University of South Carolina
Scholar Commons

Faculty Publications | Chemistry and Biochemistry, Department of

12-10-1983

# $^{13}C$ NMR Investigation of Nonenzymatic Glucosylation of Protein

Carolyn I. Neglia

Helga J. Cohen

Albert R. Garber

Paul D. Ellis

Suzanne R. Thorpe

*See next page for additional authors*

Follow this and additional works at: https://scholarcommons.sc.edu/chem_facpub

Part of the Chemistry Commons

## Publication Info
Published in *Journal of Biological Chemistry*, Volume 258, Issue 23, 1983, pages 14279-14283.
This research was originally published in the Journal of Biological Chemistry. Neglia CI, Cohen HJ, Garber AR, Ellis PD, Thorpe SR, Baynes JW. 13C NMR Investigation of Nonenzymatic Glucosylation of Protein. *Journal of Biological Chemistry*. 1983; 258:14279-14283. © the American Society for Biochemistry and Molecular Biology.

This Article is brought to you by the Chemistry and Biochemistry, Department of at Scholar Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of Scholar Commons. For more information, please contact digres@mailbox.sc.edu.

Author(s)
Carolyn I. Neglia, Helga J. Cohen, Albert R. Garber, Paul D. Ellis, Suzanne R. Thorpe, and John W. Baynes

This article is available at Scholar Commons: https://scholarcommons.sc.edu/chem_facpub/154

The Journal of Biological Chemistry
AFFINITY SITES
ASBMB
American Society for Biochemistry and Molecular Biology

# 13C NMR investigation of nonenzymatic glucosylation of protein. Model studies using RNase A.

C I Neglia, H J Cohen, A R Garber, P D Ellis, S R Thorpe and J W Baynes
*J. Biol. Chem.* 1983, 258:14279-14283.

Access the most updated version of this article at http://www.jbc.org/content/258/23/14279

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/258/23/14279.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 23, Issue of December 10, pp. 14279-14283, 1983
Printed in U.S.A.

# $^{13}$C NMR Investigation of Nonenzymatic Glucosylation of Protein

MODEL STUDIES USING RNase A*

(Received for publication, June 6, 1983)

**Carolyn I. Neglia‡, Helga J. Cohen‡, Albert R. Garber‡, Paul D. Ellis‡, Suzanne R. Thorpe‡, and John W. Baynes‡§||**

*From the ‡Department of Chemistry and §School of Medicine, University of South Carolina, Columbia, South Carolina 29208*

**Nonenzymatic glucosylation of protein is initiated by the reversible condensation of glucose in its open chain form with the amino groups on the protein. The initial product is an aldimine (Schiff base) which cyclizes to the glycosylamine derivative. The aldimine can undergo a slow Amadori rearrangement to yield the relatively stable ketoamine adduct which is structurally analogous to fructose. $^{13}$C NMR has been used to characterize these early products of nonenzymatic glucosylation, using RNase A as a model protein. C-1 of the β-pyranose anomer of the glycosylamine was identified at 88.8 ppm in the spectrum of RNase glucosylated ~1:1 with D-[1-$^{13}$C]glucose. C-1 of the Amadori product was also apparent in this spectrum, resonating as a pair of intense peaks at 52.7 and 53.1 ppm. The anomeric (C-2) resonances of the Amadori adduct were seen in the spectrum of RNase glucosylated ~1:1 with [U-$^{13}$C]glucose. This spectrum was interpreted by comparison to the spectra of reference compounds: D-fructose, fructose-glycine, $N^{\alpha}$-formyl-$N^{\epsilon}$-fructose-lysine, and glucosylated poly-L-lysine. In the protein spectrum, the most intense of the C-2 resonances was that of the β-fructopyranose anomer at 95.8 ppm. The α- and β-fructofuranose anomers were also observed at 101.7 and 99.2 ppm, respectively. One unidentified signal in the anomeric region was observed in the spectra of poly-L-lysine and RNase, both glucosylated with [U-$^{13}$C]glucose; no comparable resonances were observed in the spectra of the model compounds.**

Nonenzymatic glucosylation is the first step in a complex, poorly understood series of Maillard or browning reactions of proteins, which occur in the presence of reducing sugar (1-4). It is known that nonenzymatic glucosylation is a common post-translational modification of proteins in the body (5, 6), but it is not yet known if the subsequent Maillard reactions also occur *in vivo*. Glucosylation is initiated by condensation of glucose, in its open chain form, with protein amino groups, primarily the ε-amino groups of lysine. The initial product is an aldimine (Schiff base) derivative (Fig. 1). The equilibrium constant for this reaction is unfavorable, but the aldimine slowly undergoes Amadori rearrangement to yield a relatively stable derivative, commonly termed the ketoamine adduct (5, 6). The products obtained in subsequent Maillard reactions are necessarily dependent on the structure and reactivity of the early adducts formed between glucose and proteins; however, the actual conformations of the protein-bound sugar have not been fully characterized. Based on model studies (1-4) with small molecules, the Schiff base and Amadori adducts should exist largely in cyclic furanose and pyranose conformations, i.e. as glycosylamines and N-substituted 1-amino-1-deoxyglycoses (Figs. 1 and 2). Recently, Fischer and Winterhalter (7) concluded that Amadori adducts to hemoglobin exist almost exclusively in the ring form, because of their lack of reactivity with phenylhydrazine. In the present study, we have used $^{13}$C NMR at 9.4 Teslas (100.6 MHz) to characterize the actual conformations of glucose adducts to protein, using bovine pancreatic RNase A as a model protein.

* This work was supported by United States Public Health Service Research Grants AM-19971 and 25373. The Nuclear Magnetic Resonance Center at the University of South Carolina is supported by National Science Foundation Grant CHE 82-07445. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| Recipient of United States Public Health Service Research Career Development Award AM-00931.

## MATERIALS AND METHODS

RNase A (Type XII-B), L-lysine, and poly-L-lysine (approximate $M_r$ = 14,000) were purchased from Sigma, glycine and D-fructose from Fisher, n-butylamine from Aldrich, and D-glucose from P & L Laboratories. 6-D-[$^3$H]Glucose was obtained from New England Nuclear. D-[U-$^{13}$C]Glucose, 14.5 atom % enriched, was obtained from MSD Isotopes and D-[1-$^{13}$C]glucose, 99 atom % enriched, from Omicron Biochemicals.

*Preparation of Model Compounds*—$N^{\alpha}$-formyllysine was prepared from acetic anhydride and lysine formate according to the method of Hofmann (8). $N^{\alpha}$-formyl-$N^{\epsilon}$-fructose-lysine was synthesized using glucose and $N^{\alpha}$-formyllysine as described by Finot and Mauron (9); the product was purified by chromatography on Dowex 50W-X8 (Bio-Rad) using the linear pyridine-acetate gradient described by Chiou *et al.* (10). Glucosyl-n-butylamine was made from glucose and n-butylamine according to Mitts and Hixon (11). Fructoseglycine was prepared as described by Abrams *et al.* (12).

*Glucose Purification*—6-[$^3$H]Glucose was purified by paper chromatography (Whatman 3 MM) overnight in butanol:pyridine:water (9:5:4). The chromatogram was scanned on a Packard Model 7201 Radiochromatogram Scanner and the glucose region eluted with 1 ml of distilled water. This glucose was stored at 4 °C and used within 2 weeks of purification. In preliminary experiments, $^{13}$C-enriched glucose was preincubated for 48 h with RNase to remove any reactive contaminants, analogous to those described in preparations of radioactive glucose (13). However, no differences were observed in the NMR spectra of protein glucosylated with $^{13}$C-enriched glucose whether or not the glucose had been preincubated with protein. Thus, in the experiments reported here $^{13}$C-enriched sugars were used without further purification.

*Glucosylation Reactions*—All glucosylation reaction mixtures were sterilized by filtration through a Gelman 0.2 μ Acrodisc filter immediately prior to incubation. RNase (274 mg/2 ml, 10 mM) was glucosylated at 37 °C for 6 days with 0.25 M $^{13}$C-enriched glucose in 0.2 M phosphate buffer, pH 7.4. Poly-L-lysine (274 mg/2 ml) was glucosylated under identical conditions except that the incubation was carried out for only 3 days. At the end of the incubation period, reaction mixtures were dialyzed in Spectrapor membrane tubing ($M_r$ = 6000-8000 cutoff, Fisher) at 4 °C against 0.2 M phosphate buffer, pH 7.4, to remove free glucose.



Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014

The extent of protein modification by glucose was estimated from parallel reaction mixtures containing 6-[$^3$H]glucose. Radioactive, glucosylated protein was separated from free glucose by Sephadex G-25 (Pharmacia) chromatography; the degree of substitution was calculated from the specific activity of the 6-[$^3$H]glucose and radioactivity/mg of protein. The calculated degree of modification of RNase and poly-L-lysine was 1.2 and 3 mol of glucose/mol of polypeptide, respectively.

*NMR Spectroscopy*—$^{13}$C NMR spectra were obtained at 100.6 MHz on a Bruker WH-400 spectrometer at the National Science Foundation Regional NMR Center at the University of South Carolina, employing a standard Bruker 10-mm broadband probe. All samples contained 20% $D_2O$ (v/v) in 0.2 M potassium phosphate buffer, pH 7.4, unless otherwise stated. Sample temperature was regulated at 5 ± 1 °C.

Spectra were acquired with broadband proton decoupling (2 watts) at a sample width of 25,000 Hz (250 ppm) following a 10-μs pulse (tip angle equal to ~30°). The time domain spectrum was sampled for approximately 0.33 s (16K data points), yielding an effective resolution of about 3 Hz. Successive scans were acquired without a relaxation delay.

The 16K data table was Fourier transformed without zero filling following an application of 10-Hz line broadening. Difference spectra were obtained by digitally, subtracting the spectrum of native RNase from the sample spectrum. Chemical shifts were referenced to external dioxane (0.2 M potassium phosphate, pH 7.4, 20% $D_2O$) at 66.5 ppm to lower shielding from tetramethylsilane.

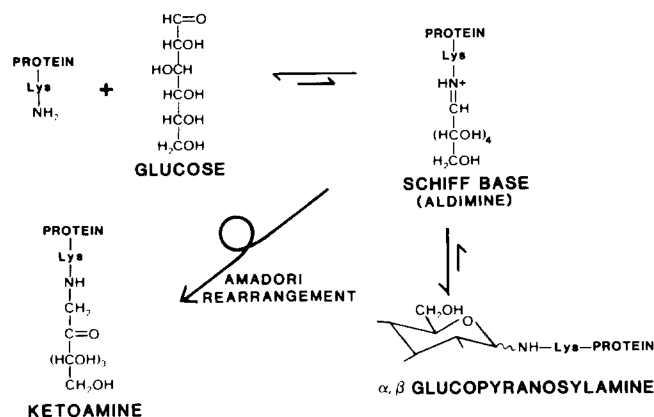


FIG. 1. **Early products of nonenzymatic glucosylation of protein.**

## RESULTS AND DISCUSSION

*Characterization of Glycosylamine Adduct to RNase*—The spectrum of native RNase A (Fig. 3*A*) contains no carbon resonances in the region characteristic of the anomeric carbons of either *O*-linked (90–110 ppm) or *N*-linked (80–90 ppm) carbohydrates (14). However, in preliminary experiments, carbohydrate resonances were not detectable in these regions in the spectrum of RNase glucosylated ~1:1 with the natural abundance [$^{13}$C]glucose. In contrast, the anomeric carbon resonances were readily seen in the spectrum of RNase glucosylated with [1-$^{13}$C]glucose (Fig. 3*B*). In order to minimize dissociation of the glycosylamine derivative, these spectra were acquired after a relatively short dialysis period (see legend to Fig. 3). Thus, the dominant resonances in the spectrum are those of C-1 of the α and β anomers of free glucose at 91.9 and 95.6 ppm, respectively. The resonance at 88.8 ppm is attributed to the anomeric carbon of the β-glucosylamine adduct to RNase (see below). This resonance is much less intense after 24-h dialysis and is not detectable after 48-h dialysis of RNase glucosylation reaction mixtures. It also disappeared completely (data not shown) after a 1-h exposure of the glucosylated RNase to pH 5 at 37 °C, a treatment known to cause rapid hydrolysis of small molecule glycosylamines (15) and to discharge glycosylamine adducts from other proteins (16).

The assignment of the resonance at 88.8 ppm in the spectrum of glucosylated RNase as the β-pyranose anomer of the glycosylamine is based on comparison to the spectrum of glucosyl-*n*-butylamine (Fig. 3, *C* and *D*). Although this compound was dissolved in buffer shortly before starting the NMR experiment, some hydrolysis is evident since the α and β anomers of free glucose are clearly visible in the spectrum in Fig. 3*C*. The prominent resonance at 89.7 ppm is assigned to the β-pyranose and that at 86.2 ppm to the α-pyranose anomer of glucosyl-*n*-butylamine. By analogy to the structure of glucose in solution, furanose anomers of the glycosylamine should be rare, but, if present, would likely be obscured by the free glucose resonances. The increased shielding (upfield shift) of the anomeric carbons of the glycosylamine, relative to glucose, is in agreement with the chemical shifts of *N*-linked carbons, in general (14). Further support for the as-

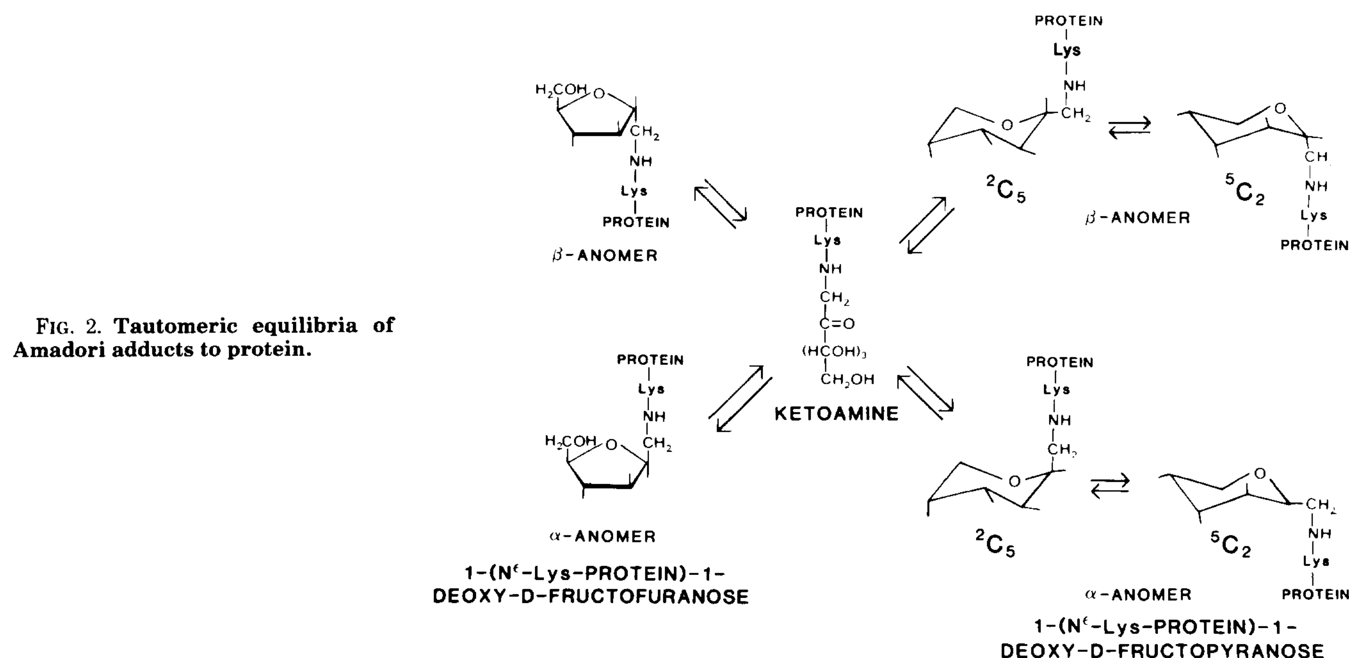


FIG. 2. **Tautomeric equilibria of Amadori adducts to protein.**

Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014

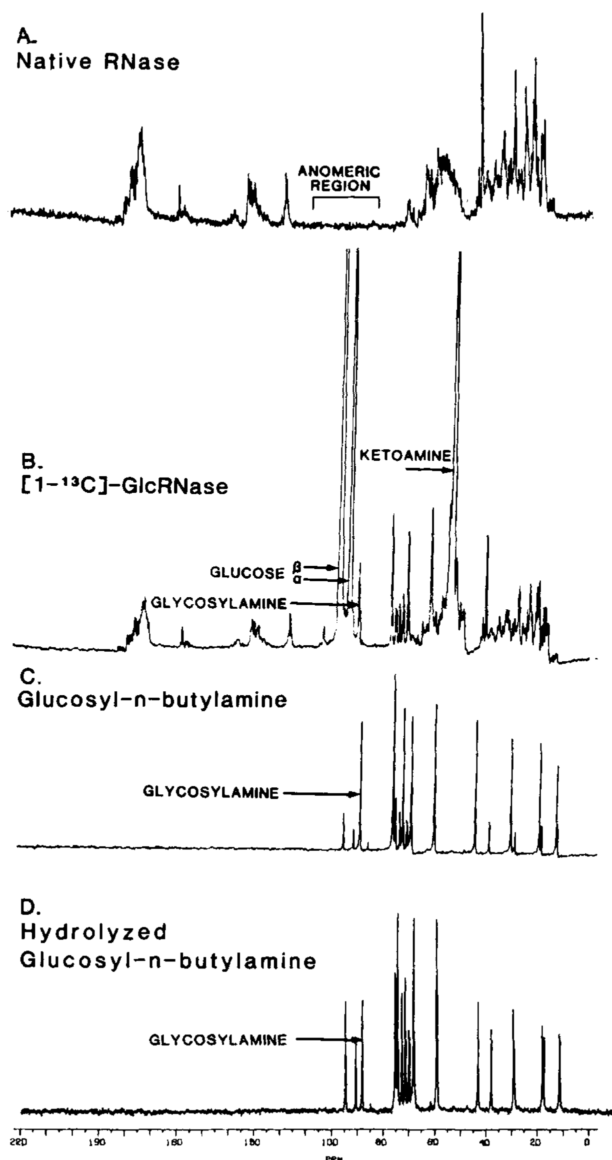


FIG. 3. **$^{13}C$ NMR spectra of native and glucosylated RNase and glucosyl-*n*-butylamine.** *A*, natural abundance $^{13}C$ NMR spectrum of native RNase: 5 mM; 217,470 scans. *B*, spectrum of RNase glucosylated with D-[1-$^{13}$C]glucose. Glucosylated RNase, 1.2 mol of glucose/mol of protein, was prepared as described under "Materials and Methods." At the end of the incubation period the protein was dialyzed for a total of 2 h against 2 changes of phosphate buffer. Final protein concentration: 4.7 mM; 217,470 scans. *C*, spectrum of glucosyl-*n*-butylamine: 0.2 M; 12,175 scans. *D*, hydrolyzed glucosyl-*n*-butylamine. This sample was allowed to incubate for 72 h at 4 °C, and then a second spectrum was obtained, as above.

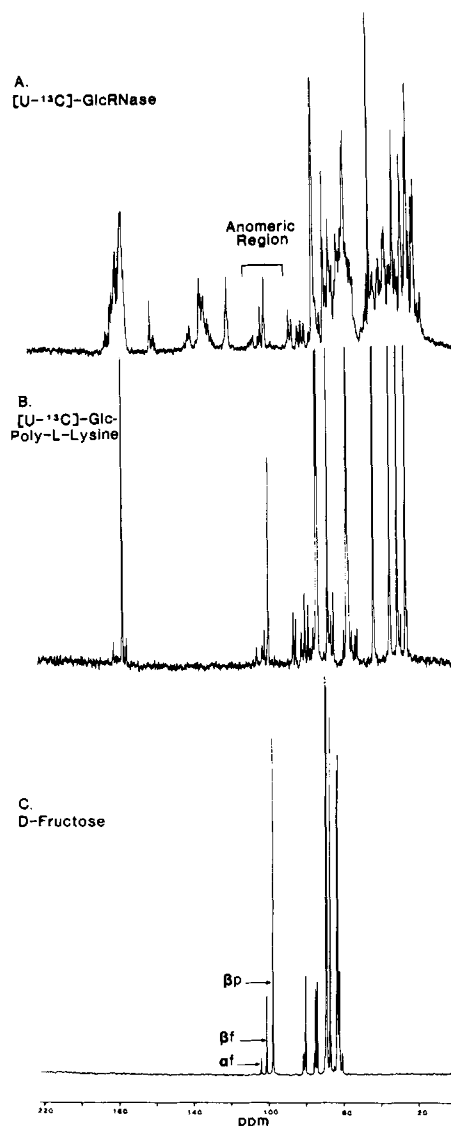


FIG. 4. **$^{13}C$ NMR spectra of RNase and poly-L-lysine glucosylated with D-[U-$^{13}$C]glucose and D-fructose.** *A*, glucosylated RNase, 1.2 mol of [U-$^{13}$C]glucose/mol of protein, was prepared as described under "Materials and Methods." Protein concentration: 7.5 mM; 217,470 scans. *B*, glucosylated poly-L-lysine, 3 mol of [U-$^{13}$C] glucose/mol of polypeptide was prepared as described under "Materials and Methods." Polypeptide concentration: 69 mg/ml; 217,470 scans. *C*, D-fructose: 0.5 M in 0.2 M phosphate buffer; 2,345 scans.

signment of the glycosylamine resonances is found in Fig. 3*D*, which is the spectrum of glucosyl-*n*-butylamine after 72-h incubation at 4 °C, after substantial hydrolysis of the glycosylamine had occurred. The hydrolysis reaction results in an increase in the free glucose anomeric resonances and a concomitant decrease in the relative intensity of the glycosylamine peaks. No resonances were observed in the azomethine ($>$C=N—) region at 145–165 ppm (17) in the spectra of either glucosylated RNase or glucosyl-*n*-butylamine. Thus, there is no evidence for a measurable concentration of the open chain, Schiff base conformation of the glycosylamines (Fig. 1).

As a methylene carbon attached to nitrogen, C-1 of the Amadori rearrangement product on RNase is expected to resonate between 40 and 60 ppm (17) (Figs. 1 and 2). This resonance is clearly evident at ~53 ppm in the spectrum of glucosylated RNase (Fig. 3*B*), and was unaffected by pH 5 treatment which discharged the glycosylamine adduct (data not shown). No signals were observed in the region of ketone resonances (175–225 ppm) (17). Thus, in agreement with the chemical studies of Fischer and Winterhalter (7), there is no evidence for the open chain, ketoamine conformation of the Amadori product. The additional peaks in the spectrum between 60 and 80 ppm in Fig. 3*B* are attributed to natural abundance resonances of carbons 2–6 from the [1-$^{13}$C]glucose in the sample. Based on the integrated intensities of the C-1 resonances of the glycosylamine (88.8 ppm) and Amadori (53 ppm) adducts (after digital subtraction of the native RNase spectrum), it could be estimated that about 14% of the pro-

Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014

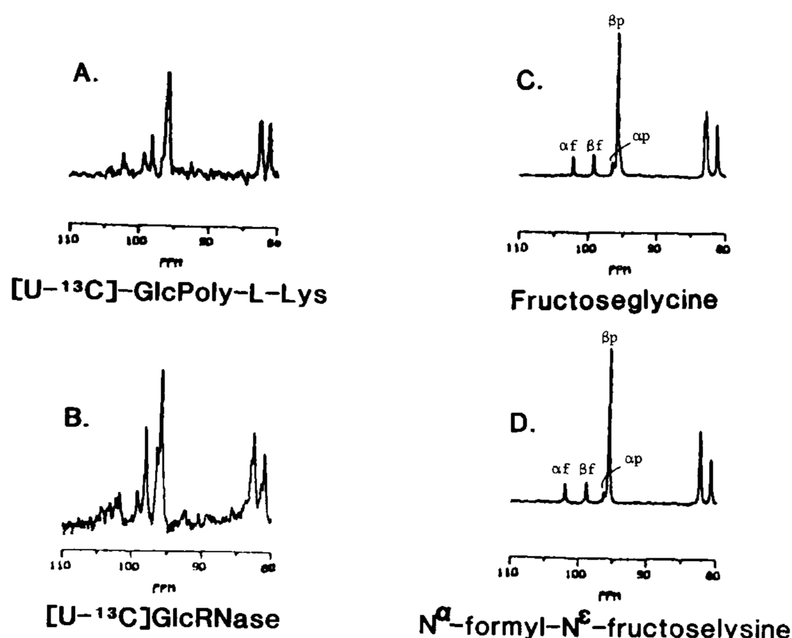


FIG. 5. **Expanded spectra of region from 80–110 ppm of U-$^{13}$C-glucosylated RNase and poly-L-lysine and model compounds.** *A* and *B*, same conditions as in the legend to Fig. 4, *A* and *B*. *C*, fructose-glycine: 60 mM; 134,211 scans. *D*, $N^\alpha$-formyl-$N^\epsilon$-fructose-lysine: 100 mM; 217,470 scans.

TABLE I

*Chemical shifts and intensities of anomeric carbon resonances of [U-$^{13}$C]Glc-RNase and model compounds*

| Compound[a] | Chemical shift of anomeric carbon | | | | | Ratio of intensities[b] |
|---|---|---|---|---|---|---|
| | α-Fructofuranose | β-Fructofuranose | Unknown | α-Fructopyranose | β-Fructopyranose | |
| | | | ppm[c] | | | |
| [U-$^{13}$C]Glc-poly-L-lysine | 101.9 | 98.8 | 97.8 | 96.5 | 95.5 | 1:1.1:1.9:1:12.6 |
| [U-$^{13}$C]Glc-RNase | 101.7 | 99.2 | 98.1 | 96.5 | 95.8 | 1:1.2:3.4:2.6:5.2 |
| $N^\alpha$-Formyl-$N^\epsilon$-fructose-lysine | 101.7 | 98.7 | — | 96.2 | 95.4 | 1.5:1.8:—:1:14.0 |
| Fructose-glycine | 101.8 | 98.9 | — | 96.1 | 95.4 | 1.3:1.4:—:1:9.0 |
| D-Fructose | 104.3 | 101.4 | — | — | 98.1 | 1:5:—:—:23 |

[a] Compounds prepared as described under "Materials and Methods."

[b] Peak heights measured on spectra in Figs. 4 and 5.

[c] Chemical shifts expressed relative to trimethylsilane, taken from spectra shown in Figs. 4 and 5.

tein-bound glucose existed as the glycosylamine adduct.[1] This is in good agreement with the observation that when glucosylated RNase from a parallel incubation with 6-[$^3$H]glucose was treated at pH 5, 12.5% of the protein-bound glucose was released.

*Conformation of Amadori Adducts to RNase*—Once the Amadori rearrangement has occurred, the product may assume several possible ring conformations, as illustrated in Fig. 2. To determine which of these structures are present, RNase was glycosylated with [U-$^{13}$C]glucose. A model polypeptide, glucosylated poly-L-lysine, was also prepared. As shown in Fig. 4, *A* and *B*, several anomeric resonances (90–110 ppm) were observed in the spectra of both glucosylated peptides. Because the Amadori adducts to protein are structurally analogous to fructose, their spectra may be interpreted by comparison to the spectrum of free D-fructose in the same phosphate buffer (Fig. 4*C*). Inspection of these spectra shows, in fact, that the anomeric carbons on the polypeptides resonate similarly to those of the α- and β-furanose and β-pyranose anomers of fructose. As with fructose, the β-pyranose anomer yields the most intense signal in the spectra of glucosylated RNase and poly-L-lysine at 95.8 and 95.5 ppm, respectively. Distinct resonances due to the α- and β-furanose anomers are also observed at 101.7 and 99.2 ppm in the protein and polypeptide spectra (see Fig. 5 for greater detail). The slight shielding (about 2 ppm) of the anomeric carbons on RNase and poly-L-lysine, compared to fructose, results from the substitution of an amine nitrogen in the Amadori compound for the C-1 hydroxyl group of fructose (Fig. 4, *A* and *B*, compared to Fig. 4*C*; see also Table I). Carbons 3, 4, and 5 of the furanose anomers on protein and poly-L-lysine appear between 74 and 83 ppm (Fig. 4, *A* and *B*), essentially the same locations as in free fructose. The pyranose ring carbons and C-6 of the furanose resonate between 64 and 70 ppm in these spectra. In general, the ratio of furanose to pyranose ring carbon intensities appears comparable to the ratio of intensities of the anomeric carbons.

While the carbohydrate resonances in the spectra of the Amadori adducts agree closely with those of free fructose, the anomeric resonance at 98.1 ppm in RNase and at 97.8 ppm in poly-L-lysine (Fig. 5) is unique to the macromolecule spectra. Its appearance in glucosylated poly-L-lysine argues against an unusual environmental effect in RNase, *e.g.* glucosylation of an active-site lysine. However, if α-amino groups were more reactive than ε-amino groups, the additional peak could result from preferential glucosylation at the α-$NH_2$-terminal lysines in both RNase and poly-L-lysine. The model compounds, fructose glycine and $N^\alpha$-formyl-$N^\epsilon$-fructose-lysine were, therefore, synthesized to determine whether glucose

[1] In order to obtain better sensitivity, all spectra were acquired without suppression of Nuclear Overhauser Enhancement. The estimate is, therefore, only approximate, and assumes a comparable Overhauser effect for C-1 of both the glycosylamine and Amadori compounds.

Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014

adducts to an $\alpha$-amino group would resonate differently from those on an $\epsilon$-amino group. The spectra of the anomeric region of these compounds, shown in Fig. 5, are identical with one another. As with fructose, the $\beta$-pyranose anomer is the most intense peak in these spectra. The relative intensities and spacing of the furanose and pyranose peaks are also comparable in the polypeptides and model compounds (Fig. 5), with the exception of the unidentified peak at ~98 ppm. Comparison of the spectra of fructose-glycine and $N^{\alpha}$-formyl-$N^{\epsilon}$-fructose-lysine indicates that the difference in substituents on the carbon $\gamma$ to the anomeric carbon does not substantially affect the resonance of the anomeric carbon. Thus, there is no evidence that the unidentified signal in the anomeric region results from glucosylation at the $\alpha$-$NH_2$ terminus.

The spectra in Fig. 5, *C* and *D* also contain a distinct resonance at 96.5 ppm, appearing as a shoulder about 1 ppm to lower shielding (downfield) of the $\beta$-pyranose peak. We have tentatively assigned this resonance to the $\alpha$-pyranose conformation. The $\alpha$-pyranose peak is also apparent as a shoulder on the $\beta$-pyranose anomer in both glucosylated RNase and poly-L-lysine (Fig. 5). The locations and intensities of the various resonances for the glucosylated polypeptides and model compounds described in Figs. 3–5 are summarized in Table I.

*Conclusions*—Overall, with the exception of the unassigned resonance at ~98 ppm, the spectra of glucose adducts to RNase were readily interpreted by reference to the spectra of the free sugars, glucose and fructose. The anomeric carbons of the glycosylamine and Amadori compounds were observed to resonate slightly upfield (to higher shielding) of the corresponding carbons in the sugars. The spectra of the Amadori adducts to RNase A and poly-L-lysine were not significantly different from one another with respect to either the location or the relative intensity of the peaks. For all of the model compounds, as well as the free sugars, the pyranose conformation appears to be the predominant structure in solution, based on the intensity of the various resonances. Thus far, RNase A has proven to be a useful model protein for characterizing the structures of the early products of the Maillard reaction by $^{13}$C NMR spectroscopy. Similar studies are in progress to identify other products and intermediates in the browning and aging of protein in the presence of reducing sugars.

REFERENCES


1. Hodge, J. E. (1953) *J. Agric. Food Chem.* **1,** 928–943
2. Ellis, G. P. (1959) *Adv. Carbohydr. Chem.* **14,** 63–134
3. Reynolds, T. M. (1963) *Adv. Food Res.* **12,** 1–52
4. Reynolds, T. M. (1965) *Adv. Food Res.* **14,** 167–283
5. Monnier, V. M., Stevens, V. J., and Cerami, A. (1981) *Prog. Food Nutr. Sci.* **5,** 315–327
6. Thorpe, S. R., and Baynes, J. W. (1982) in *The Glycoconjugates* (Horowitz, M. I., ed) Vol. III, Academic Press, New York
7. Fischer, R. W., and Winterhalter, K. H. (1981) *FEBS Lett.* **135,** 145–147
8. Hofmann, K., Stutz, E., Spühler, G., Yajima, H., and Schwartz, E. T. (1960) *J. Am. Chem. Soc.* **82,** 3727–3732
9. Finot, P. A., and Mauron, J. (1969) *Helv. Chim. Acta* **52,** 1488–1495
10. Chiou, S.-H., Chylack, L. T., Jr., Bunn, H. F., and Kinoshita, J. H. (1980) *Biochem. Biophys. Res. Commun.* **95,** 894–901
11. Mitts, E., and Hixon, R. M. (1944) *J. Am. Chem. Soc.* **66,** 483–486
12. Abrams, A., Lowy, P. H., and Borsook, H. (1955) *J. Am. Chem. Soc.* **77,** 4794–4796
13. Trueb, B., Holenstein, C. G., Fischer, R. W., and Winterhalter, K. H. (1980) *J. Biol. Chem.* **255,** 6717–6720
14. Dill, K., and Allerhand, A. (1979) *J. Biol. Chem.* **254,** 4524–4531
15. Isbell, H. S., and Frush, H. I. (1958) *J. Org. Chem.* **23,** 1309–1319
16. Bisse, E., Berger, W., and Flückiger, R. (1982) *Diabetes* **31,** 630–633
17. Breitmaier, E., and Voelter, W. (1978) in *Monographs in Modern Chemistry* (Ebel, H. F., ed) 2nd Ed., Vol. 5, pp. 67–92, Verlag Chemie, New York


Downloaded from http://www.jbc.org/ at USC SCHOOL OF MEDICINE on September 30, 2014