Immediate Elimination of Labile HbA_{1c} With Allosteric Effectors of Hemoglobin

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Our aim was to find a simple method of removing labile glycosylated hemoglobin (HbA_{1c}) from blood samples before it is measured by cation-exchange chromatography. Labile HbA_{1c} is formed by the binding of glucose to the NH_2-terminal valine of the β-chain of HbA. We sought a more competitive binder for the same site to dissociate labile HbA_{1c} to glucose and HbA. Inorganic phosphates were found to have a strong allosteric effect and a great ability to eliminate labile HbA_{1c}. We developed our method with 4 mM tetrapolyphosphate in the hemolyzing solution to eliminate labile HbA_{1c} during the automatic processing (at pH 6 and heated for 2 min at 45°C) of blood samples for HbA_{1c} estimation. This may be useful when estimating HbA_{1c} by the manual method. *Diabetes* 39:17–21, 1990

Formation of glycosylated hemoglobin (HbA_{1c}) is a two-stage nonenzymatic process (1–5). Initially, a readily reversible reaction takes place between glucose and the NH_2-terminal valine of the β-chain of HbA to form an aldimine or Schiff base (labile HbA_{1c}). This labile HbA_{1c} then either undergoes an irreversible molecular rearrangement (Amadori rearrangement) to form stable HbA_{1c} or dissociates back to glucose and HbA. The level of labile HbA_{1c} fluctuates simultaneously with blood glucose (6–10). There are many methods of estimating HbA_{1c}. Although some measure only stable HbA_{1c} and some separate out labile HbA_{1c}, most of these methods are impractical for routine use (11–14). Cation-exchange chromatography is the most commonly used laboratory method. Because it measures total HbA_{1c}, pretreatment of the sample to remove labile HbA_{1c} is required to avoid false high results (15–20). Previous methods for removing labile HbA_{1c} are lengthy and laborious (21–26), and incubation at pH 5 results in Hb instability (25,27–29).

The NH_2-terminal valine of β-chain that binds glucose is one of seven amino acids that form an allosteric binding site for 2,3-diphosphoglycerate (2,3-DPG; 30). To remove labile HbA_{1c}, we attempted to find another allosteric effector that could successfully compete with glucose at the NH_2-terminal valine and thus dissociate labile HbA_{1c}. We estimated the relative potency of the allosteric effectors by measuring their effect on the O_2-Hb dissociation curve.

RESEARCH DESIGN AND METHODS

All polyphosphates were obtained from Taihei Chemical (Osaka, Japan). All other reagents were of high analytical grade.

HbA_{1c} was measured with an automated HbA_{1c} analyzer (high-performance liquid chromatography [HPLC]; Hi-Auto A_{1c}, type 8121, Kyoto Daiichi Kagaku, Kyoto, Japan). This machine is an improved model of one described earlier (31), taking only 4 min/sample and having a sample loop surrounded by an electric thermal jacket where the specimen can be heated for 2 min at constant temperature.

With freshly prepared deionized Hb fractions, the O_2-Hb equilibria were determined with a TCS Hemox Analyzer (Southampton, PA) at 25°C with 15.5 μM Hb in 50 mM bis-tris buffer, pH 6, with 0.1 M NaCl and 62 μM of allosteric effector (with an additional 100 mM orthophosphate).

Heparinized blood (5 ml) from a healthy subject was incubated with 0.1 ml of 500 mg/ml glucose at 37°C for 3 h to generate a high concentration of labile HbA_{1c}. Erythrocytes were washed three times with isotonic saline and suspended in 1 vol saline. All allosteric effectors (10 mM each) were dissolved in 50 mM bis-tris buffer, pH 6, with 0.1% Triton X-100 (with an additional 100 mM orthophosphate). pH was readjusted to 6 with 1 N HCl or 1 N KOH. The solutes were

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serially diluted with the same buffer to make the hemolyzing solution. Erythrocytes (3 μl) were mixed with 450 μl of various kinds of hemolyzing solutions. The mixture was incubated in a water bath at 45°C for 10 min and then placed on the Auto-Sampler of the HbA_{1c} analyzer, from which the thermal jacket was detached. In this part of the study, bis-tris buffer was used instead of phosphate buffer, which has an allosteric effect on Hb (30,32).

Once we had established tetrapolyphosphate (TPP) to be very effective, we reevaluated it under two conditions: first, at room temperature without water-bath incubation or the thermal jacket, and second, with the thermal jacket at 45°C, also without water-bath incubation.

Bis-tris buffer is too expensive for routine use, so we refined our method with a phosphate buffer. Two kinds of hemolyzing solutions were prepared: an 8-mM phosphate buffer, pH 7.4, with 0.1% Triton X-100 (solution T) to measure total HbA_{1c}, and a 4-mM phosphate buffer, pH 6, with 4 mM TPP and 0.1% Triton X-100 (solution S) to measure stable HbA_{1c}. These were tested at varying temperatures and pH to establish the optimum conditions. Total Hb concentration was estimated by absorbance at 415 nm. The highest absorbance was taken as the baseline reading of 100% in each series. The absorption spectrum of Hb was measured by spectrophotometer in solutions T and S before and after incubation at 45°C for 10 min.

We compared our method with four other methods, namely those of Goldstein et al. (21), Nathan et al. (22), Mullins and Austin (25), and Edmonds and John (26). Intra-assay coefficients of variation were obtained for samples from healthy and diabetic subjects.

Fasting venous blood was then taken from three groups of subjects (healthy, diabetic, or those with impaired glucose tolerance [IGT]) classified according to WHO criteria (33). Total HbA_{1c} was measured with solution T and stable HbA_{1c} with solution S. Labile HbA_{1c} was calculated by subtraction of stable from total HbA_{1c} for each sample.

RESULTS

We determined the patterns of O_2-Hb equilibria for the allosteric effectors investigated (Fig. 1). Chromatograms of the Hb results and HbA_{1c} values are shown in Fig. 2. P_{50} (Po_2 at 50% O_2 saturation) in the presence of allosteric effectors and the minimum concentrations of allosteric effectors that would remove labile HbA_{1c} are presented in Table 1. The stronger the allosteric effect, the greater the ability to remove labile HbA_{1c} in the inorganic phosphates. We chose TPP because it is stable and safe. Minimum effective concentrations of TPP without heating and with 2 min of heating at 45°C with the thermal jacket were 6 and 0.4 mM, respectively.

Stability of the Hb and the elimination of labile HbA_{1c} at different temperatures and pH are shown in Fig. 3. Hb denaturation was observed >50°C, and below pH 5.7, Hb was destroyed so as to lose absorbance at 415 nm, as reported previously (25,27–29). There was no significant change in the Hb absorption spectrum between solutions T and S before and after incubation at 45°C for 10 min. Heating at 45°C, pH 6, for 2 min provides the optimum condition for labile HbA_{1c} removal with 4 mM TPP.

Chromatograms of HbA_{1c} from erythrocytes pretreated by various incubation methods and the HbA_{1c} values are shown in Fig. 4. Incubation at pH 5 for 30 min (required in the methods of Nathan et al. [22] and Mullins and Austin [25]) resulted in methemoglobin formation, although methemo-

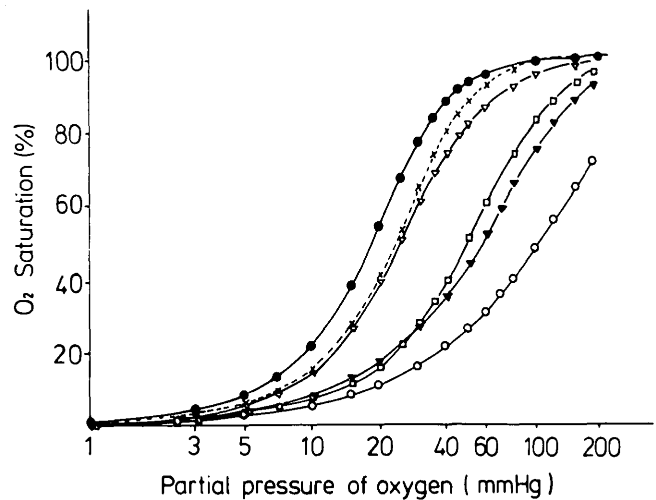

FIG. 1. O_2-equilibrium curves of Hb (15.5 μM) in presence of various allosteric effectors (62 μM) at 25°C in 50 mM bis-tris buffer, pH 6, containing 0.1 m NaCl. ●, Control; X, diphosphoglycerate; ▽, tripolyphosphate; □, tetrapolyphosphate; ▼, hexametaphosphate; ○, inositol hexaphosphate.

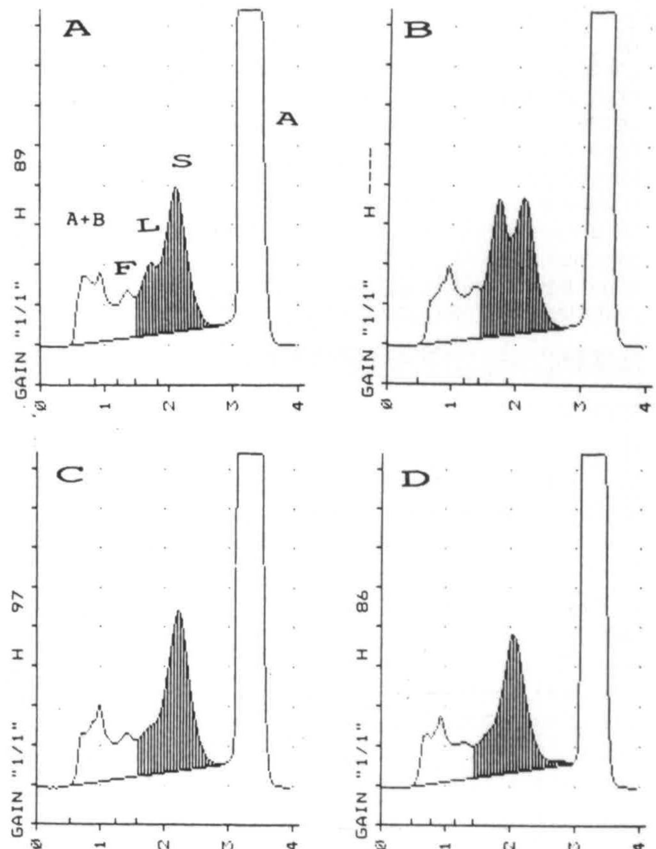

FIG. 2. Chromatograms of Hb and HbA_{1c} values. *A*: fresh heparinized blood (5.6%). *B*: control (7.35%); erythrocytes with in vitro–generated labile HbA_{1c}, incubated with 50 mM bis-tris buffer, pH 6, containing 0.1% Triton X-100 at 45°C for 10 min. *C*: tetrapolyphosphate (4.83%); same as *B* except buffer contained 0.02 mM tetrapolyphosphate. *D*: inositol hexaphosphate (4.97%); same as *B* except buffer contained 2 mM inositol hexaphosphate. A, HbA; S, stable HbA_{1c}; L, labile HbA_{1c}; F, HbF; A + B, HbA_{1a+b}; *shaded areas*, HbA_{1c} fraction.

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TABLE 1
P_{50} of Hb in presence of 62-μM allosteric effectors and minimum concentrations of allosteric effectors capable of removing labile HbA_{1c}

	P_{50} (mmHg)*	Minimum concentration (mM)†
Ultrapolyphosphate	32.3	0.4
Hexamethaphosphate	58.6	0.04
Tetrapolyphosphate	49.0	0.02
Tripolyphosphate	24.6	0.2
Pyrophosphate	20.5	0.4
Orthophosphate	18.6	60
Additional 100 mM	22.2	
2,3-Diphosphoglycerate	23.6	8
Inositol hexaphosphate	104.0	2
ATP	24.0	6
Control	18.3	0

*P_{50}, Po_2 at 50% O_2 saturations.
†Samples were exposed to allosteric effectors for 10 min at 45°C.

globin formation did not change the chromatogram. Incubation at 22°C, pH 7.4, for 18 h did not eliminate labile HbA_{1c} completely (method of Goldstein et al. [21]) (Fig. 4*D*). Letting

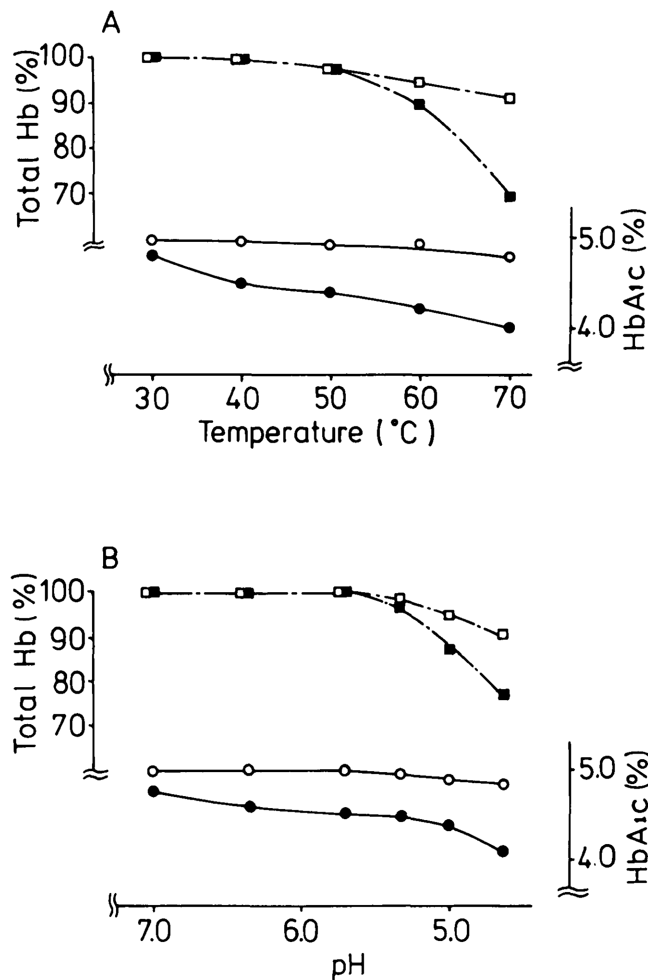

FIG. 3. Total Hb concentration and HbA_{1c} at different temperatures (*A*) and pH (*B*). ○, HbA_{1c} measured with solution T (8 mM phosphate buffer, pH 7.4, with 0.1% Triton X-100); ●, HbA_{1c} measured with solution S (4 mM phosphate buffer, pH 6, with 4 mM tetrapolyphosphate and 0.1% Triton X-100); □, total Hb measured with solution T; ■, total Hb measured with solution S.

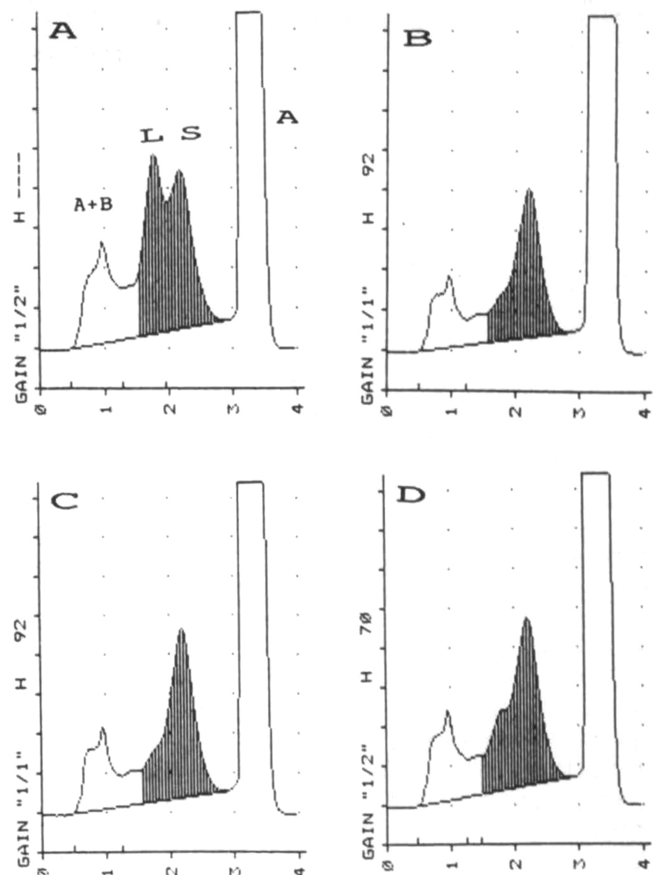

FIG. 4. Chromatograms of Hb and HbA_{1c} values. *A*: whole blood incubated with 10 mg/ml glucose (8.49%); *B*: erythrocytes from *A* treated by method of Nathan et al. (4.55%; 22). *C*: erythrocytes from *A* treated by method of Mullins and Austin (5.07%; 25). *D*: erythrocytes from *A* treated by method of Goldstein et al. (5.52%; 21). Total and stable HbA_{1c} values measured with solutions T and S (Fig. 3) in fresh blood in this experiment were 5.38 and 4.69%, respectively. A, HbA; S, stable HbA_{1c}; L, labile HbA_{1c}; A + B, HbA_{1a+b} *shaded areas*, HbA_{1c} fraction.

the fresh EDTA-treated blood stand at 4°C for 48 h (method of Edmonds and John [26]) also showed remaining labile HbA_{1c}, with 5.15% total HbA_{1c}.

Intra-assay coefficients of variations were 0.84% (mean ± SD HbA_{1c} 5.64 ± 0.05%) and 1.01% (7.74 ± 0.08%) for total HbA_{1c} and 1.12% (5.10 ± 0.06%) and 0.82% (6.98 ± 0.06%) for stable HbA_{1c} (n = 20 each). These results confirm that TPP provides a simple reliable method of removing labile HbA_{1c}.

Data on the three types of HbA_{1c} (stable, total, and labile) in the three groups of subjects investigated are in Table 2. The highest values for labile HbA_{1c} were found in the diabetic group.

DISCUSSION

It is well known that 2,3-DPG lies in the central cleft of the Hb molecule along the twofold axis of symmetry to regulate its oxygen affinity (30). The binding of 2,3-DPG to stable HbA_{1c} is impaired by the presence of glucose on the NH_2-terminal valine of the β-chain, and O_2 transport is thereby impaired (34–36). Therefore, Lowrey et al. (29) expected the inverse to apply, i.e., that the binding of glucose would be blocked by the presence of 2,3-DPG, but they failed to

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TABLE 2
Stable, total, and labile HbA_{1c} in healthy and diabetic subjects and subjects with impaired glucose tolerance (IGT)

	Healthy	Diabetic	IGT
n (M/F)	98/32	61/26	32/11
Age (mean ± SD)	57.7 ± 11.3	62.4 ± 11.0	63.0 ± 10.0
HbA_{1c} (%)			
Stable	4.77 ± 0.43	6.82 ± 1.80	5.14 ± 0.62
Total	5.43 ± 0.47	7.24 ± 2.07	5.76 ± 0.61
Labile	0.66 ± 0.19	0.92 ± 0.43	0.62 ± 0.23

Values are means ± SD.

prove this hypothesis with phosphate buffer. Even orthophosphate, which is a popular buffer system, is now regarded as a weak allosteric effector of Hb (30,32). We used bis-tris buffer in our experiment and examined orthophosphate at a high concentration. Elimination of labile HbA_{1c} by 2,3-DPG and high concentrations of orthophosphate was observed (Table 1). Benesch and Benesch (37) and Chanutin and Curnish (38) first reported the allosteric effect of polyphosphates. Our results with bis-tris buffer at pH 6 were almost same as those with phosphate buffer at pH 7 (37,38). Nonphysiological inorganic polyphosphates, which have stronger allosteric effects, showed a marked ability to eliminate labile HbA_{1c} (Table 1). Polyphosphates, with their higher affinity to the central cleft, might push out glucose to take its place in the central cleft. This mechanism is quite different from previous ones for removing labile HbA_{1c} (21–26).

We developed our method with 4 mM TPP in the hemolyzing solution at pH 6 to remove labile HbA_{1c} during automatic processing in the HPLC analyzer with a thermal jacket (2-min heating time at 45°C). However, TPP at a higher concentration removes labile HbA_{1c} within 3 min at room temperature. This may be useful when estimating HbA_{1c} without a heating system.

After removal of labile HbA_{1c} by all methods, a shoulderlike peak remained (Figs. 2, *C* and *D*, and 4, *B* and *C*). With HPLC with a longer analysis time, this appears as a small peak between stable and labile HbA_{1c}. It is not affected by incubation with glucose or elimination of labile HbA_{1c}. Our HPLC separated labile and stable HbA_{1c} peaks (Figs. 2 and 4). However, it takes 12 min/sample for the machine to separate, read, and calculate the two peaks completely, even a small peak (Fig. 4*D*). It is difficult to maintain these analysis conditions. Analysis time can be reduced to 4 min if labile HbA_{1c} is first eliminated.

The highest values of labile HbA_{1c} were observed in the diabetic group (Table 2). Diabetic patients often have high fasting blood glucose, even when they are in good diabetic control. We believe that it is especially important to eliminate the labile component before HbA_{1c} estimation (15–20), although this idea is not universally accepted (39–41). Some methods, e.g., affinity chromatography, isoelectric focusing, and thiobarbituric acid colorimetry, are not affected by labile HbA_{1c} (11–14). In the most commonly used method based on charge differences, elimination of labile HbA_{1c} is achieved very simply with our method.

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