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The lysine glycation
1. A preliminary investigation on the products arising from the reaction of protected lysine and D-glucose

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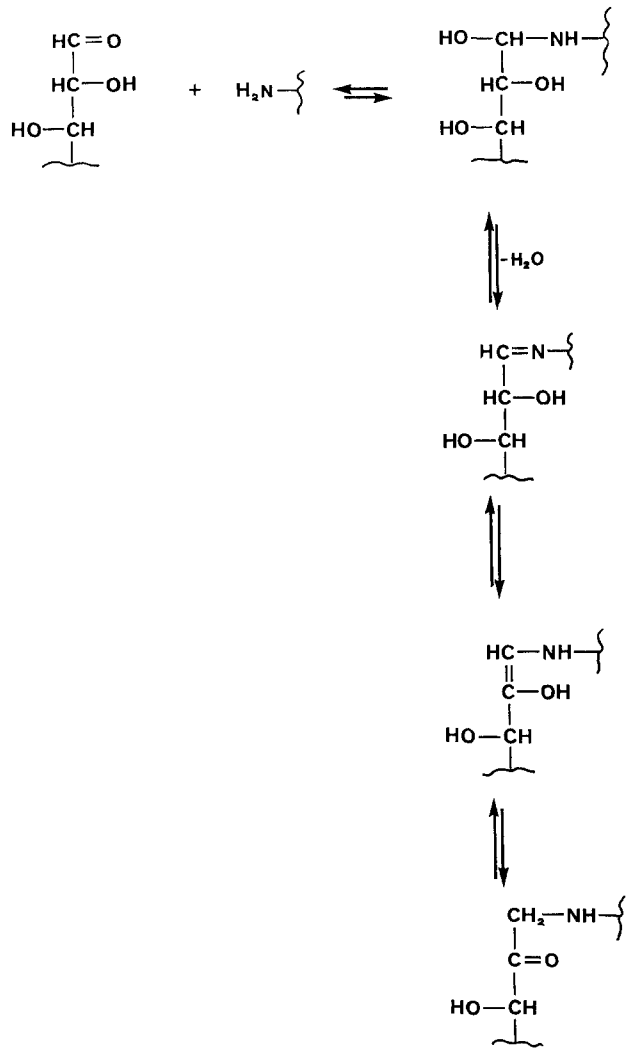
Summary. The nature of the products arising from a 10 days, sterile incubation at 37°C and pH 7.2 of a 1:1 mixture of N-α-(p-tosyl)-lysine-methylester-hydrochloride and anhydrous D-glucose was investigated by fast atom bombardment mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopies. Differently to the reactivity usually described on the basis of other analytical techniques, FAB mass spectrometric measurements indicate the occurrence of the reaction of protected lysine with more than one D-glucose molecule.

Keywords: Amino acids – Glycation processes – Mass spectrometry – Nuclear magnetic resonance

Introduction

The interaction between D-glucose and proteins and the further cross-linking reactions are usually considered the main non-enzymatic processes responsible for the long-term complications of diabetes (Monnier and Cerami, 1982).

The first step of such reactions is currently considered to originate from the interaction between ε-amino group of lysine and glucose (Maillard, 1916), giving rise to an unstable Shiff base between glucose carbonyl group and free amino group of protein. The ketoimine so formed leads to a more stable ketoamine *via* an Amadori rearrangement (see Scheme 1). The occurrence of further cyclization processes lead to intermediate species responsible for cross-linking reactions. The ultimate step of such reaction pattern leads to the so-called "Advanced Glycation End Products" (AGE) (Reynolds, 1965), real responsible for the



Scheme 1. General scheme of the Maillard reaction

tissutal alterations typical of long term diabetic complications (i.e. nephropathy, retinopathy, neuropathy and macroangiopathy) (Brownlee et al, 1984).

For the above described aspects, the study of the intimate mechanisms of the reactions between ε-amino group of lysine with sugars is an argument of high interest (Ledl, 1990), leading to precious information on the first step of the glycation processes.

For such reasons we undertook the present study, based on the reaction of protected lysine (N-α-p-tosyl-L-lysine-methylester) with D-glucose under pseudo-physiological conditions. The reaction mixture was analyzed by two different techniques, both exhibiting high information power from the structural point of view, i.e. fast atom bombardment mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance. While the former analytical approach gave precious information on the molecular weight distribution of the species present in the

reaction mixture, the latter approach gave data on the appearance and disappearance of specific functional groups during the reaction itself.

Experimental

5 g of N-α-(p-tosyl)-lysine-methylester-hydrochloride (Sigma, St. Louis, MO) were incubated in sterile conditions with 2.57 g of anhydrous D-glucose (Sigma, St. Louis, MO) in 6 ml of distilled water containing 5.5 mmol/l toluene to ensure sterility. The solution, kept at pH 7.2 by adding suitable amounts of NaOH, was maintained at 37°C for 10 days under darkness. The free glucose was then eliminated by the absorption of the aqueous solution, on an Extrelut cartridge (Extrelut 20, Merck, Gibbstown, NJ) and eluted with CH_2Cl_2. The eluated was chromatographed on a silica gel column eluting with Et_2O, to obtain fraction A.

In a different experiment the reaction mixture was passed on the Extrelut cartridge and the yellow eluate was furtherly chromatographed on a silica gel column eluting first with CH_2Cl_2, leading to colorless fraction I, then with MeOH obtaining two different fractions: brownish fraction II and yellow fraction III. The Extrelut cartridge was furtherly eluted with CH_2Cl_2 obtaining light yellow fraction IV. Finally the cartridge was eluted with H_2O yielding a last colorless fraction containing only free glucose.

Mass spectrometric measurements were performed on a VG ZAB 2F instrument (Morgan et al., 1978) operating under electron impact (EI) conditions (70 eV, 200 μA) and in FAB conditions (Barber et al., 1981) (8 keV Xe atoms bombarding glycerol solutions of samples). Collisionally activated decomposition (CAD), mass-analyzed ion kinetic energy (MIKE) (Cooks, 1978) spectra were obtained by 8 keV ions colliding with air in the collision cell placed in the second field free region (2^{nd} FFR) of the apparatus. The pressure in the collision cell was such as to reduce the main beam intensity to 60% of its usual value.

^{13}C- and ^{1}H-NMR spectra were obtained on a Bruker AC 200 spectrometer in D_2O solutions: chemical shift values are referred to sodium-3-(trimethylsilyl)propanoate (external standard).

A portion of the reaction mixture, after glucose purification, was analyzed by gel permeation chromatography (GPC) (Chromatograph Waters 600 MS, Refractive Index Detector Waters 410). This was achieved using two columns (first: linear range from 50 to 10,000; second: linear range from 50 to 10,000,000; 7.85 × 300 mm, particle diameter 7 μm) with tetrahydrofurane as eluent (1 ml/min). The UV detection was kept at 253 nm.

Results and discussion

As described in the introduction section, the glycation of proteins consists in many different steps, the latest of which leading to cross-links. What might be expected in the present case, in which just one protected aminoacid is reacted with glucose, is the occurrence of polymerization reactions corresponding, from the mechanistic point of view, to the above cited cross links.

In a previous work (Lapolla et al., 1991), the reaction products arising from the interaction between glucose and N-α-acetyl-lysine-methylester were analyzed by liquid chromatography mass spectrometry (HPLC/MS) using the plasmaspray interface (Arpino, 1990). In that case some of the detected compounds containing the lysine structure were found free of protecting groups, proving the occurrence of undesired hydrolysis processes. Consequently, in order to avoid such a behaviour, for the present study we choose the N-α-(p-tosyl)-lysine-methylester as reagent substrate.

Furthermore, in the above cited investigation (Lapolla et al., 1991), plasmaspray ionising conditions generally led to quite abundant molecular ions but

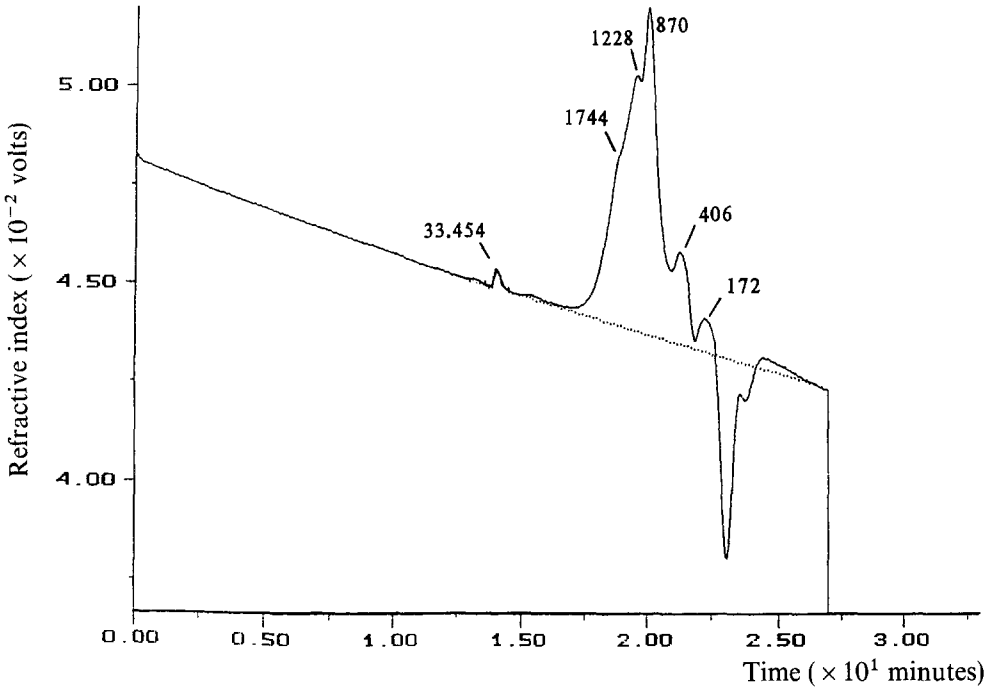


Fig. 1. Gel permeation chromatogram of the whole reaction mixture obtained by 10 days incubation of protected lysine and glucose (37°C, pH = 7.2)

scarcely abundant fragment ions. Thus, if on one hand such behaviour allowed an easy identification of the molecular weight of the compounds under study, on the other hand it gave poor information on their structure. Consequently, in order to gain more structural information, the use of other analytical approaches for the present study became of interest.

Firstly, in order to obtain reliable information on the molecular weight distribution of the products arising from the reaction protected lysine-glucose, some gel permeation experiments were undertaken on the whole reaction mixture. The results so obtained are shown in Fig. 1. Quite surprisingly, a major peak with components corresponding to mean molecular weight of 870, 1228 and 1744 Da is evidenced. Other quite abundant components of the reaction mixture have mean molecular weights of 172 and 406, while a minor (but significant) component corresponds to a mean molecular weight of 33454 Da, indicating the occurrence of extensive polymerization processes, as previously reported (Bailey and Kent, 1988).

This first screening allowed to estimate the real complexity of the reaction mixture, and consequently the use of column chromatography was considered essential to obtain different fractions containing a limitate number of components (Eichener and Kittman, 1990); such separation was achieved following the procedures reported in the experimental section.

The ethereal fraction A and the four fractions I–IV (see experimental section) were analyzed by mass spectrometry, ^{1}H- and ^{13}C-NMR spectroscopies.

Electron ionization resulted ineffective for both structural characterization and molecular weight determination of the components of the five fractions. In

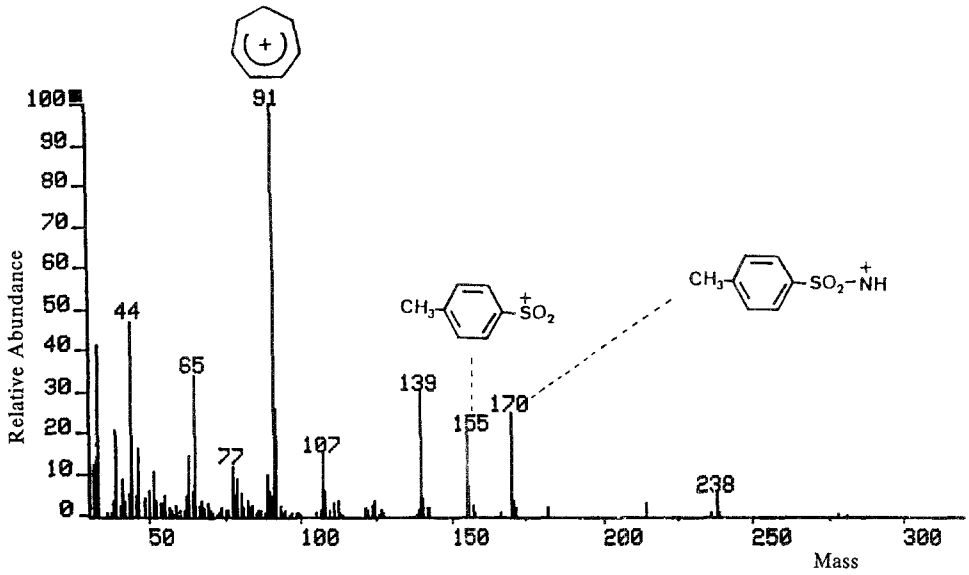


Fig. 2. EI mass spectrum of fraction A

fact the only EI generated ions detected were due to fragmentation of protected lysine (see as an example Fig. 2). This behaviour can be explained by either the low volatility of the reaction products or their possible thermal degradation (probe temperature higher than 280°C, ion source temperature 200°C).

On the contrary, FAB mass spectrometry gave interesting analytical information. The FAB mass spectrum of the fraction A, obtained by eluting with diethyl ether the reaction mixture in order to separate lower molecular weight organic products (see experimental section), evidenced the presence of a highly abundant ion at m/z 223 (see Fig. 3). The other abundant ionic species at m/z 337, 315, 283 and 238 do not have any analytical significance, being due to the presence of protected lysine.

In the above cited work (Lapolla et al., 1991) based on HPLC/MS study of glycation products of protected lysine, an ionic species at m/z 222 was found, for which structure **a** was proposed just on the basis of its molecular weight and literature (Ledl and Severin, 1982).

In the present case, the fragmentation pattern of ions at m/z 223, possibly corresponding to the protonated species at m/z 222, obtained by metastable ion studies, is more in agreement with a different structure as the **b** one, previously proposed by Ledl (1990). In fact while the loss of the side chain leads to ions at m/z 131 for both the proposed structures, the primary methyl loss, leading to ions at m/z 208, is in agreement with the structure **b** only.

The FAB mass spectrum of fraction I shows the presence of highly abundant ions at m/z 191, 219, 315 and 531 with relative abundances of 13, 40, 25 and 8% respectively. A possible molecular weight of 530 Da cannot be justified by the simple condensation of a glucose molecule on the protected lysine. In such case a molecular weight of 476 Da might be present. Hence the species at m/z 530 (531 in FAB conditions) must necessarily originate by the reaction of two glucose units with the protected lysine and further skeletal rearrangement leading to

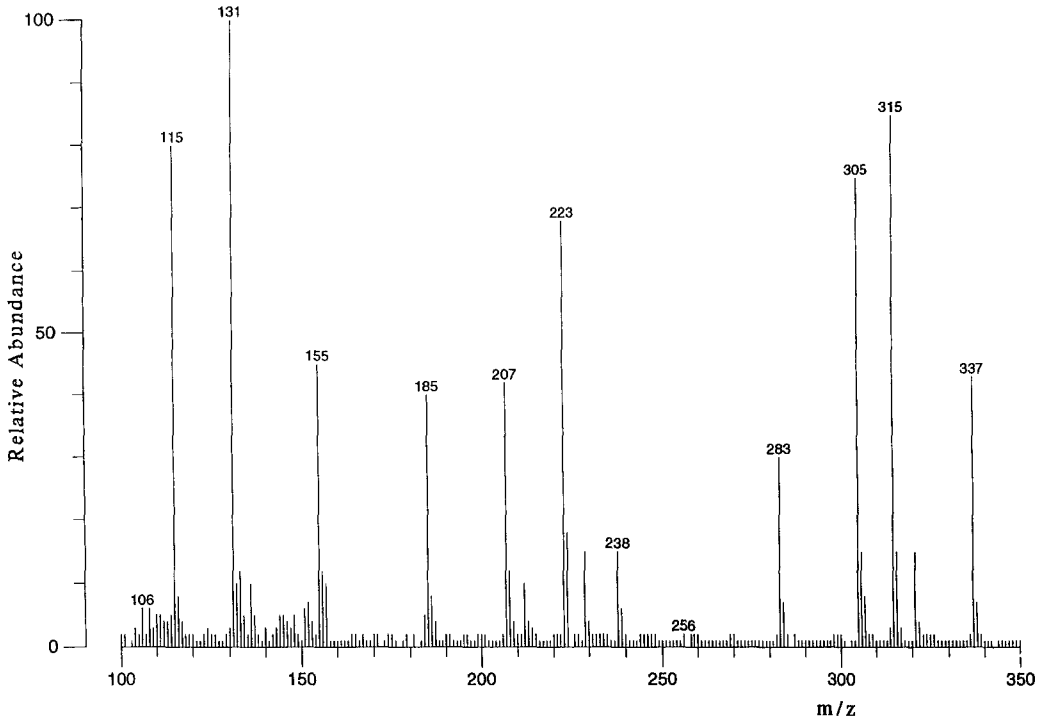


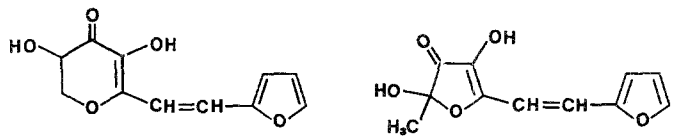
Fig. 3. FAB mass spectrum of fraction A

oxygen-containing heterocycles, always invoked in glycation processes (Ledl, 1990). The fragment ions detected in the FAB spectrum as well as the MIKE data (showing the loss of $CH_3 \cdot$ and the formation of ions at m/z 315 and 222) are in agreement with structure **c.**

In fact while the ion at m/z 315 can be explained by the cleavage 1 with H rearrangement leading to the protonated molecular ion of the original N-α-(p-tosyl)-L-lysine-methylester, the ion at m/z 476 can be rationalized by the loss of three water molecules, a typical behaviour of sugar derivatives. However, ^{1}H-NMR results more effective to establish the real structure of the condensation product.

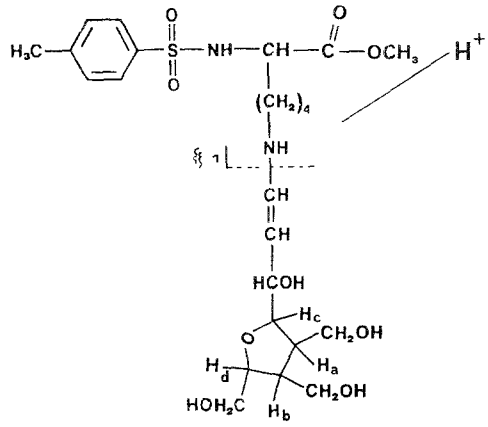
The ^{1}H-NMR spectrum in D_2O of fraction I shows, together with the signals due to the protected lysine skeleton (1.4–1.7 ppm, m, CH_2; 2.45 ppm, s, CH_3; 2.96 ppm, t, CH_2NH_2; 3.44 ppm, s, OCH_3; 3.98 ppm, dd, CH; 7.45–7.79 ppm, m, Ph), a complex multiplet centered at 5.4 ppm which can be attributed to olefinic protons (possibly a *cis*- and *trans*- isomer mixture); an AA'BB' system centered at 4.11 ppm, due to H_{a-d} (J_{HaHb} 5.6 Hz, J_{HaHd} J_{HaHc} 1.5 Hz, J_{HaHd} J_{HdHc} J_{HdHc} 0 Hz) (Betterham, 1973; Barbier et al., 1968). Finally CH_2OH protons may give rise to the multiplet centered at 4.91 ppm.

The FAB mass spectrum of fraction II shows the presence of highly abundant ions at m/z 459 (20%) and 477 (22%). The MIKE spectrum of ions at m/z 477 shows sequential losses of two water molecules giving rise to ions at m/z 459 and 441 respectively. From ions at m/z 459, species at m/z 238 and 315 are formed, indicating the presence of protected lysine skeleton. ^{1}H-NMR spectrum doesn't show the presence of signals attributable to heterocyclic systems, how-



a

b



c

ever the major polarity of this compound, soluble in MeOH, indicates the presence of the unchanged glucose molecule bonded to ε-NH_2 group of lysine, with loss of one molecule of water.

FAB mass spectrum of fractions III and IV shows abundant ions at higher values of molecular weight: at m/z 783 in fraction III and at m/z 1070 in fraction IV. In both cases highly abundant ions at m/z 477 are present, together with ions originated by protected lysine.

Ions at m/z 783 and 1070 may be formally considered originated from addition to a protected lysine of 3 glucoses (with losses of 4 H_2O) and 5 glucoses (with loss of 8 H_2O) respectively. Lower abundant ions at m/z 945 may be attributed to the condensation of 4 glucoses on a protected lysine (with loss of 5 H_2O).

^{1}H-NMR spectra of fractions III and IV show very complex patterns due to lysine together with glucose protons. Integration data are in agreement with proposed assignements of multiple additions of glucose on a molecule of lysine and not with polymerization of the Amadori product itself. Thus even if fractions III and IV are yellow, indicating the presence of "browning products" of the Maillard reactions, the most abundant component in the fraction under study is the Amadori product. This feature indicates that under experimental conditions we used (37°C, pH 7.2), pseudo-physiological, the late stage of the Maillard reaction is just initiated.

More information may be obtained by the ^{13}C-NMR spectra of fraction III, reported in Fig. 4, compared with ^{13}C-NMR spectra of protected lysine and glucose recorded at pH 7.2 in D_2O.

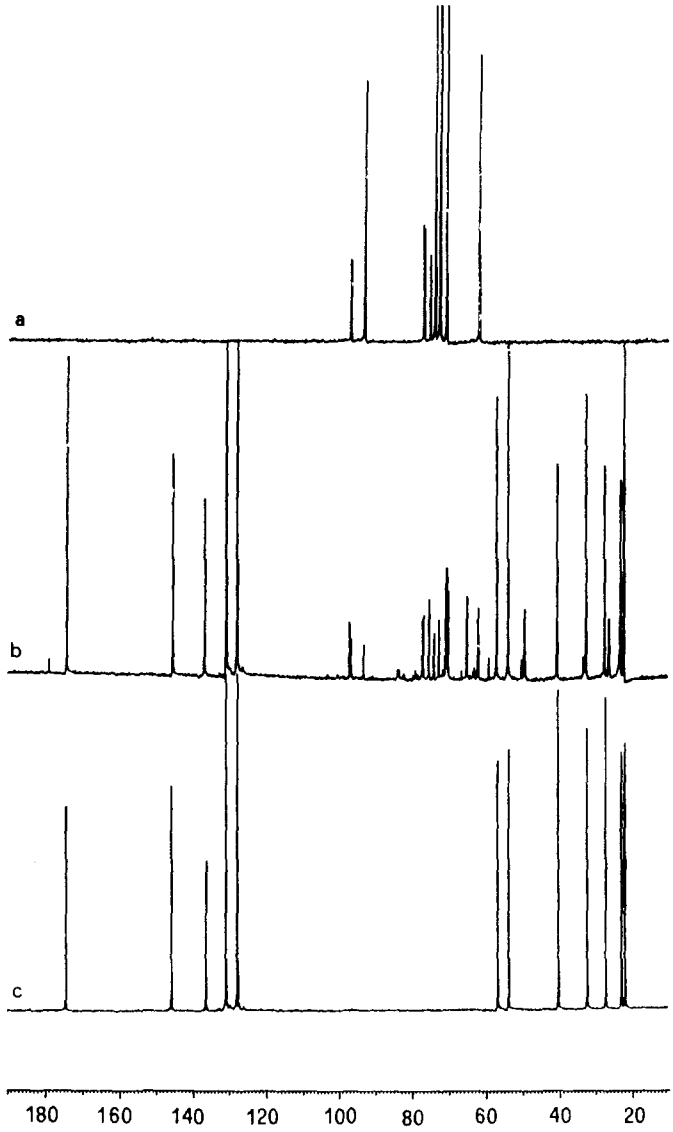
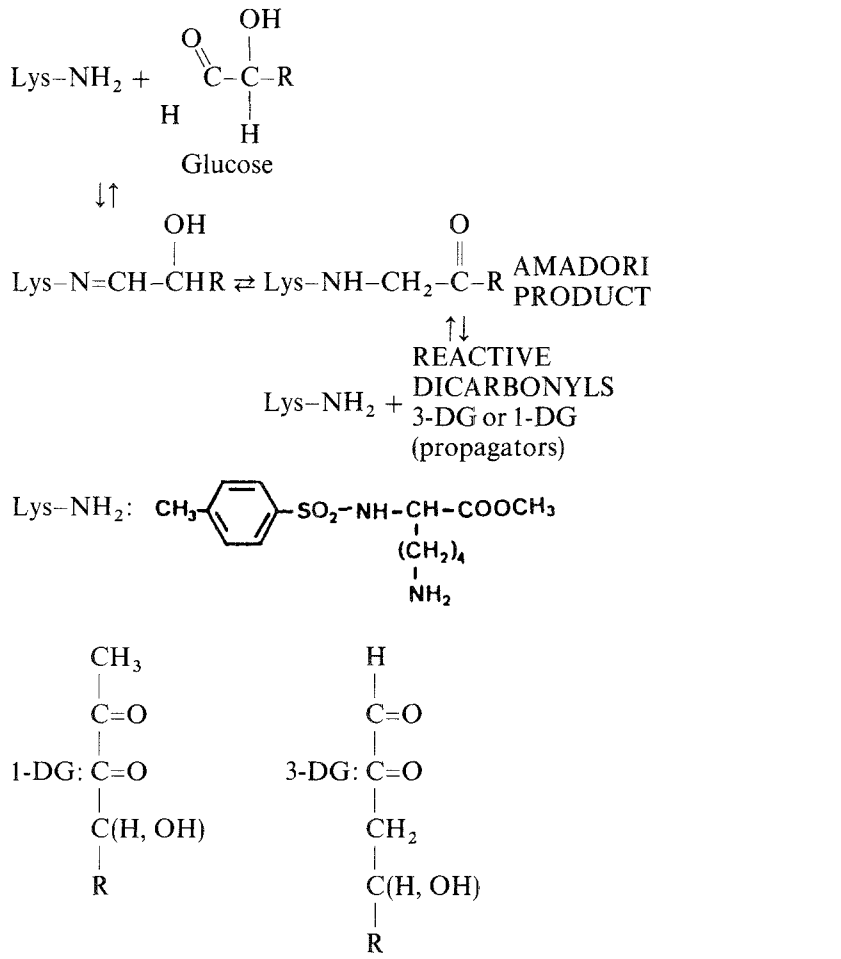


Fig. 4. ^{13}C-NMR spectra in D_2O, at pH 7.2 of: **a** protected lysine, **b** fraction III, **c** free glucose

For fraction III a general pattern close to those of protected lysine and glucose is observed, but some new signals are well evident as those at 178.96 ppm (new carbonyl), at 49.25 ppm (which may be attributed to a NH–CH_2–C=O system) and at 64.99 ppm (attributable to O=C–*C*HOH– system).

The ^{13}C-NMR spectrum of the anomeric region (100 ppm) shows, together with the signals due to free glucose (96.69 ppm and 93.17 ppm), the presence of the Amadori product (97.02 ppm) as it was previously identified in the reaction of glucose with lysine residues in RNase (Baynes et al., 1988).

FAB mass spectrometric and ^{13}C-NMR data are in agreement with the formation of the Amadori product which then may evolve into a variety of reactive dicarbonyl compounds (see Scheme 2).



Scheme 2. Amadori product and reactive dicarbonyls formation in the Maillard reaction, involving in this case, protected lysine and glucose

The adducts of higher molecular weights may be originated by further reactions of the Amadori product, as a secondary amine, with 3- and 1-Deoxyglucosones, which are reported to be the propagators of the Maillard reaction (Monnier, 1988) towards the formation of carbocyclic compounds. However, alternative pathways, involving radicals, cannot be in principle excluded (Ledl, 1990).

Definitive evidences of the structures of such multiple adducts cannot be given on the basis of the present analytical data. The understanding of this new feature of the Maillard reaction is still in progress, by means of higher resolution chromatographic procedures and two-dimension NMR experiments.

Furtherly, it was considered of interest to follow by ^{1}H-NMR the reactions at 37°C and pH 7.2 in D_2O of protected lysine with glucose in equimolar amounts and in a 2:1 molar ratio. In both cases free glucose was found even after two weeks, as evidenced by doublets at 5.25 and 4.65 ppm (see Fig. 5) due to the anomeric protons, indicating a very complex kinetic trend (Suarez, 1988). In a previous detailed study about two-dimensional J-resolved Proton Nuclear

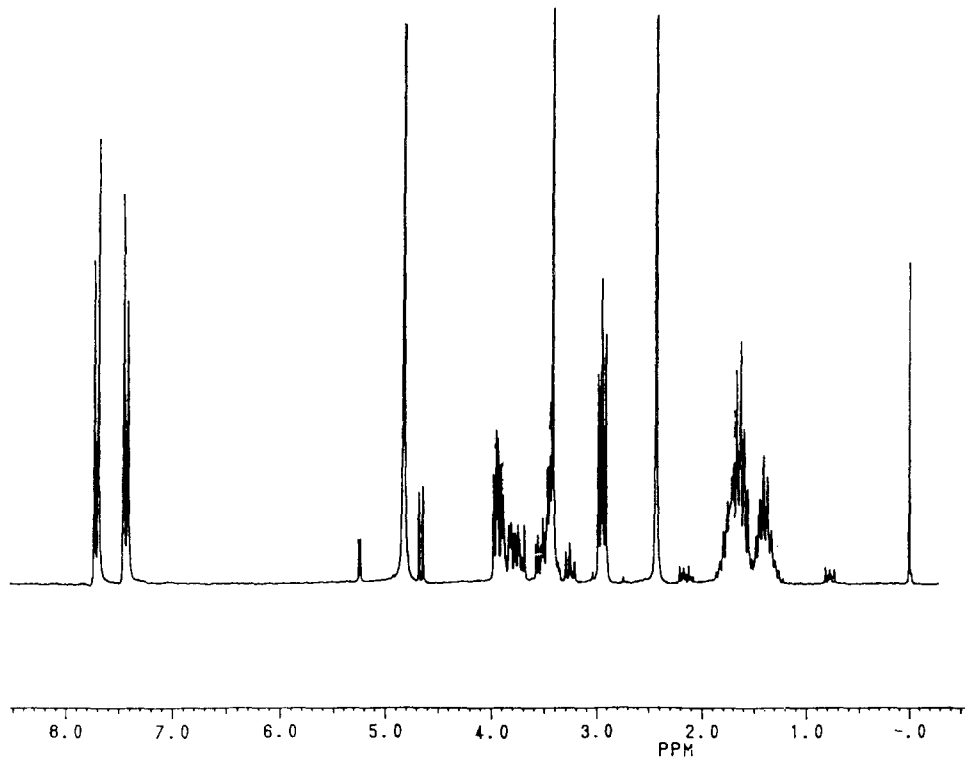


Fig. 5. ^{1}H-NMR spectrum of the reaction glucose-protected lysine in molar ratio 0.5:1 (10^{-3} M) recorded at two weeks after mixing

Magnetic Resonance Spectrometry of Hydroxyl-coupled α- and β-D-Glucose doublets at 5.25 ($^{3}J_{HH}$ 3.5 Hz) and 4.65 ($^{3}J_{HH}$ 8.0 Hz) are attributed to the O–CH_2(OH) protons of the α and β epimers respectively (Coxon, 1983).

The ^{1}H-NMR spectra of the mixtures protected lysine-glucose 1:1 and 2:1, recorded after 5 min. from mixing, show immediate interaction between the reagents. In particular, the signal at 2.92 ppm of NH_2–CH_2– protons of the protected lysine broadens (see Fig. 6) being involved in a rapid, on NMR time scale, reaction.

During the reaction, the ^{1}H-NMR spectrum shows the growing and the change of many signals, due to the formation of a mixture of products, impossible to be assigned to any individual species. Nevertheless, the growing up of new signals at 3.44 and 3.47 ppm, due to new –OCH_3 groups, together with new AA'BB' systems (see Fig. 6) in the region of phenyl protons, reveals the formation of new species containing the protected lysine moiety.

The ^{13}C-NMR coupled spectra of the reaction mixtures, recorded after 9 days, show a new C=O signal at 178.77 ppm of a new carbonylic species (see Fig. 7). The new signals, shown in the ^{13}C-NMR spectrum of the reaction mixture lysine-glucose 1:1 after 9 days, strongly resemble those observed in the ^{13}C-NMR spectrum of fraction III (see Fig. 4). A very low doublet at 97.56 ppm ($^{1}J_{CH}$ 145 Hz) with anomeric region, together with a triplet at 48.98 ($^{1}J_{CH}$ 143 Hz) and a doublet at 58.90 ppm ($^{1}J_{CH}$ 147.4 Hz) confirm the previous assignment.

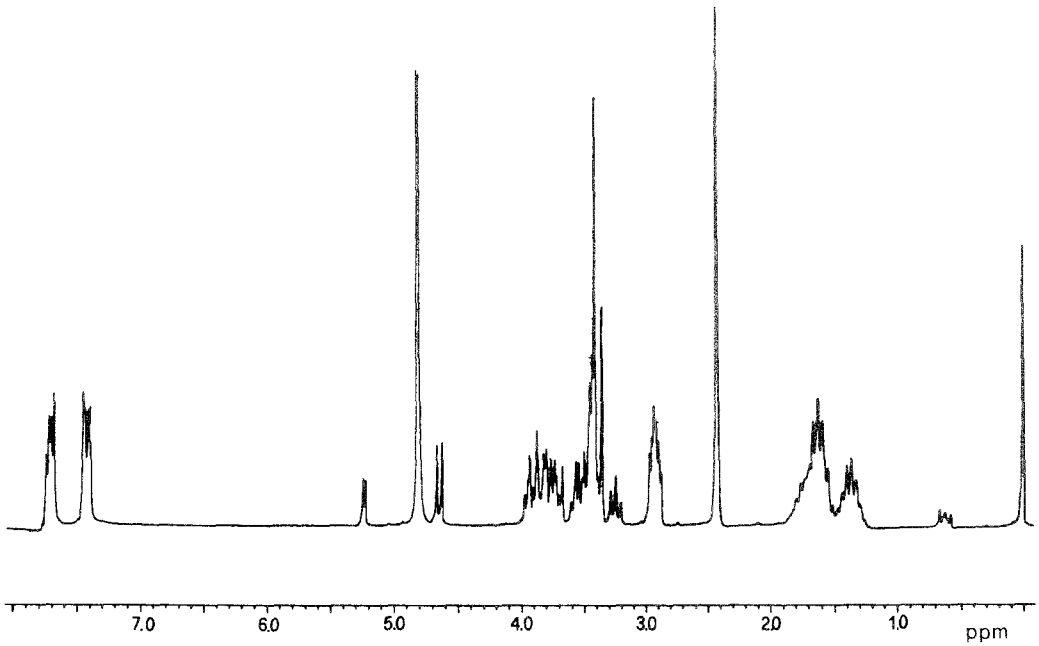


Fig. 6. ^{1}H-NMR spectrum of the reaction glucose-protected lysine in molar ratio 1:1 (10^{-3} M) recorded at five minutes after mixing

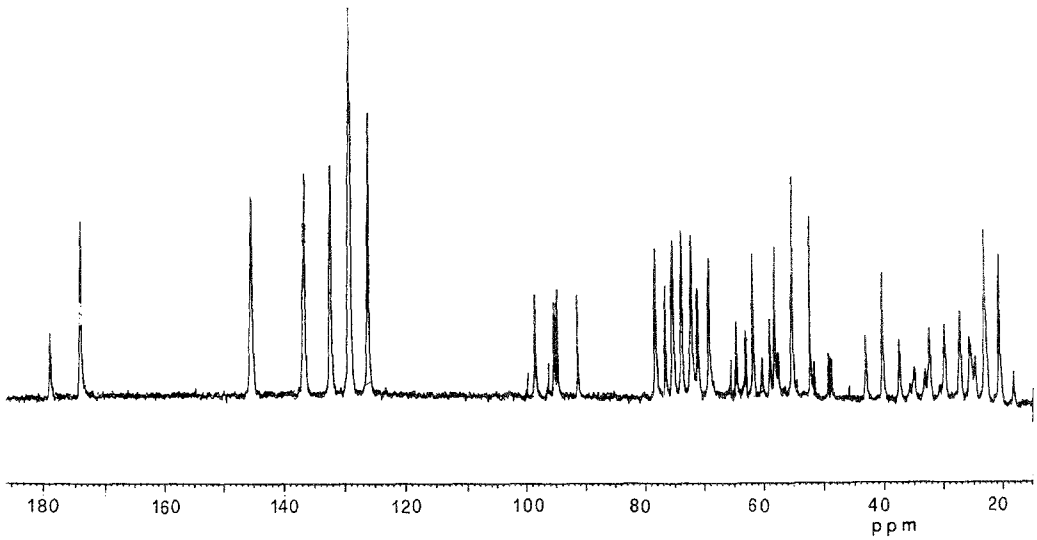


Fig. 7. ^{13}C-NMR coupled spectrum of the reaction glucose-protected lysine in a molar ratio 1:1 (10^{-3} M) recorded at 9 days after mixing

The FAB mass spectrum of the same reaction mixture in D_2O shows the presence of the Amadori product (ion at m/z 481) together with high molecular weight species due to condensation of more than one glucose molecule (ionic species at m/z 763, 827 and 932).

In conclusion from the data obtained by FAB mass spectrometry and NMR spectroscopies, a reactivity of both hydrogen of free amino group of lysine with

glucose may be proposed. Further studies are being carried out to characterize the final products, containing more than one glucose unit.

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