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INVESTIGATION OF THE HETEROGENEITY OF HEMOGLOBIN BY CATION-EXCHANGE CHROMATOGRAPHY ON BIO-REX 70

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SUMMARY

The use of Bio-Rex 70 cation-exchange resin for chromatography of normal and diabetic hemoglobin provides a reproducible pattern of the "fast components". Particular attention to the choice of sample preparation, pH of elution, and the increase of ionic strength by sodium chloride linear gradients results in the separation of Hb-A_{1b} into two components and in the isolation of a new component eluting between Hb-A_{1c} and Hb-A_0. Experiments with [^{3}H]glucose and the colorimetric test (thiobarbituric acid) normally used to determine the extent of non-enzymatic glycosylation, as well as an increase of this component in diabetic samples compared with normoglycemic ones and a significant linear correlation with Hb-A_{1c}, indicate that this component should be a part of the hemoglobins glucosylated on the ϵ-NH_2 group of the lysines of both chains and/or the hemoglobin glucosylated on the α-NH_2 of the valine of the α-chain. We propose to call this component Hb-A_{1x}, pending confirmation of its identity.

Normally Hb-A_{1x} accounts for about 3% of Hb-A, but up to 5—7% of glucosylated hemoglobins should be confined to the early part of Hb-A_0. In diabetics, the percentage of Hb-A_{1x} rises to 4—5% and that of the other glucosylated hemoglobins increases to 12—16%.

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INTRODUCTION

It has already been shown that cation-exchange chromatography on Bio-Rex 70 (or Amberlite IRC 50) of normal hemoglobin allows the separation of a number of "minor fast components", namely Hb-A_{1a-e}, with respect to the main Hb-A_0, and to a slower component designated Hb-A_2 [1]. Accurate determination of the identity of these minor fast components presents serious difficulties; at the moment not all have been structurally defined [2].

Briefly, Hb-A_{1a}, originally supposed to be a unique component, now appears to result from two components named [3] Hb-A_{1a1} and Hb-A_{1a2}. It is supposed that they derive from a slow, non-enzymatic, post-transcriptional hemoglobin modification due to the action, respectively, of fructose 1,6-diphosphate and glucose 6-phosphate on the α-NH_2 group of the terminal valines of both β-chains of hemoglobin [3]. Their identity has not been completely determined due to experimental disagreement [4]. The identity of Hb-A_{1b} is still unknown, even though its characteristics have been partially elucidated [5].

Hb-A_{1c} is the most characterized component and also the most abundant one; it is the result of the attachment by glucose on hemoglobin, described above for Hb-A_{1a1} and Hb-A_{1a2}. The aldimine adduct formed subsequently undergoes an Amadori rearrangement to form a more stable ketoamine linkage [6].

Determination of the percentage of Hb-A_{1c} in a hemoglobin sample is important in some pathological alterations of the glucosidic metabolic pathways. In red cells of patients with overt diabetes mellitus there is a two- to threefold increase in the percentage of Hb-A_{1c} (4—6% to 10—15% of total hemoglobin) [2]. It has therefore been proposed that the percentage of Hb-A_{1c} could be used in assessing the degree of diabetes, by providing an integrated measurement of blood glucose according to the red cell life span.

The Hb-A_{1c} component is not separable from Hb-F by cation-exchange chromatography [1]; hemoglobin samples with a high value of Hb-F give a falsely elevated percentage of Hb-A_{1c}. Hb-A_{1d} is probably an artifact which forms in vitro when one sulphydryl group of each of the two β-chains of Hb-A has reacted with oxidized gluthathione, and Hb-A_{1e} seems to be an artifact formed as a result of the incubation of hemoglobin at high temperatures [1].

This is further complicated by the observation [7] that the glucosylation products on the ϵ-NH_2 of the lysines of both chains and on the α-NH_2 of the valine of the α-chains, are not separable by this chromatographic technique, but remain with the early part of the Hb-A_0 peak. For this reason we have tried to perform accurate chromatographic separation to achieve good resolution of the early part of the Hb-A_0 peak. We used the preparative method of McDonald et al. [3] for analytical purposes. In fact, the exact determination of the complete glycosylation pattern of hemoglobin, including the components glucosylated on the ϵ-NH_2 of the lysines and on the α-NH_2 of the valine of the α-chain, should provide a more accurate integrated measurement of blood glucose.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade; the common reagents were Merck products, (Merck, Darmstadt, G.F.R.). Thiobarbituric acid was from Merck and Ega Chemie (Steinheim/Albuch, G.F.R.). Bio-Rex 70 (200—400 mesh); Na^+ (carboxylic resin, pK_a = 6.1, copolymeric methacrylic acid—divinylbenzene matrix) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). [^{3}H]Glucose was from The Radiochemical Centre (Amersham, Great Britain). YM 10 membranes were from Amicon, (Lexington, MA, U.S.A.). Instagel was from Packard, (Downers Grove, IL, U.S.A.).

Preparation of hemoglobin samples

Venous blood was collected in EDTA tubes from diabetic and normoglycemic volunteers and processed within 1 h of collection. All samples were tested for the presence of Hb-F by the alkali denaturation method [8]. Samples with a percentage of Hb-F greater than 1.0% were not submitted to chromatography. The red cells were washed four times with saline and lysed with 3 volumes of distilled water to 1 volume of packed erythrocytes and then stored at 4°C for 30 min. During mild stirring at 4°C, sodium chloride was added to each sample to a final concentration of about 0.4 mol/l. The solution was gently stirred for 15 min at 4°C, then centrifuged for 30 min at 4°C, 15,000 *g*, to discard the large pellet. After exhaustive dialysis against distilled water (four changes of 200 volumes), which eliminates the glucose reversibly linked to hemoglobin (aldimine adduct), the supernatant was submitted to a second centrifugation at 60,000 *g*, 60 min, 4°C. The sample was stored in lots of 3.0 ml, frozen at −20°C and chromatographed within two months.

Some hemoglobin samples, before Bio-Rex 70 chromatography, were stripped of 2,3-diphosphoglycerate by the dialysis method reported by Jelkmann and Bauer [9].

Chromatographic procedure

Eighty grams of Bio-Rex 70 were suspended in distilled water; after vigorous stirring and sedimentation, the water was aspirated off and the resin poured into a column of large diameter and equilibrated with a 0.05 mol/l potassium phosphate buffer at a pH ranging from 6.45 to 6.65 (determined at 20°C with a precision of ± 0.01 pH units). One lot of 3.0 ml of hemolysate (about 8 μmol/l in heme, equivalent to 150 mg of hemoglobin), dialysed at the chosen pH, in oxy form or with prior conversion to the carbon monoxide form, was loaded onto a 50 cm × 2 cm^2 column. The flow-rate was 40 ml/h and the fraction volume was 3.5 ml. The whole chromatographic procedure was performed at 4°C. After the exit of the non-hemoglobin protein and of Hb-A_{1a1} and Hb-A_{1a2} components (approximately 50 fractions), linear gradients of sodium chloride (0—0.10 mol/l, 0.10—0.20 mol/l, and 0.20—0.30 mol/l of sodium chloride in equilibrating buffer; 120 ml of each gradient) were applied variously as a function of pH, to elute the other components (Fig. 1). The elution pattern was determined by monitoring each fraction at 415 nm, and a few fractions, as control, at 280 nm; when the absorbance (A) was too high

for direct determination, the exact value was determined by appropriate dilution. The percentage of each component was computed as described by Schroeder and Huisman [1]. The ϵ_{max} values are those obtained from Antonini and Brunori [10]. Gradient linearity was controlled by checking the refractive index of the fractions eluted.

It should be noted that the sodium chloride-buffered solutions used for the gradients showed a pH slowly decreasing in function with concentration; this is probably due to the sensitivity of the electrode (Beckman 39501) to Na^+; correction of the pH of the sodium chloride buffers to the exact pH value of the starting buffer results in a poor separation pattern. We then left them uncorrected and, sometimes, we used the pH of the fractions as a further control of the linearity of the gradients.

Incubation of Hb-A_0 with labelled glucose

Samples of HbCO were chromatographed as described above. The central part of the Hb-A_0 peak was collected, concentrated by ultrafiltration on YM 10 membranes up to a concentration of about 10 mg/ml; 3.0 ml of this concentrated pool, after dialysis against a sodium phosphate buffer, 0.02 mol/l, pH 7.4, 0.15 mol/l in sodium chloride, were incubated with an appropriate quantity of labelled glucose and with unlabelled glucose to reach a total concentration of 25 mmol/l. The incubation was performed in an atmosphere of carbon monoxide.

At the chosen time (normally five days, as in the case reported under Results), a 1.0-ml volume of the solution was collected and filtered through a column of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden), (25 cm × 2 cm^2), equilibrated with the buffer (pH 6.55) used for the Bio-Rex 70 column, both to eliminate the free glucose from the incubation solution and to bring the sample to the exact pH required for chromatography. The labelled hemoglobin was then added to 1.0 ml of the starting whole HbCO sample and chromatographed on Bio-Rex 70 at pH 6.55 (± 0.01) as described under Chromatographic procedure.

After the spectrophotometric readings, 10 ml of Instagel were added to each fraction and the radioactivity was monitored by a Packard scintillation counter (Model 460 C D).

Colorimetric (thiobarbituric acid) test

Colorimetric analysis of the components was carried out using the thiobarbituric acid test of Fluckiger and Winterhalter [11], slightly modified as follows. The chosen hemoglobin pool was obtained by ultrafiltration on YM 10 membranes. The small quantity of the HbA_{1a-b} components never allowed their determination by this method.

To 1.0 ml of the pooled components, 1.0 ml of 1.0 mol/l oxalic acid was added; after incubation at 100°C (4.5 h), 1.0 ml of 40% (w/w) trichloroacetic acid was added to each sample and standard at 0°C. The solution was centrifuged (3000 *g*) and to 1.5 ml of the supernatant 0.5 ml of 0.05 mol/l thiobarbituric acid was added; after incubation at 40°C for 30 min and for 10 min at room temperature, the absorbance of each sample and standard at 445 nm was determined against blanks of distilled water.

RESULTS

Fig. 1 shows elution patterns for three chromatograms of the same diabetic HbCO sample at different pH values: 6.60, 6.55, 6.50 (± 0.01). By lowering the pH, the interactions between the cation-exchange resin and the hemoglobin components obviously increase. To obtain a reasonable elution time it was necessary to increase the ionic strength with the use of sodium chloride to allow good elution of each component.

The effect of the pH is different on each component; Fig. 2 illustrates the changes in $V_{e(max)}$ as a function of pH. The best resolution was at pH 6.55; therefore all analytical chromatograms were made at pH 6.55 ± 0.01. The percentages of each component at different pH values are reported in Table I; only small variations are present, probably due only to overlapping of some components.

Considering each component singly, the following can be deduced:

(1) The first chromatographic peak can be attributed to non-heme proteins or hemoglobin degradation products, as pointed out also by McDonald et al. [3], for a A_{415}/A_{280} ratio very different from that of HbCO (not reported).

(2) The Hb-A_{1a1} and Hb-A_{1a2} components, with a clear profile at pH 6.60, at lower pH have a broad elution, thus, because of their low percentage, it is difficult to recognize them precisely; nevertheless, a lower pH allows a better separation when bulky preparative samples are used.

(3) The Hb-A_{1b} component, under all the chromatographic conditions, is

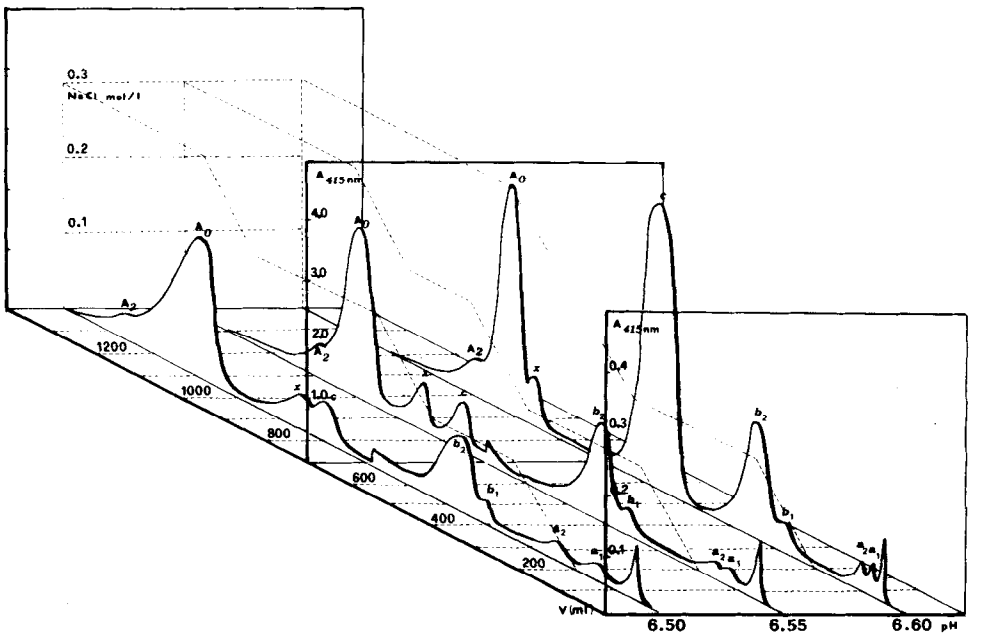

Fig. 1. Elution patterns of the same diabetic HbCO sample at different pH values. At 700 ml there is a change of absorbance scale (10 X) to include the Hb-A_0 component. Thus, the Hb-A_{1c} component which elutes at pH 6.60 with a volume of about 500 ml, at lower pH increases its elution volume with a resulting tenfold reduction in peak area.

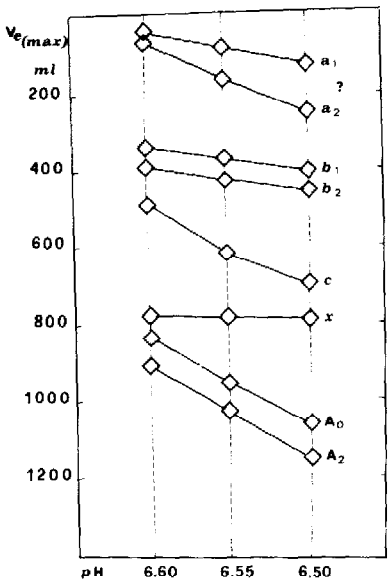

Fig. 2. Elution volume corresponding to the maximum absorbance value ($V_{e(max)}$) of each chromatographic peak as a function of pH. For Hb-A_{1a_1} and Hb-A_{1a_2} the question mark indicates difficulties in determining their exact position.

TABLE I

PERCENTAGE OF EACH COMPONENT OBTAINABLE FROM THE DIFFERENT CHROMATOGRAPHIC RUNS OF FIG. 1

More accurate values than those reported are not significant.

Component	pH 6.60	pH 6.55	pH 6.50
a_1	0.1	0.1	0.1
a_2	0.1	0.1	0.1
b_1	0.2	0.2	0.2
b_2	0.9	0.8	0.8
c	5.4	5.6	5.4
x	6.3	5.5	6.1
A_0+A_2	87.0	87.7	87.3

split into two peaks, a smaller peak always preceding a larger one. These two components are named Hb-A_{1b1} and Hb-A_{1b2}. The chromatographic resolution is poor and we did not succeed in improving it.

(4) The Hb-A_{1c} component is influenced to the greatest extent by change in pH (Fig. 2). It is well separated at pH 6.60 and 6.55, but at pH 6.50 it partially overlaps the Hb-A_{1x} component. At pH 6.60 the elution of this component is favoured by a concentration of 0.1 mol/l sodium chloride. Thus, for the elution of the last components it is advisable to apply a linear gradient increasing directly from 0.1 mol/l to 0.3 mol/l. When the pH is lowered because of the higher interaction of the Hb-A_{1c} component with Bio-Rex 70, it is convenient to apply an intermediate plateau of 0.2 mol/l sodium chloride.

(5) Between Hb-A_0 and Hb-A_{1c} there is a new component which at pH 6.60

is eluted just before the Hb-A_0 peak and at a lower pH practically does not change its $V_{e(max)}$. Therefore, pH 6.55 is the best resolutive condition for this component, because at pH 6.50 the greater retardation of Hb-A_{1c} causes overlapping of these two components. This is why it is difficult to calculate the percentage of this new component at extreme pH values (see Table I).

We propose to name this new component Hb-A_{1x}, until its identity is clearly determined.

(6) Finally, at the end of the Hb-A_0 peak eluted by 0.3 mol/l sodium chloride, the Hb-A_2 component is present, which is so overlapped with Hb-A_0 that it is difficult to separate [1].

As regards sample preparation and chromatographic conditions the following can be observed:

(1) Different sample preparations can cause very slight differences in the percentage of the components but do not change their position ($V_{e(max)}$). After comparing different sample preparations we chose the conditions described under Materials and methods, with a salting-out step after hemolysis of the erythrocytes. This step restricts the possibility of contamination by artifacts.

(2) The use of HbCO or HbO_2 does not cause noticeable variations in the percentages and in the $V_{e(max)}$ of the components, but only a slight enlargement of the peak base of the fast components and a greater one of the Hb-A_0 in HbO_2 samples. This fact, together with the well-known instability of HbO_2, advises the use of HbCO.

(3) The removal of 2,3-diphosphoglycerate from hemoglobin by dialysis [9] does not cause any changes in the chromatographic patterns, especially with regard to the major fast components. There is only a small variation in the initial part of the chromatogram with a decrease in the percentage of the non-heme peak. Although it does mean that the sample is contaminated, it is not worthwhile including this long step for a percentage determination. On the other hand, if study of Hb-A_{1a1} and Hb-A_{1a2} is the object of the experiment, it is advisable to remove the 2,3-diphosphoglycerate.

Fig. 3 shows the elution pattern of Hb-A_0 incubated with labelled glucose and chromatographed together with the starting unlabelled normal HbCO sample. The incubation generates various peaks; the first elutes at the position attributable to non-heme proteins or hemoglobin degradation products. After this, two peaks, approximately at the position of Hb-A_{1a1} and Hb-A_{1a2}, are present. A well-defined peak is present just before Hb-A_{1c}, and in the position of the Hb-A_{1x} component a broad labelled peak is evident; this peak also overlaps with the early part of Hb-A_0, where, in the leading fractions, there is a net increase of radioactivity.

The experiment with labelled glucose offers only a qualitative picture of glucosylation in vitro and the results obtained must be discussed with due consideration to the radioactive impurities probably present in the labelled glucose, as pointed out by Trueb et al. [12].

In Fig. 3 are also indicated the fractions that are usually pooled prior to concentration on Amicon YM 10 membranes for the thiobarbituric acid test. The pools are collected in accordance with the labelling experiments. The Hb-A_0 component is divided into three pools: two small ones in the leading

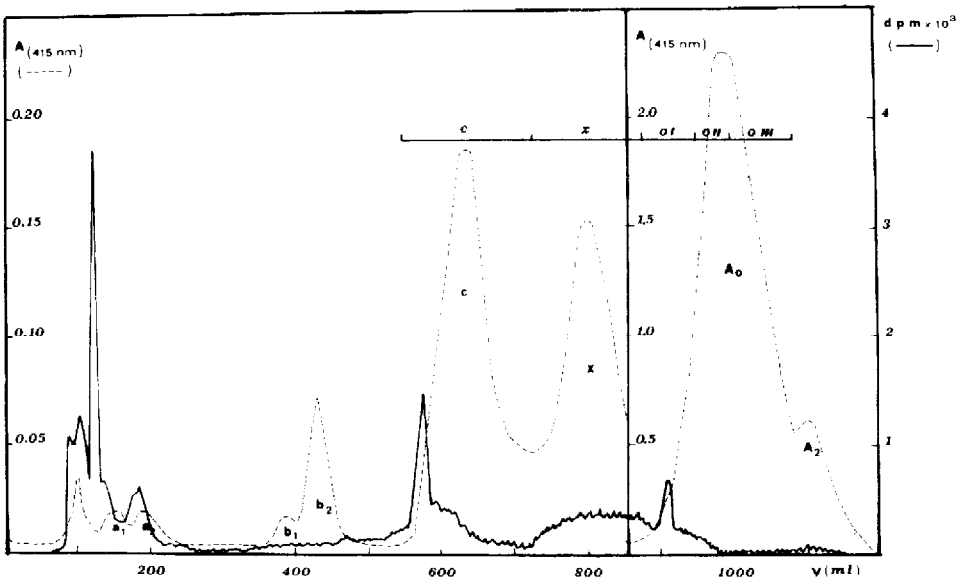

Fig. 3. Elution pattern of a normal HbCO sample at pH 6.55 ± 0.01; the dashed line (— — —) indicates absorbance (415 nm) and the solid line (——) indicates dpm obtained by incubation experiments with [^{3}H]glucose. On the top right-hand side of the figure are also indicated the pools collected for the thiobarbituric acid test.

edge of the peak and a large one at the end. Hb-A_2 is not considered in the pool collection.

The pools were named c, x, OI, OII, OIII and the results obtained with the thiobarbituric acid test are reported in Table II. The OIII pool shows a low positivity and other experiments (not reported) suggest that it may derive from some heme contaminants. In fact, the labelling experiments show that no glucose is linked in this part of the Hb-A_0; thus the A_{445} reading obtained from this component must be subtracted from the value of the other pools, not as an absolute value but as A_{445}/mol hemoglobin, assuming that the contaminant release is proportional to the total hemoglobin content.

Calculation of the other pools is made in two different ways. In the first method it is assumed that the conversion to 5-hydroxymethylfurfural of the Glu-Hb adduct (Amadori rearrangement) is equivalent to that of fructose. In effect, using fructose and 5-hydroxymethylfurfural standards, it can be observed that, under the conditions of the test, the fructose conversion is about 30%. Thus the results, expressed as mol fructose equivalent per mol Hb (tetramer), are reported for the pools in the first column of Table II. These results are a little higher than expected, as can be seen from the c pool value (2.3 mol fructose per mol Hb). Therefore, there is no reason to expect a conversion of the Glu-Hb adduct (ketoaminic form) with the same yield of fructose.

In effect, assuming that, in the second type of calculation, there is a conversion of about 36% of the ketoaminic adduct in 5-hydroxymethylfurfural, the values reported in the second column are obtained, expressed as mol Glu → Amadori per mol Hb (tetramer). Clearly, Hb-A_{1c} can be supposed to be a diglucosylated form of hemoglobin, while Hb-A_{1x} and the Hb-A_{0I} pools are

TABLE II

VALUES OBTAINED FROM THE THIOBARBITURIC ACID TEST RESULTS ON THE POOLS OF FIG. 3 (COLLECTED FROM CHROMATOGRAPHIC FRACTIONS)

In the first column the calculation is made assuming a conversion of the ketoaminic form of hemoglobin (in Amadori rearrangement) comparable with that of fructose standards (mol fructose per mol hemoglobin). In the second column the calculation is made assuming a conversion of the ketoaminic form of hemoglobin of about 36%, choosing for Hb-A_{1c} a value of 2.0 mol/mol hemoglobin (mol Amadori per mol hemoglobin). On the right hand side of the table are reported the percentage ranges for normal and diabetic samples obtained from the thiobarbituric acid test and the quantity of hemoglobin present in the pools collected.

Pool	mol fructose / mol hemoglobin	mol Amadori / mol hemoglobin	Percentage	
			Normal	Diabetic
c	2.3	2.0	4—5	8—9
x	1.4	1.2	2—3	4—5
0I	1.3	1.1	5—7	12—16
0II	0.7	0.6		
0III	—	—	—	—

monoglucosylated forms of hemoglobin. Hb-A_{0II} is a mixture in which about 60% of monoglucosylated hemoglobin is still present.

The mean value of 1.2 for Hb-A_{1x} could derive from a partial overlapping with the Hb-A_{1c} component. Obviously, the second method of calculation takes into account that Hb-A_{1c} is a diglucosylated form. Thus, considering a conversion of the ketoaminic adduct of 18%, the values obtained should be all halved.

On the right-hand side of Table II percentages of glucosylated hemoglobin in normoglycemic and diabetic samples are reported as obtained from the thiobarbituric acid test and from the total quantity of hemoglobin in each pool.

In Table III the statistical inter-assay data for the column chromatography are presented ($n = 10 \times 2$), intra-assay statistical computation is very difficult due to the length of time required for the chromatography, making it difficult to perform many experiments on the same sample.

In addition, the mean values for diabetics ($n = 28$) and for normoglycemics ($n = 15$) of each fast component are presented; Hb-A_{1b1} and Hb-A_{1b2} are computed as a single component because of difficulties in quantifying the two peaks. While the normoglycemic ranges are calculated with the aid of standard deviation and Student's t test, for the diabetic ranges, in which the distribution seems not to be of normal type, those of Hb-A_{1c} and Hb-A_{1x} are computed as the differences between the mean and the highest value and the mean and the lowest value of the range.

Also in Table III are presented the crossed correlation coefficients together with linear regression analysis among all the fast components. Hb-A_{1a1} correlates with Hb-A_{1a2}, and Hb-A_{1b}' Hb-A_{1c} and Hb-A_{1x} correlate among themselves. Our previous data on a smaller number of samples [13] did not show a significant correlation of Hb-A_{1b} with the other components, so for

TABLE III

STATISTICAL DATA OBTAINED FOR EACH COMPONENT FROM THE CHROMATOGRAPHY OF 43 SAMPLES

In the first column are shown the inter-assay coefficients of variation ($n = 10 \times 2$). In the second and third columns the normoglycemic and diabetic ranges are shown. On the right-hand side the crossed linear regression parameters are presented together with the correlation coefficient. $t \times$ S.D. is the standard deviation multiplied by the t value from the "t test" table for "95% limits".

Fast component	Inter-assay C.V. ($n = 10 \times 2$)	Normal mean ± ($t \times$ S.D.) ($n = 15$)	Diabetic mean ± ($t \times$ S.D.) ($n = 28$)	Linear regression* and correlation				
				a_1	a_2	b_{1+2}	c	x
a_1	14.4	$0.12^{+0.24}_{-0.12}$	$0.14^{+0.27}_{-0.14}$	—				
a_2	7.3	0.14 ± 0.12	0.18 ± 0.18	$a = +0.27$ $b = +0.12$ $r = +0.706$ **	—			
b_{1+2}	3.0	1.51 ± 0.66	1.81 ± 0.79	$a = -0.79$ $b = +1.83$ $r = -0.194$	$a = +0.31$ $b = +1.67$ $r = +0.029$	—		
c	4.0	4.55 ± 1.71	$8.57^{+5.95}_{-3.02}$	$a = -1.58$ $b = +6.88$ $r = -0.104$	$a = +4.54$ $b = +5.93$ $r = +0.114$	$a = +2.48$ $b = +2.37$ $r = +0.668$ **	—	
x	3.9	2.86 ± 0.91	$5.21^{+4.53}_{-2.04}$	$a = -0.79$ $b = +4.55$ $r = -0.078$	$a = +3.04$ $b = +3.96$ $r = +0.115$	$a = +1.50$ $b = +1.86$ $r = +0.687$ **	$a = +0.48$ $b = +1.28$ $r = +0.714$ **	—

*$Y = aX + b$; $n = 43$.

**$p < 0.001$.

this component it is important that the statistical analysis is performed on a larger number of samples in order to confirm the present data.

DISCUSSION

The use of Bio-Rex 70 cation-exchange chromatography to demonstrate the heterogeneity of Hb has been described [1, 3]. In fact, the preparative chromatography used by McDonald et al. [3] did not allow the separation of some components, probably due to the too high elution pH and the too steep sodium chloride gradient used in the last chromatographic step. A smoother final sodium chloride gradient and a lower pH value (6.55 ± 0.01), as well as smaller sample volumes compared to the total volume of the column, result in a more accurate separation. The interpretation of the results can be summarized as follows:

(1) The first chromatographic peak is clearly attributable to non-heme proteins because it shows a different A_{415}/A_{280} ratio to that of HbCO. In this part of the elution pattern probably a small quantity of 2,3-diphosphoglycerate hemoglobin is present along with the free glucose of the sample which has not been retained by the resin. This interpretation comes from the comparison between samples free and not free from 2,3-diphosphoglycerate and from the experiments with labelled glucose. After incubation the mixture is immediately filtered on a Sephadex G-50 column both to eliminate free glucose and to bring the labelled sample to the subsequent column pH. At this point and before the Bio-Rex column there is a lag of about 1 h which is needed for sample adjustments (addition of unlabelled hemoglobin and readings). In the meantime the reversible glucose aldimine adduct separates from hemoglobin to yield a separate labelled peak at one void volume of the column. Thus the initial peak and/or peaks observed could derive from this glucose. If true, this step could be proposed as a method for measuring the glucose reversibly linked to hemoglobin (aldimine form) which, from the results, seems to be more than previously supposed [14]. Furthermore, the possible presence of some radioactive contaminants must be considered [12]. We are performing other labelling incubation experiments to elucidate these problems.

(2) Hb-A_{1a1} and Hb-A_{1a2} are characterized with some difficulty because of their low absolute amounts. Larger samples overload the columns and the use of larger columns would be too time-consuming.

Little can be said about these two components. By lowering the pH their resolution improves. Their carbohydrate moieties [3] — fructose 1,6-diphosphate (Hb-A_{1a1}) and glucose 6-phosphate (Hb-A_{1a2}) — cannot be elucidated from our data. The fact that two labelled peaks are present at approximately the same elution volumes of these components may be explained by the above-mentioned presence of radioactive contaminants or by the presence of free labelled glucose. It is also possible that there is at this elution volume a triglucosylated form of hemoglobin which, though of little statistical importance, may be highly radioactive.

These two components correlate significantly between themselves but not with the others. This fact can be a point in favour of the given interpretation. In fact, the intra-erythrocyte concentration of the two metabolic products,

fructose 1,6-diphosphate and glucose 6-phosphate, is enzymically mediated and an increase in one of them may reflect an increase in the other.

The lack of correlation with the other components could, on the other hand, be due to a non-enzymically mediated glucose concentration. Furthermore, the mean values for normoglycemic and diabetic samples of these two components do not vary appreciably. For the very low absolute quantities of these components the results obtainable with the thiobarbituric acid test are below the sensitivity range of the system. The positive data obtained by others [3] may be artifactual contaminants from heme degradation products and, in any case, it is not clear whether the ketoaminic reversible adduct of hemoglobin with fructose 1,6-diphosphate can convert into a more stable ketoaminic form (Amadori rearrangement) sensitive to the thiobarbituric acid test.

(3) Also, very little about the identity of Hb-A_{1b} emerges from our data. This component, previously thought to be a minor homogeneous hemoglobin component, is probably the sum of two minor hemoglobin forms very similar in chromatographic properties, as shown by their parallel $V_{e(max)}$ variation as a function of pH. No labelled glucose is linked to them. From the statistical analysis no significant differences between the two mean values of normoglycemic and diabetic samples can be observed, but there is a significant linear correlation with Hb-A_{1c} and Hb-A_{1x}. An earlier statistical analysis on a smaller number of samples (n = 21) did not show a significant correlation [13]. In any case, it is not sure if the Hb-A_{1b} component(s) derive from hemoglobin modification as postulated for the other components.

(4) Hb-A_{1c} has already been mentioned as the most studied component. Our data are all in agreement with the known properties: (A) the high sensitivity of its $V_{e(max)}$ to variations in pH should indicate a pK_a of the amino group of the ketoaminic glucosidic moiety in the same range as the pH of elution; (B) the net labelled peak present in the leading edge of the Hb-A_{1c} peak in the incubation experiments. The non-exact overlapping is probably due to a partial denaturation of the glucosylated hemoglobin form during the incubation; (C) the large increase in its percentage mean value in diabetic samples compared to normoglycemic ones. It is important to note that, while in normoglycemics the percentage distribution is normal (Gaussian), in diabetics the distribution is asymmetrical and it is advisable not to use the common statistical analysis to obtain the pathological range; (D) finally, its identity is also confirmed by a high positivity in the thiobarbituric acid test; the values observed lead one to assume, in comparison with fructose standards, the presence of about 2 mol of glucose in Amadori rearrangement for 1 mol of hemoglobin, both for normal and diabetic samples; this fact shows that Hb-A_{1c} is a diglucosylated form of hemoglobin, as previously demonstrated by Bookchin and Gallop [15].

(5) Hb-A_{1x} is the new component obtained. The $V_{e(max)}$ of elution is very similar to that described for Hb-A_{1d} and Hb-A_{1e} [1]. However, we do not believe that Hb-A_{1x} is one of these two known components. Hb-A_{1d} is assumed to be an artifact formed in vitro when undialyzed hemolysates are stored and in which one sulphydryl group of each of two β-chains of Hb-A has reacted with oxidized glutathione. This is not so in our case. Hb-A_{1e} apparently is formed as a result of the incubation at 37°C, and its chromatographic properties are

different from those of Hb-A_{1d}. We do not use experimental steps in which the sample undergoes incubation at 37°C, except in the experiments with labelled glucose, where the broad peak, corresponding to the Hb-A_{1x} component, could also be thought of as a labelled Hb-A_{1e} contaminant. Hence, we indicate this component with the subscript "x" until its identity is clearly demonstrated.

We suggest, on the basis of our results, that the Hb-A_{1x} component is a glucosylated hemoglobin with blocked ϵ-NH_2 group(s) of the lateral lysines, or a hemoglobin glucosylated on the α-NH_2 of the valine of the α-chain. It has chromatographic properties ranging between those of Hb-A_{1c} and Hb-A_0, showing, therefore, that it has a positive charge lower than that of Hb-A_0 and greater than that of Hb-A_{1c}. According to the thiobarbituric acid test, the Hb-A_{1x} component shows that about 1 mol of glucose is converted in Amadori rearrangement per 1 mol of hemoglobin (tetramer), both in normal and diabetic samples. The [^{3}H]glucose incubation results in a broad peak in the position of Hb-A_{1x} (also present in the early part of the Hb-A_0 peak), so that it may correspond to a not strictly homogeneous component. Last, but not least, there is an increase in the percentage of Hb-A_{1x} in diabetic samples almost comparable to that of Hb-A_{1c} and, furthermore, the linear correlation between these two components is significant. The correlation observed is highly significant, but not in absolute value ($r = 0.714$). This is not surprising because the glucose concentration could not be the only variable affecting the percentage of these components, but it can be supposed that, owing to a very different pK_a between the amino group of the terminal valine of the β-chain (ca. 7) and the ϵ-NH_2 of lateral lysines (ca. 10), the rate of glucosylation has varying sensitivity to slight pH changes [16]. Indeed an increase of the intra-erythrocyte pH should be reflected in a variation of the rate of glucosylation more for Hb-A_{1c} than for Hb-A_{1x}, because of the greater increase in non-protonated NH_2 of the valine. Thus, the non-exact correlation may be the result of these different behaviours. These results seem to dispel every doubt on the given interpretation of the Hb-A_{1x} component, but further discussion leads to some uncertainties. The very small variation in the $V_{e(max)}$ of Hb-A_{1x} as a function of Ph shows that this component is modified on those groups interacting with Bio-Rex 70 that are particularly sensitive to pH changes, and which are present in Hb-A_0, Hb-A_{1c} and Hb-A_2. This fact seems contrary to a modification on the ϵ-NH_2 of the lateral lysines. However, as has already been said, Hb-A_{1x} could represent a peak resulting from non-homogeneous hemoglobins, so a chromatographic property which varies with pH must be justified in this respect.

Furthermore, the interpretation of the labelling experiments may be criticized for the possible presence of radioactive contaminants and for the possible presence of labelled Hb-A_{1e} in the same elution range as Hb-A_{1x}, as previously noted.

However, even with due allowance given for these uncertainties, the interpretation afforded by the other experimental results, such as the statistical analysis on normoglycemic and diabetic samples as well as the values obtained through the thiobarbituric acid test, provides a sufficiently strong demonstration.

(6) Not all the glucosylation products are confined to the Hb-A_{1c} and Hb-A_{1x} components. The labelling experiments and the thiobarbituric acid test

indicate that appreciable glucosylation is still present in the early part of the Hb-A_0 peak. Shapiro et al. [7] found that about 8—10% of normal Hb-A_0 is glucosylated at the NH_2-terminus of the α-chains or at lysine amino groups, and our mean percentage value of Hb-A_{1x} for normal samples is about 3%, so that 5—7% of glucosylated hemoglobins should still be located in the early part of the Hb-A_0 peak. From calculations of the thiobarbituric acid test, the percentage glucosylation obtained is in very good agreement with the percentage values postulated above: the leading edge of the Hb-A_0 peak contains about 5—7% of glucosylated hemoglobins, while in diabetic samples the percentage increases to about 12—16% of the whole Hb-A.

However, it is not easy to define which type of glucosylated hemoglobin is present in the early part of the Hb-A_0 peak or in the Hb-A_{1x} component. Once it is clearly established that Hb-A_{1c} is the diglucosylated form of Hb-A on the NH_2-terminus of both the β-chains, it is important to obtain information on the presence, position and percentage of the monoglucosylated form. In effect, a simple non-enzymic, non-cooperative attack of glucose should account for about 20% of the monoglucosylated form when 4% of the diglucosylated component (Hb-A_{1c}) is present. This percentage is much higher than the sum of the Hb-A_{1x} percentage and the percentage of glucosylated hemoglobins confined to the early part of the Hb-A_0 peak.

In the experiments with labelled glucose a very distinct peak can be noted in the position of the first 6—10 fractions of the Hb-A_0 peak: if the monoglucosylated form is present in this peak it should account for about the same percentage as the diglucosylated form (Hb-A_{1c}), since it has twice the specific activity of the latter. Again, this interpretation does not comply with expectations. In any case, to date there is not clear proof of a consistent presence of a monoglucosylation step for Hb-A_{1c}. This could be explained not as a demonstration of enzymic or high cooperative reaction, but rather on the assumption that this expected hemoglobin, monoglucosylated on the NH_2-terminus of the β-chains, could have a higher dimeric dissocation constant ($K_{4,2}$ splitting constant [17]) than those of Hb-A_{1c} and Hb-A.

REFERENCES

1 W.A. Schroeder and T.H.J. Huisman, in M.K. Schwartz (Editor), Clinical and Biochemical Analysis, Vol. IX, Marcel Dekker, New York, Basel, 1980.
2 H.F. Bunn, K.H. Gabbay and P.M. Gallop, Science, 200 (1978) 27.
3 M.J. McDonald, R. Shapiro, M. Bleichman, J. Solway and H.F. Bunn, J. Biol. Chem., 253 (1978) 2327.
4 M.J. McDonald, M. Bleichman, H.F. Bunn and R.W. Noble, J. Biol. Chem., 254 (1979) 702.
5 R. Krishnamoorthy, G. Gacon and D. Labie, FEBS Lett., 77 (1977) 99.
6 H.F. Bunn, D.N. Haney, K.H. Gabbay and P.M. Gallop, Biochem. Biophys. Res. Commun., 67 (1975) 103.
7 R. Shapiro, M. McManus, C. Zalut and H.F. Bunn, J. Biol. Chem., 255 (1980) 1406.
8 K. Betke, H.R. Marti and I. Schlicht, Nature (London), 184 (1959) 1877.
9 W. Jelkmann and C. Bauer, Anal. Biochem., 75 (1976) 382.
10 E. Antonini and M. Brunori, Annu. Rev. Biochem., 39 (1970) 977.
11 R. Fluckiger and K.H. Winterhalter, FEBS Lett., 71 (1976) 356.
12 B. Trueb, C.G. Holenstein, R.W. Fisher and K.H. Winterhalter, J. Biol. Chem., 255 (1980) 6717.

13 M. Castagnola, P. Caradonna, M.L. Salvi, R. Pellicano and D. Rossetti, J. Clin. Chem. Clin. Biochem., in press.
14 P.J. Higgins and H.F. Bunn, J. Biol. Chem., 256 (1981) 5204.
15 R.M. Bookchin and P.M. Gallop, Biochem. Biophys. Res. Commun., 32 (1968) 86.
16 M.H. Garner, R.A. Bogart, Jr. and F.R.N. Gurd, J. Biol. Chem., 250 (1975) 4398.
17 E. Antonini and M. Brunori, Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland, Amsterdam, 1971, pp. 110—119.