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Research Article

Aspirin (acetylsalicylic acid) inhibits platelet prostaglandin synthesis and the ADP- and collagen-induced platelet release reaction. The mechanism of the inhibitory effect is unknown but may involve protein acetylation, since aspirin acetylates a variety of substrates, including platelet protein. We have examined the relationship between protein acetylation and aspirin's physiologic effect on platelets. Suspensions of washed human platelets were incubated at 37 degrees C with (3H)aspirin, and incorporation of radioactivity into protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Exposure to (acetyl-3H)aspirin but not (aromatic ring-3H)aspirin resulted in radioactive labeling of three platelet proteins, suggesting that the drug acetylates these three proteins. The acetylation of two of the proteins (located in the supernatant fraction) was not saturable, implying that these reactions may not be physiologically significant. Acetylation of the third protein, approximate mol wt 85,000 (located in the particulate fraction), saturated at an aspirin concentration of 30 muM and was complete within 20 min. Platelets prepared from aspirin-treated donors did not incorporate any (acetyl-3H)aspirin radioactivity into the particulate protein for 2 days after drug treatment and did not show full pretreatment uptake of radioactivity for 12 days thereafter. The course of increasing incorporation of (acetyl-3H)aspirin radioactivity parralleled that of platelet turnover. Therefore, in addition to its saturability, acetylation of the particulate fraction protein by aspirin was permanent. In two […]

The Mechanism of the Effect of Aspirin on Human Platelets

I. ACETYLATION OF A PARTICULATE FRACTION PROTEIN

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ABSTRACT Aspirin (acetylsalicylic acid) inhibits platelet prostaglandin synthesis and the ADP- and collagen-induced platelet release reaction. The mechanism of the inhibitory effect is unknown but may involve protein acetylation, since aspirin acetylates a variety of substrates, including platelet protein. We have examined the relationship between protein acetylation and aspirin's physiologic effect on platelets.

Suspensions of washed human platelets were incubated at 37°C with [^{3}H]aspirin, and incorporation of radioactivity into protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Exposure to [*acetyl*-^{3}H]aspirin but not [*aromatic ring*-^{3}H]aspirin resulted in radioactive labeling of three platelet proteins, suggesting that the drug acetylates these three proteins. The acetylation of two of the proteins (located in the supernatant fraction) was not saturable, implying that these reactions may not be physiologically significant. Acetylation of the third protein, approximate mol wt 85,000 (located in the particulate fraction), saturated at an aspirin concentration of 30 μM and was complete within 20 min. Platelets prepared from aspirin-treated donors did not incorporate any [*acetyl*-^{3}H]aspirin radioactivity into the particulate protein for 2 days after drug treatment and did not show full pretreatment uptake of radioactivity for 12 days thereafter. The course of increasing incorporation of [*acetyl*-^{3}H]aspirin radioactivity paralleled that of platelet turnover. Therefore, in addition to its saturability, acetylation of the particulate fraction protein by aspirin was permanent.

In two respects, the inhibition of platelet function by aspirin correlates well with the aspirin-mediated acetylation of the particulate fraction protein. Both persist for the life-span of the aspirin-treated platelet, and both occur at a similar saturating aspirin concentration. The evidence suggests that the physiologic effect of aspirin on human platelets is produced by acetylation of a single protein located in the particulate fraction. The acetylated protein may be related to cyclo-oxygenase, the prostaglandin G_2 biosynthetic enzyme.

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INTRODUCTION

The household analgesic, aspirin (acetylsalicylic acid), has an alternate role as an inhibitor of platelet function. The effect of aspirin on platelets has been studied at many levels. Clinically, aspirin has modest effects on hemostasis in normal individuals (1) but may induce markedly prolonged bleeding times in hemophiliacs (2). When platelet function is studied in vitro, aspirin has been shown to inhibit the platelet release reaction. The inhibitory effect has several features: (*a*) Maximal inhibition is produced by relatively low concentrations ($<$50 μM) of aspirin either in vivo or in vitro. (*b*) Inhibition is permanent in that the release reaction of an aspirin-treated platelet is affected for the life-span of the platelet. (*c*) Inhibition is selective in the sense that aspirin appears to be more effective in inhibiting release induced by some agents (ADP and collagen) (3, 4) than by another (thrombin) (5).

Aspirin also inhibits the formation of prostaglandin G_2, a cyclic endoperoxide intermediate of prostaglandin biosynthesis in human platelets (6, 7). Recent studies indicate that prostaglandin G_2 production is stimulated by thrombin and that isolated prostaglandin G_2 will cause platelet aggregation and the release reaction (8, 9).

Despite these extensive investigations, the mechanism of action for aspirin's effect on platelets remains unclear. The results of previous studies have shown that aspirin can acetylate platelet protein (10). How-

ever, prior work has not demonstrated a saturable acetylation reaction between aspirin and a platelet protein, and no specific acetylated protein has been identified. Furthermore, no clear correlation has been made between protein acetylation by aspirin and the physiologic effect of the drug.

The results of the present study demonstrate that aspirin acetylates a single protein (mol wt 85,000) in the particulate fraction of human platelets. The acetylation reaction saturates at low aspirin concentrations (30 μM) and is nearly complete within 20 min. The characteristics of the reaction correlate closely with aspirin's inhibitory effect on platelet function.

METHODS

Materials

Acetylsalicylic acid (U.S.P., powder) was obtained from Merck & Co., Inc. (Rahway, N. J.). Salicylic acid (Fisher certified reagent) was obtained from Fisher Scientific Co. (Pittsburgh, Pa.). [*acetyl*-1-^{14}C]Acetylsalicylic acid. 2.38 μCi/μmol, was a gift from Merck & Co., Inc. [^{3}H]Acetic anhydride, 400 μCi/μmol (supplied in 80% benzene), and [G-^{3}H]salicylic acid,[1] 513 μCi/μmol, were obtained from New England Nuclear (Boston, Mass.).

Ion-exchange resins, AG1-x2 and AG50W-x1, were purchased from Bio-Rad Laboratories (Richmond, Calif.). Silica gel plates, chromogram, with fluorescent indicator were obtained from Eastman Kodak Co. (Rochester, N. Y.). *N,O*-bis-(Trimethylsilyl)-trifluoroacetimide was obtained from Pierce Chemical Co. (Rockford, Ill.). Commercial scintillation fluid, 3a70, was obtained from Research Products International Corp. (Elk Grove Village, Ill.). Tissue solubilizer, NCS, was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). apyrase (ADPase) was a gift of Dr. H. Joist, Washington University, St. Louis, Mo.

Methods

Aspirin

Aspirin preparations. Aspirin preparations were stored at −20°C in ethanol at concentrations of 7–100 mM. Before use in platelet experiments, the ethanol was evaporated under nitrogen, and the remaining dry aspirin was dissolved in Tyrode's solution, pH 7.3 (11). Aqueous solutions of aspirin were used within 4 h of preparation to minimize hydrolysis.

Synthesis of tritiated aspirin. (*a*) [*acetyl*-^{3}H]Aspirin was prepared by acetylating nonradioactive salicylic acid with tritiated acetic anhydride using pyridine as a catalyst. A reaction mixture containing salicylic acid, 0.25 mmol, [^{3}H]acetic anhydride (specific activity 400 μCi/μmol), 0.25 mmol, and pyridine, 0.1 ml, was incubated in the tip of a conical centrifuge tube for 2 h at 37°C. Water, 0.2 ml, was added to hydrolyze unreacted acetic anhydride, and the mixture was dried under vacuum. The dried material was dissolved in ethanol, 0.3 ml, and glacial acetic acid, 0.01 ml, and the solution was evaporated under vacuum to remove tritiated acetic acid formed by the hydrolysis of acetic anhydride. The step was performed three times. The residue was dissolved in diethyl ether, 0.3 ml. Petroleum ether (bp 38.1–47.4°C), 0.3 ml, was added, and the solution was left undisturbed for 18 h at 4°C. The fluid phase was removed and discarded. The crystals of [*acetyl*-^{3}H]aspirin (190 μCi/μmol) were dissolved in ethanol and stored.

A nonvolatile, ethanol-soluble residue from the [^{3}H]-acetic anhydride preparation was found to contaminate the crystallized [*acetyl*-^{3}H]aspirin. The following procedure was used to eliminate the residue. [*acetyl*-^{3}H]Aspirin, 71.2 μmol, was dissolved in 20 ml of 0.0136 M sodium acetate containing 50% ethanol and applied to a column (0.7 × 4.0 cm) of Bio-Rad AG1-x2 in acetate form. The column was washed successively with 10 ml of 0.01 M sodium acetate containing 50% ethanol, 10 ml of ethanol to remove the residue, and 2 ml water. Sodium acetate, 0.4 M, was used to elute the [*acetyl*-^{3}H]aspirin. Eluate fractions containing 85% of the original radioactivity were pooled and applied to a column (2.1 × 18.0 cm) of Bio-Rad AG50W-x1 in hydrogen form equilibrated with water. The second column step provided for the conversion of sodium acetate to acetic acid. [*acetyl*-^{3}H]Aspirin was eluted with water, and eluate fractions containing 71% of the original radioactivity were pooled and lyophilized to remove acetic acid. The dry product was dissolved in ethanol and stored.

The purified [*acetyl*-^{3}H]aspirin preparation contained 73% aspirin and 27% salicylic acid on a molar basis. The specific activity of [*acetyl*-^{3}H]aspirin was 197 μCi/μmol. All tritium radioactivity was present in the aspirin peak of the preparation as determined by radio-gas-liquid chromatography. The [*acetyl*-^{3}H]aspirin comigrated with salicylate when analyzed by thin-layer chromatography (TLC) in two solvent systems. The procedures used to assay the aspirin preparation are outlined later in the Methods section.

(*b*) [*aromatic ring*-^{3}H]Aspirin was synthesized by the acetylation of [G-^{3}H]salicylic acid by nonradioactive acetic anhydride using pyridine as catalyst. A reaction mixture containing [G-^{3}H]salicylic acid (200 μCi/μmol),[2] 0.050 mmol, acetic anhydride, 0.21 mmol, and pyridine, 0.01 ml, was incubated for 60 min at 37°C. As described above in (*a*), water, 0.1 ml, was added to hydrolyze unreacted acetic anhydride. Ethanol, 0.3 ml, and glacial acetic acid, 0.01 ml, was added and evaporated three times. No crystallization step was performed. The final dry material was dissolved in ethanol and stored.

The [*aromatic ring*-^{3}H]aspirin preparation contained 80% aspirin and 20% salicylic acid on a molar basis. Analysis by radio-gas-liquid chromatography indicated that 81% of tritium was present in aspirin and 19% in salicylic acid. The specific activity of [*aromatic ring*-^{3}H]aspirin was 194 μCi/μmol. The [*aromatic ring*-^{3}H]aspirin comigrated with salicylate when analyzed by TLC in two solvent systems.

Assay of aspirin. Neutral hydroxylamine reacts with the acetyl group of aspirin to form acetyl hydroxamate which can be assayed with ferric chloride reagent by measuring absorbance at 540 nm (12). Aqueous solutions of aspirin (0–10 μmol) were mixed with 0.5 ml of neutral hydroxylamine (2 M), and water was added to give a total volume of 1.5 ml. The solution was incubated for 10 min at room temperature. Ferric chloride reagent (1.5 ml) was added, and absorbance at 540 nm was determined. The concentration of aspirin in test solutions was determined by comparison to a standard curve: $E^1_{540} = 30{,}700$.

[1] G indicates that tritium is generally distributed on the carbon atoms of the benzene ring.

[2] Nonradioactive salicylic acid was added to [G-^{3}H]salicylic acid to give the specific activity of 200 μCi/μmol.

For analysis by TLC, aspirin and salicylic acid (0.1 μmol), mixed with trace amounts of radioactive aspirin (0.007–0.04 pmol), were applied to silica gel plates and subjected to TLC in two solvent systems: (*a*) *n*-butanol: pyridine: dioxane: H_2O (70:20:5:5, by volume); (*b*) Benzene: diethyl ether: glacial acetic: methanol: (120:60:18:1, by volume). Salicylate spots were visualized under ultraviolet light. Radioactivity was localized by scraping 1-cm strips of gel from the TLC sheets into scintillation fluid (3a70). The radioactivity of the suspended silica gel was measured in a liquid scintillation counter.

Salicylate preparations were analyzed by gas-liquid chromatography (GLC) using the method of Thomas, Solomonraj, and Coldwell (13). Aspirin or salicylic acid (2 μmol) was incubated with 0.05 ml *N,O*-bis-trifluoroacetimide to convert the salicylate to the trimethylsilyl derivative. By comparison with standards, the salicylic acid and aspirin contents of a synthesized tritiated aspirin preparation were determined. The content of tritium in the aspirin and salicylic acid peaks eluted from the GLC column was measured by collecting effluent gas from the column into cold ethanol (−70°C) and counting aliquots of the ethanol in a liquid scintillation counter.

Platelets

Human blood was collected into one-sixth volume of acid-citrate-dextrose (0.085 M sodium chloride, 0.065 M citric acid, 0.111 M D-glucose). Washed platelets were prepared after the method of Ardlie and Han (11), omitting bovine serum albumin from wash and resuspension buffers. For experiments on platelets suspended in acetate-containing media, sodium acetate, 0.10 M, with sodium chloride, 0.04 M, was substituted for sodium chloride, 0.14 M, the concentration generally used in Tyrode's solution. For gel electrophoresis of sonicated platelet fractions, suspensions of platelet material were made in protein-free Tyrode's solution, pH 7.3.

A "microhematocrit" method was used to estimate the number of platelets in suspensions as previously described (14).

Incorporation of radioactive aspirin into platelets

Incorporation into whole platelets and trichloroacetic acid (TCA)-treated whole platelets. The incorporation of radioactive aspirin into whole platelets and TCA precipitates of whole platelets was assessed by the following method. Platelet suspensions, 2.5×10^8–10.0×10^8 platelets/ml, were incubated at 37°C with either [*acetyl*-1-^{14}C]aspirin or [*acetyl*-^{3}H]aspirin at final concentrations of 50–500 μM. At varying times, the platelet suspension was layered above 1 ml of 0.25 M sucrose in a siliconized Hopkin's vaccine tube (Pyrex tube 8225, Corning Glass Works, Corning N. Y.) and centrifuged at 2,000 *g* for 15 min. Centrifugation of platelets through the sucrose solution served to remove nonplatelet-bound aspirin from the platelet pellet. The sucrose and media overlying the platelet pellet was removed by aspiration. The platelet pellet was dispersed in buffer, and an aliquot was assayed for radioactivity. A volume of cold 10% TCA equal to that of the remaining platelet suspension was added, and the suspension was centrifuged at 12,000 *g* for 15 min. The TCA precipitate was solubilized in NCS. Aliquots of the whole platelet suspension, TCA supernatant, and NCS-solubilized TCA precipitate were added to 3a70 and counted in a liquid scintillation counter.

Gel electrophoretic analysis of radioactively labeled platelet protein. Washed platelet suspensions containing 1×10^9 platelets/ml were incubated with aspirin for varying times at 37°C. In experiments involving platelet fractionation, the platelet suspension was cooled to 4°C after 20 min incubation, and diisopropyl fluorophosphate (10 mM) was added immediately before platelet disruption to inhibit proteolysis. Cells were broken by sonication (30 s 70% intensity) using a Biosonic sonicator with microprobe attachment (Biosonic II, Bronwill Scientific, Rochester, N. Y.) and the suspension was centrifuged at 200,000 *g* for 45 min. The supernatant fraction was decanted and saved. The pellet of particulate material was dispersed in one-half the original volume of buffer.

Radioactively labeled whole platelets and sonicated platelet fractions were subjected to sodium dodecyl sulfate (SDS)[3] polyacrylamide gel electrophoresis after the method of Weber and Osborn (15). An aliquot of labeled whole platelet suspension (0.11 ml) or sonicated platelet fraction (0.10 ml) was mixed with 0.025 ml of 20% SDS in 0.1 M sodium phosphate buffer, (pH 7.4)–0.5 M 2-mercaptoethanol, placed in a boiling water bath for 10 min, and applied to a gel of 4.9% polyacrylamide. To study labeling of whole platelets at zero time, 0.025 ml of the 20% SDS-2-mercaptoethanol solution was added initially to 0.1 ml of whole platelet suspension, and the sample was boiled for 5 min. Then 0.01 ml of aspirin solution was added, and boiling was continued for an additional 5 min. Electrophoresis was carried out in cylindrical gels (0.5 × 10 cm) at ambient temperature using 8 mA/gel for 3½ h.

Gels were stained for 12 h in 0.03% Coomassie Blue, 50% methanol, 12% TCA, and destained in 10% methanol, 7% glacial acetic acid for 24 h. Destained gels were frozen on solid CO_2 slabs and sectioned at 2.14-mm intervals using a razor blade-type gel slicer (Bio-Rad Laboratories). Gel slices were placed in scintillation vials and extracted for 24 h at 37°C after the method of Ames (16). Radioactivity was measured in a liquid scintillation counter at ambient temperature. Each vial containing radioactivity above background was counted for the time required to reach a counting error of 7% at a 95% confidence level.

The extraction of tritium radioactivity from the gel slices appeared to be complete. Removing the swollen gel slice from the vial after the extraction step did not lead to any reduction in radioactivity in the remaining scintillation fluid, indicating that the gel slice itself did not contain measurable tritium radioactivity.

Characterization of acetylated particulate fraction protein. In an effort to characterize the protein in a preliminary fashion, we treated the acetylated radioactively labeled protein in a particulate fraction prepared from 2×10^8 platelets as follows: (*a*) Incubation with pronase or trypsin, 0.2 mg/ml, at 37°C for 6 h. (*b*) Incubation with RNAse, 0.2 mg/ml, at 37°C for 6 h. Controls for each treatment were incubated at the same temperature for the same time period, but buffer was added instead of the active agent. The control and treated particulate fractions were then subjected to SDS gel electrophoresis, and the position of the radioactively labeled protein in the gel was determined as previously described.

The molecular weights of radioactively labeled platelet proteins were estimated by comparing the migration of labeled proteins in a SDS gel to that of proteins of known molecular weight. Seven known proteins were used: human platelet myosin (mol wt 230,000), *Escherichia coli* B-galactosidase (130,000), human fibrinogen alpha chain (73,000),

[3] *Abbreviation used in this paper:* SDS, sodium dodecyl sulfate.

TABLE I

Incorporation of [Acetyl-1-^{14}C]Aspirin into Whole Platelets and TCA-Treated Platelets

	Buffer anion	dpm/10^9 platelets	Molecules ASA*/platelet
Whole platelets	Chloride	5×10^3	6×10^5
	Acetate	1×10^3	1×10^5
TCA supernatant	Chloride	5×10^3	6×10^5
	Acetate	1×10^3	1×10^5
TCA precipitate	Chloride	$<0.2 \times 10^3$	$<0.2 \times 10^5$
	Acetate	$<0.2 \times 10^3$	$<0.2 \times 10^5$

Platelets were incubated with 210 μM [*acetyl*-1-^{14}C]aspirin for 30 min at 37°C. Duplicate experiments were performed in two different buffers: (*a*) Tyrode's solution with 0.14 M NaCl (chloride); (*b*) Tyrode's solution with 0.10 M Na acetate +0.04 M NaCl (acetate).

* Aspirin abbreviated ASA.

beta chain (60,000), and gamma chain (53,000), bovine serum albumin (68,000), *E. coli* alkaline phosphatase (43,000), rabbit muscle aldolase (40,000), and sperm whale myoglobin (17,200). A semilogarithmic plot of protein molecular weight vs. migration distance resulted in a straight line from which the molecular weights of labeled platelet proteins could be estimated.

RESULTS

Incorporation of [acetyl-1-^{14}C]aspirin and [acetyl-^{3}H]aspirin radioactivity into whole platelets. Initially, we repeated the work of previous investigators (10, 17). Using [*acetyl*-1-^{14}C]aspirin of relatively low specific activity, 2.38 μCi/μmol, we studied the incorporation of radioactivity from labeled aspirin into whole platelets and TCA precipitates and supernates of whole platelets. Experiments were performed in both saline- and acetate-containing buffers. The results of an initial experiment are summarized in Table I.

Aspirin in aqueous solutions is hydrolyzed to yield salicylic acid and free acetate. Therefore, incorporation of [*acetyl*-1-^{14}C]aspirin radioactivity into platelets could be due in part to cellular uptake of radioactive acetate formed by the hydrolysis of aspirin. The uptake of labeled acetate can be minimized by substituting acetate for chloride anions in platelet suspension buffers.

As seen in Table I, whole platelets take up approximately fivefold more radioactivity in saline as compared to acetate-containing media. The result suggests that hydrolysis of aspirin with subsequent incorporation of labeled acetate accounts for the major portion of whole platelet labeling in nonacetate-containing media. At least 80% of the radioactivity in whole platelets is located in the supernatant fraction after treatment with TCA, a finding that probably reflects the presence of acetate and unbound aspirin free in the cytoplasm.

The radioactivity of TCA precipitates in the experiment reported in Table I was not sufficient for accurate measurement. Radioactive aspirin of higher specific activity was used to measure incorporation of aspirin into the TCA precipitate fraction of platelets.

Experiments similar to those noted above were performed by incubating platelet suspensions for 0–200 min with [*acetyl*-^{3}H]aspirin (specific activity 197 μCi/μmol) at concentrations from 50 to 500 μM. The results with [*acetyl*-^{3}H]aspirin were similar to those shown in Table I. Whole platelets took up two- to fivefold more radioactivity in saline as compared to acetate-containing media. In both media, the major portion of whole platelet radioactivity was located in the supernate after TCA treatment. The amount of label in TCA precipitates did not saturate at aspirin concentrations between 50 and 500 μM, and labeling increased progressively with longer incubation times up to 200 min.

The experiments indicated that the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into TCA precipitates of platelets was not saturable under the conditions used. To detect a saturable acetylation reaction, radioactively labeled platelet proteins were separated by SDS gel electrophoresis, and the saturability of [*acetyl*-^{3}H]-aspirin label incorporation into each protein was studied independently.

Demonstration of a saturable acetylation reaction between aspirin and a particulate fraction protein (mol wt 85,000)

(*a*) *SDS gel electrophoresis of radioactively labeled whole platelets.* Whole platelet suspensions were in-

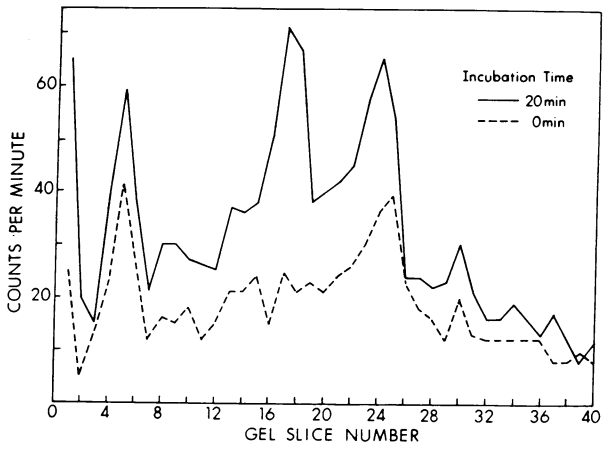

FIGURE 1 Incorporation of [*acetyl*-^{3}H]aspirin radioactivity into platelet proteins. Washed platelets (0.1 ml, 1×10^9 platelets/ml) were incubated for 0 or 20 min with 30 μM [*acetyl*-^{3}H]aspirin. The entire reaction mixture was subjected to SDS polyacrylamide gel electrophoresis. Gels were sliced at 2.14-mm intervals, and the radioactivity of each slice was measured. A background of 20 cpm was subtracted.

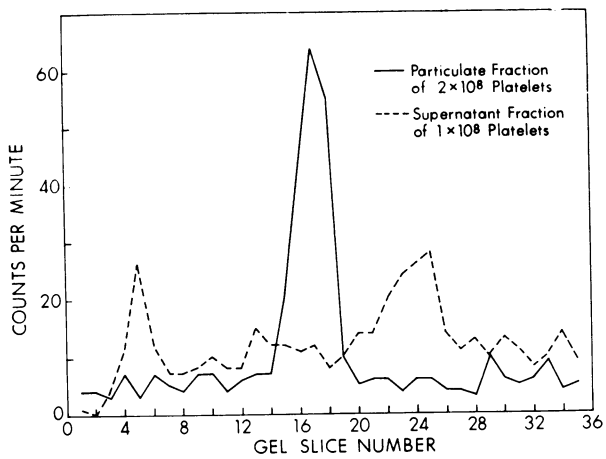

FIGURE 2 Location of radioactively labeled proteins in platelet sonicates. Washed platelets (1.5 ml, 1×10^9/ml) were incubated for 20 min with 30 μM [*acetyl*-^{3}H]aspirin and sonicated. Supernatant and particulate fractions were prepared from the sonicate. Fractions containing protein of 1×10^8 platelets (supernatant) or 2×10^8 platelets (particulate) were analyzed for radioactivity by SDS gel electrophoresis as in Fig. 1.

cubated with [*acetyl*-^{3}H]aspirin and then subjected to SDS-polyacrylamide gel electrophoresis. Three peaks of radioactivity were present at gel slices 5, 17, and 24; corresponding to mol wt of 225,000, 85,000, and 55,000, respectively (Fig. 1). Similar patterns of protein labeling were seen with platelets suspending in acetate-containing media. Therefore, acetate-containing media did not alter platelet protein labeling but, as seen in Table I, did interfere with the uptake of free acetate ions resulting from the hydrolysis of aspirin.

The time-course of uptake of aspirin radioactivity into platelet protein was different for the different radioactive protein peaks. The first and third peaks (mol wt 225,000 and 55,000) take up radioactivity at zero time, indicating that these proteins can incorporate [*acetyl*-^{3}H]aspirin radioactivity despite prior denaturation by heating in SDS solutions, and the amount of label incorporated increases progressively with incubation times up to 120 min. In contrast to the other proteins, incorporation of radioactivity into the second peak (mol wt 85,000) is nearly maximal between 20 and 60 min, varying somewhat from one platelet preparation to another. No incorporation of radioactivity was seen at zero time. Slight increases in labeling of the 85,000 mol wt protein occurred with incubation times between 60 and 120 min.

The method used for washing platelets did not affect the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into platelet proteins. A pattern of radioactive protein peaks similar to that seen in Fig. 1 was obtained using platelets washed by a different method (14).

No radioactivity was incorporated into the protein of platelets incubated with [*aromatic ring*-^{3}H]aspirin as measured using SDS gel electrophoresis. The finding indicates that the aromatic ring portion of aspirin is not transferred to platelet protein under the conditions used. Similar conclusions have been reached by other investigators (10, 17). The results imply that the uptake of [*acetyl*-^{3}H]aspirin radioactivity into platelet protein involves an acetylation reaction between aspirin and at least three platelet proteins.

Only a small percentage (<0.05%) of the total amount of [*acetyl*-^{3}H]aspirin radioactivity present in the incubation solution is incorporated into platelet protein as measured by SDS gel electrophoresis. If a relatively small content of radioactive material, undetected by the assay procedures, contaminated the [*acetyl*-^{3}H]aspirin preparation, it could account for the protein labeling observed. The possibility was explored by pretreating [*acetyl*-^{3}H]aspirin with neutral hydroxylamine (10 mM) before incubation with platelets. Hydroxylamine treatment, which hydrolyzes the acetyl group of aspirin, prevented the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into platelet protein. The observation suggests that aspirin rather than a minor contaminant is the active agent responsible for the transfer of radioactivity to platelet protein. This conclusion was further supported by the dilution experiment described below.

(*b*) *Localization experiments using sonicated platelets.* To determine the location of the radioactive proteins within the platelet, soluble and particulate fractions were prepared from sonicated labeled platelets as depicted in Fig. 2. The protein of mol wt 85,000 (second peak of Fig. 1) was located in the particulate fraction and constituted the only radioactive protein labeled by [*acetyl*-^{3}H]aspirin in this fraction. The other peaks of radioactive protein of mol wt 225,000 and 55,000 (first and third peaks of Fig. 1) were located in the supernatant fraction which contains soluble proteins.

The preparation of soluble and particulate fractions allows for distinct separation of labeled protein peaks and results in low base-line levels of radioactivity. Because of these advantages, the fractionation step was used in all later experiments involving the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into platelet proteins.

The flat base-line level of radioactivity permits an estimate of the amount of [^{3}H]acetate incorporated into the three main labeled protein peaks. From the data of Fig. 2, we estimate that 160 cpm were incorporated into the particulate fraction protein of mol wt 85,000. Repeated experiments with different platelet preparations indicated that the 85,000 mol wt protein of 2×10^8 platelets contained 160–200 cpm under the

conditions used. This amount of radioactivity corresponds to 2,000–3,000 acetyl groups per platelet associated with the 85,000 mol wt protein.

(*c*) *Effect of aspirin concentration on [^{3}H]acetate incorporation into platelet proteins.* The effect of increasing aspirin concentration on incorporation of [^{3}H]-acetate into each of the three radioactive platelet proteins is shown in Fig. 3. The incorporation of radioactivity into the 85,000 mol wt protein reaches maximal levels at aspirin concentrations between 30 and 100 μM. The saturation characteristics of the reaction suggest that it is physiologically significant since the saturating concentration of 30 μM with a 20-min time-course correlates well with in vitro observations of aspirin inhibition of platelet function (4). In contrast, the uptake of radioactivity into the two soluble platelet proteins of mol wt 225,000 and 55,000 increases progressively with increasing aspirin concentrations between 0 and 300 μM under the conditions used. The fact that the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into these two proteins is not saturable indicates that these acetylation reactions do not correlate with the physiologic effect of aspirin on platelets. The nonsaturable acetylation of the two platelet proteins resembles the effect of aspirin in acetylating a variety of other proteins such as albumin, immunoglobulins, and fibrinogen (18).

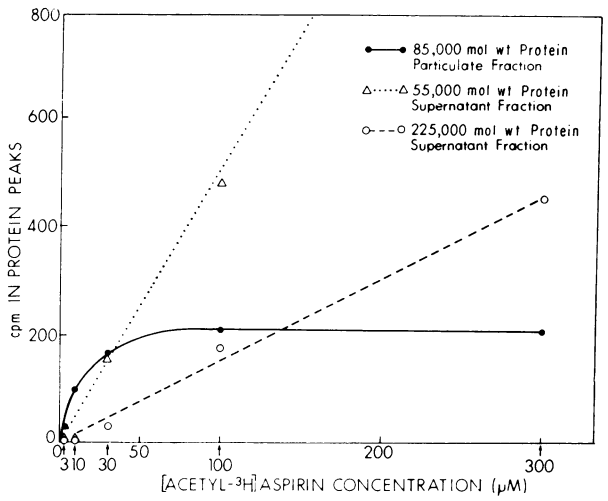

FIGURE 3 Effect of increasing [*acetyl*-^{3}H]aspirin concentrations on radioactive labeling of protein peaks. Washed platelets (1.5 ml, 1×10^9/ml) were incubated for 20 min with increasing concentrations of [*acetyl*-^{3}H]aspirin (3, 10, 30, 100, and 300 μM) and sonicated. Supernatant and particulate fractions were analyzed as in Fig. 2 (2×10^8 platelets/gel), and the number of counts per minute in each labeled protein peak was estimated. Incorporation of radioactivity into the 55,000 mol wt protein peak was linear up to 300 μM aspirin (1,550 cpm).

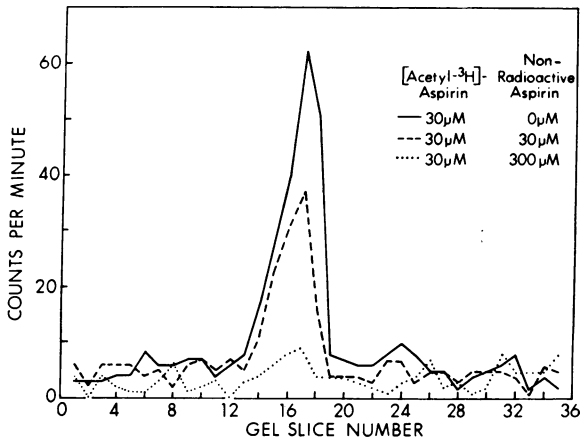

FIGURE 4 Inhibition of [*acetyl*-^{3}H]aspirin labeling of the particulate protein by nonradioactive aspirin. Washed platelets (1.5 ml, 1×10^9/ml) were incubated for 20 min with 30 μM [*acetyl*-^{3}H]aspirin alone or with added nonradioactive aspirin and treated as in Fig. 2. Incorporation of [*acetyl*-^{3}H]aspirin radioactivity into the particulate fraction protein was assessed as in Fig. 2 (2×10^8 platelets/gel).

(*d*) *Label dilution experiments.* The saturable nature of the acetylation reaction between aspirin and the 85,000 mol wt protein was demonstrated in another way using a technique of label dilution. The results of a label dilution experiment are shown in Fig. 4. The addition of unlabeled aspirin inhibited label uptake into the 85,000 mol wt protein in proportion to the relative amounts of [*acetyl*-^{3}H]aspirin and nonradioactive aspirin used in the incubations. A 1 : 1-dilution of 30 μM [*acetyl*-^{3}H]aspirin with 30 μM nonradioactive aspirin inhibited incorporation by about 50%. A 1 : 10-dilution of 30 μM [*acetyl*-^{3}H]aspirin with 300 μM nonradioactive aspirin inhibited labeling by about 90%.

The results of Fig. 4 reinforce the point that the acetylation reaction between aspirin and the 85,000 mol wt protein saturated at an aspirin concentration of approximately 30 μM. Furthermore, since nonradioactive aspirin inhibits the incorporation of [*acetyl*-^{3}H]-aspirin label, aspirin itself rather than a unique contaminant of the [*acetyl*-^{3}H]aspirin preparation was the active agent responsible for the incorporation of [^{3}H]-acetate into platelet protein. Using the same technique of label dilution, nonradioactive aspirin did not inhibit the incorporation of [*acetyl*-^{3}H]aspirin radioactivity into the two soluble platelet proteins up to 300 μM, again indicating that these acetylation reactions are not saturable. (Data not shown.)

Label dilution experiments of the same type as shown in Fig. 4 were performed using [*aromatic ring*-^{3}H]-aspirin in place of nonradioactive aspirin. Added [*aromatic ring*-^{3}H]aspirin inhibited [*acetyl*-^{3}H]aspirin labeling of the 85,000 mol wt protein to the same extent

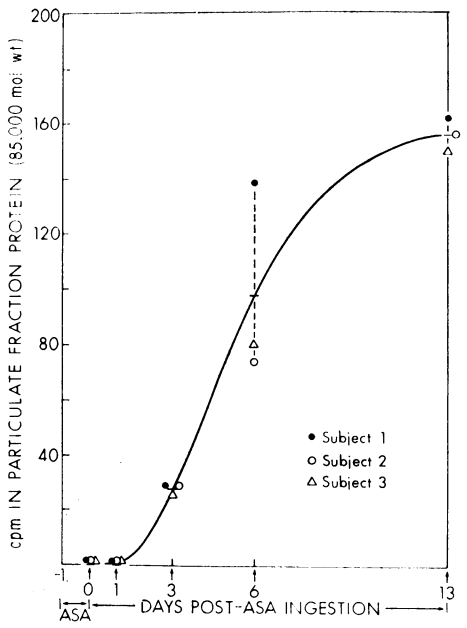

FIGURE 5 Inhibition of [*acetyl*-^{3}H]aspirin (ASA) labeling of the particulate platelet protein by aspirin ingestion. Three subjects ingested 3 g of aspirin orally over 16 h. Platelets were prepared from each subject at five times after oral aspirin (0, 1, 3, 6, and 13 days). Platelets were incubated with 100 μM [*acetyl*-^{3}H]aspirin for 20 min and treated as in Fig. 2. The incorporation of radioactivity into the particulate fraction protein was assessed as in Fig. 2 (2×10^8 platelets/gel), and the number of counts per minute incorporated was estimated. Before aspirin treatment platelets of subject 3 incorporated 160–200 cpm under the same conditions.

as did nonradioactive aspirin. The inhibitory effect of [*aromatic ring*-^{3}H]aspirin indicates that this synthesized aspirin preparation exerts the same effect as nonradioactive aspirin under the conditions used. Also, the inhibitory effect substantiates the previous observation that no incorporation of [*aromatic ring*-^{3}H]aspirin radioactivity into platelet protein occurs at aspirin concentrations up to 300 μM.

(*e*) *Preliminary characterization.* Trypsin and pronase treatment of the [^{3}H]acetate-labeled particulate fraction protein led to a disappearance of the radioactive peak on SDS gel electrophoresis of the treated fraction. The result indicates that the [^{3}H]acetate group is contained in protein. The amount of available material was insufficient for further characterization of the [*acetyl*-^{3}H]platelet protein linkage. A platelet particulate fraction containing the [^{3}H]acetate-labeled protein was subjected to SDS gel electrophoresis without added 2-mercaptoethanol. The radioactive protein migrated in the same manner as that in samples reduced with 2-mercaptoethanol, suggesting that the acetylated particulate fraction protein does not contain subunits linked by disulfide bonds.

Inhibition of [acetyl-^{3}H]aspirin labeling of particulate fraction platelet protein by aspirin ingestion. Platelets taken from subjects who ingest aspirin exhibit abnormal in vitro function tests for up to 5 days after aspirin treatment (3). This physiologic observation indicates that the effect of aspirin on platelet function may persist for the lifetime (8–12 days) (19) of an aspirin-treated platelet. If our observations account for the physiologic effect of aspirin on platelets, then the 85,000 mol wt protein of a platelet exposed to 30 μM or greater concentration of aspirin in vivo should be maximally acetylated for the life-span of the platelet. Further exposure of the platelet to aspirin would not lead to additional acetylation of the protein. Platelet turnover, however, would provide new platelets, not previously exposed to aspirin, and the protein of the new platelets could be acetylated by aspirin. The question was studied by reacting platelets which had already been exposed to aspirin in vivo to [*acetyl*-^{3}H]aspirin in vitro.

Three normal male subjects ingested a total of 3 g of aspirin over 16 hr. Salicylate levels of 10–20 mg/100 ml (500–1,000 μM) were found in the serum of the subjects after the last done (20). Platelets were collected from each subject at five times (0, 1, 3, 6, and 13 days) after the last aspirin dose. The washed platelets were incubated with 100 μM [*acetyl*-^{3}H]aspirin, and the particulate fraction of sonicated platelets was prepared and analyzed for radioactivity.

In Fig. 5, the radioactivity content of the labeled 85,000 mol wt protein is plotted against time after aspirin ingestion. No labeling of this protein was seen on day 0 or day 1, indicating that acetylation of the protein was completely inhibited. Increasing amounts of label were incorporated on days 3 and 6, and essentially full uptake was seen on day 13. The course of increasing acetylation of the particulate fraction protein parallels platelet turnover, substantiating the idea that acetylation of this protein by aspirin is a permanent effect.

DISCUSSION

The observation that aspirin acetylates proteins and other biologic substrates is not new. Aspirin (500 μM) acetylates human serum albumin at a single site leading to the formation of ε-*N*-acetyl lysine residues (21). The drug (2.0–20 mM aspirin) acetylates hemoglobin at several sites within the molecule perhaps through the formation of ε-*N*-acetyl lysines (22). Less well characterized is the acetylation by aspirin (500 μM) of RNA, hormones, and various other serum proteins (18). However, the search for a connection between protein acetylation and aspirin's physiologic effect has

been a frustrating one. The point is well illustrated by the case of aspirin's effect on platelets. The drug has a definite and well-defined role as an inhibitor of platelet function. Furthermore, aspirin acetylates platelet protein (10). However, no convincing evidence has been presented previously that the mechanism of aspirin's effect on platelets involves protein acetylation (10, 17). Such evidence could not be obtained in earlier studies due to the methodology used. Using [*acetyl*-1-^{14}C]-aspirin and [*acetyl*-^{3}H]aspirin of low specific activity, prior investigators studied the incorporation of radioactivity into whole platelets and TCA precipitates of whole platelets. These methods were not suitable for distinguishing saturable from nonsaturable acetylation reactions for two reasons. First, the absolute amount of radioactivity transferred to platelet proteins at low aspirin concentrations was relatively small and difficult to measure due to the low specific activity of the radioactive aspirin. Second, a protein acetylated by a saturable reaction could not be distinguished from one acetylated in a nonsaturable fashion since a technique for protein separation was not used. Consequently, the radioactivity incorporated by the nonsaturable reactions obscured that taken up by the concomitant saturable reaction on a simply quantitative basis.

The demonstration of an acetylation reaction which saturates at low aspirin concentrations (30 μM) is relevant to the understanding of the drug's mechanism of action. Aspirin exerts its antiplatelet effect at very low serum concentrations, 50 μM or less. Oral aspirin doses as low as 150 mg result in a permanent defect in the aspirin-exposed platelets (3). Larger doses have little additional effect, a characteristic feature of a saturable process. Furthermore, brief in vitro exposure of platelets to low aspirin concentrations results in a functional defect in the release reaction. Human platelets exposed to 40 μM aspirin for 15 min show marked inhibition of the collagen-induced release reaction (4), as do rabbit platelets incubated with 30 μM aspirin for 10 min (10). We have observed similar inhibitory effects of aspirin on the collagen-induced release reaction using washed human platelets.[4] Therefore, the physiologic effect of aspirin on platelets, measured both in vivo and in vitro, is fully expressed at low aspirin concentrations (50 μM or less). Our results show that aspirin acetylates a particulate protein, mol wt 85,000, of human platelets. The acetylation is permanent (i.e., persists for the life-span of the platelet) and saturates at low aspirin concentrations (30 μM) with a time course of about 20 min. The correlation between previous physiologic observations and the present findings is striking and implies that aspirin inhibits platelet function by acetylation of the particulate protein.

The physical characteristics and function of the acetylated particulate protein have not been thoroughly explored. The acetylation reaction may involve the formation of *N*-acetyl bonds in the protein, but the actual product of the reaction is unknown. One could speculate that the acetylated protein is related to an enzyme of prostaglandin biosynthesis. Samuelsson and co-workers have described the pathways of prostaglandin biosynthesis in several tissues (23). In particular, they have identified a cyclic endoperoxide intermediate (prostaglandin G_3) of prostaglandin synthesis in human platelets (9). The endoperoxide has been implicated as a mediator of the platelet release reaction induced by ADP and collagen (8, 9). In addition, the enzyme (cyclo-oxygenase) responsible for the synthesis of prostaglandin G_3 is located in the particulate fraction of human platelets (24) and is inhibited "permanently" by low concentrations (approximately 50 μM) of aspirin (6, 7, 25). Therefore the position within the platelet and the susceptibility to aspirin inhibition of the acetylated particulate protein described in this study correlate with the same aspects of the prostaglandin G_3 synthetic enzyme of human platelets studied by other investigators.

The acetylation of the particulate fraction protein by aspirin occurs at drug concentrations 10- to 100-fold lower than so-called "nonspecific" acetylations of albumin and hemoglobin (18, 21, 22). The striking specificity of the former reaction suggests that aspirin is a site-specific agent in acetylating the platelet protein. If the particulate fraction protein proves to be cyclo-oxygenase, aspirin may inhibit the enzyme by acetylating a single site (perhaps an active site) in the molecule. Therefore, aspirin may exert its antiplatelet effect by acetylating a residue within an active site of the prostaglandin G2 biosynthetic enzyme, cyclo-oxygenase. Finally, [*acetyl*-^{3}H]aspirin may prove to be a useful probe in detecting sites of prostaglandin synthesis and have application in studies of organ physiology.

[4] Roth, G. J., and P. W. Majerus, unpublished observations.

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