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The Mass-Spectrometric Identification of Hypoxanthine and Xanthine ('Oxypurines') in Skeletal Muscle from Two Patients with Congenital Xanthine Oxidase Deficiency (Xanthinuria)

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1. The presence of hypoxanthine and xanthine in the skeletal muscle of two patients with congenital xanthine oxidase deficiency (xanthinuria) was demonstrated by high-resolution mass spectrometry. 2. Evidence was obtained for the presence of a trace of hypoxanthine only in normal muscle. 3. Dry pulverized tissue was introduced directly into the mass spectrometer and preliminary chemical processing of the tissue was therefore unnecessary. 4. The criteria for the mass-spectrometric identification of hypoxanthine and xanthine in the tissue and the significance of the observations are discussed.

The presence of crystalline deposits in the skeletal muscles of two patients who are congenitally deficient in xanthine oxidase (xanthine–oxygen oxidoreductase, EC 2.1.3.2) was reported by Chalmers, Watts, Pallis, Bitensky & Chayen (1969*a*). Examination of the crystals by polarized light, phase-contrast and interference microscopy showed that the optical properties of the crystals were compatible with their being hypoxanthine and xanthine (Chalmers *et al.* 1969*a*; Chalmers, Watts, Bitensky & Chayen, 1969*c*).

This paper reports the mass-spectrometric identification of hypoxanthine and xanthine in the muscle tissue of these xanthine oxidase-deficient (xanthinuric) patients. The mass spectra of hypoxanthine and xanthine have been reported previously (Chalmers, Parker, Simmonds, Snedden & Watts, 1969*b*) and the use of high-resolution mass spectrometry made prior chemical separation of the purines unnecessary.

MATERIALS AND METHODS

Patients and muscle biopsy specimens. The xanthinuric patients studied were the 31-year-old male previously referred to as patient A (Chalmers, *et al.* 1969*a*,*c*) and the 26-year-old female previously referred to as patient B by these authors and in whom the enzyme defect was first demonstrated (Watts, Engelman, Klinenberg, Seegmiller & Sjoerdsma, 1964; Engelman, Watts, Klinenberg, Sjoerdsma & Seegmiller, 1964). The muscle biopsy specimens identified as 1 and 3 in this study were obtained from patients A and B respectively, a calf muscle (gastrocnemius) being used for biopsy from patient A and a thigh muscle (vastus lateralis) being used for biopsy from patient B. The control tissue biopsy specimens were from patients who were undergoing elective orthopaedic operations. Specimens 2A and 2B were from the rectus femoris muscle of a 24-year-old woman and specimen 4 was from the vastus lateralis of a 29-year-old man. Specimen 2A was frozen immediately in liquid N_2 (−196°), and specimens 2B and 4 were frozen in hexane cooled to −70°. The biopsy specimens were stored at −20° until they were analysed. They were then dehydrated by evacuation overnight at room temperature and about 10^{-3} Torr with a rotary pump (Edwards 2SC20) and a liquid N_2 trap. Previous experiments had shown that xanthine and hypoxanthine are not volatile under these conditions.

Mass spectrometry. Portions of the dry pulverized tissue (approx. 0·5 mg.) were placed in the direct insertion probe of the mass spectrometer (Varian MAT model SM1). All the spectra were obtained at a probe temperature of 250±2°. Previous experiments had shown this to be the optimum temperature for observing xanthine and hypoxanthine. Other operating conditions that applied throughout were: source temperature 230°, ionizing potential 70 v, ionizing current 300 μA. At least three separate samples of each specimen were examined to allow for inhomogeneity of the dehydrated tissues. Initially, a survey spectrum of low (about 1000) resolving power covering the mass range 40–900 was examined in each case. Small portions of the spectrum around the mass numbers (54, 81, 109, 136, 152) of the major ions characteristic of hypoxanthine and xanthine (subsequently referred to as the oxypurine ions) were then rescanned at a resolving power of 15000 to record their multiplet structure. Accurate mass measurements, with perfluorokerosine as mass reference, were made to obtain the atomic composition of each component.

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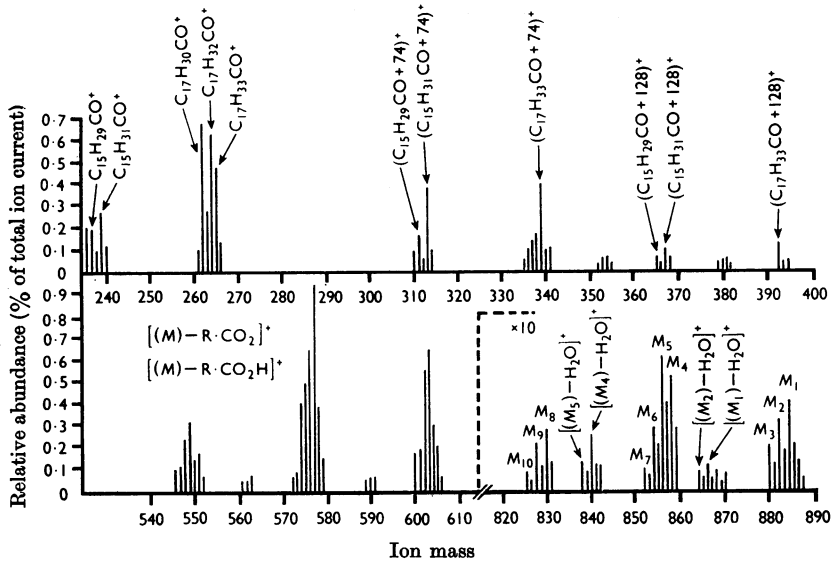

Fig. 1. Low-resolving-power (about 1000) mass spectrum obtained from dry pulverized muscle.

RESULTS

Survey spectra. All five specimens gave closely similar spectra. A portion of one showing peaks of structural significance is shown in Fig. 1. The molecular ions denoted M_1–M_{10} correspond to a complex mixture of glycerides containing predominantly C_{16} and C_{18} saturated, mono- and di-unsaturated fatty acids. The fragment ions are typical of a glyceride spectrum (Barber, Merren & Kelly, 1964). Those in the mass range 540–610 have the general formulae $(M_1-R\cdot CO_2)^+$ and $(M_1-R\cdot CO_2H)^+$, where $R\equiv C_{15-17}H_{27-35}$, and other fragments have the composition indicated. The remainder of the spectrum below mass 240 consisted of a series of moderately intense peaks at every mass number from 50 to 235 with few outstanding features. The observations were repeated at several probe temperatures in the range 200–300°. At 200° the glycerides were not sufficiently volatile to be detected and the resulting spectrum was found to be closely similar to that of cholesterol. When the sample temperature was raised to 250°, the cholesterol was rapidly pumped away and the glycerides were vaporized to give a spectrum similar to Fig. 1. On no occasion could a spectrum characteristic of xanthine or hypoxanthine be distinguished.

High-resolution spectra. In Fig. 2, tracings of the high-resolving-power spectra at masses 54, 81, 109, 136 and 152 are shown for muscle specimens from a

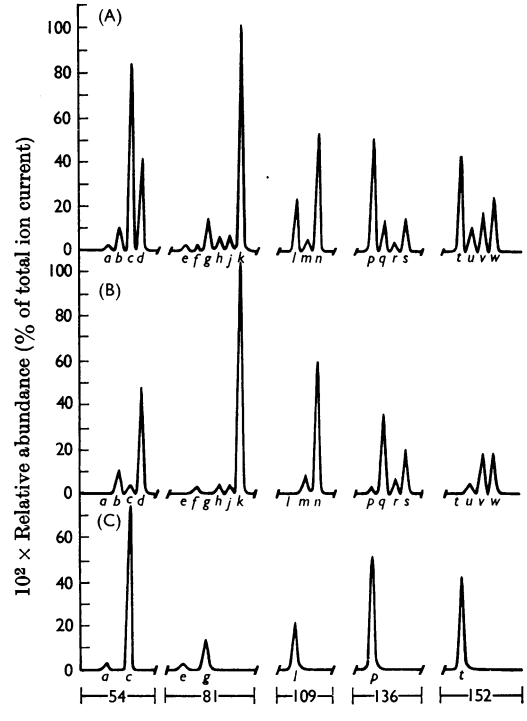

Fig. 2. High-resolving-power (about 15000) mass spectra at masses 54, 81, 109, 136, 152 for: (A) xanthinuric patient's muscle; (B) normal muscle; (C) mixture of pure hypoxanthine and xanthine.

Table 1. *Accurate masses, atomic compositions and origins of certain ions observed in the spectra of volatile constituents of muscle*

Precision of mass measurement was ±0·002 mass unit.

Ion	Mass	Assignment	Theoretical mass*	Origin
a	53.997	C_2NO	53.9980	Oxypurine†
b	54.011	C_3H_2O	54.0106	Glyceride
c	54·021	$C_2H_2N_2$	54·0218	Oxypurine
d	54·032	C_3H_4N	54·0343	Histidine
e	81·010	C_3HN_2O	81·0089	Oxypurine
f	81·021	C_4H_3NO	81·0215	
g	81·034	$C_3H_3N_3$	81.0327	Oxypurine
h	81.045	$C_4H_5N_2$	81·0453	Histidine
j	81·058	C_5H_7N	81·0578	
k	81·071	C_6H_9	81·0704	Glyceride
l	109·028	$C_4H_3N_3O$	109·0276	Oxypurine
m	109·064	C_7H_9O	109·0653	Glyceride
n	109·102	C_8H_{13}	109·1017	Glyceride
p	136·039	$C_5H_4N_4O$	136·0385	Hypoxanthine
q	136·064	$C_7H_8N_2O$	136·0637	
r	136·090	$C_9H_{12}O$	136·0888	Glyceride
s	136·126	$C_{10}H_{16}$	136·1252	Glyceride
t	152·034	$C_5H_4N_4O_2$	152·0334	Xanthine
u	152·082	$C_9H_{12}O_2$	152·0837	Glyceride
v	152·118	$C_{10}H_{16}O$	152·1201	Glyceride
w	152·156	$C_{11}H_{20}$	152·1565	Glyceride

* From Beynon & Williams (1963).

† 'Oxypurine' is used to denote ions derived either from xanthine or hypoxanthine or both.

xanthinuric patient and a normal subject, together with the spectrum of a mixture of pure hypoxanthine and xanthine under the same conditions. At each mass number a group of peaks was recorded, each peak corresponding to ions having a given exact mass. The mass, the corresponding atomic composition and the most probable origin of each ion are listed in Table 1.

For a few ions (*f*, *j*, *q*) there was insufficient evidence from the atomic composition data alone to establish unambiguously their origin. *d* and *h* were known from previous observations to be prominent in the spectrum of histidine, and *a*, *c*, *e*, *g*, *l*, *p* and *t* had the correct compositions to be derived from xanthine and hypoxanthine. The remaining ions contained only carbon, hydrogen and oxygen. Since the glycerides were the predominant species in the sample vapour, their fragmentation probably gave rise to most of these ions although fragments of other oxygen-containing species may have contributed to some extent.

The relative intensities (expressed as percentages of the total ion current) of the oxypurine and background ions in normal and xanthinuric muscle are given in Table 2. In Table 3, the oxypurine ion intensity, expressed as a percentage of the total ion abundance (oxypurine + background) at each mass number, is compared for the five specimens.

As a further check on the presence of xanthine and hypoxanthine, the observed intensities of the oxypurine fragment ions were compared with those calculated for a mixture of xanthine and hypoxanthine. The ions of masses 136 and 152 are independently characteristic of hypoxanthine and xanthine respectively. Furthermore, the intensities of the ions of masses 109, 81 and 54 relative to 136 and 152 separately were known from the spectra of pure hypoxanthine and xanthine respectively (Chalmers *et al.* 1969*b*). Hence, assuming the observed abundances of the ions of masses 136 and 152 from the muscle spectra (Table 3), the intensities of the ions of masses 109, 81, and 54 were obtained separately for hypoxanthine and xanthine. The sum of those calculated intensities was then compared for each mass number with those observed in the muscle spectra. The relevant data are assembled in Table 4 for specimens 1 and 3 in which the greatest amounts of oxypurine were observed.

Table 2. *Relative abundances of oxypurine (Ox) and background (Bg) ions*

Ion	Specimen ...	Relative abundance (% of total ion current) 1 Xanthinuric	2A (Normal)	2B (Normal)	3 (Xanthinuric)	4 (Normal)
54 *a*	(Ox)	0·03	0·00	0·00	0·08	0·00
b	(Bg)	0·11	0·09	0·11	0·15	0·13
c	(Ox)	0·87	0·03	0·04	0·88	0·00
d	(Bg)	0·43	0·51	0·51	0·33	0·44
81 *e*	(Ox)	0·03	0·00	0·00	0·02	0·00
f	(Bg)	0·03	0·00	0·03	0·00	0·05
g	(Ox)	0·15	0·00	0·00	0·15	0·00
h	(Bg)	0·07	0·03	0·07	0·04	0·05
j	(Bg)	0·08	0·05	0·05	0·05	0·10
k	(Bg)	1·03	1·09	1·00	1·10	1·16
109 *l*	(Ox)	0·24	0·01	0·00	0·31	0·00
m	(Bg)	0·05	0·09	0·10	0·05	0·08
n	(Bg)	0·54	0·60	0·61	0·61	0·69
136 *p*	(Ox)	0·51	0·04	0·03	0·31	0·03
q	(Bg)	0·14	0·37	0·37	0·22	0·19
r	(Bg)	0·04	0·07	0·08	0·07	0·07
s	(Bg)	0·15	0·22	0·21	0·22	0·24
152 *t*	(Ox)	0·43	0·00	0·00	0·63	0·00
u	(Bg)	0·11	0·05	0·05	0·06	0·04
v	(Bg)	0·18	0·20	0·18	0·11	0·14
w	(Bg)	0·25	0·18	0·20	0·16	0·31

Table 3. *Relative intensity of the oxypurine ions as a percentage of the total intensity of each multiplet*

A zero entry indicates that no ions were detected. The detection limit corresponded to a relative intensity of about 0·5 in the scale.

Ion mass	Specimen ... 1	2A	2B	3	4
54	62·5	4·8	6·3	66·7	0
81	12·8	0	0	12·0	0
109	28·6	1·5	0	36·9	0
136	63·0	6·1	4·5	38·3	5·7
152	44·3	0	0	64·9	0

DISCUSSION

Identification of hypoxanthine and xanthine. To confirm the identity of hypoxanthine and xanthine in the muscle specimens it was necessary first to detect the presence of these oxypurines in the total mass spectrum of the volatile material from each muscle specimen. However, under the necessary experimental conditions a large variety of compounds, including amino acids, steroids and glycerides, were vaporized. These gave rise to a relatively intense background spectrum from which the small contribution of xanthine and hypoxanthine had to be separated. This was most satisfactorily accomplished by examining the spectrum at high resolving power whereby ions of differing atomic compositions were distinguished by virtue of their fractional mass differences. Provided that the exact masses of the oxypurine ions are sufficiently different from the exact masses of the background ions having the same integral masses but different atomic compositions, the peaks due to the oxypurine ions will be displaced from those of the background ions. Thereby, small changes in the intensities of the oxypurine ions in the spectra of muscle samples would become readily detectable.

In the spectra of all five muscle specimens, ions were found whose atomic compositions were identical with those of the corresponding ions in the spectra of authentic hypoxanthine and xanthine. The observed oxypurine ion intensities were in reasonable agreement with those calculated on the assumption of a mixture of hypoxanthine and xanthine, as shown in Table 4. This indicated that the observed oxypurine ions were derived very largely from hypoxanthine and xanthine and that no other purine derivative was making a significant contribution.

Relative concentration of oxypurine in the different specimens. The direct insertion sample

Table 4. *Comparison of the intensities of the oxypurine ions observed in volatile constituents of muscle with those calculated for a mixture of hypoxanthine and xanthine*

R_{Hx} and R_X: the ratios of the fragment ions to the parent ions for hypoxanthine (Hx) and xanthine (X) as obtained from the spectra of the pure compounds. $[Hx]_1$, $[Hx]_3$, $[X]_1$ and $[X]_3$: the calculated intensities of the ions derived from hypoxanthine and xanthine in specimens 1 and 3 assuming the intensities of the corresponding molecular ions have the values given in Table 3. $[Ox]_1^C$, $[Ox]_1^O$, $[Ox]_3^C$, $[Ox]_3^O$: the total calculated (C) and observed (O) intensities of the oxypurine ions of specimens 1 and 3. $[Ox]^C=[Hx]+[X]$; $[Ox]^O$ is obtained from Table 3.

Ion mass	R_{Hx}	R_X	$[Hx]_1$	$[Hx]_3$	$[X]_1$	$[X]_3$	$[Ox]_1^C$	$[Ox]_1^O$	$[Ox]_3^C$	$[Ox]_3^O$
152	—	1·00	—	—	44·3	64·9	(44·3)	(44·3)	(64·9)	(64·9)
136	1·00	—	63·0	38·3	—	—	(63·0)	(63·0)	(38·3)	(38·3)
109	0·06	0·53	3·8	2·3	23·5	34·4	27·3	28·6	36·7	36·9
81	0·10	0·13	6·3	3·8	5·8	8·4	12·1	12·8	12·2	12·0
54	0·29	0·80	18·3	11·1	35·4	51·9	53·7	62·5	63·0	66·7

inlet of the mass spectrometer is a fractionating device, and the composition of the vapour in equilibrium with a solid mixture contained in the inlet will therefore vary with vaporization temperatures. Thus it was important to ensure that the vaporization conditions were the same for each specimen, so that differences in oxypurine ion intensity reflected differences in the oxypurine concentration in the muscle. Temperature fluctuations can alter (a) the relative intensities of individual components of a multiplet with respect to each other and (b) the relative intensities of each component from specimen to specimen. Comparison of the relative abundances of corresponding background ions (Table 2) shows that both types of variation are small compared with the variation in oxypurine ion intensity. Consequently it was reasonable to conclude that all five specimens were examined under essentially the same conditions and that oxypurine ion intensities could be compared meaningfully among the five specimens as shown in Table 3.

The relative abundances of the oxypurine ions from xanthinuric subjects' muscle were found to be consistently greater by at least a factor of 10 than those from normal muscle. Although a part of this variation in relative abundance may have been due to small differences in vaporization temperature, the observed fluctuations in background ion intensity suggested that such variations should be no more than a factor of about 2. Hence a substantial proportion of the observed increase in oxypurine ion intensity between xanthinuric and normal muscle must have been the result of an increased concentration of hypoxanthine and xanthine in the xanthinuric subject's muscle tissue.

Possible origin of the increased amounts of hypoxanthine and xanthine in the xanthinuric subjects' muscle. The total concentration of hypoxanthine and of xanthine in the plasma in xanthinuria is about 0·5 mg./100ml. This is considerably less than the solubility in plasma of either hypoxanthine (115mg./100ml.) or of xanthine (10mg./100ml.) reported by Klinenberg, Goldfinger & Seegmiller (1965), and suggests that these purines are formed locally in the muscle rather than precipitated from the blood and tissue fluids. Nasrallah & Al-Khalidi (1964) observed a 20-fold increase in the urinary excretion of hypoxanthine with a three-fold increase in xanthine excretion during severe muscle work, and although exertional raised blood lactic acid causes urate retention, a net increase in uric acid excretion over a 48hr. or 72hr. period during which severe muscular exercise is undertaken has been reported (Cathcart, Kennaway & Leathes, 1908). Adenylic acid is irreversibly deaminated to inosinic acid by AMP-aminohydrolase (E.C. 3.5.4.6) during severe muscle work and although reamination occurs via adenylosuccinate (Newton & Perry, 1960) the accumulation of inosine by the isolated rat diaphragm preparation *in vitro* (Alertson, Walaas & Walaas, 1958), indicates that this process may not always be complete. Free hypoxanthine could arise from hydrolysis of the nucleoside, and further oxidation by xanthine oxidase would yield xanthine and finally uric acid. The detection of hypoxanthine in the normal muscle and the accumulation of hypoxanthine and xanthine in xanthine oxidase-deficient subjects' muscle supports this suggestion and indicates the possible physiological function of the trace of xanthine oxidase activity that is normally present in striped muscle (Watts, Watts & Seegmiller, 1965; Al-Khalidi & Chaglassian, 1965). The presence of xanthine as well as hypoxanthine in the xanthinuric patients' tissue indicates that the xanthine oxidase reaction is not the only mechanism available for the oxidation of hypoxanthine to xanthine in muscle; however, there is no quantitatively important alternative mechanism to effect the

oxidation of xanthine to uric acid. Similar considerations apply to the interpretation of the urinary purine excretion pattern in the disease.

It should be noted that although the present work is confined to the detection of hypoxanthine and xanthine in muscle, the method is a general one and should be applicable to the detection of different substances in other types of tissue. Also, although histidine was the only amino acid definitely identified, it should not be assumed that no other amino acid is present. The identification of histidine was fortuitous in that two of its major characteristic ions have masses 81 and 54. If the whole mass spectrum had been examined under high resolving power, then undoubtedly more compounds would have been identified. Such a project would require the use of automatic data-handling facilities.

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