# IN VITRO SYNTHESIS OF HEMOGLOBIN $A_{Ic}$*

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## 1. Introduction

Several minor hemoglobin components in normal human erythrocytes have been described [2]. The most prominent of these, $HbA_{Ic}$, is presented as 4–6% of total hemoglobin in normal red blood cells. In erythrocytes of patients with diabetes considerably larger amounts have been found [3,4]. The amino acid sequence of $HbA_{Ic}$ is identical to that of HbA, but both the N-terminal α-amino groups of the β-chains are blocked by a carbohydrate residue containing a linkage reducible by sodiumborohydride [5,6]. The p*K* of this group is ~6.65 [5]. These results indicated the bond to be a Schiff base which, however, proved stable even at pH 4.0 [5]. The blocking group has a mass spectrum compatible with a hexose [5], but both the anthrone test and the phenol test for reducing sugars are negative [4].

The present study was undertaken to determine the structure and synthetic pathway of $HbA_{Ic}$.

## 2. Materials and methods

### 2.1. *General*

All chemicals used were reagent grade, radioactively labeled compounds were purchased from the Radiochemical Centre in Amersham, England.

### 2.2. *Hemoglobin preparation*

Blood from male volunteers or animals was drawn using heparin as an anticoagulant. The red cells were washed with saline and lysed with 5 vol. of 0.01 M phosphate buffer, pH 6.8, containing $10^{-4}$ M EDTA. After dialysis against the same buffer the hemolysate was freed from debris by centrifugation. The various hemoglobins were separated on a CM–Sephadex column (25 × 400 mm for 0.5–3.0 g hemoglobin), equilibrated with the above dialysis buffer. The elution was carried out by a gradient going from 0.01 M phosphate pH 6.8 (2:1) to 0.02 M disodium phosphate (2:1) [7]. Quantitation of $HbA_{Ic}$ was done by planimetry of the individual hemoglobin peaks obtained from spectrophotometric readings at 540 nm of all fractions of the chromatograms.

Purity was assessed by electrophoresis on Cellogel strips (12 h at 150 V) in a system consisting of 6 M urea in 0.04 M phosphate buffer pH 6.6 containing 0.05 M β-mercaptoethanol. The strips were stained for 15 min with Ponceau S (0.5 g/100 ml 5% TCA) and destained with 5% v/w acetic acid. The strips were cut into portions which were individually dissolved in 1 ml 80% acetic acid, and radioactivity was counted as follows: 1 ml of sample was mixed with 2 ml of a 1:1 toluene–isopropanol mixture. After 10 min 0.2 ml $H_2O_2$ 30% was added and after a further 40 min standing at room temperature 15 ml of a 9:1 mixture of instagel–5 N HCl was added. The same sample preparation was used for column effluents. Counting was done on a Packard B 2450 LSC scintillation counter.

### 2.3. *Incubations*

Whole blood was incubated at 37°C for up to 20 h after adding uniformly labeled [$^{14}$C]glucose mixed with unlabeled glucose to give a final concentration of 10 mg/ml.

Purified hemoglobin was incubated for 8–18 h

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in either its $O_2$ or CO form at a concentration of 4–10%. Buffers used were 0.01 M phosphate in the range of 6.0–8.0; Tris 0.1 M, pH 7.9; and 0.1 M Bis–Tris, pH 5.1–7.3.

### 2.4. *Product analysis*

Native $HbA_{Ic}$ and the analogous products from incubations were reduced with sodium borohydride [7]. α- and β-chains were spearated [8] and subsequently aminoethylated [9]. Tryptic peptide mapping and staining was done in the usual manner as described by Lehmann and Huntsman [10]. The resulting peptide spots were ringed with pencil and the fingerprint destained in a $H_2O_2$ saturated atmosphere. The peptide spots were cut out and counted in toluene containing 8 g butyl-BDP per litre. The thiobarbituric acid (TBA) reaction for determining the presence of 5-hydroxymethylfurfural was done as follows: 1 ml of a 1% globin from $HbA_{Ic}$ solution was mixed with 0.5 ml 0.3 N oxalic acid and heated for 1 h in a boiling water bath. After cooling to room temperature, 0.5 ml of 40% TCA was added and the resulting precipitate removed by filtration. After addition of 0.5 ml 0.05 TBA. the mixture was incubated at 40°C for half an hour. Spectra obtained on these samples were typical of 5-hydroxymethyl-furfural with an $\epsilon_{max}$ at 443 nm [11].

## 3. Results

Incubation of whole blood and hemolysates in the presence of high concentrations of glucose results in an increase in $HbA_{Ic}$. When isolated HbA is incubated with glucose, a hemoglobin derivative is formed which is chromatographically indistinguishable from $HbA_{Ic}$ [1]. This hemoglobin has a β-chain with the electrophoretic properties of the β-chain obtained from natural $HbA_{Ic}$.

When the incubations were carried out with $^{14}C$-labeled glucose, the resulting hemoglobin derivative had between 2.0 and 2.4 moles of labeled glucose bound per tetramer (table 1). Also given in table 1 are the amounts of $HbA_{Ic}$ formed at two different incubation times and glucose to hemoglobin ratios. When either natural or synthetic $HbA_{Ic}$ was treated with oxalic acid, 5-hydroxymethylfurfural was released from the protein, and could be detected by the colour reaction with 2-thiobarbituric acid. A sample of synthetic $HbA_{Ic}$ resulting from the incubation of [$^{14}C$]glucose with HbA and having a specific activity of 3510 cpm/mg was separated into its component polypeptide chains by electrophoresis as described in Materials and methods. 0.1 mg of the labeled protein was applied to the strip. 310 cpm were associated with the β-chain band and only 34 cpm with the α-chain band, thus showing that almost 10 times more glucose was bound to β-chains. Tryptic fingerprints of glucose labeled β chains after reduction with $NaBH_4$ [6] revealed the absence of a normal βTp-I peptide, but the presence of a peptide in the position of the βTp-I from natural similarly treated $HbA_{Ic}$ was observed. Of the total of 430 cmp recovered from the entire fingerprint 395 cpm

Table 1
Incubation of HbA with labeled glucose at 37°C, pH 7.4

| | Incubation time | |
|---|---|---|
| | 8 h | 18 h |
| Buffer (M) | 0.05 Bis–Tris | 0.01 $PO_4$ |
| CO–Hb (mM) | 0.15 | 0.8 |
| Glucose (Glc) (mM) | 55 | 55 |
| Ratio (Glc)/(Hb) | 366 | 69 |
| Specific radioactivity of glucose (μCi/mmol) | 227 | 460 |
| Specific radioactivity of $HbA_{Ic}$ (μCi/mmol) | 550 | 910 |
| Ratio Glc/$HbA_{Ic}$ | 2.4 | 2.0 |
| % $HbA_{Ic}$ formed | 4.7 | 4 |

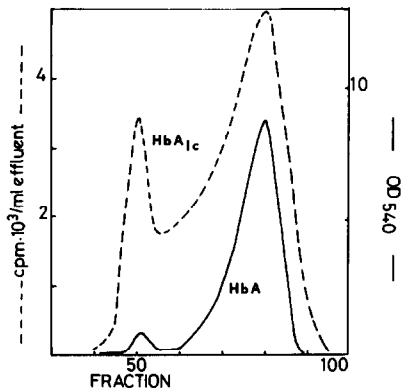


Fig.1. Chromatogram of HbA on CM–Sephadex after incubation with 1000 mg [$^{14}$C]glucose in 0.1 M phosphate buffer, pH 7.4, 37°C, 15 h. (- - - -) cpm/ml effluent × $10^3$ (———) $OD_{540}$nm

migrated with the peptide $\beta A_{Ic}$Tp-I. The remaining counts were distributed among several peptides. Thus, over 90% of the label was found in the N-terminal peptide. Figure 2 illustrates the strong dependence of the reaction on pH. Table 2 shows that the reaction is not specific for human HbA and does not depend upon the N-terminal sequence. Products analogous to $HbA_{Ic}$ were obtained with other hexoses (rhamnose, glucosamine, *N*-acetyl glucosamine, fructose-6-phosphate).

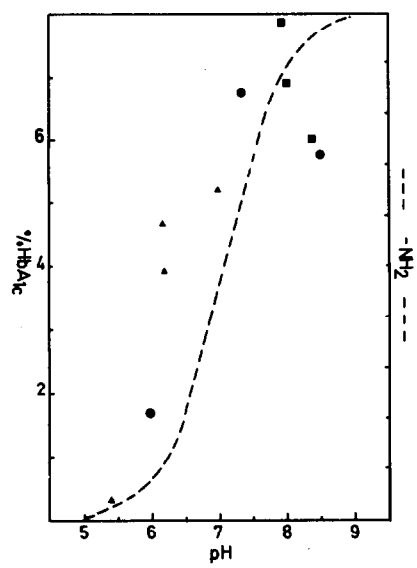


Fig.2. % $HbA_{Ic}$ formed in 8 h as function of pH. For conditions see text. (●) Phosphate 0.01 M. (■) Tris 0.1 M. (▲) Bis–Tris 0.1 M. (- - - -) Titration curve of the α-amino group of β-chains in CO–hemoglobin from ref. [13].

Table 2
Compounds analogous to $HbA_{Ic}$

| Hemoglobin | N-terminal sequence | in vivo present | in vitro synthetized |
|---|---|---|---|
| HbA | Val–His | + | + |
| $HbA_2$/HbS | Val–His | | + |
| Hb mouse obob[a] | Val–His | + | nd[b] |
| $Hb_f$,$Hb_s$ horse | Val–Gln | + | + |
| Hb cat B | Gly–Phe | + | + |

[a]obese mice suffering from diabetes
[b]nd = not done

When red blood cells were incubated with either D-[2-$^3$H]glucose or D-[5-$^3$H]glucose of identical specific activities, 3.9% $HbA_{Ic}$ was synthesized de novo in both incubations. In the case of glucose labeled in the 5 position this hemoglobin had a specific activity of 671 cpm/mg. In contrast, in the incubation with glucose labeled in the 2 position the specific activity was only 369 cpm/mg of $HbA_{Ic}$. These results indicate in the loss of label from position 2 implying the intracellular occurrence of the Amadori rearrangement. A current scheme of the reaction sequence in the Amadori rearrangement is given in fig.3.

## 4. Discussion

Our results clearly demonstrate the non-enzymic nature of the reaction of glucose with the β-N-terminal amino group of hemoglobin. This is independent of the N-terminal sequence. Contamination of our hemoglobin samples with an as yet unknown enzyme is unlikely, since the reaction occurred with hemoglobins differing widely in their chromatographic properties. Such a non-enzymatic reaction has been postulated recently by Bunn et al. [12]. This reaction is strongly pH-dependent and in view of the nucleophilic attack on the carbohydrate by the un protonated amino group is a function of the p*K* of

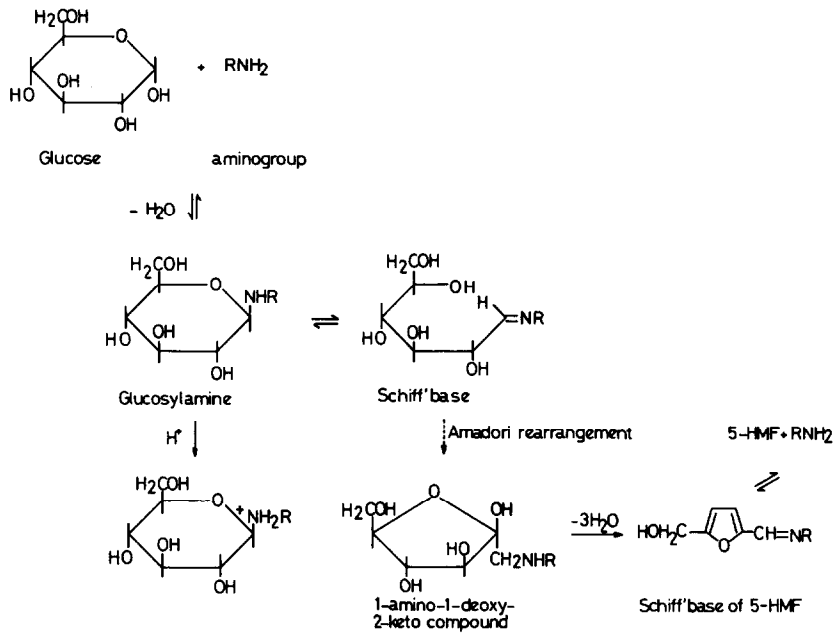


Fig.3. Simplified scheme of the proposed reaction sequence involved in the formation of $HbA_{Ic}$ and the subsequent Amadori rearrangement.

amino group involved. Therefore, this reaction must be very general in nature, both in respect to the amino groups and the carbohydrates, depending on the p$K$ of the amino group involved. This generality of the reaction implies a wide significance in biological systems and in diabetes specifically. In the case of erythrocytes, these carbohydrates are most likely the metabolic derivatives of glucose.

That other groups can similarly react, albeit to a lesser extent, is evidenced by some incorporation of radioactivity into HbA without change of chromatographic properties (fig.1). The pH dependence of the reaction illustrated in fig.2 strongly favors hemoglobin as a reactant in view of the relatively low p$K$ values of its N-terminal α-amino groups in both the R- and the T-state (table 3). This feature is, however, not able to explain the very high specificity of the reaction for the β-chain. This is particularly true, since our incubations were done with the CO or $O_2$ derivative of HbA (see table 3).

The structure of the N-terminal blocking group is most easily rationalized by the formation of an *N*-glycoside in equilibrium with a Schiff base [14]. The evidence for this is reviewed in the introduction. Both the formation of 5-hydroxymethylfurfural and the experiments with D-[2-$^3$H]glucose provide strong evidence that the initial product undergoes the Amadori rearrangement even intra-erythrocytically (see fig.3). Similar conclusions were reached by Bunn et al. [15] using a somewhat different approach. Finally, it needs to be pointed out that the course of the off-reaction obtained by the incubation of [$^{14}$C]-glucose-labeled $HbA_{Ic}$ in the presence of a high concentration of non-labeled glucose proceeds very slowly. In fact, non protein-bound radioactivity appeared very slowly in the TCA supernatant obtained after various incubation times. This is in agreement

Table 3
p$K$ values of N-terminal amino groups

| Compound | p$K$ | Method[a] |
|---|---|---|
| Hemoglobin–CO α | 6.95 | C |
| Hemoglobin–CO β | 7.05 | C |
| Hemoglobin–deoxy α | 7.79 | C |
| Hemoglobin–deoxy β | 6.84 | C |

[a]C: Cyanate reaction [13]

with recent in vivo observations on diabetics [16] showing that $HbA_{Ic}$ levels decreased after normalisation of blood sugars at a rate at which the erythrocytes were replaced by newly formed cells.

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