PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 177, 226–233 (1984)

The Effect of Acetaldehyde Concentrations on the Relative Rates of Formation of Acetaldehyde-Modified Hemoglobins (41935)

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Abstract. The effect of various concentrations of acetaldehyde (0, 0.05, 0.1, 0.25, 0.5, 1.0, and 5.0 m*M*) on the relative rates of formation of hemoglobin acetaldehyde adducts detected in fractions eluted from cation exchange high-pressure liquid chromatography (HPLC) was investigated. When the hemoglobin and acetaldehyde mixtures were incubated at 37°C for various time intervals up to 24 hr, increased amounts of HbA1c could be observed after 2 hr incubation with 1 m*M* or greater concentrations of acetaldehyde, or after 4 hr incubation with at least 0.5 m*M* acetaldehyde. An increase in the HbA1a+b fraction was not observed with 4 hr incubation time until the acetaldehyde level reached 1 m*M*. The HPLC method detected no difference in minor hemoglobins from alcoholic and normal subjects. Incubation of red blood cells at 37°C for 1 hr with six consecutive pulses of 0.05 m*M* [^{14}C]acetaldehyde showed no differences in the amounts of minor hemoglobins determined chromatographically at various pulse intervals. However, the measure of the ^{14}C-label incorporation into hemoglobin showed that adducts eluting in the HbA1a+b fraction were formed at a faster rate than those eluting in the HbA1c or HbAo fraction, respectively. The specific activities of the HbA1a+b fractions at 2, 4, and 6 pulses were 34, 128, and 949 cpm/mg hemoglobin; those of the HbA1c fraction were 15, 58, and 174 cpm/mg hemoglobin. This evidence of modification of hemoglobin by physiological levels of acetaldehyde from ^{14}C-label incorporation suggests that an assay more sensitive than chromatographic separation of adducts might be clinically useful in detecting alcoholism or monitoring alcohol detoxification programs.

Many toxic effects of alcoholism have been linked to acetaldehyde, the major metabolite of ethanol (1–4). The mechanism by which excess acetaldehyde leads to pathology remains undefined. Acetaldehyde has been shown to react with various cellular components (1–5), albumin (6), and hemoglobin (7, 8). The suggestion that hemoglobin–acetaldehyde adducts might be useful in the detection and/or monitoring of alcoholism needs further investigation. The enrichment of minor hemoglobin fractions from cation exchange chromatography with adducts was previously observed with acetaldehyde concentrations between 3 and 30 m*M* (7, 8). Recent data indicate that acetaldehyde concentrations in nondrinkers are ≤ 2 μM but may be considerably higher in alcoholic individuals (9–12). It is generally agreed that acetaldehyde levels in alcoholics remain below 100 μM (9–14). The present study was undertaken

(1) to examine the effect of physiological concentrations of acetaldehyde on the relative rates of formation of various hemoglobin–acetaldehyde adducts, using a cation exchange high-pressure liquid chromatography (HPLC) method for hemoglobin separation and detection of incorporation of [^{14}C]acetaldehyde in hemoglobin fractions;

(2) to evaluate the sensitivity of several methodologies of hemoglobin separation to changes induced by acetaldehyde; and

(3) to relate *in vitro* findings on acetaldehyde-modified hemoglobins to evidence for the formation of acetaldehyde-modified hemoglobins *in vivo* as detected by HPLC.

Materials and Methods. *Effect of varying concentrations of acetaldehyde and incubation time on the formation of acetaldehyde-modified hemoglobin as detected by HPLC.* All studies were approved by appropriate institutional review, and informed witnessed consent was obtained from all subjects. Venous blood samples were collected from five healthy subjects, aged 19–40, having no history of diabetes or alcoholism. The blood, collected in EDTA coated vacutainers, was centrifuged at 1600*g* for 10 min to remove

0037-9727/84 $1.50

plasma. The red cells were washed twice and incubated in saline overnight at 4°C to eliminate the labile glycosylated hemoglobin (15). After removal of the saline wash, the cells were lysed with an equivalent volume of distilled water. Cell debris was removed by centrifugation (23,000*g* for 30 min at 4°C) and filtration (1.2-μM Millipore MF membrane). The mean ± SEM hemoglobin concentration of the five hemolysates, determined by the Drabkin procedure (16) with commercially available reagent (Fisher Scientific), was 1.8 ± 0.007 m*M*.

Seven 1.8-ml aliquots of each hemolysate were used to react with 0.2 ml stock acetaldehyde solutions to yield mixtures containing 0–5 m*M* acetaldehyde (0, 0.05, 0.1, 0.25, 0.5, 1.0, and 5.0 m*M*). The acetaldehyde was added to the hemoglobin in a cold room (4°C), tightly sealed in test tubes, and then incubated in a 37°C water bath for 2 hr. The reactions were stopped by opening the test tubes and evaporating residual acetaldehyde under a ventilated hood. The samples were diluted with 1 m*M* sodium phosphate buffer, pH 7.0, 0.01% KCN, to 0.5 mg/ml for HPLC analysis.

The HPLC apparatus used was an automated hemoglobin Alc analyzer, Model HA-8110, manufactured by the Sekisiu Chemical Company and distributed by the Daiichi Kagaku Company, Kyoto, Japan. The hemoglobin separation is done via a column packed with spherical crosslinked methacrylic acid and methacrylate copolymers containing both ionic and hydrophobic groups which separate hemoglobin components by cation exchange and reverse-phase partition chromatography. The HPLC apparatus also includes an autosampler, a degasser-pinch valve pump for delivery of buffers in a regulated sequence, a prefilter, a thermostatic chamber for the column, a dual wavelength (415 and 500 nm) photometer for detection of eluting fractions, a built-in microcomputer to identify and calculate peak areas, and a printer for recording of chromatographic pattern and numerical data. The total analysis time for each sample is 13 min with 4 min elution with eluant A (8.4 g/liter KH_2PO_4 and 2.3 g/liter K_2HPO_4) followed by 3 min with eluant B (8.4 g/liter KH_2PO_4 and 13.0 g/liter K_2HPO_4) and 6 min reequilibration with eluant A.

To study the effect of longer incubation times on the formation of acetaldehyde-modified hemoglobin, hemolysates from five normal subjects were pooled and divided into seven duplicate 1.8-ml aliquots. To each aliquot of hemolysate was added 0.2 ml stock acetaldehyde as described above to give a final concentration of 0, 0.05, 0.1, 0.25, 0.5, 1.0, or 5.0 m*M* acetaldehyde. The samples were incubated in sealed vials at 37°C, and a small amount was withdrawn with a syringe at 0, 4, and 24 hr. The samples were diluted as above for HPLC analysis. Blood samples from 11 healthy subjects were analyzed under the same conditions to establish the 99% confidence intervals (17) for normal HbA1a+b and HbA1c values from this HPLC system.

Incubation of hemoglobin with pulses of [^{14}C]acetaldehyde and assay of adducts by comparative methods. Blood from three healthy subjects was collected in EDTA-coated vacutainers. After removal of plasma, the red cells were washed twice and resuspended in normal saline. The cells were divided into two sets of samples. Samples A were reacted with six successive pulses of 0.05 m*M* [^{14}C]acetaldehyde (New England Nuclear, 7.4 mCi/mmole) alternated with saline washes. Samples B were incubated in a second saline wash with regular mixing at room temperature for 2 hr before each additional pulse of [^{14}C]acetaldehyde was added. The B set of samples was included to document that any adducts formed were stable to dialysis for 2 hr across the red cell membrane.

The adding of labeled acetaldehyde from a tenfold solution to the cell suspensions was performed as above. Aliquots of washed cells were removed from both the A and B samples at 0, 2, 4, and 6 pulse intervals, and hemolysates were prepared as above for assays of minor hemoglobins by three different methods. These methods included the HPLC analysis as described above, a cation exchange column chromatographic method based on Trivelli's procedure (18) using 1 × 30-cm columns packed with Biorex-70 (Bio-Rad Laboratories), and an affinity chromatographic method for separation of HbA1c via disposable microcolumns and borate/phos-

phate buffer, using the procedure recommended by the column manufacturer (Bio-Rad Laboratories, Catalog No. 191-9000).

To verify that acetaldehyde–hemoglobin adduct formation had occurred, hemoglobin eluted from the Biorex-70 columns was collected with a fraction collector (LKB Redirac, Model 2112) in 4.2-ml fractions. Peak fractions, detected by a uv/visible detector (Altex Model 153) using a 405-nm filter, were pooled. The percentage area of each peak was calculated from its relative product of absorbance and volume. The collected fractions were divided and precipitated in duplicate with 10% trichloroacetic acid (Fisher Scientific). The amounts of hemoglobin precipitated for counting were in the range of 0.7–4.5 mg. After an overnight incubation at 4°C, the precipitates were washed twice with 25 ml 5% trichloroacetic acid and once with ether on a manifold filter apparatus (Millipore Sample Manifold Model 1225) using double layers of filter membranes (Whatman 3MM, 2.5-cm circles). The labeled precipitates were dried on the filter membranes and counted for 10 min in 10 ml Aquasol (New England Nuclear) in a liquid scintillation counter (Packard Tri-Carb Model 3255). The counts were not corrected for quenching as determination of a quench curve showed negligible differences for the sample sizes used.

Assays for minor hemoglobin fractions from blood samples of diabetics, alcoholics, and control subjects. Blood samples were obtained from 8 diabetic patients, 8 alcoholic patients participating in an alcohol detoxification program who had consumed alcohol within 48 hr, and 11 nondiabetic, nonalcoholic control subjects. The mean ages ± SEM of the diabetic, alcoholic, and control groups were 37 ± 8, 41 ± 4, and 34 ± 4 years. All samples were analyzed by the HPLC system described above, and by the chemical assay for glycosylation of Parker *et al.* (19) using 2-thiobarbituric acid (TBA) for the colorimetric measurement of a glucose adduct derivative.

Results. Table I shows the effect of increasing the concentration of acetaldehyde from 0 to 5.0 m*M* on the amounts of HbA1a+b and HbA1c, as percentage of total hemoglobin, by HPLC analysis. After 2 hr incubation of hemoglobin with acetaldehyde at 37°C, a significant increase ($P < 0.01$) in the amount of the HbA1a+b fraction was observed only at 5 m*M* acetaldehyde. An increase in the HbA1c fraction was observed once the level of acetaldehyde reached 1 m*M*. The HbA1a+b and HbA1c fractions are defined as the fast eluting fractions from cation exchange chromatography that predominantly contain glycosylated hemoglobins (20) but may contain other coeluting modified hemoglobins.

TABLE I. EFFECT OF INCREASING THE CONCENTRATIONS OF ACETALDEHYDE ON THE AMOUNTS OF HbA1a+b AND HbA1c FRACTIONS AS PERCENTAGE OF TOTAL HEMOGLOBIN AFTER SEPARATION BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF THE ACETALDEHYDE-MODIFIED HEMOGLOBINS

Concentration of acetaldehyde (m*M*)	Minor hemoglobins as percentage of total[a]	
	HbA1a+b	HbA1c
0	2.0 ± 0.10	4.5 ± 0.12
0.05	2.1 ± 0.09	4.5 ± 0.12
0.1	2.0 ± 0.10	4.4 ± 0.14
0.25	2.1 ± 0.09	4.6 ± 0.09
0.5	2.1 ± 0.07	4.7 ± 0.07
1.0	2.3 ± 0.05	5.6 ± 0.21[b]
5.0	6.1 ± 0.10[b]	12.1 ± 0.12[b]

[a] Values shown are means ± SEM of five determinations. The minor hemoglobins were separated by HPLC after 2 hr incubation at 37°C. The HbA1a+b and HbA1c fractions refer to fast eluting fractions that normally contain well-defined glycosylated hemoglobins but may contain other modified hemoglobins that coelute with these glycosylated hemoglobins.

[b] Significantly different from control values (shown at top for 0.0 m*M* acetaldehyde). $P < 0.01$.

Table II presents the effect of longer reaction time on the formation of acetaldehyde-modified hemoglobins eluted in the minor hemoglobin fractions from HPLC. The increases in amounts of HbA1a+b fractions from acetaldehyde-modified hemoglobins relative to the amounts of HbA1a+b from control hemoglobins were significant ($P < 0.01$) with 4 hr incubation time and acetaldehyde concentrations of 1 m*M* or higher. After 24 hr incubation, the amounts of HbA1a+b were elevated in all samples. Increased amounts of HbA1c from acetaldehyde-modified hemoglobin were observed after 4 hr incubation of hemoglobin with 0.5

TABLE II. EFFECT OF REACTION TIME ON THE FORMATION OF ACETALDEHYDE-MODIFIED HEMOGLOBIN ELUTED IN THE MINOR HEMOGLOBIN FRACTIONS FROM HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Hemoglobin fraction	Reaction time (hr)	Concentration of acetaldehyde (m*M*):	Minor hemoglobins as percentage of total[a]						
			0.0	0.05	0.1	0.25	0.5	1.0	5.0
HbA1a+b	0		2.2	2.2	2.1	2.2	2.3	2.2	2.6
	4		2.2	2.3	2.4	2.4	2.5	3.0[b]	9.6[b]
	24		4.6[b]	4.9[b]	4.9[b]	4.9[b]	5.1[b]	5.4[b]	19.4[b]
HbA1c	0		4.9	4.8	4.9	4.9	5.1	5.1	5.9
	4		4.4	4.5	4.7	4.9	5.4[b]	6.6[b]	16.8[b]
	24		4.9	5.1	4.9	5.3[b]	5.9[b]	7.0[b]	28.5[b]

[a] The amounts of HbA1a+b and HbA1c, as percentage of total hemoglobin, were determined by HPLC after incubation of hemoglobin with various acetaldehyde concentrations at 37°C for the indicated reaction times. Values shown are averages of duplicate analyses. The 99% confidence intervals for control values of minor hemoglobin fractions, determined with blood samples from 11 healthy subjects, were 1.7, 2.5% and 4.3, 5.1% for HbA1a+b and HbA1c, respectively.

[b] Significantly higher than the normal control range, $P < 0.01$.

m*M* or higher concentrations of acetaldehyde, or after 24 hr incubation with 0.25 m*M* or higher acetaldehyde. Figure 1 shows HPLC chromatograms of hemoglobin after 4 hr incubation at 37°C with either blank buffer or 1 m*M* acetaldehyde (Fig. 1A), and after 24 hr incubation at 37°C with blank buffer (Fig. 1B). Concomitant with the increase in amount of HbA1a+b seen in the control sample upon 24 hr incubation, there is the appearance of a minor unidentified peak following elution of the HbA1c peak.

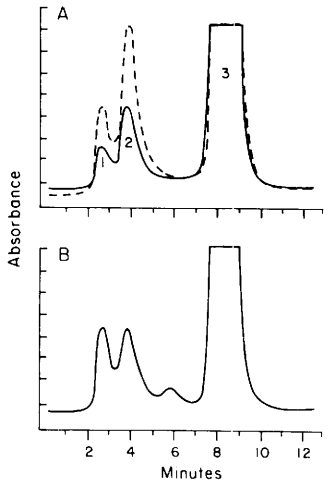

FIG. 1. Chromatograms of acetaldehyde-modified hemoglobin. (A) The dotted line represents hemoglobin after 4 hr incubation with 1 m*M* acetaldehyde at 37°C; the solid line represents hemoglobin upon incubation with blank saline solution under the same conditions. Peaks 1, 2, and 3 are the HbA1a+b, HbA1c, and HbAo fractions, respectively. (B) Chromatogram of hemoglobin after 24 hr incubation with blank saline solution at 37°C showing an increase in peak 1 with the appearance of a new peak between peaks 2 and 3. Absorbance measured by dual wavelength photometer at 415 and 500 nm.

Table III shows the amounts of HbA1a+b and HbA1c as percentage of total hemoglobin, assayed by three different chromatographic methods at various pulse intervals following exposures to 0.05 m*M* acetaldehyde. Comparison of results from the HPLC analysis of acetaldehyde-modified hemoglobins with the 99% confidence intervals for normal HbA1a+b and HbA1c values showed that the variations in minor hemoglobins were mostly within the normal range. A slight increase in HbA1a+b was observed with the B samples (which were washed and incubated in saline between exposures to acetaldehyde) after 4 and 6 pulses. There were no significant differences between amounts of minor hemoglobins recovered at various pulse intervals following chromatography on Biorex-70 or following affinity chromatography.

Figure 2 shows the specific activities of the various hemoglobin fractions separated by Biorex-70 chromatography after exposures to 2, 4, and 6 pulses of [^{14}C]acetaldehyde. There were no significant differences in results between the A and B samples. The radioactive

TABLE III. EFFECT OF INCREASING THE NUMBER OF EXPOSURES OF HEMOGLOBIN TO PULSES OF 0.05 m*M* ACETALDEHYDE ON THE AMOUNTS OF HbA1a+b AND HbA1c DETERMINED BY THREE DIFFERENT CHROMATOGRAPHIC METHODS

Sample[a]	Separation method	Pulse number	Minor hemoglobin as percentage of total	
			HbA1a+b	HbA1c
A	HPLC[b]	0	2.1	4.7
		2	2.1	4.4
		4	2.1	4.3
		6	2.3	4.2
	Biorex-70[c]	0	2.0	4.1
		2	2.3	3.9
		4	1.8	3.6
	Bio-Rad[d]	0	1.9	4.4
		6	2.2	4.0
B	HPLC[b]	0	2.3	5.0
		2	2.3	5.0
		4	2.6*	5.0
		6	2.9*	5.2
	Biorex-70[c]	0	2.2	3.4
		2	2.5	3.8
		6	2.0	4.1

[a] The red cells of the (A) samples were washed twice with saline solution after each exposure to acetaldehyde and the washed cells were lysed at indicated pulse intervals for assay of minor hemoglobins. The cells of the B samples were incubated in the second saline wash for 2 hr at room temperature with regular gentle mixing before reacting with another pulse of acetaldehyde or lysis for assay.

[b] Values shown are averages of three determinations. The 99% confidence intervals for normal control HbA1a+b and HbA1c values, determined from HPLC analysis of blood samples from 11 healthy subjects, were 1.7, 2.5% and 4.2, 5.1%, respectively. *Above the 99% confidence limits.

[c] Triplicate samples of acetaldehyde-modified hemoglobin were pooled prior to analysis by cation exchange chromatography with Biorex-70 resin. The amounts of minor hemoglobin fractions as percentage of total were determined by relative products of absorbance at 540 nm and volume of peak fractions.

[d] Separation by affinity chromatography using microcolumns and borate/phosphate buffers from Bio-Rad Laboratories. Only HbA1c values corrected for temperature using commercially available (Bio-Rad Laboratories) standards. Values shown are averages of six determinations (duplicate samples from three subjects).

label incorporated in all hemoglobin fractions increased with the number of exposures to 0.05 m*M* [^{14}C]acetaldehyde. The specific activities of the HbA1a+b fractions at 2, 4, and 6 pulses were 34, 120, and 949 cpm/mg hemoglobin, those of the HbA1c fraction were 15, 58, and 174 cpm/mg hemoglobin, and those of the HbAo fraction were 3, 6, and 17 cpm/mg hemoglobin. The [^{14}C]acetaldehyde-modified hemoglobins in the HbA1a+b fraction increased at the most rapid rate as indicated by the ratios of specific activities of the HbA1a+b and HbA1c fractions relative to that of the HbAo fraction at increasing pulse intervals. These ratios were 14:6:1 after 2 pulses, 19:9:1 after 4 pulses, and 56:10:1 after 6 pulses.

Table IV shows the comparison of amounts of minor hemoglobins from the HPLC analysis and glycosylated hemoglobins as measured by the thiobarbituric acid reaction of blood samples obtained from diabetic and alcoholic individuals, and from healthy control subjects. While the HbA1a+b and HbA1c fractions from diabetic blood samples were significantly elevated ($P < 0.01$) compared to the control values, no differences could be

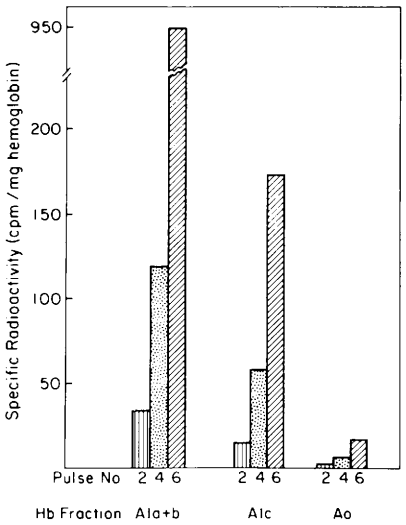

FIG. 2. Specific activities of various hemoglobin fractions from cation exchange chromatography of [^{14}C]acetaldehyde-modified hemoglobin. Red blood cells were reacted for 1 hr at 37°C with pulses of 0.05 m*M* [^{14}C]acetaldehyde, with washing of the cells with normal saline after each pulse. Incorporation of ^{14}C label into hemoglobin was determined by liquid scintillation counting of trichloroacetic acid-precipitated hemoglobin.

TABLE IV. COMPARISON OF AMOUNTS OF MINOR HEMOGLOBINS FROM DIABETIC AND ALCOHOLIC PATIENTS AND FROM HEALTHY TEETOTALERS

Group	No.	HPLC[a] % HbA1a+b	HPLC[a] % HbA1c	TBA[b] (μM fructose)	Ratio HPLC/TBA HbA1a+b	Ratio HPLC/TBA HbA1c
Diabetic	8	3.7 ± 0.5[c]	9.1 ± 0.6[d]	99.0 ± 5.9[d]	0.039 ± 0.007	0.091 ± 0.001[d]
Alcoholic	8	2.0 ± 0.1	4.5 ± 0.2	60.9 ± 1.5	0.032 ± 0.002	0.073 ± 0.003
Teetotaler	11	2.1 ± 0.1	4.7 ± 0.1	62.1 ± 1.4	0.033 ± 0.002	0.075 ± 0.002

[a] High-pressure liquid chromatography. Values shown are means ± SEM.
[b] Thiobarbituric acid assay (colorimetric method).
[c] Significantly different from the teetotaler group, $p < 0.05$.
[d] Significantly different from the teetotaler group, $p < 0.01$.

observed between the alcoholic and the control groups by either method.

Discussion. The enrichment of minor hemoglobin fractions from cation exchange chromatography with acetaldehyde-modified hemoglobin was previously observed upon incubation of hemolysates with levels of acetaldehyde ranging from 3 to 30 m*M* (8). In the present study an increase in minor hemoglobins was not observed until the level of acetaldehyde used was above 1 m*M* during a 2-hr incubation period (Table I) or 0.5 m*M* during a 4-hr incubation period (Table II). Longer incubation (24 hr) resulted in interference by probable degradation products of hemoglobin as are evidenced by the appearance of a third minor peak upon HPLC analysis and an increase in the HbA1a+b amount in the absence of acetaldehyde (Fig. 1). The clinical use of the chromatographic assay of minor hemoglobins in the monitoring of alcohol consumption appears to be limited by the insensitivity of these methods to changes induced by physiologic concentrations of acetaldehyde. Reports on blood levels of acetaldehyde have varied widely because of problems inherent with methods of determination of acetaldehyde (21, 22). However, the minimum level of acetaldehyde needed to yield an increase in the chromatographically separated minor hemoglobin exceeded the <1–100 μ*M* range of blood acetaldehyde reported by various studies (8, 11–14, 22, 23).

The experiment in which hemoglobin was reacted *in vitro* with pulses of 0.05 m*M* [^{14}C]acetaldehyde simulated the red cell environment in chronic alcoholism in an effort to detect any cumulative effect of repeated exposures of hemoglobin to low physiological levels of acetaldehyde. Assays for minor hemoglobin fractions by three different chromatographic methods showed no significant differences in amounts of HbA1a+b and HbA1c among samples collected at different pulse intervals. The slightly elevated amounts of HbA1a+b fractions from the HPLC analysis of B samples (2.6 and 2.9% after 4 and 6 pulses, respectively, as compared to the normal range of 1.7–2.5% HbA1a+b seen in a control group, Table III) may be due to the degradation of hemoglobin upon repeated incubation at room temperature. Values for HbA1a+b and HbA1c obtained at various pulse intervals from Biorex-70 chromatography were consistent with values reported for normal subjects in studies using a similar method of hemoglobin separation (24, 25).

Although the chromatographic assay of HbA1a+b and HbA1c showed significant differences between amounts of minor hemoglobins from diabetic patients and from normal subjects, the amounts of minor hemoglobins from alcoholics were indistinguishable from control values (Table IV). The ratios of minor hemoglobin values determined by HPLC to TBA-determined glycohemoglobin values, which reflect the presence of nonglycosylated modified hemoglobins coeluting with the glycosylated hemoglobins, did not show significant differences between the alcoholic and control groups. These results are consistent with the finding that a high level of acetaldehyde is required for the detection of an apparent

change in the chromatographic pattern of hemoglobin. The results are, however, in contrast to a report by Hoberman and Chiodo (26), who found a modest but statistically significant elevation in the HbA1 fraction from an alcoholic group relative to a normal control group, and findings by Stevens *et al.* (7) of an increase in hemoglobin variants that eluted in the HbA1a–c region in alcoholic patients. Recent data show that storage and handling conditions markedly influence chromatographic determinations of minor hemoglobins (27, 28), and these factors may have contributed to the findings in previous reports. In the present study, blood samples were kept at 4°C and analyzed within 48 hr. Huisman *et al.* (28), using an HPLC method, recently reported an increase in a minor hemoglobin that coeluted with HbA1c upon incubation of red cells from a normal subject with a supraphysiological level of acetaldehyde, and an elevation of this minor hemoglobin in four of nine alcoholic subjects.

Results from the experiment with pulses of [^{14}C]acetaldehyde revealed that the ^{14}C-label incorporation into hemoglobin increased significantly with each pulse of 0.05 m*M* [^{14}C]acetaldehyde (Fig. 2). The more rapid rate of formation of acetaldehyde-modified hemoglobin in the HbA1a+b fraction is consistent with the report by Tsuboi *et al.* (29) who found most of the acetaldehyde-modified hemoglobin eluting in this fraction after incubation of red cells with acetaldehyde in a medium supporting glycolytic activity. The present study utilized washed cells, thus eliminating enzymatic activity and precluding the formation of adduct with acetaldehyde derivatives such as 5-deoxy-D-xylulose-1-phosphate (DXP) which may bind to hemoglobin and increase the HbA1 fraction (26). The HbA1a+b fraction has been shown to include glycosylated, phosphorylated hemoglobin (28, 29). The relative reactivity of acetaldehyde with this form of hemoglobin as well as the mechanism of this reaction needs further investigation.

It appears that with a relatively high level of acetaldehyde, the major hemoglobin fraction, HbAo, is modified to yield hemoglobin variants that elute more rapidly and mainly in the HbA1c fraction upon cation exchange chromatography. In a medium with physiologic levels of acetaldehyde, the modification of hemoglobin by acetaldehyde may proceed preferentially and via a different mechanism with hemoglobin variants that normally elute in the HbA1a+b fraction, and to a lesser extent with those which elute in the HbA1c fraction. Furthermore, the relative stabilities of various adducts, which have not been examined in this study, may influence results obtained with various methodologies. At the low physiological concentration of acetaldehyde, when the difference in amount of adducts in the HbA1a+b and HbA1c fractions may be marginal, differences in stabilities of these adducts against dialysis across the red cell membrane may explain discrepancies in observations with cells and hemolysates.

The present study confirmed that acetaldehyde nonenzymatically forms stable adducts with hemoglobin as documented by incorporation of radioactivity. Systems which rely on changes in the isoelectric point of hemoglobin induced by physiological concentrations of acetaldehyde have been shown to be insensitive to adduct formation as documented by incorporation of radioactivity. The clinical utility of acetaldehyde–hemoglobin adducts as markers for alcoholism cannot be realized until more sensitive assays for these adducts are developed.

The authors thank Miss Liberty Walker for her dedicated technical assistance and Miss Deborah Kerr for her support in the preparation of the manuscript. This work was supported in part by Interinstitutional Nutritional Research Training Grant 5T32CAO9427, PHS 5 RO1 AA04851-02, and CRC RR00102.

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Received March 9, 1984. P.S.E.B.M. 1984, Vol. 177.
Accepted June 11, 1984.