Urinary Excretion of n-Hexanedioic and n-Octanedioic Acid in Juvenile Diabetics with Ketonuria

Jon Elling Pettersen, M.D., Oslo, Norway

SUMMARY

Urine samples from patients with newly diagnosed juvenile diabetes and predibetic coma or ketonuria, but no acid-base disturbances in the blood, have been analyzed for dicarboxylic acids by combined gas-liquid chromatography and mass spectrometry.

The precomatose patients excreted up to 210 and 38 μg./1 mg. of creatinine, respectively, of n-hexanedioic and n-octanedioic acid. The excretion was almost normalized within a few hours by insulin treatment. Nonacidotic patients with ketonuria excreted 9.2 ± 5.1 mg./24 hr. of n-hexanedioic acid, and some also excreted slightly increased amounts of n-octanedioic acid. All patients excreted normal amounts of 3-methylhexanedioic acid, which was unaffected by insulin treatment.

Our findings direct attention to a metabolic pathway which may be of importance in the pathogenesis of ketosis in diabetes mellitus, namely the formation of short-chain dicarboxylic acids (for instance, intermediates of the tricarboxylic acid cycle) by ω-oxidation of fatty acids followed by β-oxidations of the dicarboxylic acids thus formed. DIABETES *23*:16-20, January, 1974.

Due to the reduced glucose oxidation in untreated diabetes mellitus there is an increased utilization of fatty acids as energy source, accompanied by an increased production of ketone bodies. Different degrees of the metabolic derangement may occur, from ketonuria without acid-base disturbances up to diabetic coma, which is one of the most serious complications of diabetes mellitus and is characterized by a strong metabolic acidosis and the presence of large amounts of ketone bodies in the body fluids. We have recently observed considerable quantities of two short-chain dicarboxylic acids, namely n-hexanedioic and n-octanedioic acid, in urine samples from fifteen ketotic patients, of which four had diabetes mellitus.[1,2] As will be discussed at the end of this paper, dicarboxylic acid metabolism may be of importance in the pathogenesis of ketosis. This prompted us to study dicarboxylic acid excretion in diabetes mellitus.

The present study was undertaken to determine if an increased urinary excretion of n-hexanedioic and n-octanedioic acid is a constant finding in untreated juvenile diabetes mellitus, and if the excretion is changed by insulin treatment. Urine samples from diabetics with ketonuria and normal acid-base status in blood, and from patients with prediabetic coma have been examined for short-chain dicarboxylic acids by combined gas-liquid chromatography and mass spectrometry.[3]

MATERIALS AND METHODS

Chemicals and instruments. Glutaric, hexanedioic, heptanedioic and octanedioic acids were obtained from Fluka A. G., Buchs, Switzerland. 3-Methylhexanedioic acid was synthesized by the Kolbe electrolytic procedure as described elsewhere.[4] N-nitrosomethylurea was purchased from K and K Laboratories, Calif. Stationary phases for gas chromatography (BDS and OV-17) and column supports (Chromosorb W and Gas Chrom Q) were obtained from Applied Science Laboratories, Pa. Other chemicals were commercial products of analytical grade.

For combined gas-liquid chromatography and mass spectrometry an instrument consisting of a gas chromatograph (Varian 1400) with a single, coiled glass column (8 ft. x 0.25 in.) combined with a low resolution mass spectrometer (Varian CH 7) was used. The column was filled with either 10 per cent OV-17 on Gas Chrom Q (80 to 100 mesh) or 8 per cent BDS on Chromosorb W (80 to 100 mesh), and the temperature was programmed at a rate of 8° C. per minute from 80 to 300° C. and 80 to 180° C., respectively. The instrument was equipped with an ion current detector,

From the Institute of Clinical Biochemistry, University of Oslo, Rikshospitalet, Oslo 1, Norway.

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the ionization energy was 70 eV, and helium was used as carrier gas (flow 30 ml. per minute).

Patients. Five women and ten men, aged nine to thirty-five years, with newly diagnosed classic juvenile diabetes were studied before and during initial insulin treatment. They were ambulant during the day and received a fixed diabetes diet, as shown in table 1. None of them were acidotic, severely dehydrated or obese. The patients were studied twice during a twenty-four hour period before insulin treatment was started (sampling period 1) and a twenty-four hour period (sampling period 2) after four to eight days of insulin treatment. Urine was collected for twenty-four hour periods at +4° C. and immediately examined for ketone bodies and sugar (table 2). It was then kept frozen at —20° C. until examination for dicarboxylic acids. Capillary ear blood for blood sugar determination was collected at 7:30 a.m., 10:30 a.m., 1:30 p.m., 4:30 p.m. and 9:30 p.m. Blood sugar and urinary sugar were determined according to the o-toluidine method.[5] Ketonuria was determined by Ketostix.

Two previously untreated patients with prediabetic coma were examined at intervals during the first day in the hospital (table 3). Patient A was a four year old girl, patient B a six year old boy. Urine was collected once before insulin therapy was started and then repeatedly during the first twenty-four hours in the hospital. Insulin was administered immediately after the first urine sample had been collected. Acid-base status was determined in capillary blood samples with an Astrup apparatus (Radiometer, Copenhagen).

The control group consisted of six healthy persons, three women and three men, aged twenty-six to thirty-five years (laboratory staff). They received an ordinary diet.

Determination of dicarboxylic acids in urine. To a urine sample (usually 1/200 of a twenty-four hour sample or a urine sample equivalent to 6 mg. of creatinine) were added glutaric and/or heptanedioic acid as internal standards. The mixture was acidified to pH 1 by addition of hydrochloric acid and continuously extracted with diethyl ether for eighteen hours. The extract was dried over anhydrous disodic sulfate, and methylated by diazomethane liberated from N-nitrosomethylurea. The samples were then subjected to combined gas-liquid chromatography and mass spectrometry. For quantitative purposes correction factors, including both the degree of extraction and the relative responses of the ion current detector, were determined as described previously.[2]

RESULTS

Juvenile diabetics with ketonuria and normal acid-base status in blood. The clinical data of the patients can be seen in table 1. All of the patients were clinically nonacidotic. Serum electrolytes and acid-base status in blood were examined in seven subjects. All of them had normal values. None of the patients had albuminuria as evaluated by Albustix. Endogenous creatinine clearance was calculated before and during insulin treatment in twelve subjects: before treatment, 116.0 ± 24.1 ml./1 min./1.73 m^2 (mean ± S.D.); during treatment, 101.7 ± 29.1 ml./1 min./1.73 m^2. These values did not differ statistically from each other. Table 2 shows results from analyses of blood and urine samples collected before and during initial insulin treatment. In each patient the mean blood sugar values were consider-

TABLE 1

Clinical data of patients with juvenile diabetes

Patient No.	Sex	Age (yr.)	Height (cm.)	Weight (kg.)	Insulin dose	Days of insulin treatment	Diet (gm.) Carbohydrate	Fat	Protein
1	M	13	155	43	20 NPH	8	196	82	122
2	F	29	174	70	16 NPH	7	114	54	79
3	M	22	178	63	20 NPH	6	186	85	93
4	F	10	140	27	16 NPH	7	160	87	86
5	M	16	173	61	10 NPH	4	216	77	118
6	M	20	189	68	12 Reg.	4	196	92	95
7	M	24	179	70	24 NPH	6	194	89	95
8	M	35	164	61	22 NPH	6	205	58	112
9	M	19	187	62	10 Reg./10 NPH	6	228	71	118
10	M	20	184	70	20 Reg./14 Reg.	6	262	69	113
11	F	31	160	53	12 NPH	7	145	29	96
12	M	17	169	58	10 NPH	4	197	90	109
13	F	20	165	51	22 NPH	7	171	101	99
14	F	9	127	21	10 NPH	8	182	72	80
15	M	30	179	61	8 NPH	6	248	100	108

TABLE 2

Urinary excretion of dicarboxylic acids in nonacidotic juvenile diabetics before (1) and during (2) initial insulin treatment

Patient No.	Sampling period	Blood sugar (mg./100 ml.)	Glucosuria (gm./24 hr.)	Keto-nuria	Urinary dicarboxylic acids (mg./24 hr.): n-hexanedioic	n-octanedioic	3-methylhexanedioic
1	1	276 ± 62	105	3	22.0	8.2	32.6
	2	157 ± 29	18	0	2.1	traces	25.4
2	1	258 ± 30		3	12.3	4.3	25.7
	2	148 ± 48		0	traces	traces	19.9
3	1	303 ± 91	193	3	8.9	traces	20.2
	2	235 ± 50	84	0	traces	traces	17.0
4	1	337 ± 91	274	3	10.8	2.7	18.5
	2	214 ± 53		0	2.4	traces	7.8
5	1	281 ± 37	119	1	7.1	traces	22.8
	2	153 ± 57	9	0	1.5	traces	23.1
6	1	230 ± 31	75	0	4.0	traces	12.8
	2	188 ± 59	25	0	traces	traces	8.3
7	1	294 ± 46	198	2	8.2	traces	21.8
	2	214 ± 39	51	0	1.6	traces	23.9
8	1	361 ± 100	245	3	18.5	8.7	20.4
	2	204 ± 39	50	0	7.3	8.9	27.4
9	1	296 ± 55	104	2	6.3	traces	18.2
	2	207 ± 90	32	0	traces	traces	21.1
10	1	317 ± 59	207	2	8.9	traces	26.1
	2	207 ± 55	19	0	2.1	traces	20.5
11	1	223 ± 22	75	2	6.7	traces	20.0
	2	153 ± 37		0	1.8	traces	22.0
12	1	246 ± 39	101	0	5.5	traces	23.1
	2	141 ± 42	5	0	2.9	traces	14.3
13	1	262 ± 26	71	1	8.9	6.4	20.0
	2	176 ± 47		0	4.7	traces	15.8
14	1	313 ± 104	112	3	5.7	traces	12.7
	2	121 ± 58	2	0	1.6	traces	7.0
15	1	285 ± 31	108	0	4.3	traces	26.1
	2	150 ± 49	6	0	2.8	traces	19.0

ably higher before than during treatment (average 285 ± 38 and 178 ± 34 mg./100 ml. respectively). However, it is seen that during treatment the mean blood sugar values of all patients were also considerably higher than in a normal population. The degree of glucosuria was significantly decreased during initial insulin treatment. However, all of the patients had glucosuria during the second sampling period also. In some of the urine samples the amounts of sugar were not determined, but the presence of glucosuria was confirmed in all samples by Tes-Tape. On admission to the hospital all but three patients had ketonuria as evaluated by Ketostix; combined gas-liquid chromatography and mass spectrometry showed that even the three exceptions excreted pathologic amounts of 3-hydroxybutyrate. After four to eight days of insulin treatment none of the patients excreted pathologic amounts of ketone bodies in the urine.

Before insulin therapy was instituted the patients excreted 9.2 ± 5.1 mg./24 hr. (mean ± S.D.) of n-hexanedioic acid. This was significantly more ($p < 0.01$) than during the second sampling period, when insulin treatment had been given for some days (2.2 ± 1.8 mg./24 hr.). During sampling period 2 the excretion was not different, in a statistically significant manner, from the excretion in the control group (2.0 ± 0.6 mg./24 hr.). The healthy subjects excreted below 3.0 mg./24 hr. of n-hexanedioic acid. All but two of the patients showed a urinary excretion of n-hexanedioic acid in this range during insulin treatment. Before treatment five of the patients excreted more than trace amounts of n-octanedioic acid (see table 2). During insulin treatment only one of these five patients, and none of the others, showed significant n-octanedioic acid excretion. This patient (No. 8), who excreted the most n-octanedioic acid before treatment, excreted about the same amounts following insulin treatment. The persons in the control group excreted only trace amounts of this acid (less than 1 mg./24 hr.). The excretion of 3-methylhexanedioic acid was not changed significantly by insulin treatment (before, 21.4 ± 5.1 mg./24 hr.; after, 18.2 ± 6.4 mg./24 hr.). In ten of the patients the excretion decreased, and in the five others it increased. The control group excreted 24.5 ± 4.9 mg./24 hr. of 3-methylhexanedioic acid, range 16.0 to 30.4 mg./24 hr., which was even more than in the patient

TABLE 3

Urinary excretion of dicarboxylic acids in patients with prediabetic coma before and during initial insulin treatment. Insulin was administered immediately after the first urine sample (0 hr.) had been collected

Patient	Sample time	Acids in urine (μg./mg. of creatinine): n-hexanedioic	n-octanedioic	3-methylhexanedioic
A	0 hr.	160	38	22
	2 hr.	22	5	15
	4 hr.	5	traces	15
	24 hr.	3	traces	18
B	0 hr.	210	16	18
	1 hr.	85	10	20
	2 hr.	10	traces	13
	10 hr.	12	traces	16
	24 hr.	5	traces	17

group. The difference was statistically significant ($p < 0.05$) between the insulin-treated patients and the normal group. However, this difference may be due to body weight. The normal group consisted exclusively of adults, whereas children were included in the diabetic group. Patients Nos. 4 and 14, who excreted the smallest amounts of 3-methylhexanedioic acid, weighed only 27 and 21 kg., respectively.

Patients with pre-diabetic coma. Table 3 shows the results in two diabetic children admitted to the hospital in precoma. Both were strongly ketoacidotic (patient A: pH 7.13, BE —19; patient B: pH 7.02, BE —24). Blood sugar values were 470 and 660 mg./100 ml. in patients A and B, respectively. Before treatment both patients excreted n-hexanedioic and n-octanedioic acid in considerable quantities. The excretion of n-hexanedioic acid was always much greater than that of n-octanedioic acid. Following insulin therapy the urinary excretion of the two acids was reduced nearly to normal values in a few hours, concomitantly with the disappearance of ketone bodies from the urine. In contrast to these findings stands the excretion of 3-methylhexanedioic acid, which was not markedly changed by insulin treatment in either of the patients.

DISCUSSION

The present study has shown that increased excretion of n-hexanedioic and n-octanedioic acid is found in juvenile diabetics with ketonuria. Strongly ketoacidotic patients with prediabetic coma excreted considerable quantities of the two dicarboxylic acids, whereas patients with ketonuria but without acid-base disturbances in blood excreted lesser amounts of the two acids. During insulin therapy the dicarboxylic acid excretion was normalized. These findings fit well with previous investigations[2,6] in which the urinary dicarboxylic acid excretion seemed to parallel the degree of ketosis. The tendency preferably to excrete dicarboxylic acids with a chain length of about six carbon atoms is well-known.[7-14]

A dietary origin of the dicarboxylic acids proper or of medium-chain monocarboxylic fatty acids as possible precursors[15-19] has been excluded.[2] In vivo experiments with ketotic streptozotocin-diabetic rats have shown that short-chain dicarboxylic acids may be derived from long-chain monocarboxylic acids by an initial ω-oxidation followed by β-oxidations.[6] Investigations by others,[9,10,14,20-22] both in vivo and in vitro, also indicate that short-chain dicarboxylic acids may be formed by these mechanisms.

The increased urinary excretion of n-hexanedioic and n-octanedioic acid may be due to several causes. Liberation of nonesterified fatty acids from the peripheral fat stores is increased in untreated diabetics.[23] Also, increased ω-oxidation capacity has been demonstrated in liver microsomes from ketotic alloxan-diabetic rats.[24] Thus increased amounts of long-chain dicarboxylic acids may be formed. Furthermore, an augmented degradation of these acids might also participate in the production of increased amounts of short-chain dicarboxylic acids. We have recently demonstrated in vitro that long-chain dicarboxylic acids are activated by CoA and transported into the inner mitochondrial compartment as carnitine esters.[25] This transport is mediated by the enzyme hexadecanoyl-CoA:carnitine 0-hexadecanoyltransferase, which has increased activity in ketotic streptozotocin-diabetic rats.[25]

The source of 3-methylhexanedioic acid is most probably branched fatty acids produced by the microflora of the intestinal tract.[3] Apparently depot fats contain only negligible amounts of branched fatty acids, since the excretion of 3-methylhexanedioic acid in ketosis was not increased in parallel with the straight-chain dicarboxylic acids. As it is reasonable to assume that the branched-chain and straight-chain acids are metabolized by the same enzyme system, our results may indicate that the most important cause of augmented excretion of n-hexanedioic and n-octanedioic acid in ketosis is increased liberation of nonesterified fatty acids from the fat depots.

The degradation of long-chain dicarboxylic acids seems to be hampered when a chain-length of about six carbon atoms is reached. However, only a minor part of the dicarboxylic acids are probably excreted as n-hexanedioic or n-octanedioic acid, while most of them are broken down to succinyl-CoA. The or-

ganism may thus produce tricarboxylic acid cycle intermediates from fatty acids. More acetyl-CoA may then be handled by this pathway, and less ketone bodies will be produced. Thus the tendency to ketosis may be counteracted. The dicarboxylic acids excreted in urine, however, may represent a loss of precursors of tricarboxylic acid cycle intermediates. Recent studies by Wada et al.[26] with alloxan-diabetic rats seem to support our hypothesis. Rats fed long-chain dicarboxylic acids had smaller amounts of ketone bodies in the blood than rats fed long-chain monocarboxylic acids.

Therefore, in conclusion, the urinary dicarboxylic acid excretion in ketosis is probably only an "overflow" phenomenon, primarily due to the increased levels of nonesterified fatty acids in this condition. Our findings have, however, directed attention to a metabolic pathway of possible importance in the prevention of ketosis in diabetes mellitus, namely the production of succinyl-CoA from fatty acids by an initial ω-oxidation followed by β-oxidations of the dicarboxylic acids thus formed.

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