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Insulin (1 and 0.1 mU/ml) did not affect HbA_{1c} synthesis in vitro. In addition to glucose, galactose and mannose, but not fructose, served as precursors to HbA_{1c}. A good substrate for hexokinase (2-deoxyglucose) and a poor hexokinase substrate (3-O-methylglucose), were better precursors for HbA_{1c} synthesis than glucose, suggesting that enzymatic phosphorylation of glucose is not required for HbA_{1c} synthesis. Autoradiography after erythrocyte incubation with ³²P-phosphate showed incorporation of radioactivity into HbA_{1a1} and A_{1a2}, but not HbA_{1b}, A_{1c}, or A. Acetylated HbA, generated during incubation with acetylsalicylate, migrated anodal to HbA_{1c} and clearly separated from it.

Erythrocytes from patients with insulinopenic diabetes mellitus synthesized HbA_{1c} at the same rate as controls when incubated with identical glucose concentrations. Likewise, the rate of HbA_{1c} synthesis by erythrocytes from patients with cystic fibrosis and congenital spherocytosis paralleled controls. When erythrocytes from cord blood and from HbC and sickle [...]

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IN VITRO STUDY OF REGULATION

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ABSTRACT Factors that influence hemoglobin (Hb)A_{1c} synthesis by intact erythrocytes were studied in vitro. After incubation cells were lysed, and hemoglobins were separated by isoelectric focusing on polyacrylamide slab gels and quantitated by microdensitometry. HbA_{1c} increased with time, glucose concentrations (5–500 mM), and incubation temperature (4°–37°C). Low temperatures allowed prolonged incubations with minimal hemolysis. At 4°C HbA_{1c} increased linearly with time for 6 wk; after incubation at the highest glucose concentration, HbA_{1c} comprised 50% of total hemoglobin.

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when incubated with identical glucose concentrations. Likewise, the rate of HbA_{1c} synthesis by erythrocytes from patients with cystic fibrosis and congenital spherocytosis paralleled controls. When erythrocytes from cord blood and from HbC and sickle cell anemia patients were incubated with elevated concentrations of glucose, fetal Hb, HbC, and sickle Hb decreased, whereas hemoglobins focusing at isoelectric points near those expected for the corresponding glycosylated derivatives appeared in proportionately increased amounts.

INTRODUCTION

The measurement of hemoglobin (Hb)A_{1c}, a glycosylated hemoglobin that occurs in low concentration in normal erythrocytes, is gaining acceptance as an index of the long-term control of diabetes, because its concentration reflects a timed average of plasma glucose levels over the previous 2–4 wk (1–5). Whereas long-term variations in the plasma concentrations of glucose correlate with HbA_{1c} levels, the possible influence of factors other than glucose remains unknown. There has been uncertainty concerning the metabolic route of HbA_{1c} synthesis. On the basis of precursor studies in erythrocyte hemolysates, it was suggested that the synthesis of HbA_{1c} proceeds via the interaction of HbA with glucose-6-phosphate, yielding a HbA-glucose-6-phosphate intermediate, which in turn is dephosphorylated to HbA_{1c} (6, 7). HbA_{1b} was proposed as the intermediate by Stevens et al. (6), whereas Haney and Bunn (7) suggested either HbA_{1a} or HbA_{1b}. However, in a recent review of in vivo and in vitro studies, with erythrocyte hemolysates or hemoglobin solutions, Bunn et al. (8) concluded that the nonenzymatic condensation of glucose itself with the beta chains of HbA is the major route of HbA_{1c} synthesis, a pathway involving Schiff base formation followed by Amadori rearrange-

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ment (9–11). The present investigation was undertaken in intact human erythrocytes in an attempt to distinguish between these two hypotheses, and to develop a model in which the effects of several physiological parameters that may influence the rate of HbA_{1c} formation in vivo could be tested. Erythrocytes from cord blood, and from blood of HbC and sickle anemia patients were also studied to determine whether fetal Hb (HbF)¹ sickle Hb (HbS), and HbC are subject to glycosylation.

METHODS

Incubation of intact erythrocytes in buffered media was followed by separation of hemoglobins by isoelectric focusing at pH 6–8 on polyacrylamide slab gel and quantitation by microdensitometry (12). Blood was drawn from the antecubital vein of volunteers with a 16- or 19-gauge needle and placed into vials that contained citrate as anticoagulant. Erythrocytes were separated by centrifugation for 10 min at 300 g; 0.25-ml aliquots were dispersed into sterile 1.5-ml polyethylene conical vials (Brinkmann Instruments, Inc., Westbury, N. Y.) that contained 0.3 ml of the sterile incubation medium. The vials were capped and placed in horizontal racks for incubation at temperatures indicated in the text, and rotated 90° three times daily.

Systems capable of preserving human erythrocytes for prolonged periods have at least two features in common, low temperatures (4–6°C) and glucose concentrations in excess of 50 mM (13). These conditions were instituted in our first series of experiments because they were reported most appropriate for the preservation of intracellular levels of critical metabolic intermediates, e.g., ATP, NADP, 2,3-diphosphoglycerate; and reduced glutathione (13). Beutler and Wood (14) utilized the criteria of erythrocyte survival after transfusion, to demonstrate that a bicarbonate buffer system that contained 101.4 mM sodium bicarbonate, 14.3 mM disodium carbonate, 1.0 mM adenine, 1.0 mM trisodium phosphate, 0.5 mM mannitol, and 50 mM or higher glucose, (BAGPM) prolonged erythrocyte viability for up to 6 wk. This system was used in all studies shown here. The concentration of glucose and/or other sugars in BAGPM was varied as indicated in the text; the most frequently used concentrations were 50 and 250 mM. Under normal blood bank conditions in a citrate-phosphate-dextrose buffer system the final glucose concentration is approximately 20 mM.

Additions of glucose, other hexoses, insulin, acetylsalicylate, or radiolabeled compounds (New England Nuclear, Boston, Mass.) preceded passage of the media through 0.22 μm Millipore filters (Millipore Corp, Bedford, Mass.) into sterile tubes, which were stored stoppered at 4°C. Five-times recrystallized bovine insulin (25.4 U/mg) was a gift of The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

After incubation, the erythrocytes were separated from the medium by centrifugation, washed with isotonic saline, lysed by addition of 0.2 ml of deionized water with 5 min of gentle shaking, followed by 0.1 ml of 4% KCN. Lipid was extracted by shaking for 5 min with 1 ml carbon tetrachloride. After removal of the nonpolar phase, the hemolysate was diluted with 0.2 ml of a mixture of 1 M phosphate (pH 7.1)

¹Abbreviations used in this paper: BAGPM, bicarbonate buffer system that contained 101.4 mM sodium bicarbonate, 14.3 mM disodium carbonate, 1.0 mM adenine, 1.0 mM trisodium phosphate, 0.5 mM mannitol, and 50 mM or higher glucose; 2DG, 2-deoxyglucose; Hb, hemoglobin(s); HbF, fetal Hb; HbS, sickle Hb; 3MG, 3-O-methylglucose.

and glycerol (2:8) and stored at –14°C until analysis. For isoelectric focusing the final hemoglobin concentration was adjusted spectrophotometrically at 540 nm to obtain an OD of ≅0.2 for aliquots diluted 1:1300 with deionized water.

The extent of sample hemolysis was determined spectrophotometrically. The hemoglobin present in the media and in the isotonic saline wash after incubation was expressed as a percentage of that present in a nonincubated aliquot of erythrocytes (zero time control), which was hemolyzed by freezing and thawing five times.

The effect of pH on HbA_{1c} formation was examined in intact erythrocytes and in hemolysates. BAGPM that contained 250 mM glucose (pH 8.3) was added to the erythrocytes and the samples were titrated to pH 5.0, 6.5, 7.5, 8, and 9.5 by addition of sterile aliquots of 1 N HCl or 1 N NaOH. Half the samples were lysed by repetitive freezing and thawing before all samples were incubated at 30°C. At 12 h, all samples were again titrated to their preincubation pH. After 12- and 24-h incubation, the HbA_{1c} content was determined.

In experiments where the effect of acetylsalicylate was tested, erythrocyte lysates were incubated at 30°C for 4 and 6 h in BAGPM that contained 50 mM and 250 mM glucose, with or without 10 mM acetylsalicylate, pH 7.2. After incubation the lysate was freed of acetylsalicylate by passage through a Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column (15) before further processing as described above.

Hemoglobin analysis. Erythrocyte hemoglobins were separated by polyacrylamide slab gel isoelectric focusing at pH 6–8, as described (12), with the exception that thinner gels (0.75 mm) were focused by constant power application (model 2103, LKB Instruments, Inc., Rockville, Md.) of 7.5 W (maximum voltage, 200 V and maximum current, 200 mA) at 14°C for 2 h. Finally, sharpening of the separate hemoglobin bands was accomplished by application of 30 W for 20 min. The hemoglobins were measured by scanning the slab gels fixed in 12.5% trichloroacetic acid from cathode to anode on a microdensitometer at 560 nm (model 4300, Ortec Inc., E. G. & G., Inc., Oak Ridge, Tenn.).

Reference standard HbA, HbA_{1a}, HbA_{1b}, and HbA_{1c} were prepared from pooled blood samples obtained from consenting diabetic patients. Hemoglobins were isolated from washed, lysed, and cyanide-treated erythrocytes by chromatography, using the Trivelli et al. (16) modification of the Schnek and Schroeder method (17). Details of preparation have been described elsewhere (12). Hemoglobins were quantitated spectrophotometrically at 540 nm with Drabkin's (18) solutions and cyanide-treated hemoglobin (Hemoglobin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) as standards. Commercial preparations of cyanide-treated HbF and HbS were obtained from Helena Laboratories (Hemocontrols Div., Beaumont, Tex.).

Autoradiographic analysis of the distribution and quantity of ³²P in erythrocyte hemoglobin was accomplished after isoelectric focusing by drying the slab gels on medium gauge filter paper (3M Whatman Inc., Clifton, N. J.) in a Bio-Rad gel dryer (model 224, Bio-Rad Laboratories, Richmond, Calif.). The labeled gel was taped to Chromex-4 film (Dupont Instruments, Wilmington, Del.), keeping both firmly approximated by placement between two large sheets of filter paper in a large x-ray cassette. After appropriate exposure (1–3 wk) the film was developed and scanned on the microdensitometer.

Statistical analysis. Mean ± SEM are indicated. Differences between means were evaluated by Student's *t* test.

RESULTS

Incubation time and glucose concentration (Fig. 1). Intact erythrocytes from adult volunteers contained

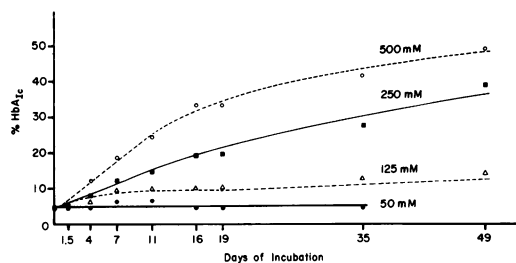


FIGURE 1 HbA_{1c} synthesis by erythrocytes in vitro is dependent on time and glucose concentration. Erythrocytes from a healthy adult male volunteer were incubated in BAGPM at 4°C. HbA_{1c} is expressed as percentage of total hemoglobin in the erythrocytes after incubation. Data points are means of three to four observations. Hemolysis was <10% after prolonged incubations.

increasing concentrations of HbA_{1c} with increasing incubation time at 4°C, provided that glucose in the medium exceeded 50 mM. Increasing glucose in the media above this level accelerated the accumulation of HbA_{1c}. At the higher glucose concentrations there was <10% hemolysis after 49-d incubation. Prolonged erythrocyte incubations at 4°C in media that contained <50 mM glucose resulted in excessive hemolysis.

When fresh, unincubated erythrocytes were examined by polyacrylamide gel isoelectric focusing, they contained <1% HbA_{1b}. "Fast" hemoglobins other than HbA_{1c} were only detected by isoelectric focusing after chromatographic fractions from pooled blood samples were highly concentrated (12). However, when erythrocytes were incubated in media that contained 250 or 500 mM glucose, fast hemoglobins other than HbA_{1c} became gradually detectable. After 3-wk incubation at 4°C with 500 mM glucose, erythrocytes contained hemoglobins migrating anodal to HbA_{1c}; (\approx 7% in the position of HbA_{1b} and 5% in that of HbA_{1a1} and HbA_{1a2}), whereas \approx 50% of the total hemoglobin was present as HbA_{1c}.

Temperature (Fig. 2). The rate of HbA_{1c} formation increased directly with increasing incubation temperature. In most studies incubations at 30°C were terminated after 1 d, to minimize erythrocyte hemolysis, which was <10% in 24 h, but increased rapidly thereafter.

The effect of pH and dialysis. No significant difference in HbA_{1c} content (22–24% of total hemoglobin after 24-h incubation) was detectable over the pH 6.5–9.5 range. After incubation at pH 5.0 HbA_{1c} accumulation decreased to $8.8 \pm 0.3\%$ in 24 h ($n = 3$). The rate of HbA_{1c} formation by erythrocyte hemolysates approximated that of intact cells under the same conditions.

Because previous work suggested that glucose-mediated glycosylation of HbA proceeded at a barely detectable rate in vitro (6), we tested the hypothesis

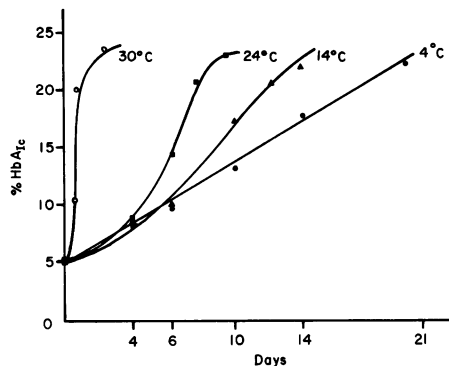


FIGURE 2 Temperature dependence of HbA_{1c} synthesis. Erythrocytes from a healthy adult male volunteer were incubated in BAGPM containing 250 mM glucose. HbA_{1c} is expressed as percentage of total erythrocytes hemoglobin after incubation. Data points are means of three to four observations. Hemolysis increased with increasing temperature, e.g. incubation at 30°C resulted in 10% hemolysis in 36 h.

that a dialyzable factor that promotes HbA_{1c} synthesis was present in our hemolysates. After 18 h dialysis at 4°C against 0.1 M phosphate buffer, pH 7.2, that contained 2% KCN, hemolysates were incubated for 24 h at 30°C in BAGPM that contained 250 mM glucose. The mean HbA_{1c} content of three dialyzed and three nondialyzed hemolysates was not significantly different; $23.3 \pm 1.9\%$ (SEM) and $27.6 \pm 1.0\%$, respectively.

Hexoses as substrates of HbA_{1c} synthesis (Table I). Erythrocytes incubated at 4°C with the aldohexoses, mannose and galactose (each 50 mM), contained slightly more HbA_{1c} than erythrocytes incubated with equimolar glucose. In contrast, the ketohexose, fructose, was not a substrate for HbA_{1c} synthesis at 4°C. To examine the effect of higher concentrations of these sugars, erythrocytes incubated in media that contained 50 mM glucose augmented by either 200 mM mannose, galactose, or fructose, were compared to cells incubated with 250 mM glucose alone. Mannose and galactose again substituted well for glucose as HbA_{1c} substrates. Galactose increased HbA_{1c} content markedly after 4-d incubation. The late decline in HbA_{1c} was not explained by hemolysis alone; it may indicate the formation of derivatives of the adduct that do not co-electrofocus with HbA_{1c}; (for references see 19). The HbA_{1c} content of erythrocytes incubated with 50 mM glucose + 200 mM fructose was no greater than that observed with 50 mM glucose alone.

Effect of insulin, diabetes, and cystic fibrosis. Erythrocytes from nondiabetic adult donors were incubated in BAGPM that contained either 0.1 mU/ml or 1.0 mU/ml insulin and 250 mM glucose. Neither the high physiological nor the pharmacological dose of insulin affected the rate of HbA_{1c} synthesis at either 30°C or 4°C (Table II).

TABLE I
HbA_{1c} in Intact Erythrocytes Incubated with Galactose, Mannose, or Fructose*

Glucose	Other hexoses	Days incubated . . .	HbA _{1c} †					Hemolysis‡					
			0	4	9	14	21	0	4	9	14	21	
mM	mM		%					%					
50	0		4.78	7.3	7.4	7.8	7.5	<1					
0	Mannose, 50		±0.16	9.0	10.0	11.1	12.0	1	4	3	7		
0	Galactose, 50			11.2	11.7	12.4	13.2	2	4	4	7		
0	Fructose, 50			5.2	2.2	3.6	3.0	1	2	2	4		
250	0			10.8	12.5	16.2	20.5	1	1	1	7		
50	Mannose, 200			11.9	14.0	16.4	16.0	2	2	3	18		
50	Galactose, 200			18.4	13.3	8.1	7.0	2	2	4	19		
50	Fructose, 200			8.3	5.7	6.4	4.8	1	1	1	1		

* Erythrocytes were incubated in BAGPM at 4°C with the additions indicated.

† Means of three to four determinations are tabulated and expressed as the percentage of HbA + HbA_{1c}. Values above 7.0 are significantly greater ($P < 0.05$) than preincubation values (zero time).

‡ Hemolysis was measured as the sum of Hb released into the medium and wash, expressed as percentage of Hb present in the cells at the beginning of incubation.

Fig. 3 illustrates the in vitro HbA_{1c} synthesis by erythrocytes from six healthy adults and six adults with juvenile-onset diabetes, whose 3- to 4-h postprandial plasma glucose at the time of sampling was 150–250

mg/100 ml. Erythrocytes from diabetic patients contained significantly more HbA_{1c} than the controls, but no difference in the rate of HbA_{1c} synthesis was detected between the two groups during incubation with 50 or 250 mM glucose.

Paulsen and Koury (20) recently reported mild elevations of HbA_{1c} in patients with cystic fibrosis and apparently normal blood glucose levels. We studied three children with this condition; they had mildly elevated HbA_{1c} levels (mean 7.33 ± 0.42 (SEM) vs. 4.73 ± 0.23 in 39 normal controls) but without clinically apparent diabetes as judged by lack of glycosuria and normal 2- to 4-h postprandial plasma glucose levels. Glucose tolerance tests were not performed. Because it was pos-

TABLE II
Insulin Fails to Affect HbA_{1c} Synthesis
by Erythrocytes In Vitro*

Incubation		Insulin	HbA _{1c} †
Temperature	Time		
°C		mU/ml	%
—	0	0	5.2±0.21
30	9 h	0	15.1±0.41
		0.1	16.6±1.88
		1.0	14.8±0.20
30	18 h	0	22.3±0.77
		0.1	22.5±2.75
		1.0	19.8±0.89
4	4 d	0	10.7±1.13
		0.1	11.5±0.80
		1.0	11.6±0.43
4	14 d	0	16.3±1.41
		0.1	17.6±0.34
		1.0	17.0±0.86
4	21 d	0	24.2±1.47
		0.1	23.4±0.55
		1.0	22.7±1.25

* Erythrocytes from two healthy adult males were incubated in BAGPM that contained 250 mM glucose.

† HbA_{1c} content is expressed as percentage of total hemoglobin in erythrocytes after incubation. Means of three to four observations ± SEM are tabulated.

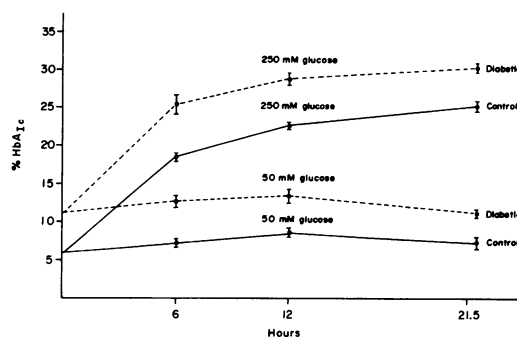


FIGURE 3 Comparison of HbA_{1c} synthesis by erythrocytes from six healthy adult volunteers and six adults with insulinopenic diabetes. Erythrocytes were incubated at 30°C in BAGPM containing 50 or 250 mM glucose. HbA_{1c} is expressed as percentage of total red cell hemoglobin at the end of incubation. Data points are means ± SEM of triplicate observations in six individuals.

sible that the elevation of HbA_{1c} in these patients represented an inherent abnormality of the erythrocyte, we studied their HbA_{1c} synthesis in vitro. Fig. 4 compares HbA_{1c} synthesis by erythrocytes from three patients with cystic fibrosis, three nonaffected first-degree relatives and one patient with hereditary spherocytosis. No differences were detected between these groups; indeed, their rate of HbA_{1c} synthesis was essentially identical to that of the previously studied normal volunteers (Fig. 4).

Incubation with nonutilizable hexoses (Fig. 5). 3-O-methylglucose (3MG) and 2-deoxyglucose (2DG) are hexoses that compete with glucose for transport into erythrocytes, but neither is appreciably metabolized. Whereas 2DG is phosphorylated by hexokinase, 3MG is not; consequently, phosphorylated 2DG and non-phosphorylated 3MG accumulate within the erythrocyte (21). 3MG, like 2DG, served better than glucose as the sole carbohydrate source for HbA_{1c} synthesis, indicating that phosphorylation by hexokinase is not a prerequisite for HbA_{1c} synthesis. The rate of HbA_{1c} synthesis in the presence of 3MG and 2DG was comparable to that observed with fivefold greater concentrations of glucose.

[³²P]Orthophosphate labeling of hemoglobins (Fig. 6). After 12-h incubation at 30°C, in media that contained 250 mM glucose and 10 μCi/ml [³²P]orthophosphate, erythrocytes contained radioactivity, which, on isoelectric focusing, migrated to positions coincident with two hemoglobin bands, both of which were considerably anodal to HbA_{1b}. The identity of the bands shown in Fig. 6 was established by parallel isoelectric focusing of standards prepared by column chromatography (12). In accordance with their isoelectric focusing

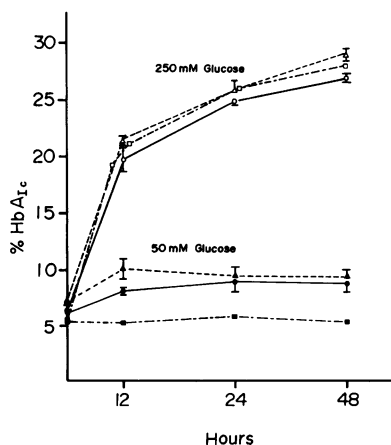


FIGURE 4 HbA_{1c} synthesis by erythrocytes from three patients with cystic fibrosis (Δ), three first-degree relatives without cystic fibrosis (○) and one patient with hereditary spherocytosis (□). Erythrocytes were incubated at 30°C in BAGPM that contained 50 or 250 mM glucose. All determinations were made in triplicate. Means ± SEM are shown.

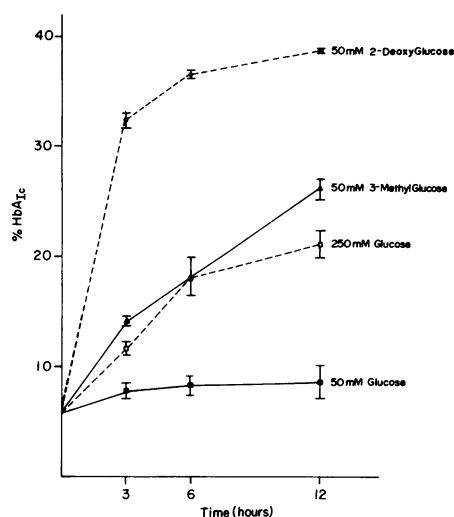


FIGURE 5 Nonmetabolizable hexoses as substrates of hemoglobin glycosylation. Erythrocytes were incubated at 30°C in BAGPM with either 50 mM 3MG (▲), 50 mM 2-deoxyglucose (●), 50 mM glucose (■), or 250 mM glucose (□). Data points are means of three observations ± SEM. HbA_{1c} is expressed as percentage of total hemoglobin in erythrocytes after incubation.

positions, the two ³²P-containing bands are designated as HbA_{1a1} and HbA_{1a2} (22). No significant ³²P radioactivity was incorporated into HbA, HbA_{1c}, or HbA_{1b}, suggesting that only HbA_{1a} forms significant amounts of adducts with phosphorylated glucose in intact erythrocytes in vitro.

Glycosylation of HbF, HbS, and HbC. HbA_{1c} is a product of the glycosylation of the amino-terminal valine of the β-chain of HbA. HbS and HbC have the same terminal amino acid sequences as HbA and may therefore be expected to be susceptible to glycosyla-

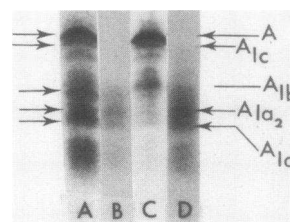


FIGURE 6 [³²P]orthophosphate incorporation into minor hemoglobins. Erythrocytes were incubated for 12 h at 30°C in BAGPM that contained 250 mM glucose and 5 μCi/ml [³²P]orthophosphate. After incubation, erythrocyte hemoglobins were separated by isoelectric focusing on polyacrylamide slab gels. Autoradiographs of the gels were prepared as described in Methods. A, unstained gel with superimposed radiograph, after 8-d exposure to the gel. B, radiograph alone after 5-d exposure to gel. C, unstained gel alone. D, radiograph alone after 8-d exposure to gel. The location of the minor hemoglobins was identified by isoelectric focusing of standards.

tion. The gamma chain of the major HbF terminates in glycine, the side group of which, an uncharged hydrogen atom constitutes less steric bulk than the isopropyl side chain of valine. A minor HbF, HbF₁, has the same amino acid sequence as HbF, except that the amino-terminal positive charge of the β -chain is neutralized by an acetyl group (23). Separation of HbF₁ from HbF and HbA by isoelectric focusing on polyacrylamide slab gels has been recently reported (24).

Fig. 7 illustrates microdensitometry tracings of cord blood and S-C hemoglobins separated by isoelectric focusing before and after incubation. The tracing from cord blood erythrocytes (Fig. 7A) shows $\cong 90\%$ of HbF, and relatively small amounts of HbA and HbF₁ before incubation. After 12-h incubation in 250 mM glucose (Fig. 7B), HbF decreased considerably and a hemoglobin that focused at an isoelectric point similar to that of HbF₁ increased correspondingly. Glycosylated HbF, having the same net charge and amino acid sequence as acetylated HbF (HbF₁) may coelectrofocus with it, and may cause the enhancement of this band seen after incubation.

Fig. 7C shows a hemoglobin tracing prepared before incubation from erythrocytes of a HbS-C patient. HbS and HbC, in nearly equal proportions, comprised $>95\%$ of the total hemoglobin. After 12-h incubation in 250 mM glucose (Fig. 7D), minor bands appeared anodal to both HbS and HbC, in positions similar to

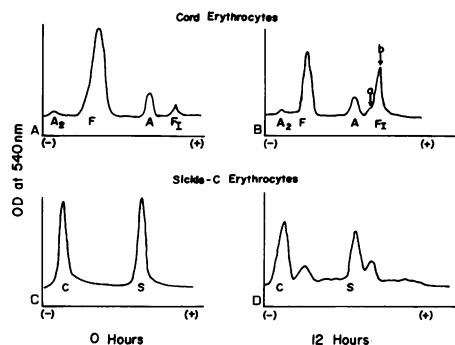


FIGURE 7 Apparent glycosylation of HbF, HbS and HbC. Erythrocytes were incubated for 12 h at 30°C in BAGPM that contained 250 mM glucose. Hemoglobins were separated by isoelectric focusing on polyacrylamide slab gels before incubation (A and C) and after incubation (B and D); densitometric tracings of the gels are shown. The upper portion of the graph shows erythrocytes obtained from cord blood. HbF decreased during incubation, whereas a hemoglobin electrofocusing in the region of HbF₁ increased. a, indicates a small shoulder on the postincubation peak b, suggesting superimposition of HbF₁ (acetylated) on glycosylated HbF. Because the concentration of HbA was very low, newly formed HbA_{1c} may not be detectable; it could however be included in "a." Incubation of erythrocytes from a patient with HbS-C disease is illustrated in the lower portion of the graph. Note the new peaks developing during incubation, which are anodal to HbS and HbC, respectively.

that of HbA_{1c} in its relation to HbA. These two newly increased bands may represent glycosylated HbS and HbC. Apparent glycosylation of HbS and HbC was also observed when erythrocytes from a patient with sickle cell anemia and from a CC homozygote were incubated with 250 mM glucose (data not shown).

Bridges et al. (15) reported that acetylsalicylate causes acetylation of HbA in vivo and in vitro, giving rise to a "fast" hemoglobin. The question arose whether or not treatment with acetylsalicylate may result in spurious elevations of HbA_{1c} estimates in diabetic patients when an isoelectric focusing technique (12) is used for quantitation. Fig. 8 shows that a fast hemoglobin that migrated anodal to HbA_{1c} increased during incubation of hemolysates with 10 mM acetylsalicylate. The presence of acetylated hemoglobin did not interfere with the quantitation of HbA_{1c} by microdensitometry. On the other hand, acetylated hemoglobin focused at or near the isoelectric point of HbA_{1b} and was not separated from the latter.

DISCUSSION

The data presented support the view of the direct, nonenzymatic interaction of glucose with the β -chain of HbA yielding HbA_{1c}. Several observations strengthen this hypothesis. Hexose phosphatase activity in erythrocytes is absent or very low (25, 26). If either HbA_{1a} or HbA_{1b} were phosphorylated intermediates in A_{1c} synthesis, then their dephosphorylation to HbA_{1c} could be rate-limiting, and the intermediate would be expected to accumulate in the course of accelerated HbA_{1c} synthesis. In experiments involving incubation of intact erythrocytes at 4°C with high concentrations of glucose HbA_{1a} and HbA_{1b} did not accumulate in significant quantities for at least 2 wk, at which time HbA_{1c} levels had increased fivefold.

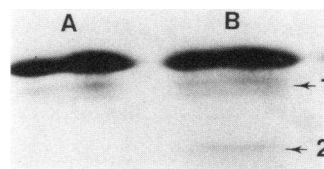


FIGURE 8 Isoelectric focusing of acetylated HbA. Erythrocytes were incubated for 4 h in BAGPM that contained 50 mM glucose alone (A) and 50 mM glucose + 10 mM acetylsalicylate (B). The heavy band is HbA. 1, HbA_{1c}. A doublet HbA_{1c} band is often seen after high-voltage zone sharpening; it may represent a mixture of HbA with both β -chains and only one β -chain glycosylated. 2, band representing acetylated HbA; the latter is well separated from HbA_{1c} but had a similar isoelectric point to HbA_{1b}. After 4-h incubation followed by column chromatography (see Methods) Hb in region 2 was $2.9 \pm 0.10\%$ (SEM, $n = 3$) of total hemoglobin in samples incubated without salicylate and 7.6 ± 0.26 after incubation with salicylate.

Similarly, freshly obtained erythrocytes from diabetic patients, with 15% or more HbA_{1c}, contained <1% HbA_{1a} and HbA_{1b} on isoelectric focusing (12).

If glucose-6-phosphate was a substrate for HbA_{1c} synthesis, then hexokinase, which is present in high concentration in mammalian erythrocytes, would be expected to catalyze it. 3MG is not phosphorylated by mammalian hexokinase (21); yet it was approximately five times more effective than glucose as a precursor for HbA_{1c} synthesis in vitro. Thus, hexose phosphorylation does not seem to be required for HbA_{1c} formation by intact erythrocytes.

It is noteworthy that incubation of intact erythrocytes with [³²P]orthophosphate revealed incorporation of radioactivity only into bands migrating with the characteristics of HbA_{1a1} and HbA_{1a2} but not HbA_{1b}, HbA or HbA_{1c}. Recently, Bunn et al. (8) reported similar results, using different techniques, namely that only HbA_{1a1} and HbA_{1a2} but not HbA_{1b} contained a phosphorylated hexose (22) the phosphate content per $\alpha\beta$ -dimer being two in HbA_{1a1}, the chromatographically faster component and one in HbA_{1a2}. When the two ³²P-labeled subfractions of HbA_{1a} were separated by isoelectric focusing in the present study, the component with the higher isoelectric point had apparently incorporated more [³²P]phosphate than the one with the lower isoelectric point (Fig. 6). Krishnamoorthy et al. (27) found no evidence of either glucose or phosphate residues in a highly purified fraction of HbA_{1b}.

Our data obtained with intact erythrocytes are in agreement with recent observations using hemolysates and purified hemoglobin A solutions, reporting greater "minor hemoglobin" formation during incubations with mannose or galactose, than with glucose (28). In the same study fructose was found to be as effective a precursor of HbA_{1c} as glucose; a finding not supported by the present data. The reason for the discrepancy is not clear. Our observations were made during incubation at 4°C. It is possible that 42-h incubation at 37°C (28) allows some fructose to be converted into other intermediates which interact with HbA, or fructose transport into the erythrocyte may be rate limiting.

In experiments where the formation of adducts between sugars and hemoglobin was measured during incubation with high sugar concentrations, the effect of glycolysis on the extra and intracellular sugar concentration was presumably negligible. The rate of penetration of the sugars into the erythrocyte and the extent to which they exist in the open vs. the ring (hemiacetal) structure may be important determinants of the rate of adduct formation between the sugars and hemoglobin. The sugars that were found to be good precursors of HbA_{1c} in this study are readily transported into the erythrocyte (21, 29). Fructose has a low affinity for the sugar transport system. There was no

obvious correlation between the affinity of individual aldohexoses for the glucose carrier system and the rates of hemoglobin glycosylation. Because the glycosylation of hemoglobin by glucose proceeded at the same rate in intact erythrocytes and hemolysates it appears that under the experimental conditions used (e.g., aldohexose concentrations exceeding the K_m for transport and prolonged incubation times), transport was not an important determinant of HbA_{1c} formation.

On the other hand, there may be a correlation between the percentage of the sugar that exists in the aldehyde form (30, 31) and the rate of hemoglobin glycosylation. Of the monosaccharides studied in both systems the highest percentage of the open structure was found in D-ribose, followed by galactose, mannose and glucose in decreasing order. D-ribose yielded the highest rate of hemoglobin glycosylation among the sugars studied by Dolhofer and Wieland (28) with erythrocyte lysates.

In the present study, a pH range between 6.5 and 9.5 did not affect the rate of HbA_{1c} synthesis in vitro, indicating that in vivo pH changes such as occur in acidosis are unlikely to influence this process. pH dependency of in vitro HbA_{1c} synthesis with purified HbA has been reported (32).

Our studies concerning the possible in vitro effect of insulin on A_{1c} synthesis were stimulated by reports suggesting that, contrary to common belief, insulin may affect the metabolism of the human erythrocyte. Thus, an insulin effect on the efflux of glucose from erythrocytes has been reported (33), as well as the presence of highly specific insulin receptors on the erythrocyte membrane (34). We did not detect an effect of insulin or diabetes on HbA_{1c} synthesis by intact erythrocytes. The latter experiments were prompted by suggestions that the metabolic milieu in the erythrocyte of the diabetic may be more favorable for HbA_{1c} synthesis than that of controls (6); however, we have not tested erythrocytes from diabetic patients in ketoacidosis.

Studies concerning in vitro synthesis of HbA_{1c} by erythrocytes of patients with cystic fibrosis were stimulated by a report suggesting that mild elevations of HbA_{1c} occurred in these patients, before the onset of diabetes (20). The three children with cystic fibrosis without clinical diabetes that we studied had essentially identical HbA_{1c} levels as the mean reported by Paulsen for this group (20). Because in vitro, erythrocytes from cystic fibrosis patients synthesized HbA_{1c} at the same rate as controls, it seems likely that the mild elevations of HbA_{1c} observed could have been caused by undetected, intermittent hyperglycemia, prodromal to developing clinical diabetes mellitus.

Graf and Porter (35) reported that erythrocytes of patients with mildly elevated glucose concentrations glycosylate proportionately more HbA than erythrocytes

exposed to marked elevations of glucose, suggesting that HbA_{1c} synthesis is not linearly related to serum glucose levels in the physiologic range in vivo. An experimental model using intact erythrocytes incubated with [¹⁴C]glucose at physiological concentrations is being developed to assess the validity of this hypothesis in vitro. Calculating the ratio of the specific activity of HbA_{1c} to that of HbA during incubation with [¹⁴C]glucose seems useful in correcting for the background activity introduced by nonspecific interaction between glucose and various sites on the hemoglobin molecule (8, 32). Whereas we detected no difference in the rate of HbA_{1c} formation in vitro between hemolysates and intact cells using very high concentrations of glucose, such differences could become apparent during incubation with glucose in the physiological range.

Abdella et al. (36) reported the binding of various reducing mono- and disaccharides to HbS. The stereochemical affinity of HbS for sugars is similar to that of HbA. As in the present study fructose is a poor precursor, whereas sucrose, lactose, galactose, mannose, and glucose (listed in decreasing order of affinity) are excellent substrates (36). Glycosylation significantly increased the minimum gelling concentration of the deoxy-conformation, raising the question whether the clinical manifestations of sickling may be less common in diabetics with homozygous S genes (36).

To our knowledge, there is no previous report of glycosylation of HbC and HbF. The latter could have functional implications, because glycosylation decreases the reactivity of hemoglobins to 2,3-diphosphoglycerate, resulting in increased affinity for oxygen (37). Thus glycosylation of HbF in the infant of the diabetic mother could promote O₂ delivery from maternal to fetal blood, but at the same time reduce the delivery of O₂ to the fetal tissues. Whether or not glycosylation of HbF indeed affects the oxygenation of fetal tissues under in vivo conditions, possibly playing a role in the neonatal complications that occur in infants of diabetic mothers, needs further study. HbF is known to have less affinity for 2,3-diphosphoglycerate than HbA (37).

By using isoelectric focusing and microdensitometry for quantitation of HbF and its putative glycosylated derivative, we could not separate glycosylated HbF from HbF_i, the acetylated derivative of HbF. Whether or not the infant of the diabetic mother has increased amounts of acetylated or glycosylated HbF, and whether or not the synthesis of either or both correlates with the maternal control of diabetes needs further investigation.

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