Hemolysis and Sulfa-drug

By

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In previous papers, the author reported, in two families of congenital nonspherocytic hemolytic anemia, an enzymatic anomaly of erythrocytes, consisting of a low content of reduced glutathione (GSH), an abnormality in the reduced glutathione stability test, and a low activity of glucose-6-phosphate dehydrogenase (G6PD). The author reported that the administration of Domian (6-sulfanilamido-2,4-dimethylpyrimidine) to the patient with nonspherocytic hemolytic anemia resulted in aggravation of hemolysis accompanied with further decrease of G6PD and GSH in the erythrocytes. From these observations the author designed to investigate the relationship between Domian and the metabolic anomaly of erythrocyte mentioned above. In this respect, there are several possibilities supposed: 1) Domian may inhibit G6PD activity in erythrocytes, 2) it may inhibit glutathione reductase activity in them, 3) it may accelerate oxidation of GSH in them, or 4) it may accelerate oxidation of TPNH or DPNH in them. Since both G6PD and glutathione reductase in erythrocyte are a conjugate reaction dependent on TPNH, any one of above possibilities may result in a decrease of the GSH in erythrocytes which substance is probably essential for structural integrity of erythrocyte. With the above-mentioned suppositions in view, the following 6 experiments were performed.

Experiment I: The effect of Domian on activity of G6PD and of glutathione reductase was in vitro investigated, using hemolysate from normal human erythrocytes; the hemolysate was prepared as described by Zinkham. The activity of G6PD was assayed by the method of Zinkham, excepting that incubation was performed at 37°C instead of 25°C. The activity of glutathione reductase was assayed by the method of Schreier et al. In controls an equimolar sodium chloride was used in place of Domian. As is shown in Fig. 1, Domian had a definitely inhibitory effect on G6PD activity at the concentration of 3.75 x 10^-3M, while it had no significant effect on glutathione reductase in the same con-

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TPNH*=reduced triphosphopyridine nucleotide

DPNH**=reduced diphosphopyridine nucleotide
Fig. 1. Effect of Domian on G6PD and glutathione reductase (Experiment I)

- ....... With addition of $3.75 \times 10^{-3}$ M Domian
- ------ Without addition of $3.75 \times 10^{-3}$ M Domian

Fig. 2. Inhibitory effect by different concentration of Domian on G6PD (Experiment I)
centration. Fig. 2 shows the inhibitory effect of different concentrations of Domian on G6PD activity. The inhibitory effect on G6PD activity was tried

<table>
<thead>
<tr>
<th>TABLE I. Inhibitory Effect of Sulfad-drugs on G6PD.</th>
<th>Inhibitory effect %</th>
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<tbody>
<tr>
<td><strong>Drugs examined</strong></td>
<td></td>
</tr>
<tr>
<td>Domian</td>
<td>![Structure of Domian]</td>
</tr>
<tr>
<td>Thiasin</td>
<td>![Structure of Thiasin]</td>
</tr>
<tr>
<td>Merian</td>
<td>![Structure of Merian]</td>
</tr>
<tr>
<td>Abcicd</td>
<td>![Structure of Abcicd]</td>
</tr>
<tr>
<td>Sulzol</td>
<td>![Structure of Sulzol]</td>
</tr>
<tr>
<td>Pasnal</td>
<td>![Structure of Pasnal]</td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>![Structure of Sulfanilic acid]</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>![Structure of Sodium benzoate]</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>![Structure of Glutamic acid]</td>
</tr>
</tbody>
</table>

Concentration of drugs used: $3.75 \times 10^{-3}$M
by using various sulfa-drugs other than Domian. It was observed that a series of sulfa-drugs had in vitro an inhibitory effect on G6PD (cf. Table I). Fig. 3 shows a Lineweaver-Burk's plot for inhibition by Domian on G6PD activity. Since there is a distinct difference in maximal velocity of enzyme activity between "with inhibitor" and "without inhibitor", the inhibition is thought to be non-competitive against substrate (glucose-6-phosphate). Fig. 4 shows the inhibition rate by Domian on G6PD activity in different concentrations of enzyme on the one hand and at a fixed concentration ($3.75 \times 10^{-3}$M) of Domian on the other. A tendency to a decrease of the inhibition rate, in proportion with increase of enzyme concentration, was observed. This may mean that the inhibitory effect by Domian will be manifested under the deficient condition of G6PD.

**Experiment II:** — 10% suspensions of normal human erythrocytes in isotonic saline-phosphate buffer (pH 7.4) were incubated, with shaking of 70 cycles per minute, at 37°C for 30 minutes with or without addition of $7.50 \times 10^{-3}$M Domian. GSH content and G6PD activity in erythrocytes were determined before and after incubation. GSH content in erythrocytes was determined by the method of Gruenert and Phillips. In case of adding Domian, a remarkable decrease of G6PD activity and a milder but significant decrease of GSH
Inhibitory effect of Domian on G6PD (Experiment I)
Concentration of Domian: $3.75 \times 10^{-3}$M

Fig. 5. Inhibitory effect of Domian on G6PD in erythrocyte (Experiment II)

content in erythrocytes were observed (cf. Fig. 5). On the other hand, no significant differences of G6PD activity and of GSH content in erythrocytes were found in case of no Domian addition. These experimental results will give us
the impression that Domian might attack G6PD activity at the first step, resulting in decrease of GSH because of deficient TPNH.

*Experiment III:* \( 10^{-3} \text{M} \) GSH in phosphate buffer (pH 7.4) was incubated, with shaking of 70 cycles per minute, at 37°C with or without addition of Domian \((3.75\times10^{-3} \text{M} \text{ or } 7.50\times10^{-3} \text{M})\). At intervals of 0, 1/2, 1, 2 and 4 hours during incubation, the concentration of GSH was measured. The results (cf. Fig. 6) indicated that there was no significant difference of GSH concentration between an addition of Domian and no addition. This will mean that Domian has in vitro no effect on oxidation of GSH.

*Experiment IV:* \( 10^{-3} \text{M} \) TPNH or DPNH in phosphate buffer (pH 7.4) was incubated, with shaking of 70 cycles per minute, at 37°C with or without addition of Domian \((3.75\times10^{-3} \text{M} \text{ or } 7.50\times10^{-3} \text{M})\). At intervals of 0, 1/2, 1, 2 and 4 hours during incubation, the concentration of TPNH or DPNH was measured spectrophotometrically at 340 m\(\mu\). The results (cf. Fig. 7) showed that there was no significant difference of concentration of TPNH or DPNH between an addition of Domian and no addition. This will mean that Domian has in vitro no effect on oxidation of TPNH or DPNH.

*Experiment V:* Normal human erythrocytes, which had been washed three times with isotonic saline, were diluted with isotonic saline-phosphate buffer (pH 7.4) to make a 2% suspension. The suspension was incubated, with shaking of 70 cycles per minute, at 37°C for one hour with or without addition of \(4\times10^{-3} \text{M} \text{ Salyrgan}\). After incubation the suspensions were centrifuged and the hemo-
globin concentration in the supernatant was measured colorimetrically by oxyhemoglobin method. Salyrgan, in the concentrations used, was found to be without significant effect on the absorption spectrum of oxyhemoglobin. In the controls, an equimolar sodium chloride was used in place of Salyrgan. The results obtained are shown in Table II. A remarkable hemolysis during the incubation took place by addition of Salyrgan, while no hemolysis occurred in case of no addition of Salyrgan. The hemolysis caused by incubation with Salyrgan was completely protected by the previous addition of an equimolar concentration of GSH.

Table II. Hemolytic Effect by Salyrgan (Experiment V).

<table>
<thead>
<tr>
<th>No.</th>
<th>Salyrgan</th>
<th>Glutathione</th>
<th>Hemolysis %</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>(−)</td>
<td>(−)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4.0 × 10^{-3}M</td>
<td>(−)</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>(−)</td>
<td>4.0 × 10^{-2}M</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4.0 × 10^{-2}M</td>
<td>4.0 × 10^{-2}M</td>
<td>0</td>
</tr>
</tbody>
</table>

Complete hemolysate | 100

This hemolytic effect by organic mercury compounds and the protection of the hemolytic effect by GSH were observed by Benesh and Benesh, using sheep erythrocytes. The present author could demonstrate the same phenomenon in human erythrocytes. This experimental results—with Salyrgan that is
Hemolysis and Sulfa-drugs

known to high affinity for sulphydryl group in GSH—will indicate that GSH is essential for the maintenance of the intact erythrocyte structure.

Experiment VI:—The suspensions of normal human erythrocytes were prepared in the same way as in Experiment V. The incubation, with shaking of 70 cycles per minute at 37°C for two hours, was performed in four different conditions (Nos. 1, 2, 3 and 4) as will be seen from Table III. The grade of hemolysis was measured in the same way as in Experiment V. Sample in which an equimolar sodium chloride replaced the test substances served as the control (No. 1). A remarkable hemolysis was observed in No. 4, while no hemolysis took place in No. 3. These results will indicate that the erythrocytes previously incubated with Domian, which would, as has been shown in Experiment II (cf. Table II), bring about a decrease of G6PD activity and of GSH content, became so fragile as to lead to hemolysis. Although the cases of hemolytic anemia following the administration of sulfa-drugs have been reported10-12), it is not yet clear why a sulfa-drug should bring about hemolysis. Through the experimental results described in this paper, it was elucidated that sulfa-drugs have in vitro an inhibitory effect on G6PD activity in erythrocytes, thus resulting in decrease of GSH in erythrocytes. Since the maintenance of GSH is dependent on G6PD activity in erythrocytes,1), 13) it may follow that the decrease of G6PD activity will result in decrease of GSH in erythrocytes. In normal individuals, G6PD activity in erythrocytes may not be in vivo affected by an usual dosage of sulfa-drug, because enzymes such as G6PD are thought to exist in an ample amount in normal erythrocytes. However, when such individuals as deficient in G6PD in erythrocytes are exposed to sulfa-drugs, the inhibitory effect by the drugs on G6PD activity may be manifested, thus resulting in a further decrease of GSH and in aggravation of hemolysis.
SUMMARY

Basing upon the observations of hemolytic effect by the administration of Domian to the patients with erythrocytes deficient in G6PD and in GSH, a relationship between sulfa-drug and metabolism in erythrocytes was investigated. The experimental results obtained are as follows.

1) Domian has in vitro an inhibitory effect on G6PD activity but no effect on glutathione reductase activity.
2) A series of sulfa-drugs have in vitro a similar effect on G6PD activity as that by Domian.
3) Inhibition of G6PD activity by Domian may be noncompetitive against glucose-6-phosphate.
4) Inhibition rate of G6PD activity by Domian tends to decrease in proportion with increase of concentration of the enzyme.
5) Domian has in vitro no effect on oxidation of GSH.
6) Domian has in vitro no effect on oxidation of TPNH or DPNH.
7) The hemolysis of human erythrocyte caused by incubation with Salyrgan is completely protected by addition of an equimolar solution of GSH.

CONCLUSIONS

When such individuals as deficient in G6PD in erythrocytes are exposed to sulfa-drug, an inhibitory effect by the drug on G6PD in erythrocytes may be manifested by such a dosage of the drug that normal individuals are not affected. Then a block of G6PD activity in erythrocyte caused by the drug in those patients may result in a further decrease of GSH in erythrocytes due to deficiency of TPNH and in aggravation of hemolysis.

References