INTRODUCTION

In 1970 we wrote: "The first major phase in the clinical investigation of vitamin B₁₂ metabolism and its derangements began with the development and application of methods for the estimation of total B₁₂ in tissues and body fluids and the assay of total serum vitamin B₁₂ has been a routine diagnostic procedure for many years. Most of the information obtainable by such methods has been gained and it is now clear that we are in the early stages of a second phase, in which the further development and application of methods for the estimation of individual cobalamins will play an important part" [1]. We now believe that this second phase is well advanced; this we will leave the reader to judge. It must be remembered that although our laboratory is the only one which has a chromatographic technique for simultaneous estimation of individual blood and tissue cobalamins in regular use, there are other (enzymatic and also indirect) methods for estimating or monitoring individual cobalamin coenzymes, and, so far, all methods have provided results in agreement. Further advances may be expected when recently developed chromatographic techniques, such as high pressure liquid chromatography [2], are introduced, though detection and estimation of picogram quantities of cobalamins (1 pg = 1 x 10⁻¹² g) will still be a problem.

In this review, references, which could run into many hundreds, have been kept to a minimum by referring, where possible, only to key papers, relevant books and reviews. Useful general references to books or reviews are [3-15].

Key words: cobalamins, cyanide metabolism, megaloblastosis, metabolic errors, neoplasia, optic atrophies, vitamin B₁₂.

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HISTORICAL BACKGROUND

Recent advances cannot be seen in perspective without some knowledge of the historical background. For many years after the isolation of 'vitamin B₁₂' (cobalamin) as cyanocobalamin (CNCbl) in 1948 by Carl Folkers and independently by Lester Smith, it was thought that 'serum B₁₂' was CNCbl, the form in which the vitamin was first isolated. It is now realized that CNCbl is primarily an artifact of isolation and normally occurs in the body only in traces.

In the early 1960s, some began to wonder whether the blood contained in addition to CNCbl a proportion of hydroxocobalamin (OHCb), a product of photolysis of the former compound [16]. The first coenzyme form of the vitamin was discovered in micro-organisms, and soon afterwards a form of cobalamin, adenosylcobalamin (AdoCb), having coenzyme activity in man, was found in human liver. In 1963 Lindstrand & Ståhlberg [17], using the technique of chromatography and bioautography (see below), showed that human serum or plasma contained several cobalamins, the chief form being methylcobalamin (MeCb), a second coenzyme which Lindstrand isolated from a large quantity of liver. Great credit is due to Lindstrand for this finding and a good deal of our early work was partly an extension of his. Lindstrand demonstrated his technique for separating cobalamins to one of us (D.M.M.) in 1966 while working in Mollin's laboratory, and we published a short paper together. His technique, involving paper chromatography and bioautography (see below) on large paper sheets, had the disadvantage of requiring very large quantities of blood (30-50 ml) and was qualitative only, the 'spots' being ill-defined areas of opalescence on a clear background. Because of this, and because the method seemed to show great promise, we set out in 1966 to refine it and place it on a quantitative basis. After several years' work, we perfected a technique needing only 2 ml of blood, which
enabled us to quantify by photometric scanning crimson-stained spots representing the individual cobalamins in blood or tissues.

Method

The coenzyme forms of vitamin B₁₂, MeCbl and AdoCbl, are extremely photolabile in aqueous or clear solutions, though more stable in whole blood and tissues. This lability makes it essential to handle samples with protection against normal light, i.e. in darkness or by red 'photographic' light. Cobalamins are extracted from the sample of plasma or homogenized tissue or cells with hot ethanol. After freeze-drying, the extract is desalted by solvent extraction, concentrated in water and the cobalamins separated by thin layer chromatography on cellulose/silica gel. Cobalamins on the chromatogram (5-50 pg per spot) are detected bioautographically by incubating the plate in contact with a layer of agar medium inoculated with a cobalamin-sensitive strain of *Escherichia coli* and a growth indicator. Crimson zones appear on the bioautogram, which are quantified by transmitted light in a scanning densitometer and compared with appropriate standards [18, 19].

Functions of the cobalamin coenzymes

Coenzyme functions are known for only two cobalamins, AdoCbl and MeCbl (Fig. 1). In man, AdoCbl is required for the conversion (isomerization) of methylmalonate to succinate and for the isomerization of α- to β-leucine [20]. MeCbl is involved in the methylation of homocysteine to methionine. Of the two cobalamin coenzymes, MeCbl appears the more important since a block in homocysteine methylation leads to a failure in DNA synthesis and megaloblastic changes which can be life-threatening.

Distribution of cobalamins in healthy subjects and simple cobalamin deficiency

In healthy persons the overall pattern of cobalamins is such that MeCbl predominates in the plasma (Plate la) and AdoCbl in the cells. In both
plasma and cells OHCbl or reduced cobalamin intermediates detected as OHCBbl are found. MeCbl normally accounts for over half the plasma total Cbl. The remainder is made up of AdoCbl and OHCBbl, with, in some individuals, a small proportion of CNCbl (up to 8%). In erythrocytes and other cells the normal cobalamin distribution differs markedly from that in plasma. MeCbl accounts for only about 10% of the total Cbl in erythrocytes, whereas CNCbl, as in normal plasma, is either absent or present only in small amounts.

In simple cobalamin deficiency, due, for example, to pernicious anaemia (Plate 1b) or veganism, there is a striking and disproportionate reduction in the plasma MeCbl, which occurs while the total plasma Cbl is still within normal limits and may therefore be a better indicator of cobalamin status than the ‘serum Bl level alone [11].

Distribution of individual cobalamins in laboratory animals

The distribution of individual cobalamins in laboratory animals is markedly different from that in man. We have made a detailed study of the baboon (see below), rat, cat and guinea pig. In particular, MeCbl is not the major Cbl in the plasma of any of these animals, AdoCbl and OHCBbl together amounting to more than 70% of the total plasma Cbl. Similarly, the proportion of MeCbl in plasma is low or very low in dog, ram and Patas monkey. We were surprised to encounter unidentified cobalamins or corrinoid compounds (compounds ‘p’ and ‘q’) in the rat and guinea pig. These were not present in traces only: in rat kidney cortex compound ‘q’ represented a greater proportion of the total Cbl than AdoCbl and OHCBbl together. Here, we think, new ground has been broken and the versatility of the method illustrated. This account is highly condensed. The original references are [21-24].

Cobalamins in food

In the rather small number of samples of proteinaceous food (our only source of dietary cobalamins) which have been examined [21,25], the main cobalamins have been AdoCbl and OHCBbl. As already mentioned, in food or tissue samples, in which the cobalamins are protein-bound and partially light-protected, the coenzyme forms are far more resistant to photolysis than in aqueous solution. Farquharson & Adams [26], who examined a variety of protein foods and meat products, found a substantial proportion of MeCbl in some, e.g. egg yolk and sardines. In some foods they found a low concentration of a compound which they considered to be sulphitocbl.

Cobalamins in Streptomyces griseus

This organism is the commercial source of CNCbl, which is extracted by a method designed to convert all cobalamin in this compound. In collaboration with The Radiochemical Centre (Amersham, U.K.) we were able to show that the organism contained mainly MeCbl with some AdoCbl. It was then possible, quite simply, to obtain these coenzyme forms labelled with 57Co at an unprecedentedly high specific activity [27].

Intestinal absorption of cobalamins

A somewhat perverse tradition has been maintained of doing all experiments on cobalamin absorption with CNCbl, a compound scarcely encountered in the diet! Fortunately, it is likely that the absorption of this compound is like that of OHCBbl and the two coenzyme forms. Our own investigation of CNCbl absorption in the guinea pig [28] showed that the bulk of the compound entered the blood unaltered, but that there was some conversion to AdoCbl, apparently in the mitochondria of the absorptive cells. In man there is no great lag in absorption of CNCbl; this compound appears in the blood no longer than 5 h after ingestion, if not earlier.

Cobalamins in pregnancy and lactation

This investigation of individual cobalamins was one of the earliest to be carried out. We think the results were interesting, but for reasons of space we will not discuss them at length; the reader is referred to the original paper [25]. We confirmed that plasma total Cbl fell towards term and rose post partum and we showed that the fall was associated with a tendency to a fall in MeCbl. Foetal blood had a higher total Cbl than maternal blood and a much higher level of MeCbl. This suggested either differential binding of cobalamins or active transport, especially of MeCbl, across the placenta in the fetal direction. Breast milk contained more AdoCbl and less MeCbl than the maternal plasma. Parenterel CNCbl appeared not only in maternal plasma, but at high dosage, in the milk. Fresh cow’s milk contained mainly AdoCbl but dried milk contained almost entirely OHCbl with a trace of CNCbl.

Cobalamins and age

We have observed, but reported only briefly [29], that the proportion of MeCbl in plasma and organs,
very high in foetal life and infancy, slowly declines through life. For example, MeCbl in the foetal liver was about 35% of the total, whereas in the elderly it was only some 2% (Fig. 2). This, together with the findings on cobalamin metabolism and tumour growth (see below), suggests an important but still ill-defined connection between MeCbl and normal and pathological growth.

Cobalamins and cyanide metabolism

Interest in this field, which was last reviewed in 1979 [11], goes back for more than 20 years, owing to the suggestion of A. D. M. Smith in 1961 that the cyanide in tobacco smoke might largely inactivate OHCoB (then thought to be the active form of cobalamin) by conversion to inert CNCbl. Though this was shown to be untrue in normal people at least [16, 30], evidence gradually accumulated showing beyond doubt that there was an interrelationship between cyanide intake and 'vitamin B₁₂'; for example, there was a strong negative correlation between total serum B₁₂ and plasma cyanide and plasma and urine thiocyanate (Fig. 3) (thiocyanate is the main detoxication product of cyanide) and an increased urinary excretion of 'vitamin B₁₂' in smokers (e.g. [31-34]).

In 1965, Wilson [35] put forward the hypothesis that Leber's disease, a heritable optic atrophy of late development, might be due to a derangement of cyanide metabolism (probably a defect of conversion of cyanide to thiocyanate) and later suggested that it should be treated with hydroxocobalamin (to detoxicate cyanide) and not with cyanocobalamin, which might be harmful [36]. The correctness of Wilson's hypothesis has recently been confirmed by a report of a deficiency of rhodanese (the enzyme converting cyanide to thiocyanate) in patients with Leber's disease [37]. Further progress in the field had to await a method for separating and estimating individual cobalamins. In 1969, we were able to report that there was frequently a substantial increase in plasma CNCbl in Leber's optic atrophy, dominantly inherited optic atrophy, tobacco-alcohol amblyopia and a number of optic atrophies of obscure origin [38]. It was later suggested that the increase in plasma CNCbl, which may be large (up to 200-300 pg/ml or more than 30% of total 'B₁₂'), was secondary to various errors in cyanide
metabolism [34]. Some of the cases respond to massive dosage with parenteral hydroxocobalamin. Clearly far more research on the underlying defects is needed.

Another condition in which plasma CNCbl is elevated is tropical ataxic neuropathy (Plate 1c), prevalent in Nigeria. In this condition an increase in liver CNCbl has also been shown. Here the increase is probably secondary to an increase in plasma cyanide due to massive overdosage with cyanide produced from cyanogenetic glycosides in the staple food cassava [39]. Here again, treatment is with massive doses of hydroxocobalamin.

It is important to note that an increase in plasma CNCbl is not necessarily pathological. A large increase in plasma CNCbl occurs after parenteral or even oral administration of this compound and may persist for some weeks. In this connection, it should be remembered that ‘vitamin B12’ is given to patients (and to animals) as a ‘tonic’ for a wide spectrum of conditions, from dental ulceration and ‘general debility’ to multiple sclerosis.

For completeness, it should be added that an increase in plasma CNCbl has been observed in some cases of pernicious anaemia, in which the cause is unknown. It may be due to raised plasma cyanide levels associated with reduced ability to detoxicate this compound. It has also been found in cases of acute myeloid leukaemia, in which the B12-binding capacity of the plasma is increased, and in acute liver failure, in which cobalamins are lost from the liver as suggested by an increased proportion of OHcbl and AdoCbl in the plasma [33]. It would not be surprising to find it also in the underinvestigated elevated total plasma Cbl of chronic renal failure [40]. Lastly, it should be mentioned that prolonged infusion of sodium nitroprusside (a hypotensive agent yielding cyanide among its metabolic products) in man disturbs cobalamin metabolism, leading to a fall in total plasma Cbl, a fall in MeCbl and a rise in CNCbl [41].

Cobalamin depletion and cyanide intoxication in baboons

The large-scale experiment touched on here is included mainly for the sake of completeness, since it is not of clear clinical interest. The experiment was carried out with 36 baboons: controls and other animals given a low cobalamin diet, some of the latter being given (a) cyanide parenterally and (b) thiocyanate by the same route. Only moderately low levels of plasma cobalamins were achieved in the cobalamin-depleted animals and there was no megaloblastosis nor neurological damage. However, abnormalities in cobalamin distribution were produced. For example, in blood plasma and tissues, MeCbl was disproportionately reduced by cobalamin depletion, this reduction being lessened or prevented by the administration of cyanide. We do not pretend to explain all the results [23, 24].

Congenital inability to synthesize coenzymes

Since we have recently published a detailed review [12] of all conditions causing cobalamin deficiency in childhood, including such conditions as dietary deficiency, juvenile pernicious anaemia and the Imerslund-Gräsbeck syndrome, in which intestinal transport of cobalamins is defective, we shall confine ourselves here to conditions in which the body cannot synthesize the cobalamin coenzymes. It is in these conditions that estimation of individual cobalamins has been extensively employed. Such conditions range in severity from the lethal, in which death occurs in infancy or childhood, to the very mild, in which the patient is normal or only rather slow-witted, but with evidence of biochemical disorder such as methylmalonic aciduria.

Combined deficiency of AdoCbl and MeCbl

It was in this syndrome that the diagnostic utility of the method was most strikingly shown. The patient (the second to be described) had long been a diagnostic puzzle. As soon as her plasma cobalamins were examined, it became obvious from the abnormal pattern that she had a disorder of vitamin B12 metabolism [42].

The patient, who died at the age of 7 years, had recurrent episodes of megaloblastic anaemia and neurological signs suggestive of cobalamin deficiency in spite of a normal total ‘serum B12’ and folate. She also had recurrent infections. For some years she was given neither cobalamins nor folates, but on one occasion when suffering from pneumonia she was given folic acid, a transfusion of packed erythrocytes and a short course of CNCbl. Just before the age of 7 years she deteriorated sharply, especially in her neurological condition, becoming unable to walk, and she died of pneumonia. The major findings were as follows. MeCbl in plasma was abnormally low despite a high normal total plasma cobalamin (Plate 1d). Erythrocyte cobalamins were depressed. Post-mortem analysis of liver, kidney, spleen and brain showed a great decrease in all forms of cobalamin (for example, total cobalamin in liver was only 2% of normal), although the proportion of OHcbl was moderately increased. There was homocystinuria and methylmalonic aciduria. Tissue extracts showed
larly, fibroblast extracts could not methylate which became normal with added AdoCbl. Similarly, fibroblast extracts could not methylate homocysteine to methionine, but their activity became almost normal in the presence of added MeCbl. Transcobalamins I and II were normal. The results suggested that some part of the common pathway leading to the synthesis of both coenzyme forms of vitamin B₁₂ was defective, but the precise site of the defect could not be determined. This is discussed at more length elsewhere [12, 42].

Several cases with the same or a similar biochemical defect have now been described, the first by Levy and his colleagues in 1970 [43], all characterized by homocystinuria and methylmalonic aciduria, and two of the cases we have helped to investigate. As the result of experiments with cultured fibroblasts, Rosenberg and his colleagues have classified the mild cases who survive as 'Cbl D mutants' and the severe cases, who if untreated die in the first few weeks of life or in childhood, as 'Cbl C mutants'. Treatment is with massive doses of OHCbl (not less than 1000-2000 µg/day) and, to be effective and have a chance of minimizing permanent damage to the central nervous system, it must be started as soon as possible. We believe that early diagnosis is greatly assisted by initial estimation of plasma and erythrocyte cobalamins and that this procedure can be life-saving. What is so bewildering about these and related conditions is that total 'serum B₁₂' is normal, even high, which can delay diagnosis for months or years.

**AdoCbl deficiency**

Cases have been described in which there is an isolated defect in the synthesis of AdoCbl, while synthesis of MeCbl is normal. Classically, the patients resemble those with methylmalonic aciduria due to apoenzyme deficiency, but they usually respond to massive doses of OHCbl (1000-2000 µg/day). Differential diagnosis is from the latter condition.

**Possible isolated MeCbl deficiency**

Such a case has not yet been described, but we should look out for one. It should be characterized by low tissue levels of MeCbl, homocystinuria responsive to OHCbl treatment (but no methylmalonic aciduria) and a liability to megaloblastic anaemia. Differential diagnosis will be from other causes of homocystinuria, including deficiency of the apoenzyme methionine synthetase.

**Transcobalamin II (TC II) deficiency**

Deficiency of the plasma cobalamin binder TC II is a potentially devastating condition, since in its fully blown form, cobalamins cannot be absorbed from the gut, taken up by the tissues nor (probably) effectively removed from the relatively inert binder, TC I, which binds the mass of the plasma cobalamins. Children with this disorder usually present with severe illness within the first few weeks or months of life, with failure to thrive, obvious grave sickness, and megaloblastosis, though the total 'serum B₁₂' is usually within normal limits. In two cases which we have investigated, the patterns of cellular cobalamins were grossly disturbed and, in one, plasma AdoCbl was low. Another anomalous feature in this case was normal absorption of cobalamins, as estimated by the Schilling test. In this case, findings did not 'tie up' with current theory, and it is obvious that more investigation is needed and that theory must be refined. Treatment of the condition is with massive doses of OHCbl; in fact it can be said of all the conditions outlined that massive dosage of OHCbl can do no harm and may do dramatic good.

**Biochemical mechanism of nitrous oxide megaloblastosis**

Amess et al. [44] reported that patients given N₂O for some hours developed megaloblastosis. It is also known that dentists addicted to N₂O inhalation can develop neurological disorders. We [45] studied the effects of N₂O exposure on human lymphocytes blast-transformed by phytohaemagglutinin. MeCbl synthesis was markedly depressed but AdoCbl synthesis was not. This supports the suggestion by Amess et al. that MeCbl is responsible for maintaining normoblastic erythropoiesis, and, if the evidence from human addicts is taken into account, it would seem that MeCbl may also be necessary for maintaining the integrity of the nervous system. The subject of N₂O megaloblastosis has recently been reviewed by Chanarin [46].

**Cobalamins in neoplastic disease**

There is an unexplained but intimate relationship between cobalamins and neoplasia [47]. Though in myelomatosis total plasma cobalamin falls and the proportion of plasma MeCbl is reduced, the proportion of this compound in erythrocytes is increased [48]. In patients with primary hepatoma, the MeCbl in tissues including brain and liver was found to be increased by the extraordinary factor of 3-25 [47]. In rats bearing transplanted Morris
hepatomas, both liver and tumour MeCbl were increased, and in rats fed with the carcinogen diethylnitrosamine there was an 80% increase in hepatic MeCbl after an acute dose [49]. If forced to generalize, one could say that MeCbl promotes tumour growth, and lack of MeCbl inhibits it [50, 51]. The potential diagnostic and therapeutic implications of these findings remain to be explored.

Experimental factors affecting cobalamin synthesis
Since MeCbl at least seems to be closely connected with tumour growth, the effect of factors affecting the synthesis of cobalamin coenzymes is of great interest, but since this is a highly specialized field, we shall be brief.

Myasishcheva et al. [50] showed that difluoro-chloro-MeCbl reduced uptake of CNCbl by phytohaemagglutinin-stimulated human lymphocytes, and caused a disproportionate reduction in synthesis of AdoCbl. MeCbl–palladium trichloride reduced uptake more effectively and reduced formation of both coenzymes and OHCbl in proportion. The results suggested that in addition to inhibiting CNCbl uptake, one or both compounds interfered directly with the synthesis of the cobalamin coenzymes.

Linnell et al. [51] studied the effects of dietary deprivation of methyl donors (CNCbl, methionine and choline) on cobalamin distribution in rat organs. Total cobalamin levels were altered by all these regimens, though MeCbl was most resistant to alteration. The results showed that CNCbl, methionine and choline exerted quite different effects on tissue levels of the individual cobalamins. The paper cited is an abundant source of references on the relationship between cobalamins and cancer.

Conclusion
We hope this account will have been of some interest, but do not expect any general reader to retain it all. When one of us told a senior medical friend that there were at least four forms of vitamin B12 etc., he merely groaned. This reaction is understandable, since modern medicine has become intolerably complex. All we would ask our readers to remember is that there exist serious and potentially lethal disorders of B12 metabolism and that a normal or high 'serum B12' level does not by any means exclude these: indeed it is characteristic of many of them. Such cases may come to the attention of the paediatrician, the haematologist, the neurologist, the psychiatrist or indeed any physician. We would suggest that if there is the slightest suspicion of a disorder of cobalamin metabolism, an expert on megaloblastic anaemias should be consulted at once. He will know how to get the case properly investigated, which may well involve international collaboration. Delay can be fatal, literally so in infants. In such cases, it would be wise to give massive doses of OHCbl as soon as possible. This can do no harm, may be life-saving, and will not preclude adequate diagnosis at a later stage.

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