AN INVESTIGATION INTO FOLATE DEFICIENCY IN HEALTHY UNDERPRIVILEGED INFANTS WITH SPECIAL EMPHASIS ON THE INTERPRETATION OF THE FIGLU TEST AFTER ORAL HISTIDINE

by

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University of Cape Town

To my Parents.

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ABBREVIATIONS USED IN THIS STUDY.

Formimino-L-glutamic acid	- Figlu
Tetrahydrofolate	- FH_4
Pteroylglutamic acid	- PGA
N-Formimino-L-glutamate : tetrahydrofolate 5-formiminotransferase	- Glutamate formiminotransferase
N^5-Formimino-tetrahydrofolate cyclodeaminase	- Cyclodeaminase
Methyl-tetrahydrofolate : L-homocysteine S-methyl transferase	- Transmethylase
Glutamate formiminotransferase and cyclodeaminase prepared according to the method of Tabor and Wyngarden, 1958	- T-C enzyme
L-Histidine monohydrochloride	- Histidine
D-Xylose	- Xylose
Streptococcus faecalis	- S.faecalis
Lactobacillus casei	- L.casei
Pedicoccus cerevisiae	- P.cerevisiae
Umbilical cord samples	- Cord samples
Serum L.casei folate assays (if S.faecalis was used, this will always be stated.)	- Serum folate assays
Red cell L.casei folate assays	R.B.C. folate assays
Figlu test after oral histidine (if the test was performed without oral histidine, this will always be stated.)	- Figlu test
Deoxyribonucleic acid	- DNA
Serum glutamic-oxaloacetic transaminase	- S.G.O.T.
Mean corpuscular haemoglobin concentration -	- M.C.H.C.
Nanograms/ml.	- ng. (10^{-9}g.)/ml.
Picograms/ml.	- pg. (10^{-12}g.)/ml.

ABBREVIATIONS USED ON TABLES AND GRAPHS

Serum	- S. or Se
Serum folate levels	- Folates, Folate or Serum Folates
Weight percentile	- Wt. %
Number	- n
Standard error of mean	- S.E. of M. or S.E. of Mean
Gastroenteritis	- G.E. or gastroent.
Upper respiratory tract infection	- U.R.T.
Sulphadiazine	- S.D.Z.

C O N T E N T S.

CHAPTER I.

INTRODUCTION.

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INTRODUCTION

In South Africa, as in other countries with a large underprivileged population group, the problem of malnutrition and its associations and complications assumes considerable importance. Until the socio-economic problems involved are solved, attention must be directed to early diagnosis, treatment and prophylaxis of malnutrition and its associations.

At the Red Cross Children's Hospital, Cape Town, a considerable percentage of admissions are for marasmus and kwashiorkor. Anaemia is a frequent association, (Walt, 1959) sometimes contributing to the mortality (Walt, Holman and Naidoo, 1957).

In 1959 it was decided to investigate the aetiology of the anaemia in kwashiorkor at the hospital because this was a controversial issue which limited effective therapy (Altmann and Murray, 1948; Adams, 1954; Woodruff, 1955; Walt, Holman and Hendrickse, 1956; Foy and Kondi, 1957; Trowell and Simpkiss, 1957; Shnier and Metz, 1959).

These early studies showed that a protein diet of high biological value did not cure the anaemia unless specific haematinics were also administered. Iron deficiency was found in nearly every case and a high proportion of the infants showed varying degrees of folate deficiency. No unequivocal vitamin B_{12} deficiency was found (Friedman, 1960; 1962).

Lanzkowsky, (1960) showed that iron deficiency was prevalent in underprivileged but otherwise healthy non-White infants in Cape Town. From this it seemed possible that both the iron and folate deficiencies encountered in kwashiorkor were a manifestation of wide-spread deficiencies occurring in healthy underprivileged infants rather than a specific effect of kwashiorkor.

In order to investigate this relationship and to acquire further information regarding the aetiology of folate deficiency in kwashiorkor, an investigation was carried out in 1963 on healthy underprivileged infants and underprivileged infants with gastroenteritis (Friedman, McKenzie, Turner and Wittmann, 1964 a, b).

Laboratory methods for diagnosing folate deficiency were by then available. The estimation of Formimino-L-glutamic acid (Figlu) excreted in urine after an oral L-histidine monohydrochloride (histidine) load was one of these methods. This was regarded as a sensitive, specific and reliable test of folate deficiency (Luhby, Cooperman and Teller, 1959 b). Moreover, a simple method was available for estimating Figlu (Kohn, Mollin and Rosenbach, 1961). For these reasons the Figlu test after the administration of oral histidine was chosen as a laboratory parameter for investigating folate deficiency.

By means of this method it was shown that approximately 64% of healthy, underprivileged infants had a positive Figlu test. This implied that a considerable number of these infants had low folate reserves. If this were so, it seemed worth while acquiring further information regarding the incidence, aetiology, significance and need for prophylaxis of latent folate deficiency in healthy underprivileged infants. This had particular relevance to the possible development of folate deficiency in kwashiorkor and marasmus.

This investigation will be prefaced by a review of information available at the time of commencement of the study (1963). Material published thereafter will be referred to only when the particular subject under review is not discussed again in later chapters.

CHAPTER II.

REVIEW OF THE LITERATURE

CHAPTER II

BRIEF HISTORICAL REVIEW OF THE RECOGNITION OF THE ROLE OF FOLIC ACID DEFICIENCY IN THE PRODUCTION OF MEGALOBLASTIC ANAEMIA.

The fundamental role of folic acid and vitamin B_{12} deficiency in the production of the megaloblastic anaemias was only recognised when these vitamins were prepared in synthetic form in 1945, (Angier et al.) and in 1948 (Rickes, Brink, Koniuszy, Wood and Folkers, 1948; Smith, 1948) respectively. This was the result of many valuable contributions made throughout the nineteenth and the first half of the twentieth centuries.

Recognition of pernicious anaemia.

Early in the nineteenth century, physicians reported case histories of patients suffering from obscure forms of anaemia (Combe, 1824; Channing, 1842) which were subsequently recognised as cases of vitamin B_{12} (Combe, 1824) and folate (Channing, 1842) deficiency. Addison (1849; 1855) and Biermer (1872) were independently responsible for precise clinical descriptions of a new entity later to be known as Addison-Biermer pernicious anaemia. This was a puzzling idiopathic anaemia distinguishable from chlorosis and other causes of secondary anaemia which were recognised at the time. The course was progressive and the outcome nearly always fatal.

Following the recognition of the function of the bone marrow in blood formation, (Bizzozero, cited by Ewing, 1904) Pepper (1875) and Cohnheim (1876) independently studied post mortem specimens of the bone marrow in pernicious anaemia. Cohnheim (1876) made most important histological observations. He identified megaloblasts and megalocytes and suggested that the megaloblastic erythropoiesis was the essential cause of the anaemia.

The macrocytic appearance of the red cells was noted by Lepine (1876; 1877) and contemporary investigators appreciated other changes in the red cells in this condition (Ewing, 1904). In 1880 Ehrlich described the presence of megaloblasts in blood smears as a characteristic of pernicious anaemia. He has been credited with the introduction of the term "megaloblast" (Dacie and White, 1949).

The introduction of a method of bone marrow biopsy suitable for use on patients (Ghedini, 1908) enabled Zadek (1922) to compare the bone marrow with the blood of patients in all phases of pernicious anaemia. He confirmed that the erythropoiesis in the bone marrow was megaloblastic (Cohnheim, 1876; Ehrlich, 1891), and that macrocytosis developed as a result. Like other investigators (cited by Muir, 1894) but unlike Cohnheim (1876) and Rindfleisch (1890), he believed that the megaloblastosis was a secondary phenomenon - possibly a manifestation of the haemolytic nature of pernicious anaemia.

Aetiology of pernicious and other macrocytic anaemias.

In all this time there had been much speculation about the aetiology of pernicious anaemia, (Ewing, 1904) but little advance in knowledge. In 1926, Minot and Murphy produced evidence which strongly suggested that pernicious anaemia was a deficiency disease. Prompted by earlier and unrelated experimental work carried out on dogs by Robscheit-Robbins and Whipple, (1925) they successfully treated patients suffering from pernicious anaemia with a standard diet low in fat and containing 120-240 g. of liver per day as well as 120 g. or more of beef or mutton and 300 g. of vegetables. Patients were permanently maintained in remission as long as liver therapy was continued.

These findings had far-reaching results.

In the first place, Castle, (1929) conducted important experiments which finally established pernicious anaemia as a deficiency disease. In

doing so, he confirmed a long suspected relationship between this anaemia and food (Combe, 1824; Habershon, 1863; Elders, 1922).

He and his colleagues concluded that a factor in normal gastric juice (the intrinsic factor) combined with a dietary factor (the extrinsic factor) to produce a haemopoietic factor which was haematologically effective in the treatment of pernicious anaemia. They believed that the essential defect leading to the development of pernicious anaemia was the lack of the intrinsic factor (Castle, 1929; Castle and Townsend, 1929; Castle, Townsend and Heath, 1930).

For many years it had been thought that "pernicious anaemia" occurred in association with pregnancy, gastrointestinal diseases, infection, neoplasia and worm infestation (Ewing, 1904). Liver therapy was successfully used in patients with pernicious anaemia of pregnancy, (Strauss and Castle, 1932 a; Wills and Mehta, 1930) tropical macrocytic anaemia (Wills, 1931) and pernicious anaemia of sprue (Castle and Rhoads, 1932).

Further study of these conditions by Wills in particular, but by Strauss and Castle as well, led to the realisation that there was a distinction between pernicious anaemia and these other macrocytic anaemias. Clinical differences were noted by Wills and Mehta (1930) and Bennett, Hunter and Vaughan (1932). Moreover, the pathogenesis and the therapeutic requirements of the other macrocytic anaemias appeared different from those of pernicious anaemia. The concept of the extrinsic factor deficiency developed from these observations which were as follows:

A single course of liver without maintenance therapy was effective in curing pernicious anaemia of pregnancy (Strauss and Castle, 1932 a). Marmite, an autolysed yeast preparation, rich in vitamin B complex,

especially B_1 and B_2 (Wills, 1931; 1934) was as effective as liver in treating tropical macrocytic anaemia which included the macrocytic anaemia of pregnancy (Wills, 1931). It was also effective in the treatment of macrocytic anaemia of sprue (Castle and Rhoads, 1932) and steatorrhea (Vaughan and Hunter, 1932). But it was ineffective in treating pernicious anaemia unless it was combined with the intrinsic factor (Strauss and Castle, 1932).

Finally it was shewn that Marmite could cure a macrocytic anaemia induced in monkeys by feeding them a deficient diet similar to that taken by patients who developed tropical macrocytic anaemia (Wills and Bilimoria, 1932).

On the above evidence, the aetiology of some of these macrocytic anaemias seemed different from the aetiology of pernicious anaemia and was attributed in most cases to a dietary deficiency of extrinsic factor (Wills, 1934; Strauss and Castle, 1932).

The clinical and aetiological differences between pernicious anaemia and other macrocytic anaemias therefore justified their differentiation (Wills, 1934).

The extrinsic factor was at one stage believed to be vitamin B_2 (Wills, 1934; Strauss and Castle, 1932; Strauss and Castle, 1933) because of a similar distribution in a wide variety of foodstuffs (Strauss, 1934).

This was subsequently disproved (Wills, 1934; Strauss, 1934). Though still thought to be a member of the vitamin B complex, the nature of the extrinsic factor was not specifically determined (Minot, 1935).

As a result of the work of Minot and Murphy (1926) and Castle, Townsend and Heath (1930), it was believed that the intrinsic and extrinsic factors combined to form a haemopoietic principle which was stored in the liver. The efficacy of liver therapy in all macrocytic anaemias led

Castle and Ham (1936) to consider that these, including pernicious anaemia, ultimately resulted from a deficiency of the single active principle stored in the liver although the aetiology of liver principle deficiency could be different (Unitarian Hypothesis). In pernicious anaemia it resulted from an intrinsic factor deficiency and required permanent replacement therapy. In other macrocytic anaemias the deficiency arose as the result of low intake of the extrinsic factor, malabsorption of extrinsic or haemopoietic factors or destruction of intrinsic, extrinsic or haemopoietic factors (Castle and Ham, 1936; Strauss and Castle, 1932). Maintenance therapy was usually not required in these cases because of the transitory nature of most of the aetiological mechanisms.

Other possible causes of macrocytic anaemia that were suggested were inability to store (Wintrobe and Shumacker, 1933) and to utilise liver principle (Minot, 1935).

The validity of the Unitarian Hypothesis of Castle and Ham (1936) was questioned, when Wills and Evans (1938) demonstrated that purified extracts of liver, effective in the treatment of pernicious anaemia, were ineffective in nutritional macrocytic anaemias though they could be cured by Marmite or crude liver extracts. This led the investigators to suggest that there might be two extrinsic factors.

<u>The concept of megaloblastic anaemia.</u>

Following the description of megaloblastic erythropoiesis in pernicious anaemia (Cohnheim, 1876; Ehrlich, 1880; Zadek, 1922), there was controversy about the meaning and importance of megaloblasts. Some morphologists (Doan, Cunningham and Sabin, 1925; Maximow, 1927; Sabin, 1928), did not distinguish between normal primitive erythroid precursors and

megaloblasts and as a consequence found megaloblasts in normal persons and in conditions besides pernicious anaemia.

The introduction of an improved method for bone marrow biopsy (Seyfarth, 1923) and a method of bone marrow aspiration (Arinkin, 1929), led to more frequent examination of the bone marrow and therefore to greater familiarity with normal haemopoiesis. Morphologists were able to define the megaloblast precisely and to recognise it as a pathological cell distinct from normal erythroid precursors and a characteristic of diseases caused by a deficiency of liver principle (Naegeli, 1931; Ferrata, 1933; Segerdahl, 1935; Jones, 1938; Rohr, 1937; Scott, 1939; Jones, 1943). This morphological concept was further supported by the observation that megaloblastosis was often associated with similar changes in the white cell precursors and megakaryocytes (Tempka and Braun, 1932; Jones, 1936; Rohr, 1937; Rohr, 1949). The rapid disappearance of these haemopoietic abnormalities following liver therapy provided further confirmation that the megaloblast was a pathological cell and that megaloblastic haemopoiesis was a primary rather than a compensatory abnormality in pernicious anaemia (Peabody, 1927; Rohr, 1937). The success of liver therapy was due to the replacement of the factor required by the cellular metabolism of haemopoietic precursors for normal haemopoiesis (Peabody, 1927).

The study of the bone marrow in different phases of remission induced by liver therapy, led to a better understanding of the origin and dynamics of megaloblastosis and an appreciation of the diagnostic importance of lesser and intermediate degrees of megaloblastosis (Lambin and de Weerdt, 1938; Davidson, Davis and Innes, 1942 a; Zuelzer, Newhall and Hutaff, 1947; Dacie and White, 1949).

More frequent examination of the bone marrow showed that macrocytosis did not specifically occur in association with megaloblastic erythropoiesis but could also be present in association with normoblastic erythropoiesis (Jones, 1943). Although megaloblastosis was always to be found in diseases caused by liver factor deficiency, macrocytosis was not a consistent feature (Segerdahl, 1941; Davidson, Davis and Innes, 1942b; Callender, 1944). Thus examination of the bone marrow was accepted as the diagnostic and therapeutic guide and the concept of megaloblastic anaemia replaced that of macrocytic anaemia (Davidson and Davis, 1947).

Folic acid metabolism.

For many years investigators were keen to identify the active liver principle. In 1940 Snell and Peterson observed that Lactobacillus casei (L.casei) required a growth factor found in yeast and liver. This factor could be adsorbed onto charcoal and was therefore called the "norite eluate factor". In 1941 Mitchell, Snell and Williams concentrated a factor from spinach which was required for growth by both L.casei and Streptococcus facaelis (S.faecalis). It was abundantly present in green leaves and was therefore called folic acid. Hogan and Parrott (1939; 1940), induced an anaemia in chicks, under defined dietary conditions, which could be cured with liver extracts. They thought that the anaemia could be attributed to a deficiency of an unidentified vitamin, possibly of the B complex group found in liver. Later Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell (1943), isolated a compound from liver which was active for L.casei and also haematologically active in chicks. Another compound identical to this was obtained by Stokstad (1943) from liver (liver L.casei factor).

Later Angier, et al. (1945) synthesised a compound which was identical to the liver L.casei factor. It consisted of a pteridine group linked to

PTEROYLGLUTAMIC ACID

[FOLIC ACID]

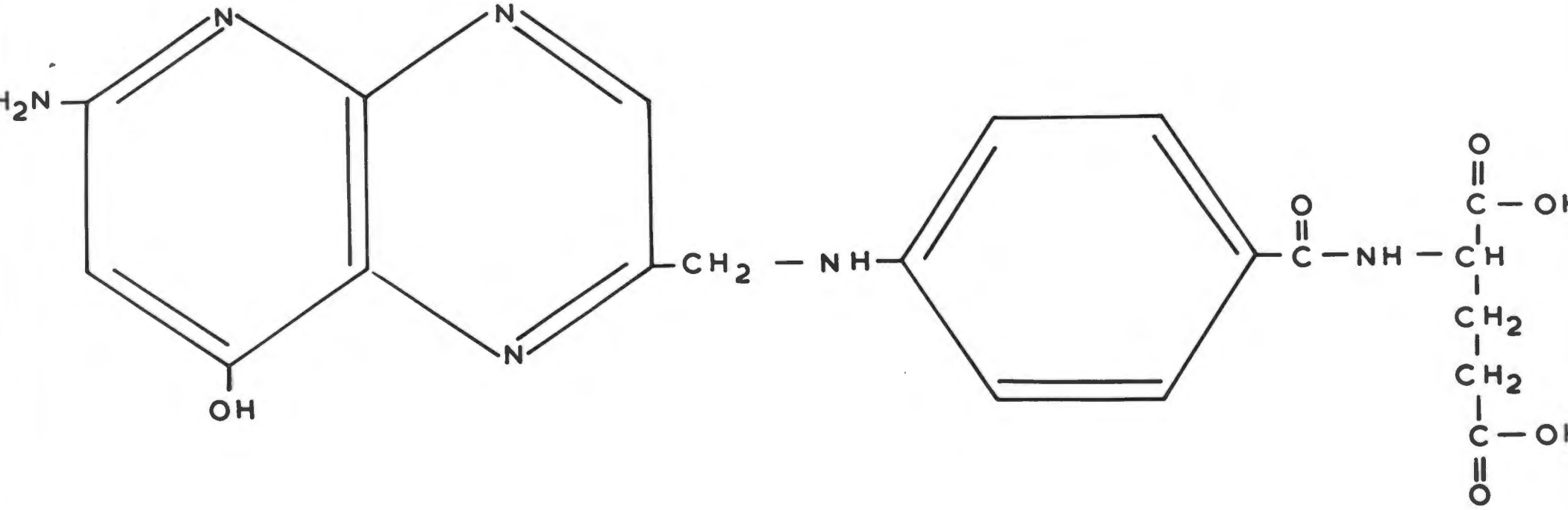

Fig. 1

p-aminobenzoic acid and a single L-glutamic acid radicle (Fig. 1) and was called pteroylglutamic acid (Angier, et al. 1946). Pteroylglutamic acid (PGA) was later found to have the same biological properties as folic acid (Metz, 1963).

At first it was thought that PGA was the naturally occurring food folate and that it was biologically active (Jukes, 1955). However, it was later shown that food folate consisted of conjugated and unconjugated forms of PGA and its derivatives (Jukes and Stokstad, 1948; Luhby and Cooperman, 1963). When folic acid or its derivatives were conjugated with γ-glutamic acid peptide chains of varying length, they were known as conjugated or polyglutamate forms (Jukes, 1955). The conjugated forms constituted the bulk of food folate (Jukes and Stokstad, 1948; Bird, Bressler, Brown, Campbell and Emmett, 1945), particularly the reduced forms (Herbert, 1967). Less than one quarter of food folate was in the monoglutamate form (Herbert, 1967). A fraction of food folate might be present as PGA (Butterworth, Santini and Frommeyer, 1963) but this was uncertain (Herbert, 1967).

The conjugated (polyglutamate) forms of folic acid and its derivatives could be converted to monoglutamates by enzymes known as conjugases (Bird, Bressler, Brown, Campbell and Emmett, 1945; Bird, Binkley, Bloom, Emmett and Pfiffner, 1945). Conjugases were widely distributed in nature, and hog kidney, liver and small intestine, and beef liver were rich sources (Bird, Binkley, Bloom, Emmett and Pfiffner, 1945). They could be present in small quantities in the succus entericus of humans (Klipstein, 1967).

The biological value of the different forms of folate is unknown (Butterworth, 1968) primarily because there is insufficient information regarding the absorbability of the different forms of folate (Herbert, 1967).

CONVERSION of FOOD FOLATE to TETRAHYDROFOLATE AND ITS COMPLEXES

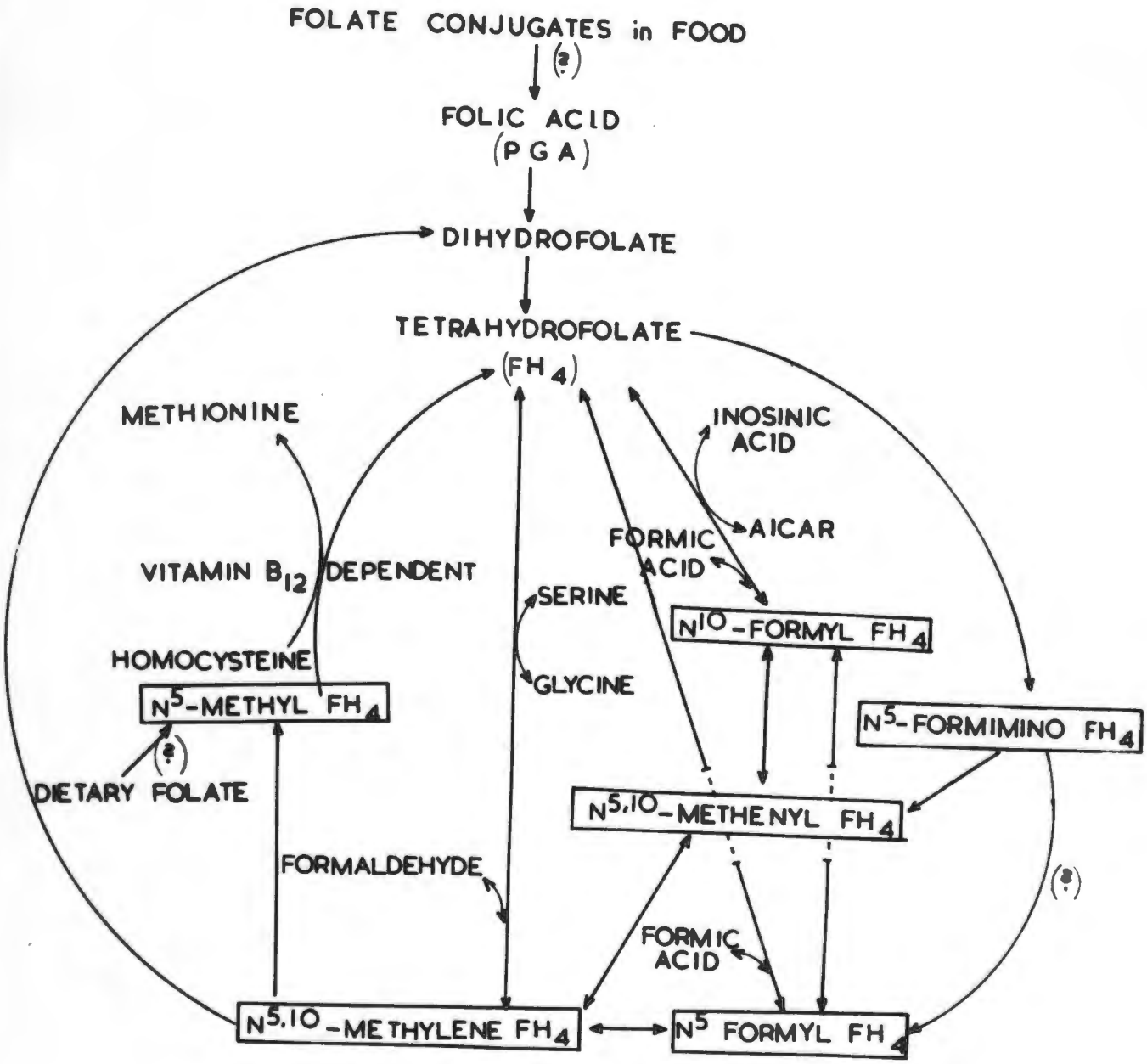

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Fig. 2

A little is known of the chemical form in which folate is absorbed. It was believed that folate was absorbed in the monoglutamate form (Herbert, 1967; Klipstein, 1967) and that intestinal conjugases might convert folate polyglutamates to monoglutamates to facilitate their absorption (Klipstein, 1967). Others found that folate could be absorbed in the conjugated form (Herbert, 1967) as diglutamate, triglutamate (Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka, 1965) or heptaglutamate (Cooperman and Luhby, 1965). The suggestion was made that folate was best absorbed as triglutamate (Baker, Frank, Sobotka, Pey-Ping, Cohen, Janowitz, Ziffer and Leevy, 1964).

Although the monoglutamate (Whitehead and Cooper, 1967), the triglutamate (Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka, 1965), and heptaglutamate forms (Cooperman and Luhby, 1965) were absorbed as such from the gut, the diglutamate form was apparently altered to a metabolically active form by the gut (Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka, 1965).

It is now acknowledged that folic acid is rapidly reduced in vivo to the biologically active form, tetrahydrofolate (FH_4) (Heunnekens and Osborn, 1959; Heunnekens, 1966; Stokstad and Koch, 1967). It combines with C_1 fragments to form six tetrahydrofolate complexes (Heunnekens, 1966), four of which are known to act as coenzymes (Ghitis, 1966). These donate the C_1 fragments for incorporation into purines, pyrimidines and some amino acids, and in so doing, revert to tetrahydrofolate. By acting as a reversible carrier of C_1 fragments, FH_4 plays a fundamental role in nucleoprotein metabolism (Heunnekens and Osborn, 1959). The conversion of food folate to the active coenzyme form and to tetrahydrofolate complexes is diagrammatically represented (Fig. 2).

In accordance with current ideas of nomenclature, the term folate will be used henceforth to refer to the multiplicity of folate compounds whereas the term folic acid will be used only to refer to PGA.

Aetiological differentiation of the megaloblastic anaemias (Folate and vitamin B_{12} interrelationships).

When folic acid was isolated in 1945, it was first thought to be either Castle's active liver principle or the extrinsic factor. Folic acid was used in therapeutic trials on patients with all types of megaloblastic anaemia. First results were very encouraging even when it was used in the treatment of pernicious anaemia (Spies, Vilter, Koch and Caldwell, 1945; Moore, Bierbaum, Welch and Wright, 1945; Darby, Jones and Johnson, 1946; Spies, 1946).

Although the efficacy of folic acid therapy in most macrocytic anaemias was later confirmed, the subsequent results of folic acid treatment in pernicious anaemia were disappointing (Vilter, Vilter and Spies, 1947). It was found to be less effective in curing the haematological and neurological manifestations of pernicious anaemia than refined liver extract (Vilter, Vilter and Spies, 1947; Heinle, Dingle and Weisberger, 1947; Spies and Stone, 1947; Ross, Belding and Paegal, 1948; Wilkinson, 1948 a, b). More disturbing was the observation that it might aggravate and even precipitate the neurological manifestations of pernicious anaemia (Heinle and Welch, 1947; Ross, Belding and Paegal, 1948; Wilkinson, 1948 a, b).

The isolation of another factor from liver which was haematologically active in pernicious anaemia - vitamin B_{12} - helped to clarify some of the aetiological and therapeutic problems of the megaloblastic anaemias.

Vitamin B_{12} was isolated and crystallised by Rickes, Brink, Koniuszy, Wood and Folkers (1948), following helpful preliminary microbiological

studies by Shorb (1947). Independently and at the same time vitamin B_{12} was purified by Smith (1948).

In therapeutic trials, intramuscular vitamin B_{12} was found to be effective in curing both the neurological and haematological manifestations of pernicious anaemia (West, 1948; Berk, Denny-Brown, Finland and Castle, 1948). Later Berk, Castle, Welch, Heinle, Anker and Epstein (1948) identified vitamin B_{12} as Castle's extrinsic factor and suggested that the function of the intrinsic factor was to absorb vitamin B_{12} from the gut. Thus it was shown that two extrinsic factors present in liver, folic acid and vitamin B_{12} played fundamental roles in the production of megaloblastic anaemia. This confirmed an earlier suggestion made by Wills and Evans (1938).

In 1935 Minot and Castle established the criteria for using the reticulocyte response under controlled conditions, as a measure of therapeutic efficacy of haematinics. They found that each therapeutic substance could provoke only one therapeutic response. Thus it was possible to compare the efficacy of two test substances in submaximal doses by using them in sequence on the same patient and comparing their effect on the reticulocyte response. (The method of the double reticulocyte response).

Once vitamin B_{12} and folic acid were available for clinical use, this method of controlled therapeutic trial was used in the aetiological differentiation of the megaloblastic anaemias. In order to prevent possible neurological damage, vitamin B_{12} was administered first (Mollin, 1957/58; Herbert, 1959). This method of aetiological diagnosis could only be used on anaemic patients and as it was time-consuming it was impractical for use on sick patients (Mollin, 1957/58; Chanarin, Mollin and Anderson 1958 a). Moreover, it was often difficult to interpret the results because the physiological requirements of the vitamins were not known and folic acid and vitamin B_{12}

were therapeutically interrelated, especially when used in pharmacological doses (Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins and Seaman, 1950; Herbert, 1959). This had been established when folic acid was found to be effective in improving vitamin B_{12} deficiency (Moore, Bierbaum, Welch and Wright, 1945; Wilkinson, 1948 a, b) and vitamin B_{12} to be effective in improving folic acid deficiency (Killander, 1958). It was clear that the development of precise laboratory techniques for the aetiological differentiation of the megaloblastic anaemias would have advantages.

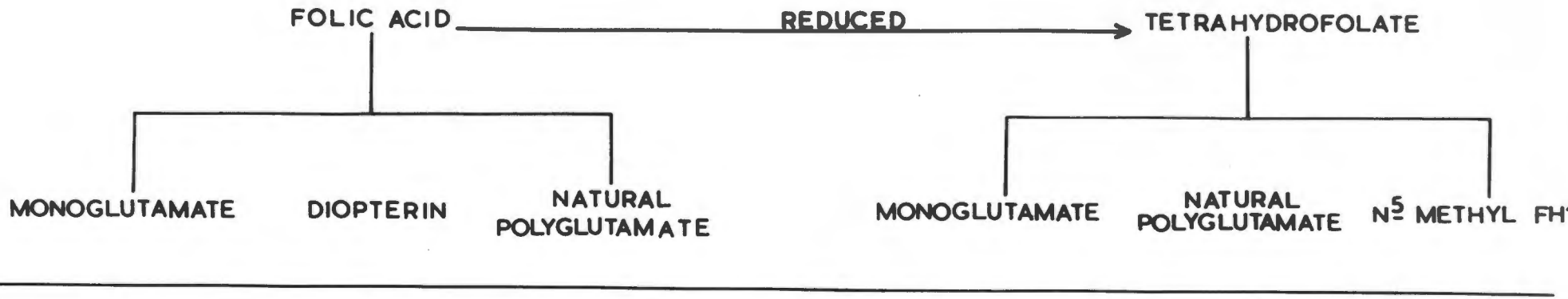
The introduction of a microbiological assay of vitamin B_{12} was a useful contribution towards this end (Shorb, 1948; Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin and Jukes, 1949; Ross, 1950; Rosenthal and Sarett, 1952; Hutner, Bach and Ross, 1956). It was hoped that diagnostic accuracy would be further improved by the introduction of laboratory methods for measuring folate. This was not easily achieved and only became possible in the late 1950's (Chanarin, Mollin and Anderson, 1958a; Herbert, Wasserman, Frank, Pasher and Baker, 1959; Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sobotka, 1959; Luhby, Cooperman and Teller, (1959b).

LABORATORY DIAGNOSIS OF FOLATE DEFICIENCY.

It was shown that L.casei (Snell and Peterson, 1940), S. faecalis (Mitchell, Snell and Williams, 1941) and P.cerevisiae (Sauberlich and Baumann, 1948) particularly, as well as a large number of lactic acid bacteria, required folate compounds for their growth (Jukes and Stokstad, 1948).

The growth response depended on the form of folate provided, since the organisms seemed to require different forms for their metabolism

GROWTH POTENTIAL of FOLATE COMPOUNDS for the 3 ORGANISMS



L. CASEI	+	−	+	+	+	+
S. FAECALIS	+	−	−	+	−	−
P. CEREVISIAE	−	−	−	+	+	−

FROM BAKER, H., ET. AL. AMER. J. CLIN. NUTR., 1965, 17, 88

Fig. 3

(Jukes and Stokstad, 1948). The diagram (Fig. 3) illustrates recent knowledge regarding the growth requirements of the three bacteria.

As early as 1947 attempts were made to utilise this information in the standardisation of a microbiological assay for blood and plasma folate (Schweigert and Pearson, 1947). This was not satisfactorily achieved until twelve years later, as a result of which indirect methods of assessing folate nutrition were introduced in the meantime. The direct methods eventually developed, and the relevant indirect methods used, were as follows:-

Direct Methods.

Folate microbiological assays.

The greatest practical difficulty in the development of a microbiological assay for folate was the low level of "folate" found in blood (Schweigert and Pearson, 1947) and plasma or serum (Schweigert and Pearson, 1947; Chanarin, Anderson and Mollin, 1958) using S.faecalis as the assay organism. The use of L.casei as the organism made no difference to the results obtained in blood (Schweigert, 1948) or serum (Spray and Witts, 1952; Spray, 1952). This limited the clinical application of the method (Niewig, Faber, de Vries and Kroese, 1954; Girdwood, 1953).

After earlier studies, Toennies, Usdin and Phillips introduced an assay procedure for folate in 1956 which overcame most of the practical difficulties. In 1953 they had obtained a good growth of L.casei in dialysates prepared from blood haemolysates. Their results were substantially higher than those previously reported (Toennies, Frank and Gallant, 1953). Thus the assay they introduced in 1956 involved the useof L.casei as the assay organism.

Another important modification resulted from earlier investigations

which had suggested that "folate" might be lost during the assay procedure (Toennies, Frank and Gallant, 1953). Ascorbic acid was thus added to the buffer to prevent loss of the "thermolabile folate" from auto-oxidation while it was heated in the assay procedure or stored at low temperatures.

Finally they showed that in contrast to the findings with red cell haemolysates there was little "folate activity" in preparations of washed red cells or plasma or serum. However, haemolysis of red cells had to be complete and had to occur in the presence of plasma before "folate activity" could be successfully measured. This suggested to them that the plasma contained a factor (probably conjugase) which made "folate" in red cells available to the assay organism.

With these modifications "folate" blood levels in normal subjects were high enough to allow further investigations of the clinical application of the method (Toennies, Frank and Gallant, 1956).

At this stage there were still theoretical objections most of which were based on doubt about the nature of the L.casei growth factor in the blood. It had been shown that in the absence of folate various purines and pyrimidines, as well as PABA acted as growth factors for the assay organism (Chanarin, 1958).

Moreover, chromatographic procedures had shown that the "folate active" material of blood haemolysates was not recognisable as folic acid or any other folate compound chemically defined at that stage. The "folate active" material supported the growth of L.casei but not S.faecalis (Usdin, Phillips and Toennies, 1956). This pattern was unlike that of folic acid but was compatible with the pattern found when folate triglutamates were provided (Jukes and Stokstad, 1948; Stokstad, 1954).

Thus although there was no evidence to the contrary, there was no proof that the L.casei growth factor was a folate. Nor was there evidence that these levels of "folate" related to folate nutrition except in the case of some animal experimental studies (Schweigert, 1949; Toennies, Frank and Gallant, 1956).

These theoretical objections were overcome as the result of two important contributions. The first was the demonstration that the microbiological assays of "folate active" material in serum (Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sobotka, 1959; Herbert, Wasserman, Frank, Pasher and Baker, 1959), or whole blood (Grossowicz, Mandelbaum-Shavit, Davidoff and Aronovitch, 1962; Grossowicz, Rachmilewitz, Izak and Zan, 1962) had clinical application in the diagnosis of folate deficiency.

The second was made after experimental observations by Donaldson and Keresztesy (1959) and Larrabee, Rosenthal, Cathou and Buchanan (1961), when Herbert, Larrabee and Buchanan (1962), identified the L.casei growth factor in serum as a folate compound - N^5-methyl FH_4. This compound, like folate triglutamates was metabolically available to L.casei but not to S.faecalis. Folate present in red cells was later identified as N^5-methyl FH_4 and its conjugates, the majority of the folate being in the conjugated form (Noronha and Aboobaker, 1963). By these means the growth factor for L.casei was identified as a folate compound.

The serum L.casei folate assay.

In spite of theoretical objections and initial practical difficulties, Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sobotka (1959) continued to develop the serum L.casei folate assay. In contrast with previous results (Toennies, Usdin and Phillips, 1956), they showed that the levels of folate in serum in normal subjects (7.5 to 24.0 ng./ml) were

high enough to make the assay a practical procedure (Herbert, Baker, Frank, Pasher, Sobotka and Wasserman, 1960). They believed that by using serum rather than blood the growth of L.casei could be attributed to "folate" rather than to other growth factors possibly present in red cells.

Thus the assay developed by Baker, Herbert, Frank, Pasher, Hutner, Wasserman and Sobotka (1959) incorporated two of the modifications suggested by Toennies, Usdin and Phillips (1956) - ascorbic acid protection and the use of L.casei as the assay organism.

Conflicting results were obtained in initial clinical studies. Herbert, Baker, Frank, Pasher, Sobotka and Wasserman (1960), and Cooper and Lowenstein (1961) were impressed with its clinical application. On the other hand, Waters and Mollin (1961) and Cooperman, Luhby and Avery (1960) were unimpressed with the value of the test in differentiating between normal and folate deficient subjects. When the method was modified by the addition of still higher concentrations of ascorbic acid to protect folate from the vagaries of uneven heating during autoclaving (Herbert, 1961; Waters and Mollin, 1961; Waters and Mollin, 1963), the validity of the serum L.casei folate assay was more generally accepted.

It could be used to differentiate between normal and folate deficient subjects (Davis and Kelly, 1962). In the diagnosis of megaloblastic anaemia due to folate deficiency the agreement between serum L.casei folate assays and bone marrow morphology was good but not absolute (Waters and Mollin, 1961). Occasional patients had normal serum L.casei folate levels in association with megaloblastosis due to folate deficiency (Cooper and Lowenstein, 1961). This problem was most often encountered in pregnancy (Waters and Mollin, 1961).

The megaloblastosis of vitamin B_{12} deficiency could be distinguished

from that caused by folate deficiency by means of the serum L.casei folate assay (Waters and Mollin, 1961; Stevens, Metz, Brandt and v.Broekhuizen, 1962; Gough, Read, McCarthy and Waters, 1963). In this context the test was especially useful because vitamin B_{12} deficiency did not lead to a non-specific lowering of serum L.casei folate levels. On the contrary there was evidence that folate utilisation was impaired in vitamin B_{12} deficiency (Herbert and Zalusky, 1962) and that this led to an elevation of serum L.casei folate levels (Waters and Mollin, 1961). For this reason minor degrees of folate deficiency in association with vitamin B_{12} deficiency might not be diagnosed unless serum L.casei folate assays were repeated following treatment with vitamin B_{12}. Also, in the presence of vitamin B_{12} deficiency a low serum L.casei folate level meant associated folate deficiency.

The test was a very sensitive index of folate deficiency because low serum L.casei folate levels preceded the development of megaloblastic change (Herbert, 1962a). Furthermore, the test was specific for folate deficiency provided that broad spectrum antibiotics were not administered, for they could interfere with the growth of the assay organism and might result in spuriously low serum L.casei folate levels (Waters and Mollin, 1961).

Summary.

During the course of twelve years a microbiological assay of folate in serum and plasma had been developed in spite of practical and theoretical difficulties. It could be used to differentiate between normal subjects and patients with megaloblastic anaemia due to folate deficiency.

The agreement of serum L.casei folate levels with megaloblastosis

due to folate deficiency was good but not absolute and this was especially noted in pregnancy. The test was valuable in distinguishing megaloblastic anaemias due to folate from those due to vitamin B_{12} deficiency because the latter resulted in a disturbance of folate utilisation which elevated rather than lowered serum L.casei folate levels. The test was very sensitive, but specific for folate deficiency provided that broad spectrum antibiotics were not administered.

Whole blood folate assays.

It was believed that folate bypassing substances might be present in red cells though the likelihood was decreased, when Usdin, Phillips and Toennies (1956) failed to identify thymine and thymidine as constituents of red cells. This encouraged Grossowicz and his colleagues to evaluate whole blood as well as serum folate levels as diagnostic parameters, especially as folate levels were much higher in blood than in serum.

Grossowicz and his colleagues developed the whole blood folate assay method introduced by Toennies, Usdin and Phillips (1956) and examined its clinical application. They introduced minor modifications (Grossowicz, Mandelbaum-Shavit, Davidoff and Aronovitch, 1962) but followed the important recommendations made by Toennies, Usdin and Phillips (1956). L.casei was used as the assay organism but they also examined the value of S.faecalis and P.cerevisiae. They employed ascorbic acid protection and haemolysed the red cells in plasma to ensure that the plasma factor made all the red cell folate available to the assay organism.

They showed that the whole blood method was a sensitive test of folate deficiency (Izak, Rachmilewitz, Sadovsky, Bercovici, Aronovitch,

and Grossowicz, 1961). Blood folate levels particularly with L.casei were of diagnostic value in nutritional megaloblastic anaemia and were regarded as more reliable than serum folate levels (Grossowicz, Rachmilewitz, Izak and Zan, 1962).

These investigators showed that the test had application in the diagnosis of folate deficiency, but there was still insufficient information to assess its reliability in regard to diagnosis and differential diagnosis of the megaloblastic anaemias.

Red cell L.casei folate assays.

Hansen and Weinfeld (1962) and Herbert (1962a), used whole blood (corrected for haematocrit) or red cell L.casei folate assays in preference to whole blood L.casei folate assays. This approach had greater meaning because uncorrected whole blood L.casei folate levels could vary with the haematocrit regardless of folate nutrition. The red cell L.casei folate assay was found to be a reliable index of folate deficiency (Hansen and Weinfeld, 1962), and of depleted folate stores (Herbert, 1962a).

In the presence of vitamin B_{12} deficiency, red cell L.casei folate levels could be low in the absence of folate deficiency, possibly because vitamin B_{12} was necessary for the transfer of folate into the developing red cell precursors (Hansen and Weinfeld, 1962). Unlike the serum L.casei folate assay this method had limitations in the differential diagnosis of the megaloblastic anaemias.

Summary.

On the basis of relatively little experience it was shown that the L.casei assay of folate in red cells had value in the diagnosis of folate deficiency. Moreover, low red cell L.casei folate levels indicated a significant depletion of folate stores. The test had little diagnostic value when vitamin B_{12} deficiency was present.

Indirect methods.

While direct microbiological folate assays seemed impractical, indirect methods were introduced to investigate folate nutrition. Two of these methods were relevant and were as follows:-

1. The folic acid clearance test.
2. FiGlu excretion after oral histidine.

The folic acid clearance test.

This was similar in concept to the folic acid utilisation tests used by Bethell, Meyers, Gould, Swendseid, Bird and Brown (1947) and Spray, Fourman and Witts (1951), and technically similar to methods used by Schweigert (1948), Spray and Witts (1953) and Butterworth, Nadel, Perez-Santiago, Santini and Gardner (1957). However, the folic acid clearance test was developed and investigated as a diagnostic parameter of folate deficiency by Chanarin and his associates. It was based on the principle that folate deficient subjects should remove intravenously injected folic acid from the circulation more rapidly than normal subjects. They administered 15 µg./kg. of folic acid intravenously and then measured the serum S.faecalis activity of blood samples taken 3, 15 and 30 minutes after the injection (Chanarin, Mollin and Anderson, 1958a).

The folic acid clearance test using S.faecalis as the assay organism.

It was shown that rapid folic acid clearance tests were not the result of increased urine loss (Chanarin, Mollin and Anderson, 1958a; Girdwood and Delamore, 1961; Chanarin and Bennett, 1962b) and could be slowed by the prior injection of folic acid (Chanarin, Mollin and Anderson, 1958a; Girdwood and Delamore, 1961). Folic acid clearance rates were very rapid in patients with megaloblastic anaemias due to

folate deficiency and differed markedly from those found in normal subjects (Chanarin, Mollin and Anderson, 1958 a, b; Sheehy, Santini, Angel and Guerra, 1962; Mollin and Waters, 1962; Metz, Stevens, Krawitz and Brandt, 1961). The 15 and 30 minute samples were particularly useful (Chanarin, Mollin and Anderson, 1958b).

Chanarin and colleagues suggested that in folate deficiency the rapid folic acid clearance tests could be attributed to tissue folate deficiency because folic acid clearance curves remained rapid after megaloblastic haemopoiesis returned to normal (Chanarin, Mollin and Anderson, 1958a), and because rapid folic acid clearance curves could precede the development of megaloblastosis (Chanarin, Mollin and Anderson, 1958b).

The use of the test was subject to certain limitations, notably in pregnancy, in vitamin B_{12} deficiency, in iron deficiency and in some drug-induced megaloblastic anaemias. It was shown that normal non-anaemic pregnant women could have rapid folic acid clearances particularly as term approached. Though these curves were more rapid than usual they did not fall in the range seen in the megaloblastic anaemia of pregnancy except in some cases of twin pregnancy (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959). Similar findings were reported by Girdwood and Delamore (1961). The reason for these curves in pregnant women was not known. They were not thought to be the result of blood volume changes alone (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959; Girdwood and Delamore, 1961) though they could have been related to foetal demand for folate (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959; Girdwood and Delamore, 1961) or latent maternal folate deficiency developing in pregnancy (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959). Thus the rapid

folic acid clearance test was believed to indicate either folate deficiency or increased demand for folate. Contradictory findings in pregnant women were noted by Grossowicz, Aronovitch, Rachmilewitz, Izak, Sadovsky and Bercovici (1962) who found normal folic acid clearance tests in both anaemic and non-anaemic pregnant women when using the same technique as Chanarin, Mollin and Anderson (1958a). Luhby, Feldman, Salerno and Cooperman (1963) did not find the test useful as a parameter of folate deficiency in pregnancy.

Abnormally fast clearance curves were found in megaloblastic anaemia resulting from vitamin B_{12} deficiency (Chanarin, Mollin and Anderson, 1958a,b; Herbert and Zalusky, 1962) particularly in the presence of severe anaemia (Chanarin, Mollin and Anderson, 1958a, b). This was thought to be the result of disturbed folate metabolism in vitamin B_{12} deficiency (Herbert and Zalusky, 1962; Chanarin, Mollin and Anderson, 1958b).

In iron deficiency moderately rapid clearances were found which they thought might have been due to associated folic acid deficiency (Chanarin, Mollin and Anderson, 1958 a, b).

On the other hand, Chanarin and his associates found that certain drugs such as primidone and barbiturates could cause megaloblastic anaemia by interfering with folate metabolism without resulting in tissue folate deficiency. In these instances folic acid clearances were normal (Chanarin, Mollin and Anderson, 1958 a, b; Chanarin, Elmes and Mollin, 1958).

The test was a sensitive test of folate deficiency because it could be abnormal before the development of megaloblastosis (Chanarin, Mollin and Anderson, 1958b; Metz, Stevens, Krawitz and Brandt, 1961; Chanarin, Bennett and Berry, 1962).

The folic acid clearance test using L.casei as the assay organism.

The folic acid clearance test was found to have the same diagnostic value in megaloblastosis due to folate deficiency when folate was measured with L.casei instead of S.faecalis. To some extent the use of L.casei as the assay organism was advantageous because the folic acid clearance rate measured with L.casei was less rapid in vitamin B_{12} deficiency than when it was measured with S.faecalis (Mollin and Waters, 1962; Herbert and Zalusky, 1962).

The folic acid clearance test using tritiated folic acid.

The use of tritiated folic acid confirmed the pattern of the folic acid clearance test found in normal subjects using microbiological methods (Johns, Sperti and Burgen, 1961). Although use of tritiated folic acid showed that urinary loss of folate was underestimated when the microbiological method was used, rapid folic acid clearances could still not be attributed to increased urinary loss (Chanarin, Belcher, and Berry, 1963). This test was also valid in the diagnosis of megaloblastic anaemia due to folate deficiency (Sheehy, Santini, Angel and Guerra, 1962; Mollin and Waters, 1962). It was rapid in the presence of vitamin B_{12} deficiency (Mollin and Waters, 1962).

All folic acid clearance tests had practical disadvantages. Several blood samples were required as well as the administration of therapeutic doses of folic acid.

Summary.

The folic acid clearance test using either microbiological or radioactive methods was a good diagnostic parameter of megaloblastic anaemia due to folate deficiency. The test could not be used to differentiate between folate and vitamin B_{12} deficiency if

S.faecalis was used as the assay organism. In this case the L.casei folic acid clearance test had greater diagnostic value. The application of the test was limited in the case of iron deficiency, and some drug-induced megaloblastic anaemias and perhaps in pregnancy. The test was a sensitive index of tissue folate deficiency because it could indicate the presence of folate deficiency before the development of megaloblastosis.

The test had certain practical disadvantages.

Figlu excretion after oral histidine.

This test was developed following observations by Bakerman and his associates in 1951, which led to an understanding of the interrelationships of histidine and folate metabolism. Bakerman, Silverman and Daft (1951) observed a significant excess of glutamic acid in the urinary amino acid pattern of rats suffering from drug-induced folate deficiency. This glutamic acid excretion could be prevented or reversed by the administration of folic acid. Later it was shown that the excretion product was not glutamic acid but a precursor of glutamic acid (Silverman, Gardiner and Bakerman, 1952) which was identified as an intermediate of histidine metabolism (Tabor, Silverman, Mehler, Daft and Bauer, 1953). It was found to have the properties of α -formamidinoglutaric acid (Formimino-L-glutamic acid - Figlu) by Borek and Waelsch (1953). The glutamic acid precursor in the urine of folate deficient rats was then identified as Figlu by Seegmiller, Silverman, Tabor and Mehler (1954). The interrelationships of histidine and folate metabolism were clarified by the recognition that Figlu provided a formimino group for transfer to tetrahydrofolate (Miller and Waelsch, 1956). This transfer was enzymatic and resulted in the formation of

INTERRELATIONS of HISTIDINE and FOLATE METABOLISM

[DIAGRAMMATIC]

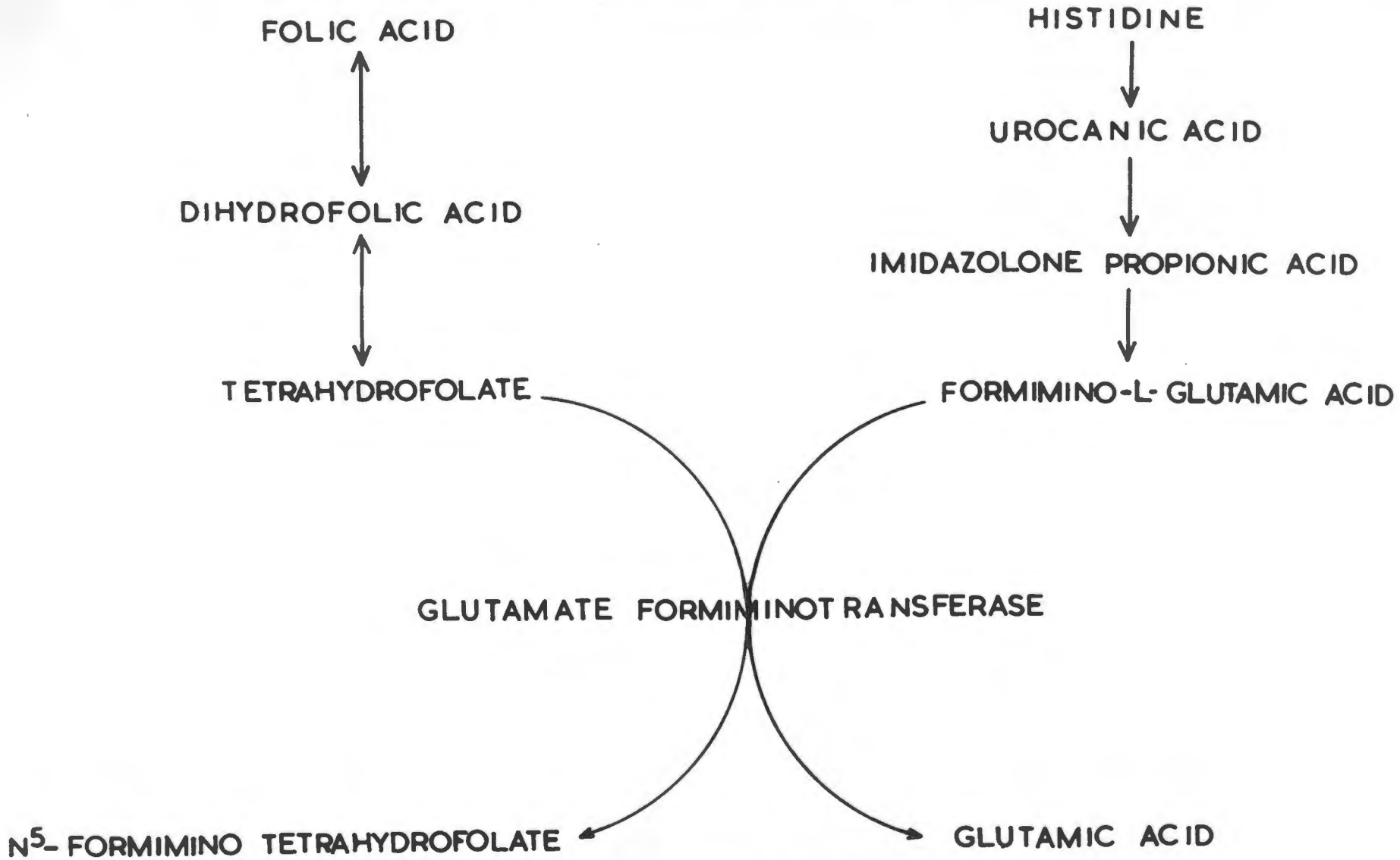


Fig. 4

N^5-formimino FH_4 and glutamic acid (Tabor and Rabinowitz, 1956). When there was not enough FH_4, as in folate deficiency, excess Figlu was excreted in the urine.

Meanwhile, the steps of a major pathway of histidine degradation had been and were being defined. It was established that in this pathway histidine was degraded first to urocanic acid (Sera and Yada, 1939; Mehler and Tabor, 1953), then to imidazole propionic acid (Revel and Magasanik, 1958) and then to Figlu (Borek and Waelsch, 1953). The diagram (Fig. 4) illustrates the interralationships of folate and histidine metabolism.

This experimental data was applied by Broquist (1956) in an attempt to find a test for folate deficiency in humans. A compound similar to Figlu was found in the urine of children receiving folate antagonists as part of the treatment of Leukemia (Broquist, 1956; Broquist and Luhby, 1957). The compound was finally identified as Figlu in 1959 (Broquist and Luhby, 1959).

Microbiological, chromatographic, enzymatic and electrophoretic methods of estimating Figlu in urine were developed. It was agreed that of these, the enzymatic method was the most sensitive (Luhby, Cooperman and Teller, 1959b; Knowles, 1962). The electrophoretic method of Kohn, Mollin and Rosenbach was relatively insensitive and concentrations of Figlu less than 100 µg./ml. could not be estimated (Knowles, 1962).

The clinical application of the test was investigated by Luhby and his colleagues.

First of all it was established in a small number of patients that the test could be used to differentiate between normal and folate deficient subjects and between folate deficiency and vitamin B_{12} deficiency (Luhby,

1957). However, the test was relatively insensitive and in order to overcome this problem an oral dose of histidine was given before the collection of urine for Figlu estimation (Luhby, Cooperman and Teller, 1959a). This modification was based on previous experimental work on rats in which it had been shown that a dietary histidine load resulted in an increase of Figlu excretion in folate deficient rats, but not in normal rats (Tabor, Silverman, Mehler, Daft and Bauer, 1953).

In humans, the Figlu test after oral histidine could still be used with success to distinguish between folate deficient subjects and normal subjects and to distinguish between the megaloblastic anaemias due to folate and those due to vitamin B_{12} deficiency. Moreover, it was a sensitive and specific test for folate deficiency which could therefore be used in the diagnosis of subclinical folate deficiency, (Luhby, Cooperman, Teller and Donnenfeld, 1958; Luhby, Cooperman and Teller, 1959 a, b). Unless otherwise stated, the Figlu test will refer to the Figlu test after oral histidine.

The amount of Figlu excreted with (Carter, Heller, Schaffner and Korn, 1961) and without a histidine load (Luhby, Cooperman, MacIver and Montgomery, 1960) was in proportion to the severity of the folate deficiency.

From this time until 1963 this experience with the Figlu test after oral histidine was only partly confirmed.

Other investigators confirmed that the test could be used with validity to distinguish between normal and folate deficient subjects with megaloblastosis (Spray and Witts, 1959; Stevens, Metz, Brandt and v.Broekhuizen, 1962; Kohn, Mollin and Rosenbach, 1961; Chanarin, Bennett and Berry, 1962), or without megaloblastosis (Knowles, Prankerd and Westall, 1960; Gräsbeck, Björkstén and Nyberg, 1961; Kohn, Mollin and

Rosenbach, 1961; Zalusky and Herbert, 1961; Chanarin, Bennett and Berry, 1962). The use of low doses of histidine (2 g.) decreased the reliability of the test (Spray and Witts, 1959). Occasionally negative tests were noted in folate deficiency for no apparent reason (Chanarin, Bennett and Berry, 1962).

The value of the Figlu test after oral histidine in distinguishing between the megaloblastic anaemias due to folate and those due to vitamin B_{12} deficiency was not confirmed in the long run in spite of some confirmatory evidence for Luhby's findings in the first place (Spray and Witts, 1959; Knowles, Prankerd and Westall, 1960). Later, a number of investigators found positive tests after oral histidine was administered in cases of vitamin B_{12} deficiency (Marshall and Jandl, 1960; Carter, Heller, Schaffner and Korn, 1961; Zalusky and Herbert, 1961; Kohn, Mollin and Rosenbach, 1961; Stevens, Metz, Brandt and v.Broekhuizen, 1962; Chanarin, Bennett and Berry, 1962; Knowles and Prankerd, 1962). The quantity of Figlu excreted in vitamin B_{12} deficiency was less than the excretion in folate deficiency (Carter, Heller, Schaffner and Korn, 1961; Stevens, Metz, Brandt and v.Broekhuizen, 1962).

These contradictory findings were difficult to evaluate at first since there had been little uniformity in the dose of histidine given, in the schedule of histidine administration, in the duration of urine collection and in the method of Figlu estimation. Certain contributions helped to establish that Figlu could be excessive in vitamin B_{12} deficiency. Hansen and Weinfeld (1962) used exactly the same experimental methods as Luhby, Cooperman and Teller (1959b) and found positive Figlu tests after oral histidine in vitamin B_{12} deficiency. Zalusky and Herbert (1961),

and Hansen and Weinfeld (1962), showed that excessive Figlu excretion after oral histidine in vitamin B_{12} deficiency increased after folic acid therapy but greatly decreased after treatment with small doses of vitamin B_{12}. Chanarin (1963) found that vitamin B_{12} was as effective as folic acid in improving Figlu excretion after oral histidine in vitamin B_{12} deficiency. Finally, Herbert and Zalusky (1962) showed that vitamin B_{12} deficiency might block the conversion of N^5-methyl FH_4 to FH_4. This produced a diminution of available FH_4 and provided an explanation of positive Figlu tests in vitamin B_{12} deficiency.

Although some later studies did not confirm the findings from which Herbert and Zalusky (1962) drew their conclusions (Chanarin and McLean, 1967; Chanarin and Perry, 1968) it remains possible that the conversion of N^5-methyl FH_4 or its conjugates to FH_4 is blocked in vitamin B_{12} deficiency (Kutzbach, Galloway and Stokstad, 1967; Chanarin and McLean, 1967; Chanarin and Perry, 1968).

Other investigators believed that the function of glutamate formiminotransferase was inhibited in vitamin B_{12} deficiency (Knowles and Prankerd, 1962; Baker, Frank, Gellene and Leevy, 1964; Vitale and Hegsted, 1967). This would also lead to a disturbance of folate metabolism in vitamin B_{12} deficiency and a diminution of available FH_4 resulting in positive Figlu tests after oral histidine.

The sensitivity of the Figlu test after oral histidine as a parameter of folate deficiency was confirmed when other investigators found that excessive Figlu excretion preceded the development of megaloblastosis due to folate deficiency (Zalusky and Herbert, 1961; Knowles, Prankerd and Westall, 1960; Kohn, Mollin and Rosenbach, 1961; Chanarin, Bennett and Berry, 1962; Gräsbeck, Björkstén and Nyberg, 1961; Knowles and Prankerd,

DIAGRAMMATIC PRESENTATION of FACTORS WHICH MAY REDUCE FORMATION of, or INTERFERE WITH THE UTILIZATION of TETRAHYDROFOLATE

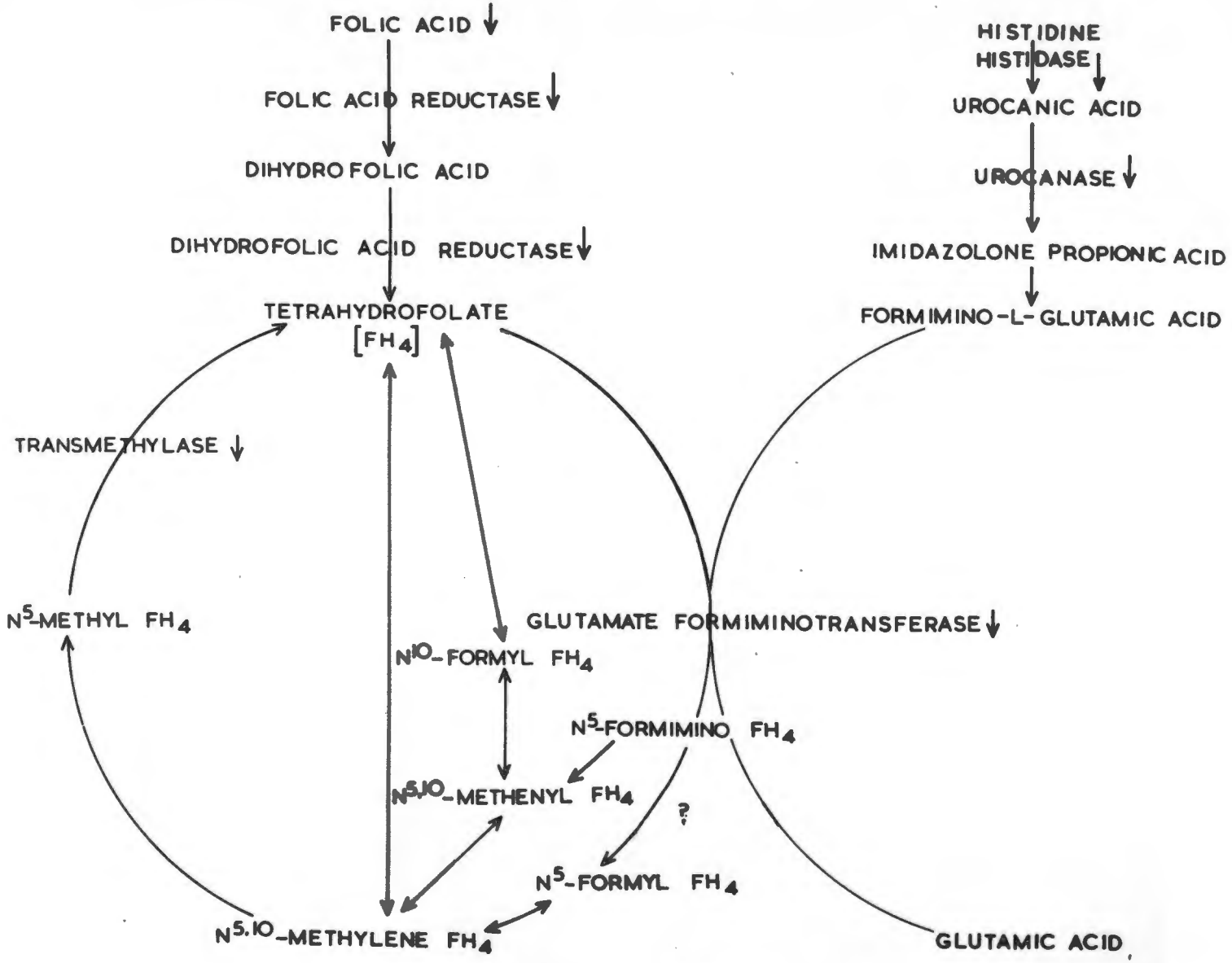


Fig. 5

1962; Herbert, 1962a).

However, it became increasingly clear that these results could not be attributed to folate deficiency without supporting evidence because the specificity of the Figlu test was no longer regarded as absolute.

As may be seen in the diagram (Fig. 5) both folate deficiency and disturbances of folate metabolism may result in positive Figlu tests. Secondary disturbances of folate metabolism may arise from interference with enzyme function (folic acid reductase, glutamate formiminotransferase or transmethylase) resulting in a diminution in the formation or availability of FH_4.

By 1963 positive Figlu tests after oral histidine not related to folate deficiency were described in the following conditions:

1. Liver disease.

A number of investigators noted positive Figlu tests after oral histidine in liver disease which did not or only partly resulted from folate deficiency (Carter, Schaffner and Heller, 1960; Merritt, Rucknagel, Gardiner and Silverman, 1960; Carter, Heller, Schaffner and Korn, 1961; Merritt, Rucknagel, Silverman and Gardiner, 1962; Knowles, Shaldon and Fleming, 1963). These positive tests were explained on the basis of a disturbance of folic acid reductase (Carter, Heller, Schaffner and Korn, 1961; Carter Schaffner and Heller, 1960) or glutamate formiminotransferase function (Carter, Schaffner and Heller, 1960; Carter, Heller, Schaffner and Korn, 1961; Merritt, Rucknagel, Silverman and Gardiner, 1962). This suggestion was made because liver disease could disturb enzyme function, and histidine and folate were mainly metabolised in the liver.

2. Congenital abnormalities.

A congenital absence of glutamate formiminotransferase resulting in

excessive Figlu excretion after oral histidine was reported by Arakawa, Ohara, Kudo, Tada, Hayashi and Mizuno, 1963.

3. Vitamin B_{12} deficiency.

Positive Figlu tests in this condition were discussed above.

4. Miscellaneous conditions.

Positive Figlu tests after oral histidine were reported in miscellaneous conditions like iron deficiency (Chanarin, Bennett and Berry, 1962; Knowles, 1962), neoplasia (Kohn, Mollin and Rosenbach, 1961; Chanarin, Bennett and Berry, 1962), haemolytic anaemia (Chanarin, Bennett and Berry, 1962), and sarcoidosis (Kohn, Mollin and Rosenbach, 1961). These results were not fully explained, but associated folate deficiency, increased demand for folate and unsuspected associated liver disease were suggested as possible explanations (Kohn, Mollin and Rosenbach, 1961; Chanarin, Bennett and Berry, 1962).

As the Figlu test after oral histidine was not only a sensitive but also a non-specific test, a positive Figlu test could be attributed to folate deficiency only if supported by confirmatory laboratory evidence or by a successful therapeutic trial with folic acid.

It was clear from past therapeutic studies that large doses of folic acid could be effective in the case of vitamin B_{12} deficiency (Moore, Bierbaum, Welch and Wright, 1945; Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins and Seaman, 1950). Furthermore, excessive Figlu excretion after oral histidine resulting from secondary disturbances of folate metabolism such as occurred in liver disease could be improved with large doses of folic acid (Carter, Heller, Schaffner and Korn, 1961; Knowles, Shaldon and Fleming, 1963). On the other hand, physiological doses of folic acid (100-200 µg. daily) were effective in the treatment of folic acid deficiency

yet did not provoke a therapeutic response in secondary disturbances of folate metabolism such as vitamin B_{12} deficiency (Hansen and Weinfeld, 1962). Because in folate deficiency, 200 µg. of folic acid daily could convert positive Figlu tests after oral histidine to normal in the majority of instances (Hansen and Weinfeld, 1962), this dose was recommended for use in diagnostic therapeutic trials. The dose (400 µg. daily), previously recommended by Marshall and Jandl (1960), could occasionally result in a therapeutic response in vitamin B_{12} deficiency (Hansen and Weinfeld, 1962). Therefore if a therapeutic trial was used as specific evidence for attributing the results of the Figlu tests after oral histidine to folate deficiency it had to be carried out with physiological doses of folic acid not exceeding 200 µg. daily.

The Figlu test and its relationship to histidine metabolism.

Urocanic acid, one of the intermediates in the degradation of histidine, was detected in the urine of humans by Acheson, Paul and Tomlinson (1958). The necessity to consider disturbances of histidine metabolism when using the Figlu test in the diagnosis of folate deficiency became apparent after a report by Merritt, Rucknagel, Gardiner and Silverman (1960). They identified urocanic acid in the urine of a patient probably suffering from liver disease, whom they were investigating for folate deficiency, using the Figlu test after oral histidine.

Later it was shown that normal subjects and patients deficient in folate and vitamin B_{12}, as well as those with miscellaneous haematological conditions (Bennett and Chanarin, 1961; Bennett and Chanarin, 1962) could all excrete Figlu or urocanic acid or both after receiving a histidine

load. The proportion of urocanic acid to Figlu could vary from 0 to 97% (Bennett and Chanarin, 1961).

From the diagram (Fig. 5) it may be seen that a metabolic disturbance of histidine metabolism resulting in a disturbance of urocanase function could result in excessive excretion of urocanic acid in the urine. In these circumstances excess Figlu might not be excreted even when there was folate deficiency. The same applied when there was a disturbance of histidase function or an alteration in the metabolism of histidine itself.

In certain pathological conditions a possible disturbance of urocanase function leading to excessive urocanic acid excretion in the urine, was reported. One of these was liver disease (Merritt, Rucknagel, Silverman and Gardiner, 1962), and another was kwashiorkor (Whitehead and Arnstein, 1961).

It was not known whether the excessive urocanic acid excretion noted in folate deficiency could be attributed to an effect of folate deficiency on urocanase function or whether the two findings were unrelated. It was suggested that urocanase required folic acid or a derivative for its function in converting urocanic acid to Figlu (Baldridge, 1958) or for its synthesis (Bennett and Chanarin, 1961), or alternatively that urocanase function might be depressed by the negative feed back caused by accumulation of Figlu (Baldridge, 1958; Bennett and Chanarin, 1961; 1962). A relationship between urocanase function and folate deficiency could not be demonstrated in a subsequent study by Hoffbrand, Neale, Hines and Mollin (1966).

As disturbances of urocanase function might interfere with the interpretation of the Figlu test after oral histidine in the investigation of folate deficiency, it was suggested that both urocanic acid and Figlu

should be estimated (Bennett and Chanarin, 1962). A modification of Silverman, Gardiner and Condit's (1958) enzymatic method for estimating Figlu, allowed the estimation of Figlu and urocanic acid (Bennett and Chanarin, 1962; Chanarin and Bennett, 1962a).

A rare congenital disturbance of histidase function was described by Auerbach, Di George, Baldridge, Tourtellotte and Brigham (1962), and Ghadimi, Partington and Hunter (1962). This had little practical application in the investigation of folate deficiency.

It was found that in pregnant women histidine absorption was prolonged, that there was increased urinary loss of histidine and probably increased utilisation of histidine for protein synthesis. These acquired disturbances of histidine metabolism in pregnancy lessened the efficacy of the histidine load and so rendered the Figlu test unreliable in this condition (Berry, Booth, Chanarin and Rothman, 1963; Chanarin, Rothman and Watson-Williams, 1963).

Summary.

The Figlu test after oral histidine was developed as the result of certain experimental observations. It was found to be a good diagnostic test of megaloblastic anaemia due to folate deficiency. It could not be used in the differential diagnosis of megaloblastic anaemia due to vitamin B_{12} or folate deficiency. Although sensitive, it was non-specific, and therefore could only be attributed to folate deficiency if supported by laboratory evidence or a successful therapeutic trial with physiological doses of folic acid. Furthermore, disturbances of histidine metabolism could lead to erroneous interpretation of the Figlu test. But with the possible exception of pregnancy, these were not of great practical significance as long as both urocanic acid and Figlu were always estimated.

THE CONCEPT OF BIOCHEMICAL FOLATE DEFICIENCY.

This concept was developed following the use of laboratory methods of diagnosing folate deficiency and the demonstration of the evolution of nutritional folate deficiency in man (Herbert, 1962a).

It had been shown that the folic acid clearance test (Chanarin, Mollin and Anderson, 1958b; Metz, Stevens, Krawitz, and Brandt, 1961; Chanarin, Bennett and Berry, 1962), the serum L.casei folate assay (Cooper and Lowenstein, 1961), and the Figlu test after oral histidine (Luhby, Cooperman and Teller, 1959b; Knowles and Prankerd, 1962; Gräsbeck, Björkstén and Nyberg, 1961) could all indicate folate deficiency before the development of megaloblastosis. This was illustrated in an interesting experiment of Herbert's (1962a) in which he produced folate deficiency in himself by taking a daily diet of 5 µg. of folate. After three weeks, serum L.casei folate levels were abnormally low; after seven weeks, hypersegmentation of the neutrophils developed; after three months, excess Figlu after oral histidine was excreted; and after four months, red cell L. casei folate levels were abnormally low. Clinical folate deficiency in the form of megaloblastic anaemia did not become apparent until approximately $4\frac{3}{4}$ months from the start of the experiment.

Diagnosis of subclinical folate deficiency.

All these laboratory parameters could be used to diagnose biochemical folate deficiency, and Herbert's experiment clarified their application. The serum L.casei folate assay was the most sensitive test, indicating folate deficiency long before stores were sufficiently depleted to result in megaloblastic anaemia. It was suggested that this test probably reflected dietary intake and absorption (Herbert, 1962a). As the Figlu test after

oral histidine and red call L.casei folate levels did not become abnormal until shortly before the development of megaloblastic anaemia, they appeared to be good indices of significant depletion of folate stores (Herbert,1962a).

The folic acid clearance test was a more sensitive test of folate deficiency than Figlu excretion after oral histidine (Chanarin, Bennett and Berry, 1962). From this observation and those of Herbert (1962a) it was likely that the sensitivity of this test and that of hypersegmentation of the neutrophils came after the serum L.casei folate assay but before Figlu excretion after oral histidine and red cell L.casei folate assays.

These laboratory tests varied in their sensitivity as indices of folate deficiency as well as in their ability to differentiate between folate deficiency and vitamin B_{12} deficiency. So, too, the specificity, reliability and simplicity of the tests varied in the ways described above. Thus none of these parameters could be regarded as unequivocally superior as diagnostic parameters of both biochemical and significant folate deficiency.

The prevalence and significance of biochemical (latent) folate deficiency in man was not known, but there was evidence that it might have considerable importance (World Health Organization Report). In an investigation of the problem it would be important to utilise at least two of the laboratory parameters in the diagnosis of the condition and in the assessment of its significance. The significance should be further assessed in relation to morphological and clinical studies.

MEGALOBLASTIC ANAEMIA OF INFANCY AND THE ROLE OF FOLATE DEFICIENCY IN ITS PRODUCTION

The development of understanding of megaloblastic anaemia in infants and the role of folate deficiency in its production followed the pattern

of the earlier discussion of the condition in adults. Major advances were as follows:

First of all there were isolated reports of macrocytic anaemia occurring in infancy and, to a lesser extent, in childhood. The earliest of these was credited to von Stark (1909). Some of the earlier studies concerned infants who developed macrocytic anaemia while on a diet of goats' milk (Glanzmann, 1926).

The next advance was the recognition that liver was haematologically effective in infantile macrocytic and pernicious anaemia (Faber, 1928).

The aetiological concept of the macrocytic anaemias occurring in infancy was outlined by Parsons and Hawksley (1933). They believed that macrocytic anaemia of infancy was a rare disease, caused either by a deficiency of the intrinsic factor as in infantile pernicious anaemia or by a deficiency of the extrinsic factor, as in coeliac disease (Parsons and Hawksley, 1933), or goats' milk anaemia (Parsons, 1933). It could also result from failure to absorb the haemopoietic factor (combined extrinsic and intrinsic factor) as in dibothriocephalus latus infestation (Parsons and Hawksley, 1933).

Bachman (1936) emphasised certain aspects of this concept. He reported a case of macrocytic anaemia of infancy which responded to a single course of liver therapy. He attributed the macrocytic anaemia to nutritional deficiency and possible malabsorption (the result of repeated gastrointestinal infection). From his experience and after a critical assessment of previous contributions, he clearly defined the entity of infantile nutritional macrocytic anaemia which he believed to be the most common cause of macrocytic anaemia occurring in infants under a

year. However he was not convinced that a single case of true infantile pernicious anaemia had been reported.

The megaloblastic nature of the haemopoiesis in infantile macrocytic anaemia was recognised by Veeneklaas (1942), Davis (1944) and Zuelzer and Ogden (1946). However, it was Zuelzer and Ogden (1946) who emphasized the diagnostic and therapeutic significance of megaloblastosis and who were mainly responsible for introducing the concept of infantile megaloblastic rather than macrocytic anaemia.

Until 1946, 40 cases of infantile macrocytic anaemia had been described (Zuelzer and Rutzky, 1953), but in 16 months Zuelzer and Ogden (1946) had recognised 24 new cases of megaloblastic anaemia occurring in infants. All of the infants appeared to be suffering from a similar syndrome.

From a study of their clinical experience, they defined the condition of infantile megaloblastic anaemia which they believed occurred with relative frequency (Zuelzer and Ogden, 1946). They conducted successful therapeutic trials with liver and folic acid, the last of which had by then just become available. Thus they related the condition to folic acid deficiency which they believed might have resulted from dietary deficiency or the effects of infection. In the discussion below the condition they described will be referred to as infantile nutritional megaloblastic anaemia.

Other megaloblastic anaemias due to other causes of folic acid and vitamin B_{12} deficiency (MacIver, 1962) and rarely to disturbances of pyrimidine metabolism (Huguley, Bain, Rivers and Scoggins, 1959), are known to occur in infants and children. Only the most important group - infantile nutritional megaloblastic anaemia will be considered below.

INFANTILE NUTRITIONAL MEGALOBLASTIC ANAEMIA.

It is now clear that this can result from folate or vitamin B_{12} deficiency. Other causes are speculative.

Clinical presentation and the aetiology of infantile nutritional megaloblastic anaemia.

Folate deficiency.

The clinical presentation, defined by Zuelzer and Ogden (1946) was as follows: Infants from 2 - 16 months, but most frequently, infants from 8 - 14 months were affected. Symptoms were related to the anaemia or preceding infection. Dietary intake was unsatisfactory in half the cases. The findings on examination were severe anaemia and its effects. Hepatomegaly was always present, splenomegaly infrequently, and purpura occasionally. Half of the infants were undernourished, a quarter had scurvy, and examination often showed evidence of infection. With occasional exceptions there was no evidence of underlying organic disease. Macrocytosis and hypersegmentation of the neutrophils were often observed on blood smears, but the essential and diagnostic feature of the condition was megaloblastic haemopoiesis which specifically and permanently responded to a single course of liver or folic acid therapy. The absence of underlying organic disease in the majority of cases, and the permanent cure afforded by treatment with folic acid suggested a relationship to a temporary deficiency of folic acid which in many cases appeared to be nutritional in origin.

In the next few years, approximately 100 further cases similar to those described by Zuelzer and Ogden (1946) were reported from the U.S.A., Italy, England, Holland and France. By 1953 the incidence, particularly in the U.S.A., had declined (Zuelzer and Rutzky, 1953), but the condition was then frequently reported from countries with large underprivileged population groups (Adams, 1954; Walt, Holman and Hendrickse, 1956; Walt, Holman and Naidoo, 1957; Shnier and Metz, 1959; Kho and Odang, 1959; MacIver and Back, 1960; Metz, Brandt and Stevens, 1962; Velez, Ghitis, Pradilla and Vitale, 1963). In this group, the presentation in slightly

older infants and in association with malnutrition and infection was emphasized (Walt, Holman and Naidoo, 1957; Shnier and Metz, 1959; MacIver and Back, 1960).

In 1946, when Zuelzer and Ogden conducted their successful therapeutic studies with folic acid, this substance was believed to be the haemopoietic factor or Castle's extrinsic factor. Thus it was believed that infantile nutritional megaloblastic anaemia could be specifically attributed to folic acid deficiency even though liver therapy had also proved successful.

It was later realised that there were two extrinsic factors present in liver, folic acid and vitamin B_{12} which, when deficient, could both result in megaloblastic anaemia. These factors were therapeutically interrelated, and though most effective when used specifically, could produce haematological improvement in megaloblastic anaemia caused either by folic acid or by vitamin B_{12} deficiency, especially if used in pharmacological doses (Wilkinson, 1948 a, b; Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins and Seaman, 1950; Killander, 1958).

Therefore it was necessary to carry out further therapeutic trials with folic acid and vitamin B_{12} to determine the aetiology of nutritional megaloblastic anaemia of infancy. This was not a successful method because not all investigators compared the therapeutic efficacy of both factors used in sequence under controlled conditions as Minot and Castle (1935) had suggested. Those who did, found most cases could be attributed to folic acid deficiency (Luhby and Wheeler, 1949; Zuelzer and Rutzky, 1953). Occasionally folic acid was present with vitamin B_{12} deficiency and rarely uncomplicated vitamin B_{12} deficiency was responsible for the

condition (Zuelzer and Rutzky, 1953). Those who used only vitamin B_{12} as the therapeutic agent found they succeeded with it and concluded that vitamin B_{12} deficiency also played a significant role in infantile nutritional megaloblastic anaemia (McPherson, Jonsson and Rundles, 1949; Sturgeon and Carpenter, 1950; Shnier and Metz, 1959; MacIver and Back, 1960).

Laboratory methods clarified the problem. It was shown that vitamin B_{12} in pharmacological doses could be used successfully to treat patients with megaloblastic anaemia, in spite of normal serum vitamin B_{12} values (Metz, Brandt and Stevens, 1962) and low serum L.casei folate levels (Zalusky, Herbert and Castle, 1962). Moreover, serum vitamin B_{12} levels were normal in the great majority of cases of infantile nutritional megaloblastic anaemia (Adams and Scragg, 1962; Kondi, MacDougall, Foy, Mehta and Mbaya, 1963; Kende, Ramot and Grossowicz, 1963; Metz, Brandt and Stevens, 1962). A few of these infants had subnormal vitamin B_{12} levels (Adams and Scragg, 1962; Metz, Brandt and Stevens, 1962; Kende, Ramot and Grossowicz, 1963) but these were not necessarily the result of vitamin B_{12} deficiency because Mollin and Waters (1962) had shown that severe uncomplicated folate deficiency might result in subnormal vitamin B_{12} levels.

In infantile nutritional megaloblastic anaemia there was unequivocal evidence for folate deficiency. Serum L.casei folate levels were low (Herbert, Baker, Frank, Pasher, Sobotka and Wasserman, 1960; Kondi, MacDougall, Foy, Mehta and Mbaya, 1963), whole blood L.casei folate levels were low (Kende, Ramot and Grossowicz, 1963) and Figlu excretion

with and without oral histidine was excessive, (Luhby, Cooperman, MacIver and Montgomery, 1960; Luhby, Cooperman, MacIver and Montgomery, 1962).

From these observations folate deficiency was shown to have been primarily responsible for producing infantile nutritional megaloblastic anaemia, as had been suggested by Zuelzer and Ogden (1946).

Goats' milk anaemia.

The presentation of this macrocytic anaemia was similar to that described by Zuelzer and Ogden (1946) but was often more severe and occurred at an earlier age (Collins, 1962).

As early as 1933 Parsons had suggested that extrinsic factor deficiency might be the cause of the severe macrocytic anaemia occurring in infants who had been fed on a diet of goats' milk. This was partly confirmed when György (1934) demonstrated the therapeutic efficacy of yeast and liver therapy in this condition. As before, therapeutic trials failed to determine whether the anaemia was due to folate or vitamin B_{12} deficiency, because both folic acid (Gasser, 1948) and vitamin B_{12} (Reale and Feliciangeli, 1958), produced positive results.

Initial laboratory studies performed on goats' milk to estimate its folate and vitamin B_{12} content, were unhelpful because of the imperfect methods then available for assaying folate (Collins, Harper, Schreiber and Elvehjem, 1951). Once the assay method was improved it was shown that the folate content of goats' milk was extremely low (Naiman and Oski, 1964; Becroft and Holland, 1966), whereas the vitamin B_{12} content was comparable to that found in human milk (Nicol and Davis, 1967).

Infants with this anaemia had low serum L.casei folate levels (Becroft and Holland, 1966), but normal vitamin B_{12} levels (Wise, Lovric and O'Gorman Hughes, 1963; Becroft and Holland, 1966). This anaemia therefore appeared to be an example of severe dietary folate deficiency and should probably not be distinguished from infantile nutritional megaloblastic anaemia. The earlier and more severe presentation characteristic of this anaemia appeared to reflect the severity of the dietary folate deficiency (Becroft and Holland, 1966).

Vitamin B_{12} deficiency.

In Italy, Gerbasi (1940) Pecorella, Burgio and Aversa (1947), and Amato (1952), described an infantile megaloblastic anaemia associated with neurological manifestations such as pyramidal and extrapyramidal lesions. Later a similar syndrome was reported by Jadhav, Webb, Vaishnava and Baker (1962) in India. The principal neurological manifestations they described were disturbed levels of consciousness, developmental regression and involuntary movements. Hyperpigmentation was an important association.

There was good evidence for attributing these megaloblastic anaemias to vitamin B_{12} deficiency. Low levels of serum vitamin B_{12} were found in the affected infants and their mothers, and in the breast milk they received (Jadhav, Webb, Vaishnava and Baker, 1962; MacIver, 1962). So, too, a single course of therapy with physiological (Jadhav, Webb, Vaishnava and Baker, 1962) and small oral doses of vitamin B_{12} cured the condition (Burgio, Russo and LoJacono, 1956; Jadhav, Webb, Vaishnava and Baker, 1962).

Later Kaul, Prasan and Chowdhry (1963), described a similar neuro-

logical syndrome with hyperpigmentation in Indian infants which was not associated with megaloblastosis or consistent evidence of vitamin B_{12} deficiency. Other investigators confirmed the existence of a syndrome of neurological manifestations, hyperpigmentation and megaloblastosis due to vitamin B_{12} deficiency, though they did not find that megaloblastosis was always present (Srikantia and Reddy, 1967). They suggested that the megaloblastic anaemia might be a late manifestation of the condition (Srikantia and Reddy, 1967).

In some parts of the world infantile nutritional megaloblastic anaemia had been ascribed to vitamin B_{12} deficiency. Its clinical manifestations may be different to those found in other forms of infantile nutritional megaloblastic anaemia but there is no agreement on this at the present time.

Vitamin C deficiency.

The association of megaloblastic anaemia and scurvy was noted both in adults and children (Jennings and Glazebrook, 1938; McMillan and Inglis, 1944; Zuelzer and Ogden, 1946; Aldrich and Nelson, 1947).

Initially a specific role for vitamin C in the production of this megaloblastosis could not be demonstrated and as a result it was concluded that vitamin C and folate deficiency were often associated (Zuelzer, Hutaff and Apt, 1949). After a series of experiments on monkeys, May and his colleagues concluded in 1953 that vitamin C deficiency resulted in increased demand for folate. This led to the production of megaloblastic anaemia when folate supplies were insufficient (May, Nelson, Lowe and Salmon, 1950; May, Hamilton and Stewart, 1953).

Further studies, most of them on adults, produced controversial

results. Some investigators believed that vitamin C deficiency could be specifically responsible for the megaloblastosis which sometimes occurred in scurvy (Bronte-Stewart, 1953; Brown, 1955), but others ascribed it to associated dietary folate deficiency (Zalusky and Herbert, 1961a; Chazan and Mistilis, 1963). Dietary conditions were not always controlled in some of the earlier studies and these came in for criticism (Zalusky and Herbert, 1961a). At present there are two schools of thought on the aetiology of the megaloblastic anaemia sometimes seen in scurvy. Cox, Meynell, Northam and Cooke (1967) believed that the megaloblastosis resulted from a disturbance of folate metabolism caused by vitamin C deficiency. The severity depended on the degree of this disturbance and the degree of associated folate depletion. Without associated folate depletion the anaemia was cured by the administration of vitamin C alone, but when it was present, vitamin C by itself had no effect. There was some biochemical evidence for the belief that vitamin C might play a role in folate metabolism. It was thought that vitamin C protected folic acid reductase and so ensured that folate coenzymes were maintained in sufficiently high concentration (Vilter, 1964).

The other view suggested by Zalusky and Herbert (1961a) and Chazan and Mistilis (1963) was that megaloblastic anaemia in scurvy only resulted from associated folate deficiency. Although the experiment of Zalusky and Herbert (1961a) was well controlled, their evidence was based on the investigation of only one patient. Chazan and Mistilis (1963) did not carry out controlled studies. The view suggested by Cox, Meynell, Northam and Cooke (1967) adequately explains the controversial findings, but there may be criticism of the dietary control in their experiments.

The role of vitamin C deficiency in the production of megaloblastic anaemia still requires further investigation.

Vitamin E deficiency.

It was reported that malnourished infants with megaloblastic anaemia (Majaj, Dinning, Azzam and Darby, 1963) and low serum L.casei folate levels (Majaj, 1966) responded to treatment with vitamin E. The improvement following vitamin E therapy was associated with a rise in serum L.casei folate levels. Thus it was believed that the anaemia could be attributed to a secondary disturbance of folate metabolism caused by vitamin E deficiency, although other explanations were also considered (Majaj, 1966). Up until 1964 there was no biochemical proof of a relationship between vitamin E and folate metabolism (Vilter, 1964).

Whitaker, Fort, Vimokesant, and Dinning (1967) carried out a controlled study and showed that vitamin E produced a therapeutic response in anaemic infants with kwashiorkor which occurred both in the presence and absence of megaloblastic anaemia. On the other hand Asfour and Firzli (1965) found that the administration of vitamin E did not have a beneficial effect in undernourished infants with iron deficiency and low serum vitamin E levels. An interrelationship between vitamin E and vitamin B_{12} has also been suggested (Oski, Myerson, Barness and Williams, 1966).

There was evidence that vitamin E might be therapeutically effective in the anaemia of kwashiorkor, but the role of this deficiency in the production of infantile megaloblastic anaemia and its relationship to folate metabolism still had to be established.

Summary.

The classical pattern of infantile nutritional megaloblastic anaemia

has been described, as well as the clinical differences observed in underprivileged communities, among infants receiving goats' milk and those with vitamin B_{12} deficiency. The common form of infantile nutritional megaloblastic anaemia was shown to have resulted from folate deficiency in the great majority of cases. In Italy and India a megaloblastic anaemia due to vitamin B_{12} deficiency was described. The role of vitamin C and vitamin E deficiencies in the production of infantile megaloblastic anaemia has not yet been proved.

The aetiology of folate deficiency in the nutritional megaloblastic anaemia of infancy.

Evidence for the belief that folate deficiency in infantile megaloblastic anaemia was nutritional in origin was both negative and positive. On the negative side there was an absence of any underlying organic disease in the infants with this condition, and on the positive side there was the therapeutic efficacy of a single course of oral folic acid (Zuelzer and Ogden, 1946).

In some instances this belief was furthersupported by a history of inadequate or incorrect dietary intake (Zuelzer and Ogden, 1946; Luhby, 1959). Moreover, the macrocytic anaemia which developed in infants who had received a diet of goats' milk was believed to be an example of pure dietary deficiency (Zuelzer and Ogden, 1946). The subseqnent reports of megaloblastic anaemia in infants from underprivileged communities in association with the clinical features of malnutrition further emphasised the nutritional character of this megaloblastic anaemia (Walt Holman and Naidoo, 1957; Shnier and Metz, 1959; MacIver and Back, 1960).

Megaloblastic anaemia also occurred in infants given calorically

adequate diets (Zuelzer and Ogden, 1946). It was believed that the folate content of all milk was low (Collins, Harper, Schreiber and Elvehjem, 1951) and that infants on a milk diet especially formula milks unsupplemented with vitamin C received less folate than other diets provided (Zuelzer and Rutzky, 1953). Luhby (1959) concluded from this that infants on a milk diet could either have low folate reserves or more commonly folate reserves within the lower limits of normal. For this reason normal infants were predisposed to the development of folate deficiency.

However, in well nourished and most malnourished infants megaloblastic anaemia seldom developed on the basis of dietary deficiency alone, especially in the younger infants (Zuelzer and Rutzky, 1953; Luhby, 1959). It usually resulted from inability to compensate for the effect of superimposed stress factors such as vitamin C deficiency (May, Nelson, Lowe and Salmon, 1950; Zuelzer and Rutzky, 1953) or infection (Zuelzer and Rutzky, 1953; Luhby, 1959). This occurred either because folate reserves were too low or even when these were normal because the effect of the stress factor such as infection was too severe (Zuelzer and Rutzky, 1953; Luhby, 1959). The significance of infection as a stress factor was appreciated in infants from both good (Zuelzer and Ogden, 1946) and poor socio-economic circumstances (Walt, Holman and Naidoo, 1957; Shnier and Metz, 1959; MacIver and Back, 1960). Of the infections, gastrointestinal infection was particularly important because it also could result in a reduction of folate intake and absorption (Zuelzer and Rutzky, 1953). May, Stewart, Hamilton and Salmon (1952) found that infection could result in folate deficiency and so induce the development of megaloblastic anaemia in monkeys maintained on what was

believed to be an adequate folate intake.

Other aetiological factors for the development of folate deficiency such as intestinal malabsorption, alterations in intestinal bacterial flora and the size of maternal stores were considered but not further investigated. (Zuelzer and Ogden, 1946; May, Nelson, Lows and Salmon, 1950).

Summary.

The evidence for the nutritional nature of the folate deficiency in infantile megaloblastic anaemia was based on clinical and therapeutic studies. The most important factors resulting in megaloblastic anaemia were thought to be a combination of poor dietary intake in association with stress factors which increased the demand for folate, or interfered with its utilisation. Of the stress factors vitamin C deficiency and infection were regarded as the most important. Infection alone could result in megaloblastic anaemia if severe enough. Pure dietary folate deficiency was uncommon but did occur in infants fed a diet of goats milk, occasionally in older infants, and in association with severe malnutrition. The role of maternal folate deficiency, intestinal malabsorption and altered intestinal bacterial flora were considered but not studied.

Prevalence of nutritional megaloblastic anaemia of infancy.

By 1963 infantile nutritional megaloblastic anaemia was still prevalent in countries with large underprivileged population groups. In Jamaica it accounted for 6.3% of all paediatric admissions (MacIver and Back, 1960). In South America, approximately 40% (Velez, Ghitis, Pradilla and Vitale, 1963), and in Kenya, approximately 17% (Kondi, MacDougall, Foy, Mehta and Mbaya, 1963) of malnourished infants had megaloblastosis

of varying severity.

South Africa was no exception. Nine percent (9%) of paediatric admissions (Walt, Holman and Naidoo, 1957) and 8.5% of all malnourished infants, had megaloblastic anaemia (Shnier and Metz, 1959).

Mortality in megaloblastic anaemia. (infantile nutritional megaloblastic anaemia)

Although the condition may easily be cured, it has been associated with a high mortality reported as follows:
10% (Kende, Ramot and Grossowicz, 1963); 12% (MacIver and Back, 1960); 24% (Walt, Holman and Hendrickse, 1956); 25% (Zuelzer and Ogden, 1946); 40% (Kho and Odang, 1959).

Occasionally deaths were due to anaemia and its effects, but usually they were attributed to the severe associated infection. It had been reported that megaloblastic anaemia predisposed to infection because of the leucopaenia and reduced antibody synthesis associated with this condition (Luhby, 1959). On these grounds megaloblastic anaemia might also have contributed to the mortality.

The condition was still prevalent in 1963 and still associated with a significant mortality. It was clear there had to be early diagnosis and treatment.

Prophylaxis of folate deficiency in infants.

Investigators recommended a balanced diet to include liver, kidney, meat and leafy vegetables as prophylaxis against folate deficiency (Zuelzer and Rutzky, 1953; Luhby, 1959; MacIver and Back, 1960). This recommendation could not be applied in the prevention of megaloblastic anaemia in underprivileged infants.

Others suggested that prophylactic folic acid should be used in stress situations such as gastroenteritis and recurrent infection (Zuelzer

and Rutzky, 1953; Luhby, 1959; Kende, Ramot and Grossowicz, 1963; Wise, Lovric and O'Gorman Hughes, 1963). But this had practical disadvantages. Investigators thought that the routine prophylaxis with folic acid in infancy was unnecessary and ignored it (Zuelzer and Rutzky, 1953; Castle, Elvehjem, May, Welch, Zuelzer and Butler, 1951). This may have been because they worked in a country where the standard of living was generally high and where the incidence of the condition had declined. The decrease in incidence of the condition in the United States of America was ascribed to the addition of ascorbic acid to formula milks (Zuelzer and Rutzky, 1953).

The concept of judicious routine prophylaxis with folic acid gained favour when it was established that the daily physiological adult requirement of folic acid, 50 µg. (Herbert, 1962b) or less (Knowles, Prankerd and Westall, 1961) was lower than the amount capable of precipitating neurological damage in patients with unsuspected vitamin B_{12} deficiency (Davidson and Jandl, 1959; Herbert, 1962b).

It was necessary to reconsider the advisability of routine prophylaxis with folic acid in the case of infants from underprivileged communities when malnutrition, infection and megaloblastic anaemia were prevalent.

LABORATORY DIAGNOSIS OF FOLATE DEFICIENCY IN INFANCY

Only a few studies using laboratory methods to diagnose folate deficiency were carried out on infants before 1963.

Normal values for these parameters were determined and their application in the diagnosis of folate deficiency in infancy and childhood was investigated.

Normal values.

Whole blood and serum L.casei folate levels in normal infants were found to approximate adult levels (Grossowicz, Mandelbaum-Shavit, Davidoff and Aronovitch, 1962; Kende, Ramot and Grossowicz, 1963). Normal infants given a histidine load of 0.12 g. per lb. body weight in three divided doses four hours apart, excreted less than 30 µg./ml. of Figlu in a 24-hour urine collection (Luhby, 1963). Figlu was estimated with the sensitive enzymatic method of Tabor and Wyngarden (1958).

Tests of folate deficiency.

Blood (Kende, Ramot and Grossowicz, 1963) and serum L.casei folate assays (Kondi, MacDougall, Foy, Mehta and Mbaya, 1963; Herbert, Baker, Frank, Pasher, Sobotka and Wasserman, 1960; Dormandy, Waters and Mollin, 1963) and the Figlu test after oral histidine (Luhby, Cooperman, MacIver and Montgomery, 1962;) were all found to be good tests of megaloblastic anaemia due to folate deficiency. The value of these tests in distinguishing between vitamin B_{12} and folate deficiency in infancy was not investigated.

BIOCHEMICAL (LATENT) FOLATE DEFICIENCY IN INFANCY.

In infants and children as in adults, folate deficiency could be diagnosed on the results of laboratory parameters before the development of megaloblastic anaemia. This was shown by three groups of investigators.

Kende, Ramot and Grossowicz (1963) demonstrated that healthy Israeli infants of good socio-economic circumstances had significantly higher blood L.casei folate levels than a comparable group of infants with a poorer socio-economic background. Dormandy, Waters and Mollin (1963) found that infants of more than twelve months, with coeliac disease, had low serum

L.casei folate levels, excessive Figlu excretion after oral histidine, and morphological evidence of folate deficiency before the development of anaemia. Although this also applied to coeliac infants of less than twelve months, a fair percentage of a control group of infants in this age group had low serum L.casei folate levels and increased Figlu excretion after oral histidine. This implied that in infants of less than twelve months, folate deficiency might be relatively common and not necessarily aetiologically related to coeliac disease.

In 1962 Luhby, Cooperman, MacIver and Montgomery showed that Figlu excretion, after oral histidine, was excessive in folate deficiency before the development of megaloblastic anaemia.

In infants, just as in adults, folate deficiency could be diagnosed by means of biochemical tests before the development of overt megaloblastic anaemia.

It was found that the sensitivity of some of these tests was different according to their use on infants or adults. Herbert (1962a) demonstrated a greater sensitivity of the serum L.casei folate assay when it was compared with Figlu excretion after oral histidine in the diagnosis of folate deficiency in adults. But in infants Luhby, Cooperman, MacIver and Montgomery (1962) found Figlu excretion after oral histidine a more sensitive test than serum L.casei folate assays. This was confirmed by Dormandy, Waters and Mollin (1963) when they studied control infants of less than 12 months. About a third of these had low serum L.casei folate levels, and 75% had positive Figlu tests.

There appeared to be two possible explanations for these contradictory results. On the one hand, differences in methodology might have been responsible. Cooperman, Luhby and Avery (1960) had been dissatisfied

with the serum L.casei folate assay as a method of diagnosis. Perhaps their conclusions were based on studies performed before satisfactory modifications for assaying serum L.casei folate levels were introduced. Dormandy, Waters and Mollin (1963) used a histidine load comparatively larger than that used in adults and exceeding the load recommended by Luhby, Cooperman, MacIver and Montgomery (1962). They thought that this might have been an important factor in young infants, where hepatic enzymes might have been immature (Dormandy, Waters and Mollin, 1963).

On the other hand, Herbert (1962a) induced folate deficiency in only one subject under strictly controlled experimental conditions. If in infants as in some adults with vitamin B_{12} deficiency, there were both a disturbance of folate metabolism and associated folate deficiency, then it was conceivable that the Figlu test after oral histidine might provide evidence of folate deficiency before the serum L.casei folate assay.

The prevalence and significance of latent folate deficiency in infancy was unknown.

SUMMARY

The existence of a condition of biochemical folate deficiency was demonstrated in infancy. In some infants this was related to socio-economic circumstances. In the diagnosis of latent folate deficiency in infants and children the Figlu test, after oral histidine, appeared to be a more sensitive test than the serum L.casei folate assay, but this required further investigation. The clinical importance of latent folate deficiency in infancy still had to be established.

CHAPTER III

ASSESSMENT OF THE PROBLEM AND THE OBJECTS OF THE THESIS

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ASSESSMENT OF THE PROBLEM AND THE OBJECTS OF THE THESIS.

In a preliminary study carried out in Cape Town and referred to in Chapter I, approximately 64% of healthy, underprivileged infants had positive Figlu tests after oral histidine (Friedman, McKenzie, Turner and Wittmann, 1964a). Luhby, Cooperman and Teller (1959b) had reported that the Figlu test after oral histidine was a sensitive, specific and reliable test of folate deficiency. From their findings it seemed likely that there was a high incidence of biochemical folate deficiency in the population group studied in Cape Town. The later findings reviewed above had made it plain that this inference had to be reconsidered.

Dormandy, Waters and Mollin (1963) suggested that an excessive dose of histidine given to infants might result in spurious positive Figlu tests. At the time the preliminary study was undertaken, information on the recommended dose of histidine for infants was not available in Cape Town. From data later available it was found that the dose used (2.5 g.) was larger than that recommended for infants by Luhby, Cooperman, MacIver and Montgomery (1962) and Luhby (1963). However, in the same study, a proportion of healthy, underprivileged infants were given a 1 g. histidine load. After this dose, 39% had positive Figlu tests. As this histidine load fell within the recommended range, it seemed that at least in 39% of cases the positive Figlu tests could not be ascribed to the effect of overloading with histidine.

The method of estimation of Figlu used in this study (Kohn, Mollin and Rosenbach, 1961) was only semi-quantitative, and insensitive in comparison with others (Knowles, 1962). Moreover, an important weakness was the necessity for a five-hour urine collection. In infants it was

difficult to ensure complete urine collection in these circumstances. This method was also used by Dormandy, Waters and Mollin (1963). They found that 75% of control infants under a year excreted excessive Figlu after a 3 g. histidine load. These findings were comparable to the 64% positive results found in healthy, underprivileged infants given a 2.5 g. histidine load in Cape Town. It seemed unlikely that the positive results could be attributed only to a semi-quantitative method and incomplete urine collection.

On these grounds it was believed that the observation in Cape Town was valid and that at least 39% of healthy, underprivileged infants had positive Figlu tests after oral histidine.

Meanwhile other investigators did not confirm the initial experience of Luhby, Cooperman and Teller (1959b) and showed that the Figlu test after oral histidine was not a specific test for folate deficiency (Zalusky and Herbert, 1961; Carter, Heller, Schaffner and Korn, 1961; Kohn, Mollin and Rosenbach, 1961; Chanarin, Bennett and Berry 1962). It was shown that a positive Figlu test could only be attributed to folate deficiency if supported by other laboratory evidence or by a successful therapeutic trial conducted with physiological doses of folic acid.

In Cape Town, in a study carried out on underprivileged infants with gastroenteritis (Friedman, McKenzie, Turner and Wittmann, 1964b) it was found that positive Figlu tests after oral histidine agreed well with bone marrow morphology. In healthy, underprivileged infants, a therapeutic trial with folic acid (400 µg. orally daily) was used to determine whether positive Figlu tests after oral histidine could be attributed to folate deficiency (Friedman, McKenzie, Turner and Wittmann, 1964a). With one

exception, all infants given a therapeutic trial with folic acid, subsequently excreted normal quantities of FigIu after oral histidine. Unfortunately it was shown that the result of a successful therapeutic trial with folic acid could only be used as unequivocal evidence for attributing positive Figlu tests to folate deficiency if it was conducted with 100-200 µg. of folic acid daily. In addition, the therapeutic trial was not controlled by a comparable study in infants not given folic acid.

The experience of other investigators with the Figlu test after oral histidine in infancy (Luhby, Cooperman, MacIver and Montgomery, 1962; Dormandy, Waters and Mollin, 1963) encouraged belief in its value as a parameter of folate deficiency in infancy. But the greater sensitivity of the test compared with the serum L.casei folate assay found in infants as compared with adults, was puzzling and required investigation.

Although folate deficiency was favoured as a possible explanation for the results, it was believed that the positive Figlu tests after oral histidine in the study in Cape Town could not be confidently attributed to this deficiency. Their interpretation required clarification.

From the work reviewed above, it appeared that there was interest in the concept of biochemical folate deficiency and its possible importance to adults and infants. There appeared to be good reason for believing that many healthy underprivileged infants might have biochemical folate deficiency.

a) It was believed that normal infants on a milk diet received less folate than other diets provided (Zuelzer and Rutzky, 1953) and that they could as a result develop subclinical folate deficiency (Luhby, 1959).

b) In healthy underprivileged infants the effects of underfeeding and faulty feeding practices might further reduce folate intake. Furthermore, most milks in South Africa were not supplemented with ascorbic acid.

For these reasons it was anticipated that latent folate deficiency might be wide spread in healthy underprivileged infants.

c) The use of laboratory methods for investigating folate deficiency had shown that infants could suffer from biochemical folate deficiency before the development of overt megaloblastic anaemia (Luhby, Cooperman, MacIver and Montgomery, 1962; Dormandy, Waters and Mollin, 1963; Kende, Ramot and Grossowicz, 1963). This might have resulted from inadequate dietary intake and in some infants was shown to have been related to socio-economic circumstances (Kende, Ramot and Grossowicz, 1963).

d) The incidence of positive Figlu tests after oral histidine (39%) in healthy underprivileged infants in Cape Town seemed to be in accord with the above findings and suggested that there was possibly a high incidence of latent folate deficiency in healthy underprivileged infants in Cape Town.

Infantile nutritional megaloblastic anaemia could result from diminished folate intake or increased demand for folate. Most commonly it resulted from inability to compensate for the increased demands for folate caused by infection and other stress factors. If in South Africa, widespread biochemical folate deficiency were present, then it might have been an important factor in the development of infantile megaloblastic anaemia commonly associated with malnutrition and infection. Because infantile nutritional megaloblastic anaemia due to folate deficiency was frequently found in South Africa, and because it was still associated with a fairly high mortality, it seemed important to find out more about latent folate deficiency in underprivileged infants, especially with regard to its relationship to the development of megaloblastic anaemia.

Thus the study was undertaken to investigate:

1. The incidence, aetiology, significance and need for prophylaxis of biochemical folate deficiency in healthy infants from poor socio-economic circumstances.

From the review above it was clear that the laboratory parameters for diagnosing folate deficiency in infants as well as adults differed in sensitivity, specificity and reliability. It would therefore be necessary to use more than one parameter in the diagnosis of biochemical folate deficiency and in determining its significance. The last would be further established in relation to clinical and morphological studies.

Aetiological studies of folate deficiency would include an attempt to study the influence of maternal on infant folate nutrition and the role of malabsorption in determining biochemical folate deficiency because little was known of these aspects. However, knowledge concerning the absorption of folate under physiological conditions was incomplete and therefore the application of absorption studies even with folic acid might be limited.

From all these studies inferences regarding prophylaxis of the condition might be possible.

In view of the uncertainty regarding the value and application of the Figlu test after oral histidine in the diagnosis of folate deficiency in infancy, a sensitive method of measuring Figlu after a standard dose of histidine would be used as a diagnostic parameter in the above study in order to investigate

2. The interpretation and validity of the Figlu test after oral histidine as a parameter of biochemical folate deficiency in healthy underprivileged infants.

CHAPTER IV

DESIGN, CONDUCT OF STUDY, PROCEDURES AND CASE MATERIAL.

CHAPTER IV.

DESIGN OF STUDY

One hundred healthy Coloured women were studied for folate deficiency from the 32nd to 40th week of gestation. At birth, umbilical cord blood samples (cord blood samples) were taken and afterwards their infants were studied for folate deficiency at four intervals until sixteen months.

As this prospective study did not provide sufficient information to make the interpretation of positive Figlu tests after oral histidine possible, further additional studies were undertaken. In these,maternal folate nutrition was not assessed, and infants were investigated only once, unless they were participating in a therapeutic trial. Infants under 7½ months were selected because, from the data in the prospective study, positive Figlu tests were most common in this age group. With one exception, only male infants were studied to facilitate ease of urine collection.

Several approaches were adopted in planning the investigations necessary to try and establish the meaning of the positive Figlu tests. The first was comparing the results of the Figlu test after oral histidine with those of other parameters of folate deficiency. The second was determining if there was an aetiological reason for folate deficiency in infants with positive Figlu tests. The third was assessing the effects of folic acid administration on Figlu excretion. The fourth was determining whether the positive tests could be attributed to the effect of diseases or deficiencies known to cause secondary disturbances of folate metabolism. In addition, an attempt was made to establish normal values in white (European) infants and to investigate the pattern of Figlu

TABLE 1

DETAILS OF INVESTIGATIONS IN PROSPECTIVE STUDY

I. Healthy pregnant Coloured women

Purpose of investigation	Method of investigation
The determination of:	
folate deficiency	Serum L. casei folate levels. Figlu excretion after oral histidine.
significant folate deficiency	Haemoglobin levels. Examination of blood smears for: (i) Hypersegmentation of neutrophils (ii) Macrocytosis
coincidental deficiencies possibly affecting folate metabolism	Serum iron levels. Serum iron-binding capacity. Serum vitamin B_{12} levels.
nutrition	Serum protein levels

II. Cord blood samples

Purpose of investigation	Method of investigation
To investigate maternal/infant folate relationships	Cord serum L. casei folate levels

III. Quarterly assessment of infants in the 2½ - 16 months period

Purpose of investigation	Method of investigation
The determination of:	
health and development of infants	History: Past and present health " " " infection Developmental progress Dietary schedule Drug administrations, etc. Examination: Health Nutrition; including nutritional deficiencies, e.g. rickets. Development
folate deficiency	Serum L. casei folate assays Figlu excretion after oral histidine *Urocanic acid excretion
significant folate deficiency	Haemoglobin levels Examination of blood smears for: (i) Hypersegmentation of neutrophils (ii) Macrocytosis + Bone marrow examination
the effect of folic acid treatment on results of Figlu excretion after oral histidine	Re-estimation of Figlu excretion after oral histidine following therapeutic trial with oral folic acid in pharmacological doses
a possible aetiology for folate deficiency in infants excreting Figlu in excess	Xylose absorption tests
coincidental deficiencies and disease possibly affecting folate metabolism	Serum iron levels Serum iron-binding capacity Serum vitamin B_{12} levels Serum glutamic oxaloacetic transaminase levels
nutrition	Serum protein levels

* Not a true test of folate deficiency

\+ Indications discussed in text

TABLE 2

A. DETAILS OF INVESTIGATION IN ADDITIONAL STUDIES

Age (months)		Purpose of investigation	Method of investigation
		The determination of:	
		health and development	History Examination
		folate deficiency	L. casei serum folate assays L. casei red cell folate assays L. casei folic acid clearance tests Figlu excretion with and without oral histidine
		Figlu excretion in 'normal' European and African infants	Four European infants Eight African infants
2½ - 4½	Coloured (30) Europeans (2) Africans (5)	significant folate deficiency	Haemoglobin levels
		the effect of folic acid treatment on Figlu excretion	Re-estimation of Figlu excretion after (i) small and physiological doses of oral folic acid (ii) pharmacological doses of oral folic acid
4½ - 7½	Coloured (30) Europeans (2) Africans (3)	a possible aetiology for folate deficiency in infants excreting Figlu in excess	Milk L. casei folate assays Folic acid absorption tests Xylose tests Reticulocyte counts Occult blood tests
		coincidental deficiencies and disease possibly affecting folate metabolism	Serum iron levels Serum iron binding capacity Serum vitamin B_{12} levels Serum glutamic oxaloacetic transaminase levels S. faecalis folic acid clearance tests Therapeutic trial with intramuscular iron
		nutrition	Serum albumin levels

B. CONTROLLED THERAPEUTIC STUDY IN 46* COLOURED INFANTS 2½ - 5 MONTHS OF AGE

Purpose of investigation	Method of investigation
The determination of:	
health and development	History Examination
folate deficiency	L. casei serum folate assays L. casei red cell folate assays Figlu excretion after oral histidine[x] Urocanic acid excretion[x]
significant folate deficiency	Haemoglobin levels
the effect of folic acid treatment on Figlu excretion	Re-estimation of Figlu excretion after oral histidine following therapeutic trial, with either pharmacological doses of folic acid and oral iron or oral iron therapy alone
a possible aetiology for folate deficiency in infants excreting Figlu in excess	Milk L. casei folate levels
coincidental deficiencies and disease affecting Figlu excretion	Serum iron levels Serum iron binding capacity Serum vitamin B_{12} levels
the effect of non-specific factors on the results of the Figlu test	Incomplete urine collections (creatinine 24-hour collections). The validity of expressing Figlu results in µg./ml. rather than mg./24 hours.

* The Figlu and folic acid clearance tests (S. faecalis and L. casei) were performed on 7 additional infants who did not participate in this trial.

[x] Figlu estimations with method of Chanarin and Bennett, 1962a

excretion in African infants. Finally, the relationship of Figlu excretion to non-specific factors like incomplete urine collection and urine concentration was examined.

At the same time the incidence and severity of folate deficiency in infancy was determined from the results of the laboratory and morphological parameters used to investigate this deficiency. Maternal, dietary and absorption studies were carried out to determine the aetiology of folate deficiency in healthy,underprivileged infants. The details of these investigations are set out in Tables 1 and 2.

CONDUCT OF THE STUDY

Selection of Subjects.

In the prospective study all healthy Coloured women in the last trimester of pregnancy attending the maternity hospitals associated with the University of Cape Town, were interviewed. The nature and purpose of the trial was explained to them and they were promised as little inconvenience as possible. Mothers and infants were provided with free transport and free accommodation during the hospitalisation necessary for the 24 hour urine collection from the infants. Selection was made on the basis of maternal health and parental co-operation.

In the additional studies selection was determined by the infant's health and parental co-operation. Mothers were approached at infant welfare clinics and the Red Cross Children's Hospital. The superintendent of a foster home gave permission for the study of seven infants there.

Composition of the groups.

In the prospective study, 86 of the original hundred infants were investigated at least once. Eighty-four were present on the first

TABLE 3

COMPOSITION OF PROSPECTIVE STUDY

MOTHERS

Numbers studied	Numbers attending with infants at least once
100	87

INFANTS

VISIT 1 : Age 2¼ - 4½ months			
Numbers studied - 85			
Interim Period			
Exclusions	(16)	Inclusions	(2)
Consent withdrawn	11	Parents give consent	1
Temporary withdrawal	4	Infant recovered from pertussis and broncho-pneumonia	1
Cerebral birth trauma *	1		
Number to study at Visit 2 = 85 + 2 - 16 = 71			

VISIT 2 : Age 4½ - 7½ months			
Numbers studied - 71			
Interim Period			
Exclusions	(37)	Inclusions	(4)
Consent withdrawn	8	Parents give consent again	4
Therapeutic trial	9		
Withdrawn **	20		
Number to study at Visit 3 = 71 + 4 - 37 = 38			

VISIT 3 : Age 7½ - 10¾ months			
Numbers studied - 38			
Interim Period			
Exclusions	(19)	Inclusions	(20)
Consent withdrawn	3	Re-admitted to study **	20
Discharged from study **	16		
Number to study at Visit 4 = 38 + 20 - 19 = 39			

VISIT 4 : Age 10¾ - 16 months
Numbers studied - 39

* Data not analysed

** Alteration made by investigator: explained in text

occasion, 71 on the second, 38 on the third and 39 on the fourth. Of the 86 infants, 16 were investigated on all four occasions, and a further 41 on three occasions.

The lack of homogeneity of the sample resulted initially from permanent or temporary withdrawal of consent by the parents. In these circumstances there seemed no point in further investigation of infants who showed little or no evidence of folate deficiency after two consecutive visits. By arrangement with the investigator after visit two, 20 infants were not required again until visit four, and after visit three, 16 infants were discharged from the study. A further 9 infants were dismissed from the study after receiving a therapeutic trial with folic acid after visit two. The details are presented in Table 3.

In all, 125 infants were investigated in the additional studies. The group comprised 113 Coloured infants (60 + 46 + 7), 8 Africans and 4 Whites(Table 2).

Method of study.

Maternity hospitals

The mothers were investigated at their antenatal visits to the maternity hospitals. The nursing staff notified the investigator when delivery was imminent so that cord blood samples could be taken.

Red Cross War Memorial Children's Hospital/Foster Home/Private Home

Infants were admitted to a Metabolic Research Unit where infection was minimal and the nursing staff was experienced in the collection of 24 hour urine samples. In a few cases infants were admitted to a general ward. Strict precautions were taken against infection, and the nursing staff were instructed how to manage the urine collection. After the

clinical assessment, the first histidine dose was administered and the 24 hour urine collection was begun. On the next day, at least two and usually four hours after a feed, a venepuncture was performed. Infants were discharged when the urine collection was completed. In the additional studies a few infants were brought back a week later as out patients for a folic acid clearance or absorption test.

After each visit parents were encouraged to bring their infants back to the hospital in the event of illness, with a view to providing free medical care and ensuring that the programme had no harmful effects on the infants. A good number of the parents took advantage of this arrangement. The same principles were applied to infants studied in a foster home (7) and a private home (1).

There were no side effects unequivocally attributable to this study. It was possible that the histidine might on occasions have aggravated, if not caused, diarrhoea. In addition, the break in routine,and investigations carried out in a strange environment,may have disturbed some of the infants, particularly those over the age of six months. It was very difficult to assess these impressions without the benefit of a controlled study. On the completion of this investigation, the infants were given iron and folic acid according to their requirements.

PROCEDURES

a) Mothers

After a venepuncture a 15g. load of histidine was administered in orange juice. Three hours later urine was passed and discarded. For the next five hours all urine was collected into bottles containing 1cc. of concentrated hydrochloric acid. On completion of the collection, more acid was added if the pH was not less than 4. The urines were stored at $0^{\circ}C$.

b) Cord blood samples

These were collected at birth, or as soon afterwards as possible. A sterile knife was used to make an incision through the separated cord. The placenta was elevated and the cord blood allowed to drain into a test tube. Care was taken to avoid milking the cord.

c) Infants

1. Figlu excretion after oral L-histidine monohydrochloride (histidine).

Histidine (0.12g./lb. or 0.26g./kg) was weighed out in three divided doses on a Mettler scale which was checked for accuracy. The crystals were dissolved in hot water and brought to the required temperature with cold water or orange juice. The doses were administered four hours apart, and feeding was withheld for one hour before and after histidine administration.

The nursing staff was given a time schedule for the administration of histidine and the feeds. They were asked to record the number and nature of stools passed, and, if relevant, the time and amount of vomiting and urine leakage. The urine was collected into perineal bags into which catheters were inserted for the extraction of urine. The nurses were successful in putting the bags on girls as well as boys, but collection from the former always required greater care. The 24 hour urine collection was begun after the administration of the first histidine dose. Urine was regularly extracted and discharged into a bottle containing 1cc. of concentrated hydrochloric acid. At the conclusion of the test, urines were further acidified if the pH was not less than 4 as assessed with the use of Merck's Reagenzien indicator paper.

Urine was first stored at 0°, but approximately six months later aliquots were taken and stored at -20°C. Thereafter aliquots of the 24-hour urines were always stored at -20°C ab initio.

Urine collections were excluded from the study if vomiting occurred in relation to the administration of histidine, or if there was a significant urine loss. A loss in excess of 10 - 15% of the total 24 hour urine volume was regarded as significant. The amount of urine lost by leakage was estimated by measuring the amount of water required to produce a similar sized stain on the sheets.

2. Figlu excretion without oral histidine

In 24 infants, samples of urine were collected before the administration of histidine. These were stored at a pH of less than 4 at -20°C. until assayed.

3. Collection of blood

Throughout the study all blood samples collected from adults, umbilical cords and infants, were taken with needles, syringes and test tubes free of folate, vitamin B_{12} and iron.

4. Venepunctures

These were performed at least two, and usually four hours after a feed. With two exceptions only superficial veins were used. In the prospective study where four admissions were contemplated, only one attempt at venepuncture was made. Thus blood samples were not always obtained, nor was sufficient blood always available for completion of the intended tests. The same procedure was followed in the additional studies, except with infants on whom folic acid clearance and absorption tests were performed. In these cases it was sometimes necessary to repeat the attempt to obtain blood.

5. Bone marrow examination (28 infants).

Bone marrow aspirations were taken from the greater trochanter of the femur following sedation and injection of local anaesthetic at the

site. The standard aseptic precautions were observed. Bone marrow examinations were performed if mothers consented, and if significant folate deficiency was suspected on clinical or biochemical grounds. In this study the clinical criterion was chronic or recurrent gastro-enteritis in association with undernutrition. The biochemical criteria were low serum L.casei folate levels and positive Figlu tests.

6. D-Xylose (xylose) absorption tests

Xylose was administered as a 10% solution in water and given in a dose of 0.5 g./kg. body weight not exceeding a total dose of 5 g. It was administered after a four hour fast, and five hours before the end of the 24 hour urine collection for Figlu. For the first two hours after xylose administration, feeding was withheld, but later infants received milk and fluid ad libitum to ensure adequate urine volume. The urine passed during these five hours was separately collected. After a small aliquot, approximately 2cc. was taken to estimate xylose, the remainder was mixed with the rest of the urine collected for Figlu estimation. Xylose did not seem to interfere with the estimation of Figlu. The test was performed on 22 infants, 15 infants in the prospective study in the age range 9 - 16 months, and 7 in the additional study group, age range $3\frac{1}{4}$ - 7 months.

7. Folic acid studies

A standard solution of folic acid for intramuscular injection ("Folvite", Lederle) was used for the folic acid absorption and clearance tests.

(i) Folic acid absorption tests (12 infants)

The procedure was modified slightly from that of Chanarin, Anderson and Mollin (1958). L.casei was used as the assay organism. In the week following their admission for

24 hour urine collection for Figlu estimation, the infants received 15 mg. of folic acid intramuscularly on two successive days at the infant welfare clinics. After an interval of 36 - 48 hours following the last dose, and a week after the original Figlu test, they attended the hospital as out-patients for the absorption test. The mothers were instructed to give a glucose feed at 5 a.m. on the morning of the test and afterwards only small glucose feeds when necessary during the four hour period before the commencement of the test at 9 a.m. At the start of the test a blood sample was taken for serum L.casei folate estimation. The infants received 40 µg./kg. body weight of folic acid given orally in water. Further blood samples were taken for folate estimation at one and two hours after the administration of folic acid.

(ii) Folic acid clearance tests (Chanarin, Mollin and Anderson, 1958a).

L.casei was used as the assay organism in all the tests (21). In nine of these tests the sera were also assayed with S.faecalis through the help of Dr. Metz.

The infants attended as out-patients one week after the urine collection for Figlu, during which time blood samples had not been taken. A solution of folic acid containing approximately 100 µg./ml. was prepared in the following way: 18 mg. of "Folvite" (1.2cc.) were injected into a bottle containing 180 ml. of sterile half normal saline for intravenous administration (Baxter) and well mixed. Blood was taken for serum folate and other haematological investigations and followed by an injection of folic acid (15 µg./kg.) through the same needle if possible. Further blood samples were taken 3, 15 and 30 minutes after the injection for measurement of serum folate. In the absence of

TABLE 4

SUMMARY OF THERAPEUTIC TRIALS

Aim of Trial	Composition of Trial Group	Therapeutic Method	Therapeutic assessment
(i) The effect of large doses of folic acid on Figlu excretion. (ii) The effect in infants with low serum folate levels and morphological changes in the bone marrow.	(a) Nine infants excreting Figlu in excess after 2nd visit. Two of these had low serum folate levels. (b) Three infants excreting Figlu in excess at the time of 4th visit. Two of these had low serum folate levels and morphological changes in the bone marrow were present in one. (c) Four African infants excreting Figlu in excess. **	Dose: 1½ mg. of oral folic acid daily, or 5 mg. b.d. if infants had gastro-enteritis. Duration: 7 - 14 days Re-examination: 21 - 28 days after therapy commenced.	Subjective*: Effect on appetite: activity: development: resistance to infection. Objective: (a) Clinical examination: Weight gain Development (b) Laboratory assessment: Haemoglobin levels Figlu tests Serum folate levels Percentage hypersegmentation× Serum iron levels× Bone marrow examination×
The effect of intramuscular iron on Figlu excretion.	Six infants from additional study group	Dose: Imferon given according to weight and haemoglobin level. Re-examination: After 14 days	
The effect of small and physiological doses of folic acid on Figlu excretion.	Four infants still excreting Figlu in excess after the above therapeutic trial with iron	Dose: 50 - 300 µg. of oral folic acid daily. Duration: 21 days Re-examination: 21 - 28 days after therapy commenced.	
The effect of oral folic acid and iron compared with that of iron alone on Figlu excretion in 46 infants	Forty six infants	Dose: Oral folic acid 1 mg. daily; oral iron according to weight and haemoglobin level. Duration: 21 days Re-examination: 21 days after therapy commenced.	

* Subjective criteria established either by voluntary statements or on direct questioning.

× If relevant.

** This also included 3 infants excreting borderline, and 1 infant excreting normal, quantities of Figlu.

visible external jugular veins, capillary or scalp vein samples were taken. Only folate free capillary tubes or disposable scalp vein sets were used for this purpose.

8. Therapeutic trials.

These were conducted with both small and pharmacological doses of folic acid. The success of the therapy was assessed by questioning the mothers and observing the effect on serum folate levels. Subjective improvement was regarded as definite if the mother said that there had been an improvement and described its nature convincingly. Clinical and laboratory criteria were used to assess objective improvement. (Table 4).

The advancing age of the infants during the first therapeutic trial and the small number available in the second trial on younger African infants, meant these trials had to be repeated before the effect of folic acid on Figlu excretion could be interpreted. The details of the therapeutic trials undertaken are summarised in Table 4. The symptomatic benefit of folic acid therapy was also assessed in all infants who were given folic acid at the end of the investigation. In the controlled therapeutic trial on 46 infants, the two therapeutic schedules were prescribed alternatively.

CASE MATERIAL AND COMMENT

Mothers.

During pregnancy the women were cared for by the obstetricians. Most of them received prophylactic iron and some vitamin B_{12}. One received folic acid and in her case the mother/infant folate relationship was not considered.

Haematological and nutritional values were analysed in the 86 mothers who brought their infants for investigation (Table 5).

TABLE 5.

MATERNAL STUDIES

Investigation	Numbers investigated	Mean	Range	Standard deviation	Standard error of mean	Numbers with abnormal results
Haematocrit vols. %	86	35.0	28.0 - 41.0	± 2.6	± 0.3	$\frac{28 - 29}{3}$ $\frac{30 - 35}{38}$
Serum albumin g.%	84	3.2	2.6 - 4.0	± 0.3	± 0.03	43
Serum iron µg./100 ml.	76	124.9	47.4 - 209.0	± 39.0	± 4.5	3
Vitamin B_{12} levels pg./ml.	82	437.0	158.0 - 1526.0	± 220.4	± 24.5	-

Thirty-eight out of the 86 were mildly anaemic (haematocrit 30-35 volumes %), and three others were moderately anaemic (28-29 volumes %). These criteria for assessing anaemia in pregnancy were used by Benjamin, Bassen and Meyer (1966). Haematocrit rather than haemoglobin concentration was used to determine anaemia as some of the haemoglobin estimations were made when the colorimeter readings were too high.

About half the mothers were hypoalbuminaemic. This finding was described in normal pregnant women (Report of a W.H.O. Expert Committee, 1965).

Only three women had low serum iron levels but the incidence might have been higher if the women had been asked to discontinue their medication a few days before the blood taking. Vitamin B_{12} deficiency was not encountered and vitamin B_{12} administration almost certainly explained the high level sometimes noted.

Infants

One infant was excluded from the study because of cerebral birth trauma. The 86 infants studied were healthy though they suffered from the disadvantages associated with their socio-economic conditions. Thus nutrition was variable and infection not uncommonly encountered. In addition there was a fair incidence of associated deficiency disease like iron deficiency and rickets. Parasitic infestation was also encountered.

1. Infant nutrition

The pattern of infant feeding in the group was fairly standard, but many infants were exclusively or partly breast fed. After $7\frac{1}{2}$ months there was a marked decline in breast feeding. From $4\frac{1}{2}$ - $7\frac{1}{2}$ months most infants received cereal or mixed feeding and from $7\frac{1}{2}$ months most infants

TABLE 6.

PATTERN OF INFANT FEEDING

(a) Milk feeding

Age (months)	Total numbers of infants	Milk feeding				
		Breast	Breast with supplementary bottle feeding	Breast and bottle feeding	Bottle feeding with supplementary breast feeding	Bottle feeding
2¼ - 4½	119*	23	10	62	13	11
4½ - 7½	106	6	-	33	-	66
7½ - 10¾	47x	1	-	8	-	38
10¾ - 16	39	-	-	5	-	34

(b) Supplementary feeding

Age (months)	Total numbers of infants	Supplementary feeding						
		Cereal	Mixed** feeding	Meat	Orange juice - vitamin C	Cod liver oil	Vitaminxx syrups	Patent medicines
2¼ - 4½	119*	44	24	3	15	46	17	19
4½ - 7½	105*	94	90	19	51	44	19	35
7½ - 10¾	47x	47	47	28	40	15	8	14
10¾ - 16	39	39	39	33	39	8	?	10

* Dietary schedule not available in 2 infants in the 2¼ - 4½ months and 1 infant in the 4½ - 7½ months age groups.

x Includes 9 infants given therapeutic trial.

** This means vegetables, eggs and fruit. None of the infants received leafy vegetables.

xx None of these contains folic acid. Some of the vitamin syrups contain vitamin B_{12}.

NUTRITIONAL PATTERN in the 4 AGE GROUPS

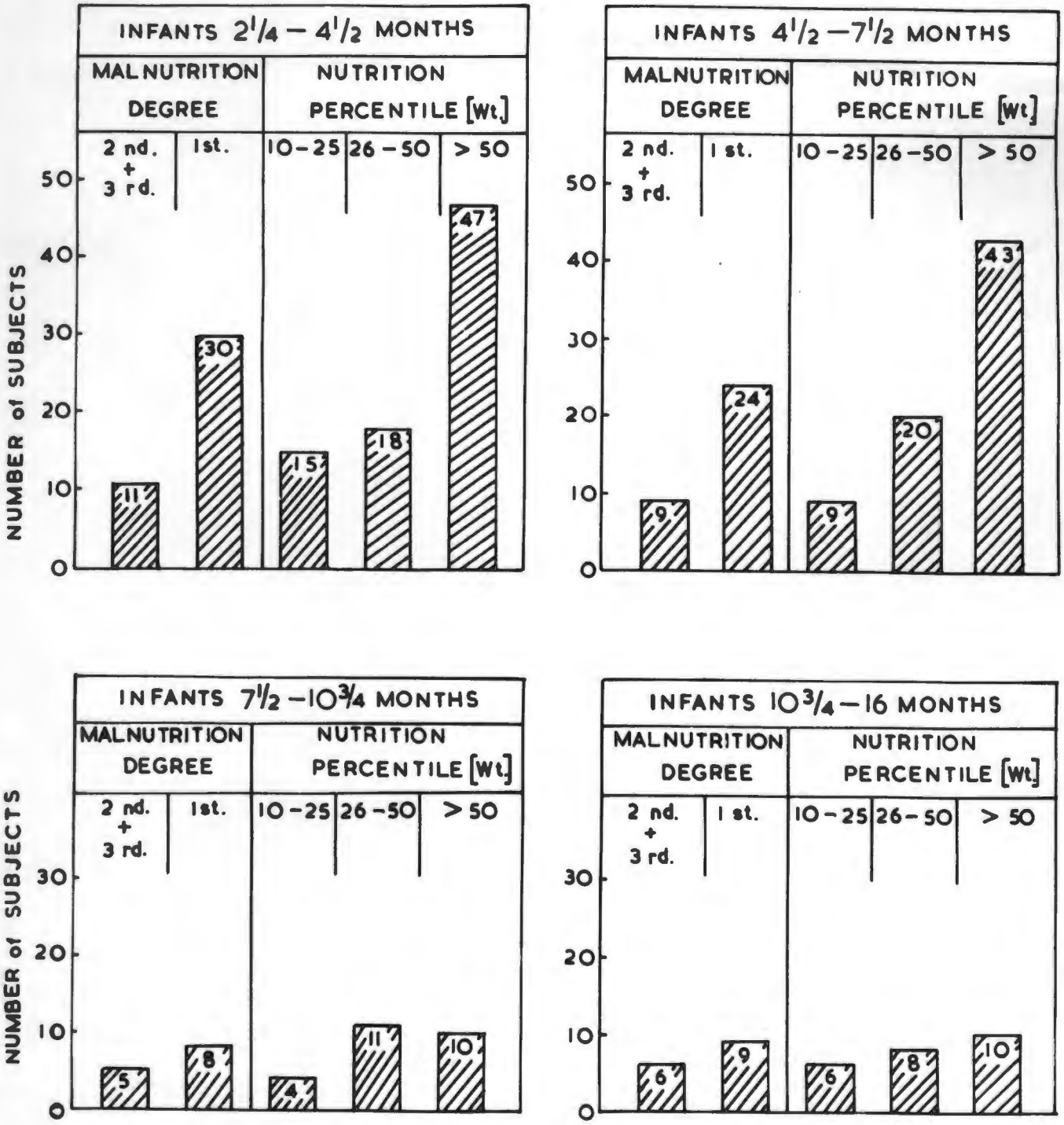
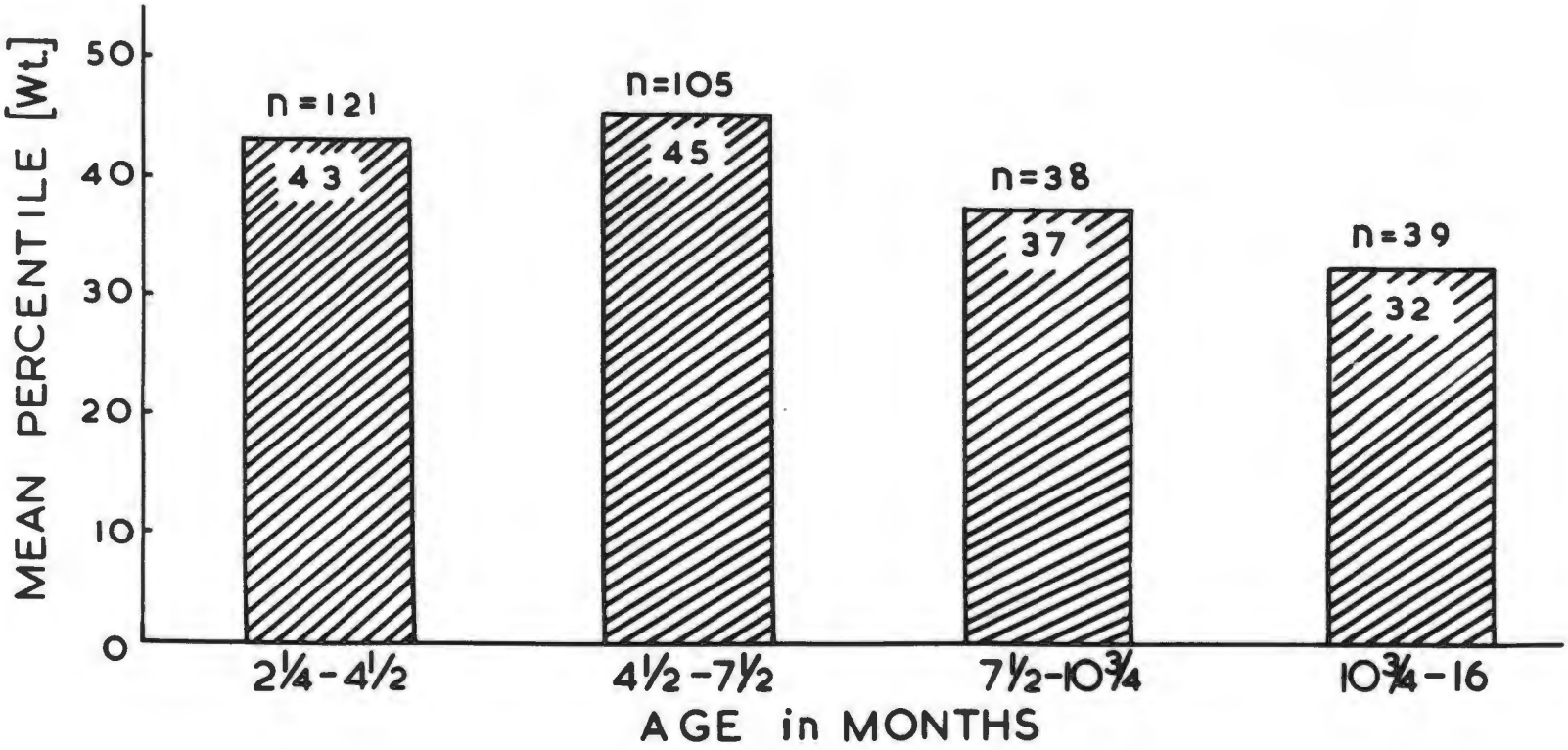


Fig. 6

MEAN WEIGHT PERCENTILE in the 4 AGE GROUPS



RANGE	2-100	2-97	2-95	2-95
S.D.	±34.0	±34.0	±30.0	±31.0
S.E. of MEAN	±3.0	±3.0	±5.0	±5.0

Fig. 7

TABLE 7.

INCIDENCE OF LOW SERUM ALBUMIN LEVELS IN INFANTS

Age (months)	Numbers of infants investigated	Numbers of infants with serum albumin levels < 3.3 g.%
2¼ - 4½	92	2
4½ - 7½	65	-
7½ - 10¾	28	-
10¾ - 16	36	3

INCIDENCE OF RICKETS : CLINICAL EXAMINATION

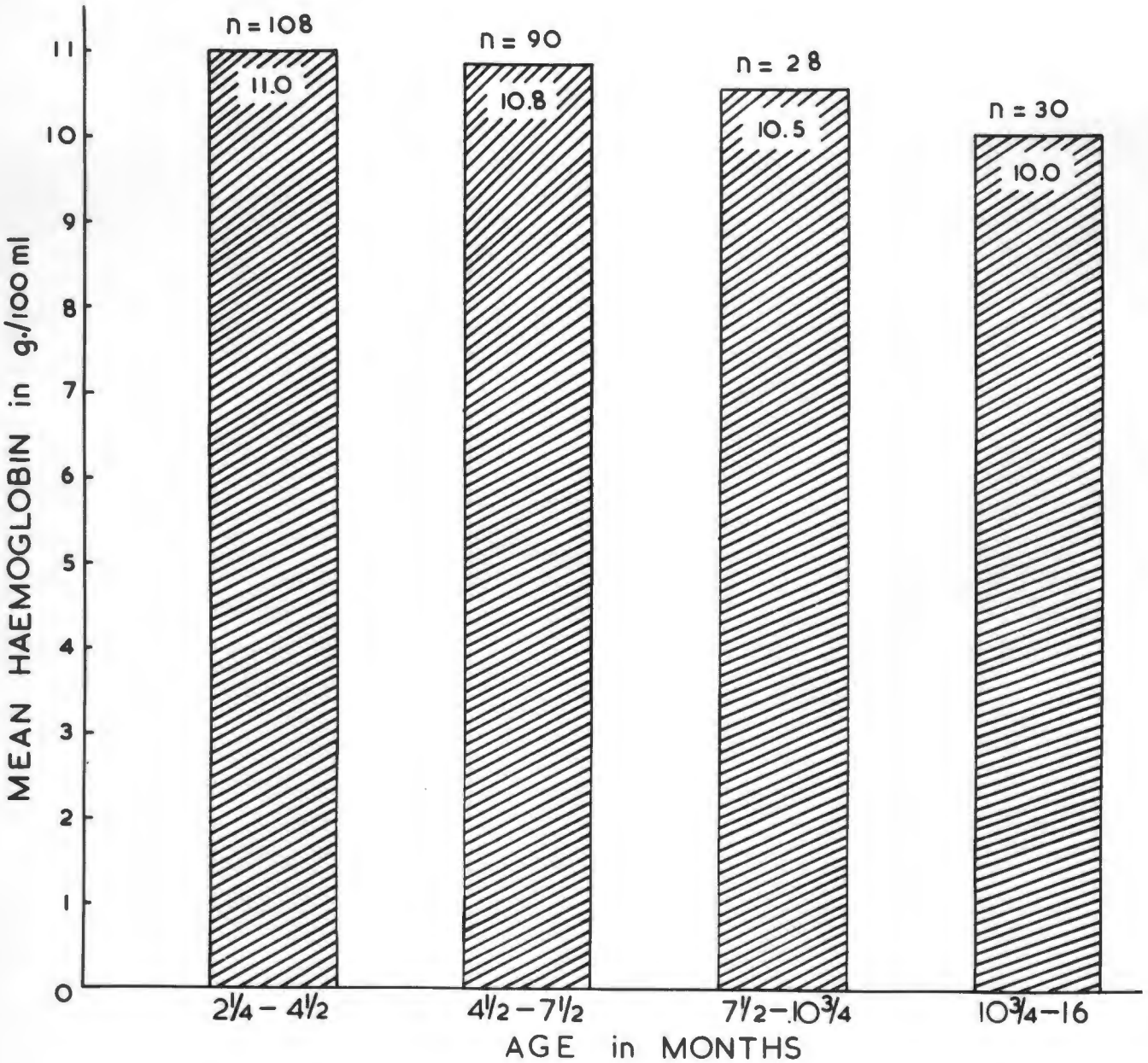
Age (months)	Studies	Numbers of infants	Incidence of rickets	Total numbers	Total incidence of rickets
2¼ - 4½	Prospective	84	12	121	19
	Additional	37	7		
4½ - 7½	Prospective	70*	7	105	15
	Additional	35	8		
7½ - 10¾	Prospective	38	2	38	2
10¾ - 16	Prospective	38*	1	38	1

* Information not available in one.

Conclusion: Prospective study -

Twenty two infants showed clinical evidence of rickets at one or other visit during the 16 months. Fifteen out of 72 infants examined on one occasion in the additional studies showed clinical evidence of rickets.

MEAN HAEMOGLOBIN LEVELS in the 4 AGE GROUPS



RANGE	8.4–13.1	7.7–13.7	7.5–13.3	7.5–12.3
S.D.	± 1.0	± 1.2	± 1.4	± 1.3
S.E. of MEAN	± 0.1	± 0.1	± 0.3	± 0.2

Fig. 8

TABLE 10.

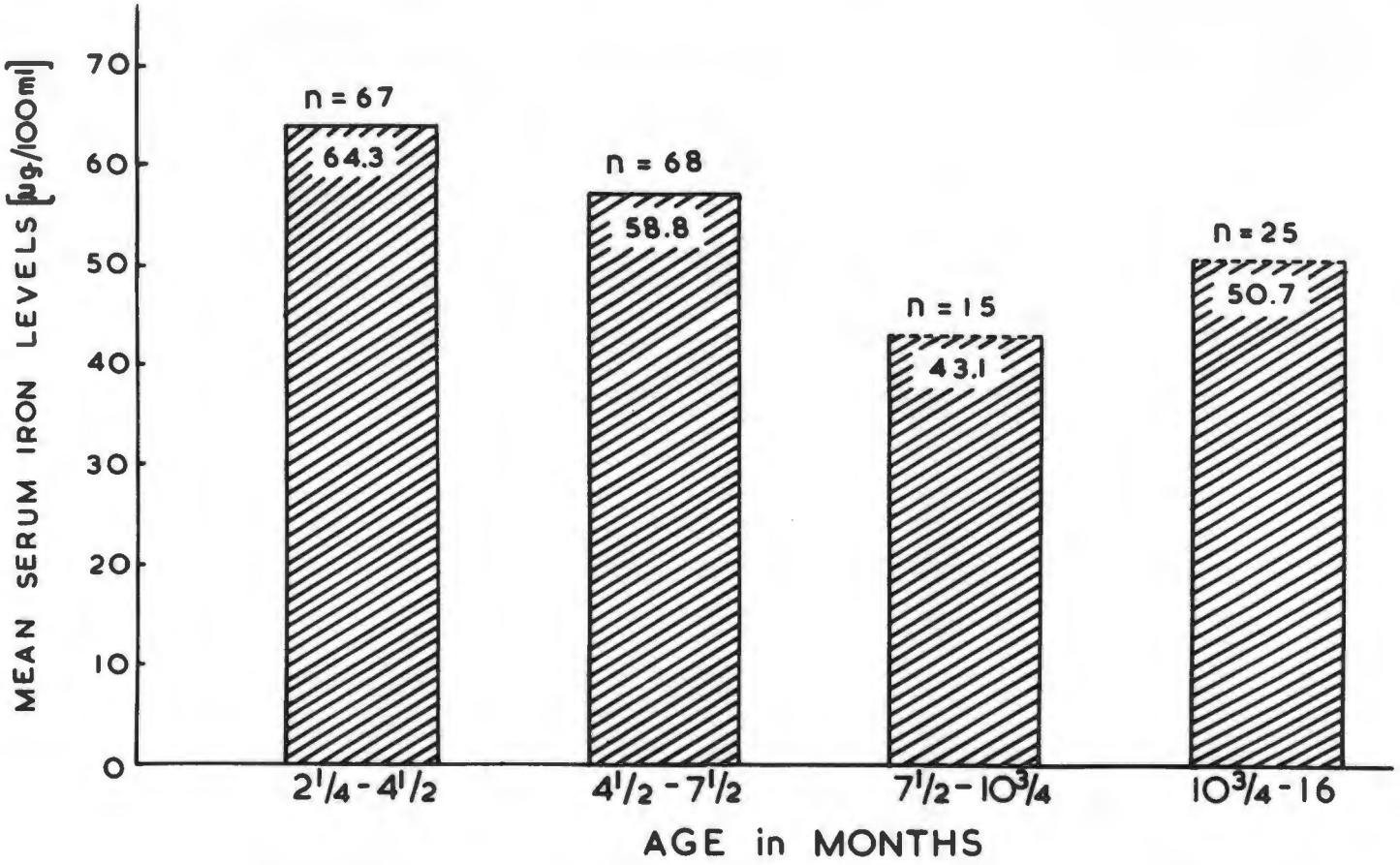
INCIDENCE OF ANAEMIA AND LOW SERUM IRON LEVELS IN THE FIRST 16 MONTHS OF LIFE

Investigation	Age (months)	Number of investigations	Incidence of abnormal results
Haemoglobin g./100 ml.	2¼ - 4½	108	60 (56%)
	4½ - 7½	90	61 (68%)
(Normal>11.1 g./100 ml.)	7½ - 10¾	28	20 (71%)
	10¾ - 16	30	25 (83%)
Serum iron levels	2¼ - 4½	67	39 (58%)
	4½ - 7½	68	45 (66%)
(Normal:	7½ - 10¾	15	14 (93%)
>64.9 µg./100 ml.)	10¾ - 16	25	22 (88%)

M.C.H.C. VALUES IN THE FIRST 16 MONTHS OF LIFE

Age (months)	Mean	Range	Standard deviation	Standard error of mean
2¼ - 4½	33 n = 108	25 - 36	± 1.7	± 0.2
4½ - 7½	32 n = 90	26 - 36	± 1.8	± 0.2
7½ - 10¾	31 n = 28	28 - 34	± 1.7	± 0.3
10¾ - 16	30 n = 30	27 - 32	± 1.8	± 0.3

MEAN SERUM IRON LEVELS in the 4 AGE GROUPS



RANGE	21.0–131.0	16.0–210.0	27.4–84.2	27.8 – 79.8
S.D.	±23.3	±31.3	±14.0	±14.4
S.E. of MEAN	± 2.9	± 3.8	± 3.7	± 2.9

------- SELECTION in SAMPLE

Fig. 9

3. Iron deficiency.

Mean haemoglobin concentration (Fig.8), mean corpuscular haemoglobin concentration (MCHC)(Table 10), and serum iron levels steadily decreased with age (Fig.9). At $10\frac{3}{4}$ - 16 months, approximately 80% of infants were anaemic and about the same proportion had low serum iron levels (Table 10).

Both in the maternal and in the infant studies, there was seldom enough blood to make estimations of iron binding capacity possible. Where percentage saturation data was available it did not negate the diagnosis of iron deficiency based on serum iron levels alone.

4. Rickets

Twenty-five percent (25%) of infants (22 out of 86) in the prospective study had unequivocal clinical criteria for diagnosing rickets at some stage in the 16 months. In the additional studies where the infants were seen only once, the incidence was approximately 20% (15 out of 72; Table 7).

5. Infestation

The incidence of parasitism was in the region of 18% in the prospective study, but this incidence might well have been higher had stools been examined more frequently (Table 8).

ANALYSIS OF THE DATA

Definition of criteria.

1. Socio-economic circumstances.

By South African socio-economic standards all the infants came from underprivileged homes. However, within the group of infants studied, there were gradations of socio-economic circumstances. These were

TABLE 8

INCIDENCE OF PARASITIC INFESTATION IN INFANTS

Investigation	Numbers		Infestation			Numbers of stools examined (range)
	Investigated	With infestation	Ascaris lumbricoides	Giardia lamblia	Trichuris trichiura	
Prospective	36	7*	6	3	1	1 - 8
Additional	5	0	0	0	0	1 - 2

* Some infants were infested with more than one parasite.

INFECTION AT TIME OF INVESTIGATION

Age (months)	Numbers investigated	Numbers with infection	Percentage with infection
$2\frac{1}{4}$ - $4\frac{1}{2}$	119*	55	46
$4\frac{1}{2}$ - $7\frac{1}{2}$	105*	43	41
$7\frac{1}{2}$ - 10	38	16	42
10 - 16	39	20	51

* Information not available in 2 infants - $2\frac{1}{4}$ - $4\frac{1}{2}$ months
1 " $4\frac{1}{2}$ - $7\frac{1}{2}$ "

TABLE 9

INFANTS WITH SIGNIFICANT INFECTION AT THE TIME OF INVESTIGATION

Infant	Age (months)	Nature of infection	Organism isolated	Pyrexia
M.R.	6	Meningitis and septicaemia	N. Meningitides	101.4°F
	13	Dysentery	Shigella flexneri III	apyrexial
N.v.W.	6	Bronchopneumonia	-	100°F
F.U.	2.8	Chronic gastro-enteritis	-	apyrexial
C.F.	2.8	Chronic gastro-enteritis	Salmonella C_2O	apyrexial
T.de B.	6	Chronic gastro-enteritis	Salmonella *	98.8°F
W.S.	3.3	Acute severe gastroenteritis	Salmonella B.O.	apyrexial

* not identified any further.

received meat. Some infants were given cod liver oil, vitamin preparations and patent medicines. The accuracy of the data concerning supplementary feeding was uncertain (Table 6).

Throughout the study some two-thirds of the infants belonged to a better nourished group, their weight percentile being more than the 9th percentile (Fig. 6). Mean weight percentile decreased with increasing age (Fig. 7). Moodie, Wittmann, Truswell and Hansen (1965) showed that in underprivileged infants the percentage of underweight infants increased with growth. This could be related to increasingly insufficient milk feeding. This might be the explanation of the decrease in mean weight percentile noted with advancing age in this study.

Although about a third of the infants were undernourished, hypoalbuminaemia was seldom found (Table 7). This was compatible with the findings of Wittmann, Moodie, Fellingham and Hansen (1967) who showed that weight for age could be a more sensitive criterion of nutritional development than serum albumin levels.

2. Infection.

Approximately half the infants were investigated at a time they had infection most of which was trivial. A small proportion were pyrexial at the time, but the temperature never exceeded 100.8°F.(Table 8). Six (6) infants were investigated at a time they had mild or moderate infections. The temperature here did not exceed 101.4°F. (Table 9). All grades of infection were treated in infants during interim periods. Of these the most notable were three cases of Salmonella gastroenteritis, one case of herpes stomatitis with local spread, specific E.coli (0127) diarrhoea, measles and pertussis. One infant had to have a tracheotomy for measles complicated by stridor and bronchopneumonia.

defined by the hospital social worker (D.B.) who also classified 27 groups of parents in the prospective study according to their socio-economic circumstances. The criteria used were as follows: all infants from good socio-economic circumstances were in the care of their mothers. The average income was R66.44 per month (£38.0.0d.) The entire income was available for the maintenance of the family and there was no period of unemployment.

In the group from average socio-economic circumstances the monthly income was approximately R54.52 (£31.8.0d.) but only R36.52 (£21.3.0d.) was available for the maintenance of the family. Infant care was undertaken by the mother or some other responsible person. There was no history of present unemployment, though this was a factor in the past.

The approximate income in those from poor socio-economic circumstances was R16.40 per month (£9.6.0d.) Infants were seldom cared for by the mother. Unemployment and the use of earnings for purposes besides the maintenance of the family were common.

Although not used as a criterion for classification, the size of the family was usually bigger as the income was lower.

2. Nutrition.

All infants weighing less than the Boston 10th percentile were taken to be suffering from first degree malnutrition. Second degree malnutrition (60 - 75% of the Boston 50th percentile) and third degree malnutrition ($< 60\%$ of the Boston 50th percentile) were defined according to recommendations made by Gomez, Galvan, Cravioto and Frenk (1955).

In the analysis of the data it was sometimes necessary to use weight percentile as a number rather than as a grade of nutrition. Percentiles had to be allotted arbitrarily in two circumstances. When weight for age fell between two percentile standards, the nearest exact percentile was estimated. All infants with a weight for age ratio less than the third percentile were taken to be on the 2nd percentile.

3. Infection.

The effect of infection was assessed according to an infection score. Whenever possible, this was determined on the basis of both history and examination, but sometimes it had to be allotted on the basis of history alone. A doubtful or mild infection was designated a half, moderate infection, one, and severe infection, two. For the purposes of this study diarrhoea was regarded as infective in origin, even when proof was lacking, because usually it was part of an infective episode or else a short and transient phenomenon associated with mild fever.

4. Age.

When used in the analysis of the data, age was expressed in months and weeks, the latter calculated to a single decimal point. Thus, for example, 4 months and 1 week was expressed as 4.3 months.

DIFFICULTIES

Subjects for study.

After three months attendance at infant welfare clinics, parents' consent was obtained for the investigation of only four white infants to determine normal values. As these four infants did not come from good socio-economic circumstances, the value of the findings as criteria of normality were doubtful. In the same period consent was obtained

for the investigation of eight African infants. Further studies of these groups were not practical.

About 10% of all Coloured parents agreed to the investigation. Though the search for subjects was time consuming, the study was a practical possibility in this group.

Procedures.

1. Serum folate assays.

Certain factors might have affected the reliability of some of the results obtained.

There were late notifications of impending delivery resulting in delay in obtaining cord blood samples. It was also found that mothers sometimes fed their infants during the 2 - 3 hour fasting period before the taking of blood for serum folate assays. When this was discovered, venepunctures were delayed for two to three hours, but it was not known whether some infants were fed without the knowledge of the investigator. To overcome this problem, venepunctures were no longer performed at the commencement of the period of hospitalisation but next morning after a strictly controlled period of fasting. The change was made when half of the first visits in the prospective study had been completed. Other factors that might have affected the reliability of some of the serum folate assays were storage of sera without ascorbic acid, administration of antibiotics, blood sampling from capillary rather than venous sites and haemolysis. Thus the effect of all these factors had to be investigated before the results of all serum folate assays could be accepted for analysis.

2. The Figlu test after oral histidine.

The histidine was first given in three divided doses eight hours apart, but later at 4-hourly intervals as a result of information received from Dr Luhby (1963). The change was made when half the first visits in the prospective study were completed. It was found that the administration of histidine in one dose as against three made no difference to the results (Chanarin, 1964). So the departure from suggested technique in a small percentage of tests was not taken to be important.

In view of the unpleasant taste of the histidine and the necessity to give it repeatedly to infants in the prospective study, it was administered through a stomach tube. In the additional studies it was usually given in orange juice with a teaspoon and only given through a stomach tube if the infant refused to take it. As administration through a gastric tube was more efficient, this might have accounted for the slightly higher incidence of positive tests in the prospective study.

3. Bone marrow examination.

A temporary inability to produce an effective enzyme (Tabor and Wyngarden, 1958) necessitated the use of Figlu results from the first visit as a guide to the need for bone marrow examinations at visit two in the prospective study. For this reason bone marrow examinations were not always performed on infants with the highest Figlu excretion.

4. Folic acid clearance test.

There were technical problems involved in the frequent sampling of blood from the infants and in the complete administration of the folic acid dose. The problems of blood sampling could be overcome to some extent by using capillary and scalp veins. The problem of administering the dose was more difficult to approach.

The infant had to be sufficiently co-operative to allow first the withdrawal of blood for basal haematological investigations, then the injection of folic acid through the same needle, and then the alternate injection and withdrawal of a couple of ml. of blood to ensure that folic acid did not remain in the syringe or needle. If the infant was not co-operative during this rather lengthy process, an unknown quantity of folic acid was sometimes injected into the tissues and the test has to be excluded on this account.

In the next series of tests, the procedure first adopted (Procedure I) was changed (Procedure II). In order to reduce the length of the process, a straight injection of folic acid was given, and any folic acid that might have remained in the needle or syringe was ignored. The results of Procedure II differed from those of Procedure I so that it seemed that the amount left in the needle and syringe might have been critical. In future it seems important to give the folic acid as a straight injection but to make allowance in the initial calculation for the amount likely to be left in the needle and syringe.

Analysis of the data.

1. Erratic attendance.

A large proportion of the parents did not keep their appointments. The necessity for frequent and sometimes repeated re-booking created problems of bed space. Hence Figlu tests excluded for technical reasons could seldom be repeated, and it became necessary to carry out investigations in the presence of trivial infection. It also resulted in the necessity to divide the age groups for analysis in the following way: $2\frac{1}{4}$ - $4\frac{1}{2}$ months, $4\frac{1}{2}$ - $7\frac{1}{2}$, $7\frac{1}{2}$ - $10\frac{3}{4}$, and $10\frac{3}{4}$ - 16 months, to allow all first, second and third and fourth visits to be grouped together.

TABLE 11.

INVESTIGATIONS AVAILABLE FOR ANALYSIS.

(a) Infants.

Investigations	Group of infants investigated	Age Group	Number of completed investigations	Exclusions	Reasons	Investigations available for analysis **
Figlu test after oral histidine	Prospective study and additional studies *	2¼ - 4½	121***	7	(a) vomiting of histidine load	114 (77 + 37)
		4½ - 7½	106***	8		98 (65 + 33)
		7½ -10¾	38	3	(b) leakage of urine above 15% of total urine output	35
		10¾ -16	39	3		36
	Controlled therapeutic study	2¾ - 5.0	46	1	Vomiting of histidine load	45
		3½ - 5¾ (after **** therapy)	46	1	Excessive excretion of urocanic acid	45
	Folic acid clearance study	3¾ - 5¾	7	0	-	7
Figlu test without oral histidine	Additional studies	3.0 - 6.5	25	1	Enzyme insufficient	24

TABLE 11 (Continued).

Investigations	Group of infants investigated	Age Group	Number of completed investigations	Exclusions	Reasons	Investigations available for analysis
Urocanic acid excretion	Prospective study	2¼ - 4½	77	-	-	77
		4½ - 7½	65	-	-	65
		7½ - 10¾	35	-	-	35
		10¾ - 16	36	-	-	36
	Controlled therapeutic study	2¾ - 5.0	46	1	Vomiting of histidine load	45
		3½ - 5¾	46	0	-	46
	Folic acid clearance study	3¾ - 5¾	7	0	-	7
Serum L. casei folate assay	Prospective study and additional studies	2¼ - 4½	108	2	Specimens not stored immediately at -20°C.	106 (71 + 35)
		4½ - 7½	92	1		91 (56 + 35)
		7½ - 10¾	28	-		28
		10¾ - 16	32	-	Identification uncertain	32
	Controlled therapeutic study	2¾ - 5.0	32	0	-	32
Red cell L. casei folate assays	Additional studies	2¼ - 4½	27	-	-	27
		4½ - 7½	28	-	-	28
	Controlled therapeutic study	2¾ - 5.0	34	-	-	34

TABLE 11 (Continued)

Investigations	Group of infants investigated	Age Group	Number of completed investigations	Exclusions	Reasmns	Investigations available for analysis
Folic acid absorption test	Additional studies	¾ - 6½ øøø	12	5	Discussed in Chapter V	7
Folic acid clearance test	Additional studies	3.0 - 6.0 øøø	14	6	Folic acid lost during injection	8
	Folic acid clearance study	¾ - 5¾	7	3		4
Milk folatæ levels	Additional studies	2½ - 7.0 øøø	31	-	-	31
	Controlled therapeutic study	2¾ - 5.0	39	-	-	39
Hypersegmentation of the neutrophils	Prospective study	2¼ - 4½	84	19	Films technically unsatisfactory	65
		4½ - 7½	71	19		52
		7½ - 10¾	38	6		32
		10¾ - 16	39	4		35
Bone marrow examinations	Prospective study	2¾ - 16.0 øøø	29	-	-	29
Reticulocyte Counts	Additional studies	2¼ - 4½	23	-	-	23
		4½ - 7½	22	-	-	22
Serum iron levels	Prospective and additional studies	2¼ - 4½	71	4	(a) infants received iron therapy	67(46 + 21)
		4½ - 7½	68	0		68(41 + 27)
		7½ - 10¾	15	0	(b) Identity could not be established	15
		10¾ - 16	25	0		25
	Controlled therapeutic study	2¾ - 5.0	20	0	-	20

TABLE 11 (Continued)

Investigations	Group of infants investigated	Age Group	Number of completed investigations	Exclusions	Reasons	Investigations available for analysis
Haemoglobin estimations	Prospective and additional studies	2¼ - 4½	109	1	Infants received iron therapy	108 (78 + 30)
		4½ - 7½	91	1		90 (60 + 30)
		7½ - 10¾	29	1		28
		10¾ - 16	30	0		30
	Controlled therapeutic study	2¾ - 5.0	34	-	-	34
Serum vitamin B_{12} levels	Prospective and additional studies	2¼ - 4½	107	-	-	107 (71 + 36)
		4½ - 7½	84	-	-	84 (51 + 33)
		7½ - 10¾	35	-	-	35
		10¾ - 16	34	-	-	34
	Controlled therapeutic study	2¾ - 5.0	29	-	-	29
Serum protein levels	Prospective and additional studies	2¼ - 4½	92	-	-	92 (67 + 25)
		4½ - 7½	65	-	-	65 (32 + 23)
		7½ -10¾	28	-	-	28
		10¾ -16	36	-	-	36
S.G.O.T. levels	Additional studies	3.0-14.5ØØØ	15	-	-	15
Blood urea estimations	Additional studies	3.0-14.5ØØØ	15	-	-	15
Xylose tests	Additional Studies	3¼ - 16.0ØØØ	22	-	-	22

Table 11 (contd.)

Investigations	Group of infants investigated	Age Group	Number of completed investi-gations.	Exclusions	Reasons	Investigations available for analysis
Occult blood tests	Additional studies	2½ - 7½	20	-	-	20
Creatinine tests	Controlled therapeutic study	2¾ - 5.0	26	-	-	26
		3½ - 5¾	26			26

(b) Maternal Studies

Investigations	Number of completed investigations	Exclusions	Reasons	Investigations available for analysis
Figlu test after oral histidine	84	0	-	84
Serum L.casei folate assay	85	1	Received folic acid therapy	84
Hypersegmentation of the neutrophils	86	1	Film technically unsatisfactory	85
Serum iron estimations	76	-	-	76
Serum vitamin B_{12} levels	82	-	-	82
Serum protein levels	84	-	-	84
Haematocrit levels	86	-	-	86

TABLE 11 (Contd.)

Investigations	Number of completed investigations	Exclusions	Reasons	Investigations available for analysis.
Cord serum L.casei folate levels	69	-	-	69

INCOMPLETE INFORMATION.

Information	Age (months)	Study	Number	
Diet	2¼ - 4½	Additional	2	
	4½ - 7½	Prospective	1 (Incomplete only in regard to supplementary feeding)	
Weight Percentile	4½ - 7½	Prospective	1	
Infection øøøøø	2¼ - 4½	Prospective	2	
	4½ - 7½	Prospective	1	
Rickets	4½ - 7½	Prospective	1	
	10 - 16	Prospective	1	
Urine Volume	2¼ - 4½	Prospective	2)	9
		Additional	7)	
	4½ - 7½	Prospective	1)	6
		Additional	5)	
	7½ - 10¾	Prospective	3	
	10¾ - 16	Prospective	4	

Table 11 (contd.)

* Additional studies do not include controlled therapeutic study and folic acid clearance study for the purposes of this table. The actual numbers of infants attending in the combined prospective and additional studies were 121 at $2\frac{1}{4}$ - $4\frac{1}{2}$ months; 106 at $4\frac{1}{2}$ - $7\frac{1}{2}$ months; 38 at $7\frac{1}{2}$ - $10\frac{3}{4}$ months; 39 at $10\frac{3}{4}$ - 16 months. In the prospective study the numbers were 84; 71; 38; 39; respectively.

** On each occasion when data were analysed the numbers involved depended on the completeness of relevant information and investigations.

*** Five infants were included in these groups when positive and negative Figlu tests were analysed. They were not included when quantitative Figlu excretion was analysed because these results were based on a single reading of a single assay (Enzyme insufficient).

**** Investigations available after therapeutic trials are only listed here if relevant. All available relevant data following therapy were analysed and are presented elsewhere.

ø Although in this age-group one infant excreted urocanic acid in the absence of excessive Figlu excretion, this was not excluded from the analysis. The test was repeated when the infant was still in this age-group and when urocanic acid excretion was no longer excessive. This result was used in the relevant analyses.

øø The results of this study were only used to determine the relationship of the folic acid clearance test (L.casei and S.faecalis) to Figlu and urocanic acid excretion.

øøø These are the actual age ranges of the infants studied but the infants were part of the prospective and additional studies. The age ranges of these were stated above. (cf. Figlu test after oral histidine, column 3).

øøøø The numbers in brackets denote the numbers available from the prospective and additional studies respectively.

øøøøø When the effect of infection score is assessed the numbers compared depend on

(i) available information

(ii) available results

(iii) comparable periods of exposure to infection.

2. Incomplete information or investigation.

Tests sometimes had to be excluded for technical reasons and investigations were sometimes incomplete because the blood could not be obtained,or not obtained in sufficient amounts. Occasionally information was incomplete. In each situation all available information has been analysed. The exclusions made and the reasons for these are detailed in the Table (Table 11).

3. Selection in the sample.

Mean results and incidence of positive results studied in the age group $7\frac{1}{2}$ - $10\frac{3}{4}$ and $10\frac{3}{4}$ - 16 months might have been affected by the selection exercised by the investigator for reasons already mentioned. These will be remarked on, whenever relevant, in the presentation of the results.

4. Age.

To overcome the effect of physiological changes that might have occurred with advancing age during the period of investigation, data was separately analysed in the four age groups.

5. Associated deficiencies and diseases.

The effect of infection, iron deficiency, rickets and parasitic infestation had to be considered in the analysis of the data.

6. Methodology.

Two methods were used for measuring Figlu in infants. The data related to these methods were separately analysed. There was a delay in obtaining all the results from the last studies conducted on the 46 and the 7 infants. With the exception of a detailed analysis of the health of the infants in these studies, all the data will be presented, but statistical analysis could only be performed on the more important data.

Overall difficulties.

Difficulties with methods, the erratic attendance of the parents, and difficulties in obtaining subjects for study, proved very time consuming. This was complicated by the fact that attention could not be focused on the study alone. While in the pathology department considerable concessions were made, but later clinical responsibilities greatly interfered with the conduct of this study.

CHAPTER V

METHODS.

HAEMATOLOGICAL METHODS

TABLE 12.

THE REPRODUCIBILITY IN COUNTING SEGMENTATION OF NEUTROPHILS.

Smear	Reading 1	Reading 2	Difference	% difference*
1	10	5	5	67
2	5	3	2	50
3	4	3	1	29
4	4	3	1	29
5	4	3	1	29
6	4	5	1	23
7	11	6	5	59
8	0	5	5	200
9	5	5	0	0
10	15	5	10	100
11	6	4	2	40
12	5	4	1	22
13	4	3	1	29
14	7	2	5	111
15	8	8	0	0
16	2	4	2	67
17	13	6	7	74
18	8	3	5	91
19	6	4	2	40
20	5	2	3	86
21	4	3	1	29
22	5	3	2	50
23	6	0	6	200
24	10	5	5	67
25	6	3	3	67
26	7	5	2	33
27	4	2	2	67
28	8	8	0	0
29	7	7	0	0
30	10	1	9	164
31	4	0	4	200
32	5	4	1	22
33	6	4	2	40

Mean difference 3

Mean % difference 63

$$* \frac{\text{Reading 1 - Reading 2}}{\text{Mean of readings}} \times 100$$

CHAPTER V

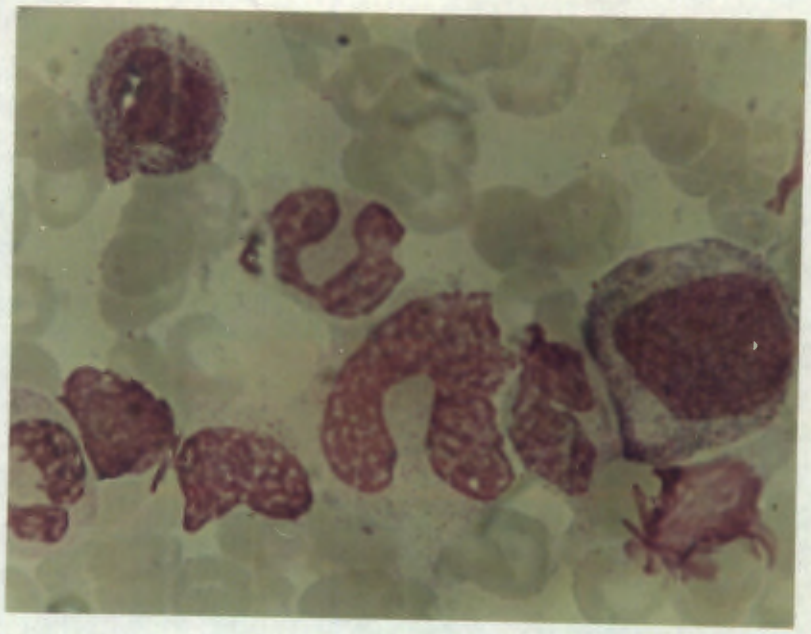
HAEMATOLOGICAL METHODS

Blood was collected into sequestrene tubes for the measurement of haemoglobin concentration, volume of packed red cells and percentage of reticulocytes. Haemoglobin was measured as oxyhaemoglobin in a Klett-Summerson photoelectric colorimeter. At regular intervals a commercial standard was used as a reference to ensure the reliability of the readings. The volume of packed red cells was measured, using a micro-haematocrit technique, and reticulocytes were counted by a dry method. Blood films were made from blood obtained by venepuncture before mixing it with sequestrene. They were stained with May-Grünwald-Giemsa's solution and then examined. Unequivocal macrocytosis was not seen in the films of infants and adults in this investigation.

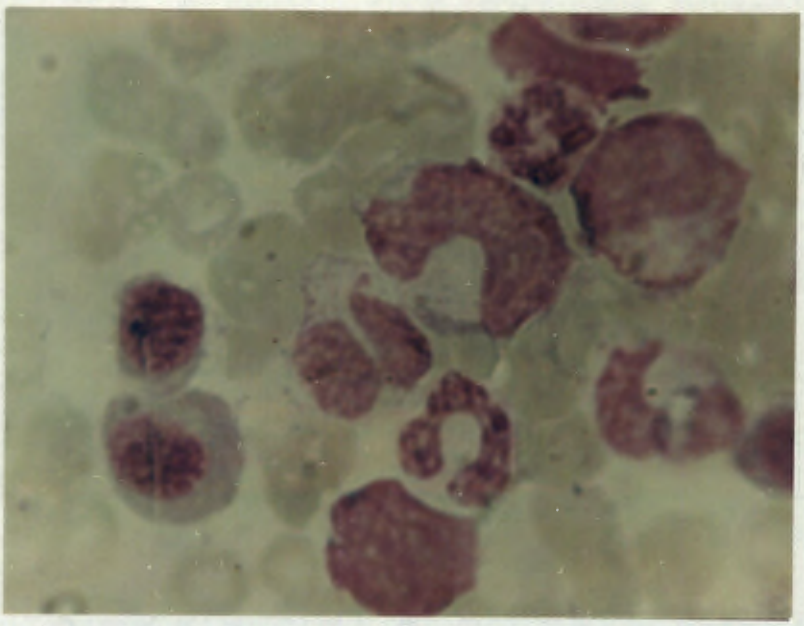
For these tests standard methods were used (Dacie, 1956; Dacie and Lewis, 1963) and normal values were taken from Wintrobe (1967).

PERCENTAGE HYPERSEGMENTATION OF THE NEUTROPHILS

The examination of the blood films included the determination of the percentage of neutrophils having 5 or more lobes to their nuclei. A 400-fold magnification was used but this was increased to a thousand-fold where there was uncertainty about nuclear separation of the neutrophils. Lobes that were clearly separate or connected by a definite chromatin bridge were counted individually. All smears were counted in duplicate. Those which showed evidence of hypersegmentation on one or both occasions were examined a third time because reproducibility between these repeat examinations was initially poor (Table 12). Hypersegmentation was present if more than 3% of neutrophils had five or more lobes (Herbert, 1959; Chanarin, Rothman and Berry, 1965).



[a]



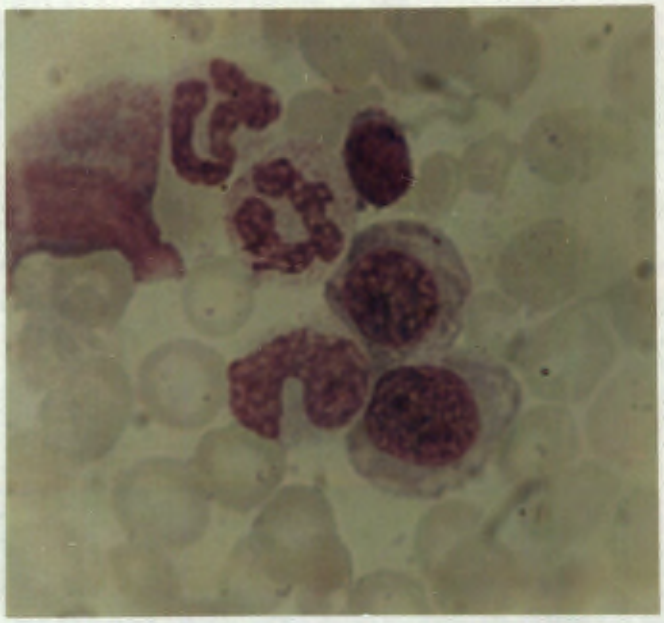
[b]

Plate 1:- Photomicrograph of the bone marrow of M.R. showing

(a) Giant stab cell;

(b) Giant metamyelocyte in association with early megaloblastic change in erythroid precursor.

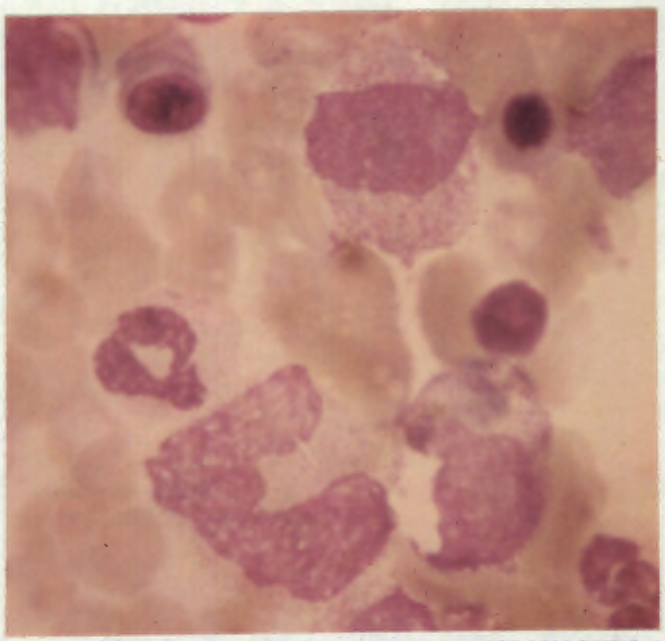
(Magnification x 800)



[a]

Plate 2:- Photomicrograph of the bone marrow of M.R. showing early megaloblastic changes in erythroid precursors as well as evidence of iron deficiency.

(Magnification x 800)



[b]

Plate 2 (continued):- Photomicrograph of the bone marrow of M.R. showing a giant metamyelocyte (Magnification X800)

Discussion.

The lobe average was an alternative method of assessing hypersegmentation (Herbert, 1962a). The use of this method necessitated the identification and enumeration of the number of lobes in a hundred neutrophils. The result was expressed as the mean lobe count per cell. Chanarin, Rothman and Berry (1965) preferred the counting of hypersegmented neutrophils to the identification and enumeration of nuclear lobes in a hundred neutrophils. This preference arose because it was sometimes difficult to distinguish between separate and folded nuclei. Percentage hypersegmentation appeared to be the more accurate method and was therefore used in this study. Edwin (1967) showed that percentage hypersegmentation was a more accurate parameter of nuclear segmentation than the lobe average but found that both these parameters were relatively inaccurate, especially if based on a single count. He introduced the use of a segmentation index which he believed was a better index of nuclear segmentation than lobe average or percentage hypersegmentation.

BONE MARROW EXAMINATIONS

Bone marrow films were stained with May-Grünwald-Giemsa's fluid and then examined. Particular attention was paid to the presence of megaloblastic changes in all elements (Zuelzer and Ogden, 1946). Three grades of megaloblastosis were recognised.

Grade 1: Megaloblastosis.

Approximately 5% of the myeloid series were giant forms and/or intermediate megaloblasts were present in convincing numbers. The photographs (Plates 1:2) are illustrations of the giant myeloid cells and early erythroid changes seen in one patient in this study.

Grade 2: Megaloblastosis.

This was characterised by the presence of both giant myeloid cells and intermediate megaloblasts in greater numbers than in Grade 1 as well as the presence of occasional true megaloblasts.

Grade 3: Megaloblastosis.

This was defined as florid megaloblastic change involving both red and white cell series.

Giant metamyelocytes were larger than 18 μ (Dawson and Bury,1961) and showed morphological characteristics described by Zuelzer and Ogden (1946).

The bone marrows were independently examined by two observers and a representative group by a third.

Discussion.

Zuelzer and Rutzky (1953) and MacIver and Back (1960), believed that the giant myeloid cells were pathognomonic of folate or vitamin B_{12} deficiency and that they were diagnostic of megaloblastosis in the absence of characteristic red cell changes. Other investigators reported these cells in the presence of iron deficiency (Foy and Kondi, 1952; Davidson, 1954; Tasker, 1959; Dawson and Bury, 1961; Hansen, 1967), chronic infection (Dawson and Bury, 1961), infection (Hansen, 1967) and neoplasia (Dawson and Bury, 1961). Although some investigators believed that the presence of giant myeloid cells in these conditions could be attributed to associated folate or vitamin B_{12} deficiency (Tasker, 1959; Dawson and Bury, 1961), this was uncertain.

In view of the iron deficiency and infection commonly encountered in this study, there was uncertainty until recently as to whether giant

myeloid cells alone could be used as evidence of megaloblastosis and, if so, in what numbers they had to be present for a valid morphological diagnosis. Contributions from other investigators were helpful.

It was reported that giant metamyelocytes were seldom found in great numbers when not the result of folate or vitamin B_{12} deficiency (Dawson and Bury, 1961). Later, Hansen (1967) reported that giant myeloid cells could only be used as evidence of folate or vitamin B_{12} deficiency if they constituted 5% or more of the myeloid series. This was recently confirmed by Hoffbrand and Newcombe (1967). They found low leucocyte folate levels in all patients whose bone marrows were normoblastic but contained more than 5% of giant metamyelocytes.

The criteria used for diagnosing megaloblastosis on the basis of giant myeloid cells were based on the findings of Hansen (1967) and Hoffbrand and Newcombe (1967).

BIOCHEMICAL METHODS FOR INVESTIGATING FOLATE DEFICIENCY

PRINCIPLE OF ENZYMATIC METHOD OF FIGLU ESTIMATION
[Tabor & Wyngarden, 1958]

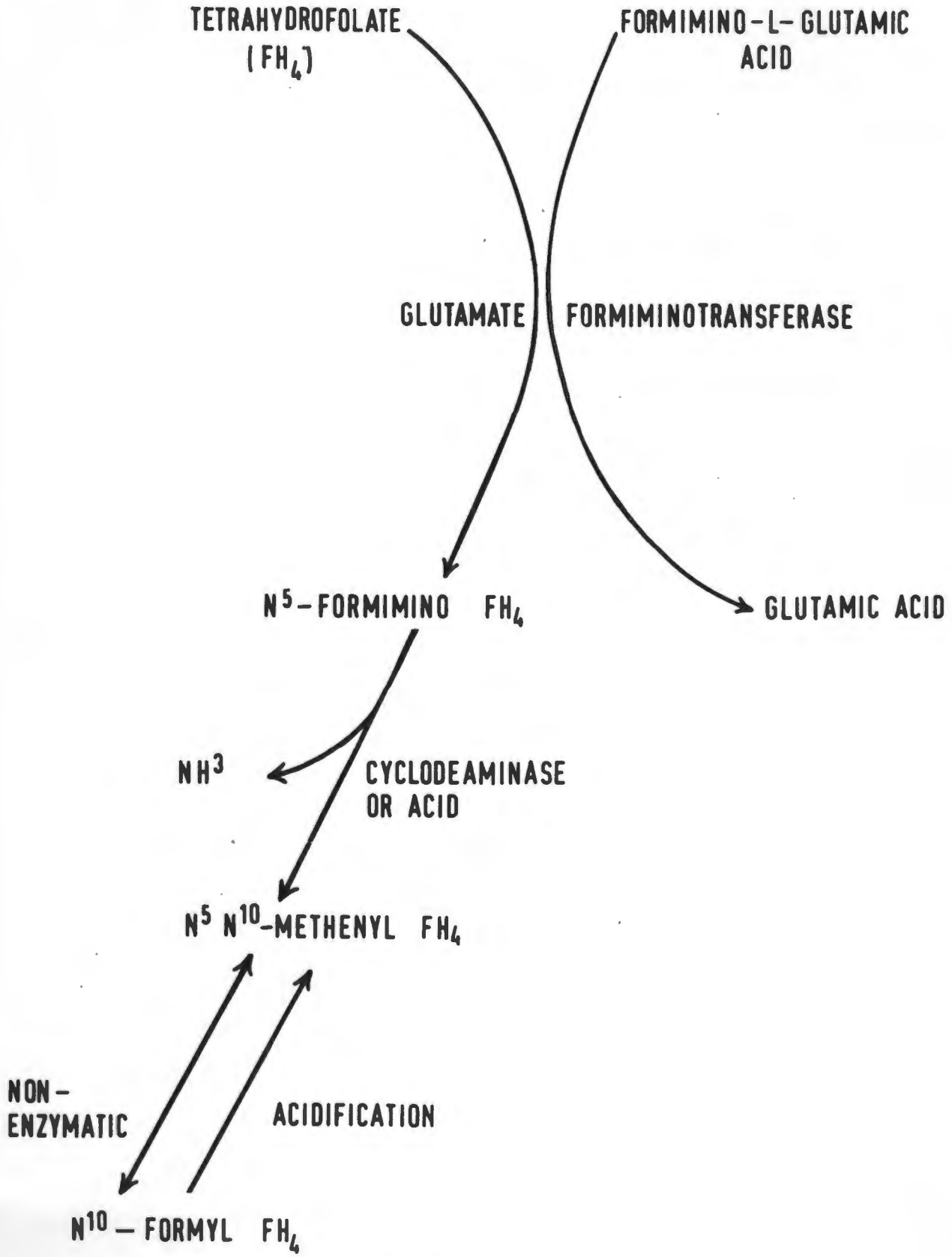


Fig. 10

BIOCHEMICAL METHODS FOR INVESTIGATING FOLATE DEFICIENCY

THE ESTIMATION OF FORMIMINO-L-GLUTAMIC ACID (FIGLU) IN URINE

Two enzymatic methods and one electrophoretic method were used to measure Figlu in urine.

Determination of Figlu in urine (Tabor and Wyngarden,1958; Luhby and Cooperman,1964).

Principle.

In the degradation of Figlu to glutamic acid, the formimino group is transferred to FH_4 by N-formimino-L-glutamate: tetrahydrofolate 5-formiminotransferase (glutamate formiminotransferase). The N^5-formimino FH_4 so formed is further changed to N^5,N^{10}-methenyl FH_4 by N^5-formimino-tetrahydrofolate cyclodeaminase (cyclodeaminase). N^5,N^{10}-methenyl FH_4 has a peak absorption at 350 mμ and the amount formed is directly proportional to the amount of Figlu in solution. During the enzymatic reaction, N^5,N^{10}-methenyl FH_4 is converted nonenzymatically to N^{10}-formyl FH_4. This is reconverted to N^5,N^{10}-methenyl FH_4 by acidification. The reactions employed in the enzymatic determination of Figlu are shown diagrammatically (Fig.10).

Preparation of the enzymes (glutamate formiminotransferase and cyclodeaminase; T-C enzyme).

Step A. Preparation of acetone powder.

Approximately 5 kg. of fresh hog liver were obtained from the abbatoirs on the morning of commencement of the procedure. The liver was chopped into ½" pieces and weighed into 50g. portions. Each portion was homogenised for 1 minute with 250 ml. of acetone (technical grade: National Chemical Products, South Africa) at room temperature in a Kelvinator homogeniser (± 3,000 r.p.m.). The homogenate was filtered through Green's Hyduro Filter Paper in a Büchner funnel and then dried by a fan at room temperature. The filter cake was rehomogenised with 250 ml. of acetone,

filtered and dried as before. The cake was powdered while moist and allowed to dry overnight at room temperature. The acetone powder was stored over a drying agent P_2O_5 in vacuo at -20°C.

Step B: Preparation of a particle free solution by ultracentrifugation.

Two hundred g. of acetone powder were gently stirred with 2,000 ml. of distilled water for 20 minutes at room temperature. The slurry was filtered through muslin and the residue discarded. The filtrate was centrifuged at 20,000 g. for 15 minutes and the supernatant solution collected. From this stage onwards all procedures were carried out at 0°C.

Step C: Ammonium sulphate fractionation.

Ammonium sulphate was added to the supernatant in small quantities with continuous stirring till a final concentration of 21.4% was obtained. The solution was left at 0-4°C. for 1 hour to allow precipitation. Thereafter it was centrifuged for 15 minutes at 20,000 g. The precipitate was collected and suspended in approximately 50 ml. of distilled water and then stored overnight.

The suspension was spun at 30,000 g. for 2 hours. The precipitate was collected and dissolved in 38 ml. of 0.2 M.sodium acetate by magnetic stirring for 2 hours. During this time the container and its contents were placed in an ice bath. The solution was cleared by low-speed centrifugation in a refrigerated centrifuge and the residue discarded. The enzyme was stored at -20°C.

Reagents.

1 M K_2HPO_4 (pH 7.18)

Figlu (California Corporation for Biochemical Research, Los Angeles, California).

Figlu solution in water (1 µmole/ml. of solution. Stable at 0°C. for up to 3 weeks).

FH_4 (Nutritional Biochemicals Corporation, Cleveland, Ohio).

TABLE 13.

TESTS, STANDARDS AND CONTROLS FOR ESTIMATING FIGLU.

(Tabor and Wyngarden, 1958).

Reagents	Urine (ml.)		Standard (ml.)		Enzyme (ml.)	
	Control	Sample+	Control	Sample+	Control	Sample+
Water *	0.70	0.45	0.70	0.45	0.80	0.55
Buffer	0.10	0.10	0.10	0.10	0.10	0.10
Urine°	0.10	0.10	-	-	-	-
Figlu	-	-	0.10*	0.10*	-	-
Enzyme	-	0.25	-	0.25	-	0.25
FH_4	0.10	0.10	0.10	0.10	0.10	0.10
Perchloric acid	0.30	0.30	0.30	0.30	0.30	0.30

* 3 standards were set up containing 0.1, 0.4, 0.8 micromoles of Figlu/ml.

+ All sample tubes set up in duplicate.

° Neutralized.

* Distilled de-ionized water.

FH_4 solution (4.4 mg./ml.). This was made up of 18 ml. of 1.1 M 2-mercaptoethanol, 0.53 ml. of FH_4, 0.47 ml. of H_2O and 1 ml. of 0.4 N KOH

KOH (0.4 N).

Perchloric acid - 10% w/v in water.

Assay Procedure.

Urine was incubated in phosphate buffer with a mixture of the enzyme and FH_4. The mixture was then acidified and the concentration of N^5,N^{10}-methenyl FH_4 calculated from the optical density at 350 mμ. The details were as follows:

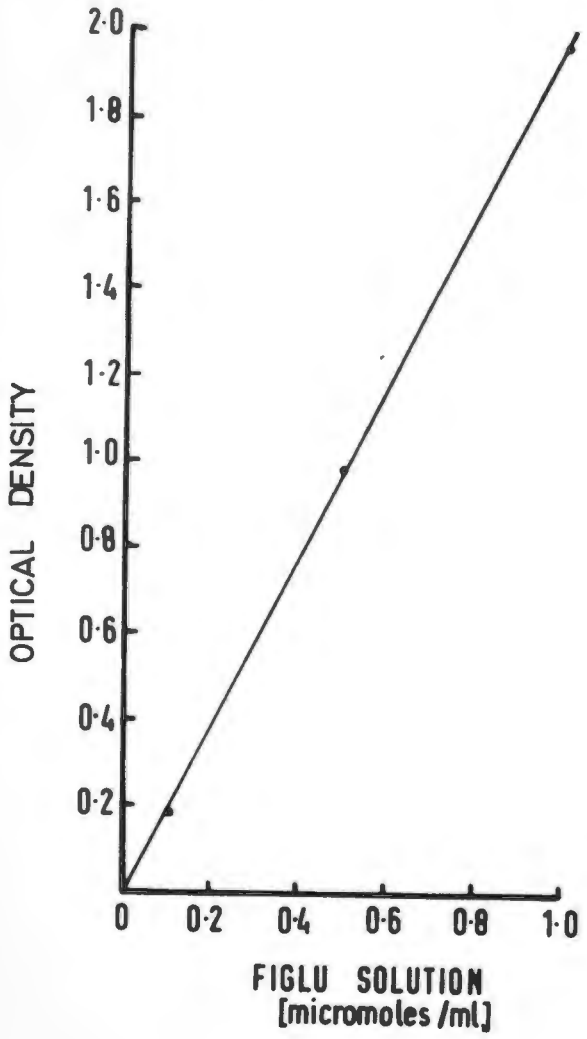
The solutions were pipetted into 10 x 75 mm. test tubes according to the details in Table 13. They were well mixed and then placed in the dark for ½ hour at 25°C. Perchloric acid 0.3 ml. of 10% solution was then added to each of the test tubes and the contents mixed again. The test tubes were re-covered with aluminium caps and placed in a boiling water bath for 1 minute. They were then rapidly cooled in iced water. After centrifugation, the protein free supernatant was decanted and the optical density of this read at 350 mμ in a Zeiss spectrophotometer in semimicro quartz cuvettes with a 1 cm. light path and a capacity of 1.5 ml. A tungsten light source was used.

The enzyme control mixture was used to set the zero of the instrument, and the optical density of the enzyme sample at 350 mμ was then determined. Likewise, the Figlu standard and test sample solutions were read against their control tubes. The optical density of the enzyme sample was subtracted from the readings of the Figlu standard and test samples respectively.

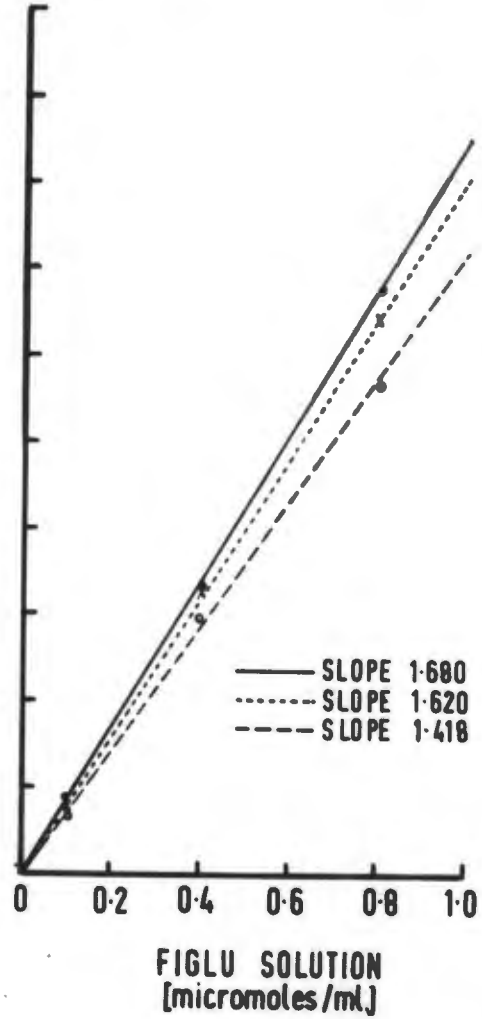
A standard curve was constructed by plotting optical density at 350 mμ. against Figlu concentration (micromoles/ml.). A linear relationship

DETERMINATION OF FIGLU [Tabor & Wyngarden, 1958]

REPRESENTATIVE CURVE



COMPARISON OF STANDARD CURVES USING ENZYMES OF DIFFERENT SLOPES



COMPARISON OF STANDARD CURVES USING THE SAME ENZYME BUT 2 FIGLU PREPARATIONS.

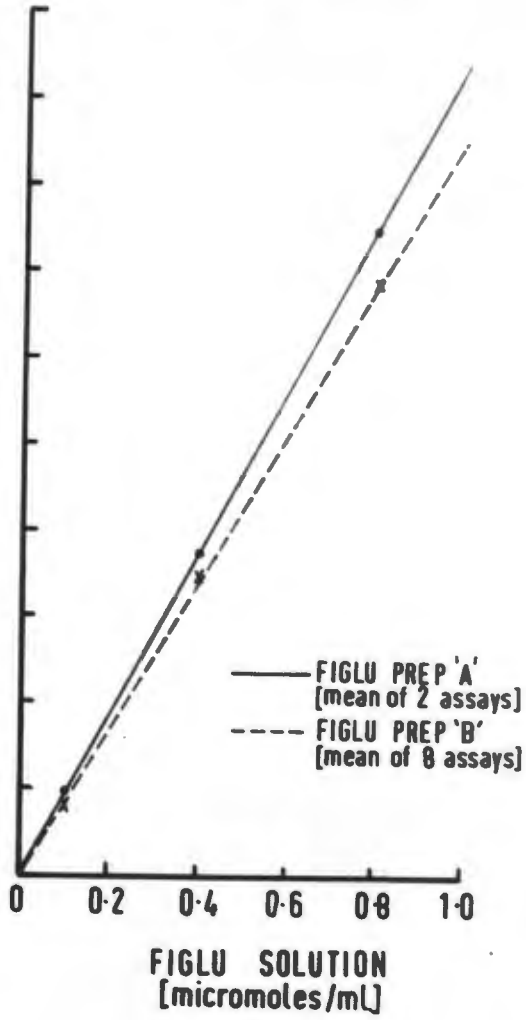


Fig. 11.

was obtained up to 1.0 µmoles/ml. (Fig.11). Since this gave optical density readings above 1.5, solutions were appropriately diluted if unknown readings were obtained above this figure. Results were expressed in µg. of Figlu per ml. of urine by converting µmoles to µg. and correcting for neutralisation and dilution of urine when necessary.

Discussion of the Method.

(i) Isolation and preparation of the enzymes.

Enzyme preparations were not always of sufficient activity for use in the test. Low activity of the final preparation was due to inactivation of the enzyme during the preparation of the acetone powders from hog liver (Step A). Various possible factors which could have affected the production of active enzyme during the acetone powder stage were investigated. It was found that the age of the pig made no difference. Only one breed of pig was used, and they all received the same stall feeding. Livers used were always fresh and activity was not improved by carrying out the entire procedure at 0°C. or by using frozen livers, or de-ionized distilled water instead of distilled water, or pure instead of technical grade acetone. There was an impression that the procedure was more successful in summer than in winter months, but this was not further investigated.

Comparatively large amounts of the enzyme were required to assay the urine. In view of the difficulties with consistent production of the enzyme, delay between repeat assays was at times inevitable. In particular, this affected the assays performed on infants who attended for the first and second assessments in the prospective study. Many of these repeat estimations were only performed after storage of the urine for 6 - 10 months. Figlu was stable in urine for at least 6 months at pH 2-3 if stored at 4°C. (Lovric and Kenrick, 1965) or for a year if stored at pH 2 or less (Luhby,

TABLE 14.

COMPARISON OF RESULTS OF ASSAYS FOR FIGLU CARRIED OUT ON 56 URINES USING ENZYMES OF HIGH AND LOWER ACTIVITY

Urine	Figlu µg./ml.	
	Standard curve slope (1.680 O.D.units/micromole/ml. of Figlu) solution.	Standard curve slope (1.620 and 1.418 O.D.units/micromole/ml. of Figlu) solution.
1	8.0	8.2
2	8.5	6.8
3	28.6	31.6
4	9.7	12.1
5	5.3	6.1
6	43.3	47.4
7	51.4	50.5
8	101.5	102.8
9	13.4	16.0
10	44.3	56.1
11	47.0	46.9
12	117.0	111.4
13	64.4	64.0
14	10.2	11.8
15	49.0	45.0
16	39.2	35.1
17	17.0	15.7
18	164.1	162.7
19	31.2	29.5
20	38.3	41.3
21	32.7	52.1
22	6.0	7.4
23	27.8	29.8
24	16.1	15.5
25	4.7	2.3
26	62.7	59.3
27	45.4	39.3
28	16.8	17.4
29	20.4	20.5
30	14.6	15.5
31	28.8	30.0

TABLE 14 (contd.)

Urine	Figlu µg./ml.	
	Standard curve slope (1.680 O.D.units/micromole/ml. of Figlu) solution.	Standard curve slope (1.620 and 1.418 O.D.units/ micromole/ml. of Figlu) solution.
32	20.5	20.9
33	88.2	68.7
34	73.5	74.8
35	59.9	60.1
36	16.7	16.4
37	22.0	18.3
38	35.5	32.4
39	56.8	59.5
40	18.4	18.8
41	16.6	18.7
42	34.0	33.7
43	48.0	48.8
44	11.9	10.3
45	4.2	2.5
46	11.2	10.9
47	42.8	41.3
48	13.1	9.5
49	55.0	55.8
50	5.1	2.5
51	30.3	29.9
52	12.5	7.6
53	4.3	5.4
54	27.6	32.7
55	19.1	20.1
56	67.9	75.4
Mean	35.0	35.1
S.D.	± 30.6	± 30.2
S.E. of mean	± 4.1	± 4.1

Difference of means $t = 0.018$

$P > 0.90$

Cooperman and Teller, 1959b). But at pH 5 or higher, urine could only be kept for short periods at -10° to 25°C. without loss of Figlu activity (Luhby, Cooperman and Teller, 1959b). Thus, if the urine pH was greater than approximately 7.5 after storage, repeat assays could not be considered. This meant that the results of 14 out of 77 urines at visit 1, 3 out of 65 at visit 2, and 1 out of 35 at visit 3 in the prospective study, were based on a single assay done in duplicate. In the additional studies, repeat duplicate estimations were performed on 67 out of 72 urines. Towards the end of the investigation the enzyme preparation was only sufficient to allow a single assay on a single sample in 5 urines. Figlu excretion was normal in these 5.

Difficulties in the production of active enzyme preparations resulted in the use of a crude liver enzyme preparation for quantitation of Figlu (Chanarin and Bennett, 1962a). There was no difficulty in the production of this crude liver enzyme, and greater amounts were produced much more easily.

(ii) Tests of enzyme function.

The standard curve showed a linear relationship between Figlu concentration and optical density up to 0.8 μmoles/ml. of Figlu solution (Fig. 11). The slope of the curve (1.680 optical density units for 1 μmole/ml. of Figlu solution) gave a conversion factor of 0.057 μmoles of Figlu per optical density unit. This was within the accepted range of 0.050-0.060 μmoles of Figlu per optical density unit (Luhby and Cooperman, (1964).

Enzyme preparations which yielded curves with slopes below this range (1.620 and 1.418 optical density units for 1 μmole/ml. of Figlu solution) and conversion factors greater than 0.060 μmoles of Figlu per optical density unit still gave comparable results on urines assayed (Fig.11; Table 14).

TABLE 15.

RECOVERY OF FIGLU ADDED TO URINE

Urines	Figlu in urine µg./ml.	Figlu added to urine µg.	Expected recovery µg./ml.	Observed recovery µg./ml.	Recovery %
1	54.5	17.4	71.9	68.7	95.6
2	1.4	17.4	18.8	19.3	102.7
3	5.0	17.4	22.4	26.8	119.6
4	17.0	17.4	34.4	31.3	91.0
5	8.7	17.4	26.1	26.7	102.2
6	36.4	69.6	106.0	99.5	93.9
7	1.0	69.6	70.6	80.8	114.4
8	12.1	139.2	151.3	132.9	87.8
9	0.3	139.2	139.5	141.6	101.5

Mean % recovery = 101
Range = 87.8 - 119.6
S.D. = ± 10.4%
S.E. of mean = ± 3.5%

TABLE 16.

REPRODUCIBILITY BETWEEN DUPLICATE ESTIMATIONS FOR FIGLU.*

Urine	Figlu µg./ml.		Difference	% difference
	Reading 1	Reading 2	µg./ml.	
1	68.1	69.2	1.1	1.6
2	55.6	57.4	1.8	3.2
3	61.3	65.0	3.7	5.9
4	65.0	62.1	2.9	4.6
5	25.8	28.3	2.5	9.2
6	28.3	31.6	3.3	11.0
7	109.0	105.9	3.1	2.9
8	115.8	111.1	4.7	4.1
9	113.2	115.8	2.6	2.3
10	67.1	67.3	0.2	0.3
11	55.5	53.5	2.0	3.7
12	60.8	62.1	1.3	2.1
13	62.9	64.5	1.6	2.5
14	28.1	25.7	2.4	0.9
15	30.4	29.9	0.5	1.7
16	108.0	107.8	0.2	0.2
17	29.2	29.8	0.6	2.0
18	27.9	27.9	0.0	0.0
19	37.9	34.9	3.0	8.2
20	38.6	38.4	0.2	0.5
21	15.4	13.0	2.4	16.9
22	12.1	13.1	1.0	7.9
23	22.1	21.1	1.0	4.6
24	23.7	20.9	2.8	12.6
25	33.4	42.0	8.6	22.8
26	32.8	33.4	0.6	1.8
27	26.2	27.0	0.8	3.0
28	26.1	26.7	0.6	2.3
29	37.0	35.7	1.3	3.6
30	37.2	35.8	1.4	3.8

Mean difference µg./ml. = 1.9
Mean % difference = 4.9

*(Tabor and Wyngarden, 1958.)

TABLE 17

REPRODUCIBILITY BETWEEN REPEAT ASSAYS FOR FIGLU ON 100 CONSECUTIVE URINES.

Urine	Figlu µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference *
		Figlu < 21 µg./ml.		
1	0.0	2.0	2.0	200.0
2	2.1	4.1	2.0	64.5
3	2.6	0.1	2.5	178.5
4	3.3	3.3	0.0	0.0
5	3.5	0.7	2.8	133.3
6	4.1	3.9	0.2	5.0
7	4.2	2.9	1.3	36.1
8	4.5	4.0	0.5	11.8
9	4.5	5.1	0.6	12.5
10	5.0	17.8	12.8	112.3
11	5.2	5.2	0.0	0.0
12	5.9	0.7	5.2	157.6
13	6.0	7.4	1.4	20.9
14	6.2	0.5	5.7	167.6
15	6.3	11.4	5.1	57.3
16	6.4	3.9	2.5	48.0
17	6.8	3.1	3.7	74.0
18	7.7	11.0	3.3	35.1
19	8.0	8.2	0.2	2.5
20	8.7	10.8	2.1	21.0
21	11.0	13.4	2.4	19.7
22	11.1	12.9	1.8	15.0
23	11.5	11.8	0.3	2.6
24	12.3	2.6	9.7	129.3
25	12.5	7.6	4.9	48.5
26	13.1	13.8	0.7	5.2
27	13.6	15.1	1.5	10.4
28	13.6	17.4	3.8	24.7
29	14.1	15.1	1.0	6.8
30	14.4	12.6	1.8	13.3

TABLE 17 (contd.)

Urine	Figlu µg./ml. Reading 1	Figlu µg./ml. Reading 2	Difference µg./ml.	% difference*
		Figlu < 21 µg./ml. (contd.)		
31	14.4	13.4	1.0	7.2
32	14.8	12.0	2.8	20.9
33	14.8	18.5	3.7	22.3
34	15.2	12.1	3.1	22.6
35	15.6	16.4	0.8	5.0
36	16.0	14.2	1.8	11.9
37	16.2	23.0	6.8	34.7
38	16.4	16.4	0.0	0.0
39	16.6	15.7	0.9	5.6
40	17.7	13.4	4.3	27.9
41	18.8	18.4	0.4	2.2
42	19.8	17.9	1.9	10.1
43	20.1	19.1	1.0	5.1
44	20.2	15.8	4.4	24.0
45	20.5	15.4	5.1	28.3
46	20.6	10.8	9.8	62.4
47	20.9	18.6	2.3	11.6
		Figlu 20-69 µg./ml.		
1	20.5	20.9	0.4	1.9
2	21.2	20.9	0.3	1.4
3	21.6	23.8	2.2	9.7
4	22.2	29.3	7.1	27.5
5	23.4	21.0	2.4	10.8
6	24.4	23.8	0.6	2.5
7	24.6	40.9	16.3	49.7
8	24.9	32.8	7.9	27.0
9	25.1	16.0	9.1	44.2
10	27.1	24.7	2.4	9.3
11	27.8	28.2	0.4	1.4
12	28.2	27.9	0.3	1.1
13	28.6	31.6	3.0	10.0
14	29.1	23.7	5.4	20.5

TABLE 17 (contd.)

Urine	Figlu µg./ml. Reading 1	Figlu µg./ml. Reading 2	Difference µg./ml.	% difference*
		Figlu 20-69 µg./ml. (contd.)		
15	29.8	35.0	5.2	16.0
16	31.8	34.7	2.9	9.0
17	33.8	34.1	0.3	0.9
18	34.1	24.1	10.0	34.4
19	34.2	26.1	8.1	26.7
20	35.2	25.8	9.4	30.8
21	36.6	31.3	5.3	15.6
22	37.2	40.7	3.5	9.0
23	38.4	41.3	2.9	7.3
24	39.9	37.8	2.1	5.4
25	41.4	43.7	2.3	5.4
26	41.4	62.9	21.5	41.2
27	41.9	40.7	1.2	2.9
28	43.1	41.2	1.9	4.5
29	47.0	46.9	0.1	0.2
30	48.6	38.9	9.7	22.1
31	53.2	37.1	16.1	35.6
32	55.0	69.1	14.1	22.7
33	55.3	67.3	12.0	19.6
34	58.1	56.2	1.9	3.3
35	59.9	60.6	0.7	1.2
36	62.5	56.6	5.9	9.9
37	63.1	63.6	0.5	7.9
38	63.5	61.4	2.1	3.4
39	64.6	63.1	1.5	2.3
40	66.2	43.0	23.2	42.5
41	66.7	62.1	4.6	7.2
42	68.7	67.2	1.5	2.2
		Figlu 62.0-128 µg./ml.		
1	69.3	62.9	6.4	9.7
2	71.9	84.2	12.3	15.7
3	73.4	67.9	5.5	7.8

TABLE 17 (contd.)

Urine	Figlu µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference*
	Figlu 62.0-128 µg./ml.(contd.)			
4	84.0	69.6	14.4	18.8
5	84.6	84.6	0.0	0.0
6	88.2	68.7	19.5	24.8
7	101.5	102.8	1.3	1.3
8	107.5	107.9	0.4	0.4
9	108.8	125.2	16.4	14.0
10	123.6	123.2	0.4	0.3
11	127.7	101.4	26.3	22.9

Overall reproducibility between repeat assays of 100 urines.

Mean difference µg./ml. = 4.6
Mean % difference = 26.4

Figlu < 21 µg./ml.

Mean difference µg./ml. = 2.8
Mean % difference = 40.7

Figlu 20-69.0 µg./ml.

Mean difference µg./ml. = 5.4
Mean % difference = 14.4

Figlu 62.0-128 µg./ml.

Mean difference µg./ml. = 9.4
Mean % difference = 10.5

* $\%\ \text{difference} = \frac{\text{Reading 1 - Reading 2}}{\text{Mean of readings}} \times 100$

Method: Tabor and Wyngarden, 1958.

(iii) Accuracy of the method.

The mean percentage recovery was 101.0% and the range 87.8-119.6% (Table 15).

The final result of studies conducted to determine reproducibility will be expressed as mean difference as well as mean percentage difference ($\frac{R1 - R2}{\text{mean of } R} \times 100$). This seemed necessary because the second is not only a measure of reproducibility but also of the concentration of the test substance.

In duplicate assays the mean percentage difference was 4.9 and the mean difference was 1.9 µg./ml. (Table 16).

In repeat assays for Figlu the mean percentage difference and the mean difference (Table 17) were as follows:

Classification		Mean % difference	Mean difference µg./ml.
100 consecutive urine samples		26.4	4.6
Figlu concentration	< 21 µg./ml.	40.7	2.8
Figlu concentration	20-69 µg./ml.	14.4	5.4
Figlu concentration	62-128 µg./ml.	10.5	9.4

Normal values.

Normal infants could not be investigated in this study. Since the experimental method used here was standardised by Luhby (1963) and Luhby and Cooperman (1964), it seemed reasonable to use their criterion of normality. This was Figlu excretion in excess of 30 µg./ml. or 35 mg. per 24 hours. They found this criterion applicable to both infants and adults. This was puzzling because the 24 hour urine volume of infants is a fraction of that of adults. It was possible to surmise that an infant excreting

35 mg. per 24 hours, would have to excrete levels higher than 30 µg./ml. to compensate for the smaller urine output. For the same reason, a result which was positive in terms of concentration (30 µg./ml.) could be normal in terms of total Figlu output (mg. per 24 hours). In reply to this question, Dr. Cooperman (1967) stated that they found the use of concentration of Figlu in urine (µg./ml.) entirely satisfactory as a criterion of biochemical folate deficiency in infancy, provided their recommendations regarding histidine administration and urine collection were followed. In their experience only infants with folate deficiency excreted more than 30 µg./ml. of Figlu. Their study included the investigation of infants of less than six months. They preferred the use of Figlu concentration (µg./ml.) to that of total Figlu output (mg. per 24 hours) as a criterion of normality because complete urine collections were difficult in infants. In the case of infants excreting extraordinarily large urine volumes, Dr. Cooperman suggested that the result should then be expressed as total Figlu output. He suspected that the abnormal value would then exceed 35 mg. per 24 hours. In the light of their experience 30 µg./ml. was used as the criterion of normality in this study.

When the data were analysed, it was clear that a considerable proportion of infants excreted Figlu in excess of 30 µg./ml. These results did not correlate with those using other parameters of folate deficiency. It therefore seemed desirable to compare the results of the assays performed here with those of Dr. Cooperman to ensure that the criterion of normality was not being invalidated by possible differences in assay techniques. Thus 5 urines previously assayed here were also assayed by Dr. Cooperman in New York.

TABLE 18.

COMPARISON OF THE RESULTS OF FIGLU ASSAYS PERFORMED IN NEW YORK AND CAPE TOWN.

Urine	New York µg./ml.	Cape Town µg./ml.
S.C.	11.3	15.4
C.C.	21.5	38.3
A.C.	53.3	71.4
A.P.	80.0	146.2 *
M.L.	84.1	123.7

* The validity of this result was in doubt since the standard graph was lower than those previously obtained and could not be checked as no further T-C enzyme was available. With the usual standard graph, result would have been 122.7.

The comparison of the results showed that the readings in New York were approximately 30 - 40% less than those in Cape Town (Table 18). There were several possibilities which could have explained the difference, though the reason was not finally ascertained. The urines were lost in transit and not refrigerated for a period of 2 weeks before being assayed by Dr. Cooperman. This might have resulted in some fall off in Figlu concentration. The values for optical density at 350 mμ. obtained by the investigator, either fell within the accepted range given by Luhby and Cooperman (1964) or within a range shown to give comparable results. Therefore the standardisation of the method did not appear to be the responsible factor. It was found that the standard curves were different when two different Figlu preparations were assayed by the same enzyme preparation (Fig.11). However, both fell within the accepted range. Using Figlu preparation A the slope was 1.870 optical density units for 1 μmole/ml. of Figlu solution (conversion factor 0.050 μmoles of Figlu per optical density unit). With Figlu preparation B. the slope was 1.710 optical density units per μmole per ml. of Figlu solution (conversion factor 0.056 μmoles of Figlu per optical density unit). These differences were not great enough to be solely responsible for the discrepancy between the results of assays in New York and Cape Town. Dr. Cooperman (1967) noted that the actual Figlu concentration in commercial preparations was often less than claimed.

In view of a possible difference in methodology accounting for the higher results in Cape Town, the upper limit of normal was arbitrarily increased by a third to 45 μg./ml. It was thought that this bias against positive results would compensate for any possible difference in assay

PRINCIPLE OF ENZYMATIC ASSAY OF FIGLU
[Chanarin & Bennett, 1962a]

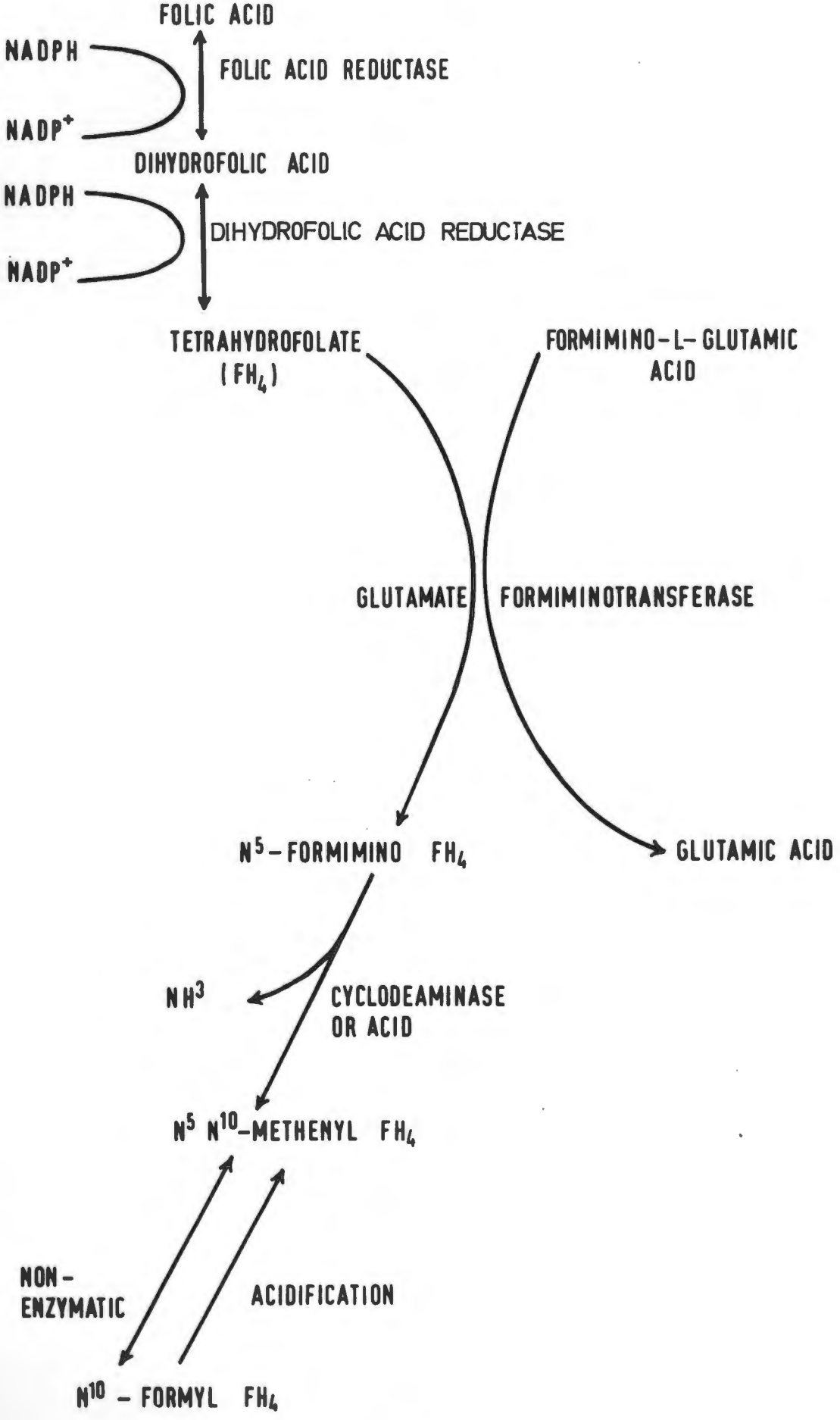


Fig. 12.

technique. The criteria used for determining positive, borderline and negative results in this study were as follows:

Classification	Figlu excretion
Positive results	>44.9 µg./ml.
Borderline results	30-44.9 µg./ml.
Negative results	<30 µg./ml.

Application.

All urines in the prospective study and the additional studies except those from 46 infants participating in the controlled therapeutic study and 7 infants on whom folic acid clearance tests were performed, were assayed using the method of Tabor and Wyngarden (1958). This method was also used to measure Figlu in the urine of infants not given a histidine load. The normal value for Figlu excretion without oral histidine was <10 µg./ml. (Luhby and Cooperman, 1964).

Determination of Figlu in urine (Chanarin and Bennett, 1962a).

Principle: This method differs from the previous one in that a crude rather than a purified enzyme is used. This crude liver preparation contains the enzymes necessary to convert folic acid to tetrahydrofolate as well as to transfer the formimino group from Figlu to tetrahydrofolate. The N^5-formimino FH_4 thus formed is converted to N^5,N^{10}-methenyl FH_4 as before (Fig.12).

Reagents.

A substrate buffer solution which contained

a) KH_2PO_4, 20m-moles (2.72 g.).
b) Citric acid ($C_3H_4(OH)(COOH)_3.H_2O$, 2m-moles (0.42 g.).
c) $MgSO_4.7H_2O$, 2m-moles (0.49 g.).
d) TPN (NADP) 12 µmoles (0.01 g.).
e) Folic acid 55 µmoles (0.024 g.).

The solution was adjusted to pH 6 with KOH (0.1N). The volume was made up to 100 ml. with water and the solution stored at -20°C. Only distilled de-ionised water was used for this solution and for the assay procedure.

Stock Figlu solution (152 µg./ml; pH 6). The test was standardised with 0.4 ml. dilutions of the stock standard Figlu solution.

Stock folinic acid solution (272.5 µg./ml. at pH 7).

Crude liver enzyme preparation.

Potassium phosphate buffer (0.1 M; pH 6).

Perchloric acid (10% w/v in water).

Preparation of the crude liver enzyme.

Approximately 2 kg. of fresh chicken liver was immediately frozen at -20°C. Next day the liver was sliced with a scalpel into 2-mm. portions. Eighty (80) ml. of 0.1 M potassium phosphate buffer were added to each 50 ml. of packed liver portions. The mixture was homogenised for 15 seconds in a Kelvinator homogeniser. The homogenate was centrifuged for 10 minutes at 30,000 g. and the sediment discarded. The supernatant was dialysed against distilled water at 0°C. The pH was adjusted to 6 with KOH (0.1 N) and the dialysate centrifuged at 30,000 g. for 10 minutes. Thereafter the supernatant was aspirated and stored at -20°C. The whole procedure was carried out at 0°C.

The assay procedure.

The original assay procedure (Chanarin and Bennett, 1962a) was slightly modified from the method of Tabor and Wyngarden (1958).

Liver and substrate solution were incubated at 37°C for two hours. Urine, water and standard Figlu solution were added to the appropriate

TABLE 19.

TEST URINES, STANDARDS AND CONTROLS FOR ESTIMATING FIGLU.

(Chanarin and Bennett, 1962(a)).

Reagents	Test urines		Standard	Enzyme	Distilled water
	Control(ml.°)	Sample(ml.°)	Sample(ml.°)	Control(ml.°)	Control(ml.°)
Crude liver enzyme	-	1.0	1.0	1.0	-
Substrate solution	-	2.0	2.0	2.0	-
Urine	0.4+	0.4+	-	-	-
Figlu standard	-	-	0.4*	-	-
Water	3.0	-	-	0.4	4.9
Perchloric acid	1.5	1.5	1.5	1.5	-

* Three standards of 38, 76 and 114 µg./ml. (i.e. 15.2, 30.4 and 45.6 µg./0.4 ml.) were set up in duplicate and included in each assay.

+ Dilutions of urine were made up to 0.4 ml., if the Figlu concentration in test urine exceeded the range covered by the standards.

° Set up in duplicate.

test tubes and mixed by inversion (Table 19). After incubation for a further 2 hours at 37°C., 10% perchloric acid was added. The tubes were re-covered with aluminium caps and the solutions thoroughly mixed. Thereafter the test tubes were placed in a boiling water bath for 1 minute and immediately cooled in ice cold water. The precipitate was sedimented by low speed centrifugation and the optical density of the supernatant read at 350 mμ in a Beckman DB spectrophotometer using a 1-cm. light path.

Test urine and standard samples were read against the enzyme control which was placed in the reference position. The urine controls were read against distilled water and the value obtained subtracted from the corresponding readings of the test urines.

The optical densities of the standard solutions were plotted against concentration of Figlu in μg./ml. The amount of Figlu in the test urine (μg./ml.) was determined from the standard curve.

Discussion.

(i) Modification.

In the original method of Chanarin and Bennett (1962a), 1.5 ml. of 3 N HCL was added to the final incubation mixture. This was mixed and allowed to stand for 15 minutes. The mixture was then filtered and the optical density of the clear filtrate read at 350 mμ. Using this procedure in the study, the filtrate remained turbid and could not be completely cleared even after low-speed centrifugation. A combination of 10% perchloric acid (1.5 ml.) and heat coagulation (1 minute at 100°C.) resulted in a clear supernatant after centrifugation (Tabor and Wyngarden, 1958). Presumably turbidities observed were due to protein precipitation.

DETERMINATION OF FIGLU [Chanarin & Bennett, 1962a]

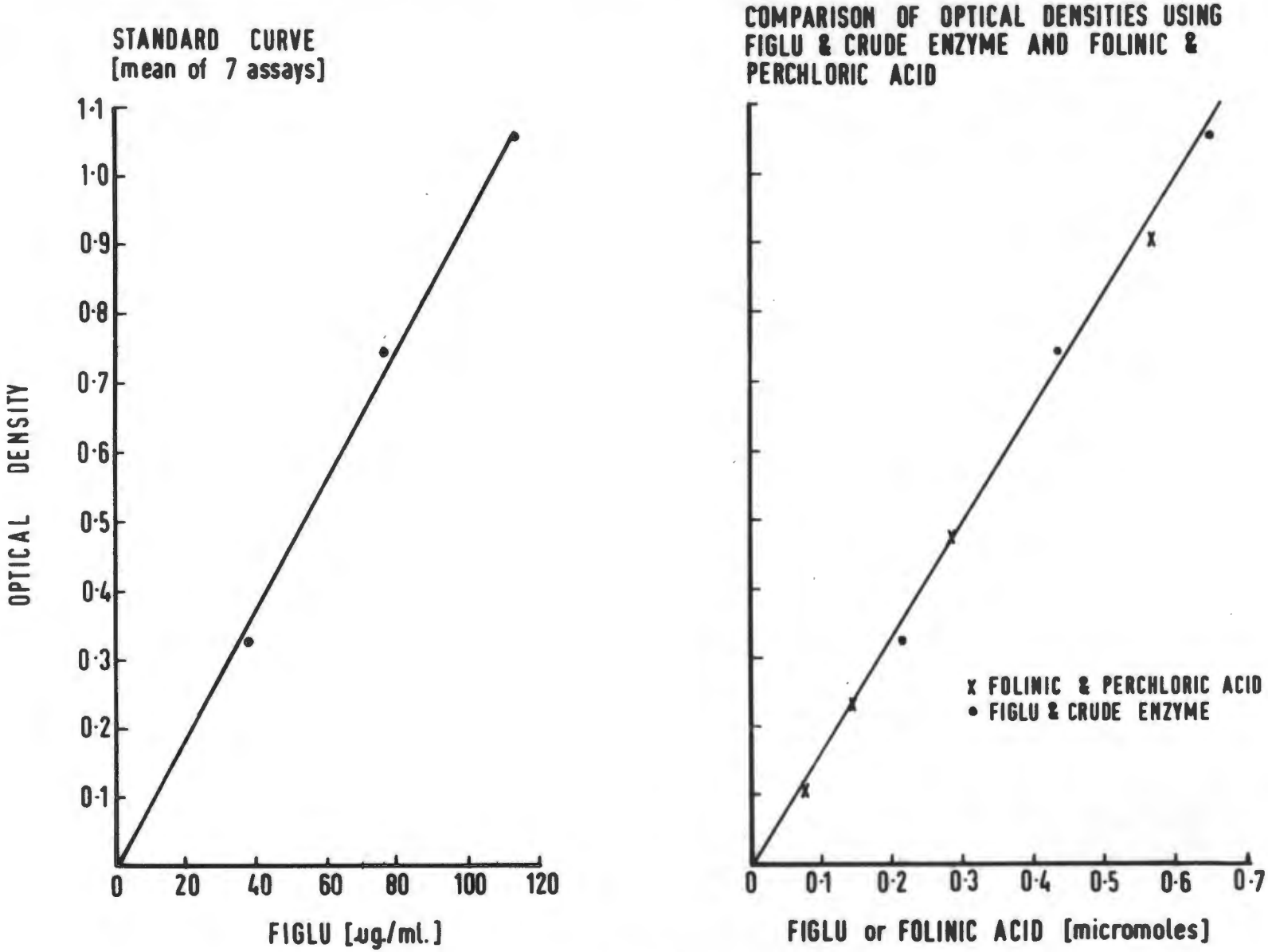


Fig. 13.

TABLE 20

REPRODUCIBILITY BETWEEN REPEAT ASSAYS FOR FIGLU
ON 118 CONSECUTIVE URINES.

Urine	Figlu (histidine derivatives) µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference*
1	13.7	21.1	7.4	42.5
2	2.0	4.1	2.1	70.0
3	10.3	12.4	2.1	18.6
4	6.2	5.8	0.4	6.6
5	7.2	4.4	2.8	48.3
6	19.5	17.4	2.1	11.4
7	22.9	22.5	0.4	1.8
8	32.6	34.2	1.6	4.8
9	7.4	10.7	3.3	36.7
10	27.8	28.8	1.0	3.5
11	6.4	6.0	0.4	6.5
12	8.6	11.7	3.1	30.7
13	14.9	12.0	2.9	21.5
14	22.4	23.4	1.0	4.4
15	18.8	21.9	3.1	15.3
16	9.2	12.0	2.8	26.4
17	39.6	44.4	4.8	11.4
18	23.2	22.4	0.8	3.5
19	64.7	50.8	13.9	24.0
20	37.0	41.6	4.6	11.7
21	12.2	9.4	2.8	25.9
22	14.1	9.3	4.8	41.0
23	84.5	86.6	2.1	2.5
24	33.9	33.1	0.8	2.4
25	7.0	8.1	0.9	12.2
26	1.1	1.9	0.8	53.3
27	26.2	26.8	0.6	2.3
28	32.2	33.7	1.5	4.6
29	17.9	17.0	0.9	5.2
30	18.5	17.8	0.7	3.9
31	29.4	26.5	2.9	10.0
32	16.7	17.2	0.5	3.0
33	38.3	39.8	1.5	3.8

TABLE 20 (contd.)

Urine	Figlu (histidine derivatives) µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference*
34	75.9	70.3	5.6	7.7
35	27.8	26.4	1.4	5.2
36	12.6	9.8	2.8	25.0
37	1.0	1.2	0.2	18.2
38	4.3	3.0	1.3	36.1
39	9.7	8.7	1.0	10.9
40	21.8	17.4	4.4	22.4
41	14.5	10.5	4.0	32.0
42	8.4	5.7	2.7	38.6
43	7.0	13.2	6.2	61.4
44	25.6	25.0	0.6	2.4
45	4.3	4.6	0.3	6.8
46	14.0	11.5	2.5	19.7
47	0.4	2.1	1.7	141.7
48	64.7	70.0	5.3	7.9
49	19.1	19.2	0.1	0.5
50	15.0	14.9	0.1	0.7
51	46.2	46.4	0.2	0.4
52	156.3	162.2	5.9	3.7
53	12.6	10.6	2.0	17.2
54	58.0	59.7	1.7	2.9
55	16.6	18.5	1.9	10.8
56	186.0	193.2	7.2	3.8
57	8.0	3.6	4.4	75.9
58	43.5	40.6	2.9	6.9
59	2.5	3.2	0.7	25.0
60	33.8	34.2	0.4	1.2
61	9.2	11.4	2.2	21.4
62	21.7	22.2	0.5	2.3
63	67.5	83.2	15.7	20.9
64	31.7	31.5	0.2	0.6
65	12.8	18.2	5.4	34.8
66	5.2	4.1	1.1	23.9
67	6.4	1.0	5.4	146.0
68	22.2	22.8	0.6	2.7

TABLE 20 (contd.)

Urine	Figlu (histidine derivatives) µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference*
69	30.2	25.5	4.7	16.9
70	5.1	4.2	0.9	19.6
71	60.0	59.0	1.0	1.7
72	5.2	2.3	2.9	78.4
73	30.6	30.6	0.0	0.0
74	2.9	3.8	0.9	27.3
75	8.6	9.7	1.1	12.1
76	7.0	9.1	2.1	26.3
77	52.0	53.1	1.1	2.1
78	1.0	0.7	0.3	37.5
79	38.4	42.4	4.0	9.9
80	31.2	30.4	0.8	2.6
81	12.4	7.5	4.9	49.5
82	12.5	13.3	0.8	6.2
83	150.1	164.0	13.9	8.9
84	141.2	106.5	34.7	28.0
85	25.7	17.4	8.3	38.6
86	94.8	94.0	0.8	0.8
87	14.7	18.7	4.0	24.0
88	8.5	8.7	0.2	2.3
89	102.7	117.6	14.9	13.5
90	4.4	10.6	6.2	82.7
91	3.6	3.4	0.2	5.7
92	23.2	25.2	2.0	8.3
93	15.0	17.0	2.0	12.5
94	15.0	20.8	5.8	32.4
95	10.9	7.8	3.1	33.3
96	13.8	13.3	0.5	3.7
97	5.2	12.2	7.0	80.5
98	21.6	21.7	0.1	0.5
99	91.1	88.5	2.6	2.9
100	13.5	17.5	4.0	25.8
101	26.2	28.2	2.0	0.7

TABLE 20 (contd.)

Urine	Figlu (histidine derivatives) µg./ml. Reading 1	Reading 2	Difference µg./ml.	% difference*
102	14.2	16.4	2.2	14.4
103	65.8	66.6	0.8	1.2
104	13.9	15.9	2.0	13.4
105	53.0	60.0	7.0	12.4
106	6.1	9.0	2.9	38.7
107	32.6	39.7	7.1	19.7
108	56.9	62.1	5.2	8.7
109	32.8	36.6	3.8	11.0
110	39.0	44.8	5.8	13.8
111	87.3	88.7	1.4	1.6
112	80.5	81.4	0.9	1.1
113	34.3	34.2	0.1	0.3
114	73.8	71.0	2.8	3.9
115	75.0	77.4	2.4	3.1
116	46.4	47.8	1.4	3.0
117	14.9	14.9	0.0	0.0
118	28.2	27.7	0.5	1.8

Mean difference = 3.1 µg./ml.

Mean % difference = 19.1

$$* \% \text{ difference} = \frac{\text{Reading 1 - Reading 2}}{\text{Mean of readings}} \times 100$$

Method: Chanarin and Bennett, 1962.(a)

(ii) Tests of Enzyme function.

The standard curve showed a linear relationship between Figlu concentration and optical density up to 114 µg./ml. of Figlu (Fig.13).

During the test if folinic acid was used in the absence of Figlu in solution or in urine, there was stoichiometric conversion to N^5,N^{10}-methenyl FH_4 which occurred during heating in the presence of perchloric acid. This could be used as a test of efficiency of enzyme activity. A solution of folinic acid (M.W. = 473.6) containing 272.5 µg./ml. (0.575 µmoles/ml.) and Figlu (M.W. = 174) containing 152 µg./ml. (0.874 µmoles/ml.) were prepared. The enzymatic conversion of folic acid to N^5, N^{10}-methenyl FH_4 was compared with that of the nonenzymatic conversion of folinic acid to N^5, N^{10}-methenyl FH_4 (Fig.13). The results were comparable in the range of 0.0 - 0.655 umoles which was equivalent to 0 - 114 µg./ml. of Figlu. From this it could be deduced that enzyme activity was in the optimal range.

(iii) Purity of Figlu solution.

Since the activity of the enzyme was optimal, the standard folinic graph could be used to establish the purity of the Figlu preparation.

(iv) Accuracy of the method.

The mean percentage difference between repeat assays was 19.1 and the mean difference between repeat assays 3.1 µg./ml. (Table 20).

Normal values.

The normal values for infants using this method and a dose of histidine of 0.26 g./kg. (0.12 g./lb.) had not been determined. Therefore to allow a consistent approach to the interpretation of the Figlu test in

DETERMINATION OF UROCANIC ACID [Chanarin & Bennett, 1962a]

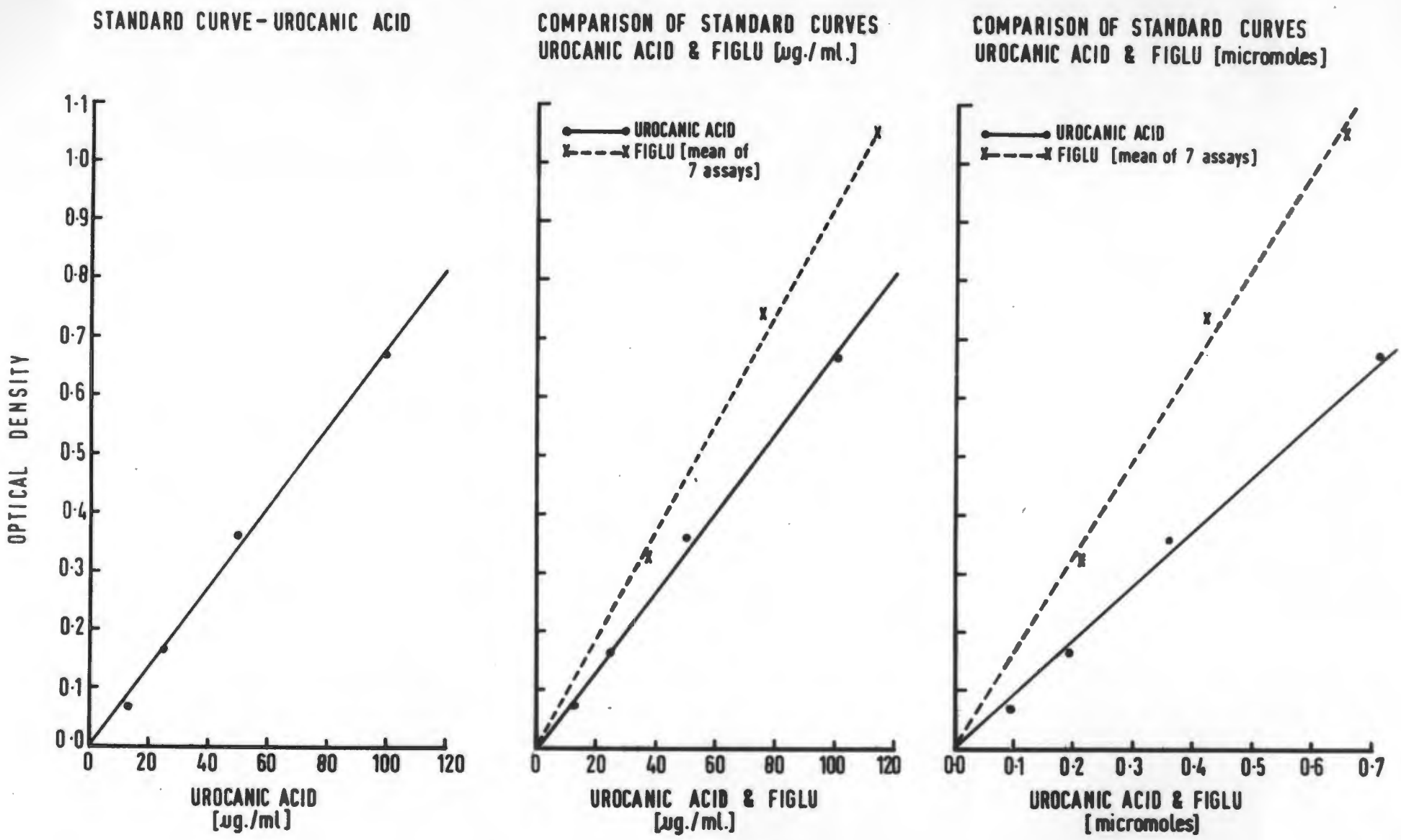


Fig. 14.

according to the method of Chanarin and Bennett (1962a) contains urocanase which converts urocanic acid to Figlu. Thereafter the same principles are employed as before (Fig.12).

Reagents.

Stock urocanic acid solution (100 µg./ml. at pH 6). The other reagents were the same as those listed for the estimation of Figlu using the method of Chanarin and Bennett (1962a).

Assay procedure.

This was modified slightly from the method of Tabor and Wyngarden (1958). In order to destroy Figlu the urine to be tested was adjusted to a pH of 11 - 12 using a measured amount of 1 N NaOH. The mixture was allowed to stand at 25°C. for 4 hours. The pH was then adjusted to 6.0 with a measured amount of 1 N HCL.

The subsequent assay technique was the same as that described for the estimation of Figlu (Chanarin and Bennett, 1962a), except that urocanic acid standards were used and appropriate correction had to be made for the urine dilution. Results of urocanic acid were expressed as µg./ml. or mg. per 24 hours.

The amount of true Figlu in urine could be determined by subtracting the urocanic value from the total amount of histidine derivatives estimated in the first part of the procedure.

Discussion.

(i) Assay procedure.

The optical densities at 350 mµ for a series of urocanic acid standards differed from those obtained with the Figlu standards (Fig.14). Thus it was necessary to compensate for this before subtracting the

this study Luhby and Cooperman's (1964) criterion of normality (30 µg./ml.) was used. In this case there was no reason for modifying it to 45 µg./ml.

Later in the study it was apparent that the use of a criterion of normality for Figlu excretion in mg. per 24 hours would be desirable. This was artibrarily determined by using Luhby and Cooperman's criterion of 30 µg./ml. as a standard of reference. Infants excreting more than 30 µg./ml. in unconcentrated urine prior to receiving therapy with folic acid and/or iron excreted more than 10 mg. of Figlu per 24 hours. Thus results were classified as follows:

Classification	Figlu excretion
Positive result (lower limit)	30 µg./ml. and 10 mg. per 24 hours
Borderline result (lower limit)	30 µg./ml. or 10 mg. per 24 hours
Negative result	< 30 µg./ml. and < 10 mg. per 24 hours

Application.

This test was used to assay all the urines of the infants participating in the controlled therapeutic trial (46) and in 7 infants on whom folic acid clearance tests were performed. All the results were the mean of repeat assays, each done in duplicate.

The determination of Figlu in urine (Kohn, Mollin and Rosenbach,1961).

Principle.

A sample of urine is subjected to electrophoresis on cellulose acetate membranes in a pyridine-acetic acid buffer (pH 5.3). Figlu and glutamic acid migrate to the same position and can be located with ninhydrin. Exposure to ammonia converts Figlu to glutamic acid and the increase in the ninhydrin colour is compared before and after exposure to ammonia on two separate membrane strips. Estimation of the concentration of Figlu is by visual comparison with a Figlu standard.

The cellulose acetate strips were dipped in ninhydrin solution and allowed to dry in air. They were then placed between two sheets of filter paper, covered by two pieces of cardboard to hold them flat, and then heated in an oven at 90 - 100°C. for five minutes. Thirty (30) minutes later the strips were examined with the aid of transmitted light. A semi-quantitative estimation of the amount of Figlu present was obtained by comparing the intensity of the colour of the urine standard spot with that of a test urine after allowing for the presence of glutamic acid on the non-ammoniated strip.

Normal values.

These were defined as a difference of intensity of the ninhydrin positive spot with and without treatment with ammonia vapour less than that of a urine standard containing 100 µg./ml. of Figlu (Dormandy, Waters and Mollin, 1963).

Application.

This test was only used to assess Figlu excretion in the urine of the pregnant women and all determinations were always performed twice.

ESTIMATION OF UROCANIC ACID IN URINE

An enzymatic and a chromatographic method were used to measure urocanic acid in urine.

Determination of urocanic acid in urine (Chanarin and Bennett,1962a; Bennett and Chanarin,1962).

Principle.

Total histidine derivatives expressed as Figlu are first determined according to the method discussed under determination of Figlu (Chanarin and Bennett, 1962a). Figlu in urine is destroyed and urocanic acid is then estimated. This is possible because the crude liver enzyme prepared

TABLE 21

THE EFFECT OF ALKALINIZATION (pH 11-12) AND STANDING (4 hrs. at 25°C) ON THE RECOVERY OF FIGLU AND UROCANIC ACID IN URINE.

Test	Urine before alkalinization and standing		Urine after alkalinization and standing	Recovery (Urocanic Acid) %	
	Figlu µg./ml.	Urocanic acid µg./ml.	Histidine derivatives (Urocanic Acid) µg./ml.		Mean
1	30	50	51.7	103.4	103%
2	30	50	51.3	102.6	

urocanic acid value from the total amount of histidine derivatives to allow final expression in terms of Figlu.

The optical density at 350 mμ for a series of urocanic acid standards was 0.0067 optical density units per μg. of urocanic acid. This compared with 0.0092 optical density units per μg. of Figlu.

From these values the contribution of urocanic acid to total histidine derivatives (expressed as Figlu) could be calculated and thus by difference the original concentration of Figlu could be established.

(ii) Modification.

In the method of Chanarin and Bennett (1962a) urocanic acid was separately estimated after Figlu was destroyed by autoclaving the urine at an alkaline pH. It was shown by Tabor and Wyngarden (1958) that if urine at pH 11 - 12 was left for 4 hours at 25°C., 97% of Figlu was degraded, but urocanic acid remains stable in these circumstances. In this study it was shown that when urine, originally containing both Figlu and urocanic acid, was adjusted to a pH of 11 - 12 and then left for 4 hours at 25°C., the amount of histidine derivatives recovered was slightly in excess of the amount of urocanic acid originally in the solution. Since this derivative was probably urocanic acid, the mean recovery of urocanic acid in these circumstances was 103% (Table 21).

Normal values.

This method had not been used to determine normal values on 24 hour urines collected from infants given a histidine load of 0.26 g./kg. (0.12 g./lb.).

Spector, Falcke, Yoffe and Metz (1966) administered 10 g. of histidine to healthy well nourished infants from 6 - 24 months, and

collected urines for 8 hours thereafter. Normal values,using the method of Chanarin and Bennett (1962a) were less than 10 µg./ml.

Vanier and Tyas (1966) used a high voltage electrophoretic method for measuring urocanic acid. Urine was collected for 6 hours following a histidine load of 0.1 - 0.3 g./kg. Normal values for infants in the first year of life were up to 50 µg./ml.

Application.

This method was used for estimating urocanic acid excretion in 46 infants participating in the controlled therapeutic trial and 7 infants on whom folic acid clearance tests were performed. Urocanic acid in urine was only estimated in infants who excreted total histidine derivatives in excess of 30 µg./ml., because only in these infants was it likely that the excretion of Figlu alone might be excessive. In these circumstances the results were separately expressed as urocanic acid and Figlu. In all other circumstances the results were expressed as the sum of the two histidine derivatives.

Determination of urocanic acid in urine (Whitehead, 1964).

Principle.

A standard method of ascending chromatography (Smith, 1958), was used to separate amino acids in urine using a butanol, acetic acid and water solvent. Urocanic acid was located with Pauley's reagent.

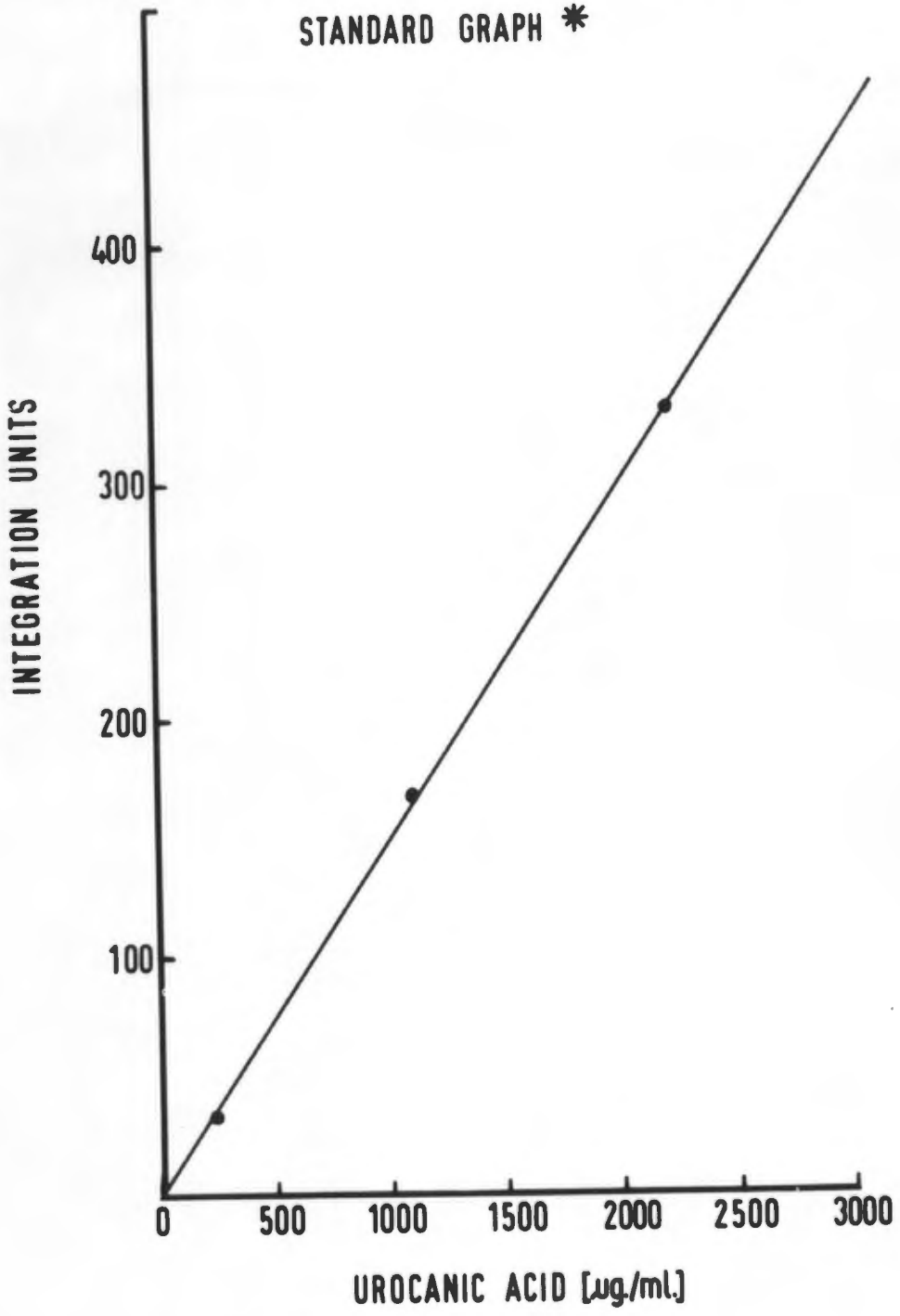
Reagents.

1. Butanol/acetic acid/water: 120/30/50 (by volume).

2a Pauley's reagent:

a) 9 g. of sulphanilic acid in 90 ml. concentrated HCL made up to 1 litre.
b) $NaNO_2$ (5% w/v).
c) Anhydrous sodium carbonate (10% w/v).

DETERMINATION OF UROCANIC ACID [Whitehead, 1964]



* Each point mean of 5 readings

Fig. 15

One volume of a), 1 volume of b) and 2 volumes of c) were mixed before use as described by Smith (1958).

3. Urocanic acid (L.Light and Co. Ltd.). A stock standard solution containing 2.2 mg./ml. in 0.1 N HCL and urine was prepared.

Procedure.

A slightly modified procedure was used. Thirty (30) µl. of each test urine collected after histidine loading were applied 3 cm. from the edge and 4 cm. apart on Whatman No.1 chromatographic paper (25 x 28 cm.). Care was taken to produce as small a spot as possible by applying small aliquots at a time and drying with a hair dryer between applications. Ascending chromatography was performed in a closed chromatographic jar for 15 - 20 hours. The sheets were then dried at room temperature. The paper was cut into strips and these dipped into Pauley's reagent. They were then dried on blotting paper. Urocanic acid appeared as a pinkish-red spot with a RF value of 0.5 - 0.53.

Standards were set up each time the test was performed. Standard solutions of urocanic acid in urine and 0.1 N HCL covering the range of 220 µg./ml. - 2,200 µg./ml. were treated in the same way as test urine.

Occasionally other Pauley positive spots not corresponding in RF value to urocanic acid were noted. These were rather faint and were not quantitated or further identified.

The densities of the urocanic acid spots were measured in a Joyce Loebl chromoscanner. A graph was constructed by plotting integration units against known concentrations of urocanic acid (Fig.15). The results were expressed in µg./ml. Urines having a concentration of urocanic acid greater than 2,200 µg./ml. were repeated in dilution.

Discussion.

Whitehead (1964) advocated the use of an elution method for quantitating urocanic acid in urine. The chromoscan method of quantitating urocanic acid was introduced here because it was not possible to achieve reproducible results with the elution method.

Application.

This method was used to estimate urocanic acid excretion in all infants in the prospective study, in 4 White and 8 African infants.

MICROBIOLOGICAL ASSAYS FOR FOLATE

MICROBIOLOGICAL ASSAYS FOR FOLATE

THE SERUM L.CASEI FOLATE ASSAY
(Waters and Mollin, 1961; Waters and Mollin, 1963)

Principle.

A folic acid dependent organism is grown under controlled nutritional and environmental conditions in both known quantities of folic acid and unknown quantities of folate in serum. The quantity of folate present in the unknown serum is determined by comparing the bacterial growth in serum with that in known quantities of folic acid. With all microbiological assays attempts are made to maintain sterility throughout.

Requirements.

Folate free glassware, test tubes, syringes, needles and aluminium caps.

Care was taken that all glassware and equipment were sterile and folate free. This was achieved by thorough cleansing in hot water with soap followed by numerous rinses in hot water and afterwards in distilled water. Thereafter the glassware and equipment were autoclaved three times while immersed in distilled water and rinsed again in glass-distilled de-ionized water. The glassware and equipment were dried and sterilised in a hot air oven at 160°C. for 60 minutes.

Glass-distilled, de-ionized water.

This was used for washing glassware and equipment, for making up solutions and in the assay procedure. The distilled water was de-ionized in an Elgastat de-ionizer (B 107) with Elgalite mixed-bed resin (C.403).

Sera.

Venepunctures were performed according to the method previously described. Blood was allowed to clot at room temperature for approximately 2 hours. Sera were separated and then stored at -20°C. until assayed.

TABLE 22.

DIFCO FOLIC ACID CASEI MEDIUM (Code 0822)

Charcoal Treated Casitone	10.0 g.
Dextrose	40.0 g.
Sodium Acetate	40.0 g.
Dipotassium Phosphate	1.0 g.
Monopotassium Phosphate	1.0 g.
dl tryptophane	200.0 mg.
l Asparagine	600.0 mg.
l Cysteine	500.0 mg.
Adenine Sulfate	10.0 mg.
Guanine Hydrochloride	10.0 mg.
Uracil	10.0 mg.
Xanthine	20.0 mg.
Tween 80	100.0 mg.
Glutathione (reduced)	5.0 mg.
Magnesium Sulfate	400.0 mg.
Sodium Chloride USP	20.0 mg.
Ferrous Sulfate	20.0 mg.
Manganese Sulfate	15.0 mg.
Riboflavin	1.0 mg.
p Aminobenzoic Acid	2.0 mg.
Pyridoxine Hydrochloride	4.0 mg.
Thiamine Hydrochloride	400.0 µg.
Calcium Pantothenate	800.0 µg.
Nicotinic Acid	800.0 µg.
Biotin	20.0 µg.

Basal medium.

Bacto-Folic Acid Casei Medium (Difco, Code 0822; Table 22) was made up immediately before required. It was free of folate but contained all the other requirements necessary for the growth of L.casei ATCC 7469.

Maintenance of stock culture, and initial preparation of the inoculum.

Lactobacillus casei ATCC 7469 was maintained in dried gelatin discs and was stored in a desiccator over phosphorous pentoxide at room temperature. A fresh gelatin disc containing the dried organism was placed in 10 ml. of Difco microinoculum broth the day before the assay procedure and incubated at 37°C. for a total of 30 hours.

Phosphate buffer and ascorbic acid (0.1M; pH 6.1).

This was constituted as follows: 26.7 ml. Na_2HPO_4(14.2 g./litre) 73.3 ml. KH_2PO_4(13.6 g./litre) and 280 mg. ascorbic acid.

Stock standard folic acid solution(5 mg./%.)

A stock folic acid solution was prepared by dissolving 10 mg. of highly purified folic acid (L.Light and Co. Ltd.) in a little of buffer solution, Na_2HPO_4. The volume was then made up to 200 ml. with water. This was dispensed in small quantities in 3 x ½" tubes and kept frozen at -20°C.

Procedure.

Phosphate buffer (3.6 ml.) was added to 0.4 ml. of the serum to be tested. The mixture was autoclaved for 2½ minutes at 15 lb./sq.in. and the coagulum precipitated thereafter by low speed centrifugation. The supernatant (0.5 ml.) was added to each of four 6 x 5/8" boro-silicate tubes. The volume in each of these tubes was made up to 2 ml. with water. A further 2 ml. of double-strength basal medium were delivered to each of the 4 tubes resulting in a dilution of 1 in 80 of the original serum.

Standards were prepared in triplicate. They were made up in water to a volume of 2 ml. from the 5 mg.% PGA solution. They contained 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ng. of PGA per tube. In addition 3 test tubes containing 2 ml. of distilled water to be used as uninoculated blanks were also prepared. Double-strenth basal medium (2 ml.) were delivered to all of these tubes.

The tubes were all covered with aluminium caps and autoclaved at 10 lb./sq.in. for 10 minutes. They were then cooled and one test tube of the four from each serum and the three test tubes with distilled water prepared with the standards were set aside as blanks. The remaining tubes were placed at random in stainless steel test tube racks.

The bacterial cells from the inoculum culture set up the day before assay were washed 3 times in 6 ml. of normal saline and afterwards suspended in 6 ml. of normal saline. A measured aliquot of this final suspension was added to 10 ml. of saline to provide an inoculum of standard optical density (0.04).

All test tubes (except the blanks) were inoculated with 1 drop of this prepared inoculum of L.casei using a syringe and a spinal needle with squared off point. Following inoculation, all test tubes were incubated at 37°C. for 18 hours.

The optical densities of the bacterial growth in the folic acid standard tubes and test sera were read against their blanks in a Unicam photoelectric colorimeter using an Ilford 625 filter (λ = 510-590 mμ.). The optical densities of the folic acid standards were plotted against the amount of folic acid per tube, and the concentration of folate in unknown sera (ng./tube) determined from the graph. The final results

TABLE 23.

REPRODUCIBILITY BETWEEN 100 CONSECUTIVE REPEAT L.CASEI SERUM FOLATE ASSAYS

Sera	Serum folate levels ng./ml. Reading 1	Reading 2	Difference ng./ml.	% difference
1	16.6	19.9	3.3	14.3
2	9.4	10.8	1.4	13.9
3	17.9	18.0	0.1	3.9
4	7.0	8.0	1.0	13.3
5	9.2	10.0	0.8	8.3
6	4.0	4.4	0.4	9.5
7	9.0	12.7	3.7	34.1
8	5.0	5.6	0.6	11.3
9	21.6	24.2	2.6	11.4
10	11.4	14.0	2.6	20.5
11	8.0	8.6	0.6	3.6
12	1.5	1.5	0.0	0
13	11.4	12.4	1.0	8.4
14	2.7	2.7	0.0	0
15	1.8	1.8	0.0	0
16	14.5	14.0	0.5	3.5
17	6.3	5.0	1.3	23.0
18	28.2	27.6	0.6	2.2
19	4.8	5.9	1.1	20.6
20	1.2	1.2	0.0	0
21	2.7	2.3	0.4	16.0
22	4.3	4.8	0.5	11.0
23	3.5	4.2	0.7	18.2
24	23.0	14.5	8.5	45.3
25	1.9	2.2	0.3	14.6
26	1.5	1.3	0.2	14.3
27	4.9	4.7	0.2	4.2
28	3.1	3.4	0.3	9.2
29	3.0	3.4	0.4	12.5
30	3.8	3.8	0.0	0
31	20.2	22.4	2.2	10.3
32	11.1	14.9	3.8	29.2
33	12.4	14.2	1.8	13.5

TABLE 23 (contd.)

Sera	Serum folate levels ng./ml. Reading 1	Reading 2	Difference ng./ml.	% difference
34	18.1	20.6	2.5	12.0
35	23.4	24.6	1.2	5.0
36	19.8	23.2	3.4	15.8
37	1.8	3.3	1.5	58.8
38	9.7	10.9	1.2	11.7
39	2.3	2.6	0.3	12.2
40	12.1	11.8	0.3	2.5
41	6.6	6.6	0.0	0
42	2.6	2.6	0.0	0
43	14.0	11.0	3.0	24.0
44	2.4	2.4	0.0	0
45	4.1	4.7	0.6	13.6
46	2.1	2.8	0.7	28.6
47	2.1	2.5	0.4	17.4
48	14.8	16.0	1.2	7.8
49	3.2	3.8	0.6	17.1
50	4.8	6.2	1.4	25.5
51	3.2	3.8	0.6	17.1
52	6.2	8.1	1.9	26.6
53	7.0	7.5	0.5	6.9
54	21.9	28.0	6.1	24.4
55	25.0	27.4	2.4	9.2
56	2.6	2.9	0.3	10.9
57	23.4	27.4	4.0	15.7
58	17.9	21.8	3.9	19.6
59	15.9	20.9	5.0	27.2
60	16.0	17.6	1.6	9.5
61	10.6	11.4	0.8	7.3
62	18.6	19.4	0.8	4.2
63	21.2	22.4	1.2	5.5
64	4.5	4.8	0.3	6.5
65.	4.1	4.6	0.5	11.5
66	2.6	3.4	0.8	26.7
67	6.6	7.8	1.2	16.7
68	4.8	5.0	0.2	4.1

TABLE 23 (contd.)

Sera	Serum folate levels ng./ml. Reading 1	Reading 2	Difference ng./ml.	% difference
69	5.4	6.1	0.7	12.2
70	6.0	7.2	1.2	18.2
71	5.4	6.6	1.2	20.0
72	5.6	10.6	5.0	61.7
73	6.9	9.4	2.5	30.7
74	21.6	25.8	4.2	17.7
75	9.4	10.5	1.1	11.1
76	13.3	20.1	6.8	40.7
77	14.1	20.7	6.6	37.9
78	13.2	19.2	6.0	37.0
79	16.4	24.4	8.0	39.2
80	2.5	2.8	0.3	11.3
81	17.6	18.1	0.5	2.8
82	4.8	5.5	0.7	13.6
83	3.5	3.6	0.1	2.8
84	8.1	9.2	1.1	12.7
85	4.7	5.3	0.6	12.0
86	11.3	11.6	0.3	2.6
87	6.5	7.8	1.3	18.2
88	14.8	16.7	1.9	12.1
89	7.7	12.4	4.7	46.8
90	6.2	10.1	3.9	47.9
91	1.1	4.8	3.7	125.4
92	3.1	1.4	1.7	61.8
93	3.6	6.6	3.0	58.8
94	3.2	5.6	2.4	54.5
95	5.8	10.0	4.2	53.1
96	4.1	5.7	1.6	32.7
97	3.3	4.6	1.3	32.9
98	8.7	12.4	3.7	35.1
99	1.6	2.7	1.1	51.1
100	5.1	6.1	1.0	17.9

MEAN % DIFFERENCE 19.4

MEAN DIFFERENCE ng./ml. 1.7

TABLE 24.

REPRODUCIBILITY BETWEEN REPEAT L.CASEI SERUM FOLATE ASSAYS IN THE RANGE 0 - 4.6 ng./ml.

Sera	Serum folate levels ng./ml.		Difference	% difference
	Reading 1	Reading 2	ng./ml.	
1	1.5	1.5	0	0
2	2.7	2.7	0	0
3	1.8	1.8	0	0
4	1.2	1.2	0	0
5	2.3	2.7	0.4	16.0
6	1.9	2.2	0.3	14.6
7	1.3	1.5	0.2	14.3
8	3.1	3.4	0.3	9.2
9	3.0	3.4	0.4	12.5
10	3.8	3.8	0	0
11	1.8	3.3	1.5	58.8
12	2.3	2.6	0.3	12.2
13	2.6	2.6	0	0
14	2.4	2.4	0	0
15	2.1	2.8	0.7	28.6
16	2.1	2.5	0.4	17.4
17	3.2	3.8	0.6	17.1
18	2.6	2.9	0.3	10.9
19	2.5	2.8	0.3	11.3
20	3.5	3.6	0.1	2.8
21	1.4	3.1	1.7	75.6
22	1.6	2.7	1.1	51.2
23	3.3	3.9	0.6	16.7
24	2.3	3.2	0.9	32.7
25	1.3	1.6	0.3	20.7
26	3.4	1.9	1.5	56.6
27	4.0	3.6	0.4	10.5
28	4.1	2.4	1.7	52.3
29	2.3	4.2	1.9	58.5
30	2.4	2.4	0	0
31	2.2	2.7	0.5	20.4
32	3.9	3.4	0.5	13.7
33	3.4	3.3	0.1	3.0
34	3.7	3.4	0.3	8.5

TABLE 24 (contd.)

Sera	Serum folate levels ng./ml. Reading 1	Reading 2	Difference ng./ml.	% difference
35	3.0	3.4	0.4	11.1
36	2.0	2.2	0.2	9.5
37	3.5	3.8	0.3	8.2
38	3.9	3.5	0.4	10.8
39	2.8	3.2	0.4	13.3
40	2.2	2.5	0.3	12.8
41	3.3	3.8	0.5	14.1
42	2.6	4.1	1.5	44.8
43	2.0	6.2	4.2	102.4
44	2.1	2.2	0.1	4.7
45	3.5	3.6	0.1	2.8
46	1.2	1.6	0.4	28.6
47	3.4	3.8	0.4	11.1
48	3.2	2.5	0.7	24.6
49	3.2	2.6	0.6	20.7
50	3.0	2.7	0.3	10.5
51	2.1	4.4	2.3	70.8
52	2.2	4.6	2.4	70.6
53	1.9	4.4	2.5	79.4
54	1.9	3.1	1.2	48.0
55	3.6	3.2	0.4	11.8
56	3.3	2.3	1.0	35.7
57	3.5	1.6	1.9	74.5
58	3.1	2.7	0.4	13.8
59	3.6	2.8	1.2	25.0
60	3.2	3.7	0.5	14.5
61	4.0	2.8	1.2	35.3
62	3.8	2.7	1.1	33.8
63	3.7	2.6	1.1	34.9
64	1.6	1.6	0.0	0
65	3.4	1.2	2.4	95.7
66	2.4	3.2	0.8	28.6
67	3.4	3.7	0.3	8.5
68	2.3	2.7	0.4	16.0
69	1.9	1.2	0.7	45.2

TABLE 24 (contd.)

Sera	Serum folate levels ng./ml. Reading 1	Reading 2	Difference ng./ml.	% difference
70	3.2	2.4	0.8	28.6
71	2.8	2.0	0.8	33.3
72	2.8	3.1	0.3	10.2
73	1.9	1.6	0.3	17.1
74	2.2	2.3	0.1	4.4
75	3.8	3.3	0.5	14.1
76	2.8	3.2	0.4	13.3
77	1.4	1.2	0.2	15.4
78	3.1	1.8	1.3	53.1
79	2.0	2.6	0.6	26.1
80	3.0	3.8	0.8	23.5
81	3.8	2.7	1.1	33.8
82	1.2	0.7	0.5	52.6
83	2.4	1.8	0.6	28.6
84	3.6	3.6	0.0	0
85	2.4	2.8	0.4	15.4
86	2.0	1.5	0.5	28.6

MEAN % DIFFERENCE 24.6

MEAN DIFFERENCE ng./ml. 0.7

were expressed as ng./ml. after carrying out the appropriate calculation. Where the serum folate activity was higher than the range covered by the standards (0 - 1.4 ng./tube) the assay of the serum was repeated in dilution.

With few exceptions,which will be referred to when relevant, results were always expressed as the mean of two assays each done in triplicate. The assays were so arranged that about half of the sera being assayed were duplicates from the previous run. In this way the sera as well as the standards were used to check the accuracy of the assay.

Discussion of the L.casei folate method.

Accuracy of the method.

Reproducible results were only achieved once a commercial basal medium and a standardised inoculum size were used (Table 23; Table 24).

	Mean difference	Mean % difference
Reproducibility of repeat assays for folate	1.7 ng./ml.	19.4
Reproducibility of repeat assays for folate in the range 0-4.6 ng./ml.	0.7 ng./ml.	24.6

Spray (1964) reported a mean % difference of 12% between repeat assays for folate using L.casei as the assay organism.

Reliability of the method.

It was necessary to investigate several factors which might have affected the reliability of some of the serum L.casei folate assays.

(a) Storage of sera.

Many of the sera from the mothers and from the umbilical cord samples were stored for approximately a year without ascorbic acid protection. Some investigators reported that the addition of ascorbic acid to sera was essential to prevent loss of L.casei folate activity

TABLE 25.

THE EFFECT OF ASCORBIC ACID PROTECTION ON FOLATE ACTIVITY (L.CASEI) IN SERA STORED AT -20C. FOR 3 MONTHS.

Sera	Serum folate ng./ml.		
	Sample A* (24 hrs.)	Sample A (3 months)	Sample B+ (3 months)
1	6.7	5.8	4.9
2	4.4	4.1	3.6
3	8.4	7.5	6.8
Mean	6.5	5.8	5.1

Significance of differences:

F = 0.46

P >0.05

* Sample A was stored with ascorbic acid 7 mg./ml.

+ Sample B was stored without ascorbic acid.

THE EFFECT OF STORAGE (-20°C.) ON FOLATE ACTIVITY (L.CASEI) IN SERA STORED WITHOUT ASCORBIC ACID FOR 3 - 6 MONTHS.

Sera	Serum folate ng./ml.			
	Sample A (24 hrs)	Sample B (3 months)	Sample B (5.5 months)	Sample B (6.0 months)
1	3.6	2.8	3.7	2.5
2	6.7	4.9	5.3	3.8
3	4.4	3.6	4.3	3.3
4	4.3	3.8	4.8	4.1
5	8.4	6.8	7.2	5.7
6	4.4	2.9	3.9	2.3
Mean	5.3	4.1	4.9	3.6

Significance of differences:

F = 1.55

P >0.05

during storage at -20°C. (Waters and Mollin, 1963; Ghanarin and Berry, 1964) and at -15°C. (Spray, 1964). On the other hand Herbert (1962;1966) and Davis and Kelly (1962) did not find it necessary to add ascorbic acid to prevent loss of L.casei folate activity in sera stored at -20°C. for periods up to 2 years and 10 months respectively.

In view of these conflicting reports and because the sera were stored without ascorbic acid protection it was necessary to determine whether these folate estimations were reliable under the experimental conditions pertaining in this study. This was examined prospectively and retrospectively.

In a prospective study 6 sera from control subjects were divided into two samples, A and B. Sample A was stored with ascorbic acid and then assayed on day 1 and then at monthly intervals. In only 3 cases was there sufficient serum to complete this procedure for a period of 3 months. Sample B was stored without ascorbic acid and assayed at 3, 5½ and 6 months after storage.

The effect of ascorbic acid protection on folate activity (L.casei) of sera stored for 3 months at -20°C.

The mean L.casei folate levels of sera after storage for 3 months were lower (5.8 ng./ml. - Sample A; 5.1 ng./ml. - Sample B) than the mean serum folate level of assays performed on day 1 (6.5 ng./ml. - Sample A). This was more obvious in sera stored without ascorbic acid protection (Sample B). The differences were not statistically significant ($P > 0.05$; Table 25).

The effect of storing sera (-20°C.) for 3-6 months without ascorbic acid protection, on L.casei serum folate levels.

The mean serum L.casei folate level in 6 sera stored with ascorbic acid and assayed after 24 hours was 5.3 ng./ml. The mean serum L.casei

TABLE 26

RETROSPECTIVE STUDY OF INFLUENCE OF STORAGE (-20°C) ON FOLATE ACTIVITY (L.CASEI)

Sera	Serum folate ng./ml.		Duration of storage prior to 1st assay (months)	Difference in time between 1st and 2nd assays (months)
	Assay 1	Assay 2		
1	8.2	10.7	2.0	4.0
2	14.6	7.1	4.0	1.5
3	14.4	14.0	4.0	2.0
4	14.3	15.0	4.0	4.5
5	26.4	25.0	4.8	3.2
6	7.1	4.1	5.0	2.0
7	5.1	6.8	5.0	2.5
8	2.9	3.0	5.0	2.5
9	2.5	2.5	5.0	3.3
10	6.0	5.6	5.5	3.0
11	2.7	2.4	5.8	1.2
12	4.3	4.3	6.0	6.3
13	4.8	4.6	6.3	2.5
14	1.7	2.9	6.3	5.7
15	6.4	5.0	6.5	2.5
16	5.5	4.3	6.5	6.0
17	3.6	2.8	6.5	6.0
18	15.4	21.0	7.0	2.0
19	16.7	18.6	7.0	3.5
20	4.0	2.8	7.0	4.5
21	3.9	4.0	7.0	4.5
22	15.4	15.9	7.0	4.5
23	22.6	15.6	7.0	5.0
24	9.6	9.5	7.0	5.5
25	3.9	4.7	7.0	5.5
26	5.5	5.3	7.0	5.5
27	3.4	3.4	8.5	2.0
28	2.8	2.2	8.8	3.2
29	2.9	2.9	8.8	3.5
30	21.3	29.6	9.0	2.0

TABLE 26 (contd.)

Sera	Serum folate ng./ml. Assay 1	Assay 2	Duration of storage prior to 1st assay (months)	Difference in time between 1st and 2nd assays (months)
31	9.8	11.8	9.0	2.0
32	25.3	29.8	9.0	2.0
33	2.4	3.2	9.0	2.5
34	5.9	4.7	9.0	3.5
35	3.1	2.6	9.0	3.5
36	19.8	10.9	9.0	5.0
37	18.8	21.4	9.3	2.2
38	15.9	16.9	9.5	2.0
39	16.5	14.7	9.5	2.0
40	20.1	17.9	9.5	2.5
41	6.3	5.3	9.5	3.5
42	3.6	4.0	9.8	3.2
43	3.7	5.9	10.0	2.0
44	7.3	14.9	10.0	2.0
45	4.7	4.9	10.0	2.5
46	20.1	18.5	10.0	3.5
47	4.2	2.9	10.0	4.0
48	4.2	3.0	10.3	3.7
49	3.4	2.3	10.5	2.0
50	4.6	4.3	10.8	3.2
51	5.2	7.1	11.0	2.5
52	2.2	1.7	11.0	3.0
53	3.7	4.6	11.0	4.0
Mean	8.8	8.8		

TABLE 27.

THE EFFECT OF MILK AND SOLID FOOD ON SERUM FOLATE ACTIVITY (L. CASEI)

Classification	Sera	Fasting (2-3 hrs.)	Serum folate ng./ml. 60 minutes after food Breast Milk	Formula Milk	Midday Meal
Infant	1	2.5	-	2.7	-
Infant	2	4.5	-	4.4	-
Infant	3	7.0	-	7.9	-
Infant	4	10.3	11.5	-	-
Adult	5	3.6	-	-	4.1
Adult	6	3.0	-	-	3.2
Adult	7	4.4	-	-	5.1
Mean		5.0		5.6	

Difference of means:

$t = 0.380$

$0.70 < P < 0.80$

folate level of the same sera but stored without ascorbic acid protection (Sample B), was lower after 3 months (4.1 ng./ml.), 5½ months (4.9 ng./ml.) and 6 months (3.6 ng./ml.).

The apparent loss of folate activity after storage of sera without ascorbic acid was not statistically significant ($P > 0.05$; Table 25), but might have been important in individual sera containing borderline concentrations of folate. Thus the effect of storage was also examined retrospectively.

In a retrospective study there was no difference in mean L.casei serum folate levels of the sera stored for 2 - 11 months before the first assay and for 1.2 - 6.3 months between repeat assays (Table 26). In individual sera containing concentrations of folate at the lower limit of normal (Samples 17, 20, 35, 47, 49), the "fall-off" of folate activity on standing did not appear to be beyond the limits of experimental error of the method. As results were nearly always expressed as the mean of two assays, any effect possibly due to loss of folate in these sera on storage was further reduced.

From these findings it seemed that the loss of L.casei folate activity which occurred in sera stored at -20°C. without ascorbic acid protection was unlikely to have been significant.

(b) The effect of feeding on serum L.casei folate levels.

The mean postprandial serum folate level was higher (5.6 ng./ml.) than the mean fasting serum L.casei folate level (5.0 ng./ml.) in 7 control subjects. This was not a statistically significant result ($P > 0.05$; Table 27).

These findings were comparable with those of Matoth, Pinkas, Zamir, Mooallem and Grossowicz (1964) who found that milk feeding did not affect

TABLE 28

A COMPARISON OF FOLATE ACTIVITY (L. CASEI) IN SERA TAKEN FROM CAPILLARY AND VENOUS SITES IN THE SAME CONTROL SUBJECTS.

Sera	Serum folate ng./ml.	
	Capillary	Venous
1	4.2	4.4
2	4.0	3.2
3	8.8	8.9
4	6.3	7.6
5	11.2	14.2
Mean	6.9	7.7

Difference of means:

$t = 0.348$

$0.70 < P < 0.80$

THE EFFECT OF ORAL PENICILLIN AND SULPHADIAZINE THERAPY ON SERUM FOLATE ACTIVITY (L. CASEI)

Sera	Serum folate ng./ml.	
	During therapy	After therapy (3 days)
1	6.2	5.0
2	8.9	4.3
Mean	7.6	4.7

whole blood folate levels in infants, and those of Frank, Baker and Hutner (1968) who confirmed that a fasting state was not essential for reliable serum L.casei folate results. Cooper and Lowenstein (1964) showed that food intake only materially affected the serum folate level if folate-rich foods were ingested. In this study the rise in serum L.casei folate level after feeding was highest in the breast fed infant, but even this was not impressive.

These findings differed from those of Cooperman (1967) who stated that serum L.casei folate levels should only be estimated after a six hour fast, and Reizenstein (1965) who showed that the L.casei folate levels in postprandial sera were significantly higher than those in sera collected after an overnight fast.

(c) <u>The effect of sampling from capillary and venous sites on the results of serum L.casei folate assays</u>.

The mean serum L.casei folate level was 6.9 ng./ml. when capillary samples were assayed and 7.7 ng./ml. when venous samples were assayed from the same control subjects. This was not a statistically significant difference ($P > 0.05$; Table 28).

(d) <u>The effect of penicillin and sulphadiazine on serum L.casei assays</u>.

Only 1 infant who had a low serum L.casei folate level received antibiotics (penicillin and sulphadiazine) at the time of the estimation.

Penicillin and sulphadiazine therapy did not lower mean serum L.casei folate levels in 2 control subjects (Table 28). Waters and Mollin (1961) also found that the administration of penicillin did not affect serum L.casei folate levels, but Reizenstein (1965) reported that penicillin could affect serum L.casei folate levels in in-vitro experiments.

TABLE 29.

THE EFFECT OF DELAY IN TAKING SAMPLES FROM UMBILICAL CORDS ON SERUM FOLATE ACTIVITY (L.CASEI)

Sera	Serum folate ng./ml.		
	5 minutes	30 minutes	60 minutes
1	17.5	16.5	22.7
2	17.5	20.7	17.2
3	15.0	17.6	14.6
4	19.9	19.4	18.4
5	13.4	19.6	19.5
Mean	16.7	18.8	18.5

Significance of differences:

$F = 1.10$

$P > 0.05$

THE EFFECT OF HAEMOLYSIS ON SERUM FOLATE LEVELS (L.CASEI)

Sera	Serum folate levels ng./ml.	
	Unhaemolysed sera	Haemolysed sera
1	8.4	7.7
2	7.7	7.5
3	3.5	3.5
4	7.3	6.2
5	3.6	3.6
6	4.3	3.9
7	4.4	4.4
Mean	5.6	5.2

Difference of means:

$t = 0.364$

$0.70 < P < 0.80$

TABLE 30

SERUM FOLATE LEVELS IN 51 NORMAL EUROPEAN SUBJECTS (L CASEI)

Serum	Reading ng./ml.
1	9.6
2	3.9
3	8.4
4	6.6
5	3.0
6	7.4
7	8.1
8	9.1
9	5.4
10	5.4
11	10.7
12	4.9
13	6.1
14	12.4
15	8.1
16	5.8
17	6.9
18	4.8
19	9.3
20	6.7
21	7.8
22	7.4
23	6.1
24	17.3
25	8.3
26	7.6
27	7.4
28	5.4
29	7.7
30	8.0
31	5.2
32	7.3
33	8.4
34	3.5
35	4.3
36	6.1
37	4.2
38	6.4
39	4.1
40	5.8
41	8.8
42	3.6
43	6.4
44	4.6
45	7.2

TABLE 30 (contd.)

Serum	Reading ng./ml.
46	5.0
47	6.6
48	11.4
49	5.6
50	5.2
51	4.2

Range 3.0-17.3 ng./ml.
Mean 6.9 ng./ml.
S.D. $\pm$ 2.5
S.E. of mean $\pm$ 0.4

The p-aminobenzoic acid in the medium probably protected against any effect of sulphadiazine on serum L.casei folate levels (Herbert, 1961; Metz, 1968).

(e) The effect of delay in collecting cord blood samples on serum L.casei folate activity.

The mean serum L.casei folate levels from cord blood samples were 16.7 ng./ml., 18.8 ng./ml. and 18.5 ng./ml. after a delay in sampling of 5, 30 and 60 minutes respectively. These differences were not statistically significant ($P > 0.05$; Table 29).

(f) The effect of haemolysis on serum L.casei folate levels.

Blood was collected from 7 control subjects and a proportion of the unhaemolysed serum was separated. Haemolysis was produced in the specimens and the haemolysed sera then separated. The mean serum L.casei folate level in unhaemolysed sera was 5.6 ng./ml. as compared to 5.2 ng./ml. in haemolysed sera. The difference was not significant ($P > 0.05$; Table 29).

It was found that haemolysis could produce a spurious increase in serum L.casei folate levels (Waters and Mollin, 1961; Davis and Kelly, 1962; Cooperman, 1967). Thus the results in this study were unexpected. It might be that haemolysis in this experiment was not sufficiently severe or complete. Vanier and Tyas (1966) found that slight haemolysis did not significantly influence serum L.casei folate levels unless these were abnormal to start with.

Normal values.

Serum L.casei folate estimations were performed on normal volunteers drawn from the technical, nursing and medical staff. The range was 3.0 - 17.3 ng./ml. and the mean and standard error were 6.9 ± 0.4 ng./ml. (Table 30). The range of serum folate levels in 28 normal healthy

TABLE 31

SERUM FOLATE LEVELS IN 28 NORMAL COLOURED WOMEN.

Serum	Reading ng./ml.
1	5.6
2	5.6
3	4.7
4	5.2
5	6.2
6	5.1
7	7.4
8	6.5
9	5.0
10	2.9
11	4.2
12	4.6
13	4.0
14	6.4
15	4.6
16	6.2
17	5.4
18	6.8
19	4.0
20	4.8
21	5.4
22	3.8
23	5.8
24	8.0
25	8.0
26	3.8
27	5.7
28	9.2

Range 2.9-9.2 ng./ml.

Mean 5.5 ng./ml.

S.D. $\pm$ 1.4

S.E. of mean $\pm$ 0.3

TABLE 32.

SERUM L.CASEI FOLATE LEVELS IN NORMAL SUBJECTS

Investigators	Subjects	Mean ng./ml.	Range ng./ml.
Cooperman, Luhby and Avery, 1960	Adults	15.9	4.0 - 45.6
	Infants	27.5	2.7 - 201.2
Waters and Mollin, 1961	Adults	9.9	5.9 - 21.0
Hansen and Weinfeld, 1962	Adults	5.4	3.7 - 9.3
Davis and Kelly, 1962	Adults	5.93	2.7 - 18.5
Grossowicz, Mandelbaum-Shavit, Davidoff and Aronovitch, 1962	Adults	8.3	3.2 - 15.0
Hogan, Maniatis and Moloney, 1964	Adults	-	5.0 - 25.0
Spray, 1964	Adults	7.8	2.1 - 28.0
Magnus, 1965	Adults	-	3.0 - 11.0
Metz, Festenstein and Welch, 1965	Adults	-	3.0 - 15.0
Reizenstein, 1965	Adults	4.6	2.5 - 7.4
Herbert, 1966	Adults	-	7.0 - 15.9
Temperley and Horner, 1966	Adults	5.1	2.1 - 9.5
Vanier and Tyas, 1966	Adults	-	7.0 - 20.0
Investigation in Cape Town	Adults (Coloured)	5.5	2.9 - 9.2
	Adults (White)	6.9	3.0 - 17.3

A COMPARISON OF REPRESENTATIVE STANDARD CURVES FOR THE L. CASEI ASSAY

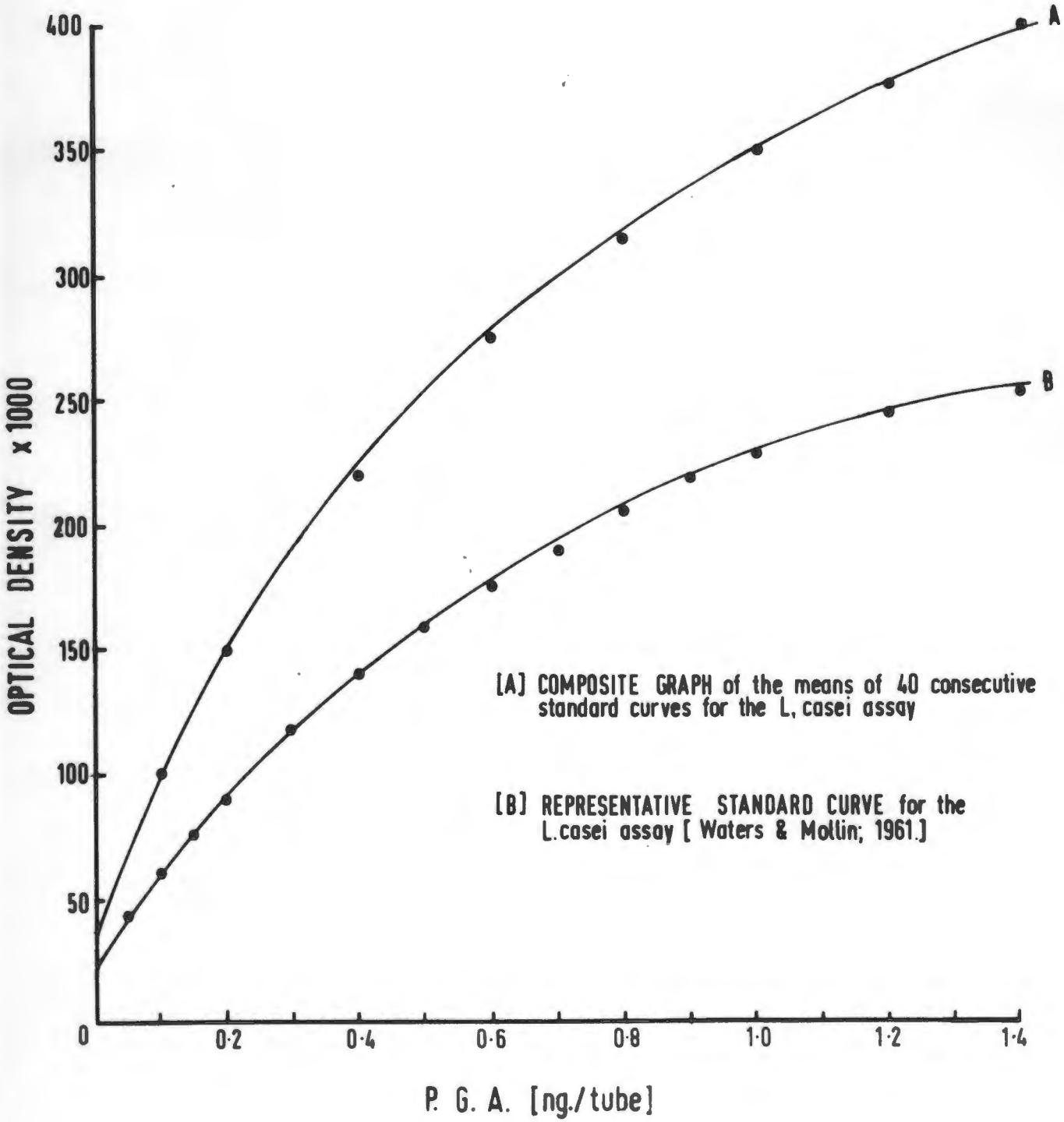


Fig. 16

Coloured nurses was 2.9 - 9.2 ng./ml. The mean and standard error were 5.5 $\pm$ 0.3 ng./ml. (Table 31). A wide range of normal levels was reported from different laboratories some of which were comparable to those found here (Table 32). The reasons for reported differences in normal values were discussed by Mollin and Hoffbrand (1965) and investigated by Temperley and Horner (1966). The latter found that the normal range of serum L.casei folate levels was lower if bacterial growth in the standard solutions was increased by adding vitamin C to the medium or by allowing prolonged incubation. These differences were not great enough to explain the wide discrepancies in the reported normal ranges.

The slope of the bacterial growth curve in the standard solution in this study was higher (30% to 40%) and showed a steeper rise than that found by Waters and Mollin (1961) (Fig. 16). The reasons for this difference were not determined but the finding might have a bearing on the difference in normal range between that reported by Waters and Mollin (1961) and that reported by this laboratory.

Abnormal values.

Waters and Mollin (1961) reported a normal range of 5.9 to 21 ng./ml. for the serum L.casei folate assay. They found that levels of 3.0 ng./ml. or less were associated with unequivocal folate deficiency, whereas serum L.casei folate levels in the subnormal range (3.0 to 6.0 ng./ml.) could be associated with megaloblastosis, minimal megaloblastic change or normal haematological findings (Mollin and Hoffbrand, 1965).

In view of their experience, serum L.casei folate values less than the lower limit of normal (3.0 ng./ml.) were regarded as evidence of biochemical folate deficiency in this study. When serum L.casei folate

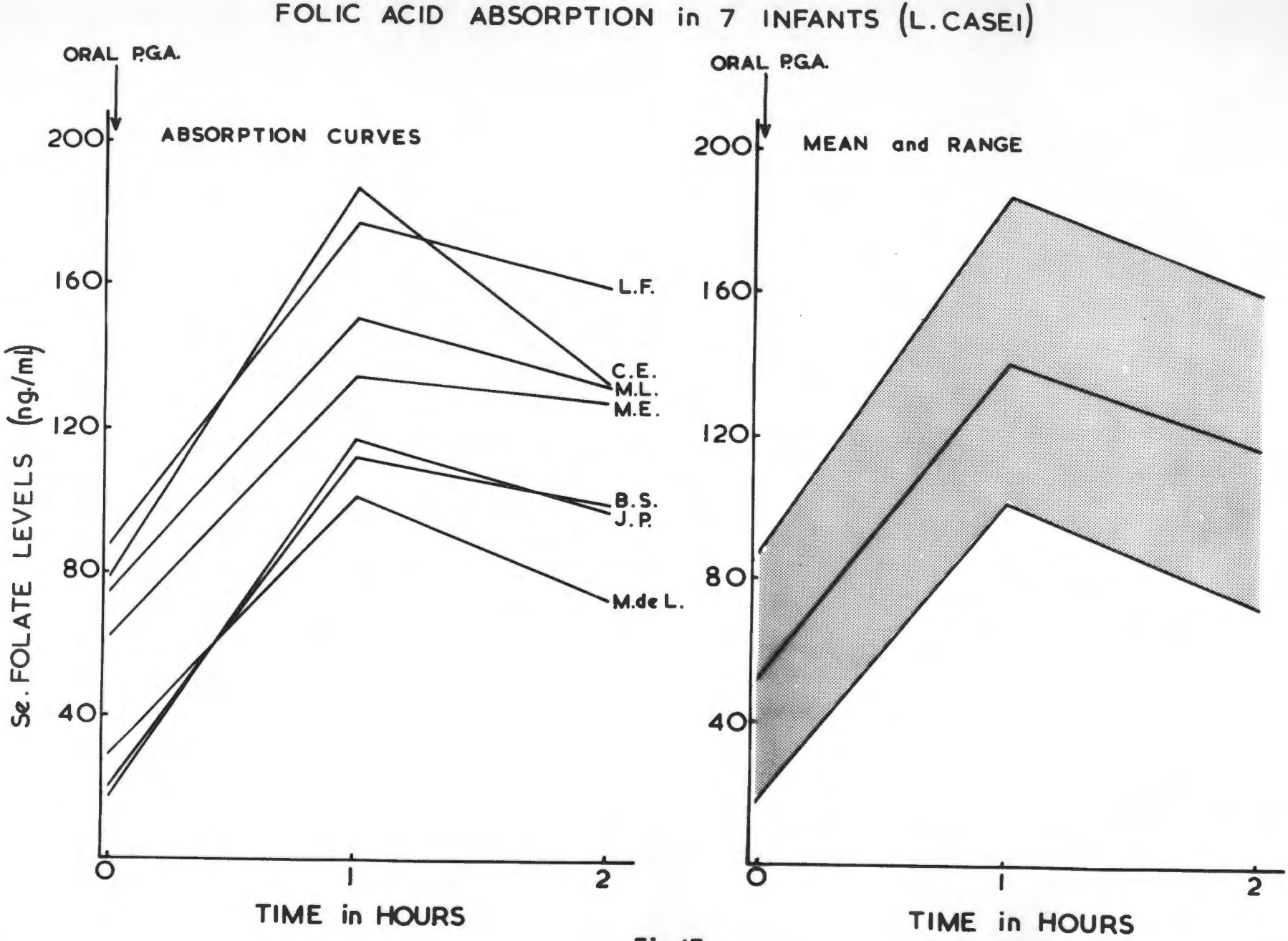

FOLIC ACID ABSORPTION in 7 INFANTS (L. CASEI)
ORAL P.G.A.
ABSORPTION CURVES
200
160
120
80
40
Se. FOLATE LEVELS (ng/ml)
L.F.
C.E.
M.L.
M.E.
B.S.
J.P.
M.de L.
0
1
2
TIME in HOURS
ORAL P.G.A.
MEAN and RANGE
200
160
120
80
40
0
1
2
TIME in HOURS

Fig. 17

FOLIC ACID ABSORPTION in 7 INFANTS

DATA in DETAIL

SUBJECT	FIGLU		FOLATE LEVELS ng./ml.				CLINICAL CONDITION
	µg./ml.	mg./24H.	Se. FOLATE	BASAL	1 Hr.	2 Hrs.	
M. de L.	84.6	25.4	10.6	26	102	73	POST GASTRO-ENTERITIS
M.E.	71.0	17.7	6.1	60	135	128	POST GASTRO-ENTERITIS
C.E.	60.0	—	19.1	75	187	134	WELL
L.F.	42.9	14.2	20.5	85	178	160	U.R.T.
B.S.	21.9	—	8.0	18	112	99	WELL
J. P.	16.9	—	1.1	14	117	98	POST GASTRO-ENTERITIS
M. L.	28.9	14.7	2.3	72	151	134	POST GASTRO-ENTERITIS
			MEAN	50	140	118	
			S.D.	± 29.7	± 33.1	± 29.3	
			S.E. of M.	± 11.4	± 12.7	± 11.3	

MEAN RISE in FOLATE ng./ml.

BASAL to 1 HOUR

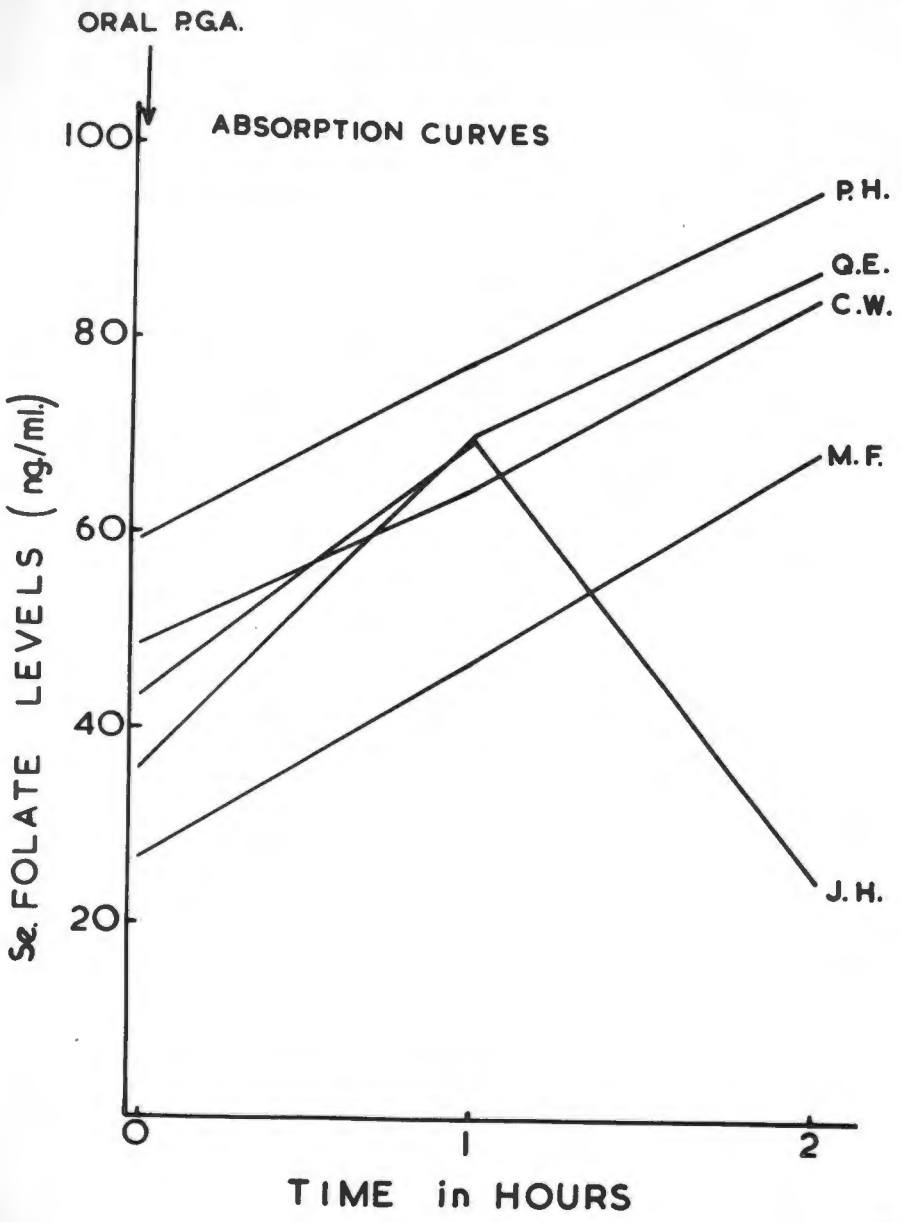
RANGE	75 — 112
MEAN	90
S.D.	± 14.2
S.E. of MEAN	± 5.5

BASAL to 2 HOURS

RANGE	46 — 84
MEAN	68
S.D.	± 13.3
S.E. of MEAN	± 5.1

TABLE 33

FOLIC ACID ABSORPTION in 5 INFANTS (L.CASEI)



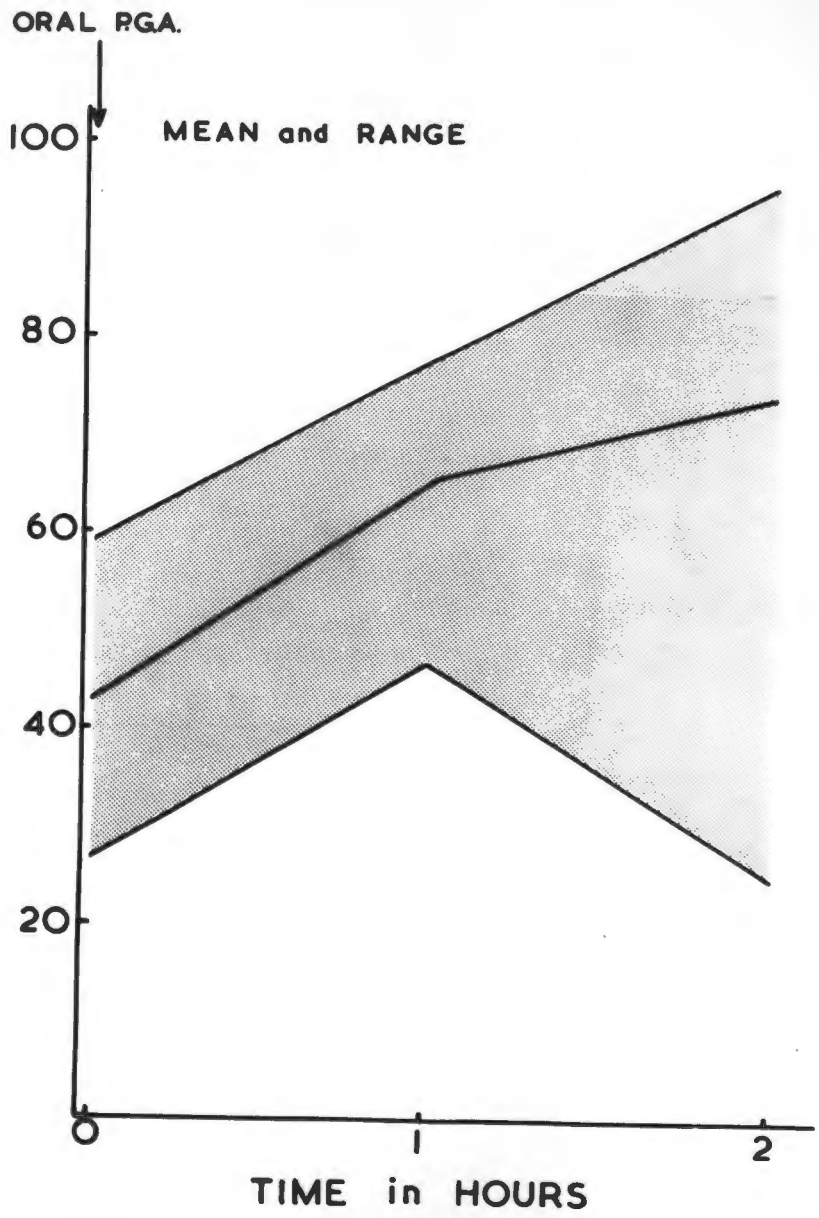


Fig. 18

FOLIC ACID ABSORPTION in 5 INFANTS

DATA in DETAIL

SUBJECT	FIGLU µg/ml.	SERUM FOLATE LEVELS ng/ml.				CLINICAL CONDITION
		Se. FOLATE	BASAL	1 HR.	2 HRS.	
Q.E.	16.0	3.4	35	70	87	GASTRO-ENTERITIS
C.W.	EXCLUDED	21.2	48	64	85	POST GASTRO-ENT.
M. F.	EXCLUDED	13.2	26	47	78	WELL
J. H.	38.1	4.1	43	70	25	GASTRO-ENTERITIS
P. H.	42.5	13.1	59	78	96	GASTRO-ENTERITIS
		MEAN	42	66	74	

MEAN RISE in FOLATE ng./ml.

BASAL to 1 HOUR 24 BASAL to 2 HOURS 32

TABLE 34

levels were correlated with the results of bone marrow examinations performed on infants not investigated in this study, it was found that unequivocal megaloblastosis was often associated with serum L.casei folate levels of 1.3 ng./ml. or less but could be associated with serum L.casei folate levels of up to 2.3 ng./ml. From this it appeared that in this study serum L.casei folate levels of 1.3 ng./ml. or less were most likely to be associated with significant folate deficiency.

FOLIC ACID ABSORPTION

Serum folate levels after the administration of oral folic acid were measured with L.casei.

Results.

Two patterns of absorption were noted in the twelve infants studied. In seven there was a good peak rise in serum L.casei folate levels which occurred after the first hour(Fig.17). The mean rise from basal to 1 hour was 90 ng./ml. (Table 33).

In five there was a poor peak rise in serum L.casei folate levels and it occurred mainly after the second hour (Fig.18). The mean rise from the basal to the second hour was 32 ng./ml. (Table 34).

Discussion.

"Malabsorption of folic acid".

The reason for the apparent malabsorption of folic acid in five infants could not be determined. It could not be attributed or related to folate deficiency because serum L.casei folate levels were normal and Figlu excretion was less than 45 µg./ml. in three of the five infants. The other two infants vomited a considerable amount of the histidine and in their case the Figlu test could not be considered (Table 34).

Gastroenteritis might have contributed to the poor absorption in some of the infants but this could not have been the only explanation (Table 34).

It was also possible that mothers had administered milk and food to their infants during the period of supposed glucose water feeding from 5 a.m. to 9 a.m. Even if this were so, it was unlikely that this explained the poor absorption, because both Baker, Frank and Sobotka (1964) and Chanarin and Bennett (1962c) permitted light meals before the folic acid absorption test was performed.

A technical reason for these results could not be found but this remained a possibility.

Because these curves were not related to folate deficiency and because there might have been a technical explanation for them, they will not be further discussed.

Folic acid absorption test.

The range of absorption of folic acid (75 - 112 ng./ml.) in seven infants (Table 33) was comparable to that found in children (70 - 140 ng./ml.) by Luhby and Cooperman (1961). In both studies L.casei was used as the assay organism.

Some of the folic acid absorption tests were carried out on infants while they were convalescing from gastroenteritis. Thus it was possible that the range and mean rise in serum L.casei folate levels after the administration of oral folic acid might have been higher if all infants were completely well at the time of investigation. It also meant that in the later analysis of the data, this complicating factor might have to be considered when the relationship of folic acid absorption to folic acid deficiency was assessed.

MEAN and RANGE of FOLIC ACID CLEARANCES — L.CASEI

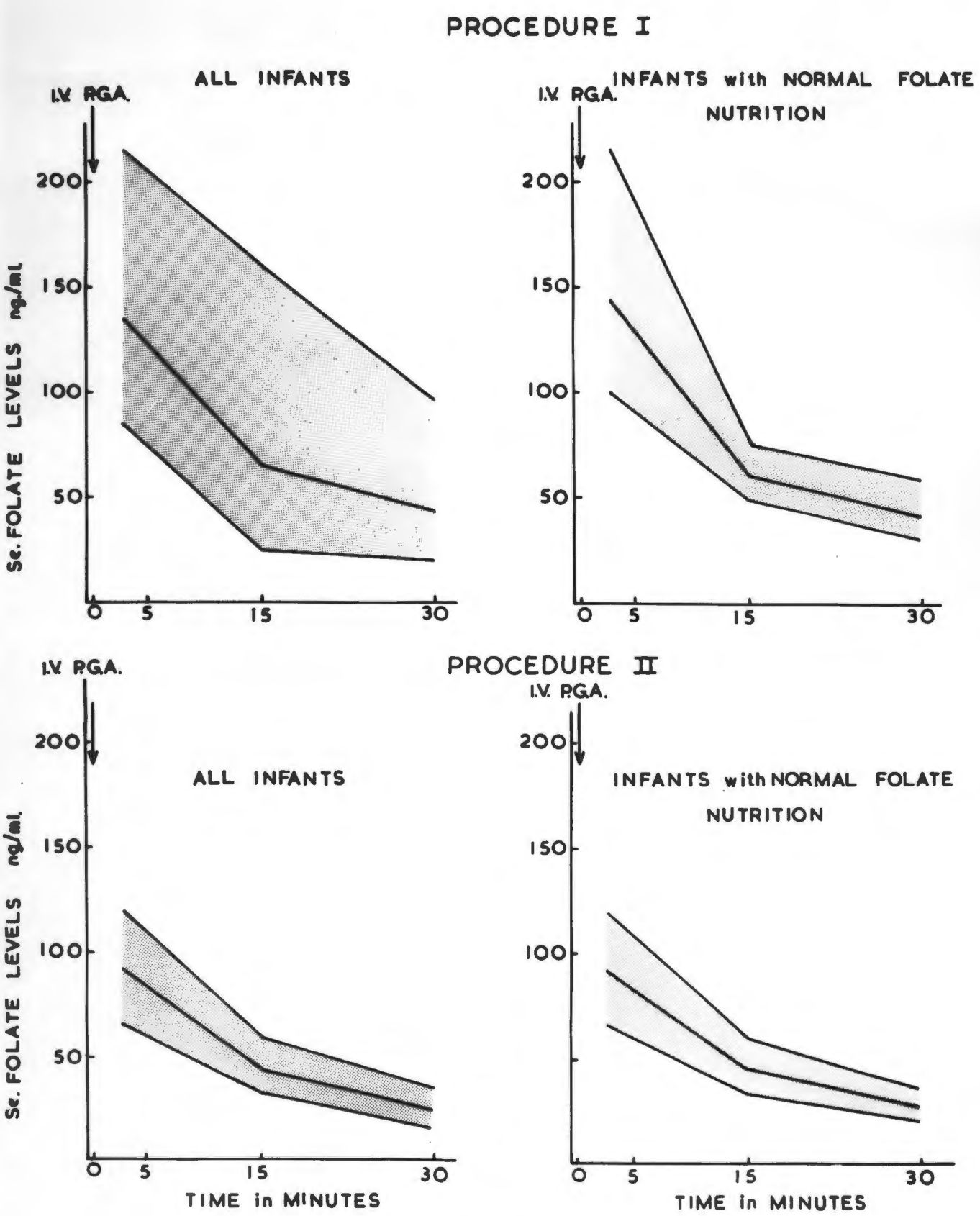


Fig. 19

FOLIC ACID CLEARANCES — L. CASEI

PROCEDURE I

ALL INFANTS

SUBJECT	AGE in MONTHS	Wt. %	Hb. g./100 ml.	FIGLU		S.IRON	FOLATE LEVELS ng./ml.				CLINICAL CONDITION
				µg./ml.	mg/24H.	µg/100 ml.	BASAL	3 MIN.	15 MIN.	30 MIN.	
R.K.	5.0	45	12.0	8.0	6.0	—	9.4	100	50	30	WELL
W.A.	4.5	97	12.2	23.2	8.4	80	13.2	214	75	58	WELL
N.S.	5.3	10	10.0	14.0	8.4	51	7.6	118	58	35	WELL
D.G.	6.0	60	12.4	42.5	12.8	—	9.6	—	50	37	WELL
G.R.	4.5	10	11.5	32.6	19.9	—	4.4	83	43	20	U.R.T.
A.K.	4.8	40	—	70.1	19.6	34	4.8	115	52	41	WELL
D.A.	3.0	50	10.6	4.4	2.6	—	27.0*	92	26	29	WELL
A.H.A.	4.0	3	11.4	103.6	17.6	—	46.8	211	160	95	WELL
					MEAN		15.4	133	64	43	
					S.D.		± 14.5	±55.5	±41.2	±23.7	
					S.E.of M.		± 5.2	± 21.3	± 14.7	± 8.3	

INFANTS with NORMAL FOLATE NUTRITION

SUBJECT	FOLATE LEVELS ng./ml.			
	BASAL	3 MIN.	15 MIN.	30 MIN.
R.K.	9.4	100	50	30
W.A.	13.2	214	75	58
N.S.	7.6	118	58	35
MEAN	10.1	144	61	41
S.D.	± 2.7	± 37.7	± 13.0	± 14.9
S.E.of M.	± 1.7	± 22.2	± 7.6	± 8.8

* ONE READING

TABLE 35

FOLIC ACID CLEARANCES — L.CASEI

PROCEDURE II

ALL INFANTS

SUBJECT	AGE in MONTHS	Wt.%	Hb. g/100 ml	FIGLU * µg./ml.	FIGLU * mg/24H.	FOLATE LEVELS ng./ml. BASAL	3 MIN.	15 MIN.	30 MIN.	CLINICAL CONDITION	DRUGS
F. F.	3.8	<3rd	11.7	27.2	7.3	6.4	65	32	19	G.E.	S.D.Z.
R.A	5.8	<3rd	8.6	52.5[x]	21.5[x]	9.6	85	34	16	BRONCHITIS	PENBRITIN
N.de J.	5.8	5th	11.6	24.2	7.4	9.6	118	59	35	U.R.T + G.E.	S.D.Z.
N.O	6.0	30th	8.5	28.9	22.8	2.4	98	40	21	U.R.T.	NIL
				MEAN		7.0	91	41	23		
				S.D.		± 3.3	± 22.3	± 12.4	± 8.4		
				S.E. of M.		± 1.7	± 11.2	± 6.2	± 4.2		

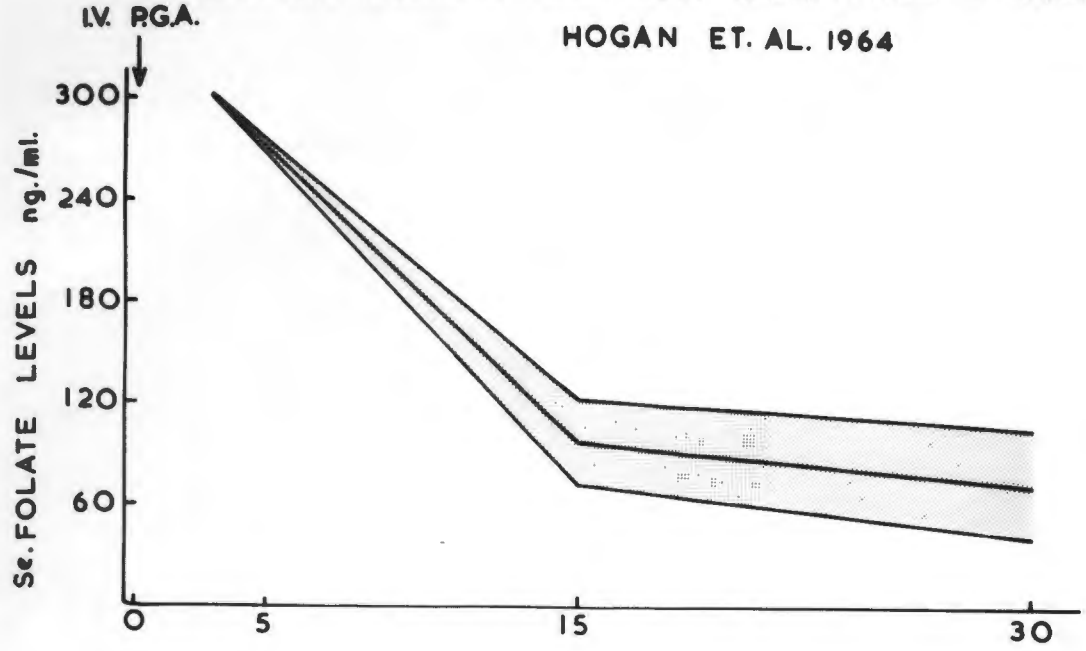
INFANTS with NORMAL FOLATE NUTRITION

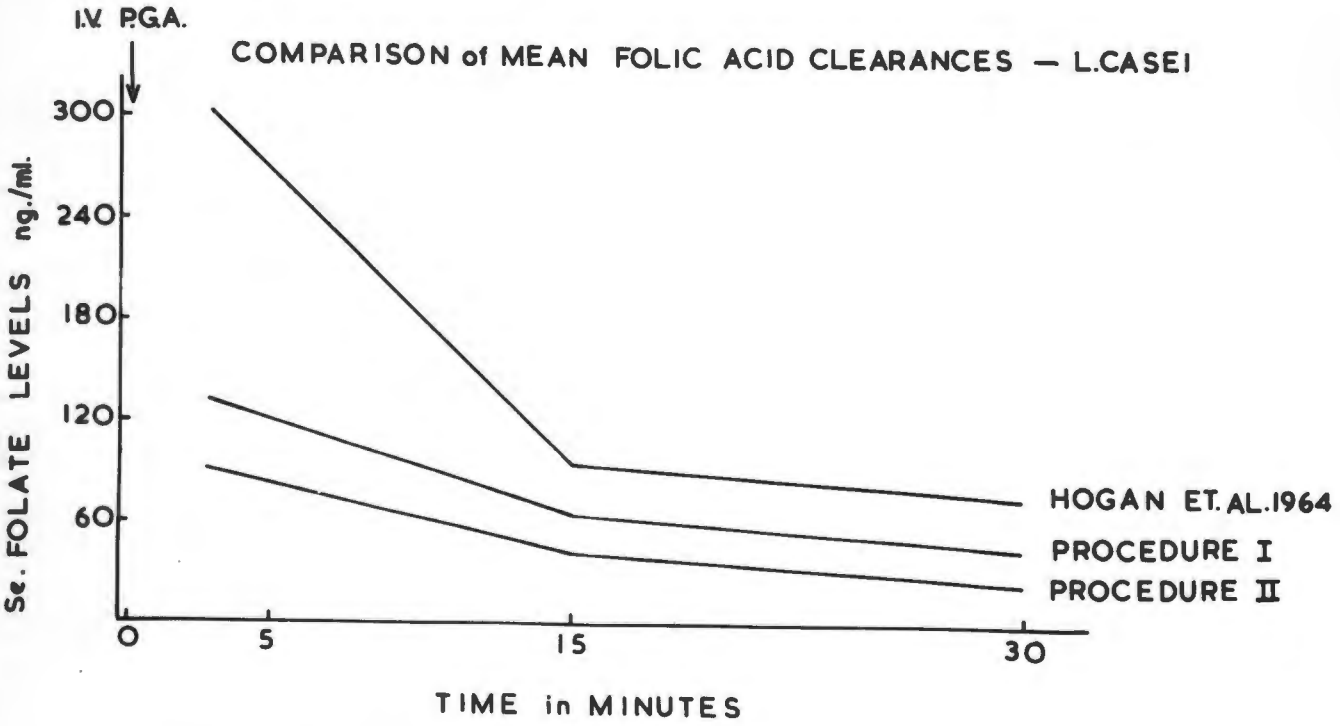
SUBJECT	FOLATE LEVELS ng./ml. BASAL	3 MIN.	15 MIN.	30 MIN.
F. F.	6.4	65	32	19
N.de J.	9.6	118	59	35
MEAN	8.0	91	45	27
S.D.	± 2.2	± 37.6	± 19.2	± 11.3
S.E.of M.	± 1.6	± 26.9	± 13.7	± 8.1

* METHOD – CHANARIN and BENNETT, 1962.(a)

x THE ONLY RESULT EXPRESSED AS FIGLU – THE OTHERS ARE THE SUM OF HISTIDINE DERIVATIVES

TABLE 36





DIFFERENCE of MEANS			
	3 MINUTES	15 MINUTES	30 MINUTES
PROCEDURE I-II	t = 0.465 0.60 < P < 0.70	t = 1.070 0.30 < P < 0.40	t = 1.629 0.10 < P < 0.20
HOGAN - PROCEDURE I	t = 9.680 P < 0.001	t = 2.420 0.02 < P < 0.05	t = 2.504 0.02 < P < 0.05

Fig. 20

Furthermore, the peak absorption of folic acid as measured here with L.casei in infants was lower than that found in adults (80-230 ng./ml.) by Ball (1966). However, no inferences could be drawn regarding a possible difference between absorption of folic acid by adults and infants because some infants were investigated in the recovery phase of gastroenteritis, and because serum L.casei folate assays from different laboratories were not necessarily comparable.

The curves will be regarded as representative of normal for this population group and valid for the purposes of comparison. They may not be representative of normal folic acid absorption in infancy.

FOLIC ACID CLEARANCE TEST

The folate activity in serum following an intravenous injection of folic acid was measured with L.casei in twelve infants and with both L.casei and S.faecalis in six infants.

Results.

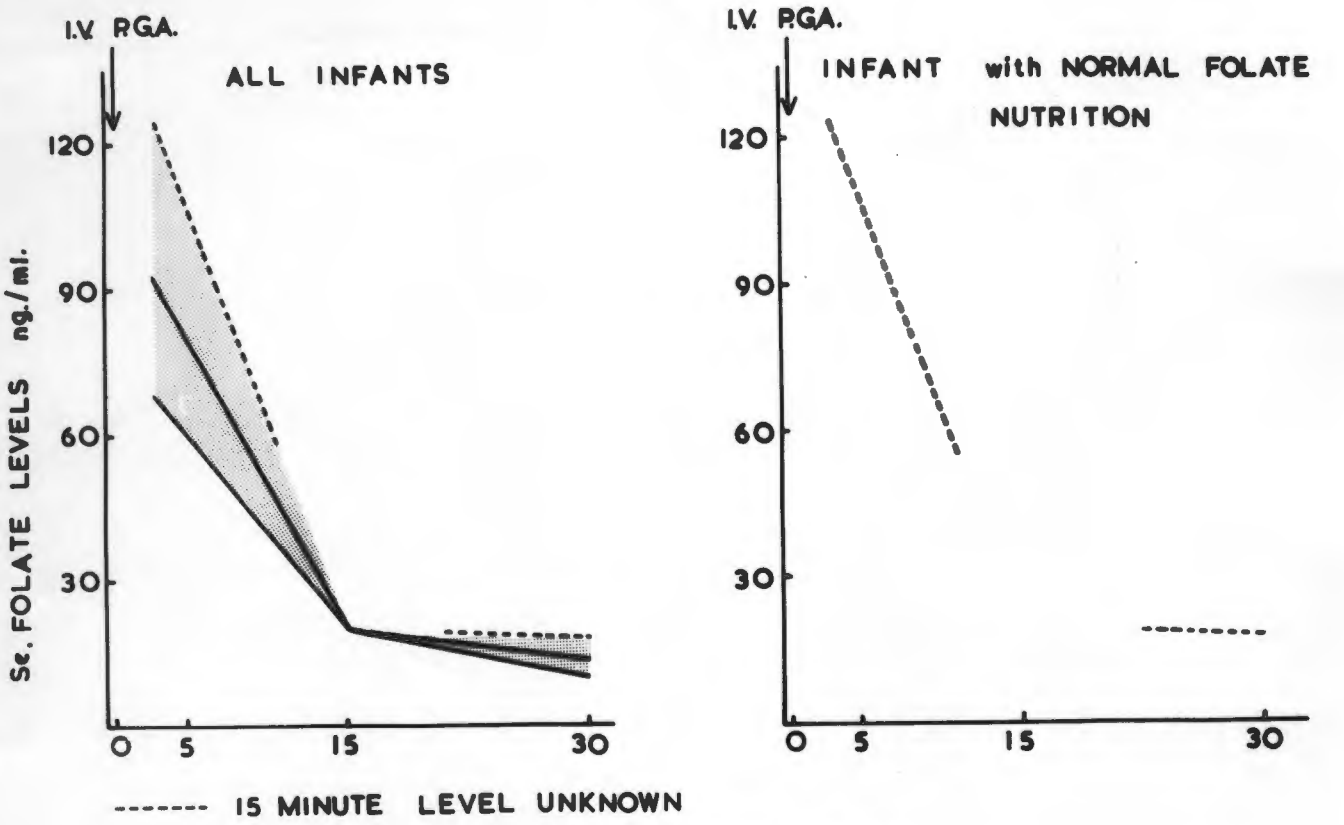
L.casei folic acid clearance curves.

The mean and range of folic acid clearance curves obtained using procedures 1 and 2 (defined in Chapter IV) in all infants and in those with normal folate nutrition (viz. serum D.casei folate levels in the normal range and normal Figlu excretion) are shown in Fig. 19. The data is presented in detail in Tables 35 and 36.

The mean folic acid clearance curve using procedure 1, was slower than that with procedure 2 but the difference was not statistically significant (Fig. 20). The folic acid clearance test is associated with a high variance and when few tests are performed, differences have to be very great before they become significant.

The mean folic acid clearance curve obtained in infants using procedure 1 was significantly faster (Fig.20) than that found in adults by Hogan, Maniatis and Moloney (1964). By these adult standards only 2 of the 8 curves obtained in infants fell within the normal range (W.A. and A.H.A.; Table 35; Fig. 20).

MEAN and RANGE of FOLIC ACID CLEARANCES S.-FAECALIS



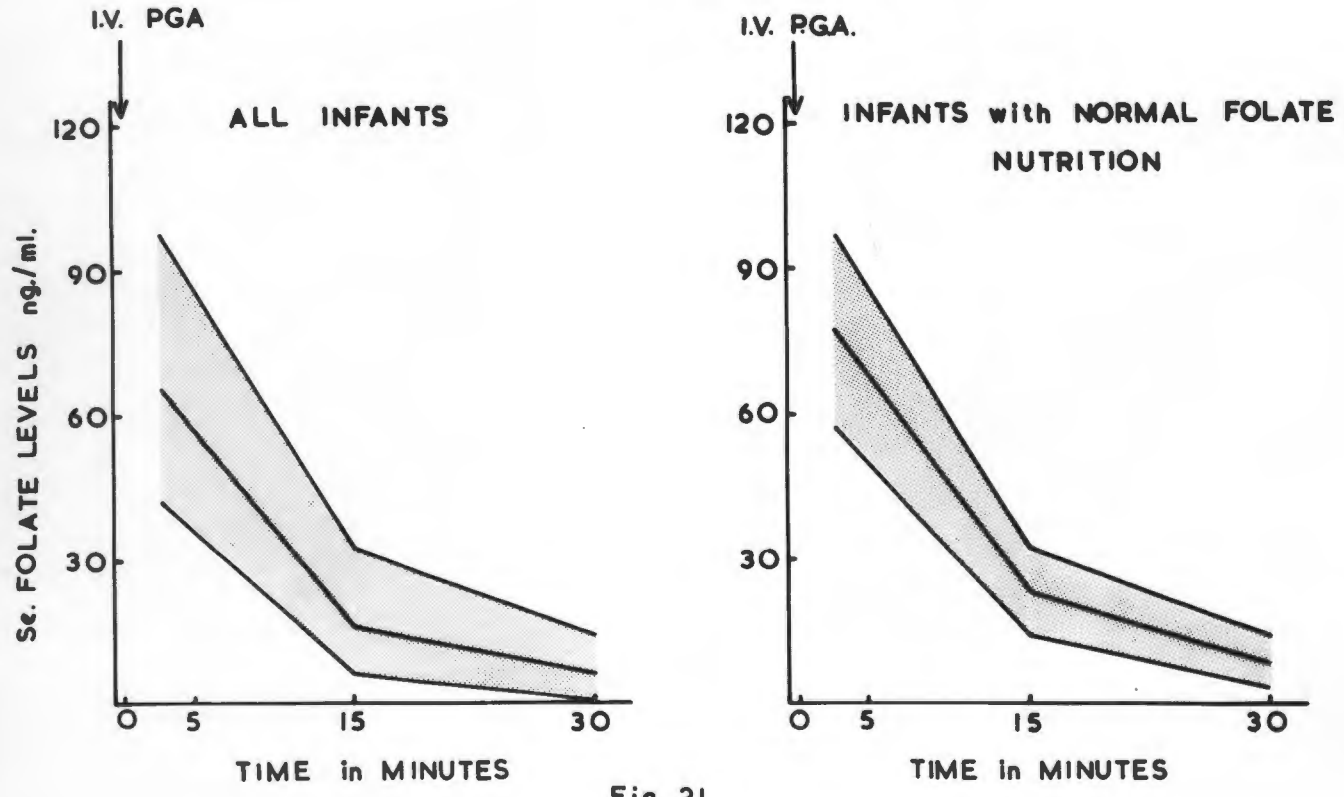


Fig. 21

FOLIC ACID CLEARANCES — S. FAECALIS

PROCEDURE I

ALL INFANTS

SUBJECT	3 MIN.	15 MIN.	30 MIN.
N.S.	123	—	18
A.K.	68	20	10
MEAN	96	20	14

INFANT with NORMAL FOLATE NUTRITION

SUBJECT	3 MIN.	15 MIN.	30 MIN.
N.S.	123	—	18

PROCEDURE II

ALL INFANTS

SUBJECT	3 MIN.	15 MIN.	30 MIN.
F.F.	57	14	3
R.A.	42	6	0
N.de J.	97	32	14
N.O	64	13	—
MEAN	65	16	6
S.D.	± 23.2	± 11.0	± 7.4
S.E. of M.	± 11.6	± 5.5	± 4.4

INFANTS with NORMAL FOLATE NUTRITION

SUBJECT	3 MIN.	15 MIN.	30 MIN.
F.F.	57	14	3
N.de J.	97	32	14
MEAN	77	23	8

TABLE 37

S.faecalis folic acid clearance curves.

The mean and range of S.faecalis folic acid clearance curves in all infants and in those with normal folate nutrition (procedures 1, 2) are shown in Fig.21 and Table 37. The mean folic acid clearance curve using procedure 1 was slower than that obtained using procedure 2 (Fig.21; Table 37).

The mean S.faecalis folic acid clearance curve obtained using procedure 1 (Table 37) was faster than the adult mean reported by Chanarin, Mollin and Anderson (1958a). They reported mean values of 127, 40 and 20 ng./ml. at 3, 15 and 30 minutes after the injection of folic acid.

Discussion.

The more rapid mean folic acid clearance curves both with L.casei and S.faecalis found in the second group of infants (procedure 2) could have been due to a slightly lower dose of administered folic acid. In view of this technical difference, and clinical differences which will be discussed below, the clearance curves performed according to procedure 2 might not have been representative of those found in healthy, under-privileged infants. Therefore whenever these tests are discussed, they will be discussed separately from those of procedure 1.

The clinical differences between the two groups were as follows: (a) Undernutrition: The second group of infants was less well nourished than the first, and two of them (F.F. and R.A.; Tables 35, 36) had the lowest weight percentiles (Wt.%) of all infants investigated using this test. It was not known to what extent undernutrition might have contributed to the more rapid folic acid clearances obtained when procedure 2 was used. However, it was noted that one infant, A.H.A. (Table 35) whose weight

percentile was nearly as low as that of F.F. or R.A. had a very slow L.casei folic acid clearance curve.

(b) Incidence of infection: (Tables 35, 36). Infection was noted in all the infants studied according to procedure 2, and in only one studied according to procedure 1. Infection was of a trivial nature and unassociated with pyrexia at the time of investigation. For this reason it seemed unlikely that it produced a greatly increased demand for folate. One infant with a low basal serum L.casei folate level (N.O.) had an L.casei folic acid clearance curve within the range found in healthy, underprivileged infants (procedure 1) despite the presence of trivial infection at the time of investigation (Tables 35, 36). This did not apply to the S.faecalis folic acid clearance curve (Table 37).

(c) The administration of antibiotics: Three of the four infants studied according to procedure 2 received antibiotics (Table 36). Of the antibiotics administered, ampicillin (penbritin) was the only one likely to have affected the folic acid clearance test. However, the normal basal serum L.casei folate level in this infant (R.A.) made this an unlikely possibility.

Last, the apparent difference between the folic acid clearance tests in infants (procedure 1) and adults was of interest. From the findings in this study it could be inferred that the infants concerned had lower folate stores than the adults investigated by Hogan, Maniatis and Moloney (1964). The normal basal serum L.casei folate levels in the infants did not exclude this interpretation because Einhorn and Reizenstein (1966) and Hogan, Maniatis and Moloney (1964) had shown that in certain conditions leading to folate deficiency, the L.casei folic acid clearance curve could be rapid in the absence of low basal serum L.casei folate levels.

However, no inferences were drawn from the apparent difference between folic acid clearance curves in infants and adults for the following reasons:

(a) Coincidental iron deficiency (Table 35): This deficiency was a common finding in infants in this study. It was shown that iron deficiency could be associated with a rapid S.faecalis folic acid clearance curve (Chanarin, Mollin and Anderson, 1958a) and Einhorn and Reizenstein (1966) believed it might result in rapid L.casei folic acid clearance curves. This second finding was not confirmed by Hogan, Maniatis and Moloney (1964).

The effect of iron deficiency on the pattern of the folic acid clearance curves obtained in the infants studied could not be excluded.

(b) Differences in techniques of assaying folate.

It was shown that differences in the technique of assaying folate existed among different laboratories (Table 32). For this reason it seemed doubtful whether a comparison between the results obtained by Hogan, Maniatis and Moloney (1964) and those obtained here was valid. In this regard it was noted that the 15 minute serum folate level of the L.casei folic acid clearance test performed on control adults was reported by Chanarin and McLean (1967) as 55.3 ng./ml. This was comparable to that found in healthy infants here (Table 35) but markedly different from that (Fig. 20) reported by Hogan, Maniatis and Moloney (1964).

Until folic acid clearance tests are performed on normal infants and normal adults and the folate levels determined by the same laboratory it will not be known whether there is a difference between infant and adult folate stores. However, the curves obtained will be regarded as representative of normal for this population group and valid for the purposes of comparison.

WHOLE BLOOD AND RED CELL L.CASEI FOLATE ASSAY
(Hoffbrand, Newcombe and Mollin, 1966)

The differences between this and the serum L.casei method will be stated below: The procedure has been slightly modified from the original method.

TABLE 38

RED CELL FOLATE LEVELS IN NORMAL EUROPEAN SUBJECTS

Red cell sample	Reading ng./ml.
1	201
2	143
3	242
4	287
5	223
6	158
7	107
8	211
9	180
10	230
11	151
12	216
13	262
14	319
15	243
16	188
17	181

Range 107 - 319 ng./ml.

Mean 208 ng./ml.

S.D. ± 54

S.E. of Mean ± 13

TABLE 39

RED CELL FOLATE LEVELS IN NORMAL COLOURED WOMEN

Red cell sample	Reading ng./ml.
1	226
2	282
3	218
4	113
5	224
6	229
7	327
8	297
9	261
10	192
11	202
12	225
13	199
14	234
15	280
16	122
17	153
18	286
19	247
20	300
21	197
22	492

Range 113-492 ng./ml.

Mean 241 ng./ml.

S.D. $\pm$ 79

S.E. of mean $\pm$ 17

TABLE 40

REPRODUCIBILITY BETWEEN REPEAT ASSAYS FOR WHOLE BLOOD FOLATE (L.CASEI)

Whole blood	Folate ng./ml.		Difference	% difference
	Reading 1	Reading 2		
1	132	124	8	6
2	148	100	48	39
3	170	150	20	13
4	124	168	44	30
5	82	82	0	0
6	28	32	4	13
7	42	36	6	15
8	78	104	26	29
9	200	100	100	67
10	40	50	10	23
11	80	116	36	37
12	46	58	12	23
13	134	96	38	33
14	76	92	16	19
15	136	164	28	19
16	128	84	44	42
17	80	96	16	18
18	118	140	22	17
19	110	130	20	17
20	50	62	12	21
21	50	58	8	15
22	112	86	26	26
23	57	54	3	5
24	106	132	26	22
25	54	40	14	30
26	146	132	14	10
27	146	134	12	9
28	142	152	10	7
29	56	82	26	38
30	90	132	42	38
31	100	128	28	25
32	96	90	6	7

TABLE 40 (contd.)

Whole blood	Folate ng./ml.		Difference	% difference
	Reading 1	Reading 2		
33	64	58	6	10
34	108	98	10	10
35	154	96	58	46
36	100	64	36	44
37	174	116	58	44
38	28	42	14	40
39	44	64	20	37
40	144	176	32	37
41	178	196	18	10
42	72	90	18	22
43	66	72	6	9
44	170	140	30	19
45	50	54	4	8
46	48	34	14	34
47	128	110	18	15
48	60	70	10	15
49	46	62	16	30
50	138	70	68	58
51	156	86	70	58
52	108	98	10	10
53	184	110	74	50

Mean difference ng./ml. 25

Mean % difference 25

Procedure.

Blood was collected into a test tube containing 1 drop of heparin. Whole blood (0.5 ml.) was slowly added to 4.5 ml. of distilled water containing 1 g.% freshly added ascorbic acid. The haemolysate was prepared on the day of collection and stored at -20°C. until the day of assay. Then it was slowly thawed at room temperature, thoroughly mixed and an aliquot of 0.4 ml. added to 3.6 ml. of 0.1 M phosphate buffer containing 280 mg.% of ascorbic acid (pH 6.1). The solution was autoclaved for 2½ minutes at 15 lb./sq.in. to precipitate the protein. The assay procedure was completed as previously described for serum L.casei folate assay.

The concentration of folate in whole blood in ng./tube was determined from the standard curve for folic acid set up with each assay. The final result was expressed as ng./ml. after performing the appropriate calculation. The following formula was used to calculate the red cell folate level from the whole blood folate reading.

$$\text{Red cell folate} = \frac{\text{whole blood folate} - \text{serum folate}\left(\frac{(1 - \text{PCV})}{100}\right)}{\frac{\text{PCV}}{100}} \text{ ng./ml.}$$

Normal Range.

The range of red cell folate levels in normal European subjects was 107 - 319 ng./ml. The mean and standard error were 208 ± 13 ng./ml. (Table 38). In normal Coloured women the range was 113 - 492 ng./ml. and the mean and standard error were 241 ± 17 ng./ml. (Table 39).

Accuracy of the method.

The mean difference and mean percentage error between repeat assays were 25 ng./ml. and 25% respectively (Table 40).

TABLE 41

REPRODUCIBILITY BETWEEN REPEAT ASSAYS FOR FOLATE IN MILK*

Milk	Folate ng./ml. Reading 1	Reading 2	Difference	% Difference
1	10	6	4	48
2	8	13	5	44
3	17	21	4	21
4	16	22	6	31
5	24	16	8	42
6	9	24	15	89
7	17	17	0	0
8	20	14	6	35
9	29	22	7	28
10	18	12	6	41
11	36	27	9	28
12	25	22	3	10
13	9	10	1	12
14	33	31	2	5
15	16	14	2	13
16	18	10	8	55
17	10	7	3	33
18	29	25	4	15
19	30	40	10	27
20	12	21	9	54
21	10	12	2	18
22	7	9	2	30
23	28	40	12	35
24	10	13	3	29
25	37	44	7	17
26	30	20	10	41
27	16	18	2	14
28	24	32	8	29
29	13	27	14	71
30	25	23	2	8

TABLE 41 (contd.)

Milk	Folate ng./ml. Reading 1	Reading 2	Difference	% Difference
31	13	18	5	32
32	16	7	9	78
33	13	14	1	9
34	16	26	10	46
35	40	32	8	22

Mean difference ng./ml. = 6

Mean % difference = 32

* Total milk folates.

ESTIMATION OF TOTAL AND TRUE MILK FOLATE USING L.CASEI
(Vanier and Tyas, 1966)

Principle.

Milk may contain substances other than folate which stimulate the growth of L.casei. In order to eliminate this, the true folate in milk is adsorbed on to charcoal. The supernatant is assayed and the activity of the non-folate growth factors determined. This value is subtracted from the total milk folate level to determine the true milk folate concentration.

The differences between the assay techniques for folate in milk and serum will be stated below.

Estimation of total milk folate.

The total milk folate level was determined using the serum L.casei assay procedure,but 0.2 ml. instead of 0.4 ml. aliquots were used.

Estimation of true milk folate.

Procedure.

Phosphate buffer (3.8 ml. of 0.1 M containing 280 mg.% ascorbic acid; pH 6.1) was added to 0.2 ml. of the milk to be assayed. This was autoclaved for 2½ minutes at 15 lb./sq.in.

The coagulum was spun down and the supernatant decanted into a tube containing approximately 16 mg. of activated charcoal. This was well mixed and allowed to stand for 20 minutes at room temperature. After centrifugation, 0.5 ml. of the supernatant fluid was added to each of four 6" x 5/8" borosilicate tubes. The procedure was followed as in the serum L.casei folate assay.

Accuracy of the method.

The mean difference between repeat assays was 6 ng./ml. The mean percentage difference was 32 (Table 41).

ADDITIONAL METHODS

TABLE 42.

BASAL MEDIUM FOR VITAMIN B_{12} ASSAY

	Wt(g)
KH_2PO_4	3.0
$MgSO_4 7H_2O$	4.0
L-Glutamic Acid	30.0
$CaCO_3$	0.8
Sucrose	150.0
DL-Aspartic acid	20.0
DL-Malic acid	10.0
Glycine	25.0
Ammonium succinate	6.0
Thiamine) HCl-Vit B_1) Aneurine)	0.006
p-Aminobenzoic acid	0.0125
"Metals Mix" - see below	0.22
Final volume with freshly collected glass distilled water	5 litres

METALS MIX

$FeSO_4 (NH_4)_2 SO_4 6H_2O$	14.0
$ZnSO_4 . 7H_2O$	4.4
$MnSO_4 . H_2O$ (or $4H_2O$)	1.55 (2.027)
$CuSO_4 . 5H_2O$	0.31
$CoSO_4 . 7H_2O$	0.48
H_3BO_3	0.57
$(NH_4)_6 Mo_7O_{24} . 4H_2O$	0.64
$Na_3VO_4 . 16H_2O$	0.093

ADDITIONAL METHODS

MICROBIOLOGICAL

The determination of Vitamin B_{12} in serum by microbiological assay.

(Anderson,1964; Hutner, Bach and Ross, 1956)

Principle.

A vitamin B_{12} dependent organism is grown under controlled nutritional and environmental conditions in both known concentrations of vitamin B_{12} and unknown concentrations of vitamin B_{12} in serum. The concentration of vitamin B_{12} present in the unknown serum is determined by comparing the bacterial growth in serum with that in known concentrations of vitamin B_{12}.

Materials.

Glassware and equipment: this was cleaned as described for the serum L.casei folate assay.

The assay medium: This is shown in Table 42.

The assay organism: The Z. strain of Euglena gracilis was used. It was maintained by weekly subculture in the liquid stock culture medium recommended by Hutner, Bach and Ross (1956).

The water bath: This differed in detail from that described by Anderson (1964), but it satisfied the important principles. It provided a uniform illumination of 50 ft. candles when measured in the recommended way. It was covered with a black cloth during the incubation phase to eliminate outside light and to ensure that the assay tubes only received light from the strip lights below. It provided a steady temperature of 27 - 28.5°C. The constant circulation of distilled water throughout the bath helped to maintain a uniform temperature.

Venepunctures: The needles, syringes and test tubes used were carefully cleaned to prevent contamination with vitamin B_{12}. The sera were separated and stored at -20°C. until assayed.

Assay procedure.

A 1 in 100 dilution of serum was prepared by adding 0.1 ml. of serum to 9.9 ml. of distilled water. Aliquots (2 ml.) were transferred to three 6" x 5/8" test tubes. This allowed each serum to be assayed in triplicate. Double-strength medium (2 ml.) was added to each tube.

Ampoules of vitamin B_{12} solution containing 100 µg./ml. ("cytamen 100"; Glaxo Laboratories Ltd.) were used to prepare the standard solutions. Standards were set up in quadruplicate to contain 0, 4.0, 8.0, 16.0, 24.0, 32.0 and 40.0 pg. per tube. To these tubes 0.1 ml. of diluted serum with a low vitamin B_{12} concentration was added and the volume made up to 2 ml. with distilled water. Thereafter, 2 ml. of double-strength medium were added to each test tube.

A solution was prepared for the future seven-day-old culture. Vitamin B_{12} (0.3 ml. of a solution of vitamin B_{12} containing 4 ng./ml.), 4.7 ml. of Tryptone (Difco) and 5 ml. of double-strength medium were pipetted in sequence into three 6" x 5/8" test tubes.

All tubes were plugged with cotton wool and heated in a boiling water bath for fifteen minutes. Thereafter they were rapidly cooled in cold water and distributed at random in the test tube racks.

The inoculum was prepared from a seven-day-old culture. This was centrifuged for one minute and thereafter the supernatant was discarded and the cells washed three times by repeated centrifugation with fresh 6 ml. amounts of a single-strength medium (double-strength diluted 1 in 2). The cells were finally suspended in 6 ml. of single-strength medium and well mixed. A 1 ml. aliquot was transferred to a tube containing 4 ml. of medium. The optical density of this 1 in 5 solution was determined by reading this against a sample of single-strength medium using the Unicam photoelectric colorimeter (SP 1300) and a red filter (Ilford 608). This

TABLE 43.

REPRODUCIBILITY BETWEEN REPEAT ASSAYS OF SERUM FOR ESTIMATIONS OF VITAMIN B_{12}

Sera	Serum vitamin B_{12} pg./ml.		Difference	% difference
	Reading 1	Reading 2		
1	1710	2065	355	19
2	700	820	120	16
3	540	590	50	14
4	550	603	53	9
5	180	190	10	5
6	562	630	68	11
7	244	320	76	27
8	1030	1236	206	18
9	400	451	51	12
10	362	433	71	18
11	84	82	2	3
12	394	450	56	13
13	532	602	70	12
14	320	392	72	20
15	540	511	29	6
16	1000	740	260	30
17	216	320	104	39
18	320	464	144	37
19	558	660	102	17
20	376	440	64	16
21	596	696	100	16
22	366	403	37	10
23	328	364	36	10
24	752	856	104	13
25	246	200	46	6
26	550	620	70	12
27	680	740	60	9
28	620	710	90	14
29	562	654	92	15
30	504	616	112	20
31	246	310	64	23
32	224	264	40	16

TABLE 43 (contd.)

Sera	Serum vitamin B_{12} pg./ml.		Difference	% difference
	Reading 1	Reading 2		
33	450	494	44	9
34	510	556	46	9
35	722	712	10	1
36	230	272	42	17
37	608	806	198	18
38	684	646	38	6
39	676	620	56	9
40	530	564	34	6
41	302	253	49	16
42	1530	1485	45	3
43	251	256	5	2
44	400	357	43	11
45	612	674	62	10
46	928	880	48	5
47	367	370	3	1
48	1209	1358	149	12
49	372	388	16	4
50	840	852	12	1
51	810	860	50	6
52	370	362	8	2
53	350	382	32	9
54	620	616	4	1
55	274	300	26	9
56	364	320	44	13
57	410	358	52	14
58	434	348	14	22
59	400	390	10	3
60	270	275	5	2
61	126	140	14	11
62	102	135	33	28
63	410	368	42	11
64	616	624	8	1
65	664	608	56	9

TABLE 43 (contd.)

Sera	Serum vitamin B_{12} pg./ml.		Difference	% difference
	Reading 1	Reading 2		
66	322	350	28	8
67	548	576	28	5
68	650	406	144	46
69	334	382	48	13
70	736	740	4	0
71	908	940	32	4
72	446	360	86	21
73	442	340	102	26
74	726	690	36	5
75	400	494	94	21
76	192	236	44	21
77	714	706	8	1
78	320	346	26	10
79	1044	1044	0	0
80	1044	1008	36	4
81	872	824	48	6
82	712	692	20	3
83	1632	1228	404	28
84	542	690	148	24
85	1244	1214	30	2
86	580	544	36	6
87	510	490	20	4
88	968	860	108	12
89	322	360	38	11
90	954	900	54	6
91	522	490	32	6
92	730	692	38	5
93	337	343	6	2
94	364	350	14	4
95	140	155	15	10

Mean difference 61 pg./ml.

Mean % difference 12

reading was used to determine the dilution of the inoculum required to produce an optical density of 0.182 using a 1 cm. light path.

All tubes containing the sera to be tested and the standard solutions were inoculated with one drop of the standardised suspension of Euglena cells using a syringe fitted with a No.20 spinal needle with a squared-off point. Three drops of the standardised suspension of Euglena cells were added to the inoculum culture tubes. The tubes were placed in the water bath for five days. They were removed on day six, well shaken and transferred to racks and read in cuvettes of 1 cm. light path in the Unicam colorimeter (SP 1300) using a red filter (Ilford 608).

A standard curve was constructed by plotting the concentration of standards against their optical densities. The concentration of vitamin B_{12} (pg. per tube) in the test sera was determined from this graph. The final result was expressed as pg./ml. after performing the appropriate calculation. Each serum was assayed twice in triplicate and the result expressed as the mean of the two assays.

Accuracy of the method.

The mean difference between repeat assays was 61 pg./ml. and the mean percentage difference was 12 (Table 43).

Normal range.

This was 140 - 900 (Mollin, 1960).

TABLE 44

REPRODUCIBILITY BETWEEN REPEAT ASSAYS FOR IRON IN SERUM.

Sera	Serum iron µg./100 ml.		Difference	% difference
	Reading 1	Reading 2		
1	114	115	1	1
2	92	93	1	1
3	136	123	13	10
4	27	33	6	2
5	153	144	9	6
6	117	113	4	4
7	49	45	4	9
8	101	101	0	0
9	123	115	8	7

Mean difference µg./100 ml. = 5

Mean % difference = 6

TABLE 45.

REPRODUCIBILITY BETWEEN REPEAT ESTIMATIONS
FOR CREATININE IN URINE

Urine	Creatinine mg./100 ml.		Difference	% difference
	Reading 1	Reading 2	mg./100 ml.	
1	7.9	6.7	1.2	16.4
2	19.8	18.3	1.5	7.9
3	5.2	5.5	0.3	5.7
4	28.7	28.7	0.0	0.0
5	3.1	3.1	0.0	0.0
6	28.0	28.4	0.4	1.4
7	18.3	18.0	0.3	1.7
8	32.4	32.6	0.2	0.6
9	7.9	8.9	1.0	11.9
10	20.7	21.0	0.3	1.4
11	22.0	23.2	1.2	5.3
12	28.0	28.4	0.4	1.4
13	9.8	11.0	1.2	11.5
14	43.7	43.4	0.3	0.7
15	7.3	8.9	1.6	19.8
16	29.9	29.6	0.3	1.0

Mean difference 0.6 mg./100 ml.

Mean % difference = 5.4

BIOCHEMICAL METHODS

Serum iron estimations (Trinder,1956).

Accuracy of the serum iron method.

The mean difference between repeat assays was 5 µg./100 ml. and the mean percentage difference was 6 (Table 44).

Iron binding capacity (Henry, Sobel and Chiamori, 1958).

Measurement of d-xylose in blood and urine.

Xylose was measured in urine according to Roe and Rice (1948) and in blood according to Lanzkowsky, Lloyd and Lahey (1963).

Normal values for xylose excretion in urine in infants were taken from Shapiro, Morgan and Cozetto (1963). These were 12+ (age in months x 0.1).

Creatinine estimation in urine.

Creatinine in urine was estimated according to Bonsnes and Taussky (1945). In this study compensation had to be made for the difference in the weights of the infants. Hence creatinine was always expressed as the ratio defined below.

$$\frac{\text{actual creatinine output}}{\text{expected creatinine per kg. bodyweight}} \times 100$$

Accuracy of the method.

The mean difference between repeat assays for creatinine in urine was 0.6 mg./100 ml. and the mean percentage difference was 5.4 (Table 45).

Total protein and serum albumin (Weichselbaum, 1946; Martin and Morris,1949).

Serum glutamic oxaloacetic transaminase (Mohun and Cook, 1957).

Occult blood test (Ames Hematest).

BACTERIOLOGICAL METHODS

Standard methods were used for the microscopic examinations and for the stool, urine and blood cultures (Baker, Silverton and Luckcock, 1962).

STATISTICAL METHODS

Standard methods were used (Bailey, 1959).

CHAPTER VI

AN INVESTIGATION OF MATERNAL FOLATE DEFICIENCY

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AN INVESTIGATION OF MATERNAL FOLATE DEFICIENCY

INTRODUCTION

Investigators who studied the problem of megaloblastic anaemia and folate deficiency in pregnancy reported that the highest incidence of megaloblastic anaemia due to folate deficiency occurred after the thirtieth week of pregnancy or in the postpartum phase (Giles and Shuttleworth,1958; Chanarin, MacGibbon, O'Sullivan and Mollin,1959; Gatenby and Lillie,1960). For this reason healthy women were investigated from the 32nd - 40th week of pregnancy in order to determine whether maternal folate deficiency contributed to the development of folate deficiency in their infants.

Laboratory methods had been introduced for diagnosing folate deficiency even in the absence of megaloblastic anaemia. Three of these methods (viz. serum L.casei folate assays, Figlu excretion after oral histidine and hypersegmentation of the neutrophils) were used as parameters for diagnosing maternal folate deficiency. When this study began (1963) and afterwards, laboratory methods were much used to investigate folate deficiency in pregnant women (Solomons, Lee, Wasserman and Malkin, 1962; Berry, Booth, Chanarin and Rothman, 1963; Luhby, Feldman, Salerno and Cooperman, 1963; Ball and Giles, 1964; Chanarin, Rothman and Berry, 1965; Metz, Festenstein and Welch, 1965; Lowenstein, Cantlie, Ramos and Brunton, 1966; Vanier and Tyas, 1966; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966; Hansen, 1967; Temperley, Meehan and Gatenby, 1968). However, it became increasingly clear that laboratory parameters for diagnosing folate deficiency before the development of megaloblastosis, might not always be valid criteria of folate deficiency in pregnancy.

TABLE 46

MATERNAL FOLATE NUTRITION

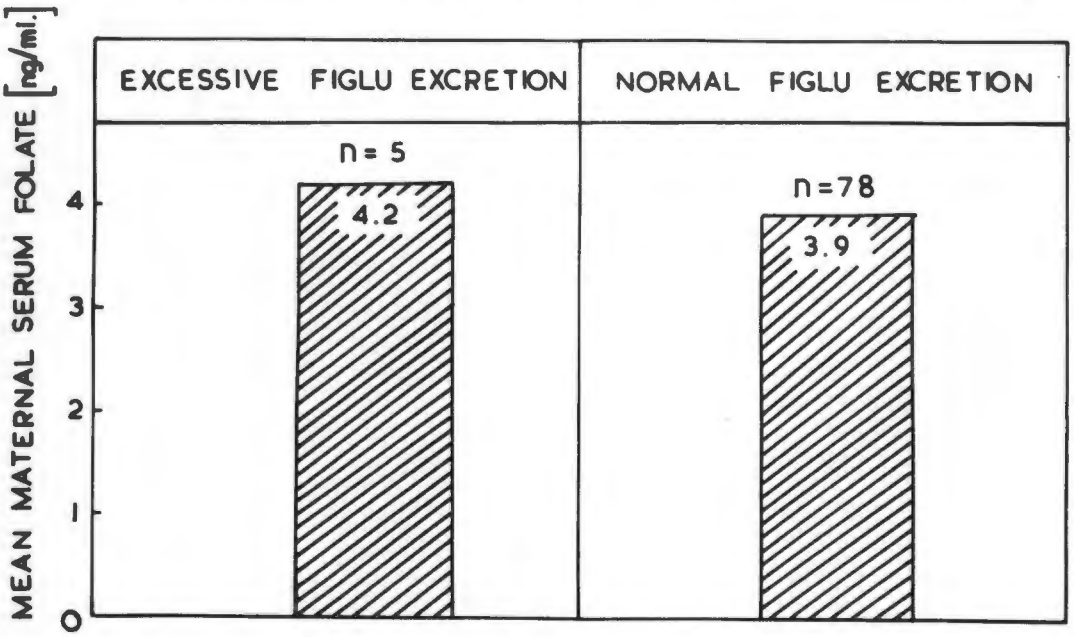
Investigation	Numbers ** investigated	Mean	Range	Standard deviation	Standard error of mean	Numbers with abnormal results
Serum folate ng./ml.	84	3.9	1.2 - 11.7	± 2.1	± 0.2	34
Cord serum folate ng./ml.	69	20.4	7.9 - 47.2	± 7.4	± 0.9	-
Figlu excretion	84	-	-	-	-	5
Percentage hyper-segmentation of neutrophils	85	-	-	-	-	7*

* Range 5 - 11%.

** In the case of the mothers, this refers to the numbers of available and technically satisfactory investigations.

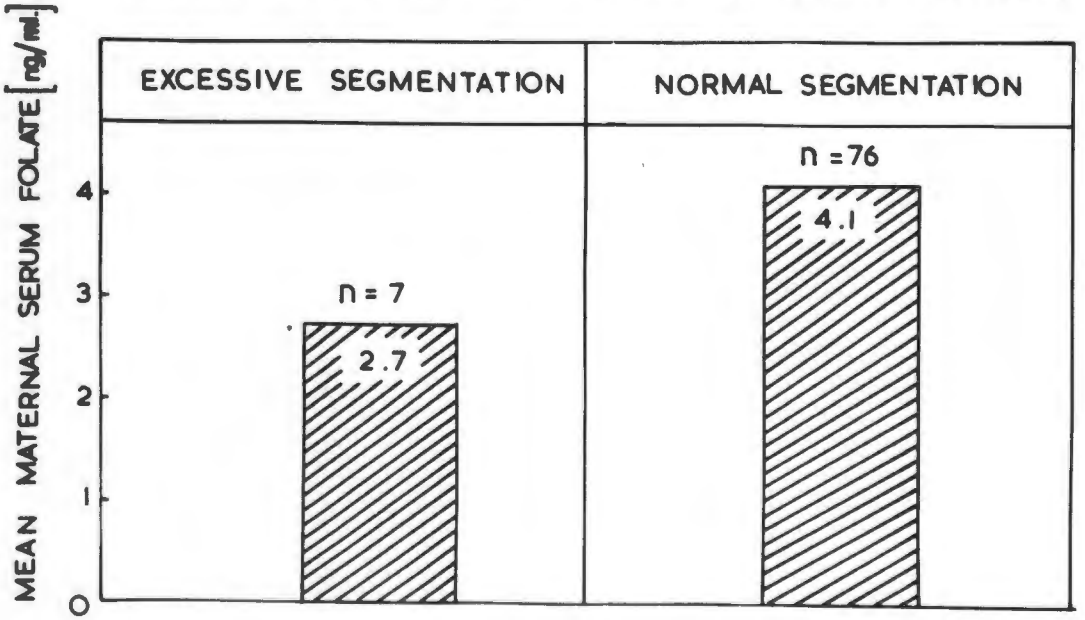
CORRELATION of MATERNAL PARAMETERS of FOLATE DEFICIENCY

MEAN MATERNAL SERUM FOLATE LEVELS
related to MATERNAL FIGLU EXCRETION



DIFFERENCE of MEANS
t = 0.300
0.70 < P < 0.80

MEAN MATERNAL SERUM FOLATE LEVELS
related to POLYMORPHONUCLEAR SEGMENTATION



DIFFERENCE of MEANS
t = 1.750
0.05 < P < 0.10

Fig. 22

TABLE 47

RELATIONSHIP OF HAEMATOCRIT TO PARAMETERS OF FOLATE DEFICIENCY IN MOTHERS

Classification (mothers)	Mean haematocrit vols. %	Test of significance	Significance
Low serum folate levels	35.0 n = 34	'STUDENT'S' t-Test	t = 1.036
Normal serum folate levels	35.5 n = 50		0.30 < P < 0.40
Positive Figlu tests	35.0 n = 5	"	t = 0.325
Negative Figlu tests	35.5 n = 79		0.70 < P < 0.80
Hypersegmentation of neutrophils	35.5 n = 7	"	No difference
Normal segmentation of neutrophils	35.5 n = 78		

Conclusion:
There is no statistical relationship between haematocrit levels and these parameters of folate deficiency in the mothers.

RELATIONSHIP OF MATERNAL AND CORD SERUM FOLATE LEVELS

Classification (mothers)	Mean cord serum folate level	Test of significance	Significance
Low serum folate levels	18.5 n = 27	'STUDENT'S' t-Test	t = 1.680
Normal serum folate levels	21.6 n = 41		0.05 < P < 0.10

Conclusion:
Low maternal serum folate levels are associated with lower cord serum folate levels. The relationship is not statistically significant.

Accordingly, in the discussion below it was necessary to determine how far the results obtained in this study could be used to diagnose maternal folate deficiency. This was necessary before maternal and infant folate interrelationships could be considered and assessed.

RESULTS

The results of the 86 women who brought their infants for study will be presented.

Approximately 40% of the women investigated had low serum L.casei folate levels (34 of 84), but only 6% (5 of 84) had positive Figlu tests after oral histidine and 8% (7 of 85) had hypersegmentation of the neutrophils (Table 46).

There was no statistical relationship between mean maternal serum L.casei folate levels and

(a) Figlu excretion after oral histidine;

(b) percentage hypersegmentation of the neutrophils (Fig. 22).

None of the women with hypersegmentation of the neutrophils excreted Figlu in excess.

There was no statistical relationship between mean haematocrit levels and

(a) low serum L.casei folate levels;

(b) positive Figlu tests after oral histidine;

(c) hypersegmentation of the neutrophils (Table 47).

There was no correlation between mean cord and maternal serum L.casei folate levels (Table 47).

TABLE 48.

SERUM L.CASEI FOLATE LEVELS IN LATE PREGNANCY
(Unsupplemented)

Investigators	Stage of pregnancy	Mean	S.D.	S.E.of Mean	Range
Ball and Giles, 1964.	Last Trimester				
	(Normoblastic)	3.19	± 0.97	-	1.6 - 5.3
	(Megaloblastic)	1.89	± 0.96	-	0.3 - 4.0
Temperley, Meehan and Gatenby, 1968	36th week	2.7	-	± 0.12	0.4 - 8.7
	40th week	1.9	-	± 0.09	0.2 - 5.0
Cape Town Investigation	32nd - 40th week	3.9	± 2.1	± 0.2	1.2 - 11.7

DISCUSSION

The results obtained by means of the laboratory tests used in this study will be discussed in sequence. In each case they will first be compared with those reported by other investigators. Afterwards the validity of each of these tests as diagnostic parameters of folate deficiency in pregnancy and their application in determining maternal and infant folate interrelationships will be discussed. The value of other diagnostic methods for folate deficiency not used in the study will also be mentioned whenever relevant.

Serum L.casei folate assays.

In this study, 40% of women from the 32nd - 40th week of pregnancy had low serum L.casei folate levels. This incidence was within the range (25 - 80%) found by other investigators who studied healthy pregnant women near term (Metz, Festenstein and Welch, 1965; Chanarin, Rothman and Berry, 1965; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966; Lowenstein, Cantlie, Ramos and Brunton, 1966; Vanier and Tyas, 1966; Hansen, 1967).

The mean and range of serum L.casei folate levels was comparable to those reported by other investigators (Table 48).

For the above-mentioned reasons, it was necessary first to examine the validity of the serum L.casei folate assay as a parameter of folate deficiency in pregnancy before one could conclude that 40% of healthy pregnant women had biochemical folate deficiency in the last trimester of pregnancy.

Investigators who carried out serial investigations for folate deficiency reported a decline in mean serum L.casei folate levels towards

term in pregnant women (Solomons, Lee, Wasserman and Malkin, 1962; Ball and Giles, 1964; Chanarin, Rothman and Berry, 1965; Metz, Festenstein and Welch, 1965; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966; Vanier and Tyas, 1966; Hansen, 1967). Similarly, an increasing number of women developed low serum L.casei folate levels as term approached (Chanarin and Rothman, 1964; Metz, Festenstein and Welch, 1965; Vanier and Tyas, 1966; Hansen, 1967).

These changes were noted in healthy pregnant women (Hansen, 1967); normal pregnant women (Chanarin, Rothman and Berry, 1965) and unselected pregnant women (Ball and Giles, 1964; Solomons, Lee, Wasserman and Malkin, 1962).

It seemed that the pattern of decline in mean serum L.casei folate levels as term approached could be attributed to any of the following:

(a) the development of maternal folate deficiency;

(b) increased demand for folate;

(c) the effect of increasing blood volume as pregnancy advanced.

From the above studies it was not possible to exclude the effect of blood volume changes in producing this finding. This was supported by the finding that small (Willoughby and Jewell, 1966; Hansen and Rybo, 1967) and large doses of folic acid (Metz, Festenstein and Welch, 1965; Hansen and Rybo, 1967) did not result in a return to normal serum L.casei folate levels in all pregnant women.

However, it did not seem that the low serum L.casei folate levels could be attributed to the effect of blood volume changes alone. The decline in serum L.casei folate levels towards term occurred in both non-anaemic (Ball and Giles, 1964; Vanier and Tyas, 1966) and anaemic women (Ball and Giles, 1964; Solomons, Lee, Wasserman and Malkin, 1962).

Also, serial studies performed during pregnancy using the folic acid clearance test (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959) and red cell L.casei folate studies (Chanarin, Rothman, Ward and Perry, 1968; Hansen, 1967) showed the same decline in mean values towards term as had been shown with the serum L.casei folate assay. The results with the first test could not be attributed to the effect of blood volume changes alone (Chanarin, MacGibbon, O'Sullivan and Mollin, 1959; Girdwood and Delamore, 1961) and red cell L.casei folate levels should not have been affected by blood volume changes.

In addition to these arguments there was positive evidence for attributing the low serum L.casei folate levels to folate deficiency or increased demand for folate. It was shown that women with low serum L.casei folate levels in the last trimester of pregnancy still had low values in the puerperium when the physiological and metabolic stresses of pregnancy were removed (Metz, Festenstein and Welch, 1965; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966; Hansen, 1967; Temperley, Meehan and Gatenby, 1968).

The administration of small doses of oral folic acid (300 µg.daily) to non-anaemic pregnant women during pregnancy maintained serum L.casei folate activity at near normal levels in the postpartum phase in 84% of women as compared with 55% in a control group not given folic acid (Willoughby and Jewell, 1966). Physiological doses of folic acid (200 µg./day) also improved mean serum L.casei folate levels, but the percentage of women who still had low serum L.casei folate levels was slightly higher (Hansen and Rybo, 1967).

As in the case of red cell L.casei folate levels (Chanarin, Rothman, Ward and Perry, 1968), there was a relationship between the L.casei serum

folate levels in early pregnancy and the subsequent development of megaloblastic anaemia (Temperley, Meehan and Gatenby, 1968). Moreover there was a good relationship between red cell L.casei folate levels and food folate intake (Chanarin, Rothman, Ward and Perry, 1968).

From these findings it appeared that low serum L.casei folate levels were primarily the result of biochemical folate deficiency. However, there might have been two factors which limited the application of the test in assessing a relationship between maternal and infant folate deficiency.

First, it seemed that low serum L.casei folate levels did not always indicate significant folate deficiency. Apart from Temperley, Meehan and Gatenby (1968), investigators did not find a relationship between low serum L.casei folate levels and haemoglobin concentration (Solomons, Lee, Wasserman and Malkin, 1962; Chisholm, 1966; Edelstein, Stevens, Baumslag and Metz, 1968).

Moreover, low serum L.casei folate levels were not necessarily associated with morphological changes in the bone marrow (Lowenstein, Cantlie, Ramos and Brunton, 1966; Chanarin, Rothman, Ardeman and Berry, 1965). Also the lower limit of reduced serum L.casei folate levels found in pregnant women with normoblastic marrows could overlap with those found in pregnant women with megaloblastic bone marrows (Ball and Giles, 1964). For these reasons the serum L.casei folate assay was regarded as an unreliable index of megaloblastosis (Chanarin, Rothman and Berry, 1965).

To overcome this problem Giles (1966) compared labile serum L.casei folate levels with megaloblastosis. Although he observed a 95% correlation, his method was criticised on technical grounds (Chanarin, Rothman, Ardeman and Berry, 1965). Hansen (1967) claimed that serum L.casei folate assays

could be successfully used as an index of megaloblastic bone marrow morphology, provided abnormal levels were defined as being lower than those found in normoblastic pregnancies. This was not confirmed by Chanarin (1967).

Thus, if low maternal serum L.casei folate levels did not always imply significant maternal folate deficiency and if it were correct that infants deprived their mothers of folate (Grossowicz, Aronovitch, Rachmilewitz, Izak, Sadovsky and Bercovici, 1960; Baker, Frank, Pasher, Ziffer and Sobotka, 1960; Zachau-Christiansen, Hoff-Jørgensen and Kristensen, 1962), it was possible that low maternal serum L.casei folate levels would not be associated with any restriction of foetal folate stores in the majority of cases.

Second, there was uncertainty as to whether serum L.casei folate levels were always low in megaloblastic anaemia due to folate deficiency in pregnancy. Some investigators were impressed with the agreement of these two parameters in pregnancy (Lowenstein, Brunton and Hsieh, 1966; Lawrence and Klipstein, 1967). Other investigators found that serum L.casei folate levels could be low or normal in the presence of megaloblastic anaemia due to folate deficiency in pregnancy (Chanarin, Rothman, Ardeman and Berry, 1965; Varadi, Abbott and Elwis, 1966), and in the postpartum phase (Edelstein, Zail, Faulding and Metz, 1967).

Most evidence was in favour of attributing the low serum L.casei folate levels in pregnancy to biochemical folate deficiency. However, the use of this investigation to determine whether maternal folate deficiency contributed to infant folate deficiency might prove disappointing for two reasons:

(i) low maternal serum L.casei folate levels might not necessarily mean reduced foetal folate stores, and

(ii) serum L.casei folate levels could sometimes be normal in spite of significant maternal folate deficiency which might have restricted infant folate stores.

Red cell L.casei folate estimations.

These were not performed in this study. Some investigators found that low red cell L.casei folate levels were a reliable index of significant folate deficiency in pregnancy (Varadi, Abbott and Elwis, 1966; Hansen, 1967). However, other investigators noted that red cell L.casei folate levels could be normal in the presence of megaloblastosis due to folate deficiency in pregnancy (Chanarin, Rothman, Ward and Perry, 1968; Lowenstein, Brunton and Hsieh, 1966). Thus the red cell L.casei folate assays might have proved a better index of depleted maternal and therefore foetal folate stores but its application would also have been limited because red cell L.casei folate levels were not always low in the presence of significant maternal folate deficiency.

The Figlu test after oral histidine.

The incidence of positive Figlu tests after oral histidine found here (8%) was slightly lower than that reported by others who used the same method of estimating Figlu (Kohn, Mollin and Rosenbach, 1961). Karthigiani, Gnanasundaram and Baker (1964) reported an incidence of 18% positive Figlu tests after oral hiatidine in healthy pregnant women near term but an incidence of 9% in those with normoblastic marrows. The incidence reported by Husain, Rothman and Ellis (1963) was 20% in unselected pregnancies near term and in the puerperium.

Using sensitive methods of estimating Figlu (enzymatic and high voltage electrophoretic methods) investigators conducted serial investigations with this test during pregnancy. From a study of the pattern of Figlu excretion during pregnancy, it seemed unlikely that the positive Figlu tests after oral histidine could be attributed to uncomplicated folate deficiency.

There was general agreement that there was a high incidence of positive Figlu tests in early pregnancy (Berry, Booth, Chanarin and Rothman, 1963; Metz, Festenstein and Welch, 1965; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966; Stone, Luhby, Feldman, Gordon and Cooperman, 1967). This finding was not only different from findings reported of the other laboratory parameters used for diagnosing folate deficiency in pregnancy, but it also implied that biochemical folate deficiency developed before pregnancy was very far advanced.

There was disagreement about the pattern of Figlu excretion after early pregnancy until term. Berry, Booth, Chanarin and Rothman (1963) noted a decline in mean Figlu excretion and a decrease in the number of positive Figlu tests after oral histidine.

The decline in mean Figlu excretion after oral histidine during pregnancy was confirmed by Metz, Festenstein and Welch (1965) and Edelstein, Stevens, Brandt, Baumslag and Metz (1966). A decrease in the incidence of positive Figlu tests after early pregnancy was noted in White patients (Metz, Festenstein and Welch, 1965), but to a much lesser extent in African patients (Edelstein, Stevens, Brandt, Baumslag and Metz, 1966), where the incidence of positive results was as high as 60% near term.

After a decline in positive Figlu results after oral histidine in the middle trimester, Stone, Luhby, Feldman, Gordon and Cooperman (1967)

noted a high incidence (60%) of positive results towards term. Their data included results from women with obstetrical complications during pregnancy.

The incidence of positive Figlu tests after oral histidine was again high in the puerperium (Berry, Booth, Chanarin and Rothman, 1963; Metz, Festenstein and Welch, 1965; Edelstein, Stevens, Brandt, Baumslag and Metz, 1966).

Those investigators who reported a decline in mean Figlu excretion as pregnancy advanced, attributed it to the following:

(a) increased used of histidine for protein synthesis (Chanarin, Rothman and Watson-Williams, 1963; Berry, Booth, Chanarin and Rothman, 1963);

(b) increased transfer of urocanic acid to the foetus (Chanarin, Rothman, Ardeman and McLean, 1965);

(c) delayed absorption of histidine in pregnancy (Chanarin, Rothman and Watson-Williams, 1963);

(d) histidinuria in pregnancy (Chanarin, Rothman and Watson-Williams, 1963).

These last two factors were of less importance, but all reduced the efficacy of the histidine load and so could render the Figlu test an unreliable test of folate deficiency in pregnant patients.

The most important evidence against attributing the results of positive Figlu tests after oral histidine to folate deficiency was the poor agreement noted between the results of positive Figlu tests after oral histidine and those of other parameters of folate deficiency in early pregnancy (Hansen, 1967), late pregnancy (Lowenstein, Cantlie, Ramos and

Brunton, 1966; Chisholm and Sharp, 1964; Chanarin, Rothman, Ardeman and Berry, 1965; Edelstein, Stevens, Baumslag and Metz, 1968), and in the puerperium (Edelstein, Zail, Faulding and Metz, 1967).

Other investigators reported a good agreement between Figlu excretion after oral histidine and other parameters of folate deficiency (Stone, Luhby, Feldman, Gordon and Cooperman, 1967; Luhby, Feldman, Salerno and Cooperman, 1963; Hibbard, 1964; Scott and Sommerville, 1965).

Although still controversial, most of the evidence did not confirm the value of the Figlu test after oral histidine as a parameter of folate deficiency in pregnancy. Whereas in experimental nutritional folate deficiency, positive Figlu tests after oral histidine could be attributed to significant folate depletion, this did not appear to be the case in pregnancy. Moreover, the test could be an unreliable index of megaloblastosis because histidine metabolism could be disturbed in pregnancy. For these reasons the application of this test as a parameter of maternal folate deficiency in the subsequent assessment of maternal and infant folate interrelationships seemed very limited.

Hypersegmentation of the neutrophils.

The majority of women received iron supplementation during pregnancy. The incidence of hypersegmentation found here (8%) was best compared with that found in pregnant women receiving iron supplementation. Chanarin, Rothman and Berry (1965) found that 4 out of 50 healthy pregnant women given iron supplements developed hypersegmentation (8%).

Again the value of this parameter in the diagnosis of folate deficiency was uncertain. It was reported that hypersegmentation was not an uncommon finding in late pregnancy amongst non-anaemic healthy women,

but that its relationship to folate deficiency in these circumstances was uncertain (Hansen, 1967). Moreover, hypersegmentation of the neutrophils (more than 3%) could be found in the absence of megaloblastosis due to folate deficiency in pregnancy and vice versa (Chanarin, Rothman and Berry, 1965; Chanarin, Rothman, Ardeman and Berry, 1965).

Lowenstein, Cantlie, Ramos and Brunton (1966),and Varadi, Abbott and Elwis (1966) showed that hypersegmentation of the neutrophils correlated with other evidence of folate deficiency only if 5% or more of neutrophils showed hypersegmentation. It seemed that the significance of hypersegmentation as a parameter of folate deficiency in pregnancy still had to be established. Until such time it was best used as a guide to the possible presence of underlying folate or vitamin B_{12} deficiency (Chanarin, Rothman and Berry, 1965). The application of this parameter as an index with which to assess the effect of maternal folate nutrition on infant folate nutrition also seemed very limited.

Bone marrow examinations.

Minimal megaloblastic bone marrow changes were found in 75% (Roberts, Waters and Mollin, 1963), and 25% (Hansen, 1967) of non-anaemic normal women in the last trimester of pregnancy. The reason for this finding and its relationship to folate deficiency were unknown. Accordingly Chanarin (1967) did not diagnose megaloblastosis in pregnancy unless there were unequivocal changes in the red cell precursors.

It was stated that none of the laboratory parameters for diagnosing folate deficiency in pregnancy could be reliably used to indicate megaloblastosis and therefore significant folate deficiency in pregnancy (Chanarin, 1967). This opinion appears to be justified after an

evaluation of the reports on the use of laboratory methods in the diagnosis of folate deficiency in pregnancy. Moreover, the validity of some of the laboratory methods for diagnosing biochemical or morphological folate deficiency in pregnancy is still uncertain. This probably explained the poor agreement between the parameters of folate deficiency used in this study. For these reasons it seemed that unequivocal megaloblastosis associated with supportive evidence of folate deficiency or a normal serum vitamin B_{12} level would have been the best test of significant folate deficiency in pregnancy. It might also have presented the best method of assessing the effect of maternal folate deficiency on infant folate stores. At the time the study was conducted this was not clear and as a consequence, bone marrow examinations on healthy pregnant women were not thought advisable.

SUMMARY.

The actual observations noted in pregnant women in Cape Town were similar to those reported by other investigators who used comparable methods. The failure to demonstrate a relationship between the parameters of folate deficiency in this study could be explained on the basis of possible differences in the interpretation of these tests in pregnancy.

Of the tests used in this study, the serum L.casei folate assay appeared to be the best parameter of maternal folate deficiency. However, its application in determining the effect of maternal on infant folate nutrition appeared to be limited because it did not appear to be a reliable index of significant maternal folate deficiency and therefore might not have been a reliable index of foetal folate stores.

The validity of the Figlu test after oral histidine and hypersegmentation of the neutrophils as parameters of folate deficiency could not be established. Their application in future studies to test the effect of maternal on infant folate nutrition was therefore even more limited. These results will be examined in future chapters only because there are investigators who believe in the value of these tests, particularly the Figlu test after oral histidine, as parameters of folate deficiency in pregnancy.

The best criterion of significant folate deficiency in pregnant women would have been the presence of unequivocal megaloblastosis supported by confirmatory evidence of folate deficiency.

CHAPTER VII

THE INTERPRETATION OF THE FIGLU TEST AFTER ORAL HISTIDINE

TABLE 49.

METHODS USED FOR MEASURING FIGLU EXCRETION AFTER ORAL HISTIDINE IN INFANCY.

Investigators	Dose of histidine	No. of doses	Number and classification of subjects	Duration of urine collection	Method of estimation	Normal Range
Vanier & Tyas, 1966.	0.1 - 0.3 g./kg.	1	24 infants	0-6 hours after histidine	Knowles, Prankerd & Westall,1960(modified high voltage electrophoresis)	>50 µg./ml. or >6 mg./6 hrs.
Shojania and Gross, 1964a.	0.333 g./kg.	1 dose daily for 3 days	6 premature infants	Between 5-8 hours after last histidine	Zalusky and Herbert, 1962.	>100 µg./ml.
Arakawa,Ohara Fujii, Hirata and Takahashi, 1965.	0.33 g./kg.	1	14 control and healthy infants	0-3 hours after histidine	Tabor and Wyngarden, 1958,or Arakawa,Ohara Fujii & Hirono, 1965	>40 µg./ml.
Speetor,Falcke, Yoffe, Metz, 1966.	10 g.	1 x daily for 2 days	10 healthy infants 6-24 months	0-8 hours after histidine	Chanarin and Bennett, 1962a.	0-76 µg./ml. (1 dose) 33-97 µg./ml. (2 doses)
Allen & Whitehead, 1965.	2 - 5 g.	One dose	12 protein malnourished infants	0-12 hours after histidine	Low voltage modification of Knowles,Prankerd & Westall,1960.	———
Naiman, 1966	0.3 g./kg.	One dose	Infants and children	0-6 hours after histidine	Knowles, Prankerd & Westall, 1960.	Semi-quantitative
Luhby and Cooperman, 1964.	0.12 g./lb.	3 divided doses 4 hrs.apart	normal infants	0-24 hours after histidine	Tabor and Wyngarden, 1958.	>30 µg./ml.

CHAPTER VII.

THE INTERPRETATION OF THE FIGLU TEST AFTER ORAL HISTIDINE.

INTRODUCTION

The infants studied were healthy but underprivileged. Thus, associated nutritional deficiencies and recurrent infection, mostly trivial, were often encountered in the group. It was therefore more unfortunate that infants from a high socio-economic group could not be investigated to establish a standard of normal Figlu excretion for the experimental conditions of the study.

Since 1963 additional investigators had used the Figlu test after oral histidine as a parameter of folate deficiency in infants. There was little uniformity in:

(a) the dose of histidine administered;

(b) the length of period during which urine was collected;

(c) the method of estimation of Figlu;

(d) the population group examined; (Table 49).

In this investigation the procedure of execution of the Figlu test after oral histidine and the method of estimating Figlu (Tabor and Wyngarden, 1958) were those recommended by Luhby (1963) and Luhby and Cooperman (1964). Accordingly, their criterion of normal Figlu excretion for infants given a histidine load was accepted. This was modified from 30 µg./ml. to 45 µg./ml. to compensate for possible differences in assay technique (Chapter V).

It was necessary to use a second method for measurement of Figlu in the study. An investigation carried out applying this method (Chanarin

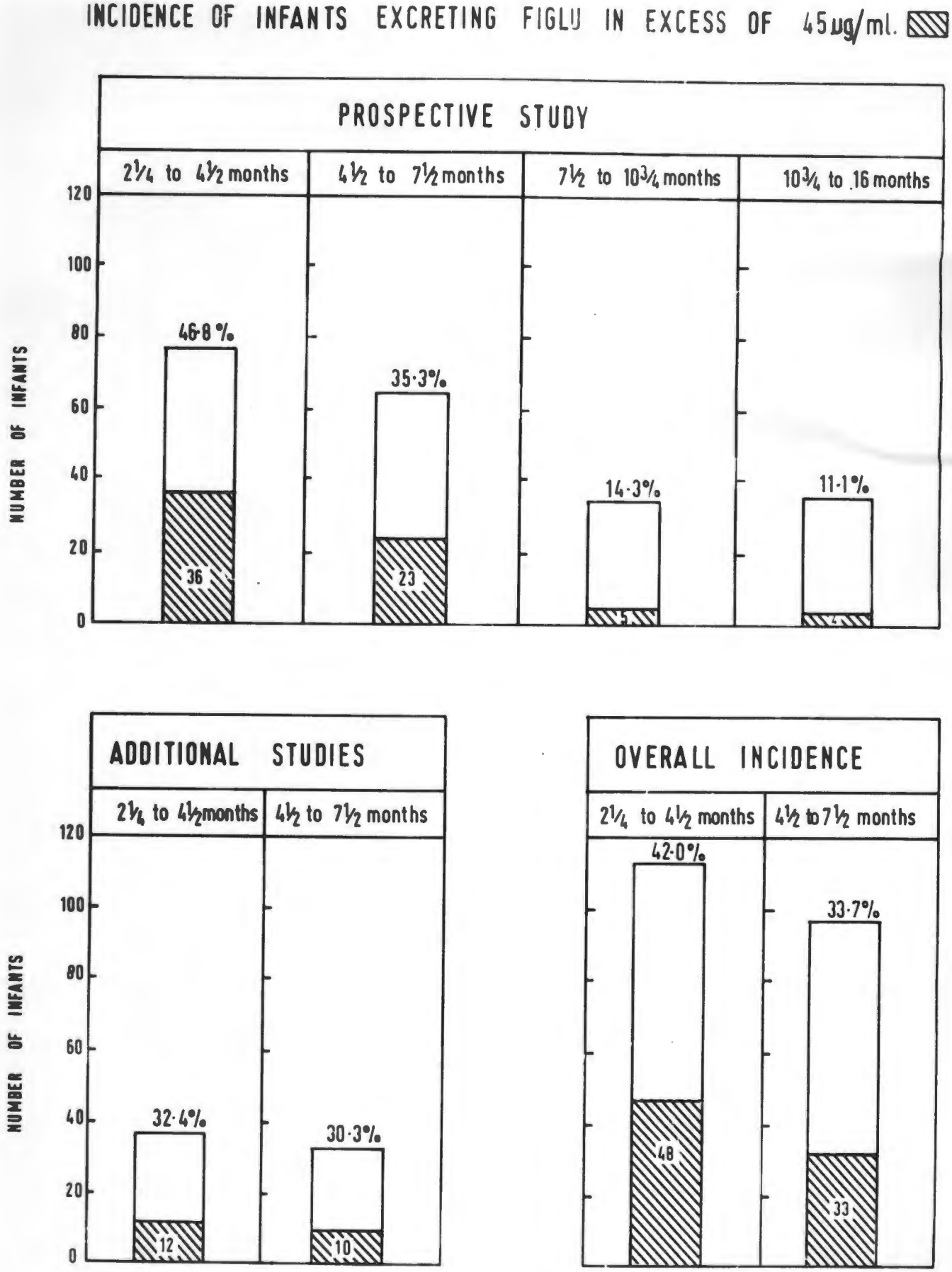

INCIDENCE OF INFANTS EXCRETING FIGLU IN EXCESS OF 45 µg/ml.
PROSPECTIVE STUDY
2¼ to 4½ months
4½ to 7½ months
7½ to 10¾ months
10¾ to 16 months
NUMBER OF INFANTS
120
100
80
60
40
20
0
46·8 %
35·3%
14·3%
11·1%
36
23
5
4
ADDITIONAL STUDIES
2¼ to 4½months
4½ to 7½ months
32·4%
30·3%
12
10
OVERALL INCIDENCE
2¼ to 4½ months
4½ to 7½ months
42·0%
33·7%
48
33

Fig. 23

TABLE 50.

CONTROLLED THERAPEUTIC TRIAL.

Number of infants excreting Figlu in excess[XX]

(a) Initial study.

Age (months)	Investigation	Positive	Borderline	Negative	Total	Percentage of positive results.
$2\frac{3}{4}$ - 5	Figlu test	8	4	33	45[X]	17.8

(b) Follow up study - after therapy.

(months)	Investigation	Positive	Borderline	Negative	Total	Percentage of positive results.
$3\frac{1}{2}$ - $5\frac{3}{4}$	Figlu test	6	7	32	45[X]	13.3

[X] One exclusion prior to therapy because of significant vomiting of histidine

One exclusion after therapy because of high urocanic acid excretion probably interfering with the interpretation of Figlu test.

[XX] Method of estimation - Chanarin and Bennett (1962a).

INCIDENCE OF INFANTS EXCRETING FIGLU IN EXCESS OF 30 µg./ml. ▨

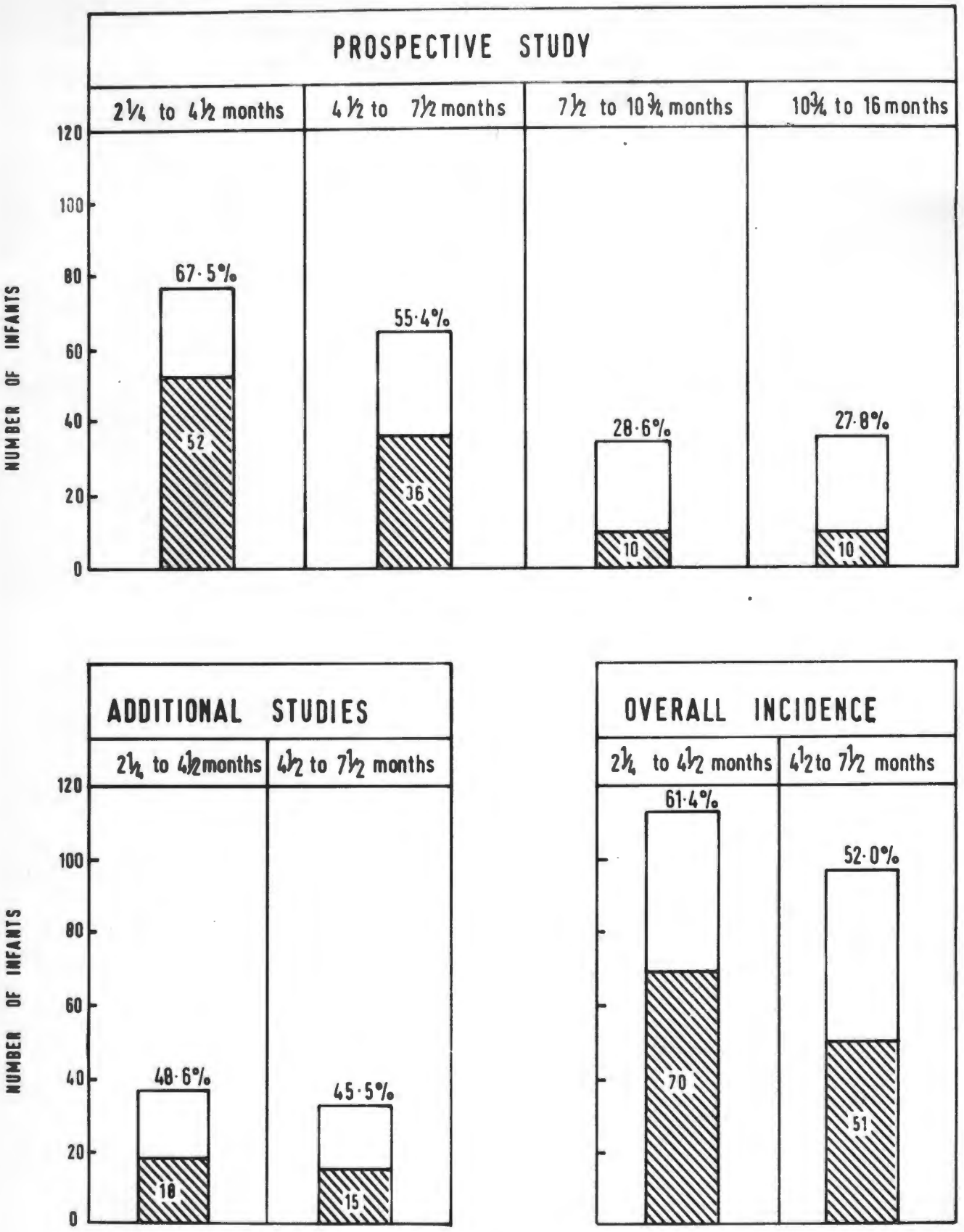


Fig. 24

and Bennett, 1962a) showed that infants who passed concentrated urine might have false positive Figlu tests if results were expressed as Figlu concentration (μg./ml.) rather than total Figlu output (mg./24 hrs.). For this reason a standard of normality comparable to that used with the method of Tabor and Wyngarden (1958) was defined in terms of total Figlu output (mg./24 hrs.). By using this criterion, spurious positive tests resulting from urinary concentration were avoided (Chapter V).

The results using the different methods were separately analysed because the method of Figlu estimation and the criterion of normality were different. The method of Tabor and Wyngarden (1958) will be referred to as method A and that of Chanarin and Bennett (1962a) as method B. The results will be discussed simultaneously with a view to determining the interpretation of the Figlu test after oral histidine.

RESULTS.

Incidence of Positive Results (Fig.23; Table 50).

The incidence of positive results was greatest in the youngest group of infants. In this age group the incidence was lower when method B was used for estimating Figlu.

Incidence of Positive Results (%)

	METHOD A (Age $2\frac{1}{4}$ - $4\frac{1}{2}$ months)	METHOD B (Age $2\frac{3}{4}$ - 5 months)
Prospective Study	46.8	
Additional Studies	32.4	17.8
Overall Study (Combined Studies)	42.0	

The incidence of positive Figlu tests after oral histidine declined with growth (Fig. 23; Table 50).

INCIDENCE OF INFANTS EXCRETING FIGLU IN EXCESS OF 30 µg./ml.

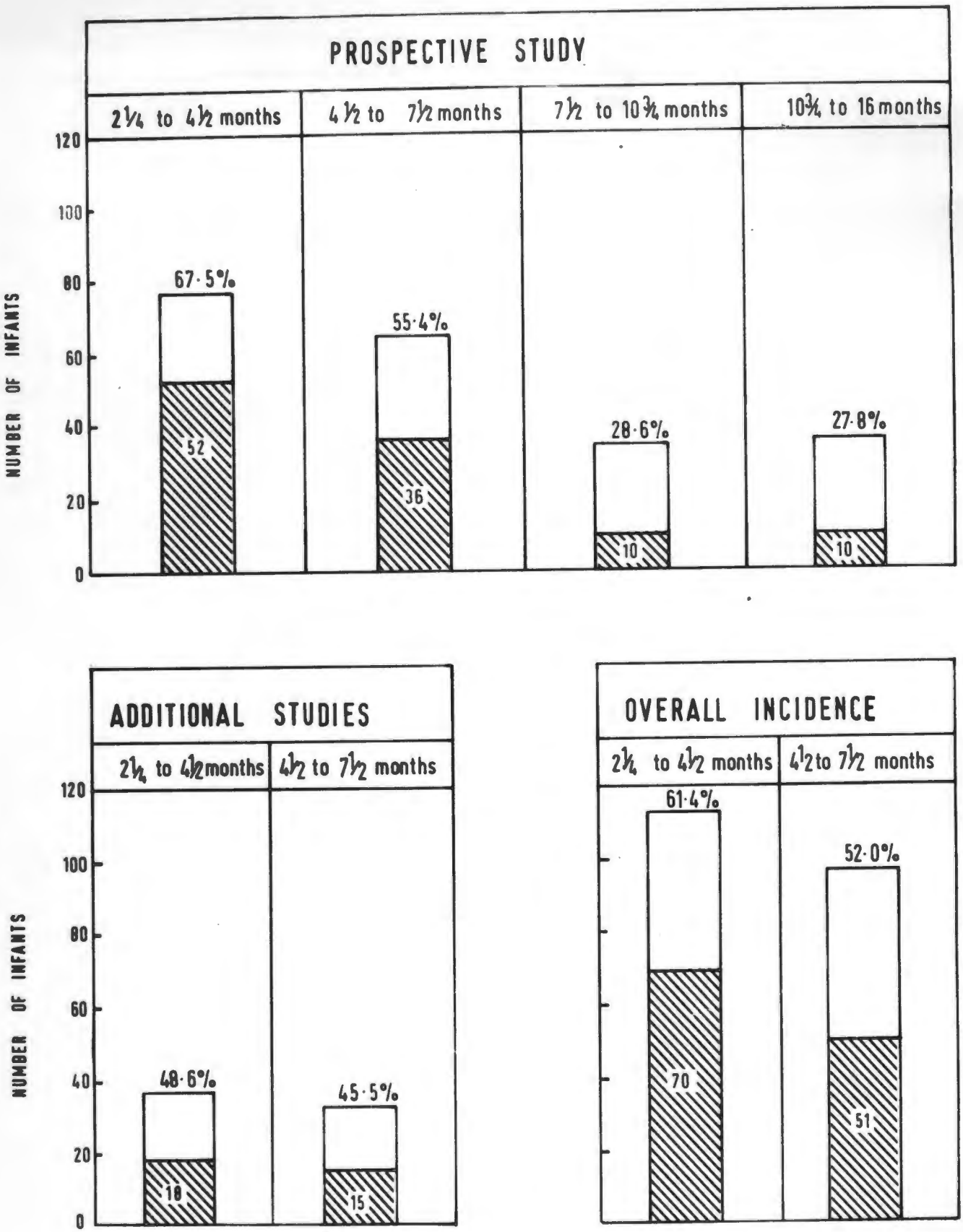


Fig. 24

MEAN FIGLU EXCRETION IN µg./ml

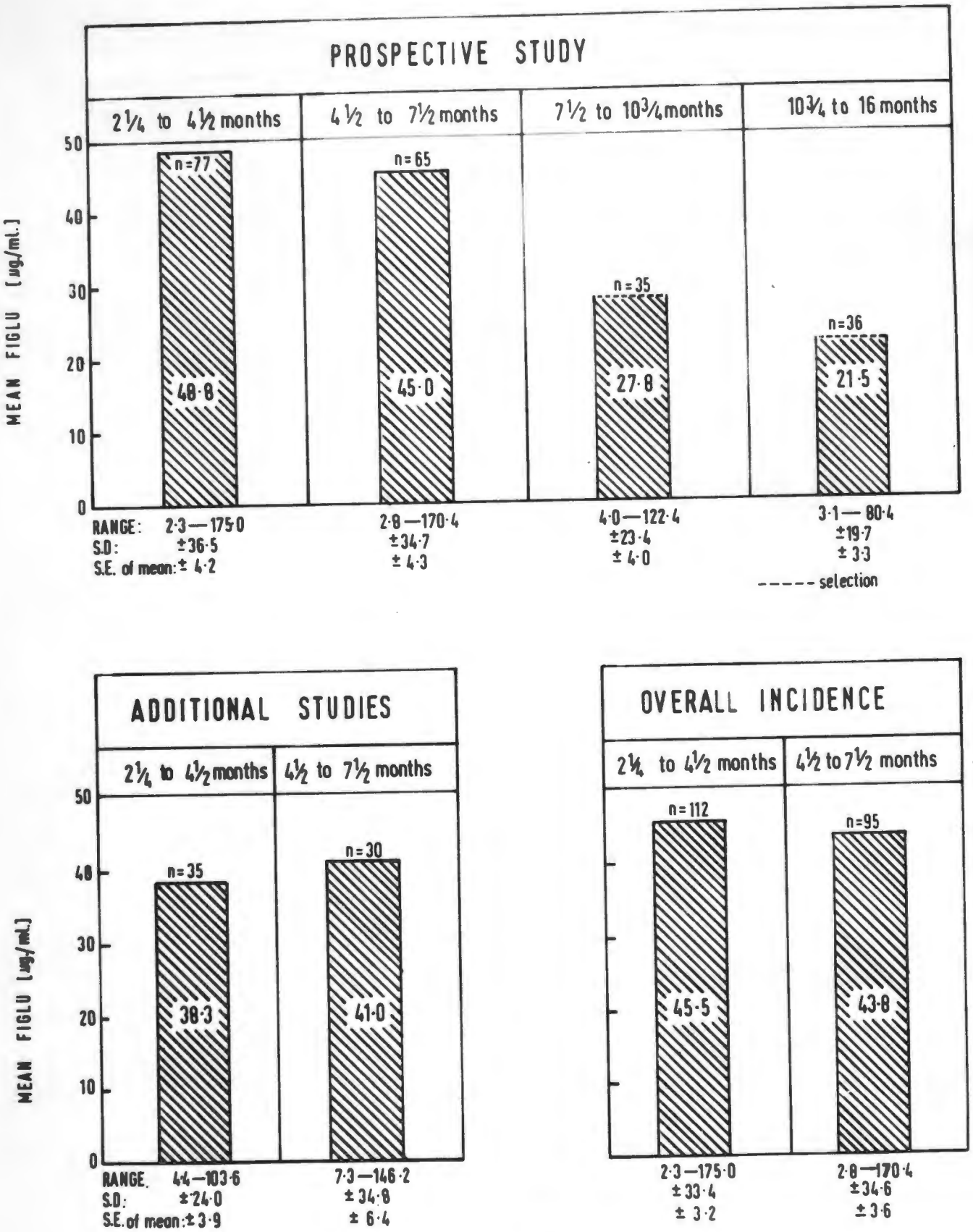


Fig. 25

MEAN FIGLU EXCRETION IN mg/24 hours

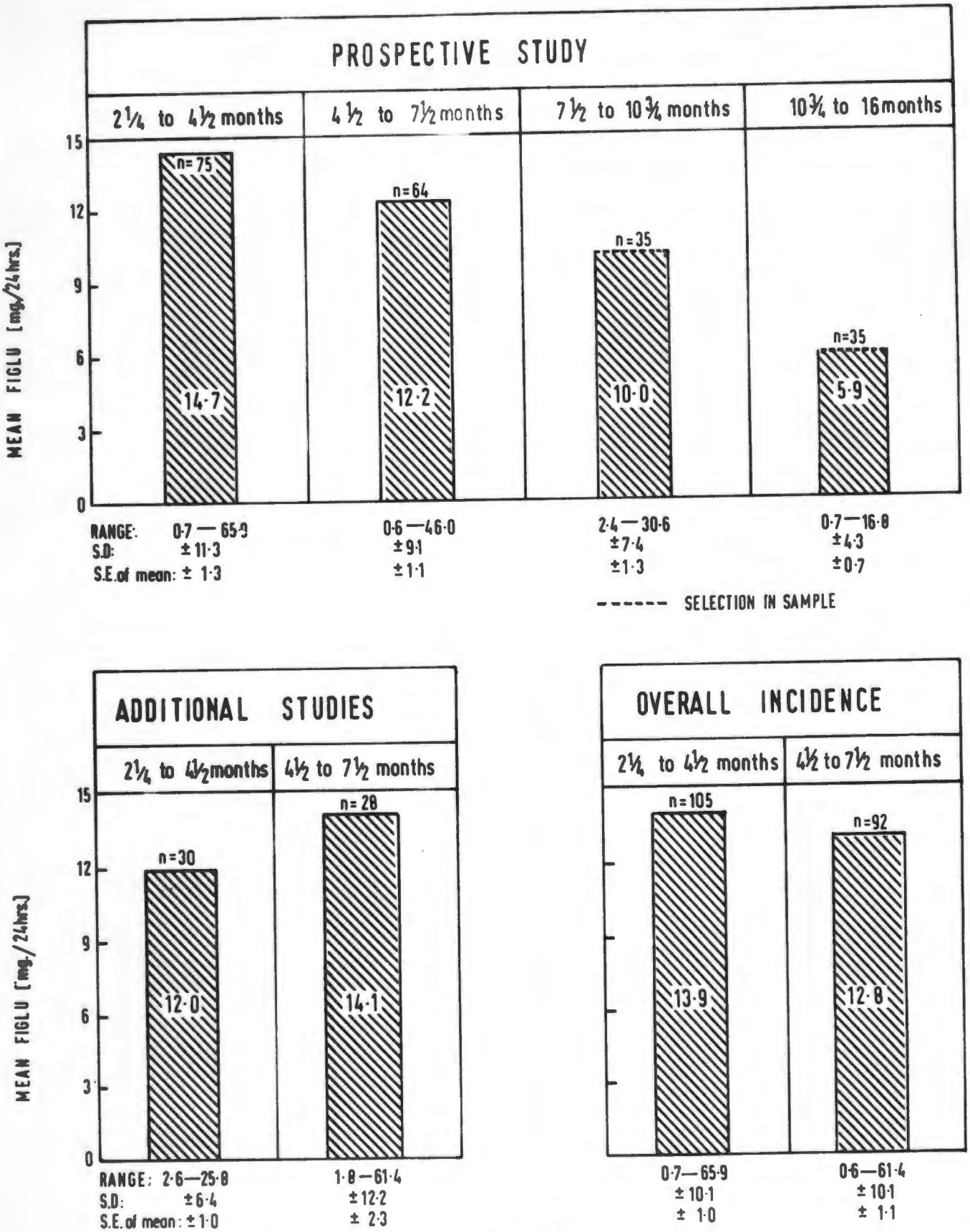


Fig. 26

TABLE 51.

MEAN AND RANGE OF FIGLU EXCRETION IN 45 INFANTS.

(a) Initial study.

Figlu µg./ml.				Figlu mg./24 hrs.			
Mean	Range	S.D.	S.E. of Mean	Mean	Range	S.D.	S.E. of Mean
22.7	1.1-181.5	± 28.5	± 4.3	7.6	0.5-27.2	± 5.6	± 0.8

(b) Follow up study - after therapy.

Mean	Range	S.D.	S.E. of Mean	Mean	Range	S.D.	S.E. of Mean
24.8	1.2-152.9	± 28.5	± 4.3	6.9	0.4-26.6	± 5.8	± 0.9

Incidence of Positive Results(%)

METHOD A (Prospective Study)		METHOD B	
$2\frac{1}{4}$ - $4\frac{1}{2}$ months	46.8	$2\frac{3}{4}$ - 5.0 months	17.8
$10\frac{3}{4}$ - 16 months	11.1	$3\frac{1}{2}$ - $5\frac{3}{4}$ months	13.3

The incidence of positive results might have been higher had 30 µg./ml. been used as the criterion of normality (Method A; Fig. 24). Also, the incidence of positive results might have been higher using method B had Figlu excretion been measured in concentration (µg./ml.) rather than in quantity (mg./24 hrs). In these circumstances in the age group $3\frac{1}{2}$ - $5\frac{3}{4}$ months the incidence would have increased from 13% to 29%.* It would not have made any difference in the age group $2\frac{3}{4}$ - 5.0 months.**

Mean Figlu Excretion after Oral Histidine (Fig.25, 26; Table 51).

The mean Figlu excretion was highest in the youngest age group. In this age group mean Figlu excretion was highest in the infants of the prospective study and lowest in those where Figlu was estimated with method B.

Mean Figlu Excretion (µg./ml.)

	METHOD A (Age $2\frac{1}{4}$ - $4\frac{1}{2}$ months)	METHOD B (Age $2\frac{3}{4}$ - 5.0 months)
Prospective Study	48.8	
Additional Studies	38.3	22.7
Overall Study (Combined Study Groups)	45.5	

*Borderline and positive tests together gave an incidence of 29% (13 of 45).

**In this group the borderline positive results were so graded because of total Figlu output and not because of Figlu concentration.

Mean Figlu Excretion (mg./24 hrs.)

METHOD A (Age $2\frac{1}{4}$ - $4\frac{1}{2}$ months)		METHOD B (Age $2\frac{3}{4}$ - 5.0 months)	
Prospective Study	14.7		
Additional Studies	12.0		7.6
Overall Study (Combined Study Groups)	13.9		

The mean Figlu excretion declined with growth although this was not obvious when Figlu concentration was measured over a time period of three weeks using method B.

Mean Figlu Excretion (µg./ml.)

METHOD A (Prospective Study)		METHOD B	
$2\frac{1}{4}$ - $4\frac{1}{2}$ months	48.8	$2\frac{3}{4}$ - 5.0 months	22.7
$10\frac{3}{4}$ - 16 months	21.5	$3\frac{1}{2}$ - $5\frac{3}{4}$ months	24.8

Mean Figlu Excretion (mg./24 hrs.)

METHOD A (Prospective Study)		METHOD B	
$2\frac{1}{4}$ - $4\frac{1}{2}$ months	14.7	$2\frac{3}{4}$ - 5.0 months	7.6
$10\frac{3}{4}$ - 16 months	5.9	$3\frac{1}{2}$ - $5\frac{3}{4}$ months	6.9

Range of Figlu Excretion (Fig.25, 26; Table 51).

The range of Figlu excretion was at its maximum in the youngest infants and comparable in the various study groups when Figlu was measured in concentration (µg./ml.).

Range of Figlu Excretion(µg./ml.)

	METHOD A (Age 2¼ - 4½ months)	METHOD B (Age 2¾ - 5.0 months)
Prospective Study	2.3 - 175.0	
Additional Studies	4.4 - 103.6	1.1 - 181.5
Overall Study (Combined Studies)	2.3 - 175.0	

Range of Figlu Excretion (mg./24 hrs.)

	METHOD A (Age 2¼ - 4½ months)	METHOD B (Age 2¾ - 5.0 months
Prospective Study	0.7 - 65.9	
Additional Studies	2.6 - 25.8	0.5 - 27.2
Overall Study (Combined Studies	0.7 - 65.9	

The range of Figlu excretion after oral histidine declined with growth.

Range of Figlu Excretion (µg./ml.)

METHOD A (Prospective Study)		METHOD B	
2¾ - 4½ months	2.3 - 175.0	2¾ - 5.0 months	1.1 - 181.5
10¾ - 16 months	3.1 - 80.4	3¼ - 5¾ months	1.2 - 152.9

TABLE 52.

FIGLU EXCRETION AFTER ORAL HISTIDINE IN EUROPEAN AND AFRICAN INFANTS

Infants aged 2½ - 4½ months

Infants	Classification	Figlu Excretion µg./ml	Figlu Excretion mg./24 hrs.	Mean Figlu excretion µg./ml.	Mean Figlu excretion mg./24 hrs.
N.H.	African	48.2	9.2		
G.T.	"	26.5	14.3		
J.Q.	"	9.0	4.6	35.6	10.9
O.M.	"	33.6	15.5		
W.S.	"	60.5	-		
A.H.	European	47.4	15.2		
L.M.	"	14.2	5.4	30.3	10.3
	Infants aged 4½ - 7½ months				
A.M.	African	96.7	30.9		
L.O.	"	52.0	14.4	69.5	19.9
M.M.	"	52.9	14.4		
A.v.D.	European	64.0	14.1		
M.H.	"	15.8	6.3	39.9	10.2

Range of Figlu Excretion after Oral Histidine (mg./24 hrs.)

METHOD A (Prospective Study)		METHOD B	
$2\frac{1}{4}$ - $4\frac{1}{2}$ months	0.7 - 65.9	$2\frac{3}{4}$ - 5.0 months	0.5 - 27.2
$10\frac{3}{4}$ - 16 months	0.7 - 16.8	$3\frac{1}{4}$ - $5\frac{3}{4}$ months	0.4 - 26.6

Figlu Tests after Oral Histidine in African and White (European) infants (Table 52).

The number of infants investigated was small.

Incidence of Positive Results (%)

African	White
62.5% (5 of 8)	50% (2 of 4)

Maximum Mean Figlu Excretion after Oral Histidine.

µg./ml.

African	White
69.5 ($4\frac{1}{2}$ - $7\frac{1}{2}$ months)	39.9 ($4\frac{1}{2}$ - $7\frac{1}{2}$ months)

mg./24 hrs.

African	White
19.9 ($4\frac{1}{2}$ - $7\frac{1}{2}$ months)	10.3 ($2\frac{1}{4}$ - $4\frac{1}{2}$ months)

Pattern of Figlu excretion.

Urine was successfully collected from 50 of the 57 infants of the prospective study who were examined 3 - 4 times during the first 16 months of life. During this period the following patterns of Figlu excretion were observed:

a) Positive Figlu tests were evident throughout the period of investigation (2).
b) Positive Figlu tests became negative at some stage during the investigation (15).

TABLE 53.

THE NUMBERS OF INFANTS EXCRETING UROCANIC ACID AND OTHER IMIDAZOLES IN EXCESS

Age (months)	Number investigated	Number with positive results	
		Urocanic acid	Imidazoles
2¼ - 4½	77	1 (F.U.)	0
4½ - 7½	65	0	1
7½ - 10¾	35	0	0
10¾ - 16	36	1 (M.R.)	3

- Method: Whitehead, R.G. 1964.

INTERRELATIONS OF NUTRITION, IMIDAZOLE AND FIGLU EXCRETION

Infant	Weight percentile	Serum Albumin g.%	Imidazole	Figlu µg./ml.
S.v.S.	-	-	Trace	74.1
K.G.	<3rd 2nd degree malnutrition	3.4	"	8.1
J.G.	55th	-	"	33.5
N.v.W.	80th	-	"	24.1

TABLE 54.

INTERRELATIONS OF

NUTRITION, UROCANIC ACID EXCRETION AND FOLATE NUTRITION

Initial study

Infant	Age (mths)	Weight percentile	Serum albumin g.%	Urocanic acid µg./ml.	Figlu µg./ml.	Serum folate ng./ml.	Bone marrow examination
F.U.	2.8	<3rd 2nd degree malnutrition	3.1	496	57.2	2.0	No megalo-blastosis
M.R.	13.0	<3rd 2nd degree malnutrition	-	1254	9.1	0.9	Megalo-blastosis (1)

Follow-up study

F.U.	5.8	<3rd 2nd degree malnutrition	-	-	96.2	-	No megalo-blastosis
M.R.	14.8	<3rd 3rd degree malnutrition	3.2	-	80.4	1.8	Megalo-blastosis (1)

TABLE 55.

RELATIONSHIP OF UROCANIC ACID TO FIGLU EXCRETION WHERE TOTAL HISTIDINE DERIVATIVES EXCEED 30 µg./ml.

Investigation	Urocanic acid excretion µg./ml.	Figlu excretion µg./ml.	Interpretation
1	10.9	22.0	Both degradation products contributed to excess of total histidine derivatives
2	15.9	14.9	
3	90.0	33.8	Both degradation products excreted in excess
4	153.0	6.3	Urocanic acid solely responsible for positive result
5	0.0	39.0	
6	3.5	54.3	
7	6.4	40.1	
8	1.1	32.3	Figlu excretion solely responsible for positive results.
9	5.4	33.9	
10	2.4	31.6	
11	4.9	37.1	
12	4.9	70.4	
13	1.1	58.4	
14	0.0	30.6	
15	3.4	69.7	
16	1.1	30.5	
17	4.1	152.9	
18	5.9	88.5	
19	2.2	65.1	
20	5.9	52.9	
21	8.1	181.5	
22	2.2	31.3	
23	0.0	42.0	
24	6.6	83.2	

c) Negative tests throughout (23).

d) Fluctuations from negative to positive results (10 infants). These fluctuations could not be related to the effect of growth, infection, known dietary or technical factors.

THE RELATIONSHIP OF DISTURBANCES OF HISTIDINE METABOLISM TO THE FIGLU TEST AFTER ORAL HISTIDINE.

The effect on the incidence of positive Figlu tests.

1) Urocanic acid excretion.

Method of Whitehead (1964).

Urocanic acid excretion was excessive in 2 of 213 instances (Table 53). In one of these, excessive excretion of urocanic acid was accompanied by excessive Figlu excretion (Table 54). The other positive test for urocanic acid was not associated with excessive Figlu excretion. In this infant the Figlu test would have been judged an unreliable parameter of folate deficiency if urocanic acid had not been estimated (Table 54). Thus, a disturbance of histidine metabolism at the level of urocanic acid could have accounted for only one false negative Figlu test.

In addition to these results of the investigations in the prospective study, urocanic acid could not be detected in the urine of 8 African and 4 White infants.

Method of Chanarin and Bennett (1962a).

Urocanic acid was separately estimated only in those instances where the excretion of total histidine derivatives exceeded 30 µg./ml. It was only in these cases that the Figlu test after oral histidine might have been positive (24 out of 91 tests). In only one of these 24 tests was urocanic acid excretion solely responsible for the excess of histidine derivatives (Table 55).

A disturbance of histidine metabolism resulting in excessive excretion of urocanic acid could have interfered with the interpretation of the Figlu test after oral histidine in one of 91 estimations for Figlu.

EFFECT OF RICKETS ON MEAN FIGLU EXCRETION

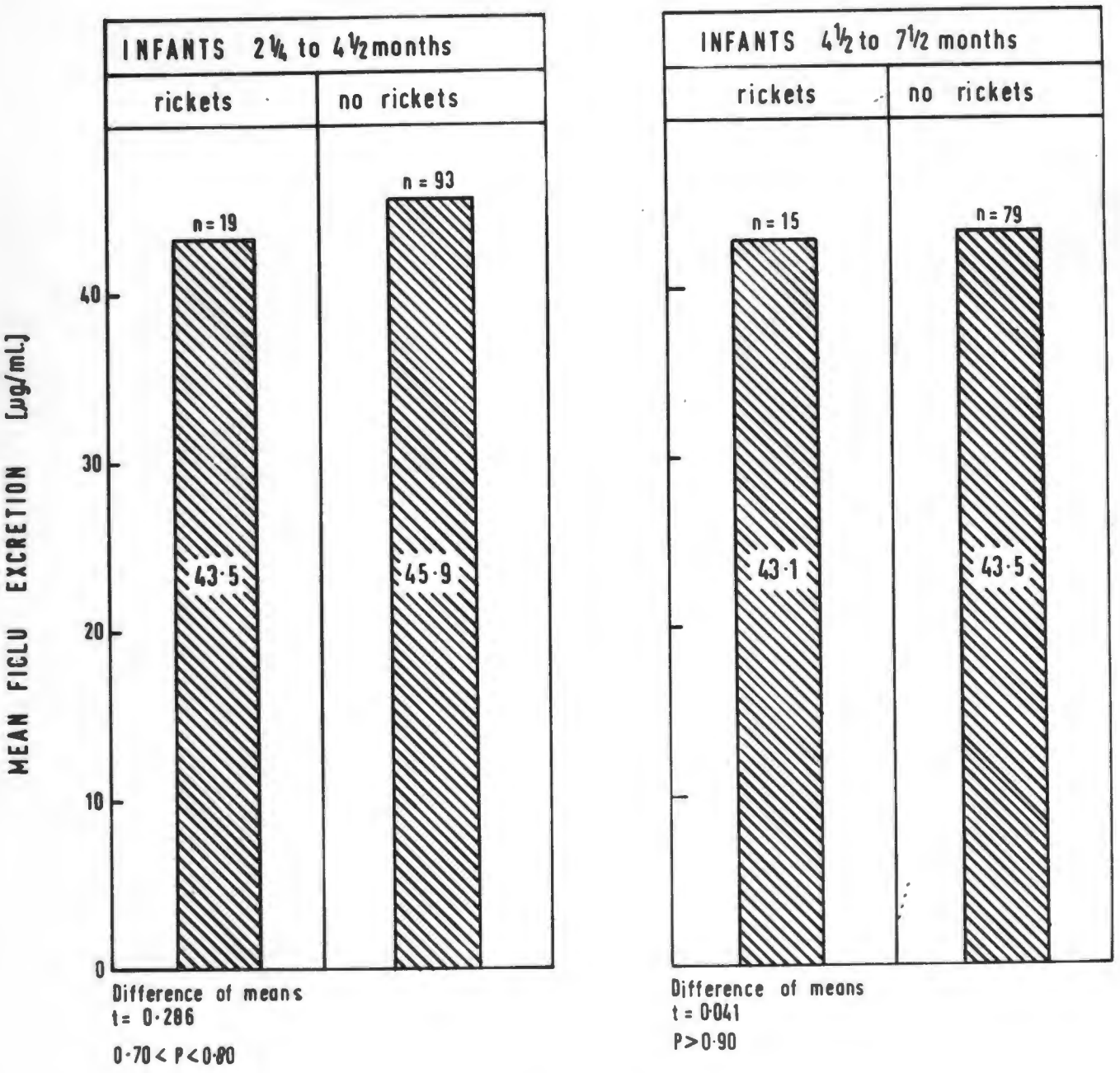


Fig. 27

Thus, even with a sensitive method of estimating urocanic acid excretion (Chanarin and Bennett, 1962a), a disturbance of histidine metabolism at the level of urocanic acid rarely resulted in a false negative Figlu test in the infants examined.

2) Urinary imidazoles.

Method of Whitehead (1964).

Four (4) infants excreted imidazoles which were not identified and in three cases this might have resulted in false negative Figlu tests (Table 53).

Even if this were so, this would not materially have influenced the incidence of positive Figlu tests.

3) Rickets.

From the following findings it was unlikely that the histidinuria sometimes found in rickets was severe enough to interfere with the efficacy of the histidine load and so result in false negative Figlu tests.

Figlu Method A (Fig.27).

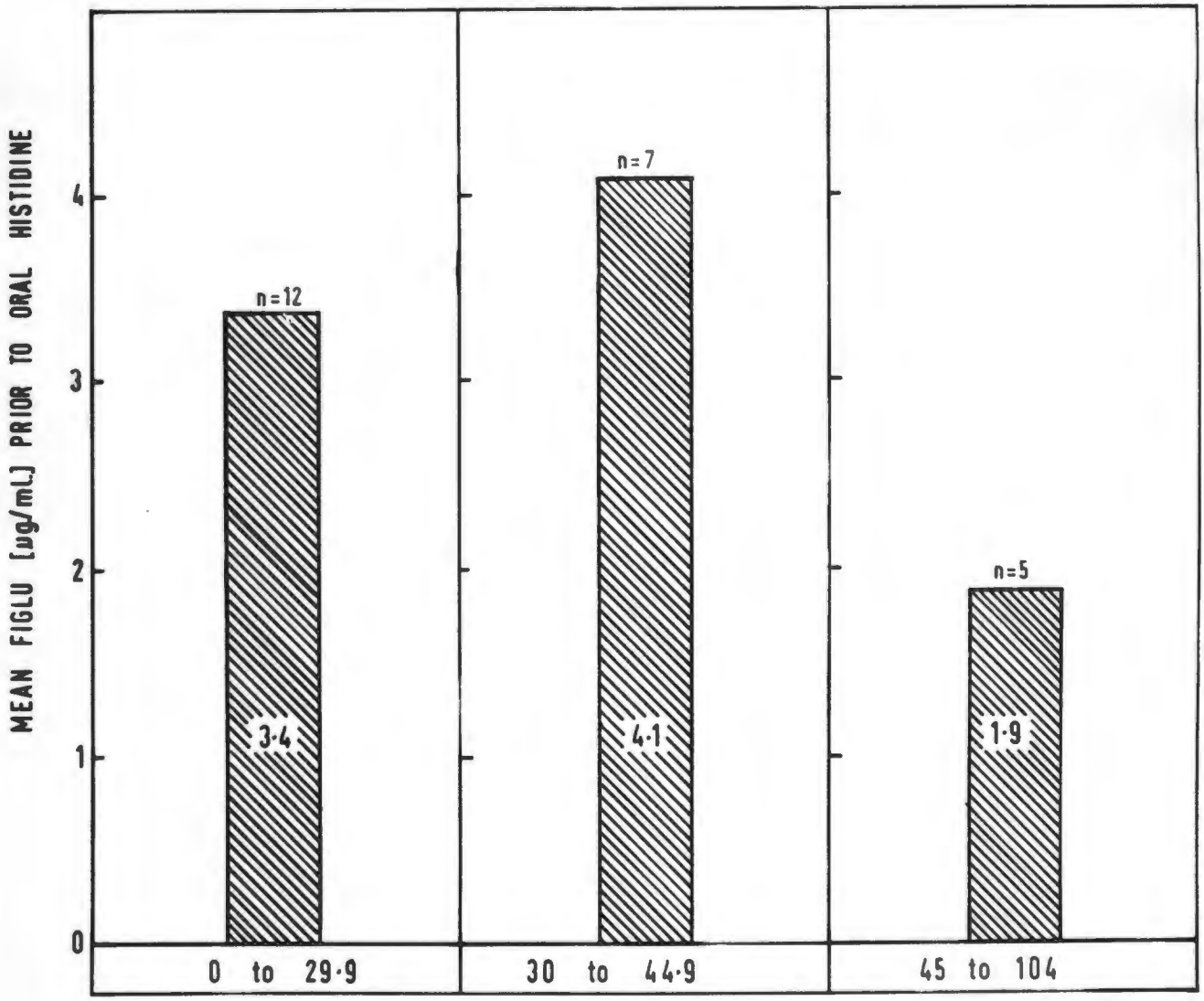
There was no significant difference in mean Figlu excretion after oral histidine in infants with or without rickets. The mean Figlu excretion was slightly lower in infants with rickets.

Thirteen (13) infants who suffered from rickets and who excreted normal quantities of Figlu were retested after receiving treatment with Ostelin Forte. Two (2) of these subsequently excreted excessive Figlu after oral histidine.

Figlu Method B.

All infants suffering from rickets excreted normal quantities of Figlu. After receiving Ostelin Forte only one of the eight excreted an excess of Figlu.

MEAN FIGLU EXCRETION PRIOR TO ORAL HISTIDINE IN INFANTS RELATED TO THEIR RANGE OF FIGLU EXCRETION AFTER ORAL HISTIDINE



RANGE:	0·0 — 8·7	0·9 — 8·1	0·0 — 3·8
S.D:	± 2·9	±2·6	±2·9
S.E. of mean:	±0 9	±1·0	±1·3

F = 1·10
P >0·05

Fig. 28

Relationship of Disturbances of Histidine Metabolism to Undernutrition.

1) Urocanic Acid Excretion and Nutrition.

Method of Whitehead (1964).

The data were insufficient and therefore inconclusive (Table 54). Hypoalbuminemia (2.9 - 3.2 g.%) was noted in 5 instances in this study (Table 7). This was associated with excessive urocanic acid excretion in only one instance (F.U.; Table 54). However, both infants who excreted excessive urocanic acid were undernourished.

The test was repeated on the two infants who originally excreted excessive urocanic acid. Although the infants remained underweight, excessive urocanic acid excretion was no longer evident. It was not known whether the degree of hypoalbuminemia had improved during this time and whether there had been any relationship to hepatic dysfunction (Table 54).

2) Imidazole excretion and nutrition (Table 53).

Method of Whitehead (1964).

The data were insufficient and no inferences could be drawn.

THE FIGLU TEST AFTER ORAL HISTIDINE AS A PARAMETER OF FOLATE DEFICIENCY.

Comparison with other parameters of folate deficiency.

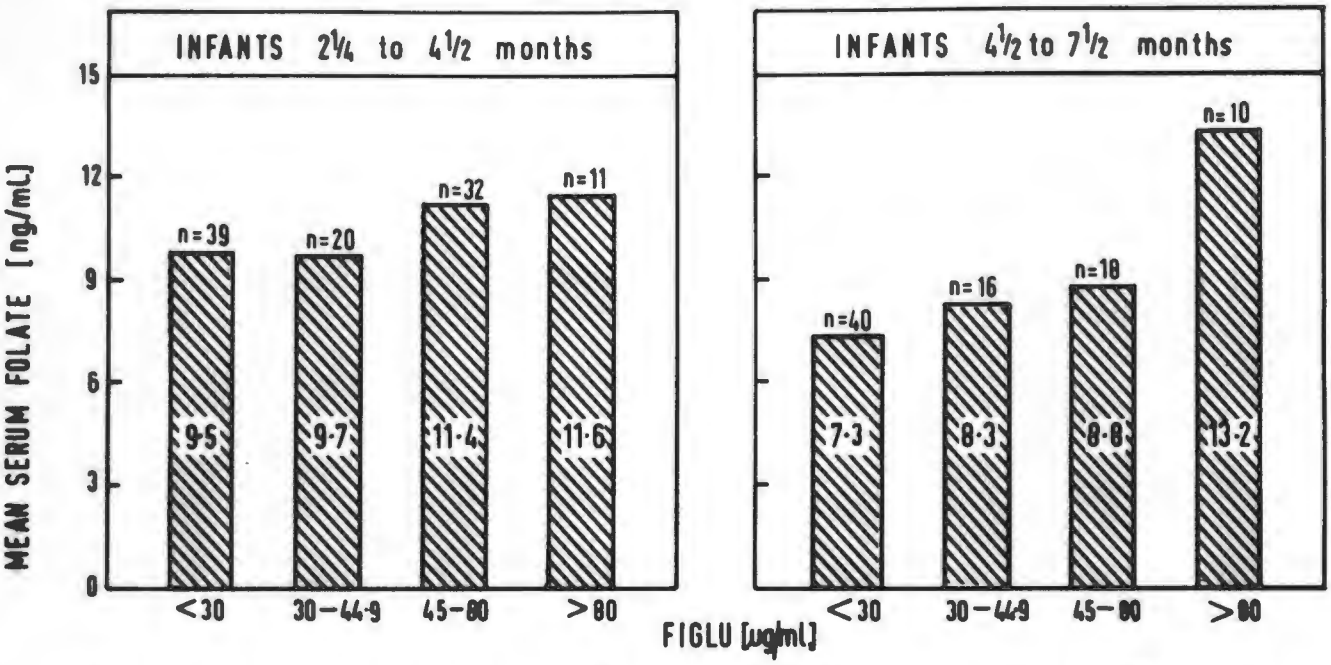
A relationship could not be demonstrated between Figlu excretion after oral histidine and:

a) Figlu excretion without a histidine load.

Method A (Fig. 28).

There was no statistical relationship between Figlu excretion before and after histidine loading. None of the infants tested excreted an excess of Figlu without an oral histidine load.

MEAN FOLATE LEVELS RELATED TO RANGE OF FIGLU EXCRETION

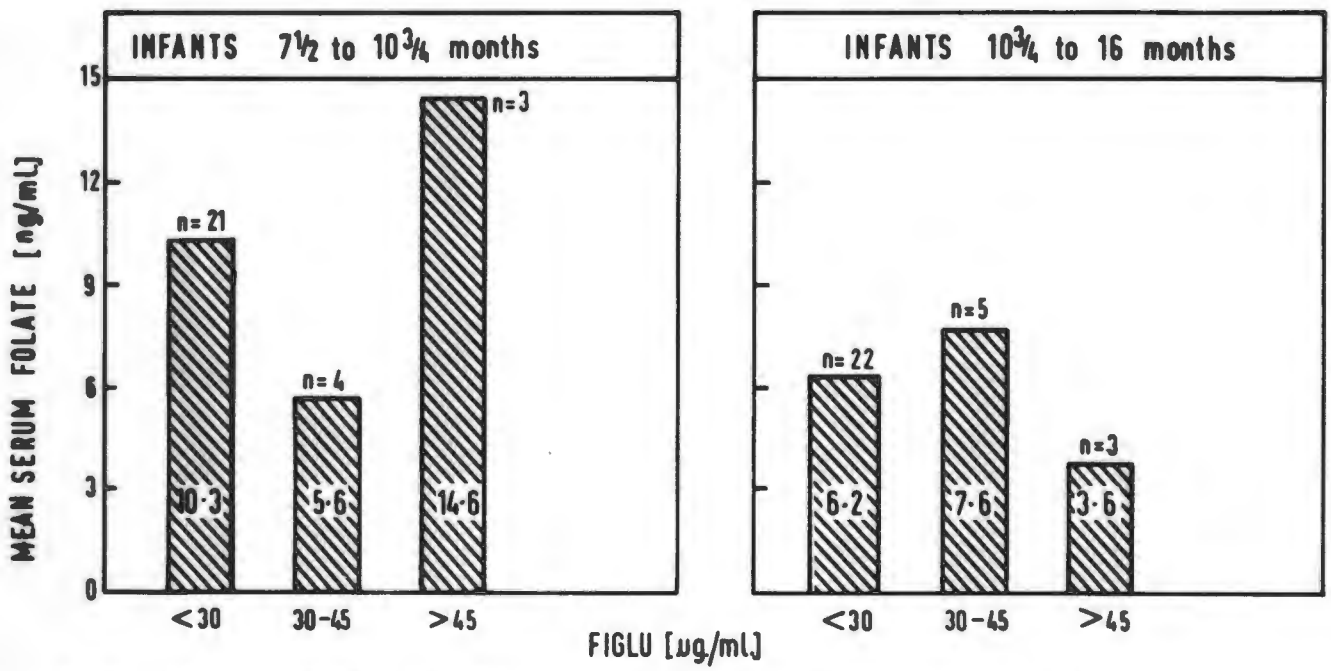


	<30	30–44·9	45–80	>80
RANGE:	1·1–29·1	1·4–30·0	2·0–28·4	3·4–46·8
S.D:	±6·8	±7·8	±6·8	±16·0
S.E. of mean:	±1·1	±1·8	±1·2	± 4·8

F = 0·48
P >0·05

	<30	30–44·9	45–80	>80
RANGE:	1·8–16·0	2·4–20·5	3·0–30·8	1·9–29·8
S.D:	±3·2	±4·7	± 7·8	± 10·2
S.E. of mean:	±0·5	±1·2	±1·9	± 3·3

F = 2·63
P >0·05



	<30	30–45	>45
RANGE:	2·9–24·0	4·0–7·0	10·5–18·3
S.D:	± 5·3	±1·3	±3·9
S.E. of mean:	± 1·2	±0·7	±2·3

F = 2·82
P >0·05

	<30	30–45	>45
RANGE:	2·1–11·4	3·6–12·5	1·8–6·6
S.D:	± 2·9	±3·4	±2·6
S.E. of mean:	±0·6	±1·5	±1·5

F = 1·64
P >0·05

Fig. 29

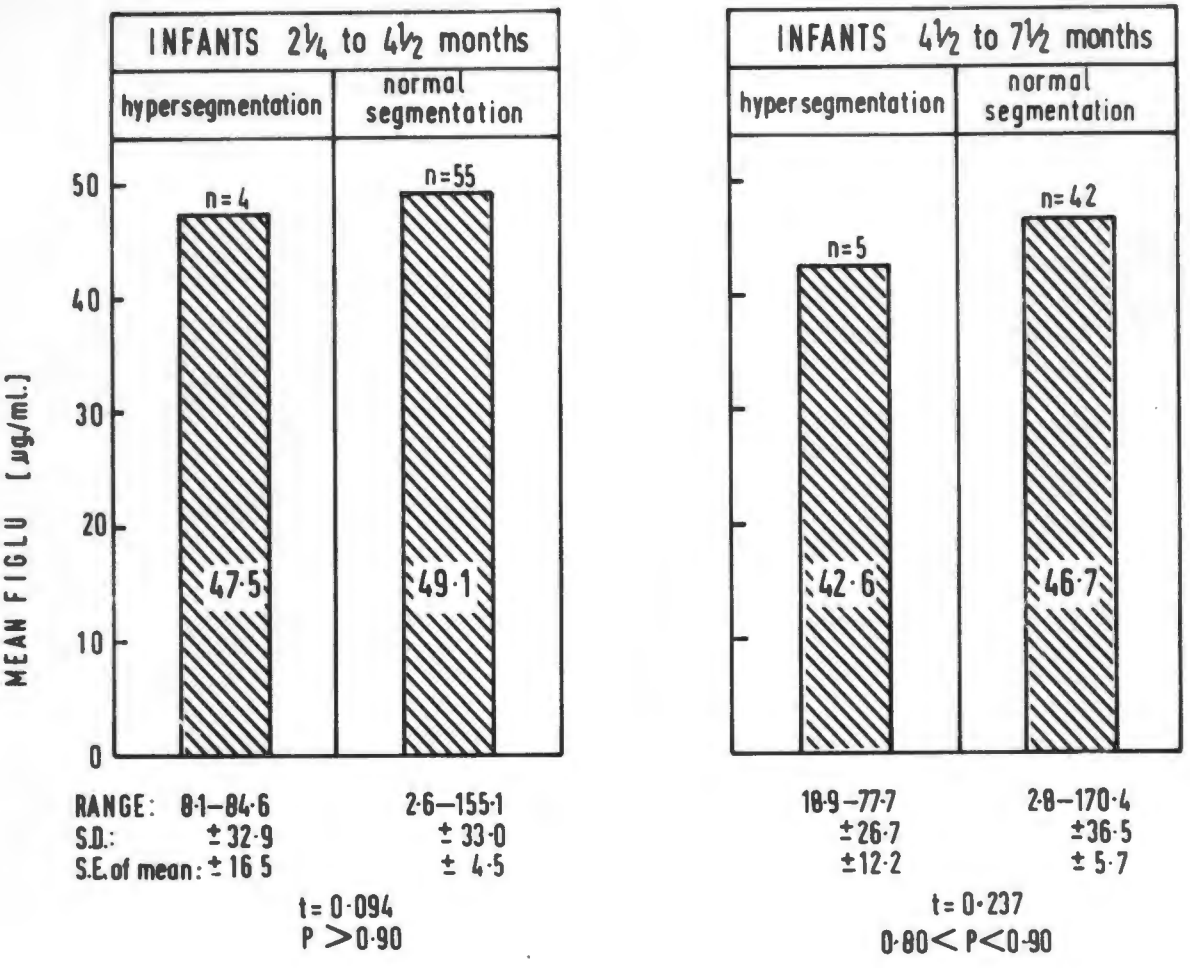
TABLE 56.

RELATIONSHIP OF FIGLU EXCRETION TO SERUM FOLATE LEVELS

Figlu excretion	Mean serum folate ng./ml.	Test of significance	Significance
Negative	5.1 n = 23	Analysis of variance	F = 3.94 0.01 < P < 0.05
Borderline	8.8 n = 4		
Positive	4.9 n = 5		

The overall differences are statistically significant. The only statistically significant individual difference is that between infants with negative and borderline positive Figlu tests. There is therefore no relationship between Figlu excretion in excess and serum folate levels.

RELATIONSHIP OF MEAN FIGLU EXCRETION TO HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS



	INFANTS 2¼ to 4½ months		INFANTS 4½ to 7½ months	
	hypersegmentation	normal segmentation	hypersegmentation	normal segmentation
RANGE:	8·1–84·6	2·6–155·1	18·9–77·7	2·8–170·4
S.D.:	± 32·9	± 33·0	± 26·7	± 36·5
S.E. of mean:	± 16·5	± 4·5	± 12·2	± 5·7
	t = 0·094 P > 0·90		t = 0·237 0·80 < P < 0·90	

	INFANTS 7½ to 10¾ months		INFANTS 10¾ to 16 months	
	hypersegmentation	normal segmentation	hypersegmentation	normal segmentation
RANGE:	13·9–63·9	4·0–122·4	5·5–9·1	3·1–80·4
S.D.:	± 21·8	± 25·3	± 1·8	± 20·9
S.E. of mean.	± 9·9	± 5·3	± 1·1	± 3·9
	t = 0·189 0·80 < P < 0·90		t = 1·303 0·20 < P < 0·30	

Fig. 30.

b) Mean serum L.casei folate levels.

Method A (Fig.29).

There was no statistical relationship between mean serum L.casei folate levels and Figlu excretion. Mean serum L.casei folate levels were higher in infants with positive Figlu tests in all age groups, but infants from $10\frac{3}{4}$ - 16 months.

Method B (Table 56).

A statistical relationship between mean serum L.casei folate levels and Figlu excretion after oral histidine was not demonstrated. Using the analysis of variance, overall differences between mean serum L.casei folate levels in infants with positive, borderline and negative tests were significantly different. However, the significant individual difference did not lie between infants with positive Figlu tests and those with either borderline or negative tests, but between infants with negative and borderline positive Figlu tests.

When the 'Student's' t-test was used to compare the difference between the mean serum L.casei folate level in infants with positive Figlu tests (4.9 ng./ml.) and the mean serum L.casei folate level of the combined group of infants with negative and borderline Figlu tests (5.6 ng./ml.), no statistical difference could be shown ($t = 0.522$; $0.60 < P < 0.70$).

c) Percentage hypersegmentation of the neutrophils.

Method A (Fig.30).

A statistical relationship could not be demonstrated between mean Figlu excretion after oral histidine and percentage hypersegmentation of the neutrophils.

RELATIONSHIP OF MEAN RBC FOLATE LEVELS TO RANGE OF FIGLU EXCRETION IN INFANTS

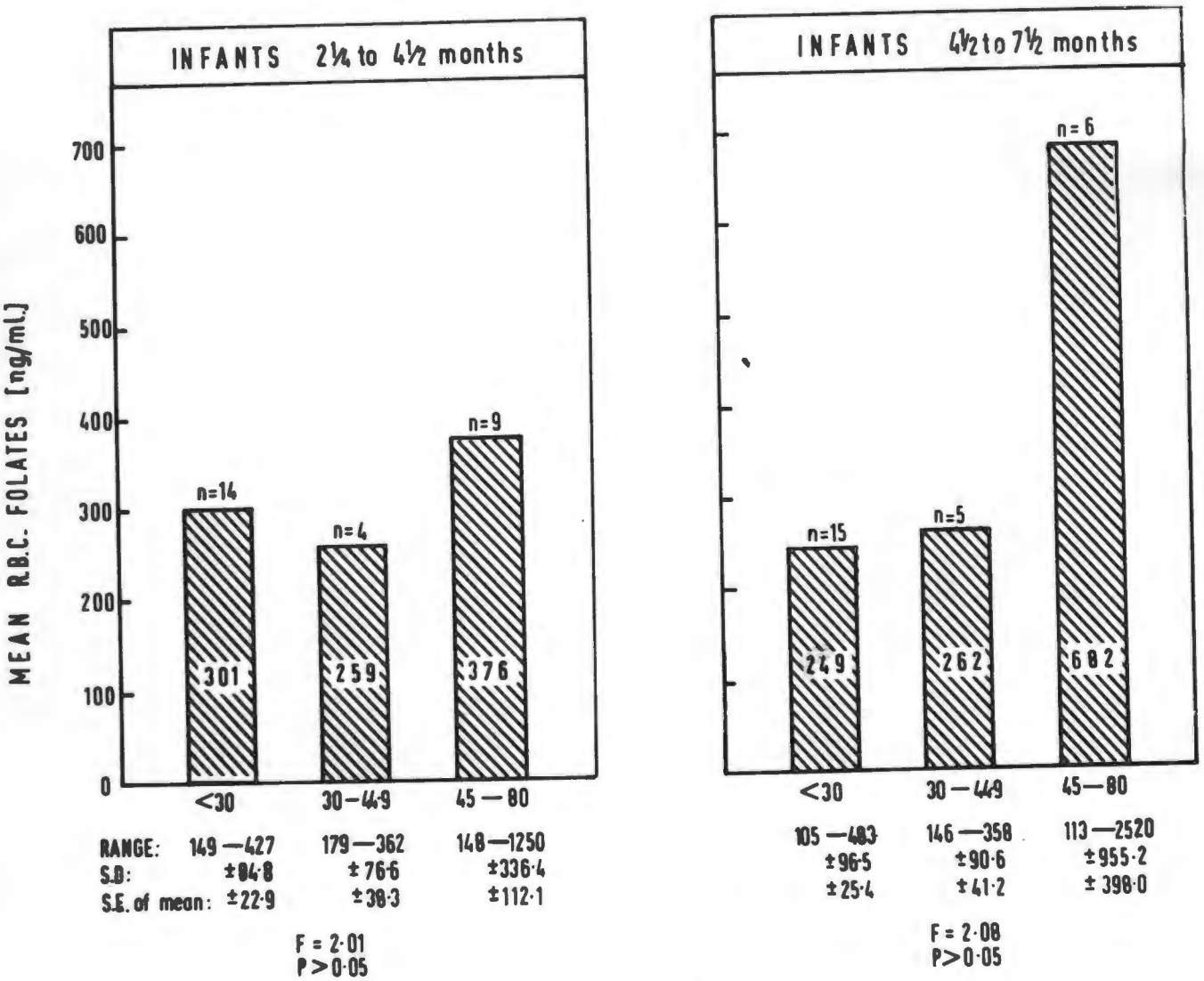


Fig. 31

MEAN and RANGE of
FOLIC ACID CLEARANCES RELATED to RANGE of FIGLU EXCRETION
L.CASEI

PROCEDURE I

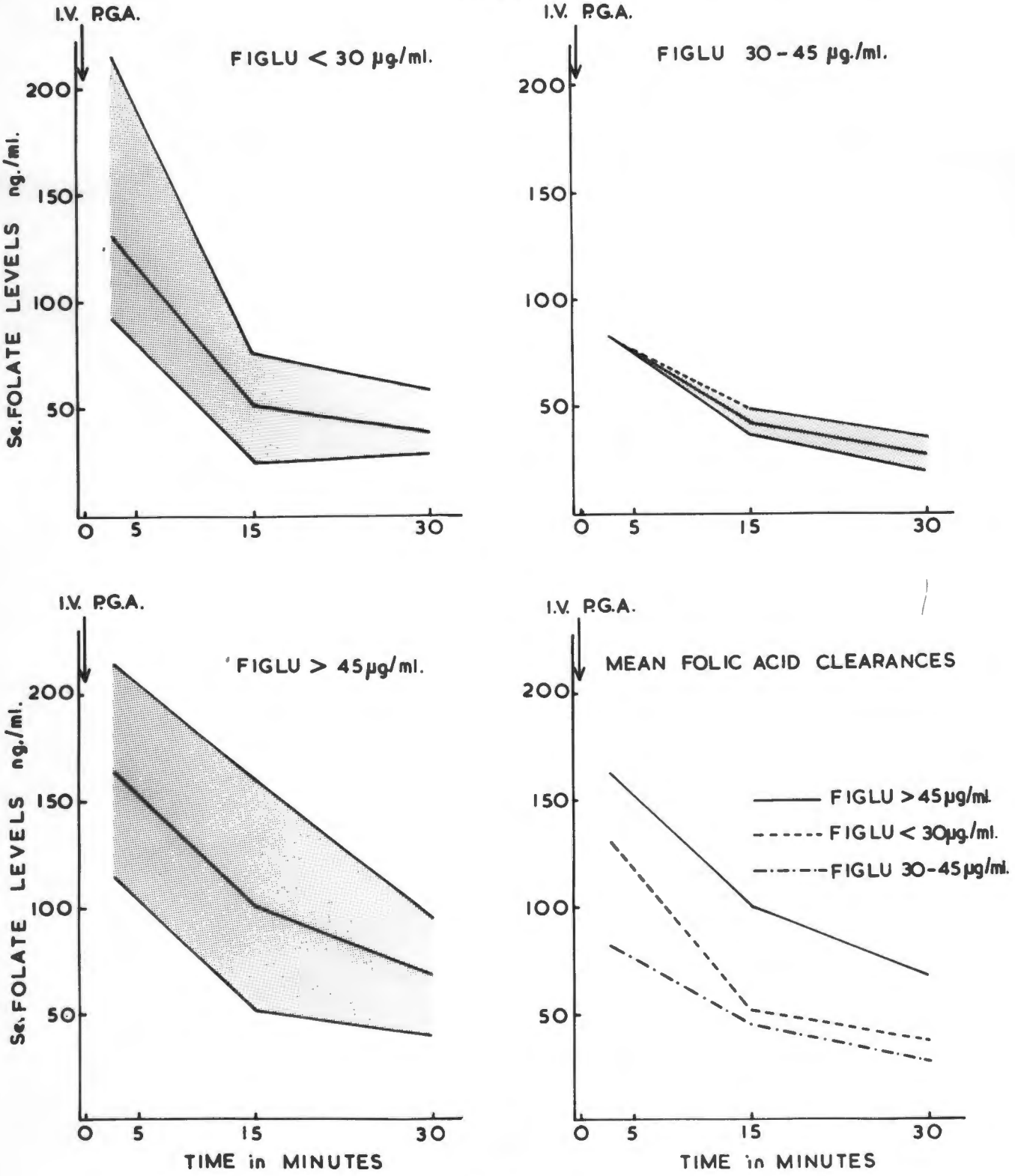


Fig. 32

MEAN and RANGE of
FOLIC ACID CLEARANCES RELATED to RANGE of FIGLU EXCRETION
L.CASEI

PROCEDURE I

FIGLU EXCRETION < 30 µg./ml

SUBJECT	FIGLUmg/24H	3 MIN.	15 MIN.	30 MIN.
R.K.	6.0	100	50	30
W.A.	8.4	214	75	58
D.A.	2.6	92	26	29
N.S.	8.4	118	58	35
	MEAN	131	52	38
	S.D.	± 56.5	± 20.5	± 13.7
	S.E. of MEAN	± 28.3	± 10.3	± 6.9

FIGLU EXCRETION 30–45 µg./ml.

SUBJECT	FIGLUmg./24H	3 MIN.	15 MIN.	30 MIN.
D.G.	12.8	—	50	37
G.R.	19.9	83	43	20
	MEAN	83	46	28
	S.D.	—	± 4.4	± 11.8
	S.E. of MEAN	—	± 3.1	± 8.4

FIGLU EXCRETION > 45 µg./ml.

SUBJECT	FIGLUmg./24H	3 MIN.	15 MIN.	30 MIN.
A.H.A.	17.6	211	160	95
A.K.	19.6	115	52	41
	MEAN	163	106	68
	S.D.	± 67.9	± 76.4	± 38.5
	S.E.of MEAN	± 48.5	± 54.6	± 27.5

SIGNIFICANCE of DIFFERENCE of MEANS

3 MIN.	15 MIN.	30 MIN.
F = 0.61	F = 1.67	F = 2.02
P > 0.05	P > 0.05	P > 0.05

TABLE 57

d) Red cell L.casei folate levels.

Method A (Fig.31).

There was no statistical relationship between excessive Figlu excretion after oral histidine and mean red cell L.casei folate levels. The mean red cell L.casei folate levels were highest in infants with excessive Figlu excretion. None of the infants studied had abnormally low red cell L.casei folate levels.

Method B.

Figlu excretion	Red cell L.casei folate levels (ng./ml.)	
	Range	Mean
Excessive	102 - 632	251 n = 5
Borderline	452 - 553	496 n = 4
Negative	63 - 542	272 n = 25

In this study 2 infants had abnormally low (< 100 ng./ml.) red cell L.casei folate levels (63 and 67 ng./ml.). Both excreted normal quantities of Figlu after oral histidine.

e) L.casei folic acid clearance test.

Method A (Fig.32; Table 57).

The mean L.casei folic acid clearance test was slower in infants with positive Figlu tests after oral histidine. The difference in the rates of clearance of injected folic acid at 3, 15 and 30 minutes in infants with normal, borderline and positive Figlu excretion tests was not statistically significant.

TABLE 58.

RELATIONSHIP OF FIGLU EXCRETION TO BONE MARROW MORPHOLOGY

Subject	Figlu µg./ml.	Figlu mg./24 hours	Bone marrow morphology
S.R.	8.0	1.8	
B.C.	10.4	4.7	
C.H.	13.5	4.4	
V.S.	25.0	10.4	
N.L.	28.6	9.7	
N.v.W.	29.7	12.6	
U.v.S.	32.5	6.2	
R.J.	32.6	15.5	
N.M.	34.8	9.0	
A.H.	35.0	6.0	
C.F.	35.3	5.9	
R.S.	35.8	15.8	
M.P.	38.3	14.7	
V.B.	40.8	16.1	No megaloblastosis
M.R.	45.1	14.7	
J.A.	53.5	9.1	
F.U.	57.2 *	14.3	
K.M.	57.4	15.6	
S.B.	57.8	-	
J.D.	58.8	11.2	
M.I.	62.9	15.1	
H.F.	64.9	7.9	
S.v.S.	74.1	11.3	
A.U.	77.7	12.7	
D.B.	92.6	40.7	
E.L.	128.8	18.4	
R.K.	136.8	36.3	
T.deB.	170.4	46.0	
M.R.	80.4	-	Megaloblastosis

* Also excreted urocanic acid (Whitehead, R.G. : 1964).

This conclusion would not have been altered if Figlu in mg./24 hrs rather than Figlu in µg./ml. was compared with the rate of folic acid clearance (Table 57; Fig.32).

Method B (Fig.34; Table 67).

The amount of injected folic acid in this group of infants might have been insufficient (cf. Chapter IV). The L.casei folic acid clearance curve in the infant excreting excessive Figlu after oral histidine (R.A.) fell within the range found in infants excreting normal amounts of Figlu after oral histidine except at the 30 minute level. Here the value in R.A. was slightly lower than that found in infants with normal Figlu excretion.

f) Bone marrow morphology.

Method A (Table 58).

Bone marrow examinations were performed on 28 infants, one of whom, M.R., had a repeat examination at the age of 13 months. At this stage the marrow showed unequivocal evidence of megaloblastosis and Figlu excretion was 80.4 µg./ml. S.B. was the only infant of a comparable age group who excreted Figlu in excess (57.8 µg./ml.) and on whom a bone marrow examination was performed. No megaloblastosis was noted. Other infants in this age group excreted up to 74.2 µg./ml. of Figlu without other evidence of folate deficiency, but bone marrow examinations were not performed on these infants.

All other infants with positive Figlu tests after oral histidine, on whom bone marrow examinations were performed, were less than 7½ months of age. Positive Figlu tests of up to 170.4 µg./ml. and 46.0 mg./24 hrs were not associated with megaloblastosis.

From these findings it was deduced that infants up to 7½ months of age could have excessive Figlu excretion after oral histidine without

TABLE 59

THE RELATIONSHIP OF MATERNAL TO INFANT FOLATE NUTRITION

(a) The relationship of maternal serum folate levels to mean Figlu excretion in infants

Age (months)	Maternal folate levels	Mean Figlu excretion in infants µg./ml.	Test of significance	Significance
2¼ - 4½	Normal	50.3 n = 45	'STUDENT'S' t-Test	t = 0.634
	Low	45.1 n = 30		0.50 < P ≤ 0.60
4½ - 7½	Normal	54.6 n = 36	"	t = 2.735
	Low	31.9 n = 27		0.001 < P ≤ 0.01
7½ - 10¾	Normal	30.0 n = 23	"	t = 0.702
	Low	23.4 n = 12		0.40 < P ≤ 0.50
10¾ - 16	Normal	24.4 n = 21	"	t = 1.813
	Low	13.7 n = 13		0.05 < P ≤ 0.10

n = Number

Conclusion:

Mean Figlu excretion is always higher in the infants of mothers with normal serum folate levels.

The differences become statistically significant at the 4½ - 7½ months age group. It is unlikely that this result has any meaning.

TABLE 59 (Contd.)

(b) The relationship of maternal Figlu excretion to mean Figlu excretion in infants

Age (months)	Maternal Figlu Excretion	Mean Figlu excretion in infants µg./ml.	Test of significance	Significance
2¼ - 4½	Excessive	44.9 n = 4	'STUDENT'S' t-Test	t = 0.242
	Normal	49.4 n = 73		0.80<P<0.90

Conclusion:

There is no statistical relationship between these two parameters.

(c) The relationship of hypersegmentation of maternal neutrophils to mean Figlu excretion in infants

Age (months)	Segmentation of maternal neutrophils	Mean Figlu excretion in infants µg./ml.	Test of significance	Significance
2¼ - 4½	Excessive	42.9 n = 5	'STUDENT'S' t-Test	t = 0.401
	Normal	49.6 n = 72		0.60<P<0.70

Conclusion:

There is no statistical relationship between these two parameters.

In studies (b) and (c) the numbers involved did not permit study beyond the age group of 2¼ - 4½ months.

It is unlikely that Maternal Folate nutrition can influence that of the infant beyond 4½ months of age, because, from Herbert's (1962a) experiment, it appeared that folate stores were only sufficient for approximately 4½ months (cf. Chapter I, page 36).

TABLE 59 (Contd.)

(d) Relationship of cord serum folate levels to infant Figlu excretion

Age of infants (months)	Statistical method			Significance
2¼ - 4½	Correlation coefficient		r = -0.027 n = 60	r does not differ significantly from zero
4½ - 7½	"	"	r = +0.038 n = 53	P > 0.10
7½ - 10¾	"	"	r = -0.383 n = 28	r is significantly different from zero 0.02 < P < 0.05
10¾ - 16	"	"	r = +0.465 n = 29	0.01 < P < 0.02

Conclusion:

Although the correlation coefficient is greater than zero in the older infants, it is unlikely that this result has any meaning because -

(i) the correlation at best is a very poor one; and

(ii) there is a fluctuation between positive and negative correlations.

TABLE 60

DIET

(a) Relationship of milk feeding to Figlu excretion *

Age (months)	Range of Figlu excretion µg./ml.	Mean milk folate ng./ml.	Test of significance	Significance
2¼ - 4½	< 30	23.3 n = 10	Analysis of variance	F = 0.13 P > 0.05
	30 - 45	21.2 n = 3		
	> 45	20.7 n = 13		

Conclusion: There is no statistical relationship between these two parameters.

(b) Pattern of milk feeding in relation to mean Figlu excretion

Age (months)	Nature of milk feeding	Mean Figlu excretion µg./ml.	Test of significance	Significance
2¼ - 4½	Breast	40.9 n = 23	Analysis of variance	F = 1.66 P > 0.05
	Breast supplemented with bottle	41.0 n = 9		
	Breast and bottle	42.2 n = 56		
	Bottle supplemented with breast	56.7 n = 11		
	Bottle	65.7 n = 11		
4½ - 7½	Breast	53.8 n = 5	"	F = 0.24 P > 0.05
	Breast and bottle	43.9 n = 32		
	Bottle	42.7 n = 58		

Conclusion: There is no consistent relationship between mean Figlu excretion and the nature of milk feeds. None of the differences are statistically significant.

*When both breast and bottle milk folate were compared with Figlu excretion the mean of the bottle and breast milk folate readings was used for the comparison.

TABLE 61

FIGLU EXCRETION IN RELATION TO AETIOLOGICAL POSSIBILITIES FOR FOLATE DEFICIENCY.

(a) Relationship of Figlu excretion to milk folate levels.*

Figlu excretion	Mean milk folate levels		Test of significance	Significance
Negative	24.1	n = 21	Analysis of variance	F = 0.84 P > 0.05
Borderline	17.3	n = 3		
Positive	20.8	n = 8		

Conclusion: There is no relationship between milk folate levels and Figlu excretion. The differences are not statistically significant.

(b) Relationship of growth (weight percentile) to Figlu excretion.

Nutrition	Mean Figlu excretion mg./24 hrs.		Test of significance	Significance
2nd & 3rd degree malnutrition	10.1	n = 5	Analysis of variance	F = 1.73 P > 0.05
1st degree malnutrition	10.9	n = 2		
10 - 25th percentile	9.4	n = 1		
26 - 50th percentile	6.3	n = 11		
> 50th percentile	7.2	n = 26		

Conclusion: Although there are some fluctuations the better nourished infants have lower mean Figlu excretion. The overall differences are not statistically significant.

*When both breast and bottle milk folate were compared with Figlu excretion the mean of the bottle and breast milk folate readings was used for the comparison.

TABLE 61 (Contd.)

(c) Relationship of infection score to Figlu excretion.

Infection score	Mean Figlu excretion mg./24 hrs.		Test of significance	Significance
0	5.9	n = 3		
½ - 2	6.6	n = 25	Analysis of variance	F = 1.39
2½ - 4½	10.2	n = 14		P > 0.05
5 and >	5.9	n = 3		

Conclusion: There is no relationship between infection score and mean Figlu excretion. The overall differences are not significant.

evidence of megaloblastosis. Information in the older age group was insufficient for definitive conclusions.

An Aetiology for Folate Deficiency and its Relationship to Positive Figlu Tests after Oral Histidine.

There was no relationship between Figlu excretion after oral histidine and:

a) Maternal folate nutrition.

Method A (Table 59).

There was no unequivocal relationship between mean Figlu excretion in the infants and maternal folate deficiency as assessed by serum L.casei folate levels, excessive Figlu excretion, hypersegmentation of the neutrophils. There was no convincing correlation between infant Figlu excretion and cord serum L.casei folate levels.

b) Dietary factors.

(i) Milk feeding.

Method A (Table 60).

Method B (Table 61).

This was assessed only in the youngest infants where milk was the exclusive or major dietary constituent. There was no statistical relationship between mean milk L.casei folate levels and excessive Figlu excretion.

(ii) Nature of milk feeding.

Method A (Table 60).

There was no demonstrable statistical relationship between mean Figlu excretion and the nature of the milk feeding.

(iii) Other dietary factors.

Method A.

The reduction of Figlu excretion with growth could not be attributed to the addition of cereal, mixed feeding, orange juice, vitamins, meat or cod-liver oil to the diet of the infants.

TABLE 62

RELATIONSHIP OF SOCIO-ECONOMIC CIRCUMSTANCES TO MEAN FIGLU EXCRETION

Age (months)	Socio-economic circumstances	Mean Figlu excretion µg./ml.	Test of significance	Significance
2¼ - 4½	Poor	49.5 n = 3	Analysis of variance	F = 0.93 P > 0.05
	Average	29.6 n = 14		
	Good	33.2 n = 10		
4½ - 7½	Poor	110.4 n = 3	"	F = 10.39 P < 0.01
	Average	38.0 n = 14		
	Good	33.6 n = 8		
7½ - 10¾	Poor	44.8 n = 3	"	F = 6.50 P < 0.05
	Average	21.2 n = 8		
	Good	37.5 n = 5		
10¾ - 16	Poor	37.9 n = 3	"	F = 1.60 P > 0.05
	Average	17.3 n = 12		
	Good	14.7 n = 9		

Conclusion:

Mean Figlu excretion is consistently higher in infants from poor socio-economic circumstances. The overall differences are significant at 4½ - 7½ and 7½ - 10¾ months.

In the analysis of the individual differences in the two groups, mean Figlu excretion in infants from poor socio-economic circumstances is significantly different from that in infants from average and good socio-economic circumstances in the age group 4½ - 7½ months. This consistent relationship is not demonstrated in infants from 7½ - 10¾ months of age where the difference in result between infants from poor and average socio-economic circumstances is statistically significant, but not that between infants from poor and good socio-economic circumstances.

It may be that there is an association between socio-economic circumstances and Figlu excretion, but it would need further investigation, involving greater numbers of infants, to prove this point.

TABLE 63

RELATIONSHIP OF NUTRITION (WEIGHT PERCENTILE) TO MEAN FIGLU EXCRETION

Age (months)	Nutritional development	Mean Figlu excretion µg./ml.		Test of significance	Significance
2¼ - 4½	2nd & 3rd degree malnutrition	54.3	n = 11		
	1st " "	40.2	n = 28	Analysis	F = 0.40
	10th - 25th percentile	46.8	n = 15	of	
	26th - 50th "	42.2	n = 17	variance	P > 0.05
	>50th "	46.3	n = 41		
4½ - 7½	2nd & 3rd degree malnutrition	77.3	n = 9		
	1st " "	29.4	n = 23		F = 3.86
	10th - 25th percentile	46.7	n = 9	"	
	26th - 50th "	34.9	n = 17		P < 0.01
	>50th "	50.2	n = 36		
7½ - 10¾	2nd & 3rd degree malnutrition	42.8	n = 4		
	1st "	24.9	n = 8		F = 0.83
	10th - 25th percentile	25.2	n = 4	"	
	26th - 50th "	31.9	n = 11		P > 0.05
	>50th "	18.6	n = 8		
10¾ - 16	2nd & 3rd degree malnutrition	19.5	n = 6		
	1st " "	34.1	n = 8		F = 1.05
	10th - 25th percentile	17.1	n = 5	"	
	26th - 50th "	17.7	n = 7		P > 0.05
	>50th "	17.6	n = 10		

Conclusion:

Infants with 2nd and 3rd degree and, in the last age group, with 1st degree malnutrition, have a higher mean Figlu excretion. These differences are significant in the age group 4½ - 7½ months.

This association will not be emphasized because (i) there is much fluctuation within groups and (ii) the individual differences in the age group 4½ - 7½ months are not consistent. The result in infants with 2nd and 3rd degree malnutrition is not significantly different from that in infants in the 10th - 25th percentile, although it is significantly different from those in all other groups.

RELATIONSHIP of FOLIC ACID ABSORPTION TO RANGE of FIGLU EXCRETION

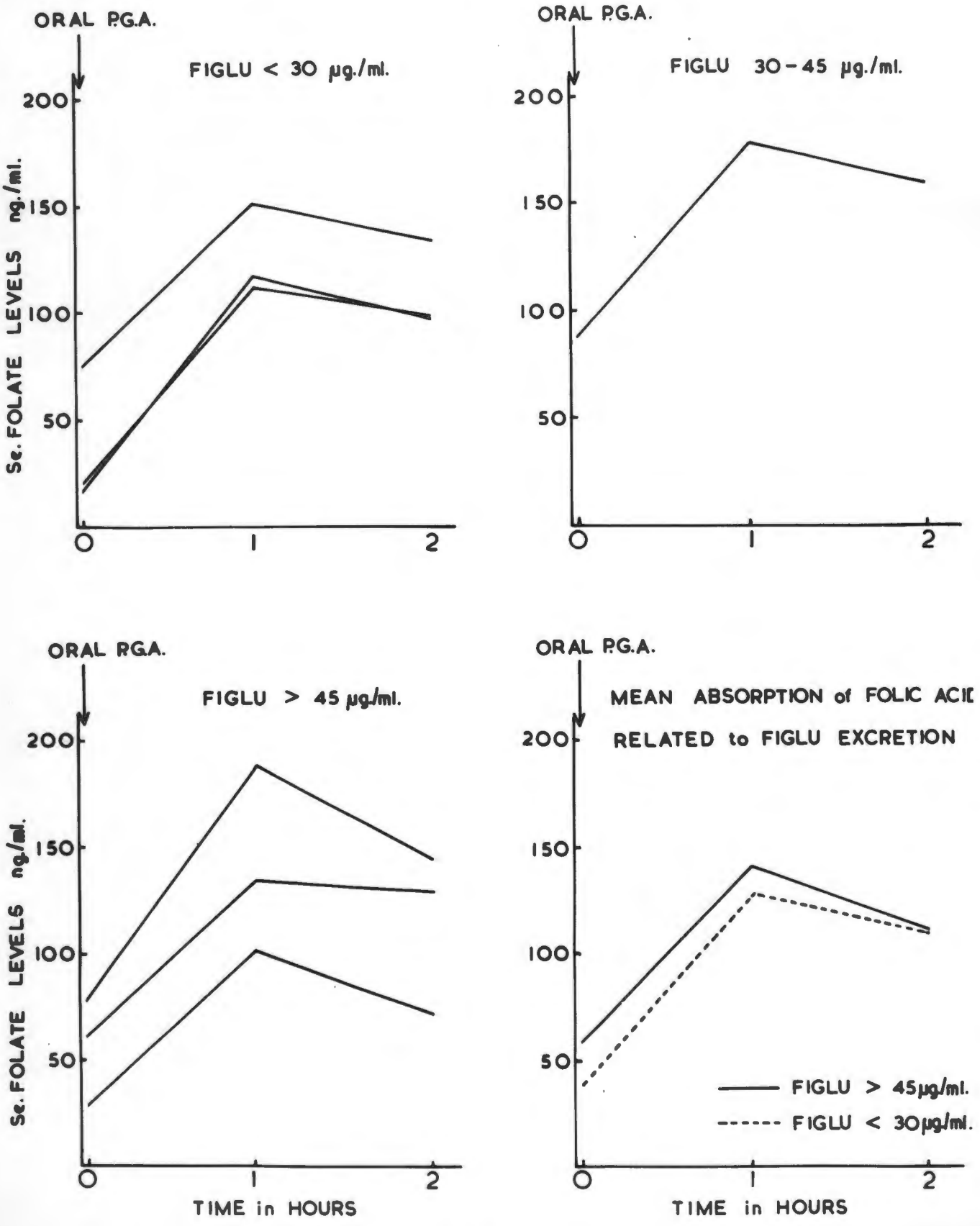


Fig. 33

RELATIONSHIP of FOLIC ACID ABSORPTION to RANGE of FIGLU EXCRETION

FIGLU < 30 µg/ml.

SUBJECT	FIGLU µg./ml.	FOLATE LEVELS ng/ml.				CLINICAL CONDITION
		Se. FOLATE	BASAL	1 Hr.	2 Hrs.	
B.S.	21.9	8.0	18	112	99	WELL
J. P.	16.9	1.1	14	117	98	POST G.E.
M. L.	28.9	2.3	72	151	134	POST G.E.
		MEAN	35	127	110	

FIGLU 30–45 µg./ml.

SUBJECT	FIGLU µg./ml.	FOLATE LEVELS ng./ml.				CLINICAL CONDITION
		Se. FOLATE	BASAL	1 Hr.	2 Hrs.	
L. F.	42.9	20.5	85	178	160	U.R.T.

FIGLU > 45 µg./ml.

SUBJECT	FIGLU µg./ml.	FOLATE LEVELS ng./ml.				CLINICAL CONDITION
		Se. FOLATE	BASAL	1 Hr.	2 Hrs.	
M. de L.	84.6	10.6	26	102	73	POST G.E.
M.E.	71.0	6.1	60	135	128	POST G.E.
C.E.	60.0	19.1	75	187	134	WELL
		MEAN	54	141	112	

RISE in FOLATE

BASAL to 1 HOUR

DATA	FIGLU > 45 µg./ml.	FIGLU < 30 µg./ml.
MEAN	88	92
RANGE	75 – 112	79 – 103
S.D.	± 21.2	±11.9
S.E. of MEAN	± 12.5	± 7.0

DIFFERENCE of MEANS $t = 0.338$

$0.70 < P < 0.80$

BASAL to 2 HOURS

DATA	FIGLU > 45 µg./ml.	FIGLU < 30 µg./ml.
MEAN	58	75
RANGE	46 – 69	62 – 84
S.D.	±11.0	±12.1
S.E. of MEAN	± 6.5	± 7.1

DIFFERENCE of MEANS $t = 1.872$

$0.10 < P < 0.20$

TABLE 64

c) Socio-economic circumstances.

Method A (Table 62).

A statistical relationship could not be proved between mean Figlu excretion after oral histidine and socio-economic circumstances.

d) Nutrition.

Method A (Table 63).

A relationship between mean Figlu excretion and nutrition could not be proved.

e) Intestinal absorption.

(i) Folic acid absorption.

Method A (Fig. 33; Table 64).

Folic acid absorption was not significantly different in infants with positive Figlu tests. The mean rise in folate from the basal level to the first and to the second hour was slightly lower in infants with positive Figlu tests.

(ii) Xylose absorption.

Method A.

These tests were performed on 21 infants on whom Figlu tests were successfully performed.

9 infants excreted Figlu in excess of 45 µg./ml.

2 infants excreted Figlu in the range 30 - 45 µg./ml.

10 infants excreted Figlu in amounts less than 30 µg./ml.

Only one infant with a positive Figlu test after oral histidine had abnormal xylose excretion (6%) which returned to normal (25.3%) after successful treatment of the associated gastroenteritis. The Figlu test after oral histidine was still positive 3 months later. Three infants with negative Figlu tests and without evidence of folate deficiency had abnormally low xylose excretion. In one of these the urine output was unsatisfactory.

TABLE 65

THE RELATIONSHIP OF FIGLU EXCRETION TO INCREASED DEMAND FOR FOLATE

(a) Increased erythropoiesis as judged by reticulocyte count (%)

Age (months)	Range of Figlu excretion µg./ml.	Range of reticulocyte count		Mean reticulocyte count
2½ - 7½	< 30	0.1 - 4.1	n = 23	1.6
	30 - 45	0.6 - 2.8	n = 6	1.7
	> 45	0.7 - 2.6	n = 16	1.5

Conclusion:

Infants who excrete excessive amounts of Figlu do not have increased erythropoiesis as judged by mean and range of reticulocyte counts.

TABLE 65 (Contd.)

(b) The effect of infection as judged by the number and severity of infections (Infection score)

Age (months)	Infection score	Mean Figlu excretion µg./ml.		Test of significance	Significance
2½ - 4½	0	42.6	n = 27		
	½ - 2	46.2	n = 69	Analysis of variance	F = 0.18
	2½ - 4½	40.7	n = 14		P > 0.05
	5 & >	57.2	n = 1		
4½ - 7½	0	38.4	n = 15		
	½ - 2	44.2	n = 54	"	F = 0.11
	2½ - 4½	42.9	n = 20		P > 0.05
	5 & >	44.0	n = 3		
7½ - 10¾	0	15.0	n = 4		
	½ - 2	33.9	n = 19	"	F = 1.18
	2½ - 4½	19.6	n = 7		P > 0.05
	5 & >	17.0	n = 1		
10¾ - 16	0	21.0	n = 3		
	½ - 2	26.1	n = 9	"	F = 4.02
	2½ - 4½	32.3	n = 5		P < 0.05
	5 & >	71.3	n = 2		

Conclusion:

There is no consistent effect of infection score on mean Figlu excretion except in the age group 10¾ - 16 months. Here Figlu excretion is significantly higher in the group with an infection score of 5 and > than in all other groups. This analysis was complicated by the fact that M.R. one of the 2 infants in the 5 and > group had low Figlu and excessive urocanic acid excretion on one occasion and on another had high Figlu excretion without excessive urocanic acid excretion, when the test was repeated while she was still in this age group. If infection score is compared with the second of these results the above statistically significant result is obtained. If it is compared with the first result there is no statistical relationship between infection score and Figlu excretion. For this reason and because the pattern is not consistent in the other age groups a relationship between infection score and Figlu excretion has not been proved.

TABLE 66.

EFFECT OF SMALL DOSES OF FOLIC ACID ON FIGLU EXCRETION.

Composite data.

Investigation	Mean value before treatment	Mean value after treatment	Test of significance	Significance
Figlu excretion mg./24 hrs.	21.6 n = 3	13.3 n = 3	'STUDENT'S' t-test	t = 1.239 0.20<P<0.30
Figlu excretion µg./ml.	80.2 n = 4	44.8 n = 4	"	t = 1.553 0.10<P<0.20

Conclusion: The improvement in mean Figlu excretion (µg./ml. and mg./24 hrs.) following folic acid treatment is not statistically significant.

Individual results.

Infant	Figlu result µg./ml.		Figlu result mg./24 hrs.	
	Before treatment	After treatment	Before treatment	After treatment
B.M.	87.3	22.9	19.6	8.5
R.L.	65.8	15.7	16.1	7.1
A.P.	68.7	108.4	29.2	24.4
T.J.	99.0	32.1	-	-

Conclusion: Results of Figlu excretion µg./ml.

Figlu excretion improved in 3 to normal or borderline levels.
Figlu excretion increased in 1.

Results of Figlu excretion mg./24 hrs.

Figlu excretion improved in all.

A relationship between Figlu excretion after oral histidine and malabsorption using the parameters in this study was not demonstrated.

f) Increased demand for folate.

(i) Increased erythropoiesis.

Method A (Table 65).

The range and mean reticulocyte counts were unrelated to Figlu excretion after oral histidine.

Occult blood tests were performed on 20 infants. None of the infants with positive Figlu tests had positive occult blood tests.

(ii) Infection score.

Method A (Table 65).
Method B (Table 61).

A statistical relationship between mean Figlu excretion after oral histidine and infection score could not be proved.

(iii) Growth.

Method A (Table 63).
Method B (Table 61).

There was no relationship between rapid growth and excessive Figlu excretion. On the contrary, mean Figlu excretion appeared higher in poorly nourished infants.

The Effect of Small Doses of Folic Acid on Figlu Excretion.

Method A. (Table 66).

Small doses of folic acid were administered (approximately 50 - 300 µg. daily) to 4 infants who had not shown significant reduction of Figlu excretion after intramuscular iron therapy. There was a marked reduction in mean Figlu excretion after oral histidine, but the decrease in both concentration (µg./ml.) and quantity (mg./24 hrs.) was not statistically different to that before treatment.

FOLIC ACID CLEARANCES RELATED to FIGLU EXCRETION
L.CASEI and S.FAECALIS

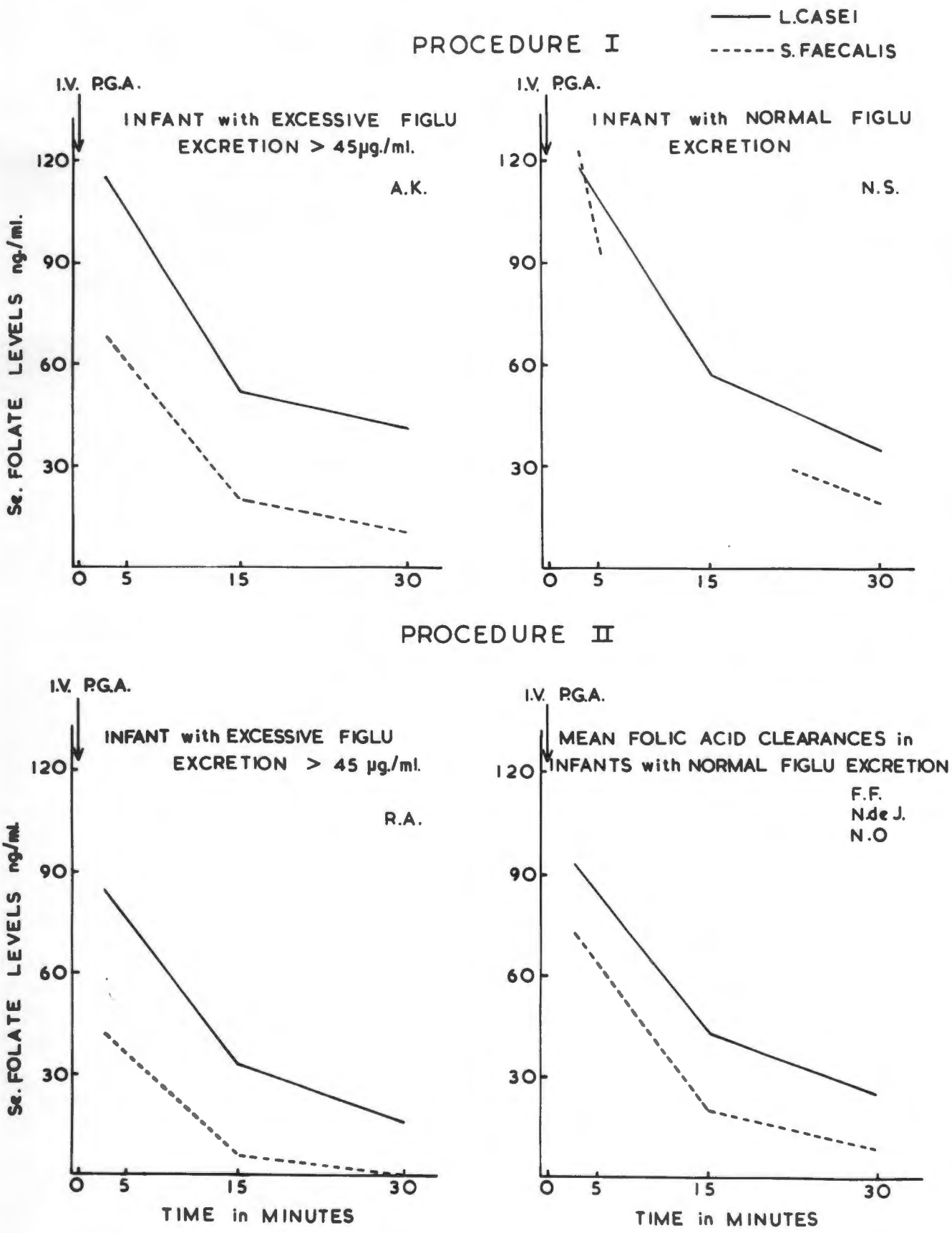


Fig. 34

FOLIC ACID CLEARANCES RELATED to FIGLU EXCRETION
L.CASEI and S. FAECALIS

PROCEDURE I

INFANT with EXCESSIVE FIGLU EXCRETION >45 µg./ml.

SUBJECT	ASSAY ORGANISM	FIGLU		Se. FOLATE LEVELS ng./ml.			
		µg./ml.	mg./24 Hrs.	BASAL	3 MIN.	15 MIN.	30 MIN.
A.K	L.CASEI	70.1	19.6	4.8	115	52	41
	S.FAECALIS			—	68	20	10

INFANT with NORMAL FIGLU EXCRETION

SUBJECT	ASSAY ORGANISM	FIGLU		Se. FOLATE LEVELS ng./ml.			
		µg./ml.	mg./24 Hrs.	BASAL	3 MIN.	15 MIN.	30 MIN.
N.S.	L.CASEI	14.0	8.4	7.6	118	58	35
	S.FAECALIS			—	123	—	18

PROCEDURE II

INFANT with EXCESSIVE FIGLU EXCRETION > 45µg/ml.

SUBJECT	ASSAY ORGANISM	FIGLU		Se. FOLATE LEVELS ng./ml.			
		µg./ml.	mg/24 Hrs.	BASAL	3 MIN.	15 MIN.	30 MIN.
R.A.	L.CASEI	51.2	21.0	9.6	85	34	16
	S.FAECALIS			—	42	6	0

FOLIC ACID CLEARANCES in INFANTS with NORMAL FIGLU EXCRETION

SUBJECT	ASSAY ORGANISM	FIGLU		Se. FOLATE LEVELS ng./ml.			
		µg./ml.	mg/24Hrs.	BASAL	3 MIN.	15 MIN.	30 MIN.
F.F.	L.CASEI	27.2	7.3	6.4	65	32	19
	S.FAECALIS			—	57	14	3
N. de J.	L.CASEI	24.0	7.4	9.6	118	59	35
	S.FAECALIS			—	97	32	14
N.O	L.CASEI	16.2	12.8	2.4	98	40	22
	S.FAECALIS			—	64	13	—
			MEAN	L.CASEI	94	44	25
				S.FAECALIS	73	20	9

TABLE 67

All individual infants showed reduced Figlu excretion when this was expressed in quantity (mg./24 hrs) and three of the four infants showed reduction when Figlu excretion was expressed as concentration (µg./ml.).

THE FIGLU TEST AFTER ORAL HISTIDINE AS A PARAMETER OF SECONDARY DISTURBANCE OF FOLATE METABOLISM.

Folic acid clearance test.

Procedure 1.

Method A (Fig.34; Table 67).

L.casei folic acid clearance curves were similar irrespective of the amount of Figlu excreted.

The S.faecalis folic acid clearance curve was faster in the infant with a positive Figlu test (A.K.). The S.faecalis clearance curves in A.K. and N.S. fell within the lower limit of the reported normal adult range (Chanarin, Mollin and Anderson, 1958a) with the exception of the 3 minute and possibly the 15 minute level in A.K.

Both infants were iron deficient but A.K.'s serum iron level was lower (Table 35).

Procedure 2.

Method B (Fig.34; Table 67).

L.casei folic acid clearance curves were comparable in infants with positive and negative Figlu tests excepting at the 30 minute level. Here the curve in the infant with the positive Figlu test (R.A.) was slightly faster than those with normal Figlu tests.

The S.faecalis folic acid clearance curve was faster in R.A. than in the other infants at all levels but particularly at the 3 minute level.

TABLE 68

RELATIONSHIP OF

FIGLU EXCRETION TO METABOLIC DISTURBANCES OF FOLATE METABOLISM

Relationship to vitamin B_{12} deficiency

Age of infant (months)	Range of vitamin B_{12} pg./ml.	Mean	S.D.	S.E. of mean
$2\frac{1}{4}$ - $4\frac{1}{2}$	118*/178 - 1236+ n = 107	469.8	± 211.0	± 20.5
$4\frac{1}{2}$ - $7\frac{1}{2}$	133*/146 - 1037+ n = 84	482.8	± 212.7	± 23.4
$7\frac{1}{2}$ - $10\frac{3}{4}$	165 - 993 n = 35	571.4	± 212.2	± 36.6
$10\frac{3}{4}$ - 16	180 - 1936+ n = 34	607.0	± 323.8	± 55.8

*There were two low readings in the whole series, one of which was found in an infant excreting >45 µg./ml. of Figlu.

+Some of these infants with high readings received vitamin B_{12}. This could not be established in all.

The slightly faster clearance of folic acid using S.faecalis at all levels and especially at the 3 minute level was a consistent finding in 2 infants with positive Figlu tests.

The folic acid clearance test is associated with a high degree of variation in normal values. The number of infants investigated was small and it was therefore not known whether the observation had any meaning, and if so, what relationship there was to associated iron deficiency.

An unequivocal relationship between excessive Figlu excretion and the rate of folic acid clearance using S.faecalis as the assay organism was not demonstrated. It was unfortunate that it was not possible to carry out these tests on a greater number of infants and especially on infants who had both high Figlu excretion and high serum L.casei folate levels.

An Aetiology for Disturbed Folate Metabolism and its Relationship to Positive Figlu Tests after Oral Histidine.

a) Vitamin B_{12} deficiency.

Method A (Table 68).

In this study only 2 infants were found to have a low vitamin B_{12} level. One excreted Figlu in excess of 45 µg./ml. and had a serum vitamin B_{12} level of 118 pg./ml. The other infant excreted normal quantities of Figlu and had a serum vitamin B_{12} level of 133 pg./ml.

Four (4) infants in the prospective study and 3 in the additional study did not have vitamin B_{12} estimations at any stage. There was no evidence for attributing all the positive Figlu tests to vitamin B_{12} deficiency.

Method B.

No vitamin B_{12} deficiency was encountered in this group.

RELATIONSHIP OF MEAN SERUM IRON LEVELS TO RANGE OF FIGLU EXCRETION IN INFANTS

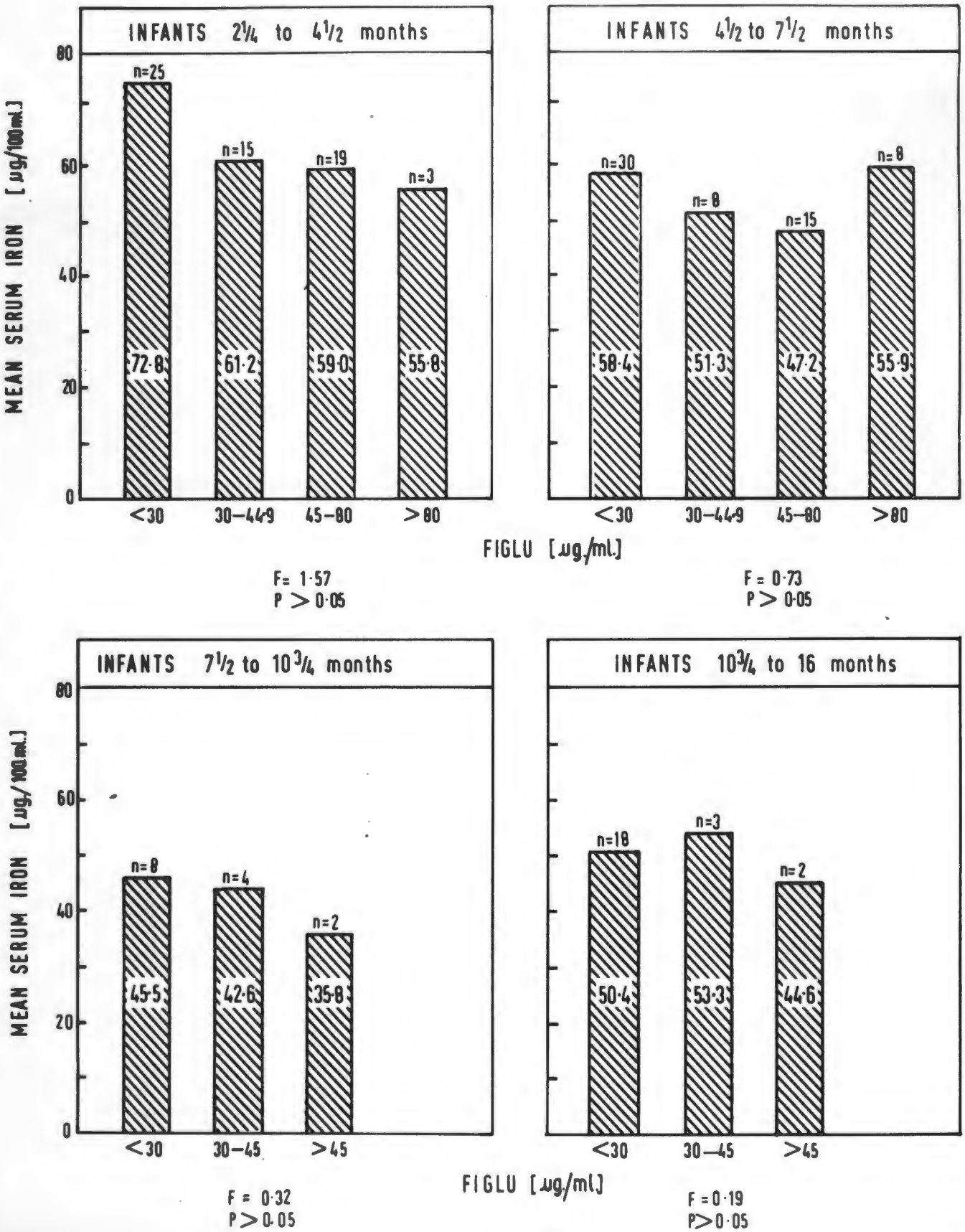


Fig. 35.

TABLE 73

THE EFFECT OF THERAPY ON FIGLU EXCRETION.

INDIVIDUAL RESULTS.

(a) Folic acid and iron therapy.

Infant	Figlu excretion µg./ml.		Figlu excretion mg./24 hrs.	
	Before treatment	After treatment	Before treatment	After treatment
A.G.	37.1	70.4	12.2	13.7
D.E.	69.7	30.5	12.5	7.3
D.H.	65.1	16.7	16.3	6.8
M.J.	181.5	9.3	27.2	2.8

Conclusion: Figlu excretion decreased in three out of four infants.

(b) Iron therapy.

Infant	Figlu excretion µg./ml.		Figlu excretion mg./24 hrs.	
	Before treatment	After treatment	Before treatment	After treatment
P.L.	32.3	33.9	13.6	15.3
M.S.	31.3	12.9	10.6	9.2
B.H.	52.9	16.0	22.2	7.0
E.B.[x]	42.0	83.2	21.0	23.3
A.F.	17.4	39.0	5.6	11.7
A.G.	19.6	152.9	7.2	22.9
B.R.	4.4	88.5	1.5	26.6

Conclusion: Figlu excretion improved in two out of seven infants.

[x] Only infant where therapy was irregular.

TABLE 74

THE EFFECT OF THERAPY ON OTHER PARAMETERS.

(a) Folic acid and iron.

Parameter	Mean value before treatment	Mean value after treatment
Haemoglobin g/100 ml.	10.2 n = 3	9.7 n = 3
Haematocrit. vols %	33 n = 3	32 n = 3
Percentile weight	18 n = 4	16 n = 4
Serum folate ng./ml.	4.1 n = 4	20.9 n = 4
Serum iron µg./100ml.	58.1 n = 2	52.1 n = 2

Conclusion: Apart from a rise in mean serum folate level therapy did not produce any improvement on the parameters.

(b) Oral iron.

Parameter	Mean value before treatment	Mean value after treatment
Haemoglobin g./100 ml.	10.5 n = 4	10.2 n = 4
Haematocrit. vols %	34 n = 5	33 n = 5
Percentile weight	67 n = 7	67 n = 7
Serum folate ng./ml.	5.9 n = 3	5.2 n = 3
Serum iron µg./100 ml.	61.0 n = 2	44.0 n = 2

Conclusion: Therapy had no beneficial effect on any of these parameters.

Figlu excretion returned to normal in these cases and also in 2 of the 4 African infants given standard doses of folic acid. These data were used to assess the therapeutic effect of folic acid on other parameters but were unhelpful in determining the effect of folic acid on Figlu excretion.

(ii) Infants less than 6 months.

Method B (Fig. 37; Table 73).

Folic acid and iron therapy.

The administration of folic acid and iron to 4 infants with positive Figlu tests after oral histidine led to a marked decrease in the mean Figlu excretion in µg./ml. and mg./24 hrs. This was not related to a change in mean 24 hour urine volume. The improvement was not statistically significant probably because only 3 of the 4 infants showed reduction in Figlu excretion.

Iron Therapy.

Oral iron was administered to 7 infants with positive Figlu tests after oral histidine. These infants showed an increase in mean Figlu excretion (µg./ml. and mg/24 hrs.) which was not related to a change in mean 24 hour urine volume. The increase in mean Figlu excretion was not statistically significant probably because Figlu excretion in individual infants either increased or decreased after this therapeutic programme.

Because oral iron appeared relatively ineffective as assessed by serum iron levels (Tables 74; 69), it was unlikely that in both groups of infants a coincidental increased demand for folate resulting from the

TABLE 75

RELATIONSHIP OF CREATININE RATIO* TO AGE

Mean age (months)	Mean creatinine ratio	Test of significance	Significance
4.2	45.9 n = 26	'STUDENT'S' t-Test	t = 2.162
4.9	61.9 n = 26		0.02 < P < 0.05

* $\frac{\text{Actual creatinine}}{\text{Expected creatinine/Kg./body weight}}$ mgs./24 hrs. X 100.

Conclusion: The increase in mean age was associated with a significant improvement in mean creatinine ratio.

RELATIONSHIP OF CREATININE RATIO TO NUTRITION (WEIGHT PERCENTILE)

Mean age (months)	Mean percentile	Mean creatinine ratio	Test of significance	Significance
4.2	53 n = 26	45.9 n = 26	'STUDENT'S' t-Test	t = 0.353
4.9	56 n = 26	61.9 n = 26		0.70 < P < 0.80

Conclusion: The mean percentile in the group studied was greater than the 50th percentile on both occasions. The minimal improvement in mean percentile value with age was not statistically significant. It did not therefore contribute to the statistically significant improvement in creatinine ratio previously shown over this time period.

iron therapy given to iron deficient infants had any effect on the Figlu excretion. Mothers reported that infants showed a great reluctance to take the oral iron (Ferlucon) though they took the folic acid well. It seemed likely that ineffective administration of the iron played a role in the apparent inefficacy of iron therapy. There was no evidence that folic acid was not administered and mean serum L.casei folate levels showed a substantial rise (Table 74).

Although the effect of the 2 therapeutic programmes on mean Figlu excretion was very different, neither the reduction of mean Figlu excretion after folic acid (pharmacological doses) and oral iron, nor the increase in mean Figlu excretion after oral iron was statistically significant. This difference in therapeutic effect might have become more or less obvious had greater numbers of infants been investigated. However, it was clear from this that the positive Figlu tests after oral histidine could not be attributed to folate deficiency but could possibly still be ascribed to a secondary disturbance of folate metabolism.

THE FIGLU TEST AFTER ORAL HISTIDINE AND NON-SPECIFIC FACTORS.

a) Incomplete urine collection.

(i) Creatinine ratio.

Method B (Table 75).

The mean creatinine ratio showed a significant increase parallel with growth. This was unrelated to the minimal associated nutritional improvement which occurred at the same time.

(ii) Creatinine ratio as an index of "completeness" of urine collection.

(1) Relationship to 24 hour urine volumes.

TABLE 76.

RELATIONSHIP OF CREATININE RATIO TO 24-HOUR URINE VOLUME

Age (months)	Creatinine ratio	Mean volume	Test of significance	Significance
4.2	< 50	439 n = 17	'STUDENT'S' t-Test	t = 1.575
	> 50	353 n = 9		0.10 < P < 0.20
4.9	< 50	340 n = 9	"	t = 0.081
	> 50	334 n = 17		P > 0.90

Conclusion:

The lower creatinine ratios are associated with higher mean volumes.

The differences are not statistically significant.

TABLE 77.

RELATIONSHIP OF CREATININE RATIO TO FIGLU EXCRETION

Mean age (months)	Figlu excretion	Mean creatinine ratio	Test of significance	Significance
4.2	Negative	40.7 n = 16	Analysis of variance	F = 3.22
	Borderline	67.5 n = 1	'STUDENT'S' t-Test	P > 0.05
	Positive	56.3 n = 8	(Negative compared with positive group)	t = 2.197 0.02 < P < 0.05
4.9	Negative	58.4 n = 15	Analysis of variance	F = 0.28
	Borderline	61.5 n = 5	'STUDENT'S' t-Test	P > 0.05
	Positive	70.8 n = 6	(Negative compared with positive group)	t = 0.725 0.40 < P < 0.50

Conclusion:

The mean creatinine ratio is consistently higher in infants with borderline and positive Figlu tests. The overall differences are not significant.

In the age group 4.2 months, the maldistribution of numbers might have influenced the statistical result. Thus the mean creatinine ratios in the positive and negative Figlu groups were compared using the 'STUDENT'S' t-Test.

At 4.2 months, the mean creatinine ratio was significantly higher in infants excreting Figlu in excess than in those excreting normal quantities. At 4.9 months, the mean creatinine ratio in infants with positive Figlu tests was not significantly higher than in infants with normal tests.

TABLE 78

NON-SPECIFIC FACTORS POSSIBLY INFLUENCING FIGLU EXCRETION

Correlation of Figlu excretion with 24-hour urine volumes

Age of infants (months)	Statistical method	Result	Significance
2¼ - 4½	Correlation coefficient	r = -.146 n = 104*	r does not differ from zero. P > 0.10
4½ - 7½	" "	r = -.314 n = 92	r differs from zero. 0.02 < P < 0.05
7½ - 10¾	" "	r = -.348 n = 35	r does not differ from zero. 0.05 < P < 0.10
10¾ - 16	" "	r = -.333 n = 35	r does not differ from zero 0.05 < P < 0.10

Conclusion:

The correlation coefficient only differs from zero at the age group 4½ - 7½ months. At best this is a very weak correlation and on the basis of the statistical result alone is unlikely to have much meaning. There is no correlation between these two parameters in any of the other age groups.

* The infant who excreted both urocanic acid and Figlu in excess was not included in this analysis.

Method B (Table 76).

There was no statistical relationship between creatinine ratio and mean 24 hour urine volume. Lower mean 24 hour urine volumes were associated with higher creatinine ratios. Thus, the volume of the 24 hour urine collection was not necessarily a reflection of "completeness" of urine collection. Small urine volumes were probably the result of urine concentration rather than incomplete urine collection.

(2) Relationship to Figlu excretion.

Method B (Table 77).

The mean creatinine ratio was consistently higher in infants with positive or borderline positive Figlu tests after oral histidine. In using the analysis of variance no statistical relationship between Figlu excretion and mean creatinine ratio was evident. However, using the 'Student's' t-test it was possible to show that at 4.2 months mean creatinine ratio was significantly higher in infants with positive Figlu tests than in infants with negative Figlu tests. The infants with borderline Figlu excretion were not considered in this statistical analysis. Although mean creatinine ratio remained higher in infants with positive Figlu tests at 4.9 months, this was not a statistically significant relationship.

The higher creatinine ratio found in infants with positive Figlu tests implied that urine collection was either as, or more complete than that of infants with negative Figlu tests. Thus,positive Figlu tests could not be ascribed to incomplete collection of urine.

b) Urine concentration and the results of the Figlu tests after oral histidine.

Method A (Table 78).

A statistical relationship between Figlu excretion and 24 hour urine volume could not be proved.

THE EFFECT OF URINE CONCENTRATION ON THE VALIDITY OF FIGLU RESULTS µg./ml. & mg./24hrs. IN THE COMBINED THERAPEUTIC GROUPS.

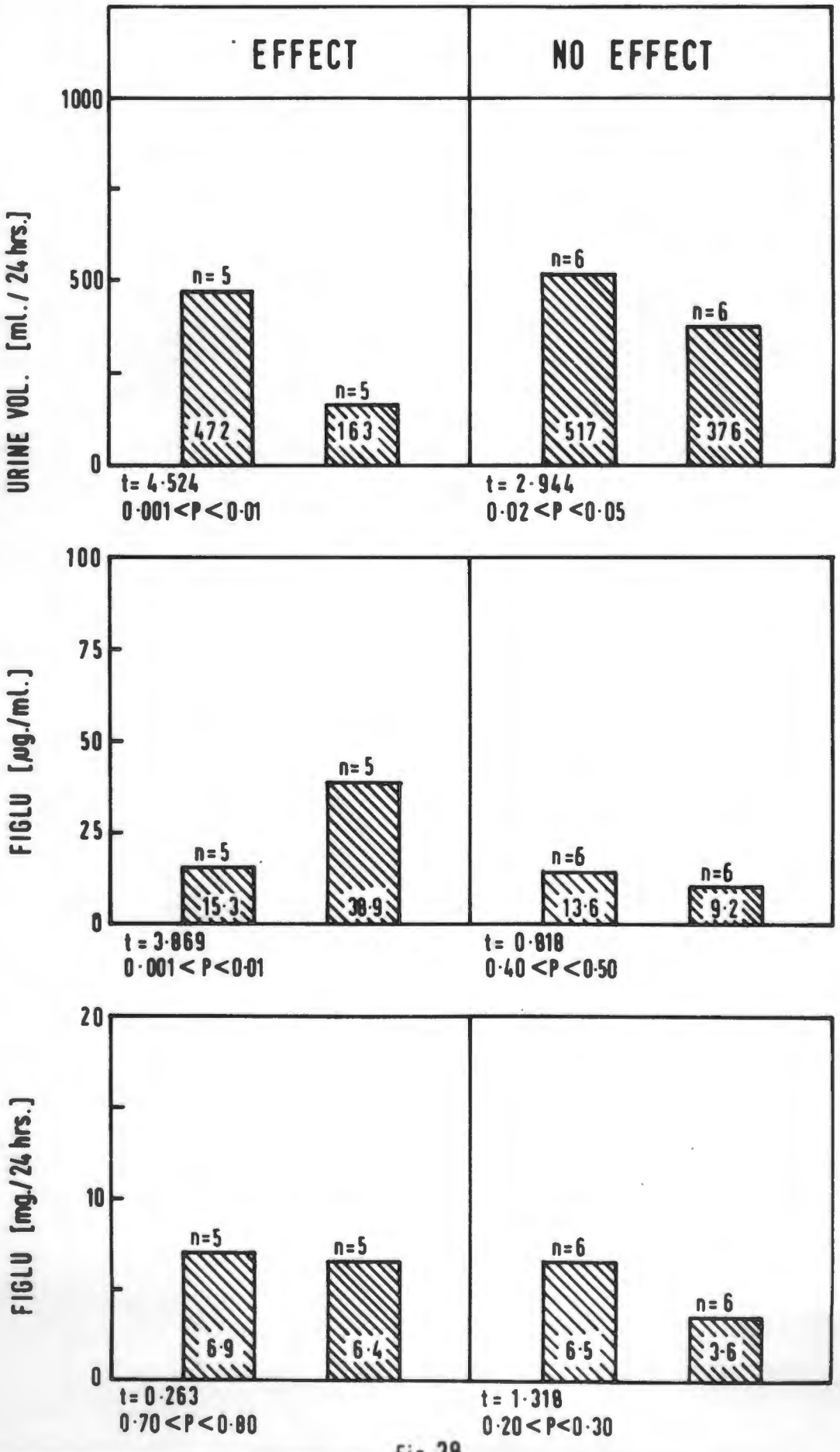


Fig. 38

THE EFFECT OF URINE CONCENTRATION ON THE VALIDITY OF FIGLU RESULTS [µg./ml. & mg./24hrs.]

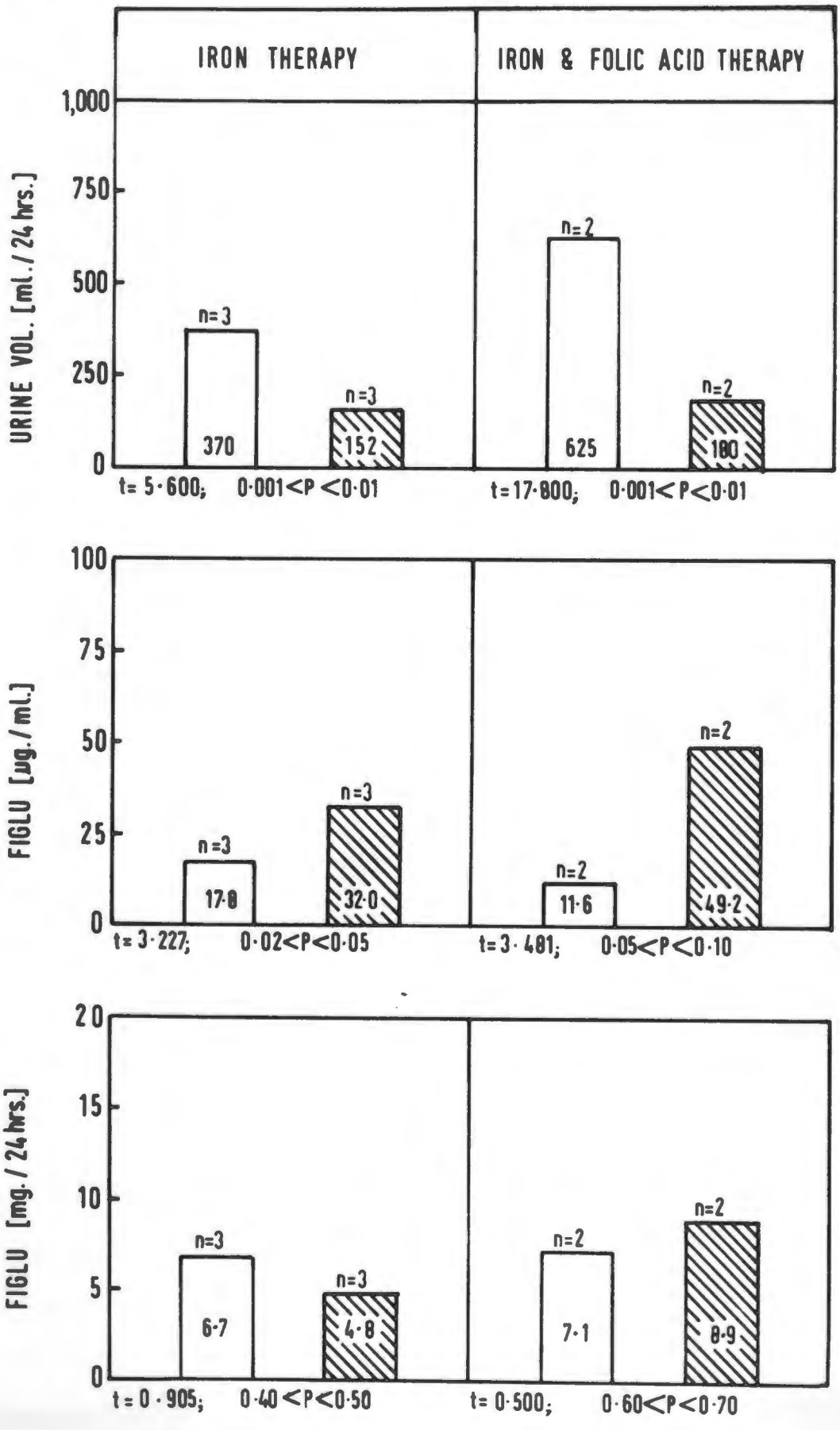


Fig. 39

Method B (Fig.38; Fig.39).

In the controlled therapeutic trial, the Figlu test was repeated 3 weeks after the administration of folic acid and oral iron. Large differences in 24 hour urine output on the two occasions were noted in some infants. The effect of urine volume on Figlu excretion was assessed in these infants in spite of the therapy administered in the interim, because it had been previously shown that this did not have any significant effect on Figlu excretion.

In one group of infants (5) a significant decrease in urine volume resulted in an increase in mean Figlu excretion expressed in concentration (µg./ml.) while total output of Figlu (mg./24 hrs.) was little changed (Fig.38 - "effect" column). Because quantity of Figlu output was unaltered, the rise in Figlu concentration (µg./ml.) was almost certainly spurious and the result of urine concentration. This pattern was similar in the 5 infants whether it was analysed according to therapy administered (Fig.39) or not (Fig.38 - "effect" column). The increase in Figlu excretion in µg./ml. under these circumstances was statistically significant in the combined therapeutic group and also in the group of infants given only iron. The failure to demonstrate a statistically significant rise in Figlu excretion in the infants given folic acid and iron, might have been related to the smaller number of infants investigated in this group.

In another group of infants, 4 of whom received folic acid and iron, and 2 of whom were given only iron (Fig.38 - "no effect" column) a significant decrease in urine volume was associated with a decrease in Figlu excretion in µg./ml. and mg./24 hours. This was not statistically significant.

TABLE 79

THE SIGNIFICANCE OF EXCESSIVE EXCRETION OF FIGLU

(a) Relationship to haemoglobin levels

Age (months)	Range of Figlu excretion µg./ml.	Mean haemoglobin g./100 ml.		Test of significance	Significance
2¼ - 4½	< 30	10.9	n = 39	Analysis of variance	F = 0.33
	30 - 44.9	11.2	n = 18		P > 0.05
	45 - 80	11.0	n = 33		
	> 80	11.1	n = 11		
4½ - 7½	< 30	10.8	n = 40		F = 0.01
	30 - 44.9	10.6	n = 17	"	P > 0.05
	45 - 80	10.6	n = 15		
	> 80	10.6	n = 10		
7½ - 10¾	< 30	10.6	n = 18		F = 0.20
	30 - 45	10.2	n = 4	"	P > 0.05
	> 45	10.1	n = 3		
10¾ - 16	< 30	9.9	n = 23		F = 0.17
	30 - 45	10.3	n = 4	"	P > 0.05
	> 45	10.1	n = 2		

Conclusion: There is no relationship between these two parameters.

(b) Relationship to growth (weight percentile)

Age (months)	Range of Figlu excretion µg./ml.	Mean weight percentile		Test of significance	Significance
2¼ - 4½	< 30	46	n = 44	Analysis of variance	F = 3.32
	30 - 44.9	42	n = 22		0.01 < P < 0.05
	45 - 80	44	n = 37		
	> 80	29	n = 11		
4½ - 7½	< 30	45	n = 47		F = 0.19
	30 - 44.9	43	n = 18	"	P > 0.05
	45 - 80	39	n = 19		
	> 80	48	n = 13		
7½ - 10¾	< 30	37	n = 25		F = 0.25
	30 - 45	30	n = 5	"	P > 0.05
	> 45	29	n = 5		
10¾ - 16	< 30	34	n = 26		F = 3.31
	30 - 45	54	n = 6		P < 0.05
	> 45	4	n = 4		

Conclusion: Growth is not consistently retarded in infants excreting Figlu in excess, although this is the case in infants in the age group 2¼ - 4½ and 10¾ - 16 months. It is unlikely that the result has any meaning because (i) of the lack of consistency in this association, and (ii) the analysis of individual differences shows that the results of Group 4 (>80) differ significantly from all other groups at age 2¼ - 4½ months but at 10¾ - 16 months the result of Group 3 (>45) is only significantly lower than Group 2 (30 - 45).

TABLE 79 (Contd.)

(c) Relationship to infection score

Age (months)	Range of Figlu excretion µg./ml.	Mean Infection score		Test of significance	Significamce
2¼ - 4½	<30	1.3	n = 44	Analysis of variance	F = 0.00
	30 - 44.9	1.2	n = 22		
	45 - 80	1.3	n = 37		P > 0.05
	> 80	1.3	n = 10		
4½ - 7½	<30	1.5	n = 46	"	F = 0.93
	30 - 44.9	1.4	n = 18		
	45 - 80	2.1	n = 19		P > 0.05
	> 80	1.7	n = 12		
7½ - 10¾	< 30	1.9	n = 22	"	F = 0.44
	30 - 45	2.0	n = 5		
	> 45	1.3	n = 4		P > 0.05
10¾ - 16	< 30	1.6	n = 11	"	F = 3.17
	30 - 45	1.9	n = 5		
	> 45	4.2	n = 3		P > 0.05

Conclusion:

Excessive excretion of Figlu does not have a consistent effect on infection score and there is no statistical relationship between these two parameters.

TABLE 80

SIGNIFICANCE OF FIGLU EXCRETION

Relationship of Figlu excretion to mean haemoglobin levels.

Figlu excretion	Mean haemoglobin levels	Test of significance	Significance
Negative	10.0 n = 25	Analysis of variance	F = 0.60 P > 0.05
Borderline	9.9 n = 3		
Positive	10.6 n = 6		

Conclusion: Infants with excessive excretion of Figlu do not have lower mean haemoglobin levels.

None of the differences between mean haemoglobin levels in the 3 groups are statistically significant.

From this it seemed that in individual infants significant urine concentration might result in spurious positive Figlu tests if the results were expressed in µg./ml. and if both urine concentration and total Figlu output were great enough. From this, it seemed more valid to express Figlu results of infants as total output (mg./24 hrs) rather than concentration of Figlu output (µg./ml.). This was therefore done when the results of the Figlu excretion test, using method B, were analysed.

THE SIGNIFICANCE OF THE POSITIVE FIGLU TEST AFTER ORAL HISTIDINE.

There was no statistical relationship between Figlu excretion and:

a) Mean haemoglobin concentration

Method A (Table 79).

Method B (Table 80).

b) Infant growth

Method A (Table 79).

There was no consistent effect of excessive Figlu excretion on mean weight percentile.

c) Infection score

Method A (Table 79).

There was no statistical relationship between positive Figlu tests and mean infection score.

The administration of folic acid did not produce

a) Convincing evidence of symptomatic benefit

Method A.

More infants with negative Figlu tests (6 of 15) had unequivocal symptomatic benefit from folic acid therapy than had infants with positive Figlu tests (3 of 13).

TABLE 81.

THE EFFECT OF

FOLIC ACID TREATMENT ON PERCENTILE AND HAEMOGLOBIN VALUES IN INFANTS EXCRETING EXCESSIVE CONCENTRATIONS OF FIGLU (> 45 µg./ml.)

Parameter	Mean value prior to treatment	Mean value post treatment	Test of significance	Significance
Haemoglobin level g./100 ml.	9.9 n = 9	10.1 n = 9	'STUDENT'S' t-Test	0.364 0.70< P <0.80
Percentile (weight)	42 n = 9	32 n = 9	-	-

Conclusion:

Neither haemoglobin level nor percentile value were improved by folic acid therapy

THE EFFECT OF FOLIC ACID TREATMENT

ON INFANTS EXCRETING NORMAL CONCENTRATIONS OF FIGLU.

Parameter	Mean value prior to treatment	Mean value post treatment
Haemoglobin level g./100 ml.	8.9 n = 3	8.9 n = 3
Percentile (weight)	39 n = 2	34 n = 2

Conclusion:

Folic acid therapy had no effect on mean haemoglobin or percentile values.

TABLE 69.

RELATIONSHIP OF FIGLU EXCRETION TO DISTURBANCES OF FOLATE METABOLISM.

(a) Relationship of Figlu excretion to iron deficiency.

(i)

Figlu excretion	Mean serum iron levels	Test of significance	Significance
Negative	60.5 n = 14	Analysis of variance	F = 0.04 P > 0.05
Borderline	58.7 n = 3		
Positive	63.4 n = 3		

Conclusion: There is no relationship between Figlu excretion and mean serum iron levels. The overall differences are not statistically significant.

Interrelationships of therapy, serum iron levels, and Figlu excretion

(ii) Therapy with oral iron.

Infant	Figlu mg./24 hrs.		Serum iron µg./100 ml.	
	Before treatment	After treatment	Before treatment	After treatment
A.G.	7.2	22.9	48.0	50.0
B.R.	1.5	26.6	74.0	38.0

Therapy with oral iron and folic acid.

Infant	Figlu mg./24 hrs.		Serum iron µg./100 ml.	
	Before treatment	After treatment	Before treatment	After treatment
D.E.	12.5	7.3	57.0	68.2
M.J.	27.2	2.8	59.2	36.0

Conclusion: Mean Figlu excretion in

(A.G.) increases while degree of iron deficiency is unchanged;
(B.R.) increases with increasing iron deficiency;
(D.E.) decreases with improvement in serum iron levels;
(M.J.) decreases with increasing iron deficiency.

There is no constant relationship between serum iron levels and Figlu excretion.

TABLE 69 (Contd.)

RELATIONSHIP OF FIGLU EXCRETION TO DISTURBANCES OF FOLATE METABOLISM.

(b) Infection at the time of investigation.

Classification	Mean Figlu excretion mg./24 hrs.	Test of significance	Significance
Infants with infection	8.3 n = 38	'STUDENT'S t-test	t = 1.842 0.05<P<0.10
Infants without infection	4.1 n = 7		

Conclusion: Mean Figlu excretion is higher in infants with infection than those without infection at the time of investigation. The difference is not statistically significant.

TABLE 70

EFFECT OF THERAPY WITH INTRAMUSCULAR IRON ON FIGLU EXCRETION.

Composite data.

Investigation	Mean value prior to treatment	Mean value after treatment	Test of significance	Significance
Figlu excretion mg./24 hrs.	21.7 n = 6	16.1 n = 6	'STUDENT'S' t-test	t = 0.800 0.40 < P < 0.50
Figlu excretion µg./ml.	64.0 n = 6	62.7 n = 6	"	t = 0.076 P > 0.90

Conclusion: Mean Figlu excretion was improved by intramuscular iron therapy. The result is not statistically significant.

INDIVIDUAL RESULTS.

Infant	Figlu result µg./ml. Before treatment	Figlu result µg./ml. After treatment	Figlu result mg./24 hrs. Before treatment	Figlu result mg./24 hrs. After treatment	Serum iron µg./100 ml. Before treatment	Serum iron µg./100 ml. After treatment	Haematocrit. Vols % Before treatment	Haematocrit. Vols % After treatment
M.E.	53.2	32.5	13.8	9.1	-	-	-	-
H.P.	43.6	23.0	10.5	4.8	-	-	-	-
B.M.	55.6	87.3	16.4	19.6	44.0	57.0	30	31
R.L.	61.8	65.8	21.6	16.1	51.0	60.0	40	38
T.J.	47.0	99.0	16.2	17.8	44.0	72.0	31	33
A.P.	122.7	68.7	51.5	29.2	79.0	75.0	28	33
Mean	64.0	62.7	21.7	16.1	54.5	66.0	32	34

Conclusion: Results in µg./ml.

Figlu excretion improved in three - Normal levels (1)
Borderline (1)
Still abnormal (1)

increased in three -

Results in mg./24 hrs.

Figlu excretion improved in 4.
Figlu excretion increased in 2.

Figlu excretion	Range of vitamin B_{12} levels
Excessive	336 - 1640 pg./ml.
Borderline	301 - 388 pg./ml.
Negative	272 - 928 pg./ml.

b) Iron deficiency.

(i) The relationship of Figlu excretion to mean serum iron levels.

Method A (Fig. 35)

Method B (Table 69)

There was no demonstrable statistical relationship between Figlu excretion in excess and mean serum iron levels.

(ii) Therapeutic trials with iron.

Method A (Table 70).

There was an overall decrease in mean Figlu excretion (µg./ml. and mg./24 hrs.) after intramuscular iron therapy. This was not a statistically significant difference probably because Figlu excretion was not reduced in all infants.

An inference drawn from this result was probably justified although the administration of iron did not cure the iron deficiency in all cases. There was evidence in only one case (R.L.) that cure of the iron deficiency might further have reduced Figlu excretion. In the others, A.P., B.M. and T.J., the change in Figlu excretion had no relation to the pattern of the serum iron levels.

Method B (Table 69)

A therapeutic trial with oral iron was relatively ineffective in improving serum iron levels. In individual infants the changes in Figlu excretion did not have a consistent relationship to the changes in the serum iron levels.

TABLE 71

SERUM GLUTAMIC-OXALOACETIC TRANSAMINASE AND BLOOD UREA ESTIMATIONS IN RELATION TO FIGLU EXCRETION.

Infant	Age (months)	Figlu result µg./ml.	S.G.O.T. (units)	Blood urea mg. %
R.K.	9.0	63.9	18.0	-
R.S.	3.5	80.3	-	29.0
P.C.	14.0	34.1	10.0	16.7
N.S.	9.3	41.0	22.0	29.8
C.C.	3.5	118.0	-	34.0
F.U.	12.8	74.2	20.0	12.9
B.W.	12.8	32.5	15.0	19.9
T.de B.	9.0	122.4	20.2	35.9
C.V.	14.5	34.9	25.0	29.0
S.B.	13.5	53.4	30.0	23.9
M.R.	13.0	9.1	40.0	14.0
A.v.D.	4.8	64.0	28.0	-
A.H.	3.0	47.4	5.0	34.4
A.M.	5.3	96.7	15.0	19.2
N.H.	3.0	48.2	35.0	-
O.M.	4.3	33.6	18.0	21.0
L.O.	7.0	52.0	15.0	21.0
W.S.	3.3	60.5	-	33.0

TABLE 72

THERAPEUTIC TRIAL WITH PHARMACOLOGICAL DOSES OF FOLIC ACID IN THE PROSPECTIVE STUDY

(a) Composite data

Infants given folic acid * Figlu µg./ml.		Infants not given folic acid * Figlu µg./ml.	
Before treatment Mean age 6.0 months	After treatment Mean age 7.7 months	Before treatment Mean age 6.0 months	After treatment Mean age 8.4 months
60.7 n = 9	29.1 n = 9	71.5 n = 22	29.3 n = 22

(b) Individual results

Subject	Age (months)	Figlu µg./ml.	Age (months)	Figlu µg./ml.	Therapy
J.A.	7.3	53.3	9.8	35.5	Oral folic acid 1.25 mg. daily for 10 days or 5 mg. b.d. in the presence of gastroenteritis. Infants were tested approximately 1 month after therapy commenced.
R.J.	6.3	32.6	8.0	33.5	
D.Br.	6.0	92.6	8.0	19.9	
E.L.	5.0	128.8	6.5	42.7	
H.F.	6.8	64.9	8.5	34.2	
R.S.	6.0	35.8	8.0	33.8	
A.U.	6.8	77.7	8.8	20.3	
C.F.	4.3	35.3	5.3	18.1	
V.S.	5.0	25.0	6.5	23.6	
J.D.	6.0	58.8	9.0	8.6	No treatment given
N.M.	6.0	34.8	8.8	18.7	
R.K.	6.0	128.1	9.0	63.9	
G.G.	6.0	38.5	8.8	11.6	
M.I.	6.0	62.9	9.5	4.0	
T.deB.	6.0	170.4	9.0	122.4	
E.D.	6.0	81.2	9.0	42.9	
P.C.	6.0	91.4	10.8	39.4	
N.L.	6.0	136.8	9.0	61.8	
D.B.	6.0	65.2	9.0	54.0	
M.R.	6.0	45.1	9.0	44.2	
N.S.	6.0	60.2	9.3	41.0	
V.B.	6.0	40.8	9.0	9.3	
K.M.	6.0	57.4	9.0	12.3	
N.v.W.	6.0	64.4	9.5	29.7	
B.C.	6.0	54.4	10.5	10.4	
N.D.	6.0	55.2	9.0	17.0	
F.U.	5.8	96.2	9.3	12.4	
C.V.	6.3	68.4	10.0	16.9	
D.Bz.	5.8	106.6	9.0	24.4	
J.E.	5.8	36.2	8.5	14.3	
S.B.	6.0	19.6	9.5	34.3	

* With one exception in the therapeutic group, this only refers to infants excreting excessive or borderline amounts of Figlu, on one or both occasions.

c) Infection at the time of investigation.

Method A (Fig.36).

Method B (Table 69).

There was no statistical relationship between infection at the time of investigation and mean Figlu excretion.

d) Hepatic and renal dysfunction.

Method A (Table 71).

Infants did not show clinical evidence of hepatic or renal dysfunction. Serum glutamic-oxaloacetic transaminase levels (SGOT) and blood urea estimations were normal when these tests were performed.

The Effect of Pharmacological Doses of Folic Acid on Figlu Excretion.

(i) Infants older than 6 months.

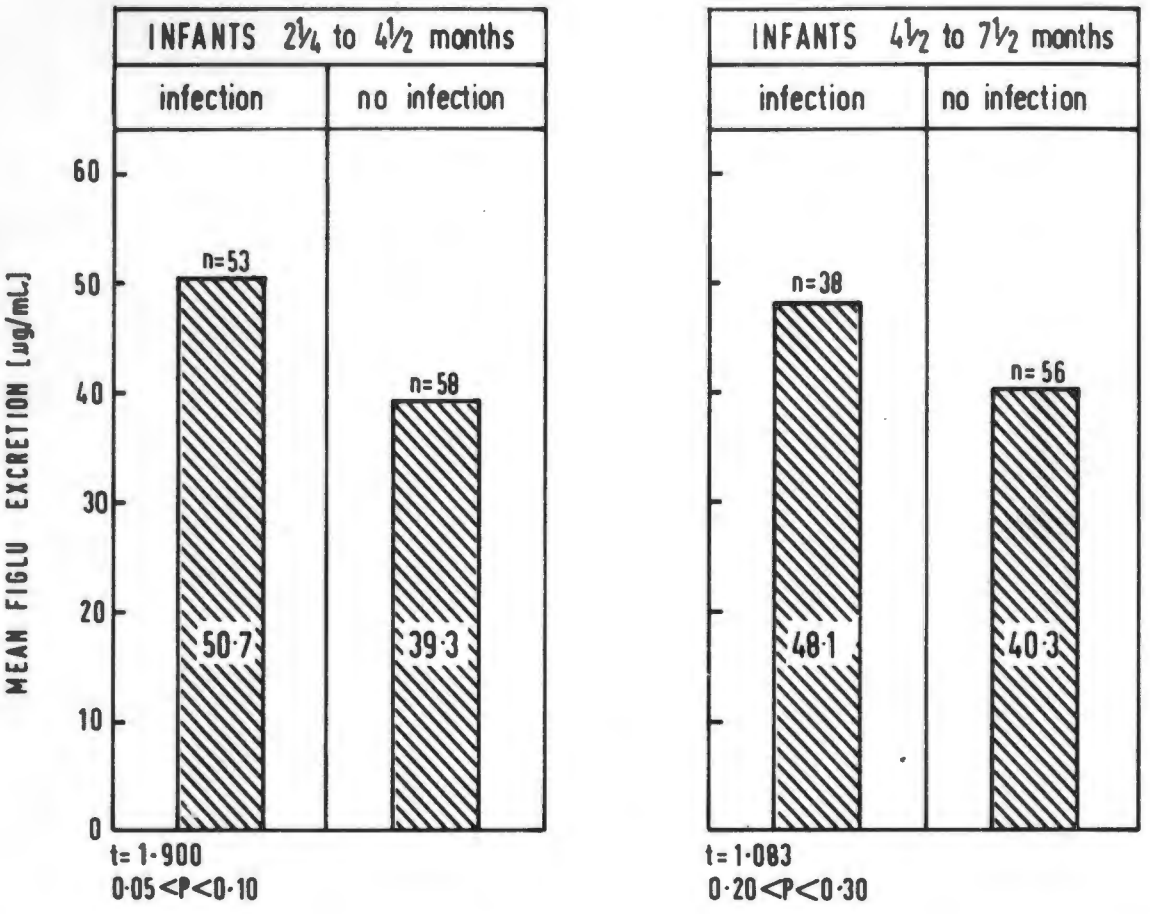
Method A (Table 72).

The mean Figlu excretion decreased whether therapy was administered or not. Because of this, reduction in Figlu excretion in the therapeutic group could not be attributed to the effect of therapy. However, all individual results were less than 45 µg./ml. in the therapeutic group although 4 infants in the non-therapeutic group still excreted Figlu in excess of this amount.

It seemed possible that coincidental physiological improvement stemming from growth and development might have contributed to the decrease in Figlu excretion. It was therefore necessary to repeat the therapeutic trial in younger infants.

By the end of the prospective study, 3 infants still had positive tests. They received a standard dose of oral folic acid, except in the case of one infant who received 100 µg. of intramuscular folic acid daily.

RELATIONSHIP OF INFECTION AT TIME OF INVESTIGATION TO MEAN FIGLU EXCRETION



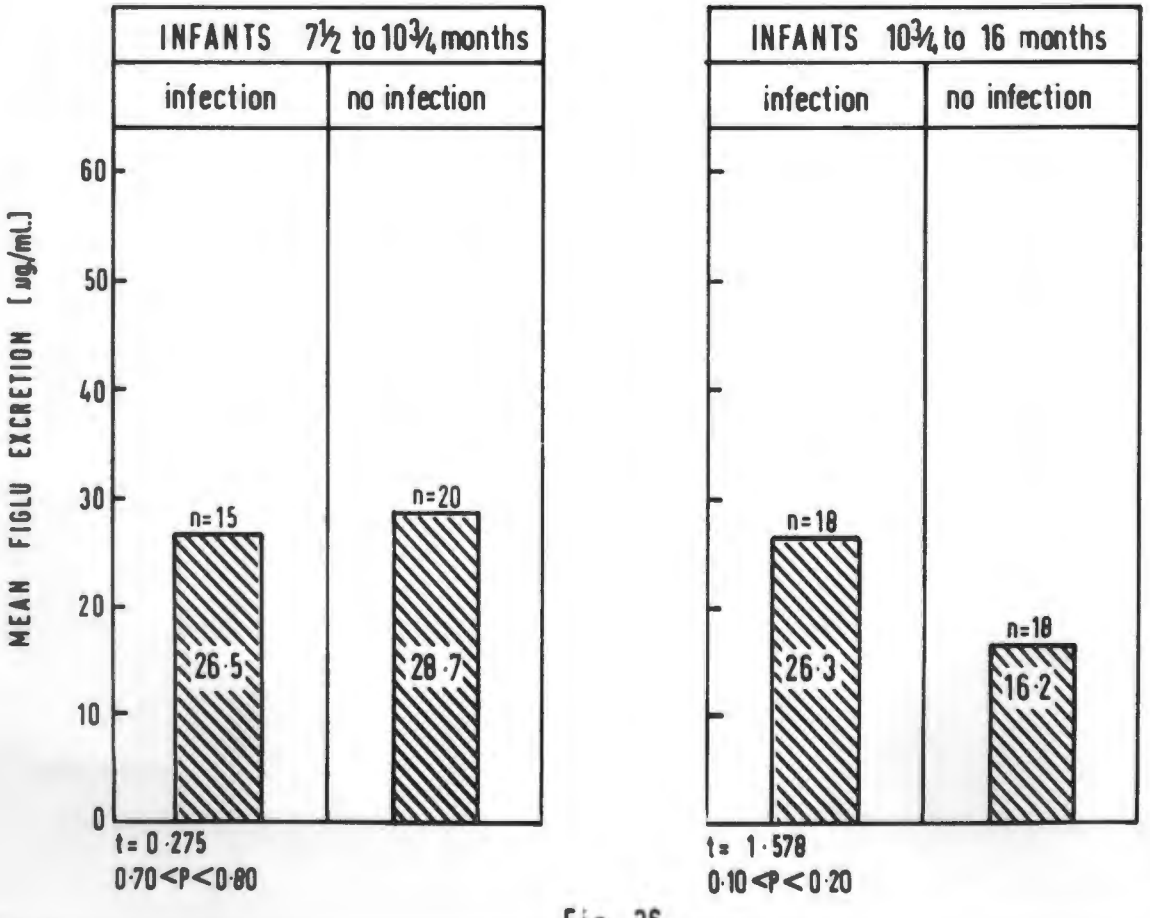


Fig. 36

EFFECT OF THERAPY ON FIGLU EXCRETION

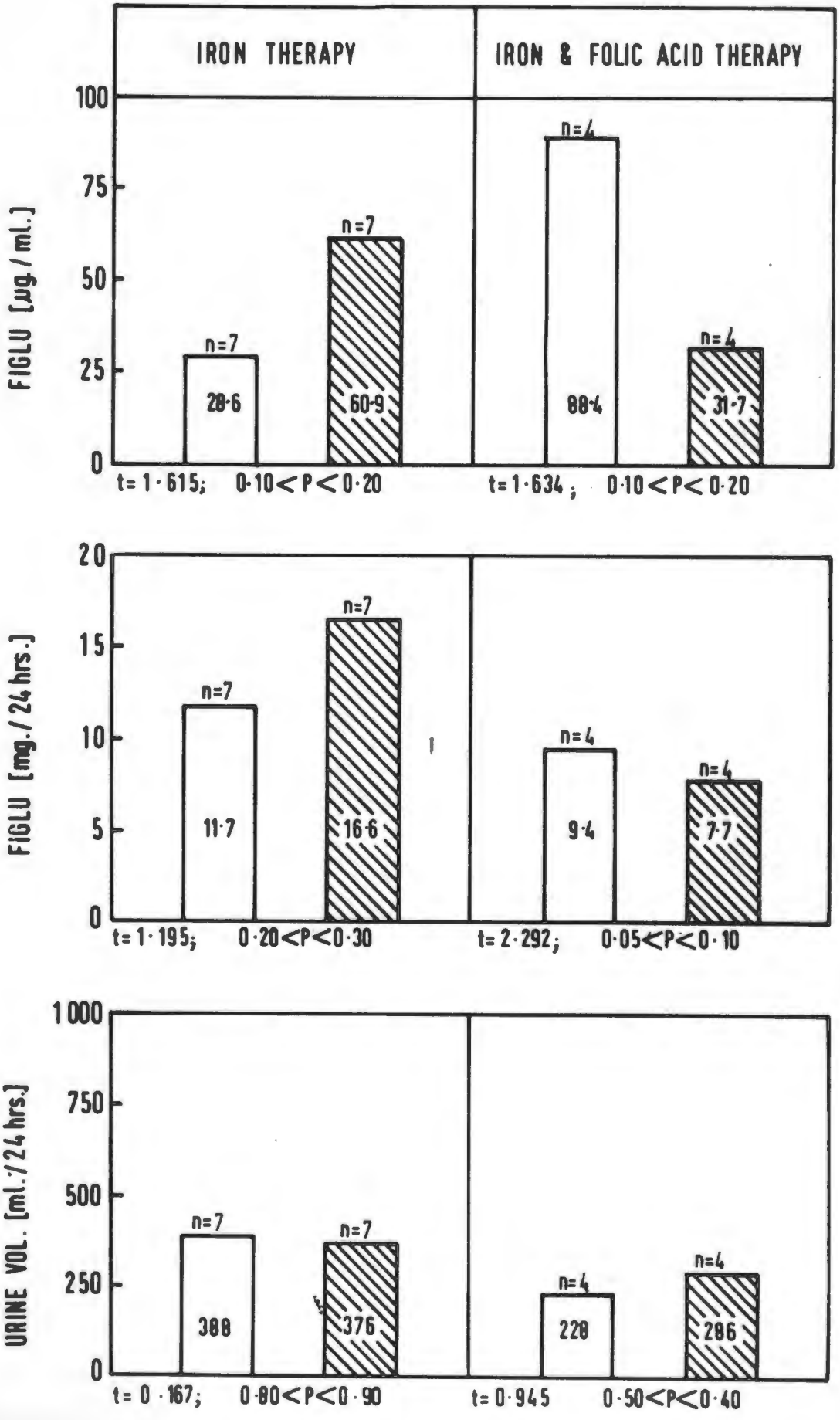


Fig. 37

Method B.

One of 4 infants given folic acid and iron, and one of 7 infants given only oral iron, showed unequivocal symptomatic benefit. This was not a statistically significant difference ($\chi^2 = 0.196$; $0.50 < P < 0.70$).

b) Significant improvement of haemoglobin concentration

Method A (Table 81).

The administration of folic acid did not produce any significant improvement of haemoglobin concentration in infants with positive Figlu tests. It did not improve haemoglobin concentration in infants with negative Figlu tests.

Method B (Table 74).

Mean haemoglobin concentration decreased in infants receiving folic acid and iron.

c) Improvement of mean weight percentile

Method A (Table 81).

Folic acid therapy did not improve the mean weight percentile of infants who had normal or abnormal Figlu tests.

This probably confirmed that the inconsistent relationship between excessive Figlu excretion and nutrition previously noted in the statistical analysis, had little meaning.

Method B (Table 74).

The mean weight percentile was not improved by folic acid and iron therapy.

d) Improvement of resistance to infection

The effect of folic acid therapy on resistance to infection in infants with positive Figlu tests could not be assessed.

From the combined results of the statistical analysis and the direct assessment of the effect of folic acid therapy it seemed that there was no relationship between excessive Figlu excretion and infant health.

SUMMARY

Two methods were used to estimate Figlu excretion. With both these methods it was found that a considerable number of healthy underprivileged infants excreted

(a) Figlu (µg./ml.) in excess of the accepted normal values.
(b) Figlu (mg./24 hrs.) in excess of normal according to criteria defined in this study.

Figlu excretion was maximal in young infants and then decreased with growth.

Disturbances of histidine metabolism did not significantly interfere with the interpretation of the Figlu test after oral histidine in the infants studied.

There was no relationship between positive Figlu tests after oral histidine and other parameters of folate deficiency. There was no relationship between Figlu excretion and an aetiology for folate deficiency. These applied whether Figlu results were expressed in terms of concentration (µg./ml.) or quantity (mg./24 hrs.). The administration of small doses of folic acid to infants with positive Figlu tests did not produce significant reduction in Figlu excretion.

There was no definite relationship between Figlu excretion and the S.faecalis folic acid clearance test. No cause for a disturbance of folate metabolism could be demonstrated. Pharmacological doses of folic acid and oral iron did not produce significant reduction of Figlu excretion.

There was no relationship between positive Figlu tests and non-specific factors such as incomplete urine collections. However, if Figlu excretion was expressed as concentration (µg./ml.) then in a small number of infants significant urine concentration might lead to spurious positive Figlu tests.

There was no relationship between excessive Figlu excretion and infant health.

DISCUSSION.

In measuring Figlu by two methods (Tabor and Wyngarden, 1958; Chanarin and Bennett, 1962a), it was shown that a fair percentage of healthy, underprivileged infants had positive Figlu tests after oral histidine. The maximum incidence and highest mean total Figlu output was noted in infants of under $7\frac{1}{2}$ months. The incidence and mean total Figlu output declined with growth and only a small percentage of infants in the $10\frac{3}{4}$ - 16 month age-group had positive Figlu tests after oral histidine.

The Figlu Test after Oral Histidine and Folate Deficiency.

It was necessary first to determine whether these positive tests could be attributed to biochemical folate deficiency (Chapter III).

In this study there was no agreement between the results of the Figlu test after oral histidine and those of the other parameters used to investigate folate deficiency.

Hoffbrand, Newcombe and Mollin (1966) confirmed Herbert's observations (1962a) that the Figlu test after oral histidine was a satisfactory test of adult folate stores, comparable in sensitivity to the red cell L.casei folate assay. It was a less sensitive parameter of folate deficiency than the following:

a) serum L.casei folate assay (Herbert, 1962a);

b) S.faecalis folic acid clearance test (Chanarin, Bennett and Berry,1962);

c) hypersegmentation of the neutrophils (Herbert, 1962a).

However, in the infants in this study, the results obtained with these sensitive parameters did not provide evidence for attributing the positive Figlu tests after oral histidine to folate deficiency. On these grounds, it might have been concluded that the positive Figlu tests after oral histidine were not manifest of biochemical folate deficiency.

However, this conclusion was not then reached because the validity of these parameters appeared controversial or were unproved in infants and children. Also some of the parameters used for investigating folate deficiency in this study were believed to be less sensitive than the Figlu test after oral histidine. This will be discussed below.

Figlu excretion without an oral histidine load was found to be a less sensitive test of folate deficiency than Figlu after oral histidine (Luhby, Cooperman and Teller, 1959a). This was apparently supported by the findings in this study. The use of a technique of concentrating urine without concentrating Figlu (Lewis and Moore, 1962a, b; Lovric and Kenrick, 1965) would have improved the value of the non-histidine loading Figlu test as a parameter of folate deficiency.

The diagnostic value of the serum L.casei folate assay in infancy was controversial. It was found that the serum L.casei folate assay was not always a reliable index of folate deficiency (Lovric and Kenrick, 1965) or megaloblastic anaemia due to folate deficiency, in infants and children (Luhby, Cooperman, MacIver and Montgomery, 1962; Pereira and Baker, 1966). Other investigators found the serum L.casei folate assay a good test of folate deficiency (Shojania and Gross, 1964a; Dormandy, Waters and Mollin, 1963; Vanier and Tyas, 1966) and noted a good agreement between serum L.casei folate levels and megaloblastic anaemia in infants and children (Dormandy, Waters and Mollin, 1963). This finding was confirmed by Spector and Metz (1966), provided infection was not present when the tests were performed.

The validity of hypersegmentation of the neutrophils as an index of folate deficiency in infancy was equally controversial. It was reported that in infants, up to 4% of neutrophils could be hypersegmented in the absence of other evidence of folate deficiency (Vanier and Tyas, 1966). In megaloblastosis in premature infants (Gray and Butler, 1965) and in infants and children (Wise, Lovric and O'Gorman Hughes, 1963), hypersegmentation of the neutrophils was an inconsistent finding. But other

investigators found a good agreement between hypersegmentation of the neutrophils and other parameters of folate deficiency in premature infants (Vanier and Tyas, 1967) and in children (Shojania and Gross, 1964b). In children with kwashiorkor and megaloblastic anaemia there was a good agreement between neutrophil lobe average and other parameters of folate deficiency (Allen and Whitehead, 1965).

The sensitivity of hypersegmentation of the neutrophils as a parameter of folate deficiency also appeared uncertain. Earlier, Herbert (1962a) had found this a sensitive parameter of folate deficiency present before folate stores were depleted. However, Chanarin, Hutchinson, McLean and Moule (1966) showed that in the presence of reduced hepatic folate stores, low serum L.casei folate levels and excessive Figlu excretion, hypersegmentation of the neutrophils was not found.

Little had been reported on the validity of the red cell L.casei folate assay as a parameter of folate deficiency in infancy. From the data collected by Vanier and Tyas (1966), it appeared that in normal infants the red cell L.casei folate assay was a more sensitive parameter of folate deficiency than the Figlu test after oral histidine. However, in premature infants it was found that the Figlu test after oral histidine was a slightly more sensitive index of folate deficiency than the red cell L.casei folate assay (Vanier and Tyas, 1967). From the little evidence available it seemed that in infants, as in adults, the Figlu test after oral histidine and the red cell L.casei folate assay might be comparable as parameters of folate deficiency. If this were so, the failure to find supportive evidence for the Figlu test after oral histidine by studying red cell L.casei folate assays was sound evidence for not attributing the results of the Figlu test to folate deficiency.

The only information found on the use of the folic acid clearance tests in infancy was not helpful in determining the interpretation of the

Figlu test after oral histidine. Hirata and Arakawa (1965) reported that in infants and children the basal serum L.casei folate levels correlated well with the level of folate in circulation 4 hours after the injection of folic acid. It was believed that the pattern of the L.casei folic acid clearance curves in adults was a reflection of the degree to which injected folic acid displaced folate from the stores (Chanarin and McLean, 1967). In patients with low folate stores, less folate was displaced and the curves appeared faster. Since the L.casei folic acid clearance curves were comparable in infants who had positive and negative Figlu tests after oral histidine it seemed that the folate stores in these two groups of infants should also be comparable. If this were so, this again was evidence against the value of the Figlu test after oral histidine as a parameter of folate deficiency and depleted folate stores in these infants.

In infants, (Luhby, Cooperman, MacIver and Montgomery, 1962; Shojania and Gross, 1964a; Lovric 1964) as in adults, (Herbert, 1962a; Chanarin,1964) there was agreement that positive Figlu tests after oral histidine might indicate the presence of folate deficiency before megaloblastosis developed. In this study, infants under $7\frac{1}{2}$ months had positive Figlu tests which were not associated with morphological evidence of folate deficiency in the bone marrow. Although this was a situation compatible with folate deficiency, this result did not in any way clarify the interpretation of the positive Figlu tests. One infant, M.R., later (at 13 months of age) developed a megaloblastic bone marrow, and in her case the positive Figlu test after oral histidine might have reflected the presence of folate deficiency. However, this could not be proved on the evidence available.

The negative evidence collected from the above studies appeared considerable but was not regarded as conclusive because the validity of the

other comparable or more sensitive parameters used for diagnosing folate deficiency in infants and children were either controversial or unproven. For this reason, other methods of investigating a relationship between positive Figlu tests after oral histidine and biochemical folate deficiency were pursued. This was done also because other investigators had reported that the Figlu test after oral histidine (Luhby, Cooperman, MacIver and Montgomery, 1962) and without oral histidine (Lovric and Kenrick, 1965) was a very reliable parameter of folate deficiency in infancy.

Infants with positive Figlu tests after oral histidine did not appear to have any reason for folate deficiency because excessive Figlu excretion could not be related to an aetiology for folate deficiency. There was no apparent relationship to maternal folate nutrition, dietary factors,, nutrition, malabsorption and increased demand for folate. A relationship to socio-economic circumstances might have existed but could not be proved. This aetiological study was complicated by certain factors. The best criterion for assessing maternal folate deficiency in pregnancy (megaloblastosis with supportive evidence for folate deficiency) was not employed in this study. Actual daily folate intake could not be measured and absorption was measured with xylose or folic acid. It is not known whether these results necessarily reflect ability to absorb food folate under physiological conditions.

Last, neither the administration of small physiological doses of folic acid known to be effective in correcting most positive Figlu tests after oral histidine resulting from folate deficiency (Hansen and Weinfeld, 1962); nor the administration of pharmacological doses of folic acid which should have converted positive Figlu tests due to

folate deficiency, resulted in a statistically significant decrease in Figlu excretion after oral histidine. Although the therapeutic trials with folic acid were conducted on an out-patient basis, there was no evidence that the infants did not receive the folic acid therapy.

In view of the failure to find support for the results of the positive Figlu tests after oral histidine from those of other parameters of folate deficiency, the failure to find an aetiology for folate deficiency in infants with positive Figlu tests and the failure of a therapeutic trial both with small and pharmacological doses of folic acid, it was concluded that positive Figlu tests after oral histidine were not the result of biochemical folate deficiency. The one method of establishing this beyond any doubt would have been to assay liver for folate content (Chanarin, Hutchinson, McLean and Moule, 1966). This was not justifiable in this study.

The Positive Figlu Tests after Oral Histidine and Secondary Disturbance of Folate Metabolism.

There was much to suggest that a disturbance of folate metabolism accounted for the positive Figlu tests.

First in contrast to the findings in normal infants (Luhby and Cooperman, 1964; Vanier and Tyas, 1966), other investigators had reported that underprivileged infants and infants with miscellaneous diseases had positive Figlu tests after oral histidine in the absence of supportive evidence of folate deficiency (Naiman, 1966; Arakawa, Ohara, Fujii, Hirata and Takahashi, 1965).

Second,in all except the last study, serum and red cell L.casei folate levels were higher, though not significantly so, in infants with positive Figlu tests after oral histidine. Elevated serum L.casei folate levels and

positive Figlu tests were described in other conditions where folate metabolism was disturbed: vitamin B_{12} deficiency (Herbert and Zalusky, 1962), infection (Spector, Falcke, Yoffe and Metz, 1966) and enzymatic disturbances such as congenital glutamate formiminotransferase (Arakawa, Ohara, Takahashi, Ogasawara, Hayashi, Chiba, Wada, Tada, Mizuno, Okamura and Yoshida, 1965) and transmethylase deficiency (Arakawa, Narisawa, Tanno, Ohara, Higashi, Honda, Tamura, Wada, Mizuno, Hayashi, Hirooka, Ohno and Ikeda, 1967). Of these conditions, infection and possibly immature enzyme function, particularly of glutamate formiminotransferase (Arakawa and Fujii, 1966) seemed most applicable to the infants in this study. Another possible cause for a disturbance of folate metabolism in these infants was iron deficiency. It was believed that iron deficiency could result in a diminution of glutamate formiminotransferase function and so lead to positive Figlu tests after oral histidine. However, raised serum L.casei folate levels were not reported to be a feature of this disturbance (Vitale, Streiff and Hellerstein, 1965; Vitale, Restrepo, Velez, Riker and Hellerstein, 1966).

Third, in conditions where folate metabolism was disturbed, it was noted that the administration of folic acid in pharmacological doses might result in a decrease in Figlu excretion after oral histidine without necessarily returning this to normal (Knowles, Shaldon and Fleming, 1963). This was similar to the findings in this study.

The relationship of positive Figlu tests to a secondary disturbance of folate metabolism was investigated by means of

(a) the folic acid clearance tests using S.faecalis as the assay organism;

(b) aetiological studies.

The use of the L.casei and S.faecalis folic acid clearance curves to try to relate positive Figlu tests to a disturbance of folate metabolism, arose from conclusions drawn by Hogan,, Maniatis and Moloney (1964) from the following data of Herbert and Zalusky (1962). In the study performed by Herbert and Zalusky (1962),, both the S.faecalis and L.casei clearance curves of injected folic acid were very rapid over the period of 0 to 30 minutes in patients with folate deficiency. Yet, over the same period in patients with vitamin B_{12} deficiency, the S.faecalis folic acid clearance test was as rapid as before but the L.casei folic acid clearance curve was within or slightly lower than the normal range. It was also believed (Herbert and Zalusky, 1962) that injected folic acid was converted to N^5-methyl FH_4 and that in vitamin B_{12} deficiency the conversion of N^5-methyl FH_4 to FH_4 was blocked. Since there was no alternative metabolic pathway for N^5-methyl FH_4, it accumulated and was trapped in the circulation. N^5-methyl FH_4 was available to L.casei and not S.faecalis so resulting in an apparently 'normal' folic acid clearance curve when L.casei was used as the assay organism in vitamin B_{12} deficiency. Presumably the rapid folic acid clearance curve with S.faecalis in vitamin B_{12} deficiency was the result of associated tissue folate deficiency. Thus, Hogan, Maniatis and Moloney (1964) inferred that the L.casei folic acid clearance curve could be used as a satisfactory test of folate deficiency particularly because it appeared to be relatively unaffected by secondary disturbances of folate metabolism. On the other hand, the S.faecalis folic acid clearance curve reflected both folate deficiency and disturbances of folate metabolism.

In a later study Chanarin and McLean (1967) used the L.casei and S.faecalis folic acid clearance tests in folate deficient,. vitamin B_{12}

deficient and control subjects, to investigate the methyl folate block hypothesis in vitamin B_{12} deficiency. It was possible to make observations from their findings which were relevant to this study. At the 15 and 30 minute levels, the mean folic acid clearance curves with S.faecalis were comparable in folate and vitamin B_{12} deficiency but were much lower than in control subjects. However, when L.casei was used as the assay organism the mean folic acid clearance levels at 15 and 30 minutes in vitamin B_{12} deficiency were only slightly higher than those in folate deficiency and both were substantially lower than those found in normal controls. From this, it seemed that the L.casei folic acid clearance curve could also be rapid in vitamin B_{12} deficiency and that the difference in the mean L.casei folate levels in folate and vitamin B_{12} deficiency was unimpressive. It seemed doubtful whether this difference had any meaning in relation to folate metabolism and whether differential folic acid clearance curves could be used to distinguish between folic acid deficiency and disturbances of folate metabolism particularly that found in vitamin B_{12} deficiency.

In this study the L.casei folic acid clearance curves were comparable in infants with positive and negative Figlu tests after oral histidine, whereas the S.faecalis folic acid clearance curves in infants with positive Figlu tests after oral histidine were slightly faster than those with negative Figlu tests. The difference was slight, the numbers investigated small and the test is associated with a high variance. Moreover, the contribution of associated iron deficiency to the rapidity of the S.faecalis folic acid clearance curves was unknown. Thus the observations were regarded as inconclusive and did not help in the identification of a possible disturbance of folate metabolism in infants with positive Figlu tests. It

was unfortunate that infants with high serum L.casei folate levels and positive Figlu tests after oral histidine could not be investigated with both the S.faecalis and L.casei folic acid clearance tests.

The data were further analysed to determine whether the positive Figlu tests could be attributed to iron deficiency, infection at the time of investigation, vitamin B_{12} deficiency or hepatic dysfunction.

Positive Figlu tests were previously reported in iron deficiency (Knowles, 1962; Chanarin, Bennett and Berry, 1962) but were not observed by Kohn, Mollin and Rosenbach (1961) and Stone, Luhby, Feldman, Gordon and Cooperman (1967). It was believed that iron deficiency might lead to folate deficiency (Chanarin, Rothman and Berry, 1965; Velez, Restrepo, Vitale and Hellerstein, 1966) or a secondary disturbance of folate metabolism (Vitale, Streiff and Hellerstein, 1965; Vitale, Restrepo, Velez, Riker and Hellerstein, 1966). Both these mechanisms might have resulted in positive Figlu tests after oral histidine.

Iron deficiency was a common occurrence in the group of infants investigated in this study, but no evidence could be found for attributing the positive Figlu results after oral histidine to this deficiency. There was no relationship between serum iron levels and Figlu excretion and therapy with intramuscular iron did not significantly improve Figlu excretion. As the infants grew older, Figlu excretion decreased in parallel to an increasing incidence and severity of iron deficiency.

Following in-vitro studies, Panders and Rupert (1965) reported that at a temperature of 39°C. the conversion of folic acid to tetrahydrofolate was inhibited. Subsequently, Spector, Falcke, Yoffe and Metz (1966) showed that malnourished infants with febrile conditions had higher Figlu

excretion than those infants without pyrexia. In this study a possible relationship between infection at the time of investigation and excessive Figlu excretion could not be demonstrated with a statistical analysis of the data. The failure to demonstrate a relationship between positive Figlu tests and infection did not contradict previous observations because none of the infants was investigated when the pyrexia was as much as 39°C (102.2°F).

Unequivocal vitamin B_{12} deficiency was not encountered in this study. One infant with a positive Figlu test after oral histidine had a slightly subnormal serum vitamin B_{12} level. This deficiency could not have accounted for all the positive Figlu tests.

There was no clinical reason for suspecting hepatic insufficiency in the infants studied. SGOT levels were normal when estimated. The incidence of rickets precluded the use of the alkaline phosphatase test and reported fatalities with the Bromsulphthalein test discouraged the use of this as a test of hepatic function (Astin, 1965).

The role of vitamin C and E deficiency in producing a disturbance of folate metabolism was not specifically investigated. On the strength of dietary information which might not have been completely reliable,* no statistical relationship was found between the administration of vitamin C and Figlu excretion.

Arakawa and Fujii (1966) showed that glutamate formiminotransferase function was low in rats and mice at birth. It increased rapidly until it reached a constant level comparable to that found in mature animals ($^7/_{52}$) at 5 weeks or later. It was possible that the positive Figlu tests after oral histidine could be attributed to greater immaturity of glutamate formiminotransferase function in infants with positive Figlu

* This data has not been presented.

tests. Estimation of glutamate formiminotransferase function was successfully performed on liver biopsy material (Arakawa, Ohara, Kudo, Tada, Hayashi and Mizuno, 1963). This was not justified in this investigation, but a method of estimating the enzyme function in erythrocyte haemolysates seemed to offer a suitable alternative (Arakawa, Fujii and Ohara, 1966). Unfortunately this study could ultimately not be undertaken. Later experience with the use of this method of estimating glutamate formiminotransferase function apparently proved disappointing (Konno, 1968).

The result of the therapeutic trial with pharmacological doses of folic acid was compatible with, but did not in any way prove a secondary disturbance of folate metabolism in infants with positive Figlu tests.

It was concluded that with the possible exception of immature glutamate formiminotransferase function, a secondary disturbance of folate metabolism was an unlikely explanation for the positive Figlu tests after oral histidine. Failure to demonstrate an aetiology for the disturbance of folate metabolism was the main reason for this conclusion.

The Figlu Test after Oral Histidine and Non-specific Factors.

A relationship between Figlu excretion after oral histidine and non-specific factors was sought in a further attempt to clarify the interpretation of the Figlu test after oral histidine.

The first factor considered was that of incomplete urine collection which might have occurred either because of leakage or incomplete voiding of urine when the collection was discontinued. However, when there was a loss of urine in excess of 10% - 15% of the total 24-hour urine volume, the urine collection was regarded as incomplete and excluded from the study (Chapter IV). Cooperman (1967) accepted a urine collection as

satisfactory if it was representative of the total urine passed during the 24-hour period. In order to examine the relationship of positive Figlu tests after oral histidine to incomplete urine collections which might have occurred as a result of incomplete voiding, creatinine was estimated in urine. This test was used because Arroyave and Wilson (1961) reported that 24-hour urine creatinine estimations could be used as a measure of 'completeness' of urine collection.

Although measurement of creatinine in serum or plasma could be inaccurate, the measurement of creatinine in urine was regarded as a satisfactory technique (Wrong, 1962). This was apparently confirmed by the reproducibility found between repeat estimations for creatinine in urine in this laboratory (Table 45). In this study creatinine in urine was expressed as a percentage of expected creatinine output per pound/kg. bodyweight in order to compensate for differences in the weight of the infants. When the results of the Figlu test after oral histidine were compared with those of creatinine excretion, it was found that the positive Figlu tests after oral histidine could not be attributed to incomplete urine collections.

The second non-specific factor considered in relation to positive Figlu tests after oral histidine, was that of urinary concentration. It was shown that a small percentage of infants could have spurious positive Figlu tests after oral histidine as a result of urine concentration if the level of the normality was set at 30 µg. per ml. This was established using method B.

Even when spurious positive Figlu tests were excluded by using quantitative Figlu output rather than Figlu concentration (µg./ml.) as

a criterion of normality, it was still not possible to attribute positive Figlu tests after oral histidine to folate deficiency or to most of the factors that might have disturbed folate metabolism.

The relationship of the Figlu Test after Oral Histidine to Infant Health.

Positive Figlu tests after oral histidine were not associated with any measurable effect on infant health.

The Interpretation of Positive Figlu Tests after Oral Histidine.

Because there was no aetiology for the positive Figlu tests after oral histidine and because excessive Figlu excretion did not produce any detectable effect on infant health, it seemed important to re-examine the criterion used for determining normal Figlu excretion in this study.

Luhby and Cooperman (1964) reported that normal infants and adults excreted less than 30 µg. per ml. or 35 mg. per 24 hours of Figlu. They preferred to use the criterion of 30 µg. per ml. in infants (Cooperman, 1967). Since the validity of 30 µg. per ml. (45 µg. per ml.) was now being questioned, the value of 35 mg. per 24 hours as a criterion of normality was examined. It was previously shown that the results of assays for Figlu in Cape Town were approximately 30% to 40% higher than those in New York (Chapter V). When the results in this study (mg. per 24 hours) were modified to compensate for this difference, it was found that Figlu excretion exceeded 35 mg. per 24 hours in only three instances. The highest level encountered was approximately 40 mg. per 24 hours (65.9 mg. per 24 hours reduced by 35%). None of the three infants showed any evidence of folate deficiency as assessed by the results of other parameters used for the investigation of this deficiency.

Noeypatimanond, Watson-Williams and Israëls,(1966) showed that normal adults excreted up to 40 mg. per 24 hours of histidine derivatives when:

a) they were given a 15 g. load of histidine in three divided doses;

b) urine was collected for 24 hours thereafter;

c) the method of Chanarin and Bennett (1962a) was used to determine Figlu excretion.

This experimental procedure was comparable to that used in this study. Because Vanier and Tyas (1966) confirmed that normal infants and adults excreted comparable total amounts of Figlu (mg./24 hrs), the result in this study could be compared with the above normal adult values for quantitative excretion of Figlu. In this study, the total output of histidine derivatives excreted by infants did not exceed 31.4 mg. per 24 hours when the method of Chanarin and Bennett (1962a) was used and total Figlu output did not exceed 27.2 mg. per 24 hours.

Thus, by using both methods of Figlu estimation and the above-mentioned criteria for normality it seemed likely that the Figlu excretion by the infants participating in this study was within normal limits for the experimental conditions pertaining here. By the use of the Figlu test after oral histidine, biochemical folate deficiency was not demonstrated in the infants studied.

This required a re-examination of the use of Figlu concentration as a criterion of normality in infants. There seemed to be agreement that total quantitative Figlu output in young infants was comparable to that in adults. Also, it was known that the average daily urine output in infants under a year was approximately a third of the average daily adult urinary output. It therefore seemed inconceivable that infants and adults could have the same criterion of normality for Figlu excretion expressed in

terms of concentration (µg. per ml.). As an example, the urine of A.P. contained 80.0 µg. per ml. when assayed by Dr. Cooperman. Total 24 hour urine output by this infant was 420 ml. i.e. within the normal range (400 to 500 ml.) for infants in this age group (Rubin, 1964). His total Figlu output was 33.6 mg. per 24 hours. The result of his Figlu test was not supported by other evidence for folate deficiency. It seemed clear that in his case, and in the case of other infants, a level of 80.0 µg. per ml. or more of Figlu was to be expected if infants were to excrete as much as 35 mg. per 24 hours in their smaller urine volumes. Moreover, the greater the urine concentration, the higher the concentration of Figlu was likely to be.

It therefore follows that if Figlu concentration is to be used as a valid criterion of folate deficiency, particularly for healthy, underprivileged infants under 7½ months, normal levels must be determined for them. Such levels should not be comparable to normal adult levels. In this study levels of as much as 181.5 µg. per ml. of Figlu were encountered without there being other evidence of folate deficiency.

It is not clear how Luhby and Cooperman (1964) found 30 µg. per ml. of Figlu a valid criterion of normality. If they used some method for producing diuresis, infants could have excreted large amounts of Figlu without this resulting in a Figlu concentration in excess of 30 µg. per ml. It is not known whether the conduct of this study in a hospital environment strange to the infants in any way contributed to the fact that some of the infants studied, passed highly concentrated urines. This may have contributed to a slightly higher incidence of positive Figlu tests (45 µg./ml.).

The relatively high total Figlu output by healthy infants, especially those under 7½ months of age, and the use of a criterion of normality for

Figlu excretion comparable to that used in adults, might partially explain the high incidence of positive results previously reported in infants (Friedman, McKenzie, Turner, Wittmann, 1964a; Dormandy, Waters and Mollin, 1963) (Chapter 111). It might also have explained why Naiman (1966) found that underprivileged infants under 2 years could have positive Figlu tests in the absence of supportive evidence for folate deficiency.*

From the results in this study it seemed clear that quantitative Figlu output (mg./24 hrs) progressively decreased as the infants grew older. The same pattern for Figlu excretion expressed as concentration was reported by Vanier and Tyas (1966) and attributed to parallel maturation of hepatic function. From experiments performed on rats and mice it seemed that this could have resulted from coincident maturation of glutamate formiminotransferase function (Arakawa and Fujii, 1966). Accordingly, normal values in infants, at least in those under 16 months, should be determined on the basis of infant age.

Moreover, the use of a criterion of normality for Figlu concentration based on adult standards might become more valid as infants mature and total Figlu output decreases. This could explain the decrease in the incidence of positive results (µg./ml.) in infants in this study and might have contributed to the explanation for the apparent efficacy of folic acid therapy in the earlier study performed on infants in Cape Town, (Friedman, McKenzie, Turner and Wittmann, 1964a) (Chapter 111).

It was originally recommended that Figlu excretion in infants should be expressed as concentration (µg./ml.) rather than quantity of Figlu excreted (mg./24 hrs) (Dormandy, Waters and Mollin,, 1963; Cooperman, 1967; Vanier and Tyas, 1966). This was suggested because it was difficult

*It would seem that all these investigators used standard semiquantitative methods for determining excessive Figlu excretion in urine but did not first establish the concentration of Figlu excretion passed by normal infants in these circumstances.

to obtain complete urine collections from infants. However, Figlu excretion reaches a maximum at some time between 3 and 10 hours after the administration of oral histidine, and then declines (Kohn, Mollin and Rosenbach, 1961; Knowles, 1962; Chanarin, 1964). It is difficult to understand the preference for expressing Figlu results as concentration because, after the administration of oral histidine, Figlu is not excreted at a uniform rate. From this it seems that the Figlu result obtained from a substantially incomplete urine collection would be inaccurate regardless of expression (µg./ml. or mg./24 hrs).

In this study it was found that urine concentration could result in spurious positive Figlu tests, using 30 µg./ml. as the criterion of normality. If the maximum Figlu excretion in µg./ml. (181.5 µg./ml.) found in this study is regarded as the maximum normality, it is clear that urine concentration could not have resulted in any false positive Figlu tests (the greatest rise was from 17.4 µg./ml. - 58.4 µg./ml.). Thus, provided normal levels are determined for infants, Figlu excretion can be assessed with equal validity, either as concentration, or as quantity of Figlu output.

In this study there was little opportunity for investigating the value of the Figlu test in the diagnosis of megaloblastosis. It might be that this test would have the same value in infants as it has in adults, provided normal values for infants are adequately defined.

There are still a few aspects to be discussed. First was the difference in the results of assays for Figlu performed in New York and Cape Town using the method of Tabor and Wyngarden (1958). The reason for this could not be determined, but it was noted that differences in assay techniques for Figlu might exist between different laboratories. The normal range for the excretion of histidine derivatives in an 8-hour urine sample collected from adults given a 15 g. load of histidine and using the

method of Chanarin and Bennett (1962a) was reported to be 0 - 17 mg. by some investigators (Chanarin, Bennett and Berry, 1962; Hoffbrand, Neale, Hines and Mollin, 1966), and 0 - 25 mg. by others (Dymock, 1964; Bashir and Biro, 1966). Although the reasons for the technical difference in this study remained unexplained, compensation was made for this when the results were expressed in µg./ml. (30 µg./ml. increased to 45 µg./ml.) and when the results in mg. per 24 hours were discussed (result reduced by 35%).

Second, the incidence of positive Figlu tests (45 µg./ml.) obtained with method A was higher than that obtained with method B (> 30 µg./ml. and > 10 mg./24 hrs). A possible reason for the higher incidence obtained with method A was the number of spurious positive tests which might have resulted from the effect of urine concentration. This was not the sole explanation because:

(a) when the results obtained with method B were expressed only in terms of concentration, the incidence of positive results was still lower than those obtained with method A;

(b) when method A was used to measure quantitative Figlu excretion in infants, the results were higher than those obtained with method B. This is tabulated below.

Maximum Figlu excretion (mg./24 hours)

Method A	Method B
40.0 mg.(65.9 reduced by 35%)	27.7

Mean Figlu excretion (mg./24 hours)

Method A		Method B	
2¼ to 4½ months	9.0 (13.9 reduced by 35%)	2¾ - 5.0 months	7.6
4½ to 7½ months	8.3 (12.8 reduced by 35%)	3½ - 5¾ months	6.9

Greater numbers of infants were investigated with method A, so that it was possible that the difference in maximum and mean Figlu excretion obtained with the two methods might have reflected a difference in the samples studied. More infants whose urines were examined with method A might have excreted total quantities of Figlu in the higher normal range. Another less likely possibility was Cooperman's (1967) claim that the method of Chanarin and Bennett (1962a) was less sensitive than that of Tabor and Wyngarden (1958) and not suitable for use in children. While there might be a difference in sensitivity between the two methods (Cooperman and Luhby, 1962) there is as yet no proof that the two methods should not produce comparable results. It was not possible to compare the two methods in this study.

Third, the reason for the change from negative to positive Figlu tests noted in some infants was of interest. It was apparent that this was largely attributable to the effect of urine concentration as judged by an incorrect criterion of normality for concentration of Figlu excretion. However, this was not the case in all instances, because in a small proportion of infants total Figlu output also increased. There might be another explanation for this. It was observed that as infants grew older there was a significant improvement in creatinine ratio. This was not related to improved nutrition and there were no clinical reasons for believing that there had been previous hepatic or renal insufficiency. It was possible that the improvement in creatinine ratio resulted from maturation of glomerular filtration function, as growth occurred. It has been stated that glomerular filtration function in infants reaches adult levels at about a year of age(McCance,1962). Since Figlu excretion was higher in infants with higher creatinine ratios, it seemed possible that

normal Figlu output in individual infants might, to some extent, be influenced by the maturity of glomerular filtration function. Clearly at this stage any inference regarding the relationship of Figlu excretion to glomerular filtration function is highly speculative and requires confirmation by further investigation using more refined techniques. Caution is especially necessary in view of the fact that Figlu is known to have a high clearance rate (Knowles, Prankerd and Westall, 1961).

Although there is strong experimental evidence for believing that normal Figlu excretion in infants might depend on maturity of glutamate formiminotransferase function, it is as yet unknown to what extent maturity of glomerular filtration function may influence normal Figlu excretion.

Disturbances of Histidine Metabolism.

In this study no attempt was made to investigate histidine metabolism in infancy. Urocanic acid and other imidazoles were estimated only in so far as these might have affected the interpretation of the Figlu test. The degree of disturbance of histidine metabolism was rarely severe enough to interfere with the interpretation of the Figlu test.

The number of infants excreting excessive urocanic acid was too small to allow investigation of the interrelationships between histidine metabolism,hepatic dysfunction and undernutrition (Dean and Whitehead, 1963; Friedman, Mckenzie, Turner and Wittmann, 1964b; Spector, Falcke, Yoffe and Metz, 1966; Davis and Kelly, 1963; Hoffbrand, Neale, Hines and Mollin, 1966).

Histidinuria had been reported in rickets (Chisholm and Harrison, 1960), but did not apparently interfere with the interpretation of the Figlu test.

SUMMARY AND CONCLUSIONS.

1. By means of two methods for estimating Figlu, it was shown that a fair proportion of healthy, underprivileged infants excreted Figlu in excess after oral histidine. When the method of Tabor and Wyngarden (1958) and an accepted criterion of normality for concentration of Figlu in urine were used, approximately 42% of infants under 4½ months had positive Figlu tests. These tests were not the result of folate deficiency, nor the result of a disturbance of folate metabolism as far as this could be ascertained, nor entirely the result of non-specific factors. Positive Figlu tests were not associated with any detrimental effect on infant health.
2. When the method of Chanarin and Bennett (1962a) was used, a criterion of normality for quantity of Figlu excretion (mg./24 hrs) was derived, using 30 µg./ml. as the standard of reference. In this way, spurious positive Figlu tests resulting from urine concentration, were eliminated. Even under these circumstances 17.8% of infants under 5 months had positive Figlu tests which could not be attributed to any of the causes above. Nor was there any relationship between these positive Figlu tests and infant health.
3. It was found that the total quantitative Figlu output in all but three infants with positive Figlu tests after oral histidine did not exceed the normal values found in adults. Because it had been reported that total Figlu output in normal infants was comparable to that of adults, it was concluded that the Figlu excretion found in the infants in this study was within normal limits for the experimental conditions of this study. It reached 181.5 µg./ml. and 65.9 mg./24 hours without being indicative of folate deficiency. Accordingly the results of this test did not reveal unequivocal biochemical folate deficiency.

4. The previously accepted criterion of normality for infant Figlu excretion expressed as concentration was the same as that of adults. It was concluded that this was invalid because it did not allow for the smaller urine volume passed by infants and that this explained the "positive Figlu tests" (expressed as µg./ml.) found in this study.
5. Total Figlu output declined with growth. This meant:
   (a) that the use of a criterion of normality such as 30 µg./ml. (45 µg./ml.) became more valid with growth;
   (b) normal Figlu excretion in infants should be related to age.
6. The application of the Figlu test as a diagnostic parameter of megaloblastosis must still be established. It might have a similar application to that in adults, provided normal levels are adequately defined.
7. In this study, disturbances in histidine metabolism were not investigated as such. They were rarely severe enough to result in a significant decrease of Figlu excretion in healthy underprivileged infants.
8. Two methods were used for estimating Figlu. The method of Chanarin and Bennett (1962a) appeared to have two advantages over the method of Tabor and Wyngarden (1958):
   (i) The enzyme was more easily prepared in greater amounts during a shorter period of time;
   (ii) the method allows for the separate and combined estimation of urocanic acid and Figlu. This may be an advantage when this test is used to investigate folate deficiency in malnourished infants.

CHAPTER VIII

HYPERSEGMENTATION OF THE NEUTROPHILS AS A PARAMETER OF FOLATE DEFICIENCY.

CHAPTER VIII

HYPERSEGMENTATION OF THE NEUTROPHILS AS A PARAMETER OF FOLATE DEFICIENCY.

INTRODUCTION.

Arneth (1904) was the first investigator to consider the importance of the number of nuclei present in polymorphs and to recognise the increased segmentation of polymorphs evident in pernicious anaemia (Arneth, 1907). This shift to the right in nuclear segmentation was later confirmed by v.Decastello (1913), Brosamlen (1913), Briggs (1914), Naegeli (1917) and Cooke (1927, 1929).

At first it was believed that the number of nuclei in polymorphs was a manifestation of the age of the cell (Arneth, 1904). Although there was further speculation about the origin of the hypersegmented cells (Cooke, 1927; 1929), the first positive contribution was made when Tempka and Braun (1932) noted pathological white cell precursors in the bone marrow in pernicious anaemia. Later Segerdahl (1935) and Jones (1937) showed that the hypersegmented neutrophils developed from these pathological white cell precursors in the bone marrow.

The original method of assessing hypersegmentation was both detailed and complex (Arneth, 1904), but was later modified (Briggs, 1914; Cooke and Ponder, 1927) and simplified (Hynek, 1909). Despite the improvement in the method and the recognition of the diagnostic value of the test Briggs, 1914; Heck and Watkins, 1933; Cooke, 1927; 1929; Jones, 1937) this parameter was apparently little used.

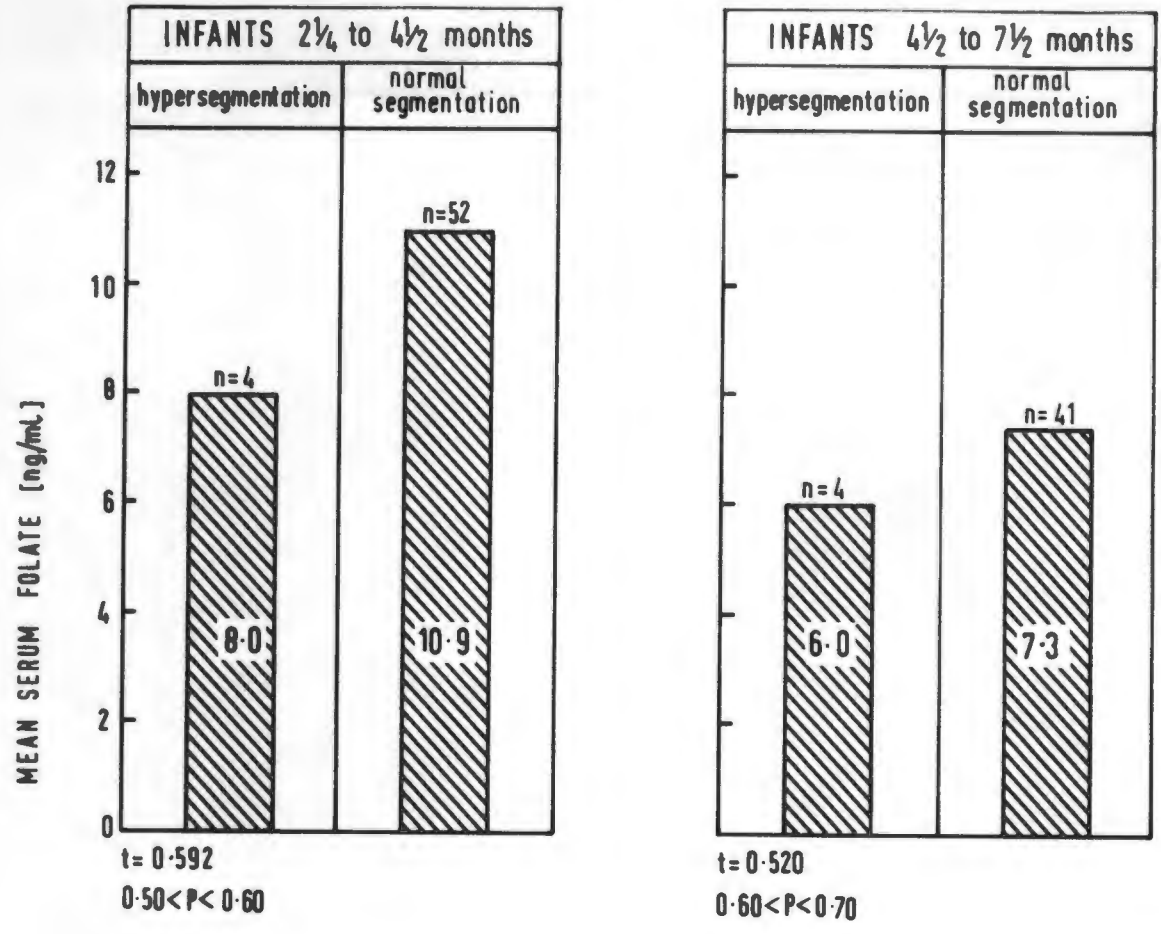
In 1959 the value of the test as an index of folate and vitamin B_{12} deficiency was again emphasised (Herbert, 1959). Thereafter, two methods for assessing nuclear segmentation were frequently used. One of these was the lobe average (Herbert, 1962a), and the other the percentage of

TABLE 82.

INCIDENCE AND PATTERN OF HYPERSEGMENTATION OF THE NEUTROPHILS IN INFANTS

Age (months)	Positive results		Numbers of Investigations	Percentage of infants with Hypersegmentation
	Infant	% Hypersegmentation		
$2\frac{1}{4}$ - $4\frac{1}{2}$	R.J.	5	65	6.2
	N.M.	7		
	A.B.	4		
	T.F.	13		
$4\frac{1}{2}$ - $7\frac{1}{2}$	S.W.	5	52	11.5
	H.F.	5		
	A.U.	7		
	L.v.R.	7		
	J.C.	7		
	T.F.	8		
$7\frac{1}{2}$ - $10\frac{3}{4}$	S.W.	5	32	12.5
	R.K.	4		
	J.E.	8		
	C.H.	6		
	C.V.	7		
$10\frac{3}{4}$ - 16	A.B.	4	35	8.6
	T.F.	10		
	S.D.	7		

RELATIONSHIP OF MEAN SERUM FOLATE LEVELS TO HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS



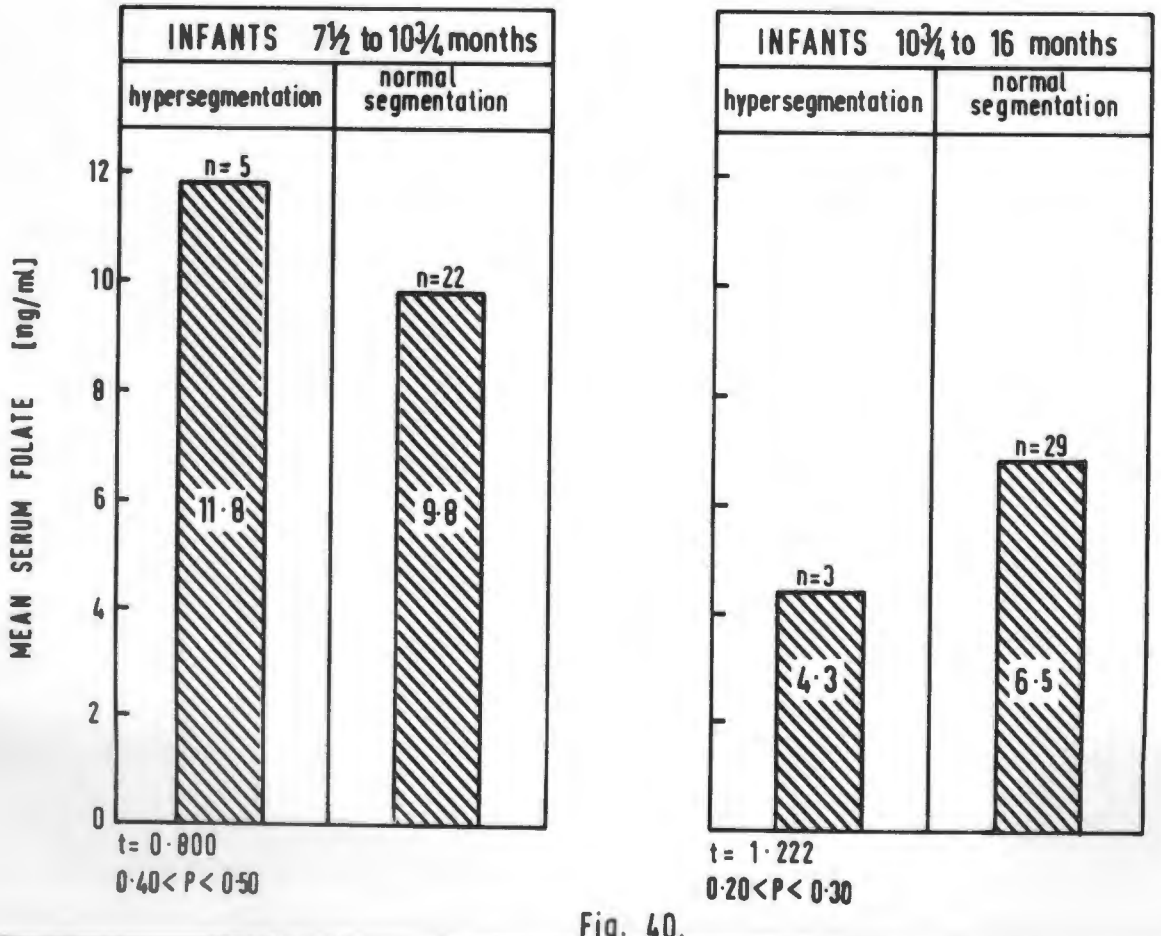


Fig. 40.

TABLE 83.

A COMPARISON OF PERCENTAGE SEGMENTATION OF NEUTROPHILS AND BONE MARROW EXAMINATION AS PARAMETERS OF FOLATE DEFICIENCY.

(a) Morphology of the bone marrow in infants with hypersegmentation of neutrophils.

Infant	% Hypersegmentation	Bone marrow morphology
H.F.	5	No megaloblastosis
A.U.	7	No megaloblastosis
L.v.R.	7	No megaloblastosis

(b) Percentage hypersegmentation of neutrophils in infants with megaloblastic changes in the bone marrow.

Infant	% Hypersegmentation	Bone marrow morphology
M.R.	3	Grade 1 megaloblastosis

neutrophils with five or more lobes - percentage hypersegmentation (Chanarin, Rothman and Berry, 1965). In this study, percentage hypersegmentation was used as an index and its validity as a parameter of folate deficiency will be evaluated in this chapter.

RESULTS.

Incidence of Hypersegmentation (Table 82).

In the four age groups, from 6.2% - 12.5% of infants had hypersegmented neutrophils.

Pattern of Hypersegmentation (Table 82).

Hypersegmented neutrophils were consistently found only in T.F. However, this could not be adequately assessed in A.B., S.W., J.E., J.C. and N.M. because blood films taken from these infants were not always technically satisfactory.

HYPERSEGMENTATION OF THE NEUTROPHILS AS A PARAMETER OF FOLATE DEFICIENCY.

Hypersegmentation of the Neutrophils compared with Serum Folate Assays (Fig.40).

There was no statistical relationship between mean serum L.casei folate levels and hypersegmentation of the neutrophils.

A Comparison of Hypersegmentation of the Neutrophils with Bone Marrow Morphology (Table 83).

Three infants who had hypersegmented neutrophils did not have megaloblastosis. One infant with megaloblastosis did not have hypersegmented neutrophils.

In all other instances (22), where bone marrow examinations were performed and where these two parameters could be compared, there was evidence of neither megaloblastosis nor of hypersegmentation of the neutrophils.

THE RELATIONSHIP OF HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS (2¼ - 4½ months) TO MATERNAL FOLATE NUTRITION

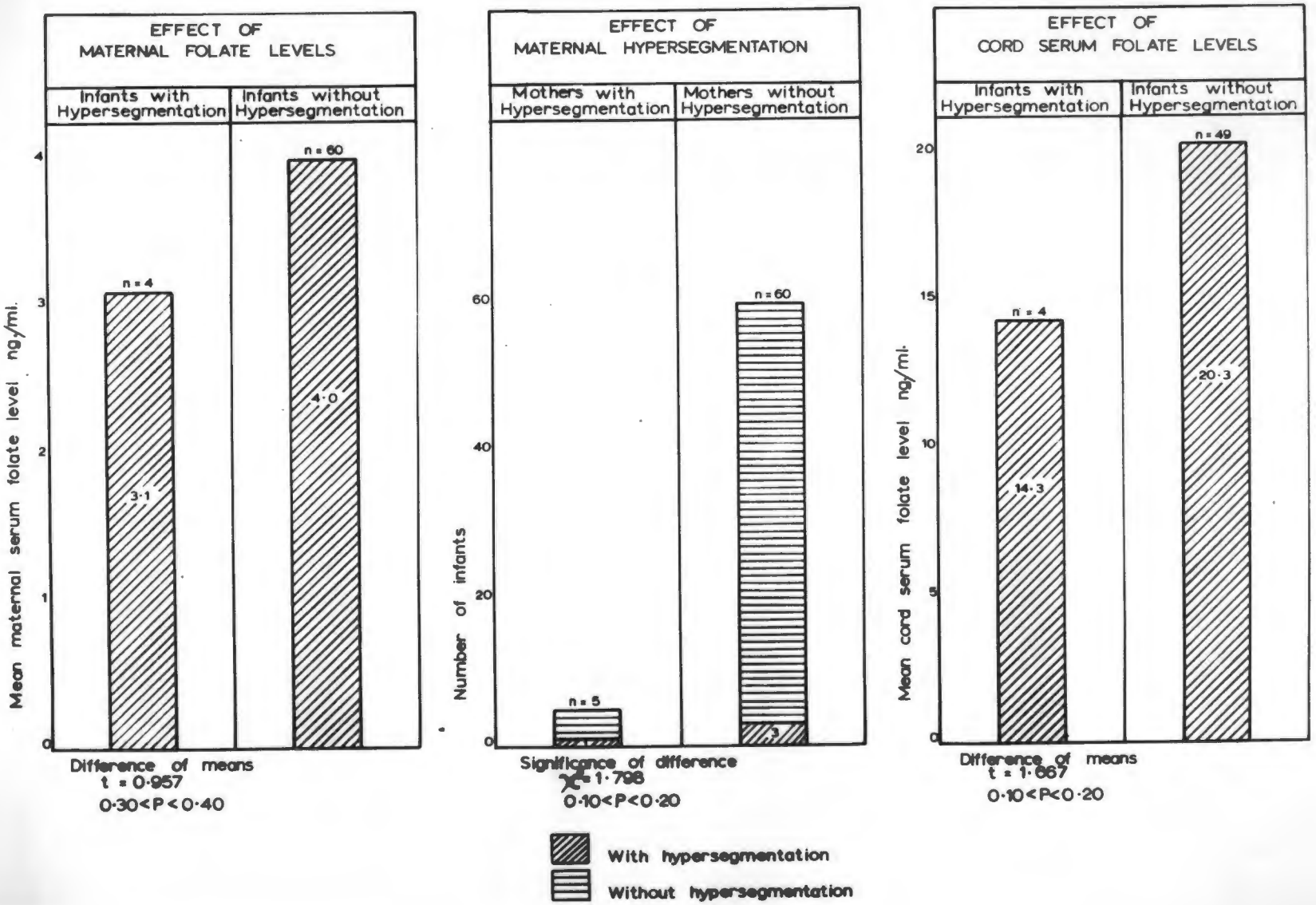


Fig. 41

THE EFFECT OF THE NUMBER & SEVERITY OF INFECTIONS (INFECTION SCORE) ON THE PRODUCTION OF HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS.

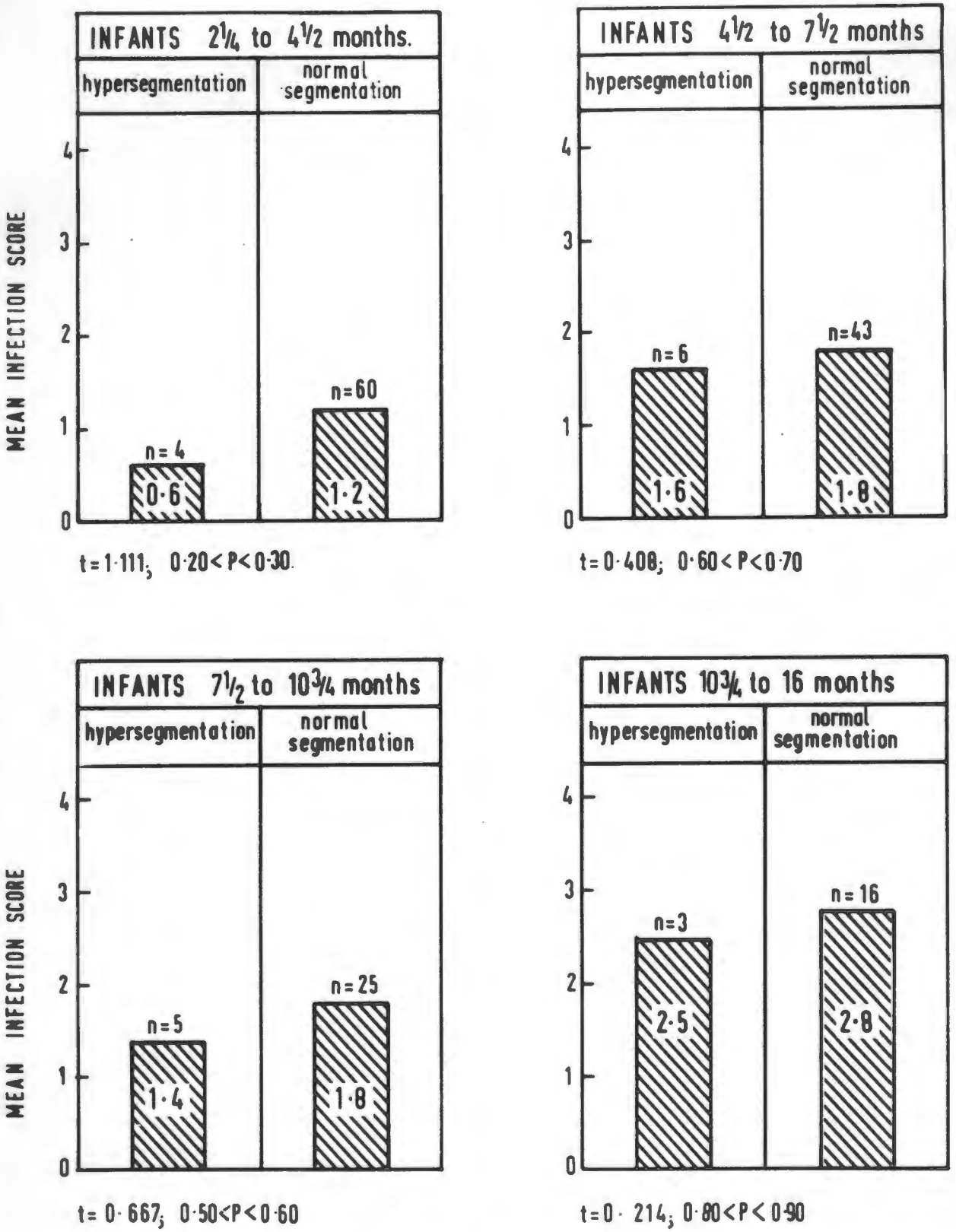


Fig. 42

RELATIONSHIP OF NUTRITION TO HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS.

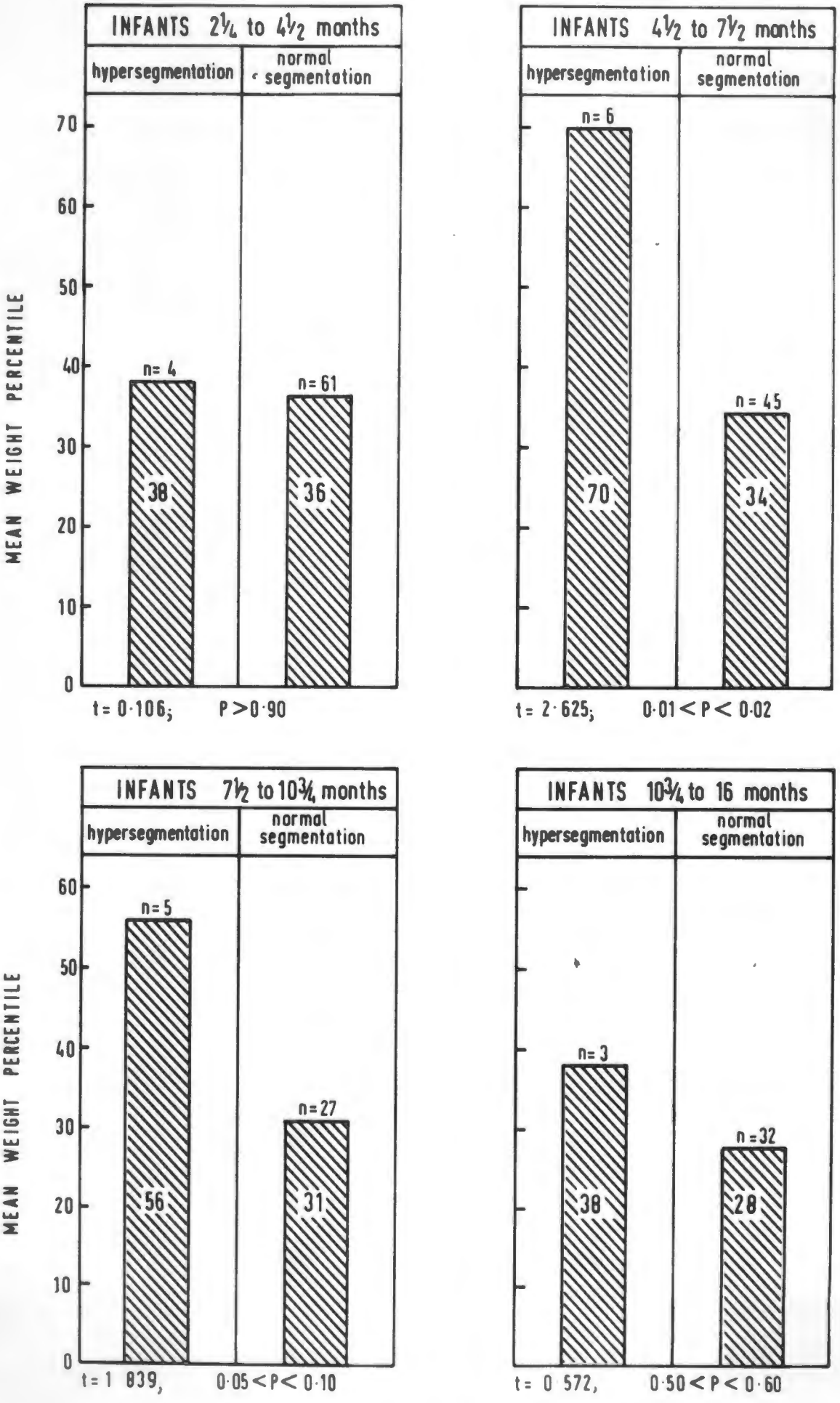


Fig 43

AETIOLOGICAL POSSIBILITIES FOR FOLATE DEFICIENCY IN INFANTS WITH HYPERSEGMENTED NEUTROPHILS

Maternal Factors (Fig. 41).

There was no statistical relationship between hypersegmentation of the neutrophils in infants and

(a) mean maternal serum L.casei folate levels;
(b) hypersegmentation of the neutrophils in their mothers;
(c) mean cord serum L.casei folate levels.

None of the infants whose mothers excreted excessive Figlu had hypersegmented neutrophils.

Dietary Factors.

From $2\frac{1}{2}$ - $4\frac{1}{2}$ months all infants with hypersegmented neutrophils received virtually the same diet. This consisted of a combination of breast and formula milk and, in three of the four infants, cereal and a mixed diet. This did not provide any evidence for further investigation along these lines.

Malabsorption.

Xylose excretion was normal in one infant who had hypersegmented neutrophils.

Increased Demand for Folate.

1. Infection Score (Fig. 42). There was no statistical relationship between hypersegmentation of the neutrophils in infants and mean infection score.
2. Growth and Nutrition (Fig. 43).

   Infants with hypersegmented neutrophils had higher mean weight percentiles. This relationship was statistically significant only in infants from $4\frac{1}{2}$ - $7\frac{1}{2}$ months.

RELATIONSHIP OF SERUM IRON LEVELS TO HYPERSEGMENTATION OF NEUTROPHILS IN INFANTS

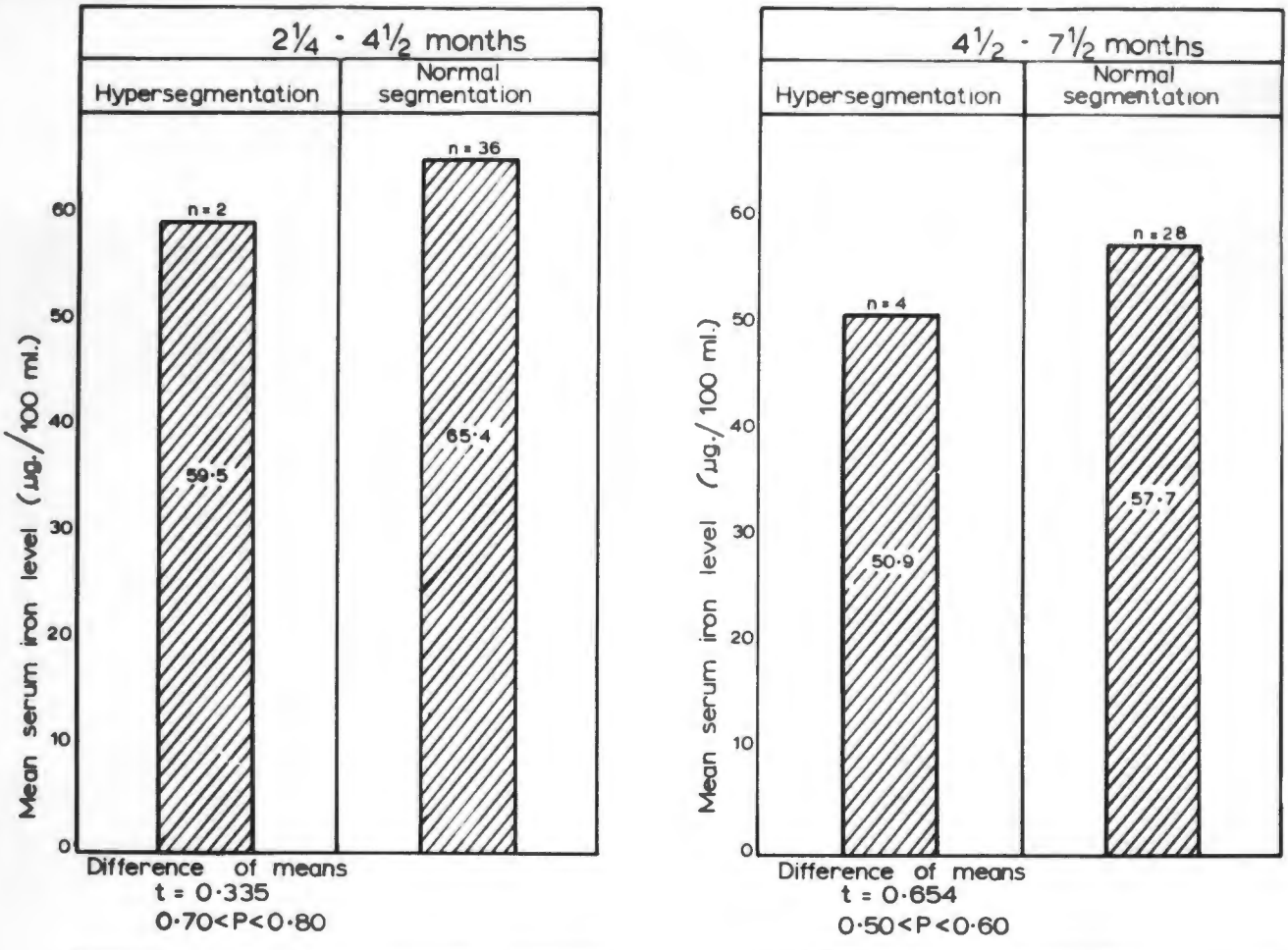


Fig. 44.

THE EFFECT OF INFECTION AT THE TIME OF INVESTIGATION ON THE PRODUCTION OF HYPERSEGMENTATION OF THE NEUTROPHILS

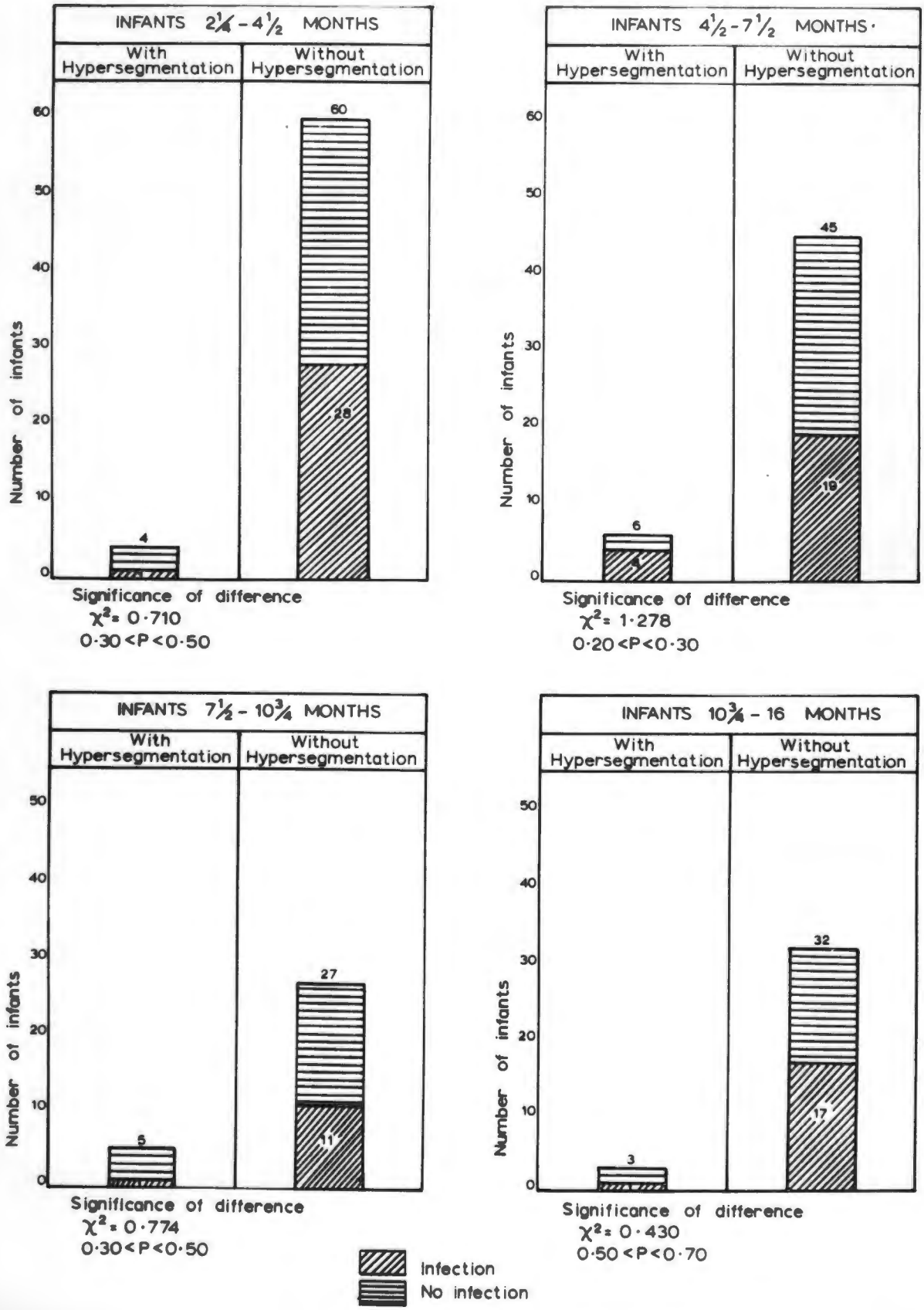
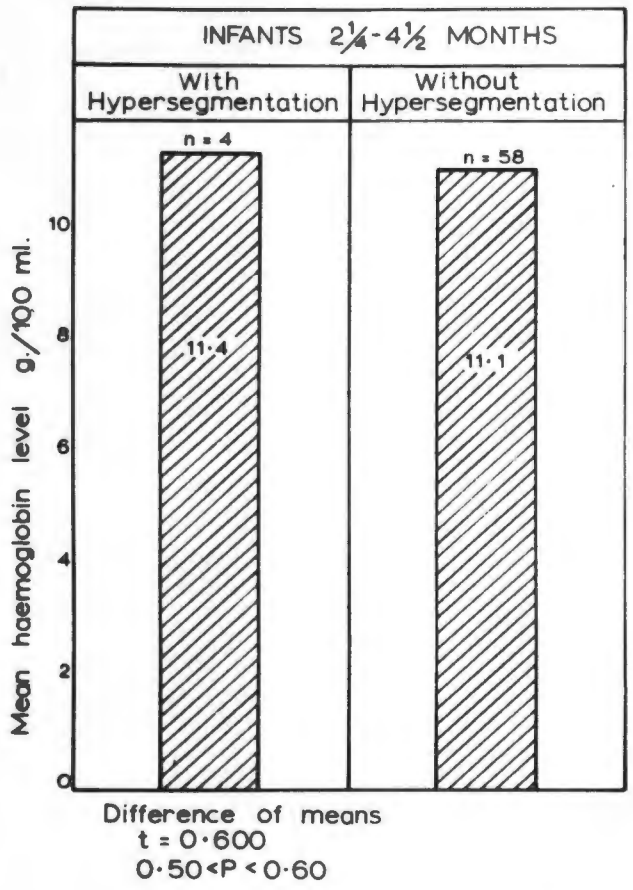
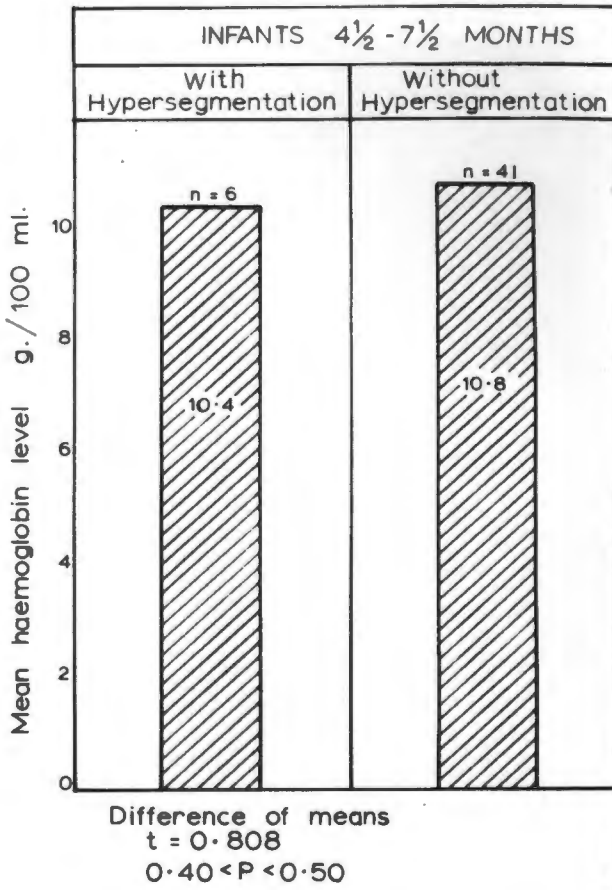
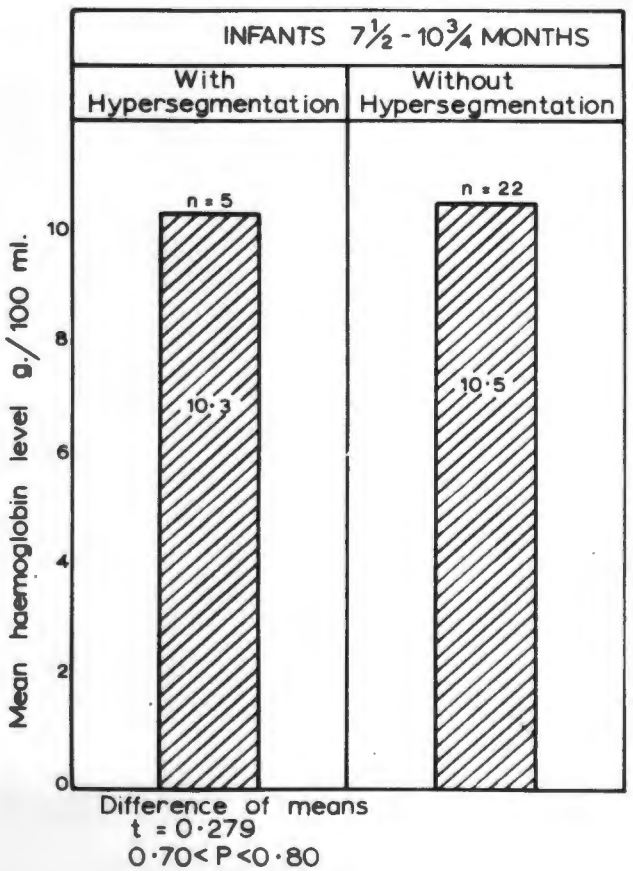


Fig. 45

RELATIONSHIP OF HYPERSEGMENTATION OF THE NEUTROPHILS IN INFANTS TO HAEMOGLOBIN LEVELS







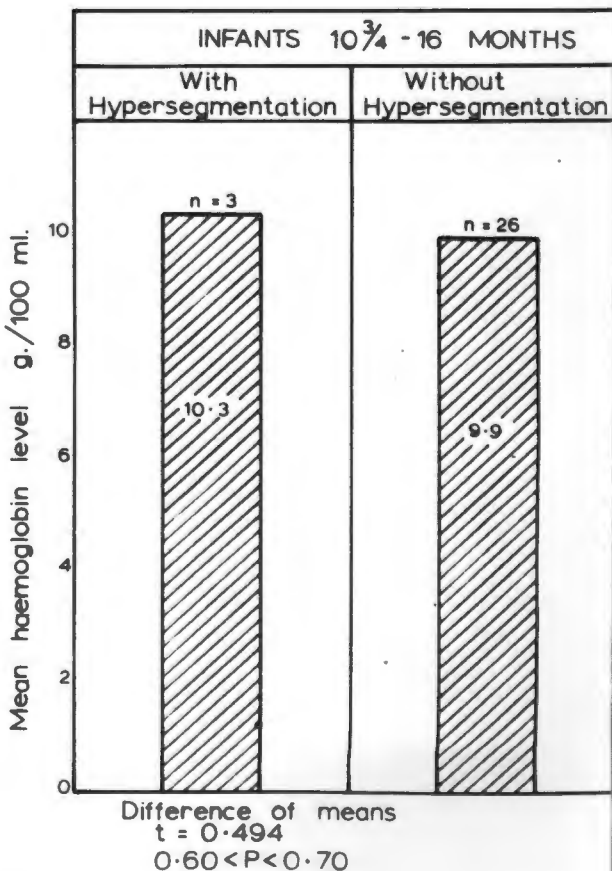


Fig. 46

From these findings it appeared that rapid growth leading to increased demand for folate was the only possible aetiology for folate deficiency in infants with hypersegmented neutrophils. However, there was statistical support for this only in infants of one age group. Thus, the possibility that rapid growth had resulted in increased demand for folate and therefore caused folate deficiency in infants with hypersegmented neutrophils was doubtful.

THE RELATIONSHIP OF HYPERSEGMENTATION TO OTHER POSSIBLE AETIOLOGICAL FACTORS

Iron Deficiency (Fig. 44).

There was no statistical relationship between hypersegmentation of the neutrophils and mean serum iron levels.

Infection at the time of investigation (Fig. 45).

A statistical relationship between infection at the time of investigation and hypersegmentation of the neutrophils in infants could not be demonstrated.

Congenital Hypersegmentation.

The parents of the infant who consistently showed evidence of hypersegmentation did not have hypersegmented neutrophils.

None of the infants with hypersegmented neutrophils had an increase of 4-lobed neutrophils greater than 50%.

HAEMATOLOGICAL IMPORTANCE OF HYPERSEGMENTATION OF THE NEUTROPHILS

Relationship to Haemoglobin Concentration (Fig. 46).

There was no statistical relationship between mean haemoglobin concentration and hypersegmentation of the neutrophils.

HYPERSEGMENTATION AS ASSESSED WITH BRIGGS' (1914) CRITERION

The highest count noted was 5% and only 3 infants had counts in excess of 3% (4%; 4% and 5%). Serum L.casei folate levels and the Figlu test after oral histidine were normal in the infants with 4% hypersegmented neutrophils but were not available for comparison in the infant with 5% hypersegmented cells. These 5% hypersegmented neutrophils were not associated with megaloblastosis. The serum iron level was low in one infant (4% hypersegmented cells) where this was available.

DISCUSSION.

Hypersegmentation of the neutrophils was not often present in the infants in this investigation and in most cases was an inconsistent finding. Even in the presence of megaloblastosis, hypersegmentation of the neutrophils could be a transient finding (Chanarin, Rothman, Ardeman and Berry, 1965).

The hypersegmented neutrophils noted in this study did not show any of the following morphological changes:

(a) increase in size of the cells (Cooke, 1927; 1929);

(b) increased nuclear-cytoplasmic ratio (Herbert, 1964);

(c) multiple bridging between nuclear lobes (Luhby and Cooperman, 1964).

Furthermore there was no proof that the hypersegmented neutrophils noted in this study were a manifestation of folate deficiency. The relationship of hypersegmented neutrophils to the serum L.casei folate assays and bone marrow morphology was poor. Also there was no unequivocal aetiology for folate deficiency in the infants with hypersegmented neutrophils.

From other studies it appeared that hypersegmentation of the neutrophils was not always a successful parameter of folate deficiency. In adults, when hypersegmentation was defined as an increase of neutrophils having more than 4 lobes to their nuclei, a poor relationship between this parameter and serum L.casei folate assays was found (Hurdle and Picton Williams, 1966; Kremenchuzky and Hoffbrand, 1965). However, when 6 or more lobed neutrophils were compared with the serum L.casei folate assays, there was agreement between these parameters (Kremenchuzky and Hoffbrand, 1965; Roberts, Hoffbrand and Mollin, 1966).

There was a poor relationship between hypersegmented neutrophils and megaloblastosis (Reynolds, Milner, Matthews and Chanarin, 1966).

In infants, the value of percentage hypersegmentation of the neutrophils was controversial. Some investigators found agreement between hypersegmentation of the neutrophils and the serum L.casei folate assays (Vanier and Tyas, 1967; Shojania and Gross, 1964b) while others found a poor agreement (Vanier and Tyas, 1966). Hypersegmentation was reported as both a consistent (Vanier and Tyas, 1967) and an inconsistent association (Gray and Butler, 1965; Wise, Lovric and O'Gorman Hughes, 1963) of megaloblastosis due to folate deficiency.

It was believed that hypersegmentation of the neutrophils was not pathognomonic of folate and vitamin B_{12} deficiency (Cooke, 1929; Tempka and Braun, 1932; Herbert, 1964; Edwin, 1967; Hansen, 1967). This might partly have explained the conflicting reports of this test.

In this study, other possible aetiologies for hypersegmentation of the neutrophils were iron deficiency, infection and a congenital abnormality of nuclear segmentation. There was no statistical evidence for attributing hypersegmentation of the neutrophils to either iron deficiency or infection. However, it was asserted that hypersegmented neutrophils might remain in the circulation long after the pathological cause had been treated (Neuberger, 1927; Watkins and Berglund, 1927) although this was not confirmed by Fleming (1929). If this were so, it might have complicated the interpretation of the statistical data. Only a direct study of the effect of infection and iron deficiency on nuclear segmentation conducted under controlled conditions would have provided conclusive evidence with which to assess a relationship between percentage hypersegmentation and iron deficiency and infection.

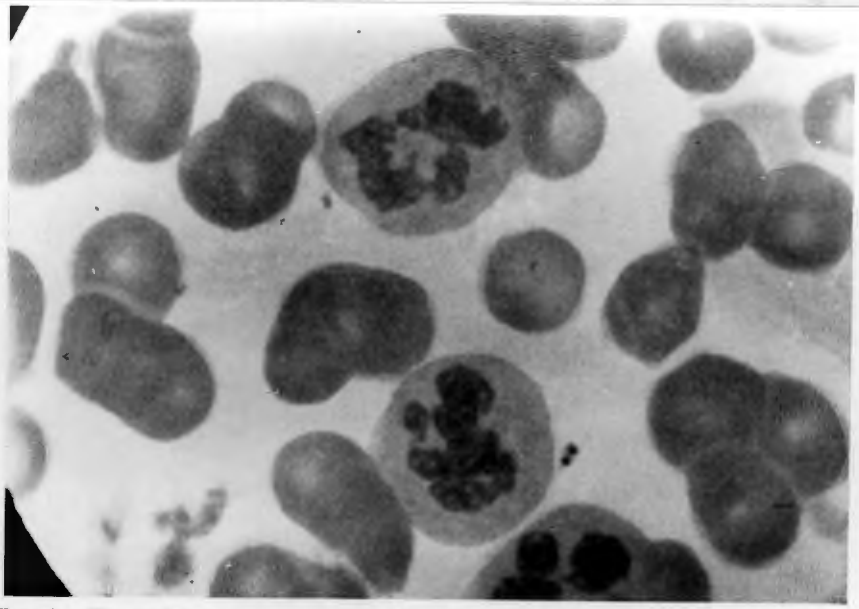


FIG. 4.—Typical hypersegmented polymorphonuclear leukocytes due to vitamin B_{12}

[a]

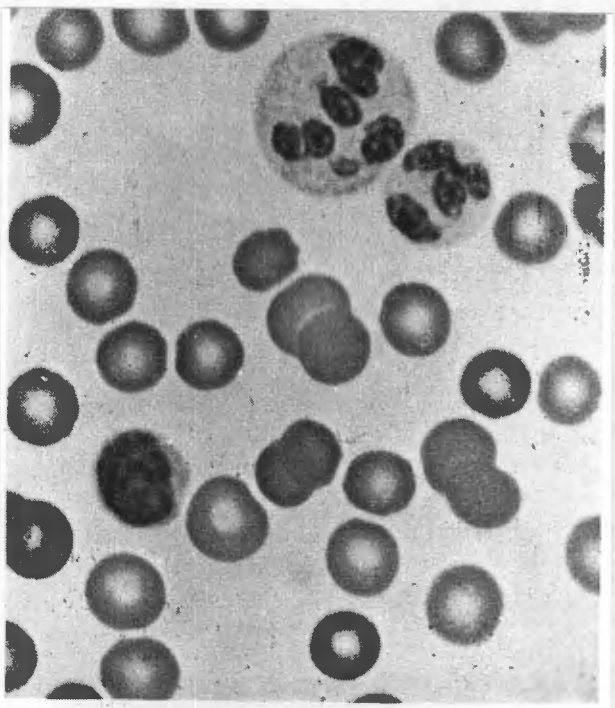


Fig 3 *'Twinning deformity' next to four-lobed neutrophil (peripheral blood of normal man after seventy-eight days of deprivation of dietary folate)*

[b]

Plate 3:- Illustrations of hypersegmented neutrophils according to

(a) Herbert, 1959;

(b) Herbert, 1964.

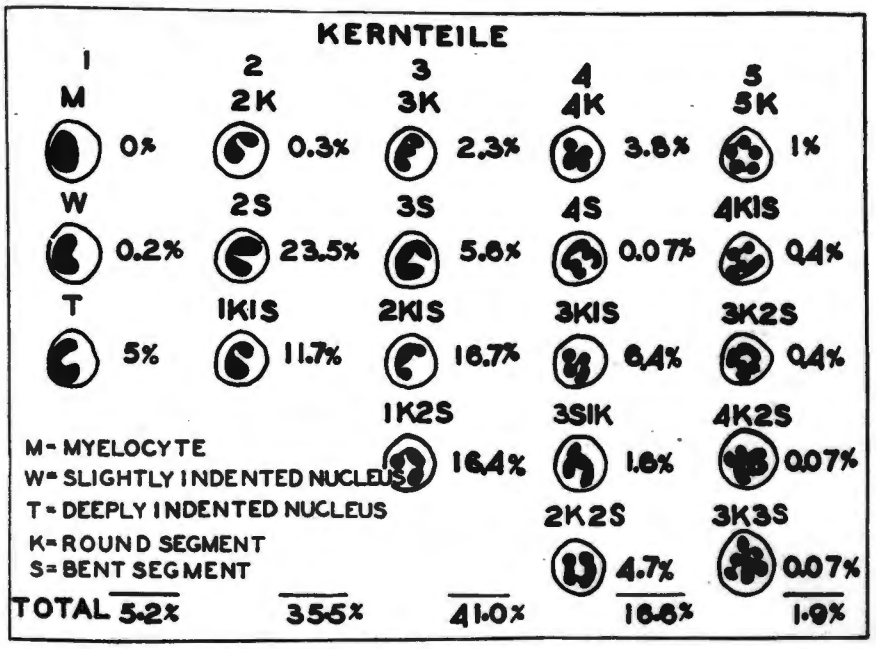


FIG. 45.—Arneth's classification of neutrophilic leukocytes. The number of each of these cell types which he considered normal is shown in per cent. (Wintrobe, courtesy of Bull. New York Acad. Med.)

[a]

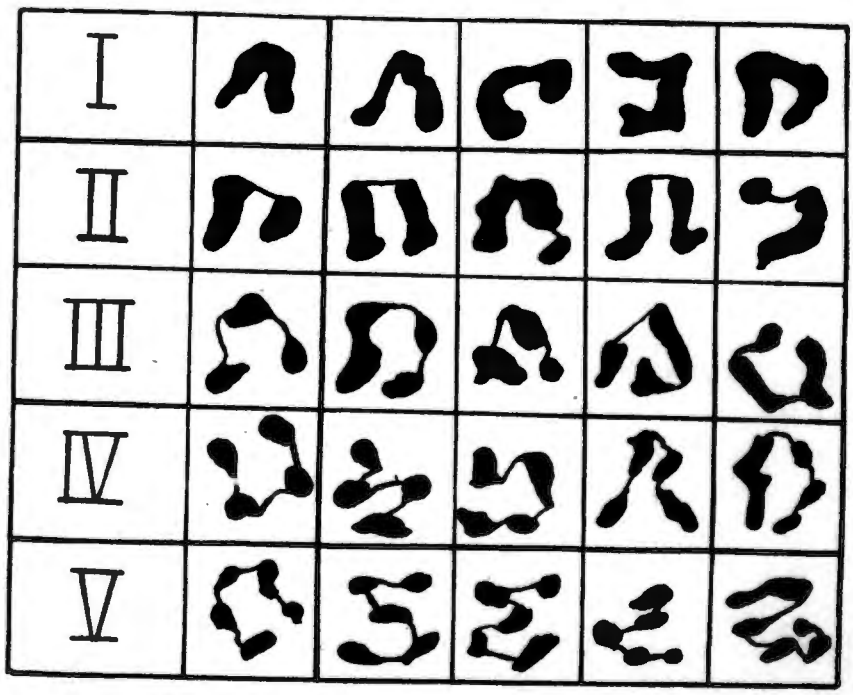


FIG. 14.—The classification of neutrophils or number of lobes.

[b]

Plate 4:- Illustrations of hypersegmented neutrophils according to

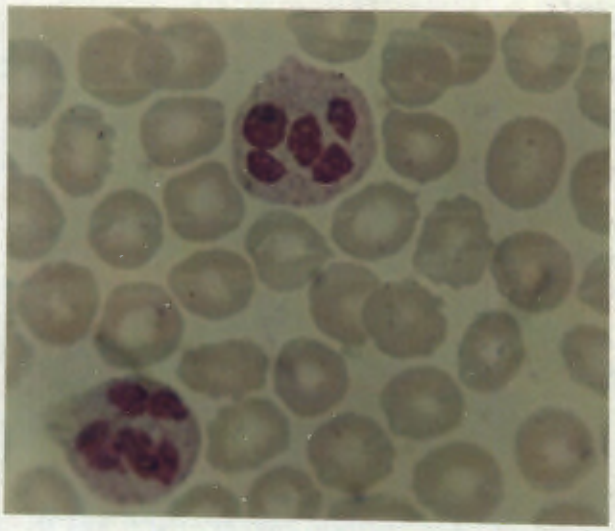
(a) Arneth's classification (Wintrobe, 1956);

(b) Delaney, 1960.

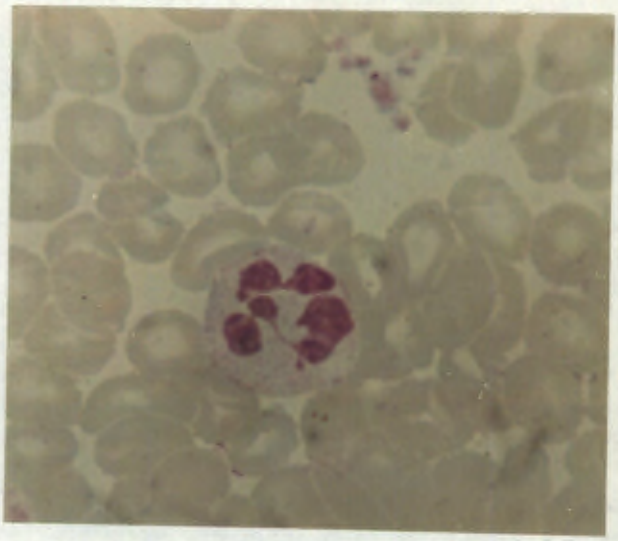
A congenital cause for hypersegmentation was excluded in most infants because the finding was an inconsistent one and because there was no associated increase in four-lobed neutrophils (Undritz, 1958; Herbert, 1965). It could also be excluded in the one infant in whom hypersegmentation was consistently found, because his parents did not show evidence of this anomaly (Undritz, 1958).

From the data in this study, iron deficiency and infection could not be entirely excluded as causes for hypersegmentation but there was a likelier explanation for this finding. This concerned the lack of clarity regarding the definition of separate lobulation. This difficulty prompted Herbert (1964) to suggest that each laboratory determine their own normal values. The wide range of reported normal values for percentage hypersegmentation is probably a measure of the disagreement which exists concerning separate lobulation. The upper limit of hypersegmented neutrophils in normal subjects was set at 2% by Arneth (1904) and Cooke (1927; 1929), at 3% by Heck and Watkins (1933) and Herbert 1959), at 4% by Hurdle and Picton Williams (1966) and Willoughby (1967), and at 5% by Lowenstein, Cantlie, Ramos and Brunton (1966) and Herbert (1967).

In attempting to define separate lobulation in this study it was found that investigators differed about the width of the inter-lobular chromatin bridge necessary for individual lobe-counting. Nuclear lobes were individually counted when they were separated by a thin chromatin thread (Briggs, 1914; Heck and Watkins, 1933; Chanarin, Rothman and Berry, 1965) or a bridge of chromatin not greater than 50% of the largest diameter of the lobes on either side (Edwin, 1967). Moreover, illustrations of nuclear segmentation such as those depicted in Plates 3 and 4 were confusing.

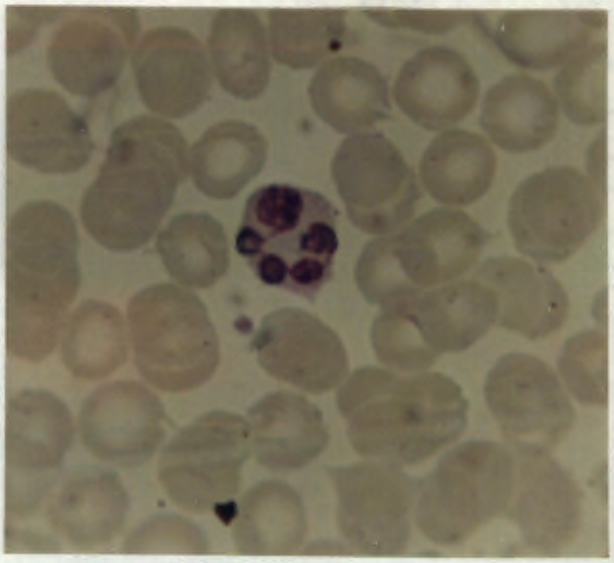


[a]

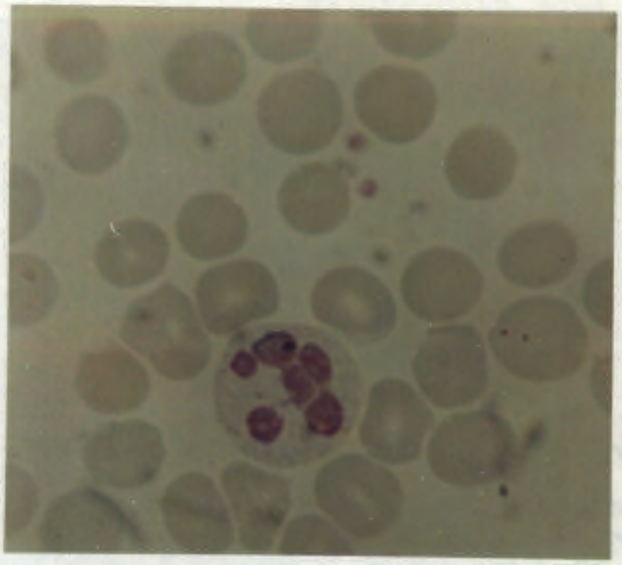


[b]

Plate 5:- Examples of neutrophils which were regarded as hypersegmented but which would not qualify for this if Briggs' (1914) criterion were used.



[a]



[b]

Plate 6:- Examples of neutrophils which were regarded as hypersegmented but which would not qualify for this if Briggs' (1914) criterion were used.

From a study of some of the above reports and the illustrations presented here, it was decided to define separate lobulation as lobes separated by a definite bridge of chromatin. This seemed a reasonable way of satisfying most definitions. However, it was too flexible a definition resulting in inconsistent application especially because long periods of time elapsed between repeat counts of percentage hypersegmentation. Thus, initial reproducibility between repeat counts was poor (Table 12). When the definition was applied with greater consistency, reproducibility improved but was still occasionally disappointing. When the cause for percentage hypersegmentation of the neutrophils in this study was not clear, smears showing hypersegmented neutrophils were recounted using Briggs' (1914) criterion for nuclear separation. Briggs (1914) counted nuclear lobes as single if they were not clearly separate or if they were connected by anything more than the finest chromatin thread: doubtful cells were not counted. With the use of this criterion none of the infants had hypersegmented neutrophils in excess of 5%. In Plates 5 and 6, examples of neutrophils are shown which were regarded as hypersegmented but which would not qualify for this description using Briggs' (1914) criterion. It seems clear that the levels obtained with this method will vary according to the definition of hypersegmentation*.

The criterion for nuclear segmentation used by Briggs (1914) was preferred to that used in the beginning of the study, because it is less equivocal and therefore provides greater possibilities for reproducible results. These could possibly be further improved by counting more than 100 cells on each occasion. With the use of this criterion, up to 4% (perhaps 5%) of hypersegmented neutrophils could be present in the absence of folate deficiency, and with this parameter folate deficiency was not diagnosed in the infants studied. The upper limit of normal has been set

* separate lobulation.

at 3% by investigators who used a similar criterion of nuclear separation (Heck and Watkins, 1933; Chanarin, Rothman and Berry, 1965). The slightly higher levels found here of 4% (perhaps 5%) might have resulted from associated iron deficiency or infection. The numbers concerned were too few to allow clarification of this possibility.

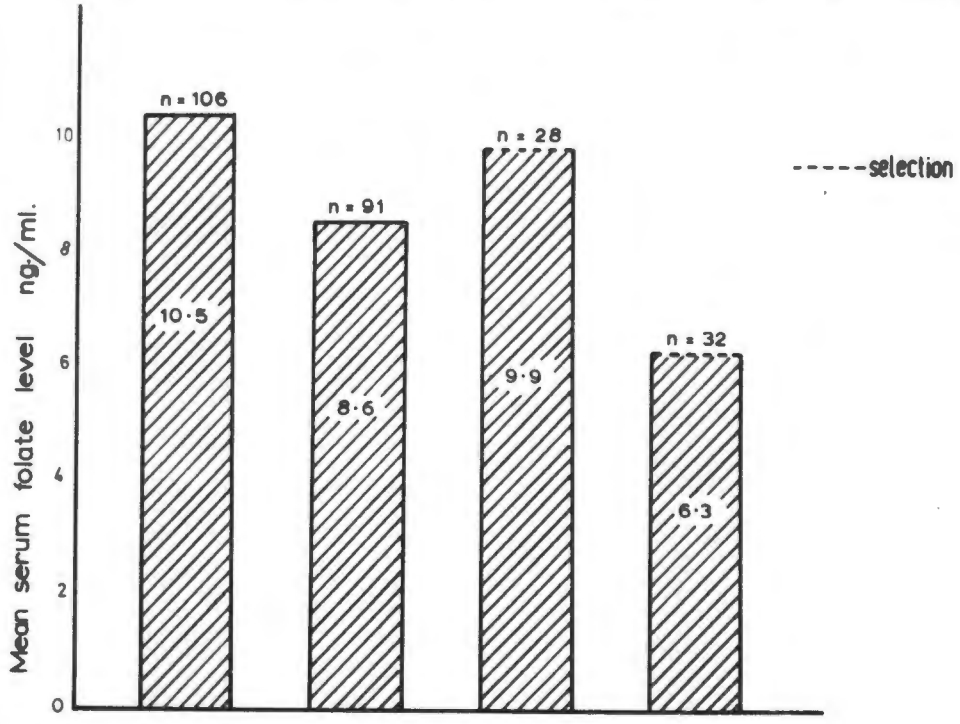
SUMMARY

By means of percentage hypersegmentation as a parameter, it was not possible to establish that infants in this study had morphological folate deficiency. The use of a criterion for nuclear separation suggested by Briggs (1914) provided the best opportunity for reproducible results with this method. Up to 4% (possibly 5%) of neutrophils could be hypersegmented in the absence of folate deficiency.

CHAPTER IX

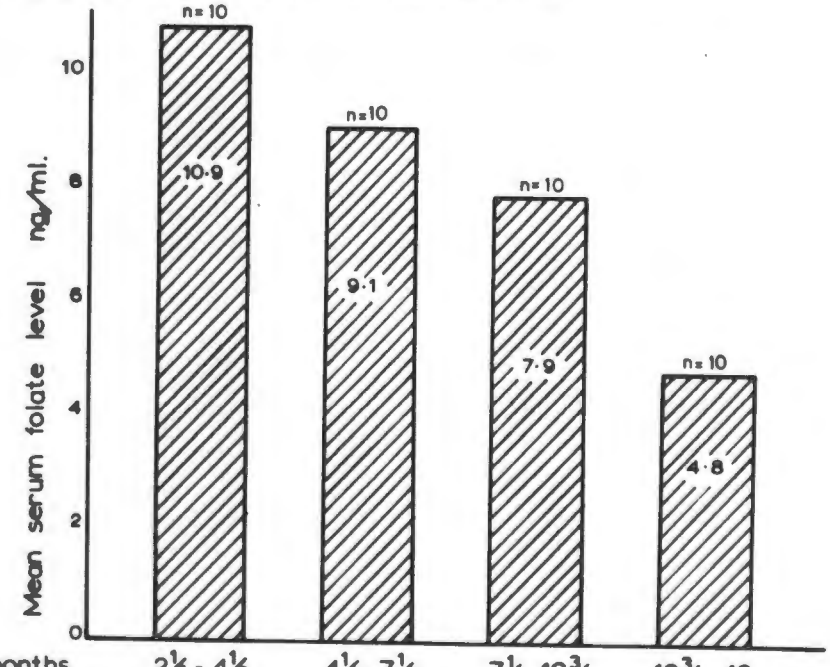
FOLATE STUDIES.

MEAN SERUM FOLATE LEVELS OF ALL INFANTS INVESTIGATED DURING FIRST 16 MONTHS



Age in months	2¼ - 4½	4½ - 7½	7½ - 10¾	10¾ - 16
Range	1·1 - 46·8	1·8 - 30·8	2·9 - 24·0	0·9 - 12·7
S.D.	± 7·9	± 6·0	± 5·2	± 3·1
S.E. of M.	± 0·8	± 0·6	± 1·0	± 0·6

MEAN SERUM FOLATE LEVELS OF THE 10 INFANTS ASSESSED AT ALL FOUR PERIODS DURING FIRST 16 MONTHS



Age in months	2¼ - 4½	4½ - 7½	7½ - 10¾	10¾ - 16
Range	3·0 - 26·8	1·9 - 29·8	3·4 - 14·9	0·9 - 12·4
S.D.	± 7·1	± 7·5	± 3·7	± 3·3
S.E. of M.	± 2·3	± 2·4	± 1·2	± 1·1

Fig. 47.

CHAPTER IX

FOLATE STUDIES

INTRODUCTION.

It was not possible to prove the presence of biochemical folate deficiency in the infants in this study, using either the Figlu Test after oral histidine or hypersegmentation of the neutrophils as parameters of folate deficiency. In this chapter the results of microbiological assays for folate particularly in serum will be assessed. In the light of these findings the incidence, aetiology, significance and need for prophylaxis of folate deficiency in healthy underprivileged infants will be discussed.

RESULTS OF THE SERUM L.CASEI FOLATE ASSAYS.

Pattern of serum L.casei folate estimations (Fig.47).

(i) All infants.

The mean serum L.casei folate level in infants from $2\frac{1}{4}$ - $4\frac{1}{2}$ months was higher (10.5 ng./ml.) than that in adults (6.9 ng./ml.). The mean serum L.casei folate level showed a fluctuating decline with growth until $10\frac{3}{4}$ - 16 months, when it fell below the mean adult L.casei folate level.

(ii) Ten Infants.

A gradual decline of mean serum L.casei folate levels was noted in association with growth in ten infants from whom serum was obtained for L.casei folate assays on all four occasions of investigation. Because there was no fluctuation in the decline of the mean serum L.casei folate level in this group, the fluctuation noted above was ascribed to selection in the sample (cf. Chapter IV).

TABLE 84

INFANTS WITH HIGH SERUM FOLATE LEVELS

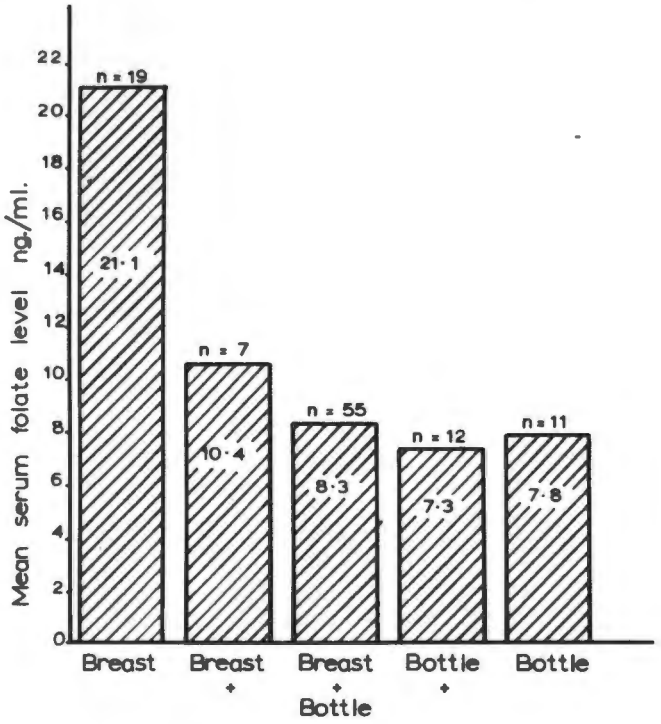
2¼ - 4½ months

Infant	Age (months)	Serum folate level ng./ml.	Number of high serum folate estimations	Total number of serum folate estimations
J.A.	3.5	19.0		
C.H.	3.0	28.4		
P.C.	2.8	26.8		
C.M.	2.3	30.0		
M.J.	3.0	25.6		
S.W.	3.0	27.9		
J.S.	3.0	29.1		
N.H.	3.0	27.6		
A.H.A.	4.0	46.8		
J.E.	3.0	17.5	19	106
C.E.	3.8	19.1		
G.J.	3.0	17.7		
H.L.	3.0	17.6		
A.H.	3.3	19.6		
D.B.	3.0	18.0		
C.A.	3.3	18.2		
C.P.	3.5	22.9		
D.D.	3.5	23.8		
D.A.	3.0	27.0 *		
		4½ - 7½ months		
P.C.	6.0	29.8		
R.K.	6.0	22.5		
A.P.	4.8	32.4		
C.W.	5.0	21.2	7	91
L.F.	6.5	20.5		
M.E.	6.5	27.2		
L.O.	7.0	30.8		
		7½ - 10¼ months		
R.K.	9.0	18.3		
N.D.	9.0	24.0	3	28
S.W.	9.0	17.5		

* One reading only

SERUM FOLATE LEVELS RELATED TO BREAST AND BOTTLE FEEDING

INFANTS 2¼ - 4½ MONTHS OF AGE



MILK FEEDING

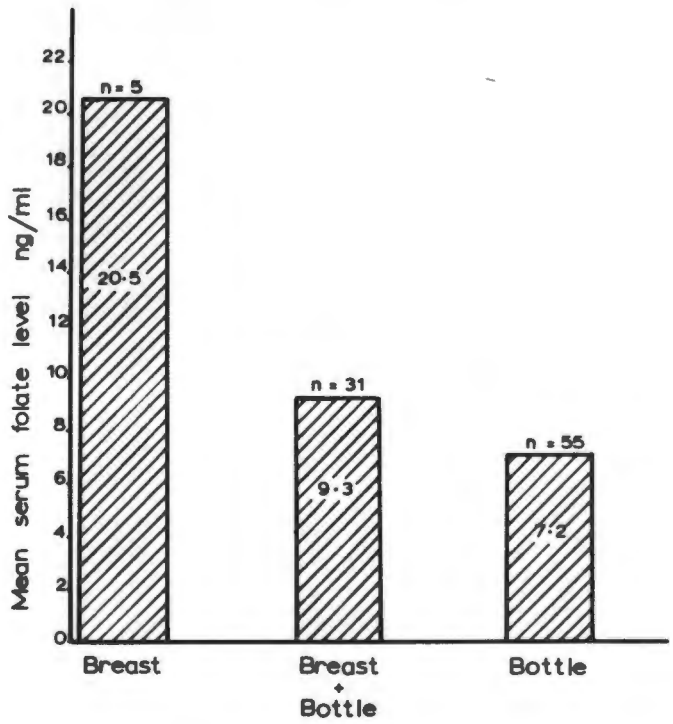
Difference of individual means

t = 3·963 ; P < 0·001

Significance of difference of the means

F = 16·63
P < 0·01

INFANTS 4½ - 7½ MONTHS OF AGE



MILK FEEDING

Difference of individual means

t = 4·480 ; P < 0·001

Significance of difference of the means

F = 14·64
P < 0·01

Fig. 48

Range of serum L.casei folate levels (Fig.47).

The range of serum L.casei folate levels found in infants extended below (0.9 ng./ml.) and above (46.8 ng./ml.) the normal range found in adults (3.0 - 17.3 ng./ml.).

HIGH SERUM L.CASEI FOLATE LEVELS.

Incidence (Table 84).

The maximum incidence of high serum L.casei folate levels occurred in the youngest group of infants. The percentage incidence was as follows:

$2\frac{1}{4}$ - $4\frac{1}{2}$ months - 18% (19 of 106)

$4\frac{1}{2}$ - $7\frac{1}{2}$ months - 8% (7 of 91)

$7\frac{1}{2}$ - $10\frac{3}{4}$ months - 10% (3 of 28).

No infant of more than $10\frac{3}{4}$ months had a serum L.casei folate level above the normal adult range.

Aetiology of high serum folate levels.

With the exception of

(a) the folic acid absorption tests

(b) infection at the time of investigation

the aetiology was first assessed only in infants from $2\frac{1}{4}$ - $4\frac{1}{2}$ months.

It was further assessed amongst the older infants only where the numbers allowed it and where a definite cause for the high serum L.casei folate levels had been found.

It was found that high serum L.casei folate levels could be related to dietary factors.

Milk feeding (Fig.48).

Breast-fed infants who received no other milk supplements had significantly higher mean serum L.casei folate levels than infants who

RELATIONSHIP OF HIGH SERUM FOLATE LEVELS TO MILK FEEDING

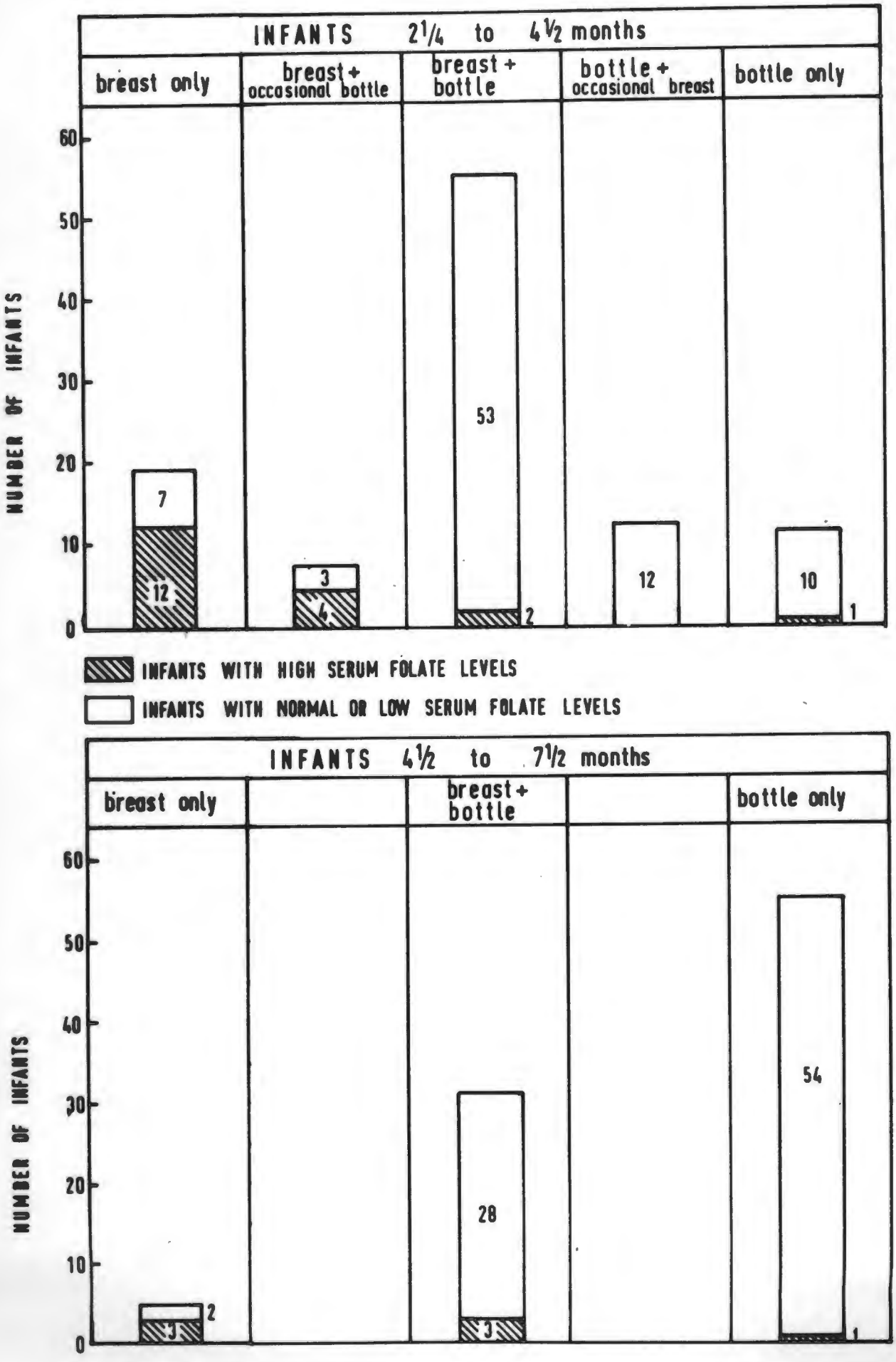


Fig 49

TABLE 85

THE RELATIONSHIP OF DIETARY FACTORS TO MEAN SERUM FOLATE LEVELS IN INFANTS $2\frac{1}{4}$ - $4\frac{1}{2}$ MONTHS.

Dietary factor	Mean serum folate level ng./ml.	Significance test	Significance
Infants given cereal	8.3 n = 39	'STUDENTS' t-test	t = 2.342
Infants not given cereal	12.0 n = 65		$0.02 < P < 0.05$
Infants given mixed diet	8.2 n = 21	"	t = 1.600
Infants not given mixed diet	11.2 n = 83		$0.10 < P < 0.20$
Infants given orange juice or Vitamin C	8.8 n = 11	"	t = 0.800
Infants not given orange juice or Vitamin C	10.8 n = 93		$0.40 < P < 0.50$
Infants given cod liver oil	10.0 n = 38	"	t = 0.635
Infants not given cod liver oil	11.0 n = 66		$0.50 < P < 0.60$
Infants given vitamin syrup	9.5 n = 16	"	t = 0.619
Infants not given vitamin syrup	10.8 n = 88		$0.50 < P < 0.60$
Infants given patent medicines	11.0 n = 18	"	t = 0.350
Infants not given patent medicines	10.5 n = 86		$0.70 < P < 0.80$

Conclusion: **The mean serum folate level is not significantly higher in infants receiving any of these dietary factors.**

Infants not receiving cereal have significantly higher mean serum folate levels than those receiving cereal.

TABLE 86

COMPARISON OF FOLATE CONTENT* OF BREAST MILK WITH THAT OF MILKS PREPARED BY THE MOTHERS.

Classification	Range	Mean	Test of significance	Significance
Breast milk	8.4 - 58.8	28.7 n = 21	'STUDENT'S' t-test	t = 2.633
Prepared milk	3.1 - 40.4	20.8 n = 49		$0.01 < P < 0.02$

*All were assessed as total milk folate. True milk folate values were determined on 39 of the milks. However the range of non-folate growth factors was negligible (0.1 - 1.4 ng./ml.)

Conclusion: The total folate content of breast milk was significantly higher than that of prepared milks.

FOLATE ASSAYS PERFORMED ON MILK PREPARED* IN THE RED CROSS HOSPITAL MILK KITCHEN.

Classification	Folate content ng./ml.
Lactogen	14.5
S-26	5.2
Nespray	29.2
SMA	7.0
Cows milk	23.6

*Preparation of formula milks:- The powdered milk was reconstituted in boiled water after cooling this to a suitable temperature. This was bottled and then autoclaved for 3-4 minutes at 5 lb./sq.in. at 228°F.

Preparation of cows milk feeds:- Pasteurized milk was boiled for 20 minutes and then bottled and autoclaved as above.

TABLE 87

THE EFFECT OF AUTOCLAVING ON FOLATE ACTIVITY IN SERA (L.CASEI) NOT PROTECTED WITH ASCORBIC ACID DURING ASSAY PROCEDURE

Sera	Autoclaved with ascorbic acid protection	Autoclaved without ascorbic acid protection
1	19.1	2.8
2	27.2	1.6

RELATIONSHIP OF INFECTION AT TIME OF INVESTIGATION TO MEAN SERUM FOLATE LEVELS

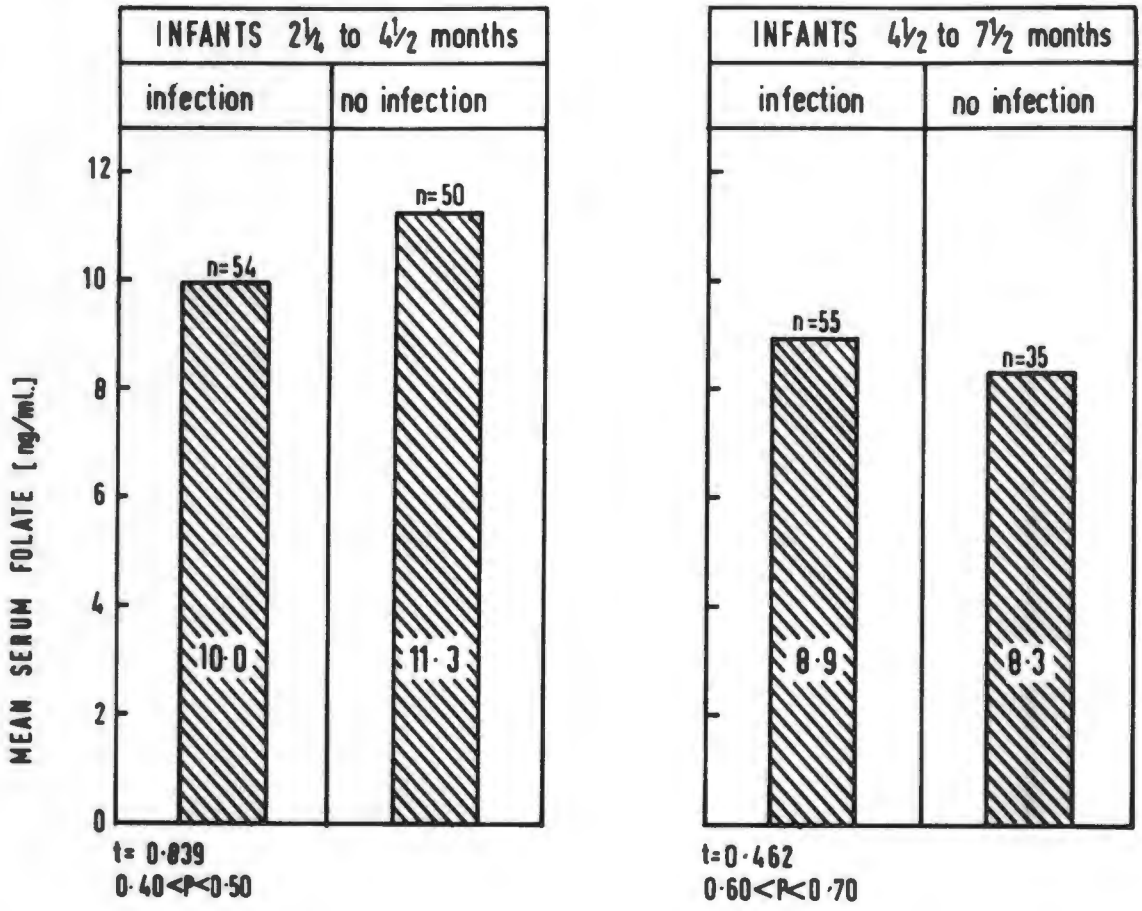


Fig. 50.

RELATIONSHIP OF MEAN CORD SERUM FOLATE, HAEMOGLOBIN, URINARY FIGLU AND SERUM IRON LEVELS TO HEIGHT OF SERUM FOLATE. (INFANTS 2¼ - 4½ MONTHS)

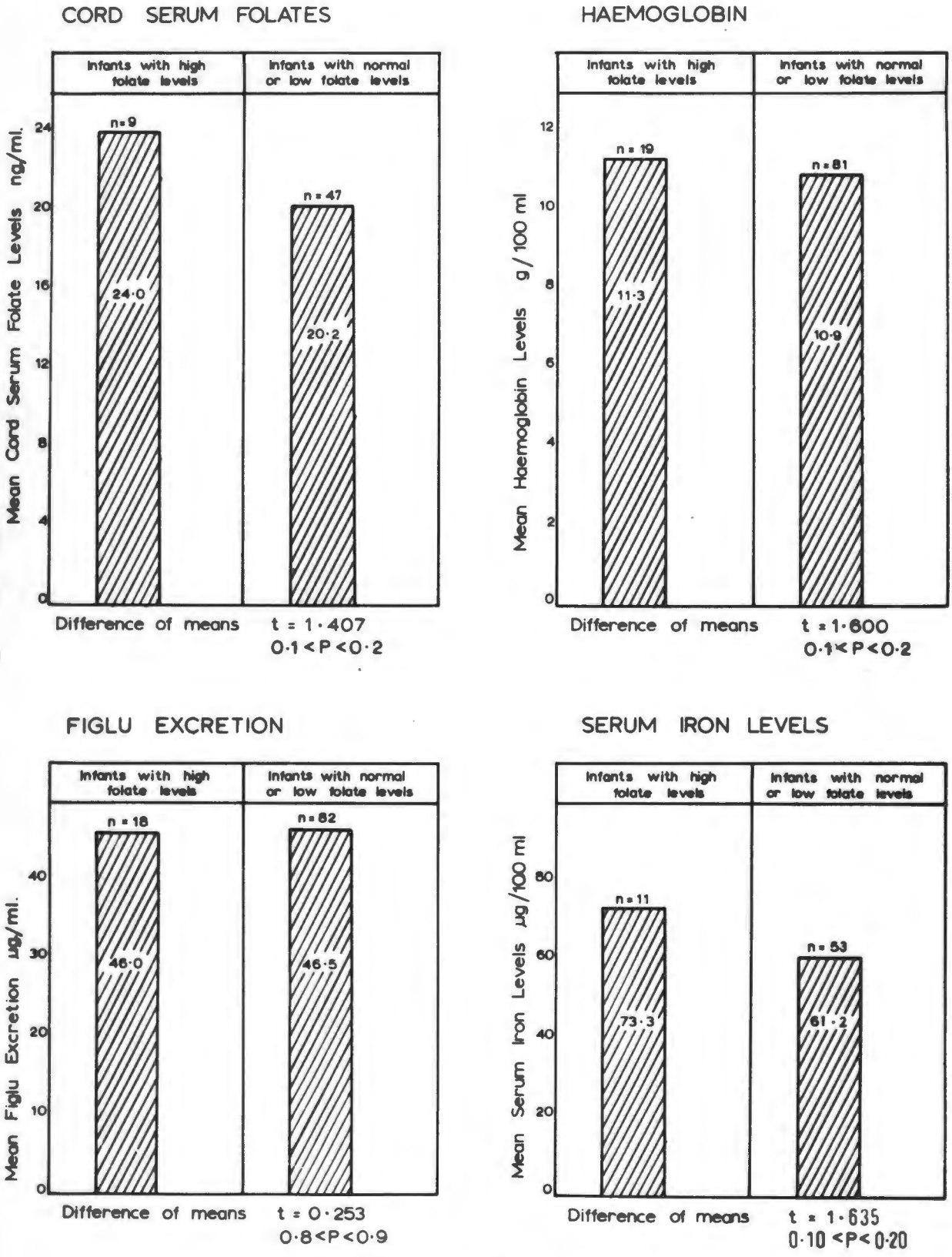


Fig. 51.

received a combination of breast and artificial milk feeding or artificial milk feeding alone. This applied to infants from 2¼ - 4½ months and 4½ - 7½ months.

In the same age group the greatest incidence of high serum L.casei folate levels was found in infants who received breast milk and no other milk supplements (Fig.49).

Supplementary Feeding (Table 85)

None of the additional dietary supplements given to the infants produced a significant rise in mean serum L.casei folate levels.

Folate Content of Milk (Table 86)

The mean L.casei folate content of breast milk (28.7 ng./ml.) was significantly higher than that of prepared milk (20.8 ng./ml.).

The L.casei folate content of some of the proprietary milk feeds prepared in the Red Cross War Memorial Children's Hospital's diet kitchen ranged from 5.2 ng./ml. - 29.2 ng./ml.

High serum L.casei folate levels could not be related to:

(a) Contamination with folic acid (Table 87).

Two sera containing high concentrations of L.casei folate activity were later assayed without ascorbic acid protection. This resulted in a great reduction of measurable folate.

(b) Infection at the time of investigation (Fig.50).

There was no statistical difference between mean serum L.casei folate levels in infants with and without infection at the time of investigation.

(c) Cord serum L.casei folate levels (Fig.51).

The mean cord serum L.casei folate levels were not statistically different in infants with and without high serum L.casei folate levels.

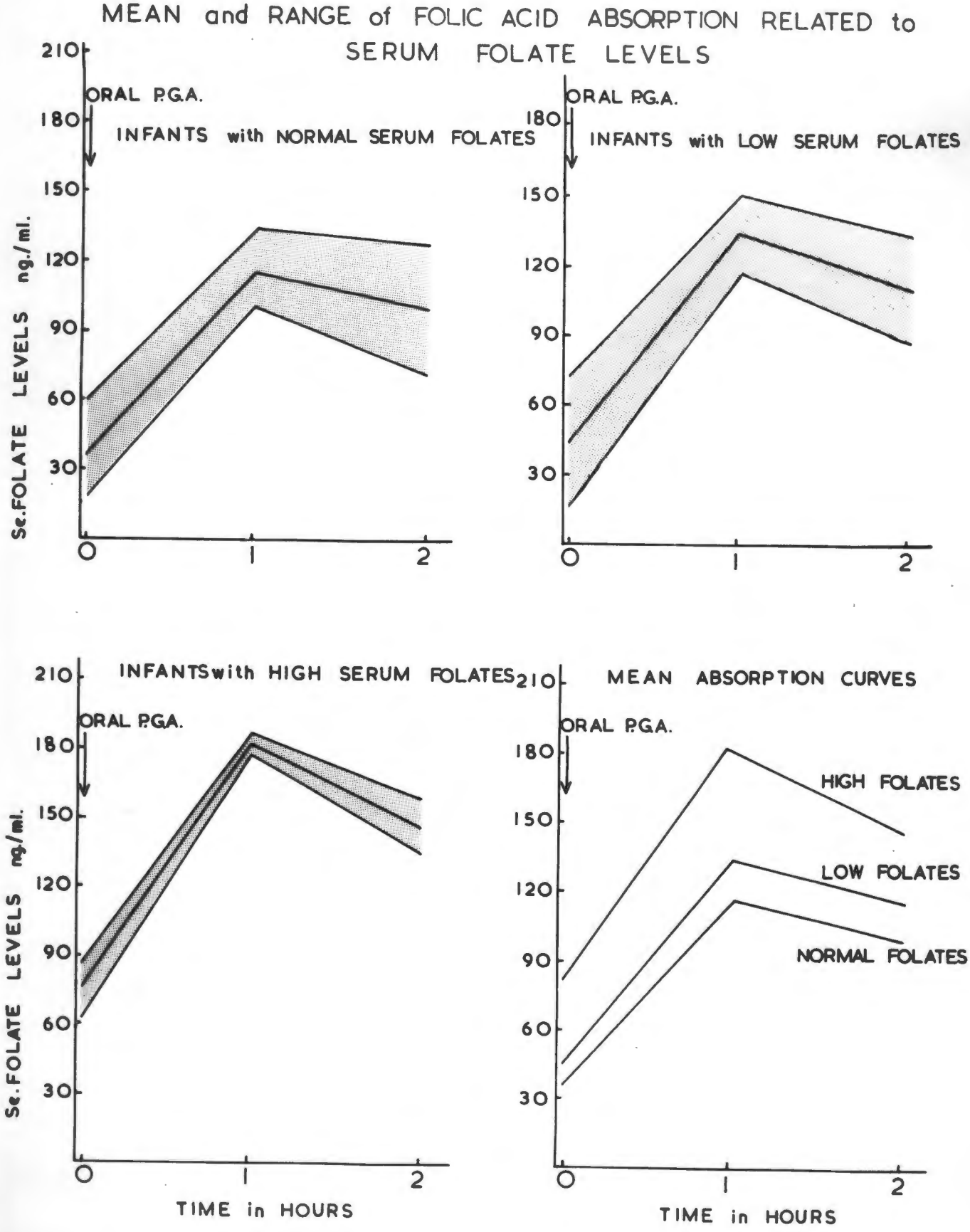

MEAN and RANGE of FOLIC ACID ABSORPTION RELATED to SERUM FOLATE LEVELS
ORAL P.G.A.
INFANTS with NORMAL SERUM FOLATES
INFANTS with LOW SERUM FOLATES
INFANTS with HIGH SERUM FOLATES
MEAN ABSORPTION CURVES
HIGH FOLATES
LOW FOLATES
NORMAL FOLATES
Se. FOLATE LEVELS ng./ml.
TIME in HOURS
210
180
150
120
90
60
30
0
1
2

Fig. 52

MEAN and RANGE of FOLIC ACID ABSORPTION RELATED to SERUM FOLATE LEVELS

INFANTS with NORMAL SERUM FOLATES

SUBJECT	FOLATE LEVELS ng./ml.			
	Se. FOLATE	BASAL	1 Hr.	2 Hrs.
M.de L.	10.6	26	102	73
M.E.	6.1	60	135	128
B.S.	8.0	18	112	98
	MEAN	35	116	100

INFANTS with LOW SERUM FOLATES

SUBJECT	FOLATE LEVELS ng./ml.			
	Se. FOLATE	BASAL	1 Hr.	2 Hrs.
J. P.	1.1	14	117	98
M.L.	2.3	72	151	134
	MEAN	43	134	116

INFANTS with HIGH SERUM FOLATES

SUBJECT	FOLATE LEVELS ng./ml.			
	Se. FOLATE	BASAL	1 Hr.	2 Hrs.
C.E.	19.1	75	187	134
L.F.	20.5	85	178	160
	MEAN	80	183	147

RISE in S. FOLATES

BASAL to 1 HOUR

DATA	NORMAL FOLATE	LOW FOLATE	HIGH FOLATE
RANGE	75 — 94	79—103	93—112
MEAN	82	91	103

SIGNIFICANCE of DIFFERENCES of MEANS

$F = 1.48$ $P > 0.05$

BASAL to 2 HOURS

DATA	NORMAL FOLATE	LOW FOLATE	HIGH FOLATE
RANGE	46 — 81	62 —84	58 —76
MEAN	65	73	67

SIGNIFICANCE of DIFFERENCES of MEANS

$F = 0.13$ $P > 0.05$

TABLE 88

TABLE 89

RELATIONSHIP OF NUTRITION TO HEIGHT OF SERUM FOLATE LEVEL IN INFANTS AT $2\frac{1}{4}$ - $4\frac{1}{2}$ MONTHS OF AGE.

Height of serum folate levels in infants	Mean Percentile (weight)
High serum folate levels	42.0 n = 19
Normal or low serum folate levels	42.0 n = 87

No relationship between growth and height of serum folate level.

RELATIONSHIP OF ERYTHROPOIESIS AS ASSESSED BY RETICULOCYTE COUNT TO HEIGHT OF SERUM FOLATE IN INFANTS $2\frac{1}{4}$ - $4\frac{1}{2}$ MONTHS OF AGE.

Infant serum folate level	Range of reticulocyte count	Mean reticulocyte count
High	1.1 - 2.5 n = 3	2.0
Normal or low	0.5 - 4.1 n = 20	1.8

There is no obvious relationship between decreased erythropoietic activity and height of serum folate level.

TABLE 90

RELATIONSHIP OF HIGH SERUM FOLATE LEVELS TO SERUM VITAMIN B_{12} LEVELS

Classification	Mean serum vitamin B_{12} pg./ml.	Test of significance	Significance
High serum folate levels	445 n = 19	'STUDENT'S' t-test	t = 0.550
Normal or low serum folate levels	474 n = 85		$0.50 < P < 0.60$

Conclusion: Mean serum vitamin B_{12} levels are lower in infants with high serum folate levels but this is not a statistically significant result.

(d) Folic acid absorption (Fig.52: Table 88).

There was no statistically significant difference in the absorption of folic acid between infants with high serum L.casei folate levels and those with serum L.casei folate levels in the normal or low range.

(e) Decreased demand for folate.

(i) Growth (Table 89): There was no relationship between high serum L.casei folate levels and poor growth. Mean percentile levels were the same in infants with and without high serum L.casei folate levels.

(ii) Erythropoiesis: A relationship between high serum L.casei folate levels and decreased erythropoiesis was not demonstrated.

(a) Mean reticulocyte count in infants with high serum L.casei folate levels was similar (2%) to that found in infants with normal or low serum L.casei folate levels (1.8%) (Table 89).

(b) There was no statistical difference between mean haemoglobin concentration in infants with and without high serum L.casei folate levels. (Fig. 51)

(f) Secondary disturbance of folate metabolism.

There was no statistical difference between

(i) mean Figlu excretion (Fig.51);

(ii) mean serum iron levels (Fig.51);

(iii) mean serum vitamin B_{12} levels (Table 90)

in infants with and without high serum L.casei folate levels.

FOLIC ACID CLEARANCES in INFANTS with HIGH and LOW SERUM FOLATE LEVELS
SUPERIMPOSED on RANGE of CLEARANCES in INFANTS with NORMAL FOLATE NUTRITION
L. CASEI

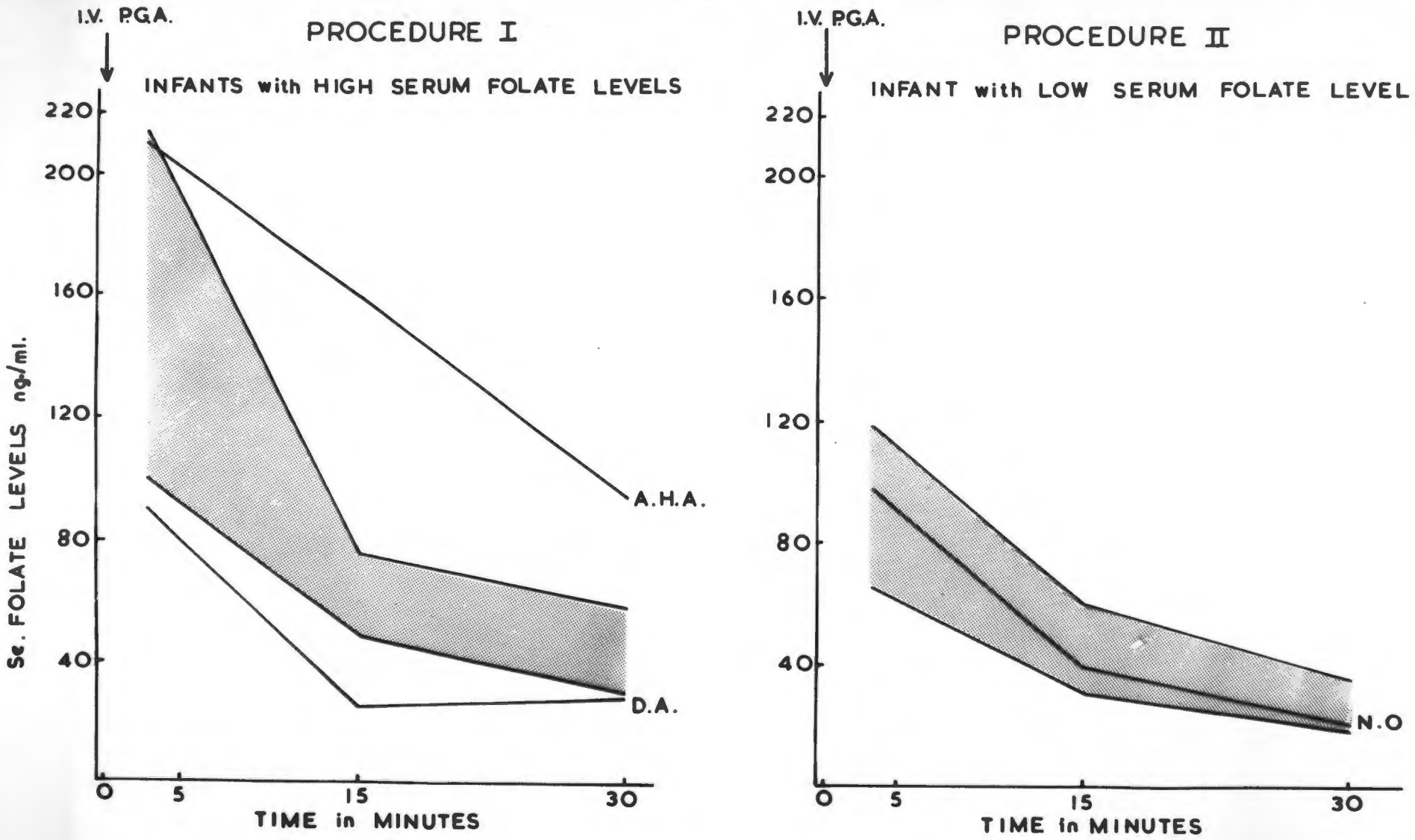


Fig. 53

TABLE 91

INCIDENCE OF LOW SERUM FOLATE LEVELS IN INFANTS.

Nature of Study	Age (months)	Number of estimations	Number of low estimations < 3.0 ng./ml.
Prospective Study	2¼-4½	71	4
	4½-7½	56	4
	7½-10¾	28	1
	10¾-16	32	4
Additional Studies	2¼-7½	112	8

Pattern of L.casei folic acid clearance test in infants with high serum folate levels (Fig.53).

The rate of clearance of folic acid was different in the two infants with high serum L.casei folate levels. In one infant, A.H.A., the clearance of folic acid was slower and in the other, D.A., the clearance of folic acid was faster than in infants with normal folate nutrition. No inference could be drawn from these findings.

LOW SERUM L.CASEI FOLATE LEVELS

Incidence.

Prospective Study (Table 91).

Only an approximate incidence of low serum L.casei folate levels can be given in this study because of several complicating factors:

(i) Serum L.casei folate estimations could not be performed on all infants at all times of investigation.

(ii) Twenty-nine (29) of the original 86 infants were withdrawn from the study at the end of the second visit.

Of the 86 infants, 10 had low serum L.casei folate levels only once or more often. On this basis (10 of 86) the approximate incidence was 11.6%.

Additional Studies (Table 91).

Of the infants under 7½ months of age om whom investigations were performed only once, 7.1% (8 of 112 estimations) had low serum L.casei folate levels.

THE SERUM L.CASEI FOLATE ASSAY AS A PARAMETER OF FOLATE DEFICIENCY.

Comparison with L.casei Folic Acid Clearance Test (Fig.53).

The L.casei folic acid clearance curve in the infant with a low serum L.casei folate level was comparable to those in infants with normal serum L.casei folate levels.

TABLE 92

RELATIONSHIP OF LOW SERUM L.CASEI FOLATE LEVELS TO BONE MARROW MORPHOLOGY.

Infant	Serum folate ng./ml.	Bone Marrow Morphology
S.R.	2.4	No megaloblastosis
R.J.	2.4	No megaloblastosis
B.C.	2.9	No megaloblastosis
C.F.	1.4	No megaloblastosis
F.U.	2.0	No megaloblastosis
S.B.	1.4	No megaloblastosis
T.de B.	1.9	No megaloblastosis
M.R.	0.9	Grade 1 Megaloblastosis

TABLE 93 (Contd.)

(c) The relationship of maternal Figlu excretion to infant serum folate level.

Age (months)	Figlu excretion in mothers	Mean infant serum folate level
2¼ - 4½	Excessive	11.2 n = 4
	Normal	11.2 n = 66

There is no relationship between these two parameters.

TABLE 94

RELATIONSHIP OF SOCIO-ECONOMIC FACTORS TO SERUM FOLATE LEVELS

Age (months)	Socio-economic conditions	Mean serum folate ng./ml.		Test of significance	Significance
$2\frac{1}{4}$ - $4\frac{1}{2}$	Good	12.5	n = 9	Analysis	F = 2.00
	Average	8.7	n = 11	of	P > 0.05
	Poor	4.7	n = 3	variance	
$4\frac{1}{2}$ - $7\frac{1}{2}$	Good	10.6	n = 8		F = 1.21
	Average	7.0	n = 9	"	P > 0.05
	Poor	1.9	n = 1		
$7\frac{1}{2}$ - $10\frac{3}{4}$	Good	8.5	n = 4		F = 0.05
	Average	8.4	n = 7	"	P > 0.05
	Poor	10.5	n = 1		
$10\frac{3}{4}$ - 16	Good	7.1	n = 9		F = 0.65
	Average	6.0	n = 11	"	P > 0.05
	Poor	4.4	n = 2		

There is no consistent relationship or statistical association between socio-economic conditions and serum folate levels.

TABLE 93

RELATIONSHIP OF MATERNAL FOLATE NUTRITION TO INFANT SERUM FOLATE LEVELS

(a) Correlation between maternal and infant serum folates.

Age (months)	Statistical method	Result	Significance
2¼ - 4½	Correlation coefficient	r = -0.081 n = 70	r does not differ significantly from zero in any of these. P > 0.10
4½ - 7½	"	r = -0.053 n = 55	
7½ - 10¾	"	r = +0.073 n = 28	
10¾ - 16	"	r = -0.006 n = 32	

Conclusion: There is no statistical correlation between these two parameters.

(b) The relationship of cord to infant serum folate levels.

Age (months)	Statistical method	Result	Significance
2¼ - 4½	Correlation coefficient	r = +0.131 n = 56	r does not differ from zero in any of these. P > 0.10
4½ - 7½	"	r = -0.117 n = 45	
7½ - 10¾	"	r = -0.220 n = 22	
10¾ - 16	"	r = +0.314 n = 28	

Conclusion: There is no statistical correlation between these two parameters.

Comparison with Red Cell L.casei Folate Assays.

Red cell L.casei folate assays were performed on 6 infants with low serum L.casei folate levels. One of the 6 infants had a low red cell L.casei folate level (67.0 ng./ml.). However, red cell and serum L.casei folate levels were available for comparison in a further 80 infants who had normal serum L.casei folate levels. The red cell L.casei folate level was low (63.0 ng./ml.) in one of these infants who had a serum L.casei folate level of 4.0 ng./ml.

Comparison with Bone Marrow Morphology (Table 92).

One infant with a megaloblastic bone marrow had a low serum L.casei folate level (0.9 ng./ml.). This was the lowest level found in this study. Seven other infants with low serum L.casei folate levels did not show unequivocal evidence of megaloblastosis on examination of the bone marrow.

AETIOLOGY OF LOW SERUM L.CASEI FOLATE LEVELS.

1. A statistical analysis of the relationship between aetiological factors for folate deficiency and low serum L.casei folate levels.

A statistical relationship between low serum L.casei folate levels and the following factors could not be demonstrated:

(a) Maternal folate nutrition (Table 93).

There was no correlation between

(i) maternal and infant serum L.casei folate levels;

(ii) cord and infant serum L.casei folate levels.

There was no statistical relationship between maternal Figlu excretion and mean infant serum L.casei folate levels.

None of the infants with low serum L.casei folate levels ($2\frac{1}{4}$-$4\frac{1}{2}$ months) had mothers whose neutrophils were hypersegmented.

(b) Socio-economic circumstances (Table 94).

There was no statistical relationship between socio-economic conditions and mean serum L.casei folate levels in the infants.

TABLE 95

RELATIONSHIP OF NUTRITION TO SERUM FOLATE LEVEL

Age (months)	Nutritional development (weight percentile)	Mean serum folate ng./ml.		Test of significance	Significance
2¼ - 4½	2nd & 3rd degree malnutrition	11.8	n = 10	Analysis of variance	F = 0.62 P > 0.05
	1st degree malnutrition	10.5	n = 29		
	10 - 25th percentile	9.6	n = 14		
	26 - 50th percentile	13.2	n = 14		
	>50th percentile	9.7	n = 39		
4½ - 7½	2nd & 3rd degree malnutrition	5.1	n = 7	"	F = 0.96 P > 0.05
	1st degree malnutrition	7.9	n = 22		
	10 - 25th percentile	9.8	n = 10		
	26 - 50th percentile	8.4	n = 13		
	>50th percentile	9.5	n = 38		
7½ - 10¾	2nd & 3rd degree malnutrition	7.2	n = 4	"	F = 3.99 P < 0.05
	1st degree malnutrition	5.8	n = 8		
	10 - 25th percentile	11.3	n = 2		
	26 - 50th percentile	13.9	n = 8		
	>50th percentile	11.5	n = 6		
10¾ - 16	2nd & 3rd degree malnutrition	4.8	n = 6	"	F = 1.66 P > 0.05
	1st degree malnutrition	5.3	n = 7		
	10 - 25th percentile	5.1	n = 5		
	26 - 50th percentile	7.9	n = 8		
	>50th percentile	8.0	n = 6		

There is no consistent relationship between nutrition and mean serum folate level but the overall differences become significant in the age group 7½-10¾.

In this group the only significant individual difference is that between 2nd and 3rd degree malnourished infants and those in the 26-50th percentile. In view of this inconsistent pattern it is unlikely that the result has any meaning.

TABLE 96

RELATIONSHIP OF INCREASED DEMAND FOR FOLATE TO SERUM FOLATE LEVELS.

(a) Relationship of infection score to serum folate levels.

Age (months)	Infection score	Mean serum folate levels		Test of significance	Significance
2¼ - 4½	0	10.0	n = 25	Analysis of	F = 0.85
	½ - 2	11.4	n = 66		
	2½ - 4½	8.7	n = 12	variance	P > 0.05
	5 &>	2.0	n = 1		
4½ - 7½	0	9.3	n = 17	Analysis of	F = 0.21
	½ - 2	9.0	n = 48		
	2½ - 4½	7.9	n = 20	variance	P > 0.05
	5 &>	8.7	n = 3		
7½ - 10¾	0	6.9	n = 3	Analysis of	F = 3.91
	½ - 2	10.4	n = 19		
	2½ - 4½	6.6	n = 3	variance	P < 0.05
	5 &>	24.0	n = 1		
10¾ - 16	0	12.4	n = 1	Analysis of	F = 4.46
	½ - 2	6.1	n = 8		
	2½ - 4½	4.5	n = 4	variance	P < 0.05
	5 &>	0.9	n = 1		

There is no consistent relationship between infection score and mean serum folate levels. The overall differences in the age groups 7½-10¾ and 10¾-16 are statistically significant.

In the age group 7½-10¾ mean serum folate levels are significantly lower in infants with an infection score of 0 than in infants with an infection score of 5 &> . This does not therefore suggest a relationship between increased demand for folate caused by infection and low serum folate levels. In the age group 10¾-16 months the only significant difference is that between infants with an infection score of 0 and ½-2.

The maldistribution of numbers in the groups may account for these statistical results. It is unlikely that the inconsistent associations shown here have any meaning.

TABLE 96 (Contd.)

(b) Relationship between low serum folate levels and increased erythropoiesis as judged by reticulocyte counts.

Age (months)	Serum folate levels in infants	Range of reticulocyte count %	Mean reticulocyte count %
$2\frac{1}{4}$ - $7\frac{1}{2}$	Low	0.5 - 1.9 n = 4	1.4
	Normal or high	0.1 - 4.1 n = 41	1.6

There is no relationship between increased erythropoiesis (mean reticulocyte count) and low serum folate levels.

TABLE 97

RELATIONSHIP OF IRON DEFICIENCY TO SERUM FOLATE LEVELS

Age (months)	Serum folate levels	Mean serum iron µg./100 ml.	Test of significance	Significance
2½ - 4½	Low	76.0 n = 4	'STUDENT'S'	t = 1.110
	Normal	62.9 n = 60	t-test	0.20< P<0.30
4½ - 7½	Low	68.3 n = 4	"	t = 0.623
	Normal	58.2 n = 64		0.50< P<0.60
10¾ - 16	Low	56.8 n = 3	"	t = 0.767
	Normal	49.9 n = 22		0.40< P<0.50

Conclusion: Mean serum iron levels were higher in the infants with low serum folate levels at all ages.

The differences were not statistically significant.

The infant with a low serum folate level at 7½ - 10¾ months did not have a serum iron estimation.

(c) Nutrition (Table 95).

A convincing statistical relationship was not demonstrated between nutrition and mean serum L.casei folate levels in the infants.

(d) Increased demand for folate.

(i) Growth (Table 95).

There was no apparent relationship between rapid growth in infants and low mean serum L.casei folate levels. On the contrary, after 4½ months the better nourished infants had higher mean serum L.casei folate levels.

(ii) Infection (Table 96).

There was no convincing statistical evidence for attributing low mean serum L.casei folate levels to the effect of increased demand for folate as the result of infection.

(iii) Erythropoiesis (Table 96).

Infants with low serum L.casei folate levels did not have increased erythropoiesis as assessed by mean reticulocyte counts.

None of the infants with low serum L.casei folate levels had positive occult blood tests.

(e) Secondary disturbances of folate metabolism (Table 97).

There was no statistical relationship between low serum L.casei folate levels and mean serum iron levels.

II. Aetiological factors for folate deficiency in individual infants (18) (Table 98: 99).

Dietary Factors.

Of the 18 infants whose serum L.casei folate levels were low, none received breast milk without other milk supplements. Thus the folate intake

TABLE 98

POSSIBLE AETIOLOGICAL FACTORS IN INDIVIDUAL INFANTS WITH LOW SERUM FOLATE LEVELS

PROSPECTIVE STUDY

(a) Infants without morphological folate deficiency

Infant	Age (mths)	Serum folate ng./ml.	Maternal folate nutrition (parameter)	Nature of milk feeding	Socio-economic status	Nutritional development (percentile)	Absorption	Infection score	Mean infection score	Parasitic infestation	Serum iron µg./100 ml	Iron therapy	Comment
S.B.	3.0	10.8	Normal	Breast 1 mth.then bottle	Average	5th 1st degree malnutrition	-	2.0	1.3	-	80.6	-	Diet History of dietary deprivation.
	6.0	6.1	"	Bottle	"	10th	-	5.0	1.7	-	35.5	-	Recurrent infection
	9.5	5.2	"	"	"	10th	-	0.5	1.7	-	27.4	-	Infection score higher than mean infection score at the time that serum folate levels were low. Infection may therefore have contributed to the biochemical folate deficiency.
	13.5	2.5	"	"	"	< 3rd 1st degree malnutrition	-	4.0	2.5	Ascariasis and Giardiasis	61.4	Possibly at clinic	
	16.0°	1.4	"	Bottle but no milk for 2 wks.prior to study	"	< 3rd 2nd degree malnutrition	36.7% (xylose)	4.0		-	47.5	-	
T.de B.	3.0	3.0	Normal	Breast 3 days then bottle	Poor	< 3rd 2nd degree malnutrition	-	1.0	1.3	-	-	-	Diet An undernourished infant whose folate intake may have been suspect from the start.
	6.0	1.9	"	Bottle	"	"	-	2.0	1.7	-	28.4	-	Infection and malabsorption At six months chronic salmonella gastroenteritis may have resulted in malabsorption and further reduction of folate level.
	9.0	10.5	"	"	"	"	-	1.0	1.7	-	35.8	-	
	13.5	2.1	"	"	"	"	64.7% (xylose)	3.0	2.5	-	44.5	-	
	16.0°	4.1	"	"	"	"	-	2.0		-	-	-	Iron deficiency This may have contributed.
B.C.	2.6	2.7	Normal	Breast 1 mth.then bottle	Average	9th borderline 1st degree malnutrition	-	1.0	1.3	-	-	-	Diet An undernourished infant whose dietary intake may have been suspect.
	6.0	3.0	"	Bottle	"	10th	-	2.0	1.7	-	34.3	-	
	10.5	2.9	"	"	"	7th 1st degree malnutrition	-	2.0	4.2 (1.7 + 2.5 7½-16 mths)	-	-	Iron therapy	Iron deficiency This may have contributed.
	14.0°	3.2	"	"	"	< 3rd 2nd degree malnutrition	-	2.5		-	61.8	-	

Table 98
Continued

Infant	Age (mths)	Serum folate ng./ml.	Maternal folate nutrition (parameter)	Nature of milk feeding	Socio-economic status	Nutritional development (percentile)	Absorption	Infection score	Mean infection score	Serum iron µg./100 ml.	Iron therapy	Comment
S.D.	3.0	9.9	Normal	Breast and bottle	Average	97th	-	1.0	1.3	-	-	Diet Change in diet associated with decreasing weight and serum folate levels
	6.3	10.1	"	"	"	85th	-	1.0	1.7	-	-	
	12.8	2.6	"	Bottle	"	18th	-	5.0	4.2 (1.7 + 2.5 7½ - 16 mths.)	64.6	-	
E.H.	3.0	2.7	Low maternal serum folate	Breast 2 mths.then bottle	Good	97th	-	0	1.3	100.8	-	Diet Dietary folate may have been low.
	6.3	1.8[x]	"	Bottle	"	75th	-	1.0	1.7	86.4	-	Maternal folate deficieny
	14.0	6.9	"	"	"	85th	-	1.0	4.2 (1.7 + 2.5 7½ - 16 mths.)	79.8	-	This may have contributed.
F.U.	2.8	2.0	Excessive Figlu excretion	Breast 1 mth.then bottle	Poor	<3rd 2nd degree malnutrition	6% (xylose)	5.0	1.3	40.2	Iron therapy	Diet F.U. & C.F. Undernourished infants from poor socio-economic circumstances possibly receiving insufficient dietary folate.
	12.8[oo]	6.6	"	Bottle	"	7th 1st degree malnutrition	-	4.5	5.9 (1.7 + 1.7 + 2.5 4½ - 16 mths)	27.8	"	Infection and malabsorption Low serum folate levels noted when infants were recovering from chronic gastroenteritis. Infection and malabsorption probably contributed.
C.F.	2.8	-	Low serum folate. +ve Figlu test	Breast 1 mth.then bottle	Poor	5th 1st degree malnutrition	-	1.0)))	1.3	-	-	Maternal folate deficieny Doubtful role (F.U.) Possible role (C.F.)
	4.3	1.4	"	Bottle	"	<3rd 1st degree malnutrition	-	3.0)))		-	-	
R.J.	4.0	11.4	Serum folate low. Hypersegmentation of neutrophils	Breast and bottle	No assessment	50th	-	2.0	1.3	-	-	R.J. & S.R. Diet Insufficient folate intake is the only obvious cause
	6.3	2.4	"	Bottle	"	30th	-	0.5	1.7	78.8	-	Maternal folate deficieny At this age a maternal contribution is doubtful
S.R.	6.5	2.4	Low serum folate	Bottle	"	<3rd 2nd degree malnutrition	-	3.5	3.0 (1.3 + 1.7 2½ - 7½mths.)	79.6	-	

Table 98
continued

(b) Low serum folate levels in association with a megaloblastic bone marrow

Infant	Age (mths)	Serum folate ng./ml.	Maternal folate nutrition (parameter)	Nature of milk feeding	Socio-economic status	Nutritional development (percentile)	Absorption	Infection score	Mean infection score	Parasitic infestation	Serum iron µg./100 ml.	Iron therapy	Comment
M.R.	3.5	12.7	Low serum folate	Breast and bottle	Poor ♂	6th 1st degree malnutrition	-	2.5	1.3	-	-	-	Undernourished infant from very adverse socio-economic circumstances. Serum folate levels declined with change in diet and markedly so after deprivation of milk. Recurrent infection The infection score was much greater than the mean value in the period 10½-16 months of age. Half the infection score was accounted for by gastrointestinal infection. Infection was almost certainly a contributory factor. Maternal deficiency It is unlikely that there was any contribution at this age - 13.0 months.
	6.0	8.6	"	"	"	10th	-	2.5	1.7	-	-	-	
	9.0	4.0	"	Bottle. breast occasionally	"	10th	-	2.0	1.7	-	48.2	-	
	13.0	0.9	"	Virtually no milk feeding in this time	"	<3rd 2nd degree malnutrition	-	7.0)))))	2.5	-	-	-	
	14.8*	1.8	"	Bottle	"	<3rd 3rd degree malnutrition	16.2% (xylose)	6.0)		Ascariasis and Giardiasis	37.3	-	

* These infants re-attended in the period 10½ - 16 months because of low serum folate levels.
The data of the second of these visits has not been included in the statistical analysis of the data for this period.

** Blood samples not obtained at second and third assessments.

♂ Personal assessment.

x Result of a single serum folate assay.

TABLE 99

POSSIBLE AETIOLOGICAL FACTORS IN INDIVIDUAL INFANTS WITH LOW SERUM FOLATE LEVELS

ADDITIONAL STUDIES

Infant	Age (months)	Serum folate ng./ml.	Nature of milk feeding	Nutritional development (percentile)	Absorption	Infection score	Mean infection score	Serum iron µg./100 ml.	Comment
J.P.	3.8	1.1[x]	Breast and bottle	90th	Normal folic acid absorption test	2.0	1.3	91.0	
M.L.	3.3	2.3	Breast 2 months then bottle	10th	Normal folic acid absorption test	2.0	1.3	-	
O.M.	4.3	2.7	Breast 6 weeks then bottle	75th	16.7% xylose	2.5	1.3	72.0	None of the infants received breast milk without other milk supplements at the time serum folate levels were low. There was no other obvious aetiological factor in these infants
R.S.	4.3	2.4	Breast 1 month then bottle	25th	-	0	2.3[ø]	-	
V.L.	4.5	1.0	Breast 2 months then bottle	55th	-	2.0	2.3	88.0	
A.J.	4.3	2.9	Bottle	< 3rd 2nd degree	-	2.0	2.3	27.0	
S.H.	4.3	2.7	Breast 2 months then bottle	25th	-	2.0	2.3	45.0	
N.O.	5.3	2.4	Bottle	30th	-	•	-	-	

[x] Result of a single serum folate assay

• Information incomplete

[ø] This represents the mean infection score of the infants in the controlled therapeutic study. This data could not be included with data previously analysed.

POSSIBLE AETIOLOGICAL FACTORS IN INDIVIDUAL INFANTS WITH LOW RED CELL FOLATE LEVELS

ADDITIONAL STUDIES

Infant	Age (months)	Serum folate ng./ml.	Nature of milk feeding	Nutritional development (percentile)	Absorption	Infection score	Mean infection score	Serum iron µg./100 ml.	Comment
V.L.	4.5	1.0	Breast 2 months then bottle	55th	-	2.0	2.3	88	Neither of these infants received breast milk without other milk supplements. There was no other obvious aetiological factor.
C.S.	4.5	4.0	Breast 2 months then bottle	85th	-	1.0	2.3	-	

TABLE 100

THE RELATIONSHIP OF LOW SERUM FOLATES AND MORPHOLOGICAL FOLATE DEFICIENCY TO XYLOSE ABSORPTION AND PARASITIC INFESTATION

Infant	Serum folate ng./ml.	Bone marrow examination	Xylose absorption % excretion	Blood xylose mg.%				Parasitic infestation	Clinical condition
				Minutes					
			6	30	60	90	120		
O.M.	2.7	-	16.7	-				-	Well
F.U.	2.0	No megaloblastosis		2	5	7.5	11	-	Moderately severe chronic gastro-enteritis
			25.3	18	29	30	26		14 days after cure of gastroenteritis
S.B.	1.4	No megaloblastosis	36.7	-				Ascariasis and giardiasis treated prior to this examination	Mild salmonella gastroenteritis
T.deB.	2.1	-	64.7	-				-	Intermittent loose stools
M.R.*	1.8	Megaloblastosis (1)	16.2	-				Ascariasis treated prior to this examination. Giardiasis still present.	-

* Investigated after milk feeding and treatment of infection had raised serum folate level from 0.9 to 1.8 ng./ml.

might have been lower in these infants, so resulting in, or contributing to the development of biochemical folate deficiency.

Also, there was circumstantial evidence for dietary folate deficiency in 3 infants. In 2 (M.R. and S.B.) there was a clear history of deprivation of food and therefore folate. In the other infant (S.D.) serum L.casei folate levels declined as nutrition deteriorated.

Nutrition.

There was no consistent relationship to nutritional development. Seven of the 10 infants in the prospective study were undernourished while only one of the eight infants investigated in the additional studies was undernourished.

Malabsorption.

This was investigated in seven infants with low serum L.casei folate levels. The folic acid absorption test was used in two, and the d-xylose absorption test in five.

(i) Folic acid absorption test (Table 99, 88; Fig. 52).

There was no statistical difference in the mean absorption of folic acid between infants with low serum L.casei folate levels and infants with normal or high serum L.casei folate levels.

(ii) D-Xylose absorption test (Table 100, 98, 99).

Four of five infants with low serum L.casei folate levels excreted a normal percentage of xylose.

The fifth infant (F.U.) who had a low serum L.casei folate level and associated chronic gastroenteritis, excreted an abnormally low percentage of xylose (6%). This returned to normal (25.3%) when the associated gastroenteritis had been effectively treated. In this infant similar changes were observed in blood xylose levels.

Because, in F.U., gastroenteritis was shown to have been associated with malabsorption, it was possible that this contributed to the development of the low serum L.casei folate level. At the time that their serum L.casei folate levels were low, another two infants (T.de B. and C.F.) had chronic gastroenteritis (Table 98). From this it was inferred that malabsorption associated with chronic gastroenteritis might have contributed to the biochemical folate deficiency.

Parasitic infestation was found in two infants with low serum L.casei folate levels. No relationship was demonstrated between parasitic infestation and malabsorption in M.R. (Table 100).

Recurrent infection (Table 98).

In two infants with low serum L.casei folate levels the incidence and severity of infection (S.B. 8, and M.R. 13) much of which was gastrointestinal, was much greater than that found in other infants of a similar age group (mean infection score in the age group 2.5). In these two infants (M.R. and S.B.) it was probable that recurrent infection contributed to the development of low serum L.casei folate levels. This could have occurred as a result of restricted folate intake, increased demand for folate or malabsorption of folate when the infection was gastrointestinal.

Maternal Folate Deficiency, Socio-economic circumstances, Iron Deficiency (Table 98, Table 99).

The role of these factors in producing low serum L.casei folate levels in the infants was uncertain.

Conclusion.

From the study of the aetiological factors that might have contributed to the development of low serum L.casei folate levels in individual infants, there was evidence that:

TABLE 101

SIGNIFICANCE OF LOW SERUM FOLATE LEVELS

(a) Effect of low serum folate levels on haemoglobin levels.

Age (months)	Folate levels in infants	Mean haemoglobin levels		Test of significance	Significance
2¼ - 4½	Low	10.7	n = 7	'STUDENT'S' t-test	t = 0.790
	Normal or high	11.0	n = 93		0.40 < P < 0.50
4½ - 7½	Low	11.2	n = 4	"	t = 0.678
	Normal or high	10.8	n = 78		0.50 < P < 0.60
7½ - 10¾	Low	8.5	n = 1	"	t = 1.467
	Normal or high	10.5	n = 25		0.10 < P < 0.20
10¾ - 16	Low	9.5	n = 4	"	t = 2.014
	Normal or high	10.4	n = 25		0.05 < P < 0.10

There is no consistent relationship or statistically significant association between these two parameters.

TABLE 101 (Cont.)

(b) The effect of low serum folate levels on nutrition.

Age (months)	Serum folate levels in infants	Mean percentile (weight)		Test of significance	Significance
2¼ - 4½	Low	41	n = 7	'STUDENT'S' t-test	t = 0.074
	Normal or high	42	n = 99		P > 0.90
4½ - 7½	Low	27	n = 4	"	t = 1.098
	Normal or high	46	n = 86		0.20< P <0.30
7½ - 10¾	Low	7	n = 1	"	t = 0.282
	Normal or high	34	n = 27		0.70< P< 0.80
10¾ - 16	Low	6	n = 4	"	t = 1.257
	Normal or high	32	n = 28		0.05< P< 0.10

In all age groups infants with low serum folate levels have a lower mean weight percentile. None of the differences are statistically significant.

(c) The effect of low serum folate levels on infection score.

Age (months)	Serum folate levels in infants	Mean infection score		Test of significance	Significance
2¼ - 4½	Low	2.3	n = 7	'STUDENT'S' t-test	t = 2.771
	Normal or high	1.2	n = 97		0.001< P < 0.01
4½ - 7½	Low	1.2	n = 3	"	t = 0.552
	Normal or high	1.6	n = 85		0.50< P< 0.60
7½ - 10¾	Low	2.0	n = 1	"	t = 0.320
	Normal or high	1.6	n = 25		0.70< P< 0.80
10¾ - 16	Low	5.1	n = 3	"	t = 5.667
	Normal or high	1.7	n = 11		P< 0.001

In all age groups infection score is higher in infants with low serum folate levels. The differences become significant at 2¼- 4½ and 10¾ - 16 months of age.

(i) dietary deficiency existed. This was based on the following findings:-

(a) None of the infants received breast milk without other milk supplements at the time that serum L.casei folate levels were low.

(b) There was a clear history of dietary deprivation in two infants.

(ii) malabsorption of folate might have contributed to the development of low serum L.casei folate levels in three infants.

(iii) recurrent infection apparently contributed to the development of low serum L.casei folate levels in two infants.

SIGNIFICANCE OF LOW SERUM L.CASEI FOLATE LEVELS

Statistical Assessment (Table 101).

There was no statistical relationship between low serum L.casei folate levels and

(a) mean haemoglobin concentration;

(b) mean weight percentile.

Although infants with low serum L.casei folate levels had a higher incidence and severity of infection, this was statistically significant only in infants from $2\frac{1}{4}$ - $4\frac{1}{2}$ months and $10\frac{3}{4}$ - 16 months. There may be a statistical association between these two factors but this has not been proved.

Therapeutic Assessment.

(a) Infants with low serum L.casei folate levels without morphological folate deficiency.

Symptomatic Improvement.

Four of seven infants with low serum L.casei folate levels had symptomatic benefit from folic acid therapy, and five of twenty-one with

TABLE 102

THE EFFECT OF BIOCHEMICAL FOLATE DEFICIENCY ON THE INFANTS

I. INFANTS WITH LOW SERUM FOLATE LEVELS WHO DID NOT RECEIVE FOLIC ACID THERAPY

(a) Infants with iron deficiency

Study	Age (months)	Mean serum folate ng./ml.	Mean haemoglobin g./100 ml.	Percentile (weight)
Initial	4.2 n = 2	2.3 n = 2	10.9 n = 2	6 n = 2
Follow-up study	12.0 n = 2	2.5 n = 2	8.0 n = 2	5 n = 2

Infants with low serum folate levels and iron deficiency showed little change in mean serum folate levels and percentiles, but a drop in haemoglobin over a mean of 7.8 months.

(b) Infants without iron deficiency

Study	Age (months)	Mean serum folate ng./ml.	Mean haemoglobin g./100 ml.	Percentile (weight)
Initial	2.9 n = 2	2.3 n = 2	10.7 n = 2	49 n = 2
Follow-up study	13.4 n = 2	6.7 n = 2	11.1 n = 2	46 n = 2

Infants with low serum folate levels without iron deficiency or in the presence of treated iron deficiency showed a rise in mean serum folate levels, mean haemoglobin and a slight decrease in mean percentile levels over a period of 10.5 months.

Conclusion:

In the absence of iron deficiency there is no evidence that low serum folate levels affect haemoglobin concentration.

No effect of low serum folate levels on mean percentile could be demonstrated.

II. INFANTS WITH LOW SERUM FOLATE LEVELS TREATED WITH FOLIC ACID *

Parameter	Mean value before treatment	Mean value after treatment
Haemoglobin g./100 ml.	10.0 n = 3	9.5 n = 3
Percentile (weight)	12 n = 3	13 n = 3
Serum folate ng./ml.	1.7 n = 3	21.3 n = 3

* Treated with 1¼ mg. folic acid daily for 10 days or 5 mgm. b.d. if gastroenteritis present. Re-examined after a mean of about 6 weeks after therapy commenced.

Conclusion:

Folic acid therapy did not result in an improvement in haemoglobin values. The minimal improvement in mean percentile could not be dissociated from the effects of cure of coincidental gastroenteritis in one of the 3 infants.

TABLE 103.

FOLIC ACID THERAPY IN AN INFANT WITH MORPHOLOGICAL FOLATE DEFICIENCY AND ASSOCIATED IRON DEFICIENCY

Parameter	Before treatment	After treatment *
Haemoglobin g./100 ml.	9.2	8.5
Percentile (weight)	2	3
Bone marrow morphology	Megaloblastosis	Scanty giant metamyelocytes

* Folic acid 100 µg. intramuscularly daily for 10 days assessed 3 weeks after treatment commenced.

Conclusion:

Folic acid therapy did not affect haemoglobin level but did greatly improve bone marrow morphology. The improvement in weight cannot be dissociated from the effect of improved dietary intake.

TABLE 104

PATTERN OF RED BLOOD CELL FOLATE LEVELS
IN INFANTS OF 2¼ - 7½ MONTHS OF AGE

Age (months)	Range of red blood cell folate ng./ml.	Mean	Standard deviation	Standard error of mean
2¼ - 4½	148 - 1250 n = 27	320	± 202.3	± 39.6
4½ - 7½	105 - 2520 n = 28	362	± 456.3	± 87.8

normal serum L.casei folate levels enjoyed the same benefit from this therapy. This was not a statistically significant result ($\chi^2 = 2.674$; $0.10 < P. < 0.20$).

Objective Improvement (Table 102).

Therapy with folic acid did not improve mean haemoglobin concentration, nor did it have a substantial effect on weight percentile. The mean haemoglobin concentration and mean weight percentile were not affected when a group of infants with low serum L.casei folate levels were not given folic acid in the absence of iron deficiency.

(b) Folic acid therapy in an infant with biochemical and morphological folate deficiency (Table 103).

Following folic acid therapy there was considerable improvement of the morphological changes in the bone marrow. Folic acid therapy did not produce a beneficial effect on haemoglobin concentration and the apparent improvement in weight percentile following this therapy could not be dissociated from a coincident effect of improved feeding and treatment of associated infection.

RESULTS OF THE RED CELL L.CASEI FOLATE LEVELS

Pattern of Red Cell L.casei Folate Levels (Table 104).

Eighty-nine red cell L.casei folate estimations were performed. The mean range and standard error of this investigation were analysed in 55 infants in the earlier additional studies. As with the serum L.casei folate assays, the mean values in infants under 7½ months were higher than that of the adult mean (208ng./ml. in White and 241 ng./ml. in Coloured adults).

Red cell L.casei estimations were also performed on a further 34 infants who participated in a controlled therapeutic study. The range of the red cell L.casei folate assays encountered here was 63.0 ng./ml. - 632.0 ng./ml. and the mean was 295 ng./ml.

In this last group only one infant from whom blood could be taken for folate estimations received breast milk without other milk supplements. This might have explained these generally lower red cell folate levels.

Red cell L.casei folate assay as a parameter of folate deficiency.

Red cell L.casei folate levels were low in two of the 89 infants. In one of these infants, the red cell L.casei folate level was associated with a low serum L.casei folate level but not in the other.

Aetiology of low red cell L.casei folate levels (Table 99).

Neither of the two infants who had a low red cell L.casei folate level, received breast milk without other milk supplements. There was no other cause for these low red cell L.casei folate levels.

DISCUSSION

Pattern of serum L.casei folate estimations.

In this study it was noted that the mean serum L.casei folate level was high in young infants. It declined with growth to approximate the mean adult level. This pattern had been described by Vanier and Tyas (1966) using the serum L.casei folate assay, and Matoth, Pinkas, Zamir, Mooallem and Grossowicz (1964) using the whole blood L.casei folate assay.

A point of difference between the three studies was the age at which the mean L.casei folate levels dropped below mean adult levels. This was reported to have occurred at 8 weeks by Matoth, Pinkas, Zamir, Mooallem and Grossowicz (1964) and at 3-4 months by Vanier and Tyas (1966). In this study it was noted only after 10¾ months.

The serum L.casei folate levels of some of the infants in this investigation were either higher or lower than the normal adult range in Cape Town. The cause for the high serum L.casei folate levels found in some infants was investigated.

High serum L.casei folate levels.

It was shown that infants who received breast milk without other milk supplements had statistically significantly higher mean serum L.casei folate levels than infants who received the following:

a) formula milks;

b) a combination of formula and breast milk;

c) breast milk and cow's milk. Three (3) infants received this last combination, but in none of these infants was the serum L.casei folate level high.

It was previously noted that the serum (Vanier and Tyas, 1966) and the whole blood (Matoth, Pinkas, Zamir, Mooallem and Grossowicz, 1964) L.casei folate levels of breast-fed infants were higher than those of infants fed with formula milks.

The higher mean serum L.casei folate levels found in breast-fed infants in this study appeared to be due to the higher concentration of folate present in breast milk. It was found that the mean L.casei folate content of breast milk was significantly higher than that of reconstituted formula milks prepared by the mothers for their infants. Similarly, reconstituted powdered milk feeds prepared in the diet kitchen of the Red Cross War Memorial Children's Hospital showed a lower range of L.casei folate content than that found in breast milk.

This confirmed an earlier finding by Matoth, Pinkas, and Sroka (1965), who noted that breast milk had a higher L.casei folate content than reconstituted powdered milks. This finding was contradicted by Nicol and

Davis (1967) who reported higher L.casei folate levels in reconstituted powdered milk than in breast milk. Also the levels of L.casei activity in breast milk found by Ramasastri (1965) were lower than those reported in this study and by Matoth, Pinkas and Sroka (1965). Karlin, Hours, Bertoye, Vallier and Berry (1967) showed that the L.casei folate content of breast milk was initially low and reached its maximum in the fourth month of lactation. This might be one likely explanation for some of the above differences.

Although the range of L.casei folate content of breast milk was higher than that of reconstituted powdered milk, high values of up to 40.4 ng./ml. were obtained with some powdered milks. Conversely, values as low as 8.4 ng./ml. werefound in breast milk. This was probably why infants who received formula milks occasionally had high serum L.casei folate levels and also why breast-fed infants did not always have high serum L.casei folate levels.

Apart from the higher L.casei folate content of breast milk compared to formula milks, it might be that two other factors contributed to the high serum L.casei folate levels found in breast-fed infants.

First, the folate in breast milk might have greater biological accessibility for the infants because of a more favourable folate composition. Although the biological availability of food folate must still be determined (Herbert, 1967; Chanarin, Rothman, Perry and Stratfull,1968) it is believed that "free folate" (folate immediately available to L.casei without conjugase treatment) is available to humans (Herbert, 1967). Since the free L.casei folate activity in fresh cow's milk was higher than that in breast milk (Matoth, Pinkas and Sroka, 1965) it seemed unlikely that breast milk contained folate of greater biological availability. This possibility might finally be excluded when more is known of the exact folate composition of the different milks and of the biological availability of the different forms of folate.

The second possibility for the apparently greater efficacy of breast milk was a suggestion made by Matoth, Pinkas, Zamir, Mooallem and Grossowicz (1964) and Matoth, Pinkas and Sroka (1965). They considered that the intestinal bacterial population of breast-fed infants might have a greater capacity to synthesise folate or less tendency to consume folate than the bacterial population found in artificially fed infants. It was known that intestinal bacteria could synthesise folate (Donaldson, 1964), and that this could occur in humans (Hoffbrand, Tabaqchali and Mollin, 1966; Klipstein and Samloff, 1966). Moreover, in pathological conditions, serum L.casei folate levels could be elevated as a result of the synthesis of folate by intestinal bacteria (Hoffbrand, Tabaqchali and Mollin, 1966). However, the role of intestinal bacteria in infant folate nutrition is uncertain and in general the extent to which the synthesis of folate by intestinal bacteria contributes to the nutrition of the host, remains to be determined (Klipstein and Samloff, 1966).

Apart from breast-feeding, no cause for the high serum L.casei folate levels could be found. They were not related to coincident supplementary feeding, contamination of serum with folic acid, or to the effect of trivial infection at the time of investigation. It had been reported that serum L.casei folate levels could be elevated in the presence of infection (Spector and Metz, 1966; Spector, Falcke, Yoffe and Metz, 1966). The high serum L.casei folate levels could also not be ascribed to:

a) differences in the initial cord serum L.casei folate levels;

b) increased absorption of folic acid;

c) decreased demand for folate;

d) a secondary disturbance of folate metabolism as a result of

(i) vitamin B_{12} deficiency;
(ii) iron deficiency.

The higher serum L.casei folate levels were therefore attributed to the effect of breast-feeding. The slow decline in mean serum L.casei folate levels with growth could be attributed to the higher incidence of breast-feeding found in this study (23 of 119; 19.3%) as compared to the following:

(a) 8.4% - 23 of 373 (Matoth, Pinkas, Zamir, Mooallem and Grossowicz,1964);

(b) 8.3% - 2 of 24 (Vanier and Tyas, 1966).

The satisfactory L.casei folate content of breast milk (Matoth, Pinkas and Sroka, 1965) and of fresh cow's milk (Naiman and Oski, 1963; Luhby and Cooperman, 1963) was unexpected in the light of earlier beliefs regarding the low folate content of milk (Zuelzer and Rutzky, 1953; Luhby, 1959). Luhby (1959) believed that normal infants might have low folate reserves and in some instances biochemical folate deficiency because milk was a poor source of folate. These concepts were partially based on the results of folate assays performed on milk at that time (Collins, Harper, Schreiber and Elvehjem, 1951).

Shortly after this study had commenced, Naiman and Oski (1963) disproved the results of previous assays for folate in milk. They improved the assay technique by using:

a) L.casei as the assay organism, and

b) ascorbic acid as protection for the labile folate during assay.

By this method they showed that fresh cow's milk contained approximately 60 µg. of folate/quart * and not the previously reported 2.2 µg. of folate/quart *. The surprisingly satisfactory folate content of milk made widespread biochemical folate deficiency in infancy seem a much less likely possibility.

* These are the stated figures but are probably meant to be µg./litre.

Afterwards many investigators confirmed the satisfactory L.casei folate content of fresh cow's milk (Naiman and Oski, 1964; Matoth, Pinkas and Sroka, 1965; Ghitis, 1966; Becroft and Holland, 1966) and found that it was higher than that of breast milk (Matoth, Pinkas and Sroka, 1965).

However, it seemed that the folate content of milk could be markedly reduced by boiling (Ghitis, 1966; Matoth, Pinkas and Sroka, 1965) and by autoclaving (Naiman and Oski, 1964; Ghitis, 1967) but was little affected by pasteurisation (Matoth, Pinkas and Sroka, 1965; Ghitis, 1966). Thus, depending on the procedure employed, folate could be lost during the manufacture of powdered milks (Matoth, Pinkas and Sroka, 1965; Ghitis, 1966). This was the probable explanation for the generally lower folate content of reconstituted powdered milk when compared to that of fresh cow's milk (Naiman and Oski, 1964; Matoth, Pinkas and Sroka, 1965; Ghitis, 1966; Becroft and Holland, 1966) or breast milk (Matoth, Pinkas and Sroka, 1965). The folate content of cow's milk and reconstituted powdered milk might further be reduced by feeding practices such as overdilution or boiling of milk. However, the labile folate could be protected during autoclaving and boiling by the addition of vitamin C in sufficient concentration. This was found to be 10 mg./100 ml. for autoclaving (Ghitis, 1967) and 40 mg./litre for boiling (Nicol and Davis, 1967).

The daily total folate requirements of infants have not been determined. It was found that the daily therapeutic requirements of folic acid for infants not receiving a folate free diet were 5-20 µg. (Velez, Ghitis, Pradilla and Vitale, 1963) or 50 µg. (Sullivan, Luhby and Streiff, 1966). It had been inferred that the physiological daily requirements of folic acid were 20-50 µg. (Sullivan, Luhby and Streiff, 1966) and that the daily

folate requirements of infants might be 20 µg. (Matoth, Pinkas and Sroka, 1965) or less than 50 µg. (Becroft and Holland, 1966). From Table 86, it appeared that on average a litre per day of breast milk provided 28.7 µg. of folate to infants exclusively receiving breast milk. This figure represented only the "free folate" content of breast milk and was therefore probably an underestimation. Karlin, Hours, Bertoye, Vallier and Berry (1967) showed that approximately a third of the total folate content of breast milk consisted of folate polyglutamates which would be available to L.casei only after treatment of milk with conjugase. Although there is some information about the biological availability of certain folate polyglutamates (Herbert, 1968) nothing is known of those in milk. If all were available to the infant, a litre of breast milk would have provided the average infant with approximately 43 µg. of folate daily. Since none of the infants who received breast milk without other milk supplements developed low serum L.casei folate levels, this amount appeared to be adequate for the needs of these healthy underprivileged infants. It seemed probable that normal daily requirements were less than 43 µg./day because -

a) the concentration of folate in breast milk was sometimes much lower than the average of 28.7 ng./ml. Also, all infants would not have received as much as a litre of milk;

b) the folate content of breast milk was not constant throughout lactation. It was found to be low immediately post partum, to rise rapidly by the end of the first month, and to reach a maximum in the 4th month of lactation (Karlin, Hours, Bertoye, Vallier and Berry, 1967). During the first month when breast milk folate levels are low, the high serum L.casei folate levels (derived from the mothers*) may provide protection against folate deficiency.

* most cord serum folate levels were high

A small proportion of infants who received reconstituted powdered milk or a combination of milk feeds developed low serum L.casei folate levels. Their folate intake, especially those receiving only redonstituted milk feeds, might have been inadequate. Some might have received less than 5 µg. of total folate daily (Table 86).

LOW SERUM L.CASEI FOLATE LEVELS

The serum L.casei folate levels of a small number of infants were lower than those found in normal adults. Should the serum L.casei folate assay be accepted as a valid parameter of folate deficiency, these low levels may be attributed to biochemical folate deficiency.

The serum L.casei folate assay as a parameter of folate deficiency.

It was established that the serum L.casei folate assay was a sensitive parameter of folate deficiency which could indicate the presence of this deficiency before

a) neutrophils became hypersegmented,

b) Figlu excretion after oral histidine became excessive,

c) red cell L.casei folate levels decreased,

d) bone marrow morphology became megaloblastic (Herbert, 1962a; Mollin and Hoffbrand, 1965).

In most instances it was also a more sensitive parameter of folate deficiency than the L.casei folic acid clearance test (Hogan, Maniatis and Moloney, 1964).

For the above reason, the validity of the serum L.casei folate assay could not be gainsaid by negative results obtained with other parameters for folate deficiency.

It was known that some non-specific factors could spuriously lower serum L.casei folate levels (Reizenstein, 1965). Of these, the administration of antibiotics was the only relevant factor in this study and even this was excluded (Chapter V). In infants, unexplained spuriously low serum L.casei folate levels had not been reported. The low serum L.casei folate levels were unlikely to have been a manifestation of inhibitors in the serum of the infants because a serum-extraction method of estimating L.casei folate activity was used in this study (Cowan, Hoffbrand and Mollin, 1966).

Because there was no other reason for the low serum L.casei folate levels, and because there was a good relationship between serum L.casei folate levels and dietary folate intake, low serum L.casei folate levels were attributed to biochemical folate deficiency. This finding is of some importance as Leevy(1967) showed that there was a good relationship between serum L.casei folate levels and hepatic folate content. Also, serum L.casei folate levels were found to provide a better index of hepatic folate content than red cell L.casei folate levels (Leevy, 1967). Similarly it was found that in folate deficiency of recent onset in pregnant women, red cell L.casei folate levels underestimated the frequency and degree of folate deficiency. This might have been because folate was locked in red cells till the end of their life span (Chanarin, Rothman, Ward and Perry, 1968).

The reliability of the serum L.casei folate assay as an index of folate deficiency in infancy was controversial because normal serum L.casei folate levels were reported in the presence of folate deficiency(Lovric and Kenrick, 1965) and megaloblastosis not due to vitamin B_{12} deficiency (Luhby,Cooperman, MacIver and Montgomery,1962; Pereira and Baker,1966). In this study the only infant who had a megaloblastic bone marrow had a low serum L.casei folate level (0.9 ng./ml.- the lowest level encountered). However, the agreement between serum L.casei folate assays and red cell L.casei folate assays was disappointing. Two infants had red cell L.casei folate levels below the normal range but in only one was the serum L.casei folate level low. Vanier and Tyas (1966) reported a similar experience. They stated that the interpretation of low

red cell L.casei folate levels (whole blood/ml. of red cells) was difficult unless these were very low because an occasional normal patient had a red cell L.casei folate level (whole blood/ml. of red cells) which was below the normal range. They cited Hansen (1964) who had made similar observations. Thus the lack of agreement between red cell L.casei folate levels and the serum L.casei folate levels found in this study did not prove the serum L.casei folate assay to be an unreliable index of folate deficiency. The reliability of the serum L.casei folate assay in the diagnosis of megaloblastosis in infancy must still be established.

INCIDENCE OF BIOCHEMICAL FOLATE DEFICIENCY

This could not be accurately determined in the prospective study because the same sample could not be studied at all times. Of the original 86 infants, 10 showed evidence of biochemical folate deficiency resulting in an approximate incidence of 11.6%.

Of the infants under 7½ months who were investigated, only once 7.1%, had low serum L.casei folate levels.

Furthermore, the incidence of biochemical folate deficiency might have been inaccurate for two reasons. The infants participating in the study were selected on the basis of health and parent co-operation. For these reasons it was not known whether these figures are representative of all healthy underprivileged infants in Cape Town, particularly the less privileged members of this population group. Also, because the incidence of biochemical folate deficiency was determined solely from the serum L.casei folate assay, the inherent margin of error of the serum L.casei folate method must be considered.

Vanier and Tyas (1966) carried out a prospective investigation on 24 normal European infants whose mothers received folic acid supplements during pregnancy. Under these optimum circumstances, 4 of the 24 infants ($\pm$ 16.7%) showed evidence of biochemical folate deficiency (low serum L.casei folate levels) at some stage of the first year of life. The lower incidence,in this study, of 11.6%, in infants in poorer circumstances might have resulted from:

a) changes in the sample studied prospectively; or

b) the higher incidence of breast-feeding in healthy underprivileged infants in Cape Town.

AETIOLOGY OF BIOCHEMICAL FOLATE DEFICIENCY

This was first investigated by a statistical analysis of the data.

It was not possible to demonstrate a relationship between maternal and infant folate nutrition. However, of the parameters used for investigating maternal folate deficiency, probably only the serum L.casei folate assay had validity (Chapter VI). This was a sensitive criterion of folate deficiency and was not necessarily related to significant maternal folate deficiency. From the findings in this study it could therefore be concluded only that there was no relationship between minimal maternal biochemical folate deficiency and the development of folate deficiency in infants whose dietary folate intake was not controlled.

Accordingly, this study did not exclude the possibility that significant maternal folate deficiency might

a) contribute to the development of folate deficiency in infants receiving an inadequate folate intake;

b) be primarily responsible for the development of folate deficiency in infants receiving an adequate folate intake.

This seemed less likely if it were correct that infant folate stores were formed to the detriment of maternal folate nutrition.

Recently Luhby, Feldman, Gordon and Cooperman (1967) claimed that significant folate deficiency in mothers resulted in reduced folate stores in their infants (birth - 12 weeks). Although a control group of infants apparently did not show this abnormality, the results might have been more convincing had there been further evidence of folate deficiency as support for the positive Figlu tests in 4 of the 5 infants. The only infant who did have supportive evidence for the Figlu test also had erythroblastosis foetalis. Moreover, it was not clear whether these infants had received adequate folate intake up to the time the investigation was undertaken.

Willoughby (1967) investigated the relationship between maternal and infant folate status. He assessed the effect of maternal folic acid supplementation on the whole blood L.casei folate levels in their infants at 6 weeks of age. He concluded that if the daily diets of pregnant women were supplemented with 300 - 450 µg. of folic acid and iron, the whole blood folate levels in their infants were significantly higher than in those whose mothers had been supplemented with iron and smaller doses of folic acid. However, these results were based on whole blood folate levels which do not make allowance for differences in haematocrit. Furthermore, the daily folate intake of the infants was not controlled and might have varied widely. Last, it was not clear, from his data (Table), whether the whole blood folate levels in the infants whose mothers had received daily folic acid (300 - 450 µg.) and iron were statistically significantly higher than those of infants whose mothers did not receive any supplementation at all during pregnancy.

It is apparent that the relationship of significant maternal folate deficiency to the development of folate deficiency in infants requires further investigation under controlled conditions.

There was no demonstrable relationship between biochemical folate deficiency and socio-economic differences within the group of under-privileged infants. A relationship between whole blood L.casei folate levels and socio-economic circumstances in a group of infants who were mainly artificially fed was demonstrated by Matoth, Pinkas, Zamir, Mooallem and Grossowicz (1964). They were not able to demonstrate the same relationship in breast-fed infants (Matoth, Pinkas and Sroka, 1965). The poor dietary intake which is often associated with poor socio-economic circumstances might have been offset in this study by the high incidence of breast feeding which provided a satisfactory folate intake.

Similarly, a relationship between nutrition and biochemical folate deficiency could not be established. It could be inferred, from the findings of Vanier and Tyas (1966), that a diet which provided sufficient calories might be deficient in folate. Conversely, from the findings in this study, it became evident that a calorie restricted diet, such as a limited supply of breast milk, might still contain enough folate to maintain normal serum L.casei folate levels. This was seen in under-nourished infants receiving breast milk without other milk supplements. For this reason it might not be possible to demonstrate a statistical relationship even though folate deficiency was dietary. Matoth, Zamir, Bar-Shani and Grossowicz (1964) demonstrated a relationship between whole blood L.casei folate levels and undernutrition in infants who, for the most part, received reconstituted formula milk.

It was generally recognised that increased demand for folate from infection (Roberts, Hoffbrand and Mollin, 1966; Hoffbrand, Stewart,

Booth and Mollin, 1968), or increased haemopoiesis (Lindenbaum and Klipstein, 1963; Alperin, 1967; Forshaw, Moorhouse and Harwood, 1964; Kremenchuzky and Hoffbrand, 1965) could result in folate deficiency. Recently, the increased demand for folate associated with increased haemopoiesis (Izak, Rachmilewitz, Chirasiri and Grossowicz, 1966) or DNA synthesis (Leevy, 1967) was demonstrated experimentally . It was not possible to demonstrate a statistical relationship between biochemical folate deficiency and increased demand for folate resulting from rapid growth or recurrent infection. This did not negate such a relationship because dietary folate intake was variable in the group of infants studied and some infants might have compensated for the increased demand for folate by increased dietary intake where this was available.

It was shown, that in the presence of iron deficiency serum L.casei folate levels might be low in the absence of supporting evidence for folate deficiency (Hansen, 1967). It was believed that iron deficiency might lead to folate deficiency (Chanarin, Rothman and Berry, 1965) possibly by interfering with folate metabolism (Vitale, Streiff and Hellerstein, 1965). In this study, no statistical relationship between serum iron and serum L.casei folate levels was found.

In view of all the factors which might have invalidated a statistical investigation, it was deemed necessary to examine aetiological possibilities in the individual infants with biochemical folate deficiency.

In the 18 infants with low serum L.casei folate levels and in the 2 infants with low red cell L.casei folate levels, one consistent aetiological factor was noted: none of these infants received breast milk without other milk supplements at the time biochemical folate deficiency

was diagnosed. Since the concentration of folate in reconstituted powdered milk was lower than that in breast milk, it seemed probable that dietary deficiency was either primarily responsible for or contributed to the development of biochemical folate deficiency in these infants. Also in 2 infants, there was a clear history of deprivation of food and therefore folate. Furthermore, in some of these infants there was circumstantial evidence for dietary folate deficiency based on

a) an association with under nutrition (in infants who did not receive breast milk without other milk supplements), or

b) increasingly poor nutrition in association with developing biochemical folate deficiency.

The concept of a dietary cause for folate deficiency was recently emphasised (Forshaw, Moorhouse and Harwood, 1964; Gough, Read, McCarthy and Waters, 1963; Read, Gough, Pardoe and Nicholas, 1965; Hurdle and Picton Williams, 1966). However, the frequency of its occurrence was uncertain (Girdwood, 1964; Girdwood, Thomson and Williams, 1967). It had previously been found that dietary folate deficiency could result in the production of megaloblastosis in man under experimental conditions (Herbert, 1962a),but a recent experiment did not confirm this (Velez, Restrepo, Vitale and Hellerstein, 1966).

Folic acid and d-xylose absorption tests were used as parameters for assessing absorption in infants with biochemical folate deficiency. Of these, the folic acid absorption test was believed to be the better test, especially when xylose absorbed was measured as percentage urinary excretion rather than blood-xylose (Chanarin and Bennett, 1962c). They achieved their results using S.faecalis as the folate assay organism.

Presumably the same conclusion would apply if L.casei were used as the assay organism. As the folic acid absorption test necessitated the administration of a therapeutic dose of folic acid, it could not be used as a test of absorption in the prospective study.

Absorption studies were performed on 7 infants with low serum L.casei folate levels. Folic acid absorption was within "normal limits" in two infants even though the tests were performed in the convalescent phase of gastroenteritis (Table 33). Xylose excretion was normal in 4 of 5 infants. In the 5th infant xylose excretion was abnormal in association with chronic gastroenteritis. Cure of the gastroenteritis resulted in the return to normality of the xylose absorption test. It seemed probable that temporary malabsorption of folate might have contributed to biochemical folate deficiency in this infant (F.U.) It was inferred that this was also the case in 2 other infants, T. de B. and C.F., who had chronic gastroenteritis at the time of diagnosis of biochemical folate deficiency. Only in these 3 infants was there evidence for attributing the development of biochemical folate deficiency to malabsorption of folate.

In the absence of chronic gastroenteritis there was no relationship between malabsorption and the development of biochemical folate deficiency in healthy underprivileged infants. However, this could not be entirely excluded by the evidence in this study because the absorption of folate under physiological conditions was not investigated. Until more is known of the nature of folate absorbed under physiological conditions, absorption studies performed with food folate (Markkanen, 1968) might provide more accurate information than those performed with synthetic PGA.

It seemed probable that recurrent infection contributed to the development of biochemical folate deficiency in 2 infants (M.R. and S.B.) because their infection score was much higher than the mean for infants of a similar age.

The study of aetiological factors in individual infants did not provide any convincing evidence for attributing biochemical folate deficiency to the effects of maternal folate deficiency or iron deficiency.

Dietary folate deficiency apart, no other cause was evident for the low red cell L.casei folate levels in C.S. and V.L.

Thus in all the infants with biochemical folate deficiency, dietary deficiency was the only consistent aetiological factor. It appeared to be primarily responsible for the folate deficiency in approximately 13 of the 18 infants with low serum L.casei folate levels. In three infants, biochemical folate deficiency might have resulted from a combination of dietary folate deficiency and malabsorption. In the other two infants, dietary folate deficiency, recurrent infection and, perhaps, malabsorption from recurrent gastrointestinal infection might have contributed to the development of folate deficiency.

AETIOLOGY OF FOLATE DEFICIENCY IN THE INFANT WITH MEGALOBLASTOSIS

One (1) of the 18 infants with biochemical folate deficiency also had morphological evidence of folate deficiency. In this infant (M.R.) the biochemical and morphological evidence of folate deficiency developed

a) after a period of severe deprivation of virtually all food and therefore folate, and

b) in association with the effect of recurrent infection, at least half of which was gastrointestinal.

The folate deficiency developed in parallel with clinical evidence of marasmus which was objectively confirmed (less than 60% of the expected body weight) shortly after the megaloblastosis had been diagnosed.

SIGNIFICANCE OF BIOCHEMICAL FOLATE DEFICIENCY

An attempt was made to assess the importance of biochemical folate deficiency in these infants by studying its haematological and clinical effects.

Fourteen (14) of the 18 infants with biochemical folate deficiency were investigated for evidence of depleted haematological folate stores. One (1) of 6 infants had a low red cell L.casei folate level and in 1 of the other 8 infants the bone marrow was megaloblastic. As it was uncertain whether the low red cell L.casei folate levels could be used as concrete evidence of depleted red cell L.casei folate stores, it was concluded that in only 1 of 14 infants was there unequivocal evidence for significant haematological folate depletion. Moreover, there was no relationship between biochemical folate deficiency and haemoglobin concentration.

Biochemical folate deficiency apparently did not have any adverse clinical effects. The administration of folic acid to infants with biochemical folate deficiency did not produce symptomatic benefit or detectable improvement of haemoglobin concentration or weight percentile. There was a clinical impression which could not be further assessed, that the administration of folic acid to these infants did not produce any effect on resistance to infection. Conversely, when folic acid was not administered to infants with biochemical folate deficiency, there was no adverse effect on haemoglobin concentration, weight percentile or apparently on resistance to infection.

Biochemical folate deficiency could not be statistically related to:

a) haemoglobin concentration, and

b) weight percentile.

However, the infection score was always highest in infants with low serum L.casei folate levels and the statistical association was significant in some age groups. This might have been due to a lowered resistance to infection caused by biochemical folate deficiency but the evidence from the therapeutic studies with folic acid did not support this. The other possible explanation for the association was that infection contributed to low serum folate levels in some infants.

A relationship between biochemical folate deficiency and haemoglobin concentration was not demonstrated by Shojania and Gross (1964a), Vanier and Tyas (1967) and Matoth, Zamir, Bar-Shani and Grossowicz (1964). Shojania and Gross (1964a) could not demonstrate any clinical effect of biochemical folate deficiency in premature infants but Matoth, Zamir, Bar-Shani and Grossowicz (1964) were impressed by the symptomatic benefit of folic acid therapy in infants with biochemical folate deficiency. Some of these infants might have had associated morphological folate deficiency.

The effect of folic acid therapy on the infant with megaloblastosis was more difficult to assess because improvement could not be dissociated from a coincidental improvement caused by better feeding and treatment of infection. Considerable improvement of the megaloblastosis was associated with the administration of folic acid.

PROPHYLAXIS

The incidence of biochemical folate deficiency in healthy underprivileged infants (11.6%) followed prospectively, was not high. It might conceivably have been higher had the study been more fully representative and the same sample studied at all times.

The condition had little demonstrable clinical and haematological importance and in this study did not lead to the subsequent development of megaloblastic anaemia. However, under adverse circumstances megaloblastic anaemia would be more likely to develop in such infants than in those with normal folate nutrition.

It would seem that, in South Africa, healthy underprivileged infants with or without biochemical folate deficiency are particularly likely to develop megaloblastic anaemia under two common sets of circumstances:

a) chronic gastroenteritis; a relationship between biochemical folate deficiency and chronic gastroenteritis was shown in this study. However, because gastroenteritis was promptly treated it was not possible to demonstrate its relationship to megaloblastosis.

b) Underfeeding and incorrect feeding: one infant from very poor socio-economic circumstances, developed megaloblastosis in parallel with the development of marasmus. Both probably occurred for the same reasons - deprivation and the effect of associated infection.

Chronic gastroenteritis, marasmus and kwashiorkor are still important medico-socio-economic problems in South Africa. The association of megaloblastic anaemia with kwashiorkor and marasmus has been frequently reported in South Africa (Adams, 1964; Walt, Holman and Naidoo, 1957; Shnier and Metz, 1959; Metz, Brandt and Stevens, 1962).

Until malnutrition and gastroenteritis are no longer public health problems in South Africa, it would seem reasonable to suggest that all healthy underprivileged infants receive milk containing adequate amounts of folate. This could be achieved by:

a) encouraging breast feeding;
b) prevention of overboiling and overdilution of powdered and cow's milk feeds and;
c) the addition of folic acid to formula milks.

This was shown to be effective both in vitro (Ghitis, 1967) and in vivo, as was inferred from the effect of a folic acid supplemented proprietary food, in a study by Vanier and Tyas (1966). A similar but less acceptable effect might be achieved by adding ascorbic acid in sufficient concentration to the milk before processing. In infants from poor socio-economic circumstances it would be of greater practical benefit to suggest the addition of folic acid to milk rather than to vitamin syrups. It is believed that the addition of folic acid to milk could be achieved without adding to the cost of preparation for the consumer (Hager, 1968).

If the above suggestions were realized, the folate stores of underprivileged infants might be maintained sufficiently to prevent the development of megaloblastic anaemia in those infants who are likely to develop chronic gastroenteritis, kwashiorkor and marasmus but who are not completely deprived of milk. Simultaneously the unnecessary condition of biochemical folate deficiency in healthy underprivileged infants would be eliminated.

SUMMARY AND CONCLUSIONS

The serum L.casei folate assay appeared to be a satisfactory and the most sensitive criterion of biochemical folate deficiency. Latent folate deficiency was diagnosed using this parameter in 11.6% of infants investigated prospectively and 7.1% of infants investigated on one occasion. Conclusions regarding aetiology, significance and need for prophylaxis of latent folate deficiency appeared to be as follows:

1) Infants who received breast milk without other milk supplements had significantly higher mean serum L.casei folate levels than those who received formula or a combination of milk feeds.

2) It was found that the folate content of breast milk was significantly higher than that of formula milk.

3) Healthy, underprivileged infants who received breast milk and no other milk supplements, received enough folate for normal growth, development and overcoming recurrent trivial infections.

4) The lower folate content of formula milk feeds probably contributed to the development of biochemical folate deficiency in a small

percentage of healthy underprivileged infants who received either formula milks or a combination of milk feeds.

5) Other factors which appeared to contribute to the development of biochemical deficiency in some infants were

a) deprivation of food;

b) malabsorption;

c) recurrent infection.

The relationship of maternal folate deficiency and iron deficiency in the infants to the development of biochemical folate deficiency in infants could not be determined in this study.

6) The condition of biochemical folate deficiency was not associated with unequivocal clinical or haematological effects and did not progress to the development of significant folate deficiency.

7) One (1) infant from very adverse socio-economic circumstances developed biochemical and morphological folate deficiency in association with the development of marasmus. This was certainly the result of deprivation to which the effects of infection might have been added.

8) Because many infants in underprivileged population groups in South Africa are likely to develop chronic gastroenteritis, kwashiorkor or marasmus, all of which could be associated with the development of megaloblastosis, it is suggested that attempts be made to prevent biochemical folate deficiency.

9) Prevention of biochemical deficiency could be achieved by:

a) encouraging breast feeding;

b) discouraging the overboiling and overdilution of milks, and

c) the addition of small quantities of folic acid to artificially prepared formula milks, especially those with low folate content.

CHAPTER X

RÉSUMÉ

This study was undertaken because it was believed that there might be a high incidence of biochemical folate deficiency in healthy underprivileged infants in Cape Town. This belief was based on the results of a preliminary study in which the Figlu test after oral histidine was used. Although it became increasingly evident that the Figlu test after oral histidine was not a specific test of folate deficiency, the concept of biochemical folate deficiency in healthy underprivileged infants was encouraged by other theories held at that time. These were that:

a) milk (the major dietary constituent of the infants) had a low folate concentration (Zuelzer and Rutzky, 1953; Luhby, 1959);

b) a condition of biochemical folate deficiency existed in infants under a year who were used as controls. They were healthy infants but were suffering or convalescing from mild infections (Dormandy, Waters and Mollin, 1963);

c) low whole blood L.casei folate assays indicative of biochemical folate deficiency were demonstrated in healthy infants and related to poor socio-economic circumstances (Kende, Ramot and Grossowicz, 1963).

Because it was believed that the state of biochemical folate deficiency might render the infants liable to the development of megaloblastic anaemia, it was decided to investigate the following:

a) the incidence, aetiology, significance and need for prophylaxis of biochemical (latent) folate deficiency in healthy underprivileged infants;

Moreover, because there was uncertainty regarding the specificity and application of the Figlu test after oral histidine as a diagnostic parameter of biochemical folate deficieney in infancy:

b) The validity of this test as a parameter of biochemical folate deficiency was investigated in healthy underprivileged infants.

Two groups of infants were investigated for biochemical folate deficiency. The first group of infants was studied prospectively at four intervals up to the 16th month of life. Their mothers had been previously investigated for folate deficieney in late pregnancy. The second group of infants were investigated on only one occasion.

Serum and red cell L.casei folate assays, folic acid clearance tests and the Figlu test after oral histidine were used to diagnose biochemical folate deficiency. Hypersegmentation of the neutrophils and bone marrow morphology were the criteria used to establish the presence of morphological folate deficiency. The clinical effects of folate deficiency were assessed in relation to haemoglobin concentration, growth and infection. The conclusions drawn from the investigation were as follows:

<u>The Figlu test after oral histidine</u>.

By means of sensitive methods for measuring Figlu and using the recommended dose of oral histidine, it was found that a considerable number of infants under 7½ months had positive Figlu tests using an accepted criterion of normality for concentration of Figlu excretion. These results could not be attributed either to biochemical folate deficiency or to a disturbance of folate metabolism.

The results of this study confirmed that healthy infants under 7½ months without folate deficiency could excrete total quantities of Figlu comparable to that found in adults. Although it is known that the 24 hour urine volume of infants under a year of age is considerably

smaller than that of the average adult, the accepted criterion of normality for concentration of Figlu excretion is the same for both infants and adults. Accordingly if infants excrete the same total quantity of Figlu as adults do, but in smaller urine volumes, it follows that the normal concentration of Figlu excreted by infants must be greater than that in adults. From this it is clear that the criterion of normal concentration of Figlu excretion should not be the same in infants and adults and must be determined for infants before the Figlu test after oral histidine (µg./ml.) can be used as a valid diagnostic parameter of folate deficiency. The high incidence of "positive Figlu tests" (µg./ml.) after oral histidine, obtained in this study from infants who were not folate deficient, has been accepted as evidence of this belief.

The total 24 hour Figlu excretion (mg./24 hours) after oral histidine, found in the infants in this study, appeared to be within normal limits and therefore unequivocal biochemical folate deficiency was not found by means of this test.

Figlu excretion declined with growth; actual Figlu output within the range of normality might have depended on the maturity of glutamate formiminotransferase function. This suggested that the standards of normal Figlu excretion (µg./ml. and mg./24 hours) might depend on infant age. Accordingly this should be considered when the Figlu test after oral histidine is used in the diagnosis of megaloblastic anaemia in infants, particularly those under 16 months.

Hypersegmentation of the neutrophils.

Morphological folate deficiency could not be demonstrated using this parameter. It was found that this parameter could be used with

accuracy and reproducibility only when nuclear separation was defined according to criteria used by Briggs (1914). Using this criterion it was found that up to 4% neutrophils could be present without there being evidence of folate deficiency.

Folate microbiological assays.

The serum L.casei folate assay was found to be the most sensitive index of biochemical deficiency in this study. Low levels were attributed to this deficiency. However, it was uncertain whether low red cell L.casei folate levels found in 2 of 89 infants could be used as evidence of biochemical folate deficiency. This deficiency was not evident from the results of the L.casei folic acid clearance tests, but definitive conclusions could not be drawn as normal values for infants could not be established.

Incidence of latent folate deficiency.

The incidence of this deficiency was determined from the results of the serum L.casei folate assays. It was approximately 11.6% in infants followed prospectively and 7.1% in infants under 7½ months investigated on one occasion.

The aetiology of latent folate deficiency.

The folate content of breast milk was found to be generally higher than that of reconstituted powdered milks. This confirmed the findings of certain investigators. It appeared that the lower folate content of most powdered milks could be attributed to the loss of folate during processing.

The higher folate content of breast milk appeared to account for the significantly higher mean serum L.casei folate levels in infants who received breast milk without other milk supplements. This also confirmed a finding established by other investigators in the interim. The folate

content of breast milk appeared to be adequate for the normal growth and development of healthy underprivileged infants because none of those who received breast milk without other milk supplements developed biochemical folate deficiency. On the other hand the folate present in reconstituted powdered milk and boiled cow's milk might not always have been sufficient for infants in similar circumstances, because a small percentage of those who did not receive breast milk as the sole source of milk, developed biochemical folate deficiency. This was in accordance with the very low folate content found in some of the prepared milks in which the folate content might have been further reduced by overdilution and boiling. Because of the apparently adequate folate content of most milk and especially of breast milk, and the high incidence of breast-feeding among the underprivileged, the incidence of biochemical folate deficiency might have been lower than was anticipated.

In this study, other factors which apparently contributed to the development of biochemical folate deficiency were malabsorption from associated gastroenteritis and recurrent infection. The effect of maternal folate nutrition on the subsequent folate nutrition of their infants could not be established in this study. In an infant with biochemical and morphological folate deficiency, deprivation and probably infection appeared responsible for the condition.

<u>The significance of latent folate deficiency</u>.

The condition of biochemical folate deficiency was not associated with:

a) significant haematological folate deficiency in the majority of cases;
b) detectable adverse clinical effects.

Although, in this study the condition of biochemical folate deficiency did not appear significant in itself, infants with this condition are probably more prone to the development of megaloblastic anaemia than those with normal folate nutrition.

Significant folate deficiency.

However, one infant developed megaloblastosis and therefore significant folate deficiency. In this infant who came from very poor socio-economic circumstances, the diagnoses of marasmus, and biochemical and morphological folate deficiency were made simultaneously. Both the folate deficiency and the marasmus appeared to result from the effects of deprivation and probably also the effect of recurrent infections. Thus it might be that significant folate deficiency develops in kwashiorkor and marasmus because the conditions have a similar aetiology.

Prophylaxis of latent folate deficiency.

The addition of folic acid to milk and improved feeding practices are recommended as prophylactic measures against the development of biochemical and overt folate deficiency in underprivileged infants because megaloblastosis is still an important problem in South Africa. By these means folate stores in infants who are not totally deprived of milk might be sufficiently maintained to prevent the development of megaloblastic anaemia, despite the prevalence of malnutrition and stress factors (viz. infection and chronic gastroenteritis) in South Africa.

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