

Effect of Vitamin C on Glycosylation of Proteins

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Twelve nondiabetic subjects consumed 1 g/day vitamin C for 3 mo. A fasting blood sample was taken at the start of the study and at the end of each month for the measurement of plasma and intraerythrocyte glucose, vitamin C, glycosylated hemoglobin (affinity chromatography and electrophoresis), and glycosylated albumin (affinity chromatography). Although there were no significant changes in fasting glycemia, glycosylated hemoglobin (affinity chromatography) decreased 18%, from $6.18 \pm 0.48\%$ (mean \pm SD) at the start to $5.05 \pm 0.50\%$ ($P < 0.0001$) after 3 mo, whereas, HbA_{1c} measured by electrophoresis increased 16%, from 6.17 ± 0.61 to $7.16 \pm 0.59\%$ ($P < 0.0001$) in this period. Glycosylated albumin decreased 33%, from 1.56 ± 0.24 to $1.04 \pm 1.01\%$ ($P < 0.0001$) after 3 mo. This discrepancy between glycosylated hemoglobin measured by electrophoresis and affinity chromatography was due to methodological differences between the two techniques, with affinity chromatography measuring "true" glycosylated hemoglobin. The greater decrease found with glycosylated albumin was probably due to the different distribution of vitamin C between plasma and within the erythrocyte, levels after 1 mo of supplementation being 109 ± 19 and $59 \pm 9 \mu\text{M}$, respectively ($P < 0.001$). This indicates that administration of oral vitamin C may inhibit the glycosylation of proteins in vivo by a competitive mechanism. *Diabetes* 41:167-73, 1992

Vitamin C, in particular dehydroascorbic acid, has long been known to food chemists to react with amino groups of both amino acids and proteins (1-3). Studies with electron-spin resonance showed that ascorbic acid could bind to proteins via an ionic interaction (4). In physiological fluids, it is likely that dehydroascorbic acid can form a reversible Schiff base with amino groups (5). It is also possible that the carbonyl groups of ascorbic acid and its further oxidation product, diketogulonic acid, could also react

with amino groups to form Schiff bases (Fig. 1). Thus, all three compounds, (ascorbic acid, dehydroascorbic acid, and diketogulonic acid) could compete with glucose for binding to proteins and thereby inhibit glycosylation.

Several studies have shown that vitamin C affects glycosylation of proteins (6,7). However, the effects found were confusing and conflicting. In preliminary work, Stolba et al. (6,8) investigated the effect of ascorbic acid and its metabolite dehydroascorbic acid on the glycosylation of bovine serum albumin and collagen in the presence of varying concentrations of glucose. They found that, although ascorbic acid inhibited glycosylation, dehydroascorbic acid apparently enhanced glycosylation. Khatami et al. (7) also studied the effect of ascorbic acid and dehydroascorbic acid on the glycosylation of bovine serum albumin in vitro but found that both compounds inhibited glycosylation. There was a difference between the two studies in the concentration of vitamin C used. Stolba et al. (6) used concentrations up to 1 mM, whereas, Khatami et al. used 5 mM ascorbic acid and dehydroascorbic acid. Stolba et al. also performed an in vivo study in which the diet of 10 insulin-dependent diabetic subjects was supplemented with 1.5 g/day vitamin C for 3 wk. A significant fall in fructosamine concentration occurred, thus suggesting inhibition of glycosylation of plasma proteins by vitamin C because no change in glycemia was observed.

Although Stolba et al. (6) showed a significant decrease in fructosamine concentration in vivo with vitamin C supplementation, the effect on glycosylation of intra-

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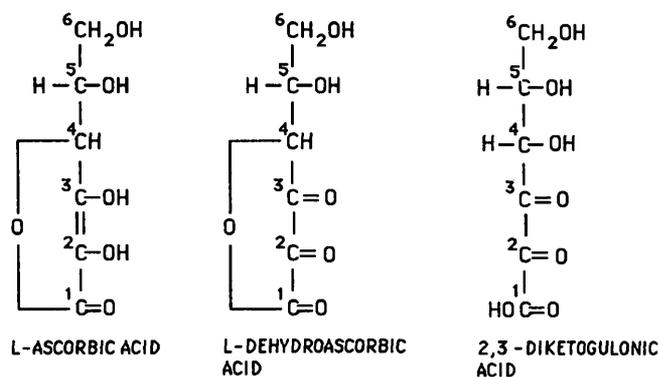


FIG. 1. Structure of ascorbic acid and its metabolites dehydroascorbic acid and diketogulonic acid.

cellular proteins such as hemoglobin was not determined, and no attempt was made to elucidate the mechanisms involved. The fructosamine assay is also unreliable, with many unidentified nonspecific interferences (9,10). Therefore, to study the mechanisms in more detail, the effect of oral vitamin C on the glycosylation of proteins in a group of healthy nondiabetic volunteers was investigated. We also examined the effect of vitamin C in vitro on methodologies for measuring glycosylated proteins.

RESEARCH DESIGN AND METHODS

All the reagents were of "AnalaR" grade and obtained from BDH (Poole, UK) unless stated otherwise.

Twelve healthy volunteers (7 men and 5 women aged 22–50 yr) participated in the study. Ascorbic acid in 500-mg tablets was supplied by Roche (Welwyn Garden City, UK). The subjects were asked to take 1 g/day (2 500-mg tablets) of vitamin C for 3 mo. Fifteen-milliliter fasting venous blood samples were collected at the start of the study and at the end of each month. These samples were taken 12–15 h after the last dosage of vitamin C. Two further samples were obtained at 1 mo (15 ml) and 2 mo (5 ml) after cessation of the vitamin C supplementation (6 samples total). The subjects were asked to follow their usual diet but to refrain from consumption of excess alcohol and ingestion of aspirin.

The 15-ml blood samples were each divided into two

aliquots. Ten milliliters of blood was transferred into a lithium-heparin tube, and 5 ml was transferred into a potassium-EDTA tube. Some of the lithium-heparin whole blood was deproteinized immediately to measure glucose. The remaining lithium-heparin whole blood was centrifuged at $3000 \times g$ at room temperature for 10 min. Some of the resulting plasma was deproteinized for measurement of glucose and vitamin C. The remaining plasma was stored at -80°C in aliquots for analysis of insulin, fructosamine, and glycosylated albumin up to 3 mo later. The packed erythrocytes were also deproteinized to measure vitamin C. The whole-blood and plasma extracts for glucose were stored at 4°C and analyzed within 6 h of collection. The plasma and erythrocyte extracts for measurement of vitamin C were stored at -80°C and analyzed within 1 wk. A small amount of the potassium-EDTA whole blood was used immediately to measure hematocrit (Hct). The rest was stored at 4°C for 1 wk before analysis of glycosylated hemoglobin by affinity chromatography and agar-gel electrophoresis (with removal of the Schiff base). The 5-ml blood sample collected 2 mo after cessation of the vitamin C supplementation was transferred into a potassium-EDTA container. It was stored at 4°C and analyzed for glycosylated hemoglobin by affinity chromatography and electrophoresis within 1 wk of collection.

For in vitro investigation of the effect of vitamin C on glycosylated hemoglobin methodologies, fasting blood samples (5 ml) from five healthy nondiabetic volunteers were collected in lithium-heparin tubes and centrifuged at $3000 \times g$ for 10 min. The erythrocytes were washed twice in normal saline and then incubated in the presence or absence of 25 mM glucose in 0.1 M phosphate buffer (final pH 7.4) and 0.001% sodium azide. Some of the incubations were done in the presence of 1 mM ascorbic acid. After incubation for 3 days at 37°C , the mixtures were dialyzed against 0.9% saline before glycosylated hemoglobin was measured on each of the incubates by both affinity chromatography and agar-gel electrophoresis (with removal of Schiff base).

Glycosylated hemoglobin was measured by affinity chromatography with the method of Gould et al. (11). HbA_{1c} was assayed by agar-gel electrophoresis, with the removal of the labile Schiff base, with the Glytrak kit (Corning Medical, Halstead, UK).

Glycosylated albumin was measured by first separat-

TABLE 1

In vitro effect of ascorbic acid on methodologies used to measure glycosylated hemoglobin

	Phosphate buffer (0.1 M)	Glucose (25 mM)	Ascorbic acid (1 mM)	Glucose (25 mM) + ascorbic acid (1 mM)
Glycosylated hemoglobin (%)	4.89 ± 0.59	7.58 ± 1.16*	5.02 ± 0.72	6.94 ± 1.19†
Increase (%)		54.8 ± 9.8	3.3 ± 2.3	42.0 ± 10.2
HbA _{1c} (%)	6.40 ± 0.62	9.43 ± 0.96*	13.48 ± 1.08*	12.40 ± 3.02‡
Increase (%)		47.50 ± 4.04	96.8 ± 19.5	75.8 ± 26.9

Values are means ± SD. Erythrocytes from 5 nondiabetic individuals were incubated for 3 days at 37°C in phosphate buffer with the additions shown above. Glycosylated hemoglobin and HbA_{1c} values were measured by affinity chromatography and agar-gel electrophoresis, respectively.

* $P < 0.001$, † $P < 0.01$, ‡ $P < 0.05$, vs. phosphate buffer alone (paired Student's *t* test).

TABLE 2
Fasting plasma and erythrocyte vitamin C levels before, during, and after supplementation with vitamin C

	Plasma vitamin C (μM)	Erythrocyte vitamin C (μM)	Plasma-erythrocyte ratio
Basal	73.3 \pm 13.9	59.5 \pm 17.3*	1.26 \pm 0.13
Supplementation			
1 mo	109.2 \pm 18.6†	59.1 \pm 9.5‡	1.92 \pm 0.17†
2 mo	119.1 \pm 19.9†	73.8 \pm 17.1‡§	1.55 \pm 0.23
3 mo	93.3 \pm 16.8	83.6 \pm 10.8†	1.14 \pm 0.22
1 mo after end of supplementation	59.2 \pm 12.9§	43.3 \pm 16.8*§	1.26 \pm 0.35

Values are means \pm SD.

* $P < 0.01$, † $P < 0.001$, vs. plasma.

‡ $P < 0.001$, § $P < 0.05$, || $P < 0.01$, vs. basal.

ing the nonglycosylated and glycosylated albumin by affinity chromatography with the method of Gould et al. (12) followed by specific analysis of the albumin content of each fraction by the method of Laurell (13). One hundred microliters of lithium-heparin plasma was diluted in 1.5 ml distilled water. One hundred fifty microliters of the diluted plasma was then applied to a 1-ml affinity column containing *m*-aminophenylboronate-agarose gel (Pierce and Warriner, Chester, UK), which had been equilibrated with 5 ml wash buffer (200 mM ammonium acetate, 50 mM magnesium chloride, 3 mM sodium azide [pH 8.5] at 20 \pm 1°C). This was followed by 1 ml wash buffer and then a further 8 ml wash buffer. The entire eluate containing the nonglycosylated albumin was collected and freeze-dried. The glycosylated albumin was eluted from the column with 3 ml elution buffer (200 mM sorbitol, 50 mM disodium EDTA, 100 mM tris-[hydroxymethyl]-methylamine, 3 mM sodium azide [pH 8.5] at 20 \pm 1°C), which was also freeze-dried. The nonglycosylated albumin fraction was reconstituted in 20 ml deionized water and the glycosylated fraction in 0.5 ml deionized water.

Immunoelectrophoresis was done with 1% agarose-gel plates containing 0.002% (vol/vol) sheep anti-human albumin (Guildhay Antisera, Guildford, UK). Reconstituted samples (10 μl) of human albumin standard (SPSO1, Sheffield Protein Reference Unit, Sheffield, UK, diluted in 0.9% saline to produce a range of standards 2.5–40 mg/L) were pipetted into 4-mm-diam wells. Electrophoresis was carried out with 0.06 M barbital buffer (pH 8.6) at 100 V for 16 h. Then the gel was compressed, dried, stained with Coomassie Brilliant Blue C (Sigma, Poole, UK), destained with methanol-glacial acetic acid-deionized water (1:2:17), and dried again. The percentage of glycosylated albumin was calculated as

$$\% \text{ glycosylated albumin} = \frac{[\text{glycosylated albumin}]}{[\text{glycosylated albumin}] + 40[\text{nonglycosylated albumin}]} \times 100$$

Plasma fructosamine was measured with the method of Johnson et al. (14) adapted onto the Cobas Bio centrifugal analyzer (Roche) (15).

Plasma and whole-blood glucose were measured after deproteinization with 0.6 M perchloric acid with a hexokinase kit (Roche). Intraerythrocyte glucose was calcu-

lated from the whole-blood and plasma measurements with the formula

$$[\text{Glucose}]_E = [\text{Glucose}]_P - \frac{[\text{Glucose}]_P - [\text{Glucose}]_{WB}}{\text{Hct}}$$

where *E*, *P*, and *WB* are the erythrocyte, plasma, and whole-blood glucose concentrations, respectively (16).

Plasma insulin was measured by a standard competitive radioimmunoassay with polyclonal antisera (Guildhay).

Plasma and intraerythrocyte vitamin C were measured by a manual fluorometric method first introduced by Deutsch and Weeks (17) and adapted for the measurement of vitamin C in physiological fluids by Brubacher and Vuilleumier (18). The method is based on the conversion of ascorbic acid to dehydroascorbic acid with iodine, which is then reacted with *o*-phenyldiamine to form a fluorescent derivative.

Values are means \pm SD. The results were analyzed with the paired Student's *t* test.

RESULTS

One millimolar ascorbic acid had no significant effect on levels of glycosylated hemoglobin in the absence or presence of glucose when affinity chromatography was used (Table 1). However, when agar-gel electrophoresis was used to measure HbA_{1c}, there was a significant increase in the presence of ascorbic acid, which was significantly greater than that found with glucose alone. The results shown in Table 1 were obtained with washed erythrocytes, but similar changes were observed when hemoglobin replaced erythrocytes in the incubation (data not shown).

The total fasting plasma vitamin C levels increased by 49% after 1 mo and remained elevated during supplementation. The percentage by which the vitamin C levels were elevated above the basal level varied from month to month. After cessation of the vitamin C supplementation, the plasma levels of vitamin C were lower than the original basal level (Table 2).

The erythrocyte levels of vitamin C followed a different pattern from the plasma levels with supplementation. After 1 mo, there was no significant change, but the erythrocyte level of vitamin C rose by 21% after 2 mo and by 40% after 3 mo. After 1-mo cessation of vitamin C,

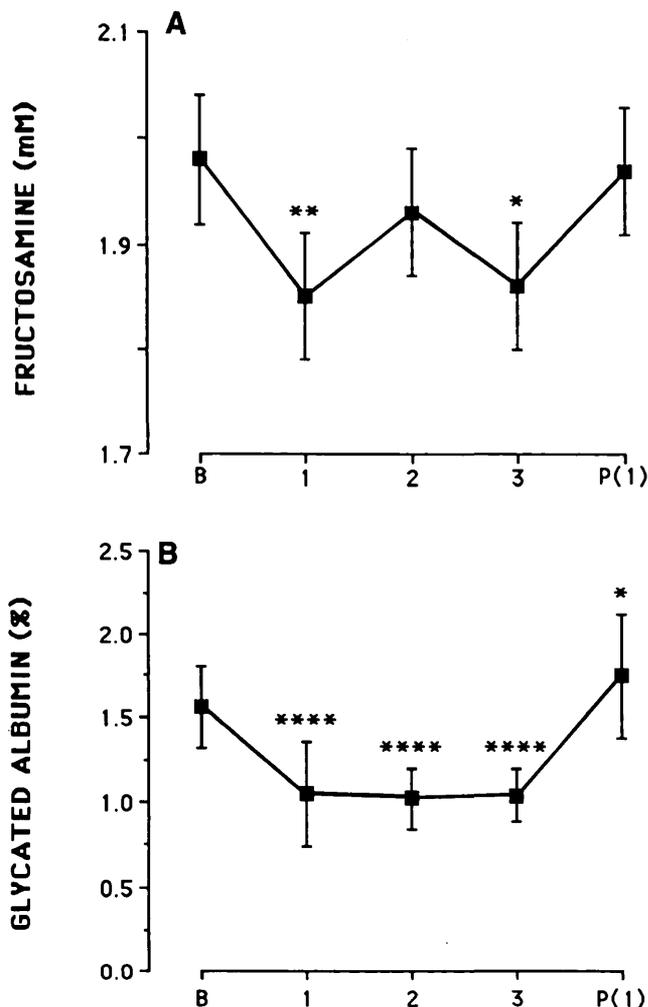


FIG. 2. Effect of vitamin C on fructosamine (A) and glycated albumin (B) measurements. Values are means \pm SD for 12 healthy nondiabetic volunteers. B, basal at 1, 2, and 3 mo of 1-g/day vitamin C supplementation; P(1), 1 mo post-vitamin C supplementation. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ (paired Student's *t* test).

the levels of vitamin C within the erythrocyte were lower than the basal level (Table 2). As a consequence of these changes, the ratio of plasma to erythrocyte vitamin C showed substantial changes during the study (Table 2).

There were no significant changes in either fasting plasma or erythrocyte glucose concentration throughout the study. Similarly, there were no significant changes in fasting plasma insulin levels (data not shown).

Supplementation with vitamin C produced a small decrease in fructosamine concentration. At 1 and 3 mo, the decreases were 6% ($P < 0.01$) and 5% ($P < 0.05$), respectively, but at 2 mo, the decrease was not statistically significant (Fig. 2A). One month after cessation of vitamin C, the fructosamine concentration returned to the basal level.

In contrast, the mean level of glycosylated albumin decreased by 33% ($P < 0.0001$) after 1 mo and remained at this level during supplementation with vitamin C. After 1-mo cessation of vitamin C, the glycosylated albumin level was increased to 12% above basal ($P < 0.05$; Fig. 2B).

The glycosylated hemoglobin level measured by affinity chromatography decreased by 18% ($P < 0.0001$) at 1 mo and remained at about this level during supplementation and even at 1 mo after cessation of vitamin C. Two months after the end of vitamin C supplementation, the glycosylated hemoglobin level had almost returned to the basal level (Fig. 3A).

The HbA₁ levels measured by agar-gel electrophoresis initially fell by 11% ($P < 0.01$) after 1 mo but then increased progressively until they were 16% ($P < 0.0001$) above basal at 3 mo. The HbA₁ levels remained raised even 1 mo after vitamin C supplementation had ended ($P < 0.0001$) but had almost returned to the basal level 2 mo after vitamin C supplementation had ended (Fig. 3B).

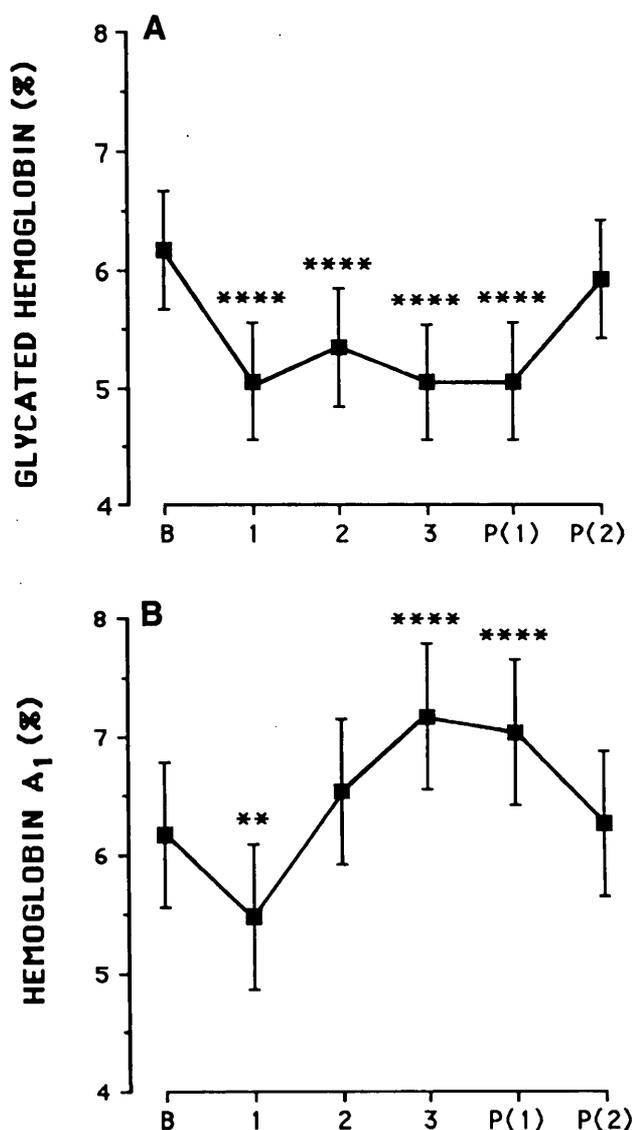


FIG. 3. Effect of vitamin C on glycosylated hemoglobin measured by affinity chromatography (A) and electrophoresis (B). Values are means \pm SD for 12 healthy nondiabetic volunteers. B, basal at 1, 2, and 3 mo of 1-g/day vitamin C supplementation; P(1) and P(2), 1 and 2 mo post-vitamin C supplementation. ** $P < 0.01$, **** $P < 0.0001$ (paired Student's *t* test).

DISCUSSION

This study has confirmed previous work that vitamin C affects the glycosylation of proteins. It has also provided a better insight into the possible mechanisms involved in vivo and shown that the methodology used for the measurement of glycosylated proteins has important consequences on interpretation of the results.

The in vitro experiment showed that when erythrocytes or hemolysates were incubated with vitamin C in the absence or presence of glucose, measurement of glycosylated hemoglobin by electrophoresis and affinity chromatography produced very different results. Ascorbic acid at 1 mM had no apparent effect on the glycosylation of hemoglobin as measured by affinity chromatography but apparently increased the levels of HbA_{1c} by >90% when measured with electrophoresis. This discrepancy between electrophoresis and affinity chromatography is probably because they measure different species because the two methods rely on different properties of glycosylated hemoglobin for their measurement. Affinity chromatography relies on the presence of cis-diols (19) to separate glycosylated from nonglycosylated hemoglobin. The electrophoresis method separates on the basis of small charge differences. These findings suggest that vitamin C in one form or another was reacting with hemoglobin to produce a charge change similar to the addition of glucose to hemoglobin. For the electrophoresis method, the Schiff base is removed before the assay of glycosylated hemoglobin. Therefore, the increase in glycosylated hemoglobin measured in the presence of vitamin C must be the result of vitamin C reacting with hemoglobin to form a stable linkage, analogous to the ketoamine formed with glucose. With the affinity-chromatography method, no change in glycosylated hemoglobin in the presence of vitamin C was found, implying that the vitamin C-hemoglobin complex does not have the correct structure or configuration for binding to the boronate gel. Therefore, it is apparent that affinity chromatography is measuring "true" glycosylated hemoglobin, whereas, electrophoresis is measuring vitamin C bound to hemoglobin in addition to "true" glycosylated hemoglobin. Vitamin C did not significantly inhibit the addition of glucose to hemoglobin in this in vitro situation, unlike the inhibition of glycosylation of albumin by ascorbic acid noted by Stolba et al. (6) and Khatami et al. (7). This may be partly due to differences in the relative concentrations of vitamin C and glucose and the different proteins used in the experiments.

Supplementation of the subjects' diet with vitamin C during the in vivo study produced a significant decrease in glycosylated hemoglobin when measured by affinity chromatography, whereas, electrophoresis measured a significant increase in glycosylated hemoglobin (except at 1 mo after supplementation). The in vitro study provides the likely explanation for this discrepancy and implies that affinity chromatography is measuring the true decrease in glycosylated hemoglobin. Plasma glycosylated protein concentrations were also significantly decreased during vitamin C supplementation as measured by fructosamine, as was glycosylated albumin measured

specifically by affinity chromatography. The decrease in fructosamine concentrations was much less marked than for glycosylated albumin. This is probably partly due to the fact that fructosamine is measuring the glycosylation of many proteins that may be affected to different extents by vitamin C (20,21). Also, the variable contribution of nonspecific components to the fructosamine reaction (10), thought partly to account for the variability of the measurement (9), may mask the effect of vitamin C on glycosylation. The decrease in glycosylated albumin and glycosylated hemoglobin during supplementation suggests that vitamin C was inhibiting glycosylation by some mechanism. The decrease in glycosylated albumin was approximately twice that of glycosylated hemoglobin. This may be due to structural differences of the two proteins and availability of sites for glycosylation or vitamin C binding but is probably mainly due to the higher concentration of vitamin C in the plasma compared with the erythrocyte during the first 2 mo of supplementation.

One month after cessation of vitamin C, vitamin C levels were significantly below basal levels in both plasma and erythrocytes. This overcompensation is a recognized phenomenon (22). The rapid elimination of excess vitamin C would allow glycosylation to proceed normally. Thus, 1 mo after cessation of vitamin C, glycosylated albumin (and fructosamine) had returned to their basal levels or above. However, glycosylated hemoglobin (measured by both techniques) did not return to the basal level after 1 mo but had done so 2 mo after finishing the vitamin C supplementation. This is probably due to the different rates of turnover of erythrocytes, which have a mean life of 120 days, and albumin, which has a half-life of 17–23 days (23).

The mechanism for the effect of vitamin C on glycosylation is complex. The in vitro experiment suggested that vitamin C could react with hemoglobin to form a stable linkage. Therefore, the most likely explanation for the decrease in glycosylated proteins measured during supplementation with vitamin C in vivo is the competition of vitamin C with glucose for reaction with the protein amino groups, thereby inhibiting glycosylation. Vitamin C has previously been shown to react with amino groups on proteins, possibly via an ionic interaction (4). The structures of ascorbic acid and dehydroascorbic acid would suggest that they could only form a Schiff base, whereas, the structure of diketogulonic acid may allow it to form three different Schiff bases, only one of which could undergo the Amadori rearrangement to form a ketoamine (Fig. 1). It is likely therefore that all three vitamin C-related compounds will compete with glucose for binding to proteins, thereby decreasing glycosylation. The reaction of diketogulonic acid with proteins to form a ketoamine would explain the apparent increase in glycosylated hemoglobin as measured by electrophoresis both in vitro and in vivo. Although the incubation solutions contained only ascorbic acid at the start, further oxidation probably occurred during the 3-day incubation. When similar incubation conditions were used by Ortwerth and Olesen (24), they observed that <30% of the original ascorbic acid remained after 24 h. Cox and Whichelow (25) found

that, in nondiabetic subjects, diketogulonic acid made up a surprising 20% of the total plasma vitamin C concentrations, whereas, dehydroascorbic acid made up only 5% of the total. Furthermore, Chatterjee et al. (26) found that supplementation with 2–4 g vitamin C produced a much greater increase in plasma levels of dehydroascorbic acid than ascorbic acid. Diketogulonic acid was not measured in that study, but it is possible that diketogulonic acid levels may increase similarly to dehydroascorbic acid. Although glycosylated hemoglobin measured by electrophoresis increased progressively after 2 mo of supplementation with vitamin C, at 1 mo, a significant decrease was recorded. At 1 mo, the fasting erythrocyte ascorbic acid concentration remained unchanged from the basal level. Thus, it is possible that not enough diketogulonic acid was produced within the erythrocyte to react with hemoglobin to form a charged product, although the postdosage rise in erythrocyte ascorbic/dehydroascorbic acid was probably sufficient to compete with glucose and therefore reduce the amount of glycosylated hemoglobin measured by both electrophoresis and affinity chromatography.

If vitamin C reacts with proteins to produce a stable linkage, it may have deleterious effects similar to glucose. In vitro, ascorbic acid reacts with lens crystallin proteins, eventually producing extensive cross-linking analogous to glucose in nonenzymic glycosylation (24,27). However, these experiments used concentrations of 20–100 mM ascorbic acid compared to normal plasma concentrations of 40–90 μ M. Also, under the conditions used, ascorbic acid would be rapidly oxidized to several uncharacterized, highly reactive products, many of which are probably absent or maintained at very low concentrations in vivo. Therefore, these in vitro findings cannot be extrapolated to the in vivo situation. Moreover, an in vivo experiment in which mice were fed a diet high in vitamin C for 1 yr had no effect on lens crystallin proteins (27). However, further work is required to investigate the long-term effects of high dosages of vitamin C in vivo on both short- and long-lived proteins.

If vitamin C can inhibit the glycosylation of proteins at levels unlikely to cause deleterious effects, it may be of use in the treatment of diabetes. Some of the chronic complications occurring in diabetes are thought to be due mainly to the further reactions of glycosylated proteins (28), and administration of vitamin C may be a simple way of slowing their progression. If glycosylation is also involved in the normal process of aging (29), long-term vitamin C supplementation may even slow down this natural process.

In conclusion, this study has shown that, in nondiabetic subjects, vitamin C reduces the glycosylation of short-lived proteins such as albumin and hemoglobin in vivo. Competition of vitamin C with glucose for reaction with protein amino groups appears to be the main explanation for this effect. However, the reaction of ascorbic acid, dehydroascorbic acid, and diketogulonic acid with protein amino groups also needs to be studied to determine whether they bind irreversibly or reversibly. This may have important consequences on whether vitamin C

could be used therapeutically to delay or prevent the chronic complications occurring in diabetes.

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