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CHROMATOGRAPHIC PROFILING OF URINARY VOLATILE AND ORGANIC ACID METABOLITES OF NORMAL AND DIABETIC C57BL/Ks MICE

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SUMMARY

Capillary gas chromatography and gas chromatography—mass spectrometry were used to examine the urinary volatile and organic acid metabolites in normal and diabetic C57BL/Ks male mice. Quantitative differences in the excretion of these metabolites were assessed in the animals from 5 to 24 weeks of age. Statistically significant differences were examined with respect to known metabolic abnormalities in the diabetic animals and to the possible toxic effects of elevated levels of certain metabolites. A number of aldehyde metabolites and aromatic acids, as well as most other organic acids, were found at consistently higher levels in diabetic urine. Several ketone metabolites, linalool, and 2-*sec.*-butylthiazoline were found at consistently low levels throughout the study.

INTRODUCTION

Considerable research has been conducted concerning the chromatographic profiling of volatile and acidic metabolites in urine with association to diabetes mellitus [1—9]. Various aspects of the relationship between the production of these metabolites and diabetes have been investigated in humans and in suitable animal models. Such research has explored differences in the excretion of urinary acid and volatile metabolites [1—3], including alcohols [4], the application of computerized pattern recognition algorithms in distinguishing normal and diabetic volatile urinary profiles [5], as well as the effect of the induction of alloxan and streptozotocin diabetes in the rat on volatile urinary metabolites [6, 7]. In addition, the possibility of a link between the excretion of certain ketones and diabetic polyneuropathy [8] has also been examined.

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In certain cases, the observed differences in the excretion of organic acid or volatile metabolites have been associated with specific alterations in metabolic pathways due to the diabetic condition. For example, the increased ω-oxidation of long-chain fatty acids [10] and the enhanced degradation of branched-chain amino acids [11] observed in ketoacidotic subjects are reflected by higher urinary levels of several dicarboxylic acids and additional acids, such as 3-hydroxyisovaleric and 3-hydroxyisobutyric acids. In many other cases, however, the metabolic origins of the acidic and volatile urinary metabolites remain obscure.

Attempts to elucidate the more subtle differences in the composition of normal and diabetic urine and to better define the relationships between the excretion of the volatile and acidic metabolites and diabetes in humans are hampered by the considerable inter-individual variability encountered with human subjects and the influence of such factors as diet, medication, physical state, etc., which are often not easily controlled. The potential importance of uncovering these subtle differences is underscored by recent reports suggesting a connection between the excretion of certain ketones and diabetic neuropathy, as well as indications that certain metabolic processes, such as lipid peroxidation, which are increased in diabetics may yield toxic metabolites [8, 12]. Thus, the use of animal models of diabetes mellitus, such as the db/db mouse and alloxan or streptozotocin rats, provides the opportunity for greater control over experimental variables, allowing a greater potential for assessing slight differences in the excretion of urinary metabolites.

The present study examines differences in the excretion of acidic and volatile urinary metabolites in normal (m^+/m^+ or db/m^+) and diabetic (db/db) mice of the C57BL/Ks strain. The diabetes in this strain is caused by an autosomal recessive gene with full penetrance in the homozygote [13] and is characterized by abnormal early obesity followed by hyperglycemia, polyuria and glycosuria [13, 14]. Chromatographic profiling of the urinary metabolites of the mice began at 5 weeks of age and continued until the mice were 24 weeks of age. The study was designed to examine consistent differences in the urinary profiles of the diabetic and normal animals with the twin objective of (a) relating the observed differences to known metabolic abnormalities in the diabetic animals; and (b) examining metabolites which appear at significantly higher levels in the diabetic animals with regard to the possible toxicity of such compounds.

EXPERIMENTAL

Sample collection

Ten normal (m^+/m^+ or db/m^+) and ten diabetic (db/db) male mice of the C57BL/Ks strain were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at weanling age (4 weeks). The mice were housed in smooth-bottomed plastic cages (two animals) with adequate bedding materials and were allowed free access to food and water.

Urine samples (24-h) were collected from the animals weekly from 5 to 10 weeks of age and biweekly from 10 to 24 weeks of age. During urine collection, the mice were housed in standard metabolism cages and the urine was collected

over dry ice. Urine columns and animal weights were recorded at the end of each collection period; blood glucose values were also monitored. Following urine collection, the samples were quickly thawed, filtered, diluted to a standard volume, divided into suitable aliquots, and refrozen until analysis. No preservatives were used.

Analysis of volatile metabolites

Analysis of the volatile constituents of the diabetic and normal urine samples was accomplished using a headspace concentration method followed by capillary gas chromatography (GC) as previously described [15]. In this method, volatile compounds are purged from the heated urine headspace (100°C) with high-purity helium and absorbed onto the porous polymer Tenax-GC (2,6-diphenyl-*p*-phenylene oxide polymer; Applied Science Labs., State College, PA, U.S.A.). The porous polymer was contained in platinum micro-baskets which were subsequently encapsulated for injection [15].

Separation of the volatile compounds was performed on an automated capillary GC system [15]. Reproducibly prepared glass capillary columns (60 m × 0.25 mm I.D.) coated with 0.2% UCON-50-HB2000 (Applied Science Labs.), a polar stationary phase, containing 0.015% benzyltriphenylphosphonium chloride (BTPPC) as surfactant were employed for chromatographic analysis. A Perkin-Elmer 3920 gas chromatograph equipped with an automatic injector (Perkin-Elmer, AS41), a flame ionization detector, a mass-flow controller (Brooks, Model 5840) and a data acquisition system (PEP-2, Perkin-Elmer) was utilized for chromatographic analysis. All samples were run at a flow-rate of 2 ml/min and the columns were temperature-programmed from 28 to 160°C.

Analysis of organic acid metabolites

Chromatographic profiles of the urinary organic acids were obtained using a two-step solvent extraction method and two derivatization techniques. In this procedure a 2-ml aliquot of urine is first made basic (pH 10) with sodium hydroxide (6 *M*) and 200 μl of a 20 mg/ml solution of methoxylamine hydrochloride (Applied Science Labs.) are added, as well as 10 μl of the 2 mg/ml solution of *p*-chlorobenzoic acid used as internal standard. The sample is then heated to 60°C for 45 min to allow formation of the methoxime derivatives of the urinary ketoacids. Following this derivatization, the basic sample is extracted once with a 4-ml portion of ethyl acetate. The organic layer is discarded and the aqueous sample is saturated with sodium chloride and acidified with 6 *M* hydrochloric acid. The urinary acids are extracted into an organic solvent using two 4-ml extractions with ethyl acetate, followed by one extraction using 4 ml of diethyl ether. Th organic extracts are combined and evaporated to dryness using a gentle nitrogen stream. Trimethylsilyl derivatives are formed by adding 50—200 μl of bis-trimethylsilyltrifluoroacetamide (BSTFA, Pierce, Rockford, IL, U.S.A.) and heating in tightly sealed vials for 45 min at 60°C. Following derivatization, 0.1—0.2 μl aliquots of each sample are analyzed by capillary GC.

A Varian Model 1400 gas chromatograph, modified for use with capillary columns and employing flame ionization detection was used in the analysis of

the derivatized acids. Peak area and retention time data were provided by a Sigma 10 (Perkin-Elmer) chromatographic data acquisition system. Separation of the sample constituents was accomplished using a glass capillary column (40 m × 0.25 mm I.D.) coated with SE-30 (Applied Science Labs.), a non-polar methylsilicone stationary phase. Following an initial 2-min isothermal period at 50° C, temperature programming was employed from 50 to 250° C at 4° C/min.

Identification of profile constituents

Structural identification of urinary volatile and acidic metabolites was accomplished using capillary GC—mass spectrometry (MS). Low-resolution electron impact ionization spectra were obtained at 70 eV with a Hewlett-Packard Model 5982 combined gas chromatograph—dodecapole mass spectrometer—computer system. The glass capillary column (SE-30- or UCON-coated) was directly interfaced with the mass spectrometer ion source (maintained at 220° C). All spectra were run at a scan-rate of 100 a.m.u./sec using the same chromatographic conditions which were employed in the analysis of the volatile or acidic fractions of urine. Whenever possible, proposed structures were verified through comparison of mass spectra and retention times of authentic compounds.

Data analysis

Quantitative comparisons of diabetic and normal urinary metabolites were carried out using peak area values obtained with the computerized data acquisition system. The average peak area of each measured peak in the diabetic profiles was calculated as a percent of the corresponding normal value plus or minus the standard error. Statistically significant differences in the excretion of any metabolites were assessed using the Student's *t*-test.

RESULTS AND DISCUSSION

Upon arrival at 4 weeks of age, the diabetic (db/db) and heterozygote (db/m^+) normal mice were barely distinguishable on the basis of size and weight. By 7 weeks of age, the average diabetic weight was 7 g (ca. 30%) more than that of the normal average. The 24-h urine excretion for the diabetic animals averaged 15—20 ml at 7 weeks compared with 0.5—1.0 ml per 24 h for the normal mice. The diabetic animals reached their maximum weights between 12 and 16 weeks of age followed by a slow decline in body weight and in some cases, death. Four diabetic animals died prior to the end of the study; one at 16 weeks, two at 18 weeks and one at 24 weeks of age. Blood glucose values for the diabetic mice ranged from 586 to 772 mg per 100 ml of blood during the acute later stages of the syndrome. The normal values ranged from 106 to 143 mg per 100 ml of blood.

The use of the C57BL/Ks db/db mouse in this study has provided a valuable opportunity to evaluate alterations in the excretion of acidic and volatile urinary metabolites due to the development of a condition similar to the maturity-onset diabetes. Since many of the morphological and metabolic abnormalities of this mutant diabetic strain have been well characterized [14], it is of note that the alterations in physiological parameters (body weight, urine

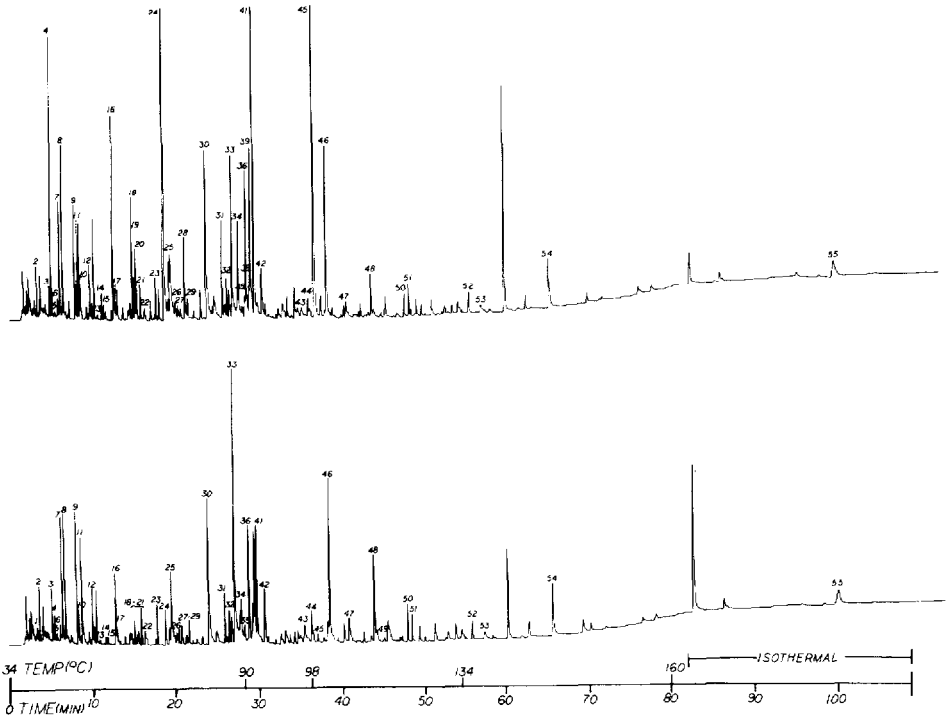

Fig. 1. Urinary volatile metabolic profiles of normal (top) and diabetic (bottom) mice at 5 weeks of age. Peak numbers correspond to the metabolite identifications of Table I.

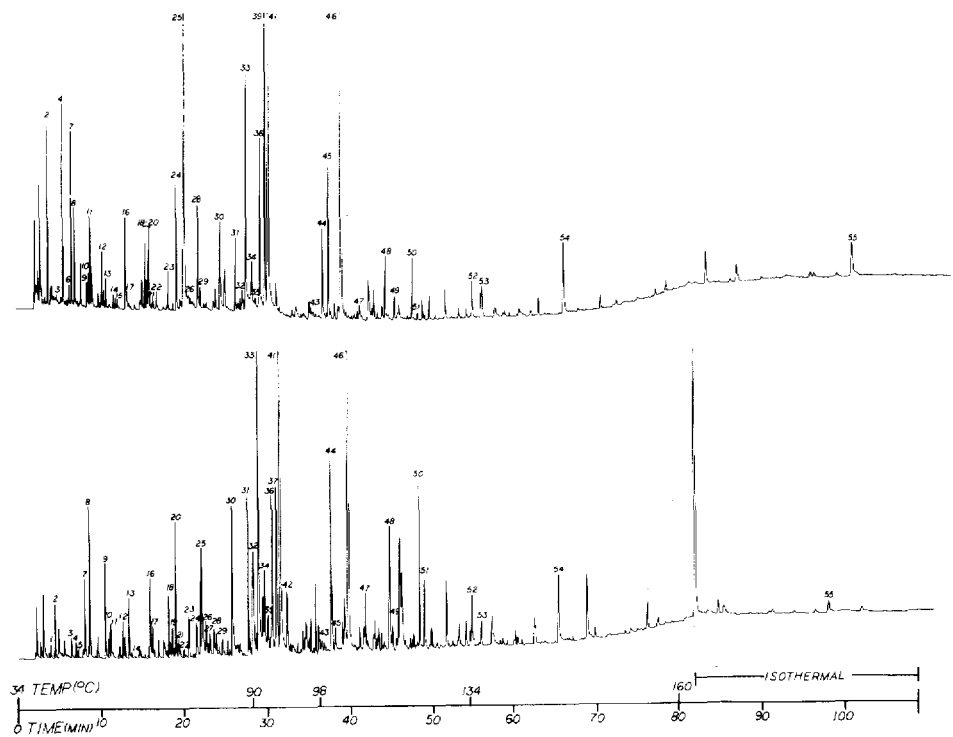

Fig. 2. Urinary volatile metabolite profiles of normal (top) and diabetic (bottom) mice at 10 weeks of age. Peak numbers correspond to the metabolite identifications of Table I.

TABLE I

SIGNIFICANT DIFFERENCES IN VOLATILE METABOLITE EXCRETION IN DIABETIC MICE AS PERCENTAGE OF MEAN AGE-MATCHED CONTROL VALUE

Values reported as percentage of mean age-matched control value ± standard error. Only significant ($p \leq 0.05$ Student's t-test) differences are reported. Peak numbers correspond to those of Figs. 1 and 2.

Peak No.	Structural identification	Age (weeks)								
		5	6	7	8	10	12	14	20	24
3	Pentanal			219 ± 96	327 ± 117					
4	Dehydration product I from 6-hydroxy-6-methyl-3-heptanone	16 ± 36		14 ± 60	34 ± 54			20 ± 70		
9	Hexanal		164 ± 49	282 ± 100		230 ± 31		270 ± 67	277 ± 130	293 ± 93
11	Dehydration product II from 6-hydroxy-6-methyl-3-heptanone	25 ± 39	32 ± 60	9 ± 25	8 ± 53	16 ± 23		12 ± 33		34 ± 43
13	2,4-Pentadienal*	238 ± 74	305 ± 171	345 ± 160	201 ± 59	284 ± 76	215 ± 80	160 ± 45	297 ± 84	368 ± 215
16	2-Heptanone	49 ± 16				175 ± 22	180 ± 67	77 ± 10	292 ± 64	173 ± 41
18	Styrene + *trans*-5-hepten-2-one	34 ± 24		39 ± 23	46 ± 37					
20	*trans*-4-Hepten-2-one	20 ± 36	14 ± 19	20 ± 8	23 ± 54	39 ± 35		14 ± 15		
28	6-Methyl-6-hepten-3-one	33 ± 32	33 ± 17	24 ± 51	28 ± 62	52 ± 40		46 ± 18		34 ± 50
30	Furfural					141 ± 21		176 ± 30		
33	Nonanal	248 ± 55	330 ± 95	160 ± 37	138 ± 19	134 ± 23	202 ± 39	215 ± 41	289 ± 67	242 ± 83
35	2-Octenal	42 ± 44			192 ± 51	163 ± 30	237 ± 100		190 ± 54	
38	N-Acetylpyrrole			748 ± 199	632 ± 163	582 ± 240	631 ± 238	898 ± 320	990 ± 510	468 ± 52
41	2-*sec*.-Butylthiazoline	25 ± 43	30 ± 29	35 ± 26	31 ± 56	31 ± 34	61 ± 23	36 ± 28	61 ± 30	53 ± 38
42	5-Methylfurfural*	201 ± 57	235 ± 93	231 ± 83	199 ± 63	350 ± 90	265 ± 79	264 ± 48	388 ± 130	276 ± 59
44	2-Nonenal	194 ± 37	197 ± 43	200 ± 54	231 ± 42	203 ± 37	216 ± 52	325 ± 82	408 ± 87	317 ± 68
45	Linalool	6 ± 14	17 ± 35	25 ± 10	21 ± 49	30 ± 32	29 ± 33	19 ± 48	25 ± 36	9 ± 48
46	Acetophenone					63 ± 32		39 ± 36		
48	Phenylacetone	167 ± 32	366 ± 100			159 ± 35	146 ± 38	158 ± 50	197 ± 62	205 ± 42
50	Unidentified	226 ± 77	343 ± 142	204 ± 84	257 ± 77	277 ± 105	264 ± 88	376 ± 170	363 ± 120	
51	Unidentified		177 ± 64	201 ± 73	191 ± 41	191 ± 52			206 ± 67	
53	Indole	430 ± 60	348 ± 155	231 ± 107	221 ± 69		296 ± 143			881 ± 199

Additional peaks exhibiting no significant differences

1	Acetone	19	6-Methyl-3-heptanone	34	2-Acetylfuran
2	Acrolein	21	2-(Methylthio)furan	36	Benzaldehyde
5	2-Methyl-3-buten-2-ol	22	2-Pentylfuran	37	4,6-Octadiene-3-one*
6	Toluene	23	3-Hepten-2-one	39	Unidentified
7	2-Ethyl-5-methylfuran*	24	2,5-Dimethylpyrazine	40	2-Methyl-5-vinylpyrazine*
8	2-Methylbutanenitrile*	25	2-Propylisoxazole*	43	3-Nonen-2-one
10	3-Penten-2-one	26	2-Heptenal	47	4-(Methylthio)-2-butenal
12	4-Heptanone	27	Octanal	49	2-Decenal*
14	3-Heptanone	29	6-Methyl-5-hepten-2-one	52	Benzothiazole
15	Dehydration product III from 6-hydroxy-6-methyl-3-heptanone	31	Dehydrobrevicomin	54	Unidentified
		32	3-Octen-2-one	55	Unidentified
17	Heptanal				

*Tentative identification.

volume, blood glucose values and morbidity) observed in the diabetic animals in this study are in agreement with previous reports [14, 15]. This consonance allows the correlation of observed urinary excretion patterns with an established sequence of metabolic events.

Volatile metabolites

The profiling results involving volatile metabolites reflect the physiological and metabolic differences between the normal and diabetic mice. The representative chromatograms shown in Figs. 1 and 2 visually demonstrate these differences in the two sets of animals at 5—10 weeks of age. This evaluation is confirmed by the data in Table I, which give the average peak area value of the components in the diabetic profiles as a percentage of the corresponding average peak area value in the normal profiles throughout the 24-week study. Only peaks which gave significantly different values ($p \leqslant 0.05$) in the diabetic and normal profiles were included in Table I.

Upon examination of the chromatograms in Figs. 1 and 2 and the data in Table I, it is immediately obvious that large quantitative differences exist in the urinary excretion of volatile metabolites in the normal (m^+/m^+ and db/m^+) and diabetic (db/db) mice, even as early as 5 weeks of age. At this stage in the syndrome, the db/db mice have blood glucose levels which are only slightly above normal, they are hyperinsulinemic, and display elevations in certain hepatic enzymes as well as abnormal fat deposition [13, 14]. These early abnormalities of the diabetic syndrome are reflected in the urinary excretion of volatile metabolites by the significantly higher levels of nonanal, 2-nonenal, 5-methylfurfural, indole, phenylacetone and the unidentified substance represented by peak number 50, as well as the dramatically lower levels of several branched-chain and/or unsaturated ketone metabolites (5-hepten-2-one, 3-hepten-2-one and 6-methyl-6-hepten-2-one) as well as linalool (peak 45) and 2-*sec.*-butylthiazoline (peak 41).

By the seventh week of the study, pentanal, hexanal and the tentatively identified 2,4-pentadienal and 2-octenal have risen to levels that are two to three times normal, while the levels of many ketone metabolites, linalool and 2-*sec.*-butylthiazoline remain low. Insulin levels are peaking at this age in the diabetic [14] and blood glucose levels are generally in the range of 300—400 mg per 100 ml blood [13]. Increased gluconeogenesis is occurring as evidenced by elevated activities of enzymes such as glucose-6-phosphatase, fructose-1,6-diphosphatase and others [14, 16].

Based on available information concerning the alterations in metabolism occurring in the diabetic mice and the results of similar profiling studies in alloxan and streptozotocin diabetic rats [6, 7], it is possible to relate these observed differences in the excretion of volatile metabolites with the metabolic alterations occurring in this early stage of the diabetic syndrome. One of the most striking differences observed between the early diabetic and normal urine volatile profiles is the elevation of several straight-chain aldehyde metabolites. Although the metabolic origin of these compounds has not definitely been established, the structures suggest that they arise through a pathway related to fat metabolism. More specifically, the abnormally high levels of such compounds found in urine may be a result of the increased lipid peroxidation

activity reported in diabetics [12, 17], since similar compounds are known to be metabolic products of this process [18].

In contrast to the observed elevation of the aldehyde metabolites in the early weeks of the diabetic syndrome, the diabetic volatile profiles display drastically lower levels of 5-hepten-2-one, 3-hepten-2-one and 6-methyl-6-hepten-2-one. Similar compounds have been observed in the urine of alloxan and streptozotocin diabetic rats [6, 7]. In long-term studies, these were found to be excreted in significantly lower amounts in the diabetic animals with depleted fat stores [7]. In this case, the production of such metabolites would appear to be related to a deficiency in fat breakdown. Their presence in significantly lower amounts in the urine of diabetic mice probably reflects the hyperinsulinemia and correspondingly reduced fat catabolism occurring in these animals in the early stages of the syndrome [14].

Another metabolite which was found at characteristically lower levels throughout the 24-week study is 2-*sec.*-butylthiazoline. The excretion of this compound has been reported to be sex-related and unique to the mouse [19]; the lower excretion observed in the diabetic males is probably related to the infertility and lack of sexual development in this model [13].

The period between 10 weeks and 16 weeks of age in the diabetic mice is characterized by a gradual decline of insulin levels to normal, coupled with a continued high rate of gluconeogenesis [14]. Many of the aldehyde metabolites were still excreted at elevated levels, while the ketones (with the exception of 2-heptanone) are found at lower-than-normal levels. Other metabolites, such as linalool, 2-*sec.*-butylthiazoline or N-acetylpyrrole were found at consistently lower or higher amounts than normal. The relationships between the altered excretion of linalool and N-acetylpyrrole and the diabetic state have not yet been firmly established, although certain pyrrole derivatives, including N-acetylpyrrole have been reported as increased in alloxan and streptozotocin diabetic rats [6, 7].

Around 16 weeks of age, the drop in insulin secretion in the diabetic mice is pronounced and the animals cease to gain weight. Increased gluconeogenesis and reduced glucose oxidation combine to yield continued severe hyperglycemia and the condition of the animals rapidly deteriorates. The urinary volatile profiles obtained during the last stages of the syndrome retain the characteristic elevation of several aldehydes, phenylacetone, peak 50, and indole. Correspondingly, the excretion of linalool, 2-*sec.*-butylthiazoline, 3-hepten-2-one and 6-methyl-6-hepten-2-one remains depressed. However, it is worthwhile to note that the excretion of some of the previously depressed branched-chain and/or unsaturated ketones (such as 5-hepten-2-one) appeared at near normal levels. This corresponds with the low insulin secretion and increased fat catabolism occurring in this stage [14].

In examining the consistent differences in the excretion of volatile metabolites in the normal and diabetic mice throughout the study, the most striking abnormalities were the increased excretion of a number of aldehyde metabolites, N-acetylpyrrole, 5-methylfurfural and indole in the diabetics. Equally dramatic were the initially lower levels of branched-chain and/or unsaturated ketones and the consistently low levels of linalool and 2-*sec.*-butylthiazoline found in the diabetic animals. The alterations in the excretion of these

compounds appear to be characteristic for the diabetic syndrome in this model.

The possible metabolic relationships between the excretion of most of these compounds and the diabetic syndrome have already been discussed, but it is important to consider possible physiological effects of increased levels of these compounds in the diabetic animals. Long-term diabetes in man is associated with many pathological complications including neuropathy, retinopathy, early development of vascular and circulatory difficulties and renal failure [20]. The metabolic abnormalities underlying these degenerative processes are generally poorly understood and considerable research is currently focussed in these areas. A recent report has suggested that patients with metabolic neuropathies such as diabetic polyneuropathy may produce abnormal neurotoxic metabolites which may be involved in the pathogenesis of the disorder [21]. Other reports suggest that increased lipid peroxidation may account for degenerative changes in the retina of diabetics [22] or contribute to other diabetic complications [12].

In this regard, the elevation of the aldehyde metabolites observed in the diabetic animals deserves further consideration. Such or structurally similar compounds, which have been reported to be products of lipid peroxidation [18], have also been reported as having cytotoxic effects as well as the ability to inhibit S-adenosylmethionine decarboxylase activity [23], and to alter the excitability and conduction properties of isolated nerves [24]. Further investigation is certainly warranted regarding the possible toxic effects of chronically elevated blood levels of such compounds, especially with reference to the later complications of diabetes.

Organic acid metabolites

Alterations in urinary organic acid excretion due to the diabetes in the mouse can be clearly observed in Figs. 3 and 4 (representative chromatograms). In Fig. 3 which presents chromatographic profiles of the urinary organic acids of a normal and diabetic mouse at 5 weeks of age, clear differences in the excretion of these metabolites are already apparent, although the blood glucose values for the diabetics are only slightly above normal. By 10 weeks of age (Fig. 4), the excretion of organic acid metabolites has increased tremendously in the diabetics; insulin levels are elevated and severe hyperglycemia is present. This dramatic increase in the excretion of organic acids continues until at least 24 weeks of age, as can be ascertained by examining the contents of Table II. The data in Table II are presented in the same manner as those of Table I: the average peak area for each of the numbered peaks in the diabetic profiles is given as the percent of the corresponding mean peak area from the normal profiles.

No significant reduction in the excretion of acid metabolites was observed in the diabetic animals. Instead, a general increase in the excretion of most acid metabolites was observed by 6 weeks of age. There are differences in the degree of elevated excretion observed; in some cases, such as with glyoxylic acid, the acid is excreted in amounts two to three times those of the normal animals. Other acids, such as 3-hydroxypropionic acid are found at levels 50—100 times higher than normal throughout the study.

At 5 weeks of age, significantly higher levels of several intermediates of

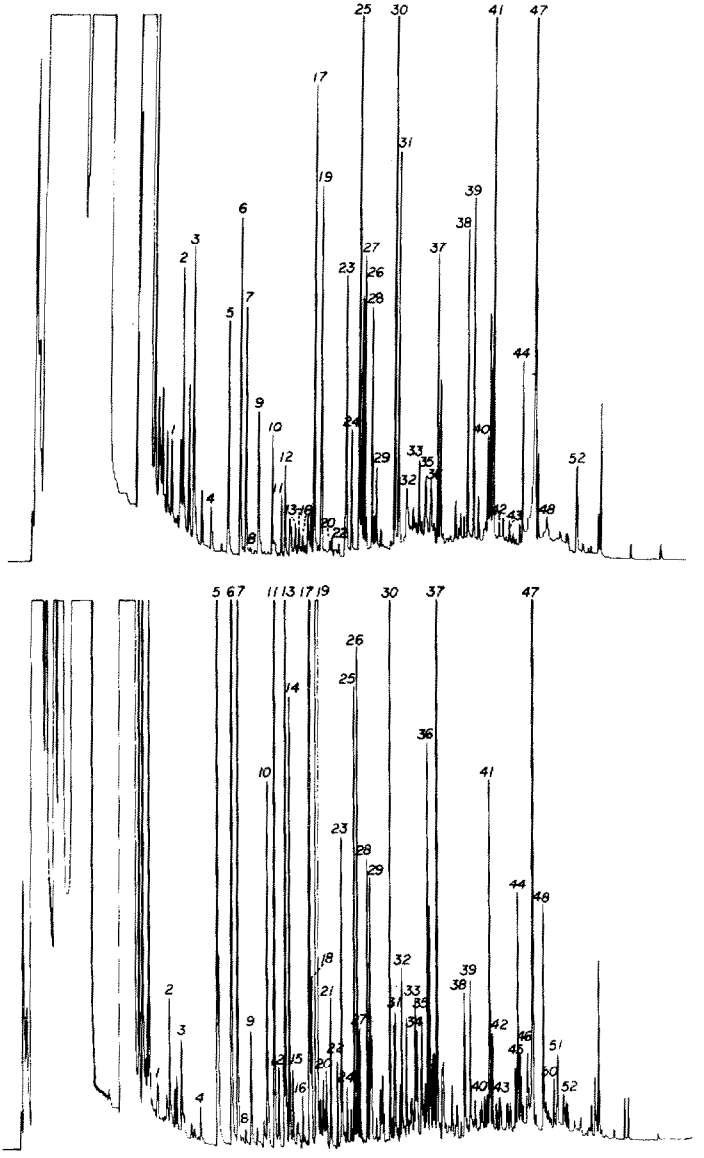

Fig. 3. Urinary organic acid profiles of normal (top) and diabetic (bottom) mice at 5 weeks of age. Peak numbers correspond to the identified metabolites of Table II.

glucose metabolism were observed; lactate, pyruvate, glyceraldehyde, dihydroxyacetone, 3-hydroxypropionate and glycerate are all elevated to levels 10—60 times normal. As blood glucose continues to rise in the diabetics, these metabolites are excreted in continually increasing amounts and, with the exception of lactate, pyruvate and 3-hydroxypropionate, reach peak levels between 7 and 10 weeks of age. Lactate, pyruvate and 3-hydroxypropionate are found at progressively higher levels until 10 weeks of age, reflecting the persistently high rate of gluconeogenesis in the diabetics [14].

A trio of tentatively identified furanoic acids are found among the acids in

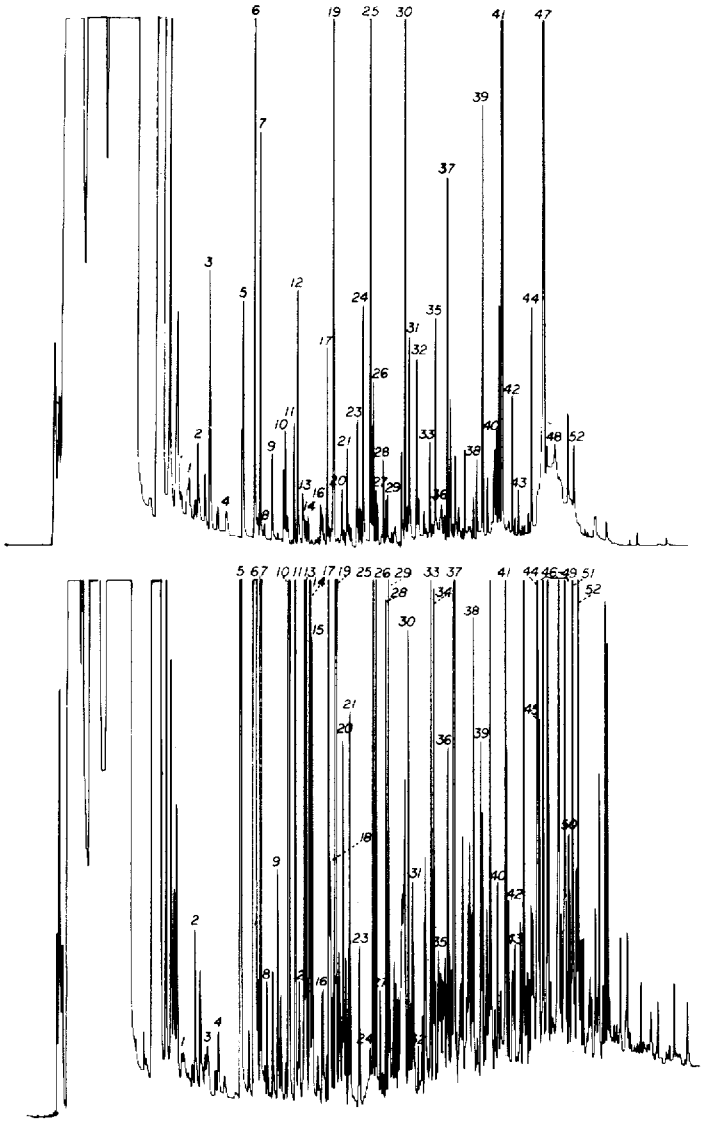

Fig. 4. Urinary organic acid profiles of normal (top) and diabetic (bottom) mice at 10 weeks of age. Peak numbers correspond to the identified metabolites of Table II.

urine (which are only slightly elevated in the diabetics at 5 weeks); however, these were later found in concentrations 40 times greater than normal. These derivatives probably arise from the metabolism of glucuronic acid [23] and ultimately from glucose. Their elevation roughly parallels the rising blood glucose levels in the diabetic animals as would be expected.

Several acids which are elevated during the middle stages of the diabetic syndrome (oxaloacetic, succinic, aconitic, 2-hydroxyglutaric acid), or throughout the entire study (malic, 2-ketoglutaric acid) are intermediates in the tricarboxylic acid cycle. Since the early and middle stages of the diabetic state are associated with increases in the activities of insulin-dependent enzymes such as

TABLE II

SIGNIFICANT DIFFERENCES IN THE URINARY EXCRETION OF ORGANIC ACIDS IN DIABETIC MICE RELATIVE TO AGE-MATCHED CONTROLS

Values presented with diabetic as percentage of mean age-matched control value ± the standard error. Peak numbers correspond to those of Figs. 3 and 4.

Peak No.	Structural identification	Age (weeks)		
		5	6	7
2	Glyoxylate	250 ± 78		780 ± 570
4	Phenol	398 ± 66	205 ± 62	222 ± 83
5	Pyruvate	1740 ± 890	3130 ± 630	3930 ± 640
6	Lactate	921 ± 353	1810 ± 1160	1380 ± 220
7	Glycolate	705 ± 140	1300 ± 527	1950 ± 750
9	Levulinate*	289 ± 64	1820 ± 700	940 ± 570
10	2-Hydroxybutyrate	581 ± 250	2220 ± 410	1360 ± 380
11	3-Hydroxypropionate	6970 ± 2430	8600 ± 1990	8550 ± 1690
13	Unidentified	3320 ± 2200	5270 ± 710	3920 ± 1990
14	Unidentified		2428 ± 920	5790 ± 2600
17	Glyceraldehyde I	3090 ± 780	10312 ± 1700	7480 ± 1600
19	Glyceraldehyde II	5920 ± 1480	9893 ± 1790	8550 ± 2230
20	Unidentified	1290 ± 430	643 ± 140	2303 ± 1180
21	Dihydroxyacetone*	3210 ± 1650	3390 ± 1300	3460 ± 1350
23	Oxalacetate*	463 ± 116		496 ± 187
25	Succinate		186 ± 42	
26	3-Hydroxy-2-furoate*		1360 ± 840	4670 ± 2150
28	4-Hydroxy-2-furoate*	228 ± 98	1930 ± 890	727 ± 310
29	Glycerate	960 ± 470	2740 ± 880	4030 ± 1320
31	Unidentified	168 ± 48	930 ± 320	411 ± 103
32	3-Hydroxy-5-methyl-2-furoate*	270 ± 120	1021 ± 760	406 ± 160
33	2-Methylglycerate	410 ± 145	620 ± 63	575 ± 150
35	Methylcatechol	401 ± 91	981 ± 362	659 ± 300
36	Unidentified	1580 ± 407	3145 ± 1400	1521 ± 390
37	Malate	767 ± 450	3575 ± 380	1000 ± 200
38	2-Ketoglutarate	329 ± 190	2255 ± 1060	1320 ± 610
39	2-Hydroxyglutarate*			
40	*o*-Ethylhydroxybenzoate	211 ± 54	1050 ± 690	1250 ± 900
41	Atrolactate*			394 ± 95
43	3,5-Dihydroxy-2,4-heptadienoate*			733 ± 141
45	Catechol		2250 ± 390	2790 ± 1160
47	Aconitate			499 ± 261

Additional identified peaks exhibiting no significant differences.

1	Acetophenone	34	2,3-Dihydroxybutyrate*
3	2,3-Butanediol	42	2-Ketoglutarate
8	2-Ketobutyrate	44	3-Hydroxypentanedioate*
12	Pyruvate	46	Vanillate
15	3-Furoate*	48	3-Deoxyhexuronic acid derivative*
16	5-Methyl-2-furoate*	49	Pyrogallol
18	Benzoate	50	Hippurate
22	3-Hydroxy-2-hydroxyethylfuran	51	Protocatecheate
24	Glycerol	52	Citrate
27	2-Methylglycerol*		
30	*p*-Chlorobenzoate, internal standard		

*Tentative identification.

glucokinase, glucose-6-phosphate dehydrogenase, citrate lyase and acetyl-CoA synthetase and, hence, glucose oxidation [13, 25], it is not surprising to find abnormally high levels of the Krebs cycle intermediates in the urine. By 18 weeks of age, insulin levels have fallen, and lower levels of glycolytic and pentose-phosphate shunt enzymes are found. Correspondingly, normal urinary levels of oxaloacetate, succinate and 2-hydroxyglutarate were observed at this time.

8	10	12	16	20	24
232 ± 94	287 ± 160	245 ± 105	289 ± 61	330 ± 66	
345 ± 115	429 ± 176			259 ± 87	
3500 ± 1350	3860 ± 950	2790 ± 1000	2250 ± 730	5370 ± 580	1630 ± 440
3900 ± 1600	5280 ± 3160	1200 ± 500	7510 ± 3790	22100 ± 5300	5920 ± 2600
2450 ± 940	4270 ± 2080	1900 ± 420	4748 ± 2200	6150 ± 1050	1750 ± 670
1210 ± 440	741 ± 200	527 ± 146	286 ± 98	288 ± 70	
1770 ± 590	3520 ± 1400	1770 ± 660	5240 ± 1700	9340 ± 4600	1940 ± 1200
8540 ± 1500	8240 ± 3200	5360 ± 1640	4510 ± 1150	9100 ± 710	1280 ± 370
5460 ± 1700	2830 ± 1570	1030 ± 460	2640 ± 500	8490 ± 4210	
11200 ± 6000	22900 ± 9900	5060 ± 2200	11500 ± 3500	25300 ± 5200	
6240 ± 600	4430 ± 2200	3690 ± 2300	1940 ± 860	8820 ± 1600	386 ± 120
5340 ± 400	3530 ± 1800	3000 ± 2100	1420 ± 670	8560 ± 2200	1406 ± 650
3870 ± 2600	2920 ± 1970	464 ± 251	1960 ± 490	3522 ± 2000	
1620 ± 450	793 ± 315	613 ± 320	370 ± 115	1301 ± 240	209 ± 88
470 ± 170	534 ± 186	376 ± 150	288 ± 160		
240 ± 88	264 ± 75	473 ± 258	154 ± 45		
4310 ± 2500	2110 ± 660	1800 ± 670	4960 ± 3200	2530 ± 750	422 ± 180
745 ± 180	1390 ± 727	786 ± 360	815 ± 400	1340 ± 35	354 ± 160
4230 ± 870	4480 ± 1170	1520 ± 420	6810 ± 2200	6760 ± 820	690 ± 250
880 ± 230		490 ± 210	234 ± 107	240 ± 30	197 ± 82
1420 ± 700	237 ± 64	332 ± 105	660 ± 190	1800 ± 1000	
732 ± 250	967 ± 319	602 ± 150	1410 ± 680	1170 ± 200	895 ± 380
351 ± 146	219 ± 55	254 ± 93	185 ± 53	915 ± 35	
1610 ± 230	2480 ± 570	4280 ± 1800	2510 ± 400	4070 ± 620	653 ± 190
2530 ± 540	2700 ± 300	3300 ± 1400	2930 ± 870	3290 ± 310	829 ± 430
1410 ± 390	1350 ± 270	2100 ± 700	1150 ± 570	1060 ± 510	252 ± 100
420 ± 260	556 ± 250	478 ± 190			
1050 ± 760	414 ± 186	847 ± 199		840 ± 300	1144 ± 800
716 ± 150	503 ± 112	710 ± 193	358 ± 115	1030 ± 170	
1160 ± 390	325 ± 128	391 ± 104	2460 ± 1900	1680 ± 300	
1850 ± 430	878 ± 217	1130 ± 700	3780 ± 1000	5400 ± 1800	
353 ± 120		352 ± 186	291 ± 98	442 ± 125	

It should be noted that no ketone bodies or other acids derived specifically from increased fat oxidation were found to be elevated in the diabetic urine. This finding is in agreement with reports of decreased lipolytic acitivity and enhanced lipogenesis in the diabetic mice as a result of high insulin levels [16].

The remainder of the compounds which have been identified and which are elevated in diabetic urine are phenolic compounds (phenols, catechol, methylcatechol) or aromatic acids (O-ethylhydroxybenzoic, atrolactic acid). Addition-

ally, several similar compounds were found exclusively in the urine of diabetic mice, including vanillate, pyrogallol and protocatechuate. These compounds are probably intermediates in the catecholamine metabolism although it is not clear if plasma catecholamines are elevated in the diabetic mouse. Studies of norepinephrine levels in isolated organs innervated by sympathetic nerves revealed significantly reduced concentrations in the heart, kidney and salivary glands of older diabetic mice, but normal concentrations in the spleen and adrenal glands [26]. However, a massive elevation of monoamine oxidase, an enzyme that degrades norepinephrine to inactive products, was found in the kidneys of older diabetic mice [26]. It is possible that the elevated levels of degradation products of catecholamines observed in diabetic urine may be related to enhanced catecholamine catabolism. Alternatively, the inappropriately high glucagon levels found in the diabetic mice [14] may stimulate the metabolism of tyrosine and phenylalanine through the induction of tyrosine dehydroxylase [27], thus contributing to the appearance of phenolic metabolites in the urine.

In either case, a significant consideration in the observation of elevated urinary levels of phenolic metabolites is the possible consequence(s) of correspondingly elevated plasma levels of these toxic compounds. Recently, the elevation of several phenolic and polyphenolic acids has been reported in analyses of uremic hemofiltrate [28, 29]. These studies have commented on the general toxicity of such compounds and their possible involvement in the pathogenesis of such uremic symptoms as central nervous system dysfunction, anemia, and impaired blood coagulation. Curiously, a polyneuropathy virtually identical to diabetic polyneuropathy in its course develops in cases of severe uremia and alcoholism [30]. A toxic assault on the nerve has been implicated in the etiology of both neuropathies, although the toxin(s) responsible have yet to be identified. The further investigations of the elevation of phenolic acids in urine and in plasma of diabetic subjects would seem to be warranted in this regard.

Although further interpretation of the urinary organic acid data was hampered by difficulties in the unambiguous assignment of structures from the mass spectra of trimethylsilyl derivatives, the trends in the excretion of these compounds in the normal and diabetic mouse are fairly evident and will serve to direct future investigations.

CONCLUSIONS

As indicated in the introduction, the intent of this paper has been to go beyond the mere reporting of observed differences in the excretion of certain urinary metabolites among normal and diabetic mice. This investigation has also attempted to relate observed differences in the excretion of certain urinary metabolites to known metabolic abnormalities of the diabetic animals and to examine the data in terms of possible implications of elevated levels of some potentially toxic metabolites. In particular, the observed increased urinary levels of several aldehyde and phenolic metabolites warrant further investigation with regard to possibly elevated blood levels and the toxic effects of such compounds.

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