Vitamin C Metabolomic Mapping in Experimental Diabetes With 6-Deoxy-6-Fluoro-Ascorbic Acid and High Resolution ^{19}F-Nuclear Magnetic Resonance Spectroscopy

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Metabolomic mapping is an emerging discipline geared at providing information on a large number of metabolites as a complement to genomics and proteomics. Here we have probed ascorbic acid homeostasis and degradation in diabetes using 6-deoxy-6-fluoro ascorbic acid (F-ASA) and 750 MHz ^{19}F-nuclear magnetic resonance (NMR) spectroscopy with proton decoupling In vitro, Cu^{2+}-mediated degradation of F-ASA revealed the formation of 4 major stable degradation products at 24 hours. However, when normal or diabetics rats were injected with F-ASA intraperitoneally (IP) for 4 days, up to 20 fluorine-labeled compounds were observed in the urine. Their composition resembled, in part, metal catalyzed degradation of F-ASA and was not explained by spontaneous degradation in the urine. Diabetes led to a dramatic increase in urinary F-ASA loss and a relative decrease in most other urinary F-compounds. Diabetes tilted F-ASA homeostasis toward oxidation in liver ($P < .01$), kidney ($P < .01$), spleen ($P < .01$), and plasma ($P < .01$), but tended to decrease oxidation in brain, adrenal glands, and heart. Surprisingly, however, besides the major oxidation product fluoro-dehydroascorbic acid (F-DHA), no F-ASA advanced catabolites were detected in tissues at 5 μ mol/L sensitivity. These findings not only confirm the key role of the kidney in diabetes-mediated loss of ascorbic acid, but demonstrate that only selected tissues are prone to increased oxidation in diabetes. While the structure of most degradation products needs to be established, the method illustrates the power of high resolution ^{19}F-NMR spectroscopy for the mapping of complex metabolomic pathways in disease states.

METABOLOMIC MAPPING is a rapidly evolving "post-genomic" science whose goal is to develop integrated databases of metabolite concentrations across human and research animal populations. It provides an invaluable tool for determining the distributions of metabolite concentrations in humans, the relationship of these metabolite concentrations to disease, and the extent to which nutrition can modulate metabolite concentrations.[1,2] While various techniques are being developed toward that goal, we have used below nuclear magnetic resonance (NMR) spectroscopy to assess the catabolic pathways of ascorbic acid in vivo. One advantage of this technique is that it can detect and quantitate individual compounds without prior separation, assuming these contain an isotope whose abundance is present in sufficiently large concentrations for detection. NMR applications for metabolomics have been recently reviewed by Reo.[3]

L-ascorbic acid (ASA) plays a key role in the oxidant defense of the body by trapping of free radicals.[4] Evidence suggests that oxidative stress is increased in diabetes.[5,6] ASA level in plasma and tissue of diabetic animals and humans is lower than normal,[7,8] and this abnormality may contribute to an acceleration of oxidative damage in diabetes, leading to diabetic complications, such as cardiovascular and renal disease. The administration of vitamin C might have therapeutic benefits for diabetic individuals,[7,9] but ASA can also have prooxidant effects depending on the milieu.[10,11] Despite considerable information on plasma levels of ascorbic acid in diabetes, little information on its homeostasis and degradation in the tissue is available. This lack of knowledge, in part, reflects the technical difficulty of making comprehensive measurements of all ASA degradation products.

ASA is oxidized into dehydro-L-ascorbic acid (DHA) via monodehydro-L-ascorbic acid. Normally DHA is quickly reduced to ASA in the cell. Under oxidative stress, DHA accumulates and undergoes irreversible delactonization to diketo-L-gulonic acid (DKG).[12,13] The latter undergoes further degradation (Fig 1) into carbonyl compounds, such as L-xylosone,[14] L-erythrulose,[15] and L-threose,[16] although the existence of some of these compounds has been contested.[15] The degradation compounds are similar to those observed during degradation of sugars and are thought to promote the formation of advanced glycation end-products (AGEs) by reacting with proteins (Maillard reaction).[14,17,18] Some of the products that have been identified in vivo include pentosidine,[19] carboxymethyl-lysine (CML),[20] vesperlysine A,[21] and oxalic acid monoamide.[22] It is likely that ASA participates in the formation of these compounds, especially in tissues in which ASA concentrations are high[16,19,23,24] and especially in end-stage renal disease (ESRD) in which oxidative events are dramatically increased.[25]

Utilizing diabetes as a paradigm of cellular oxidant stress, the study below was initiated with the goal of investigating the extent to which advanced ascorbic acid degradation takes place in vivo and how this degradation is affected by intracellular oxidant stress. One critical question, for example, is whether

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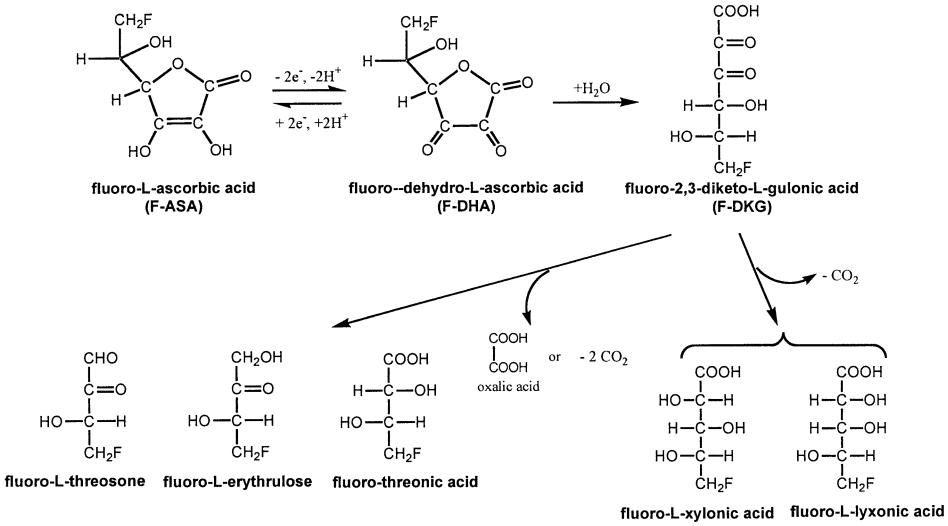

Fig 1. Possible degradation pathways of F-ASA. Except for oxalic acid, most degradation compounds are expected to have a fluorine atom in the original position 6 of ASA and thus to be detectable by ^{19}F-NMR spectroscopy.

ascorbic acid degradation in diabetes results from metal or nonmetal catalyzed oxidation and how this is reflected in the composition of the degradation products distal from dehydroascorbic acid. This question can only be addressed with isotopically-labeled ascorbic acid. However, the use of the commercially available L-[1-^{14}C]ASA to study these questions is inadequate because the labeled atom is lost in the form of $^{14}CO_2$ at the first stage of DHA degradation. A similar approach using uniformly radiolabeled ascorbic acid would be extremely expensive and would still require tedious chromatographic methods to identify the degradation products. While recent studies have been performed in vitro with ^{13}C-labeled ascorbic,[26] the sensitivity of this isotope was deemed insufficient for in vivo studies by comparison with ^{19}F, which is about 10 times more sensitive. We have therefore evaluated 6-deoxy-6-fluoro-ascorbic acid (F-ASA) and ^{19}F-NMR spectroscopy as tools for in vivo studies of ascorbate catabolism.[27] Preliminary studies showed that F-ASA was structurally identical with ascorbic acid and behaved similarly in terms of degradation kinetics.[27] These studies also showed that 5 to 7 metal catalyzed oxidation products of F-ASA could be observed by ^{19}F-NMR without separation by chromatography. Below, we have applied this fluorinated form of ASA to investigate the effects of diabetes on ascorbic acid homeostasis and obtain insight into the number and type of degradation products that are expected to accumulate, if at all, in normal and diabetic tissues.

MATERIALS AND METHODS

Materials

F-ASA was synthesized as previously reported.[27] D_2O, phosphate buffer tablets (pH 7.4) and 6-deoxy-6-fluoro-D-glucose (F-glucose) were from Sigma-Aldrich (St Louis, MO). Other chemicals of the highest analytical grade available were from Fisher Scientific (Pittsburgh, PA).

NMR Spectroscopy

^{13}C 188-MHz and ^{19}F 705-MHz NMR spectra were obtained on a Varian Unity Plus-750 MHz spectrometer equipped with a Varian 5-mm ^{1}H/^{13}C/^{19}F PFG triple resonance probe (hardware configured for ^{19}F detection and ^{1}H/^{13}C decoupling). Data processing was performed on a Sun SPARC station-10 (Sun Microsystems, St Clara, CA) using Varian (Palo Alto, CA) VNMR software. The 1-dimensional ^{19}F spectra were measured using 16.8 μs ($\sim$ 90°) ^{19}F pulse width, 0.8-second acquisition time, 256 to 512 transients with a relaxation delay of 1 second. The data were weighted with 2-Hz exponential line broadening and zero-filled before Fourier transformation. Two-dimensional (2D) ^{1}H-decoupled ^{19}F-^{13}C Hetero Nuclear Single Quantum Coherence (2D-gHSQC, from Varian pulse sequence library) spectra were measured at 25°C. A sweep width of 9,172 Hz in ^{19}F dimension and 32,025 Hz in ^{13}C dimension were used. The acquired data consists of 1,834 data points for ^{19}F and 272 data points for ^{13}C dimension, with 96 transients for each increment. The data were weighted with shifted sine bell window functions in both dimensions and zero-filled with 4,096 $\times$ 2,048 data points before the final 2D transformation. An internal standard consisting of F-D-glucose in D_2O was added to be 0.1 mmol/L immediately before the measurement at 25°C. For calibration, the chemical shift of the downfield furanose conformer of F-glucose was set at -219 ppm based on the shift of CCl_3F (δ_F = 76 ppm) as the external standard. The other F-glucose signal at -218.3 ppm likely corresponds to pyranose conformer as judged by the 60% to 40% ratio of these 2 signals. For quantitation, the sum of both signals was assumed to represent 0.1 μmol/L or 1.0 mmol/L fluoro compound, as described for each experiment, respectively.

F-DKG Analysis

The F-DKG potassium salt was synthesized from F-ASA using the same method as for the DKG potassium salt[28,29] and obtained as a white hygroscopic powder that was stored at -55°C before NMR analysis. The F-DKG potassium salt was analyzed by ^{13}C-NMR, ^{19}F-NMR, and 2D-NMR between ^{13}C and ^{19}F to confirm its structure.

F-ASA Degradation In Vitro

F-ASA was dissolved in phosphate-buffered saline (PBS, pH 7.4) that was treated with Chelex-100 resin according to the manufacturer's protocol (BioRad, Hercules, CA) to remove metal ions. For the oxidative experiment of 1 mmol/L F-ASA with 10 μmol/L $CuCl_2$, 50 μL $CuCl_2$ solution (0.4 mmol/L) was added to 1.8 mL PBS, followed by 0.2 mL F-ASA (10 mmol/L). The solution was incubated at 37°C for 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 4 days. After the incubation, 50 μL EDTA solution (2.1 mmol/L) was added to

stop the reaction. The oxidative experiment of F-ASA without $CuCl_2$ was performed the same way as for the incubation of F-ASA with $CuCl_2$, but 50 μL distilled water was added instead of 50 μL $CuCl_2$ solution (0.4 mmol/L). After the incubation, 50 μL EDTA solution (2.1 mmol/L) was added. For the time 0 sample, 50 μL EDTA solution (2.1 mmol/L) was added before addition of F-ASA. To all incubated samples were added 1/4 volume of 20% metaphosphoric acid (MPA), and the pH was adjusted to pH 3.0 with aqueous HCl, and measured by ^{19}F-NMR with proton decoupling. Some samples were incubated for 24 hours with and without $CuCl_2$ and were subjected to ^{19}F-NMR without proton decoupling.

Animals and Diet

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing about 150 g were obtained for all studies. They were randomly separated into 2 groups, consisting of normal (N, n = 9) and diabetic rats (DB, n = 15). DB was induced by intravenous injection of streptozotocin (STZ; 65 mg/kg body weight) at 2 months of age. Two rats per cage were maintained on rotating 12-hour dark/light cycle at an ambient temperature of about 24°C and were provided with free access to a diet poor in vitamin C (Rodent AIN-76A; Bio-Serv, Frenchtown, NJ). Blood glucose and glycohemoglobin (GHb) levels were determined 3 months after the STZ injection. The average ± SD of blood glucose levels and GHb in N (n = 9) and DB (n = 15) were 3.77 ± 0.37 and 17.37 ± 3.27 mmol/L and GHb(%) = 3.14 ± 1.10 and 20.88 ± 2.63, respectively. F-ASA was dissolved in saline and filtered sterile immediately before injection to rats.

Plasma Preparation

At 25 weeks of age and 4 months of diabetes F-ASA was injected IP to N (n = 7) and DB rats (n = 9) at a dosage of 4 mg/rat/d for 5 days. Mean ± SD body weights were 531.94 ± 28.23 g and 307.01 ± 37.09 g, respectively. Blood (<0.5 mL) was collected from the tail vein without anesthesia 30 minutes after the last injection of F-ASA. Whole blood was centrifuged for 20 minutes at 1,500 × *g* (4°C) and plasma was separated as supernatant. To plasma (200 μL) was added 50 μL 10% MPA, and centrifugation was performed for 10 minutes at 10,000 × *g* (4°C). The supernatant was filtered using 0.45-μm syringe filters. The sample was stored at −80°C until the measurement by ^{19}F-NMR.

Tissue Preparation

At 33 weeks of age and 6 months of diabetes duration, rats (n = 6 for N and DB) were injected with F-ASA (4 mg/rat) twice per day for 5 days. They were killed by cardiac puncture under anesthesia with pentobarbital (50 mg/kg body weight) 15 hours after the last injection of F-ASA. Liver, spleen, kidney, adrenal glands, heart, lung, and brain were removed, weighed, and quickly frozen. Body and tissue weights are shown in Table 1. Tissues that were stored at −80°C were homogenized in 1 vol 5% (vol/wt) MPA with cooling on ice water. The samples were centrifuged for 10 minutes at 10,000 × *g* (4°C). The supernatant was filtered and measured by ^{19}F-NMR.

Urine Studies

F-ASA was injected to N (n = 5) and DB (n = 6) (25 weeks old, 4 months after onset of diabetes) at a dosage of 4 mg/rat/d for 4 days by IP. Using metabolic cages, urine was collected for 24 hours into plastic tubes containing 1 mL 20% MPA starting 30 minutes after the last injection of F-ASA. Each sample was freeze-dried and adjusted to 2 mL with D_2O. After filtration over 0.45-μm filters, they were stored at − 80° C until measurement by ^{19}F-NMR. For the recovery determination of total fluoro-compounds into urine, F-ASA was injected to N (n = 5) and DB (n = 6) (33 weeks old, after 6 months of diabetes in DB) at a dosage of 4 mg/rat/d for 4 days by IP. The urine of N and DB was collected into plastic tube containing 1 mL 20% MPA from 30 minutes after the last injection of F-ASA until 24 hours. The urine was freeze-dried and prepared for the measurement by ^{19}F-NMR as above.

Table 1. Body and Tissue Weights of Normal and Diabetic Rats

	Normal (g)	Diabetic (g)
Body weight	590.67 ± 23.24 (n = 6)	287.71 ± 28.42 (n = 7)*
Brain	1.866 ± 0.205 (n = 5)	1.860 ± 0.172 (n = 5)
Adrenal glands	0.083 ± 0.034 (n = 4)	0.067 ± 0.006 (n = 6)
Liver	14.697 ± 1.303 (n = 6)	12.229 ± 2.336 (n = 4)†
Heart	1.548 ± 0.084 (n = 5)	1.227 ± 0.182 (n = 6)*
Spleen	0.640 ± 0.031 (n = 5)	0.273 ± 0.099 (n = 6)*
Kidney	1.537 ± 0.110 (n = 5)	1.894 ± 0.275 (n = 5)†

NOTE. Body weights were measured before sacrifice. Results are expressed as means ± SD. *$P < .01$,
†$P < .05$ compared with normal rats.

Effect of Urine on F-ASA Degradation In Vitro

F-ASA (1 mmol/L) was dissolved in normal rat urine (2 mL), incubated at 37° C for 1 day and filtered with a 0.45-μm syringe filter prior to assay for degradation by ^{19}F-NMR spectroscopy with proton decoupling.

Statistical Analysis

Difference of means between diabetic and controls was assessed using Student's *t* test.

RESULTS

F-2,3-Diketogulonic Acid Assignment

The F-DKG potassium salt was synthesized following the procedure in the literature.[28,29] Some minor contaminants were observed in the F-DKG potassium salt that was synthesized as white powder, but the chemical shifts of F-DKG in ^{13}C-NMR and ^{19}F-NMR were identified by 2D-NMR between ^{13}C and ^{19}F. The chemical shifts were $\delta_F = -213.90$ ppm, $\delta_C = 172.94$ (C1), 101.94 (C2), 100.44 (C3), 74.85 (C3), 78.68 (C4), 83.53 (C6) ppm. The chemical shifts of F-DKG in the ^{13}C-NMR spectrum were very similar to DKG.[30]

Kinetics of F-ASA Degradation Under Oxidative Conditions

Figure 2A shows the proton decoupled NMR spectra of F-ASA undergoing Cu^{2+} catalyzed degradation in PBS as a function of time. The kinetics data are plotted in Fig 2B. F-ASA was completely degraded within 2 hours of incubation. From the spectrum of the sample incubated for 96 hours, 4 very stable, yet unidentified degradation compounds from F-ASA ($\delta_F = -211.55, -212.45, -213.31, -214.20$ ppm) were observed. The 2 compounds at −211.55 and −214.18 ppm were degradation products formed at a late stage in the oxidation of F-ASA. On the other hand, compounds such as those at −212.19 and −214.0 ppm (F6 at 15, 30, and 60 minutes) and −218.2 ppm (at 30 and 60 minutes) were clearly intermediate compounds. Figure 2B (inset) shows that the major stable degradation compound accounting for 30% of total F-ASA was at −212.45 ppm.

The kinetics of F-ASA degradation were compared with those of F-DHA under identical experimental conditions to find

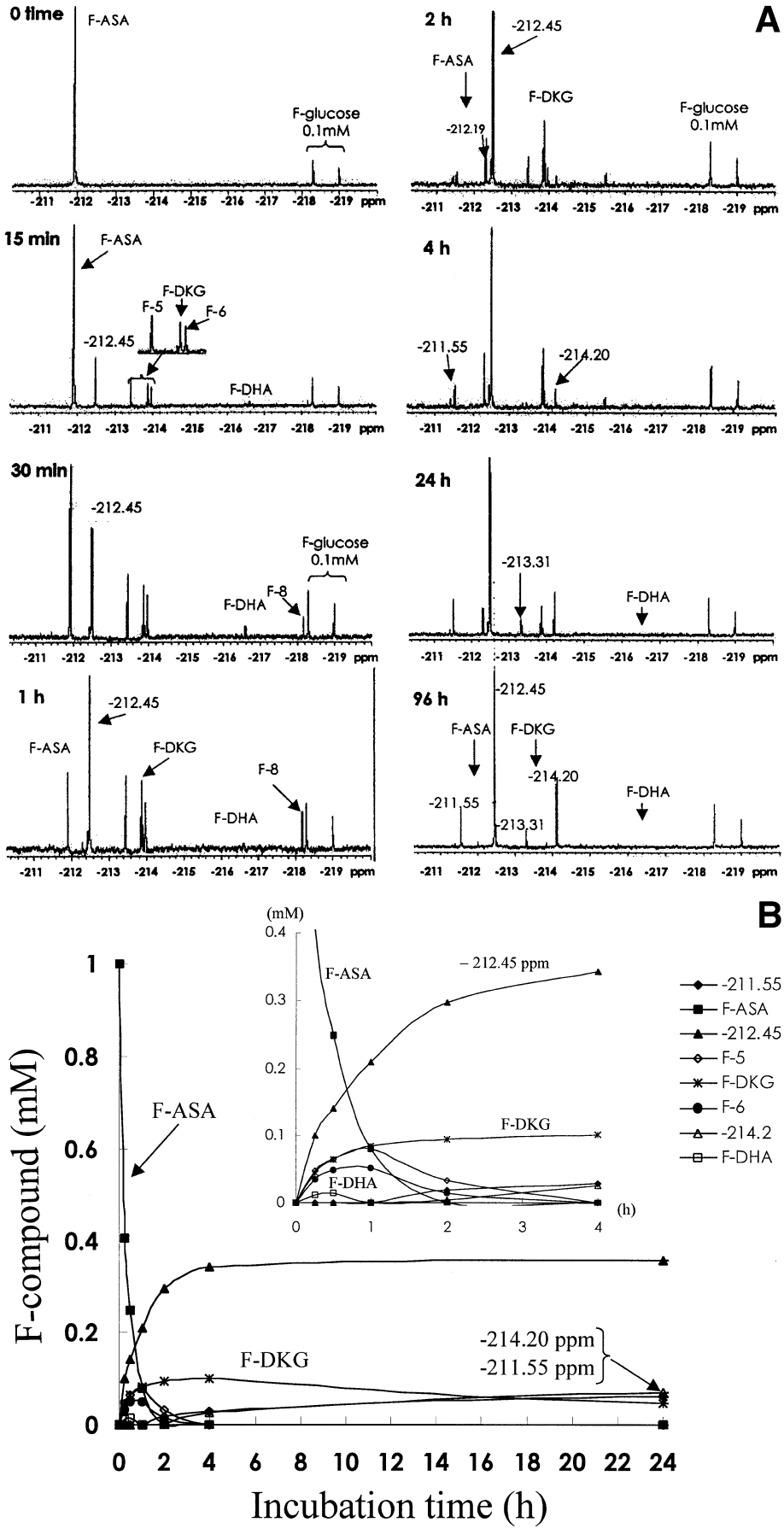

Fig 2. ^{19}F-NMR spectra showing the in vitro kinetics of F-ASA degradation and formation of F-degradation compounds. F-ASA (1 mmol/L) was incubated in PBS (pH 7.4) containing 10 μmol/L $CuCl_2$ at 37°C for 0 minute, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 4 days. (A) NMR spectra. (B) Quantitative analysis based on the added internal standard (0.1 mmol/L 6-F-glucose).

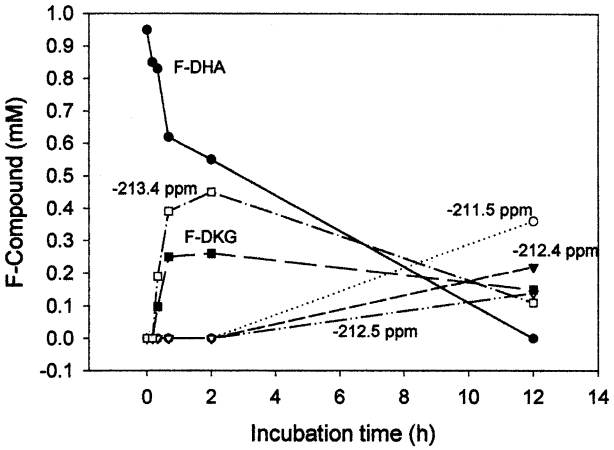

Fig 3. In vitro kinetics of F-DHA degradation and formation of F-degradation compounds. F-DHA (1 mmol/L) was incubated in PBS (pH 7.4) containing 10 μmol/L $CuCl_2$ at 37°C for 0 minute, 10 minutes, 20 minutes, 40 minutes, 120 minutes, and 12 hours.

out if the products formed were identical (Fig 3). To our surprise however, while the major compound from F-ASA was the unknown compound at −212.4 ppm, the major F-DHA degradation product was a compound at −213.4 ppm. This compound appeared to be progressively converted into the −211.5 and/or −212.4 ppm compounds, but only very slowly. However, because the −212.4 ppm compound formed rapidly from F-ASA, this experiment clearly suggests the presence of a novel F-ASA degradation pathway that is not dependent on F-DHA formation.

Body and Tissue Weights

Table 1 shows body and tissue weights of N and DB at sacrifice. The body weight of N was about twice as high as in DB. Whereas the weights of brain and adrenal glands were not significantly different between N and DB, liver weight was significantly lower and that of the kidney was significantly higher in DB ($P < .05$). Surprisingly, both heart and spleen weights from DB were much smaller than those of N ($P < .01$).

Concentration of F-ASA and F-DHA in Plasma and Tissues

Rats were killed and their tissues were taken 15 hours after the last injection of F-ASA (2 × 4 mg/rat/d, for 5 days). Based on previous reports suggesting that plasma ascorbate levels are low in diabetic rats.[31] it was decided to inject them with the same total dose of F-ASA as the control rats, ie, approximately a twice higher dose per body weight, to ensure the highest possible tissue levels. Figure 4 shows typical ^{19}F-NMR spectra of the tissue samples. All samples were measured with proton decoupling, and had to be scanned for at least 30 minutes to detect F-ASA degradation products that were expected only in small concentrations. Under such conditions, the smallest visible signal in a spectrum corresponded to a concentration of approximately 5 μmol/L F-compound based on the internal standard consisting of added 100 μmol/L 6-F-D-glucose. The NMR signals for the latter were split into 2 peaks corresponding to the expected pyranose and fyranose conformers present in 60% to 40% ratios, respectively.

Table 2 summarizes in quantitative form the data shown in Fig 4. The concentration of total F-ASA in liver, heart, brain, kidney, and spleen was statistically not different between N and DB. On the other hand, the total F-ASA concentration was highest in adrenal glands and more than twice higher in DB than in N ($P < .01$). The concentration of total F-vitamin C in plasma 30 minutes after the injection of F-ASA was much lower in DB than in N (42.24 ± 16.38 μmol/L (N) *v* 27.00 ± 9.92 μmol/L (DB) ($P < .05$) despite lower body weight. Absolute F-ASA levels were also significantly lower in kidney, spleen, and plasma, but not in heart, brain, and adrenal glands. The ratio of F-DHA to F-ASA varied considerably among individual tissue. Figure 5 shows the percentage of oxidized F-ASA ie the F-DHA to total F-vitamin C (F-ASA + F-DHA) ratio, a parameter reflecting either oxidant stress or low DHA reduction capacity. F-ASA was kept almost entirely in its reduced form in liver, brain, and adrenal glands of N and DB, but the liver of DB was much more tilted toward oxidation than N ($P < .01$). On the other hand, oxidation of F-ASA was increased in heart in both N and DB, but diabetes had no additional effect. Levels of F-ASA were also significantly worse in plasma ($P < .05$), kidney, and spleen (both, $P < .01$) from diabetic rats. Surprisingly, except for F-DHA, no other degradation products were detected at about 5 μmol/L sensitivity in any tissue or plasma samples of N and DB.

Urinary Elimination

Urine was collected into metaphosphoric acid for 24 hours starting 30 minutes after the last injection of F-ASA (4 mg/rat/d for 4 days) at 25 weeks of age (after 4 months of diabetes). Figure 6 shows typical ^{19}F-NMR spectra of deproteinized N and DB urine. The main fluorodegradation compounds in urine from N and DB were much the same and included F-DKG, compounds F-1 to F-7, but very little F-DHA, especially in diabetic rat. The signal of F-ASA floated from −211.9 ppm to about −212.2 ppm compared with the samples in which F-ASA was incubated in PBS (Fig 2A). The signals of F-ASA in all urine samples were confirmed by addition of F-ASA. The quantity of total fluoro-compounds from F-ASA eliminated into urine was 4.19 ± 0.38 μmol/24 hours (N) *v* 8.93 ± 1.42 μmol/24 hours (DB). After 24 hours, the recovery of F-ASA injected IP was 18.6% (N) and 39.7% (DB). Thus, the urinary loss of F-ASA in DB was much worse than in N ($P < .05$). Interestingly, it even appears that the ^{19}F-NMR pattern of urinary products seen in DB is somewhat simpler than that of normal rats in that several minor signals between F-4 and F-7 are missing. This could be the result of hyperfiltration or the impaired ability of the diabetic kidney to perform certain detoxification reactions. Using the data in Table 2 and Fig 7, and an average 24-hour urinary volume of 8 and 50 mL that was recorded in N and DB, respectively, the F-ASA clearances in N and DB mice were found to be 0.016 and 0.11 mL/min, respectively, ie, 6.8-fold higher in diabetes.

F-ASA Incubation in Urine

Finally, experiments were performed to investigate whether the pattern of the observed urinary products from diabetic and

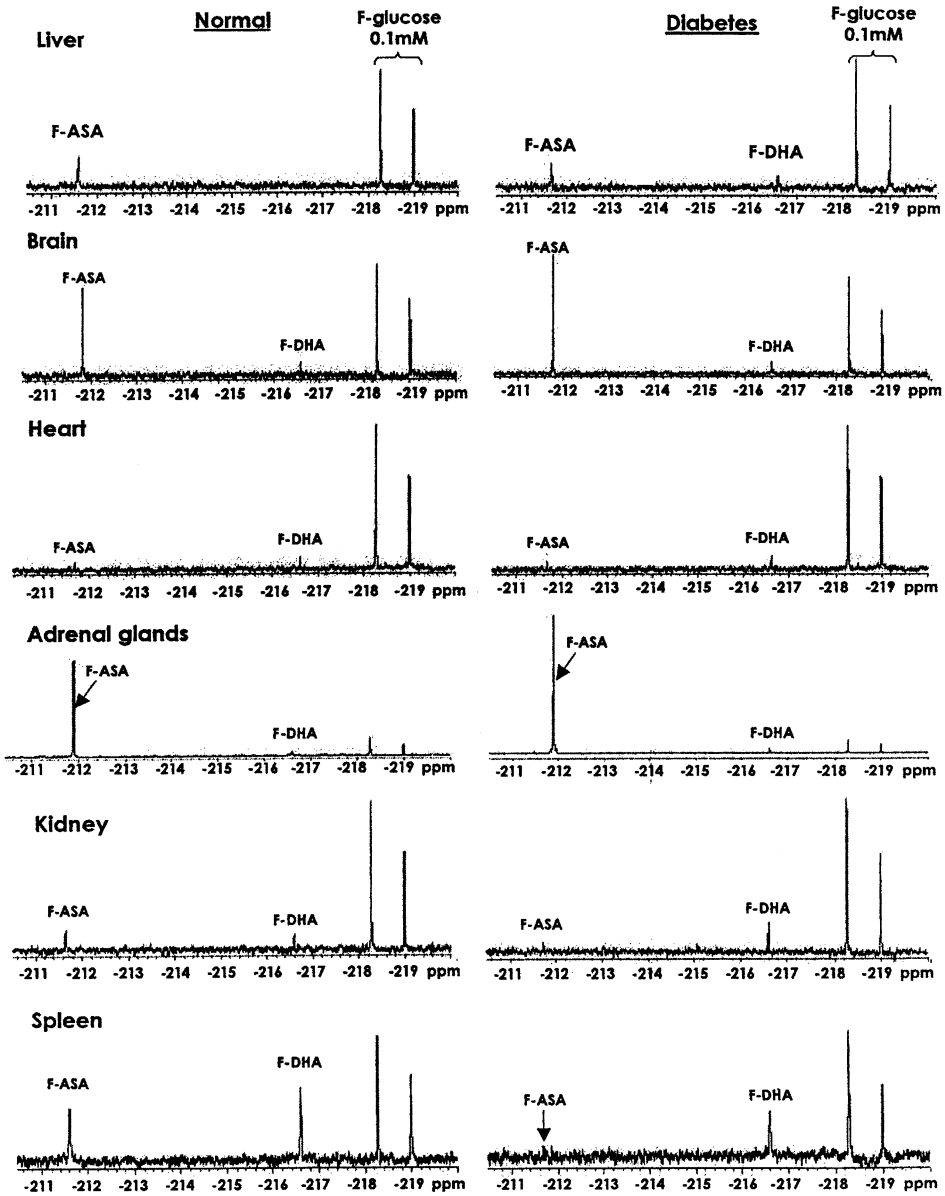

Fig 4. Typical ^{19}F-NMR spectra of normal and diabetic rat tissues. All samples were measured for 30 minutes by ^{19}F-NMR with proton decoupling. 6-F-Glucose (0.1 mmol/L) was added as the internal standard.

normal rats resembled primarily that of metal or nonmetal catalyzed ascorbic acid degradation. Figure 8 shows the ^{19}F-NMR spectra of F-ASA incubated for 24 hours in N urine to mimick the effects of urine itself on F-ASA degradation. F-ASA in N urine was completely degraded into essentially a single product at −212.45 ppm. This peak was also the dominant peak in F-ASA that underwent Cu^{2+} catalyzed degradation (Fig 2A, Fig 8) and likely corresponds to F-3 in urine from normal and diabetic rats (Fig 6). However, it is clear that none of the fluorodegradation compounds detected in N and DB urine after IP injection of F-ASA (F-1, F-2, F-3, F-5, F-6, and F-7) were present in urine that was left standing for 24 hours with 1 mmol/L F-ASA. Thus, it is apparent that the signals observed in urine from diabetic and normal rats did not originate from in situ degradation of F-ASA during the 24-hour collection, and therefore must be the result of biologic degradation. Furthermore, the pattern of urinary F-ASA degradation products has some features in common with metal catalyzed degradation (F-3, F-5, F-6, F-DKG), while others are quite different (F-1, F-2, F-4) and may represent the effects of reductases and oxidases onto intermediate degradation products of F-ASA.

DISCUSSION

The need to assay a large number of metabolites as a complement to genomics and proteomics is expected to grow dramatically in coming years. In that regard, the utilization of isotopically labeled ASA, such as 6-deoxy-6-fluoro ascorbic acid, has for the first time allowed us to explore in a comprehensive manner ASA homeostasis and degradation in an animal

Table 2. F-ASA and F-DHA Concentration in Normal and Diabetic Rat Tissues and Plasma Calculated From ^{19}F-NMR Spectral

	F-ASA	F-DHA	Total F-ASA
Liver			
N	4.80 ± 1.20	N.D.	4.80 ± 1.20
DB	4.05 ± 0.71	0.77 ± 0.35*	4.83 ± 0.67
Brain			
N	10.10 ± 1.80	0.81 ± 0.13	10.91 ± 1.81
DB	12.45 ± 3.40	0.72 ± 0.45	13.17 ± 4.37
Adrenal glands			
N	154.77 ± 26.86	8.91 ± 1.97	163.68 ± 28.64
DB	372.61 ± 121.30†	15.49 ± 7.17	388.10 ± 126.53†
Heart			
N	0.30 ± 0.20	0.74 ± 0.17	1.04 ± 0.14
DB	0.56 ± 0.54	0.72 ± 0.44	1.29 ± 0.60
Kidney			
N	4.62 ± 1.34	4.70 ± 1.59	9.32 ± 2.17
DB	0.97 ± 1.36*	7.30 ± 4.33*	8.27 ± 4.71
Spleen			
N	1.45 ± 0.34	0.67 ± 0.17	2.12 ± 0.44
DB	0.48 ± 0.29*	1.41 ± 0.24*	1.89 ± 0.29
Plasma			
N	25.33 ± 14.65	16.91 ± 9.65	42.24 ± 16.39
DB	9.55 ± 11.76†	17.45 ± 11.05	27.00 ± 9.92†

NOTE. Tissue: μmol/100 g tissue weight; plasma: μmol/L.
*$P < .01$,
†$P < .05$ with normal rats.

model of intracellular oxidant stress, ie, the streptozotocin diabetic rat. The structure of F-ASA and its oxidation kinetics by copper ion were very similar to those of native ASA.[27] Like ASA, F-ASA was degraded into F-DHA, F-DKG, and other compounds (Fig 2), and F-ASA was the major form of vitamin C that accumulated in adrenal glands[32] (Table 2).

Obviously, one major strength of the approach used in this study is that all fluorinated products could be assayed and quantitated simultaneously with minimal loss or alteration due to sample processing. One potential disadvantage, however, is that the fluorine in position 6 may interfere with the biology of F-DHA. Studies by Rumsey et al[33] have shown that ASA fluorinated in position 6 is an effective inhibitor of ASA transport, and that F-ASA is taken up into cells by a sodium-dependent ASA transporter. In contrast, F-DHA is unable to form a stable cyclic hemiketal structure, thus preventing its uptake by GLUT1 or GLUT3 transporters and slightly reducing its half-life compared with native DHA.[27] However, we have demonstrated in the lens that F-DHA, like F-ASA, is also taken up by a sodium-dependent transporter, most likely SVCT2 and immediately and quantitatively reduced to F-ASA, provided the cellular redox system is intact.[34] Thus, because both compounds are taken up by the same transporter, the tissue F-DHA/F-ASA ratio is expected to accurately reflect the cellular redox

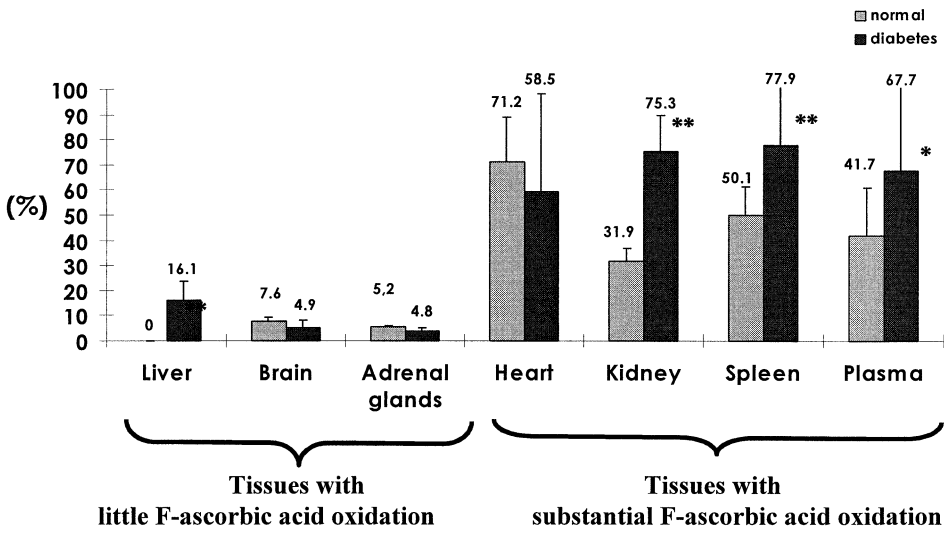

Fig 5. The mean percentage ± SD of F-DHA to total F-ASA (F-ASA+F-DHA) in tissues and plasma. **$P < .01$, *$P < .05$ with normal rats. The numbers above the bars indicate the percentage in numeric form.

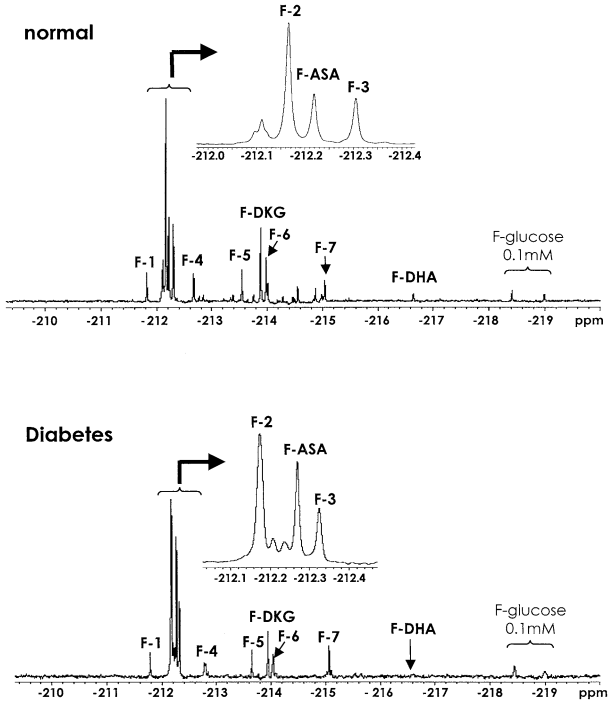

Fig 6. Typical ^{19}F-NMR spectra of normal and diabetic rat urine. Normal and diabetic rats were injected F-ASA (4 mg/d/rat) for 4 days before urine collection. Urine was collected into metaphosphoric acid for 24 hours starting 30 minutes after the last injection of F-ASA. The main degradation products of F-ASA are labeled F1 to F7.

state of ascorbic acid, regardless of whether F-DHA is taken up or formed from in situ oxidation of F-ASA.

Our data confirm observations obtained with other methods on the effects of diabetes on plasma and kidney levels of ASA in rats.[7,8,35,36] Most interestingly, despite injecting a higher dose of F-ASA per unit of body weight to diabetic than control rats, F-ASA plasma levels 30 minutes after injection were much lower in diabetic than normal rats, while plasma F-DHA levels were almost identical (Table 2). Thus, the elevated F-DHA/F-ASA ratio would suggest the presence of oxidant stress in the plasma (Fig 5). However, while the intracellular F-DHA/F-ASA ratio likely reflects intracellular oxidant stress, the tilting of this ratio toward oxidation in plasma appears to be strongly influenced by the massive urinary loss of F-ASA (Fig 7). Therefore, one cannot interpret the increased F-DHA/F-ASA plasma ratio as necessarily reflecting increased oxidative activity in the plasma, although the loss of plasma F-ASA would contribute toward weakening of the total free radical trapping activity of the plasma. Indeed studies have shown that plasma ASA is a contributor to total human and rat plasma antioxidant status in the total radical-trapping antioxidant potential (TRAP) assay.[37,38]

A major finding in this study is that none of the many F-ASA advanced degradation compounds that were observed during in vitro metal catalyzed oxidation or during spontaneous degradation in the urine (Fig 6) were detected in plasma or the tissues assayed in this study. In both normal and diabetic rats, tissue total F-vitamin C was almost entirely in form of F-ASA and F-DHA. In 3 tissues, ie, liver, brain, and adrenal glands, more than 80% of F-compounds were present in reduced form in both normal and diabetic rat (Fig 5), and both brain and adrenal glands were highly resistant toward oxidation in diabetes. Similar data were found in brain and liver by Kashiba et al.[35] In contrast, even in the absence of diabetes, baseline oxidation activity as reflected by F-DHA levels was much higher in heart, kidney, and spleen than liver, brain, and adrenal glands. No comparative data on this relationship could be found in the literature.

There may be various reasons why some tissues have higher levels of oxidized ASA and are more or less susceptible to the effects of hyperglycemia. These include increased oxidation by redox active metal copper complexes,[39-41] redox active methylglyoxal protein adducts,[42] increased oxidation by mitochondria-derived superoxide radicals,[43] increased F-ASA oxidation by peroxynitrite radical,[44] and decreased antioxidant defenses. Evidence for the presence of redox active metal complexes has been described in human lenses and arteries from normal or diabetic human and monkey[39,45,46] peritoneal fluid[25] and plasma from humans with ESRD.[47] However, in diabetic humans without ESRD, there is no evidence for a net increase in metal or nonmetal catalyzed oxidation in plasma and extracellular fluid as reflected by, eg, methionine oxidation or plasma CML.[25,48] As discussed above, the most likely explanation for the low plasma levels of F-ASA in diabetic rats appears to be renal loss.

One of the main protective mechanisms responsible for keeping ASA in reduced form is glutathione (GSH). In diabetic rats, GSH is easily depleted from lens, red blood cells, nerves, and kidney and is more oxidized in the heart,[49-52] although no

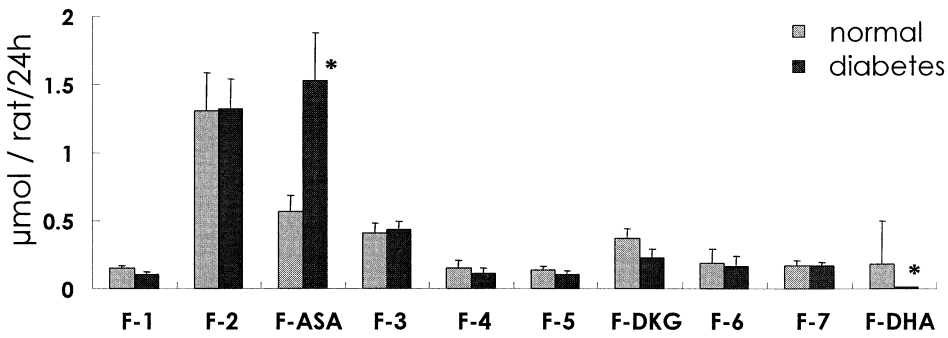

Fig 7. Comparative quantitative analysis of F-ASA and its degradation products in the urine collected from normal and diabetic rats for 24 hours after the last injection. *$P < .05$ with normal rat urine.

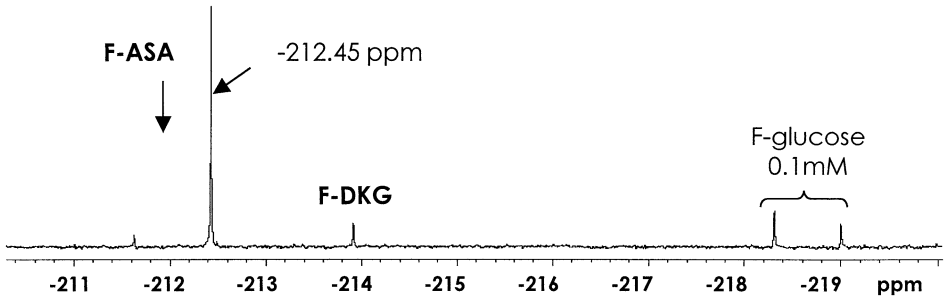

Fig 8. The ^{19}F-NMR spectra of F-ASA (1 mmol/L) incubated in urine of normal rat at 37°C for 24 hours to mimick potential F-ASA degradation in stagnant urine.

change in kidney, brain, and heart were observed by Rausher et al.[53] Thus, loss of GSH may be an important contributory factor for the increased oxidant stress in diabetes that is reflected in the increased F-DHA/F-ASA ratio.

Finally, individual differences in uptake capacity and retention of ASA may adversely affect the F-ASA/F-DHA tissue ratio. The highest F-ASA concentration was found in adrenal glands,[32,54] whereby the F-ASA level in adrenal glands from diabetic rats was surprisingly more than twice higher than in normal rat. This finding may reflect the relatively higher dose of injected F-ASA per unit of body weight, since F-ASA values were half as high when the doses were decreased by 50% (not shown). This tissue, however, must have a very high-affinity transport system because ASA levels were normal despite a 50% lower plasma level in diabetic compared with normal rats. Indeed, the presence of the SVCT-2 transporter was reported in adrenal glands.[55] Thus, it may be that there is a compensatory mechanism that protects adrenal glands from oxidative stress due to hyperglycemia.

As discussed previously, hyperglycemia might inhibit DHA uptake via competitive inhibition of GLUT1 and GLUT3.[56] This, however, is not expected to be an issue in this study, because F-DHA is apparently not taken up by these transporters. Nevertheless, some inhibition of F-ASA uptake may have occurred based in Ngkeekwong's findings that ASA transport into lymphoblasts was inhibited at km of 20 mmol/L glucose, while mean glucose concentration in our rats was 21 mmol/L on the average. Does this mean there is another ASA transporter or simply a downregulation of SVCT-2 in diabetes?

One peculiar finding was the extreme decrease in the weight of the spleen and the heart of diabetic rats. This finding has, to our knowledge, not been previously reported. While the heart weight is known to correlate with body weight, the low weight of the spleen in experimental diabetes has not been reported. The dramatic decrease in tissue weight together with the increased F-DHA/F-ASA ratio is compatible with an increase in free radical-mediated apoptosis in this tissue,[57] possibly due to mitochondrial superoxide formation.[43] One triggering factor could be an increase in ACTH leading to corticosterone-induced apoptosis of lymphocytes.[58,59]

Except for F-DHA and F-DKG, the chemical nature and tissue origin of most degradation compounds found in the urine remains to be established. Previous studies by others on ASA catabolism using 1-^{14}C-ASA showed that oxalate, ascorbate-2-sulfate,[60,61] and 2-O-methylascorbic acid[62] could be recovered in the urine. However, most of these compounds were minor, and mainly 1-^{14}C-ASA was excreted in the urine. In contrast, in our study the concentration of F-2 was much higher than that of F-ASA. While this study was in progress, Ortwerth et al[15] have used deuterated ascorbic acid and reported the formation of erythrulose as the major degradation product of ascorbic acid under nonoxidative conditions. It may well be that the major peak at −212.45 is identical to F-erythrulose[15] (Fig 8) and corresponds to F3 (Fig 6). If so, however, it is clear that F-erythrulose is but one of many degradation products of ascorbic acid in vivo. Other F-compounds present in the urine could include F-erythritol or F-erythronic acid, both of which are known to occur in urine.[63]

A prominent finding in this study is the rapid urinary loss of F-ASA. At 24 hours, 40% of injected F-ASA appeared in the urine from DB rats, while only 18% was excreted by the normal rat. Urinary volume of diabetic rats was on the average 6.25-fold than from the control rats. The single major reason for the loss would thus appear to be increased filtration, most likely coupled with a decreased tubular reuptake of F-ASA. Quantitation of the sodium-dependent ASA transporters in proximal renal tubules will be needed to clarify the extent to which saturation of these receptors has occurred. The latter hypothesis would appear less likely, because total F-ASA level in plasma, kidney, and spleen was much lower in diabetic than in normal rats, suggesting that F-ASA level in diabetic rats did not reach the saturation limit.

In summary, this study demonstrates that F-ASA is a very powerful tool for the clarification of the ascorbic acid homeostasis and catabolism in vivo and suggests that increased clearance of F-ASA is a major reason for the loss in plasma levels found in diabetes. While some tissues, such as brain and adrenal glands are able to preserve ascorbic acid in reduced form, accelerated ascorbic acid oxidation is apparent in kidney, spleen, heart, and liver. Surprisingly, except for the lens,[34] advanced ascorbic acid degradation products are found only in the urine, suggesting once formed they are rapidly eliminated from the tissue or possibly bound to protein, eluding thus detection.

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