The Inhibitory Effect of Salicylate on the Acetylation of Human Albumin by Acetylsalicylic Acid

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The transacetylation reaction between aspirin and human serum albumin (HSA) is partially inhibited by pharmacologic concentrations of salicylate anion. This inhibition was noted with respect to the inhibition of enhanced acetrizoate binding and by autoradiography of peptide maps of aspirin-acetylated HSA. The acetylation of a single lysine residue in a peptide “A” has been found to be responsible for the previously reported, enhanced binding of acetrizoate by aspirin-altered albumin. At least 5 other peptides are also acetylated, but they do not influence acetrizoate binding. The results are discussed in view of the unique pharmacologic activities of aspirin, and also, with respect to biologic reactions ascribed to aspirin therapy.

Recently, it has been established that acetylsalicylic acid (aspirin) acetylates in vitro a wide variety of body constituents—plasma proteins, hormones, enzymes, DNA and RNA (1-3). Furthermore, it has been demonstrated that the ingestion of therapeutic amounts of aspirin results in the preferential acetylation in vivo of a particular lysine residue present in human albumin (4). Aspirin has been reported to be pharmacologically superior to sodium salicylate with respect to its analgesic (5), anti-inflammatory (6) and antipyretic properties (7). Aspirin, but not salicylate, has been shown to enhance specifically the binding capacity of human albumin for a marker anion, acetrizoate (8), and to induce the clinical features of “aspirin disease” (9). In addition, recent evidence indicates that aspirin interferes with certain aspects of platelet aggregation (10-14). The preceding findings indicate that a transacetylation reaction of various receptor sites may be responsible for the variety of reported physiologic actions of aspirin. The present studies, therefore, were undertaken to characterize the qualitative and quantitative nature of the site
specificity for acetylation of human serum albumin (HSA) by aspirin.

**MATERIALS AND METHODS**

The effect of temperature upon the rate of the acetylation of HSA with acetyl-1-C-14-salicylic acid. Acetyl-1-C-14-salicylic acid* had a specific activity of 2.38 mc/mM HSA,† 14 mg/ml Cohn Fraction V, was dialyzed against 0.20 M sodium phosphate buffer pH 7.30 for 24 hr. Twelve milliliters of the HSA solution were equilibrated at 37° C, and 12 ml of HSA were equilibrated in an ice-water bath at 0° C. At various intervals a 1 ml sample was withdrawn from each reaction mixture and dialyzed exhaustively at 0° C against 0.15 M NaCl containing 0.01 M sodium salicylate as carrier to remove ionically bound labeled aspirin. Each sample was counted for C-14 activity in a Packard Tri-Carb liquid scintillation counter;‡ protein concentration was determined by the Micro-Kjeldahl method for nitrogen analysis.

The effect of sodium salicylate upon the acetylation of HSA with acetyl-1-C-14 salicylic acid. HSA, 28 mg/ml, Cohn Fraction V was dialyzed against 0.20 M sodium phosphate buffer, pH 7.30 for 24 hr. Concentrations of sodium salicylate ranging from 4.0 × 10⁻³ to 4.0 × 10⁻² M were prepared in 0.20 M sodium phosphate buffer pH 7.30. To a series of test tubes, 0.25 ml of the HSA solution was added followed by 0.25 ml amounts of the various concentrations of sodium salicylate. The mixtures were incubated for 1 hr at 37° C, and then 0.50 ml of 1.0 × 10⁻⁸ M acetyl-1-C-14 salicylic acid in distilled water were added to each tube. After incubating at 37° C for 24 hr, the samples were dialyzed for 72 hr against multiple changes of 0.15 M NaCl containing 0.01 M sodium salicylate. Each sample was counted for C-14 activity, and protein concentration was determined as in the first experiment. These HSA samples were also studied for their capacity to bind the marker anion, 1³¹ labeled sodium acetazolamide.

Acetylation of HSA with acetil-1-C-14 anhydride. Acetic-1-C-14 anhydride§ had a specific activity of 27.6 µc/mM, HSA 70 mg/ml, Cohn Fraction V, was dialyzed against 0.10 M Na₂HPO₄ for 24 hr. The HSA solution was cooled to 0° C, and the pH was adjusted to 9.0 with 1.0 M NaOH. To a series of test tubes placed in a 0° C ice-water bath, increasing microliter amounts of acetic-1-C-14 anhydride were added, and then immediately 3.0 ml of the cooled HSA solution were added. The reaction mixtures were incubated at 0° C for 2 hr; dialyzed for 24 hr against borate buffered saline, pH 7.4, containing 0.05 M sodium acetate as carrier; and dialyzed against borate buffered saline pH 7.4 for 24 hr. Each sample was counted for C-14 activity, analyzed for protein content and the binding of ¹³¹ labeled sodium acetazolamide was determined.

Determination of ¹³¹ labeled sodium acetazolamide binding by HSA. ¹³¹ labeled sodium acetazolamide (3-acetamido-2, 4, 6-triiodobenzoic acid) had a specific activity of 149 mc/mM. The various HSA or acetyl-HSA derivatives, at a concentration of 7 mg/ml, were dialyzed against borate buffered saline, pH 7.40. Binding studies were carried out by equilibrium dialysis (15), and the data were expressed as the bound/free ratio of acetazolamide when the free acetazolamide concentration was 1.0 × 10⁻⁵ M. Duplicate 1 ml amounts of each HSA sample were placed in washed Visking 8/32 inch dialysis tubing. The dialysis bags were then placed in 4 liters of borate buffered saline pH 7.4 containing 1.0 × 10⁻³ M ¹³¹ labeled sodium acetazolamide and 1.0 × 10⁻⁴ M NaI as carrier. The samples were dialyzed with constant mixing for 18 hr at room temperature. Samples of 0.50 ml from each bag were counted in a well-type gamma scintillation spectrometer with a 2-inch sodium iodide crystal. The concentration of free acetazolamide was obtained from borate-saline controls and the outer buffer pool.

The preparation of acetyl-1-C-14 salicylic acid acetylated HSA for peptide mapping. The preparation of the acetyl-1-C-14-HSA derivatives was carried out as previously described, in the presence or absence of sodium salicylate. HSA, 7 mg/ml, was acetylated with either 5.0 × 10⁻⁴ M or 1.0 × 10⁻⁴ M acetyl-1-C-14-salicylic acid, in the presence or absence of 1.0 × 10⁻³ M or 1.0 × 10⁻⁴ M sodium salicylate. The reactions were carried out at 37° C for 24 hr, and the samples were dialyzed for 72 hr against multiple changes of 0.15 M NaCl containing 0.01 M sodium salicylate as carrier. The samples were then dialyzed for 48 hr against distilled water and lyophilized.

Peptide mapping of the acetyl-1-C-14-HSA derivatives. Peptide mapping was performed as pre-
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Previously described (4). The various HSA samples were reduced with 2-mercaptoethanol and carboxymethylated with iodoacetic acid. 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 milligram of protein. Peptide maps were prepared using 5 mg of the purified tryptic digests as follows: Descending chromatography was performed in a Shandon chromatography tank* with a normal butanol-acetic acid-water solvent (200:30:75). The chromatography papers were dried and immersed in a pyridine-acetic acid-water buffer (1:10:589), pH 3.55. High voltage electrophoresis was performed at 90° to the direction of chromatography at 2000 V for 70 to 80 min in a Savant apparatus.† The peptide maps were stained with 0.25% ninhydrin in acetone and placed directly on Kodak No-Screen Medical X-ray Film‡ for 2–8 days.

RESULTS

The rate of acetylation of HSA by aspirin at physiologic pH and temperature is seen in Fig 1. During the first 6–8 hr, there was a constant rate of acetylation of 0.07 M acetyl residues/M HSA/hr. After 24 hr there was an average of 1 M acetyl residue/M HSA, and this value increased to 1.2 after 48 hr. In contrast, no acetylation of HSA took place during the 24 hr test period when HSA was exposed to aspirin at 0°C.

The inhibitory effects of sodium salicylate on the acetylation of HSA by aspirin are shown in Fig 2. It should be pointed out that while human albumin was exposed to 5.0 × 10⁻⁴ M acetyl-1-C-14 labeled aspirin, it was simultaneously exposed to varying concentrations of sodium salicylate. Sodium salicylate at concentrations from 1.0 × 10⁻⁶ to 1.0 × 10⁻⁴ did not appreciably inhibit the acetylation of HSA by aspirin. At concentrations of salicylate greater than 10⁻⁴ M, there was a logarithmic inhibition of the acetylation of HSA by aspirin. Figure 2 also shows the effect of this inhibition on acetrizoate binding. The decrease of enhanced binding of acetrizoate by aspirin-altered HSA paralleled the inhibition of acetylation of HSA by sodium salicylate. When the concentration of sodium salicylate was 1.0 × 10⁻² M, an average of 0.4 M acetyl residues/M HSA was observed; the bound/free ratio of acetrizoate was 5.32. This bound/free ratio is within the normal range of acetrizoate binding for normal HSA not previously exposed to aspirin.

The effect of the acetylation of HSA by

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†Savant Instruments, Inc, Hicksville, NY.
‡INS-54 T, Kodak Corp, Rochester, NY.

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acetic anhydride on acetozoate binding is seen in Fig 3. The degree of acetylation varied from 2.8 to 32 M of acetyl residues/M HSA, and the bound/free ratio of acetozoate was enhanced to the same degree throughout the entire range of acetylation studied.

The effect of sodium salicylate on the qualitative nature of the acetylation of HSA by aspirin was assessed by peptide mapping. A peptide map and the respective autoradiograph of HSA acetylated with 1.0 × 10^{-4} M acetyl-1-C-14 labeled aspirin are seen in the upper half of Fig 4. The autoradiograph shows that at least 7 distinct peptides are acetylated by aspirin. As previously noted, 1 peptide, designated “A,” is acetylated to a far greater degree than the other peptides (4). Peptide “A” formation is a result of aspirin’s preferential acetylation of a lysine residue in either peptides “B” or “C.” From this acetylation, a trypsin-resistant, acetylated lysine residue appears, thereby forming the new peptide. The peptide map and the respective autoradiograph of HSA acetylated with acetyl-1-C-14 labeled aspirin in the presence of 1.0 × 10^{-4} M sodium salicylate are seen in the lower half of Fig 4. On both the stained peptide map and the autoradiograph, the presence of peptide “A” is greatly diminished, while the relative intensities of other acetylated peptides seen on the autoradiograph are not affected. The peptide maps and autoradiographs of HSA acetylated with 5.0 × 10^{-4} M acetyl-1-C-14 labeled aspirin in the presence or absence of 1.0 × 10^{-2} M sodium salicylate show that these reactions almost completely inhibited the formation of peptide “A” without affecting the acetylation of other peptides.

**DISCUSSION**

In vitro aspirin is an acetylator of HSA (1,2) and many other body constituents such as plasma proteins, hormones, enzymes, DNA and RNA (3). This fact was established from data obtained in parallel experiments using aspirin labeled with carbon 14, either at the carboxyl carbon or at the acetyl-1-carbon. HSA treated with acetyl-labeled aspirin retained substantial acetyl-C-14 activity. The covalent nature of the HSA–acetyl-1-C-14 bond was demonstrated by the persistence of acetyl-1-C-14 activity after treatment with 8 M urea or 6 M guanidine. In addition, we have demonstrated by peptide mapping procedures that at least one lysine residue in the HSA molecule is acetylated in vivo after the ingestion of therapeutic amounts of aspirin, and that this reaction is similar to that observed in vitro (4).

The present studies were undertaken to characterize the mechanisms of aspirin-mediated transacetylation and to relate such mechanisms to the known physiologic actions of aspirin. Specifically, the site specificity of the transacetylation reaction between aspirin and HSA was assessed with respect to: (1) the accessibility of the acetyl-receptor sites; (2) the rate at which the transacetylation reaction occurs; (3) the degree to which the reaction depends upon secondary bonding between aspirin and the
Acetyl-receptor sites ("affinity-labeling"), and whether structurally related compounds would inhibit the reaction ("affinity protection"); and (4) whether a receptor site might be functionally modified as a result of acetylation.

The acetrizoate binding data obtained from the sodium salicylate inhibition experiment provide initial evidence for the site specificity of the acetylation of HSA by aspirin. As seen in Fig 2, maximum, enhanced acetrizoate binding was observed when HSA was acetylated by aspirin in the absence of added salicylate. The degree of acetylation at this point was 1.4 M acetyl residues/M HSA. When the molar concentration of sodium salicylate was $1.0 \times 10^{-2}$, enhancement of acetrizoate binding was completely inhibited, although there were still 0.4 M acetyl residues/M HSA. Therefore, it can be assumed that a minimum of two qualitatively different sites of acetylation exist on the HSA molecule: (1) a specific site responsible for enhanced ace-
Fig 5. Proposed mechanism for the acetylation of the peptide “A” region of HSA by aspirin. Also shown are the “affinity protection” caused by salicylate and the enhancement of acetrizoate binding by the acetylated “A” peptide region.

The peptide mapping data presented in Fig 4 support the above assumptions about the site specificity of aspirin-mediated acetylation of HSA. At least 7 peptides in HSA are acetylated by aspirin in the absence of added sodium salicylate. The addition of a tenfold excess of sodium salicylate significantly diminished acetylation of 1 of these peptides, designated as “A.” This finding demonstrates that sodium salicylate specifically blocks the acetylation of a lysine residue in peptide “A,” leaving this peptide susceptible to tryptic hydrolysis and resulting in the subsequent formation of peptides “B” and “C” (4). This observation of the “affinity protection” of peptide “A,” combined with data on acetrizoate inhibition directly demonstrates that the enhanced acetrizoate binding in HSA exposed to aspirin, either in vitro or in vivo (8, 16), is the result of the acetylation of a lysine residue in peptide “A.” In addition, the unpublished studies of one of us (RSF) demonstrate that acetrizoate itself could prevent aspirin from altering HSA. Thus, it may be concluded that the peptide “A” region of the HSA molecule contains an acetrizoate binding site in addition to a salicylate binding site. Figure 5 shows the character of this proposed site specificity of the transacetylation reaction between aspirin and the peptide “A” region of HSA.
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It is known that most of a dose of acetyl-
salicylic acid given to humans is converted
to salicylate within 2 hr after ingestion
(17). Further, in the plasma of persons
receiving continuous therapeutic doses of
aspirin, there is 5–100 times more salicylate
than acetylsalicylate. Coupling these facts
with the data presented in Fig 1, which
indicate that the rate of the acetylation
reaction is relatively slow, one could pre-
dict that relatively high concentrations of
plasma salicylate would prevent, to some
degree, a subsequent dose of aspirin from
acetylating peptide “A” in HSA. In sup-
port of this expectation, clinical experience
has shown that maximum enhancement of
acetrizoate binding by HSA (ie, the acety-
lation of a lysine residue in peptide “A”) is
not observed in the sera of patients taking
large daily doses of aspirin until several
weeks later (19). Therefore, if the ability
of aspirin to acetylate body constituents is
responsible for its purported enhanced an-
algesic, anti-inflammatory and antipyretic
properties as well as its other biologic reac-
tions, the presence of sodium salicylate
might antagonize these reactions by inhibit-
ing the acetylation of receptor sites.

Clinically, the action of aspirin is
markedly different from that of sodium
salicylate in two areas. Therefore, it is in
these areas that the ability of aspirin to
acetylate biologic materials is, at present,
most potentially significant.

One of the most striking effects of aspirin
is its ability to interfere with certain aspects
of platelet aggregation. Specifically, it abol-
ishes the second wave of aggregation which
takes place after the addition of adenosine
diphosphate (ADP), as well as the second
wave of aggregation normally occurring
when adrenalin is added (11–14). In addi-
tion, there is a poor and incomplete re-
sponse to collagen (11). All of these chan-
ges are produced by the ingestion of a
single dose of aspirin, as low as 150 mg.
Furthermore, it has been demonstrated
that the effects on platelet aggregation per-
sist for many days after the ingestion of a
single dose of aspirin (11, 13). Not only
does the effect on platelets appear to occur
fairly rapidly, but it appears to last for the
life of the platelet. Only the addition of
platelet-rich plasma can correct the abnor-
malities, which are based on interference
with the release of intrinsic platelet ADP.
While a variety of other aromatic anti-
flammatory acids produce comparable
changes, they are less striking than those
produced by aspirin (11).

One possible explanation for this phe-
nomenon may be derived from the informa-
tion presented in this paper concerning the
effect of aspirin vis-à-vis sodium salicylate
on a single receptor site. When aspirin is
bound, the receptor site is acetylated
(“affinity labeling”), thereby inducing per-
manent change. However, when salicylate
is bound, “affinity protection” of the site is
produced leaving an unaltered receptor
site after the salicylate anion dissociates
(Fig 5). Since most other platelet functions
are not altered except for the intrinsic
release of ADP, presumably, only a single
surface receptor site or enzyme is perma-
nently affected.

Recently, it has been shown that signifi-
cant binding of carbon-14 occurs when
platelets are interacted with aspirin labeled
with carbon-14 in the acetyl position, but
not with that in the carboxyl position (20).
As in the case of albumin and other biolog-
ic materials (1–3), this occurrence strongly
suggests that a transacetylation reaction has
taken place. Of interest is the fact that
acetic anhydride, a potent acetylating
agent, produced identical inhibitory effects
on platelet function (20). The ability of
aspirin to acetylate some, as yet undeter-
dined, component in the platelet may ac-
count for the in vivo and in vitro effects mentioned above. The full significance of the potency of aspirin in this regard is seen in patients with basic coagulation defects such as hemophilia. In these patients, adding the aspirin-induced defect can produce a serious, if not life-threatening, hemostatic abnormality.

The other clinical difference between aspirin and sodium salicylate is aspirin’s ability to induce the symptoms of “aspirin disease” (9). Patients with this disorder frequently have asthmatic attacks immediately after they ingest a single dose of aspirin, while sodium salicylate produces no effect on them. Again, it is possible that the release of chemical mediators responsible for bronchospasm and other physiologic aberrations in asthma is related to aspirin’s acetylating capacity.

Aspirin may thus prove to be a highly useful tool for elucidating certain, basic physiologic mechanisms such as those involved in platelet aggregation and in the release of chemical mediators of inflammation.

REFERENCES
19. Hench PK: (Personal communication)