

## PD FLUIDS CONTAIN HIGH CONCENTRATIONS OF CYTOTOXIC GDPs DIRECTLY AFTER STERILIZATION

Martin Erixon,<sup>1,2</sup> Torbjörn Lindén,<sup>1</sup> Per Kjellstrand,<sup>1</sup> Ola Carlsson,<sup>1</sup> Malin Ernebrant,<sup>1</sup>  
Gunita Forsbäck,<sup>1</sup> Anders Wieslander,<sup>1</sup> and Jan Åke Jönsson<sup>2</sup>

Corporate Research,<sup>1</sup> Gambro AB; Analytical Chemistry,<sup>2</sup> University of Lund, Lund, Sweden

◆ **Objective:** Glucose degradation products (GDPs) in peritoneal dialysis (PD) fluids are cytotoxic and affect the survival of the peritoneal membrane. One of the most reactive GDPs in PD fluids is 3,4-dideoxyglucosone-3-ene (3,4-DGE). 3,4-DGE has been reported as an intermediate between 3-deoxyglucosone (3-DG) and 5-hydroxymethyl furaldehyde (5-HMF) during degradation of glucose. In PD fluids, 3,4-DGE exists in a temperature-dependent equilibrium with a pool of unidentified substances. The aim of this study was to explore this equilibrium and its temperature dependence during the first months of storage after the sterilization procedure.

◆ **Methods:** GDPs and inhibition of cell growth (ICG) were measured directly after sterilization of the PD fluid and during storage at different temperatures for 60 days. The following GDPs were analyzed: 3-DG, 3,4-DGE, 5-HMF, formaldehyde, acetaldehyde, glyoxal, and methylglyoxal.

◆ **Results:** Immediately after sterilization, the concentration of 3,4-DGE was 125  $\mu\text{mol/L}$ . During the first weeks of storage, it decreased by about 80%. At the same time, the 3-DG concentration increased. None of the other GDPs were significantly affected. Cytotoxicity correlated well with the concentration of 3,4-DGE. When pure 3,4-DGE was substituted for the lost amount of 3,4-DGE after 30 days of storage, the initial ICG was almost completely regained.

◆ **Conclusions:** Heat sterilization of PD fluids promotes the formation of large quantities of 3,4-DGE, rendering the fluid highly cytotoxic. During storage, the main part of 3,4-DGE is reversibly converted in a temperature-dependent manner to a less cytotoxic pool, consisting mainly of 3-DG. Cytotoxicity seems to be dependent exclusively on 3,4-DGE. In order to avoid higher levels of 3,4-DGE concentrations, PD fluids should not be used too soon after sterilization and should not be stored at temperatures above room temperature.

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**KEY WORDS:** Cytotoxicity; 3,4-DGE; sterilization; storage; glucose.

Correspondence to: M. Erixon, Gambro AB, Box 10101, S-220 10 Lund, Sweden.

martin.erixon@gambro.com

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In order to remove waste products and water from patients with renal failure, peritoneal dialysis (PD) fluids contain an osmotic agent. The most widely used is glucose because it is cheap and nontoxic. It is easily metabolized and is the most important energy source for living cells.

Before use, PD fluids are sterilized. This is normally carried out through exposure of the fluids to high temperatures (approximately 120°C). After sterilization, the fluids are stored for up to 2 years before being used by patients. According to manufacturers, the storage temperature should be room temperature or below. The temperatures may possibly be considerably higher during transport, at the clinics, and at the patients' homes.

Glucose is known to degrade to carbonyl compounds during heat sterilization and during subsequent storage (1–3). These are generally referred to as glucose degradation products (GDPs). These GDPs have been reported as cytotoxic in a great variety of *in vitro* and *in vivo* models (4–10). The clinical relevance of GDPs has been investigated in several studies showing positive effects of effluent markers of peritoneal membrane integrity, such as cancer antigen 125, procollagen-1-C-terminal peptide, procollagen-3-N-terminal peptide, and hyaluronan (11,12). Furthermore, in some cases high concentrations of GDPs have been associated with chemical peritonitis in PD patients (13).

Several GDPs have been identified, including glyoxal, methylglyoxal, formaldehyde, 3-deoxyglucosone (3-DG), 3,4-dideoxyglucosone-3-ene (3,4-DGE), and 5-hydroxymethyl furaldehyde (5-HMF) (1–3,14), but the number of identified GDPs represents only a fraction of a large number of compounds that can be generated from glucose. 3,4-DGE was recently identified as the most biologically reactive GDP in PD fluids (2). The degradation pathway from glucose to 3,4-DGE is not known in detail, but 3,4-DGE has been reported as an intermediate between 3-DG and 5-HMF (15), and to exist in a temperature-dependent equilibrium with a pool of unidentified substances (2). The aim of the present study was to further explore this equilib-

rium and its temperature dependence during the first months of storage after the heat sterilization procedure.

## MATERIAL AND METHODS

### FLUIDS

Laboratory-made PD fluids were produced and the pH was adjusted to 5.5 with HCl. The fluids contained 83 mmol/L D-glucose (1.5%), 132 mmol/L sodium, 1.75 mmol/L calcium, 0.75 mmol/L magnesium, 102 mmol/L chloride, and 35 mmol/L lactate. In the experiments with conventional fluids, Gambrosol (Gambro AB, Lund, Sweden) was used.

### EXPERIMENTAL PROCEDURES

Glass bottles (500 mL) filled with PD fluid were heat sterilized in a steam autoclave (Getinge AB, Getinge, Sweden) at 121°C for 40 minutes ( $F_0 = 40$ ). Details on the sterilization procedure and  $F_0$  calculation have been described previously (16). After sterilization, the bottles, together with sterile-filtered controls, were incubated at 25°C and 40°C in the dark for a period of 60 days. During the incubation period, samples were aseptically collected on days 0, 1, 3, 6, 8, 15, 24, 30, 45, and 60, and immediately frozen at -80°C (on day 0 the sample was taken approximately 20 minutes after sterilization). On day 30, 3,4-DGE (80  $\mu\text{mol/L}$ ) was added to one set of bottles and the incubation continued for another 30 days. For this part of the experiment, samples were aseptically collected at days 30, 32, 35, 45, and 60. In a separate experiment, fluid from a conventionally manufactured 1.5% PD bag was incubated for 14 days at 5°C, 25°C, 30°C, 40°C, and 60°C.

### CHEMICAL ANALYSIS OF GDPs

Analyses of 3-DG, 3,4-DGE, 5-HMF, methylglyoxal, glyoxal, acetaldehyde, and formaldehyde were done using a high performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany) equipped with UV detection (1-3). The standards, 5-HMF, 3-DG, methylglyoxal, glyoxal, acetaldehyde, and formaldehyde were diluted in water; 3,4-DGE standard was extracted from a heat-sterilized glucose-containing fluid according to a previous description (2). Each sample collected was analyzed on three separate occasions. Values are given as mean  $\pm$  standard error of the mean (SEM).

### PROLIFERATION TESTS

Mouse L-929 fibroblast cells (CCL-1; ATCC, Rockville, Maryland, USA) were cultured to conflu-

ence in 25-cm<sup>2</sup> tissue culture flasks with cell culture media (MEM; Gibco, Paisley, Scotland) completed with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, Utah, USA), 1% glutamine, and 1% nonessential amino acids.

Human peritoneal mesothelial cells (HPMC) were obtained from the overnight dwell peritoneal effluent. The effluent was centrifuged at 1800g at 4°C for 10 minutes in 225-mL tubes. The pellet was washed twice in phosphate-buffered saline (PBS; Gibco) before it was suspended in 10 mL M199 medium (Gibco) supplemented with 10% vol/vol FCS and 0.1% gentamicin (Gibco). All experiments were performed using cells from at least five separate donors and in second or third passage.

The cells were seeded in a 96-well tissue culture plate at densities of 2500 (L-929) and 7000 - 12 000 (HPMC) cells/well. After 24 hours, the medium was removed and the samples to be tested were added. The samples consisted of freshly thawed PD fluid mixed either 1:1 or 3:2 with growth medium containing 20% FCS, nonessential amino acids, and 2% L-glutamine. The plates were incubated for 72 hours at 37°C before inhibition of cell growth (ICG) was determined by the neutral red assay (17).

Percent ICG was calculated using a control containing only growth medium as reference. Values of ICG for the differently stored samples are given as the difference between a heat-sterilized sample and corresponding filtered sample.

### STATISTICAL ANALYSES

All data are expressed as mean  $\pm$  SEM. Statistical evaluations were performed using one-way ANOVA and *post hoc* tested using Bonferroni test for multiple comparisons. The chosen level of significance was  $p < 0.05$ .

## RESULTS

### CHANGES IN GDPs AFTER STERILIZATION

Figure 1 demonstrates that, immediately after heat sterilization,  $125 \pm 4 \mu\text{mol/L}$  3,4-DGE was present in the laboratory-made PD fluid. The concentration rapidly decreased during the first weeks of storage. The decrease was faster at 40°C compared to 25°C. At 25°C, equilibrium was reached after 30 days; only about a week was needed at 40°C. After 8 days of storage, the level of 3,4-DGE was significantly higher ( $p < 0.05$ ) at 25°C compared to 40°C (no other GDPs were significantly affected). Equilibrium of 3,4-DGE was stabilized at a lower final value at 25°C than at 40°C. 3-DG increased by 77  $\mu\text{mol/L}$  during the first 30 days of

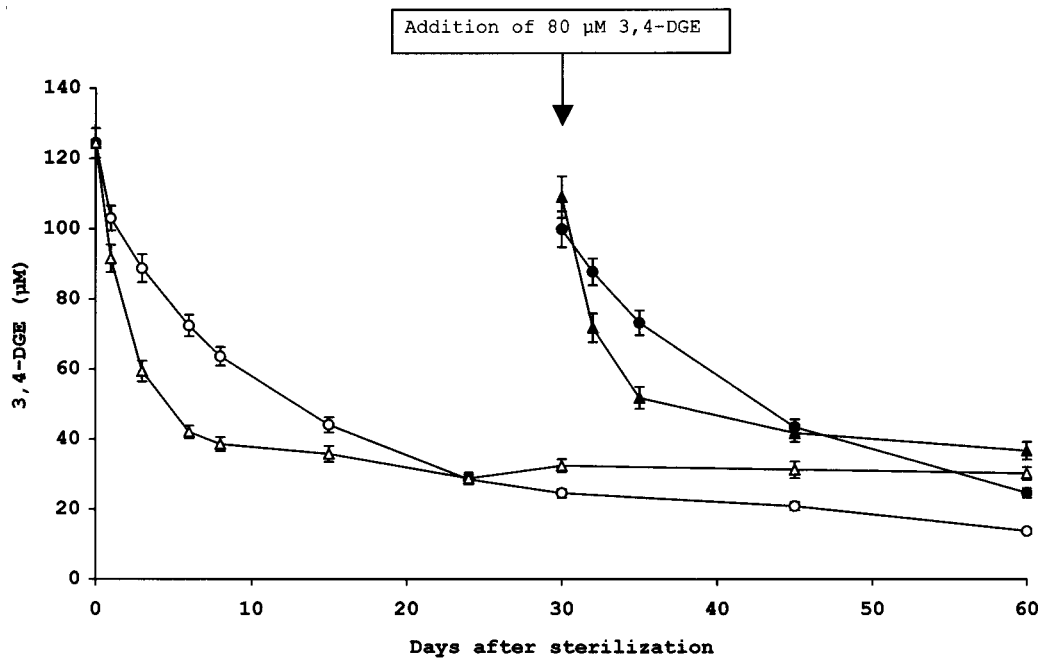


Figure 1 — 3,4-Dideoxyglucosone-3-ene (3,4-DGE) concentration in laboratory-made 1.5% peritoneal dialysis fluids incubated for 60 days at 25°C (open circles) and 40°C (open triangles). After 30 days, 80 µmol/L pure 3,4-DGE was added: 25°C (closed circles), 40°C (closed triangles). (Mean ± SEM, *n* = 3)

incubation at 25°C. At 40°C, 3-DG increased by 56 µmol/L after 1 week of incubation. At the same time as 3-DG increased, 3,4-DGE decreased by 100 and 86 µmol/L respectively. No other GDP except 3-DG went through alterations comparable to those of 3,4-DGE (Table 1). There were no detectable amounts of GDPs in sterile-filtered controls.

ADDITION OF 3,4-DGE

Addition of 80 µmol/L 3,4-DGE to the fluids incubated for 30 days restored the concentration to approximately 100 µmol/L at 25°C and approximately 110 µmol/L at 40°C. During the following 30 days, the concentration of 3,4-DGE decreased in the same way as during the preceding 30 days (Figure 1).

TABLE 1

Glucose Degradation Products (GDPs) (µmol/L) in 1.5% Peritoneal Dialysis Fluids Stored at 25°C or 40°C After Sterilization. At 25°C, equilibrium was reached after 30 days; 8 days was needed at 40°C.

Storage	25°C			40°C	
	0 days	8 days	30 days	8 days	30 days
3,4-DGE	125±4	64±3 <sup>a</sup>	25±1 <sup>a</sup>	39±2 <sup>a</sup>	32±2 <sup>a</sup>
3-DG	230±6	273±2 <sup>a</sup>	307±7 <sup>a</sup>	286±3 <sup>a</sup>	265±12 <sup>a</sup>
5-HMF	14±0	14±0	14±0	17±1 <sup>a</sup>	21±1 <sup>a</sup>
Methylglyoxal	1±1	1±1	1±1	1±1	3±1
Glyoxal	12±1	10±0	9±0 <sup>a</sup>	10±0	9±0 <sup>a</sup>
Formaldehyde	4±1	4±0	4±1	6±0	6±1 <sup>a</sup>
Acetaldehyde	84±7	93±3	83±2	86±1	106±3 <sup>a</sup>

3,4-DGE = 3,4-dideoxyglucosone-3-ene; 3-DG = 3-deoxyglucosone; 5-HMF = 5-hydroxymethyl furaldehyde.

<sup>a</sup> *p* < 0.05.

Statistical comparisons were made between the values of each substance at 0 days against 8 and 30 days respectively. Values are given as mean±SEM. Each value represents the mean of three separate analyses (*n* = 3).

CYTOTOXICITY

Figure 2 demonstrates (using L-929 cells) that cytotoxicity of fluids incubated at 40°C decreased in parallel with 3,4-DGE. This was found irrespective of whether 3,4-DGE was created during sterilization or pure 3,4-DGE was added to the fluids after 30 days. Similar results were obtained for incubation at 25°C. Good correlation was also found for ICG (L-929) versus the concentration of 3,4-DGE for all different PD fluids tested in this study (Figure 3).

In another test, HPMC were used. The fluids to be tested were incubated for 60 days at 40°C. The ICG decreased in parallel with the L-929 results. The HPMC turned out to be more sensitive, giving rise to higher initial ICG values compared to the L-929 cells (Figure 2).

CONVENTIONAL PD FLUIDS INCUBATED AT DIFFERENT TEMPERATURES

An increase in incubation temperature resulted in an increase in the concentration of 3,4-DGE (Table 2).

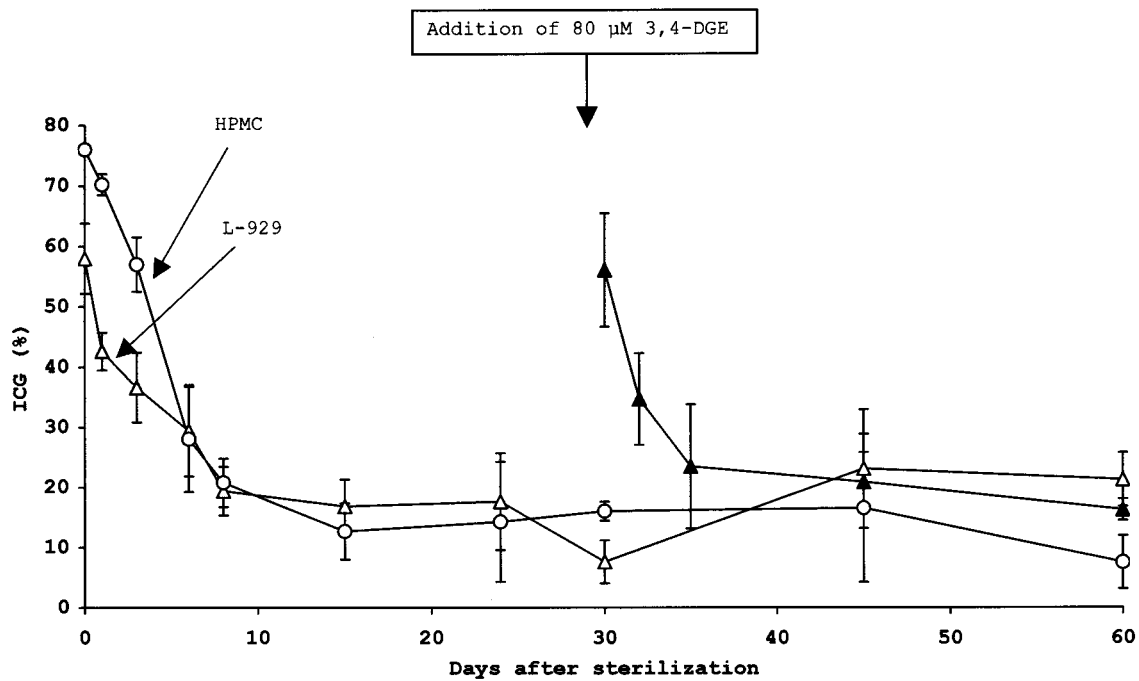


Figure 2 — Inhibition of cell growth (ICG), using L-929 cells (open triangles) and human peritoneal mesothelial cells (HPMC; open circles), caused by laboratory-made 1.5% peritoneal dialysis fluids incubated for 60 days at 40°C. In one set of samples, 80 μmol/L 3,4-dideoxyglucosone-3-ene (3,4-DGE) was added to the L-929 cells after 30 days of incubation (closed triangles). (Mean ± SEM, n = 5)

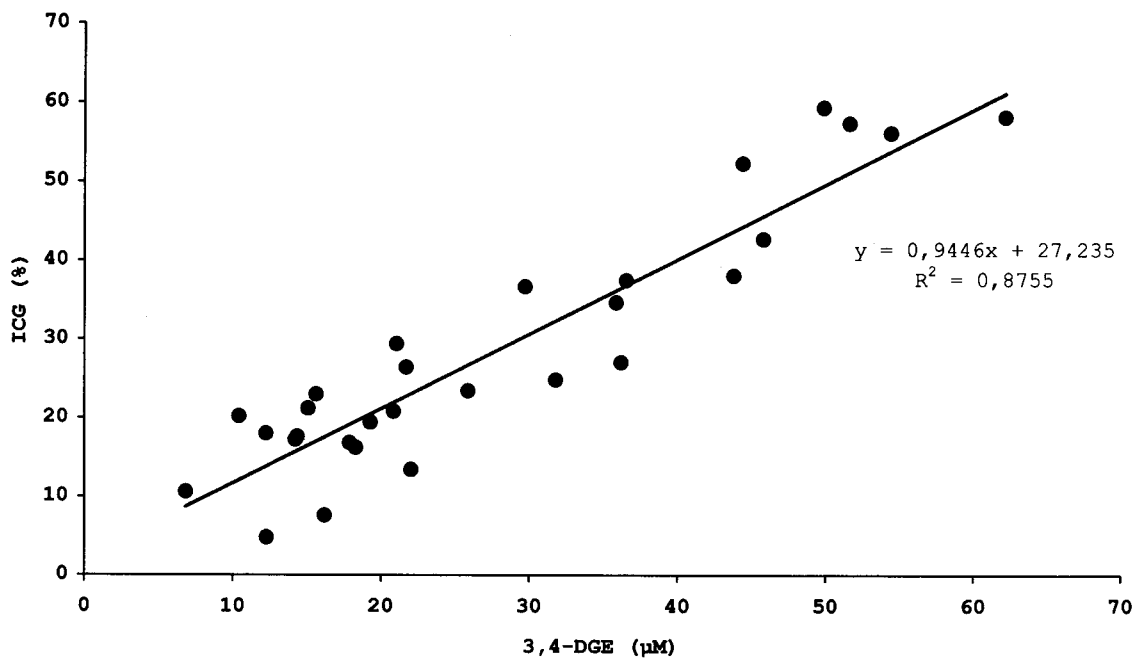


Figure 3 — Inhibition of cell growth (ICG), using L-929 mouse fibroblasts, caused by laboratory-made 1.5% peritoneal dialysis fluids (PDF) stored at 25°C and 40°C and containing different concentrations of 3,4-dideoxyglucosone-3-ene (3,4-DGE). All 3,4-DGE values presented in this graph were diluted 1:1 (1 part PDF and 1 part medium) prior to ICG testing. [Mean ± SEM; n = 5 (ICG) and n = 3 (3,4-DGE)]

Raising the temperature from room temperature to 60°C for 2 weeks more than tripled the concentration of 3,4-DGE, from  $11 \pm 1$  to  $38 \pm 3$  μmol/L (for the cell

tests, the fluid was diluted 3:2, which corresponds to  $7 \pm 0$  to  $26 \pm 2$  μmol/L). Cytotoxicity increased corresponding to the 3,4-DGE concentrations.

TABLE 2  
Conventional 1.5% Peritoneal Dialysis Fluid (PDF) Incubated for Two Weeks at Different Temperatures.  
The cell tests were performed using PDFs diluted 3:2 with cell growth medium.

	5°C	25°C	30°C	40°C	60°C
3,4-DGE (μmol/L)	11±1	12±1	16±1	24±2	38±3
3,4-DGE after dilution (μmol/L)	7	8	10	16	26
ICG (%)	22±3	25±4	30±4	32±3	44±4

3,4-DGE = 3,4-dideoxyglucosone-3-ene; ICG = inhibition of cell growth.

3,4-DGE values are given as mean±SEM (*n* = 3); ICG values are given as mean±SEM (*n* = 6).

## DISCUSSION

Immediately after heat sterilization, the laboratory-made PD fluid contained a 10-times higher concentration of 3,4-DGE compared to what is normally present in the commercially produced fluids. During storage, 3,4-DGE decreased rapidly. The decrease at 25°C was slower and 3,4-DGE reached a lower final value compared to that of the fluids stored at 40°C. The most likely explanation for this is that, when energy (heat) is added during sterilization, a dehydration of 3-DG occurs and 3,4-DGE ( $\alpha$ ,  $\beta$ -unsaturated carbonyl) is formed (Figure 4).

The formation of 3-DG from 3,4-DGE is a reversible reaction. To drive the reaction in the direction of 3,4-DGE, heat is essential. After sterilization, when the system loses energy as it cools, the equilibrium is reversed, resulting in an increase in 3-DG and a decrease in 3,4-DGE. The speed of the reaction depends (in an exponential way) on temperature. Thus, the higher the temperature, the faster the equilibrium establishes. Levels of 3,4-DGE are higher at 40°C than at 25°C. The reason for this difference is that

more energy is available for the formation of 3,4-DGE at the higher temperature. However, 3,4-DGE may be lost not only in the direction of 3-DG but also through further dehydration and removal of water, forming 5-HMF (15). A loss in the latter direction must thus not necessarily be noticed as an increase in the 5-HMF concentration, since 5-HMF may then be further degraded.

We found that 3-DG increased by 55 – 69 μmol/L when 3,4-DGE decreased by 86 – 100 μmol/L, implying that the increase in 3-DG thus explains most of the disappearance of 3,4-DGE.

Degradation of glucose is very complex and there are multiple routes leading to the formation of 3-DG and 3,4-DGE. One way is through the Maillard reaction, where glucose reacts with the amino groups of proteins, probably functioning as catalysts, producing highly reactive carbonyl compounds (18). In our case, this route is not at hand due to no amino groups being available. A more likely way available during heat sterilization is degradation of carbohydrates to furaldehydes, where 3-DG is an intermediate (15,19). This reaction is probably based on the same principles as the Maillard reaction, the difference being that no catalyzing amino groups are present.

Immediately after sterilization, cytotoxicity of the PD fluids was high. It decreased in parallel with the decrease in 3,4-DGE concentrations. This was found both after sterilization and when the concentration was restored by the addition of pure 3,4-DGE, strongly supporting 3,4-DGE as the main cytotoxic GDP in PD fluids. The initial decrease in cytotoxicity was also confirmed by use of HPMC. The ICG values using HPMC were initially higher compared to the L-929 cells. This is in line with earlier work indicating that the HPMC is a more sensitive system (6). However, as the differences between the cell types were small, L-929 was chosen for testing cytotoxicity because the method is rapid and offers a much simpler screening.

Our results emphasize that patients should not use glucose-containing PD fluids too soon after sterilization. A quarantine period of at least 1 month at room temperature should be ensured before the patient

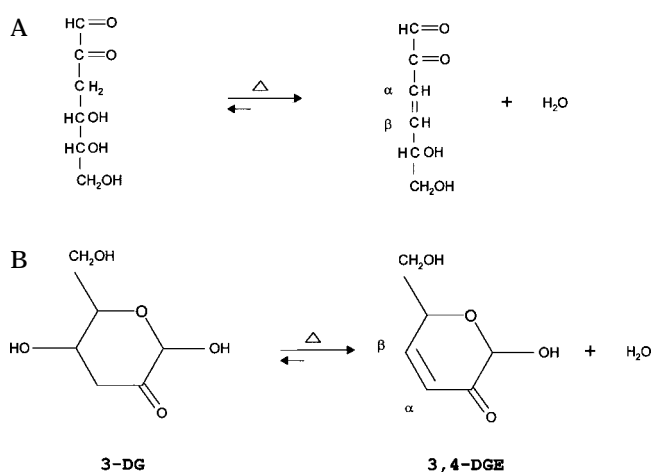


Figure 4 — Dehydration of 3-deoxyglucosone (3-DG) occurs when heat (open triangles) is applied, resulting in the loss of a molecule of water. This moves the equilibrium toward 3,4-dideoxyglucosone-3-ene (3,4-DGE). A = ring opened, B = ring closed.

uses the fluids. Another aspect of our findings is that, if the fluid is stored at high temperatures, even for as short a period as 14 days prior to use, it will contain more 3,4-DGE and be more cytotoxic. At temperatures above 40°C, this period might be considerably shorter than 14 days.

It was demonstrated earlier that GDPs are transported over the peritoneal membrane into the blood (11,12,20). One of the GDPs that have been found to increase in the circulation after infusion of PD fluid is 3-DG (12,21). As the reaction between 3-DG and 3,4-DGE is reversible, 3-DG might, in the circulation, be converted to the highly reactive 3,4-DGE. This may then react with and cause damage to tissues. A safe way to reduce negative side effects related to GDPs seems to be to prevent their formation by using modern manufacturing techniques and by storing the product in a suitable way (22–24).

We conclude that, immediately after sterilization, glucose-based PD fluids contain high levels of 3,4-DGE and are extremely cytotoxic. Upon storage, 3,4-DGE seems to be converted back into mainly 3-DG, in a reversible way. Peritoneal dialysis fluids must thus be produced with low total GDP content and, in order to avoid higher levels of 3,4-DGE, they should not be used too soon after sterilization and should not be stored at temperatures above room temperature.

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