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Cobalamin and the Enzymic Formation of a Factor Concerned in the Synthesis of Methionine by *Escherichia coli*

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The formation of methionine from homocysteine and serine by extracts of acetone-dried *Escherichia coli* PA 15 (a serine- or glycine-requiring auxotroph) is dependent on the presence of some form of folic acid for the transfer of the one-carbon-atom unit and this can be supplied by an extract of the heated organism (Cross & Woods, 1954; Szulmajster & Woods, 1960). (The term folic acid is used in this paper in general reference to the family of compounds; specific members will be referred to by the names in common use, e.g. tetrahydropteroylglutamic acid.) Tetrahydropteroylglutamic acid could not replace this heated extract unless the source of enzyme was organisms which had been harvested from a medium containing cobalamin (Kisliuk & Woods, 1957, 1960). However, enzyme-containing extracts prepared by ultrasonic disruption of the same organism were able to use tetrahydropteroylglutamic acid if free cobalamin was added to the reaction mixture; with an extract of heated *E. coli* as source of folic acid no cobalamin was required (Guest, Helleiner, Cross & Woods, 1960). The synthesis of methionine by ultrasonic extracts of *E. coli* strain 121/176 (a cobalamin or methionine-requiring auxotroph) depended on the presence of cobalamin when either tetrahydropteroylglutamic acid or extract of heated *E. coli* was used as source of folic acid (Helleiner, Kisliuk & Woods, 1957; Guest *et al.* 1960).

A heat-labile and non-diffusible material was partially purified from acetone-dried powders of strain PA 15 cultured in the presence of cobalamin; this material (hereinafter called X) enabled tetrahydropteroylglutamic acid to be used as the co-factor for C_1 transfer in methionine synthesis by extracts of acetone-dried powders of the same organism grown in the absence of cobalamin (Woods, 1958; Kisliuk & Woods, 1960).

It appeared possible that ultrasonic extracts were able to form X in the presence of cobalamin, and that treatment of the organism with acetone destroyed an enzyme or enzymes required for its formation. Indirect evidence for the formation of X by ultrasonic extracts was also obtained by using inhibitory analogues of cobalamin. The action of cobalamin in methionine synthesis was inhibited by the anilide of the monocarboxylic acids of cobalamin, but with extracts of organisms grown in the presence of cobalamin this was not the case (Guest, 1960).

In this paper direct evidence is given for the formation of X by ultrasonic extracts together with some properties of the system. Brief accounts of this work have been published (Guest, 1959; Woods, 1958).

Towards the end of this work, the existence of a cobalamin-containing enzyme, forming part of the complex of enzymes catalysing the transfer of C-3 of serine to homocysteine by a cobalamin-requiring auxotroph of *E. coli*, was reported (Hatch, Takeyama & Buchanan, 1959).

METHODS AND MATERIALS

Extracts of Escherichia coli

Organisms. The strain (PA 15) of *E. coli* used for most of the work was an auxotroph requiring serine or glycine for growth. Strain 121/176 (Davis & Mingioli, 1950) required cobalamin or methionine, whereas strain 3/62 required methionine and did not respond to either cobalamin or homocysteine; the latter strain was isolated from *E. coli* 518 (ATCC 9723) in this Laboratory by the technique of Adelberg & Meyers (1953). All strains were maintained on tryptic meat–agar slopes, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

Ultrasonic extracts. These were used mainly as source of enzyme in studies of the formation of X. Their preparation was as described by Guest *et al.* (1960); for strains 121/176 and 3/62 the original growth medium was supplemented with 0·5 mM-DL-methionine.

Extracts of acetone-dried organisms. These were used mainly in the assay of X. Large-scale production of acetone-dried *E. coli* PA 15 was as described by Szulmajster & Woods (1960). For a few experiments the organism was grown in the presence of cobalamin; in this case the vitamin (13 μm-moles) was added with the inoculum to the surface of each tray containing 500 ml. of solidified medium.

The acetone-dried organisms were extracted by mixing with water (10 ml./g. of powder) in a Potter–Elvehjem homogenizer. After centrifuging (25 000 *g*, 45 min.) at 0° the supernatant fluid was dialysed for 18 hr. at 3° against 0·05 M-phosphate buffer, pH 7·8 (750 ml./g. of dried organism); the dialysis fluid was changed once. The product was the extract of acetone-dried organisms normally used. In some experiments the procedure was modified by subjecting the homogenized material to

ultrasonic vibration (25 kcyc./sec., 2·5 min. at 600 w with an ultrasonic generator type E7590B of Mullard Ltd., London) before centrifuging and dialysing. Such extracts were only used with organisms grown without cobalamin.

Extract of heated E. coli. This was prepared as described by Guest *et al.* (1960).

General. Enzymic extracts were sometimes treated with Dowex-1 resin (chloride form, ×8, 100–200 mesh) as described by Kisliuk & Woods (1960). Extracts have been stored at −14° for up to 4 weeks with only moderate loss of activity, though they were normally used immediately after preparation. Their protein content was estimated spectrophotometrically according to Layne (1957).

Formation of the factor

Ultrasonic extracts of organisms were incubated with cobalamin in reaction mixture *A*, which contained (per ml.): glucose, 15 μmoles; adenosine triphosphate (ATP), 7·5 μmoles; $MgCl_2$, 7·5 μmoles; diphosphopyridine nucleotide (DPN), 0·75 μmole; potassium phosphate buffer, pH 7·8, 167 μmoles. The concentration of ultrasonic extract used was equivalent to 7·5 mg. of protein/ml. and the reaction was carried out in a final volume of 3–6 ml. under an atmosphere of H_2 at 37°. In early experiments the concentration of cobalamin was 50 μμmoles/ml. and incubation was for 3–4 hr. Later the concentration of the vitamin was increased to 0·5 μm-mole/ml. and the time of incubation decreased to 45 min. The reaction was stopped by cooling the mixture to 0°.

Assay of the factor

During the first part of the work the method used was based on the work of Kisliuk & Woods (1960). A modified method was developed during the present work and will be described in the Results section.

Separation of the factor. Partly purified material, containing the bulk of the X formed during the reaction, was obtained by treatment with protamine sulphate. This reagent (2%; 0·1 ml./ml. of reaction mixture) was stirred in and the mixture stood for 20 min. at 0° before centrifuging at 25 000 *g* for 5 min. The residue (first protamine precipitate, P1) was normally discarded and the supernatant fluid dialysed against 0·05 M-phosphate buffer pH 7·8 for 18 hr. at 3° (150 ml. of buffer/ml. of original reaction mixture); the dialysis fluid was changed twice. During the dialysis a fine white precipitate formed (second protamine precipitate, P2). After centrifuging (15 000 *g*, 10 min.) P2 was suspended in half the original reaction volume of 0·2 M-phosphate buffer, pH 7·8.

Estimation. Determination of the content of X in P2 depended on its ability to promote methionine synthesis by extracts of acetone-dried organisms (cultured without cobalamin) in a system containing tetrahydropteroylglutamic acid as cofactor for the C_1 transfer (Kisliuk & Woods, 1960). The samples used were generally equivalent to 6 mg. of the protein of the ultrasonic extract used to produce X.

The reaction mixture (*B*) used to follow the synthesis of methionine contained (in 2·2 ml. final volume): DL-homocysteine, 10 μmoles; L-serine, 10 μmoles; fructose 1:6-diphosphate, 15 μmoles; ATP, 10 μmoles; $MgSO_4$, 10 μmoles; DPN, 1 μmole; pyridoxal phosphate, 1 μmole; tetrahydropteroylglutamic acid, 1 μmole; potassium phosphate buffer, pH 7·8, 250 μmoles. The enzyme system (dialysed extract of acetone-dried organisms) was added in an amount equivalent to 10 mg. of protein (normal extract) or 20 mg. of protein (ultrasonic-treated extract). Incubation was for 3 hr. at 37° in an atmosphere of H_2. After heating at 100° for 3 min. to stop the reaction, the precipitated protein was removed by centrifuging, and the supernatant fluid assayed for methionine, by the microbiological method of Gibson & Woods (1960), with *Leuconostoc mesenteroides* P60.

In each assay there were controls in which the enzyme was incubated with and without cobalamin in reaction mixture *B* in the absence of a sample containing X; the values for methionine obtained were deducted from the experimental values. Controls in which the samples containing X were incubated in reaction mixture *B* without the extract of acetone-dried organisms were also carried out as a routine.

Expression of results. The activity of the enzymic extract used for assaying X varied slightly from batch to batch and the conditions for the assay were not completely reproducible; it was not possible therefore to define an absolute unit for X. Each complete experiment included a sample of X formed under standard conditions, and this was titrated for its effect on methionine synthesis; the titration of other samples was compared with this. The arbitrary unit of activity of X chosen was that amount which caused the synthesis of 0·1 μmole of L-methionine in a particular assay. In some experiments the results are recorded simply as the total amount of methionine (μm-moles) formed.

Assay of cobalamin

The method described by Kisliuk & Woods (1960) with *Euglena gracilis* var. *bacillaris* was adopted.

Chemicals

The source of cobalamin was 'Anacobin' (British Drug Houses Ltd., Poole, Dorset). The methylamide, ethylamide and anilide derivatives of the monocarboxylic acids of cobalamin (Smith, Parker & Gant, 1956) were kindly provided by Dr E. Lester Smith, who also gave the sample of Factor B (the analogue of cobalamin lacking the nucleotide residue). 5:6-Dimethylbenzimidazolylcobamide-coenzyme was a gift from Dr H. A. Barker; precautions were taken to exclude light in experiments with this compound. The cobalamins were all used as aqueous solutions and were assayed spectrophotometrically by the methods quoted by Guest (1960).

Protamine sulphate (from herring roe) was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and fructose 1:6-diphosphate from British Drug Houses Ltd., Poole, Dorset. Other chemicals used and their sources have been described by Guest *et al.* (1960).

RESULTS

Trial experiments showed that if ultrasonic extracts were first incubated with cobalamin in the presence of glucose, ATP, Mg^{2+} ions, DPN and phosphate, then the initial rate of synthesis of methionine, on subsequent addition of homocysteine, serine and tetrahydropteroylglutamate, was double that attained when cobalamin was

omitted from the first incubation but was present during the second. This suggested that metabolism of cobalamin led directly or indirectly to the production of material which was more immediately active than cobalamin itself in promoting the utilization of tetrahydropteroylglutamate as cofactor for the C_1 transfer.

Production and properties of the factor

Synthesis by ultrasonic extracts. The assay of X depends upon its ability to promote synthesis of methionine by extracts of acetone-dried organisms when the form of folic acid supplied is tetrahydropteroylglutamate; free cobalamin is not active. In order to obviate the possibility of X being formed during the assay procedure it was first necessary to separate the X formed by the ultrasonic extracts as far as possible both from the enzymes concerned in its formation and from free cobalamin; it was also desirable to remove from the samples other enzymes which participate in the conversion of serine and homocysteine into methionine. This was achieved by treatment of the reaction mixture with protamine sulphate as described in the Methods section.

With relatively small samples of partly purified X (second protamine precipitate, P 2) synthesis of methionine in the assay system showed a linear relationship to the size of the sample (Fig. 1), though with larger samples a proportionate increase was not obtained; possibly some other factor or enzyme becomes rate-limiting. A satisfactory assay of X was obtained by using the range over which the response was linear. Samples equivalent to 4·5 or 6 mg. of protein of the original ultrasonic extract were normally used; these caused the synthesis of less than 1 μmole of L-methionine.

The addition of free cobalamin to the assay system had no significant effect (Fig. 1). There was a small but significant formation of methionine in the control in which cobalamin had been omitted from the system for synthesis of X but added to the assay system (Fig. 1). The validity of the assay was not affected by this since free cobalamin in the samples would have been largely removed by dialysis in the course of their preparation. It is probable that the treatment with protamine did not completely remove the enzymes concerned in the formation of X and that this proceeds slowly during the assay procedure if sufficient free cobalamin is present. The partly purified samples of X did not cause the synthesis of any methionine in the assay system if the enzyme preparation (extract of acetone-dried organisms) was omitted. There was no synthesis of X during its separation from the reaction mixture. This was shown by adding cobalamin immediately after incubation to a control reaction mixture not containing the vitamin and isolating the P 2 fraction; it had less than 5% of the activity of a similar fraction isolated after incubation with cobalamin.

The first protamine precipitate (P 1, see Methods section) and the supernatant fluid remaining after removing P 2 had no activity, nor did they affect the activity of P 2. Furthermore, the activity of P 2 was apparently the same as that of an equivalent quantity of the reaction mixture which had simply been dialysed. It is probable therefore that all of the X is precipitated with the P 2 fraction. When the suspension of P 2 in 0·2 M-potassium phosphate buffer pH 7·8 was centrifuged, all the activity was recovered in the supernatant fluid. An overall purification of X of four- to five-fold was achieved during its separation; this was shown by dry-weight determinations after exhaustive dialysis of the fractions against water.

It is concluded, from the above experiments, that incubation of cobalamin with ultrasonic extract, in a suitable reaction mixture, leads to the production of material with similar biological activity to that extracted by Kisliuk & Woods (1960) from acetone powders of organisms grown in the presence of cobalamin.

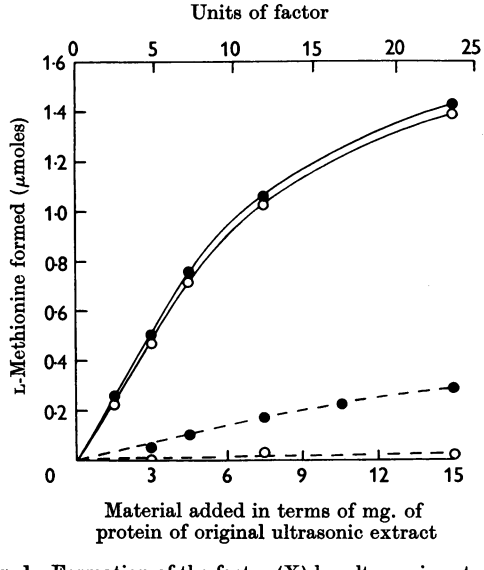

Fig. 1. Formation of the factor (X) by ultrasonic extracts. Reaction mixture *A*, with cobalamin (50 $\mu\mu$moles/ml.) present (—) or absent (- - -), was incubated for 4 hr. The reaction mixtures were then treated to obtain the second protamine precipitates (P 2) and the latter assayed for 'X' by using reaction mixture *B* with normal extract of acetone-dried organisms equivalent to 10 mg. of protein and with cobalamin (65 $\mu\mu$moles) present (●) or absent (○); incubated 3 hr. Units of factor activity were deduced from the curve ○—○.

Other properties of the factor. These tests were carried out with the partly purified P 2 fraction. The cobalamin content as measured with *Euglena gracilis* was 7 % of the total cobalamin added to the original reaction mixture. For a sample of the size used for assay this corresponds to only about 2 $\mu\mu$moles of cobalamin compared to the 65 $\mu\mu$-moles added to the assay system in the controls of the experiments of Fig. 1. It is not known whether the cobalamin in fraction P 2 is free or bound; the fraction had, however, been extensively dialysed.

The activity of X was not decreased by dialysis for 48 hr. against 0·05 M-potassium phosphate buffer pH 7·8. It was heat-labile; 70 % of the activity was lost after 5 min. at 50° and 100 % after 5 min. at 60°. The preparations of X were unstable at pH values below 5 and above 8·5; for example, half the activity was lost in 30 min. at 0° at either pH 4 or 9·8. These properties are again in accord with those of the material separated from cobalamin-grown organisms by Kisliuk & Woods (1960) and suggest that X is a protein or at least closely associated with protein.

Effect of analogues of cobalamin

Certain analogues of cobalamin inhibit the action of cobalamin in promoting methionine synthesis by ultrasonic extracts when tetrahydropteroylglutamate is the cofactor for the C_1 transfer. However the anilide analogue does not inhibit methionine synthesis if the enzymic extracts are prepared from organisms grown with cobalamin, though synthesis was reduced if the analogue as well as cobalamin was added to the growth medium (Guest, 1960). These results suggested that the analogues may interfere with the formation of X, though not with its activity once formed.

Effect of analogues of cobalamin on the activity of the factor. The anilide of the monocarboxylic acids of cobalamin had no effect on the activity of the partly purified preparation of X derived from the incubation of cobalamin with ultrasonic extract (Table 1). A more extended series of tests was carried out with a crude preparation of X derived from acetone-dried organisms grown in the presence of cobalamin (Kisliuk & Woods, 1960). The amount of the preparation used itself catalysed the formation of negligible quantities of methionine and contained less than 15 $\mu\mu$moles of cobalamin. The anilide analogue again had no effect on the activity of the X (Table 1); this was also the case with the methylamide and ethylamide analogues. Factor B (the analogue of cobalamin containing no nucleotide residue) did however inhibit about 40 % at relatively high concentrations; the inhibition (by 70 μm-moles) was not relieved by cobalamin (6·5 μm-moles). The inactivity of the anilide analogue was not due to its destruction in the test system. Furthermore, in controls in which X was omitted the analogues did not themselves promote methionine synthesis in the assay system.

Effect of analogues of cobalamin on the formation of the factor. All the analogues tested inhibited the formation of X completely if sufficiently high concentrations were used (Table 2). The ratios of concentration of cobalamin to analogue resulting in 50 % inhibition were similar to those reported by Guest (1960) for the inhibition of the activity of cobalamin in methionine synthesis. The analogues themselves, when incubated with ultrasonic extracts, did not give rise to significant X activity (Table 2). Only in the case of Factor B was it possible that a carry-over of the test substance into the final assay system could vitiate the results by inhibiting the function of X itself (see last paragraph): it is unlikely, however, that inhibitory concentrations would remain after the treatment, with protamine sulphate and dialysis, used in preparing the samples for assay.

The conclusion that the anilide analogue inhibited the formation of X, though not its subsequent activity, was supported by the experiment of Table 3. Cobalamin was required for the synthesis of methionine by ultrasonic extracts with tetrahydropteroylglutamate as the cofactor for the C_1 transfer, and such synthesis was completely inhibited by the anilide analogue. A preparation of X replaced cobalamin, but in this case the anilide analogue was not inhibitory. For this experiment a more purified preparation of X derived from acetone-dried organisms grown with cobalamin was used (M. A. Foster & K. M. Jones, unpublished work in this Laboratory); it had a higher ratio of

Table 1. *Effect of analogues of cobalamin on the activity of the factor*

Source of factor. (I) Second protamine precipitate, P 2, after incubation of cobalamin (50 $\mu\mu$moles/ml.) with sonic extract in reaction mixture *A* for 4 hr.; sample used was equivalent to 6 mg. of protein of the sonic extract. (II) Dialysed extract (equivalent to 2 mg. of protein) of acetone-dried organisms grown in the presence of cobalamin.

Assay of activity of the factor. Reaction mixture *B* plus (I) normal acetone-powder extract (10 mg. of protein) or (II) ultrasonic-treated acetone-powder extract (20 mg. of protein). Incubated 3 hr.

Source of factor	Analogue (μm-moles)	L-Methionine formed (μm-moles)
I	None	1050
	Anilide (5·7)	1070
	(57·0)	1070
II	None	575
	Anilide (1·2)	580
	(57·0)	590
	Factor B (3·5)	465
	(70·0)	345

specific activity to cobalamin content than the usual P 2 preparation so that even less free cobalamin was added with the sample of X.

No evidence could be obtained for the production of an inhibitory analogue of X when the anilide analogue was incubated with ultrasonic extract under the same conditions as led to the production of X from cobalamin. A second protamine precipitate fraction was isolated from this reaction mixture; when added to the assay system at five times the concentration of a similar preparation of X it did not inhibit the methionine production caused by the latter.

Table 2. *Effect of cobalamin analogues on the synthesis of the factor*

Synthesis of factor. Reaction mixture *A* plus cobalamin (50 $\mu\mu$moles/ml.) when present and analogues as stated. Incubated 3·5 hr. Samples assayed were the P2 fractions (see text) and were equivalent to 6·0 mg. of original ultrasonic protein.

Assay of factor. Reaction mixture *B* plus normal extract of acetone-dried organisms (10 mg. of protein). Incubated 3 hr.

Analogue (μm-moles/ml.)	Units of factor formed with cobalamin: Absent	Present
None	0·5	12·0
Methylamide (4·4)	2·4	3·0
(44·0)	2·2	2·1
Ethylamide (1·3)	—	5·7
(43·0)	1·3	1·8
Anilide (0·8)	—	3·6
(42·0)	1·3	1·3
Factor B (2·6)	—	4·4
(51·0)	0·7	0·8

Table 3 *Effect of the anilide analogue of cobalamin on the synthesis of methionine by ultrasonic extracts*

Reaction mixture for the synthesis of methionine (Guest, 1960) plus tetrahydropteroylglutamate (1 μmole) and ultrasonic extract of *E. coli* (equivalent to 10 mg. of protein). Cobalamin (65 $\mu\mu$moles) and the anilide analogue (30 μm-moles) when present. Purified preparation of factor X (see text) equivalent to 0·1 mg. of protein. Incubated 4 hr.

Additions	L-Methionine formed (μm-moles/mg. of protein) with anilide analogue: Absent	Present
None	4	10
Cobalamin	70	10
Factor X	38	40
Cobalamin + factor X	110	42
Factor X (no ultrasonic extract)	2	—

Use of analogues of cobalamin in the assay of the factor

The results of the preceding section suggested a more convenient method for the assay of X in which it would not be necessary to separate it (as the P 2 fraction) from the reaction products. Addition of the anilide analogue to the reaction mixture (*B*) for the assay should prevent the formation of X during the assay itself from cobalamin carried over with the sample, provided that the concentration of the cobalamin is not too great; dialysis alone should reduce the concentration sufficiently.

Ultrasonic extract was incubated with cobalamin in the usual reaction mixture. One portion of the mixture after incubation was simply dialysed against 0·05 M-potassium phosphate buffer pH 7·8 for 18 hr. at 3° (150 ml. of buffer/ml. of reaction mixture); the dialysis fluid was changed twice. The other portion was treated with protamine and a P 2 fraction obtained as in Methods. Both types of sample were assayed for X activity in the usual test system (see Methods) but with the anilide analogue either present or absent. Two special controls were done. In one, cobalamin was omitted from the reaction mixture for synthesis of the factor, and, in the other, cobalamin at a relatively high concentration (30 $\mu\mu$moles/ml.) was added to the reaction mixture for assay. The activities of the two types of sample were similar (Table 4) whether the anilide analogue was present or not in the assay system. However, slight doubt was cast on the validity of the assay (with anilide absent) on the sample which had been dialysed only, because the control in which cobalamin had been absent from the synthesis system but present in the assay system gave a higher value than a similar control for the P 2 fraction. This apparent X activity was greatly decreased by the addition of the anilide analogue to the assay system (Table 4).

The main disadvantage of this method for assay of X was that when the normal enzyme preparation (extract of acetone-dried organisms) was omitted from the assay system there was a small synthesis of methionine; this can be attributed to the enzymes of the ultrasonic extract carried over with the sample. Under the conditions normally used this was equivalent to 2–3 units of X. The difficulty was overcome (*a*) by ensuring that all systems for assay of X contained the same amounts of protein of similar origin and (*b*) by increasing the amount of the main enzyme source (extract of acetone-dried organisms) so that its concentration could not be rate-limiting.

The final procedure for this alternative assay of X (which was adopted for most of the work still to be described) was to dialyse samples against

Table 4. *The anilide analogue of cobalamin and the assay of the factor*

Synthesis of factor. Reaction mixture *A* with cobalamin (50 $\mu\mu$moles/ml.) when present. Incubated 4 hr. Samples of factor tested (each equivalent to 6 mg. of protein of the original ultrasonic extract): (I) Second protamine precipitate (P2) fraction from reaction mixture, (II) reaction mixture dialysed only.

Assay of factor. Reaction mixture *B* with cobalamin and anilide analogue as shown; normal extract of acetone-dried organisms equivalent to 10 mg. of protein. Incubated 3 hr.

			Assay of the factor		
			Units of factor activity in the sample in presence of anilide analogue (μm-moles)		
Sample	Cobalamin (during synthesis of factor)	Cobalamin (65 $\mu\mu$moles)	0	5·7	57·0
(I) (P2 fraction)	Present	Absent	10·8	11·0	11·0
		Present	11·2	—	—
	Absent	Absent	0·2	—	—
		Present	1·8	0·5	0·2
(II) (Dialysed only)	Present	Absent	11·5	—	10·8
		Present	11·3	—	—
	Absent	Absent	0·4	—	0·8
		Present	3·2	1·5	0·9

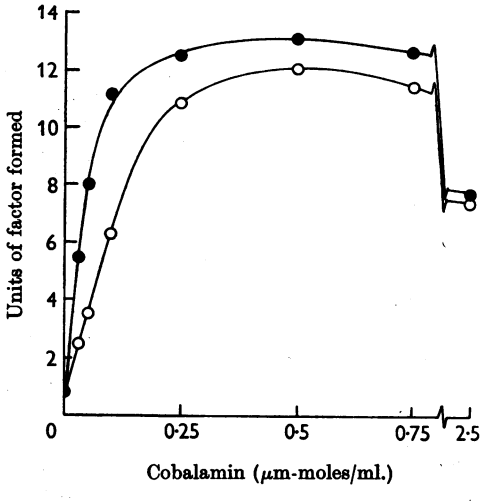

Fig. 2. Effect of cobalamin concentration on the synthesis of the factor by ultrasonic extracts. Synthesis of factor: reaction mixture *A* plus Dowex-1-treated ultrasonic extract and cobalamin; incubated 1 hr. (○) or 3 hr. (●). Dialysed samples equivalent to 6 mg. of protein of the original ultrasonic extract were used for assay. Assay of factor: reaction mixture *B* plus extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (46 μm-moles); incubated 3 hr.

0·05 M-potassium phosphate buffer pH 7·8, and to assay them in the reaction mixture *B* in the presence of the anilide analogue (23 or 46 μm-moles) and with the source of enzyme (extract of acetone-dried organisms) increased to the equivalent of 20 mg. of protein. The curve relating size of sample to amount of methionine synthesized was similar to that of Fig. 1 for the original method of assay, except that the linear range was slightly reduced.

Addition of cobalamin to the assay system did not increase the methionine produced by either experimental or control samples of the reaction mixture for the synthesis of X.

The new method of assay for X enabled its formation to be studied when changes were made in the synthesis system, either by purification of the ultrasonic extract or by altering the composition of reaction mixture *A*, which made protamine treatment no longer practicable.

Properties of the system forming the factor

Effect of the concentration of cobalamin. An optimum synthesis of X during the first hour of incubation was obtained with 0·5 μm-mole of cobalamin/ml. (Fig. 2); synthesis was proportional to time up to 45 min. With higher concentrations of cobalamin, although the initial rate was increased, synthesis of X stopped after a short period; the reason for this is not known. In subsequent experiments a cobalamin concentration of 0·5 μm-mole/ml. and 45 min. incubation was used as a routine and 46 μm-moles of the anilide analogue was added to the assay system. The cobalamin content of the resultant dialysed samples, used for assay of X, was estimated by assay with *Euglena gracilis* as 0·1 μm-mole.

5:6 - *Dimethylbenzimidazolylcobamide - coenzyme.* This is one of several coenzyme forms of the cobalamins which functions in the formation of β-methylaspartate from glutamate by *Clostridium tetanomorphum* (Weissbach, Toohey & Barker, 1959; Barker *et al.* 1960). It is 2–3 times as active as cobalamin in the synthesis of methionine by ultrasonic extracts of *E. coli* and is also less sensitive to inhibition by the anilide analogue of cobalamin (Guest *et al.* 1960; Guest, 1960).

The coenzyme did not replace X for its activity in the present test system nor did it affect the activity of X. It did however replace cobalamin for the formation of material with the activity of X (Table 5). Such synthesis was inhibited 80 % by a concentration of the anilide analogue which completely prevented the synthesis of X in the presence of cobalamin. Other requirements for the formation of X from the cobamide-coenzyme were similar to those for cobalamin (Table 6).

Other requirements for the synthesis of the factor. Omission of any of the constituents of reaction mixture *A* decreased the rate of formation of X, the effect of ATP + Mg^{2+} ions and of glucose being the most pronounced (Table 6). Absence of DPN decreased synthesis by about 40 %. Reduced DPN however replaced both DPN and glucose, and at higher concentrations also eliminated the requirement for ATP (Table 6). Addition of the substrates and the cofactor for the synthesis of methionine itself (homocysteine, serine and tetrahydropteroylglutamate) had no significant effect on the formation of X.

The presence of substances possibly required for protein synthesis (mixtures of amino acids, and mixtures of amino acids, purines, pyrimidines and B-group vitamins) did not increase synthesis of X, nor was such synthesis inhibited by *p*-fluorophenylalanine (mM), 4-methyltryptophan (mM) and 8-azaguanine (0·2 mM). Chloramphenicol (0·14 mM) inhibited by 30 % only; it could not be used at higher concentrations since it then inhibited the growth of the organism used for the assay of methionine.

Activity of the ultrasonic extract in synthesizing X. The synthesis of X increased linearly with the concentration of ultrasonic extract up to the limit tested (11 mg. of protein/ml.). Different batches of the extract varied in activity by an overall factor of two, but there was generally no loss of activity on storage for up to 6 weeks at −14°. The extract lost 90 % of its activity on heating to 45° for 3 min. Treatment with Dowex-1 resin caused only a 10–15 % decrease in activity which could be restored by the addition of the heated extract of organisms.

Ultrasonic extracts of *E. coli* strain 121/176 and 3/62 also catalysed the synthesis of X, though the activity of strain 3/62 was only 10–20 % of that of strains PA 15 and 121/176.

Table 5. 5:6-*Dimethylbenzimidazolylcobamide-coenzyme and the synthesis of the factor by ultrasonic extracts*

Synthesis of factor. Reaction mixture *A*, cobalamin or 5:6-dimethylbenzimidazolylcobamide-coenzyme (each 50 μμmoles/ml.) and anilide analogue (95 μm-moles/ml.) when added. Incubated 4 hr. Samples assayed were P2 fractions equivalent to 6 mg. of protein of the ultrasonic extract.

Assay of factor. Reaction mixture *B* plus ultrasonic-treated extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (23 μm-moles). Incubated 3 hr.

Additions	Units of factor formed
None	0·7
Cobalamin	10·0
Cobalamin + anilide analogue	0·9
Anilide analogue	0·9
Cobamide-coenzyme	11·2
Cobamide-coenzyme + anilide analogue	3·0

DISCUSSION

The difference in behaviour of extracts of acetone-dried organisms and ultrasonic extracts appears to lie in the ability of the latter to utilize free cobalamin in the formation of a factor (X), which is required for the synthesis of methionine by cell-free extracts of *E. coli* PA 15 when tetrahydropteroylglutamate is provided as the cofactor for C_1 transfer. Treatment with acetone appears almost completely to destroy the capacity of the organism

Table 6. *Requirements for the formation of the factor by ultrasonic extracts of* E. coli PA 15

Synthesis of factor. Reaction mixture *A* plus ultrasonic extract (treated with Dowex-1; see Methods) and cobalamin (0·5 μm-mole/ml.) or 5:6-dimethylbenzimidazolylcobamide-coenzyme (0·4 μm-mole/ml.). Incubated 45 min. Samples for assay were equivalent to 4·5 mg. of protein of the ultrasonic extract.

Assay of factor. Reaction mixture *B* plus ultrasonic-treated extract of acetone-dried organisms (20 mg. of protein) and anilide analogue (46 μm-moles). Incubated 3 hr. $DPNH_2$, reduced diphosphopyridine nucleotide.

Omissions from reaction mixture *A*	Additions	Units of factor formed with: Cobalamin	Units of factor formed with: Cobamide-coenzyme
None	—	9·5	11·0
Cobalamin or cobamide-coenzyme	—	0·6	0·9
Glucose	—	2·2	4·0
DPN	—	6·0	5·0
ATP and Mg^{2+} ions	—	2·4	3·2
Glucose and DPN	$DPNH_2$ (4 μmoles)	9·5	—
Glucose, DPN and ATP	$DPNH_2$ (16 μmoles)	9·0	—

to form X, though acetone does not affect X once formed. Extracts of such acetone-dried organisms cannot synthesize methionine with tetrahydropteroylglutamate as source of folic acid and do so only very feebly on the addition of free cobalamin. Extracts of acetone-dried organisms may therefore be used for the assay of the X which is formed either by growing the organisms in the presence of cobalamin or, as in the present work, incubating cobalamin with ultrasonic extract in the presence of glucose, ATP and DPN.

The properties of the factor formed in the two different ways are closely similar and suggest that it is either a protein or firmly associated with a protein. Although the material contains cobalamin it cannot be said at the present low degree of purification whether this is adventitious or whether X contains a cobalamin residue. The fact that one analogue of cobalamin (Factor B) at relatively high concentrations partially inhibits the activity of X provides some evidence that the latter may have a cobalamin residue at its active centre; other analogues of cobalamin do not, however, inhibit. All the analogues inhibited the formation of X; this could be interpreted either as inhibition of the incorporation of a cobalamin residue into X or as inhibition of a catalytic function of cobalamin in the synthesis of X.

The formation of X requires reducing power and possibly a source of energy. Although *de novo* synthesis of protein cannot be excluded since there was slight inhibition by chloramphenicol it is more likely that some pre-existing protein is modified; synthesis was not increased by mixtures of amino acids, purines and pyrimidines and was not inhibited by analogues of these substances which would be expected to inhibit protein synthesis.

5:6-Dimethylbenzimidazolylcobamide-coenzyme replaced cobalamin for the synthesis of X and may be more active. However no direct evidence was obtained for or against the cobamide-coenzyme being an intermediate in the function of cobalamin in the formation of X.

If X is indeed a protein the question arises as to whether it has enzymic activity in some reaction or whether it has catalytic activity in some other sense, perhaps analogous to the function of cytochrome. No direct enzymic action of X on, for example, tetrahydropteroylglutamate has so far been detected. The function of X in methionine synthesis, and particularly its relationship to the utilization of tetrahydropteroylglutamate, is not yet known; various hypotheses have been discussed previously (Kisliuk & Woods, 1960; Guest *et al.* 1960; Guest, 1960). Hatch *et al.* (1959) have shown a cobalamin-containing enzyme to be part of the complex of enzymes catalysing the transfer of the β-carbon atom of serine to homocysteine by a cobalamin auxotroph of *E. coli*; tetrahydropteroylglutamate was the source of folic acid in this system also.

Ultrasonic extracts of a strain (121/176) of *E. coli* requiring cobalamin or methionine for growth also form X when incubated with cobalamin. Extracts of this organism require cobalamin for methionine synthesis even when folic acid is supplied by an extract of heated *E. coli* (Guest *et al.* 1960); it is likely therefore that the function of X in methionine synthesis is not solely concerned with the utilization of tetrahydropteroylglutamate.

E. coli strain 3/62 has an absolute requirement for methionine for growth, and cell-free extracts have no ability to synthesize it. Nevertheless ultrasonic extracts do form X, though in less quantity than the other strains. This emphasizes that concurrent synthesis of methionine is not necessary or helpful to the formation of X; it will be recalled that the addition of homocysteine, serine and tetrahydropteroylglutamate had no effect on the formation of X by extracts of strain PA 15.

SUMMARY

1. A heat-labile, non-diffusible material has been partly purified from the products of the incubation of ultrasonic extracts of *Escherichia coli* strain PA 15 with cobalamin, glucose, ATP, Mg^{2+} ions, DPN and phosphate buffer. This factor is essential for the synthesis of methionine by extracts of acetone-dried organisms of the same strain when tetrahydropteroylglutamate acts as source of the necessary folic acid cofactor for the C_1 transfer reaction.

2. The factor has similar properties to material with the same biological activity previously isolated from the same organism grown in the presence of added cobalamin.

3. Monosubstituted cobalamin-amide analogues inhibit the formation of the factor by the ultrasonic extracts but have no effect upon its function in methionine synthesis. Factor B partially inhibits both the formation and the function of the factor.

4. A method for assaying the factor formed by ultrasonic extracts is described. It makes use of the ability of analogues of cobalamin to inhibit formation of the factor.

5. 5:6-Dimethylbenzimidazolylcobamide-coenzyme has no activity as the factor but replaces cobalamin for its formation.

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The Metabolism of C_2 Compounds in Micro-organisms

4. SYNTHESIS OF CELL MATERIALS FROM ACETATE BY *ASPERGILLUS NIGER**

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Many fungi grow readily on C_2 compounds as the sole source of carbon and excrete, into their growth media, intermediates of the tricarboxylic acid cycle or compounds derived therefrom. The tricarboxylic acid cycle has been shown to be the major route both for the catabolism of C_2 compounds such as acetate or ethanol and for the provision of the carbon skeletons of most cellular constituents and of compounds excreted (for review, see Chain, 1955; Foster, 1958; Kornberg, 1959). Its continued operation under these conditions necessitates the occurrence of reactions, ancillary to the tricarboxylic acid cycle, which lead to the net formation of intermediates of that cycle from the C_2 growth substrate.

The main purpose of this paper is to show that *Aspergillus niger* growing on acetate as sole carbon source incorporates isotope from [2-^{14}C]acetate in a manner which excludes the primary formation of succinate from acetate (Thunberg, 1920) and which indicates that the required syntheses of intermediates of the tricarboxylic acid cycle are effected via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957). Cell-free extracts contain a high activity of the requisite enzymes of the glyoxylate cycle and can catalyse the net formation of malate and succinate from acetyl-coenzyme A and *iso*citrate. Extracts of *A. niger*, grown on substrates the metabolism of which does not necessitate such syntheses of C_4 compounds from acetate, are virtually devoid of a key enzyme of the glyoxylate cycle, *iso*citratase: this supports the view that that cycle plays an essential role in growth on acetate.

This work has been presented in part to the Biochemical Society (Kornberg & Collins, 1958).

* Part 3: Kornberg & Madsen (1958).

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METHODS

Maintenance and growth of the mould. The strain of *A. niger* studied had a high ability for producing citric acid. The organism was maintained on slopes consisting of 50 mM-ammonium acetate, 20 mM-sodium–potassium phosphate buffer, pH 7·4 (prepared by mixing 80·4 vol. of 20 mM-Na_2HPO_4 with 19·6 vol. of 20 mM-K_2HPO_4), salts (25 mg. of $MgSO_4,7H_2O$, 0·1 mg. of $FeSO_4,7H_2O$, 0·1 mg. of $ZnSO_4,7H_2O$, 10 μg. of $MnSO_4,4H_2O$, 1 μg. of $CuSO_4,5H_2O$ and 0·2 μg. of $K_2Cr_2O_7$/100 ml. of medium), 0·05% (w/v) of 'Difco' yeast extract and 2% (w/v) of agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures were grown at 30° until sporulation had taken place (4–7 days), and were then stored at 2°. For growth in liquid medium, a suspension of spores in sterile water was used to inoculate Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of medium which, with the omission of the agar, was identical with that used for the slopes. The flasks were shaken in air at 30° for 20–