucleoside cleavage was reached with PNP alone, as can be judged from the case of guanosine: as red cells do not contain guanase, guanine accumulates and inhibits the further degradation of guanosine (Fig. 4).

To exclude a possible formation of allopurinol-1-riboside by added PNP from unmetabolized allopurinol contained in the urine and ribose-1-phosphate generated by nucleoside cleavage, samples were preincubated with xanthine oxidase for 1 hr to degrade any allopurinol before addition of PNP—exactly the same result was obtained as after simultaneous addition of xanthine oxidase and PNP (Fig. 4b).

The quantitative excretion of allopurinol metabolites in the urines of our patient as measured by

Table 2. Urinary excretion of allopurinol metabolites (mmole/day and % of total urinary metabolites) in our PNP-deficient patient receiving 120 mg (0.88 mmole) allopurinol (3–5 November 1981 and 6–7 May 1982) and 175 mg (0.96 mmole) guanine hydrochloride/day (3–5 November 1981) as determined by HPLC method 1 and by the method of Simmonds [13]

Day	24 hr urine vol. (ml)	Oxipurinol	Allopurinol-1-riboside	% of dose recovered in the urine
HPLC method 1				
3.11.81	379	0.35 (57.4%)	0.26 (42.6%)	70
4.11.81	284	0.37 (61.7%)	0.23 (38.3%)	68
5.11.81	271	0.39 (61%)	0.25 (39%)	73
6.5.82	100	0.27 (62.8%)	0.16 (37.2%)	49
7.5.82	145	0.25 (64.1%)	0.14 (35.9%)	44
Method of Simmonds [13]				
6.5.82	100	0.27 (56.3%)	0.21 (43.7%)	54.5
7.5.82	145	0.26 (57.8%)	0.19 (42.2%)	51

No unchanged allopurinol or oxipurinol-7-riboside was detected with either method.

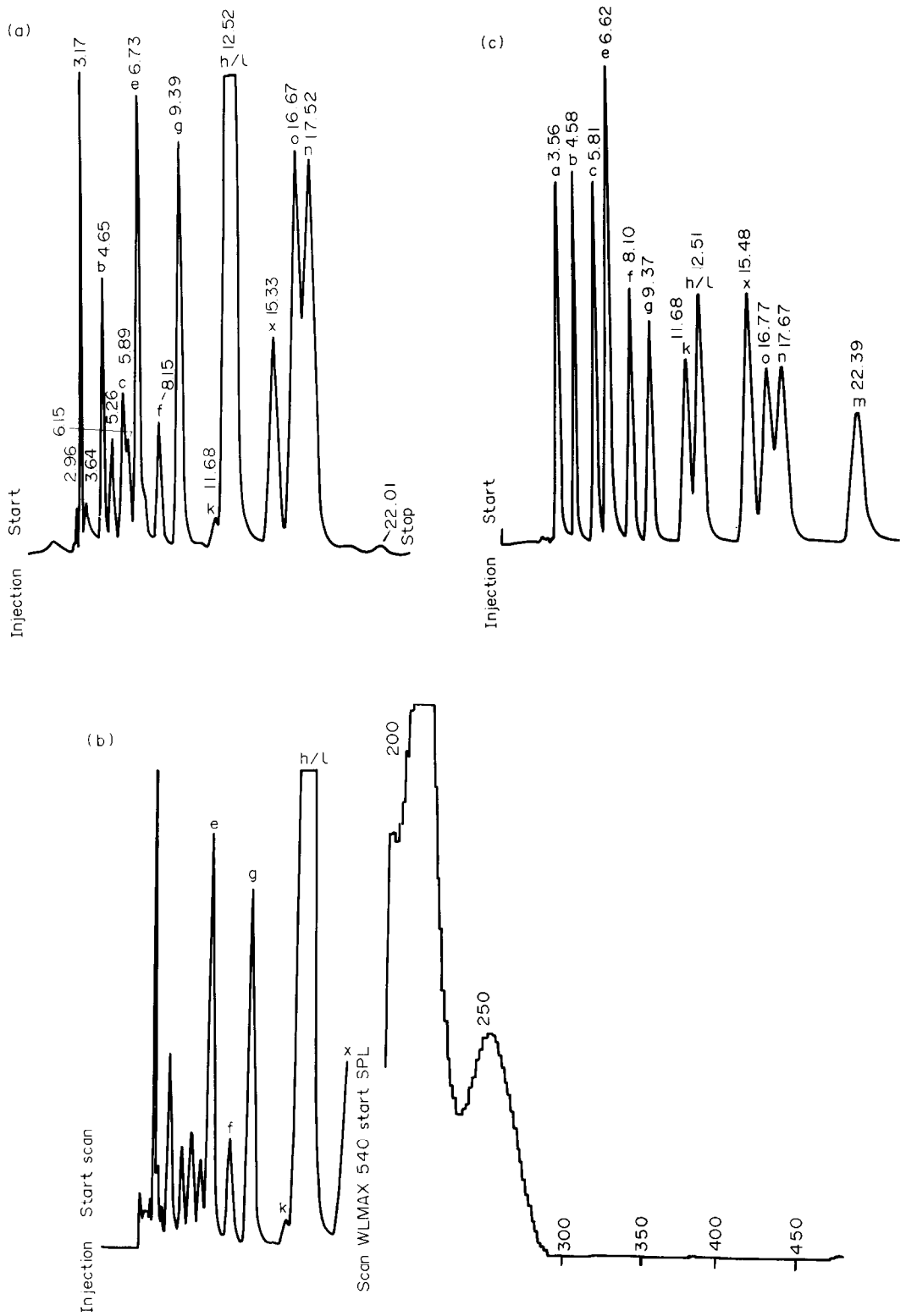

Fig. 5. (a) The same urine as in Figs. 1b and 2b (4 November 1981) chromatographed under isocratic conditions using method 4. Injection volume 0.05 ml; urine pre-separated on a small anion exchange column resulting in a 1:15 dilution. Absorbance at 260 nm. For details, see Materials and Methods; for peak identification, see Table 1. Hypoxanthine (e) may have arisen partly from deoxyinosine (n), which is unstable in the acid eluate of the anion exchange chromatography. Inosine and guanosine were not separated (h/l). A trace of unchanged allopurinol (k) was detected. (b) Stop-flow UV spectrum of the peak (x) identified as allopurinol-1-riboside. Same sample and conditions as in a. (c) Separation of a standard mixture by method 4. Same conditions as in a.

HPLC method 1 is given in Table 2. The results show that all allopurinol recovered in the urines was excreted as oxipurinol (57–64% of urinary metabolites) or allopurinol-1-riboside (36–43% of urinary metabolites); no unchanged allopurinol or oxipurinol-7-riboside could be detected by HPLC method 1. The different total recovery of allopurinol metabolites during the two periods of treatment (68–73% in November 1981 as compared to 44–49% in May 1982) could be due to the simultaneous administration of guanine, which may have influenced the absorption of allopurinol from the gut. Nevertheless, it seems not to have influenced the metabolism of allopurinol, since during both periods the ratio of oxipurinol to allopurinol-1-riboside excretion was similar.

Two other HPLC methods (methods 3 and 4) were used to confirm the reported results. These methods provided a more clear and direct separation of allopurinol and allopurinol-1-riboside as compared to HPLC method 1 (Table 1). With method 3, no unchanged allopurinol could be detected. In the chromatograms recorded using method 4, a trace of unchanged allopurinol could be seen (Fig. 5a). A stop-flow UV spectrum of the allopurinol-1-riboside peak separated by the same method is shown in Fig. 5b. It corresponded exactly to the UV spectrum of standard allopurinol-1-riboside.

Fractionation of nucleosides by boronate affinity gel chromatography followed by HPLC separation using method 4 showed that the peak identified as allopurinol-1-riboside was eluted together with the ribosides orotidine, pseudouridine and inosine/guanosine in the formic acid fraction (Fig. 6b), whereas the purine bases (including oxipurinol and allopurinol) and deoxynucleosides (deoxyinosine and deoxyguanosine) appeared in the first ammonium acetate washing (Fig. 6a). Obviously this latter fraction contained a small amount of inosine, most likely due to overloading of the affinity gel column.

Acid hydrolysis converted the nucleosides contained in the urine of our patient to the corresponding bases guanine, hypoxanthine and allopurinol (Fig. 7) thus again confirming the occurrence of allopurinol-1-riboside in our patient's urine.

In addition to these HPLC experiments, the occurrence of allopurinol-1-riboside and nearly complete absence of unchanged allopurinol in the urines of our patient was proved by the method of Simmonds [13] using conventional anion exchange column chromatography followed by two-dimensional high voltage thin-layer electrophoresis. This clearly

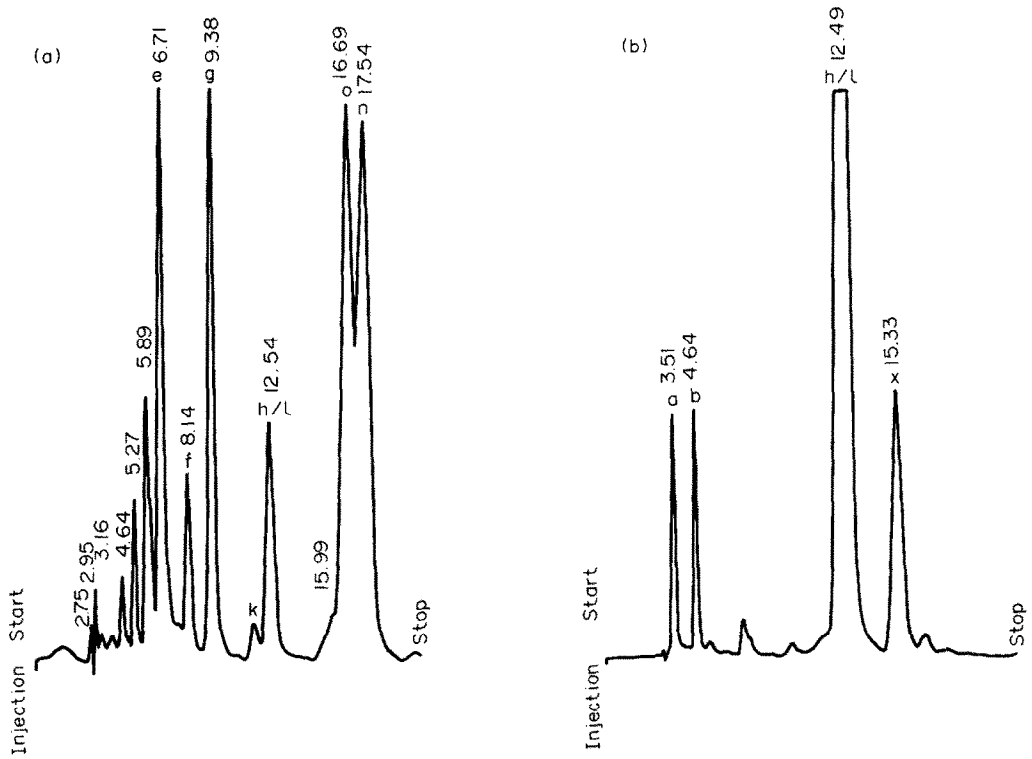

Fig. 6. Fractionation of the same urine as above (4 November 1981) by boronate affinity gel chromatography (see Materials and Methods) followed by HPLC using method 4. Injection volume 0.01 ml; attenuation 2^4; absorbance at 260nm. (a) The first ammonium acetate washing (1 ml sample volume + 4 ml ammonium acetate) contained besides hypoxanthine (e), xanthine (f), oxipurinol (g) and unchanged allopurinol (k), the deoxynucleosides deoxyinosine (n) and deoxyguanosine (o) which were not bound to the affinity gel. The presence of inosine/guanosine (h/l) was most likely due to overloading the column. (b) The formic acid fraction contained orotidine (a), pseudouridine (b), inosine + guanosine (h/l) and the peak identified as allopurinol-1-riboside (x) thus confirming its nucleoside character.

separated allopurinol and allopurinol-1-riboside as allopurinol moved towards the cathode whereas allopurinol-1-riboside migrated towards the anode. Furthermore, the UV spectrum of the two compounds at pH 2.0 and 10.0 was very different. The results obtained by this method are given in Table 2.

DISCUSSION

As mentioned above, allopurinol-1-riboside can be formed by two separate pathways both *in vitro* and *in vivo*. The direct synthesis by PNP is commonly thought to predominate *in vivo* [8]. This opinion is based on studies which show a higher PNP— than HGPRT-activity in monkey tissues [17] and low intracellular levels of allopurinol-1-ribotide as compared to the much higher tissue and plasma concentrations of allopurinol-1-riboside after intravenous administration of radiolabelled allopurinol of high specific activity in rats [6].

The studies comparing PNP- and HGPRT-activities were conducted in phosphate-free mediums using high ribose-1-phosphate concentrations for the PNP- assay; they do not take into account the high intracellular phosphate and low ribose-1-phosphate concentrations found in most tissues which favour nucleoside breakdown rather than synthesis by PNP *in vivo* [9].

It is not known how quickly allopurinol-1-ribotide is dephosphorylated under *in vivo* conditions. As this latter reaction is irreversible and allopurinol-1-riboside is not further degraded in higher animals, the low ratio of allopurinol-1-ribotide to allopurinol-1-riboside found in rat tissues [6] does not necessarily exclude the possibility that allopurinol-1-riboside is formed to a greater extent via the nucleotide *in vivo*. The excretion of allopurinol-1-riboside by our PNP-deficient patient (with a complete deficiency of the enzyme in both the synthetic and degradative directions [10]), clearly demonstrates this latter possibility, as allopurinol-1-riboside could only have been synthesized exclusively by the indirect pathway catalysed by HGPRT and a phosphatase in our patient.

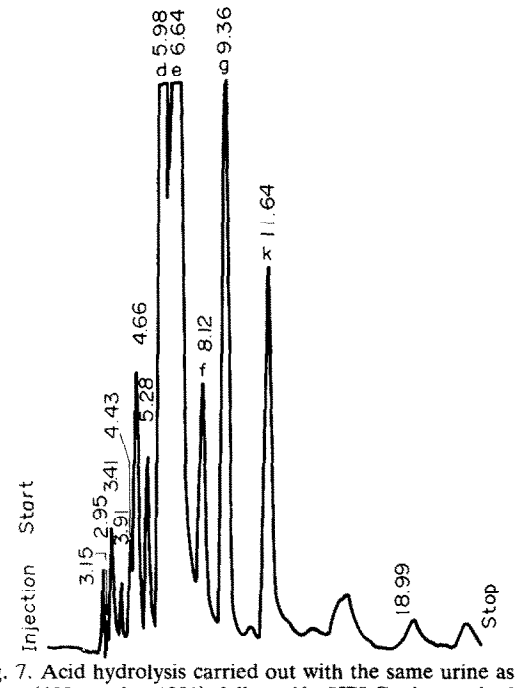

Fig. 7. Acid hydrolysis carried out with the same urine as above (4 November 1981), followed by HPLC using method 4; conditions as in Fig. 6. Inosine and deoxyinosine were converted to hypoxanthine (e), guanosine and deoxyguanosine to guanine (d). The appearance of an allopurinol-peak (k) confirmed the occurrence of allopurinol-1-riboside in the urine of our PNP-deficient patient.

The very low hypoxanthine and guanine levels, together with the elevated intracellular phosphoribosylpyrophosphate (PRPP) levels found in PNP deficiency [18–20], obviously favour this pathway in our patient so much that all the non-oxidized allopurinol was converted to allopurinol-1-riboside via allopurinol-1-ribotide, resulting in a greatly increased formation of this metabolite as compared with normal subjects. Since allopurinol-1-ribotide is not a substrate for IMP-dehydrogenase [21], rapid degradation to allopurinol-1-riboside would be anticipated.

Our observation does not allow the conclusion that allopurinol-1-riboside is synthesized exclusively via allopurinol-1-ribotide under normal *in vivo* conditions, i.e. in the presence of PNP and a normal intracellular PRPP concentration. However, the fact that allopurinol-1-riboside is not excreted by Lesch–Nyhan children [22], who cannot convert allopurinol to allopurinol-1-ribotide because of their HGPRT deficiency, would support our observations. The latter fact has been explained by competitive inhibition of allopurinol-1-riboside formation by PNP due to accumulating hypoxanthine [8], which is a much better substrate for PNP than allopurinol [23]. An argument against this explanation is the observation of Simmonds *et al.* [24] that large oral doses of guanine (1 mmole/kg) in the presence of allopurinol to allopurinol-1-ribotide because of their have no influence on the formation of allopurinol-1-riboside despite the fact that xanthine is also a much better substrate for PNP than allopurinol [23]. As can be concluded from the kinetic data collected in Fig. 8 [5, 23], the absence of any effect of xanthine on the formation of allopurinol-1-riboside agrees much more with the indirect synthetic pathway catalysed by HGPRT, xanthine being a very poor substrate for HGPRT.

From our results in a child with proven PNP deficiency in both the synthetic and degradative directions [10], and the above considerations, concerning especially the function of PNP *in vivo*, we believe that the indirect biosynthesis of allopurinol-1-riboside is more likely to predominate *in vivo* than the currently accepted route of direct biosynthesis by PNP.

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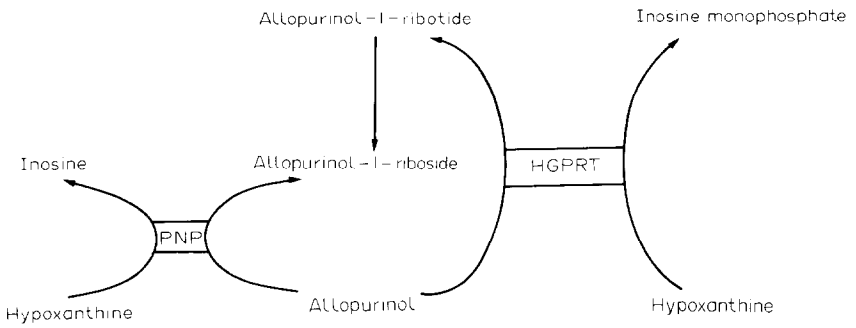

	Purine nucleoside phosphorylase		Hypoxanthine guanine phosphoribosyltransferase	
	$K_i \times 10^3$	Relative V_{max}	$K_i \times 10^3$	Relative V_{max}
Hypoxanthine	0.01	10	0.0024	36.7
Guanine	0.022		0.0018	44
Xanthine	0.039	5	0.25	0.022
Allopurinol	0.97	1	0.99	1

Fig. 8. Pathways of allopurinol-1-riboside biosynthesis. Kinetic data for PNP from Krenitsky [23]; for HGPRT from Krenitsky *et al.* [5].

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