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EXCRETION OF URINARY VOLATILE METABOLITES IN RESPONSE TO ALLOXAN INDUCED DIABETES OF SHORT DURATION IN RATS

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SUMMARY

Alterations in urine volatile metabolites due to the induction of alloxan diabetes in the rat were examined by capillary gas chromatography and gas chromatography—mass spectrometry for the five days immediately following the onset of chronic hyperglycemia. Elevations of a number of metabolites were observed including several short chain ketones, acetophenone, 2-acetylfuran and indole. The value of urine volatile metabolic profiles as characteristic indicators of the diabetic condition is demonstrated through profiles obtained from a diabetic animal which spontaneously reverted to normal.

INTRODUCTION

Complex metabolic pathways altered by a disease state can often be followed quantitatively by modern chromatographic methods. This approach provides simultaneous multicomponent analysis of different classes of metabolically important constituents of physiological fluids. Among various body fluid constituents, volatile metabolites of relatively low molecular weight have been of recent interest [1—3]. The volatile metabolites include by-products, intermediates and terminal products of a variety of metabolic processes. However, this fact itself does not diminish their potential importance as valuable

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indicators of certain metabolic pathways altered by a disease condition. Structurally, the volatile fraction of urine and serum is mostly composed of small organic neutral metabolites such as ketones, aldehydes, alcohols, terpene compounds, furan and pyrrole derivatives, as well as other small heterocyclic substances.

As several reports indicate [4—9], small organic (volatile) molecules may be of special importance in diabetic conditions as useful indicators of the associated metabolic abnormalities. For example, 4-heptanone and certain aliphatic alcohols [6, 7] have been shown to be associated with diabetes mellitus in humans; some other types of molecules are suspect [4, 8, 9]. An accurate assessment of the importance of additional metabolites in human diabetes is complicated by variations in genetic background, diet, medication, and other exogenous factors; however, advanced computational techniques designed for the treatment of multicomponent analytical data may simplify this complex problem [8—10].

Despite these studies, however, knowledge concerning the metabolic origin of these small neutral organic metabolites and their relationship to the characteristically altered metabolic conditions of diabetes mellitus is relatively incomplete and further investigations are warranted.

One useful approach to a better understanding of volatile metabolism and its correlation with diabetes mellitus is the utilization of suitable diabetic animal models. Using such animal models, metabolic circumstances and manipulations which are not practically feasible with human subjects may be examined. Additionally, since genetic and other exogenous factors (diet, age, etc.) can be carefully controlled, experiments with diabetic animal models can be carried out relatively free from the large normal variation encountered with human subjects.

Toward that end, the present study reports on the effect of diabetes mellitus, of a short-term duration, on the urinary volatile metabolites of alloxan-diabetic rats. The alloxan-diabetic rat has long been considered a valuable model of human diabetes mellitus [11] and our experience has shown that humans and rats share many common volatile metabolites [12]. Since the severity and onset of disease can be carefully controlled [11], we have been able to acquire comparative qualitative and quantitative data on the alteration of urinary volatile metabolites in alloxan-diabetic rats referenced to suitable control animals, immediately following the chemical induction of diabetes mellitus. In a previous report [13], we examined the effect of long-term disease duration on the volatile urinary metabolic profiles of alloxan-diabetic rats.

MATERIALS AND METHODS

Sprague-Dawley male rats (Harlan Industries, Indianapolis, IN, U.S.A.) weighing between 150 and 170 g were utilized in the described experiments. The rats were divided into two groups of eight animals each. One group received a single injection, after a 24-h fast, of 40 mg/kg alloxan (alloxan monohydrate; Sigma, St. Louis, MO, U.S.A.) in saline solution. Solution concentrations were adjusted to keep injection volumes of approximately 1 ml. Control rats received a single injection of 1 ml of saline solution. All injections were

intravenous, using the tail vein of the rat. Blood glucose values were measured with a Beckman Glucose Analyzer. Values were determined at 24 and 48 h after alloxan injection and every other day thereafter for one week. No insulin or other treatment was utilized in the maintenance of these animals. Normal rat chow (Labblox, Wayne Feeds, Bloomington, IN, U.S.A.) and water were supplied ad libitum throughout the duration of experiments. During the subsequent urine collection, the blood glucose values for the control animals ranged between 70—100 mg per 100 ml, while those for the diabetic animals were between 429—579 mg per 100 ml.

Twenty-four-hour urine samples were collected from the uncontrolled diabetic animals beginning at the onset of chronic hyperglycemia, and continued for five consecutive 24-h periods. Twenty-four-hour urine samples were also collected from each of the eight control animals. The urine samples were collected in polycarbonate metabolism units (Maryland Plastics, Federalsburg, MD, U.S.A.). Interim samples were kept frozen over dry ice during urine collection. The samples were then brought to room temperature and immediately filtered, divided into suitable aliquots, and refrozen at −20°C until analysis. No preservatives were used.

Analysis of the volatile constituents of the urine samples was accomplished utilizing a headspace concentration method followed by automated glass capillary gas chromatography (GC). The procedures and instrumentation utilized for the acquisition of these metabolic profiles have been previously described [14]. In the only modification of prior procedures, the thermostated sampling bottles were scaled down to accommodate a smaller sampling volume of 2 ml of urine.

Reproducibly prepared glass capillary columns (60 m × 0.25 mm I.D.) coated with 0.2% UCON-50—HB-2000 (Applied Science Labs., State College, PA, U.S.A.), a polar stationary phase, containing 0.015% benzyltriphenylphosphonium chloride (BTPPC) as surfactant were employed for chromatographic analysis. Separation was carried out on 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 (Perkin-Elmer, PEP-2). All samples were run at a flow-rate of 2 ml/min and temperature programmed from 28—160°C as indicated in Figs. 1—4. Quantitative variations of volatile metabolites were calculated from the integrated peak areas of each chromatogram from the individual diabetic and control animals.

Identification of volatile constituents was accomplished using the same glass capillary GC column attached to either a low- or high-resolution mass spectrometer. Low-resolution electron impact ionization spectra were obtained at 70 eV with a Hewlett-Packard Model 5980A combined gas chromatograph—dodecapole mass spectrometer—computer system. The glass capillary column was directly interfaced with the mass spectrometer ion source (maintained at 220°C). All spectra were recorded at scan rates of 100 a.m.u./sec. Capillary GC—high-resolution mass spectrometry was performed on a Varian-MAT 311A instrument. Electron impact ionization spectra were obtained at 70 eV at a scan rate of 5 sec/decade from *m/e* 47—210. High-resolution mass spectral data were recorded with an SS-1000 data system (Varian). Chromatographic con-

TABLE I

EFFECTS OF ALLOXAN DIABETES OF SHORT-TERM DURATION ON THE CONCENTRATION OF RAT URINARY VOLATILE CONSTITUENTS

Animals were followed for five days after the onset of chronic hyperglycemia. Concentrations reported as percent of the control value ± S.E. NS = not significant.

Compound	Peak	Control	Day				
			1	2	3	4	5
2-Pentanone	A	100.0 ± 8.3	279.0 ± 21.2	268.0 ± 17.2	328.2 ± 24.1	311.0 ± 28.6	380.6 ± 30.4
2-Hexanone	B	100.0 ± 5.6	167.3 ± 7.2	269.4 ± 19.4	203.1 ± 16.3	270.4 ± 22.6	210.6 ± 14.6
3-Penten-2-one	C	100.0 ± 7.8	NS	NS	245.6 ± 17.3	274.8 ± 23.4	184.3 ± 13.8
4-Heptanone	D	100.0 ± 10.3	168.0 ± 12.6	192.0 ± 11.9	210.0 ± 10.3	237.0 ± 18.1	210.0 ± 25.2
2-Heptanone	E	100.0 ± 12.4	161.3 ± 13.4	183.4 ± 16.3	195.6 ± 14.6	270.3 ± 15.1	200.4 ± 10.2
5-Hepten-2-one	F	100.0 ± 13.2	234.3 ± 36.4	318.4 ± 40.3	626.0 ± 50.2	481.0 ± 48.3	421.1 ± 39.2
3-Ethyl-4-methyl-2-hexanone	G	100.0 ± 12.1	248.8 ± 23.2	315.4 ± 20.2	359.7 ± 23.9	405.6 ± 32.8	398.4 ± 34.2
2-Acetylfuran	N	100.0 ± 11.2	242.3 ± 18.6	274.7 ± 20.6	638.1 ± 23.4	362.3 ± 22.6	348.2 ± 28.3
Acetophenone	R	100.0 ± 9.4	NS	NS	240.3 ± 18.9	270.2 ± 16.2	252.1 ± 23.6
4-Phenyl-2-butanone	U	100.0 ± 14.3	382.7 ± 26.2	504.5 ± 34.9	1761.3 ± 50.6	918.8 ± 41.2	412.1 ± 25.6
Indole	W	100.0 ± 8.6	290.3 ± 16.4	483.2 ± 18.5	362.1 ± 20.4	215.1 ± 14.6	260.3 ± 21.2
2-Heptenal*	H						
6-Methyl-5-hepten-2-one*	I						
Furfural*	J						
5-Methyl-5-octen-4-one*	K						
3-Octen-2-one*	L						
Pyrrole*	M						
Benzaldehyde*	O						
N-Acetylpyrrole*	P						
3-Methyl-N-acetylpyrrole*	Q						
4-Methylthio-2-butenal*	S						
Nitrogen compound ($C_{16}H_{21}NO$)*	V						

*Identified metabolite not significantly altered from normal.

ditions were the same as above. Proposed structures were verified through comparison of mass spectra and retention times of authentic compounds.

RESULTS AND DISCUSSION

The methodological aspects of urinary headspace analysis [14, 15] have been previously established, indicating the validity of the method for the routine analysis and comparison of volatile metabolic profiles. The data presented here represent the average variations of urinary volatile metabolites from eight alloxan-diabetic rats, referenced to suitable control animals.

Figs. 1, 2, and 3 illustrate typical metabolic profiles of the volatile urinary compounds of an alloxan-treated rat followed for five days after the onset of hyperglycemia (day 1 corresponds to chromatogram 1, day 2 to chromatogram 2, etc.); the profile of a control animal is also shown (Fig. 1, C). While minor variations may occur among the individual animals, the chromatograms displayed are representative of the profiles obtained in the study.

Table I lists the identified volatile metabolites, along with the peak numbers in Figs. 1—3 to which they correspond. Also shown in Table I are the average percent variations from normal of the urinary volatile metabolites of the

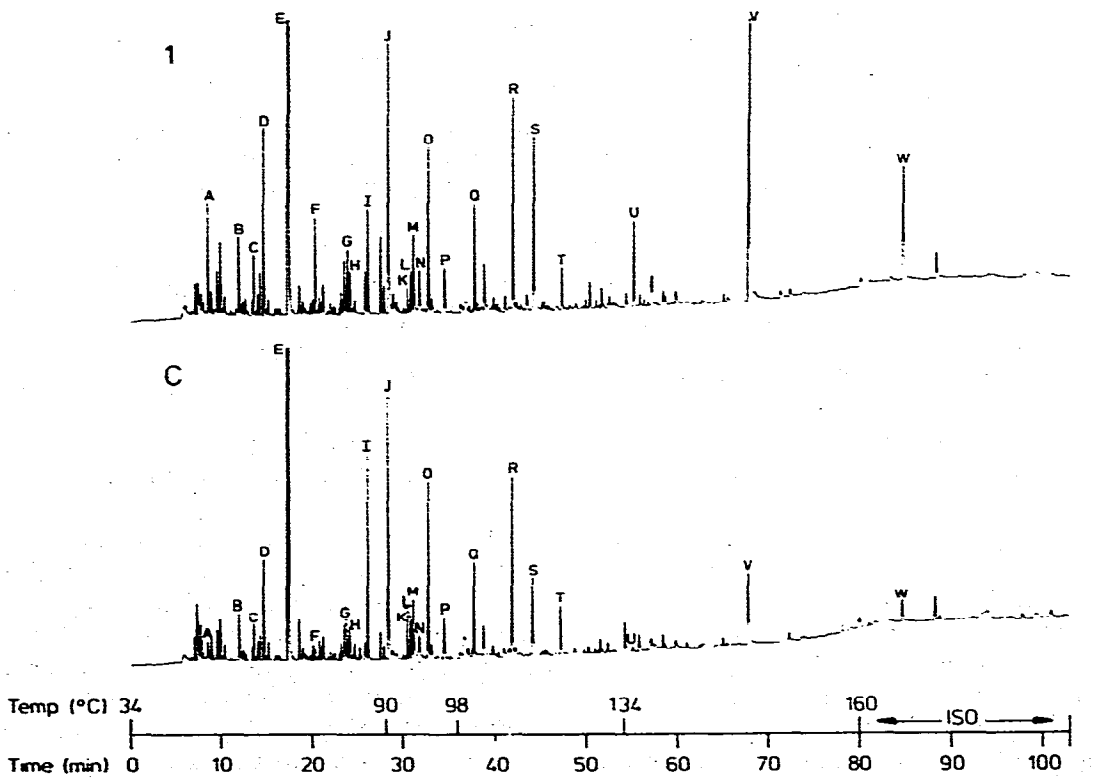

Fig. 1. Urinary volatile chromatograms of a control (C) and alloxan-diabetic rat 1 day after the onset of chronic hyperglycemia (1). Chromatographic conditions were as described in the text and as indicated in the temperature scale. Identified chromatographic peaks are listed in Table I.

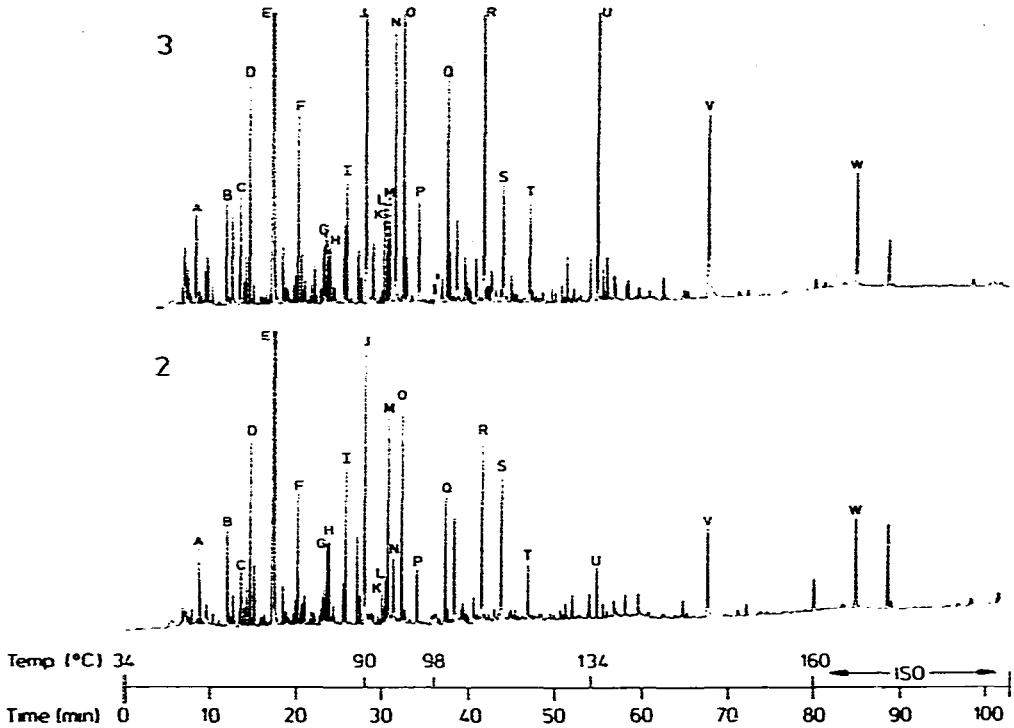

Fig. 2. Urinary volatile chromatograms of an alloxan-diabetic rat 2 (2) and 3 (3) days after the onset of chronic hyperglycemia. Chromatographic conditions were as described in the text and as indicated in the temperature scale. Identified chromatographic peaks are listed in Table I.

diabetic animals for each of the five days examined following induction of disease. Quantitative data are displayed only for those compounds which were judged significantly altered from control values (Student's *t*-test, $p < 0.05$). The data were calculated as the percent of control average (i.e., 100 implies the equivalence of the average diabetic and control values for a given metabolite).

While we observed no consistent qualitative differences, a number of demonstrably consistent quantitative changes from normal can be ascertained from Figs. 1—3 and Table I.

The most obvious changes from normal in the urine of alloxan-diabetic rats examined here occur in the concentration of several aliphatic ketones. There was an immediate and sustained elevation in the concentration of these metabolites over the full 5-day period examined. Previous analytical results with human subjects suffering from diabetes mellitus [6—9] indicated increased urinary levels of 4-heptanone and 2-heptanone; these results are corroborated in our animal study. Additionally, a number of other aliphatic ketones are elevated in the urine of alloxan-diabetic rats to a similar extent as 4- and 2-heptanone, demonstrating, at least under the metabolic conditions examined here, a general trend rather than a phenomenon restricted to one or two metabolites. This general increase in ketone metabolites was observed in each animal examined in this work. Similar results have not been reported with human

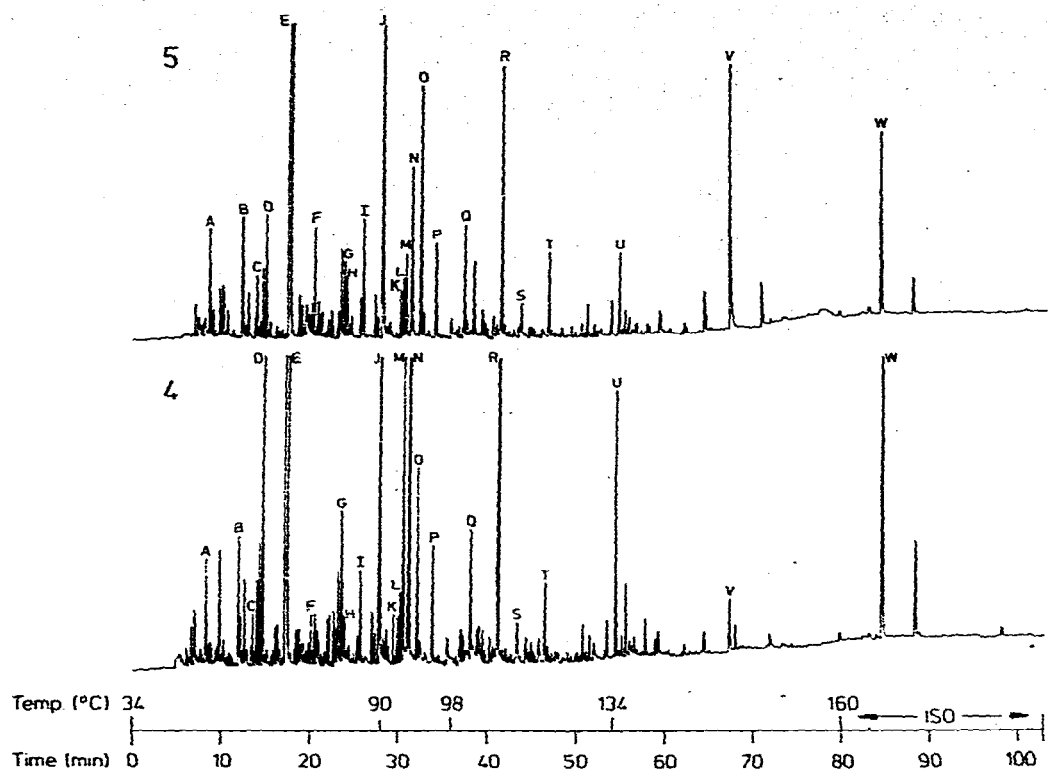

Fig. 3. Urinary volatile chromatogram of an alloxan-diabetic rat 4 (4) and 5 (5) days after the onset of chronic hyperglycemia. Chromatographic conditions were as described in the text and as indicated in the temperature scale. Identified chromatographic peaks are listed in Table I.

subjects, although such studies have involved patients on dietary or medicinal control, and thus a metabolic state different from the animals examined here. The observation of severe uncontrolled alloxan diabetes in the rat, with a minimum of exogenous variables, undoubtly maximizes volatile metabolite alterations allowing a clear pattern to be discerned.

The aliphatic ketones are thought to arise from the decarboxylation of keto-acids, in a manner similar to the well known formation of acetone. This seems reasonable, since the increase in concentration of the urinary aliphatic ketones observed in this study correlates with the temporary elevation of free fatty acids and ketone bodies known to occur in alloxan-diabetic rats shortly after disease induction [11]. Further support for keto-acids and fat metabolism as the source of urinary aliphatic ketones was provided by a previous report from this laboratory [13], which demonstrated greatly reduced urinary aliphatic ketone levels in uncontrolled alloxan-diabetic rats of long-term disease duration (2—12 months) and the correlation of these results to the markedly reduced fat stores in the long-term uncontrolled diabetic animals.

Interestingly, the only urinary ketones not elevated in the short-term alloxan-diabetic animals examined here are of longer chain length and are unsaturated. No explanation can be offered for this at present although similar results have also been observed with human diabetics [9].

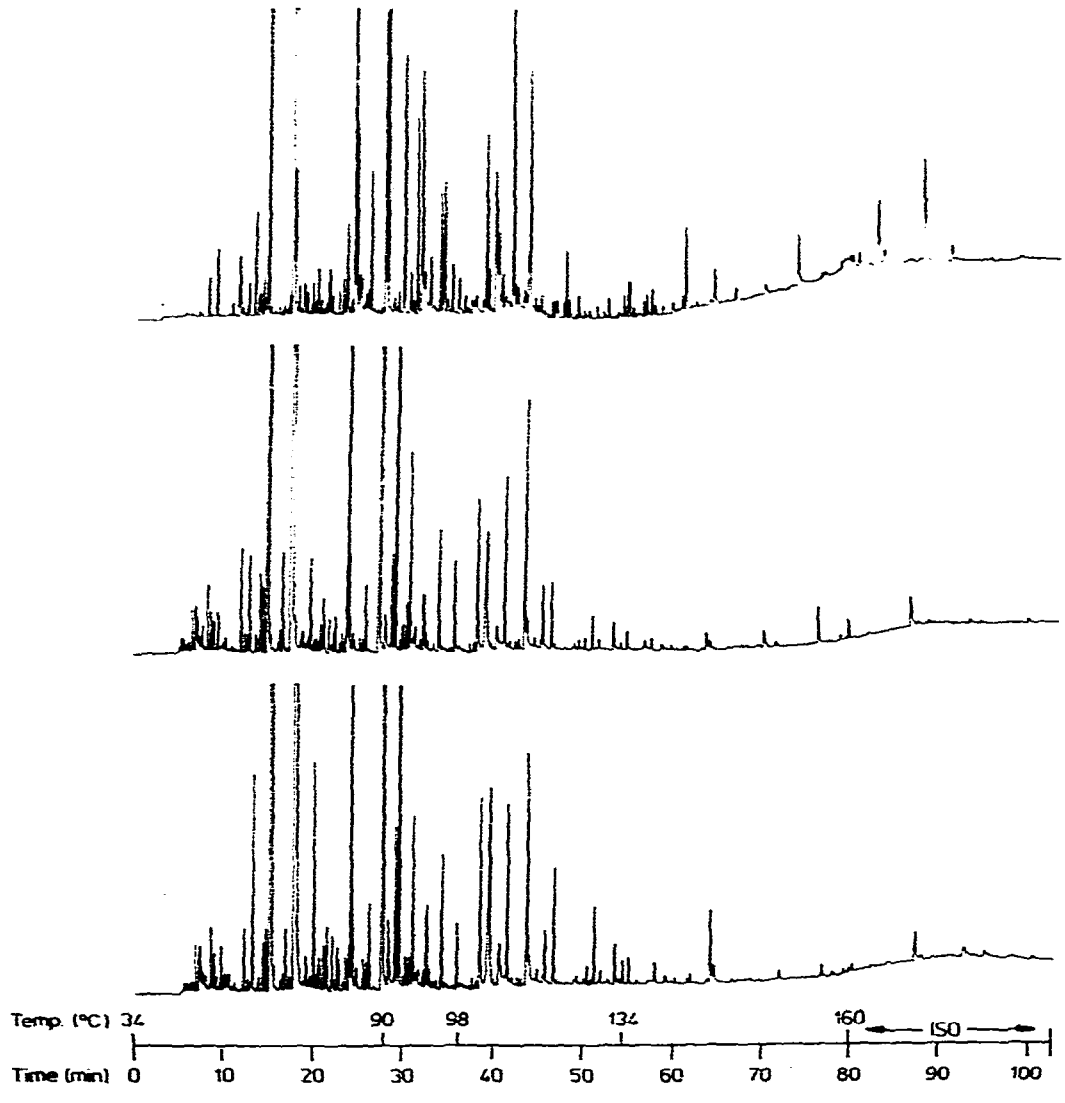

Fig. 4. Urinary volatile metabolite chromatograms of an 8-month experimentally diabetic rat (top), and that same animal after spontaneous reversion to normal at 12 months (middle). Also shown is the profile of a 12-month control animal (bottom). Chromatographic conditions were as described in the text and as indicated in the temperature scale.

The increased urinary level of indole observed in this study has also been reported in long-term alloxan-diabetic rats and human diabetics [8, 9, 13]. Indole is probably formed from L-tryptophan [16], and the well-documented increase in protein catabolism encountered with alloxan-diabetic rats and human diabetics would account for its increased levels.

Acetophenone has also been reported as being elevated in concentration in the urine of long-term alloxan-diabetic rats [13]. Acetophenone is known to arise from phenyl-β-oxypropionic acid, which is in turn formed from phenylpropionic acid and, possibly, cinnamic acid [16]. These latter substances may

also be elevated in concentration in the urine of alloxan-diabetic rats.

2-Acetylfuran and 4-phenyl-2-butanone were also observed to be increased in concentration in the urine of short-term alloxan-diabetic rats. The metabolic source of these compounds is obscure at present.

The urine volatile metabolic profiles obtained for the short-term alloxan-diabetic and the long-term diabetic animals examined in a previous report [13] are easily distinguished from those of normal rats and appear to be indicative of the metabolic states of these animals. This finding is supported by Fig. 4, which displays chromatograms of an interesting case in which an uncontrolled experimentally diabetic rat spontaneously reverted to normal between 8 and 12 months after disease induction. Spontaneous reversion to normal may sometimes occur due to regeneration of pancreatic β-cells. The top chromatogram displays the urinary volatile profile of the animal at 8 months, at which time blood glucose (539 mg per 100 ml), weight (200 g), and urine volume indicated that the animal was clearly diabetic. The middle chromatogram displays the volatile profile of this same animal after reversion to near normal blood glucose (123 mg per 100 ml) and body weight (420 g). The lower chromatogram is that of a 12-month control rat. The 8-month profile of this diabetic rat displays the diabetic profile consistent with the previous findings, but the 12-month profile following reversion to normal, is almost identical to the normal control.

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REFERENCES

1 A. Zlatkis and H.M. Liebich, Clin. Chem., 17 (1971) 592.
2 L. Pauling, A.B. Robinson, R. Teranishi and P. Carey, Proc. Nat. Acad. Sci. U.S., 68 (1971) 2374.
3 M. Novotny, M.L. McConnell, M.L. Lee and R. Farlow, Clin. Chem., 20 (1974) 1105.
4 A. Zlatkis, W. Bertsch, H.A. Lichtenstein, A. Tishbee, F. Shunbo, H.M. Liebich, A.M. Coscia and N. Fleischer, Anal. Chem., 45 (1973) 763.
5 H.M. Liebich and O. Al-Babbili, J. Chromatogr., 112 (1975) 539.
6 H.M. Liebich, J. Chromatogr., 112 (1975) 551.
7 H.M. Liebich, O. Al-Babbili, A. Zlatkis and K. Kem, Clin. Chem., 21 (1975) 1294.
8 M.L. McConnell, G. Rhodes, U. Watson and M. Novotný, J. Chromatogr., 162 (1979) 495.
9 G. Rhodes, M. Miller, M.L. McConnell and M. Novotný, Clin. Chem., 27 (1981) 580.
10 A. Zlatkis, K.Y. Lee, C.F. Poole and G. Holzer, J. Chromatogr., 163 (1979) 125.

11 C.C. Rerup, Pharmacol. Rev., 22 (1970) 485.
12 M. Novotný, unpublished results.
13 G. Rhodes, M. Holland, D. Wiesler, M. Novotný, S.A. Moore, R.G. Peterson and D.L. Felten, Experientia, (1982) in press.
14 A. Zlatkis, R.S. Razell and C.F. Poole, Clin. Chem., 27 (1981) 789.
15 M.L. McConnell and M. Novotný, J. Chromatogr., 112 (1975) 559.
16 B.L. Goodwin, Handbook of Intermediary Metabolism of Aromatic Compounds, Wiley, New York, 1976, p. A13.