The nucleotide can be removed from vitamin B_{12} 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 B_{12} 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 B_{12}, 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 B_{12} 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 B_{12} 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 B_{12} have been isolated from pig and calf manure. These are factors *A*, *B* and *C* of Ford & Porter (1952) and ψ-vitamin B_{12} 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 B_{12} only in having 2-methyladenine in place of 5:6-dimethylbenziminazole.

3. On deamination, factor *A* is converted into a naturally occurring factor *H*, which yields 2-methylhypoxanthine on mild hydrolysis.

4. Similarly, ψ-vitamin B_{12} is deaminated to a naturally occurring hypoxanthine containing factor *G*.

5. ψ-Vitamin B_{12} and factors *A*, *G* and *H* resemble vitamin B_{12} 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 B_{12}-Like Compounds*

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We have shown (Ford, Holdsworth, Kon & Porter, 1953) that the 'vitamin B_{12}' 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 B_{12}. 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 B_{12}' 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).

the nucleotide can be removed from cyanocobalamin, factor *A* and pseudovitamin B_{12}. In each instance the 'denucleotided' substance produced was identified as factor *B*. Further tests established that both factor *A* and pseudovitamin B_{12} differ from cyanocobalamin solely in the nature of their nucleotide components.

It is not known what nucleotide, if any, is present in factor *C*. In cyanocobalamin the nucleotide is 5:6-dimethylbenziminazole-α-D-ribofuranose phosphate (Buchanan, Johnson, Mills & Todd, 1950*a*,*b*); the base of pseudovitamin B_{12} has been identified as adenine (Dion, Calkins & Pfiffner, 1952) and that of factor *A* as 2-methyladenine (Brown & Smith, 1954; Dion, Calkins & Pfiffner, 1954). Fig. 1 shows the structural formulae of the three bases. All the compounds are active as cyanocobalamin for *Escherichia coli* 113–3 (Davis), a vitamin B_{12}-requiring mutant strain. Cyanocobalamin, factor *A* and pseudovitamin B_{12} appear to be intrinsically active, and are not necessarily interconverted in the metabolism of the organism. Thus, they can largely be recovered unchanged after passage through the organism. Factor *B*, on the other hand, is normally recovered as factor *C*, sometimes accompanied by traces of the other B_{12} vitamins (Ford, Kon & Porter, 1952). By adding to the growth medium both factor *B* and the nucleotide or the nucleotide base of cyanocobalamin, pseudovitamin B_{12} or factor *A*, we were able to direct *Esch. coli* to the synthesis of the corresponding substances (Ford & Holdsworth, 1954). Thus, with factor *B* and adenine, pseudovitamin B_{12} was produced, in accord with the finding that adenine is present in its nucleotide; and with factor *B* and a number of compounds related to adenine or benziminazole, certain new, 'unnatural' analogues of 'vitamin B_{12}' were produced. We report here the preparation and some properties of these compounds.

EXPERIMENTAL

To each of a series of penicillin culture flasks were added 250 ml. of the vitamin B_{12}-free basal medium of Burkholder (1951), modified by substituting thiomalic for thioglycollic acid. The flasks were plugged with cotton wool and sterilized by autoclaving. To each was then added aseptically 0·5 ml. of a solution of factor *B* (8 μg./ml.) in 30% (v/v) ethanol. To one flask no further addition was made, while to each of the others was added one or another of the different test compounds listed in Table 1, usually in amounts of 5 mg. However, only 1 mg. of cyanocobalamin nucleotide was available for these experiments, and only a few micrograms of factor *A* nucleotide, representing the purified product of the hydrolysis of 0·4 mg. of the factor. The flasks were then each inoculated with 0·2 ml. of a culture of *Esch. coli* 113–3 grown with limiting cyanocobalamin. After incubation for 16 hr. at 30° the cultures were centrifuged. Microbiological test showed that the supernatant liquors contained no vitamin B_{12} activity, and they were discarded. The harvested

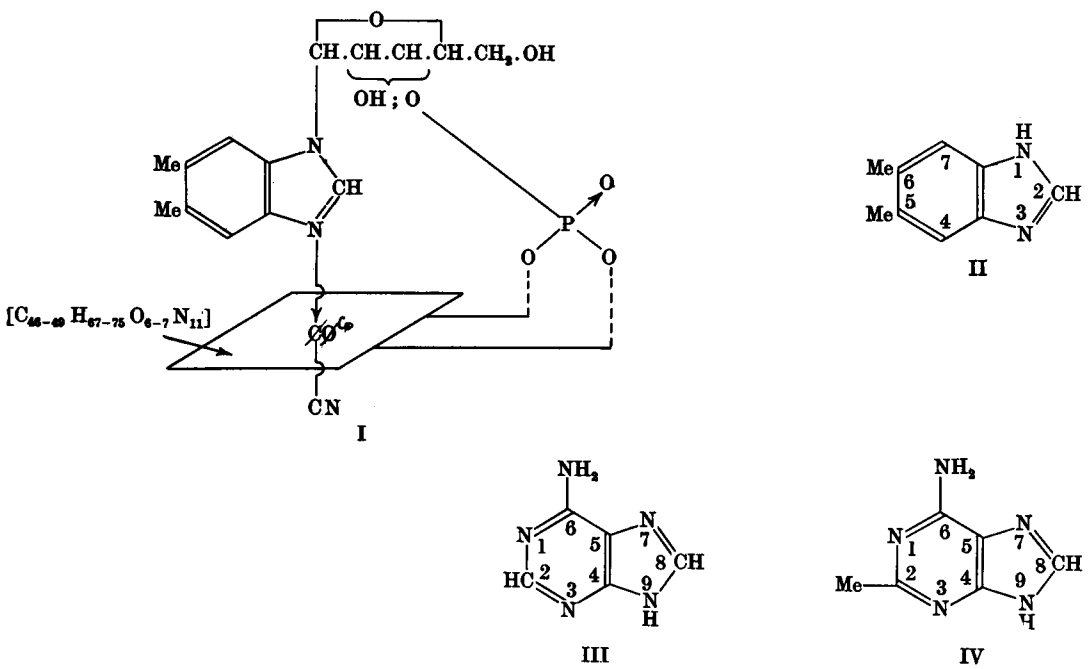

Fig. 1. I, partial formula of cyanocobalamin (Armitage *et al.* 1953); II, 5:6-dimethylbenziminazole; III, adenine; IV, 2-methyladenine.

cells were taken up in 3 ml. 0·033 N-HCl and 0·2 ml. of a 1% solution of NaCN, and heated at 100° for 30 min. The extracts were made to 5 ml. with water and clarified by centrifuging. They were then assayed for 'vitamin B_{12}' with *Esch. coli* 113–3 and with *Ochromonas malhamensis* (see below).

The forms of 'vitamin B_{12}' in the extracts were examined by a bioautographic technique, after paper chromatography and after ionophoresis on paper.

Certain of the experiments were repeated, using radioactive factor *B*, labelled with ^{60}Co. In these instances the extracts were examined by an autoradiographic technique, after chromatography on paper.

Microbiological assay methods

Tests with Esch. coli 113–3. The technique used was that described by Burkholder (1951). The basal medium was modified by substituting thiomalic for thioglycollic acid, and by adding 1 mg. NaCN/100 ml. of 'five-times strength' medium.

Tests with Ochromonas. The technique described by Ford (1953) was used. The 'five-times strength' basal medium was supplemented with 1 mg. NaCN/100 ml.

Other methods

Chromatography. Two solvent systems were used: (*a*) *sec.*-butanol–acetic acid–water–5% (w/v) KCN (100:1:50:0·25, by vol.); (*b*) *sec.*-butanol–aq. NH_3 (sp.gr. 0·88)–water–5% (w/v) KCN (100:1:50:0·25, by vol.). The chromatography was done at 35° on Whatman no. 4 paper. Solutions of cyanocobalamin, pseudovitamin B_{12} and factors *A*, *B* and *C*, each containing 0·2 μg./ml., were used for reference and they and the cell extracts were applied to the paper in 0·005 ml. amounts.

Ionophoresis. The reference standards and cell extracts were examined by ionophoresis on paper with N acetic acid containing 0·01% (w/v) KCN (Holdsworth, 1953).

Bioautographic technique. Esch. coli 113–3 was used as an indicator of vitamin B_{12} activity on the paper chromatograms, in a highly sensitive bioautographic technique described in an earlier publication (Ford & Holdsworth, 1953).

Autoradiographic technique. In the experiments with factor *B* labelled with ^{60}Co, chromatograms of the cell extracts were placed in contact with Ilfex (Ilford, Ltd.) X-ray film for 7 days. The film was then processed as recommended by the manufacturers.

Sources of intermediates. The factor *B* used in these experiments was kindly given to us by Dr Lester Smith of Glaxo Laboratories Ltd., Greenford. It had been prepared by partial hydrolysis of cyanocobalamin (Gant *et al.* 1954) and purified by chromatography and ionophoresis. Dr Lester Smith also provided the nucleotides of cyanocobalamin and factor *A*.

We are indebted to Professor A. R. Todd, F.R.S., for gifts of 2-methyladenine, 2:8-dichloroadenine and 2-methylthioadenine, and to Dr R. E. F. Mathews and Dr J. D. Smith for the 8-azapurines. Certain of the substituted benziminazoles were made by the general procedure outlined by Beaven *et al.* (1949). The remaining substances were available commercially.

RESULTS

In all, forty-eight compounds were tested. Some of them had little or no effect upon the conversion of factor *B* into factor *C*, and these are marked with an asterisk in Table 1. Others modified this process, by 'shunting' the synthesis to one or another of a variety of 'natural' or 'unnatural' B_{12} vitamins. The results obtained with these compounds are set out in Table 2.

It is evident that, when given factor *B* and the nucleotide of factor *A* or of cyanocobalamin, *Esch.*

Table 1. *List of compounds tested for their effect on the biosynthesis of B_{12} vitamins by* Esch. coli

Compounds related to benziminazole	Compounds related to adenine	Various
5:6-Dimethylbenziminazole	Adenine	Guanine*
4:5-Dimethyl-1:2-diaminobenzene	Adenosine*	Guanylic acid*
5-Methylbenziminazole	Adenosine 2′-phosphate*	8-Azaguanine*
3:4-Dimethyl-6-D-ribitylaminobenzene*	Adenosine 3′-phosphate*	Hypoxanthine*
1-Amino-3:4-dimethyl-6-D-ribitylaminobenzene	Adenosine 5′-phosphate*	2-Methylhypoxanthine*
Riboflavin	Adenosine triphosphate*	8-Azahypoxanthine*
Benziminazole	2-Methyladenine	Inosine*
5:6-Dichlorobenziminazole	8-Azaadenine	Xanthine*
4:5-Dichloro-1:2-diaminobenzene	2:6-Diaminopurine	Uracil*
5-Aminobenziminazole	2:8-Dichloroadenine	Uridine*
5-Nitrobenziminazole	2-Methylthioadenine	Cytidine*
4-Chloro-1:2-benztriazole		Thymidine*
Benzthiazole		Thymine*
		5-Methylcytosine*
		Orotic acid*
		Thiamine*
		Nicotinic acid*
		Nicotinamide*
		p-Aminobenzoic acid*
		Folic acid*
		Citrovorum factor*
		Pyridoxamine*
		Riboflavin phosphate*

* These compounds had little or no effect on the conversion of factor *B* into factor *C*.

coli reconstitutes the corresponding vitamin. We had none of the nucleotide of pseudovitamin B_{12} to test, but it seems likely that it would be combined with factor *B* in the same manner. The free nucleotide bases were also effective in 'shunting' the biosynthesis, as is evident from Fig. 2, which illustrates the findings of experiments with factor *B* labelled with ^{60}Co. The autoradiograph shows that, when grown with factor *B* alone, *Esch. coli* converted it into factor *C*, but when adenine was added to the system relatively little factor *C* appeared, and most of the radioactivity was in the pseudovitamin B_{12} position. Adenosine and adenosine 2'-, 3'- and 5'-phosphates were much less effective than the free base in directing the biosynthesis of pseudovitamin B_{12}.

Fig. 3 illustrates again, in diagrammatic form, the effects of certain intermediates in 'directing' the biosynthesis of B_{12} vitamins. It further illustrates the use of ionophoresis on paper, combined with the bioautographic technique, in differentiating between the different forms of 'vitamin B_{12}'.

Table 2. *Biosynthesis of B_{12} vitamins by* Esch. coli 113–3 *when given factor* B *and different compounds structurally related to benziminazole or adenine*

To each flask were added factor *B* (4·0 μg.) and one of the different test compounds (usually 5 mg.) listed in column 1. The flasks were then inoculated with *Esch. coli* as described in the text. The R_F values were obtained with the two solvent systems, the detailed composition of which is given in the Experimental section. The figures for recovery of vitamin B_{12} activity in extracts of cells grown with factor *B* and various test compounds (last two columns) indicate only the relative activities of the different compounds formed for the two micro-organisms. It must be noted that the different B_{12} vitamins have widely different activities for each individual test micro-organism (Ford, 1953). On a weight basis, factor *B* is approximately 20% as active as cyanocobalamin in the *Esch. coli* tube test. It is quite inactive for *Ochromonas*.

Test intermediates in addition to factor *B*	B_{12} vitamins in cell extracts*	R_F		Recovery of vitamin B_{12} activity (expressed as μg. cyanocobalamin) by assay	
		sec-Butanol–acetic acid–water	*sec*.-Butanol–NH_3–water	*Esch. coli*	*Ochromonas*
Factor *B* alone (control)	Factor *C*	0·02	0·04	0·16	0·04
+5:6-dimethylbenziminazole	Cyanocobalamin	0·25	0·30	0·97	1·05
+cyanocobalamin nucleotide	Cyanocobalamin	0·25	0·30	0·90	0·82
+5-methylbenziminazole	New factor	0·19	0·28	0·76	0·76
+benziminazole	New factor	0·17	0·24	0·31	0·35
+3:4-dimethyl-6-D-ribityl-aminobenzene	Factor *C*	0·02	0·04	0·13	0·03
+1-amino-3:4-dimethyl-6-D-ribityl-aminobenzene	Factor *C* and cyanocobalamin	0·02 0·25	0·04 0·50	0·50	0·36
+riboflavin	Factor *C* and cyanocobalamin	0·02 0·25	0·04 0·30	0·30	0·18
+5:6-dichlorobenziminazole	New factor	0·35	0·41	0·27	0·46
+4:5-dichloro-1:2-diaminobenzene	New factor	0·35	0·41	0·30	0·50
+4-chloro-1:2-benztriazole	New factor	0·26	0·40	0·19	0·10
+5-aminobenziminazole	New factor	†	0·14	0·22	0·05
+5-nitrobenziminazole	New factor	†	0·29	0·34	0·23
+benzthiazole	Factor *C* and new factor	0·02 0·15	0·04 0·24	0·23	0·15
+adenine	Pseudovitamin B_{12}	0·11	0·085	0·85	0·07
+adenosine	Factor *C* and trace of pseudovitamin B_{12}	0·02 0·11	0·04 0·085	0·20	0·04
+adenosine 2'-phosphate	Factor *C*	0·02	0·04	0·20	0·04
+adenosine 3'-phosphate	Factor *C*	0·02	0·04	0·16	0·04
+adenosine 5'-phosphate	Factor *C*	0·02	0·04	0·25	0·05
+adenosine triphosphate	Factor *C*	0·02	0·04	0·20	0·04
+2-methyladenine	Factor *A*	0·13	0·12	0·28	0·007
+factor *A* nucleotide	Factor *A* and trace of cyanocobalamin	0·13 0·25	0·12 0·30	0·50	0·10
+8-azaadenine	Factor *C* and factor *A* (?)‡	0·02 0·13	0·04 0·12	0·14	0·03
+2:6-diaminopurine	New factor(s)	0·04 (0·06)	0·04 (0·07)	0·12	0·004
+2:8-dichloroadenine	New factor	0·22	0·26	0·17	0·13
+2-methylthioadenine	New factor	0·26	0·17	0·25	0·03

* The compounds listed were sometimes accompanied by other factors in traces.

† No value available.

‡ New spot inseparable from factor *A* by chromatography or ionophoresis. Evidence of incorporation of azaadenine must await isolation of factor, hydrolysis and identification of the base.

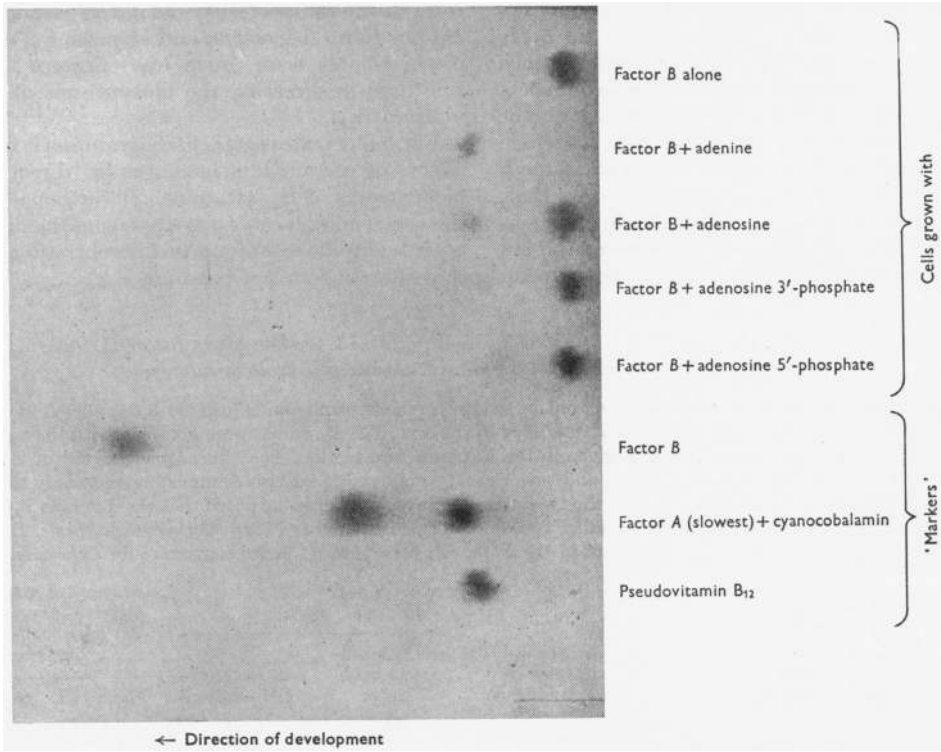

Fig. 2. Autoradiograph of paper chromatogram of factors *A* and *B*, pseudovitamin B_{12} and cyanocobalamin, 'labelled' with ^{60}Co; and of extract of cells of *Esch. coli* grown with 'labelled' factor *B*, and with adenine or certain of its derivatives. Chromatogram developed with *sec.*-butanol–acetic acid–water at 35°. Cells grown in factor *B* alone contain factor *C*.

From the analogues of benziminazole tested, a number of new compounds were produced, some highly active microbiologically for both *Esch. coli* and *Ochromonas*. The latter organism has hitherto been regarded as highly specific in its requirement for cyanocobalamin (Ford, 1953), and it may well prove to display the same pattern of specificity as birds and mammals. We have now undertaken the preparation of certain of the benziminazole derivatives in pure form and on a larger scale, and hope in due course to report the results of growth tests with higher animals. The vitamin B_{12}-like compound formed with benziminazole has already been isolated in pure form. For *Ochromonas* it proved 36 % as active as cyanocobalamin, and in *Esch. coli* (tube) assays the compound was 27 % as active as cyanocobalamin.

Several other new B_{12} vitamins were produced by adding certain substituted purines to the growth medium (see Table 2).

DISCUSSION

It seems likely that in the mutant strain of *Esch. coli* used in these experiments the requirement for 'vitamin B_{12}' is imposed by a defect in the synthesis of factor *B*. Given this factor and the base of any one of a number of alternative nucleotides, the organism completes the synthesis of the form of 'vitamin B_{12}' containing that particular nucleotide. In contrast, for certain other micro-organisms requiring 'vitamin B_{12}', e.g. *Euglena gracilis*, *Lactobacillus leichmannii* and *Ochromonas malhamensis*, factor *B* is inactive. In the series of compounds tested as intermediates in nucleotide synthesis, two main aspects are of interest: first, the ability of the compound to be incorporated; and secondly its suitability for the formation of substances active for *Ochromonas*.

In the benziminazole series, all the compounds tested gave rise to different forms of 'vitamin B_{12}'. Owing to tautomerism in the symmetrical benziminazole molecule N-1 and N-3 atoms are similar. But with benziminazoles unsymmetrically substituted in the benzene ring, two different nucleotides would presumably be formed by the attachment of the ribotide to the different nitrogen atoms. From factor *B* and 5- (or 6-) methylbenziminazole it seemed that only one new form of 'vitamin B_{12}' was produced. This new substance appeared homogeneous on chromatography and ionophoresis, but

it must be stressed that, if in fact the two monomethyl derivatives were formed they would almost certainly be extremely difficult to separate.

A new form of 'vitamin B_{12}' was produced with the compound 4-chloro-1:2-benztriazole, in which a nitrogen atom replaces the CH of the iminazole ring. This compound, however, was less effective than the benziminazoles in 'shunting' the synthesis.

The appearance of a new form of 'vitamin B_{12}' with benzthiazole was unexpected, since neither the sulphur nor the nitrogen of the thiazole would appear to have a free valency which could combine with ribose to form a nucleotide.

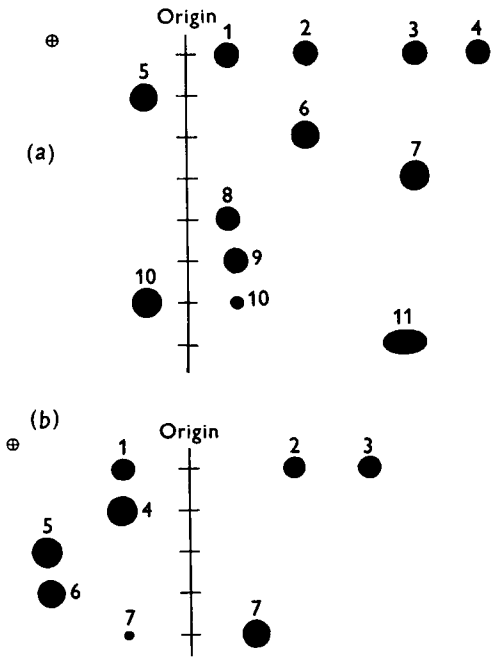

Fig. 3. Ionophoresis on paper of B_{12} vitamins extracted from *Esch. coli* grown with factor *B* and various intermediates. Diagrammatic representation of the bioautographs. (*a*) Ionophoresis at pH 2·2 for 16 hr. at 8 v/cm. in N acetic acid containing 0·01% (w/v) KCN. Marker spots: 1, cyanocobalamin; 2, pseudovitamin B_{12}; 3, factor *A*; 4, factor *B*. Spots obtained with extracts of *Esch. coli* grown with factor *B* and different intermediates: 5, factor *B* alone; 6, factor *B* + adenine; 7, factor *B* + 2-methyladenine; 8, factor *B* + 5:6-dimethylbenziminazole; 9, factor *B* + benziminazole; 10, factor *B* + riboflavin; 11, factor *B* + 2:6-diaminopurine. (*b*) Ionophoresis at pH 6·4 for 16 hr. at 8 v/cm. in phosphate–citrate buffer containing 0·01% KCN. Marker spots: 1, factor *C*; 2, cyanocobalamin; 3, factor *B*. Spots obtained with extracts of *Esch. coli* grown with factor *B* and different intermediates: 4, factor *B* alone; 5, factor *B* + 5:6-dichlorobenziminazole; 6, factor *B* + 5-nitrobenziminazole; 7, factor *B* + 4-chloro-1:2-benztriazole.

More substances were available in the purine series for testing. Adenine, 2:8-dichloroadenine, 2-methyladenine and 2:6-diaminopurine gave vitamin B_{12}-like substances, but with intermediates having a hydroxyl group in position 6, e.g. hypoxanthine, xanthine, guanine and 2-methylhypoxanthine, there was no significant shift of the synthesis from factor *C*.

None of the adenine nucleotides tested was as effective as the free base in directing the synthesis of pseudovitamin B_{12}. Owing to the unsymmetrical nature of adenine, two different sugar derivatives could be formed by combination at positions 7 and 9 (see Fig. 1). Furthermore, the commonly occurring adenylic acids contain the ribose in a β-linkage, whereas cyanocobalamin nucleotide contains an α-linked sugar molecule. Thus there would seem to be at least four theoretically possible adenine nucleotide derivatives, the 7α- and 7β- and the 9α- and 9β-ribose phosphates. Of these only the 9β compounds have been tested and found not to produce pseudovitamin B_{12}. That the isomerism of the purine–sugar link is important is suggested by the work of Tamm, Folkers, Shunk & Horsfall (1954), who tested the ability of a variety of benziminazole derivatives to inhibit the multiplication of influenza virus. Of all the compounds tested, 5:6-dichloro-1-β-D-ribofuranosylbenziminazole proved especially active, much more so than the dichlorobenziminazole itself. This high activity is very largely conferred by the β-D-ribofuranosyl component of the molecule, and it would be of interest to learn whether the corresponding α-D-riboside is also active.

In view of the specificity of *Ochromonas malhamensis*, which resembles that shown by animals, for 'vitamin B_{12}', it was of particular interest to establish whether any of the new compounds were active or inhibitory for this organism. It appeared that with the loss of methyl groups from the dimethylbenziminazole nucleotide of cyanocobalamin, activity for *Ochromonas* diminished. The compound produced with benziminazole itself had only 36% of the activity of cyanocobalamin. The compound produced with 5-methylbenziminazole has not yet been isolated in pure form and therefore no precise figure is available, but it seems likely that it is somewhat less active than cyanocobalamin.

We were surprised to find that with 5:6-dichlorobenziminazole (or the corresponding diamine) a new factor was produced having the same order of activity as cyanocobalamin for *Esch. coli*, *Lb. leichmannii* and *Ochromonas*. The 5-nitrobenziminazole, but not the 5-aminobenziminazole, led to the production of *Ochromonas* activity. In the purine series, pseudovitamin B_{12}, containing adenine, is inactive for *Ochromonas*, the 2-methyl derivative (factor *A*) has a small but significant

activity and the 'unnatural' analogue from 2:8-dichloroadenine is much more active. The number and variety of compounds so far examined is too small to allow speculation about the kind of nucleotide grouping that confers *Ochromonas* activity, but presumably the active compounds have certain common features either of molecular size or shape or electronic character. It should perhaps be stressed that our work provides no direct proof that these intermediates are actually incorporated into the new factors. The evidence is on the change of activities towards the two test organisms and on the appearance of substances with new chromatographic and ionophoretic properties. But Fantes & O'Callaghan (1954) have shown that when given benziminazole, *Streptomyces griseus* produces a new form of vitamin B_{12} containing this base in its nucleotide.

Such of the B_{12}-group of compounds as are active for *Esch. coli* or *Ochromonas* (with the exception of factor *B* and possibly also factor *C*) can very largely be recovered unchanged after passage through *Esch. coli* or *Ochromonas*. Certain of the 'unnatural' compounds have this property in common with the naturally occurring factors. Thus the compounds obtained from benziminazole and dichlorobenziminazole are intrinsically active and are not first converted into cyanocobalamin.

Our observation that cyanocobalamin was produced when riboflavin was included in the medium gives some support to the view of Woolley (1950, 1951), that the metabolic paths of these two vitamins are closely interconnected. Of course, it need not mean that riboflavin is actually broken down to provide the precursor of cyanocobalamin. It could equally well be that a common precursor of riboflavin and cyanocobalamin nucleotide is normally available in limiting amounts and is used preferentially in the synthesis of riboflavin. Thus the provision of preformed riboflavin might exert a sparing action. Certainly 4:5-dimethyl-1:2-diaminobenzene, which Woolley considers to be the common precursor, is capable of 'shunting' synthesis to cyanocobalamin very effectively.

The finding that riboflavin can promote the synthesis of cyanocobalamin prompts speculation on several reports that it will also stimulate the growth of rats and chicks on vitamin B_{12}-deficient diets (Cooperman, Tabenkin & Drucker, 1952; Hartman, Dryden & Cary, 1951). Certain substituted benziminazoles were also active in the same manner (Emerson *et al.* 1950), and it seems likely that the effect is in each instance due to an enhanced gut synthesis of forms of vitamin B_{12} active for higher animals.

We have found that certain 'wild' strains of *Esch. coli* and *Aerobacter aerogenes* when grown in a medium containing dichlorodiaminobenzene in the amount of 20 μg./ml. synthesize a new form of 'vitamin B_{12}' apparently identical with that formed by the *Esch. coli* mutant from factor *B* and dichlorobenziminazole. In view of this, it is interesting to note that Woolley (1951) found dichlorodiaminobenzene to have relatively little growth inhibitory action on coliform organisms.

SUMMARY

1. In *Escherichia coli* 113–3 the requirement for 'vitamin B_{12}' is probably imposed by a defect in the synthesis of factor *B*, which represents the non-nucleotide portion of the molecule in several of the B_{12} vitamins (Gant *et al.* 1954). Given factor *B*, the micro-organism can be directed to the synthesis of cyanocobalamin, factor *A* or pseudovitamin B_{12} by the provision of the appropriate nucleotide or nucleotide base. For example, with adenine, pseudovitamin B_{12} is synthesized.

2. A variety of compounds related in their chemical structure to adenine or to 5:6-dimethylbenziminazole have been examined for their effect on 'vitamin B_{12}' synthesis in this system. In this way new 'unnatural' analogues of vitamin B_{12} have been obtained. Certain of these new analogues are active for *Ochromonas malhamensis*, an organism previously thought to be specific for cyanocobalamin.

3. Certain other organisms, e.g. wild strains of *Esch. coli* and *Aerobacter aerogenes*, will produce these analogues when the appropriate intermediate is included in the growth medium.

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The Application of the Ceric Sulphate-Arsenious Acid Reaction to the Detection of Thyroxine and Related Substances

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During a study of the urinary excretion products in connexion with the clinical trials of the antithyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (Fraser & Maclagan, 1953; Maclagan & Wilkinson, 1951, 1954) it was observed that the urine from patients receiving the drug decolorized the ceric sulphate–arsenious acid reagent of Kolthoff & Sandell (1934). Although a trace of iodide was present, this reagent could not be used for its quantitative estimation because the principal metabolic product, 4-hydroxy-3:5-diiodobenzoic acid, appeared to catalyse the reduction of ceric sulphate by arsenious acid almost as effectively as iodide. Subsequently it was found that many diiodophenols, including thyroxine and diiodotyrosine, gave similar reactions, and it seemed possible that, with the employment of this reagent, a highly sensitive method for the detection of thyroxine and related substances might be developed.

Existing methods for the detection of these compounds, e.g. in paper chromatography, depend either upon the reactions of their amino acid side chains with ninhydrin or diazotized sulphanilic acid, or on autoradiography for ^{131}I-labelled materials only. The former procedure is not sufficiently sensitive to make visible spots containing less than 10 μg. of thyroxine or other iodine-containing amino acids. Moreover, the presence of metabolites such as iodide, which are not amino acids, cannot be demonstrated by this method. The use of the ceric sulphate–arsenious acid reagent would overcome these disadvantages, and the present paper describes the development of such a method, a brief account of which has already appeared (Bowden & Maclagan, 1954).

EXPERIMENTAL

Preparation of reagents. Unless otherwise specified, reagents of the 'AnalaR' grade were used, and all solutions were prepared with 'iodine-free' water (Riggs & Man, 1940). In our experience, water which had been distilled from glass apparatus proved satisfactory without further treatment.

Three primary solutions have been used, the final reagents being made by diluting or mixing these as described below:

(1) Ceric sulphate (10%, w/v) was prepared by adding $Ce(SO_4)_2$, $4H_2O$ (B.D.H. 'low in other rare earths'; 10 g.) to N-H_2SO_4 (100 ml.), previously cooled to 0–5°. The cloudy solution was refrigerated for 1 hr., then clarified by centrifuging or filtration and stored in a refrigerator.

(2) The arsenious acid solution (5%, w/v) was prepared by adding sodium arsenite (Hopkin & Williams's laboratory reagent grade; 5 g.) in small portions to vigorously stirred N-H_2SO_4 (100 ml.) at 0°. If the temperature was allowed to rise or if stirring was omitted, dehydration occurred leading to the precipitation of arsenious oxide. Alternatively, the sodium arsenite (5 g.) was dissolved in N-NaOH (30 ml.) and the solution run dropwise into mechanically stirred 2N-H_2SO_4 (65 ml.) at 0–5°. The volume was then adjusted to 100 ml. with water.

(3) Ceric sulphate (2%, w/v) was prepared by adding $Ce(SO_4)_2$, $4H_2O$ (2 g.) to 4N-H_2SO_4 (100 ml.).

For ordinary paper chromatograms the reagent was prepared by mixing equal volumes of solutions 1 and 2 immediately before use (reagent *A*).

If the paper chromatograms are required for photoelectric scanning, then solutions 1 and 2 were diluted to one-fifth the strength with N-H_2SO_4 before mixing (reagent *B*).

For detection of iodine-containing compounds in aqueous solutions, the same one-fifth strength reagents are used as described. For the detection of iodide in urine, however, solution 3 is used in conjunction with the 1% (w/v) sodium arsenite produced by dilution of solution 2 with N-H_2SO_4.