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URINARY EXCRETION OF METHYLATED PURINES IN MAN AND IN THE RAT AFTER THE ADMINISTRATION OF THEOPHYLLINE

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SUMMARY

Chromatographic characteristics of urinary metabolites of theophylline were studied by two-dimensional thin-layer chromatography, high-performance liquid chromatography and gas chromatography—mass spectrometry. Quantitative data for the urinary metabolites of theophylline in asthmatic children are given. It was shown that 1,3-dimethyluric acid is the predominant excretory product. In addition, smaller amounts of 1-methyluric acid, 3-methylxanthine and unchanged theophylline were found.

Excretory patterns after theophylline ingestion before and during the administration of allopurinol in asthma patients and in rats suggest the existence of three metabolic pathways of theophylline. The administration of this drug to a patient with xanthine oxidase deficiency resulted in the excretion of 1-methyluric acid in addition to 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and unchanged theophylline. It was concluded that in man the oxidation of theophylline is not catalysed by xanthine oxidase.

INTRODUCTION

Theophylline is used extensively for the treatment of bronchial and cardiac asthma, cardiac and coronary insufficiency, and obstructive lung disease. Moreover, it has been applied recently for the management of apnea and of bradycardic spells in premature infants.

In man and rat theophylline is converted into 1,3-dimethyluric acid (1,3-diMeU), 1-methyluric acid (1-MeU) and 3-methylxanthine (3-MeX), which are excreted in the urine [1–3]. When present in human urine which is screened for inborn errors of purine and pyrimidine metabolism, these compounds can be expected to interfere. This screening is indicated especially in patients showing the characteristics of severe immune deficiency disease as a result of adeno-

sine deaminase (ADA) deficiency [4, 5] and purine nucleoside phosphorylase (NP) deficiency [6, 7].

We felt there was a need to study the chromatographic parameters of these compounds in detail. The methods employed include two-dimensional thin-layer chromatography (TLC) as described previously [8] and high-performance liquid chromatography (HPLC). As far as we know gas chromatography—mass spectrometry of the trimethylsilyl (TMS) derivatives of the methylated uric acids has not yet been described.

As little is known about the quantitative aspects of theophylline metabolism, we also investigated urinary concentrations in patients under treatment. The metabolic pattern does suggest that xanthine oxidase (XO) is a catalyst for the in vivo oxidation of theophylline. However, milk XO does not have this activity in vitro [1]. We had the opportunity to study the effect of allopurinol on the metabolism of this drug in asthma patients under treatment and in rats. Also the oxidation of theophylline in a patient with XO deficiency could be investigated. In this paper we present the data obtained from these studies.

METHODS

Screening for urinary metabolites of theophylline

Screening was performed by two-dimensional TLC, after isolation of these substances from the urine by anion-exchange column chromatography as described previously [8]. Elution was performed with 5 ml of 0.1 *M* ammonia (fraction I), 40 ml of water (fraction II), 150 ml of 0.01 *M* formic acid (fraction III) and 150 ml of 4 *M* formic acid (fraction IV).

Preparative TLC

The same stationary and mobile phases as described for the screening procedure were used. Unknown compounds were scraped off and extracted from the cellulose with 0.1 *M* ammonia. The extracts were centrifuged and investigated by UV spectrometry at various pH values.

High-performance liquid chromatography

HPLC was performed as described previously [8]. For identification of the peaks a Perkin-Elmer Model LC-55 UV—Vis spectrophotometric detector with scanning accessory was used. Quantitative HPLC of urinary 1,3-dimethylxanthine (1,3-diMeX), 3-MeX and 1-methylxanthine (1-MeX) was performed on fraction III, and HPLC of 1-MeU and 1,3-diMeU on fractions III and IV of the isolation procedure using as the mobile phase acetonitrile (UV grade)—sodium acetate/acetic acid buffer (10 mmol/l, pH 4.0) (7:93, v/v) (see ref. 9). A solvent flow-rate of 2.0 ml/min was used and continuous monitoring of the eluent was performed at 280 nm. At the top of the peaks UV spectra were scanned for confirmation.

Calibration curves were prepared and a linear relationship between extinction peak area and concentration was found. For determination of the recoveries the synthetic compounds were added to a preanalyzed urine.

1-Methyluric acid

1-Methyluric acid was synthesized by the oxidation of 0.2 m*M* 1-MeX in oxygen-saturated water, adjusted to pH 7.4 with ammonia, and an excess of xanthine oxidase (from milk, Boehringer, Mannheim, G.F.R.). The course of the reaction was monitored by UV spectrometry. Isolation of 1-MeU from the reaction mixture was performed by anion-exchange column chromatography as described for urine [8].

Gas—liquid chromatography—mass spectrometry

The 70 eV mass spectra of the trimethylsilyl (TMS) derivatives of 1,3-diMeU, 1-MeU and 3-MeU were recorded on a Jeol JGC-20 KP/JMS-D 100/W-JMA system combination at an ion source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA.

For this purpose the extracts obtained from preparative TLC were evaporated to dryness under reduced pressure at 40°. The extracted or synthesized compounds were converted to TMS derivatives with a mixture of N,O-bis(trimethylsilyl)acetamide (BSA) and pyridine (1 : 3) plus 1% trimethylchlorosilane (TMCS) [10, 11] in a tightly closed glass tube at 70° for 30 min. Gas chromatographic conditions were as follows: dual glass columns (8 ft. × 1/8 in. I.D.) packed with 5% GE SE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP); carrier gas, helium, 30 ml/min; oven temperature 250°

Uric acid

Uric acid was determined with uricase.

RESULTS

Identification of 1,3-dimethyluric acid and 1-methyluric acid

During the isolation of the purines from the urine by anion-exchange chromatography, 1,3-diMeU was mainly eluted with fraction III, to a lesser extent with fraction IV. Theophylline (1,3-diMeX) was eluted completely in fraction III. Fig. 1 represents the chromatogram of fraction III from the urine of a six-month-old boy with the characteristics of severe combined immune deficiency disease (SCID) under treatment with theophylline. A marked spot (48) in the position of 1,3-diMeU is present. Only a small spot of 1,3-diMeX can be seen. Both substances gave a blue to pink colour with mercuric acetate—diphenylcarbazone. The UV absorption spectrum of compound 48, isolated from the two-dimensional chromatogram, was identical with that of authentic 1,3-diMeU. Characteristic UV absorption data of the methylated uric acids are given in Table I. In HPLC the isolated compound eluted in the position of 1,3-diMeU. The

TABLE I

UV ABSORPTION MAXIMA (nm) IN 0.1 *M* HYDROCHLORIC ACID (I), 0.05 *M* SODIUM PHOSPHATE, pH 7.4 (II) AND 0.1 *M* AMMONIA (III)

	I	II	III
1,3-diMeU	286	294	295
1-MeU	283	288	292
3-MeU	286	292	292

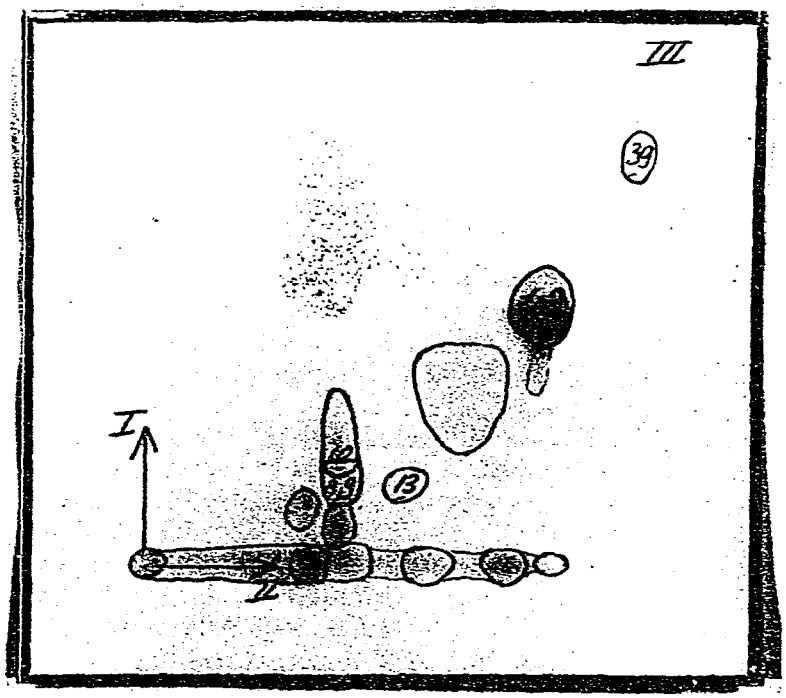

Fig. 1. Two-dimensional chromatogram of fraction III from the urine of the patient with severe combined immune deficiency disease. Sorbent: cellulose 0.1 mm on DC-Alufolien (Merck, Darmstadt, G.F.R., No. 5552). Solvent 1 = isopropanol—5% ammonia (8:2, v/v). Solvent 2 = butanol—acetic acid—water (8:2:2, v/v/v). Chromatogram is developed twice by the ascending technique in both solvents. Large spot 48 represents 1,3-di-MeU; spot 39 = 1,3-diMeX. See also ref. 8.

UV spectrum of the eluate corresponding to the peak of the isolated compound matched that of 1,3-diMeU. The mass spectrum obtained after GLC of the TMS derivative (see Fig. 2a) was identical with that of 1,3-diMeU obtained from Fluka (Buchs, Switzerland).

1-Methyluric acid was isolated from the urine of an asthma patient under treatment with theophylline. Isolation and identification of this compound was performed using the same procedures as for 1,3-diMeU. The mass spectra of 1-MeU and 3-MeU are given in Fig. 2b and c.

Quantitative analysis

Fig. 3 shows representative chromatograms on the μBondapak C_{18} column of fractions III and IV from a patient on theophylline therapy. 3-MeU, 1-MeU, 3-MeX, 1-MeX, 1,3-diMeU and 1,3-diMeX are eluted after 131, 166, 183, 205, 237 and 400 sec and their overall recoveries are 93, 88, 92, 92, 89 and 92% respectively (n=5).

Allopurinol and its metabolites oxipurinol, allopurinol riboside and oxipurinol-7-riboside are eluted after 143, 134, 145 and 174 sec, respectively. Hypoxanthine and xanthine are eluted after 128 and 133 sec, respectively, and uric acid is eluted after 102 sec. So these compounds do not interfere in the analysis of theophylline metabolites.

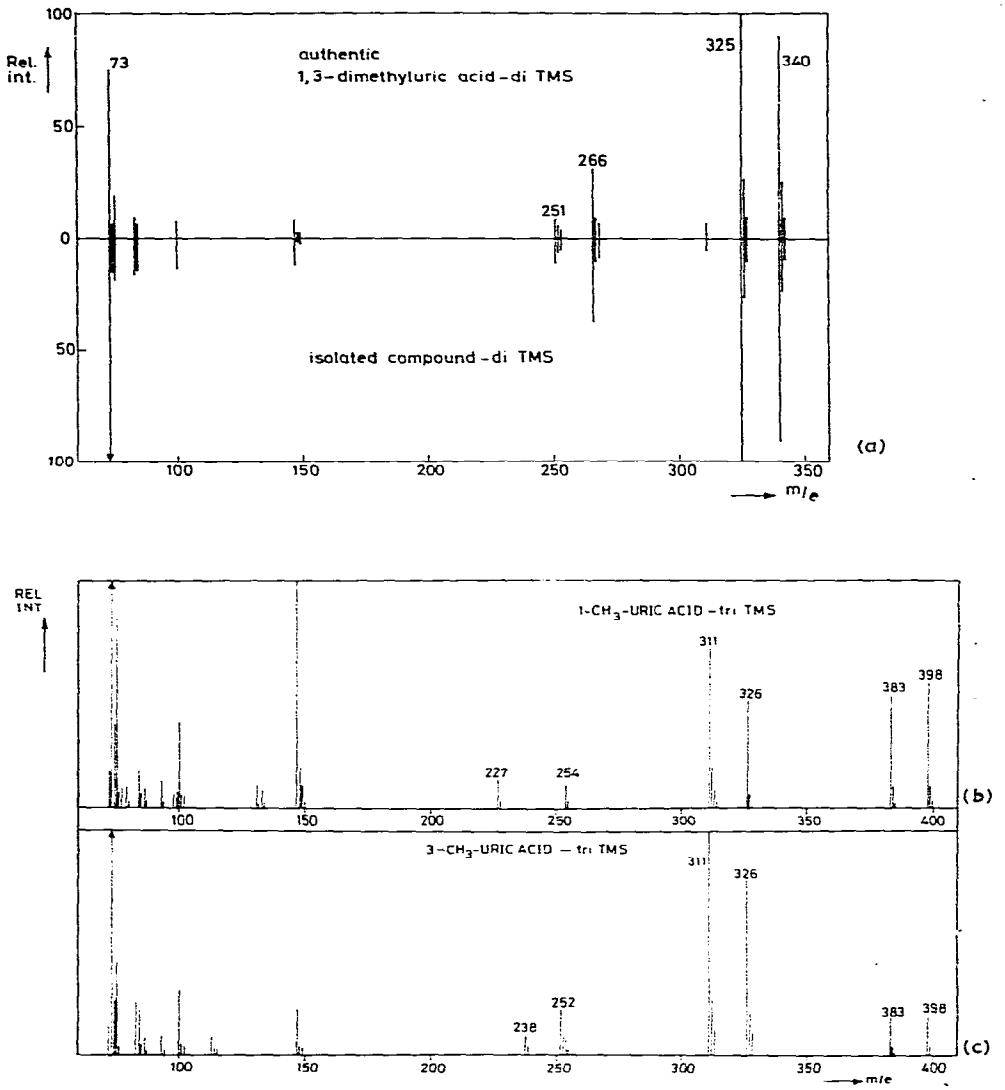

Fig. 2. Mass spectra of the trimethylsilyl derivatives of 1,3-diMeU (a), 1-MeU (b) and 3-MeU (c) using a GLC inlet system. 1,3-diMeU was isolated from the urine by preparative TLC. Chromatographic and mass spectrometric conditions as described in Methods.

Comparison of the HPLC elution profiles of fractions III and IV from the urine of patients and rats before and during therapy with theophylline revealed that, in the urine without theophylline, some UV absorbing compounds were eluted in front of 1-MeU. However, the area of the chromatogram occupied by theophylline and its metabolites was practically empty and as confirmed by "peak top UV spectrometry" there was no interference by other substances.

Excretion values

Excretion data for the SCID patient and five asthma patients under treatment with theophylline are summarized in Table II. In four of them 24-h urine

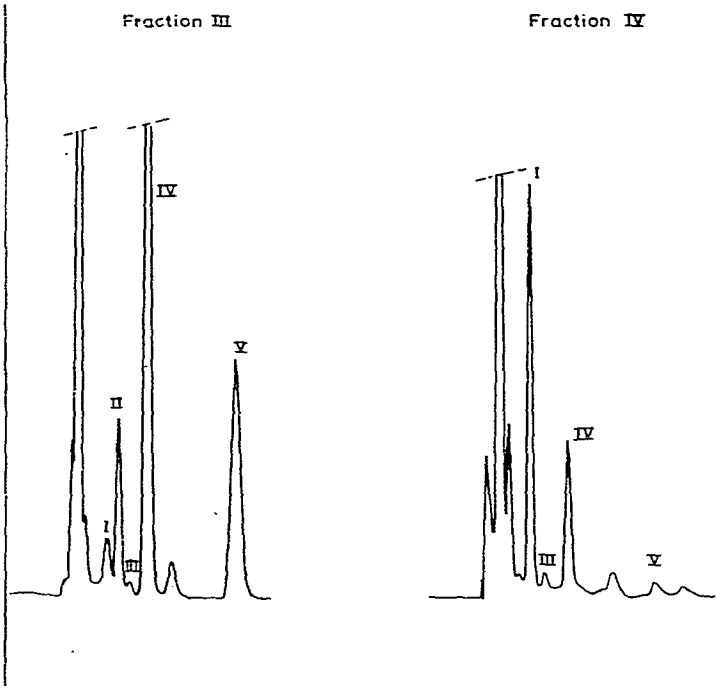

Fig. 3. HPLC of urinary N-methylpurines in fractions III and IV from a patient on theophylline therapy. Column: μBondapak C_{18}, 30 cm × 4 mm I.D. Eluent: acetonitrile (UV grade)—sodium acetate/acetic acid buffer (10 mmol/l, pH 4.0)(7:93, v/v). Flow-rate: 2.0 ml/min. UV detection at 280 nm. Peaks: I = 1-MeU; II = 3-MeX; III = 1-MeX; IV = 1,3-diMeU; V = 1,3-diMeX.

samples were collected and excretion values were related to the intake (see Table III). The results demonstrate that 1,3-diMeU is the main excretion product, followed by 1-MeU, 3-MeX and unchanged 1,3-diMeX. Only traces of 1-MeX were seen. However, in the SCID patient only small amounts of 1-MeU and trace amounts of 3-MeX were present; 1-MeX was absent.

Excretion patterns in rats following oral administration of theophylline before and during loading with allopurinol

Five male WAG rats each weighing approximately 300 g were maintained on a fixed diet during the course of the experiment. A 24-h control urine from each rat was collected and stored in a freezer (−20°) until analyzed. Subsequently 10 mg of theophylline in 2 ml of water were administered to each of four rats by stomach tube three times a day on two successive days. The remaining rat served for comparison. On the three following days two rats of the group received 15 mg of theophylline two times a day, the remaining two rats 15 mg of theophylline two times a day as well as 60 mg and 30 mg allopurinol, respectively, in one dose. From each rat 24-h urine samples were collected daily and analyzed for theophylline metabolites by two-dimensional TLC and HPLC. The quantitative results are summarized in Fig. 4. After the ingestion of theophylline a large amount of 1,3-diMeU, moderate amounts of 1-MeU and theophylline and small amounts of 3-MeX were excreted. In all samples trace

TABLE II

URINARY EXCRETION OF THEOPHYLLINE, 1,3-DIMETHYLURIC ACID, 1-METHYLURIC ACID, 1-METHYLXANTHINE AND 3-METHYLXANTHINE IN PATIENTS RECEIVING THEOPHYLLINE

In H.O. (SCID patient) and K.T. (asthma patient) random urine samples, and in the others 24-h samples, were analyzed. H.O. received theophylline 4 × 154 μmol per day, and K.T. 2 × 528 μmol per day (as suppositories). For the other patients see Table III.

Subject	Creatinine (mmol/l)	1,3-diMeX (μmol/l)	1,3-DiMeU (μmol/l)	1-MeU (μmol/l)	1-MeX (μmol/l)	3-MeX (μmol/l)
H.O. (1)	1.9	174	684	56	n.d.*	19
H.O. (2)	1.9	246	872	52	n.d.	20
H.O. (3)	2.3	169	1684	46	n.d.	28
K.T. (1)	3.7	169	571	466	n.d.	173
K.T. (2)	3.1	76	361	407	23	163
K.T. (3)	6.3	140	811	709	52	257
R.C.	11.4	79	786	772	n.d.	218
R.H.	4.1	89	628	604	8	335
D.A.	4.9	212	401	204	6	71
H.P.	8.3	151	2306	346	15	675

*n.d. = Not detected.

amounts of 1-MeX were present before, as well as during, the loading with the drugs. After administration of allopurinol there was a decrease in the excretion of 1,3-diMeU. However, the excretion of 1-MeU was reduced to zero and a sharp increase in the excretion of 1-MeX was noted; 3-MeX was unaltered. Uric acid became negligible, demonstrating that XO was strongly inhibited.

TLC patterns of the urinary purines before and after the administration of allopurinol to rats receiving theophylline were in agreement with the HPLC data shown in Fig. 4.

Excretion patterns in asthma patients on treatment with theophylline before and during loading with allopurinol

Two boys aged 10.5 and 6.8 years were treated for their asthma with aminophylline 3 × 200 mg per day orally and 3 × 150 mg per day as suppositories, respectively. During this treatment 24-h urine samples were collected and stored in a freezer (−20°) until analyzed. Subsequently, on the three following days, the patients received allopurinol 200 mg per day orally in addition to their theophylline and 24-h urine samples were collected on the third day. Theophylline metabolites were analyzed by both two-dimensional TLC and HPLC. Quantitative excretion values are given in Table IV. After the administration of allopurinol in addition to theophylline an obvious increase in urinary 1-MeX was observed in both patients. But the excretion of 1-MeU was decreased while 1,3-diMeU remained almost unchanged. A reduction in urinary 3-MeX was found in one patient only.

TABLE III

EXCRETION VALUES OF THEOPHYLLINE METABOLITES IN RELATION TO THE AMOUNT OF THEOPHYLLINE ADMINISTERED

Subject	1,3-diMeX administered* (μmol)	Excretion (%)					
		1,3-diMeX	1,3-diMeU	1-MeU	1-MeX	3-MeX	Total
R.C.	1972 (s)	2.5	25.3	24.8	0	7.0	59.6
R.H.	4557 (i)	2.2	15.7	15.1	0.2	8.4	41.6
D.A.	5583 (i)	9.7	18.3	9.3	0.3	3.2	40.8
H.P.	594 (s)	3.4	52.4	7.9	0.3	15.3	79.3

*s = As suppositories; i = infusion.

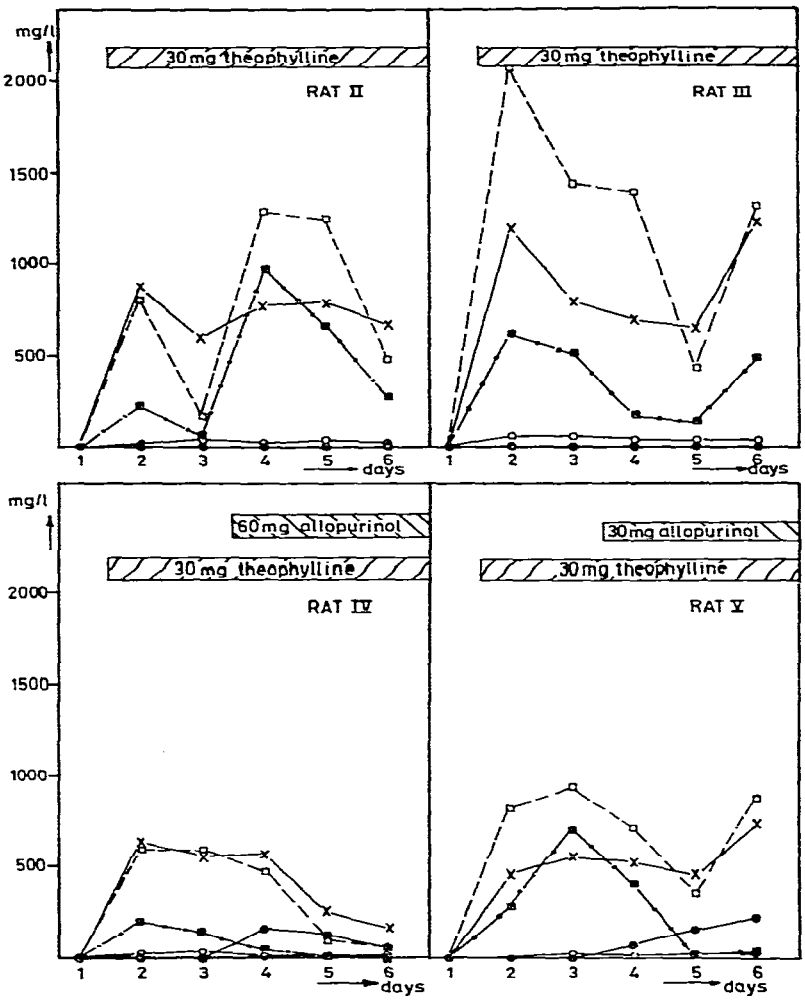

Fig. 4. Urinary theophylline metabolites in rats following oral administration of theophylline before and during loading with allopurinol. x——x, Theophylline; □– – –□, 1,3-diMeU; ■ —•—•—■, 1-MeU; ○——○, 3-MeX; ●——●, 1-MeX.

Excretion pattern in a patient with XO deficiency after ingestion of theophylline

In XO deficiency xanthine is not oxidized to uric acid. XO is also necessary for the oxidation of hypoxanthine to xanthine. A xanthinuric girl, aged 15 months (described elsewhere [12]), was given a single dose of 25 mg euphyllin (3 mg per kg body weight) as a suppository and a 24-h urine sample was collected in order to investigate the metabolic fate of theophylline.

1,3-diMeU was found to be the most prominent metabolite of theophylline in the urine. Also moderate amounts of 1-MeU and 1-MeX were present. This was confirmed by the UV spectra scanned at the tops of the peaks during HPLC. Quantitative data are given in Table V.

TABLE IV

URINARY EXCRETION OF N-METHYLATED PURINES AND URIC ACID IN TWO ASTHMA PATIENTS UNDER TREATMENT WITH THEOPHYLLINE BEFORE AND DURING LOADING WITH ALLOPURINOL

For doses see text.

Medication		Theophylline			Theophylline + allopurinol		
Patient	Compound	μmol/l	μmol/g creatinine	μmol per 24 h	μmol/l	μmol/g creatinine	μmol per 24 h
M.S.	1,3-diMeX	42	39	38	169	226	220
	1,3-diMeU	1260	1162	1123	944	1266	1227
	1-MeU	518	478	461	209	280	271
	1-MeX	n.d.*	n.d.	n.d.	320	429	416
	3-MeX	580	534	516	383	513	497
	Uric acid	3100	2858	2759	1300	1743	1690
J.R.	1,3-diMeX	57	41	16	31	43	23
	1,3-diMeU	1398	990	391	404	567	303
	1-MeU	769	545	215	78	110	59
	1-MeX	n.d.	n.d.	n.d.	126	177	94
	3-MeX	1229	870	344	214	300	160
	Uric acid	4000	2832	1120	1800	2528	1350

*n.d. = Not detected.

TABLE V

URINARY EXCRETION OF N-METHYLPURINES IN A PATIENT WITH XO DEFICIENCY BEFORE AND AFTER THE ADMINISTRATION OF THEOPHYLLINE (110 μmol)

Excretion product	Without theophylline			Theophylline administered		
	μmol/l	μmol/g creatinine	μmol per 24 h	μmol/l	μmol/g creatinine	μmol per 24 h
Theophylline	n.d.*	n.d.	n.d.	36.1	118	9.4
1,3-diMeU	n.d.	n.d.	n.d.	268	879	69.7
1-MeU	n.d.	n.d.	n.d.	214	702	55.6
3-MeX	4.8	9.3	1.1	45.2	148	11.8
1-MeX	26.5	86.9	6.2	78.3	257	20.4

*n.d. = Not detected.

DISCUSSION

By two-dimensional TLC screening of urinary purines and pyrimidines in a patient with SCID, an abnormal high excretion of an unknown compound, later identified as 1,3-diMeU, was observed. It is known from the literature [1—3] that 1,3-diMeU is the main metabolite of theophylline and indeed the SCID patient appeared to have been treated with theophylline. In addition, 1-MeU [2, 3] and 3-MeX [3, 13] are reported to be theophylline metabolites. Both

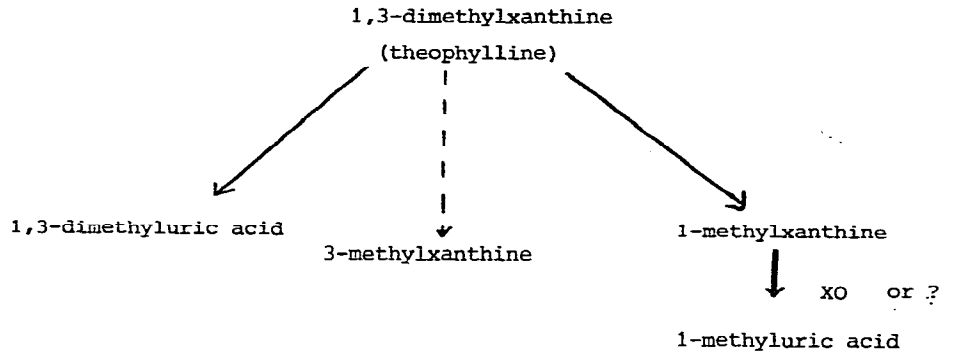

Fig. 5. Metabolic pathways of theophylline in man and in the rat.

compounds are excreted in moderate amounts in the urine of adults receiving theophylline. However, our patient excreted only small amounts of 1-MeU and traces of 3-MeX. This raised the question of whether theophylline in children and adults could follow different metabolic pathways. Such a hypothesis was supported by the fact that in children the plasma theophylline clearance is approximately 40% greater than that in adults [14]. Investigation of the urines of five asthmatic children treated with theophylline revealed the excretion of considerable amounts of 1,3-diMeU, moderate amounts of 1-MeU and unchanged theophylline and small amounts of 3-MeX and 1-MeX. These findings argue against the theory of different pathways for theophylline in children and adults. The higher plasma clearance in children could be explained by a greater capacity of the liver in children to metabolize theophylline. The very low urinary concentrations of 1-MeU, 3-MeX and 1-MeX in the terminal phase of the patient with SCID, may be caused by a decreased metabolic activity of his liver resulting in a reduced N-demethylation of theophylline.

The results of the experiments with theophylline and allopurinol in rats and in asthma patients point to probable metabolic pathways of theophylline in rats and in man being those shown in Fig. 5. The oxidation of theophylline without demethylation appears to be the major route. For a small part theophylline is demethylated at position 1 giving 3-MeX, which does not seem to be metabolized further because urinary 3-MeU was not found. On the other hand, theophylline is demethylated at position 3 to give 1-MeX, which is excreted in small amounts.

Moreover, the moderate excretion of 1-MeU indicates that 1-MeX may be an intermediate in the formation of 1-MeU. This is supported by the significant increase of 1-MeX and the decrease of 1-MeU after the administration of allopurinol in the theophylline-treated patients and rats. These findings also indicate that 1-MeU is formed by the oxidation of 1-MeX, catalyzed by XO or another enzyme which is inhibited by allopurinol and not by demethylation of 1,3-diMeU, as was suggested in the literature [3]. It can be concluded from in vitro experiments [1], our experiments in rats and the results from our patient with XOD, that the XO-catalyzed oxidation of theophylline cannot be the sole pathway of 1,3-diMeU formation. With XO from milk and from human liver 3-MeX cannot be converted into 3-MeU either, but 1-MeU and 7-MeU do arise from 1-MeX and 7-MeX, respectively [15, 16]. Apparently, a methyl substitution on

position 3 prevents oxidation by XO. This is supported by the fact that allopurinol did not suppress the excretion of 1,3-diMeU to zero level in the rats.

In our XO-deficient patient a considerable amount of theophylline is oxidized to 1,3-diMeU and to 1-MeU. It is not known which enzyme activity is responsible for this oxidation, but the results from our patient suggest that it is not XO. Demethylation of 1,3-diMeU at position 3 might explain the excretion of 1-MeU, but in this case it is difficult to understand why it should not happen in the asthma patients and the rats on theophylline and allopurinol.

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