Acetylsalicylic Acid-Induced Hemolysis and Its Mechanism

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ABSTRACT Acetylsalicylic acid (ASA) is known to cause severe hemolytic anemia in some glucose-6-phosphate-dehydrogenase-deficient (G-6-PD-deficient) individuals. To study its mechanism, erythrocytes from an ASA-sensitive patient were transfused into a normal compatible recipient. The administration of 2,5-dihydroxybenzoic (gentisic) acid, a known ASA metabolite with redox properties, to the recipient resulted in a marked decrease in the survival of the patient's erythrocytes. Similar studies with red cells from individuals with A- and Mediterranean variants of G-6-PD revealed no alteration in the erythrocytes' survival. Further studies disclosed that both salicylate and gentisate competitively inhibited the G-6-PD from the ASA-sensitive patient resulting in a marked change in the $K_m$ for NADPH. These drugs also inhibited the A- and Mediterranean variants of G-6-PD. The magnitude of inhibition, however, was comparatively small and not different from that observed with a normal enzyme.

The above studies suggested that enzyme inhibition by salicylate and gentisate may play an important role in ASA-induced hemolysis. Such an inhibition would further curtail NADPH regeneration, rendering the cells more vulnerable to oxidants. In this connection, gentisate seems to play a major role in ASA-induced hemolysis for it is both a G-6-PD inhibitor and an "oxidant."

INTRODUCTION

To evaluate the potential toxicity of various drugs suspected to cause hemolysis in glucose-6-phosphate-dehydrogenase-deficient (G-6-PD-deficient) individuals, most clinical studies have involved subjects with A- variant. As a result of these studies, some drugs such as primaquine and pamaquine are incriminated in causing dangerous to severe hemolysis in G-6-PD-deficient individuals, and others, such as acetylsalicylic acid (ASA), are classified as mild hemolytic agents even in high dosage (1). However, severe red cell destruction following ASA ingestion has been reported on several occasions, mostly in Caucasians (1-4). In the majority of the patients (2-4) the severe hemolytic process had occurred after the ingestion of small therapeutic doses not exceeding 2 g/day. Since in all instances ASA had been given to alleviate fever, infection rather than ASA itself had been incriminated as the cause of the severity of the hemolytic process (1). Recently, however, it was demonstrated that the survival of the red cells from a G-6-PD-deficient patient markedly decreased in a healthy compatible recipient when the latter was given a daily dose of 1.8 g of ASA (4). This great individual variation in the severity of ASA-induced hemolysis and its mechanism remains unexplained. The present investigation was undertaken in an attempt to elucidate the mechanism of ASA-induced hemolysis in G-6-PD-deficient individuals and to ascertain the reasons for the individual variations in the degree of hemolysis.

METHODS

The clinical material comprised five male subjects with erythrocyte G-6-PD deficiency and seven healthy adult males who either gave blood for enzyme investigation or volunteered for homologous erythrocyte survival studies. Of patients with G-6-PD deficiency, two were adult Negroes with no evidence of hemolysis. The remaining three, aged 8, 14, and 17 yr respectively, were Caucasian and had hereditary nonspherocytic hemolytic disease (HNSHD). All these individuals had received ASA on several occasions without any apparent clinical and hematological side effects except for the 8 yr old boy who on several occasions developed severe hemolysis following the ingestion of 1.2-1.5 g of ASA per day. The hemolytic episodes were manifested by a significant drop in the hemoglobin concentration, dark urine, and the presence of Heinz bodies in the circulating erythrocytes. The history and some of the laboratory data of this patient have been previously described (4).

Erythrocyte survival studies were performed using $^{51}$Cr as outlined by Read, Wilson, and Gardner (5). Methemoglobin and sulfhemoglobin were measured by the method of Evelyn and Malloy (6). The reduced glutathione was determined using 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Beutler, Duron, and Kelly (7). The assay for G-6-PD was based on a method described by Glaser and

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Brown (8) and modified by Zinkham (9). The changes in the absorbancy at 340 nm were measured using a DU spectrophotometer adapted for recording and scale expansion (Gilford). The NADP reduction was measured at 25°C in a 3 ml cuvette with 1 cm light path containing Tris-buffer pH 8, 300 μmoles; G-6-P, 2 μmoles; MgCl₂, 20 μmoles; NADP, 0.6 μmoles; hemolysate, 0.1 ml; and water to a total volume of 3 ml. 1 U of enzyme activity was defined as the amount of enzyme generating 1 μmole of NADPH per min. Specific activity is defined as the units of enzyme per 10⁶ RBC. To prepare the hemolysate fresh defibrinated blood was centrifuged at 3000 g for 15 min, the plasma and the Buffy coat were removed, and the red cells were washed three times with cold isotonic saline. 0.1 ml of the washed erythrocytes was mixed with 3.9 ml of cold water containing mercaptoethanol and EDTA, each at the final concentration of 10⁻³ mole/liter and NADP at the final concentration of 2 × 10⁻³ mole/liter. After 5 min the hemolysate was frozen and thawed twice, and centrifuged at 10,000 g for 30 min to remove the stroma. 0.1 ml of the same sample of erythrocytes was mixed with 3.9 ml of isotonic saline and the cells were counted in a Coulter counter.

Partial purification of the erythrocytes' G-6-PD was done according to the procedure outlined by Kirkman and associates (10, 11) in a cold room at 4°C. Deionized double distilled water was used throughout the experiments.

Vertical starch-gel electrophoresis of the G-6-PD was performed according to the method of Kirkman and Hendrickson (12). Thermostability studies were performed on diluted ammonium sulfate suspensions according to the procedure described by Kirkman, Rosenthal, Simon, Carson, and Brinson (11).

Kinetic studies were done with and without sodium salicylate and in some instances sodium gentisate. Because of the interference of these drugs with fluorometric determination of NADPH, spectroscopic measurements were performed. The absorbency of NADPH was determined at 365 nm rather than 340 nm to avoid interference caused by absorption of salicylate and gentisate at this wave length. To increase the absorbency 30-ml cylindric cuvettes with 100-mm light path were used and were housed in a special chamber connected to the Beckman DU adapted to a Gilford scale expander and recorder. The substrate mixture contained 1 ml 0.3 M MgCl₂; 1 ml 0.01 M G-6-P; 10 ml 0.2 M Tris pH 8; 0.275-5.5 ml 6 × 10⁻⁴ M NADP; and water to a final volume of 33 ml. The mixtures were prepared in a 50 ml test tube which was placed in a 25°C water bath. After equilibration of the temperature, the content was transferred to the cuvette. The reaction was started with the addition of the enzyme extract (0.1 ml–0.3 ml according to the activity) by means of a Hamilton syringe. The reaction mixture was rapidly but gently mixed by allowing a small air bubble to travel several times back and forth the length of the cuvette. The data were plotted as reciprocal plots (Lineweaver-Burk method) (13) using the Fortran computer programs developed by Cleland (14). These programs make the least square fit to equations in enzyme kinetic studies.

Incubation studies. All incubation studies were performed in 25-ml Erlenmeyer flasks placed in a Dubnoff metabolic shaker at 37°C. 2 ml of washed erythrocytes were suspended in an equal volume of isotonic buffered saline pH 7.4 (15) containing 2 mg/ml of dextrose. The drugs were dissolved in the buffered saline immediately before incubation. Sodium salicylate and sodium gentisate were obtained commercially. The purity of both compounds was verified by the melting point of these compounds in the stock solution used for kinetic studies were ascertained enzymatically.

**Table I**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age and sex</th>
<th>Designation</th>
<th>Activity of G-6-P % of normal</th>
<th>Kₘ G-6-P</th>
<th>Kₘ NADP</th>
<th>2 deoxy-G-6-P utilization</th>
<th>Thermal stability</th>
<th>pH optimum</th>
<th>Electro-photometric pattern</th>
</tr>
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<tbody>
<tr>
<td>Normals</td>
<td>5 adults M</td>
<td>B</td>
<td>75–125*</td>
<td>50–80</td>
<td>3–6</td>
<td>4</td>
<td>Normal</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>G. R. Adult M</td>
<td>A−</td>
<td>10</td>
<td>58.3</td>
<td>3.8</td>
<td>3.3</td>
<td>Normal</td>
<td>9</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td>H. A. Adult M</td>
<td>A−</td>
<td>8</td>
<td>62.5</td>
<td>2.2</td>
<td>4</td>
<td>Normal</td>
<td>9</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td>D. L. 17 yr M</td>
<td>HNSHD</td>
<td>0.9</td>
<td>14.2</td>
<td>1.3</td>
<td>24.5</td>
<td>Labile</td>
<td>9−5</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>J. L. 14 yr M</td>
<td>HNSHD</td>
<td>3.0</td>
<td>11.9</td>
<td>1.5</td>
<td>29.2</td>
<td>Labile</td>
<td>6−5</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>R. O. 8 yr M</td>
<td>HNSHD</td>
<td>0.4</td>
<td>224†</td>
<td>17.6</td>
<td>3.7†</td>
<td>Normal†</td>
<td>8†</td>
<td>Slow†</td>
</tr>
</tbody>
</table>

HNSHD: hereditary nonspherocytic hemolytic disease.
* Based on values obtained in our laboratories on 60 healthy adults and children of both sexes.
† Determination made by Dr. H. N. Kirkman and reported previously by Westring and Pisciotta (4).

G-6-P and NADP were purchased from Sigma Chemical Co. The concentrations of these compounds in the stock solution used for kinetic studies were ascertained enzymatically.

**Acetylsalicylic Acid–Induced Hemolysis and Its Mechanism** 1335
and isopropanol-ammonia patients transfused into FIGURE 1

a qualitative apparent the summarizes phase of point and acid-water run.

While from NADP has been small therapeutic doses of ASA (22%)-water and isopropanol-ammonia (22%)-water 16:1:3 for the second run.

RESULTS

Quantitative and qualitative enzyme studies. Table I summarizes the quantitative and qualitative aspects of G-6-PD from normal and enzyme-deficient subjects. While the enzyme from Negro deficient exhibited no apparent qualitative differences from the normal, that from patients with HNSHD showed significant abnormalities. The patients D. L. and J. L. showed an enzyme pattern indistinguishable from the Mediterranean variant (16). The G-6-PD from patient R. O. with a markedly high $K_w$ value for both G-6-P and NADP has been designated as Milwaukee variant (17).

The effect of the drugs on the erythrocytes' survival in vivo. Previous studies demonstrated a rapid decrease in the survival of erythrocytes from patient R. O. in a normal compatible recipient when the latter was given small therapeutic doses of ASA (4).

Since ASA is not an oxidizing "redox" compound, it seemed likely that 2,5-dihydroxybenzoic acid (gentisic acid), an ASA biotransformation product with oxidizing properties, might be responsible for erythrocyte destruction. It has been estimated (18, 19) that approximately 3-8% of the ingested salicylic acid is normally converted to gentisic acid. It has been shown that this compound decreases total glucose consumption in the red cell but increases the percentage metabolized through the pentose phosphate pathway (20). Consequently, its effects on patient's red cells were ascertained both in vivo and in vitro. Fig. 1 shows the survival of the $^{51}$Cr-labeled red cells from patient R. O. in a normal compatible adult volunteer before and after ingestion of various amounts of gentisic acid. As seen, the daily ingestion of 75 mg of gentisic acid did not result in any change in the erythrocytes' survival. However, when the gentisic acid was given in a dosage of 150 mg/day, the $^{51}$Cr half-life ($t_1/2$) decreased by 50%. The administration of 225 mg of gentisic acid per day resulted in a further decrease in the $^{51}$Cr $t_1/2$ to 23 hr which is approximately $1/4$ of the initial value. Similar experiments were performed by injecting $^{51}$Cr-labeled erythrocytes from two individuals with A-variant and one of the patients with Mediterranean variant to normal adult volunteers. The administration of gentisic acid in a daily dose as high as 450 mg to the recipients had no effect on the survival of erythrocytes from these patients.

The effect of sodium gentisate on erythrocytes in vitro. Sodium gentisate in concentration of 6 mmoles/liter under the experimental condition described caused significant GSH and hemoglobin oxidation (Fig. 2). As seen, the concentration of methemoglobin in the red cells from one of the subjects with A-variant (G. R.) increased steadily upon incubation with sodium gentisate. At the end of 3 hr the methemoglobin reached 18% and GSH dropped to approximately half its initial value. The accumulation of sulfhemoglobin was not significant. Experiments with red cells from normal individuals exhibited similar amounts of methemoglobin under the same condition although the drop in GSH was less than 15% of the initial values. Similar concentration of gentisate resulted in a higher concentration of methemoglobin and lower levels of GSH when red cells from patient R. O. were used (Fig. 3). As seen, the GSH was completely oxidized at the end of 3 hr of incubation. There was also a small accumulation of sulfhemoglobin. The incubation of ASA or salicylate at the final concentration of 10 mmoles/liter with red cells from normal or G-6-P; deficient individuals did not result in hemoglobin or GSH oxidation. It is known, however, that salicylate inhibits several NAD- and NADP-dependent dehydrogenases (21) including G-6-PD (22). Dialysis experiments have shown that the inhibition of NAD and NADP dehydrogenases by salicylate is reversible (23). To assess whether such an inhibition would en-
METHGB
SULFHGB
GSH
mg/100ml RBC
50-100

0 20 40 60 80
20

\[ \text{HOURS} \]

\[ \bullet \bullet \bullet \ \text{GENTISATE + SALICYLATE} \]
\[ \bullet \ \text{GENTISATE ALONE} \]

**Figure 2** Showing the effect of gentisate (6 mM) alone and in combination with salicylate (10 mM) on the red cells from G. R. (A- variant). Note the additive effect of salicylate on the oxidation of reduced glutathione (GSH) and hemoglobin. Salicylate alone (10 mM) demonstrated no oxidative properties. 2 ml of erythrocytes suspended in equal volume of a buffered saline solution (15) containing 2 mg/ml of dextrose were incubated with and without drugs at 37°C in 2.5-ml Erlenmeyer flasks placed in a Dubnoff metabolic shaker. The data represent the mean values for three separate determinations.

**Figure 3** Illustrating the effect of gentisate alone and in combination with salicylate on red cells from patient R. O. The experimental conditions were similar to those described in Fig. 2.

hance the gentisate-induced hemoglobin and GSH oxidation by curtailing NADPH regeneration, similar experiments were performed in the presence of sodium salicylate. As shown in Figs. 2 and 3 respectively, the addition of salicylate at the concentration of 10 mmoles/liter enhanced hemoglobin and GSH oxidation by gentisate in the red cells from patients G. R. and R. O. The enhancement of the oxidative properties of gentisate by salicylate, however, was much greater when the red cells from patient R. O. were used.

**Inhibition studies.** Fig. 4 shows the effects of 10 mM sodium salicylate on the partially purified G-6-PD from a normal individual and some of the patients with G-6-PD deficiency. As seen, in all instances, the slope of the curve is greater in the presence of sodium salicylate suggesting inhibition. Since the curves intercept the vertical line at the same point, the inhibition is competitive resulting in an apparent increase in the $K_a$ which is designated as $K_i$. It is also apparent that the degree of inhibition is not the same. Table II summarizes the values for $K_a$, $K_p$, and $K_i$ for all individuals tested. The $K_i$ which is the enzyme-inhibitor
The dissociation constant was computed from the equation:

\[ K_i = \frac{1}{K_p/K_m - 1} \]

As seen, the degree of inhibition was quite similar in all individuals except in patient R. O. whose enzyme was markedly inhibited by salicylate resulting in a shift in \( K_m \) from 17.6 to 123 \( \mu \)moles/liter. Consequently, the \( K_i \) was markedly low as compared to normal or other G-6-PD mutants tested. It has been shown that gentisate exerts similar inhibitory effects on NAD and NADP dehydrogenases including G-6-PD (24). To verify this possibility, similar experiments were performed with sodium gentisate as the inhibitor. This compound was found, in all instances, to be a more potent inhibitor in that 5 \( \mu \)moles/liter produced an apparent change in \( K_m \) similar to that obtained with 10 \( \mu \)m salicylate (Table II). Kinetic studies were also performed using crude extract of sonicated fibroblast from skin cultures of patients D. L., J. L., and R. O. with HNSHD. The results were similar to those obtained using partially purified G-6-PD obtained from the red cells.
to assess inhibitors used. However, after experiments of presence of patient R. the concentration of NADP at the maximum in the presence of salicylate, by virtue of its dehydroxy group in para position, possesses oxidative properties (20). The data further suggest that qualitative abnormality of G-6-PD may play an important role in sensitivity to drug-induced hemolysis. As shown in Table I, the enzyme from patient R. O. with extremely high $K_m$ for both G-6-P and NADP functions at a great disadvantage even in the absence of any inhibition. Further increase in the $K_m$ for NADP as caused by competitive inhibition results in further decrease in NADPH regeneration rendering the cell more vulnerable to the effects of oxidizing agents. To function at its $\frac{1}{2}$ maximum in the presence of salicylate at the concentration of 10 mmole/liter, the G-6-PD from patient R. O. requires approximately 3 times more NADP than is actually available in the erythrocytes. Similar NADP concentrations are required in the presence of 5 mM gentisate. It is obvious that the concentration of salicylate and gentisate used in these experiments are in excess of blood levels usually reached after therapeutic doses of ASA. In addition, other factors such as cellular permeability may affect the concentration of these drugs within the intact cells. However, the primary aims of the inhibition studies were to assess the enzyme inhibition pattern in various G-6-PD mutants and to compare their affinity for the inhibitors used. According to the equation $K_i = K_m (1 + i/K_i)$, salicylate at the concentration of 1 mmole/liter would result in a change in $K_m$ from 17.6 to 28.1 which is a 60% increase for patient R. O. As judged by the $K_i$ values, similar concentration of salicylate would result in negligible change in $K_m$ for the G-6-PD for all other individuals tested. Competitive and noncompetitive inhibition of G-6-PD by various hemolytic compounds have been investigated by Desforges, Kalaw, and Gilchrist (25). Information regarding the inhibitory effects of hemolytic agents and their biotransformation products on human G-6-PD mutants is not available.

As suggested by erythrocyte survival studies (Fig. 1), gentisate seems to play a major role in red cell destruction induced by ASA in patient R. O.; this compound alone caused significant decrease in the survival of red cells from this patient. This is probably due to the fact that gentisate acts both as an inhibitor of G-6-PD and as an oxidizing agent. Salicylate, if present, may further increase the magnitude of enzyme inhibition. While gentisate seems to be the main biotransformation product of ASA with oxidative properties, the role of other di- or trihydroxybenzoic acids cannot be ruled out. However, according to available information (26) the extent for formation of these compounds from ASA in man is negligible.

ASA has been shown to acetylate numerous human proteins (27, 28). It is conceivable that ASA could acetylate red cell membrane causing damage. However, the fact that gentisate alone can lead to red cell destruction in vivo indicates that acetylation does not play a major role in red cell damage.

### Table II

<table>
<thead>
<tr>
<th>Subject</th>
<th>Designations</th>
<th>$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td></td>
<td>3.3</td>
<td>13.0</td>
</tr>
<tr>
<td>G. R.</td>
<td>A</td>
<td>3.8</td>
<td>13.3</td>
</tr>
<tr>
<td>H. A.</td>
<td></td>
<td>2.2</td>
<td>16.2</td>
</tr>
<tr>
<td>D. L.</td>
<td>HNSHD (Mediterranean)</td>
<td>1.3</td>
<td>34.5</td>
</tr>
<tr>
<td>J. L.</td>
<td></td>
<td>1.5</td>
<td>9.4</td>
</tr>
<tr>
<td>R. O.</td>
<td>HNSHD (Milwaukee)</td>
<td>17.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Note the apparent $K_m(K_i)$ for NADP in the presence of salicylate and gentisate. The enzyme-inhibitor dissociation constant ($K_i$) was computed according to the equation outlined in the text.

### DISCUSSION

The present investigation indicates that both salicylate and its biotransformation product gentisate play a role in ASA-induced hemolysis. Both drugs exert an inhibitory effect on G-6-PD. In addition, gentisate, by virtue of its dihydroxy group in para position, possesses oxidative properties (20). The data further suggest that qualitative abnormality of G-6-PD may play an important role in sensitivity to drug-induced hemolysis. As shown in Table I, the enzyme from patient R. O. with extremely high $K_m$ for both G-6-P and NADP functions at a great disadvantage even in the absence of any inhibition. Further increase in the $K_m$ for NADP as caused by competitive inhibition results in further decrease in NADPH regeneration rendering the cell more vulnerable to the effects of oxidizing agents. To function at its $\frac{1}{2}$ maximum in the presence of salicylate at the concentration of 10 mmole/liter, the G-6-PD from patient R. O. requires approximately 3 times more NADP than is actually available in the erythrocytes. Similar NADP concentrations are required in the presence of 5 mM gentisate. It is obvious that the concentration of salicylate and gentisate used in these experiments are in excess of blood levels usually reached after therapeutic doses of ASA. In addition, other factors such as cellular permeability may affect the concentration of these drugs within the intact cells. However, the primary aims of the inhibition studies were to assess the enzyme inhibition pattern in various G-6-PD mutants and to compare their affinity for the inhibitors used. According to the equation $K_i = K_m (1 + i/K_i)$, salicylate at the concentration of 1 mmole/liter would result in a change in $K_m$ from 17.6 to 28.1 which is a 60% increase for patient R. O. As judged by the $K_i$ values, similar concentration of salicylate would result in negligible change in $K_m$ for the G-6-PD for all other individuals tested. Competitive and noncompetitive inhibition of G-6-PD by various hemolytic compounds have been investigated by Desforges, Kalaw, and Gilchrist (25). Information regarding the inhibitory effects of hemolytic agents and their biotransformation products on human G-6-PD mutants is not available.

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ASA has been shown to acetylate numerous human proteins (27, 28). It is conceivable that ASA could acetylate red cell membrane causing damage. However, the fact that gentisate alone can lead to red cell destruction in vivo indicates that acetylation does not play a major role in red cell damage.
In analogy to ASA, sulfadiazine has been found to cause hemolysis in some patients with G-6-PD deficiency (2). Similar kinetic studies in sulfadiazine-susceptible individuals are highly desirable. It should be emphasized, however, that abnormal enzyme kinetics are not the sole mechanism responsible for increased sensitivity to drug toxicity. Alteration in drug biotransformation (29, 30) may play an equally important role.

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