

# Acetaldehyde Adducts with Hemoglobin

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**ABSTRACT** Clinical studies on the minor hemoglobins (hemoglobin A<sub>1a-c</sub>) have suggested that a novel adduct may form in people abusing alcohol. Such patients were found to have an elevated concentration of minor hemoglobins, but normal or subnormal amounts of glycosylated hemoglobin (hemoglobin A<sub>1c</sub>) as determined by radioimmunoassay. Acetaldehyde, a reactive metabolite of ethanol, was postulated to form adducts with hemoglobin A that change its chromatographic properties.

At physiological concentrations, acetaldehyde was found to form adducts with hemoglobin that were stable to extensive dialysis for several days. The amount of hemoglobin adducts formed were a function of the concentration and number of exposures to acetaldehyde. The reaction of acetaldehyde with hemoglobin A produced chromatographic variants, some of which migrated in the hemoglobin A<sub>1a-c</sub> region. Analysis of stable acetaldehyde-hemoglobin adducts demonstrated that valine, lysine, and tyrosine residues of globin were sites of reaction. The acetaldehyde-modified amino acid residues appear to exist in interconvertible conformations, only one of which is reducible by sodium borohydride. The amount of these adducts was found to be significantly elevated in hemoglobin from alcoholics as compared with normal volunteers.

## INTRODUCTION

Clinical interest in the heterogeneity of hemoglobin A has been stimulated by the observation that post-translational adducts of the protein form spontaneously with reducing sugars (1, 2). The resulting minor hemoglobins (HbA<sub>1a-c</sub>)<sup>1</sup> have been used increasingly

as a means of monitoring hyperglycemia in diabetes mellitus (3, 4). One of these components, hemoglobin A<sub>1c</sub>, has been shown to form from the reaction of glucose with the N-terminal valine of beta chains of hemoglobin A (5). An initial Schiff-base adduct is formed and is stabilized by a subsequent Amadori rearrangement from the aldimine to a ketoamine (6, 7). Clinical studies have shown that either hemoglobin A<sub>1c</sub> or the whole fast-eluting fraction, HbA<sub>1a-c</sub>, integrates the mean blood glucose over the previous 3 wk (4, 8).

In the course of clinical studies on minor hemoglobins, we found that certain patients who had a history of alcohol abuse had higher levels of minor hemoglobins than controls, yet were not diabetic. Acetaldehyde, a primary metabolite of ethanol, is present in the blood stream of persons consuming alcohol at concentrations of 1–50  $\mu$ M (9). It has also been reported to reach concentrations higher in persons chronically abusing ethanol than in control individuals (9, 10). We hypothesized that acetaldehyde, like reducing sugars, might modify hemoglobin A and contribute to the concentration of HbA<sub>1a-c</sub> in persons abusing alcohol. Such adducts could prove to be clinically important in assaying the recent consumption of alcohol or as a biochemical mechanism by which some of the sequelae of alcoholism develop.

The following study was conducted with hemoglobin as a model protein to determine whether acetaldehyde would form stable adducts, the nature of such adducts, their effect on the hemoglobin molecule, and what evidence of adduct formation might be detected in alcoholic patients.

## METHODS

**Hemoglobin.** Hemolysates were prepared from anticoagulated blood obtained from human volunteers. The erythrocytes were separated from plasma by centrifugation (1,000 g for 10 min) and washed three times with 0.15 M NaCl. The erythrocytes were lysed by the addition of 1/4 vol of toluene and mixed for 30 min. The toluene phase was separated by centrifugation (1,000 g for 30 min) and

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<sup>1</sup>Abbreviations used in this paper: HbA<sub>1a-c</sub>, hemoglobin A<sub>1a</sub> plus hemoglobin A<sub>1b</sub> plus hemoglobin A<sub>1c</sub>;  $\alpha$ -t-Box, alpha N-tert-butoxycarbonyl.

aspirated from the hemolysate. The concentration of hemoglobin was determined by Drabkin's ferricyanide method (11). For experiments with purified hemoglobin A, the hemolysate was fractionated by column chromatography on Biorex 70 (Bio-Rad Laboratories, Richmond, Calif.) after the method of Trivelli et al. (12). Hemoglobin A was concentrated by ultrafiltration and verified as uniform by isoelectric focussing (pH range, 6–8).

**Adduct reaction.** Acetaldehyde adduct formation with hemoglobin was tested in erythrocytes, hemolysate, and hemoglobin A using labeled [1,2- $^{14}\text{C}$ ]acetaldehyde (5 mCi/mmol sp act; New England Nuclear, Boston, Mass.). The reaction mixture consisted of 1–2 mM hemoglobin, various concentrations of [1- $^{14}\text{C}$ ]acetaldehyde, and 0.01 M bis-Tris buffer (pH 7.0). The mixture was incubated at 37°C in a sealed vaccine vial and aliquots of 0.5 ml were removed with a syringe at appropriate time intervals to determine the amount of adduct formation. The aliquots of hemoglobin were immediately reduced with 400 M excess (compared with acetaldehyde) of sodium borohydride (Sigma Chemical Co., St. Louis, Mo.) contained in 0.5 M sodium phosphate buffer (pH 7.0) for 3 min, to obtain the total amount of acetaldehyde adducts. Both stable and unstable adducts were present in the total. The stable [1- $^{14}\text{C}$ ]acetaldehyde adducts were identified by dialyzing aliquots of the reaction mixture for several hours against 4 liters of 0.001 M sodium phosphate buffer (pH 7.0) before the reduction with sodium borohydride. Globin was prepared from these reduced samples by precipitating the hemoglobin in a 25 vol excess of 2% HCl in acetone at 4°C (13). The precipitate was washed three times in cold acetone and a fourth time in ether before being dried. Globin samples were dissolved in water and normalized for protein concentration by determining the absorbance at 280 nM (1 mg globin = 0.65 OD) in a Zeiss P-6 spectrophotometer (Carl Zeiss, Inc., New York). 1 mg of protein was precipitated in 10% trichloroacetic acid and collected on a 25-mm Millipore filter, pore size 0.8  $\mu\text{M}$  (Millipore Corp., Bedford, Mass.). The filters were washed three times with 20 ml of 5% trichloroacetic acid, dried, and counted in a Packard #3375 beta counter (Packard Instrument Co. Inc., Downers Grove, Ill.), using 10 ml Aquasol scintillation fluid (New England Nuclear).

The relationship of hemoglobin adduct formation to the concentration of acetaldehyde was determined by incubating various concentrations of labeled acetaldehyde (3.0, 0.3, 0.03, and 0.03 mM) with hemoglobin A. Aliquots of the mixture were removed after 30 min of reaction and processed as described above for preparing the stable adducts with hemoglobin.

Adduct formation with hemoglobin after a sequence of discrete pulses of [1- $^{14}\text{C}$ ]acetaldehyde was tested by incubating hemolysate in 15 or 30  $\mu\text{M}$  [1- $^{14}\text{C}$ ]acetaldehyde for 30 min at 37°C. The reaction mixture was then dialyzed for 1 h before reducing an aliquot of the mixture with borohydride to measure the amount of stable adduct. The remaining unreduced mixture was again incubated in 15 or 30  $\mu\text{M}$  [1- $^{14}\text{C}$ ]acetaldehyde and the sequence was repeated for five or six pulses. Globin was prepared from each pulse aliquot and the radioactivity in 1 mg of protein was measured as described above.

**Chromatography.** The effect of acetaldehyde adduct formation on the chromatographic behavior of hemoglobin was evaluated by incubating hemolysate (2.5 mM hemoglobin) at 37°C for 2 h alone or with 0.3, 3, or 30 mM [1- $^{14}\text{C}$ ]acetaldehyde. The reaction mixtures were then placed in stretched dialysis tubing, and dialyzed twice against 4,000 vol of 0.001 M bis-Tris buffer, pH 7.0, for 24 h at

4°C. 20 mg of reacted, dialyzed hemolysate was chromatographed on Biorex 70 with 0.05 M sodium phosphate buffer (pH 6.72). The hemoglobin concentration of the chromatographed fractions (1 ml) was determined by measuring the absorbance at 420 nm. The amount of radioactivity of the fractions was measured in 10 ml Aquasol as described above.

**Adduct analysis.** Globin was prepared from hemoglobin which had been reacted with [1- $^{14}\text{C}$ ]acetaldehyde and reduced with borohydride. An aliquot of 4 mg of the protein was hydrolyzed in 6 N HCl at 110°C for 12 h and chromatographed on a Beckman 119C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.), using a stream division of the eluant. A major portion (80%) of the eluant was collected in 2-min fractions (1 ml) with a LKB 7000 fraction collector (LKB Instruments, Inc., Rockville, Md.). The amount of radioactivity was determined as described above. The peaks of  $^{14}\text{C}$  activity in the hydrolyzed globin were identified by comparing the elution time of each peak with synthesized standards of borohydride-reduced, acetaldehyde-modified amino acids. These standards were synthesized from [1- $^{14}\text{C}$ ]acetaldehyde and either valine, *N*-(1-deoxy-1-glucitolyl)valine,  $\alpha$ -*tert*-butoxycarbonyl lysine ( $\alpha$ -*t*-Box lysine), *N*<sup>6</sup>-(1-deoxy-1-glucitolyl)-*N*<sup>2</sup>-*tert*-butoxycarbonyl lysine (*N*<sup>6</sup>-[1-glucitolyl] $\alpha$ -*t*-Box lysine) or  $\alpha$ -*t*-Box tyrosine. The reaction mixture, containing 0.1 mM amino acid, 0.1 mM [1- $^{14}\text{C}$ ]acetaldehyde, and 0.5 mM sodium cyanoborohydride ( $\text{NaCNBH}_3$ ) in 1 ml of 0.05 M sodium phosphate buffer (total volume 2 ml) pH 7.0, was incubated at 37°C for 2 d. The solution was then chromatographed on Dowex 50 according to the procedure of Schwartz and Gray (14). The standards were deblocked for 3 h at 110°C in 4 N HCl, and the time of the elution of  $^{14}\text{C}$  activity after amino acid analysis was determined as described above. All standards eluted as single separate peaks on amino acid analysis. The elution times of these peaks were not altered by acid hydrolysis (6 N HCl, 110°C, 12 h).

The recovery of [1- $^{14}\text{C}$ ]acetaldehyde adducts after acid hydrolysis of globin was also examined by continuous reduction with sodium cyanoborohydride. Hemolysate that was reacted with 0.3 mM [1- $^{14}\text{C}$ ]acetaldehyde was dialyzed two times against 4 liters of 0.001 M sodium phosphate buffer (pH 7.0) to remove all free acetaldehyde. The hemolysate was then incubated with 100 M excess of sodium cyanoborohydride, and aliquots were taken at various times. Globin was precipitated from the hemolysate and acid hydrolyzed for amino acid analysis with stream division as described above. The amount of radioactivity in fractions eluted from the amino acid analyzer was measured as described above.

**Effect on oxygen affinity.** A hemoglobin solution was divided into aliquots and incubated with varying concentrations of acetaldehyde (0.3–30 mM) or buffer alone for 2 h. The reaction mixtures were then dialyzed for 4 h and deoxygenated under nitrogen. Oxygen-affinity determinations were performed by exposing the deoxygenated hemoglobin to increasing partial pressures of oxygen. Measurements of oxygen saturation were made utilizing a Hemo-Scan apparatus (American Instrument Co., Travenol Laboratories, Inc., Silver Spring, Md.) (15).

**Patient studies.** To determine minor hemoglobin concentrations in the blood of alcoholic patients, anticoagulated blood was obtained from individuals admitted to an alcohol detoxification program. All patients had consumed alcohol within the previous 4–24 h. Total fast hemoglobins ( $\text{HbA}_{1a-c}$ ) were determined using the high-performance liquid chromatographic method of Cole et al. (16), and hemoglobin  $\text{A}_{1c}$  was determined by radioimmunoassay according to the procedure of Javid et al. (17).

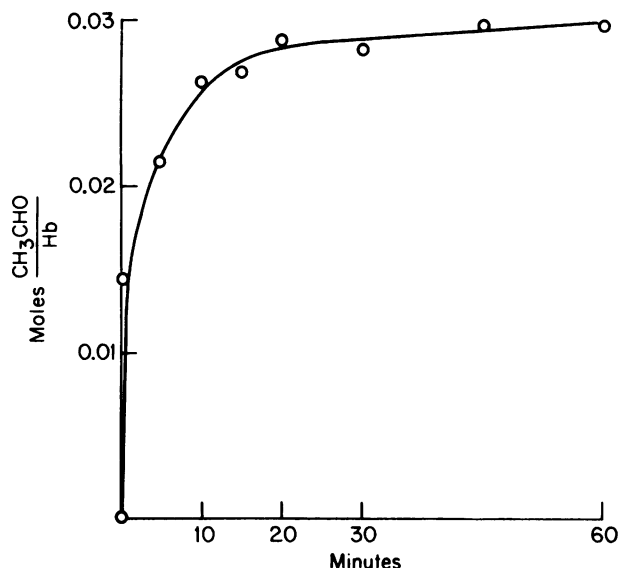


FIGURE 1 On-rate of acetaldehyde adduct with hemoglobin. Destomatized hemolysate (2 mM hemoglobin) was reacted with 0.3 mM [ $^{14}C$ ]acetaldehyde in a vaccine stoppered reaction vial at 22°C. Aliquots were withdrawn with a syringe at various times and reduced with a 400-fold excess of NaBH<sub>4</sub>. Globin was prepared from the reduced aliquots and the radioactivity associated with 1 mg of protein was determined as described in Methods. Almost 20% of the [ $^{14}C$ ]acetaldehyde had reacted with hemoglobin after 60 min.

The amount of acetaldehyde adducts with amino acids of hemoglobin of alcoholic patients compared with control individuals was also investigated. Well-dialyzed hemolysates from alcoholic patients or controls were reduced with

tritiated sodium cyanoborohydride (NaCNB<sup>3</sup>H<sub>3</sub>; 95% pure, 0.8 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) to stabilize the adduct and incorporate tritium label into the adducts. At various times aliquots of the hemolysate were processed for amino acid analysis as described above. The determination of relative amounts of radioactivity in the various amino acid adducts eluted from the analyzer was made for each aliquot as described above. All studies were approved by the appropriate hospital committees, and informed consent was obtained in each case.

## RESULTS

The ability of acetaldehyde to form adducts with hemoglobin was tested by incubating hemolysate with 0.3 mM [ $^{14}C$ ]acetaldehyde at 22°C. As a Schiff-base was assumed to be the primary product of the reaction, the total amount of adduct formed at various times was assessed by reducing an aliquot of the reaction mixture with sodium borohydride (Fig. 1). Under these conditions, the reaction was virtually complete within 10 min at 22°C. The present recovery of [ $^{14}C$ ]acetaldehyde reacted with hemoglobin was 20%. Dialysis of the reacted hemoglobin before borohydride reduction demonstrated that both freely reversible adducts and more stable adducts were present (Fig. 2). The rapid loss of ~75% of the reducible adducts after 30 min of dialysis was followed by a more gradual loss of counts to a relatively stable level of 15–20% of the total adducts formed. The adducts stable to dialysis were also stable to 5% trichloroacetic and found to remain associated with the precipitated protein. This fraction, which resists extensive dialysis and acid precipitation,

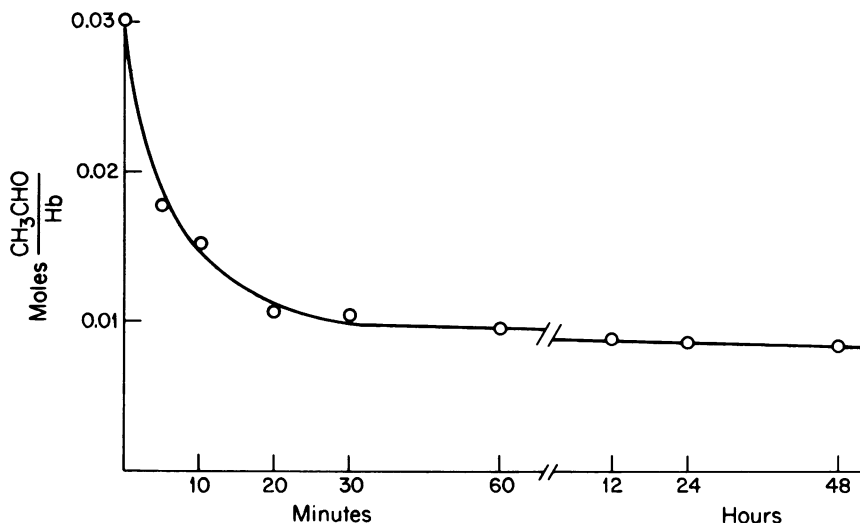


FIGURE 2 Off-rate of acetaldehyde adduct with hemoglobin. A portion of the hemolysate reacted with 0.3 mM [ $^{14}C$ ]acetaldehyde, shown at 60 min in Fig. 1, was dialyzed against 4 liters of sodium phosphate buffer, pH 7.0. Aliquots were withdrawn at the time intervals noted and reduced with sodium borohydride. Globin was prepared from the aliquots and the radioactivity in 1 mg was measured. About 6.6% of the [ $^{14}C$ ]acetaldehyde remained with the hemoglobin after 24 h.

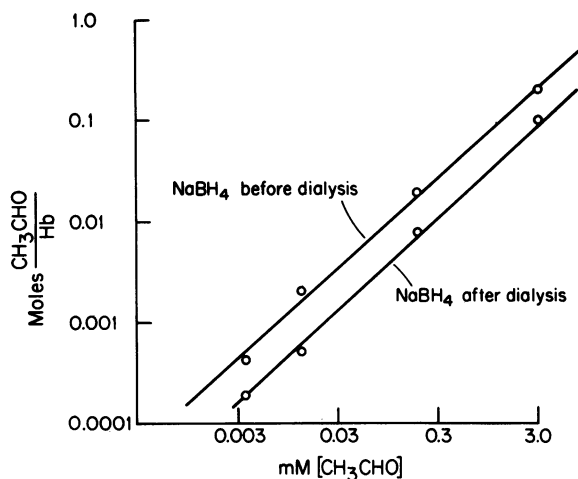


FIGURE 3 The stable hemoglobin adducts formed at various concentrations of acetaldehyde. Hemoglobin (2 mM) and [<sup>14</sup>C]acetaldehyde were reacted at 37°C for 30 min. The solution was dialyzed for 3 h and reduced with NaBH<sub>4</sub>. Globin was prepared from the reduced mixture and the radioactivity of 1 mg of trichloroacetic acid-washed protein was determined.

we refer to as the stable adduct and constitutes the basis of the investigations reported here.

The amount of stable adducts formed with hemoglobin was investigated over a [<sup>14</sup>C]acetaldehyde concentration range of 3  $\mu$ M to 3 mM (Fig. 3). The adducts that were stable to dialysis were directly proportional to the concentration of [<sup>14</sup>C]acetaldehyde in the reaction mixture over 4 log units. A single exposure of [<sup>14</sup>C]acetaldehyde in the concentration range of 30–300  $\mu$ M led to a stable modification of 0.2–1.0% of the total hemoglobin. Incubation of [<sup>14</sup>C]acetaldehyde with washed erythrocytes also resulted in the incorporation of radioactivity into hemoglobin in amounts comparable to that of hemolysate (not shown). The amount of stable [<sup>14</sup>C]acetaldehyde adducts formed with hemoglobin was also a function of the number of pulses of [<sup>14</sup>C]acetaldehyde given at intermittent intervals (Fig. 4). Exposure of hemoglobin to 15- $\mu$ M pulses of [<sup>14</sup>C]acetaldehyde, each of which was separated by dialysis, lead to a dose-related increase in the amount of stable adducts formed with hemoglobin. In effect, the cumulative hemoglobin adducts appeared to integrate the exposure to acetaldehyde over the course of these experiments.

Amino acid residues of hemoglobin that were susceptible to modification by [<sup>14</sup>C]acetaldehyde were investigated with an amino acid analyzer. Preliminary experiments showed that, to retain the [<sup>14</sup>C]acetaldehyde adduct during acid hydrolysis of the globin protein, it was first necessary to reduce hemoglobin with sodium borohydride or with sodium cyano-

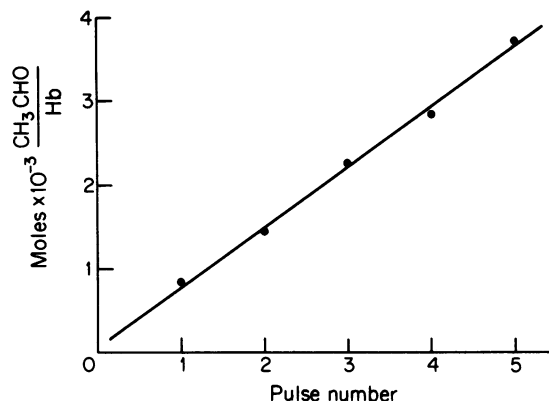


FIGURE 4 Accumulation of hemoglobin adducts with sequential pulses of acetaldehyde. Pulses of 15  $\mu$ M [<sup>14</sup>C]acetaldehyde were reacted with hemoglobin at sequential intervals. Each sequence consisted of a pulse of acetaldehyde, incubation at 37°C for 30 min and dialysis for 1 h. An aliquot of the dialyzed solution was taken and reduced with sodium borohydride. The remainder of the solution was again pulsed with 15  $\mu$ M acetaldehyde and the sequence repeated. Globin was prepared from the reduced aliquots and analyzed for radioactivity per milligram protein.

borohydride. After amino acid chromatography, radioactivity from the labeled adducts was found distributed in two minor and three major peaks, which corresponded to derivatives of valine, lysine, and tyrosine (Fig. 5).

The first peak, which appeared at 18–20 min, was found to elute at the same time as the synthesized, borohydride-reduced adduct of [<sup>14</sup>C]acetaldehyde plus valine. The third peak eluted at the same time as the borohydride-reduced adduct of N<sup>6</sup>-(1-deoxy-1-gluci-

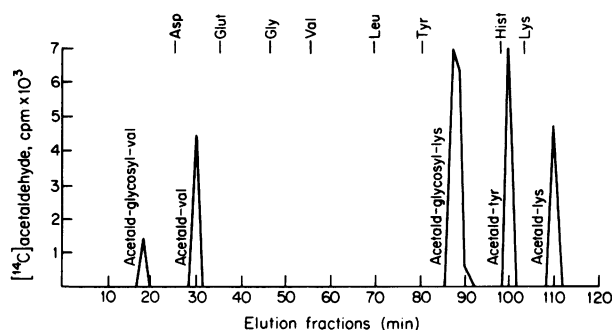


FIGURE 5 Elution profile of [<sup>14</sup>C]acetaldehyde modified amino acids hydrolyzed from globin. Globin derived from NaBH<sub>4</sub>-reduced, acetaldehyde-reacted hemoglobin was hydrolyzed and subjected to amino acid analysis. The peaks are labeled according to the chromatographic pattern of synthesized standards of borohydride-reduced, [<sup>14</sup>C]acetaldehyde-modified amino acids with which they coeluted at the times indicated. Standard amino acid elution times determined by split-stream division and ninhydrin are indicated above the peaks.

tolyl) lysine and [ $^{14}\text{C}$ ]acetaldehyde. The fourth peak coeluted with a borohydride-reduced  $\alpha$ -*t*-Box tyrosine adduct with [ $^{14}\text{C}$ ]acetaldehyde, while the fifth coeluted with the reduced [ $^{14}\text{C}$ ]acetaldehyde adduct formed with the  $\epsilon$ -amino group of lysine. It was necessary to reduce the modified globin protein with sodium borohydride in order to recover any of the amino acid adducts with [ $^{14}\text{C}$ ]acetaldehyde after acid hydrolysis. However, even with reduction, the recovery of the [ $^{14}\text{C}$ ]acetaldehyde adducts amino acid analysis was only 5% of those which were stable to dialysis. This suggested that many of the stable hemoglobin adducts were not in a form reducible by sodium borohydride. This was tested by incubating the stable hemoglobin [ $^{14}\text{C}$ ]acetaldehyde adducts with sodium cyanoborohydride over weeks (22°C) (Fig. 6). The cumulative reduction of the adducts over time by sodium cyanoborohydride increased the recovery of [ $^{14}\text{C}$ ]acetaldehyde adducts on amino acids to 40% of those on hemoglobin at the start of the experiment. Addition of additional cyanoborohydride led to no increase in labeling, and borohydride was verified to be active because  $\text{H}_2$  was produced on acidification of the incubation mixture. Apparently some of the stable [ $^{14}\text{C}$ ]acetaldehyde adducts on tyrosine, lysine, or valine occur in a form that is not reducible by sodium borohydride or sodium

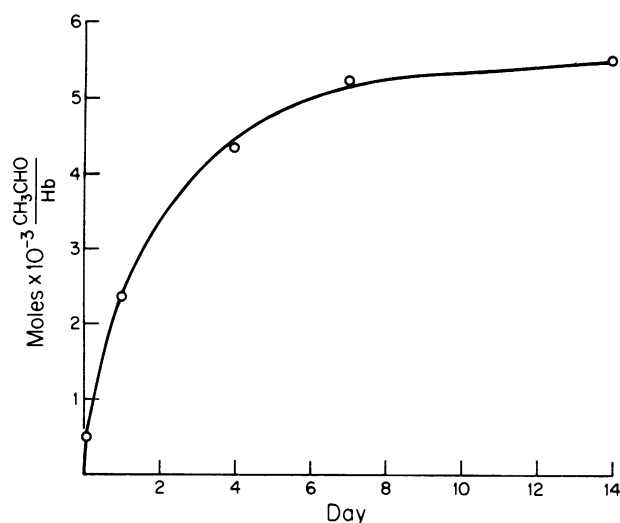


FIGURE 6 The recovery of [ $^{14}\text{C}$ ]acetaldehyde in amino acid adducts of globin following continuous sodium cyanoborohydride reduction. [ $^{14}\text{C}$ ]Acetaldehyde adducts of hemoglobin which were stable to dialysis were incubated with sodium cyanoborohydride ( $\text{NaCNBH}_3$ ) in buffer, pH 6.0 at 22°C. Aliquots were taken at various times and globin was prepared from them. 4 mg of globin was acid hydrolyzed and run on an amino acid analyzer using split-stream division. The amount of radioactivity associated with the various acetaldehyde adducts with amino acids was determined at each time.

cyanoborohydride. Only after slow conversion to a reducible form is the adduct fixed by the sodium cyanoborohydride reduction.

The effect of the stable acetaldehyde adducts on the chromatography of hemoglobin on Biorex 70 cation-exchange resins was assessed (Fig. 7). After incubation of [ $^{14}\text{C}$ ]acetaldehyde with destromatized hemolysate at 37°C, the labeled protein with the highest specific activity was found in the fast-eluting hemoglobin components. To determine whether [ $^{14}\text{C}$ ]acetaldehyde had modified the chromatographic elution profile of hemoglobin A or had preferentially formed adducts with the minor hemoglobins, the experiment was repeated with purified hemoglobin A. The results of incubating chromatographically purified hemoglobin A with 3 or 30 mM acetaldehyde are shown in Fig. 8. There was an increase of almost 3% of the total hemoglobin in the fast-eluting fraction after incubation of hemoglobin A with 3 mM acetaldehyde. This value increased to 23% of the total with 30 mM acetaldehyde. Despite these changes in net charge, incubation of acetaldehyde (0.3–30 mM) with hemoglobin resulted

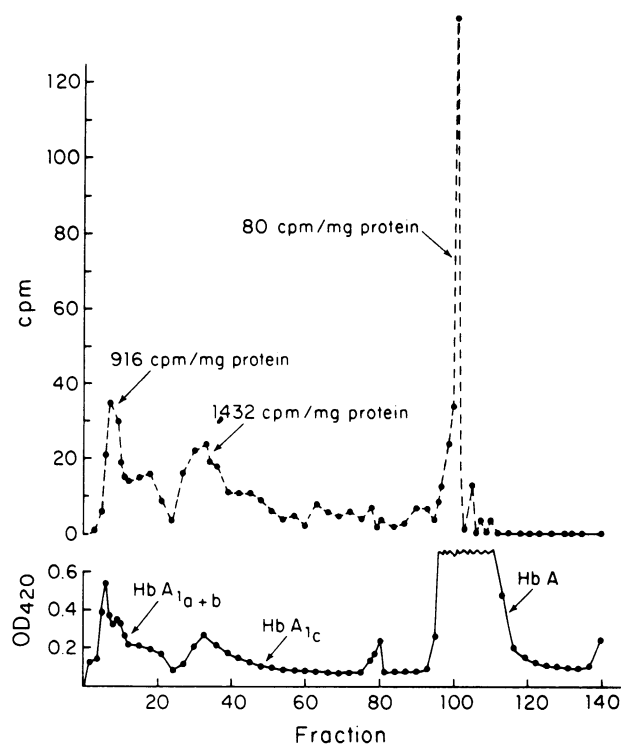


FIGURE 7 Hemolysate (2 mM hemoglobin) which has been incubated at 37°C with 3 mM [ $^{14}\text{C}$ ]acetaldehyde was dialyzed for 6 h before being chromatographed on Biorex 70, a cation-exchange resin. The upper panel represents the radioactivity eluted while the lower panel details the absorbance of the elution of 420 nm.

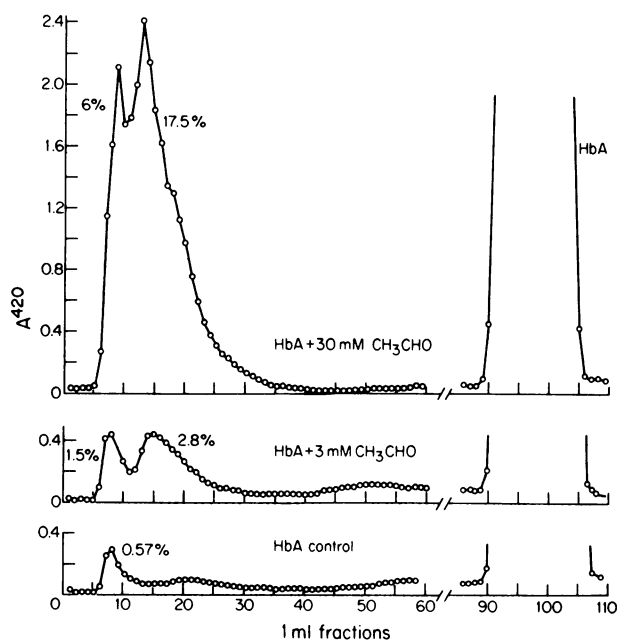


FIGURE 8 Hemoglobin A (1 mM) was incubated with 3 or 30 mM acetaldehyde or alone at 37°C. After dialysis for 6 h, the hemoglobin was chromatographed on Biorex 70.

in no detectable change in oxygen affinity ( $P_{50}$ ) of whole blood or hemolysate.

To assess whether acetaldehyde-modified hemoglobins might occur in patients chronically consuming ethanol, blood was obtained from patients admitted for alcohol detoxification. Hemolysates were prepared and analyzed for the amount of total fast hemoglobin as determined chromatographically, and the amount of hemoglobin  $A_{1c}$  was determined by radioimmunoassay. Table I details these results. The hemoglobin  $A_{1c}$  levels in persons consuming excess alcohol as determined by radioimmunoassay are lower than those seen in nonalcoholic subjects ( $P < 0.05$ ). However, the total fast hemoglobin component determined by high-performance chromatography according to Cole et al. (16) is elevated when compared with controls ( $P < 0.001$ ). This suggests that the fast hemoglobin components are enriched by nonglucosyl adducts.

Evidence that these differences in the minor hemoglobins of alcoholics were due to acetaldehyde adducts was found by the reduction of hemoglobin samples with tritiated sodium cyanoborohydride ( $NaCNB^3H_3$ ). As described in Fig. 5, this procedure was found to give the highest recovery of stable acetaldehyde adducts. After 21 d of reduction with

TABLE I  
Comparison of Alcoholic Patients and Controls for Concentrations of Fast Hemoglobins ( $HbA_{1a-c}$ ) by Chromatography and for Hemoglobin  $A_{1c}$  by Specific Radioimmunoassay

Alcoholic patient	Hemoglobin	Mean corpuscular volume	Chromatographic fast hemoglobin	Radioimmunoassay hemoglobin $A_{1c}$
	gm/dl	fl	%	%
1	14.2	90	12.9	2.6
2	14.2	106	11.1	3.2
3	13.5	103	9.0	2.3
4	12.8	112	11.1	2.3
5	14.1	94	6.8	4.0
6	13.0	103	15.0	2.3
7	13.3	75	13.6	4.5
8	12.3	96	16.4	3.6
9	14.6	103	11.9	4.4
10	11.0	113	13.4	1.8
Alcohol detoxification patients				
Mean	13.3	99.5	12.1*	3.10†
SD	1.1	11.3	2.8	0.98
Normal controls				
Mean			8.8	4.33
SD			±0.8	±0.50

\*  $P < 0.05$  vs. control.

†  $P < 0.001$  vs. control.

**TABLE II**  
*The Radioactivity Associated with the Reduction of Acetaldehyde-Amino Acid Adducts after Incubating Hemoglobin from Alcoholics and Controls with NaCNB<sup>3</sup>H<sub>3</sub>*

Patient	Hemoglobin in peaks of acetaldehyde-modified			
	Valine and glycosyl-valine	Glycosyl-lysine	Tyrosine	Lysine
	<i>cpm <sup>3</sup>H/mg</i>			
Alcoholic				
A	916	3,856	4,718	663
B	3,113	7,401	5,418	3,033
C	1,806	4,659	3,562	1,716
Mean*	1,945	4,147	4,565	1,804
Control				
A	524	1,401	1,350	139
B	371	1,671	1,685	63
C	656	1,195	1,268	108
D	620	2,068	1,520	316
Mean	543	1,584	1,455	157

The hemolysate of these subjects was dialyzed against 4 liters of 0.05 sodium phosphate buffer, pH 7.0, and reduced with tritiated sodium cyanoborohydride for 21 d at 22°C. The incorporation of tritium into acetaldehyde adducts had stabilized at this time. Globin was prepared and acid was hydrolyzed. The hydrolyzate was chromatographed on an amino acid analyzer with the stream division mode of operation. Radioactivity eluting with peaks of acetaldehyde adducts of amino acids was normalized for the amount of protein chromatographed. Peak times of elution were determined with [<sup>14</sup>C]acetaldehyde adducts from hydrolyzed hemoglobin and identified with synthesized standards (see Fig. 5).

\*  $P < 0.001$  vs. controls for all four columns of amino acid adducts.

cyanoborohydride, globin was prepared and processed for amino acid analysis. The radioactivity was recovered in five peaks and the amount of the elution peaks of the various acetaldehyde adducts is shown in Table II. The alcoholic patients' hemoglobin had several times more acetaldehyde adducts than did normal controls.

## DISCUSSION

Acetaldehyde, an important metabolite of ethanol, has been demonstrated to form adducts with human hemoglobin, a portion of which (15–25%) is stable to dialysis. The reaction is nonenzymatic and occurs with purified hemoglobin A. The amount of stable hemoglobin adducts formed is proportional to the amount of acetaldehyde to which it is exposed, or to the number of intermittent pulses. The hemoglobin adducts of [<sup>14</sup>C]acetaldehyde that were stable to dialysis were also stable to precipitation by trichloroacetic acid, but were

lost after acid hydrolysis unless they had first been reduced with sodium borohydride.

Reaction of hemoglobin A with 3 or 30 mM acetaldehyde was observed to increase significantly the amount of minor hemoglobins recovered after chromatography on cation-exchange resin. Although these are supra-physiological concentrations of acetaldehyde, the accumulation of smaller pulses of acetaldehyde adducts on hemoglobin over the 120-d lifespan of the erythrocyte may elevate the values for minor hemoglobins found in alcoholics. The actual concentrations of acetaldehyde found in the bloodstream of individuals consuming alcohol is controversial, because of problems of methodology (9, 21). It would appear, however, that most investigators would presently agree on a physiological range of 5–50  $\mu$ M acetaldehyde (9, 10). The accumulation of adducts would appear to explain the fact that alcoholics in this study had elevated minor hemoglobins but below normal levels of hemoglobin A<sub>1c</sub> by specific radioimmunoassay. Gel chromatography and electrophoresis revealed no evidence for cross-linked hemoglobin after reaction with 0.3 mM acetaldehyde but cross-linked hemoglobin has been reported at higher concentrations of acetaldehyde (24). As minor hemoglobin levels are being clinically employed as a means of diagnosing and monitoring diabetes mellitus, the alcohol consumption habits of patients should also be considered.

The amino acid residues of hemoglobin with which acetaldehyde formed stable adducts were analyzed by reducing them with sodium borohydride. Unreduced adducts were not stable to acid hydrolysis. Acetaldehyde adducts with hemoglobin involve at least three different amino acid residues, valine, lysine, and tyrosine, in addition to the two modified residues glucosyl-valine and glucosyl-lysine. The acetaldehyde appears to be reacting with the  $\epsilon$ -amino group of lysine and  $\alpha$ -amino group of valine probably through an initial Schiff's base reaction. The secondary amines of glycosylated valine or glycosylated lysine residues are also proposed to be the sites of reaction with acetaldehyde. Disubstitution of amino groups is known to occur with hexose sugars (14) and by analogy, acetaldehyde may also react with the secondary amine of glycosylated residues. However, participation of the hexose moiety in adduct formation is also possible and the exact structures need to be determined. Acetaldehyde adduct formation with tyrosine residues may involve either a nucleophilic attack by the third or fifth carbon of the phenolic ring, by analogy to formaldehyde modification of proteins (18), or alternatively by reaction with the hydroxyl group of tyrosine. The exact structures of these adducts are presently under investigation.

Only a portion of the stable hemoglobin-acetaldehyde adducts that were stable to 24 h of dialysis could be irreversibly fixed by sodium borohydride reduction. A greater portion, however, appeared to be in non-reducible (noncarbonyl, nonimine) form. Up to 45% of the dialysis stable adducts could be reduced by sodium cyanoborohydride and be hydrolyzed to amino acid adducts, if given either sufficient time (2–3 wk at 22°C) or increased temperature (1–2 d at 50°C). An increase in adduct recovery occurred in all five residues by amino acid analysis. This suggests that the adducts form and reverse through a reducible (e.g., Schiff-base) form, but that most of the time the stable adduct occurs in a nonreducible state.

Reduction of blood from alcoholics for several weeks with tritiated sodium cyanoborohydride is consistent with the presence of stable acetaldehyde adducts in vivo. Tritium incorporation into hemoglobin adducts increased logarithmically over the course of 3 wk. Alcoholics had several times more radioactivity incorporated into amino acid adducts than control individuals. The experiments with [<sup>14</sup>C]acetaldehyde in vitro would suggest that this still only accounts for 40% of the adducts present on hemoglobin in vivo. The fact that normal control individuals also have such adducts may reflect occasional consumption of ethanol or more probably a basal level of acetaldehyde production from intestinal bacteria and metabolism (19). As the blood was refrigerated and thoroughly dialyzed before incubation with NaCNB<sup>3</sup>H<sub>3</sub>, artifactual formation of acetaldehyde is not believed to be a factor (20). The significant differences in the amount of adducts detected between the blood of alcoholic patients and controls raise the possibility that specific assays could be developed which would serve as markers for alcohol consumption in the way that hemoglobin A<sub>1c</sub> integrates blood glucose (4). However, the fact that normal people also have what appears to be acetaldehyde bound to hemoglobin necessitates the development of more specific assay methods.

The acetaldehyde adducts with hemoglobin or other proteins, which are stable but slowly reversible, may partially explain why measurements of whole blood acetaldehyde have been so difficult (9). A rapid disappearance artifact (21) and elevation of acetaldehyde levels with hemolysis in vitro (22) have been described. Recently, it has been reported that the immediate precipitation of blood proteins helps to minimize these changes (23). The present studies may provide an explanation for these findings.

Acetaldehyde has been shown to crosslink erythrocyte membrane proteins (24), to brown with albumin (25), and in this report to react with hemoglobin by adduct formation. At least as many adducts are recovered in the plasma proteins as in hemoglobin when

whole blood is reacted with [<sup>14</sup>C]acetaldehyde (not shown). The formation of adducts between acetaldehyde and hemoglobin may serve as a model for investigating functional changes in various tissues (e.g., nerve, muscle, lens) involved in the sequelae of alcoholism. The origin of some of these sequelae may be explained by acetaldehyde adducts with proteins, just as some of the sequelae of diabetes are believed to result from postsynthetic modification of proteins by glucose (26, 27).

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