DEVELOPING AN ANIMAL MODEL FOR THE STUDY OF FAVISM

by

Rosemary L. Milne

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Presented to the University of Manitoba
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This thesis is dedicated to

John M. Key

ABSTRACT

Dietary manipulations and chemical drugs have been utilized to create an animal model with low blood GSH concentrations so as to mimic the situation in the favic red blood cell. Four methods were used to deplete blood GSH levels in rats, including feeding a riboflavin deficient diet, feeding a riboflavin antagonist, (galactoflavin), orally administering a GSH depleting chemical agent (vinylidene chloride) and finally administering intraperitoneal injections of BCNU, an irreversible inhibitor of erythrocyte glutathione reductase. The rat blood models were then challenged with vicine, to determine whether the same response can be produced as with a favic individual.

The galactoflavin treated rats proved to be the most successful in vivo animal model. The rats rapidly became acutely deficient in riboflavin after 28- days. Galactoflavin had the effect of lowering the activity of erythrocyte glutathione reductase and hence the reducing capacity of the cell. The rat model, as a consequence, responded to the effects of vicine with a significant decline occurring in blood GSH ($p < .0005$), hemoglobin ($p < .006$), hematocrit ($p < .0001$) and an increase in spontaneous hemolysis ($p < .0001$). The riboflavin deficient animal model, in comparison, was only partially successful, as the rats did not become as deficient and so erythrocyte glutathione reductase activites were not diminished to the same extent. However, blood GSH concentrations were lowered ($p < .0001$) and the rats did show a small but significant decline in blood GSH ($p < .006$), hemoglobin

($p < .085$) and hematocrit ($p < .084$) and an increase in spontaneous hemolysis ($p < .088$), when fed vicine.

The BCNU animal model had the lowest blood GSH concentrations compared to any of the other blood models, with erythrocyte glutathione reductase activites being depressed by 83% in 48 hours. The rats, however, showed no response to dietary vicine ($p > .05$). This may have been due to an insufficient amount of vicine being hydrolysed in the hind gut, as the rats suffered from a loss of appetite and diarrhea. Vinylidene chloride had no effect on blood GSH levels and has no potential for use as a model for studying favism.

It may be concluded that the galactoflavin animal model sensitizes erythrocytes to the effects of the pyrimidine aglycones in vivo and can be produced in a shorter period of time compared to the riboflavin animal model. BCNU treated rats provide a very useful blood model for studying the effects of vicine in vitro and has potential, in vivo, if divicine can be administered in some other way and not via the gut.

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List of Abbreviations

AC	activity coefficient
C	Celsius
cm	centimetre
BCNU	1,3-bis (2-chloroethyl)-1-nitrosourea
EGR	erythrocyte glutathione reductase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidised glutathione
HMP	Hexose Monophosphate Pathway
H_2O_2	hydrogen peroxide
I.P.	intraperitoneal
kg	kilogram
ml	milliliter
min	minute
M	molar
mM	millimolar
µm	micromole
$^1O_2$	singlet oxygen
O_2^-	superoxide
nm	nanometre

List of Abbreviations continued

$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
6PG	6-phosphogluconate

Introduction

The utilization of faba beans as a cheap and abundant protein source in North Africa and the Middle and Far East presents certain problems due to the presence of various toxic constituents which can cause favism in certain susceptible individuals. Favism is characterized as an acute hemolytic anemia which can be fatal (Belsey, 1973; Luisada, 1941). The major factor predisposing an individual to favism is an inherited deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD)(Carson et al., 1956) which, due to its inability to function normally, causes a subnormal level of blood GSH (Yoshida, 1973; Kirkman et al., 1975). The active factors which are now generally accepted to be causative agents of favism are the aglycones of vicine and convicine, namely divicine and isouramil respectively (Chevion et al., 1982; see Mager et al., 1980). The hemolytic crisis is thought to be caused by the oxidative effect divicine and isouramil impose on the already low GSH levels (Mager et al., 1965; Chevion et al., 1982; Arese et al., 1981).

Since it is impraticable to depend on blood from a G6PD deficient subject to study how the aglycones exert their effect, it would be very useful to create a biological animal model. The first successful animal model which has been developed is the laying hen (Muduuli et al., 1981b). However, although this model proved to be very effective, it is limited when being compared to the human situation since it is not mammalian and therefore results cannot necessarily be extrapolated to the human situation. This necessitates the development of a mammalian animal model whose erythrocytes are a mimic of a G6PD deficient individual.

Homeostasis of GSH in the red blood cell is maintained by a closely linked series of reactions. Glucose-6-phosphate dehydrogenase is responsible for the generation of NADPH which is required by erythrocyte glutathione reductase (EGR) to convert oxidized glutathione to reduced glutathione (see Beutler, 1978; Beutler, 1971). The GSH generated is subsequently utilized by glutathione peroxidase for the detoxification of peroxides (Beutler, 1971). The regeneration of GSH can be impaired by lowering the activity of EGR. This can be achieved by feeding rats a riboflavin deficient diet or one incorporated with a riboflavin antagonist such as galactoflavin, thereby removing the precursor for the synthesis of FAD which is the cofactor for this enzyme (Meister & Anderson, 1983). Another method which can be employed is to administer to rats the cancer chemotherapy drug BCNU , which is an irreversible inhibitor of EGR (Frischer & Ahmad, 1977). Blood GSH levels can be lowered directly by using chemical depleting agents such as vinylidene chloride (Younes & Siegers, 1980). The riboflavin deficient animal model was the model used by D'Aquino et al. (1979) to study the effect divicine and isouramil had in vitro on erythrocytes derived from these rats.However, the effect vicine has in vitro may differ under physiological conditions in vivo. In this study, the 4 models mentioned were used in an attempt to reduce blood GSH levels and thereby sensitize the erythrocytes to the in vitro effects of vicine.

REVIEW OF LITERATURE

Etiology of Favism

Brief History and Description of the Disease

Favism was the term used by an Italian physician, Montaro, in 1894 to describe the acute hemolytic disorder caused in certain glucose-6-phosphate dehydrogenase (G6PD) deficient individuals following the ingestion of faba beans (Sansone et al., 1958). The disease dates as far back as the ancient Greeks where the mathematician Pythagoras recognized the presence of toxic constituents in the beans and based a religion on the sinfulness of eating them (Waldron, 1973). The Romans also noted the lethargic and dulling effect the beans had (Renfrew, 1973). The hemolytic crisis has not only been reported to be induced by the ingestion of faba beans but also by inhalation of the bean pollen (Hedayat et al., 1971). The main symptoms characterising favism include headache, malaise, dizziness, nausea, vomiting, pallor, abdominal pain and fever (Luisada, 1941; Hedayat et al.,1971). The onset of the symptoms is usually within 5-24 hours after ingestion of the faba beans and within seconds after inhalation of the bean pollen (Luisada, 1941). Symptoms associated with severe hemolytic attacks tend to be more specific and include hemoglobinuria appearing between 5-30 hours following pollen or bean exposure (see Beutler, 1972a), jaundice and anemia,which varies depending on the severity of the attack (Luisada, 1941; Kattamis et al., 1969) and occassionally acute renal failure (Schmitz & Fritz, 1968). Kattamis et al. (1969) reported in a study carried out on 120 hospitalized patients suffering from a favic attack that 81% had a hemoglobin concentration

below 6 g/100ml and in 30% it was less than 4 g/100ml. The hemoglobin range in some of the severest cases was between 1.8 & 3 g/100ml but adequate blood transfusion enabled a swift recovery. The duration of an attack lasts between 2-6 days, the acute stage occurring in the first 2 days followed by a rapid spontaneous recovery (see Mager et al., 1980). Fatalaties usually occur on the second day with very few thereafter (Luisada, 1941).

Epidemiology of the Disease

Favism is very prevalent in Mediterranean and Middle East regions where both the enzyme defect and faba bean consumption are widespread (Belsey, 1973). Ten to fifteen per cent of the world's faba beans are grown in Western Asia and North Africa where it represents an important source of dietary protein (Hawtin & Stewart, 1979). Countries incurring frequent outbreaks of favic attacks include Italy (Sansone et al., 1958), Greece (Kattamis et al., 1969), Rhodes (Kattamis et al., 1969), Sardinia (Crosby, 1956), Cyprus (Joannides, 1952), Lebanon (Shahid, 1960), Algeria (Messerschmitt et al., 1967), Egypt (Belsey, 1973), Iraq (Amin-Zaki et al., 1972), Iran (Lapeyssonnie & Keyhan, 1966) and the Sudan (Hassan, 1971). The occurrence of Favism in other parts of the world is a rarity but cases have been reported from France (Auquier et al., 1968), Germany (Gehrmann et al., 1963), Poland (Sroczynska & Sychlowy, 1973), Thailand (Panich & Na Nakom, 1973) and England (Holt & Sladden, 1965).

Epimediological surveys in countries predisposed to Favism have shown a seasonal pattern in the incidence of the disease corresponding to the harvesting of the bean (Crosby, 1956; Kattamis et al., 1969).

Predisposition to Favism

a) Age

Favism tends to occur more frequently in children with a variation in the age distribution (Belsey, 1973). Kattamis et al. (1969) observed in a survey carried out in Greece that 65% of the cases belonged to the 2 - 5 year age group with a steady decline in cases occuring in the 10-15 year age group. Favism occurring in breast fed infants, caused by the unaffected mother ingesting fababeans and transmitting the toxic constitutents via the milk, is not a rare occurrence and such cases have been well documented (Kattamis et al., 1969, 1971; Emanuel & Schoenfeld, 1961). Mortality is highest in children with relatively few deaths occurring in adults (Luisada, 1941). Lapeyssonnie & Keyhan (1966), finding a strong correlation between mortality and age, classified the pair into three main groups: 4.7% for children less than 2 years, 1.11% for those aged between 2-4 years and zero for those aged between 5-16 years.

b) Sex

Favism has a strong predilection for males. The ratio between male and female cases ranged from 3.2:1 in Iran (Hedayat et al., 1971), 21:1 in Cyprus (Joannides, 1952) and 6.2:1 in Greece (Kattamis et al., 1969). These apparent ratios can be explained by the fact that G6PD deficiency is a sex-linked trait with intermediate dominance (Childs et al., 1958). A gene located on the X chromosome is responsible for the transmission of the G6PD deficiency (see Mager et al., 1980). This deficiency will manifest itself and be fully expressed in the hemizygous male ($\overline{X}Y$) as the normal allele (Y) does not override the effect of the mutant gene ($\overline{X}$) (see Mager et al., 1980). In the heterozygous female ($\overline{X}X$) studies conducted

by Motulsky (1964) have shown G6PD activity to be normal or totally absent. Larizza et al (1960), however, reported that the vast majority of heterozygous females had an intermediate enzyme deficiency. These individuals were found to have a mixture of normal and G6PD deficient erythrocytes (see Beutler, 1972b). This cellular mosaicism can be explained in terms of the Lyon theory where either the paternal or maternal X chromosome is inactivated in the cell at morphogenesis (Lyon, 1961). The severity of the hemolytic attack will ultimately depend on the relative ratio of G6PD deficient and normal erythrocytes in the blood (see Mager et al., 1980). Full expression of G6PD deficiency in the female is uncommon because as the gene is recessive a homozygous mutant genotype ($\overline{XX}$) would have to appear and this is a statistically rare occurrence (see Mager et al., 1980).

c) Glucose-6-Phosphate Dehydrogenose Deficiency

G6PD deficiency has been estimated to affect 100 million people of varying races in the world (Carson, 1960). This defect was first discovered when American Negro soldiers developed an acute hemolytic anemia when taking primaquine, an antimalarial drug (Carson et al., 1956). The degree to which this intrinsic abnormality of the erythrocyte occurs varies considerably in different ethnic groups, the trait having the highest frequency in Kurdish, Iranian and Iraqi Jews (Szeinberg et al., 1958; Szeinberg & Sheba, 1960), Sardinians (Siniscalco et al., 1961), Greek Cypriots (Plato et al., 1964) and in certain African and Asian populations (see Mager et al., 1980). Nearly all of these populations reside in malarial areas, probably due to the selective advantage this inheritant disorder has in offering some degree of immunity to

falciparum malaria (Motulsky, 1964). The trait is virtually absent in Eskimos and some American Indians, Japanese, Northern European and American caucasians and Ashkenazic Jews (of European descent) (Motulsky, 1960).

Characteristics Between Normal and Mutant G6PD

G6PD is responsible for catalysing the initial step in the Hexose Monophosphate Pathway (HMP) of carbohydrate metabolism, converting glucose-6-phosphate (G6P) to 6-phosphogluconate (6PG), with the subsequent reduction of $NADP^+$ to NADPH (Figure 1) (see Beutler, 1978). G6PD exists in several oligomeric forms but mainly in the dimeric state, which is the catalytic active species (Yoshida, 1973). The HMP accounts for approximately 10% of glucose metabolism in the red blood cell, the remaining 90% being metabolised via the Embden-Meyerhoff pathway (see Beutler, 1978). The main function of the HMP is to maintain $NADP^+$ in its reduced form as NADPH (see Beutler, 1978). NADPH is required by glutathione reductase to act as a coenzyme in the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH) (Figure 2) and acts as a reducing agent for the cleavage of mixed disulphides (Beutler, 1971). GSH plays an important role in reductive processes which are essential for the synthesis of the deoxyribonucleotide precursors of DNA, the formation of proteins and the regulation of enzymes (Meister & Anderson, 1983). GSH also functions in the protection of cells against reactive oxygen compounds and free radicals, and is required by the enzyme glutathione peroxidase to detoxify hydrogen peroxide (H_2O_2) (Beutler, 1971).

The HMP is regulated mainly by the availability of $NADP^+$. The ratio of NADPH/$NADP^+$ is maintained at a very high level under normal

Figure 1. The Hexose Monophosphate Pathway (HMP)

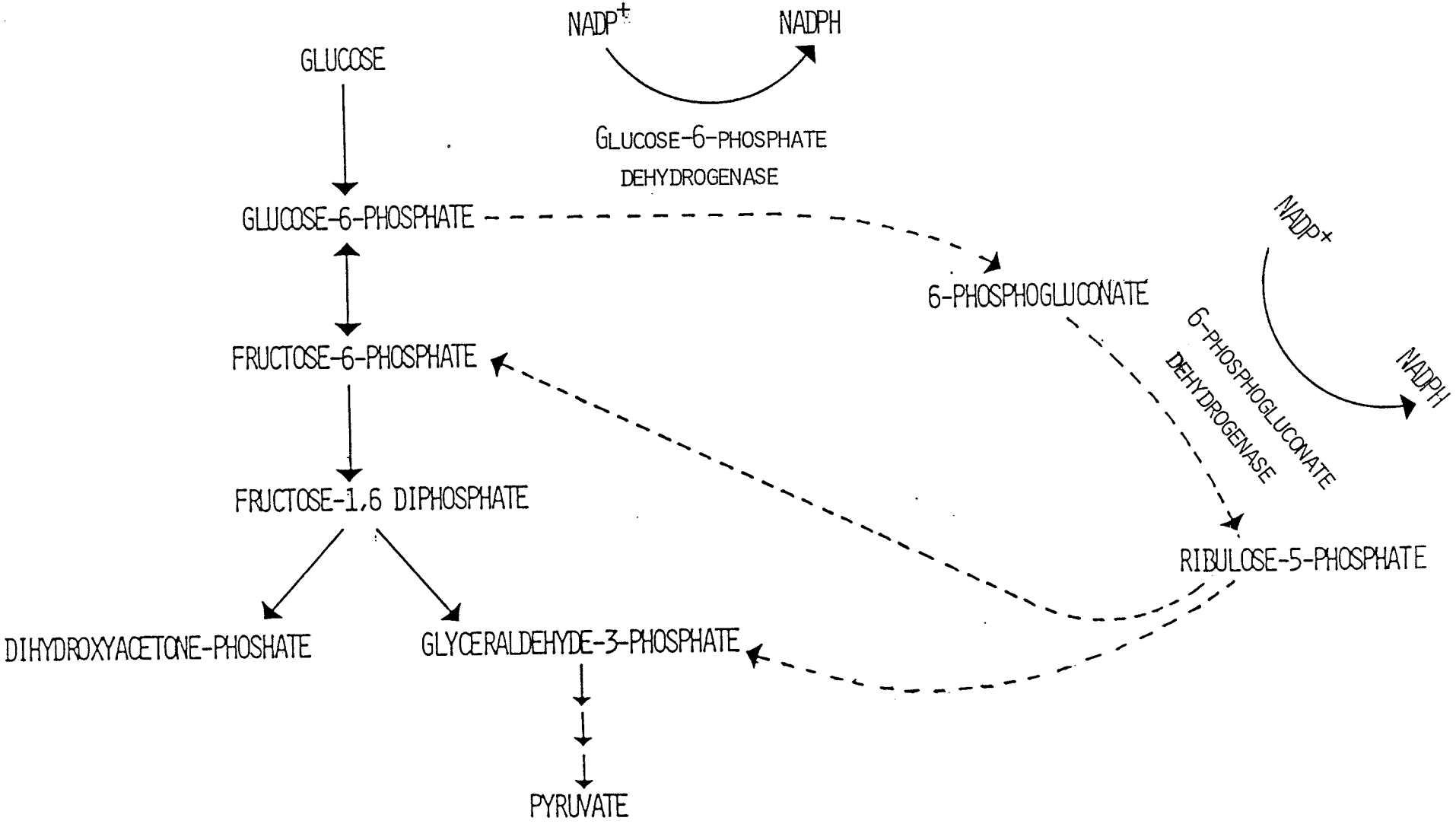
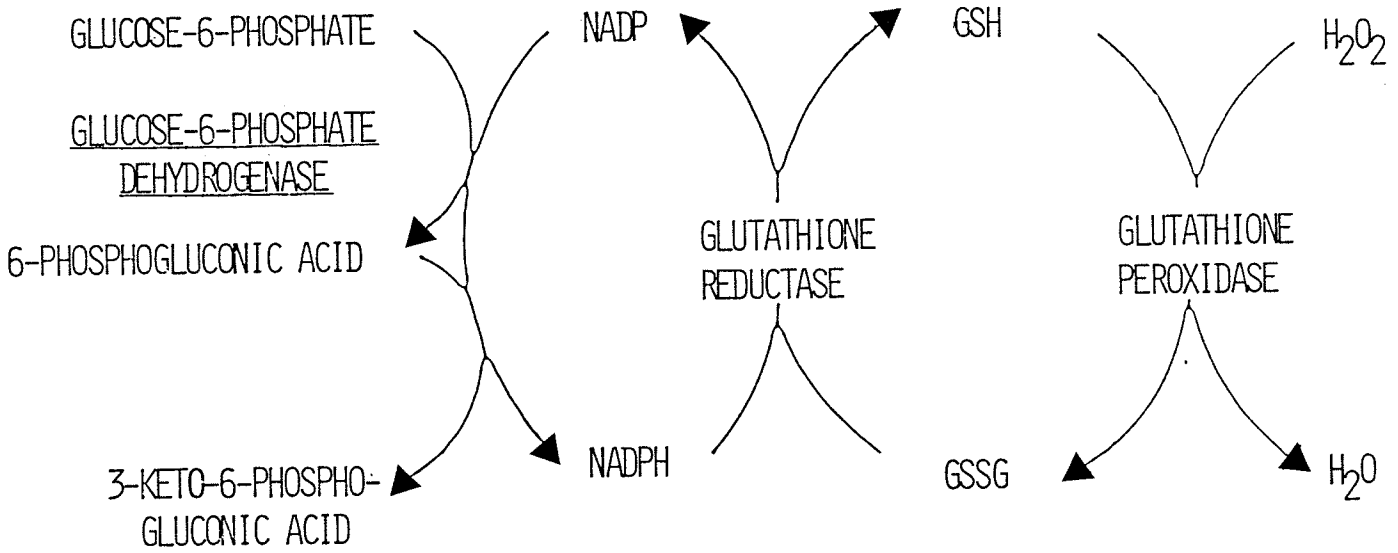

NADP+
NADPH
GLUCOSE
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
GLUCOSE-6-PHOSPHATE
6-PHOSPHOGLUCONATE
NADP+
NADPH
6-PHOSPHOGLUCONATE DEHYDROGENASE
FRUCTOSE-6-PHOSPHATE
FRUCTOSE-1,6 DIPHOSPHATE
RIBULOSE-5-PHOSPHATE
DIHYDROXYACETONE-PHOSHATE
GLYCERALDEHYDE-3-PHOSPHATE
PYRUVATE

Figure 2. Reactions maintaining red blood cell GSH homeostasis.


GLUCOSE-6-PHOSPHATE
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
6-PHOSPHOGLUCONIC ACID
3-KETO-6-PHOSPHO-GLUCONIC ACID
NADP
NADPH
GSH
GSSG
GLUTATHIONE REDUCTASE
GLUTATHIONE PEROXIDASE
H_2O_2
H_2O

conditions (see Beutler, 1978). Under these circumstances G6PD is inactive because it is strongly inhibited by ATP and NADPH (Luzzatto, 1967; Yoshida, 1973). When the red blood cell is challenged with an oxidant stress, the intracellular concentration of $NADP^+$ will rise causing the NADPH/ $NADP^+$ ratio to fall, thereby releasing G6PD from inhibition. Hence, more glucose will be diverted to flow through the HMP, whose activity will also be markedly increased (see Beutler, 1978). The oxidant effect is thus overcome as the stimulation of G6PD leads to the generation of a sufficient supply of NADPH to reduce the elevated level of GSSG. In the G6PD deficient erythrocyte, NADPH and GSH levels are low (Kirkman et al., 1975; Beutler et al., 1955) but the cell is still capable of carrying out its metabolic activities. When challenged with an oxidant drug these cells cannot generate sufficient NADPH to meet the increased demand for GSSG reduction (Carson et al., 1956). Since this will ultimately lead to GSH homeostasis being affected, the cell is rendered vulnerable to oxidative damage. Erythrocytes are almost totally dependent on the HMP for their supply of NADPH as they, unlike other tissues, do not possess Krebs's oxidative enzymes and so cannot obtain NADPH from this cycle (Beutler, 1971). This increases a G6PD deficient individual's vulnerability to the effect of lesions involving the HMP pathway.

More than 150 variants of G6PD have been discovered (see Beutler, 1978) all of which can be distinguished on the basis of differing kinetic characteristics, electrophoretic mobilities, substrate specificity, Michaelis constant values for G6P & $NADP^+$ and heat stability (Yoshida, 1971, Kirkman et al., 1964). Approximately 35 of these variants cause a severe enzyme deficiency in erythrocytes rendering them susceptible to

hemolysis when challenged with exogenous agents such as drugs and faba beans (see Beutler, 1978; Yoshida, 1971).

d) Other Factors

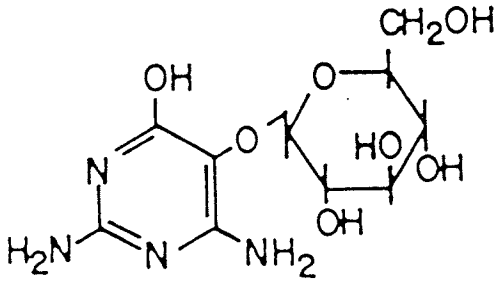
It has been inferred that some other factor in addition to G6PD deficiency are important for the development of favism as not all G6PD deficient individuals develop favism. Stamatoyannopoulos et al. (1966) postulated that this factor was inherited as an autosomal gene in the heterozygous state. Factors including blood glucose level and pre-existing hemoglobin levels may affect the severity of the attack in susceptible individuals (Belsey, 1973). Malnutrition, particularly protein deficiency may also be significant due to the resultant effect it may have on enzyme systems and glutathione synthesis (Belsey, 1973).

Causative Agents and their Role in the Etiology and Pathogenesis of Favism

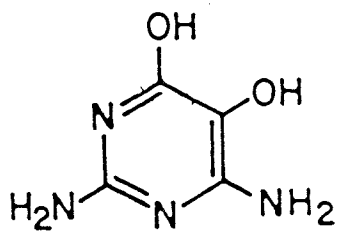
Causative Factors

Crosby in 1956 was the first to point out the close resemblance between favism and primaquine induced hemolytic anemia. The main factors in faba beans which have been implicated as being possible hemolytic principles include the pyrimidine aglycones of vicine and convicine & 3, 4-dihydroxyphenylalanine (DOPA). The aglycones, divicine & isouramil are derived from their respective glycosides by enzymatic splitting with β-glucosidase or mild acid hydrolysis(see Mager et al,1980,Figure 3).Vicine is known to occur in vetch seeds (Ritthausen & Kreusler, 1870), beet juice and peas (Schulze, 1893 & Bendich & Clements, 1953) as well as faba beans. Virtually nothing is known of the metabolic origin of vicine or what

Figure 3. Structure of Vicine & Convicine & their Aglycones



Hydrolysis by Acid or β-glucosidase



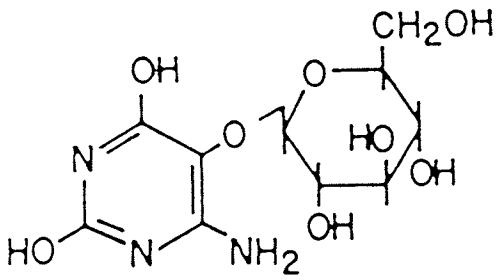
+ Glucose

Vicine ($C_{10}H_{16}O_7N_4$)

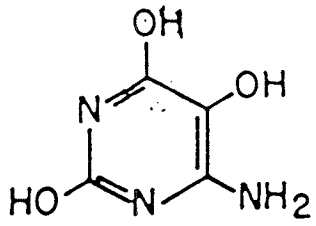
(2,6-diamino-4,5-dihydroxy pyrimidine -5(β-D-glucopyranoside))

Divicine

(2,6-diamino-4,5-dihydroxy pyrimidine)



Hydrolysis by Acid or β-glucosidase



+ Glucose

Convicine ($C_{10}H_{15}O_8N_3$)

(2,4,5-trihydroxy, 6-amino pyrimidine) -5(β-D-glucopyranoside)

Isouramil

(2,4,5-trihydroxy, 6-amino pyrimidine)

role it plays in the plant (Brown & Roberts, 1972). The structures of vicine (2, 6-diamino - 4, 5-dihydroxy pyrimidine 5-[β-D-glucopyranoside]) was first revealed by Bendich & Clements (1953) and that of convicine (2,4,5 - trihydroxy-6-aminopyrimidine 5-[β-glucopyranoside]) was reported later by Bien et al. (1968). The structures of vicine and convicine and their aglycones divicine (2,6-diamino-4,5-aminopyrimidine) & isouramil (2,4,5-trihydroxy, 6-aminopyrimidine) can be seen in Figure 3. Studies carried out by Hegazy & Marquardt (1984) with rats revealed that no dietary vicine and convicine appeared in the blood, liver, kidney or muscle tissue,but a certain amount of these glycosides were very rapidly hydrolysed by digesta in the large intestine and cecum, with a slow rate of hydrolysis occurring in the stomach and small intestine. In vitro studies showed that no hydrolysis of vicine and convicine occurred in liver, kidney, muscle and cecal wall homogenates. When high levels of these compounds were incorporated into the diet, small quantities appeared in the urine and feces, thereby suggesting that the remaining glycosides were hydrolysed mainly in the hind gut. Micro-organisms present in the hind gut possessing a β-glucosidase-like enzyme are thought to be responsible for the hydrolysis of vicine and convicine to their respective aglycones (Frohlich & Marquardt, 1983). The aglycones can also be produced in vitro by mild acid hydrolysis using 0.IM HCl at $37^{o}C$ but this rate of hydrolysis is much slower compared to the enzymatic splitting action (Frohlich & Marquardt, 1983).

Divicine and isouramil are both strong reducing agents as can be seen by their capacity to readily reduce alkaline solutions of phosphomolybdate and dichlorophendophenol (Bendich & Clements, 1953). They are highly unstable in the presence of oxygen, the oxidative breakdown being

most rapid at an alkaline pH (Chevion et al., 1982). The strong reducing tendencies and marked instability of divicine and isouramil can be attributed to these structures possessing an enolic hydroxyl group at C-5 on the pyrimidine ring (Bendich & Clements, 1953). However, the reducing tendencies are further enhanced by the presence of the hydroxyl group on C_4. These authors went on to show by various structural substitutions that vicine and convicine share none of these properties because the enolic hydroxyl group at C-5 is blocked by the glycosidic linkage. They are not reducing agents and are very heat stable in solution.

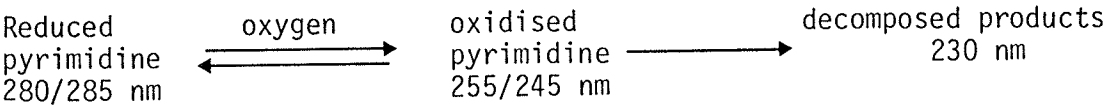
DOPA occurs in large quantities in faba beans, mainly in the free state (Andrews & Pridham, 1965). It has also been considered as being one of the active factors although to a much lesser extent compared to vicine and convicine. DOPA and ascorbate may act synergistically with vicine and convicine (Beutler, 1970).

Effect of Divicine and Isouramil on Red Blood Cell Metabolism

a) Reaction with Oxygen

An aqueous solution of isouramil and divicine in atmospheric nitrogen exhibits a single absorption peak at 280 nm and 255 nm, respectively, in the ultraviolet spectrum (Bendich & Clements, 1953). The pyrimidine aglycones are in the reduced form at this absorption band, the spectrum remaining constant providing oxygen is absent. Studies carried out by Chevion et al. (1982) revealed the shift in absorbance to 255 nm and 245 nm for isouramil and divicine upon exposure to oxygen. The peaks then disappeared with an increase in non-specific "end absorption" occurring due to further oxidative decomposition of the pyrimidine ring. A summary of these changes in absorption in the ultraviolet spectrum are

given below.



(Chevion et al., 1982).

The rate of the above reaction is strongly pH dependent and was found to be optimal at an alkaline pH (Chevion et al., 1982). In a subsequent series of experiments these authors found that H_2O_2 is formed as a by-product of this reaction but does not react with either the oxidised or reduced pyrimidine. This was demonstrated by adding catalase (100µg/ml) to the aqueous isouramil and oxygen reaction mixture. The kinetics of the oxidation reaction was not seen to alter even when the concentration of H_2O_2 was as high as 1mM.

b) Reaction with GSH

When isouramil and divicine are incubated with normal and G6PD deficient red blood cells, GSH levels drop rapidly being swiftly followed by a drop in ATP (Mager et al., 1965). In a similar experiment, carried out by Arese et al. (1981), GSH levels were found to be rapidly re-generated in normal but not in G6PD deficient erythrocytes.

When GSH is placed in an oxygen saturated solution at room temperature and pH7, its oxidation is very slow (Brownlee et al., 1977). However, the reaction can be accelerated considerably by adding a catalytic amount of isouramil so that 80% of the GSH was oxidised within 15 minutes (Chevion et al., 1982). A similar result was obtained by Razin et al., (1968) who noted a 30 - 50% decrease in GSH content when 0.2 µmol/ml erythrocytes of isouramil was added to a red blood cell suspension over a 3-hour period. Even when the ratio of GSH to pyrimidine is

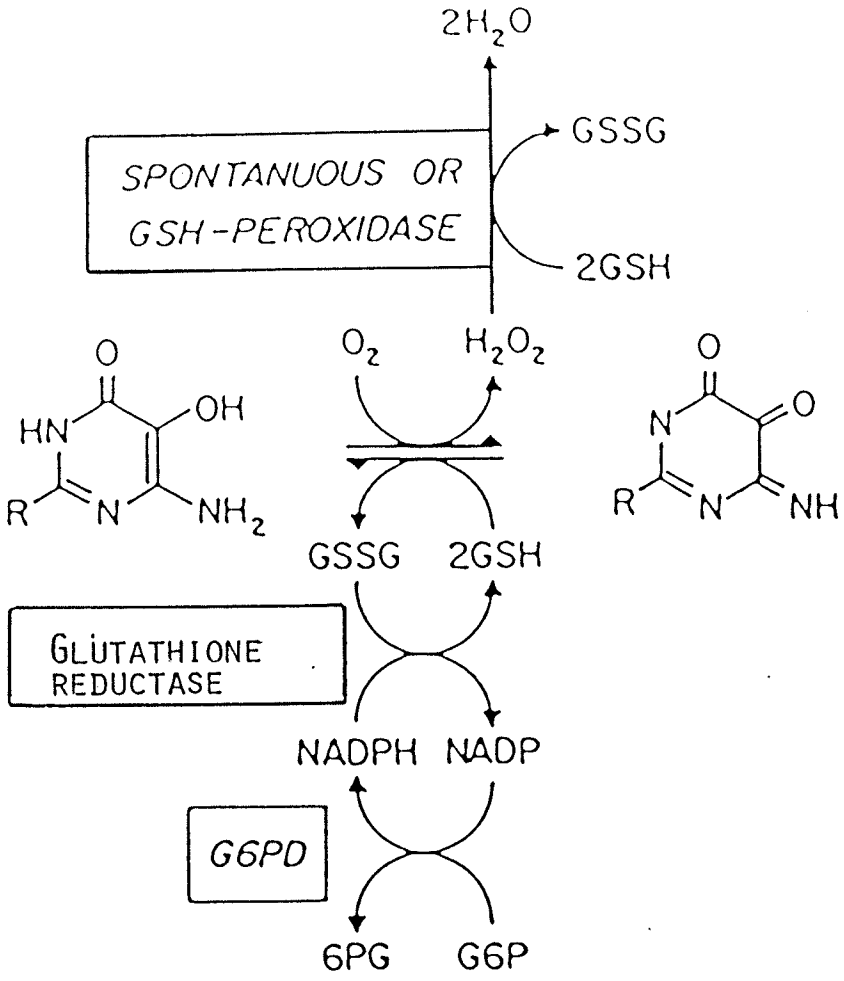
10 times or higher on a molar basis, the reaction still proceeds to completion,providing oxygen is present (Mager et al., 1965). The amount of H_2O_2 generated in this reaction did not exceed 10-15% of the oxidised GSH (Chevion et al., 1982). This low percentage can be accounted for by the H_2O_2 reacting with 2 molecules of GSH as follows:

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2H_2O$$ (Brownlee et al., 1977).

The non-stoichiometric nature of the reaction of the pyrimidine with GSH in the presence of oxygen suggests that it is mediated by successive reductions of isouramil or divicine by GSH, their subsequent reoxidation occurring by atmospheric oxygen (Razin et al., 1965). This mechanism is similar to the GSH ascorbic acid redox system (Borsook et al., 1937). Figure 4 illustrates the proposed catalytic oxido-reduction mechanism of faba pyrimidines in the red blood cell (Chevion et al., 1982). When a molecule of divicine or isouramil is oxidised in molecular oxygen, one molecule of H_2O_2 is liberated. Two molecules of GSH are dissipitated with the reduction of H_2O_2 to water. In order to complete the oxido-reduction cycle another 2 molecules of GSH are used to reduce the pyrimidine. Hence,overall 4 molecules of GSH are utilized in one oxido-reduction cycle of a faba pyrimidine. The rate of oxidation of GSH by isouramil was found to be lower in the presence of catalase,due to the acceleration of the conversion of H_2O_2 to water. This provides further evidence supporting this mechanism (Chevion et al., 1982).

Isouramil also reacts with GSH,forming a complex which is similar to that formed between structurally related alloxan and GSH (Patterson

Figure 4. The proposed mechanism for the action of isouramil and divicine in the red blood cell. (Chevion et al., 1982).


2H2O
GSSG
SPONTANUOUS OR
GSH-PEROXIDASE
2GSH
O2
H2O2
O
HN
OH
R
N
NH2
O
N
O
R
N
NH
GSSG
2GSH
GLUTATHIONE
REDUCTASE
NADPH
NADP
G6PD
6PG
G6P
ISOURMIL: R=OH
DIVICINE: R=NH2

et al., 1949). This can be observed on the ultra violet spectrum where the oxidised pyrimidine, indicated by the 255 and 245 nm absorption peak, forms a new complex absorbing at 305 nm upon exposure to GSH (Razin et al., 1968). As the new complex formed, the 305 nm absorbance peak increased being accompanied by a parallelled fall in the 255, 245 nm absorption peak because the oxidised pyrimidine is the required molecular species for reacting with GSH (Razin et al., 1968) which is also the case with the alloxan-GSH complex (Patterson et al., 1949). The complex formation can be inhibited by adding an excess of GSH or cysteine ,causing a rapid reduction of the pyrimidine,so that it is no longer in the correct form to react with GSH (Razin et al., 1968).

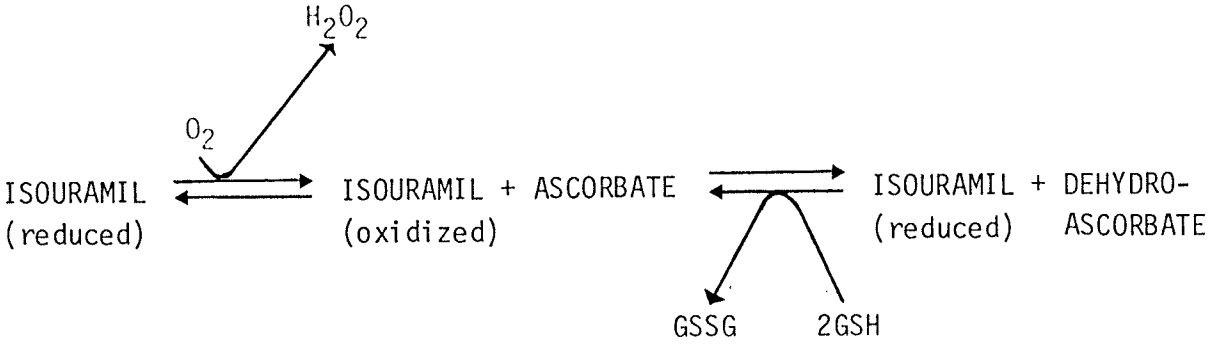
Concerning the overall oxido-reduction cycle of isouramil and divicine, the complex formation between GSH and isouramil only constitutes a side reaction (Razin et al., 1968). The principal function of the pyrimidines in the oxido-reduction cycle is the oxidation of GSH which will be retarded because a certain amount of the GSH will be complexed and so is unable to participate in this cycle (Chevion et al., 1982).

c) Reaction of DOPA and Ascorbate with GSH

Razin et al. (1968) when using DOPA at a concentration of 1.0 mM in conjunction with isouramil (0.2 mM),noted that the drop in GSH was twice that when isouramil was used alone. DOPA when used by itself caused no change in intracellular GSH in G6PD deficient red blood cells (Beutler, 1970) even when a concentration as high as 10 mm was used (Razin et al., 1968). These authors suggested that this was due to DOPA synergistically enhancing the oxidant effect of isouramil on

GSH. Conflicting results have been obtained by Kosower & Kosower (1967) who found that concentrations as low as 0.75 μmoles/ml of DOPA when used alone can lower intracellular GSH levels in G6PD deficient cells. The reason for this discrepancy is not yet known.

Ascorbate is a strong reducing agent and its antioxidant properties play an important role in living tissues, protecting them against the detrimental effects of free radicals (see Klaui & Pongracz, 1981). Chevion et al. (1982) noted that ascorbate when incubated with isouramil tended to have a protective and stabilizing effect on the solution. However, other authors have found that ascorbate tends to potentiate the oxidation of GSH by isouramil acting in a synergistic fashion. Arese et al. (1981) showed GSH oxidation in deoxygenated G6PD deficient red cells to be increased 4.5 times at a 0.05 mM concentration of divicine when ascorbic acid (0.2mM) was added. Similar findings were reported by Razin et al. (1968) who also noted a very significant increase in red cell GSH oxidation by ascorbate. The synergistic enhancement by ascorbic acid with isouramil on GSH oxidation is due to a coupled cyclic system involving the oxidation of ascorbate by isouramil (reduced) to dehydroascorbate and its subsequent reduction by GSH (Razin et al., 1968). The cycle is illustrated below:



This cycle is dependent on the oxygen required autooxidation of the reduced isouramil as described previously. Hence isouramil is capable of oxidising GSH directly as well as through the coupled cyclic system.

Mechanism of hemolysis

a) Possible Role of Divicine & Isouramil in the Etiology of Favism

The pyrimidine aglycones have a powerful capacity to function as reversible redox systems rapidly causing the irreversible oxidation of intracellular GSH in vitro (Chevion, et al., 1982, Mager et al., 1965). It has been proposed that a similar mechanism may exist in vivo,leading to the hemolytic crisis in susceptible favic individuals (Chevion et al., 1982; see Mager et al., 1980). Drug induced hemolysis is also thought to occur by a similar chain of events (Beutler et al., 1957; Flanagan et al., 1955; see Harris & Kellermeyer, 1970).

In the normal cell the oxidant stress imposed by the pyrmidine aglycones or oxidant drugs is counteracted by an increase in NADPH concentration by the NADPH-generating HMP and the NADPH-linked erythrocyte glutathione reductase (EGR) (see Mager et al., 1980, see Beutler, 1978). The rise in NADPH concentration is stimulated by the release of G6PD inhibition due to the fall in NADPH/$NADP^+$ ratio (see Beutler, 1978). Deficient G6PD cells whose sole source of NADPH is through the HMP are unable to reduce $NADP^+$ to NADPH at a normal rate,but are capable of supplying enough GSH to sustain the necessary metabolic activities taking place within the cell (see Mager et al., 1980). This vulnerable metabolic equilibrium is very easily upset by a challenging reducing agent,as is the case with the pyrimidine aglycones. The G6PD deficient erythrocyte is unable to generate sufficient NADPH and so the increased demand for GSSG reduction by NADPH

dependent EGR is severely impaired (see Mager et al., 1980). The consequences of the rapid depletion of GSH,which inevitably results from the action of isouramil and divicine and the suggested enhancement by ascorbic acid and DOPA (Razin et al., 1968; Beutler, 1970),could cause irreversible cellular alterations leading to the eventual destruction of the cell (Chevion et al., 1982). The cells are then removed by intravascular hemolysis or the reticuloendothelial system (Gaetani et al., 1979; Chevion et al., 1982).

b) Factors contributing to the Rupture of Erythrocytes

i) Reduced Glutathione Peroxidase Activity and Oxidative Radical Accumulation

GSH dependent glutathione peroxidase catalyses the reduction of H_2O_2 (Mills, 1957) and other hydroperoxides (see Flohé et al., 1976). It is a selenoenzyme (Flohé et al., 1973) and plays a crucial role in protecting the erythrocytes from H_2O_2 induced hemolysis oxidation of hemoglobin (Cohen & Hochstein, 1963). Hydrogen peroxide, superoxide(O_2^-) and singlet oxygen ($^1O_2$) are produced in a number of biochemical mechanism and they can also indiscriminately attack a variety of biological structures due to the high reactivity of these oxygen species (Cohen & Heikkila, 1974; Loschen et al., 1974). H_2O_2 is also produced with the interaction of hemolytic drugs with oxyhemoglobin (Cohen & Hochstein, 1963 & 1964), which may also be the case with the aglycones in vivo. It is imperative to regulate the detoxification of H_2O_2 and the other oxidising radicals. Calalase is also important in eliminating H_2O_2 from the cell (Sies, 1974) but it does not protect the cell membrane from oxidative attack (see Flohé, 1982; McCay et al., 1976; Cohen & Hochstein, 1963) as does glutathione peroxidase. As the activity of glutathione peroxidase declines in a G6PD deficient individual subjected to an oxidative attack, due to the inability of EGR to reduce GSSG to GSH, the rate of detoxification of H_2O_2 will fall. This will ultimately lead to the

accumulation of H_2O_2 in the cell, rendering it vulnerable to lipid peroxidation and oxidation of sulfhydryl groups of proteins and membrane proteins (Yamaguchi et al., 1983; Zipursky et al., 1974; Cohen & Hochstein, 1963). Studies carried out by Brownlee et al. (1977) show that the intracellular decomposition of H_2O_2 to the hydroxyl radical (·OH) is necessary for hemolysis whereas the concurrent formation of the $^1O_2$ is necessary for lipid peroxidation. These two oxidant species are generated when O_2^- reacts with H_2O_2. The generation of O_2^- from the autooxidation of oxyhemoglobin is elevated in the presence of a strong reducing agent (Goldberg & Stern, 1975; Goldberg et al., 1976). Superoxide dismutase, the enzyme responsible for the detoxification of this radical, is unable to cope with the increased concentration. The rise in O_2^- contributes to the oxidative damage of the cell by reacting with hemoglobin causing it to break down and adhere to the membrane (Goldberg & Stern, 1977). Studies carried out by D'Aquino et al., (1983) using riboflavin and tocophorol deficient rats so as to mimic the favic situation (i.e., a low blood GSH level), and found that the main cause hemolysis was due to lipid peroxidation. The peroxyl radicals formed during oxidation of the lipid components were unable to be removed by the dual effect of glutathione peroxidase and vitamin E which protects the membrane from an oxidative attack.

The important role glutathione peroxidase plays in protecting the cell from peroxide radicals if further substantiated by the hemolytic effect caused in individuals who have a hereditary disorder whereby they partially lack the enzyme (Necheles et al., 1970). Similarly selenium, an important catalytic component of the enzyme is known to protect the erythrocyte membrane from oxidative damage and denaturation of hemoglobin (Rotruck et al., 1972).

ii) Heinz Body Formation

The pyrimidine aglycones have been shown to induce the formation of methemoglobin and Heinz bodies (Mager et al., 1983). According to D'Aquino et al. (1983), the formation of Heinz bodies represents the last event leading to hemolysis in tocopherol and riboflavin deficient rats. The denaturation of the hemoglobin is brought about by the oxidation of the sulfhydryl group and the formation of mixed disulphides arising from GSH complexing with the hemoglobin itself (see Beutler et al., 1972b). This occurs with the cysteine sulfhydryl group on the beta position of hemoglobin (Beutler, 1971). In normal erythrocytes this complex would be reduced through NADPH dependent EGR (Srivastava & Beutler, 1970). The defective NADPH generating system in the G6PD deficient individual retards the reduction of this complex so leading to its build up in the cell. The complex, which is unstable, then loses its heme group with the concurrent precipitation of hemoglobin (Jacob & Winterhalter, 1970). Once this has occurred the hemoglobin is irreversibly damaged and the precipitated globin adheres to the cell membrane through disulphide bridges (Beutler, 1971). These denatured aggregates of hemoglobin are Heinz bodies and are removed by intravascular hemolysis or by the reticuloendothelial system (Chevion et al., 1982; Cohen & Hochstein, 1964).

iii) Increased Rigidity of Red Blood Cell Membrane

The formation of disulphide bonds between spectrin molecules (spectrin diamers) and spectrin and protein molecules as a result of membrane polypeptide sulphydryl oxidation was discovered in G6PD deficient individuals suffering from chronic hemolytic anemia and it was suggested that these aggregates make a contribution to hemolysis and abnormal red cell morphology (Johnson et al., 1979). These authors established that the diamers have a

molecular weight of approximately 4.4 x 10^5 daltons and the spectrin/protein aggregate a molecular weight greater than 50 x 10^6 daltons. However, no such structures were discovered in D'Aquino et al's (1983) experiments with isouramil treated blood from tocopherol and riboflavin deficient rats.

iv) Hexokinase Inhibition

The accumulation of GSSG in red blood cells has been shown to have a pronounced inhibitory effect on hexokinase activity (Mager et al., 1964). Hexokinase marks the first step in the utilization of glucose in the Embden-Meyerhoff pathway. The reaction involves the phosphorylation of glucose, ATP serving as a phosphate donor. When normal and G6PD deficient red blood cells were incubated with phenylhydrazine, GSH and ATP levels fell and glycolysis was inhibited (Kosower et al., 1964). This effect was alleviated if glucose was added but not in the case of G6PD deficient erythrocytes. Similar results were obtained by Mager et al (1965) when using isouramil and divicine. This inhibition of glycolysis was due to the irreversible inhibition of hexokinase caused by the high concentration of GSSG produced as a result of the oxidant action of acetylphenylhydrazine, isouramil and divicine on GSH in G6PD deficient cells. Hexokinase inhibition will retard glycolysis and ATP generation so leading to the premature shortening of the red blood cells life span (Brewer et al., 1964; Simon, 1967). This may have certain consequences with regards to red cell rupture but this has yet to be established. GSSG accumulation also effects other enzymes including G6PD, inorganic pyrophosphatase (Scheuch et al., 1961) and ATPase (Kutscher, 1961).

v) Disorders in Glutathione Metabolism

Disorders in glutathione metabolism and synthesis have been known to predispose individuals to favic and drug-induced hemolytic attacks (Boivin & Garland, 1965; Oort et al., 1961). Individuals suffering from a hereditary GSH synthetase deficiency incur hemolytic attacks and defective brain functioning (Meister, 1983). The enzyme plays an important role in the γ-glutamyl cycle and is responsible together with γ-glutamylcysteine synthetase for the synthesis and turnover of GSH (see Meister, 1975). In severe glutathione sythetase deficiencies, blood GSH levels are very low with high 5-oxoproline levels occurring in the blood, cerebrospinal fluid and urine (Meister & Anderson, 1983). In mild cases of GSH synthetase deficiency the enzyme synthesized with the genetic defect is unstable but the deficiency is compensated to a certain degree by the rapid regeneration of the enzyme in certain tissues but not in blood because no protein synthesis can take place. Hence these individuals are still susceptible to hemolytic attacks (Meister & Anderson, 1983; Minnich et al., 1971). Individuals lacking γ-glutamylcysteine synthetase also experience hemolytic attacks (Meister & Anderson, 1983).

These defects in GSH metabolism, which are analogous to G6PD deficiency, further substantiates the critical role GSH plays in mediating the structural intactness of the red cell. The oxidant action of the pyrimidine aglycones may play a very important role in the pathogenesis of favism, as it is generally accepted that there is a connection between GSH oxidation and hemolysis.

Search for an Animal Model

Further research to ascertain unequivocally the role the aglycones play in the events leading to the hemolytic attack has been hampered by lack of a suitable animal model. It would be potentially dangerous to use sensi-

tive human subjects to induce a hemolytic attack. An animal model would not only contribute to the identification of the causative agents in favism but would also help in the elucidation of their mechanism of action and provide an invaluable aid for a prophylactic and therapeutic approach to be established.

The laying hen is the first suitable animal model to be used for studying favism due to its high sensitivity to the effects of vicine. Studies carried out by Muduuli et al. (1981a and 1981b) demonstrated that when laying hens were fed vicine egg size, egg production, egg mass, yolk weight and yolk mass were all depressed. These authors also showed that plasma lipid levels, plasma lipid peroxide levels and spontaneous hemolysis of erythrocytes increased with a subsequent fall occurring with hemoglobin and hematocrit levels. Fertility and hatchability of eggs, which were also depressed in vicine fed laying hens, were improved considerably following vitamin E supplementation. The pronounced effect vicine had on the metabolism of the laying hen, including the decline in hemoglobin and hematocrit levels and the rise in hemolysis, are similar to the hemolytic effects observed in G6PD deficient individuals upon ingestion of faba beans. These results tend to support previous conclusions that vicine and possibly convicine and their aglycones are the causative factors of favism. In the laying hen large quantities of NADPH are utilized for the synthesis of fat which is deposited in the yolk. Muduuli et al.(1981b) suggested that the laying hen is incapable of meeting the demand for NADPH in the presence of a challenging oxidant stress such as divicine. This challenge will have the effect of not only retarding fat synthesis but also GSH synthesis which will result in increased fragility of the yolk and erythrocyte membrane due to the reduced protection provided by GSH against free radicals. These

conclusions are further supported by the results with vitamin E which acts as a scavanger of free radicals. It appears from these studies that the laying hen is a highly suitable model which could be used for studying the mode of action of vicine and convicine.

It would be advantageous if a mammalian animal model could be established to study favism,as it is more closely related to the situation in man, compared to the hen. An ideal animal model would be an animal which was deficient in G6PD and when sufficiently challenged with an oxidant stress suffered from a hemolytic attack in much the same way as a favic individual does. Animals possessing low levels of blood GSH concentrations or any other hereditary disorder upsetting GSH homeostasis could also have potential as animal models. The other approach would be to mimic the situation existing in the favic erythrocyte.

Glucose-6-Phosphate Dehydrogenase or GSH Deficient Animals

Several authors have shown ovine erythrocytes to be deficient in G6PD (Cheun, 1966; Kaneko & Smith, 1964; Budtz-Olsen et al., 1963; Thompson & Todd, 1964). The mean enythrocyte activity of G6PD determined by Cheun (1966) were found to be 1.20 ± 0.37 and 1.98 ± 0.27 U.O.D. 340 mμ/min/g hemoglobin for the sheep and goat respectively. Other experimental animals screened for G6PD deficiency, in the same study had the following activities: 13.82 ± 1.19 for the rat, 11.0 ± 2.70 for the dog and 11.86 ± 1.52 U.O.D. 340 mμ/min/ghemoglobin for the rabbit. The discrepancy between sheep and goats and other animals which have a normal G6PD activity is very pronounced. Cheun (1966) reported no evidence of any hemolytic anemia occurring during the studies. Carson (1960) was unable to induce hemolysis in sheep erythrocytes in vivo and in vitro when administering large doses of primaquine. The HMP pathway was less

active in the 5 sheep studied compared to humans and both G6PD and 6PGD were unstable. The enzyme is apparently active enough to be capable of overcoming a challenging oxidant stress (Smith, 1968).

An inherited erythrocyte GSH deficiency has been reported in a number of different breeds of sheep (Agar, 1975). The deficiencies were found to be of two kinds. One, which occurs in the Finnish-Landrace, involves impaired membrane transport causing cysteine uptake to be effected and hence GSH synthesis as it is a precursor (Young et al., 1975). The other type of deficiency is associated with a reduced activity of γ-glutamyl-cysteine synthetase and occurs in the Tasmanian Merino (Smith et al., 1973; Young & Nimmo, 1975). Sheep which manifest both types of deficiency have been produced by selective breeding experiments (Tucker et al., 1976). The low erythrocyte GSH concentrations do not appear to have any clinical effects (Young & Nimmo, 1975).

It appears from the studies carried out on G6PD deficient sheep that a sheep model for studying favism is limited on the basis that it is not sensitive enough to oxidant stresses. However, there does lie some potential in glutathione deficient sheep, although its suitability as a favic model has not been tested.

Naik and Anderson (1971) used a "brilliant cresyl blue" decolorization test to determine G6PD activity in cattle. The cattle were originally found to be deficient but this deficiency later proved to be a NADPH diaphorase deficiency because the test involved the action of both enzymes and did not discriminate between them (Paniker & Beutler, 1972). A dog was found to have a G6PD activity which was 44% of normal but had no litter mates or offspring and so could not participate in further studies (Smith et al., 1976). A rat strain with G6PD deficiency was successfully bred and

persisted for several generations until they all perished in an animal house infection (Werth & Muller, 1967). No suitable G6PD deficient animal model has been established to study drug induced types of hemolytic anemia and favism.

GSH Depletion in Erythrocytes

a) Inhibiting GSH Synthesis

Sulfoximines are selective powerful inhibitors of either glutamine synthetase or γ-glutamyl cysteine synthetase or both, causing the depletion of intracellular GSH levels and glutamate in a variety of tissues (Griffith et al., 1979; Griffith & Meister, 1979). Methionine sulfoximine was first shown to inhibit glutamine synthetase and then γ-glutamylcysteine synthetase (Meister & Tate, 1976). If the methyl group in methionine sulfoximine is replaced with a bulkier group such as an ethyl or propyl, a decline in the inhibition of glutamine synthetase occurs but not with γ-glutamylcysteine synthetase (Griffith et al., 1979). The substitution of an even bulkier butyl group giving buthionine sulfoximine, resulted in the inhibition of γ-glutamylcysteine synthetase which proved to be 100 times more effective compared to methionine sulfoximine. The inhibition is caused by the alkyl moiety binding to the active site of γ-glutamylcysteine synthetase which would ordinarily bind to the acceptor amino acid (Griffith & Meister, 1979). Buthionine sulfoximine has proven to be the most potent inhibitor of this enzyme and due to its high specificity it has more potential for use as an inhibitor of GSH synthesis (Meister & Anderson, 1983). Buthionine sulfoximine when fed to mice rapidly reduced GSH levels in the kidney, liver, plasma, pancreas and muscle as well as a variety of other tissues and did not cause convulsions which was a side effect of the other sulfoximine drugs. Injecting 32 mmol/kg of buthionine sulfoximine into mice lowered kidney

GSH levels to less than 20% of the controls (Griffith & Meister, 1979). The rate of fall of GSH in tissues was due to the rate at which they were utilized (Meister, 1983). It appears that if blood GSH was reduced by the same magnitude then buthionine sulfoximine would be an extremely useful agent in creating a situation in the erythrocyte mimicing that of the favic erythrocyte.

D'Aquino et al. (1979) fed rats a diet deprived of protein for 30 days with the aim of impairing glutathione synthesis. This favic model however did not prove to be very successful because GSH levels were not sufficiently depressed and so no hemolytic response was obtained when erythrocytes were challenged with isouramil.

b) Chemical GSH Depleting Agents

Two of the most widely used tissue GSH depleting agents are diethylmaleate and phorone which are α,β-unsaturated carbonyl compounds. These agents cause GSH depletion by reacting with GSH in the presence of glutathione transferases (Boyland & Chasseaud, 1970). An intraperitoneal injection (I.P.) of 0.6-1.0 ml/kg of diethylmaleate very rapidly reduced hepatic GSH levels in the rat by 6-20% in 30 minutes (Young & Siegers, 1980). This low level was maintained for 4 hours and then returned to normal within 24 hours. Phorone also induces a similar effect (Young & Siegers, 1981). When 250 mg/kg was injected intraperitoneally into rats, hepatic GSH values were depressed to 9% of the control values in 2 hours, after which it rapidly rose. Reactive electrophiles formed by cytochrome P-450-dependent monooxygenase activity can be used to deplete tissue GSH levels in vivo, for example, vinylidene chloride. Daily injections of phenobarbital are usually given to the animals as a pretreatment to stimulate the microsomal enzymes and so enhance the GSH depleting effect (Jollow et al., 1972; Mitchell et al., 1973). Oral administration of 0.5g/kg of vinylidene chloride reduced

GSH levels in the liver by 95% compared to controls in 6 hours (Younes & Siegers, 1980). GSH oxidising agents also lower glutathione levels by oxidising GSH to GSSG. These oxidants, for example diamide, are mainly used for depleting GSH levels in cell preparations (Goldstein & Livingston, 1978; Harris et al., 1971).

Aside from the rapid GSH depleting action these agents have, there are a number of drawbacks associated with their use. The effects are often very short lived due to GSH synthesis invariably being stimulated after an acute depression (Meister & Anderson, 1983). Many toxic effects are ramifications of the non specific interaction of the α,β-unsaturated carbonyl compounds (Meister, 1983). Similarly, these oxidising agents are non specific and will readily oxidise other cellular components (Meister & Anderson, 1983). Such dramatic declines in GSH can also give rise to spontaneous lipid peroxidation and cell lysis in hepatocytes (Young & Siegers, 1980; Anumdi et al., 1979). Hence chemical methods employed to deplete GSH are limited due to their toxicity, unspecific cellular interactions and effect on other cellular parameters.

Factors Effecting Glutathione Reductase Activity

The primary function of the flavo protein, glutathione reductase is to regenerate GSH from GSSG which has been formed through the GSH peroxidase reaction, from peroxides and radicals, thiol-disulphide exchange reactions and other redox reactions (see Flohé & Günzler, 1976). The enzyme can completely carry out all of its reducing functions providing an adequate supply of NADPH is available (Benohr & Waller, 1974). The activity of the enzyme is increased when NADPH generation is impaired (Yawata & Tanaka, 1971), which suggests that GSSG reduction is mediated by glutathione reductase (see Flohé & Günzler, 1976). Glutathione reductase exists as a

diamer, consisting of 2 identical subunits with binding positions for NADPH & GSSG occurring at opposite sides of each subunit (Meister & Anderson, 1983). The activity of the enzyme is dependent on the cofactor flavinadenine dinucleotide (FAD) which is located in the centre of each subunit (Meister & Anderson, 1983). FAD and a disulphide at this position comprises the active site of the enzyme (Massey & Williams, 1965). In order for the catalytic reaction to proceed the active site must be reduced by NADPH causing the formation of a semiquinone of FAD which mediates the transfer of reducing equivalents (Massey & Williams, 1965). The overall sequence of events involving the attachment and release of substrates and products is not well understood. It is not clear whether the $NADP^+$ formed after the reduction of the active site is released before or after GSSG reduction (Worthington & Rosemeyer, 1976). The reaction sequence may involve a branch type mechanism (Mannervick, 1973). As the enzyme has an active site consisting of 2 binding sites, the 2 substrates will associate themselves with the binding sites at random so causing the reaction path to be determined by their relative concentrations (Worthington & Rosemeyer, 1976).

Riboflavin (Figure 5) is the precursor of both FAD and flavin mononucleotide (FMN) (Figure 6). The structure of riboflavin consists of a conjugated system of nitrogen containing fused rings which is attached via one of the nitrogens to a sugar alcohol, D-ribitol. FMN has a phosphate group attached at the hydroxyl end of the ribitol and is derived from riboflavin by the action of flavokinase (see Goldsmith, 1975). FAD is formed from FMN by FAD pyrophosphorylase and ATP (see Goldsmith, 1975). Riboflavin is obtained either in the diet or in part from the feces in the case of coprophagous animals, where enteric bacteria in the large intestine

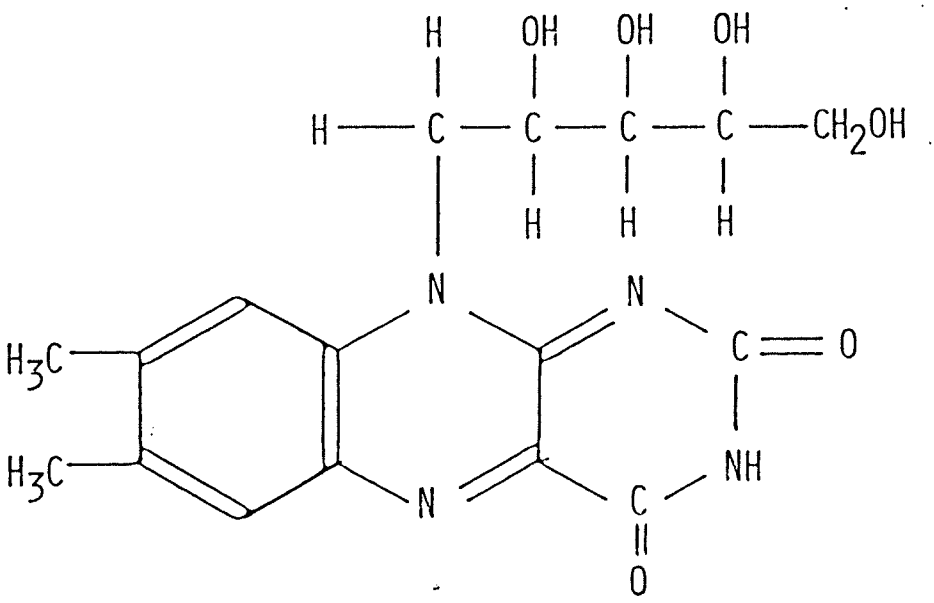
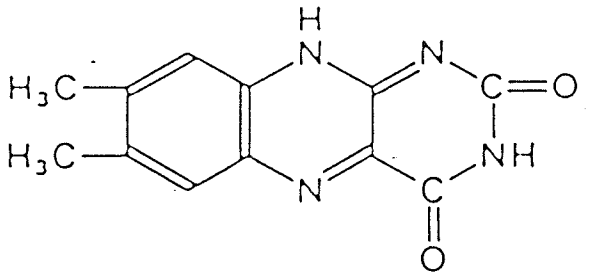
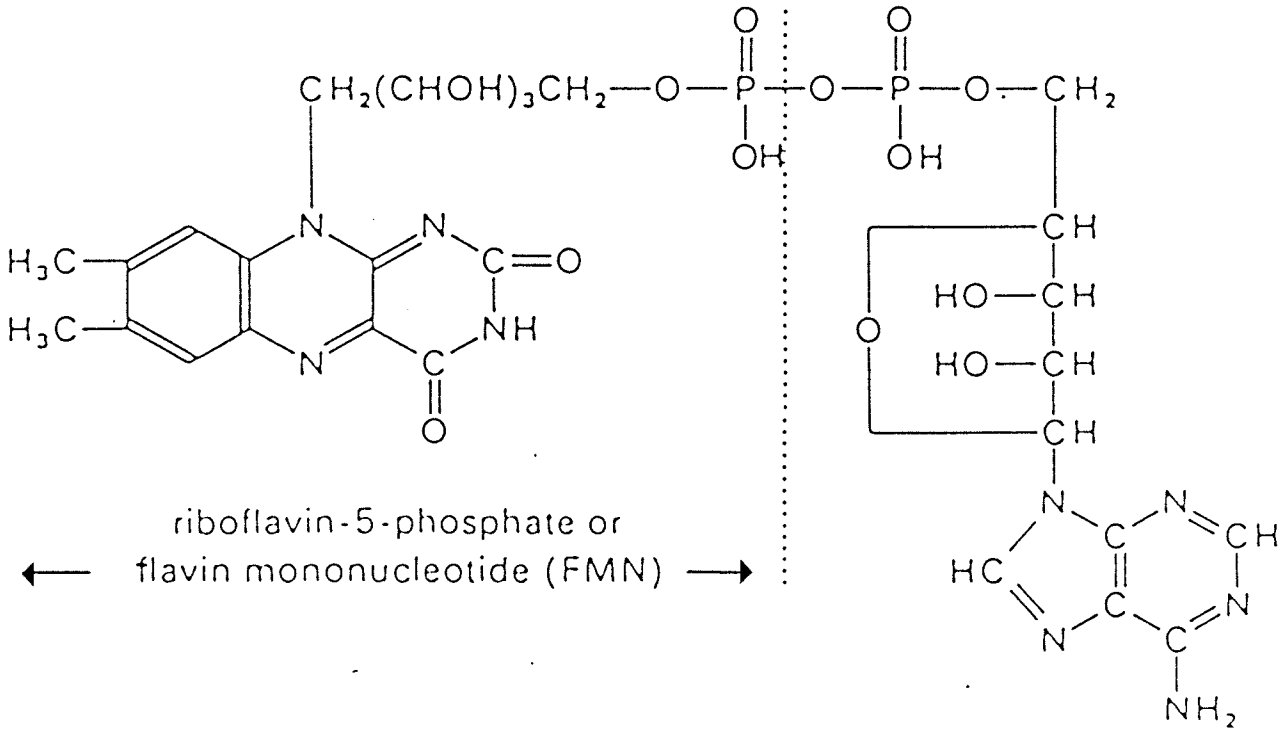


Figure 5. Structure of riboflavin (vitamin B_2)

Figure 6. Structure of flavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).



flavin



riboflavin-5-phosphate or
flavin mononucleotide (FMN)

flavin adenine dinucleotide (FAD)

synthesize certain vitamins (see Jayne-Williams & Fuller, 1971).

a) Diet Induced Riboflavin Deficiency.

A riboflavin deficiency will ultimately lead to an impaired synthesis of FMN and FAD and an overall decline in tissue concentrations (see Goldsmith, 1975). Two key flavoprotein enzymes in the erythrocyte, namely EGR and NADH-methemoglobin reductase are affected in a riboflavin deficiency. These enzymes are the only two in the erythrocyte requiring FAD (Beutler,1969a) Erythrocyte glutathione reductase is now widely used in both epidemiological and experimental studies as a sensitive indicator or riboflavin status in both humans and rats (Tillotson and Sauberlich, 1971; Sauberlich et al., 1972; Tillotson & Baker, 1972; Glatzle et al., 1970; Glatzle et al., 1968). Beutler(1969b) & Glatzle et al.,(1968) have shown that in vivo EGR activity is effected by riboflavin in the diet and in vitro the enzyme activity is altered by FAD. The degree to which the enzyme is stimulated in vitro is dependent on the FAD saturation of the enzyme which is ultimately dependent on the availability of riboflavin. Glatzle et al. (1970) suggested that by measuring the degree to which EGR is stimulated in vitro with the addition of FAD will provide a measure of the riboflavin status of an individual. This stimulatory effect is referred to as the activity coefficient (AC) and is defined as the reduction in absorbance of NADPH with FAD in a 10 minute period divided by the reduction in absorbance without FAD. An AC value of 1.0 indicates no stimulation and an AC value of 1.2 represents a 20% stimulation in EGR activity. An AC value of greater than 20% is indicative of a riboflavin deficient state (Glatzle et al., 1970). Activity coefficients obtained by other authors in riboflavin deficiency experiments using rats have been in the range of 2-4 (Tillotson & Sauberlich, 1971; Hassan & Thurnham, 1977; Prentice &

Bates, 1981). Prentice & Bates (1981) created a state of acute riboflavin deficiency by feeding rats a riboflavin deficient diet for a period of 7 weeks. Mean AC at the end of this period were approximately 4.0 representing a 300% stimulation in EGR activity. The values these authors obtained were greater than those obtained by the aforementioned authors. Prentice & Bates (1981) attributed this to the prevention of coprophagy using tail cups.

Since EGR activity is reduced in riboflavin deficient rats and humans (Glatzle et al., 1973; Paniker et al., 1970) this will lead to an impaired glutathione reducing capacity in the cell which has been demonstrated by Hassan & Thurnham (1977). These authors created a state of riboflavin deficiency (AC = 1.96) in male weanling rats and then subjected blood samples in vitro to a H_2O_2-peroxide generating system for 45 minutes measuring the degree to which the samples were hemolysed. The percent hemolysis was found to be significantly greater in riboflavin deficient rats compared to control rats fed an ordinary diet. This hemolysis was attributed to the drop in blood GSH resulting from the lowered activity of EGR caused by the riboflavin deficiency.

Creating a riboflavin deficiency in the rat leading to a fall in the regeneration of GSH from GSSG was the red blood cell model used by D'Aquino et al. (1979) to study the toxic factors in faba beans. Weanling rats were fed a riboflavin free diet for 60-90 days. Blood samples were taken and assayed for EGR activity which was found to be 6% of the control values and blood GSH which had decreased slightly. The blood samples were then incubated with varying concentrations of isouramil which caused a very dramatic fall in GSH, hemolysis also occurring. It was suggested by these authors that GSH homeostasis in the riboflavin deficient rat can be

maintained by the impaired activity of EGR. However, this homeostasis breaks down when challenged with a strong oxidising agent such as isouramil because the enzyme cannot generate GSH at a sufficient rate to overcome the oxidising effect. There does appear to be potential in the riboflavin deficient red blood cell model as it behaves in the same way as the G6PD deficient red erythrocyte with the response of GSH to isouramil.

b) Riboflavin Antagonists

A wide range of riboflavin antagonists exist which can be used to induce a riboflavin deficiency state. The antagonists exert their action in a variety of different ways including competing with the prosthetic group of the flavoenzymes, preventing riboflavin penetration in cells and effecting coenzyme functions (Kearny, 1952; McCormick et al., 1963). Riboflavin antagonists include derivatives and isomers of riboflavin for example D-araboflavin and isoriboflavin, homologs of riboflavin such as 5' deoxyriboflavin, D-galactoflavin and 6 ethylflavin and finally analogues such as lumiflavin and 6,7 dimethyl-9-(B-hydroxyl-ethyl) isoalloxazine (for review see Lambooy, 1975). The homologs of riboflavin have among their group some of the most potent riboflavin antagonists (see Lambooy 1975). Galactoflavin, largely due to its availability, is a useful and effective inducer of riboflavin deficiency and accelerating the onset (Prosky et al., 1964). The structure of galactoflavin is illustrated in Figure 7. It is structurally very similar to riboflavin, the only difference being that riboflavin possesses a ribityl group as the sugar moiety of the molecule and galactoflavin possesses a dulcityl group (Emerson et al., 1945). Galactoflavin has been shown to completely retard growth in rats when fed 1.08 or 2.16 mg. of galactoflavin/day (Emerson et al., 1945; Prosky et al., 1964). Prosky et al.(1964) compared the growth rate of rats fed galactoflavin

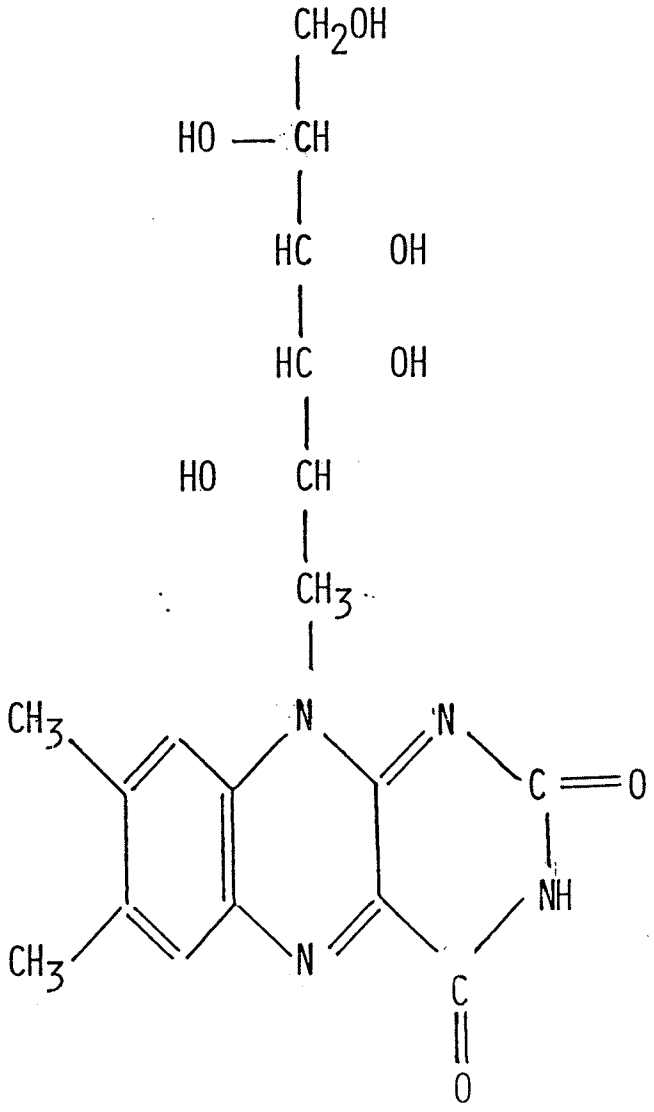


Figure 7. Structure of 7,8- dimethyl-10-(1-D-dulcityl) isoalloxazine (galactoflavin)

& no riboflavin with rats fed on a riboflavin free diet alone over a period of 5 weeks & found that the former group weighed 66% as much as those on the riboflavin free diet. Lane (1964) reported that the onset of symptoms of a typical riboflavin deficiency were greatly accelerated with rats fed galactoflavin. Liver weights were also found to be larger with the galactoflavin treated rats compared to the control rats fed a simple riboflavin free diet. It is not fully understood how galactoflavin exerts its antagonistic action. Prosky et al. (1964) suggest 3 possible mechanisms which include the inhibition of riboflavin phosphorylation, the interference of riboflavin uptake in tissues & the acceleration of riboflavin excretion. However, Kearny (1951) showed that galactoflavin did not inhibit the phosphorylation of riboflavin by yeast flavokinase. These results cannot be extrapolated to animals because flavokinase properties may differ. Studies carried out by Lane (1964) on riboflavin & individual flavin components of the liver revealed that in a simple riboflavin deficiency, riboflavin, FMN & FAD levels were depressed but when galactoflavin was incorporated into the diet, these levels were depressed even further. The major reduction was produced with liver riboflavin, with less marked reductions occurring with FMN & FAD. These flavin components had declined by as much as 60.2% & 35.3% respectively after 3 weeks. Galactoflavin was also noted to have accumulated in the liver. Similar results were obtained by Miller et al. (1962) who fed galactoflavin incorporated into a riboflavin deficient diet to pregnant rats over a period of 2 weeks & noted that liver FAD levels were depressed by 50% compared to a 34% decline with pregnant rats fed on a simple riboflavin free diet. Lane (1964) suggests that galactoflavin, due to its incorporation into the liver may result in the displacement of riboflavin

from the binding sites or inhibit hepatic incorporation of riboflavin. Hence the overall effect was a decline in the synthesis of FMN and FAD.

c) Hypothyroidism and Riboflavin Deficiency

When rats are fed a low iodine diet so as to induce a state of hypothyroidism, hepatic FAD levels were found to be two thirds of the normal value (see Rivlin & Langdon, 1966). Similarly hepatic FMN also dropped to low levels (Rivlin et al., 1968). The fall in these flavins can be partly attributed to the reduced activity of flavokinase, which converts riboflavin to FMN, and is regulated by thyroid hormones (see Rivlin & Langdon, 1966). In the riboflavin FAD pathway, flavokinase is the only enzyme which significantly decreases in activity in the hypothyroid animal (Rivlin et al., 1968). Many biochemical similarities exist between riboflavin deficiency and hypothyroidism including low FAD, FMN and riboflavin concentrations in the liver, decreased activities of enzymes requiring FMN and FAD as cofactors particularly glutathione reductase (Rivlin et al., 1968; Rivlin, 1970). The magnitude of the changes occurring are usually greater in the riboflavin deficient rat compared to hypothyroid rats (see Rivlin, 1975). Riboflavin deficiency also affects thyroid hormone metabolism, for example hormogenesis (Nagasaka et al., 1971) and reducing the deiodination of thyroxine (Galton & Ingbar, 1962). Since riboflavin metabolism is subject to thyroid regulation, a combined riboflavin and iodine free diet may further reduce FAD levels and glutathione reductase as opposed to using either one alone.

d) Erythrocyte Glutathione Reductase Inhibition Using 1,3-bis (chloroethyl) -1-nitrosourea (BCNU)

BCNU is a drug used in cancer chemotherapy for the treatment of multiple myeloma and primary brain tumors. Frischer & Ahmad (1977) noted that patients receiving therapeutic doses of the drug acquired a rapid deficiency of EGR within minutes of drug administration. In some patients, the drop in EGR activity was 90%. Blood GSH levels also fell with a disturbed H_2O_2 removal. These effects were greatly enhanced in G6PD deficient erythrocytes rendering them even more susceptible to oxidative hemolysis. As H_2O_2 removal in the erythrocytes is dependent on EGR and glutathione peroxidase rather than catalase, detoxification was severly limited after BCNU treatment. Frischer & Ahmad went on to suggest that as the effects of BCNU are compounded in the G6PD deficient individuals, then it is advisable for the physician to exercise extreme caution when such patients require BCNU treatment, thereby minimizing the risks of oxidative hemolysis. Similar results were obtained by Sagone & Burton (1979) who studied the effect BCNU had on normal and G6PD deficient erythrocytes. They compared the response of BCNU on normal erythrocytes with G6PD deficient erythrocytes with the effect of an oxidant stress on HMP activity and GSH stability. BCNU treated erythrocytes in vitro behaved in the same way as G6PD deficient erythrocytes, that is, the BCNU red blood cell was a mimic of the G6PD deficient red blood cell. None of the other 19 erythrocytic enzymes were noted to alter in activity (Frischer & Ahmad, 1977). These authors suggested that BCNU exerts its potent and specific inhibitory effect through alkylation and this was irreversible. Erythrocyte glutathione reductase activity returned to normal when the bone marrow had synthesized new erythrocytes to replace the enzyme defective old ones.

Effecting the Draining of Peroxides

Another step to manipulate in the erythrocyte to create a situation similar to that existing in a favic one is to affect the draining of peroxides through the action of glutathione peroxidase. This will have an effect on GSH homeostasis in the red blood cell,causing a fall in intracellular GSH. Glutathione peroxidase activity can be impaired by feeding a selenium deficient diet to rats which has been shown to make them susceptible to drug-induced hemolysis (Rotruck et al., 1972).

Vitamin E is capable of protecting the cell from hemolysis by preventing lipid peroxidation and sulfhydryl group oxidation. It does this by interacting with H_2O_2 derived radicals (Brownlee et al., 1977). D'Aquino et al. (1981) mimicked a favic situation in rats by feeding them a diet deficient in riboflavin and tocopherol thereby making this model more sensitive compared to the last one which was riboflavin deficiency alone. The resulting low activity of EGR leads to impaired GSH regeneration and lack of vitamin E made erythrocytes, when challenged with an oxidant stress, more susceptible to hemolysis (D'Aquino et al., 1981). The GSH levels in these riboflavin and tocopherol deficient rats after a period of 5 months were found to be much lower compared to control rats who were fed a normal diet. When erythrocytes from the rats were treated in vitro with 0.5mM of isouramil, GSH concentrations dramatically dropped by 89 percent (D'Aquino et al., 1981). They used this model to determine whether or not other oxidative factors besides vicine and convicine are present in faba bean extracts. This was investigated by comparing the oxidant effect different faba bean extracts had on the riboflavin and tocopherol deficient erythrocytes, expressing the results in terms of convicine equivalents, with the values obtained from the chemical method using vicine

and convicine. By treating extracts with and without β-glucosidase they could determine if oxidising agents other than the aglycones are present in faba beans. This model was capable of detecting and quantifying all of the oxidising agents which can cause hemolysis both free and glucoside-bound. Other oxidising factors were found to exist in faba bean extracts in the free state besides vicine and convicine. These authors also used the same model for studying the oxidative mechanism vicine and convicine have on inducing erythrocyte hemolysis (D'Aquino et al., 1983).

Experimental Objectives

The objectives of this study were to create an animal model for studying favism by lowering GSH concentrations in the rat erythrocyte so that it will respond to vicine in the same way as a favic red cell does in vivo. Four different blood models were attempted to lower blood GSH levels.

1. Riboflavin-deficient Animal Model

   A riboflavin deficient diet was fed to rats with the objective of lowering EGR activity by removing the precursor necessary for the synthesis of this enzymes cofactor, FAD. This will have the effect of lowering the GSH reducing capacity of the cell, rendering it highly sensitive to an oxidant challenge.

2. Galactoflavin Animal Model

   The same type of effect was attempted as in the riboflavin deficient animal model only rats were fed galactoflavin, a riboflavin antagonist, in addition to being fed the riboflavin deficient diet.

3. Vinylidene Chloride Animal Model

   Vinylidene chloride was used as a chemical means of rapidly depleting GSH levels and so creating a favic animal model in a short period of time.

4. BCNU Animal Model

   An attempt to lower blood GSH levels was made using the cancer chemotherapy drug BCNU which has the side effect of causing the swift irreversible inhibition of EGR.

MATERIALS & METHODS

MATERIALS

Sodium phosphate, FAD, GSSG, NADPH & Drabkins reagent was purchased from Sigma Chemical Co., St. Louis and sodium citrate, sodium chloride, tris (hydroxy methyl) aminomethane (Tris) from Baker Chemical Co., N.J.. 5.5'Dithiobis-2 (2-nitrobenzoic acid) was obtained from Eastman Kodak, Rochester, N.Y.. Glacial metaphosphoric acid, ethylene diamide tetra-acetate (EDTA), potassium phosphate, gooch rubber tubing, heparinized micro-hematrocrit capillary tubes were all purchased from Fischer Scientific Co., Rochester, N.Y.. Brass shim metal was bought from H. Paulin & Co., Ltd., Ontario and boston round bottles from Nalge Co., Rochester, N.Y.. 1, 1 dichloroethylene (vinylidene chloride) was purchased from Aldrich Chemical Co., Milwaukee, Wis.. 1, 3-bis (2-chloroethyl) -1-nitrosourea (BCNU) was obtained from Bristol Laboratories of Canada, Ontario and 7, 8 - dimethyl -10- (1-D-dulcityl) isoalloxazine (galactoflavin) was supplied by Merck Sharp & Dohme, Rahway, N.Y.. Vicine was isolated from faba beans by the procedure developed by Marquardt et al., 1983.

GENERAL METHODS

Animals and Management

Male Sprague-Dawley rats were obtained from Central Animal Care at the University of Manitoba. The rats, upon arrival, were given a 5-day acclimatization period prior to each experiment during which they were fed a stock diet of Purina Chow (Ralston, Purina Co., St. Louis) & water was supplied ad libitum. The rats were housed individually in wire mesh cages in an electrically heated room maintained at a thermostatically controlled

temperature of 25-27^{o}C. and a relative humidity of between 50-55%. The lighting management employed was 10 hours of light in a 24-hour period (0800-1800).

Assays

A Beckman, Model DU-8 automatic recording spectrophotometer was used to read all absorbance measurements.

GSH Determination

GSH was assayed colorimetrically according to Beutler et al. (1963) on the supernatant which was derived from the erythrocytes after precipitating the proteins with metaphosphoric acid. DTNB was the reagent used to react with the SH groups of GSH and the resulting product was assayed at 412 nm on the spectrophotometer.

Erythrocyte Glutathione Reductase Assays

i). Erythrocyte glutathione reductase activity was determined by the method of Glatzle et al. (1970) as modified by Tillotson & Sauberlich (1971) which has been used to evaluate the riboflavin status of individual subjects. A value for the activity coefficient (AC) of the enzyme was obtained by determining the reduction in absorbance at 340nm and 37^{o}C of NADPH with and without FAD in a 10-minute period. The AC can be expressed as the following equation:

$$AC = \frac{\text{Reduction of absorbance with added FAD/10 minutes}}{\text{Reduction of absorbance without added FAD/10 minutes}}$$

The AC was used to assess the riboflavin status of the rats.

ii). A similar assay based on the method of Long & Carson (1961) expresses EGR activities as the number of micromoles of NADPH oxidised per minute per gram of hemoglobin at 37^{o}C. thereby enabling a quantitative comparison to be made between different samples. This method was modified

slightly as the assay was run with and without FAD. The hemolyzate was prepared by washing 0.3ml of erythrocytes three times with 5 volumes of 0.145M NaCl, and centrifuging for 10 minutes at 1000xg at 4^{o}C. The red cells were then diluted with 3-4 volumes of 0.067M potassium phosphate buffer, pH 7.6, and were frozen and thawed twice using dry ice and a warm water bath. The hemolyzate was diluted again so that when 0.5ml was added to the assay medium, the final reaction mixture had a hemoglobin concentration of 0.105g/100ml. The assay medium without FAD contained 2.2 ml 0.13M Tris buffer pH 7.6; 0.1ml 0.032M EDTA; 0.1ml 5.3×10^{-3}M GSSG; 0.1ml 2.9×10^{-4}M NADPH; 0.1ml of distilled water and 0.5 ml hemolysate. The 0.1ml of water was replaced with 0.1ml 10^{-4}M FAD when the assay was run with FAD. The blank consisted of the above solutions omitting the hemolysate and replacing it with 0.5ml of Tris buffer. NADPH was used to start the assays.

Hemolysis

Draper and Csallany's test (1969) was used to determine spontaneous hemolysis. This was calculated as the optical density at 415nm in the phosphate-saline buffered sample divided by a completely hemolysed sample in water x 100 after a 4-hour incubation period.

Hemoglobin

The cyanomethemoglobin method, according to Crosby et al. (1954) was used to determine hemoglobin.

Hematocrit

Blood samples were collected in heparinized micro-hematocrit capillary tubes and were centrifuged for 10 minutes at maximum speed using an International Micro-Capillary Centrifuge (Model MB) and readings were obtained using an International Micro-Capillary Reader (Model CR).

Experiment 1. The Effect of Feeding 2% Crude Vicine to Riboflavin Deficient Rats.

Experimental Plan and Diets

Twenty-four male weanling rats weighing approximately 60-70g were divided into 4 experimental groups. Groups I and II were fed on a complete diet whereas Groups III & IV were fed on a riboflavin deficient diet. The basal diet with and without riboflavin is shown in Table 1, all the nutrient requirements being met according to the National Research Council (NRC) (1976). A sucrose based diet was used as the main energy source as opposed to a straw based diet in order to minimize microbial intestinal synthesis of riboflavin (Mannering et al., 1944). The rats, after an initial weighing, were randomly assigned to individually marked cages. Rats in Groups I & III were then fitted with tail cups to prevent coprophagy and Groups II & IV with sham cups. The appropriate feeds and water were provided ad libitum. The rats were maintained on their respective diets until the rats on the riboflavin deficient diet had gained 5g or less in 14 consecutive days and had actually lost weight during the last 7 of these 14 days (see Lambooy, 1975). The duration of the depletion period was between 29 and 120 days. The weight of each rat was recorded every week at 1100 hours using a triple bar balance.

Preparation of Tail and Sham Cups

Tail cups based on a design by Barnes et al. (1957, 1963) were used with the introduction of certain modifications. Boston round plastic bottles having a capacity of 30ml were used. The neck of the bottle was removed by sawing, widened until the hole had a diameter of 1.5cm and was then smoothed by melting using a low heat bunsen burner. A smaller hole

Table 1. Formula of Basal Diet.

Composition	%
Vegetable Oil	10
Vitamin Free Casein	18
Sucrose	66.5
Salt Mix Draper 4164[1]	4.0
Vitamin Mix[2]	1.0
Methionine Mix[3]	0.5

[1]The composition of the salt mix in g per Kg of diet was: calcium carbonate, 16.356g; cupric sulfate .5H_2O, 0.018g; calcium diphosphate, 35.55bg; potassium diphosphate, 7.733g; ferric citrate .3H_2O, 1.6g; magnesium carbonate, 4.089g; manganese sulfate .H_2O, 0.138g; potassium citrate H_2O, 23.653g; potassium iodide, 0.004g; sodium chloride, 10.809g; zinc carbonate, 0.044g.

[2]The vitamin mix per Kg of diet was: choline chloride, 2000 mg; calcium pantothenate, 20mg; thiamine hydrochloride, 4mg; pyridoxine hydrochloride, 9 mg; nicotinamide, 25mg; biotin, 1mg; pteroyglutamic acid, 1mg; cyanocobalamin, 0.05mg; menadione, 9mg; α-tocopherol, 250mg; vitamin D, 1200 iu; retinyl acetate, 21.mg; riboflavin, 3mg. The riboflavin was omitted when making up the riboflavin free diet.

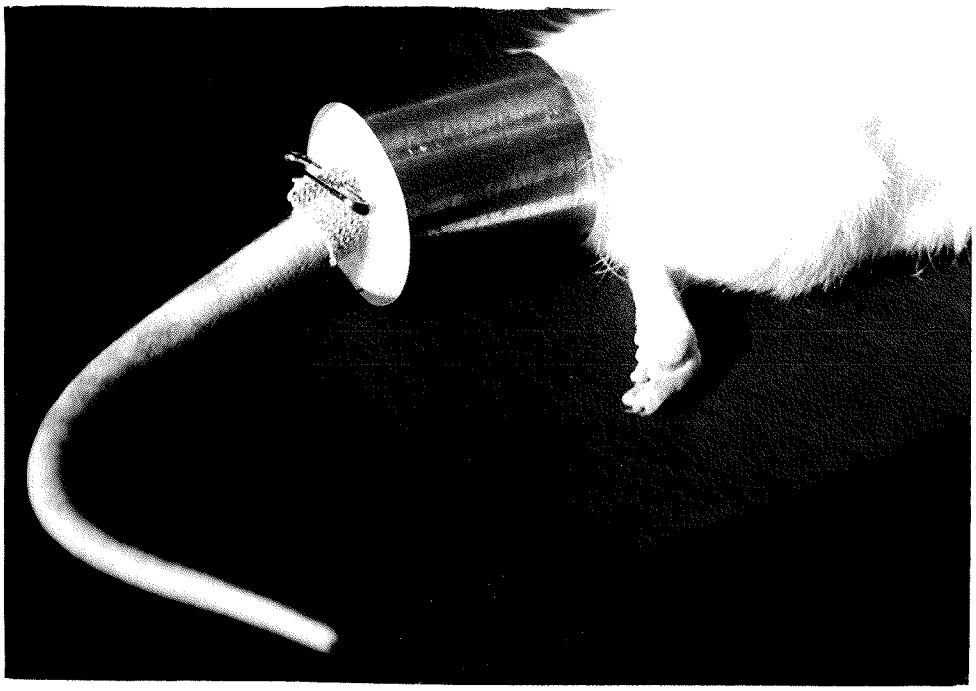
[3]Methionine added per Kg of diet was 1.9g.

having a diameter of between 0.75cm and 1cm was made in the other end of the bottle using scissors and once again was smoothed by melting. A strip of brass shim metal (0.004mm in width) was placed around the length of the bottle to discourage the rats from chewing it. A 7cm length of gooch rubber tubing (having a flat diameter of 2.5cm) was pulled over the bottle with an overlap of 1.5 cm at the base of the bottle. A similar cup referred to as a sham cup whose total weight equalled that of the tail cup was made for the control animals. The hole at the top of the bottle was not enlarged, as it was for the tail cup, but was left with a diameter of 1cm. The gooch rubber tubing was allowed to overlap by 2cm at both ends. The major modification introduced in this method was the technique employed to attach the tail and sham cups to the rats. In the case of the tail cup, a 3cm length of elastoplast having a width of 2cm was placed around the rats' tail approximately 3cm from the anus. The tail was then passed through both of the holes in the bottle, the larger one encompassing the anus. A safety pin was secured to the elastoplast protruding from the proximal end of the cup so as to hold it comfortably in place (Figure 8). The sham cup was secured in the same way but two pieces of elastoplast were placed 1cm apart, the first being approximately 3cm from the anus. The cup was attached, using two safety pins, to each of the protruding pieces of elastoplast (Figure 9). In this position the rats were capable of consuming their feces upon extrusion from the anus. The diameter of the holes in the cups did vary depending on the size of the rat and thickness of the tail. Both sham and tail cups were replaced as soon as they has been chewed by the rats.

Figure 8. Fecal collection tail cups for the prevention of coprophagy

a). Tail cup secured to the rat by means of an elastoplast and safety pin. Brass shim metal was placed around the cup to prevent chewing.

b). Entire assembly with gooch rubber tubing covering the cup.



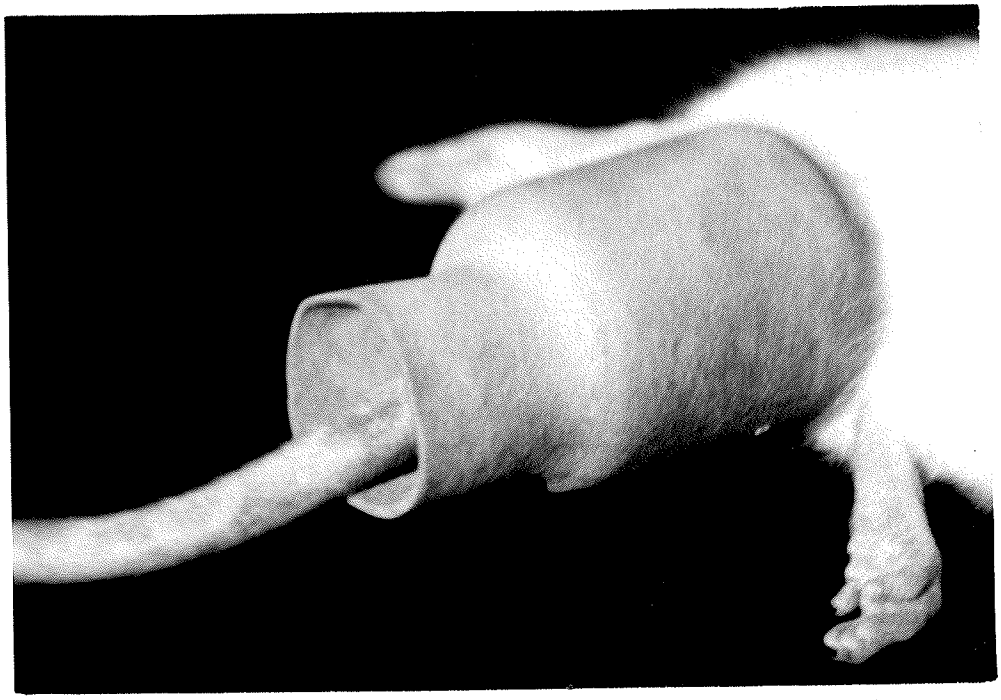
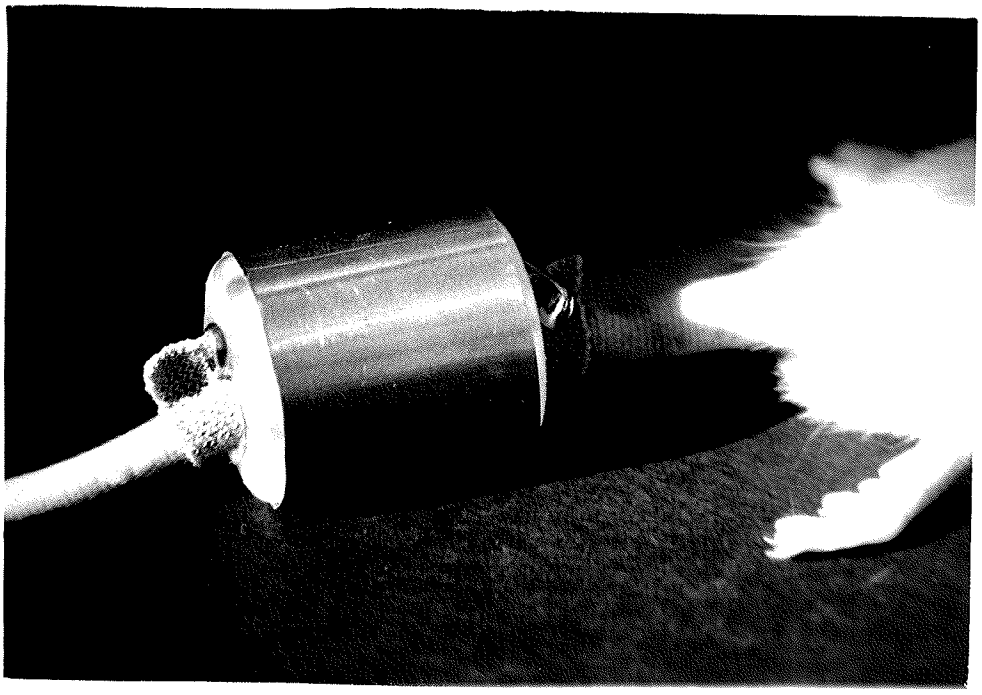
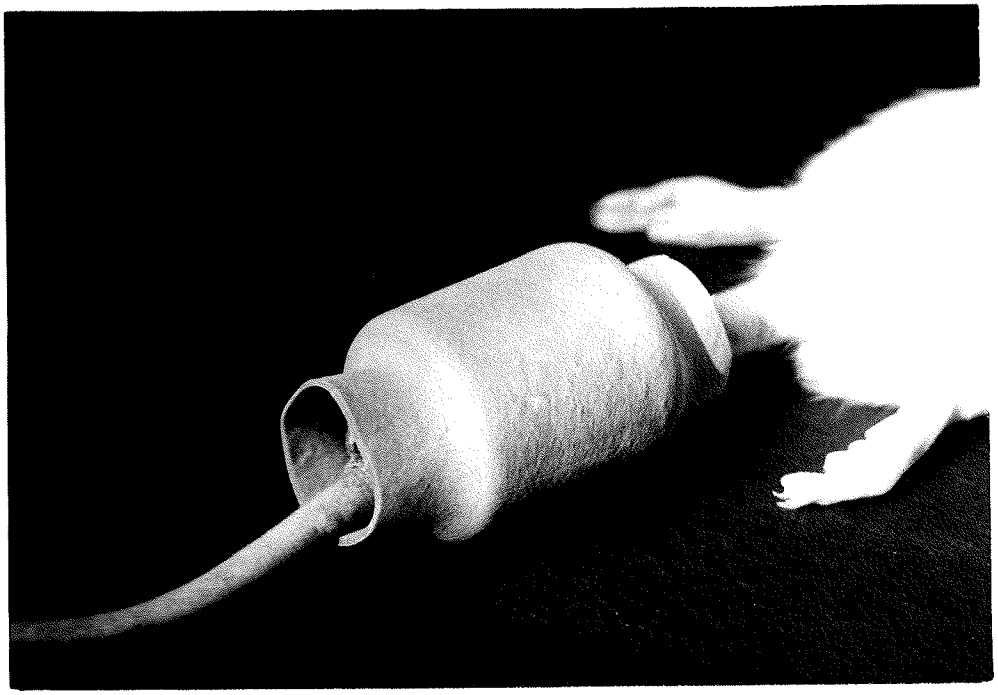


Figure 9. Sham cups permitting coprophagy.

a). Sham cup secured to the tail.

b). Entire assembly





Experimental Procedure for Feeding Vicine

When the riboflavin deficient rats in Groups III and IV began to lose weight, one from each group together with one randomly chosen rat from Groups I and II were selected so as to yield a set of 4 animals. A portion of the tail was then removed from each rat using a sterile blade and 0.25ml of bloodwas thendrawn into heparinized micro-hematocrit tubes by capillary action. The blood was deposited into a heparinized tube and placed in an ice water bath. An assay for blood GSH was then carried out on each of the samples. The 2 rats from Groups I and III were fed their respective diets with the incorporation of 2% crude vicine and the remaining 2 rats in Groups II and IV were fed the original diet. These diets were fed for 48 hours and a record of total feed consumption was made during this period. The rats were then mildly anesthetised using chloroform and were slaughtered by decapitation. As much blood as possible was collected in a heparinized beaker and the liver of each rat was removed and weighed. The following assays were then carried out in duplicate on the blood samples: GSH, EGR (Glatzle et al., 1970), spontaneous hemolysis, hemoglobin and hematocrit. The next set of rats to be assayed were selected in the same way, only the 2 rats from Groups II and IV were fed the crude vicine with Groups I and III remaining on their original diets. In this way the crude vicine was fed to both tail and sham-cupped rats alternately. This procedure was carried out until all of the rats had been assayed.

Experiment 2. The Effect of Feeding 5% Crude Vicine to Rats Treated with Galactoflavin.

Experimental Procedure

Twenty male weanling rats weighing between 70 - 90g were allowed to adapt to their new environment. Sixteen rats were then divided into 4 equal groups. Groups I and II were fed the riboflavin deficient diet (Table 1) containing 400mg/kg of galactoflavin and Groups III and IV the same diet,only it contained 200mg/kg of galactoflavin. The remaining 4 rats were placed on the riboflavin containing diet and were to be used for comparing growth rates and feed consumption patterns with rats fed on the galactoflavin supplemented diet. The rats were then randomly assigned to marked cages. Food consumption was determined daily and the weight of each rat was recorded on a weekly basis. After 25 days, rats in Groups I and II were starved for 24 hours and were then presented with the same diet as before only 5% crude vicine was incorporated into the diet fed to Group II. This diet was fed for a period of 48 hours, total feed consumption being recorded, after which the rats were decapitated and the following assays in duplicate were carried out: blood GSH, EGR activity (Long & Carson, 1961), spontaneous hemolysis, hemoglobin and hematocrit. The same procedure was carried out on the next day for rats in Groups III and IV, Group IV rats being presented with 5% crude vicine in the diet.

Experiment 3. The Effect of Vinylidene Chloride on Blood GSH levels in the Rat.

Preparation of Dose Response Curve

Eight rats weighing between 300-350g were fed the complete diet throughout the whole of the experimental period, after the usual 5-day

adjustement period. The rats were then divided into 4 groups, each group having 2 rats per treatment. Rats in Group I were to act as controls. Microsomal enzymes in all of the rats were induced by giving 7 daily I.P. injections of 60mg/kg of phenobarbital, the control rats being given an equivalent dose of saline. On the seventh day, 300, 400 and 500mg/kg of vinylidene chloride was given orally in 2ml/kg of olive oil to rats in Groups II, III & IV respectively. Control rats were given 2ml/kg of olive oil with no vinylidene chloride. Oral administration was performed by allowing the rats to swallow the vinylidene chloride and olive oil from a 2ml syringe as the plunger was slowly pressed in. Blood GSH levels were then determined in duplicate 2, 4, and 6 hours after administering the vinylidene chloride.

Experiment 4. The Effect of Feeding 5% Crude Vicine to Rats treated with BCNU.

Preparation of Dose Response Curve

Eight rats weighing approximately 300g were fed the complete diet (Table 1) for 4 days ad libitum, after the usual acclimatization period. The rats were then divided into 4 groups, each group having 2 rats per treatment. Rats in Groups II, III and IV were given an I.P. injection of 30, 60 and 90mg/kg of BCNU, respectively. Group I rats, which were to act as controls, were given an equivalent dose of saline. After 0, 4, 24, and 48 hours, 0.7ml of blood was taken from the tail of each rat and EGR activities were assayed in duplicate according to the method of Long and Carson (1961).

Experimental Plan and Procedure for Feeding 5% Crude Vicine

Twelve rats weighing approximately 300-350g were allotted to 4

groups, Groups I & II having 2 rats each and Groups III & IV having 4 rats,respectively. All of the rats were fed the complete diet (Table 1) for a period of 5 days,which was presented to them at 1800 hours and was removed at 0800 hours. Rats in Groups III & IV were then given an I.P. injection of 100mg/kg of BCNU and Groups I & II rats an equivalent dose of saline as they were to act as controls. Twenty four hours later, blood was taken from the tail vein & was assayed for the concentration of GSH and for EGR activity (Long & Carson, 1961). On the same day as these assays were performed, the rats in Groups I and III were presented with the complete diet as usual but Groups II and IV received the same diet with the addition of 5% crude vicine. The high level of vicine (5%) was incorporated into the diet so as to ensure a sufficient quantity was consumed, as the side effects of the drug include diarrhea, nausea, vomiting and loss of appetite. Forty-eight hours later, the total feed consumed over this period was recorded. The rats were then decapitated with the following assays being performed in duplicate on the blood samples: GSH, EGR activity (Long & Carson, 1961), spontaneous hemolysis, hemoglobin and hematocrit.

Statistical Analysis

Data was analysed using the Analysis of Variance (ANOVA) and General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute Inc., Cary, N.C). Significant differences between treatment means were tested using the Student-Newman Keul (SNK) multiple range test (Snedecor & Cochran , 1967).

RESULTS

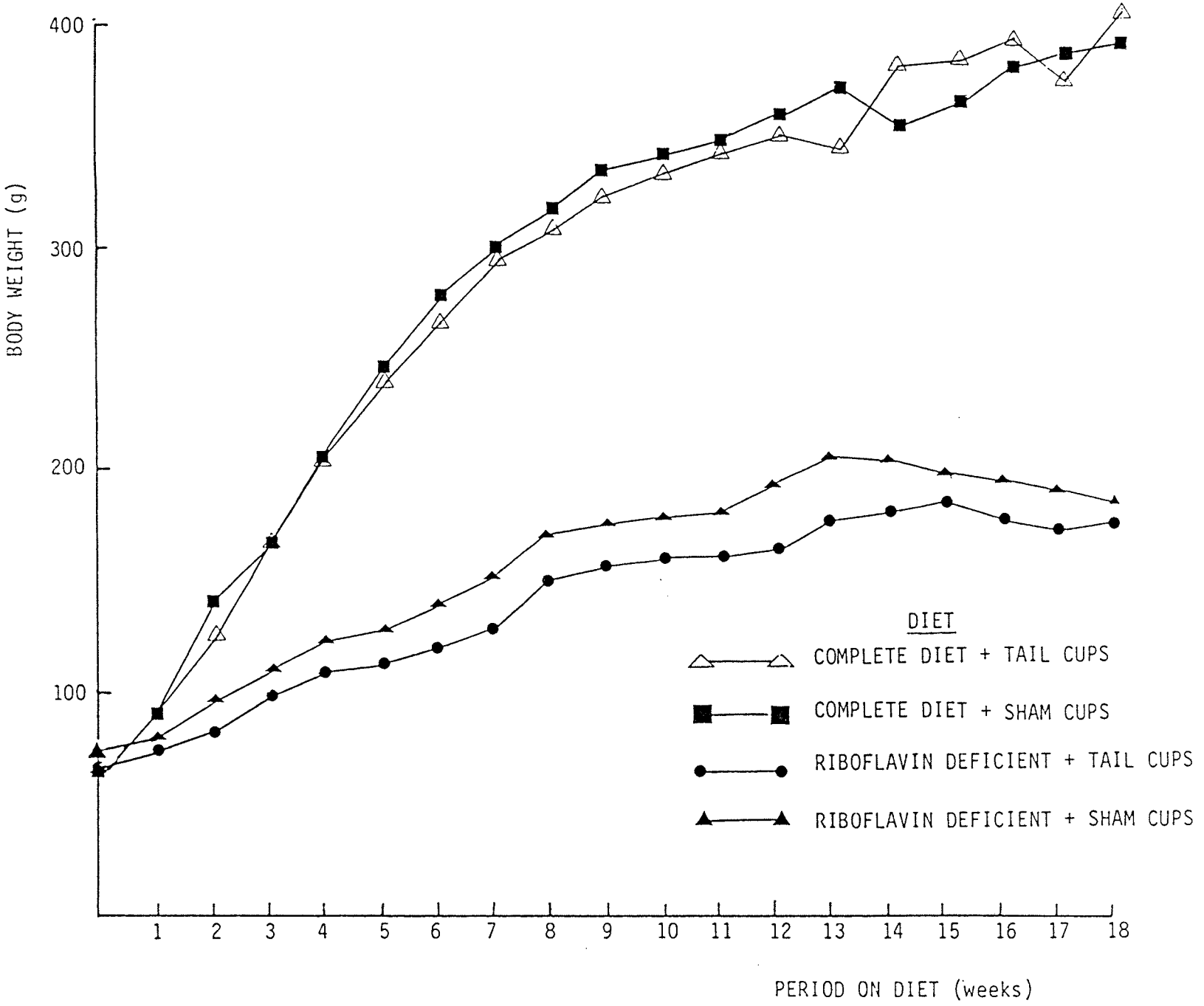
Experiment 1. The Effect of Feeding 2% Crude Vicine to Riboflavin Deficient Rats.

Within one week of initiation of the riboflavin deficient diet, the rats showed clinical symptoms of a riboflavin deficiency. The first symptom to manifest itself was that of growth retardation. Figure 10 illustrates the mean growth rate of the rats in the 4 treatment groups over the experimental 18-week period. As each set of 4 rats was selected and slaughtered for the assay procedures at varying periods throughout the experiment, the average body weight of the remaining rats were taken in order to plot the growth rate (Table 1A). The mean growth rates of the rats on the riboflavin deficient diet was significantly lower ($p < .0001$, Table 2A), than rats fed on the complete diet. After one week on the deficient diet the average change in weight for rats in Groups I and II was 24.9 g and 25.3 g respectively and for Groups III and IV was only 6.8g and 9.9g. During the acute stage of the deficiency, growth virtually ceased altogether but the time taken to reach this stage varied considerably among the rats.

Rats fitted with sham cups appeared to grow at a slightly faster rate compared to rats fitted with tail cups although this difference was found to be non significant ($p > .05$, Table 2A). Both sham and tail cups had to be replaced approximately every 2 days and daily in some cases as the rats succeeded in rapidly chewing through the gooch tubing and the plastic bottle at both the proximal and distal ends of the cups, thereby gaining access to their feces. The sham cups were chewed to a lesser extent. The cups on the whole caused little or no irritation or constriction to the tail.

The quantity of feed consumed by the riboflavin deficient rats and

Figure 10. Mean growth rate of rats fed a riboflavin deficient and complete diet fitted with either tail or sham cups over a period of 18-weeks.


400
300
200
100
BODY WEIGHT (g)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
PERIOD ON DIET (weeks)
DIET
COMPLETE DIET + TAIL CUPS
COMPLETE DIET + SHAM CUPS
RIBOFLAVIN DEFICIENT + TAIL CUPS
RIBOFLAVIN DEFICIENT + SHAM CUPS

rats fed the complete diet over the 48-hour period prior to death, is shown in Table 3A. Feed consumption was the same for rats fed both diet types ($p > .05$, Table 4A). The incorporation of vicine into the diet and the effect of tail and sham cups also did not significantly effect feed consumption (Table 4A).

After the third and fourth week, the characteristic symptoms of a riboflavin deficiency became increasingly evident. These included dermititis occurring on the feet, nose and tail, a dull and ragged appearance of the fur, which, in some cases, was accompanied by hair loss occurring in patches, and conjunctivitis which was noted on 4 of the rats. After 28 - 35 days, the lips appeared to be red and swollen and a red-brown exudate was noted on the whiskers and surrounding mouth area. This later appeared on the rats' feet and fur, presumably caused as a result of preening. Physical lesions appeared on the back and feet on approximately 25% of the rats after 35 days on the deficient diet. All of these symptoms increased in severity as the rats were maintained on the diet. There was considerable variation with regards to the degree to which each individual rat was inflicted with the clinical symptoms. Table 5A illustrates the mean liver weights for rats fed both diet types with and without vicine. Mean liver weights/100g body weight were much greater and were highly significant for rats on the deficient diet compared to rats fed the complete diet ($p < .0001$, Table 6A).

Table 2 and figure 11 illustrates the values obtained for the mean AC of EGR. During the 18-week study, the AC of the rats fed the complete diet with and without vicine was 1.2 and 1.3 respectively, which was considered to be within the normal range in this study. These AC differed significantly from rats fed the riboflavin free diet with and without vicine

Table 2. The effect of feeding vicine to riboflavin deficient rats on blood GSH spontaneous hemolysis, hemoglobin, hematocrit and erythrocyte glutathione reductase (EGR) activity

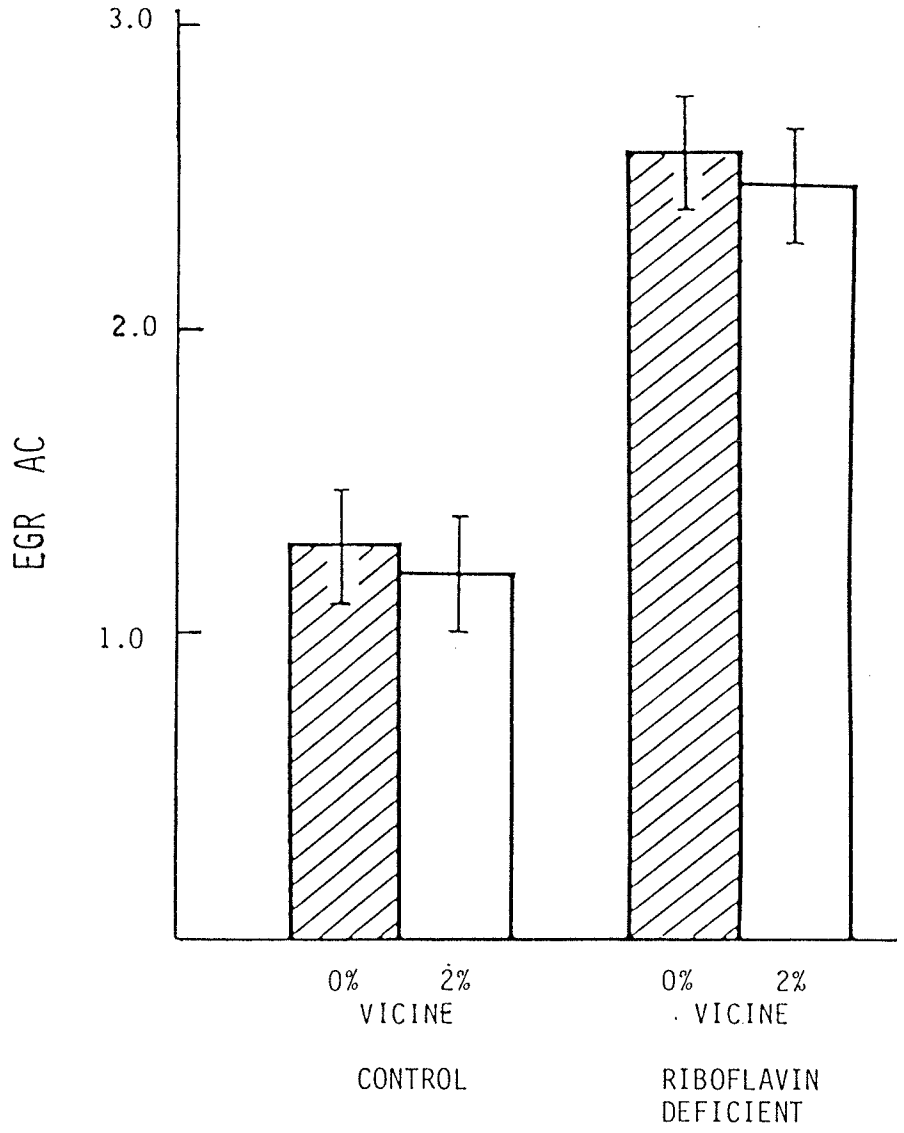
	Response Criteria				
Interactions	GSH (mg/100ml blood)	Hemolysis (%)	Hemoglobin (%)	Hematocrit (%)	EGR AC
Riboflavin x 0% vicine	41.9[c]	6.8[b]	13.9[b]	43.9[a]	1.3[b]
Riboflavin x 2% vicine	38.0[c]	7.3[b]	13.8[b]	42.7[a]	1.2[b]
0% riboflavin x 0% vicine	32.5[b]	9.3[b]	13.1[b]	42.0[a]	2.6[a]
0% riboflavin x 2% vicine	27.8[a]	15.3[a]	11.9[a]	36.7[a]	2.5[a]
SE	1.3	1.8	0.4	1.8	0.2

Summary of Analysis of Variance

		GSH		Hemolysis		Hemoglobin		Hematocrit		EGR AC	
Source of variation	df	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F
Riboflavin (R)	1	53.04	.0001	171.95	.008	10.65	.002	93.02	.044	10.81	.0001
Vicine (v)	1	10.03	.006	62.66	.088	2.73	.085	65.84	.084	0.06	.525
Tail Cup (T)	1	0.02	.897	2.73	.709	0.25	.586	16.25	.374	0.12	.384
RxV	1	0.08	.776	49.02	.128	1.77	.158	23.50	.288	0.00	.903
RxT	1	0.18	.678	2.52	.720	0.69	.774	13.13	.423	0.23	.229
VxT	1	2.15	.162	57.66	.100	3.78	.046	6.25	.578	0.00	.903
RxVxT	1	1.60	.224	0.18	.923	0.56	.420	28.71	.242	0.01	.779
Error	16	10.84		18.97		0.81		19.42		0.14	

[a]Means in each column not sharing a common superscript differ significantly ($p < .05$)

Figure 11. The effect of feeding vicine to riboflavin deficient rats on erythrocyte glutathione reductase (EGR) activity. Enzyme activities were expressed as activity coefficients (Glatzle et al. (1970).


3.0
2.0
1.0
EGR AC
0%
2%
VICINE
0%
2%
VICINE
CONTROL
RIBOFLAVIN
DEFICIENT

which had a mean AC of 2.5 and 2.6 respectively ($p < .0001$). The mean stimulation of EGR by FAD was therefore 150 % and 160 %. Since a 20% stimulation is indicitive of a riboflavin deficient state (Glatzle et al., 1970), the riboflavin status of these rats was obviously low. The highest AC recorded in this study for the riboflavin deficient rats was 3.1 and the lowest was 1.6. Vicine had no significant effect on mean EGR AC values for rats fed the complete or riboflavin deficient diet ($p > .05$)

The figures recorded in Table 2 represent the values of the blood parameters assayed from the treatment groups. Blood from rats fed the riboflavin deficient diet (Groups III & IV) showed a significant decrease in blood GSH concentrations ($p < .0001$) as compared to control animals on the riboflavin supplemented diet (Groups I & II). Normal levels of these parameters in the blood taken from rats fed the complete diet without vicine were found to be 41.9mg/100ml of blood for blood GSH, 13.9% for hemoglobin and 43.9% for hematocrit.

A slight response to feeding vicine ($p < .09$) was found with each blood parameter measured. Figures 12 and 13 illustrate the trends obtained for blood GSH and spontaneous hemolysis, hemoglobin and hematocrit for each of the treatment groups. There appeared to be more of a decline in blood GSH when the riboflavin deficient rats were fed vicine ($p < .006$). These rats had a mean GSH value of 32.5 mg/100ml blood whereas rats who were fed vicine had a mean GSH value of 27.8mg/100ml blood. Comparison of these treatment means using the SNK test revealed that this difference was significant ($p < .05$). Blood GSH values for the riboflavin deficient rats showed a significant decline ($p < .05$) from 36.2mg/100ml blood to 27.8mg/100ml blood after the inclusion of vicine in the diet (Table 7A & 8A). These rats

Figure 12. The effect of feeding vicine to riboflavin deficient rats on blood GSH and spontaneous hemolysis.

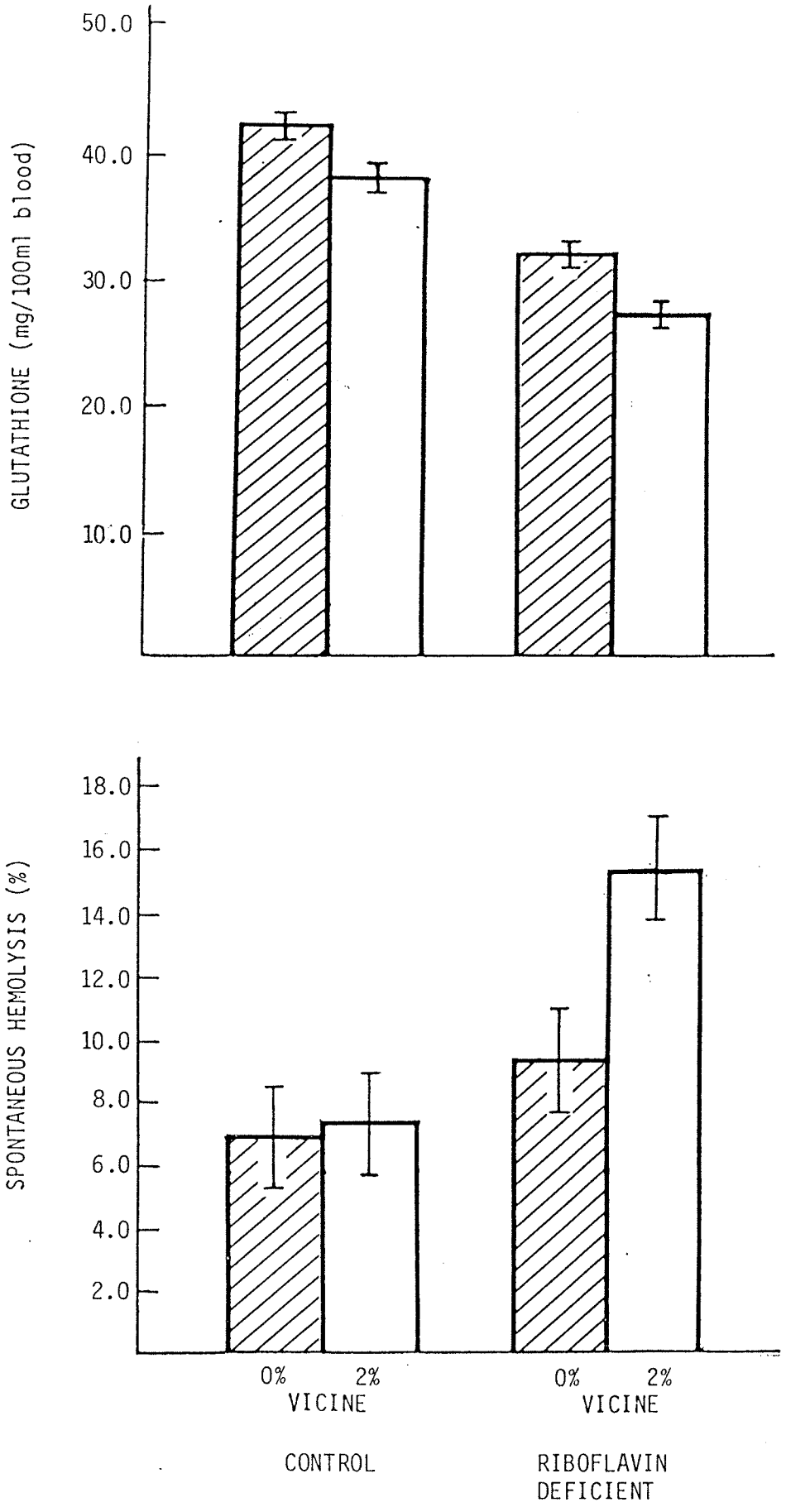
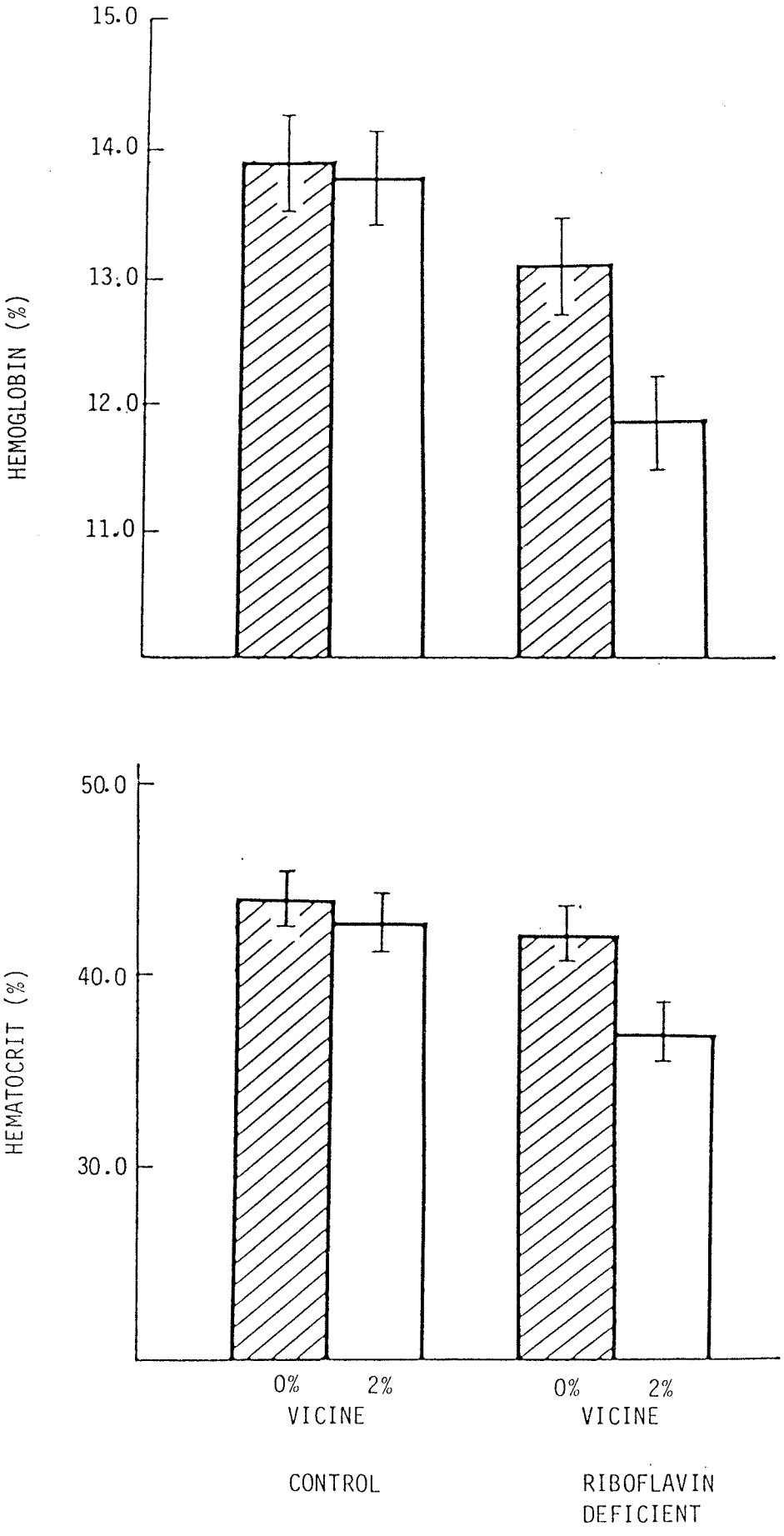

GLUTATHIONE (mg/100ml blood)
50.0
40.0
30.0
20.0
10.0
SPONTANEOUS HEMOLYSIS (%)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0%
2%
VICINE
0%
2%
VICINE
CONTROL
RIBOFLAVIN
DEFICIENT

Figure 13. The effect of feeding vicine to riboflavin deficient rats on hemoglobin and hematocrit values.

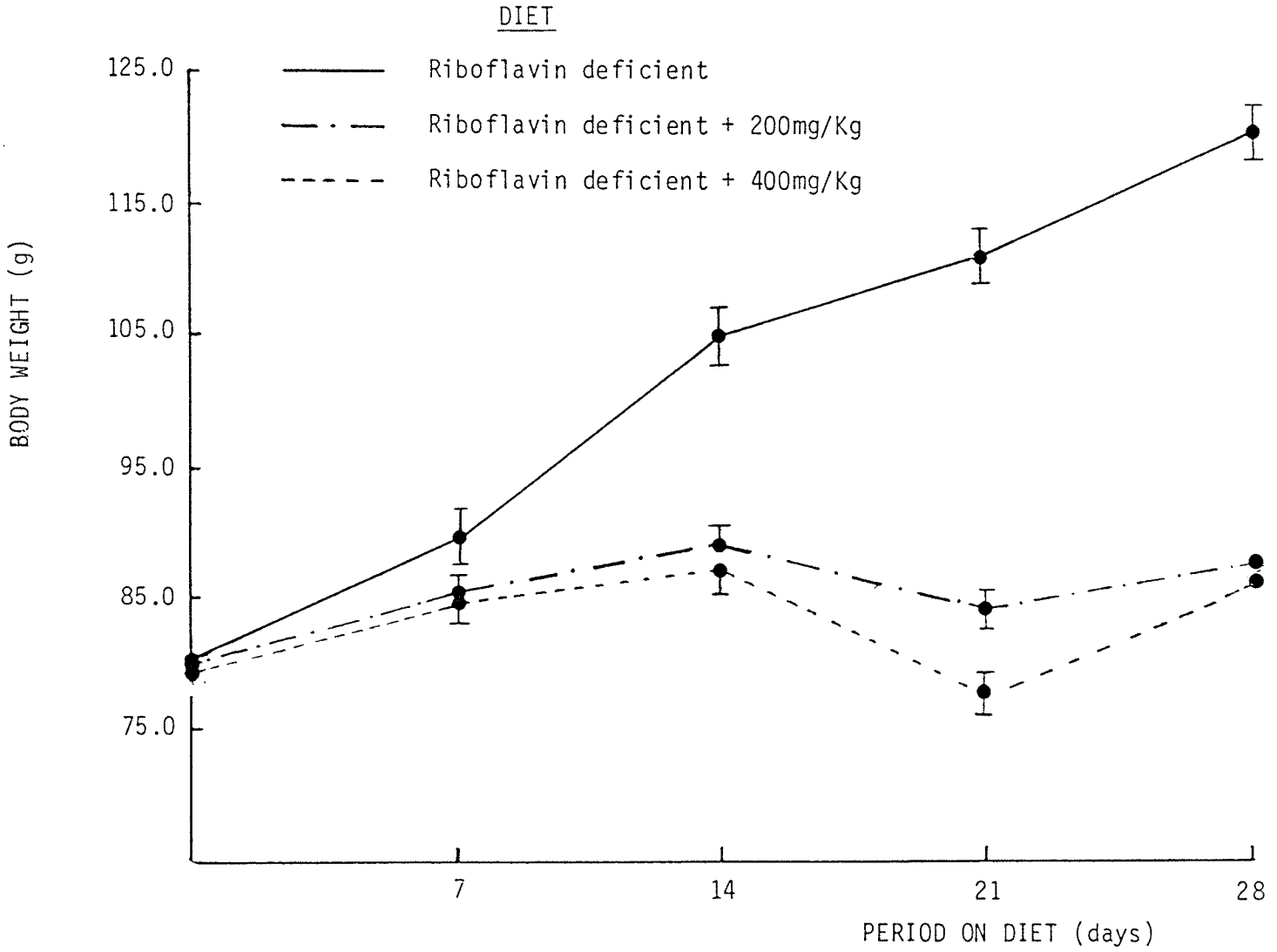

15.0
14.0
13.0
12.0
11.0
HEMOGLOBIN (%)
50.0
40.0
30.0
HEMATOCRIT (%)
0%
2%
VICINE
0%
2%
VICINE
CONTROL
RIBOFLAVIN
DEFICIENT

also responded with an apparent increase in spontaneous hemolysis ($p < .008$), and a decline in hemoglobin ($p < .085$). The highest value recorded for spontaneous hemolysis was 17.8% and the lowest value was 4.8%. Treatment means for spontaneous hemolysis and hemoglobin, when analysed using the SNK test, for all groups, revealed that a significant difference ($p < .05$) was evident for riboflavin deficient rats fed vicine compared to the remaining groups. Hemoglobinuria was observed in 3 of the riboflavin deficient rats, indicating the presence of ruptured erythrocytes in the urine, one day after their diets had been supplemented with vicine. No significant difference was obtained for treatment means for hematocrit values ($p > .05$). The SNK test revealed that there was no significant difference between treatment means for any of the parameters assayed for rats fed the complete diet ($p > .05$) with and without the incorporation of vicine.

Experiment 2. The Effect of Feeding 5% Crude Vicine to Rats Treated with Galactoflavin.

The growth rate of rats fed the simple riboflavin deficient diet and the riboflavin deficient diet supplemented with 200 and 400mg/Kg of galactoflavin over the 28-day experimental period is shown in Figure 14. The rats fed these levels of galactoflavin showed no growth during this period. The mean initial weights of rats fed 200 and 400mg/Kg of galactoflavin respectively were 80.3g and 79.2g and at the end of the experimental period were 87.3g and 86.4g. The growth rates of rats fed 200mg/Kg of galactoflavin appears to be marginally greater than those fed 400mg/Kg but comparison of the treatment means using the SNK test revealed no

Figure 14. Mean growth rate of rats fed a riboflavin deficient diet, and a riboflavin deficient diet supplemented with 200mg/Kg and 400mg/Kg of galactoflavin.


DIET
Riboflavin deficient
Riboflavin deficient + 200mg/Kg
Riboflavin deficient + 400mg/Kg
125.0
115.0
105.0
95.0
85.0
75.0
BODY WEIGHT (g)
7
14
21
28
PERIOD ON DIET (days)

a/

significant difference ($p > .05$). The striking difference in growth rates with rats fed the riboflavin antagonist can be observed by comparison with rats fed the riboflavin free diet ($p < .0034$, Table 9A).

Figure 15 illustrates the feed consumption pattern of the rats fed the 2 levels of galactoflavin and the riboflavin deficient diet alone. The rats fed galactoflavin consumed very little food, the average daily feed intake for Groups II and III combined being 4.2g /day, which was significantly lower than rats eating the riboflavin deficient diet ($p < .0001$, Table 10A).

Clinical symptoms typical of a riboflavin deficiency developed at a far greater rate with galactoflavin supplementation. Not only was the growth rate more severely retarded compared to rats on the riboflavin deficient diet but acute deficiency symptoms manifested themselves at an earlier stage. After 2 weeks, nearly all of the rats exhibited signs of an acute deficiency with matted ruffled hair, dermatitis occurring on the ears, nose and feet and brown exudate appearing on the lips. Rats on the riboflavin deficient diet without galactoflavin supplementation did not develope these symptoms until the end of the third week.

Table 3 presents the results obtained for blood GSH levels, spontaneous hemolysis, hemoglobin and hematocrit values and EGR AC. The EGR AC resulting from this study were much greater than those obtained for rats fed only a riboflavin deficient diet in Experiment 1. The values of the AC for each of the respective groups were 3.7, 4.0, 4.9, and 3.0 which corresponds to a mean EGR stimulation by FAD of 270%, 300%, 390% and 200%,respectively. The highest AC recorded in this study was 5.3 and the lowest was 3.0. Since a normal rat has a percent stimulation for

Figure 15. Mean daily feed consumption of rats fed a riboflavin deficient diet, and a riboflavin deficient diet supplemented with 200mg/Kg and 400mg/Kg of galactoflavin.

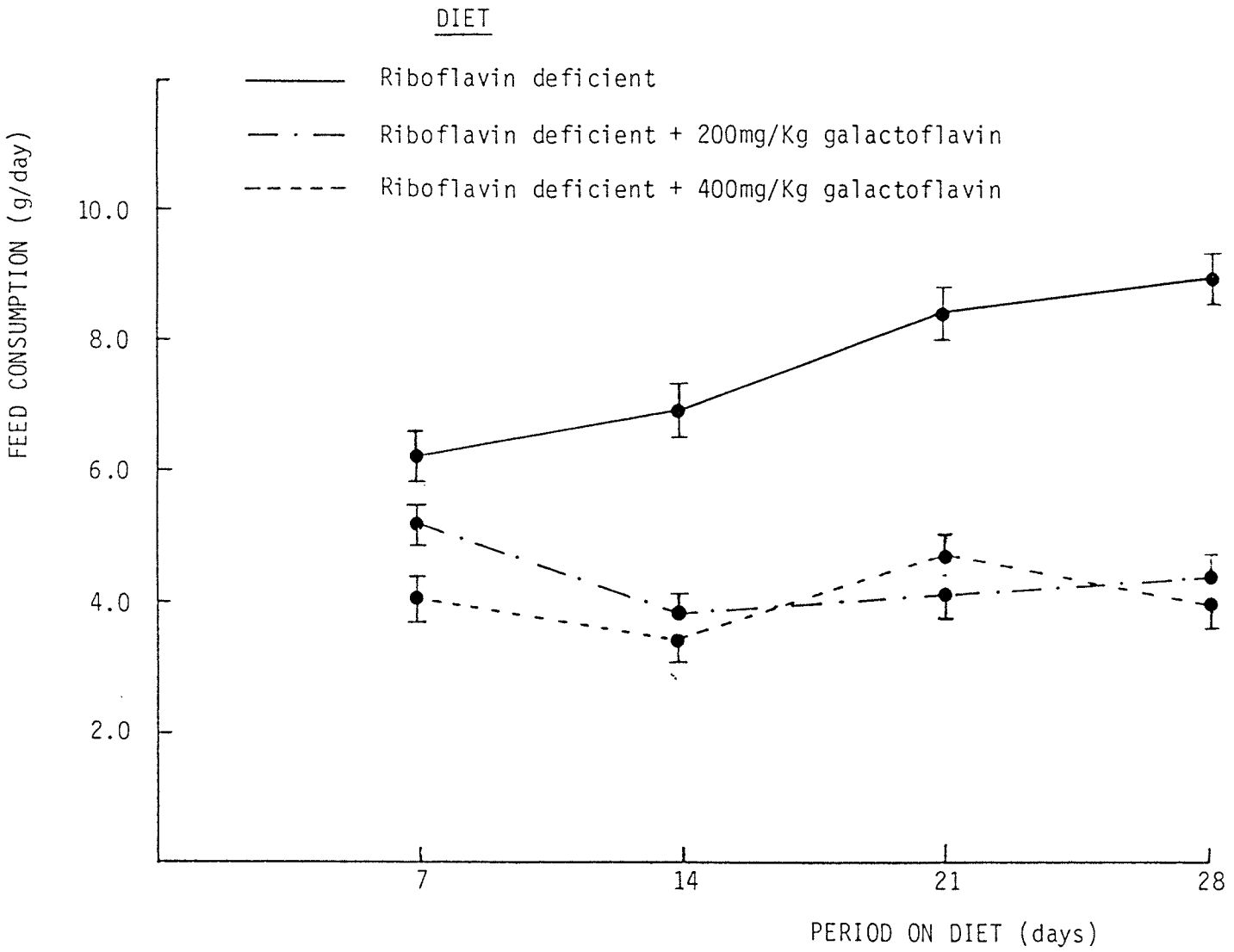

DIET
Riboflavin deficient
Riboflavin deficient + 200mg/Kg galactoflavin
Riboflavin deficient + 400mg/Kg galactoflavin
FEED CONSUMPTION (g/day)
10.0
8.0
6.0
4.0
2.0
7
14
21
28
PERIOD ON DIET (days)

Table 3. The effect of feeding vicine & galactoflavin to rats on blood GSH, spontaneous hemolysis hemoglobin, hematocrit & erythrocyte glutathione reductase (EGR) activity.

	Response Criteria				
	GSH (mg/100ml blood)	Hemolysis (%)	Hemoglobin (%)	Hematocrit (%)	EGR AC
Interactions					
400mg/Kg galactoflavin x 0% vicine	42.5	3.6	13.2	36.3	3.7 [a]
400mg/Kg galactoflavin x 5% vicine	32.8	17.2	12.7	31.3	4.0 [a]
200mg/Kg galactoflavin x 0% vicine	36.7	3.4	14.0	37.3	4.9 [a]
200mg/Kg galactoflavin x 5% vicine	32.0	24.2	11.8	30.0	3.0 [a]
SE	1.5	2.6	0.4	1.1	0.4

Summary of Analysis of Variance

		GSH		Hemolysis		Hemoglobin		Hematocrit		EGR	
Source of Variation	df	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F
Galactoflavin (G)	1	41.93	.052	44.76	.224	.0009	.971	.11	.882	1.06	.229
Vicine (V)	1	204.35	.0005	1182.33	.0001	7.08	.006	151.91	.0001	0.16	.633
G x V	1	22.99	.137	52.42	.1902	2.61	.067	5.41	.301	0.74	.312
Error	12	9.03		27.19		0.64		4.62		0.66	

[a]Means in each column not sharing a common superscript differ significantly ($p < .05$).

EGR in the range of 20%, the riboflavin status of the rats was very low. Activity coefficient values for EGR in the 4 treatment groups were not found to differ significantly when compared using the SNK test ($p > .05$).

No significant difference ($p > .05$) was found in any of the 4 assays carried out between the 2 different levels of galactoflavin supplemented to the diet. The addition of vicine to the diet, however, caused blood GSH levels to fall ($p < .0005$), spontaneous hemolysis values to rise ($p < .0001$) and hemoglobin ($p < .006$) and hematocrit ($p < .0001$) levels to significantly drop. Figure 16 illustrates the trends for each of the parameters assayed for Groups I & II and III & IV combined as an average.

Experiment 3. The Effect of Vinylidene Chloride on Blood GSH Concentrations in the Rat.

Blood GSH concentrations obtained when varying doses of vinylidene chloride were given to rats are summarized in Table 4. The level of vinylidene chloride administered to the rats over the 6-hour period had no significant effect on blood GSH($p > .05$). The rats appeared to be withdrawn and subdued during the 6-hour period. Since vinylidene chloride had no effect on erythrocyte GSH levels, no further experiments involving challenging rats with vicine in the diet were carried out. A higher dose of vinylidene chloride was not given due to the lethality of the drug.

Figure 16. The effect of feeding vicine and galactoflavin to rats on blood GSH, spontaneous hemolysis, hemoglobin and hematocrit. Each bar represents an average value of the low (200mg/Kg) and high (400mg/Kg) galactoflavin doses.

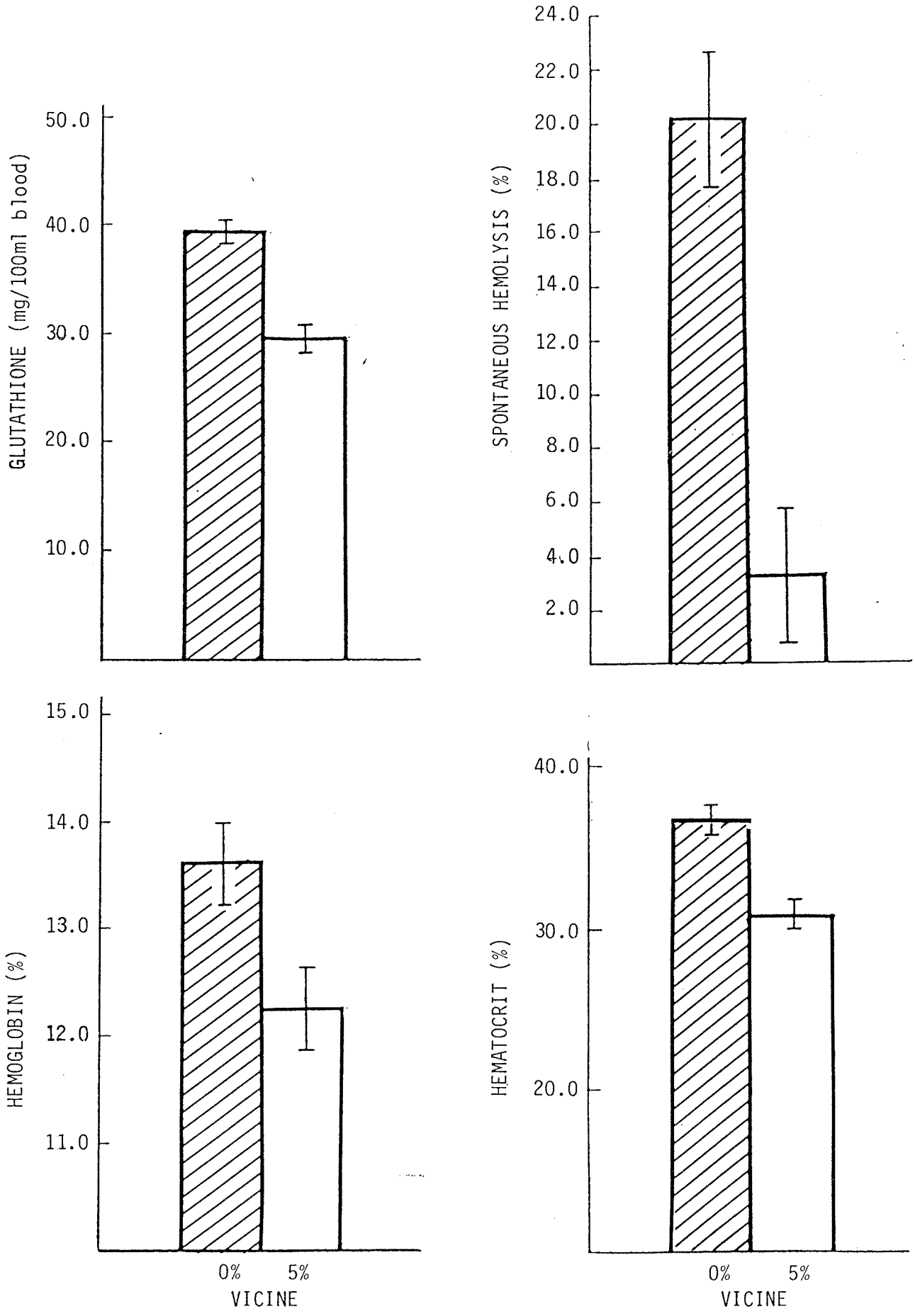

GLUTATHIONE (mg/100ml blood)
50.0
40.0
30.0
20.0
10.0
SPONTANEOUS HEMOLYSIS (%)
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
HEMOGLOBIN (%)
15.0
14.0
13.0
12.0
11.0
HEMATOCRIT (%)
40.0
30.0
20.0
0%
5%
VICINE
0%
5%
VICINE

Table 4. Blood GSH levels at varying doses of vinylidene chloride in male rats

Time (hours)	Blood GSH levels (mg/100ml blood) Dose of vinylidene chloride (mg/kg) 0	300	400	500
2	35.0±1.0	34.3±0.9	36.1±1.5	38.4±1.4
4	40.9±1.8	38.0±2.5	39.4±3.2	33.2±1.2
6	31.1±1.6	35.6±3.1	37.1±3.0	36.6±0.9

Summary of Analysis of Variance

Source of variation	df	MS	PR> F
Vinylidene chloride	3	12.15	0.618
Time	2	7.34	0.590
Error	6	6.36	

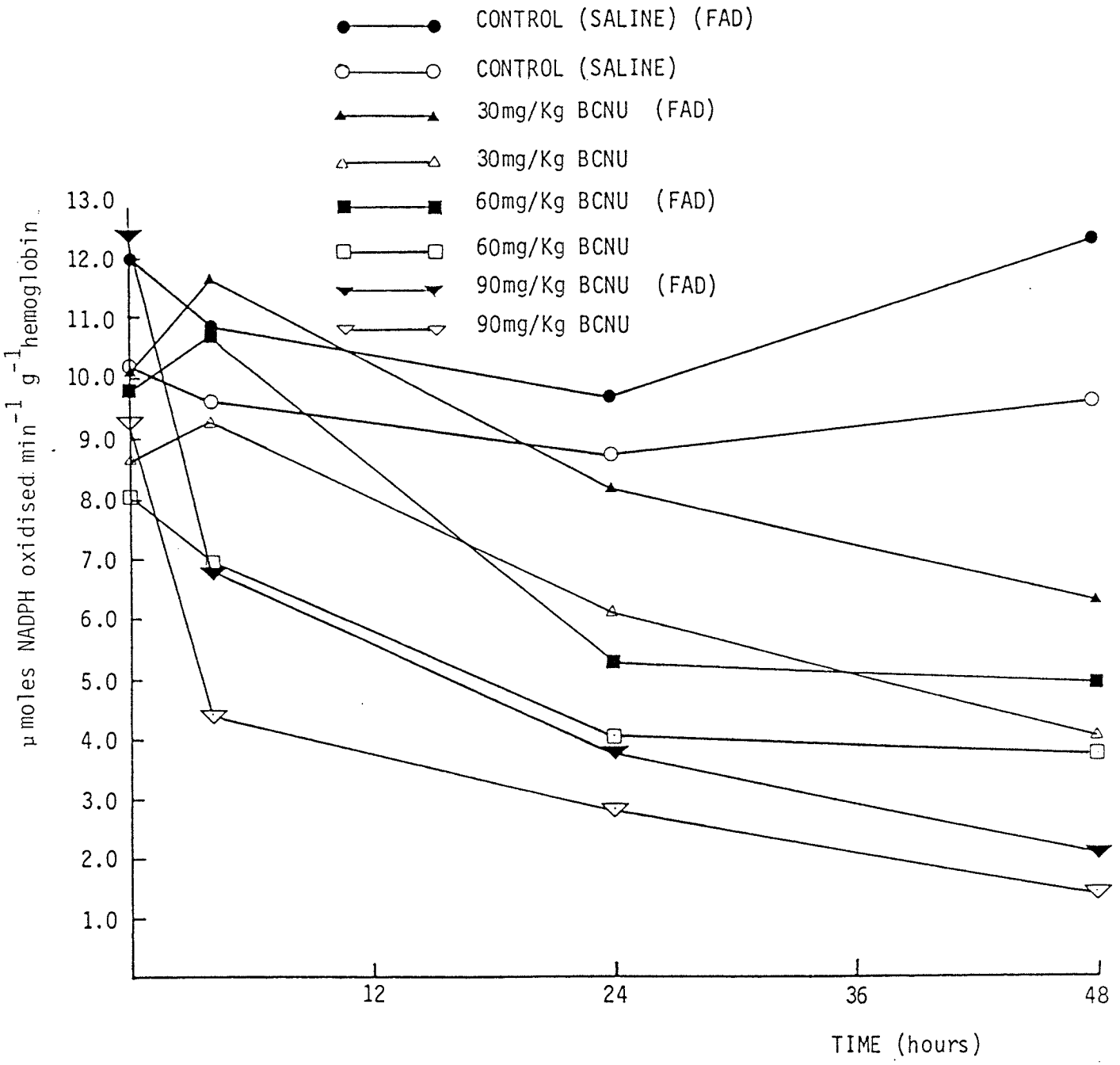
Experiment 4. The Effect of Feeding 5% Crude Vicine to Rats treated with BCNU.

Figure 17 illustrates the dose response curve of BCNU on EGR activity, with and without FAD, over a 48-hour period. The actual values obtained for EGR activities are tabulated in Table 11A. Enzyme activities with FAD were always greater compared to activities without FAD, due to the enzyme being fully saturated with its cofactor. Control rats in Group I injected with saline had mean EGR activities of 11.0 and 10.2 μmoles NADPH oxidized $min^{-1} \cdot g^{-1}$ hemoglobin with and without FAD respectively. Rats in Groups II, III and IV all showed a decline in EGR activity 4 hours after being administered the drug. This response continued in a fairly rapid downward trend for another 20 hours after which the decline in activity was only marginal and tended to level off over the next 24 hours. The percentage drop in enzyme activity over the 48-hour period was 38.0% for a 30mg/kg dose, 50.2% for a 60mg/kg dose and 83.4% for a 90mg/kg dose of BCNU.

During this period the rats did not suffer from any observable adverse side effects with the exception of Group IV which did develope a mild form of diarrhea. Diarrhea is one of the characteristic side effects of therapeutic doses of BCNU given to humans.

Mean EGR activities 24 hours after the administration of saline and 100mg/kg of BCNU were 10.4, 12.4, 3.9 and 3.5 μmoles NADPH oxidized $min^{-1} \cdot g^{-1}$ hemoglobin with the addition of FAD for Groups I-IV respectively (Table 12A). A significant decrease in enzyme activities was found for rats in Groups III and IV treated with BCNU compared to Groups I and II which were untreated with the addition of FAD ($p < .0003$, Table 13A)

Figure 17. The effect of administering varying intraperitoneal doses of BCNU to rats on erythrocyte glutathione reductase activity with and without the addition of FAD.


CONTROL (SALINE) (FAD)
CONTROL (SALINE)
30mg/Kg BCNU (FAD)
30mg/Kg BCNU
60mg/Kg BCNU (FAD)
60mg/Kg BCNU
90mg/Kg BCNU (FAD)
90mg/Kg BCNU
μ moles NADPH oxidised min⁻¹ g⁻¹ hemoglobin
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
12
24
36
48
TIME (hours)

and without the addition of FAD ($p < .0007$, Table 14A). The decline in enzyme activities over the 24-hour period in Groups III and IV were approximately the same as those values obtained using 90mg/kg of BCNU in the preliminary dose response curve. Although the dose of BCNU used in this experiment was greater than the 90mg/kg dose used in the response curve, the drop in activity of the enzyme was surprisingly no greater.

Table 5 presents the mean blood GSH, spontaneous hemolysis, hemoglobin and hematocrit values and EGR activities obtained from each of the 4 treatment groups 72 hours after BCNU administration. Erythrocyte glutathione reductase activities still remained significantly lower ($p < .0004$) in rats treated with BCNU but these activities, 6.0 and 5.1 μmoles NADPH oxidized $min^{-1}g^{-1}$ hemoglobin for Groups III and IV, were not as low as anticipated and had actually shown no significant change over the 24 to 72-hour period (Tables 15A and 16A).

Mean blood GSH values for rats in Groups III and IV were significantly lower (p .0001) than those for Groups I and II. No significant differences were found between treated and untreated rats with BCNU for spontaneous hemolysis, hemoglobin and hematocrit values, the latter two of which remained within normal ranges. Spontaneous hemolysis values did appear to be greater with rats treated with BCNU (Table 5).

The inclusion of 5% vicine into the diet of rats in Groups II and IV had no effect on any of the parameters measured ($p > .05$). Figure 18 illustrates the trends obtained for blood GSH and spontaneous hemolysis when the rats were treated with BCNU and fed vicine.

The quantity of feed consumed by the rats in the 4 groups is illustrated in Table 17A. BCNU caused a significant drop in appetite ($p < .013$), with vicine having no effect ($p > .05$, Table 18A). The average

Table 5. The effect of feeding vicine to rats treated with BCNU on blood GSH spontaneous hemolysis, hemoglobin, hematocrit and erythrocyte glutathione reductase (EGR) activity.

Response Criteria

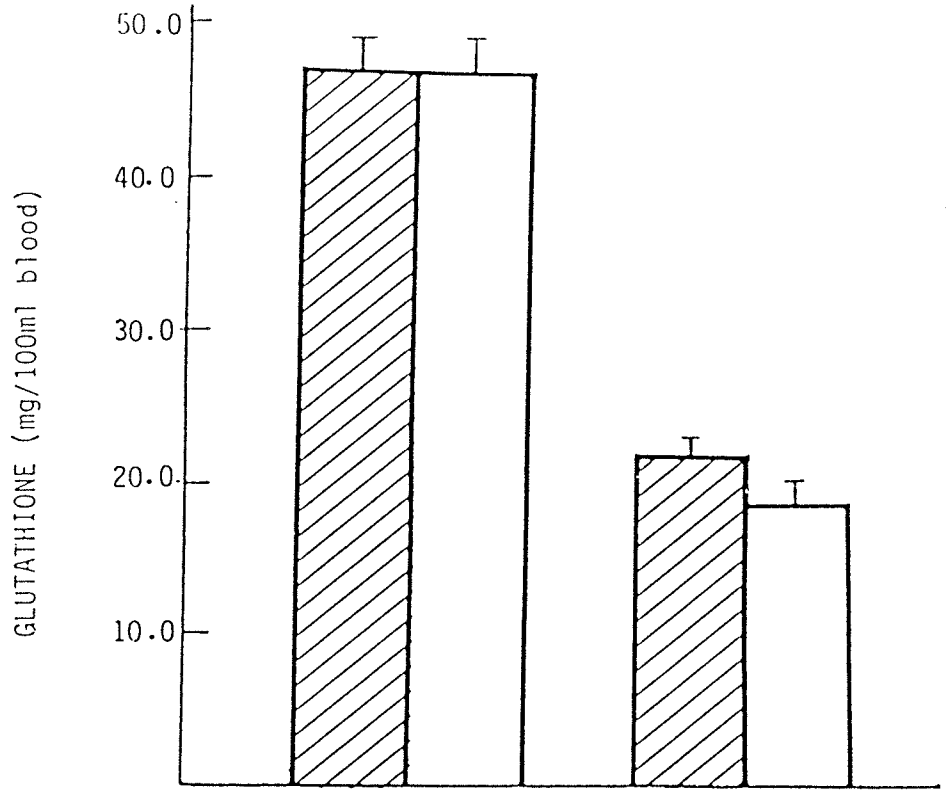
	GSH (mg/100ml blood)	Hemolysis (%)	Hemoglobin (%)	Hematocrit (%)	EGR activity μmoles NADPH oxidized min^{-1} g^{-1} hemoglobin	
					FAD	NO FAD
CONTROL x 0% vicine	46.7	1.68	11.2	42.8	12.3	8.7
CONTROL x 5% vicine	46.7	2.55	14.1	42.3	12.2	8.8
100mg/Kg BCNU x 0% vicine	21.8	5.5	13.6	37.3	6.0	4.7
100mg/Kg BCNU x 5% vicine	18.9	10.48	14.4	41.9	5.1	4.3
[a]SE	2.6	4.7	0.7	3.8	1.3	1.0
	1.8	3.3	0.5	2.7	0.9	0.7

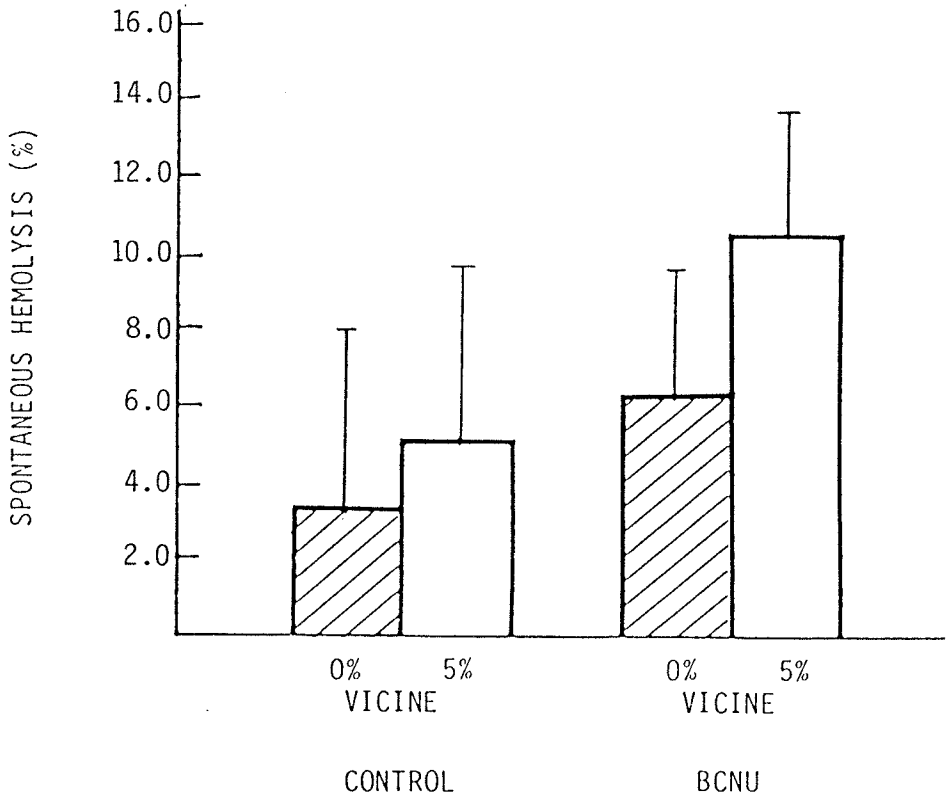
Summary of Analysis of Variance

		GSH		Hemolysis		Hemoglobin		Hematocrit		EGR activity FAD		EGR activity NO FAD	
Source	df	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F	MS	PR>F
BCNU (B)	1	1849.95	.0001	92.71	.186	0.02	.879	23.01	.396	117.84	.0004	48.45	0.0013
Vicine (V)	1	5.18	.550	38.52	.379	0.77	.371	11.34	.546	1.28	.565	0.16	.787
B x V	1	5.79	.528	11.00	.632	0.59	.428	17.51	.457	0.49	.719	0.22	.753
Error	8	13.32		44.39		0.86		28.6		3.55		2.08	

[a]Standard errors for Groups I & II and III & IV respectively

Figure 18. The effect of feeding vicine to rats after administering intraperitoneal injections of 100mg/Kg of BCNU on blood GSH and spontaneous hemolysis.


GLUTATHIONE (mg/100ml blood)
50.0
40.0
30.0
20.0
10.0


SPONTANEOUS HEMOLYSIS (%)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0%
5%
VICINE
0%
5%
VICINE
CONTROL
BCNU

daily feed consumption for rats in Groups I and II, III and IV was 5.5, 5.0, 3.5 and 3.1g/100g body weight respectively. No abnormal behaviour was observed in any of the rats. Diarrhea did, however, occur in the BCNU treated rats which was quite severe in some cases.

Discussion

The riboflavin deficient animal model responded to the challenge of vicine in a manner similar to that of a favic red blood cell with declines in blood GSH ($p < .006$), hemoglobin ($p < .085$) and hematocrit ($p < .084$) concentrations and an increase in spontaneous hemolysis ($p < .088$) These trends were not as pronounced as the symptoms exhibited in severe cases of favism but nevertheless were apparent.

The trends resulting from this animal model may have been more pronounced if the rats had become more deficient in riboflavin. This would have lowered EGR activity and the glutathione reducing capacity of the cell even further, thereby rendering the cell even more vulnerable to the oxidant effects of the pyrimidine aglycone. The mean AC values of the riboflavin deficient rats obtained from the current study were not as high as those reported by other authors who also carried out similar riboflavin deficient studies on rats (Tillotson & Sauberlich, 1971; Hassan & Thurnham, 1977; Prentice & Bates, 1980 & 1981). These latter studies were of shorter duration compared to the current study and no attempt to prevent coprophagy, with the exception of Prentice & Bates (1980, 1981) had been made. Certain individual rats did, however, exhibit high AC of 3 and above, which were in the same order of magnitude as some of the values obtained by other authors. The lower mean AC for the riboflavin deficient rats can be attributed to several factors. Two of the sets of rats assayed were performed prematurely after only 29 and 48 days on the deficient diet. These AC values proved to be low, indicating that the rats were not acutely

deficient. This would have resulted in a less dramatic response in all assayed parameters with the vicine treated rats, since the reducing capacity of the cell had not been sufficiently decreased.

The initial weights of the rats at the start of the experiment were greater relative to those reported by other authors due to the increase in weight that incurred over the 5-day acclimitization period when the rats were fed rat chow. This may have influenced the time taken to create an acute riboflavin deficient state, as riboflavin body reserves may have been increased during this period.

It appears that partial refection resulting from an increase in riboflavin supply to riboflavin deficient rats through coprophagy could have occurred despite the fact that a purified diet was used together with tail cups. Faulker and Lambooy (1961) have estimated that rats on a purified riboflavin free diet are capable of synthesizing 3µg of riboflavin/day by the microflora in their gastrointestinal tract. The remission from deficiency was made possible by the fact that some of the rats wearing tail cups had a rapacious appetite for chewing their tail cups and gaining access to their feces. This refection may account for the small but continuous growth rate and the lower AC values. The lack of a significant effect of tail cups on riboflavin status of the animal would suggest that the tail cups were ineffectual and therefore can be dispensed with in the future or a more effective means of preventing their destruction should be devised.

All of these factors would influence the values obtained for the AC and give an explanation as to why the overall means were not as high as was expected. Although the rats did exhibit signs of an acute riboflavin deficiency state, higher AC would have been expected. However, studies carried out by Tillotson & Baker (1972) indicated that the symptoms in a riboflavin

deficiency do not always correlate well with AC values. Liver hypertrophy was very apparent in this study and according to Prentice & Bates (1981) this is indicative of a severe riboflavin deficiency state. Burch et al. (1956) interpreted this as a compensatory adaptation for the metabolic inadequacy.

The AC produced in this study did result in a decreased glutathione-reducing capacity. Blood GSH levels in rats fed the diet devoid of riboflavin had dropped significantly compared to control rats fed the complete diet ($p < .0001$). This was presumably caused by the impaired activity of EGR resulting from diminished FAD levels. These results are in accordance with those of Hassan & Thurnham (1977) who found an increased instability and a decrease in glutathione reducing capacity in erythrocytes obtained from rats fed a riboflavin deficient diet. D'Aquino et al. (1979) also noted that spontaneous hemolysis in riboflavin deficient erythrocytes was 13% compared to 7% for control rats indicating an increase in erythrocyte membrane fragility.

The degree to which the riboflavin deficient erythrocyte was challenged and the severity of the hemolytic attack will not only depend on the ability of the cell to regenerate GSH but also on the quantity of vicine hydrolysed in the cecum and large intestine to divicine and to the amount of divicine absorbed into the blood stream. A diet devoid of riboflavin has been reported to cause a reduction in appetite (see Harper & Boyle, 1976). Feed intake in this study was not found to differ significantly ($p < .05$) for rats fed either diet. However, the amount of vicine which was hydrolysed in the hind gut may not have been as high as anticipated due to the nature of the diet. The low fibre content of the diet may have caused a reduction in intestinal microflora and hence the amount of vicine hydrolysed.

D'Aquino et al.'s (1979) riboflavin deficient animal model clearly demonstrated that isouramil concentrations of between 2mM and 5mM when added to rat erythrocytes in vitro, caused a dramatic decline in GSH concentrations. The in vitro model is analagous to the in vivo situation but the physiological concentration of pyrimidine aglycone challenging the erythrocyte in vivo would be much lower. In vitro, D'Aquino et al. (1979) had already prepared the aglycone of convicine, isouramil, by the action of β-glucosidase prior to the addition of incubated erythrocytes. In vivo, the formation of divicine from vicine is dependent on the hydrolytic action of bacteria in the hind gut which must then be absorbed into the blood stream before an oxidant challenge can be made(see Mager et al.,1980). It appears from the results of this investigation that the quantity of vicine hydrolysed in the gut over the 48-hour period may have been insufficient to impose a very high oxidant challenge on the already lowered GSH reducing capacity of the riboflavin deficient cell, which would partly account for the small trends obtained. The data do illustrate that the riboflavin deficient erythrocyte was unable to cope completely with the challenge of divicine and, as a result, GSH concentrations decreased and spontaneous hemolysis increased. This was due to the inability of EGR to generate a sufficient amount of GSH to combat the oxidant effect of divicine. As GSH is known to protect the cell from oxidative attack (Beutler, 1971), this leads to irreversible damage to the erythrocyte membrane causing it to rupture and hemolyse. The occurrence of hemoglobinuria in 3 of the riboflavin deficient rats treated with vicine further supports the inability of the diminished blood GSH levels to neutralize the toxic effects of divicine.

The degree to which hemolysis occurs in vivo may not appear to be very great because the ruptured cells may also have been removed, not only by the

intravascular system but also the reticuloendothelial system and hence would escape detection in the hemolysis assay (Chevion et al.,1982). A greater decline, however, in hematocrit values would be anticipated if this were the case but there was no such trend in the data in this study.

These trends are similar to the results obtained by Muduuli et al. (1981b) involving feeding vicine to the laying hen where a pronounced increase in spontaneous hemolysis and decline in hemoglobin and hematocrit levels was evident. These authors attributed these effects to the oxidant effect vicine had on the metabolism of the laying hen. Since the laying hen requires a high level of NADPH for fat synthesis, it was unable to meet the demand in the presence of vicine. This would lead to impaired fat synthesis and also GSH synthesis, resulting in peroxidative damage to the erythrocyte membrane.

The incorporation of 2% vicine into the diet of non riboflavin deficient rats resulted in an insignificant response in blood GSH, hemoglobin, hematocrit and spontaneous hemolysis ($p > .05$). These results are supported by Hegazy & Marquardt (1984) who reported that spontaneous hemolysis was low in rats fed 1% and 3% vicine in their diets and was only slightly greater than control values. This apparent insensitivity of the rat to the effects of vicine can be attributed to the rats' ability to generate a sufficient amount of GSH, thereby neutralizing the oxidant effect of divicine.

To summarize, the riboflavin deficient rat did provide a reasonable in vivo animal model for studying favism. However, erythrocytes derived from the riboflavin deficient rat would have become a closer mimic of a G6PD deficient erythrocyte if they had become more deficient. This could have been achieved more successfully by using younger rats or by using the surviving offspring derived from a riboflavin deficient mother (see Lambooy, 1975). A tocopherol and riboflavin deficient diet would also have the combined effect

of lowering GSH regeneration and retarding the removal of peroxides and enhance erythrocytes susceptibility to an oxidant attack. However, it took D'Aquino et al. (1983) 140 days to produce erythrocytes in rats that were suitable for in vitro studies. The potential that such an animal model would have in vivo is limited because of problems involving the ingestion and absorption of vicine due to the probable loss of appetite and general condition of the animal.

Galactoflavin treated rats not only created erythrocytes which were sensitive to an oxidant challenge but provided a very successful animal model for studying the effects of vicine in vivo.

The effect galactoflavin had on growth rate, feed consumption and clinical symptoms were very similar to results obtained by Lane (1964), Prosky et al. (1964) and Emerson et al. (1945). The onset of the deficiency was very rapid and pronounced when compared to rats on a simple riboflavin deficient diet and rats from Experiment I. The AC obtained in this study for both levels of galactoflavin were high,indicating that the in vitro addition of FAD produced a very high stimulatory response from EGR. The AC in this experiment were comparable to the ones obtained by Prentice & Bates (1981). However, in this study it took only 4 weeks to reach an AC value of 4 whereas with these authors it took 7 weeks. and the rigorous prevention of coprophagy to arrive at the same AC value. It appears from the EGR AC data that galactoflavin rapidly exerts its antagonistic effect, creating a state of acute deficiency in the rat and causing a considerably diminished activity in EGR and fall in blood GSH levels ($p < .052$).

Erythrocytes from galactoflavin treated rats also proved to be highly sensitive to the effects of vicine. It appears that despite feed intake being low, a sufficient amount of vicine was hydrolysed in the gut in order

to produce the deleterious effects on the erythrocytes. The animal model produced the same effect with vicine as occurs in a G6PD deficient individual. The divicine would have had the effect of depleting GSH concentrations via successive reductions as explained by Chevion et al. (1982) in the literature and since the overall GSH reducing capacity of the cell was lowered by the impaired activity of EGR, the erythrocyte is unable to generate sufficient GSH to neutralize this effect. A large significant increase in spontaneous hemolysis ($p < .0001$) thus resulted with a significant decline in hemoglobin ($p < .006$) and hematocrit ($p < .0001$) levels, presumably due to the rupture of the erythrocytes. The data for hemoglobin does not suggest that the rats were anemic after the 28-day period on the galactoflavin diet. Anemia is often associated with acute riboflavin deficiency (Prentice & Bates, 1981). The changes occurring with the 4 parameters measured were surprisingly less for rats treated with the higher level of galactoflavin. This may be due to the fact that these rats had not consumed as much feed and hence vicine.

The effects of vicine in the galactoflavin animal model were more dramatic compared to the riboflavin deficient animal model. Spontaneous hemolysis was probably greater than it appeared to be from the assay as some of the ruptured cells may have been removed by the spleen (Chevion et al., 1982). Hematocrit values do indicated that a pronounced fall in erythrocytes had resulted. The galactoflavin animal model was also produced in a shorter period of time compared to the previous model. This animal model has provided a very closely related in vivo situation comparable to a G6PD individual and responded in much the same way as a susceptible enzyme deficient subject would upon ingestion of faba beans.

Lowering blood GSH levels using vinylidene chloride in the rat does

not provide a suitable animal model for studying favism. Vinylidene chloride has been documented as exerting its main toxic effect on the liver (Younes & Siegers, 1980; Siegers et al., 1979). Although it causes a very rapid hepatic depletion of GSH (Younes & Siegers, 1980), it appeared to have no effect on blood GSH in this study. It was felt that the administration of a higher dose of this GSH depleting agent would have proven to be too hepatotoxic to the rat. In retrospect, the use of certain depleting agents in rats would not necessarily provide a suitable animal model due to their high hepatotoxicity effect on other cellular parameters (Meister & Anderson, 1983; Younes & Siegers, 1980) and lack of effect on erythrocyte GSH concentration.

The action of BCNU proved to be very effective in creating a situation in the erythrocyte similar to that of a favic red blood cell. High doses of BCNU caused a very rapid pronounced depletion of EGR activity; enzyme activities were depressed by as much as 83% 48 hours after drug administration. The severe EGR deficiency created in the erythrocyte by BCNU impaired GSH regeneration causing a decline in intracellular GSH concentrations. Although the experiments carried out by Frischer & Ahmad (1977) and Sagone & Burton (1979) were on human blood samples, EGR inhibitions in the rat, in this study, were of the same order of magnitude. Blood GSH concentrations were depleted to a greater extent in this experiment, whereas Frischer & Ahmad's (1977) data revealed only a small but significant decline.

The high doses of BCNU used in this study, by having a pronounced effect on EGR and hence the glutathione pathway, should be able to render the erythrocyte very sensitive to an oxidant stress. However, the incorporation of vicine into the diet did not prove to have any significant

effect on the parameters measured. The data obtained for spontaneous hemolysis does indicate an increase for rats treated with BCNU, the vicine treated rats being marginally greater relative to the controls. Since BCNU is affecting the major peroxide detoxification pathway in the erythrocyte by inhibiting EGR, then the HMP will be unable to respond to the elevated H_2O_2 levels and low GSH levels. This may account for the apparent increase in sensitivity of the BCNU treated erythrocytes rendering them more fragile and vulnerable to rupture. However, as the standard errors for the spontaneous hamolysis data are large, no firm conclusions regarding trends can be made.

Erythrocyte glutathione reductase activities revealed no significant change in activity during the 24-72 hour period after drug administration. The administration of 100 mg/kg BCNU did not depress EGR activities to a greater extent compared to the 90 mg/kg dose used in the dose-response curve. The reason for this apparent discrepancy is not clear. The dose-response curve reveals that EGR activities were still being marginally depressed 48 hours after drug administration. Assuming this were the case in the actual study carried out with feeding vicine, the EGR activities remained the same in the period 24 hours prior to death. Presumably the drug at this stage had imposed its major inhibitory effect on EGR. The rats were also possibly responding to the effect of the drug by releasing an influx of newly synthesized erythrocytes from the bone marrow into the blood stream. This may explain why there was no significant change in EGR activity over the 24 - 72-hour period as the release of new erythrocytes would have partially cancelled out the inhibitory effect that BCNU had on EGR. The release of new erythrocytes may, in part, account for the insignificant response of vicine in the BCNU treated rats as the normal erythrocytes

released with normal EGR activity may have been capable of counteracting the oxidant effect of divicine by generating sufficient GSH. Frischer & Ahmad (1977) also noted the reappearance of EGR activity 3-4 days after drug administration and suggested that it may be attributed to the release of a subpopulation of erythrocytes from the bone marrow.

Diarrhea and loss of appetite was observed in rats treated with BCNU, which are some of the characteristic side effects of the drug. These two factors would have the combined effect of reducing, firstly the amount of feed passing through the gut and secondly, diarrhea would cause the digesta to pass through the intestines more quickly, thereby hindering the hydrolysis of vicine by the microorganisms in the hind gut. Under these circumstances, the amount of vicine hydrolysed in the hind gut may have been very small. This could have been the major reason as to why vicine had no effect on blood GSH, spontaneous hemolysis, hemoglobin and hematocrit values on the rats treated with BCNU.

The erythrocytes derived from the BCNU animal model would be highly suitable for in vitro studies because it did create a situation similar to that of a G6PD-deficient red blood cell. This model also has the added advantage of being able to be produced in 48 hours in contrast to D'Aquino et al.'s (1981, 1983) riboflavin and tocopherol deficient animal model which took 140 days to create. The two models are comparable as the BCNU animal model caused a 83% drop in EGR activity compared to a 90% drop in EGR activity in D'Aquino et al.'s models (1981,1983). The BCNU animal, since it is easier and quicker to produce, has obvious advantages over D'Aquino's et al.'s (1981, 1983) animal models when producing erythrocytes in rats suitable for in vitro studies involving faba beans. The drug has obvious limitations for creating an in vivo animal model due to the deleterious side

effects. Hence the main drawback of the model may be due to the problem of hydrolysis of vicine in the gut and absorption into the blood stream. This could be overcome by injecting divicine directly into the blood stream.

In summary, the BCNU treated rat does have possibilities for use as an animal model to study the effects of vicine both in vivo and in vitro. The drug does not seem to affect any of the other erythrocyte enzymes except EGR (Frischer & Ahmad, 1977); this should reduce complications when interpreting results after experiments involving vicine. The overall declines in blood GSH in this animal model were much greater compared to the riboflavin deficient and galactoflavin animal model and were achieved in a far shorter period of time.

Summary & Conclusions

1. Rats treated with galactoflavin provide a suitable animal model for studying the mode of action of the toxic factors in faba beans in vivo. Galactoflavin due to its effect on riboflavin metabolism, impaired the activity of EGR to such an extent that blood GSH levels were unable to cope with the oxidant challenge invoked by divicine. As a result blood GSH ($p < .0005$) hemoglobin ($p < .006$) and hematocrit ($p < .0001$) values declined with an increase in spontaneous hemolysis ($p < .0001$) occurring. Hence the erythrocytes derived from this model responded in the same way as a favic erythrocyte does to the pyrimidine aglycones.

2. The riboflavin deficient animal model did not prove to be as successful as the aforementioned model. The activity of EGR was not impaired to the same extent due to difficulties encountered in preventing coprophagy and lowering the riboflavin status of the rats. The GSH reducing capacity of the cell was, however, lowered and the rats did respond to the effects of vicine. These trends were not very dramatic but a small and significant decline did occur with blood GSH ($p < .006$), hemoglobin ($p < .085$) and hematocrit ($p < .084$) and increase in spontaneous hemolysis ($p < .088$). The major limitation of this animal model is that EGR activities cannot be lowered beyond a certain point unless more effective means are employed to prevent refection from occurring. This model also takes a considerably longer period of time to create compared to the galactoflavin animal model. Galactoflavin treated rats have more potential for use as an in vivo model for studying favism.

3. Rats treated with vinylidene chloride do not provide a suitable blood model since the drug has no depleting effect on GSH levels ($p > .05$).

4. BCNU treated rats resulted in erythrocytes not only having the lowest GSH concentrations but also achieved this depletion in a very short period of time compared to the other models. Erythrocyte glutathione reductase activities were depressed by 83% using a 100mg/kg dose of BCNU in 48 hours. This animal model has limitations as an in vivo model if the challenge to the erythrocyte is to be made by ingesting vicine. However, it does have considerable potential if divicine is administered in some other way for example, directly into the blood stream. It should be very suitable for swiftly mimicing favic erythrocytes for in vitro studies.

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APPENDIX

Table 1A. Raw data for the mean live weights of rats fed a riboflavin deficient diet and complete diet fitted with either sham or tail cups for a period of 18 weeks.

Period on diet	Day assays were performed		Riboflavin		0% Riboflavin	
	Day	Set	Tail cup	Sham cup	Tail cup	Sham cup
0			68.0	67.0	69.0	72.1
7			92.9	92.3	75.8	82.0
14			126.2	141.9	81.3	100.3
21			168.1	168.1	100.0	112.0
28	29	1	206.8	209.4	110.6	126.7
35			237.2	245.4	112.6	131.6
42			267.6	277.9	120.9	141.8
48	50	2	296.4	299.9	128.2	154.5
56			308.4	318.1	150.1	173.0
63			323.6	335.2	156.0	178.2
70			330.8	341.6	158.9	178.1
77			339.0	346.0	162.5	183.1
84			350.4	357.0	166.1	194.1
91	91	3	345.7	370.2	178.5	200.8

. . . cont'd.

Table 1A (cont'd.)

Period on diet	Day assays were performed		Riboflavin		0% Riboflavin	
	Day	Set	Tail cup	Sham cup	Tail cup	Sham cup
98	101	4	380.2	355.1	180.2	200.5
105	103	5	385.0	365.1	185.2	198.6
112			394.4	382.1	178.2	198.8
119			375.0	385.2	174.3	190.0
126	126	6	407.0	395.0	175.3	186.0.

Table 2A. Analysis of variance for the growth rate of rats fed a riboflavin deficient diet and complete diet fitted with either sham or tail cups for a period of 18-weeks.

Source of Variation	df	MS	PR>F
Riboflavin (R)	1	354065.99	.0001
Tail cups (T)	1	2014.37	.573
R x T	1	1087.51	.679
Error	72	6290.95	

Table 3A. Mean feed consumption for rats fed a riboflavin deficient and complete diet with and without vicine over a 48-hour period

	Mean feed consumption ($g\ day^{-1}\ 100g\ body\ weight^{-1}$)
Riboflavin x 0% vicine x tail cups	3.6
Riboflavin x 0% vicine x sham cups	4.6
Riboflavin x 2% vicine x tail cups	5.1
Riboflavin x 2% vicine x sham cups	4.5
0% riboflavin x 0% vicine x tail cups	6.9
0% riboflavin x 0% vicine x sham cups	5.2
0% riboflavin x 2% vicine x tail cups	6.0
0% riboflavin x 2% vicine x sham cups	4.0
SE	0.8

Table 4A. Analysis of variance for the feed consumption of rats fed a riboflavin deficient and complete diet with and without vicine over a 48-hour period.

Source of variation	df	MS	PR>F
Riboflavin (R)	1	6.96	.067
Vicine (V)	1	0.2	.727
Tail cups (T)	1	4.13	.150
R x V	1	4.66	.127
R x T	1	6.57	.074
V x T	1	1.14	.439
R x V x T	1	0.53	.594
Error	16	1.80	

Table 5A. Mean liver weights for rats fed a riboflavin deficient diet and complete diet with and without vicine

	Mean Liver Weight (g/100g body weight)
Riboflavin x 0% vicine	3.4
Riboflavin x 2% vicine	3.3
0% riboflavin x 0% vicine	5.4
0% riboflavin x 2% vicine	4.8
SE	0.5

Table 6A. Analysis of variance of liver weights of rats fed a riboflavin deficient diet and complete diet supplemented with and without Vicine.

Source of variation	df	MS	PR>F
Riboflavin (R)	1	17.68	0.0001
Vicine (V)	1	0.95	0.251
R x V	1	0.37	.471
Error	16	0.67	

Table 7A. Mean blood GSH values for rats fed a riboflavin deficient diet and a complete diet prior to the incorporation of vicine into the diet.

	Blood GSH (mg/100ml blood)
Riboflavin x 0% vicine	41.5
Riboflavin x 2% vicine	40.3
0% riboflavin x 0% vicine	36.2
0% riboflavin x 2% vicine	36.2
SE	2.1

Table 8A. Analysis of variance for blood GSH values of rats fed a riboflavin deficient diet and a complete diet before and after the incorporation of vicine in the diet.

Source of variation	df	MS	PR>F
Riboflavin (R)	1	627.56	.0001
Vicine (V)	1	72.37	.011
Tail cups (T)	1	3.91	.535
Time (S)	1	147.14	.0005
R x V	1	0.13	.909
R x T	1	0.07	.933
R x S	1	78.59	.008
V x T	1	21.25	.153
V x S	1	38.95	.056
T x S	1	1.86	.668
R x V x T	1	2.93	.591
R x V x T x S	1	34.30	.496
Error	32	9.93	

Table 9A. Analysis of variance for the growth rate of rats fed a deficient diet and a riboflavin deficient diet supplemented with 200 and 400 mg/Kg of galactoflavin over a 28-day period.

Source of Variation	df	MS	PR>F
Diet	2	1295.23	.0034
Error	17	86.89	

Table 10A. Analysis of variance for feed consumption for rats fed a riboflavin deficient diet and a riboflavin deficient diet supplemented with 200 and 400mg/Kg of galactoflavin over a 28-day period.

Source of Variation	df	MS	PR>F
Diet	2	43.73	.0001
Error	17	0.78	

Table 11A. Erythrocyte glutathione reductase activities at varying doses of BCNU with and without the addition of FAD.

	EGR activity with FAD				EGR activity without FAD			
	(μmoles NADPH oxidized/min/g hemoglobin)							
	DOSE OF BCNU (mg/Kg)							
Time (hours)	Saline	30	60	90	Saline	30	60	90
0	11.0 ±3.7	10.1 ±1.2	9.8 ±0.5	12.4 ±1.8	10.2 ±3.3	8.6 ±1.2	8.1 ±1.8	9.3 ±1.9
4	10.8 ±1.8	11.6 ±1.5	10.7 ±0.8	6.8 ±1.0	9.6 ±1.7	9.3 ±0.8	6.9 ±0.9	4.4 ±1.0
24	9.6 ±3.0	8.2 ±1.4	5.3 ±0.4	3.8 ±0.9	8.7 ±2.3	6.1 ±1.2	4.0 ±0.6	2.8 ±0.4
48	12.3 ±2.1	6.3 ±0.8	4.9 ±0.8	2.1 ±0.3	9.6 ±1.5	4.7 ±0.7	3.7 ±1.0	1.5 ±0.3

Table 12A. Erythrocyte glutathione reductase activites 24 hours after the administration of BCNU with and without the addition of FAD.

	EGR activity with FAD	EGR activity without FAD
CONTROL x 0% vicine	10.4	8.5
CONTROL x 5% vicine	12.4	11.5
100mg/Kg BCNU x 0% vicine	3.9	2.9
100mg/Kg BCNU x 5% vicine	3.5	3.3
[a]SE	1.4 1.0	1.5 1.0

[a]Standard errors for Groups I & II and III & IV respectively.

Table 13A. Analysis of variance of erythrocyte glutathione reductase activities with the addition of FAD 24 hours after intraperitoneal injections of 100mg/Kg BCNU.

Source of variation	df	MS	PR>F
BCNU (B)	1	155.96	.0003
Vicine (V)	1	0.53	.730
B x V	1	3.60	.379
Error	8	4.15	

Table 14A. Analysis of variance of erythrocyte glutathione reductase activities without the addition of FAD 24 hours after intraperitoneal injections of 100mg/Kg BCNU.

Source of Variation	df	MS	PR>F
BCNU (B)	1	126.18	.0007
Vicine (V)	1	4.80	.326
B x V	1	4.78	.327
Error	8	4.38	

Table 15A. Analysis of variance of erythrocyte glutathione reductase activities with the addition of FAD 72 hours after intraperitoneal injections of 100mg/Kg BCNU.

Source of variation	df	MS	PR>F
BCNU	1	272.46	.0001
Vicine	1	0.082	.886
Time	1	13.71	.078
Error	16	3.85	

Table 16A. Analysis of variance of erythrocyte glutathione reductase activities without the addition of FAD 72 hours after intraperitoneal injections of 100mg/Kg BCNU.

Source of Variation	df	MS	PR>F
BCNU	1	165.50	.0001
Vicine	1	1.59	.492
Time	1	1.52	.502
Error	16	3.23	

Table 17A. Mean feed consumption for rats treated with and without BCNU and fed with or without vicine over a 48-hour period.

	Mean feed consumption ($g\ day^{-1} 100g\ body\ weight^{-1}$).
CONTROL x 0% vicine	5.5
CONTROL x 5% vicine	5.0
100mg/Kg BCNU x 0% vicine	3.5
100mg/Kg BCNU x 5% vicine	3.1
[a]SE	0.7 0.5

[a]Stanadard errors for Groups I & II and III & IV respectively

Table 18A. Analysis of variance for the feed consumption of rats treated with and without BCNU and fed with and without vicine over a 48-hour period.

Source of Variation	df	MS	PR>F
BCNU (B)	1	10.45	.013
Vicine (V)	1	0.48	.516
B x V	1	0.02	.901
Error	11	1.03	