THE USE OF FOOD FORTIFICATION TO PREVENT FOLATE DEFICIENCY IN POORLY-NOURISHED COMMUNITIES

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This is to certify that
this thesis is my own work and has not
been submitted for a degree at any
other University.

NEVILLE COLMAN

M.B., B.Ch. (Rand)

To those South Africans who have concerned themselves with the problems of poverty and malnutrition.

A C K N O W L E D G E M E N T S

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NOMENCLATURE

1. Folic acid, PGA and folate

In this thesis, the term "folic acid" is used exclusively to denote the compound monopteroylglutamic acid, in accordance with the nomenclature policy of The Journal of Nutrition (Nomenclature, 1973). The term "PGA" is used synonymously with the term "folic acid". The term "folate" is used as the generic descriptor for folic acid and related compounds exhibiting qualitatively the biological activity of folic acid.

2. Negro

The indigenous black people of South Africa are officially called "Bantu", and this term has been used in numerous scientific publications. For a number of reasons, "Bantu" is an unsatisfactory name to use to describe these people.

In The Oxford History of South Africa, Wilson and Thompson (1969) abandoned the use of this term because "it is disliked by those to whom it is applied". Tobias (1971) has marshalled the evidence to show that it is biologically unjustified as well.

A symposium on "Peoples of South Africa", sponsored by the South African Institute for Medical Research and the Royal Society of South Africa, was held in Johannesburg in June, 1971. Authorities in the fields of ethnology,

linguistics, social and physical anthropology, archaeology, human biology and human genetics discussed the question of nomenclature under the chairmanship of Professor Monica Wilson. The participants agreed that the term "Negro" was that most appropriate to describe the people referred to by other workers as "Bantu", "Natives", "Africans", and "Bantu-speaking Negro". The nomenclature agreed upon at this symposium is that used in this thesis.

C H A P T E R 1

STATEMENT OF THE PROBLEM

I Historical note

Well over a century ago, Channing (1842) drew attention to a severe form of anaemia in pregnancy which was rapidly progressive and sometimes fatal. Osler (1919) subsequently noted that the condition could persist, or present for the first time, during the post partum state, and that the blood picture resembled that found in Addisonian pernicious anaemia.

Wills and Mehta (1930) presented a preliminary report of a form of macrocytic anaemia common in pregnant women which was associated with gastrointestinal disturbances. They attributed the disease to an antecedent dietary defect and Wills (1931) subsequently showed that it could be cured by the administration of yeast extract.

In 1943, folic acid was first purified (Stokstad, 1943) and isolated in crystalline form from liver (Pfiffner et al., 1943). Moore et al. (1945) demonstrated response to large doses of folic acid in patients with megaloblastic anaemia of pregnancy, and Lowenstein et al. (1955) were able to prevent the development of the disease by giving pregnant women three mg of folic acid daily.

When deficiency of a substance is suspected of being the cause of a disease, response to therapy with the

substance is usually an effective means of proving that suspicion. An exception to this was found when attempting to differentiate between vitamin B_{12} and folate deficiency. Patients with pernicious anaemia were shown to respond to folic acid (Vilter et al., 1950) and anaemic patients with normal serum vitamin B_{12} levels and apparent folate deficiency responded to treatment with vitamin B_{12} (Killander, 1958). These observations cast doubt on the value of the findings of Moore et al. (1945) and Lowenstein et al. (1955) as proof that folic acid deficiency was the cause of megaloblastic anaemia in pregnancy.

Subsequently, however, Marshall and Jandl (1960) showed that specific optimal haematological response to the deficient vitamin could be obtained by limiting the dose given in a therapeutic trial. They reasoned that "there should be a different dose/response relationship when folic acid is given to patients with cyanocobalamin deficiency as compared to patients deficient in folic acid." They described three patients with folic acid deficiency who responded to what they termed a "physiological dose" of folic acid (400 µg daily). These patients did not have a secondary reticulocyte response when they were subsequently given 15 mg folic acid daily. Three patients with vitamin B_{12} deficiency were subjected to the same regime and showed little or no response to the lower dose of folic acid, but did respond optimally to the higher dose and had no secondary response to 30 µg

cyanocobalamin given daily thereafter. None of these patients were pregnant and their ages ranged from 51 to 84 years. Thus patients with folic acid deficient megaloblastic anaemia respond to small doses of folic acid but not to small doses of vitamin B_{12}.

Zalusky and Herbert (1961) confirmed the general findings of Marshall and Jandl but were able to reduce the "physiological dose" level of the vitamin by effecting response to as little as 50 µg folic acid daily in megaloblastic anaemia due to folate deficiency. Response to folic acid has been effected in vitamin B_{12} deficiency with a dose of 400 µg daily (Herbert, 1963b) but not with smaller doses.

Microbiological assays were being used concurrently with the above investigations, and Chanarin and his coworkers (1959) published a paper which stated that folic acid deficiency was the cause of megaloblastic anaemia in pregnancy. Their evidence for folic acid deficiency was an increased rate of clearance of injected folic acid from the serum. On the basis of this test, the degree of "saturation" of the tissues with folic acid was greatest in a group of healthy women between the ages of 18 and 40, intermediate in a group of pregnant women without anaemia, and lowest in patients with twin pregnancies and in patients with megaloblastic anaemia in pregnancy. Chanarin et al. interpreted these findings to mean that the factors producing folic acid deficiency in normal

pregnancy were responsible for more severe folic acid deficiency in pregnant patients with megaloblastic anaemia.

In South Africa, Metz and his coworkers reported several studies of urban and rural Negro women during the early part of the last decade. They investigated pregnant and lactating women, some of whom had megaloblastic anaemia, by means of microbiological assays for serum folate and vitamin B_{12}, folic acid clearance tests, folate absorption tests, measurement of urinary formiminoglutamic acid (Figlu) excretion after a histidine load, Schilling tests, augmented histamine tests, and therapeutic trials of folic acid and vitamin B_{12} (Metz et al., 1961; Metz et al., 1962a; Metz et al., 1962b; Metz et al., 1963; Stevens and Metz, 1964; Shapiro et al., 1965; Combrink et al., 1966; Edelstein et al., 1966). The conclusions drawn were that folate deficiency was the cause of megaloblastic anaemia in all the pregnant and lactating patients who had this disease.

II Non-haematological effects of folate deficiency

(a) Obstetric complications

There is a wealth of conflicting literature and a resultant confusion about the role that folate deficiency plays in relation to a number of complications of pregnancy. It has been considered as a causative factor in abruptio placentae, abortion, eclampsia,

premature labour and low birthweight, perinatal mortality, and foetal abnormalities.

As the role of folic acid in these conditions is germane to the purpose of this study, the subject is reviewed in detail hereunder.

(i) Abruptio placentae

Since Hourihane et al. (1960) first reported that the incidence of abruptio placentae was five times greater in patients with megaloblastic anaemia than in other pregnant women, and recommended marrow biopsy in all cases of accidental haemorrhage, there have been a number of groups investigating this relationship.

Abruptio placentae was found twice as often in patients with megaloblastic anaemia than in the general population of pregnant women (MacKenzie and Abbot, 1960; Ainley, 1961). Coyle and Geoghegan (1962) and Hibbard (1964) investigated patients with abruptio placentae and reported the presence of megaloblastic anaemia in 45 per cent and 64 per cent of them respectively. Hibbard and his coworkers supported the association between folate deficiency and abruptio placentae in pregnant women in England (Hibbard and Hibbard, 1963) and other areas (Hibbard et al., 1969). Streiff and Little (1967) reported red cell folate deficiency at term in 18 per cent of all patients and 94 per cent of patients with abruptio placentae, and a significant association was found in a similar study in the Philippines (Tantengco et al., 1971). Some studies which based the

diagnosis of folate deficiency on increased urinary excretion of Figlu (Hibbard, 1964; Hibbard and Jeffcoate, 1966) have not been of great value because of evidence that this is an unreliable index of folate nutrition during pregnancy (Berry et al., 1963).

Hibbard's theory was that folate deficiency in early pregnancy impairs cell growth, resulting in instability of the placental attachment to the uterine wall and subsequent premature separation.

In contrast to the above reports, numerous workers were unable to demonstrate an association between abruptio placentae and megaloblastic anaemia in pregnant women in a number of different countries (Gatenby and Lillie, 1960; Giles, 1966; Varadi et al., 1966; Menon et al., 1966; Thambu and Llewellyn-Jones, 1966; Whalley et al., 1969; Hall, 1972b).

Fletcher et al. (1971) and Hall (1972b) were unable to demonstrate significant reduction in the incidence of accidental haemorrhage in a total of 1247 women given folic acid compared with 2236 pregnant controls. As these supplements were only given after the patients had presented at antenatal clinic, these findings do not conflict with Hibbard's theory that the underlying pathology commences at the time of trophoblast implantation.

It is possible that there is a casual rather than causal relationship between the two conditions, as both are said to occur in patients of high parity (Hibbard and

Hibbard, 1963; Rae and Robb, 1970). The relationship is at best highly controversial, and there is no convincing evidence that any connection exists at all.

(ii) Abortion

On the basis of the Figlu test, patients admitted to hospital with abortions have a fourfold increase in the incidence of folate deficiency when compared with pregnant controls (Hibbard, 1964; Hibbard et al., 1965). As mentioned above, however, the value of this test as a measure of folate deficiency in pregnancy is questionable. Martin et al. (1965) reported a high incidence of low serum folate levels in patients with abortion, but their study did not include a control group. Streiff and Little (1965) and Chanarin et al. (1968) reported similar levels of serum folate and red cell folate in patients with abortion and pregnant controls, and similar serum folate levels were found in aborting patients and controls in Venezuela (Diez de Ewald and Molina, 1970).

It is concluded that no association has been demonstrated between folate deficiency and abortion.

(iii) Toxaemia of pregnancy

Gatenby and Lillie (1960) reported that toxaemia was four times more common in patients with megaloblastic anaemia than in pregnant controls. Once again, some workers were able to confirm these findings (Mackenzie and Abbott, 1960; Ainley, 1961; Stone et al., 1967) while others were not (Hourihane et al., 1960;

Giles, 1966).

A recent reviewer of the subject has endorsed the statement of a World Health Organisation Expert Committee that "there seems to be no scientific basis for believing that deficiency or excess of any essential dietary nutrient predisposes to pre-eclampsia and eclampsia" (Davies, 1971). It is noteworthy, however, that on the basis of animal experiments (Stamler, 1959; McKay and Wong, 1962; McKay et al., 1967), at least one eminent worker believes that the question of a nutritional cause for eclampsia is still open (Page, 1972).

(iv) Premature labour and low birthweight

A possible relationship between folate deficiency and premature labour was first suggested by Callender (1944). Gatenby and Lillie (1960) reported that one eighth of infants of patients with severe megaloblastic anaemia were of low birthweight, compared with one twelfth of all deliveries. A number of subsequent studies failed to confirm any association with folate deficiency (Hourihane et al., 1960; Karthigani et al., 1964; Chanarin et al., 1965 and 1968; Abramovicz and Kass, 1966; Giles, 1966; Varadi et al., 1966). Whiteside et al. (1968) found significant correlation between low birthweight and the combined effects of iron, folate and vitamin B_{12} deficiency, but no single factor contributed significantly. Harrison and Ibeziako (1973) found birthweight to be decreased in the infants of Nigerian mothers

who were anaemic in pregnancy, and further decreased if they were still anaemic at term. There was a highly significant correlation between foetal birthweight and haematocrit when the latter was less than 30 per cent.

Baumslag and her coworkers (1970) suggested that the relationship might be more significant in populations with poor folate nutrition. They compared 183 South African Negroes who had a suboptimal diet with 172 Whites on a good Western diet. Both samples were randomised into three groups, the groups receiving supplements of iron alone, iron and folic acid, or iron, folic acid and vitamin B_{12}. The White patients received supplements from the 24th week of pregnancy, and the Negro patients from the 28th week. In the Negro sample, 20 per cent of the infants of mothers in the group receiving iron alone weighed less than four lb. at birth, while all the 110 infants in the folic acid-supplemented groups weighed more than four lb. In the White sample three to four per cent of the infants born of mothers in each of the groups weighed less than five lb.

The striking difference between the Negro and White patients explains the failure of some workers to demonstrate a relationship between folate deficiency and prematurity in well-nourished populations. The mean birthweight in the Negro group receiving iron alone was lower than 5.5 lb., which is generally accepted as the dividing line between low and normal birthweight. The highly significant reduction in the incidence of prematurity in

patients receiving folic acid is strong evidence for a causal relationship between folic acid deficiency and low birthweight in populations with suboptimal folate nutrition.

(v) Perinatal mortality

Perinatal mortality was found to be twice as high in megaloblastic anaemia as in controls by Hourihane et al. (1960), Ainley (1961) and Giles (1966). Other workers found an increase of neonatal deaths but not of stillbirths (Gatenby and Lillie, 1960), while Pritchard et al. (1970) found no increase in either.

The question of whether folate deficiency without anaemia increases perinatal mortality is far more controversial. Martin et al. (1967) and Pritchard et al. (1969) could not demonstrate any relationship.

Nelson and Haltalin (1972) demonstrated marked growth retardation and an increased susceptibility to bacterial infection in the neonatal period in guinea pigs fed a folate-deficient diet. Other animal studies using folate antagonists (Chepenik and Waite, 1972) are not considered here because the syndrome produced by these agents may not be analogous to pure nutritional folate deficiency (Herbert, 1965), since the antagonists produce cytoplasmic inclusion bodies in the mucosa of the small bowel, which are not seen in pure nutritional folate deficiency but have been observed following radiation and a variety of noxious agents (Trier, 1962).

(vi) Foetal abnormalities

Nelson et al. (1952) fed pregnant rats a low folate diet from the ninth day after conception and produced foetal death, while feeding the diet from the eleventh day resulted in 95 per cent of the litter having congenital abnormalities, namely oedema, skeletal malformation, visual atrophy, cataracts, and anaemia. Rats fed the diet from the twelfth day produced litters with mild defects, while those on the diet from the fifteenth day had normal litters. Severe folate deprivation was subsequently confirmed to have a consistent teratogenic effect dependent on time of deprivation in a number of animal studies (Johnson et al., 1963; Johnson, 1964; Monie and Nelson, 1963; Stempak, 1965; Armstrong and Monie, 1966; Woodward and Newberne, 1967).

These findings were used as a basis for the use of folate antagonists as abortifacients in man (Thiersch, 1952; Goetsch, 1962), but it is not clear whether the action of these drugs was mediated by their effect on folate metabolism.

The relevance of the effect of the rather gross deprivation produced in animals to the relatively mild deficiency usually seen in pregnant women is not established. Folate deficiency is associated with lower oestrogen levels in the mother (Martin et al., 1963 and 1964). Malformations have been found in 29 per cent of infants of a group of patients with megaloblastic anaemia (Fraser

and Watt, 1964), and women with anencephalic foetuses excreted excessive amounts of Figlu four times more commonly than pregnant controls (Hibbard et al., 1965; Hibbard and Smithells, 1965).

Pritchard et al. (1970) studied 86 women with megaloblastic anaemia and reported that foetal malformation was not increased in patients with folate deficiency, and the same group have reported that folate status was no worse in mothers of malformed foetuses than in controls in late pregnancy (Scott et al., 1970). It is not possible to assess whether folate deprivation occurred in these patients early in pregnancy when the foetus was susceptible to teratogenic influences, but this is unlikely to be the case unless the patients were subsequently given folic acid supplements.

Hall (1972a) measured serum folate levels during the second and third trimesters in 2949 pregnancies and found no association with foetal malformation. She concluded that a causal relationship was most unlikely on the basis of an unsubstantiated statement that the serum folate level "at 30 weeks of pregnancy reflects closely what the level was earlier in the pregnancy".

It is feasible that the degree of folate deprivation in populations with a high incidence of folate deficiency may become so great in some members as to simulate conditions seen in animal experiments. However, there is no direct evidence that this ever occurs.

(vii) Conclusion

It is concluded that folate deficiency is a causative factor in prematurity, and that the incidence of low birthweight infants, in the South African Negro population at least, can be decreased by folate replenishment. Perinatal mortality is increased in at least some populations in the infants of mothers with frank megaloblastic anaemia but this may be the result of anaemia per se, and foetal abnormalities may possibly be more common when folate deprivation is severe early in pregnancy.

There is no direct evidence for a causal relationship between folate deficiency and abruptio placentae, abortion, or toxaemia of pregnancy.

(b) The gastrointestinal tract

Folate deficiency affects all proliferating cells and changes may therefore occur throughout the length of the gastrointestinal tract. These changes are essentially the same as those in the marrow, consisting of an increase of nuclear and cytoplasmic material, a tendency to multiple nuclei, and alterations in the chromatin pattern. These changes have been demonstrated in the buccal mucosa and tongue of patients with folate deficiency (Boddington and Spriggs, 1959). Graham and Rheault (1954) demonstrated similar changes in the buccal mucosa and stomach of patients with pernicious anaemia. Abnormalities in the epithelium of the jejunum have been noted by Veiger et al. (1965) in patients with folate

deficiency associated with sprue.

Common clinical manifestations of folate deficiency are acute and chronic glossitis, diarrhoea, anorrhexia, and cachexia. There is evidence that gastrointestinal changes occur later in the disease than haematological abnormalities. At the time that anaemia appeared after a period of experimental folate deprivation, no morphological or functional change in the gastrointestinal tract was observed by Herbert (1962a). It is possible that the intestinal cells have a greater avidity for folate than does haemopoietic tissue, or that their requirement is smaller (Herbert, 1965).

The epithelial surfaces of the respiratory and genito-urinary tracts have been shown to manifest the same changes as those seen in the gastrointestinal tract (Boddington and Spriggs, 1959).

(c) The skin

Rose (1971) reported that serum folate levels in patients with angular cheilosis were significantly lower than those in controls. Nine of the fifty affected patients in his study had serum folate levels in the deficient range. He postulated a causative association and stated that two patients were successfully treated with folic acid.

Increased urinary Figlu excretion was found in a third of patients with various dermatoses (Knowles et al., 1963), but was thought to be secondary to the skin disease.

(d) The nervous system

The possibility that folate deficiency might cause neurological disease has attracted much attention because of the fact that vitamin B_{12} deficiency causes both megaloblastic anaemia and disorders of the nervous system. A standard textbook of neurology recommends that "estimation of the serum folate should be carried out in cases of unexplained neuropathy, myelopathy, and dementia, although the exact role of folate deficiency in these disorders remains to be determined" (Brain and Walton, 1969).

During a period of experimental folate deprivation, irritability, insomnia and amnesia were noted (Herbert, 1962a).

Grant et al. (1965) reported ten cases of megaloblastic anaemia in association with peripheral neuropathy, of whom seven had folate deficiency. They doubted that folate deficiency was a causative factor, and suggested that neurological disease had caused anorrhexia with resultant folate deficiency.

A recent case report has purported to show an association between folate deficiency and a neurological syndrome similar to that seen in vitamin B_{12} deficiency (Pincus et al., 1972). This has been severely criticised on the basis that vitamin B_{12} deficiency and alcoholism were not excluded as causes of the syndrome (Herbert, 1972b). The same group has reported that patients with folate deficiency had an increase in neurological disease over controls with normal serum folate levels (Reynolds

et al., 1973). This finding may be explained on the same basis as those of Grant et al. (1965).

There have been other isolated reports of neurological disease occurring in patients with folate deficiency (Anand, 1964; Hansen et al., 1964).

There has been a suggestion that visual abnormalities might be caused by folate deficiency (Adams et al., 1967). This is based on the observation that two of four patients with folate deficiency were found to have these abnormalities. In one case, it is possible that thrombocytopaenia caused the abnormality, and little emphasis can be given to the association noted in the single remaining patient.

The absence of any convincing demonstration of folate deficiency as a cause of neurological disease is hardly surprising, as folate is selectively concentrated in the cerebrospinal fluid (Herbert and Zalusky, 1961).

III Nutritional requirements for folic acid

The subject of nutritional requirements for folic acid has been reviewed by Herbert (1968a). The term "nutritional requirement" is defined by Herbert as the minimal daily adult requirement, i.e. that quantity of the nutrient which would be adequate to prevent the development of any clinical or laboratory evidence of deficiency in patients with depleted body stores. This concept is a convenient

one to use for the purpose of gaining information, as most of the published information on this topic is based on subjects with folate deficiency.

However, as Herbert (1968a) points out, this requirement may not be the same as the quantity of the nutrient which should be consumed by groups of people in order to maintain a desirable state of nutrition. In the United States, this latter amount has been given the title "Recommended Dietary Allowance", and these allowances "are intended to serve as goals for planning food supplies and for the interpretation of food consumption by groups of people ..... The purpose for establishing a national dietary standard is not the same in all countries. At the same time, it must be recognised that the 'reference' individual will vary from country to country" (National Academy of Sciences - National Research Council, 1968). It is not known what quantity of folic acid should be recommended for daily consumption by groups of people, but it is reasonable to base any assumptions in this regard on the nutritional requirement as defined above. Problems in interpreting this information for use in programmes designed to improve folate nutrition will be dealt with in the following chapter.

The nutritional requirement for folic acid is about 50 µg. This daily dose was adequate to maintain the serum folate level of a healthy adult female on a low folate diet for over a month, while the serum folate fell in a similar subject receiving 25 µg and rose in one

receiving 100 µg daily (Herbert, 1962b). Fifty µg folic acid daily produces a relatively rapid conversion of bone marrow morphology from megaloblastic to normoblastic, with concomitant haematological improvement (Zalusky and Herbert, 1961), and prevented the development of increased urinary Figlu excretion in a patient on a folate-deficient diet (Fleming et al., 1963). The red cell folate level in a patient with experimental folate deficiency fell by approximately 90 per cent in 137 days (Herbert, 1962a), and Herbert (1968a) has calculated that this represents a daily utilisation of 50 µg folic acid, apart from the five µg of food folate present in the diet.

It has been emphasised that the nutritional requirement for folic acid may be dependent on body weight, and that requirements increase with a raised metabolic rate, as in hyperthyroidism, and increased cell turnover, as in haemolytic anaemia (Herbert, 1968a). The increased requirement for folic acid during pregnancy and lactation is the cause of folate depletion in large numbers of women, resulting in some cases in the disease which forms the subject of this study. A daily dose of 150 µg folic acid caused optimal haematological response in only three of five patients with megaloblastic anaemia of pregnancy, and on the basis of serum folate levels appeared inadequate prophylaxis in eight of twenty pregnant patients in Manchester (Dawson, 1966). At the

other extreme, Lowenstein et al. (1966) found that 500 µg folic acid daily was adequate for prophylaxis. Two hundred µg daily failed to cause optimal response in two patients studied by Alperin et al. (1966).

The study conducted by Willoughby and Jewell (1966) has provided extremely useful information. They divided 350 pregnant women in Glasgow into five groups which each day received, respectively, no haematinics, iron (105 mg) alone, iron plus 100 µg folic acid, iron plus 300 µg folic acid, and iron plus 450 µg folic acid. This study showed that 300 µg folic acid daily prevented the development of megaloblastic anaemia and maintained serum folate levels in a range similar to that of non-pregnant subjects, 100 µg folic acid daily was inadequate to prevent either megaloblastic anaemia or reduced serum folate, and 450 µg daily resulted in supranormal levels of serum folate.

This finding is of great significance, as a daily dose of 400 µg folic acid may produce haematological response in some patients with pernicious anaemia (Herbert, 1963), although others only respond to greater doses (Marshall and Jandl, 1960). It appears probable that administration of 300 µg folic acid daily may be effective prophylaxis in pregnancy without causing diagnostic problems in patients with pernicious anaemia.

Information on the requirement for folic acid in the lactating female has been reviewed by Metz (1970). He noted that although maternal serum folate levels

improve after delivery, this improvement is short-lived in populations whose dietary folate intake is suboptimal (Edelstein et al., 1966). After South African Negro subjects had lactated for twelve weeks, serum folate levels were less than 2.5 ng per ml in 38 per cent and there was increased urinary Figlu excretion in 49 per cent (Shapiro et al., 1965). A daily dose of 200 µg folic acid failed to produce haematological response in lactating patients (Stevens and Metz, 1964). On the basis of serum folate levels, the nutritional requirement for folic acid in the lactating female is between 200 µg and 300 µg daily (Metz and Hackland, 1968).

The minimum daily folate requirement in infants can not be extrapolated on a weight for weight basis from the adult requirement (Herbert, 1968a). Newborn infants of low birth weight show a fall in serum folate concentration which can be prevented, without resulting in supranormal serum folate levels, by the daily administration of 50 µg folic acid (Samuel et al., 1973). Burland et al. (1971) have calculated that week-old infants of low birthweight were likely to receive less than 15 µg food folate per day from their diet.

Velez et al. (1963) found that children with megaloblastic anaemia in kwashiorkor responded haematologically to 20 µg folic acid daily. However Kamel et al. (1972) reported suboptimal haematological responses in children with kwashiorkor and megaloblastic anaemia given graded

doses of folic acid up to 50 µg per day. They calculated that recovery from folate deficiency in most children recovering from kwashiorkor would be effected by a daily dose of 5.8 µg free folate per kg body weight in the diet plus 5.4 µg folic acid per kg body weight. Maximal therapeutic response would require more than 13 µg folic acid per kg body weight daily.

It has been pointed out that there is a sharply increased folate requirement during periods of rapid growth (Herbert, 1968a), and the figures quoted for children should thus be treated with reserve.

IV The incidence of folate deficiency and megaloblastic anaemia in pregnancy and in the general population

(a) The incidence in pregnancy

Until the second half of the twentieth century, megaloblastic anaemia was thought to be rare in pregnancy. Since 1950, there have been numerous reports of the incidence of the disease in pregnant women in different areas. The stated incidence has increased in recent years, but this can probably be attributed to greater awareness of the condition. Unfortunately, the criteria used for diagnosis of the disease have varied in different studies, so the reports are not strictly comparable. In addition, it is necessary to differentiate carefully between the incidence of anaemia due to megaloblastic haemopoiesis, the frequency of megaloblastic change on

bone marrow screening, and the incidence of folate deficiency.

In the United States, an early report suggested that 0.5 per cent of a group of poor Negro women had megaloblastic anaemia in pregnancy (Lund, 1951), but twelve years later 15 per cent of pregnant women were reported to have the disorder (Luhby et al., 1963). Herbert et al. (1973) studied a group of poor women in New York and reported low red cell folates in 23 per cent in the second trimester of pregnancy and 40 per cent in the third trimester. There was supportive evidence that the low red cell folates represented true tissue deficiency.

In Liverpool, megaloblastic anaemia was reported in 0.6 per cent of anaemic pregnant women in 1957 (Forshaw et al.), but in 1964 the condition was found to be present in 5.4 per cent of all antenatal patients (Hibbard, 1964). Between 1958 and 1967, reports from various parts of Britain suggested an incidence of one to four per cent (Giles and Shuttleworth, 1958; MacKenzie and Abbott, 1960; Ainley, 1961; Hussain et al., 1963; Varadi et al., 1966; Willoughby, 1967). After most of these reports had appeared, Chanarin and his coworkers (1965 and 1968) found megaloblastic change in 16.6 per cent of antenatal patients and 13 per cent of patients receiving iron supplements.

In Dublin, the incidence of megaloblastic anaemia was reported to be three to four per cent of all

pregnancies and only 0.4 per cent of anaemic patients in the early 1960's (Hourihane et al., 1960; Coyle and Geoghegan, 1962; Gatenby and Lillie, 1960). A few years later, Temperley et al. (1968) examined the marrows of fifty patients, of whom 15 were anaemic, and found megaloblastic change in 30 per cent.

The disorder is reported to be more common in areas where malnutrition is endemic. In India, Menon et al. (1966) found that 17 per cent of anaemias in pregnancy were due to folate deficiency and Karthigaini et al. (1964) classified 54 per cent of marrows taken from an unselected group of pregnant women as megaloblastic.

Yusufji et al. (1973) studied 1000 women from Southern India. Sixty per cent of marrow aspirates from 740 unselected women in the group were megaloblastic, the incidence increasing with the duration of pregnancy. Haemoglobin concentrations less than 10 g per 100 ml were present in one third of their patients, but it is not stated how many of these were associated with iron deficiency, which was also common. Thambu and Llewellyn-Jones (1966) reported megaloblastic anaemia in 18 per cent of antenatal patients in Malaysia, and the incidence appeared higher in the Indian group.

Hibbard and Hibbard (1972) reported on three ethnic groups in Singapore and found subnormal red cell folate levels in 36 per cent of pregnant Indian women, 7 per cent of Malays and 6 per cent of Chinese. All these

women were assessed to be in the 37th week of gestation.

In Venezuela, Diez-Ewald and Molina (1972) recorded low red cell folate levels in 47 per cent of women in the third trimester and 66 per cent of those post partum. Anaemia was present in 36 per cent of the pregnant and 38 per cent of the post partum patients, and about one half of each group had low red cell folate levels.

In many African countries, particularly in West Africa, folate deficiency is the main cause of nutritional anaemia in pregnancy. In Nigeria, iron deficiency is rare in pregnancy and 30 per cent of primiparous women show evidence of folate deficiency in the second trimester. The incidence increases to 85 per cent at term, and 30 per cent have frankly megaloblastic erythropoiesis (Fleming et al., 1968). Bone marrow aspirates of 136 anaemic Nigerian antenatal patients were examined by Fleming et al. (1969), who reported that 11 per cent were normoblastic, 14 per cent showed early transitional megaloblastic change, and the remaining 75 per cent were classified as either essentially megaloblastic or grossly megaloblastic. Haemolysis, probably due to chronic malaria, was an aggravating factor and patients receiving antimalarial drugs had a higher mean serum folate level (Fleming et al., 1968). In Kenya, it has been reported that the most common form of anaemia in pregnancy is megaloblastic, with almost half the cases being associated with malaria (Mati et al., 1971).

In Johannesburg, iron deficiency is a more frequent cause of anaemia in pregnancy than is folate deficiency. Turchetti et al. (1966) studied fifty pregnant Negro women with subnormal haemoglobin levels and found evidence of body iron deficiency in over 90 per cent. Of the patients described, 28 per cent showed evidence of folate deficiency associated with iron deficiency and a further 8 per cent were folate deficient with normal iron nutrition. Metz (1966) reported that 25 per cent of Negro patients at risk admitted to a Johannesburg hospital had megaloblastic haemopoiesis post partum. Edelstein et al. (1966) found evidence of biochemical folate deficiency in 25 to 30 per cent of all their Negro patients in late pregnancy, rising to between 35 and 45 per cent post partum.

(b) The incidence of folate deficiency in the general population

Although megaloblastic anaemia due to folate deficiency is most commonly seen in pregnant women, there is a considerable body of evidence that it may attain significant incidence in other sections of the population. This is particularly true of many of the economically disadvantaged people of the world. Subjects at the extremes of life appear most susceptible.

Zuelzer and Ogden (1946) first clearly defined the syndrome of megaloblastic anaemia in infants, and concluded that significant aetiological factors included nutritional deficiency, prematurity, infection and

maternal anaemia. It has since been shown that serum and red cell folate levels drop rapidly in all infants after birth, but that the fall is accelerated in premature infants (Matoth et al., 1964; Shojania and Gross, 1964; Vanier and Tyas, 1967). It has been mentioned earlier in this chapter that the folate intake of premature infants is unlikely to meet the requirement of 20 - 50 ug folic acid, which on a weight for weight basis is four to ten times higher than the adult requirement (Matoth et al., 1964; Sullivan et al., 1966). A recent study of 54 premature infants revealed megaloblastic haemopoiesis in seven cases and probable megaloblastosis in a further four subjects (Strelling et al., 1966). Tan and Ong (1972) investigated 96 children under the age of two with nutritional anaemia in Singapore and found that almost all had iron deficiency, but that half also had concomitant megaloblastic anaemia. The relative ethnic incidence corresponded with observations in pregnant women the same area (Hibbard and Hibbard, 1972).

The question of whether premature infants should be given prophylactic folic acid is unresolved. Hoffbrand (1970) recommends that supplements be given to all infants weighing less than 1500 g at birth, and to all neonates who have impaired milk intake or have had exchange transfusions.

The recognition that malnutrition, particularly kwashiorkor, is associated with folate-deficient

megaloblastic anaemia is now widespread. Walt et al. (1956) and Shnier and Metz (1959) described a total of 97 cases of megaloblastic anaemia in South African children, almost all of whom were malnourished, infected, or both.

An association between malnutrition, particularly kwashiorkor, infection and folate-deficient megaloblastic anaemia has been found in studies in Jamaica (MacIver and Back, 1960), Indonesia (Lien-Keng and Odang, 1959), Colombia (Velez et al., 1963), India (Pereira and Baker, 1966), Egypt (Kamel et al., 1972), and in a further South African study (Spector and Metz, 1966). It is not intended to review these and other similar surveys in detail here. Haematological response following the administration of folic acid has been demonstrated, by Spector and Metz (1966) in South African Negro children, and in numerous studies in other areas.

Kamel et al. (1972) gave graded doses of folic acid to children with kwashiorkor and anaemia who were receiving a diet containing about six /ug dietary folate (before conjugase treatment) per kg body weight. They concluded that a daily dose of 11.2/ug of total free dietary folate per kg body weight is sufficient to promote recovery from folate deficiency in most, but not all, children recovering from kwashiorkor.

Reports of megaloblastic anaemia due to nutritional folate deficiency in adults have concerned elderly patients

mainly, but this is not a uniform finding. In Britain, Gough et al. (1963) reported seven cases from Bristol, all females, of whom only one was over the age of 65. In Sheffield, a report of 333 cases of megaloblastic anaemia stated that 17 per cent of all these cases were due to nutritional folate deficiency, which was two-thirds as common as pernicious anaemia in patients between the ages of 70 and 79, but more common over the age of 80 years (Varadi and Elwis, 1964). Once again females predominated and not all of the patients were elderly. Variations in the incidence of nutritional folate deficiency in different parts of Britain have been stressed by Girdwood (1969).

In the United States, Moore et al. (1944) reported 56 patients with nutritional megaloblastic anaemia from Alabama, but considered that some of these might have had tropical sprue.

Low serum folate levels were reported in 45 per cent of indigent patients admitted to an American hospital (Leevy et al., 1965). It appears, however, that the incidence of folate deficiency in hospital patients is considerably higher than that in the general population (Girdwood, 1969). Nevertheless, Girdwood (1969) concluded that "there is good evidence for the belief that, quite apart from pregnancy, folate deficiency is a real problem both in the United States and the United Kingdom".

Prior to this study, there has been little information about the frequency of folate deficiency in the adult population in areas with a high incidence of megaloblastic anaemia of pregnancy. Metz et al. (1961) reported abnormally rapid clearance of an injected dose of PGA in four of eight Negro women and two of eight Negro men. Clearance was normal in thirty-two white men and women. It was thought that this information could be interpreted to mean that subclinical folate deficiency was not uncommon in Negro females in Johannesburg. While this suggestion is confirmed by clinical impressions in this city, there has been no published survey of the incidence of folate deficiency in South African adults.

V The administration of folic acid tablets to prevent megaloblastic anaemia in pregnancy and the puerperium

(a) The efficacy of folic acid prophylaxis

There is no doubt that megaloblastic anaemia is much less common, if not abolished, in pregnant women who take folic acid tablets. None of the women given folic acid by Lowenstein et al. (1955) and Giles and Burton (1960) developed megaloblastic anaemia. Special care was taken to ensure that these patients took their tablets.

In areas where folic acid prophylaxis has been initiated, the incidence of megaloblastic anaemia in pregnancy has been substantially reduced. A good example of this phenomenon has been described by Giles (1966) from

Stoke-on-Trent. During 1957, three per cent of all women delivering in the obstetric unit had megaloblastic anaemia. This incidence decreased to 1.2 per cent in 1960 after prophylactic folic acid was initiated for all antenatal clinic patients. As general practitioners in the area began to dispense folic acid to their patients, the incidence decreased further and was 0.6 per cent in 1964. Giles (1966) comments that although these figures are comparable with each other, "they are almost certainly an underestimate of the true incidence of the disease".

(b) Reasons for the persistence of folate deficiency and megaloblastic anaemia in pregnancy and the puerperium

The fact that megaloblastic anaemia in pregnancy was seen at all in 1964 in Giles' study illustrates an important point. As the disease "disappeared among the regular attenders at the hospital antenatal clinics," and yet the incidence was only reduced by 80 per cent, it can be assumed that a fifth of all patients at risk did not actually take folic acid supplements.

The knowledge that megaloblastic anaemia of pregnancy is easily prevented by the administration of folic acid tablets has not meant that the disease has disappeared. Herbert (1972a), lecturing to the International Congress of Haematology 27 years after Moore and his co-workers (1945) demonstrated response to folic acid, said that "studies in various countries suggest that up to as much as a third of all the pregnant women in the world

have folate deficiency" and suggested studies directed towards "developing public health programs to wipe out such deficiency". It is clear that the problem of preventing folate deficiency in pregnancy has shifted from the fields of diagnostic and therapeutic medicine to that of health care delivery.

A factor contributing to the high incidence of folate deficiency in pregnant women is probably the reluctance of pregnant women to take their tablets. Bonnar and his coworkers (1969) found that significant numbers of their antenatal patients did not take their iron tablets. It might be argued that iron, unlike folic acid, discolours the stool and can cause gastrointestinal problems, and that these factors would not discourage women from taking folic acid tablets. However, as folic acid in pregnancy is almost invariably given in the form of tablets containing iron as well, the same problems would be expected to arise.

Metz (1966) has commented on the "insurmountable difficulties" encountered in attempts to administer folic acid to South African Negro women. He said, "Many of the patients fail to take the tablets issued to them; many first attend antenatal clinics late in pregnancy, so that supplementation begins only in the last trimester .... folic acid supplementation during pregnancy failed appreciably to reduce the incidence of anaemia at term."

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The failure of pregnant women to attend antenatal clinics regularly is not limited to populations in developing countries. There have been several surveys indicating that underprivileged American mothers show similar behaviour patterns. Antenatal care was either inadequate or was not sought at all in the large numbers of women in major American cities detailed in Table 1.1.

TABLE 1.1

Proportion of women who received little or no antenatal care

Percentage	City	Year	Characteristics
62.1	Boston	1956	Income less than $2 000 p.a.[1]
63	Boston	1956	Less than 8 years schooling[1]
47	Baltimore	1958	Municipal hospital [2]
47	New York	1961	Municipal Hospital [3]
20-50	Atlanta	1966	Low income [4]

Sources 1 - Donabedian and Rosenfeld, 1961
2 - Buetow, 1961
3 - Schoeneck, 1964
4 - Lesser, 1968.

Other studies in the United States have indicated that antenatal attendance falls off with increasing parity, and that it is the woman of high parity from a deprived socioeconomic background who is susceptible to folate deficiency (Schonfield et al., 1962; Gallagher, 1967; Kahn et al., 1970).

The author has not been able to ascertain what proportion of South African Negro women receive antenatal care, but it is apparent that this figure would be very low, particularly in rural areas. Problems arising in the recently proclaimed "Bantu homelands" of South Africa are discussed in the following chapter, but it can be stated here that the prospects for rapid improvement in antenatal facilities for South African Negro women are not good. The same is probably true of most areas in Africa, Asia and Latin America where folate deficiency is common. It would appear likely that the immediate problem of folate undernutrition in pregnancy will have to be solved outside the sphere of antenatal clinics.

C H A P T E R 2

AIM OF THE PRESENT STUDY

I Introduction

The broad aim of the present study is to solve the problem, posed in the previous chapter, of preventing folate deficiency in pregnancy by means other than the distribution of folic acid tablets at antenatal clinics. This is necessary because significant numbers of pregnant women do not attend these clinics, and of those who do attend a significant proportion do not take the tablets. These two factors are particularly common in areas where megaloblastic anaemia of pregnancy and lactation is prevalent, the evidence for this having been reviewed in the previous chapter.

It is the intention of the author to develop a means of increasing the dietary folate intake of pregnant women. However, there is no reason to believe that pregnant women would be more willing or able to comply with any such alternative measure aimed specifically at them than they are to take folic acid tablets at antenatal clinics. Therefore, a successful alternative will result in increased folate intake in non-pregnant members of the population as well. It is for this reason that the prevalence of folate deficiency in non-pregnant groups has been reviewed in the previous chapter.

The main aim of this study is to develop the above-mentioned ideas for specific use in the South African context. It is hoped, however, that ancillary investigations will facilitate the use of data presented here in developing similar programmes in other areas where these problems are encountered.

The necessity for a study along the lines of this one has been previously recognised. Metz and his co-workers (1970) made preliminary suggestions after asking the question "Can the folate content of maize be increased in order to reduce the incidence of deficiency in maize-eating populations?" Streiff (1971b) introduced a report with the words "folate deficiency in man is more prevalent than seems reasonable, particularly when considering the many rich dietary sources of folate that are readily available". Herbert (1972a) suggested investigations aimed at the elimination of folate deficiency by dietary measures, concluding that "such a study has not yet been carried out, and would appear to be both urgent and of enormous public health benefit". Jelliffe and Jelliffe (1972) reviewed nutritional problems, including folate deficiency, in lactating mothers and expressed the view that "research into these important interactions should be a priority in world nutrition."

The supply of specific nutrients could be increased by the addition of preparations such as the "Nutricube"

(Roche) to the cooking pot (Smith and Unglaub, 1972), but most authorities believe that "this would offer little or no advantage over the provision of direct supplements to each member of the target group" (FAO/WHO, 1971). The FAO/WHO Expert Committee on Nutrition recommended that, where possible, inadequate nutrition should be rectified using natural food supplies (FAO/WHO, 1971).

II The possibility of supplying foods rich in natural folate to populations at risk

(a) Previous work

The possibility that low folate diets might be improved using natural foodstuffs has been considered previously. Baumslag and Metz (1970) concluded their report of a therapeutic trial of whole lettuce in megaloblastic anaemia of lactation with the suggestion that "the consumption of adequate amounts of unprepared green leafy vegetables in pregnancy and lactation appears of major importance in the prevention of megaloblastic anaemia". Jelliffe (1967) prepared "multimixes" from legumes and dark-green leafy vegetables for use in weaning infants. He subsequently suggested the use of these preparations, which are rich in folate as well as amino-acids and other nutrients, to improve the "diet of young children, pregnant and lactating women, and, indeed, whole communities in less developed, largely tropical regions" (Jelliffe and Jelliffe, 1971). Streiff (1971b) suggested that frozen orange juice, which he found to have a high content of unconjugated

folate (50 ug per 100ml), would be a "reasonable and stable dietary supplement for people needing additional amounts of folate". This suggestion appears unacceptable in view of the subsequent observation that the folate in orange juice is poorly absorbed (Tamura and Stokstad, 1973). In a discussion of the problem of folate deficiency in developing populations, it was suggested that this could be "eliminated from almost any population group by daily ingestion of one fresh raw fruit, fruit juice or vegetable daily" (Herbert, 1972a).

(b) The availability of food folate

It is uncertain what proportion of the food folate activity determined by microbiological assay is available to man. Herbert (1963a) suggested that the L. casei activity of foods without conjugase treatment was a closer approximation of the available folate than the postconjugase activity. Subsequent studies suggested that conjugated forms of yeast folate were absorbed and utilised to about one-third of the extent of unconjugated forms at doses greater than 200 ug, but that absorption of the two forms was similar at a dose of 200 ug (Streiff and Rosenberg, 1967; Hoffbrand and Necheles, 1968; Perry and Chanarin, 1968; Hoffbrand and Peters, 1970).

Most studies of food folate absorption have used yeast or yeast derivatives. Retief (1969) studied folate absorption from several different foods and showed that there was wide variation in the availability of folate

from food to food. His results suggested that both conjugated and unconjugated forms of folate are efficiently absorbed from at least some dietary items, such as calf's liver, spinach and peas, whereas absorption is delayed and less efficient from tomato, cauliflower and pumpkin. The observations of Retief have been confirmed by Tamura and Stokstad (1973), who demonstrated that the folate in banana, lima beans, liver and brewer's yeast was readily absorbed, while that in orange juice, romaine lettuce, egg yolk, cabbage, defatted soybean and wheat germ was poorly absorbed. Baumslag and Metz (1964) reported marked haematological response in a lactating patient with megaloblastic anaemia who ate three heads of lettuce per day for seven days, suggesting that lettuce folate absorption in this particular situation was good.

The reason why availability of the same folate compounds should vary from food to food is not clear. For example, the principal form of folate in orange juice is monoglutamate (Streiff, 1971b) which is known to be easily absorbed, and the juice contains large amounts of glucose, which has been shown to enhance monoglutamate absorption (Gerson et al., 1971). Despite these properties, Tamura and Stokstad (1973) reported that only 35 per cent of orange juice folate was available for absorption, and that increasing the pH had little effect. Retief (1969) suggested that the urinary excretion

methods used by himself and other workers to assess absorption of folate, which were also used subsequently by Tamura and Stokstad (1973), could be criticised because they required the use of relatively large doses of folate. He considered that it would be physiologically more sound to assess the absorption of food folate by means of therapeutic trials such as that conducted by Baumslag and Metz in a patient with folate-deficient megaloblastic anaemia.

(c) The folate content of modern diets

There have been numerous studies of the folate content of foods since Toepfer and his coworkers published their original extensive study in 1951. The folate content of uncooked foods is greatly diminished by methods used for preparing food, and this subject is reviewed in Chapter four. After cooking, the average western diet contains about 600 - 700 µg of folate daily, of which about a quarter is in the unconjugated form (Butterworth et al., 1963; Hurdle et al., 1968; Perry, 1971). It would be expected that this type of diet would be adequate to supply the 300 µg folic acid required daily during pregnancy, and that folate deficient megaloblastic anaemia in pregnancy would be rare in populations subsisting on this diet. This would appear to be true in some relatively well-nourished western women (Fletcher et al., 1971).

However, dietary histories obtained from women in impoverished circumstances have suggested that their food contains very little folate. For example, Edelstein et al. (1966) obtained dietary histories from 235 Negro women in Johannesburg and found that maize meal comprised the major item of diet. Although the maize meal was often supplemented by animal products and by vegetables boiled for long periods, these were invariably eaten in small quantities only. It has been shown that even before cooking, the most popular brands of South African maize meal contain less than 40 µg total folate per kg, of which about two-thirds is unconjugated (Metz et al., 1970). It is not surprising that megaloblastic anaemia of pregnancy and lactation has been reported to be prevalent in these women, whose staple item of diet is a folate-poor cereal.

(d) Economic factors affecting the nutrition of South African Negroes

Poverty is the main cause of malnutrition, including folate deficiency, in the South African Negro, although there is little doubt that an intensive health education programme would be required even if low cost foods rich in folate were freely available. Studies of wages and prices in South Africa are almost entirely limited to urban areas.

On the basis of wage surveys conducted by the University of South Africa's Bureau of Market Research

in 1970, it has been estimated that at least 79 per cent of Negro workers in urban areas were being remunerated at a rate below that necessary to pay for the minimum amounts of food, fuel, lighting, household cleaning, rent and transport to work (Poverty Datum Line); only 3 - 7½ per cent received wages above the level required to meet more long-term needs such as medicine, education and recreation (Horrell et al., 1973).

Two more recent studies allow a less dated analysis. The University of the Orang. Free State reported a wage survey, conducted during the first half of 1972, of 110 Bloemfontein firms employing 14,302 workers, of whom 59.4 per cent were Negroes (van Breda and Langenhoven, 1972). Of the Negro sample, 61.1 per cent earned R5 to R9 weekly, and 35.3 per cent earned R10 to R19 weekly. The consumer price index rose by 9.98 per cent from June 1972 to June 1973 (South Africa. Department of Statistics, 1973). In April 1973, a comprehensive survey of the Poverty Datum Line was conducted in urban centres of the Republic, and the figure derived for Bloemfontein was R74-55 monthly (Potgieter, 1973). This corresponds to a weekly requirement of R15-64 at the time that the wage survey was conducted in that city, accounting for the lower consumer price index.

It must be emphasised that the above example is a most conservative one. Firstly, the consumer price index does not include items such as rent, and the

Poverty Datum Line would thus have been higher than the figure calculated by the author. Secondly, Potgieter's figures are the lowest of several estimations of the Poverty Datum Line conducted in the areas he surveyed (Horrell et al., 1974). Finally, as Potgieter concludes, "The Poverty Datum Line ... is at best a conservative estimate or a bare theoretical minimum, taking only short-term considerations into account and assuming the wisest of expenditure .... I sincerely believe that to offer wages less than the PDL under present living conditions is inhuman, whatever the economic circumstances".

There is a dearth of similar information about rural areas, particularly the recently-proclaimed "Bantu homelands". According to the 1970 South African Census, there were approximately 15 million Negro inhabitants of the Republic at that time, of whom 7 million were living in the homelands. The average population density of the homelands was 119 per square mile, compared with a figure of 35 persons of all races per square mile in the rest of the Republic; since that time there have been large tracts of land added to the homelands and many Negroes have been moved into these areas, adding greatly to the population (Horrel et al., 1973). Despite the fact that a large proportion of the Republic's Negro population lives in these areas, the author has been unable to locate any survey of wages or prices there conducted within the past five years. The results of preliminary investigations conducted at the

site of the present study are reported elsewhere.

It is apparent that Negroes could not have paid for more nutritious food at the time that the information above was reported. During 1973 there have been major increases in the wages of many Negroes, but it is as yet unclear whether these will be adequate to account for rising prices (Horrell et al., 1974).

It is theoretically possible, however, that folate intake could be greatly increased by other means. Fifteen years ago, the National Nutrition Research Institute of the Council for Scientific and Industrial Research recommended that the Government establish permanent machinery to devise nutritional programmes, subsidise certain foods, and encourage agricultural production in areas with large Negro populations (C.S.I.R., 1959).

A possibility which has received considerable publicity, with some good results, is the provision of surplus fruit to undernourished people. In the middle of the 1967 citrus season, it was reported that 770 tons of citrus fruit were being left to rot on a single South African farm (The Star, Johannesburg, 11th July, 1967). One year later, 600 tons of citrus fruit had been given to charity (The Star, 18th July, 1968). During the 1973 season, a drought and a high export demand combined to keep surplus citrus off the local

market (Rand Daily Mail, Johannesburg, 1st August, 1973). An unexpected beneficial effect of unfavourable circumstances occurred later in the season when a British dock strike and a boycott of South African fruit resulted in increased local availability (The Star, 17th August 1973; Rand Daily Mail, 30th August 1973).

Poor organisation still results in wastage of food. During the first week of December 1973, South African farmers ploughed 960 tons of surplus bananas into the ground after failing in attempts to give them to charities in exchange for transport costs (Sunday Tribune, Durban, 9th December 1973). The basic price of the fruit had been paid by the Banana Control Board. Based on the availability of banana folate (Tamura and Stokstad, 1973), and allowing for 25 per cent by weight of the fruit being skin and stalk (personal observation on two bunches of bananas), this represents the total folate requirement for more than 5 000 pregnancies.

Whereas it is clear that attempts are being made to provide surplus foods to poor people, at this time these efforts are inadequate. Until government agencies assume responsibility for this task, it is unlikely that this source will provide the folate that is lacking in the diet of South African Negroes. On the basis of the buying power of Negroes and the history of subsidised schemes, it is concluded that it is not economically or practically feasible to increase the supply of food

folate to populations at risk. The long-term prospects are promising but inconclusive. In this situation, it has been recommended that authorities should "improve the quality of the food supply immediately, without necessarily changing food habits, by adding nutrients directly to food" (FAO/WHO, 1971).

III The prospects for improvement of folate nutrition by food fortification

(a) History of food fortification

The fortification of salt with iodine compounds was first recommended some fifty years ago, and has since been adopted by many countries. This was followed by the use of natural sources of vitamins A, D, and the B-group to fortify various foodstuffs in the period 1920-1940 (FAO/WHO, 1971).

The United States was the first country to add iron to food, a policy continued from 1941 (National Research Council, 1944) until the present, when proposals to increase the amount of iron used to fortify flour are being considered (Finch and Monsen, 1972). Flour has been enriched with iron in Britain since 1953 (Elwood, 1963). Rice was fortified with iron in the Philippines in 1948 (National Research Council, 1950) and similar programmes have been conducted in Japan, Taiwan and Papua - New Guinea (FAO/WHO, 1971). Ten years ago, it was estimated that 250 million people in the western

world were eating fortified bread (Roche, 1963).

In South Africa, iron deficiency in Negroes was thought to be rare (Gerritsen and Walker, 1953) and fortification of food with iron was not considered. It can be mentioned in passing that the abovementioned belief was questioned and iron deficiency was shown to be not uncommon in pregnant Negro females, and particularly common in malnourished infants (Cassel and Metz, 1958; Greig, 1959; Turchetti et al., 1966).

The high incidence of protein-calorie malnutrition motivated a decision by the South African Department of Nutrition in 1952 to fortify brown bread with a mixture of defatted groundnut flour, skim milk powder and calcium. Fish flour was subsequently used as an alternative on an experimental basis in some parts of the Western Province (C.S.I.R., 1959). This experiment was abandoned following the negative report of the National Nutrition Research Institute (C.S.I.R., 1959).

More recently, analysis of biochemical data has indicated a high incidence of riboflavin and nicotinamide deficiency among Negro schoolchildren (du Plessis, 1967) and a successful trial has been conducted using maize meal fortified with these two vitamins (du Plessis et al., 1971). The report on this trial concludes, "it is strongly recommended that a compulsory national maize meal enrichment scheme should be introduced with the least possible delay." The Department of Health of the

South African Government has accepted this recommendation in principle and is assessing the practical implications before making an official recommendation to the Cabinet (Raymond, 1972).

(b) Principles of food fortification as defined by the World Health Organisation

The National Nutrition Research Institute (C.S.I.R., 1959) considered the policies for adding nutrients to foods of the United States, Britain and other countries and concluded that the following principles had been used to govern such policies:

"Evidence that there is a need for the nutrients to be added by enrichment.

Evidence that the enrichment of a staple food has advantages over the use of natural foods as a means of improving the diet.

Evidence that the amounts of enriching substances provided are sufficient to satisfy the proven need.

Proof that the enrichment medium is regularly consumed in important quantities by those in need of enrichment.

Evidence that enrichment can be easily carried out in practice.

Evidence that the enriching substances or preparations do not appreciably alter the appearance, taste or physical characteristics of the medium.

Evidence that the enriching substance or preparations are free from bacterial contamination or toxicity.

Evidence that the cost of the enriching material is low in relation to their nutritive value.

In cases where all the nutritional deficiencies are not satisfied simultaneously, evidence that the particular enriching nutrients will not fail to exert their beneficial effects."

These criteria correspond closely with those set out by the Joint FAO/WHO Expert Committee on Nutrition (FAO/WHO, 1971), which are discussed hereunder and used as the main reference for this study.

According to the Expert Committee, the objective of a food fortification programme should be decided and then the programme designed with due consideration for the definition of nutrient needs and target groups, the selection of a vehicle, the level of fortification and technological aspects. The design of such a programme should also include methods of assessing its effectiveness, of legislating for and controlling the programme, and of ensuring that economic considerations are not neglected.

The primary objective of a food fortification programme should be to improve and maintain the health of individuals in the population. The programme may "be designed to meet the needs of people in particular geographic areas, of particular socioeconomic groups, or of particular age and sex groups". The Committee noted that there is no unanimity about the role food

fortification should play in solving nutrition problems. Some of the points which have led to these differences of opinion are that knowledge of human nutrient requirements remains inadequate, that fortification with a small number of nutrients is often embarked upon whereas other nutrients are neglected, that some nutrients previously used, notably vitamin D, have proved harmful in certain situations, and that fortification programmes may divert limited resources and efforts from more important approaches such as the maintenance of the basic quality of food products and the development of nutrition education programmes.

The definition of nutrient needs and target groups is the next step. It is pointed out that food consumption surveys are usually based at the family level, thus leaving unanswered the question of distribution within the family. Where there is clinical evidence of a deficiency disease, the prevalence of the disease itself will serve to describe the primary target group. It is then only necessary to establish that the disease will respond to the nutrient in question and to determine whether other segments of the population have evidence of deficiency, thus constituting a secondary target group. Wherever possible, biochemical criteria should be used to reinforce other studies. Where fortification is accepted as a possible solution, and the programme proves relatively inexpensive, it may be more economical

to avoid spending additional money to define the target group precisely, and to aim at a rather wide group of the population. It is pointed out that the cost of adding a second or third nutrient may be relatively low, once the initial programme is established.

In selecting a vehicle for fortification, it is desirable to choose a food used preferentially by the target group. In general, staple foods which are consumed in relatively large amounts are chosen as vehicles, and sugar, soft drinks and alcoholic beverages are avoided. The level of fortification is calculated according to the estimated nutritional need for the nutrient and the distribution of individual intakes. In practice it is impossible to ensure that those with the lowest intakes of the vehicle food receive adequate quantities of the nutrient, but it is necessary to know the upper range of intake of the vehicle food when potentially toxic nutrients are used.

The most important technological factor to be considered in designing a fortification programme is the stage in the processing of the vehicle at which the nutrient can be added. It is desirable to minimise the number of centres at which the fortification takes place, possibly by making use of premixes. Tests should be run periodically to ensure a uniform rate of addition and thorough mixing.

It must also be established that the nutrient is

biologically available when the vehicle is eaten, and that it does not confer unwanted properties (colour, taste, flavour, cooking properties) or unduly increase the cost of the product. The effect of home cooking practices upon the retention of the nutrient should be checked.

Methods of legislating for, controlling, and monitoring established food fortification programmes will not be considered.

Economic considerations will be discussed after description of the field work.

(c) Folic acid and the W.H.O. recommendations on food fortification

The principles enumerated above are here considered in relation to the proposal to fortify food with folic acid.

The need to increase the folate intake of pregnant and lactating women has been dealt with in the previous chapter. There is no question that this need is urgent in South Africa and many other countries. Pregnant women thus constitute the primary target group. In addition, the evidence of folate deficiency in other sections of the population has been reviewed. The prevalence of protein-calorie malnutrition in South Africa has been mentioned, and the association between this disease and folate deficiency has been discussed, as well as the high incidence of infantile megaloblastic anaemia due to folate deficiency. The evidence for

folate deficiency among non-pregnant females and elderly patients has been outlined. Thus the whole South African Negro population, with the exception of young adult males, might be said to constitute a secondary target group, although the need for increased folate intake in infants and children is probably far greater than in other non-pregnant groups. However, as has been pointed out, there has been no comprehensive survey of the incidence of folate deficiency outside of pregnancy and lactation in healthy South African Negroes.

The possibility of using natural folate to increase intake of the vitamin has been considered. Whereas special mixes have been used in some specific situations, the fact that these entail a change in dietary habits limits their use among unsophisticated rural populations. Economic and political factors militate against the use of surplus fruits and vegetables to supply folate to populations at risk. Folic acid (pteroylmonoglutamic acid) has several inherent properties which suggest that it would be more suitable than food folate for use in a food fortification programme. Firstly, it is available in pure form at economic prices, and is manufactured commercially in large quantities. Secondly, unlike food folate which is destroyed by boiling (Herbert, 1963a), it is stable at temperatures usually used for cooking (Herbert, 1965) and baking (Dawson et al., 1969). Thirdly, the addition of the substance to the diet would

not necessitate a change in dietary habits, which would entail a costly and problematical health education programme. The response of the deficiency disease to the nutrient has been well known for almost thirty years.

In South Africa, the choice of a vehicle for the nutrient poses no problem. Maize was introduced into Africa by the Portuguese (Casalis, 1861), who had brought it from America, where it was regarded as an inferior human food (Mangelsdorf, 1958). The first record of the plant in South Africa was made in Jan van Riebeeck's diary on 24th July 1658, when he received a sample of grain from New Guinea on the ship Hasselt, which also conveyed a number of slaves to whom the first culture of the crop in South Africa was entrusted (Quin, 1959). The ground meal of the plant is now the staple food of South African Negroes and is selectively eaten in large quantities by this group (Raymond, 1972). The food thus fulfils the criteria for an ideal vehicle for fortification.

The biological availability of folic acid when eaten with this vehicle, the effect of home cooking practices, and the effect of the nutrient on the colour, odour, taste and cooking properties of maize meal form part of the investigations undertaken in this study. Practical factors will be discussed after presentation of the results of the study.

(d) Evidence for harmful effects of folic acid

There has been a single report suggesting that folic acid might have adverse effects when given in pharmacological doses to healthy volunteers. Of fourteen subjects taking 15 mg folic acid daily for one month, eight experienced gastrointestinal symptoms, nine altered sleep patterns, and eight behaviour changes; only one patient remained unaffected throughout (Hunter et al., 1970). This report caused considerable concern, as these doses of folic acid had previously been given to pregnant women and anaemic patients without untoward effect (e.g. Marshall and Jandl, 1960). Double-blind controlled studies subsequently failed to demonstrate any harmful effect of folic acid in doses equal to or double those used by Hunter et al. (Hellström, 1971; Richens, 1971). The work of Hunter et al. has been severely criticised because it was not controlled and the symptoms described were of a type usually associated with apprehension and anxiety (Salter, 1971).

An allergic sensitivity reaction to folic acid has been reported in a single subject after ingestion of 20 mg folic acid by mouth; this reaction was confirmed by intradermal tests (Chanarin et al., 1957). This phenomenon has never been reported again, despite the large number of patients receiving folic acid. It is the opinion of the author that, in the context of this study, this isolated case report can be regarded

as no more important than the numerous types of food allergy.

In epilepsy, the administration of folic acid appears to have been associated with increased fit frequency in isolated individuals (Chanarin et al., 1960; Reynolds, 1967), but no such effect has been observed in five separate controlled studies (Grant and Stores, 1970; Jensen and Olesen, 1970; Ralston, Snaith, and Hirley, 1970; Moore and Ball, 1972; Mattson et al., 1973). Hawkins and Meynell (1958) and Neubauer (1970) reported that fit frequency decreased after folic acid therapy. There is experimental evidence that folic acid may act as a cerebral excitant when injected into rat brain (Spector, 1972), but this may be irrelevant to physiological studies.

Reynolds (1967) and Neubauer (1970) noted an improvement in mental condition after folate therapy in 50 of 76 folate-deficient epileptics on anticonvulsants. No behavioural response was noted in the five controlled studies mentioned above (Grant and Stores, 1970; Jensen and Olesen, 1970; Ralston, Snaith and Hirley, 1970; Moore and Ball, 1972; Mattson et al., 1973). Dow (1971) documented electroencephalogram changes in a patient with folate deficiency without anaemia, which returned to normal after folic acid therapy.

It is concluded that folic acid has no adverse effect when given to the vast majority of epileptics,

but that pharmacological doses may possibly increase fit frequency in occasional patients.

The only situation in which folic acid is known to cause adverse effects in man is in patients with vitamin B_{12} deficient megaloblastic anaemia, such as pernicious anaemia. It has been mentioned that the vitamin effects haematological response in this disease when given in pharmacological doses, and that some patients may respond to as little as 400 µg daily. However, there is evidence that 50 per cent of these patients subsequently show haematological relapse (Will et al., 1959) and it has been disputed whether the blood film ever reverts to normal (Herbert, 1963b). It is recognised, however, that the administration of folic acid in sufficient dosage to patients with undiagnosed pernicious anaemia might lead to a delay in diagnosis, by virtue of raising the haemoglobin level. This could allow neurological changes to progress in an occasional patient.

The question of whether folic acid administration hastens the development of neurological disease in vitamin B_{12} deficiency is more controversial. There has been a suggestion that anaemia may protect against vitamin B_{12} neuropathy by reducing the activity of red cell "thiocyanate oxidase" (Wilson and Langman, 1966). Haematological response to folic acid would remove this protective mechanism. This suggestion depends on the hypothesis that chronic cyanide intoxication is responsible

for some vitamin B_{12} neuropathy. The proposers of this hypothesis were unable to confirm this theory when it was tested (Wells et al., 1972).

Clinical evidence for increased vitamin B_{12} neuropathy after folic acid therapy can be examined by comparing the reported incidence of this phenomenon with that in patients not receiving folic acid. Of 466 patients with pernicious anaemia, Cox (1961) found that 38 per cent had evidence of spinal cord damage, 23 per cent had parasthesiae alone, and 39 per cent had no evidence of neurological involvement. These figures can be compared with two reports of long-term treatment with folic acid in pernicious anaemia. Schwartz et al. (1950) reported on 85 patients with this disease treated with 5 mg folic acid daily for $3\frac{1}{2}$ years. At the end of this period, 37 per cent had some evidence of neuropathy and only 14 per cent remained free of any symptoms. Will et al. (1959) treated 36 patients for up to ten years with 30 mg folic acid daily, of whom 8 per cent remained well. Sixteen patients (44 per cent) developed neurological disease, more than half of these being associated with haematological relapses. Parasthesiae were considered to be evidence of neurological relapse. Twenty-one patients with haematological relapses were treated with folic acid in doses increasing to either 50 or 100 mg daily, and all of these eventually developed neurological relapses.

Three conclusions can be drawn from the above reports. The first is that the incidence of neurological disease in patients with pernicious anaemia (61 per cent) was no higher in groups of patients receiving daily folic acid doses of 5 mg (37 per cent) or 30 mg (44 per cent) for up to ten years. Secondly, the administration of folic acid in doses of 50 - 100 mg daily was associated with neurological relapse in all patients with the disease. A final point, which has profound significance for the present study, is that only 8 and 14 per cent of the two groups of patients receiving folic acid remained well, the remainder developing haematological relapses, glossitis, weight loss and a "loss of well-being". This suggests that only a very small proportion of patients with undiagnosed pernicious anaemia receiving very large doses of folic acid would remain undiagnosed by virtue of failing to seek medical advice.

It must be emphasised that the doses of folic acid used in the above studies ranged from 15 - 300 times those being considered in the present study. It appears unlikely that a daily dose of folic acid of the order of 300 µg would maintain full, symptom-free haematological remission in patients with primary vitamin B_{12} deficiency. Since studies of long-term low-dosage folate therapy in pernicious anaemia cannot be justified, as this would entail withholding vitamin B_{12}, it is highly unlikely that firm evidence on this point will become available.

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It is concluded that available data do not exclude the possibility that low dose folic acid therapy may have adverse effects in an occasional patient with undiagnosed pernicious anaemia. The question of whether it is ethical to fortify foods in order to prevent widespread deficiency when an occasional patient might be prejudiced has received attention in relation to food fortification with iron. In general, it is felt that fortification is justified, and that harmful effects could be avoided by measures such as the provision of unfortified foods to patients with rare diseases (FAO/WHO, 1971; Finch and Monsen, 1972; Darby, 1972). Some workers remain opposed to the use of any potentially hazardous material, no matter how few patients are affected (Crosby, 1973). This subject receives further attention in the final chapter of this thesis.

(e) The level of fortification

The amount of nutrient to be added to food in order to prevent deficiency poses one of the major problems in designing food fortification programmes. It is known that the amount of iron added to American wheat products for the past thirty years has been inadequate to prevent deficiency (Council on Foods and Nutrition, 1972), and the Food and Drug Administration has proposed that this amount should be tripled (F.D.A., 1971). This example is given in order illustrate the degree of error which can be made, even when a

country with advanced medical technology is dealing with a widespread deficiency which affects an estimated 30 million women in the United States, of whom about one sixth have consequent anaemia (Finch and Monsen, 1972).

The desirable level of fortification depends on two factors, namely the consumption of the vehicle food by the population and the dose of nutrient which should be delivered to the population. In relation to the amount of the vehicle food consumed, it has been mentioned that authorities consider that it will be impossible to ensure that those with the lowest intakes receive adequate quantities of the nutrient (FAO/WHO, 1971). As large doses of folic acid may be potentially harmful in subjects with vitamin B_{12}-deficient megaloblastic anaemia, the upper range of consumption must also be considered to ensure that the safe dose of nutrient is not exceeded. Dietary surveys in South Africa have usually expressed only the mean maize meal intake (C.S.I.R., 1959; Quin, 1959). Du Plessis et al. (1971) give more information by stating "the mean intake was found to be 474 $\pm$ 127 g per adult per day". From this the confidence limits may be calculated but not the range of consumption. One of the objectives of the present study will be to conduct a survey of maize meal consumption at the site of the study. This will be done by determining the amount of maize meal bought in the home, as this method has been found to be much more reliable than weighing the

maize meal cooked for a particular meal (du Plessis et al., 1971). The validity of this method will be further tested by comparing the stated consumption of maize meal with the observed consumption of fortified maize by families given the latter for use in the house.

Previous workers experienced considerable difficulty in determining the quantity of a nutrient which should be consumed by groups of people. The nutritional requirements for folic acid have been discussed in the previous chapter, where it was stated that the daily requirement of pregnant women (300 µg) exceeded that of other groups. The Recommended Dietary Allowances for the United States have been mentioned (National Academy of Sciences - National Research Council, 1968).

Beaton (1972) showed convincingly that standards such as the Recommended Dietary Allowances should be interpreted with caution, when relating these to the expected incidence of deficiency. He obtained dietary histories from 825 Canadian women, and found that 74 per cent were consuming less than the 14 mg iron daily recommended for women by the FAO/WHO (FAO/WHO, 1970). Assuming that these histories were reliable, this might suggest that iron deficiency would develop in the majority of women. However, Beaton then used the data on menstrual blood loss carefully quantitated by Hallberg et al. (1966) to predict the expected incidence of iron deficiency. He calculated that

although 74 per cent of the women had an estimated iron intake below recommended levels, only 12.5 per cent would be expected to develop iron deficiency (Table 2.1).

TABLE 2.1

Prediction of prevalence of iron "deficiency"*

Iron intake	Observed number	Probability of "deficiency"	Predicted number of "deficients"
mg/day			
Below 4.0	3	1.00	3
4.0 - 4.9	6	0.92	6
5.0 - 5.9	12	0.75	8
6.0 - 6.9	18	0.50	9
7.0 - 7.9	48	0.32	15
8.0 - 8.9	57	0.23	13
9.0 - 9.9	85	0.16	14
10.0 -10.9	107	0.12	13
11.0 -11.9	93	0.08	8
12.0 -12.9	100	0.06	6
13.0 -13.9**	73	0.05	4
14.0 -14.9	53	0.04	2
15.0 -15.9	51	0.03	2
16.0 -16.9	114	0.01	1
TOTAL	825		184

Predicted prevalence = 12.5 per cent

* Taken from Beaton (1972)

** The recommended iron intake for women is 14 mg per day.

Hegsted (1972) cites the work of Beaton (1972) and the fact that nutrient requirements vary in different people as evidence for his belief that the nutritional

status of a given population could not be accurately assessed by calculating the differences between dietary intake and recommended intake. In view of these comments, the author will assess nutritional folate status and the value of different levels of fortification by means of a sensitive laboratory index of folate nutrition viz. the red cell folate concentration. These observations will be mainly concerned with the primary target group, pregnant women.

IV Site of the present study

The present study was based at Nqutu, which is within the boundaries of the recently proclaimed homeland of KwaZulu, South Africa (Figure 2.1). The town is set in hilly country and is at an altitude of 4,400 feet above sea level (South Africa. Trigonometrical Survey, 1955). It is 50 km from the White town of Dundee and approximately 420 km from Johannesburg. This area was selected because the Charles Johnson Memorial Hospital, which serves the area, routinely admits healthy pregnant women to its lodging facilities for the last few weeks of pregnancy, in order to avoid the hazards of travelling long distances once labour has been initiated. These women are lodged in a hall. Antenatal care is given at the hospital and at a number of outlying clinics, which are in general staffed for one day in each fortnightly cycle. The hospital provides a unique

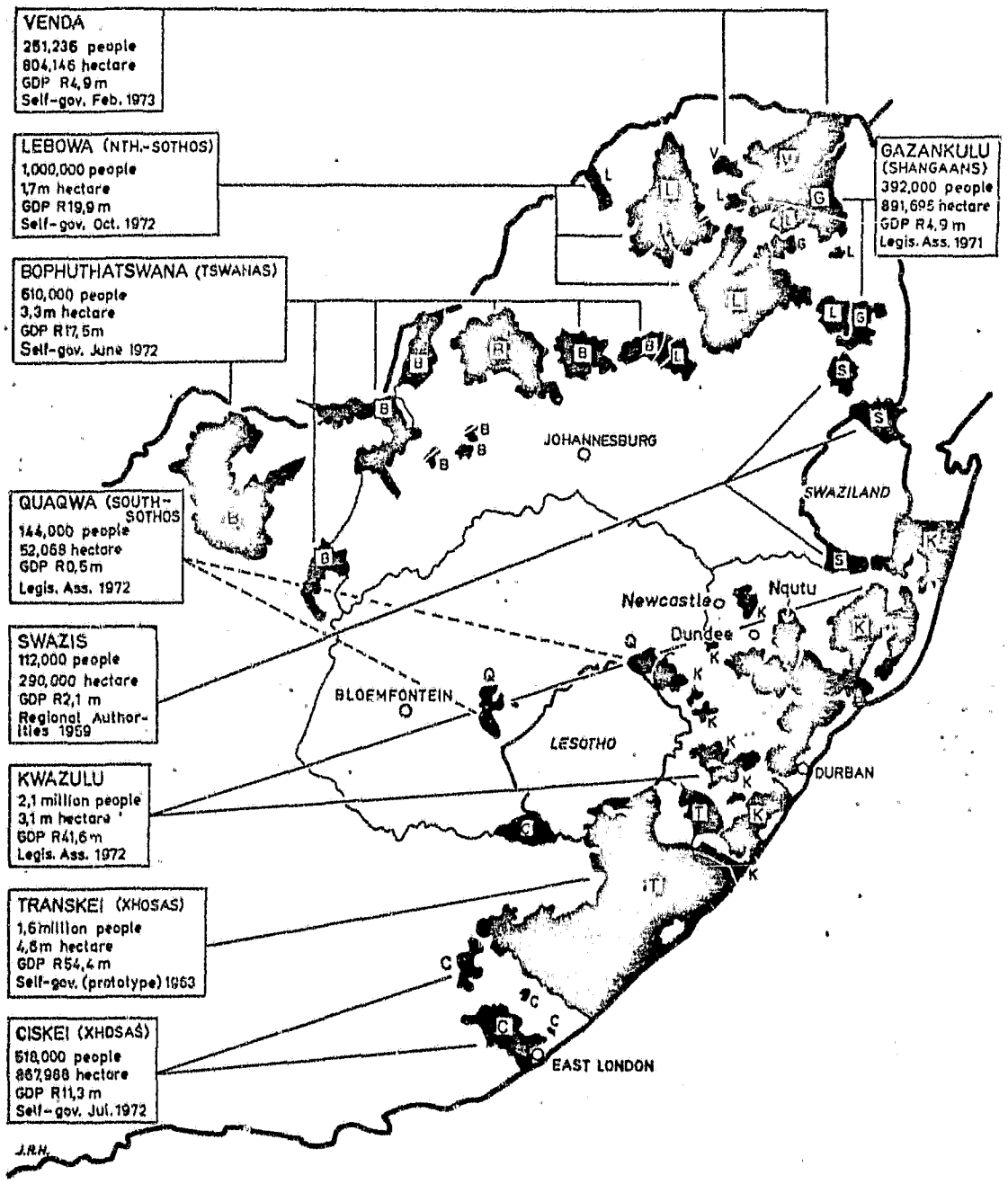

FIGURE 2.1. Map of South Africa showing the "Bantu homelands" and the location of the Magisterial District of Nqutu. (Adapted from The Star, October 19, 1972).

opportunity to supervise and control the diet of healthy pregnant women.

The magisterial district of Nqutu is in many ways representative of a typical homeland district. The district encompasses an area of approximately 700 square miles (E.A. Barker, personal communication), within which 79,690 Negroes and 408 Whites were enumerated by the 1970 South African Census (South Africa. Department of Statistics, 1970) which was double the population enumerated by the 1951 Census (South Africa. Department of Statistics, 1951). This represents a population density of 114 Negroes per square mile.

The hospital staff have attempted to assess the cash income and requirements of the population. During 1972 a preliminary survey of 517 families revealed a mean monthly cash income of R11-44 per family, with a mean family size of 7.26 members (Financial Mail, Johannesburg, 15th June 1973).

During 1973, 150 randomly selected families were surveyed, of whic 120 came from the more established areas and 30 from the resettlement areas to which people had been removed from White areas (E. Clarke, personal communication). The mean monthly cash income was R12-37, with a mean family size of 6.9 members. Of this income, 81 per cent was earned by migrant labourers in White areas and the remainder in the homeland as wages or payment for handiwork, produce, and other sources. The extent to which this income

was supplemented by livestock and agricultural produce was determined. The livestock owned by the 150 families is shown in Table 2.2, which also shows the number of families to whom the animals belonged. Six of the 67 families who owned cows received a regular milk supply from their animals. Maize was grown by 48 families, whose combined annual harvest was 345 bags, each containing 180 lb (81.54 kg) whole maize.

At the same time that the above survey was conducted, an attempt was made to establish a Poverty Datum Line in the area. Using the same criteria for food as those used in urban areas by Potgieter (1972), the data indicate that the family food supply alone required a cash income of not less than R54 per month (E. Clarke, personal communication). The analysis of the Poverty Datum Line data had not been completed at the time of writing this thesis. It has been pointed out that shoppers are limited to a few general stores, where prices are higher than in urban supermarkets used to calculate urban Poverty Datum Lines (E.A. Barker, personal communication).

As mentioned earlier, one of the aims of the present study was to conduct a survey of cash income of subjects in the study. At the time that the survey was started, there had been no information on wages or prices in the homelands since 1968. The survey was carried out from June 1972 to November 1973, in collaboration with the staff of the Charles Johnson Memorial Hospital.

TABLE 2.2

Livestock owned by 150 families at Nqutu.

Type	Number of animals	Number of families which owned these animals
Cows	300	67
Oxen	800	102
Sheep	438	37
Goats	487	50

CHAPTER 3

METHODS

The methods described here relate to investigations carried out in most parts of the study. Methods exclusive to particular stages of the study are detailed in the relevant chapters.

I Collection of samples and preparation for storage

At designated times during the absorption studies and therapeutic trials, and each week during the clinical trials, approximately 10 ml of venous blood were taken from subjects, using either disposable needles and syringes or vacuum tubes.

A minimum of 3 ml was mixed in a sterile tube with 4 mg ethylene-diamine tetra-acetate (Sequestrene). An aliquot of the anticoagulated specimen was haemolysed and stored at $-20^{o}C$ for assay of red blood cell folate activity. The remainder of the anticoagulated specimen was used for haematological investigations.

The rest of the blood specimen was allowed to clot in a glass tube at room temperature for not less than four hours, after which serum was separated by centrifugation. A one ml aliquot of serum was transferred to a sterile tube containing 10 mg ascorbic acid for folate assay, the remainder being kept for vitamin B_{12}

assay. Both serum samples were stored at -20°C until the day of assay.

Folate and vitamin B_{12} concentrations of all specimens were assayed within 3 months of collection, and all samples from a particular patient were assayed within the same batch.

At other times during the absorption studies and therapeutic trials, venous blood was taken in the normal way for haematological and other investigations. Bone marrow aspirates were taken from patients for therapeutic trial before any therapy had been given.

II Haematological methods

(a) Haematocrit

The haematocrit was estimated by the micro-method detailed by Dacie and Lewis (1968), using an International microhaematocrit centrifuge model MB (International Equipment Company, U.S.A.) and Yankee microhaematocrit tubes No. 1021 (Beckton, Dickinson and Company, New Jersey).

(b) Haemoglobin, red cell count, mean cell volume and mean cell haemoglobin concentration

These indices were measured on a Coulter Counter Model S (Coulter Electronics, Inc., Florida), after standardising the machine against a commercial standard supplied by the manufacturer. The machine measures

haemoglobin by a cyanmethaemoglobin technique, and red cell count and cell volume by recording the interference caused by a dilute stream of blood being drawn through an electric current. The usual method is then used to calculate mean cell haemoglobin concentration.

(c) Staining of peripheral blood smears

Peripheral blood smears were stained automatically with a Hema-tek Slide Stainer (Ames Company, Elkhart, Indiana), which used a modification of Wright's stain.

(d) Reticulocyte counts

Reticulocyte counts were done by the method described by Dacie and Lewis (1968), using brilliant cresyl blue. Cells were counted in each specimen until at least 1000 cells and 100 reticulocytes had been observed.

(e) Platelet counts

Platelets were counted either by visual direct means as described by Dacie and Lewis (1968) using a formal-citrate red-cell diluent, or automatically using a Technicon autocounter (Technicon Instruments Corporation, Tarrytown, New York). This latter instrument records the number of optical disturbances caused by particles larger than a predetermined size when a stream of haemolysed blood is passed through the focal point of a light beam.

(f) Staining of bone marrow smears

Smears of aspirated bone marrow were stained by the method used for peripheral blood smears. Staining for non-haem iron was done as described by Dacie and Lewis (1968).

III Microbiological assay for folate using Lactobacillus casei

(a) Serum L. casei folate activity

This was measured by a modification of the methods of Herbert (1961) and Waters and Mollin (1961), using the serum which had had ascorbic acid added before storage.

Medium

The medium used was Difco folic acid casei medium No. 0822-15 (Difco Laboratories, Detroit).

Stock culture and preparation of inoculum

The organism L. casei (A.T.C.C. 7469) was maintained on dried gelatin discs (Stamp, 1947; Chanarin, Anderson and Mollin, 1958), a fresh disc being used for each assay batch. The disc was incubated in 10 ml Difco micro inoculum broth No. 0320-02 for $\pm$ 30 hours at 37°C. The cells were then washed six times in 20 ml broth and suspended in 20 ml of the medium for inoculation.

Standard solutions

Standard solutions were prepared in concentrations of pteroylglutamic acid (PGA) ranging from 0-1.4 ng per tube, at intervals of 0.2 ng per tube.

Preparations of serum extracts

Sera were diluted 1:40 with 0.1M phosphate buffer pH 6.1, containing 100 mg per cent ascorbic acid, and then autoclaved for $2\frac{1}{2}$ minutes at 15 lb per square inch. The coagulated serum proteins were then centrifuged down.

Assay method

Standards and specimens were set up in triplicate and the assay was carried out in a total volume of 4 ml per tube. Tubes were autoclaved for five minutes at 15 lb per square inch, two of the three tubes inoculated with the organism, and the three tubes were incubated for 18 hours at 37°C. Optical density was measured on a Unicam photoelectric colorimeter, and the colorimeter reading of the uninoculated sample was subtracted from the mean of readings of the corresponding inoculated samples. The folate activity of the sample was then obtained by reference to the standard curve.

(b) Red blood cell L. casei folate activity

This was measured by a modification of the method described by Hoffbrand, Newcombe and Mollin (1966).

An 0.5 ml aliquot of anticoagulated blood was pipetted slowly into 4.5 ml of distilled deionised water containing 1 g per cent of freshly added ascorbic acid. The resulting haemolysate was stored at -20°C until the day of assay, when it was thawed slowly and an aliquot of 0.5 ml was pipetted into 19.5 ml of 0.1M phosphate buffer pH 6.1 containing 100 mg per cent ascorbic acid.

The remaining stages of the assay were the same as those for assay of serum folate activity.

When the folate activity of the anticoagulated blood sample had been calculated, red cell folate concentration was calculated from the formula:

$$\text{Red cell folate} = \frac{\text{Whole blood folate} - \text{serum folate}^{\left(1 - \frac{PCV}{100}\right)}}{\frac{PCV}{100}}$$

IV Microbiological assay method for folic acid using Streptococcus faecalis

Folic acid was assayed with Streptococcus faecalis using a modification of the method of Teply and Elvehjem (1945).

Medium

The medium used was the same as that used in the assay for folic acid using L. casei.

Organism

The test organism was S. faecalis (A.T.C.C. 8043), which was maintained on dried gelatin discs. A new disc was planted on the morning of the day on which each assay batch was set up.

Standard solutions

Standard solutions were prepared from the same PGA used to make up the solutions of PGA and to fortify the foodstuffs which were assayed or administered to patients in the absorption tests. The concentration of PGA in the standard tubes ranged from 0-5 ng per tube.

Preparation of sera, solutions and maize meal for assay

Sera and PGA solutions were diluted from 1:50 - 1:250 according to the results expected and five ml of the dilution plus five ml of double strength medium were added to each of four tubes.

In order to ensure that the growth of S. faecalis was not affected by the presence of serum, each assay batch contained three samples of pooled human serum, to which PGA had been added to achieve final concentrations of respectively, 100, 40, and 20 ng per ml.

Specimens of fortified maize meal were prepared for assay according to the method followed by Metz et al. (1970), based on the method of Herbert (1963a), without using chicken pancreas conjugase.

Assay method

The tubes were autoclaved at 15lb per square inch for five minutes and three of the four tubes inoculated. All the tubes were incubated for 18 hours at 37°C. The density of the tubes was read on a Unicam photoelectric colorimeter and the result obtained by subtracting the colorimeter reading of the uninoculated tube from the mean of the three inoculated tubes. The folic acid content was read from the standard curve.

V Measurement of serum vitamin B_{12} concentration by radioisotopic dilution

The serum vitamin B_{12} concentration was measured by the method of Green et al. (1974)[1] which is a modification of the method of Lau et al. (1965) in which chicken serum is used as the source of vitamin B_{12}-binding protein.

The conditions for this assay were carefully selected on the basis of thorough investigation of the critical steps in the radioisotopic dilution assay for vitamin B_{12}. Newmark et al. (1973) have detailed these investigations, which showed that the binding capacity of chicken serum remained constant over the range of B_{12} concentrations measured, provided that a

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1. At the time of writing this thesis, this paper had not yet been published, and for this reason the method is described in detail here.

high ionic strength and alkaline pH, preferably about pH 9.0, were maintained. This work also demonstrated that the assay was not affected by the presence of denatured human serum, and that the properties of chicken serum as a B_{12}-binding protein are compatible with the use of protein-coated charcoal to separate excess B_{12} from B_{12} bound by chicken serum.

Green et al. (1974) found a normal range, using this method, of 400-1020 pg B_{12} per ml serum, which is higher than that of any previously reported vitamin B_{12} assay. Excellent discrimination was obtained between normal subjects and those with pernicious anaemia. The serum vitamin B_{12} levels measured by this method in 596 subjects were compared with the results of microbiological assay using either Euglena gracilis or Lactobacillus leichmannii. The chicken serum method consistently gave higher results, and these were especially higher in many pregnancy and cord sera, as well as in certain diseases.

Reagents.

All chemicals AR and solutions are made or diluted with glass-distilled, deionized water.

^{57}Co-cyanocobalamin. High specific activity (100-300 uCi/ug) ^{57}Co-cyanocobalamin (^{57}Co-B_{12}) (obtainable

from the Radiochemical Centre, Amersham) is diluted to a B_{12} concentration of 100 pg per ml and stored at 4°C in a dark bottle. This solution is used in the assay in 0.5 ml aliquots, each containing 50 pg vitamin B_{12}.

Standard cyanocobalamin (Std. B_{12}). A solution of cyanocobalamin containing 1000/ug per ml ("cytamen" - Glaxo) is diluted to give standard solutions of 1000, 500, 250 and 125 pg per ml.

Na-Acetate/HCl extracting buffer. pH 4.0. 0.4I. A solution of 0.4 M Na-acetate is titrated to pH 4 with 0.4 N HCl. KCN is added to give a cyanide concentration of 20/ug per ml.

Glycine/NaOH/NaCl buffer. pH 10, 0.8I. A 0.8 M solution of glycine is made up in 0.8 M NaCl. This is titrated to pH 10.0 with 0.8 N NaOH.

Saline. 0.9 per cent (0.154 M) sodium chloride.

Chicken Serum (CS). Serum, separated from chicken blood which has been allowed to clot at 37°C, is suitably diluted with saline, and its unsaturated vitamin B_{12}-binding capacity ($UB_{12}BC$) is determined. This is done by a modification of the method of Ian et al. (1965) in which binding is carried out in the presence of equal volumes of both the extracting and neutralising buffers and in a reaction volume which is equal to that used in the assay (2ml) for 60 minutes at ambient temperature.

Based on the $UB_{12}BC$, the CS is then appropriately diluted so that 0.25 ml will bind between 40 per cent and 60 per cent of the 50 pg $^{57}Co\text{-}B_{12}$ present in the assay. This may be achieved by obtaining a binding curve with increasing concentrations of CS using the same amount (50 pg) of $^{57}Co\text{-}B_{12}$. The $UB_{12}BC$ of undiluted CS used in the studies of Green et al. varied between 180 000 and 500 000 pg per ml depending on the source of the serum and the dilution necessary was therefore between 1 in 1 500 and 1 in 5 000.

Stored at -20°C, the $UB_{12}BC$ of diluted CS remains unchanged for at least 4 months; undiluted CS stored at -20°C appears to be stable indefinitely (Newmark et al., 1973).

Protein-coated charcoal is made up according to Lau et al. (1965). A solution of haemoglobin is prepared from washed red cells by hypotonic lysis, and the concentration adjusted to 10 g per 100 ml. This is then dispensed into 5 ml volumes and stored at -20°C. Before use the contents of a tube are diluted to 200 ml with water, and added to 200 ml of an aqueous suspension of 10 g Norit A, neutral activated charcoal (Amend). Coated charcoal stored at 4°C may be used for 1 week after preparation.

Principle

Vitamin B_{12} in the sample is removed from its endogenous binding protein by boiling at acid pH. A known amount of ^{57}Co-B_{12} is then allowed to compete with the extracted B_{12} for binding to a constant limited amount of B_{12}-binding protein. Finally, bound and free portions of B_{12} are separated. Measurement of the amount of ^{57}Co-B_{12} that is bound allows determination of the degree of radioisotope dilution and hence the amount of serum B_{12} present, by reference to a curve constructed from a series of standards containing known amounts of cyanocobalamin.

Assay method

The assay protocol is shown in Table 3.1. All tubes in the assays were set up in duplicate. Solutions and sera were added with automatic dispensing equipment; coated charcoal was dispensed with an automatic sampler, with continuous stirring of the charcoal suspension using a magnetic stirrer. Extraction was carried out by placing tubes in a boiling water bath for 15 minutes. During this stage, evaporation was prevented by capping tubes with spring loaded metal "Capotest" covers (supplied by C.S. Payne Ltd., London SW 4 OHS). Optimal and consistent binding was achieved by addition of the neutralising buffer, which renders the pH alkaline

TABLE 3.1

Assay Protocol (volumes given in ml)

	^{57}Co-B_{12} (100pg/ml)	Test Serum	Saline	Standard B_{12} (125-1000 pg/ml)	Extracting buffer		Neutralising buffer		Diluted chicken serum	Saline		Protein coated charcoal	
Unknown Sample	0.5	0.25	-	-	0.5	Cap, boil x 15 min.	0.5	Cool, mix	0.25	-	Mix, incubate x 60 min. (room temp.)	1.0	Mix, centrifuge, remove and count 1 ml supernatant
Supernatant Control* (Sample)	0.5	0.25	-	-	0.5		0.5		-	0.25		1.0	
Standard Curve	0.5	-	0.25+	or 0.25	0.5		0.5		0.25	-		1.0	
Supernatant Control (Standard)	0.5	-	0.25	-	0.5		0.5		-	0.25		1.0	
Total Counts	0.5	-	0.25	-	0.5		0.5		-	1.25		-	

* A single sample supernatant control prepared from pooled serum may be used to correct all samples in a particular batch.

\+ In addition to standard tubes containing 0.25 ml of standard B_{12} (125, 250, 500, 1000 pg/ml) a set of duplicate tubes containing no added standard B_{12} is included to serve as zero points on the standard curve.

(ca 9.3) and maintains high ionic strength, followed by incubation with CS for 60 minutes at room temperature without shaking the tubes (Newmark et al., 1973). After the addition of the neutralising buffer, the CS and the charcoal, the tube contents were thoroughly mixed using a Gallenkamp "Whirlimixer". After the addition of charcoal which removed the free portion of B_{12}, the tubes were centrifuged for 10 minutes at 1850 g. One ml supernatant, containing bound B_{12}, was taken off for counting. This step was facilitated by the use of an automatic diluter which draws up and delivers the required volume together with a 2 ml water wash between samples to prevent carry-over of radioactivity between samples. In the present study, samples were counted in a gamma spectrometer (Packard autogamma) for 10 minutes each, which allowed a maximum counting error of 5 per cent on statistical grounds.

Charcoal supernatant controls. In tubes to which CS had been added, the radioactivity present in the supernatant after treatment with coated charcoal represents the ^{57}Co-B_{12} bound by CS. However, a small proportion of unbound ^{57}Co-B_{12} is not removed by coated charcoal and it is therefore necessary to correct the supernatant counts for the presence of some radioactivity not bound to CS (Lau et al., 1965). For this purpose,

supernatant control tubes were included both for the test serum and the standard tubes (see Table 3.1). These control tubes did not receive CS, but were treated with charcoal. The counts in both samples and standards are corrected by subtracting the counts in the appropriate supernatant control tubes. In practice, Green et al. found that all sera give very similar controls (always less than 2.5 per cent of the total counts added). It is therefore sufficient to include a single control, prepared from pooled serum, for any particular batch of samples instead of the individual sample controls found to be necessary by Lau et al. (1965) and Frenkel et al. (1970).

Calculation of results

Following the principle of radioisotope dilution, the bound radio-activity present in the supernatant diminished in the presence of increasing amounts of non-radioactive B_{12}. The standard curve is constructed by plotting the ratio of total counts added to counts bound (after subtraction of controls) on the ordinate (y) against the amount of standard B_{12} added on the abscissa (x). Constructed in this way, the standard curve yields a straight line when the system obeys the principle of radioisotope dilution. This linear form facilitates the calculation of results. From the equation for a

straight line, $y = bx + a$, the amount of B_{12} present in an unknown test serum sample (x) may be calculated as: $x = {}^{1}/b\ (y-a)$ where b is the slope, at the y-axis intercept, and y the value of total counts/counts bound for that sample. Since 0.25 ml serum is used in the assay:

$$B_{12}\ (pg/ml) = {}^{4}/b\ \left(\frac{\text{total counts}}{\text{counts bound}} - a\right)$$

In the results obtained in this study a desktop computer (Olivetti Programma 101) was used to calculate the parameters of the best line of fit for points on the standard curve (regression line by the method of least squares) and a second programme was devised which then calculated serum B_{12} levels from the ratio of total counts added to counts bound according to the formula given above.

VI Statistical methods

Standard statistical techniques were used in this thesis, according to the methods described by Hoel (1963) in his "Introduction to Mathematical Statistics".

C H A P T E R 4

PROPERTIES OF FOLIC ACID RELEVANT TO FOOD FORTIFICATION

I Introduction

Before serious consideration could be given to the question of whether folic acid was a suitable nutrient for use in food fortification programmes, it was necessary to investigate certain properties of the substance. The aim of this investigation was to assess the importance of factors which might modify the efficacy of the fortified food between the time of mixing of food with folic acid and the time of absorption into the bloodstream.

The time period between mixing of the food and absorption can be separated into four stages, namely storage, cooking, eating, and actual absorption of the folic acid.

Each of these stages was considered separately.

II Investigation of properties of folic acid

(a) Stability of added folic acid during storage of fortified food

It was decided to test the effect of storage on the folate activity of fortified maize meal by storing the meal under conditions which simulated those which obtain when commercially-produced maize

meal is stored. The folate activity of the stored maize was then compared with that of newly-mixed fortified maize, and with the amount of added folic acid.

Method

On three separate occasions, fifty Kg of "Impala Special" brand white maize meal (80 per cent extraction)[1] were mixed with folic acid (Sigma) for twelve hours, using a Y-blender to achieve homogeneity. The first 50 kg batch of fortified meal, prepared eighteen months before folate content of the meal was measured, contained 1667 mg folic acid. The second batch, prepared ten months before assay, contained 833 mg, and the third batch, prepared seven months before assay, contained 500 mg. The reason for using these proportions of folic acid to maize meal was that this meal was to be given to patients at Nqutu in 30 g helpings, which would therefore contain 1000 µg, 500 µg, and 300 µg folic acid per helping respectively.

Immediately after mixing each batch, the meal was placed in an airtight fibre-glass drum and railed to Nqutu, where the drum was opened once daily for a few minutes to remove meal. After three months, an aliquot

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1. "Impala Special" brand maize meal is prepared by removing the bran from the maize seed and then progressively grinding and sifting the whole seeds until a fine granular product is obtained. It is one of the two most common maize products bought by South African Negroes, the other being a similar product which is degerminated before grinding.

of the remaining meal was removed and placed into a translucent plastic bucket, which was exposed to light, in a room which admitted sunlight, until the time of assay. The three aliquots were therefore in the buckets for fifteen, seven, and four months respectively.

At the time of assay, aliquots of the stored maize meal and of newly mixed fortified meal containing the same proportions of added folic acid were prepared for folate assay by the method described by Herbert (1963a). The method consisted of homogenising 3 g samples in phosphate buffer containing ascorbic acid in a concentration of 350 mg per 100 ml. The samples were not treated with conjugase. The samples were then assayed for folic acid activity using Lactobacillus casei as the test organism. Each sample was assayed twice, and on each occasion duplicate tubes and an uninoculated blank were used.

Results

The results of folate assay of both stored and newly-mixed fortified maize meal are depicted in Figures 4.1 and 4.2.

Figure 4.1 shows the effect of storage for different periods of time on folate content of the meal. The time of storage had no detectable effect on the percentage of added folic acid recovered from the meal ($r = 0.1714$; $p > 0.5$).

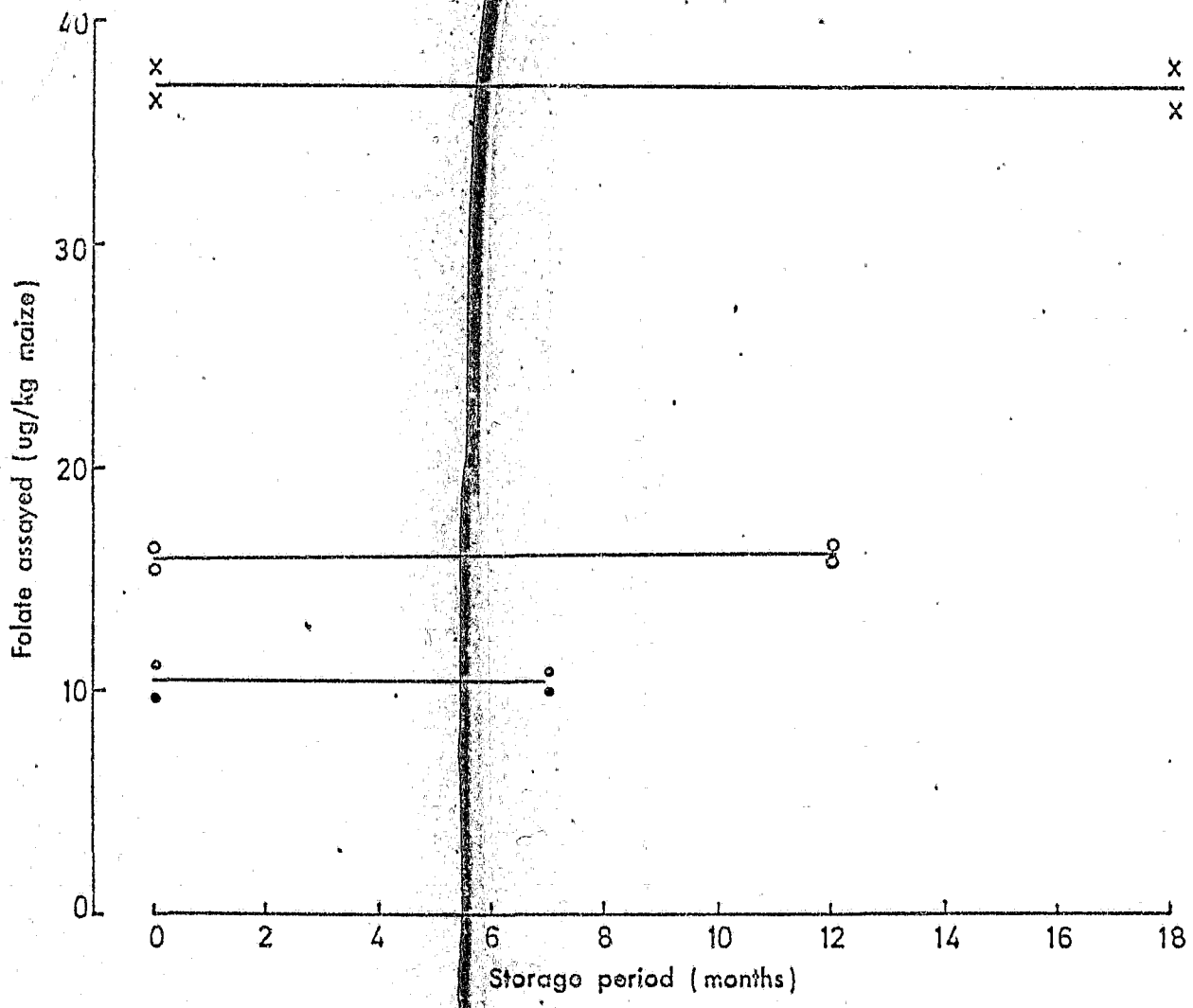

FIGURE 4.1 Effect of storage on folate recovery from maize fortified with folic acid in three different concentrations. The lines join the means of results obtained on samples with the same proportion of added folic acid.

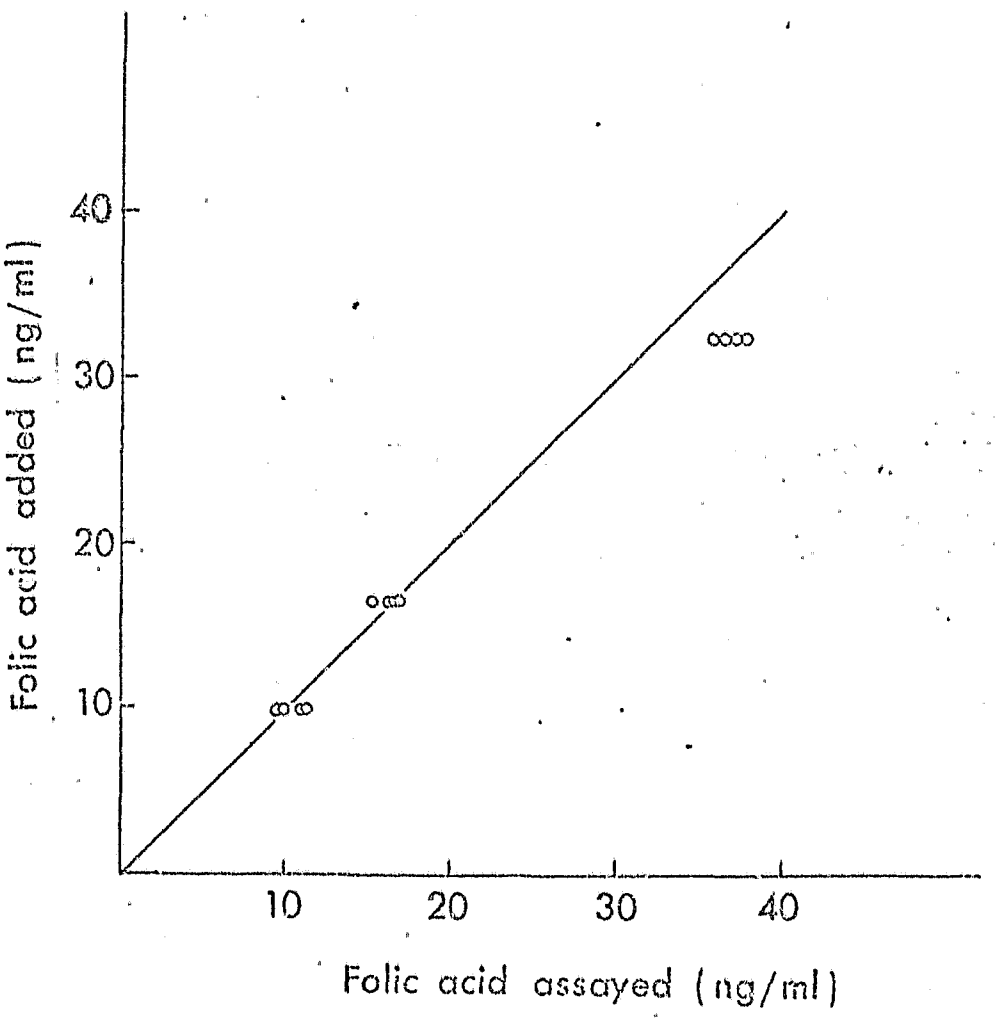

FIGURE 4.2. Folate recovered from twelve samples of maize fortified with folic acid in three different concentrations.

Figure 4.2 compares folate recovery from both stored and newly-mixed maize meal with the amount of added folic acid. The recovery of folate from the meal correlated closely with the amount of added folic acid ($r = 0.9953$; $p < 0.001$)

Discussion

The finding that the folate content of uncooked folic acid-fortified maize meal is entirely dependent on the added folic acid is in accordance with the report of Metz et al. (1970) that "Impala Special" contains 27 µg of folate activity per kg, or 0.81 µg per 30 g. As the smallest proportion of folic acid added to maize in this study was 300 µg/30 g maize meal, the amount contributed by endogenous folate activity is insignificant.

No reduction in folate activity of the fortified meal was detectable after storage for periods up to eighteen months.

Stokstad et al. (1947) reported that when a solution of folic acid was exposed to ultraviolet light, there was rapid cleavage of the molecule at the 9, 10 position into a pteridine and a free aromatic amine. More recently Anderson and Cowan (1968) found that exposure of standard solutions of folic acid and of serum extracts to bright reflected sunlight for three hours had no significant effect on their folate content,

whereas direct sunlight apparently reduced folate levels in both folic acid solutions and in sera. Commercial producers usually supply folic acid in opaque containers and recommend that these be protected from light.

The maize meal stored for use in this investigation was exposed to direct sunlight for a few hours virtually every day, and to daylight every day, for periods of fifteen, seven and four months after it had been placed in plastic buckets. The stability of folic acid in these circumstances can be explained by the fact that only the outermost layer of the meal is exposed to light, while the major part is beneath this layer of opaque meal and thus protected by it.

South African millers usually pack maize meal in hessian or linen bags, which are then stored in an environment protected from damp and rodents. Most but not all storage methods afford protection of the meal from light, fortuitously rather than by design. Grain storage procedures are such that meal is not normally exposed to damp. The method of storage used in this study thus simulated conditions which obtain when commercially-produced maize is stored. It is concluded that, when conventional storage procedures are used, the folic activity of the fortified meal is stable at ambient temperature for at least eighteen months.

(b) Stability of folic acid during the cooking process

Numerous reports describe the effect of different methods of food preparation on the folate content of a variety of foods. Folate content is diminished by boiling food in large volumes of water (Herbert, 1963a). The canning of fruit juices reduced folate content by about 80 per cent, steaming of vegetables resulted in a reduction of up to 97 per cent, and frying of meat and fish caused a decline in folate activity of 62-95 per cent (Hardinge and Crooks, 1961; Cheldelin et al., 1943; Schweigert et al., 1946).

The reduction of the folate of vegetables during boiling was found by Hurdle (1967) to be due to destruction of folate rather than to loss into the boiling water, and most of this process occurred during the first minute. The decline in folate content of cabbage, broccoli, oat porridge, potatoes and eggs after boiling varied from 80-90 per cent.

The knowledge that food folate is highly labile may have discouraged investigation of folic acid as a possible micro-nutrient for use in food fortification programmes. Even when Metz and his co-workers (1970) considered the possibility of fortifying maize with folic acid, they suggested this "would be of limited value unless coupled with the addition of ascorbic acid to protect the folic acid during cooking."

Herbert (1965) found that folic acid is stable for one hour at 100°C in solutions above pH 5, but unstable at pH values below 4. Chanarin (1969) states that pteroyl-glutamic acid loses two molecules of water on heating above 140°C, but does not discuss the effect of this on biological activity. The decomposition point of pteroylglutamic acid is 250°C (Dawson et al., 1969). The staple foods of most people are either boiled, or else baked at temperatures lower than 250°C. It was therefore decided to assess the effect of boiling and baking on the folic acid activity of aqueous solutions at an alkaline pH.

As Streptococcus faecalis responds more specifically to P.G.A. than does L. casei (Blakley, 1969), it was decided to use the former as the assay organism in this experiment.

Method

Six flasks were filled with distilled deionised water containing, respectively, 0, 0.2, 0.4, 0.6, 0.8 and 1.0 µg folic acid per litre. All the solutions were brought to a neutral pH by adding NaOH. A twenty ml aliquot of each solution was then pipetted into each of eight sterile McCartney bottles. The bottles were arranged in groups, each of which comprised one bottle of each solution, and one group was retained for use as a standard curve. The other seven groups were placed in a boiling water bath for seven different periods of time, namely ten, twenty, thirty, forty-five, sixty, ninety and 120 minutes.

Aliquots of the same solutions were then prepared in the same manner and baked in an electric oven at 230°C (the temperature often used for baking bread) for forty-five, sixty, ninety and 120 minutes.

During the baking process, some of the bottles lost water despite their seals. The volume was made up to a mark indicating original 20 ml volume by adding distilled deionised water after cooking.

The solutions were then assayed in triplicate for folic acid using S. faecalis.

Results

The colorimeter readings of the aliquots of water (0 ng per ml) were identical after all periods of boiling and baking.

The activity of the PGA solutions was expressed as a percentage of the expected content. Each of the three readings obtained on solutions taken from the same bottle was expressed independently. The percentages of added folic acid recovered from the boiled solutions, as assayed by S. faecalis, are shown in Table 4.1, and those of the baked solutions are shown in Table 4.2. The mean recoveries of all the solutions subjected to the same cooking period are shown. The significance of the difference of these means from 100 per cent was calculated in each case using a one-tailed t-test, and the probability (p) values obtained are shown in the tables.

TABLE 4.1

Effect of boiling on aqueous solutions of folic acid

Folic acid concentration of solution(ng/ml)	Percentage recovery of folic acid after boiling for																				
	10 minutes			20 minutes			30 minutes			45 minutes			60 minutes			90 minutes			120 minutes		
0.2	90	85	100	95	100	110	80	100	90	120	100	80	100	95	100	90	100	110	110	80	80
0.4	103	103	103	108	108	99	95	92	103	116	125	116	95	103	108	108	125	103	116	108	108
0.6	118	97	110	97	97	101	95	90	105	97	90	105	105	110	110	101	105	101	90	87	87
0.8	113	118	105	95	101	105	95	91	98	101	98	113	101	95	105	105	85	105	91	89	76
1.0	103	90	108	100	100	103	108	94	103	87	90	100	100	103	108	87	90	103	100	114	114
Mean	103.06			101.27			95.93			102.53			102.53			101.20			96.67		
p	> 0.1			> 0.15			< 0.05			> 0.2			< 0.05			> 0.3			> 0.15		

S. faecalis was used as the test organism. The three readings for each solution after each boiling period refer to the triplicate in a single assay batch. The p values refer to the results of one-tailed t tests comparing the observed percentages with the expected value of 100%.

TABLE 4.2

Effect of baking on aqueous solutions of folic acid

Folic acid concentration of solution (ng/ml)	Percentage recovery of folic acid after baking for											
	45 minutes			60 minutes			90 minutes			120 minutes		
0.2	130	120	120	105	90	95	105	95	90	80	60	70
0.4	82	78	78	100	100	115	100	105	85	80	95	90
0.6	120	113	120	93	101	111	83	87	87	80	87	80
0.8	91	88	99	103	99	115	84	91	88	81	88	84
1.0	120	100	114	105	89	108	89	86	86	82	79	79
Mean	104.87			101.93			90.73			81.00		
p	>0.15			>0.15			<0.0005			<0.0005		

S. faecalis was used as the test organism. The three readings for each solution after each baking period refer to the triplicate results in a single assay batch. The p values refer to the results of one-tailed t-tests comparing the observed percentages with the expected value of 100%.

When the mean recoveries of the boiled solutions, arranged according to cooking time, are examined, it can be seen that none of these differed significantly from 100 per cent at the 99 per cent confidence level. Although the thirty and sixty minute means differed from expected values at the 95 per cent confidence level, one being greater and one less than expected values, this may be attributed to the fact that the procedure of doing a number of t-tests tends to increase the risk of exaggerating the significance of data by committing a type 1 statistical error (Miller, 1966).

There was no correlation between the boiling time and recovery of folic acid ($r = -0.1045$; $0.1 \leq p < 0.2$).

In contrast to this, baking at 230°C did cause a reduction in folic acid recovery. This effect could not be detected up to 60 minutes, but after 90 minutes the mean recovery had dropped to 90.7 per cent, which was significant at the 99.5 per cent level. A further thirty minutes' baking caused a further drop to 81 per cent, which was also highly significant ($p < 0.005$). In accordance with these observations, there was a strong negative correlation between the baking period and folic acid recovery ($r = -0.6564$; $p < 0.001$). Ninety minutes' baking reduced the folic acid activity of solutions with a concentration of 0.6 ng per ml and greater, while all solutions had reduced activity after two hours.

The distributions of these results are diagrammatically depicted in Figures 4.3 and 4.4, together with the corresponding regression lines.

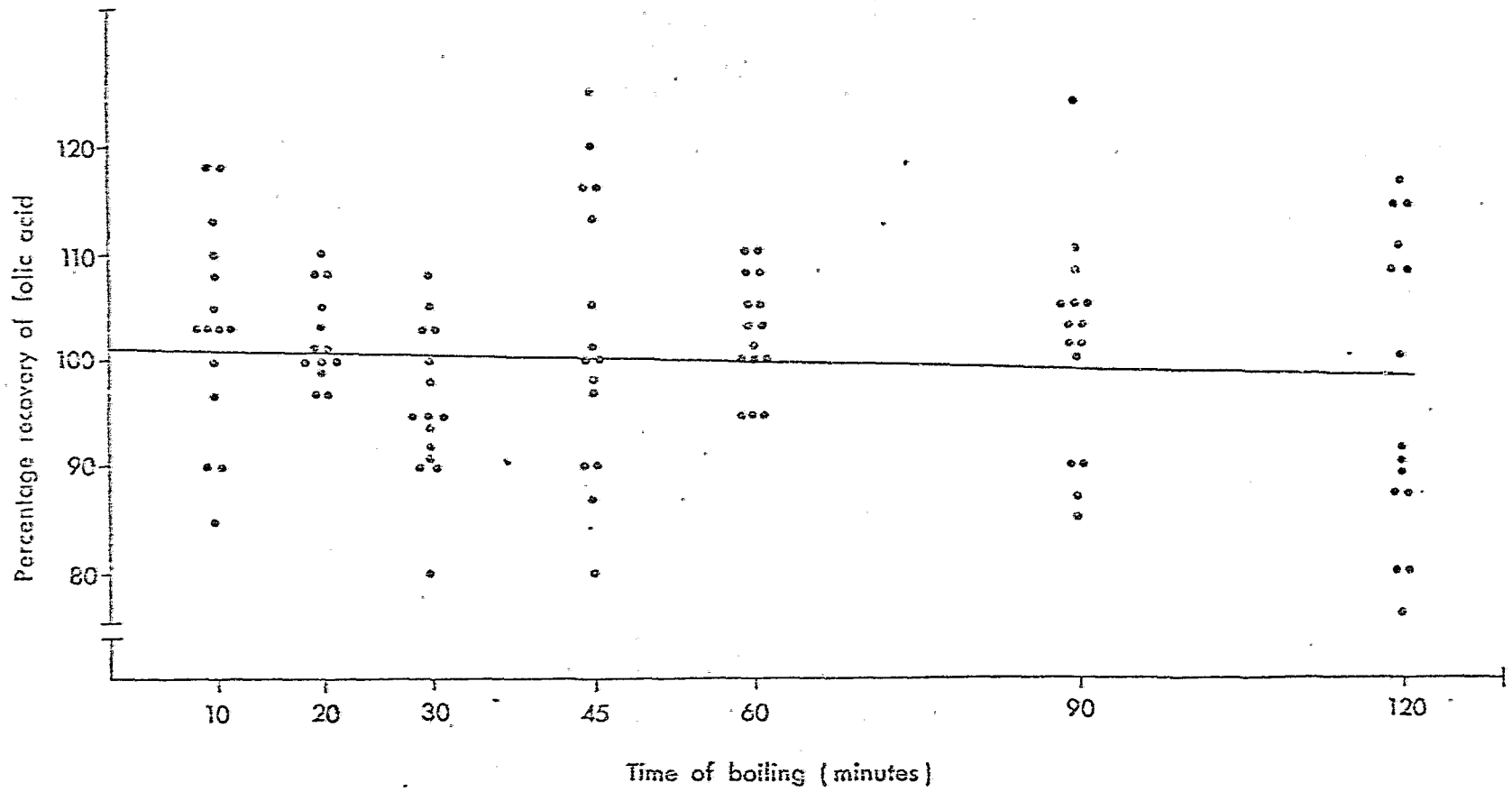

FIGURE 4.3 Effect of boiling for varying periods on the recovery of folic acid from aqueous solutions. Details of the recovery from solutions of different concentrations are shown in Table 4.1.

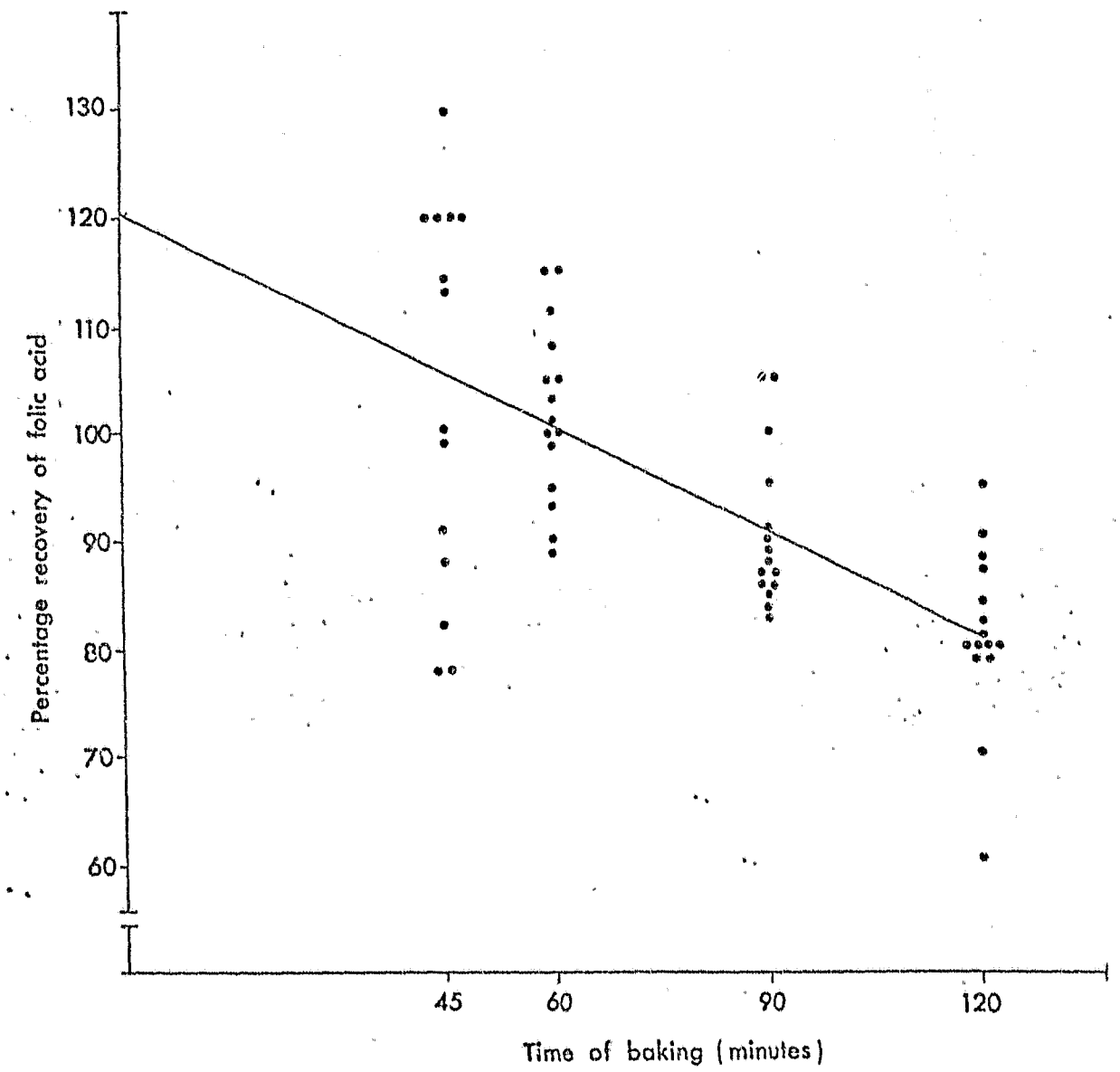

FIGURE 4.4. Effect of baking at 230°C for varying periods on the recovery of folic acid from aqueous solutions. Details of the recovery from solutions of different concentrations are shown in Table 4.2.

Discussion

Unlike food folate, which is rapidly destroyed by boiling (Hurdle, 1967), folic acid has been shown to be stable for one hour in solutions with pH values greater than 5 (Herbert, 1965). This investigation has shown that at neutral pH, solutions of folic acid are stable for at least two hours. This period is longer than that likely to be used by the vast majority of people for cooking staple foods, which would seldom have pH values below 5.

These results do not contradict the fact that the vitamin, being water-soluble, is likely to be lost in all cooking procedures which involve the decanting of water, such as those used by Herbert (1963a). Water is not decanted in the cooking of maize, and this is usually true of most cooking methods for rice as well.

Baking at 230^{o}C reduced folic acid activity after one hour. This observation may be due to the effect of temperatures greater than 140^{o}C mentioned by Chanarin (1969). The bread used in the absorption study conducted by the author, and reported hereunder, was baked for fifty-five minutes. Folic acid activity should thus have remained unaffected, but longer baking periods may reduce activity. This would necessitate adjustment of the proportion of the nutrient to be used for fortifying flour in some areas, depending on the cooking practices prevalent in the locality.

(c) <u>The effect of added folic acid on the acceptability of food to the consumer</u>

The Joint FAO/WHO Expert Committee on Nutrition (FAO/WHO, 1971) emphasised that nutrients used for fortifying foods should not "convey unwanted characteristics to the food (colour, taste, flavour, cooking properties)".

The fortified maize meal used in the trials at Nqutu, described in a subsequent chapter, contained proportions of folic acid ranging from ten to thirty-three parts per million by weight. None of the patients who received this meal was able to ascribe to it any characteristic which differed from those of unfortified maize meal to which they were accustomed.

Similarly, volunteers participating in the absorption studies and therapeutic trials described in subsequent chapters were unable to detect any effect of folic acid on the colour, odour or taste of bread, rice or maize meal.

While the yellow colour of folic acid was not detectable even in the white maize meal used in the studies conducted by the author, recent legislation in South Africa makes the possibility of detectable discoloration even more remote. Since August 1973, it has not been permissible to market white maize meal without mixing this with yellow maize meal. As a result, all commercial maize meals in South Africa are now yellow in colour, and would not be further discoloured by folic acid.

Finally, neither the author nor his assistant at Nqutu were able to discern any effect of added folic acid on the cooking properties of maize meal, bread or rice.

(d) Absorption of added folic acid from fortified maize meal, bread and rice

In order to determine the effect of food on the absorption of folic acid, it was decided to compare folic acid absorption from fortified maize meal, bread and rice with absorption of an aqueous solution of folic acid, in healthy volunteers.

Methods

Preparation of the fortified food.. Folic acid was added to each food just before cooking or baking, and care was taken to homogenise the food to achieve equal distribution of the added nutrient throughout.

Whole wheat bread was made using one part by weight of salt and dried yeast to two parts of sugar, five of sunflower seed oil, and seventy-five of whole wheat flour. This was prepared by conventional techniques using water, and baked at 230°C for fifteen minutes and then 200°C for forty minutes. Rice was boiled in three times its volume of water for thirty minutes, at which time all of the water had either been absorbed or had evaporated. The rice and bread to be given to all volunteers were each prepared in a single batch, divided

into equal parts by weight, and frozen at -20°C until the night before administration, when they were allowed to thaw at room temperature.

Maize meal porridge was prepared by boiling the meal in water for thirty minutes immediately before administration.

The dose of folic acid added before cooking was such that each helping of maize meal porridge, bread and rice contained one mg folic acid.

The absorption test was done by a modification of the method outlined by Chanarin and Bennett (1962). Seven healthy Caucasian volunteers were used after ensuring that all were of normal haematological status and that iron and folate nutrition were normal (Table 4.3).

TABLE 4.3

Haematological values of volunteers for absorption study

Volunteer	1	2	3	4	5	6	7
Sex	M	F	F	M	M	M	F
Haemoglobin (g%)	17.4	14.4	14.8	15.8	15.9	16.1	14.3
Mean cell volume (μ^3)	83	89	89	82	84	87	84
Serum iron (μg%)	149	174	146	154	147	169	165
Transferrin saturation (%)	38	37	38	38	42	41	49
Red cell folate (ng/ml)	296	478	273	300	321	357	234

To saturate their folate stores, the volunteers were given 15 mg folic acid by mouth daily for three days, the last dose being given 36 hours before the first test was carried out. All tests were carried out after fasting for at least ten hours.

On the next four consecutive days, volunteers were given either
1. one mg folic acid in 25 ml water (F)
2. fortified maize (M)
3. fortified rice (R)
4. fortified bread (B)

Each helping of maize, rice and bread contained one mg folic acid. The order in which these were given was rotated to avoid any effect of giving a single food more than 36 hours after the saturating dose (Table 4.4).

TABLE 4.4

Order of administration of fortified food

Volunteer	Day 1	Day 2	Day 3	Day 4
1	F	B	R	M
2	F	R	M	B
3	M	R	F	B
4	M	B	F	R
5	B	F	R	M
6	R	M	B	F
7	B	F	M	R

B = bread M = maize, R = rice, F = folic acid solution

Blood was taken before administration of the food or folic acid and one and two hours thereafter. The serum was frozen until the day of assay, when all sera from a particular volunteer were assayed by both L. casei and S. faecalis after diluting sufficiently to allow them to be read on the sensitive portion of the standard curve. The folate level of the specimen taken before the oral dose was subtracted from the corresponding one and two hour levels, which were then summated. The sum of the one and two hour levels after maize, bread and rice were then expressed as percentages of the levels after administration of the aqueous solution of folic acid.

Results

The sum of the increase in serum folic acid over basal levels at one and two hours was calculated for each absorption test. The results are depicted in Figures 4.5 (S. faecalis) and 4.6 (L. casei).

When S. faecalis was used as the assay organism, the increase ranged from 20 to 93 ng per ml (mean 57.6 ng per ml) after administration of the aqueous solution of folic acid. The mean percentage after maize was 56.3 per cent (range 33-80), after rice was 54.4 per cent (range 38-75), and after bread was 28.7 per cent (range 14-40).

The summated increase in L. casei folic acid activity after administration of the folic acid solution ranged from 37 to 146 ng per ml (mean 97 ng per ml). The

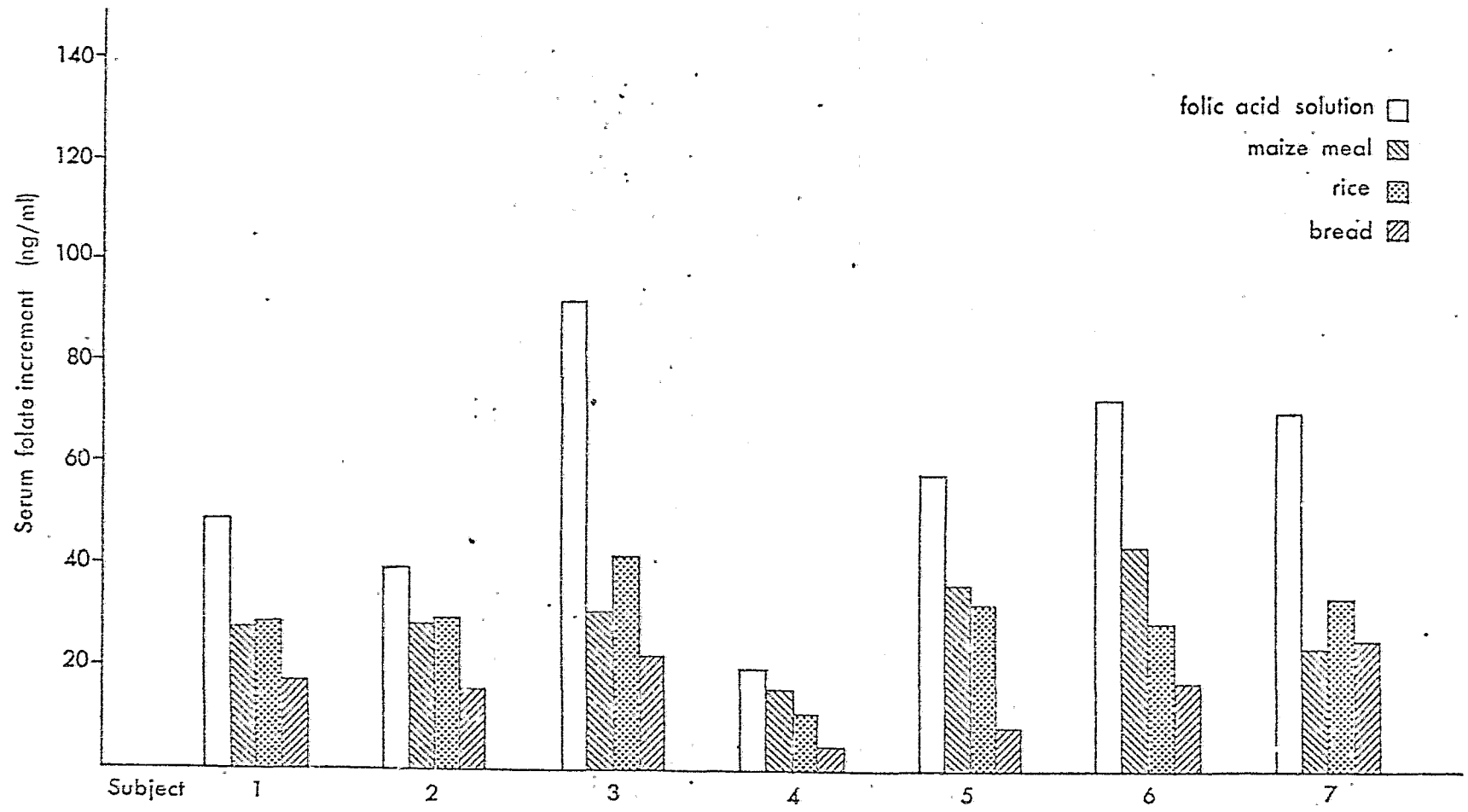

FIGURE 4.5. The absorption of folic acid from fortified staple foods - S. faecalis assay. Sum of rise in serum folate one and two hours after ingesting one mg folic acid alone or cooked with food.

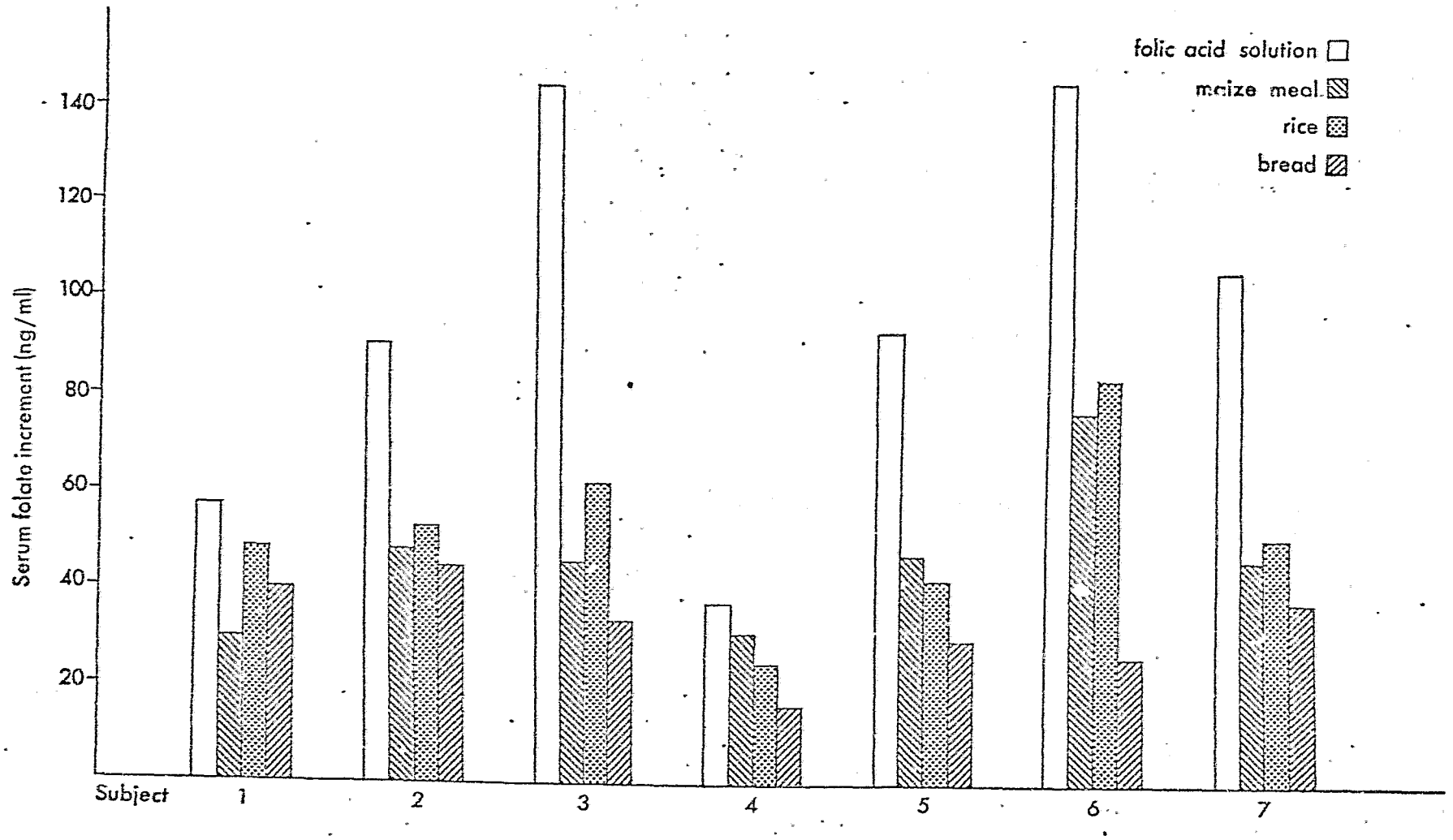

FIGURE 4.6. The absorption of folic acid from fortified staple foods - L. casei assay. Sum of rise in serum folate one and two hours after ingesting one mg folic acid alone or cooked with food.

corresponding mean percentages were 52.7 per cent after maize (range 32-77), 57.6 per cent after rice (range 42-84), and 38.3 per cent after bread (range 18-68).

All sera had a greater activity for L. casei than for S. faecalis.

Discussion

The measurement of serum folate levels after an oral loading dose of folic acid is a satisfactory method of assessing folate absorption in man (Chanarin, 1969; Hoffbrand, 1972).

The technique used here differed in several aspects from the method outlined by Chanarin and Bennett (1962). Firstly, the loading dose of folic acid was not adjusted to the subjects' body weights, and was in all cases less than the recommended 40 µg per kg body weight. Secondly, the loading dose of folic acid was dissolved in distilled deionised water instead of NaOH, which is reported to augment folate absorption (Perry and Chanarin, 1972).

The reason for altering the technique was to simulate as far as possible conditions which would obtain when folic acid-fortified food is eaten. Alkalinising the foods may have invoked influences which would not be present under natural conditions of food ingestion.

When serum levels are used as an index of folic acid absorption, peak serum folate is utilised as an index rather than the sum of the levels. While the

test is regarded as satisfactory on the basis of clinical observations, as has been mentioned above, it is reasonable to postulate that results would be distorted by differences in gastric motility, as folic acid is absorbed from both the duodenum and jejunum (Hepner et al., 1968). The effect that the different foods used in this study have on gastric motility and the rate, rather than the amount, of absorption, could not be predicted. It was considered that the use of the sum of serum levels at one and two hours, rather than the peak level, might tend to reduce possible distortion caused by the effect of food on gastric motility and the rate of absorption.

The wide range of S. faecalis activity measured in the seven subjects (20-93 ng per ml) is in accordance with the findings of Anderson et al. (1960), who reported that the absorption of a 200/ug dose of ^{3}H-folic acid varied from 41 to 91 per cent when assessed by measuring unabsorbed radioactivity in the faeces.

The measurement of serum folate levels with L.casei does not give an absolute index of the actual quantity of folic acid absorbed from the bowel, because variable amounts of tissue folate are displaced by absorbed folate. However, the pattern of absorption as assessed by L. casei (Figure 4.6) was similar to that found using S. faecalis (Figure 4.5).

In all subjects, S. faecalis activity of serum after administration of the aqueous solution of folic acid was greater than that after administration of the fortified foods. In all but one subject, S. faecalis activity after bread was less than after both maize and rice. The mean percentage activity was similar after maize and rice, both approximating to 55 per cent. The mean percentage activity after bread was considerably lower.

In the light of the findings reported earlier in this chapter, it is unlikely that the methods used for preparing the foods used in this study decreased folic acid activity. It is possible, however, that the presence of the food may augment the effect of baking at high temperatures and result in a more rapid manifestation of the effect seen after one hour when folic acid solution are baked.

The results presented above give some idea of the dose of folic acid that should be used for food fortification. In order to ensure that nutritional folate requirements are met in all people, it would be necessary to take into account the lower limits of the range of percentage absorption of fortified foods. The dose of folic acid added to the food could then be adjusted accordingly.

It would appear necessary to add three times the required dose to maize, between $2\frac{1}{2}$ and three times the dose to rice, and seven times the dose to bread.

III Summary and conclusions

It was considered necessary to investigate factors which might modify the efficacy of food fortified with folic acid between the time that the vitamin was added to the food and its absorption into the bloodstream.

The effect of storage on folic acid in fortified maize meal was assessed by comparing the L. casei folate activity of three mixtures of maize, containing between ten and thirty-three parts per million of folic acid, which had been stored for 7 - 18 months, with the activity of newly prepared maize meal and with the amount of added folic acid. There was no detectable effect of storage on folate activity.

The effect of cooking procedures was tested by boiling and baking folic acid solutions for up to 2 hours. Boiling had no effect on S. faecalis folic acid activity of the solutions. Baking at 230°C had no effect up to sixty minutes, but a highly significant reduction had occurred at ninety minutes and activity was further reduced during the next 30 minutes.

There was no effect of folic acid on the colour, odour, taste or cooking properties of maize meal, rice

and bread. This precludes these factors from making the food less palatable or unacceptable to the consumer.

Finally, absorption of folic acid from fortified maize, rice and bread was compared with absorption of a folic acid solution, using a modification of the method of Chanarin and Bennett (1962). The mean absorptions from maize and rice were similar, and approximated to 55 per cent, while mean absorption from bread was measured at 28.7 per cent, when compared with absorption of a folic acid solution in each patient.

C H A P T E R 5

TRIALS OF FOLIC ACID-FORTIFIED MAIZE MEAL IN PREGNANT WOMEN AT NQUTU, INCLUDING A COMPARISON WITH THE EFFECT OF FOLIC ACID TABLETS

I Introduction

The evidence presented in Chapter 4 was considered adequate to justify a clinical trial of maize meal fortified with folic acid. It was decided to feed this meal to pregnant women, because their folate requirements exceed those of other groups in the population. The Charles Johnson Memorial Hospital at Nqutu was chosen as the site for the trials because healthy women are admitted to its lodging facilities after the 36th week of pregnancy.

A pilot study conducted on twelve pregnant women lodging in the hospital during February 1972 confirmed the expectation that folate deficiency was common (see Table 5.1). In five of the twelve subjects, red cell folate concentrations were in the deficient range (less than 160 ng per ml), serum folate levels were low (below 3 ng per ml), hypersegmented polymorphs were present in the peripheral blood and, in four of the five, there were red cell changes suggestive of megaloblastic haemopoiesis. A further two subjects had low serum folate levels.

All women are given iron supplements from the time of their first antenatal attendance at the hospital. The form in which these supplements were given varied during the course of this study, but was always 100 - 200 mg elemental iron daily. Folic acid tablets

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All women are given iron supplements from the time of their first antenatal attendance at the hospital. The form in which these supplements were given varied during the course of this study, but was always 100 - 200 mg elemental iron daily. Folic acid tablets

(5 - 15 mg daily) are given to all pregnant women with obstetrical or medical complications, including infections, or a haemoglobin level less than 11 g per 100 ml as measured at the hospital with a field visual haemoglobinometer.

TABLE 5.1

Results of preliminary survey of twelve pregnant women lodging at C.J.M. Hospital in February 1972

Haemoglobin (g per 100ml)	Blood film		Folate (ng per ml)	
	Hyper-segmented polymorphs	Red cells	Serum	Red cell
11.1	+	macrocytes teardrops	2.0	90
11.5	-	normal	3.6	228
13.0	-	normal	3.2	319
11.0	-	normal	1.8	270
13.2	+	normal	1.4	167
14.0	+	teardrops	1.8	62
12.1	+	megaloblasts	2.8	135
13.5	-	normal	4.2	178
11.7	-	normal	5.2	171
10.0	-	hypochromic	3.2	167
12.5	+	oval macro-cytes	2.0	106
14.1	+	normal	2.2	126

The trials were divided into two main parts. From June to September 1972, fortified maize meal was fed to a test group, and unfortified meal to a control group.

During 1973, sequential trials were conducted using different levels of fortification of maize meal, and the results compared with those of subjects given folic acid tablets. The second series of trials were planned sequentially rather than concurrently because it was possible that the use of more than one source of folic acid at any one time would have been difficult to control and might have led to confusion.

II Material and methods

(a) Subjects studied

The subjects were healthy pregnant women admitted to the lodging facilities at the hospital. Subjects less than 37 weeks pregnant were selected, as judged by clinical examination. By virtue of the hospital's practice of giving folic acid tablets to all complicated cases, the subjects were all free of any detectable illness or obstetric complication, and were not receiving antibiotics. If complications developed in any subject, she was excluded from the trials. On admission to the study all subjects had haemoglobin concentrations of at least 11 g per 100 ml as measured by a visual haemoglobinometer, but the trials continued in occasional patients whose levels were lower than this when measured on the Coulter Counter Model S in Johannesburg. Subjects who delivered within two weeks of the commencement of the trial were excluded from the

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calculations, as were two patients who delivered twins.

A total of 750 blood samples were taken from the remaining 122 subjects, who were studied for a mean period of 29.4 days. Details of the five groups of subjects are shown in Table 5.2, together with details of the folic acid supplement given to each group. Groups I and II were studied concurrently from June to September 1972, subjects being allocated to one or other group on a random number basis. The remaining groups were studied for three consecutive thirteen week periods from February to November 1973. Group V was larger than the other groups admitted to the trial during equivalent time periods. This is explained by the fact that the annual return of migrant labourers to the homelands during the Christmas vacation results in a marked elevation of the birthrate in September and October each year (Barker, 1959), during which months these subjects were studied.

TABLE 5.2

Groups of pregnant women participating in trials.

Group	Number of subjects	Mean duration of study (days)	Folic acid supplement Daily dose (/ug)	Vehicle
I	18	33.1	0	-
II	20	25.9	1000	maize meal
III	27	30.6	500	maize meal
IV	23	30.7	300	maize meal
V	34	27.2	300	tablets

(b) Provision of fortified maize meal

"Impala Special" brand maize meal (undegerminated, 80 per cent extraction) was mixed with crystalline folic acid in powder form (Sigma) for 12 hours, using a Y-blender to achieve homogeneity. The proportion of folic acid to maize meal in each batch was such that 30 g of the mixture would contain the daily dose of added folic acid intended for use in each trial, as shown in Table 5.2. Each batch of fortified maize was adequate for the whole duration of the particular trial.

After mixing, the maize meal was placed in a fibre-glass drum and taken to the site of the study, where it was kept in the lodging hall. The drum was opened each day for a few minutes in order to remove aliquots of meal, and was then resealed. Each subject received a daily helping of 30 g dry weight of the fortified meal, cooked for 30 minutes in approximately four times its weight of boiling water over a gas flame, and served as a soft porridge with milk and sugar. No water was decanted during the cooking process, in accordance with usual practice in cooking maize meal.

It was decided to use a daily dose of 1000 µg added folic acid in group II on the basis of the absorption tests described in Chapter 4. In these tests, the absorption of 1000 µg added folic acid from 30 g fortified maize meal porridge was 33 - 80 per cent of the absorption of the same dose in aqueous solution

in the same subjects. In order to ensure that all subjects in group II received at least the minimum daily PGA requirement of 300 µg, it was deemed necessary for the daily helping of fortified maize to contain 1000 µg crystalline PGA before cooking. The dose of folic acid was reduced in the maize meal administered to groups III and IV in order to compare the effects of different levels of fortification.

(c) Provision of folic acid tablets

During the period that group V was studied, the administration of iron syrup to pregnant women in the hospital was suspended. All pregnant in-patients, including those not being studied, were given three "Pregamal" (Evans) tablets in a single daily dose, each tablet containing 100 µg folic acid and 200 mg ferrous fumarate.

(d) Procedure

The nature and purpose of the trial were explained, and those subjects who gave consent completed a questionnaire which dealt with health and socio-economic status. Blood was drawn by venipuncture on two consecutive days between Sunday and Tuesday to establish baseline levels, and the folic acid, either as fortified maize meal or tablets, was given from the third day. Subsequent blood samples were drawn each Tuesday after 11 a.m., until the subject delivered.

The fortified maize meal or folic acid tablets were given to subjects at 7 a.m. each day except for Tuesdays, when the supplement was given at 3.30 p.m. after blood samples had been taken. There was therefore a period of not less than 28 hours between the last dose of folic acid on Monday at 7 a.m. and venipuncture on Tuesday after 11 a.m. Special care was taken to ensure that subjects actually ingested the folic acid each day. Subjects receiving fortified maize meal ate this within an enclosed area under direct supervision. Subjects receiving folic acid tablets took the tablets immediately they were dispensed, under the supervision of the dispenser.

Control subjects ate unfortified maize meal only.

All subjects received the normal hospital diet, which contains copious quantities of maize meal.

(e) Blood samples

Blood samples were collected into tubes containing dried sequestrene and into plain tubes as described in Chapter 3. Serum was separated from the clotted blood and frozen and the tubes of serum and anticoagulated whole blood were then packed in a vacuum flask with ice bags. In order to prevent freezing of the whole blood samples, these were separated from the ice bags and serum samples by a thick layer of cotton wool.

Each Wednesday morning, the flask was taken 50 km by the weekly hospital bus to Dundee Station, from

which it was railed 370 km to Johannesburg.

On Thursday morning in Johannesburg the sera were thawed, and a 1 ml aliquot from each sample was added to a tube containing 10 mg ascorbic acid, and this and the remaining serum were frozen pending folate and vitamin B_{12} assay. An aliquot of whole blood was haemolysed by the method described in Chapter 3, and then frozen pending red cell folate assay. The remainder of the whole blood sample was used for microhaematocrit determination and for haemogram in the Coulter Counter according to the methods described in Chapter 3. Samples for folate and vitamin B_{12} assay were stored at $-20^{o}C$.

The procedure followed in the initial trial (groups I and II) differed from the above description in two ways. Firstly, the serum was added to a tube containing ascorbate immediately after separation at Nqutu. This procedure rendered the serum unsuitable for vitamin B_{12} assay, which was the reason for changing it in subsequent trials. Secondly, the microhaematocrit procedure for the first study was done at the Dundee Laboratories of the Natal Blood Transfusion Service on a separate specimen of sequestrene blood, for fear that the cells would swell during the trip to Johannesburg. This procedure was abandoned after comparison of the Dundee and Johannesburg results showed that they were no different.

(f) Assay procedure

Folate levels were measured by microbiological assay using L. casei as the test organism, and serum vitamin B_{12} by radioisotope dilution, using chicken serum as the source of vitamin B_{12} binder, according to the methods described in Chapter 3. All samples from a particular subject were assayed within the same batch. Most samples were assayed within six weeks and all within twelve weeks of collection, that is within the period over which serum and red cell folate are stable (Waters and Mollin, 1961; Hoffbrand et al., 1966).

(g) Reporting of results

In this chapter, the changes observed in the five groups of women during the course of the trials are reported in order to compare the effects of the fortified maize meal and the folic acid tablets with observations in subjects not receiving folic acid. Although the folate and vitamin B_{12} levels on admission to the study are listed in order to illustrate these changes, the incidence of vitamin deficiency and of anaemia in the pregnant subjects is not discussed here, but is compared with the findings in non-pregnant groups in Chapter 8.

Results obtained from the two initial blood samples were averaged and expressed as the baseline value for each subject. In calculating time relationships, those

samples which had been taken between Sunday and Tuesday were expressed as if they had been taken on the Tuesday, and the results obtained on subsequent samples were expressed as changes from the initial baseline result in each subject (e.g. change in red cell folate concentration at week 6 = red cell folate concentration at week 6 minus baseline red cell folate concentration).

III Red cell folate and serum folate concentrations

(a) Results

(i) Red cell folate concentration

The mean red cell folate concentrations in each group on admission to the trials and at delivery are depicted together with the corresponding standard errors in Figure 5.1. The mean for each group on admission to the trials was statistically similar to the combined mean for all subjects ($p > 0.5$), which was 201.2 ng per ml. At the time of delivery, the mean red cell folate concentration in the group receiving unfortified maize (group I) had fallen by 42 ng per ml into the deficient range, and was significantly lower than that in each of the groups receiving folic acid supplements (for all, $p < 0.005$).

During the period of study, the mean red cell folate concentration in group II rose by 60 ng per ml, in groups III and V by 50 ng per ml, and in group IV by approximately 30 ng per ml.

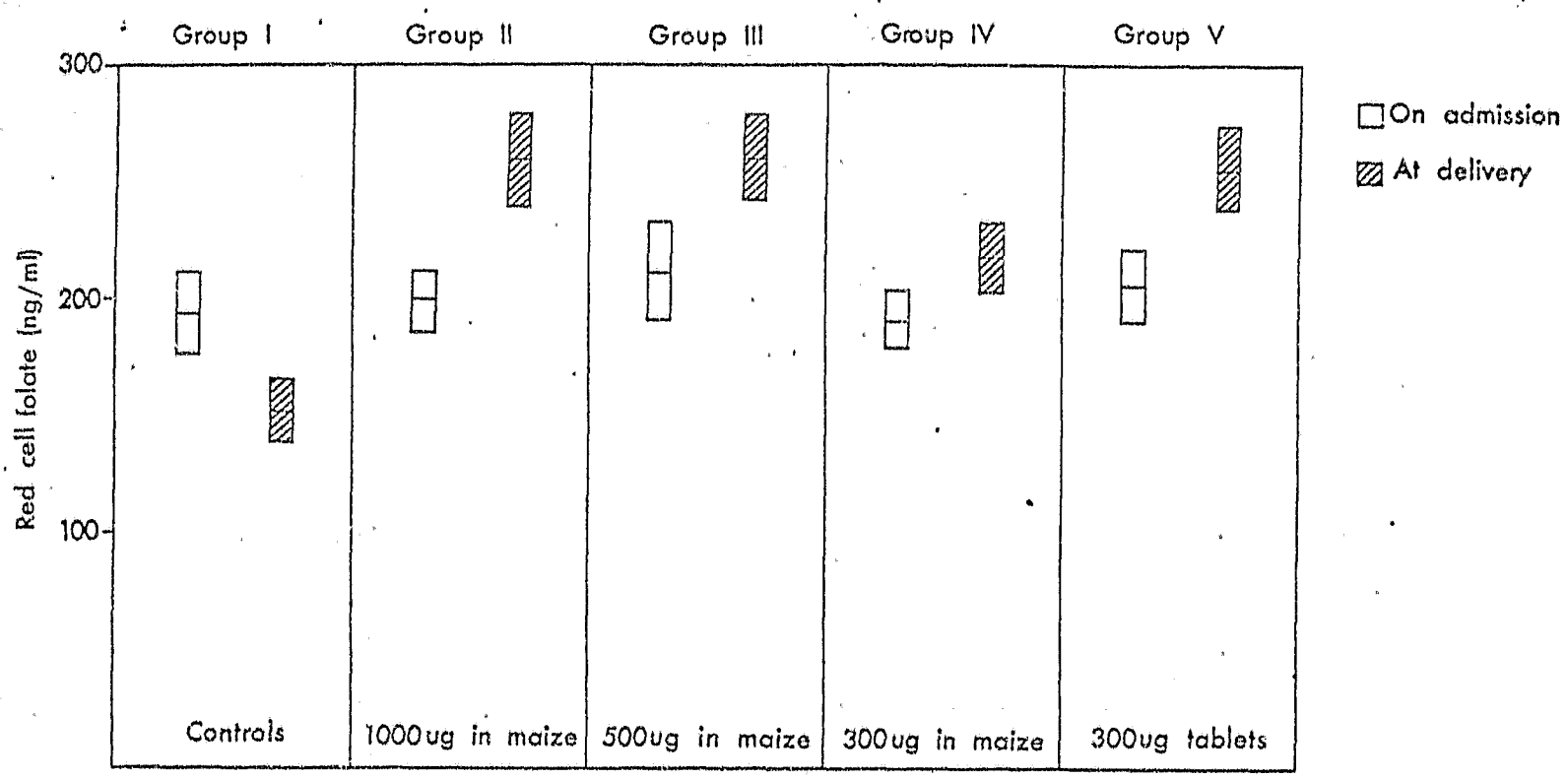

FIGURE 5.1. Red cell folate concentration (mean + standard error) in five groups of pregnant women. The daily doses of folic acid and the vehicles for the supplements are shown at the foot of each column.

When the red cell folate concentrations in each subject were expressed as deviations from the corresponding baseline levels, the mean change at each week was calculated for each of the five groups and a regression line for the group was calculated using the method of least squares. These means and regression lines for groups I and II are shown in Figure 5.2, and for groups III, IV and V in Figures 5.3, 5.4 and 5.5. The regression equations are shown in the legends of these figures.

In group I, which received unfortified maize meal, the mean had fallen significantly by the end of the first week ($p < 0.01$), and it continued to fall with increasing duration of the study. Examination of the regression line for this group revealed that the fall in red cell folate concentration during the trial was highly significant ($p < 0.001$).

In the groups receiving folic acid, either in maize meal or as tablets, all the weekly means were above baseline levels. The means for groups II, III and V were significantly raised after two weeks, and continued to rise with increasing duration of the trials except that during the fourth week of study, the mean for group V fell. As more than a third of the subjects in this group delivered during the fourth week, this fall is difficult to interpret. The regression lines showed that the rise in red cell folate concentration in groups II, III and V during the course of the study were all

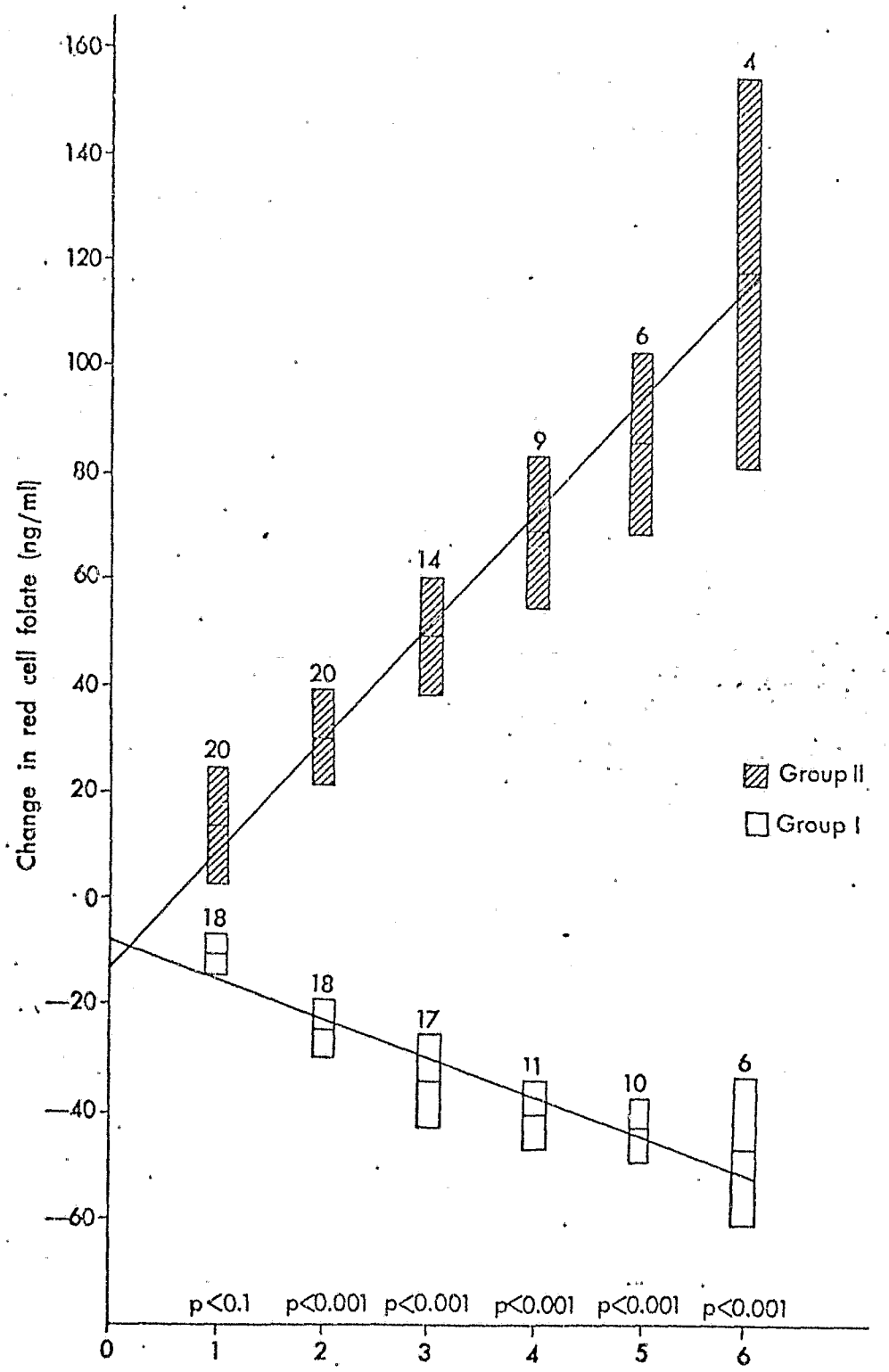

FIGURE 5.2. Change in red cell folate from value recorded on admission to the study (mean ± standard error) in subjects receiving unfortified maize (group I) and maize containing 1000 µg folic acid daily (group II). Regression equations - group I: $y = -7.56 - 7.36x$ ($r=-0.4263$; $p < 0.001$); group II: $y = -12.70 + 21.0x$ ($r=0.6022$; $p < 0.001$).

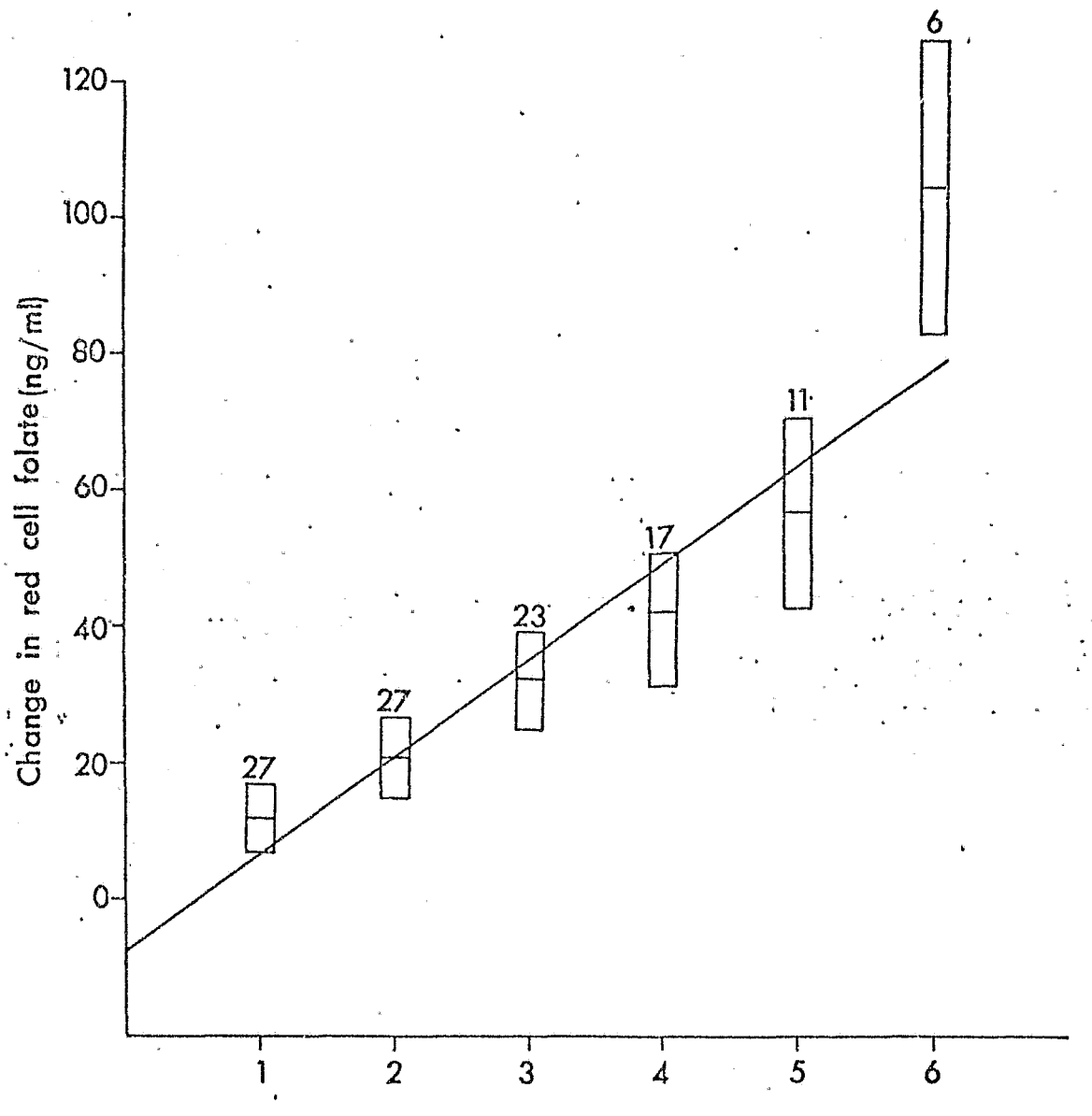

FIGURE 5.3. Change in red cell folate from value recorded on admission to the study (mean ±standard error) in subjects receiving maize meal containing 500 ug folic acid daily (group III). Regression equation -
$y = -6.76 + 14.11x$ ($r = 0.5798$; $p < 0.001$)

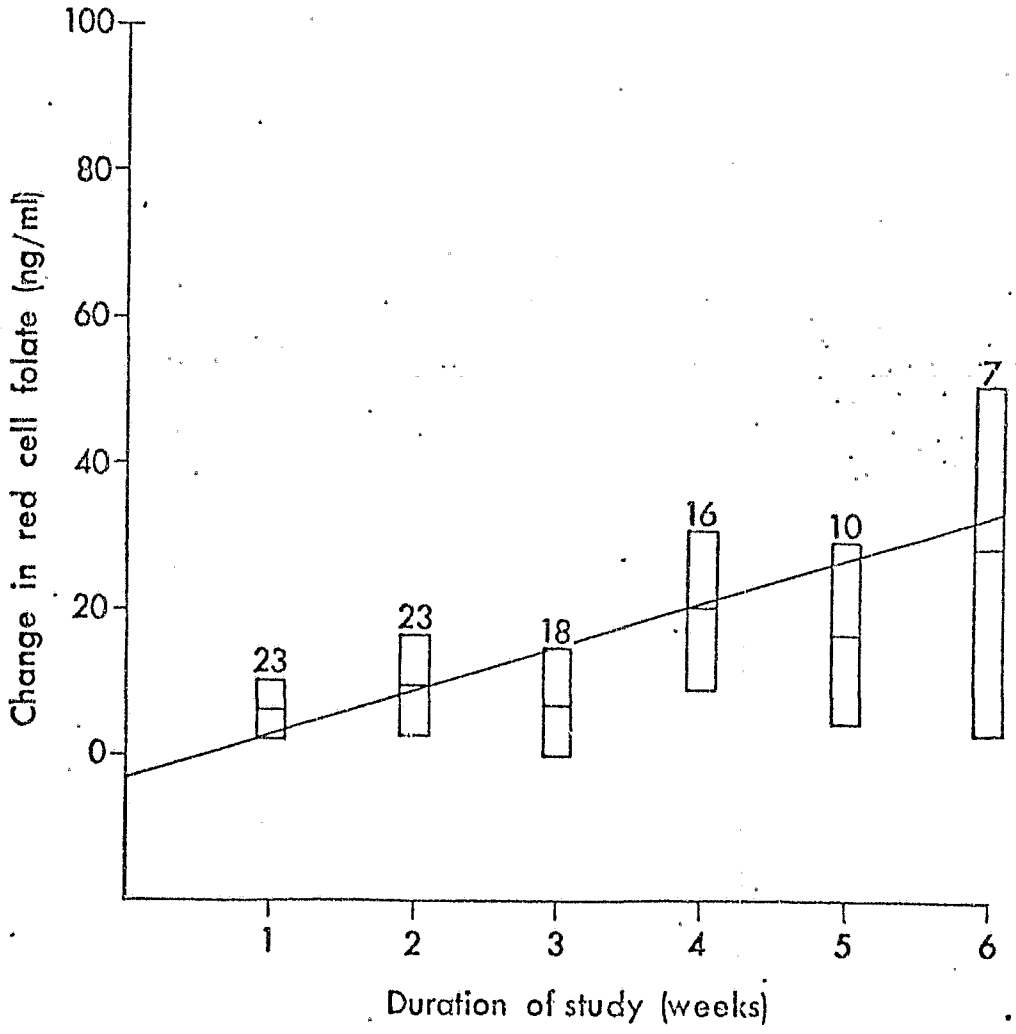

FIGURE 5.4 Change in red cell folate from value recorded on admission to the study (mean ± standard error) in subjects receiving maize meal containing 300 ug folic acid daily (group IV).
Regression equation -
$y = -3.27 + 6.00x$ ($r = 0.2580$; $p < 0.01$).

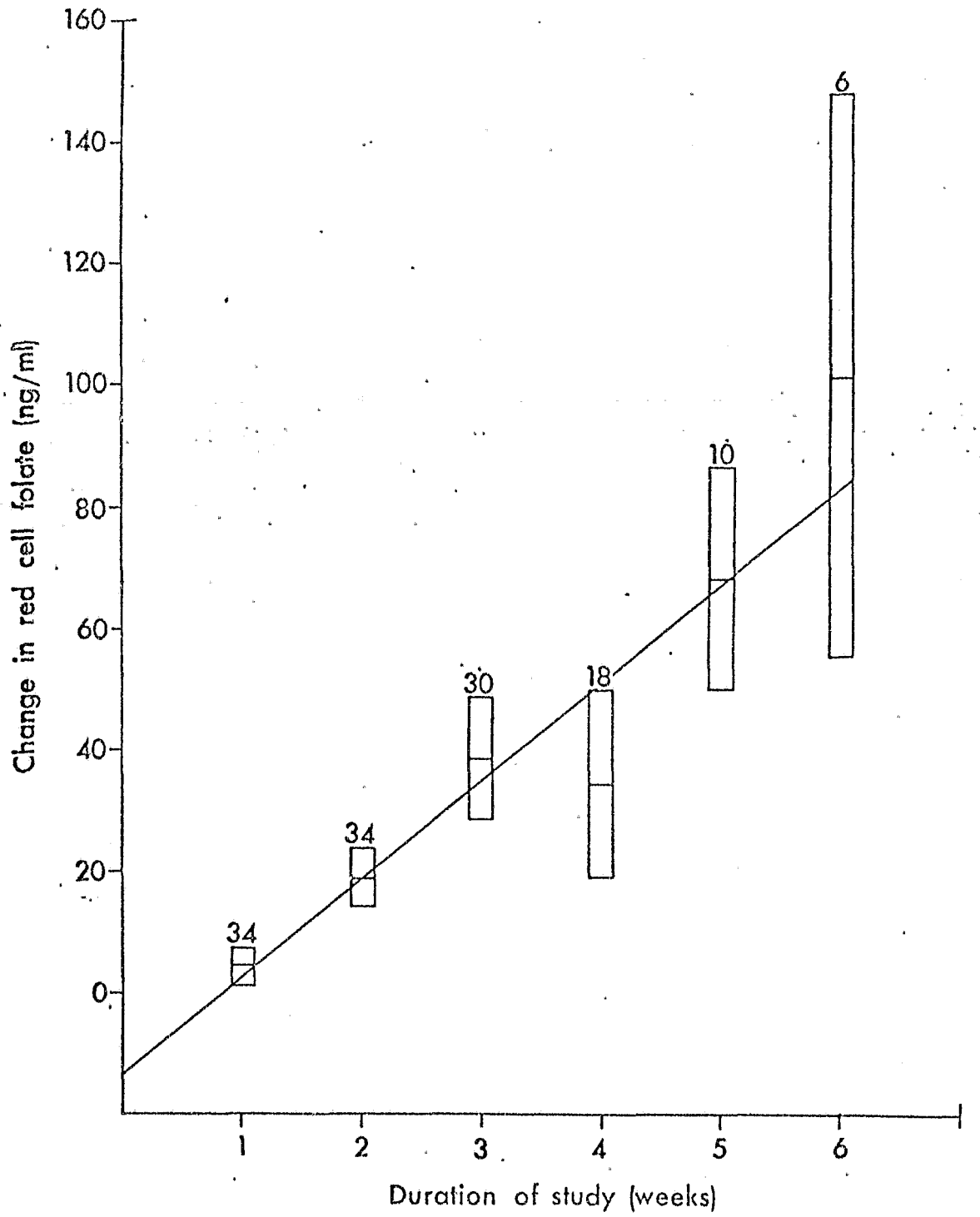

FIGURE 5.5 Change in red cell folate from value recorded on admission to the study (mean ±standard error) in subjects receiving 300 ug folic acid daily as tablets (group V). Regression equation - $y = -12.59 + 15.81x$ ($r=0.4278$; $p < 0.001$).

highly significant (for all, $p < 0.001$).

The elevation of the mean red cell folate concentration in group IV, which received maize porridge containing 300 µg folic acid daily, was not significant at any particular week (for all, $p > 0.05$), and the means did not rise consistently with increasing duration of the study. However, analysis of the regression line revealed that over the period of study as a whole the rise was significant, although not to the same extent as in the other groups receiving folic acid ($0.005 < p < 0.01$).

The slope of the regression lines for subjects receiving fortified maize meal increased with increasing doses of added folic acid. The slope of the line for group V, which received 300 µg folic acid tablets daily, was similar to that for group III, which received fortified maize containing 500 µg folic acid daily.

The regression equations for the groups receiving folic acid were examined in order to determine at which time these rose above baseline levels. Thus for example, the regression line for group II (slope = 21.20, intercept = -12.70) rose above the abscissa after the fourth day (at 0.60 weeks = 4.2 days). Using this method, it was calculated that red cell folate levels rose above baseline levels between the third and sixth days in groups II to V (at 4.2, 3.4, 3.8 and 5.6 days respectively).

(ii) Serum folate concentration

The mean serum folate concentrations in the five groups on admission to the trial and at delivery are shown in Figure 5.6, together with the corresponding standard errors. The mean for each group at the time of admission was statistically similar to the mean for all subjects, which was 4.65 ng per ml ($p > 0.3$).

During the period of study, the mean serum folate concentration in group I fell by 1.4 ng per ml. At the time of delivery, it was significantly lower than the mean for the other four groups (for all, $p < 0.001$). The mean level in group II rose by six ng per ml, and in groups III, IV and V by approximately three ng per ml.

The weekly means and regression lines calculated for the change in serum folate concentrations from baseline levels are shown in Figures 5.7 (groups I and II), and 5.8 to 5.10 (groups III to V). The regression equations are shown in the legends of these Figures.

In group I, the mean serum folate levels did not fall consistently and the regression showed that there was no significant change with duration of the study ($p > 0.95$).

The weekly means rose with increasing duration of the study in all groups receiving folic acid with the exception of the fifth week of study in group III. The regression lines for these groups showed that there was a significant rise during the period of study in

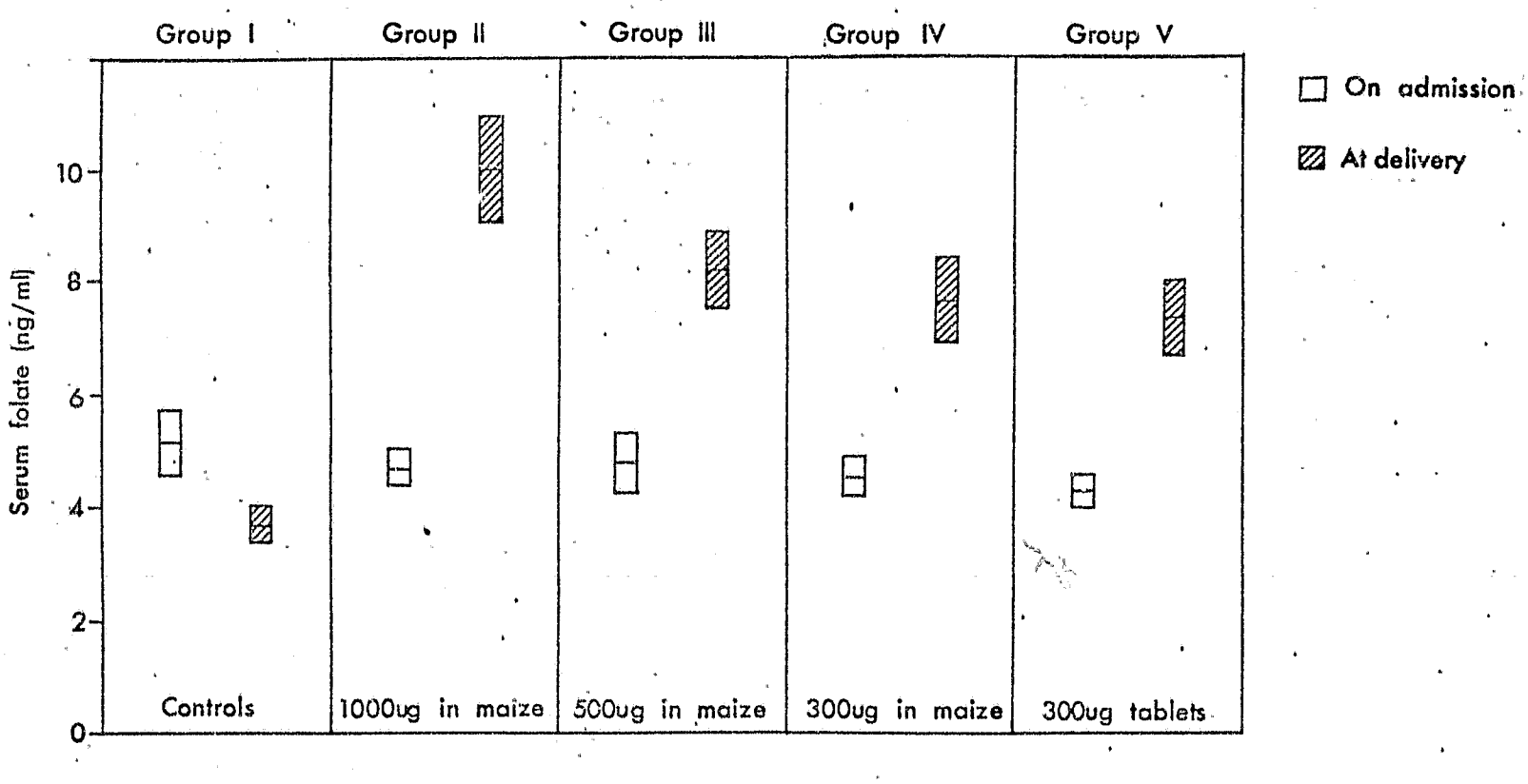

FIGURE 5.6. Serum folate concentration (mean ± standard error) in five groups of pregnant women. The daily doses of folic acid and the vehicles for the supplements are shown at the foot of each column.

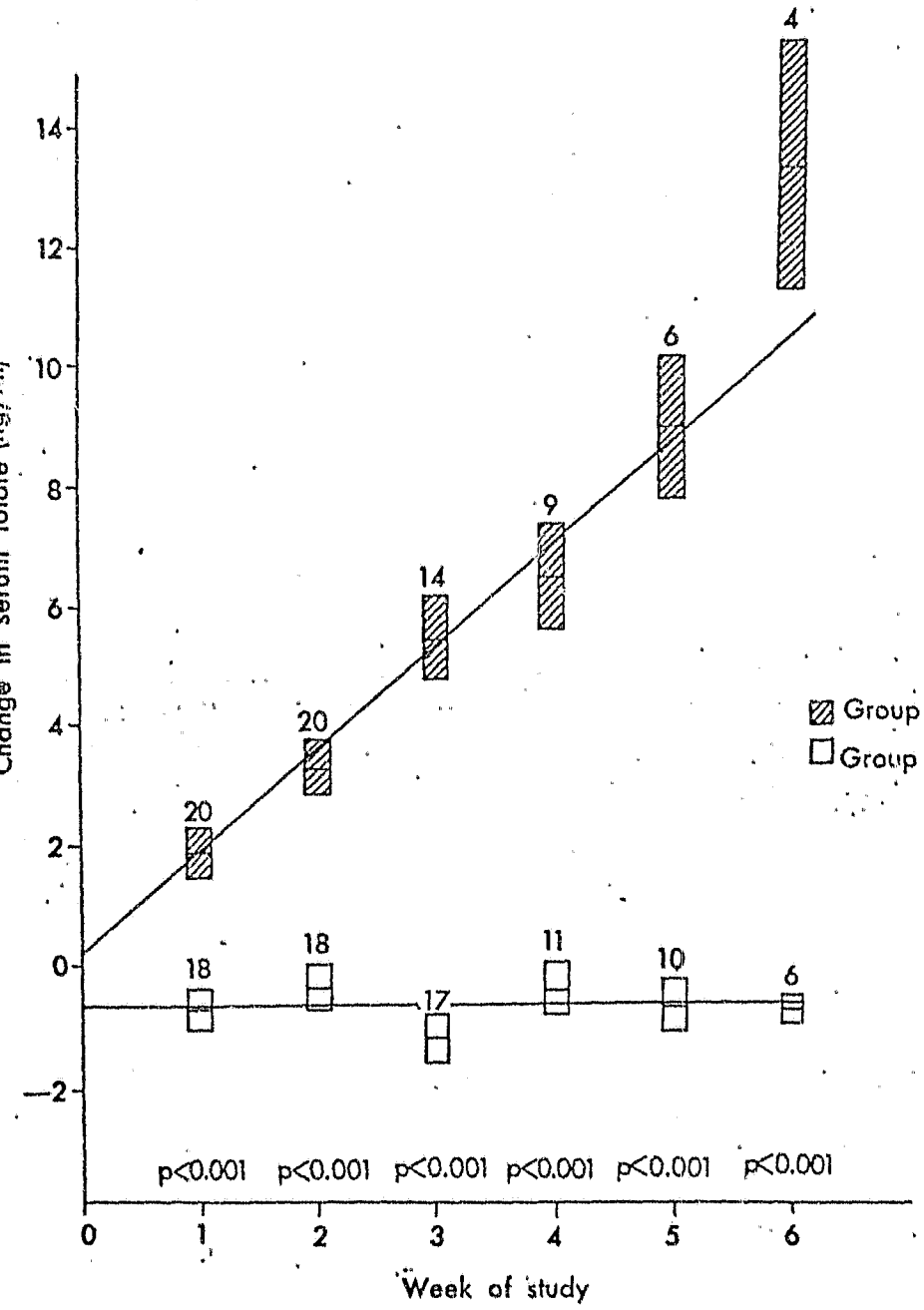

FIGURE 5.7. Change in serum folate from value recorded on admission to the study in subjects receiving unfortified maize (group I) and maize containing 1000/ug folic acid daily (group II). Regression equations - group I: $y = -0.673 - 0.006x$ ($r = 0.0063$; $p > 0.95$) group II: $y = 0.135 + 1.695x$ ($r = 0.7189$; $p < 0.001$).

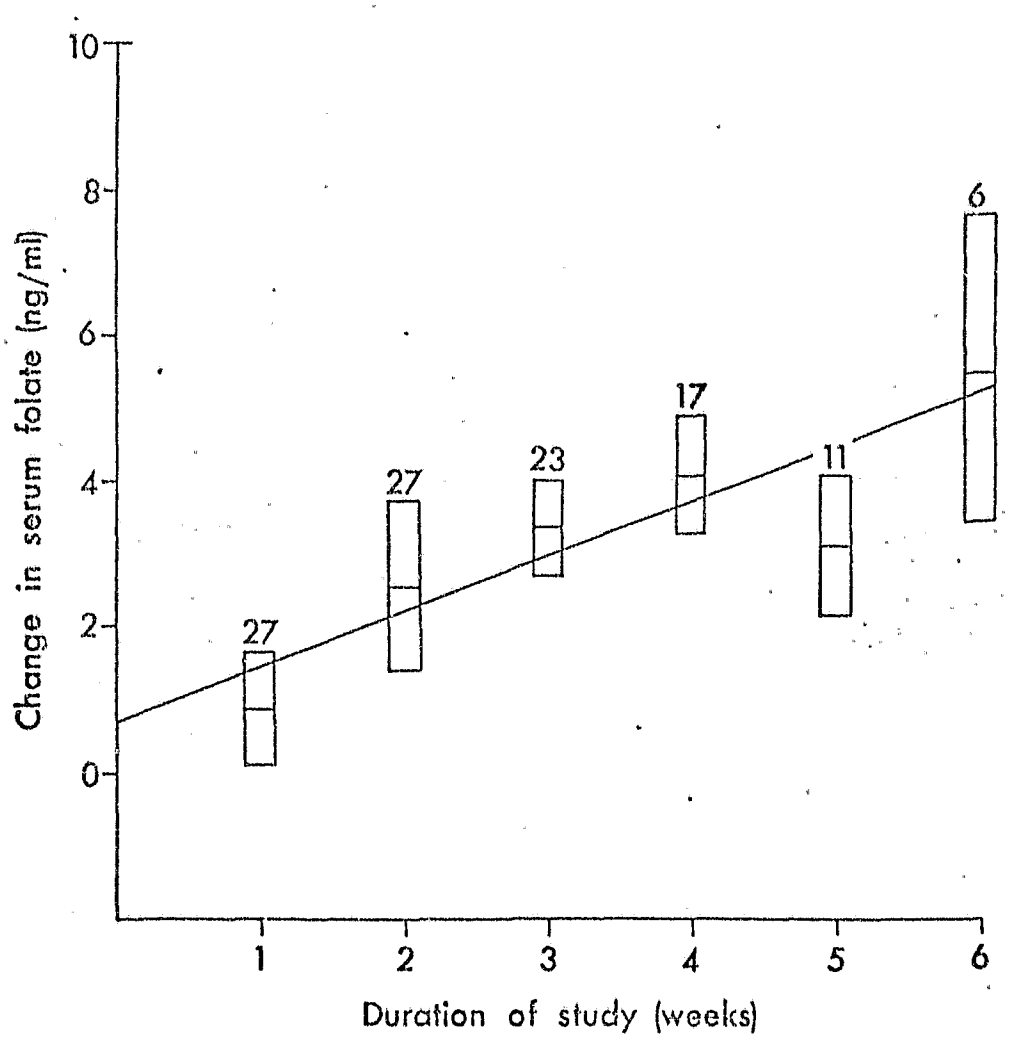

FIGURE 5.8 Change in serum folate from value recorded on admission to the study (mean ± standard error) in subjects receiving maize meal containing 500 ug folic acid daily (group III). Regression equation - $y = 0.685 + 0.752x$ ($r=0.3026$; $p < 0.005$).

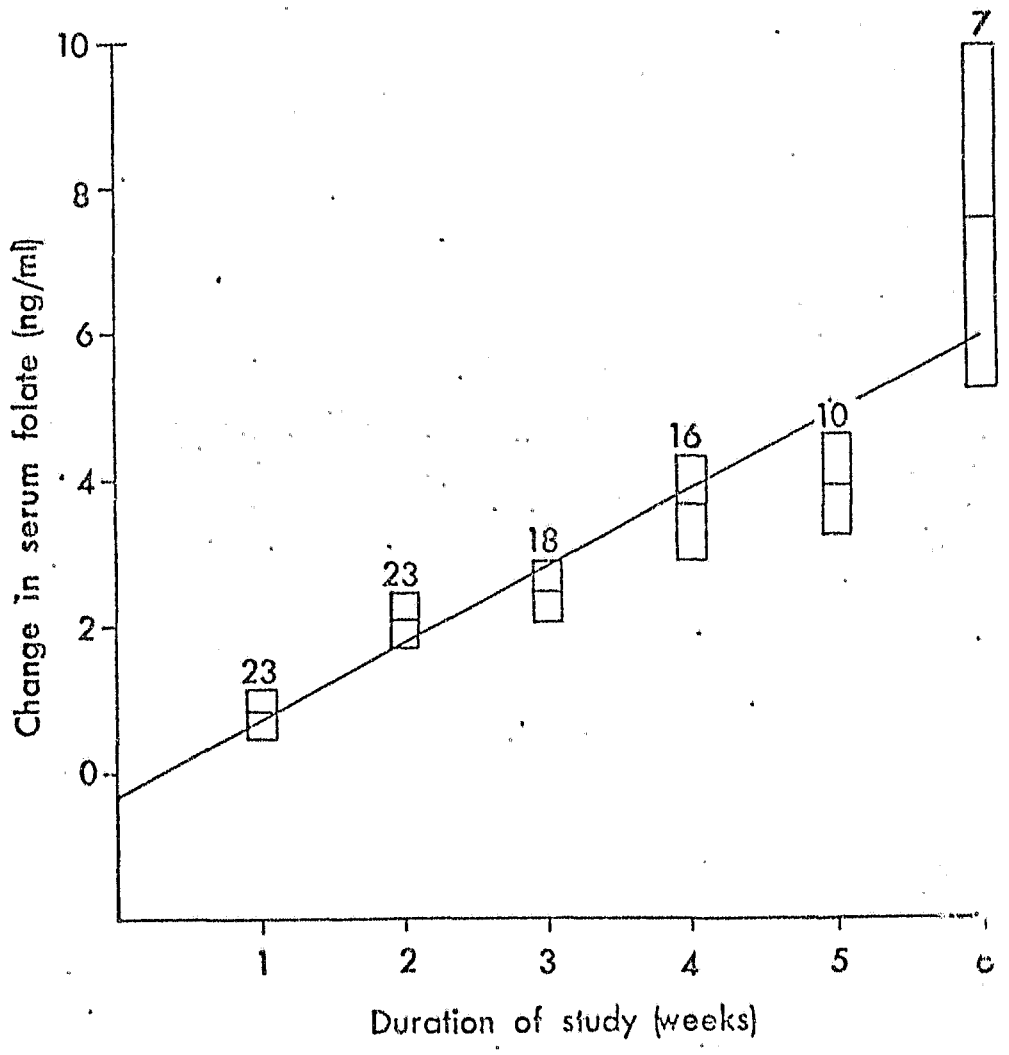

FIGURE 5.9 Change in serum folate from value recorded on admission to the study (mean ± standard error) in subjects receiving maize meal containing 300 ug folic acid daily (group IV). Regression equation - $y = -0.271 + 1.029x$ ($r=0.5575$; $p < 0.001$)

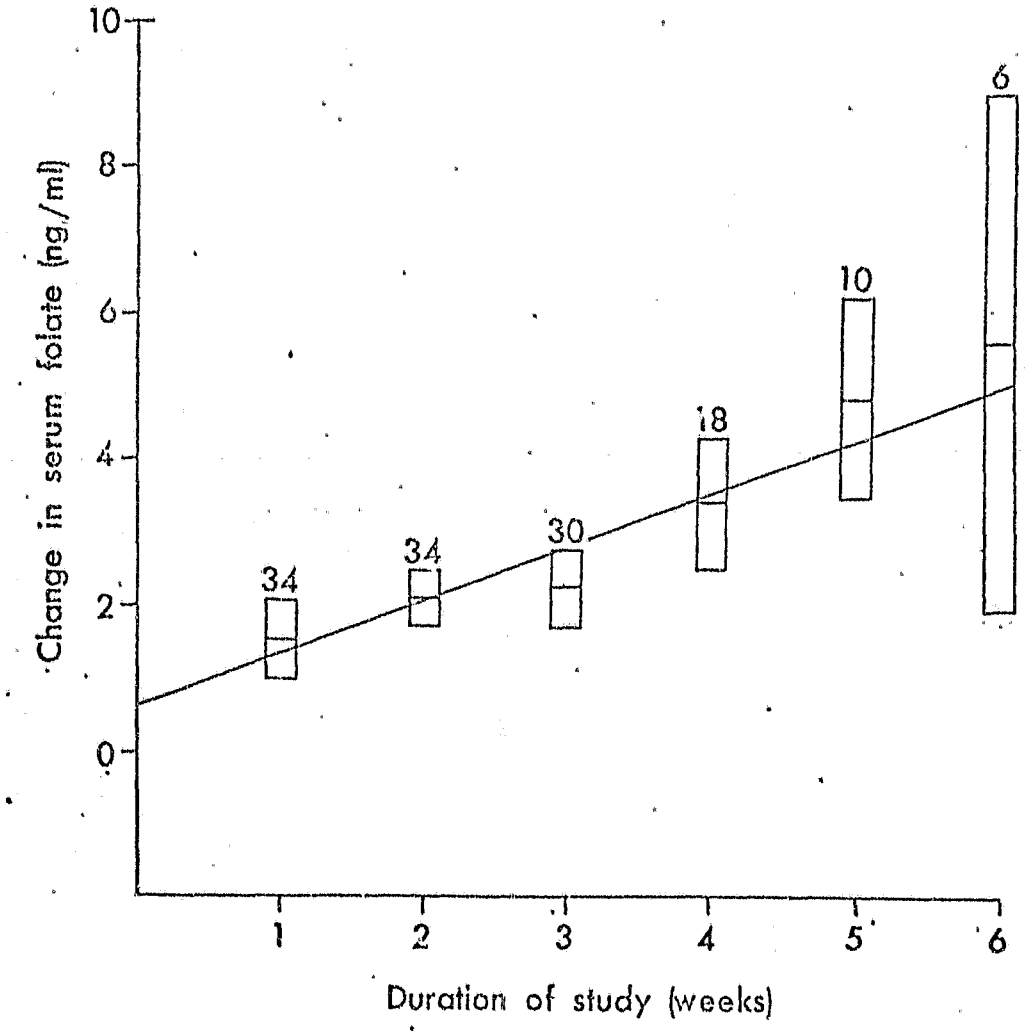

FIGURE 5.10 Change in serum folate from value recorded on admission to the study (mean ± standard error) in subjects receiving 300/ug folic acid daily as tablets (group V). Regression equation - $y = 0.643 + 0.713x$ ($r=0.2914$; $p<0.001$).

all four groups (for all, $p < 0.005$), and that the slope of each of these regression lines was significantly greater than the slope of the line for group I (for all, $p < 0.005$).

(iii) The prevention of folate depletion in individual subjects

The red cell folate and serum folate concentrations in each subject on admission to the trial and at delivery are shown in Tables 5.3 to 5.7, together with the duration of study. The subjects in each group are arranged in ascending order according to the red cell folate level on admission to the study.

The differences between these levels in each subject on admission to the study and at delivery were calculated. It was assumed that the difference was insignificant if red cell folate levels changed by 10 ng per ml or less, and if serum folate levels changed by 0.5 ng per ml or less. The subjects were then assigned to separate categories according to whether red cell folate and serum folate levels rose, fell, or remained unchanged. The number of subjects in each of the five groups assigned to each category is shown in Table 5.8.

The chi-square method was used to analyse the results shown in Table 5.8. In the case of both red cell folate and serum folate concentrations, there was no significant difference between groups II, III, IV, and V in the number of subjects whose levels rose, fell or remained unchanged

TABLE 5.3

Folate levels in pregnant subjects receiving unfortified maize meal (group I)

No.	Red cell folate (ng per ml)		Serum folate (ng per ml)		Duration of study
	On admission*	at delivery	on admission*	at delivery	(weeks)
1	80	72	3.7	3.8	6
2	108	89	3.7	5.0	5
3	111	106	4.0	4.4	4
4	135	95	3.8	3.6	6
5	141	135	5.5	2.2	3
6	147	131	3.1	1.2	6
7	150	84	6.8	4.0	5
8	152	110	2.2	2.0	5
9	171	126	7.4	4.0	3
10	197	140	5.2	4.2	7
11	210	209	5.6	3.6	4
12	211	155	3.4	3.4	6
13	221	217	5.2	3.4	3
14	247	184	4.4	3.6	3
15	253	155	1.8	1.2	6
16	263	219	8.0	8.0	5
17	321	277	6.8	4.0	5
18	369	225	13.0	6.8	3

*Mean of two observations

TABLE 5.4

Folate levels in pregnant subjects receiving fortified maize meal containing 1000 µg folic acid daily (group II)

No.	Red cell folate (ng per ml)		Serum folate (ng per ml)		Duration of study
	on admission*	at delivery	on admission*	at delivery	(weeks)
19	103	175	4.6	11.6	5
20	107	182	4.0	11.8	3
21	115	130	4.5	6.4	4
22	137	338	4.3	11.6	7
23	144	112	6.4	11.8	2
24	157	192	4.8	18.0	6
25	173	234	3.2	7.6	4
26	176	174	4.8	7.4	3
27	182	218	4.1	7.4	2
28	187	186	2.7	6.0	2
29	196	400	2.9	20.2	6
30	217	241	4.4	15.2	4
31	217	301	6.3	21.6	6
32	219	352	5.5	12.4	3
33	234	252	3.7	7.6	3
34	238	238	4.6	7.0	2
35	263	397	4.8	7.4	5
36	266	370	7.3	6.0	2
37	315	300	4.9	10.4	3
38	326	387	7.2	10.8	2

*Mean of two observations

TABLE 5.5

Folate levels in pregnant subjects receiving fortified maize meal containing 500 ug folic acid daily (group III).

No.	Red cell folate (ng per ml.)		Serum folate (ng per ml.)		Duration of study
	on admission*	at delivery	on admission*	at delivery	(weeks)
39	75	115	2.2	2.6	3
40	84	169	1.6	6.0	6
41	90	184	3.2	5.2	3
42	119	146	6.6	10.2	5
43	120	280	2.0	5.0	7
44	128	128	5.1	6.6	3
45	134	211	2.6	12.2	3
46	135	131	3.6	6.8	3
47	149	196	3.6	8.8	4
48	149	218	5.3	14.8	4
49	155	171	2.5	4.0	5
50	162	204	3.4	7.2	6
51	162	362	4.3	10.4	6
52	166	190	5.2	15.2	3
53	179	264	4.4	7.2	4
54	229	230	4.8	2.6	3
55	234	237	7.0	9.0	4
56	238	259	4.4	5.6	5
57	246	320	2.7	5.6	4
58	269	351	3.9	11.8	10
59	271	347	4.4	7.0	5
60	276	355	3.2	4.2	6
61	313	364	5.9	6.4	2
62	349	365	6.0	13.6	5
63	404	383	10.2	12.4	4
64	405	400	4.8	6.4	2
65	479	445	16.1	17.4	3

*Mean of two observations

TABLE 5.6

Folate levels in pregnant subjects receiving fortified maize meal containing 300 µg folic acid daily (group IV).

No.	Red cell folate (ng per ml)		Serum folate (ng per ml)		Duration of study
	on admission*	at delivery	on admission*	at delivery	(weeks)
66	73	161	5.7	8.2	2
67	103	109	2.4	2.4	4
68	113	135	3.4	5.4	5
69	125	135	4.0	4.0	4
70	130	151	4.2	4.8	3
71	146	178	2.7	5.0	5
72	148	252	4.8	12.0	6
73	151	159	3.7	4.0	3
74	163	195	2.3	6.2	6
75	169	186	3.3	5.6	4
76	177	199	5.2	7.2	2
77	183	199	4.0	7.6	3
78	200	169	5.1	8.8	2
79	202	191	2.4	4.4	3
80	210	282	5.4	11.2	6
81	224	310	5.6	19.2	7
82	229	230	4.2	6.8	4
83	235	263	6.0	11.6	4
84	247	351	4.3	4.4	4
85	265	389	8.9	10.0	7
86	266	181	3.2	8.0	6
87	305	270	6.2	10.8	6
88	312	297	7.4	8.2	5

*Mean of two observations

TABLE 5.7

Folate levels in pregnant subjects receiving 300/ug folic acid tablets daily (group Y).

No.	Red cell folate (ng per ml)		Serum folate (ng per ml)		Duration of study
	on admission*	at delivery	on admission*	at delivery	(weeks)
89	92	175	2.8	6.0	6
90	100	150	3.0	4.2	3
91	104	130	4.3	5.2	6
92	106	86	2.0	4.6	2
93	110	137	3.4	6.2	5
94	111	168	2.7	4.0	6
95	123	452	2.6	18.6	6
96	126	167	4.1	5.0	4
97	133	252	9.0	18.2	3
98	136	206	1.6	2.4	3
99	138	147	2.8	4.0	2
100	143	206	7.1	14.0	6
101	147	164	4.0	6.0	3
102	151	223	3.0	8.0	5
103	152	164	3.9	6.4	3
104	160	180	3.4	4.4	3
105	170	169	5.0	3.6	4
106	187	245	3.5	5.4	3
107	203	292	5.3	7.2	4
108	218	230	2.5	5.6	4
109	231	355	6.2	6.2	3
110	235	254	3.4	9.2	5
111	235	283	6.8	9.0	4
112	240	252	9.0	18.2	3
113	245	311	4.7	8.0	3
114	253	283	4.5	5.0	3
115	263	315	5.1	6.8	6
116	270	261	4.3	6.4	2
117	285	320	2.2	6.4	2
118	348	589	7.4	7.8	3
119	353	374	8.4	17.2	4
120	384	443	3.6	7.0	3
121	396	460	4.8	8.2	5
122	446	252	4.6	6.0	4

*Mean of two observations.

TABLE 5.8

Number of subjects in whom red cell folate (RCF) and serum folate levels (SF) rose or fell during the study.

Group	Folic acid supplement		Number of subjects in whom RCF			Number of subjects in whom SF			Number of subjects in whom neither RCF nor SF rose**
	daily dose (ug)	Vehicle	fell	was unchanged	rose	fell	was unchanged	rose	
I	0	-	13(72)*	5(28)	0	11(61)	6(33)	1(6)	17(94)
II	1000	maize	2(10)	3(15)	15(75)	1(5)	0	19(95)	0
III	500	maize	2(7)	5(19)	20(74)	1(4)	2(7)	24(89)	1(4)
IV	300	maize	4(17)	3(13)	16(70)	0	4(17)	19(83)	3(13)
V	300	tablets	2(6)	3(9)	29(85)	1(3)	3(9)	30(88)	1(3)

* The figures shown in parentheses are percentages

** Serum folate levels rose in all subjects in groups II to V in whom red cell folate levels fell.

(for both measures, $p > 0.95$). When group I was compared with the other groups, it was found to differ significantly (for both measures, $p < 0.001$).

Similarly, when the groups were divided into those subjects in whom either red cell folate or serum folate levels rose, and those subjects in whom neither rose, there was no difference between the groups receiving folic acid supplements ($p > 0.5$) but group I differed significantly from the other groups ($p < 0.001$). It is worthy of note that in all subjects in groups II to V in whom red cell folate levels fell, serum folate levels rose.

(b) Discussion

These results show that food fortified with folic acid can be used to increase folate stores in pregnancy, and so safeguard against the development of deficiency. The administration of fortified maize porridge containing three different concentrations of folic acid resulted in a rise in mean red cell folate and serum folate concentrations in each group to levels significantly higher than those of subjects receiving unfortified maize.

The observation that serum folate levels did not fall significantly in group I probably reflects the fact that these subjects had been in a state of negative folate balance since early pregnancy. It has been shown

previously that this situation causes a fall in serum folate concentration to its lowest level within five weeks (Herbert, 1962a).

Red cell folate concentration is generally regarded as a good measure of tissue folate stores (Herbert and Zalusky, 1962; Chanarin, 1969; Streiff, 1971b). The decrease in red cell folate in subjects receiving unfortified maize, shown by the regression line for group I having a slope of minus seven ng per ml per week, thus reflects the extent of the drain on reserves in late pregnancy. This drain is due mainly to increased requirements, as excretion of folate, although increased in pregnancy, is insufficient to significantly affect body stores (Fleming, 1972).

The lag period of three to six days which preceded the rise in red cell folate in groups II to V may reflect the time taken for newly-formed red cells to be released from the marrow. In groups II, III and V, the change in red cell folate concentration became significant at the second week. This is compatible with the previous observation that there is a detectable rise in red cell folate levels within nine days of folic acid administration (Moore and Ball, 1972), although this rise may be delayed as long as five weeks in individual subjects with severe folate deficiency (Herbert, 1962a).

A composite diagram of the regression lines for red cell folate in the five groups is shown in Figure 5.11.

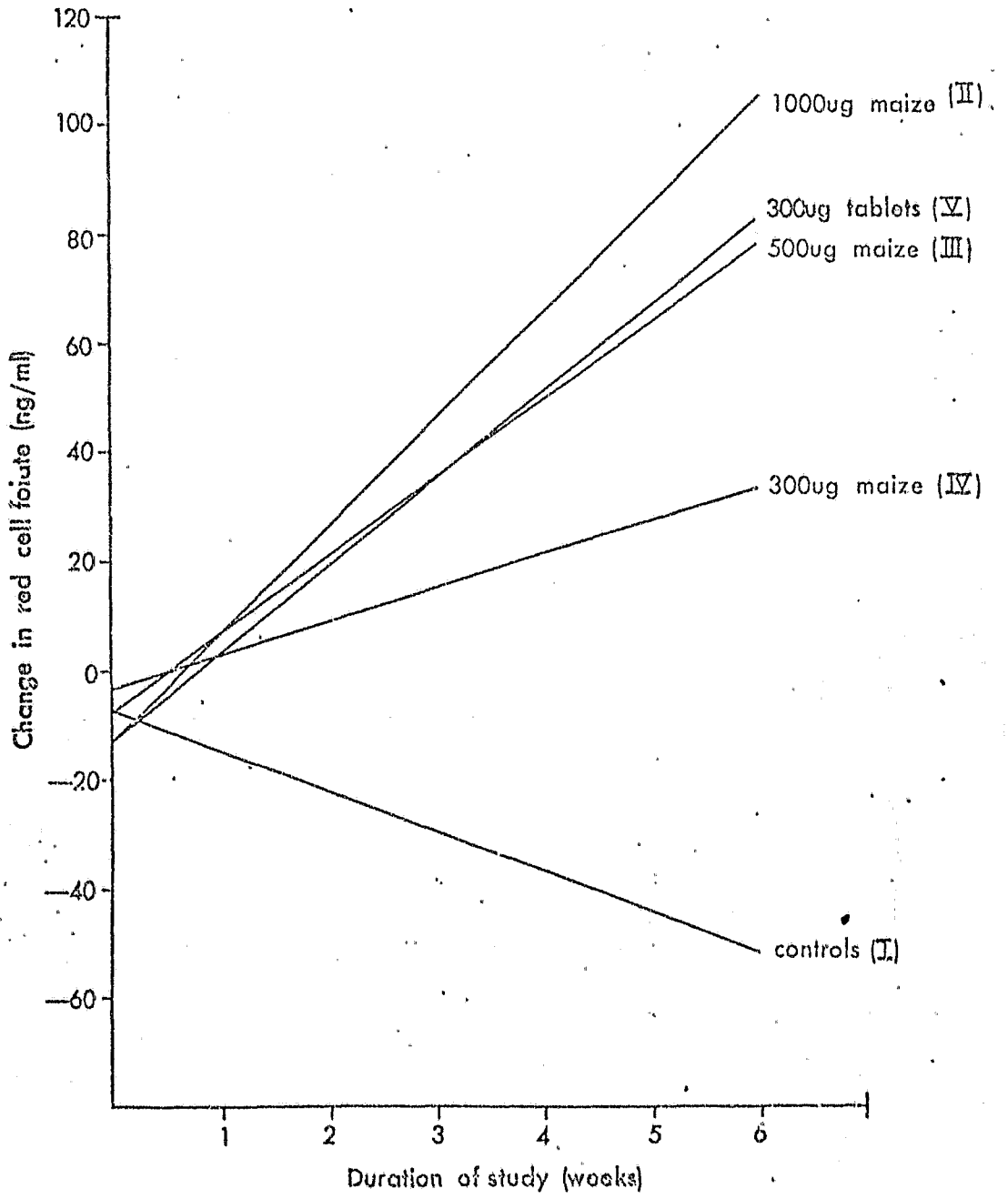

FIGURE 5.11 Composite diagram showing regression lines for the change in red cell folate in five groups of pregnant women. The daily dosage of folic acid, vehicles for the supplement, and group numbers are shown to the right of the corresponding regression lines.

If it is assumed that the regression line for red cell folate in group I reflects what would have occurred in all groups in the absence of folic acid supplements, the slopes of the regression lines for the other groups can be corrected to account for this fall. Thus, for example, it may be said that the addition of 1000 µg folic acid daily to the maize fed to group II resulted in the slope of the regression line being increased by 7.363 ng per ml per week to the horizontal, and by a further 21.197 ng per ml per week to the observed slope. The corrected slope would thus be 28.560 ng per ml per week. The slopes of the regression lines for groups II to V, corrected in this way to account for the slope expected in the absence of folic acid supplements, are shown in Table 5.9.

TABLE 5.9

Slopes of regression lines for red cell folate corrected to account for slope in the absence of folic acid supplements (group I).

Group	Folic acid supplement daily dose (µg)	vehicle	Corrected slope (ng per ml per week)
II	1000	maize	28.560
III	500	maize	21.470
IV	300	maize	13.367
V	300	tablets	23.176

These corrected slopes can be used to compare the incorporation of folic acid into red cell folate in the different groups. The increase in red cell folate in group IV, receiving fortified maize containing 300 ug added folic acid daily, was 57.7 per cent of that for group V, which received the same dose of folic acid in tablet form. Similarly, if it is predicted that increasing the dose of folic acid tablets by two-thirds would result in a corrected slope two-thirds greater than that observed in group V, then the corrected slope for group III is 55.6 per cent of that predicted for subjects receiving the same dose of folic acid as tablets. These two percentages concur closely with the observation made in Chapter 4 that the mean absorption of folic acid from fortified maize porridge was 56.3 per cent of that from an aqueous solution of folic acid. This tends to validate the methods used in that absorption study, and lends further credence to the figures quoted there for absorption of folic acid from fortified rice and bread. The abovementioned figures also suggest that decreased absorption of folic acid from fortified maize porridge is the sole reason for the rise in red cell folate levels being slower than in subjects receiving equivalent doses of folic acid tablets. Once the folic acid from maize is absorbed, its metabolism in the body appears to be identical to that of folic acid tablets.

The corrected slopes can also be used to compare the utilisation of different doses of folic acid in fortified maize. The corrected slope for group IV was 62.2 per cent of that for group III, corresponding to the fact that the dose of folic acid in the maize received by the former was 60 per cent of that in the maize received by the latter. This is interpreted to mean that the 300 µg and 500 µg daily doses of added folic acid were absorbed with equal efficiency.

However, the corrected slope of the regression line for group II, receiving 1000 µg added folic acid daily, was only about two-thirds (66.5 per cent and 64.1 per cent) of that expected if this higher dose of folic acid had been utilised as efficiently as the 500 µg and 300 µg doses in maize meal.

It can thus be said that single doses of folic acid in fortified maize meal porridge are utilised with similar efficiency up to a dose of 500 µg, but that if the amount of folic acid is increased to 1000 µg, only one-third of the additional quantity is utilised. The proportion of a 1000 µg dose of folic acid absorbed from fortified maize compared with aqueous solutions (Chapter 4) was similar to the proportion of a 300 µg dose incorporated into red cell folate from fortified maize compared with tablets. It is therefore likely that the administration of folic acid tablets to pregnant women in doses greater than

500/ug would result in inefficient utilisation of the amount in excess of this figure. Since the effect of 300/ug folic acid tablets was similar to that of 500/ug in maize meal, it can be argued that folic acid tablets taken in single doses greater than 300/ug would be inefficiently utilised.

Two qualifications can be expressed when interpreting the above observations for use in food fortification programmes. The first is that most staple foods are eaten more than once daily, and the daily folic acid intake from fortified food would thus not be taken in a single dose. Secondly, it is questionable whether it would ever be necessary to fortify any food with the intention of providing a folic acid supplement greater than that equivalent to 300/ug in tablet form.

In all groups receiving folic acid supplements, there were some subjects in whom red cell folate levels fell during the course of the trial. It is likely that these subjects had greater folic acid requirements than others, possibly related to an increased avidity of the foetus for the vitamin. The fact that serum folate levels rose in all of these subjects suggests that they were absorbing the folic acid, but that the total dose received during the few weeks of the trial was insufficient to significantly improve body stores.

It is of value to consider the relative efficacy of the different strengths of fortified meal. The rate of rise in red cell folate and the mean rise during the whole period of the study increased with increasing doses of folic acid. However, the number of subjects in whom red cell folate and serum folate rose, were maintained, or fell, was statistically similar in all groups receiving folic acid. It can thus be concluded that the maize containing a 300 µg daily dose of added folic acid was as effective as the more concentrated maize in preventing the progression of folate depletion in late pregnancy. In the light of the observation that added folic acid in maize is about one half to two-thirds as available as folic acid in tablets, this suggests that folic acid supplements of 200 µg daily as tablets would be adequate for prophylaxis in this population.

This inference is in accordance with some observations made in other populations. In Sweden and London, daily doses of 100 µg folic acid maintained red cell folate levels in late pregnancy (Hansen and Rybo, 1967; Chanarin *et al.*, 1968), but in Glasgow they did not (Willoughby, 1967), and 200 µg folic acid daily failed to evoke optimal haematological responses in two patients with megaloblastic anaemia in pregnancy studied by Alperin *et al.* (1966). In Canada, supplements of 200 µg folic acid daily maintained red cell folate

levels in pregnant women (Cooper et al., 1970), and Cooper (1973) has suggested that 200 ug folic acid daily would be an adequate supplement for pregnant women with a diet poor in folate.

IV Haemoglobin and serum vitamin B_{12} concentration

(a) Introduction

The pregnant women studied in these trials comprised a selected group, in that none had haemoglobin concentrations lower than 11 g per 100 ml on admission to the study, as measured by a visual haemoglobinometer.

Haemoglobin concentration was measured in all of the whole blood samples obtained from subjects in the five groups, and vitamin B_{12} concentration was measured in the sera obtained on admission and just before delivery from subjects in groups III, IV and V.

(b) Results

(i) Haemoglobin concentrations

The mean haemoglobin levels in the five groups on admission to the study and at delivery are shown in Table 5.10, which also lists the number of subjects in each group in whom haemoglobin levels rose, fell or were maintained.

The mean haemoglobin level in each group on admission was similar to the combined mean for all subjects (for all, $p > 0.2$), which was 12.46 g per 100 ml. At the time

TABLE 5.10

Change in haemoglobin levels (Hb) in groups and individual subjects.

Group	Folic acid supplement		Mean Hb level (g per 100ml)		Number of subjects in whom Hb		
	daily dose (/ug)	vehicle	on admission	at delivery	fell	was unchanged	rose
I	0	-	12.44	11.75	15	2	1
II	1000	maize	12.71	13.21	5	1	14
III	500	maize	12.34	13.19	2	1	24
IV	300	maize	12.60	13.12	5	0	18
V	300	tablets	12.32	12.93	4	2	28

TABLE 5.10

Change in haemoglobin levels (Hb) in groups and individual subjects.

Group	Folic acid supplement		Mean Hb level (g per 100ml)		Number of subjects in whom Hb		
	daily dose (µg)	vehicle	on admission	at delivery	fell	was unchanged	rose
I	0	-	12.44	11.75	15	2	1
II	1000	maize	12.71	13.21	5	1	14
III	500	maize	12.34	13.19	2	1	24
IV	300	maize	12.60	13.12	5	0	18
V	300	tablets	12.32	12.93	4	2	28

of delivery, the mean haemoglobin concentration in subjects receiving unfortified meal was significantly lower than the combined mean and than the mean for each of the groups receiving folic acid supplements (for all, $p < 0.001$). None of the means for the groups receiving folic acid was different from any of the other means in these groups.

The number of subjects in whom haemoglobin levels rose, fell, or remained unchanged were analysed using the chi-square test. When the groups receiving folic acid supplements in maize or as tablets were compared, this test did not detect any difference between the groups. However, when group I, which received unfortified maize, was included in the chi-square there was a highly significant difference between the groups ($p < 0.001$). This is interpreted to mean that the number of subjects in whom haemoglobin levels fell during the trial was statistically much greater in group I than in all the other groups.

Similarly, when regression lines were calculated on the change in haemoglobin levels at each week from the corresponding baseline values, the slope of the line was found to be negative in the case of group I but positive in the other groups. The correlation coefficients for the lines are listed in Table 5.11, together with their significance and the conclusions drawn. The fall in haemoglobin levels in group I and

TABLE 5.11

Correlation between weekly change in haemoglobin concentration and duration of study.

Group	Folic acid supplement daily dose (µg)	vehicle	Correlation coefficient	p value	conclusion
I	0	-	-0.0645	> 0.5	fall insignificant
II	1000	maize	0.3934	< 0.001	rise significant
III	500	maize	0.4415	< 0.001	rise significant
IV	300	maize	0.1581	> 0.05	rise insignificant
V	300	tablets	0.3057	< 0.001	rise significant

the rise in group IV were not significant when assessed by this method, but the rise of the regression line in groups II, III and V was highly significant (for all, $p < 0.001$).

(ii) Serum vitamin B_{12} concentrations

Table 5.12 shows the mean serum vitamin B_{12} levels on admission and at delivery, and the number of subjects in whom the levels rose, fell and were unchanged. In calculating these numbers, it was assumed that a change in concentration of less than 50 pg per ml was insignificant.

The mean serum vitamin B_{12} level in each group was similar to the combined mean for all the subjects, on admission to the study ($p > 0.1$) and at delivery ($p > 0.1$). In no group did the mean change significantly during the course of the study (for all, $p > 0.3$). The number of subjects in each group whose levels changed or were maintained was analysed by the chi-square test, which revealed that the differences between the groups were not statistically significant ($p > 0.2$).

It was concluded that serum vitamin B_{12} levels did not change significantly during the course of the trials in subjects receiving fortified maize and folic acid tablets.

(c) Discussion

The finding that haemoglobin levels were

TABLE 5.12

Change in serum vitamin B_{12} levels (B_{12}) in groups and in individual subjects.

Group	Folic acid supplement		Mean B_{12} level (pg per ml)		Number of subjects in whom B_{12}		
	daily dose (/ug)	vehicle	on admission	at delivery	fell	was unchanged	rose
III	500	maize	655.2	699.3	10	11	6
IV	300	maize	611.7	665.7	9	12	2
V	300	tablets	729.9	724.7	12	10	12

significantly affected in the subjects studied by the administration of folic acid supplements was somewhat unexpected, as they were a selected group, with haemoglobin levels of 11 g per 100 ml or greater as measured on a visual haemoglobinometer, and were followed for only a few weeks. It should be noted that the visual haemoglobinometer provided a relatively rough approximation, as haemoglobin levels measured by the Coulter Counter Model "S" were 9.5 - 10 g per 100 ml in two subjects and 10 - 11 g per 100 ml in four on admission to the trial.

The rise in haemoglobin levels in subjects receiving folic acid supplements suggests that haemopoiesis in these subjects may have been limited by folate deficiency. This is regarded as additional evidence that the provision of folic acid supplements to this population should be regarded as a priority.

The observation that serum vitamin B_{12} concentration did not change in subjects receiving folic acid supplements is in accordance with previous reports that the administration of these supplements, in doses ten times greater than those used in the present study, does not significantly affect serum vitamin B_{12} levels (Metz et al., 1965).

C H A P T E R 6

PILOT TRIAL OF FOLIC ACID-FORTIFIED MAIZE MEAL IN THE HOME

I Introduction

In order to prove conclusively th value of a food fortification programme, it is necessary to demonstrate an improvement in nutritional status in populations who store, cook and eat the fortified food in the home environment according to their custom. The ideal way of bringing about this situation is to fortify food and then offer it for sale to the consumer through the usual commercial channels.

A most convincing trial of this nature has been conducted in South Africa using maize meal fortified with riboflavin and nicotinamide (du Plessis et al., 1971). Maize was fortified at a mill which was the only source of maize meal in a certain area, and a group of schoolchildren living in the area were then compared with a control group living in an adjacent village. On the basis of clinical and biochemical observations, it was concluded that the fortified maize meal had significantly diminished the incidence of deficiency of the two vitamins in the population eating this meal.

The patients described in Chapters 5 and 7 received, under hospital supervision, fortified meal cooked according to local custom. In this chapter, the effect of the meal in the home environment was studied by giving folic acid-fortified maize meal to selected families for home use. It was considered that this investigation would reveal whether any aspect of local custom had been unaccounted for in the hospital-based trials.

II Materials and methods

(a) Subjects studied

Eight index patients were selected for the trial from the Charles Johnson Memorial Hospital at Nqutu. Seven were healthy women in the 28th week of pregnancy who had come to the antenatal clinic for the first time, and one was a healthy lactating woman who was studied from the eighth week post partum. None of the patients had received folic acid supplements prior to being studied. The pregnant women were all given iron supplements from the start of the study, and the lactating subject had received iron during pregnancy. These subjects were selected for study by virtue of the fact that they lived in relatively close proximity to the hospital.

The oldest member of each index subject's family was also studied. All of these older subjects were women. It was decided to study the oldest member of each family in order to assess the effect of the folic acid-fortified food on members of the age group most prone to develop pernicious anaemia.

(b) Calculation of dietary intake

Individual dietary intake was assessed by relating family size to the amount of maize meal bought monthly. In order to correct for varying consumption within each family, children above the age of sixteen years were taken to be adults, those between two and sixteen years were assumed to be consuming half as much as adults,

and children below two years of age were disregarded. This method was used because it has been shown that the amount of maize meal bought each month, corrected for varying consumption on the basis of age, is a very reliable basis for estimating maize meal intake, whereas weighing the amount of maize meal cooked for a single meal is a much less satisfying basis for this calculation (du Plessis et al., 1971).

(c) Provision of fortified maize meal

Separate batches of fortified maize meal were prepared for each family. The amount of folic acid added to each batch was such that the weight of maize meal calculated to be consumed in one day by each adult in the particular family contained 500 µg of added folic acid. The method of mixing the folic acid with maize meal was identical to that described in Chapter five.

Based on the calculated dietary intake, each family was given enough meal to supply its total demand for six weeks, and was instructed to use this meal exclusively until it had all been consumed. The families were not told how long the meal was expected to last, and were asked to report when they had used all of it.

(d) Procedure

Blood was taken for folate and vitamin B_{12} assay and haemoglobin estimation from the index subject and

the oldest member of each family on the two days before the start of the trial and weekly thereafter. All samples from a particular subject were assayed for folate and vitamin B_{12} within the same respective batch. Two pregnant women and the senior members of their respective families failed to report at the second week, and refused to continue in the study when approached in their homes. These subjects all stated that the reason for their refusal was a reluctance to submit to blood sampling.

III Results

Two subjects from each of six families were studied for the full six week period. Table 6.1 shows the ages of the subjects studied, the calculated number of adults in each family, and the period each family took to consume the maize meal intended to last for six weeks.

The results of laboratory investigations are summarised in Table 6.2. The haemoglobin levels and serum vitamin B_{12} concentrations remained essentially unchanged in all subjects. Serum folate levels in the subjects studied showed wide variation from week to week. This phenomenon was attributed to the fact that the blood samples were taken at variable times after the patients had eaten the fortified meal. The serum folate levels have therefore been disregarded.

The red cell folate concentrations in the subjects at each week during the study are shown in Figure 6.1. The

TABLE 6.1

Details of families given folic acid-fortified maize meal.

Family	Age of subject		"Adults" in family**	Time taken to consume fortified maize meal***
	Index*	Oldest		
1	26	58	3	43 days
2	16	60	4.5	40 days
3	28	56	4.5	71 days
4	30	63	4.5	39 days
5	33	76	5	41 days
6	24	58	5	46 days

* The index subject in family 1 was lactating, and the remaining index subjects were pregnant.

** The method of calculating the number of adults in each family is described in the text.

*** The meal was fortified on the assumption that it would last for 42 days.

TABLE 6.2

Laboratory findings before and after eating folic acid-fortified maize meal for 6 weeks.

Family	Subject	Haemoglobin (g/100 ml)		Vitamin B12 (pg/ml)		Red cell folate (ng/ml)	
		Before*	After	Before	After	Before*	After
1	a	13.0	12.7	584	551	116	383
	b	13.6	13.6	966	971	310	396
2	a	13.7	13.5	766	796	166	355
	b	13.8	13.9	1114	1115	127	187
3	a	11.0	12.0	696	686	381	405
	b	14.7	15.0	1484	1156	228	275
4	a	11.7	11.8	903	876	159	493
	b	15.0	16.0	1670	1889	248	242
5	a	12.2	12.2	891	789	208	369
	b	13.2	13.1	659	707	533	603
6	a	12.0	12.2	608	640	170	257
	b	12.4	12.7	739	853	216	294

a - index subject. b - oldest member of family. * - mean of two observations.

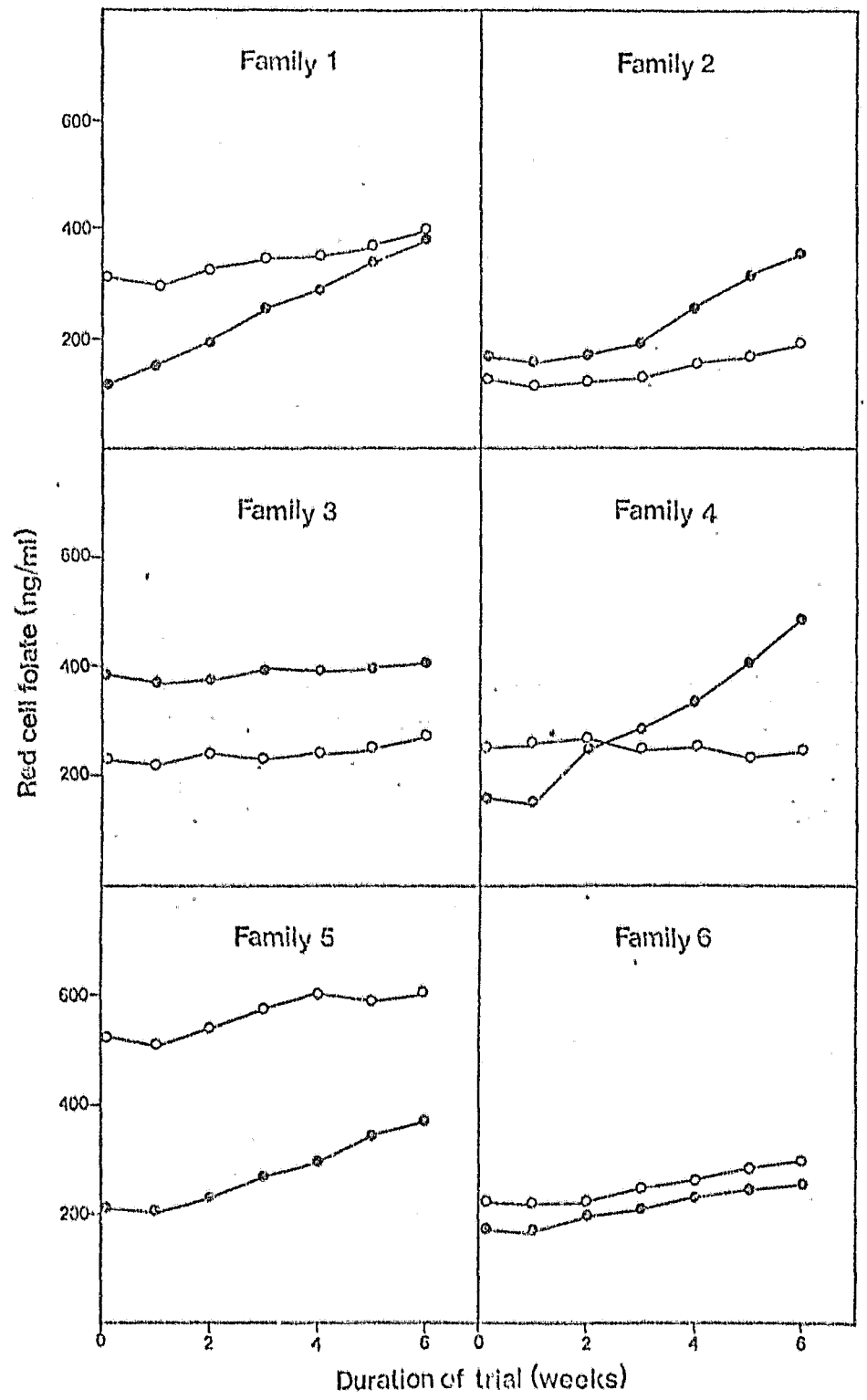

FIGURE 6.1 Weekly red cell folate concentration in the index subject (closed circles) and the oldest member (open circles) of six families given a supply of fortified maize meal designed to provide 500 µg folic acid daily.

fortified maize meal did not appreciably raise the red cell folate concentration in the index subject of family number 3, which consumed the meal at approximately 60 per cent of the predicted rate, i.e., a calculated folic acid intake of 300 /ug per day for each adult. This patient was pregnant, and the fact that her red cell folate concentration rose slowly is in accordance with the effect of a 300 /ug daily dose noted in the trial described in Chapter five.

In the index subjects of all the other families there was a marked rise in red cell folate concentration. The change in this value was calculated by the same method as that used in Chapter five, using linear regression and expressing each weekly value as the change from the initial value in that subject. Using this method, the regression line for the four subjects whose families consumed the amount of maize meal predicted (families 2,.4, 5 and 6) was $y = 39.3x - 47$ ($r = 0.7861$), where y = red cell folate concentration in ng per ml, and x = time in weeks. This reflects a significantly greater rate of rise than that observed in pregnant subjects fed fortified maize meal containing 500 /ug folic acid daily under supervision at the hospital ($u = 3.677$; $p < 0.001$). Figure 6.2 shows the weekly mean of the change in red cell folate in these four index subjects, compared with the mean $\pm$2S.E. in the hospital subjects receiving 500 /ug added folic acid daily. It would be predicted with greater than 95 per cent confidence that the mean of any comparable group would fall with-

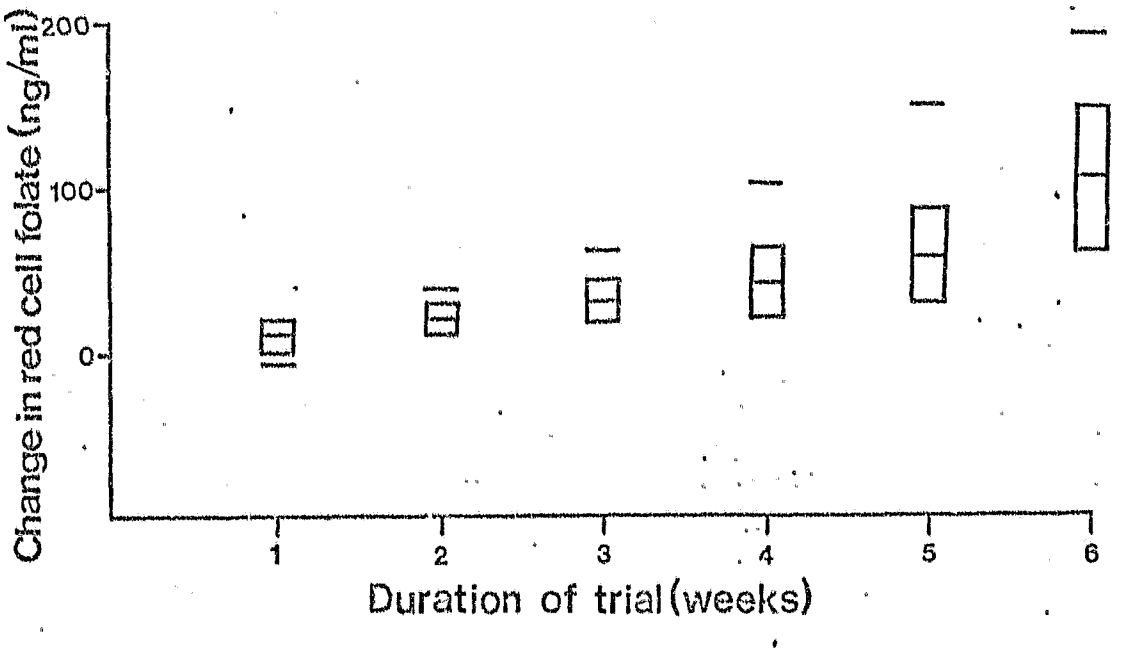

FIGURE 6.2 Mean change in red cell folate in pregnant subjects supplied with fortified maize meal for home use (bars), compared with mean ± 2 S.E. in pregnant subjects (group III) receiving the meal under supervision at a hospital (blocks). The hospital subjects received 500/ug added folic acid daily, and the meal provided for home use was designed to deliver the same dose.

in the range of the original mean ±2S.E. The rate of rise in the lactating subject was greater than that of the group of pregnant subjects ($y = 46.4x - 12.7$; $r = 0.9973$).

The corresponding regression line for the older subjects in the six families was $y = 12.7x - 19.8$ ($r = 0.7540$). When this line was compared with the regression line for all six index subjects, it was found that the rate of rise in the index subjects was significantly greater than that in the older subjects ($u = 3.34$; $p < 0.01$). This is mainly due to the fact that there was a much greater rise in the index subjects of families 1, 2, and 4 than in the corresponding older subjects. This fact is illustrated in Table 6.3, which lists the ratios of the rise in red cell folate over the six week period in the index subject to that in the older subject in each family.

Finally, the rise in red cell folate concentration in each subject over the six week period was compared with the concentration before eating the fortified maize meal. There was no correlation between these two values ($r = -0.4679$; $p > 0.1$).

IV Discussion

The rise in red cell folate in the pregnant subjects whose families consumed the predicted amount of maize meal was significantly more rapid than that in pregnant subjects receiving a comparable dose of added folic acid

TABLE 6.3

Ratio of the rise in red cell folate (ng/ml) in index subjects to that in oldest members of families receiving fortified maize meal for six weeks.

Family member	Ratio (index/oldest)
1	3.10:1
2	3.15:1
3	0.51:1
4	334:-6
5	2.30:1
6	1.12:1

under supervision at the hospital. A further finding was that the red cell folate level in the index subjects of the families rose much more rapidly than in the corresponding older woman in the family.

These two observations suggest that the consumption of maize meal was not uniform in the adults in each family. It appears that the younger, pregnant or lactating females may have consumed more than the average intake in the family. There is information that elderly people have reduced food consumption, and the reasons for this have been reviewed by Mayer (1972).

It is possible to explain these observations on a different basis. The possibility of defective folate absorption in the older patients cannot be entirely discounted. The pregnant subjects described here were studied from the 28th week of pregnancy, whereas those in the hospital-based trial were in a more advanced stage of pregnancy and may have had greater folate requirements.

If this pilot trial reflects the general pattern of food consumption in the population, this has considerable significance in planning for food fortification with folic acid. It has been mentioned previously that the main hazard of fortifying food with folic acid for consumption by the general population is that haematological response might result in an occasional patient with undiagnosed megaloblastic anaemia due to vitamin B_{12} deficiency. The smallest daily dose of folic acid which