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MODEL STUDIES PERTAINING TO THE HYDRAZINOLYSIS OF GLYCOPEPTIDES AND GLYCOPROTEINS: HYDRAZINOLYSIS OF THE 1-*N*-ACETYL AND 1-*N*-(L-β-ASPARTYL) DERIVATIVES OF 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYLAMINE

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ABSTRACT

The products of hydrazinolysis of the 1-*N*-acetyl and 1-*N*-(L-β-aspartyl) derivatives of 2-acetamido-2-deoxy-β-D-glucopyranosylamine could not be converted quantitatively into 2-amino-2-deoxy-D-glucose under mild conditions. Proton and ^{13}C-n.m.r. measurements indicated that the hydrazone of 2-amino-2-deoxy-D-glucose was a major product of the hydrazinolysis of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine. Control experiments showed that acetohydrazide is slowly converted into 4-amino-3,5-dimethyl-1,2,4-triazole under the conditions of hydrazinolysis, and that 2-amino-2-deoxy-D-glucose reacts slowly with acetohydrazide in dilute acetic acid. The implications of these results in relation to the hydrazinolysis of glycopeptides and glycoproteins are discussed.

INTRODUCTION

The hydrazinolysis–nitrous deamination procedure[1] for the selective degradation of glycoproteins and glycopeptides is useful in structural studies of such compounds and, indeed, of any compounds that contain 2-acetamido-2-deoxy-D-glucopyranosidic and -D-galactopyranosidic moieties[2,3]. Hydrazinolysis has also been used to isolate the carbohydrate moieties of glycoproteins[4,5]. Early difficulties with the hydrazinolysis step, such as partial decomposition of the substrate[6] and incomplete *N*-deacetylation[7], were overcome by the discovery that hydrazinium sulphate catalysed the *N*-deacetylation[8]. It was also found[9] that more-vigorous conditions were required to effect the complete *N*-deacetylation of a 2-acetamido-2-deoxy-D-glucopyranoside glycosylated at position 3. The difference in reactivity of the latter compound and the corresponding 3-hydroxy compound can be ascribed to intramolecular catalysis by HO-3.

When applied to glycoproteins or glycopeptides in which the carbohydrate is linked to asparagine, hydrazinolysis also cleaves the *N*-glycosylasparagine amide-

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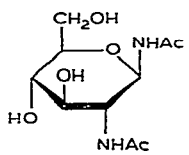
bond. In a study of the application of the hydrazinolysis–deamination procedure to such glycoproteins and glycopeptides, Bayard and Montreuil[10,11] used uncatalysed hydrazinolysis, and established the optimum conditions for the reaction (100° for 36 h) by using methyl 2-acetamido-2-deoxy-α-D-glucopyranoside as a model compound. However, the products of hydrazinolysis of glycopeptides and glycoproteins have not been fully characterised. Kaverneva and Lapuk[12] found that the ratio (5:5) of hexose to 2-amino-2-deoxy-D-glucose in the hydrazinolysis product of ovomucoid was different from that (5:8) in the glycoprotein. Bayard and Montreuil[11] recovered 79% of 2-amino-2-deoxy-D-glucose after hydrazinolysis of α_1-acid glycoprotein, and they attributed the apparent loss of hexosamine to incomplete *N*-acetylation prior to analysis by acid hydrolysis. The high yield of free 2,5-anhydro-D-mannose obtained[11,13] by sequential hydrazinolysis–nitrous deamination of α_1-acid glycoprotein suggests that, whatever the nature of the hydrazinolysis products, the terminal reducing-sugar residues are converted by nitrous acid into 2,5-anhydro-D-mannose. The hydrazinolysis product from pineapple-stem bromelain contained 0.4 residue less of 2-amino-2-deoxy-D-glucose than the original glycoprotein, and it was shown that the 2-amino-2-deoxy-D-glucose residue which had been modified was that linked to asparagine[4].

It has been assumed by some investigators that the terminal reducing-sugar in the hydrazinolysis product is 2-amino-2-deoxy-β-D-glucopyranosylamine[14] or 2-amino-2-deoxy-D-glucose[15]. We thought that the glycosylamine primary product of hydrazinolysis would probably react further with hydrazine, and we now report on model studies which support this belief.

RESULTS

The glycosylamine derivatives **1** and **2** were chosen as models for the 2-acetamido-2-deoxy-β-D-glucopyranosylasparagine linkage in glycopeptides and glycoproteins. The diacetamide **1** was prepared by a modification of the literature method[17]. Because of the tendency of the amine **3** to form a bisglycosylamine during storage and recrystallisation[17,18], it was acetylated *in situ* by addition of acetic anhydride to the solution of the amine immediately after its preparation by hydrogenation of the corresponding glycosyl azide. *O*-Deacetylation then gave the diacetamide **1** which had physical constants different from those reported (see Experimental).



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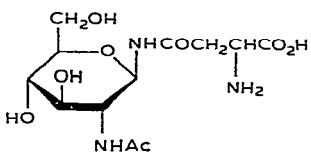

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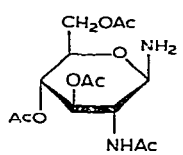

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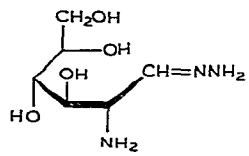
Hydrazinolysis of compounds **1** and **2** was performed in the presence of hydrazinium sulphate in sealed glass tubes at 105 ±2° for 5 h. The hydrazine was removed *in vacuo* over conc. sulphuric acid, and the residue was dissolved in water

or 15% aqueous acetic acid prior to analysis. Quantitative analysis for 2-amino-2-deoxy-D-glucose was performed by the colorimetric procedure of Dische and Borenfreund[19] and by using an amino acid analyser.

Hydrazinolysis of the diacetamide **1**. — Qualitative analysis of the product by paper electrophoresis at pH 6 showed three components, corresponding to hydrazine, 2-amino-2-deoxy-D-glucose, and acetohydrazide, similar results being obtained 0, 2, 4, and 24 h after dissolving the product in 15% aqueous acetic acid. The amino acid analyser gave two peaks, one (194 min) corresponding to 2-amino-2-deoxy-D-glucose, and the other having a slightly longer retention time (202 min). The latter peak predominated when the analysis was carried out immediately after dissolving the hydrazinolysis product in water. An attempt was made to improve the yield of 2-amino-2-deoxy-D-glucose, and 15% aqueous acetic acid at 50° was found to be superior to 0.01M hydrochloric acid at 20°. The yield was thus apparently improved from 46 ±5% to 62 ±6% (Dische and Borenfreund procedure) after 2 days at 50°. Increasing the time of this reaction, for example to 5 days, led to the appearance of a third component having a retention time of 209 min on the amino acid analyser. A control experiment showed that this component was an artefact arising from 2-amino-2-deoxy-D-glucose.

Because of the high retention-times (>3 h) and the possible occurrence of hydrolysis or other acid-catalysed reactions during chromatography on the column of the amino acid analyser (the column was eluted with buffers of pH 3.25 and then 4.30, at 57°), the analyses were repeated using the short column, which was eluted with a buffer of pH 5.25. With this column, only one peak was detected in the region of 2-amino-2-deoxy-D-glucose (21 min), and hydrazine was eluted as an asymmetric peak at 45 min. The asymmetry of this peak and the fact that the peak recorded by the 440-nm photocell had a retention time of 46 min suggested the presence of a compound having a retention time similar to, but slightly different from, that of hydrazine. The apparent yield of 2-amino-2-deoxy-D-glucose obtained with the short column was 70 ±7% after 2 days at 50°.

The ^{1}H-n.m.r. spectrum (D_2O) of a freshly prepared solution of the hydrazinolysis (100°) product from the diacetamide **1** showed signals which could be assigned to the hydrazone **4** (H-1 at τ 2.74, d, J 5.5 Hz) and the pyranoid tautomer **5** (H-1 at τ 5.94, d, J 9.5 Hz). The ^{1}H-n.m.r. spectrum (D_2O) of the hydrazinolysis product from *N*-acetyl-β-D-glucopyranosylamine showed doublets for the hydrazone (H-1 at τ 2.73, J 6 Hz) and β-pyranoid tautomer (H-1 at τ 5.94, J 9 Hz). After neutralisation of the solution with acetic acid, the spectrum showed two low-field doublets at 2.76 and 2.73. Further addition of acetic acid diminished the intensity of these low-field doublets and, to a lesser extent, of the doublet at 5.94 which shifted to lower field with decreasing pH. At pH 5–6, the low-field doublets were absent. It should be noted that none of the acyclic, imine tautomer was detected in the p.m.r. spectrum of β-D-glucopyranosylamine, and the anomeric-proton doublet (τ 5.90, J 9 Hz) was absent 30 min after the addition of 10 μl ($<10^{-3}$ molar equiv.) of acetic acid, hydrolysis to D-glucose having occurred.



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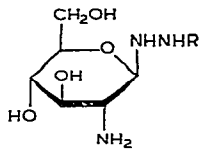
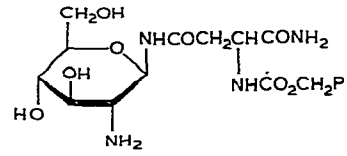


5 R = H
6 R = Ac



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These studies were complicated by the possibility of tautomerism in solution and of an exchange reaction between the hydrazinolysis product and acetohydrazide during the removal of hydrazine, which took 2 days at room temperature. Therefore, ^{13}C-n.m.r. measurements were performed before and after removal of the hydrazine; the behaviour in solution of a typical, crystalline, monosaccharide acetylhydrazone, namely D-galactose acetylhydrazone, was also examined. The spectrum of the hydrazinolysis (100°) reaction mixture showed the following significant features: (*i*) it closely resembled the spectra of the hydrazinolysis product from 2-acetamido-2-deoxy-D-glucose and of a *solution* of 2-amino-2-deoxy-D-glucose hydrochloride in hydrazine; (*ii*) the spectrum had a strong signal at 149.0 p.p.m. which may be assigned to C-1 of a hydrazone derivative such as **4**. After removal of hydrazine, and dissolution of the residue in deuterium oxide, the carbon spectrum contained a strong signal at 93.9 p.p.m. in addition to the low-field signal (146.5 p.p.m.). A signal at 24.8 p.p.m. was assigned to the methyl group of the partially *N*-deacetylated product, 1-*N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosylamine. This carbon nucleus was shown to have a much smaller relaxation time than that of the methyl carbon nucleus of acetohydrazide (22.6 p.p.m.) by the inversion–recovery, two-pulse sequence (using a delay of 2.5 sec between the π and $\pi/2$ pulses), suggesting that the carbon atom (24.8 p.p.m.) was part of a molecule larger than acetohydrazide. The methyl carbon atoms of acetohydrazide and D-galactose acetylhydrazone were differentiated in a similar experiment.

After addition of acetic acid to the deuterium oxide solution to a concentration of 15%, the signal at 146.5 p.p.m. in the ^{13}C-spectrum had disappeared, and the anomeric-carbon region contained two weak signals (at 95.6 and 92.0 p.p.m.) due to 2-amino-2-deoxy-αβ-D-glucose, and a strong signal at 87.2 p.p.m. The solution was kept at 50° for 24 h, and the spectrum then showed, in addition to the signals for 2-amino-2-deoxy-D-glucose, a new signal at 88.7 p.p.m. When the hydrazinolysis was repeated using a longer reaction time, namely 10 h, the spectrum contained no signal at 24.8 p.p.m., but a new minor signal at 13.9 p.p.m. was present together with several other weak signals between 127 and 158 p.p.m. After adding tetradeuterioacetic acid to a concentration of 15%, the ^{13}C- and ^{1}H-n.m.r. spectra, measured before and after warming the solution for 1 day at 50°, again showed that 2-amino-2-deoxy-D-glucose was present, but it was not the only product. Quantitative estimation using the amino acid analyser (long column) gave apparent yields of 47 ±5% and 63 ±6% for 2-amino-2-deoxy-D-glucose after 2 days at room temperature and

1 day at 50°, respectively. For each analysis, the baseline was elevated after the peak for 2-amino-2-deoxy-D-glucose. However, a control experiment, in which a solution of equimolar quantities of 2-amino-2-deoxy-D-glucose hydrochloride and acetohydrazide in deuterium oxide containing 15% of tetradeuterioacetic acid was warmed at 50° for 16 h, showed that a reaction occurred under these conditions. The product was provisionally formulated as the glycosylhydrazide **6**, on the basis of the ^{1}H-n.m.r. (H-1 at τ 5.57) and ^{13}C-n.m.r. (C-1 at 88.7 p.p.m.) spectra, and a small amount of this compound was also detected after the solution had been kept for 16 h at room temperature. The glycosylhydrazide **6** had the same retention time as 2-amino-2-deoxy-D-glucose on both long and short columns of the amino acid analyser. Paper chromatography revealed one strong spot having the same mobility as 2-amino-2-deoxy-D-glucose, and only traces of other compounds.

Another artefact was identified in a control experiment in which acetohydrazide was exposed to the conditions of hydrazinolysis: ~13% of the acetohydrazide was converted into 4-amino-3,5-dimethyl-1,2,4-triazole after hydrazinolysis at 100° for 4.5 h. After reaction at 105° for 16 h, ~70% conversion had occurred and the triazole was isolated in crystalline form.

Crystalline D-galactose acetylhydrazone was shown, by n.m.r. measurements, to tautomerise in deuterium oxide solution. The proton spectrum of a freshly prepared solution showed a doublet (J 5 Hz) at τ 2.40 for H-1, which integrated for less than one proton, and which slowly decreased in intensity by a factor of five during 24 h. Similarly, the ^{13}C-spectrum of the solution showed that mainly two tautomers, namely the hydrazone (C-1 at 155.3 p.p.m.) and a cyclic tautomer (C-1 at 92.9 p.p.m.), were present after 1 h, whereas the cyclic tautomer preponderated after 24 h.

The effectiveness of hydrazinium sulphate as a catalyst was shown by an experiment in which it was omitted. The p.m.r. spectrum of the hydrazinolysis product in deuterium oxide contained signals which could be assigned to the hydrazone (**4**, H-1 at τ 2.74), to unchanged starting material (**1**, H-1 at τ 4.93), and to the partially *N*-deacetylated compound, 1-*N*-acetyl-2-amino-2-deoxy-β-D-glucopyranosylamine (H-1 at τ 5.11).

*Hydrazinolysis of 2-acetamido-1-*N-(L-*β-aspartyl)-2-deoxy-β-*D-*glucopyranosylamine* (**2**). — Hydrazinolysis of the asparagine derivative[20] (**2**) also gave a product which could not be converted quantitatively into 2-amino-2-deoxy-D-glucose under mild conditions. Analysis of the product by paper electrophoresis gave three ninhydrin-positive spots having M_{GLCN} 3.0, 1.0, and 0.2. The last corresponded to L-aspartic acid 4-hydrazide, which was shown to be present by using the amino acid analyser and by paper chromatography. Starting material was absent. When a freshly prepared, aqueous solution of the hydrazinolysis product was assayed on the amino acid analyser (long column), a peak corresponding to 2-amino-2-deoxy-D-glucose was detected (at 194 min), but the baseline was elevated after this peak. This result was reproducible and is probably due to a reaction occurring during chromatography. A very low yield (2%) of 2-amino-2-deoxy-D-glucose was detected using the

short column of the amino acid analyser immediately after dissolving the hydrazinolysis product in 15% aqueous acetic acid. Analysis by the Dische and Borenfreund procedure gave a yield of 41% for the same solution. A similar difference was observed for the analysis of the product from the diacetamide **1**. The Dische procedure suffers from the disadvantage that it is not specific for 2-amino-2-deoxy-D-glucose; any compound that can be converted into a 2,5-anhydrohexose on reaction with nitrous acid would give a positive reaction. There was considerable streaking during paper chromatography, but strong spots were detected for 2-amino-2-deoxy-D-glucose and L-aspartic acid 4-hydrazide after a solution of the product in 15% acetic acid had been kept at 50° for 1 day. The apparent yield of 2-amino-2-deoxy-D-glucose was 60 ±6% after 1 or 2 days at 50°, and a yield of 74 ±7% was obtained after 4 days at 50° using the long column of the amino acid analyser, when no elevated baseline was observed. The artefact previously detected at 209 min was present; after the solution had been kept for a further 24 h at 80–85°, this peak had increased and the yield of 2-amino-2-deoxy-D-glucose had decreased. Yields of 57 ±6% and 76 ±8% were obtained (amino acid analyser, long column) when the product of a 10-h hydrazinolysis was kept in 15% aqueous acetic acid for 2 days at room temperature and for 1 day at 50°, respectively. However, the Dische and Borenfreund procedure gave yields of 25% and 33%, respectively.

DISCUSSION

The n.m.r. measurements indicate that the main product of the hydrazinolysis of the diacetamide **1** is the hydrazone **4**, which is less readily hydrolysed than simple glycosylamines. Aliphatic hydrazones are somewhat unstable[21], and the hydrazone **4** tautomerises in aqueous solution to give the glycosylhydrazine **5**. The hydrazones of substituted D-ribose derivatives exist as equilibrium mixtures of acyclic and cyclic tautomers[22]. The quantitative estimation of 2-amino-2-deoxy-D-glucose in the products is not straightforward, and the most useful results were those obtained using the short column of the amino acid analyser. Thus, very little 2-amino-2-deoxy-D-glucose was detected in freshly prepared solutions of the hydrazinolysis products. The control experiment in which 2-amino-2-deoxy-D-glucose was found to react with acetohydrazide showed that warming the dilute acetic acid solution was not a satisfactory procedure for optimising the yield of 2-amino-2-deoxy-D-glucose. It should be noted that the products of the hydrazinolysis of glycopeptides and glycoproteins are normally separated from acetohydrazide and amino acid hydrazides by gel filtration. The ^{13}C-n.m.r. spectrum of a solution of the hydrazone of 2-amino-2-deoxy-D-glucose in 15% aqueous acetic acid, in the absence of acetohydrazide, showed that the glycosylhydrazine **5** was still the major component, the upfield shift of the C-1 signal (94.3 in aqueous solution to 87.3 p.p.m. in 15% aqueous acetic acid) being due to the effect of protonation of the NH_2 groups. The other main components were 2-amino-2-deoxy-α- and -β-D-glucose. After this solution had been kept at 50°

for 24 h, 2-amino-2-deoxy-D-glucose was the main component, but **5** was still present together with smaller amounts of other components.

These results are relevant to structural studies of the carbohydrate moieties isolated from glycoproteins or glycopeptides by hydrazinolysis. Standard procedures for determining monosaccharide composition, *e.g.*, *N*-acetylation followed by acid hydrolysis, would not reveal the presence of a modified 2-amino-2-deoxy-D-glucose residue such as the hydrazone. Thus, variable results may be expected from periodate-oxidation and methylation studies, depending on the conditions to which the hydrazinolysis product has been exposed. In a methylation study of oligosaccharides obtained by hydrazinolysis of porcine thyroglobulin, 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol, which originated from the terminal reducing-sugar, was isolated in 60% yield[4]. It is also noteworthy that a low yield of the partially methylated derivative was obtained from the terminal reducing-sugar of the hydrazinolysis product of a virus glycoprotein[16]. Interpretations of the results of periodate oxidation of carbohydrates isolated *via* hydrazinolysis should take into account the behaviour, at present unknown, of the hydrazone of 2-amino-2-deoxy-D-glucose.

Our results suggest that quantitative yields of 2,5-anhydro-D-mannose would not be obtained from asparagine-linked 2-acetamido-2-deoxy-D-glucose by sequential hydrazinolysis and nitrous acid deamination. However, Bayard and Fournet[13] have reported 80–100% yields for 2,5-anhydro-D-mannose from the hydrazinolysis product of α_1-acid glycoprotein. These authors also referred to the results of sequential hydrazinolysis and deamination of compound **2**, but the reference[10] cited does not describe the experiments.

The asparagine derivative **2** may not be an entirely satisfactory model for glycoproteins, in which the asparagine is linked to other amino acids. In preliminary experiments, the substituted derivative **7** has been found to behave similarly to **1** and **2**, the hydrazinolysis product being converted into 2-amino-2-deoxy-D-glucose in apparent yields of 70% (amino acid analyser, long column) or 40% (Dische and Borenfreund procedure) when kept in 15% aqueous acetic acid at 50° for 1 day[23].

EXPERIMENTAL

General. — Melting points were measured on a Kofler hot-stage and are corrected. P.m.r. and ^{13}C-n.m.r. spectra were recorded on Varian HA-100 and XL-100 spectrometers. ^{13}C-Spectra were measured with proton noise decoupling and a pulse repetition time of 1.6 sec; intensities, where cited, are peak heights and are given in parentheses following each chemical shift. Some proton spectra were measured on the XL-100 instrument with solvent suppression[24] and a digital resolution of 3 points/Hz. ^{13}C-Spectra of hydrazine solutions were measured using a fluorine external lock. Tetramethylsilane and sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate were used as the internal references in deuteriochloroform and deuterium oxide solutions, respectively. The apparent coupling-constants are the directly observed line-spacings.

Analytical methods. — Paper chromatography was performed on Whatman No. 1 paper with 1-butanol–pyridine–water (4:3:4). Paper electrophoresis was conducted on Whatman No. 3 paper with sodium acetate buffer at pH 5. Ninhydrin and silver nitrate spray-reagents were used to locate spots. The Dische and Borenfreund procedure[19], involving dichromatic readings at 492 and 520 nm, was used as described, except that 3 ml instead of 2 ml of ethanol was added. A Beckman amino acid analyser was used for quantitative estimations of 2-amino-2-deoxy-D-glucose, a standard sample being run before or after the unknown.

*2-Acetamido-1-*N*-acetyl-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosylamine.*— 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylazide[25] (2.34 g) was hydrogenated in ethanolic solution over platinum oxide (0.2 g) for 4 h; excess of acetic anhydride (2.3 g) was then added and the mixture kept overnight. The mixture was warmed to dissolve the crystals which had formed, and then filtered. Evaporation of the filtrate gave the title product (2.35 g) which, after one recrystallisation from ethanol, had m.p. 235–236°; lit.[17] m.p. 236–237° and[25] 241°; yield, 2.28 g (93%).

*2-Acetamido-1-*N*-acetyl-2-deoxy-β-*D*-glucopyranosylamine* (**1**). — The foregoing compound (1.81 g) was deacetylated according to ref. 17. Recrystallisation of the product (**1**) from methanol containing a little water gave material (235 mg) having m.p. 262–264°. Addition of ether to the mother liquor and cooling gave more **1** as chunky prisms (670 mg), m.p. 262–266°; lit.[17] m.p. 232–233° and[25] 240–243° (for the monohydrate). Both crops were identical and had $[\alpha]_D^{20}$ +27° (*c* 0.76, water); lit.[17] $[\alpha]_D^{19}$ −24° (*c* 2.0, water) and[25] +43.7° (water) for the monohydrate. N.m.r. data (D_2O): ^1H, τ 4.93 (d, 1 H, *J* 9 Hz, H-1), 6.0–6.5 (m, 6 H), and 7.98 (s, 6 H, MeCO); ^{13}C, 177.6, 177.5 (CO), 81.2 (C-1), 80.3, 77.0, 72.3, 63.3 (C-6), 57.1 (C-2), 24.82 (*Me*CO), and 24.77 p.p.m. (*Me*CO).

Anal. Calc. for $C_{10}H_{18}N_2O_6$: C, 45.8; H, 6.9; N, 10.7. Found: C, 46.2; H, 7.1; N, 10.8.

Hydrazinolysis procedure. — In a typical experiment, the diacetamide **1** or the asparagine derivative[20] **2** (100 μmol) and hydrazinium sulphate (100 μmol) were heated in anhydrous hydrazine (0.5 ml) in a sealed Pyrex-tube at 105 ±2° (or 100° in a few experiments) for 5 h. The cooled tube was opened, and hydrazine was removed in a vacuum desiccator over conc. sulphuric acid during 2 days.

N.m.r. measurements. — (*a*) The product from the hydrazinolysis at 100° of the diacetamide **1** (200 μmol) was dissolved in deuterium oxide; the p.m.r. spectrum, measured immediately, showed signals at τ 2.74 (d, 0.3 H, *J* 5.5 Hz, H-1 of hydrazone), 5.94 (d, *J* 9.5 Hz, H-1 of β-pyranose), 7.28 (t superimposed on multiplet, *J* 9.5 Hz, H-2 of β-pyranose), 7.61, 7.91, 7.98, and 8.04 (4 s, ratio 1:1:3:20, 6 H, 2 Me). Little change occurred during 24 h, but addition of acetic acid to a concentration of 15% (v/v) caused the doublet at τ 2.74 to disappear. ^{13}C-N.m.r. spectra were also measured before and after removal of hydrazine. For the hydrazine solution, the signal of the methyl carbon (at 22.6 p.p.m.) of acetohydrazide was used as the reference. The five strongest signals were at 149.0, 73.9, 66.0, 57.9, and 22.6 p.p.m.; several weaker signals were present between 82 and 57, and at 174.4, 173.0 (C=O), and 24.7 p.p.m.

(*Me*CONH). Measurements were repeated after removing the hydrazine and dissolving the residue in deuterium oxide (spectrum A, pH 8–9), then adding acetic acid to a concentration of 15% (v/v) (spectrum B), and again after warming this solution for 24 h at 50° (spectrum C). The most significant changes were in the 82–150-p.p.m. region, and the partial ^{13}C-data were as follows.

Spectrum	*Chemical shift (p.p.m.)*					
A	146.5(31),	93.9(44),		24.8(39),	22.6[a](100)	11.8(14)
B		95.6(7), 92.0(9)	87.2(35),	24.9(31),	22.7(46),	11.7(8)
C		95.6(9), 92.0(13),	88.7(27), 87.2(15),	25.0(43),	22.8(60), 22.6(39),	11.8(11)

[a]This peak was negative in a spectrum measured using the two-pulse sequence (τ, 2.5 sec–τ/2, 25 sec).

The product from the hydrazinolysis at 105° for 10 h of the diacetamide **1** was similarly examined by n.m.r. measurements. The proton spectrum of the solution in D_2O differed in the methyl region from that of the product after hydrazinolysis at 100° for 5 h: τ 7.61, 8.04, and 8.15 (3 s, ratios 2 : 8 : 1), and there was an extra, weak singlet at τ 2.30 (~25% of the intensity of the doublet at τ 2.74). Addition of tetradeuterioacetic acid (20 μl) diminished the intensity of the low-field signals and removed the singlet at τ 8.15. Addition of more CD_3CO_2D (50 μl, to give a concentration of 15% v/v) removed the low-field signals completely; after the solution had been kept for 24 h at 50°, and then for 6 days at room temperature, the spectrum showed a signal for H-1 of 2-amino-2-deoxy-α-D-glucose at τ 4.48 (d, J 3.5 Hz). Other prominent signals were present at τ 5.52 (d, J 9.5 Hz), 5.27 (d, J 9.5 Hz), and 2.59 (d, J 3 Hz). The ^{13}C-spectrum of the hydrazine solution differed from that of the product after hydrazinolysis at 100° for 5 h, in that several signals were absent, including those at 174.4 and 24.8 p.p.m. The strongest signals were as before, and weak signals were also detected at 154.3, 131.2, 130.7, 127.7, 96.4, and 13.9 p.p.m. The spectrum of the product in deuterium oxide was very similar to spectrum A above, but lacked peaks at, *inter alia*, 177.5 and 24.8 p.p.m., and contained additional, weak peaks at 157.5, 156.3, 139.8, and 13.9 p.p.m. Addition of CD_3CO_2D (20 μl) removed the signals at 157.5, 139.8, and 13.9 p.p.m., the region below 100 p.p.m. now containing signals at 156.3 and 142.1 p.p.m. Addition of more CD_3CO_2D (50 μl) gave a spectrum similar to spectrum B above, but lacking a peak at 24.9 and with an extra peak at 156.4 p.p.m. After the solution had been warmed at 50° for 24 h, the spectrum contained the following peaks: 177.3 (28), 175.9 (27), 175.6 (73, Me*C*ONHNH$_2$), 156.4 (36), 95.6 (15), 92.0 (20), 88.7 (55), 87.2 (21), 81–55 (several peaks), 22.7 (151), and 11.8 p.p.m. (109); the CD_3CO_2D peaks are excluded.

(*b*) The ^{13}C-spectrum of the hydrazine solution after the hydrazinolysis of 2-acetamido-2-deoxy-D-glucose (200 μmol) contained 5 strong singlets, the chemical

shifts being identical to those in the spectrum of the product from **1**; there were no signals at 24.8 and 174.4 p.p.m.

(*c*) The ^{13}C-spectrum of a freshly prepared solution of 2-amino-2-deoxy-D-glucose hydrochloride in hydrazine showed two singlets in the 150-p.p.m. region, but after the solution had been kept overnight, the signals at 149.0, 73.9, 66.0, and 57.9 p.p.m. predominated (the strongest peak, at 73.9 p.p.m., was used as the reference, this being the strongest peak also in the spectra of the hydrazinolysis products from 2-acetamido-2-deoxy-D-glucose and from **1**). There were no signals in the 25–10-p.p.m. region. Measurements were repeated after removing the hydrazine and dissolving the residue in D_2O (spectrum D), then adding CD_3CO_2D to a concentration of 15% (v/v) (spectrum E), and again after warming the solution for 24 h at 50° (spectrum F). The significant changes were as follows.

Spectrum	*Chemical shift* (*p.p.m.*)				
D	148.2(47),	94.3(103)			
E	143.7(8), 142.1(11)	95.6(19),	91.9(30),	90.7(17),	87.8(14) 87.3(79)
F		95.6(30),	92.0(45),		87.8(12) 87.3(28)

Spectrum F contained several additional, weak peaks in the 87–150 p.p.m. region, and the solution was pale yellow.

The anomeric carbon nuclei of a solution of 2-amino-2-deoxy-D-glucose hydrochloride in D_2O resonated at 95.4 and 91.8 p.p.m. After adjustment of the pH to 8–9 with aqueous potassium hydroxide, these resonances were at 99.0 and 94.5 p.p.m.

(*d*) The product from the hydrazinolysis of *N*-acetyl-β-D-glucopyranosylamine [50 mg (222 μmol), m.p. 258–261° (dec.)], when dissolved in D_2O, showed ^1H signals at τ 2.73 (d, *J* 6.4 Hz), 5.69 (dd, $J_{1,2}$ 6.4, $J_{2,3}$ 7.0 Hz, H-2 of hydrazone), 5.94 (d, *J* 8.6 Hz, H-1 of β-pyranose), 6.0–6.8 (m), 7.91 (s), and 8.04 (s, $CH_3CONHNH_2$). The pH was changed from 9 to 7 by addition of glacial acetic acid (30 μl); the spectrum then contained an extra, weak doublet (*J* 6 Hz) at τ 2.76, and the doublet at τ 5.94 had moved to 5.87. Addition of more acetic acid (10 μl) to a pH of 5–6 gave, after 1 day, a spectrum containing no low-field doublets, and the doublet at τ 5.86 had moved to 5.73.

Hydrazinolysis of the diacetamide **1** *in the absence of hydrazinium sulphate.* — The product of the hydrazinolysis of **1** (28.6 mg) in hydrazine (1 ml), when dissolved in D_2O (0.5 ml), showed the following ^1H resonances: τ 2.74 (d, *J* 5.5 Hz), 4.93 (d, *J* 9 Hz), and 5.11 (d, *J* 9 Hz) (integral ratios 2:7:3). After changing the pH from 9 to 5 by addition of acetic acid and warming the solution at 50° for 24 h, the doublet at τ 2.74 was absent, that at τ 5.11 had moved to 4.69, and there were weak doublets at

τ 5.59 (J 9.5 Hz) and 4.55 (J 3.5 Hz), the latter corresponding to H-1 of 2-amino-2-deoxy-α-D-glucopyranose.

D-*Galactose acetylhydrazone.* — The following n.m.r. data (D_2O) were obtained for D-galactose acetylhydrazone (m.p. 171.5°): ^1H, τ 2.40 (d, ~0.3 H, J 5 Hz), 5.8–6.7 (m), 8.02 (s, plus 4 smaller singlets, total 6 H, CH_3CO); after 24 h, the doublet at τ 2.40 was much weaker, and a doublet (J 8 Hz) at τ 5.94 was prominent; ^{13}C (after 1 h), 176.1 and 173.3 (carbonyl), 155.3 (CH=N), 96.4–63.8 (several peaks), and 23.1 and 22.8 (CH_3); after 24 h, the following peaks predominated: 176.1, 92.9, 78.9, 76.0, 71.7, 70.9, 64.1, and 22.8 p.p.m.

Acetohydrazide. — Acetohydrazide (m.p. 64–66°) gave the following n.m.r. data (D_2O): ^1H, τ 8.04; ^{13}C, 175.5 and 22.6 p.p.m. The product of the hydrazinolysis (100° for 4.5 h) of acetohydrazide (30 mg) in the presence of hydrazinium sulphate (25 mg), when dissolved in D_2O, showed the following n.m.r. signals: ^1H, τ 7.61 and 8.04 (2 s, intensity ratio 1:7); ^{13}C, 175.7 (41), 22.7 (98), and 11.8 (18) p.p.m.; the signal of the triazole ring-carbon was too weak to be seen under the conditions used. After hydrazinolysis under more vigorous conditions (105° for 16 h), acetohydrazide was a minor constituent of the product mixture, and the product, 4-amino-3,5-dimethyl-1,2,4-triazole, was isolated from the solvent-free residue by extraction with hot chloroform. Addition of light petroleum (b.p. 40–60°) gave prisms, m.p. 199–200°; lit.[26] m.p. 196.5–197.5°. N.m.r. data (D_2O): ^1H, τ 7.61 (s); ^{13}C, 156.4 and 11.8 p.p.m.; (D_2O–CD_3COOD, 85:15): ^1H, τ 7.48 (s); ^{13}C, 156.6 and 11.7 p.p.m.

The reaction of 2-amino-2-deoxy-D-glucose hydrochloride with acetohydrazide. — 2-Amino-2-deoxy-D-glucose hydrochloride (50 mg) and acetohydrazide (17 mg) were dissolved in deuterium oxide (0.46 ml) containing 15% of acetic acid-d_4. After 16 h at room temperature, the ^{13}C-spectrum showed a weak signal at 88.7 p.p.m. in addition to those for 2-amino-2-deoxy-αβ-D-glucose. The ^1H-spectrum showed a weak doublet (J 9.5 Hz) at τ 5.57. After the solution had been kept at 50° for 16 h, the ^{13}C-spectrum included signals at 177.4 (41), 175.9 (15), 95.5 (50), 92.0 (79), 88.7 (99), 22.8 (96), and 22.6 (71) p.p.m., and the doublet at τ 5.57 was much stronger in the ^1H-spectrum. This solution gave only one peak, with the same retention time as 2-amino-2-deoxy-D-glucose, when analysed on both the long and short columns of the amino acid analyser. Paper chromatography also gave a single spot that had the same mobility as 2-amino-2-deoxy-D-glucose.

Paper chromatography. — The hydrazinolysis products streaked badly when analysed by paper chromatography, but better results were obtained after adding acetic acid and warming the solution at 50°. The product from the diacetamide **1** then gave spots for 2-amino-2-deoxy-D-glucose and acetohydrazide (R_{GLCN} 1.35, yellow with ninhydrin), and other ill-defined, faint spots at R_{GLCN} <1.0. Similar results were obtained for the product from the asparagine derivative **2**, except that there was a strong spot (R_{GLCN} 0.55 with ninhydrin) corresponding to aspartic acid 4-hydrazide.

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REFERENCES

1 B. BAYARD AND D. ROUX, *FEBS Lett.*, 55 (1975) 206–211.
2 J. M. WILLIAMS, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 73–77.
3 B. A. DMITRIEV, Y. A. KNIREL, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 40 (1975) 365–372.
4 M. FUKUDA, T. KONDO, AND T. OSAWA, *J. Biochem. (Tokyo)*, 80 (1976) 1223–1232.
5 M.-L. RASILO AND O. RENKONEN, *Biochim. Biophys. Acta*, 582 (1979) 307–321.
6 Z. YOSIZAWA AND T. SATO, *Biochim. Biophys. Acta*, 52 (1961) 591–593.
7 M. L. WOLFROM AND B. O. JULIANO, *J. Am. Chem. Soc.*, 82 (1960) 2588–2592.
8 Z. YOSIZAWA, T. SATO, AND K. SCHMID, *Biochim. Biophys. Acta*, 121 (1966) 417–420.
9 B. A. DMITRIEV, Y. A. KNIREL, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 29 (1973) 451–457.
10 B. BAYARD, Ph.D. Thesis, University of Science and Technology, Lille, France, 1974.
11 B. BAYARD AND J. MONTREUIL, *Colloq. Int. C.N.R.S.*, 221 (1974) 209–218.
12 E. D. KAVERNEVA AND V. K. LAPUK, *Biokhimiya*, 31 (1966) 137–142.
13 B. BAYARD AND B. FOURNET, *Carbohydr. Res.*, 46 (1976) 75–86.
14 Ref. 4, footnote on p. 1224.
15 Ref. 16, Fig. 3.
16 C. L. READING, E. E. PENHOET, AND C. E. BALLOU, *J. Biol. Chem.*, 253 (1978) 5600–5612.
17 C. H. BOLTON, L. HOUGH, AND M. Y. KHAN, *Biochem. J.*, 101 (1966) 184–190.
18 C. H. BOLTON, M. Y. KHAN, AND L. HOUGH, *Carbohydr. Res.*, 1 (1966) 493–494.
19 Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 184 (1950) 517–522.
20 G. S. MARKS, R. D. MARSHALL, AND A. NEUBERGER, *Biochem. J.*, 87 (1963) 274–281.
21 P. A. S. SMITH, *Open Chain Nitrogen Compounds*, Vol. II, Benjamin, New York, 1966, p. 157.
22 R. R. SCHMIDT, J. KARG, AND W. GUILLIARD, *Chem. Ber.*, 110 (1977) 2433–2444.
23 M. S. SAEED AND J. M. WILLIAMS, unpublished work.
24 S. L. PATT AND B. D. SYKES, *J. Chem. Phys.*, 56 (1972) 3182.
25 A. YAMAMOTO, C. MIYASHITA, AND H. TSUKAMOTO, *Chem. Pharm. Bull.*, 13 (1965) 1036–1041.
26 R. M. HERBST AND J. A. GARRISON, *J. Org. Chem.*, 18 (1953) 872–877.