by Badenoch *et al.* (1955). As they suggest, the cause of megaloblastic anaemia of pregnancy seems to be a resistance to the action of haematopoietic factors rather than a deficiency state.

Conclusion

1. Inadequate diet is an important cause of iron-deficiency anaemia in pregnancy. Nevertheless iron therapy itself is effective in the individual case and there is some suggestion that iron therapy in one pregnancy will prevent or ameliorate iron deficiency in the subsequent pregnancy; presumably by replenishing depleted stores.
2. Inadequate diet is not likely to be the cause of megaloblastic anaemia of pregnancy, at least in European climates. Temporary failure to utilize certain haematopoietic factors is a more probable explanation of this disease.

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The role of pteroylglutamic acid and related compounds in macrocytic anaemia

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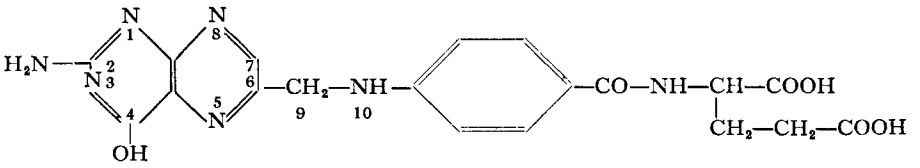
As dietary aspects in the narrow sense form such a small part of this subject and will be partly covered by other speakers, I have made this paper rather more general in its scope. It is impossible in a limited space to survey all the literature, and more details can be found in the reviews of Jukes & Stokstad (1948), Welch & Heinle (1951), Girdwood (1952*a*), Stokstad, Harris & Bethell (1954), and others.

Nomenclature and chemistry

Pteroylglutamic acid (PGA) is the chemically precise name for a compound isolated from liver and yeast, with the following structure:



The name folic acid was originally given to a substance concentrated from spinach but never completely purified (Mitchell, Snell & Williams, 1941). This substance had similar properties to PGA, and for this reason the term folic acid is often used in reference to PGA, a situation which causes some confusion. Some authors use the two names synonymously, whereas others reserve PGA for the pure substance, and use folic acid to refer to all compounds with the biological activity of PGA. I propose to adopt the latter convention in this paper.

Later work has shown the existence of various derivatives of PGA, all of which play some part in biological processes. These include 5-formyl-5:6:7:8-tetrahydro PGA, known variously as the citrovorum factor, folinic acid, or leucovorin; 10-formyl PGA, 5:6:7:8-tetrahydro PGA, and 5-hydroxymethyl-5:6:7:8-tetrahydro PGA.

PGA, and probably the citrovorum factor, also occur as peptides with glutamic acid, the so-called conjugates. These include pteroylhexaglutamylglutamic acid ('yeast conjugate'), containing six γ-linked glutamic-acid residues attached to the one already present in PGA; and pteroyldiglutamylglutamic acid, containing two such residues. Both these compounds occur naturally, and the diglutamyl conjugate has been synthesized. There is also the synthetic pteroylglutamylglutamic acid.

Only PGA, the citrovorum factor and the conjugates have been studied in connexion with human anaemia, and all seem to have the same potency on a molar basis against macrocytic anaemia. Unless specific statements to the contrary are made, therefore, it will be assumed that similar effects to those attributed to PGA have usually been observed for the related compounds.

History

The isolation of PGA from liver and yeast (Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan & O'Dell, 1943; Stokstad, 1943) was the outcome of several unrelated lines of research, including studies of the nutritional requirements of micro-organisms, chicks, monkeys, and man. The most significant work on man was that of Wills, who in 1931 described tropical macrocytic anaemia, a condition in Indian women with a blood picture similar to that in pernicious anaemia but lacking the other characteristic symptoms of pernicious anaemia. This anaemia responded to autolysed yeast (Marmite) (Wills, 1931), but not to purified liver extracts

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which were effective in pernicious anaemia (Wills & Evans, 1938). This distinguished the haematopoietic factor in yeast from the anti-pernicious-anaemia factor of liver.

It is still not clear whether the effects of Marmite were due solely to its content of folic acid, or whether there is an unidentified 'Wills factor'. Yeast products are effective in some cases of pernicious anaemia (Wintrobe, 1939).

These observations made it logical to study the effects of PGA in macrocytic anaemia when the synthetic material became available. Reports quickly accumulated showing that it caused blood regeneration in macrocytic anaemias such as Addisonian pernicious anaemia, nutritional macrocytic anaemia, pernicious anaemia of pregnancy, and the megaloblastic anaemias in infancy and in sprue. More details of these and later findings are considered in the next section.

Therapeutic effects

PGA is closely related in its anti-anaemic effects to vitamin B_{12} and vitamin C, and it is necessary to refer also to the role of these substances in the various conditions. Deficiency of vitamin B_{12} and of vitamin C can be detected by relatively simple tests, the determination of vitamin B_{12} in serum or of vitamin C in white cells. No comparable test exists for folic acid, and a normal level of vitamin B_{12} in the serum in megaloblastic anaemia is generally taken to indicate a deficiency of folic acid.

It is usually assumed that an haematopoietic response to folic acid indicates a disturbance in the metabolism of the factor. This disturbance may be due either to deficiency, caused by inadequate dietary intake, faulty absorption from the gut, inability to utilize dietary forms of folic acid, or excessive demand; or to impaired utilization, caused by lack of other substances necessary for proper utilization, or by the presence of factors interfering with normal utilization. The various conditions can be explained on this basis.

Nutritional macrocytic anaemia. This anaemia appears to be due to dietary deficiency of folic acid. In India, where the disease responds to PGA (Das Gupta & Chatterjea, 1946), the patients' diet was deficient in calories, animal protein, fresh fruit, and green vegetables (Wills & Talpade, 1930). In the southern United States, patients also had a low intake of animal protein (Moore, Vilter, Minnich & Spies, 1944), and PGA was effective against the anaemia (Vilter, Spies & Koch, 1945). In parts of Africa where megaloblastic anaemia occurs, the diet is again low in animal protein, and rich in bulky carbohydrates (Foy, Kondi & Manson-Bahr, 1955). Thus in all these areas there is a low intake of animal protein and probably also of other dietary sources of folic acid. A deficiency of vitamin B_{12} may also occur in this condition (Das Gupta, Chatterjea & Basu, 1953).

The sprue syndrome. An early report (Spies, Milanes, Menendez, Koch & Minnich, 1946) showed that PGA produced great improvement in the form of sprue seen in Cuba. Davidson, Girdwood & Innes (1947) showed that folic acid did not cause an haematological remission in tropical sprue and idiopathic steatorrhoea unless the bone marrow was megaloblastic at the beginning of treatment. The absorption of many substances from the gut is defective in steatorrhoea, and although in-

adequate dietary intake may play some part in the aetiology of the disease in some patients (Spies, Milanes *et al.* 1946), deficient absorption is probably the primary factor. We (Spray & Witts, 1952*b*) found evidence confirming the existence of a deficiency of PGA in steatorrhoea, and Girdwood (1953), by comparing the urinary excretion of folic acid after oral and parenteral administration of PGA, showed that absorption was defective in tropical sprue and idiopathic steatorrhoea. In these anaemias also, there may be a shortage of vitamin B_{12} as well as of folic acid (Nieweg, van Buchem & Kroese, 1952).

Pernicious anaemia of pregnancy. This condition responds to PGA (Davidson, Girdwood & Clark, 1948; Badenoch, Callender, Evans, Turnbull & Witts, 1955). Moderate doses of vitamin B_{12} are usually ineffective (Ungley, 1955) unless given in combination with vitamin C (Holly, 1951). Gatenby (1956) has discussed the effect of large doses of vitamin B_{12}, and the possible roles of poor diet and resistance to the action of haematopoietic factors. Girdwood (1954) found no evidence of inadequate absorption of PGA from the gut in eighteen patients. Perhaps the extra demands of pregnancy bring about sufficient deficiency of folic acid to cause anaemia in susceptible individuals.

Megaloblastic anaemia in infancy. PGA corrects this anaemia (Zuelzer & Ogden, 1946). Deficient intake of vitamin C probably plays a major part in the pathogenesis (May, Nelson, Lowe & Salmon, 1950), and vitamin B_{12} is not effective in all patients (Sturgeon & Carpenter, 1950).

Megaloblastic anaemia associated with epilepsy. An obscure anaemia in two epileptic women was reported by Badenoch (1954). Other cases have since been described (Chalmers & Boheimer, 1954, and others). Several patients responded to PGA after proving refractory to vitamin B_{12}, but the latter was effective in one of Badenoch's cases. A common feature of this condition seems to be the use of phenytoin to control the epilepsy, and it has been suggested that this substance may be antagonistic to folic acid. There is no proof of this yet.

Addisonian pernicious anaemia. The problem of folic acid in classical pernicious anaemia has interested us in Oxford for some years. The disease is caused by a deficiency of vitamin B_{12}, due to impaired absorption of this vitamin from the gut. However, PGA produces haematological remissions (Vilter *et al.* 1945; Wilkinson, Israëls & Fletcher, 1946), although the improvement may be only temporary (Schwartz, Kaplan & Armstrong, 1950). Under this treatment, however, the other principal sign of the disease, the subacute combined degeneration of the spinal cord, does not improve as it does with liver extracts or vitamin B_{12}, in fact it sometimes becomes worse (Ross, Belding & Paegel, 1948; Schwartz *et al.* 1950).

Evidence of a deficiency of folic acid in pernicious anaemia has been obtained by several authors. Bethell, Meyers, Andrews, Swendseid, Bird & Brown (1947) showed that after oral doses of PGA, patients with pernicious anaemia in relapse excreted less folic acid in the urine than did normal subjects. We have confirmed and extended these observations (Spray, Fourman & Witts, 1951; Spray & Witts, 1952*a*,*b*, 1953) by measuring folic acid in plasma and urine after oral or intravenous doses of PGA or citrovorum factor. In all these tests patients with pernicious anaemia

in relapse showed less folic acid in both plasma and urine than either normal subjects or patients (sometimes the same patients) in remission. Thus the deficiency of folic acid in untreated pernicious anaemia seems to be corrected by treatment with vitamin B_{12}, and the question arises of why there is this deficiency in a condition caused by lack of vitamin B_{12}. This problem is considered in connexion with theories about the biochemical action of folic acid (see below).

Rare cases of megaloblastic anaemia are encountered in association with scurvy, intestinal lesions such as diverticula or blind loops left after surgery, liver disease, and gastrectomy, but information about the action of folic acid in these diseases is not very precise and need not be mentioned here.

Biochemical defects in macrocytic anaemia

The most plausible hypothesis yet advanced is that of Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins & Seaman (1950), who suggested that folic acid and vitamin B_{12} take part in reactions leading to the synthesis of the nucleoproteins of blood cells from simple precursors (Fig. 1). If vitamin B_{12} is deficient, as in pernicious anaemia, there is an excessive demand for folic acid, which keeps the reactions

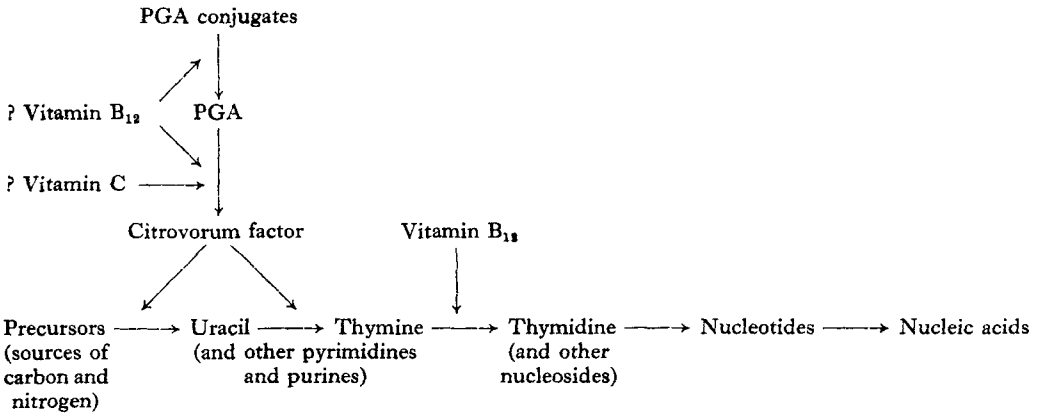

Fig. 1. Hypothetical interrelationships between haematopoietic factors. A more comprehensive version of this scheme, embodying findings in many fields, has been drawn up (Mueller & Will, 1955), but this simplified scheme is sufficient for the present purpose.

going by 'mass action'. This ultimately causes a deficiency of folic acid. When the lack of vitamin B_{12} is made good, the demand for folic acid falls and the deficiency disappears. Large doses of folic acid increase blood formation by raising the concentration of pyrimidines or purines, so that the later reactions can proceed despite the shortage of vitamin B_{12}. This process uses up the remaining small stores of vitamin B_{12}, causing exacerbation of the nervous degeneration, because vitamin B_{12} is apparently essential for the integrity of the nervous system as well as of the haematopoietic system. Sooner or later insufficient vitamin B_{12} is left for proper blood formation even in the presence of large amounts of folic acid, which explains why patients with pernicious anaemia on PGA relapse haematologically. Increasing the dose of

folic acid produces a further temporary remission (Vilter *et al.* 1950), presumably by a repetition of the same process.

Several observations support this hypothesis. Large doses of thymine (Spies, Frommeyer, Vilter & English, 1946; Vilter *et al.* 1950) and of thymidine (Hausmann, 1951) cause responses in pernicious anaemia, as would be expected if the products of the early reactions in the series are in short supply. I was unable to demonstrate a deficiency of thymine in untreated pernicious anaemia by giving test doses of this substance (Spray, 1956), but this may be because only small amounts of exogenous thymine are utilized for synthesis of nucleoproteins (Holmes, Prusoff & Welch, 1954). Folic acid plays some part in the synthesis of nucleoproteins and their constituents, because in megaloblastic anaemia the bone marrow contains more ribonucleic acid in proportion to deoxyribonucleic acid, and more uracil in proportion to thymine, than normal. The proportions become normal on treatment with PGA or vitamin B_{12} (Vilter, Glazer, Mueller, Jarrold, Sakurai & Will, 1953).

This hypothesis suggests a reciprocal relationship between folic acid and vitamin B_{12} in blood formation. The therapeutic effects of the vitamins in different megaloblastic anaemias support it. PGA aggravates vitamin B_{12} deficiency in pernicious anaemia (see above), and the reverse process may occur in other types of megaloblastic anaemia (Harris, 1955). Patients occasionally fail to respond to vitamin B_{12} until PGA is given (Ungley, 1952), or vice versa (Nieweg *et al.* 1952). Against this view, Reisner & Weiner (1952) suggested that small oral doses of PGA and vitamin B_{12} together may have a synergistic effect in pernicious anaemia, and Tasker (1955) found evidence of a direct relationship between the factors in nutritional megaloblastic anaemia.

There have been suggestions that the metabolism of folic acid is partly controlled by vitamin B_{12}. Patients with untreated pernicious anaemia have partial or complete inability to utilize conjugates of folic acid, a defect which is corrected by treatment (Welch, Heinle, Nelson & Nelson, 1946; Bethell *et al.* 1947; Sharp & Vonder Heide, 1947). Bethell *et al.* attributed this to the presence in their preparations of inhibitors of the enzymes (conjugases) which release PGA from its conjugates. The anti-anaemic factor in liver extract was supposed to overcome the action of these inhibitors. However, conjugases have not been detected in gastric or duodenal juice (Buyze & Engel, 1948; Welch *et al.* 1946), and normal people absorb very little folic acid from crude sources (Swendseid, Bird, Brown & Bethell, 1947; Spray, 1952), so that there is little evidence to support this hypothesis. We had hoped to collect further data on this point, but we have been unable to obtain any pteroylhexaglutamylglutamic acid.

The conversion of PGA to the citrovorum factor was also supposed to be controlled by vitamin B_{12}. After test doses of PGA, untreated patients with pernicious anaemia excreted less citrovorum factor in the urine than normal subjects or the same patients in remission (Spray & Witts, 1952*a*). The liver of a patient with untreated pernicious anaemia apparently contained a considerable amount of PGA as well as of citrovorum factor, whereas normal livers only contained citrovorum factor (Girdwood, 1952*b*). These observations appear to favour this hypothesis, but the citrovorum factor is

no more effective weight for weight than PGA in pernicious anaemia (Ellison, Wolfe, Lichtman, Ginsberg & Watson, 1951), and Burchenal (1951) stated that patients with pernicious anaemia convert PGA to the citrovorum factor effectively.

Questions still to be answered

The hypothesis of Vilter *et al.* (1950) provides a useful working basis, but we are still a long way from a complete understanding of the role of folic acid in blood formation. There is no indication whether the functions of folic acid suggested by work in other fields, such as its role in the transfer of 'one-carbon fragments', have any relevance in human blood formation.

Even though there are obvious limitations on research that can be carried out on human subjects, there are many surprising gaps in our knowledge, as well as loopholes in work already done. Most studies have been unphysiological because the doses of PGA were much larger than the normal intake, and because PGA seldom occurs naturally in the free state. We know almost nothing of the utilization of folic acid from the diet, or about its fate in the body. Is it stored in the liver, or elsewhere, like vitamin B_{12}? Is it excreted in a form not detected by methods used hitherto? How much is destroyed in food by cooking? Does folic acid synthesized by intestinal bacteria contribute to the supplies utilized for haematopoiesis in man? What is the true mechanism of the anti-anaemic action of yeast? Information on these and related questions would help a lot in unravelling this problem.

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The absorption of vitamin B_{12} in the megaloblastic anaemias

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The labelling of vitamin B_{12} with radioactive cobalt has permitted application of the tracer technique to studies of the absorption of vitamin B_{12} and eliminated the major difficulties inherent in earlier methods. This technique enjoys the unique advantages that it can be carried out on patients who have received treatment and that it gives a moderately accurate quantitative assessment of absorption with doses of vitamin B_{12} similar to the daily requirements of man. In Professor Witts's depart-

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