plasma glycolate concentrations found previously (15), because heating may promote glycolate generation from precursors in plasma or contamination from the acid. Despite the differences between our earlier and current techniques, the recovery of [14C]glycolate was the same in each (~97%).

The technique described here for measuring plasma oxalate and glycolate is efficient, because a single sample ultrafiltrate can be used for both assays. Furthermore, the technique is sensitive, accurate, and reproducible.

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References

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Interference of Carbamylated and Acetylated Hemoglobins in Assays of Glycohemoglobin by HPLC, Electrophoresis, Affinity Chromatography, and Enzyme Immunoassay

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In vitro-synthesized carbamylated and acetylated hemoglobin interfered in assays of glycohemoglobin by HPLC and electrophoresis but had no effects on results obtained by affinity chromatography and enzyme immunoassay. Correlations between long-term serum urea concentrations and glycohemoglobin percentages revealed that, in vivo, carbamylated hemoglobin equivalent to 0.063% of total hemoglobin is formed for every 1 mmol/L of serum urea. The use of acetylsalicylate, either chronically or in small doses (200–300 mg/day) or for 1 week at 2000 mg/day, did not cause significant interference from acetylhemoglobin, formed in vivo. We conclude that interference from carbamylated hemoglobin explains only a small part of existing discrepancies between results of glycohemoglobin assays in current use. The interfering effect of acetylsalicylate formed in vivo with acetyl-CoA as substrate is as yet unknown.

Additional Keyphrases: variation, source of urea, acetylsalicylate

Glycohemoglobin is widely accepted as a valuable indicator for long-term diabetic control (1). However, the various methods currently used for its assay in clinical chemical laboratories show systematic differences between reported values (2). Solving this problem has become a major priority of the National Diabetes Group in the United States (3). In a European external
quality-control program, we showed that the discrepancies are partially caused by differences in standardization (4). Especially in the lower concentrations of glycohemoglobin, interferences from carbamylated and acetylated hemoglobins may become important, particularly for glycohemoglobin results obtained by ion-exchange chromatographic and electrophoretic methods (5).

The in vivo reaction of hemoglobin with urea-derived isocyanate and the possible interference of the resulting carbamylated hemoglobin in hyperuremic patients have been reported (6). Interpretation of the degree of interference is, however, hampered by two factors that may affect glycohemoglobin percentages in hyperuremic patients, i.e., decreases in erythrocyte survival and glucose intolerance (7, 8). These conditions may be expected to cause a decrease and increase of glycohemoglobin percentages, respectively. Analogous to fetal hemoglobin, ~10% of which is known to become acetylated, adult hemoglobin (HbA0) can be converted into carbamylated HbA (9). Both acetyl-CoA and acetylsalicylate can act as substrates. Relatively high percentages of acetylated hemoglobin have been noted in nondiabetic pregnant women (1.9%), in alcoholic subjects (2.7%) (10), and after ingestion of acetylsalicylate (11).

We studied the analytical interference of in vitro carbamylated and -acetylated hemoglobins in four major methods for the assay of glycohemoglobin: high-performance liquid chromatography, electrophoresis, affinity chromatography, and enzyme immunoassay. To determine the effects of in vivo-produced carbamylated and acetylated hemoglobins, respectively, we investigated blood samples from hyperuremic patients and from patients and control subjects taking acetylsalicylate.

Subjects and Methods
Patients and Control Subjects

We studied venous blood samples, both EDTA-anticoagulated and coagulated, from the following groups of patients and control subjects:

- Twenty-one nondiabetic patients (mean age 69 years; range 49–86), who had exhibited a broad range in mean serum urea concentrations (median: 12.3 mmol/L; range: 4.4–46.2) in the preceding period. For each patient the mean plasma urea concentration was calculated from all samples collected in the preceding 3 months (minimum of two samples taken at least 6 weeks apart).
- Twenty-one cardiology patients (group A200; mean age 63 years; range 49–74), who had been taking a daily dose of 200 mg of acetylsalicylate for at least 3 months. They had had normal serum urea and blood glucose concentrations in the previous year.
- Twelve patients (group A300; mean age 74 years; range 55–92) with transient ischemic attacks, who had been taking a daily dose of 300 mg of acetylsalicylate for at least 3 months. They had had normal blood glucose and normal to slightly increased serum urea concentrations (mean 10.1 mmol/L; range 4.7–17.3) in the previous year.
- Twenty-five patients (group C; mean age 63 years; range 45–83) who served as age-matched controls for the groups that used acetylsalicylate (groups A200 and A300). Selection criteria were: comparable age, no use of acetylsalicylate in the previous 3 months, and normal serum urea and blood glucose concentrations during the previous year.
- Four healthy volunteers (mean age 37 years; range 24–46) with normal serum urea and blood glucose concentrations. Three of them took 2000 mg of acetylsalicylate for one week; the fourth served as an untreated control. Blood samples were collected at the start, at days 4 and 7 during medication, and 1 and 2 weeks after its discontinuation.

The study was approved by the local medical ethics committee and was in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Procedures

In vitro carbamylation of a hemolysate. Three erythrocytes were isolated from EDTA-anticoagulated blood by centrifugation and washed with an equal volume of isotonic saline (NaCl, 150 mmol/L). To 4.5 mL of washed packed cells we added 4.0 mL of 0.01 mol/L NaCN solution, 8.0 mL of 0.01 mol/L KOCN solution, and 0.5 mL of chloroform. The mixture was shaken for 1 min and centrifuged for 10 min at 1500 × g. To allow formation of carbamylated hemoglobin, we incubated the supernate at 37 °C for 1 h. Excess KOCN was removed by dialyzation against water for 4 h, with use of molecular porous membrane tubing (molecular mass cutoff 12–14 kDa) from Spectrum Medical Industries, Los Angeles, CA. Formation of carbamylated hemoglobin was confirmed by the method of Manning (12). Briefly, carbamylated hemoglobin is converted into carbamylated globin with hydrochloric acid/acetone and hydrolyzed in acetic acid/hydrochloric acid at 100 °C to form valine-hydantoin. The latter is identified by gas chromatography.

In vitro acetylation of a hemolysate. Three washed erythrocytes were obtained as described above. To 4.5 mL of washed packed cells we added 4.0 mL of 0.01 mol/L NaCN solution and 8.0 mL of 40 mmol/L acetylsalicylate solution in 0.01 mol/L NaH2PO4 buffer (pH adjusted to 6.9 with 4 mol/L HCl after the addition of the acetylsalicylate) (11). To allow formation of acetylated hemoglobin, we incubated the mixture at 37 °C for 16 h, then removed excess acetylsalicylate by dialyzation against water for 6 h.

Analytical methods. We quantified glycohemoglobin by performing the following commercially available tests according to the manufacturers' instructions: HPLC (Diamat; Bio-Rad Laboritories, Hercules, CA) (13), electrophoresis (Diatrac; Beckman Instruments, Brea, CA) (14), affinity chromatography (Glycotest II; Pierce, Rockford, IL) (15), and enzyme immunoassay (Novoclon; Dako, Cambridge, UK) (16). Glucose was measured by the hexokinase assay and urea by the

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3Small quantities of synthetic carbamylated and acetylated hemoglobins are available from the first author.
conductometric assay, both with the Beckman Synchron CX7 analyzer.

Statistics. Differences were analyzed with Student's *t*-test. *P* values <0.05 were considered significant.

Results

Influence of in vitro carbamylation and acetylation. Figure 1 shows HPLC and electrophoresis patterns of a hemolysate before and after in vitro carbamylation and acetylation. Glycohemoglobin is defined as the sum of all glycated hemoglobins for affinity chromatography and as HbA1c for HPLC, electrophoresis, and immunoassay. In neither technique was carbamylated hemoglobin separated from HbA1c. HPLC did not separate acetylated hemoglobin from HbA1c, whereas electrophoresis partially resolved the acetylated hemoglobin. The quantitative results are shown in Table 1. By HPLC and electrophoresis the percentages of apparent glycohemoglobin became substantially greater after both in vitro carbamylation and acetylation. The affinity chromatographic and enzyme immunoassay methods did not show changes in glycohemoglobin percentages.

Influence of in vivo carbamylation. The glycohemoglobin percentages of 21 patients exhibiting a broad range of serum urea concentrations were determined by each of the four assay methods. Figure 2 shows the absolute differences between results of two methods as a function of mean serum urea concentration in the preceding 3 months. Statistically significant effects of long-term serum urea concentration on glycohemoglobin percentage differences were found between HPLC and affinity chromatography, HPLC and enzyme immunoassay, electrophoresis and affinity chromatography, and electrophoresis and enzyme immunoassay. There were no significant differences between affinity chromatography and enzyme immunoassay or between HPLC and electrophoresis.

Influence of in vivo acetylation. Table 2 shows the glycohemoglobin percentages and serum urea and blood glucose concentrations determined in two groups of patients taking acetylsalicylate daily (A200 and A300) and in the group of age-matched control subjects (C). Glycohemoglobin was determined by all four methods. Results of neither method showed statistically significant differences in glycohemoglobin percentages between the patients' groups (A200 and A300) and the age-matched control group C. Glucose was slightly higher in groups A200 and A300, and urea was higher in group A300.

Three volunteers took 2000 mg of acetylsalicylate daily for 1 week. Glycohemoglobin percentages were determined by Diamat-HPLC before (5.1%, 5.5%, 5.6%) and after (5.3%, 5.5%, 5.7%) this period. In an untreated volunteer the respective percentages were 5.7% and 5.8%. Considering the within-series CV of 2–3% (4),

### Table 1. Mean Glycohemoglobin Percentages in Hemolysate before and after In Vitro Carbamylation and Acetylation

<table>
<thead>
<tr>
<th>Method</th>
<th>Basal</th>
<th>Carbamylated</th>
<th>Acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>5.5</td>
<td>14.3*</td>
<td>21.0*</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>5.7</td>
<td>12.9*</td>
<td>6.7*</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>4.6</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Enzyme immunoassay</td>
<td>3.2</td>
<td>3.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>

All samples analyzed in triplicate. * Significantly different from basal hemolysate (*P* <0.05).
none of the subjects showed statistically significant changes in glycohemoglobin percentages that could be related to the use of acetylsalicylate.

Discussion

The present data (Figure 1, Table 1) show that carbamylated and acetylated hemoglobins prepared in vitro interfere with the HPLC and electrophoretic methods widely used to assay glycohemoglobin. The hemoglobin derivatives have no effects on methods that make use of affinity chromatography and enzyme immunoassay. These results are in agreement with previous observations by Bannon et al. (5) and Engbaek et al. (16).

By studying nondiabetic hyperuremic patients, we could evaluate the interfering effect of in vivo-formed carbamylated hemoglobin on the assay of glycohemoglobin. There are, however, three pitfalls in comparing these results with those expected for nondiabetic normouremic controls. First, in hyperuremic patients, glucose tolerance is often impaired (8), which may have the effect of increasing the percentage of true glycohemoglobin. Second, erythrocyte turnover may be increased in hyperuremic subjects (7); this has the effect of decreasing the glycohemoglobin percentage. Third, analogous to the relation between glycohemoglobin percentage and blood glucose concentration, the carbamylated hemoglobin percentage may depend on the mean plasma urea concentration of the preceding 3 months—which might be quite different from the serum urea concentration in the sample in which the glycohemoglobin percentage is determined. To eliminate the in vivo effects of hyperuremia on the true glycohemoglobin percentage, we merely studied the differences between results for glycohemoglobin obtained by the various methods and related these to the mean serum urea concentrations.

The regression lines in Figure 2 show that only glycohemoglobin differences related to the mean serum urea concentrations are the differences between results of carbamylated hemoglobin-sensitive (HPLC and electrophoresis) methods and those of carbamylated hemoglobin-insensitive (affinity chromatography and enzyme immunoassay) methods. The slopes of the four possible combinations were almost identical (mean slope 0.063% per mmol/L; range 0.060–0.064), suggesting that when the mean serum urea concentration increases by 1 mmol/L, the glycohemoglobin percentage will increase by 0.063%, because of the presence of carbamylated hemoglobin. This outcome is in reasonable agreement with results that can be calculated from previous reports. Applications of carbamylated hemoglobin-sensitive and carbamylated hemoglobin-insensitive glycohemoglobin methods to normo- and hyperuremic patients showed results of 0.07%, 0.06% (5), and 0.09% (16). Kwan et al. developed a direct HPLC method for carbamylated hemoglobin (17) and applied it to normo- and hyperuremic subjects (18). From their data we calculated a glycohemoglobin increase of 0.08% (from carbamylated hemoglobin) per mmol/L concentration of serum urea.

The interfering effect of in vivo-formed acetylated hemoglobin on the assay of glycohemoglobin was studied in patients being chronically treated with acetylsalicylate (Table 2) and in volunteers taking a large dose for 1 week. However, these groups showed no differences from the age-matched control group (Table 2) or from basal values, in agreement with previous observations. Compared with the control subjects, patients with rheumatoid arthritis who were treated with 4 g of salicylate per day had a 1.5% (absolute difference) greater glycohemoglobin percentage, as determined by HPLC (17). One volunteer showed an absolute increase of 0.4% after

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**Fig. 2.** Relation between mean plasma urea concentrations and the difference in glycohemoglobin percentages obtained by two methods. Elec, electrophoresis; Aff, affinity chromatography; EIA, enzyme immunoassay. n.s. = not significant.
daily ingestion of 1.4 g of acetylsalicylate for 4 weeks (II). Estimation of the reaction rate revealed that, in vitro, 27% of acetylated hemoglobin is formed per mol/L of acetylsalicylate per hour. The reaction rate in vivo was <6% in our in vivo experiment and 2.4% in the experiment of Nathan et al. (II), based on the assumption that an acetylsalicylate dose of 4 g/day results in a blood concentration of 110 mg/L. The reaction rate in vitro exceeds the reaction rate in vivo by about 10-fold. Probably the in vitro conditions are more favorable to acetylation than are in vivo conditions; alternatively, acetylated hemoglobin may be more rapidly degraded or removed from the circulation in vivo.

We conclude that both in vitro- and in vivo-synthesized carbamylated hemoglobins interfere in glycohemoglobin assays that make use of HPLC and electrophoresis. With a glycohemoglobin increase of 0.063% of total hemoglobin per mmol/L of long-term serum urea concentration, normal serum urea concentrations (≈3–7 mmol/L) would lead to formation of enough carbamylated hemoglobin to cause an absolute glycohemoglobin increase of 0.2–0.4%. Interference from carbamylated hemoglobin may reach clinically relevant amounts in uremic patients. Chronic use of small (300–300 mg/day) doses of acetylsalicylate or brief use of a higher dose (2000 mg/day for 1 week) does not seem to cause clinically relevant increases of glycohemoglobin percentages from in vivo-produced acetylated hemoglobin. The variables that govern the in vivo formation of acetylated hemoglobin with acetyl-CoA as a substrate are as yet unknown. Further studies are necessary to elucidate the underlying causes of the discrepancies between glycohemoglobin percentages obtained by currently available techniques.

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References