The Heinolytic Effect of Primaquine and Related Compounds: a Review

By ERNEST BEUTLER

IT HAS LONG BEEN KNOWN that when certain, ordinarily harmless drugs are administered to some individuals an acute hemolytic anemia results. An opportunity arose to study sensitivity to a drug-induced hemolytic anemia in greater detail than had previously been possible when the Army Malaria Research Unit at Stateville Penitentiary, Illinois, undertook to investigate the hemolytic effect of the antimalarial drug, primaquine [8-(4 amino 1-methylbutylamino)-6-methoxyquinoline].

Study of this hemolytic anemia revealed an entirely new type of intrinsic red cell abnormality, an enzyme deficiency which is apparently harmless unless the red cells are challenged in some way, most usually by administration of one of a large group of drugs. Relatively simple in vitro tests for the detection of this red cell abnormality have made possible extensive study of its biochemistry, genetics and distribution.

It is the purpose of this communication to review the investigations that have led to the elucidation of this red cell defect and the subsequent studies of the defect that have been carried out recently in many laboratories in the United States, Israel and Italy. A brief review of the earlier portions of the work of the Army malaria research project on primaquine sensitivity has been published in English.49 Some of the work has also been reviewed in Italian.78

HISTORICAL*

In 1926, when Mühlen7 first described the use of the 8-aminoquinoline, plasmochin (pamaquine) in the treatment of naturally acquired malaria, he stated “that plasmochin has no damaging effect on the red blood cells themselves is suggested to me not only by the significant increase in hemoglobin during treatment and the often rapid and significant regression of splenomegaly but also through its use in cases of blackwater fever...” This erroneous conclusion was soon to be corrected. It was in the same year that the first cases of acute hemolytic anemia caused by pamaquine were described by Cordes.32 In a preliminary report, he referred to 4 cases of acute hemolytic

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*See footnote on page 104.
anemia occurring in 72 Negro patients receiving pamaquine. One case was reported in some detail. These, and two additional cases in a series totaling 250 were presented in greater detail in subsequent publications. Blood smears from one of his patients were examined by Professor Victor Schilling, who observed marked oligocytosis, anisocytosis, and poikilocytosis, many normoblasts and some polychromatophilia. Dr. F. B. Mallory examined the organs of one patient who died and found, among other things, many red cells in the endothelial leukocytes of the spleen. In 1927, Eislerberg, Menk, Brosius and Manson-Bahr reported cases of acute hemolytic anemia during pamaquine administration. In the next few years many additional cases were reported. Roskott and Seno observed three cases and carried out osmotic fragility tests. The results were within the range of normal, except that in two of the patients, hemolysis did not begin until .38 per cent saline. No controls were reported. Of particular interest is a report by Palma of a probable case of mild hemolytic anemia occurring during pamaquine treatment, with the appearance of Heinz bodies in the red cells. Palma’s 1928 observations escaped our notice and that of most other investigators. We noted independently the appearance of this abnormality in the red cells of sensitive individuals receiving primaquine. This observation subsequently formed the basis for the first in vitro method for the detection of primaquine sensitivity.

Between 1930 and 1940 more reports of hemolysis caused by pamaquine were published. Ficacci reported a case of pamaquine hemolysis and found that the results of osmotic fragility tests and the Donath-Lansteiner test were normal and that bleeding and clotting times were normal. Immersing the patient in cold water did not elicit any further hemolysis. Readministration of pamaquine 26 days after the original exposure to the drug caused a milder hemolytic episode. Additional possible cases were reported by Blackie and Dixon. In 1939, Strauss reviewed some of the earlier literature and stated that he regarded hemolytic anemia as a rare toxic effect that was almost purely of theoretical interest.

Since 1940, there has been a resurgence of interest in pamaquine and the
newer 8-aminoquinolines due to the requirements of two wars. Several attempts were made to determine the mechanism of hemolysis. In 1943, Mann\(^85\) described the presence of a nonspecific hemagglutinin in the blood of one patient and conjectured, incorrectly, that abnormal plasma factors were responsible for hemolysis. It is of interest, in this respect, that Earle et al.\(^48\) later reported finding autoagglutinins in three patients receiving pamaquine; only one developed a hemolytic anemia. In 1945, Swantz and Bayliss\(^121\) found that the Donath-Landsteiner test gave negative results in a patient undergoing hemolysis caused by pamaquine. In 1946, Dimson and McMartin\(^46\) reported 25 cases of pamaquine-induced hemoglobinuria. They studied the intradermal response to pamaquine and also made some observations on its in vitro hemolytic activity. Readministration of pamaquine to their patients after 30 days caused recurrent hemolytic anemia. The most ambitious studies were made by Feldman et al.\(^52\) and by Earle et al.\(^48\). Feldman's group observed 2 cases of acute hemolytic anemia among 11 Negro patients given pamaquine. One of these patients was given the drug again after three months, and a second hemolytic episode was observed. Special studies were carried out in one patient during the hemolytic episode. Red cell fragility was normal, and the authors were unable to reproduce hemolysis in vitro by incubating the patient's cells with homologous plasma, with plasma from a patient who had recently received pamaquine, plasma to which varying amounts of pamaquine hydrochloride had been added, or by incubating the red cells from a control patient with the patient's plasma. Earle and his associates studied the blood of patients receiving pamaquine for mechanical and osmotic fragility of the red cells, isoagglutinins, hemolysins, cold hemagglutinins, and autoagglutinins, without detecting any difference between sensitive and nonsensitive subjects. They also noted that there was no correlation between plasma pamaquine levels and hemolysis. They concluded that “pamaquine or, more likely, one of its metabolic products, acts as a precipitating factor capable of producing hemolysis when certain predisposing factors are present.” They failed, however, to elucidate the “predisposing factors” except in that they noted the susceptibility of Negroes. Jones et al.\(^71\) examined the red cells of one primaquine-sensitive Negro for sickling and osmotic fragility and obtained normal results. Zylmann,\(^147\) Birnbaum et al.\(^17\) and Mer et al.\(^90\) studied the in vitro hemolytic effect of pamaquine without elucidating the mechanism of hemolysis. Hockwald et al.\(^69\) studied a number of patients sensitive to primaquine and established that these patients were also sensitive to pamaquine. They performed sickling and osmotic fragility tests, obtaining normal results. They found no correlation between methemoglobin formation and hemolysis. Further clinical reports, without any experimental studies, were also published.\(^19,30,31,65,73,81,132,137\) Turchetti\(^182\) called attention to the familial nature of pamaquine sensitivity. Gennis et al.\(^59\) observed marked anemia in one of two subjects receiving primaquine. They mistakenly concluded that the anemia was not caused by the drug because readministration of primaquine nine days later failed to cause recurrence. We now recognize that such temporary insensitivity to the hemolytic effect...
of primaquine is characteristic following a hemolytic episode in a sensitive subject (vide infra).

Racial Susceptibility to Hemolysis (Table 1)

A very great difference in the susceptibility of Caucasians and American Negroes to the hemolytic activity of primaquine has been demonstrated in the past few years. The difference is so striking that it is somewhat surprising that it had not been recognized more generally in the first twenty years of clinical use of these compounds. The nature of most reports of hemolytic anemia makes it impossible to draw any valid conclusions regarding sex incidence of sensitivity. Most of the cases reported were males, but since the reports often originated from military installations and plantations, there is no way of assessing the sex factor. The incidence by race and sex of positive in vitro tests for sensitivity is considered separately (table 2).

Caucasians: It was observed by Manifold,84 Amy3 and Smith,118 and more recently by Dimson and McMartin,46 that British troops were less susceptible to the hemolytic action of pamaquine than were Indian troops. The cause of this difference was not always appreciated. In the Proceedings of the Conference of Medical Specialists,51 the observation was made that all cases of hemolysis occurred in Indians and Burmese. This was attributed merely to the lower body weight of these individuals. Reports by Hawking,67 Monk93 and Dixon47 indicate that no hemolysis occurred respectively among 200, 160 and 600 British troops receiving pamaquine. Most et al.91 and Warthin et al.134 observed no hemolysis among 100 and 163 Caucasians receiving pamaquine, and Strauss and Gennis,129 no cases among 49 Caucasians given pentaquine. Clayman et al.29 reported no acute hemolytic anemia among 575 Caucasians receiving 30 to 240 mg. of primaquine daily. Gennis et al.59 observed no hemolysis among 99 carefully studied Caucasian subjects receiving 15 mg. of primaquine daily.

However, hemolysis in Caucasians other than Indians has been reported. Kliger and Reitler76 treated 364 Bedouins with pamaquine for three or more days. One case of hemoglobinuria and three cases of icterus were observed. Missiroli and Marino12 reported 10 possible cases of hemolytic anemia among 1043 inhabitants of Sardinia given pamaquine and quinine. Alving6 reported 2 acute hemolytic crises among 300 Caucasians receiving 60 mg. of pentaquine daily. No hemolysis was observed among 500 Caucasians receiving only 30 mg. daily. Loeb41 reported severe anemia in 1 of 171 Caucasians receiving pentaquine. Coatney et al.30 have reported 1 case of acute hemolysis occurring in a Jewish male receiving pentaquine, and Smith118 refers to cases of hemolysis in 2 Jews, 1 Greek, 1 white Rhodesian, as well as in 7 Indians. The anemia during pamaquine administration reported by Atchley et al.4 occurred in an Italian male. Motulsky96 has observed primaquine-induced hemolysis in an Italian patient.

American Negroes: Cordes35 observed 6 cases of hemolysis among 250 Negroes given pamaquine in 1928, and Menk80 suggested that Haitian Negroes could not tolerate as much pamaquine as other racial groups. However, the striking susceptibility of a relatively high proportion of American Negroes
to the hemolytic effect of these compounds was not noted until 1945 when Swantz and Bayliss\textsuperscript{12} reported that all 10 cases of hemolysis that they observed following pamaquine treatment were in Negroes, although of the 3,000 individuals receiving the drug most were Caucasians. Subsequently Earle et al.\textsuperscript{48} reported 6 acute hemolytic reactions among 76 "pigmented subjects." Five of these men were Negroes, one Chinese. Of 81 Caucasians, there was one "slowly developing hemolytic anemia." This striking difference between susceptibility of Caucasians and American Negroes was later confirmed in the case of primaquine by Hockwald et al.\textsuperscript{60} who observed 5 cases of acute hemolysis among 105 Negro volunteers given 30 mg. of primaquine daily.\textsuperscript{8} Jones et al.\textsuperscript{71} observed 1 case of hemolysis among 14 Negroes receiving primaquine. Of 199 consecutive Negro volunteers, to whom Dern et al.\textsuperscript{43} have administered 30 mg. of primaquine daily, 22, or approximately 11 per cent, developed acute hemolytic anemia.

\textbf{African Negroes}: There is considerably less available information regarding the incidence of sensitivity of African Negroes to the hemolytic effect of 8-aminoquinolines than is the case for American Negroes. Of particular interest is the report by Henderson\textsuperscript{8} who gave 160 Sudanese villagers 30 mg. of pamaquine daily for 14 days (or correspondingly lower doses for children), a dose sufficient to elicit severe hemolytic episodes in sensitive subjects. No "untoward symptoms" were observed. Rice\textsuperscript{104} noted no symptoms among 42 West African natives given 20 mg. of pamaquine daily. There are, however, isolated references to the occurrence of hemolytic anemia in African Negroes receiving pamaquine. Smith\textsuperscript{118} refers to cases in a Basuto and an East African native. Mann\textsuperscript{85} refers to one case occurring in a Bantu.

\textbf{Other Racial Groups}: Roskott and Seno\textsuperscript{105} observed hemolysis in 2 of 38 Chinese patients and in 1 of 9 East Indies natives given pamaquine. A case of probable primaquine hemolysis in a Javanese was reported by Baermann and Smits.\textsuperscript{5} Earle et al.\textsuperscript{48} and Keng\textsuperscript{73} have each observed hemolysis in a Chinese subject given pamaquine. Namikawa\textsuperscript{98} observed black-water fever-like symptoms among 3 of 25 subjects given pamaquine in Formosa. Thaeler et al.\textsuperscript{131} observed no hemolysis among 200 Nicaraguan Miskito Indians who received 15 to 20 mg. of primaquine daily. Although this dose is not sufficient to cause \textit{clinical} signs of hemolysis in many sensitive subjects,\textsuperscript{69} the repeated hematologic examinations that were performed would have been sufficient to permit detection of sensitivity. Brosius\textsuperscript{23} has reported hemoglobinuria in a Costa Rican patient.

\textsuperscript{*}In reviewing the records of Hockwald's patients it has been found that several of the 17 regarded originally as developing "mild anemia" actually underwent acute hemolytic reactions. These men were not recognized as sensitive in the original study because the change in hemoglobin level after 14 days of drug administration was used as an index of blood destruction. When subsequent studies demonstrated that the natural course of the hemolytic reaction was a self-limited one, a review of the original data revealed that several men had an early sharp fall in the hemoglobin level associated with dark urine, but that considerable recovery had occurred by the 14th day of drug administration. Readministration of the drug to these men revealed that they were "sensitive."
TABLE 1.—Racial Incidence of 8-aminoquinoline-induced Hemolytic Anemia

<table>
<thead>
<tr>
<th>Author</th>
<th>Drug</th>
<th>Race</th>
<th>No. of Subjects Surveyed</th>
<th>No. of Developing Hemolytic Anemia</th>
<th>Approximate % Developing Hemolytic Anemia</th>
</tr>
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<td>Corder et al.</td>
<td>Pamaquine</td>
<td>Negro</td>
<td>250</td>
<td>6</td>
<td>2</td>
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<td>Pamaquine</td>
<td>Negro (Sudanese villagers)</td>
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<td>0</td>
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<td>Pamaquine</td>
<td>Negro</td>
<td>11</td>
<td>2</td>
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<td>Pamaquine</td>
<td>“Pigmented subjects”</td>
<td>76</td>
<td>6</td>
<td>8</td>
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<td>Negro</td>
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<td>.3-1.1</td>
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<td></td>
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<td>(Bedouins)</td>
<td></td>
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<td>Caucasian (British Troops)</td>
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<td>.15*</td>
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<td>Caucasian (Indian Troops)</td>
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<td>.26*</td>
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<td>Caucasian (British Troops)</td>
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<td>Pamaquine</td>
<td>Caucasian (Sardinia)</td>
<td>1048</td>
<td>10</td>
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<td>22,000</td>
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<td>Caucasian</td>
<td>575</td>
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<td>Primaquine</td>
<td>Caucasian</td>
<td>99</td>
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<td>0</td>
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<td>Primaquine</td>
<td>Mongolian</td>
<td>38</td>
<td>2</td>
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<td></td>
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<td>(Chinese)</td>
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<td>Pamaquine</td>
<td>Mongolian</td>
<td>25</td>
<td>3</td>
<td>12</td>
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<tr>
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<td></td>
<td>(Formosan)</td>
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<td>Pamaquine</td>
<td>East Indies natives</td>
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<tr>
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<td>69</td>
<td>6</td>
<td>8.7</td>
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<tr>
<td></td>
<td></td>
<td>(Chinese)</td>
<td>72</td>
<td>8</td>
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*Frank jaundice.
†Frank hemoglobinuria.

CLINICAL COURSE OF THE HEMOLYTIC REACTION

When a primaquine-sensitive individual is given 30 mg. of primaquine daily, no evidence of hemolysis occurs until 2 or 3 days after the first dose of drug. In contrast to what might be expected in immunologic sensitivity, subsequent administration of primaquine does not shorten this latent period.

After two or three days the urine begins to darken. In mild cases the patient may observe no other abnormality. When hemolysis is more severe the patient feels weak and complains of abdominal and back pain, the sclerae become icteric and the urine may be nearly black. The hemoglobin, red blood count and hematocrit fall rapidly, and a reticulocytosis begins to develop. Heinz bodies appear in many of the red cells, but no alteration in fragility or shape of the cells is observed. The Coombs' test is negative. Even if primaquine administration is continued, however, this "acute hemolytic
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Phase” terminates spontaneously in about one week, and the “recovery phase” begins. The patient feels better, the color of the urine rapidly becomes normal, the hemoglobin, red count and hematocrit begin to rise, and, although the reticulocyte count remains high at first, it begins to decline. Red cells with Heinz bodies are no longer seen, and the Coombs’ test remains negative. Red cell fragility remains essentially unaltered. Finally, the peripheral blood picture returns entirely to normal and the patient is entirely asymptomatic in spite of continued administration of primaquine. This apparent tolerance to primaquine lasts for only a few weeks after withdrawal of the drug, however, since re-administration of primaquine always causes an acute hemolytic reaction. There is no tendency for successive attacks to become milder if the intervals between administration of drug are sufficiently great.

Although there is some variability in the intensity of the hemolytic response, sensitivity to 30 mg. of primaquine per day, as observed in healthy male volunteers, is essentially an all-or-none phenomenon. This is true both clinically and in regard to the survival of labeled erythrocytes. Red cell survival studies have been carried out on several subjects whose hemoglobin gradually declined approximately 2 Gm. per 100 cc. of blood during the administration of primaquine. There was no evidence whatsoever of accelerated red cell destruction in these studies. While the most extensive observations on the course of the hemolytic reaction have been carried out in patients with primaquine-induced hemolysis, the course of events is quite similar when drugs such as pamaquine, acetanilid, sulfanilamide or nitrofurantoin (Furadantin) are administered. When phenylhydrazine is given, however, the onset of hemolysis is more gradual and in favism, onset is more sudden than is the case with primaquine.

METHEMOGLOBINEMIA AND HEMOLYSIS

It is not our purpose to review the methemoglobin-forming properties of 8-aminoquinolines. Nevertheless there has been much confusion concerning the relationship of methemoglobin formation to hemolysis, and this subject deserves brief consideration. Cyanosis is mentioned as an outstanding toxic symptom in most of the reports dealing with the use of pamaquine and other 8-aminoquinolines. Sioli and Mühlen, in introducing pamaquine to clinical medicine, noted cyanosis but failed initially to recognize the fact that it was due to methemoglobinemia, assuming that it was due to circulatory disturbances. Fisher and Weise demonstrated that methemoglobin was the cause of the dusky color of patients who received pamaquine. Although pamaquine and other 8-aminoquinolines may cause hemolysis or methemoglobin formation, or both, this does not prove any causal relationship between the two. However, many authors have implicitly or explicitly assumed that such a relationship exists. For example, Goodman and Gillman, in reviewing the hemolytic action of phenylhydrazine, stated “Methemoglobin-containing cells apparently are more vulnerable to physiological stress and to destruction by the reticulo-endothelial system. The result is the disintegration of erythrocytes.” We know of no experimental support for such a view, and, on the

*This section has been eliminated from the most recent edition.
contrary, Clark and Morrisey28 have demonstrated that this is not the case in dogs. However, as recently as 1955, Wilcocks139 illustrated the confusion between methemoglobin formation and hemolysis by misquoting Hansen64 as having reported primaquine-induced hemolysis in a Caucasian, when actually the report was one of methemoglobinemia. The essentially independent nature of these toxic effects is evident from the following considerations:

1. The production of marked degrees of methemoglobinemia induced by the administration of sodium nitrite has failed to cause any hemolysis of primaquine-sensitive or nonsensitive red cells, although the amount of methemoglobin formed was greatly in excess of that formed by administration of 8-aminoquinolines.43

2. Administration of methylene blue, which has a marked influence in effecting the reduction of methemoglobin due to drugs,140 has failed to influence the course of hemolysis.14,69

3. Some potently hemolytic aniline derivatives to which these cells are sensitive, such as phenylhydrazine, cause little or no methemoglobin formation.43,54

4. Spectrophotometric measurement of methemoglobin formation in sensitive and nonsensitive Negroes and in Caucasians has indicated that methemoglobin formation is more marked in Caucasians,43 although hemolysis is rare, and that methemoglobin formation in sensitive Negroes is no greater than in nonsensitive Negroes.

**Primaquine Sensitivity as an Intrinsic Abnormality of the Erythrocyte**

The finding that some individuals develop an acute hemolytic anemia when exposed to primaquine could be explained either on the basis of the production of abnormal extracorpuscular factors developed in response to the administration of primaquine or as a manifestation of unique susceptibility of the erythrocyte. Studies by Dern, Weinstein et al.44,136 established in 1953 that sensitivity to primaquine was due to an intrinsic abnormality of the erythrocytes of susceptible subjects. The erythrocytes from sensitive individuals were labeled with Cr51 and transfused into nonsensitive recipients who were subsequently given primaquine (fig. 1). Conversely, Cr51-labeled red cells from nonsensitive individuals were transfused into sensitive individuals, and primaquine was given to the recipients (fig. 2). These studies localized the defect of primaquine sensitivity to the erythrocyte. They established that sensitivity to primaquine was not due to abnormal degradation of the drug or development of abnormal immune mechanisms by sensitive individuals.

**The Nature of the Red Cell Defect**

Having demonstrated that sensitivity to primaquine was due to an intrinsic defect of erythrocytes, studies were carried out to determine whether this defect was identical with or a variant of defects such as the abnormal hemoglobin syndromes, thalassemia, paroxysmal nocturnal hemoglobinuria or congenital hemolytic anemia, or whether it represented a previously undescribed red cell abnormality. The latter alternative proved to be correct. Sensitive and nonsensitive red cells were compared by a variety of techniques, including hemoglobin electrophoresis, mechanical and osmotic fragility before and during in vivo exposure to primaquine and other agents, and saponin and taurocholate hemolysis with and without acceleration with naph-
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Fig. 1.—Chromium\(^\text{51}\)-labelled red cell survival studies carried out with red cells from a primaquine-sensitive donor. The sensitive red cells were transfused into four recipients. One (open circles) was given no drug. The others were given 30 mg. of primaquine daily. Rapid destruction of primaquine-sensitive red cells in recipients receiving primaquine is noted. (From Dern et al.,\(^4\) courtesy J. Lab. & Clin. Med.)

Thalene and primaquine. The methemoglobin content of sensitive cells was measured. The antigenic pattern was determined, and a test for susceptibility to acid hemolysis was carried out. No difference could be demonstrated between sensitive and nonsensitive cells by any of these means.\(^1\) It was possible to demonstrate, however, that when incubated in the presence of oxygen with a variety of substances, including acetylphenylhydrazine, hydroxylamine, phenylhydrazine and ascorbic acid, sensitive cells developed a consistently different pattern of Heinz body (denatured protein particles\(^\text{135}\)) formation than did nonsensitive cells.\(^\text{13}\) The manner in which these compounds, which are reducing substances, cause oxidation of red cell components is not clear. It is possible that they may first become oxidized in vivo and then cause oxidation of the red cell component; or, alternatively, that they may undergo coupled oxidation with hemoglobin. Details of a test procedure utilizing this difference in pattern of Heinz body formation, which makes possible reliable differentiation of sensitive from nonsensitive individuals, have been published by Beutler et al.\(^\text{13}\) Five or more Heinz bodies were found invariably
in more than 40 per cent of the erythrocytes of sensitive subjects after heparinized blood had been incubated in a phosphate buffer containing glucose and acetylphenylhydrazine. With few exceptions, less than 30 per cent of red cells from nonsensitive subjects contained 5 or more Heinz bodies under identical conditions (fig. 3). These findings were later confirmed by Kimbro et al.,74 who studied cells from patients sensitive to nitrofurantoin, by Sansone,107 studying cells from subjects with naphthalene sensitivity and by Larrizza et al.,78 studying the red cells of favism. On the other hand, Josephson et al.72 state in a recent paper that they have been unable to confirm the finding that most red cells from normal subjects form only one or two Heinz bodies when incubated with acetylphenylhydrazine, as in this
Fig. 3.—Heinz body formation in sensitive (upper field) and nonsensitive (lower field) erythrocytes after incubation with 100 mg.% buffered acetylphenylhydrazine for 4 hours as described by Beutler et al. Wet preparations stained with crystal violet. Magnification x 1300. (From Beutler et al., J. Lab. & Clin. Med.)

test. Their failure to do so is probably due to their use of oxalate as the anticoagulant instead of heparin as originally described and to the fact that no glucose was included in the incubation medium. The test is quite sensitive to changes in oxygen tension and to changes in the hematocrit and has
therefore been found to be somewhat less satisfactory under field conditions than when carried out on healthy volunteers. Flanagan et al. have proposed correction of the hematocrit to normal values prior to performance of the test.

Biochemical examinations of primaquine-sensitive erythrocytes suggested that primaquine sensitivity is not caused by a defect in glycolysis, in catalase activity, in carbonic anhydrase or in cholinesterase of sensitive cells. However, Beutler et al. demonstrated that the reduced glutathione content of sensitive erythrocytes tended to be below normal. Furthermore, poisoning of nonsensitive cells with iodoacetate or arsenite (which binds sulfhydryl groups) caused them to react like sensitive cells in vitro with respect to Heinz body formation.

Szeinberg and Chari-Bitrou have reported that sensitive cells also have a decreased content of oxidized glutathione (GSSG), but their findings have been disputed by Flanagan et al. because of the relatively nonspecific method (iodometric titration) that they employed. Flanagan and his associates found the GSSG of sensitive cells to be normal. Larizza et al., using iodometric titration for the determination of GSSG, report decreased GSSG during and shortly following hemolytic crises in subjects with favism.

Beutler reported that when red cells from primaquine-sensitive men were incubated with acetylphenylhydrazine, there was a marked fall in the GSH level (fig. 4). No such fall occurred in normal cells. Similar destruction of GSH was observed when primaquine, phenylhydrazine, ascorbic acid, analine and hydroxylamine were used. Subsequently, similar results have been reported by others using nitrofurantoin, α and β naphthol, α and β naphthoquinone and certain vitamin K derivatives. Naphthalene has been ineffective.

The finding that the GSH of sensitive red cells is uniquely sensitive to destruction forms the basis for a means of differentiating sensitive from nonsensitive cells in vitro, the “glutathione stability test.” When originally applied by Beutler to the red cells of 5 sensitive and 7 nonsensitive male subjects, the GSH of all of the nonsensitive subjects remained above 44 mg.%, while that of the sensitive subjects fell to less than 12 mg.%. In its first application to a random population, this test was carried out on the blood of 159 subjects, 127 Negroes and 32 Caucasians. Two distinct responses were seen. All of the samples from Caucasian donors and 121 of the 127 samples from Negro donors were found to have stable GSH, the GSH concentration after incubation with acetylphenylhydrazine being greater than 35 mg.% in each of these cases. In 6 subjects, all Negro, the GSH was found to be unstable, the final GSH concentration falling below 16 mg.% in each case.

Flanagan et al. have proposed certain modifications in the means of de-

*It was pointed out that the incidence of positive tests was lower than the known incidence of primaquine sensitivity. It was proposed that this might be due to a high degree of racial mixture in the sample. A review of these cases revealed that several allegedly Negro subjects, whose blood had been obtained from another hospital, were actually Caucasian. Appropriate corrections are made in the data presented in table 2.
Fig. 4.—The GSH content of red blood cells from seven nonsensitive and five sensitive male subjects before and after incubation with 5 mg. acetylphenylhydrazine per ml. (From Beutler,4 courtesy J. Lab. & Clin. Med.)

termining GSH. While these modifications may well increase the precision of the GSH estimation, other investigators27,83,74,78,88,90,100,122,143 have found the original method to be satisfactory for the purposes of the GSH stability test, and we have continued to use it without modification in our own laboratory. The essentially bimodal response of red cell GSH to incubation with acetylphenylhydrazine in random populations has been confirmed by several investigators.27,122 In addition, it has been found that the female relatives of subjects whose red cells have unstable GSH sometimes display an intermediate degree of instability.27,63,78,78,99,122 We have also observed one such case in our laboratory.7 Alving et al.2 have presented data which indicate that this test is less reliable in predicting sensitivity to hemolysis in females than in males. Some females with normal or intermediate GSH values after incubation with acetylphenylhydrazine were found to have red cells which hemolyzed in vivo when exposed to primaquine, while some had red cells which did not. Szeinberg et al. have detected some cases in which the results of the test were made more clear when glucose was added, and these authors recommend the routine addition of glucose (final concentration, 400 mg%) to each
tube. While we have not encountered an instance in which a fresh blood sample from an adult required addition of glucose, the addition of glucose does not affect the GSH stability of red cells from sensitive subjects, and the suggestion is probably a sound one. We now use acid-citrate-dextrose (ACD) solution as anticoagulant, rather than heparin. This makes unnecessary the concern about the glucose level of the blood and obviates the necessity for performing the test within a short time after the blood samples are drawn.

Extensive studies using the GSH stability test have been carried out in infants. It has been found that the GSH concentration of cord blood of both "sensitive" and "nonsensitive" infants is somewhat elevated, and in the case of nonsensitive infants, it is stable to acetyl phenylhydrazine. At the age of 30 to 75 hours, however, the red cell GSH of all infants becomes unstable, in spite of the fact that the concentration of the enzymes glucose-6-phosphate dehydrogenase (G-6-P.D.) and glutathione reductase are not diminished. In nonsensitive infants, this instability can be corrected with glucose or inosine. The effect is apparently due to low blood sugar levels and extraordinarily rapid utilization of glucose by the infant's red cells, which is accentuated in the presence of acetylphenylhydrazine. Zinkham has shown that a marked decrease in glucose level precedes the fall in GSH. In sensitive infants, correction of GSH instability with glucose or inosine cannot be made. G-6-P.D. deficiency has also been detected in cord blood obtained from sensitive infants. Red cells from eight-day-old nonsensitive infants are again able to protect their GSH against acetylphenylhydrazine.

The GSH stability test has been found to be normal in subjects with acute leukemia, polycythemia vera, myeloid metaplasia, symptomatic acquired hemolytic anemia, disseminated lupus erythematosus, reticulum cell sarcoma, lymphosarcoma, macroglobulinemia of Waldenström, diabetes mellitus, hyperthyroidism, leprosy, iron-deficiency anemia, galactosemia, erythroblastosis fetalis, glycogen storage disease, plumbism, mesantoin-induced agranulocytosis after splenectomy and in women in labor. Positive tests have been obtained in nonspherocytic congenital hemolytic anemia.

Heparinized blood stored as long as four but not for seven days at 4 C. will give satisfactory results with the GSH stability test if the blood is incubated with .05 parts of 10 per cent glucose solution for one-half hour before initiation of the test. Studies of banked blood in ACD solution indicated that the results of the GSH stability test are valid if the blood has been stored for three or four weeks.

The GSH stability test has provided a means for further in vitro studies of the mechanism of GSH destruction and protection in red cells. An increase in the oxidized glutathione of sensitive red cells was found to occur

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*The terms "sensitive" and "nonsensitive" will be used in the remainder of this paper to refer to the presence or absence of the red cell defect which makes the subject drug-sensitive, as demonstrated by in vitro test, whether or not the subject or his red cells have been exposed to drug in vivo.
as the GSH disappeared. Zinkham\textsuperscript{143} has demonstrated that when the GSH of normal infant red cells fell, following depletion of the blood sugar, addition of glucose subsequently resulted in a rise in GSH. Beutler et al.\textsuperscript{8,15} demonstrated that destruction of the GSH of sensitive cells incubated with acetyl phenylhydrazine occurred only when the red cell suspension was oxygenated. When carbon dioxide or carbon monoxide was passed through the red cell suspension prior to incubation with acetyl phenylhydrazine, marked or complete inhibition of the destruction of GSH was observed. Incubation of GSH with acetylphenylhydrazine alone resulted in no fall in the GSH level, but if a solution of oxyhemoglobin was added to the system in moderately high concentrations, rapid destruction of GSH took place. Carboxyhemoglobin, however, was inert in this respect.\textsuperscript{15} These findings led Beutler et al.\textsuperscript{15} to suggest that the destruction of GSH in sensitive red cells might also be mediated through the action of acetylphenylhydrazine on oxyhemoglobin. Hemoglobin solutions prepared from red cells which had been incubated with acetylphenylhydrazine were shown to destroy GSH. Their activity was not modified by carbon monoxide. The active compound has not been identified.\textsuperscript{15} Recent description of the enzyme GSH peroxidase in red cells by Mills\textsuperscript{9} suggests that this enzyme could mediate the oxidative destruction of GSH by a hemoglobin-peroxide compound.

Beutler et al.\textsuperscript{15} demonstrated that washed red cells suspended in a saline-phosphate buffer did not protect their GSH against the effect of acetylphenylhydrazine. When glucose was present in the system, however, red cell GSH was again protected. Inosine had a similar effect, but ribose, pyruvate, fumarate, maleate and lactate were ineffective substrates for GSH protection (fig. 5). Szeinberg et al.\textsuperscript{130} have confirmed the necessity for the presence of glucose for protecting red cell GSH against acetylphenylhydrazine. Studies by Beutler et al.\textsuperscript{15} of the capacity of hemolysates incubated with acetylphenylhydrazine to protect their GSH in the presence of various substrates revealed no consistent differences between the red cells of sensitive and nonsensitive individuals. Glucose was an ineffective substrate, but substances such as glucose-6-phosphate, fructose-1, 6-diphosphate, ribose-5-phosphate or inosine were effective in protecting GSH against destruction in hemolysates from both sensitive and nonsensitive cells. However, Carson et al.\textsuperscript{24} in an attempt to explain the abnormalities of GSH levels in drug-sensitive cells, used a more dilute system and succeeded in demonstrating that hemolysates from sensitive subjects could reduce GSSG when either TPNH or phosphogluconate was present, but that when glucose-6-phosphate was used as the substrate, GSSG reduction was markedly impaired. These important findings indicated that a deficiency in glucose-6-phosphate dehydrogenase (G-6-P.D.) existed in the sensitive cells. This deficiency was best demonstrated when the hemolysate was permitted to incubate with a nondialyzable stromal factor present in normal or sensitive cells.\textsuperscript{25} Several groups of investigators,\textsuperscript{40,63,78,90,111,107,127,129,142} using different assay procedures, have confirmed that G-6-P.D. is decreased considerably in red cells with positive GSH stability tests, in red cells from subjects or their families with drug-induced hemolytic anemias and in subjects with favism. In addition, Waller et al.\textsuperscript{133} misinterpreting Carson’s findings,
have reported total absence of G-6-P.D. in the red cells of an Iranian patient with intermittent jaundice. Although their patient was regarded as having an active hemolytic anemia by the authors, this seems quite unlikely in view of the clinical and laboratory findings presented. Newton and Bass\textsuperscript{99} have reported GSH instability, a total absence of glucose-6-phosphate dehydrogenase activity and positive in vitro tests, using Heinz bodies, in the blood of three Caucasian children with nonspherocytic congenital hemolytic anemia. These results have been confirmed in three other patients by Zinkham.\textsuperscript{144}

According to Szeinberg et al.\textsuperscript{128} storage of blood for 30 days in ACD solution does not affect appreciably the G-6-P.D. content of either sensitive or nonsensitive cells. It has been shown that cells with deficient glucose-6-phosphate dehydrogenase produce decreased quantities of CO\textsubscript{2} from the 1-carbon of glucose,\textsuperscript{70} and that methemoglobin reduction in the presence of methylene blue\textsuperscript{40} is impaired in these cells. LeRoy et al.\textsuperscript{80} have found decreased...
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utilization of the 1-carbon of glucose administrated intravenously to two sensitive subjects.

Studies of other enzyme systems in primaquine-sensitive cells have also been carried out. Although the original report by Carson et al. suggested that GSH reductase activity might be normal in the red cells of sensitive individuals, subsequent more quantitative studies by Schrier et al. showed that a slight increase in the activity of this enzyme was actually present. Similarly, the activity of aldolase has been reported to be increased in primaquine-sensitive cells. Studies of other enzyme systems in primaquine-sensitive cells have also been carried out. Although the original report by Carson et al. suggested that GSH reductase activity might be normal in the red cells of sensitive individuals, subsequent more quantitative studies by Schrier et al. showed that a slight increase in the activity of this enzyme was actually present. Similarly, the activity of aldolase has been reported to be increased in primaquine-sensitive cells.

It has been pointed out by Beutler et al. that the GSH instability of drug-sensitive cells is probably a result of their deficiency in G-6-P-D. Figure 6 illustrates the expected effect of this enzymatic defect in the metabolism of the red cell. It is apparent that the defect in glucose-6-phosphate dehydrogenase would result in impaired reduction of TPN (tri-phosphopyridine nucleotide). This, in turn, would impair reduction of GSH, which is generally
believed to occur in a TPN-linked system, although evidence has been presented recently that in a hemolysate, di-phosphopyridine nucleotide may substitute for TPN.

On the other hand, it should be evident that GSH stability could also be impaired as a result of other metabolic lesions, such as a decrease in GSH reductase activity or defects in the phosphorylation and/or transport of glucose. Thus, the result of the GSH stability test and assay of glucose-6-phosphate dehydrogenase activity do not have identical meanings. In point of fact, where both have been carried out on the red cells of a single individual, the correlation is quite good, although dissociation of results has been observed. Infants with unstable red cell GSH, as was discussed above, may have normal or elevated red cell G-6-P.D. Other, less well explained, discrepancies have also been observed. A few subjects with normal enzyme levels have been found to have unstable GSH, and a few subjects with decreased enzyme level have been found to have normal or only slightly reduced GSH stability.

Kinetics of the Hemolytic Reaction

Studies were made to determine why “tolerance” to primaquine develops when the drug is administered to sensitive individuals for several weeks. By measuring the survival of Cr-labeled red cell populations, Dern et al. determined that (1) a sensitive individual who had developed such “tolerance” to primaquine retains an undiminished capacity to destroy transfused sensitive red cells and (2) the red cells of a sensitive individual who had developed such “tolerance” were not sensitive to primaquine, even in a recipient who had never previously been given the drug. “Tolerance” is therefore not due to altered metabolism of primaquine. Rather, it appears that the red cell population of a sensitive individual is heterogeneous, that about one-half is sensitive and one-half insensitive to the hemolytic action of primaquine.

That this is actually the case was demonstrated by labeling a segment of the red cell population of narrow age range with Fe. Beutler et al. demonstrated that young red blood cells are insensitive to the hemolytic action of primaquine, while older ones are highly sensitive (fig. 7).

Based on this finding, the events observed when primaquine is administered to a sensitive individual for a prolonged period of time may be explained readily. When primaquine is first administered, the older half of the red cell population is sensitive. This half is destroyed rapidly over a period of about one week (the “acute hemolytic phase” of Dern et al.). At the end of this time, only the younger, insensitive cells remain. As these age, they are destroyed gradually, but for about 60 days at a rate not exceeding the normal rate of red cell destruction. The new cells produced by the bone marrow to replace those destroyed in the acute hemolytic phase are insensitive until they, too, are 60 days old. If primaquine administration is continued for over 60 days, these cells would be expected to age sufficiently to become sensitive and to be destroyed also through the action of primaquine. Since they have been produced over a period of several weeks, however, only a small proportion becomes susceptible to destruction daily, and this would be a clinically imperceptible event.
The biochemical changes that occur in red cells during primaquine administration to sensitive and nonsensitive subjects have been investigated by Flanagan and associates.\textsuperscript{56,57} They found that there was rapid destruction of red cell GSH during the first few days of drug administration, but that while the major portion of hemolysis occurred, a rise in the GSH occurred. This may indicate that GSH is destroyed in the susceptible cells and that the GSH-depleted cells are then destroyed.\textsuperscript{15} During the fall in GSH, no rise in GSSG was observed, even when blood samples were examined every two to four hours.\textsuperscript{57} Similar observations have been made by Larizza et al.\textsuperscript{78} in favism. This is in contrast with our findings in vitro, in which a transient rise in GSSG was seen.\textsuperscript{15} Flanagan et al.\textsuperscript{57} conclude that the failure of GSSG levels to rise perceptibly means that GSH is not converted to GSSG during drug administration. This conclusion does not seem entirely warranted since it is possible that the size of the GSSG pool in erythrocytes is strictly limited and that GSSG destruction occurs whenever new GSSG is formed from GSH. However, it does raise a question as to what is the final fate of GSH of sensitive cells. This problem requires further study. Flanagan et al.\textsuperscript{57} report the results of glutathione stability tests carried out on three normal and four sensitive...
subjects, before, during and after primaquine administration. It is concluded that no significant change in GSH stability occurred. Nonetheless, there was a slight increase in GSH stability in all four sensitive and in two of the three nonsensitive subjects. However, Szeinberg et al.\textsuperscript{122} and Larizza et al.\textsuperscript{78} have reported transient loss of GSH instability after fava bean-induced hemolysis. Dawson et al.\textsuperscript{49} have also observed relatively stable GSH immediately following naphthalene-induced hemolysis, with instability observed several months later. It is likely that this discrepancy is due to the fact that the hemolytic episodes induced by fava beans or naphthalene are more severe than those observed when primaquine is given.

The data presented by Flanagan et al. reveal that the in vitro test for sensitivity, using Heinz bodies, remained positive even after the drug had been administered for a sufficiently long period of time so that the remaining red cells were no longer sensitive. Glucose-6-phosphate dehydrogenase levels remained unchanged in normal individuals. In sensitive individuals, a slight increase in enzyme activity was observed with reticulocytosis, but G-6-P.D. activity soon returned to the original low levels, even while the patient remained refractory to further hemolysis.\textsuperscript{57} Similar observations have been reported by Larizza et al.\textsuperscript{78} in favism. While these observations do not support the concept that young cells are more resistant to hemolysis because they contain more G-6-P.D. than do older cells, such a supposition is supported by the report by Johnson and Marks\textsuperscript{90} that G-6-P.D. activity is higher in young than in older cells and that the difference is particularly large in primaquine-sensitive cells. The assay procedure used by Marks and by Flanagan et al. differ; this may explain the discrepancy. Flanagan has measured the rate of reduction of GSSG, while Marks\textsuperscript{87} has measured directly the reduction of TPN. If a higher glucose-6-phosphate dehydrogenase level in young cells is not the cause of their resistance to hemolysis, the explanation may be found in some other pathway, more available in young than in old cells. It may enable the young cells to resist damage, even when glucose-6-phosphate dehydrogenase activity is reduced. Thus one might draw an analogy between a sensitive red cell and a leaky boat equipped with a pump. The leak (G-6-P.D. deficiency) may not enlarge in size, but if the pump (compensatory mechanism) wears out, the boat will sink.

The Relationship of Primaquine Sensitivity to the Hemolytic Effect of Other Compounds

A. Aminoquinolines

It was recognized by Hockwald and co-workers\textsuperscript{80} that individuals sensitive to primaquine are also sensitive to pamaquine. These observations have been extended to other aminoquinoline derivatives to determine the portion of the molecule that is responsible for the hemolytic properties of these compounds. The investigations have been limited necessarily by the relatively small number of aminoquinoline derivatives that may be administered safely to man. It has been possible to conclude, however, that hemolytic potency is influenced by not just one but rather by a considerable number of structural alterations. The hemolytic potencies of five 6-methoxy 8-amino-quinolines, differing only
in the structure of the side chain at the 8-amino position, have been found to differ greatly. 4-Amino-1-methylbutylamino, 4-diethylamino-1-methylbutylamino and 5-isopropylaminoamylamino side chains, forming primaquine, pamaquine and pentaquine, respectively, formed compounds highly hemolytic in doses of 30 mg. daily. A 4-aminobutylamino or 2-amino-1-methyllethylamino side chain, forming S.N. 3883 and C.N. 1110, respectively, formed compounds requiring three to six times the dose of primaquine to cause comparable hemolysis.\textsuperscript{10,43}

Alteration of the 6-position substituent in the quinoline nucleus markedly influences hemolytic potency. Pentaquine caused considerable hemolysis when 60 mg./day was administered. S.N. 15,324, identical to pentaquine except for the fact that in the latter a hydroxy group has replaced the methoxy group in the 6-position of the quinoline nucleus, was less hemolytic. If, instead of the 6-methoxy group, a 7-methyl group was present on the quinoline nucleus, the resulting compound (S.N. 15,305) had almost no hemolytic potency, even when 500 mg. per day was given. S.N. 3294, the 4-amino isomer of pamaquine, was nonhemolytic as was chloroquine, another 4-amino quinoline.\textsuperscript{10,43}

B. Other aniline derivatives

Extension of these studies to aniline derivatives related less closely to primaquine (fig. 8) demonstrated that primaquine-sensitive cells are also uniquely sensitive to the daily administration of 3.6 Gm. sulfanilamide, 3.6 Gm. acetanilid, 30 mg. phenylhydrazine, 300 mg. sulfoxone (Diasone) and 3.6 Gm. Phenacetin, 5.0 Gm. N\textsubscript{4} acetyl sulfanilimide, 5.0 Gm. sulfacetamide and 6.0 Gm. thiazolsulfone, \textit{but not} to 30 mg. aniline, 100 mg. para-aminophenol, 3.6 Gm. para-hydroxyacetanilid (Tralgon), 8.0 Gm. para-aminobenzoic acid, 3.6 Gm. acetylsalicylic acid, 2.0 Gm. chloramphenicol, 300 mg. diphenylhydramine (Benadryl), 400 mg. Antistine, 3.0 Gm. Pronestyl, 5 Gm. Sulfadiazine, sulfamerizine, or sulfathiazole or 300 mg. of tripelemamine (Pyribenazine). The more distantly related compounds, quinine and Daraprim, were not hemolytic.\textsuperscript{10,42,43} Ascorbic acid, which induces in vitro Heinz body formation and which destroys GSH in sensitive cells in vitro,\textsuperscript{8} was not hemolytic.\textsuperscript{43} None of these compounds caused significant acute hemolysis in nonsensitive individuals in the dosage indicated. The hemolytic reaction that was induced by the compounds tested in sensitive volunteers was very similar clinically to that occurring when primaquine was given to these individuals, including the spontaneous recovery that occurs during continued administration of the drugs. Furthermore, when primaquine was administered to these volunteers after they had reached the “recovery phase,” no further hemolysis occurred.\textsuperscript{42} These observations indicate that primaquine is merely one member of a large group of compounds that elicits a hemolytic reaction in sensitive individuals. All the compounds tested that were found to be hemolytic were aromatic amino compounds.

With the availability of in vitro means for the detection of drug sensitivity it has become possible for physicians to test blood of patients with a history of drug-induced hemolytic anemia to determine whether glutathione instability or G-6-P.D. deficiency is present. Kimbro and associates have demon-
strated glutathione instability of the red cells of two subjects who had developed a hemolytic anemia following the administration of nitrofurantoin (Furadantin). They also found glutathione instability of red cells in subjects who developed hemolytic anemia when exposed to antipyrine, phenacetin and probenecid. A patient with gantrisin-induced pancytopenia did not have the red cell defect.74 West and Zimmerman75 have reported a case of Furadantin-induced hemolysis in a Negro subject which was quite typical of the type of hemolysis observed in individuals with primaquine-sensitive cells. However, no studies have been carried out on red cells of this individual.74 Naphthalene, which has been known for many years to cause a hemolytic anemia, particularly in Negro children who have ingested naphtha-
lene moth balls, has also been shown to be among the compounds which will cause hemolysis in sensitive individuals. Zinkham and Childs found positive glutathione stability tests in the erythrocytes of four Negro patients with this type of hemolytic anemia. These results were confirmed by Zuelzer, Gross et al., McGovern et al., Dawson et al. and Sansone. G-6-P.D. was found to be decreased markedly in the red cells of these patients. Dawson et al. have observed hemolytic anemia in subjects with a positive glutathione stability test after the ingestion of Superanhist and possibly after Orinase. It has also been suggested that the hemolytic effect of vitamin K derivatives on newborns may be related to the red cell defect of primaquine sensitivity or to the GSH instability which occurs in normal newborns.

In 1948, Turchetti called attention to certain superficial similarities between favism and plasmoquine-induced hemolytic anemia. In 1956, Crosby suggested carrying out the newly developed in vitro tests for primaquine sensitivity on red cells from subjects with a history of favism. The independent findings of Sansone and Segni and Szeinberg et al. that the glutathione content of red cells of patients with a history of favism was low gave the first experimental evidence linking these disorders. Subsequently these authors have applied the glutathione stability test to the red cells of subjects with a history of favism and have always obtained positive results; they have also assayed the red cells for G-6-P.D. and found a marked decrease in the concentration of this enzyme. The results have been confirmed by Zinkham et al., Panizon and Pujatti, Gross et al. and Larizza et al. Carcassi et al. also found the GSH of red cells from subjects with a history of favism to be low, but because their results did not reach an adequate level of statistical significance, they concluded that this difference was not real. Such a conclusion is clearly unwarranted. Further similarity to primaquine-induced hemolysis is the fall of GSH observed in the early phase of the clinical episode, the similar hereditary pattern and the susceptibility of the red cells to in vitro Heinz body formation. Thus it would appear that subjects susceptible to the hemolytic effect of the fava bean have an erythrocytic defect that is identical with that of subjects who are sensitive to the hemolytic effect of primaquine. Final proof of this has been obtained by Larriza et al. who produced a typical hemolytic episode with primaquine in a subject with a history of favism. It is probable that other factors, possibly immunologic, are also involved in the response to fava bean that eventuates in hemolysis. Indeed, Szeinberg et al. have referred to several instances in which subjects known to have this red cell defect have eaten fava beans with impunity.

Aside from susceptibility to drugs and fava beans, there is reason to suppose that primaquine-sensitive cells may hemolyze more readily under the stress of infection. We have observed a severe hemolytic anemia in a subject with this red cell defect who developed Clostridium welchii septicemia following a septic abortion. Szeinberg et al. have called attention to the fact that the incidence of hemolytic anemia following influenza is much higher in Sephardic Jews, who have a high incidence of this defect, than in Ashkenazy Jews, who do not have this defect.
GENETICS AND INCIDENCE OF THE DEFECT

It was assumed early in the study of primaquine sensitivity that a red cell abnormality with marked difference in racial incidence would be genetically transmitted. Turchetti had emphasized the familial nature of primaquine sensitivity. The availability of in vitro methods for the detection of this abnormality has made possible extensive studies of its genetic transmission. Sansone and Segni, Szeinberg et al., Gross et al., Alving et al., Dawson et al., Larizza et al. and Beutler have all observed that the incidence of the red cell defect is greater in families of its carriers than in the population-at-large. However, the most definitive studies of the genetic transmission of the defect have been done by Childs, Browne et al. These investigators have applied the glutathione stability test to an impressive number of families (fig. 9). They found that while male relatives could be divided clearly into reactor and nonreactor groups, the distribution of post-incubation glutathione values among family females was continuous. It appeared that an intermediate group was present. This group has also been detected by Sansone and Segni, Szeinberg et al., Larizza et al.

Fig. 9.—Pedigrees of families in which members were tested by the glutathione stability test. Black circles and squares are female and male reactors. The partially black circles are females whose red cells gave an intermediate reaction with the glutathione stability test. Those marked with an X are members who were not tested. (From Childs et al., courtesy Bull. Johns Hopkins Hosp.)
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and Gross et al.\textsuperscript{63} who used both the GSH stability test and the assay for G-6-P.D. Gross et al.\textsuperscript{63} for example, found that 23 of 24 subjects with G-6-P.D. values in the intermediate range were women. Because the affected individuals appeared in as many as three generations, it was suggested by Childs et al.\textsuperscript{27} that the gene was dominant rather than recessive. It was evident from the findings that there were less “reactor” females than males, that intermediate degrees of glutathione stabilities were present in females but not in males and that this could not be simple autosomal dominance. It was apparent that the gene was either a sex-limited autosomal gene or a sex-linked gene. It was found that of 18 fathers and 25 mothers upon whom the glutathione stability test had been done and who were randomly detected, only 4 fathers but 17 mothers indicated some evidence of the trait.\textsuperscript{27} Similar results have been reported by Larizza\textsuperscript{78} and Gross et al.\textsuperscript{63} This peculiar distribution is much more consistent with a sex-linked gene than with an autosomal sex-limited gene. From Childs’ studies it would therefore appear that the gene for this defect is sex-linked with variable expressivity. The male hemizygote and the female homozygote would be expected to cause marked glutathione instability, while the female heterozygote might show all degrees of expression from marked loss of glutathione stability to apparent complete normality of the red cells. That the latter type of heterozygote does occur is suggested by the observation\textsuperscript{27,63,126} that some subjects with abnormal glutathione stability (always males) have normal parents. That the heterozygous female may show marked loss of G-6-P.D. activity is demonstrated by the observation of Gross et al.,\textsuperscript{63} that one such mother had four normal sons and that some female offspring with markedly reduced enzyme activity had normal fathers. Studies by other investigators tend to support this mode of inheritance. Alving\textsuperscript{2} has pointed out that actual in vivo hemolysis of red cells in a recipient is a different phenotype than that given by in vitro testing of the red cells. Using this method, he reports an approximately equal number of positive reactors among males and females. Such a finding is, of course, not incompatible with the mode of inheritance proposed by Browne, Childs et al.\textsuperscript{22,27} on the basis of the GSH stability test, if the two times higher incidence of the gene expected in the female were equally balanced by lower penetrance. While the results obtained by Childs et al.,\textsuperscript{27} using the GSH stability test, are quite convincing, further genetic studies using this test, an assay for G-6-P.D. and possibly in vivo challenge of the red cells with drug are indicated.

The application of the GSH stability test and assays for G-6-P.D. have made available further data regarding the incidence and distribution of this defect. Such data are summarized in table 2.

**THE OCCURRENCE OF DRUG-INDUCED HEMOLYTIC ANEMIA IN INDIVIDUALS WITHOUT DEMONSTRABLE DEFECTS OF THEIR RED CELLS**

Male volunteers given 30 mg. of primaquine daily manifest an all-or-none response: some individuals develop an acute hemolytic anemia, all others show no evidence of hemolysis. It has been reported, that if the daily dose of primaquine is increased to 120 mg, some individuals who were classified
Table 2.—Incidence of Positive in Vitro Tests for Red Cell Defect Associated with Drug Sensitivity in Various Racial Groups, Random Surveys

<table>
<thead>
<tr>
<th>Author</th>
<th>Method of Detection</th>
<th>Group Surveyed</th>
<th>No. of Subjects</th>
<th>% Pos.</th>
<th>% Neg.</th>
<th>% Intermediate 20-35 mg.%</th>
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<td>Beutler\textsuperscript{2,6,10}</td>
<td>Glutathione stability test</td>
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\textsuperscript{*}Not given.
\textsuperscript{f}23-40 mg. %.

As "nonsensitive" on the basis of their response to 30 mg. of primaquine would develop mild hemolysis.\textsuperscript{42}

It is perfectly clear, however, that nonsensitive cells do not have the same degree of resistance to the hemolytic action of some of the other compounds studied. For example, although 30 mg. of phenylhydrazine administered daily caused little or no hemolysis in nonsensitive individuals,\textsuperscript{42} it is well known that larger doses of phenylhydrazine cause acute hemolysis in all recipients. Flanagan et al.\textsuperscript{57} have pointed out certain differences between phenylhydrazine-induced hemolysis in normal individuals and hemolysis in sensitive individuals by primaquine, sulfanilamide and acetanilid administration. Severe hemolysis has been produced in normal individuals by combined administration of 3.6 Gm. of acetanilid and 90 mg. of primaquine, and gradual
mild hemolysis has been produced by the administration of 3.6 Gm. of acetanilid alone.\textsuperscript{42} It appears, then, that the resistance of “nonsensitive” cells to hemolysis induced by the different hemolytic compounds studied varies. In the case of phenylhydrazine, at least, it is possible that the basic mechanism of hemolysis of normal cells may differ from that of primaquine-induced hemolysis of sensitive cells.

While it is probably true that many or most cases of hemolysis during administration of analine derivatives that have been reported are in patients with primaquine-sensitive red cells, this cannot be assumed to be the case always. There are surely individual differences in the absorption and perhaps in the metabolism of some of these compounds. Evidence that this is so was obtained by detailed studies of the erratic response to thiazolsulfone. In different recipients, Dern et al.\textsuperscript{42} found that the percentage of primaquine-sensitive cells from the same donor that were destroyed varied from 0 to 76 per cent. It was observed that the greatest thiazolsulfone-induced destruction of primaquine-sensitive cells occurred in a normal recipient who developed a marked methemoglobinemia, abdominal pain, nausea, headache and slight anemia during drug administration. While this recipient extensively hemolyzed transfused primaquine-sensitive cells when given only 3 Gm. of thiazolsulfone daily, another recipient, who developed no toxic symptoms, failed to hemolyze any primaquine-sensitive cells even when given 18 Gm. of thiazolsulfone daily. Similar observations were made with respect to the occurrence of hemolysis in a nonsensitive individual after administration of 3.6 Gm. acetanilid daily. It is beyond the scope of this review to discuss in detail other mechanisms involved in the induction of hemolytic anemia by drugs. Most efforts to demonstrate immune mechanisms in drug-induced hemolytic anemias have ended in failure or in equivocal results, as has been described in part in the “Historical” section of this paper. It should be pointed out, however, that excellent evidence has been presented by Harris that at least one drug-induced hemolytic anemia, that produced by Fuadin, has an immunologic basis.\textsuperscript{66} While the available evidence suggests that the majority of drug-induced hemolytic anemias are associated with the same intrinsic red cell defect as is present in primaquine-sensitive cells, other factors may in some instances be responsible.

**DISCUSSION AND SUMMARY**

It has been recognized for a long time that 8-aminoquinoline compounds may cause hemolytic anemia in certain individuals, but until recently the mechanism of such sensitivity has remained obscure. The use of modern hematologic technics for the study of primaquine sensitivity has resulted in the discovery of a new intrinsic red cell defect. Cells with this defect are sensitive to hemolysis by a large number of aromatic amino compounds, including primaquine and other 8-aminoquinoline derivatives.

Administration of primaquine to sensitive subjects results in destruction of the older members of the red cell population. Available evidence suggests that the administration of a hemolytic drug causes oxidative damage to either the hemoglobin and/or the stroma of the sensitive cell. Heinz bodies are
visible manifestation of such damage. The damaged red cells are removed from the circulation by in vivo mechanisms, presumably by the reticuloendothelial system.

Red cell glutathione has been found to be related in some way to sensitivity to these compounds: (1) the glutathione level of sensitive cells is consistently lower than that of nonsensitive cells; (2) poisoning of the sulphydryl groups of red cells causes nonsensitive cells to react like sensitive cells in vitro with respect to Heinz body formation; (3) a rapid fall in the red cell glutathione level occurs in vivo when primaquine is administered to sensitive individuals but not to nonsensitive ones; and (4) a rapid fall in GSH level occurs in sensitive but not in nonsensitive cells when they are incubated with acetyl phenylhydrazine and many other compounds. These observations indicate that there is a mechanism that protects GSH in the nonsensitive but not in sensitive cells. This mechanism was found to require presence of glucose or inosine. In sensitive cells, this mechanism is defective and the GSH of the older cells is destroyed. The GSH destructive effect appears in vitro, at least, to be exerted through the oxyhemoglobin.

Primaquine-sensitive red cells have been found to be deficient in glucose-6-phosphate dehydrogenase activity. Glucose-6-phosphate dehydrogenase is involved in TPN reduction. TPN is a coenzyme for GSH reduction. Thus, a deficiency in glucose-6-phosphate dehydrogenase could result in defective GSH reduction and may therefore serve as an explanation of the GSH instability of drug-sensitive red cells.

It is not clear whether GSH serves merely as a convenient indicator of important changes within the cell that actually lead to cell death or whether GSH depletion plays a primary role in cell death and hemolysis. The role of GSH in the red cell is unknown, and evidence that GSH depletion leads to hemolysis has been obtained only by means which may be grossly injurious to the red cell in many other ways. It is entirely possible that another effect of G-6-P.D., such as TPNH deprivation, leads to cell damage in some entirely different way. Inability to reduce TPN might, for example, interfere with lipid synthesis in the red cell.

It cannot even be considered clearly established that either the GSH instability or G-6-P.D. deficiency of these red cells is the primary defect leading to susceptibility to hemolysis. If the level of G-6-P.D. alone governs the red cell's resistance to hemolysis, one might expect mild enzyme deficiency to result in mild susceptibility to hemolysis. According to preliminary data reported by Alving et al., this is not the case. The possibility must be considered, therefore, that not only GSH changes but even the G-6-P.D. changes in sensitive cells may be associated defects rather than of primary etiologic significance.

Primaquine-sensitive red cells are also uniquely sensitive to the hemolytic effect of many other compounds, including acetanilid, Furadantin and other drugs commonly used in medicine. Yet, it would appear that many of these drugs can also on occasion cause hemolysis of normal red cells. Subjects who are sensitive to the fava bean have also been shown to have the same defect in GSH stability and glucose-6-phosphate dehydrogenase as primaquine-
sensitive individuals display, but here other, as yet unknown, predisposing factors would seem to be involved.

The red cell defect of primaquine-sensitivity is genetically transmitted, probably as a sex-linked gene with intermediate penetrance.

It has thus been shown that a drug-sensitivity reaction is intimately related to a genetically transmitted enzyme deficiency. It is entirely possible, as has been pointed out so effectively by Motulsky,95 that other drug sensitivities may have a similar basis.

SUMMARIO IN INTERLINGUA

Es cognoscite depost longo que compositos de 8-aminoquinolina es capace a producer anemia hemolytic in certe individuos, sed usque recentemente le mechanismo de ille sensibilitate remaineva obscur. Le uso de moderne technicas hematologic in le studio de sensibilitate a primaquina ha resultate in le discoperta de un nove intrinsec defecto erythrocytic. Cellulas con iste defecto es sensibile a hemolyse per un grande numero de aromatic amino-compositos, incluse primaquina e altere derivatos de 8-aminoquinolina.

Le administration de primaquina a subjectos sensibile resulta in le destruction del plus vetule membros del population de erythrocytos. Le datos usque nunc accumulate suggere que le administration de un droga hemolytic causa damnos oxydative in le hemoglobina e/o le stroma del cellulas sensibile. Corpores de Heinz es un manifestation visible de tal damnos. Le afficite erythrocytos es eliminate ab le circulation per mechanismos in vivo, probablemente in le systema reticulo-endothelial.

Ha essite constatate que glutathiona erythrocytic es relationate in un maniera o un altere al sensibilitate a iste compositos. (1) Le nivello de glutathiona in le cellulas sensibile es uniformemente plus basse que in le cellulas non-sensibile. (2) Le invenenamento del gruppos sulfhydrylic de erythrocytos induce cellulas non-sensibile a reager in vitro como le cellulas sensibile quanto al formation de corpores de Heinz. (3) Un reduction rapide del nivello de glutathiona erythrocytic occurre in vivo quando primaquina es administrate a individuos sensibile sed non quando illo es administrate a individuos non sensible. (4) Un diminution rapide del nivello de glutathiona reducec (GSH) occurre in cellulas sensibile sed non in cellulas non sensibile quando illos es incubate con acetyl-phenylhydrazina e multe altere compositos. Iste observationes indica que il existe un mechanismo que protege GSH in cellulas non sensibile sed non in cellulas sensibile. Esseva trovate que iste mechanismo require le presentia de glucosa o de inosina. In cellulas sensibile, iste mechanismo es defective e le GSH de plus vetule cellulas es destruite. Le effecto del destruction de GSH pare—al minus in vitro—esser exercite per le oxyhemoglobina.

Ha essite constatate que cellulas sensibile a primaquina ha un deficientia del activitate de dishydrogenase de glucosa-6-phosphato. Dishydrogenase de glucosa-6-phosphato es interessate in le reduction de nucleotido de triphosphopyridina (NTP). NTP es un co-enzyma in le reduction de GSH. Assi, un deficientia de dishydrogenase de glucosa-6-phosphato resulta in le defec-
tive reduction of GSH and the instability of GSH in erythrocytes pharmaco-sensible.

Il non è chiaro se GSH è solamente un indicio patente di importante alterazioni intra le cellule le quali dunque al morte del cellula o se la deplezione di GSH ha un ruolo primario in la morte del cellula e in la hemolyse. Le funzione di GSH in le erythrocyto è incognosciute, e le prova che la deplezione di GSH risulta in hemolyse ha essite effetuate solmente per modi che è possibilemente grossiermente inuosi pro le erythrocyto in multe altre razioni. Il è completamente possibile che un altere effetto di dishydrogenase di glucosa-6-phosphato—per exemplo deprivation di NTPH—risulta in la dannification del cellula in un maniera completamente differente. Per exemple, le incapacità de reducere NTP poterea obstruer le synthese lipidic in le erythrocyto.

Il è non mesmo possibile considerar come clarmente establiete que le instabilitate de GSH o le deficiencia di dishydrogenase di glucosa-6-phosphato in iste erythrocytos es le defecto primari que explica lor susceptibility de esser hemolyse. Si le nivel de dishydrogenase di glucosa-6-phosphato sol governava le resistentia erythrocyto contra le effecto hemolytic, on expectarea que un leve deficiencia del enzyme resultarea in un leve susceptibility de esser hemolyse. Secondo le datos preliminari reportate per Alving et al., esse non es le caso. Per consequente, le possibilitate debe esser prendite in consideration que non solmente le alteraciones del GSH in cellulas sensible sed mesmo le alteraciones del dishydrogenase di glucosa-6-phosphato in illos es defecto associate plus tosto que factores de primari signification etiologic.

Erythrocytos sensible a primaquina es etiam distintivamente sensible al effecto hemolytic de numerose altre compositos, incluse acetanilido, Furadantino e altere drogas de uso commun in le practica medical. Tamen, il pare que multes inter iste drogas es etiam capace, in certe casos, a causar le hemolyse de erythrocytos normal. Subjectos qui es sensibile a Vicia fava ha manifestate le mesme defecto in le stahilitate de GSH e in dishydrogenase di glucosa-6-phosphato como individuos sensible a primaquina, sed il pare que in tal casos altere e non ancora cognoscite factores predisponenti es interessate.

Le defecto erythrocytic del sensibilitate a primaquina es transmittite geneticamente, probabilmente como gen a ligation sexual con penetrantia intermediari.

Assi il ha essite monstrate que un reaction de pharmaco-sensibilitate es intinemente relationate con un deficiencia enzymatic de transmission genetic. Il è completamente possibile—como Motulsky—who ha signalate si plausibilemente—que altere sensibilitates drogal ha bases del mesme genere.

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The Hemolytic Effect of Primaquine and Related Compounds: a Review

ERNEST BEUTLER