simultaneous presence of phenylenediamine and relatively low levels of diaminodimethylbenzene, makes it most probable that a mechanism of competition is operative. Whether the actual synthesis of diaminodimethylbenzene (or a more complex derivative of it) is suppressed by phenylenediamine, or whether diaminodimethylbenzene is synthesized at its normal level irrespective of the presence of phenylenediamine, but not incorporated into the vitamin B₁₂ molecule if excess phenylenediamine is present is not known.

The new substance is almost certainly identical with the one obtained independently by Ford, Holdsworth & Kon (1954), who incubated *Esch. coli* and factor B in the presence of benziminazole and then demonstrated by chromatography the formation of a new compound with an *R_F* value just less than that of vitamin B₁₂.

SUMMARY

1. Fermentations of *Streptomyces griseus* on a complex or a synthetic medium in the presence of *o*-phenylenediamine gave rise to the production of a new analogue of vitamin B₁₂.

2. The new substance has been isolated in the form of red needles.

3. Its absorption spectrum is practically identical with that of vitamin B₁₂.

4. Benziminazole has been identified as a hydrolytic product.

5. The new material is microbiologically (plate assay) more active than vitamin B₁₂: the greater activity probably explains the apparently increased

'vitamin B₁₂' titres obtained by Dulaney & Williams (1953).

6. The biosynthesis of true vitamin B₁₂ is competitively inhibited by phenylenediamine.

7. The biosynthesis of the new material in the presence of phenylenediamine is competitively inhibited by 1:2-diamino-4:5-dimethylbenzene. The inhibition index lies somewhere between 160 and 320.

The authors wish to express their thanks to Miss R. A. Brill and to Miss C. M. Lovejoy for valuable technical assistance.

Note added in proof. Clinical trials kindly carried out by Professor L. J. Witts and Dr Sheila Callendar at the Radcliffe Infirmary, Oxford, showed that the new material was fully active in three pernicious anaemia patients.

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The Vitamin B₁₂ Group. Presence of 2-Methyl Purines in Factors A and H and Isolation of New Factors

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The main cobalt-containing substance in the rumen content and faeces of calves has been called 'factor A' by Ford & Porter (1952, 1953). The absorption spectrum and microbiological activity suggested its close relation to vitamin B₁₂. Preliminary reports showed that in fact the difference lies only in replacement of the 5:6-dimethylbenziminazole of vitamin B₁₂ by 2-methyladenine, a purine not previously found in nature (Brown & Lester Smith, 1954; Gant, Lester Smith & Parker, 1954). Dion, Calkins & Piffner (1954) have independently characterized as 2-methyladenine the base present in factor A, which they call pseudo (*ψ*)-vitamin B_{12a}.

By extracting portions totalling 170 kg. of pig manure and about 180 kg. of calf manure, we were able to isolate not only factor A and vitamin B₁₂ itself but also small amounts of other factors, namely, *ψ*-vitamin B₁₂ (Dion, Calkins & Piffner, 1952), factors B and C (Ford & Porter, 1952) and several new red substances that we call factors D–I. Some of them were separated only by employing both chromatography and electrophoresis on paper, successively but in either order. Factors G and H could not be separated except by taking advantage of slight differences in the behaviour of their purple dicyano complexes. Factor I appears identical

with 'factor III' of Friedrich & Bernhauer (1953). The chemical relationships between some of these factors have been revealed by characterizing the products of their hydrolysis under various conditions.

EXPERIMENTAL

Isolation of factors

The methods used were adapted, as the work proceeded, from those used for the isolation of vitamin B₁₂ from liver and fermentation liquors (Fantes, Page, Parker & Lester Smith, 1949). Close collaboration was maintained with workers at the National Institute for Research in Dairying, who have already reported typical procedures (Ford & Porter, 1953).

In brief outline the process comprised the following steps. The manure as received was extracted with boiling ethanol (about 50%, v/v, allowing for water present in the material) containing a trace of cyanide, filtered with kieselguhr, concentrated under reduced pressure, filtered after adjustment to pH 7 and addition of ethanol to 60% (v/v), and concentrated under reduced pressure. It was adsorbed by percolation through a bed of charcoal, the adsorbate was washed with 6% (v/v) aqueous phenol, eluted with hot 60% (v/v) aqueous acetone, and concentrated under reduced pressure. Impurities were then removed by passage through a column of acid-washed alumina, which was developed with water.

Partition chromatography was used for the final stages of purification and for partial separation of the factors. At first this operation was carried out on columns of kieselguhr containing half its weight of water, with water-saturated *n*-butanol for development. Later it was found preferable to streak the partially purified extract across sheets of Whatman 3 MM filter paper and to develop downwards for 24–48 hr. at 27° in the dark, with water-saturated *sec.*-butanol; a small beaker of aq. HCN was present in the tank to keep the factors in their cyano-coordination states. The solvent front ran off the bottom of the paper (serrated with pinking shears to ensure even flow), so that *R_F* values can only be quoted relative to that of a marker spot of vitamin B₁₂. Red zones were eluted and further fractionated by electrophoresis on Whatman no. 2 filter paper or Whatman seed-test paper in 0.5*N* acetic acid (pH 3.0) as supporting electrolyte (Armitage *et al.* 1953; Holdsworth, 1953). A voltage gradient of about 5 v/cm. for about 16 hr. usually sufficed to produce distinct zones of the basic factors (*A*, *B* and ψ -vitamin B₁₂), separate from each other and from the group of neutral factors. For special purposes electrophoretic separations under similar conditions were carried out with other electrolytes, namely 0.05 *M* sodium phosphate buffer containing 0.01% KCN at pH 6.5 or 7.5, or 0.05 *M* KCN (pH 10 approx.). Repetition of either chromatography or electrophoresis or both was necessary to ensure complete removal of other factors; identification of the known factors was simultaneously accomplished by running spots of authentic specimens alongside.

The earlier stages of fractionation were controlled by microbiological plate assays with the B₁₂-requiring *Escherichia coli* mutant (Harrison, Lees & Wood, 1951) and also by chromatography and electrophoresis of small amounts on filter paper, the positions of the microbiologically active factors (i.e. all except factor *D*) being revealed by the bioautographic technique (Ford & Holdsworth, 1953). The purified factors were also assayed with the more selective

micro-organisms *Lactobacillus leichmannii* (Ford, 1953*a*) and *Ochromonas maltheensis* (Ford, 1953*b*).

Factor *D* was seen as a slow-running pink band on chromatographing partially purified calf-manure extracts on alumina from an aqueous solution. It was microbiologically inactive and came through the column after all the active fractions had been eluted. Factor *D* was purified further by paper chromatography and obtained as a deep red solid that could not be induced to crystallize. It behaved as a base on electrophoresis in acetic acid. The absorption spectrum suggests a close relationship to the B₁₂ vitamins, of which it is perhaps a metabolic product.

The other factors are described briefly in the order in which they separate on paper chromatography; some of their properties are given in Table 1.

Factor *C* (Ford & Porter, 1952; Holdsworth, 1953) was revealed on bioautographs of both source materials as the slowest chromatographic zone (*R_F* relative to vitamin B₁₂, about 0.2). The zone often appeared as a doublet, suggesting the presence of two factors. Electrophoresis in neutral or alkaline buffers revealed the acidic character of this factor (or factors). Purification proved exceedingly difficult and so far only brown concentrates have been obtained from these sources.

Factors *A*, *G*, *H* and ψ -vitamin B₁₂ appeared together on paper chromatograms with *R_F* values (relative to vitamin B₁₂) of 0.35–0.45; the *R_F* values appeared to be affected by impurities present in the concentrates. On paper electrophoresis in acetic acid, the mixture could be partially resolved. Factors *G* and *H* remained together near the origin, while factor *A* and ψ -vitamin B₁₂ moved towards the cathode, factor *A* being the faster moving and more basic of the two.

Factors *G* and *H* proved more difficult to separate than any other members of this group so far encountered. Indeed, the factors themselves behaved identically on chromatography or electrophoresis under all the conditions tested. However, like cyanocobalamin itself, these factors take up an additional molecule of cyanide, signalled by a colour change to purple, in alkaline cyanide solutions. These acidic dicyano complexes of factors *G* and *H* could be separated by paper chromatography in an atmosphere containing HCN using *sec.*-butanol containing 0.2% of NH₃ (sp.gr. 0.88) as developing solvent, when factor *H* travelled slightly the faster. The purple complexes could not be separated by electrophoresis in 0.05 *M* KCN at pH 10, but separation did occur with 0.05 *M* sodium phosphate buffer containing cyanide at pH 7.5.

At this pH, factor *H* was more completely converted into the acidic double cyanide than was factor *G*, so that its effective mobility was slightly greater: correspondingly, the colour of the zone was purplish mauve, whereas that of factor *G* was pinkish mauve.

Factor *I* appeared as a red zone with *R_F* (relative to B₁₂) of 0.65, on chromatographing both calf- and pig-manure concentrates. It was electrophoretically neutral. This factor appeared to be identical with 'factor III' isolated by Friedrich & Bernhauer (1953) from sewage sludge. We are indebted to Professor Bernhauer for a reference sample of their crystalline preparation. Clinical trials in Germany showed that it was active in pernicious anaemia. In clinical trials kindly carried out by Dr Sheila Callender at the Radcliffe Infirmary, Oxford, the German preparation gave an optimum response at 50 μ g. while our factor *I* from pig manure gave a suboptimum response at 25 μ g.

Factor *F* was found in manure from pigs, but only in traces from calf manure; it appeared to be the main factor, however, in chicken faeces. Its R_F value (relative to B_{12}) was 0.77; it was electrophoretically neutral.

Factor *E* appeared below the vitamin B_{12} zone, with a relative R_F of 1.35–1.5. It did not move from the origin on electrophoresis in acetic acid, but in phosphate buffer at pH 6.5 it moved towards the anode, and separated into two red zones of slightly different mobilities.

Factor *B* moved the furthest on chromatography, with relative R_F 1.6–1.7. On electrophoresis in acetic acid it moved towards the cathode, but faster than the other basic factors, *A* and ψ -vitamin B_{12} . Factor *B* differs from vitamin B_{12} only in the absence of the nucleotide portion (Armitage *et al.* 1953).

An alternative fractionation scheme followed for part of the material comprised electrophoresis in acetic acid as the first step yielding four fractions, namely (in order of separation from the cathode end) factor *B*, factor *A*, ψ -vitamin B_{12} and all the neutral factors. These last were then separated from one another by paper chromatography with the ammoniacal solvent mixture.

Factors *A*, *F*, *G*, *H*, *I* and ψ -vitamin B_{12} were obtained crystalline from aqueous acetone. It is hoped to report in more detail later, upon factors *C*, *D*, *E*, *F* and *I*.

Deamination

Factor *A* (4.2 mg.) was dissolved in water (1 ml.) and glacial acetic acid (0.05 ml.). The solution was held at 75° for 15 min., NaNO_2 (2 portions of 0.02 g.) being added initially and after 7.5 min. respectively. The warm solution was held briefly at reduced pressure to remove nitrous fumes and was then extracted with a few drops of phenol. The washed phenol extract was shaken with a few drops of water and 2 ml. of ether. Acetone was added to the aqueous layer until it became faintly turbid; crystals were deposited on keeping overnight. ψ -Vitamin B_{12} was deaminated similarly on a smaller scale.

Hydrolysis: isolation of purine base and preparation of picrate

Factor *A* (1.3 mg.) was dissolved in 2N-HCl (1.5 ml.) and the solution was heated under reflux in a boiling-water bath for 2 hr. The red hydrolytic products were removed by extraction with *n*-butanol (3 $\times$ 1 ml. portions), the extracts being washed in turn with 0.5 ml. of N-HCl. The aqueous layers were taken to dryness under reduced pressure to remove acid; the residues from this and similar hydrolyses were used for chromatography, electrophoresis and absorption spectroscopy. Part of the material was converted into the picrate by adding a slight excess of picric acid; the precipitate was collected by centrifuging and recrystallized from hot 25% (v/v) acetic acid. The infrared absorption spectrum in Nujol mull was compared with that of adenine picrate.

Other factors were hydrolysed similarly.

Hydrolysis: isolation of factor B

Factor *A* (1.2 mg.) was dissolved in conc. HCl (0.1 ml.) and the solution was held at 65° for 4.5 min. Acid was removed by evaporation under reduced pressure (bath temperature 60°) and the residue was dissolved in 1% KCN (0.5 ml.). The solution was acidified after 5 min. standing

and extracted with phenol-benzene mixture (1:2, by vol.; 2 portions of 0.5 ml.). The extracts were shaken with a few drops of water and ether-acetone mixture (1:1, by vol.; 2 ml.). The red material in the aqueous layer was compared with a preparation made similarly from the same weight of vitamin B_{12} ; the two alongside were submitted to paper chromatography and electrophoresis and to the microbiological plate assay with *Esch. coli* mutant, as described above.

Chromatography of purines

The purine bases released on hydrolysis with hot 2N-HCl as described earlier were chromatographed alongside appropriate 'markers' by descending development on Whatman no. 4 paper. After preliminary trials, two solvent systems were selected. The first consisted of *n*-butanol- NH_3 (sp.gr. 0.88) (97:3, by vol.) with just enough water to saturate the mixture. The second contained *n*-butanol-diethylene glycol-water (4:1:1, by vol.); an ammonia atmosphere was provided by a small beaker of NH_3 (sp.gr. 0.88) in the tank (Vischer & Chargaff, 1948).

RESULTS AND DISCUSSION

A property of factor *A* that proved invaluable in the course of its purification was its slight mobility towards the cathode during filter-paper electrophoresis in dilute acetic acid. This indication of a weakly basic group was confirmed by treatment with nitrous acid, which converted factor *A* into a different substance which was red and could be crystallized; this material, although it could not be distinguished from factor *A* by chromatography with *sec*-butanol, was electrophoretically neutral. This behaviour was consistent with deamination of an amino group in the original substance. Moreover, the 'semi-synthetic' product appeared to be identical with a natural factor we have isolated in trace amounts from pig manure and call 'factor *H*' (Table 1). It is not necessarily synthesized directly by rumen micro-organisms, for it may arise by enzymic deamination of factor *A*. Hydrolysis of both factors, *A* and *H*, with hot 2N-HCl yielded (among other products) substances that appeared to be purines, for on chromatography they gave spots that quenched the fluorescence of the filter paper in ultraviolet light at 254 $\text{m}\mu$., and they responded to staining reactions with cuprous ions (Holdsworth, private communication) or with silver chromate (Reguera & Asimov, 1950).

However, the R_F values in some solvent mixtures differentiated them from known natural purines (Table 2). Moreover, the purine from factor *A* displayed characteristic ultraviolet absorption maxima in neutral, acid and alkaline solution. Consideration of R_F values and electrophoretic mobilities suggested that this purine might well be an alkyl adenine, while that from the deaminated factor *A* could be the corresponding alkyl hypoxanthine. A step in the biogenesis of purines is

completion of the pyrimidine ring by reaction of the precursor 4-aminoimidazole-5-carboxamide with 'formate' (Buchanan & Wilson, 1953). It seemed possible that the abundant acetic acid in the rumen might partially displace formate from this role, leading to 2-methyl purines. We were fortunate to obtain synthetic specimens of 2-methyladenine and 2-methylhypoxanthine from Sir Alexander Todd, F.R.S.: by all the tests we were able to apply to quantities of less than 1 mg., their properties tallied with those of the bases from factors *A* and *H* respectively. In addition to those recorded in Table 2, the infrared absorption spectra of the crystalline picrates of 2-methyladenine and of the base from factor *A* in Nujol mull showed numerous corresponding peaks, in positions different from those given by adenine picrate.

The chromatograms from the hydrolysate of factor *A* showed, in addition to the main 2-methyladenine zone, a minor zone in the position of 2-methylhypoxanthine. Material eluted from this zone had an absorption maximum in acid solution at 249 m μ ., the expected position for 2-methylhypoxanthine (Table 2), which doubtless arises from partial deamination of 2-methyladenine during the acid hydrolysis.

The identification has been neatly confirmed by

Ford & Holdsworth (1954), who have shown that when, and only when, synthetic 2-methyladenine or the nucleotide from factor *A* is included in the medium, an *Esch. coli* mutant elaborates mainly factor *A* instead of other B₁₂ vitamins.

In a preliminary search, we have failed to find 2-methyladenine in other natural products, namely, the crude purines precipitated by cuprous salts from rumen extract and from fish solubles (kindly prepared by Dr E. S. Holdsworth), crude rumen nucleic acids and yeast nucleic acids. A proportion greater than 1% of the adenine present would have been detected by the methods used.

A precisely analogous relationship exists between another pair of B₁₂ vitamins. We have isolated a few mg. of pure ψ -vitamin B₁₂ from pig manure and have confirmed that it yields adenine on acid hydrolysis (Dion *et al.* 1952). It also can be deaminated with hot nitrous acid, yielding a neutral, red, crystallizable and microbiologically active substance, which again appears to be identical with a substance that we have isolated in minute yield from pig manure and call 'Factor *G*'. Deaminated ψ -vitamin B₁₂ and factor *G* both yield the expected base, hypoxanthine, on acid hydrolysis (Brown & Lester Smith, 1954). Deamination of ψ -vitamin B₁₂ has also been reported by Pfiffner, Calkins & Dion (1954).

Table 1. *Physical and microbiological properties of some B₁₂ vitamins*

Factor	Absorption maxima (m μ .)	Electrophoretic behaviour at pH 3	Microbiological potency (in % relative to that of B ₁₂)			
			<i>Esch. coli</i> mutant†		<i>L. leichmannii</i> tube assay†	<i>Ochromonas malhemensis</i> ‡
			Plate assay	Tube assay		
<i>A</i>	361, 520, 548	Slightly basic Less basic than factor <i>A</i>	85	30	25	1
ψ -Vitamin B ₁₂	—		120	20	50	0.2
<i>G</i> (and deaminated ψ -B ₁₂)	359, 516, 540	Neutral	160	—	20–100*	0.3–3*
<i>H</i> (and deaminated factor <i>A</i>)	358.5, 517, 540	Neutral	280	40	15–40*	0.7–2*
<i>F</i>	—	Neutral	130*	50	—	5
<i>I</i>	295, 561, 518, 550	Neutral	125–150*	45–60	35	40–60*

* Non-linear response.

† Ford (1953*a*).

‡ Ford (1953*b*).

Table 2. *Properties of purines from factors A, G and H*

The exact composition of the two solvent systems is given in the text.

Purine	Absorption maxima (m μ .)			<i>R_F</i> values in <i>n</i> -butanol plus	
	pH 2	pH 7	pH 10	NH ₃	Diethylene glycol + NH ₃
Base from factor <i>A</i>	266	262.5	270	0.52	0.65
2-Methyladenine	266	262	270	0.53	0.65
Base from deaminated factor <i>A</i>	—	—	—	0.27	0.49
Base from factor <i>H</i>	249	249.5	261.5	0.25	—
2-Methylhypoxanthine	249.5	249.5	261.5	0.27	0.50
Base from factor <i>G</i>	248	—	—	0.15	—
Hypoxanthine	248	248	262.5	0.15	—
Adenine	262.5	259	268	0.38	0.51

The nucleotide can be removed from vitamin B₁₂ with little attack on other labile groups by brief treatment with warm concentrated hydrochloric or perchloric acid (Armitage *et al.* 1953). The main cobalt-containing part of the B₁₂ molecule so released is a substance still exhibiting microbiological activity towards the *Esch. coli* mutant; it is identical with factor *B* previously isolated from calf faeces and from fermentation liquors (Ford & Porter, 1952). When the treatment with hydrochloric or perchloric acid was applied to ψ -vitamin B₁₂, to factor *A* or to their deamination products, factor *B* was always produced. The latter was identified by microbiological activity, behaviour on paper electrophoresis at various pH values and paper chromatography with *sec.*-butanol. The total hydrolysate from each of the factors was run against a similar B₁₂ hydrolysate made with concentrated HCl. Not only were the *R_f* values of the main (factor *B*) zones identical, but several other zones tallied in the parallel chromatograms: these zones arose from acids produced by partial hydrolysis of labile amide groups (Armitage *et al.* 1953). It is therefore clear that all these B₁₂ vitamins are built up by addition of appropriate nucleotides to the one fundamental substance, factor *B*.

SUMMARY

1. By suitable fractionation procedures a number of red cobalt-containing substances other than vitamin B₁₂ have been isolated from pig and calf manure. These are factors *A*, *B* and *C* of Ford & Porter (1952) and ψ -vitamin B₁₂ of Dion, Calkins & Pfiffner (1952). Others are designated Factors *D*, *E*, *F*, *G*, *H* and *I*.

2. Factor *A* of Ford & Porter (1952) differs from vitamin B₁₂ only in having 2-methyladenine in place of 5:6-dimethylbenzimidazole.

3. On deamination, factor *A* is converted into a

naturally occurring factor *H*, which yields 2-methylhypoxanthine on mild hydrolysis.

4. Similarly, ψ -vitamin B₁₂ is deaminated to a naturally occurring hypoxanthine containing factor *G*.

5. ψ -Vitamin B₁₂ and factors *A*, *G* and *H* resemble vitamin B₁₂ in yielding one fundamental cobalt-containing substance, factor *B*, on removal of their nucleotides with warm concentrated hydrochloric acid.

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The Biosynthesis of Vitamin B₁₂-Like Compounds*

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We have shown (Ford, Holdsworth, Kon & Porter, 1953) that the 'vitamin B₁₂' in extracts of gut contents and faeces is composed in the main of cyanocobalamin and four related compounds, factors *A*, *B* and *C*, and pseudovitamin B₁₂. In general, extracts of animal tissues contain preponderantly cyanocobalamin, whereas in natural

materials subjected to bacterial fermentation the other compounds are often present in relatively larger amounts. For example, more than 70 % of the 'vitamin B₁₂' in the rumen contents of calves is in the form of factor *A*, whereas the liver contains mainly cobalamin, with sometimes a trace of factor *A* (Ford, Holdsworth & Porter, 1953).

In a recent publication, Gant, Smith & Parker (1954) disclosed that on controlled acid hydrolysis

* Read in part before the Biochemical Society on 16 July 1954 (Ford, Holdsworth & Kon, 1954).