1st September, 1962.

This is to certify that the thesis "Primaquine Sensitivity: Some Epidemiological and Biochemical Aspects" presented for the degree of Doctor of Medicine at the University of the Witwatersrand is my own work and has not been presented at any other University.

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PRIMAQUINE SENSITIVITY:

SOME EPIDEMIOLOGICAL AND BIOCHEMICAL

ASPECTS

by

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of the Witwatersrand.

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TO MY WIFE

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Introduction

INTRODUCTION

Plasmoquine (plasmochin, pamaquin) was the first synthetic antimalarial drug, and it aroused a great deal of interest when it became available in 1926. Dixon (1933) estimated that at least 415 papers relating to plasmoquine had appeared in the first 4 years following its introduction. Thereafter the volume of communications decreased somewhat, until fresh interest was stimulated by the Second World War and the Korean War with fighting in malarious areas.

Haemolysis in some patients treated with plasmoquine had been reported from the earliest days of its clinical use. From time to time attempts were made to explain why some individuals were sensitive to the drug, whereas most patients did not develop haemolytic anaemia. However, it was only in 1954 that any real progress was made in the understanding of the condition. In that year Beutler, Dern, Alving and their co-workers at the Army Malaria Research Unit at Stateville Penitentiary, Illinois, published the first of a series of papers which largely elucidated the problem of

plasmoquine sensitivity. These workers established that susceptibility to haemolysis was due to a new variety of intrinsic red cell defect, characterised by several biochemical abnormalities, but morphologically indistinguishable from the normal by conventional techniques. The trait is believed to be harmless under normal circumstances, but exposure to a number of extraneous substances including primaquine and plasmoquine results in massive haemolysis. The condition is known today as 'primaquine sensitivity' or 'glucose-6-phosphate dehydrogenase deficiency'. Many aspects of the metabolism of these abnormal cells are still being intensively studied, since in this way insight into normal erythrocyte metabolism is gained. In addition, the exact way in which the drugs produce haemolysis is still not unequivocally established.

HISTORICAL REVIEW

In 1926 Muhlens (1926) and Sioli (1926) first described the use of plasmoquine in human malaria, and one of Soli's original patients, a syphilitic under treatment with therapeutic vivax malaria, developed methaemoglobinuria on plasmoquine in a dose of 75 mgms. daily (Sioli, 1926). In the same year Cordes reported plasmoquine haemolysis in the treatment of natural malaria (Cordes, 1926 (a); 1926 (b)), and further reports followed almost immediately (Eiselsberg, 1927; Cordes, 1927; Brosius, 1927; Manson-Bahr, 1927). In an attempt to explain the haemolysis Roskott and Seno (1928) carried out osmotic fragility tests on the red cells of 3 patients with plasmoquine haemolysis, but did not find any abnormality. Cordes (1928), Menk (1928), Brosius (1928) and Manikawa (1928) reported further instances of haemolytic anaemia. Of great interest in the light of later developments is the report of Palma (1928), who observed Heinz bodies in the red cells of an individual with mild haemolytic anaemia associated with the administration of plasmoquine. However, the significance of the observation was not appreciated until the independent demonstration

by Beutler, Dern and Alving in 1954 of the association between Heinz body formation and drug sensitivity.

Reports of pamaquine haemolysis continued to appear (Baermann and Smits, 1929; Freiman, 1929; Kligler and Reitler, 1929; Manai, 1929; Huisman, 1932; Banerjee and Brahmachari, 1933; Blackie, 1935). There was confusion between pamaquine haemolysis and blackwater fever. Manifold (1931) ascribed 3 cases of haemolysis in 1915 Indian patients treated with pamaquine to blackwater fever, although no haemoglobinuria was observed in 1298 British patients. Amy (1934), reviewing 10 cases of haemoglobinuria on the Indian frontier associated with plasmoquine treatment, pointed out that the cases had occurred in districts not regarded as blackwater areas, and that all cases had occurred in Indians, not British troops (which was very unlike blackwater). Furthermore, falciparum malaria had not been demonstrated in all the cases. Nevertheless, Amy rejected the possibility of a racial idiosyncrasy to the drug, and concluded that "the sooner plasmoquine is connected up with blackwater fever in much the same way as quinine is now, the better". However, Fernán-Núñez (1936) made a clear distinction

between the two conditions in his review of 52 cases of blackwater fever.

Investigating his case of pamaquine haemolysis, Ficacci (1935) found normal osmotic fragility, bleeding and clotting times, and a normal Donath-Landsteiner test. The administration of pamaquine 26 days after the original exposure resulted in a second, though milder, haemolytic episode. Amy and Boyd (1936) reported haemoglobinuria in a British soldier in India associated with the taking of plasmoquine, while haemolytic anaemia with haemoglobinuria on the 4th day of exposure to plasmoquine was observed by Sein (1937). His patient, an Indian soldier, was taking the drug prophylactically, and there was no evidence of associated malaria.

Stauss (1939) reviewed the literature on plasmoquine toxicity, and concluded that haemoglobinuria was rare. He ascribed the reported cases to overdosage, and claimed that in doses of 10 mgms./10 Kg. body weight per day, plasmoquine was nonhaemolytic: however, haemolysis had in fact been reported with this dosage (Manifold, 1931; Amy, 1934; Sein, 1937; etc.).

The Second World War and the Korean war, with the necessity for maintaining armies in malarious areas, rekindled interest in pamaquin and the newer antimalarials, and further attempts were made to unravel the mechanism of pamaquine haemolysis. Mer et al. (1941) studied the lysis of red cells by bile in vitro without discovering the mechanism of drug-induced haemolysis. Mann (1943) described an agglutinin in the serum of his patient, and thus thought that plasma factors were responsible. Zylmann (1944) attempted without success to produce in vitro haemolysis with plasmoquine. Swantz and Bayliss (1945) noted that all 10 subjects with haemoglobinuria following pamaquine administration were Negroes, although the number of Caucasians treated had been much greater. In their patients the Donath-Landsteiner and Kahn tests were negative. Birnbaum et al. (1946) found that pamaquine, quinine and atabrine accelerated bile haemolysis in vitro.

In 1947 Dimson and McMartin studied 25 Indian soldiers with pamaquine haemolysis and reported that the intradermal injection of pamaquine did not produce an allergic reaction. In vitro haemolysis could only be

produced by concentrations of the drug far in excess of therapeutic dosage. The administration of pamaquin to their patients 30 days after the original haemolytic episode produced a second, milder, haemolytic response.

Feldman et al. (1947) studied 2 cases of haemolysis among 11 Negroes given pamaquine. A second massive haemolysis was produced by re-administration of the drug 3 months after the original episode. Red cell fragility was found to be normal, and haemolysis could not be produced by incubation of the patient's cells in normal plasma containing pamaquine, or normal cells in the patient's plasma.

Earle et al. (1948) distinguished between two toxic effects of the drug, methaemoglobin formation and haemolysis, and noted that the latter was much more common in 'pigmented subjects' (Negroes and Chinese). Haemolysis was not related to the concentration of pamaquine in the plasma, and a study of mechanical and osmotic fragility, isoagglutinins, haemolysins, cold haemagglutinins and autoagglutinins revealed no abnormality Their conclusion was that the drug acted as a precipitating factor, producing haemolysis when certain predisposing

factors were present, although the nature of the latter remained obscure.

Turchetti (1943) reported the important observation that pamaquine sensitivity was familial in nature. However, the significance of his communication was ignored for some years.

Hockwald et al. (1952) observed haemolysis in 17 of 110 Negroes given primaquine mgms. 30 daily for 14 days. There were no similar episodes in Caucasian subjects. These workers established that an individual who was sensitive to primaquine was also sensitive to other 8-aminoquinoline derivatives, such as pamaquine. They found no correlation between methaemoglobin formation and haemolysis, and showed that the erythrocytes manifested normal osmotic fragility. Similar observations were reported by Jones et al. (1953).

Gennis et al. (1954) ré-administered primaquine 9 days after the haemolytic episode to one of their 2 subjects, and mistakenly concluded that primaquine had not been responsible for the original attack since it failed to produce a recurrence. The reason for the temporary

insensitivity to the drug shortly after a haemolytic episode was not understood until the publication of the work of the Chicago group (Dern, Beutler and Alving, 1954).

Further reports of haemolysis during this period without experimental work were published by Smith (1943), West and Henderson (1944), Thirlby (1944), Braun and de Vries (1944), Loeb (1945), Hardgrove and Applebaum (1946), Keng (1948), and Coatney et al. (1950).

Up to 1954, then, the nature of 8-aminoquinoline haemolysis remained obscure, although observations indicating the genetic basis of the condition had been published. The varying racial susceptibility to the condition had been observed by several workers (Swantz and Bayliss, 1945; Dimson and McMartin, 1946; etc) but this had been explained at the Conference of the Medical Specialists, Central Command and North-Western Army, at Lahore in 1944 as a consequence of the lower body weight of Indians and Burmese compared with British troops. The familial nature of susceptibility had also been observed by Turchetti (1948). However, very little

progress in understanding the condition had been made in spite of a considerable amount of investigation.

The Work of the Chicago Group

In 1954 Dern and his co-workers published the first of a series of papers which succeeded in elucidating the problem to a large extent. Firstly, they showed that susceptibility to haemolysis was a characteristic of the cells of sensitive individuals, and not due to a plasma factor (Dern, Weinstein et al., 1954). This was achieved by cross-transfusion experiments. When ^{51}Cr-labelled cells from a sensitive subject were introduced into the circulation of a compatible normal recipient, the labelled cells rapidly disappeared from the circulation when primaquine was exhibited. In contrast, labelled normal cells introduced into a susceptible individual were not destroyed when the recipient was given primaquine. Dern, Beutler and Alving (1954) then investigated the insensitivity to further haemolysis which had been observed shortly after a haemolytic episode (Gennis, 1954). They administered primaquine to susceptible volunteers in the Stateville Penitentiary, and did not stop the adminis-

tration even when massive haemolysis developed. In spite of the continued exhibitioh of the drug haemolysis ceased on about the 10th day, and thereafter the haemoglobin rose to normal levels by the 20th - 30th day. These workers showed that the resistance which developed was due to a change in the erythrocytes rather than to the development of a detoxifying system elsewhere in the body, since labelled cells from a subject in the 'resistant phase' were not destroyed by primaquine when introduced into a compatible normal circulation. In addition, cells from a second sensitive subject were promptly destroyed by primaquine when in the circulation of a sensitive individual in the 'resistant phase'.

In a third paper, Beutler, Dern and Alving (1954)(a) studied sensitive erythrocytes during all phases of the haemolytic episode. No abnormality in morphology, antigenic characteristics, susceptibility to acid haemolysis, type of haemoglobin, mechanical, osmotic or chemical fragility was detected, apart from the appearance of Heinz bodies in the cells at the commencement of haemolysis, on about the 4th day of administration. The Heinz bodies were a transient phenomenon, disappearing

as haemolysis progressed. It thus seemed that the red cell abnormality of primaquine sensitivity was different from previously described intrinsic red cell defects.

Beutler, Dern and Alving (1954)(b) then showed that only the older cells in the circulation of a susceptible individual were destroyed when primaquine was exhibited. This was established by labelling erythrocytes entering the circulation from the marrow over a few days with ^{59}Fe, thus tagging a particular 'generation' of cells. The administration of primaquine during the 3rd week after the appearance of the labelled cells produced the usual haemolysis, but did not result in the destruction of any of the tagged cells. A second course of primaquine when the labelled cells were 80 days old, however, did result in their destruction. It was thus shown that the resistance to haemolysis which develops during continuous administration of primaquine was due to the elimination of the vulnerable erythrocytes from the circulation, i.e. the older cells. Once only the younger cells remained haemolysis ceased.

Dern, Beutler and Alving (1955) then showed that

cells sensitive to primaquine were also sensitive to other substances, such as acetanilid, sulphanilamide, phenylhydrazine, phenacetin, sulfoxone and thiazolsulfone. They next described a method of production of Heinz bodies in vitro, and showed that sensitive cells formed Heinz bodies which differed morphologically from the Heinz bodies formed in normal cells (Beutler, Dern and Alving, 1955). The test could be used to predict primaquine sensitivity, and was the first in vitro method of diagnosing the condition to be described. Although Josephson et al. (1958) have challenged the validity of the Heinz body test, their objections cannot be accepted since they used oxalated blood with no added glucose 4 hours after collection. If the technique recommended by Beutler et al. is followed the test is valid, although several more recently described tests for primaquine sensitivity are more convenient.

Biochemical study of the sensitive cells then followed (Beutler, Dern, Flanagan and Alving, 1955). The reduced glutathione (GSH) content of sensitive cells was found to be slightly below normal, and it was found that poisoning of normal cells with iodoacetate or

arsenite (which bind the sulphydryl groups of GSH) caused them to produce the type of Heinz bodies in the in vitro test which were characteristic of sensitive cells. This suggested that the slight GSH deficiency of sensitive cells was closely connected with the mechanism of primaquine haemolysis. Glycolysis, catalase, carbonic anhydrase and cholinesterase were also studied and reported to be normal. However, later work (Tarlov and Kellermeyer, 1959) has established that there is a deficiency of catalase activity in sensitive cells, which can be demonstrated only when the very rapid reaction is slowed down.

A major discovery was the demonstration of defective glucose-6-phosphate dehydrogenase activity by Carson and his co-workers in 1956. Following on the demonstration of diminished GSH in sensitive cells, these workers showed that activity of the enzyme glutathione reductase was normal in haemolysates of sensitive cells, as assessed by the rate of formation of GSH from added oxidised glutathione (GSSG) and reduced coenzyme II (TPNH). In the presence of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD), TPN is reduced

to TPNH, so that substitution of TPN with G6P for TPNH in the above experiment provides a measure of G6PD activity. When this was done a marked defect of G6PD activity was found in sensitive cells. Substitution of 6-phosphogluconic acid for G6P restored the rate of GSH formation to normal, implying normal 6-phosphogluconic dehydrogenase activity.

Shortly afterwards Schrier, Kellermeyer, Carson and Alving (1957) reported that glutathione reductase activity was in fact greater in haemolysates of sensitive cells than in normal cells. This was interpreted as a compensatory phenomenon: because the hexose monophosphate shunt pathway is the only known mechanism for the reduction of TPN in erythrocytes, and since glutathione reductase is TPN-dependent, deficiency of G6PD (the first enzyme on the shunt pathway) results in defective reduction of glutathione. Hence increased GSSG reductase activity would tend to compensate for the deficiency of TPN reduction. The metabolic pathways involved are shown in the diagram.

Of great interest in view of the relationship of

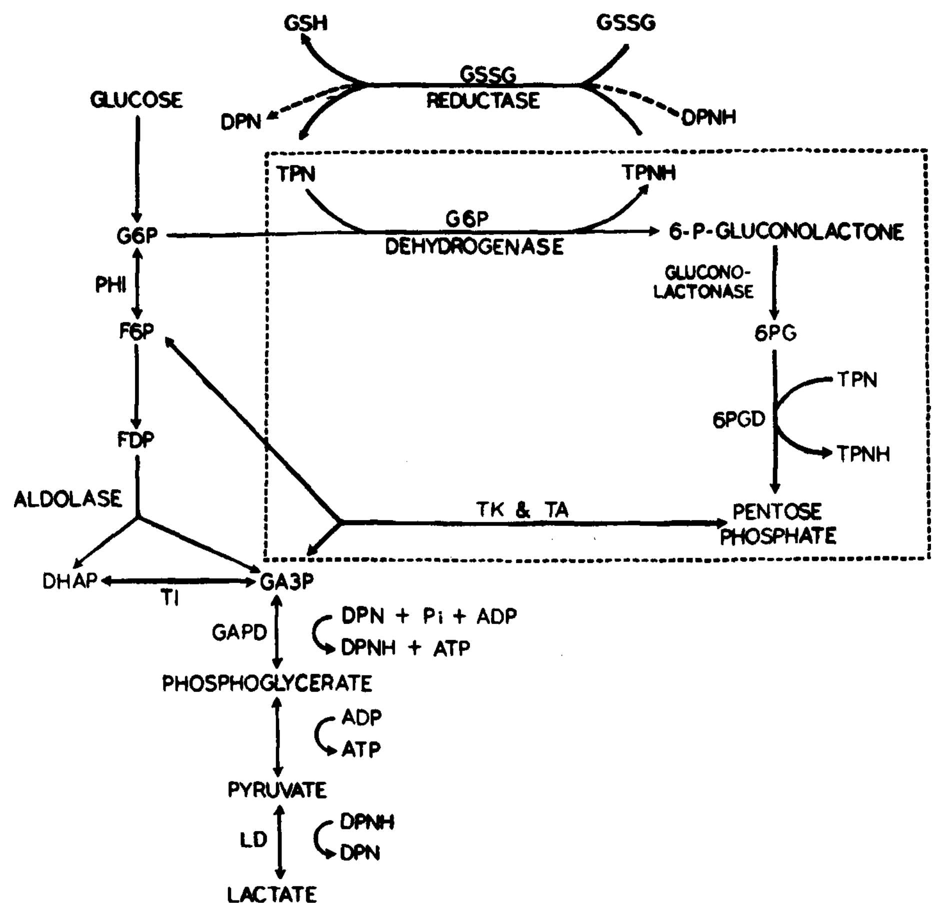


Fig. 1. A diagrammatic representation of the pathways of glucose metabolism in human erythrocytes. The dotted line encloses the pentose phosphate pathway (hexose monophosphate shunt pathway). Anaerobic glycolysis (Embden-Meyerhof) is depicted to the left of the diagram. G6P: glucose 6-phosphate; TPN: oxidised triphosphopyridine nucleotide; TPNH: reduced triphosphopyridine nucleotide; GSSG: oxidised glutathione; GSH: reduced glutathione; DPN: oxidised diphosphopyridine nucleotide; DPNH: reduced diphosphopyridine nucleotide; 6PG: 6-phosphogluconic acid; 6PGD: 6-phosphogluconic dehydrogenase; TK: transketolase; TA: transaldolase; PHI: phosphohexoseisomerase; F6P: fructose-6-phosphate; FDP: fructose 1-6-diphosphate; DHAP: dihydroxacetone phosphate; TI: triose isomerase; GA3P: glyceraldehyde-3-phosphate; GAPD: glyceraldehyde-3-phosphate dehydrogenase; Pi: inorganic phosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; LD: lactic dehydrogenase.

cell age to primaquine haemolysis was the demonstration by Marks (1957) of lesser activity of both G6PD and 6-phosphogluconic dehydrogenase in older cells compared with younger cells.

A second in vitro test for primaquine sensitivity was then described by Beutler (1957). It was shown that the incubation of blood from susceptible individuals with acetylphenylhydrazine (in the presence of oxygen) resulted in a marked fall in the concentration of GSH, whereas the GSH content of normal blood remained unaltered. This 'GSH stability test' was found to be accurate in predicting primaquine sensitivity, and more reliable than the Heinz body test. It is still a standard test for primaquine sensitivity.

In attempting to elucidate the mechanism of red cell destruction in sensitive persons in the light of these discoveries, Beutler, Robson and Buttenwieser (1957) estimated GSH, GSSG and total glutathione in blood from sensitive individuals during incubation with acetylphenylhydrazine in vitro. While the concentration of GSH decreased, the level of GSSG increased slightly

at first but later also fell. The total glutathione content diminished less rapidly than did the GSH level. The GSH concentration did not decrease if oxygen was excluded. It was concluded that GSH was destroyed by a process of oxidation. Beutler and his co-workers also showed that acetylphenylhydrazine alone did not oxidise GSH, but that oxyhaemoglobin incubated with acetylphenylhydrazine formed a new compound which would destroy GSH. Methaemoglobin and carboxyhaemoglobin could not be substituted for oxyhaemoglobin, and the oxyhaemoglobin derivative could not be identified. In a further series of experiments these workers showed that the GSH of normal blood became 'unstable' on standing unless glucose was added. In addition, the GSH instability of normal cells incubated without glucose for 4 hours could be restored to normal by adding not only glucose, but also inosine. However, lactate, malate, pyruvate, fumarate or ribose were not effective. It thus seemed that GSH stability was dependent upon the metabolism of glucose via the oxidative pathway.

CHAPTER 2

Substances Known to Provoke Haemolysis in Primaquine Sensitive Subjects

SUBSTANCES KNOWN TO PROVOKE HAEMOLYSIS IN PRIMAQUINE SENSITIVE SUBJECTS

In addition to the drugs reported by Dern et al., (1955) listed on page 13, several further haemolytic agents have been recognised. Kimbro (1957) and Kimbro et al. (1957) found the condition in 2 Negroes who developed haemolytic anaemia while taking nitrofurantoin, and showed that the concentration of GSH fell on incubation of their blood with nitrofurantoin in vitro. They were unable to show the formation of Heinz bodies with this drug, however. Zinkham and Childs (1957)(a) demonstrated glutathione instability of the red cells of 4 Negro patients who had developed haemolytic anaemia after exposure to naphthalene. On in vitro incubation with naphthalene the GSH was not altered, although when certain naphthalene derivatives were tested it fell abruptly. It was suggested that certain metabolites of naphthalene were responsible for the haemolysis rather than naphthalene itself. Naphthalene haemolysis was also reported by Dawson et al. (1958). Zinkham and

Childs (1958)(b) also showed that a vitamin K analogue, menadione sodium bisulphite (a naphthalene derivative) had a similar action. In addition, sulfisoxasole ('Gantrisin') was found to produce Heinz bodies in vitro, but not to lead to destruction of GSH. These workers also noted that the blood of all infants in the immediate postnatal period manifests glutathione instability. This was later shown to be a manifestation of hypoglycaemia, and can be corrected by the addition of glucose before incubation (Zinkham, 1959).

Kellermeyer et al. (1958) have added Furoxone, Azulfidine, Kynex and aspirin in large doses to the list of drugs which may provoke haemolysis. Szeinberg, Kellermeyer et al. (1960) observed haemolytic jaundice in a G6PD deficient individual related to the taking of aspirin, and found that the GSH of his red cells fell on incubation with high concentrations of aspirin in vitro. However, in view of the high incidence of the trait in Israel these workers are surprised that haemolysis associated with aspirin is not more often seen, and they wonder whether a second hitherto unrecognised factor might not have been involved in this case. Houston and

Barlow (1959) have reported haemolytic anaemia associated with the taking of phenacetin. In a second communication Szeinberg et al. have reported differences between different sulphonamide drugs with regard to the frequency with which haemolysis is provoked by the drugs in sensitive individuals (Szeinberg, Sheba et al., 1960). In their experience sulphapyridine invariably causes haemolysis while sulphadiazine does so only sometimes.

In a recent review, Tarlov et al. (1962) have added mepacrine (Atabrine), dimercaprol (BAL), probenecid (Benemid), methylene blue and trinitrotoluene to the group of haemolytic drugs. These workers state that certain drugs will produce haemolysis in primaquine sensitive Caucasians but not in sensitive Negroes. In this category are chloramphenicol, quinidine and quinine.

Recently it has been claimed that haemolysis may be provoked by factors other than drugs or other chemical substances. Szeinberg, Sheba et al.(1960) have observed haemolysis in sensitive subjects associated with unspecified viral and bacterial infections, while Marks (1960) has reported haemolysis in 4 sensitive

individuals with virus hepatitis and 3 with infectious mononucleosis. Gant and Winks (1961) have observed haemolytic anaemia in a primaquine sensitive subject complicating diabetic acidosis in whom there was no evidence of infection or of exposure to drugs.

FAVISM

In 1948 Turchetti had remarked upon certain similarities between favism and primaquine sensitivity, and in 1956 Crosby had suggested that the recently reported tests for primaquine sensitivity should be applied to subjects with a history of favism. In 1957 Sansone and Segni (1957), Szeinberg and Chari-Bitrou (1957) and Szeinberg et al. (1957) found low blood GSH in these patients, and subsequently demonstrated positive GSH stability tests (Szeinberg, Asher and Sheba, 1958) and deficiency of G6PD. (Szeinberg, Sheba and Adam, 1958 (a)). This has been confirmed by other workers (Zinkham, Lenhard and Childs, 1958; Gross, Hurwitz and Marks, 1958; Larizza et al., 1958). Final confirmation of the identity of the red cell defect in favism and in primaquine sensitivity is provided by the observation of

Larizza et al. (1959) that administration of primaquine to an individual with a history of favism produced the typical haemolytic reaction. In addition, Bowman and Walker (1961) have shown that certain extracts of the beans will cause a fall in red cell GSH on incubation in vitro. Nevertheless, while it seems that all cases of favism which have been examined to date have manifested the red cell abnormalities associated with primaquine sensitivity, it has been well established that primaquine sensitive individuals have eaten fava beans without suffering haemolysis (Roth and Frumin, 1960; Greenberg and Wong, 1961).

While this thesis was being prepared the opportunity to study a case of favism became available. Favism is rare in South Africa, only one other case having been reported in the literature (Senior and Braudo, 1955). That patient was a Greek immigrant, and the present case is the first to be described in an Afrikaans speaking white child. Because of the rarity of the condition in South Africa, and because many of the typical features of favism are illustrated, the case

report is included here.

<u>Case History</u>

The patient, a 6-year old white male child, was first seen on the morning of 23.9.61, complaining of tiredness and intermittent abdominal pain of 4 days' duration. The symptoms had progressively increased, and the day before he was seen the parents noticed that he was pale and put him to bed. The next day the child was worse and medical aid was sought.

No history of significant previous illness was obtained. Seventeen days previously he had been given 3 doses of a sulphonamide suspension for a mild upper respiratory tract infection. The day before the commencement of the present illness (18.9.61) the family had eaten cooked broad beans which were subsequently identified as Vicia faba. These beans had been eaten regularly by the family, including the patient, for a month previously, and the patient had been seen to eat raw beans in the garden on several occasions prior to the present episode without ill effect. Although careful enquiry was made, no history of exposure to any other possible haemolytic agent could be obtained.

On examination there was marked pallor of the mucous membranes with slight icterus of the sclerae. The oral temperature was 99° F., the pulse rate 140 per minute, and respiratory rate 30 per minute with

prominent movement of the alae nasi. Examination of the cardiovascular and respiratory systems was negative. There was generalised abdominal tenderness but no guarding, and the tip of the spleen was felt. The urine was dark red in colour and albumin was present (++++).

On examination of the blood the haemoglobin concentration was 6.7 Gm.% and there were 2.8 million erythrocytes/cu.mm. The reticulocyte count was 16.5%. The leucocyte count was 20,400 of which 74% were neutrophils. A normal number of platelets were seen, and no malarial parasites were observed. The red cells showed diffuse polychromasia, aniso- and poikilocytosis, and some spherocytosis. Occasional normoblasts were present. No Heinz bodies were seen. The Coombs test was negative. Osmotic fragility was normal and the Donath-Landsteiner and Ham tests were negative. The serum bilirubin was 3.1 mgm.% (total) and Schumm's test was positive. The screening test for erythrocyte glucose-6-phosphate dehydrogenase (G6PD) (Motulsky and Campbell, 1960) showed markedly deficient enzyme activity.

Porphyrins were not found in the urine, but oxyhaemoglobin was present in addition to the marked albuminuria. On microscopic examination granular casts were seen, together with 8 pus cells and 3 red cells per high power field.

By the evening of 23.9.61 the child appeared

even more distressed, and air hunger was present. A second blood count showed similar findings to those previously noted. A transfusion of 1,000 ccs. blood was commenced and continued slowly over the succeeding 24 hours. In addition, cortisone was given parenterally for the first 24 hours and then replaced by oral prednisone. The patient's condition rapidly improved. The output of urine remained good, and by 27.9.61 it was of normal colour and free of albumin. On 28.9.61 the haemoglobin was 9.5 Gm.%. The glutathione (GSH) stability test (Beutler, 1957), was negative, the initial level of 34 mgms./100 ml. erythrocytes not falling on incubation with acetylphenylhydrazine. A temporarily negative GSH stability test is not unexpected during or immediately after a haemolytic episode in sensitive individuals (Szeinberg, Asher and Sheba, 1958). When the test was repeated 3 months later the initial concentration of 34 mgm.% fell to 4 mgms.% on incubation, a markedly positive result.

On 2.10.61 the child was discharged from hospital. He has since remained well, and a haemoglobin estimation on 24.1.62 was normal for his age (12.9 Gm.%).

DISCUSSION

An acute haemolytic episode may be provoked in individuals with enzyme-deficient erythrocytes by exposure to a variety of extrinsic agents (Beutler, 1959).

These include primaquine and pamaquine, phenacetin, vitamin K analogues, nitrofurantoin, certain sulphonamides and broad beans. Haemolysis commences at varying intervals after exposure to these different agents, but in no case longer than 5 days. In the present case, therefore, the administration of the sulphonamide preparation 17 days before the child was seen can be excluded as a precipitating factor. The ingestion of the beans, however, occurred immediately prior to the onset of the illness, and this time relationship is characteristic of favism (Luisada, 1941). No history of exposure to any other known precipitating agent could be obtained. The absence of Heinz bodies in the erythrocytes during the acute haemolytic crisis is a further point in favour of the beans having been the precipitating factor. These structures, which are almost certainly derived from denatured haemoglobin (Jandl et al., 1960) are typical of the drug-induced haemolytic anaemias (Beutler, 1959) but are not seen in favism (Greenberg and Wong, 1961).

Certain other members of the patient's family had eaten the beans without ill effects although they were subsequently found to be markedly enzyme deficient.

The ingestion of favus beans by primaquine sensitive individuals without provoking haemolysis has been well documented (Szeinberg, Sheba et al., 1957; Szeinberg, Asher and Sheba, 1958; Roth and Frumin, 1960), and represents a further point of difference between the haemolytic anaemia of favism and that produced by drugs, since primaquine and the other drugs in sufficient dosage will always cause haemolysis in G6PD deficient subjects.

Sensitivity to favus beans may vary from time to time in the same individual. As in the present case, a G6PD deficient subject may eat the beans without ill effect on many different occasions, and then eventually haemolyse (Jacobs, 1950; Wasserman and Chapman, 1951; McPhee, 1956). It has also been observed that individuals formerly sensitive to the beans may spontaneously become resistant (Luisada, 1941).

Consideration of these facts has led many workers to conclude that other factors in addition to the red cell defect must be involved in the pathogenesis of favism, though the defective red cells seem to be essential. An allergic basis has long been postulated

(Luisada, 1941) and is still current (Beutler, 1959). The massive haemolysis which may follow within seconds after a sensitive subject inhales the pollen from flowering beans (Luisada, 1941) seems difficult to explain on other than an allergic basis. Recent work, however, has indicated that the additional factor distinguishing favism from primaquine haemolysis may be a protective plasma constituent, which is absent in those enzyme-deficient individuals who are sensitive to the beans. Thus Roth and Frumin (1960) were able to produce haemolysis in vitro by incubation of cells from a patient with favism with an extract of the beans. This haemolysis was prevented by normal plasma, but not by the patient's own plasma. The patient's father had eaten the beans without ill effect although his erythrocytes were markedly enzyme-deficient, and it was of great interest that the father's plasma also prevented haemolysis in the in vitro system. Further evidence is provided by the experiments of Vullo and Panizon (1959) and Panizon and Vullo (1961). These workers labelled erythrocytes from favism patients with Cr^{51} and introduced them into the circulation of compatible normal recipients. When the

recipient then ate fava beans there was usually no destruction of the labelled cells, although in a few experiments this did occur. When sensitive cells were introduced into other favism sufferers, however, consumption of the beans resulted in rapid disappearance of the tagged cells on every occasion. Greenberg and Wong (1959), (1961) have confirmed these findings, and in addition showed that administration of primaquine, in contrast to ingestion of the beans, produced rapid disappearance of the enzyme-deficient cells from the circulation of a normal individual. These observations were compatible with the concept of a protective plasma factor, as suggested by the work of Roth and Frumin, although other explanations (such as a different metabolic degradation of the beans in favism sufferers) are also possible.

It thus seems that the pathogenesis of haemolysis in cases of favism is complex. *All cases so far tested have manifested G6PD deficiency and GSH instability. However, since the beans do not cause haemolysis in all persons with these defective red cells, and since sensitivity to the beans varies in the same individual*

from time to time, other factors must clearly be operating.

SUMMARY

A case of favism in a white South African child is reported, and the pathogenesis of this disease is briefly discussed.

C H A P T E R 3

Genetics and Racial Distribution

GENETIC ASPECTS

In 1948 Turchetti had drawn attention to the familial nature of sensitivity to pamaquine, and the marked difference in racial incidence had led several workers to assume that the red cell defect would prove to be genetically transmitted (Beutler, Dern and Alving, 1955; Dameshek, 1955). The first definitive study of the genetics of primaquine sensitivity was that of Browne (1957) and Childs, Zinkham, Browne, Kimbro and Torbert (1958). Investigating the relatives of individuals with unstable GSH, they found that 72% of the mothers of these persons showed some degree of GSH instability, while the incidence in the fathers was no higher than in the general male population (12%). This suggested that the gene was sex-linked, and since the condition was observed in 3 generations it was considered to be dominant. Most of the females, however, did not manifest the marked degree of GSH instability found in the males: on incubation of their blood with acetylphenylhydrazine the GSH concentration decreased somewhat, but sometimes the decrease was slight and only occasionally was it as

profound as in the males. The sons of those women in the latter category all carried the trait, while only some of the sons of the less severely affected women manifested the condition. It was thus postulated that the women with marked GSH instability were homozygous for the trait while the less severely affected females were heterozygous. The mode of inheritance was thus via a sex-linked gene of intermediate dominance.

However, from a study of several families, Beutler (1960) has shown that not all females with profound GSH instability are homozygous, since certain such individuals have borne sons who were not primaquine sensitive. Trujillo et al. (1961) established that the marked degree of primaquine sensitivity in one such heterozygous female was not due to an abnormal sex-chromosome constitution such as XO or XY, and also that there was no detectable morphological abnormality of the X-chromosome in this condition.

The incomplete GSH instability of most affected females was confirmed by Gross, Hurwitz and Marks (1958), who found in addition moderately diminished G6PD activity

in these individuals, and also by Larizza et al. (1958) and by Szeinberg, Asher and Sheba (1958). The lack of close correlation in these females between the degree of GSH instability and of G6PD deficiency and the liability to haemolysis was pointed out by Alving et al. (1958), who observed some females, sensitive to primaquine, with normal GSH stability, and also some with normal G6PD activity. This has been confirmed by Tarlov et al. (1962), who state that in only about 75% of heterozygous females can diminished G6PD activity be demonstrated. In some heterozygotes haemolysis on exposure to primaquine could only be demonstrated by isotopic labelling, and Tarlov et al. suspected that some were not detectable even in this way, since the incidence found in the Negro population was somewhat lower than would be expected from the number of hemizygotes.

Several reports of linkage between primaquine sensitivity and colour-blindness have recently appeared (Siniscalco et al., 1960; Adam, 1961; Porter et al., 1961). Since colour-blindness is well known to be sex-linked, these observations confirm the earlier theories

(Childs et al., 1958; Alving et al., 1958; Motulsky et al., 1959 (a)) that primaquine sensitivity is transmitted via a sex-linked gene.

During the preparation of this thesis the distribution of primaquine sensitivity throughout a very large family was studied. Since the mode of inheritance of the condition is well illustrated the study is included at this point.

Materials and Methods: Blood was collected in acid-citrate-dextrose solution (ACD) and sent by road to the laboratory where it was tested as soon as possible, but in all cases sooner than 24 hours after collection. It has been established that the tests are valid as much as several days after collection of blood in ACD (Beutler, 1959). G6PD activity was estimated by the dye decolorization technique of Motulsky and Campbell (1960). The glutathione (GSH) stability test was performed by the method of Beutler (1957) as modified by Flanagan et al. (1958). These tests are described in detail in Chapter 5.

Enzyme activity was estimated on all specimens.

When deficient activity of G6PD was observed a GSH stability test was performed. The GSH stability of several of the samples showing normal enzyme activity was also tested as a control.

Results: The results are shown in the Table. Blood from male members of the family was either normal or markedly positive to both tests. In the case of some of the females, however, slightly abnormal results were obtained to one or both of the tests. The interpretation of these results cannot be regarded as unequivocal in all cases. Tarlov et al. (1962) have recently discussed methods of identification of female heterozygotes and have concluded that none of the in vitro techniques available at the present time is capable of detecting more than 80% of the affected females. In their experience the GSH stability test was falsely negative in 30 - 60% of cases, and they quote Allison (1960) as having found a high proportion of false negatives using the Motulsky technique. However, Allison regarded as abnormal only those samples not decolorized by 120 minutes, i.e. he used the same criterion which is applied to males. In my hands the Motulsky test has given very constant results,

Subject	Sex	Age	Relationship to Propositus	G6PD Activity Maximum Normal Decolourisation Time 75 minutes	GSH Stability Normally no decrease on incubation		Interpretation
					Before	After	
John. L	M	6	Propositus	250 +	34	4	Hemizygous
El.L	F	13	Sister	70	43	43	Normal
M.L.	F	11	Sister	65	-	-	Normal
Hap. L	M	10	Brother	250 +	31	12	Hemizygous
Bu. L	M	7	Brother	60	-	-	Normal
F.L.	M	4	Brother	55	-	-	Normal
A.L.	F	2	Sister	90	54	57	Heterozygous
Jan. L	M	9/12	Brother	250+	35	11	Hemizygous
F.L.L.	F	34	Mother	120	58	38	Heterozygous
K.L.	M	36	Father	55	56	57	Normal
E.L.F.	M	65	Maternal grandfather	250 +	35	10	Hemizygous
E.J.S.F.	F	64	Maternal grandmother	60	-	-	Normal
E.J.S.F	F	45	Maternal aunt	90	34	23	Heterozygous
C.E.F.	M	41	Maternal uncle	60	-	-	Normal
L.E.F.	M	39	Maternal uncle	75	51	48	Normal
F.F.	M	10	son of L.E.F.	75	62	63	Normal
A.F.	M	15	" " "	60	-	-	Normal
E.F.	M	17	" " "	60	-	-	Normal
Y.F.	F	9	Daughter of L.E.F.	60	-	-	Normal
H.E.F.	M	36	Maternal uncle	60	-	-	Normal
Els. F	F.	10	Daughter of H.E.F.	60	-	-	Normal
An.F	M	6	Son of H.E.F.	60	-	-	Normal
Eli. F.	F	7	Daughter of H.E.F.	60	-	-	Normal
J.E.F.	M	30	Maternal uncle	60	-	-	Normal
J.H.	F	29	Maternal aunt	75	103	36	Heterozygous
R.H.	F	7	Daughter of J.H.	60	33	28	Probably heterozygous
F.H.	M	5	Son of J.H.	60	48	50	Normal
E.J.E.	F	26	Maternal aunt	90	38	31	Heterozygous
A.P.E.	M	1 9/12	Son of E.J.E.	60	39	37	Normal
C.F.F.	M	24	Maternal uncle	60	-	-	Normal
A.S.v.d. M.F.	F	21	Maternal aunt	75	57	44	Probably heterozygous
H.F.	M	66	Great uncle	360 +	32	3	Hemizygous
H.B.F.	M	35	Son of greatuncle H.F.	60	-	-	Normal
M.F.	F	15	Daughter of H.B.F.	55	-	-	Normal
S.F.	F	12	Daughter of H.B.F.	55	-	-	Normal
D.F.	F	10	Daugher of H.B.F.	60	-	-	Normal
H.F.	M	12	Son of H.B.F.	55	-	-	Normal
L.F.	M	31	Son of great uncle H.F.	55	-	-	Normal
L.F. Junior	M	10	Son of L.F.	55	-	-	Normal
K.F.	M	1 9/12	Son of L.F.	60	-	-	Normal
A.v.R	F	43	Daughter of greatuncle H.F.	95	36	35	Heterozygous
M.v.R	M	20	Son of A.v.R	60	56	58	Normal
J.v.R	M	15	Son of A.v.R	65	60	56	Normal
P.v.R	M	8	Son of A.v.R	55	44	46	Normal
I.W.	F	21	Daughter of A.v.R	125	28	20	Heterozygous
A.W.	F	2	Daughter of I.W.	70	42	42	Normal
M.O.	F	42	Daughter of greatuncle H.F.	80	35	30	? Normal
H.O.	M	21	Son of M.O.	65	40	40	Normal
S.O.	F	15	Daughter of M.O.	60	47	50	Normal
O.R.	F	24	Daughter of M.O.	110	35	31	Heterozygous
M.L.	F	23	Daughter of M.O.	100	30	16	Heterozygous
C.L.	M	2	Son of M.L.	360 +	28	2	Hemizygous
G.J.v.R	F	38	Daughter of greatuncle H.F.	95	38	38	Heterozygous
S.J.v.R	F	19	Daughter of G.J.v.R	75	45	45	Normal
G.J.v.R Jnr.	F	18	Daughter of G.J.v.R	125	34	28	Heterozygous
S.J.v.R	M	17	Son of G.J.v.R	60	55	55	Normal
J.J.v.R	M	14	Son of G.J.v.R	65	38	42	Normal
G.J.v.R	M	14	Son of G.J.v.R	60	68	67	Normal
L.J.v.R	F	10	Daughter of G.J.v.R	90	35	23	Heterozygous
M.J.v.R	F	7	Daughter of G.J.v.R	95	34	17	Heterozygous
La. J.V.R	F	4	Daughter of G.J.v.R	55	54	54	Normal
V.F.	F	39	Daughter of greatuncle H.F.	75	36	31	? Normal
P.F.	M	17	Son of V.F.	55	60	64	Normal
M.F.	F	14	Daughter of V.F.	85	45	26	Heterozygous
S.F.	M	8	Son of V.F.	180 +	42	-6	Hemizygous
W.O.	F	34	Daughter of greatuncle H.F.	75	30	25	? Normal
C.O.	M	7	Son of W.O.	65	66	64	Normal
H.O.	M	3	Son of W.O.	55	56	56	Normal
F.P.	F	32	Daughter of greatuncle H.F.	110	35	21	Heterozygous
Mar. P	F	14	Daughter of F.P.	145	28	21	Heterozygous
May. P	F	11	Daughter of F.P.	85	34	27	Heterozygous
R.P.	M	9	Son of F.P.	65	64	66	Normal
J.P.	M	6	Son of F.P.	360 +	34	3	Hemizygous
W.P.	F	4	Daughter of F.P.	115	30	16	Heterozygous
El.F.	M	62	Greatuncle	360 +	36	0	Hemizygous
E.A.S.	M	58	Son of greataunt E.S.	55	74	70	Normal
R.S.	M	23	Son of E.A.S.	60	50	50	Normal
L.S.	F	27	Daughter of E.A.S.	55	48	50	Normal
A.v.S	F	31	Daughter of E.A.S.	60	36	36	Normal
J.v.S	M	12	Son of A.V.S.	55	46	44	Normal
B.v.S	M	3	Son of A.V.S.	60	60	58	Normal
S.v.S	F	9	Daughter of A.V.S.	65	45	40	Normal
E.V.	F	57	Daughter of greataunt E.S.	75	40	40	Normal
L.V.	F	20	Deughter of E.V.	75	36	40	Normal
M.C.	F	14	Granddauehter of E.V.	110	28	21	Heterozygous

normal specimens decolorizing the dye within 75 minutes at the outside, and usually within 65 minutes. I have thus regarded decolorization times of greater than 75 minutes as being of significance in the presence of a normal haematocrit.

Similarly, in normal blood the GSH concentration after incubation is seldom more than a few milligrams per cent less than before, and in my opinion a fall in the GSH level greater than this probably indicates abnormality, particularly when associated with a low initial concentration. We have thus considered these slightly abnormal results to be indicative of the heterozygous state. It is of interest that females whose blood is not abnormal on in vitro testing may nevertheless be sensitive to primaquine. Alving et al. (1958) reported 3 females with normal GSH stability who developed haemolysis on administration of primaquine, and these workers have also observed other sensitive females with normal G6PD activity.

DISCUSSION

The pattern of inheritance of sex-linked

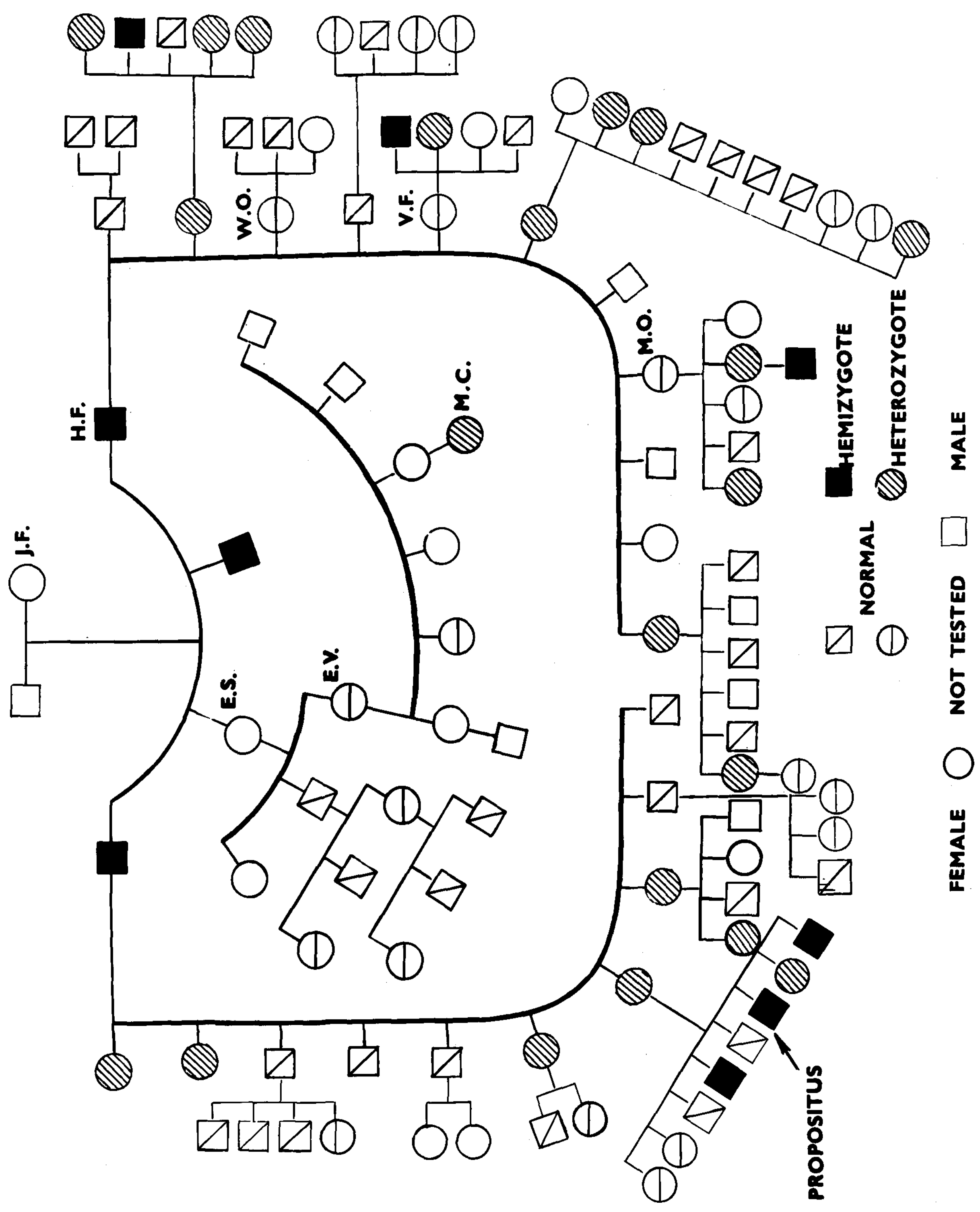


Fig. 2. The family of a child with favism, illustrating inheritance of primaquine sensitivity as a sex-linked trait of intermediate dominance. The initials indicate individuals referred to in the discussion.

conditions is well known. An affected male can only have inherited the gene from his mother, since it is carried on the X-chromosome. Similarly, he can only transmit the gene to his daughters, since his sons will receive his Y-chromosome, not his X-chromosome. Affected females, however, may inherit from either parent, and transmit to approximately 50% of their children of both sexes.

In the present family this pattern is well demonstrated as shown in the diagram. Thus the mother of the propositus is affected, not his father; in addition about half his siblings are abnormal, as expected. The mother could have inherited the gene from either parent; in fact, her father, the grandfather of the propositus, is affected. None of his sons, the uncles of the propositus, manifest the condition, while there is evidence that all his daughters are heterozygous.

Both the brothers of the maternal grandfather were found to be enzyme-deficient. Their sister, E.S. (deceased) could not be tested, but since one of her

descendants (M.C.) was affected it can be assumed that she too, carried the gene. Her daughter E.V. must also be a carrier for the same reason, even though on testing her blood neither G6PD deficiency nor GSH instability could be demonstrated. E.V. is thus a heterozygote not detectable by these two techniques (Tarlov et al., 1962).

The family of greatuncle H.F. also manifests the pattern of sex-linkage. Neither of the two available sons is affected, while there is evidence that all the daughters (except one) carry the gene. Although the results of the tests on M.O. and V.C. are not unequivocal, both have children who are definitely abnormal and therefore they must themselves be heterozygous. The exception is W.O., neither of whose sons is affected. However, it is obviously likely that she too is heterozygous in spite of the lack of definite evidence.

The distribution of primaquine sensitivity in this large family, therefore, is consistent with sex-linkage and intermediate dominance of the gene. This being so it is possible to infer that the grandfather of the propositus inherited the condition from his mother (J.F.),

not his father. She is said to have been of French extraction and to have come to South Africa from Mauritius. The incidence of primaquine sensitivity varies widely in different parts of the world (Beutler, 1959) and no reports of its occurrence in Mauritius or in France have been encountered. It is rare in the white population of South Africa (Zail and Charlton, 1962).

SUMMARY

The available members of the large family of a child with favism were tested for the associated red cell defect.

The pattern of distribution of erythrocyte glucose-6-phosphate dehydrogenase deficiency and glutathione instability in the family was found to be consistent with inheritance via a sex-linked gene of intermediate dominance.

RACIAL INCIDENCE

The varying racial incidence of primaquine and pamaquine haemolysis had been observed by several of the earlier workers (Manifold, 1931; Amy, 1934; Swantz and Bayliss, 1945; Earle et al., 1948; etc.). Application of the in vitro tests to different populations has confirmed that the distribution of the trait differs markedly in different parts of the world. Thus it has been shown by several workers that the incidence in American Negroes is about 3 - 14%, whereas the condition is very uncommon in white Americans (if special ethnic subgroups, such as Sardinians, be excluded) (Beutler, 1957; Kimbro et al., 1957; Childs et al., 1958; Alving et al., 1958). Szeinberg, Sheba et al. (1958)(b) in Ismael found the defective erythrocytes in non-Ashkenazic subjects only, with a higher incidence (20%) in Iraqi Jews than in Yemenite and North African communities (5%). Beutler, Yeh and Necheles (1959) found no positive reactors among 77 Chinese students in the U.S.A., and 1 positive out of 9 Thais. However, Vella (1961) found that 2.5% of Chinese in Singapore were positive. In

addition, the trait was present in 0.6% of Malays and 3% of the Indian population of the island. Budtz-Olsen and Kidson (1961) found no individuals with G6PD deficiency among Australian aborigines. Walker and Bowman (1959) reported an incidence of 9.8% in Iranian males, while the distribution in various parts of Africa has been studied. Gilles et al. (1960) found 9 positives among 139 Ibo and 11 among 65 Yomba in Nigeria. Allison studied the distribution of the trait in both East and West Africa, and found a high incidence (15 - 28%) except in two East African tribes, the Kikuyu and the Masai, where the condition was found in less than 3% of individuals (Allison, 1960; Allison et al., 1961). This unequal distribution follows that of the sickle trait. A similar incidence of these two conditions was also found in the Congo (Sonnet and Michaux, 1960), where 21.5% of the males were deficient in G6PD activity and 21.8% carried the sickle trait. It has been suggested (Motulsky et al., 1959 (b); Motulsky, 1960; Allison, 1960) that both types of erythrocyte abnormality confer a degree of immunity to falciparum malaria, and that the high incidence of the potentially lethal primaquine

sensitivity and sickle disease in endemic malarious areas throughout the world is a consequence of this favourable action. It has been shown (Trager, 1941) that plasmodia utilise both GSH and the oxidative glucose pathway of the erythrocyte, so that it is reasonable to suppose that cells deficient in these respects would be less hospitable to the parasite. Close sorrelation between the altitude (and thus the incidence of malaria) and not only G6PD deficiency but also thalassaemia has been reported by Siniscalco et al. (1961) in various Sardinian communities. An interesting sidelight is the observation that in individuals with both thalassaemia and G6PD deficiency a history of favism was less common than in those with primaquine sensitivity alone. Tarlov et al. (1962) suggest that the chronic haemolytic state of thalassaemia produces a cell population with a younger mean age, and therefore less susceptible to the fava principle. In Greece (Choremis et al., 1962) further support for the malaria hypothesis has been reported, the incidence of G6PD deficiency being much higher in the malarious areas of Petromagula and Pyrgos-Amalias than in Athens. Kidson (1961)

reported an association between the enzyme deficiency and malaria in New Britain and New Guinea, although the sickle trait was not observed. However, Best (1959) found no G6PD deficiency in a group of Peruvian Indians, even though falciparum malaria was endemic in the district.

In an attempt to show the protective effect of the enzyme deficiency more directly, Allison and Clyde (1961) measured the malarial parasite rates and densities in very young African children in Tanganyika, and found these to be significantly lower in those children with G6PD deficiency. They explained the equivocal results obtained by Motulsky (1960) in a similar study as being due to the effect of acquired resistance to malaria: it was in order to minimise this influence that Allison and Clyde studied only very young children.

C H A P T E R   4

Recent Advances

G6PD ACTIVITY IN TISSUES OTHER THAN ERYTHROCYTES

Deficient G6PD activity in tissues other than erythrocytes has been reported in primaquine sensitive individuals. Thus Ramot, Fisher et al. (1959) found decreased enzyme activity in leucocytes of sensitive individuals in Israel, and this group of workers has also reported similar findings with regard to platelets (Ramot, Szeinberg et al., 1959) and saliva (Ramot, Sheba et al., 1960). Recently Wurzel et al. (1961) reported decreased G6PD activity in platelets from sensitive American Negroes. Zinkham (1960) has observed deficient G6PD activity in lenses of sensitive subjects. Whole body studies by Carson (1960) suggest a generally diminished activity of the oxidative shunt pathway of glucose metabolism, since formation of $C^{14}O_2$ after injection of glucose-1-C^{14} occurs at only half the normal rate. Further evidence of a generally abnormal metabolism in primaquine sensitive individuals is the observation of Tarlov et al. (1962) that primaquine sensitive Negroes have raised levels of serum cholesterol. The administration of primaquine produced a fall in the

concentration of esterified cholesterol, although no comparable effect was observed in normals.

VARIETIES OF G6PD DEFICIENCY

Marks and Gross (1959) have pointed out that there are certain differences between G6PD deficiency in American Negroes and in Caucasians. Thus enzyme activity is normal in the leucocytes of primaquine-sensitive Negroes (Marks, Gross and Hurwitz, 1959) and reduced in Caucasians. In addition, affected Caucasians have even less G6PD activity than affected Negroes, and there is no decline of the already low activity with cell ageing although this can be demonstrated in the case of Negroes. Recently, Tarlov et al. (1962) have maintained that certain drugs such as quinine, quinidine and chloramphenicol will produce haemolysis in Caucasians only, not in Negroes. In addition, Oski et al. (1962) have demonstrated a deficiency of phosphomonoesterase activity in the erythrocytes of G6PD deficient Caucasians, whereas in primaquine sensitive Negroes activity of this enzyme was normal.

Lohr and Waller (1958), Newton and Bass (1958), Zinkham and Lenhard (1959), and Shahidi and Diamond (1959) have demonstrated GSH instability and deficiency of erythrocyte G6PD in patients with congenital non-spherocytic haemolytic anaemia, i.e. persons with a chronic haemolytic state in whom no exogenous factor appeared to be operating. This condition thus appears to be a third variety of G6PD deficiency.

Most recently, Marks, Gross and Banks (1961) have observed an Italian family with moderately diminished G6PD activity (the deficiency in other Caucasians being much more marked) in whom the electrophoretic mobility and the affinity for TPN of the purified enzyme was different from the normal. Enzyme from other affected Caucasians, affected Negroes, and a case of congenital non-spherocytic haemolytic anaemia was normal in these respects. Marks et al. (1961) have also detected no differences between normal and primaquine sensitive G6PD with regard to affinity for TPN and G6P, competitive and noncompetitive inhibitors, pH optimum electrophoretic mobility, and various aspects of the stability properties. However, in an earlier study Motulsky et al. (1959)(a)

found that the pH optimum and heat lability of enzyme from sensitive individuals was different from the normal. Carson et al. (1956) had also found 'sensitive' G6PD to be more heat labile than normal. Kirkman (1959) has reported no differences between enzyme from primaquine-sensitive subjects (presumably American Negroes) and normals with regard to stabilisation by various substances in solution and anion-exchange column chromatography characteristics. However, enzyme from a case of congenital non-spherocytic haemolytic anaemia was significantly different from both the normal and primaquine-sensitivity in Km values and pH optimum (Kirkman et al., 1960).

It thus appears that some of the varieties of G6PD deficiency are due to qualitative differences between the enzyme proteins. Recently Rimon et al. (1960) have shown that stroma from normal erythrocytes is capable of activating G6PD from primaquine-sensitive cells. The inference is that, in the variety of primaquine sensitivity seen by these workers in Israel at least, absence of a stromal enzyme activator may be the primary defect rather than quantitative or qualitative deficiency of G6PD. Marks et al. (1961) have been unable

to confirm these observations in American Negroes, and have suggested that the effect of added normal cell stroma is due to small amounts of contaminating G6PD. However, this suggestion has been refuted by Ramot et al. (1961) for various reasons. A possible explanation for the conflicting observations is that the groups of workers are dealing with different varieties of primaquine sensitivity.

The genetic implications of these different varieties of G6PD deficiency have been discussed by Childs (1961). Either control of G6PD is exercised by at least three different genes, or else the variants of G6PD deficiency are produced by only one mutant gene, the effects of which are modified by different genetic environments.

PRESENT BIOCHEMICAL CONCEPTS

In addition to the low GSH, GSH instability, deficient G6PD activity and increased GSSG reductase activity already mentioned, further biochemical abnormalities have recently been demonstrated. Thus Schrier and Kellermeyer (1958) have demonstrated increased

aldolase activity in primaquine-sensitive cells. This finding has been interpreted by these workers and by Alving et al. (1958) as a compensatory phenomenon, allowing a more rapid rate of metabolism of glucose via the anaerobic Embden-Meyerhof pathway, and hence a greater supply of reduced DPN and of high-energy phosphate bonds. This enzyme is considered to be the rate-limiting factor in this pathway, since in normal red cells large quantities of fructose diphosphate (its substrate) are found whereas no triose phosphate (the next compound in the pathway) is detectable (Schrier et al., 1959). Alving et al. (1958) suggest that the additional DPNH formed by increased glycolytic activity might be utilised by GSSG reductase for the reduction of GSSG in the absence of adequate supplies of TPNH. The increased GSSG reductase found in sensitive cells seems to be readily interpretable as compensatory. It has also been reported by Larizza et al. (1958) that lactic dehydrogenase activity is increased in primaquine sensitive cells, although this has not been confirmed by Johnson and Marks (1958). However, another possible explanation of the increased activity of this enzyme and of aldolase in enzyme deficient blood

is the higher proportion of young cells present as a consequence of the somewhat shortened cell life, as suggested by Carson (1960).

Schrier, Kellermeyer, Carson et al (1958) showed that the haemolytic action of primaquine was not due to inhibition of GSSG reductase. This observation was confirmed by Buzard et al. (1960), and extended to the other known haemolytic agents with the exception of nitrofurantoin, which they found to inhibit GSSG reductase both in normals and in sensitive individuals. This property of nitrofurantoin may well account for the haemolytic effect of this substance, therefore. The absence of Heinz bodies in the red cells during nitrofurantoin haemolysis support the hypothesis that the mechanism of erythrocyte destruction is different in the case of this substance.

The demonstration of increased TPN (Schrier, Kellermeyer and Alving, 1958) and diminished TPNH (Tarlov and Kellermeyer, 1959) in the abnormal cells was to be anticipated as a consequence of the disorder of the mechanism of TPN reduction consequent on the G6PD

deficiency. Kellermeyer et al. (1958) have also shown that DPNH, in addition to TPNH, is diminished, and that the concentration of DPN is increased. The demonstration by Tarlov and Kellermeyer (1959) that catalase activity in deficient cells is only 55 - 75% of normal was unexpected, however. The activity of this enzyme, in contrast to that of G6PD, was found to decrease still further during primaquine administration to sensitive individuals, although normals were unaffected. After the haemolytic crisis the return of enzyme activity to normal levels is very slow. Catalase is an iron-containing enzyme and it has been postulated that the ferrous iron of catalase is oxidised to the ferric form during the oxidative damage to the cell produced by the haemolytic agents (Tarlov et al., 1962). This process would parallel the conversion of haemoglobin to methaemoglobin. Tarlov et al. have demonstrated an 'anticatalase factor' in the plasma of subjects 4 - 6 hours after the ingestion of primaquine. It thus seems feasible to suppose that deficiency of this enzyme may be of importance in the mechanism of haemolysis, though Carson (1960) doubts whether this degree of deficiency of catalase can be

significant in view of the extremely rapid removal of hydrogen peroxide by human erythrocytes. Indeed, deficiency of this enzyme was initially overlooked because of the speed of the reaction (Beutler et al., 1955).

Johnson and Marks (1958) studied oxygen consumption and formation of $^{14}CO_2$ from glucose-1-^{14}C in normal and in enzyme deficient cells. Since certain enzymes of the Krebs cycle are not present in mature erythrocytes, $^{14}CO_2$ is formed from glucose-1-^{14}C only by oxidative decarboxylation of the 1-carbon via the pentose phosphate pathway. If the 6-carbon is labelled instead of the 1-carbon only 1/200 of the quantity of $^{14}CO_2$ results. Consumption of oxygen and formation of $^{14}CO_2$ thus provide measures of the activity of the shunt pathway, and this was borne out by the results obtained. The deficient cells, with 17% of normal G6PD activity, consumed 35% as much oxygen and formed 57% as much $^{14}CO_2$ as normal cells. When the cells were subdivided on the basis of cell age, it was found that young normal cells with 19.1 units G6PD activity consumed 529 µL oxygen/hour/Gm haemoglobin, whereas old normal cells manifested 16.0 Units G6PD and consumed 370 µl/hour/Gm. Young primaquine-sensitive cells

contained 11.4 Units G6PD and utilised 223 µL O_2, whereas the old erythrocytes had only 2.2 Units of the enzyme and consumed 47 µL O_2. The decrease in G6PD activity with cell age reported by Marks in 1957 was confirmed by these observations, and was shown to be associated with a considerable decrease in the oxidative metabolism of glucose.

Mohler and Williams (1961) have studied the effect of phenylhydrazine upon the concentration of adenosine triphosphate (ATP) in normal and sensitive cells. Whereas in the presence of glucose the ATP concentration of normal erythrocytes remains constant on incubation in vitro, in enzyme deficient erythrocytes the level falls. These workers also noticed that phenylhydrazine increased the utilization of glucose and produced an accumulation of pyruvic acid. A similar effect of acetylphenylhydrazine was reported by Sternschuss et al. (1961). Szeinberg and Marks (1961) found that not only acetylphenylhydrazine but also primaquine, nitrofurantoin, α - and β - naphthol, fava beans extract, ascorbic acid and cysteine, increase the production of $^{14}CO_2$ from glucose-1-^{14}C, i.e.

increase metabolism of glucose via the shunt pathway. The effect was shown to be due to oxidation of TPNH, mediated by diaphorase. This stimulation of the shunt pathway by the haemolytic substances is similar to the effect of redox dyes such as methylene blue (Harrop and Barron, 1928), and in fact Brewer and Tarlov (1961) have reported that methylene blue does produce haemolysis in primaquine sensitive persons. These workers also noted that a degradation product of primaquine stimulated the pentose phosphate pathway by oxidising TPNH.

METHAEMOGLOBIN AND PRIMAQUINE SENSITIVITY

The addition of methylene blue markedly stimulates the reduction of methaemoglobin by normal erythrocytes. It is thought that under these circumstances TPNH-linked methaemoglobin reduction is enhanced. Ross and Desforges (1959) observed that the rate of methaemoglobin reduction in primaquine sensitive erythrocytes was not increased by the addition of dye, and a test for primaquine sensitivity based on this difference from the normal has been introduced (Brewer et al., 1960). It is claimed that this test is more accurate in predicting susceptibility to haemolysis

in heterozygous females than other methods (Tarlov et al., 1962).

Since the agents which provoke haemolysis of primaquine sensitive erythrocytes also cause methaemoglobinaemia to a greater or lesser extent, and defective reduction of methaemoglobin is a characteristic of enzyme deficient cells, it is reasonable to postulate that methaemoglobin formation may be directly related to the haemolytic process.

However, it is not yet entirely clear whether an increased concentration of methaemoglobin within a cell reduces its ability to withstand stress, or whether oxidation of haemoglobin occurs independently of the weakening of the cell membrane. It has been stated that the methaemoglobin content of erythrocytes is not related to their susceptibility to haemolysis (Clark and Morrissey, 1951), and that even sulphaemoglobinaemia does not shorten red cell survival (Jope, 1946). In his review of primaquine sensitivity, Beutler (1959) stated that the processes were unrelated, but he has recently investigated the problem directly (Beutler and

Mikus, 1961). He has shown that in rats sodium nitrite did not decrease the survival of ^{51}Cr-labelled cells, although as much as 50% of the red cell haemoglobin was methaemoglobin. However, when instead of sodium nitrite the methaemoglobin producing agent was para-amino-propriophenone, a considerable shortening of red cells survival was observed even though rather less methaemoglobin was produced by this substance than by the nitrite. This together with the observations of Harley and Mauer (1961), which are discussed below, suggests that the association between the oxidation of haemoglobin and cell fragility is not a direct one. Beutler and Mikus note that there must be species differences, since the erythrocytes of mice have been reported to form Heinz bodies and to be destroyed when the animals are fed sodium nitrite (Richardson, 1941). The relationship of methaemoglobin formation to haemolysis is further discussed in Chapter 6.

HEINZ BODIES

In a review of earlier work, Webster (1949) concluded that the formation of Heinz bodies in erythro-

cytes was evidence of injury to the cell which might proceed to haemolysis. The Heinz bodies were newly formed particles, originating either from the cell membrane or from the protoplasm, in the course of irreversible injury by a toxic agent. Recent work by Harley and Mauer (1960; 1961), and by Jandl, Engle and Allen (1960) and Allen and Jandl (1961), has elucidated the process of formation of these bodies when erythrocytes are exposed to the group of compounds which provoke haemolysis in primaquine sensitive individuals. Harley and Mauer (1960) measured oxyhaemoglobin and methaemoglobin in human blood incubated in vitro with a variety of the haemolytic agents. In this study 'intact haemoglobin' was defined as the sum of oxy- and methaemoglobin, and a decrease in the sum of these two pigments was used as an index of destruction of haemoglobin by further oxidation. Some of the haemolytic agents caused destruction of 'intact haemoglobin' as well as methaemoglobin formation, while others only produced methaemoglobin, although in some instances increasing the concentration of the haemolytic substance was found to lead to disappearance of 'intact haemoglobin' as well. Destruction of 'intact haemoglobin' was never observed unless

methaemoglobin was also formed. Addition of glucose to the system always diminished the effects. In their second paper these workers showed that the formation of Heinz bodies was proportional to the quantity of 'intact haemoglobin' which disappeared. They were also able to produce coccoid bodies resembling Heinz bodies by incubating a destromatised haemoglobin solution with the haemolytic agents in vitro. Harley and Mauer thus concluded that Heinz bodies are formed from haemoglobin, and that the formation of methaemoglobin was an essential step in the process.

Jandl and his co-workers, working independently, showed that incubation of intact erythrocytes, or solutions of crystalline oxyhaemoglobin, with acetylphenylhydrazine or the other haemolytic substances resulted in a sequence of chemical changes in the haemoglobin. Methaemoglobin was first formed, and then an unnamed fast-moving haemoglobin component detected on protein electrophoresis. This was followed by a group of soluble, poorly soluble and insoluble brown to green denatured pigments, which represented the generic term "sulphaemoglobin". Finally a precipitate of coccoid

bodies closely resembling Heinz bodies were formed from the haemoglobin solution. The same sequence of changes was observed when intact cells or haemoglobin solution were incubated with oxygen alone, although the process was much slower. It thus seemed that this group of substances formed redox bridges between oxygen and haemoglobin, and greatly accelerated oxidative degradation of haemoglobin.

In a second paper, Allen and Jandl (1961) investigated the rôle of thiol compounds in oxidant drug action. When the cell is exposed to one of the haemolytic compounds, reduced glutathione is oxidised, and in the course of this reaction some glutathione becomes bound to haemoglobin by forming mixed disulphides with available SH groups of the globin fraction. After the direct oxidation of haemoglobin to methaemoglobin, the two reactive SH groups of the haemoglobin molecule are oxidised, resulting in a loss of the normal configuration of the molecule. The new compound formed exhibits different electrophoretic mobility and a different oxygen dissociation curve, and with the alteration of the mole-

cule new SH groups become susceptible to oxidation. Various brown and green haemochromes then form; these correspond to sulphaemoglobin, which is not a specific compound. Finally the denatured haemoglobin molecules polymerise to form Heinz bodies.

These workers consider that the action of GSH is to buffer the haemoglobin against oxidation; they showed that addition of GSH to the haemoglobin solution slows the appearance of Heinz bodies, and that these are fewer and larger than those formed without GSH. This observation correlates well with the well-established difference between the type of Heinz bodies formed in normal and in GSH-deficient erythrocytes. Allen and Jandl feel that GSH probably provides similar protection to other oxidatively labile cellular constituents, such as the SH groups of certain enzymes and the cell membrane. Harley and Mauer (1961) also observed that GSH inhibited Heinz body formation, but pointed out that Heinz body production was not the sole determinant of haemolysis, since different agents produced different effects with regard to Heinz body formation and increase in osmotic

fragility. Thus acetylphenylhydrazine caused marked Heinz body formation but only slightly increased osmotic fragility, while primaquine markedly increased osmotic fragility though there was only slight destruction of haemoglobin with the formation of small, discrete Heinz bodies. In vivo, acetylphenylhydrazine produces a slow extravascular haemolysis while primaquine causes an explosive intravascular haemolytic crisis.

The haemolytic activity of certain possible metabolic derivatives of sulphanilamide and hydroquinone had been investigated by Emerson, Ham and Castle as far back as 1941. These workers had concluded that oxidant breakdown products of the drugs caused methaemoglobin formation and increased the volume and the fragility of the red cells. In 1957 Mills described an enzyme, glutathione peroxidase, which catalysed the oxidation of GSH by hydrogen peroxide. He believed that this enzyme was more effective than catalase in protecting haemoglobin from oxidative degradation by ascorbic acid, and that it was an important mechanism in the maintenance of the cell (Mills and Randall, 1958). However, Carson (1960) considers that it has not yet been established whether

this system is physiologically important. Nevertheless, it has been shown that hydrogen peroxide is formed during the reaction between oxyhaemoglobin and phenylhydrazine (Restorfer and Cormier, 1957), and since catalase activity is diminished in primaquine sensitive erythrocytes (Tarlov and Kellermeyer, 1961) it is possible that the glutathione peroxidase system may be significant.

GLUTATHIONE

The significance of the disorder of glutathione metabolism in primaquine sensitive red cells with regard to the susceptibility to haemolysis is still not definitely established. Glutathione is a tripeptide, γ-glutamyl-cysteinylglycine, and is found in all living cells (Isherwood, 1959). It exists in a reduced (GSH) and an oxidised (GSSG) form (Lymann and Barron, 1937), and enzyme systems affecting both the oxidation of GSH and the reduction of GSSG are found in both plant and animal cells (Mapson, 1959). It seems likely that glutathione may play a part in a respiratory pathway for some components of the cell, but at the present time the evidence indicates that the proportion of total respiration

passing over this route is small. Nevertheless, this role may be significant for the particular cellular components with which it is associated (Mapson, 1959). Of greater importance physiologically, in all probability, is the protective action of GSH towards other cellular thiol-containing compounds, particularly enzymes. It is well known that many hydrolytic, oxidising and reducing, and transferring enzymes are dependent on one or more intact sulphydryl groups for their activity (Barron, 1951). Experimentally the addition of a thiol such as GSH to oxidatively denatured -SH containing enzymes restores the activity of the enzymes (Mapson, 1959). Besides this general function, GSH acts as a specific cofactor to the enzymes formaldehyde dehydrogenase, glyoxalase, maleylacetoacetate isomerase, and probably glyceraldehyde-3-phosphate dehydrogenase (Jocelyn, 1959). However, it is of interest that activity of both glyceraldehyde-3-phosphate dehydrogenase and glyxoalase is normal in primaquine sensitive erythrocytes (Schrier et al., 1959; Carson, 1960). Other functions of glutathione include involvement in detoxification mechanisms in the liver (Jocelyn, 1959),

and protection against radiation injury (Hope, 1959). It has been established that erythrocytes are capable of incorporating labelled glycine into GSH (Dinant et al., 1955; Elder and Mortensen, 1956), and that there is a fairly rapid turnover with a half-life of about 65 hours (Mortensen et al., 1956). Biosynthesis of GSH by erythrocytes has been confirmed by Kasbekar and Sreenivasan (1959). It has been shown that in sensitive cells the rate of incorporation of glycine-^{14}C into GSH is lower than normal (Szeinberg, Adam, Ramot et al., 1959).

The relation between GSH and haemolysis has been directly studied by a number of workers. Fegler (1952) correlated GSH levels with the number of erythrocytes haemolysed per hour in vitro on incubation with both oxygen and iodine, and showed that haemolysis increased sharply as the concentration of GSH approached zero. Sheets et al. (1956) reported that incubation of cells with a sulphydryl inhibitor, p-chloromercuribenzoic acid, resulted in haemolysis which could be prevented by the addition of -SH containing compounds, particularly GSH. Sulphydryl inhibition has been much more fully investigated by Jacob and Jandl (1962), who compared the effect

of two sulphydryl inhibitors, one permeable (N-ethylmaleimide), and one non-permeable (p-mercuribenzoic acid). The permeable inhibitor blocked cellular GSH, inhibited glycolysis, and caused the erythrocytes to lose potassium and gain sodium and water, with resultant spherocytosis and haemolysis. In contrast, the nonpermeable substance produced no effect upon GSH or glycolysis, but caused nevertheless the same shifts in electrolytes and water and hence also haemolysis. These workers concluded therefore that the shape and viability of red cells is related to the sulphydryl activity of the cell membrane, not of intracellular structures. Direct evidence of the importance of GSH in the maintenance of the integrity of the red cell has, however, been afforded by the observations of Carson et al. (1961). These workers have investigated a patient with normal G6PD activity but a partial deficiency of glutathione reductase (57%). Although GSH content was rather low, the GSH stability (Beutler, 1957) was normal. This individual was sensitive to primaquine. It seems, therefore, that the disorder of glutathione reduction in these erythrocytes was responsible for their susceptibility to haemolysis, even though the in vitro test for gluta-

thione 'stability' (i.e. resistance to oxidation) was not abnormal. Further evidence of the importance of an adequate supply of GSH in maintaining the integrity of the cell is the demonstration by Buzard et al. (1960) that one of the haemolytic drugs, nitrofurantoin, inhibits glutathione reductase, and it seems reasonable to postulate that it is this action of the drug which produces haemolysis. It therefore seems possible at least that normal supplies of GSH are important in preventing haemolysis.

In a study of intracellular sulphydryl metabolism, Rapoport and Scheuch (1960) have reported that destruction of GSH or interference with oxidative glucose metabolism (to which GSSG reduction is linked) results in diminished activity of the enzyme pyrophosphatase (which has a sulphydryl group). In a second communication (Scheuch et al., 1961) these workers showed that, in addition to pyrophosphatase, hexokinase, GSSG reductase and G6PD are inactivated under conditions of oxidation of GSH by phenylhydrazine in vitro. The addition of glucose partially protects the enzymes. They conclude that the

pentose phosphate pathway is therefore concerned in the protection of these enzymes, a mong other thiol compounds, from oxidative destruction, and that cells in which this pathway is defective will be less able to withstand oxidative damage. Scheuch et al. feel that the inactivation of hexokinase, with resultant failure of energy production by the cell, is the proximate cause of cellular disintegration, whereas Jacob and Jandl (1962) would on the basis of their experiments place the emphasis upon direct damage to the cell membrane.

OTHER ASPECTS OF THE METABOLISM OF PRIMAQUINE SENSITIVE ERYTHROCYTES

Reviewing the known functions of reduced TPN, since deficiency of this substance is the metabolic consequence of G6PD deficiency, Alving, Tarlov et al. (1960) consider the reduction of GSSG, the metabolic degradation of drugs (Brodie et al., 1958), the reduction of methaemoglobin (Brewer et al, 1960) and the reductive synthesis of amino acids and fatty acids. If inadequate supplies of TPNH are directly responsible for the increased susceptibility to haemolysis of primaquine-sensitive cells, deficiency of one

or more of these functions, or some other function still unrecognised, may be the cause of disintegration of the cell. These workers draw attention to two further aspects of primaquine sensitivity not previously described. The first is the slightly shortened red cell survival even in the absence of any extrinsic precipitating factor. This constantly increased red cell destruction is easily compensated by slightly increased activity of the erythroid marrow, so that no anaemia results. The decreased survival even in the absence of an extrinsic agent is evidence that some aspect of shunt pathway activity is of importance with regard to the maintenance of the integrity of cell. The second is the fall in total red cell lipids associated with the acute haemolytic episode provoked by one of the extrinsic agents, and followed by a very slow return to normal levels. A comparable degree of anaemia produced by phlebotomy is not associated with this change in erythrocyte lipids. In addition, Tarlov et al. (1962) have shown that total erythrocyte lipids are reduced in primaquine sensitive compared with normal subjects. These workers also found raised serum cholesterol levels in G6PD deficient subjects, and observed

that the administration of primaquine would reduce the cholesterol concentration although there was no effect in normals.

In a study of pentose metabolism in primaquine sensitive erythrocytes, Kellermeyer, Carson, et al. (1961) demonstrated decreased formation of pentose in vitro compared with normal cells. However, the total pentose content of normal and deficient erythrocytes was not different. In addition, the sensitive cells were found to exhibit a normal capacity for the reversible conversion of pentose phosphate to triose phosphate and hexose monophosphate by the transaldolase and transketolase reactions of the pentose phosphate pathway.

In a recent review, Tarlov, Brewer, Carson and Alving (1962) have listed the metabolic characteristics of primaquine sensitive erythrocytes. As the position at the time of writing is summed up in a convenient form, their assessment is given below.

I. Diminished G6PD activity, the primary disorder.

II. Diminished regeneration of TPNH secondary to I.

A. Decreased TPNH and increased TPN content

B. Decreased response to redox dyes, with regard to

(i) oxygen consumption

(ii) methaemoglobin reduction

(iii) pentose formation

(iv) glucose utilisation

(v) reduction of dye

III. Abnormalities related to the defective pentose phosphate pathway.

A. Decreased GSH content

B. Vulnerability of GSH to oxidation.

C. Increased activity of glutathione reductase.

D. Increased methaemoglobin formation during the administration of nitrite

E. Susceptibility to Heinz body formation in vivo and in vitro.

F. Decreased lipid content.

IV. Abnormalities related to Embden-Meyerhof glycolysis.

A. Increased Aldolase activity.

B. Decreased DPNH and increased DPN content.

C. Fall in adenosine triphosphate content on incubation with acetylphenylhydrazine in vitro.

V. Diminished catalase activity, with further diminution during in vivo haemolysis.

Tarlov et al. (1962) visualize the steps leading to haemolysis after exposure to haemolytic drugs as follows. The drug is metabolised and converted into an active redox compound, which bridges the physiological hiatus between molecular oxygen and intracellular electron donors. The transfer of electrons from these compounds, including TPNH, GSH, oxyhaemoglobin and other thiol substances, is thus mediated by the drug metabolite, and these cellular constituents are oxidised. In the abnormal cell the normal protective mechanisms, namely GSH, catalase and the TPNH-generating system, are deficient, and the oxidation proceeds to irreversible stages. Heinz bodies form, in the manner described by Allen and Jandl (1961), and metabolic processes diminish to levels at which vital functions can no longer be carried out. Alterations

in the lipoprotein membrane occur and intravascular lysis results.

MORPHOLOGY OF PRIMAQUINE SENSITIVE ERYTHROCYTES

Although by conventional techniques no morphological abnormality of primaquine sensitive erythrocytes is detectable (Beutler et al., 1954), Danon et al. (1961) have observed a difference between normal and sensitive blood on electron microscopy. In normal blood a proportion of the erythrocyte cell membranes appear 'smooth', while the remainder are 'granular'. Danon et al. established that the younger cells have granular membranes, while the older members of the erythrocyte population appear smooth. In primaquine sensitive blood a larger proportion of the cell membranes are smooth than in normal blood. The morphological appearances thus suggest premature ageing of the erythrocytes in primaquine sensitivity. Since the activity of many enzymes, including G6PD, diminishes with ageing of the cell, and since primaquine sensitivity is associated with decreased G6PD activity,

this concept fits in well with current theories.

THE HAEMOLYTIC EPISODE IN VIVO

Various aspects of haemolysis in vivo have been studied. Flanagan et al. (1958) followed four parameters of the abnormal erythrocytes during the administration of primaquine to sensitive individuals. Firstly, the concentration of reduced glutathione fell sharply during the first 3 days of administration of the drug, before any haemolysis had occurred. During the period of major haemolysis, from the 4th to the 12th day of exposure, the concentration of GSH in the remaining cells rose rapidly, but had not reached the prehaemolytic level by the 9th day, the day of peak reticulocyte response. The concentration of GSSG was not found to vary during a haemolytic episode. Flanagan et al. concluded that the outpouring of young cells by the marrow was not the only factor involved in the return of GSH content to normal levels, and that the lack of change in the level of GSSG did not support the concept that GSH is destroyed by oxidation. However, if GSSG were removed at the same rate at which it were formed the level would not rise.

The second parameter observed was GSH stability,

and this was found not to alter during the haemolytic episode. However, Szeinberg et al. (1958) have reported that the GSH stability test may temporarily become negative during and immediately following an acute haemolytic episode. In addition, Zannos-Mariolea and Kattamis (1961) found that GSH instability was not nearly so marked during attacks of favism, although some abnormality was usually demonstrable. Thirdly, typical 'sensitive' Heinz bodies could be produced in vitro throughout the period of exposure to the drug. Finally, the activity of glucose-6-phosphate dehydrogenase, which was of course very low prior to the haemolysis, rose slightly during the reticulocytosis and then decreased to the previous low level.

Kellermeyer et al. (1961) have reinvestigated the 'resistant phase' (Dern et al., 1954) which follows the haemolytic crisis. Dern et al. showed that continued administration of the drug during this phase did not produce further overt haemolysis. However, Kellermeyer et al. reported that haemolysis could be produced during this period if the dose of drug were increased. In addition, they established the fact that the survival

of the remaining erythrocytes could be progressively shortened by increasing doses of primaquine. Thus in addition to cell age, haemolysis is also dependent upon the concentration of the haemolytic agent.

Tarlov et al. (1962) have drawn attention to other factors influencing the severity of the haemolysis. Coexistent liver or kidney disease, by delaying detoxification or excretion, may produce a higher blood level of the haemolytic substance. Concurrent viral or bacterial infection may enhance the haemolytic effect, as may diabetic acidosis. Hypoglycaemia, especially in the neonate, may reduce the resistance of the cells. (Szeinberg, Ramot et al., 1958). Finally, the haemolytic effect of primaquine may be diminished by administering the drug intermittently rather than continuously. Since the antimalarial action is enhanced if the drug is administered in this way, this schedule has been recommended by Alving et al. (1960).

NEONATAL JAUNDICE

It seems clear that some at least of the varieties of G6PD deficiency of the erythrocytes may cause severe neonatal jaundice. The cases of congenital neospherocytic haemolytic anaemia with marked G6PD deficiency reported by Newton and Bass (1958), Zinkham and Lenhart (1959) and Shahidi and Diamond (1959) had all manifested hyperbilirubinaemia in the neonatal period.

There is also good evidence that primaquine sensitivity, as well as congenital nonspherocytic haemolytic anaemia, may be associated with neonatal jaundice. Vella (1959), in a survey of G6PD deficiency in Singapore, noticed that among the group of reactors were 4 Chinese infants with neonatal jaundice, and Smith and Vella (1960) subsequently reported 14 cases of kernicterus in Chinese infants in whom G6PD deficieny was demonstrated, there being no demonstrable blood group incompatibility. Weatherall (1960) also noticed the association between neonatal jaundice and G6PD deficiency in Malayan children, and commented that no external

precipitating factor

appeared to be operating. Gilles and Arthur (1960) detected G6PD deficiency associated with unexplained neonatal jaundice in West Africa.

Doxiadis, Fessas and Valaes (1961)(a) showed that many of the full-term babies with severe neonatal jaundice seen in Athens in whom there was no blood-group incompatibility were deficient in G6PD activity, and from their paper there seems to be no doubt that enzyme deficiency does cause neonatal jaundice in Greece. In most of their cases no extrinsic precipitating factor could be identified, although some were associated with the administration of Vitamin K (Doxiadis et al., 1961 (b)).

In the neonatal period, even in infants with normal G6PD, GSH instability can be demonstrated. This is corrected if glucose is added (Szeinberg, Ramot et al., 1958, Zinkham, 1959) and presumably the temporary instability of GSH is related to hypoglycaemia. It seems reasonable to suppose, therefore, that primaquine sensitive erythrocytes are particularly vulnerable to haemolysis at this time (Tarlov et al., 1962). Possibly

a minimal insult, or no extraneous influence at all, may provoke disintegration and hence a haemolytic jaundice.

C H A P T E R 5

Primaquine Sensitivity in Various Racial Groups in Southern Africa

The incidence of primaquine sensitivity varies considerably in different parts of the world. The known racial distribution of the condition in various countries has been discussed in Chapter 3. Since no previous study of the incidence in Southern Africa has been reported, it was decided to determine the distribution of primaquine sensitivity in certain racial groups.

MATERIAL AND METHODS

A. THE HEINZ BODY TEST

This was the first in vitro test for primaquine sensitivity to be described (Beutler, Dern and Alving, 1955). When erythrocytes are incubated under aerobic conditions with acetylphenylhydrazine, Heinz bodies form in the cells from the oxidatively denatured haemoglobin (Jandl et al., 1960). The Heinz bodies formed in primaquine sensitive cells differ in size and number from those formed in normal cells, and with a little experience it is easy to distinguish an enzyme deficient sample of blood from the normal. It was found that better preparations were obtained if

the technique described by Beutler et al. (1955) was slightly modified, as indicated below.

MATERIALS

1. Buffer. Mix 1.3 parts $M/_{15}$ KH_2PO_4 with 8.7 parts $M/_{15}$ Na_2HPO_4, to produce a solution pH 7.6. Add glucose to produce a concentration of 200 mgms.%. This solution is stable in the refrigerator for a considerable time.

2. Acetylphenylhydrazine solution. Dissolve enough acetylphenylhydrazine in the buffer solution at room temperature to make a concentration of 100 mgms.%. This solution must be used within one hour.

3. Crystal violet solution. Add 2 Gm. crystal violet to 100 ml. aqueous 0.73% NaCl solution at room temperature. Shake for 5 minutes and then filter. This solution is said to be stable for 5 months; however, fading of the dye was observed after 2 months and fresh solution was made up.

METHOD

Blood is drawn into a heparinized syringe, and then transferred to a plain tube. The test must be started within 1 hour of collection.

0.1 ml. blood is pipetted into 2.0 ml. acetylphenylhydrazine solution in a test tube. Mixing and aeration is achieved by drawing about 0.1 ml. back into the pipette and blowing it, together with a small quantity of air, back into the tube. This is repeated 2 - 3 times. The pipette is left in the uncapped tube, which is placed in a water bath at 37° C. After incubation for 2 hours the mixture is again aerated as before. After a further 2 hours the pipette is gently blown through yet again in order to mix the cell suspension, and then a small drop is placed

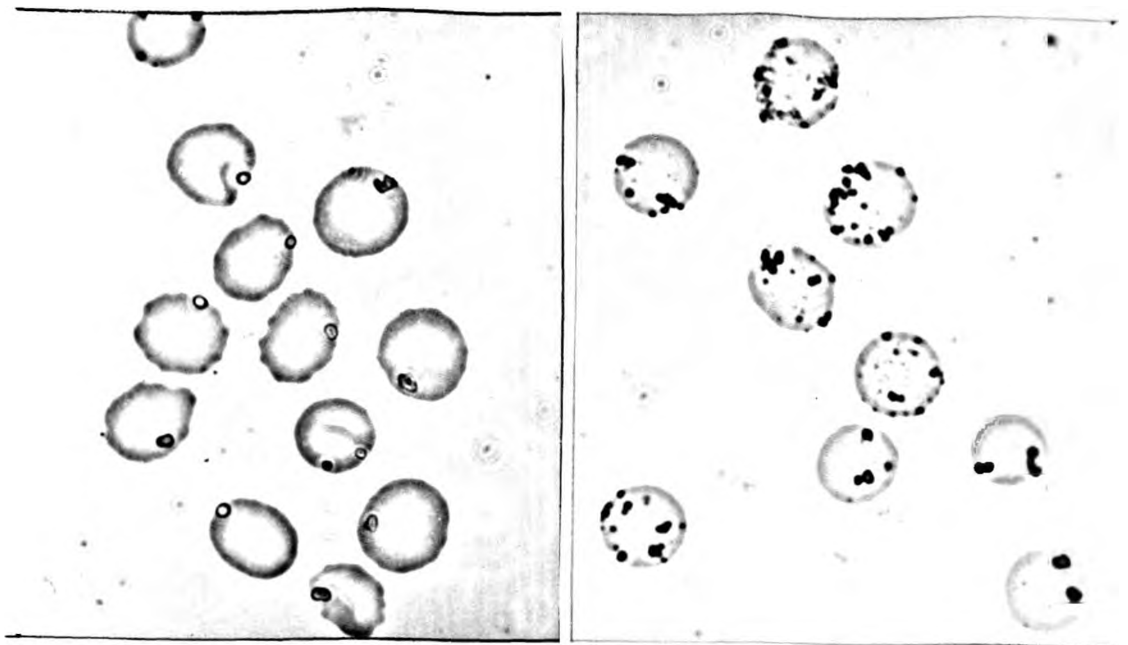


Fig. 3 Heinz body formation in erythrocytes after incubation in vitro with acetylphenylhydrazine. In normal blood (left) most of the cells contain one or two large peripheral Heinz bodies whereas in primaquine sensitive blood (right) the Heinz bodies are small, multiple and scattered throughout the cell.

on a glass coverslip. A larger drop of the crystal violet solution is placed on a slide, and the coverslip put on top of it so that the fluids mingle. After about 10 minutes a suitable field is selected under oil immersion and the cells are examined.

In primaquine sensitive blood most of the cells contain many small Heinz bodies, while in normal blood the majority of cells contain one or two marginal, large, well-rounded Heinz bodies. Typical fields are shown in the photomicrographs (Fig. 3).

It was found that better preparations were obtained by using a different technique. After incubation with acetylphenylhydrazine as described above, the cell suspension and the crystal violet solutions were mixed in a tube in approximately the proportions indicated above. After 10 minutes a drop of plasma was added, and then a smear was made on a glass slide using a glass spreader, as in making ordinary blood smears. When the smear had dried the cells were examined under oil as before.

B. THE GLUTATHIONE STABILITY TEST.

The method of Beutler (1957) as modified by Flanagan et al. (1958) was used, and further slight modifications were introduced. In this test the reduced glutathione content of a sample of blood is measured before and after incubation with acetylphenylhydrazine. The blood is haemolysed and a protein-free filtrate is prepared. Free sulphydryl groups are then measured colorimetrically by the reaction with nitroprusside (Gruenert and Phillips, 1951). Since there are insigni-

ficant amounts of other free SH-containing compounds in erythrocytes this reaction represents the reduced glutathione content. In normal blood the GSH concentration changes little, if at all, whereas in primaquine sensitive blood the initial concentration (which tends to be somewhat lower than normal) falls markedly after incubation. A concentration after incubation of 20 mgms./100 ml. erythrocytes or less is regarded as positive, and in males a positive GSH stability test correlates well with sensitivity to primaquine (Beutler, 1960). In females the test gives very variable results; this has been discussed in detail in Chapter 3.

MATERIALS

1. Saturated aqueous NaCl.

2. 2% Sodium nitroprusside. This solution is light-sensitive and must be kept in an amber bottle. Even when this precaution is taken it does not keep indefinitely, decomposition being detectable by a change in colour.

3. 0.067M sodium cyanide in 1.5M sodium carbonate, Dissolve 328 mgms. NaCN in 100 ml. 15.9% Na_2CO_3 solution.

4. 4.5% Metaphosphoric acid. B.D.H. Laboratory Reagent metaphosphoric acid sticks were used. This

reagent was found to deteriorate rapidly, and fresh solutions were made up every few days.

5. <u>Test tubes containing 10 mgms. acetylphenylhydrazine.</u> Dissolve 100 mgms. acetylphenylhydrazine in 2 ml. methyl alcohol. Pipette 0.2 ml. into each tube and allow to evaporate.

METHOD

If heparinized blood is used, the test must be commenced within 3 hours of collection. Szeinberg, Asher and Sheba (1958) recommend the addition of glucose before incubation in order to prevent occasional false positive results. If blood is collected into acid-citrate-dextrose (ACD) solution it is possible to perform the test satisfactorily some days after collection (Beutler, 1959), and this was found to be much more convenient than using heparinized blood. In the majority of instances, therefore, this method was followed.

1. Incubation

2 ml. blood is placed in a tube containing 10 mgms. acetylphenylhydrazine. By stirring with a glass rod and tapping the tube against the palm of the hand the acetylphenylhydrazine is dissolved and the blood well oxygenated. The tube is placed in a water bath at 37° C., and after 1 hour the tube is again agitated by tapping as above. After 2 hours the GSH content of the incubated blood is determined, and compared with the concentration in the unincubated specimen.

2. Estimation of GSH

Flanagan et al. (1958) recommend that the

laboratory temperature during the estimation of GSH be maintained at 20 - 22° C., and that the solutions used in the determination should stand in a waterbath at 20° C. It was not possible to control the laboratory temperature, but satisfactory results were obtained by the use of a waterbath as described.

1.5 ml. blood is pipetted into a 50 ml. Erlenmeyer flask containing 5.5 ml. distilled water. After mixing, the flask is allowed to stand for 10 - 15 minutes until haemolysis is complete. 5.0 ml. metaphosphoric acid is added with constant swirling of the flask to produce an even precipitate. Enough solid NaCl is added to saturate the solution (approximately 4.75 gm.). The flask is sealed with Parafilm and vigorously shaken for 2 minutes, after which the mixture is filtered through Whatman No.2 filter paper. 2 ml. filtrate are added to 6 ml. saturated NaCl solution in a test tube and placed in the waterbath at 20° C.

As a reagent blank, 1.5 ml. distilled water is subjected to the above procedure, and 1.5 ml. of 40 mgm.% GSH solution is used as a standard. The preparation of the GSH solution is further discussed in the next section.

To the tube containing the 6 ml. saturated NaCl and 2 ml. filtrate is added 1 ml. nitroprusside followed immediately by 1 ml. cyanide/carbonate solution (using a syringe pipette). The mixture is at once poured into an Evelyn tube, adequate mixing occurring with the transfer of fluid, and the optical density at 520 mμ is measured in an Evelyn electrophotometer which has been set to read 100 with the blank solution. The blank reads 87 against distilled water. The GSH concentration of the test solution may readily be calculated by comparison of its optical density with that of the standard. Determination of the haematocrit (Wintrobe, 1956) of the blood specimen then permits expression of the GSH content of the blood as X mgm. per 100 ml. erythrocytes, which eliminates distortion due to anaemia or to dilution of the blood by

the ACD solution.

3. Preparation of the standard curve.

This is done in the usual manner using various concentrations of GSH. However, Flanagan et al. (1958) report that different samples of commercial GSH do not give the same results, and that this is due to incomplete reduction of the glutathione. They advise electrolytic reduction of the GSH solution by a modification of the method of Dohan and Woodward (1939) before measurement. The reduction is carried out on 10 ml. of the filtrate, i.e. at the stage in the method immediately before the cyanide is added and the rose colour develops.

The apparatus is shown in the diagram (Fig. 4)

The KCl agar bridge (A) is constructed by dissolving 4 Gm. agar in 100 ml. saturated aqueous KCl, boiling, and pouring the warm solution into a polythene tube 1 cm. in diameter, where it is allowed to set. Polythene was found to be more convenient than glass as it could easily be bent into the required shape, and shortened when the collection of hydrogen bubbles between the agar and the wall of the tube at the cathode end made it necessary.

The glass paddle stirrer (B) is rotated by an electric motor at approximately 60 r.p.m. Since reduction occurs only at the surface of the mercury, mixing of the overlying solution is important.

The 4% sulphosalicylic acid solution in the anode beaker (C) rapidly becomes yellow and then brown, when it is replaced.

The mercury cathode in its beaker (D) is kept covered with distilled water when not in use. Prior to

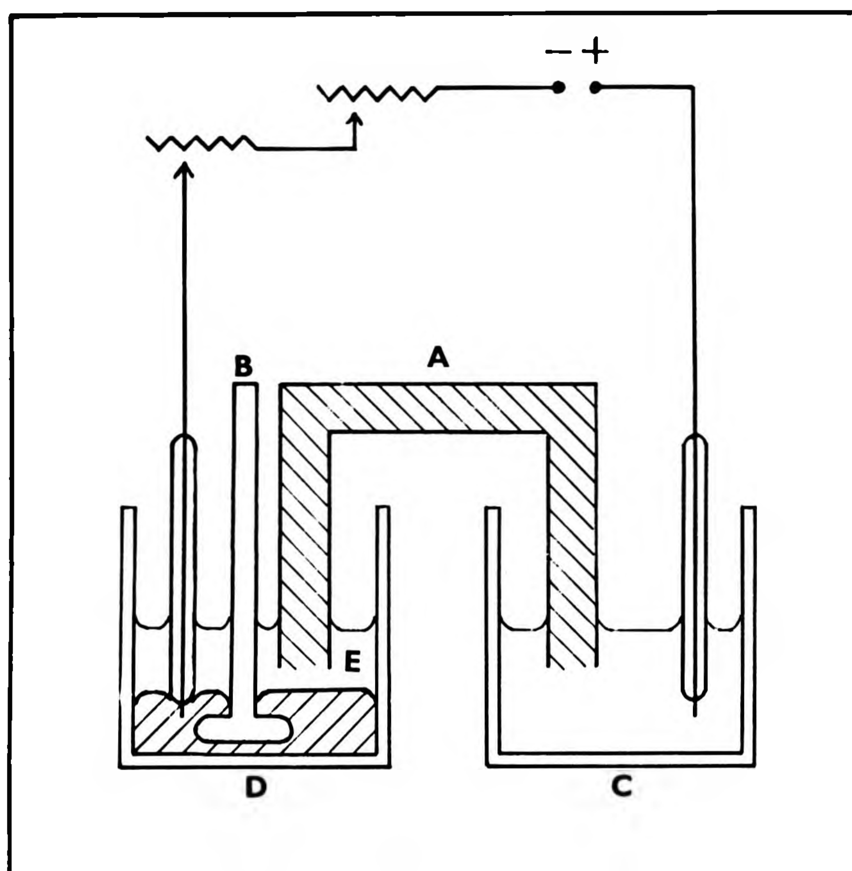
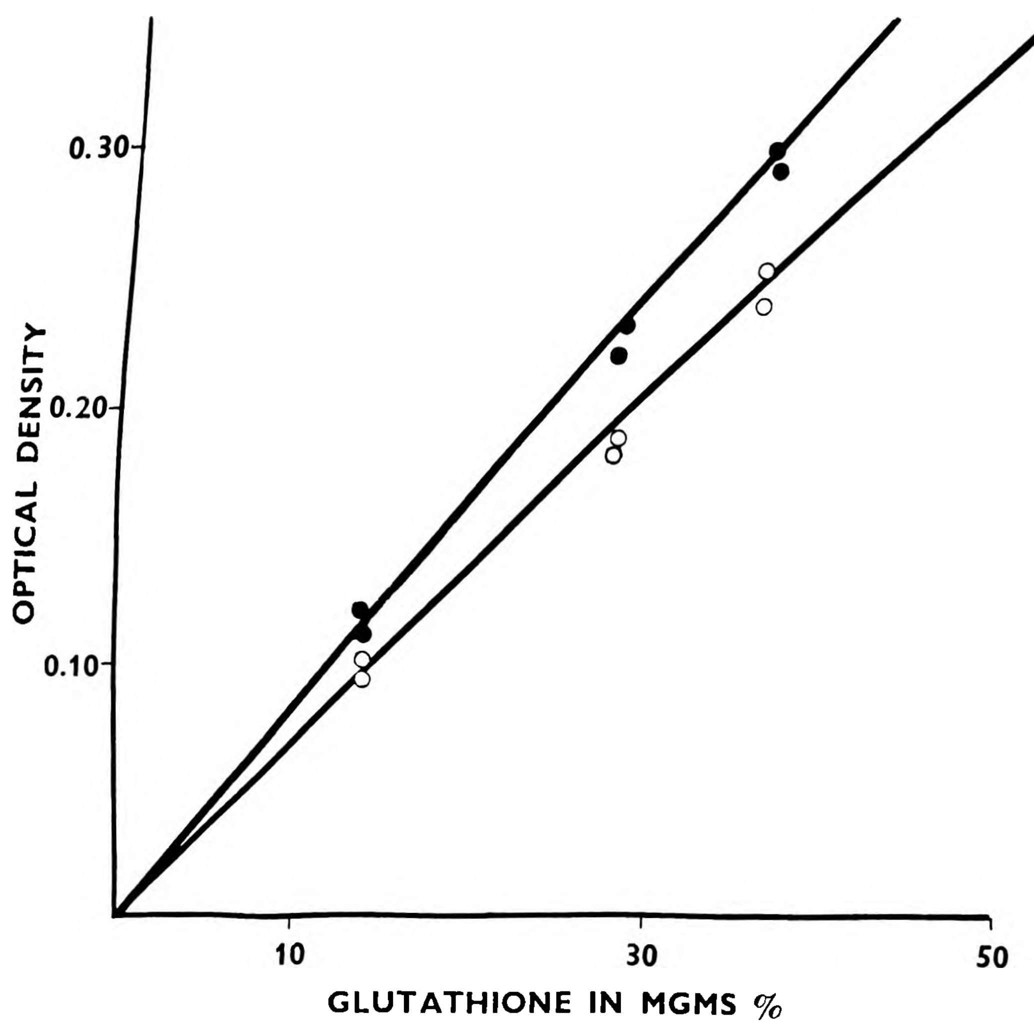


Fig. 4. The apparatus required for the electrolytic reduction of GSH. A: KCl agar bridge; B: glass paddle stirrer; C: anode beaker; D: cathode beaker; E: GSH solution.



<u>Fig. 5</u>. Standard curves used in the GSH stability test. The closed circles are the values obtained after electrolytic reduction of the GSH standard solutions.

each reduction of solution, the cathode itself is "reduced" by passing current through the circuit for 30 minutes with the agar bridge immersed in the mercury (Flanagan et al., 1958). After reduction of a solution the mercury is washed many times with distilled water.

<u>Method</u>. The mercury and the agar bridge are carefully dried with filter paper. 10 ml. of the solution to be reduced (E) is then pipetted onto the surface of the mercury. Current at 50 volts and 36 milliamps is passed through the circuit, with the agar bridge raised above the mercury so that it dips into the solution, and the paddle stirrer rotating. Flanagan et al. state that reduction is complete after 15 minutes; however, higher values are obtained by allowing current to flow for 30 minutes, although reduction for longer than this had no further effect. 2 ml. of the reduced filtrate are then added to 6 ml. saturated NaCl solution, as before, and after the nitroprusside and cyanide/carbonate is added the optical density is measured.

The standard curves obtained are shown in Fig. 5.

C. <u>THE SCREENING TEST FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY</u>. (Motulsky and Campbell, 1960).

This test depends on the coupling of a dye to the dehydrogenation of glucose-6-phosphate by a haemolysate of the blood to be tested. Excess substrate and coenzyme are added to the reaction mixture so that the rate of the reaction is proportional to the enzyme activity of the

blood sample. The time taken to decolorize a fixed quantity of the dye is thus a measure of the G6PD activity. The reaction is shown diagrammatically in Fig. 6.

Blood may be collected in ACD, in citrated, oxalated or heparinized tubes, or drawn directly into the pipette after digital puncture. The test may be performed several days after collection of venous blood.

MATERIALS

1. Buffer. 0.74 M tris (hydroxymethyl) aminomethane pH 7.5 (8.95%).

2. Sodium glucose-6-phosphate (G6P) 5 µM solution (1.65%). 1 ml. is enough for 20 tests. Made up freshly for each batch of tests.

3. Triphosphopyridine nucleotide (TPN) 0.1% solution. 1 ml. is enough for 20 tests. Made up freshly each day.

4. Brilliant crystal blue (BCB) 1 µM solution (0.032%). Keeps indefinitely.

The G6P and TPN were obtained from the Sigma Chemical Co. of St. Louis, Mo., U.S.A. The BCB was obtained from the National Aniline Division of the Allied Chemical and Dye Corporation, 40 Rector Street, New York, N.Y., U.S.A. Samples of BCB obtained from other sources were found not suitable for the test.

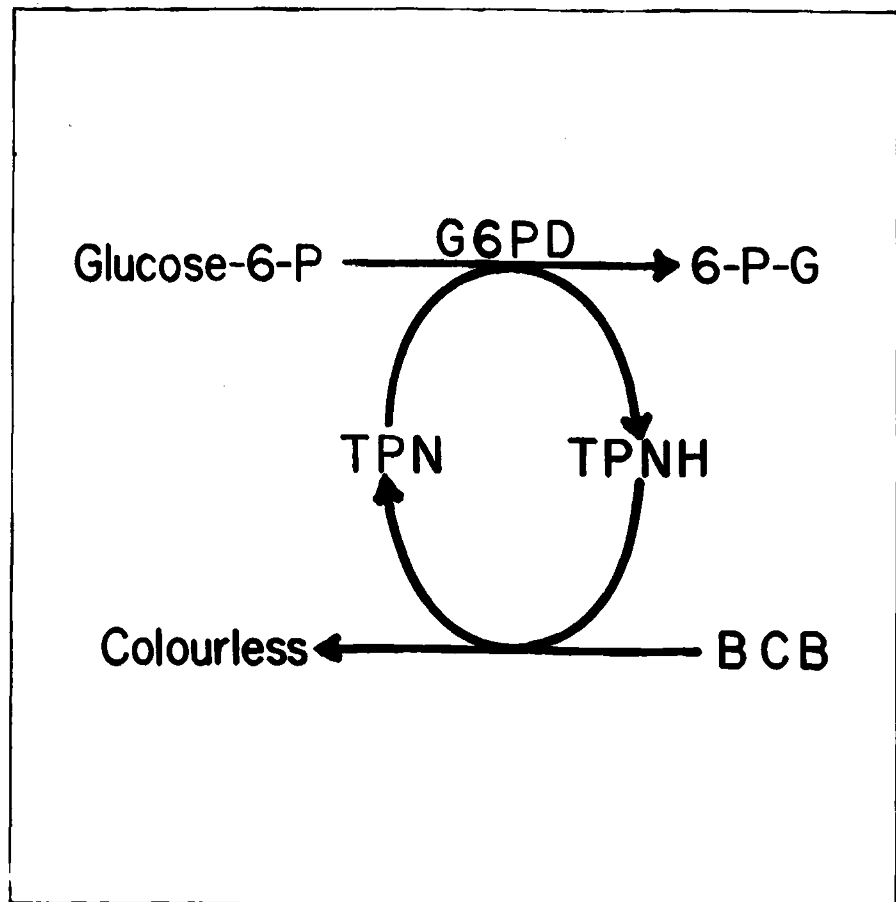


Fig. 6. A diagrammatic representation of the screening test for G6PD activity (Motulsky and Campbell, 1960). Glucose-6-P: glucose-6-phosphate; G6PD: glucose-6-phosphate dehydrogenase; 6-P-G: 6-phosphogluconic acid; TPN: oxidised triphosphopyridine nucleotide; TPNH: reduced triphosphopyridine nucleotide; BCB: brilliant cresyl blue.

METHOD

0.02ml. blood is pipetted into a tube containing 1 ml. distilled water. When haemolysis is complete (after 10 - 15 minutes), 0.2 ml. buffer is added, and then 0.05 ml. of both the G6P and the TPN solutions. 0.25 ml. BCB solution is then added and the contents of the tube well mixed by swirling and shaking. A layer of liquid paraffin is poured on top of the mixture to secure anaerobic conditions, and the tube is placed in an incubator or a waterbath at 37° C. The end point is obvious; when the blue dye is all decolorized the tubes appear pink from the haemoglobin solution. With most normal bloods decolorization was complete in 65 minutes, although occasionally the reaction was prolonged to 75 minutes. Enzyme deficient blood failed to decolorize the dye within 120 minutes and this time was often much longer. There was thus a clear distinction between normal and primaquine sensitive samples.

VALIDATION OF TECHNIQUES

In order to be sure that abnormal Heinz bodies, glutathione instability and deficiency of glucose-6-phosphate dehydrogenase detected by the application of these techniques did in fact indicate primaquine sensitivity, two approaches were used.

In the first, blood from a child with a history of favism (Senior and Braudo, 1955) was subjected to the three tests. Favism occurs only in primaquine sensitive subjects (Tarlov et al., 1962), and if the diagnosis was

correct the in vitro tests for primaquine sensitivity should be positive. The cells were shown to form the typical abnormal Heinz bodies, and the initial GSH concentration of 51 mg./100 ml. erythrocytes fell to 10.6 mg./100 ml. on incubation with acetylphenylhydrazine. In addition, markedly deficient enzyme activity was observed, the dye not having been decolorized after 360 minutes incubation.

In the second, red cells from an individual whose blood was positive to the 3 tests were labelled with ^{51}Cr as described by Mollison and Veall (1955), and introduced into the circulation of a normal compatible recipient. Measurement of radioactivity in the blood of the recipient was compatible with a normal survival of the labelled donor cells for 8 days after introduction. Primaquine 30 mgms. daily was then administered to the recipient, and on the third day of administration a sharp increase in the rate of disappearance of the donor cells was observed. The graph of red cell survival is illustrated (Fig. 7). It was concluded that cells giving positive results to the 3 tests were in fact

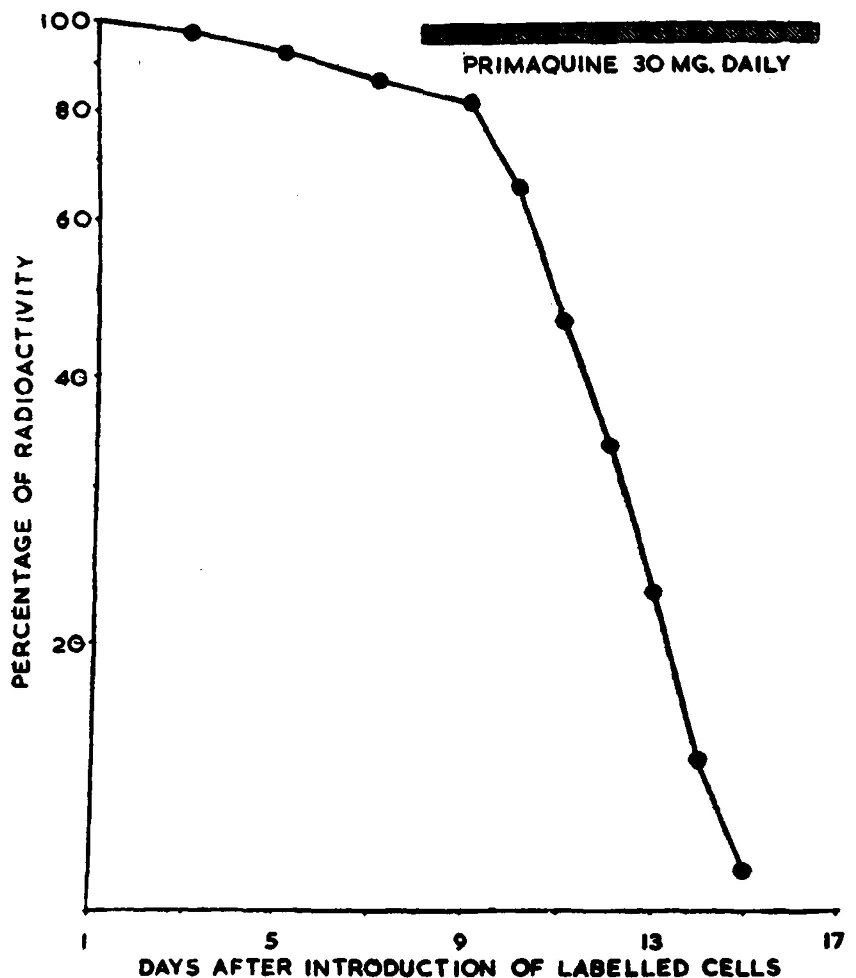


Fig. 7. The survival of ^{51}Cr-labelled erythrocytes in a compatible, normal recipient. The erythrocytes had been shown to form Heinz bodies in vitro typical of primaquine sensitivity, to be deficient in G6PD activity, and to manifest GSH instability. On administration of primaquine in a dosage of 30 mgms. daily the rate of destruction of the labelled cells was sharply accelerated, as shown. It was therefore established that erythrocytes giving positive results to the 3 tests were in fact sensitive to primaquine.

sensitive to primaquine.

SUBJECTS STUDIED

Primaquine sensitivity can be detected with considerable accuracy in male subjects, as distinct from females, by the application of the preceding tests (Beutler, 1959; Tarlov et al., 1962). In males there is a marked difference between normals and abnormal individuals; in females, however, there is a considerable degree of variation in the response to the tests. Some female carriers of the gene manifest markedly deficient G6PD activity and unstable GSH, some are normal to the tests, while others exhibit all degrees of enzyme deficiency and GSH instability (Tarlov et al., 1962). The problem of the identification of female heterozygotes has been discussed in Chapter 3. In order to assess the incidence of the condition in a population it is therefore usual to examine the blood of males only.

Four racial groups were studied, namely Bantu, Indians, Cape Malays and Bushmen. In the case of the Bantu, Indians and Malays, only males were tested, but as

very few Bushmen were available for study both males and females were examined.

I. BANTU. 310 Bantu subjects belonging to a number of different tribes were studied. Of these, 129 were patients in hospital in Johannesburg suffering from a variety of medical and surgical conditions. The remaining 181 were healthy blood donors.

II. INDIANS. 100 Indian patients in King Edward VII Hospital, Durban, were tested.

III. MALAYS. 53 Cape Malay patients in Groote Schuur Hospital, Cape Town, were examined.

IV. BUSHMEN. 70 Specimens of blood were collected in the Kalahari desert by members of Professor P.V. Tobias' 1960 Kalahari expedition, refrigerated, and flown to Johannesburg where they were tested. Of these 47 (16 males and 31 females) belonged to the Central Kalahari group of Bushmen, while 23 (13 males and 10 females) were of the Northern group. During the 1962 expedition 25 further specimens were obtained from Bushmen of the Southern group. Of these 21 were males and 4 female.

PROCEDURE

In all cases positive results were confirmed by applying a second test. Initially glutathione stability tests were performed, and the Heinz body test was used in confirmation. When the screening test for G6PD became available it was used as the initial procedure, and deficiency was confirmed by assessing glutathione stability.

RESULTS

I. BANTU

In the first 182 specimens examined the glutathione stability test was applied, and 5 positive results were obtained. The average concentration of reduced glutathione after incubation in these 5 samples was 12.3 mg./ 100 ml. erythrocytes (range 8.2 - 18 mg./100 ml.), whereas in the case of the 177 negative reactors the average was 54.4 mg./100 ml. with a range of 31 - 156.8 mg./100 ml. In the 5 subjects with unstable glutathione the Heinz body test was found to be positive.

In a further 128 blood specimens the test for

glucose-6-phosphate dehydrogenase activity was used, and 5 samples were found to be deficient in the enzyme. When these 5 specimens were incubated with acetylphenylhydrazine the reduced glutathione concentration after incubation was found to range between 1 and 11 mg./100 ml. erythrocytes.

Thus of 310 Bantu males tested 10 were considered to be primaquine sensitive. The tribal origins of the subjects studied are given in Table II, and the glutathione concentrations before and after incubation in the 187 specimens tested are shown in Fig. 8.

II. INDIANS

Blood specimens from 100 Indian males were tested for glucose-6-phosphate dehydrogenase activity. Enzyme deficiency was not detected.

III. CAPE MALAYS

Samples from 53 male Cape Malays were subjected to the enzyme test, and 2 specimens were found to be deficient in glucose-6-phosphate dehydrogenase. After incubation with acetylphenylhydrazine the reduced

TABLE II

Tribe	No. Tested	No. Positive
Msutu	74	2
Zulu	73	1
Xhosa	43	1
Twana	25	2
Shangaan	18	1
Pedi	11	0
Swazi	8	1
Nyasa	7	1
Coloured (mixed origins)	13	1
Remainder (Baca, Pondo, Venda etc.)	38	0
TOTAL	310	10

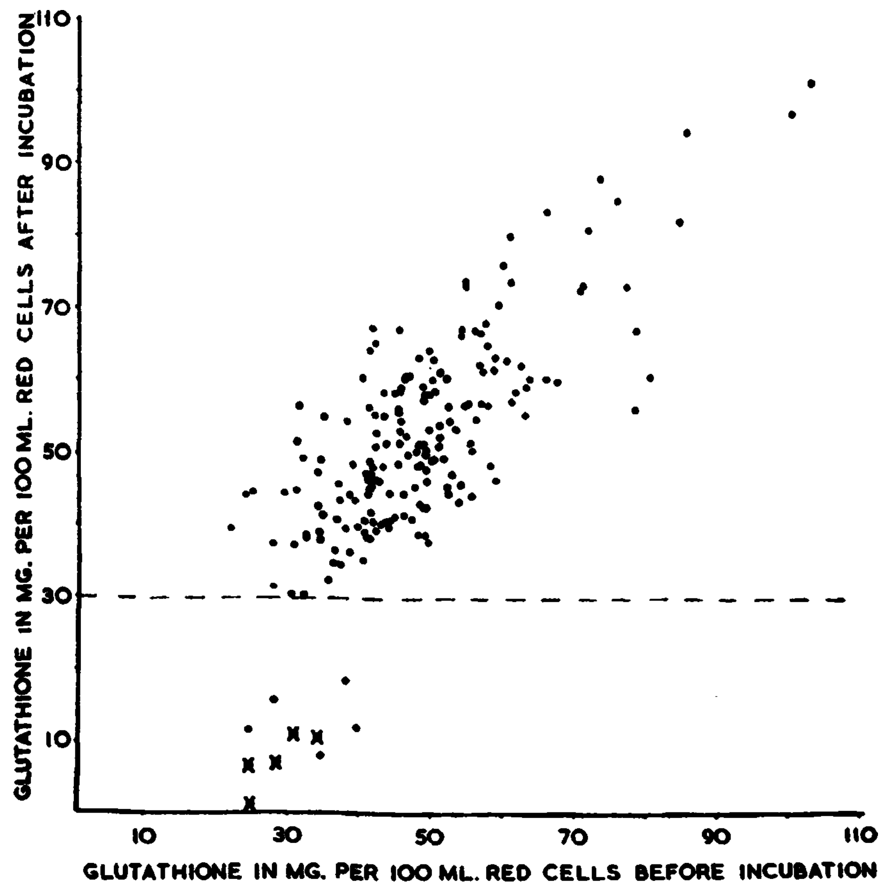


Fig. 8. GSH concentrations before and after incubation of blood specimens from 187 Bantu males. In addition to the first 182 tests of which 5 were positive, the results of the 5 sensitive individuals detected by application of the G6PD screening test to a further 128 subjects have been plotted (crosses).

glutathione concentrations of these 2 specimens were 7.4 and 4.5 mg./100 ml. red cells respectively.

IV. BUSHMEN

Blood from 50 males and 45 females was tested for enzyme activity, and in 2 instances (1 male and 1 female) deficiency was detected. The reduced glutathione concentrations in these 2 specimens after incubation were 0 and 10 mg./100 ml. red cells respectively. Both positive reactors were members of the Central Kalahari group.

DISCUSSION

The results of this investigation suggest that the incidence of primaquine sensitivity of the red blood cells in the Bantu of Southern Africa is approximately 3%. When the subjects studied are subdivided on the basis of their tribal origins, the 10 positive reactors appear to be randomly distributed among a number of different tribes. While the numbers are not large enough for the different tribal incidences to be determined, there do not appear to be marked differences in frequency.

The finding of this condition in the Bantu was to be expected, since pamaquine haemolysis has previously been observed in a Nyasa soldier (Mann, 1943) and in a Basuto (Smith, 1943). The incidence is approximately the same as that found by Allison (1960) in the Masai and Kikuyu tribes of East Africa, but much less than in other East African tribes (Allison, 1960) and in the Congo (Sonnet and Michaux, 1960) and West Africa (Gilles et al., 1960). The incidence in other parts of Africa has been discussed in Chapter 3.

The number of Cape Malays and Bushmen available for testing was too small to allow estimations of the incidence of the trait, other than to say that the condition does occur with approximately the same order of frequency as in the Bantu. The Cape Malays are not a pure racial group, being crossbred descendents of Asiatics, Bantu, Hottentots, Bushmen and Europeans (du Plessis, 1944). Comparison of the incidence in this group with that found in Malaya (Vella, 1961) is thus not valid.

None of the 100 Indians tested was found to be deficient in red cell G6PD activity. However, it is evident from the literature that the trait does occur in India, since pamaquine haemolysis has been reported in Indians by several workers (Manifold, 1931; Amy, 1934; Sein, 1937; Proc. Conf. Med. Spec., 1944; Dimson and McMartin, 1946). Possibly the trait is present in South African Indians but was not encountered in the present study. Even if this is not so, it is very likely that the incidence of the condition varies in different parts of India, as it does in Africa. The Indians of

Natal are for the most part descendants of immigrants from Madras (Shafa'at Ahmad Khan, 1946), while most of the reported cases of pamaquine haemolysis were in Northern India.

CHAPTER 6

Methaemoglobin Formation and Reduction in Primaquine Sensitive Erythrocytes

Methaemoglobinaemia associated with plasmoquine administration was recorded by many workers, and was in fact regarded as a manifestation of toxicity comparable with haemolysis by many of the earlier authors. However, Beutler (1959) strongly denied a causal relationship between these two consequences of the administration of plasmoquine (and the other drugs in this group). He quoted Clark and Morrisey (1951) who showed that in dogs the survival of methaemoglobin-containing cells was not shorter than normal. Other workers have shown that even sulphaemoglobin formation is not associated with shortened red cell survival (Jope, 1946). Beutler also pointed out that some of the substances producing marked haemolysis in susceptible individuals caused little or no methaemoglobin formation, one such substance being phenylhydrazine. As a third point against a relationship between the formation of methaemoglobin and haemolysis, Beutler mentioned the unpublished observations of Dern, Beutler and Alving that methaemoglobinaemia following the administration of primaquine was more marked in normal persons than in

sensitive subjects.

However, there is other evidence which suggests that the oxidative degradation of haemoglobin may be intimately linked with the mechanism of haemolysis. Thus haemolysis induced in sensitive individuals by primaquine and most of the other substances is preceded by the appearance of Heinz bodies in the erythrocytes, and Jandl and Allen (1960) have shown that Heinz bodies are formed from oxidatively denatured haemoglobin. During this process haemoglobin is converted first to methaemoglobin and then to sulphaemoglobin. Furthermore, the enzymatic reduction of methaemoglobin has been shown to be defective in sensitive cells (Ross and Desforges, 1959). In addition, the deficiency of reduced glutathione (Beutler et al., 1955) and of catalase (Tarlov and Kellermeyer, 1959) are both thought to weaken the defences of primaquine sensitive cells against oxidative damage. Finally, most if not all of the substances provoking haemolysis in primaquine sensitive individuals do cause methaemoglobinaemia as well. It seems reasonable to suppose, therefore, that there may be a connection between

the methaemoglobin-producing action of this group of substances and their haemolytic action.

It was thus thought to be of interest to study methaemoglobin formation in vitro in primaquine sensitive cells, in order to compare the rate of formation with that in normal blood. In addition, it was decided to determine whether enzymatic methaemoglobin reduction in vitro is inhibited by primaquine, in either sensitive or normal erythrocytes.

A. FORMATION OF METHAEMOGLOBIN IN VITRO

METHODS

I. Estimation of Methaemoglobin and Total Haemoglobin

The method of Evelyn and Malloy (1939) was used.

Solutions

1. 0.067 M Phosphate Buffer pH 6.6

Mix 37.5 ml. 0.067 M Na_2HPO_4 with 62.5 ml. 0.067 M KH_2PO_4.
This solution keeps well.

2. 0.0168 M Phosphate Buffer. Dilute 1 part 0.067 M Buffer with 3 parts distilled water. Must be freshly

made up every day.

3. Neutral sodium cyanide. Mix equal volumes of 10% NaCN and 12% acetic acid; this should be carried out in the fume cupboard in an icebath because of the danger of poisoning by HCN gas. This reagent is made up every day. The NaCN and acetic acid solutions are kept in amber bottles in the refrigerator.

4. 10% NaCN solution.

5. Concentrated NH_4OH solution.

6. 20% potassium ferricyanide solution.

METHOD

Add 0.1 ml. oxalated blood to 10 ml. 0.0168 M buffer in an Evelyn tube. After standing for 5 minutes read in an Evelyn colorimeter against a water blank, using the 636 filter. This reading is L_1.

Add one drop neutral cyanide solution. After 2 minutes read again using the same setting. This reading is L_2.

Add one drop of concentrated NH_4OH solution. Read against a water blank with the 620 filter. This reading is L_3.

To 2 ml. of the above mixture add 3 ml. 0.067 M buffer. Add one drop 20% potassium ferricyanide. After 2 minutes add one drop 10% NaCN solution. After standing for a further 2 minutes read using the 540 filter,

against a blank of distilled water to which a drop of both the potassium ferricyanide and the NaCN solutions have been added. This reading is designated L_4.

Calculations:

Total Haemoglobin = $\frac{100 \times L_4}{2.38}$ Gm.%

Methaemoglobin = $\frac{100 (L_1 - L_2)}{2.77}$ Gm.%

Sulphaemoglobin = $(100 \times L_3) - (8.5 \times \text{methaemoglobin} + 4.4 \times \text{total haemoglobin})$ Gm.%

II Incubation of Cells

Primaquine sensitive subjects were identified by means of the Motulsky screening test for glucose-6-phosphate dehydrogenase deficiency. Heparinized venous blood was used, experiments being started within 30 minutes of collection of the blood samples. 1 ml. samples of blood were incubated in test tubes 9 x 1.5 cm. covered with Parafilm. 0.1 ml. of a 5% glucose solution was added to each tube. Acetylphenylhydrazine was added in solid form and allowed to dissolve, as was nickel sulphate and

sodium fluoride in the different experiments, but brilliant cresyl blue (BCB) was added as a solution in normal saline. Before incubation at 37° C. was commenced the tubes were inverted 20 times in order to mix and oxygenate the contents. 0.1 ml. samples were taken at intervals for estimation of methaemoglobin and total haemoglobin.

III. Establishment of a suitable test system

In order to develop a suitable in vitro system with which to assess the rates of methaemoglobin formation in normals and primaquine sensitive subjects, certain preliminary experiments were performed.

It was found that the addition of BCB to produce final concentrations of dye of 0.25 µM/ml. or more resulted in the formation of significant amounts of methaemoglobin. However, the shape of the graphs obtained suggested that an equilibrium between the rates of formation and reduction of methaemoglobin rapidly resulted, with stabilization of the concentration of methaemoglobin even though incubation was continued for several hours (Fig. 9).

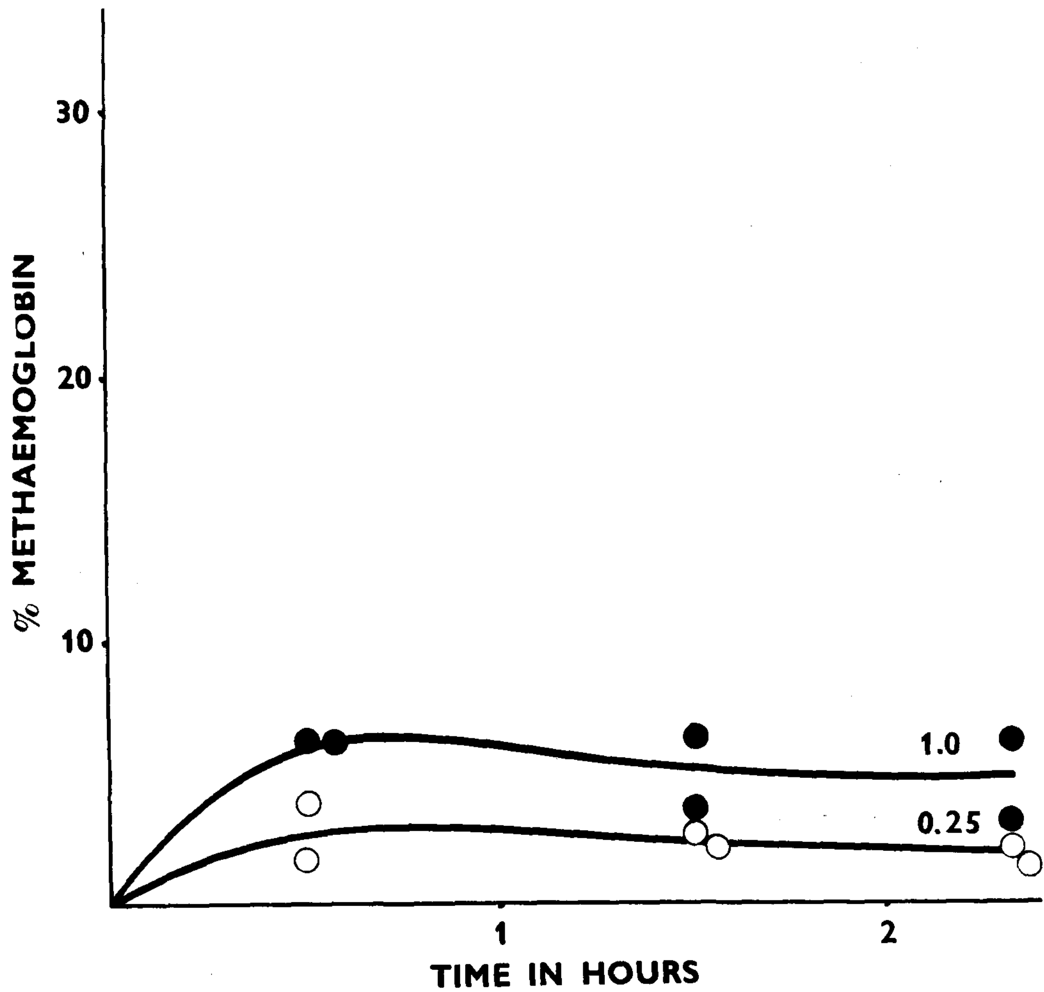


Fig. 9. Methaemoglobin formation in normal erythrocytes incubated with BCB in concentrations of 0.25 and 1.0 μM/ml. respectively.

Since it was felt that a system in which there was a steady accumulation of methaemoglobin would be more likely to reveal a difference between primaquine sensitive and normal cells, the effect of adding various concentrations of certain enzyme inhibitors was next examined. Firstly, increasing concentrations of sodium fluoride were used (Fig. 10). As shown in the graph, the addition of increasing concentrations of this substance resulted in the accumulation of increasing quantities of methaemoglobin, even though the concentration of BCB was not changed. Sodium fluoride in the absence of dye did not produce any methaemoglobin. A similar effect was observed when increasing concentrations of nickel sulphate were used (Fig. 11). However, the addition of sodium azide in a concentration of 50 µM/ml. to the system did not cause a greater accumulation of methaemoglobin than the dye alone.

As a result of these experiments a system containing 0.5 µM/ml. BCB and 20 µM/ml. nickel sulphate was selected for the comparison between normal and primaquine sensitive cells.

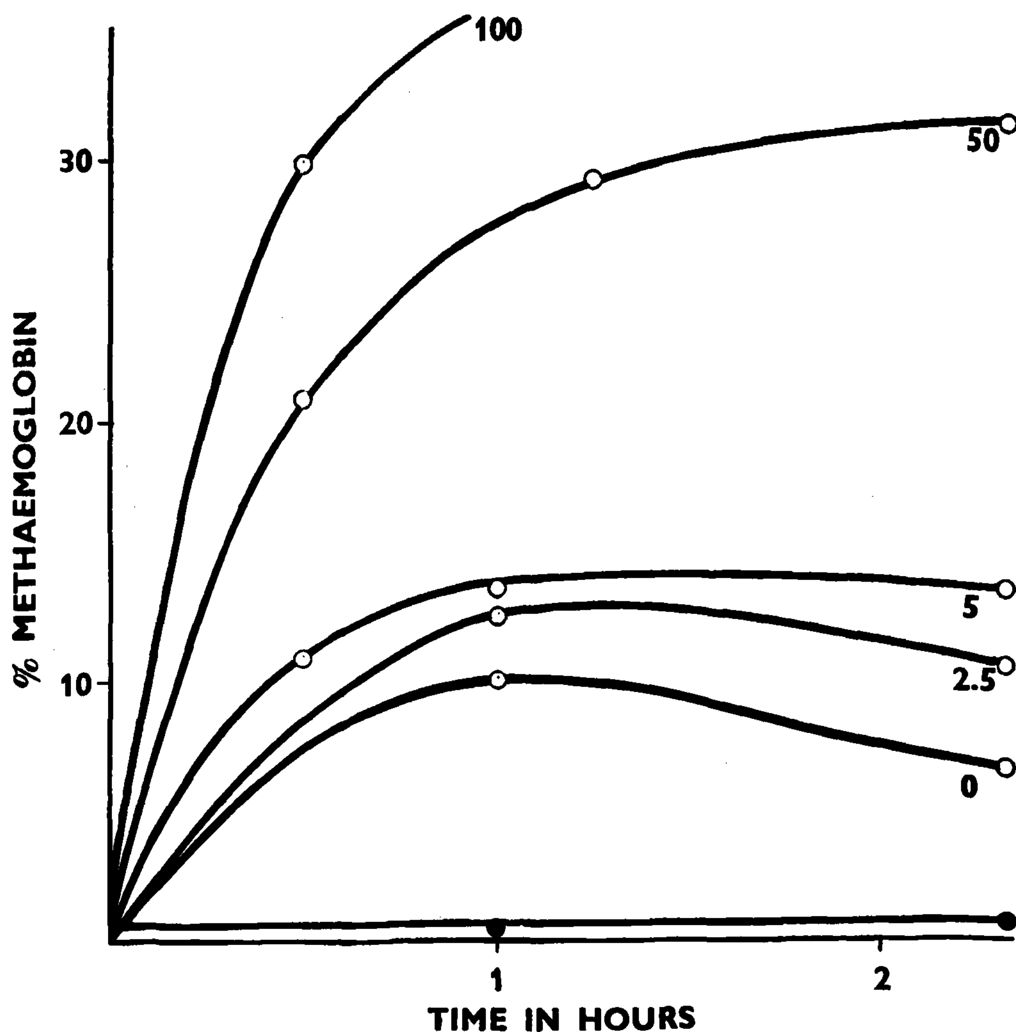


Fig. 10. The effect of various concentrations of NaF upon methaemoglobin formation in normal erythrocytes incubated with BCB in a concentration of 1.0 µM/ml. The figures indicate the concentration of NaF in µM/ml. The closed circles represent the effect of NaF in a concentration of 50 µM/ml. without added BCB.

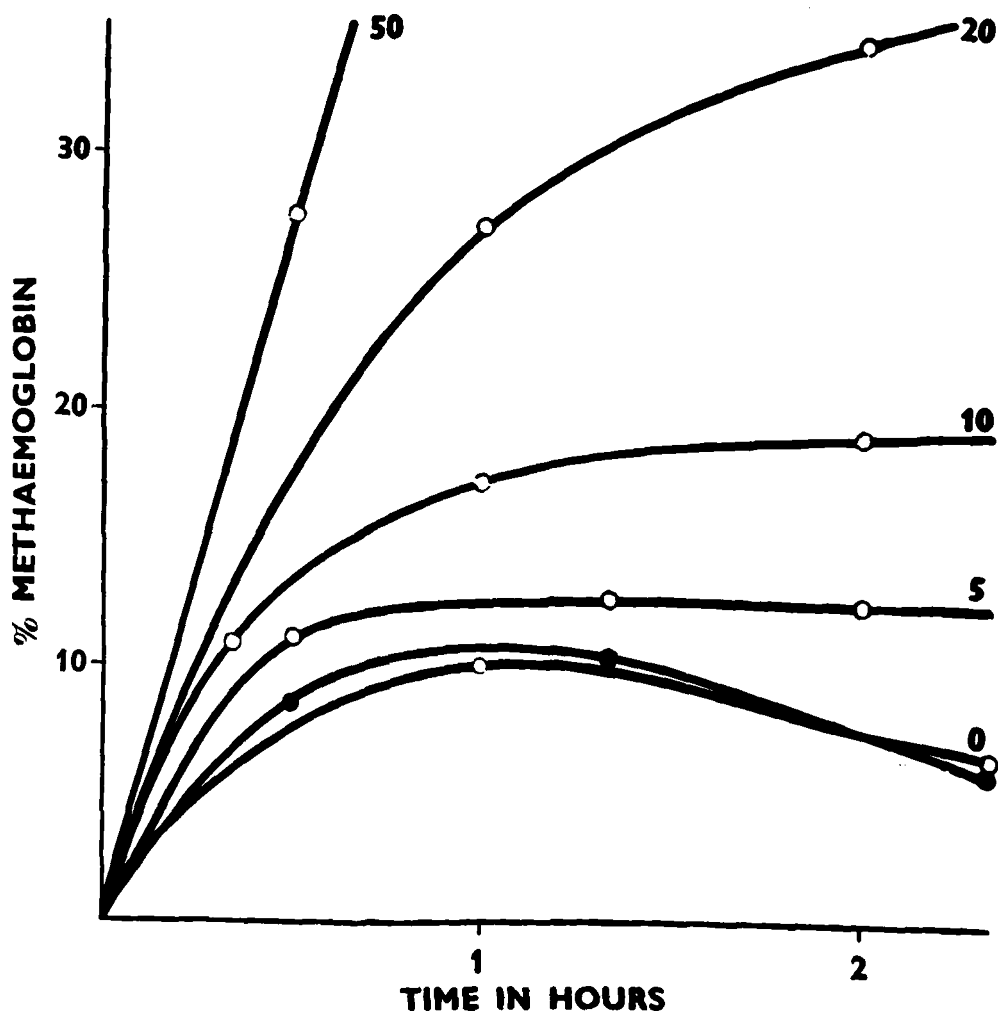


Fig. 11. The effect of increasing concentrations of $NiSO_4$ upon the formation of methaemoglobin in normal erythrocytes incubated with BCB 1.0 μM/ml. The figures indicate the concentration of $NiSO_4$ in μM/ml. No methaemoglobin accumulated when the cells were incubated with $NiSO_4$ alone. The closed circles represent the effect of sodium azide in a concentration of 50 μM/ml. in the presence of BCB 1.0 μM/ml.

After comparing the performance of normal and deficient cells in this system it was decided to assess the rate of methaemoglobin formation in a second system as well, and acetylphenylhydrazine was chosen as the methaemoglobin-producing substance. Since obvious methaemoglobin formation occurs during incubation of blood with this compound in the glutathione stability test (Chapter 5), the same concentration of acetylphenylhydrazine (33 μM/ml.) was tried and was found to be suitable for present purposes.

RESULTS

As shown in the graph (Fig. 12) it was found that greater concentrations of methaemoglobin accumulated in primaquine sensitive cells than in normal cells when compared in the BCB/$NiSO_4$ test system. The mean methaemoglobin concentration after 60 minutes was 30% in the enzyme deficient blood and 21% in normal blood.

In the acetylphenylhydrazine system similar results were obtained (Fig. 13), the mean methaemoglobin concentration after 60 minutes being 28% in sensitive blood and 21% in normal blood.

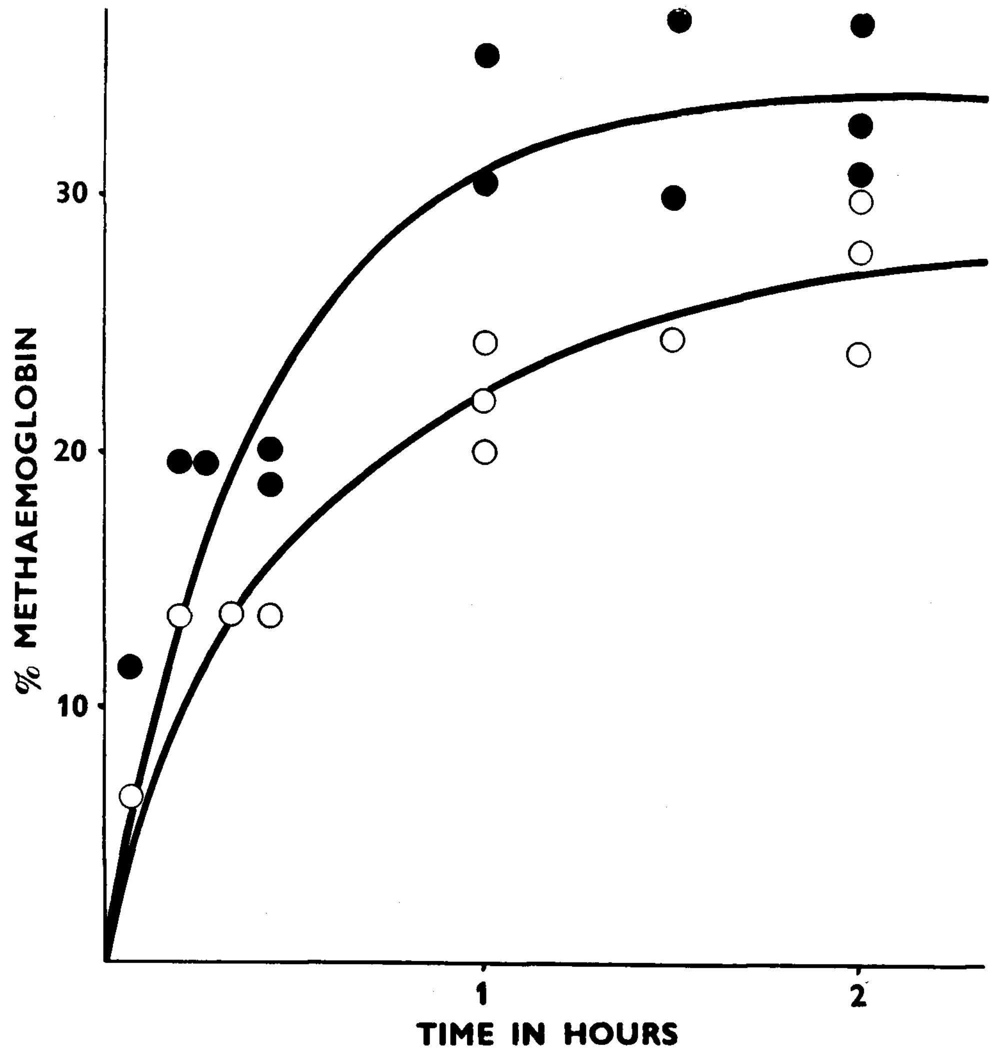


Fig. 12. A comparison between methaemoglobin formation in normal (open circles) and primaquine sensitive (closed circles) erythrocytes incubated in vitro. The test system contained BCB and $NiSO_4$ in concentrations of 0.5 µM/ml. and 20 µM/ml. respectively.

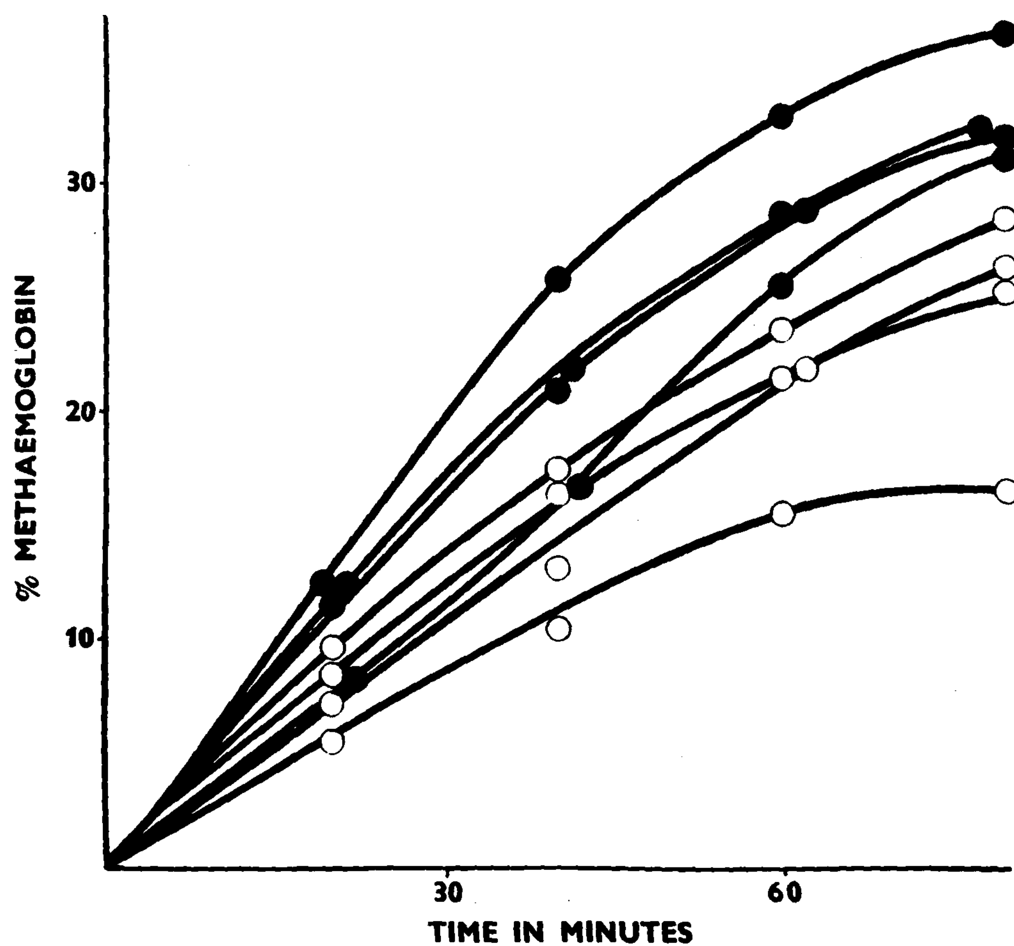


Fig. 13. A comparison between methaemoglobin formation in normal (open circles) and primaquine sensitive (closed circles) erythrocytes incubated in vitro with acetylphenylhydrazine in a concentration of 33 µM/ml.

In these two in vitro systems, then, approximately 38% more methaemoglobin accumulates in primaquine sensitive erythrocytes than in normal cells.

DISCUSSION

Small amounts of methaemoglobin are demonstrable in normal blood (van Slyke et al., 1946). It is thought that haemoglobin is continually being oxidised to methaemoglobin, and that the erythrocyte enzymatic reduction systems in their turn convert the methaemoglobin to haemoglobin again. The concentration of methaemoglobin in the blood thus represents the resultant of these opposing reactions, and hence is related both to the rate of formation of methaemoglobin and to the rate of its reduction.

Under conditions in which the stimulus to methaemoglobin formation is identical, therefore, it is to be anticipated that in cells partly deficient in their ability to reduce methaemoglobin, greater concentrations of the pigment will accumulate. Of the two known enzymatic reduction systems (Heunnekens et al., 1957) it has been established that the reaction linked to TPNH is deficient in primaquine-sensitive cells (Ross and Desforges, 1959). It was thus to be expected that these cells would be more susceptible to the action of methaemo-

globin-producing agents than normal, even though it is thought that of the two enzymes the DPNH-linked one is the more important. Thus it is the DPNH-linked reduction which is deficient in cases of congenital methaemoglobinaemia, and these patients are cyanosed in spite of an intact TPNH-linked system, Gibson, 1948; Scott and Hoskins, 1958; Jaffe, 1959). On the other hand, primaquine-sensitive individuals who are deficient in the TPNH-linked system, are not cyanosed. Indeed, even less methaemoglobinaemia is observed in G6PD deficient subjects exposed to primaquine than in normals (Earle et al., 1948; Hockwald et al., 1952; Beutler, 1959). This anomaly has been explained only very recently. Brewer et al. (1960) confirmed that primaquine does not produce more methaemoglobinaemia in sensitive persons, but pointed out that the drug haemolyses the older members of the red cell population in these individuals, whereas no cells are destroyed in normal persons. If these older cells contain more methaemoglobin than the younger cells, as claimed by Brewer et al., their disappearance from the circulation lowers the overall percentage of methaemoglobin in the blood. Jalavisto

and Solantera (1959) have shown that older cells have less capacity to reduce methaemoglobin than younger cells, although Beutler and Mikus (1961) were unable to demonstrate a higher methaemoglobin content in older cells fractionated by differential osmotic haemolysis. However, the observation of Brewer et al. (1960) that the administration of nitrite, instead of primaquine, does cause more methaemoglobinaemia in enzyme deficient subjects than in normal persons supports their contention strongly, since nitrite does not cause haemolysis.

The relationship between methaemoglobin formation and haemolysis has become clearer recently. Beutler and Mikus (1961) have confirmed that in rats administration of nitrite does not shorten red cell survival, even though considerable methaemoglobinaemia occurs. However, a second methaemoglobin-producing agent, para-aminoproprio-phenone (PAPP) does accelerate red cell destruction. Nitrite produces methaemoglobin by a stoichiometric chemical reaction, whereas PAPP mediates the oxidation of a far greater quantity of haemoglobin molecules in a catalytic manner. The agents producing haemolysis in primaquine-sensitive individuals, or their metabolic

products, mediate oxidation in the second way (Emerson et al., 1949). This suggests strongly that their haemolytic effect is related to this oxidant action. In addition, many of the known metabolic consequences of the biochemical defects in the primaquine sensitive erythrocytes appear to be related to mechanisms defending the cell against oxidative damage. These include the deficiency of catalase (Tarlov and Kellermeyer, 1959), the deficiency of reduced glutathione (Beutler et al., 1955) and the defective methaemoglobin reduction (Ross and Desforges, 1959). It seems highly probable, therefore, that haemolysis is the consequence of oxidative damage to a cell in which the defence against oxidation is deficient. On the other hand, the presence of considerable concentrations of methaemoglobin or even sulphaemoglobin does not of itself shorten red cell survival (Jope, 1946; Clark and Morrissey, 1951). It follows, then, that if haemolysis is the result of oxidation of the haemoglobin, the process must proceed beyond the sulphaemoglobin stage. It seems more probable, however, that oxidative damage to other cellular components, such as enzymes or the cell membrane itself, produces the destruction of the cell.

This view has recently been championed by Jacob and Jandl (1962) and by Scheuch et al. (1961).

The actions of sodium fluoride and nickel sulphate in enhancing methaemoglobin accumulation in the test systems are of interest. Fluoride was originally selected as an inhibitor of glycolysis, through its inhibition of enolase. However, it also inhibits adenosine triphosphatase (ATPase). Since nickel sulphate has been reported to inhibit ATPase, but not enolase (Clark and Hubscher, 1961), it was substituted for the fluoride in order to discover which inhibition was causing the accumulation of methaemoglobin. Since methaemoglobin formation was quite as striking with nickel sulphate as with fluoride, it seems that inhibition of ATPase is the critical action of both substances, provided that inhibition of an enzyme is responsible for the methaemoglobin-promoting effect. It has been shown that neither substance alone caused methaemoglobin formation (Fig.10 and Fig.11), so that a direct chemical action on haemoglobin is excluded. However, the possibility remains that the chemicals interact with

the brilliant cresyl blue to produce a more actively methaemoglobin-producing substance. This possibility was not examined.

B. THE EFFECT OF PRIMAQUINE ON THE RATE OF ENZYMATIC METHAEMOGLOBIN REDUCTION IN NORMAL AND IN SENSITIVE CELLS

MATERIALS and METHODS

The methaemoglobin reduction test for primaquine sensitivity as described by Brewer, Tarlov and Alving (1960) was used for this study.

MATERIALS

1. Acid-citrate-dextrose solution (ACD). 2.48% glucose, 0.8% citric acid and 2.2% trisodium citrate.

2. 0.18 M sodium nitrite solution (1.25%). Keep in a tightly-stoppered amber bottle.

3. 0.0004 M methylene blue chloride solution. Dissolve 149.5 mg. trihydrated methylene blue chloride (Mallinckrodt; M.W. 373-92) in 100 ml. distilled water. Dilute 10 ml. of this solution with normal saline to make a final volume of 100 ml. Make up fresh each month.

4. Solutions for determination of methaemoglobin and total haemoglobin.

METHOD

Venous blood was collected in ACD solution (0.15 ml./ml. blood) and the test started within 30 minutes of

collection.

0.1 ml. of the 0.18 M sodium nitrite solution are added to 2.0 ml. blood in a test tube. When the TPN-linked reductase was to be studied this was followed by 0.1 ml. of the 0.0004 M methylene blue solution. The solutions were mixed by inversion 12 times and placed in a water-bath at 37° C. A 0.1 ml. pipette was left in each tube. At 60 and 120 minutes after the start of the incubation the mixture was agitated by stirring with the pipette, and one breath of air was gently blown through the pipette. This aeration is critical and must be exactly observed. 0.1 ml. samples were removed at intervals and total haemoglobin and methaemoglobin concentrations measured as described above.

RESULTS

The rate of methaemoglobin reduction in normal and in sensitive cells in the absence of primaquine was first studied. It was found, as reported by Ross and Desforges (1959), that whereas methylene blue greatly accelerated the rate of methaemoglobin reduction in normal cells, there was little or no increase in rate of reconversion to oxyhaemoglobin in the case of sensitive cells. The rates of reduction are plotted on the graph (Fig. 14).

The effect of the addition of primaquine 30 micrograms/ml. to the incubation mixtures was then

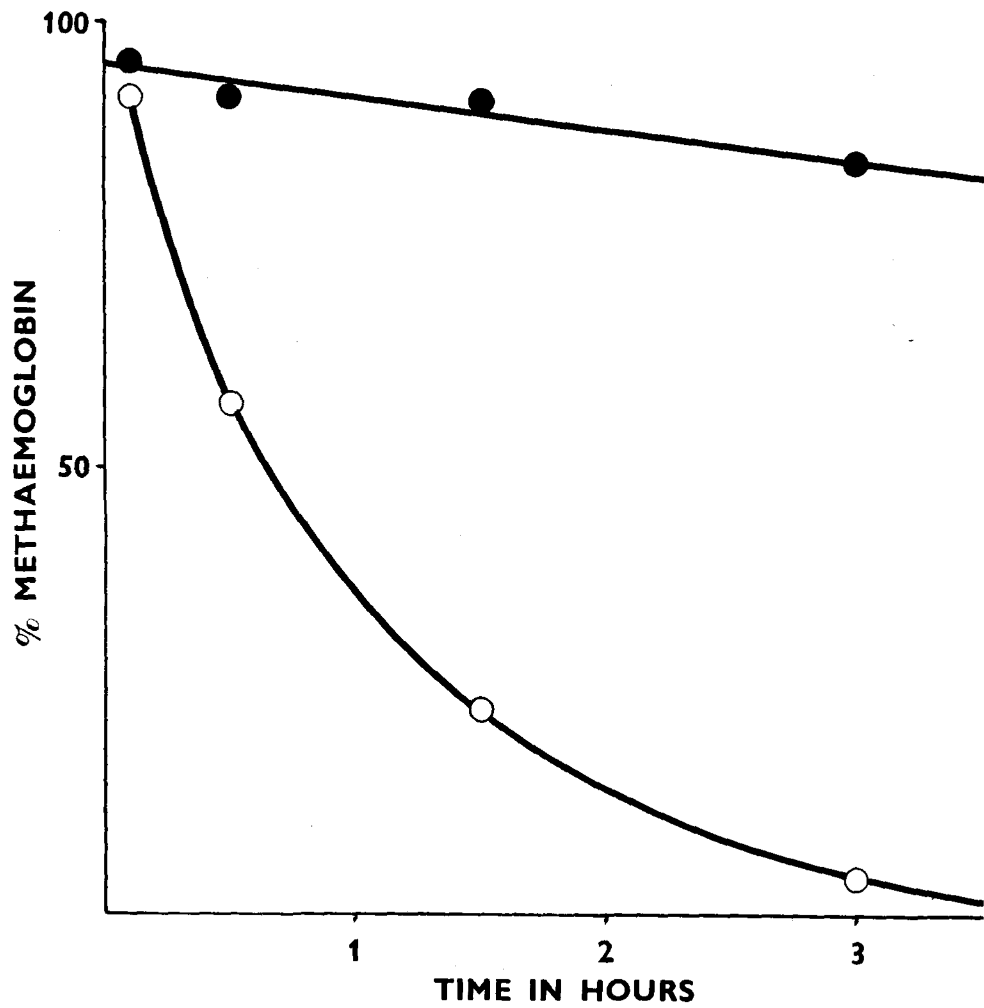


Fig. 14. Methaemoglobin reduction in normal (open circles) and primaquine sensitive (closed circles) erythrocytes incubated with methylene blue.

studied. It was found that there was no significant effect on the rate of reduction over 3 hours whether methylene blue was present or not. This was true of both normal (Fig. 15) and sensitive (Fig. 16) cells.

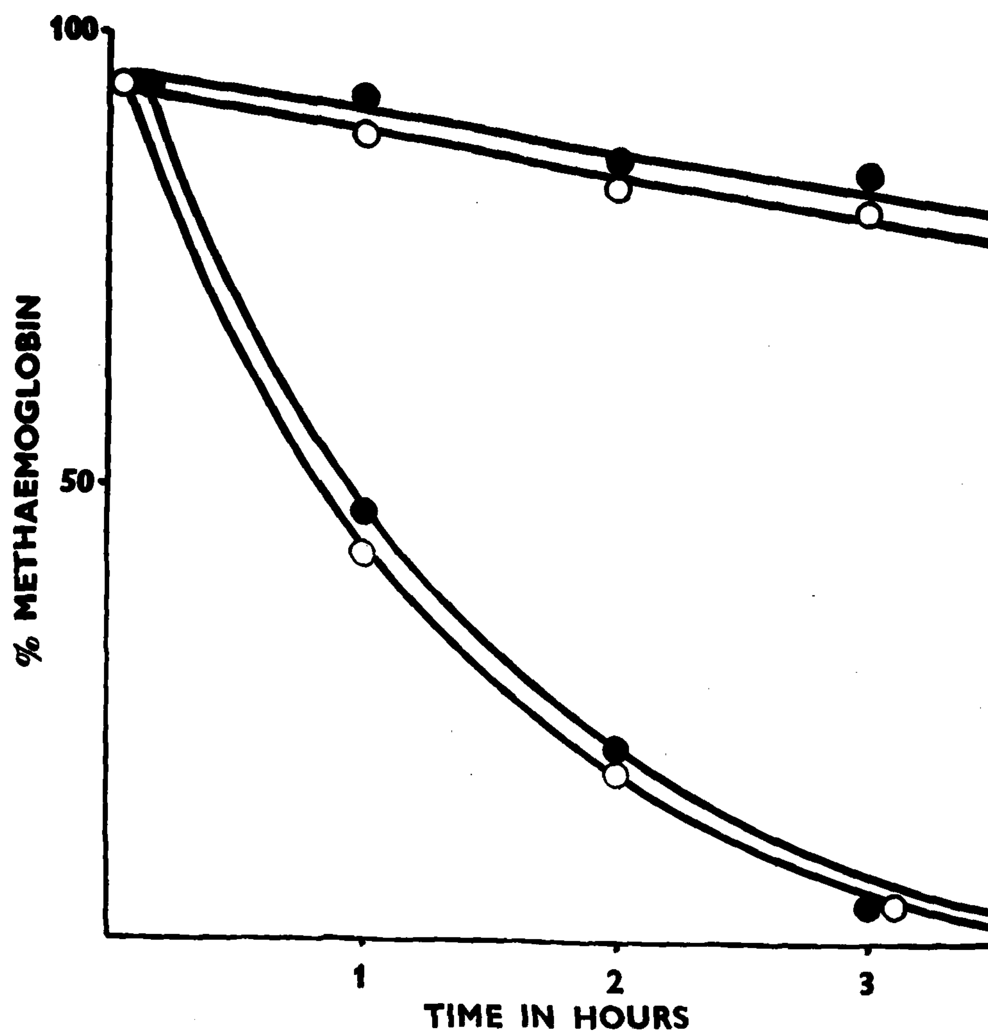


Fig. 15. Methaemoglobin reduction in normal cells with and without the addition of methylene blue. The presence of primaquine in a concentration of 30 micrograms/ml. (closed circles) does not affect methaemoglobin reduction.

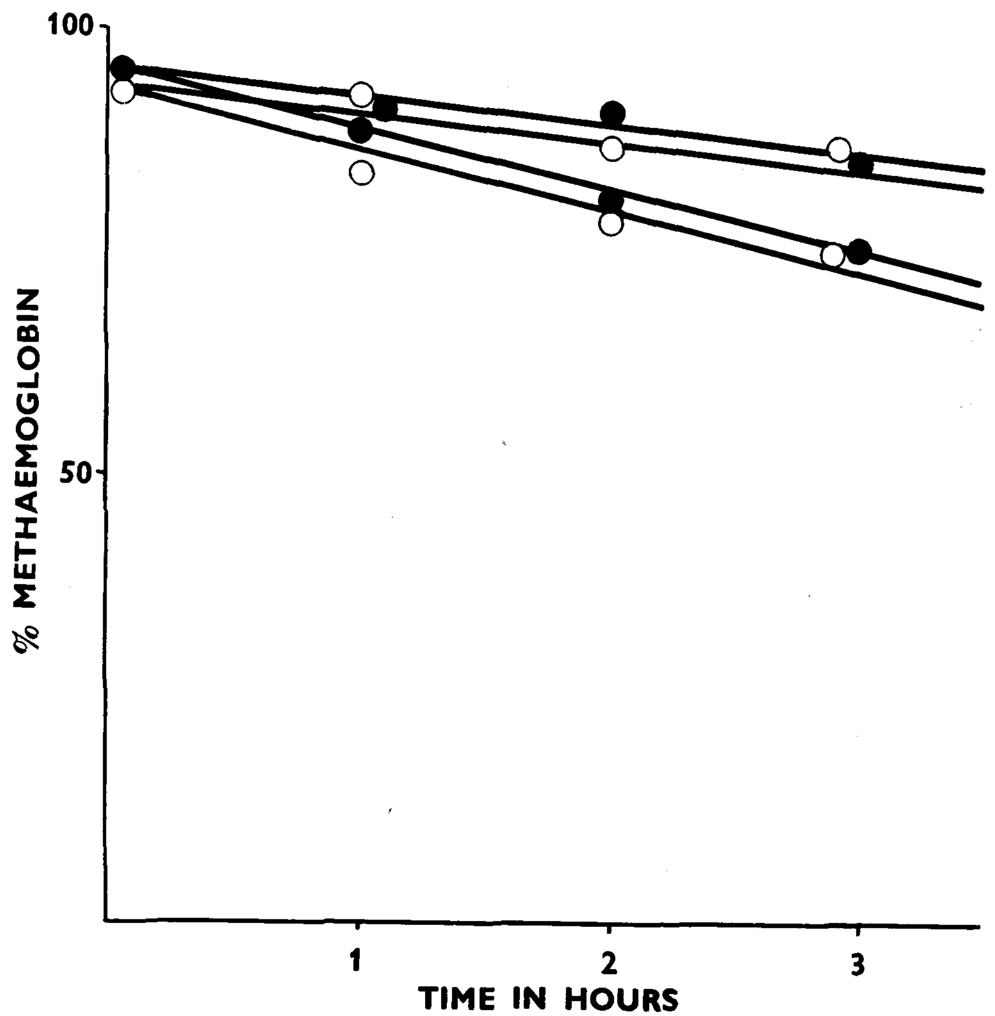


Fig. 16. Methaemoglobin reduction in primaquine sensitive cells with and without the addition of methylene blue. The presence of primaquine in a concentration of 30 micrograms/ml. (closed circles) does not affect methaemoglobin reduction.

DISCUSSION

Even though no inhibition by primaquine of methaemoglobin reduction has been demonstrated by these studies, it remains possible that a product of the metabolism of primaquine by the body might have such an effect. Thus, it has been shown that metabolic intermediates of pamaquine in chickens have a marked methaemoglobin-producing action in vitro (Josephson et al. 1951). In addition, Brewer et al. (1962) have reported that an 'anticatalase factor' can be demonstrated in the plasma of both normal and sensitive individuals 4 - 6 hours after the administration of primaquine. It is probable that this is a metabolite of the drug, since the anticatalase activity is not related to the concentration of undegraded primaquine in the plasma; it is possibly an oxidant quinone (Brewer et al., 1962). Furthermore, in studies in vitro Carson (1960) has enhanced the oxidant effect of primaquine by exposing the drug to ultraviolet light. The possibility that a metabolite producing inhibition of methaemoglobin reduction may be formed in vivo, therefore, cannot be excluded. However, there is

no particular reason to suspect that this does happen. The methaemoglobinaemia commonly associated with administration of primaquine, to normal subjects as well as to sensitive individuals, is easily explained as the consequence of the oxidant action of the drug or its metabolic products on oxyhaemoglobin. Indeed, there is evidence that a degradation product of primaquine, far from inhibiting the enzyme, actually accelerates the rate of reduction of methaemoglobin in normal red cells in vitro (Brewer et al., 1960). In primaquine sensitive erythrocytes, however, only a very slight effect was noticed. This derivative of primaquine caused a marked fall in the concentration of GSH when incubated with deficient cells. Its action thus resembles that of methylene blue and brilliant cresyl blue very closely, since the dyes may both cause the accumulation of methaemoglobin and accelerate its reduction. However, no comparable effect of primaquine itself was noted in the present study.

C H A P T E R   7

The Effect of Primaquine Upon Glucose-6-Phosphate Dehydrogenase Activity in Vitro

In 1960 it was reported that primaquine and some of the other haemolytic substances could be demonstrated to inhibit the action of glucose-6-phosphate dehydrogenase in vitro (Desforges, Kalaw and Gilchrist, 1960). From the shape of the graphs obtained it was deduced that the inhibition was competitive in some instances and non-competitive in others. These observations seemed to be of such importance with reference to the mechanism of haemolysis in primaquine sensitivity that it was thought necessary to attempt to repeat them, particularly since certain experiments reported in Chapter 8 appeared to indicate that the action of primaquine was to stimulate rather than to inhibit the hexose monophosphate shunt pathway in red cells.

MATERIALS AND METHOD

A modification of the method of Marks (1958) was used.

Materials

1. 0.1 M $MgCl_2$. Kept at room temperature
2. 0.25 M Glycyl-glycine buffer pH 7.6. Store at 4° C.

3. 0.85% KCl. Adjust pH to 7.4 with KOH

4. 2.3×10^{-3} M Triphosphopyridine nucleotide (TPN) i.e. 2 mgm. in 1 ml. Made up each day and kept at 4° C.

5. 0.125 M sodium glucose-6-phosphate (G6P). Made up each day.

Preparation of Haemolysate. Blood from both normal and primaquine sensitive subjects was used. Approximately 8 ml. heparinized venous blood in a 1 cm. tube is placed immediately after collection in ice. It is then centrifuged for 10 minutes at 1500 × g at 0° C., and the plasma and Buffy layer removed. The remaining red cells are washed with the isotonic KCl solution at 0° C., re-centrifuged, and the uppermost layer of red cells removed with the washings. This washing is repeated, after which the cells are diluted to 3 times their volume with the cold KCl solution. A haematocrit (Wintrobe, 1956) is performed at this stage (it is approximately 30%). The erythrocytes are then haemolysed by freezing and thawing twice, immersing the tube in a dry ice/acetone mixture. The solution must not be shaken during the freezing and thawing procedure. The haemolysate is kept frozen until used.

METHOD

A Quartz spectrophotometer cuvette is then filled with the following ingredients in the order indicated:-

0.5 ml. 0.1 M $MgCl_2$ solution

0.6 ml. 0.25 M glycyl-glycine buffer

0.1 ml. 2.3×10^{-3} M TPN solution

0.53 ml. distilled water

0.02 ml. haemolysate

The cuvette is then placed in a water bath at 25° C., together with the 0.125 M G6P solution in a tube. After 10 minutes the cuvette is removed and dried, and the reaction started by the addition of 0.25 ml. G6P solution. The development of optical density at 340 mμ is measured in the spectrophotometer against a blank cuvette which contains all the ingredients except the TPN. Readings are taken every minute. If the laboratory temperature is more than one or two degrees away from 25° C. the cuvettes should be returned to the water bath after 3 readings have been taken. After a few minutes in the bath a further set of 3 readings may be made. From these readings a graph can be constructed and the activity of the enzyme expressed as Δ O.D./minute/ml. erythrocytes.

Primaquine was dissolved in the buffer solution and the pH re-adjusted to 7.6. Both primaquine base and primaquine diphosphate were used. The effect of concentrations varying from 0.0001 M to 0.001 M was noted. In one experiment the solution containing primaquine was also incubated with the haemolysate for 30 minutes before the other ingredients were added, as recommended by Desforges et al. (1960).

RESULTS

No inhibitory effect of primaquine could be demonstrated upon glucose-6-phosphate dehydrogenase activity in haemolysates of normal or primaquine sensitive erythrocytes. Neither primaquine base nor primaquine diphosphate in concentration of 0.0001 M - 0.001 M had any observable effect even when incubated for 30 minutes with the haemolysate prior to starting the experiment. Only if the pH of the buffer was not brought back to

7.6 was some inhibition noted.

The change in optical density observed is indicated in Fig. 17

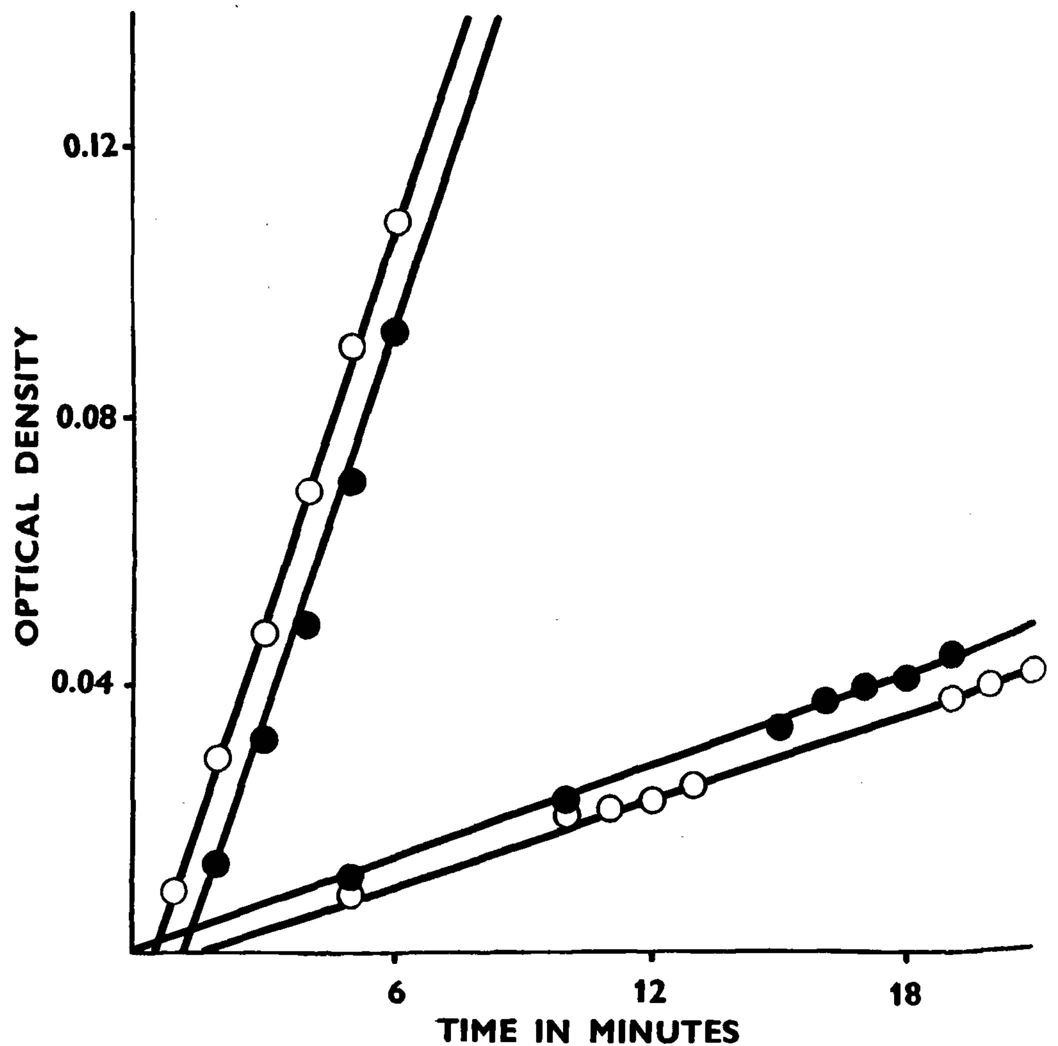


Fig. 17. Glucose-6-phosphate dehydrogenase activity in haemolysates of normal and primaquine sensitive erythrocytes by the method of Marks (1958). The haematocrits before haemolysis were 22% and 21% respectively. Optical density at 340 mμ develops much more rapidly in the normal haemolysate (Δ O.D./minute/ml. erythrocytes = 4.50) than in the sensitive haemolysate (Δ O.D. /minute/ml. erythrocytes = 0.54). The addition of primaquine in concentrations varying from 0.0001 M - 0.001 M did not inhibit activity of the enzyme (closed circles).

DISCUSSION

At the present time it is thought that primaquine and the other haemolytic compounds stimulate the metabolism of glucose via the shunt pathway (Szeinberg and Marks, 1961). Some experimental observations in support of this concept are reported in Chapter 8. It was difficult to reconcile a stimulation of the shunt pathway with inhibition of the first enzyme on this pathway, as reported by Desforges, Kalaw and Gilchrist (1960). It was therefore not entirely unexpected that the observations of these workers were not confirmed, at any rate with respect to primaquine.

It is difficult to explain the results obtained by Desforges et al. In the present study it was found that the enzyme was inhibited only if the pH was incorrect. Since the addition of primaquine to the buffer markedly changes the pH, it seems possible that the inhibition reported by Desforges et al. was due to the pH effect, not to the primaquine, as these workers do not state that the pH of the buffer was restored to normal after addition of the primaquine in their experiments.

CHAPTER 8

Metabolism of Glucose and Pyruvic Acid In Vitro in Primaquine Sensitive Erythrocytes

It is well known that dyes such as methylene blue and brilliant cresyl blue (BCB) stimulate the utilisation of glucose and the consumption of oxygen by erythrocytes in vitro (Harrop and Barron, 1928), and that pyruvic acid accumulates under these circumstances. It was to be anticipated that cells in which the oxidative metabolism of glucose is defective because of deficiency of an enzyme of the oxidative pathway would behave differently under the influence of these dyes, and it was therefore thought to be of interest to characterize this behaviour. It was thus decided to compare glucose utilisation and the accumulation of pyruvic acid in normal and in sensitive cells incubated with BCB.

MATERIALS AND METHODS

A. Preparation of red cell suspensions. Fasting venous blood from normal subjects and from primaquine sensitive individuals was defibrinated. It was centrifuged for 15 minutes at 800Xg and the serum and buffy layer aspirated. The remaining cells were washed with normal saline, recentrifuged, and the uppermost cells again aspirated. The erythrocytes were then suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 µM/ml. glucose, to produce a haematocrit of 30 - 35%. BCB dissolved in the phosphate buffer was added to the appropriate tubes to produce a final concentration of 0.25 µM/ml., an equivalent volume of buffer being added

to the control tubes. Primaquine dissolved in buffer, the pH being restored to its original value, was added to the appropriate tubes to produce a final concentration of 30 micrograms/ml. The white cell count of suspensions prepared in this way was less than 0.1% of the original count.

Incubation. 3 ml. of the cell suspension was placed in 80 x 15 mm. tubes which were sealed with stoppers covered with Parafilm. The sealed tubes were attached to a vertical wheel 20 cm. in diameter rotating at 20 r.p.m. inside an incubator at 37° C. After an initial 15 minute equilibration period, samples were removed for the estimation of glucose, pyruvic acid and haematocrit. Incubation was then continued for 3 hours, when further samples were taken.

B. Determination of glucose. Glucose was estimated by the glucose oxidase method as described by Salomon and Johnson (1959) using the Somogyi (1945) deproteinisation procedure.

Solutions

1. Acetate buffer. Mix 1 part 2 M sodium acetate solution with 2.5 parts 2 M acetic acid to give a solution pH 4.1 - 4.2.

2. Composite Reagent. Add 700 mg. O-toluidine hydrochloride dissolved in a few ml. water to 150 ml. acetate buffer. Dissolve 2 mgm. crude glucose oxidase and 15 mgm. horseradish peroxidase in a small quantity of the buffer solution and add to the mixture. Make up to 300 ml. with buffer and filter. Store at 4° C. in a dark bottle. The reagent can be used for 4 weeks. If turbidity develops the solution should be filtered again. A reagent blank prepared with fresh composite reagent should have an absorption at 635 mμ of less than 0.01.

3. Deproteinisation solutions. 5% $ZnSO_4 7H_2O$ and 0.3N Ba $(OH)_2$. The concentration of these solutions must be adjusted so that the barium hydroxide exactly neutralises the zinc sulphate, volume for volume. This is assessed by titration with phenolphthalein as indicator. 10 ml. $ZnSO_4$ solution is placed in a conical flask and diluted with 100 ml. distilled water. The BA $(OH)_2$ solution is run in drop by drop with continued agitation until a pink colour persists for 1 minute.

Procedure. Add 0.4 ml. of the erythrocyte suspension to 3.6 ml. water. Then add 2 ml. $ZnSO_4$ solution followed by 2 ml. Ba $(OH)_2$ solution, the mixture being vigorously shaken after each addition. Centrifuge briefly at high speed. To 3 ml. distilled water in an Evelyn tube add 1 ml. supernatant and 3 ml. composite reagent. Allow to stand for 1 hour at 25° C. and measure optical density at 635 mμ against a reagent blank containing distilled water instead of supernatant. Standard solutions of glucose are also subjected to the procedure as a control. The concentration of glucose is proportional to the intensity of blue colour developed and may be read from a previously prepared standard curve.

C. Determination of pyruvic acid. Pyruvic acid was measured by the method of Friedeman and Haugen (1943).

Solutions

1. 15% trichloracetic acid.

2. 2,4 Dinitrophenylhydrazine (DNP) solution. Add 100 mgm. DNP to 85 ml. distilled water, followed by 15 ml. concentrated HCl. Shake for 30 minutes. A deposit settles out and the supernatant solution is used.

3. 10% Na_2CO_3 solution.

4. 1.5 N NaOH solution

Procedure. Mix 0.6 ml. red cell suspension with 0.5 ml. distilled water, and add 2 ml. 15% trichloracetic acid. Mix thoroughly, centrifuge and add 2 ml. of the supernatant to 0.4 ml. DNP solution. After standing for 5 minutes add 3 ml. benzene, stopper, and shake vigorously for 2 minutes. Centrifuge again and add 3 ml. 10% Na_2CO_3 solution to 2 ml. supernatant. After thorough mixing, centrifuge again. Aspirate and discard the supernatant. To 2.5 ml. of the lower layer add 3.5 ml. 1.5 N NaOH solution and mix. After 10 minutes measure the optical density at 520 mμ against a reagent blank containing 0.6 ml. distilled water instead of the cell suspension. 0.6 ml. of a standard solution containing 1 mgm. pyruvate/100 ml. is also subjected to the procedure.

Determination of Haematocrit. The haematocrit was determined by the method of Wintrobe (1956).

RESULTS

The alteration in glucose utilisation produced by BCB in this system in normal cells and in primaquine sensitive erythrocytes is shown in Fig. 18. In normal cells consumption of glucose was increased by a mean of 50% when BCB was present. In contrast, the effect of the dye in primaquine sensitive cells was to reduce the utilisation of glucose by a mean of 17%.

The effect of primaquine upon glucose utilisation of normal and deficient cells is shown in Table III. The action was similar to that of BCB, but less marked.

% Change in Glucose Consumption		Concentration of Pyruvic Acid	
Normal	Deficient	Normal	Deficient
+ 10.8	- 3.6	0.2	0.5
+ 21.4	- 15.4	0.3	0.0
+ 14.3	- 5.2	0.2	0.2
+ 11.0	- 5.0	0.0	1.7
+ 13.1	- 10.4	0.3	0.8
+ 17.1	- 6.3	0.6	0.4
+ 6.4	- 11.1	0.5	0.6
+ 13.3	- 3.2	1.0	0.9
+ 15.5	- 8.4	0.3	0.3
+ 20.9	- 9.0	0.2	0.6
+ 14.9	- 3.3	0.4	0.6

TABLE III

The effect of primaquine in a concentration of 30 micrograms/ml. on glucose utilisation and the accumulation of pyruvic acid in normal and enzyme deficient erythrocytes

Once again the difference between the behaviour of deficient and normal cells was noticed, primaquine increasing the glucose consumption of normal cells by an average of 14.9%, but decreasing utilisation in the sensitive cells, the mean decrease being 8.3%

The concentrations of pyruvic acid after incubation of normal and deficient cells in the BCB system are also indicated in Fig. 18 . In a proportion of cases there was a significantly greater concentration of pyruvate in primaquine sensitive cells than in normal erythrocytes.

The effect of primaquine on the accumulation of pyruvate is shown in the table. There was a small amount of pyruvic acid present after incubation of both normal and sensitive cells with primaquine, although much less than with BCB. No significant difference between normal and sensitive cells was found.

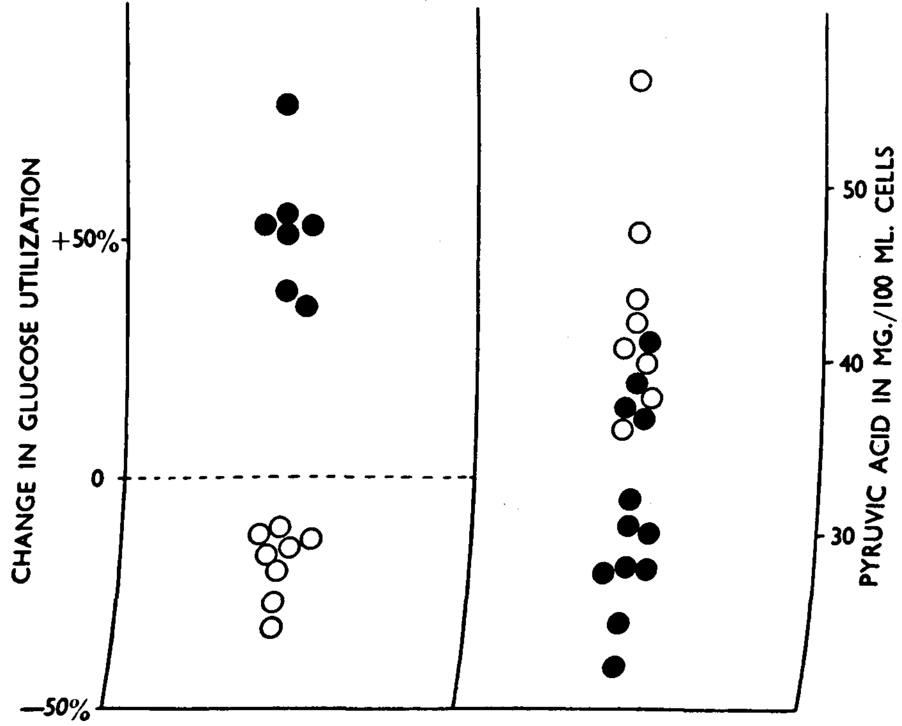


Fig. 18. The effect of BCB upon the consumption of glucose and the accumulation of pyruvic acid on incubation of normal (closed circles) and primaquine sensitive (open circles) erythrocytes in vitro.

DISCUSSION

The decrease in overall glucose consumption produced by BCB in enzyme-deficient erythrocytes was not anticipated. Since the shunt pathway is defective in primaquine sensitivity, it was expected that the effect of BCB would be to increase glucose utilisation only slightly or not all. However, the diminution which was observed was constant, and recently similar observations have been reported by Carson (1960).

The decrease in glucose consumption must be due to inhibition either of oxidative glucose metabolism or else of anaerobic glycolysis. The available evidence is against the first possibility. Firstly, Murphy (1957) has estimated that only 10 - 20% of the glucose metabolised by normal cells at pH 7.5 passes via the shunt pathway, and it is reasonable to suppose that the proportion is even smaller in cells in which the pathway is deficient. The degree of inhibition observed in the present study (averaging 17%) therefore appears to be too great to be accounted for by inhibition of oxidative glucose metabolism alone. Secondly, there is evidence that the shunt

pathway is active in primaquine sensitive cells incubated with a redox dye, methylene blue. Johnson and Marks (1958) studied oxygen consumption and formation of $^{14}CO_2$ from ^{14}C-1-glucose by sensitive cells incubated with the dye. In the absence of an intact tricarboxylic acid cycle in the mature red blood cell, it is generally accepted that the only source of $^{14}CO_2$ from glucose labelled with ^{14}C in the 1-position is the hexose monophosphate shunt, so that the recovery of $^{14}CO_2$ by Johnson and Marks in their study is evidence that the shunt does function under these conditions. These workers found oxygen consumption to be 35% and CO_2 formation 50% of normal.

It seems probable, therefore, that the overall diminution in glucose utilisation observed in the current study cannot be accounted for by inhibition of the shunt pathway by BCB. It must therefore be postulated that the dye produces an inhibition of anaerobic glycolysis. In support of this is the recent work of Murphy (1960) showing an inhibition of anaerobic glycolysis by methylene blue even in normal cells. The

stimulation of oxidative glucose metabolism in normal cells by the dye is well established (Harrop and Barron, 1928). The overall increase in consumption of glucose by normal cells must therefore result from the sum of these two opposing effects, i.e. the increased metabolism via the oxidative pathway must be greater than the degree of inhibition of anaerobic glycolysis. It is suggested, therefore, that in primaquine sensitive cells inhibition of the Embden-Meyerhof pathway of dye is greater than the stimulation of the defective shunt pathway, and that the net effect is to diminish the overall consumption of glucose.

The mechanism of the inhibition of anaerobic glycolysis by the dye is not established. However, it resembles the inhibition of the pathway produced by increasing concentrations of oxygen (Murphy, 1960).

In a proportion of cases greater concentrations of pyruvic acid accumulated when primaquine sensitive cells were incubated with BCB than when normal cells were used. Although the amount of pyruvic acid formed by cells from the same individual on different occasions

was reasonably constant, there appeared to be a wide range of values in both normal and enzyme deficient cells. If the difference is real, its significance is not obvious. A possible explanation would be increased lactic dehydrogenase activity in primaquine sensitive cells. This has in fact been reported by Larizza et al. (1958) although Johnson and Marks (1958) found no difference from the normal.

The effect of primaquine in a concentration of 30 micrograms/ml. was found to resemble that of BCB upon glucose consumption by both normal and enzyme deficient erythrocytes. Although the change in utilisation was less marked, more glucose was metabolised by normal cells and less by primaquine sensitive cells when the drug was present. Small but significant concentrations of pyruvic acid were also observed after incubation with primaquine. In this system, therefore, primaquine stimulates oxidative glucose metabolism and depresses anaerobic glycolysis in the same way as the redox dyes. Similar conclusions have recently been reached by other workers as well. Thus Brewer and Tarlov (1961) reported that a derivative of

primaquine stimulated the pentose phosphate pathway of normal but not of sensitive individuals by oxidising TPNH to TPN. Szeinberg and Marks (1961) showed that not only primaquine, but also nitrofurantoin, acetylphenylhydrazine, α- and β-naphthol, an extract of fava beans, and certain other substances as well increased the rate of formation of $^{14}CO_2$ from glucose-1-^{14}C in both normal and deficient erythrocytes. These workers found that the action of these substances was to increase the rate of oxidation of TPNH, the reaction being mediated by diaphorase. Mohler and Williams (1961) reported that phenylhydrazine increased the utilisation of glucose by normal cells and produced an accumulation of pyruvic acid in vitro. An accumulation of pyruvic acid on incubation with acetylphenylhydrazine was also observed by Steinschuss et al. (1961).

If methylene blue and BCB have the same effect upon cellular metabolism as primaquine, it seems possible that the dyes may provoke haemolysis when administered to primaquine sensitive subjects. This has, in fact, recently been reported (Carson, 1960; Brewer and

Tarlov, 1961) and is in favour of the concept that it is the redox nature of the haemolytic compounds which is responsible for their haemolytic action.

C H A P T E R   9

Fat Metabolism in Primaquine Sensitive Erythrocytes

While several workers have claimed that the mature human erythrocyte is not capable of the biosynthesis of lipid in vitro (Marks et al., 1960; Buchanan, 1960), Mendelsohn (1961) has demonstrated a synthesis of fat from carbohydrate by normal human red blood cells (essentially free from leucocytes) under in vitro conditions. The addition of a redox dye such as methylene blue or brilliant cresyl blue (BCB) to the in vitro system greatly enhanced the biosynthesis of lipids from glucose.

Recent work has revealed the importance of an adequate supply of reduced coenzyme II (TPNH) in the reductive synthesis of fatty acids in tissue cells (Langdon, 1957; Siperstein, 1959). The hexose monophosphate shunt pathway is believed to be the only source of TPNH in the erythrocyte, and since it is known that redox dyes markedly stimulate shunt activity in red cells (Brin and Yonemoto, 1958) it was suggested that an intimate relationship exists between the oxidative metabolism of glucose and the synthesis of lipids. Since the hexose monophosphate shunt pathway is defective in

primaquine sensitive erythrocytes because of diminished activity of glucose-6-phosphate dehydrogenase, it was thought to be of interest to compare lipid synthesis in the abnormal cells with the normal.

MATERIALS AND METHODS

Preparation of Red Cell Suspensions. This technique has been described in Chapter 8.

Determination of Free Fatty Acids (FFA). The higher free fatty acids were determined as described by Mendelsohn (1958).

Determination of Total Fatty Acids (TFA). Total erythrocyte fatty acids were estimated by the method of Mendelsohn (1962). Briefly, lipid was extracted from one volume of red cell suspension with 5 volumes chloroform: methyl alcohol (1:1). The extraction was repeated 3 times. After removal of the solvent, saponification and acidification, total fatty acids were extracted into low boiling point petroleum ether. The extract was then taken to dryness and the fatty acids dissolved in benzene. An aliquot was removed for colorimetric estimation using rosaniline reagent (Mendelsohn, 1958).

Incubation with radioactive glucose. In the appropriate experiments, uniformly labelled glucose (glucose-U-C14) was added to the red cell suspension to produce a final concentration of 0.4 µc/ml. The extraction and separation of red cell lipids for assay of radioactivity after incubation was carried out as described by Mendelsohn (1962), weighed aliquots of the extracted fatty acids being plated on to planchettes for counting at infinite thinness in a windowless gas flow counter. A zero time control was put through the same liquid extraction procedure, and the radioactivity of the lipid fraction

before incubation subtracted from the counts recorded in the lipid fraction after 3 hours' incubation.

RESULTS

The incubation of normal red cells in this in vitro system resulted in an increase in the concentration of erythrocyte free fatty acids (FFA). In contrast, enzyme deficient cells produced no significant increase in the level of FFA, and in fact in some instances a fall in the concentration was observed.

In addition, a marked difference between normal and primaquine sensitive erythrocytes with respect to total red cell fatty acids (TFA) was observed. There was an increase in the concentration of TFA when normal cells were incubated, whereas incubation of primaquine sensitive cells resulted in a marked decrease in TFA.

After incubation with uniformly labelled glucose, radioactivity was detected in the total fatty acid fraction of both normal and sensitive cells. The specific activity of the fatty acids was somewhat higher in the case of the sensitive cells.

The results are summarised in Fig. 19.

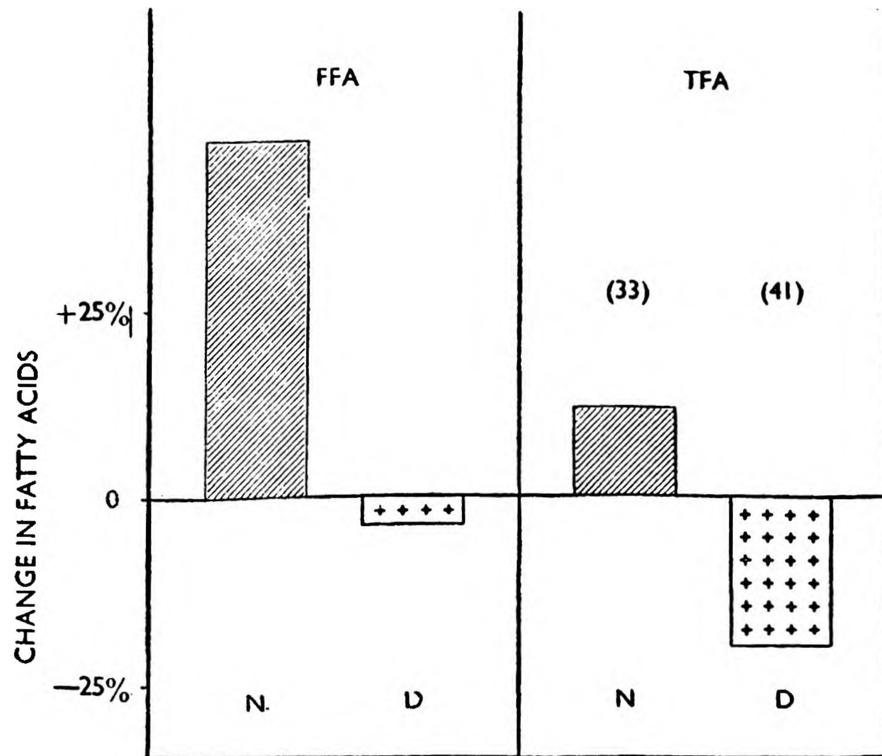


Fig. 1[illegible]. The change in erythrocyte free fatty acids (FFA) and total fatty acids (TFA) upon incubation of normal (N) and primaquine sensitive (D) erythrocytes with glucose and BCB. The figures in brackets represent the average TFA specific activity after incubation with glucose-U-^{14}C.

DISCUSSION

The evidence for a disordered lipid metabolism as defined in this study seems clear. The incubation of normal erythrocytes with glucose and brilliant cresyl blue resulted in a net increase in both free fatty acids and total fatty acids. Confirmation of a net synthesis of fat from glucose was provided by the incorporation of ^{14}C from uniformly labelled glucose into erythrocyte fatty acids. Although this incorporation of radio-activity into red cell lipids also occurred when enzyme deficient erythrocytes were incubated, little or no increase in the concentration of FFA was observed, together with a pronounced fall in TFA. The recovery of activity from the lipid fraction of primaquine sensitive erythrocytes must imply some synthesis of fat; indeed, the specific activity observed was somewhat greater than in normal cells. However, the decrease in total erythrocyte fatty acids on incubation must equally mean that a greater degree of catabolism than of metabolism had occurred.

Since it has been shown that TPNH is necessary

for the biosynthesis of fatty acids, it is perhaps not surprising that cells in which the capacity to reduce TPN is defective should manifest disordered lipid metabolism. Nevertheless, on the basis of the results of the present study it is apparent that the relationship between TPN reduction and lipid synthesis in primaquine sensitive cells is not a direct one.

This aspect of the aberrant metabolism in primaquine sensitive cells may be related to the susceptibility of these erythrocytes to haemolysis in vivo. Alving et al. (1960) have reported that total red cell lipids decrease during the acute haemolysis produced by the administration of primaquine to sensitive individuals, with very slow recovery to previous levels. Danon et al. (1961) have recently reported morphological differences on electron microscopy between the cell membranes of primaquine sensitive and normal erythrocytes, and it seems possible that a lipid constituent of the membrane is defective, since 90% of erythrocyte lipids are in the membrane (Tarlov et al., 1962). The report by Prankerd (1958) that the lipid content of old normal erythrocytes

is lower than that of young cells may be relevant, since on electron microscopy the cell membrane of old cells has a different appearance to that of younger cells. In primaquine sensitive blood the abnormality which has been observed is that the proportion of cells having the appearance of old cells is increased (Danon et al., 1961). Tarlov et al. (1962) have in fact reported that the total lipid content of enzyme deficient erythrocytes is diminished, although Löhr and Waller (1958) found no abnormality in stromal lipid fractions of erythrocytes from a case of congenital non-spherocytic haemolytic anaemia (in which the deficiency of G6PD was even more marked than in primaquine sensitive cells). Further evidence of abnormal lipid metabolism in primaquine sensitivity is the report by Tarlov et al. (1961),(1962) of raised serum cholesterol levels in affected Negroes. The administration of primaquine to these individuals produced a fall in the level of esterified cholesterol, although there was no similar effect in normal subjects.

CHAPTER 10

Summary and Conclusions

At the time of writing investigations are still in progress in many fields related to primaquine sensitivity and fresh communications are appearing each month. It has therefore only been possible to include papers published before April 1962 in the critical review of the literature. The investigations recorded in this thesis were carried out between June 1959 and March 1962. Some of the conclusions reached have since been confirmed by other workers.

In summary, it was decided first of all to survey various racial groups in Southern Africa in order to discover whether primaquine sensitivity could be demonstrated, and, if so, to determine the incidence of the trait in certain sections of the population. On investigation the condition was found in 3% of the Bantu population, with no discernible tribal predilection, and was also found in Cape Malays and Kalahari Bushmen with approximately the same frequency. No primaquine sensitive subjects were found among 100 Natal Indian males examined.

Once it had been established that primaquine

sensitivity was reasonably common in South Africa, a study of various biochemical aspects of the abnormal erythrocytes was undertaken. The formation and enzymatic reduction of methaemoglobin were first investigated. It was established that under the same oxidant stress in vitro, the rate of accumulation of methaemoglobin was more rapid in primaquine sensitive erythrocytes than in normal cells. At the time that this study was carried out it was believed that methaemoglobin formation was not greater in sensitive cells, since it was known that methaemoglobinaemia was less marked in primaquine sensitive subjects exposed to the drug than in normals (Beutler, 1959). Recently it has been shown that this is due to destruction of the older cells by the drug, these cells containing most of the methaemoglobin, and that on exposure to nitrite methaemoglobinaemia is more marked in sensitive subjects than in normals, since nitrite does not shorten erythrocyte survival (Tarlov et al., 1962). This work fits in well with the in vitro studies reported here.

In addition to the investigation of methaemoglobin formation in sensitive cells, the effect of primaquine

upon enzymatic methaemoglobin reduction in both sensitive and normal cells was examined.

No inhibiting or stimulating action was demonstrated. Subsequently, however, it has been reported that a degradation product of primaquine stimulates the reduction of methaemoglobin in a manner similar to the effect of redox dyes such as brilliant cresyl blue (Beutler et al., 1960).

Glucose and pyruvate metabolism in primaquine sensitive erythrocytes was then investigated, and it was shown that the utilization of glucose was decreased in the presence of the dye brilliant cresyl blue, although it is well known that the effect of this substance is markedly to stimulate glucose consumption in normal cells. Carson (1960) has reported similar observations. From a consideration of the available evidence it seems probable that the action of the dye in both normal and primaquine sensitive cells is to inhibit anaerobic glycolysis and to stimulate oxidative glucose metabolism. In normal erythrocytes the net effect is to increase the overall consumption of glucose. In sensitive red cells,

however, the oxidative pathway is defective, due to the deficient glucose-6-phosphate dehydrogenase activity, so that glucose utilization is decreased in the presence of brilliant cresyl blue. Somewhat greater concentrations of pyruvic acid accumulated when sensitive erythrocytes were incubated with the dye than in the case of normal cells but the difference was not marked.

In addition, it was shown that the effect of primaquine upon glucose utilization was similar to that of the redox dye, consumption being increased in normal cells but diminished in sensitive cells. This has subsequently been confirmed by other workers (Brewer and Tarlov, 1961; Szeinberg and Marks, 1961). Since primaquine has been found to increase the utilization of glucose in normal cells, it was decided to repeat the work of Desforges et al. (1960) showing an inhibition of glucose-6-phosphate dehydrogenase activity by primaquine. It was difficult to reconcile the stimulation of glucose consumption which had been observed with inhibition of an enzyme on one of the pathways of glucose metabolism. However, on investigation no

inhibition of the enzyme by primaquine could be demonstrated, whether G6PD from normal or from sensitive cells was studied.

Certain aspects of lipid metabolism in primaquine sensitive cells were then examined. On incubation with glucose and brilliant cresyl blue no change in erythrocyte free fatty acid was observed, although a marked increase was constantly found in the case of normal cells. In addition, total red cell fatty acids decreased in sensitive erythrocytes on incubation although the concentration increased in normal cells. However, radioactivity could be demonstrated in the fatty acid fraction of both normal and deficient erythrocytes after incubation with glucose labelled with ^{14}C. It is suggested that the disordered lipid metabolism revealed by these studies is related to inadequate generation of TPNH in primaquine sensitive cells, since it is generally believed that adequate supplies of TPNH are required for the reductive synthesis of fatty acids. Abnormal lipid metabolism has recently been characterized in other ways in primaquine sensitive subjects (Alving et al. 1960; Tarlov et al. 1962), and may be of importance with regard to the

mechanism of haemolysis since 90% of the erythrocyte lipids are in the cell membrane.

Finally, during the course of these studies, the blood of a child suspected of favism was examined for G6PD deficiency and glutathione instability, and the diagnosis was confirmed. Since the child belonged to a very large family, the available relatives were tested for the associated red cell defect, and it was found that the distribution of the trait throughout several generations was consistent with transmission as a sex-linked gene of intermediate dominance.

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