Biochem. J. (1989) 261, 871–878 (Printed in Great Britain)

Identification of glycation at the *N*-terminus of albumin by gas chromatography–mass spectrometry

Donald A. ROBB,* O. Samson OLUFEMI,* David A. WILLIAMS†‡ and John M. MIDGLEY†
Departments of *Bioscience and Biotechnology and †Pharmacy, University of Strathclyde, George Street, Glasgow G1 1XW, Scotland, U.K.

Amino groups in human albumin are modified *in vivo* by glucose in a non-enzymic reaction, and previous studies have implicated lysine residues as exclusive participants. An investigation using g.c.–m.s. was undertaken to ascertain whether or not the *N*-terminus was also involved. Appropriate model compounds [*N*-(1-deoxyglucitol-1-yl) and *N*-(1-deoxymannitol-1-yl) adducts of aspartic acid] were synthesized and the diagnostic fragment ions of suitable derivatives were established under electron-impact and negative-chemical-ionization conditions. Characteristic fragment ions were identical with those obtained from the model compounds in the mass spectra of derivatives prepared from hydrolysates of reduced albumin. A purified mixture of the model compounds was also obtained from such hydrolysates. Use of radioisotopic incorporation demonstrated that the relative extent of glycation of the ε-amino and α-amino groups in albumin was approx. 8:1. *N*-1-Deoxyhexitol adducts of aspartic acid were also identified in reduced and hydrolysed peptides of human urine.

INTRODUCTION

Post-transitional and non-enzymic modification of proteins by glucose (glycation) is an established phenomenon in animals, an adventitious consequence of the constant presence of glucose in the circulation. Some examples of the proteins affected are haemoglobin, albumin, β-lipoprotein, fibrinogen, collagen, lens crystallin and myelin [1]. The chief mechanism of glycation is thought to be an example of the Maillard or browning reaction in which the Schiff base formed between the amino group of a protein and glucose undergoes an Amadori rearrangement to produce a stable fructose–amino acid adduct, although an additional autoxidative process has also been proposed [2]. The fructose–amino acid complex may react further to produce an 'advanced glycosylation end product' [3], which can cross-link with the same or a different protein. The amino acid residues which could take part in glycation are expected to be the α-amino group of the *N*-terminus and the side-chain ε-amino group of lysine and, since a low pK_a enhances reactivity, one might anticipate that the α-amino group would be the more reactive. The glycation of haemoglobin was the first example to be characterized, and g.c.–m.s. combined with peptide fractionation helped to show that the α-amino group of the β-chain was indeed the principal site involved [4]. However, it has been much easier to show that the ε-amino group is modified in the other proteins listed above. In albumin for example, Lys^{525} is principally involved, and previous attempts to demonstrate *N*-terminal glycation by classical methods have been unsuccessful [5,6]. Since the extent of glycation depends on glycaemic status and survival time of the protein [1], the clinical application (in which the glycated protein serves as a reliable index of a patient's glycaemic status) has attracted much interest [7]. Changes in metabolism have also been noted [8], and glycated compounds can be recovered from urine, but their cellular origin is not known [9].

The standard approach used to characterize glycated residues is to reduce the glycated protein (usually with a hydride donor) so that both the Schiff base and the Amadori complex are stabilized as the *N*-1-(1-deoxyhexitol)–amino acid adduct. After hydrolysis, part of the sample is subjected to amino acid analysis or peptide fractionation and part is derivatized and characterized by g.c.–m.s. [4,10–13]. Reduction of the Amadori compound with borohydride produces both C-2 epimers, *N*-1-(1-deoxymannitol)– and *N*-1-(1-deoxyglucitol)–, of the amino acid adduct. Reduction of the Schiff base with either borohydride or cyanoborohydride entails reduction at C-1 and gives the corresponding glucitol derivative as the sole product. These reduced compounds are more stable (e.g. to hydrolysis) than the parent compounds. However, exposure to the strongly acidic conditions usually used to hydrolyse proteins causes dehydration of the hexitol to give a mixture of anhydro derivatives, as shown previously for lysine [10].

We have reported that reduction of human albumin with [^{3}H]borohydride followed by acid hydrolysis yielded three significant fractions on amino acid analysis [14]. Whereas it was possible to confirm that most of the radioactivity originated from glycated lysine, the composition of the other two fractions was not elucidated. In this study the use of g.c.–m.s. has resulted in the unequivocal identification of glycated aspartic acid in a second of the fractions; it is reasonable to assume that

Abbreviations used: e.i., electron-impact; n.c.i., negative chemical ionization; t.i.c., total ion chromatogram; MASP and GASP, *N*-(1-deoxymannitol-1-yl) and *N*-(1-deoxyglucitol-1-yl) adducts of L-aspartic acid; TFA-OMe, trifluoroacetyl methyl ester; TMS, trimethylsilyl; NHDAA, *N*-(1-deoxyhexitol-1-yl)-amino acids.

‡ On sabbatical leave from the Department of Chemistry and Physics, Massachusetts College of Pharmacy and Allied Health Sciences, Boston, MA 02115, U.S.A.

this compound arises from the reaction of glucose with the *N*-terminus of albumin, and we have also identified the same substance in human urine.

EXPERIMENTAL

1. Preparation of model compounds

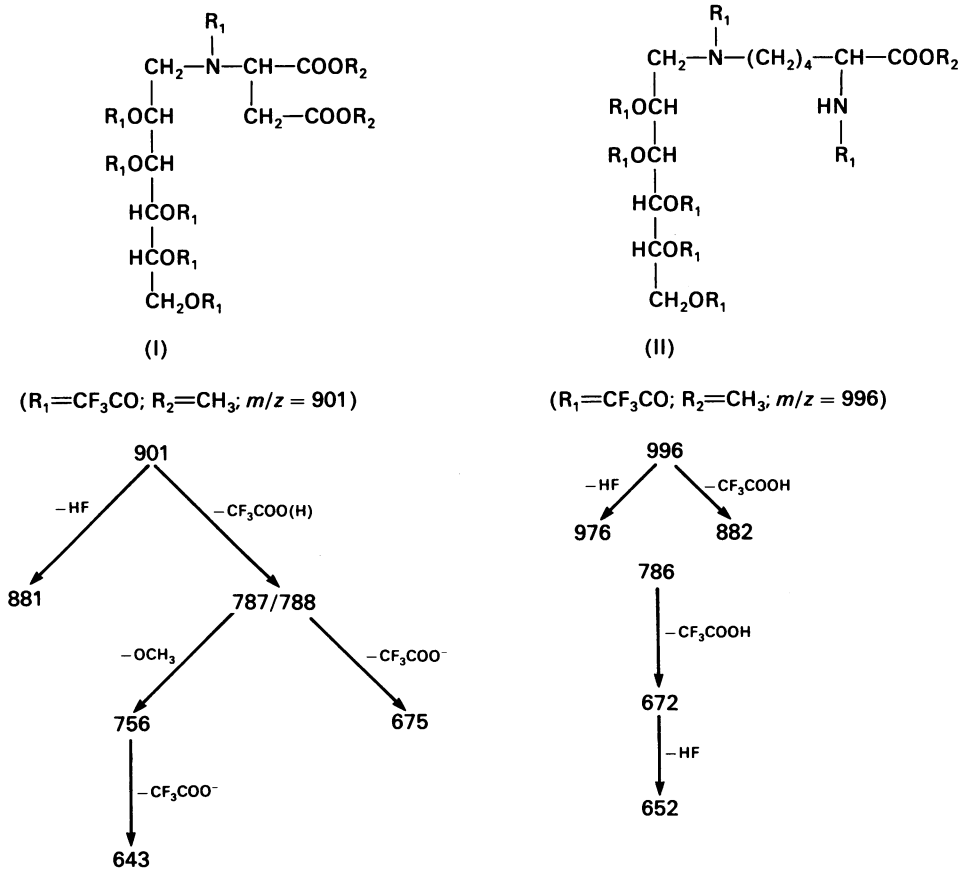
***(a)* Synthesis of *N*-(1-deoxy-D-glucitol-1-yl)-L-aspartic acid (GASP; Scheme 1, structure I, R_1 and R_2 = H).** A solution of L-aspartic acid (5 g) and D-glucose (9 g) in water (50 ml) was incubated at 80 °C and pH 8.5 for 30 min. The solution was cooled to 37 °C, $NaBH_4$ (1 g) was added in small portions and the mixture was incubated for a further 3 h at 37 °C. An equal volume of ethanol was added and the resulting solution was poured on to a column of Dowex-50 resin (200 ml) previously equilibrated with ethanol/water (1:1, v/v). After the column had been washed with the equilibration solution (300 ml) and water (100 ml), the hexitol–amino acid product was recovered, together with a small amount of aspartic acid, by elution with aq. 0.1 M-NH_3 and collection of the effluent with a pH between 7 and 9. This fraction was evaporated to dryness under reduced pressure, and the ion-exchange step repeated. The ammoniacal solution was evaporated to dryness, redissolved in water and freeze-dried to yield a hygroscopic product (1 g). A sample of this was purified further by boronate affinity chromatography (as described in subsection 3 below) to obtain the adduct free from aspartic acid. The purified GASP was dissolved in ^2H_2O and analysed on a Bruker WM 250 MHz Fourier-transform-n.m.r. spectrometer with t-butanol as the internal reference. The p.m.r. spectrum showed peaks at 3.09 p.p.m. (m, 2H, β-H of aspartate); 3.29 p.p.m. (m, 1H,CH_A-N); 3.38 p.p.m. (m,1H,CH_B-N); 3.6–3.9 p.p.m. (m, 6H, α-H of aspartate and H-3,4,5,6) and 4.13 p.p.m. (m, 1H, H-C_2-O).

***(b)* Synthesis of glucitol- and mannitol–aspartic acids by reductive amination.** The procedure employed was based on that described by Schwartz & Gray [15]. Aspartic acid (20 mg) was incubated with an equimolar amount of either D-glucose or D-mannose in phosphate-buffered saline (0.5 ml), which contained 0.9% NaCl, 0.02% NaN_3 and 0.01% Na_2EDTA in 0.01 M-sodium phosphate, pH 7.4, for 2 h at 37 °C. Sodium cyanoborohydride (15 mg) was added and the mixture was incubated for 5 days at 37 °C. The pH was adjusted to 4.0 with acetic acid and the solution stored at −20 °C until required.

***(c)* Synthesis of glucitol and mannitol adducts of lysine [16] (Scheme 1, structure II, R_1 and R_2 = H).** Poly-L-lysine (27 mg, residue number > 100; Sigma, Poole, Dorset, U.K.) was incuated with the appropriate hexose (27 mg) in phosphate-buffered saline (0.5 ml) for 2 h at 37 °C before sodium cyanoborohydride (5 mg) was added and the incubation continued.

After 5 days a gelatinous material had formed; the pH of the reaction mixture was adjusted to 4.0 with acetic

Scheme 1. Structures and possible modes of fragmentation of the TFA-OMe derivatives of *N*-1-(1-deoxyhexitol)-aspartate (I) and *N*-1-(1-deoxhexitol)-lysine (II) under n.c.i. conditions



acid and the material was dialysed for 48 h against two changes of water before hydrolysis (see subsection 3 below).

2. Reduction of albumin

Two protocols were used. In order to reduce the glycated groups in albumin, the protein (20 mg; from Sigma or from Behring, Marburg, W. Germany, or isolated as described previously [14]) was dissolved in phosphate-buffered saline (0.5 ml) and a solution of sodium borohydride (0.5 ml; 5 mg dissolved in 0.01 M-NaOH) was added to give a > 400 molar excess of borohydride. The mixture was incubated for 2 h at room temperature before the pH was adjusted to 4.0 with acetic acid. It was then dialysed for 72 h at 4 °C against 0.9% saline to remove excess reagents.

3. Hydrolysis and recovery of reduced adducts obtained from polylysine and albumin

Hydrolysis of the appropriate adduct (see subsections 1*c* and 2 above) was performed in a sealed tube under N_2 with 6 M-HCl for 18 h at 110 °C. After removal of excess acid by evaporation (N_2 stream), the residue was dissolved in water and the pH adjusted to 9.0 with 4 M-NaOH. The resultant solution was applied to a column (16 cm × 1.5 cm) of boronate gel (Affigel 601; Bio-Rad, Watford, Herts., U.K.) previously equilibrated with 25 mM-sodium phosphate buffer, pH 9.0. The column was washed with the equilibration buffer (350 ml) before it was developed with 25 mM-HCl. Fractions (5.5 ml) were collected, and those containing ninhydrin-positive material were pooled and freeze-dried.

4. Isolation of glycation substances from urine

This was achieved by the method of Brownlee *et al.* [17], which used the same column and reagents as those described in subsection 3 above, and resulted in the recovery of Amadori compounds. This material was freeze-dried, dissolved in phosphate-buffered saline (1 ml), reduced with borohydride (12 mg per 100 ml of urine processed) and treated as described in subsection 3 above.

5. Preparation of radioactive samples

NaB^3H_4 (specific radioactivity 10 Ci/mmol; Amersham International) was used to reduce both albumin (as specified in subsection 2 above) and the glycated fraction isolated from urine by affinity chromatography (see subsection 4 above). In each case a solution (0.5 ml) of the reducing agent (5 mg and 0.5 mCi) in 0.01 M-NaOH was employed, and excess radioactivity was removed from the albumin sample by dialysis. The radioactive solution originating from urine was poured on to a column of Dowex-50 (8 ml), which was washed with water (180 ml); glycated components were recovered by elution with a solution of aq. 1.5 M-NH_3 (50 ml) and were freeze-dried.

6. Amino acid analysis

The method was based on that of Robins [18] and was described previously [14].

7. Preparation of trifluoroacetyl methyl esters (Scheme 1; structures I and II, $R_1 = CF_3CO$ and $R_2 = CH_3$)

The trifluoroacetyl methyl esters (TFA-OMe) of the *N*-deoxyhexitol–amino acids were prepared as described by Cruikshank & Sheehan [19]. The freeze-dried product (approx. 5 μmol) was treated with anhydrous methanol [0.2 ml, containing 3% (w/v) dry HCl gas] at 40 °C for 5 min. The mixture was evaporated to dryness (N_2 stream) before trifluoroacetic anhydride (0.2 ml) and dry ethyl acetate (0.4 ml) were added to the residue. The mixture was incubated at 70 °C for 30–45 min until a clear solution was obtained. The residue produced by evaporation under an N_2 stream was dissolved in ethyl acetate to give the desired concentration for g.c.–m.s. analysis. For standards, residues were dissolved in 1 ml of ethyl acetate and 1 μl was used for analysis; for test samples the 1 ml solution was usually evaporated under N_2 to about 10 μl, and 1 μl of this solution was used for analysis.

8. G.c.–m.s. conditions

The instrument used was a Hewlett–Packard model 5988A with electron-impact (e.i.), negative-chemical-ionization (n.c.i.), positive-chemical-ionization and selected-ion-monitoring facilities; this was interfaced with an HP RTE-6/VM data system. The column was a fused silica capillary column (0.2 mm × 12 m) with a cross-linked bonded methylsilicone phase (HP-1, 0.33 μm film thickness) and utilized helium as the carrier gas (flow rate: 1 ml/min). The temperature program required maintenance of the oven temperature at 150 °C for 30 s, followed by a gradient from 150° to 250 °C at 20 °C/min and then maintenance at 250 °C for 10 min. For e.i. the source temperature was 200 °C and the electron energy was 70 eV. For n.c.i. analysis, with methane as the reagent gas, the source temperature was 150 °C and the electron energy was 200 eV.

RESULTS AND DISCUSSION

The two potential targets for glycation in albumin are the α-amino group of aspartic acid and the ε-amino group of lysine, and hence authentic mannitol and glucitol derivatives of aspartic acid and poly-L-lysine were prepared by reductive amination. Polylysine was chosen in preference to lysine because it offered a convenient starting material for the preparation of epimers substituted at the ε-amino group and also of their degradation products, which are formed on acid hydrolysis [10,11]. GASP was prepared by an unequivocal route (i.e. one that avoided Amadori rearrangement to the fructose–aspartic acid adduct, which would have yielded a mixture of epimers at C-2 on subsequent reduction). The structure of GASP was confirmed by the fragmentation pattern of the TFA-OMe derivative on g.c.–m.s. and the ^{1}H-n.m.r. spectrum. In the latter, the appearance of signals arising from the methylene hydrogens attached to the deoxy carbon (C-1) at δ 3.29 p.p.m. and 3.38 p.p.m., and the chemical-shift difference of 0.09 p.p.m. between them, are characteristic of *N*-deoxy-D-*gluco*–amino acids [12]. For all practical purposes, the mannitol epimer was absent from GASP, since the resonance at ~ δ 3.5 p.p.m. (which corresponds to H-1A of the *N*-deoxy-D-*mannitol*-1-yl–amino acid [12]) was absent.

Walton *et al.* [12] prepared model *N*-(1-deoxyhexitol-1-yl)–amino acids (NHDAA) and found that TMS derivatives afforded structurally diagnostic ions on g.c.–m.s. [13]. Ahmed *et al.* [20] have applied the same technique to hydrolysates of glycated proteins. Walton *et al.* [13] have reported that e.i.–m.s. of TMS derivatives

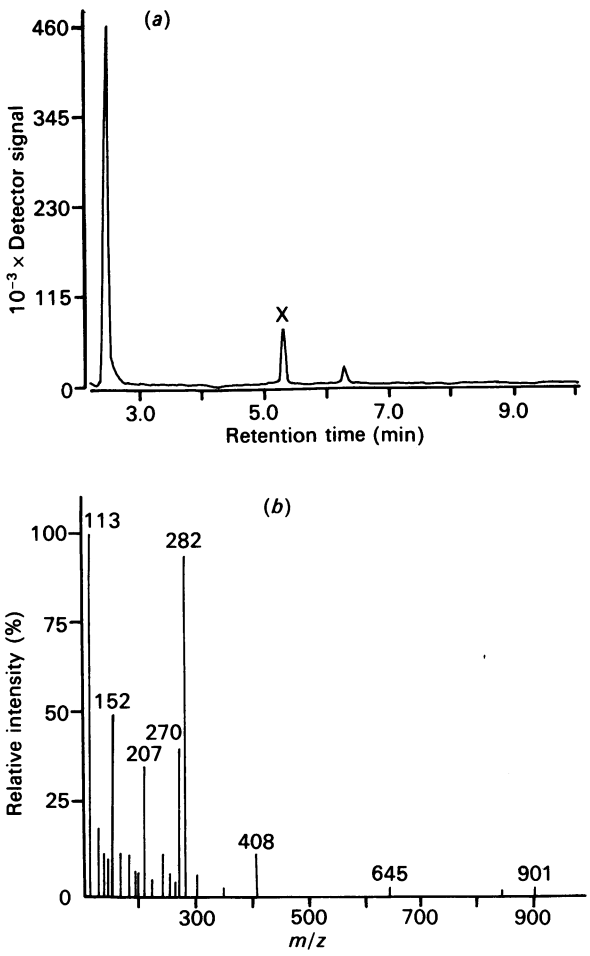
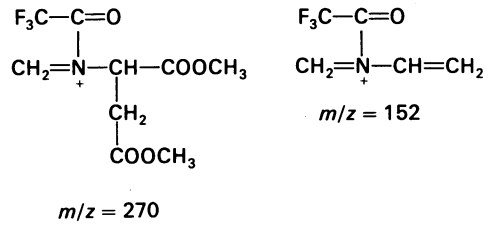


Fig. 1. (*a*) T.i.c. obtained for the TFA-OMe derivative of a sample of GASP utilizing e.i. ionization, and (*b*) e.i. mass spectrum of peak X in (*a*)

of NHDAA resulted in cleavage between the deoxy carbon (C-1) and C-2 of the hexitol moiety and between the carbonyl carbon of the carboxyl group and the α-carbon atom to form characteristic ions which allow the amino acid portion of the molecule to be identified unequivocally; ions derived from the sugar residue comprise most of those remaining, but these are not diagnostic [13]. It has been shown that e.i.–m.s. of TFA–aspartic acid esters also results in cleavage between the α-carbon atom and the carbonyl carbon of the ester, loss of the alkoxy group from the α-ester and loss of both alkoxycarbonyl groups [21].

We have found that TFA-OMe derivatives were more suitable for g.c. (i.e. were more stable, homogeneous and volatile) than trimethylsilylated derivatives. Moreover, the halogen atoms provide a substantial cross-section for electron capture, which allowed their detection by negative-ion chemical-ionization m.s. (n.c.i.m.s.). N.c.i. is an extremely valuable adjunct to e.i.-induced methods, since it produces different fragmentation patterns from the same compound. Often the molecular ion is the base peak and the technique can be 10–100-fold more sensitive than e.i. methods, since it yields fewer fragments [22].

The e.i. total ion chromatogram for GASP–TFA-OMe (Fig. 1*a*) showed a singlet peak at 5.3 min, and its mass spectrum (Fig. 1*b*) displayed ions of *m/z* 270 and 152 relating to structures which are characteristic for the aspartic acid portion:



The other intense ions of *m/z* 282 and 207 are attributable to fragments of the trifluoroacetylated glucitol group and that of *m/z* 113 to the reagent-specific trifluoroacetate ion. The prominent peak at 2.5 min (Fig. 1*a*) is due to the TFA-OMe derivative of aspartic acid, and the peak at 6.3 min has not as yet been conclusively identified. Its e.i. mass spectrum exhibits ions of *m/z* 270 and 152 (characteristic of the aspartate moiety), but the fragmentation pattern is distinct from that yielded by GASP–TFA-OMe; it probably represents an anhydro derivative of GASP which is formed during dehydration of the hexitol moiety during esterification with methanolic HCl. In the n.c.i. total-ion chromatogram of GASP–TFA-OMe (Fig. 2*a*) the peak with retention time of 5.28 min afforded a mass spectrum (Fig. 2*b*) which contained the expected molecular ion (of *m/z* 901) in

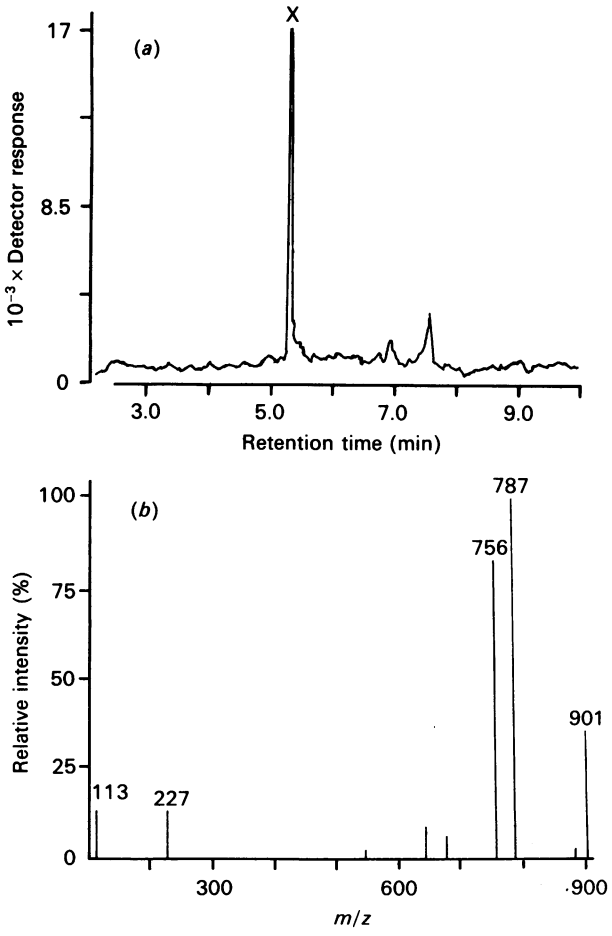


Fig. 2. (*a*) T.i.c. of TFA-OMe–GASP afforded by n.c.i., and (*b*) n.c.i. mass spectrum of TFA-OMe–GASP (peak X in *a*)

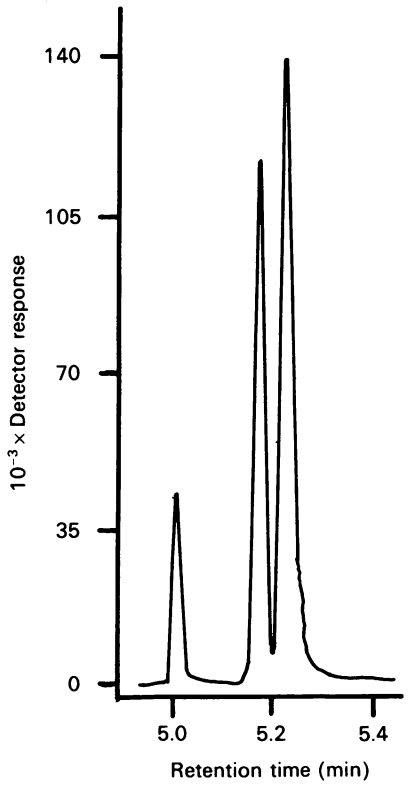


Fig. 3. N.c.i. chromatogram obtained when ions of *m/z* 901, 881, 787 and 756 were monitored

The chromatogram demonstrates that the TFA-OMe derivatives of GASP and MASP were separated under the g.c. conditions employed.

32% abundance relative to the base peak of *m/z* 787. Comparison of the mass spectra obtained by e.i. and n.c.i. (Figs. 1 and 2) clearly demonstrated the advantages of c.i. methodology. E.i.-induced ionization of GASP–TFA-OMe produced a considerable number of low-intensity fragment ions (including the molecular ion), and the ions of high abundance tended to be of low *m/z* values, which limited their diagnostic value for the complex as opposed to its components. In contrast, the n.c.i. spectrum exhibited a number of high-intensity ions of high *m/z* values in addition to the molecular ion of the adduct. A mixture of the TFA-OMe derivatives of MASP + GASP was also analysed as a model system in order to determine whether or not these substances could be identified independently of each other in protein hydrolysates. For this purpose, n.c.i. was chosen with selected ion monitoring. The chromatogram obtained for the mixture displayed two prominent peaks with retention times of 5.18 and 5.23 min (Fig. 3), with identical n.c.i. mass spectra. Comparison of Fig. 2(*a*) with Fig. 3 indicated that the peaks at 5.18 and 5.23 min corresponded to MASP and GASP respectively. The small peak at 5.02 min (Fig. 3) gave a mass spectrum similar to those of the TFA-OMe derivatives of MASP and GASP, and it is attributed to a corresponding C-3 isomer.

A derivatized hydrolysate of polylysine, which had undergone prior reductive amination with glucose, was also analysed under n.c.i. conditions. The total ion chromatogram contained two principal peaks, with retention times of 6.74 and 8.13 min. The n.c.i. mass spectrum of the peak at 6.74 min afforded abundant ions of *m/z* 996, 976 and 882 (cf. Fig. 4*b* above); data consistent with its identification as the TFA-OMe derivative of *N*-(1-deoxyglucitol-1-yl)–lysine (Scheme 1); the other peak contained ions of *m/z* 786, 766, 689 and 672, which suggested that it represented an anhydro analogue of deoxyglucitol–lysine. The corresponding mannitol derivative produced two equivalent peaks at slighter shorter retention times (see Table 1) and the total-ion chromatogram for the mixture of epimers (expected to be present in protein hydrolysates) showed all four peaks well resolved from each other (Fig. 4*a*). An additional peak (retention time 6.57 min) was also observed: it afforded ions characteristic for 1-deoxyhexitol–lysine and possibly is due to a C-3 isomer.

Robins & Bailey [10], using g.c.–m.s., have previously

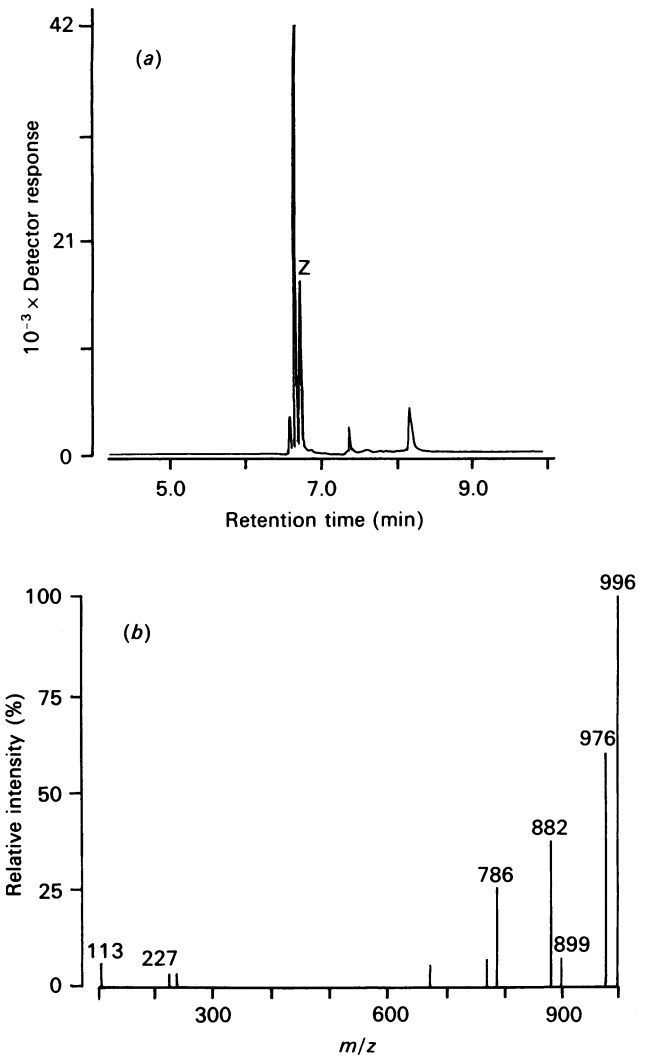


Fig. 4. (*a*) T.i.c. obtained under n.c.i. conditions for a mixture of N^{ϵ}-1-deoxymannitol and N^{ϵ}-1-deoxyglucitol adducts of lysine, and (*b*) n.c.i. mass spectrum of the TFA-OMe derivative of N^{ϵ}-(1-deoxyglucitol-1-yl–lysine (peak Z in *a*)

In (*a*) adducts were prepared by reductive amination of poly-L-lysine with mannose and glucose, hydrolysis and derivatization.

Table 1. Comparison of retention times and mass ions observed in g.c.–n.c.i.m.s. of TFA-OMe derivatives of *N*-1-(1-deoxyhexitol)–amino acids

Model compound*	Retention time (min)	Principal fragment ions (*m/z*)
N-1-(1-Deoxymannitol)–aspartic acid	5.18	901‡, 881‡, 788, 787‡, 756‡, 675, 643, 227, 113
N-1-(1-Deoxyglucitol)–aspartic acid	5.23	
N-1-(1-Deoxymannitol)–lysine	6.62	996‡, 976‡, 882‡, 786, 672, 652, 227, 113
N-1-(1-Deoxyglucitol)–lysine	6.74	
Anhydro derivative of:		
N-1-(1-Deoxymannitol)–lysine†	7.42	786‡, 766‡, 689‡, 672, 558, 227, 113
N-1-(1-Deoxyglucitol)–lysine†	8.13	

* From which derivatives were prepared.
† Formed during acid hydrolysis and derivatization.
‡ Ions used in selected ion monitoring.

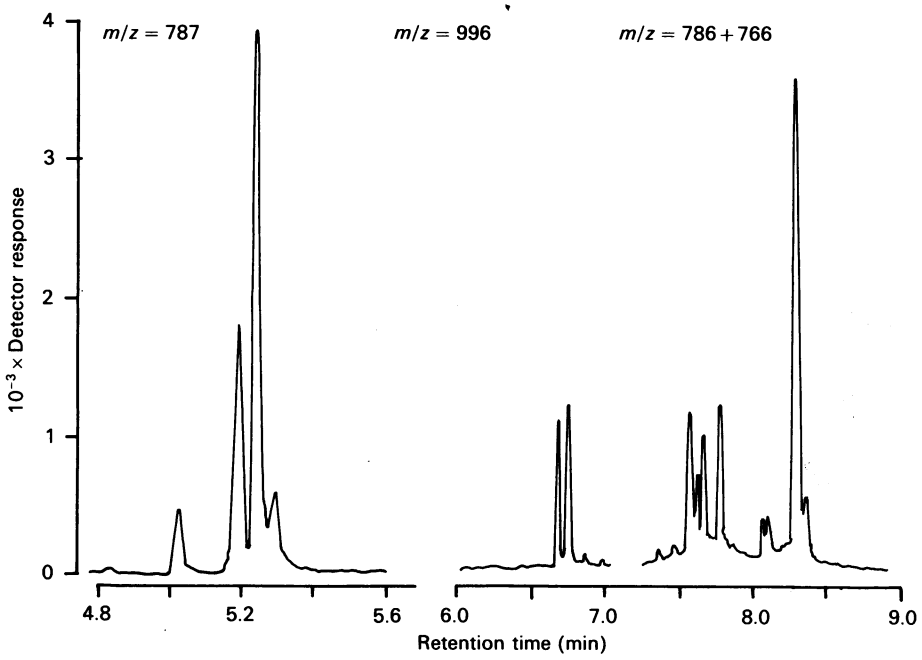


Fig. 5. Selected-ion traces obtained with n.c.i. for a derivatized hydrolysate of reduced albumin

The ion of *m/z* 787 was monitored for the first 6 min, and ions of *m/z* 996, 786 and 766 were selected thereafter.

reported that anhydro derivatives of glycated lysine are formed on acid hydrolysis of collagen, but they did not furnish any mass-spectral data. It was also postulated that the anhydro compounds were present as furanose structures, formed by ring closure between either C-2 and C-5 or between C-3 and C-6 of the hexitol moiety [18]. The anhydro derivatives are artefacts, and their formation can be attributed to the severity of the acid conditions (particularly of time and temperature) to which the deoxyhexitol–amino acids were subjected, either during hydrolysis of the protein or during derivatization. Data relating to the model compounds are collated in Table 1, and the proposed fragmentation in the n.c.i. mode is depicted in Scheme 1.

Hydrolysates of albumin (which had been previously reduced with borohydride) were subjected to affinity chromatography to obtain a fraction enriched in glycated components. A typical total-ion chromatogram produced by n.c.i. analysis of the TFA-OMe derivatives present in such a fraction contained more than ten peaks, some with retention times appropriate for the derivatives of hexitol–aspartic acid and hexitol–lysine. Therefore selected ion monitoring (using the appropriate ions listed in Table 1) was undertaken to confirm that the glycated components were present, clearly demonstrating the power of the method for the specific detection of glycated amino acids in protein hydrolysates. For example, when the base ion (of *m/z* 787) for the aspartic acid derivative was monitored, two intense peaks (with retention times of 5.19 and 5.24 min and indicative of the presence of the TFA-OMe derivatives of 1-deoxymannitol– and 1-deoxyglucitol–aspartic acid) were obtained, together with two minor peaks with retention times of 5.03 and 5.30 min (Fig. 5). Whereas the species eluted at 5.03 min probably arises from the C-3 epimer, the identity of the peak at 5.30 min has not been established. A simple trace

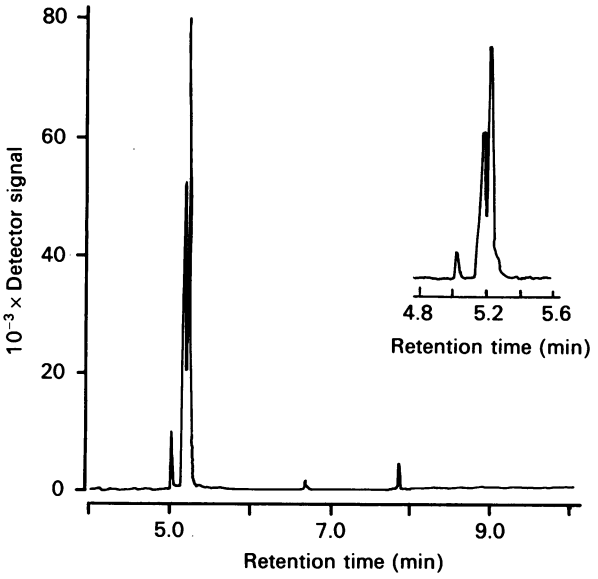


Fig. 6. A selected-ion chromatogram (generated under n.c.i.) for the derivatized 'breakthrough fraction' furnished by amino acid analysis of a reduced albumin hydrolysate

The monitoring conditions used were identical with those given in the legend to Fig. 3.

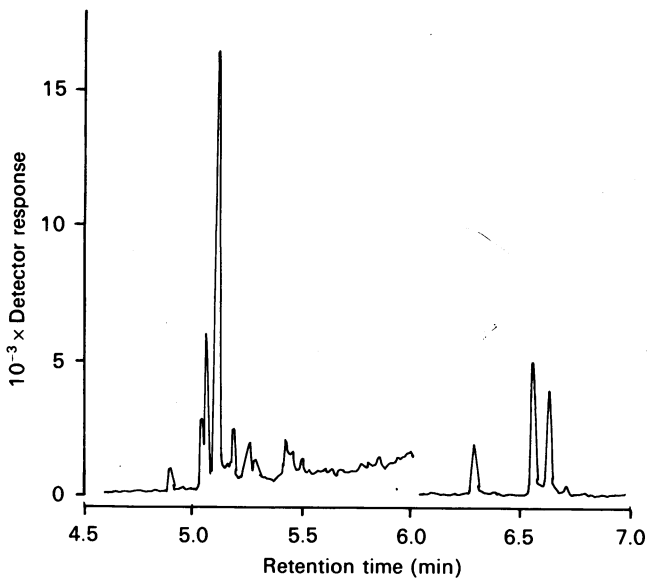


Fig. 7. Selected-ion traces produced under n.c.i. for the TFA-OMe derivatives present in an acid hydrolysate of glycated urinary peptides

Two ions were monitored: that of *m/z* 787 for the first 6 min, then the ion of *m/z* 996 thereafter.

also resulted when the molecular ion (of *m/z* 996) of the lysine adduct was utilized. It contained only two peaks, which were eluted at 6.68 and 6.75 min (Fig. 5), and which corresponded to the 1-deoxymannitol and 1-deoxyglucitol isomers derived from lysine. Analysis of the anhdyro forms of lysine proved more difficult. The characteristic ions (of *m/z* 786 and 766) appeared in several peaks with long retention times; the dominant peak possessed a retention time of 8.3 min and is attributed to an anhydro form of the 1-deoxyglucitol–lysine. Further purification was also undertaken by subjecting the glycated fraction obtained by affinity chromatography to amino acid analysis [14] and collecting material weakly bound to the cation-exchange resin. The chromatogram obtained on subsequent g.c.–m.s. analysis (Fig. 6) showed that the glycated aspartic acid could be isolated by this procedure and that it consisted of both epimers (Fig. 6, inset; cf. Fig. 3). It is suggested that the epimers are formed by non-stereospecific reduction of a fructose–aspartic acid complex present in albumin.

Glycated components were also recovered, by affinity chromatography, from human urine. These were reduced with borohydride and purified by cation-exchange chromatography; samples were hydrolysed, derivatized as described above and subjected to g.c.–m.s. analysis in the n.c.i. mode. A complex total-ion chromatogram was obtained initially, and it was not possible to reach firm conclusions regarding the presence of glycated amino acids. However, using the base ion of *m/z* 787, a clearer trace resulted (Fig. 7). The major peak (retention time 5.11 min) was derived from GASP with a significant contribution from the TFA-OMe derivative of MASP (retention time 5.06 min). Analysis using the molecular ion of the lysine derivatives (*m/z* 996) produced two prominent peaks (retention times 6.57 and 6.63 min) corresponding to the mannitol and glucitol isomers of glycated lysine. (The retention times for this analysis were slightly shorter because the g.c. column was 5 cm shorter than that used previously).

Radioactive samples of albumin and urinary peptides were also prepared with NaB^3H_4. Hydrolysates were purified by affinity chromatography (in order to remove non-specifically labelled substances), subjected to amino acid analysis, and the distribution of radioactivity was determined. For albumin, 12 and 78% of the ^3H recovered was eluted in positions expected for hexitol and anhydrohexitol derivatives of aspartic acid and lysine respectively. With urinary peptides, the corresponding values were 28 and 65%, and in both cases the overall recovery of radioactivity from the analyser exceeded 85%. It has been shown previously [14] that the albumin used in the present study was homogeneous and contained 0.36 glycated groups per mol. By means of g.c.–m.s. and amino acid analysis it is now possible to show that aspartic acid is glycated in addition to lysine. Fig. 6 indicates that glycated aspartic acid is present in the breakthrough fraction obtained by amino acid analysis and, if all radioactivity associated with the fraction is due to this residue, it can be calculated that it contributes a maximum of 0.04–0.05 glycated groups per mol of albumin and represents glycation at the *N*-terminus. Therefore it is not surprising that the conventional methods of analysis previously used were not sensitive enough to detect this small contribution to the overall level of glycation of albumin. The current list of proteins which undergo *N*-terminal glycation is short and comprises osteocalcin [23], albumin (the present study) and each chain of haemoglobin [24], but it is anticipated that this will increase as more sophisticated techniques, such as g.c.–m.s., are applied more widely. The factors governing the rate of formation of the Amadori complex are not fully understood, but the pK_a of the target amino acid is undoubtedly an important parameter in Schiff-base formation. However, there must be additional determinants, because the valine residue at the

N-terminus of the β-chain of haemoglobin forms the fructose adduct more readily than the valine at the same position in the α-chain [24], in spite of their similar pK_a values [25]. The presence of a neighbouring group which is able to participate in the Amadori rearrangement may be an important consideration [6,26,27]. In addition, *in vivo*, the proteins may form complexes with metabolites or cofactors which render a potential site inaccessible to glucose. For example, it is known that Cu(II) binds at the *N*-terminus of albumin [28], and the Cu(II)–albumin complex present *in vivo* may not undergo glycation.

The occurrence of glycated amino acids in urine may reflect intracellular catabolism or may be due to dietary intake of glycated proteins [9]. We have indirect evidence from a clinical study that the presence of glycated aspartic acid in the urine represents turnover of albumin. This study involved serial analyses of glycated components in 24 h urine samples provided by diabetic subjects during a period when their glycaemic status improved. There was a significant correlation between the amount of glycated components in urine and the corresponding level of glycation of plasma proteins (D. Talwar, M. Small, A. Cameron, O. S. Olufemi, D. A. Robb & A. C. MacCuish, unpublished work) and a poor relationship between the former and the extent of glycation of haemoglobin. Our preliminary experiments using g.c.–m.s. to detect glycated valine in urine have been unsuccessful, suggesting also that haemoglobin metabolism is not an important source of urinary glycated components.

D.A.W. gratefully acknowledges the financial support of both the Burroughs Wellcome Fund (Research Triangle Park, NC, U.S.A.), and the University of Strathclyde. O.S.O. is indebted to the European Economic Community for the provision of a studentship, and J.M.M. gratefully acknowledges financial support from the Scottish Home and Health Department.

REFERENCES

1. Cohen, M. P. (1986) Diabetes and Protein Glycosylation: Measurement and Biologic Relevance, Springer, New York
2. Wolff, S. P. & Dean, R. T. (1987) Biochem. J. **245**, 243–250
3. Brownlee, M., Cerami, A. & Vlassara, H. (1988) New Engl. J. Med. **318**, 1315–1321
4. Bookchin, R. M. & Gallop, P. M. (1968) Biochem. Biophys. Res. Commun. **32**, 86–93
5. Garlick, R. L. & Mazer, J. S. (1983) J. Biol. Chem. **258**, 6142–6146
6. Iberg, N. & Fluckiger, R. (1986) J. Biol. Chem. **261**, 13542–13545
7. Mayer, T. K. & Freedman, Z. R. (1983) Clin. Chim. Acta **127**, 147–184
8. Kesaniemi, Y. A., Witztum, J. L. & Streibrecher, U. P. (1983) J. Clin. Invest. **71**, 950–959
9. Rucklidge, G. J., Bates, G. P. & Robins, S. P. (1983) Biochim. Biophys. Acta **747**, 165–170
10. Robins, S. P. & Bailey, A. J. (1972) Biochem. Biophys. Res. Commun. **48**, 76–84
11. Bailey, A. J., Robins, S. P. & Tanner, M. J. A. (1976) Biochim. Biophys. Acta **434**, 51–57
12. Walton, D. J., Ison, E. R. & Szarek, W. A. (1984) Carbohydr. Res. **128**, 37–49
13. Walton, D. J., Szarek, W. A., MacLean, D. B. & Gerard, R. V. (1985) Carbohydr. Res. **137**, 31–38
14. Olufemi, O. S., Talwar, D. & Robb, D. A. (1987) Clin. Chim. Acta **163**, 125–136
15. Schwartz, B. A. & Gray, G. R. (1977) Arch. Biochem. Biophys. **181**, 542–549
16. Trueb, B., Hughes, G. J. & Winterhalter, K. H. (1982) Anal. Biochem. **119**, 330–334
17. Brownlee, M., Vlassara, H. & Cerami, A. (1980) Diabetes **29**, 1044–1047
18. Robins, S. P. (1982) Methods Biochem. Anal. **28**, 329–379
19. Cruikshank, P. A. & Sheehan, J. C. (1964). Anal. Chem. **36**, 1191–1197
20. Ahmed, M., Thorpe, S. R. & Baynes, J. W. (1986) J. Biol. Chem. **261**, 4889–4894
21. Gelpi, E., Koenig, W., Gilbert, J. & Uro, J. (1969) J. Chromatogr. Sci. **7**, 604–613
22. Hunt, D. F. & Crow, F. W. (1978) Anal. Chem. **50**, 1781–1784
23. Gundberg, C. M., Anderson, M., Dickson, I. & Gallop, P. M. (1986) J. Biol. Chem. **261**, 14557–14561
24. Shapiro, R., McManus, M. J., Zalut, C. & Bunn, H. F. (1980) J. Biol. Chem. **255**, 3120–3127
25. Garner, M. H., Bogardt, R. A. & Gurd, F. R. N. (1975) J. Biol. Chem. **250**, 4398–4404
26. Baynes, J. W., Thorpe, S. R. & Murtiashaw, M. H. (1984) Methods Enzymol. **106**, 88–98
27. Mori, N. & Manning, J. M. (1986) Anal. Biochem. **152**, 396–401
28. Peters, T. (1975) in Plasma Proteins, 2nd edn. (Putnam, F. W., ed.), vol. 1, p. 133, Academic Press, New York

Received 22 November 1988/3 March 1989; accepted 9 March 1989