METABOLIC EFFECTS OF PYRIMIDINES DERIVED FROM FAVA BEAN GLYCOSIDES ON HUMAN ERYTHROCYTES DEFICIENT IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Susceptibility to favism, an acute hemolytic crisis following ingestion of fava beans, has been shown to be associated with a genetically determined propensity to drug-induced hemolysis (see Tarlov et al., 1962). The inborn error responsible for the abnormal vulnerability of the susceptible RBC was traced to their deficiency in G6PD (Carson et al., 1956). The blockade of the main route of supply of NADPH inherent in the enzymic defect and the consequent shut-off of the NADPH-linked GSSG reductase accounted for the characteristic "GSH instability" of the drug-sensitive RBC, i.e. the inability of glucose to obviate the fall of their GSH content occurring upon exposure to a challenging drug, such as APH (Beutler et al., 1957).

In view of the essential identity of the pathogenetic mechanism underlying both favism and "drug sensitivity", it appeared reasonable to anticipate that the causative agent of favism might be a component of the fava beans endowed with a pronounced GSH-oxidizing capacity. To our knowledge, however, apart from some presumptive

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Abbreviations: RBC, red blood cells; G6PD, glucose-6-phosphate dehydrogenase; APH, acetylphenylhydrazine.

evidence for the occurrence of the postulated factor in crude fava bean extracts (Walker and Bowman, 1960), no clue as to its chemical nature has been afforded until now

In the course of fractionation of fava bean extracts (Bien et al.), the search for the active principle was guided by its discriminatory property of inducing GSH depletion in G6PD-deficient but not in normal RBC, when incubated in the presence of glucose. Some of the isolated fractions conforming to this criterion, exhibited in the presence of air a tendency to undergo rapid oxidation, attended by a loss of activity. The structural instability linked to a characteristic redox pattern and the relatively low solubility in water of the active fractions were reminiscent of the properties of divicine (2,4-diamino-5,6-dihydroxypyrimidine) and isouramil (4-amino-2,5,6-trihydroxy-pyrimidine), substances known as the aglycon moieties of the β-glycosides occurring in fava beans, vicine and convicine respectively (Bendich and Clements, 1953). As part of a continuing effort towards the characterization of the active fractions isolated from the fava beans, it was decided to explore the effects of the pyrimidine aglycons on certain metabolic parameters, which had been implicated previously in the mode of action of APH (Mager et al., 1964).

RESULTS

As shown in Table I, suspensions of washed human RBC exhibited in the course of incubation with the pyrimidine aglycons of vicine and convicine (to be referred to as "fava pyrimidines") a drastic fall of GSH, followed by a marked decline of their ATP content. The specificity of the mode of action of these compounds was indicated by the failure of added glucose to modify the course of events in G6PD - deficient RBC, contrasting with its pronounced protective effect on normal erythrocytes. Thus, the fava pyrimidines as well as their synthetic congener, isouramil, shared with APH and ascorbic acid the ability to differentiate between G6PD - deficient and normal RBC, when examined under conditions similar to those prevailing in whole blood. The

TABLE I

Differential effects of fava pyrimidines on GSH and ATP levels in G6PD-deficient and normal erythrocytes; prevention of ATP decline by inosine.

Substances tested μmoles per ml incubation mixture	G6PD-deficient RBC				Normal RBC			
	with glucose		with inosine		with glucose		without glucose	
	GSH	ATP	GSH	ATP	GSH	ATP	GSH	ATP
None (control)	1.10	0.95	1.10	0.90	1.70	0.97	1.55	0.64
Isouramil (synthetic), 2	0.31	0.41	0.29	0.68	1.50	0.98	0.30	0.30
Aglycon of convicine, 2	0.36	0.38	0.29	0.66	1.60	1.02	----	----
Aglycon of vicine, (divicine), 2	0.29	0.38	0.33	0.65	1.55	0.99	----	----
Convicine, 2	1.10	0.95	----	----	1.7	0.98	1.55	0.65
Vicine, 2	1.10	0.95	----	----	1.72	0.97	1.60	0.63
Ascorbic acid, 30	0.19	0.53	0.11	0.85	1.7	0.92	0.25	0.29
Acetylphenylhydrazine, 30	0.28	0.30	0.22	0.52	1.2	0.80	0.07	0.18
Alloxan, 2	0.7	0.95	----	----	1.30	0.91	1.36	0.64
Dialuric acid, 2	----	----	----	----	1.21	0.94	0.85	0.62

Erythrocytes twice washed with a solution of isotonic saline - 0.01 M K-phosphate buffer (pH 7.4) were suspended in an equal volume of the same medium, supplemented with the various substances tested, as specified. Where indicated, glucose or inosine were added at a final concentration of 0.01 M. Following incubation with continuous shaking for 3 hours at 37°C, the cells were washed again, resuspended in phosphate-buffered saline and samples were taken for determining GSH (Ellman, 1959) and ATP (Strehler and Totter, 1954). The results were computed in terms of μmoles per ml packed RBC. The packed cell volumes of the samples were estimated by the conventional microhematocrit procedure. Convicine and vicine were isolated from fresh fava beans by a modified procedure of Ritthausen (1896). The aglycons were prepared by incubating the fava glycosides under nitrogen with a suitable amount of β-glucosidase (almond emulsin, obtained from "Calbiochem") in 0.01 M phosphate buffer until complete splitting was achieved. The extent of enzymic hydrolysis was gauged by the amount of glucose released, as determined with the "Glucostat" reagent ("Worthington Biochemical Corporation"). Isouramil was synthesized as described by Davoll and Laney (1956).

activity of the pyrimidines, however, as tested in the present system, was roughly ten or twenty-fold higher, on a molar basis, than that of APH or ascorbic acid, respectively. On the other hand, the divergent behavior of dialuric acid, structurally related to isouramil, appears to be due to the tendency of its oxidized derivative,

alloxan, to form with GSH a characteristic addition compound (Patterson et al., 1949), rather than to function as a hydrogen acceptor.

Addition of inosine prevented to a considerable extent the loss of ATP elicited by the pyrimidines (Table I). It was inferred from this observation that the impairment of energy metabolism underlying the ATP decay, may be attributable to GSSG - induced inhibition of hexokinase, similar to that previously described by us in APH - treated RBC (Mager et al., 1964). This conclusion was further corroborated on the enzymic level by the demonstration of hexokinase inhibition in the stroma-free hemolyzates derived from pretreated RBC (Table II).

TABLE II

Fall of GSH and ATP and inhibition of hexokinase activity

Substances added to the RBC suspensions at the start of preincubation	GSH at the end of preincubation	ATP at the end of preincubation	Hexokinase activity
μmoles per ml incubation mixture	μmoles per ml RBC	μmoles per ml RBC	μmoles GSH produced per hour per amount of hemolyzate equivalent to 1 ml RBC
None (control	1.80	0.73	9.5
Isouramil, 2	0.19	0.53	1.8
Ascorbic acid, 50	0.18	0.51	1.2
Acetylphenylhydrazine, 30	0.19	0.33	0.8

Suspensions of normal RBC in phosphate-buffered saline (pH 7.4) were supplemented with the various substances tested, as specified. At the end of 3 hours' incubation with shaking at 37°C, the RBC were washed twice with saline-phosphate and hemolyzed by adding an equal volume of distilled water to the packed cells. The hemolyzates were spun for 15 min at 21,000xg and the stroma-free supernatant fluids were assayed for hexokinase activity (see Mager et al., 1964) in the following reaction mixture: 0.5 ml hemolyzate, 25 μmoles tris buffer (pH 7.4), 1.5 μmoles $MgSO_4$, 5 μmoles glucose, 0.75 μmoles NADP, 0.75 μmoles ATP, 2.5 μmoles creatine phosphate, 10 μg creatine kinase, 2.5 μmoles GSSG; total volume 0.75 ml. The values were corrected for these obtained in "no glucose"-controls. Other details as in Table I.

The fava pyrimidines were able to oxidize GSH in pure solution, in contradistinction to APH and primaquine which require the presence of RBC or hemoglobin for catalyzing GSH oxidation (Beutler et al., 1957). The oxidation of GSH by the pyrimidines proceeded to completion even at a ten-fold or higher molar ratio of GSH to pyrimidine. Furthermore, it was considerably enhanced by shaking in air and completely suppressed in an atmosphere of pure nitrogen. The non-stoichiometric nature, as well as the apparent oxygen-dependence of the oxidation of GSH by the pyrimidines, suggested that this reaction is mediated by a catalytic oxido-reduction mechanism, similar to the well known GSH-ascorbic acid redox system (Borsook et al., 1937). The latter conclusion was also consistent with the virtual inertness displayed in this system by convicine and vicine (see Table I), in which the readily oxidizable 5-hydroxyl group of the pyrimidine aglycons is blocked by the β-glycosidic linkage. It may be mentioned in this connection that the fundamental similarity in the chemical properties of the fava pyrimidines and ascorbic acid has been noted by Bendich and Clements (1953) and ascribed by them to the structural resemblance of the carbonyl-conjugated aminoenol and enediol rings of these compounds.

DISCUSSION

The overall pattern of metabolic disturbances resulting from incubation of G6PD-deficient RBC with the pyrimidine aglycons of vicine and convicine is essentially identical to that elicited by treatment with APH. These pyrimidines may arise from the parent fava glycosides either in the beans or in the digestive tract through the hydrolytic action of β-glycosidases.

The powerful capacity for oxidizing GSH exhibited by the pyrimidine aglycons in vitro, is consistent with a possible causative role of these substances in precipitating the favic crises. Moreover, the conceivable vicissitudes in the availability of suitable conditions for enzymic release of the aglycons from the fava glycosides, as well as the particular lability of these compounds might account for the puzzling

irregularity characterizing the occurrence of favism in susceptible individuals, irrespective of the degree of their exposure to the noxious agent (see Tarlov et al., 1962).

The true significance of fava pyrimidines in the etiology of favism cannot be assessed, however, without recourse to proper clinical tests.

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