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URINARY PROFILES OF ORGANIC ACIDS AND VOLATILE METABOLITES DURING THE STARVATION PROCESS IN RATS

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SUMMARY

Capillary gas chromatographic procedures were used to quantify the volatile and acidic compound profiles in the urinary samples of Sprague—Dawley rats during the starvation and refeeding periods. Numerous metabolites, identified through mass spectrometry, showed significant variations due to these physiological processes. Correlations are attempted with the previously studied biochemical processes in diabetic animals.

INTRODUCTION

Modern chromatographic methods have often been used [1—7] to investigate metabolic changes which arise due to diabetic conditions in humans and experimental animals. The results of this laboratory with experimentally induced diabetic rats [4, 5] and genetically diabetic mice [6] established that significant changes occur in the excretion of various urinary volatile and acidic metabolites which may parallel certain biochemical processes in human diabetes.

Acquisition of meaningful data from the metabolic studies is often complicated by genetic, dietary, and environmental variations. The use of advanced computational techniques for the treatment of data from complex chromatograms obtained with humans [7] has been only partially successful. Diabetic animal models seem to provide greater hope for the process of unraveling the basic biochemical abnormalities, since the disease conditions, nutrition, dietary modification, age, etc., can all be more easily controlled.

Our investigations with alloxan-induced diabetic rats revealed increases of

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various urinary ketones during the first week of diabetes [4]. Long-term studies [5] conducted on the same disease model, however, demonstrated that the same compounds were depressed in concentration, while aldehydes and pyrrole derivatives became elevated. In the C57BL/Ks (db/db) mouse (an esablished model of maturity-onset diabetes), we observed significant concentration changes in several classes of compounds: increases in aldehyde metabolites, aromatic acids and most other organic acids, but general decreases in ketones [6].

Up to this time, a major difficulty in the interpretation of data from metabolic profiling studies has been the lack of information regarding the metabolic origin of the compounds found to be altered by the diabetic process. In order to study this problem, a single, specific metabolic condition should be established in the test animal. This condition must involve a physiologically well defined alteration of carbohydrate, lipid, or protein metabolism which is similar to that existing in diabetes. Physiological similarities between diabetes and starvation are often considered [8—10]. The starvation process has been well documented [8, 11—13] as a series of metabolic events concerning the availability of fuel, carbohydrate balance, lipid and protein catabolism, etc.

In general, the response to starvation includes (a) the maintenance of glucose production to meet the needs of a glucose-consuming tissue such as the brain; (b) maximized utilization of fat, the major source of stored fuel; and (c) minimized dissipation of body protein, preserving the skeletal muscle [14].

The chronological events of starvation, similar in many ways to diabetes [8—10], have been fairly well characterized [8, 11—13]. In the rat, the body will quickly exhaust any reserve glycogen stores, usually within 12 h [11, 12]. The resulting lowered blood glucose level in turn causes a drop in the insulin level, initiating the release of triglycerides as evidenced by increases in plasma free fatty acids, ketone bodies, and glycerol levels. Peak concentrations for the rat are reached by day 2 or 3 of starvation, following which gradual decreases occur during the remainder of the starvation period [12]. Eventually, the lipid stores are also exhausted and the protein must be used. Urinary nitrogen excretion increases progressively after the third day of starvation [12].

The aim of this work has been to investigate the starvation process as a series of metabolic events by monitoring the volatile and acidic metabolites in the urine. Hypothetically, the metabolic profiles obtained during the first day of starvation should reflect the exhaustion of the glycogen stores. Days 2 and 3 should exhibit alterations due to the increased lipid mobilization, while the remainder of the starvation may yield information on the use of body protein as the primary metabolic fuel. To allow comparison with the previously conducted alloxan diabetes experiments [4, 5] and investigations on the role of intestinal flora [15], male Sprague—Dawley rats were used in this research, while the urinary volatile and acidic metabolites were chromatographically determined throughout the starvation/refeeding experiment.

EXPERIMENTAL

Sample collection

Ten male Sprague—Dawley rats (Harlan Industries, Indianapolis, IN, U.S.A.),

weighing between 250 and 275 g, were individually caged. Urine samples were collected for 24-h periods throughout the study. The urine was collected using standard metabolism cages. The animals were supplied with food (Lab-Blox, Wayne Feeds, Indianapolis, IN, U.S.A.) and water ad libitum for three days, while urine was collected to serve as control samples. All food was removed from the animals for the next five days during the starvation period. Refeeding occurred on the sixth day of the experiment, and the urine samples were collected for three additional days.

The body weights and urine volumes were measured each day. The urine samples were frozen over dry ice during the collection. Following urine collection, the samples were quickly thawed, filtered, diluted to a standard volume, and refrozen until analysis. No preservatives were used.

Analysis of volatile metabolites

The semi-automated method for the analysis of urinary volatile metabolites has previously been described by McConnell and Novotny [16]. First, a headspace sampling technique is used that involves trapping the volatile compounds purged from a heated urine sample (100°C) by passing purified helium over the sample. The volatile organics are trapped at room temperature in a porous polymer trap (Tenax-GC, Applied Science Labs., State College, PA, U.S.A.) contained in a platinum microbasket. After the sampling is complete, the basket is encapsulated, awaiting chromatographic analysis. The compound separation is accomplished through an automated capillary gas chromatographic system [16], using a glass capillary column (60 m × 0.25 mm I.D.) coated with 0.2% UCON-50-HB2000 (Applied Science Labs.) containing 0.015% benzyltriphenylphosphonium chloride as a surfactant. The essential parts of the system consist of a modified Perkin-Elmer 3920 gas chromatograph, an automatic injector (Perkin-Elmer AS41), and a flame ionization detector. The column was temperature-programmed from 32 to 160°C.

Analysis of acidic metabolites

The analytical method for the urinary organic acids, used in this study, has been described in regard to our previous investigations [6, 15]. Briefly, this procedure involves a solvent extraction to isolate the organic acids from the urine. Acids are converted to trimethylsilyl esters, any keto groups present having been first converted to oximes.

Separation of the acid derivatives was accomplished using a glass capillary column (40 m × 0.25 mm I.D.) statically coated with 0.4% SE-30 (Applied Science Labs.). The gas chromatograph used in the study was a Varian Model 1400, modified for use with capillary columns and equipped with a flame ionization detector. The column was temperature-programmed from 50 to 250°C at 4°C/min following an initial 2-min isothermal period at 50°C.

Compound identification and quantitation

Identification of both volatile and acid metabolites was accomplished using a Hewlett-Packard Model 5980A combined gas chromatograph—dodecapole mass spectrometer coupled to a Finnigan Incos computer system. Electron-impact ionization spectra were obtained at 70 eV at a scan-rate of 100 a.m.u./s.

The chromatographic conditions were the same as indicated previously, with the capillary column directly interfaced to the mass spectrometer ion source. Integrated peak areas were obtained using a Perkin-Elmer Sigma 10 chromatography data station.

RESULTS AND DISCUSSION

Parrilla [12, 13] observed that starved rats lose about 9% of their body weight during the first day, followed by losses of about 5% each day for the next five days. This general trend was also seen in the present study. The loss for the first day was 11.0%, while an average loss for the consecutive four days was 6.7%. The large weight loss on the first day has been attributed to a rapid loss of hepatic glycogen, elimination of gastrointestinal content, and dehydration [12].

Volatile metabolites

For the sake of brevity, only representative chromatograms of the volatile metabolites for selected days of the starvation/refeeding process are shown in Fig. 1, while identities of the numbered peaks are revealed in Table I.

While some urinary metabolites tended to fluctuate throughout the experiment, we have also observed numerous quantitative changes that were statistically significant alterations due to starvation (Table II). Values in Table II represent the average peak areas as a percentage of the animals in their original state ± the standard error for those cases where significant changes occurred on the subsequent days. No entry is given for those days where no significant difference from the control values occurred. This allows for a clearer indication of the observed variations during the starvation period.

Obviously, the volatile fraction of urine contains a variety of compounds. The analysis of our data was most conveniently accomplished by examining a single-compound class, while looking for alterations and trends.

With one of the abundant metabolite classes, ketones, two general trends could be observed. Some of the ketones demonstrated a sharp increase during the first two or three days of starvation. Their levels then returned to near the control values until the animals were refed. At that time, their levels dropped to less than half the normal values. This group of ketones included 2-pentanone, 4-heptanone, 3,4-dimethyl-3-penten-2-one (tentative), 5-methyl-5-octen-4-one (tentative), and 4-decanone. 2-Pentanone and 4-decanone, however, were found at normal levels during refeeding.

The ketones which exhibited a different trend showed near normal levels for the first two to three days of starvation, followed by a drop to well below normal for the remaining starvation period into the refeeding period. This group of ketones included 2-methyl-3-hexanone (tentative), 2-heptanone, 2-hepten-4-one (tentative), 3-ethyl-4-methyl-2-hexanone, 6-methyl-5-hepten-2-one, and 1-nonen-6-one (tentative).

The data appear suggestive of two different metabolic sources for the two groups of ketones. Increases during the first few days for the first group correspond to the elevated levels of free fatty acids and their oxidation [11—13]. Their decline during the remainder of the starvation correlates well

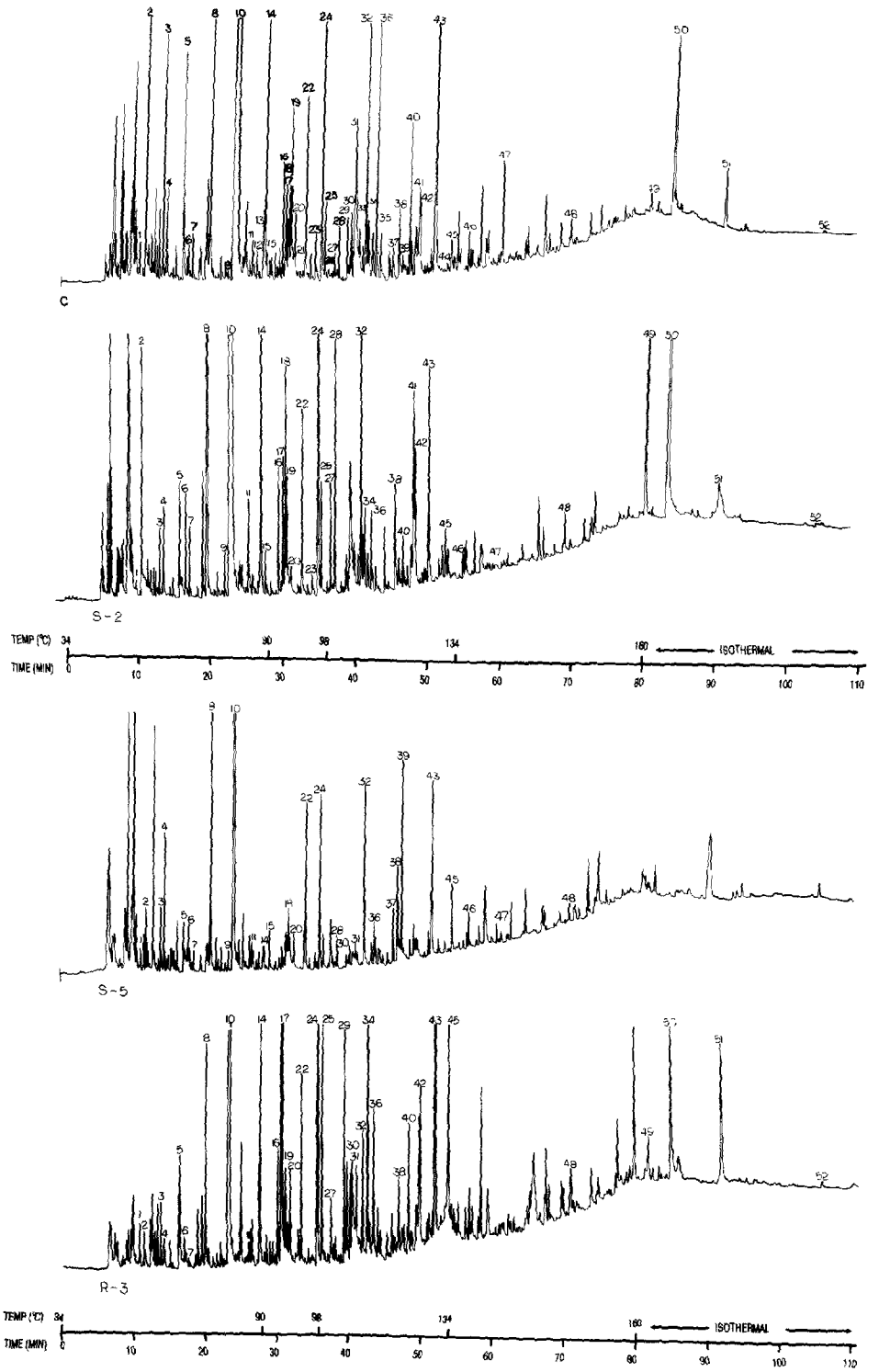

Fig. 1. Representative gas chromatograms of urinary volatile metabolites during various phases of starvation. (C) Control; (S-2) starvation day 2; (S-5) starvation day 5; (R-3) refeeding day 3.

TABLE I

VOLATILE COMPOUNDS IDENTIFIED IN THE URINE OF NORMAL AND STARVED RATS

Peak No.	Structural identification	Peak No.	Structural identification
1	Benzene	27	1-Nonen-6-one*
2	2-Pentanone	28	5-Methyl-5-octen-4-one*
3	Toluene	29	Pyrrole
4	Dimethyl disulfide	30	2-Acetylfuran
5	Hexanal	31	Nonanal
6	3-Penten-2-one	32	Benzaldehyde
7	2-Methyl-3-hexanone*	33	2-Methyl-6-vinylpyrazine*
8	4-Heptanone	34	2-Methyl-5-(2-methyl-1-butenyl)furan*
9	A cyclic vinyl ether	35	1-Octen-3-ol*
10	2-Heptanone	36	N-Acetylpyrrole
11	3,4-Dimethyl-3-penten-2-one*	37	5-Methylfurfural
12	5-Hepten-2-one	38	5,7-Nonadien-4-one*
13	4-Hepten-2-one	39	5-Methyl-2,4-heptadienal*
14	2-Hepten-4-one*	40	3-Nonen-2-one
15	Unidentified	41	4-Decanone
16	3-Hepten-2-one + 2,5-dimethyl-pyrazine	42	2-Nonenal
		43	Acetophenone
17	Ethylpyrazine*	44	1-Octanol*
18	3-Ethyl-4-methyl-2-hexanone	45	4-Methylthio-2-butenal
19	Octanal	46	Phenylacetone
20	Unidentified	47	1-Nonanol*
21	6-Methyl-5-hepten-3-one*	48	Benzothiazole
22	6-Methyl-5-hepten-2-one	49	*p*-Cresol
23	Unidentified	50	Formanilide
24	Furfural + 4-nonanone	51	*p*-Ethylphenol
25	2-Methyl-5-ethylpyrazine*	52	Indole
26	Trimethylpyrazine		

*Tentative identification.

with the exhaustion of the lipid stores [12, 13]. Results for the second group show a drop during the fourth and fifth days of starvation which may still result from the depletion of fats.

It is of considerable interest that several of the ketones from both groups were found to be elevated in the diabetic rat [4, 5]. 2-Pentanone, 4-heptanone, 2-heptanone, and 3-ethyl-4-methyl-2-hexanone were all increased during the first five days of alloxan-induced diabetes [4]. However, the levels of these compounds were near normal or diminished after one month, at the time when the diabetic rats experienced a considerable weight loss. Whereas there may be two metabolic routes for the formation of these ketones as evidenced by this starvation study, both pathways appear to be acting in unison during the diabetic process.

Both groups of ketones quantitated in this study showed a significant decline during the refeeding period. This could be attributed to the carbohydrate intolerance in the "starvation diabetes" following starvation [10, 17]. In a different diabetic model (the db/db mouse), many of the urinary ketones were

TABLE II

ALTERATIONS IN VOLATILE METABOLITES DUE TO PROGRESSIVE STARVATION FOLLOWED BY REFEEDING IN THE RAT

Values expressed as percentage of mean control (fed) peak-area value ± standard deviation Entries given for only those values in which significant differences from control levels existed ($p \leq 0.05$, Student's t-test)

Peak No.	Structural identification	Day 1 starvation	Day 2 starvation	Day 3 starvation	Day 4 starvation	Day 5 starvation	Day 1 refeeding	Day 2 refeeding	Day 3 refeeding
2	2-Pentanone	186 ± 3	301 ± 98	262 ± 100					
5	Hexanal					170 ± 39	200 ± 52	184 ± 35	200 ± 76
7	2-Methyl-3-hexanone*					60 ± 29	31 ± 36	26 ± 14	19 ± 37
8	4-Heptanone	294 ± 140	400 ± 153	282 ± 121			49 ± 37	29 ± 35	33 ± 36
9	A cyclic vinyl ether*	278 ± 99	342 ± 136	251 ± 89	173 ± 62			66 ± 22	57 ± 33
10	2-Heptanone		81 ± 17	61 ± 19	25 ± 14	14 ± 13	14 ± 13	18 ± 14	18 ± 12
11	3,4-Dimethyl-3-penten-2-one*	198 ± 81	193 ± 81					37 ± 29	47 ± 36
14	2-Hepten-4-one*		65 ± 33	44 ± 45	28 ± 32	29 ± 29	59 ± 14		65 ± 26
15	Unidentified	218 ± 64	269 ± 134						
17	Ethylpyrazine*				41 ± 29	45 ± 23			184 ± 67
18	3-Ethyl-4-methyl-2-hexanone					33 ± 16	24 ± 65	13 ± 34	15 ± 24
20	Unidentified	50 ± 34	23 ± 26	20 ± 26	23 ± 26	35 ± 41			
22	6-Methyl-5-hepten-2-one			55 ± 23	37 ± 22	27 ± 21	28 ± 22	56 ± 31	72 ± 12
23	Unidentified				69 ± 25		211 ± 67	156 ± 31	
24	Furfural + 4-nonanone				38 ± 44	33 ± 45	37 ± 45		
25	2-Ethyl-5-methylpyrazine*			39 ± 46	34 ± 41	20 ± 26			
27	1-Nonen-6-one*				57 ± 39	37 ± 26	43 ± 26	46 ± 38	49 ± 39
28	5-Methyl-5-octen-4-one*	218 ± 109	241 ± 119				50 ± 16	34 ± 50	43 ± 26
29	Pyrrole	38 ± 20	14 ± 20	6 ± 19	10 ± 21	14 ± 21	34 ± 31		
30	2-Acetylfuran			24 ± 51	18 ± 27	21 ± 52			
33	2-Methyl-6-vinylpyrazine*			23 ± 46	6 ± 25	5 ± 15	3 ± 45	1 ± 45	1 ± 45
36	N-Acetylpyrrole					61 ± 21	56 ± 28	48 ± 28	
41	4-Decanone	331 ± 128	338 ± 121	237 ± 99					
42	2-Nonenal						66 ± 34	62 ± 32	
43	Acetophenone	49 ± 33	18 ± 29	11 ± 29	12 ± 30	11 ± 19	17 ± 29	52 ± 34	
46	Phenylacetone			40 ± 28	48 ± 37	29 ± 28	45 ± 39		
48	Benzothiazole			46 ± 32	45 ± 26		44 ± 40		
51	*p*-Ethylphenol		16 ± 40	6 ± 39	5 ± 24	5 ± 38	9 ± 38	40 ± 24	

*Tentative identification.

also found [6] to be depressed below normal levels in comparison to non-diabetic mice. This, coupled with the fact that the db/db mouse exhibits increased lipogenesis rather than the lipolysis seen in the alloxan rat [18], seems to indicate that these ketones do indeed arise from the catabolism of lipids.

Another class of volatile compounds to be discussed here are the aldehydes. While long-term increases were previously seen for several aldehydes in the experimentally diabetic rat and the db/db mouse [5, 6], no consistent alterations were detected for these same compounds in the starvation/refeeding study. Whereas hexanal was increased from day 5 of starvation until the third day of refeeding and 2-nonenal was decreased during the first two days of refeeding, octanal, nonanal, and several other aldehydes showed no significant changes during this study.

It has been suggested that several aldehydes arise from the breakdown of lipid peroxides [19, 20]. While lipid peroxidation is known to be elevated during diabetes [21—23], studies concerning the level of lipid peroxidation during starvation are somewhat difficult to interpret due to a variety of results found. A study by Nakakimura et al. [24] has shown lipid peroxidation to be incrased for the first 16 h of starvation, then decreased during the next 40 h. Lemeshko et al. [25] report significant increases in lipid peroxidation for an undetermined starvation period, but Gatsko et al. [26] demonstrated no

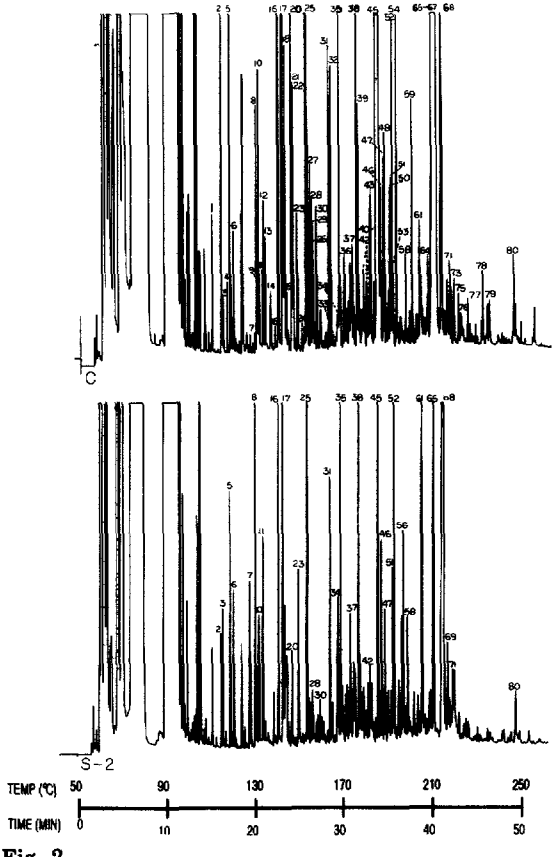

Fig. 2.

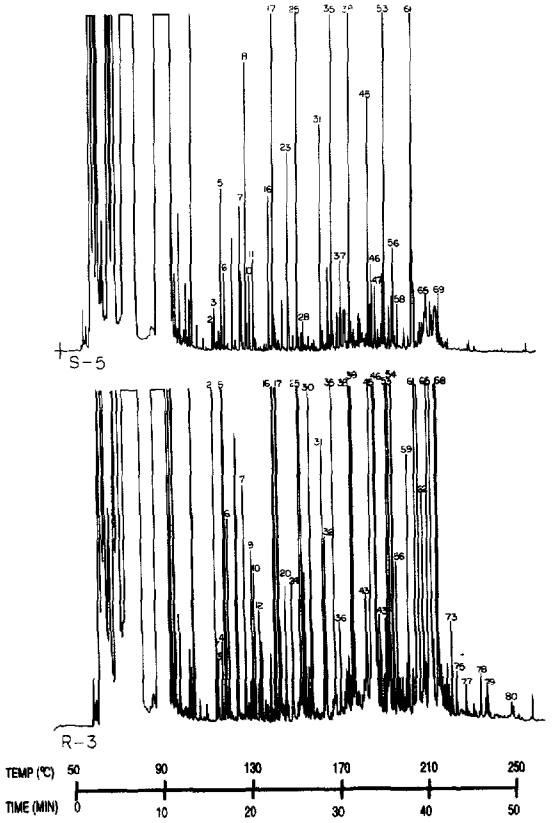

Fig. 2. Representative gas chromatograms of urinary acidic metabolites during various phases of starvation. (C) Control; (S-2) starvation day 2; (S-5) starvation day 5; (R-3) refeeding day 3.

change during 48 h of starvation. Additional experiments must be designed to elucidate such processes in future studies.

Yet another class of metabolites obviously affected by the starvation process are nitrogen-containing compounds, pyrroles, pyrazines, and benzothiazole. The levels of these compounds generally dropped to below normal for the entire starvation period and slowly rose during the refeeding process. It is of interest that the pyrrole derivatives were seen at normal levels during the first five days of alloxan-induced diabetes in the rat [4] but were elevated past the first month for the duration of the animals' life [5]. These data are difficult to explain in relation to the starvation and diabetic information available. At present, there is little biochemical information as to the origin of these nitrogen-containing compounds. One might postulate a relationship of pyrrole derivatives to the metabolism of carbonyl compounds along the lines of the known synthesis of pyrroles from diketones and primary amines. However, other metabolic routes may also be involved.

The similarity between the excretion trends of pyrrole and acetophenone are also noteworthy. Acetophenone is known to arise in part due to the action of the intestinal microflora [15]. Thus, the rapid decline in acetophenone concentration during our experiments may correspond to the elimination of the

contents of the gastrointestinal tract. Likewise, heme catabolism could also play a part in the presence of pyrroles in the urine. Bilirubin, a catabolism product of heme [21], is further broken down into mono- and dipyrrolic products via a route including the intestinal microflora [21]. Additional studies, such as those involving isotopically labeled compounds, are needed.

Acidic metabolites

For the sake of brevity, only representative chromatograms of the urinary acid profiles for selected days of the starvation/refeeding process are shown in Fig. 2; structures corresponding to the numbered peaks are listed in Table III. Table IV shows the alterations of acidic metabolites due to starvation in the same manner as Table II does for the volatiles. Obviously, the urinary acidic fraction contains a variety of compounds which could not be effectively resolved without capillary gas chromatography.

Dicarboxylic acids constitute a major group of urinary acidic metabolites. This group of acids includes the straight-chain (C_3—C_9) compounds with both odd and even carbon numbers. Several hydroxy dicarboxylic acids have also been evident. As seen in Table IV, the primary pattern for these acids has been a decrease in their levels, starting at about the third day of starvation. The odd-carbon, straight-chain metabolites are somewhat exceptional, since their levels appear decreased already on the first starvation day.

The dicarboxylic acids are known to arise from the ω-oxidation of fatty acids [8, 27—29]. The level of ω-oxidation has been shown to increase in liver preparations of starved and diabetic rats [8]; however, our data show that the levels of dicarboxylic acids are lowered during the starvation process. This agrees with the earlier results of Verkade et al. [28]. As described by Wada et al. [30] the level of ketone bodies rises in a diabetic or starved rat due to the decreased utilization of glucose and increased utilization of lipids. The terminal product of β-oxidation is acetoacetyl coenzyme A (CoA) whereas that of ω-oxidation is succinyl-CoA. The latter, a member of the tricarboxylic acid (TCA) cycle, acts to lower the ketone body levels [28] and can be used for the production of energy in the starved rat. During starvation, the demand for energy production is very high and the body must make the most efficient use of its available fuels [12, 13]. Although the dicarboxylic acids are produced at an increased rate during starvation, they are quickly metabolized via the TCA cycle, resulting in the decreased levels shown here.

The reason why the odd-carbon numbered dicarboxylic acids are at the low levels by the first day of starvation is still unknown. The ability of an even-carbon-number dicarboxylic acid to reduce the level of ketone bodies is evident; however, Wada and Usami [8] have shown that feeding starving rats odd-carbon-number dicarboxylic acids also lowers the level of ketone bodies. The terminal product of the bilateral β-oxidation of an odd-carbon-number dicarboxylic acid is malonic acid. The metabolic pathway involved in the decrease of ketone bodies by malonic acid is not clear [8].

Alterations for certain dicarboxylic acids have also been evident in the diabetic rat [31, 32] and mouse [6]. Mortensen [31] and Pettersen et al. [33] have shown that adipic and suberic acids are elevated in diabetic rats and humans. Mortensen [31] has also shown that the urinary excretion of succinic acid decreased prior to elevated ketosis in the diabetic rat.

TABLE III

ACIDIC COMPOUNDS IDENTIFIED IN THE URINE OF NORMAL AND STARVED RATS

Peak No.	Structural identification	Peak No.	Structural identification
1	Pyruvic acid	39	Adipic acid + malic acid
2	Phenol	41	3-Methyladipic acid
3	2,3-Butanediol (stereoisomer)	42	5-(Hydroxymethyl)furoic acid*
4	2,3-Butanediol (stereoisomer)	43	2-Ketoglutaric acid (*cis* oxime)
5	Lactic acid	44	*o*-Hydroxyphenylacetic acid
6	Glycolic acid	45	2-Ketoglutaric acid (*trans* oxime)
7	Oxalic acid	46	Phenyllactic acid
8	*p*-Cresol	47	2-Hydroxyglutaric acid
9	3-Hydroxypropionic acid	48	Pimelic acid + *m*-hydroxyphenyl-acetic acid
10	Pyruvic acid (TMS oxime)		
11	3-Hydroxy-2-methylpropionic acid + 3-hydroxybutyric acid	50	3-Hydroxy-3-methylglutaric acid
		51	*p*-Hydroxybenzoic acid
12	2-Oxobutyric acid (*syn* oxime)	52	*p*-Hydroxyphenylacetic acid
13	2-Oxobutyric acid (*anti* oxime)	53	2-Ketoglutaric acid (*cis* TMS oxime)
14	2-Oxo-4-butenoic acid		
16	Methylmalonic acid* + glyceraldehyde (*syn* oxime)	54	2-Ketoglutaric acid (*trans* TMS oxime)
17	Glyceraldehyde (*anti* oxime)	56	N-Formylanthranilic acid
18	5-Hydroxypentanoic acid*	57	2-Hydroxy-2-methylglutaric acid
19	Malonic acid		
20	Dihydroxyacetone	58	Suberic acid + 3-hydroxyadipic acid
21	Levulinic acid (*syn* oxime)		
22	Levulinic acid (*anti* oxime)	59	3'-Hydroxyhydrocinnamic acid
23	Phenylacetic acid	60	2-Hydroxypimelic acid
24	Phosphoric acid	61	Aconitic acid
25	Succinic acid	62	*p*-Hydroxycinnamic acid*
26	Catechol	63	*p*-Hydroxymandelic acid
27	Methylsuccinic acid	64	Azelaic acid
28	3,4-Dihydroxybutanal	65	Hippuric acid
29	3,5-Dihydroxy-4-pentalactone	66	3,4-Dihydroxybenzoic acid
30	Fumaric acid	68	Citric acid
31	*p*-Chlorobenzoic acid (internal standard)	69	*m*-Hydroxycinnamic acid*
		71	3,5-Dimethoxy-4-hydroxybenzoic acid
32	Glutaric acid		
35	Contaminant found in blank	73	Ferulic acid
36	2,4-Dihydroxybutyric acid	74	*p*-Hydroxyphenyllactic acid
37	3,4-Dihydroxy-2-methylbutanoic acid*	75	*m*-Hydroxyphenyllactic acid
		77	4-Hydroxyatrolactic acid
38	Contaminant found in blank	78	4-Hydroxy-3-methoxymandelic acid

*Tentative identification.

The data from the starvation and diabetic studies appear to agree in that the ω-oxidation provides an alternative pathway when the diabetic or starved animal lacks carbohydrates and uses fats as the primary energy source. During diabetes, the energy production system is not challenged to the same extent as during acute starvation, hence the increased production of dicarboxylic acids is evidenced by elevated levels of these compounds in the urine. However, during starvation, these acids are more efficiently utilized as succinate in the TCA cycle.

TABLE IV

ALTERATIONS IN ACIDIC METABOLITES DUE TO PROGRESSIVE STARVATION FOLLOWED BY REFEEDING IN THE RAT

Values expressed as percentage of mean control (fed) peak-area value ± standard deviation. Entries given for only those values in which significant differences from control levels existed ($p \leq 0.05$, Student's *t*-test).

Peak No.	Structural identificaton	Day 1 starvation	Day 2 starvation	Day 3 starvation
8	*p*-Cresol	262.5 ± 45.1	405.7 ± 40.6	292.4 ± 8 0
9	3-Hydroxypropionic acid			
11	3-Hydroxy-2-methylpropionic acid + 3-hydroxybutyric acid		140.3 ± 8.8	137.3 ± 11.9
12	2-Oxobutyric acid (*syn* oxime)	28.9 ± 8.6	33.6 ± 5.6	15.9 ± 4.4
13	2-Oxobutyric acid (*anti* oxime)			6.6 ± 3.7
14	2-Oxo-4-butenoic acid			7.1 ± 4.4
15	Unidentified			
16	Methylmalonic acid* + glyceraldehyde (*syn* oxime)	33.7 ± 7.8	35.2 ± 6.3	16.1 ± 1.9
18	5-Hydroxypentanoic acid*			0.0 ± 0.0
19	Malonic acid*			53.6 ± 5.8
21	Levulinic acid (*syn* oxime)	28.3 ± 9.5	6.7 ± 6.0	0.0 ± 0.0
24	Phosphoric acid		519.6 ± 65.0	
25	Succinic acid			36.8 ± 1.8
26	Catechol		50.8 ± 9.0	21.5 ± 1.0
27	Methylsuccinic acid		51.0 ± 10.4	19.4 ± 1.2
29	3,5-Dihydroxy-4-pentalactone	46.6 ± 18.5	25.4 ± 10.0	27.2 ± 8.4
30	Fumaric acid			0.0 ± 0.0
32	Glutaric acid	52.6 ± 7.7	40.8 ± 11.0	16.7 ± 1.3
33	Unidentified	4263 ± 306	5749 ± 425	3059 ± 765
34	Unidentified		144.4 ± 21.8	130.7 ± 8.8
39	Adipic acid + malic acid			9.4 ± 3.7
40	Unidentified	627.0 ± 174.2	871.0 ± 62.0	490.4 ± 112.9
41	3-Methyladipic acid			
43	2-Ketoglutaric acid (*cis* oxime)			12.2 ± 2.1
44	*o*-Hydroxyphenylacetic acid	39.6 ± 10.5	23.1 ± 12.7	0.0 ± 0.0
45	2-Ketoglutaric acid (*trans* oxime)		26.6 ± 10.3	3.9 ± 0.6
47	2-Hydroxyglutaric acid	38.5 ± 9.2	42.4 ± 3.2	20.9 ± 5.5
48	Pimelic acid + *m*-hydroxyphenylacetic acid	21.1 ± 18.9	4.4 ± 4.0	13.8 ± 7.6
49	Unidentified			
50	3-Hydroxy-3-methylglutaric acid			
51	*p*-Hydroxybenzoic acid			
52	*p*-Hydroxyphenylacetic acid		29.3 ± 26.2	0.0 ± 0.0
53	2-Ketoglutaric acid (*cis* TMS oxime)			
54	2-Ketoglutaric acid (*trans* TMS oxime)			12.7 ± 3.9
55	Unidentified			542.5 ± 31.3
56	N-Formylanthranilic acid			
58	Suberic acid + 3-hydroxyadipic acid			

Day 4 starvation	Day 5 starvation	Day 1 refeeding	Day 2 refeeding	Day 3 refeeding
222.0 ± 30.4				
19.7 ± 2.2				
10.2 ± 2.9		51.8 ± 2.5	60.2 ± 14.4	
5.7 ± 3.2	14 0 ± 5 6			
14.0 ± 5.2	4.0 ± 3.6			
24.7 ± 16.0	11.4 ± 6.2			
13.2 ± 1.0	14.3 ± 1.1	33.5 ± 9.0	51.6 ± 8.3	47.1 ± 11 9
21.5 ± 12.3	18.1 ± 10.1			
48.3 ± 9.2	13.7 ± 8.2	39.0 ± 7.6	34.2 ± 13.2	52.7 ± 7.1
0.0 ± 0.0	0.0 ± 0.0			25.7 ± 4.3
31.4 ± 2.4	31.9 ± 2.3	26.6 ± 4 7	63.9 ± 9.6	
13.6 ± 3.3	10.5 ± 3.8	26.5 ± 4.0		
14.6 ± 0.4	12.3 ± 3.2	14.9 ± 6.8	65.4 ± 6.9	
15.7 ± 6.8	10.1 ± 6.7	32 6 ± 9.4		72.1 ± 8.8
0.0 ± 0.0	2.7 ± 2.4	19 2 ± 5.4		134.1 ± 43.5
11.9 ± 1.4	5.4 ± 3.0	30.3 ± 2.2		
2773 ± 613	1674 ± 277			
107.0 ± 14.9	78.8 ± 8.8			
8.7 ± 3.3	4.6 ± 2.5	31.5 ± 3 1		
				435.0 ± 112.9
		32.9 ± 9.4		
7.2 ± 2.8	12.4 ± 4.8	8.8 ± 0.5		
0.0 ± 0.0	0.0 ± 0.0	4.3 ± 3.8	18.5 ± 7.2	21.2 ± 11.64
2.7 ± 0.3	2 0 ± 0 4	4.2 ± 1.1		
33.2 ± 3.6	24.2 ± 2.2	39.8 ± 1.5		
12.3 ± 7.0	7.8 ± 6.9	0.0 ± 0.0	28.2 ± 17.3	
8.7 ± 7.7	0.0 ± 0 0	0.0 ± 0.0		
	11.6 ± 7 0			
	20.2 ± 13.2			
0.0 ± 0.0	27.3 ± 10.5		33.6 ± 15.1	
14 8 ± 2 8	9.8 ± 3.4			
6.4 ± 1.5	4.2 ± 2.4	19.3 ± 5.0		
391.6 ± 25.9	356.0 ± 28.6			
		25 3 ± 2.4		
	29.3 ± 12.8			

(Continued on p. 16)

TABLE IV (continued)

Peak No.	Structural identificaton	Day 1 starvation	Day 2 starvation	Day 3 starvation
59	3'-Hydroxyhydrocinnamic acid	59.9 ± 11.6	42.4 ± 12.6	14.8 ± 3.2
60	2-Hydroxypimelic acid			17.1 ± 5.2
61	Aconitic acid			11.8 ± 0.6
62	*p*-Hydroxycinnamic acid*	22.4 ± 5.7		7.5 ± 4.6
63	*p*-Hydroxymandelic acid			16.9 ± 9.6
64	Azelaic acid	69.2 ± 6.0	55.4 ± 14.5	14.2 ± 7.9
65	Hippuric acid	53.9 ± 14.9	42.0 ± 13.8	8.5 ± 0.9
66	3,4-Dihydroxybenzoic acid		0.0 ± 0.0	10.2 ± 9.2
67	Unidentified		0.0 ± 0.0	15.2 ± 13.6
68	Citric acid			25.8 ± 9.0
70	Unidentified			
71	3,5-Dimethoxy-4-hydroxy-benzoic acid	51.1 ± 19.1		39.2 ± 6.1
72	Unidentified			29 6 ± 3.8
73	Ferulic acid		34.8 ± 11.9	
74	*p*-Hydroxyphenyllactic acid			16.3 ± 14.6
75	*m*-Hydroxyphenyllactic acid	51.9 ± 9.9	42.3 ± 13.5	4.1 ± 3.7
76	Unidentified	20.4 ± 13.2	39.4 ± 17.6	0.0 ± 0.0
77	4-Hydroxyatrolactic acid			0.0 ± 0.0
79	Unidentified			
80	Unidentified			0.0 ± 0.0

*Tentative identification.

Another group of acids which showed significant alterations are those with aromatic functionality, most also being hydroxy aromatic compounds. These include various hydroxybenzoic, -phenylacetic, -phenyllactic, and -hydrocinnamic acids, as well as several di- and trihydroxy aromatic acids. The primary pattern for these acids, as seen in Table IV, was a decrease in level during the third through fifth days of starvation. A few acids were decreased from the first day whereas others remained at low levels into the refeeding period. One compound which did not fit this trend was *p*-cresol, which was elevated from the first through fourth days of starvation. Several of these acids are elevated during the diabetic process in the mouse [6] and the rat [33].

The catabolism of phenylalanine and tyrosine is one possible source for these acids. These amino acids are known to be metabolized to certain phenolic acids [34, 35]. Proteins are generally spared during the early starvation; however, their catabolism increases starting about day 3 in the rat when the lipid stores are finally exhausted. Based on this, it was expected that the metabolites of these amine acids would be found at decreased levels during the first three days of starvation and would increase during the final days. This was not the case for these acids.

Phenylalanine is metabolized into a variety of important compounds including thyroxine, melanin, and the catecholamines [21]. Its normal degradation also includes the formation of acetoacetate and fumarate [21]. Again, the energy production requirements during the late stages of starvation are more demanding than in diabetes so that by the time protein is utilized as the primary source of energy, the starvation process is quite advanced and the most efficient use of fuels must be made [12, 13]. The acetoacetate produced by

Day 4 starvation	Day 5 starvation	Day 1 refeeding	Day 2 refeeding	Day 3 refeeding
13.9 ± 2.9	8.4 ± 2.5	51.0 ± 5 8		
	5.1 ± 2.9			
9.2 ± 1.0	8.9 ± 1.9			
11.2 ± 8.3	2.5 ± 1.4			
13.6 ± 7.5	15.7 ± 9.3			
15.9 ± 9.0	9.4 ± 5.3	62.4 ± 2.9		
5.6 ± 0.7	6 3 ± 0.5	6.8 ± 2.2		
0.0 ± 0.0	9.5 ± 8.5	6.2 ± 2.8	22.6 ± 8.6	16.4 ± 10.6
0.0 ± 0.0				0.0 ± 0 0
9.2 ± 3.0	11.8 ± 4.0	18 1 ± 8 2		
19 9 ± 7.4				
24.0 ± 6.1	27.6 ± 4.9			
25.3 ± 7.2	19.1 ± 6.1	43.7 ± 3.8		56.9 ± 10.2
18.6 ± 2.8	36.9 ± 11.7			
9.0 ± 8.0	0.0 ± 0.0			
0.0 ± 0 0	0.0 ± 0.0			
0.0 ± 0.0	0.0 ± 0.0	12.9 ± 11.6		20.0 ± 17.9
0.0 ± 0.0	0.0 ± 0.0			
	13.2 ± 7.5			
0.0 ± 0.0	0.0 ± 0 0			

phenylalanine enters the TCA cycle for the production of energy. Fumarate may also act to maintain the TCA cycle. This would result in the decreased levels of the aromatic acids and the increased level of energy production. It is interesting to note that fumarate was at very low levels during the third to fifth days of starvation which may also indicate its preferential use in the TCA cycle. Other members of the TCA cycle identified in this study are also at diminished levels during the late stages of starvation.

It is interesting that certain metabolites which are altered in starving humans are not significantly altered in the starving rat. There are several key differences between rats and humans. Humans have a higher percentage of body fat than rats along with a slower metabolic rate [12]. This leads to less protein conservation in the rat than in humans. The rat, with a higher level of gluconeogenesis, does not show the increased level of ketone bodies seen in humans. According to Parrilla [12], starvation places a much greater demand on the rat for energy production than on humans, with a supply which is much less. These differences prevent the direct comparisons of the level of urinary metabolites between humans and rats.

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