CRYSTALLINE ANTI-PERNICIOUS-ANAEMIA FACTOR

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The anti-pernicious-anaemia factor (vitamin B₁₂) having at last been isolated in the pure crystalline state, it may reasonably be asked why this took so long. Within a few weeks of one another, independent announcements of the achievement were made by American and British teams (Rickes *et al.*, 1948a; Lester Smith and Parker, 1948). Yet it is twenty-two years since Minot and Murphy (1926) first discovered the effectiveness of liver therapy for pernicious anaemia. During the intervening years many new vitamins and hormones have been discovered, and nearly all of them have been concentrated, purified, and analysed and have had their chemical constitutions elucidated and confirmed by synthesis. All the well-defined vitamins have been prepared synthetically with the partial exception of vitamins D₂ and D₃, which have been "synthesized" only from biologically inert natural sterols.

In the light of these achievements the fact that the anti-pernicious-anaemia factor still remained to be isolated became an increasing challenge to biochemists. The delay was certainly not due to lack of interest in the problem. In a review on the factor published in 1945 SubbaRow, Hastings, and Elkin were able to cite no fewer than 129 relevant references.

The prime necessity for successful isolation of a natural biologically active substance is a means for its assay. The quicker and the more reliable the assay method the more rapid is progress likely to be, because there is usually no great difficulty in devising fractionation techniques that may be applicable. Indeed, for the water-soluble vitamins there are available a number of techniques that have become almost standard practice. One recalls, for example, such methods as precipitation with salts of lead, silver, and mercury, fractionation of phosphotungstates, adsorption on charcoal, and so on. For all the vitamins that were isolated within a short period of their initial discovery satisfactory tests were available. Thus, aneurin could be assayed by growth-rate methods, using rats; riboflavin could be assayed very easily by its intense fluorescence in ultra-violet light; ascorbic acid even more easily by titration with iodine or still more specific oxidizing agents.

For the anti-pernicious-anaemia factor, however, the only available test until very recently was the assay on clinical cases. Moreover, only a small fraction of these cases were suitable for assay purposes. Much ingenuity was expended in attempting to devise animal tests, but until very recently none of them has proved reliable. It now seems that it may be possible to base an assay technique on the observation that chicks need the factor. The American workers (Rickes *et al.*, 1948a) had the advantage of a microbiological assay technique with *Lactobacillus lactis* Dorner (Snorb, 1948), but the news of this came too late to be of any assistance to our British team. Thus an adequate number of clinical tests was vital to the success of the

work. It is probably true to say that no previous investigator in this field had the advantage of so many carefully conducted clinical tests as we were afforded by our collaborator, Dr. C. C. Ungley.

A Review of Earlier Work

The more outstanding contributions to the chemical fractionation of liver are perhaps worth reviewing again in the light of present knowledge. The first important advances were made by Cohn, Minot, *et al.* (1928). They showed that the factor could be extracted from liver with water at faintly acid pH, and that proteins could be removed and other impurities separated by addition of alcohol to about 70% concentration. After evaporation to small bulk the active fraction could be thrown out with strong alcohol; this was the famous "Cohn's fraction G." This insolubility in alcohol was due to co-precipitation with other substances present in the crude extract, because at a later stage of purification the factor became soluble in alcohol. Cohn, Minot, *et al.* also showed that the active principle was precipitated by phosphotungstic acid but not by lead acetate, and that it could be extracted with butanol.

These techniques were successfully used again by West and Nichols (1928). West and Howe (1930) claimed the isolation of an active crystalline quinine salt, but later they withdrew this claim.

Dakin and West (1935) and Dakin, Ungley, and West (1936) reported an extensive investigation in which a number of new procedures were developed. They showed that impurities could be precipitated with alcoholic calcium acetate, and that the active principle could be precipitated with Reinecke acid and also with uranium acetate. They showed, too, that the factor could be salted out from neutral solutions by ammonium or magnesium sulphate, and from acid solution by sodium chloride.

New Techniques

Valuable new techniques were introduced in 1936 by Laland and Klem. They reported that the active factor could be extracted from aqueous solutions with liquefied phenol and also that it could be adsorbed on activated charcoal and eluted with liquefied phenol.

By the repeated use of these procedures, together with salting out by ammonium sulphate and fractionation with alcohol and ether, they obtained fractions reported by Strandell to be clinically active in doses of a milligram or so. These remarkable claims were received initially with some scepticism, especially as the novel techniques introduced were not described in sufficient detail to permit exact replication by independent workers. Moreover, when the use of techniques alleged to be those of Laland and Klem, but in effect not closely similar, led to the

separation of much less active fractions, there was a tendency to discredit the Norwegian workers. Thus they have never received proper recognition for genuine achievements that were not bettered during the next ten years. Moreover, their description of their most active fraction as a bright reddish-yellow substance acquires new significance in the light of present knowledge.

We were accorded the privilege of examining their case reports at the time; although in several instances the responses were inadequate with the very small doses given, there seemed no doubt that the fractions tested were very much more active than anything previously obtained. In addition, we were able to secure independent clinical tests on some of their less highly purified fractions, which proved fully active in single doses of less than 10 mg. It should be added that their methods of fractionation have provided an invaluable foundation for our own work. Karrer and his colleagues (1938) also reported the preparation of fractions fully active in doses of 10 mg., or rather less, by methods that were not described in detail.

It is difficult to assess the work of Jacobson, SubbaRow, and others published between 1935 and 1938 (see SubbaRow *et al.*, 1945). These workers claimed that successful treatment of pernicious anaemia called for the simultaneous administration of several factors. One of these was identified as *L*-tyrosine, but the others were not fully purified. Their theory was supported by a reasonable weight of clinical evidence, but it has not been widely accepted. Ungley has now presented ample evidence showing that the pure crystalline anti-pernicious-anaemia factor alone, in adequate doses, gives a classical response in cases of pernicious anaemia. One is inclined to suggest, therefore, that the supplementing action of Jacobson and SubbaRow's accessory factors may have been due to contamination with small proportions of their primary factor (presumably the anti-pernicious-anaemia factor in crude form); satisfactory responses might well have been obtained with larger doses of the primary factor alone.

No further significant advance was reported until 1945, when Emery and Hurran reported a concentrate fully active in a dose of 5.5 mg. In the following year Emery and Parker (1946) reported further concentration to a preparation active in a single dose of only 1 mg. These fractions were made by application of known techniques—in particular, repeated adsorption on charcoal, elution with phenol or with hot 65% alcohol, and salting-out with ammonium sulphate.

It is something of a tragedy to recall the considerable amount of analytical work that has been performed by some groups of investigators on material now known to have contained as little as 0.01% to 1% of the pure factor. It must be confessed that we fell into the same error ourselves in making preliminary chemical investigations on material containing only about 3% of the factor, under the impression that it must be approaching purity. It seemed appropriate at the time to carry out the various analyses, for there was no reason at all to anticipate the phenomenal potency of the crystalline factor. We now know that the human requirement is only about 0.001 mg. a day. The only substances known to be physiologically active in smaller amounts are such poisons as plutonium and botulinus toxin.

Partition Chromatography

Reviewing the situation in 1946 it seemed to us unlikely that any further advance would be made without the introduction of new techniques. There was also little doubt that the new approach required was some form of chromatography, preferably perhaps the recently introduced parti-

tion chromatography. This method employs a tube packed with an inert porous powder capable of soaking up a considerable proportion of water without appearing damp; the material to be separated is applied to this column dissolved in an organic solvent not completely miscible with water, and development is continued with the same solvent. These columns do not differ in appearance from the familiar adsorption chromatograms using alumina, for example, but the mechanism of separation is different: it depends on differences in the partition of the various substances present between water (held in the pores of the solid) and the solvent, instead of upon differences in adsorbability. Such partition chromatograms, using damp silica and butanol, fulfilled our expectations. Liver extracts, sufficiently purified by the methods already outlined, separated readily into three coloured zones. Yellow material travelled quickly down the column; a pink zone travelled very much more slowly; a brown zone remained near the top of the column, with a tendency to overlap somewhat into the pink zone. Nearly colourless material was also present between the pink and yellow zones.

It was an obvious step to use these colours as a guide in cutting up the chromatogram, but we were surprised to find it so effective, for nearly all the clinical activity was concentrated in the pink fraction. The same thing happened in other experiments. Nevertheless we expected that this would turn out to be merely an adventitious association of a red pigment with the active principle, and a faulty interpretation of some later experiments seemed to confirm this view. Already we had reached the stage of purification where a ton or so of liver was needed to provide a workable amount of material, and the lengthy procedures for preliminary concentration as well as inevitable delays in clinical testing made progress very slow.

About this time we received temporary discouragement from the announcements that folic acid was effective in pernicious anaemia, and that it could be made synthetically. It was soon clear, however, that our liver concentrates, though still impure, were weight for weight much more effective than folic acid. We were therefore dealing with a different substance, and its isolation was still of academic interest at least. Then, as the clinical shortcomings of folic acid gradually came to light, interest in the liver factor revived and indeed intensified.

The Active Red Factor

By now we were satisfied that colour and clinical activity were inseparable, but our pink material obstinately refused either to crystallize or to respond further to attempted purification by repeated partition or adsorption chromatography, fractional salting-out, or fractional precipitation by solvent mixtures. After a long period of frustration, further progress came suddenly from the observation that treatment with proteolytic enzymes like trypsin would break down persistent peptide impurities. On repurification by partition chromatography the product from the red band was eight times as intensely coloured as any previous fraction; it was later shown to be correspondingly more active clinically.

After a few more weeks spent in further purifying the minute amounts of red fractions remaining, we had the final satisfaction of watching the pure substance crystallize from aqueous acetone. But during those last weeks we were forestalled by the announcement that the Merck team under Karl Folkers had added one more vitamin to their conquests by isolating the same substance in crystalline form. Following proof of identity by exchange of

specimens (Lester Smith, 1949) we have now adopted the name vitamin B₁₂ suggested by the American workers.

It is worth noting that the methods we have used for the isolation of the anti-pernicious-anaemia factor are almost exclusively physical rather than chemical (Fantes, Page, Parker, and Lester Smith, 1949). In other words, we have depended on such physical properties as solubility in salt solutions and solvent mixtures, adsorption on charcoal, alumina, and silica, and partition between water and solvents. The countercurrent principle, upon which both adsorption and partition chromatography depend, enabled us to exploit the small differences in such properties between the factor and associated impurities.

This has been necessary because the factor is singularly devoid of characteristic chemical properties. The only chemical precipitants that have been used with advantage are Reinecke acid and phosphotungstic acid, but these are not at all specific, as both will precipitate many basic compounds. Moreover, neither is essential to the isolation of the pure factor.

Here, then, is another reason why the pure factor could hardly have been isolated in earlier years before some of these physical methods were perfected.

Standardization of Dosage

One considerable advantage that will follow from this work is the ability to express the dosage of liver extracts and other concentrates of anti-pernicious-anaemia factor in accurate and logical terms. The microbiological assay method using crystalline vitamin B₁₂ as a standard permits standardization in terms of vitamin B₁₂ activity. There will no longer be any excuse for stating dosages as the number of millilitres of a liver extract or the milligrams of crude solids administered, or in "liver equivalents" or the weight of liver from which the dose is prepared. Clearly, all these are misleading on account of variations in the potency of the original liver, variable yields in the course of extraction and purification, and differences in the extent of purification as between one liver extract and another. On the other hand, the growing body of evidence that liver extracts owe their efficacy in macrocytic anaemias solely to their vitamin B₁₂ activity, which can be accurately assessed microbiologically, puts this method of standardization on a very firm foundation.

Three Interesting Points

Interest did not slacken, even temporarily, with the isolation of crystalline vitamin B₁₂. For one thing, we were able to provide evidence (Lester Smith, 1948a), since reinforced (Cuthbertson and Lester Smith, 1949), for the existence of a second red factor, also clinically active in pernicious anaemia; indeed, it now appears probable that there are three such factors (Lester Smith and Cuthbertson, 1949). It is not known whether the others are more complex substances incorporating the molecule of vitamin B₁₂ (analogous to the various conjugates of folic acid with glutamic acid) or whether they are chemical modifications of vitamin B₁₂ (analogous to the various kinds of vitamin D or of penicillin).

The isolation of one of these factors in crystalline form has recently been announced from the Lederle Laboratories (Pierce *et al.*, 1949). It has been named vitamin B_{12b}, the name B_{12a} having already been used for an artificially produced hydrogenation product of vitamin B₁₂.

Secondly, the chemical nature of vitamin B₁₂ is gradually being unfolded as supplies of this very scarce material become available. (See Lester Smith, 1948b; Rickes *et al.*, 1948b; Kaczka *et al.*, 1949; Brink *et al.*, 1949;

Ellis *et al.*, 1949; and Brink and Folkers, 1949.) The most surprising discovery, announced almost simultaneously by the British and American teams, was the presence of an atom of cobalt in the molecule—the first time this metal has been found in a pure substance of biological origin. This has given rise to a crop of speculations on the function of cobalt in nutrition, and the relation (if any) between vitamin B₁₂ and the already known need of ruminants for cobalt. The molecule of vitamin B₁₂ contains some readily hydrolysable group essential to its microbiological activity, since it is slowly inactivated by cold acid or alkali. This might be a phosphate ester group, although the phosphorus present is completely split off only on long heating with acid. This treatment also liberates a substance giving a positive ninhydrin reaction, but leaves the greater part of the molecule intact and still retaining the cobalt, though with altered solubilities. This has been disrupted only by fusion with alkali, when pyrrolic substances are released, suggesting some structural relationship with chlorophyll and haem pigments.

A third point of interest is a promising new source of vitamin B₁₂. This arose from an entirely different research on the "animal protein factor" required by chicks and some other animals reared on a vegetable-protein diet. It now appears likely that this factor is identical with vitamin B₁₂, but meanwhile it had been established that the factor is synthesized by some intestinal bacteria. This raised the possibility of production by a fermentation process, and the Merck group, doubtless turning first to fermentations already established, found that the factor is produced by *Streptomyces griseus* and succeeded in isolating crystalline vitamin B₁₂ from the metabolism fluid of this organism (Rickes *et al.*, 1948c). Whilst the isolation of the crystalline product remains at present a complex and expensive procedure, the industrial production in this country of a purified vitamin B₁₂ concentrate from fermentation liquors is now at an advanced stage. It is expected to become generally available shortly.

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