provided. Tetrahydropteroylglutamate can, however, only be used if cobalamin is present.

4. The formaldehyde derivative of tetrahydropteroylglutamate can serve with both strains both as source of folic acid coenzyme and of C_1 units provided again that cobalamin is present.

5. Growth of either strain in the presence of cobalamin enhances the ability of ultrasonic extracts of the organisms to methylate homocysteine.

6. Reduced diphosphopyridine nucleotide abolishes the requirement for both glucose and diphosphopyridine nucleotide.

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Inhibition of Methionine Synthesis in *Escherichia coli* by Analogues of Cobalamin

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A relationship between cobalamin and the synthesis of methionine by *Escherichia coli* was first established by the isolation of auxotrophic strains requiring either cobalamin or methionine for growth (Davis & Mingioli, 1950). Cobalamin has since been found to increase methionine synthesis from homocysteine and serine by washed suspensions of many strains of *E. coli* (Gibson & Woods, 1960); such synthesis is also increased several-fold by the addition of cobalamin to ultrasonic extracts of an auxotrophic strain, *E. coli* 121/176, which requires cobalamin or methionine for growth (Helleiner & Woods, 1956). More

recently ultrasonic extracts of another auxotrophic strain (PA15), which does not require cobalamin for growth, have been found to require the vitamin for methionine synthesis only when the source of the cofactor for the transfer of the C_1 unit is tetrahydropteroylglutamic acid and not when heated extract of the organism is used. With ultrasonic extracts of strain 121/176, cobalamin is essential with both sources of the folic acid cofactor (Helleiner, Kisluk & Woods, 1957; Guest, Helleiner, Cross & Woods, 1960).

Several analogues of cobalamin have been prepared (Smith, Parker & Gant, 1956) and reported

to inhibit the growth of the cobalamin-requiring auxotroph *E. coli* 113/3 (Cuthbertson, Gregory, O'Sullivan & Pegler, 1956). Baker *et al.* (1959), however, have not found them to be active in this respect. These analogues have also been shown to inhibit a possible function of cobalamin in amino acid activation and protein synthesis (Wagle, Mehta & Johnson, 1958*a, b*); however, a direct action of cobalamin here has not been confirmed by Fraser & Holdsworth (1959) or by Arnstein & Simkin (1959). Recently the analogues have been found to inhibit the activity of a cobalamin-containing enzyme of *E. coli* required for methionine formation from serine and homocysteine (Hatch, Takeyama & Buchanan, 1959).

In the present work the effect of analogues and derivatives of cobalamin on the synthesis of methionine by ultrasonic extracts of *E. coli* has been studied in an attempt to elucidate further the role of cobalamin.

MATERIALS AND METHODS

Organisms and extracts. Stock cultures of *E. coli* were maintained on slopes of tryptic meat-agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. Four strains have been used; two of these were auxotrophic strains (121/176 and 113/3), which require cobalamin or methionine for growth (Davis & Mingioli, 1950), and another was their parent (strain W). The greater part of the work, however, was with strain PA15, which requires serine or glycine for growth and was originally obtained from Dr Barbara Wright.

The growth medium and the preparation of the ultrasonic extract have been described by Guest *et al.* (1960). The medium was supplemented with glycine (10 mM) for strain PA15 and DL-methionine (0.5 mM) for strain 121/176. In a few experiments cobalamin or its analogues were added to the complete medium for strain PA15; they were sterilized separately by Seitz-filtration.

Extract of heated organisms was prepared by heating a washed suspension (170 mg. dry wt./ml.) at 100° for 3 min., cooling and centrifuging. The supernatant fluid will be referred to as extract of heated *E. coli*, and is a source of natural folic acid coenzyme.

Study of methionine synthesis. The solution used for testing methionine synthesis was similar to reaction mixture B of Guest *et al.* (1960) and contained (in 2 ml. of 0.125M-potassium phosphate buffer, pH 7.8): L-serine, 10 μ moles; DL-homocysteine, 10 μ moles; glucose, 20 μ moles; MgCl₂, 10 μ moles; adenosine triphosphate (ATP), 10 μ moles; diphosphopyridine nucleotide (DPN), 1 μ mole; pyridoxal phosphate, 0.5 μ mole. In experiments with the formaldehyde derivative of tetrahydropteroylglutamic acid (tetrahydro-PtGA-CH₂O) the amount of DL-homocysteine was decreased to 5 μ moles and pyridoxal phosphate and serine were omitted (Kisliuk & Woods, 1960).

When extract of heated *E. coli* was added the amount was equivalent to 17 mg. dry wt. of original organisms. The volume of ultrasonic extract used in all experiments contained 10 mg. of protein; the latter was

estimated spectrophotometrically according to Layne (1957).

The complete reaction mixture was incubated at 37° for 4 hr. under an atmosphere of H₂. Methionine synthesis was stopped by heating at 100° for 3 min. After centrifuging to remove precipitated protein the supernatant fluid was assayed for methionine with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 (Gibson & Woods, 1960). Presence in the basal medium of cobalamin (or of any of its analogues used in this work) at concentrations equivalent to those in the samples of reaction mixture to be assayed, had no effect on the response of the organism to methionine.

The amount of L-methionine formed is given as μ m-moles/mg. of protein of the ultrasonic extract. The value obtained for a control, containing all components of the reaction mixture but heated at zero time, was subtracted from the experimental and other control values.

Experiments with growing cultures. Liquid-culture technique was adopted for studying the growth of strains of *E. coli* in the presence of cobalamin and its analogues. The medium described previously (Guest *et al.* 1960) was used at half the final concentration of all constituents. Duplicate tests were carried out in 150 mm. $\times$ 20 mm. hard-glass tubes containing, with the appropriate supplements and inoculum, a final volume of 2 ml. Solutions of cobalamin and its analogues were sterilized by Seitz-filtration and added to the medium, which had been autoclaved at 115° for 10 min.

The inoculum was prepared from a tryptic meat-agar-slope culture of the organism and harvested after incubation for 16 hr. at 37°. The organisms were washed once with 10 ml. of water and each tube of the tests was inoculated with about 3.5 μ g. dry wt. of organisms (in 0.1 ml.). Incubation was for 24 or 48 hr. at 37° with the tubes sloped to increase aeration.

The extent of growth was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) with a neutral-density filter; the uninoculated medium was used to give the zero setting. With high concentrations of cobalamin analogues, the value for an uninoculated control was deducted from the corresponding experimental values. The instrument was calibrated in terms of mg. dry wt. of organisms/ml. of culture and results are expressed in this way. The inhibition index for half-maximal and complete inhibition of growth is defined as the ratio, concentration of analogue : concentration of cobalamin.

Chemicals. The source of cobalamin was Anacobin (British Drug Houses Ltd., Poole, Dorset); it was assayed spectrophotometrically with the extinction coefficients quoted by Brink *et al.* (1949). The methylamide, ethylamide and anilide of the monocarboxylic acids of cyanocobalamin (Smith *et al.* 1956) were provided by Dr E. Lester Smith; they will be referred to as the methylamide, ethylamide and anilide analogues. They contained less than 0.02% of cobalamin itself (E. L. Smith, personal communication). Crystalline samples were dissolved in water and examined spectrophotometrically in visible and ultraviolet light. The spectra were identical with cobalamin except that the anilide analogue had a higher absorption in the 220–275 m μ region, owing to the presence of one additional benzene ring in the molecule. The molecular extinction coefficients at the three wavelengths of maxi-

mum absorption (278, 361, 550 μ m) were assumed to be the same as those of cobalamin.

Factor B, which has no nucleotide residue, was also provided by Dr E. Lester Smith and was assayed spectrophotometrically by assuming that the molecular extinction coefficient in 0.01% KCN solution at the absorption maximum (367 μ m) is the same as for cobalamin under the same conditions.

A specimen of 5:6-dimethylbenzimidazole B₁₂-coenzyme was given by Dr H. A. Barker and it was also assayed spectrophotometrically (Weissbach, Toohey & Barker, 1959).

The values given for the concentration of cobalamin and analogues have been corrected for their probable purities, which were: cobalamin, 88%; 5:6-dimethylbenzimidazole B₁₂-coenzyme, 100%; methylamide, 82%; ethylamide, 84%; anilide, 83% and factor B, 70%.

Other chemicals and their sources were as described by Guest *et al.* (1960).

RESULTS

Methionine synthesis by cell-free extracts

The synthesis of methionine from homocysteine by ultrasonic extracts of strains of *E. coli* was studied under three conditions: (I) serine as the donor of the C₁ unit and tetrahydropteroylglutamic acid (tetrahydro-PtGA) as the folic acid cofactor; (II) the formaldehyde derivative of tetrahydro-PtGA (tetrahydro-PtGA-CH₂O) as both donor of the C₁ unit and as the cofactor; (III) serine as donor and extract of heated *E. coli* as source of folic acid cofactor. With ultrasonic extracts of strain 121/176, cobalamin is required in every case, but, for strain PA15, cobalamin is only essential when tetrahydro-PtGA is the cofactor for transferring C₁ units (i.e. conditions I and II).

Factor B and the methylamide, ethylamide and anilide derivatives of the monocarboxylic acids of cobalamin were tested for their ability to replace or inhibit the action of cobalamin in these three conditions for methionine synthesis.

Methionine synthesis in the presence of serine and tetrahydropteroylglutamic acid. The analogues of cobalamin replace the requirement for cobalamin to a limited extent, but only at relatively high concentrations (Table 1). The analogues, however, were also inhibitors of the action of cobalamin and decreased the synthesis of methionine to the value obtained with the analogue alone. The order of increasing activity in this respect was methylamide < factor B < ethylamide < anilide (Table 1). The two extreme cases of the analogue activities are illustrated in Fig. 1. Because the analogues have some ability to replace cobalamin, and because this is different for each analogue, it is not possible to express their capacities for inhibition in precise quantitative terms. The anilide analogue was chosen for more detailed studies because it combines high inhibitory activity with a weak ability to replace the vitamin.

Fig. 1. Effect of the methylamide and anilide analogues of cobalamin on methionine synthesis. Reaction mixture plus ultrasonic extract of strain PA15 and tetrahydro-PtGA (1 μ mole). Cobalamin: $\square$, present (0.032 μ m-mole/ml.); ▨ , absent. Incubated for 4 hr.

Table 1. *Effect of analogues of cobalamin on methionine synthesis by ultrasonic extract of Escherichia coli in a system containing serine and tetrahydropteroylglutamic acid*

Reaction mixture (see Materials and Methods section) plus ultrasonic extract and tetrahydro-PtGA (1 μ mole); cobalamin, 0.032 μ m-mole/ml. when present. Incubated for 4 hr.											
		Cobalamin absent					Cobalamin present				
		Concn. of analogue (μ m-moles/ml.)									
		0	0.032	0.32	3.2	32.0	0	0.032	0.32	3.2	32.0
Strain of <i>E. coli</i>	Analogue	L-Methionine formed (μ m-moles/mg. of protein)									
PA15	Methylamide	4	7	18	28	24	59	63	58	40	25
	Ethylamide	5	7	13	20	21	73	72	63	33	22
	Anilide	3	4	9	10	12	55	54	38	19	12
	Factor B	3	5	7	8	9	62	61	59	24	11
121/176	Anilide	2	3	5	7	8	68	68	34	10	8

Treatment of the enzymic extract with Dowex-1 resin as described by Kisluk & Woods (1960) had no effect on the inhibitory action of the anilide analogue.

In order to determine the type of inhibition, the rate of methionine formation was measured over a

range of cobalamin concentrations (0-0.32 $\mu\text{m-moles/ml.}$) in the presence of two concentrations of the anilide analogue (0.73 and 1.45 $\mu\text{m-moles/ml.}$). The results were plotted by the method of Lineweaver & Burk (1934) and show (Fig. 2) that the anilide analogue is a competitive inhibitor of cobalamin. A second experiment of the same type with different concentrations of the anilide (0.29 and 2.9 $\mu\text{m-moles/ml.}$) confirmed this conclusion.

Fig. 2. Effect of the anilide analogue on the rate of formation of methionine by ultrasonic extracts of strain PA 15. Reaction mixture plus tetrahydro-PtGA (1 μmole). S, Cobalamin concentration ($\mu\text{m-moles/ml.}$); V, rate of methionine formation ($\mu\text{m-moles}$ of L-methionine/mg. of protein/4 hr.). Anilide analogue: $\circ$, nil; $\bullet$, 0.73 $\mu\text{m-mole/ml.}$; $\bullet$, 1.45 $\mu\text{m-mole/ml.}$

Table 2. Effect of analogues of cobalamin on methionine synthesis by ultrasonic extract of *Escherichia coli* in a system containing the formaldehyde derivative of tetrahydropteroylglutamic acid

Reaction mixture (modified as described in the Materials and Methods section) plus enzymic extract of strain PA 15 and tetrahydro-PtGA-CH₃O, (3.3 μmoles); cobalamin, 0.032 $\mu\text{m-mole/ml.}$ when present. Incubated for 4 hr.

Analogue		L-Methionine formed ($\mu\text{m-moles/mg. of protein}$)	
Nature	Concn. ($\mu\text{m-moles/ml.}$)	Cobalamin absent	Cobalamin present
None	—	6	62
Methylamide	3.0	34	44
	30.0	28	32
Ethylamide	1.0	21	51
	30.0	24	25
Anilide	0.5	12	53
	29.0	19	24
Factor B	1.7	7	36
	34.5	8	10

Table 3. Effect of analogues of cobalamin on synthesis of methionine by ultrasonic extract of *Escherichia coli* in a system containing serine and heated extract of *Escherichia coli*

Reaction mixture plus Dowex-treated ultrasonic extract and heated extract of the corresponding strain (see Materials and Methods section). Cobalamin, 0.032 $\mu\text{m-mole/ml.}$ when present. Incubated for 2 hr. (strain PA 15) or for 4 hr. (strain 121/176).

Strain of <i>E. coli</i>	Analogue		L-Methionine formed ($\mu\text{m-moles/mg. of protein}$)	
	Nature	Concn. ($\mu\text{m-moles/ml.}$)	Cobalamin absent	Cobalamin present
PA 15	Methylamide	0	54	—
		3.0	59	—
		30.0	57	—
	Ethylamide	0	54	—
		1.0	57	—
		30.0	60	—
	Anilide	0	67	70
		0.5	71	71
		29.0	70	74
	Factor B	0	54	60
1.7		58	58	
34.5		56	56	
121/176	Anilide	0	5	37
		0.5	7	18
		29.0	10	10

Methionine synthesis in the presence of the formaldehyde derivative of tetrahydropteroylglutamic acid. Two concentrations of analogue only were used for testing their activity when serine and tetrahydro-PtGA were replaced by tetrahydro-PtGA-CH₂O. These were those giving half and maximum inhibition under condition I. The results were essentially the same as those for condition I both in replacing cobalamin and in inhibiting its action (Table 2).

Methionine synthesis in the presence of serine and extract of heated E. coli. The synthesis of methionine by ultrasonic extracts of strain PA15 with extract of heated *E. coli* as source of folic acid coenzyme was not inhibited by the analogues whether or not cobalamin was added (Table 3). With enzymic extracts of strain 121/176 and extract of heated *E. coli*, methionine synthesis occurred only if cobalamin was added, and here the anilide analogue was inhibitory; the degree of inhibition was the same whether the source of folic acid cofactor was the heated extract or tetrahydro-PtGA.

These results suggest that if cobalamin is present in the heated extract of strain PA15 it is not in a form that can function in the synthesis of methionine. However, there may be a more active form of cobalamin whose action is not inhibited by the anilide analogue. This possibility was investigated by interchanging the enzymic and heated extracts of the two strains of *E. coli* (Fig. 3). Dialysed and Dowex-treated extracts of strain PA15 showed no requirement for cobalamin if the source of heated extract was prepared from either strain PA15 or strain 121/176, whereas similar enzymic extracts of strain 121/176 required cobalamin even if the

heated extract was derived from strain PA15. In addition, the synthesis of methionine by enzymic extracts of strain PA15 with heated extract derived from strain 121/176 was not affected (either in the presence or absence of cobalamin) by concentrations of the anilide analogue which were inhibitory in systems in which cobalamin is required.

Effect of the anilide analogue upon methionine synthesis when cobalamin is replaced by 5:6-dimethylbenzimidazole B₁₂-coenzyme. Under condition I (serine, tetrahydro-PtGA system) 5:6-dimethylbenzimidazole B₁₂-coenzyme, a factor required for the metabolism of glutamate by *Clostridium tetanomorphum* (Barker, Weissbach & Smyth, 1958; Weissbach *et al.* 1959), is two to three times as active as cobalamin in promoting the synthesis of methionine (Guest *et al.* 1960). Much higher concentrations of the anilide analogue were required to inhibit the action of the B₁₂-coenzyme than those effective with an equimolar concentration of cobalamin (Fig. 4). In order to determine the type of inhibition here, two concentrations of B₁₂-coenzyme, one three times as high as the other, were chosen, and the effect of a series of concentrations of the anilide analogue was studied. The percentage inhibition of the action of B₁₂-coenzyme was constant for a constant ratio of anilide to B₁₂-coenzyme concentration (Table 4). The inhibition is therefore, as with cobalamin, of the competitive type over the range of concentrations tested.

Fig. 3. Activity of heated extracts of strains of *Escherichia coli*. Progress of methionine formation by ultrasonic extracts of (a) strain 121/176 and (b) strain PA15. Reaction mixture plus Dowex-treated enzymic extract with: ○, heated extract of strain 121/176; ●, heated extract of strain PA15; cobalamin: —, present (32 μ moles/ml.); ---, absent.

Fig. 4. Inhibition by the anilide analogue of methionine synthesis in the presence of cobalamin and 5:6-dimethylbenzimidazole B₁₂-coenzyme. Reaction mixture plus Dowex-treated extract of strain PA15 and tetrahydro-PtGA (1 μ mole); ○, No addition; ●, cobalamin (32 μ moles/ml.); ●, 5:6-dimethylbenzimidazole B₁₂-coenzyme (32 μ moles/ml.). Incubated for 4 hr.

Effect of the anilide analogue upon methionine synthesis by extracts of organisms grown in the presence of cobalamin. When cobalamin ($4\text{ }\mu\text{moles/ml.}$) is added to the growth medium for strain PA 15, the ability of enzymic extracts to synthesize methionine without added cobalamin becomes just limiting (Guest *et al.* 1960). Under these conditions the anilide analogue, even at $29\text{ }\mu\text{moles/ml.}$, had no inhibitory action. If the anilide was added instead to the growth medium together with cobalamin ($4\text{ }\mu\text{moles/ml.}$), the activity of the enzymic extract obtained was decreased (Fig. 5). Considerable activity, however, was present in extracts of organisms harvested after growth in the presence of the anilide only. When the source of folic acid coenzyme was extract of heated *E. coli*, enzymic extracts of organisms derived from media containing the anilide analogue with or without added cobalamin had the same activity for methionine formation.

Anilide analogue and growth of Escherichia coli

Over the range of concentrations tested ($0\cdot029\text{--}29\cdot0\text{ }\mu\text{moles/ml.}$), the anilide analogue did not

Table 4. *Inhibition by the anilide analogue of the synthesis of methionine promoted by 5:6-dimethylbenzimidazole B₁₂-coenzyme by ultrasonic extract of Escherichia coli*

Reaction mixture plus ultrasonic extract of strain PA 15 and tetrahydro-PtGA ($1\text{ }\mu\text{mole}$). Incubated for 4 hr. in the dark.

Anilide analogue ($\mu\text{moles/ml.}$)	B_{12} -coenzyme ($\mu\text{mole/ml.}$)	
	0·011	0·032
	Inhibition (%) of the action of B_{12} -coenzyme in methionine synthesis	
0	0	0
0·29	20	0
0·87	40	23
2·61	54	38
7·83	70	56
23·49	—	73

support the growth of strains requiring cobalamin or methionine, nor did it inhibit growth when an optimum concentration of methionine ($0\cdot5\text{ mM}$) was present. The anilide analogue had little effect on the growth of strain PA 15 in the presence of glycine (5 mM) or of strain W.

When cobalamin was added to the basal medium at a concentration greater than optimum for the growth of strain 121/176, the anilide analogue inhibited the growth of strains 121/176 and 113/3 (Table 5). The inhibition indices for 50 % inhibition of growth at 24 hr. at three concentrations of cobalamin ($0\cdot13$, $0\cdot65$ and $6\cdot5\text{ }\mu\text{moles/ml.}$) were 550, 570 and 370 for strain 121/176 and 550, 580 and 390 for strain 113/3. For full inhibition the values were about 1000 for both strains in every case. For strains PA 15 and W, which do not

Fig. 5. Effect of growing *Escherichia coli* PA 15 in the presence of the anilide analogue on the ability of ultrasonic extracts to synthesize methionine. Reaction mixture plus tetrahydro-PtGA ($1\text{ }\mu\text{mole}$) and extract of organisms cultured with cobalamin ($\square$, $4\text{ }\mu\text{moles/ml.}$ of growth medium) or without cobalamin (▨). Incubated for 1 hr. 40 min.

Table 5. *Effect of the anilide analogue on the growth of Escherichia coli*

Basal medium was supplemented with glycine (5 mM) and cobalamin ($0\cdot65\text{ }\mu\text{mole/ml.}$) where stated.

Strain of <i>E. coli</i>	Additions to basal medium	Time of incubation (hr.)	Anilide analogue ($\mu\text{moles/ml.}$)				
			0	0·029	0·29	2·9	29·0
			Growth (mg. dry wt. of organisms/ml.)				
W	—	24	0·7	0·7	0·7	0·8	0·9
	Cobalamin	24	1·2	1·1	0·7	0·7	0·7
	Cobalamin	48	2·5	2·5	2·6	2·6	2·8
PA 15	Glycine	24	0·4	0·4	0·4	0·4	0·4
	Glycine, cobalamin	24	0·5	0·5	0·4	0·3	0·3
	Glycine, cobalamin	48	1·7	1·7	1·6	1·7	1·7
121/176	Cobalamin	24	0·5	0·5	0·3	0·0	0·0
113/3	Cobalamin	24	0·6	0·6	0·4	0·0	0·0

depend on added cobalamin for growth, addition of the vitamin increased the early growth (Table 5). This effect was more pronounced after 16 hr. and was still apparent after 24 hr., but cobalamin had no effect on the extent of growth at 48 hr. The anilide analogue (at concentrations which inhibited the growth of strains 121/176 and 113/3) also inhibited this increase in early growth, but it had no effect on the extent of growth at 48 hr.

DISCUSSION

Synthesis of methionine is inhibited by the analogues of cobalamin whenever cobalamin is essential for such synthesis. The anilide analogue shows a competitive relationship with cobalamin and the inhibitory activity is thus related specifically to the function of cobalamin. The ability of the analogues both to replace cobalamin and inhibit its action makes it difficult to interpret the results in a quantitative manner. There are several possible explanations for this phenomenon. First, the analogues may have sufficient structural similarity to cobalamin to function as cobalamin at low efficiency. Secondly, the enzymic extracts may convert a proportion of the analogue into cobalamin. The contamination of the analogue with cobalamin is less than 0.02% (E. L. Smith, personal communication) and this would not account for their ability to replace cobalamin.

Escherichia coli 113/3, which requires cobalamin for growth, can grow in the presence of factor B, which is converted into complete cobalamins (Ford & Porter, 1953; Ford, Holdsworth & Kon, 1955). Factor B in the presence of 5:6-dimethylbenzimidazole is weakly active in methionine synthesis with extracts of *E. coli* strain PA 15 (Guest *et al.* 1960). However, alone it had low activity in replacing cobalamin whereas at high concentrations it proved to be an active inhibitor of the action of cobalamin.

A coenzyme form of cobalamin, 5:6-dimethylbenzimidazole B₁₂-coenzyme, which is more active than cobalamin in the synthesis of methionine, was found to require much higher concentrations of the anilide analogue to inhibit its activity. It is possible that cobalamin may first be transformed into such a compound, but if this is so it is unlikely to be the ultimate functional material in the synthesis of methionine since (a) inhibition of the activity of the coenzyme by the anilide analogue is still competitive in nature and (b) extracts of strain PA 15 cultured in the presence of cobalamin can synthesize methionine and this is insensitive to the anilide.

The ability of enzymic extracts of strain PA 15, grown in the presence of cobalamin, to synthesize methionine was decreased if high concentrations of the anilide analogue were also added to the growth

medium. The relative concentrations of the anilide to cobalamin which produce this effect were of a similar order to those which inhibit the growth of cobalamin-requiring strains. However, the inhibition is not absolute because the extracts of organisms grown in the presence of the anilide with no added cobalamin have considerable activity. On the data available for the upper limit of possible contamination of the anilide analogue with cobalamin this could be due to such contamination; it becomes a factor of possible significance with the high concentrations of analogue relative to cobalamin used in these experiments.

Two experiments indicate that cobalamin is not present in the heated extract of *E. coli* PA 15. The anilide analogue did not inhibit the synthesis of methionine by enzymic extracts of strain PA 15 when extract of heated *E. coli* was present. Furthermore, heated extract derived from strain PA 15 did not alone promote methionine synthesis by enzymic extracts of the cobalamin auxotroph, strain 121/176, which requires cobalamin for this purpose.

The ability of the anilide analogue to inhibit the growth of strains dependent upon cobalamin for growth is in agreement with the findings of Cuthbertson *et al.* (1956). The critical ratio, concentration of anilide : concentration of cobalamin, is higher for growth than for the synthesis of methionine by enzymic extracts. This may reflect differences in the permeability of cobalamin and the analogue for the whole organism, a higher external concentration of analogue being needed to achieve an inhibitory concentration at the site of cobalamin action.

SUMMARY

1. Under certain conditions cobalamin is required for the synthesis of methionine by ultrasonic extracts of strains of *Escherichia coli*. The action of cobalamin is inhibited by the methylamide, ethylamide and anilide of the monocarboxylic acids of cobalamin and by factor B.

2. The anilide analogue inhibits competitively both the action of cobalamin and of 5:6-dimethylbenzimidazole B₁₂-coenzyme, but is less active against the latter.

3. When an extract of heated organisms is used as source of folic acid coenzyme with enzymic extracts of a strain not requiring cobalamin for growth, cobalamin is not required for methionine synthesis and the analogues are not inhibitory. Extracts of organisms grown in the presence of cobalamin do not require added cobalamin for methionine synthesis and the anilide analogue again does not inhibit.

4. The anilide analogue inhibits the growth of strains requiring cobalamin or methionine for

growth when cobalamin is the growth factor, but not when methionine is supplied. It does not inhibit the growth of strains which do not require cobalamin for growth.

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Chemical Changes in Rabbit Aorta and Ear Cartilage Induced by Papain and Calciferol

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It has been shown both histochemically (Thomas, 1956) and chemically (Tsaltas, 1958) that the intravenous administration of a solution of crude papain decreases the mucopolysaccharide content of rabbit-ear cartilage.

With regard to the action of papain *in vivo*, it has been shown by McCluskey & Thomas (1958) that the injection of crystalline papain results in depletion of cartilage matrix only if the enzyme is inactivated by oxidation or sulphhydryl blocking agents before administration. Cysteine-activated crystalline papain produces little effect on cartilage when injected. The changes produced by injection of inactivated crystalline papain were found to be indistinguishable from those produced by crude papain. These workers suggested that the reason for the failure of activated papain to enter cartilage

after intravenous injection is that it probably reacts with one or more substrates present in blood. Oxidized or inactivated papain, on the other hand, appears to be taken up by cartilage and converted into the active form. In the present study a solution of crude non-activated papain (L. Light and Co. Ltd) in 0.9% sodium chloride solution was used.

It has also been shown that after toxic doses of calciferol in rats, there is an increase in total hexose and hexosamine content of the aorta, which parallels the aortic mineralization which occurs (Gillman, Grant & Hathorn, 1960). Because of the possibility that mucopolysaccharides may play an important role in the process of calcification, it was decided to test the effects of papain on the action of calciferol given simultaneously.