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## Effect of Phosphate on the Kinetics and Specificity of Glycation of Protein\*

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The glycation (nonenzymatic glycosylation) of several proteins was studied in various buffers in order to assess the effects of buffering ions on the kinetics and specificity of glycation of protein. Incubation of RNase with glucose in phosphate buffer resulted in inactivation of the enzyme because of preferential modification of lysine residues in or near the active site. In contrast, in the cationic buffers, 3-(*N*-morpholino)propanesulfonic acid and 3-(*N*-tris(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid, the kinetics of glycation of RNase were decreased 2- to 3-fold, there was a decrease in glycation of active site *versus* peripheral lysines, and the enzyme was resistant to inactivation by glucose. The extent of Schiff base formation on RNase was comparable in the three buffers, suggesting that phosphate, bound in the active site of RNase, catalyzed the Amadori rearrangement at active site lysines, leading to the enhanced rate of inactivation of the enzyme. Phosphate catalysis of glycation was concentration-dependent and could be mimicked by arsenate. Phosphate also stimulated the rate of glycation of other proteins, such as lysozyme, cytochrome c, albumin, and hemoglobin. As with RNase, phosphate affected the specificity of glycation of hemoglobin, resulting in increased glycation of amino-terminal valine *versus* intrachain lysine residues. 2,3-Diphosphoglycerate exerted similar effects on the glycation of hemoglobin, suggesting that inorganic and organic phosphates may play an important role in determining the kinetics and specificity of glycation of hemoglobin in the red cell. Overall, these studies establish that buffering ions or ligands can exert significant effects on the kinetics and specificity of glycation of proteins.

Nonenzymatic glycosylation or glycation is a common post-translational modification of proteins in the body (1), resulting from reaction between glucose and the amino groups on protein. Glycation proceeds through formation of a Schiff base intermediate, followed by an Amadori rearrangement to yield a relatively stable ketoamine adduct to the protein (*cf.* Fig. 6 under "Discussion") (2, 3). Although nucleophilic, low

$pK_a$  amino groups, *e.g.* the  $\alpha$ -amino groups on protein, should be most reactive in forming Schiff base adducts, it is now recognized that other factors beside the  $pK_a$  of the amino group determine the final distribution of ketoamine adducts to proteins. Thus, for hemoglobin (Hb),<sup>1</sup> despite the similar  $pK_a$  values of the valine residues at the termini of the  $\alpha$ - and  $\beta$ -chains (4), the  $\beta$ -chain valines are about 10 times more reactive with glucose both *in vivo* and *in vitro* (5). Similarly, we have observed (6) that although *N*<sup>ε</sup>-Lys-1 was the primary site of formation of Schiff base adducts to ribonuclease (RNase), other amino groups, including *N*<sup>ε</sup>-Lys-1, were up to 10 times more reactive in forming ketoamine adducts.

Some progress has been made in identifying factors affecting the specificity of glycation of proteins. Shapiro *et al.* (5) noted increased glycation of lysine residues adjacent to acidic amino acids in Hb, suggesting that the carboxyl groups of adjacent aspartate or glutamate residues could serve as neighboring group, intramolecular catalysts of the Amadori rearrangement. Lysines 1 and 37 are adjacent to acidic amino acids in RNase and were also among the more reactive sites of glycation of this protein (6). Studies with Hb (5), human serum albumin (7, 8), and lysozyme<sup>2</sup> have shown that the lysine residues in Lys-Lys and other basic amino acid sequences are more highly glycated than other lysines in these proteins. In recent work on the glycation of RNase (6, 9), we showed that the reactive lysine residues in this enzyme were located in or near the phosphate binding region of the active site, a fairly basic region of the protein, rich in lysine, histidine, and arginine residues. Since our experiments were done in phosphate buffer, we suggested that some of the specificity of glycation of RNase might derive from the binding of phosphate ions, which could serve as a local catalyst of the Amadori rearrangement on lysine residues in or near the active site. In this paper we describe the effects of phosphates on the kinetics and specificity of glycation of RNase and several other proteins and present a general theory on how buffering ions and ligands affect the kinetics and specificity of glycation of proteins.

### EXPERIMENTAL PROCEDURES

**Materials**—RNase A (Type XII-A), horse heart cytochrome c (Type VI), chicken egg white lysozyme (Grade I), human serum albumin, sodium borohydride, sodium cyanoborohydride, 2,3-diphosphoglycerate (DPG), MOPS, and TAPSO were obtained from Sigma. D-Glucose was purchased from P-L Biochemicals, and [<sup>3</sup>H]sodium

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<sup>1</sup> The abbreviations used are: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RNase, bovine pancreatic ribonuclease; TAPSO, 3-(*N*-tris(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid; HPLC, high pressure liquid chromatography.

<sup>2</sup> C. J. Hull, S. R. Thorpe, and J. W. Baynes, unpublished observations.

borohydride and D-[6-<sup>3</sup>H]glucose from Du Pont-New England Nuclear. Radioactive glucose was purified by column chromatography on Aminex HPX-87C (Bio-Rad), as described previously (6). [<sup>3</sup>H]-Glucitolvaline was prepared by incubating D-[6-<sup>3</sup>H]glucose (250  $\mu$ Ci, 0.1 M) with L-valine (0.1 M) in the presence of NaBH<sub>3</sub>CN (0.2 M) in 0.2 M phosphate buffer for 3 days at 37 °C. Excess NaBH<sub>3</sub>CN was discharged by addition of 1 N HCl to pH 3, and the product isolated by cation exchange chromatography, as described by Schwartz and Gray (10).

**Glycation of Proteins**—General procedures for glycation of proteins and characterization of products were as described previously for RNase (6). Briefly, proteins (25 mg/ml) were incubated with 0.4 M glucose in 0.2 M buffer (phosphate, MOPS, or TAPSO), pH 7.4, containing 3 mM sodium azide. Solutions were preincubated for 1 h at 37 °C, the pH readjusted to 7.4, as needed, and the reaction mixture sterilized by filtration through a Gelman 0.2- $\mu$ m filter (Acrodisc) into a sterile 1.5-ml plastic centrifuge tube (Sarstedt). Aliquots were removed at various times and frozen at -20 °C until assayed.

**Measurement of Glucose Adducts to Protein**—For measuring Schiff base adducts to RNase, the protein was incubated for 3 h with radioactive glucose, a sufficient time to achieve Schiff base equilibrium (11), but prior to extensive ketoamine modification on the protein. The reaction mixture was then cooled on ice, and an aliquot (100  $\mu$ l, 2 mg of protein) was fractionated by gel exclusion chromatography on a 0.5  $\times$  30 cm column of Sephadex G-25 (Pharmacia) equilibrated in 0.1 M Tris-Cl buffer, pH 7.4. The chromatography was conducted at low temperature to minimize dissociation of Schiff base adducts, and the extent of Schiff base formation calculated from the specific activity of the glucose and the glucose radioactivity and protein content of the void volume fractions, as described previously (11). Glucose concentration was measured by a glucose oxidase-peroxidase assay (Amresco) and protein concentration by absorbance at 280 nm. Corrections were applied for the limited extent of ketoamine formation during the incubation period. For measurement of ketoamine adducts to RNase and other proteins, an aliquot of the reaction mixture was diluted to 1 ml with 0.1 N sodium acetate, pH 5, incubated at 37 °C for 1 h in order to discharge Schiff base adducts (12), then chromatographed, and analyzed as above. Procedures for labeling glycated RNase by reduction with [<sup>3</sup>H]NaBH<sub>4</sub> (6), for separating the tryptic peptides of RNase by reversed-phase HPLC (6), and conditions for NMR spectrometry (13, 14) have been described.

**Glycation of Valine Versus Lysine Residues in Hemoglobin**—Hb was isolated following hemolysis of saline-washed human red cells in 2 volumes of distilled water for 1 h at room temperature. The hemolysate was centrifuged for 20 min at 10,000  $\times$  g to remove cell membranes, and the supernatant was gassed with carbon monoxide. Hb-CO concentration was determined by absorbance at 540 nm ( $E_{\text{mM}} = 13.4$ ) (15). Aliquots of this solution were then diluted 100-fold into 0.2 M incubation buffer (phosphate, TAPSO, or TAPSO plus 10 mM DPG, pH 7.4), containing 0.4 M glucose, and incubated at 37 °C under a CO<sub>2</sub> atmosphere. After incubation, the protein solution was dialyzed against phosphate-buffered saline to remove free glucose, then reduced with [<sup>3</sup>H]NaBH<sub>4</sub>. Excess borotritide was destroyed and heme simultaneously extracted by addition of 20 volumes of 0.25 M HCl in acetone (16). Globin was isolated by centrifugation, then hydrolyzed and analyzed for its hexitolvaline and hexitollysine content by cation exchange chromatography, as previously described (6, 14).

## RESULTS

To evaluate the effects of phosphate on the specificity of glycation of RNase, we compared reactions in phosphate with those in MOPS (17) and TAPSO (18), which have pK<sub>a</sub> values similar to phosphate (7.2 and 7.4 at 37 °C, respectively), but are cationic, rather than anionic buffers. As shown in Fig. 1A, the kinetics of glycation of RNase, *i.e.* the rate of formation of the ketoamine adduct, varied significantly with the nature of the buffering species. The kinetic differences were maximal at early times, but even after 1 week of incubation the rate of reaction was measurably faster in phosphate than in MOPS or TAPSO. The slight difference in rate between MOPS and TAPSO was reproducible, and the slowest rates of glycation were consistently obtained in TAPSO buffer. The pH of the reaction mixtures was checked regularly in these experiments using a microelectrode (Lazar Research, model 1416) and

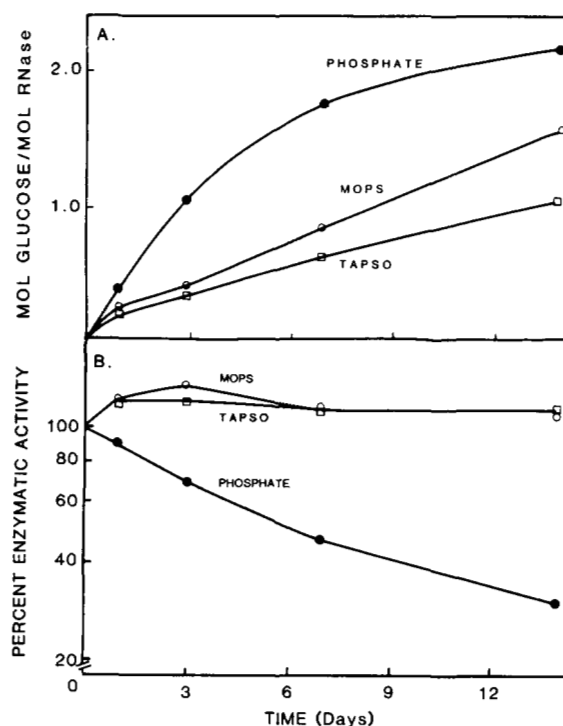


FIG. 1. Effect of buffering ions on the kinetics of glycation and inactivation of RNase. RNase was incubated with 0.4 M glucose in each of the three buffers (0.2 M) at pH 7.4 and samples removed at indicated times for assay. A, the extent of glycation (formation of the ketoamine adduct) was measured by chromatography on Sephadex G-25 after mild acid treatment to discharge Schiff base adducts, as described under "Experimental Procedures." B, the percent of initial specific enzymatic activity of RNase was determined using yeast RNA as substrate (19).

required occasional adjustment with 1 N NaOH during the first few days. However, pH drift never exceeded 0.1 pH units so that the kinetic differences were not the result of pH variations in the three buffer systems.

As shown in Fig. 1B, the kinetics of inactivation of RNase were also significantly different in the three buffers. The enzyme lost about 35% of its activity after glycation with 1 mol of Glc/mol of protein in phosphate buffer, *i.e.* after 3 days' reaction. In contrast, RNase glycated to the same extent in MOPS or TAPSO buffer, *i.e.* after incubation for 1-2 weeks, retained essentially 100% of its original enzymatic activity. The reason for the initial increase in RNase activity in the MOPS and TAPSO incubations is unknown, but it was observed consistently in separate experiments. Since the inactivation of RNase by glucose in phosphate buffer was associated with glycation of lysine residues in or near the active site (6), the results in Fig. 1 suggested that the decreased rate of glycation and inactivation of RNase in cationic buffers might be associated with alterations in the sites of glycation of the enzyme.

The tryptic peptide maps, shown in Fig. 2, indicated that the pattern of modification of RNase is significantly different in phosphate *versus* TAPSO buffer, with the peptide map in MOPS being nearly identical to that in TAPSO (data not shown). Notably, no new labeled peptides appeared in the MOPS or TAPSO chromatograms, but clearly, compared to Lys-37, the relative extent of glycation of Lys-1, 7, and 41 was significantly increased in phosphate *versus* MOPS and TAPSO buffers. Although Lys-37 was the primary site of glycation of RNase in the cationic buffers, its absolute rate of modification was similar in all three buffers, *i.e.* about 3% per

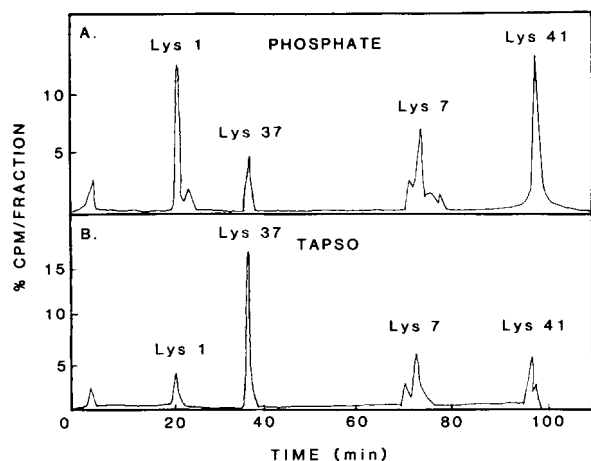


FIG. 2. Tryptic peptide maps of RNase glycosylated in phosphate and TAPSO buffers. RNase was incubated with 0.4 M glucose in either phosphate (top) or TAPSO (bottom) buffer for 3 or 14 days, respectively, in order to obtain glycation with 1 mol of Glc/mol of protein. After discharging Schiff base adducts and dialysis, glycosylated protein was reduced with  $[^3\text{H}]\text{NaBH}_4$ , oxidized with performic acid, then digested with trypsin. The tryptic peptides were resolved by reversed-phase HPLC (6). The labels over the peaks identify the sequence number of the lysine residue glycosylated in each peptide. These procedures and the identification of the lysine residues were reported in detail previously (6).

day. Thus, RNase glycosylated for 3 days in phosphate buffer contained 1 mol of Glc/mol of RNase, and Lys-37 accounted for 8.4% of the total peptide radioactivity (Fig. 2A), *i.e.*  $\sim 2.8\%$  glycation of Lys-37 per day in phosphate buffer. Although a 14-day incubation period was required to obtain 1 mol of Glc/mol of RNase in TAPSO buffer, Lys-37 accounted for 40.6% of peptide radioactivity in this preparation (Fig. 2B), yielding  $\sim 2.9\%$  glycation of Lys-37 per day, *i.e.* the same rate as in phosphate buffer. In contrast, by a similar analysis there was about a 10-fold increase in the rate of modification of Lys-1 and 41 in phosphate *versus* TAPSO and about a 4-fold increase in glycation of Lys-7. The constant reactivity of Lys-37 with glucose in the various buffers is probably attributable to catalytic effects of the adjacent Asp-38 residue (5), independent of the buffering species. Analysis of the hexitollysine isomers from RNase glycosylated in phosphate buffer yielded 15%  $N^\alpha$ -hexitollysine in the protein (6); however, only traces ( $<3\%$ ) of  $N^\alpha$ -hexitollysine were detectable in RNase glycosylated in MOPS or TAPSO (data not shown). The overall increase in glycation of Lys-1 in phosphate indicated that phosphate stimulated the glycation of both the  $\alpha$ - and  $\epsilon$ -amino groups of Lys-1. The results of the peptide and hexitollysine analyses were confirmed by  $^{13}\text{C}$ -NMR spectroscopy. As shown in Fig. 3, resonances assigned to the anomeric carbons of glucose adducts to  $N^\alpha$ -Lys-1 and  $N^\epsilon$ -Lys-41 (14) were absent from the spectrum of RNase glycosylated in either MOPS or TAPSO. Overall, the peptide mapping and NMR results indicate that phosphate stimulates the glycation of Lys-1, 7, and 41 in RNase but does not affect the glycation of Lys-37.

Phosphate ions could affect the kinetics or specificity of glycation of RNase either by altering the equilibrium concentration of Schiff base adducts at various sites on the protein or by differential catalysis of the Amadori rearrangement at these sites. To determine the effect of phosphate on Schiff base formation, the protein was incubated with glucose for short periods of time, and the concentration of Schiff base adducts was measured by gel exclusion chromatography at low temperature (11). These data were used to derive an overall equilibrium constant for Schiff base formation be-

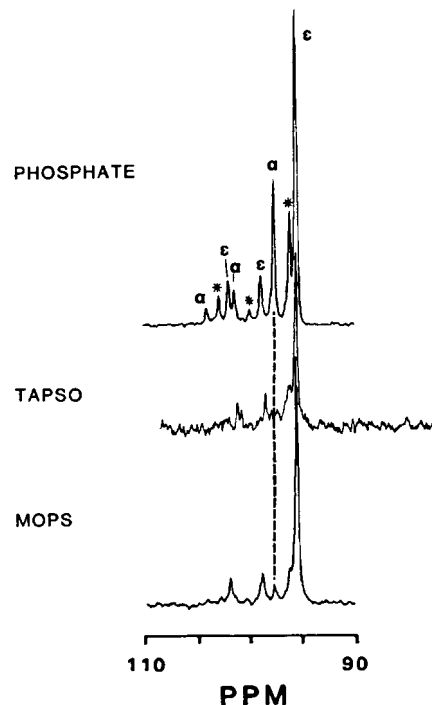


FIG. 3. Anomeric region of Carbon-13 NMR spectra of RNase glycosylated in various buffers. RNase was incubated with  $[2\text{-}^{13}\text{C}]\text{glucose}$  in various buffers in order to obtain modification with 1 mol of glucose/mol of RNase. NMR spectra were acquired on a Bruker WH-400 NMR spectrometer under conditions previously described (13, 14). Sets of three resonances ( $\alpha$ - and  $\beta$ -furanose and  $\beta$ -pyranose) are observed for the anomeric carbon of glucose adducts to each of the three types of amino groups in RNase (20):  $\alpha$  =  $N^\alpha$ -Lys-1,  $pK_a \sim 7.5$ ;  $\epsilon^*$  =  $N^\epsilon$ -Lys-41,  $pK_a \sim 8.8$ ; and  $\epsilon$  = peripheral  $\epsilon$ -amino groups,  $pK_a \sim 10.5$ .

TABLE I

Effect of buffers on formation of Schiff base adducts to RNase

All incubations were conducted in 0.2 M buffer, pH 7.4, for 3 h and analyzed for Schiff base and ketoamine content as described under "Experimental Procedures." The amount of ketoamine adduct was less than 10% of Schiff base content in all three incubations.

Buffer	Schiff base mol Glc/mol RNase	Estimated $K_{eq}^a$ $M^{-1}$
Phosphate	0.53	1.3
MOPS	0.78	1.9
TAPSO	0.62	1.6

<sup>a</sup> The  $K_{eq}$  was determined using the equation,  $\text{Glc} + \text{RNase} \rightarrow \text{Glc}=\text{RNase}$ , where  $\text{Glc}=\text{RNase}$  refers to the Schiff base adduct to RNase.

tween glucose and RNase, *i.e.* a composite  $K_{eq}$  for all amino groups in the enzyme. As shown in Table I, the equilibrium constant for Schiff base formation did not vary greatly among the three buffers, and was, in fact, somewhat higher in MOPS and TAPSO than in phosphate, despite the more rapid overall rate of glycation in phosphate. The data indicate that in 0.4 M glucose at pH 7.4 more than half of the protein molecules contain glucose in the form of a Schiff base adduct. As reported previously (6), the primary site of Schiff base formation is at the  $\alpha$ -amino group of Lys-1, whereas, regardless of the buffers used, the pattern of glycation of the protein indicates that other sites are more reactive in undergoing the Amadori rearrangement. Clearly, the slow step in the glycation reaction is the Amadori rearrangement, and phosphate appears to exert its effect by catalysis of this reaction.

As shown in Table II, the phosphate effect is concentration-

TABLE II  
Glycation of RNase in various buffers

RNase was incubated with 0.4 M glucose for 3 days in the various buffer systems and analyzed as described in the legend to Fig. 2.

Buffer	mol Glc/mol RNase
0.2 M phosphate	1.02
0.2 M TAPSO	0.33
0.2 M TAPSO + 0.01 M phosphate	0.38
0.2 M TAPSO + 0.05 M phosphate	0.62
0.2 M TAPSO + 0.2 M phosphate	1.01
0.2 M TAPSO + 0.2 M arsenate	1.56
0.2 M TAPSO + 0.2 M NaCl	0.29

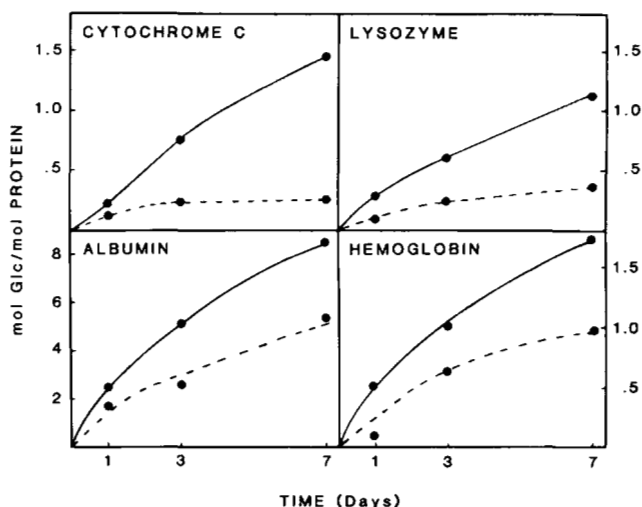


FIG. 4. Kinetics of glycation of various proteins in phosphate versus TAPSO buffer. Incubations and analyses were carried out as described in the legend to Fig. 2.

dependent, with an indication that half-maximal stimulation is achieved at about 50 mM phosphate concentration. This is remarkably close to the dissociation constant for phosphate binding to RNase, estimated as ~40 mM (20). Arsenate, which is anionic and similar in geometry and  $pK_a$  to phosphate, was more effective than phosphate as a catalyst of glycation, but it is not clear whether this results from a difference in binding affinity or the slight, 0.4-unit decrease in the  $pK_a$  of arsenate versus phosphate. In a control experiment, the addition of 0.2 M NaCl, a nonbuffering salt at neutral pH, had no effect on the rate of glycation in TAPSO buffer (Table II).

The experiments described thus far have focused on the analysis of buffer effects on glycation of a model protein, RNase, which has a well-characterized phosphate-binding site. We had expected that the kinetics of glycation of proteins without unique phosphate-binding sites would be relatively insensitive to buffer effects. As shown in Fig. 4, however, whereas the magnitude of buffer effects on glycation varied with different proteins, phosphate stimulated the glycation of all proteins studied. The greatest effects were noted with very basic proteins such as RNase, lysozyme, and cytochrome *c*, while less pronounced effects were seen with more acidic proteins such as albumin and Hb.

The glycation of Hb was studied in greater detail by assessing buffer and ligand effects on the specificity of glycation of amino-terminal valine versus intrachain lysine residues in this protein. Hb was reacted with non-radioactive glucose for 3 days in various buffer systems, and the distribution of glucose between valine and lysine residues was quantitated by reduction with [ $^3$ H]NaBH<sub>4</sub> and measurement of radioac-

tivity in hexitolvaline and hexitollysine fractions. Fig. 5, A–C shows the results obtained on glycation of Hb in 0.2 M TAPSO, 0.2 M phosphate, and 0.2 M phosphate containing 10 mM DPG, respectively. In agreement with kinetic data in Fig. 4, comparison of Fig. 5, A and B, indicates an approximate 2.5-fold increase in glycation of Hb in phosphate versus TAPSO buffer. Although phosphate increased the overall extent of glycation of both valine and lysine residues in the protein, there was also a significant and reproducible increase in the relative extent of glycation of valine residues. In TAPSO the ratio of hexitolvaline to hexitollysine in Hb was 1:16 (8.4% hexitolvaline), whereas in phosphate the ratio was 1:8 (12.6% hexitolvaline). We reasoned that phosphate might exert selective enhancement of valine glycation through its binding in the allosteric, DPG-binding site of Hb, which includes the  $\beta$ -chain amino-terminal valine residues. To study possible effects of the natural ligand, DPG, on glycation of Hb, the protein was also glycated in TAPSO buffer in the presence of DPG. The concentration chosen, 10 mM, was at the upper limit of the physiological range of organic phosphate concentration in the red cell (21). As can be seen in Fig. 5C, even at low concentrations in TAPSO buffer, DPG exerted a significant effect on the kinetics of glycation of both valine and lysine residues in Hb (Fig. 5, C versus A). In addition, the relative extent of glycation of valine residues was even further increased, compared to both the TAPSO and phosphate incubations, *i.e.* hexitolvaline/hexitollysine = 1:5, or 17.1% hexitolvaline. These results are consistent with both a general effect of DPG on the glycation of both valine and lysine residues in Hb, as well as a selective enhancement of the glycation of valine residues. Further studies are now in progress to evaluate the concentration dependence of phosphate and DPG effects on the kinetics and specificity of glycation of both valine and lysine residues in hemoglobin.

## DISCUSSION

Glycation is an early step in a complex series of Maillard of browning reactions which occur between reducing sugars and proteins or other amines (1, 22, 23). Phosphate is known to enhance the overall rate of this reaction, as measured by the kinetics of development of brown color or fluorescence (24, 25). Phosphate also increases the proportion of the open chain, reactive form of reducing sugars in solution (26) and

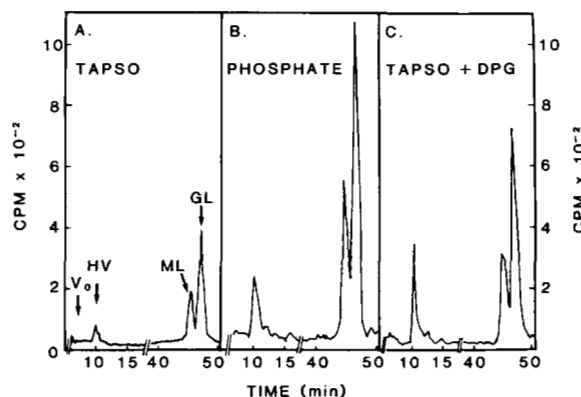


FIG. 5. Specificity of glycation of hemoglobin in various buffer systems. In each case hemoglobin was glycated for 3 days in 0.4 M glucose. After acid treatment and dialysis, proteins (200  $\mu$ g) were reduced with [ $^3$ H]NaBH<sub>4</sub>. Following acid hydrolysis, equal amounts of protein were fractionated on an amino acid analyzer and fractions assayed for radioactivity. A, Hb glycated in 0.2 M TAPSO. B, Hb glycated in 0.2 M phosphate. C, Hb glycated in 0.2 M TAPSO buffer containing 10 mM DPG. HV, hexitolvaline; ML, N<sup>6</sup>-mannitollysine; GL, N<sup>6</sup>-glucitollysine.

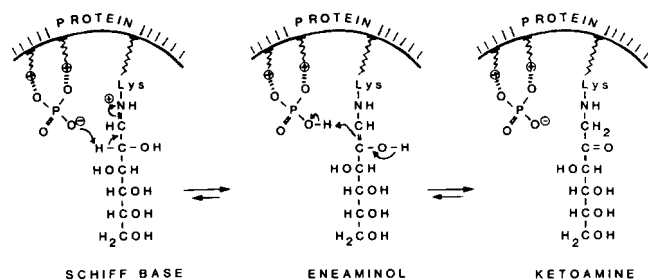


FIG. 6. Reaction pathway for glycation of protein, showing the catalytic effect of phosphate bound in a basic microenvironment in the structure of a protein. The mechanism for catalysis by phosphate is adapted from Refs. 2 and 3. The hypothetical phosphate-binding microenvironment consists of an array of at least 2 basic residues. The binding site may be small, such as a Lys-Lys sequence (including the lysine residue to be glycated), or a larger feature of a protein, such as the active site of RNase or allosteric site of Hb. The effect of phosphate on the specificity of glycation is achieved by its binding and function as a neighboring group catalyst of glycation in specific microenvironments of the protein.

promotes the enolization of sugars in solution (27). Since its  $pK_a$  is near neutral pH, phosphate can also serve as an acid-base catalyst of numerous reactions under physiological conditions, including the Amadori rearrangement. Thus, it was not surprising that this ion, which can affect sugar structure and reactivity, would affect the kinetics of glycation of protein in a concentration-dependent fashion. The unexpected observation in this work was that inorganic and organic phosphate also altered the specificity of modification of proteins by glucose. We conclude that the effectiveness of phosphate as a catalyst of glycation is promoted by its binding to unique, basic microenvironments in proteins (Fig. 6), and that the specificity of phosphate binding affects the specificity of glycation of protein. Thus, preferential glycation of lysines in or near the active site of RNase and of  $\beta$ -chain-terminal valine residues in Hb can be explained by the binding of phosphate in the active and allosteric sites of these proteins, respectively. While we have not shown conclusively at this point that phosphate catalyzes only glycation of the  $\beta$ -chain valines in the allosteric site of Hb, leading to the formation of HbA<sub>1c</sub> (1,5), this is likely, based on analogies between the phosphate-binding sites of RNase and Hb (28). Like the reactive lysines of RNase, the  $\beta$ -chain valines of Hb are located in a basic cleft or cavity in the protein. These regions in RNase and Hb have a functional role in the binding of phosphorylated molecules, *i.e.* the substrate, RNA, or the allosteric effector, DPG, respectively. The effects of phosphate on the specificity of glycation of these proteins suggest that organic phosphate-binding active and allosteric sites may also be reactive sites for glycation of other proteins and enzymes.

Since DPG exerts such a pronounced effect on both the overall kinetics and the specificity of glycation of Hb *in vitro* (Fig. 5, C versus A) the binding of organic phosphates to Hb is probably also significant in determining the rate and pattern of glycation of the protein *in vivo*. Several investigators (29–31) have described strong positive correlations between DPG concentration and the kinetics of formation of HbA<sub>1c</sub> *in vivo* and *in vitro*. Our results support these observations by showing that both phosphate and DPG enhance the rate of glycation of the  $\beta$ -chain valine residues of Hb *in vitro*. Smith *et al.* (29) also noted increased rates of HbA<sub>1c</sub> formation *in vitro* when red cells were deoxygenated, an observation which might now be explained, in part, by the increased binding of DPG to deoxyhemoglobin (15). In addition to effects on glycation of valine, it is apparent in Fig. 5 that phosphate and

DPG also alter the kinetics, and possibly the specificity, of glycation of lysine residues in Hb. Effects on glycation of lysine might result, for example, from weak binding of DPG, as well as phosphate to microenvironments on the surface of hemoglobin. Indeed, the ratio of valine to lysine glycation in the presence of 10 mM DPG (Fig. 5C, 17% valine) is significantly different from that observed in TAPSO buffer alone but closer to that observed for Hb *in vivo* (26% valine) by Bunn *et al.* (32). Our results do not exclude competing or complementary roles for both organic and inorganic phosphate since the concentration of free phosphate in the red cell,  $\sim 0.5$  mM (21), is sufficiently high, *e.g.* relative to the binding constant for phosphate to hemoglobin ( $K_d \sim 1$  mM) (33), that fractional saturation of the DPG-binding site with phosphate is also likely. The effects of phosphates may not be limited to reactions between glucose and Hb, since Acharya *et al.* (34, 35) have shown that glyceraldehyde forms Schiff base adducts equally with Val-1( $\alpha$ ) and Val-1( $\beta$ ) in Hb, whereas Amadori adducts are formed at least 20 times more rapidly at Val-1( $\beta$ ) than Val-1( $\alpha$ ) in phosphate buffer.

Besides amino groups in or near high-affinity phosphate-binding sites in proteins lysine residues in Lys-Lys or other basic sequences have also been identified as reactive site for glycation of Hb (5), albumin (7, 8), and lysozyme.<sup>2</sup> In recent work on the glycation of albumin, which appeared while this paper was in preparation, Iberg and Flückiger (8) noted that lysine residues glycated in albumin were frequently located in such sequences of basic amino acids and suggested that clusters of basic amino acids in protein may act as neighboring group catalysts of the Amadori rearrangement on adjacent lysine residues. Our observations suggest that the catalysis of the Amadori rearrangement in basic microenvironments is more likely to result from the binding of phosphate, bicarbonate, or other buffering ions in body fluids and that the buffering milieu and the protein's structure are equally important in determining the specificity of glycation of proteins *in vivo* and *in vitro*. Once the glycation reaction is completed, the continued binding of anionic buffering ions in the vicinity of the Amadori adduct may also serve to catalyze further browning and cross-linking reactions characteristic of the later stages of the Maillard reaction. Additional studies on the specificity of glycation of proteins in anionic versus cationic buffers may provide insights into the distribution of anion-binding sites on proteins and their possible role in the browning stages of the Maillard reaction.

Finally, as a cautionary note, the observation of buffer-dependent specificity of glycation suggests that proteins glycated in "physiological buffers" *in vitro* may not always be good models for the same proteins glycated *in vivo*. Shapiro *et al.* (5) have reported differences in the pattern of glycation of lysine residues in Hb glycated *in vitro* versus *in vivo*, which might be attributable to differences in their buffer system (Krebs-Ringer phosphate) versus the milieu interieur of the red cell. Our results emphasize that the pattern of glycation of proteins glycated *in vitro* should be compared to that of the naturally glycated protein before drawing conclusions regarding the relevance of glycation *in vitro* to effects on the structural and functional properties of the protein *in vivo*.

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