THE FOLATE STATUS OF PLASMA AND ERYTHROCYTES IN HEALTH AND DISEASE.

by

A. Omer, M.B. Ch.B. (Khartoum), M.R.C.P. (Edin.).

Thesis presented for the degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine.

August, 1967.

LIBR. UNIV. EDINBURGH

Statement in terms of Ph.D. Regulation 13 of the University of Edinburgh.

Some of the results described in Chapter II were presented as a communication before the Scottish Society for Experimental Medicine on the 3rd June, 1967.

CONTENTS.

GENERAL INTRODUCTION.

HISTORICAL REVIEW.

When reviewed in the light of modern knowledge, it becomes obvious in retrospect, that Wills made the first observation of folic acid deficiency in man.

Thus in (1931) she reported that a macrocytic anaemia observed in Hindu women in Bombay and often but not necessarily associated with pregnancy responded either to "Marmite", a concentrated extract of autolysed yeast or to a crude liver extract.

Later studies by the same author (Wills and Bilimora 1932, Wills 1933, 1934, Wills and Stewart 1935) showed that a similar megaloblastic anaemia could be produced in monkeys fed on a diet similar to that consumed by the affected Indian natives, and that the anaemia was also curable by yeast and liver extracts.

A few years later it was shown that Anahaemin, a more refined anti-pernicious anaemia liver extract, was without effect on nutritional anaemia either in man or the monkey and that it differed in this respect from Marmite or crude liver extract (Wills, Clutterbuck and Evans 1937 (a) and (b), Wills and Evans 1938). This was an observation of great importance and interest since it showed that Wills factor and the extrinsic factor (Castle 1926) were not identical.

It has been shown since that the folic acid content of Marmite is about 60ug/g. (Welch and Heinle 1951, Kodicik and/

and Carpenter 1948) which means that Wills was giving her patients from 0.5 - 1mg. of folic acid daily, an amount well in excess of the dose necessary for a therapeutic response in folic acid deficiency (Hansen and Weinfield 1962). Microbiological assays of crude liver extracts gave a folic acid content ranging between 0.7 - 4.5ug/ml. (Girdwood 1954). The presence of "folic acid substances" in Marmite and crude liver extract, and a report appearing later from India describing the remission of macrocytic anaemia of pregnancy following treatment with synthetic pteroylglutamate acid under conditions relating to earlier observations (Benjamin 1946) lend strong support to the concept that Wills factor was folic acid.

ISOLATION, SYNTHESIS AND NOMENCLATURE OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS.

Bacterial requirements for "folic acid":-

Rapid progress in the characterisation of "folic acid substances" came with the use in assays of micro-organisms unable to synthesise folic acid for their own needs. Snell and Patterson (1940) pioneered the field by showing that *Lactobacillus casei* required a growth factor present in liver and yeast, which could be adsorbed and eluted from charcoal. It was referred to as the norite eluate factor and the liver *Lactobacillus casei* factor (Stockstad 1943). The term folic acid (Latin folium = a leaf) was introduced by Mitchell et al. (1941) to denote a material/

material extracted from spinach and the green leaves of many plants which was also present in yeast, kidney and liver and which supported the growth of Streptococcus lactis R.

Yet another name, vitamin Bc, was given to a factor in liver extract potent against deficiency anaemia in chicks (Hogan et al. 1940) and isolated later in a crystalline form and shown to support the growth of Lactobacillus casei (Pfiffner et al. 1943). In 1943 Keresztesy and co-workers reported the isolation of a factor active for S. lactis R. "now known as S. faecalis", but relatively inactive for Lactobacillus casei.

The issue was complicated further by the discovery that yeast concentrates had a potent curative effect in anaemic chicks but little microbiological activity for either L. casei or S. faecalis unless previously treated enzymatically (Binkley et al. 1944, Pfiffner et al. 1945, Mims et al. 1944). The terms Bc conjugate and Bc conjugase were used to denote these substrate and enzyme systems respectively (Bird et al. 1945).

Names that were now being used included the norite eluate factor, L. casei factor, folic acid, S.L.R. factor, vitamin Bc, vitamin M. (Day et al. 1938), vitamin B10 and B11 (Briggs et al. 1943) and vitamin Bc conjugate.

The possibility that these factors were the same substance was suggested (Mallroy et al. 1944, Briggs et al. 1945) and it was subsequently found that they could be replaced in bacterial nutrition by pteroylglutamic acid or related

substances/

substances.

The chemical structure and synthesis of the L. casei liver factor was announced by Angier and co-workers in two successive publications in 1945-46 (Angier et al. 1945 and 1946). They showed its molecular configuration to be a pteridine grouping linked through p-aminobenzoic acid to a single glutamic acid residue and suggested the name "pteroyl-glutamic acid". Soon the chemical characterisation of more related compounds followed in quick succession. These included:-

1. Pteroyltriglutamic acid (Angier et al. 1946, Lampen and Jones 1946) (L. casei fermentation factor) a three glutamic acid radicle conjugate active for L. casei but has no activity for S. faecalis R.
2. Pteroylheptaglutamic acid (pteroylhexa-glutamyl glutamic acid (Pfiffner 1946) Vitamin Bc conjugate) was isolated from yeast. It has no activity for either L. casei or S. faecalis R. However an enzyme present in many animal tissues will liberate assayable monoglutamates from it (Juke and Stockstad 1948).
3. Pteroic acid. This was synthesised by omitting the glutamic acid grouping from PGA molecule; it is exclusively active for S. faecalis R.
4. Rhizopterin (10 formylpteroic acid, S.L.R. factor) was isolated from the fermentation of Rhizopus nigricans and is active for S. faecalis R.and inactive for both L. casei and animals.

5. Folinic acid (Citrovorum Factor) (CF).

In (1948) Sauberlich and Baumann made the observation that unless a liver "Factor" was added, Leuconostoc citrovorum (pediococcus cerevisiae) failed to grow on a synthetic medium. The synthesis of a substance with similar properties and able to reverse the toxicity of 4 amino-pteroylglutamic acid in mice, was announced in (1950) by Brockman and coworkers. In the same year Nichol et al. demonstrated the ability of liver slices in vitro to convert PGA to citrovorum factor (Nichol and Welch 1950). They showed that the reaction was augmented by ascorbic acid and gluco-ascorbic acid. Later work suggested that citrovorum factor, a relatively stable compound, is a non-enzymatic degradation product of a highly labile metabolically active folate compound (Nichol et al. 1955).

THE DEVELOPMENT OF MICROBIOLOGICAL TECHNIQUES FOR FOLIC ACID ESTIMATION.

1. The Assay Media:-

Most microbiological procedures for folic acid assay are based on the method described by Teply and Elvehjem (1945) for S. faecalis and L. casei. These workers used a titemetric method, for L. casei, utilising acid hydrolysed casein in the basal medium and requiring a 30-72 hr. incubation period. Robert and Snell (1946) substituted enzymatically digested casein for the acid hydrolysate and were able to reduce the incubation period to 16 hours. This/

This was followed by various minor modifications until the question was to a large extent settled by the recent advent of satisfactory commercial media for carrying out assay with the three micro-organisms.

2. The extraction of folates from biological material:-

The demonstration by Toennies et al. (1953) that folate activity in biological material is destroyed by heat but is fully preserved when autoclaved in the presence of ascorbic acid (Toennies and Usdin 1956) marked a major advance in the area of microbiological folic acid estimation, a procedure which involves autoclaving at least once. It has provided investigators with a simple method for obtaining protein free folate extracts from biological fluids and tissues. Since then ascorbic acid concentrations varying from .05 to 0.3g.% in phosphate buffer have been used by various workers to prepare serum or blood extracts (Luhby and Coopermann 1965).

3. The folate content of serum and whole blood as diagnostic aids:-

Attempts by earlier workers to measure the basal folate content of human serum or blood were uniformly unsuccessful in demonstrating a significant difference that would distinguish normality from deficiency. This is understandable in part since most workers used S. faecalis (Schweigert et al 1947, Nieweg et al. 1954, Chanarin et al. 1957 and 1958a, Cox et al. 1960), a micro-organism shown later not to respond to the major component of serum or blood folates. Inadequate protection/

protection of labile folate must have been the major cause of low levels obtained in L. casei assays (Schweigert et al. 1948, Simpson et al. 1949, Spray 1952 (a) and (b)). These results led some workers (Chanarin 1958, Luhby 1959) to doubt the validity of folate estimations as diagnostic tools. Indeed some doubted the existence of assayable folates in the plasma at all (Chanarin 1958).

However, in 1959 Baker et al. published a L. casei assay method, by which they found that folate deficient patients had significantly lower serum levels than normal subjects or patients with uncomplicated Vitamin B_{12} deficiency. Baker's method paved the way for further studies in the microbiological activity of human serum. Thus Herbert (1961) found that 150mg.% ascorbic acid in the phosphate buffer afforded a better protection for the labile folates, and that the folate content of plasma is the same as that of serum. Waters and Mollin (1961) showed a clear cut difference between folate levels in deficient and normal subjects and demonstrated the protective effect of ascorbic acid on stored serum.

Provided the assay is done under standard conditions, it has since been possible to obtain reasonably reproducible results. Causes of unsatisfactory results in microbiological assays are adequately tabulated by Girdwood (1963).

However, results obtained in different laboratories employing the L. casei assay vary widely. This is illustrated/

illustrated below where the normal ranges of serum folate in eight published series are shown.

Serum folate uug/ml. Normal range.	Reference.
2.1 - 28.0	Spray (1964).
2.1 - 9.5	Temperly et al. (1966).
2.3 - 18.6	Deller et al. (1966).
2.7 - 18.3	Davies and Kelly (1962).
3.4 - 11.6	Ball and Giles (1964).
4.6 - 18.4	Kershaw and Girdwood (1964).
5.9 - 21.0	Waters and Mollin (1961).
7.0 - 15.9	Herbert (1966).

The normal range of serum folate in eight published series.

THE CHEMICAL NATURE OF THE SERUM FOLATE COMPOUND(S).

Herbert's observation that human serum contains folate activity, that supports the growth of L. casei but not S. faecalis or L. citrovorum, led him to believe that the serum folate material must be pteroyl-triglutamate (Herbert 1961); a conclusion supported by previous observations on the growth response of the three micro-organisms/

organisms to this folate compound (Usdin 1949, Stockstad 1954, Rabinowitz 1960). However, Larrabee et al. (1961 (a) and (b).) isolated a monoglutamate (N^5-methyltetrahydrofolic acid) as an intermediate product of methionine biosynthesis and Keresztesy et al. (1961) and Donaldson et al. (1961) isolated a compound with similar properties from horse liver. Both compounds produced the same effect on the growth of the three micro-organisms as the serum folate derivative. Working on these lines, Herbert and co-workers by applying chromatography and conjugase treatment to serum was able to prove that the serum folate compound is N^5-methyltetrahydrofolic acid monoglutamate (Herbert et al. 1962a.).

THE FOLATE COMPOUNDS OF WHOLE BLOOD AND THEIR RELEASING FACTORS.

In 1949 Simpson and Schweigert showed that incubation at 37°C. and within a pH optimum of 6-8 markedly increased the folate content of blood haemolysates obtained from different species including man. They attributed this effect to the presence in blood of "conjugases" which they defined as "enzymes that liberate microbiologically available folic acid from more complex forms of the vitamin". They also demonstrated the effectiveness of "blood conjugases" in releasing folic acid from pteroylheptaglutamate. The folate released, either from whole blood alone or from whole blood with added pteroylheptaglutamate, gave considerably higher values when assayed with L. casei than when measured with/

with S. faecalis R. They condemned the use of takadiastase as an exogenous conjugase because they pointed out that "blood conjugases" are capable of releasing variable amounts of folic acid from conjugates in this substance.

In 1953 Toennies, Frank and Gallant, using dialysis rather than heat to separate the folic acid released in blood haemolysates, found that dialysates obtained at low temperatures and against .05 M phosphate or acetate buffer of pH 5.7 - 6.0 gave L. casei levels which were 20 to 100 times greater than previously reported values.

Later the same group of workers showed that washed red blood cells and plasma when assayed separately under conditions which yield high folic acid activity for whole blood, are nearly inactive (Toennies et al. 1956). From this they formulated the concept that the released folic acid in blood haemolysates is the result of interaction of an inactive red blood cell folate precursor and an enzymatic plasma factor(s). In support of their postulated substrate-enzyme system they pointed out that:-

(a) It is the red blood cell content of the folate precursor rather than the plasma factor that determines the folate yield, and that under certain conditions the precursor gives rise to small amounts of assayable folates without the action of plasma.

(b) In accord with its protein nature, the plasma factor is damageable by heat and is present in the highly purified plasma protein Cohn fractions IV and V.

A./

A. THE ERYTHROCYTE FOLATE PRECURSOR SUBSTANCE (FPS).

1. General Properties:-

In 1964 Iwai et al. speculated on the possible protein nature of the erythrocyte folate precursor substance (FPS) and the similarity of its molecular size to haemoglobin on the basis of:-

(a) When haemolysates, prepared from washed erythrocytes, were applied to a Sephadex column, FPS and haemoglobin moved through the column at the same rate indicating that they were of similar molecular size.

(b) 100% precipitation of FPS occurred at 80% saturation of haemolysates with ammonium sulphate. Further, when haemolysates were dialysed, all the potential folate activity remained in the impermeable matter. Both of these findings lend support to the proposed protein nature of FPS. Furthermore, when a solution of a crude lyophylised preparation of FPS was applied to a DEAE column and the effluent analysed for its protein and folate content, the fractions were found to have a protein bound folate content of approximately 1%.

The predominant L. casei activity in these fractions suggested to the workers the possible presence of N^5-methyltetrahydrofolic acid, an observation to be confirmed later (Noronha and Aboobaker 1963).

2. Chemical Nature:-

Usdin and co-workers (1956) analysed a concentrated preparation of the erythrocyte folate precursors by paper chromatographic and tetrazolium bioautographic techniques.

A/

A complex situation was found in which none of the isolated predominantly L. casei active fraction was identical with any of the chemically well defined folate compounds at the time, namely PGA, C.F. and pteroyltriglutamic acid.

Later Usdin (1959) subjected the folate material to triethylamine ethyl cellulose column chromatography. Nine peaks of folate activity were obtained, all of which were identified by him as formylated folate compounds of various structure. This led him to consider the folate precursors as formylated polyglutamyl derivatives.

However, Noronha and Aboobaker (1963) by extracting blood folates under a rigorously controlled reducing environment, presented evidence that the erythrocyte folate precursors are conjugated forms of N^5-methyltetrahydrofolic acid which on enzymatic degradation yield N^5-methyltetrahydrofolic acid monoglutamates. This conclusion was based on the almost exclusive activity of these extracts to L. casei (a known characteristic of the N^5 methyl derivatives of folic acid), and the similarity of their behaviour on DEAE-cellulose columns to the pattern shown to be characteristic of the synthetic counterparts (Silverman et al. 1961, Wittenberg et al. 1962). Usdin's finding of formylated compounds was attributed by the same workers to his failure to maintain a reducing atmosphere during extraction, a hazard that may cause oxidative alteration of the C-1 moiety (methyl to formyl).

The presence of N^5-methylmonoglutamate in serum (Herbert/

(Herbert et al. 1962a) and the polyglutamate forms in the red blood cells (Noronha et al. 1963) as well as the recent demonstration of this folate derivative in human liver (Chanarin et al. 1966) makes it the main circulating and storage folate form in the human body.

B. THE FOLATE RELEASING FACTORS.

1. Exogenous Enzymes:

(a) The chicken pancreas conjugase.

Laskowski, Mims and Day (1945) described the presence of an enzyme in chicken pancreas that can release S. faecalis active folates from their inactive precursors in yeast. The enzyme was shown later to act optimally in the presence of calcium ions, at a pH of 7.8 and a temperature of 32°C. (Mims et al. 1945). Based on the observation of Pfiffner and co-workers (1946) that the enzyme must be a carboxy-peptidase, Mims et al. (1948) classified it as γ-glutamic acid carboxy-peptidase by showing its specificity for hydrolysing terminal glutamic acid which has both of its carboxyl groups free and which is linked through its amino groups to the γ-carboxyl group of the preceding glutamic acid.

(b) Hog kidney conjugase.

This is another folate releasing enzyme differing from the previous one by not being affected by calcium ions and by having a pH optimum of 4.5 (Toennies et al. (1959).

Both conjugases have been used by a number of workers to obtain assayable folate from various microbiologically/

biologically inactive conjugates.

2. The Plasma Factor:

Toennies et al. (1959) investigated some of the properties of the plasma factor using a crude preparation of the latter obtained from Cohn fraction IV. Rapid loss of enzyme activity occurred on standing in dilute aqueous solution. The complete regeneration of activity by reagents like hydrogen sulphide, reduced glutathione and mercaptoethanol suggested the presence of easily oxidisible thiol groups that are vital for enzyme action. When a solution of the enzyme was fractionated on a DEAE column there was a marked variation of enzyme activity in the obtained fractions, a finding that may suggest the presence of more than one enzyme form in plasma.

To date one is not aware of any advance on Toennies' original observations concerning this enzyme and its identity remains obscure.

CAUSES OF FOLIC ACID DEFICIENCY IN MAN.

Conditions that may be complicated with folic acid deficiency are listed below. In the majority of these the exact aetiological factors leading to the deficiency are well recognised. In a small group, however, these remain to be established.

1. Malabsorptive disease:
   - (a) Idiopathic steatorrhoea. (Frequently gluten enteropathy.)
   - (b) Coeliac disease. (Usually gluten enteropathy).
   - (c) Tropical Sprue.
   - (d) Resection or organic disease of a large part of the small bowel.
2. Dietary deficiency.
3. Pregnancy.
4. Conditions associated with increased cellular turnover:
   - (a) Lympho-and myeloproliferative disorders.
   - (b) Haemolytic anaemias.
   - (c) Sideroblastic anaemias.
   - (d) Leukaemias and reticuloses.
   - (e) Carcinomatosis.
5. Complicating the administration of certain drugs:
   - (a) Folic acid antagonists.
   - (b) Anticonvulsants or barbiturates.
   - (c) Certain antimalarials.
6. Hepatic cirrhosis.
7. Rheumatoid arthritis.
8. Chronic infections.

THE DIAGNOSIS OF FOLIC ACID DEFICIENCY.

1. Exclusion of B_{12} deficiency:-

With the exception of a few rare conditions, the finding of a normal serum vitamin B_{12} in a patient presenting with megaloblastic erythropoiesis is virtually diagnostic of folic acid deficiency. The reverse is not true, however, since megaoblastic anaemia may result from combined folate and B_{12} deficiency and also because some folate deficient subjects may have "marginal" B_{12} levels. The occurrence of such cases makes the assessment of the folate status of patients essential for reaching a confident diagnosis.

2. The Serum Folate:-

Despite the excessive variation in the serum folate values reported from different units, there is general agreement that megaloblastic folate deficient anaemias have subnormal serum values, whereas in vitamin B_{12} deficient anaemias there is a tendency for the active L. casei material to accumulate and therefore for the serum folate levels to be higher than controls. However, it is not uncommon to encounter B_{12} deficient subjects with marginal or subnormal folate levels.

Herbert (1962) showed that the serum folate is the first parameter to be effected during the development of experimental nutritional folate deficiency in man. Thus it dropped to abnormal levels three weeks after starting a folate deficient diet, whereas macrovalocytosis, reduction in the red blood cell folate content, a positive FIGLU and the/

the development of megaloblastic erythropoiesis took more than three months to manifest itself. Reduction in the serum folate level thus precedes the development of tissue depletion by some time and may therefore be regarded as a sensitive index of folate deficiency. This may account for the occurrence of the occasional non-anaemic case in which a low serum folate level is found in the absence of any biochemical evidence of folate deficiency. Even so, evaluation of such a situation can pose a difficult question as to whether such a case is an early "subclinical" folate deficiency or not. This will be discussed later in relation to rheumatoid arthritis in which such a situation may be encountered.

3. The red blood cell folate content:-

Reported series have shown that the red blood cell folate level may be low in either folate or B_{12} deficiency (Hansen and Weinfield 1962, Cooper and Lowenstein 1964, Hoffbrand et al. 1966a). On its own such a parameter appears to be useless in differentiating between the two deficiencies, a point which will be evaluated later. Combined with the serum folate and serum vitamin B_{12} estimations, it is claimed to be a good index of the folate status of body tissues (Hoffbrand et al. 1966a).

4. The Clearance Test:-

The value of the S. faecalis clearance test as an index of the tissue folate saturation has been extensively studied by many workers (Chanarin et al. 1958b, Metz et al. 1961/

1961, Hansen and Nystrom 1961, Grossowicz et al. 1962). It is well established that rapid clearance occurs not only in folate deficiency but also in some vitamin B_{12} deficient cases. However, of more recent interest is the comparison between the S. faecalis and L. casei clearances in vitamin B_{12} and folate deficiency. Herbert et al. (1962) and later Hogan et al. (1964) found a rapid S. faecalis clearance in both deficiencies. The L. casei clearance, however, was also rapid in folate deficiency but markedly delayed in vitamin B_{12} deficiency. This suggested to Herbert that vitamin B_{12} deficiency, the injected PGA (which is available for both organisms) is rapidly converted, perhaps in the liver, to a form which is only available for L. casei. The new folate derivative "piles up" in the serum probably because vitamin B_{12} is required for its utilisation. The test is certainly of academic interest in as far as the metabolic inter-relationships of the two vitamins is concerned. However, one sees no justification for its use as a diagnostic test for differentiating the two deficiencies when simpler and equally reliable tests are available.

5. Formiminoglutamic acid (FIGLU) excretion:-

Folate deficient patients excrete high amounts of FIGLU in the urine, when given loading doses of histidine. This has been used as a test of folate deficiency by many investigators (Broquist 1956, Luhby 1957, Tabor and Wyngarden 1958, Broquist et al. 1959, Knowles et al. 1960,

Kohn/

Kohn et al. 1961, Zalusky and Herbert 1962, Chanarin and Bennet 1962). FIGLU excretion, however, is not specific for folate deficiency and a positive result may occur in B_{12} deficiency, liver disease and other conditions. As pointed out by Kershaw and Girdwood (1964), a positive result is only of value if interpreted with other diagnostic parameters.

FIGLU can be determined electrophoretically, spectrophotometrically, by microbiological methods or by paper chromatography.

6. The Therapeutic Trial:-

The efficacy of this as a diagnostic test in folate deficiency is adequately discussed by Luhby and Cooperman (1965). They state that if done properly it is a very sensitive and specific test of folate deficiency but that certain conditions must be strictly observed, namely:-

(1) The folic acid dose must not exceed .05 - 0.25mg. daily, to avoid a non-specific response in vitamin B_{12} deficiency, conditioned by a "mass action".

(2) The patient must be put on a well controlled folate deficient diet.

(3) For a significant response to occur, the reticulocyte count must reach a peak between the 7th and 10th day of the trial followed by a significant rise in the red blood cell count and haemoglobin concentration.

It is obvious that such a test is only feasible in units where controlled metabolic studies can be conducted.

INTRODUCTION TO THE THESIS.

The present work is primarily concerned with the investigation of factors that may influence the release of assayable folate from erythrocytes and the evaluation of the folate status of plasma and erythrocytes in health and disease.

A description is given in Chapter I of the microbiological techniques used in analysing the various samples. The major part concerns the procedure employed for estimating the folate content of plasma and whole blood extracts.

Chapter II embodies a series of experiments designed to evaluate the effect of various factors on folate release from erythrocytes. In addition the collection, storage and preparation of whole blood samples prior to extraction is described.

The folate status of patients with rheumatoid arthritis is analysed in Chapter III and compared to that of normal subjects as well as that of patients with megaloblastic erythropoiesis and iron deficiency anaemia.

CHAPTER I.

MICROBIOLOGICAL METHODS.

MICROBIOLOGICAL METHODS.

A. Deionised water was used throughout.

See Appendix A (I).

B. The cleaning of glassware used for folate and vitamin B_{12} estimation is described in Appendix A (II).

C. STOCK CULTURES.

Micro-organisms:-

1. Lactobacillus casei (NCI 8010).
2. Streptococcus faecalis (ATCC 8123).
3. Euglena gracilis Z strain (Algae and protozoa collection, Cambridge).

a) Fluid Maintenance Media:

The preparation and storage of fluid maintenance media for the three micro-organisms is described in Appendix A (III).

b) Maintanenace of Stock Cultures:

i. L. casei.

ii. S. faecalis.

Both micro-organisms are maintained at 37°C. by subculture on alternate days from fluid media to bacto-microassay culture agar. An agar stab with a 24 hours old culture of either micro-organisms was also stored at 4°C. and changed every week. Cultures have been maintained by this method for long periods without evident mutation. The procedure is shown below.

	37°C.	4°C.
Day I	Fluid medium	
Day II	Agar stab (1)	Agar stab (2) changed weekly.
Day III	Fluid medium	
Day IV	Agar stab	
	continued on alternate days.	

iii. E. gracilis:

Subcultures of E. gracilis were made every 48 hours by adding 4 drops from a 4-5 days old culture to a bottle of maintenance medium. These cultures were maintained in a water bath (aquarium tank), the temperature of which is kept at 28-30°C. by a circotherm heater. The appropriate light intensity is provided by a double strip fluorescent lighting system (40w) placed under the transparent bottom of the tank.

c) PREPARATION OF ASSAY INNOCULAE:-

1. L. casei and S. faecalis:-

The micro-organisms were sub-cultured from agar to maintenance fluid media and grown at 37°C. for 24 hours.

The Lactobacillus casei culture was then transferred aseptically into a sterile universal container and centrifuged for ½ hour at 3000g. The supernatant was decanted and the growth/

growth residue washed three times in physiological saline by resuspension and centrigugation. Depending on the density of the final suspension, 0.5 to 0.8 ml. of this was added with a sterile one ml. pipette to a universal bottle containing 20 ml. of sterile physiological saline, to provide the required innoculum. Using a sterile Pasteur pipette (25 drops/ml.) one drop of the innoculum was delivered into each of the assay tubes.

Centrifugation was found unnecessary for washing S. faecalis. The liquid in the culture was decanted and the cells at the bottom of the tube resuspended in physiological saline. This was repeated three times and the 4th suspension used for innoculating the assay tubes with a Pasteur pipette, as described for L. casei.

2. E. Gracilis:-

A 4-5 days old culture was washed six times in physiological saline by resuspension and centrifugation and a drop of the 7th suspension delivered by a sterile Pasteur pipette into each assay tube.

THE LACTOBACILLUS CASEI ASSAY OF THE FOLATE CONTENT OF PLASMA AND WHOLE BLOOD EXTRACTS.

The folate content of plasma and whole blood extracts was measured by a slight modification of the method of Waters and Mollin (1961).

The principles of the method are:-

1. Basal Medium:-

Difco folic acid casei medium was rehydrated by adding 9.4g./100ml. of deionised water and boiling for 2-3 minutes.

2. The standard folic acid solutions:-

(a) The stock solution has a concentration of 4mg.% of folic acid and is prepared by dissolving 20mg. of crystalline pteroyl-glutamic acid (Lederle) in 500 ml. of water. 1 or 2 pellets of sodium hydroxide were usually added to help solution.

(b) The working solution, always freshly prepared, has a folic acid concentration of 1 mug/ml., achieved by making a 1/40000 (1/200 x 1/200) dilution of the stock solution in water. The dilutions were made in volumetric flasks and each flask was always used for the same folic acid concentration.

3. Preparations of samples for assay:-

(a) Plasma:

Heparinised plasma stored at -20°C. with 10mg. of dry ascorbic acid/ml., was thawed at room temperature and diluted 1:20 (in a universal bottle) with 0.1 M phosphate buffer containing/

containing 200mg.% ascorbic acid. The 1:20 plasma buffer solution was autoclaved for 2½ minutes at 15 p.s.i.* cooled, centrifuged and filtered. The protein-free extract was then diluted with an equal volume of water and assayed on the same day, two ml. being mixed with an equal volume of basal medium giving a final plasma dilution of 1:80.

A duplicate extract was prepared from each sample and assayed on a different day and the mean of at least two results agreeing within 10% taken to represent the folate content of the sample.

(b) Whole blood:

Whole blood haemolysates were prepared in different ways e.g. by varying the pH, ascorbic acid concentration, temperature etc. These methods are described in detail in Chapter II.

For extraction, however, and unless specified, haemolysates were mixed with 0.1 M phosphate ascorbate buffer to give a final blood dilution of 1:100 and a final ascorbic acid concentration of 200mg.%. When haemolysates contained differing amounts of ascorbic acid, the ascorbic acid content of the buffer was adjusted accordingly to give the desired 200mg.% in the final mixture.

The samples were autoclaved for 5 minutes at 15 p.s.i. to effect complete protein precipitation. The extracts, separated as for plasma, were stored at -20°C. until assayed at a final dilution of 1/600 to 1/800. Samples were always extracted/

* pounds per square inch.

TABLE (1).

		Innoculated tubes set up in duplicate.								
Tube No.	1	2	3	4	5	6	7	8	9	10
Basal medium ml./tube	2	2	2	2	2	2	2	2	2	2
Autoclaved ascorbate phosphate buffer ml./tube	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Folic acid mug/tube	0	0	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Water ml./tube	1.4	1.4	1.3	1.2	1.0	0.8	0.6	0.4	0.2	0
Final volume ml.	4	4	4	4	4	4	4	4	4	4

The standard tubes (L. Casei method).

Tube No. 1 = uninnoculated blank.

extracted and assayed independently at least twice, and the average of two results that agreed within 10% taken as the folate content of the blood sample.

20 ml. amounts of ascorbate phosphate buffer to be added later to standard tubes were autoclaved with each batch of plasma or whole blood samples.

The preparation of phosphate buffer is described in Appendix A (IV).

4. Assay procedure:

The assay was carried out in 6 x ¾" Pyrex test tubes. Four tubes were used for each sample. Two ml. of the basal medium were delivered with an automatic dispensor into each tube. Using a five ml. pipette, two ml. of the diluted plasma or whole blood extract were added to each of the four tubes which were then covered with oxoid caps and placed in order in wire racks.

A control "pooled normal" plasma was included in each assay batch.

The standard tubes:- Table (1).

The standard row included nine tubes set up in duplicate and containing 0 to 1.4 mug. of folic acid as well as one uninnoculated blank containing NO folic acid.

Autoclaved ascorbate phosphate buffer was found to stimulate L. casei growth and was therefore added to standard tubes (0.6 ml/tube).

The standard was placed with the sample tubes in the wire/

wire racks and the whole assay sterilised for 10 minutes at 10 p.s.i. and allowed to cool to room temperature.

INNOCULATION, INCUBATION AND CALCULATION OF THE RESULTS:-

Plasma and whole blood extracts are usually colourless. One tube selected at random from a sample was therefore set aside as a blank for each dilution of either extract. The remaining tubes were innoculated as described.

The assay was incubated at 37°C. for 16-18 hours and growth density measured with a Hilger Spekker using a green filter (Kodak No. 3·M/μ500).

Standard growth was plotted on arithmetic graph paper against the amount of folic acid per tube.

The optical density (O.D.) of the blank was subtracted from that of the sample tubes and the amount of folic acid per tube read from the standard curve. The average of each of the four tubes was then taken and multiplied by the dilution factor to obtain the sample's folate content in mug/ml.

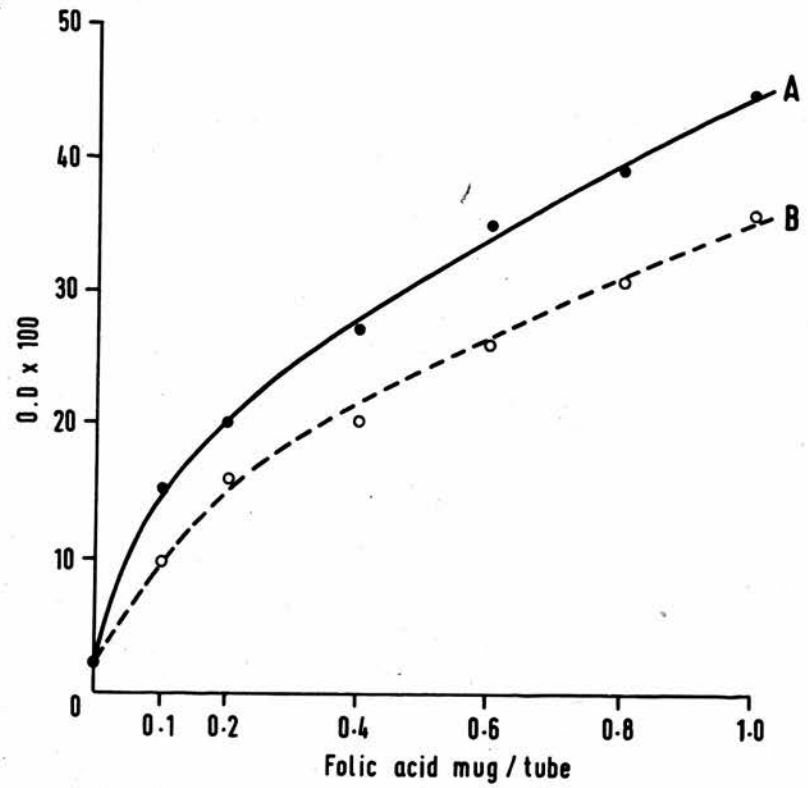
THE EFFECT OF AUTOCLAVED ASCORBATE PHOSPHATE BUFFER ON L. CASEI GROWTH.

Herbert (1961) showed that L. casei growth was stimulated by both ascorbic acid and phosphate buffer. Spray (1964) and Temperly et al. (1966) obtained similar results by adding ascorbic acid to the basal medium. Waters and Mollin (1961) on the other hand found no effect on growth when autoclaved ascorbate phosphate buffer was added to standard tubes.

The basal medium used in the present work contains no ascorbic acid. If L. casei growth is enhanced by ascorbate phosphate buffer, this must be added to standard tubes to avoid the occurrence of erroneously high folate results due to its presence in sample tubes. This was investigated as follows:-

Five standards containing 0, 0.3, 0.45, 0.6 and 0.9 ml. respectively of autoclaved ascorbate phosphate buffer per tube were prepared on the same day. Each was set up as in Table 1 except that the folic acid content ranged from 0 to 1mug. per tube and, depending on the amount of buffer added, the quantities of water were changed accordingly to give final volumes of 4 ml. per tube. The buffer quantities were selected to cover those calculated to be present in the final assay dilutions usually employed for plasma or whole blood extracts (Table 2).

FIGURE 1.



A - 0·6 ml of autoclaved ascorbate phosphate buffer / tube.
B - No ascorbate phosphate buffer.

The effect of autoclaved ascorbate phosphate buffer on the growth of L. casei in standard tubes.

TABLE (2).

Calculated buffer quantity ml./assay tube.	Final dilution of sample.	
	Plasma	Blood
1.8	1:40,	1:200
0.9	1:80*	1:400
0.67	1:120*	1:600*
0.45	1:160	1:800*

The calculated quantities of ascorbate phosphate buffer per assay tube at various assay dilutions of plasma or whole blood.

* Usual assay dilutions.

Results:-

1. Table (3) compares microbiological growth in the five standards containing the different quantities of autoclaved ascorbate phosphate buffer.
2. Two standard curves set up with and without autoclaved ascorbate phosphate buffer are shown in Figure (1). The folate results of 16 plasma samples assayed concurrently with and read from both curves are shown in Table (4).

TABLE (3).

Standard	A	B	C	D	E
Autoclaved ascorbate phosphate buffer ml./tube.	0	0.3	0.45	0.6	0.9
Folic acid mug/tube	Optical density x 100				
0.0	2	4	3	2	3
0.1	10	12	14	15	15
0.2	16	19	21	20	20
0.4	20	25	27	27	28
0.6	26	31	33	35	34
0.8	31	35	38	39	40
1.0	36	40	44	45	45

The effect of autoclaved ascorbate phosphate buffer on the growth of L. casei in standard tubes.

N.B. Each standard was set up in duplicate. The figures represent the average of the two readings which showed very little difference.

TABLE (4).

Sample No.	Folate µg/ml. of plasma.	
	Standard with ascorbate buffer.	Standard without ascorbate buffer.
1	5.2	8.8
2	5.0	8.5
3	3.9	6.4
4	3.7	6.1
5	3.8	6.0
6	3.0	5.2
7	3.5	6.1
8	13.6	21.8*
9	8.0	13.2*
10	9.0	14.2*
11	8.9	14.4*
12	5.3	8.9*
13	4.7	8.0
14	4.5	8.0
15	11.0	16.8*
16	4.3	7.3

Duplicate folate results of 16 plasma samples calculated from the two standard curves shown in Fig. (1).

* Megaloblastic vitamin B_{12}-deficient subjects.

COMMENT:-

Table (3) and Figure (1) show that autoclaved ascorbate phosphate buffer causes a marked stimulation of micro-organismal growth. It can also be seen in Table (3) that while 0.45 - 0.9 ml. of buffer per tube gave similar responses, less growth occurred in the presence of 0.3 ml. per tube. Autoclaved ascorbate phosphate buffer prepared with each batch of samples during extraction was therefore added to standard tubes throughout the present work. The volume selected 0.6 ml. per tube (Table 1) was technically convenient and had the same stimulant effect on bacterial growth as the quantities calculated to be present in the usually employed dilutions namely 0.45 to 0.9 ml. per tube.

The lower results obtained by this method (Table 4) should represent a better approximation of the folate content of samples than those calculated from standards containing no ascorbate phosphate buffer.

ACCURACY OF THE METHOD:

1. Reproducibility of results in duplicate assays:-

(a) Plasma:

35 random samples were assayed twice on two consecutive days. The range of difference between the duplicate assays was 1 to 20% with a mean of 8% and SD ± 5.9%

(b) Whole blood:

Duplicate assays were carried out in the same way on 47 whole blood samples. The difference ranged from 0 to 25% with a mean of 8.4% and SD ± 6.2%.

2. Recovery of added folic acid:-

Folic acid was added to five different plasma samples. The recovery varied from 89 to 100% when 5mug. were added to each and 85 to 97% when 10mug. were added.

3. Variation in the same assay:-

(a) A plasma sample was assayed 15 times in one assay. The coefficient of variation was 10%.

(b) A whole blood sample was treated in the same way. The coefficient of variation was 12%.

TABLE (5).

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Plasma µmg/ml.	5.4	9.9	4.8	9.6	6.5	6.4	3.7	5.1	4.6	5.4	2.4	5.0	5.8	2.7	8.9	6.5
Serum µmg/ml.	5.6	9.3	4.0	10.2	6.1	7.0	4.0	4.7	4.7	5.6	2.2	5.0	6.4	2.8	9.2	6.5

The plasma and serum folate levels of 16 subjects.

THE FOLATE LEVEL OF PLASMA COMPARED TO THAT OF SERUM.

METHOD:

20 ml. of blood were obtained from each of 16 subjects. 10 ml. were added to a universal container and allowed to clot for 2 hours at room temperature. The remaining 10 ml. were delivered into a heparinised bottle and plasma separated by immediate centrifugation. Plasma and serum were stored at -20°C. with 10mg. of ascorbic acid per ml. and the 32 samples assayed later in one batch as described.

RESULTS:

The results of such an assay are shown in Table 5. These indicate as previously reported by Herbert (1961) that the folate levels of plasma and serum obtained from the same subject at the same time are similar. As a 20 ml. heparinised blood sample was sufficient for estimating both plasma and whole blood folate contents and as all subjects studied in the present work had both estimations done, plasma was preferred to serum for folate estimation.

THE EFFECT OF VARYING THE LENGTH OF AUTOCLAVING WITH PRESSURE ON THE FOLATE CONTENT OF WHOLE BLOOD.

Grossowicz et al. (1962) extracted whole blood folate by autoclaving for 20 minutes at 15 p.s.i. Hoffbrand et al. (1966) on the other hand, recommended 2½ minutes at 10 p.s.i. but found that protein precipitation was not always complete. The effect of varying the length and pressure of autoclaving on whole blood folate was therefore determined in order to select conditions at which complete protein precipitation occurs

without/

TABLE (6).

Autoclaving	Pressure	10 p.s.i.		15 p.s.i.			Mean	SD±
	Time	2½'	5'	2½'	5'	15'		
	Blood Sample No.	Folate		mug/ml. packed cells.				
	1	235	224	225	236	235	231	5.5
	2	150	146	143	139	150	146	4.7
	3	166	156	155	169	154	160	6.5

The effect of the duration and pressure of autoclaving on whole blood folate.

without affecting folate stability.

METHOD:

Each of three blood specimens was haemolysed at a dilution of 1:10 by adding 1½ ml. of blood to 13½ ml. of a freshly prepared 1g.% ascorbic acid solution. After ten minutes at room temperature, the three haemolysates were stored at -20°C. for one hour and then thawed at room temperature. Two ml. of each haemolysate were then added to each of five universal bottles containing 18 ml. of 0.1 M phosphate buffer with 100mg.% assorbic acid. The final blood dilution in all samples was 1/100 and the final ascorbic acid concentration 200mg.%.

Triplicate bottles (representing the three blood specimens) were then autoclaved for periods ranging from 2½ minutes at 10 p.s.i. to 15 minutes at 15 p.s.i. and the folate content of the extracts determined as described.

RESULTS:

Similar folate values were obtained in each of the three blood specimens when subjected to the different autoclaving conditions (Table 6). This indicates that under these circumstances, folate yield was not affected by either the duration or pressure of autoclaving.

All extracts obtained by heating for 2½ minutes at 10 p.s.i. and some of these prepared at 10 p.s.i. for 5 minutes or 15 p.s.i. for 2½ minutes had a yellowish tinge indicating incomplete protein precipitation. The remaining extracts were colourless.

Autoclaving for 5 minutes at 15 p.s.i. was therefore adopted for extracting whole blood samples throughout the present work.

FOLIC ACID CLEARANCE.

The clearance of injected folic acid was performed by the method of Chanarin et al. (1958). A solution of folic acid in physiological saline (1mg./ml.) was given intravenously, the dose being 15 ug./kg. of body weight. 10 ml. of blood were obtained by venepuncture at 3, 15 and 30 minutes respectively after the intravenous dose and allowed to clot at room temperature; the serum was then separated aseptically and stored at -20°C. until assayed, using S. faecalis as the test organism.

For assay, 4 dilutions in water were prepared aseptically from each sample and 2 ml. of each dilution added to a tube containing 2 ml. of DIFCO folic acid assay medium previously sterilised by autoclaving for 10 minutes at 10 p.s.i. The final assay dilutions were as follows:-

1. 1/250, 1/175, 1/125 and 1/100 respectively for the 3 minutes sample.
2. 1/100, 1/75, 1/50 and 1/40 for both the 15 and 30 minutes samples.

A standard row containing 0 - 4mug. of folic acid per tube was included and the assay innoculated as described.

Microbiological growth was measured after a 16 - 18 hours incubation period at 37°C. and the results calculated as described for the L. casei method.

A second assay was always performed and the dilutions changed/

changed if necessary. The test gave the following values in normal controls:-

175 - 95mug. of folic acid/ml. of serum at 3 minutes.
70 - 40 " " " 15 minutes.
50 - 25 " " " 30 minutes.

FOLIC ACID ABSORPTION.

The test used for folic acid absorption was that described by Girdwood (1953, 1960). The patient was given 5mg. folic acid (Lederle) subcutaneously and the urine collected in a brown bottle toluene for 24 hours (Specimen A.). A 5mg. dose was then given orally and a second 24 hour urine collection made (Specimen B.). The folic acid in the urine was measured with S. faecalis. Samples were diluted with water and 5 ml. added to each of three tubes containing 5 ml. of Difco folic acid assay medium. A standard containing 0-10mug. of folic acid per tube and final volumes of 10 ml. per tube was included. The assay was sterilised by autoclaving for 10 minutes at 10 p.s.i. Innoculation, incubation and calculation of results were as described for the clearance test.

The folic acid excretion index:

$$\frac{\text{Output of folic acid in the urine after 5mg. orally.}}{\text{Output of folic acid in the urine after 5mg. subcut.}} \times 100$$

was calculated.

A folic acid output of less than 1.5mg/24 hours after the oral dose and an excretion index of less than 75% indicated malabsorption.

THE VITAMIN B_{12} CONTENT OF SERUM.

The preparation of samples for assay, the final assay volume and the setting of the standard tubes were exactly as described by Anderson (1964). The basal medium was different, however. DIFCO Euglena B_{12} assay medium was used rather than the basal medium recommended by Hunter et al. (1956). The assay was innoculated and incubated for 4 - 5 days as described. Growth was measured by a Hilger Spekker using a red filter (Kodak No. 8mμ680).

The range of the serum vitamin B_{12} concentration in normal controls is 150 - 1000 uug./ml. Megaloblastic vitamin B_{12} - deficient subjects always have values less than 100mug./ml. and in 26 cases investigated in the present study the levels ranged from 0 - 88uug./ml.

CHAPTER II.

THE INVESTIGATION OF FACTORS INFLUENCING THE RELEASE OF ASSAYABLE FOLATE FROM ERYTHROCYTES.

INTRODUCTION.

Toennies and co-workers (1956) showed that a factor present in plasma is important for the release of assayable folate from inactive precursors in erythrocytes and that this release is enhanced by the incubation of whole blood at 37°C. in phosphate buffer (pH 6 - 6.1), prior to precipitation of the proteins by autoclaving.

These conditions for "maximal" folate release i.e. incubation at 37°C. and pH 6 - 6.1 have since been adopted by most workers who have estimated the folate content of whole blood (Grossowicz et al. 1962, Hansen and Weinfield 1962, Cooper and Lowenstein 1964, Grzesinkowicz et al. 1966).

Since the commencement of the present work, however, Hoffbrand et al. (1966a) have found that incubation does not increase the folate content of haemolysates prepared in 1g.% ascorbic solution. This finding of "maximal" folate release at room temperature and pH 3.6 - 3.8 is at variance with the original observations of Toennies et al. (1953) who found that folate yield fell almost to zero at pH 4 and was maximal within a narrow pH range around 5.7. This led them in later work (1956) to consider pH 6.0 as optimal for plasma factor action.

The prime object of this part of the Thesis was to investigate factors which may influence folate release from erythrocytes. This was achieved by studying the effects of the/

the following factors on the assayable folate content of whole blood haemolysates:-

1. Ascorbic acid concentration.
2. pH.
3. Temperature, time and degree of haemolysis.
4. Relative concentration of erythrocytes to that of plasma.
5. The folate releasing ability of plasma from patients with vitamin B_{12} or folate deficiency as compared to normal.

General procedures for investigating folate release in whole blood haemolysates.

The following methods apply to the majority of experiments to be described in this Chapter.

1. Collection of blood:-

Heparinised blood samples were used throughout.

40 ml. of blood were obtained by venepuncture from healthy subjects with a normal peripheral blood picture and 20 ml. transferred into each of two sterile universal containers (Vol. 25 ml.) containing 0.1 ml. of heparin (25,000 units/ml.). The plasma was separated by immediate centrifugation for ½ hour at 1500g. 5-6 ml. of the plasma to be used for folate estimation was stored at -20°C. after the addition of 10mg. of dry ascorbic acid per ml. The remainder was recombined with enough of the red cells to give a final packed cell volume of 30% ± 2%. A fixed packed cell volume was adopted to standardise conditions in/

in different experiments.

2. Temperature:-

Many of the experiments were conducted at room temperature. Unless specified this means a temperature of 21 ± 2°C. (daily temperature readings were taken during the execution of these experiments).

3. pH determination:-

A Beckman model 72 pH meter was used to determine the pH of all haemolysates. To avoid "carry over" from one sample to another, pH determination was always done after aliquots had already been removed for extraction.

4. Extraction (protein precipitation):-

Haemolysates were always extracted in duplicate by adding 2 ml. to each of two universal containers each containing 18 ml. of 0.1 M phosphate buffer pH 6.0 - 6.1. The ascorbic acid concentration in the blood buffer solution was always kept constant at 200mg%. For haemolysates containing little or no ascorbic acid (0 - 50mg%) a 200mg% ascorbate phosphate buffer was used. When haemolysates contained 100 - 1000mg% ascorbic acid the buffer ascorbic acid content was reduced accordingly to give the desired 200mg% in the final mixture.

5. The haematological methods used were those of Dacie and Lewis (1963).

PRELIMINARY EXPERIMENT.

This is essentially a verification of the findings of Hoffbrand et al. (1966a), that maximal folate release can be achieved without incubation, by the haemolysis of whole blood in a solution containing 1g.% ascorbic acid.

METHOD:

9 ml. volumes of a freshly prepared 1g.% ascorbic acid solution were dispensed into each of 9 pairs of sterile universal containers.

To each of the pairs was then added a 1 ml. aliquot of heparinised blood obtained from each of 9 normal subjects thus giving a 1/10 dilution of each haemolysate.

One set of haemolysates was incubated at 37°C. for 90 minutes; the duplicates were frozen at -20°C. for one hour and then thawed at room temperature.

The pH of all frozen samples and three of the incubated ones was measured as described.

Haemolysates were extracted in duplicate with phosphate buffer and assayed as in Chapter I. The folate values were calculated as mug/ml. of packed cells (See Appendix B.II for formula).

RESULTS:

The folate value obtained after freezing and thawing was expressed in each case as a percentage of that obtained after incubation at 37°C. (Table 7). The Table also shows the/

the pH values of frozen haemolysates.

TABLE (7).

Blood Sample No.	1	2	3	4	5	6	7	8	9	Mean	SD ±
% folate yield after freezing and thawing.	110	103	97	103	108	96	90	93	103	100.3%	7
Haemolysate pH.	3.6	3.6	3.7	3.6	3.7	3.6	3.6	3.6	3.7		

1. Folate release after freezing and thawing expressed as a percentage of that after incubation.

2. Haemolysate pH.

COMMENT:

The mean folate value of the frozen samples is 100.3% ± 7% which is not significantly different from that of the incubated standards. These results confirm those of Hoffbrand et al. (1966) that incubation is not necessary for maximal folate release providing that the blood is treated by the method described. The pH values of the three frozen samples were identical with those of their incubated duplicates.

All haemolysates turned brown within three minutes of the initial dilution and the pH values in Table 7 suggest that this colour change may be due to the formation of acid haematin. The/

The pH 3.6 - 3.7 of these haemolysates is of interest as it means that the plasma factor is active at very low pH values.

Based on these results, a detailed study of the effect of ascorbic acid on folate release was first made.

OPTIMAL OR 100% FOLATE RELEASE.

Folate release was frequently assessed and compared in a single blood specimen under a variety of experimental conditions. In order to facilitate analysis, the folate results of each blood specimen were related as percentages of its "arbitrary optimal or 100% folate release", defined as the amount of folate (mug/ml. of packed cells) obtained when a blood sample is haemolysed at a dilution of 1/10 in 1g.% ascorbic acid solution and the haemolysate is incubated for 90 minutes at 37°C.

SECTION I.

THE EFFECT OF ASCORBIC ACID ON THE FOLATE CONTENT OF WHOLE BLOOD HAEMOLYSATES.

This section consists of two experimental series (A. and B.) designed to investigate the effect of differing ascorbic acid concentrations on the release of assayable folate in whole blood haemolysates.

The blood dilution was 1/5 in Series A. and 1/10 in Series B. Complete haemolysis was ensured in all samples by freezing and thawing. All samples were extracted and assayed without incubation at 37°C.

EXPERIMENTAL SERIES A.

METHOD:

1. Nine blood specimens each with an approximate volume of 30 ml. and a packed cell volume of 30% ± 2% were used. No more than two specimens were analysed each day.

2. Fresh ascorbic acid aqueous solutions of concentrations 0, 25, 50, 100, 250, 500, 750 and 1000mg.% respectively were prepared.

3. Haemolysis was carried out in sterile universal containers by combining 3 ml. volumes of a blood specimen with 12 ml. of each ascorbic acid solution. In this way 8 haemolysates with ascorbic acid concentrations of 0, 20, 40, 80, 200, 400, 600 and 800mg.% were prepared from each specimen. The final blood/

blood dilution in each case was 1/5 and the final haemolysate volume 15 ml.

4. After 10 minutes at room temperature all haemolysates were stored at -20°C. and one hour later allowed to thaw at room temperature. Complete thawing usually took 40-50 minutes. However all samples were given one hour at room temperature.

5. The degree of haemolysis was always tested for after thawing by removing 3 ml. volumes from haemolysates containing 0, 20, and 40mg.% ascorbic acid, centrifuging for 5 minutes at 3000g. and estimating the haemoglobin content of the supernatant after appropriate dilution. These values were then compared to the haemoglobin content of the blood sample and complete haemolysis assumed when the two values were found to be equal.

An "optimal" (100%) folate release preparation was made from each blood specimen by incubating a 1/10 haemolysate in 1g.% ascorbic acid for 90 minutes at 37°C. (p. 49).

The pH of all haemolysates was determined as described. Haemolysates were extracted with phosphate buffer and assayed as in Chapter I and the folate results of each set of haemolysates calculated as percentages of "optimal folate release" in the parent blood specimen.

RESULTS:

Each horizontal set of values in Table 8 represents individual percentages of folate release in a single blood specimen calculated as described and arranged under the appropriate ascorbic acid concentrations. The folate percentages/

MED. LIBR. UNIV. EDINBURGH

percentages are also grouped in eight columns under the different ascorbic acid concentrations and the mean (SD±1) calculated for each group. The significance of these results is summarised in Table 9.

Haemolysate pH:-

The pH was fairly constant within any group of haemolysates prepared at a given ascorbic acid concentration. The mean values (Table 8) ranged from 7.47 in the absence of ascorbic acid to 4.25 in haemolysates containing 800mg.%.

Series A. (Blood dilution 1/5).

TABLE (8).

Group No.	1	2	3	4	5	6	7	8
Haemolysate ascorb. acid concn. mg.%	0	20	40	80	200	400	600	800
Blood specimen No.	Folate release % of optimum.							
1	40	44	44	59	90	100	93	100
2	45	39	52	61	90	96	93	96
3	38	34	48	70	103	95	100	95
4	41	46	51	64	91	97	95	100
5	42	46	46	62	97	100	100	100
6	47	45	48	55	95	100	97	98
7	63	63	68	68	95	102	95	97
8	60	60	67	71	96	91	100	100
9	48	49	51	58	99	97	98	98
Mean	47	47	53	63	95	98	97	98
SD±	8.8	9.2	8.7	5.6	4.3	3.4	2.9	1.9
Mean haemolysate pH	7.47	7.35	7.25	6.90	5.73	4.75	4.40	4.25

The relationship between haemolysate ascorbic acid concentration, pH and percentage folate release.

Series A.

TABLE (9).

Means of groups compared.	t	P	Significance.
1 - 2	0	-	Not sig.
2 - 3	1.428	-	"
3 - 4	2.941	P .01	Significant
4 - 5	12.705	P .001	Highly sig.
5 - 8	1.550		Not sig.
6 - 8	0.8621		"
7 - 8	0.7516		"

Comparison between the mean percentage folate yields of the various groups shown in Table 8.

COMMENT:

Tables 8 and 9 show that:-

1. Raising the haemolysate ascorbic acid concentration from 0 to 40mg% did not alter the folate content significantly.

2. The first significant rise in the mean percentage folate yield occurred in the presence of 80mg% ascorbic acid ($P<.01$). At 200mg% "maximal folate" values were obtained in Samples 3 and 9; the mean percentage folate release in this group is not significantly different from that of haemolysates containing 800mg%/

800mg% ascorbic acid.

3. There is a progressive fall in pH with increasing ascorbic acid concentration and the highest folate yields - over 90% - occurred at pH values less than 6.0.

Factors which may be responsible for this pattern of folate yield:

(a) Haemolysis:

Haemolysis was complete when tested in the first three groups. Inadequate haemolysis can therefore be excluded as a cause of low folate yield at low ascorbic acid concentrations.

(b) Potentiation of the plasma factor by higher ascorbic acid concentrations:

It is possible that increased ascorbic acid concentrations may directly affect the plasma factor with resultant increased folate release. Toennies et al. (1963) concluded that thiol groups were important for the plasma factor activity. In their experiments, enzyme activity quickly dropped on standing at room temperature in aqueous solution, but was restored by hydrogen sulphide or reduced glutathione. In the same way, these labile SH groups may well be protected by the reducing action of ascorbic acid when present in higher concentrations.

(c) A pH effect?

It is conceivable that the high folate release at higher concentrations of ascorbic acid is due to its acidifying action. The role of ascorbic acid here would be to reduce the pH of the reaction to optimum values for the plasma factor.

This/

This would suggest that the pH optimum of the plasma factor is in fact much lower than that usually accepted namely 6.0 - 6.1.

(d) Lability of the released folate?

Folate may have been released equally in all haemolysates but ascorbic acid concentrations in excess of 80mg.% are necessary to prevent oxidation.

(e) Maximal folate release was obtained in haemolysates containing ascorbic acid concentrations of 200mg.% or more without incubation. Possibly this does not apply to lower ascorbic acid concentrations when a temperature of 37°C. may be important.

(f) Haemolysates were combined with phosphate buffers containing appropriate ascorbic acid concentrations to give a final concentration of 200mg.% during autoclaving. Better protection of folate during extraction or a greater stimulation of bacterial growth by higher ascorbic acid concentrations cannot therefore be a factor in producing higher folate values.

EXPERIMENTAL SERIES B.

This differs in only one respect from Series A, namely blood was haemolysed at a dilution of 1/10 instead of 1/5. Blood specimens No. 1, 2 and 3 in this series were taken from the same subjects as No. 5, 6 and 7 respectively in Series A.

METHOD:

10 blood specimens were haemolysed at a dilution of 1/10 by combining 1½ ml. aliquots of each with 13½ ml. quantities of freshly prepared ascorbic acid solutions covering the following concentrations:- 0, 25, 50, 100, 250, 500, 750 and 1000mg.% respectively. Haemolysis was carried out in sterile universal containers and the haemolysate ascorbic acid concentrations were 0, 22.5, 45, 90, 225, 450, 675 and 900mg.% respectively.

4 blood specimens were haemolysed over the whole range of ascorbic acid concentrations thus obtaining 8 haemolysates from each.

In 3 specimens the 50mg.% ascorbic acid solution was not used and therefore only 7 haemolysates were obtained from each of them. Only 5 haemolysates were prepared from each of the remaining three specimens.

Haemolysates were treated by freezing and thawing as in Series A and their pH values determined as described.

An "optimal folate release" haemolysate was prepared from/

Series B. (Blood dilution 1/10).

TABLE (10).

Group No.	1	2	3	4	5	6	7	8
Haemolysate Ascorb. acid concn. mg.%	0	22.5	45	90	225	450	675	900
Blood specimen No.	% Folate release.							
1	42	61	69	96	93	93	100	100
2	41	62	70	90	94	94	100	95
3	68	76	81	95	100	102	98	100
4	34	60	68	96	100	100	100	100
5	46	58	-	90	91	92	92	92
6	26	48	-	97	90	100	94	97
7	42	73	-	96	100	100	108	104
8	25	43	75	100	-	-	-	103
9	42	67	82	100	-	-	-	100
10	33	61	78	92	-	-	-	92
Mean	40	61	75	95	95	97	99	98
SD ±	12	10	6	3.6	5.8	4.0	5.2	4.2
Mean haemolysate pH	7.29	6.87	6.63	5.83	4.48	4.13	3.9	3.67

Relationship between haemolysate ascorbic acid concentration, mean pH and percentage folate release.

from each specimen as described. Extraction, assay and calculation of results were as described.

RESULTS:

The percentage folate values obtained for the 10 blood specimens are shown in Table 10. They are arranged as in Series A except that groups 1, 2, 4 and 8 comprise 10 haemolysates each while the remaining groups contain 7 haemolysates each. The mean and standard deviation were calculated for each group and tests of significance applied to compare the means in a similar way to that described for Series A; The results are shown in Table 11.

The mean haemolysate pH values ranged from 7.29 in the absence of ascorbic acid to 3.67 in the 900mg.% haemolysates.

Series B.

TABLE (11).

Means of groups compared.	t	P	Significance
1 - 2	4.1176	$<.001$	Highly sig.
2 - 3	3.3180	$<.001$	" "
3 - 4	9.6375	$<.001$	" "
4 - 8	1.000	-	Not sig.
5 - 8	1.2397	-	" "
6 - 8	0.2405	-	" "
7 - 8	0.1917	-	" "

Comparison between the mean percentage folate yields of the various groups shown in (Table 10).

TABLE (12).

Group No.	Series A (1/5)[x]			Series B (1/10)[x]			
	Haemolysate ascorb. acid concn. mg.%	pH	Percentage folate release.	Haemolysate ascorb. acid concn. mg.%	pH	Percentage folate release	Difference in folate release. P =
1	0	7.47	47	0	7.29	40	Not sig.
2	20	7.35	47	22.5	6.87*	61*	<.005
3	40	7.25	53	45	6.63	75	<.001
4	80	6.9*	63*	90	5.83+	95+	<.001
5	200	5.84+	95+	225	4.48	95	Not sig.
6	400	4.73	98	450	4.13	97	Not sig.
7	600	4.41	97	675	3.90	99	Not sig.
8	800	4.27	98	900	3.67	98	Not sig.

Comparison between the mean percentage folate release and pH values of Series A. and B.

x Blood dilution.

* , + Relationship between pH and folate release.

COMMENT:

Examination of the results in Table 10 shows that the folate and pH values exhibit a trend differing from that observed for Series A. (Table 8). It may thus be more informative to analyse the results of the present series by comparing them to those of Series A.

Table 12 compares the mean haemolysate pH values and the mean folate release percentages obtained for both series at different ascorbic acid concentrations.

pH:-

It can be seen (Table 12) that there is a progressive fall in haemolysate pH in both series with increasing ascorbic acid concentration but that for a given ascorbic acid concentration the fall in pH is greater in Series B than in Series A. The lower pH values in Series B. may be explained by the fact that since a blood dilution of 1/10 contains a larger volume of ascorbic acid solution per unit volume of blood than a dilution of 1/5, it follows that at any given ascorbic acid concentration the buffering capacity of blood has to counteract more hydrogen ions in the former case thus giving a lower pH value than in the latter case.

Folate release:-

At ascorbic acid concentrations of 22.5, 45 and 90mg.%, folate release is significantly higher in Series B. than at comparable concentrations in Series A. namely 20, 40 and 80mg.%/

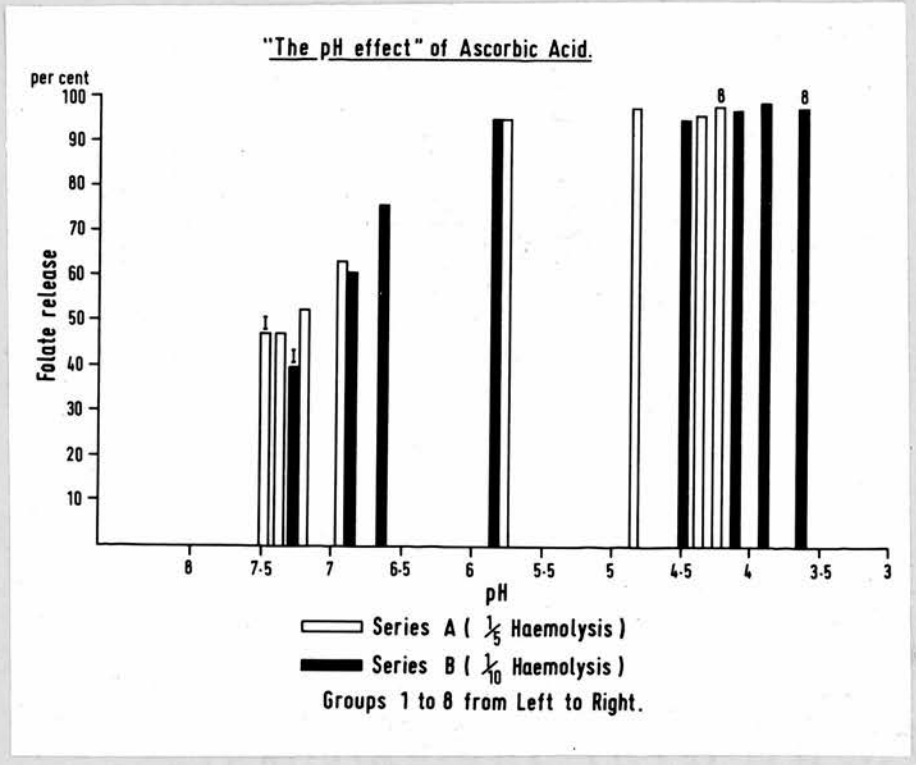
80mg.% respectively. The folate percentages obtained in Series B. at 22.5 and 45mg.% ascorbic acid are also significantly higher than those occurring in Series A. at ascorbic acid concentrations of 40 and 80mg.% respectively ($P < 0.05$ and 0.005 respectively).

On the other hand a close relationship is shown between haemolysate pH and folate release irrespective of ascorbic acid concentration. Thus in Series A. the pH of 6.9 and the percentage folate release of 63 found in the presence of 80mg.% ascorbic acid are virtually identical to the pH and folate values occurring in Series B. at 22.5mg.%, namely 6.87 and 61% respectively. Similarly the percentage folate yields at a pH of 5.84 in group 5 of Series A. (200mg.% ascorbic acid) and a pH of 5.83 in group 4 of Series B. (90mg.% ascorbic acid) are identical.

These findings indicate that pH alone irrespective of the haemolysate dilution or ascorbic acid concentration may profoundly influence folate release. By neglecting the ascorbic acid concentration in both series therefore and relating folate yield as a sole function of haemolysate pH, a partial assessment of the role played by the latter on folate release may be obtained.

This is shown in Figure 2 where the mean percentage folate release in both series is plotted against the corresponding pH values. It is obvious from the figure that/

FIGURE 2.



The mean percentages of folate obtained at differing ascorbic acid concentrations for Series A. (Table 8) and Series B. (Table 10) are plotted against the corresponding mean haemolysate pH. Group No. indicate ascorbic acid concentration. (See Tables

that folate release is closely related to pH thus:-

(i) A gradual rise in folate release which is clearly dissociated from ascorbic acid concentration occurs between pH 7.4 and 5.8. All folate values occurring at a pH of 5.8 and less are persistently over 94%.

(ii) The previously mentioned similarities between the two series in folate yield at pH values of 6.9 and 5.8 are clearly shown.

(iii) A pH of 6.63 (occurring in group 3 of Series B.), which has no counterpart in Series A., gives a percentage folate release that is intermediate between the values obtained at pH 6.9 and 5.8 respectively.

CONCLUSIONS.

These observations suggest that a pattern of folate release as obtained from Series A. and B. may be due entirely to the pH change rather than to a direct effect conditioned by the presence of ascorbic acid. For such a conclusion to be reached, however, ascorbic acid must if possible be excluded from the system and folate release assessed in a series of haemolysates covering a similar pH range artificially adjusted with HCl or NaOH. If under such circumstances folate release is found to conform to the pattern found in the presence of/

of ascorbic acid, a "pure" pH effect secondary to the presence of ascorbic acid can then be deduced as responsible for the folate results observed for Series A. and B.

Experiments designed on these lines to investigate the effect of pH on folate release will now be described.

SECTION II.

THE EFFECT OF pH ON FOLATE RELEASE.

PRELIMINARY EXPERIMENT.

An initial study was made on the effect of pH on the folate content of whole blood haemolysates. This was designed with two purposes:-

1. To assess folate release in haemolysates prepared at various pH values without ascorbic acid and to compare the results with:-

2. Folate release in haemolysates prepared in ascorbic acid solutions of 25 and 100mg.% respectively with added $\frac{N}{1}$ NHCl to give final haemolysate pH values comparable to those in (1).

METHOD:

Three sets of haemolysates were prepared from each of two blood specimens in $\frac{N}{1}$ NHCl containing deionised water, 25mg.% and 100mg.% ascorbic acid solutions respectively, as follows:-

9 ml. of deionised water or ascorbic acid solution were added to each of 10 sterile universal containers followed by appropriate volumes of $\frac{N}{1}$ NHCl (Appendix A5). The bottles were shaken and 1 ml. of each blood sample added to each of 15 universal containers (with the three different haemolysing fluids). The 1/10 haemolysates were thereafter treated as in Series A. and B. i.e. 10 minutes at room temperature, 1 hour at -20°C. followed by 1 hour at room temperature. pH determination/

determination, extraction and assay were as described.

An "optimal" folate release preparation was included simultaneously for each blood specimen.

RESULTS:

Table 13 (A. and B.) relates percentage folate release in both samples to the three pH ranges. Haemolysate No.5 in each case was prepared without the addition of HCl and therefore the pH and folate values here should be comparable to the appropriate values in Series B. (1/10 haemolysates) Table 12.

1. It can be seen from Table 13 that in the absence of ascorbic acid folate release was 100% at pH 5.0 in Sample I and 94% at pH 5.1 in Sample II.

2. In the presence of 25mg.% ascorbic acid, a reduction in haemolysate pH to 3.3 - 4.9 was paralleled in both samples by a marked increase in percentage folate release to values ranging from 92% to 102%. At pH 3.0 however, folate release is only 67% (Sample I).

3. The very low folate values obtained in the absence of ascorbic acid at pH 4.1 and less (both samples) are in marked contrast to those obtained at comparable pH values in haemolysates containing 25mg.% ascorbic acid. Thus while folate yields of 97% or more were obtained at pH 3.3, 3.5 and 4.0 (Sample I, 25mg.% ascorbic acid) only 2%, 4% and 33% were obtained respectively for the same sample when haemolysis was/

TABLE (13).

SAMPLE I.

A.

	No Ascorbic Acid					25mg.%					100mg.%				
Haemolysate No.	1	2	3	4	5*	1	2	3	4	5*	1	2	3	4	5*
pH	3.3	3.6	4.1	5.0	7.1	3.3	3.5	4.0	4.9	7.0	3.5	3.6	4.1	4.3	5.9
% Folate release	2	4	33	100	48	97	100	97	102	60	103	103	104	100	97

SAMPLE II.

B.

	No Ascorbic Acid					25mg.%					100mg.%				
Haemolysate No.	1	2	3	4	5*	1	2	3	4	5*	1	2	3	4	5*
pH	2.8	3.5	3.9	5.1	7.2	3.0	3.3	3.9	4.5	6.8	2.6	3.0	3.6	4.0	5.7
% Folate release	2	6	28	94	42	67	94	92	92	51	53	92	94	100	92

Percentage folate release at various pH values for Samples I and II. in the three haemolysing fluids.

* pH not adjusted.

was done over a comparable pH range in the absence of ascorbic acid. For a 100mg.% of ascorbic acid "maximal folate release is maintained within a pH range of 3-6.

CONCLUSIONS.

These preliminary results supported the presumptive evidence discussed previously that folate release is largely pH dependent. The statement is, however, only true for pH 5.0 and 5.1 where "maximal" folate release occurred in the absence of ascorbic acid. At lower pH values, the presence of ascorbic acid seems to be important for either the protection or release of folate. Folate release over a more detailed pH range has been studied and the results shown in the next series of experiments.

A DETAILED STUDY OF THE EFFECT OF pH ON FOLATE RELEASE.

These experiments were designed on similar lines to the previous one except that wider and more detailed pH ranges were covered.

METHOD:

1. Folate release was assessed within the following pH ranges:-

No.	pH range.	Haemolysing fluid + HCl or NaOH.
1	2.8 - 9.0	Deionised water.
2	2.3 - 7.6	25mg.% ascorbic acid
3	2.3 - 9.0	100 " " "

2. The pH was adjusted by adding either HCl or NaOH to the haemolysing fluid in a universal container prior to the addition of blood (the quantities of HCl or NaOH required to be added for obtaining the various haemolysate pH values are shown in Appendix A5).

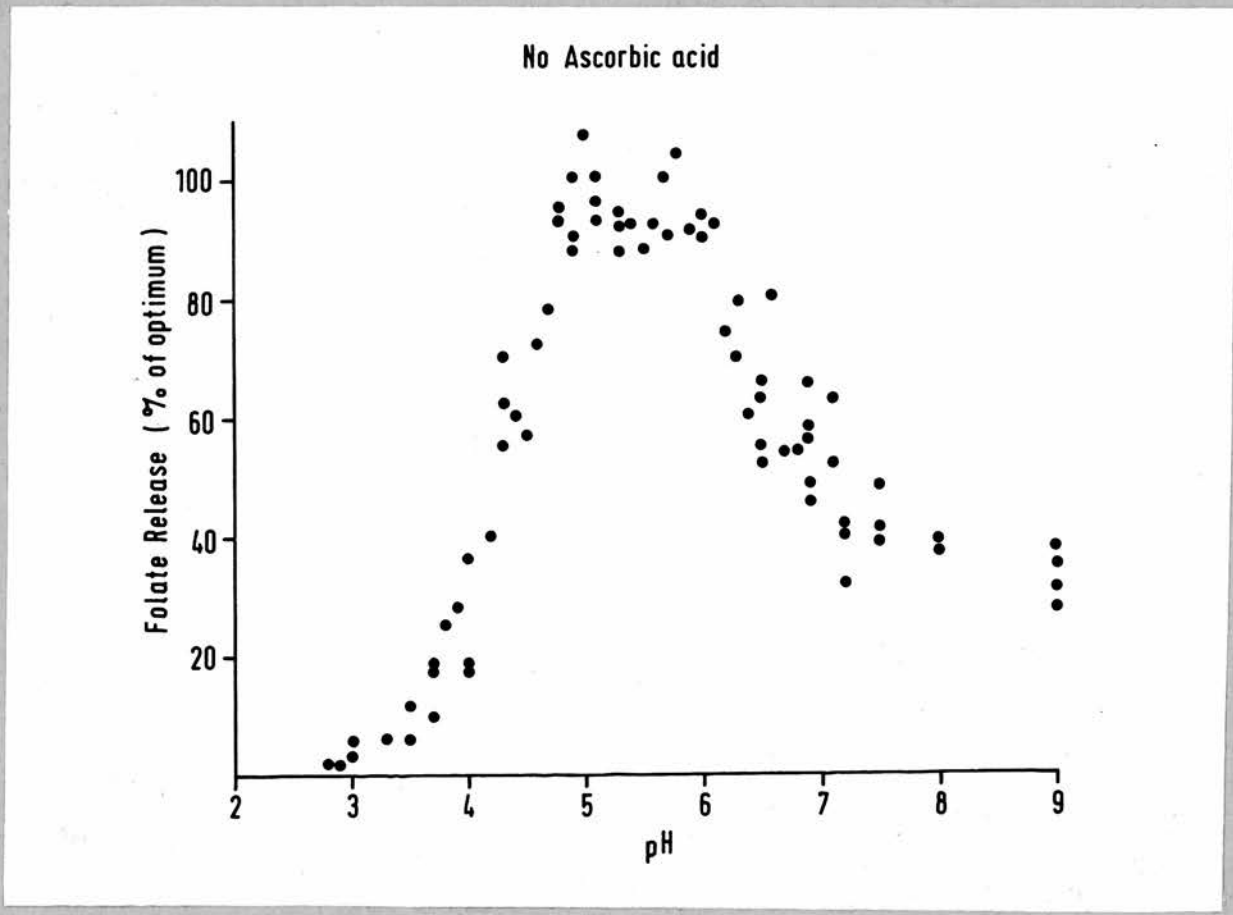
3. Folate release was assessed at pH intervals as close as possible, usually 0.2 - 0.3 apart, within each pH range. To achieve this the following experiment was done using 5 blood specimens each with an approximate volume of 60 ml. and a packed cell volume of 30% $\pm$ 2%:-

(a) A series of haemolysates was prepared from each sample to cover -

14 - 16 pH values in range I (no ascorbic acid).

10/

FIGURE 3.



The release of folate at various pH values in the absence of ascorbic acid (HCl or NaOH was added to water prior to addition of blood).

10 - 15 pH values in range II (25mg.% ascorbic acid).

12 - 15 pH values in range III (100mg.% ascorbic acid).

(b) By preparing the three sets of haemolysates of a given blood sample at slightly different pH values to those obtained for the preceding sample, it was possible to cover each pH range and therefore folate release at 0.2 - 0.3 intervals.

4. It was only possible to analyse one sample per day.

5. Haemolysis was at a blood dilution of 1/10, and all haemolysates were treated by freezing and thawing as for Series A. and B.

6. An optimal folate release preparation was assayed concurrently with each blood sample as described previously.

7. Extraction, assay and calculation of the results were as described.

RESULTS:

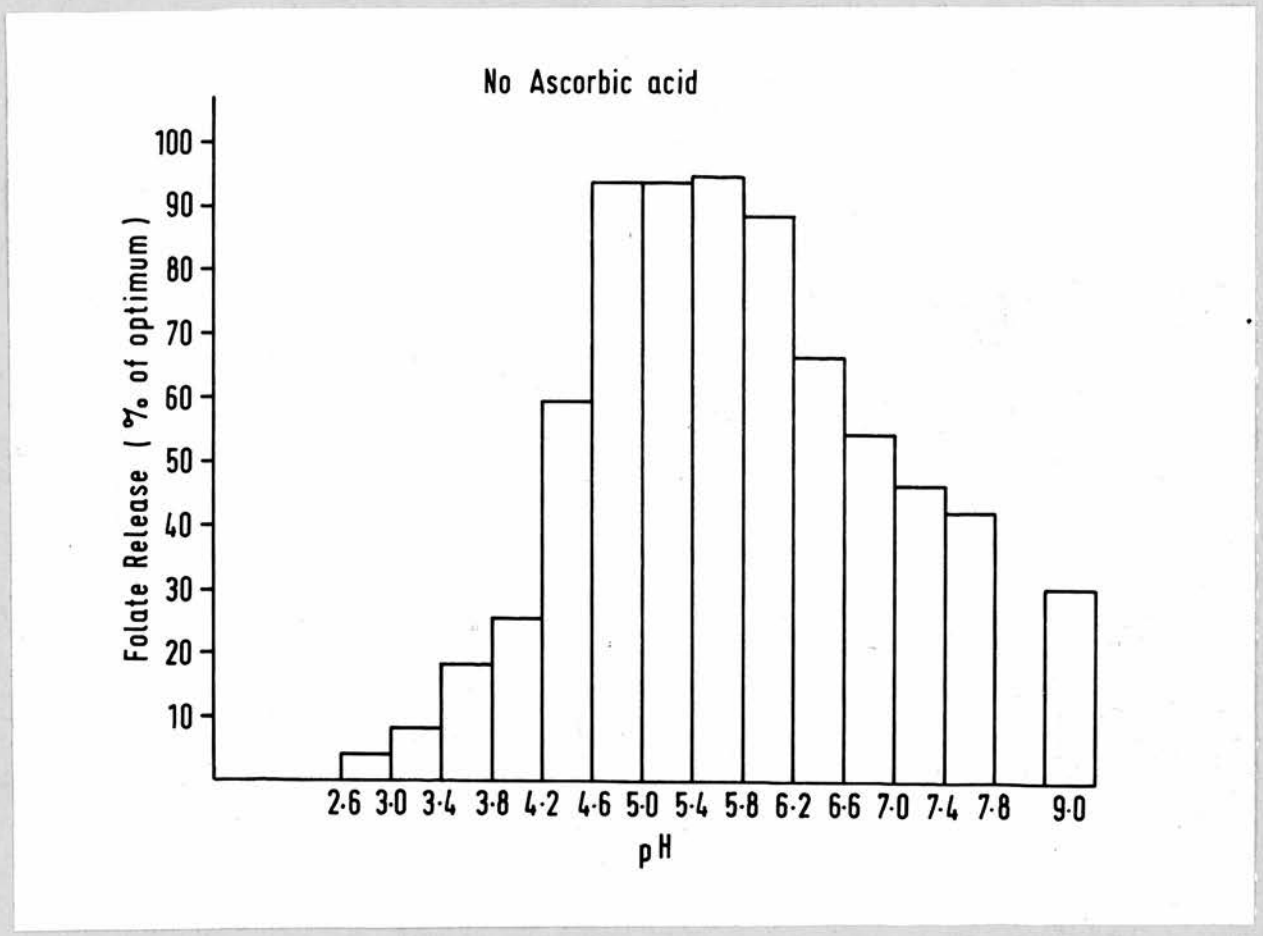
Folate release in pH range I (no ascorbic acid):

75 folate results were obtained from the five blood samples investigated within this pH range. These are plotted individually against their respective pH values in Figure 3.

For convenience the folate values were grouped at 0.4 pH intervals and the mean calculated for each group. The histogram in Figure 4 relates the mean folate values, calculated in this way over pH intervals of 0.4.

The scatter diagram in Figure 3 shows almost 100% folate "release" over the pH range of 4.8 - 6.1. There is almost/

FIGURE 4.



The means of the folate values in (Figure 3) calculated at 0.4 pH intervals.

almost no assayable folate at pH 3.0. At pH 9 the folate yield ranges from 25 - 39%. These features are shown in the mean percentage folate yield plotted in Figure 4.

Folate release in pH range II (25mg.% ascorbic acid).

The scatter diagram in Figure 5 demonstrates a change from the previous situation in that maximal folate release occurs within the pH range of 3.1 - 6.1. A sharp fall in folate yield is shown at pH values of less than 3.1 and a more gradual one at pH values of more than 6.1. These findings are substantiated in Figure 6 where a mean percentage folate release of 90 - 96 is found for the pH range of 3.1 - 5.8. A slight drop to 85% occurred for the pH interval (5.9 - 6.2).

Folate release in pH range III (100mg.% ascorbic acid).

The 64 results results are presented in a similar manner in Figures 7 and 8 respectively.

The pattern for folate release is similar to that in pH range II, the highest values occurring within the pH range of 3.1 - 6.1.

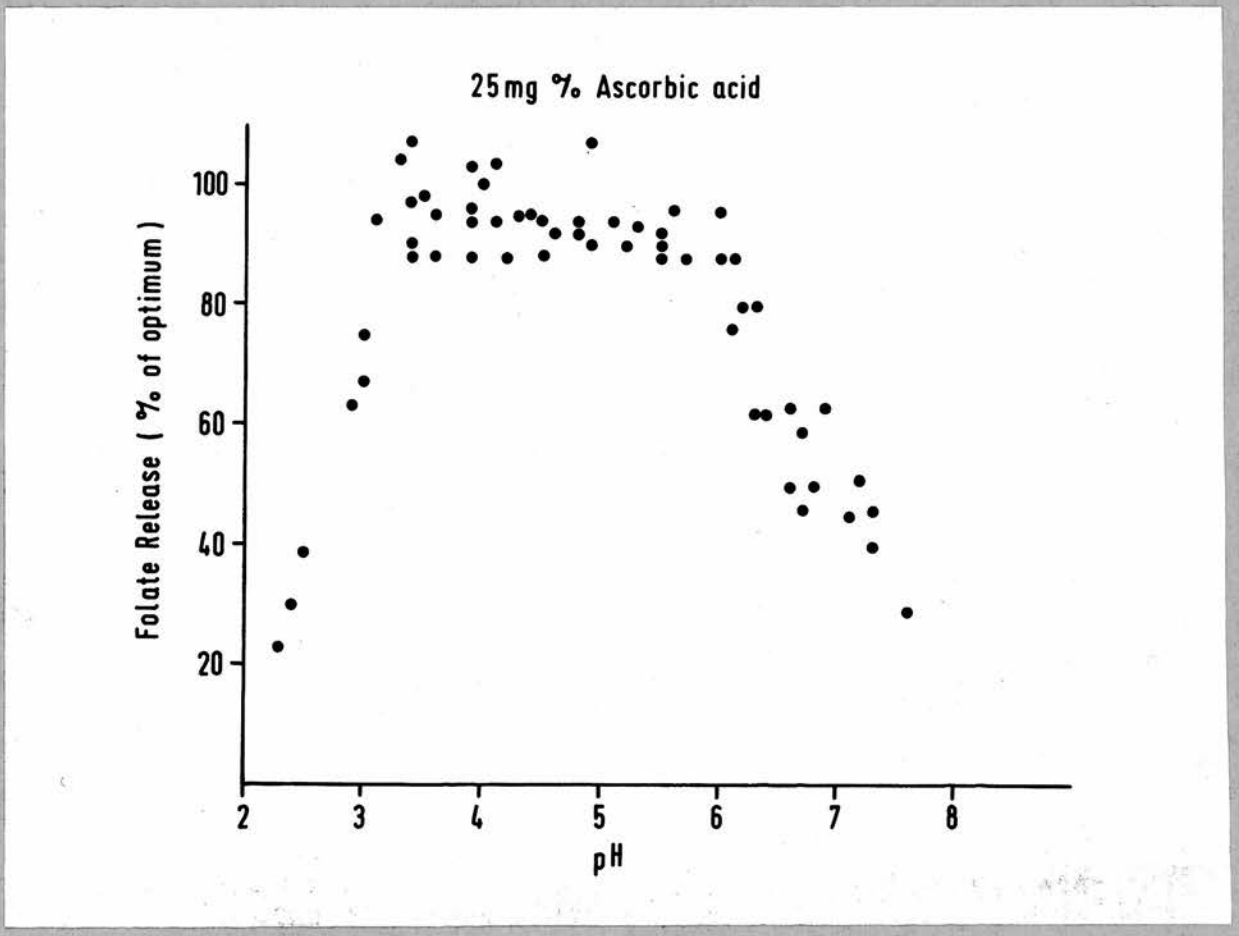
COMMENT:

The results in Figures 3 and 4 show that a modification of pH alone affects folate release. The maximal effect is in the pH range of 4.8 - 6.1. Such a range must represent the pH optimum for folate release in haemolysates prepared in the way described (no ascorbic acid).

The presence of ascorbic acid in the system appears

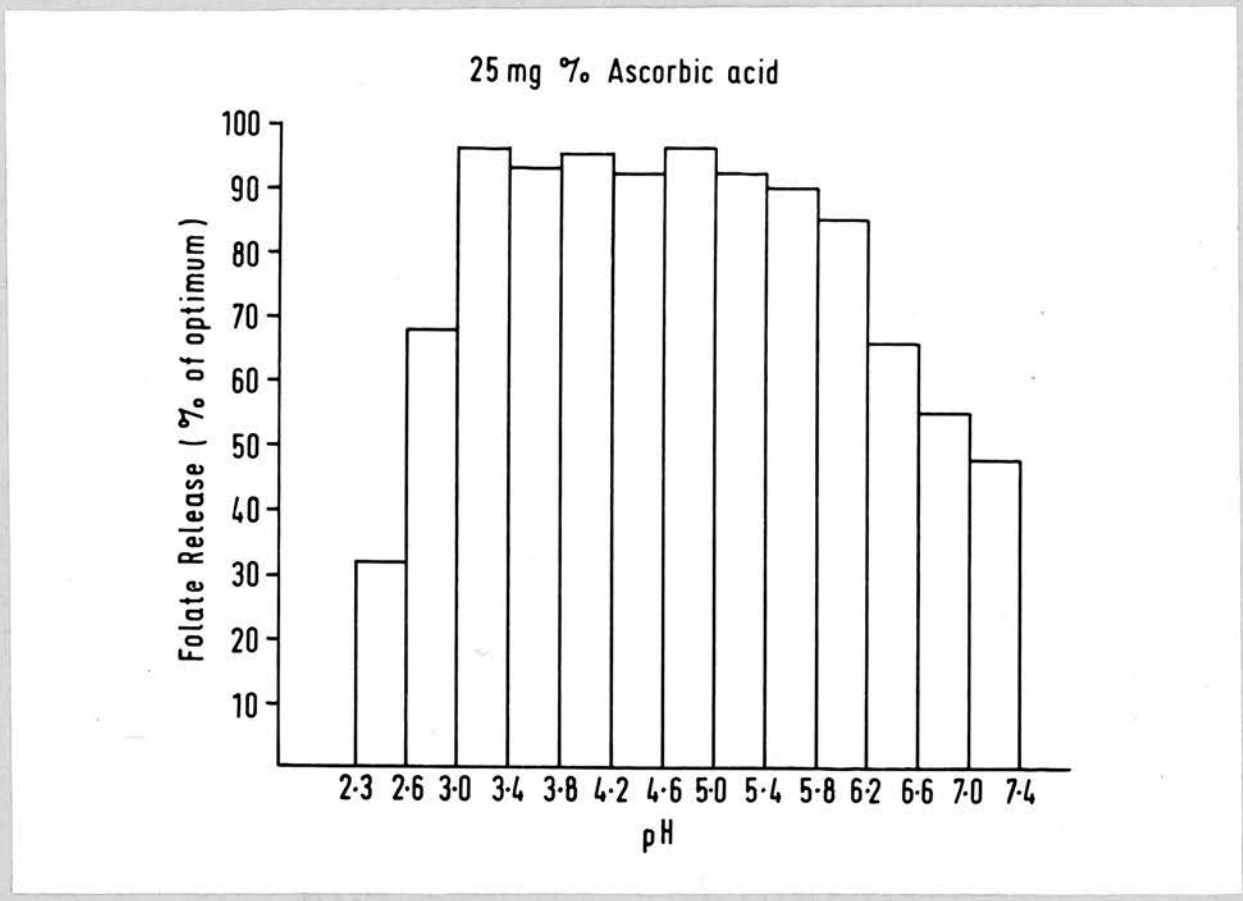
to/

FIGURE 5.



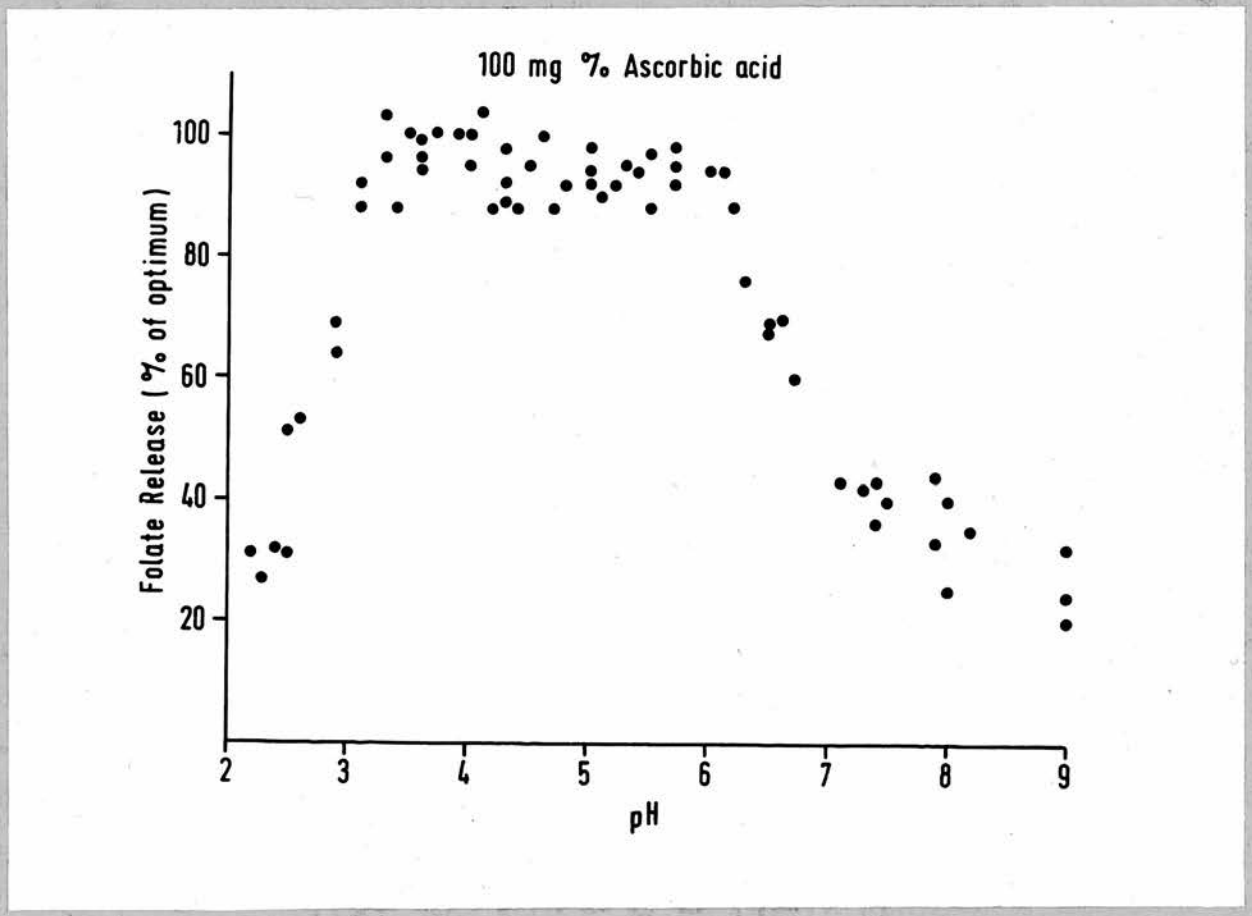
Folate release related to pH in the presence of 25mg.% ascorbic acid.

FIGURE 6.



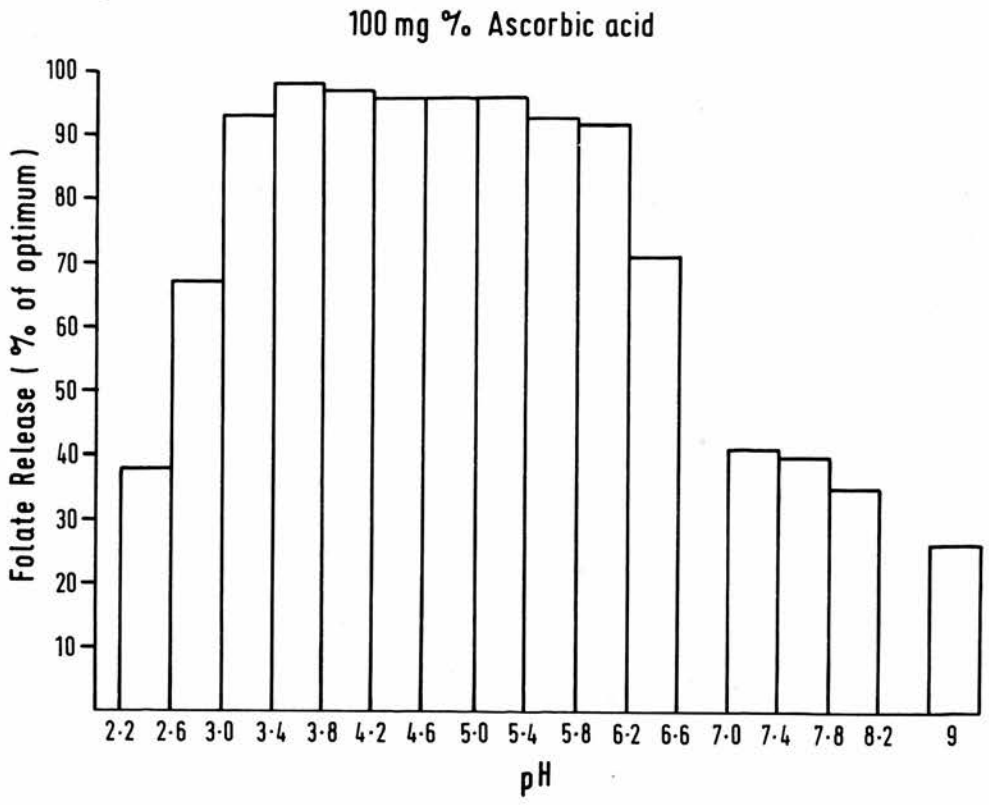
The means of the folate values in (Figure 5) at 0.4 pH intervals.

FIGURE 7.



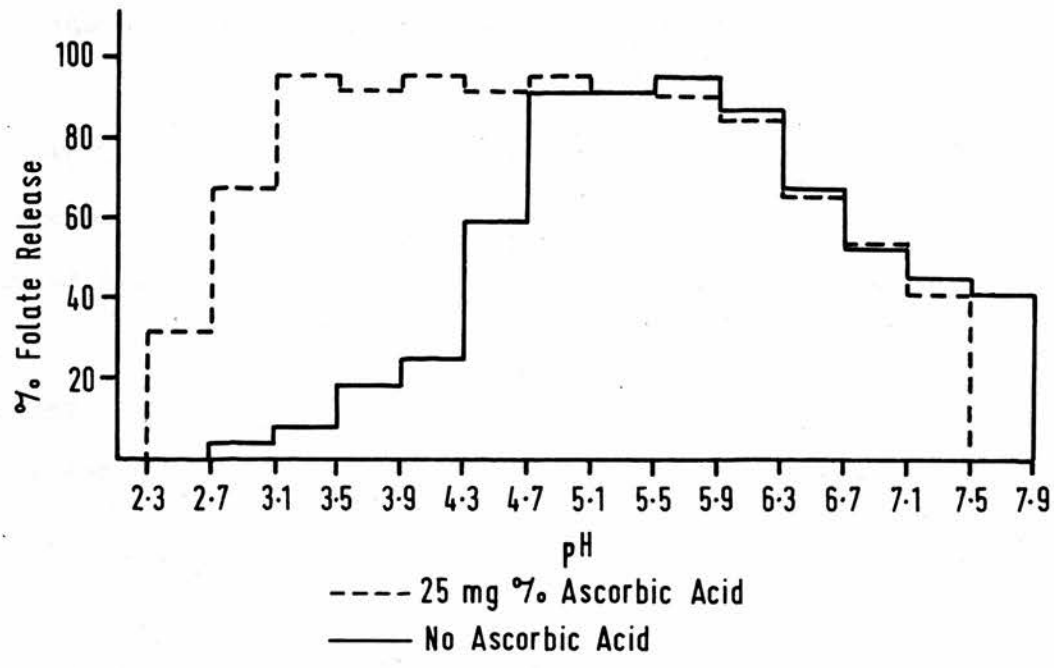
Folate release related to pH in the presence of 100mg.% ascorbic acid.

FIGURE 8.



The means of the folate values at 0.4 pH intervals.

FIGURE 9.



The pH-folate release pattern in the absence of ascorbic acid compared to that obtained in the presence of 25mg.% ascorbic acid.

to enhance folate release or stability at a more adid pH namely 4.6 - 3.1. There is no significant difference in folate release, at any of the pH intervals, between experiments where either 25 or 100mg.% ascorbic acid was used (Figures 6 and 8).

Comparison between folate release in pH range I. (no ascorbic acid and pH range II (25mg.%).

The results obtained within the two pH ranges are shown together in Figure 9.

In the pH range 4.7 - 7.4 the two sets of results are indistinguishable. At a more acid pH than 4.7, however, much higher folate values are obtained if ascorbic acid is present in the haemolysates.

The possible causes of the discrepancy in folate yield at acid pH between the two systems may be one or both of the following:-

1. Ascorbic acid may be essential for the protection of folate released at low pH values. Folate is known to be unstable at extremes of pH but more so at acid than alkaline pH.

2. Ascorbic acid may be protecting or potentiating the plasma factor action at low pH values.

Experiments investigating the stability of "released folate" at various pH values in the absence of ascorbic acid will now be described.

THE STABILITY OF FOLATE AT VARIOUS pH VALUES.

METHODS:

1./

1. 135 ml. of deionised water were delivered into a sterile MRC bottle, to which NHCl was added, followed by 15 ml. of blood (P.C.V. 30%). The final haemolysate volume was 150 ml. and its "expected" pH 5.0 ± 0.2 (Appendix A5).

2. After 10 minutes at room temperature the haemolysate was subjected to freezing and thawing as described.

3. 4 ml. were then extracted to determine folate release. This is "control No.1", being the haemolysate's "basal" folate content. pH determination was done on a small aliquot.

4. To determine the effect of pH on folate stability, the haemolysate was treated as follows:-

(a) 10 ml. aliquots were added to 10 sterile universal containers. NHCl or N NaOH was added to each to give haemolysate pH values ranging from 3 - 11.

(b) 5mg. of ascorbic acid were added to 20 ml. of the original haemolysate and the pH brought to 3.5 by adding NHCl. This is control No. 2 where the pH is lowered in the presence of ascorbic acid. All universal containers were subjected to freezing and thawing again as described.

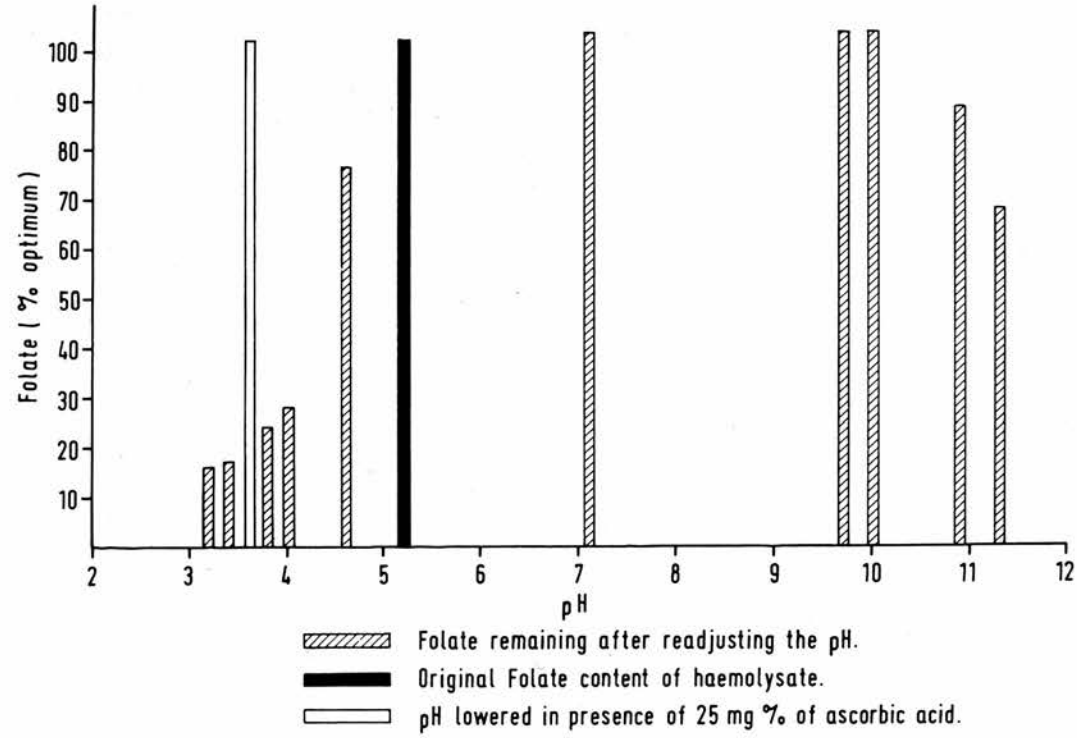
pH determination was carried out on each haemolysate. Extraction and assay were as described.

An optimal folate release was done as described.

RESULTS:

The percentage of folate recovered after readjusting the haemolysate pH over a wide range is compared in Table 14 and/

FIGURE 10.



The percentage stability of released folate determined at various pH values, in the absence of ascorbic acid.

and Figure 10 with the original haemolysate folate content and the amount obtained when the pH was lowered in the presence of 25mg.% ascorbic acid.

TABLE (14).

pH	3.2	3.4	3.8	4.0	4.6	5.2*	7.1	9.7	10	10.9	11.3	3.5x
% folate recovered.	16	17	24	28	76	100	102	103	103	88	69	102

Percentage folate stability at various pH values.

* Control I (original haemolysate pH).
x Control 2 (pH lowered in presence of 25mg.% ascorbic acid).

COMMENT:

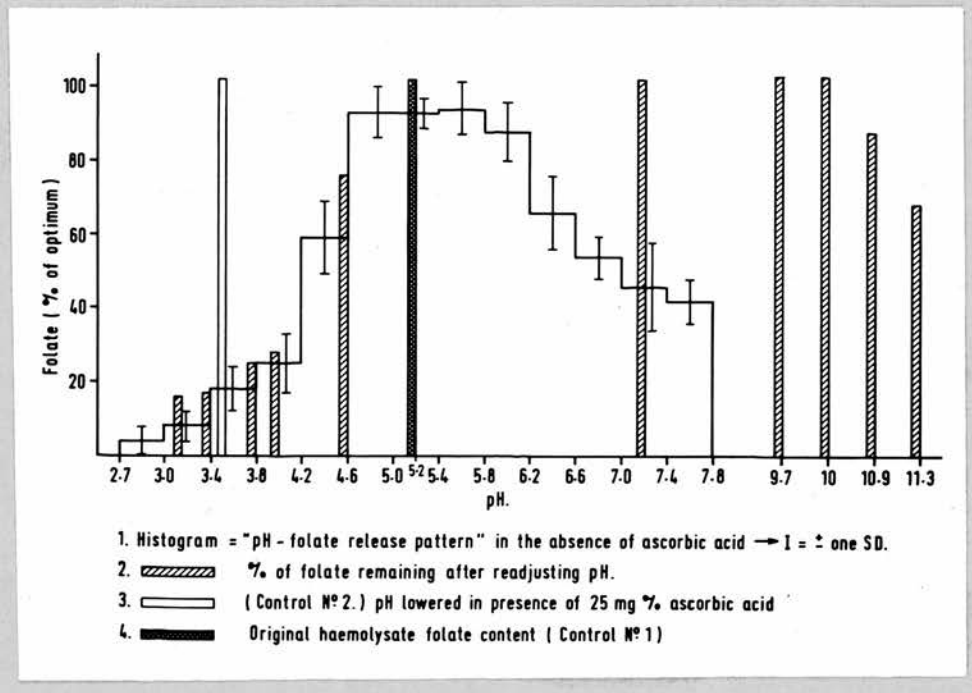
The original haemolysate had a pH of 5.2 which is within the optimal range for folate release. It was found to have a folate content of 102% as given by control No.1 (Table 14).

When the haemolysate pH was readjusted over a wide range, a marked loss of folate occurred at low pH values. Thus when the haemolysate pH was dropped to 3.2, 85% of its folate was lost.

On the other hand, folate seems to be reasonably stable when exposed to alkaline pH. It is completely stable for example up to a pH of 10 and possibly more. Even at a pH of 11.3 the folate loss is only 31%.

Of especial interest is the complete protection of folate when 5mg. of ascorbic acid was added immediately before the addition of HCl (control No. 2, Table 14 and Figure 10) which/

FIGURE 11.



Comparison between percentage folate release and stability patterns determined at various pH values in the absence of ascorbic acid.

which proves that ascorbic acid has a protective effect for folate at low pH values.

The percentage folate stability and the release patterns obtained at various pH values in the absence of ascorbic acid are shown together in Figure 11.

It is clear from the figure that at comparable pH values and within a pH range of 3.1 - 5.2 the "release" and stability patterns are closely matched. This suggests that the low percentage folate "release" found within the pH range 3.1 - 4.6 reflects folate instability rather than reduced plasma factor action. The fact that ascorbic acid has been shown to protect folate at low pH values explains the discrepancy in folate "release" between pH ranges No. I and II observed at pH values less than 4.6 (Figure 9).

At higher pH values (more than 6.2) on the other hand, where folate is shown to be fully stable, a suboptimal enzyme action must be responsible for the lower percentage folate release.

<u>The effect of modifying the pH on the folate yield of haemolysates containing 900mg.% ascorbic acid</u>:

When haemolysis was carried out in the presence of 25 and 100mg.% respectively maximal folate release occurred within a pH range of 3.1 - 6.1 (Figures 5 and 7).

In order to see whether a higher ascorbic acid concentration would alter this pH range, haemolysates were prepared from 4 blood samples in 1g.% ascorbic acid solution, to/

to which either HCl or NaOH was added to give haemolysate pH values distributed about the range of 3.1 - 6.1.

Samples were haemolysed at a blood dilution of 1/10 and treated by freezing and thawing as described. The haemolysate ascorbic acid concentration was 900mg.%.

Extraction, assay and calculation of results were as described.

RESULTS:

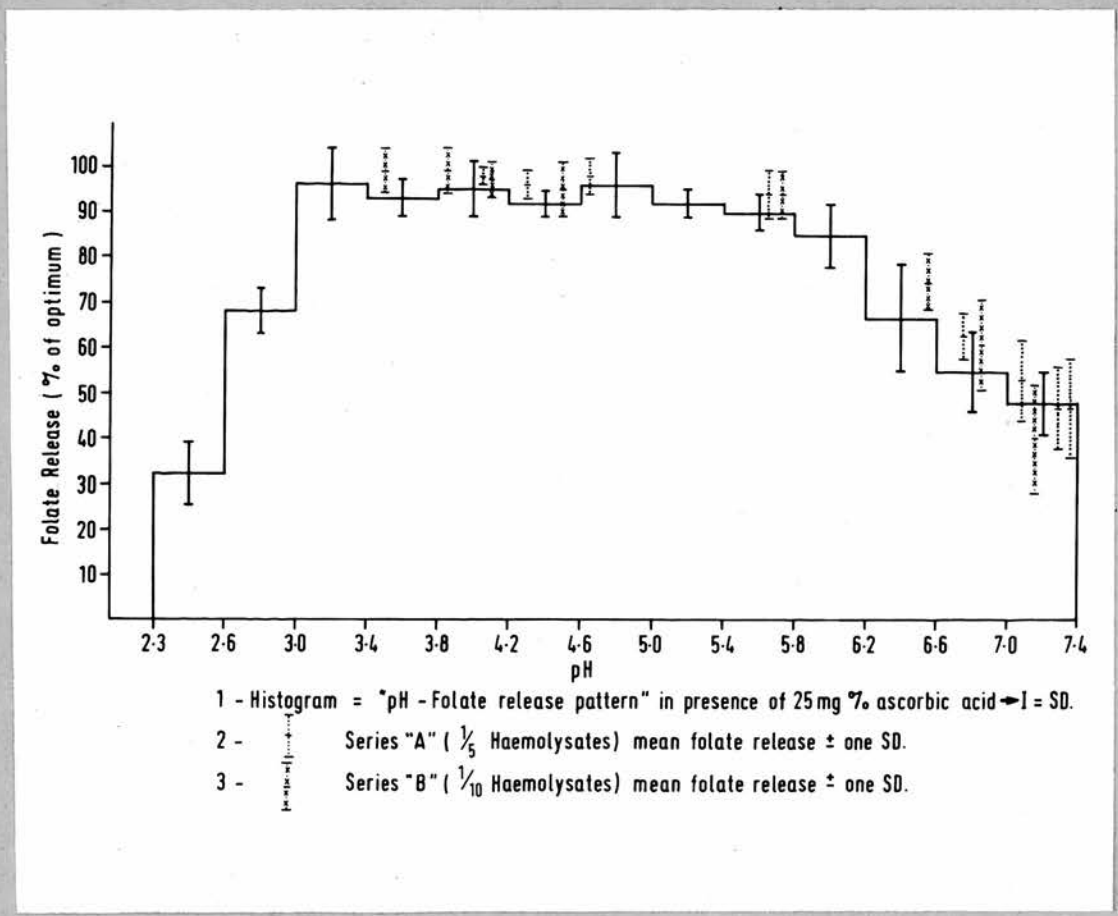
The results are shown in Table 15 as percentages of optimal folate release against the appropriate haemolysate pH values. Folate release is "suboptimal" in all samples and comparable to that obtained in the presence of 25mg.% ascorbic acid at similar pH values (Figures 5 and 6). This indicates that raising the ascorbic acid concentration to 900mg.% does not alter the pH range for "maximal" folate release determined previously in the presence of 25mg.% ascorbic acid.

TABLE (15).

Blood Sample No.	Haemolysate pH value.	% folate release.	Haemolysate pH	% folate release.
1	2.9	60	6.8	54
2	2.8	57	6.8	61
3	2.6	20	7.0	29
4	2.6	19	7.0	34

Folate release at different pH values in haemolysate containing 900mg.% ascorbic acid.

FIGURE 12.



Comparison between the effect of ascorbic acid concentration and pH on folate release.

Comparison between the effect of ascorbic acid concentrations and pH on folate release.

No significant difference was shown when the mean percentage folate values of haemolysates containing 0 - 900mg.% ascorbic acid (Series A. and B.) were compared with those obtained at comparable pH values but in the presence of 25mg.% ascorbic acid. This is illustrated in Figure 12.

To exclude the possibility that the use of different blood samples in experiments investigating the role of ascorbic acid or pH on folate release may have influenced the results shown in Figure 12, a large blood specimen was obtained from a single donor and haemolysates prepared from the sample by the methods described previously, namely:-

1. 1/5 haemolysates containing 0 - 800mg.% as in Series A.
2. 1/10 haemolysates containing 0 - 900mg.% as in Series B.
3. 1/5 haemolysates prepared at pH values comparable to those in (1) but containing 25mg.% ascorbic acid.
4. 1/10 haemolysates prepared at pH values comparable to those in (2) but containing 25mg.% ascorbic acid.

The folate content of haemolysates was determined and calculated as described and related in Figure 13 to pH.

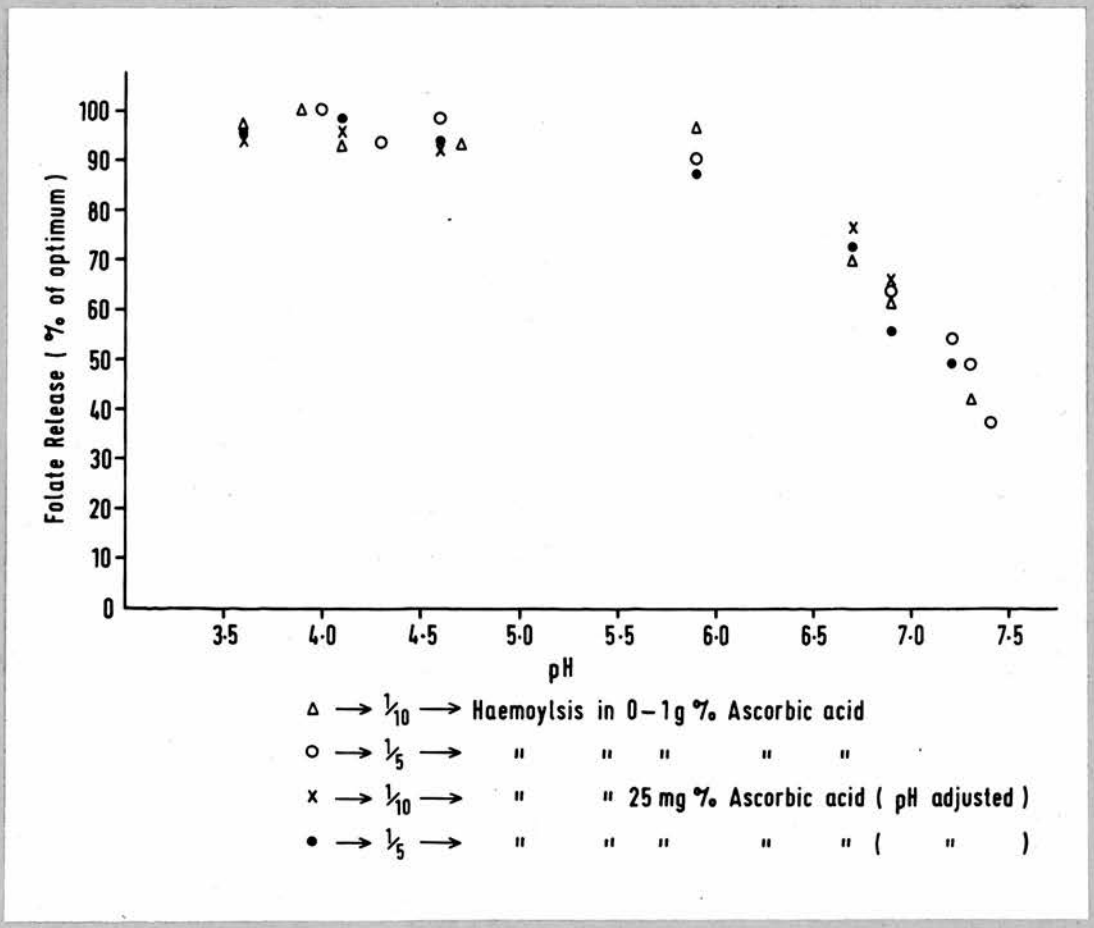
The findings are the same as in Figure 12 in that it is the pH and not the ascorbic acid concentrations which determines folate yield.

THE EFFECT OF pH ON FOLATE RELEASE FROM WASHED ERYTHROCYTES.

OBJECT OF EXPERIMENT:

To/

FIGURE 13.



Comparison between the effect of ascorbic acid concentration and pH on folate yield in a single blood sample.

To determine the effect of varying the pH in the absence of plasma on the release of assayable folate from erythrocytes.

METHOD:

A 20 ml. heparinised blood sample was centrifuged, plasma removed and the red cells washed four times in acid citrate dextrose (ACD) by centrifugation for ½ hour at 3000g. each time. Care was taken to remove as completely as possible the supernatant ACD after each wash.

Using the same method for whole blood (p. 70), six haemolysates containing 25mg.% ascorbic acid were prepared from the washed cells at a dilution of 1/10 and within a pH range of 3.0 - 6.0.

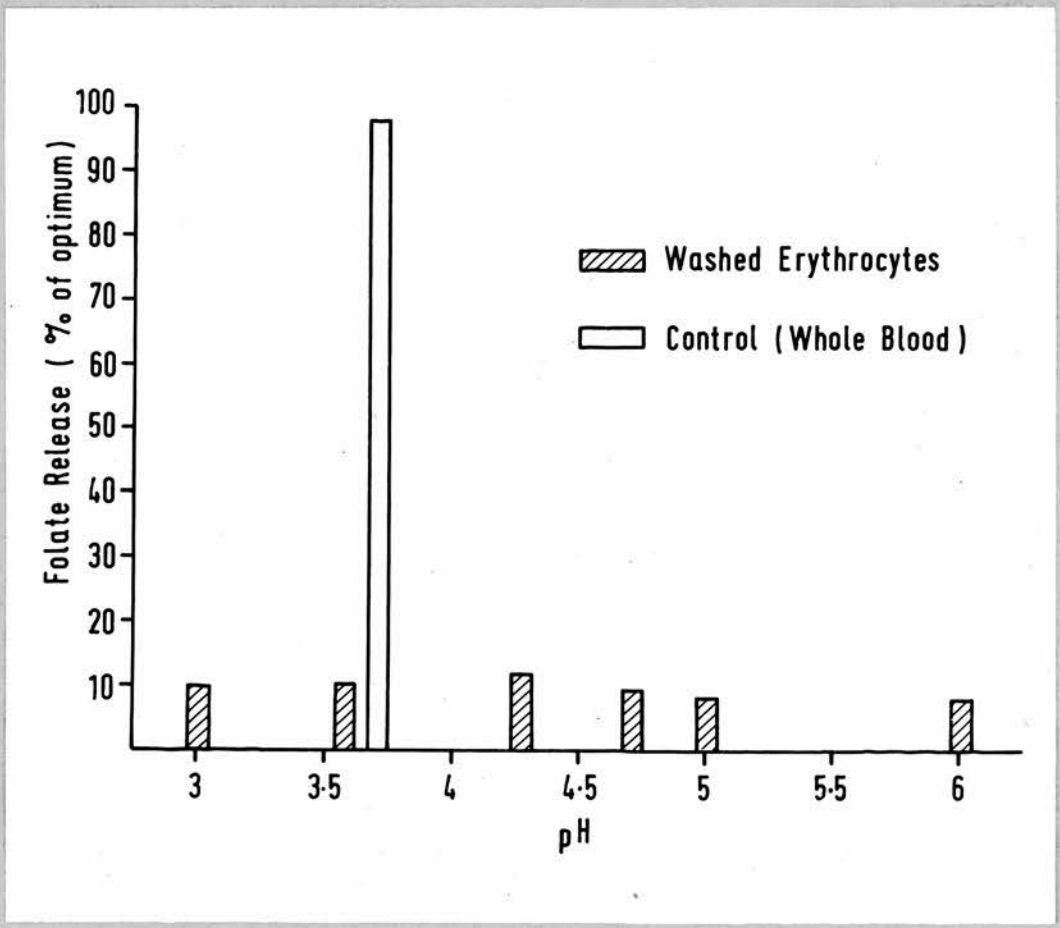
A control was included where the cells and plasma were recombined, the P.C.V. determined and the sample haemolysed at pH 3.7. All haemolysates were frozen and thawed, extracted and assayed as described.

RESULTS:

The folate content of the washed erythrocytes determined at the various pH values was expressed as a percentage of the value obtained (mug/ml. packed cells) for the control (cells + plasma). The results are shown in Figure 14.

It is clear from Figure 14 that washed erythrocytes yield negligible amounts of folate under these circumstances. Percentage folate yield is similar at the various pH values and/

FIGURE 14.



The effect of modifying the pH in the absence of plasma on folate release from washed erythrocytes.

and is less than 10% of that obtained for whole blood, indicating that the release of maximal folate from erythrocyte at all pH values depends on the presence of plasma.

CONCLUSIONS.

The results of the experiments in Sections I and II indicate that:-

1. Maximal release of assayable folate from erythrocytes occurs within a much wider pH range (3.1 - 6.1) than previously reported and that the presence of 25mg.% ascorbic acid or more is important for maintaining the released folate in an assayable form.
2. Folate release is plasma dependent at all pH values.

SECTION III.

THE EFFECT OF TEMPERATURE AND HAEMOLYSIS ON FOLATE RELEASE.

EXPERIMENT I.

Previous experiments (Section I, Series B.) had shown that folate yield was "suboptimal" when whole blood haemolysates prepared at a dilution of 1/10 by freezing and thawing contained ascorbic acid concentrations ranging from 0 - 45mg.%. The present experiment was designed to determine whether incubation at 37°C would alter the folate yield of such haemolysates.

METHOD:

Duplicate haemolysates were prepared at a dilution of 1/10 from each of six blood specimens in a series of ascorbic acid solutions containing 0, 25, 50 and 100 mg.% respectively as described in (Section I, Series B.). In this way four pairs of haemolysates containing 0, 22.5, 45 and 90mg.% ascorbic acid respectively were obtained from each blood specimen. 4 haemolysates representing the different ascorbic acid concentrations were transferred after 10 minutes at room temperature to an incubator at 37°C. for 90 minutes. Their duplicates were treated by freezing and thawing as described for Series A. and B. (Section I).

An "optimal" folate release preparation was included with each specimen and the folate content determined as described.

RESULTS/

RESULTS:

Table 16 compares the mean percentage folate release obtained from haemolysates prepared by incubation with that obtained by freezing and thawing. At the various ascorbic acid concentrations, the mean folate values obtained after incubation did not differ significantly from those obtained after freezing and thawing. Mean folate values of 95% or more were obtained as expected in haemolysates containing 90mg.% ascorbic acid. These findings indicate that a temperature of 37°C. is not an important requisite for folate release providing that haemolysates were frozen and thawed as described.

TABLE (16).

Ascorbic acid mg.%	0	20	22.5	90
Mean % folate release after incubation at 37°C. for 90'	41	62	76	97
SD±	8.9	6.4	10.1	4.8
Mean % folate release after freezing and thawing	38	59	78	95
SD±	9	11.3	2	6.2

Mean percentage folate release in six blood specimens after incubation at 37°C. compared to that after freezing and thawing in the presence of differing ascorbic acid concentrations.

EXPERIMENT II.

This experiment was designed to investigate the factors influencing folate release in whole blood incubated in phosphate buffer at 37°C. Toennies et al. (1956) had reported an increasing folate yield with increasing incubation time under such conditions and this he had attributed to an enhanced plasma factor action. The technique adopted was changed from that of Toennies and co-workers to determine whether their haemolysis was complete. The modifications included:-

(i) The rate of haemolysis was determined concurrently with that of folate release during the incubation time.

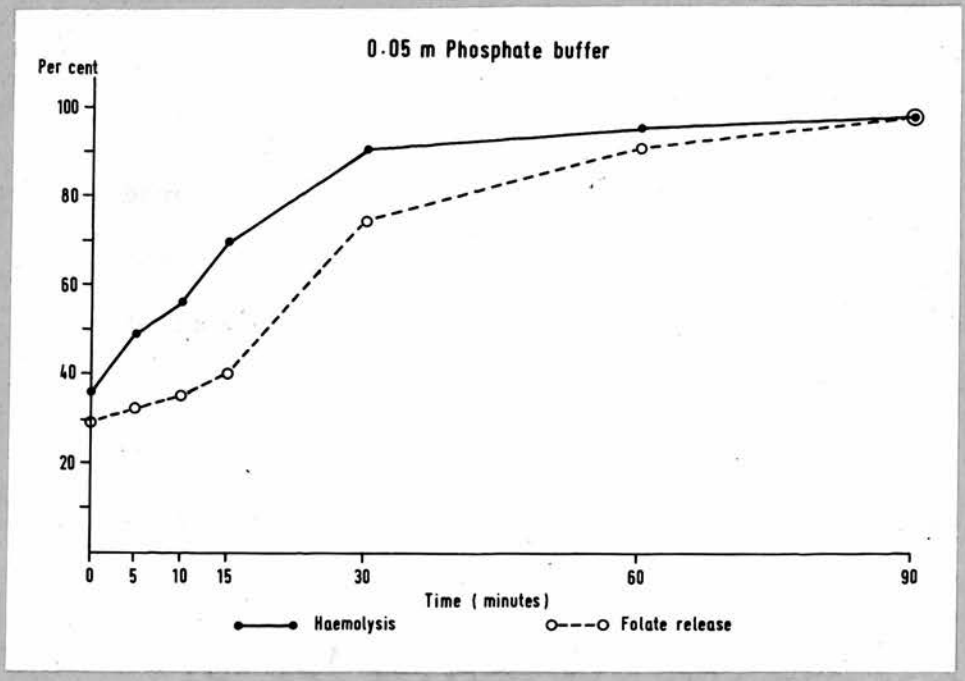
(ii) Preliminary experiments had shown that the folate yield from a whole blood dilution of 1/750 (used by Toennies et al.) was too small for accurate measurement especially at short incubation periods and hence a 1/500 dilution was adopted instead.

METHOD:

19.6 ml. of a freshly prepared 0.05 M phosphate buffer pH 6.0 containing 50mg.% ascorbic acid were pipetted into each of 21 sterile universal containers which were then incubated at 37°C. for 15 minutes to effect temperature equilibration.

0.04 ml. of a freshly drawn heparinised blood sample was then added to each container, the final blood dilution being/

FIGURE 15.



Percentage haemolysis and folate release determined at various periods during the incubation of whole blood in .05 M phosphate buffer at 37°C.

being 1/500. After each of the following incubation periods:- 0, 5, 10, 15, 30, 60 and 90 minutes three bottles were removed from the incubator, shaken thoroughly and centrifuged at 1500g. for 5 minutes and the supernatants pipetted into clean containers. One of each of these containers was set aside for measurement of haemolysis. The ascorbic acid content of the remaining pair was adjusted to 200mg.% and the samples extracted in the usual way.

The optical density of haemolysates was measured in a Unicam calorimeter at 540 and the degree of lysis at the various incubation periods calculated as a percentage of that obtained after 90 minutes incubation.

Extracts were assayed in duplicate without further dilution and their folate content calculated as a percentage of that of the sample incubated for 90 minutes.

RESULTS:

The relation between percentage haemolysis and folate release is shown for different incubation periods in Figure 15.

EXPERIMENT III.

OBJECT:

To determine the rates of haemolysis and of folate release in whole blood at room temperature when the pH is varied over the optimum range (pH 3.1 - 6.1).

pH values of 3.6 and 5.8 were chosen arbitrarily for this purpose.

METHOD/

METHOD:

1. THE RATE OF FOLATE RELEASE.

A. At pH 3.6:

(i) Using 1g.% aqueous ascorbic acid solution:-

13½ ml. volumes of a freshly prepared 1g.% ascorbic acid solution were dispensed into each of 3 sterile universal containers. To each was then added 1½ ml. of a different blood specimen giving a blood dilution of 1/10 and a haemolysate ascorbic acid concentration of 900mg.%. The contents of each bottle were mixed and left at room temperature. 2 ml. aliquots were then removed from each bottle after 5, 10, 15 and 30 minutes respectively, added to 18 ml. of 0.1 M phosphate ascorbate buffer and extracted by immediate autoclaving.

An optimal folate release preparation was prepared simultaneously from each blood specimen.

(ii) Using 100mg.% ascorbic acid solution adjusted for pH 3.6 with HCl:-

Two separate blood specimens were haemolysed in this solution at a dilution of 1/10 and folate release timed in exactly the same manner as in (i). The haemolysate ascorbic acid concentration was 90mg.%.

B. At pH 5.8:

Using 100mg.% ascorbic acid solution:-

The two blood samples from A (ii) were used and folate release timed as in A (i) and (ii).

2. THE RATE OF HAEMOLYSIS.

With/

TABLE (17).

Incubation time at room temp. (mins.)	5	10	15	30
Sample No.	Folate	% of	optimum.	
1	44	100	98	102
2	86	94	95	97
3	70	96	98	99

The rate of folate release at room temperature using 1g.% aqueous ascorbic acid solution pH 3.6.

TABLE (18).

	Sample I.		Sample II.	
pH	3.6	5.8	3.6	5.8
Time at room temp.(mins)	%	folate	release.	
5	52	34	62	25
10	99	47	102	31
15	97	66	95	38
30	100	78	96	47

The rate of folate release at room temperature using 100mg.% aqueous ascorbic acid solution (pH 5.8) and adjusted with HCl (pH 3.6).

With one of the blood specimens used in A. (ii) and B., the rate of haemolysis was determined using the two haemolysing solutions (100mg.% ascorbic acid solutions pH 3.6 and 5.8 respectively).

METHOD:

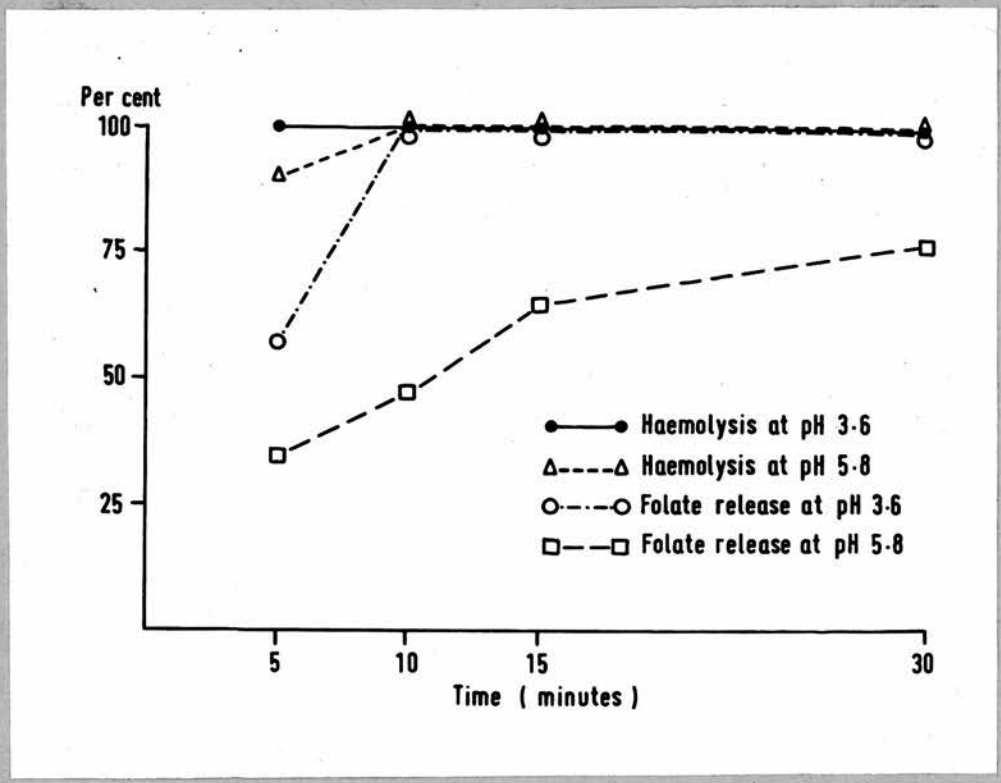
3.8 ml. of each solution were pipetted into each of 6 tubes and paired. 0.2 ml. of blood was then added to each tube and after 5, 10, 15 and 30 minutes at room temperature a pair of tubes was centrifuged for 3 minutes at 1500g. and the supernatants pipetted into clean tubes. The 6th pair was frozen at -20°C. for 1 hour then thawed at room temperature and their supernatants taken to represent 100% lysis for the appropriate pH value. Haemolysates were diluted 1/200 with water before reading in a Unicam colorimeter at 540mμ.

RESULTS:

1. Table 17 shows the rate of folate release at room temperature and pH 3.6 in haemolysates containing 900mg.% ascorbic acid.
2. Table 18 compares the rate of folate release at pH 3.6 and 5.8 respectively at room temperature in haemolysates containing 90mg.% ascorbic acid.
3. The rates of haemolysis and folate release at both pH values (3.6 and 5.8) are shown together in Figure 16.

COMMENT/

Figure 16.



The rates of haemolysis and folate release determined at pH 3.6 and 5.8 at room temperature.

COMMENT:

Figure 15 shows that when whole blood is incubated at 37°C. in 0.05 M phosphate buffer, haemolysis, incomplete initially, increases markedly with time during the first 30 minutes and thereafter very slowly up to 90 minutes. This implies that the folate release pattern in this experiment is markedly dependent on the rate of haemolysis as well as on the plasma factor-substrate interaction. From the figure it is also clear that plasma factor action develops more slowly than the degree of haemolysis.

In contrast, Table 17 shows that maximal folate release occurs within 10 minutes at room temperature when 1/10 haemolysis was carried out in 1g.% aqueous ascorbic acid solution. That this is largely a pH phenomenon is shown in Table 8 where similar folate results were obtained in haemolysates at the same pH (3.6) but containing 90mg.% ascorbic acid instead. Figure 16 shows that whereas rapid haemolysis occurs at both pH values, the rate of folate release at pH 5.8 is much slower than at pH 3.6, a finding which suggests that folate is released at a variable rate within the optimum pH range 3.1 - 6.1. The implications of this will be discussed later.

CONCLUSIONS.

The results of these experiments indicate:-

(i) Providing that whole blood is adequately haemolysed

in/

in water, incubation at 37°C. is not required for the release of assayable folate from erythrocytes. Toennies observations (1956) were explained by the initial incomplete haemolysis in .05 M phosphate buffer and its enhancement with increasing incubation time.

(ii) "Maximal" folate yield can be obtained within a short period at room temperature by haemolysing whole blood at low pH in aqueous ascorbic acid solutions.

SECTION IV.

THE "FOLATE RELEASING PROPERTIES" OF PLASMA IN HEALTH AND DISEASE.

The ability of plasma to release assayable folate from washed erythrocytes was studied in the following groups:-

(a) 32 normal subjects.

(b) 19 megaloblastic vitamin B_{12} deficient patients.

(c) 19 " folate " "

(d) 25 cases of rheumatoid arthritis.

The details of the patients are shown in Appendix (C.).

The investigations comprised two parts:-

Part I:-

Folate release was compared in each group when the washed erythrocytes of each subject were combined in turn with:-

(i) A "control" plasma (supplied by the same donor throughout the study) and used for all cases investigated.

(ii) The subject's own plasma.

Part II:-

Differing quantities of pooled plasma (0.05-0.4ml.) obtained from each group were combined with aliquots of a normal haemolysate each equivalent to one ml. of washed packed cells and the resulting folate release "patterns" compared.

METHOD.

Part I:-

(a) Control plasma:

Heparinised/

Heparinised blood samples were obtained from the same donor every 3-4 weeks. Plasma was separated, divided into 1.5 ml. aliquots, and stored at -20°C. without ascorbic acid in sterile universal containers.

(b) Collection of blood from subjects:

40 ml. were obtained by venepuncture from fasting subjects. 2 ml. were delivered into a sequestrene bottle for determination of the haematological indices, 18 ml. into a universal container and allowed to clot for 2 hours at room temperature, before serum was separated and stored at -20°C. for vitamin B_{12} estimation. The remaining 20 ml. were added to a heparinised universal container from which plasma was separated by centrifugation at 3000g. for 30 minutes and then divided into three sterile universal containers and stored at -20°C. as follows:

(i) 4 ml. with 10mg. of dry ascorbic acid per ml. for folate estimation.

(ii) 1.5 ml. without ascorbic acid to be recombined 2-3 hours later with the subjects washed erythrocytes.

(iii) The remainder was stored for other studies.

(c) Preparation and storage of whole blood samples:

The packed cells were washed three times with acid citrate dextrose (ACD) by centrifugation at 3000g. for 30 minutes. Two universal containers with 1.5 ml. aliquots of "control" and "subject" plasma respectively were thawed at room temperature and 0.8 ml. of the washed packed cells added to each. The samples were mixed thoroughly and their micro-haematocrit/

microhaematocrit values determined. The P.C.V. ranged from 27-30%. 0.4 ml. of each sample was then pipetted into each of three sterile universals containing 10mg. of dry ascorbic acid. The bottles were shaken thoroughly to dissolve the ascorbic acid and stored at -20°C. until extracted.

1.5 ml. of "control" plasma to which 15mg. of ascorbic acid had been added was stored with each batch of samples for folate estimation.

(d) Extraction of whole blood folate:

25-30 samples were extracted at a time. For each sample two bottles each containing 0.4 ml. of blood (one with "control" and the other "subject's" plasma) were thawed at room temperature. 1.6 ml. of deionised water were then added to each, the contents mixed and left at room temperature for 15 minutes followed by the addition of 18 ml. of 0.1 M phosphate buffer containing 150mg. per cent ascorbic acid. The final blood dilution was 1/50 and the final ascorbic acid concentration 200mg. per cent.

The samples were autoclaved for 5 minutes at 15 p.s.i. and the extracts stored at -20°C. until assayed.

Each sample was extracted at least twice and independent assays were performed on the extracts. The mean folate value of these was expressed as mug/ml. of packed cells as described in Appendix B2.

Part II:-

Haparinised blood samples (20 ml. each) were obtained from/

from 4 normal subjects. The packed cells were washed four times by centrifugation in ACD at 3000g for 30 minutes taking care each time to remove as completely as possible the supernatant ACD.

30 ml. of the pooled washed cells were added to 270 ml. of a g. per cent ascorbic acid solution contained in an MRC bottle to give a 1/10 dilution and the haemolysate frozen and thawed as described. 10 ml. volumes (= 1 ml. of packed cells) were dispensed into each of 25 sterile universal containers.

The following quantities of "control" and pooled plasma obtained from each of the groups under investigation were added successively to each of six universals containing the haemolysate:-

0.05 0.1 0.15 0.2 0.3 0.4 ml.

A "control" haemolysate with no plasma was included.

All bottles were transferred to an incubator at 37°C. and 30 minutes later extracted and assayed in duplicate as described and the folate content of each haemolysate expressed as mug/ml. of packed cells.

RESULTS.

1. Effect of storage on "the folate releasing ability" of plasma:

Four samples of washed erythrocytes were combined with "control" plasma that had been subjected to various periods of storage at -20°C. and 4°C. respectively and folate release assessed. The results in Table 19 which are expressed as percentages/

percentages of folate release by fresh plasma, show that storage up to a period of 4 months at 4°C. and 4½ months at -20°C. does not affect the folate releasing properties of human plasma.

TABLE (19).

	Period and temperature of storage.				
	Fresh	-20°C.			+ 4°C.
		2/52	3/52	4½/12	4/12
Washed RBCs. Sample No.	%	Folate yield.			
1	100	109	93	106	106
2	100	96	99	110	97
3	100	-	100	106	94
4	100	-	108	95	103

Folate release by "control" plasma after different periods of storage at -20°C and +4°C expressed as % of that released by fresh plasma.

N.B. The "control" plasma is the same as used for folate release in the study.

The effect of storage at -20°C. on whole blood folate:

The folate levels of 12 blood samples prepared in the way described in Part I. were estimated before and after storage with ascorbic acid at -20°C. for 3 months. The results are shown in Table 20 as percentage rise or fall in folate content after/

after storage. The folate levels obtained after 3 months were not significantly different from the initial values $P < 0.5$.

TABLE 20.

Blood specimen No.	% Rise or fall in W.B. folate after 3/12 storage at -20°C.
1	+ 5
2	+ 8
3	- 4
4	- 6
5	- 2
6	+ 7
7	+ 2
8	+ 1
9	- 3
10	+ 3
11	- 5
12	+ 6

$$t = \frac{\bar{d}}{\sqrt{\frac{\text{var.}(d)}{n}}} \quad * \qquad = \quad 0.6897$$

$(P < 0.5)$

The effect of storage on whole blood folate.

* Appendix B1.

Comparison between folate release by "control" and "subject's" plasma:-

Part I:-

The folate values (mug/ml. packed cells) obtained by the use of the "control" and "subject's" plasmas against the subject's/

TABLE 21.

A = Control plasma B = Subject's plasma

Case	Normal Subjects		Megaloblastic Folate def.		Megaloblastic B_{12} def.		Rheumatoid Arthritis	
Total No.	32		19		19		25	
Serial No.	A	B	A	B	A	B	A	B
1	228	230	36	35	166	159	120	105
2	184	183	37	25	163	147	183	160
3	164	155	112	105	108	103	103	111
4	245	279	-	-	247	250	209	198
5	156	145	50	43	171	168	121	112
6	160	143	37	38	204	181	88	70
7	178	161	32	34	49	45	194	166
8	149	143	30	24	148	146	290	305
9	195	190	98	83	213	202	282	255
10	147	140	24	23	-	-	199	177
11	170	185	61	60	98	88	225	179
12	190	186	32	31	151	155	251	215
13	185	164	42	40	88	79	189	150
14	199	170	99	98	130	150	109	117
15	150	162	-	-	188	179	175	130
16	159	177	53	55	61	57	109	91
17	175	186	-	-	105	99	163	131
18	166	167	24	26	148	153	136	118
19	175	184	-	-	-	-	163	164
20	151	152	51	54	104	115	172	169
21	225	195	40	43	50	54	208	188
22	168	152	130	132	-	-	259	259
23	146	147	23	26	-	-	135	129
24	170	167	-	-	-	-	304	263
25	239	235	-	-	-	-	208	188
26	269	231	-	-	-	-	-	-
27	200	176	-	-	-	-	-	-
28	238	240	-	-	-	-	-	-
29	129	138	-	-	-	-	-	-
30	186	204	-	-	-	-	-	-
31	152	174	-	-	-	-	-	-
32	168	186	-	-	-	-	-	-
Mean	182	179	53	51	137	134	183	166
SD ±	34	34	32	31	56	55	61	59

The erythrocyte folate values (mug/ml.) obtained in each group by the use of "subjects" and "control" plasma against subjects erythrocytes.

subject's erythrocytes are detailed for each group in Table 21.

Folate release by these plasmas is compared statistically in Table 22.

TABLE (22).

	Normal	Folate deficient	Vit. B_{12} deficient	Rheumatoid Arthritis.
No of cases.	32	19	19	25
t	0.2837	1.7250	1.4936	4.9016
P	0.8	0.1	0.1 0.2	0.001

The significance of the difference in folate release by "control" and "subject's" plasma in each of the four groups studied.

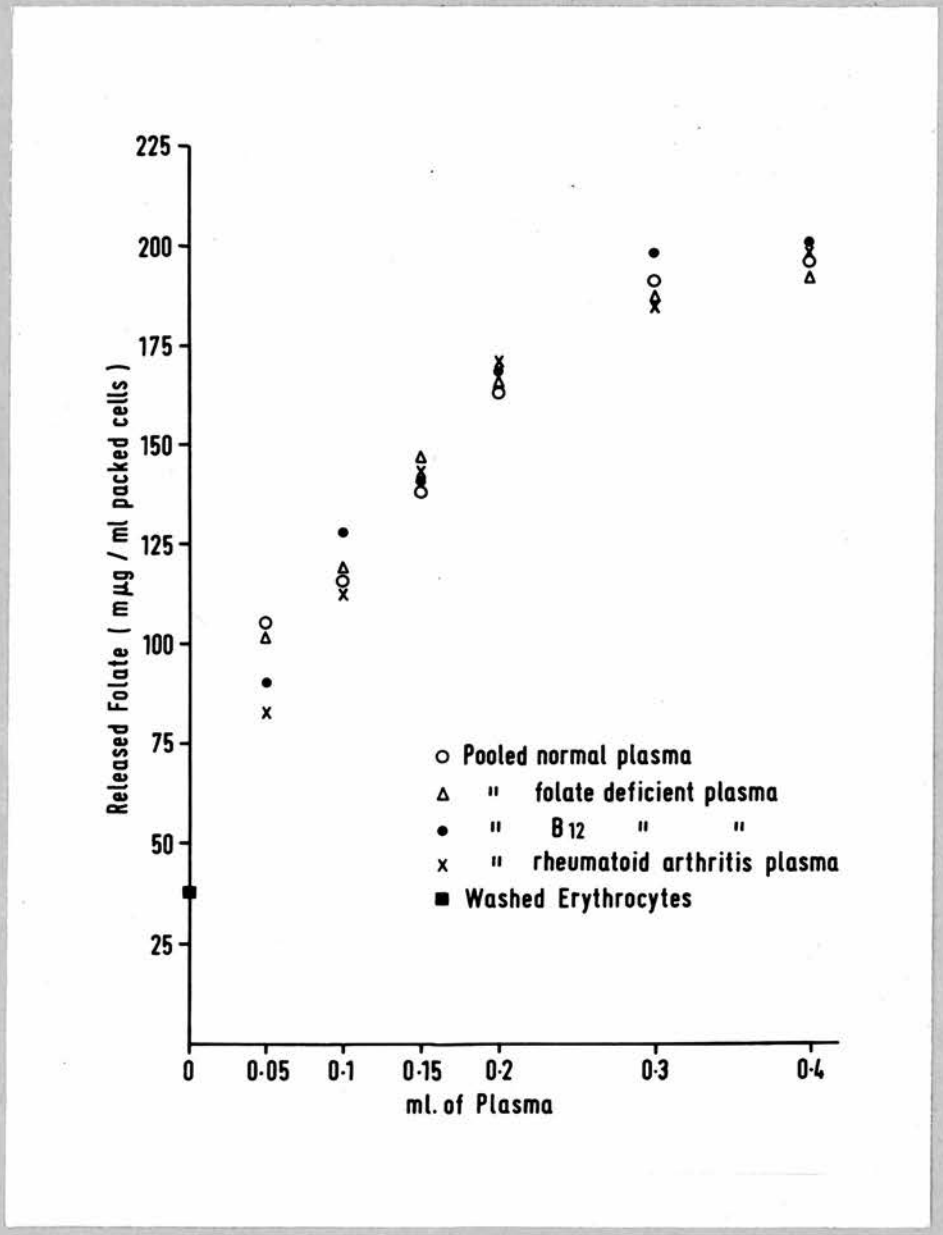
Part II:-

Figure 17 compares the folate release patterns obtained when differing quantities of either "control" or pooled plasma from the three groups of patients were combined with a constant haemolysate volume.

COMMENT.

Table 22 shows that folate release by normal plasma as well as that obtained from patients with megaloblastic erythropoeisis/

FIGURE 17.



The folate release patterns obtained when differing quantities of either "control" or pooled plasma from the three groups of patients were combined with a constant volume of haemolysate prepared from washed normal erythrocytes.

erythropoiesis is not significantly different from that obtained by the use of "control" plasma. Release by rheumatoid plasma on the other hand is significantly reduced when compared to control plasma $P < 0.001$. Thus in four cases (11, 13, 15 and 17) in the latter group (Table 21), folate release by the patient's plasma is less than 85% of that by control plasma and in two of these namely Cases No. 15 and 17, the use of the patient's plasma brought down the erythrocyte folate content to deficiency levels (less than 135mug/ml. packed cells). These findings indicate that in rheumatoid arthritis, the plasma folate releasing ability is reduced to a variable extent and that in some cases the low erythrocyte folate levels resulting from the use of whole blood obtained from such patients may be indicative of a reduced plasma factor action rather than a decrease in the erythrocyte folate precursors.

The Vitamin B_{12} and folate deficiency states on the other hand do not seem to alter significantly the plasma folate releasing characteristics.

When differing quantities of "control" or pooled plasma (from the three disease states) were combined with a constant volume of normal haemolysate, similar folate release patterns were obtained (Figure 17).

The "release curves" overlap at various points and there was no significant difference between the folate yield by the four plasmas at any of the volumes used. This is at variance with the finding (Tables 21, 22) that a lower folate yield/

yield is obtained by recombining plasma and erythrocytes from patients with rheumatoid arthritis than by using normal plasma.

DISCUSSION.

The effect of ascorbic acid and pH on folate release:

When the release of assayable folate was assessed in two series of haemolysates containing differing quantities of ascorbic acid and in both of which complete haemolysis was ensured by freezing and thawing, it was found that the "maximal" yields occurred at ascorbic acid concentrations of 90mg.% or more when the blood dilution was 1/10 and 200mg.% or more in haemolysates prepared at a dilution of 1/5.

Further, at comparable ascorbic acid concentrations folate release was significantly higher in the 1/10 than the 1/5 haemolysates. Analysis of the results showed that these dissimilarities in folate yield were due to different pH patterns in the two series of haemolysates. Thus for a given ascorbic acid concentration the pH was lower at the higher blood dilution; also irrespective of ascorbic acid concentration or blood dilution similar folate values were obtained at similar pH values. This suggested that ascorbic acid might influence folate release by virtue of its acidifying effect rather than by a direct action on the folate releasing mechanism. In terms of pH the maximal folate values in these haemolysates occurred within a range of 5.8 to 3.6. The concept of a "pH" effect was partly supported by finding that a modification of pH alone with HCl in the absence of ascorbic acid markedly affected folate release. Under these circumstances "maximal" folate release/

release occurs within a pH range of 4.7 to 6.0 with very little measurable folate at pH values less than 4.0. When haemolysates were prepared at similar pH values but made to contain 25mg.% ascorbic acid as well, maximal amounts of folate were released within a much wider pH range namely 3.1 to 6.1 (Figure 9). This discrepancy in folate yield between the two experiments was explained by the extreme lability of folate at pH values less than 4.7 and its complete protection by ascorbic acid.

One is not aware of a reference in the literature to the protective effect of ascorbic acid at low pH on assayable folate. The underlying mechanism is bound to be chemical and related to the preservation of the integrity of the molecular configuration of the assayable N^5- methyltetrahydrofolic acid monoglutamate.

A modification of pH alone in the absence of plasma was ineffective in releasing folate from washed erythrocytes. It would appear, therefore, that plasma is capable of releasing assayable folate from erythrocytes maximally within a pH range of 3.1 to 6.1 and that at pH values less than 4.7 ascorbic acid is needed for protecting the released folate. The latter finding would explain why Toennies et al. (1953), who did not include ascorbic acid in their experiments, found that folate yield approached zero at pH 4.0 and that optimal yield was around pH 5.7. Even within the optimal pH range 3.1 to 6.1 folate is apparently released at a variable rate. Thus in two samples investigated the/

the amount of folate released after 10 minutes at room temperature was 99% and 102% respectively at pH 3.6 as compared to 47% and 31% at pH 5.8 (Table 18). This might indicate that there is more than one plasma factor each of which has different kinetics and pH requirements. The possible multiplicity of the plasma factor had been suggested previously by Toennies and Phillips (1959) who found that the stepwise elution of a crude plasma factor preparation from DEAE cellulose gave 4 fractions with different plasma factor activities. On the other hand there may be only one enzyme-substrate system which can, within its own optimum pH range, reach equilibrium much more rapidly at lower than at higher pH values. Folate is present inside the red cell as conjugates of N^5-methyltetrahydrofolic (Noronha and Aboobaker 1963). It is not known for certain how or to what these precursors are attached inside the cell although it has been suggested that they may be protein bound (Iwai et al. 1964). As a fall in pH alone in the absence of plasma does not release assayable monoglutamates from these conjugates, it is possible that pH facilitates folate yield by releasing the substrate from its binding inside the cell and so exposing it to enzymatic action.

Incubation at 37°C.:-

The observations of Hoffbrand et al. (1966a) that maximal folate release occurs in haemolysates prepared in 1g.% ascorbic acid solution without incubation at 37°C. has been/

been confirmed in these studies. The folate yields in subsequent experiments have been assessed after freezing and thawing and calculated as percentages of the folate released after the incubation of haemolysates containing 900mg.% ascorbic acid for 90 minutes at 37°C. The occurrence therefore in these experiments, e.g. those investigating the effects of ascorbic acid or pH, under optimal conditions of mean folate values ranging from 90 to 99% indicates that folate release after freezing and thawing is comparable to that after incubation. Further incubation did not increase significantly the "suboptimal" folate values obtained by freezing and thawing haemolysates containing 0 - 50mg.% ascorbic acid. It would seem, therefore, that a temperature of 37°C. is not an important requisite for folate release in whole blood haemolysates prepared by the described methods.

The pattern of folate yield described by Toennies et al. (1956) during the incubation of whole blood at 37°C. in 0.05 M phosphate buffer was partly explained by the initial incomplete haemolysis of blood and its enhancement with increasing incubation time. The 90 minutes incubation period recommended by these workers for maximal folate yield is in marked contrast to the few minutes required at room temperature for such a release to occur when haemolysis was carried out at a pH of 3.6 and a blood dilution of 1/10. The adoption by Toennies et al. of a pH of 6.1 as well as an excessively high blood dilution (1/750) may have, in addition to incomplete haemolysis/

haemolysis, contributed to the marked delay in reaching the folate peak.

The choice of a method for whole blood estimation:-

The results of the study indicate that the method described by Hoffbrand et al. (1966a) is to be preferred to other published methods. In this method haemolysis is carried out at low pH (approximately 3.6) and in the presence of ascorbic acid both of which conditions have been shown to cause rapid folate release. The authors had shown that the folate content of these haemolysates is not affected by storage for up to 5 months at -20°C.

SUMMARY.

1. The release of assayable folate from erythrocytes occurs maximally within pH range 3.1 - 6.1. This optimum pH range is much wider than previously reported.

2. In the absence of ascorbic acid the released folate is extremely "labile" at pH values less than 4.7. Ascorbic acid concentrations of 25mg.% or more afford complete protection.

3. Folate is released much more rapidly at lower than at higher pH values within the optimum pH range (3.1 - 6.1). The possible causes of this are discussed.

4. A modification of pH alone i.e. in the absence of plasma was not associated with the yield of assayable folate from erythrocytes.

5. Incubation was found unnecessary for folate release from erythrocytes even when tested at different pH values or ascorbic acid concentrations. Explanations were offered for the findings of Toennies et al. (1956).

6. Plasma from patients with megaloblastic erythropoeisis is as efficient as normal plasma in releasing folate; but folate release by plasma from patients with rheumatoid arthritis is significantly reduced when compared to normal.

7. The method described by Hoffbrand et al. (1966,a) for the estimation of whole blood folate is recommended.

CHAPTER III.

CLINICAL STUDIES.

INTRODUCTION.

Moderate anaemia of a hypochromic normocytic type is a common feature of rheumatoid arthritis. Macrocytic anaemia on the other hand has been reported less frequently. Macrocytosis was regarded as coincidental by Short, Bawer and Reynolds (1957) or due to the use of anticonvulsant drugs (Chanarin, Laidlaw, Loughridge and Mollin, 1960). In the study of the bone marrow of 64 patients with rheumatoid arthritis, Richmond, Gardiner, Roy and Duthie (1956) found no consistent abnormality apart from a moderate increase in plasma cells. Reviewing the literature, these authors found that megaloblastic erythropoiesis had been reported only once in patients with rheumatoid arthritis (Marmount 1948). Partridge and Duthie (1963) found an incidence of macrocytic anaemia attributed to vitamin B_{12} deficiency in 1.38% of 2544 patients with rheumatoid arthritis as compared to an incidence of 0.27% in controls. Other authors (Forshaw 1963, Mollin, Waters, Gough and Read 1963) commenting on this paper suggested that folate deficiency may have existed. Gough et al. (1964) in a study of 46 patients with rheumatoid arthritis reported that 65% had subnormal folate levels and that in 33% the levels fell within the range encountered in megaloblastic folate deficiency. The same study also revealed that 6 of the patients had megaloblastic erythropoiesis due to folate deficiency and that 73% of the patients with/

with subnormal serum folate levels excreted abnormal amounts of FIGLU. Similar percentages of low serum folate levels were reported by Dellar et al. (1966).

It was decided in the present study to undertake a fuller investigation of the folate status in rheumatoid arthritis. Various tests of folate deficiency including erythrocyte folate levels were assessed in the rheumatoid arthritis group. Comparisons were also made with the plasma and erythrocyte folate levels in a group of normal subjects and in patients with megaloblastic erythropoiesis due to folate or vitamin B_{12} deficiency as well as in chronic iron deficiency anaemia.

MATERIAL AND METHODS.

Subjects studied:

Group I:-

The control group consisted of 59 healthy subjects who were either members of the laboratory staff or blood donors; 24 of them were females and 35 males and their ages ranged from 19 to 57 years.

Group II:-

This group comprised 23 patients with megaloblastic folate deficiency (details of cases in Appendix C1). The patients fell in the following aetiological sub-groups:-

(a) 4 cases with primary malabsorptive disease. The diagnosis was established where possible by demonstrating/

demonstrating abnormal absorption of xylose, fat and folic acid and characteristic mucosal changes in jejunal biopsies.

(b) 5 cases with nutritional folate deficiency. The diagnosis of this condition was based on a history of inadequate diet in patients with a normal intestinal absorption of folic acid and in whom no other cause for folate deficiency could be found.

(c) 6 cases forming a mixed group of patients in whom folate deficiency was attributed by exclusion to coexisting diseases known to cause folate deficiency such as lymphoproliferative disorders, drugs, disseminated malignancy etc.

(d) 1 case associated with pregnancy.

(e) In 6 patients investigations were inadequate and therefore the cause of folate deficiency was not determined.

Group III:-

This group consisted of 26 patients with megaloblastic vitamin B_{12} deficiency (See Appendix C2 for details of patients). All cases had histamine fast achlorhydria and impaired absorption of radioactive vitamin B_{12}. The diagnosis of Addisonian pernicious anaemia was confirmed in 16 patients either by showing correction of the abnormal Achilling test by simultaneous exogenous intrinsic factor administration or by demonstrating both a reduced intrinsic secretion/

secretion and total body uptake of radioactive vitamin B_{12}.

Group IV:- (Details in Appendix C3).

This group consisted of 18 cases of severe iron deficiency anaemia. All had a hypochromic peripheral blood picture, low serum iron, high total iron binding capacity and no demonstrable stainable iron in the bone marrow.

Group V:-

This group comprised 37 patients with rheumatoid arthritis (Details of patients in Appendix C4). This diagnosis was based on the criteria laid down by the American Rheumatism Association (Ropes, Bennett, Cobb, Jacox and Jessar 1959).

All patients were admitted to the M.R.C. Rheumatic Diseases Unit, Northern General Hospital, Edinburgh and a full history including details of dietary habits, duration of disease, drugs used in treatment taken. Patients on drugs other than aspirin or corticosteroids were excluded from the study. The disease activity was classified as Grade I (inactive), Grade II (moderately active) or Grade III (very active). The classification was based on the criteria of Duthie et al. (1957) (Table 23) except that the degree of anaemia was not taken into account; this was thought advisable in a study investigating the deficiency of a haemopoietic factor.

Effect of ACTH therapy:-

The folate status of five patients was assessed on admission/

TABLE (23).

Grades of severity (Duthie et al. 1957).

Grade of Severity.	Blood* sedimentation rate mm/hr. (Westergren).	Haemoglobin %	Joint* Involvement	Systemic* disturbance.
I	20 or under	85 or over	No symptoms due to inflammation in joints.	None
II	20-60	65-85	Activity in several joints.	Moderate, Weight steady.
III	over 60	65 or under	Signs of acute inflammation in many joints	Marked, with loss of weight.

* Criteria used for grading of severity.

admission and also after a remission of activity of the disease had been induced by a 4 weeks course of ACTH (20 I.U. intramuscularly daily during the first two weeks followed by 10 I.U. daily during the remaining two weeks). These five patients had generalised joint involvement and systemic manifestations of the disease on admission and none received folic acid during hospitalisation.

Follow-up study:-

Seven patients, none of whom had folic acid during the initial investigation, were recalled 3 months or more after discharge from hospital and their plasma and erythrocyte folate levels re-estimated.

METHODS.

1. Collection of blood:-

As described in Section IV, Chapter II.

2. Plasma folate and serum vitamin B_{12} estimations, folic acid absorption and clearance studies were as described in Chapter I.

3. Erythrocyte folate levels were those obtained using a "control" plasma, as described in Section IV, Chapter II.

4. Haematological methods were those described by Dacie and Lewis (1963).

5. Serum iron and total iron binding capacity were measured by the method of Ramsay (1957).

6. Bone marrow slides were examined independently by two observers/

observers and classified as normoblastic, early megaloblastic or frankly megaloblastic. These observations were based on the overall marrow picture particularly affecting the erythroid and myeloid series. Patients whose only marrow abnormality was the presence of a few giant metamyelocytes were not included in the megaloblastic group.

7. The absorption of ^{58}Co labelled vitamin B_{12} was determined by the Schilling test (Schilling 1954). The patient fasted overnight and after an oral dose of 0.5ug. of ^{58}Co-vitamin B_{12} was given a parenteral injection of 1000ug. of non-radioactive vitamin B_{12} one hour later. The patient fasted for a further hour and collected the total urine passed for 24 hours from the time of the oral dose. Normal subjects excreted more than 12% of the oral dose while patients with pernicious anaemia excreted less than 7.5%.

8. Combined formimino-glutamic acid (FIGLU) and urocanic acid were measured in an 8 hours collection of urine after an oral histidine load of 15g. given to a patient who had fasted overnight. The spectrophotometric method of Chanarin et al. (1962) was used. Urocanic acid and not FIGLU was used as a standard. Urocanic acid was also estimated separately after destroying FIGLU by autoclaving the urine at alkaline pH.

9. Jejunal biopsies were obtained using the Crosby capsule. Specimens were initially examined under a dissecting microscope and then histological sections were prepared.

10./

TABLE (24).

Cases	Normal Controls	Megaloblastic folate deficiency.	Megaloblastic vitamin B_{12} deficiency.	Iron deficiency	Rheumatoid arthritis.
No.	59	23	26	17	37
Range	2.6-10.4	0.3-2.0	2.4-18	2.4-7.2	0.7-6.1
Mean	4.3	1.0	7.5	4.4	2.4
SD±	1.6	0.17	3.9	1.4	1.1

Plasma folate mug/ml.

10. An augmented histamine test meal was carried out as described by Kay (1953).

11. The sensitised sheep cell test (SSCT) for rheumatoid factor was performed by the method of Ball (1952) modified by the use of M.R.C. haemagglutination plates in place of test tubes.

Investigations were carried out in the following order:-

Collection of blood, bone marrow puncture, FIGLU test, folic acid clearance and finally folic acid absorption.

RESULTS.

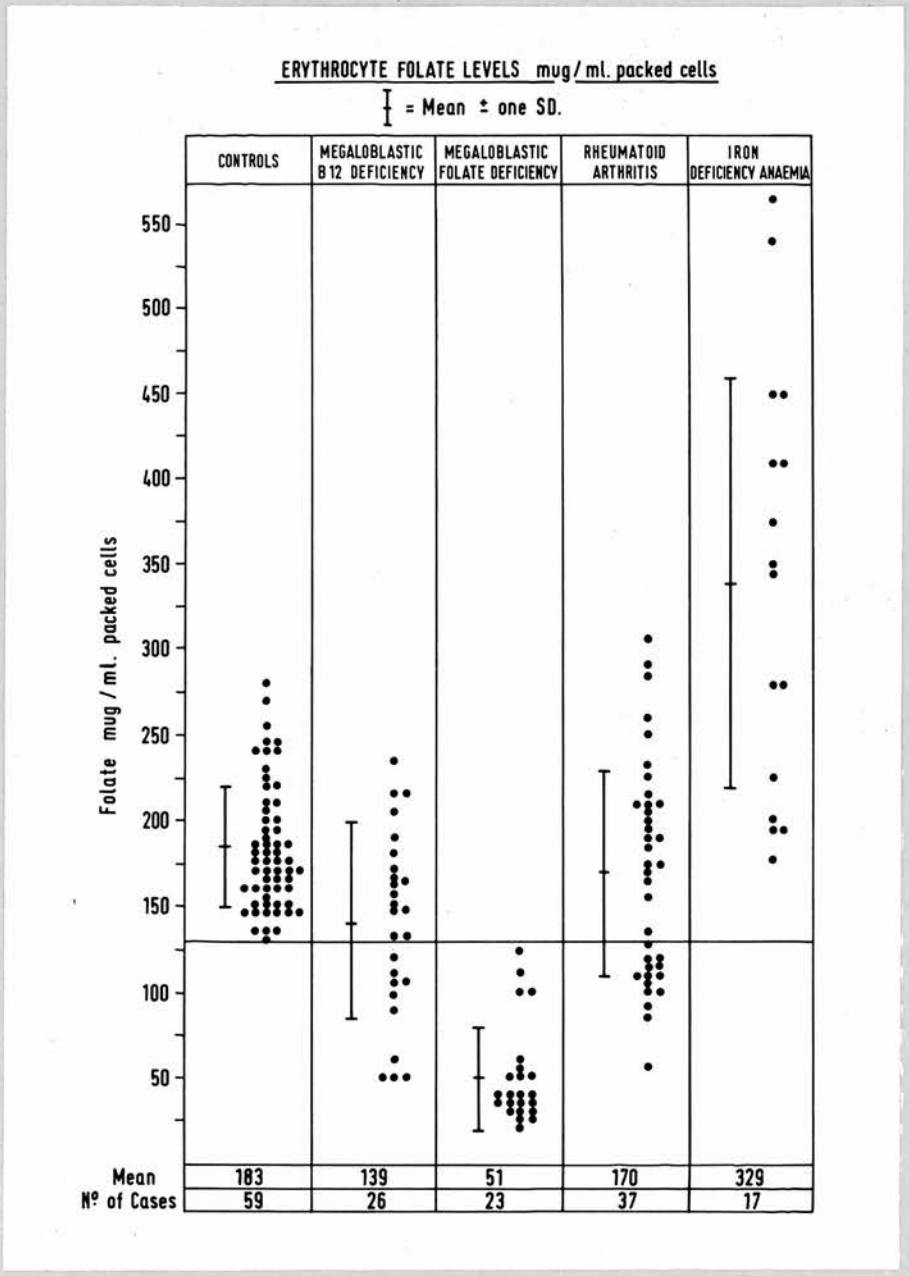
Plasma folate levels:-

The mean and range of the plasma folate content in each group is shown in Table 24.

The erythrocyte folate levels:-

Figure 18 shows the erythrocyte folate values (mug/ml. packed cells) found for all groups studied.

FIGURE 18.



1. **Megaloblastic Anaemia:-**

(i) **Folate deficient:**

All cases had subnormal erythrocyte folate levels (Figure 18). In 83% (19 cases) the levels were less than 60mug/ml. packed cells. There is poor correlation between the degree of anaemia and erythrocyte folate content, $r = 0.3$ $P > 0.1$ (Figure 19).

FIGURE 19.

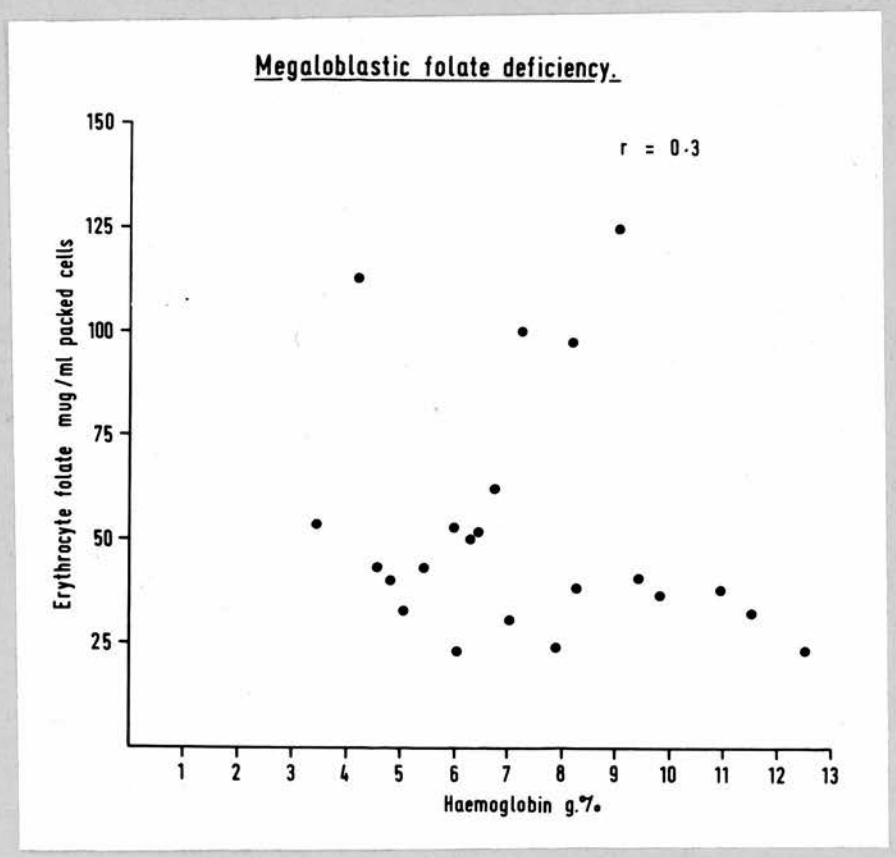
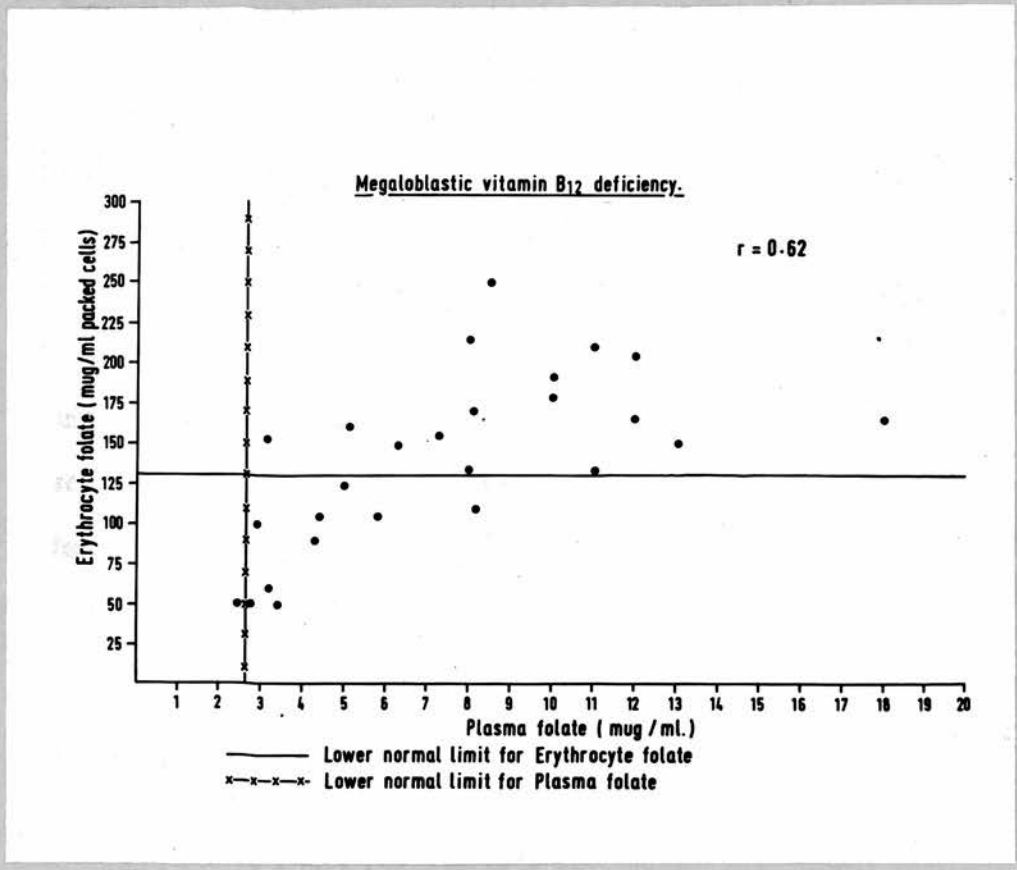


FIGURE 20.



The relationship between plasma and erythrocyte folate content in patients with megaloblastic anaemia due to vitamin B_{12} deficiency. $r = 0.62$ $P < 0.001$.

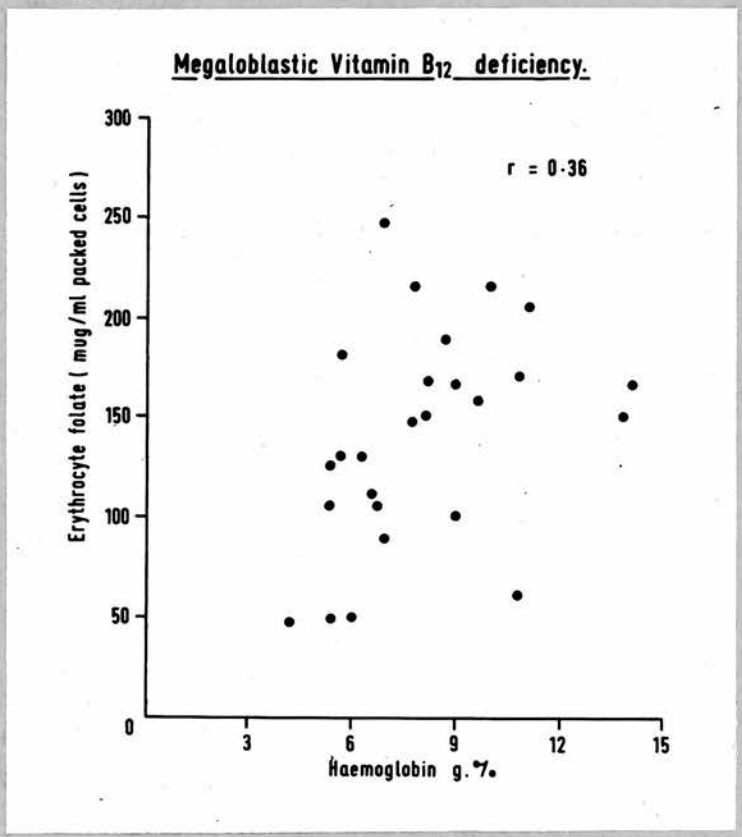
(ii) Vitamin B_{12} deficient:

Figure 18 shows that in 10 cases (38%) the erythrocyte folate levels are subnormal. However, only 4 cases had levels comparable to those encountered in the majority of the folate deficient subjects i.e. less than 60mug/ml. of packed cells.

Figure 20 relates the plasma and erythrocyte folate levels and shows a good correlation between the two $r = 0.62$ $P < .001$.

There is poor correlation between the haemoglobin concentration (g. per cent) and the erythrocyte folate levels (mug/ml. packed cells) (Figure 21) $r = 0.36$ $P < 0.1 > 0.05$.

Figure 21.



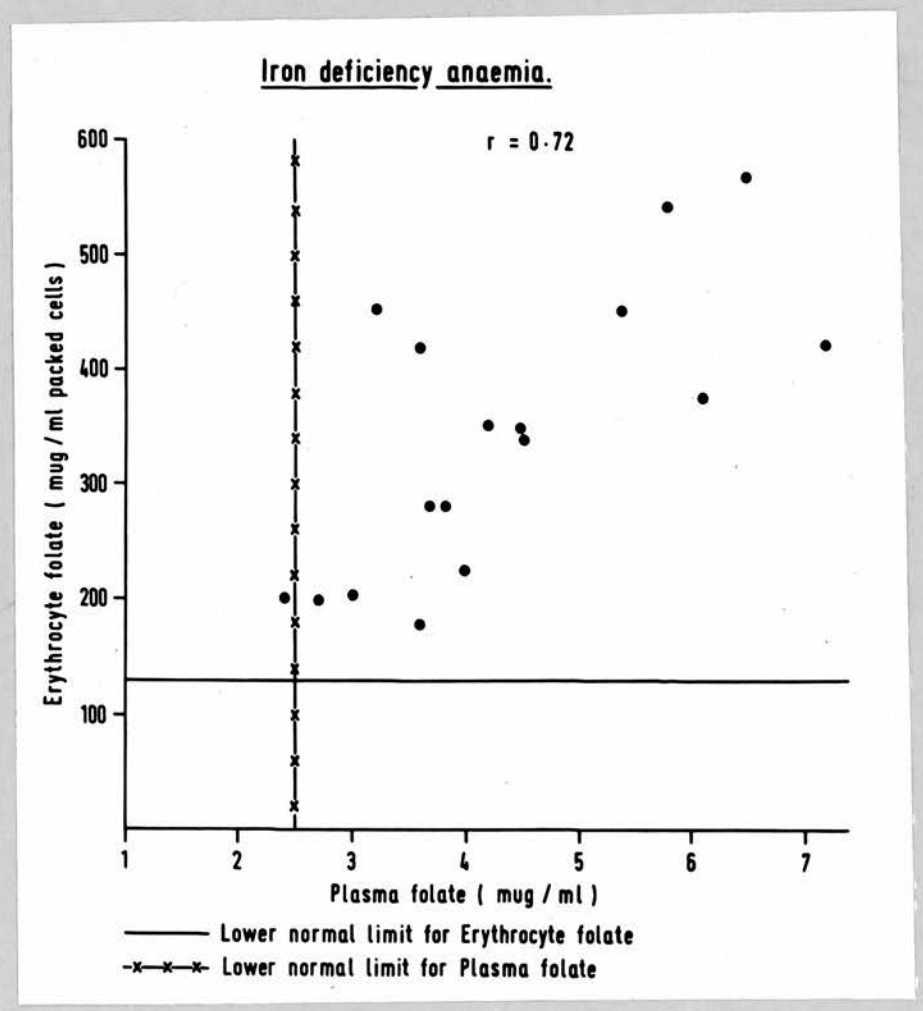
Haemoglobin concentration (g.%) related to erythrocyte folate content in megaloblastic vitamin B_{12} deficiency.
$r = 0.36$ $P < 0.1 > 0.05$.

II. Iron Deficiency Anaemia.

The highest erythrocyte folate levels (mug/ml. packed cells) were observed in this group (Figure 18). The mean 329 SD ± 126 is significantly higher than that of the control group $P < .001$.

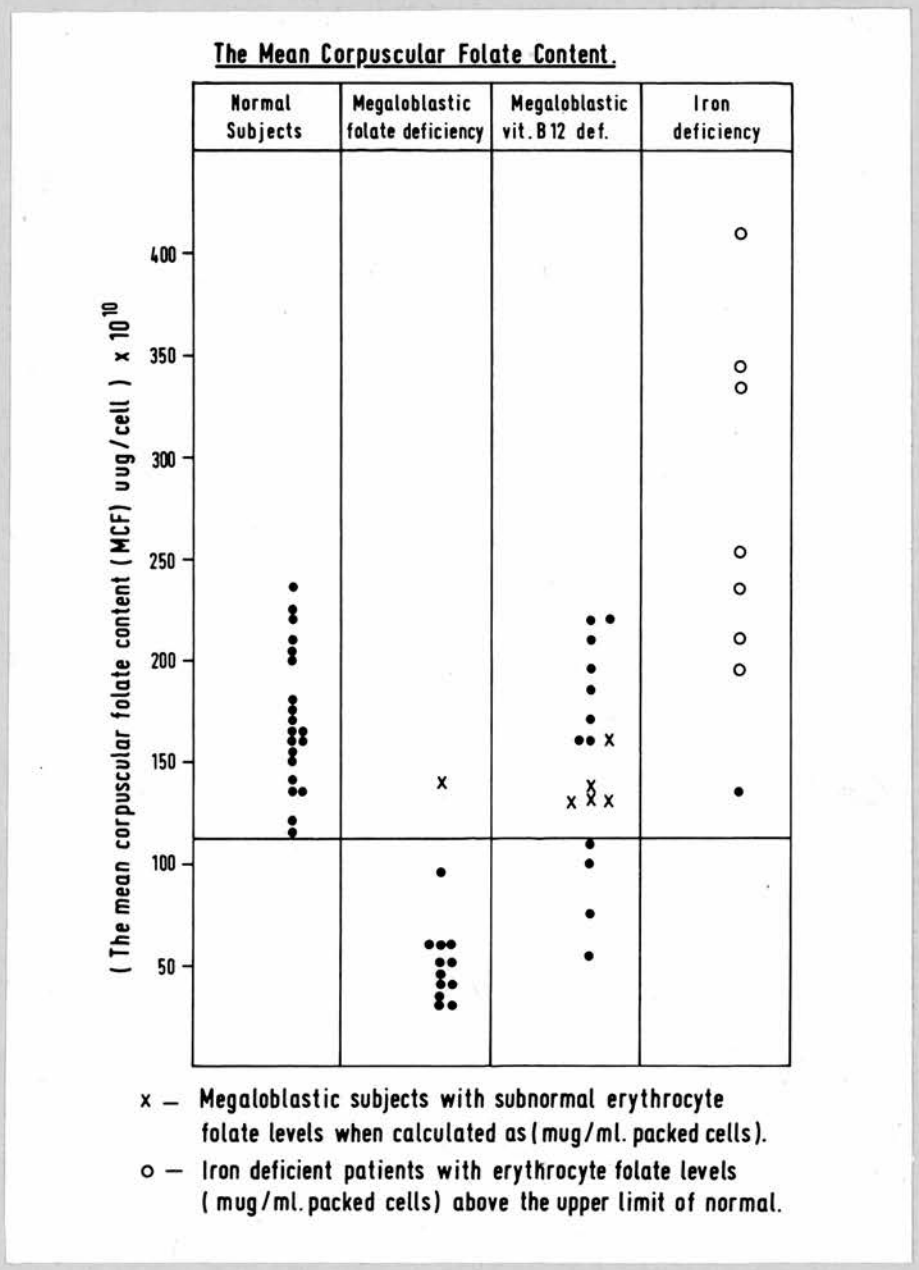
The plasma folate content (mug/ml.) shows a good correlation with the erythrocyte folate level $r = 0.7$ $P < 0.001$ (Figure 22).

FIGURE 22.



$r = 0.7$ $P < 0.001$.

FIGURE 23.



The closed circles represent subjects in whom both erythrocyte folate (mug/ml. packed cells) and MCF values were either normal or subnormal.

THE MEAN CORPUSCULAR FOLATE CONTENT (MCF).

The mean folate content per red cell was calculated as follows:-

$$\text{MCF (uug)} = \frac{\text{Folate uug/ml. of packed cells.}}{\text{Number of erythrocytes/ml. of packed cells.}}$$

The number of erythrocytes in one ml. of packed cells was calculated as follows:-

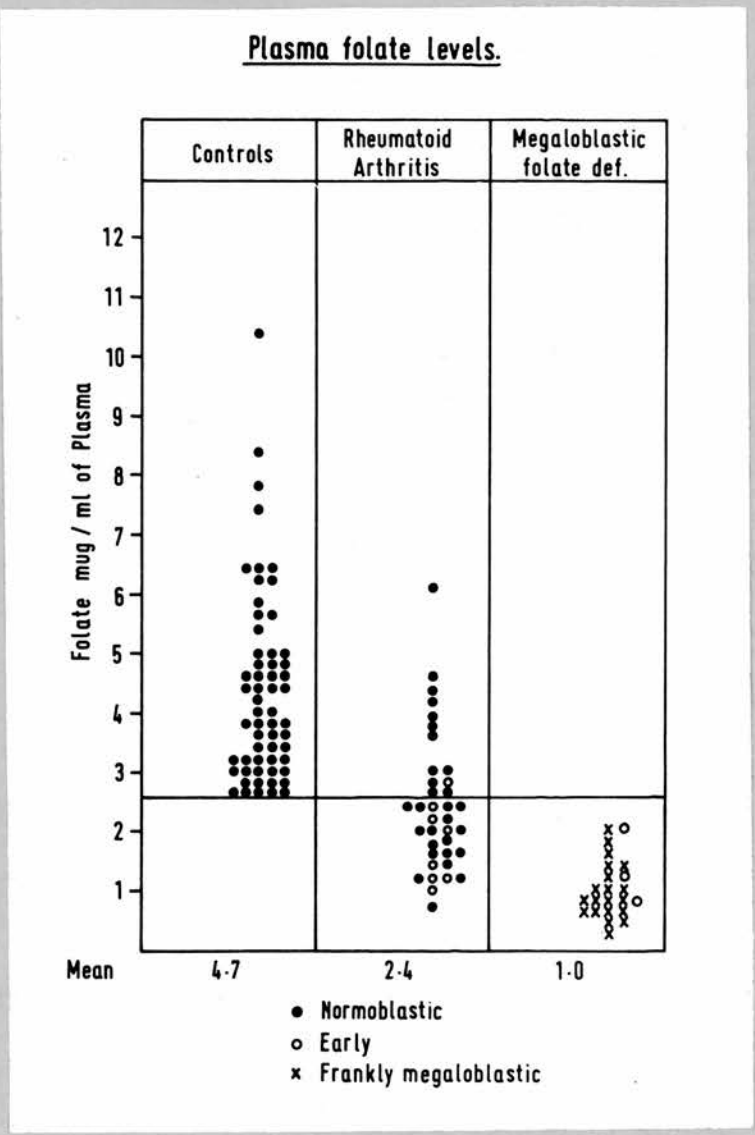
$$\frac{\text{RBC/cumm of blood} \times 100 \times 10^3}{\text{packed cell volume.}}$$

The MCF in 20 normal subjects varied from 1.16 to 2.35×10^{-8} uug. The index was also determined in 13 folate deficient subjects, 17 vitamin B_{12} deficient subjects and 8 cases with iron deficiency anaemia.

All MCF values were multiplied $\times 10^{10}$ and compared in Figure 23.

6 megaloblastic cases (5 vitamin B_{12} deficient and 1 folate deficient) who had subnormal erythrocyte folate levels (mug/ml. packed cells) (Figure 18) were found to have normal MCF values. Conversely 3 iron deficient patients whose erythrocyte folate levels (mug/ml. packed cells) were above the upper limit of normal had MCF values within the normal range. Calculation of the erythrocyte folate content in mug/ml. of packed cells does not take into account the mean corpuscular volume and gives no information therefore on the folate content per cell. The MCF on the other hand stands for the absolute mean folate content per cell.

FIGURE 24.



The open circles shown in this and subsequent figures stand for patients with an early megaloblastic change in the bone marrow.

III. Rheumatoid Arthritis.

Plasma folate levels:-

These are compared in Figure 24 with the levels found for normals and megaloblastic folate deficient subjects. 24 cases (65%) in the rheumatoid arthritis group had subnormal levels i.e. less than 2.6mug/ml. In 17 of these the plasma folate levels fell within the range found for the megaloblastic folate deficient patients i.e. 2.0mug/ml. or less; only five cases in the latter group showed megaloblastic changes in the bone marrow, and in all of these the abnormality was classified as early.

Erythrocyte folate levels:-

Figure 18 shows that 14 cases (38%) have subnormal levels ranging between 55 and 120mug/ml. packed cells.

(a) Related to plasma folate content:-

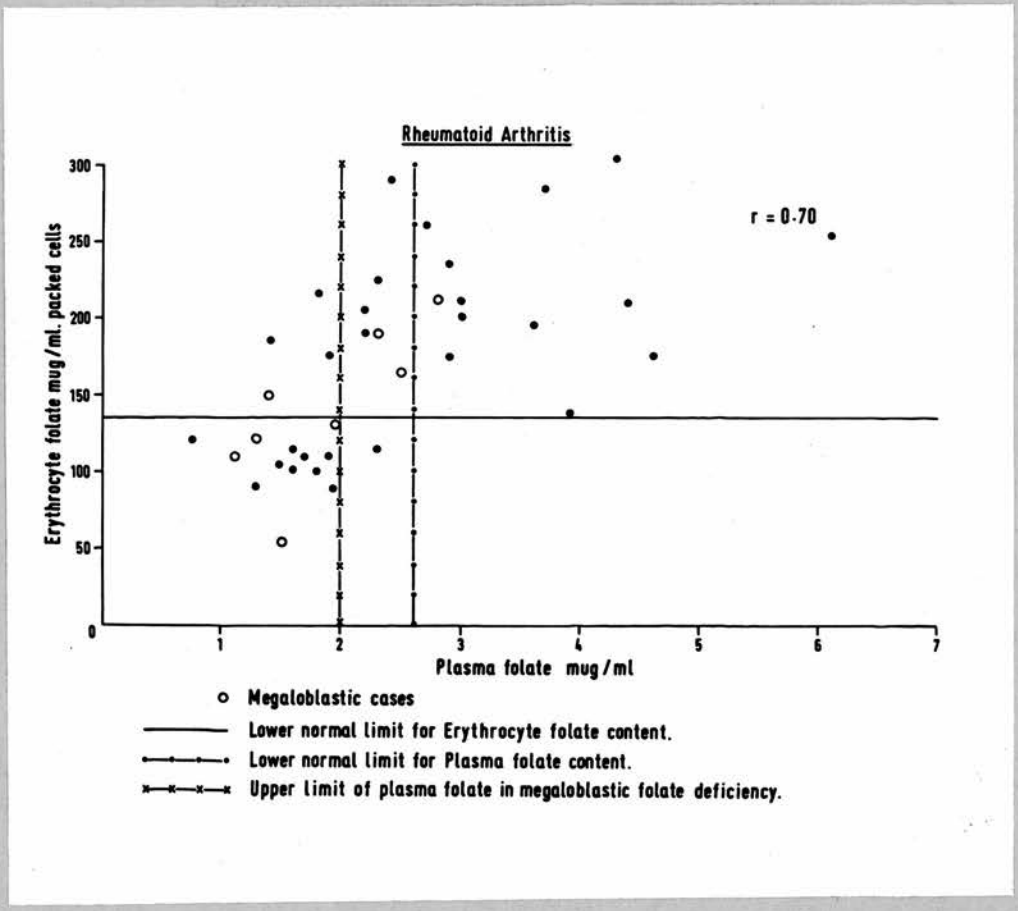
A good correlation is shown between the plasma and erythrocyte folate levels $r = 0.75$ $P < .001$ (Figure 25). The figure also shows that whereas all 14 cases with subnormal erythrocyte folate levels have reduced plasma folate values, there are 10 cases in whom a subnormal plasma folate level is associated with a normal erythrocyte level.

(b) Related to haemoglobin concentration (g.%):-

No relationship was shown in rheumatoid arthritis between the haemoglobin concentration (g.%) and the erythrocyte folate content (mug/ml. of packed cells).

$r = 0.03$ (Figure 26).

FIGURE 25.



Plasma folate content related to erythrocyte folate content in rheumatoid arthritis.

r = 0.70 P < 0.001.

FIGURE 26.

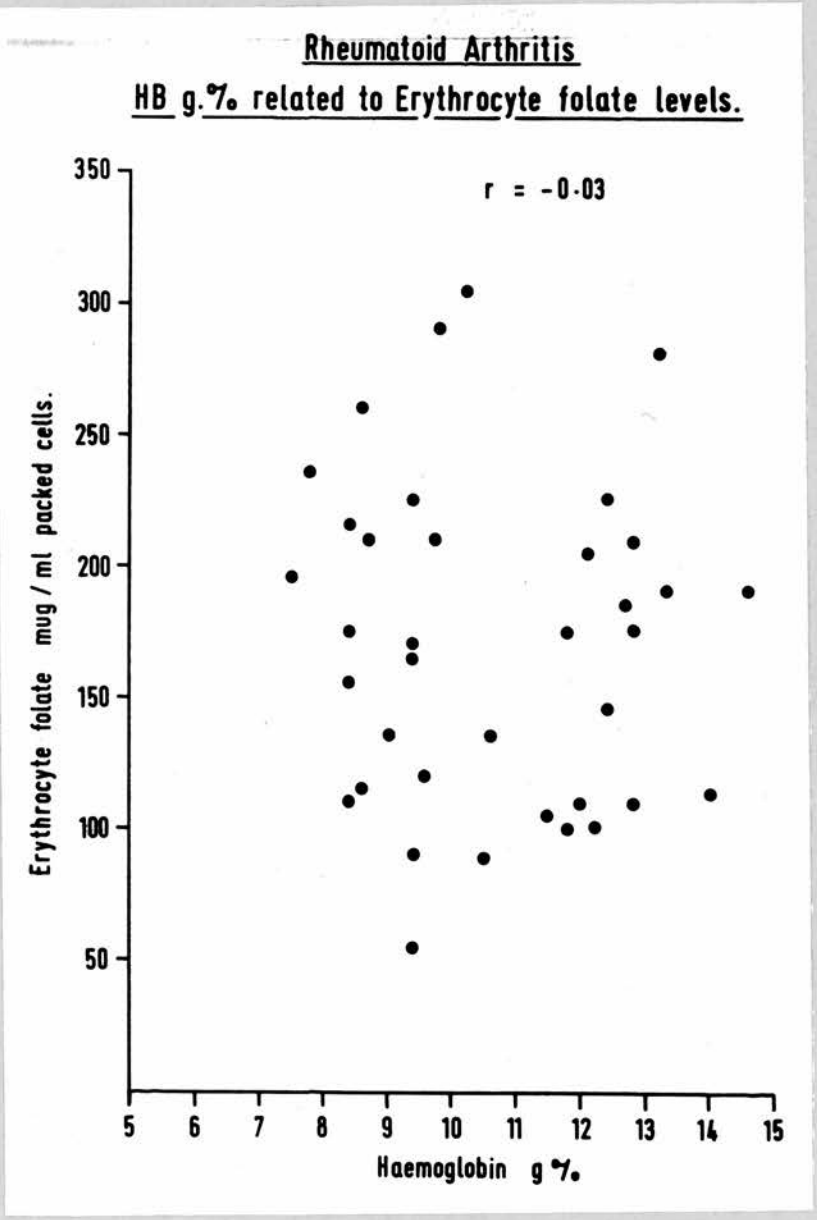
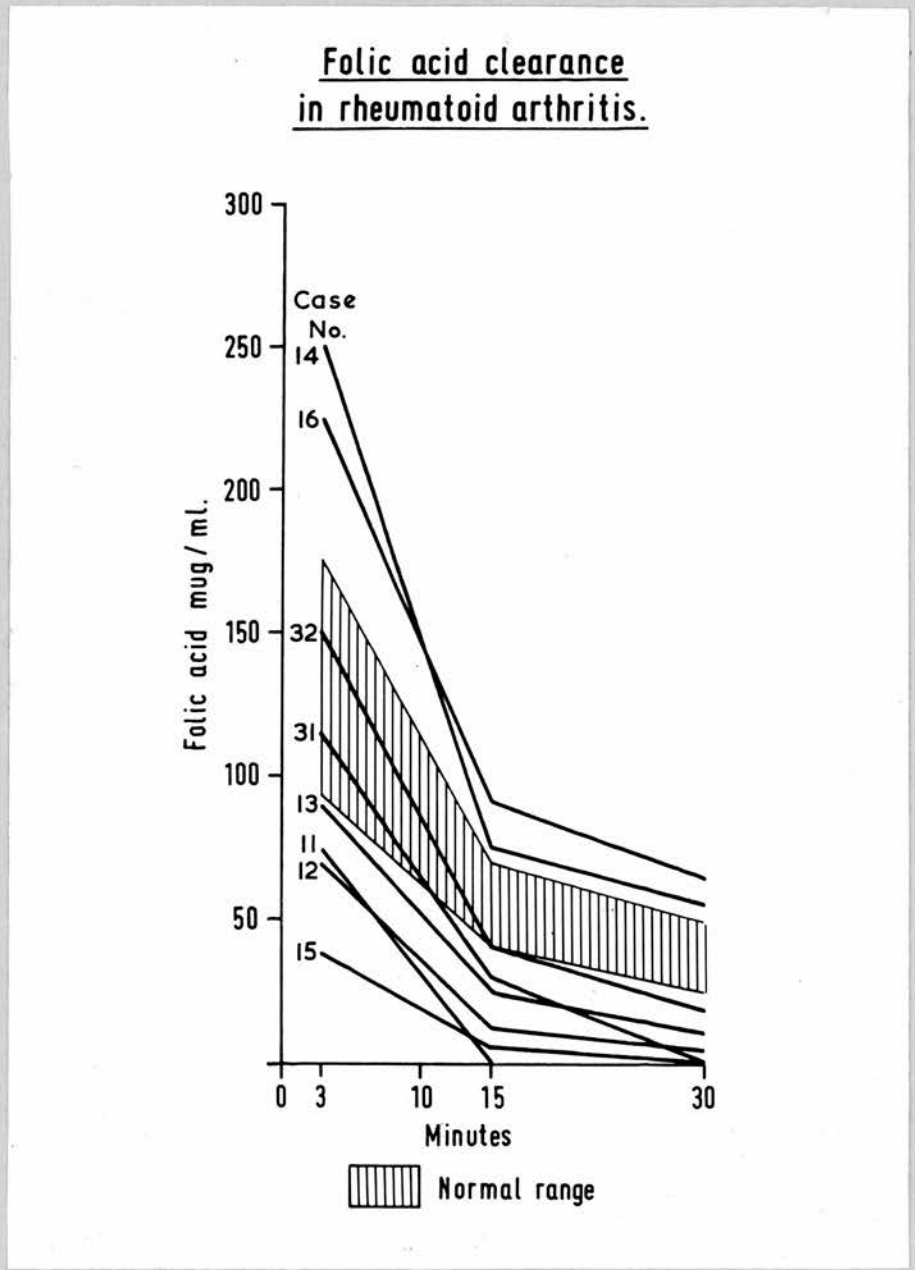

Rheumatoid Arthritis
HB g.% related to Erythrocyte folate levels.
r = -0·03
Erythrocyte folate mug / ml packed cells.
350
300
250
200
150
100
50
5
6
7
8
9
10
11
12
13
14
15
Haemoglobin g %.

FIGURE 27.


Folic acid clearance
in rheumatoid arthritis.
300
250
200
150
100
50
Folic acid mug/ml.
Case
No.
14
16
32
31
13
11
12
15
0
3
10
15
30
Minutes
Normal range

Bone Marrow Findings:-

The bone marrow was normoblastic in 29 cases (80%). The remaining 8 cases showed an early megaloblastic change. The haematological data of these cases are shown in Table 25.

Folic Acid Clearance:-

This test was performed on 8 of the total cases. The results are shown on Figure 27. Table 26 gives the haematological data on these patients. Erythropoeisis was normoblastic in all patients studied. Cases 3, 30 and 5 who showed a very rapid clearance of folic acid also had subnormal plasma and erythrocyte folate levels and two of them (30 and 5) excreted high quantities of FIGLU and urocanic acid in the urine. A rapid clearance (especially at 15 and 30 minutes) was also obtained in Case 4 in whom the only abnormality was excessive excretion of histidine metabolites and Case 20 who had a subnormal plasma but normal erythrocyte folate level.

Histidine Metabolites in Rheumatoid Arthritis.

The range for FIGLU excretion in 13 normal subjects studied was 0 - 20 mg/8 hours, and for urocanic acid 0 - 4mg/8 hours. The upper limit of normal for combined FIGLU and urocanic acid excretion or FIGLU alone is taken as 25mg/8 hours while that for urocanic acid is considered as 5mg/8 hours.

TABLE (25).

Case No.	Duration of disease.	Activity	Hb. G%.	Bone marrow	Plasma folate mug/ml.	R.B.C. folate mug/ml. packed cells.	mg/8 hrs. FIGLU	mg/8 hrs. Urocanic acid.	Folic Acid Absor.	Serum B12 uug/ml.
33	8 yrs.	++	9.4	early megalo-blastic.	1.5	55	28	17	Normal	212
1	-	++	12.4	"	1.3	120	35	184	Normal	168
13	8 yrs.	++	14.6	"	2.3	189	12	4	-	288
16	2 "	+++	12.0	"	1.1	109	55	2	-	300
17	15/12	+++	8.2	"	1.4	153	60	0	Normal	472
18	3/12	++	10.6	"	2.0	130	67	615	-	240
25	7 "	+	12.8	"	2.8	208	58	26	-	244
19	4 "	+++	9.4	"	2.5	163	138	15	-	148

Data on megaloblastic cases.

TABLE (26).

Case No.	Duration of disease.	Activity	Hb. G.%	Bone Marrow	Pl. folate mug/ml.	R.B.C. folate mug/ml.	mg/8 hrs. FIGLU	mg/8 hrs. Urocanic Acid	Folic Acid clearance.	SE. B12 uug/ml.
3	1 yr.	++	11.5	Normoblastic	1.5	103	24	13	Fast	256
30	10 "	++	8.6	"	2.3	113	110	75	"	168
4	1 "	++	8.7	"	4.4	209	217	211	"	308
28	6 "	+	12.1	"	2.2	205	0	0	Normal	168
5	7 "	++	9.6	"	0.7	120	132	62	Fast	488
6	14 "	++	10.5	"	2.0	88	20	11	Normal	424
20	8 "	+++	9.4	"	1.9	172	46	13	Fast	320
21	1 "	++	9.7	"	3.0	208	616	281	Normal	392

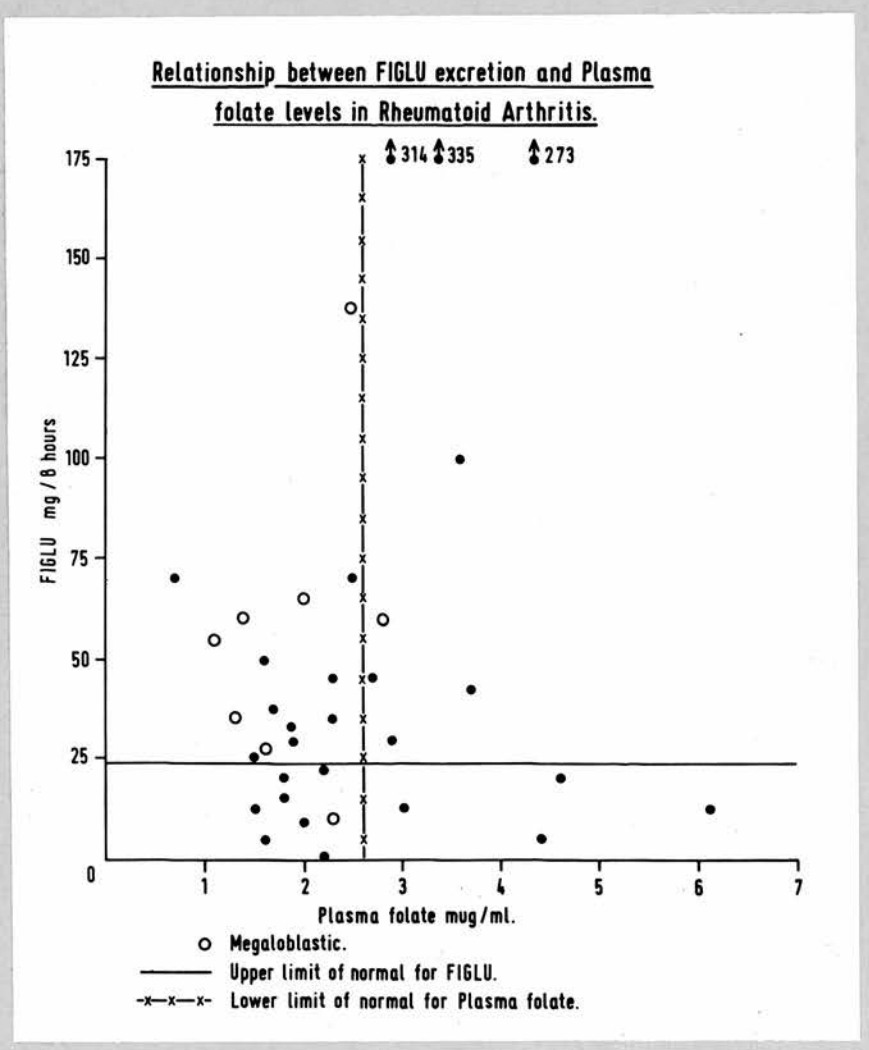
Data on patients on whom folic acid clearance studies were carried out.

FIGLU Excretion in Rheumatoid Arthritis.

(i) Relation to plasma folate:-

23 of 35 cases investigated (64%) excreted abnormal quantities of FIGLU in the urine (more than 25mg/8 hrs). 8 of these had normal plasma folate levels (more than 2.6mug/ml). Conversely FIGLU excretion was normal in 8 cases with subnormal plasma folate levels (Figure 28).

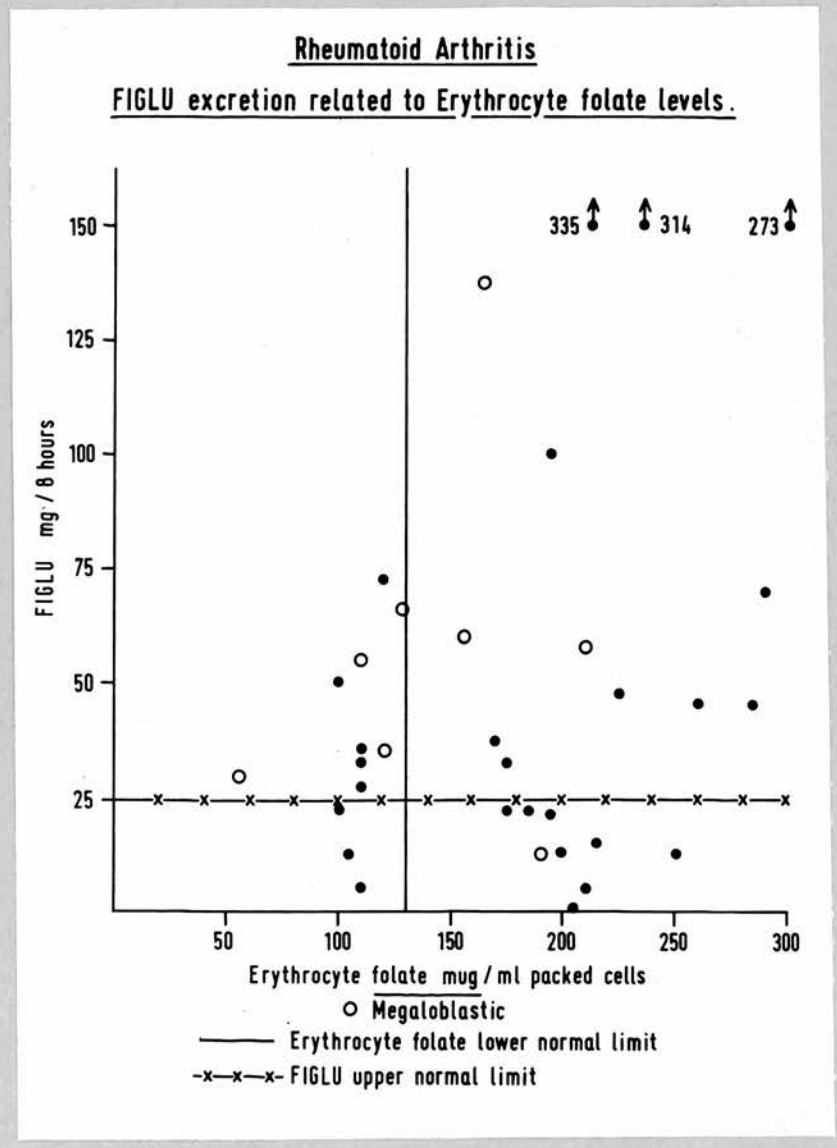
FIGURE 28.



(ii) Relation to erythrocyte folate levels:-

Figure 29 shows that 13 of those who excreted abnormal quantities of FIGLU had normal erythrocyte folate levels and that the highest quantities excreted fell in this group. Also three cases with subnormal erythrocyte folate levels excreted normal quantities of FIGLU.

FIGURE 29.



Urocanic Acid Excretion:

23 cases (66%) excreted urocanic acid in excess of 5mg/8 hours (Figure 30). Excessively high levels (more than 150mg/8 hours) were obtained in 4 cases. Two of these had a normal bone marrow morphology and erythrocyte folate levels.

FIGURE 30.

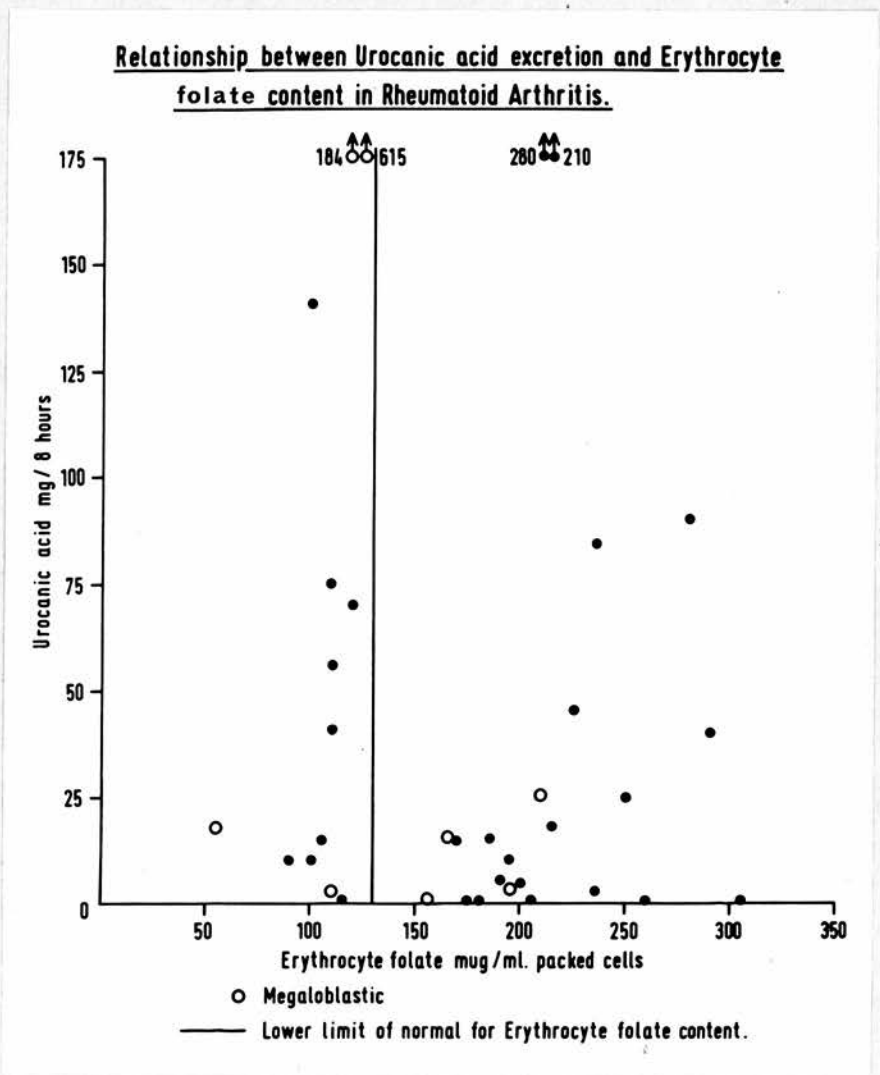
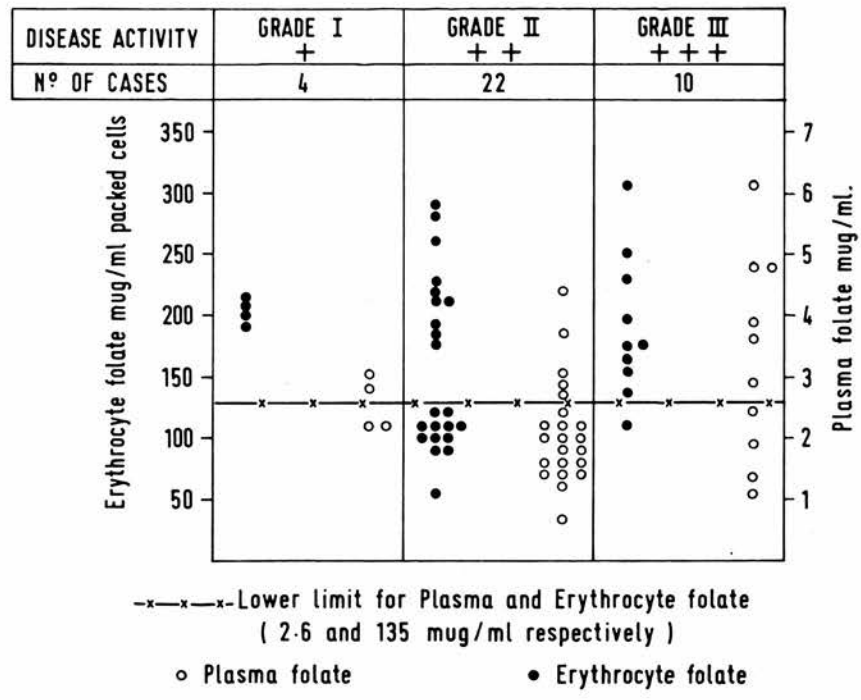


FIGURE 31.

Relation of Plasma and Erythroyte folate content to disease activity in rheumatoid arthritis.



Relation of various parameters to activity and duration of of disease in rheumatoid arthritis.

1. Disease Activity:-

(a) The erythrocyte and plasma folate content are related to disease activity in Figure 31. There appears to be no correlation of either measurement with disease activity. Thus of the 22 cases listed as Grade II, 50% had subnormal erythrocyte folate levels and 77% subnormal plasma folate levels. The corresponding percentages in Grade III are 10 and 40 respectively.

(b) The relation of total FIGLU and urocanic acid to disease activity is shown in Figure 32. The number of patients with Grade I disease activity is too small to allow statistical comparison with the other groups. All patients with Grade III disease activity excreted abnormal quantities of histidine metabolites in the urine as compared to 80% (18 cases) of those with Grade II. The much smaller number of patients in the former Grade, however, makes the significance of such a finding doubtful.

(c) The effect of ACTH therapy on the plasma and erythrocyte folate values:-

The haematological indices and the folate values assessed in five subjects before and after a remission of the disease activity had been induced by ACTH therapy are shown in Table 27.

FIGURE 32.

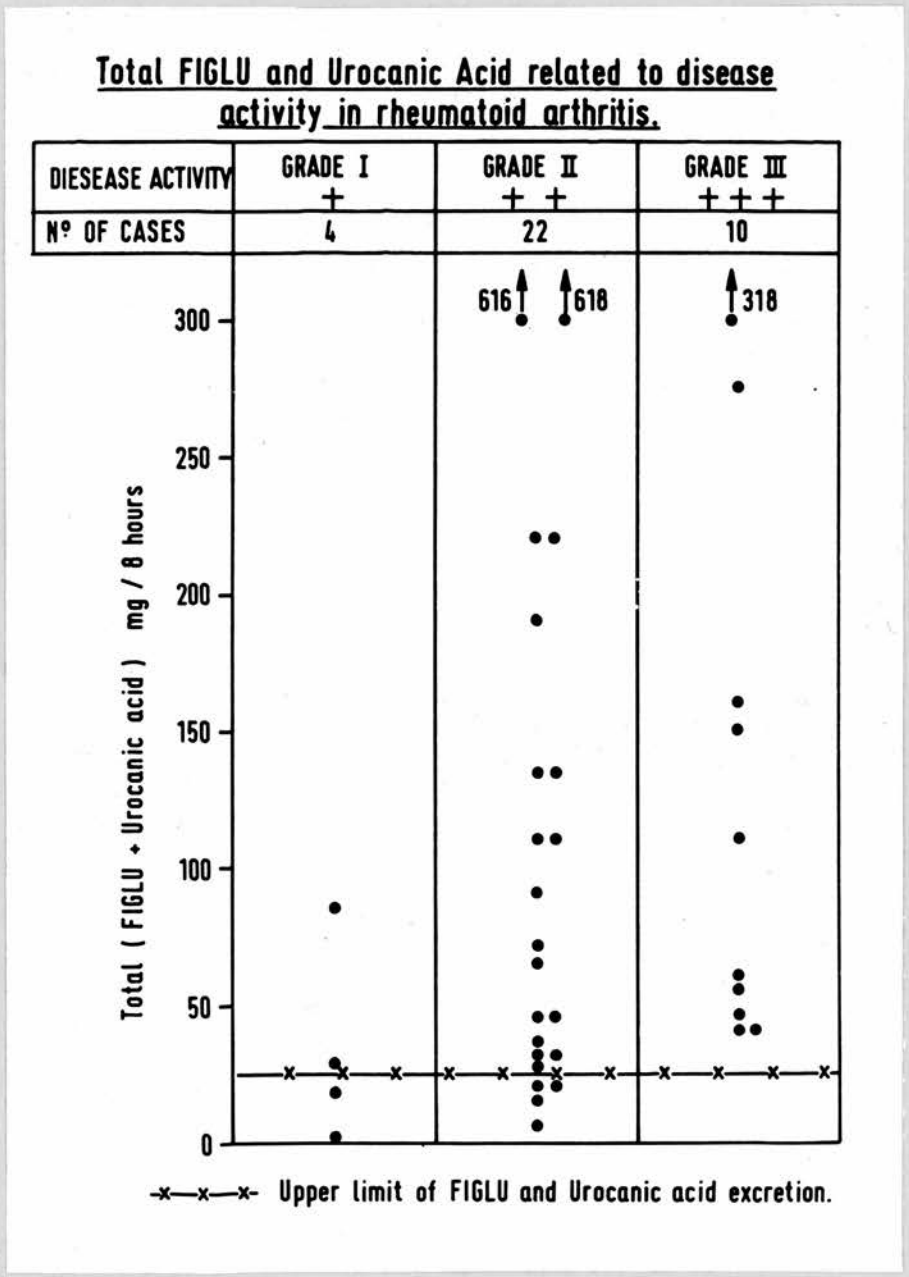

Total FIGLU and Urocanic Acid related to disease activity in rheumatoid arthritis.
DIESEASE ACTIVITY
GRADE I
+
GRADE II
+ +
GRADE III
+ + +
Nº OF CASES
4
22
10
616
618
318
300
250
200
150
100
50
0
Total (FIGLU + Urocanic acid) mg / 8 hours
Upper limit of FIGLU and Urocanic acid excretion.

TABLE (27).

Case No.	A. before ACTH B. after "	Hb. G%	E.S.R. mm/st hr. West.	Grade of severity.	Plasma Folate mug/ml.	Erythrocyte folate mug/ml.cells
30	A	9.6	70	III	1.4	153
	B	14.6	10	I	1.3	125
27	A	8.2	87	III	2.5	163
	B	11.4	25	I	2.2	155
33	A	8.6	48	II	2.7	259
	B	13.5	4	I	2.3	245
35	A	10.7	74	III	3.9	135
	B	15.4	6	I	4.0	177
34	A	9.0	77	III	4.3	304
	B	10.2	18	I	4.5	286

The "initial" and post ACTH data on the five subjects studied.

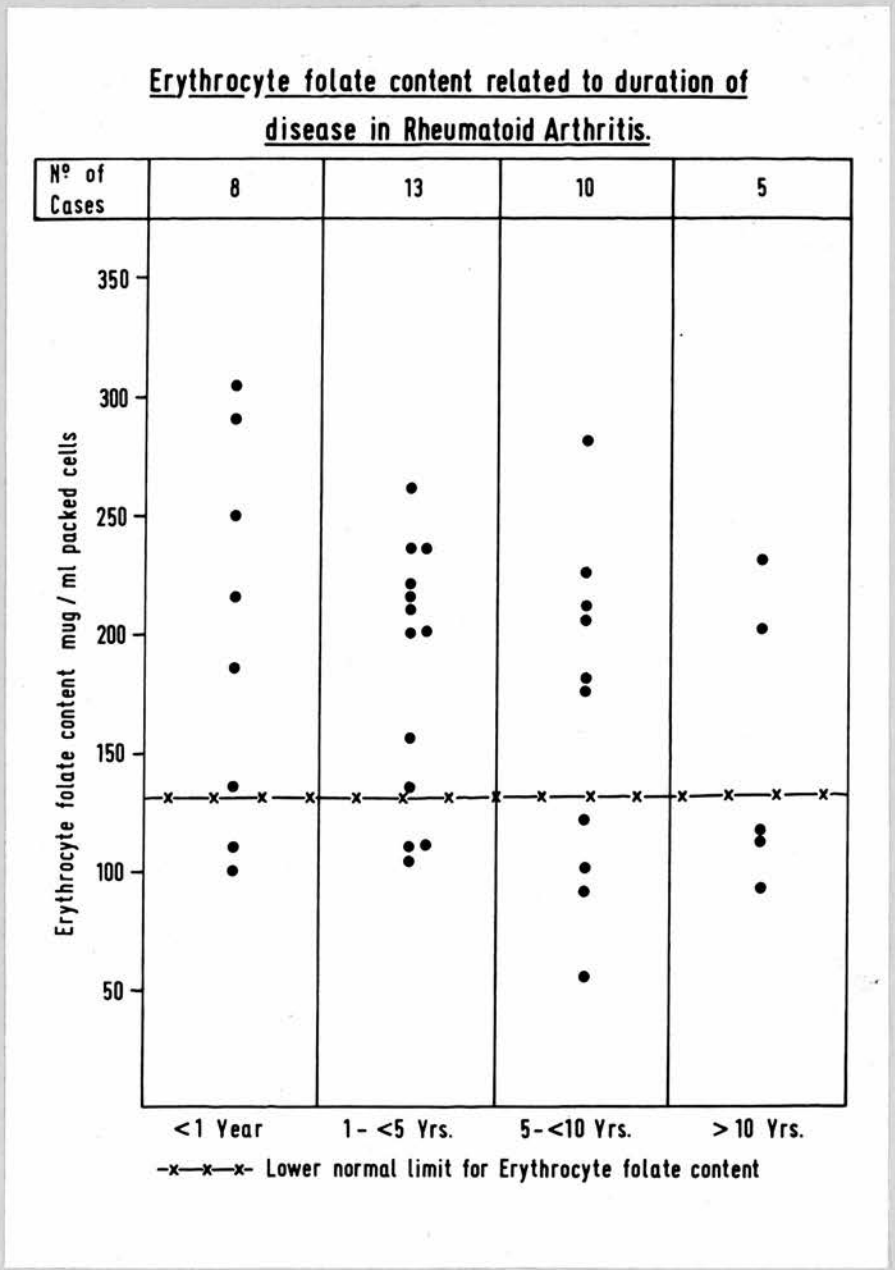
The differences between the initial and post ACTH plasma and erythrocyte folate values were not significant.

2. Relation of erythrocyte folate level and combined FIGLU and urocanic acid excretion to duration of disease.

(i) Erythrocyte folate levels:-

Figure 33 shows that there is no relationship between chronicity of the disease and the erythrocyte folate content and that there is considerable overlap between the four groups.

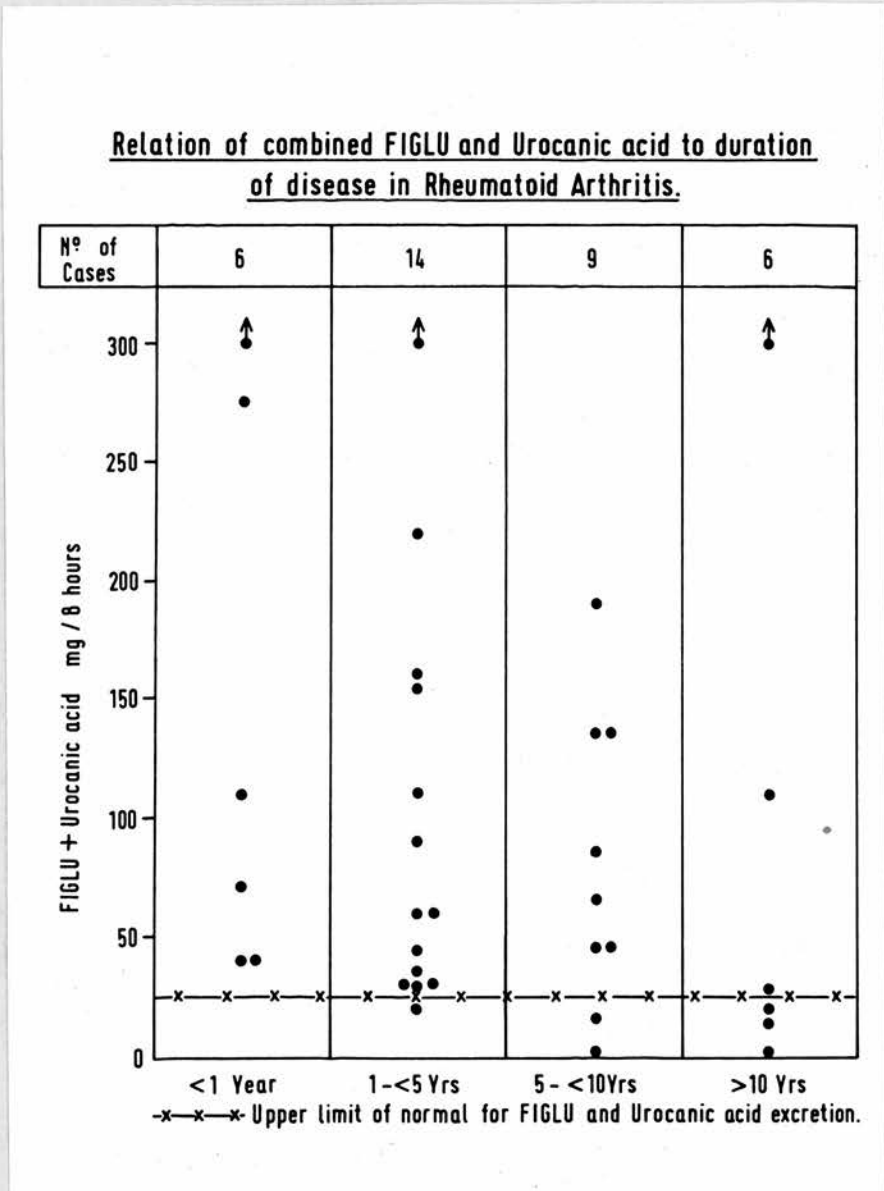
FIGURE 33.



(ii) FIGLU and urocanic acid:-

Similarly no correlation was shown between this parameter and duration of disease (Figure 34). Four of the cases who had the disease for more than 10 years (13, 14, 25 and 30 years respectively) excreted normal quantities while two cases who had it for 3 and 4 months respectively excreted 110 and 680mg/8 hrs.

FIGURE 34.



FOLLOW-UP STUDY.

The plasma and erythrocyte folate values were determined for 7 patients with rheumatoid arthritis three months after discharge from hospital and compared in Table 28 with those which were obtained at the beginning of the study. None of these patients had received any folic acid.

TABLE (28).

Initial and follow-up plasma and erythrocyte folate levels.

	Plasma folate mug/ml.		Erythrocyte folate mug/ml. packed cells.	
	Initial	F. up	Initial	F. up
1	2.3	1.9	225	233
2	6.1	4.7	251	185
3	2.4	1.5	290	205
4	1.9	2.0	109	160
5	2.3	2.3	189	144
6	3.7	2.7	282	222
7	3.0	3.5	199	204

$$t = \frac{d}{\sqrt{\frac{(var)d}{n}}} = 1.700 \ (df,6) \qquad 1.465 \ (df,6)$$

$< 0.1 \qquad < 0.2$

* See Appendix B1.

The follow up plasma and erythrocyte folate values of cases 2, 3 and 6 are lower than the initial ones. Statistically however, the difference in either parameter is not significant for the number of patients studied.

The excretion of histidine metabolites in rheumatoid arthritis patients with normal erythropoiesis and folate values.

Twelve patients who had normal plasma and erythrocyte folate values and in whom erythropoiesis was normoblastic were found among the 37 cases of rheumatoid arthritis studied. 10 of these excreted abnormal quantities of either FIGLU or urocanic acid or both in the urine (Table 29). Excessive amounts (more than 100mg/8 hours) of combined histidine metabolites were obtained in 4 cases with Grade III and 3 cases with Grade II disease activity respectively. Case 10 who was only mildly affected excreted normal amounts. Five of a total of 10 cases with generalised severe disease (Grade III) (Figure 31) belong to the group of patients in Table 29.

TABLE (29).

Case No.	Duration of disease, yrs.	Activity I, II, III + ++ +++	mg/ 8 hours. FIGLU	Urocanic Acid	Total.
4	1	++	6.0	211	217
7	2	+++	99	9.0	108
9	9	++	42	91	133
12	7/12	+++	14	25	29
21	1	++	335	280	616
22	18/12	++	46	0	46
23	1	+++	60	100	160
24	6/12	+++	273	0	273
34	22	+++	314	3.0	318
10	13	+	13.0	4.0	17
27	1	++	21	0	21
15		++	32	0	32

Excretion of histidine metabolites in rheumatoid arthritis patients with normal folate values and bone marrow morphology.

See Appendix C4 for more data on these patients.

The effect of plasma from rheumatoid arthritis patients on the folate content of normal plasma.

METHOD.

Fresh plasma samples were obtained as described previously from a normal subject and three rheumatoid arthritis patients. Each patient's plasma was combined with differing quantities of normal plasma to give mixtures containing 20%, 50% and 80% respectively of the former. 10mg. of dry ascorbic acid per ml. was added to all plasma mixtures as well as to the original 4 samples. All samples were incubated for 30 minutes at 37°C. and then extracted and assayed as described.

RESULTS.

The folate values (mug/ml.) obtained for the nine combined plasma samples are shown in Table 30. They are compared with the "expected" values which were calculated from the folate contents and relative percentages of normal and patient plasmas in the various mixtures. The assayed and expected values are similar even in samples containing 80% of patient plasmas, which indicates that plasma from patients with rheumatoid arthritis has no inhibitory effect in the utilisation of folate by L. Casei.

TABLE (30).

R/A plasma %	100	0	20%		50%		80%	
Normal " %	0	100	80%		50%		20%	
Sample No.			Assayed	Expect.	Assayed	Expect.	Assayed	Expect.
1	2.6	4.4	4.0	4.0	4.0	3.5	2.9	3.0
2	2.8	-	4.4	4.1	3.8	3.6	3.6	3.1
3	2.8	-	4.1	4.0	3.7	3.5	3.4	3.0

R/A = rheumatoid arthritis.
Expect. = expected or calculated value.

Assayed and calculated folate values in combination of normal and rheumatoid arthritis plasmas.

DISCUSSION.

The present study shows and confirms previous reports by Hansen and Weinfield (1962), Cooper and Lowenstein (1964), Mollin and Hoffbrand (1965), Hoffbrand et al. (1966,a) that reduced erythrocyte folate levels are found in megaloblastic anaemia due to either folate or vitamin B_{12} deficiency. The lowest levels were observed in the folate deficient group where 85% of patients had values less than 60mug/ml. packed cells (lower normal limit is 135mug/ml. packed cells). In contrast only 15% of the vitamin B_{12} deficient patients had such low values.

As reported previously by Cooper and Lowenstein (1964) and Hoffbrand et al. (1966,a) the present study also shows that the erythrocyte folate content in vitamin B_{12} deficiency is relatively low when compared to the corresponding plasma folate value. Thus the mean erythrocyte/plasma folate ratio in normal controls is 42:1 as compared to 18:1 in vitamin B_{12} deficiency. Further, 9 patients with normal plasma folate values had subnormal erythrocyte folate levels. Cooper et al. (1964) suggested that this altered serum/erythrocyte folate relationship may indicate interference with the transport of folate into the maturing red cell precursor. Although the disordered folate metabolism known to occur in vitamin B_{12} deficiency may be in the form suggested by Cooper et al., the demonstration of a good correlation between plasma and erythrocyte folate levels in this condition also noted by these/

these workers, suggests that the folate content of mature erythrocytes in vitamin B_{12} deficiency may in addition depend on the amount of folate available for the developing red cell. There is no doubt that reduced erythrocyte folate values may occur in vitamin B_{12} deficient patients whose plasma folate levels are well within the normal range (Figure 20), which indicates a predominant metabolic block to folate utilisation.

On the other hand the fact that all 4 cases who had erythrocyte folate values less than 60mug/ml. also had low normal or marginal plasma folate levels indicates that the occurrence of such erythrocyte folate values in vitamin B_{12} deficiency may result from an associated folate deficiency. This is especially so since in view of the tendency for the serum folate activity to accumulate in vitamin B_{12} deficiency low normal or marginal serum levels may indicate a significant degree of folate deficiency (Herbert et al. 1962b.).

The mean corpuscular folate content (MCF).

The erythrocyte folate content when expressed as mug/ml. packed cells is a measure of folate concentration per unit volume of cells. Due to variation of the mean corpuscular volume in different types of anaemia it does not provide accurate information on the absolute folate content per cell. Such an index, the mean corpuscular folate content (MCF), was calculated for normals, patients with megaloblastic anaemia and for iron deficient subjects in order to see whether the/

the intracellular folate is the same or different in these conditions. Normal (MCF) values were obtained in 6 megaloblastic cases (5 B_{12} deficient and 1 folate deficient) who had subnormal erythrocyte folate levels (in mug/ml. packed cells) showing that although in some cases the macrocytes of vitamin B_{12} deficiency and to a lesser extent those of folate deficiency, have a low intracellular folate concentration, they do in fact have a normal folate content. A true intracellular folate deficiency was shown to exist, however, in all but one of the folate deficient group and in 4 out of the 17 vitamin B_{12} deficient patients.

Arguing on the same lines the abnormally high erythrocyte folate values observed for some of the iron deficient subjects may be explained by the presence per unit volume of a larger number of cells than normal providing that the microcytes of iron deficiency have normal MCF values. This was the case in 3 subjects all of whom had erythrocyte folate values above the upper normal limit in Figure 18 and normal MCF values in Figure 23. In 4 subjects, however, the MCF values were higher than normal. The reticulocyte count in this group varies from 1 - 3% and none of the patients had a significant blood loss at the time of investigation (Appendix C3). It is tempting to postulate as an explanation for this that the developing iron deficient red cell precursor receives its normal complement of folate and that by producing, during the different stages of maturation, a cell with a small nucleus/

nucleus and cytoplasmic mass, it utilises less folate than normal thus giving rise to a mature erythrocyte with an increased intracellular folate content. The lack of either iron or folate interferes with proper utilisation of the other and in combined deficiency treatment with the one haemopoietic agent precipitates the full blown deficiency picture of the other. Further, the accumulation of folate in the iron deficient cell may have the same significance as the occurrence of sideroblasts and siderocytes in uncomplicated megaloblastic erythropoiesis. Finally it is of interest to note that Cooper and Lowenstein (1964) noted an unexplained reduction in the serum folate for the corresponding erythrocyte folate (mug/ml. packed cells) in iron deficiency anaemia.

THE FOLATE STATUS IN RHEUMATOID ARTHRITIS.

The finding of subnormal plasma folate levels in the majority of the rheumatoid arthritis patients investigated in the present study is in agreement with previous reports (Gouch et al. 1964, Dellar et al. 1966). The serum folate is a sensitive index of folate deficiency and had been shown to fall to deficiency levels within 3 weeks from the consumption of a folate deficient diet while a subnormal erythrocyte folate content and megaloblastic erythropoiesis took 4 and 4½ months respectively to develop (Herbert 1962, 1965). Clinical studies have also shown that patients may continue for long periods with subnormal serum folate levels without manifest evidence of tissue depletion (Hellman et al. 1964) or easily recognisable morphological bone marrow changes (Mollin and Hoffbrand 1965). In a series of non-anaemic patients with subnormal serum folate levels Hoffbrand et al. (1966a) found a good correlation between the serum and erythrocyte folate levels; further serum folate levels in the unequivocal deficiency range (less than 3.0mug/ml. in their series) were almost invariably associated with subnormal erythrocyte folate levels with variable degrees of megaloblastic change in the bone marrow.

Both experimental and clinical studies would appear to indicate that a low serum folate may precede by a long time a reduction in the folate content of haemopoietic tissue as indicated by a reduced erythrocyte folate level and abnormal bone/

bone marrow morphology. The significance of a low serum folate level is best therefore assessed by estimating the erythrocyte folate content and looking for haematological changes.

The plasma folate level in rheumatoid arthritis correlated well with the erythrocyte content but the mean erythrocyte/plasma folate ratio in this condition (72:1) is much higher than in controls (42:1) indicating a greater than normal but proportionate reduction in the plasma folate activity to that of erythrocytes in rheumatoid arthritis. Further the erythrocyte folate levels were normal in 10 cases with subnormal plasma levels ranging from 1.5 - 2.5 mug/ml. and in 4 of whom the levels were less than 2.0mug/ml. i.e. within the range found for megaloblastic folate deficiency. There was no significant drop in either parameter in 7 patients reinvestigated 3 months after the completion of the initial study. It is obvious therefore that rheumatoid arthritis patients may continue having reduced plasma folate activity in the face of slight or no deficiency in the folate content of haemopoietic tissue. This reduction in the plasma folate activity is genuine and inhibitors to L. casei growth have been excluded in this and other studies (Cowan et al. 1966). In the light of the previously referred to experimental and clinical studies, this disturbed plasma/erythrocyte folate ratio may be partly explained by a "subclinical" or developing folate deficiency. The occurrence, however, of normal erythrocyte folate levels in some patients with unequivocally/

unequivocally deficient plasma values may indicate that the plasma folate activity in rheumatoid arthritis does not have the same significance as it does in other folate deficient states.

There is also a striking paucity of megaloblastic change in patients who should theoretically have a significant degree of folate deficiency as manifested by both a subnormal plasma and erythrocyte folate level. Thus, of 14 such cases, 13 of whom had plasma folate values of 2.0mug/ml. or less, only 4 showed bone marrow changes suggestive of megaloblastosis and in all of these including one case (No. 33 Appendix C4), with plasma and erythrocyte folate levels of 1.5 and 55mug/ml. respectively, the abnormality was classified as early. Erythropoiesis and myelopoiesis were entirely normal in the remaining 9 cases. Three of these cases (No. 5, 30 and 3) in addition to being deficient in both plasma and erythrocyte folate values showed a fast clearance of injected folic acid and excreted abnormal quantities of histidine metabolites in the urine. In the series of rheumatoid arthritis patients reported by Gough et al. (1964) mild to moderate megaloblastic changes were found in only 6 out of 15 patients with serum folate levels in the unequivocal deficiency range. Comparison with the other folate deficient states in this study and with the series reported by Hoffbrand et al. (1966,a) shows that megaloblastic changes in rheumatoid arthritis is by far less frequent and when present less severe/

severe than would be expected from the degree of folate deficiency. Such a comparison has its limitations because whereas folate deficiency is the main cause of anaemia in the 23 megaloblastic cases included in this study (Appendix C1) and of the haematological abnormalities in those reported by Hoffbrand et al. (1966,a), the cause of anaemia in rheumatoid arthritis is not known and the aetiological significance of folate deficiency in its pathogenesis uncertain. A co-existent impairment of haemoglobin synthesis as occurs in iron deficiency or haemoglobinopathies may mask the overt megaloblastic changes of folate deficiency (Herbert 1965). In the same way the hypochromic element of rheumatoid arthritis may be responsible for the discrepancy between the morphological bone marrow changes and the results of other tests of folate deficiency.

Histidine Metabolites.

The compound formiminoglutamic acid (FIGLU) arises from the in vivo degradation of histidine and is normally converted to glutamic acid by donating its formimino group (CH=NH) to tetrahydropteroylglutamic acid in a reaction involving the enzyme FIGLU transferase. A lack of the folate coenzyme - as occurs in folate deficiency - causes FIGLU to accumulate in the urine (Broquist and Lubby 1959). The abnormality is easily demonstrated by loading the metabolic pathway with histidine (Lubby et al. 1959). A high FIGLU excretion/

excretion is a constant finding in megaloblastic anaemia requiring treatment with folic acid, an exception being the anaemia of pregnancy where due to abnormality in histidine metabolism the test is unreliable (Chanarin et al. 1963). A disadvantage of the test is its lack of specificity in that a positive result may occur in vitamin B_{12} deficiency (Zalusky and Herbert 1961, Kohn et al. 1961, Kershaw and Girdwood 1964) and in conditions with neither folate nor B_{12} deficiency e.g. liver disease (Carter et al. 1961) and malignancy (Kershaw and Girdwood 1964, Watson-Williams and Israels 1966).

Another histidine metabolite and a precursor of FIGLU is urocanic acid. Urocanic acid may be the major or only histidine metabolite in the urine of folate deficient subjects (Chanarin and Bennett 1962, Chanarin 1963, Mohmed and Roberts 1966). Abnormal urocanic acid excretion had also been reported in patients with status asthmaticus (Kerr 1963), Kwashiorkor (Whitehead et al. 1961) and thyrotoxicosis (Mohmed and Roberts 1965).

A relationship has been shown in folate deficiency between FIGLU excretion and the serum folate level. Thus in 70 patients suffering from intestinal malabsorption, Crohn's disease or who had undergone partial gastrectomy reported by Mollin and Hoffbrand (1965) a positive FIGLU test was shown in those with serum folate levels in the unequivocal deficiency range and a negative result in all of those with normal/

normal serum values. Similarly all 26 patients with primary malabsorptive disease and subnormal folate values reported by Kershaw and Girdwood (1964) excreted large quantities of FIGLU in the urine.

The urinary excretion of histidine metabolites in rheumatoid arthritis follows a different pattern from that described for other folate deficient states. There was no clear cut relationship for example between the plasma folate activity and FIGLU excretion. Thus, while in some patients with subnormal folate levels FIGLU excretion was normal, 8 out of 12 patients with normal plasma folate values excreted large quantities of FIGLU in the urine (Figure 28). An even greater dissociation was observed between FIGLU excretion and erythrocyte folate content where abnormal FIGLU excretion, sometimes excessive, occurred in a large group of patients with normal erythrocyte folate levels. The latter finding is at variance with the findings of Hoffbrand et al. (1966) who observed a correlation between the two parameters in the majority of patients with folate deficiency due to causes other than rheumatoid arthritis. Further, abnormal excretion of combined FIGLU and urocanic acid (with a variable preponderance of either) was observed in 10 out of 12 patients who had normal plasma and erythrocyte folate values and in all of whom erythropoeisis was normoblastic (Table 29). One of these patients (Case 21) who excreted the highest amount/

amount of histidine metabolites (616mg/8 hrs.) in the whole group of patients investigated, showed in addition a normal clearance of injected folic acid. All 10 patients had generalised joint and systemic manifestations of the disease (Grades II and III severity).

The excretion of abnormal quantities of histidine metabolites by rheumatoid arthritis patients in the face of a normal folate status is difficult to explain. A similar situation was observed in malignant disease by Watson-Williams and Israels (1966) where large quantities of FIGLU were excreted by patients who had normal serum folate values and normoblastic bone marrows.

The excretion of histidine metabolites is not related to the duration of disease in rheumatoid arthritis. The proper evaluation of its relationship to disease activity is hampered by the presence of only 4 patients with Grade I, as compared to 32 patients with Grades II and III disease severity (Figure 32). However, in two of the mildly affected patients (Grade I) the urinary excretion of histidine metabolites was normal and was only marginally raised in a third patient. The fourth patient who eliminated a total of 83mg/8 hrs. showed also an early megaloblastic change in the bone marrow (Case 25, Table 25). In contrast all patients with Grade III disease activity and 18 out of 22 cases with Grade II excreted abnormal quantities of combined FIGLU and urocanic/

urocanic acid. It is possible therefore that the excretion of abnormal quantities of histidine metabolites is commoner in the more severely affected patients.

Most observers would agree that a positive FIGLU test indicates disordered folate metabolism due to either lack or interference with function of the folate coenzyme or of FIGLU transferase.

Folate deficiency may therefore be responsible for the excretion of large quantities of FIGLU in the urine by those rheumatoid arthritis patients who had other criteria of the deficiency e.g. reduced folate levels or morphological bone marrow changes suggestive of megaloblastic erythropoiesis. The occurrence of a positive FIGLU test on the other hand in patients who have a normal folate status may indicate a dysfunction of FIGLU transferase possibly related to disease activity.

Since folate compounds are not directly involved in the breakdown of urocanic acid, the mechanism whereby folate deficiency causes a high excretion of this metabolite is obscure. Allan and Whitehead (1965) produced evidence that the excessive excretion of urocanic acid observed in children with protein malnutrition is caused by the hypoproteinaemia and not folic acid deficiency. They suggested that the hypoproteinaemia may depress urocanase synthesis. Similarly Hoffbrand et al. (1966,b) found that patients who had undergone partial gastrectomy and had subsequently developed/

developed low serum albumin levels excreted large quantities of urocanic acid and that this was reversed to normal by intravenous albumin infusion. Reduced serum albumin levels are frequent in patients with rheumatoid arthritis (Gutman 1948). The degree of reduction correlates with the clinical activity of the disease and degree of anaemia (Engstedt et al. 1966). It is possible on purely speculative grounds that urocanic acid excretion in rheumatoid arthritis is linked with the plasma protein disturbance frequently seen in this disease.

Of interest is the finding by Edozien and Obasi (1965) of a correlation between the excretion of urocanic acid after a histidine load and of xanthurenic acid after a typtophan load in patients with protein malnutrition (kwashiorkor). They argued that since the excretion of xanthurenic acid after tryptophan loading is a reliable test of pyridoxine deficiency (Greenberg et al. 1949) and since the enzyme urocanase has pyridoxal requirements (Gupta and Robinson 1961), the excretion of abnormal quantities of urocanic acid after a histidine load in these patients reflected pyridoxine deficiency. They suggested that urocanic acid excretion after a histidine dose is a sensitive index of pyridoxine deficiency.

Abnormalities of tryptophan metabolism in patients with rheumatoid arthritis have been reported (Bett 1962,a and b) and a relative deficiency of pyridoxine suggested as causing/

causing these (McKusick et al. 1964, Bett 1966).

This provides another possible explanation for the raised excretion of urocanic acid in rheumatoid arthritis if one accepts the postulate of these workers.

Aetiological factors:-

Gough et al. (1964) found that the dietary intake of folic acid in patients with rheumatoid arthritis did not differ significantly from that of controls and that the intestinal absorption of the vitamin was normal in the 6 megaloblastic cases found in the group.

Histories of adequate dietary intake were obtained in all cases in the present series with the exception of two (No. 33 and 15, Appendix C4); the intestinal absorption of folic acid was also shown to be normal in the 18 cases investigated. Further, preliminary results have also shown that the urinary loss of folic acid in rheumatoid arthritis is not different from that in controls.

Increased utilisation of folic acid by the cellular proliferation in the joints in rheumatoid arthritis was suggested by Gouch et al. (1964). Utilisation of folate by the autoimmune process and the hyperactive reticulo-endothelial system may in addition increase the demands for the vitamin. This concept of increased utilisation is very possible but difficult to prove.

Neither the incidence nor the degree of folate deficiency/

deficiency could be related to the duration or severity of the disease. The plasma and erythrocyte folate levels remained unchanged in 5 patients in whom a remission of disease activity and a significant rise in haemoglobin concentration were produced by ACTH therapy.

The plasma folate releasing factor:-

The significance, in relation to folate deficiency, of the reduced folate releasing ability of plasma in rheumatoid arthritis shown in Section IV of Chapter II is uncertain. Grzesiukowicz et al. (1966) demonstrated a reduced "plasma factor activity" in megaloblastic anaemia of pregnancy and suggested that this may lead to less utilisation of the "folate activity precursor" from erythrocytes thus reducing the available folate in the circulation and contributing to the aetiology of folate deficiency. There are two main criticisms of this concept:-

1. The invitro conditions under which optimal folate release occurs e.g. pH, are far removed from those prevailing in the human body. The conclusion therefore that the results of such experiments represent the in vivo interrelationship between the folate precursor and the releasing factor is not justifiable.

2. The concept that the folate content of the erythrocyte is eventually re-utilised in the body is acceptable. However, since the destruction of red cells occurs normally in the reticuloendothelial system, it is more likely that the/

the folate precursors are removed and stored there in a way similar to haemoglobin iron and that the polyglutamate forms broken to monoglutamates by "conjugases" in the reticuloendothelial system. Herbert (1962) had shown that the polyglutamate forms of folate are not present in human plasma.

SUMMARY.

1. In agreement with previous reports, reduced erythrocyte folate values were found in megaloblastic anaemia due to either folate or Vitamin B_{12} deficiency.

2. The mean corpuscular folate content (MCF) was determined in normal controls, patients with megaloblastic anaemia and in patients with iron deficiency anaemia and its significance evaluated in the various groups.

3. The occurrence of folate deficiency in rheumatoid arthritis was confirmed. Evaluation of the various findings showed that:-

(a) Subnormal plasma folate values are common in rheumatoid arthritis, occurring in 24 out of 37 patients investigated. The erythrocyte folate levels were reduced in only 14 of these patients indicating that a reduced plasma folate activity in this disease is not necessarily associated with a significant degree of folate depletion.

(b) Both the incidence and severity of the megaloblastic change in the folate deficient rheumatoid arthritis patients are less than would be expected from the degree of the folate deficiency.

(c) Histidine metabolism is abnormal in rheumatoid arthritis and in many cases unrelated to the folate status. Possible causes for this were discussed.

(d) /

(d) The possible causes of folate deficiency and the significance of the reduced plasma factor activity in rheumatoid arthritis were discussed.

Eden Grove Bond
TUB SIZED & AIR DRIED

APPENDICES.

APPENDIX A.

I.

DEIONISED WATER.

An Elgastat portable dioniser type B. 102 was used. Deionised water was used for rinsing glassware, preparation of media, buffers, standard folic acid solutions, haemolysates, ascorbic acid solutions, dilution of extracts prior to assay and the preparation of enzyme and substrate for FIGLU and urocanic acid estimations.

II.

CLEANING OF GLASSWARE.

(i) Glassware used for folate estimation:

(a) Pipettes were soaked in a detergent "Pyroneg" for at least 12 hours, transferred to an automatic washer overnight, rinsed with deionised water and dried in a hot air oven.

(b) Universal containers, test tubes, flasks, measuring cylinders etc. -

Universal containers and test tubes were first brushed thoroughly with hot Pyroneg solution, then together with the rest of glassware filled with Pyroneg for six hours before they were washed well in running tap water, rinsed with deionised water and dried in a hot air oven.

(c) Aluminium caps were soaked in a weak Pyroneg solution/

solution, washed in running tap water first then deionised water and finally dried in a hot air oven.

(ii) Glassware used for Vitamin B_{12} estimation:

This was always acid cleaned. The acid solution was prepared as follows:-

Potassium dichromate	:	150 g.
Conc. sulphuric acid	:	500 ml.
Distilled water	:	1350 ml.
Final volume	:	2000 ml.

The glassware was first filled with the acid solution and left overnight, then washed in running tap water, rinsed with deionised water and dried in a hot air oven.

III.

FLUID MAINTENANCE MEDIA.

(a) L. casei:-

DIFCO microinnoculum broth was rehydrated by dissolving 37g. in 1000 ml. of deionised water. 8 ml. aliquots of the medium were dispensed into 5' x 5/8" test tubes which were plugged with cotton wool, sterilised at 15 p.s.i. for 10 minutes and stored at 4°C. until used.

(b) S. faecalis:-

To 400 ml. of DIFCO folic acid assay medium prepared by dissolving 7.5g./100ml. of water were added 3.2ug. of folic acid (8 ml. of 1/100 dilution of the stock folic acid solution). After boiling for 2-3 minutes the medium was dispensed/

dispensed, sterilised and stored as for the L. casei maintenance medium.

(c) E. gracilis:-

Prepared as follows:-

0.4% Tryptone	:	475 ml.
DIFCO Euglena B_{12} medium	:	524 "
Vitamin B_{12} solution (2mug/ml.)		1 "
Total volume	:	1000 "

10 ml. aliquots were dispensed into universal containers, sterilised by steaming at 100°C. for 15 minutes and stored at 4°C.

IV.

PREPARATION OF PHOSPHATE BUFFER.

Stock Solutions:- The reagents used were "Analar" grade.

Solution A.

0.2 M sodium dihydrogen orthophosphate ($Na H_2 PO_4 2H_2O$) prepared by dissolving 32.2g. in a litre of deionised water.

Solution B.

0.2 M Di-sodium hydrogen orthophosphate ($Na_2H PO_4 12H_2O$) prepared by dissolving 71.7g. in a litre of deionised water.

The stock solutions were kept at room temperature, and 0.1 M phosphate buffer pH 6.1 - 6.2 was prepared when required/

required by adding 85 ml. of solution A. to 15 ml. of solution B. and diluting with an equal volume of water. When such a buffer contained 200mg.% ascorbic acid the pH varied from 6.0 - 6.1.

V.

Quantities of HCl and NaOH required for obtaining haemolysates at different pH values.

The number of drops of HCl or NaOH added by a Pasteur pipette (15 drops/ml.) to 9 ml. of water or ascorbic acid solution in order to obtain different haemolysate pH values (blood dilution 1/10), are detailed in the table shown in the following page.

APPENDIX AV.

Quantities of HCl and NaOH added to obtain various haemolysate pH values.

Deionised water.		25mg.% ascorbic acid solution.		100mg.% ascorbic acid solution.	
Expected pH range.	No. of drops of HCl or NaOH.	Expected pH range.	No. of drops of HCl or NaOH.	Expected pH range.	No. of drops of HCl or NaOH.
2.8 - 3.0	7 $\frac{N}{1}$ HCl	2.3 - 2.4	8 $\frac{N}{1}$ HCl	2.2 - 2.3	9 $\frac{N}{1}$ HCl
3.1 - 3.3	6 "	2.5 - 2.6	7 "	2.4 - 2.5	8 "
3.4 - 3.5	5 "	2.7 - 2.9	6 "	2.7 - 2.8	7 "
3.6 - 3.8	4 "	3.0 - 3.3	5 "	2.9 - 3.0	6 "
3.9 - 4.1	3 "	3.4 - 3.6	4 "	3.1 - 3.3	5 "
4.2 - 4.5	2 $\frac{N}{1}$HCl+1$\frac{N}{2}$ HCl	3.7 - 3.9	3 "	3.4 - 3.5	4 "
4.7 - 4.9	2 $\frac{N}{1}$HCl+1$\frac{N}{4}$ HCl	4.0 - 4.3	2 $\frac{N}{1}$HCl+1$\frac{N}{2}$ HCl	3.6 - 3.7	3 $\frac{N}{1}$HCl+1$\frac{N}{2}$ HCl
5.0 - 5.3	2 $\frac{N}{1}$ HCl	4.4 - 4.6	2 $\frac{N}{1}$ HCl	3.8 - 4.0	3 N HCl
5.4 - 5.7	1 $\frac{N}{1}$HCl+1$\frac{N}{2}$ HCl	4.7 - 5.0	1 $\frac{N}{1}$HCl+1$\frac{N}{2}$ HCl	4.1 - 4.2	2 "
5.8 - 6.0	1 $\frac{N}{1}$ HCl	5.1 - 5.4	" +1$\frac{N}{4}$ HCl	4.3 - 4.5	1 $\frac{N}{1}$HCl+ $\frac{N}{2}$ HCl
6.0 - 6.5	1$\frac{N}{2}$ HCl+1$\frac{N}{4}$ HCl	5.5 - 6.0	1 $\frac{N}{1}$ HCl	4.6 - 5.0	1 $\frac{N}{1}$ HCl
6.6 - 7.0	1 $\frac{N}{2}$ HCl	6.0 - 6.5	1 $\frac{N}{2}$ HCl	5.1 - 5.5	1 $\frac{N}{2}$ HCl
7.1 - 7.5	None	6.7 - 6.9	None	5.6 - 5.8	None
7.6 - 8	1$\frac{N}{2}$ NaOH	7.0 - 7.5	1$\frac{N}{2}$ NaOH	6.0 - 6.5	1$\frac{N}{2}$ NaOH
8.1 - 8.5	1 $\frac{N}{1}$ NaOH	7.5 - 8.0	1 $\frac{N}{1}$ NaOH	6.6 - 7.0	1$\frac{N}{2}$ " NaOH+1$\frac{N}{4}$ NaOH
9.0	2 $\frac{N}{1}$ NaOH	8.5 - 9.0	2 $\frac{N}{1}$ NaOH	8.0 - 8.3	1 $\frac{N}{1}$ NaOH
				8.6 - 9	1$\frac{N}{1}$NaOH+1$\frac{N}{2}$ NaOH
				9.0	2$\frac{N}{1}$NaOH

N.B. HCl or NaOH was added prior to addition of blood and the quantities in the list were found by trial and error in preliminary experiments.

APPENDIX B.

I.

STATISTICAL METHODS.

1. Arithmetic Mean ($\bar{x}$).

$$\bar{x} = \frac{\Sigma x}{n}$$

Where "Σx" is the sum and "n" the number of sample results.

2. The Standard Deviation (SD).

$$SD = \sqrt{\text{variance}} = \sqrt{s_x^2}$$

$$s_x^2 = \frac{1}{n-1}\left(\Sigma x^2 - \left(\frac{\Sigma x}{n}\right)^2\right)$$

Where:-

n = number of results.

Σx^2 = the sum of the squares of individual results.

$(\Sigma x)^2$ = the square of the sum of total results.

3. The Significance Tests.

A. The Student's "t" test.

This test was used to compare sets of results conforming to the following conditions:-

(i) Sample results must be independent.

(ii) The two population variances are normally distributed.

(iii) The two population variances are equal. This is tested by calculating the "F" value of the two variances, as follows:-

$$F = \frac{s_a^2}{s_b^2}$$

Where Sa^2 is the variance and n_a the number of the first set of results and S_b, n_b the variance and number of the second set of results respectively.

For the "t" test to be applicable, the two variances must not be significantly different as judged by looking at the calculated "F" values in 5% "F" tables with degrees of freedom of = (n_a-1) and = (n_b-1).

Formula for the "t" test:-

$$t = \frac{\bar{x}_a - \bar{x}_b}{\sqrt{\frac{s_p^2}{n_a} + \frac{s_p^2}{n_b}}}$$

"degrees of freedom" = $(n_a + n_b - 2)$

Where:-

(i) $\bar{x}_a$, $\bar{x}_b$ are the means and n_a, n_b the number of the two sample results compared respectively.

(ii) s_p^2 is the pooled variance of the two sample results, calculated as follows:-

$$s_p^2 = \frac{(n_a - 1) \times s_a^2 + (n_b - 1) \times s_b^2}{n_a + n_b - 2}$$

When the sample results are not independent e.g. comparison between the initial and follow up folate values of the same patients, the "t" test cannot be used. Instead the following formula is applicable:-

$$t = \frac{\bar{d}}{\sqrt{\frac{\text{variance } (d)}{n}}}$$

"t" has 'degrees of freedom' of n - 1.

$\bar{d}$ = mean of differences between pairs of results/

results.

n = number of differences.

* Lutz, W. (1967).

4. Correlation Coefficient "r".

$$r = \frac{Sxy}{\sqrt{s_x^2 \cdot s_y^2}}$$

Where:-

1. x and y stand for individual values in the two sets of results respectively.
2. s_x^2 and s_y^2 are the variances of the two sets of results respectively.
3. Sxy = variance of x.y

Calculated as follows:-

$$Sxy = \frac{1}{n-1}\left(\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{n}\right)$$

Where

n = number of pairs of x.y values.

The equation of the best fitting straight "regression" line is $y = A + B x$, where

$$B = \frac{Sxy}{s_x^2}$$

$A = \bar{y} - B\bar{x}$, $\bar{y}$ and $\bar{x}$ being the means of the two sets of results.

II.

Formula for calculating whole blood folate as mug/ml. of packed red cells.

* Erythrocyte folate mug/ml. packed red cells = $\dfrac{\text{whole blood folate - serum folate}\left(1-\frac{PCV}{100}\right)}{\frac{PCV}{100}}$

* (Mollen and Hoffbrand 1965).

APPENDIX C1.

Megaloblastic folate deficient cases.

Case	Age	Sex	Hb. g.%	PCV %	RBC cumm 10^6	MCHC	MCV	Plasma folate mug/ml.	RBC folate mug/ml.	Se B12 uug/ml.	F/A absorb.	Xylose gm/5 hr.	Faec. fat gm/day 5 days coll.	Jejunal biopsy.	Bone marrow	Diet	Aetiology.
1	50	F	9.8	28	-	35	-	0.6	36	208	normal	-	-	normal	frankly megalo.		Drug induced ? Barbiturates + anticonvulsants
2	34	F	8.8	28	2.11	31	133	0.6	37	252	normal	-	-	normal	"	poor	Diet def.
3	41	F	4.2	14	-	30	-	1.0	112	296	normal	-	-	normal	"	poor	Diet def.
4	60	M	5.8	18	1.57	32	115	0.6	39	688	malabs.	-	12	failed	"	good	Malabsorption + Thyrotoxicosis.
5	61	F	6.3	18	-	35	-	0.3	50	135	malabs.	-	-		"	good	Primary malabsorb. dis.
6	23	F	10.9	34	-	32	-	0.9	37	1000	-	-	4	-	early megalo.	poor	Crohn's Dis.
7	41	F	11.5	38	-	3	-	2.0	32	136	malabs.	-	2.6	normal	"	good	Partial gastrectomy.
8	52	F	7.0	20	1.9	35	105	0.9	30	688	malabs.	1.2	4.8	-	frankly megalo.	good	Malabsorption.
9		F	8.1	26	-	31	-	2.0	98	151	normal	-	5	-	early megalo.	good	Ca. Rectum.
10	84	F	7.9	24	1.96	33	124	1.1	24	140	normal	-	-	-	frankly megalo.	poor	Diet def.
11	64	M	6.7	22	2.33	30	94	1.7	61	536	normal	-	4	-	"	poor	Erythraemic myelosis.
12	85	F	6.0	20	-	30	-	1.4	32	120	normal	-	3	-	"	poor	Diet def.
13	32	F	4.5	13	-	35		0.4	42	125	-	-	-	-	frankly megalo.	poor	?
14	50	F	9.2	27	2.82	34	96	1.2	99	208	-	-	-	-	"	good	Diverticulitis ?
15	35	F	3.9	13	1.11	30	117	0.6	52	140	-	-	-	-	"	poor	Pregnancy.
16	87	M	6.0	18	1.63	33	110	1.0	53	136	normal	-	3.5	-	"	poor	Diet def.
17	60	M	8.5	27	-	32	-	1.4	39	129	normal	-	-	-	"	good	Hodgkins Dis.
18	75	F	12.5	37	-	34	-	0.4	24	144	-	-	-	-	"	poor	? Diet def.
19	85	F	5.11	18	1.75	30	103	0.8	37	168	-	-	-	-	"	poor	? Diet def.
20	60	M	6.4	19	1.88	34	101	0.7	51	126	malabs.	-	-	-	"	good	?Malabsorption.
21	70	M	9.4	29	2.88	33	101	0.8	40	520	normal	12	4	-	early megalo.	good	Lymphosarcoma
22	50	F	9.0	27	2.63	33	103	1.6	130	736	-	-	-	-	"	good	?
23	60	M	6.0	18	1.51	33	119	1.0	23	126	-	-	-	-	frankly megalo.	good	?

APPENDIX C2.

Data on megaloblastic vitamin B_{12} deficient cases.

Case No.	Hb. g.%	PCV %	RBC /cumm x 10^6	Plasma B12 uug/ml.	Plasma folate mug/ml.	RBC folate mug/ml.	Histamine test meal	Schill. test % recov.	Schill.* test + I.F. % recov.	P. Hist. hr. I.F. ng units.	Total x Body uptake Co^{58}-B12 %	Bone Marrow	Diagnosis
1	14.0	41	2.93	8	12	166	Hist.fast achlor.	4.9	-	101	4	Early megalo.	PA
2	9.0	30	-	20	18	163	"	3.9	-	-	-	Frankly "	? PA
3	6.6	17	1.43	16	8.2	108	"	4.5	13.0	-	-	" "	PA
4	6.8	19	-	16	8.5	247	"	4.0	-	-	-	" "	? PA
5	10.6	33	3.35	88	8.1	171	"	6.6	12.0	-	-	" "	PA
6	11.0	37	3.60	18	12.0	204	"	2.0	10.0	-	-	" "	PA
7	6.0	20	-	24	2.7	49	"	5.0	19.0	-	-	" "	PA
8	19.9	39	3.61	32	13.0	148	"	1.8	-	210	14	Early megalo.	PA
9	7.9	23	2.26	4	11.0	213	"	3.0	12.0	-	-	Frankly "	PA
10	4.3	15	1.29	24	3.4	47	"	2.2	10.9	-	-	" "	PA
11	9.0	29	2.95	16	2.9	98	"	3.6	12.1	-	-	" "	PA
12	8.2	21	1.99	0	3.1	151	"	1.0	34.0	-	-	" "	PA
13	7.0	23	1.60	0	4.3	88	"	2.0	-	-	-	" "	? PA
14	5.8	18	1.49	25	8.0	130	"	4.0	-	-	-	" "	? PA
15	8.8	27	-	21	10.0	188	"	4.5	-	23	2	Early megalo.	PA
16	10.8	32	2.65	16	3.2	61	"	5.0	-	-	-	Frankly "	? PA
17	5.4	15	1.18	32	4.4	105	"	3.0	-	-	-	" "	? PA
18	7.8	21	1.57	8	6.3	148	"	3.1	-	-	-	" "	? PA
19	8.2	24	-	40	5.1	167	"	2.8	-	-	-	" "	? PA
20	7.7	23	2.20	48	5.8	104	"	2.2	15.7	-	-	" "	PA
21	5.4	16	-	8	2.4	50	"	1.3	-	1.8	26	" "	PA
22	5.8	19	1.57	32	10.0	181	"	4.0	14	-	-	" "	PA
23	5.4	20	1.85	8	5.0	124	"	2.9	-	-	-	" "	? PA
24	6.3	19	-	16	11.0	130	"	0.59	-	273	6	" "	PA
25	9.7	25	2.14	24	7.3	157	"	1.6	-	33	0.0	" "	PA
26	9.9	28	-	20	8.0	215	"	3.3	-	-	-	" "	? PA

* The lower normal limit of the post histamine intrinsic factor output is 300 ng units.

x The total body uptake of Co^{58}-B_{12} in pernicious anaemia is less than 30%.

* x Finlayson and Shearmen, 1967.

APPENDIX C3.

Data on iron deficient patients.

Case No.	Age	Sex	Hb. g.%	PCV %	RBC $/mm^3 \times 10^6$	MCHC	MCV	Retics. %	Plasma folate mug/ml.	RBC folate mug/ml.	S.I.* uug.%	TIBC* uug.%	Bone Marrow Iron.	Aetiology.
1	40	F	5.5	19	2.48	29	77	-	5.8	539	15	350	No staining Fe	Menorrhagia
2	74	M	5.6	22	3.60	25	61	3.0	4.5	346	30	400	"	C.L.L. *
3	40	F	8.0	29	5.01	28	58	1.5	4.5	335	10	480	"	? - FOB *
4	16	F	5.9	25	3.99	24	63	3.6	3.6	409	20	400	"	D.U. *
5	72	M	9.4	31	4.62	30	67	<1.0	4.2	350	15	450	"	Malabsorption.
6	56	F	7.5	29	4.09	26	71	3.0	5.4	447	15	450	"	Ca. Caecum.
7	79	M	5.8	20	3.11	29	64	1.5	7.2	412	10	285	"	Dietary ? -ve FOB.
8	15	M	8.8	33	5.49	27	50	2.0	4.0	224	27	535	"	Malabsorption.
9	50	F	6.8	23	3.86	29	60	2.6	6.5	565	15	500	"	? -ve FOB
10	79	F	7.1	28	-	26	-	1.0	3.6	177	30	450	"	Dietary ? -ve FOB
11	15	M	10.9	38	-	30	-	3.5	3.0	196	20	390	"	? -ve FOB
12	35	F	5.4	23	-	25	-	-	3.8	380	30	300	"	? -ve FOB No blood loss
13	69	F	5.1	19	-	27	-	-	6.1	375	10	375	"	No blood stools.
14	59	M	5.9	24	-	25	-	1.0	3.2	447	15	480	"	Haemorrhoids.
15	50	M	7.1	27	-	26	-	2.6	2.7	195	25	390	"	D.U.
16	45	M	6.9	25	-	28	-	-	2.4	197	20	360	"	D.U.
17	73	F	6.1	25	-	24	-	4.5	3.6	278	25	600	"	Hiatus hernia.

* C.L.L. = Chronic lymphocytic leukaemia.

D.U. = Duodenal ulcer.

FOB = Faecal occult blood.

S.I. = Serum iron.

T.I.B.C.= Total iron binding capacity.

APPENDIX C4.

Data on rheumatoid arthritis patients.

Case No.	Age	Sex	Activity 0,+,++,+++	Duration yrs.	Hb. g.%	PCV %	ESR mm/hr. West.	Blood Film	Bone Marrow	Plasma folate mug/ml.	RBC folate mug/ml	Se B12 uug/ml	mg/8 FIGLU	hrs. Uroc.A	Sensitised sheep cell titre +,-	Diet +,-	F/A absor
1	53	M	++	-	12.4	41	70	Normal	Early megalo.	1.3	120	168	35	185	1/512+	+	Norm
2	64	F	++	6/12	12.7	-	32	"	Normoblastic	1.4	183	560	25	13	1/128+	+	"
3	62	F	++	30	11.5	37	48	"	"	1.5	103	256	12	12	1/1024+	+	"
4	78	F	++	1	8.7	-	52	"	"	4.4	209	308	16	212	1/16-	+	"
5	42	F	++	7	9.6	31	50	"	"	0.7	121	488	70	62	1/32-	+	"
6	65	F	++	14	10.5	34	30	"	"	2.0	88	424	9	11	1/1024+	+	-
7	73	F	+++	2	7.3	28	107	"	"	3.6	194	220	99	9	1/16-	+	-
8	71	M	++	4/12	9.8	36	58	Hypochromic	"	2.4	290	448	69	39	1/256+	+	-
9	42	M	++	9	13.2	-	70	Normal	"	3.7	282	672	42	91	1/1024+	+	-
10	57	M	+	13	12.8	38	20	"	"	3.0	199	568	13	4	1/32-	+	-
11	51	F	++	8	9.4	34	70	"	"	2.3	225	566	14	25	1/32-	+	-
12	64	M	+++	7/12	12.4	39	43	"	"	6.1	251	472	14	25	1/256+	+	-
13	70	M	++	8	14.6	45	22	"	Early megalo.	2.3	189	288	44	22	1/128+	+	-
14	42	M	++	5/12	12.8	-	28	"	Normoblastic	1.9	109	448	28	41	1/1024+	+	-
15	74	F	++	3	8.4	31	53	"	"	2.9	175	300	32	0	1/256+	+	-
16	52	F	+++	2	12.0	=	81	"	Early megalo.	1.1	109	500	54	2	1/16=	+	=
17	64	F	+++	15/12	8.2	26	112	"	"	1.4	153	472	60	0	1/128+	+	-
18	69	F	+++	3/12	10.6	33	112	"	"	2.0	136	240	67	614	1/512+	+	-
19	35	M	+++	4	9.4	34	72	"	"	2.5	163	148	138	15	1/32-	+	-
20	35	M	+++	8	9.4	32	75	"	Normoblastic	1.9	172	320	33	13	1/256+	+	-
21	51	F	++	1	9.7	34	30	"	"	3.0	208	392	335	281	1/32+	+	-
22	40	F	++	18/12	8.6	32	48	"	"	2.7	259	251	46	0	1/1024+	+	-
23	57	F	+++	1	9.0	-	77	"	"	3.9	135	552	60	100	1/16-	+	-
24	33	F	+++	6/12	10.2	-	74	"	"	4.3	304	386	273	0	1/512+	+	-
25	63	M	+	7	12.8	40	22	"	Early megalo.	2.8	208	244	58	26	1/256+	+	-
26	49	M	++	25	14.0	-	32	"	Normoblastic	1.6	113	473	0	4	1/512+	+	-
27	23	M	++	1	11.8	-	34	"	"	4.6	175	443	0	21	1/128+	+	-
28	62	M	+	6	12.1	38	22	"	"	2.2	205	168	0	0	1/1024+	+	Norm.
29	16	F	++	6	9.4	31	52	"	"	1.3	91	1000	-	-	1/64-	+	"
30	46	F	++	10	8.6	29	62	"	"	2.3	113	168	35	75	1/512+	+	"
31	73	F	++	3	8.4	26	90	Hypochromic	"	1.7	111	392	37	55	1/16-	+	"
32	26	F	+	4	13.3	-	39	Normal	"	2.2	192	220	23	5	1/64-	+	"
33	63	M	++	8	9.4	26	36	Occ.macrocy.	Early megalo.	1.5	55	212	28	17	1/16-	-	"
34	62	F	+++	22	7.8	26	135	Hypochromic	Normoblastic	2.9	232	291	35	3	1/256+	+	"
35	54	M	++	18/12	11.8	-	-	Normal	"	1.8	99	272	21	9	1/256+		"
36	59	M	++	6	12.2	-	67	"	"	1.6	99	496	49	140	1/256+	+	"
37	73	F	++	18/12	8.4	30	70	Hypochromic	"	1.8	215	132	15	18	1/128+	+	"

APPENDICES C1 - C4.

REFERENCES.

REFERENCES.

Allan, D.M. and Whitehead, R.G. (1965).
Blood. 25, 283.

Anderson, B.B. (1964).
J. clin. Path. 17, 14.

Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H., Semb, J., Stockstad, E.L.R., Subbarrow, Y., Waller, E.W., Cosulich, D.B., Fahrenbach, M.J., Hultquist, M.E., Kuh, E., Northey, E.H., Seeger, D.R., Sickels, J.P. and Smith, J.M. Jr. (1945).
Science. 102, 227.
(1946) ibid. 103, 667.

Baker, H., Herbert, V., Frank, O., Pasher, I., Hunter, S.H., Wasserman, E.R. and Sabotka, H. (1959).
Clin. Chem. 5, 275.

Ball, J. (1952).
Ann. rheum. Dis. 11, 97.

Ball, E.W. and Giles, C. (1964).
J. clin. Path. 17, 165.

Benjamin-Allan, A. (1946).
J. Ass. Med. Women in India. 34, 9.

Bett, I.M. (1962,a).
Ann. rheum. Dis. 21, 63.
(1962,b). ibid. 21, 388.

Bett, I.M. (1966).
Ann. rheum. Dis. 25, 556.

Binkley, S.B., Bird, O.D., Bloom, E.S., Brown, R.A., Calkins, D.G., Campbell, C.J., Emmett, A.D. and Pfiffner, J.J. (1944).

Science. 100, 36.

Bird, O.D., Binkley, S.B., Bloom, E.S., Emmett, A.D. and Pfiffner, J.J. (1945).

J. biol. Chem. 159, 631.

Borquist, H.P. (1956).

J. Amer. Chem. Soc. 78, 6205.

Borquist, H.P. and Luhby, A.L. (1959).

Proc. Soc. exp. Biol. Med. 100, 349.

Briggs, G.M., Lucky, T.D., Elvehjem, C.A. and Hart, S.B. (1943).

J. biol. Chem. 148, 163.

Briggs, G.M., Lucky, T.D., Elvehjem, C.A. and Hart, S.B. (1945).

J. biol. Chem. 158, 303.

Brockman, J.A. Jr., Roth, B., Borquist, H.P., Hultquist, M.E., Smith, J.M. Jr., Fahrenbach, M.J., Cosulich, D.B., Parker, R.P., Stockstad, E.L.R. and Jukes, T.H. (1950).

J. Am. Chem. Soc. 72, 4325.

Carter, F.C., Heller, D., Schuffner, G. and Korn, R.J. (1961).

Arch. int. Med. 108, 41.

Castle, W.B. (1926).

Amer. J. Med. Sc. 178, 748.

Chanarin, I. (1958).
Brit. med. J. 1, 1179.

Chanarin, I. (1963).
Brit. J. Haemat. 9, 141.

Chanarin, I., Anderson, B.B. and Mollin, D.L. (1958a),
Brit. J. Haemat. 4, 156.

Chanarin, I. and Bennett, M.C. (1962).
Brit. med. J. 1, 27.

Chanarin, I., Fenton, J.C.B. and Mollin, D.L. (1957).
Brit. med. J. 1, 1162.

Chanarin, I., Hutchinson, M., McLean, A. and Moule, M. (1966).
Brit. med. J. 1, 396.

Chanarin, I., Laidlaw, J., Loughbridge, L.W. and Mollin, D.L. (1960).
Brit. med. J. 1, 1099.

Chanarin, I., Mollin, D.L. and Anderson, B.B. (1958b).
Brit. J. Haemat. 4, 435.

Chanarin, I., Rothman, D. and Watson-Williams, E.J. (1963).
Lancet. 1, 1068.

Cooper, B.A. and Lowenstein, L. (1964).
Blood. 24, 502.

Cowan, J.D., Hoffbrand, A.V. and Mollin, D.L. (1966).
Lancet. 1, 11.

Cox, E.V., Meynell, M.J., Cooke, W.T. and Gaddie, R. (1960).

Clin. Sci. 19, 219.

Dacie, J.V. and Lewis, S.M. (1963).

Practical Haematology, 3rd ed. Churchill, London.

Davies, R.E. and Kelly, A. (1962).

Aust. J. exp. Biol. Med. Sci. 40, 437.

Day, P.L., Williams, C.L. and William, J.D. (1938).

Proc. Soc. exp. Biol. Med. 38, 360.

Dellar, D.J., Urban, E., Ibbotson, R.N., Horwood, J., Millazzo, S. and Robson, H.N. (1966).

Brit. med. J. 1, 751.

Donaldson, K.O. and Keresztesy, J.C. (1961).

Biochem. Biophys. Res. Com. 5, 289.

Duthie, J.J.R., Brown, P.E., Knox, J.D.E. and Thomson, M. (1957).

Ann. rheum. Dis. 16, 411.

Edozein, J.C. and Obasi, M.E. (1965).

Clin. Sci. 29, 1.

Engstedt, L., Johansson, S. and Strandberg, O. (1966).

Acta. med. scand. (suppl.) (454) 92.

Finlayson, N.D.C. and Shearmen, J.C. (1967).

Personal Communication.

Forshaw, J. (1963).

Brit. med. J. 1, 328.

Girdwood, R.H. (1953)

Lancet. ii 53

Girdwood, R.H. (1954).
"Some aspects of disordered folic acid metabolism in man" in Chemistry and Biology of Pteridines. p.p. 385 - 404. Churchill, London.

Girdwood, R.H. (1960).
Scot. med. J. 5, 10.

Girdwood, R.H. (1963).
J. Lab. med. Tech. 20, 26.

Girdwood, R.H. and Delamore, I.W. (1961).
Scot. med. J. 6, 44.

Gough, K.R., McCarthy, C., Read, A.E., Mollin, D.L. and Waters, A.H. (1964).
Brit. med. J. 1, 212.

Greenberg, L.D., Bohr, D.R., McGarth, H. and Rinehart, J.F. (1949).
Arch. Biochem. 21, 237.

Grossowicz, N., Arnovitch, J., Rachmilewitz, M., Izack, G., Sadovsky, A. and Bercovici, B. (1962).
J. Lab. clin. med. 60, 375.

Grossowicz, N., Mandelbaum-Shavit, F., Davidoff, R. and Aronovitch, J. (1962).
Blood. 20, 609.

Grzesinkowicz, H., Jennison, R. and Gowenlock, A.H. (1966).
J. clin. Path. 18, 599.

Gupta, N.K. and Robinson, W.G. (1961).
Fed. Proc. 20, 4.

Gutman, A.B. (1948).
"The plasma proteins in disease" in Advances in Protein Chemistry. Volume 4, p. 155. Academic Press (New York).

Hansen, H.A. and Nystrom, B. (1961).
Geront. clin. 3, 173.

Hansen, H.A. and Weinfield, A. (1962).
Acta. med. scand. 172, 427.

Hellman, S., Innattoi, A.T. and Bertino, J.R. (1964).
Ca. Res. 24, 105.

Herbert, V. (1961).
J. clin. Invest. 40, 81.

Herbert, V. (1962).
Trans. Ass. Amer. Physcns. 75, 307.

Herbert, V. (1965).
Ann. Rev. Med. 16, 359.

Herbert, V. (1966).
J. clin. Path. 19, 12.

Herbert, V., Larrabee, A.R. and Buchanan, J.M. (1962a).
J. clin. Invest. 41, 1134.

Herbert, V. and Zalusky, R. (1962b).
J. clin. Invest. 41, 1263.

Hoffbrand, A.N., Neale, G., Hines, J.D. and Mollin, D.L. (1966b).
Lancet. 1, 1231.

Hoffbrand, A.V., Newcombe, B.F.A. and Mollin, D.L. (1966a).

J. clin. Path. 19, 17

Hogan, A.G. and Parrot, E.M. (1940).

J. biol. Chem. 132, 507.

Hogan, J.A., Maniatis, A. and Maloney, W.C. (1964).

Blood. 24, 187.

Hunter, S.H., Bach, M.K. and Ross, G.I.M. (1956).

J. Protoz. 3, 101.

Iwai, K., Luttner, P.M. and Toennies, G. (1964).

J. biol. Chem. 239, 2365.

Jukes, T.H. and Stockstad, E.L.R. (1948).

Physiol. Revs. 28, 51.

Kay, A.W. (1953).

Brit. med. J. 2, 77.

Keresztesy, J.C., Rickes, E.L. and Stokes, J.L. (1943).

Science. 97, 465.

Keresztesy, J.C. and Donaldson, K.O. (1961).

Biochem. Biophys. Res. com. 5, 286.

Kerr, J.W. (1963).

Lancet. 2, 709.

Kershaw, D.W. and Girdwood, R.H. (1964).

Scot. med. J. 9, 201.

Knowles, J.P., Prankerd, T.A.J. and Westall, R.G. (1960).

Lancet. 2, 347.

Kodicek, E. and Carpenter, K.J. (1948).
Biochem. J. 43, i

Kohn, J., Mollin, D.L. and Rosenbach, L.M. (1961).
J. clin. Path. 14, 345.

Lampen, J.O. and Jones, M.J. (1946).
J. biol. Chem. 164, 485.

Larrabee, A.R., Rosenthal, S., Cathn, R.E. and Buchanan, J.M. (1961a).
J. Amer. chem. Soc. 83, 4094.

Larrabee, A.R., and Buchanan, J.M. (1961b).
Fed. Proc. 20, 9.

Laskowski, M., Mims, V. and Day, P.L. (1945).
J. biol. Chem. 157, 731.

Luhby, A.L. (1957).
Clin. Res. Proc. 5, 8.

Luhby, A.L. (1959).
J. Paed. 54, 617.

Luhby, A.L., Cooperman, J.M. and Teller, D.N. (1959).
Proc. Soc. exp. Biol. (N.Y.) 101, 350.

Luhby, A.L. and Cooperman, J.M. (1965).
Advances in Metabolic Disorders. Vol. I.
pp. 264 - 328. Academic Press. New York.
London.

Lutz, W. (1967).
Personal Communication.

Mallroy, M.E., Mims, V., Totter, J.R. and Day, P.L. (1944).

J. biol. Chem. 156, 317.

Marmount, A. (1948).

Arch. E. Maraghamo. Pat. clin. 3, 1289.

McKusick, A.B., Sherwin, R.W., Jones, L.G. and Hsu, J.M. (1964).

Arthr. and Rheum. 7, 636.

Metz, J., Stevens, K., Krawitz, S. and Brandt, V. (1961).

J. clin. Path. 14, 622.

Mims, V. and Laskowski, M. (1945).

J. biol. Chem. 160, 493.

Mims, V., Kazenteo, A. and Laskowski, M. (1948).

J. biol. Chem. 173, 217.

Mims, V., Totter, J.R. and Day, P.L. (1944).

J. biol. Chem. 155, 401.

Mitchell, H.K., Snell, E.E. and Williams, R.J. (1941).

J. Amer. chem. Soc. 36, 2284.

Mohmed, S.D. and Roberts, M. (1965).

Lancet. 2, 933.

Mohmed, S.D. and Roberts, M. (1966).

J. clin. Path. 19, 37.

Mollin, D.L. and Hoffbrand, A.V. (1965).

Series Haematologica. 3, I

Mollin, D.L., Waters, A.H., Gouch, K.R. and Read, A.E. (1963).

Lancet. 1, 609.

Nichol, C.A. and Welsh, A.D. (1950).

Proc. Soc. exp. Biol. Med. 74, 52.

Niewig, H.O., Faber, J.G., de Vries, J.A. and Kroese, W.F.S. (1954).

J. lab. clin. Med. 44, 118.

Noronha, J.M. and Aboobaker, V.S. (1963).

Arch. Biochem. Biophys. 101, 445.

Partridge, R.E.H. and Duthie, J.J.R. (1963).

Brit. med. J. 1, 89.

Pfiffner, J.J., Binkley, S.B., Bloom, E.S., Brown, R.A., Bird, O.D., Emmett, A.D., Hogan, A.G. and O'Dell, B.L. (1943).

Science. 97, 404.

Pfiffner, J.J., Calkins, D.G. and O'Dell, B.L. (1946).

J. Amer. chem. Soc. 68, 1392.

Pfiffner, J.J., Calkins, D.G., O'Dell, B.L., Bloom, E.S., Brown, R.A., Campbell, C.J. and Bird, O.D. (1945).

Science. 102, 228.

Rabinowitz, J.C. (1960).

"Folic acid" in the enzymes.

2nd Ed. D.D. Boyer, H. Lardy and K. Myrback, Eds. New York Academic Press. Vol. 2 pp. 185 - 252.

Ramsay, W.N.M. (1957).

Clinica Chem. Acta. 2 (3) 214.

Richmond, J., Gardiner, D.L., Roy, L.M.H. and Duthie, J.J.R. (1956).
Ann. rheum. Dis. 15, 217.

Roberts, E.C. and Snell, E.E. (1946).
J. biol. Chem. 163, 499.

Ropes, M.W., Bennet, C.A., Cobb, C., Jacox, R. and Jessar, R. (1959).
Ann. rheum. Dis. 18, 49.

Sauberlich, H.E. and Baumann, C.A. (1948).
J. biol. Chem. 176, 165.

Schilling, R.F. (1954).
Fed. Proc. 13, 769.

Schweigert, B.S. (1948).
J. lab. clin. Med. 33, 1271.

Schweigert, B.S. and Pearson, P.B. (1947).
Amer. J. Physiol. 148, 319.

Silverman, M., Law, L.W. and Kaufman, B. (1961).
J. biol. Chem. 236, 2530.

Simpson, R.E. and Schweigert, B.S. (1949).
Arch. Biochem. 20, 32.

Snell, E.E. and Patterson, W.H. (1940).
J. Bact. 39, 273.

Short, C.L., Bauer, W. and Reynolds, W.E. (1957).
"Rheumatoid Arthritis". A definition of the disease and a clinical description on a numerical study of 293 patients.
Harvard University Press. Cambridge, Mass.

Spray, G.H. (1964).

J. clin. Path. 17, 660.

Stockstad, E.L.R. (1943).

J. biol. Chem. 149, 573.

Stockstad, E.L.R. (1954).

"Pteroylglutamic acid" in the Vitamins.

Chemistry, Physiology, Pathology.

W.H. Sebrell, Jr. and R.S. Harris, Eds.

New York Academic Press.

Vol. III. pp. 89 - 217.

Tabor, H. and Wyngarden, L. (1958).

J. clin. Invest. 37, 824.

Temperly, I.J. and Horner, N. (1966).

J. clin. Path. 19. 43.

Teply, L.T. and Elvehjem, C.A. (1945).

J. biol. Chem. 157, 303.

Toennies, G., Frank, H.G. and Gallant, D.L. (1953).

J. biol. Chem. 200, 23.

Toennies, G., Phillips, P.M. (1959).

J. biol. Chem. 234, 2369.

Toennies, G., Usdin, E. and Phillips, P.M. (1956).

J. biol. Chem. 221, 855.

Usdin, E. (1959).

J. biol. Chem. 234, 2373.

Usdin, E., Phillips, P.M. and Toennies, G. (1956).

J. biol. Chem. 221, 865.

Waters, A.H. and Mollin, D.L. (1961).
J. clin. Path. 14, 335.

Watson-Williams, E.J. and Israels, M.C.G. (1966).
Lancet. 1, 454

Welsh, A.D. and Heinle, R.W. (1951).
Pharmacol. Rev. 3, 345.

Whitehead, R.G. and Arnstein, H.R.V. (1961).
Nature (Lond.) 190, 1105

Wills, L. (1931).
Brit. med. J. 1, 1059.

Wills, L. (1933).
Lancet. 1, 1283.

Wills, L. (1934).
Indian J. of med. Res. 21, 667.

Wills, L. and Bilimora, H.S. (1932).
Indian J. of med. Res. 20, 391.

Wills, L., Clutterbuck, P.W. and Evans, D.D.F. (1937a).
Biochem. J. 31, 2136.

Wills, L., Clutterbuck, P.W. and Evans, P.D.F. (1937b).
Lancet. 1, 311.

Wills, L. and Evans, P.D.F. (1938).
Lancet. ii, 416.

Wills, L. and Stewart, A. (1935).
Brit. J. of Exp. Path. 16, 444.

Wittenberg, J.P., Noronha, J.M. and Silverman, M. (1962).

J. Biochem. 85, 9

Zalusky, R. and Herbert, V. (1961).

J. clin Invest. 40, 1091.

Zalusky, R. and Herbert, V. (1962).

Lancet. 1, 108.

ACKNOWLEDGEMENTS.

I am deeply indebted to Professor R.H. Girdwood for providing me with the opportunity of carrying out the research for this Thesis in the Department of Therapeutics, University of Edinburgh, and for his continued encouragement and interest.

My sincere thanks to Dr. S.H. Davies, Consultant Haematologist, Royal Infirmary, Edinburgh, for his valued advice, guidance and criticism during my work and for allowing me to investigate patients under his care.

My thanks are due to Dr. J.J.R. Duthie and his colleagues of the M.R.C. Rheumatic Diseases Unit, Northern General Hospital, Edinburgh for allowing me to study patients under their care.

I wish also to thank my colleagues in the academic and laboratory technical staff of the Department of Thereapeutics for their advice and help.

Finally my deepest gratitude to Mrs. E. Wilson for accomplishing the difficult task of typing the Thesis with patience and efficiency.
