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SECOND EDITION

TOXIC CONSTITUENTS OF PLANT FOODSTUFFS

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CHAPTER 9

Favism

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I. FAVISM AS AN INBORN ERROR OF METABOLISM: EPIDEMIOLOGICAL, GENETIC, AND ENZYMOLOGICAL ASPECTS OF THE DISEASE

A. Brief Description and Epidemiology of the Disease

The term "favism" was coined in 1894 by the Italian physician L. Montano to designate an acute hemolytic anemia following ingestion of broad beans (fava beans) or inhalation of pollen of the *Vicia faba* plant (Sansone *et al.*, 1958). It is possible that the ancient Greeks had already recognized the presence of toxic constituents in broad beans. The mathematician Pythagoras founded a religion based on the tenets of the transmigration of souls and the sinfulness of eating the broad bean (Russell, 1965; Waldron, 1973). The first authentic descriptions, however, of this disease in the medical literature date back to the mid-1850s (see Aurichio, 1935). Since then, a rapidly growing number of case reports and clinical studies of favism have contributed to establish its status as a separate nosological* entity and to corroborate the etiological role of fava beans.

In a series of 1211 cases of favism in Italy reviewed by Fermi and Martinetti (1905), 725 were due to ingestion of broad beans, 459 were attributed to inhalation of pollen, whereas the remaining 27 cases were of undetermined origin. In a recent epidemiological survey of 579 cases of favism in Iran, only 4 (0.7%) were thought to be attributable to exposure to fava plant pollen, whereas consumption of broad beans was held to be responsible for the remainder of the cases (Hedayat *et al.*, 1971). Other reports failed to confirm the occurrence of pollen-induced outbreaks of favism (Chung, 1965; Kattamis *et al.*, 1969; Belsey, 1973). Thus, definitive and unequivocal evidence for the causative role of pollen is still lacking. Hemolytic crises due to ingestion of dry or cooked beans were found to be usually of a rather moderate intensity, whereas the most severe attacks were observed after eating of fresh raw fava seeds (Luisada, 1941). In rare instances, plants other than *Vicia faba*, e.g., seeds of *Pisum sativum* (garden peas) and pollen of *Anagyris foetida* and *Verbena hybrida*, have been incriminated as a cause of a hemolytic syndrome closely similar to favism (see Larizza *et al.*, 1960). Favism has been observed in individuals who had previously consumed fava beans with no untoward effects; others developed the disease on first exposure (Angelova and Andrev, 1959; Kattamis *et al.*, 1969; Belsey, 1973). Recurrent attacks of favism are not uncommon (Belsey, 1973).

Epidemiological studies in Italy revealed a seasonal incidence of the disease, characterized by two peaks: in April-May, when the plant blossoms, and in July-August, when the fresh ripe beans appear on the market (Luisada, 1941). In a more recent investigation, Donoso *et al.* (1969) found a pronounced seasonal

*Nosology is defined as the science of the classification of diseases.

peak in the Caspian littoral sea region between the middle of May and the middle of June, whereas in Abadan the highest incidence of favism occurred in April; in both instances, the seasonal peaks coincided with the harvesting of fava beans in the respective regions.

Data accumulated over the past 10 years reveal that favism is much more common in children under the age of 10 than in adults, with the highest frequency observed in the age group of 2-4 years, probably resulting from the first exposure of these infants to the beans. There are quite a few reports, however, on favism occurring during the first year of life, and even in breast-fed infants, apparently because of transmission of the noxious agent through the mother's milk (Angelov and Andrev, 1959; Chung, 1965; Kattamis, 1969, 1971).

The disease shows a marked predilection for the male sex, the male to female ratio varying in different series studied between 21:1 and 2.7:1 (Hedayat *et al.*, 1971; Belsey, 1973).

The clinical picture of favism is governed by the symptomatology inherent in the hemolytic event, the major manifestations being pallor, fatigue, dyspnea, nausea, abdominal or back pain, fever, and chills. The hemolysis is of variable intensity and gravity, the more severe cases being attended by hemoglobinuria and jaundice (see Beutler, 1972) and occasionally by acute renal failure (Schmitz and Fritz, 1968; Symvoulidis *et al.*, 1972). The onset of hemolysis may be extremely rapid and abrupt, especially in cases attributed to pollen inhalation, with symptoms starting within a few minutes following the noxious exposure. In the vast majority of cases, an interval of about 5-24 hr intervenes between the ingestion of the broad beans and the first manifestations of the hemolytic attack, whereas in rare instances the onset of the symptoms may be delayed until the third day or later following the consumption of broad beans (Luisada, 1941; Hedayat *et al.*, 1971).

The course of the disease is usually self-limited, the acute stage lasting 24-48 hr, and is then followed by prompt, spontaneous recovery. In contrast to the usually benign outcome of the disease in adults, a 6-8% case fatality rate was noted in children under 6 years of age (Fermi and Martinetti, 1905). More recently, however, the mortality figures have been greatly reduced on account of the advent of blood transfusion therapy (Crosby, 1956; Hedayat *et al.*, 1971).

B. Geographic Distribution of Favism

Favism exhibits a striking prevalence in the insular and littoral regions in the Mediterranean area and in the Middle East (Sardinia, Sicily, southern provinces of the Italian peninsula, Greece, Rhodes, Cyprus, Turkey, Lebanon, Israel, Iraq, Spain, the Balearic Islands, Algeria, Egypt, and Sudan) (Luisada, 1941; Hedayat *et al.*, 1971; Belsey, 1973; Amin-Zaki *et al.*, 1972; Hassan, 1971). The disease is also frequently encountered in China (Chung, 1965) and Bulgaria (Angelov

and Andrev, 1959). Sporadic cases have been reported from Germany (Gehrmann *et al.*, 1963, Johannsen *et al.*, 1968), France (Auquier *et al.*, 1968), Poland (Rockicka-Milewska *et al.*, 1968; Rożynkova *et al.*, 1970, 1971), Rumania (Schneer, 1968), Yugoslavia (Vince-Ribarić, 1962), and Singapore (Wong, 1972) (see also Table I).

The highest incidence was observed on the island of Rhodes (Kattamis *et al.*, 1969). The disease was found to be unevenly distributed on the island, with regions in which up to 40 cases per 1100 male inhabitants were reported. The overall prevalence of favism in the total population of the island during the years 1952-1965 was found to be 1.7-5.4 per thousand. A similarly high prevalence was recorded in Sardinia, with about five cases of favism per 1000 inhabitants (Crosby, 1956). This highly selective geographic distribution of favism is particularly puzzling since fava beans are grown and consumed almost all over the world as a cheap and popular staple food, distinguished by its relatively high content of carbohydrates and proteins (58 and 25%, respectively) per edible portion of mature dry seeds (see U.S. Department of Agriculture, 1963).

C. Role of Glucose-6-Phosphate Dehydrogenase Deficiency in the Etiology of Favism

The various concepts advanced in the earlier literature on the infectious (see Luisada, 1941), toxic (Gasbarrini, 1915), or immunological (Manai, 1929; Dacie, 1954) etiology of favism appear today to be of historic interest only. These views, apart from being devoid of solid experimental ground, failed to account for some of the most salient features of the disease, namely, its restricted geographic and ethnic distribution and its pronounced familial tendency (Luisada, 1941).

The door to an understanding of the true pathogenetic nature of favism was opened by the elucidation of the nature of the inborn error of metabolism underlying the so-called drug sensitivity, i.e., an abnormal propensity of certain individuals to develop acute hemolysis in response to treatment with primaquine and a variety of other drugs (see Beutler, 1972). Shortly after the discovery by Dern *et al.* (1954) that "primaquine sensitivity" is determined by an intrinsic abnormality of the erythrocytes, it was revealed by Beutler *et al.* (1955, 1957) that the susceptible red blood cells exhibit a relatively low content of reduced glutathione (GSH) and an enhanced rate of GSH destruction on incubation with 1-acetyl-2-phenylhydrazine in the presence of glucose. This so-called glutathione instability of drug-sensitive red blood cells was then shown by Carson *et al.* (1956) to be due to a deficiency of the nicotinamide adenine dinucleotide phosphate (NADP)-linked glucose-6-phosphate dehydrogenase (G6PD) and the resultant incapacity of these cells to maintain an adequate supply of NADPH to cope with the increased demand for GSSG (oxidized glutathione) reduction im-

posed by the challenging drugs (see Section III). In normal erythrocytes exposed to an oxidant stress, the enhanced rate of GSSG formation is compensated by a parallel increase in the rate of its reduction to GSH as a result of the concomitant stimulation of the G6PD activity, governed by an intrinsic regulatory mechanism (see Yoshida, 1973). The selective vulnerability of the red blood cells to this enzyme deficiency is accounted for by their critical dependence on the pentose phosphate shunt as the sole mechanism for NADPH generation, due to the lack of the alternate pathways for NADPH supply that are present in other cells (Beutler, 1971).

Crosby (1956), in a brief and brilliant report concerned with the clinical and epidemiological aspects of favism in Sardinia, was the first to point out the resemblance of this disease to the primaquine-induced hemolytic anemia insofar as in both instances the red blood cells are capable of normal survival unless they are challenged by the noxious agent. He suggested, by analogy to primaquine sensitivity, that a hereditary enzymatic deficiency may be the underlying cause of the susceptibility to favism.

The essential correctness of Crosby's idea was soon substantiated by direct experimental evidence obtained independently by Sansone and Segni (1956, 1957a,b, 1958) in Italy and by Szeinberg *et al.* (1957, 1958a,b) in Israel. These workers found that the GSH content of erythrocytes from persons known to have been affected by favism tends to be significantly lower (mean values below 50 mg %) than in normal individuals (mean range, 60-88 mg %) (Sansone and Segni, 1956; Szeinberg *et al.*, 1957). More significantly, the red cell GSH level was found to decline sharply during the acute phase of favism. (Szeinberg and Chari-Bitron, 1957; Larizza *et al.*, 1958), concomitant with the frequent appearance of methemoglobin and intracellular inclusions called Heinz bodies, similar to those observed in drug-induced hemolysis (Panizon and Pujatti, 1957; Larizza *et al.*, 1960). Furthermore, in all persons with a past history of favism the erythrocyte GSH proved to be unstable in Beutler's acetylphenylhydrazine test *in vitro* (Sansone and Segni, 1957a; Szeinberg *et al.*, 1958a). Finally, and most important, the GSH instability was invariably associated with a pronounced G6PD deficiency of the red blood cells (Sansone and Segni, 1958; Szeinberg *et al.*, 1958b; Larizza *et al.*, 1958; Zinkham *et al.*, 1958).

D. Ethnic Distribution of G6PD Deficiency and Favism

G6PD deficiency is probably the most common genetically determined enzymatic defect in human beings, affecting, according to a rough estimate by Carson (1960), about 100 million people of all races throughout the world. Its geographic distribution closely parallels that of malaria, presumably because of the selective advantage offered by the enzymatic deficiency in increasing the resistance of the red blood cells to infestation by *Plasmodium falciparum* (Allison and

TABLE I
INCIDENCE OF G6PD DEFICIENCY AND OCCURRENCE OF FAVISM IN DIFFERENT ETHNIC GROUPS

Ethnic groups	G6PD Deficiency		Favism	
	Incidence (%)	Reference ^a	Occurrence ^b	Reference ^a
Negroes				
United States	13	1		
Canada (Nova Scotia)	3-27	2		
Congo (Kinshasa)	18-23	3		
Pygmies	4	3		
Bantu	2-4	4		
Nigeria	10	5		
Ghana	24	6		
Gambia	15	6		
Sudan	7.1-8.3	7	+	7
Mediterraneans and Asians				
Sardinia (males)	4-48	8	+	9
Sicily, southern Italy			+	10
Greece (mainland and Crete)	0.7-3	11	+	11
Rhodes (Greeks)	22	12	+	12
Cyprus (Greeks)	7-11	13	+	13
Ashkenazic Jews	0.2			
Oriental Jewish communities (males)				
Kurdish	53	14		15
Iraqi	24	16		17
Iranian	15			
Yemenite	5			
North African	2			
Israeli Arabs	4			
Lebanon	3	18		
Egypt (males)	26	19	+	19,26
Turkey	11	20	+	
Iraq (males)	8.9	21	+	21
Kuwait (adults)	20	22		
Saudi Arabia (adults)	15-24	23		
Iran	10	24	+	25,26
India	6	3		
Philippines	12	3		
China	5.5	27	+	28,29
Singapore	3-4	30	+	30
Papua	6	31		
Melanesia	0-29	32		
Micronesia	0-9	33		

^aKey to references: 1. Marks and Gross (1959); 2. Langley *et al.* (1969); 3. Motulsky (1960); 4. Charlton and Bothwell (1959); 5. Gilles *et al.* (1960); 6. Allison *et al.* (1961); 7. Hassan (1971); 8. Siniscalco *et al.* (1961); 9. Crosby (1956); 10. Luisada (1941);

Clyde, 1961; Luzatto *et al.*, 1969). The frequency of occurrence of this inborn error of metabolism is widely dissimilar in the different ethnic groups. As shown in Table I, the highest figures of incidence of G6PD deficiency have been recorded, in decreasing order, in some of the oriental Jewish communities of Israel, Sardinians, Cypriot Greeks, American Negroes, and certain African populations. On the other hand, the abnormal trait is extremely rare or virtually absent in northern European nations and among Ashkenazic Jews (of European descent), North American Indians, and Eskimos (see Motulsky, 1960). It should be stated, however, that the available statistical data are on the whole rather incomplete and practically nonexistent in many underdeveloped areas lacking the elementary facilities for the detection of G6PD deficiency.

No detailed statistics are available concerning the ethnic distribution of favism. As may be seen, however, from Table I, the occurrence of favism does not parallel the frequency of the G6PD-deficient trait in the different populations. Most conspicuous in this respect is the complete absence of favism in North American Negroes (see Beutler, 1971).

E. Mode of Inheritance of G6PD Deficiency

G6PD deficiency is transmitted by a gene located in the X chromosome. This mode of inheritance was deduced from extensive family studies (Childs *et al.*, 1958; Szeinberg *et al.*, 1958c; Larizza *et al.*, 1960) and from the parallel segregation pattern observed when the G6PD-deficient trait and some other sex-linked anomaly, such as color blindness (Porter *et al.*, 1962) or hemophilia A (Boyer and Graham, 1965), happened to coexist in the same individual (see also Aebi, 1967). Accordingly, the enzyme deficiency is fully expressed in the hemizygous ($\bar{X}Y$) male, because the mutant gene ($\bar{X}$) is not counteracted by the normal allele (X). On the other hand, full expression is rather uncommon in females, since it depends on the statistically rare occurrence of a homozygous mutant genotype ($\bar{X}\bar{X}$). In the majority of affected females the G6PD deficiency is found to be of a partial or intermediate nature, in accordance with the expected preponderance of the heterozygous ($\bar{X}X$) constellation (Larizza *et al.*, 1960).

TABLE I (Continued)

11. Zannos-Mariolela and Kattamis (1961); 12. Kattamis *et al.* (1969); 13. Plato *et al.* (1964); 14. Szeinberg *et al.* (1958b); 15. Bogair (1951); 16. Szeinberg and Sheba (1960); 17. Efrati (1952); 18. Taleb *et al.* (1964); 19. Ragab *et al.* (1966); 20. Say *et al.* (1965); 21. Amin-Zaki *et al.* (1972); 22. Shaker *et al.* (1966); 23. Gelpi (1965); 24. Walker and Bowman (1959); 25. Hedayat *et al.* (1971); 26. Belsey (1973); 27. Chan *et al.* (1964); 28. Du (1952); 29. Vella (1959); 30. Wong (1972); 31. Ryan and Parsons (1961); 32. Kidson and Gorman (1962); 33. Kidson and Gajdusek (1962).

^bPlus (+) sign indicates that outbreaks of favism have been reported in the respective populations; blank space indicates that, to our knowledge, no such occurrences have been reported.

By the use of ingenious cytochemical techniques capable of detecting G6PD deficiency in single cells, it could be shown that erythrocytes of females with a heterozygous trait of intermediate enzyme deficiency constitute a mixture of normal and G6PD-deficient cells (Beutler *et al.*, 1962; Sansone *et al.*, 1963). This so-called cellular mosaicism bears out the prediction of Lyon's "X chromosome inactivation" theory (Lyon, 1961). According to this concept, a random loss of functional activity of one of the X chromosome pair (of either paternal or maternal origin) takes place in each individual somatic cell early in the course of morphogenesis, with a perpetuation of the inactivation pattern in the progeny. It follows, therefore, that the degree of susceptibility to drug-induced or fava hemolysis in heterozygous females will be critically dependent on the relative proportions of the G6PD-deficient and normal erythrocytes in their blood.

F. Molecular Characteristics of Normal and Mutant G6PD

During the last decade, G6PD has been the subject of intensive enzymological investigations. The normal enzyme was found to exist in several oligomeric forms (composed of two, four, or six identical subunits), the degree of aggregation depending on a variety of physicochemical factors, e.g. ionic strength, pH, and protein concentration of the enzyme solution (Yoshida, 1966, 1967b; Yoshida and Hoagland, 1970). The catalytically active molecular species that appears to be predominantly in the dimeric form is stabilized by its tight association with NADP or NADPH, whereas in the absence of the coenzymes the apoenzyme tends to dissociate into the inactive monomers (Yoshida, 1973).

Starch gel electrophoresis of normal red cell hemolyzates revealed the existence of two major molecular variants of G6PD, characterized as a fast migrating band A and a slow band B (Boyer *et al.*, 1962). The more common form B is found in Caucasian subjects and in American Negroes, whereas type A occurs among Negroes only (Boyer *et al.*, 1962; Kirkman and Hendrickson, 1963). In subsequent studies, about 80 additional genetic variants of G6PD were differentiated by a combination of various enzymological criteria, such as catalytic rate, electrophoretic mobility, substrate specificity, and particularly the ability to utilize 2-deoxy-D-glucose, K_m values for glucose 6-phosphate and NADP, pH optimum, and heat stability (Kirkman *et al.*, 1964; Yoshida *et al.*, 1971; Beutler, 1971).

In nearly 40 variants that are not associated with any clinical symptoms, the catalytic activity is within the normal range and in one instance (G6PD Hektoen) is even severalfold higher than the normal average level. About 20 variants exhibit severe red cell enzyme deficiency, which manifests as hemolytic anemia in response to oxidative stress by drugs or fava beans. Another group, comprising about 20 G6PD-deficient mutants, is associated with "chronic nonspherocytic

tic congenital hemolytic anemia," occurring spontaneously in the absence of any extraneous challenging agent (Yoshida, 1970).

The most common G6PD-deficient mutants are as follows: the Mediterranean type, which is prevalent among Sephardic Jews, Italians, and Greeks; the A type common among Negroes; the Canton type found primarily among southern Chinese and other Oriental populations; and the Debrousse type most frequent among Arabs.

Three variants of human G6PD that were isolated in molecularly homogeneous form and subjected to "fingerprinting" were found to differ from one another in a single amino acid residue. Thus, an asparagine residue present in variant B is replaced by aspartic acid in variant A (Yoshida, 1967a). Similarly, G6PD Hektoen was found to differ from the B variant by a substitution of histidine for tyrosine (Yoshida, 1970).

By applying a density gradient centrifugation procedure to separating red blood cells into different age groups, it was found that the G6PD activity in a 5% fraction of youngest cells from a blood sample of the deficient A⁻ variant was practically identical to that of normal erythrocytes (Yoshida *et al.*, 1967). It was concluded that the G6PD A⁻ enzyme has normal initial specific activity, but the rate of its inactivation and eventual loss from the cell is considerably enhanced. This conclusion was further corroborated by immunological methods (Marks and Gross, 1959; Piomelli *et al.*, 1968; Yoshida *et al.*, 1968). On the other hand, by the use of similar procedures, the Mediterranean mutation was found to involve both a decrease in the number of molecules and a diminished specific activity of the enzyme (Yoshida *et al.*, 1968).

G. Correlation between the Degree of G6PD Deficiency and the Severity of Clinical Symptomatology

The severity of clinical manifestations among the various G6PD mutants does not correlate well with the differences in the extent of G6PD deficiency, as measured *in vitro* under the conventional assay conditions. Yoshida and Lin (1973) suggested that the apparent inconsistencies could be resolved by taking into account the regulatory mechanisms governing the G6PD activity under the physiological conditions prevailing within the red blood cell. It was found that the activity of the oxidative pentose phosphate shunt in the red cell is strongly suppressed, representing only about 0.1–0.2% of the maximal potential catalytic rate of G6PD, as determined in cell-free hemolyzates. The low intracellular activity of the pentose shunt is attributable to the strong inhibitory effect of NADPH and ATP on the activity of G6PD, which constitutes the rate-limiting step in the overall oxidative pentose phosphate pathway. The various G6PD-deficient mutants differ in their affinities for NADP (K_m), as well as in the degree

of their susceptibility to inhibition by ATP and NADPH (K_i). Thus, the spontaneously hemolytic variants (e.g., Manchester, Tripler, or Alhambra) are strongly inhibited by ATP and NADPH, so that under the conditions existing in the human erythrocyte (NADP/NADPH ratio close to 1:50 and 1:5 mM ATP) these variants are virtually nonfunctional, although their potential G6PD activity is about 20% of the normal. In contrast, the nonhemolytic mutants (e.g., Mediterranean or A⁻) are resistant to inhibition by NADPH and ATP, and, therefore, under simulated physiological conditions, their catalytic activity varies between 30 and 50% of the average activity of the normal major variants (A and B).

H. Differences between Caucasian and Negro Types of G6PD Deficiency

The considerable genetic polymorphism and biochemical heterogeneity of the enzymatic defect brought to light by the above studies may at least partly account for the marked differences and variations in the biological and clinical manifestations of the inborn error observed in different ethnic groups. The differences are particularly striking between the affected American Negroes and Caucasians (Italians, Greeks, Sephardic Jews, etc.). Thus, in G6PD-deficient Negro males, the range of the erythrocytic G6PD activity is about 10–21% of the normal mean, whereas in Caucasian males the enzyme activity is only 0–6% of the normal level (Marks and Gross, 1959; Marks and Banks, 1965). This disparity in the extent of the enzyme deficiency seems to be correlated with the relatively milder clinical course of the drug-induced hemolysis usually observed in Negroes (see Beutler, 1972; Pannaciulli *et al.*, 1965). Furthermore, the G6PD activity of the young red cell population (following a hemolytic crisis) is considerably elevated in Negroes but not in Caucasians (see Marks and Banks, 1965). The enzyme deficiency appears to be more widely distributed in the different tissues of affected Caucasians than in Negroes (Brunetti *et al.*, 1960; Chan *et al.*, 1965; Marks *et al.*, 1959). The vestigial G6PD activity in deficient Caucasians is usually represented by the variant B, whereas in deficient Negroes the enzyme shows invariably A-type characteristics (Boyer *et al.*, 1962).

I. Possible Role of Genetic Determinants Other Than G6PD Deficiency in the Pathogenesis of Favism

The existence of an extracorporeal factor was postulated by Stamatoyannopoulos *et al.* (1966) to explain the relatively low incidence of favism in G6PD-deficient subjects, its total absence in American Negroes, as well as its bizarre and apparently unpredictable mode of occurrence, which shows no corre-

lation with the frequency of exposure to *Vicia faba* or the degree of enzyme deficiency. A study conducted by these authors on the incidence of favism in three different areas of Greece showed that overt episodes of favism do not occur at random in G6PD-deficient subjects, but there is a tendency for regional and familial aggregation of cases. Thus, in the area of Karditsa with a frequency of G6PD deficiency of about 27%, favism is rare, whereas on Corfu Island, with an approximately 5% incidence of this enzyme deficiency, favism is of relatively frequent occurrence. In both areas, the consumption of fava beans is very common. The authors concluded that the family data they collected are consistent with "the hypothesis of Mendelian segregation of an autosomal gene which in the heterozygous state enhances the susceptibility to favism of G6PD-deficient individuals." The functional role of the hypothetical gene remains to be defined. Its influence on the susceptibility of the red blood cells to hemolysis or alternatively its modifying effect on the absorption, detoxication, or excretion of the causative agent of favism are among the various possibilities to be considered (Stamatoyannopoulos *et al.*, 1966; see also Tarlov *et al.*, 1962). A preferential association between favism and certain acid phosphatase phenotypes (types A and C) has been described (Bottini *et al.*, 1971). However, the pathogenetic significance of this correlation is not clear.

J. Methods of Detection of G6PD Deficiency

1. Glutathione Stability Test

The test designed by Beutler (1957) is based on his observation that the GSH content of G6PD-deficient but not of normal erythrocytes declines markedly in the course of aerobic incubation with acetylphenylhydrazine and glucose under standardized conditions. The recommended method for glutathione determination is the procedure of Ellman (1959) as adapted by Beutler *et al.* (1963).

2. Spectrophotometric Determination of G6PD

The assay is based on the measurement of the rate of increase of absorbance at 340 nm due to formation of NADPH in a reaction system consisting of glucose 6-phosphate, NADP, buffer solution, and suitably diluted hemolyzate (see Carson, 1960).

3. Methemoglobin Reduction Test

The assay introduced by Brewer *et al.* (1962) is based on the observation of Dawson *et al.* (1958) that the rate of methemoglobin reduction by G6PD-

deficient erythrocytes in the presence of methylene blue is considerably slower than normal. A modification using Nile blue sulfate (instead of methylene blue), a dye that does not permit interaction between cells, was designed for the detection of intermediary states of G6PD deficiency in heterozygous females (Beutler and Baluda, 1963).

4. Indicator-Linked Screening Methods

In these tests, the G6PD-linked reduction of NADP is measured indirectly by following the rate of reduction of a suitable dye serving as an artificial terminal hydrogen acceptor. The original procedure of Motulsky and Campbell-Kraut (1961), in which the decolorization time of brilliant cresyl blue is determined, was modified by the use of 3-(4,5-dimethylthiazolyl-1,2)-2,5-diphenyltetrazolium bromide (MTT), which is reduced by the G6PD-linked system to a purple insoluble formazan derivative. The latter reagent can be used in a spot-test technique (Fairbanks and Beutler, 1962).

5. Fluorescent Screening Methods

This screening procedure, introduced by Beutler and Mitchell (1968), takes advantage of the fact that NADPH (generated by G6PD) fluoresces when illuminated with ultraviolet light, whereas NADP does not. A small volume of blood is hemolyzed with saponin and mixed with a buffered solution of suitable amounts of glucose 6-phosphate, NADP, and GSSG. Following incubation for 5–10 min, the mixture is spotted on filter paper and examined under an ultraviolet lamp for fluorescence. An automated, quantitative version of this method adapted to use with the Technicom AutoAnalyzer instrument has been described (Dickson *et al.*, 1973).

6. Ascorbate-Cyanide Test

This test, devised by Jacob and Jandl (1966), depends on the ability of ascorbate to undergo oxidation in the presence of oxyhemoglobin with formation of H_2O_2 . When catalase is inhibited by cyanide, the generated H_2O_2 can be detoxified only by GSH peroxidase. Any enzymatic deficiency interfering with the proper functioning of the GSH peroxidase system (G6PD, GSH, GSH reductase, and GSH peroxidase) causes H_2O_2 to accumulate. The hydrogen peroxide reacts with hemoglobin, producing hemochromes with resultant brown discoloration detectable by visual inspection.

II. THE SELECTIVE TOXICITY OF FAVA BEANS: SEARCH FOR THE CAUSATIVE AGENT OF FAVISM

A. Effect of Ingestion of Fava Beans on the Life Span of G6PD-Deficient Erythrocytes

Efforts to obtain direct experimental evidence for the selective toxicity of broad beans for G6PD-deficient cells are greatly handicapped by the lack of a susceptible laboratory animal and the potential health risk inherent in the induction of a hemolytic disorder in sensitive volunteers. To circumvent this difficulty, some authors have adopted the experimental stratagem of Dern *et al.* (1954), consisting of the transfusion of ^{51}Cr -labeled G6PD-deficient erythrocytes into normal compatible recipients and the determination of the effect of orally administered broad beans on the survival of the tagged cells. These studies have yielded conflicting results in the hands of different investigators. Thus, both Greenberg and Wong (1961) and Davies (1962) failed to detect any significant decrease in the life span of the transfused erythrocytes following ingestion of broad beans by healthy recipients. The negative outcome of these trials, however, is open to criticism and may be ascribed perhaps to the inadequacy of the experimental conditions, such as insufficient size of the challenging dose of the fava beans or loss of their noxious activity due to cooking or prolonged storage. On the other hand, Panizon and Vullo (1962) concluded from their well-documented experiments that fresh fava beans or juice prepared from them induced in the majority of cases a definite shortening of the survival of the transfused G6PD-deficient erythrocytes. According to the authors' estimate, however, the effective dose of the fava beans was surprisingly high, i.e., about 50,000 times larger, on a weight basis, than the minimal hemolytic dose of primaquine (Panizon and Vullo, 1962). Furthermore, the hemolytic effect of the fava beans was much more pronounced when fava-sensitive individuals (in the early stage of recovery from a favic crisis) rather than normal volunteers served as recipients. These results, therefore, seem to indicate that G6PD-deficiency and ingestion of fava beans are not sufficient by themselves to bring about a full-blown hemolytic crisis and point to the adjuvant role of an extracorporeal factor in the pathogenesis of this disease (Panizon and Vullo, 1961, 1962; Panizon, 1967).

B. Studies with Crude Fava Bean Extracts

Attempts to ascertain unequivocally the presence of a noxious factor in fava beans with a specific effect on drug-sensitive erythrocytes have thus far met with a moderate degree of success.

Mela and Perona (1959) noted a decrease of GSH in G6PD-deficient red blood cells incubated in the presence of broad bean juice prepared by squeezing with a hydraulic press. Walker and Bowman (1960) reported that crude aqueous extracts of fava beans, as well as saline extracts of fava pollen and pistils (Bowman and Walker, 1961), caused a rapid and significant drop in the GSH level of sensitive erythrocytes but had little or no effect on normal erythrocytes. The activity of the broad bean extracts was destroyed by boiling for 90 min. No effect was detected with extracts prepared from peas or other leguminous seeds tested.

Results similar to those described above, though less clear-cut, were also obtained by Contu *et al.* (1961). Furthermore, Panizon and Zacchello (1965) found that fava bean juice or whole bean homogenates induced a fall of GSH and a definite impairment of the survival of ^{51}Cr -labeled G6PD-deficient red blood cells transfused into normal recipients, whereas normal erythrocytes remained unaffected. Panizon and his collaborators (Panizon, 1967; F. Panizon, personal communication, 1968) isolated from fava beans a material soluble in lipid solvents that induced a very marked decline of the intraerythrocytic GSH accompanied by formation of methemoglobin and Heinz bodies; in contrast to primaquine, this material also acted on GSH in the absence of hemoglobin. The authors concluded that their data are incompatible with an allergic pathogenesis of favism, a view still held by certain investigators (Carcassi, 1958; Kantor *et al.*, 1962). They also suggested that fava beans and primaquine give rise to hemolysis through an essentially similar mechanism. More recently, Bottini and his associates (Bottini *et al.*, 1970; Bottini, 1973) described the separation of crude fava bean extracts into two distinct fractions, both capable of oxidizing GSH in pure solution and in G6PD-deficient erythrocytes.

Various serum abnormalities were observed in patients during an acute favic episode, such as atypical antibodies against their own erythrocytes (Marcolongo *et al.*, 1950) or antibodies against fava bean extracts (Kantor and Arbesman, 1959; Kantor *et al.*, 1962). Some investigators described the occurrence in saline extracts of broad beans of a heat-labile substance causing agglutination of both normal and G6PD-deficient erythrocytes (Roth and Frumin, 1960; Greenberg and Wong, 1961) and counteracted by a normal serum factor residing in the IgA fraction of the γ -globulins (Creger and Gifford, 1952). Frumin and his co-workers reported that the factor neutralizing the fava bean hemagglutinin was absent in the serum of a number of favic patients tested both in the acute hemolytic stage and during the remission (Perera and Frumin, 1965; Nathan *et al.*, 1974). These findings are at variance with the observation of Greenberg and Wong (1961) that both favic and normal sera exhibited the ability to inhibit hemagglutination by fava bean extracts. The paucity of the data does not permit a critical assessment of their significance and their possible relevance to the pathogenesis of favism.

C. Fractionation of Fava Bean Extracts

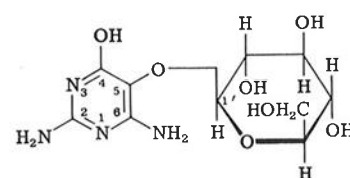
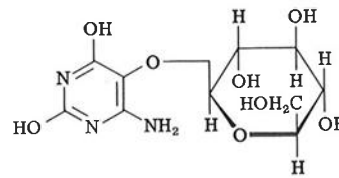
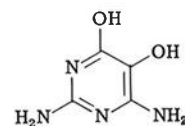
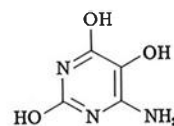
In a study carried out in our laboratory, the quest for the causative agent of favism in broad bean extracts was guided by its presumed discriminatory capacity for oxidizing GSH in G6PD-deficient but not in normal erythrocytes, when incubated *in vitro* in the presence of glucose. In fact, several fractions conforming to this criterion could be isolated from aqueous broad bean extracts by use of ion-exchange chromatography. Some of the purified fractions were sparingly soluble in water and exhibited in neutral solution a rapid loss of GSH-oxidizing activity, concomitant with a change in their spectral characteristics. The structural instability of these substances appeared to be inherent in their tendency to undergo spontaneous oxidation in air, since the deterioration could be largely prevented by storage under nitrogen (S. Bien, M. Noom, G. Glaser, A. Roigin, and J. Mager, unpublished results). The properties of the active fractions were reminiscent of those described for some pyrimidine derivatives known to occur in fava beans in the form of aglycones of the β -glycosides termed "vicine" and "convicine." Our subsequent work, therefore, was concerned primarily with exploring the possible role of these aglycones in the causation of favism.

D. Structure and Properties of Vicine, Convicine, and Their Aglycones

Vicine was first isolated by Ritthausen and Kreusler (1870) from *Vicia sativa* seeds by a method involving extraction with dilute H_2SO_4 and precipitation with HgSO_4 ; the final yield of the crystalline material was about 0.35% (Ritthausen, 1876). Vicine was subsequently found in other species of *Vicia* including *Vicia faba* (Winterstein and Somló, 1933), beet juice, and peas (Schulze, 1891; Bendich and Clements, 1953). The glycosidic nature of this compound was recognized by Ritthausen (1896), who also succeeded in isolating the aglycone divicine (Ritthausen, 1899a,b). The pyrimidine nucleoside structure was assigned to vicine by Johnson (1914) and confirmed by Levene (1914). The correct formulation, however, of vicine as 2,6-diamino-4,5-dihydropyrimidine 5-(β -D-glucopyranoside) (I) was arrived at only several decades later by Bendich and Clements (1953).

Convicine was also discovered by Ritthausen (1881) in *Vicia sativa*. It was identified by Johnson (1914) as a β -glycoside of isouramil, and the correct position of the glycosidic bond was established by Bendich and Clements (1953). The formulation of convicine as 2,4,5-trihydroxy-6-aminopyrimidine 5-(β -D-glucopyranoside) (II) was confirmed by unambiguous evidence (Bien *et al.*, 1968).

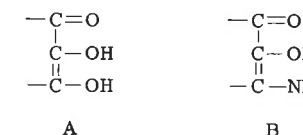
The aglycones divicine (III) and isouramil (IV) can be obtained from the respective glycosides (vicine and convicine) by mild acid hydrolysis or by enzymatic splitting with β -glucosidase (emulsin). In recent years, several synthetic

Vicine
(I)Convicine
(II)Divicine
(III)Isouramil
(IV)

procedures for preparing these compounds have also been developed (Davoll and Laney, 1956; McOmie and Chesterfield, 1956; Chesterfield *et al.*, 1964; Bien *et al.*, 1968; Ikeda *et al.*, 1973).

Vicine and convicine, as well as the corresponding aglycones, react with the Folin-Ciocalteu phenol reagent, yielding a blue color similar to that produced by tyrosine or tryptophan. This property was employed by Higazi and Read (1974) as a basis for developing a quantitative assay of vicine in plant material and blood. In our hands, however, the method failed to fulfill the authors' claim for its specificity and, therefore, did not lend itself to direct determination of the pyrimidine glycosides in natural materials. Both divicine and isouramil reduce vigorously alkaline solutions of 2,6-dichlorophenolindophenol, phosphomolybdate, or phosphotungstate and elicit an intense blue color reaction with ammoniacal ferric chloride solution, indicative of the presence of an enolic hydroxyl group in the molecule. The aglycones are highly unstable in the presence of oxygen; the rate of their oxidative breakdown is most rapid at alkaline pH and falls off with decreasing pH values. At room temperature, the half-lives of divicine and isouramil in neutral solutions are of the order of 30–40 min. The breakdown of the pyrimidine aglycones is accelerated by traces of copper (Cu^{2+}) and other heavy metals; both compounds are almost instantaneously destroyed by boiling (Bendich and Clements, 1953; M. Chevion *et al.*, results to be published).

The closely similar ultraviolet spectra of divicine and isouramil are characterized by a peak at 280 nm (reduced form), shifting upon exposure to oxygen to a less prominent absorption maximum at 255 nm (oxidized form) (Razin *et al.*, 1968). The disappearance of the characteristic peak and the concomitant increase



in nonspecific "end absorption" occurring in the course of the above-mentioned oxidative decomposition of these compounds are suggestive of the rupture of the pyrimidine ring structure.

Bendich and Clements (1953) pointed out in their monumental study that some of the distinctive features of divicine and its congeners, namely, their powerful reducing properties, spectral characteristics, and molecular instability, are strikingly similar to those of ascorbic acid. Furthermore, these authors inferred from a study of different substitutions that the common structural denominator underlying these properties is a carbonyl-conjugated enediol (A) or amino-enol (B) system. Consequently, all the characteristic properties of the aglycones are abolished by substitution of the hydroxyl group at C-5, such as that represented by the glycosidic linkage present in vicine and convicine. In fact, these glycosides show none of the reducing properties of their aglycones, are remarkably heat stable in solution, and their ultraviolet spectra differ significantly from those of their constituent pyrimidines (Bendich and Clements, 1953; Bien *et al.*, 1968; S. Bien, M. Noam, G. Glaser, A. Razin, and J. Mager, unpublished results).

E. Effects of Divicine and Isouramil on Red Cell Metabolism *in Vitro*: Synergistic Interaction of Isouramil and Ascorbic Acid

Incubation of human red cell suspensions in phosphate-buffered isotonic saline (pH 7.4), supplemented with isouramil or divicine (to be referred to as aglycones), resulted in a rapid fall of their GSH level, followed by a slower decline of their ATP content (Mager *et al.*, 1965). Addition of glucose prevented the injurious action of the aglycones on normal erythrocytes but had no protective influence on G6PD-deficient cells. This behavior of the aglycones contrasted with the virtual inertness displayed in the same system by convicine and vicine, in which the readily oxidizable enolic hydroxyl group at C-5 of the pyrimidine moiety is blocked by the β -glycosidic bond. The activity of the aglycones, as gauged by their effects on the GSH and ATP contents of the erythrocytes, was roughly 20–30 times higher than that of acetylphenylhydrazine (APH). In contrast, the structurally related pyrimidine, dialuric acid, was relatively ineffective in the test system used, presumably due to its pronounced tendency to interact with GSH by producing an addition compound with a characteristic absorption peak at 305 nm (Patterson *et al.*, 1949).

In contradistinction to APH and primaquine, which require the presence of

hemoglobin for catalyzing GSH oxidation (Beutler *et al.*, 1957), the fava pyrimidine aglycones were able to oxidize GSH in pure solution. The oxidation of GSH by these compounds proceeded to completion even at a tenfold or higher molar ratio of GSH to pyrimidine; it was considerably enhanced by shaking in air and completely suppressed in an atmosphere of nitrogen. The nonstoichiometric nature, as well as the oxygen dependence of the oxidation of GSH by the aglycones, suggested that this reaction is mediated by a catalytic oxidoreduction mechanism, similar to that of the well-known GSH-ascorbic acid redox system (Borsook *et al.*, 1937). This analogy is further emphasized by the observation that sodium ascorbate at relatively high concentrations (5–10 mM) was capable of producing a marked decrease (50–80%) in the GSH content of G6PD-deficient erythrocytes incubated in phosphate-buffered saline solution (pH 7.4) for 3 hr at 37°C (see also Waller and Benöhr, 1973; Prins and Loos, 1969). Moreover, a mixture of 0.05 mM isouramil and 0.5 mM ascorbate caused an approximately 70% decline of the intracellular GSH level under similar incubation conditions, whereas the same amounts of each compound alone were totally ineffective in this system. Essentially similar results were obtained with normal human or rabbit erythrocytes, provided that the incubation was carried out in the absence of glucose. The supraadditive nature of the combined affect of isouramil and ascorbate on erythrocyte GSH was shown to represent a net outcome of a series of reactions, namely, oxidation of GSH by its direct interaction with the rapidly autoxidizable isouramil and the concomitantly generated H_2O_2 , as well as indirectly by a cyclic system involving oxidation of ascorbate to dehydroascorbate by isouramil and the renewed reduction of dehydroascorbate to ascorbate by GSH (Razin *et al.*, 1968). It is not unlikely that such a synergistic interrelationship may play a crucial role in the pathogenesis of favism.

In a subsequent series of experiments (G. Glaser *et al.*, results to be published) designed to test the effect of isouramil on red cell survival, glucose-deprived rabbit erythrocytes were labeled with ^{51}Cr and incubated in phosphate-buffered saline at 37°C for different periods of time in the presence of various amounts of isouramil. The cells were then reinfused into the donor rabbits, and their ^{51}Cr half-life was determined. It was found that incubation of the erythrocytes with 2 mM isouramil for 75 min resulted in a reduction of their ^{51}Cr half-life to less than 5% of the normal value (9 hr and 9 days, respectively). Lower levels of isouramil or shorter incubation periods gave rise to correspondingly less pronounced impairment of the red cell survival. Furthermore, here again the effect of isouramil was found to be synergistically enhanced by addition of ascorbate.

Shortly after the publication of our study (Mager *et al.*, 1965), the earlier investigations of Lin and Ling (1962a,b,c) on the possible relation of vicine to favism, came to our attention through summaries appearing in *Chemical Abstracts* in 1966. The Formosan workers (Lin and Ling, 1962b) observed a transient hemoglobinuria occurring in puppies 3 hr after the oral administration

of vicine (0.2 gm/kg body weight), which was isolated from *Vicia faba* by a modified procedure of Levene (1914; Lin and Ling 1962a). These authors found also that vicine exerts some minor inhibitory effects on the activities of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in human red cell hemolysates (Lin and Ling, 1962c). However, the significance of these effects in the pathogenesis of favism is rather doubtful.

In another paper, Lin (1963) described some chemical interactions between divicine and sulfhydryl compounds. With an excess of GSH relative to divicine, a maximal absorption appeared at 305 nm, whereas with a mixture of divicine and cysteine two peaks, at 285 and 245 nm, were observed. In both instances, the amounts of sulfhydryl compounds that disappeared were severalfold higher, on a molar basis, than the quantity of divicine added. In our opinion, the complex absorbing at 305 nm plays no essential part in mediating the catalytic oxidation of GSH by divicine, but rather represents a side reaction analogous to that observed with alloxan (Patterson *et al.*, 1949). Furthermore, according to our data, the spectrum of the pyrimidine-cysteine mixture appears to be determined by the molecular structure of divicine per se stabilized by the reducing action of cysteine (Razin *et al.*, 1968).

F. Possible Etiological Role of Divicine and Isouramil in Favism

The overall pattern of metabolic disturbances resulting from incubation of G6PD-deficient red cells with the aglycones of vicine and convicine is essentially identical to that elicited by treatment with acetylphenylhydrazine (Beutler *et al.*, 1957; Mager *et al.*, 1965). The powerful capacity for oxidizing GSH exhibited by the pyrimidine aglycones *in vitro* as well as the observed deleterious effect of isouramil on red cell survival are consistent with a possible causative role of these substances in precipitating the favic crises. The free aglycones may arise from the parent fava glycosides either in the beans or in the digestive tract through the hydrolytic action of β -glucosidase. The conceivable vicissitudes in the availability of the requisite conditions for enzymatic release of the aglycones from the glycosides, as well as the particular lability of these compounds, might account for the puzzling irregularity that characterizes the occurrence of favism in susceptible individuals, irrespective of the degree and frequency of their exposure to the noxious agent (see Luisada, 1941).

It may be pertinent to mention in this connection that divicine has been in the past named as the causative agent of neurolathyrism (Anderson *et al.*, 1925) because of its parenteral toxicity to experimental animals (Kleiner, 1912). This view, however, was discredited by the finding that oral administration of divicine at a level as high as 1% of the diet to rats (Lee, 1950) and chicks (Arscott and Harper, 1963) produced no adverse effects other than growth retardation. As pointed out by Liener (1966), it was probably because of this lack of specific

toxicity of divicine by oral route that its possible significance as the causative principle of favism has escaped the attention of the earlier investigators in this field.

G. Effect of 3,4-Dihydroxyphenylalanine (Dopa) on Erythrocyte GSH and Critical Evaluation of Its Postulated Role in the Etiology of Favism

Kosower and Kosower (1967) put forward the hypothesis that 3,4-L-dihydroxyphenylalanine (dopa) may be one of the active principles responsible for the ability of fava beans to induce hemolysis in G6PD-deficient individuals. This substance, known to be a moderately strong reducing agent, is present in broad beans in substantial amounts [about 0.25% of the fava pods (Guggenheim, 1913)], mainly in the free state and partly in the form of its β -glycosidic derivative (Pridham and Saltmarsh, 1963; Andrews and Pridham, 1965). Kosower and Kosower (1967) found that significant losses of GSH occurred in G6PD-deficient erythrocytes, when incubated at 37°C for 3 hr in a glucose-containing medium supplemented with dopa in amounts ranging from 0.75 to 3 μ moles/ml. In contrast, oxidation of GSH by dopa in normal red blood cells was demonstrable only in the absence of glucose.

Careful scrutiny of the data of Kosower and Kosower reveals that the amounts of GSH that disappeared (were oxidized) were related to the amounts of dopa added by a roughly 1:10 molar ratio. Thus, contrary to the authors' claim, these results do not seem to support the notion of a nonstoichiometric (catalytic) oxidation of GSH by dopa. The latter conclusion is also in line with our observation that no appreciable oxidation of GSH took place when a mixture of GSH (2 mM) and dopa (4 mM) in 0.01 M phosphate buffer (pH 7.4) was incubated for 30 min at 37°C with continuous shaking in air. Moreover, comparative experiments showed that dopa at a concentration as high as 10 mM failed to affect the GSH level in normal washed human erythrocytes incubated for 3 hr at 37°C in the absence of added glucose, whereas 1 mM isouramil caused almost complete disappearance of the intracellular GSH under the same conditions. On the other hand, combined addition of 1 mM dopa and 0.2 mM isouramil resulted in nearly 80% destruction of the erythrocytic GSH, whereas each compound alone was without perceptible effect (Razin *et al.*, 1968).

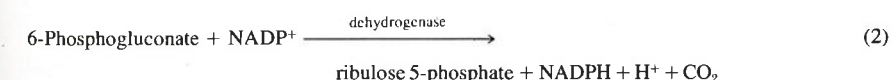
Similar results were obtained in experiments performed with glucose-starved rabbit erythrocytes. In addition, however, it was found that, although dopa potentiated the oxidant action of isouramil on the intracellular GSH, it failed to enhance the effect of isouramil in shortening the survival of ^{51}Cr -tagged erythrocytes treated *in vitro* and reinfused into the donor rabbit (G. Glaser *et al.*, results to be published).

More recently, the hypothesis of Kosower and Kosower (1967) was endorsed by Beutler (1970) and modified to suggest that dopaquinone, the oxidation prod-

uct of dopa, rather than dopa itself is the active factor responsible for the fava bean-induced hemolysis. This claim is based on the observation that a mixture of dopa (0.15 mM) and tyrosinase (presumed to generate dopaquinone) caused a rather inconspicuous decrease of the GSH content in G6PD-deficient but not in normal erythrocytes. However, the postulated role of dopa in the pathogenesis of favism was not borne out by the outcome of an experiment *in vivo* indicating that the survival of ^{51}Cr -labeled G6PD-deficient erythrocytes transfused into a normal individual was not impaired by repeated intravenous administration of dopa to the recipient (Gaetani *et al.*, 1970). Furthermore, as pointed out by Beutler himself, since L-dopa is being used extensively in rather large doses for treating Parkinson's disease, some of the Parkinsonian patients (with coexistent G6PD-deficiency) in favism-prone areas would be at risk of developing hemolytic crises in the course of therapy. To our knowledge, however, so far no single case has been reported in the literature to substantiate this expectation.

III. THE MECHANISM OF THE BIOCHEMICAL LESION UNDERLYING RED CELL DESTRUCTION IN DRUG-INDUCED HEMOLYSIS AND FAVISM

The hemolytic effect of the noxious drugs on G6PD-deficient erythrocytes appears to be attributable to their ability to function as reversible redox systems mediating the oxidation of the intracellular GSH (Emerson *et al.*, 1949; Beutler *et al.*, 1957). This property is shared also by divicine and isouramil, the pyrimidine aglycones of the fava bean glycosides vicine and convicine. In the normal red blood cell the oxidant effect of the drug is readily overcome by the coordinate action of the NADPH-generating pentose phosphate pathway and the NADPH-linked GSSG-reductase according to the following reaction scheme: [Eqs. (1)–(3)]:

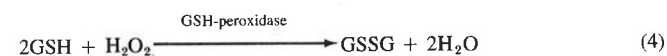


Under physiological conditions, the vestigial G6PD activity and perhaps also the limited capacity of GSSG reductase to use NAD as an alternate hydrogen

donor (Francoeur and Denstedt, 1954) enable the enzyme-deficient erythrocyte to maintain an adequate level of GSH compatible with a nearly normal or moderately reduced survival (Brewer *et al.*, 1961). This precarious metabolic equilibrium, however, breaks down under the stress conditions imposed by the oxidant compound. The resultant irreversible oxidation of GSH and the attendant catabolism of GSSG (Beutler, 1957) seem to constitute the major metabolic lesion leading to the eventual destruction of the enzyme-deficient erythrocyte. The validity of this concept, implying a vital role of GSH in preserving the structural integrity of the red blood cells, is strongly supported by the finding that the virtual absence of GSH in the blood cells of individuals affected with an inborn defect of its biosynthesis predisposes them to drug-induced hemolysis and favism (Oort *et al.*, 1961; Waller and Gerok, 1964; Boivin and Galand, 1965; Prins *et al.*, 1966; Minnich *et al.*, 1971). Similarly, congenital GSSG-reductase deficiency likewise manifests itself by drug sensitivity (Loehr and Waller, 1962; Waller *et al.*, 1965, 1969).

A major manifestation of the oxidant action of the drugs both *in vivo* and *in vitro* is the formation of methemoglobin and the concomitant appearance of Heinz bodies, which, according to Allen and Jandl (1961), represent a product of hemoglobin denaturation resulting from oxidation of its SH groups with concurrent formation of a mixed glutathione disulfide and loss of the heme group (see also Srivastava and Beutler, 1970; Bunn and Jandl, 1966; Jacob and Winterhalter, 1970; Jacob, 1970; Rachmilewitz *et al.*, 1969; Nagel and Ranney, 1973).

Cohen and Hochstein (1961, 1963, 1964) indicated that the oxidant drug or its active metabolite interacts with oxyhemoglobin, producing hydrogen peroxide. The relatively low but potentially harmful levels of peroxide cannot be efficiently destroyed by catalase and are normally eliminated through the action of GSH peroxidase (Mills, 1957, 1959, 1960; Mills and Randall, 1958), which catalyzes the following reaction [Eq. (4)]:



The sustained operation of this system is ensured by the concomitant regeneration of GSH, mediated by the NADPH-linked GSSG-reductase. Thus, the integrated pathway consisting of the oxidative pentose phosphate shunt (as a source of NADPH supply), GSSG-reductase, and GSH-peroxidase serves to detoxify the hydrogen peroxide, so as to obviate its deleterious effects on the red cell membrane and hemoglobin (see also Cohen, 1966; Flohé and Brand, 1969).

This concept, assigning a vital function to GSH-peroxidase in protecting the cell from the oxidative insult by the peroxide-forming drugs, has gained additional support from the recognition of a hereditary deficiency of this enzyme and its causative role in certain cases of spontaneous or drug-induced hemolytic anemia (T. Necheles *et al.*, 1968; Steinberg *et al.*, 1970; T. F. Necheles *et al.*,

1970; Boivin *et al.*, 1969, 1970). Furthermore, the recent discovery that GSH-peroxidase contains selenium as an integral and catalytically essential component of its molecule (Rotruck *et al.*, 1973; Flohé *et al.*, 1973) has led to the understanding of the biochemical mechanism underlying the protective effect of dietary selenium against hydrogen peroxide-induced hemolysis (Rotruck *et al.*, 1972).

Jacob and Jandl (1962a,b), in studying the effects of SH-binding compounds (*p*-hydroxymercuribenzoate, *N*-ethylmaleimide) on erythrocytes, emphasized the essentiality of the surface sulfhydryl groups for the structural intactness and normal survival of these cells. It should be pointed out, however, that contrary to the typical thiol reagents used in the above studies, primaquine and related drugs (Panizon and Zacchello, 1966; Beutler, 1966), as well as the fava bean pyrimidine aglycones (Mager *et al.*, 1965), do not induce an overt lysis *in vitro* but appear to exert their deleterious effect *in vivo* by rendering the red cells vulnerable to destruction by the reticuloendothelial system in the liver and spleen (Rifkind, 1965, 1966; Beutler, 1971). Consequently, it is not clear to what extent the conclusions drawn from the model experiments of Jandl and his associates are applicable to drug-induced hemolysis and favism.

Some investigators (Kosower *et al.*, 1969; Flohé *et al.*, 1971) suggested that in certain instances free radicals, rather than H_2O_2 , generated in the course of the metabolism of the noxious agent, may interact with GSH and protein thiols with the resultant formation of GSSG and protein S—S linkages.

Other studies have been concerned with the possible derangement of the energy-yielding metabolism as part of the mechanism underlying the drug-induced red cell hemolysis. It was observed by several authors that aerobic incubation of erythrocyte suspensions in the presence of primaquine or acetylphenylhydrazine resulted in a pronounced inhibition of glycolysis (Loehr and Waller, 1961; Kosower *et al.*, 1964) and a progressive decrease in the ATP level of the cells (Mohler and Williams, 1961; Loehr and Waller, 1961; Mager *et al.*, 1964). Essentially similar effects were obtained on incubating the red cells in the presence of divicine or isouramil (Mager *et al.*, 1965). Addition of glucose obviated the deleterious effects of the drugs in normal but not in G6PD-deficient erythrocytes. The primary site of the antimetabolic action of APH was traced to hexokinase (Kosower *et al.*, 1964; Mager *et al.*, 1964). Furthermore, the APH-induced inhibition of hexokinase was shown to be mediated by GSSG formation, thus representing a particular case of so-called disulfide poisoning previously described by Eldjarn and Bremer (1962).

While the potential significance of hexokinase inhibition in shortening the life span of the red cells need scarcely be elaborated, it remains to be seen whether this metabolic derangement plays an essential part in the actual mechanism of red cell destruction occurring in drug-induced hemolysis and favism (see Brewer *et al.*, 1964).

A number of enzymatic alterations in G6PD-deficient erythrocytes were re-

ported to occur independently of their exposure to noxious drugs (Schrier *et al.*, 1958, 1959; Larizza *et al.*, 1958). Observations on the decrease in the activities of NADPH-diaphorase (Jaffé, 1963), phosphomonoesterase (Oski *et al.*, 1963; Bottini and Modiano, 1965), and pyrophosphatase (Scheuch *et al.*, 1961; Brunetti *et al.*, 1962a,b) appear to be of particular interest. The reduced activity of these enzymes, which are known to be SH dependent, may be due to the inclement environment created by the diminished GSH level. Furthermore, it has been shown that GSSG inhibits the activity of a variety of enzymes, such as glucose-6-phosphate dehydrogenase, inorganic pyrophosphatase, triosephosphate dehydrogenase (Scheuch and Rapoport, 1962), hexokinase (Eldjarn and Bremer, 1962; Mager *et al.*, 1964), and ATPase (Kutscher, 1961).

IV. CONCLUDING REMARKS

The data reviewed in this chapter clearly indicate that, despite the considerable progress achieved in the research on favism, there are still serious gaps in our understanding of the pathogenesis of this disease. Particularly perplexing is the inadequacy of our current knowledge to account for the sporadic and rather capricious incidence of favism, as well as the absence of a clear-cut correlation between the degree of exposure of the susceptible individuals to the noxious principle of the fava plant and the occurrence of the hemolytic syndrome. It appears reasonable to surmise that the epidemiology of favism is governed not only by genetic factors, but also by a number of environmental determinants, such as variations in the amount of the toxic principle present in different varieties of the *Vicia faba* plant, as well as differences in the food and cooking habits of the G6PD-deficient subjects in the various populations.

The major and most urgent issue, however, is the definitive establishment of the chemical identity of the causative agent of favism. It is obvious that the achievement of this goal would greatly contribute to the elucidation of other aspects of this disease and might also provide a rational basis for a prophylactic and therapeutic approach.

ACKNOWLEDGMENTS

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CHAPTER 10

Allergens

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I. INTRODUCTION

Allergens hold a somewhat anomalous position in a discussion of naturally occurring food toxins. The true toxins are undesired constituents of some foods and exhibit their effects on anyone who consumes them. The severity of such toxic effects is roughly proportional to the quantity consumed. On the other hand, allergens are usually normal food constituents, and the abnormality rests in