REVIEW ARTICLE

FOLIC ACID, VITAMIN B_{12} AND ANÆMIA

I. CHEMICAL ASPECTS*

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ONE of the most fascinating stories in the whole realm of Biochemistry is the story of the attack on pernicious anæmia and other macrocytic anæmias; that is to say, those anæmias characterised by red blood cells that are too large but too few, in contrast to the microcytic anæmias in which the red cells are too small, owing generally to iron deficiency.

The story begins in 1926 with the discovery by Minot and Murphy[1] that the so-far incurable pernicious anæmia would respond to large amounts of raw or lightly cooked liver given by mouth. The story has culminated in the isolation and synthesis of folic acid and the isolation of vitamin B_{12}. An interesting feature is that it represents the convergence of a large number of different researches, some having entirely different original objectives.

The discovery of the effectiveness of liver treatment in pernicious anæmia was an immediate challenge to biochemists and medical men; first to produce a less nauseating treatment, then to isolate the active principle in liver, and finally to discover its mode of action. The first objective was accomplished within a few years, but the second and third have remained for over twenty years problems that are not fully solved even now. It was soon found possible to make aqueous extracts of liver and to purify them somewhat by fractionation with alcohol and by other means. The early crude extracts had to be taken by mouth, but the more purified preparations could be given by injection. The active principle appeared to be much more efficiently utilised when given by injection, so that this treatment has remained the standard practice.

The problem of purifying these liver extracts and finally isolating the active principle itself proved exceptionally difficult. This was due not only to inherent difficulties—although it is probably true that the isolation of vitamin B_{12} has been more troublesome than that of almost any other vitamin—but mainly to the lack of a satisfactory assay method. In this country we had to rely entirely on clinical tests on cases of pernicious anæmia in relapse, although in America the final stages of the work were made easier by Shorb's development[2] of a microbiological assay technique.

Space will not permit a review of the earlier stages of this work beyond recalling the names of those concerned. The first contributions by Cohn, Minot, West, Nichols, Howe, Dakin, Jacobson and SubbaRow came from America, and this early work has been fully reviewed in the literature[3,4,5,6]. Further valuable contributions were made by Laland and

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Klem[7] in Norway, while Karrer in Switzerland worked on the problem for a time. This covers the period up to about 1938, by which time purified preparations had been obtained which were fully active in a single dose of a few milligrams.

In order to tell this complicated story in roughly chronological fashion, it is necessary next to consider another series of researches which, until they were practically complete, seemed to have no connection with human anæmias. This work was reported between the years 1939 and 1945. Several groups of American workers had prepared, from liver and from yeast, concentrates that were characterised by two tests. They enhanced the growth rate of *Lactobacillus casei* and other micro-organisms, and they promoted normal growth of chicks on certain deficient diets. These investigations were carried out by research teams in two American industrial laboratories, Parke Davis and Co. and Lederle, and in several academic institutions[3,4,5,6]. A little later a similar or possibly identical factor was isolated in a nearly pure state from spinach, and was named folic acid because it was obtained from leaves. In 1943 the Parke Davis group also obtained crystals from liver, for which they retained their own nomenclature vitamin B_C. Almost simultaneously the Lederle team obtained a crystalline substance from liver, for which they retained the name *L. casei* factor. It soon appeared that these products were identical and a little later it was shown that the same substance could be obtained in crystalline form from yeast.

These commendable achievements did not entirely resolve the intense confusion in this field. It had arisen through the use by different groups of investigators of different animal and microbiological assay techniques; they also used different procedures for the isolation of their factors, which appeared to be distinct, though possibly related, entities. Thus among the animal assays in use were those involving growth rate, anæmia, and feathering in chicks, and anæmia in monkeys, while two different organisms were used in the microbiological work. In addition to folic acid, vitamin B_C and the *L. casei* factor, all the following names have been coined: vitamin B_{10}, vitamin B_{11}, vitamin M, Norit eluate factor, factor S, factor R and factor U.

Many of the discrepancies were cleared up with the discovery of the vitamin B_C conjugates. It was shown that from yeast in particular could be obtained preparations fully active in chick tests, but nearly inactive against *L. casei*. Microbiological activity was released, however, on treating these preparations with enzymes obtained variously from rat liver, hog kidney, chicken pancreas and almond. However, this advance was complicated by the fact that often only part of the total *in vivo* activity was released by some enzyme preparations. This difficulty was later traced to the presence in some materials like yeast extract of an inhibitor for the enzyme.

In 1944 Hutchings, Stokstad[8] and others prepared one of the conjugates in crystalline form from a new source material described as a fermentation residue. It was later shown that it could be broken down enzymatically to folic acid and two molecules of glutamic acid.

Pfiffner and others[9] isolated another conjugate from yeast which could similarly be broken down to folic acid plus six molecules of glutamic acid.

The procedures used for the preparation of these substances were not published until several years after their isolation had been announced. The methods used by the two independent teams were rather similar. Pfiffner and others[10] prepared an aqueous extract of the source material after autolysis or enzymatic digestion. The active principle was then adsorbed on the ion exchange resin Amberlite IR-4 and eluted with aqueous ammonia. The product was readsorbed on Super Filtrol and then on Norit SG-11, elution from each being with aqueous or alcoholic ammonia. An aqueous solution was then extracted with butyl alcohol, first at *p*H 5·6 to remove impurities, and then at *p*H 3 to extract the folic acid. Further purification was effected through the barium and zinc salts. Stokstad and others[11] also employed adsorption on Norit A and Super Filtrol. They also purified the product via the barium salt, but their next step was the preparation of the methyl ester which was then purified by chromatography on Super Filtrol. The ester was finally hydrolysed and the folic acid was crystallised from water.

The crowning achievement in this field was the synthesis of folic acid[12,13], announced in 1945, only two years after its isolation. The intense effort put into this project by the Lederle group is indicated by the fact that this paper had no less than sixteen authors. In the following year the constitution of the substance was revealed. It contains a pteridine nucleus (found also in xanthopterin—a yellow pigment of butterflies' wings) linked to a molecule of *p*-aminobenzoic acid, which is linked in turn to a molecule of glutamic acid. This elucidation of the structure was marked by the introduction of yet another name for the substance—pteroylglutamic acid. Subsequently the conjugates have also been synthesised and it has been proved that the successive glutamyl residues are all linked in the γ position[13,14,15,16,17]. Finally it was concluded that folic acid, vitamin B_C, or *L. casei* factor, whether isolated from yeast, liver, or spinach, was identical with the synthetic product, while all the other factors showing different microbiological activities were almost certainly glutamic acid conjugates.

It was only after the synthesis of folic acid had been accomplished that evidence of its value in human macrocytic anæmias was published[18]. In other words, all this intensive research was directed towards substances appearing at the time to be of value only to micro-organisms, chicks and monkeys. However, the demonstration of its value in human anæmias created a considerable stir in medical and other scientific circles. It seemed at first that it would soon displace liver extract. It was quickly evident, however, that folic acid could not be identical with the active principle in liver because already at that time liver concentrates had been obtained that were far more active, weight for weight, than pure folic acid. Then gradually evidence accumulated as to the clinical shortcomings of folic acid. Naturally this revived interest in

the liver factor and research on its isolation was intensified in this country and America.

Further stages of purification were effected by my own colleagues Hurran, Emery and Parker[19,20], at Glaxo Laboratories, and reported in 1945 and 1946. They carried the work to the stage where a clear-cut and generally maximal response in pernicious anæmia followed a single injection of only 1 mg. of material. At the time this seemed a rather remarkable achievement, but we now know that their product contained only about 1 per cent. of vitamin B_{12}. Nothing further appeared in the literature until 1948 when the Merck team[21], directed by Karl Folkers, dropped a bombshell by announcing their isolation of the anti-pernicious anæmia factor of liver in the form of a deep red crystalline substance; this they christened vitamin B_{12}. In the following week there appeared my own publication[22] on the isolation from liver of two highly active red amorphous preparations, while a few weeks later Parker and I[23] announced the isolation of crystalline material, probably identical with vitamin B_{12}.

Other papers appeared in quick succession on both sides of the Atlantic. The most interesting concern the simultaneous announcement[21,22] of the presence in vitamin B_{12} of an atom of cobalt, an element not previously found in any organic substance of biological origin. On the clinical side[26] it became clear that vitamin B_{12} along with our related red substance represented the true liver principle, active in pernicious anæmia and its associated neurological complications. Moreover this substance set up a new record among the vitamins and hormones by its great potency; the human requirement was assessed at something under 1 μg. per day.

The method used for isolating vitamin B_{12} from liver has now been published by our team[27]. It follows from the unexpectedly high potency of vitamin B_{12} that the proportion present in liver is extremely small. In fact it is of the order of one part per million. Another reason for the difficulty of the isolation was that the substance appears to have no chemical properties that can be utilised (such as the acidity of folic acid, for example). Accordingly our methods were almost exclusively physical. We relied upon repeated adsorption on charcoal and elution with either 80 per cent. phenol or with aqueous alcohols, and adsorption chromatography on alumina and silica. Another invaluable step was partition chromatography on damp silica from butyl alcohol or other solvents. It was desirable to interpose at some stage enzymic proteolysis to break down persistent peptide impurities. The vitamin was finally crystallised in a hydrated form from aqueous acetone.

The structure of vitamin B_{12} has not yet been fully elucidated. Its empirical formula[28] is approximately $C_{61-64}H_{86-92}N_{14}O_{13}PCo$, its molecular weight about 1300. The substance is not a peptide as earlier work with impure preparations had suggested. There is some indication that it may be related structurally to chlorophyll and the pigment of hæmoglobin. It appears to have nothing in common with folic acid. Two fragments have been "chipped off" the molecule, according to

recent reports from England and America; one was thought to be β-aminopropanol, but its true identity remains unknown, and the other a dimethylbenzimidazole[29,30,31,32].

Many vitamins and antibiotics are now known to occur in two or more varieties, closely related chemically. Thus there are several forms of vitamins A. D and E, several varieties of penicillin and two of streptomycin. Vitamin B_{12} is no exception; we have already published evidence for the existence of one and probably two additional closely related red biologically active substances. It has just been announced that one of these has been obtained in crystalline form by Jukes and his colleagues at the Lederle laboratories[33]. It has been named vitamin B_{12B}, the name B_{12A} having already been used for an artificially produced hydrogenation product of vitamin B_{12}.

It is now time to turn to still another group of researches that originally appeared to have no connection with human anæmia. During the wartime scarcity of animal feeding stuffs it was found that chicks raised on vegetable diets grew poorly. Since this trouble was cured or prevented by giving adequate amounts of animal protein, the term animal protein factor was coined. Hammond and Titus[34] showed that dried cow manure was a rich source of this factor. Rubin and others[35,36] showed that hens on an inadequate soya bean meal diet produced eggs of low hatchability, a phenomenon prevented by a purified fraction from cow manure. Independently Zucker[37] in 1948 presented evidence that a factor in animal proteins, which he called zoopherin, improved the growth of rats on a deficient diet. Robblee and others[38] presented evidence for a chick growth factor in fish solubles and described some of its chemical properties. The work of Cary and others[39] had shown that liver extracts active in pernicious anæmia contained a rat growth factor, and by 1948 it seemed probable that all these factors were identical with one another and with the anti-pernicious anæmia factor. Meanwhile it had been observed that chicks seemed to have less need for the factor during the summer months when they were out in the open and had access to food materials contaminated with their own fæces. This observation, along with the activity of cow manure, suggested that the factor might be synthesised by micro-organisms. Finally Stokstad and others[40] showed that the factor could indeed be synthesised by an organism isolated from hen fæces. They showed in addition that this bacterial preparation was effective in cases of pernicious anæmia.

This discovery immediately gave rise to the idea that the pernicious anæmia factor might be obtained by a fermentation process similar to those used for making antibiotics. This was duly followed by the even more exciting idea that the factor might be produced incidentally in some fermentations already in progress industrially. This proved to be so and late in 1948 the Merck team announced the isolation of crystalline vitamin B_{12} from the fermentation products of *Streptomyces griseus*, the organism used to make streptomycin[41].

The discovery that vitamin B_{12} contains cobalt appeared to link up this work with yet another group of researches carried out in Australia in

1935 and onwards. I refer to the independent discoveries by Filmer and Underwood[42], and by Marston[43], that cobalt is a necessary trace element for sheep and cattle. These ruminants when kept on certain pastures in Australia, and indeed in many other parts of the world, develop a fatal sickness that can only be cured by frequent ingestion of minute amounts of cobalt. On some Australian pastures there is an additional requirement for traces of copper. Our first guess was that the animals needed cobalt in order to synthesise vitamin B_{12}. Accordingly we provided Marston with a little of the crystalline vitamin B_{12} for trials on sheep. He has recently reported that the results were entirely negative[44]. The same conclusion was reached independently by Becker and others[45], so it seems that after all this work on cobalt had no direct connection with vitamin B_{12}. Various lines of evidence supported the view that the cobalt was not required by the animal directly, but rather by the rumen bacteria that help it to digest its food. Thus injected cobalt was found to be useless, while on the other hand Albert[46] had demonstrated the unique requirements of some bacteria for cobalt, and more directly Gall[47] and others had very recently shown that oral cobalt administration increases the number, diversity and viability of rumen bacteria in cobalt deficient sheep.

Nevertheless these researches may be linked in another fashion. Vitamin B_{12}, or the animal protein factor, has so far been found only in natural products of animal origin, and yet there is no evidence that animals can synthesise it for themselves; indeed the fact that several species require the substance in their diet is evidence that these at least cannot synthesise it. We are led to suggest, therefore, that vitamin B_{12} originates in nature only through bacterial synthesis, and that it finds its way into the liver and other tissues of herbivorous animals as a result of bacterial synthesis in the rumen or intestine.

The fact that intestinal synthesis of a vitamin is going on within an animal or human being does not necessarily imply no requirement for an external source of that vitamin; if the synthesis occurs too low down in the intestinal tract, then the vitamin may either not be released from the bacterial cells or if released, it may not be absorbed. It has lately been shown, for example, that some pernicious anæmia patients excrete in their fæces amply sufficient vitamin B_{12} to keep them in health if only it could find its way into the blood-stream[48].

It is now known that folic acid is also synthesised by the intestinal flora. Deficiencies of both anti-anæmic factors can be induced, in pigs for example, by giving deficient diets along with suitable sulphonamides to keep down bacterial fermentation in the intestine; folic acid antagonists have sometimes been added as well. In this way there has been demonstrated the need for both vitamin B_{12} and folic acid for normal growth and red cell regeneration. It is probable that both factors are needed by other species and by humans.

The problem of the biogenesis of vitamin B_{12} calls to mind another early group of investigations on pernicious anæmia which I am deliberately taking out of their proper chronological order; I refer to the

work of Castle and his colleagues[49]. They showed conclusively that pernicious anæmia can be treated without having recourse to liver preparations. The alternative treatment consisted in giving by mouth, along with suitable foodstuffs, either normal gastric juice or a preparation from the lining of a hog's stomach. These materials were supposed by Castle to contain an intrinsic factor, which reacts with an extrinsic factor present in certain foodstuffs to produce either the liver factor itself or something equally effective. The value and mechanism of this therapy was rapidly confirmed[50]. Ungley[51], in particular, showed that it was useless to give the hog's stomach extract alone between meals; it was effective only when given simultaneously with the sources of extrinsic factor, although previous incubation of the two was not necessary. It was argued that if the product of the interaction of these factors was identical with the anti-pernicious anæmia factor of liver, then it should prove possible to work up the product from incubating the two factors (using techniques known to be effective with liver) to make a preparation active by injection. Many attempts were made to do this but without success. Castle's suggestion that some intermediate product was formed and required further digestion to convert it into the liver factor could also not be proved experimentally.

There the matter remained until recently, but meanwhile evidence accumulated that some at least of the known sources of extrinsic factor like meat and milk contained low concentrations of vitamin B_{12} itself. When the crystalline vitamin became available it was tested orally and found to be relatively ineffective. On giving it orally, however, along with normal gastric juice, vitamin B_{12} proved to be nearly as effective as when given by injection[52]. This led to the idea that extrinsic factor and vitamin B_{12} are one and the same thing. If this conclusion is accepted, then it solves one mystery—only to pose another; that is, to explain by what means the intrinsic factor renders vitamin B_{12} effective by the oral route. Presumably it must in some fashion facilitate its passage through the wall of the intestinal tract, but it is hard to imagine the details of the mechanism.

This is only one of the mysteries still remaining to be elucidated. In the field of animal nutrition some recent investigations[53] have thrown doubt on the complete identity of the animal protein factor and vitamin B_{12}. In addition another apparently related factor[54] has been isolated from distillers' solubles and christened vitamin B_{13}.

In 1931 Lucy Wills and her colleagues[55] were working on nutritional macrocytic anæmia in India and on a related anæmia in monkeys induced by giving deficient peasant diets. They found that these conditions could be successfully treated with liver fractions that did not appear to contain the anti-pernicious anæmia factor. Independently Ungley[51] has shown that pernicious anæmia could be treated with large oral doses of yeast extracts without intrinsic factor. In the light of subsequent knowledge various reviewers have sought to explain away these findings on the grounds that the effective preparations contained either some vitamin B_{12}, or some folic acid or its conjugate. They very likely did, but the question

remains whether they contained enough of these factors to explain their efficacy. A critical review of the data makes this seem improbable, in which event the nature of the Wills factor still remains to be elucidated.

Finally it remains to be explained how and in what cells of the body this peculiar cobalt compound vitamin B_{12} accomplishes its work of regenerating the red blood cells. This includes the problem of how an injection of only some 10 μg. finds it way to the proper site of action and produces such rapid and dramatic results. Alongside remains also the problem of how larger amounts of a totally different substance, folic acid, achieve, in part at least, the same distant ends.

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