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ANALYSIS OF THE GLOBINS FROM FAST HUMAN HAEMOGLOBINS BY ISOELECTROFOCUSING ON POLYACRYLAMIDE GEL RODS

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SUMMARY

The globins from all fast haemoglobin (Hb) components obtainable by Bio-Rex 70 cation-exchange chromatography were examined by isoelectrofocusing on polyacrylamide gel rods with 8.0 mol/l urea. From this analysis HbA_{1a_1} and HbA_{1a_2} seem to be very heterogeneous components. HbA_{1b} is separable into two components, one of which is varied in both the β chains. Between HbA_{1b_2} and the well-known HbA_{1c} components two chromatographic peaks are separated, one with a noticeable percentage of glucosylated β chain and one that probably contains HbF. HbA_{1c} has both β chains glucosylated, while HbA_{1x} seems to be a β monoglucosylated Hb form. Finally, the early part of the HbA_0 peak has a large amount of glucosylation on both α and β chains.

INTRODUCTION

We recently showed, by cation-exchange chromatography, that normal human haemoglobin can be separated into a number of fast components, more than previously known [1, 2]. One of these components, HbA_{1c}, is structurally defined as glucosylated on the α-NH_2 of the terminal valines of the β chains [3]. Some components (HbA_{1a1}, HbA_{1a2}, HbA_{1x}) are awaiting confirmation of their identity [1, 2, 4]. Others (HbA_{1b} species) are still structurally unknown [5].

In this present study we describe the globin analysis of all these components, also concerning the early part of HbA_0 peak, where glucosylation on ϵ-NH_2 of lateral lysines is postulated [1, 6]. For the analytical separation of globin chains we improved the isoelectrofocusing by using polyacrylamide gel rods in 8.0 mol/l urea.

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Some authors [7—9] performed similar globin separations by isoelectrofocusing on slab gels, with or without non-ionic detergents, choosing to screen a large number of samples. The aim of our study, however, was to check a reduced number of samples with a large separation between globin chains, thus we preferred to use the higher separation properties of rod isoelectrofocusing. In fact, in comparison to slab isoelectrofocusing [9], the rod method allows the simultaneous characterization of modifications on α and β globin chains.

MATERIAL AND METHODS

Reagents

All common reagents were analytical grade from Merck (Darmstadt, F.R.G.) and from Carlo Erba (Milan, Italy). Bio-Rex 70 (200—400 mesh, Na^+) and AG 501-X8 (D), (20—50 mesh) mixed-bed resin were from Bio-Rad Labs. (Richmond, CA, U.S.A.). YM 10 membranes were from Amicon (Lexington, MA, U.S.A.). Ampholine 6—8 and 7—9 were from LKB (Bromma, Sweden). Fibrous cellulose powder CF12 was from Whatman (Maidstone, Kent, U.K.). N,N,N'N'-tetramethyl-1,2-diaminomethane (TEMED) was from BDH (Poole, U.K.) and Coomassie Brillant Blue R-250 (C.I. 42,660) was from Serva (Heidelberg, F.R.G.).

Preparation of haemoglobin samples and chromatographic separation of fast components

The haemolysis of Hb samples and their chromatography on Bio-Rex 70 were performed on the basis of the method previously described [1]. In order to obtain a quantity of small components sufficient for the subsequent isoelectrofocusing, the sample load was 25 ml of hemolysate and column dimensions were 7 cm^2 × 60 cm. The volume of elution buffer was proportionally increased as shown in the results. Elution with 0.10 mol/l sodium chloride in 0.05 mol/l potassium phosphate buffer (pH 6.55 ± 0.02) was increased with respect to the previous method to provide better resolution in the zone of the HbA_{1b} components (see Fig. 1). Each chromatographic component was pooled and concentrated, under nitrogen pressure, by Amicon YM 10 membranes.

Separation of globin from haemoglobin components

The globin from each Hb chromatographic component was obtained on the basis of the acid acetone method of Rossi-Fanelli et al. [10]. The dried globin samples were kept at —20°C.

Isoelectrofocusing in polyacrylamide gel rods with 8.0 mol/l urea

Before the gel preparation 10.0 mol/l urea was filtered on fibrous cellulose powder CF12 and subsequently eluted on a small column of AG 501-X8 (D) mixed-bed resin. Then 2.425 g of acrylamide and 0.075 g of N,N-methylene-bis-acrylamide were dissolved in 30 ml of 10.0 mol/l urea solution; 250 mg of AG 501-X8 (D) resin were added to this solution and left under mild stirring for 30 min. After lint filtration 1.6 ml of Ampholine 6—8 and 1.6 ml of Ampholine 7—9, 5.0 ml of glycerol, 10.0 ml of 10.0 mol/l urea and distilled

water were added up to a total volume of 49.5 ml. This solution, after stirring, was degassed under vacuum for 15 min.

Then 50 μl of TEMED and 0.5 ml of ammonium persulphate (freshly prepared) at a concentration of 18 mg/ml (20°C) were added; in the various preparations this persulphate concentration was slightly changed depending on room temperature. Immediately, 2.0-ml aliquots of this solution were poured into tubes (bottom capped) 5 mm (diameter) × 120 mm and 100 μl of distilled water were gently layered on the surface. The gels were left to polymerize overnight.

Isoelectrofocusing was performed in a vertical jacketed chamber (Minivolt, Rome, Italy). The gel surface was washed once with a small volume of 10.0 mol/l urea and the top of the tube was filled with a solution obtained by mixing 12 ml of 10.0 mol/l urea, 2 ml of glycerol, 0.5 ml of Ampholine 6—8 and 0.5 ml of Ampholine 7—9. The bottom chamber was filled with 0.01 mol/l ethanolamine and the upper chamber with 0.01 mol/l glutamic acid. Prefocusing was performed in the precooled chamber at a constant current of 1 mA/gel (LKB 2197 power supply) (top, anode; bottom, cathode) for 20 min. The protective upper solution was then discharged and 20 μl of the sample were layered on the surface of the gel.

The samples were prepared by dissolving 2.0 mg/ml of each globin in a solution obtained by mixing 0.8 ml of 10.0 mol/l urea, 20 μl of β-mercaptoethanol, 40 μl of Ampholine 6—8, 40 μl of Ampholine 7—9 and 100 μl of glycerol.

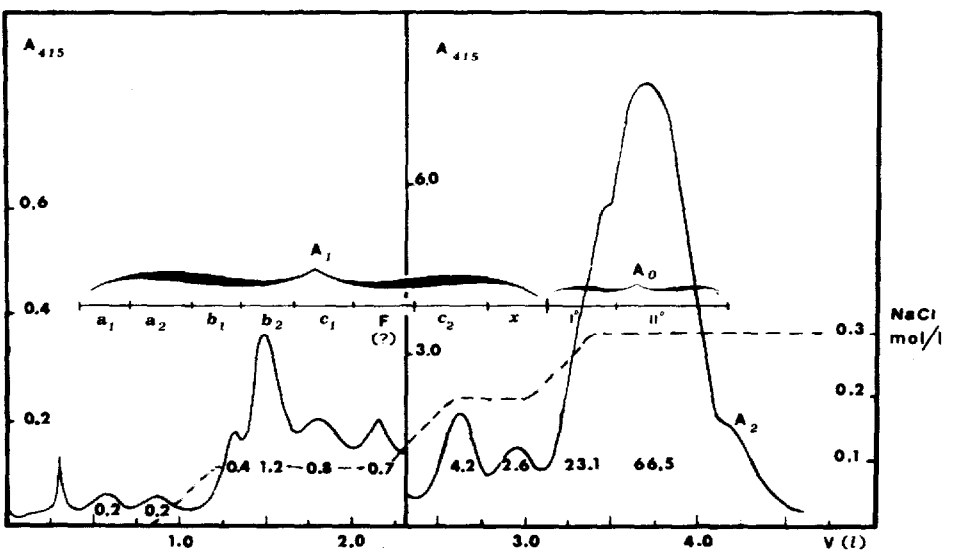

Fig. 1. Bio-Rex 70 cation-exchange chromatography of a normal Hb sample. The elution buffer was a potassium phosphate solution 0.05 mol/l, pH 6.55 ± 0.02 [1]. The dashed line represents the progressive increase of sodium chloride concentration in the buffer. The central line shows how the division of the pools is performed. The name assigned to each pool is also reported; the separation of two new peaks between HbA_{1b_2} and $HbA_{1c(2)}$ caused some problems in name assignation. Thus it was preferred, on the basis of the following experiments, to call the first peak HbA_{1c_1} and the second HbF, and transform the HbA_{1c} denomination to HbA_{1c_2}. The percentage of each pool (component) is reported on the peak base.

Isoelectrofocusing was performed for 210 min (10°C) at a constant current of 2 mA/gel with a maximal cut-off of 500 V. At the end of the run the gels were removed from the rods by rimming with a water jet. A small piece of gel was cut obliquely, at the bottom of the gel, to determine the gel orientation. The gels were kept for 1 h in fixing solution (17.25 g of 5-sulphosalicylic acid and 57.5 g of trichloroacetic acid dissolved in 150 ml of methanol and 350 ml of distilled water). Then they were stained overnight with Coomassie Brillant Blue R-250 (115 mg in 100 ml of destaining solution) and finally destained in a solution of 250 ml of ethanol, 80 ml of acetic acid and 670 ml of distilled water.

Before the fixing step, one of the gels was cut into slices of 2 mm and the pH of each slice was measured after shaking in 2.0 ml of distilled water until pH stabilization. Each stained gel was scanned by a DD2 Kipp and Zonen densitometer with automated integrator.

RESULTS

In Fig. 1 the pattern obtained using Bio-Rex 70 chromatography of Hb normal samples is shown. The pattern is slightly different than that previously reported [1]: after the HbA_{1b2} component two peaks are now obtained. This is due to longer use of the plateau with 0.10 mol/l sodium chloride in potas-

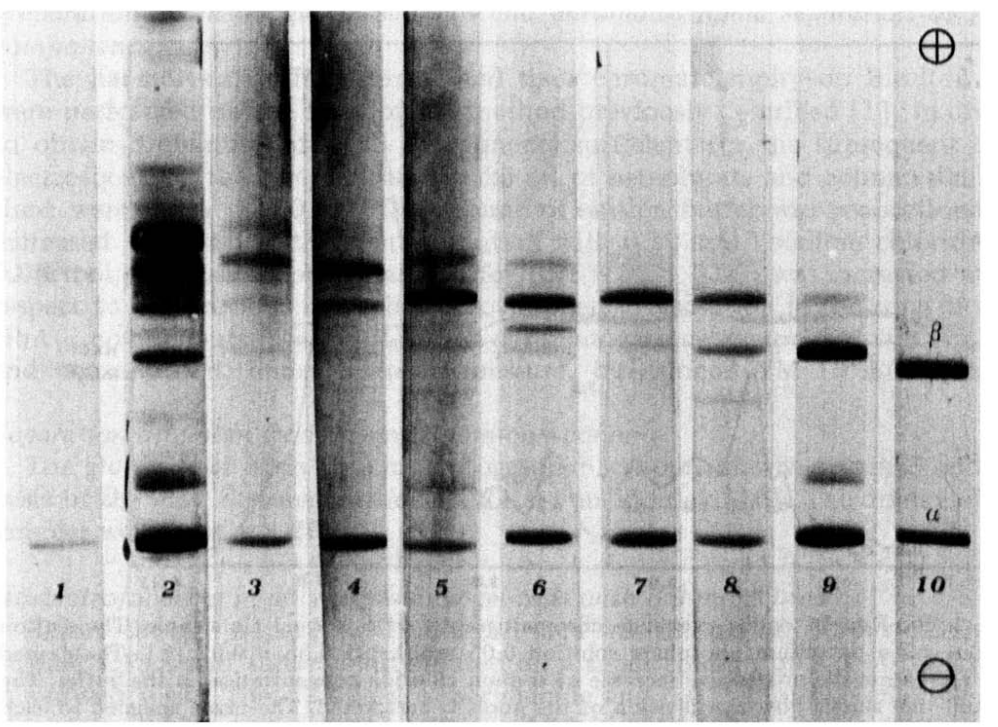

Fig. 2. Isoelectrofocusing rod gels of the globins obtained from the chromatographic pools. The numbers from 1 to 8 represent, in sequence, globin patterns from HbA_1: 1 = a_1; 2 = a_2; 3 = b_1; 4 = b_2; 5 = c_1; 6 = HbF; 7 = c_2; 8 = x. The numbers 9 and 10 are the globin patterns from HbA_0 pools: 9 = 0I°; 10 = 0II°.

sium phosphate buffer. This makes it difficult to assign a name to these components. It is possible to attribute these components to either HbA_{1b} or HbA_{1c} species. On the basis of the subsequent isoelectrofocusing we assign the first to HbA_{1c} species and suppose that in the second HbF is present. Thus, HbA_{1c} is divided into two components, named HbA_{1c1} and HbA_{1c2}, with the F component in between. In any case the nomenclature for the Hb components at this point in time seems to be inadequate and a revision is advisable.

In Fig. 1 the choice of the pools collected is indicated; the HbA_0 peak is divided into two pools, in line with both the thiobarbituric acid colorimetric test [1, 11] and the in vitro incubation experiments with labelled glucose [1]. HbA_{1b} is divided into two components and HbA_{1x} is a component which seems to be monoglucosylated [1]. At the bottom of each peak the percentage of the pool with respect to the total Hb sample is shown. The values of the A_{1b1} and A_{0I° pools do not provide accurate percentages since these peaks are poorly separated from nearby components. The final percentage (HbA_{0II° pool) also includes the HbA_2 component, although it is not considered for pool collection.
sidered for pool collection.

The collected pools are concentrated on Amicon YM 10 and the usual globin separation by the acetone–HCl method [10] is then performed. The yield of globin for each pool is very good except for HbA_{1a2}, where it is very low, and for HbA_{1a1} and HbA_{1x} components, which provide abnormally high yield.

The globins obtained are then examined by isoelectrofocusing on polyacrylamide gel rods with 8.0 mol/l urea solutions. The results are presented in Fig. 2 and in the following. Fig. 2 is a photo of the gels, and Figs. 3—5 are the densitometric scans from the gels, obtained by a scanning densitometer with integrator. In the scans, using as a reference globin from the HbA_{0II° pool, we prefer to report the sequence of components in the opposite order to that of the chromatographic elution (i.e. starting with HbA_{0II° and finishing with HbA_{1a1}). The bottom scale represents the distance (mm) from the top (anode) of the gels. Thus, each peak can be identified simply by its position.

The upper scale represents the pH measured along the gel. The cut-slice method used affords a pH value somewhat different from that obtained by Francina et al. [9] by direct measurement. Furthermore, we do not apply any correction for the presence of urea. In fact, the aim of our separation is principally to obtain a good separation simultaneously for α and β chain modifications and not to determine exact pI_{app} (apparent isoelectric point) values.

The use of Ampholine of two different ranges (6—8 and 7—9) causes an overlap in the middle and is not equivalent to the use of a unique range from 6 to 9. This middle Ampholine excess reflects an amplification in the middle pH zone which has a broader variation than the extreme ones, with the best separation in the zone between 6.75 and 7.50 pH units. Thus, the upper pH scale is not linearly related to the distance from the top of the gel.

The peak height represents the band intensity as measured by the scanning densitometer and the relative percentage of each peak is reported as measured

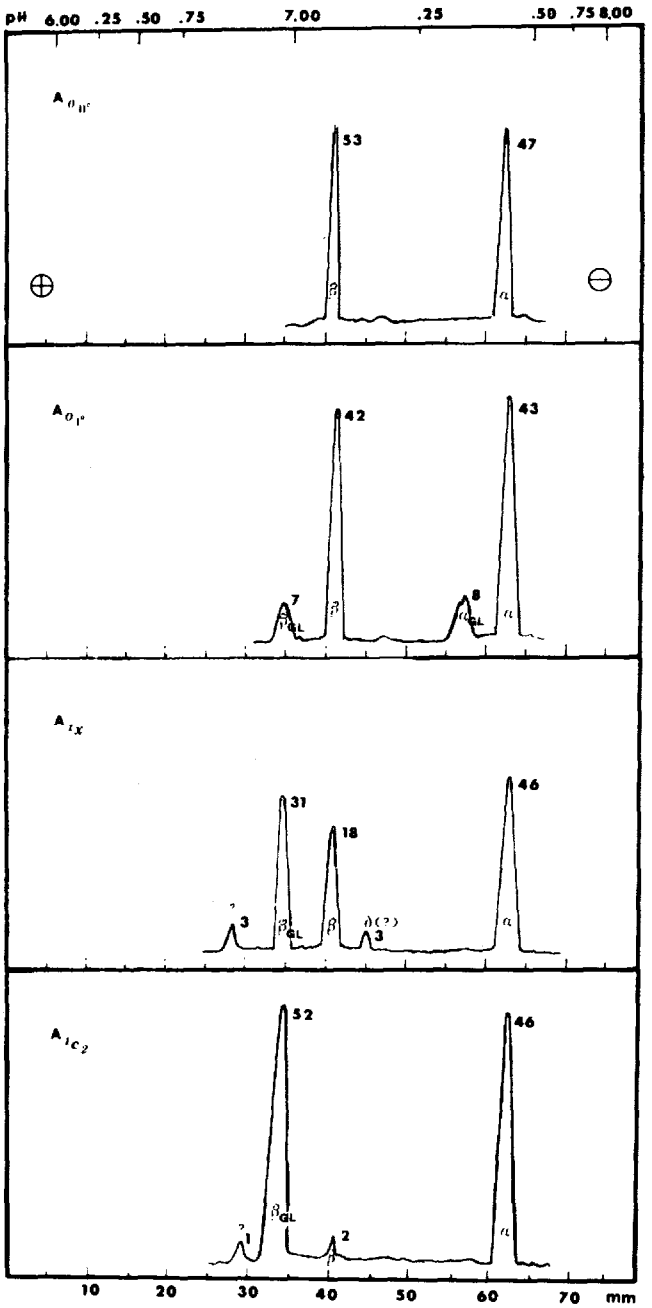

Fig. 3. Scanning of the 10, 9, 8, 7 gels from Fig. 2 by densitometer and integrator. The bottom scale represents the distance of each band from the top of the gel (+ = anodic electrode). The total gel measures 95 mm, thus only the significant zone is reported. The upper scale represents the pH measured by the cut-slice method. The pH scale is not linearly related to the distance owing to the use of Ampholine of two different ranges, with an overlap in the middle zone. The percentage of each peak is reported with respect to the total as considered within each globin sample. α and β subscripts are reported only when it is possible to assign, with some certainty, also on the basis of previous results [1, 11], the band identity. Thus α_{gl} and β_{gl} are for α and β globins glucosylated on the α-NH_2 of terminal valines or on unidentified ϵ-NH_2 of lateral lysines.

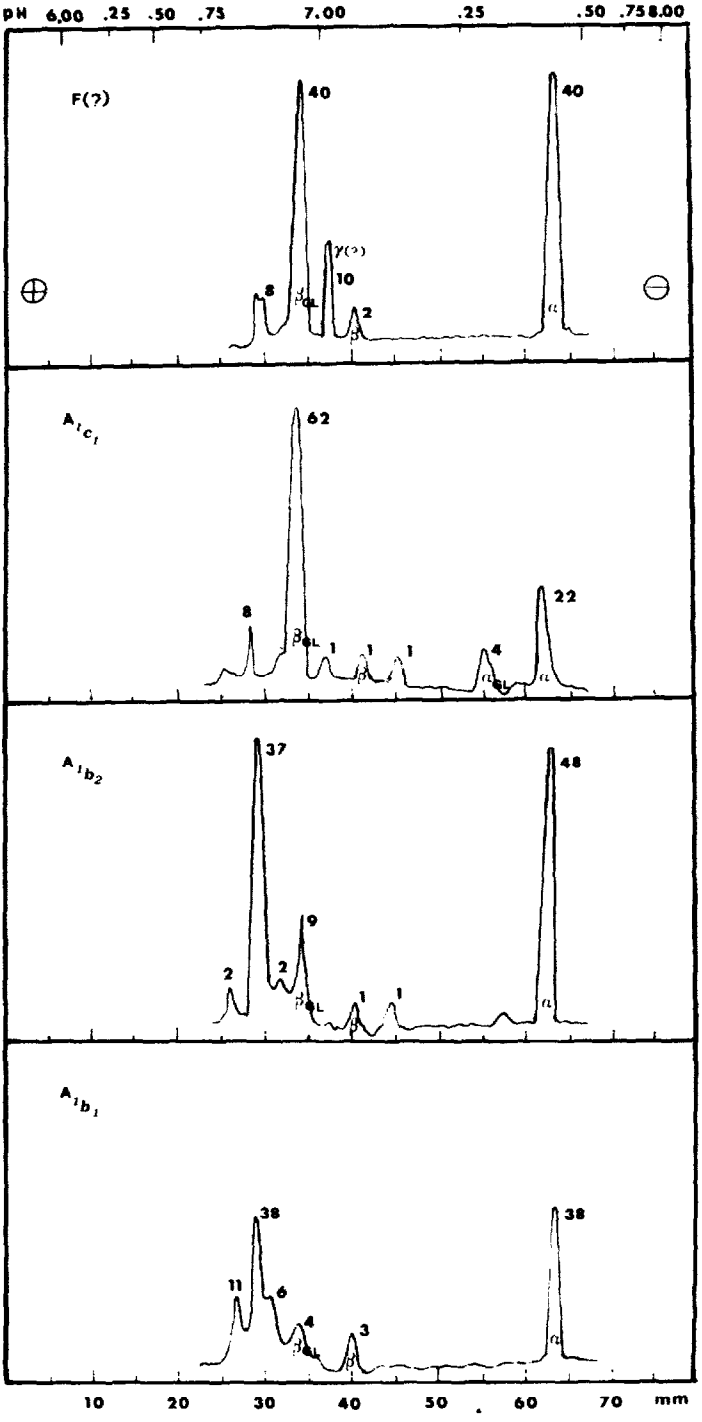

Fig. 4. Scanning of the 6, 5, 4, 3 gels from Fig. 2. The specifications are as in Fig. 3.

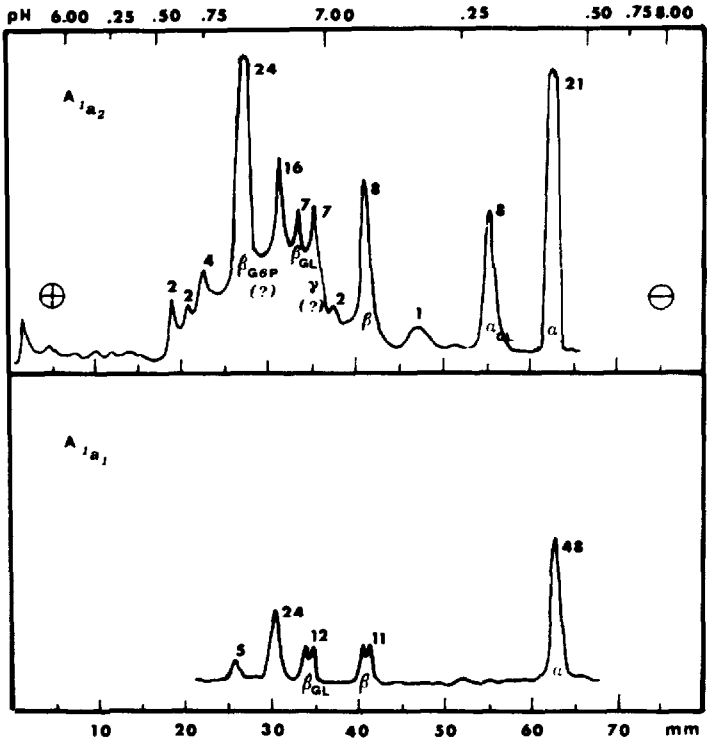

Fig. 5. Scanning of the 2, 1 gels from Fig. 2. The specifications are as in Fig. 3. β_{G_6P} is a β globin chain with a glucose-6-phosphate molecule linked to the α-NH_2 of the terminal valine.

by the integrator. The intensity values are consistent only within the same globin sample; from one scan to another the relative intensity reflects only approximately the variations between the gels because of slight adjustments made to the amplitude of the absorbance scale of the densitometer to avoid too high values in highly stained gels and too low values in poorly stained gels.

The differences in staining among the gels is not due to different quantities of total protein in the samples applied (always 40 μg for each sample) but rather to the possible presence in the sample of some protein contaminants (non-haemoglobin proteins: NHP) which have a pI so different from globin species that they are excluded from the gel ranges. We believe that NHP is definitely present in the globin from the HbA_{1a1} component and, perhaps, in the globin from the HbA_{1x} component. This supposition is confirmed, in the globin separation of these components, by a yield higher than expected.

The position of normal α and β globin chains from the HbA_{0II^0} pool is slightly different from those of the other pools, unfortunately, because this gel is from a different focusing run than the others. This is the best comparison that we can provide, due to various gel losses during the rimming from the tubes. For this reason it is advisable to refer the exact position of normal α and β globin chains to the major bands of the globin from the HbA_{0II^0} pool.

Finally, it must be noted that, until now, no great differences in the globin patterns among the pools collected from normoglycemic and diabetic samples are present.

DISCUSSION

We prefer, as mentioned above, to start our discussion with the HbA_{0II^c} pool in the opposite order to the elution of the components.

HbA_0 is normally assumed to be the pure HbA form. Shapiro et al. [6] reported that about 10% of HbA_0 is glycosylated primarily at the ϵ-amino groups of the lysines. Their experiments allowed also the prevalent sites of glycosylation to be determined, but were partly disproved by the observations of Trueb et al. [12], for the presence of labelled contaminants in the radioactive glucose used in the experiments.

Our recent experiments [1, 11] have confirmed some of their observations and allow the separation, by Bio-Rex 70 chromatography, of a new component, named HbA_{1x}, which was postulated to be monoglucosylated by the thiobarbituric acid colorimetric test (TBA test), by in vitro incubation with labelled glucose and by a significant increase in diabetic samples compared to normoglycemic ones. For the same reasons further glucosylation seems to be present in the early part of the HbA_0 peak.

Thus we attempted to find an improved method for the analysis of modifications on both globin chains, for a control of the sites and the percentage of the glucosylation process. Isoelectrofocusing of globin in polyacrylamide gel rods with 8.0 mol/l urea solutions proved to be a very sensitive method for our purposes.

Application of the method to the two pools collected from the HbA_0 peak provides verification that the HbA_{0II^c} pool, freed from its early (HbA_{0I^c}) and late (HbA_2) parts seems to be completely pure, showing only two bands (in the same percentage) which obviously represent the α and β normal chains. The pI_{app} is 7.05 for β chain and 7.45 for α chain, with some uncertainty resulting from the method of measurement and the presence of urea.

Both α and β chains of the globin from the HbA_{0I^c} pool present an appreciable percentage of modified chain with a position exactly expected for a monoglucosylated chain, as also shown from the position of the β globin chain from HbA_{1c2}. The percentages obtained by peak integration indicate that about 14% of the β chain (7% with respect to the total) and 16% of the α chain (8% with respect to the total) are modified. These values account for about 60% of the total monoglucosylation on Hb from this pool. Since the pool percentage is about 23% of the total Hb, this glucosylated Hb should account for about 13% of the total Hb, half for α and half for β glucosylation. These results are obtained in a normal sample and previous results on diabetics seem to reveal an increase in these percentages. The data described above agree very well with those obtained by the TBA test on the HbA_{0I^c} pool [11].

The glucosylation affects probably the ϵ-NH_2 of the lateral lysines, because it involves groups which keep, even after glucosylation, a positive charge at the pH of the cation-exchange chromatography (pK_a of the lysines = 10). Thus it is very difficult to separate these glucosylation products by chromatography at the pH normally in use. Combining the results of the globin analysis with the results obtained in previous experiments [11] we can confirm that HbA_{1x} is monoglucosylated on the β chain.

The percentages of the peaks of the β and glucosylated β (β_{gl}) chains show contamination by the HbA_{1c2} component. These results confirm the value

of 1.2 mol of glucose per mole Hb tetramer previously obtained [1, 11].

Obviously, from these data, it is impossible to determine the glucosylation site. If this component represents the monoglucosylation product in order to obtain HbA_{1c2}, it accounts for a lower total percentage than expected. In fact for 4% of the diglucosylated form (HbA_{1c2}), statistically 20% of the monoglucosylated form (HbA_{1x}) is expected. This fact could be explained assuming a splitting constant, $K_{2,4}$, for the monoglucosylated form much greater than of HbA_0 and HbA_{1c2}. This assumption should be still more consistent if the glucosylation on HbA_{1x} component is on a different site than the α-NH_2 of the valine of the β chain.

The two slight β bands, of the same percentage, could account for a second Hb component in this chromatographic zone; while that with a pI_{app} lower than that of β_{gl} could derive from a modification similar of that observed for HbA_{1b} components; that with a pI_{app} higher than β should be the δ chain.

No glucosylation on the α chain appears in this component. Finally, we believe that some NHP is also present in this component because of too high a yield of globin (by the acetone method) and a low staining of the bands on the gel. HbA_{1c} is known to be the most abundant fast component of Hb. Holmquist and Schroeder [13] postulated that it is a normal Hb ($\alpha_2\beta_2$) with a blocked N-terminal residue in one of the β chains. Bookchin and Gallop [14] subsequently proposed that both β chains are blocked by N-terminal hexose. Finally, Koenig et al. [15] showed that glucose is the blocking group and after attachment by aldimine linkage it undergoes an Amadori rearrangement which transforms it into a more stable ketoaminic group.

Our results are consistent with this proposal and clearly confirm that HbA_{1c} is glucosylated on both β chains, because of the presence in the globin pattern of only β_{gl} chain, except for a trace amount of normal β chain. The fact that two new peaks appear before this component, during the chromatographic elution, obliges us to introduce a slight variation into the nomenclature of Hb fast components; thus we change the name HbA_{1c} to HbA_{1c2}.

In usual cation-exchange chromatography of Hb it is known that HbF is not easily separable from the HbA_{1c} peak [16]. In the globin analysis of the HbA_{1c2} peak, obtained by the chromatographic conditions described, no peak near the β_{gl} chain is present which justifies the existence of a γ chain. In the globin analysis of the minor peak, eluted before the HbA_{1c2} peak, we find two globin bands with a pI_{app} strictly related to that of the β_{gl} chain (6.87; 6.92 β_{gl}; 7.02). The band with a pI_{app} of 7.02 seems to correspond to the γ chain, also from a preliminary comparison against globin samples from pure HbF preparations (data not reported). Thus we ascribe the chromatographic peak to the HbF component, although a noticeable quantity of HbA_{1c2} is still present in this peak and some uncertainty exists as to the exact position of the γ globin chain.

The globin from the HbA_{1c1} chromatographic peak reveals a very high presence of β_{gl} chain to which an equivalent amount of α chain does not correspond. Moreover, the α chain has an appreciable quantity of glucosylation and some other chains are present in the β position, the most abundant of which is the β chain related to the position of the β globin from HbA_{1b} species. This could derive more from chromatographic overlapping than from a complex Hb component. It is difficult to explain these results and we are performing

further experiments to clarify the globin patterns obtained from this Hb peak and the following ones. In any case, an appreciable quantity of $Hb(\beta_{gl})_4$ should be present in the HbA_{1c1} peak. It is known that HbH has a β_4 composition, thus also the β_{gl} chain could exist in a stable tetrameric form; it could have a stability greater than β_4 and, perhaps, replace the latter, by dissociation equilibrium, with a little disappearance of HbA_{1c2}. In fact no genetic excess of β_{gl} can be supposed. Because of the high percentage of β_{gl} chain we prefer to name this peak HbA_{1c1}, assigning it to HbA_{1c} rather than to HbA_{1b} species.

In the HbA_{1b2} chromatographic component the most abundant chain has a pI_{app} more acidic than β_{gl} (6.88 pH units), as expected from its chromatographic properties. A large percentage of β_{gl} chain is still present in this component, while no appreciable quantity of β chain is present. These results disprove the finding of Krishnamoorthy et al. [5], who, on the basis of electrophoretic data, postulated this Hb component as an asymmetrical hybrid, i.e. with a β normal and a β modified chain. It is possible that neither their chromatographic separation on Hb nor electrophoretic and chromatographic globin separations are able to distinguish the slight differences among the Hb components and between β_{gl} and β chains. If the β_{gl} chain is not due to contamination from a subsequent chromatographic peak, the asymmetrical hybrid must be considered at most, as $\alpha_2\beta_{gl}\beta_{1b}$.

In this case it is also possible that, at the pH of our chromatography, the dissociation equilibrium of Hb always favours glucosylated components instead of the normal ones. On the other hand, the HbA_{1b1} component is probably a Hb modified on both β chains, i.e. $\alpha_2(\beta_{1b})_2$.

In the examination of globin patterns of the two small HbA_{1a2} and HbA_{1a1} components, the first fact to discuss is the large differences in band staining. The HbA_{1a2} component is very intense in all the bands because of the great difficulty in exactly weighing its absolute amount after globin separation by the acetone method (0.2 mg); its yield in globin is very low, thus some contaminants should be present in this peak, but they do not precipitate during the globin separation. Thus the slight overload in the gel is due principally to difficulties in the globin weighing. The HbA_{1a1} component, on the other hand, has very faint bands, even though the acetone precipitate is very abundant; thus, in this peak, a large quantity of NHP, excluded from the gel by their pI, must be present.

The HbA_{1a2} globin pattern is surprisingly complex. This confirms that the rod isoelectrofocusing method has a high resolving power, but leads to the impossibility of being able to interpret the results exactly. It is possible to recognize both α and β modifications, with high percentages of α_{gl} chain, β_{gl} chain and β_{1b} chain; but the most abundant of the β chains is a band with a pI_{app} of 6.81 which corresponds well to a β_{G6P} (glucose-6-phosphate) chain, as expected for this Hb component [2]. HbA_{1a1} also has a complex pattern, but its faint bands do not allow us to verify if a $\beta_{F1,6DP}$ (fructose-1,6-diphosphate) chain is present. In any case, the presence of high heterogeneity in these chromatographic peaks, as previously postulated by McDonald et al. [4], is widely demonstrated.

CONCLUSIONS

Globin analysis of the peaks from Bio-Rex 70 chromatography of normal

Hb leads to a complex picture. The chromatographic peaks are often highly heterogeneous, either because of the presence of many components in the same peak or because of partial overlapping among the peaks. Thus, β_{gl} globin is present, either in high proportions or in trace amounts, in almost all the globin samples examined, while its characteristic peaks should be HbA_{0I^o}, HbA_{1x}, HbA_{1c2}, HbA_{1c1}. The same fact could be observed for the β_{1b} chain which, obviously present in high percentage in the HbA_{1b1} and HbA_{1b2} components, is still present, in trace amounts, from the HbA_{1a2} even to the HbA_{1x} component.

The chromatographic separation is more related to β chain modifications, while α modifications seem to affect only slightly the chromatographic properties of Hb. In fact, the globin analysis shows a continuous increase of the pI_{app} of the β chain in relation to the progressive elution.

Glucosylation on the α chain provides an earlier elution of the Hb within the peak itself, and not net peak separation. This is clear for the HbA_{0I^o} pool but can be postulated also for the HbA_{1a2} and HbA_{1c1} components. These characteristics agree well with the properties of the NH_2 of the terminal valine of the β chain, which seems to be the NH_2 group more prone to carbonyl attachment, either because its pK_a ($\sim$7) is very different from that of the NH_2 of lateral lysines ($\sim$10), or because of its external position which is different from that of the NH_2 of the terminal valine of the α chain.

The relatively easy use of rod electrofocusing makes this method a powerful tool for structural Hb analysis. The higher resolution of the rod compared with that of slab gels must be ascribed more to the higher Ampholine concentration in the rod gel, which allows a stable pH gradient, than to the greater dimension of the rod gels. Application of this method could be extended to many other Hb analysis problems, but, obviously, for some Hb variants, the Ampholine ranges should be varied to obtain maximum separation power.

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