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NON-ENZYMIC GLYCATION OF PROTEINS: ANALYSIS OF *N*-(1-DEOXYHEXITOL-1-YL)AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for determining the extent of non-enzymic glycation (originally called "glycosylation") of both lysyl and *N*-terminal residues of a protein is described. The glycated protein is treated with sodium borohydride, and is then subjected to acid-catalysed hydrolysis. The resulting *N*-(1-deoxy-D-hexitol-1-yl)-amino acids are separated by cation-exchange high-performance liquid chromatography (l.c.), and detected by a post-column reaction with periodate. The method has been applied successfully to samples of human hemoglobin and human serum albumin, for measurement of numbers of valine-attached and of lysine-attached *N*-(1-deoxy-D-fructos-1-yl) groups per protein molecule.

INTRODUCTION

Several mammalian proteins acquire *N*-(1-deoxy-D-fructos-1-yl) groups by reactions of their free amino groups with D-glucose, and subsequent Amadori rearrangement of the resulting glucosylamines[1]. This process, which used to be called "non-enzymic glycosylation"[1], but has been renamed "non-enzymic glycation"[2], can lead to the crossing-linking of proteins[3] and is implicated in the pathogenesis of diabetes[4].

A good analytical procedure should allow the determination of the extent of non-enzymic glycation of each type of amino acid residue in the protein concerned. Most published methods fall short of this ideal[5]. Several of them, such as those which depend upon charge-dependent separations or immunological assays of intact proteins[6], or upon measurement of 5-hydroxymethylfurfural released by acid treatment[7], give only comparative values of the extent of glycation of a specific protein. In contrast, the fluorimetric assay of periodate-released formaldehyde[8] and the l.c. assay of acid-released N^{ε}-(2-furoylmethyl)-L-lysine[9] can be used to determine absolute quantities of *N*-(1-deoxyfructos-1-yl) groups attached to the whole protein, or to ε-amino groups of L-lysyl residues, respectively.

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In principle, glycated amino acid residues may be determined by reduction of *N*-(1-deoxyfructos-1-yl) groups with sodium borohydride, hydrolysis of the protein, and analysis of the resulting *N*-(1-deoxyhexitol-1-yl)amino acids by ion-exchange chromatography. This method has been used for measuring N^{ε}-(1-deoxyfructos-1-yl)-L-lysine residues, when the mixed N^{ε}-(1-deoxy-D-glucitol-1-yl) and N^{ε}-(1-deoxy-D-mannitol-1-yl) derivatives of L-lysine were detected by reacting with ninhydrin[8] or *o*-phthalaldehyde[10]. However, the procedure cannot be used for the analysis of *N*-(1-deoxyhexitol-1-yl) derivatives of the other common amino acids, because a low colour yield is obtained with ninhydrin[11] and *o*-phthalaldehyde does not react with secondary amines[12]. This problem has been overcome by using sodium borotritide for reduction of a glycated protein, followed by hydrolysis and radioactivity monitoring of the resulting, labelled, *N*-(1-deoxyhexitol-1-yl)amino acids in ion-exchange column effluents[13]. Although this method has usually been used mainly for measuring glycated lysine, it should be applicable to any glycated amino acid. However, non-specific reduction of the polypeptide leads to the formation of unidentified radioactive compounds which can complicate the analyses[14].

We now describe an improved method for the identification and determination of *N*-(1-deoxyhexitol-1-yl)amino acids. The latter are fractionated by cation-exchange l.c., and detected by a post-column oxidation with periodate, which is measured as a decrease of absorbance at 260 nm. This detection technique, which was developed for l.c.of carbohydrates[15], depends mainly upon the susceptibility of the hexitolyl portion of the molecule to periodate oxidation. Problems associated with the use of tritium or ninhydrin are avoided, and the method is suitable for the assay of the *N*-(1-deoxyhexitol-1-yl) derivatives of neutral and basic amino acids.

EXPERIMENTAL

Materials. — Glyco-Gel B and Affi-Gel 601 boronate gels were purchased from Pierce Chemical, Rockford, IL and Bio-Rad Laboratories, Richmond, CA, respectively. Human serum albumin and 2-deoxy-2-methylamino-D-glucitol (*N*-methyl-D-glucamine) were obtained from Sigma Chemical Company (St. Louis, MO). Glyco-hemoglobin A_0 was a gift from Dr. R. L. Garlick, Hematology Division, Brigham and Women's Hospital, Boston, MA. It was the fraction remaining after removal of hemoglobin A_1 and non-glycated hemoglobin from human hemoglobin by cation-exchange and boronate affinity chromatography, respectively. Synthetic *N*-(1-deoxyhexitol-1-yl)amino acids (except for tyrosine derivatives) were prepared by reductive amination of D-glucose or D-mannose with amino acids, as described previously[16]. The tyrosine derivatives were synthesized from tyrosine ethyl ester, rather than tyrosine itself, owing to the low solubility of the latter in water, as follows.

N-*(1-Deoxy-D-glucitol-1-yl)-L-tyrosine.* — A solution of L-tyrosine ethyl ester (0.5 mmol) and sodium cyanoborohydride (0.75 mmol) in water (3.5 mL) was adjusted to pH 7.0 with 0.2M hydrochloric acid. D-Glucose (1.5 mmol) was added,

and the resulting solution was heated in a sealed reaction vial for 4 h at 100°. Sodium hydroxide (4M, 1.06 mL) was added, and the solution was left for 1 h at 22°. Sodium ions and unreacted glucose were removed with Dowex 1 (acetate) ion-exchange resin, as previously described[16]. Purification was effected with a column (41 × 2 cm) of silica gel (60–200 mesh; Davison Chemical Co.), when tyrosine and *N*-(1-deoxy-D-glucitol-1-yl)-L-tyrosine were eluted with 1-propanol–5.9M ammonia, 22:3 (v/v) and 21:4 (v/v), respectively. The pure product (69 mg, 40%) was obtained by recrystallization from 5:1 ethanol–water; ^{1}H-n.m.r. [D_2O, $Me_3Si(CH_2)_3SO_3Na$, 400 MHz]: δ 3.12, 3.17 (AB of ABX, 8 lines, 2 H, $J_{1A,1B}$, 13.0, $J_{1A,2}$ 3.7, $J_{1B,2}$ 8.8 Hz, H-1B and -1A), 3.15 (d, overlaps 3.12 and 3.17 signals, 2 H, $J_{\alpha,\beta}$ 6.6 Hz, 2 H-β), 3.56–3.82 (m's 5 H, H-3, -4, -5, and 2 H-6), 3.87 (t, 1 H, H-α), 4.04 (X of ABX, ddd, 1 H, $J_{2,3}$ 4.7 Hz, H-2), and 6.87 and 7.18 (2 d's, 4 H, J 8.4 Hz, C_6H_4OH).

N-*(1-Deoxy-*D*-mannitol-1-yl)-*L*-tyrosine* was prepared similarly from L-tyrosine ethyl ester and D-mannose; ^{1}H-n.m.r. [D_2O, $Me_3Si(CH_2)_3SO_3Na$, 400 MHz]: δ 3.07, 3.36 (AB of ABX, 8 lines, 2 H, $J_{1A,1B}$ 12.8, $J_{1A,2}$ 3.8, $J_{1B,2}$ 8.6 Hz, H-1B and -1A), 3.16 (d, 2 H, $J_{\alpha,\beta}$ 6.6 Hz, 2 H-β), 3.61–3.85 (m's 5 H, H-3, -4, -5, and 2 H-6), 3.88 (t, 1 H, H-α), 3.94 (X of ABX, ddd, 1 H, $J_{2,3}$ 8.6 Hz, H-2), and 6.87 and 7.18 (2 d's, 4 H, J 8.4 Hz, C_6H_4OH).

Solutions for l.c. — *Buffer* A, 0.1M sodium acetate, adjusted to pH 2.7 with acetic acid; *buffer* B, 0.5M sodium acetate, adjusted to pH 7.0 with acetic acid; 1.0mM sodium metaperiodate made up in buffer *A*.

Preparation of hemoglobin samples. — A hemolysate was prepared from blood taken from a non-diabetic subject. Hemoglobin (200 mg) was divided into glyco- and non-glyco fractions by affinity chromatography on a 30-mL column of Glyco-Gel B[17]. The resulting solutions were then dialysed against water, and lyophilized.

Determination of N-*(1-deoxyfructos-1-yl) groups by colorimetry.* — The procedure of Gallop *et al.*[8] was applied to fresh hemolysates and to aqueous solutions of serum albumin. The absorbance[18] at 410 nm rather than its fluorescence[8], was used to obtain the concentration of 3,5-diacetyl-1,4-dihydrolutidine (DDL). Extinction coefficients ($E^{1\%}_{1cm}$ at 280 nm) of 8.5 and 5.5 were used for calculating weights of globin and human serum albumin, respectively.

Processing of proteins for l.c. — A solution of each hemoglobin sample (25–75 mg) in 5.5 mL of sodium phosphate buffer (0.1M; pH 7.5) was passed through a 0.45-μm Millipore filter, cooled to 0°, and adjusted to pH 8 with 0.1M sodium hydroxide. Sodium borohydride (6 mg) was added, and the resulting solution was kept for 15 min at 22° and 1 h at 4°. The borohydride was decomposed, and the heme was removed simultaneously by precipitating the reduced globin with acidified acetone[19]. An aqueous solution of the product was dialysed and lyophilized. Human serum albumin was reduced similarly, except that borohydride was decomposed by the addition of 5M acetic acid. Each reduced protein (up to 10 mg) was hydrolysed with 4M hydrochloric acid for 24 h at 110°. An aqueous solution

of *N*-methylglucamine (maximum, 70 nmol) was added, and the solution was dried with a stream of nitrogen. The residue was dissolved in 0.5 mL of ammonium acetate buffer (50mM in acetate; pH 9). The resulting solution was readjusted to pH 9 by addition of aqueous ammonia, and applied to a column (4 mL) of Affi-Gel 601 equilibrated with the same pH 9 buffer. Free amino acids were washed from the column with 20 mL of pH 9 buffer, flowing at 0.5 mL/min. *N*-(1-Deoxyhexitol-1-yl)amino acids, and the internal standard, were eluted with 15 mL of 0.1M acetic acid. Acetic acid and ammonium acetate were removed by lyophilization, dissolution of the residue in water, and re-lyophilization. The residue was dissolved in buffer *A* in preparation for analysis by l.c.

L.c. conditions. — The l.c. system was built from a Dionex amino acid analyser kit (Model MBN/SS) which included a programmed multi-port valve for selection of buffer solutions. Solutions of *N*-(1-deoxyhexitol-1-yl)amino acids in buffer *A* were applied with a syringe-loading injector with a 100-μL loop. Buffers (*A* for the first 30 min; *B* thereafter) were pumped, at 0.3 mL/min and 600 p.s.i., through a Bio-Rad Aminex A-9 column (25 × 0.4 cm; sodium form) maintained at 85° with an electrically heated jacket. Sodium metaperiodate solution was continuously added to the post-column effluent at 0.3 mL/min via 36 m of 0.3-mm i.d. Teflon tubing, which served as a pulse dampener. The mixed solutions then flowed through a reaction coil (18 m of 0.3-mm Teflon tubing immersed in boiling water) for ~2 min, connected to a Waters model 481 u.v. detector (1-cm flow-cell) set at 260 nm. The detector was connected to a potentiometric recorder and to an Apple IIe computer equipped with an ADALAB interface card. Chromatochart software (Interactive Microwave, State College, PA) was used for peak integration and storage of data.

Special conditions for determination of tyrosine derivatives. — The periodate-oxidation method of detection was unsuitable for estimating tyrosine derivatives, as the aromatic absorbance at 260 nm decreased the size of the signal, giving small or negative peaks. The following modifications were therefore made: (*a*) The addition of *N*-methylglucamine was omitted. (*b*) D-[U-^{14}C]Glucitol (10 nmol) was added to the protein hydrolysate. The recovery of glucitol from the Affi-Gel 601 column, which was estimated by counting the acetic acid eluate, was assumed to be the same as that of the hexitolyltyrosines. (*c*) L-Tyrosine (20 nmol) was added to the acetic acid eluate, to act as an internal standard during l.c. (*d*) The post-column addition of periodate was omitted. (*e*) The detector wavelength was set at 276 nm.

RESULTS AND DISCUSSION

Well-defined peaks were obtained, as shown in Fig. 1, and retention times, relative to that of *N*-methyl-D-glucamine (Table I), were reproducible (~2% error at the 95% confidence level). Partial resolution of *manno* and *gluco* epimers was achieved (see Fig. 1).

The conditions used for periodate oxidation were based upon those described

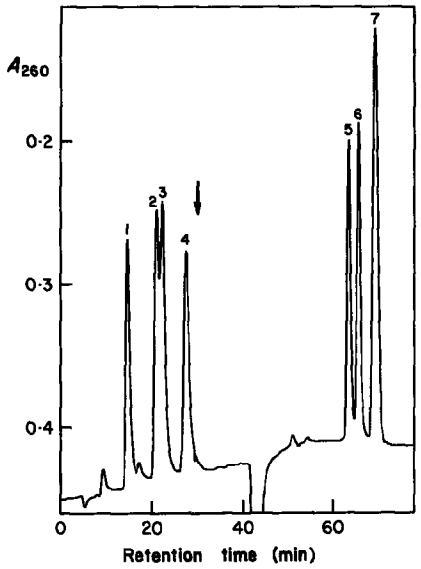

Fig. 1. Elution profile of synthetic *N*-(1-deoxyhexitol-1-yl)amino acids (~55 nmol of each), and *N*-methyl-D-glucamine (68 nmol): (1) *N*-(1-deoxy-D-mannitol-1-yl)-L-serine, (2) *N*-(1-deoxy-D-mannitol-1-yl)-L-valine, (3) *N*-(1-deoxy-D-glucitol-1-yl)-L-valine, (4) *N*-(1-deoxy-D-glucitol-1-yl)-L-alanine, (5) *N*-(1-deoxy-D-mannitol-1-yl)-L-lysine, (6) N^{ε}-(1-deoxy-D-glucitol-1-yl)-L-lysine, (7) *N*-methyl-D-glucamine. The arrow shows the change from buffer *A* to buffer *B*.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF *N*-(1-DEOXYHEXITOL-1-YL)AMINO ACIDS

Compound[a]	*Relative retention time*[b]	*Correlation coefficient*[c]	*Relative molar response*[d]	*Compound*[a]	*Relative retention time*[b]	*Correlation coefficient*[c]	*Relative molar response*[d]
ManH-Thr	0.202	0.999	0.71	GlcH-Tyr[g]	0.406	[e]	[e]
GlcH-Tyr	0.224	0.997	0.83	ManH-Ala	0.396	0.992	0.74
ManH-Ser	0.274	0.999	0.84	GlcH-Ala	0.417	0.988	0.73
GlcH-Ser	0.292	0.998	0.80	ManH-Gly	0.431	0.982	0.77
ManH-Val	0.308	0.989	0.79	GlcH-Gly	0.454	0.994	0.73
GlcH-Val	0.330	0.997	0.74	ManH-His	0.809	[e]	[e]
ManH-Met	0.293	[e]	[e]	GlcH-His	0.852	[e]	[e]
GlcH-Met	0.325	0.989	0.99	ManH-Lys[f]	0.912	1.000	0.95
ManH-Leu	0.362	1.000	0.79	GlcH-Lys[f]	0.941	0.996	0.93
GlcH-Leu	0.376	0.994	0.77	ManH-Val′	0.254	[e]	[e]
ManH-Phe	0.371	[e]	[e]	GlcH-Val′	0.292	[e]	[e]
GlcH-Phe	0.397	0.993	0.57	ManH-Gly′	0.375	[e]	[e]
ManH-Tyr[g]	*0.385*	[e]	[e]	GlcH-Lys′	1.082	[e]	[e]

[a]ManH = 1-deoxy-D-mannitol-1-yl; GlcH = 1-deoxy-D-glucitol-1-yl. A prime indicates a degradation product formed by heating the named compound with 4M HCl. [b]Retention time ÷ retention time of *N*-methyl-D-glucamine. The retention time of the latter is ~70 min. [c]For standard curve for 5–100 nmol, for example Fig. 2. [d]Molar response of compound ÷ molar response of *N*-methyl-D-glucamine. Slope of response curve. [e]Not determined. [f]1-Deoxyhexitol-1-yl attached to ε-amino group. [g]Retention times, relative to that of tyrosine, are ManH-Tyr 0.423, and GlcH-Tyr 0.451.

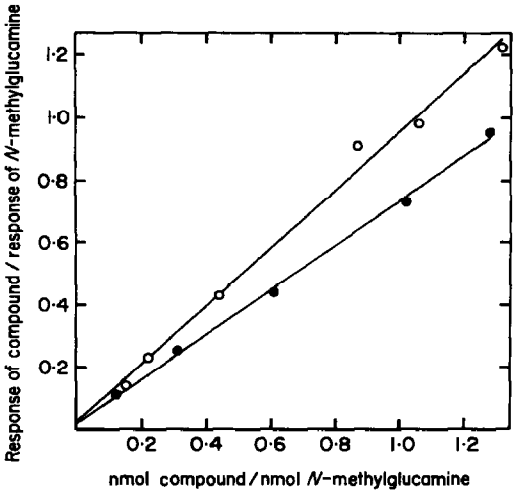

Fig. 2. Response curves of *N*-(1-deoxy-D-mannitol-1-yl)-L-threonine (closed circles) and N^{ϵ}-(1-deoxy-D-glucitol-1-yl)-L-lysine (open circles) obtained by least-squares analysis. The slope of each line is the molar response, relative to that of *N*-methyl-D-glucamine, the internal standard; 68.0 nmol of the latter was included in each injection.

by Nordin[15], who observed a relatively high periodate consumption by neutral sugars in his post-column reactor. Likewise, we found that *N*-(1-deoxy-D-glucitol-1-yl)-L-valine consumed 4.2 mol/mol of periodate in the post-column reactor (calculated from peak area and flow rate), compared with 3.0 mol/mol in buffer *A* at room temperature (measured spectrophotometrically). (The value of 3, rather than 5 mol/mol may be due to the involvement of the 2-hydroxyl group in lactonization, but this has not been substantiated.)

For the range of 5–100 nmol, response curves were linear for all the compounds examined (see Fig. 2 and correlation coefficients in Table I). Molar responses, relative to that of *N*-methylglucamine, obtained from slopes of the curves, were all fractional (Table I). The accuracy of the method was examined by performing sets of ten determinations of identical weights of *N*-(1-deoxy-D-glucitol-1-yl)-L-valine. Thus, the error incurred in a single determination of 10 or 100 nmol was estimated to be 15 or 5%, respectively, at the 95% confidence level. The lower limit of the assay is 5 nmol, but for hydrolysates of reduced proteins it is somewhat higher (see below). For the determination of tyrosine derivatives, special procedures were adopted to take into account the aromatic absorption at 260 nm (see Experimental section).

Hydrolysis of glycated proteins was conducted with 4M, rather than the usual 6M hydrochloric acid, to minimize acid-catalysed degradation of *N*-(1-deoxyhexitol-1-yl)amino acids[20]. Despite this precaution, losses of *N*-(1-deoxyhexitol-1-yl) derivatives of valine, glycine, and lysine were 14, 11, and 21%, respectively, and this was taken into account in subsequent analyses of protein hydrolysates. Some of the by-products absorbed strongly at 260 nm, giving negative peaks, as shown in Fig. 3. No attempt was made to quantitate each by-product.

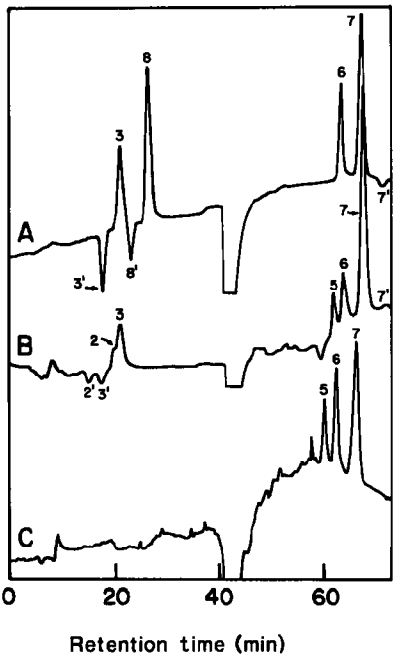

Fig. 3. A: L.c. of a mixture of *N*-(1-deoxy-D-glucitol-1-yl)-L-valine (3), -L-glycine (8), -L-lysine (ε derivative, 6), and *N*-methyl-D-glucamine (7), pretreated with 4M hydrochloric acid for 24 h at 110°. The resulting by-products are designated by primes. B and C: L.c. of hydrolysates of reduced glycohemoglobin (3.6 mg) and reduced human serum albumin (12.2 mg), respectively. Both hydrolysates were spiked with *N*-methyl-glucamine and subjected to boronate affinity chromatography. Compounds are numbered as in Fig. 1. The smaller retention times in C are attributable to the use of a different, newly-packed, column. Note that positive peaks represent a fall in absorbance, as in Fig. 1.

As free amino acids are susceptible to periodate oxidation, they gave peaks that overlapped many of the *N*-(1-deoxyhexitol-1-yl)amino acid peaks. Therefore, protein hydrolysates were usually freed of amino acids by selective adsorption of the hexitolylamino acids on phenylboronate gel[21]. Recoveries were at least 95%, and ratios of weights of hexitolylamino acids to *N*-methylglucamine were unaffected. The phenylboronate-adsorption step proved to be unnecessary for the analysis of the epimers of N^{ε}-(1-deoxyhexitol-1-yl)lysine derived from serum albumin, as they were not co-eluted with amino acids (see Table II).

L.c. of hydrolysates of reduced proteins (Fig. 3) gave peaks attributable to epimeric pairs of *N*-(1-deoxyhexitol-1-yl) derivatives of valine and/or lysine, all *gluco:manno* ratios being greater than unity. For each analysis, the degree of glycation of a particular amino acid was calculated from the sum of the areas of the *gluco* and *manno* peaks. Peaks due to by-products formed during hydrolysis were present.

Similar values were obtained when l.c. and colorimetry were both used to determine the degree of glycation of hemoglobin or serum albumin (Table II). The ratio of valine-attached to lysine-attached 1-deoxyfructose groups in hemoglobin,

TABLE II

N-(1-DEOXYFRUCTOS-1-YL) CONTENT OF HUMAN PROTEINS

Protein	*mol* N-*(1-deoxyfructos-1-yl)/mol protein*[a]			
	L.c.[b]			*DDL assay*
	Val-attached	*Lys-attached*	*Total*	*Total*
Hemoglobin	0.04	0.04	0.08	0.09[c]
Glyco-hemoglobin[d]	0.52	0.44	0.96	[e]
Non-glyco-hemoglobin[d]	0	0	0	[e]
Glyco-hemoglobin A_0[f]	0	0.54	0.54	[e]
Serum albumin:				0.49
analysis after Affi-Gel 601	0	0.40	0.40	—
analysis of hydrolysate[g]	[h]	0.46	0.46	—

[a]Hemoglobin data based on mol $\alpha\beta$ dimer. [b]Analysis of *N*-(1-deoxyhexitol-1-yl)amino acids. [c]DDL assay on hemolysate. [d]Glyco- and non-glyco-hemoglobins were fractions respectively retained or excluded by Glyco-Gel B. [e]DDL assay of lyophilized hemoglobin samples gave inconsistent results. [f]For method of preparation, see Experimental. [g]L.c. performed directly on hydrolysate. Affi-Gel step omitted. [h]Value unobtainable due to interference by amino acids.

~1:1, was similar to that reported for the analysis of borotritide-reduced hemoglobin[21], but different from an earlier estimate[22] of 1:2.5. The absence of glycated valine in glyco-hemoglobin A_0 showed that the A_1 component had been completely removed by ion-exchange chromatography.

The estimate of lysine-attached 1-deoxyfructosyl groups in human serum albumin (0.40–0.46 mol/mol protein; Table II) was higher than a reported[23] value of 0.29 mol/mol and was close to values of[23] 0.25 and[24] 0.40 mol/mol predicted from kinetic measurements.

We have therefore demonstrated that this l.c. method is suitable for the simultaneous analysis of glycated *N*-terminal and lysyl residues of a protein. For an acceptable signal-to-noise ratio, the sample of hydrolysate injected into the chromatograph should contain at least 10 nmol of an *N*-(1-deoxyhexitol-1-yl)amino acid. If a protein contains <0.02 groups of a an *N*-(1-deoxyfructos-1-yl)amino acid per 100 residues, too large a weight of reduced protein must be hydrolysed, and an erratic baseline is sometimes obtained. In this case the glycated protein should be purified by boronate chromatography prior to borohydride reduction. L.c. retention times of *N*-(1-deoxyhexitol-1-yl)amino acids may be used for their identification. Confirmation by mass spectrometry[25] may be useful in some cases.

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