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URINARY EXCRETION OF 3-METHYLXANTHINE AND RELATED COMPOUNDS IN CHILDREN

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SUMMARY

When screening urine of psychomotorically retarded children by 2-dimensional paper chromatography we found large amounts of an unknown product. After isolation this compound was identified as 3-methylxanthine by using multiple-analytical techniques. Ultraviolet spectra and the major mass spectra peaks are reported for the identified compound and also for 1-methylxanthine. The latter substance also occurs in urine, especially at older age. Another urinary compound is 7-methylxanthine. Evidence is presented that 3-methylxanthine and 7-methylxanthine are metabolites from theophylline and theobromine, occurring in stimulants such as tea and chocolate; 1-methylxanthine probably originates from coffee.

Some quantitative data concerning high excretory levels of 3-methylxanthine and 1-methylxanthine are given.

INTRODUCTION

When studying the urinary excretion of purines and pyrimidines in the urine of mentally retarded or neurologically disturbed children by 2-dimensional paper-chromatography, in some children we found large amounts of a product which behaved chromatographically and spectrophotometrically like a purine. After isolation and purification, mass spectrometric analysis revealed that this compound was identical to 3-methylxanthine (3-MeX), a compound which has received little attention in previous literature. Young *et al.*[1], Mrochek *et al.*[2] and Butts *et al.*[3] described the urinary excretion of this compound in adults. It was supposed[1,3] that 3-MeX is of exogenous origin, as it disappears from the urine after institution of a synthetic diet. Butts *et al.*[3] stated an excretion of 3 mg a day, a level much lower than we found in our children with an exaggerated excretion. At that time the compound had not

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been identified, and we wondered whether this abnormal excretion could be related to the developmental and neurological disturbances. However, the fact that the high excretions were not permanent was an argument against this theory. Later, when the compound had been identified, experimental evidence was obtained that 3-MeX was derived from theobromine or theophylline, which occur in chocolate and tea, respectively, when these food stuffs were given to the children on the day of collection of urine. 3-MeX is a main metabolite of these compounds; it is formed by demethylation at N-7 and N-1, respectively. In our high excretors, 7-methylxanthine was found in comparable amounts.

In this paper the isolation and identification of 3-MeX from the urine of the above-mentioned patients is described. Some quantitative data concerning high excretory levels are given. These data were obtained by automated cation-exchange column chromatography. Further information on the occurrence of this compound and also of 1- and 7-MeX in the urine of children and adults is given.

METHODS

A. *2-Dimensional paper chromatographic screening of purines and pyrimidines isolated from the urine by charcoal extraction according to Dalgliesh*[4]

25 ml of a 24-hour urine sample were acidified to pH 3.5 with glacial acetic acid and shaken with 375 mg deactivated charcoal. After adsorption the charcoal was filtered, washed with distilled water and then extracted with 5.5% aqueous phenol until the eluate was colourless. A second extraction was carried out with a mixture of 25% ammonia – 96% alcohol – water (6:25:69, v/v/v). Both eluates were combined and evaporated to dryness at 50 to 60° in a rotating vacuum evaporator. The dry residue was dissolved in 0.25 ml distilled water; 0.02 ml of this concentrate, corresponding to 2 ml urine, was subjected to 2-dimensional chromatography on Whatman filter paper no. 1, 23×23 cm. First solvent: isopropanol–5% ammonia (8:2, v/v) for 16 h. Second solvent: butanol–acetic acid–water (8:2:2, v/v/v) for 8 h. After drying of the chromatogram an ultraviolet contact photo was taken (Dalcoflex 90; Philips UV-lamp TUV, 15W; exposure 5 seconds at a distance of 50 cm). Then the chromatogram was sprayed with a solution of 100 mg of diazotized sulphanilic acid in 20 ml 10% sodium carbonate. A second chromatogram was sprayed with a solution of 0.25 g mercuric acetate in 100 ml 96% alcohol to which 5 drops of glacial acetic acid were added, followed by spraying with a solution of 0.05 g diphenylcarbazone in 100 ml 96% alcohol. The chromatogram was heated at 120° until complete decolouration of the background occurred[5].

B. *Quantitative determination*

3-MeX and 1-MeX can be estimated by column chromatography on a cation exchanger as is described for urinary imidazoles[6]. Elution is performed at 60° by a gradient procedure with three citrate buffers of pH 3.0, pH 5.0 and pH 6.0, respectively. Continuous analysis of the column eluate with diazotized sulphanilic acid and sodium carbonate is done by an Autoanalyzer technique. The extinction of the final solution is measured at 505 and 440 nm. A linear relation between extinction peak area and concentration was found. Both compounds are eluted between *N*-acetylhistidine and imidazolelactic acid. For chromatographic data see Table I.

TABLE I

COLUMN CHROMATOGRAPHIC AND COLORIMETRIC DATA FOR 3-MeX, 1-MeX AND HISTIDINE

Compound	*Mean Rt (min)*	*Extinction peak areas calculated per µg MeX*		E_{505}/E_{440}
		505 nm	*440 nm*	
3-Methylxanthine	65.7	6.9	37.0	0.186
1-Methylxanthine	74.4	16.8	37.8	0.444
Histidine	157.1	33.5	32.8	1.021

Both compounds were added to a pre-analyzed urine. The recovery was 97% for 3-MeX and 95% for 1-MeX ($n = 3$). It is not possible to determine 7-MeX by this method as no colour with diazotized sulphanilic acid is produced.

C. *Isolation of 3- and 1-methylxanthine*

From 5 l urine of two adults on a free diet, 118 mg 3-MeX and 204 mg 1-MeX were isolated (being only a part of the total quantities) according to the following procedure: (1) charcoal extraction; (2) ethyl acetate extraction of the dried charcoal extract in order to remove phenolic substances; (3) purification by cation-exchange chromatography (Dowex 50W X8, H^+ 50–100 mesh; elution with 3.5% ammonia after washing with water). With column chromatography on filter paper columns (Chromax LKB Sweden), elution was carried out with isopropanol–5% ammonia (8:2, v/v) butanol–acetic acid–water (8:2:2, v/v/v) and then with butanol saturated with water. Final purification by 2-dimensional paper chromatography on Whatman 3 MM paper was carried out using the first two solvents.

D. *Mass spectroscopy*

70 eV mass spectra of the isolated compounds were recorded with an AEI-MS9 mass spectrometer at an ion chamber temperature of 115–135°. The spectra are presented in Figs. 1a and 2a. The empirical formulas of the base peaks were established by exact mass measurement and were in accordance with the composition of the molecular ions of monomethyldioxypurines. This was in agreement with the xanthine-like behaviour of these compounds in chromatography. The position of the methyl group was established by comparison with the reference compounds 1-methyl-, 3-methyl-, 7-methyl- and 9-methyl-xanthine (1-MeX, 3-MeX, 7-MeX and 9-MeX, respectively).

The spectrum given in Fig. 1a is identical with that of 1-MeX (Fig. 1b) obtained from Fluka A. G. Buchs. It differs significantly from that of 3-MeX, synthesized in our laboratory (Fig. 2b)[7–9], 7-MeX, obtained from Fluka A. G. Buchs (Fig. 3) and from 9-MeX (gift, see Acknowledgement) (Fig. 4). The mass spectrometric frag-

TABLE IIa

DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS IN THE MASS SPECTRUM OF 1-METHYLXANTHINE

m/e	*Measured*	*Calculated*	*Empirical*	*Ion*
166	166.0487	166.0491	$C_6H_6N_4O_2$	M^+
109	109.0272	109.0276	$C_4H_3N_3O$	M^+ minus $H_3C{-}N{=}C{=}O$

TABLE IIb

INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF 1-METHYLXANTHINE

m^*	$m_1^+ \rightarrow m_2^+$	*Eliminated group*
114.7	166 → 138	C=O
71.6	166 → 109	$H_3C–N=C=O$
60.5	109 → 81	C=O

TABLE IIIa

DETERMINED AND CALCULATED m/e VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF 3-METHYLXANTHINE

m/e	*Measured value*	*Calculated value*	*Empirical formula*	*Ion*
166	166.0491	166.0491	$C_6H_6N_4O_2$	M^+
135	135.0307	135.0307	$C_5H_3N_4O$	?*
123	123.0432	123.0433	$C_5H_5N_3O$	M^+ minus HNCO
95	95.0482	95.0483	$C_4H_5N_3$	123 minus CO
95	95.0243	95.0245	$C_4H_3N_2O$	123 minus HCN+H
68	68.0375	68.0374	$C_3H_4N_2$	$C_4H_5N_3$ minus HCN

* Due to a contamination.

TABLE IIIb

INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF 3-METHYLXANTHINE

m^*	$m_1^+ \rightarrow m_2^+$	*Eliminated group*
133.7	166 → 149	OH (enolic form)
91.1	166 → 123	HNCO
73.4	123 → 95	CO
48.6	95 → 68	HCN

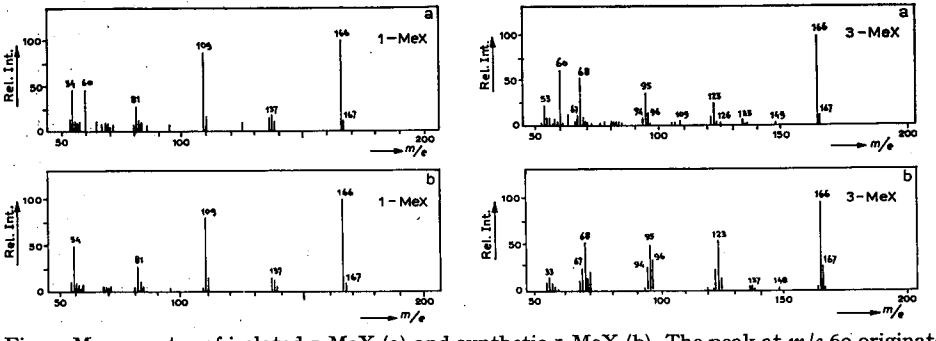
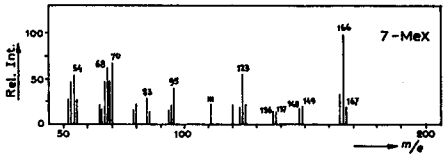


Fig. 1. Mass spectra of isolated 1-MeX (a) and synthetic 1-MeX (b). The peak at m/e 60 originates from acetic acid used in preparative chromatography.

Fig. 2. Mass spectra of isolated 3-MeX (a) and synthetic 3-MeX (b). The peak at m/e 135 is probably derived from a contamination.

mentation of the monomethylxanthines proceeds along similar routes as those of caffeine, theobromine and theophylline[10,11]. The fragmentation pattern (Fig. 5) of 1-MeX was deduced on the basis of exact mass measurements and metastables as given in Tables IIa and IIb.



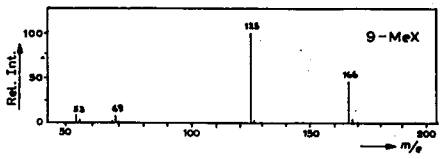
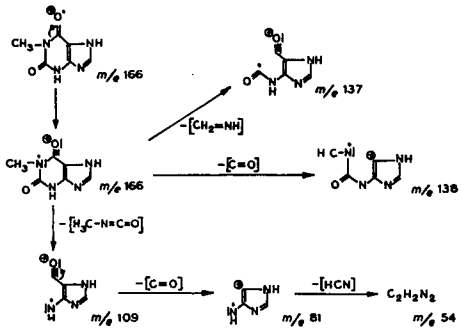


Fig. 3. Mass spectrum of 7-MeX.

Fig. 4. Mass spectrum of 9-MeX.



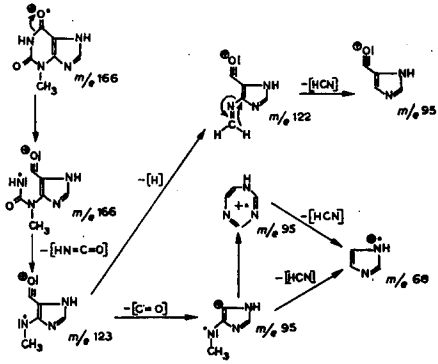


Fig. 5. Fragmentation pattern of 1-MeX.

Fig. 6. Fragmentation pattern of 3-MeX.

The spectrum of the other compound is identical with that of 3-MeX and clearly differs from that of 9-MeX. However, there is a striking resemblance with the spectrum of 7-MeX. Therefore the ultimate identification of the unknown component as 3-MeX was performed by IR and UV spectroscopy. The fragmentation pattern (Fig. 6) of 3-MeX has been based on exact mass measurements and metastables as given in Tables IIIa and IIIb.

E. *Other special techniques*

The ultraviolet and the infrared absorption spectra of the isolated and the synthetic compounds were in good agreement. In Fig. 7 ultraviolet spectra at different pH values of the isolated 3-MeX and 1-MeX are shown.

RESULTS

Occurrence of 3-methylxanthine and 1-methylxanthine in the urine of children

Paper chromatographic screening of a large number of children with various disorders revealed that 3-MeX and 1-MeX excretion was rather frequent and variable. No characteristic correlation with disease could be observed. In Table IV the occurrence of 3-MeX and 1-MeX in 175 24-h urine samples of children of various ages, but all below 15 years, are listed. Of these patients 163 were examined in connection with their psychomotoric retardation; the remaining 12 had other complaints requiring chromatographic screening. At all ages the frequency of the occurrence of 3-MeX alone equalled that of 3-MeX and 1-MeX in combination. The occurrence of 1-MeX alone, however, was exceptional at young age, but in children older than 10 years,

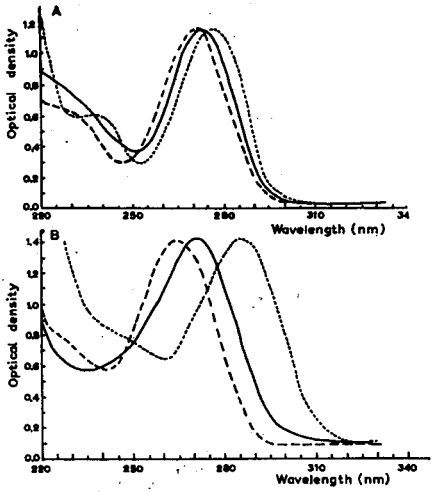


Fig. 7. Ultraviolet spectra at different pH values of 3-methylxanthine (A) and 1-methylxanthine (B). The solid lines are the spectra measured in 0.1 M sodium phosphate buffer, pH 7.5. The broken lines are the spectra measured in 0.1 M HCl, pH 1.0. The dotted lines are the spectra measured in 0.1 M NaOH, pH 13.0.

TABLE IV

THE OCCURRENCE OF URINARY 3-MeX AND 1-MeX IN RELATION TO AGE

Age (years)	*Number of patients examined*	*Number of patients with excretion of*		
		3-MeX	*3-MeX+1-MeX*	*1-MeX*
0–1	17	4	—	1
1–2	19	9	3	—
2–3	26	6	9	1
3–4	19	7	3	—
4–5	18	5	7	—
5–6	5	2	3	—
6–7	4	1	1	—
7–8	4	2	—	—
8–9	3	2	1	—
9–10	7	4	—	—
>10	53	6	23	13

1-MeX was more frequently found than 3-MeX. In adults, 1-MeX is found to be excreted with a higher frequency than 3-MeX.

Simmonds[12] and Skupp *et al.*[13] reported the urinary excretion of 1-MeX and 7-MeX, the former occurring in adults.

It is known that these compounds are metabolites of caffeine[14,15]. We supposed that 3-MeX, 1-MeX and 7-MeX in our children could arise from caffeine, theophylline or theobromine. In order to investigate the correctness of this hypothesis, a normal adult on a "methylxanthine-free" diet was given 200 mg/day of caffeine, theophylline and theobromine, respectively. Loading with caffeine resulted in the excretion of mainly 1-MeX; smaller amounts of 7-MeX were found, and even smaller amounts of 3-MeX, 3,7-di-MeX (theobromine) and 1,7-di-MeX (paraxanthine) were found (the dimethylxanthines were only identified by means of their positions on the 2-dimensional paper chromatograms). Theophylline loading resulted in the excretion of

TABLE V

URINARY EXCRETION OF 3-MeX AND 1-MeX IN RELATION TO THE INTAKE OF DIETARY METHYLATED XANTHINES

For comparison uric acid has also been determined.

Dietary compound	*3-Mex*			*1-MeX*			*Uric acid*		
	mg/l	*mg/g creatinine*	*mg/24 h*	*mg/l*	*mg/g creatinine*	*mg/24 h*	*mg/l*	*mg/g creatinine*	*mg/24 h*
Caffeine 200 mg/day adult	<10*	—	—	29	20	32	560	394	61(
Theophylline 200 mg/day adult	69	28	49	<8*	—	—	672	270	477
Theobromine 200 mg/day adult	28	22	38	<6*	—	—	364	288	499
Coffee 1.5 l/day adult	<10*	—	—	46	34	47	480	350	494
Tea+chocolate child, 2 years	50–100*	—	—	13	19	7	372	572	219
Tea+chocolate child, 2 years	83	95	31	<25*	—	—	566	651	209

* Inaccurate determination because of a contamination with unknown compound.

mainly 3-MeX; a small amount of 1-MeX was found and no 7-Mex was excreted. After loading with theobromine, the main excretory product was 7-MeX; less 3-MeX was seen and still less 1-MeX. Quantitative excretory data of 3-MeX and 1-MeX can be seen in Table V. In this table quantitative excretory data are also given, obtained from two children who ingested large amounts of tea and chocolate, and one adult was given a large amount of coffee (1.5 l/day). Administration of tea and chocolate resulted in a high urinary excretion of 3-MeX and 7-MeX; 1-MeX was excreted at a lower level than 3-MeX. After coffee consumption the excretion of 1-MeX was higher than of 3-MeX; 7-MeX was excreted at approximately the same level as 1-MeX (estimated by visual inspection of the chromatograms). For comparison the excretion of uric acid is also given in Table V.

Excessive urinary excretion of 3-MeX and 1-MeX in children

In some of our patients with psychomotoric retardation excessive concentrations of 3-MeX and 1-MeX were seen on the chromatograms as large comet-shaped spots. In Table VI some quantitative data are given.

DISCUSSION

By 2-dimensional paper chromatographic screening of urinary metabolites in a number of psychomotorically retarded children, abnormally high excretions of an unknown compound, later identified as 3-MeX, were observed. This could be affirmed by quantitative column chromatographic analysis. The excretions we observed were

TABLE VI

EXCESSIVE EXCRETION OF 3-MeX AND 1-MeX IN 6 CHILDREN

Patient	*Age*	*3-MeX*		*1-MeX*	
		mg/l	*mg/g creatinine*	*mg/l*	*mg/g creatinine*
J.M	8 years	62	148	0	0
	8 years 4 months	81	193	0	0
	8 years 5 months	90	213	0	0
	9 years 3 months	14	34	6	15
J.v.H.	18 years 2 months	6	12	34	71
	18 years 4 months	0	0	77	35
M.O.W.	4 years 7 months	34	64	11	21
C.W.	4 years 10 months	13	98	16	12
S.B.	13 years 5 months	53	62	0	0
D.H.	11 years 6 months	41	19	14	6

much higher than those reported in the literature for 3 MeX[1–3]. Methylated xanthines could theoretically be formed endogenously from 1-methyladenine, 3-methyladenine and 7-methylguanine, components of transfer RNA[16,17]. For other work on methylated purine and pyrimidine bases reference can be made to Sørensen[18], Craddock[19], Vanyushin[20], Ludlum[21], Mandel[22] and Park[23].

However, from a theoretical point of view the developmental disturbances in our children were not easily correlated with excessive methylation of DNA and RNA. Moreover, the high excretory levels were not permanent; in many patients 3-MeX had dropped to a very low level or had disappeared when the patient was re-inspected after some time. The excessive methylxanthinuria is more probably of dietary origin. Dietary products such as caffeine, theophylline and theobromine have been demonstrated to give rise to excessive urinary excretion of methylated xanthines.

When these dietary products were omitted from the diet the excretion decreased to very low levels or even became zero. Thus unrestricted consumption of methylated purine-containing products, such chocolate and tea, by the young patients analyzed, seems to be the most plausible explanation for the high excretions of 3-methylxanthine and related compounds.

It can be questioned whether stimulants containing methylated purines are harmless for young children. It is well known that methylxanthines inhibit a phosphodiesterase, resulting in accumulation of tissue cyclic 3′,5′-AMP, an important regulator of the cellular metabolism and an intracellular mediator of the physiological effects of many hormones[24–30]. Apart from this, methylated purine-containing stimulants have to be omitted for analytical reasons, when young patients are screened for inborn errors of metabolism. Physicians and nurses have to be informed about this fact.

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