Acetaldehyde reacted with hemoglobin at neutral pH and 37 °C to form adducts that were stable to dialysis and that were not reduced by sodium borohydride. Hemoglobin tetramers having 2, 3, and probably 4 molar eq of bound aldehyde were isolated by cation exchange chromatography. The sites of attachment of the aldehyde were the free amino groups of the N-terminal valine residues of the α and β chains of hemoglobin. Derivatization of the β chains caused a greater increase in the acidity of the hemoglobin than did derivatization of the α chains. Derivatization of the β chains was also preferred over that of the α chains. Acetaldehyde derivatives of the N-terminal octapeptide of hemoglobin S (β9-1 peptide), Val-Gly-Gly, and tetraglycine were formed readily, contained 1 M eq of acetaldehyde/mol of peptide, and were not reduced by sodium borohydride. In contrast, Ala-Pro-Gly failed to form a 1:1 adduct with acetaldehyde. 13C NMR analysis of the peptide adducts formed with [1,2-13C]acetaldehyde indicated that tetrahedral diastereomeric derivatives were produced. The 13C chemical shifts of the adducts formed between hemoglobin and [1,2-13C]acetaldehyde were identical to those of the peptide adducts although resonances from the individual diastereomeric adducts at each hemoglobin site could not be resolved. The results cited above as well as other evidence indicate that acetaldehyde reacts with the amino termini of hemoglobin to form stable cyclic imidazolidinone derivatives. An exchange of acetaldehyde residues between peptides was also documented.

Modified hemoglobins resembling the known minor hemoglobins in their chromatographic and electrophoretic properties are formed when acetaldehyde is added to hemolysates, solutions of purified hemoglobin, or erythrocytes (1-4). The possibility that metabolically produced acetaldehyde, i.e., acetaldehyde generated by oxidation of ingested ethanol, might react directly with hemoglobin (1) or add indirectly to hemoglobin in the form of 5-deoxy-d-xylulose-1-phosphate, a product of the metabolism of acetaldehyde by erythrocytes (2, 5), was supported by the observation that, in some alcoholic individuals, the proportion of minor hemoglobins was abnormally elevated (1, 6). A modified nonglycosylated hemoglobin fraction having chromatographic properties similar to those of hemoglobin A4c appeared to account for this increase (7). Whether produced directly by addition of acetaldehyde or indirectly by the addition of the deoxypentose phosphate, it was appreciated that the modified hemoglobin might serve to evaluate alcoholic consumption, much as levels of hemoglobin Ac4 are used to estimate the degree of chronic hyperglycemia in diabetes mellitus (1, 2).

The reaction of aldehydes with hemoglobin has received attention for other reasons, chiefly because of the effects of aldehydes on oxygen binding, gelation of hemoglobin S, and sickling (8-10). Sodium borohydride reduction of the Schiff base formed between deoxyhemoglobin and pyridoxal phosphate yielded a modified hemoglobin whose affinity for oxygen was permanently increased and unaffected by 2,3-diphosphoglycerate (8). Having stabilized the Schiff base derivative by reduction, it was possible to demonstrate by applying degradative procedures that pyridoxal phosphate had become bound to the α-amino group of the N-terminal valine residue of β chains (8, 11).

Reductive alkylation, as exemplified above, has been the method of choice for localizing sites of binding of aldehydes to proteins (12). The procedure has been applied to elucidate sites of attachment to hemoglobin of glucose (13), glyceraldehyde (14), and glycolaldehyde (15). The reduced products are stable to denaturation and enzymatic and acid hydrolysis so that binding sites can be identified by chain analysis, peptide mapping, and amino acid analysis. A further advantage of reductive alkylation is that radioactive labeling of the sites can readily be accomplished with the use of either tritiated borohydrides as the reductant or labeled aldehyde.

The first step of reductive alkylation of proteins is formation of a Schiff base between the aldehyde and a free amino group of the protein. In the case of glucose and other carbonyl compounds having a neighboring hydroxyl (glyceraldehyde and glycolaldehyde), rearrangements of the Amadori type follow formation of the imine, stabilizing the linkage between added group and protein (16). Like their Schiff base precursors, the rearranged products are susceptible to borohydride reduction. It is noteworthy, however, that the reductive step influences the course of addition of the carbonyl compound. Incorporation of glyceraldehyde, for example, was more rapid in the presence of the reductant than in its absence. However, in the absence of cyanoborohydride, it formed rearrangement-stabilized adducts to β chain termini 10 times more rapidly than to α chain termini whereas, in the presence of the reductant, addition occurred to both chains at approximately the same rate. Furthermore, in the absence of cyanoborohydride, addition of glyceraldehyde to the ε-amino group of lysine residues was preferred over addition to α-amino groups (15).

Although reductive alkylation, keeping the foregoing limitations in mind, is a reliable technique for identifying reducible sites in proteins, it is not useful for localizing sites of addition of carbonyl compounds that form nonreducible adducts. Furthermore, it cannot be assumed that sites of aldehyde addition identified by reductive alkylation will necessar-

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The Ala+b fraction was not observed whereas the AI, fraction into Ala+b and AI, fractions and found that, until the acetaldehyde concentration reached 1 mM or above, an increase in its chromatographic behavior, increased from 4.3% when the acetaldehyde concentration was 3 mM to 23.5% when the acetaldehyde concentration was 30 mM. Virtually 100% of the hemoglobin in metabolizing human erythrocytes was transformed to and stabilized by borohydride reduction, our concern here is with aldehyde-modified proteins that are formed more slowly than Schiff bases, are relatively stable to dialysis, and are not susceptible to reduction by borohydrides.

While the nature of the primary reaction between aldehydes and proteins is relatively simple, namely, nucleophilic attack of the free amino nitrogen on the aldehyde carbonyl, the ultimate effect of aldehydes on protein structure and function is complex. This is due not only to the intricacies of protein structure itself, but also to the influence of kinetic parameters on the structure, yield, and stability of the final product. Both the yield and nature of modified hemoglobins formed by reaction of acetaldehyde with hemoglobin are functions of the acetaldehyde concentration and the duration and temperature of reaction. At 37 °C and a reaction time of 30 min, Stevens et al. (1) observed that the yield of fast moving hemoglobin (FMH), a fraction resembling the entire hemoglobin A fraction in its chromatographic behavior, increased from 4.3% when the acetaldehyde concentration was 3 mM to 22.5% when the concentration was 30 mM. Virtually 100% of the hemoglobin in metabolizing human erythrocytes was transformed to FMH when the cells were exposed overnight at 37 °C to 15 mM acetaldehyde (3). Nguyen and Peterson (17), who incubated hemolysates with increasing concentrations of acetaldehyde at 37 °C for 24 h, resolved the FMH fraction into A1erm and A2erm fractions and found that, until the acetaldehyde concentration reached 1 mM or above, an increase in the A2erm fraction was not observed whereas the Aerm fraction increased above normal values with as low a concentration as 0.5 mM.

The work described in this report was undertaken with the aim of elucidating the stoichiometry of addition and identifying sites of attachment of acetaldehyde to hemoglobin in stable adducts and to throw light on the mechanism of the addition reaction. Our findings disclose that stable adducts of hemoglobin and acetaldehyde are mimizolindinone derivatives.

### MATERIALS AND METHODS

In order to define the conditions necessary to generate acetaldehyde-modified hemoglobins in good yield and having a range of ionic charge similar to that of the minor hemoglobins, 1 mM hemoglobin A0 in 0.002 M bis-Tris, 0.15 M NaCl, pH 7.5, was reacted with 0, 0.2, 1.0, and 5.0 mM [1-3H]acetaldehyde for 20 h at 37 °C. The amount of FMH, estimated by chromatography on microcolumns of Bio-Rex 70 (22), was found to increase roughly in linear proportion to the acetaldehyde concentration. The yields were 3.8, 5.0, 17.4, and 55.1%, respectively. Selecting for further analysis the reaction mixture containing FMH in highest yield, preparative cation exchange chromatography was performed by the method of Abraham et al. (20), giving the results shown in Fig. 1. Seven acetaldehyde-modified fractions were isolated, accounting for 70% of the total hemoglobin and having ionic charges ranging from the most to the least basic of the known minor hemoglobins. The specific activity and amount of each fraction were determined and the stoichiometry of aldehyde addition was calculated from the specific activities of the hemoglobin fractions and acetaldehyde. The values obtained for Fractions II–VII are shown in Table I, column 4. Fraction I was omitted from these calculations since it contained some degraded material and labile radioactivity.

Hemoglobin having 2 or 3 molar eq of bound acetaldehyde/mol of tetramer were the chief products. Yields of Fractions III–VII were strikingly similar (Table I, column 2) with the order of elution from Bio-Rex 70 appeared to be determined by the number of bound aldehyde residues/m eq of hemoglobin. The total yield of modified hemoglobins based on the amounts of hemoglobin in the collected fractions was greater than given by the microcolumn procedure due to separation of Fraction VII from hemoglobin A0, a degree of resolution not realized by the latter method. It is noteworthy that adducts having the same number of equivalents of bound aldehyde per tetramer nevertheless appeared to possess structural differences affecting the order of their elution from cationic exchangers. A somewhat higher degree of resolution of acetaldehyde-modified hemoglobins and a far more rapid analysis was obtained by cation exchange chromatography on a Synchronpak CM-300 column, following the procedure of Huisman et al. (4). Fig. 2 shows the results of analyzing a dialyzed reaction mixture of 1 mM hemoglobin A0 and 4.2 mM [1,2-3H]acetaldehyde (specific activity 1.2 x 106 dpm/pmol).

### RESULTS

The abbreviations used are: FMH, fast moving hemoglobin; HPLC, high pressure liquid chromatography; 6H-F1, the octapeptide Val-His-Leu-Thr-Pro-Val-Glu-Lys; ACh, acetaldehyde; Cbz-, benzoxycarbonyl; TPCK, 1-tosyl-amido-2-phenylethyl chloromethyl ketone.

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1 Portions of this paper (including "Materials and Methods," Figs. 2, 3, 5, 7, and 9, and Tables II–IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3493, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Acetaldehyde and Hemoglobin

Summary of stoichiometries of addition of acetaldehyde to hemoglobin tetramers, α and β chains

| Fraction | % of total Hb | Specific activity | Stoichiometry | No. of modified α chains | No. of modified β chains |
|----------|---------------|------------------|---------------|--------------------------|-------------------------|
| II       | 3.3           | 7.86             | 2.8           | 1                        | 2                       |
| III      | 13.0          | 9.41             | 3.3           | 1                        | 2                       |
| IV       | 10.5          | 9.22             | 3.3           | 1                        | 2                       |
| V        | 13.2          | 5.70             | 2.0           | 0                        | 2                       |
| VI       | 11.9          | 5.87             | 2.1           | 0                        | 2                       |
| VII      | 13.2          | 4.88             | 1.7           | 2                        | 0                       |
| VIII     | 29.3          | 0.94             | 0.23          | 0                        | 0                       |

* Specific activity of [1-3H]acetaldehyde 2.82 × 10^6 dpm/μmol.

After 20 h of incubation at 37 °C, Fractions IV and VII of Fig. 1 are each seen in Fig. 2 to have 2 components. As before, a total of 70% of the total hemoglobin had become modified. An aliquot taken at 4 h contained 53% modified hemoglobin with the most notable reductions in the yields of Fractions II-IVa.

The results in Table I, column 4, indicate that the relative acidity of acetaldehyde-modified hemoglobins, as evidenced by the order of their elution from cationic exchangers, increases with the number of chains to which the aldehyde is bound. This was confirmed by analysis of the fractions by isoelectric focusing, as shown in Fig. 3. The reaction mixture giving the results shown in Fig. 1 was found to focus as 4 discrete bands. The least acidic band was found in the same position as unmodified hemoglobin A0. Following in order of increasing acidity (net negative charge) were bands attributable to Fraction VII, Fractions V and VI, which were unresolved, and Fractions II-IV, which were also unresolved. In general the order of elution from cationic exchangers correlated with their isoelectric points, although the chromatographic methods provided finer resolution.

To ascertain to which chains acetaldehyde was bound, chain analyses of the hemoglobin fractions were performed by reverse phase HPLC. The results of analyzing a known sample of hemoglobin A0 and aliquots of Fractions III, V, and VII are shown in Fig. 4. Chromatograms in column A include assays of radioactivity as well as UV absorbance. In column B are shown chromatograms of the fractions after adding hemoglobin A0 to provide unmodified α and β chains as markers. Chain analyses of Fractions II and IV were indistinguishable from those of Fraction III while the results of analyzing Fraction VI were identical to those given by Fraction V. The number of modified α and β chains in each hemoglobin fraction are listed in columns 5 and 6 of Table I. Thus, from these results Fractions II, III, and IV are found to have the composition ααββ, Fractions V and VI, the composition αβββ, and Fraction VII, the composition ααββ, where x denotes modification due to acetaldehyde binding. These results show that modification of β chains brings about a greater increase in the relative acidity of the modified hemoglobin than does modification of α chains, and that multiple modifications, such as in Fraction II-IV, result in the largest increase. The results also suggest that β chains were more reactive than α chains, in that the amount of hemoglobin having β modifications was larger than that having α modifications. This was also indicated by the results obtained when 1.0 mM hemoglobin A0 was reacted with 0.2 mM [1,2-3H]acetaldehyde for 20 h at 37 °C. With the lower concentration of acetaldehyde fewer chains were found to bind the aldehyde and the most acidic fractions were much reduced in size. At the lower aldehyde concentration no significant formation of adducts having more than 2 modified chains was observed and hemoglobin tetramers having 2 modified β chains (Fractions V and VI) were formed in larger amount than those having 2 modified α chains (Fraction VII).
Reactivity of Hemoglobin $A_\alpha$ with Acetaldehyde—Fractions V and VI, like hemoglobin $A_\alpha$, have modified $\beta$ chains and resemble hemoglobin $A_\delta$, in their chromatographic and electrophoretic behavior. Having 1 mol eq of bound acetaldehyde per $\beta$ chain, the possible site of addition of the aldehyde is limited to the $\alpha$-amino group of the N-terminal valine residue or to a free $\epsilon$-amino group of a lysine residue. In an earlier study hemoglobin $A_\delta$ was found to incorporate no radioactivity when erythrocytes were exposed to 0.180 mM $[1^\text{H}]$acetaldehyde suggesting that glycosylation of the $\beta$-1 valine residue of hemoglobin blocked addition of acetaldehyde to hemoglobin. This inference was tested by reacting 55 $\mu$M hemoglobin $A_\delta$, which had been purified by chromatography on aminophenylboronated agarose (Glyzogel B), with 5.0 mM $[1,2-\text{H}]$acetaldehyde at pH 7.4 for 20 h at 37°C. After dialysis to remove unbound acetaldehyde, chain analysis was performed, collecting 1-ml fractions each minute for counting. The results of this analysis, shown in Fig. 5, reveal modification and labeling of the $\alpha$ chains of hemoglobin $A_\delta$ but not of the $\beta$ chains. This finding clearly shows that the $\alpha$-amino group of the N-terminal valine residue is the major if not the only site of stable attachment of acetaldehyde to the $\beta$ chains of hemoglobin. It should be emphasized that, despite the fact that the $\epsilon$-amino group of lysine residues in hemoglobin has been shown to form a Schiff base with acetaldehyde, glyceraldehyde, or glycolaldehyde, acetaldehyde does not stably bind to this site in the absence of reducing agents.

It was interesting to note that the binding of acetaldehyde by the $\alpha$ chains of hemoglobin $A_\delta$ caused the glycosylated hemoglobin to elute earlier from Bio-Rex 70 than normal hemoglobin $A_\delta$, again illustrating the effect of multiple chain addition, even of an uncharged group, on the net negative charge of the glycosylated hemoglobin. This suggests that, when radioactive acetaldehyde is incubated with hemolysates or erythrocytes, radioactive fractions eluting from cationic exchangers before hemoglobin $A_\delta$ may well be $\alpha$ chain modifications of the glycosylated hemoglobin. Like hemoglobin $A_\delta$, these adducts would be borohydride-reducible.

Borohydride Reduction of Acetaldehyde-Hemoglobin Adducts—Having established the stoichiometry of acetaldehyde binding by hemoglobin $A_\delta$, the question arose whether new reduction sites, equivalent in number to the added aldehyde residues, had been created. To answer this question acetaldehyde-hemoglobin $A_\delta$ adducts were prepared as before but with the use of unlabeled rather than labeled acetaldehyde.

After exhaustive dialysis to remove unbound acetaldehyde, the reaction mixture was reduced with standardized tritiated sodium borohydride, dialyzed to remove labile tritium, and after concentration by ultra-filtration, chromatographed on Bio-Rex 70 by the Abraham procedure to separate acetaldehyde-hemoglobin adducts. Using the data of Table I to assign the stoichiometry of aldehyde addition and the specific activities of the fractions and of the reduced standard to calculate the number of reduced sites, the results shown in Table II were obtained. Although each fraction took up a significant amount of tritium, the number of reduced sites falls far short of the number of added acetaldehyde residues.

Except for the control reductions of hemoglobin $A_\delta$, which added even more than the expected 2 atom of tritium, and hemoglobin $A_\alpha$, whose uptake of tritium is attributable to its content of glycosylated $\alpha$ chains (31), the uptake of radioactivity by the acetaldehyde-modified hemoglobins cannot be accounted for. This type of nonspecific reduction emphasizes the importance of using standardized radioactive borohydrides. The results which were obtained indicate in any case that carbonyl unsaturation of the aldehyde is lost on its addition to hemoglobin.

Tryptic Hydrolysis of Acetaldehyde-Hemoglobin Adducts—Since, other than those formed as Schiff bases, the bonds between acetaldehyde and hemoglobin are not reduced by borohydride and because the adducts are not stable to acid hydrolysis (1), a mixture of radioactive acetaldehyde-modified hemoglobins was subjected to tryptic hydrolysis with the intention of isolating and identifying labeled peptides and thereby localizing the site(s) of attachment of the aldehyde. An aliquot of the hemoglobin mixture having the composition shown in Fig. 2 was hydrolyzed with trypsin for 24 h and the resulting tryptic peptides were separated by reverse phase HPLC. It was noted that, in the course of denaturation and hydrolysis of the hemoglobin, more than half of the bound acetaldehyde was recovered in the distillate collected during lyophilization of the digest. The results of peptide analysis of the lyophilized digest are shown in Fig. 6A. Contrary to expectation, radioactive peaks were scattered throughout the chromatogram. Having observed earlier that addition of the aldehyde to the $\beta$ chains of hemoglobin $A_\delta$ appeared to be limited to the free amino group of the $\beta$-1 valine residue and that this was likely to be true of the addition of the aldehyde to $\alpha$ chains as well, the appearance of a multiplicity of radioactive peaks suggested that, during the incubation with trypsin, acetaldehyde was transferred from the $\epsilon$-amino group of the $\beta$-1 peptide, the N-terminal octapeptide of $\beta$ chains, to the $\epsilon$-amino group of N-terminal amino acid residues of the other peptides released by trypsin. On this assumption the time of tryptic digestion was shortened in the expectation that less exchange would occur. The results of a 30-min digestion of the same sample are shown in Fig. 6B. Except for labile radioactivity at the start of the chromatogram and radioactivity associated with undigested polypeptides at the end, only 2 prominent peaks of radioactivity are seen. The radioactive peaks 1 and 2 from several runs were pooled and purified by rechromatography using ammonium acetate-acetonitrile elution. The results of amino acid analyses of the 2 peptide fractions are shown in Table III. Peak 1 was observed to have the same composition as $\beta$-1, confirming that the site of stable addition of acetaldehyde to $\beta$ chains is the $\epsilon$-amino group of the valine 1 residue. Peak 2 had the composition of $\alpha$-1 + 2 indicating that the free amino group of the N-terminal valine residue of $\alpha$ chains is also the site of stable acetaldehyde modification.

Fig. 6. Reverse phase HPLC analysis of soluble tryptic peptides of acetaldehyde-hemoglobin adducts formed by incubating 1 mM hemoglobin $A_\delta$, with radioactively labeled acetaldehyde. $A$, after 24-h hydrolysis; $B$, after 30-min hydrolysis.
Transfer of Acetaldehyde between Peptides—The transfer of acetaldehyde from one peptide to another was demonstrated as follows. The acetaldehyde derivative of β5T-1 octapeptide was prepared by reacting a 12 mM solution of the peptide with 45 mM [1,2-13C]acetaldehyde for 72 h at 37 °C. The reaction mixture was lyophilized and then analyzed by reverse phase HPLC, giving the results shown in Fig. 7A. The acetaldehyde derivative of the peptide gave the radioactive peak and double peak of UV absorbance shown. The specific activity of the adduct, calculated from the total radioactivity of the collected fractions and the UV absorbance of the doublet showed that it contained 1 molar eq of acetaldehyde/molar eq of peptide. The product yield was 60% based on the starting amount of peptide. The adduct was stable to repeated lyophilization but unstable to incubation for 24 h at 37 °C at pH 3 or 8, decomposing to the extent of 90% and 40%, respectively. The transfer of acetaldehyde from the β5T-1 adduct was demonstrated by incubating a 1.2 mM solution containing 60% adduct and 40% underivatized octapeptide with 12 mM Val-Gly-Gly at pH 8 and 37 °C for 24 h, i.e., under the conditions of time, temperature, and pH employed for tryptic hydrolysis. Peptide analysis of the reaction mixture at the end of the incubation gave the chromatogram shown in Fig. 7B. From the amount of radioactivity in the labeled fractions it was estimated that 90% of the radioactive aldehyde had been transferred from β5T-1 peptide to the tripeptide. Corresponding change in the peak areas of β5T-1 and Val-Gly-Gly remaining at the end of the reaction can be seen to have taken place.

The acetaldehyde derivative of β5T-1 peptide, like the adducts of hemoglobin A0, was not susceptible to reduction by sodium borohydride, indicating that the acetaldehyde derivative of β5T-1 was a suitable model for a study of the mode of attachment of the aldehyde to the N-terminal valine residues of hemoglobin.

13C NMR Studies—The [1,2-13C]acetaldehyde derivative of β5T-1 was prepared by incubation of 100 mM isotopic aldehyde and 6 mM β5T-1 peptide at 37 °C for 72 h. Analysis of the reaction mixture by HPLC showed that 80% of the octapeptide was derivatized. The proton decoupled 13C NMR spectrum of the sample containing 2.4 mM adducts is shown in Fig. 8A. The spectrum consists of resonances from free acetaldehyde and from the peptide adducts. Thus, the doublets at δ = 88.3 and 23.2 ppm arise from the C-1 and C-2 carbons, respectively, of the free acetaldehyde in the hydrated form. Only about 15% of the free acetaldehyde exists in the carbonyl form with chemical shifts of 207 and 30.2 ppm for C-1 and C-2, respectively (Fig. 8D). The resonances from the C-1 carbons of the adducts appeared as a pair of doublets at δ = 70.3 and 69.5 ppm indicating the presence of two doublets. These probably correspond to the two closely spaced HPLC peaks observed for the adducts. No resonances were observed in the region of 140–170 ppm where the resonances for the imine carbons of Schiff bases are known to occur, and with chemical shifts of 70 ppm the C-1 carbons of the adducts were clearly not sp2 hybridized, confirming the results of sodium borohydride reductions. In the proton-coupled spectrum of the β5T-1 adducts, the resonances from the C-1 carbons appeared as a pair of quadruplets, indicating a singly directly bonded hydrogen (Fig. 8B).

When Val-Gly-Gly was reacted with [1,2-13C]acetaldehyde at pH 7.2 under the same conditions, 100% of the peptide was converted to the acetaldehyde derivative. The proton decoupled spectrum of this sample containing 2.7 mM adduct is shown in Fig. 8C. Again a pair of doublets are observed for the C-1 carbons of the adducts at 70.0 and 71.7 ppm. The results imply that a peptide with a free α-amino group is the structural requirement for stable adduct formation. Because of their similarity in intensity and chemical shift, the pair of resonances were postulated to be due to a pair of diastereomeric adducts. To test this, the [1,2-13C]acetaldehyde adduct of tetraglycine was prepared and examined. A doublet from a single adduct was observed supporting this contention. The 13C NMR assignments are summarized in Table IV.

To test whether the adducts formed between acetaldehyde and hemoglobin were the same as those formed with peptides, a solution of 1 mM hemoglobin A0, and 5 mM [1,2-13C]acetaldehyde was incubated under standard conditions, dialyzed, and analyzed by CM 300 HPLC. Hemoglobin-acetaldehyde adducts I–VII were formed in the same proportions as before and as a whole represented about 70% of the total hemoglobin. The unfraccionated reaction mixture was made 0.01 M in sodium phosphate, pH 7.4, containing 20% D2O and 0.01 M KCN for a final concentration of 0.6 mM hemoglobin for 13C NMR analysis. The KCN was added to eliminate the potential
line broadening and chemical shift effects on the carbon resonances from the adducts due to the presence of paramagnetic methemoglobin (32). A sample of 0.6 mM hemoglobin \( A_\infty \) was prepared in the same way to provide a measure of the background of the \(^{13}\)C NMR resonances due to the naturally occurring \(^{13}\)C nuclei of the protein. The proton decoupled \(^{13}\)C NMR spectrum of the hemoglobin-[\(^{13}\)C]acetaldehyde reaction mixture is shown in Fig. 9B. The observation of the broad resonance in the region of 70 ppm indicating the presence of acetaldehyde adducts similar to those made with peptides. The naturally abundant spectrum of unreacted hemoglobin \( A_\infty \) at the same concentration is shown in Fig. 9B where the resonance at 70 ppm is notably absent. To confirm the observation, the difference spectrum (spectrum A minus spectrum B) was computed and is shown in Fig. 9C. Two broad resonances are clearly observed in the regions of 70 and 20 ppm which coincide exactly with the chemical shifts of the resonances from the C-1 and C-2 carbons, respectively, of the peptide adducts. With two sites of acetaldehyde addition to hemoglobin and two diastereomeric adducts forming at each site, the intrinsically broad line widths of the resonances from the different protein bound adducts could not be resolved from one another even in the spectrum obtained after 40,000 accumulations.

**DISCUSSION**

In reacting with free amino groups of hemoglobin \( A_\infty \), acetaldehyde was observed to form tetrahedral addition products that were stable to prolonged dialysis in the cold and to lengthy preparative and analytical procedures. Schiff bases, which other observers have detected by reductive alkylation of hemoglobin in the presence of an excess of acetaldehyde, have been identified by dialysis. It is noteworthy, however, that, after dialyzing acetaldehyde-hemoglobin reaction mixtures to remove any free acetaldehyde, Stevens et al. found that the yield of reductively alkylated products of acid hydrolysis of the reduced modified hemoglobin increased with the length of time of reduction. Thus, during the reduction, slow transformation of stable adducts to Schiff bases must have occurred.

The relative basicity of acetaldehyde-hemoglobin adducts, as judged by the order of their elution from cationic exchangers or by isoelectric focusing, appears to be determined by the number of chains and the type of chain to which the aldehyde becomes bound. The most basic adduct, which eluted immediately adjacent to or with the leading edge of the hemoglobin \( A_\infty \) peak, was found to have 2 derivatized \( \alpha \) chains and normal \( \beta \) chains. The same chromatographic behavior is exhibited by hemoglobin \( A_\infty \) having glycosylated \( \alpha \) chains and normal \( \beta \) chains (31). The most acidic adduct of known composition had 3 chains to which acetaldehyde was bound (\( \alpha_\alpha\beta_\beta \)). Having properties intermediate between these extremes was a hemoglobin adduct having acetaldehyde bound to the N-terminal valine residues of both \( \beta \) chains, an adduct closely resembling in its chromatographic behavior hemoglobin \( A_\infty \), whose \( \beta \) termini are also the sole sites of chain modification. Although no hemoglobin adduct was identified having acetaldehyde bound to all 4 chains, it is likely that Fraction 1 (Fig. 1) contained an adduct of this composition.

With increasing acetaldehyde concentration not only does the amount of modified hemoglobin increase but so does the relative acidity of the product. Thus, virtually 100% of hemoglobin in erythrocytes exposed to 15 mM acetaldehyde was transformed to products which, according to the order of their elution from Bio-Rex 70, had lost much of their native basicity (3). Similar results have been reported by Stevens et al. (1) and by Abraham et al. (10). The present results indicate that the most acidic of these products are adducts having 3 and probably 4 derivatized chains.

The tendency of \( \beta \) chains to form acetaldehyde adducts more readily than \( \alpha \) chains has been observed in this laboratory and elsewhere. Since values of the pK of the \( \alpha \)-amino group of the N-terminal valine residue of both \( \alpha \) and \( \beta \) chains are closely similar (33), it cannot be argued that the formation of \( \beta \) chain adducts is favored by a higher concentration of free base at the attachment site. Furthermore, since the present experiments were carried out with oxyhemoglobin, salt bridges and hydrogen bonds that are present in deoxyhemoglobin are broken, so that differences in reactivity at the two sites cannot be said to be due to differences in their accessibility to the aldehyde. It is noteworthy that while the amino terminal groups of both chains are equally reactive in forming Schiff bases with glucose or glyceraldehyde, rearrangement of the Schiff base to the stable ketoamine is far more favored in the case of \( \beta \) than \( \alpha \) chains (14). This has been attributed to the influence of the different local environments of the \( \beta \)-1 and \( \alpha \)-1 valine residues on the rate-limiting rearrangement of the aldimine to the ketoamine (14). Similarly the preferential reactivity of \( \beta \)-1 valine towards acetaldehyde may be due to environmental or conformational factors affecting the rate-limiting ring closure that is the final step in the synthesis of imidazolidinones (see below).

Because the stable adducts formed from acetaldehyde and hemoglobin were not susceptible to reduction, localization of the sites of attachment of the aldehyde could not be accomplished by the usual degradative procedures. On reacting acetaldehyde with hemoglobin \( A_\infty \), however, it became evident that the \( \alpha \)-amino group of the N-terminal valine residues was
the probable site of addition of the aldehyde to α and β chains.

Tryptic hydrolysis, so long as it was relatively brief, provided information from which it was possible to deduce that acetaldehyde became bound only to the α-amino group of the N termini of both α and β chains. Our results indicate that, at most, 4 M eq of acetaldehyde can form stable bonds with hemoglobin A.

The intermolecular transfer of acetaldehyde residues from one peptide to another is reason for caution in interpreting the results of experiments using the labeled aldehyde to pinpoint sites of attachment. Whether or not such transfers take place between the N termini of globin chains remains to be determined.

The stability to dialysis and the nonreducible nature of the bond between acetaldehyde and the N-terminal valine residues of globin chains rule out in these adducts Schiff base linkages between the aldehyde and amino groups. The results of 13C NMR spectrometric analysis of peptide and hemoglobin adducts of [1,2-13C]acetaldehyde support that conclusion. Schiff base resonances were absent while resonance peaks attributable to a tetrahedral structure were clearly seen. Pairs of resonance peaks showed that addition of the aldehyde to the free α-amino group of the N-terminal valine residue of β-T1 peptide and to Val-Gly-Gly had created a new chiral center at the α-carbon of the attached aldehyde. Accordingly, diastereoisomers of the peptide adducts were produced. The same must also be true in the case of hemoglobin adducts. While formation of a carbinolamine, as in the reaction of formaldehyde with free amino groups of proteins, would account for these observations, other experimental evidence more strongly suggests that acetaldehyde-peptide and acetaldehyde-hemoglobin adducts are imidazolidinones. In this regard recent studies of the reaction of acetaldehyde with pentapeptide enkephalins are most pertinent (34).

A hypothetical reaction scheme for the synthesis of a 2-methylimidazolidin-4-one from acetaldehyde and an N-terminal valine peptide is shown in Fig. 10. The reaction involves the initial formation of a Schiff base between the aldehyde and the α-amino group of the peptide followed by nucleophilic attack of the amide nitrogen of the peptide bond between the first and second residues of the peptide on the electrophilic imine carbon to form the cyclic imidazolidinone. In the cases of the enkephalins and the related Tyr-Gly-Gly, the structure of the adduct was proposed based on the loss of the proton NMR resonance of the amide hydrogen of the second residue and the appearance of resonances from the added CH₂CH₂ bridging group (35). We have confirmed this observation by proton NMR of the acetaldehyde derivative of Val-Gly-Gly. The structure was confirmed by analysis of the two-dimensional proton NMR spectrum (36). The adducts also resembled in stability the imidazolidinone derivative formed between oxytocin and acetone, the structure of which was characterized by infrared spectrometry and confirmed by synthesis (37, 38).

The observation by 13C NMR spectrometric analysis of a pair of adducts formed by reaction of acetaldehyde with ββ-T1 and Val-Gly-Gly and only a single adduct of tetracycline is consistent with the proposed formation of diastereoisomeric imidazolidinones from the aldehyde and the N-terminal valine peptides and an imidazolidinone enantiomer from the aldehyde and tetracycline. The 13C NMR chemical shifts for the C-1 carbon of the aldehyde residue (C-2 of the imidazolidinone) are also consistent with a carbon bonded to amino and amido functions. However, we are not aware of 13C NMR studies of compounds proved by other methods of analysis to possess the imidazolidinone ring.

We found that Ala-Pro-Gly, which is unable to react with acetaldehyde to form an imidazolidinone, did in fact fail to yield a stable addition product with acetaldehyde whereas no difficulty was encountered in synthesizing stable acetaldehyde adducts of ββ-T-1, Val-Gly-Gly, and tetracycline.

The α-amino group of lysine residues of hemoglobin were found to be unreactive in forming stable adducts with acetaldehyde although Schiff base adducts of the same group have been demonstrated by reductive alkylation. This implies that the microenvironment of the reacting amino group is a factor affecting the production of stable acetaldehyde adducts. Whereas the distance from an α-amino group of a lysine residue to the nearest amide group is out of range for stable interaction, the nitrogen of the peptide bond between the N-terminal and adjoining amino acid residue is precisely situated to form a stable 5-membered ring with the postulated Schiff base or carbinolamine intermediate.

We have observed that the acetaldehyde adduct of ββ-T-1 octapeptide is not split by thermolysin, an endopeptidase that readily cleaved the undervariated octapeptide between histidine 2 and leucine 3 and which hydrolyzes such N-terminal blocked substrates as Chz-Gly-Phe-amine and Chz-Thr-Leu-amine between the first and second amino acid residues (29). This suggests that the acetaldehyde adducts of ββ-T-1 are not simple N-alkylated derivatives. On the other hand, the conformational restraints imposed upon the peptide by the imidazolidinone ring might well prevent proper binding of the peptide to the enzyme and thus block hydrolysis.

The foregoing evidence strongly supports the view that the structure of the attachment site of stable acetaldehyde-hemoglobin adducts is that of a 2-methylimidazolidin-4-one.

It has been known for some time that hemoglobins differing when mixed in solution and that the plane of cleavage between the dimers is always the same (40). In the course of preparative or analytical separations of a mixture of modified hemoglobins, dissociation into dimers takes place with like dimers seeking the same position along the axis of

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**Fig. 10. Reaction scheme illustrating the formation of a 2-methylimidazolidin-4-one derivative from a peptide and acetaldehyde.**

R₁ and R₂ represent the side chains of the first and second amino acid residues of the peptide. Note that two diastereomeric carbinolamines and imidazolidinones are formed in the course of the reaction (only one of each shown).
separation. Reassociation of like dimers in these zones then occurs with the formation of symmetrical tetramers. The separation process itself has become the means by which like dimers, formed from unlike tetramers, segregate and reassociate.

In none of the experiments reported here was a hemoglobin adduct having a single modified chain detected among the products of the reaction between acetaldehyde and hemoglobin. Since 2 diastereoisomeric adducts are formed at each reaction site, four types of hemoglobin tetramers having a single chain modification are possible: $\alpha'\alpha\beta\beta$, $\alpha'\alpha\beta\beta$, $\alpha\alpha'\beta\beta$, and $\alpha\alpha'\beta\beta$, where $x$ and $y$ are diastereoisomers. The absence of hemoglobin tetramers of this form is regarded as evidence of dissociation into and reassociation of hemoglobin dimers. Subunit exchange occurring during the isolation process would yield only symmetrically modified tetramers, as illustrated: $2\alpha'\alpha\beta\beta \rightarrow 2\alpha'\beta\beta + \alpha\alpha'\beta\beta$. Accordingly, we assign the compositions $\alpha'\alpha\beta\beta$ and $\alpha\alpha'\beta\beta$ to Fractions V and VI and $\alpha\alpha'\beta\beta$ to Fractions VIIa and VIIb.

Doubly modified dimers, such as $\alpha'\beta\beta$, would be expected to form a hemoglobin tetramer having 4 modified chains. Although an adduct having this composition was not isolated in any of our experiments, it is more than likely that tetramers having this composition were formed but were eluted in Fraction I which, because of its high content of labile radioactivity and what was believed to be degraded hemoglobin, was not thoroughly analyzed. However, tetramers having 3 modified chains were identified. Although structures of this type would appear to contradict the principle of association of like dimers, the existence of these structures would seem to be evidence of incomplete resolution of dimers having similar but not identical chromatographic properties. Thus, the dimer $\alpha'\beta\beta$ might very well reassociate with the dimer $\alpha\beta\beta$ since, as indicated earlier on, modification of $\beta$ chains by acetaldehyde increases the acidic character of the chains to a far greater degree than does the same modification of $\alpha$ chains. Accordingly, the structures $\alpha'\alpha\beta\beta$ and $\alpha'\alpha\beta\beta$ are assigned to Fractions II and III and $\alpha'\alpha\beta\beta$ and $\alpha'\alpha\beta\beta$ are assigned to Fractions IVa and IVb. The formation of asymmetrically modified tetramers such as $\alpha'\alpha\beta\beta$ cannot be excluded from consideration. It is evident that to an indeterminate extent the method of separation is itself an important factor in directing the reassociation of hemoglobin dimers and thus in the final composition of modified hemoglobins.

Examination of the $^{13}$C NMR spectra of separated fractions of [13C]acetaldehyde-hemoglobin adducts is expected to shed further light on this interesting problem.

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REFERENCES

1. Stevens, V. J., Fantl, W. J., Newman, C. B., Sims, R. V., Cerami, A., and Peterson, C. M. (1981) J. Clin. Invest. 67, 361-369
2. Hoberman, H. D. (1980) Adv. Exp. Med. Biol. 132, 265-273
3. Tsuboi, K. K., Thompson, D. J., Rush, E. M., and Schwartz, H. C. (1981) Hemoglobin 6(3), 241
4. Huisman, T. H. J., Henderson, J. B., and Wilson, J. R. (1983) J. Lab. Clin. Med. 102, 163-175
5. Bartlett, G. R. (1968) Biochim. Biophys. Acta 156, 254-265
6. Hoberman, H. D., and Chioldo, S. M. (1981) Alcohol Clin. Exp. Res. 6, 260-266
7. Hoberman, H. D. (1983) Biochem. Biophys. Res. Commun. 113, 1004-1009
8. Benesch, R. E., Benesch, R., Renthall, R. D., and Maeda, N. (1972) Biochemistry 11, 3576-3582
9. Zaugg, R. H., Walder, J. A., and Klotz, I. M. (1977) J. Biol. Chem. 252, 8542-8545
10. Abraham, E. C., Stallings, M., Abraham, A., and Garbutt, G. J. (1982) Biochim. Biophys. Acta 705, 76-81
11. Benesch, R., Benesch, R. E., Kwong, S., Acharya, A. S., and Manning, J. M. (1982) J. Biol. Chem. 257, 1320-1324
12. Feeney, R. E., Blankenhorn, G., and Dixon, H. B. F. (1975) Adv. Protein Chem. 29, 136-203
13. Bookchin, R. M., and Gallop, P. M. (1968) Biochem. Biophys. Res. Commun. 32, 86-93
14. Acharya, A. S., and Manning, J. M. (1980) J. Biol. Chem. 255, 7218-7224
15. Acharya, A. S., and Sussman, L. G. (1983) J. Biol. Chem. 258, 13761-13767
16. Bunn, H. F., Haney, D. N., Gabbay, K. H., and Gallop, P. M. (1975) Biochem. Biophys. Res. Commun. 67, 103-109
17. Nguyen, L. B., and Peterson, C. M. (1984) Proc. Soc. Exp. Biol. Med. 177, 226-233
18. Hoberman, H. D. (1979) Biochem. Biophys. Res. Commun. 90, 757-763
19. Drakkin, D. L. (1946) J. Biol. Chem. 164, 703-723
20. Abraham, E. C., Stallings, M., Abraham, A., and Clardy, R. (1983) Biochim. Biophys. Acta 744, 335-341
21. Mallia, A. K., Herrmannson, G. T., Krohn, R. L., Fujimoto, E. K., and Smith, P. K. (1981) Anal. Lett. 14, 649-661
22. Abraham, E. C., Huff, T. A., Cope, N. D., Wilson, J. B., Jr., Bransome, E. D., Jr., and Huisman, T. H. J. (1978) Diabetes 27, 931-937
23. Van Assendelft, O. W. (1970) Spectrometry of Hemoglobin Derivatives, Charles C Thomas, Springfield, IL
24. Winter, A., Ek, K., and Anderson, U.-B. (1977) LKB Application Note 250, pp. 1-8, LKB Produkter AB, Bromma, Sweden
25. Shelton, J. B., Shelton, J. R., and Schroeder, W. A. (1982) Hemoglobin 6(5), 451-464
26. Schroeder, W. A., Shelton, J. B., Shelton, J. R., and Powars, D. (1979) J. Chromatogr. 174, 385-392
27. Sprinson, D., and Rittenberg, D. (1949) J. Biol. Chem. 180, 709-714
28. Schroeder, W. A., Shelton, J. B., and Shelton, J. R. (1981) Advances in Hemoglobin Analysis, pp. 1-22, Allan R. Liss, New York
29. Flickinger, R., and Gallop, P. M. (1984) Methods Enzymol. 106, 77-87
30. Paz, M. A., Hensol, E., Rombauer, R., Abrah, L., Blumenfeld, O. A., and Gallop, P. M. (1970) Biochemistry 9, 2123-2127
31. Shapiro, R., McManus, M. J., Zalut, C., and Bunn, H. F. (1980) J. Biol. Chem. 255, 3120-3127
32. Fabry, M. E., and San George, R. C. (1983) Biochemistry 22, 4119-4125
33. Garner, M. H., Bogardt, R. A., Jr., and Gurd, F. R. N. (1975) J. Biol. Chem. 250, 4398-4404
34. Summers, M. C., and Lightman, S. L. (1980) Biochem. Pharmacol. 30, 1621-1627
35. Summers, M. C., and Sanders, M. J. (1980) FEBS Lett. 111, 307-310
36. Gidley, M. J., Hall, L. D., Sanders, J. K. M., and Summers, M. C. (1981) Biochemistry 20, 1880-1885
37. Yamashiro, D., and du Vigneaud, V. (1968) J. Am. Chem. Soc. 90, 487-490
38. Hruby, V. J., Yamashiro, D., and du Vigneaud, V. (1968) J. Am. Chem. Soc. 90, 7106-7110
39. Matsubara, H. (1970) Methods Enzymol. 19, 642-651
40. Guidotti, G. (1967) J. Biol. Chem. 242, 3694-3703
Acetaldehyde and Hemoglobin

Supplemental Material to
EJCTION OF ACETALDEHYDE WITH HEMOGLOBIN

Richard C. San George and Henry D. Robertson

MATERIALS AND METHODS

(1,2-3H) Acetaldehyde was prepared by oxidation of (2,3-3H) alanine (described in detail elsewhere). (1,2-3H) Acetaldehyde was assayed enzymatically and the specific activity determined by counting aliquots of the samples. The specific activities of the preparations used for the experiments reported here were 5.8 x 10^6 and 3.2 x 10^6 dpm/umole of 1,2-3H acetaldehyde, respectively.

Bio Rex-70 (Bio Rad Labs) using the elution method described by Abraham et al. (2).

In all experiments, the final pH of 8.1. Tripin TPEC (Worthington Diagnostics, Inc.) in 0.125 M NaCl with 10% w/v of 1,2-3H acetaldehyde was added to a final concentration in one of the solutions of 2.5 x 10^4 dpm/umole. The reaction mixtures were incubated with stirring at 37°C for times ranging from 30 min to 24 hrs. The reactions were stopped by addition of 20 ml of trichloroacetic acid and the tubes then lyophilized. In some cases procedures were carried out on the same day from (2) and (26) was added for released acetaldehyde after each lyophilization step. For amounts of hemoglobin greater than 25 mg, conditions of the hydrolysis were scaled up proportionately.

The tryptophan content of the samples was taken up in the appropriate buffer and analyzed by reverse phase HPLC using phosphoacetate-acetic gradient elution (26). Solvent A was 46% MeOH containing 0.37 ml of 0.5 NaHPO_4 and solvent B was acetic acid. A gradient from 100% A to 1% B over 60 min, to 100% B over 30 min was used. Fractions from 100 to 200 were collected. The flow rate was 1.0 ml/min at 10^6 dpm/umole with detection at 220 nm and typically about 0.5 mg of depleted was injected. Radioimmunoassays were identified by counting aliquots of 1 ml fractions of HPLC eluate. Preparative HPLC was performed using a 7.8 x 300 mm Water C18 reversed phase column.

The optimum ethanol level for tritiated borohydride was determined by incubating solutions of 1 mg hemoglobin A2, 0,2 and 5.0 M Me acetate for 20 hours at 37°C. The reaction mixtures were analyzed by thin layer chromatography to determine the 24,24'-dialdehyde fraction A1 fraction and by radioimmunoassay as described below. Preparative chromatography of the mixture obtained after reaction with 5.0 M (1,2-3H) acetaldehyde was performed on columns of Bio-Gel P-60 equilibrated against 0.01 M KH2PO4 buffer and analyzed by HPLC using the phosphate-acetonitrile gradient described by Shelton et al. (4). The reaction mixtures were then lyophilized in a nitrogen atmosphere and the residues dissolved in 1 ml of water. Aliquots of the latter and of the distillates were counted. An aliquot of the solution containing the peptide acid and incubated peptide was diluted in 0.37 NaHPO_4 buffer and analyzed by HPLC using the phosphate-acetic gradient elution as described. The product yield and stoichiometry of aldehyde formation was calculated from the data of Shelton et al. (4) and the assumption that 1 atom of aldehyde is produced for each peptide bond.

The radioimmunoassays of (1,2-3H) acetaldehyde and tetraglycine, prepared by (26), were performed using a 7.8 x 30 mm Whatman I IIA alkyl amino acid analyzer using ninhydrin detection.

The otoglycine val-his-leu-thr-pro-val-gly-gly-lys which was the sequence of the first eight residues of the b chain of hemoglobin S and is designated (1,2-3H) tetraglycine, was synthesized and lyophilized and analyzed by reverse phase HPLC using acetonitrile-acetic acid gradient elution (26). Solvent A was 10% acetonitrile in water and solvent B was 100% acetonitrile and the gradient was started at 100% A and 30% B at 100 000, and at 30 000, 2% per minute to 100% B and 10% A at 100 000, 7% per minute to 100% A. A gradient from 100% A to 100% B over 00 min was used at flow rates of 1 ml/min using a 7.8 x 300 mm column of Bio-Gel P-60. The effluent was collected with 1.25 x 10^6 dpm/umole and lyophilized and thus free from contaminating unmodified peptides and from phosphate salts which interfere with subsequent analysis. 100-fold less activity was observed in the absence of background activity.

For comparison of the rate of formation of an acetaldehyde-peptide adduct at acid and neutral pH, 12.5 mmol val-gly-gly and 45 mmol (1,2-3H) acetaldehyde at pH 5.9, the pH of the unbuffered reaction mixture, and the mass concentration of the reactants at pH 7.6 (0.01 M sodium phosphate buffer) were allowed to react for 72 hours, withdrawing aliquots for analysis at appropriate intervals.

Acetaldehyde adducts of (1,2-3H) val-gly-gly and tetraglycine were prepared essentially as described above.

For 12C HPLC spectrometric analysis the residues of the lyophilized mixtures were dissolved in 2 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 200 pmole. An aliquot of each reaction mixture was also analyzed by HPLC.

Reduction of hemoglobin preparations with tritiated sodium borohydride was performed essentially as described by Fidlerick and Gallop (27). Tritiated sodium borohydride (Amersham, dissolved in 0.01 M NaCl was diluted with mild sodium chloride to the concentration of the sodium borohydride stock solution. The concentration of the diluted compound was 5 mmol/ml. To 1 ml of 0.1-1.0 mg hemoglobin sample in 0.1 M potassium phosphate buffer, pH 7.4, was added a 10-fold excess of the sodium borohydride solution and the reaction mixture allowed to proceed for 1 hr at room temperature. The radioactive solution was dialyzed and directed against Dialysat 6 at 4°C until the dialyze contained a negligible number of counts.

The reduction of boron atoms was studied by the following procedures: 0.2 ml of the radioactive borohydride solution was added to 1.0 ml of 1.0 ethanol-0.01 M phosphate buffer, pH 6.5, in which 10-15 mg of proteinase K (Calbiochem, Brockton, MA) had been dissolved. After 1 hr at room temperature excess borohydride was decomposed with NaCl and the solution was diluted to about 15 ml with water. This solution was extracted 3 times with 50 ml portions of ethyl acetate. The extract was dried with anhydrous sodium sulfate and the solvent removed by flash evaporation. The residue was dissolved
in 1 ml of ethyl acetate and 100 ml of this solution then spotted on a silica gel TLC plate. The chromatogram was developed with ethyl acetate-benzene (3:2). The spots were visualized by UV light, outlined in pencil, and scraped. The Rf of the radioactive p-nitrobenzyl alcohol was 0.44 and of the aldehyde, 0.64. The concentration of p-nitrobenzyl alcohol in the extract was determined from its absorbance at 270 nm using a m extinction coefficient of 12.5. An aliquot from the same cuvette was removed for counting. Correction for counting efficiency was made with the use of appropriate standards.

$^{13}$C NMR spectra were recorded on a Varian XL-200 FT-NMR spectrometer operating at 50.3 MHz for $^{13}$C. A standard single 90° pulse sequence was used (64 scans pulse width typically) with a 3.6 (peptides) or 1.0 (hemoglobin) second delay between pulses and with continuous proton decoupling. A 10,000 Hz spectral width was examined. In some cases the proton-coupled spectra were also recorded. Chemical shifts were measured relative to external TMS and an internal diastere standard was also used in some cases. All samples contained 20% D$_2$O for spectrometer lock and spectra were recorded at 20-22°C. 3.5 (peptides) or 20°C (hemoglobin) line broadening was applied to all spectra.

In (A) HPLC analysis of the chains of acetaldehyde-modified hemoglobin A$_1$C formed by reacting 55 mM hemoglobin A$_1$C, separated from unglycosylated hemoglobin by affinity chromatography, with 5 mM $[1,2-^{3}H]$ acetaldehyde. In (B) is shown chain analysis of acetaldehyde-modified hemoglobin A$_0$ formed by the reaction of hemoglobin A$_0$ with radioactive acetaldehyde under the same experimental conditions as in (A). Attention is directed to the modification later elution times than unmodified chains. It will also be noted that 2 different $\alpha$ chains modifications were observed. Solid lines represent absorbance.

**Table I**

| Fraction | Stoichiometry of Act addition | Specific Activity after $^{13}$NaBH$_4$ reduction | Stoichiometry of reduction |
|----------|-----------------------------|-----------------------------------------------|---------------------------|
| II + III | 3                           | 6.18 x 10$^{-6}$                               | 1.2                       |
| II       | 3                           | 7.30 x 10$^{-6}$                               | 1.1                       |
| III      | 2                           | 4.30 x 10$^{-6}$                               | 0.45                      |
| VI       | 1                           | 1.60 x 10$^{-6}$                               | 0.46                      |
| VII + VIII | 0-2                        | 4.42 x 10$^{-6}$                               | 0.46                      |
| A$_0$ control | 0                          | 7.32 x 10$^{-6}$                               | 0.45                      |
| A$_0$    | 0                           | 17.2 x 10$^{-6}$                               | 0.46                      |

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**Table III**

Amino Acid Analyses of $^{13}$H Acetaldehyde-Labeled Tryptic Peptides

| Amino Acid | Peak 1 Found$^a$ | Peak 2 Found$^b$ |
|------------|-----------------|-----------------|
| Asp + Asn  | 0               | 2               |
| Thr        | 1.1             | 1.0             |
| Ser        | 0               | 1.1             |
| Gln + Gln  | 1.9             | 0               |
| Pro        | 0.9             | 0.8             |
| Cys        | 0               | 0               |
| Gly        | 0.4             | 0.1             |
| Ala        | 0.1             | 1               |
| Val        | 1.6             | 2               |
| Ile        | 0               | 0               |
| Leu        | 1.1             | 1.2             |
| Tyr        | 0.2             | 0               |
| Phe        | 0               | 0               |
| His        | 0.7             | 0               |
| Lys        | 0.4             | 1.4             |
| Arg        | 0               | 0               |

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$^a$ - Average of 4 determinations.

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**Figure 2.** Cationic exchange HPLC of modified hemoglobins formed by reaction of 1mM hemoglobin A$_0$ and 4.2 mM $[1,2-^{3}H]$ acetaldehyde. Finer resolution of peaks IV and VII is seen. Solid lines represent absorbance.

**Figure 3.** Isoelectric focusing of Fractions I-VII of figure 1. Lanes VIII and IX are those of hemoglobin A$_0$ and hemoglobin A$_2$, respectively. Total R. M. denotes total unfraccionated reaction mixture and A$_0$ is the pattern given by a separately isolated sample of hemoglobin A$_0$. See text for further comment.
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TABLE IV

| Signal | ppm | Assignments |
|-------|-----|-------------|
| A     |     | C-1, hydrated acetaldehyde |
| B     |     | C-1, diastereomer I |
| C     |     | C-1, diastereomer II |

Table IV: Summary of 13C NMR Assignments

Figure 7. (A) reverse phase HPLC peptide analysis of 857-1 octapeptide (val-his-leu-thr-pro-val-glu-lys) after 3 days incubation at 37°C with 45 mM [1,2-3H] acetaldehyde. (B) HPLC peptide analysis of a 1.2 mM solution of the reaction mixture in (A) after incubation with 12 mM val-gly-gly, pH 8, for 24 hours at 37°C. Attention is called to the disappearance of radioactivity and UV absorbance due to the 857-1 adduct and the appearance of a new radioactive peak and a small UV absorbance peak due to formation of the acetaldehyde derivative of val-gly-gly. Solid lines represent absorbance.

Figure 8. (A) 50.3 MHz 13C NMR spectra of acetaldehyde-modified hemoglobin. (A) 13C-decoupled 13C NMR spectrum of the reaction mixture prepared by incubation of 1 mM hemoglobin A₂ and 5 mM [1,2-13C] acetaldehyde which was dialyzed and made 0.5 M sodium phosphate pH 7.4, 0.01 M KCN, 20% D₂O for a final hemoglobin concentration of 0.4 mM. (B) 13C-decoupled natural abundance 13C NMR spectrum of 0.6 mM hemoglobin A₂ under the same conditions. 3050 accumulations for spectra A and B. (C) the difference spectrum (spectrum A minus spectrum B).

Figure 9. (A) 50.3 MHz 13C NMR spectra of acetaldehyde-modified hemoglobin. (A) 13C-decoupled 13C NMR spectrum of the reaction mixture prepared by incubation of 1 mM hemoglobin A₂ and 5 mM [1,2-13C] acetaldehyde which was dialyzed and made 0.5 M sodium phosphate pH 7.4, 0.01 M KCN, 20% D₂O for a final hemoglobin concentration of 0.4 mM. (B) 13C-decoupled natural abundance 13C NMR spectrum of 0.6 mM hemoglobin A₂ under the same conditions. 3050 accumulations for spectra A and B. (C) the difference spectrum (spectrum A minus spectrum B).