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Differential Modification of Hemoglobin Chains by Acetaldehyde (42237)

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Abstract. Acetaldehyde–hemoglobin adducts have been suggested as potential markers for alcohol consumption. These adducts were formed *in vitro* with [^{14}C]acetaldehyde and separated into hemoglobin subunits by cation-exchange chromatography to examine the relative modification of the α- and β-chains. The effect of varying concentrations of acetaldehyde on the relative amounts of polypeptide adducts and on the specific radioactivities of undissociated hemoglobin (Hb) following reaction with hydroxymercurybenzoate (HMB) was also studied. There were linear relationships ($P < 0.05$) between increasing levels of [^{14}C]acetaldehyde (0.0, 0.1, 0.2, 0.5 m*M*) and the radioactivities of the α- and one of the two β-chain adducts (22, 25, 53 dpm/mg Hb and 151, 272, 626 dpm/mg Hb, respectively). Increases in radioactivities of a minor unidentified hemoglobin adduct fraction were also observed. The ratios of specific radioactivities of β- to α-chain (8.8 ± 1.2 SEM) did not vary with the concentrations of acetaldehyde. Although the amounts of undissociated hemoglobin following reaction with HMB did not increase with increasing concentrations of acetaldehyde, the significant increase of specific radioactivities of this fraction (152, 1967, and 6562 dpm/mg Hb for 0.1, 0.2, and 0.5 m*M* acetaldehyde, respectively) suggested possible crosslinks within the tetramer or dimer. The amino acid analysis of α- and β-subunit adducts formed with 0.1 and 0.5 m*M* acetaldehyde showed that unreacted cysteine residues were more often detected at the higher acetaldehyde concentration consistent with the formation of cysteine adducts labile to acid hydrolysis or the shielding of cysteine residues in acetaldehyde-modified Hb against the subunit separation by HMB treatment. Thus acetaldehyde reacts differentially with the α- and β-hemoglobin subunits and with the undissociated hemoglobin molecule.

Two of the major problems in alcohol research and treatment have been a lack of objective and specific criteria with which to characterize the condition, and a lack of a biochemical basis for the understanding of the numerous secondary sequelae of the disease. Recent studies have shown that acetaldehyde, a metabolite of ethanol found to be elevated in chronic alcoholics (1–4), reacts with hemoglobin and other proteins to form a number of stable protein–acetaldehyde adducts (5–14). The formation of acetaldehyde adducts with proteins suggests that certain sequelae of alcoholism may be the result of post-translational modification of proteins at specific sites and that some of these adducts might serve as a marker for chronic alcohol consumption.

Acetaldehyde adducts formed *in vitro* with hemoglobin were found to coelute with the minor hemoglobin fraction HbA_1 upon cation-exchange chromatography (8–14). The detection by chromatographic separation methods of acetaldehyde adducts formed *in vivo* with hemoglobin was reported by some investigators (11, 15) but not confirmed by others (16). The development of a specific, sensitive, and clinically useful assay method for hemoglobin–acetaldehyde adducts is dependent on a better understanding of the mechanism of reaction and/or chemical nature of these adducts. This study was undertaken to (i) examine the relative reactivity of acetaldehyde with the two major polypeptide chains of hemoglobin from normal human subjects, (ii) study the effect of varying near physiological concentrations of acetaldehyde on modification of the hemoglobin chains, and (iii) detect any effect of acetaldehyde on the stability of the hemoglobin molecule in the process of subunit separation.

Materials and Methods. *Formation of hemoglobin–acetaldehyde adducts.* Venous blood samples were obtained from three normal volunteers, ages 24–40, with informed witnessed consent. After three washes with normal saline, the red cells were lysed with distilled water and destromatized by centrifugation at 27,000*g* for 30 min at 4°C. The

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hemoglobin concentration of the pooled hemolysate determined by the Drabkin procedure (17) using commercially available reagent (Drabkin reagent, Fisher Scientific) was 1.38 m*M*. Tenfold stock solutions of [1,2-^{14}C]acetaldehyde (8.0 mCi/mmole, New England Nuclear) were added to yield mixtures with final acetaldehyde concentrations of 0.0, 0.1, 0.2, or 0.5 m*M*. The mixtures were added at 4°C, tightly sealed with rubber stoppers, and incubated in a 37°C water bath for 1 hr. Residual acetaldehyde was allowed to evaporate under a hood, and the labeled hemoglobin solutions were saturated with carbon monoxide.

Separation of hemoglobin subunits by cation-exchange chromatography. The [^{14}C]-acetaldehyde-labeled hemoglobin samples were reacted with *p*-hydroxymercurybenzoate (HMB; Sigma Chemical Company) for subunit separation (17–19) and the α- and β-HMB chains were separated by cation-exchange chromatography using carboxymethylcellulose resin (CM-52, Whatman) according to the procedure by Acharya and Manning (19). With each concentration of acetaldehyde, 120–200 mg of ^{14}C-labeled HMB–hemoglobin was loaded on 1 × 30-cm columns previously equilibrated with 10 m*M* KH_2PO_4/K_2HPO_4 buffer (pH 7.9). All buffers were saturated with carbon monoxide and contained 1 m*M* disodium EDTA. Each sample was chromatographed at 4°C in triplicate or quadruplicate runs. The eluates were collected at 20 ml/hr (LKB 2132 MicroPerpex pump) in 10-ml fractions (LKB 2112 Redirac fraction collector) and hemoglobin peaks were detected at 405 nm by an Altex uv/visible detector Model 153.

Peak fractions from cation-exchange chromatography were pooled, and duplicate aliquots containing 2 mg labeled HMB–hemoglobin were brought up to a constant volume with distilled water and precipitated at 4°C overnight with 10% trichloroacetic acid (TCA). The precipitated hemoglobins were collected on filter papers (2.4-cm GF-C filters, Whatman), washed twice with 5% TCA and finally with anhydrous ethyl ether on a manifold filtering apparatus (Millipore 1225 sampling manifold). The samples were dried under a hood and counted with the filters by liquid scintillation (Tri-Carb Packard 3255) in 10 ml Aquasol (New England Nuclear) for 10 min. The counts were corrected for efficiency by the channel ratio method (20) using normal Hb as quenching agent and [^{14}C]toluene (4.0 × 10^5 dpm/ml, New England Nuclear) as standard. The specific radioactivities of HMB-hemoglobins modified by 0.1, 0.2 and 0.5 m*M*[^{14}C]acetaldehyde prior to subunit separation by cation-exchange chromatography were 100 ± 14, 165 ± 5, and 409 ± 71 dpm/mg Hb, respectively. These values represent about 0.4% of the activity of [^{14}C]acetaldehyde initially added to hemoglobin.

Amino acid analysis of acetaldehyde–hemoglobin adducts. The concentration of HMB–hemoglobin adduct from various peak fractions was determined spectrophotometrically with an extinction coefficient for hemoglobin monomer of 13.4 mM^{-1} cm^{-1} (21). Aliquots containing 0.1 mg adduct were precipitated with 5% TCA, washed with TCA and ether, and dried under a hood. The dried samples were hydrolyzed in 0.2 ml 6 *N* HCl *in vacuo* at 110°C for 22 hr and chromatographed (22) with a Dionex 500 amino acid analyzer. Amino acid values were corrected for recovery using leucine as an internal standard. The amino acid composition of each peak was compared to the known composition of α- and β-chains (23, 24).

Statistical analysis of data. The differences in specific radioactivities among hemoglobin adducts with varying concentrations of [^{14}C]acetaldehyde were examined by one-way analysis of variance and linear regression procedures (25). Differences in modification of the α- and β-HMB–hemoglobin chains were detected by the comparison of 99% confidence intervals for mean specific radioactivities. Data for each acetaldehyde concentration are shown as means and standard errors of triplicate or quadruplicate chromatographic runs.

Results. Figure 1 shows a chromatogram of normal hemoglobin modified by 0.2 m*M* acetaldehyde and treated with HMB prior to separation by cation-exchange chromatography. The same pattern but different specific radioactivity values were observed with other concentrations of acetaldehyde. The mean recovery of control and acetaldehyde-modified HMB–hemoglobins from the columns was 73 ± 4%. The mean recovery of the ^{14}C-label was 82 ± 3%. The four main peaks were identified by amino acid analysis to be β_1-chain, β_2-

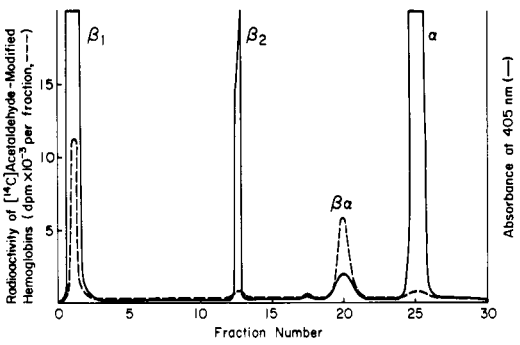

FIG. 1. Chromatogram of normal hemoglobin modified by 0.2 mM [^{14}C]acetaldehyde and treated with hydroxymercurybenzoate prior to separation by cation-exchange chromatography. Size of CM-52 columns: 1 × 30 cm, sample size: 120–200 mg HMB-hemoglobin, elution buffers: linear gradient from 150 ml 10 mM potassium phosphate (pH 5.8) to 150 ml 15 mM potassium phosphate (pH 7.9), fraction volume: 10 ml. The four peaks (β_1-chain, β_2-chain, undissociated $\beta\alpha$ hemoglobin, and α-chain) were identified by amino acid analysis (see Table I). Incorporation of radioactive label into trichloroacetic acid-precipitated hemoglobin adducts was detected by 10-min liquid scintillation counting and corrected for counting efficiency by the channel ratio method.

chain, undissociated $\beta\alpha$-dimer or tetramer, and α-chain, in order of elution time. A barely discernible and unidentified minor peak, not consistently observed in the chromatography of fresh control HMB-hemoglobin, was observed between the β_2-chain and $\beta\alpha$ peaks of the acetaldehyde-modified HMB-hemoglobin samples.

The means and standard errors of specific radioactivities of the β_1 fractions were 151 ± 2, 272 ± 17, 626 ± 80 dpm/mg Hb with 0.1, 0.2, and 0.5 mM[^{14}C]acetaldehyde, respectively. This increase in specific radioactivities was significant ($P < 0.01$) as was the increase in specific radioactivities of the α-chain (22 ± 5, 25 ± 3 and 54 ± 4 dpm/mg Hb with the same increasing concentrations of acetaldehyde). Figure 2 shows the relative increases in specific radioactivities of various adducts with increasing concentrations of [^{14}C]acetaldehyde. The linear regression of label incorporation as a function of acetaldehyde concentration showed a greater slope for the β_1-chain (1185 dpm/mg Hb) than for the α-chain (81 dpm/mg Hb), but the ratios of β_1-chain to α-chain specific radioactivities remained constant with varying concentrations of acetaldehyde (ratio of 10.1 ± 1.3). The specific radioactivities of the β_2 fractions did not change significantly ($P > 0.05$) in the range of acetaldehyde concentrations used and averaged 119 ± 26 dpm/mg Hb. When the β_1 and β_2 fractions were combined to represent the β-chain subunit, the β to α ratio of specific radioactivities was 8.8 ± 1.1 dpm/mg Hb.

The $\beta\alpha$ peak, representing undissociated hemoglobin, showed a significant increase ($P < 0.05$) in specific radioactivities with increasing acetaldehyde concentrations (152 ± 14, 1967 ± 443, 6562 ± 1804 dpm/mg Hb). The slope of the least-square line for this fraction was 1.6 × 10^4 dpm/mg Hb, the largest of all observed slopes. The amounts of HMB-hemoglobin recovered as undissociated hemoglobin in the $\beta\alpha$ fractions were 1.3 ± 0.1% with 0.1 mM acetaldehyde and 1.8 ± 0.1% and 1.8 ± 0.3% with 0.2 and 0.5 mM acetaldehyde, respectively. The change in recoveries of undissociated hemoglobin was not statistically significant. The minor peak eluting prior to the $\beta\alpha$ peak did not show a detectable increase in amounts of hemoglobin recovered

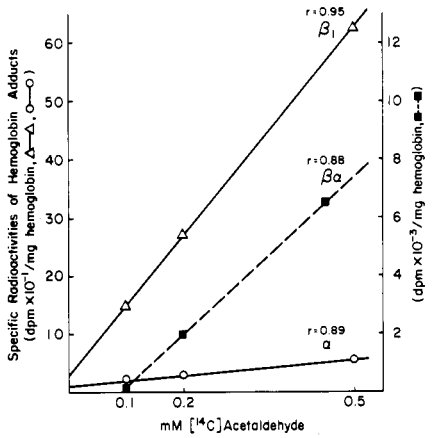

FIG. 2. Relative specific radioactivities of various cation-exchange chromatography fractions of [^{14}C]acetaldehyde-modified hydroxymercurybenzoate (HMB)-treated hemoglobins. Each point represents a mean of three chromatographic runs, each run analyzed in duplicate samples. Adducts were prepared by incubating varying concentrations of [^{14}C]acetaldehyde with normal hemolysate at 37°C for 1 hr prior to separation of hemoglobin adducts into subunits by HMB treatment and chromatography with CM-52 columns. The radioactivities of adducts were measured by 10-min liquid scintillation counting of dried samples after precipitation by trichloroacetic acid.

TABLE I. AMINO ACID COMPOSITION OF ACETALDEHYDE-MODIFIED HYDROXYMERCURYBENZOATE HEMOGLOBINS AFTER CATION-EXCHANGE CHROMATOGRAPHY

Amino acid	Expected[a]			Observed with hemoglobin–acetaldehyde adducts							
				0.1 m*M* acetaldehyde				0.5 m*M* acetaldehyde			
	β	α	$\beta\alpha$	β_1	β_2	$\beta\alpha$	α	β_1	β_2	$\beta\alpha$	α
Asp + Asn	13	12	12.5	13.5	13.6	13.6	12.4	13.4	13.6	12.3	11.7
Thr	7	9	8.5	7.6	7.7	9.0	9.7	7.5	7.7	8.9	9.3
Ser	5	11	8.0	5.7	5.8	8.0	10.5	5.7	5.5	9.0	10.2
Glu + Gln	11	5	8.0	10.8	10.6	7.6	5.0	8.1	10.8	6.6	6.1
Pro	7	7	7.0	8.8	8.2	9.9	9.6	7.9	7.9	7.6	9.5
Gly	13	7	10.0	14.1	13.5	10.1	7.8	12.4	12.9	9.1	7.2
Ala	15	21	18.0	17.2	16.2	21.6	23.9	15.2	17.3	19.2	22.8
Cys	2	1	1.5	1.0				0.6	0.7		0.3
Val	18	13	15.5	18.6	16.8	16.2	14.4	17.8	18.2	15.1	13.6
Met	1	2	1.5	1.3	1.1	1.6	2.0	1.0	1.0	1.9	1.9
Leu	18	18	18.0	18.2	17.6	18.9	18.2	17.8	18.6	18.9	18.0
Tyr	3	3	3.0	3.3	2.6	2.7	2.9	2.8	2.8	3.2	3.0
Phe	8	7	7.5	8.3	7.8	8.1	7.5	8.2	8.4	7.9	7.1
His	9	10	9.5	9.5	8.7	9.7	10.2	8.8	9.3	9.4	9.7
Lys	11	11	11.0	11.3	10.9	11.4	11.3	11.1	11.3	11.0	11.0
Arg	3	3	3.0	3.0	3.1	2.9	2.9	3.1	2.9	2.9	3.0

[a] Refs. (23, 24).

with increasing acetaldehyde concentrations, but showed high and increasing specific radioactivities (516 ± 71, 609 ± 69, and 1747 ± 270 dpm/mg Hb for 0.1, 0.2, and 0.5 m*M* acetaldehyde, $P < 0.01$).

The results of amino acid analysis of various chromatography fractions of hemoglobins modified by 0.1 and 0.5 m*M* acetaldehyde are shown in Table I. The unusually high values of proline in certain peaks were unexplained. No significant change was observed in the amino acid composition of hemoglobin adducts relative to that of the control sample and published data for normal hemoglobin. Cysteine, which is expected to be modified preferentially by HMB (17), could still be partially detected in one of the four samples modified with 0.1 m*M* acetaldehyde and in three of the four samples modified with 0.5 m*M* acetaldehyde.

Discussion. Acetaldehyde has been suggested to contribute to various sequelae of alcoholism due to its capacity to react with functional groups of proteins and other cellular components (5–7, 26). Previous studies demonstrated that the formation *in vitro* of hemoglobin–acetaldehyde adducts was dependent upon the concentration of acetaldehyde, the incubation time, and the pH condition of the reaction medium (8, 9, 12, 14). Although the knowledge of the detailed structure of such adducts is important in the understanding of the mechanism of action of acetaldehyde in alcoholism and is useful in the development of more sensitive methods to assay for adducts formed *in vivo*, work on the chemical structures of hemoglobin acetaldehyde adducts is still lacking. Stevens *et al.* (9) showed that the amino acid residues involved in the reaction with acetaldehyde included valine, lysine, tyrosine, and glycosylated valine and lysine, but no further attempt to locate more specific sites of hemoglobin modification or to compare the relative reactivity of acetaldehyde with the α- and β-chains was reported.

This study showed that acetaldehyde reacts preferentially to the β-chains of hemoglobin. A higher reactivity to the β-subunit relative to the α-subunit of hemoglobin has been reported for other aldehydes (19, 27). When the hemoglobin–acetaldehyde adducts were analyzed for amino acid composition, no losses of valine, lysine, or tyrosine were observed, even at the supraphysiological concentration of 0.5 m*M*. This may be due to the lability of the hemoglobin–acetaldehyde linkage during

the acid hydrolysis used in the amino acid analysis procedure. Acharya and Manning (19) observed a higher reactivity of the β-chains relative to α-chains of carbonmonoxyhemoglobin S with glyceraldehyde. They attributed this enhanced reactivity to terminal valine residues as well as active lysine residues in the β-chains. Since the α- and β-chains of hemoglobin have an equal number of lysine residues which can form Schiff base adducts with aldehydes via the ε-amine groups, the enhanced reactivity of one chain over the other reflects the influence of the chemical nature of residues neighboring the active amino acids and/or the conformation of the active sites in the native hemoglobin molecule.

Only about 0.4% of the radioactivity of [^{14}C]acetaldehyde initially added remained bound to hemoglobin after treatment with HMB and extensive dialysis to remove excess HMB. The concentration of acetaldehyde in the blood of alcoholics has been reported to be in the 50–100 μ*M* range (1–4). Although only a few micromoles of hemoglobin per liter of blood would be modified into stable acetaldehyde adducts according to the data of this study, extrapolation to the *in vivo* situation is at best speculative because of the unknown effect of the microenvironment on the stability of adducts formed *in vivo*. Sodium borohydride or cyanosodium borohydride can be used to stabilize the adducts against acid hydrolysis (9, 28) but may also introduce artifactual degradation products (14, 29, 30) and therefore these agents were not used in this study. Tsuboi *et al.* (10), using metabolizing human erythrocytes, reported 18% chromatographically altered hemoglobin with 68 hr incubation in 0.5 m*M* acetaldehyde and over 90% chromatographically altered hemoglobin with overnight incubation in 15 m*M* acetaldehyde. Thus "acetaldehyde adducts" detected experimentally may differ significantly in the amounts formed and/or chemical nature depending on the condition of the medium.

The linear increases in specific radioactivities of the [^{14}C]acetaldehyde-modified α- and β-chains are consistent with previous observations with whole blood hemolysates (8, 9, 14). Although the affinity of acetaldehyde with the β-chains is 9–10 times greater than with the α-chains, the undissociated βα fractions showed the largest amounts of label incorporation at each concentration of acetaldehyde (Fig. 2). The possibility of acetaldehyde participating in crosslinks within the dimer or tetramer, though not directly investigated in this study, is not ruled out in view of reports of acetaldehyde-induced crosslinks in other cellular components (5, 26). However, Jentoft and Dearborn (29) found no evidence for crosslinking by formaldehyde in model proteins and further research is needed to address the issue of crosslinking more specifically. The higher frequency of detection in cysteine in hemoglobin adducts modified by 0.5 m*M* acetaldehyde relative to adducts modified by 0.1 m*M* acetaldehyde (Table I) and to the control sample suggests that acetaldehyde adduct formation may shield certain cysteine residues against exposure to and reaction with HMB. The differential reaction of acetaldehyde with the α- and β-chains and the high specific radioactivity in the undissociated hemoglobin fraction following HMB treatment and cation-exchange chromatography suggest the need for additional study to further localize the sites of acetaldehyde modification and examine the significance of the above observations *in vivo*.

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1. Korsten MA, Matsuzaki S, Feinman L, Lieber CS. High blood acetaldehyde levels after ethanol administration. N Engl J Med **292**:386–389, 1975.
2. Lindros KO, Stowell A, Pikkarainen PH, Salaspuro M. Elevated blood acetaldehyde in alcoholics with accelerated ethanol elimination. Pharmacol Biochem Behav **13**:119–124, 1980.
3. Nuutinen H, Lindros KO, Salaspuro M. Determinants of blood acetaldehyde level during ethanol oxidation in chronic alcoholics. Alcohol Clin Exp Res **7**:163–168, 1983.
4. Eriksson CJP. Human blood acetaldehyde concentration during ethanol oxidation. Pharmacol Biochem Behav **18**(Suppl 1):142–168, 1983.
5. Gaines KC, Salhany JM, Tuma DJ, Sorrell MF. Reaction of acetaldehyde with human erythrocyte membrane proteins. FEBS Lett **75**:115–119, 1977.
6. Nomura F, Lieber CS. Binding of acetaldehyde to rat liver microsomes: Enhancement after chronic alcohol consumption. Biochem Biophys Res Commun **100**: 131–137, 1981.

7. Donohue TM Jr, Tuma DJ, Sorrell MF. Acetaldehyde adducts with proteins: Binding of ^{14}C-acetaldehyde to serum albumin. Arch Biochem Biophys **220**:239–246, 1983.
8. Fantl WJ, Stevens VH, Peterson CM. Reactions of biologic aldehydes with proteins. Diabetes **31**(Suppl 3):15–21, 1982.
9. Stevens VJ, Fantl WJ, Newman CB, Sims RS, Cerami A, Peterson CM. Acetaldehyde adducts with hemoglobin. J Clin Invest **67**:361–369, 1981.
10. Tsuboi KK, Thompson DJ, Rush EM, Schwartz HC. Acetaldehyde-dependent changes in hemoglobin and oxygen affinity of human erythrocytes. Hemoglobin **5**:241–250, 1981.
11. Hoberman HD. Post-translational modification of hemoglobin in alcoholism. Biochem Biophys Res Commun **113**:1004–1009, 1983.
12. Abraham EC, Stallins M, Abraham A, Garbutt GJ. Modification of sickle hemoglobin by acetaldehyde and its effect on oxygenation, gelation and sickling. Biochim Biophys Acta **705**:76–81, 1982.
13. Huisman THJ, Hensen JB, Wilson JB. A new high-performance liquid chromatography procedure to quantitate hemoglobin Alc and other minor hemoglobins in blood of normal, diabetic and alcoholic individuals. J Lab Clin Med **102**:163–173, 1983.
14. Nguyen LB, Peterson CM. The effect of acetaldehyde concentrations on the relative rates of formation of acetaldehyde-modified hemoglobins. Proc Soc Exp Biol Med **177**:226–233, 1984.
15. Hoberman HD, Chiodo SM. Elevation of the hemoglobin A_1 fraction in alcoholism. Alcohol Clin Exp Res **6**:260–266, 1982.
16. Mobley R, Huisman THJ. Minor hemoglobins (HbA_1) in chronic alcoholic patients. Hemoglobin **6**:79–81, 1982.
17. Bucci E, Fronticelli C. A new method for the preparation of α and β subunits of human hemoglobin. J Biol Chem **240**:PC551–552, 1965.
18. Njikam N, Jones WM, Nigen AM, Gillette PN, Williams RC Jr, Manning JM. Carbamylation of the chains of hemoglobin S by cyanate in vitro and in vivo. J Biol Chem **248**:8052–8056, 1975.
19. Acharya AS, Manning JM. Reactivity of the amino groups of carbonmonoxy-hemoglobin S with glyceraldehyde. J Biol Chem **255**:1406–1412, 1980.
20. Wang CH, Willis DL, Loveland WD. Radiotracer Methodology in the Biological, Environmental, and Physical Sciences. Englewood Cliffs, N.J., Prentice-Hall, 1975.
21. Antonini E, Brunoni M. Hemoglobin and Myoglobin in their Reactions with Ligands. New York, Elsevier, p19, 1971.
22. Moore S, Stein WH. Chromatographic determination of amino acids by the use of automatic recording equipment. Methods Immunol **6**:819–831, 1963.
23. Braunitzer G, Gehring-Muller R, Hilschmann N, Hilse K, Hobom G, Rudloff V, Wittmann-Liebold B. Die konstitution des normalen adulten humanhamoglobins. Z Physiol Chem **325**:283–286, 1961.
24. Hill RJ, Konigsberg W. The structure of human hemoglobin. J Biol Chem **237**:3151–3156, 1962.
25. Neter J, Wasserman W. Applied Linear Statistical Models. Homewood, N.J., Richard D. Irvin, 1974.
26. Ristow H, Obe G. Acetaldehyde reduces cross-links in DNA and causes sister chromatid changes in human cells. Mutat Res **58**:115–119, 1978.
27. Shapiro R, McManus MJ, Zalut C, Bunn HF. Sites of nonenzymatic glycosylation of human hemoglobin. J Biol Chem **255**:3120–3127, 1980.
28. Means GE, Feeney RE. Reductive alkylation of amino groups in protein. Biochemistry **7**:2193–2201, 1968.
29. Jentoft N, Dearborn DG. Labeling of proteins by reductive methylation using sodium cyanoborohydride. J Biol Chem **254**:4359–4365, 1979.
30. Crestfield AM, Moore S, Stein WH. The preparation and enzymatic hydrolysis of reduced and S-carboxylated proteins. J Biol Chem **238**:622–627, 1963.

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