

The Acetylation of Hemoglobin by Aspirin

IN VITRO AND IN VIVO

KENNETH R. BRIDGES, GEOFFREY J. SCHMIDT, MICHAEL JENSEN,
ANTHONY CERAMI, and H. FRANKLIN BUNN

From the Divisions of Hematology and the Departments of Medicine, Peter Bent Brigham Hospital and the Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts 02115, and the Rockefeller University, New York 10021

ABSTRACT The chemical modification of hemoglobin by aspirin (ASA) has been studied, both in intact human red cells and in purified hemoglobin solutions. After incubation of red cells with 20 mM [*acetyl*-1-¹⁴C]ASA, incorporation of radioactivity into hemoglobin was observed, in agreement with the results of Klotz and Tam (1973, *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1313-1315). In contrast, no labeling of hemoglobin was seen when [*carboxyl*-¹⁴C]ASA was used. These results indicate that ASA acetylates hemoglobin. The acetylated hemoglobin was readily separated from unmodified hemoglobin by both gel electrofocusing and by column chromatography. Quantitation of the extent of acetylation by densitometric scanning of gels agreed very well with estimates obtained from radioactivity measurements. Hemolysates prepared from red cells incubated with ASA showed normal oxygen affinity and heme-heme interaction. Purified acetylated hemoglobin had a slightly increased oxygen affinity and decreased heme-heme interaction. There was no difference in the rate of acetylation of oxy- and deoxyhemoglobin. ASA acetylated column-purified hemoglobin A more readily than hemoglobin in crude hemolysate, but less rapidly than purified human serum albumin. The rate of acetylation of hemoglobin increased with pH up to approximately pH 8.5.

Structural studies were done on hemoglobin incubated with 2.0 mM and 20 mM [*acetyl*-1-¹⁴C]ASA. α - and β -chains were acetylated almost equally. Tryptic digests of purified acetylated subunits were fingerprinted on cellulose thin layer plates and autoradiographed. Both α - and β -chains showed a number of radioactive

spots that were either ninhydrin negative or weakly ninhydrin positive. These results indicate that hemoglobin is acetylated at a number of sites, probably at the ϵ -amino group of lysine residues.

To determine whether ASA acetylates hemoglobin in vivo, hemolysates of 14 patients on long-term high-dose ASA therapy were analyzed by gel electrofocusing and compared to specimens of individuals not receiving ASA. The ASA-treated group had a twofold increase in a minor hemoglobin component having an isoelectric point lower than that of hemoglobin A, and indistinguishable from the minor component which appears when hemoglobin is incubated with ASA in vitro.

INTRODUCTION

Although aspirin (acetylsalicylic acid, ASA)¹ is the most widely used medication in the United States, its mode of action is still poorly understood. ASA has been shown to modify the structure of a number of plasma proteins by a transacetylation reaction. In addition, the drug has been shown to result in acetylation of platelet proteins. Despite the fact that hemoglobin is the most abundant and physiologically important protein in the blood, the possibility of its modification by ASA has not been explored until recently. In 1973, Klotz and Tam (1) reported that after the incubation of normal human red cells with [*acetyl*-1-¹⁴C]ASA, hemoglobin became labeled. They found a substantial increase in the oxygen affinity of ASA-treated red cells and hemolysates prepared from these specimens. They suggested that the acetylation of hemoglobin by ASA might provide a therapeutic approach to sickle cell anemia. Subsequently

¹ Abbreviations used in this paper: ASA, acetylsalicylic acid; CM-cellulose, O-(carboxymethyl)cellulose.

Received for publication 25 September 1974 and in revised form 9 December 1974.

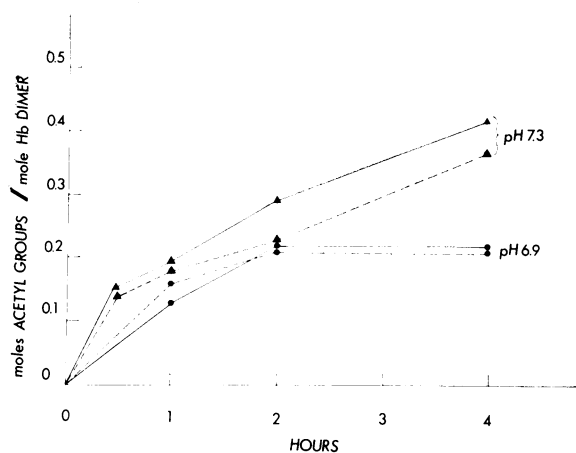


FIGURE 1 Incorporation of ^{14}C -acetyl groups into hemoglobin. Red cells were incubated with 20 mM [*acetyl-1- ^{14}C*]ASA in isotonic phosphate buffer, pH 7.3 (\blacktriangle) and pH 6.9 (\bullet), at 37°C for 4 h. The acetylation of hemoglobin was estimated from radioactivity measurements (—) and gel scanning (---).

de Furia, Cerami, Bunn, Lu, and Peterson (2) confirmed that hemoglobin reacted extensively with ASA, but were unable to show any alteration in oxygen affinity of red cells exposed to the drug *in vitro* or *in vivo*. Furthermore, no significant effect on sickling was seen. This report describes detailed structural and functional studies on ASA-treated hemoglobin. We have attempted to determine whether specific sites on the molecule become acetylated and whether this structural modification occurs in patients who are treated with high doses of ASA. A preliminary report of this work has appeared elsewhere (3).

METHODS

Normal human blood, collected in heparin, was used for all the *in vitro* experiments. Unlabeled ASA was obtained from Aldrich Chemical Co., Inc., Cedar Knolls, N. J. [*Acetyl-1- ^{14}C*]ASA was prepared by Mallinckrodt Chemical Works, St. Louis, Mo., from salicylic acid and [$1-^{14}\text{C}$]acetic anhydride. [*Carboxyl- ^{14}C*]ASA was purchased from New England Nuclear, Boston, Mass.

Incubations were performed within 2 h after the blood was drawn. Washed red cells were suspended in 2-8 parts isotonic phosphate buffer, pH 7.4. Immediately before the incubation, unlabeled ASA was dissolved in isotonic phosphate buffer to a concentration of 30 mM and then titrated to the desired pH. A small amount of dry radioactive ASA was then added and thoroughly mixed to insure complete dissolution. A measured volume was added to the incubation mixture at time zero; dilutions of the remainder were used for counting standards. The mixtures were incubated in a Dubnoff metabolic shaker at 37°C . At measured time intervals during the incubations, aliquots of the red cell suspensions were rapidly centrifuged, washed twice with cold isotonic saline, and then lysed in cold distilled water. Hemoglobin was then separated from free ASA by gel filtration on G-25 Sephadex (Pharmacia Fine Chemicals

Inc., Piscataway, N. J.). In some experiments, hemoglobin solutions of known concentration were used instead of washed red cells. Hemolysates were "stripped" of organic phosphate by dialysis in a stretched cellulose membrane against 0.1 M NaCl (4). The reaction between ASA and hemoglobin in solution was stopped at measured time intervals by rapidly passing an aliquot of the reaction mixture through G-25 Sephadex. Hemoglobin concentration was determined from the absorbance of duplicate dilutions in Drabkins solution ($\epsilon_{\text{CNM} \text{ at } 540 \text{ nm}} = 1.1 \times 10^4$). Radioactivity of bleached hemoglobin solutions was determined as previously described (4). Approximately equal amounts of unlabeled hemoglobin were added to the [^{14}C]ASA standards in order to equalize the counting error due to color quenching in the sample and standard. From the radioactivity and concentration of the hemoglobin samples and the radioactivity of the standard, the number of acetyl groups per hemoglobin molecule could be calculated. Hemoglobin solutions were analyzed by isoelectric focusing on polyacrylamide gels (5). This method has proved very effective in resolving minor hemoglobin components which cannot be demonstrated by other electrophoretic systems. The separated hemoglobins were quantitated by scanning the unstained gels at 540 nm with a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a scanning attachment. Acetylated hemoglobin was separated from unmodified hemoglobin by chromatography at 4°C on a *O*-(carboxymethyl)cellulose (CM-cellulose) column equilibrated with 0.01 M phosphate, pH 6.7, and developed with a linear pH gradient. Oxygen equilibria were determined spectrophotometrically on phosphate-free hemoglobin solutions (0.1 mM tetramer) in 0.1 M chloride, 0.05 M bis-Tris buffer, pH 7.2, at 20°C (6).

Globin was prepared from ^{14}C -acetylated hemoglobin by precipitation in cold acid acetone (7). Acetylated and unmodified alpha and beta chains were separated on a CM-cellulose column in 8 M urea by using a linear sodium phosphate gradient, pH 6.7. Fractions comprising the ^{14}C -acetylated subunit peaks were amino-ethylated, digested with TPCK trypsin (Worthington Biochemical Corp., Freehold, N. J.) and lyophilized. 1.5-mg samples containing between 5,700 and 6,300 cpm were fingerprinted on 20×40 -cm cellulose thin layer plates (Analtech Inc., Newark, Del.). Electrophoresis was performed at 1,000 V in pyridine-acetic acid-water (300:10:2,700) pH 6.5, for 3 h at 4°C

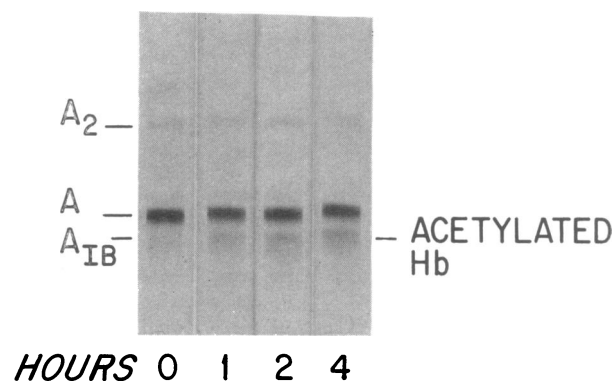


FIGURE 2 Separation of acetylated hemoglobin from normal hemoglobin A by isoelectric focusing on polyacrylamide gel. Red cells were incubated with 20 mM ASA in isotonic phosphate buffer, pH 6.9, at 37°C for 4 h. The results of quantitative scanning of these gels are shown in Fig. 1.

on a Savant electrophoresis cooling plate (Savant Instruments, Inc., Hicksville, N. Y.). Ascending chromatography was performed in pyridine-water-isobutanol-1-propanol-amyl alcohol (30:30:10:10:10) and the plates were stained with commercially prepared ninhydrin spray reagent, 0.3% (Gelman Instrument Co., Ann Arbor, Mich.). Autoradiography was done for 2 wk with RP-X-Omat X-ray film (Eastman Kodak Co., Rochester, N. Y.).

RESULTS

Red cells incubated in the presence of 20 mM ASA labeled in the acetyl group showed a progressive increase in hemoglobin radioactivity over several hours (Fig. 1) whereas no incorporation of label was observed when carboxyl-labeled ASA was used. These observations indicated that a transacetylation had occurred in which the acetyl group of ASA became covalently linked to hemoglobin. The studies of Klotz and Tam (1) and de Furia et al. (2) employed only acetyl-labeled ASA and do not rule out the possibility that the entire ASA molecule bound to hemoglobin. From the radioactivity data, the average number of acetyl groups per hemoglobin molecule could be calculated (see Methods). Since hemoglobin tetramer readily dissociates into dimers under physiological conditions ($\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$), and the $\alpha\beta$ -dimer is the smallest unit of hemoglobin which can be separated electrophoretically or chromatographically under physiological conditions, we have expressed the results as the number of acetyl groups bound per $\alpha\beta$ -dimer. From the results shown in Fig. 1, it is apparent that acetylation took place more readily at pH 7.4 than at pH 6.9. (More complete data on the effect of pH on this reaction is presented below.) Isoelectric focusing of the hemolysates of ASA-treated red cells

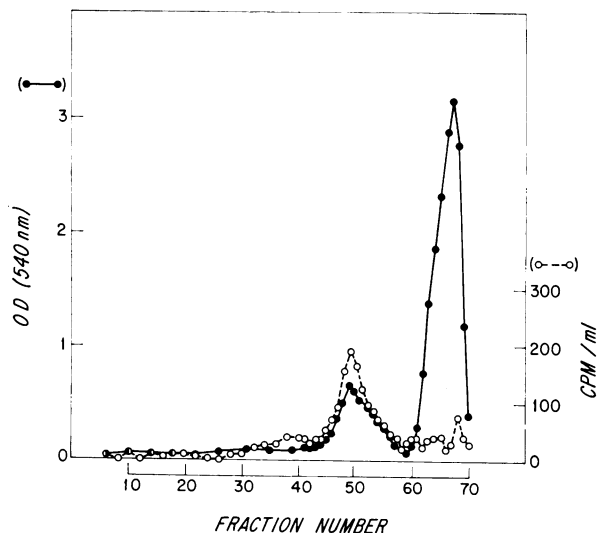


FIGURE 3 Separation of acetylated from nonacetylated hemoglobin by chromatography on CM-cellulose. No radioactivity was detected in the main hemoglobin A peak.

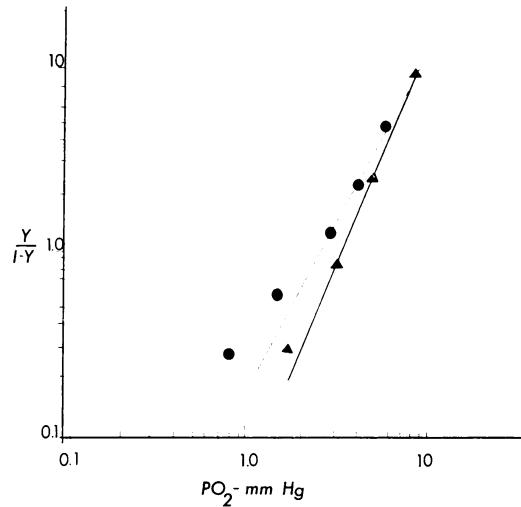


FIGURE 4 Oxygen equilibria on acetylated (●) and non-acetylated (▲) hemoglobins isolated by column chromatography (see Fig. 3). 0.1 mM hemoglobin tetramer in 0.1 M Cl^- , 0.05 M bis-Tris, pH 7.2, 20°C.

revealed an increase in a broad band clearly separable from normal hemoglobin A and having a lower isoelectric point (Fig. 2). This new hemoglobin component comigrated with hemoglobin A_{1B} (5, 8), a minor component, the structure of which is unknown. The banding pattern shown in Fig. 2 provided indirect evidence that positively charged groups on globin were acetylated. This new electrophoretic component probably contains one acetyl group per dimer (two acetyl groups per tetramer).² Densitometric scanning of the gels provides an independent measure of the extent of acetylation of hemoglobin. As Fig. 1 shows, there is good agreement between measurement of acetylation from radioactivity data and quantitation by gel scanning. After 2 and 4 h incubation at pH 7.3, estimation of acetylation by radioactivity exceeded that by gel scanning. It is likely that at these higher levels of acetylation, there are some hemoglobin dimers that contain more than one acetyl group. These would be included in the overall radioactivity data but not in the scanning data because their added negative charge would result in a separation from the main acetylated peak.

The clean separation of acetylated from nonacetylated hemoglobin was also achieved by column chromatography on CM-cellulose, shown in Fig. 3. No radioactivity was found in the main peak of A hemoglobin. The acetylation of hemoglobin by ASA apparently results in a significant reduction in the overall surface

² Assymetrical hemoglobins having one acetyl group per tetramer would not be isolated by any system dependent upon charge differences since the molecule would split into two unlike dimers which would sort with others of like charge.

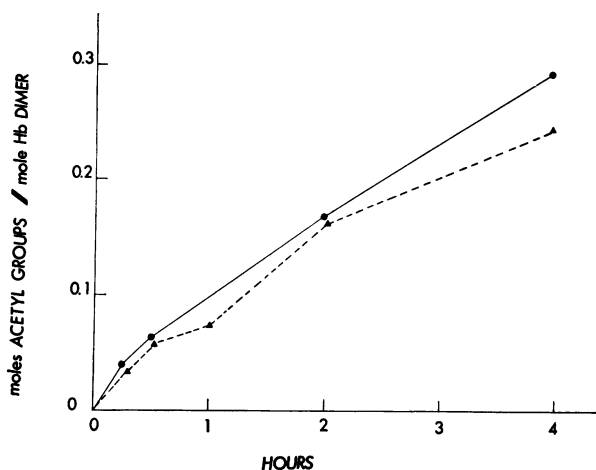


FIGURE 5 Acetylation of oxy- (●) and deoxyhemoglobin (▲). 1.0 mM hemoglobin tetramer was incubated with 20 mM [*acetyl*-1-¹⁴C]ASA in isotonic phosphate buffer, pH 6.9, 37°C.

charge, readily allowing its isolation. Specific activity measurement indicated that the radioactive acetylated peak contained 1.1 acetyl groups per hemoglobin dimer.

Oxygen equilibria were measured on the preparations shown in Figs. 1 and 3. No significant alteration in oxygen affinity or heme-heme interaction was noted in the phosphate-free hemolysates obtained from red cells incubated in 20 mM ASA, pH 6.9, for 1, 2, and 4 h. As Fig. 4 shows, the purified acetylated hemoglobin had a slightly higher oxygen affinity and lower heme-heme interaction (*n* value) when compared to that of the unmodified hemoglobin isolated from the same column. It is possible that acetylation resulted in a mixture of chemically modified hemoglobins including a small proportion with increased oxygen affinity.

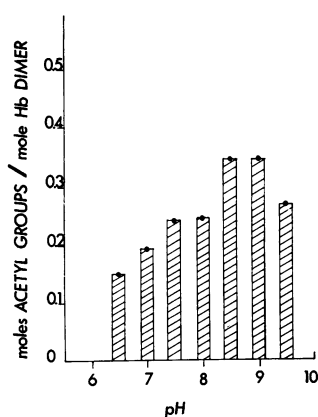


FIGURE 6 Effect of pH on the acetylation of hemoglobin. 1.0 mM hemoglobin tetramer was incubated for 60 min with 20 mM [*acetyl*-1-¹⁴C]ASA in 0.05 M Tris or bis-Tris buffers, 37°C.

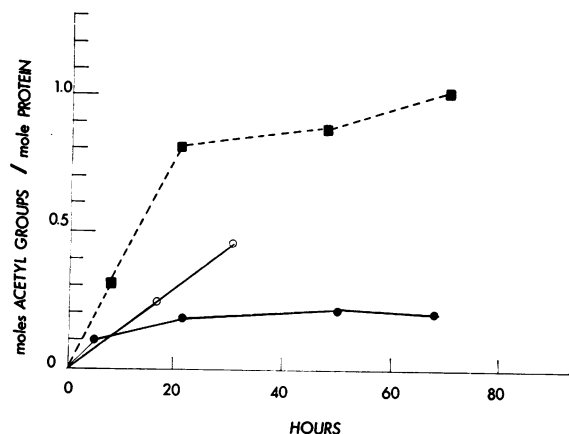


FIGURE 7 Comparison of acetylation of human hemolysate (●), purified hemoglobin A (○), and human serum albumin (■). 1.0 mM hemoglobin tetramer or albumin was incubated with 2.0 mM [*acetyl*-1-¹⁴C]ASA in 0.1 M phosphate buffer, pH 7.3, 37°C.

The state of oxygenation of the hemoglobin did not appear to affect the rate at which acetylation occurred (Fig. 5). Hemoglobin solutions were incubated with 20 mM ASA, 0.1 M phosphate, pH 6.9, after deoxygenation in paired tonometers. One tonometer was reoxygenated at the beginning of the incubation. The lower pH value was chosen in order to maximize the formation of salt bonds that stabilize deoxyhemoglobin (9). Among these are the N-terminal amino groups of the α - and β -chains. The lack of any significant difference in rates of acetylation of oxy- and deoxyhemoglobin indicates that these groups are not principal sites of acetylation.

The rate of acetylation of hemoglobin was found to be strongly pH dependent. As Fig. 6 shows, the reaction was enhanced with increasing pH. However, above pH 8.5, the reaction rate leveled off and decreased at around pH 9.5. The rate of hydrolysis of ASA in aqueous solutions is quite constant from pH 5 to 9 (10). Under more alkaline conditions, however, the rate of hydrolysis increases markedly (10). Thus the leveling off noted in Fig. 6 may be due to depletion of reactant by hydrolysis rather than to an inhibition of acetylation.

We compared the relative rates of acetylation of human hemoglobin and human albumin. Long-term incubations with a low (2 mM) concentration of [*acetyl*-1-¹⁴C]ASA in 0.1 M PO₄, pH 7.3 (37°C), were employed in order to approximate the conditions used by Hawkins, Pinckard, Crawford, and Farr (11). The incubation mixture contained either 1.0 mM albumin or 1.0 mM hemoglobin tetramer (2.0 mM hemoglobin dimer). Since the molecular weights of hemoglobin and albumin are about the same, total protein concentrations in the incubations were similar, permitting a direct comparison of the relative rates of acetylation. As Fig. 7

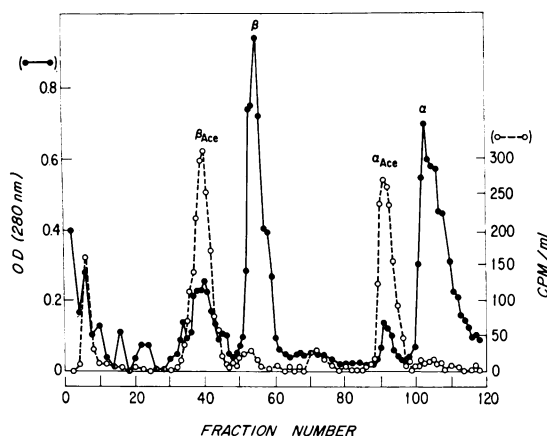


FIGURE 8 Separation of acetylated and nonacetylated globin subunits by chromatography on CM-cellulose in 8 M urea. Purified human hemoglobin A was incubated for 20 h with 2.0 mM [*acetyl*-1-¹⁴C]ASA in 0.1 M phosphate buffer, pH 7.3, 37°C.

shows, albumin was acetylated relatively rapidly, approaching one acetyl group per molecule. Hemoglobin reacted more slowly, particularly when crude hemolysate was tested rather than column-purified hemoglobin. This may be due to hydrolysis of ASA mediated by some erythrocyte factor. ASA is broken down more readily in the presence of red cells (12). The hydrolytic activity contributed by red cells is probably not due to either acetylcholinesterase (13) or to carbonic anhydrase. Even though the latter enzyme has some nonspecific esterase activity (14, 15), we were not able to show any difference in the rate of acetylation of hemoglobin when red cells or crude hemolysates were incubated with ASA in the presence and absence of the carbonic anhydrase inhibitor, acetazolamide.

Attention was directed toward determining the site(s) at which acetylation was taking place. After hemoglobin A had been purified by chromatography on DEAE-cellulose, it was incubated in 0.1 M phosphate buffer, pH 7.3, at 37°C, with 20 mM [*acetyl*-1-¹⁴C]ASA for 4 h or 2 mM [*acetyl*-1-¹⁴C]ASA for 24 h. The tenfold difference in ASA concentration had no apparent effect on the structural results that were obtained. After incubation, globin was prepared and applied to a CM-cellulose-urea column. A representative elution pattern is shown in Fig. 8. The acetylated α - and β -chains were clearly separated from the nonacetylated chains. When hemoglobin was incubated in 20 mM ASA, the ratio of α -chain radioactivity to β -chain radioactivity was 0.778, while the ratio was 0.817 in the 2.0 mM ASA incubation. Thus, the two subunits were about equally acetylated. Tryptic digests of the acetylated and nonacetylated chains were analyzed by finger printing on cellulose thin layers. The ninhydrin-stained peptide maps of the acetylated

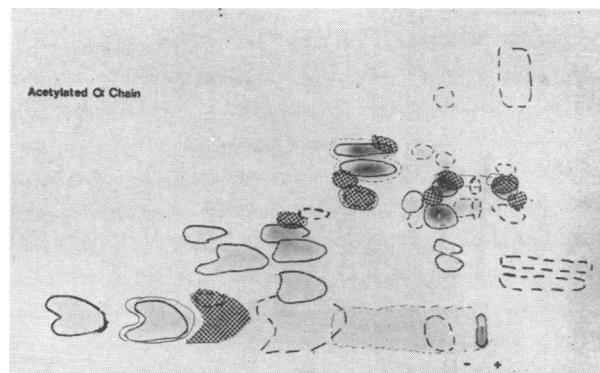


FIGURE 9 Fingerprint of ¹⁴C-acetylated α -chains. After cellulose thin layer plate was stained with ninhydrin, the positive spots were circled. The plate was then autoradiographed for 15 days. The radioactive spots are indicated by stippled areas.

chains were not consistently different from those of the nonacetylated chains. This is strong, although indirect, evidence that no single site on either subunit was preferentially attacked. Autoradiograms demonstrated eight radioactive spots for the α -chain (Fig. 9) and five radioactive spots for the β -chain (Fig. 10). The radioactive spots (indicated in these figures by stippling) were either ninhydrin negative, faintly ninhydrin positive, or overlapped part of a ninhydrin positive spot. These patterns provide further evidence that the α - and β -chains were acetylated at a number of sites. As expected from the overall decrease in isoelectric point of acetylated hemoglobins, the acetylated peptides tended to migrate more slowly to the cathode than the nonacetylated peptides.

To ascertain whether hemoglobin was acetylated by ASA *in vivo*, we obtained fresh blood specimens from 15 patients on long-term high-dose ASA (3–6 g per day p.o. for at least 4 mo) and 11 normal individuals who

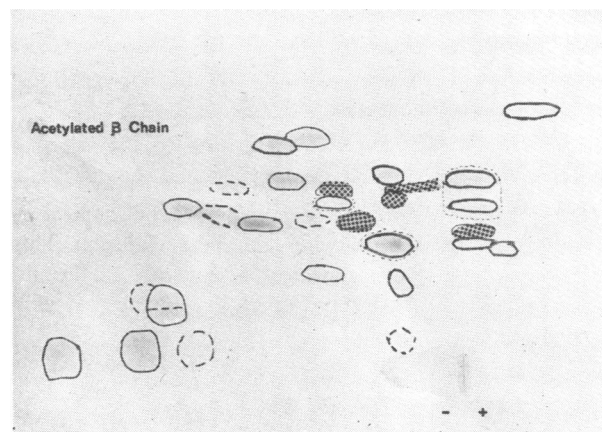


FIGURE 10 Fingerprint of ¹⁴C-acetylated β -chains. Same conditions as Fig. 9.

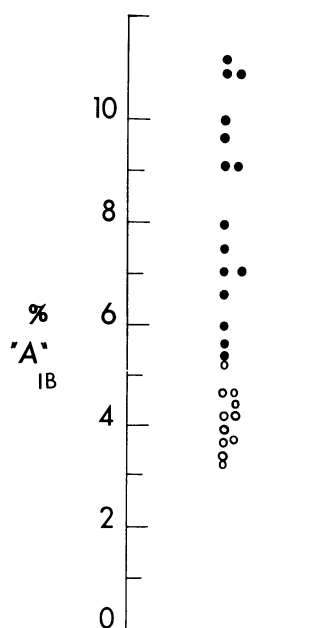


FIGURE 11 Comparison of percent minor hemoglobin component "A_{1B}" in normal individuals (○) and patients taking long-term high dose ASA (●). Hemolysates were analyzed by gel focusing followed by densitometric scanning at 540 nm.

do not take ASA. Hemolysates were prepared on the same day, dialysed overnight against 0.1 M phosphate, pH 7.2, and were then analyzed by gel electrofocusing and densitometric scanning (as in Figs. 1 and 2). The banding pattern for ASA-treated patients was indistinguishable from that obtained after treatment of normal red cells *in vitro* with ASA. Since acetylated hemoglobin co-migrates with the normal minor component A_{1B}, the results are reported as "A_{1B}"-percent of total hemoglobin. As Fig. 11 shows, the ASA-treated patients had about twice as much "A_{1B}" hemoglobin as normals. These data strongly suggest that a small proportion of hemoglobin becomes acetylated in patients who ingest large quantities of ASA.

DISCUSSION

ASA is absorbed intact in the gastrointestinal tract (16, 17). As the drug circulates in the plasma it is readily hydrolyzed to salicylic acid and acetate ion. This reaction proceeds very slowly at neutral pH in the absence of enzyme (10). Hydrolysis is enhanced during passage through the liver and other organs (17). In addition, hydrolysis of ASA may be facilitated by contact with erythrocytes (12). Patients who ingest large doses of ASA can have plasma salicylate levels exceeding 2 mM but the concentration of acetyl salicylic acid is probably less than 0.2 mM (17).

Since patients who are on full dosage of the drug have significant plasma levels of ASA, it is important to determine whether proteins in the blood are chemically modified. Hawkins et al. (11) showed that a specific lysine residue of human serum albumin is acetylated both *in vitro* and *in vivo*. They also demonstrated significant acetylation of a number of other plasma proteins after *in vitro* incubations with ASA (18). The chemical modification of platelets by ASA affects their function (19, 20) and can occasionally lead to a significant clinical disorder.

Surprisingly, the possibility that hemoglobin could be chemically modified by ASA has not been investigated until recently. The results of Klotz and Tam (1) and de Furia et al. (2) show that hemoglobin incorporates radioactivity after incubation with [*acetyl*-1-¹⁴C]ASA. The results presented above confirm this finding. In contrast, we found no incorporation of radioactivity after incubations with [*carboxyl*-¹⁴C]ASA. Therefore, like albumin and other plasma proteins, hemoglobin becomes acetylated during incubation with ASA. Our experiments employed two concentrations of ASA: 2 mM and 20 mM. It was not feasible to study this reaction with a concentration of ASA that can be achieved *in vivo* (~0.2 mM). Nevertheless, our structural results indicate that the sites on hemoglobins at which acetylation takes place are not affected by a ten-fold difference in ASA concentration. Since the red cell has a 120-day life span, it is likely that circulating levels of ASA which can be achieved clinically result in a similar structural modification of the hemoglobin. The gel scanning data shown in Fig. 11 indicate that this is true. The acetylation of hemoglobin by ASA *in vivo* is a hitherto unrecognized form of acquired hemoglobinopathy. Some patients with plumbism also have an electrophoretically distinct hemoglobin component (21), but its structure has not yet been worked out. Other acquired hemoglobin abnormalities represent alterations in the proportion of minor components that are normally present (22).

The structural studies reported here indicate that, unlike human albumin,³ the acetylation of hemoglobin does not involve any single residue. α - and β -chains appear to be almost equally reactive and the autoradiographs of the peptide maps indicate that a number of sites on each subunit are acetylated. Because there were multiple reaction sites, each radioactive peptide was present in very low yield (as shown by the absent or weak ninhydrin staining); therefore the structural analysis of these acetylated peptides would be technically difficult. It is likely that the positively charged ϵ -amino groups of lysine residues are the primary sites at which

³ In contrast to human albumin, bovine albumin is acetylated by ASA at a number of sites (23).

acetylation occurs. The modification results in a rather uniform lowering of the isoelectric point of the hemoglobin (see Figs. 2 and 3) indicating a more negative overall surface charge. This would not occur if uncharged or negatively charged groups were modified by the neutral acetyl moiety. Secondly, the change of acetylation rate with pH is compatible with the ϵ -amino of lysines being the reactive site. It is likely that the non-protonated form of the amino group carries out a nucleophilic attack on the carbonyl carbon of the acetyl group. If so, this reaction should proceed faster with increasing pH. The fact that the reaction levels off above pH 8.5 may be due to pH-dependent hydrolysis of ASA. Klotz and Tam (1) predicted that the N-terminal amino groups of the α - and β -chains would be selectively acetylated. Our results indicate that this is not the case. The fact that acetylated hemoglobin has nearly normal oxygen affinity and that oxy- and deoxyhemoglobin are acetylated at equal rates provides strong evidence against any significant structural modification at these two functionally important sites. Since ASA acetylates hemoglobin at a number of sites, it would not be expected to be effective as an antisickling agent. The experimental results of de Furia et al. (2) indicate that this is indeed true.

Note added in proof. After submission of this manuscript, the report of Shamsuddin, Mason, Ritchey, Honig, and Klotz (24) appeared. They have also found that aspirin acetylates hemoglobin at a variety of sites on the α - and β -chains. They provided direct evidence that lysine groups were modified.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant HL 16927-01 and contract No. 1-HB-42973 and by the Nehemias Gorin Foundation.

REFERENCES

- Klotz, I. M., and J. W. O. Tam. 1973. Acetylation of sickle cell hemoglobin by aspirin. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1313-1315.
- de Furia, F. G., A. Cerami, H. F. Bunn, Y. S. Lu, and C. M. Peterson. 1973. The effect of aspirin on sickling and oxygen affinity of erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 3707-3710.
- Bridges, K. R., F. G. de Furia, Y. S. Lu, C. M. Peterson, G. J. Schmidt, M. Jensen, A. Cerami, and H. F. Bunn. 1974. The acetylation of hemoglobin by aspirin. *In Proceedings of the First National Symposium on Sickle Cell Disease*. U. S. Department of Health, Education, and Welfare Publication No. (NIH) 75-723. 185-186.
- Jensen, M., D. G. Nathan, and H. F. Bunn. 1973. The reaction of cyanate with the α and β subunits in hemoglobin. Effects of oxygenation, phosphates, and carbon dioxide. *J. Biol. Chem.* **248**: 8057-8063.
- Drysdale, J. W., P. Righetti, and H. F. Bunn. 1971. The separation of human and animal hemoglobins by isoelectric focusing on polyacrylamide gel. *Biochem. Biophys. Acta.* **229**: 42-50.
- Bunn, H. F., and R. W. Briehl. 1970. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. *J. Clin. Invest.* **49**: 1088-1095.
- Rossi Fanelli, A., E. Antonini, and A. Caputo. 1958. Studies on the structure of hemoglobin. Physicochemical properties of human globin. *Biochim. Biophys. Acta.* **30**: 608-615.
- Allen, D. W., W. A. Schroeder, and J. Balog. 1958. Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin: a study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. *J. Am. Chem. Soc.* **80**: 1628.
- Perutz, M. F. 1970. Stereochemistry of cooperative effects in haemoglobin. *Nature (Lond.)*. **228**: 726-734.
- Kelly, C. A. 1970. Determination of the decomposition of aspirin. *J. Pharm. Sci.* **59**: 1053-1079.
- Hawkins, D., R. N. Pinckard, I. P. Crawford, and R. S. Farr. 1969. Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid. *J. Clin. Invest.* **48**: 536-542.
- Harthorn, L., and M. Hedström. 1971. Hydrolysis of salicylsalicylic acid in human blood and plasma: a comparison with acetyl salicylic acid. *Acta Pharmacol. Toxicol.* **29**: 155-163.
- Vincent, D., and R. Lagren. 1950. Action comparée des sérums de diverses espèces sur l'acetylcholine et sur l'acide acétylsalicylique ("pseudocholinestérase" et "aspirine-estérase"). *C. R. Seances Soc. Biol.* **144**: 641-643.
- Tashjian, R. E., D. P. Douglas, and Y. L. Yu. 1964. Esterase and hydrase activity of carbonic anhydrase-I from primate erythrocytes. *Biochem. Biophys. Res. Commun.* **14**: 256-261.
- Pocker, Y., and J. T. Stone. 1967. The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of p-nitrophenyl acetate. *Biochemistry.* **6**: 668-678.
- Mandel, H. G., N. M. Cambos, and P. K. Smith. 1954. The presence of aspirin in human plasma after oral administration. *J. Pharmacol. Exp. Ther.* **112**: 495-500.
- Leonards, J. R. 1962. Presence of acetylsalicylic acid in plasma following oral ingestion of aspirin. *Proc. Soc. Exp. Biol. Med.* **110**: 304-308.
- Pinckard, R. N., D. Hawkins, and R. S. Farr. 1968. *In vitro* acetylation of plasma proteins, enzymes and DNA by aspirin. *Nature (Lond.)*. **219**: 68-69.
- Weiss, H. J., L. M. Aledort, and S. Kochwa. 1968. The effect of salicylates on the hemostatic properties of platelets in man. *J. Clin. Invest.* **47**: 2169-2180.
- Al-Mondhiry, H., A. J. Marcus, and T. H. Spaet. 1970. On the mechanism of platelet function inhibition by acetylsalicylic acid. *Proc. Soc. Exp. Biol. Med.* **133**: 632-636.
- Charache, S., and D. J. Weatherall. 1966. Fast hemoglobin in lead poisoning. *Blood.* **28**: 377-386.
- Bradley, T. B., and H. M. Ranney. 1974. Acquired disorders of hemoglobin. *Prog. Hematol.* **8**: 77-98.
- Spencer, G. M., and T. P. Key. 1971. Isoelectric heterogeneity of bovine plasma albumin. *J. Biol. Chem.* **246**: 201-208.
- Shamsuddin, M., R. G. Mason, J. M. Ritchey, G. R. Honig, and I. M. Klotz. 1974. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 4693-4697.