STRUCTURE OF HEMOGLOBIN A_{Ic}: NATURE OF THE N-TERMINAL β CHAIN BLOCKING GROUP

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The largest of the several minor components of normal human adult hemoglobin isolated by Schroeder and his associates (Allen et al., 1958; Clegg and Schroeder, 1959) by chromatography on the ion exchange resin IRC-50 was designated Hb A_{Ic}, and comprised 5-7% of the hemoglobin found in normal adult erythrocytes. Recent studies of Holmquist and Schroeder (1966) showed that Hb A_{Ic} differed from the major component (Hb A_{II}, $\alpha_2 \beta_2$) by having a compound which was not identified, attached to the N-terminal of the β^{AIc} chain by a $NaBH_4$-reducible Schiff base linkage. The tetramer was believed to contain one unblocked β chain, with a presumed quaternary structure $\alpha_2\beta\beta^{AIc}$. The studies described in the present report indicate that a hexose is linked to the N-terminal of the β^{AIc} chain — the possible attachment of other structures to the hexose has not been excluded — and suggest that the Hb A_{Ic} tetramer may not contain β chains with free N-terminals. Hemoglobin A_{Ic} would appear to be unique both as a hemoglobin glycoprotein and in the mode of attachment of the sugar to the protein.

Hemoglobin A_{Ic} was isolated chromatographically on the ion exchange resin Bio-Rex 70 as described by Holmquist and Schroeder (1966) and comprised about

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6% of hemolysates of normal adult erythrocytes. On starch gel electrophoresis at pH 8.6 (Fig. 1) it migrated slightly anodal to the major component (Hb A_{II}) as noted by Huisman and Horton (1965). Hemoglobin A_{Ic} and Hb A_{II} were treated with parahydroxymercuribenzoate (PMB) at pH 5.8 by a modification of the method of Bucci and Fronticelli (1965) described previously (Bookchin et al., 1967), and on subsequent starch gel electrophoresis the PMB-β chains from Hb A_{Ic} migrated as a single band clearly anodal to PMB-$\beta^{A_{II}}$ chains (Fig. 1). Comparison of peptide maps of the soluble tryptic peptides of PMB-β chains from Hb A_{Ic} and from Hb A_{II} (Fig. 2) revealed that β chains from Hb A_{Ic} showed only trace amounts of peptide in the position of normal βTpI (numbering system of

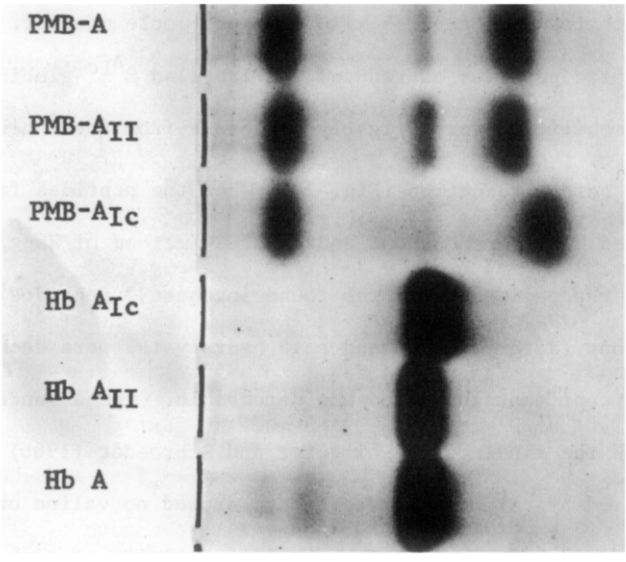

Fig. 1. Vertical starch gel electrophoresis, Tris-EDTA-borate buffer, pH 8.6; anode on right; stained with amido black. The position of Hb A_{Ic} slightly anodal to Hb A (or Hb A_{II}) is apparently due to a charge difference between the $\beta^{A_{Ic}}$ chains and the $\beta^{A_{II}}$ chains. Note that the PMB-A_{Ic} preparation shows no band in the position of the β chains of the PMB-A_{II} preparation.

Gerald and Ingram, 1961), and 2 new peptides, migrating anodal to normal βTpI, were prominent. Following elution and acid hydrolysis both of these new peptides had the amino acid composition of βTpI.

Hemoglobin A_{Ic} and Hb A_{II} (1.5 gm Hb per 100 ml, in 0.1M phosphate buffer, pH 7) were each reacted for 10 minutes at room temperature and 50 minutes at 10° with a 200 molar excess of ^3H-labelled $NaBH_4$ of effective specific activity 2.04×10^6 dpm per μmole (as defined and calibrated by Blumenfeld and Gallop (1966) and Gallop et al., (1968)). Following extensive dialysis, and acid-acetone precipitation of the globins (Anson and Mirsky, 1930), the A_{Ic}-globin had a specific activity of 4.63 to 5.25×10^6 dpm per μmole tetramer, as compared with 0.64×10^6 dpm per μmole tetramer for the A_{II}-globin.

The α and β chains of reduced, ^3H-labelled Hb A_{Ic} were isolated, after treatment with PMB, by electrophoresis on starch granules. The specific activity of the α chains was 0.15 to 0.19×10^6 dpm per μmole monomer, and that of the β chains 1.97 to 2.25×10^6 dpm per μmole monomer. Peptide maps of soluble tryptic peptides of reduced, ^3H-labelled $\beta^{A_{Ic}}$-globin showed 2 peptides with mobilities very slightly different from the 2 new βTpI peptides seen with $\beta^{A_{Ic}}$ before reduction (Fig. 2). When the peptides from the fingerprint of reduced $\beta^{A_{Ic}}$ were cut out and counted (method of Wang and Jones, 1959), most of the radioactivity was found in these 2 peptides, whose amino acid compositions (after elution and acid hydrolysis) were each the same as that of βTpI except that no valine was detectable. The absence of valine was consistent with the findings of Holmquist and Schroeder (1966) that reduced $\beta^{A_{Ic}}$TpI (isolated by column chromatography) showed no valine on amino acid analysis after acid hydrolysis. The specific activity of each of these 2 peptides was approximately 2×10^6 dpm per μmole (referred to lysine yield on amino acid analysis — there is one lysine residue per peptide), indicating a single one stage reduction whereby one non-exchangeable $-\overset{|}{\underset{|}{C}}-{}^3H$ linkage was formed; the same specific activity of the β chains of Hb A_{Ic} suggests a single one stage reduction of each of the β chains at the linkage of the chemical group with the α-amino group of β^1 Valine.

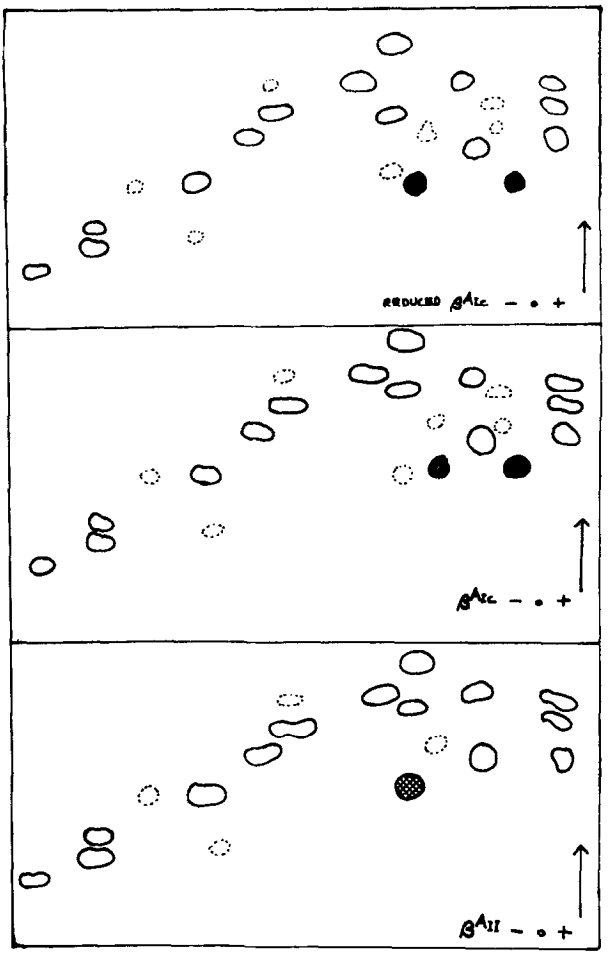

Fig. 2. Diagrams of ninhydrin-stained peptide maps of soluble tryptic peptides of the PMB-β chains of Hb A_{II}, Hb A_{Ic}, and Hb A_{Ic} after reaction with ^{3}H-labelled $NaBH_4$. Electrophoresis in pyridine acetate buffer at pH 4.7, 3000 volts for 100 min; ascending chromatography in n-butanol: acetic acid: pyridine: water (15:3: 10:12) by the methods of Clegg et al., (1966) with minor modifications. The shaded peptide in the map of the $\beta^{A_{II}}$ digest is βTpI; only a trace of peptide is seen in this position on ninhydrin-stained maps of $\beta^{A_{Ic}}$ digest. Both of the shaded peptides in the $\beta^{A_{Ic}}$ map have the amino acid composition of βTpI.

Separate preparations of A_{Ic} and A_{II} globin were acid-hydrolyzed and each was chromatographed on the ion exchange resin AG-IX8 (OH-form), eluting sequentially with water, 0.4N acetic acid, and 1N HCl; about 80% of the radioactivity was recovered with the amino acids in the acetic acid eluate. Aliquots of the acetic acid eluates from Hb A_{Ic} and from Hb A_{II} were chromatographed on the Beckman 120B amino acid analyzer (Resin type AA-15), employing a split-

stream to compare location of amino acid peaks with radioactivity and to obtain the radioactive fractions for further study. The hydrolysate of reduced A_{II} globin showed a diffuse distribution of traces of radioactivity associated with many amino acids, but that of A_{Ic} globin showed 2 prominent ninhydrin-negative radioactive peaks, one eluted before and one overlapping the beginning of the elution of aspartic acid. The first eluted radioactive material, after evaporation of the buffers, was insoluble in ethyl acetate, but soluble in warm butanol. After this material, presumed to be a tritiated N-alkylated valine, was treated with ethanol-HCl to form the ethyl ester, it was then partially soluble in acetone; but upon attempts at mass spectrometry it decomposed before volatilizing. A separate portion of the radioactive ester was further treated with acetic anhydride in pyridine for 1 hour to acetylate the secondary amine and any hydroxyl groups; it was then dissolved in ethyl acetate which was extracted with aqueous $NaHCO_3$ and water and then dried over Na_2SO_4. The material was chromatographed on a silicic acid column developed with benzene

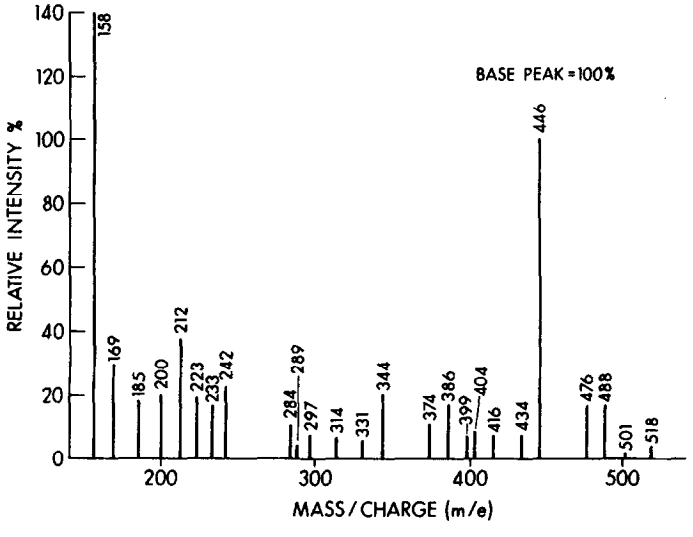

Fig. 3. Mass spectrum of N-,O-acetylated ethyl ester of N-alkyl valine isolated from ^3H-labelled reduced Hb A_{Ic}, obtained at inlet temperature 80°-100° on Hitachi Perkin-Elmer model RMU-6E mass spectrometer. Relative peak heights are referred to base peak at m/e = 446, the most abundant species with m/e over 200. Relative peak intensities varied moderately in runs of different preparations (e.g. on one run the peak at m/e = 386 exceeded the peak at m/e = 446), possibly due to incomplete acetylation of variable portions of the sample.

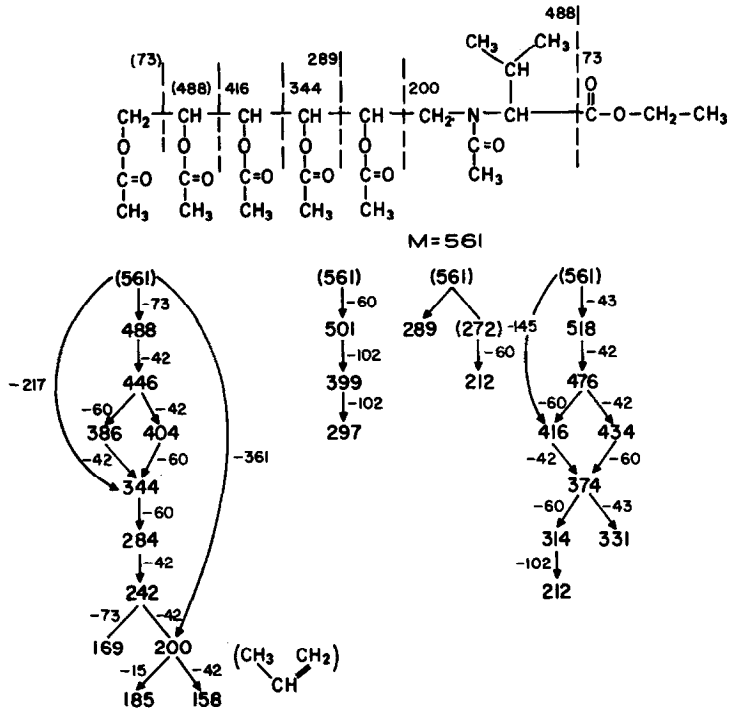

Fig. 4. Possible modes of fragmentation of N-,O-acetylated ethyl ester of N-1-(1-deoxyhexitol)-valine. Certain mass losses could occur by more than one mechanism. Preliminary mass spectra of the N-,O-propionylated derivative of N-alkyl-valine ethyl ester from ^{3}H-labelled reduced Hb A_{Ic} confirm the proposed structure and suggest that the peak at m/e = 488 (M-73) represents primarily the loss of the ethyl ester rather than the $H_2\overset{\uparrow}{C}$-O-Ac at C_6 of the hexose, and that the peak at m/e = 518 (M-43) may represent either (or both) the loss of valyl side chain or the loss of an acetyl fragment.

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and with benzene: ethyl acetate mixtures. The majority of radioactive material was eluted with 50% benzene: 50% ethyl acetate, and yielded the mass spectrum shown in Fig. 3. The spectrum is consistent with the structure shown in Fig. 4 which, prior to esterification and acetylation, would be N-1-(1-deoxyhexitol)-valine. The fragmentation in the mass spectrometer proposed for the present derivative is shown in Fig. 4. A similar mode of fragmentation of polyacetates of hexoses, with losses of acetic acid (60 mass units) and ketene (42 mass units), or 102 mass units in one step (equivalent in mass to acetic anhydride), was observed by Biemann et al., (1963), who also did not observe the molecular ions for such compounds. Similar treatment of the second eluted radioactive frac-

tion (which overlapped the elution of aspartic acid on the amino acid analyzer column) resulted in a mass spectrum whose main peaks were identical with those of the first fraction.

To confirm this structure, N-1-(1-deoxygalactitol)-valine was synthesized by reacting galactose oxime[1] overnight with a 5 molar excess of ^{3}H-labelled $NaBH_4$ to obtain galactamine-1-^{3}H [1-amino-(1-deoxygalactitol), 1-^{3}H)] which was then reacted at pH 11 in aqueous solution with α-Br-isovaleric acid. The desired compound, represented by ninhydrin-negative radioactive material obtained in low yield, was eluted from the Beckman amino acid analyzer column at positions slightly earlier than those of the radioactive peaks from the hydrolysate of reduced A_{Ic} globin. Following esterification and acetylation (as described above), the polyacetyl derivative of N-1-(1-deoxygalactitol)-valine co-chromatographed with the corresponding derivative from Hb A_{Ic} on silicic acid thin-layer chromatography in 20% ethyl acetate: 80% benzene, with an Rf of approximately 0.8; when chromatographed separately the synthetic material moved slightly faster than the derivative from Hb A_{Ic}. The mass spectrum of N-,O-acetylated ethyl ester of synthetic N-1-(1-deoxygalactitol)-valine, isolated from thin-layer chromatograms on silicic acid, contained many prominent peaks identical with those seen in the mass spectrum of the Hb A_{Ic} derivative, including the base peak (m/e = 446), but the spectra were not entirely identical, suggesting an isomeric relationship between the synthetic derivative and that from Hb A_{Ic}.

These results indicate that a hexose is linked to the N-terminal valine of the β^{AIc} chains. Since this structure was defined after $NaBH_4$ reduction and acid hydrolysis, the possibility of attachment of other structures to the hexose (which might be labile under the conditions employed for isolation) requires further study, and is under investigation. The significance of the 2

1. Galactose oxime was chosen because of ready availability; the particular hexose attached to Hb A_{Ic} is not known.

$\beta^{A_{Ic}}$TpI peptides seen on the peptide map is not yet clear.

Several of the findings are inconsistent with the quaternary structure of Hb A_{Ic} proposed by Schroeder and his co-workers ($\alpha_2\beta\beta^{A_{Ic}}$) and suggest rather that there may be no "normal" β chain (with free N-terminal) in the tetramer: (1) the migration of PMB-β chains of Hb A_{Ic} as a single band on starch gel electrophoresis, (2) the finding of only trace amounts of normal βTpI on peptide maps of the soluble tryptic peptides from β chains of Hb A_{Ic}, and (3) a comparison of the specific activities of ^{3}H-labelled, reduced Hb A_{Ic}, $\beta^{A_{Ic}}$, $\beta^{A_{Ic}}$TpI and tritiated N-1-(1-deoxyhexitol)-valine, in each case consistent with a singlefold reduction of each β chain of Hb A_{Ic} at the Schiff base linkage between the hexose and the α-amino group of $\beta^{1\ \text{Valine}}$. Further studies are in progress to establish more definitively the quaternary structure of Hb A_{Ic}.

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REFERENCES

Allen, D.W., Schroeder, W.A. and Balog, J., J. Am. Chem. Soc., 80,1628, (1958).
Anson, M.L. and Mirsky, A.E., J. Gen. Physiol., 13,439 (1930).
Biemann, K., DeJongh, D.C. and Schnoes, H.K., J. Am. Chem. Soc., 85,1763 (1963).
Blumenfeld, O.O. and Gallop, P.M., Proc. Nat. Acad. Sci., 56,1260 (1966).
Bookchin, R.M., Nagel, R.N. and Ranney, H.M., J. Biol. Chem., 242,248 (1967).
Bucci, E. and Fronticelli, C., J. Biol. Chem., 240,PC551 (1965).
Clegg, J.B., Naughton, M.A. and Weatherall, D.J., J. Mol. Biol., 19,91 (1966)
Clegg, M.D. and Schroeder, W.A., J. Am. Chem. Soc., 81,6065 (1959).
Gallop, P.M., Blumenfeld, O.O., Henson, E. and Schneider, A.L., Biochemistry, in press.
Gerald, P.S. and Ingram, V.M., J. Biol. Chem., 236,2155 (1961).
Holmquist, W.R. and Schroeder, W.A., Biochemistry, 5,2489 (1966).
Huisman, T.H.J. and Horton, B.F., J. Chromat., 18,116 (1965).
Wang, C.H. and Jones, D.E., Biochem. Biophys. Res. Commun., 1,203 (1959).