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THE BIOCHEMISTRY OF VITAMIN B_{12}

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INTRODUCTION

By Sir Alexander Todd

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That we are today holding a symposium on vitamin B_{12} is a tribute at once to the importance of this vitamin, or better, to this group of vitamins, and to the large amount of chemical and biological work which has been devoted to it during recent years. My own interest in the field goes back almost twenty years, for it was in the early days of vitamin B chemistry that the late Dr H. D. Dakin—one of the pioneers in chemical work on the anti-pernicious anaemia factor—drew my attention to it. That I did not then follow it up was because the prospect of isolation studies with only a clinical test to guide them discouraged me. Fortunately others were not so discouraged, and as a result vitamin B_{12} was finally isolated in crystalline form in 1948. It is interesting to note that, as has happened with other vitamins and hormones, the isolation was achieved independently and wellnigh simultaneously in two different laboratories—by Dr Lester Smith at the Glaxo laboratories and by Dr Folkers and his colleagues in the laboratories of Merck and Company. Today vitamin B_{12} is available for both clinical and scientific study as a result of its commercial production from *Streptomyces* fermentations.

This morning's session is devoted to chemical and analytical aspects of vitamin B_{12}. The extreme complexity of its molecular structure has slowed down chemical investigation of the structure of the vitamin, as also has the fact that it occurs in very low concentration in most natural sources—a fact which is not surprising when we remember its extremely high biological activity. Structural work has been carried out by Folkers and his group in America, by Petrow and his assistants at British Drug Houses Limited and by my own colleagues in Cambridge associated with Dr Lester Smith and with Dr Dorothy Hodgkin of Oxford on the X-ray crystallographic side. This work will be reviewed by Dr Lester Smith in his paper, and I shall invite Dr Hodgkin to follow him by giving a brief report on recent results she has obtained in her brilliant X-ray crystallographic studies on vitamin B_{12} and on a crystalline cobalt-containing degradation product isolated at Cambridge. Largely as a result of this work we are now close to a definite formulation of the vitamin and a final solution of the chemical problem should not be far away.

INTRODUCTION

The multiplicity of factors related to vitamin B_{12} which are found in sewage sludge, bird and animal faeces etc., present fascinating chemical and biological problems although their existence greatly complicates the measurement of vitamin B_{12} in natural materials. A consideration of these factors and their estimation forms the content of the remaining papers in the present session.

1. ISOLATION AND CHEMISTRY OF VITAMIN B_{12}

By E. Lester Smith

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Ox liver usually contains less than one part per million of cobalamins. We now know that these are present loosely bound to protein and that much of the activity is released in forms other than cyanocobalamin during the preparation of liver extracts. In retrospect, therefore, it may seem less surprising that the isolation of vitamin B_{12} was so long delayed than that it was ever accomplished with clinical tests as the only guide (Lester Smith & Parker, 1948; Fantes, Page, Parker & Lester Smith, 1949). My colleagues and I at Glaxo Laboratories were denied either the microbiological assay that helped forward the prior isolation in America by Rickes and colleagues of Merck, or the lucky accident of choosing cyanide-activated papain for proteolysis, which assisted the subsequent isolation by Wijmenga of Organon in Holland; the cyanide converted other forms of the vitamin into the more stable and readily crystallizable cyanocobalamin (Rickes, Brink, Konuiszy, Wood & Folkers, 1948; Wijmenga, Lens & Middelbeek, 1949). Vitamin B_{12} was also isolated later in the same year (1948) by Ellis, Petrow & Snook (1949) of The British Drug Houses.

Isolation from liver. The multi-stage purification procedures will not be discussed in detail, especially since no comparison is possible with the American ones, which have not been disclosed; indeed it is not even clear whether the original isolation was made from liver or from fermentation liquor. It is almost certain that the isolation of vitamin B_{12} could not have been accomplished without the help of chromatography. The nearest previous approaches were concentrates, which we now know to have contained around 1% of the vitamin, obtained by Emery & Parker (1946) and earlier by Laland & Klem (1936) in Norway; it is possible, however, that a significant proportion of the orange-red colour of the latter preparation was due to vitamin B_{12}. Even in these investigations, however, shallow charcoal beds were used in chromatographic fashion. In the later work adsorption columns of alumina, silica and bentonite were used, with aqueous or aqueous-alcoholic solutions. We used columns of damp silica developed with wet *n*-butanol; although the separation was due largely to partition chromatography, adsorption played a part also. We also tried starch columns and in more recent work on the isolation of related factors and hydrolysis products we have preferred to use kieselguhr, which comes closer to the ideal of a purely mechanical support for the

aqueous phase (Brown, Cain, Gant, Parker & Lester Smith, 1955). Paper chromatography with *n*- or better *sec*-butanol has also proved valuable, and in Germany, Friedrich & Bernhauer (1953) (Bernhauer & Friedrich, 1954) have used cellulose columns.

Other steps used in the original isolations were extraction with phenolic solvents, sometimes made more selective by dilution with relatively inert organic liquids such as benzene, chloroform or butanol. Salting-out with ammonium sulphate was a step taken over from early work on liver-extract fractionation, as also was exhaustive extraction with butanol, but two British teams independently combined these steps with fruitful results. Precipitation of impurities with lead acetate, and of the active principle with phosphotungstic acid, were other tried procedures pressed into service. The million-fold purification called for a combination of steps and the repetition of some, with a final crystallization from aqueous acetone.

Commercial production. Liver is entirely uneconomic as a source material for commercial production. Sewage sludge, and especially activated sludge, contains a useful amount of vitamin B_{12} and has been investigated in America, Sweden, Germany and this country (Sewage Commission, City of Milwaukee, 1954; Sjöström, Neujahr & Lundin, 1953). A grave disadvantage is its content of B_{12} analogues, that so far as is at present known cannot be separated from the B_{12} except by partition chromatography. The only commercial production is by fermentation, as practised for antibiotic manufacture. Vitamin B_{12} can, indeed, be recovered from the spent fermentation liquors of streptomycin or aureomycin manufacture, or special fermentations can be carried out with various streptomyces species, or with bacteria, of which *Bacterium megatherium* has been recommended (Hester & Ward, 1954; Garibaldi, Ijichi, Snell & Lewis, 1953). It is important to select species that make vitamin B_{12} exclusively; several manufacturers have been led astray by organisms that gave high yields of related factors such as pseudo-vitamin B_{12}. The vitamin tends to be concentrated in the cells; if convenient these can be harvested for subsequent extraction, e.g. with benzyl or propyl alcohols in presence of excess cyanide. Alternatively, activity can be released into the liquor, e.g. by boiling or acidification. Charcoal as an adsorbent has been largely displaced by synthetic resins; for example, the carboxylic acid resin IRC.50 can be used in the acid stage of the cycle, and the vitamin can be recovered by washing with dilute alkali.

Phenolic solvents are valuable for purification and volume reduction, either alone or preferably diluted with inert solvents such as benzene or carbon tetrachloride. The most selective extractant known is benzyl alcohol, especially when the aqueous phase is alkaline and contains excess cyanide (Rudkin & Taylor, 1952). The purple dicyanocobalamin is then readily extracted, acidic impurities tending to remain

behind as salts. A number of other purification steps are described in the literature and especially in patent specifications, but they are not necessarily in large-scale use. These include, for example, extraction with halogenated or alkyl phenols, or with higher fatty acids such as *iso*butyric acid, precipitation of impurities with a zinc salt and alkali, and alumina chromatography of aqueous methanol solutions.

Nothing seems to have supplanted the original aqueous acetone for crystallization, although subsequent recrystallization from hot water is useful for final purification.

The cobalamins. The molecule is moderately large, with a molecular weight of about 1,350 for the anhydrous material. Consequently micro-analytical methods are inadequate to fix the empirical formula with certainty. It is believed, however, to lie within the limits $C_{61-64}H_{84-92}O_{13-14}N_{14}PCo$. The cyano group attached to the central cobalt atom can be removed by photolysis or by treatment with hydrogen and a catalyst or with sulphurous acid; it can then be replaced by certain other acid radicals, or by hydroxyl, to give a series of cobalamins, e.g. thiocyanato-cobalamin, nitrito-cobalamin (vitamin B_{12c}) and hydroxo-cobalamin (vitamin B_{12b}), which in acid solution changes to the basic aquo-cobalamin with a co-ordinated water molecule. Vitamin B_{12a} differs slightly from both these forms in its absorption spectrum; possibly the vacuum dehydratation used in its preparation caused elimination of water between two molecules of hydroxo-cobalamin. Also the Co atom can be co-ordinated with ammonia, amines and some amino-acids to give a series of 'cobalichromes'. (For review see Lester Smith, Ball & Ireland, 1952.)

The nucleotide. After hydrolysis with hot acid, paper chromatography in butanol-acetic acid gives a pattern of three colourless but fluorescent spots. These were described first as the α, β and γ components, and it became clear from rough kinetic studies that the α component was released first and then broke down further to yield the β and γ components successively. The γ component was soon identified as 5 : 6-dimethylbenziminazole. A series of brilliant investigations from Merck & Co. Inc., The British Drug Houses and the University of Cambridge showed that the other fluorescent components were a 'nucleoside' and a 'nucleotide' of this compound. These were both characterized by analysis and also by synthesis: the nucleotide is in fact 5 : 6-dimethyl-1-(α-D-ribofuranosyl) benzimina-zole-3'-phosphate. The unusual α configuration for the riboside is noteworthy. (For review see Armitage, Cannon, Johnson, Parker, Lester Smith, Stafford & Todd, 1953.)

Vigorous hydrolysis with acid also releases D*g*-1-aminopropan-2-ol (almost certainly one mole only, although the first semi-quantitative estimation suggested two moles), five or six moles of ammonia and an acidic red gum. For some time this red fraction proved intractable,

but more recently it has begun to yield up its secrets to attacks by three entirely different techniques. These are, respectively, vigorous oxidation, controlled hydrolysis and X-ray crystallography.

Oxidation. First the 'sledge-hammer' approach of vigorous oxidation with permanganate has yielded to Karrer and his colleagues a series of simple and methyl-substituted aliphatic acids, namely, acetic, oxalic, succinic, methylsuccinic and dimethylmalonic acids, and also some larger uncharacterized acidic fractions, but none containing nitrogen (Schmid, Ebnöther & Karrer, 1953). It is impossible to build up any tentative structural formula merely by piecing together these small fragments. However, these observations will have their value in checking any structure deduced from other evidence, for obviously it must be capable of yielding these acids on oxidation.

Removal of nucleotide: Factor B. Secondly, gentle step-wise hydrolysis, followed by separation of the numerous products by electrophoresis and chromatography on paper, has given vital information about what might be called the peripheral chemistry of the molecule; this approach has been pioneered jointly by Glaxo Laboratories and the University of Cambridge (Armitage *et al.*, 1953).

We have shown that brief treatment with warm concentrated mineral acids, preferably with perchloric acid, removes the intact nucleotide (characterized as a reaction product) and does little damage to the rest of the molecule. The main product turns out to be the naturally-occurring Factor B. It had been isolated previously as a microbiologically-active factor, by my own colleagues from fermentation liquor and by Porter, *et al.* from calf manure. We have shown, moreover, that this Factor B is common to all the B_{12} factors so far characterized; they differ from vitamin B_{12} only in the nature of the basic component of the nucleotide (Brown, *et al.*, 1955).

Hydrolysis of amide groups. Hydrolysis with cold dilute acid or alkali, on the other hand, takes predominantly a different course. Amide groups are attacked in succession, to yield a series of red carboxylic acids containing 1 to 4 acidic groups. These can be elegantly separated by electrophoresis on paper. For example, with phosphate buffer at pH 6·5 containing a trace of cyanide, a few hours electrophoresis at about 300 volts suffices to separate a hydrolysis mixture into a series of equi-distant red bands. Unchanged vitamin B_{12} is neutral and remains at the origin (except for electro-endosmotic flow), while the acids travel distances proportional to the number of carboxyl groups present.

In alkaline solutions containing cyanide vitamin B_{12} takes up a second CN group to form the purple dicyanocobalamin. Since the acidic CN group displaces a co-ordination linkage between cobalt and the $N_{(3)}$ of the benziminazole, the whole molecule becomes acidic. Thus on electrophoresis in alkaline buffer (pH >8) containing cyanide,

dicyanocobalamin moves as a monobasic acid. The carboxylic acids also change to the purple dicyano configuration, which confers one additional negative charge upon each. A partial hydrolysate therefore gives a series of equi-distant purple zones on electrophoresis under these conditions, each moved one space towards the anode compared with the series of red zones at pH's around neutrality.

Several lines of evidence point to the fact that these acidic groups arise from carboxylic amides. The most convincing is the resynthesis of vitamin B_{12} from the acids. This we accomplished by treatment under anhydrous conditions in dimethylformamide solution with ethyl chloroformate and triethylamine, and then with ammonia. This reaction was applied in turn to the mono-, di- and tri- carboxylic acids, and the main crystalline product was characterized as vitamin B_{12} by its physical constants, including the infrared absorption spectrum and by microbiological assay with several organisms. It should be mentioned that the acids themselves are inactive towards the *Escherichia coli* mutant, but show slight activity towards *Lactobacillus leichmanii*. Thus the loss of a single molecule of ammonia from this large structure completely destroys growth-promoting activity for some micro-organisms and also for B_{12}-deficient rats. Activity for other micro-organisms may well be due to biological re-amidation of the acid.

Random hydrolysis of 3 labile amide groups should lead to groups of isomeric acids, namely, 3 mono-, 3 di- and one tri-basic. Paper chromatography with *sec.*-butanol did in fact fractionate the acids in this fashion; e.g. the electrophoretically homogeneous monobasic acid yielded three main bands differing in R_F values. Approximate estimates of the relative abundance of the red acids produced by hydrolysis for various periods led to the conclusion that two of the amide groups are highly labile and the third somewhat less so, whereas a fourth is considerably more stable. Similar conclusions were reached by Brierly, Sealock & Diehl (1954) from measurements of the rate of release of ammonia on acid hydrolysis; they suggested steric effects as the cause of these differences.

If one attempts to press the attack further by increasing the time of hydrolysis, the concentration of acid, or the temperature, then the nucleotide tends to be removed, as well as ammonia. This leads to a second series of acids lacking the nucleotide, derived from the parent substance, Factor B. Such a hydrolysate, containing a complex mixture of both series of acids, can be sorted out fairly easily by paper electrophoresis. At pH 6·5 the acids without nucleotide travel a little faster than corresponding ones in the first series, owing to their lower molecular weight; also their colour on the paper at this pH is not red but purple, changing to orange on drying. The mixture, therefore, yields a pattern of alternate red and purple bands, partly overlapping. This is because the purple dicyano configuration is more

stable at slightly acid pH in absence of the nucleotide. On drying the paper in the light, however (or on strongly acidifying an aqueous solution), cyanide is lost and the colour changes to orange. It should be noted, however, that the parent substance of this series, Factor B, is neutral in the dicyano form (not acidic like dicyanocobalamin ; see later for the explanation of this phenomenon). Surprisingly, therefore, adjacent red and purple zones contain the same number of carboxyl groups. Alternatively, the two series can be separated first by electrophoresis in acetic acid. The carboxyl groups are then non-ionized and make no contribution to mobility. The red acids therefore all behave as neutral bodies, but those without nucleotide lose cyanide and become orange and basic in the acid solution.

We have shown that the monobasic acid in the latter series can be converted chemically to its amide, namely, Factor B, just as vitamin B_{12} can be re-synthesized from the red monobasic acid (Armitage, *et al.*, 1953). Bernhauer & Friedrich (1954) have applied this reaction to the polycarboxylic acids without nucleotide and they adduce evidence for 4 $CONH_2$ groups in Factor B (aetiocobalamin, in their nomenclature) and for a fifth carboxyl group *not* present as a simple amide. Thus the presence of 4 $CONH_2$ groups is established with some certainty. Vigorous hydrolysis leads however to fractions with a maximum of 7 acidic groups, although this heptabasic acid is always accompanied by hexa- and penta- basic ones.

Hydrolysis with nitrous acid. A valuable key to the state of combination in the B_{12} molecule of these additional potential acid groups has come from hydrolysis with nitrous acid. Hydrolysis with cold 2N-hydrochloric acid (though not with weaker acid) is greatly accelerated by addition of a little nitrite, conditions known to split simple and substituted amide groups. We have recently re-examined this reaction and now find that, under suitable conditions, 6 red acids (i.e. with 1 to 6 carboxyl groups) can be separated from the reaction mixture. This evidence, considered with the amount of ammonia released on hydrolysis, suggests a total of 6 $CONH_2$ groups. The last two are much more resistant to hydrolysis than the others. At any rate it is clear that 6 acidic groups can become free while the nucleotide is still attached.

The nitrous acid hydrolysis mixture also yields, however, not 6 but 7 orange-coloured acids that *have* lost the nucleotide. Evidently, therefore, the nucleotide is attached via a seventh carboxyl group ; also from the acceleration of the hydrolysis by nitrous acid we may conclude that it is present as a substituted amide group. This is consistent with the earlier hypothesis that the aminopropanol constitutes this link, as amide at one end and phosphoric ester at the other, a formulation that does not conflict with the latest (unpublished) evidence from X-ray crystallography.

The phosphorus linkages. Two of the phosphoric acid hydroxyls are thus accounted for by attachment to ribose and aminopropanol respectively ; the earlier supposition of a third ester linkage was based on purely negative evidence, the lack of an acidic function in vitamin B_{12}. Recent work at Cambridge now makes it clear that such a compound, namely, a phosphoric tri-ester with an adjacent free hydroxyl on the ribose chain, would be extremely unstable. This formulation must, therefore, be rejected because vitamin B_{12} is relatively stable. It was in any event unsatisfactory, because it offered no explanation for the basicity of Factor B, arising on removal of the nucleotide. This difficulty could not be met by postulating linkage of phosphate to a basic group on the cobalt-containing fragment ; a primary or secondary base would react with nitrous acid, whereas Factor B is unchanged by mild treatment with this reagent. Moreover, such a plurality of links would inevitably lead to step-wise hydrolysis, whereas no intermediate compounds have in fact been detected after partial removal of the nucleotide. Internal salt formation with a quaternary base might be considered, but the new evidence for 7 CO—NH-linkages leaves no nitrogen to spare for any kind of basic group.

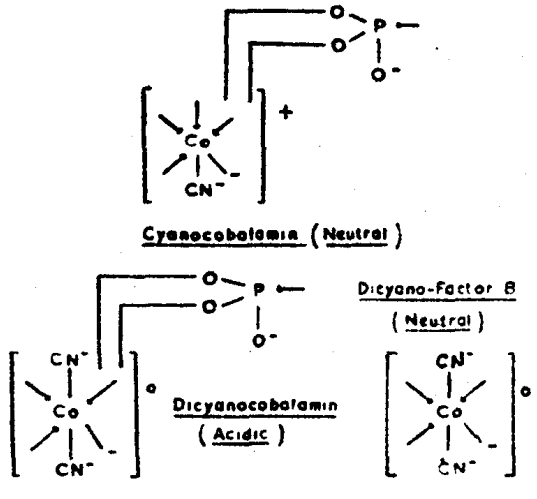

Fig. 1.

The clue to the puzzle was the realization that no new basic function was created on removal of the nucleotide, but that instead an acidic function disappeared. This can be seen most clearly by considering the purple dicyanocobalamin, which is acidic (see Fig. 1). On removing the nucleotide we are left with dicyano-Factor B, which is also purple, with almost the same absorption spectrum, and therefore presumably with the same co-ordination state, yet it is neutral. Evidently then the original acidic function is due to the phosphoric acid, for it is lost along with the nucleotide. In other words, the third

hydroxyl is free in dicyanocobalamin ; it must, therefore, be masked by internal salt formation in vitamin B_{12} itself. A direct Co-O-P link is inadmissible on X-ray evidence, and would in any event disturb the co-ordination on rupture. The only remaining possibility is that the entire co-ordination complex is basic and is neutralized by the phosphoric acid group on the rest of the molecule. This state of affairs, is illustrated in the partial formulae in Fig. 1. There seem to be no objections to this hypothesis on chemical or X-ray crystallographic grounds.

Incidentally the red mono- to tetra-basic acids (8 in all, with isomers) have all been obtained crystalline at Glaxo Laboratories, whereas Factor B and the related series of acids have remained obstinately amorphous. The only exception is the hexabasic acid crystallized at Cambridge and proving most useful for X-ray crystallography (Cannon, Johnson & Todd, 1954).

X-ray crystallography. This third line of apprach to the structural problem, non-destructive analysis by X-ray crystallography, has proved astonishingly fruitful in the gifted hands of Dorothy Hodgkin and her colleagues. No substance anything like so complex, and of completely unknown structure at the time, has ever been tackled before, so that new techniques had to be developed as the work proceeded. This approach admirably complements the chemical ones by working from the centre outwards ; the relatively heavy cobalt atom helps to reveal most clearly the positions of the immediately surrounding groups of atoms. X-ray crystallographic analyses of selenocyanato-cobalamin, and of the hexacarboxylic acid degradation product, disclose the same picture, namely, a structure around the cobalt that is almost but not quite like that of a porphyrin. The main discrepancy lies in the absence of one only of the usual 4 meso carbons linking the four rings : in vitamin B_{12} two of the rings are directly linked without any bridging atom (Brink, Hodgkin, Lindsey, Pickworth, Robertson & White, 1954). Detailed analysis still proceeding at Oxford is revealing various side-chains on the 5-membered rings. This technique can scarcely distinguish between the almost equally heavy carbon, oxygen and nitrogen atoms, and hydrogens cannot be detected. It cannot, therefore, completely replace chemical degradation ; on the other hand, the finer features of the molecular structure of vitamin B_{12} might never have been elucidated without the help of X-ray crystallography. Besides revealing this unique and unexpected ' near-porphyrin ' structure, the X-ray method has, for example, fixed the point of attachment of the phosphate on the ribose chain. Chemically it is almost impossible to decide between the 2 and 3 positions, owing to the intermediate formation of a cyclic phosphate on hydrolysis. Dr Hodgkin finds, however, that only the 3-phosphate can fit into the crystal pattern (see also Kaczka & Folkers, 1953).

Isolation of pyrrole derivatives. Tentative evidence for a pyrrole structure was presented early in the history of vitamin B_{12}, and it had always seemed likely that the cobalt was surrounded by 4 pyrrole rings. On the other hand, the porphyrin structure that this would normally imply was contra-indicated by the absence of a Soret band from the absorption spectrum and of maleinimides from the oxidation products. This anomaly has been resolved by the X-ray work, but conclusive chemical evidence for at least one reduced pyrrole ring has only very recently been provided in a note from the Merck Laboratories (Kuehl, Shunk & Folkers, 1955).

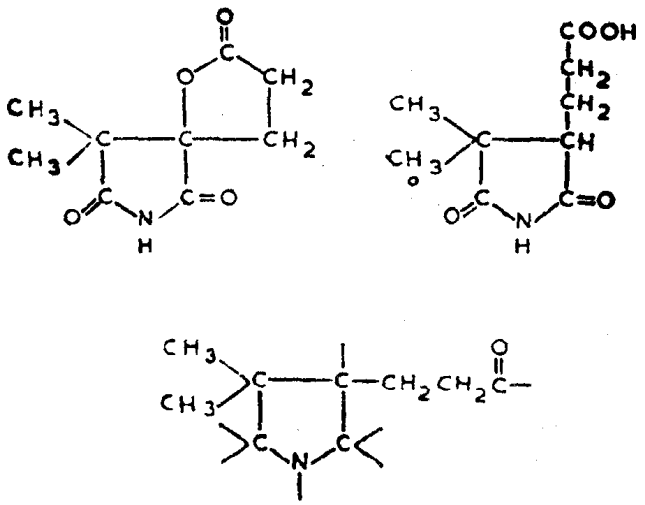

Fig. 2.

Oxidation of a vitamin B_{12} hydrolysate with chromate in acetic acid and subsequent counter-current fractionation yielded two closely related crystalline substances (see Fig. 2). Their structures were confirmed by synthesis and both are believed to arise from a moiety with pyrrolidine, pyrroline or pyrrolenine structure ; its partial formula is shown in Fig. 2 (3rd formula). Several of the acids identified by Karrer *et al.* could arise by further oxidation of such a compound.

If we piece together all the evidence that can at present be expressed in a formula without violating either chemical or X-ray data, we can already get a fairly complete picture of this extraordinary molecule (Fig. 3.) Recent progress at Oxford (outlined in Dr Hodgkin's contribution to the discussion) would permit the insertion of some additional side-chains with reasonable certainty.

Halogenation. Before the complete structure of the vitamin can be written down, however, some further puzzling observations will have to be fitted into the picture. The team from The British Drug Houses have shown that two atoms of chlorine or bromine enter the molecule

with extreme facility (Ellis, Petrow, Beaven & Holiday, 1953). If they add across a double bond, it must be one that is not part of the conjugated system constituting the chromophore, because the colour is deepened, not diminished, by halogenation: the (non-crystalline) products are purple, turning to rich blue with excess cyanide.

Reaction with alkali. At Glaxo Laboratories we have recently re-investigated the effect of alkali on vitamin B_{12}. Brief boiling with dilute alkali gives as the major product a red neutral crystallizable substance hardly distinguishable from vitamin B_{12} except by its inactivity on microbiological assay. Oxidation appears to be involved in this change, because, if air is excluded or if a reducing agent such as thioglycollate is added, then the vitamin is partially protected from destruction. Besides its chemical interest, this observation has a

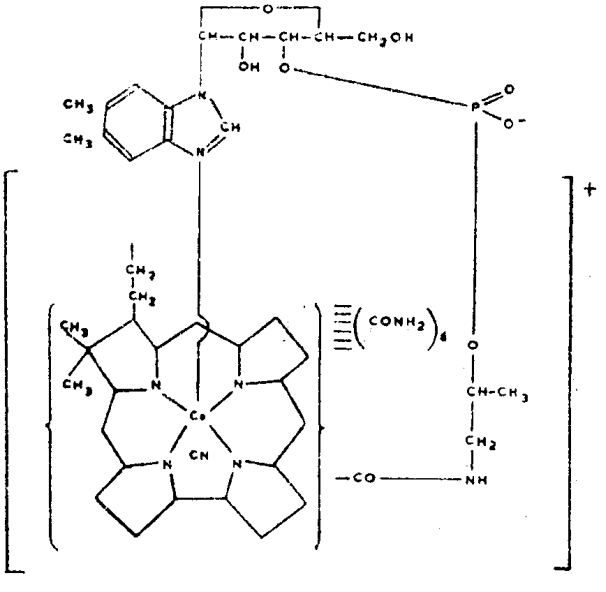

Fig. 3.

bearing on microbiological assay techniques. On the one hand, it may explain the stabilizing effect of some thio-acids on the vitamin during autoclaving; on the other, the presence of reducing agents in liver extracts and other materials may explain occasional failures to destroy B_{12} activity completely by heating with alkali.

Radioactive vitamin B_{12}. The molecule lends itself readily to labelling with various radioactive isotopes. It has recently been confirmed that slight radioactivity is induced in the cobalt and phosphorus atoms by direct irradiation of crystalline vitamin B_{12} in the nuclear pile (Maddock & Coelho, 1954). Carbon-14 can be introduced chemically into the —CN group, either by exchange with $K^{14}CN$ or by treating vitamin B_{12b} with this reagent. This tracer has been used to study the cyanide exchange reaction at different pH values (Lester Smith, Ball & Ireland, 1952). Potassium thiocyanate, labelled with radioactive sulphur, has

been used in the same way to study exchange with $KCN^{35}S$. Carbon-14 has also been introduced into the iminazole carbon biosynthetically. A fermentation with *Streptomyces griseus* was carried out in presence of 2-^{14}C-5 : 6-dimethylbenziminazole (Weygand, Klebe & Trebst, 1954). This result suggests that the mould can utilize preformed dimethylbenziminazole and build it into the B_{12} molecule, although the extent of incorporation was rather low in this experiment. However, the point has been settled by the biosynthetic preparation of known and new B_{12} analogues, as described in the next communication.

Radioactive phosphorus has also been introduced by fermentation in the presence of ^{32}P-phosphate. Unfortunately it was imperative to dilute the carrier-free isotope heavily with normal phosphate to promote growth of the organism. Nevertheless the specific activity achieved was sufficient for some biological experiments on absorption and excretion (Lester Smith, 1952).

Radioactive cobalt has been more extensively used. (For review see Lester Smith, Hockenhull & Quilter, 1951.) Cobalt-60 can be obtained at fairly high specific activity and can be used without dilution. We have made numerous batches of ^{60}Co-cyanocobalamin with specific activities up to 0·8 mc. per mg. The half-life of 5·3 years is convenient for analytical purposes, and the material is in daily routine use for determining vitamin B_{12} in fermentation broths and concentrates by isotope dilution assay (Bacher, Boley & Shonk, 1954). It is also useful in studying extraction yields, the labelled vitamin being added to a pilot run and followed through the process by 'counting' at each stage.

Haematologists at the Postgraduate Medical School, Hammersmith, have, however, been dissatisfied with this product on two scores: the half-life of cobalt-60 is inconveniently long for use in human subjects, e.g. for diagnostic uses: also the specific activity attainable by neutron bombardment of cobalt in the pile is not high enough for some purposes. In consultation with Dr J. E. Bradley, therefore, they have turned to transmutation reactions. Cobalt-58 (half-life 72 days) can be made, though not readily in quantity, by neutron bombardment of nickel (Bradley, Lester Smith, Baker & Mollin, 1954). Proton bombardment of iron in a cyclotron appears to yield under some conditions mainly cobalt-56 (half-life 72 days) and under others largely cobalt-57 (half-life 270 days). It happens, therefore, that we have been called upon to label the vitamin with all 4 available cobalt isotopes. A recent batch of ^{56}Co-cyanocobalamin had the remarkably high specific activity of 12 mc. per mg.; its preparation involved the extraction of pure crystalline vitamin B_{12} (about 200 μg.) from 2 litres of broth of very low titre (0·2 μg. per ml.) owing to deliberate use of a limiting level of cobalt to ensure high efficiency of conversion.

My thanks are due to colleagues in our fermentation research laboratories for preparing the broths from which the radioactive vitamin B_{12} has been extracted.