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CARBAMYLATION OF HUMAN HbF ON CARBOXY METHYL CELLULOSE COLUMN IN 8 M UREA pH 6.7

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Summary: Biochemical and immunochemical methods were utilized to establish whether a protein fraction eluted from a carboxy methyl cellulose column by the Clegg technique was a modified gamma (Y) chain of human HbF and should be included with the alpha/non-alpha ratios used to calculate balanced globin synthesis in determination of thalassemia. This component which eluted prior to the gamma chain fraction was found to be different from the gamma chain of human HbF judged by isoelectric point and electrophoretic mobility. However, it was similar to the gamma chain by immunochemical reactivity. Thus, this component should be incorporated into the calculations of the gamma chain when fractionations of hemoglobin by the Clegg column procedure is used to establish the diagnosis of thalassemia.

INTRODUCTION

The Clegg column is used to separate the alpha and non-alpha chains of hemoglobin in order to determine the ratios of alpha to non-alpha globin synthesis in humans (1). Data from these fractionation procedures are then used to interpret the clinical status of the patient with relevance to the possibility of thalassemia. Separation of alpha from non-alpha chains by the Clegg procedure requires urea to keep the globins soluble and dissociated while they are eluted from the carboxy methyl cellulose column. Limitations on the technique are the narrow range over which the differentiation of normal to abnormal protein synthesis is made. The incorrect assignment of small components can easily result in erroneous calculations if the nature of these small components is not know.

Attention was directed to the presence of a component eluting in front of the gamma chain of HbF by the Clegg procedure. This component has been observed by numerous others (1-6).

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Methods: Fresh human cord blood and adult blood was collected in EDTA. Hemolysates were prepared according to Lingrel and Borsook (7), globin as described by Anson and Mirsky (8), and HbF by Kristoffersen (9). The purity of the later preparation was confirmed by cellulose acetate electrophoresis at pH 8.8 (10). Globin preparations were fractionated on carboxy methyl cellulose column as described by Kan et al. (2). The method is a minor modification of the Clegg procedure (1). Prior to use of the column the solutions of 8 M urea were deionized through Dowex columns. All column separations were completed within 8 hours. Electrophoresis of the hemoglobin chains was performed on agarose (Pol - E - Film, Pfizer-Diagnostic) in 4 x 10^{-2}M Sodium Acetate buffer, 6 M urea (deionized), pH 5.8 containing 6 x 10^{-4}M dithiotreitol. Gels were stained with 0.2% Amido black in 5% acetic acid and destained in 5% acetic acid.

Molecular weight estimates were performed on polyacrylamide gels containing 10% sodium dodecyl sulfate (11). Amino acid analysis was performed by standard procedure (12), and terminal analysis and sequential analysis by methods described by Weiner et al. (13). Tryptic digests of chains, not aminoethylated, were performed as decribed elsewhere (1,14). Isoelectric points of the chains were estimated by dissolving them in a series of acids and bases and determining the pH at which each chain was least soluble. Radioactive globin was formed by incubating blood rich in reticulocytes with ^{14}C isoleucine (Amersham Searle, S.A. 342 mCi/mole) (6).

Antiserum to the gamma chain of HbF was produced in New Zealand rabbits. Prior to use the serum was decomplemented and adsorbed with alpha and beta chains and tested for specificity by double diffusion (15).

Carbamylation of the hemoglobin chains was performed as described by Stark (16). Hydantoin production was analyzed by the method of Hagel and Gerding (17).

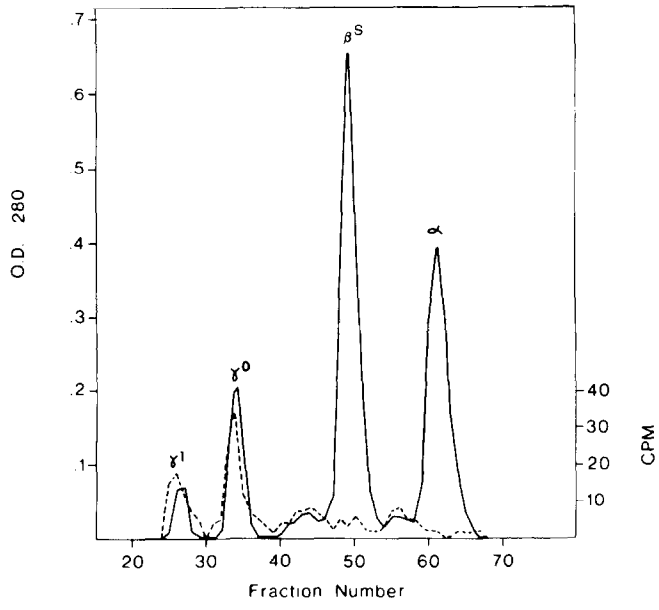

Figure 1: Elution pattern of hemoglobin from patient with HbS disease with elevated levels of HbF. Note the two peaks labeled Y^1 and Y^o both incorporate ^{14}C isoleucine.

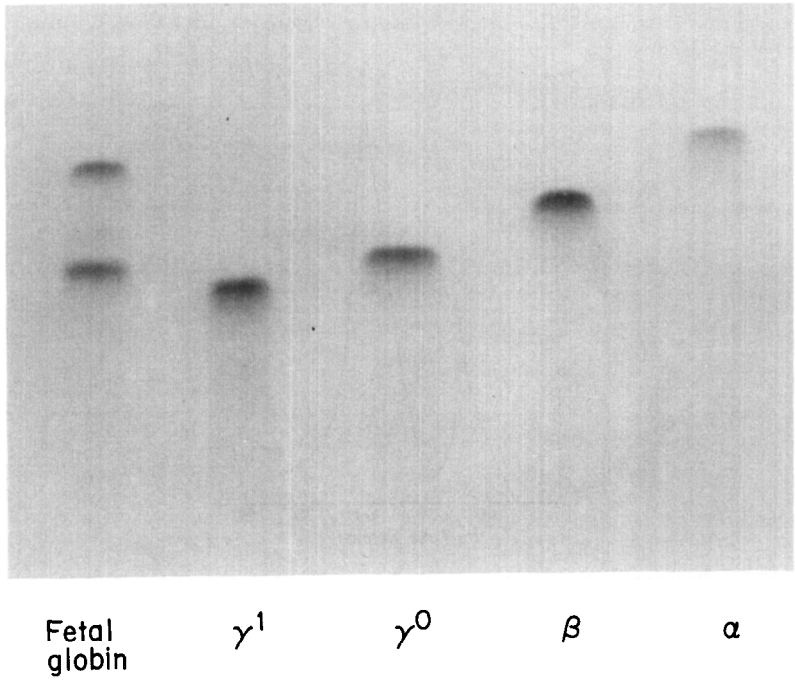

Figure 2: Agarose electrophoresis (pH 5.8, 6 M urea) of the zones from a CMC column. Note that the Y^1 peak migrates anodic to the Y° peak (anode at bottom).

RESULTS

Chromato-graphic patterns of human globin prepared by incubating fetal and adult cells with ^{14}C isoleucine are shown in Figure 1. The leading component was significant compared to the total fetal hemoglobin. This faster eluting component (Y^1) was isolated from the column and further characterized. This component was not the N acetyl derivative of the gamma chain of HbF, which has been previously reported (18), since N terminal analysis showed this group to be glycine.

The Y^1 component had an electrophoretic mobility more anoidic than the Y° chain, and in addition, had an isoelectric point 0.6 pH units less than that of the Y° chain (Figure 2). These observations were consistent with its faster elution from the Clegg column. SDS gel electrophoresis showed both fractions to be 16,000 daltons.

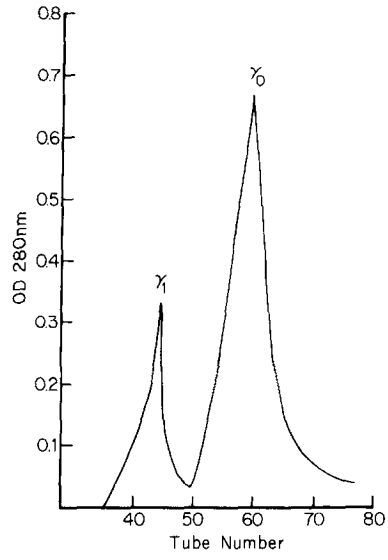

Figure 3: Rechromatography of the Y° fraction from the Clegg column results in an additional minor peak eluting in front of the Y° peaks. End terminal analysis of this peak reveals glycine. Electrophoresis in 6 M urea at pH 5.6 demonstrates that the peak migrates anodic to the Y° fraction (not shown).

Antisera prepared to the gamma chain and reacted with the Y^1 and Y^2 fractions using the Ouchterlony double diffusion method showed them to be confluent with no spurs. This immunochemical analysis provided confirmation of the identity of this material as a derivative of the gamma chain.

Further proof that this component was derived from the Y° chain was obtained by rechromatography of this chain on the Clegg column. These results are shown in Figure 3. A Y^1 fraction was formed on rechromatography of the Y° component and showed an N terminal that glycine was present in 80% of the expected amount. Finally to exclude the possibility of partial carbamylation occurring on the N terminal glycine while the proteins were on the column, the fractions were subjected to conditions favorable to the formation of glycine hydantoins. Neither the Y^1 nor Y° fraction produced N terminal glycine hydantoins while on the column. On the other, hand when both fractions were carbamylated under defined conditions in the test tube, equal amounts of these hydantoins were

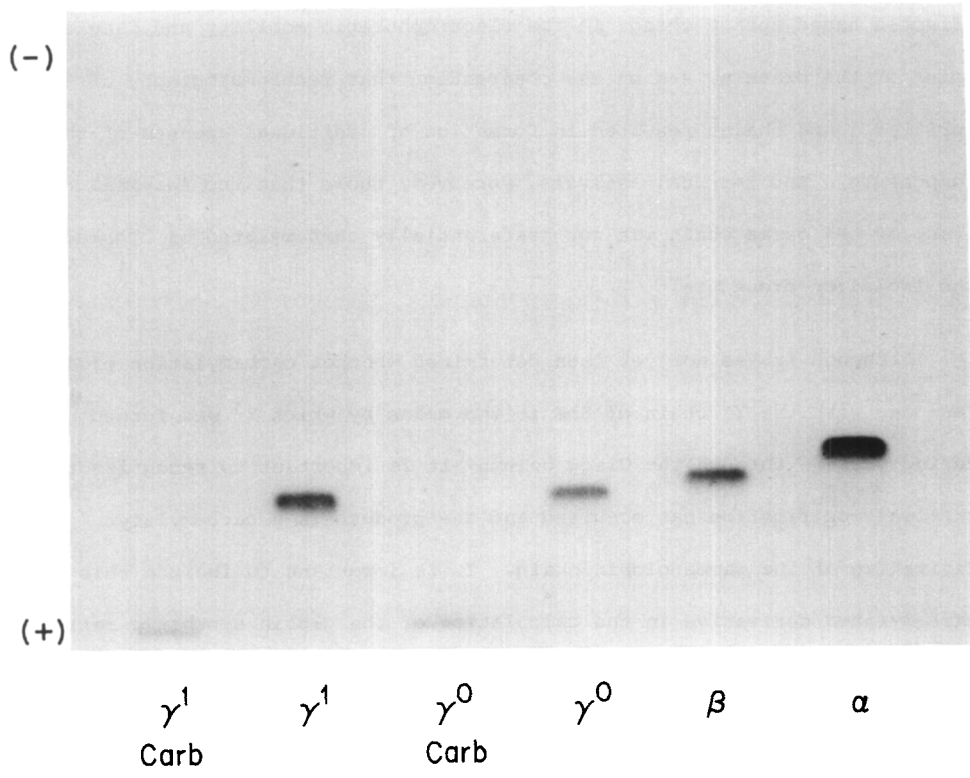

Figure 4: Agarose electrophoresis (pH 5.8, 6 M urea) of the gamma globins zones after carbamylation in the presence of 0.6 M KCNO in 8 M urea pH 8.0. Both Y^1 and Y^0 migrate as descrete bands to the same portion on the agarose plate.

obtained. Furthermore, when these carbamylated proteins were subjected to agarose electrophoresis, both carbamylated chains migrated as discrete band to identical positions (Figure 4).

DISCUSSION

The Clegg column is used to isolate the individual polypeptide chains of hemoglobin. Implicit in the separation is the use of 8 M urea and routinely precautions are made to decrease the amount of cyanate present in the buffer. Our observations show, and are consistent with those of others, that a minor component is eluted off the column when this system is used for the analysis of the chains. The minor Y^1 peak may be attributed to carbamylation of one or more lysyl residues known to be present in the gamma chain. This is consistent with observations that charged residue was

affected based upon a change in the electrophoretic mobility and isoelectric point of the protein, and on the observation that rechromatography of purified gamma chains resulted in formation of additional amounts of the components. End terminal analysis, moreover, shows that the terminal glycyl group of the gamma chain was not preferentially carbamylated or blocked by the isolation procedure.

Although it has not yet been determined whether carbamylation of lysine residues with the Y^{o} chain of HbF is the means by which Y^{1} was formed during passage through the Clegg column, it is important to recognize that a chemical modification has occurred and the product is a carbamylated derivative of the gamma globin chain. It is important to include this carbamylated derivative in the calculation of the globin synthetic ratio so that the ratio is an accurate reflection of all the globin chain produced.

Whether the calculatd ratio will be of aid in the diagnosis of the thalassemic syndrome has been recently questioned (19). In neonates it has been shown that the most accurate means of establishing the diagnosis of thalassemia is with classical hematological methods and electrophoresis, not with column chromatography and globin chain ratios.

Knowledge of the existence of the modified gamma globin is important for another reason. A recent study, employing the Clegg column, reports that the gamma chain of HbF is not synthesized in circulating adult reticulocytes but it is synthesized in circulating burst forming erythroid units (20). Review of the elution profile from this study demonstrates an unmarked peak in the position of the carbamylated gamma globin described above. This suggests that a small amount of HbF may in fact be synthesized in adult reticulocytes as well as in burst forming erythroid units.

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