Aspirin-induced Hemolysis: The Role of Concomitant Oxidant (H₂O₂) Challenge

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Summary

Studies were conducted in an attempt to determine the hemolytic potential of acetylsalicylic acid (ASA) on normal intact red blood cells (RBCs). ASA (25 mg/100 ml) did not impair RBC G-6-PD, glutathione peroxidase or catalase activity, glutathione stability, or hexose monophosphate shunt activity. Hemolysis was not observed after incubation with ASA alone but was quickly noted after exposure to ASA (25 mg/100 ml) and H₂O₂ (1.2%). Hemolysis did not occur with H₂O₂ alone but was directly proportional to the ASA concentration. Hemolysis was preceded by peroxidation of membrane lipid and was inhibited by the presence of the antioxidant, thymol. With ASA and H₂O₂ a marked reduction in membrane phosphatidyl ethanolamine occurred with oxidation of 20:4 and 22:6 fatty acids. No effect on phospholipids or fatty acids occurred with either ASA or H₂O₂ alone. Salicylic acid, singly or with H₂O₂, does not exhibit these effects.

Speculation

This study indicates that ASA alone appears to have no significant effect on RBCs in salicylate concentrations comparable to those achieved *in vivo*. In the presence of an oxidant challenge, however, ASA will cause lysis of RBCs. Acetylation appears to be important in this process. It is suggested that if ASA is harmful when administered to G-6-PD-deficient subjects, these effects would only be seen in the presence of a significant concomitant oxidative stress to the RBC.

The role of aspirin (acetylsalicylic acid) in inducing hemolysis in individuals with G-6-PD deficiency is controversial. Although caution against its usage is advised by some in subjects with G-6-PD deficiency (12, 31, 32), the experience of others is that ASA ingestion alone does not result in any difficulties (7). In fact, there is little data to suggest that ASA should be capable of causing hemolysis in routine therapeutic doses.

Reported here are studies designed to evaluate the role of ASA on normal intact red blood cells at concentrations of salicylate which are readily achievable *in vivo*. These studies demonstrate that ASA can, in combination with hydrogen peroxide, induce red blood cell hemolysis by a final mechanism of membrane lipid peroxidation. This effect appears to be specific for ASA, and not its analogs, and results primarily from membrane damage. This *in vitro* ASA-hydrogen peroxide model may aid in the clarification of ASA as a hemolytic agent.

MATERIALS AND METHODS

The hemolytic effects of ASA, hydrogen peroxide (H₂O₂), and ASA + H₂O₂ were determined using washed intact normal adult red blood cells (RBCs). ASA-containing solutions were prepared in equal parts of 0.9% NaCl and 0.09 M phosphate buffer, pH 7.4; H₂O₂ solutions were prepared as 2.4% solutions in equal parts of 0.9% NaCl and 0.09 M phosphate buffer, pH 7.4. When ASA +

H₂O₂ solutions were used, these consisted of adding ASA to the H₂O₂ solution to achieve the desired concentration.

To observe the possible hemolytic effects of ASA, H₂O₂, and ASA + H₂O₂, a washed 5% suspension of RBCs was incubated at 37° for varying periods of time with equal volumes of solutions of ASA (5 to 50 mg/100 ml), H₂O₂ (2.4%), or ASA (5-50 mg/100 ml) + H₂O₂ (2.4%). These ASA-containing solutions represent 0.325-3.25 mM concentrations of ASA. The actual washing of the RBCs and the incubation volumes employed are the same as in the standard peroxide hemolysis test (20). After the incubation, the cells were spun and the percentage of hemolysis was noted.

In order to determine whether the hemolysis observed during the incubation of RBCs with ASA + H₂O₂ could be reproduced by other similar agents, 5% suspensions of RBCs were additionally incubated for up to 2 hr at 37° with equal volume solutions of 3.25 mM acetic anhydride, 3.25 mM gentisic acid, and 3.25 mM salicylic acid prepared in manner similar to that for the ASA-containing solutions, all with and without the addition of 2.4% H₂O₂. After the incubations, the cells were studied for evidence of hemolysis.

These experiments were repeated with washed RBCs suspended in high molecular weight (average 200,000) 2 mM dextran in phosphate buffer to observe any potential inhibitory effect of this colloid on hemolysis.

In order to determine whether ASA in combination with H₂O₂ prolonged the detoxification of H₂O₂ by intact RBCs and thus increased the time of exposure to H₂O₂, an analysis of supernatant H₂O₂ after exposure of RBCs to ASA + H₂O₂ or to H₂O₂ alone was performed by two methods. In one, the determination of H₂O₂ was by titration of 1 ml supernatant with 2 × 10⁻⁵ M KMnO₄ using ferroin as an indicator (15). In the other, supernatant was added to RBCs suspended in a phosphate buffer containing aminotriazole and the percentage of inhibition of RBC catalase was determined as a measure of the quantity of H₂O₂ present (4).

RBCs were additionally incubated in the presence of ASA (50 mg/100 ml) and two agents which generate intracellular H₂O₂. In this instance, a 5% RBC suspension was incubated with either acetylphenylhydrazine (5 mg/ml) or menadione (4 µg/ml) plus glucose (10 mM final concentration). The incubations were carried out at 37° for 2 hr, after which the percentage of hemolysis was noted.

The ability of the RBC to deacetylate ASA (RBC esterase activity) was evaluated by serial assay of free salicylate determined spectrophotometrically after exposure of a 5% RBC suspension to ASA (50 mg/100 ml in equal parts of 0.9% NaCl and 0.09 M phosphate buffer, pH 7.4 at 37°).

Alterations of RBC glutathione and glutathione recovery were determined by the method of Beutler (2) after a 5-min exposure of a 5% RBC suspension to an equal volume of ASA (50 mg/100 ml) H₂O₂ (2.4%) or ASA (50 mg/100 ml) + H₂O₂ (2.4%). The effect of these agents on certain RBC enzymes including G-6-PD (1), catalase (4), glutathione peroxidase (19), and superoxide dismutase (33) was studied after the same exposure.

The effect of ASA + H₂O₂ on hexose monophosphate shunt

(HMP) activity was estimated following a 15-min incubation at 37° of a 2.0 ml, 5% cell suspension with 2.0 ml solutions of ASA (50 mg/100 ml), H₂O₂ (2.4%), or ASA (50 mg/100 ml) + H₂O₂ (2.4%) prepared as described previously. After these incubations, the RBCs were washed and resuspended in phosphate buffer pH 7.4 with 10 mM glucose. HMP activity was determined by measurement of CO₂ derived from cell suspensions incubated with [1-¹⁴C]glucose (New England Nuclear) (10). Briefly, incubation bottles were fitted with removable plastic wells suspended above the incubation mixture. The incubation was begun with the addition of tracer amounts of labeled glucose to cells suspended in the buffered medium. Approximately 0.2 µCi [1-¹⁴C]glucose was added to a 2-ml cell suspension. After a 1-hr incubation at 37°, the reaction was stopped by the addition of 0.5 N perchloric acid to each flask, after which 0.3 ml Hyamine was added to each well to trap evolved CO₂. The well was transferred to 15 ml Liquifluor and counting was performed in a Packard liquid scintillation counter. From the counts of ¹⁴CO₂ evolved, the glucose metabolized through the HMP was calculated and expressed as percent increases in comparison to specimens to which no ASA or H₂O₂ had been added. All incubations were carried out in duplicate.

The chromagens associated with lipid peroxide formation, primarily malonaldehyde (17, 24, 29), were determined as follows: a 5% RBC suspension was exposed to an equal volume of ASA (50 mg/100 ml) and H₂O₂ (2.4%) as described previously, after which 1 ml of this mixture was added to 1 ml 10% trichloroacetic acid. This was filtered and 0.5 ml of the filtrate was added to 0.6 ml freshly prepared 0.67% thiobarbituric acid (TBA). The TBA-filtrate solution was placed in a boiling water bath for 15 min, then cooled, and the optical density at 535 nm determined.

The protective effects of the antioxidant thymol on ASA + H₂O₂-induced hemolysis were observed after the incubation of a 5% RBC suspension with ASA (50 mg/100 ml) and H₂O₂ (2.4%) in the presence of 0.2 ml of a saturated solution of thymol in phosphate buffer.

The effects of ASA + H₂O₂ on RBC membrane lipids was measured by incubating a 5% suspension of RBCs with ASA (50 mg/100 ml) and H₂O₂ (2.4%). At varying time intervals, duplicate aliquots were removed, washed three times in saline phosphate buffer, and used for subsequent analysis. Membrane lipids were extracted in isopropanol-chloroform and total lipid phosphorus determined (23). Individual phospholipids were isolated using thin layer chromatography on Silica Gel H plates obtained from Brinkmann Instruments Incorporated, Westbury, NY (25). Phospholipids were scraped from each plate, eluted in isopropanol-chloroform, and used for further lipid analysis. Methyl esters of fatty acids in each component were prepared using boron trifluoride (18). Gas liquid chromatography was performed on 7% EGSSX as previously described. Peak areas were calculated by triangulation and were corrected in proportion to the molecular weights of the component fatty acids (6).

RESULTS

HEMOLYTIC EFFECTS OF ASA AND H₂O₂

Figure 1 illustrates the hemolytic effects of varying concentrations of ASA and H₂O₂. The onset of hemolysis occurred approximately 5 min after exposure to ASA + H₂O₂ and the rate with which hemolysis occurs was proportional to the ASA concentration. Hemolysis did not occur with any concentration of ASA when incubations were carried out with ASA alone. In the concentrations used, H₂O₂ alone did not induce hemolysis, but at fixed concentrations of ASA, ASA + H₂O₂-induced hemolysis was also proportional to the H₂O₂ concentration (Table 1). Even very low final concentrations H₂O₂ (0.0003% solutions) would cause hemolysis in the presence of ASA. The substitution of salicylic acid, gentisic acid, and acetic anhydride in equimolar concentrations to the ASA used in these studies did not result in hemolysis when incubated with RBCs alone or in combination with H₂O₂.

Removal of H₂O₂ from the ASA + H₂O₂ solution by the

addition of purified catalase yielded a solution which was incapable of causing hemolysis, demonstrating that an intermediary or third product was not the active cause of hemolysis.

Exposure of a 5% RBC suspension to 50 mg/100 ml ASA at 37° resulted in the rapid conversion of ASA to salicylic acid. Incubation with agents capable of generating intracellular H₂O₂, such as acetylphenylhydrazine or menadione, failed to cause hemolysis in the presence of ASA solutions. This may have resulted from the rapid deacetylation of ASA by normal RBCs so that no ASA was continuously available as H₂O₂ was being slowly generated by these agents.

If RBCs were first incubated with either ASA or H₂O₂ and then were exposed to the other agent, no hemolysis was observed; this indicated the need for simultaneous exposure to both ASA + H₂O₂. Exposure first to H₂O₂ followed by ASA does not result in

Fig. 1. At time-dose curve for the hemolytic effects of varying concentrations of ASA + 1.2% H₂O₂ (final concentration) on a 5% suspension of RBC. No hemolysis occurs if ASA or H₂O₂ are used alone.

Table 1. Percentage of hemolysis observed in a 5% cell suspension mixed with equal volumes of 50 mg/100 ml ASA and varying dilutions of 2.4% H₂O₂ after 1 hr incubation at 37°

H ₂ O ₂ dilution	% Hemolysis
1:4	90.1
1:16	47.1
1:64	36.2
1:256	28.3
1:1024	11.3
1:4096	2.4

Fig. 2. The effect of ASA (25 mg% final concentration) + H₂O₂ (1.2% final concentration) on G-6-PD, GSH peroxidase, catalase, and superoxide dismutase after a 5-min exposure at 37° expressed as percentages of control enzyme activity (cells in buffer only).

hemolysis because of the rapid destruction of H_2O_2 by RBC catalase.

No hemolysis will occur if the above incubations are carried out in the presence of high molecular weight dextran.

EFFECTS OF ASA AND H_2O_2 ON ENZYME AND HMP ACTIVITY

The effect of ASA + H_2O_2 on the activities of RBC G-6-PD, glutathione peroxidase, catalase, and superoxide dismutase may be seen in Figure 2. In the concentrations used neither ASA nor H_2O_2 alone altered the activities of these enzymes. ASA + H_2O_2 reduced G-6-PD activity to 10% of normal.

The effect of ASA + H_2O_2 and gentisic acid + H_2O_2 on RBC-reduced glutathione (GSH) levels may be seen in Table 2. De-

creases in GSH occurred with both ASA + H_2O_2 and gentisic acid + H_2O_2 . Return of GSH to normal was observed after a 2-hr incubation with glucose. The fall in GSH levels was not preventable by thymol and the recovery in the presence of glucose was not inhibited by the addition of 25 mg/100 ml ASA.

The overall effect of ASA, H_2O_2 , and ASA + H_2O_2 on HMP shunt activity may be seen in table 2. Resting shunt activity was only slightly depressed by ASA. As expected, after exposure to H_2O_2 a burst of shunt activity was observed which was only minimally inhibited by the presence of ASA + H_2O_2 .

EFFECT OF ASA AND H_2O_2 ON RBC MEMBRANE LIPID

The addition of the phenolic antioxidant thymol within 5 min of exposure of RBCs to ASA + H_2O_2 completely blocked the onset of hemolysis (Fig. 3). Evidence of lipid peroxidation as measured by the TBA reaction occurred before the onset of ASA + H_2O_2 -induced hemolysis. No lipid peroxidation was observed with either ASA or H_2O_2 alone. The presence of thymol completely blocked the formation of lipid peroxides in addition to preventing hemolysis. High molecular weight dextran, while preventing hemolysis, did not alter the rate of formation of lipid peroxides.

To determine the precise relationship of ASA + H_2O_2 induced hemolysis to membrane lipid peroxidation, alterations of total membrane phospholipid, individual phospholipid fractions, and the composition of fatty acids within each phospholipid fraction were investigated. Concomitant with the rise in TBA activity and preceding the onset of hemolysis, a decrease in membrane lipid P/ 10^8 cells was noted (Table 3). Associated with this loss of total membrane lipid was a marked decrease in the percentage of phosphatidyl ethanolamine and an increase in the percentage of phosphatidyl choline (Table 3). When these percentages are calculated in relation to the decrease in the overall phospholipid content of the RBC, it is apparent that these changes represent a decrease in the absolute quantity of phosphatidyl ethanolamine while the remainder of the phospholipids remained normal. Analysis of alterations of the individual fatty acid fractions demonstrated a loss primarily of 20:4 and 20:6 fatty acids (Table 3) in the phosphatidyl ethanolamine fraction. Similar decreases in polyunsaturated fatty acids were not as marked in other phospholipid fractions.

DISCUSSION

The present investigation demonstrates that aspirin *in vitro*, in concentrations comparable to those capable of being achieved in humans, will induce red cell hemolysis if a simultaneous stress in the form of H_2O_2 is present. ASA alone will not increase red cell destruction in the absence of such an oxidative challenge.

The known metabolic effects of salicylates are complex. Unfortunately, most *in vitro* studies have evaluated the role of salicylates, failing to differentiate between ASA and its unacetylated analog. Smith and associates observed no significant alterations of glucose consumption or of hexose monophosphate shunt activity at blood salicylate levels of 5 mM (70 mg/100) (28). At much higher

Table 2. Alterations of RBC glutathione levels and HMP activity after exposure to combinations of ASA, gentisic acid, and H_2O_2 and recovery with and without ASA.¹

	Glutathione, mg/100 ml				HMP activity, % of control
	A	B	C	D	
Control	59.2	54.0	59.1	58.9	100
ASA	60.7	55.1	56.1	57.1	98
H_2O_2	47.0	44.0	56.7	56.4	360
ASA + H_2O_2	17.1	32.6	60.25	59.1	270
Gentisic acid	60.5				
Gentisic acid + H_2O_2	29.5				

¹ These data represent the mean of five experiments. A: 5% cell suspension after incubation at 37° × 15 min where ASA = 25 mg%, H_2O_2 = 1.2%, gentisic acid = 1.62 mM; B: washed cells from the incubation of "A" after a 30-min incubation without glucose (100 mg/100 ml); C: washed cells from the incubation of "A" after a 30-min incubation with glucose (100 mg/100 ml); D: washed cells from the incubation of "A" after a 30-min incubation with glucose (100 mg/100 ml and ASA (25 mg/100 ml).

Fig. 3. The protective effects of the antioxidant thymol and the formation of lipid peroxides (TBA reaction) following incubation of a 5% cell suspension with equal volumes of ASA (50 mg/100 ml) and H_2O_2 (2.4%).

Table 3. Effect of ASA and H_2O_2 on 5% RBC suspension after a 30-min incubation: Alterations in total membrane phospholipid (10 mg/100 ml ASA + 1.2% H_2O_2), phospholipid fractions (30 mg/100 ml ASA + 1.2% H_2O_2) and levels of 20:4 and 22:6 lipid fractions¹

	Total lipid P (P/ 10^8)	Phospholipid composition ²				Fatty acid ³	
		PC	PE	SP	PS	20:4	22:6
Control cells	1.6	35.3	25.8	33.8	5.1	16.6	7.8
Cells + ASA		37.6	24.7	35.1	2.7	14.2	6.6
Cells + H_2O_2		35.8	26.3	33.4	4.5	13.2	6.6
Cells + H_2O_2 + ASA	1.05	41.2	19.2	29.4	10.2	8.4	3.3

¹ PC: phosphatidyl choline; PE: phosphatidyl ethanolamine; SP: sphingomyelin; PS: phosphatidyl serine.

² Expressed as percentage of total.

³ Expressed as percentage of total; other fatty acid fractions unchanged.

concentrations of salicylates (such as 100 mM) marked alterations of hexose monophosphate shunt activity began to be noted (28). At these higher concentrations it appears that the glycolytic pathway is more sensitive to the inhibitory effects of salicylates than is the HMP shunt (28). The major metabolic disturbance in each of these pathways appears to be mediated through inhibition of various pyridine nucleotide linked dehydrogenases (5). Hexokinase will additionally be inhibited at very high salicylate concentrations *in vitro* (30), whereas lesser concentrations may decrease red cell 2,3-diphosphoglycerate (14).

Plasma salicylate levels ideally sought in the management of disorders such as rheumatoid arthritis may be as high as 20–30 mg/100 ml (1.5–2.2 mM) (16). Even in severe salicylate poisoning, the plasma salicylate level does not rise above 10 mM (27), and at whole blood concentrations of 10 mM there is no effect upon the hexose monophosphate pathway of normal red cells and only slight inhibition of this pathway in G-6-PD-deficient red cells (28). It must be concluded that although it is possible to inhibit hexose monophosphate shunt activity *in vitro*, the concentrations required are not achievable *in vivo* and that if ASA is a cause of hemolysis in G-6-PD deficient its mechanism of action is not solely by enzyme inhibition.

Realizing that salicylates, *per se*, on the basis of enzyme inhibition alone, can not account for G-6-PD associated hemolysis, Shahidi and Westring (22) evaluated the role of gentisic acid in this process. When aspirin is ingested, it is quickly "deacetylated" by a variety of different esterases which hydrolyze ASA largely to salicylic acid. The plasma $T_{1/2}$ for ASA itself is as short as 3.5 min after intravenous injection (21, 26). When ingested orally 30% of ASA is already converted to salicylic acid before the drug enters the blood. Neither ASA or salicylic acid is an oxidizing "redox" compound, whereas gentisic acid, a biotransformation product of ASA, does possess oxidizing properties by virtue of its dihydroxy grouping in the para position. Approximately 4–8% of ingested salicylate is converted to gentisic acid. Gentisic acid is similar to salicylic acid in its degree of various enzyme inhibitions but in addition presents an oxidant challenge to the RBC. Although no hemoglobin or GSH oxidation occurs with ASA or salicylate at concentrations of 10 mM, 6 mM gentisic acid will cause marked oxidation of hemoglobin and GSH. If salicylic acid is added to gentisic acid, a greater effect is observed because of curtailment of NADPH regeneration. Although gentisic acid is capable of inducing hemolysis *in vitro*, the dose of oral ASA required for this purpose would require blood salicylate levels of 90 to 180 mM (1260–2520 mg/100 ml) since only a small fraction of the ASA is actually metabolized to gentisic acid. Glader (7) demonstrated that only at concentrations of 35 mg/100 ml or higher could gentisic acid enhance [14 C]glucose oxidation and that the maximal gentisic acid concentrations achievable with high therapeutic doses of salicylate administration should not exceed 3.5 mg/100 ml which has no metabolic consequences for the red cell. Because *in vivo* ASA administration to both G-6-PD A⁺ and B variants failed to provoke hemolysis in Glader's study, it was concluded that ASA-related hemolysis is caused by associated problems such as infection rather than solely by the drug.

Our studies verify certain of these earlier findings but do suggest that ASA may be capable of causing hemolysis under certain circumstances in doses that are achieved with routine oral administration. It was found that ASA with *in vitro* concentrations of 10–25 mg/100 ml did not affect the activity of red cell G-6-PD, glutathione peroxidase, superoxide dismutase, or catalase. Reduced glutathione levels remained normal despite prolonged incubation with ASA and no inhibition of resting shunt metabolism occurred. Similar findings were observed with equimolar concentrations of salicylate and gentisic acid.

If RBCs are exposed to ASA and H₂O₂ simultaneously, hemolysis occurs promptly. The final concentration of H₂O₂ (1.2%) is identical to the concentrations used in the peroxide hemolysis test which alone will not cause hemolysis (20). It is not possible to correlate the concentrations of H₂O₂ used in these experiments

with the unknown quantities that may be generated *in vivo*. However, concentrations of H₂O₂ as low as 0.0003% *in vitro* will cause hemolysis within 1 hr in the presence of ASA. The substitution of salicylic acid for ASA when H₂O₂ is added fails to induce hemolysis stressing that acetylation may be prominent in ASA + H₂O₂-induced hemolysis. Prior treatment of red cells with H₂O₂ followed by the addition of ASA produces no abnormalities because of the rapid destruction of H₂O₂ by RBC catalase. Likewise, the prior exposure of RBCs to ASA followed by H₂O₂ is not associated with hemolysis. This is most likely the result of the extremely rapid conversion of ASA to salicylic acid noted in the presence of red cells. In our studies it appears that the RBC as with many other tissues will deacetylate ASA by virtue of some esterase like activity on or within the RBC. It does not appear that the combination of ASA and H₂O₂ produces a metabolically active third product since the addition of purified catalase to the ASA + H₂O₂ solution, which removes all H₂O₂, leaves a solution which has no hemolytic effects on the RBC. Cells exposed to ASA + H₂O₂ for brief periods do appear to decrease their G-6-PD activity, and GSH levels fall precipitously, but overall HMP activity is only slightly inhibited suggesting that enzyme inhibition is not the major cause of hemolysis. ASA does not prolong the detoxification of H₂O₂ when ASA and H₂O₂ are added to normal RBCs since essentially no H₂O₂ is detectable within 5 min of the exposure of cells to ASA + H₂O₂.

The major abnormality caused by ASA + H₂O₂ is disruption of the RBC membrane associated with membrane lipid peroxidation. Evidence for the presence of lipid peroxidation was noted by observing the generation of lipid peroxides over a period of time preceding hemolysis. The fact that peroxidation of membrane lipids preceded hemolysis and that the addition, before the onset of hemolysis, of the antioxidant thymol could prevent RBC destruction, strongly suggests that lipid peroxidation is the cause of hemolysis. In these experiments thymol was added 5 min after exposure to ASA and H₂O₂, a time when the hydrogen peroxide had been destroyed and at a time when the process responsible for hemolysis had already been completely initiated. If lipid peroxidation and the process leading to hemolysis were independent events then inhibition of lipid peroxidation at this time should have no effect on hemolysis. Peroxidation of lipids *in vitro* involves cleavage of unsaturated fatty acids at their double bonds and the depletion of membrane lipid phosphorus is specifically related to depletion of phosphatidyl ethanolamine as observed in these studies. The destruction of 20:4 and 22:6 fatty acids resulted in the formation of short chained fatty aldehydes (mainly malonaldehyde) as measured by their color reaction products with thiobarbituric acid. Jacob and Lux (11), studying the vitamin E-deficient rat erythrocyte, demonstrated that the loss of phosphatidyl ethanolamine resulted in sufficient membrane damage to result in colloid osmotic lysis and that high molecular weight dextran would inhibit this hemolysis as was observed in these studies.

It appears clear that hemolysis resulting from exposure to the combination of ASA and H₂O₂ is in part resulting from the presence of the acetyl moiety of the acetylsalicylic acid molecule, since substitution of salicylic acid will produce no hemolysis. The exact manner in which ASA in the presence of H₂O₂ will initiate membrane lipid peroxidation and cell lysis remains unclear. Although ASA in unphysiologically high concentrations will affect the RBCs ability to handle oxidant challenges by inhibition of HMP shunt activity this mechanism can not explain the *in vivo* hemolysis resultant from ASA or the hemolysis induced by ASA and H₂O₂ *in vitro*. It is interesting to speculate that acetylation of some portion of the RBC membrane results in a greater sensitivity to peroxidative damage by H₂O₂. Aspirin is known to acetylate many different proteins, including albumin (8) and hemoglobin itself (3, 13).

Finally, it is necessary to reconcile the diverse clinical findings among individuals who ingest ASA. The actual incidence of hemolysis with severe G-6-PD deficiency is probably small under any circumstance since in a study of 203 subjects, Herman and

Ben-Meir (9) found only two instances of overt hemolysis from any cause. Although ASA is generally classified as a mild hemolytic agent, severe red cell destruction has been reported on several occasions (12, 31). However, since in all instances ASA had been given to alleviate fever at times of infection, the exact cause of hemolysis remained unclear. Although diminished survival of RBCs from a G-6-PD-deficient patient was observed after transfusion into a healthy compatible recipient when the latter was given 1.8 g ASA daily, Glader (7) found no fall in Hb or rise in bilirubin or reticulocytes of G-6-PD-deficient subjects given 50 mg/kg ASA/day. The ability of a G-6-PD-deficient subject to ingest ASA without harm on some occasions while experiencing hemolysis with similar doses of ASA at other times of stress could be explained by the ASA + H₂O₂ model in the studies presented here. Except at the time of ingestion of drugs such as primaquine, which generate large quantities of H₂O₂, or at the time of severe metabolic stress, the G-6-PD-deficient RBC would have no great excess of undetoxified H₂O₂. Thus ASA administration at times of well being would not result in hemolysis. With infection, peroxides generated by activated phagocytes, as well as microbial metabolic products, overwhelm the detoxification capacity of the HMP shunt of G-6-PD-deficient individuals creating a surplus of H₂O₂ which in the presence of ASA may result in severe RBC membrane damage. This hypothesis does not require that ASA in any way inhibits or limits the ability of the RBC to detoxify H₂O₂, although the partial inhibition of G-6-PD in the presence of ASA + H₂O₂ may contribute to this problem.

It is suggested that if ASA is harmful when administered to subjects with G-6-PD deficiency, the basis of its effect is not because of any intrinsic oxidant properties of ASA or because of inhibition of various enzyme systems, but rather because of an interaction between ASA and H₂O₂ which results in RBC membrane damage.

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- Received for publication July 6, 1977.
- Accepted for publication November 16, 1977.