with branch links on every sixth, seventh and eighth anhydroglucose unit respectively will yield, on hydrolysis with *Lb. bifidus* dextranase, oligosaccharides in unequal proportions. The approximately equal quantities of isomalto-triose, -tetraose and -pentaose produced indicate that the branching of this dextran is random. The presence in the digest of much material containing more than six glucosyl units is further support for a random branching of the dextran molecule.

It is significant that the 'branching' in tetrasaccharide A and pentasaccharide B consists of only one glucosyl unit. 3^3-Glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II) could arise from a part of the dextran molecule where the branching was a single glucosyl unit, or from parts of a randomly branched dextran molecule. Bovey (1959), on the grounds of physical measurements, has suggested that 80% of the branches in *L. mesenteroides* (NRRL-B512) dextran consist of only one glucosyl unit.

SUMMARY

1. *Lactobacillus bifidus* dextranase has been shown to hydrolyse *Leuconostoc mesenteroides* (Birmingham strain) dextran to a complex mixture of oligosaccharides.

2. In addition to isomalto-triose, -tetraose, -pentaose, -hexaose and unresolvable material of degree of polymerization greater than 6, a tetrasaccharide (A) and a pentasaccharide (B) were isolated.

3. Tetrasaccharide (A) was shown to be a mixture of 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose. Pentasaccharide (B) contained one glucose unit joined through a 1:3-linkage to a glucose unit, other than the reducing one, of isomaltotetraose and is probably a mixture of isomers.

4. The dextranase has been shown to hydrolyse 6-*O*-α-isomaltohexaosyl- and 6-*O*-α-isomaltoheptaosyl-sorbitol at two and three alternative glucosidic linkages respectively.

5. The implications of the results, as far as dextranase action and dextran structure are concerned, have been discussed.

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The Purification and Properties of a Factor containing Vitamin B_{12} concerned in the Synthesis of Methionine by *Escherichia coli*

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Evidence has been obtained that cobalamin and folic acid are concerned in the synthesis of methionine from homocysteine by *Escherichia coli* strain PA15, a serine- or glycine-requiring auxotroph (Gibson & Woods, 1960; Cross & Woods, 1954; Szulmajster & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). (In this paper, the terms folic acid and vitamin B_{12} will be used in general reference to the two families of compounds; names in common use will be used in reference to specific

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members of these families, e.g. tetrahydropteroylglutamic acid, cyanocobalamin.) The folic acid cofactor for the transfer of a C_1 unit from serine to homocysteine is supplied to cell-free enzyme-containing extracts by an extract of heated organisms. The behaviour of this heated extract is imitated by tetrahydropteroyltriglutamic acid and also by its N^5- and N^{10}-formyl derivatives; tetrahydropteroylglutamic acid is inactive and inhibits the action of the heated extract and of the synthetic tetrahydropteroyltriglutamic acids (Kisliuk & Woods, 1960; Guest *et al.* 1960; Jones, Guest & Woods, 1961). However, tetrahydropteroylglutamic acid is used as the folic acid cofactor by extracts of acetone-dried organisms if these organisms are harvested from a growth medium containing cobalamin (Kisliuk & Woods, 1957, 1960). Ultrasonic extracts of strain PA15 use tetrahydropteroylglutamic acid as cofactor if free cobalamin is added to the reaction mixture; there is no requirement for cobalamin with the extract of heated organisms as a source of folic acid (Guest *et al.* 1960). However, ultrasonic extracts of *E. coli* strain 121/176 (a cobalamin- or methionine-requiring auxotroph) require cobalamin for the synthesis of methionine when the source of folic acid is either the extract of heated *E. coli* or tetrahydropteroylglutamate (Helleiner, Kisliuk & Woods, 1957; Guest *et al.* 1960).

The presence of a non-diffusible, heat-labile material was demonstrated in extracts of acetone-dried powders of strain PA15 cultured in the presence of cobalamin (Kisliuk & Woods, 1960). Evidence has also been presented that ultrasonic extracts of *E. coli* are able to form this factor from free cobalamin, or, more efficiently, from 5:6-dimethylbenzimidazolylcobamide coenzyme (Woods, 1958; Guest, 1960; Guest & Woods, 1960). The factor, derived either from the extract of acetone-dried organisms grown in the presence of cobalamin or by incubation of free cobalamin with ultrasonic extracts, enabled tetrahydropteroylglutamic acid to be used as the source of cofactor for C_1-transfer in methionine synthesis by extracts of acetone-dried powders of the same organism grown in the absence of cobalamin; under these conditions, free cobalamin was without effect (Woods, 1958; Kisliuk & Woods, 1960; Guest & Woods, 1960). Several analogues of cobalamin inhibited the formation of the factor from cobalamin by ultrasonic extracts, but they had no effect on the activity of the factor.

In this paper a method is presented for the purification of this factor from extracts of acetone-dried organisms of strain PA15 cultured in the presence of cobalamin, together with an investigation of some of its properties.

While this work was in progress, the existence of a cobalamin-containing enzyme, forming part of the enzyme complex catalysing the synthesis of methionine from homocysteine and serine by a cobalamin- or methionine-requiring auxotroph of *E. coli*, was reported (Hatch, Takeyama & Buchanan, 1959). Brief descriptions have also appeared of the purification of this enzyme (Hatch, Cathou & Larrabee, 1960) and its formation *in vitro* from vitamin B_{12} and an apoenzyme prepared from extracts of the auxotrophic organisms grown in the absence of vitamin B_{12} (Takeyama & Buchanan, 1960). A short report has also appeared on the purification of a vitamin B_{12}–protein concerned in methionine synthesis by ultrasonic extracts of *E. coli* PA15 grown in the presence of vitamin B_{12} (Kisliuk, 1960).

MATERIALS AND METHODS

Escherichia coli strain PA15, an auxotroph requiring serine or glycine for growth, was used as a source of both the factor and the enzyme system for its assay. The organism was maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. It was originally obtained from Dr Barbara Wright and the parent strain was isolated from sewage (B. E. Wright, personal communication).

Bulk production of acetone-dried powders of organisms was carried out as described by Szulmajster & Woods (1960). Organisms cultured in the presence of cobalamin were obtained by the addition of 20 μg. of cobalamin to the inoculum for each tray containing 500 ml. of agar medium, and incubating for 18 hr. at 37°. Organisms cultured in the absence of cobalamin were incubated for 40 hr. at 37°.

The powders, in batches of about 100 g., representing the yield from 200–400 trays of medium, were stored in sealed containers at $-15°$; there was no detectable loss of activity for at least 6 months.

Preparation of extracts. Dialysed extracts of acetone-dried organisms grown in the absence of cobalamin were prepared as described by Jones *et al.* (1961). On some occasions the extracts were treated with Dowex-1 X8 resin (Cl form; 100–200 mesh) as described by Kisliuk & Woods (1960). This extract will be referred to as the assay extract.

Crude factor-containing extracts of acetone-dried organisms grown in the presence of cobalamin were prepared by mixing the powder with water (10 ml./g. of powder) in a stainless-steel Potter–Elvehjem homogenizer. The mixture was subjected for 75 sec. to ultrasonic vibration (25 kcyc./sec. at 600 w, produced by an ultrasonic generator type E7590B, Mullard Ltd., London), diluted with a further 5 ml. of water/g. of powder and then centrifuged (78 000*g*, 60 min. at 0°) in a Spinco model L ultracentrifuge. The supernatant fluid was stored at $-15°$ to 0° for up to 18 hr. before further treatment.

Estimation of protein. This was determined spectrophotometrically according to Layne (1957); the nucleic acid content was determined similarly. Measured in this way, the protein concentrations of a variety of solutions obtained during the fractionation procedure agreed, to within 10%, with values obtained by the biuret method (Layne, 1957), the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) and the method of Stickland (1951), with

crystalline bovine serum albumin (Armour Laboratories, Hampden Park, Eastbourne, Sussex) as standard.

Materials for column chromatography. Celite 545 (Johns–Manville, London) was graded to remove fine particles and washed exhaustively by decantation with large volumes of distilled water. Columns were poured as homogeneous slurries in water and washed with several column volumes of 20 mM-potassium phosphate buffer, pH 7·8.

Carboxymethylcellulose (Whatman CM-70 cellulose powder) was washed with 1 vol. of 0·1 N-HCl, 5–6 vol. of water (until free of HCl), 1 vol. of 0·5 M-KCl in 0·5 M-KOH and finally 5–6 vol. of water (until free of KOH). Columns were poured as homogeneous slurries in water and washed with 20 vol. of 160 mM-potassium phosphate buffer, pH 7·8.

Sephadex G-50 dextran gel (L. Light and Co. Ltd., Colnbrook, Bucks.) was graded to remove fine particles and washed well by decantation with 5 mM-potassium phosphate, pH 8. The column (prepared from 20 g. of dry gel) was poured as a slurry and allowed to settle with slow flow under gravity.

Diethylaminoethylcellulose floc (DEAE-cellulose) was a gift from Dr E. Lester Smith. It was washed with 2 vol. of N-KOH, followed by water until the supernatant fluid was neutral. Fine particles were removed by repeated decantation in water, and larger particles were rejected by sucking the suspension through glass tubing of 2 mm. internal bore. Columns were prepared from slurries of the graded floc in water and allowed to settle under gravity flow. Potassium phosphate (0·4 M, pH 8·0) was run through the column until the pH of the effluent reached 8·0; this was followed by 20 vol. of 5 mM-potassium phosphate, pH 8·0.

All columns were poured in glass tubes plugged with glass wool; the use of sintered-glass disks produced columns with very low flow rates. Prepared columns were capped with closely fitting disks of Whatman no. 1 filter paper and transferred to a cold room at 0–4°.

Buffers. Phosphate buffers were prepared from KH_2PO_4 and K_2HPO_4 or KOH. Acetate buffers were made from sodium acetate trihydrate and acetic acid, citrate buffers from trisodium citrate dihydrate and citric acid, and borate buffers from $Na_2B_4O_7,10H_2O$ and H_3BO_3.

Chemicals. Tetrahydropteroylglutamic acid was prepared as described by Kisliuk & Woods (1960) and stored as the solid under H_2. Cobalamin was crystalline material (Anacobin) obtained from British Drug Houses Ltd., Poole, Dorset; it was 88% pure by spectrophotometric assay. Adenosine triphosphate, diphosphopyridine nucleotide, reduced diphosphopyridine nucleotide and riboflavin phosphate were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. DL-Homocysteine was from either Mann Research Laboratories Inc., New York, N.Y., U.S.A. or Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Protamine sulphate (from herring roe) was from L. Light and Co. Ltd. Factor B (cobinamide), a form of vitamin B_{12} without a nucleotide residue, was as described by Guest (1960). 5:6-Dimethylbenzimidazolylcobamide coenzyme was a gift from Dr H. A. Barker; in experiments with this compound precautions were taken to exclude light.

Assay of the factor

This was based on the work of Kisliuk & Woods (1960). Determination of the factor content of protein fractions depended on their ability to promote methionine synthesis by extracts of acetone-dried organisms grown without cobalamin in a reaction mixture containing tetrahydropteroylglutamic acid as folic acid cofactor. This system does not respond either to cobalamin itself or to 5:6-dimethylbenzimidazolylcobamide coenzyme.

The standard reaction mixture contained enzyme-containing extract (assay extract) equivalent to 10 mg. of protein and (μmoles in a final volume of 2 ml.): potassium phosphate buffer, pH 7·8, 240; DL-homocysteine, 20; L-serine, 10; hexose diphosphate 15; $MgSO_4$, 10; ATP, 10; DPN, 1; pyridoxal phosphate, 1. In the later part of this work, 0·2 mM-riboflavin phosphate (flavin mononucleotide, FMN) was found to increase the activity of the partially or extensively purified factor by 25–40%, but to be without effect when crude preparations of the factor were used; a requirement for a flavin coenzyme in the synthesis of methionine has been observed with partially purified enzyme fractions from *E. coli* strain 113/3 (Hatch, Takeyama, Cathou, Larrabee & Buchanan, 1959). Consequently, 0·4 μmole of FMN was always added to each assay tube. It was also found that there was a pronounced lag in the synthesis of methionine during the first 30 min. of incubation; this was abolished if reduced DPN were added to the reaction mixture. In a few experiments, $DPNH_2$ (1 μmole/2 ml.) was used in place of DPN.

The reaction mixtures were incubated at 37° for 3 hr. in an atmosphere of H_2 and the reaction was stopped by bringing to 100° for 3 min. After centrifuging, the supernatant fluids were used for the estimation of the methionine formed. This was assayed microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 by the method of Gibson & Woods (1960). DL-Methionine was used as standard; the organism responds only to the L-isomer, and Kisliuk & Woods (1960) have given information on the specificity of the assay.

Before assaying, fractions containing factor were dialysed for 15 hr. at 3° against 50 mM-potassium phosphate buffer, pH 7·8; those fractions containing Ca^{2+} ions (see below) were dialysed first against 50 mM-potassium phosphate buffer, pH 7·8, containing 5 mM-disodium ethylenediaminetetra-acetate, for 6 hr. and then against 50 mM-potassium phosphate buffer, pH 7·8. Fractions obtained after step IV of the purification were assayed without prior dialysis.

The crude extracts containing factor were themselves able to synthesize methionine to a limited extent, under the conditions used, without the addition of the assay extract. However, a difference curve may be constructed, representing the ability of the factor to promote methionine synthesis by the assay extract (Fig. 1). In practice, the amounts of factor-containing extracts taken for assay were sufficiently small to have negligible ability to synthesize methionine in the absence of assay extract; in effect this meant that only the first portion of the difference curve was used and an almost linear response resulted. On purification, the ability of the preparations to synthesize methionine was rapidly abolished.

The assay for the factor led to rather variable amounts of methionine with any one preparation of the factor, even under strictly controlled conditions; it was therefore considered more satisfactory to define 1 unit of factor activity as that possessed by 1 mg. of protein of the extract prepared from one particular batch of acetone-dried powder by the carefully standardized procedure described above.

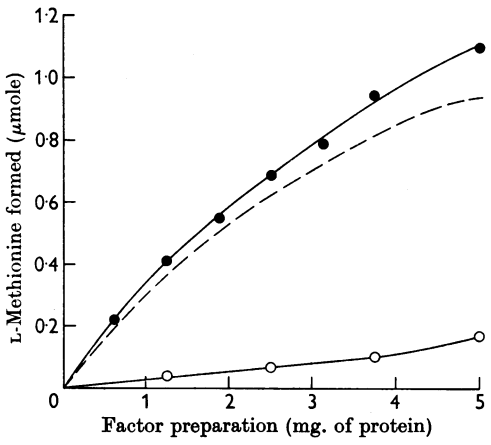

Fig. 1. Assay of the factor. Crude factor was incubated for 3 hr. with the assay system. Main source of enzymes (assay extract, see Materials and Methods section) absent, ○; main source of enzymes (equivalent to 10 mg. of protein) present, ●; difference curve, – – –.

Extracts of fresh batches of powder were standardized by direct comparison with the original powder. The units defined are approximately the same as those used by Guest & Woods (1960) in the investigation of synthesis of the factor by ultrasonic extracts of *E. coli*, i.e. 1 unit of factor increases the synthesis of methionine by 10 mg. of protein of the assay extract to the extent of about 100 μmoles of L-methionine/3 hr.

In each assay of factor activity, a standard curve was constructed over the range 0–5 units, with the crude factor-containing extract of known activity. A response curve of identical shape was given by the factor at all stages of purification. At some steps in the purification the preparations of the factor contained sufficient salts to reduce methionine formation by up to 15%. In such cases the same concentration of the same salts was added to the standards.

Assay of cobalamin

Cobalamin was assayed microbiologically with either *Euglena gracilis* var. *bacillaris* or *E. coli* strain 113/3, a cobalamin- or methionine-requiring auxotroph (Davis & Mingioli, 1950).

With *Euglena gracilis* the procedure adopted was that of Kisliuk & Woods (1960), with the exception that the inoculum was derived from a 4-day culture on basal medium not supplemented with cobalamin; the organisms were centrifuged, resuspended in 3 culture vol. of 0·9% NaCl and 0·1 ml. of this suspension was added to each assay tube. This modified procedure enabled the incubation time to be reduced from 10 to 8 days; it also led to less growth of organisms in tubes devoid of cobalamin. Growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a red filter no. 608.

The method with *E. coli* 113/3 was essentially that of Burkholder (1951). The organism was maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. The basal medium was as described by Burkholder (1951). Tween 80 (Honeywill and Stein Ltd., London, W. 1) was added to a final concentration of 0·01% immediately before using the medium; this reduced the tendency of the organisms to aggregate during growth.

The medium (final volume 2 ml., in 150 mm. × 20 mm. tubes) was autoclaved (7 min. at 115°) after the addition of cobalamin or experimental samples. Standards were included in duplicate in each assay; samples were assayed at three concentrations. The inoculum was derived from a 10 hr. culture on the basal medium containing 0·1 mM-DL-methionine. The organisms were centrifuged, resuspended in the culture volume of water and diluted 50-fold with water; 0·1 ml. of this suspension was added to each assay tube. Incubation was for 15–20 hr. at 37° on a rotary shaking machine (220 rotations/min., radius 2 cm.). Growth was assessed in an EEL colorimeter with a neutral density filter. A reproducible and almost linear curve relating growth to cobalamin concentration was obtained over the range 0–0·6 μmg. of cobalamin/ml. The accuracy of the assay was about ±10%.

As standard, an aqueous solution containing 150 μg. of cobalamin/ml. was assayed spectrophotometrically, the extinction coefficient quoted by Brink *et al.* (1949) being used. This was diluted monthly with KCN solution to give a stock solution containing 1·5 μg. of cobalamin and 1·5 mg. of KCN/ml., and stored at 4°; it was diluted further with water to an appropriate concentration just before use.

Purification of the factor

All manipulations during the purification procedure, including the column chromatography, were done at 0–4°. During the first four steps of the purification particular care was taken to control the pH to within at least ±0·03 unit of the figures quoted below, since even small deviations outside these limits caused a serious decrease in the degree of purification finally achieved. A Pye Type 11087 Dynacap pH meter (W. G. Pye and Co. Ltd., Cambridge), with standard glass electrodes, was suitable for this purpose.

Reagents were added to protein solutions dropwise, with stirring.

Step I; removal of nucleoprotein with calcium chloride. The initial extract of factor, prepared as described earlier, was brought from pH 7·0 to pH 5·65 with N-acetic acid (about 0·7 ml./100 ml. of extract). Material usually precipitated, but redissolved on continued stirring. Calcium chloride (M) was added to a final concentration of 25 mM; the pH fell to 5·35, but rose slowly to 5·55 on stirring for 30 min. The mixture was centrifuged at 24 500*g* for 20 min. and the precipitate discarded.

Step II; removal of protein at pH 5. The supernatant fluid from step I was adjusted to pH 5·00 by the addition of 0·2 M-sodium acetate buffer, pH 4·0 (about 3 ml./100 ml. of initial extract), and stirred for 30 min. The precipitate was removed by centrifuging at 24 500*g* for 20 min. and discarded.

Step III; precipitation of factor at pH 4·4. The supernatant fluid from step II was adjusted to pH 4·40 with N-acetic acid (about 1·9 ml./100 ml. of initial extract) and immediately centrifuged at 10 000*g* for 10 min. The supernatant fluid was discarded and the centrifuge tubes were wiped as free as possible from fluid. The precipitate was dissolved rapidly in 20 mM-potassium phosphate buffer,

pH 7·8 (about 20 ml./100 ml. of initial extract). This solution was adjusted to a protein concentration of 10 mg./ml. by the further addition of 20 mM-potassium phosphate buffer, pH 7·8. The pH was adjusted to 7·0, if necessary, by the addition of a few drops of N-KOH.

Step IV; treatment with protamine sulphate. Residual nucleic acid and some inert protein were removed from the solution from step III with protamine sulphate (20 mg./ml. in 20 mM-potassium phosphate buffer, pH 7·5), 0·55 ml./10 ml. of protein solution being used. After standing for 60 min. with occasional stirring, the mixture was centrifuged at 4000*g* for 15 min. and the precipitate discarded. The supernatant solution could be stored at −15° with only small loss of activity (20% in 1 month). If purification was interrupted at this stage more inert material separated out and could be removed by centrifuging again.

Step V; chromatography of protamine complexes on Celite 545. Supernatant fluid (40 ml.) from step IV was mixed with Celite 545 (1 g.) and treated with the protamine sulphate solution (1 ml./100 mg. of protein). The mixture was allowed to stand for 60 min. with occasional stirring and then poured on to a 2 cm. × 2 cm.^{2} column of Celite 545 in 20 mM-potassium phosphate buffer, pH 7·8. The effluent was caused to run through a second column (40 cm. × 2 cm.^{2}) of Celite 545 prepared in the same way. Both columns were then washed with 20 mM-potassium phosphate buffer, pH 7·8, until the effluent was free from protein; about 150 ml. of buffer was required.

Both columns were washed with 75 mM-potassium phosphate buffer, pH 7·8, until the effluent was again free from protein (about 160 ml. was required). The shorter column was then detached and discarded, and elution of the remaining column continued with 120 ml. of 120 mM-potassium phosphate buffer, pH 7·8. Factor was then eluted from the column with 150 ml. of 160 mM-potassium phosphate buffer, pH 7·8.

The effluent from the columns was collected continuously in fractions of either 5 or 10 ml. with an automatic fraction collector. The flow rate of the columns was usually 30–35 ml./hr., but on a few occasions pressure (1–2 lb./in.^{2}; N_2) was applied to the top of the columns to maintain the flow rate at this value.

Fractions were assayed for activity as soon after delivery as practicable and stored at −15° until the results of the assay were available (about 36 hr.). Fractions were then pooled to give 80 ml. of solution CL, an arbitrary balance being made between the total quantity of factor activity taken and the specific activity.

Step VI; removal of protamine. Solution CL contained protamine, which was removed by pouring slowly through a 2·5 cm. × 2 cm.^{2} bed of CM-70 cellulose in 160 mM-phosphate buffer, pH 7·8; 90 ml. of effluent (CM) was collected.

Step VII; reduction of salt concentration. This was achieved by the gel-filtration technique of Porath & Flodin (1959). A 30 cm. × 4·5 cm.^{2} column of Sephadex G-50 gel was prepared in 5 mM-potassium phosphate, pH 8·0; the void volume (40 ml.) and inner volume (75 ml.) were determined in a control run with a mixture of inert protein and potassium phosphate. Two successive 45 ml. quantities of solution CM were brought to 5 mM-potassium phosphate and 2 vol. of 55 ml. were collected from the column; these were combined to give solution SF.

Step VIII; chromatography on diethylaminoethylcellulose. The factor in SF was loaded on to a 5 cm. × 1·5 cm.^{2} column of DEAE-cellulose, prepared in 5 mM-potassium phosphate, pH 8·0, by allowing the solution to run through under gravity. The column was washed with 50 ml. of 5 mM-potassium phosphate, pH 8·0.

Stepwise elution of the column with increasing, but relatively varying, concentrations of phosphate and chloride was done with 70 ml. portions of three solutions obtained by mixing volumes of 5 mM-potassium phosphate, pH 8·0 (A) and 0·1 M-K_2HPO_4 in 0·5 M-KCl (B), in the proportions (A:B) of (*a*) 16:3, (*b*) 9:5, (*c*) 9:10. Effluent was collected in fractions of either 5 or 10 ml. with the automatic fraction collector; the smaller fractions were taken when the factor was expected to be eluted. Factor activity was eluted by solution (*c*) as a single peak. Fractions containing factor were pooled to give 20 ml. of solution DF.

Step IX; reduction of salt concentration. This was done with the 30 cm. × 4·5 cm.^{2} column of Sephadex G-50 gel, equilibrated in this case with 50 mM-potassium phosphate, pH 8·0. Effluent (30 ml.), containing purified factor, was collected as solution F and stored at −15°.

RESULTS

Purification of the factor

The overall results of a typical preparation of the purified factor are summarized in Table 1, which shows the specific activity and yield at each step of the procedure.

Table 1. *Purification of a factor containing vitamin* B_{12} *from extract of acetone-dried* Escherichia coli

Stage	Material tested	Volume (ml.)	Total protein (mg.)	Total factor activity (units)	Specific activity (units/mg. of protein)	Overall yield of factor (%)
	Crude extract	127	2530	3210	1·27	100
I	$CaCl_2$ supernatant	110	835	2000	2·39	62
II	pH 5·0 supernatant	108	445	1560	3·51	49
III	pH 4·4 precipitate	27·7	280	1345	4·8	42
IV	Protamine sulphate supernatant	27·5	230	1300	5·6	40
V	Celite 545 eluent, CL	74	1·78	173	97	5·4
VI	CM-70 cellulose eluent, CM	90	1·53	160	104	5·0
VII	First Sephadex gel eluent, SF	110	1·54	160	104	5·0
VIII	DEAE-cellulose eluent, DF	20	0·62	84	136	2·6
IX	Second Sephadex gel eluent, F	30	0·60	93	155	2·9

Acetone-dried organisms. In spite of attempts to produce a uniform starting material by rigid control of the conditions of growth and acetone-drying of the organisms, considerable variation was found in the properties of the acetone-dried powders. Activity varied from 450 to 650 units of factor/g. of acetone-dried powder, though the variation per milligram of extracted protein was somewhat greater (0·7–1·3 unit). Extracts prepared from different powders also showed considerable variation in their content of nucleic acid, as judged by the ratio of the absorption at 280 mμ to that at 260 mμ. These differences are more probably due to unrecognized factors in the acetone-drying procedure rather than in the culturing of the organisms.

Acetone-dried organisms derived from medium to which cobalamin had been added before instead of after autoclaving the other constituents had a much reduced factor content. When the organism was grown with less than the usual 20 μg. of cobalamin/500 ml. of medium, or for 40 hr. instead of 18 hr., the acetone-dried powders had relatively low activity (250–450 units/g.) without any increase in the yield of dried organisms.

Factor B (cobinamide), the analogue of cobalamin with no nucleotide residue, supports the growth of cobalamin auxotrophs of *E. coli* (Ford & Porter, 1953) and is also weakly active in methionine synthesis with ultrasonic extracts of *E. coli* PA15 (Guest, 1960). It was possible that growth on Factor B might yield organisms with increased factor activity since the organism might then synthesize any special form of vitamin B_{12} required; however, replacement of cobalamin in the growth medium by an equal amount of Factor B gave an acetone-dried powder containing only 140 units/g.

Acetone-dried powders of ox liver were also weakly active, containing about 10 units/g. of powder; the specific activity of the extracted protein was about 0·03 unit/mg. and purification of the factor from this source was not therefore undertaken.

Preparation of the extract. Ultrasonic treatment of the aqueous suspension of the acetone-dried powder for a short period gave increased extraction of activity over that obtained by the simpler procedure of Kisliuk & Woods (1960). Although the specific activity of the extract was decreased by 10–30 % by ultrasonic treatment for 75 sec., the yield of extracted material was increased by 1·5- to 2-fold. Treatment for more than 75 sec. resulted in little more activity being extracted and a sharp drop in the specific activity of the extracted material.

Extraction of the powder with acidic or alkaline buffers, or with 1 % cetyltrimethylammonium bromide, was no better than extraction with water. Autolysis or extraction in the presence of butan-1-ol (Morton, 1950) resulted in decreased extraction and specific activity.

Step I; removal of nucleoprotein with calcium chloride. The extract of factor, adjusted to 20 mg. of protein/ml., contained 5–8 mg. of nucleic acid/ml. This was reduced by 70 % in the treatment with $CaCl_2$. The precipitation of nucleic acid was maximum at a final $CaCl_2$ concentration of about 25 mM; the optimum concentration of $CaCl_2$ varied slightly with extracts from different batches of acetone-dried powder and was determined in a pilot experiment with each batch. The procedure also removed 45–70 % of the protein of the extract, with rather variable losses of activity (0–35 %). Considerable and sharp loss of activity occurred if the treatment was carried out at pH values below 5·65, whereas above this value removal of nucleic acid and protein was poor.

Steps II and III; pH *fractionation.* Material precipitated between pH 5·00 and 4·40 contained 10–20 % of the protein of the initial extract and only small amounts of activity were lost in the discarded fractions. Fine control of the pH was necessary for satisfactory fractionation, and prolonged exposure to pH 4·40 resulted in a large loss of activity.

Step IV; treatment with protamine sulphate. The nucleic acid remaining after the $CaCl_2$ treatment was concentrated in the active fraction during steps II and III, but could be almost completely removed by treatment with protamine sulphate at pH 7·0 in 20 mM-potassium phosphate buffer; 15–25 % of the remaining protein was also removed with very small loss of activity.

Step V; chromatography on Celite 545. Addition of an excess of protamine sulphate to the supernatant solution from step IV caused complete precipitation of factor together with a high proportion of the protein. It was possible to extract selectively the factor–protamine complex from the precipitated material with phosphate buffer, pH 7·8, and to chromatograph this on a Celite 545 column. The most satisfactory procedure was that described in the Materials and Methods section, and the results of such a chromatogram are shown in Fig. 2*a*.

The sequence of events on the columns was deduced from a study of protein, protamine and factor distributions obtained when steps in the procedure were omitted, the formation of a precipitate on dialysis overnight against 20 mM-potassium phosphate, pH 7·8, being used as an indication of the presence of protamine.

Addition of protamine sulphate to the step IV supernatant produced a solid phase containing protamine, factor and inert protein, with excess of protamine and inert protein in the liquid phase.

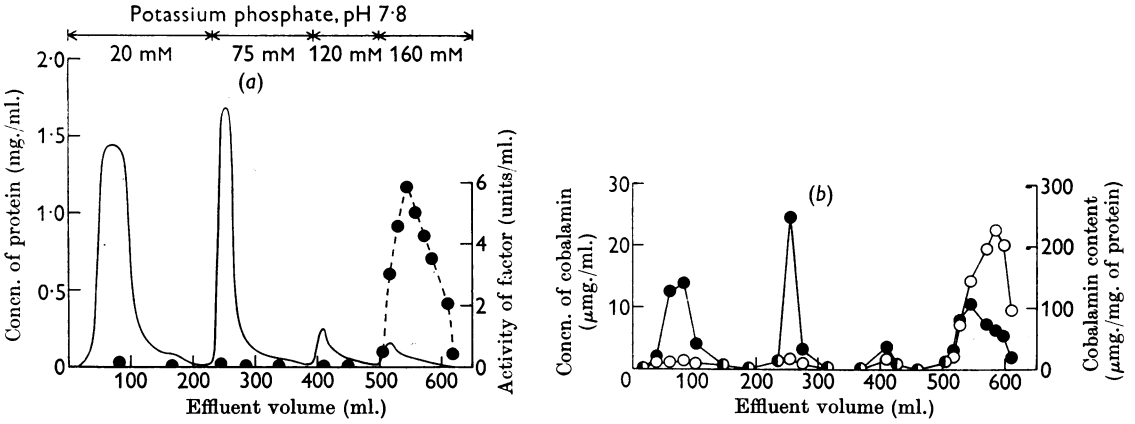

Fig. 2. Chromatography of step IV supernatant fluid on Celite 545. The solution (40 ml.; 27 units/ml.; specific activity, 5·0 units/mg. of protein) was chromatographed as described in the Materials and Methods section. (*a*) Distribution of protein and factor. Protein (—) was estimated for each fraction; points are omitted for clarity. Factor concn., ●--●. (*b*) Distribution of vitamin B_{12}. The vitamin B_{12} content of fractions was measured directly with *E. coli* 113/3 and expressed as μmg. of cobalamin/ml. (●—●) or μmg. of cobalamin/mg. of protein (○).

Before forming the solid phase into a short column a little Celite 545 was used to disperse the protein particles into a bed through which buffer solution could percolate freely. The liquid phase was washed (with 20 mM-potassium phosphate, pH 7·8) through the longer column of Celite 545. This column adsorbed the excess of soluble protamine but very little of the inert soluble protein. Some inert protein and all the factor–protamine complex of the solid phase in the shorter column could be brought into solution with 75 mM-potassium phosphate, pH 7·8; as this ran through the longer column, factor was selectively adsorbed on the protamine-coated Celite 545. The inactive shorter column was then discarded and some inert material washed out of the longer column with 120 mM-potassium phosphate, pH 7·8. The factor–protamine complex was completely eluted with 160 mM-potassium phosphate, pH 7·8.

The bulk of the factor was eluted from the column slightly behind the peak of protein washed out with the 160 mM-potassium phosphate (Fig. 2*a*), and it was necessary to determine its exact location for each chromatogram. Since the factor became increasingly unstable to storage after purification beyond step IV, it was necessary to determine the factor distribution as rapidly as possible. The routine quantitative assay required 3 working days, but an adequate qualitative picture could be obtained by reading the methionine assay with *L. mesenteroides* P 60 after growth for only 18 hr. Alternatively the distribution of vitamin B_{12} activity for *E. coli* 113/3 was used (see below).

The combined active fractions had a specific activity of 80–110 units/mg. They accounted for only 13–60 % of the total activity derived from step IV, the remainder being lost by inactivation during the procedure.

This step could also be performed with a single column of Celite 545 or Celite 535 and a continuous concentration gradient of potassium phosphate buffer. This sometimes gave up to 95 % recovery of activity, with purified material occasionally of specific activity as high as 150 units/mg. However, there was very wide variation (15-fold) in the specific activity obtained and this method was abandoned.

Steps VI and VII; removal of protamine and reduction of salt concentration. Before reduction of the phosphate content of the purified fraction from step V, protamine was conveniently removed by treatment with CM-70 cellulose; 14 % of the protein was removed in this way with negligible loss of activity. Reduction of the phosphate concentration to 5 mM was rapidly achieved with the Sephadex gel-column technique without the loss of activity found with the alternative dialysis procedure.

Step VIII; chromatography on diethylaminoethylcellulose. The protein from step VII was held completely by DEAE-cellulose equilibrated with 5 mM-potassium phosphate, pH 8·0, and could be eluted completely as three separate peaks with a continuous gradient up to 0·1 M-K_2HPO_4–0·5 M-KCl. Only the third peak had factor activity. A stepwise gradient was always used to develop the chromatogram (Fig. 3) since this gave sharper

peaks. The active peak had a constant specific activity of about 130 units/mg. of protein (Fig. 4). Only about 50% of the step VII activity was recovered from the column; this was mainly attributable to the increasing instability of the factor.

Step IX; reduction of salt concentration. The active solution DF from chromatography on DEAE-cellulose contained 55 mM-potassium phosphate and 263 mM-KCl; this concentration of salt was sufficient to cause some inhibition of methionine synthesis in the assay system for the factor. The treatment with Sephadex gel exchanges this salt for 50 mM-potassium phosphate, pH 8·0, without any loss of protein and a slight increase in the specific activity.

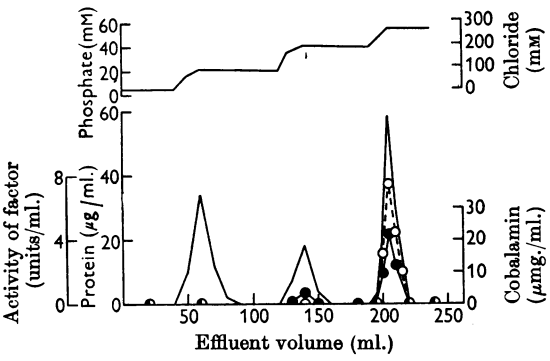

Fig. 3. Chromatography of solution SF on DEAE-cellulose. The solution (110 ml.; 1·45 unit/ml.; specific activity, 104 units/mg. of protein) was chromatographed as described in the Materials and Methods section. The solvent gradient was determined by estimation of the effluent phosphate content according to Fiske & Subbarow (1925). Protein (—) was estimated for each fraction; points are omitted for clarity. Factor concn., ○. The vitamin B_{12} content of fractions was measured directly with *E. coli* 113/3 (●).

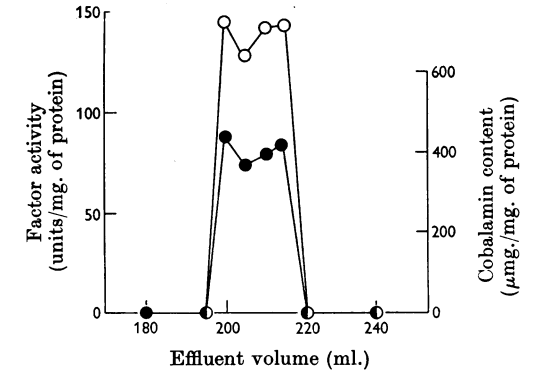

Fig. 4. Active peak of chromatogram on DEAE-cellulose. Conditions were as described for Fig. 3. Specific activity of factor, ○. Vitamin B_{12} content, ●.

The purified factor solution F contained only 3% of the activity extracted initially from the acetone-dried organisms. The specific activity of the product was always 145–155 units/mg., although derived from different batches of acetone-dried powders showing considerable variation in properties.

The ultraviolet-absorption spectrum of the purified preparation has a maximum at 275 mμ and a 280 mμ/260 mμ ratio of 1·6.

At each stage of the purification procedure combinations of fractions were assayed for factor activity but no evidence was found of the resolution of the factor into two components acting synergistically.

Stability of the purified factor

Acetone-dried organisms, crude extracts or preparations purified as far as the end of step IV, retained their activity during storage at −15° for at least 3 months. However, preparations taken beyond step IV became increasingly unstable, the final product having a half-life of about 3 days at −15° (Table 2). Storage in the presence of glutathione, cysteine, 2-mercaptoethanol, potassium ascorbate (all at 5 mM) or disodium ethylenediaminetetra-acetate (1 mM) did not improve the stability. Attempts to concentrate the factor solution (by desalting with Sephadex gel and freeze-drying) in the hope of increasing the stability to storage, resulted in considerable denaturation and loss of activity with the formation of an orange amorphous powder.

Kisliuk & Woods (1960) reported that the factor present in crude extracts was destroyed by heating. This thermolability was shown by the purified factor to an increased extent (Fig. 5). Crude preparations of the factor were reasonably stable to both acid and alkali, but the purified factor showed a sharp pH optimum for stability at pH 8·0–8·5 (Fig. 6).

Factor present in crude extracts or purified by steps I–IV was completely non-diffusible. Dialysis of more purified preparations resulted in loss of

Table 2. *Stability of factor to storage*

Solutions of factor-containing fractions of known activity were stored at −15° for periods varying from 2 days to 3 months. Day-to-day variations in the amounts of methionine formed in the assay system permit only approximate estimations of the half-life of the factor during purification.

Fraction	Half-life of factor activity
Acetone-dried organisms; crude extract	> 3 months
Step IV supernatant	3 months
Step V solution CL	9 days
Step VII solution SF	6 days
Step IX solution F	3 days

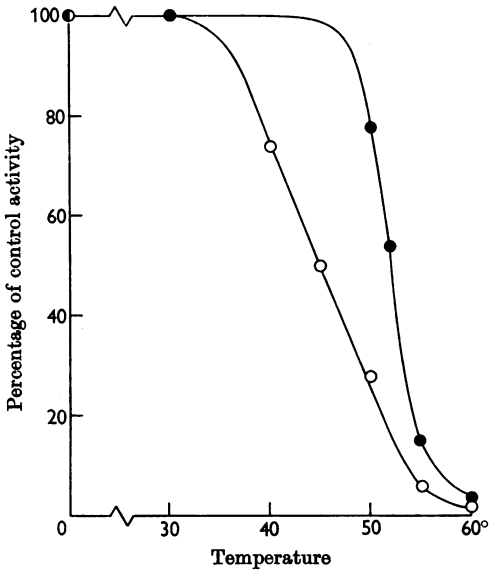

Fig. 5. Effect of heat on the factor. Samples (3 ml.) of crude factor (specific activity, 1·8 unit/mg. of protein; 60 units/ml. of 50 mM-potassium phosphate buffer, pH 7·8) or purified factor (specific activity, 143 units/mg. of protein; 5 units/ml. of 50 mM-potassium phosphate buffer, pH 8·0) were heated rapidly from 0°, maintained for 5 min. at a fixed temperature and cooled rapidly to 0°. Results are expressed as percentage of factor activity of control tube maintained at 0°. Crude factor, ●; purified preparation, ○.

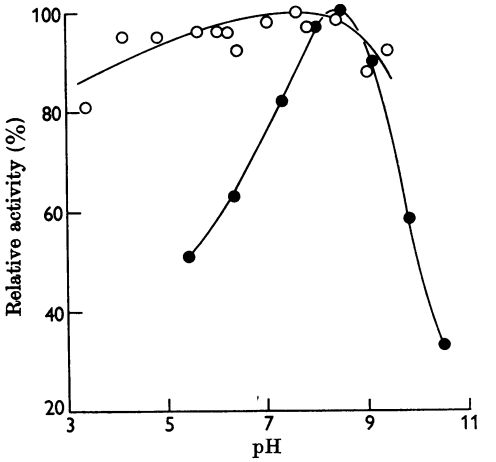

Fig. 6. Effect of pH on the stability of the factor. Samples (1·8 ml.) of crude factor (specific activity, 2·3 units/mg. of protein; 23·5 units/ml. of 50 mM-potassium phosphate, pH 7·8) were adjusted to the pH shown by addition of 0·2 M-sodium borate (pH range 7·6–9·4), 0·2 M-potassium phosphate (pH range 6·1–7·8) or 0·2 M-sodium citrate (pH range 3·4–6·2). Samples (2·5 ml.) of purified factor (139 units/mg. of protein; 3·6 units/ml. of 50 mM-potassium phosphate, pH 8·0) were adjusted to the pH shown with acetic acid or KOH. After keeping for 40 min. at 0° the pH was checked and readjusted to 7·8 with 0·5 M-potassium phosphate, pH 7·8, before assay of factor. Results are expressed as a percentage of the maximum activity; crude factor, ○; purified preparation, ●.

activity. For example, dialysis of solution CM from step VI against 5 mM-potassium phosphate, pH 8·0, at 0° for 12 hr. caused 60% inactivation; dialysis of solution DF from step VIII against the same buffer at 0° for 15 hr. resulted in 90% inactivation. Since there was no concurrent loss of vitamin B_{12} activity, the loss of factor activity in these cases is more probably a reflexion of the increased lability of the purified material to heat and storage, rather than to the factor having become diffusible. Addition of cobalamin, 5:6-dimethylbenzimidazolylcobamide coenzyme or extract of heated *E. coli* (prepared according to Guest, 1960) did not restore the activity of purified preparations partially inactivated by dialysis.

Cobalamin content of the factor

Assays for vitamin B_{12} with either or both *Euglena gracilis* and *Escherichia coli* 113/3, were performed on all the fractions produced by the purification procedure. There was little correlation between vitamin B_{12} activity and factor content of fractions obtained in steps I–IV (Table 3); there were also variable losses of vitamin B_{12} on dialysis, under conditions where there was no loss of factor.

However, examination of the distribution of vitamin B_{12} in the fractions obtained in the Celite 545 chromatogram showed that, although all the protein-containing fractions contained vitamin B_{12}, the peak of factor activity was associated with protein containing a much higher proportion of vitamin B_{12} (Fig. 2, compare *a* and *b*). With cyanocobalamin as standard, the same picture of vitamin B_{12} distribution was obtained with either assay organism and was not altered by prior dialysis of the fractions against 50 mM-potassium phosphate, pH 7·8. Inactive protein from the column contained 5–20 μmg. of cobalamin/mg. of protein, and the active peak contained up to 230 μmg./mg. of protein.

The cobalamin content of the active material was not reduced by treatment with CM-70 cellulose (step VI) or with Sephadex gel (step VII). This latter treatment would be expected to remove any vitamin B_{12} activity due to relatively small molecules, such as free cobalamin or simple derivatives of it.

Vitamin B_{12} activity was almost entirely associated with factor activity in the DEAE-cellulose chromatogram (Fig. 3). The third peak had uniform proportions of protein, factor and vitamin B_{12}

Table 3. *Cobalamin content of the factor at various stages of purification*

The cobalamin content of fractions was assayed with *Euglena gracilis* or *Escherichia coli* 113/3. Where indicated, samples were first dialysed as detailed in the Materials and Methods section for the assay of factor activity: +, dialysed; −, not dialysed.

Assay organism	Purification step	Dialysis	Cobalamin content (μmg./mg. of protein)		Factor content (units/mg. of protein)		Cobalamin/factor (μmg./unit)	
			Precipitate	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant
Euglena gracilis	Crude extract	+	.	14·5	.	1·15	.	12·6
	I	+	7·3	16·6	0·25	1·45	29·0	11·4
	II	+	11·0	17·8	1·10	1·55	10·0	11·5
	III	+	20·5	16·7	3·35	0·02	6·1	835
	IV	+	7·6*	29·6	1·30*	4·70	5·8*	6·3
Escherichia coli 113/3	IV	+	.	16·8	.	5·0	.	3·4
	V	−	.	185	.	105	.	1·8
	VI	−	.	200	.	105	.	1·9
	VII	−	.	200	.	104	.	1·9
	VIII	−	.	400	.	130	.	3·1
	IX	−	.	425	.	143	.	3·0

* Calculated by difference.

Table 4. *Cobalamin content of the purified factor*

Cobalamin assays were with samples of purified factor with a specific activity 143 units/mg. of protein. Samples were (i) autoclaved directly in growth medium, (ii) added unheated to autoclaved medium (after treatment with 2 vol. of ethanol and sterile dilution for the *Euglena gracilis* assay), (iii) digested for 24 hr. at 27° with crystalline papain (1 mg./mg. of protein of factor) in 0·25 M-potassium phosphate, pH 8·0, containing 1 mM-disodium ethylenediaminetetra-acetate and 5 mM-cysteine, and (iv) heated at 100° for 30 min. in a sealed tube with 0·1 M-sodium acetate buffer, pH 4·5, containing 0·1 mM-KCN.

Treatment of samples	Cobalamin content of factor (μmg./mg. of protein) assayed with *Escherichia coli* 113/3	Cobalamin content of factor (μmg./mg. of protein) assayed with *Euglena gracilis*
(i) Autoclaved in medium	425	550
(ii) Not autoclaved in medium	94	550
(iii) Digested with papain	495	510
(iv) Extracted with HCN	575	560

activity (Fig. 4). These results are strong evidence that the factor is a derivative of vitamin B_{12}.

The cobalamin content of fractions was usually determined after autoclaving a sample in the growth medium of the assay organism (i.e. at pH 6·8 or 4·5). If the factor was assayed (*E. coli* 113/3) unheated, the apparent cobalamin content was only one-quarter of that when it was heated in the medium (Table 4). Presumably the vitamin B_{12} bound in the factor is largely, or even completely, unavailable to *E. coli* 113/3, but is rendered available by heating.

The purified factor was also digested with crystalline papain; this treatment released slightly more vitamin B_{12} than autoclaving (Table 4). After heating the factor at 100° with HCN the *E. coli* 113/3 assay showed a still higher value (Table 4).

With the *Euglena gracilis* assay treatment of the solution DF with 2 vol. of ethanol before sterile dilution and assaying without heating gave the same cobalamin content as that obtained by heating in the medium (Table 4). This may represent release of vitamin B_{12} activity either by the ethanol or during the long growth period of *Euglena gracilis* rather than reflecting the activity of the native factor for this organism. Digestion with papain or heating with HCN as described above gave essentially similar cobalamin values with *Euglena gracilis* (Table 4).

The crude extracts of acetone-dried organisms contained 15 μmg. of non-diffusible cobalamin/mg. of protein, assayed with *Escherichia coli* 113/3; no attempt was made to release bound vitamin B_{12} except by the autoclaving of the samples in the growth medium. Thus the factor can account for only 16% of the bound cobalamin of the initial extracts. This result is to be expected since the organisms were grown in the presence of a higher concentration of cyanocobalamin than is required to satisfy the vitamin B_{12} requirement of *E. coli* auxotrophs (Davis & Mingioli, 1950).

Further properties of the factor

Effect of proteolytic enzymes. Digestion of the purified factor with crystalline papain caused complete loss of activity (Table 5). Similar results were obtained with crystalline trypsin.

Effect of cyanide. Factor purified as far as the end of step V was exposed to various concentrations of cyanide, which was subsequently removed

Table 5. *Digestion of purified factor by papain and trypsin*

In Expt. 1 a sample (3 ml.) of purified factor (20 μg. of protein/ml.; specific activity 155 units/mg. of protein) was digested for 3 hr. at 25° with crystalline papain (10 μg./ml.) in 50 mM-potassium phosphate, pH 8·0, containing 1 mM-disodium ethylenediaminetetra-acetate and 5 mM-cysteine. In Expt. 2 a second sample (3 ml.) was digested for 1 hr. at 25° with crystalline trypsin (20 μg./ml.) in 50 mM-potassium phosphate, pH 8·0. Samples (0·6 ml.) of digested factor were taken for assay. Controls were (i) samples incubated in the absence of proteolytic enzyme, and (ii) and (iii), proteolytic enzyme added in the assay of the factor to tubes with and without 0·6 ml. of factor solution. All values are corrected for a L-methionine content (30 μm-moles) of a tube, containing the digested factor and the assay system, heated at 100° for 3 min.

Expt.	Addition to assay system	Total L-methionine formed (μm-moles/3 hr.)	L-Methionine formed corrected for that formed by proteolysis during factor assay (μm-moles/3 hr.)
1	Papain-digested factor	63	6
	Undigested factor	238	238
	Undigested factor + papain	277	220
	Papain alone	57	.
2	Trypsin-digested factor	88	10
	Undigested factor	274	274
	Undigested factor + trypsin	332	254
	Trypsin alone	78	.

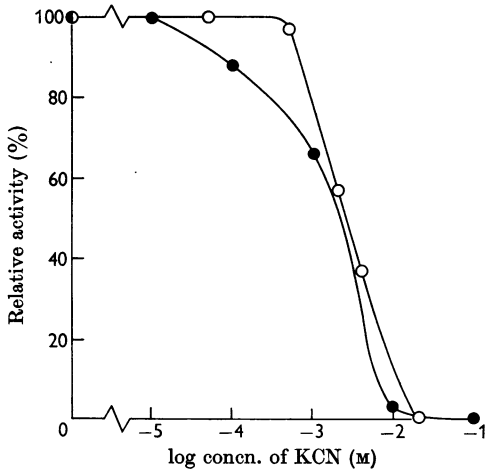

Fig. 7. Effect of cyanide on the factor. In Expt. 1 (●) samples (3·0 ml.) of purified factor (specific activity, 135 units/mg. of protein; 5·4 units/ml. of 50 mM-potassium phosphate, pH 8·0) were treated with KCN (brought to pH 8·0 with HCl) at 22° for 30 min. Cyanide was removed by dialysis for 13 hr. against five changes of 50 mM-potassium phosphate, pH 8·0, before assay of factor. In Expt. 2 (○), the KCN was added directly to the assay system for the factor.

by dialysis. Loss of activity varied with the concentration of cyanide (Fig. 7), 2·2 mM-KCN causing 50 % inactivation. Similar concentrations of KCN also inhibited the activity of the factor in catalysing the synthesis of methionine by the assay system; 2·5 mM-KCN was required for 50 % inhibition (Fig. 7).

Effect of light. Irradiation for 1 hr. of a purified factor preparation (containing 157 units/mg. in 0·1 M-potassium phosphate, pH 8·0) with light from a 100 w tungsten lamp, placed 25 cm. distant, was without effect on the activity. When irradiated in a silica cuvette placed 25 cm. in front of a u.v. lamp (7 w, principal emission at 2537 Å), the solution lost 67 % of its activity compared with a non-irradiated control sample. This loss of activity, or that caused by KCN, was not restored by cobalamin, 5:6-dimethylbenzimidazolylcobamide coenzyme or an extract of heated organisms.

Charcoal treatment. No activity was lost by the most purified preparation (solution F) when a sample (3 ml.) was stirred for 20 min. with 1·5 mg. of ground and acid-washed Nuchar-C activated charcoal (West Virginia Pulp and Paper Co., New York, N.Y., U.S.A.) and the charcoal removed by centrifuging.

Treatment of crude extracts of the factor with activated charcoal according to the method of Barker *et al.* (1960*a*) caused 27 % inactivation.

DISCUSSION

The factor when purified 150-fold is non-diffusible and unstable to heat; it also shows an instability towards both acid and alkali, being most stable in the pH range 8·0–8·5. Treatment of the purified factor with the proteolytic enzymes papain and trypsin causes complete loss of activity. These facts, together with the 280 mμ/260 mμ absorption ratio of 1·6, indicate that the factor activity is associated with a protein. Preparations of ribonucleic acid made from the crude extracts by the method of Kirby (1956) had no activity.

The factor can promote the formation of at least five times its own weight of L-methionine in the assay system used and so, on the basis of the

definition given by Dixon & Webb (1958), it is probably an enzyme concerned in the synthesis of methionine by the system studied.

The factor responds to purification techniques which have been applied to enzymes, e.g. isoelectric precipitation and chromatography on DEAE-cellulose. Less efficient purification could also be obtained by fractionation with ammonium sulphate or acetone, or by selective adsorption on calcium phosphate or alumina Cγ gels (unpublished observations). The use of calcium chloride to remove nucleoprotein from the crude extracts is a novel procedure which was found preferable to the use, at this stage, of protamine sulphate, manganese chloride or cetyltrimethylammonium bromide. The chromatography of the factor on Celite 545 with protamine sulphate is also novel. Partition chromatography of enzymes on Celite adsorbents has usually met with little success (Porter, 1955), but a similar procedure to that described here has been used successfully in the fractionation of deoxyribonucleic acids (Brown & Watson, 1953).

The instability of the purified factor has so far prevented the production of preparations sufficiently concentrated to permit studies on its homogeneity or the investigation of its absorption spectrum in the visible region.

The present results provide evidence that vitamin B_{12} activity is closely associated with the factor, i.e. that the latter is a vitamin B_{12}-containing enzyme. The form in which vitamin B_{12} is present is not yet known. Relative to cyanocobalamin, the intrinsic vitamin B_{12} content is largely unavailable for *E. coli* 113/3 but is present in some combined form from which it can be released by heating, papain digestion or extraction with hydrogen cyanide. As well as facilitating the extraction of vitamin B_{12}, hydrogen cyanide probably protects it during autoclaving by converting it into the stable cyano forms (Ford, 1953). The intrinsic vitamin B_{12} of the factor may be completely available to *Euglena gracilis*. The material obtained after autoclaving at pH 4·5 is as effective for this organism as that released by papain digestion or cyanide extraction.

The minimum measured vitamin B_{12} content of the factor (1·9 μmg. of cobalamin/unit) corresponds to a 'turnover number' of the vitamin in methionine synthesis of about $4{\cdot}6 \times 10^2$ moles of L-methionine/mole of cobalamin/min. in the factor assay system. However, the significance of this value is dubious since (*a*) the true vitamin B_{12} content may not be that measured on the assumption that the vitamin is present as cobalamin, and (*b*) the absolute amounts of L-methionine produced in the factor assay system vary for any given amount of factor.

Several coenzyme forms of the cobalamins have been obtained from *Clostridium tetanomorphum* and *Propionibacterium shermanii* for the formation of β-methylaspartic acid from glutamic acid in cell-free extracts (Weissbach, Toohey & Barker, 1959; Barker *et al.* 1960*a*, *b*). A coenzyme form of vitamin B_{12} has also been shown to act in the isomerization of succinyl-coenzyme A to methylmalonylcoenzyme A by cell-free extracts of *P. shermanii* (Stadtman, Overath, Eggerer & Lynen, 1960) or of mammalian tissues (Gurnani, Mistry & Johnson, 1960; Stern & Friedman, 1960; Marston, 1960). Guest & Woods (1960) found that the 5:6-dimethylbenzimidazolylcobamide coenzyme was more effective than cobalamin in the synthesis of the present factor by ultrasonic extracts of *E. coli* PA 15, but it would not replace the activity of the factor in promoting methionine synthesis in the presence of tetrahydropteroylglutamic acid. It seems possible that the factor is an enzyme containing a cobamide coenzyme (or a derivative) as prosthetic group.

In support of this hypothesis, the factor is destroyed by ultraviolet light and inactivated by cyanide ion, as are the cobamide coenzymes. However, it is not affected by light from a tungsten lamp, nor by treatment with activated charcoal as one might expect from the results of Barker *et al.* (1960*a*).

No evidence has been presented here for the nature of the function of the factor in methionine synthesis. The possibilities have been discussed fully by Kisliuk & Woods (1960) and Guest *et al.* (1960). It is difficult to envisage a mechanism for the action of cobalamin and its derivatives common to all the enzyme systems *in vitro* which have been studied.

SUMMARY

1. *Escherichia coli* strain PA 15 contains, when grown in the presence of cobalamin, a factor which enables tetrahydropteroylglutamic acid to serve as the C_1-transfer cofactor for methionine synthesis by extracts of acetone-dried organisms grown without cobalamin. A method is described for the assay of this factor and the preparation of extracts rich in it.

2. The purification (150-fold) of the factor is described. It includes removal of nucleoprotein with calcium chloride, pH fractionation, column chromatography on Celite in the presence of protamine and chromatography on diethylaminoethylcellulose.

3. The factor is non-diffusible and when purified is unstable to heating and to storage even at low temperatures. It is most stable at pH 8·0–8·5.

4. The protein nature of the factor is indicated by the 280 mμ/260 mμ absorption ratio of 1·6 and the loss of activity on digestion with trypsin and papain.

5. During the later stages of purification the factor is closely associated with bound vitamin B_{12}. Chromatography on diethylaminoethylcellulose produces an apparently homogeneous peak with a constant ratio of factor:protein:vitamin B_{12}.

6. The vitamin B_{12} of the factor is largely unavailable for *Escherichia coli* strain 113/3 (a cobalamin or methionine auxotroph) but can be released by heating, digestion with papain or extraction with hydrogen cyanide.

7. The purified factor is destroyed by cyanide. Irradiation with ultraviolet light inactivates the factor, but irradiation with visible light or treatment with activated charcoal is without effect.

8. Since the factor is a protein and it acts catalytically and contains vitamin B_{12}, it is suggested that it is an enzyme with a prosthetic group related to cobalamin and concerned in the conversion of homocysteine into methionine under the conditions studied.

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