Interrelations Between Two Pathways of Methionine Biosynthesis in *Aerobacter aerogenes*

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SUMMARY

Two pathways for methionine methyl formation, one cobalamin-dependent and one cobalamin-independent, corresponding to those observed in *Escherichia coli* PA 15 have been found in *Aerobacter aerogenes*. An initial difficulty in showing the cobalamin-dependent pathway in cell-free extracts proved to be due to the presence of enzymes which caused the removal of adenosylmethionine, a cofactor required for this system. In contrast to *E. coli* PA 15, *A. aerogenes* contains holocobalaminmethyltransferase even when grown in the absence of cobalamin. When cobalamin is added to the growth medium, the cobalamin-independent pathway is repressed.

INTRODUCTION

Escherichia coli has two enzymic mechanisms of methyl group transfer from 5-methyltetrahydropteroylglutamate derivatives to homocysteine to form methionine (Woods, Foster & Guest, 1964). A major difference in these two pathways is that one shows a requirement for a cobalamin derivative and the other does not (Kisliuk & Woods, 1960; Kisliuk, 1961). The cobalamin-requiring system also requires reduced flavin adenine dinucleotide and adenosylmethionine as cofactors (Rosenthal & Buchanan, 1963; Buchanan *et al.* 1964). Either 5-methyltetrahydropteroyltrimethylglutamate or 5-methyltetrahydropteroylglutamate can serve as methyl donor. The cobalamin-independent pathway does not require adenosylmethionine or reduced flavin adenine dinucleotide and will utilize only 5-methyltetrahydropteroyltrimethylglutamate as methyl donor (Guest, Friedman & Foster, 1962).

To demonstrate the cobalamin-dependent system the vitamin must be added to the growth medium or to enzymic extracts. Lacking a cobalamin supplement, *Escherichia coli* PA 15 does not synthesize detectable holocobalaminmethyltransferase (Kisliuk, 1961) although apoenzyme is readily demonstrable (Guest, Friedman, Dilworth & Woods, 1964). A role for cobalamin has not as yet been established for any reaction in *E. coli* other than methionine biosynthesis. *Aerobacter aerogenes* was selected for further study because this organism has been shown to require a cobalamin derivative for the dioldehydrase reaction (Lee & Abeles, 1963). The relative amounts of methionine synthesis were determined under various conditions of growth.

METHODS

Organisms. The strain of *Aerobacter aerogenes* (ATCC 8724) used in these studies was the same organism as used to study the cobamide coenzyme requiring dioldehydrase reaction (Lee & Abeles, 1963). *Escherichia coli* PA 15 is an auxotroph which requires serine or glycine for growth.

Growth conditions. The glucose inorganic salts medium of Davis & Mingioli (1950) was used for the growth of *Aerobacter aerogenes*. This medium was supplemented with 4 μ g. cobalamin/l. when cobalamin-grown organisms were required. Growth was aerobic for 17 hr at 37°. The inoculum was 10 ml. of a 24 hr culture per 15 l. medium. The organisms were harvested in a refrigerated Sharples centrifuge.

Escherichia coli was grown in the same way except that the medium was supplemented with glycine (0.8 g./l.).

The organisms were maintained on agar slopes prepared from the media indicated.

Preparation of extracts. Acetone-dried organisms were prepared and extracted as described by Kisiuk & Woods (1960) except that 1 g. dried organisms were extracted with 20 ml. water and the extracts were not dialysed. Sonic extracts were prepared and fractionated as described by Kisiuk (1961).

The *Escherichia coli* holocobalaminmethyltransferase used in the present work was an ammonium sulphate fraction (0–35% saturation) of a sonic extract of cobalamin-grown organisms (Kisiuk, 1961) containing 16 m μ g. cobalamin/mg. protein. An amount of material containing 22 m μ g. cobalamin was added to each incubation mixture where indicated.

Conditions for methionine synthesis in enzyme extracts. The standard reaction mixture used to study the overall formation of methionine from serine and homocysteine via 5-methyltetrahydrofolate consists of diphosphopyridine nucleotide 5×10^{-4} M, adenosine triphosphate 5×10^{-3} M, pyridoxal phosphate 5×10^{-4} M, fructose-1,6-diphosphate 5×10^{-3} M, MgSO_4 5×10^{-3} M, L-serine 5×10^{-3} M, DL-homocysteine 10^{-2} M, potassium phosphate (pH 7.8) 7.5×10^{-2} M.

The reaction mixtures (2 ml. final volume) were incubated for 4 hr at 37° in Thunberg tubes filled with hydrogen. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. After centrifuging down the precipitate, the supernatant fluid was assayed for methionine.

Assays. L-Methionine was determined microbiologically with *Leuconostoc mesenteroides* (ATCC 8042) by using Difco methionine assay medium. Cobalamin was determined with *Lactobacillus leichmannii* (ATCC 7830) by using Difco B₁₂ assay medium. The thymidine content of all samples was negligible as determined after destroying cobalamin by autoclaving samples in 0.2 N-NaOH for 30 min.

Protein was determined spectrophotometrically (Layne, 1957).

Materials. Adenosine triphosphate, diphosphopyridine nucleotide, flavin adenine dinucleotide and fructose-1,6-diphosphate were products of the Sigma Chemical Company. S-Adenosyl-L-methionine iodide was obtained from the California Corporation for Biochemical Research. Pteroyltriglutamic acid was supplied by Lederle Laboratories through the courtesy of Dr T. H. Jukes. Tetrahydropteroyltriglutamic acid was prepared by catalytic reduction (Kisiuk, 1957). Tetrahydropteroyltriglutamate was prepared similarly except that 10 mg. were reduced with

10 mg. catalyst in 10 ml. glacial acetic acid. After removal of the catalyst by filtration under hydrogen the acetic acid was removed by lyophilization.

RESULTS

Methionine synthesis by bacterial suspensions

Suspensions of *Aerobacter aerogenes* and *Escherichia coli* PA 15 were tested for their ability to synthesize methionine from homocysteine, with serine as the precursor of the methyl group under conditions similar to those described by Gibson & Woods (1960). These workers observed that cobalamin added to suspensions of organisms grown in the absence of cobalamin stimulated methionine synthesis. When cobalamin was included in the growth medium, the harvested organisms no longer were stimulated by its addition (Table 1). Suspensions of organisms grown in the absence of cobalamin synthesized considerably more methionine when cobalamin was added to the suspending medium than did the corresponding cobalamin-grown organisms. This suggests that when the cobalamin-dependent pathway operates maximally during growth the cobalamin-independent pathway is repressed. When cobalamin was added to suspensions of organisms grown in its absence, it probably combined with apocobalaminmethyltransferase present in the organisms, thus enabling them to synthesize methionine by both pathways simultaneously. The results given in Table 1 are in agreement with the conclusion of Rowbury & Woods (1961) that substances which enhance methionine synthesis during growth decrease the ability of suspended organisms to synthesize this amino acid.

Table 1. *Methionine synthesis by suspensions of Aerobacter aerogenes and Escherichia coli*

The suspending medium consisted of potassium phosphate, (pH 7.4), 0.1 M; DL-homocysteine, 0.01 M; D-glucose, 0.02 M; and where indicated L-serine, 0.005 M; cobalamin 7×10^{-6} M. The organisms added were equivalent to 7 mg. dry weight/ml. Incubated at 37° for 3 hr.

Addition to suspension	L-Methionine (μ mmoles/mg. dry wt. organism)	
	Growth without cobalamin	Growth with cobalamin
(1) <i>Aerobacter aerogenes</i>		
None	6.2	10.2
L-serine	7.4	25.4
L-serine + cobalamin	36.0	22.6
(2) <i>Escherichia coli</i>		
None	2.4	2.9
L-serine	11.7	13.1
L-serine + cobalamin	20.3	14.4

Methionine synthesis by extracts of Aerobacter aerogenes

With extracts of acetone-dried *Aerobacter aerogenes* grown in the absence of cobalamin, substantial synthesis of methionine occurred, this synthesis being inhibited by the addition of tetrahydropteroylglutamate (Table 2). However, this

inhibition was not annulled by the addition of cobalaminmethyltransferase as it is in the corresponding *Escherichia coli* PA 15 system (Kisliuk, 1961). With extracts of cobalamin-grown *Aerobacter aerogenes*, methionine synthesis was very small and was further suppressed by tetrahydropteroylglutamate. This again contrasts with the situation in *Escherichia coli* PA 15 where methionine synthesis in extracts of cobalamin-grown organisms was not inhibited by tetrahydropteroylglutamate (Kisliuk & Woods, 1960). These results are consistent with the suggestion above that the presence of cobalamin in the growth medium repressed the cobalamin-independent pathway. Earlier evidence obtained with *E. coli* PA 15 (Kisliuk & Woods, 1960) on the effect of cobalamin in the growth medium in depressing methionine synthesis in extracts may be interpreted in the same manner. Cobalamin-methyltransferase, although present, was not active in extracts of *Aerobacter aerogenes* under the conditions used for Table 2 (see below).

Table 2. *Inhibition of methionine synthesis by tetrahydropteroylglutamate*

The standard reaction mixture was incubated with extracts of *Aerobacter aerogenes* prepared from acetone-dried organisms grown without or with added cobalamin (3.5 mg. protein/incubation in each case). Tetrahydropteroylglutamate 1.8×10^{-4} M.

Extract of	Additions to reaction mixture	L-Methionine (μ mmoles)
(1) <i>A. aerogenes</i> grown without cobalamin	None	300
	+ tetrahydropteroylglutamate	0
	+ tetrahydropteroylglutamate and <i>E. coli</i> cobalaminmethyltransferase	0
(2) <i>A. aerogenes</i> grown with cobalamin	None	20
	+ tetrahydropteroylglutamate	0

Table 3. *Requirements for methionine synthesis by Aerobacter aerogenes extracts*

The complete system consisted of the standard reaction mixture supplemented with flavin adenine dinucleotide and tetrahydropteroyltriglutamate (both 1×10^{-4} M). An extract of acetone-dried *A. aerogenes* (grown in the absence of cobalamin) treated with Sephadex G-50 was used as a source of enzyme (3.8 mg. protein added).

	L-Methionine (μ mmoles)
Complete system	160
Minus serine	10
Minus homocysteine	0
Minus tetrahydropteroyltriglutamate	55
Minus DPN	115
Minus ATP	160
Minus fructose-1,6-diphosphate	35
Minus pyridoxal phosphate	40
Minus Mg^{2+}	60
Minus flavin adenine dinucleotide	145

Requirements for methionine synthesis in Aerobacter aerogenes

Extracts of acetone-dried *Aerobacter aerogenes* (grown in the absence of cobalamin) when treated with Sephadex G-50 (Kisliuk, 1960) and supplemented with all the required cofactors except a folate derivative, showed a negligible methionine

synthesis (Fig. 1). As observed with *Escherichia coli* PA 15 (Kisliuk & Woods, 1960) tetrahydropteroylglutamate did not promote methionine synthesis but tetrahydropteroyltriglutamate did reactivate the system (Jones, Guest & Woods, 1961). The additional requirements for the *A. aerogenes* system are shown in Table 3. In this experiment an absolute requirement was observed only for the substrates serine and homocysteine. The extract was only partially depleted of tetrahydropteroyltriglutamate, diphosphopyridine nucleotide, fructose-1,6-diphosphate, pyri-

Fig. 1

Fig. 2

Fig. 1. The effect of reduced folate derivatives on methionine synthesis in Sephadex-treated extracts of *Aerobacter aerogenes*. The standard reaction mixture was incubated with Sephadex-treated extracts of acetone-dried *A. aerogenes* grown in the absence of cobalamin (3.8 mg. protein) for 4 hr at 37° in a total volume of 2 ml. ●—●, tetrahydropteroylglutamate; ○—○, tetrahydropteroyltriglutamate.

Fig. 2. Assay of fractions of *Aerobacter aerogenes* grown in the presence of cobalamin for cobalaminmethyltransferase activity. Assay conditions are the same as described in Table 5 with *Escherichia coli* cobalamin enzyme omitted. ○—○, fraction 14; ●—●, fraction 14 plus 7.5×10^{-5} M. adenosylmethionine; △—△, fraction 15; ▲—▲, fraction 15 plus 7.5×10^{-5} M adenosylmethionine.

doxal phosphate, Mg^{2+} and flavin adenine dinucleotide. A requirement for adenosine triphosphate would not be expected since the cobalamin-independent pathway proceeds without this cofactor (Guest *et al.* 1962).

Inhibition of the cobalaminmethyltransferase reaction by extracts of Aerobacter aerogenes

When extracts of *Aerobacter aerogenes* and *Escherichia coli* PA 15 were mixed under conditions appropriate for the expression of the cobalamin-independent pathway (Table 4, part (a)) methionine synthesis occurred. When cobalaminmethyltransferase was added (Table 4, part (b)) to *E. coli* extracts, methionine synthesis was greatly stimulated, probably because both pathways were operating simultaneously. There was no corresponding stimulation in *A. aerogenes* extracts nor in a

mixture of *A. aerogenes* and *E. coli* PA 15 extracts. The cobalaminmethyltransferase was apparently inactive in the presence of *A. aerogenes* extracts. This suggestion is substantiated by the results obtained under conditions where the cobalamin-independent pathway was inhibited and only the cobalamin dependent pathway operates, i.e. in the presence of tetrahydropteroylglutamate (Table 4, part (c)). The addition of *A. aerogenes* extract under these conditions greatly decreased methionine synthesis.

Table 4. *The effects of extracts of Aerobacter aerogenes on methionine synthesis by Escherichia coli extracts*

The standard reaction mixture was incubated with extracts of acetone-dried *A. aerogenes* on *E. coli* (grown in the absence of cobalamin) 5.3 and 4.2 mg. protein per incubation, respectively. When the extracts were combined half of each amount was added. Tetrahydropteroylglutamate 1.8×10^{-4} M where indicated.

Type of extract	Additions	L-Methionine (μ mmoles)
(a) <i>E. coli</i>	None	450
<i>A. aerogenes</i>	None	300
<i>E. coli</i> + <i>A. aerogenes</i>	None	500
(b) <i>E. coli</i>	<i>E. coli</i> cobalaminmethyltransferase	1850
<i>A. aerogenes</i>	<i>E. coli</i> cobalaminmethyltransferase	300
<i>E. coli</i> + <i>A. aerogenes</i>	<i>E. coli</i> cobalaminmethyltransferase	500
(c) <i>E. coli</i>	Tetrahydropteroylglutamate	30
<i>E. coli</i>	Tetrahydropteroylglutamate + <i>E. coli</i> cobalaminmethyltransferase	700
<i>E. coli</i> + <i>A. aerogenes</i>	Tetrahydropteroylglutamate + <i>E. coli</i> cobalaminmethyltransferase	100

Table 5. *Annulment of the inhibition of cobalaminmethyltransferase by addition of adenosylmethionine*

The standard reaction mixture was incubated with extracts of acetone-dried *Escherichia coli* PA 15 (6 mg. protein), *E. coli* PA 15 cobalaminmethyltransferase, tetrahydropteroylglutamate 1.8×10^{-4} M and, where indicated, a sonic extract of *Aerobacter aerogenes* (0.66 mg. protein), adenosylmethionine 7.5×10^{-5} M (150 μ mmoles), L-methionine 2.5×10^{-4} M (500 μ mmoles).

Addition	L-Methionine (μ mmoles)
None	250
Adenosylmethionine	250
<i>A. aerogenes</i> extract	30
Adenosylmethionine + <i>A. aerogenes</i> extract	200
Adenosylmethionine + <i>A. aerogenes</i> extract minus L-serine	30
<i>A. aerogenes</i> extract + L-methionine	550

The nature of the inhibitor in Aerobacter aerogenes extracts

When extracts of *Aerobacter aerogenes* were heated for 5 min. at 100° they were no longer inhibitory, suggesting that the inhibitor is an enzyme. It was considered that the inhibition might be due to enzymic removal of methionine; however, this appeared not to be the case since added methionine was recovered at the end of the incubation (Table 5). It was then observed that the inhibition was annulled by the addition of adenosylmethionine (Table 5). It seems likely therefore that the inhibition of cobalaminmethyltransferase by *A. aerogenes* extracts was due to the ability of these extracts to metabolize adenosylmethionine which is required for this

system in catalytic amounts. Shapiro (1962) observed that extracts of this organism metabolized adenosylmethionine by (1) methylating homocysteine to methionine and adenosylhomocysteine, and (2) decomposition to adenine, methylthioribose and homoserine. The results of Table 5 indicate that the former reaction might account for 20 % of the breakdown of adenosylmethionine (30 of 150 μ mmoles). The remainder was presumably destroyed by the latter reaction.

*Isolation of cobalaminmethyltransferase from Aerobacter aerogenes
grown in the absence and presence of cobalamin*

To show conclusively the presence of cobalaminmethyltransferase in extracts of *Aerobacter aerogenes*, sonic extracts were fractionated with ammonium sulphate and calcium phosphate gel by the method of Kisliuk (1961). The distribution of

Table 6. *Fractionation of sonic extracts of Aerobacter aerogenes*

Fraction no.	Protein (mg.)	Cobalamin (μ mg./mg. protein)	Protein (mg.) for 50 % inhibition*
(a) Organisms grown without cobalamin			
(NH ₄) ₂ SO ₄ fractionation:			
	Sonic extract (45 mg. protein/ml.)	7050	2.4
1	0-30 % saturation	240	5.5
2	30-40 % saturation	1560	3.3
3	40-50 % saturation	1136	3.5
4	50-60 % saturation	140	2.8
Ca ₃ (PO ₄) ₂ gel:			
	(NH ₄) ₂ SO ₄ , 30-40 % saturation	1190	3.3
5	Buffer supernatant, 0.01 M	35	1.6
6	Buffer supernatant, 0.05 M	122	10.4
7	Buffer supernatant, 0.10 M	171	2.3
8	Buffer supernatant, 1.0 M	343	2.2
(b) Organisms grown with cobalamin			
(NH ₄) ₂ SO ₄ fractionation:			
	Sonic extract (41 mg. protein/ml.)	6953	15.2
9	0-30 % saturation	432	9.1
10	30-40 % saturation	1134	21.0
11	40-50 % saturation	1280	22.0
12	50-60 % saturation	304	15.3
Ca ₃ (PO ₄) ₂ gel:			
	(NH ₄) ₂ SO ₄ , 30-40 % saturation	1134	21.0
13	Buffer supernatant, 0.01 M	46	34.6
14	Buffer supernatant, 0.05 M	158	43.8
15	Buffer supernatant, 0.10 M	110	15.5
16	Buffer supernatant, 1.0 M	384	2.7

* To determine this value, the fraction was added at various concentrations to an assay system containing the standard reaction mixture + tetrahydropteroylglutamate (1.8×10^{-4} M), an extract of acetone-dried *E. coli* PA 15 (7 mg. protein) and *E. coli* PA 15 cobalaminmethyltransferase. In each case the inhibition could be annulled with adenosylmethionine.

† Results with these fractions are shown in Table 7.

‡ Results with these fractions are shown in Fig. 2.

cobalamin in these fractions (Table 6) was similar to that obtained with cobalamin-grown *Escherichia coli* (Kisliuk, 1961). Only fraction 6 (Table 6) appeared to be entirely free from inhibitor (Table 7). Fractions 7, 14, 15 showed inhibition which

was annulled with adenosylmethionine (Table 7, Fig. 2). In subsequent trials fractions equivalent to fraction 14 were also obtained free from inhibitor.

Two fractions (Table 7) obtained from organisms grown in the absence of cobalamin were tested for cobalaminmethyltransferase activity (adenosylmethionine added) separately. Activity was demonstrable in both fractions, showing that holocobalaminmethyltransferase was endogenously synthesized. In other organisms so far studied, *Escherichia coli* (Kisliuk, 1961), pigs (Loughlin, Elford & Buchanan, 1964) and chickens (Dickerman, Redfield, Bieri, & Weissbach, 1964), it is formed from exogenous cobalamin.

Table 7. Assay of fractions of *Aerobacter aerogenes* grown in the absence of cobalamin for cobalaminmethyltransferase activity

Assay conditions are the same as described in Table 5. Incubations 1 to 5 contain adenosylmethionine at 7.5×10^{-5} M.

	L-Methionine (μ mmoles)
(1) <i>E. coli</i> PA 15 cobalaminmethyltransferase omitted	0
(2) + fraction 6 (6.6 μ mg. cobalamin)	460
(3) + fraction 6 (18.2 μ mg. cobalamin)	570
(4) + fraction 7 (2.4 μ mg. cobalamin)	165
(5) + fraction 7 (4.8 μ mg. cobalamin)	280
(6) <i>E. coli</i> PA 15 cobalaminmethyltransferase included	270
(7) + fraction 6 (6.6 μ mg. cobalamin)	410
(8) + fraction 6 (18.2 μ mg. cobalamin)	590
(9) + fraction 7 (2.4 μ mg. cobalamin)	310
(10) + fraction 7 (4.8 μ mg. cobalamin)	180

The cobalamin content of the various fractions was considerably enhanced by growth on cobalamin. However, the specific activity did not change. The $m\mu$ moles methionine formed per $m\mu$ g cobalamin when 5 $m\mu$ g cobalamin was added to the assay system was 61 for the material prepared from cobalamin grown organisms (Fig. 2) and 57 for the corresponding preparations from organisms grown in the absence of cobalamin (Table 7). The activity of the *Escherichia coli* preparation used in the present work (12 $m\mu$ moles/ $m\mu$ g.) was somewhat lower because of deterioration in storage.

DISCUSSION

During this work, the late Professor D. D. Woods kindly informed us of experiments made in his laboratory in which methylcobalamin served as a methyl donor to homocysteine when incubated with extracts of *Aerobacter aerogenes*. This provides further evidence that cobalaminmethyltransferase is present in this organism. Although the cobalamin-dependent pathway was inactive in extracts because of removal of adenosylmethionine it apparently does function *in vivo*, otherwise one would not expect the observed repression of the cobalamin-independent pathway due to increased synthesis of methionine in cobalamin-grown organisms.

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