

Structural Changes in Human Serum Albumin Induced by Ingestion of Acetylsalicylic Acid

DAVID HAWKINS, R. NEAL PINCKARD, IRVING P. CRAWFORD, and
RICHARD S. FARR

*From the Division of Allergy, Immunology, and Rheumatology and Division
of Microbiology, Scripps Clinic and Research Foundation,
La Jolla, California 92037*

ABSTRACT Acetylsalicylic acid (aspirin) acetylates human serum albumin under physiologic conditions in vitro. These investigations were done to determine whether this phenomenon occurs in vivo and to delineate the site(s) of acetylation on the albumin molecule.

Albumin was reacted in vitro with aspirin labeled with ¹⁴carbon at the acetyl-1 or the carboxyl carbon. The altered albumin was hydrolyzed with trypsin and peptide mapping performed. Albumin so treated contains a unique peptide, designated "A," and shows diminution of two normal peptides, designated "B" and "C." Peptide "A" is never seen in normal albumin. Amino acid analyses indicate that peptide "A" equals the sum of peptides "B" and "C." Furthermore all three peptides contain lysine but lack arginine. Thus peptide "A" is formed by the acetylation of a lysine residue which is normally susceptible to trypsin and yields peptides "B" and "C." Radioautography of the peptide maps show most of the acetyl-1-¹⁴C activity in peptide "A." This indicates that one of the lysine residues in this peptide is the preferential site for the transacetylation reaction.

Peptide "A," used as a marker for acetylation, is found in albumin from patients who take aspirin but is not demonstrable in albumin from one of these patients while she was taking sodium salicylate. A transacetylation reaction between aspirin and human albumin occurs in vivo and is similar to that observed in vitro.

Dr. Hawkins' present address is: Division of Clinical Immunology, Montreal General Hospital, Montreal 25, Quebec, Canada. Dr. Pinckard's present address is: Department of Microbiology, College of Medicine, University of Arizona, Tucson, Arizona 85721.

Received for publication 25 September 1968 and in revised form 12 November 1968.

INTRODUCTION

Previous studies demonstrated that acetylsalicylic acid (aspirin) acetylates human serum albumin (HSA) and a variety of other body constituents in vitro (1-3). In addition HSA acetylated with aspirin has enhanced capacity to bind a marker anion, acetrizoate (2). Since the HSA from persons ingesting therapeutic doses of aspirin also have an enhanced capacity to bind this marker anion, it was postulated that aspirin acetylates HSA in vivo (1, 2, 4).

The purpose of the present study was to provide direct evidence that aspirin acetylates HSA in vivo and to characterize the site(s) of acetylation on the HSA molecule. Previous studies demonstrated that the acetyl-HSA derivative formed in vitro was stable at pH 10-11, a finding indicating that an *N*-acetyl rather than an *O*-acetyl derivative had been formed. The ϵ -amino group of lysine was considered a probable site for this reaction. A protein or peptide containing this derivative would be resistant to tryptic hydrolysis at this site (5) and would yield a peptide pattern different from the native protein on mapping. If such a peptide difference exists in HSA acetylated by aspirin in vitro, then this alteration would provide a means of demonstrating whether HSA is similarly acetylated in vivo by patients ingesting aspirin.

METHODS

Albumins. HSA (Cohn Fraction V, E. R. Squibb & Sons, New York) was kindly provided as a standard by the American Red Cross. Other HSA was obtained from Behringwerke, AG, through the Diagnostic Reagents Department, Hoechst Pharmaceutical Company, Kansas City, Mo. In addition, HSA was isolated from sera of the following subjects: (a) R.M., age 34, a healthy adult male with a

history of no aspirin ingestion for over 2 yr; (b) C.J., age 56, a female with rheumatoid arthritis. A serum sample was obtained after the patient had been taking 4 g of sodium salicylate daily for 5 months. The sodium salicylate was discontinued, she was then placed on aspirin 4 g/day, and 5 wk later a second serum sample was obtained; (c) W.H., age 70, a male with rheumatoid arthritis who had been ingesting 6.6 g of aspirin daily for 8 months. HSA was isolated from these patient's sera and the control serum by the sodium sulfate fractionation method of Kekwick (6). 1 vol of 36% (w/v) sodium sulfate was added slowly to 1 vol of whole serum with constant mixing at 25°C. After 30 min the precipitate was centrifuged and discarded. The supernate was exhaustively dialyzed against distilled water and lyophilized. The crude HSA preparations were purified further by Pevikon electrophoresis in barbital buffer, pH 8.6, $\gamma/2 = 0.05$, for 18 hr at 4°C (7). The purity of all HSA preparations, including those purchased commercially, was determined by immunoelectrophoresis against Hyland goat anti-whole human serum (8).

Alteration of HSA by aspirin *in vitro*. Acetyl-1-¹⁴C-salicylic acid with a specific activity of 2.38 mc/mmole was kindly provided by Merck, Sharp & Dohme, West Point, Pa. Acetylsalicylic acid-carboxyl-¹⁴C with a specific activity of 3.26 mc/mmole was obtained from Nuclear-Chicago Corporation, Des Plaines, Ill. The HSA samples obtained from Squibb and Behringwerke were altered *in vitro* with the labeled or unlabeled aspirin preparation as described previously (2, 3). Equal volumes of 2.0×10^{-4} M purified HSA aliquots and 1.0×10^{-3} M aspirin were reacted in 0.10 M phosphate buffer, pH 7.4, at 37°C for 24 hr and then dialyzed exhaustively against 0.15 M NaCl containing 0.01 M unlabeled sodium salicylate to remove aspirin bound noncovalently. After the exposure to sodium salicylate duplicate 0.10-ml samples were counted for ¹⁴C activity. The HSA reacted with acetyl-¹⁴C-labeled aspirin retained an average of 1.2 acetyl groups per molecule. Albumin reacted with carboxyl-¹⁴C-labeled aspirin had an average of 0.02 salicyloyl groups per molecule.

Peptide preparation. In each peptide preparation a normal HSA sample was processed simultaneously with aspirin-altered albumin. The purified albumins were reduced and carboxymethylated according to the method of Crestfield, Moore, and Stein (9) with minor modification. Ethylenediaminetetraacetate (EDTA) was not employed, and immediately after carboxymethylation the samples were dialyzed exhaustively against distilled water at 25°C and lyophilized. Enzymatic hydrolysis was performed in the presence of 2.0 M urea in a 0.1 M (NH₄)₂CO₃ buffer, pH 9.0, to which was added 0.01 ml of aqueous trypsin solution (2.5 mg/ml) per mg of protein. Trypsin, 2 times recrystallized, was obtained from Worthington Biochemical Corp., Freehold, N. J. The digestion proceeded with continuous, gentle agitation for 2 hr at 25°C. The digest was diluted 5- to 10-fold with distilled water and applied to a column containing Dowex AG 50W-X 2, 100-200 mesh with a resin bed volume of 4 ml/5 mg of digest. After the column was washed free of urea with distilled water, the peptides were eluted with 4 N NH₄OH and brought to dryness with H₂SO₄ under vacuum in a desiccator.

Peptide mapping. Peptides from approximately 50 mg of trypsin-digested albumin were resuspended in distilled water, and approximately 5 mg was applied in 10- to 30- μ l aliquots to No. 3 MM Whatman chromatography paper. Descending chromatography was performed in a Shandon chromatography tank with normal butanol:acetic acid:water

(200:30:75) as solvent. The papers were dried and then immersed in a pyridine:acetic acid:water buffer (1:10:589), pH 3.55. High-voltage electrophoresis was performed under Varsol at 90° to the direction of chromatography with 2000 v for 70-80 min in a Savant apparatus. The dried peptide maps were stained with 0.3% ninhydrin in acetone or stained for specific amino acids as described by Easley (10).

Radioautography. The position of peptides containing either the acetyl-1-¹⁴C or carboxyl-¹⁴C label was determined by placing ninhydrin-stained peptide maps directly on Kodak No-Screen Medical X-Ray Film (NS-54 T) for 5-10 days.

Amino acid analysis. The peptide maps used to isolate peptides for amino acid analysis were stained lightly with 0.01% ninhydrin in acetone and heated briefly to 70-80°C. The desired peptides were cut out and eluted from the paper with 30% acetic acid. Peptides from six to eight peptide maps were pooled, dried, and 1 ml of 6 N HCl was added to the dried peptides in ampules which were then sealed under a vacuum. The peptides were hydrolyzed at 110°C for 20 hr. Amino acid analysis was performed in a Beckman Model 120C Amino Acid Analyzer with a coupled Beckman 125 Integrator. Amino acid values in nanomoles per total sample were determined by comparison with standards.

RESULTS

Structural changes in albumin altered by aspirin *in vitro*. The peptide map of HSA acetylated with acetyl-1-¹⁴C-salicylic acid under physiologic conditions *in vitro* is seen in Fig. 1. A unique peptide designated "A" was demonstrated consistently. This peptide was never seen in normal albumin, whether obtained from Squibb (Cohn Fraction V), Behringwerke, or normal individuals who had not recently ingested aspirin. In addition, peptides designated "B" and "C" were consistently found to be absent or diminished in aspirin-altered albumin (Fig. 1).

The absence of detectable amounts of peptide "A" in pooled normal HSA and the greater prominence of the "B" and "C" peptides is seen in the map of untreated normal albumin (Fig. 2). Although some experiment-to-experiment variations were noted on other areas of the peptide maps, only the differences involving peptides "B," "C," and "A" were reproducible.

Fig. 3 is a radioautograph of the peptide map shown in Fig. 1. Peptide "A" contains most of the acetyl-¹⁴C activity in the labeled preparation because the prominent density on the radioautograph corresponds to the position of peptide "A." Several other radiopacities were noted, but they were much less intense than peptide "A" and did not correspond to any peptide which could be identified by ninhydrin, chlorination, or specific amino acid stains.

As expected, the peptide maps of carboxyl-¹⁴C-labeled aspirin-treated HSA were identical with those of HSA treated with acetyl-¹⁴C-labeled aspirin. However, radioautography of the peptide map of carboxyl-¹⁴C-labeled aspirin-treated HSA revealed a distinct radiopacity which was different from any of those seen in Fig. 3. This radiopacity was located in a more hydrophobic

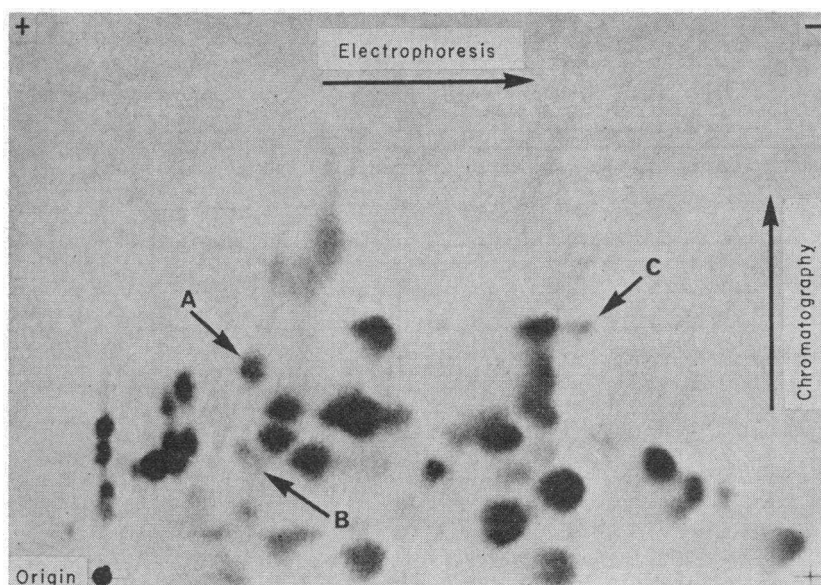


FIGURE 1 Peptide map of human serum albumin (HSA) treated with acetyl-1- ^{14}C -salicylic acid. The positions of peptides "A," "B," and "C" are indicated on this map which was stained with 0.3% ninhydrin.

position above peptide "A," and was in the area of a peptide only faintly visible after ninhydrin staining.

Stains of the peptide maps for specific amino acids showed that peptides "A," "B," and "C" lack arginine, tyrosine, tryptophan, histidine, and sulfur-containing amino acids. All were stained by chlorination, however. Peptides "A" and "B" migrate during electrophoresis to the area where neutral peptides and amino acids are

found. In contrast, peptide "C" migrates more cathodically, a finding which indicates that it is positively charged.

Amino acid composition of peptides "A," "B," and "C." The results of the amino acid analyses are seen in Table I. The peptides lay in close proximity to other ninhydrin- or chlorination-positive areas, and therefore, the amino acid compositions observed reflect some con-

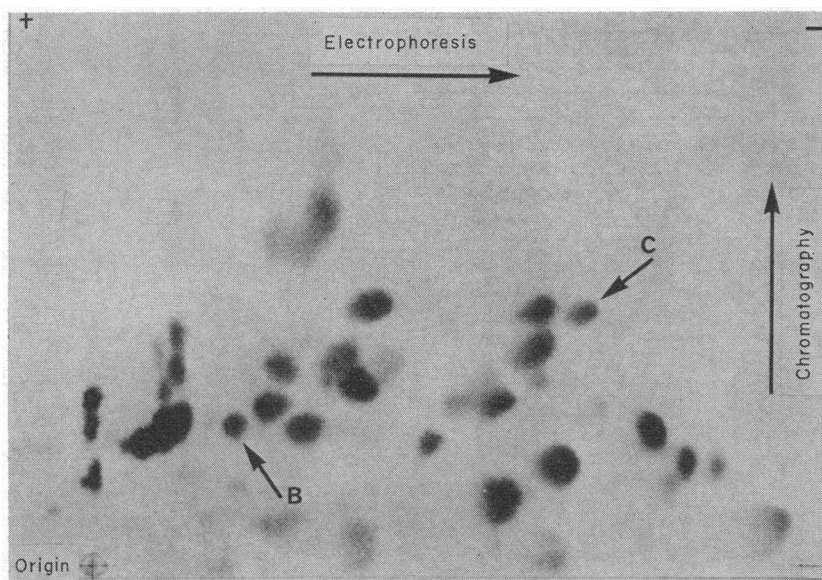


FIGURE 2 Peptide map of normal HSA (Squibb, Cohn Fraction V). The positions of peptides "B" and "C" are indicated.

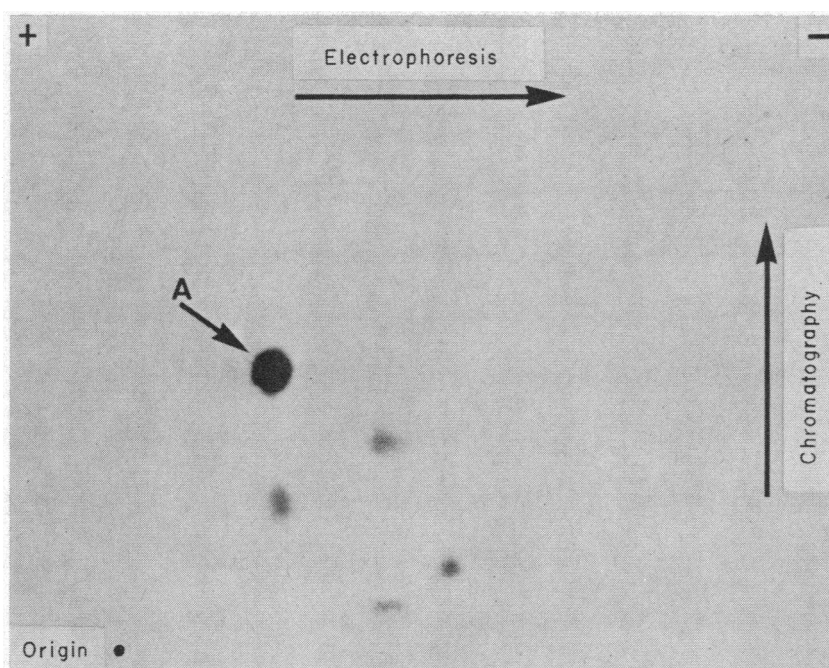


FIGURE 3 Radioautograph of the peptide map shown in Fig. 1, developed after 5 days exposure. The prominent opacity corresponds to peptide "A."

tamination. Nevertheless, five amino acids are common to peptides "A" and "B," i.e., lysine, serine, glutamic acid, alanine, and leucine. Peptide "C" contains significant quantities of only two amino acids, lysine and leucine. The values given for the moles of each amino acid residue are based on the necessity for one lysine residue in peptide "B" and one in peptide "C," and for two lysine residues in peptide "A": the carboxy terminus and a trypsin-resistant *N*-acetyl derivative. The relatively low

value for serine obtained in peptides "A" and "B" doubtless reflects partial destruction of the amino acid which occurs under the hydrolysis conditions used. The light (0.01%) ninhydrin staining was important because significantly lower values were obtained for leucine in peptides "A" and "C" and for serine in peptide "B" when heavier ninhydrin stains (0.25–0.3%) were employed to identify peptides. This suggests that these residues may occur at the amino terminal position in their respective peptides.

Structural changes in HSA in patients ingesting aspirin. Peptide mapping was performed on purified HSA from two patients, C.J. and W.H., who had ingested therapeutic doses of aspirin for a prolonged period. The results are seen in Fig. 4, which demonstrates the presence of peptide "A" in HSA from patient C.J. A similar pattern including peptide "A" was observed with HSA from patient W.H. Peptide "A" was less intense and peptides "B" and "C" were more prominent in the albumins acetylated in vivo than in albumin altered by aspirin in vitro. Peptide maps of albumin from patient C.J. while she was taking sodium salicylate, and from subject R.M. who did not take aspirin, showed no evidence of peptide "A." The latter maps were identical with that shown in Fig. 2.

DISCUSSION

Peptide maps of normal albumin consistently yield over 70 distinct peptides, at least 19 of which are arginine

TABLE I
Amino Acid Composition of Isolated Peptides

Amino acid	Peptide "A"	Peptide "B"	Peptide "C"
	<i>moles/mole*</i>	<i>moles/mole†</i>	<i>moles/mole‡</i>
Lysine	2.00§	1.00	1.00
Arginine	0.21	—	—
Aspartic acid	0.37	0.31	—
Threonine	0.22	0.19	—
Serine	0.88	0.70	—
Glutamic acid	1.39	1.16	—
Alanine	1.20	1.11	—
Valine	0.43	0.19	—
Leucine	1.76	0.95	0.89
Phenylalanine	0.23	—	—

* Moles of amino acid per 2 moles of lysine.

† Moles of amino acid per mole of lysine.

§ Italicized data are considered significant.

|| Values not corrected for destruction during hydrolysis.

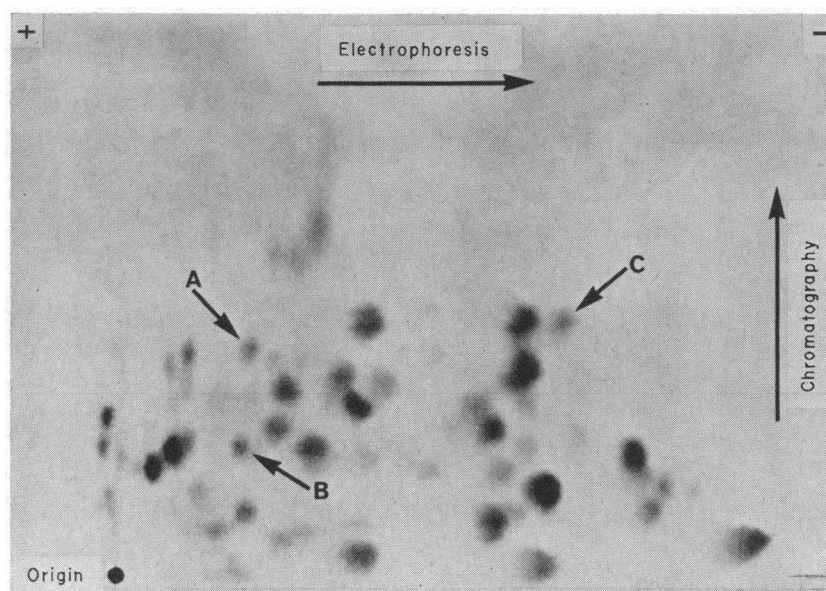


FIGURE 4 Peptide map of HSA isolated from serum of patient C.J. This sample was obtained 5 wk after initiation of aspirin therapy. The positions of peptides "A," "B," and "C" are indicated.

positive. This is compatible with the theoretical yield of 79 peptides from an HSA molecule possessing 55 lysine and 23 arginine residues (11). Peptide maps of HSA exposed to aspirin under physiologic conditions *in vitro* reveal the presence of a unique peptide, "A," and the diminution of two normal peptides, "B" and "C." Peptide "A" was never seen on maps of normal HSA, and peptides "B" and "C" were always more prominent on maps of untreated HSA than of aspirin-altered albumin. The fact that peptides "A," "B," and "C" all lack arginine strongly suggests that a lysine residue in peptide "A" is acetylated by aspirin to render it resistant to the tryptic hydrolysis which ordinarily results in the formation of peptides "B" and "C." This has been confirmed by amino acid analysis, for peptide "A" equals the sum of peptides "B" and "C."

From these analyses, peptide "C" must have the composition and sequence leucyl-lysine; its position on the map is in good agreement with this, for as mentioned earlier it bears a net positive charge. Peptide "B" is neutral, which indicates that the glutamic acid observed after hydrolysis almost certainly did not arise from glutamine. To be neutral a tryptic peptide with carboxyl terminal lysine, contributing one positive charge from its ϵ -amino group and one negative charge from its free carboxyl group, must have one negatively charged side chain to balance the positive charge on the amino terminus of the peptide. Glutamic or aspartic acid would provide this negative charge. The estimated amino acid residues of peptide "B" are one each of serine, glutamic acid, alanine, leucine, and lysine. Taking advantage of

the partial destruction of the amino terminal residue by heavier ninhydrin staining, we may hypothesize that the structure of this peptide is ser-(glu, ala, leu)-lys. By use of the same arguments plus the fact that peptide "A" is the sum of peptides "B" and "C" containing one *N*-acetyl-lysine, the structure of peptide "A" should be leu-*N*-acetyllysine-ser-(glu, ala, leu)-lysine. Alternatively the carboxyl terminal lysine in peptide "B" may be the one acetylated by aspirin, in which case the leu-lys of peptide "C" would be attached at the carboxy terminus of peptide "B." Peptide "A" is slightly more hydrophobic than peptide "B," as evidenced by the increased mobility in the organic chromatography solvent, and this probably due to the presence of the additional leucine residue and the acetylation of the ϵ -amino group of one of the lysine residues.

In radioautographs of peptide maps of HSA altered by acetyl-1- ^{14}C -labeled aspirin, the major radiopacity was observed in peptide "A." The six weaker opacities in Fig. 3 not associated with peptide "A" indicate that there are at least six other possible sites of acetylation on the HSA molecule. The relative position and intensities of these opacities were reproducible and probably represent additional unique peptides. The amounts of these peptides formed were generally insufficient to be detected by the usual staining techniques.

The radioautographic data, though qualitative, demonstrate that peptide "A" is the preferential site of acetylation. There are several possible explanations for preferential acetylation of this lysine residue. The tertiary structure of the HSA molecule may expose the ϵ -amino

group of this lysine in a way that makes it uniquely susceptible to attack by acetylating agents such as aspirin. Alternatively, or perhaps in addition, the lysine residue in peptide "A" may be located very near the aspirin binding site, thereby enhancing the likelihood of its acetylation, a reaction analogous to affinity labeling described by Wofsy, Metzger, and Singer (12).

The data discussed above established that peptide "A" can be used as a marker to assess whether the ingestion of aspirin results in acetylation of HSA in vivo. The albumin from patient W.H. showed peptide "A" and also a diminution of peptides "B" and "C." This suggested that the ingestion of aspirin had resulted in the acetylation of HSA, although the possibility of a peptide anomaly due to rheumatoid arthritis per se was not excluded. However, the peptide map of HSA from rheumatoid arthritis patient C.J. taking sodium salicylate revealed a normal peptide pattern. On the other hand, when she was placed on aspirin therapy for 5 wk, peptide maps of her albumin revealed the presence of peptide "A" and a diminution of peptides "B" and "C." Hence, exposure of albumin to aspirin either in vitro or in vivo does result in the formation of peptide "A" as well as the enhanced capacity of albumin to bind the marker anion, acetrizoate. It must be concluded that the presence of peptide "A" in the albumin from those patients resulted from the ingestion of aspirin, and that an *N*-acetyl-lysine derivative had been formed in vivo. The possibility that other medications and (or) metabolites have the capacity to interact with the reactive lysine in peptide "A" requires further study.

Radioautography of the peptide map of HSA treated with carboxyl ¹⁴C-labeled aspirin demonstrated a radiopacity situated above peptide "A." Previously we suggested that aspirin could form a salicyloyl amide derivative of HSA (2), as this reaction had been demonstrated by reacting aspirin and glycine (13). Although the composition of this salicyloyl peptide is not known, it is possible that it may be formed from peptides "B" and "C," because a salicyloyl group in place of an acetyl group could render peptide "A" more hydrophobic without altering its electrophoretic mobility. Low concentrations of this peptide in our preparations precluded confirmation of this by amino acid analysis. The trans-acetylation of lysine on the "A" peptide is the predominant reaction when aspirin interacts with albumin in vitro, but there are also 0.02 moles of salicyloyl albumin formed per mole of albumin. If this salicyloyl formation is also occurring in vivo, it could represent the source of a significant amount of potentially antigenic material. For example, if only 0.005 moles of salicyloyl albumin per mole of albumin were formed in vivo, then this would still represent 0.2 mg of salicyloyl albumin per ml of serum or a total body burden of approximately 1.4 g of altered albumin.

The biological consequences of acetylation of albumin in vivo are not known; however recent findings suggest that this phenomenon may be of significance. Minden and Farr (14) have shown that the IgG in the sera of many persons can bind ¹²⁵I-iodine-labeled HSA which has been previously treated with aspirin. This binding may reflect an alteration in the antigenicity of albumin as a result of acetylation or salicyloyl amide formation. The syndrome of "aspirin intolerance" characterized by asthma, rhinitis, and nasal polyps (15) is induced by aspirin but by no other salicylate.

Fulop and Brazeau (16) demonstrated that the administration of sodium salicylate to jaundiced bile duct-ligated dogs increased the dialyzability and urinary excretion of plasma-conjugated bilirubin. This was attributed to competition between salicylate and bilirubin for plasma protein binding sites. Since albumin is the major bilirubin-binding plasma protein it is conceivable that a bilirubin binding site, a salicylate binding site, and the preferential site for acetylation by aspirin are all shared. This hypothesis is supported in part by the fact that equimolar concentrations of sodium salicylate can largely inhibit in vitro the aspirin-induced formation of peptide "A" and the enhancement of acetrizoate binding by albumin.¹

There is increasing evidence that aspirin differs significantly from the other salicylates in its effects on hemostasis in humans. Quick has shown that a small oral dose of aspirin, but not sodium salicylate, may prolong the bleeding time, even in normal subjects (17). He implicates the acetyl linkage in this action and suggests that aspirin acts as a competitive inhibitor of cholinesterase, which permits excessive acetylcholine activity and vasodilation (18). Aspirin, but not sodium salicylate, when taken by mouth or added to platelet-rich plasma, inhibits platelet aggregation and blocks the release of adenosine diphosphate from these cells (19-21).

Finally aspirin has a strong anticomplementary action in vitro in normal therapeutic doses with respect to guinea pig complement, while sodium salicylate has no action at all (22).

The acetyl group of aspirin appears to be implicated in most of these physiologic phenomena. The unique capacity of aspirin to form acetyl and salicyloyl amide derivatives of human proteins and other body constituents may well play a role in these reactions.

ACKNOWLEDGMENTS

We thank Dr. L. Jackson for his help with the peptide mapping and Mrs. M. Young for her valuable technical assistance.

This investigation was supported in part by National Institutes of Health (NIH) Training Grant No. AI00214, NIH Research Grant No. AI06894 from National Institute

¹ Pinckard, R. N. Unpublished observations.

of Allergy and Infectious Diseases, and a grant-in-aid from the San Diego Area Chapter of the Arthritis Foundation.

REFERENCES

1. Pinckard, R. N., D. Hawkins, and R. S. Farr. 1968. Permanent alteration of human serum albumin by acetylsalicylic acid. *Fed. Proc.* **27**: 543.
2. Hawkins, D., R. N. Pinckard, and R. S. Farr. 1968. Acetylation of human serum albumin by acetylsalicylic acid. *Science*. **160**: 780.
3. Pinckard, R. N., D. Hawkins, and R. S. Farr. 1968. *In vitro* acetylation of plasma proteins, enzymes and DNA by aspirin. *Nature (London)*. **219**: 68.
4. Hench, P. K., and R. S. Farr. 1967. The alteration of serum albumin by acetylsalicylic acid. *Arthritis Rheum.* **10**: 283.
5. Canfield, R. E., and C. B. Anfinsen. 1963. Concepts and experimental approaches in the determination of the primary structure of proteins. In *The Proteins*. H. Neurath, editor. Academic Press Inc., New York. 2nd edition. **4**: 311.
6. Kekwick, R. A. 1940. The serum proteins in multiple myelomatosis. *Biochem. J.* **34**: 1248.
7. Muller-Eberhard, H. J. 1960. A new supporting medium for preparative electrophoresis. *Scand. J. Clin. Lab. Invest.* **12**: 33.
8. Scheidegger, J. J. 1955. Une micro-méthode d'immuno-electrophorèse. *Int. Arch. Allergy Appl. Immunol.* **7**: 103.
9. Crestfield, A. M., S. Moore, and W. H. Stein. 1963. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **238**: 622.
10. Easley, C. W. 1965. Combinations of specific color reactions useful in the peptide mapping technique. *Biochem. Biophys. Acta.* **107**: 386.
11. Hughes, W. L., Jr. 1954. Interstitial proteins: the proteins of blood plasma and lymph. In *The Proteins*. H. Neurath and K. Bailey, editors. Academic Press Inc., New York. 1st edition. **2 B**: 663.
12. Wofsy, L., H. Metzger, and S. J. Singer. 1962. Affinity labeling—a general method for labeling the active sites of antibody and enzyme molecules. *Biochemistry*. **1**: 1031.
13. Schwartz, M. A., and G. L. Amidon. 1966. Reaction of aspirin with amines. Potential mechanism for aspirin allergy. *Pharm. Sci.* **55**: 1464.
14. Minden, P., and R. S. Farr. 1967. Human antibodies against acetylsalicylic acid-altered human serum albumin. *Arthritis Rheum.* **10**: 299.
15. Samter, M., and R. F. Beers, Jr. 1967. Concerning the nature of intolerance to aspirin. *J. Allergy*. **40**: 281.
16. Fulop, M., and P. Brazeau. 1964. The renal excretion of bilirubin in dogs with obstructive jaundice. *J. Clin. Invest.* **43**: 1192.
17. Quick, A. J. 1966. Salicylates and bleeding: the aspirin tolerance test. *Amer. J. Med. Sci.* **252**: 265.
18. Quick, A. J. 1967. Aspirin and platelet stickiness. *Lancet*. **i**: 386.
19. Weiss, H. J., and L. M. Aledort. 1967. Impaired platelet/connective tissue reaction in man after aspirin ingestion. *Lancet*. **ii**: 495.
20. O'Brien, J. R. 1968. Effects of salicylates on human platelets. *Lancet*. **i**: 779.
21. O'Brien, J. R. 1968. Effect of anti-inflammatory agents on platelets. *Lancet*. **i**: 894.
22. Van Oss, C. J., J. C. Friedmann, and M. Fontaine. 1961. Anticomplementary action of aspirin. *Nature (London)*. **189**: 147.