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QUANTITATIVE ALTERATIONS IN THE METABOLISM OF CARBONYL COMPOUNDS DUE TO DIET-INDUCED LIPID PEROXIDATION IN RATS

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SUMMARY

Following the dietary induction of lipid peroxidation in rats (verified by the levels of malonaldehyde and glutathione peroxidase), the urinary carbonyl compounds were followed chromatographically. Through a headspace gas chromatographic procedure, increases of several aldehydes and furan derivatives were noticed. Liquid chromatography of the dinitrophenylhydrazone derivatives of urinary carbonyls provided a more definitive experiment, in which the increased-peroxidation animals could be compared to those maintained on a control diet. Several carbonyl metabolites, identified by mass spectrometry, were elevated following the lipid peroxidation induction.

INTRODUCTION

Various carbonyl compounds, such as simple ketones and ketoacids, have long been associated with various diabetic conditions [1, 2]. A suggestion [3] that small ketone-like molecules may participate in certain neurological complications of diabetes, the so-called diabetic polyneuropathy, is of a relatively recent date. Since toxicological experiments [3—5] have actually implicated *n*-hexane, 2-hexanone, and 2,5-hexanedione as very potent axonal neurotoxic agents, the hypothesis that these or chemically similar compounds are produced in vivo due to the diabetic condition has been tested [6—8].

Indeed, a variety of carbonyl structures have been detected in the urinary profiles of volatile substances in humans [9, 10]. Whereas quantitative correlations in humans are somewhat complicated by variations in genetic background and dietary modifications, the use of advanced computational techniques (pattern recognition) for the evaluation of metabolic profiles did suggest some increases in concentrations for certain carbonyl compounds due to the diabetic

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condition [9]. These preliminary findings were later paralleled by more extensive investigations made by our laboratory with experimentally diabetic rats [6, 8] and diabetic C57BL/Ks mice [7].

While alloxan-induced diabetic rats exhibited elevated levels of C_5—C_7 urinary ketones immediately following the onset of chronic hyperglycemia [8], these same compounds were depressed in concentration during the long-term experiments [6] where considerable weight loss occurred. However, an aldehyde, 2-heptenal, was among a few characteristically elevated urinary metabolites. During another set of experiments [7], a number of aldehydic metabolites were found at consistently high levels in the urine of diabetic (db/db) mutants of the C57BL/Ks mouse strain as compared to appropriate genetic controls.

Because certain aldehydes have been reported to have cytotoxic effects [11—13], their possible effects during the diabetic condition have been considered [6]. Since the metabolic origin of such compounds has been uncertain, we have designed an experiment, reported here, to test a simple hypothesis that lipid peroxidation may be involved in their formation.

Biochemical lipid peroxidation has been widely studied. Free radicals produced during the peroxide formation, such as $ROO^{\cdot}$, $RO^{\cdot}$, and $HO^{\cdot}$, may, by hydrogen abstraction and a variety of addition reactions, damage enzymes, DNA, other lipids, etc. [14—16]. Antioxidants, which trap free radicals, are useful in retarding peroxidation damage. Vitamin E is a classic example of an antioxidant which is readily available in a normal diet.

Animals which are deficient in vitamin E are particularly susceptible to tissue damage by peroxidation [17, 18]; however, animals which ingest or are injected with peroxides do not accumulate such compounds [19, 20]. Glutathione has been linked with the disappearance of excess lipid peroxides. Reduced glutathione (GSH) will react with peroxides in the presence of glutathione peroxidase (GSH-Px) to yield oxidized glutathione (GSSG) and the corresponding hydroxy acid. Rotruck et al. [21] have proposed that selenium also functions as an integral part of GSH-Px activity. GSH-Px activity in rats increases as a function of the logarithm of the dietary selenium concentration [21]. Animals given a diet deficient in selenium will also show the signs of lipid peroxide damage.

In order to stimulate lipid peroxidation in rats, we have subjected one group of animals to a diet deficient in both vitamin E (α-tocopherol acetate) and selenium; a control group of animals was fed the same diet to which controlled levels of vitamin E and sodium selenite were added. To ensure that the lipid peroxidation process did occur, previously suggested criteria — malonaldehyde levels [22] and GSH-Px activity in erythrocytes [23] — were measured throughout the duration of this experiment. Metabolic differences between the experimental and control groups were followed by (a) a capillary gas chromatographic (GC) method, intended for monitoring volatile metabolites; and (b) a liquid chromatographic (LC) procedure by which excreted carbonyl compounds are determined as their 2,4-dinitrophenylhydrazones. In both profiling procedures, structures were elucidated with the aid of mass spectrometry (MS).

EXPERIMENTAL

Animals and sample collection

Twenty Sprague—Dawley male rats, 21 days old, were divided into two groups of equal size. Each group was fed a special diet. The lipid peroxidation group was provided with a diet containing corn oil (material rich in polyunsaturated fatty acids) that was known to be deficient in both vitamin E and selenium (United States Biochemical Corp., Cleveland, OH, U.S.A.). The control group was fed the above diet plus vitamin E (*d,l*-α-tocopherol acetate) and selenium (as sodium selenite) at the levels of 200 I.U./kg and 0.2 ppm, respectively.

Blood was collected from the rats on a regular basis. The rats were anesthetized by placing them in a chamber containing Metofane (Pitman-Moore, Washington Crossing, NJ, U.S.A.). The blood samples were collected using a variation of the interorbital sinus bleeding technique. A standard disposable Pasteur pipet was positioned behind the eye and with a twisting and scraping motion, blood vessels were broken causing the bleeding. The blood was then collected in the pipet. As much as 1.5 ml of blood could be obtained from behind one eye of each rat.

Using the procedure of Paglia and Valentine [23] for the analysis of malonaldehyde and glutathione peroxidase, the blood was immediately mixed with a one fifth volume of 5% polyvinylpyrrolidone, containing 2.5% sodium citrate. The red blood cells were separated by centrifugation and washed three times with a cold isotonic saline solution, then resuspended in isotonic saline at a concentration of approximately $3 \cdot 10^6$ cells per μl. The cells were counted manually using a hemacytometer and a microscope, and lysates were prepared by adding 200 μl of cell suspension to 800 μl of water, followed after 10 min by freezing and thawing three times in a dry ice—ethanol bath. The lysate was mixed with an equal volume of double-strength Drabkin's reagent (0.0016 *M* KCN, 0.0012 *M* $K_3Fe(CN)_6$, 0.0238 *M* $NaHCO_3$) to convert the hemoglobin to the stable cyanomethemoglobin form.

During urine collection, the animals were housed in standard metabolism cages and the urine was collected over dry ice. Following urine collection, samples were quickly thawed, filtered, diluted to a standard volume, divided into suitable aliquots, and refrozen until analysis. No preservatives were used.

Tests for lipid peroxidation

Malonaldehyde. The thiobarbituric acid (TBA) test used for the quantitation of malonaldehyde is an adaption of several methods [19, 22, 23]. A 1.5-ml volume of the sample solution described above and an equal volume of 0.8% 2-thiobarbituric acid (Sigma, St. Louis, MO, U.S.A.) in 10% trichloroacetic acid were thoroughly mixed in test tubes. The tubes were placed in a boiling water bath for 10 min and then allowed to cool. After centrifugation, the absorbance of the clear supernatant was measured at 532 nm and the amount of TBA reactants was calculated based on the molar absorptivity of the TBA—malonaldehyde complex, $1.56 \cdot 10^5$ [19].

Glutathione peroxidase. The quantitation of GSH-Px is based on a previously reported method [23]. A 100-μl volume of the prepared lysate was added to

2.60 ml (pH 7.0) phosphate buffer containing 0.005 *M* EDTA. Additional solutions were added in the following order: 100 μl of 0.0084 *M* NADPH, 10 μl of GSSG-R (100 enzyme units (E.U.)/ml), 10 μl of 1.125 *M* NaN_3, and 100 μl of 0.15 *M* GSH. The enzymatic reaction was initiated by the addition of 100 μl of 0.0088 *M* hydrogen peroxide. The conversion of NADPH to NADP was monitored by recording the change in absorbance at 340 nm for a period of 10 min after the reaction was initiated. The activity of GSH-Px was calculated on the basis of the molar absorptivity of NADPH at 340 nm of $6.22 \cdot 10^6$ [24].

Analysis of urinary carbonyl compounds

Volatile constituents of the urine samples were profiled using a headspace concentration method followed by capillary GC as previously described [25]. Although this method was not intended to determine carbonyl compounds specifically, they were previously shown to comprise a substantial fraction of rat urine volatiles [6, 8]. In this method, the volatiles are purged from the heated urine headspace with high-purity helium and adsorbed onto the porous polymer Tenax-GC (Applied Science Labs., State College, PA, U.S.A.) placed in an injection precolumn.

Following the sample concentration, the precolumn is heated and the volatiles are thermally desorbed into a capillary column (60 m × 0.25 mm I.D.) coated statically with 0.2% UCON-50-HB 2000 stationary phase (Applied Science Labs.) containing 0.015% benzyltriphenylphosphonium chloride as a surfactant. A Perkin-Elmer Sigma 3 gas chromatograph equipped with a flame ionization detector was programmed from 30 to 160°C at 2°C/min and a Sigma 10 data acquisition system was utilized for all GC analyses. A combined gas chromatograph—mass spectrometer (Hewlett-Packard Model 5980A) and Finnigan Incos data acquisition system were used for compound identification.

As discussed below, the capillary GC method proved somewhat limited for monitoring the carbonyl metabolites. Consequently, an LC method, in which the sample preparation was based on the report of Benedetti et al. [26], was set up to provide additional information.

A 5.0-ml aliquot of urine was made basic by the addition of 10 drops of 5 *M* sodium hydroxide so as to isolate the neutral carbonyl compounds from any keto acids upon extraction (twice) with 5 ml methylene chloride. As an internal standard, 10 μl of a 2-pentadecanone dinitrophenylhydrazone (DNPH) solution (2 mg/ml) was added. The DNPH derivatives of the urinary carbonyl compounds were formed by adding 25 ml of a dinitrophenylhydrazine solution (48 mg per 100 ml of 10% acetic acid in methylene chloride) and allowing the reaction to proceed in darkness for 16—24 h. The solvent was then removed by rotary evaporation and the residue dissolved in a minimum amount of methylene chloride.

Preparative-scale thin-layer chromatography (TLC) was used to remove the excess derivatization reagent. The solution was streaked onto a 20 cm × 10 cm × 0.2 mm silica gel 60 TLC plate (E. Merck, Darmstadt, F.R.G.) and developed two times with methylene chloride. The silica gel was removed from the aluminum layer for the region where $R_F > 0.5$ and was packed into a disposable Pasteur pipet plugged on the bottom with silylated glass wool. The

derivatized carbonyl compounds were eluted with 20% methanol in methylene chloride. The solvent was removed using a gentle stream of nitrogen at room temperature. The residue was redissolved in 300 μl methanol and filtered through a 0.45-μm filter (Millipore HV, Bedford, MA, U.S.A.). The derivatives were chromatographed on a 25 cm × 4.2 mm C_{18} high-performance liquid chromatography (HPLC) column (Phenomonex, Palos Verdes Estates, CA, U.S.A.), using a gradient from 50% aqueous methanol to 100% methanol, in 1 h. Detection was by UV absorbance at 340 nm.

The effluent for each peak was collected, the solvent evaporated, and the residue subjected to direct-probe MS. The tentative identifications obtained from the analysis of the resulting spectra were confirmed where possible by coinjection of DNPH standards.

RESULTS AND DISCUSSION

To validate our analytical results on the level of excreted urinary carbonyl compounds, the state of lipid peroxidation had to be first verified by the established criteria [22, 23]: the levels of malonaldehyde and the GSH-Px activity in blood.

While valid data on malonaldehyde were not obtained until the eighth week of the study, the levels in the lipid peroxidation group were convincingly high compared to the control. The lipid peroxidation group malonaldehyde level rose further from 2.8 U (μmol per 10^{12} cells) to 8.6 U between weeks 8 and 11. A sharp drop followed at week 13, just one week after a normal diet was restored. The control group exhibited a nearly constant level at about 1 U (see Fig. 1).

Data for the GSH-Px were obtained during the entire study. Both groups started at nearly the same level, 5.0 E.U. (1 E.U. will catalyze the oxidation of

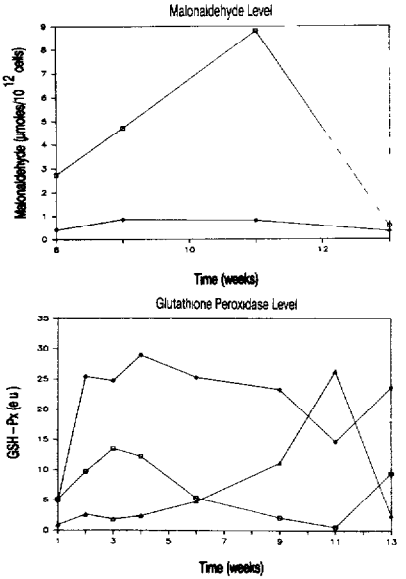

Fig. 1. Malonaldehyde and glutathione peroxidase levels versus time. (◇) Control; (□) lipid peroxidation; (△) ratio control/lipid peroxidation.

1.0 μmol of reduced glutathione by hydrogen peroxide to oxidized glutathione per min at pH 7.0 and 25°C). The level rose for both groups until the fourth week, but that for the control group was noticeably higher. At this point the group given the deficient diet showed decreasing levels until week 11, where the level was near zero. Upon being refed a normal diet, the lipid peroxidation group experienced a significant rise in level. The relative standard deviations for this test are about 25%, but the variation from week to week as seen in Fig. 1 is much greater than this. To correct for these fluctuations possibly due to the aging process, a ratio was taken between the two groups. The ratio, starting at 1.0, rose to a maximum of about 27 at the 11-week point, but dropped sharply to just over 2 at one week after the animals were refed a normal diet.

Clearly, lipid peroxidation was initiated by control of the diet. These results, while not our primary goal, do validate the use of this diet for the intended purpose.

Lipid peroxidation, in general, can result in a number of reaction products. While the above mentioned "wet chemistry" assays can be used as criteria of the process, they provide little information concerning the multitude of possible metabolic pathways and products. It has been reported [17, 27] that, depending on the type of major fatty acids in the diet, either ethane or pentane will be generated in the breakdown of the peroxides. Our further discussion will be restrained to the carbonyl products because of their potential toxicity in accordance with the major emphasis of this study.

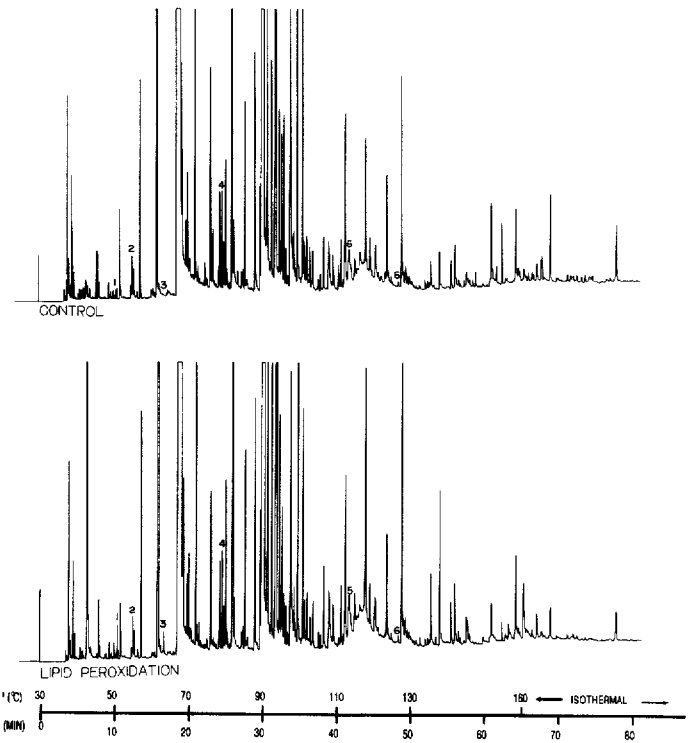

Fig. 2. Representative gas chromatograms of urinary volatile metabolites. Numbered peaks are identified in Table I. See text for conditions.

TABLE I

PEAK AREAS FOR THOSE VOLATILE METABOLITES WITH SIGNIFICANT CHANGES IN CONCENTRATIONS DUE TO LIPID PEROXIDATION

Peak No.	Name	Control	Lipid peroxidation	Lipid peroxidation as percentage of control
1	2,5-Dimethylfuran	202 ± 143	888 ± 356	440
2	Hexanal	1701 ± 137	2784 ± 281	164
3	2,4-Pentadienal	N.D.*	355 ± 13	N.D.*
4	2-Pentylfuran	966 ± 218	1843 ± 756	191
5	2-Furylmethanol	2914 ± 158	6700 ± 901	230
6	2-Decenal	510 ± 193	728 ± 144	143

*N.D. = not detected in control.

Representative chromatograms of urinary volatiles are shown in Fig. 2. Quantitative comparisons between the experimental and control groups resulted in implication of only a few components as being elevated during the lipid peroxidation process. The peaks showing consistent elevations are marked in the chromatogram and listed in Table I along with the quantitative data. Basically, three aldehydes are shown together with three furan derivatives. A reader interested in the additional profile components (those not being altered in concentration as a result of the present lipid peroxidation experiment) is referred to our previous publications [6, 8].

It is quite likely that the source of some furan derivatives may be the cyclization and dehydration of various hydroxyaldehydes, as has been previously described [28, 29]. Whether such dehydration and cyclization processes occur during sampling or in the injection port of a gas chromatograph remains unknown at this time. It has been suggested, however, that it is unlikely that they are formed in vivo [28].

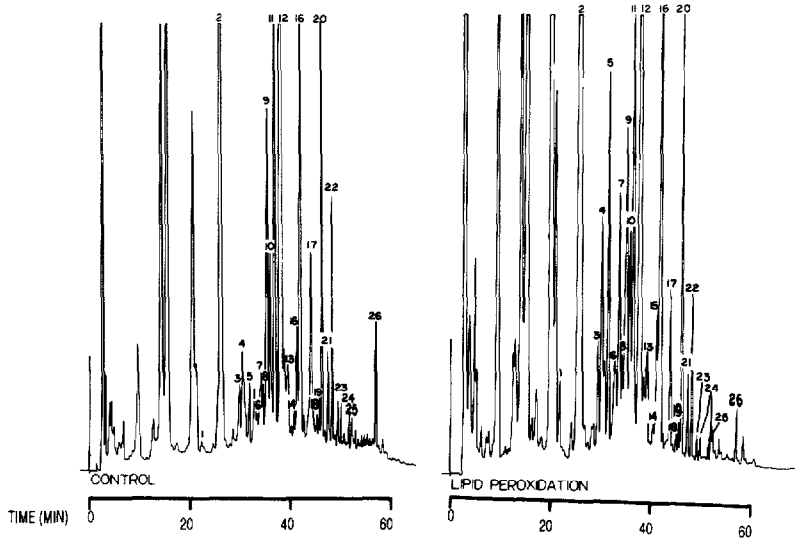

Fig. 3. Representative liquid chromatograms of dinitrophenylhydrazones of urinary carbonyl compounds. Numbered peaks are identified in Table II. See text for conditions.

Far more complete information on the excretion of urinary carbonyl compounds was obtained by LC of the DNPH derivatives on a reversed-phase column. Representative chromatograms are shown in Fig. 3. Those carbonyl compounds which were identified in the lipid peroxidation experiment are listed in Table II. Concentrations were approximated by assuming a constant molar absorptivity between all dinitrophenylhydrazone derivatives and by comparing peak areas to that of the internal standard. Duplicate entries in this table are due to two derivative isomers being formed for certain carbonyl compounds.

The results indicated that C_5–C_{11} straight-chain aldehydes, with the exception of hexanal, were all significantly elevated during the lipid peroxidation experiment, as were also hydroxyacetaldehyde, furfural and benzaldehyde. Additional compounds exhibiting elevations are several ketones, including 2-pentanone, 3-hexanone, and 2-heptanone.

Whereas butanone and octanal form two derivative isomers, only one of each

TABLE II

CONCENTRATIONS FOR THOSE CARBONYL COMPOUNDS WITH SIGNIFICANT CHANGES IN THE CONCENTRATIONS DUE TO LIPID PEROXIDATION

Peak No.	Name	Concentration (μg/ml of urine)		Lipid peroxidase as percentage of control
		Control	Lipid peroxidation	
1	Hydroxyacetyladehyde*	1.1 ± 0.9	7.3 ± 2.1	676
2	Acetone	326.4 ± 204.4	859.2 ± 447.4	—**
3	Butenone	4.1 ± 0.5	8.7 ± 3.4	—**
4	Butanone	6.8 ± 1.1	22.3 ± 3.6	327
5	Butanone	3.9 ± 3.1	6.0 ± 4.9	—**
6	Furfural	2.8 ± 2.1	13.6 ± 5.9	487
7	Benzaldehyde	4.2 ± 0.2	22.4 ± 11.2	538
8	Pentanal	3.1 ± 0.6	8.1 ± 2.0	264
9	2-Pentanone	22.6 ± 6.7	37.7 ± 6.1	166
10	3-Pentanone	12.8 ± 4.4	20.1 ± 5.4	—**
11	2,4-Hexadienal*	49.3 ± 19.6	47.1 ± 2.9	—**
12	A hexanone*	77.8 ± 18.9	140.8 ± 17.8	181
13	3-Hexanone	5.2 ± 4.2	13.7 ± 2.6	261
14	2-Heptenal	1.7 ± 0.2	2.9 ± 1.2	—**
15	Heptanal	3.9 ± 0.4	10.7 ± 2.6	271
16	2-Heptanone	35.0 ± 7.9	58.6 ± 8.7	167
17	Octanal	1.4 ± 0.3	2.1 ± 0.2	152
18	Octanal	0.5 ± 0.1	1.5 ± 1.3	—**
19	2-Nonenal	1.0 ± 0.4	0.7 ± 0.2	—**
20	Nonanal	12.8 ± 2.5	36.8 ± 2.0	289
21	Nonanal + methylnonanal	2.3 ± 0.7	5.5 ± 0.3	236
22	Decanal	5.2 ± 1.2	11.1 ± 1.4	215
23	Decanal	1.1 ± 0.4	1.9 ± 0.1	176
24	Undecanal	1.1 ± 0.1	2.2 ± 0.5	204
25	Dodecanal	0.8 ± 0.6	1.1 ± 0.1	—**
26	2-Pentadecanone (internal standard)			

*Tentative identification.
**Not a statistically significant change.

is listed as being elevated by a significant amount. Both isomers are elevated, but due to the small size of the peak and high variance in peak areas, the elevations are not statistically significant for the smaller peak.

Both capillary GC and LC results appear to implicate aldehydes (both normal and unsaturated) and related compounds, furan derivatives, as characteristic products of lipid peroxidation. Elevated aldehyde levels were also noticed in our earlier investigations of urinary metabolites of both long-term diabetic rats [6] and genetically diabetic mice [7]. Since an increased lipid peroxidation process has been associated with the diabetic condition [30, 31], it is not suprising that known peroxidation metabolites should be more abundant in diabetic than normal urine samples. Minor structural differences within the class of carbonyl compounds do occur between mice and rats, but these could be explained by both genetic and dietary variations.

The elevation in certain urinary ketones during our lipid peroxidation experiments is somewhat more difficult to explain in relation to diabetes. During alloxan-induced diabetes of short duration in rats [8], such compounds were significantly increased during the early stage of disease which coincides with a temporary elevation of free acids and ketone bodies in blood. These urinary ketones most likely arise from fat breakdown and a simple decarboxylation of keto acids. Not surprisingly, long-term diabetic rats with severely decreased weights and depleted adipose tissue were characterized by decreased ketone levels [6]. It remains unclear whether the ketones elevated in the present study originate from the lipid peroxidation process itself, or if the dietary insufficiency also affects the normal metabolic turnover of fats.

Increased lipid peroxidation clearly results in a greater production of metabolites that are either proven or suspected neurotoxins. Future studies will aim at the assessment of relative toxicity for different carbonyl structures.

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