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Gas chromatographic–mass spectrometric analysis of erythrocyte 3-deoxyglucosone in hemodialysis patients

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Abstract

The erythrocyte levels of 3-deoxyglucosone (3-DG) were measured by a selected ion monitoring method of gas chromatography–chemical ionization mass spectrometry using [$^{13}C_6$]-3-DG as an internal standard. Because the erythrocyte levels of 3-DG measured after deproteinization using ethanol were 18 times higher than those using ultrafiltration, we used ethanol deproteinization for measurement of total 3-DG in the erythrocytes. The concentration of 3-DG was significantly elevated in hemodialysis (HD) patients compared with healthy subjects. Although HD treatment could remove the erythrocyte 3-DG efficiently, its post-HD levels were still elevated compared with the healthy subjects.

Keywords: 3-Deoxyglucosone

1. Introduction

In the early stage of the Maillard reaction, glucose reacts nonenzymatically with protein amino groups to form Schiff base adducts which are then converted to stable Amadori products. In the intermediate stage of the Maillard reaction, the Amadori products can then undergo multiple dehydration and rearrangements to produce highly reactive dicarbonyl compounds such as 3-deoxyglucosone (3-deoxy-D-erythro-hexos-2-ulose: 3-DG) [1,2]. 3-DG reacts again with free amino groups, and leads to the formation of advanced glycation end products (AGEs) in the late stage of the Maillard reaction. Several AGEs are demonstrated to be formed by reacting 3-DG with proteins, such as imidazolone [3–7], pyrraline [8], N^{ε}-carboxymethyllysine (CML) [9], and pentosidine [10]. AGEs detected in vivo show a number of biological activities, and are involved in the pathogenesis of diseases such as aging, diabetes and uremia [11–13].

Glyoxal and methylglyoxal are also proposed to be important reactive precursors for AGE formation. However, any studies have never reported their increased plasma concentrations in uremic patients. On the other hand, 3-DG levels are measured in plasma and urine, and its plasma levels are increased in uremia as well as diabetes. Further, 3-DG not merely stimulates the formation of AGEs, but also has some biological activities in vitro [14–16]. Based on these evidences, we consider that 3-DG is a more clinically relevant precursor for AGE formation relating to the occurrence of diabetic and uremic complications.

Although the presence of 3-DG in vivo was suspected, its detection was difficult in blood because of its instability as the other dicarbonyls such as methylglyoxal and glyoxal. Knecht et al. [17]

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measured plasma 3-DG levels in healthy subjects using gas chromatography–mass spectrometry (GC–MS) after its reduction to 3-deoxyhexitol with $NaBH_4$ or $NaBD_4$. Niwa et al. [18] developed a simple method for the measurement of serum 3-DG using GC–MS and a stable isotope-labeled 3-DG ([$^{13}C_6$]-3-DG), and first demonstrated that serum 3-DG levels are increased in diabetic patients than in healthy subjects. Yamada et al. [19] measured 3-DG in rat plasma by high-performance liquid chromatography (HPLC) using 3,4-hexanediene as an internal standard. They monitored UV and fluorescence of 2-(2,3,4-trihydroxybutyl)-benzo[g]quinoxaline which was produced by reacting 3-DG with 2,3-diaminonaphthalene. Fujii et al. [20] reported an HPLC method with fluorescence detection in which 3-DG was first converted to 2-keto-3-deoxygluconic acid by oxoaldehyde dehydrogenase, although the method is inadequate for 3-DG in blood samples. However, the plasma 3-DG levels in healthy subjects varied 30-fold depending on the methods. Recently, Lal et al. [21] clearly demonstrated that the discrepancy comes mainly from the different deproteinization methods.

In this study, we measured erythrocyte 3-DG levels in hemodialysis (HD) patients and healthy subjects by GC–MS using [$^{13}C_6$]-3-DG as an internal standard. Further, to compare the effects of different deproteinization methods on the erythrocyte 3-DG levels, they were measured by deproteinizing the erythrocyte samples using ethanol or ultrafiltration.

2. Experimental

2.1. Patients

Blood samples were obtained from 12 HD patients (4 males, 8 females; 67.2±12.8 years old, mean±SD) before and after HD, and 11 healthy subjects (6 males, 5 females). The patients had been treated by HD for 6.3±5.3 years. HD was performed for 4 h three times a week. The heparinized blood was centrifuged to separate erythrocytes from plasma. After removing plasma and buffy coat, erythrocyte fraction was kept at −20°C until sample preparation.

2.2. Sample preparation for GC–MS

3-DG levels in erythrocytes were measured by using GC–MS, according to the modified method of Niwa et al. [18,22–24]. After addition of [$^{13}C_6$]-3-DG (1.19 nmol) as an internal standard, hemolyzed erythrocytes (100 μl) were diluted with distilled water (900 μl). The mixture was deproteinized by adding ethanol (2 ml), and subsequently centrifuged at 1000 *g* for 10 min at 4°C.The supernatant was applied to a Bond Elut SCX cartridge (cation-exchange, 100 mg in 1 ml; Analytichem International, Harbor City, CA, USA), and eluted with distilled water (3 ml). The collected eluate was then applied to a Bond Elut SAX cartridge (anion-exchange, 100 mg in 1 ml; Analytichem International), and eluted with distilled water (3 ml). The eluate was collected, and lyophilized. The carbonyl groups of the residue were transformed to their methoxime (MO) derivatives at 70°C for 30 min with 1% methoxylamine hydrochloride (Sigma Chemical, St. Louis, MO, USA) in pyridine (200 μl). After evaporation over nitrogen stream, the residues were kept at −20°C until GC–MS analysis.

2.3. GC–MS analysis

The hydroxyl groups in the residues were converted to their trimethylsilyl (TMS) derivatives at 60°C for 20 min with *N*,*O*-bis(trimethylsilyl)trifluoroacetoamide (BSTFA) (20 μl) containing 1% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, USA). After cooling to room temperature, the sample (2 μl) was subjected to GC–MS (GCMS-QP5000; Shimadzu, Kyoto, Japan) equipped with a capillary column (30 m×0.25 mm ID, 0.23 μm thick membrane; J&W Scientific, Folsom, CA, USA). The column temperature was programmed at 10°C/min from 100°C to 250°C, and the entrance pressure was 80 kPa with 20 ml/min of helium. For chemical ionization (CI), isobutane was used as a reactant gas. Quantitation of 3-DG was performed by selected ion monitoring (SIM) of *m*/*z* 337 for 3-DG and *m*/*z* 343 for [$^{13}C_6$]-3-DG as an internal standard.

2.4. Calibration for quantification of 3-DG

To quantify 3-DG in the erythrocyte samples, calibration lines were processed with SIM chromato-

grams. Amounts of 3-DG ranging from 50 to 500 ng were added to 200 ng of [$^{13}C_6$]-3-DG as an internal standard, these mixtures were processed as described above and analyzed by GC–MS. Calibration lines relating the concentrations of 3-DG to its peak area ratios at m/z 437 (3-DG) to the internal standard at m/z 443 ([$^{13}C_6$]-3-DG) were obtained from the SIM chromatograms. Correlation coefficient of the calibration line for 3-DG was 0.9998 ($y = 1.072x - 0.014$, y: concentration ratio, x: peak area ratio). The intra- and interassay coefficients of variation were 9.7% ($n = 3$) at a concentration of 4.5 μmol/l, and 16.7% ($n = 3$) at a concentration of 1.7 μmol/l, respectively. Recovery of 3-DG spiked to an erythrocyte sample at a concentration of 3.1 μmol/l was 91.1±6.0% (mean±SD, $n = 3$).

2.5. Comparison of deproteinization methods

Erythrocyte samples from patients were deproteinized in two ways: by ethanol as described above or by ultrafiltration as follows. Briefly, erythrocyte samples (100 μl) were diluted 20 times with distilled water. The samples were ultrafiltrated for deproteinzation by centrifuging at 1000 g for 30 min at 4°C using Centriflo filters (CF25, Amicon, Danvers, MA, USA). After addition of [$^{13}C_6$]-3-DG (1.19 nmol) as an internal standard, they were passed through the ion-exchange resins (SCX and SAX), and then the eluates were converted to MO-TMS derivatives.

To determine if 3-DG is absorbed by the filter or not, 3-DG solution in distilled water (1.23 nmol/ml) was ultrafiltrated using CF-25, and 3-DG concentration in the collected ultrafiltrate was measured by GC–MS. Recovery of 3-DG filtered by CF25 was 115%. This result demonstrates that 3-DG is not at all absorbed by the filter.

2.6. Statistics

Non-paired t test was applied to the comparison between healthy subjects and HD patients. Paired t test was used in the comparisons between before and after HD in HD patients. Significance was found in the case when the P value was less than 0.05.

3. Results

Fig. 1 shows a reconstructed ion chromatogram of 3-DG. In the chromatogram two major peaks are recognized between 12 and 13 min, which consist of 3-DG isomers. A major peak (A) is detected at a retention time of 12.1 min, and a minor peak (B) at 12.5 min. Fig. 2 show the mass spectra of 3-DG (A,B) and [$^{13}C_6$]-3-DG (C, D). The base ions are observed at m/z 437.4 for 3-DG, and at m/z 443.4 for [$^{13}C_6$]-3-DG. Fig. 3 shows SIM chromatograms of 3-DG and [$^{13}C_6$]-3-DG in a standard sample and erythrocyte samples. The peak area ratio of the peaks at m/z 437 to m/z 443 at a retention time of 12.1 min was used for quantitation of 3-DG. Fig. 4 shows the calibration line for quantification of 3-DG.

Table 1 lists the erythrocyte levels of 3-DG in HD patients and healthy subjects. The HD patients showed significantly elevated erythrocyte levels of 3-DG as compared with the healthy subjects. In the HD patients, erythrocyte 3-DG levels did not show any significant correlation with age, serum

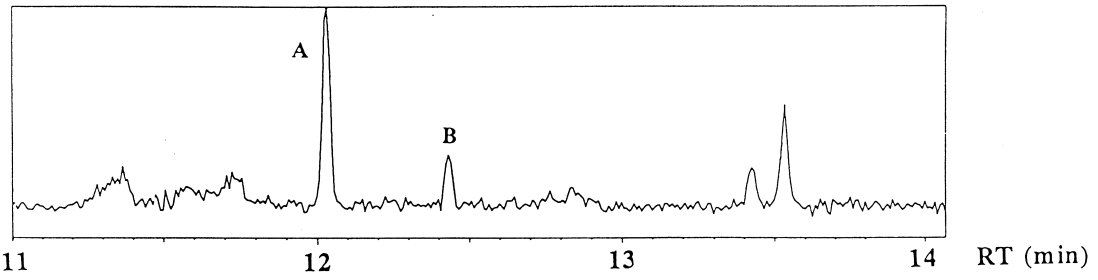

Fig. 1. Reconstructed ion chromatogram (RIC) for 3-DG obtained by gas chromatography–mass spectrometry (GC–MS) using chemical ionization (CI). Peaks A and B were identified as the methoxime-trimethylsilyl (MO-TMS) derivatives of 3-DG.

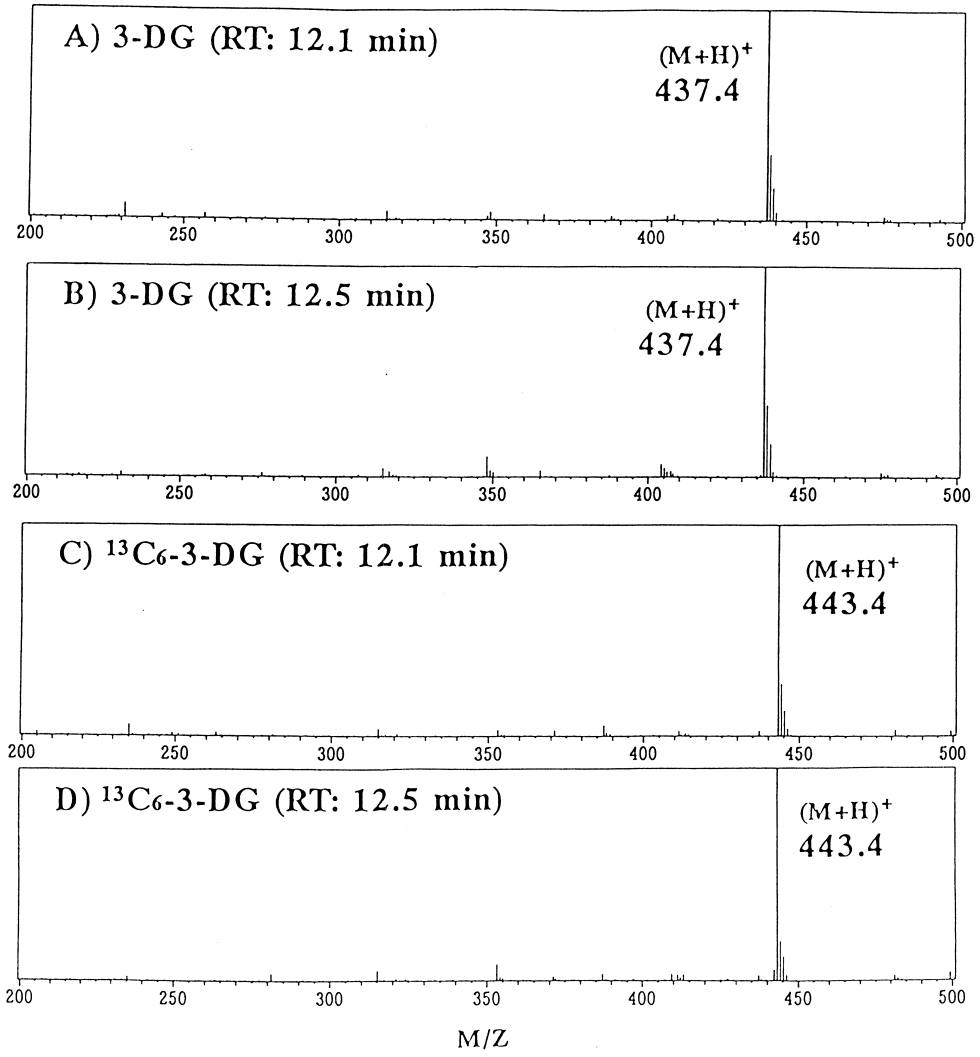

Fig. 2. Chemical ionization (CI) mass spectra of 3-DG (A and B) and [$^{13}C_6$]-3-DG (C and D).

creatinine, or HD duration. The erythrocyte levels of 3-DG decreased after HD with a mean reduction rate of 49%, which is similar to those of serum 3-DG or creatinine. Even after HD, the erythrocyte levels of 3-DG in HD patients were significantly higher than those in normal subjects.

Table 2 shows comparison of erythrocyte 3-DG levels between two different deproteinization methods. The levels of erythrocyte 3-DG measured after deproteinization using ethanol were 18 times higher than those by ultrafiltration.

4. Discussion

The presence of 3-DG in vivo had been suggested for a long time, since it was generated under physiological conditions from incubation of sugars and amino acid [1,2]. Knecht et al. [17] measured the levels of 3-DG and 3-deoxyfructose in plasma and urine for the first time. Their method for plasma samples is as follows: [1,2-D]-3-DH, which is formed from reduction of 3-DG by $NaBD_4$, was added to the plasma sample as an internal standard

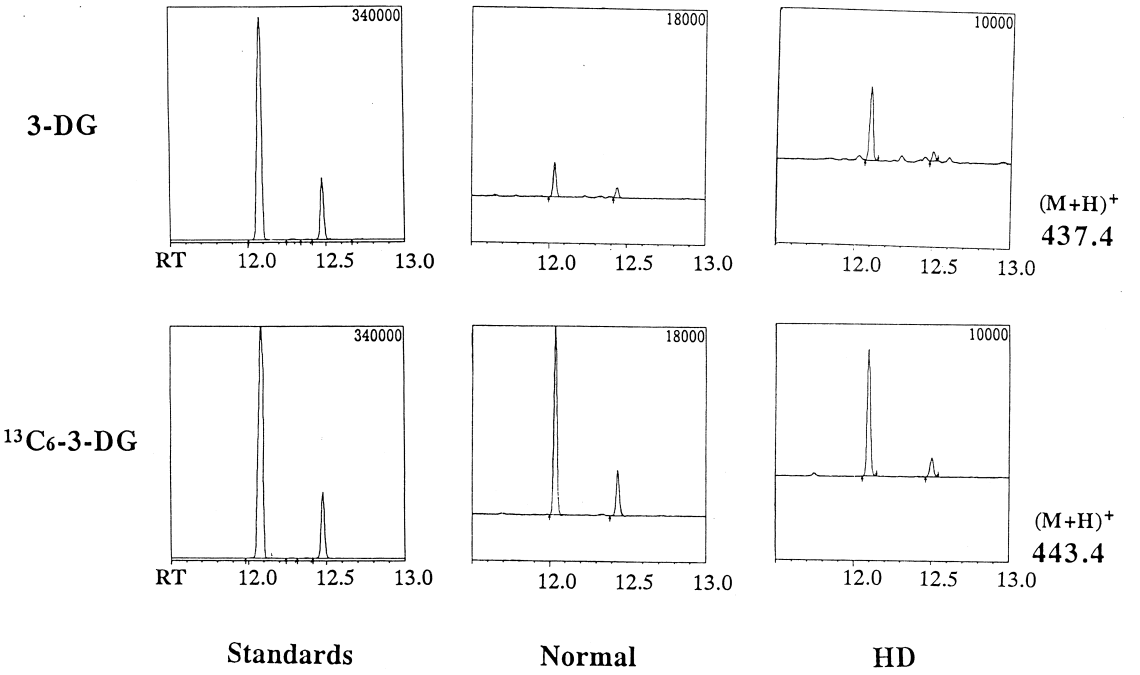

Fig. 3. Selected ion monitoring (SIM) chromatograms of 3-DG and [$^{13}C_6$]-3-DG in standards and erythrocytes from a normal subject and an hemodialysis (HD) patient.

for GC–MS analysis. In order to improve chromatographic resolution, glucose oxidase and catalase were added to the plasma sample to convert endogenous glucose to gluconic acid, and then incubated at 37°C. 3-DG and 3-deoxyfructose in plasma are reduced with $NaBH_4$ or $NaBD_4$. Protein was precipitated by addition of trichloroacetic acid. The supernatant extracted with diethyl ether was desalted on ion-exchange resins. The alditols were converted to per-*O*-acetyl derivatives, and then analyzed by GC–MS. This method enables fairly exact identification and quantitation of 3-DG, while a lot of procedures are required in sample pretreatment, and 3-deoxyfructose has to be subtracted from total 3-DH amount to obtain 3-DG level.

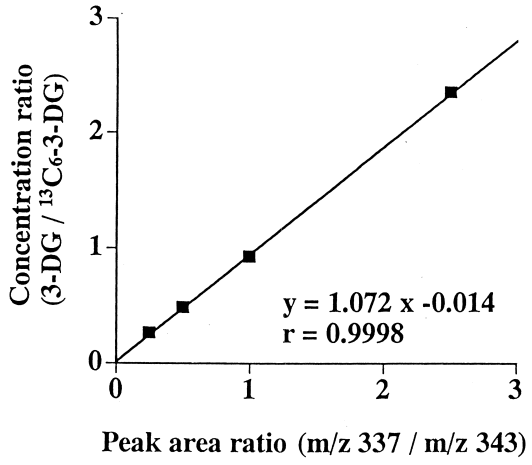

Fig. 4. Calibration line for measurement of 3-DG using selected ion monitoring (SIM) gas chromatography–mass spectrometry (GC–MS).

Niwa et al. [18,22–24] developed an alternative simple method using GC–MS and [$^{13}C_6$]-3-DG, and first demonstrated that serum 3-DG levels are increased not only in diabetic patients but also in uremic patients compared with healthy subjects.

Yamada et al. [19] reported measurement of 3-DG in rat plasma with a quite different method from those described above. Proteins in plasma sample were precipitated with perchloric acid. After addition of 2,3-diaminonaphthalene, the supernatant was incubated at 4°C overnight in the presence of 3,4-hex-

Table 1
Erythrocyte levels of 3-DG in HD patients and healthy subjects[a]

	Normal ($n=11$)	HD ($n=12$)	
		Before HD	After HD
Serum creatinine (μmol/l)	80±9[b]	850±70	320±30[b]
Erythrocyte 3-DG (nmol/g Hb)	13.8±1.6[b]	69.3±8.4	35.2±3.6[c]

[a] Values are expressed as mean±SE.
[b] $P<0.05$.
[c] $P<0.01$ as compared with before HD by non-paired t or paired t test.

anediene as an internal standard. Thus, 3-DG was converted by reacting with 2,3-diaminonaphthalene to a stable compound, 2-(2,3,4-trihydroxybutyl)-benzo[g]quinoxaline. The products were extracted by ethyl acetate, reconstituted with methanol, and used for HPLC. The effluent was monitored at UV (268 nm) or fluorescence (excitation: 271 nm, emission: 503 nm). Recently, Hamada et al. [25] investigated the effects of glycemic control on plasma 3-DG levels in diabetic patients according to the Yamada's method.

Fujii et al. [20] developed an enzymatic method in which 3-DG was first converted to 2-keto-3-deoxygluconic acid by oxoaldehyde dehydrogenase and detected as a fluorescent derivative of the acid using HPLC. They demonstrated an inhibitory effect of aminoguanidine on glycation. It is a pity that the technique is inadequate for 3-DG in blood since both human erythrocytes and plasma contain considerable amounts of 2-keto-3-deoxygluconic acid [26].

The methodology for measurement of 3-DG has been controversial. For example, plasma 3-DG levels in these reports varied 30-fold. Knecht et al. [17] and Hamada et al. [25] reported that the levels of plasma 3-DG in normoglycemics were 62 and 79 nmol/l, respectively. In contrast to these, Niwa et al. [18] reported that the serum 3-DG level was 1900 nmol/l in healthy subjects. To resolve this disagreement, Lal et al. [21] developed another GC–MS procedure using [$^{13}C_6$]-3-DG as an internal standard. 3-DG was reacted with 2,3-diamino naphthalene, and then converted to a silyl ether. 3-DG levels in normal plasma were compared between two different methods, ultrafiltration or ethanol, and were found to be 58 nmol/l and 1700 nmol/l, respectively. These results demonstrate that the discrepancy mainly comes from the deproteinzation methods, and that the greatly increased 3-DG by ethanol extraction is likely to be due to ethanol-mediated release of 3-DG bound to plasma macromolecules, probably proteins. We obtained results similar to Lal's conclusion [21] by deprotenizing the samples using a filter or ethanol, and excluded the possibility that 3-DG is absorbed by the filter. Our results also suggest that 3-DG measured by deproteinization using ethanol include free and protein-bound forms in the erythrocytes.

Further, the other problems in the quantification of 3-DG may arise during its extraction and derivatization from plasma samples. An internal standard should always behave just like endogenous 3-DG during the steps. If stable-isotope-labeled 3-DG is used, its behavior is just the same as 3-DG at each step. If another compound is used as an internal standard, its behavior during the steps may be affected by its own chemical properties different from 3-DG, and consequently may influence the concentration of 3-DG calculated by the ratio of 3-DG to the internal standard. An isotope-labeled 3-DG is ideal as an internal standard, because it does

Table 2
Erythrocyte levels of 3-DG: comparison of deproteinization methods[a]

	Ethanol ($n=4$)	Ultrafiltration ($n=4$)
Erythrocyte 3-DG (nmol/g Hb)	64.8±9.8	3.6±0.3

[a] Values are expressed as mean±SE.

not affect the ratio of 3-DG to the isotope-labeled 3-DG after sample pretreatment, and it can be easily distinguished from 3-DG by using mass spectrometry.

3-DG levels may change during sample preparation. In order to remove glucose for better chromatographic resolution on GC–MS, glucose oxidase and catalase were added to plasma samples, and then incubated at 37°C. During this incubation, generation of 3-DG in vitro has been mentioned [27,28]. However, this generation of 3-DG does not account for the discrepancy in plasma 3-DG levels reported by Knecht [17] and Niwa [18], because both used the same incubation conditions. On the other hand, 3-DG may be lost during the incubation because of its rapid reaction with amino groups such as arginine or lysine.

This study had revealed that the mean level of erythrocyte 3-DG in healthy subjects is 6.2 μmol/l, which is three times higher than that in plasma (1.9 μmol/l) [18]. Erythrocyte 3-DG could be removed by HD with the reduction rate of 49%, which was similar to that of plasma [22,24]. Fujii et al. [26] demonstrated that 3-DG easily passes through erythrocyte membranes from incubation medium. However, the presence of a specific device to transport 3-DG across the membranes is not yet known. Glucose transporter 1 or such might work selectively, since sorbitol with the molecular weight of 182 does not transfer through erythrocyte membrane. Anyhow these results suggest that the bond between 3-DG and protein in erythrocyte is so labile that 3-DG binding to protein becomes free to be dialyzed. Further, the production of 3-DG in erythrocytes may be very rapid, since there is a marked difference between erythrocyte and plasma 3-DG levels despite the easy transition of 3-DG across the erythrocyte membranes.

3-DG is produced mainly via degradation of Amadori products in the Maillard reaction. However, another possibility for the production of 3-DG was pointed out by the discovery of fructose 3-phosphate in rat lens [27,28] and in human erythrocytes [29]. Fructose 3-phosphate, a potent crosslinking intermediate, yields 3-DG non-enzymatically [30]. Recently, Petersen et al. [31] reported that erythrocyte fructose is phosphorylated to fructose 3-phosphate. We consider that fructose and its metabolite, fructose 3-phosphate, play an important role in the formation of erythrocyte 3-DG especially in diabetic patients, because the polyol pathway is enhanced in hyperglycemia. However, we do not have a convincing evidence to explain for increased erythrocyte 3-DG levels in HD patients. 3-DG is detoxified to 3-deoxyfructose by 3-DG-reducing enzymes, and to 2-keto-3-deoxygluconic acid by oxoaldehyde dehydrogenase. The activities of these 3-DG-detoxifying enzymes in erythrocytes may be decreased in uremic patients.

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