The Synthesis of Methionine by Suspensions of *Escherichia coli*

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(Received 14 July 1959)

The first indication that *p*-aminobenzoic acid is concerned in the synthesis of methionine by micro-organisms was the observation that methionine, under appropriate conditions, overcame inhibition of growth by sulphonamides in a non-competitive manner (Harris & Kohn, 1941; Kohn, 1943; Bliss & Long, 1941). Since then methionine has frequently been shown with various organisms to be an essential component of mixtures which replace *p*-aminobenzoic acid either in overcoming sulphonamide inhibition or in supporting growth of organisms requiring this vitamin (e.g. Lampen, Jones & Roepke, 1949; Strehler, 1950; Cutts & Rainbow, 1950). There is thus strong presumptive evidence that *p*-aminobenzoic acid has a function at one or more steps in methionine synthesis.

A role for cobalamin in the same synthesis was first found by Davis & Mingioli (1950), who isolated mutants of *Escherichia coli* requiring for growth either methionine or cobalamin; the relative concentrations required suggested that cobalamin might function as a catalyst in the synthesis of methionine.

Studies of the nutritional properties of mutant strains of bacteria and moulds requiring methionine for growth have provided strong presumptive evidence that this amino acid is derived from cysteine through the intermediary formation of cystathionine and homocysteine; the last step is thus the methylation of homocysteine (Horowitz, 1947; Teas, Horowitz & Fling, 1948; Lampen, Roepke & Jones, 1947; Gots & Koh, 1950; Gots, 1951; Plough, Miller & Berry, 1951). Since homocysteine will not replace methionine in the sparing of either *p*-aminobenzoic acid or cobalamin, it is likely that the function of both these vitamins is at the last, or methylation, step in the formation of methionine. The work of Emerson (1950) with *Neurospora* suggests that this stage may be complex.

The object of the present work was to obtain a simpler system for the synthesis of methionine than the whole growing culture, and one in which all the reactants could be clearly defined. It should then be possible to study with greater precision the roles of *p*-aminobenzoic acid and cobalamin, and to

build up a background of knowledge from which to extend the work to isolated enzyme systems. At the present stage two points were of particular importance: (a) the nature of the ultimate source of the one-carbon-atom unit which gives rise to the methyl group of methionine; (b) the question whether the function of *p*-aminobenzoic acid is exerted after its conversion into a member of the folic acid group; there was no evidence on this point at the time the work was begun.

E. coli was chosen for the present work because (a) a number of auxotrophic mutants were available in which it was possible to control experimentally the presence or absence of a number of the metabolites in question, and (b) preliminary tests showed that suspensions did not destroy added methionine to any appreciable extent.

The results with whole organisms in washed suspensions are here reported in some detail since they form an essential background to later work with cell-free enzyme preparations. Part of the present work has been the subject of preliminary reports (Gibson & Woods, 1952; Gibson, 1952).

METHODS

Organisms and media

Escherichia coli. A number of strains (Table 1), many of them mutants requiring amino acids or vitamins for growth, were used. All were maintained by monthly subculture on tryptic digest of meat-agar slopes stored at 4° after incubation for 18 hr. at the required temperature (Table 1); they were stable during the period of the work.

The growth medium (A) for the preparation of suspensions of organisms was that of Davis & Mingioli (1950), except that the glucose was autoclaved with the other constituents. It was supplemented when necessary with the required growth factor at the concentrations given in Table 1. The supplement used for the growth of mutants requiring *p*-aminobenzoic acid in the partial or complete absence of that factor was (per litre of final medium): either (a) acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld 1945), equivalent to 1.5 g. of original casein; thymine, 20 mg.; adenine, guanine and xanthine, each 5 mg. (supplement E); or (b) glycine, 20 mg.; DL-serine, DL-threonine, DL-lysine hydrochloride, DL-histidine hydrochloride and DL-tyrosine, each 40 mg.; DL-methionine, 10 mg.; thymine, 30 mg.; adenine, guanine, xanthine and uracil, each 5 mg. (supplement F). Both supplements were adjusted to pH 7.0.

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Table 1. *Strains of Escherichia coli used*

Strain no.*	Requirement for growth on medium A		Incubation temp.	Inoculum for bulk cultures†
	Substance	Concn. used		
273/384 (A.T.C.C. 9723a)	<i>p</i> -Aminobenzoic acid or Replacement mixture (see text)	μM See text	37° 37	0.1 ml.; 1 in 75
200 (from C 181)	Cobalamin or Methionine	1 $\mu\text{mg./ml.}$ 0.13 mM (DL-)	30 30	0.1 ml.; 1 in 75
113/3	Cobalamin or Methionine	1 $\mu\text{mg./ml.}$ 0.13 mM (DL-)	37 37	0.1 ml.; 1 in 75
PA 15	Serine or Glycine	10 mM (DL-) 10 mM	37 37	0.1 ml.; 1 in 10
55/5	Serine or Glycine	10 mM (DL-) 10 mM	37 37	0.1 ml.; 1 in 10
	None	—	25	0.2 ml.; undiluted
518 (A.T.C.C. 9723)	None	—	37	0.1 ml.; 1 in 75
86 (N.C.T.C.)	None	—	37	0.1 ml.; 1 in 75

* Strains 200, 113/3 and 55/5 were from the collection of Dr B. D. Davis; strains 273/384 and 518 were provided by Dr R. Roblin and PA 15 by Dr Barbara Wright.

† Volume and dilution of inoculum development culture (see text) used to inseminate 100 ml. cultures for the preparation of suspensions of organisms.

Leuconostoc mesenteroides P 60. This organism (American Type Culture Collection no. 7881) was used for the assay of methionine and glycine; it was maintained as described by Lascelles, Cross & Woods (1954). The basal medium was modified from that used by these authors for serine assay and contained (per litre of final medium): DL-alanine, 500 mg.; L-arginine hydrochloride, 200 mg.; DL-aspartic acid, 200 mg.; DL-asparagine, 800 mg.; L-cysteine hydrochloride, 130 mg.; DL-glutamic acid, 600 mg.; DL-histidine hydrochloride, 60 mg.; DL-isoleucine, 240 mg.; DL-leucine, 240 mg.; DL-lysine hydrochloride, 100 mg.; DL-phenylalanine, 100 mg.; DL-proline, 50 mg.; DL-serine, 210 mg.; DL-threonine, 60 mg.; DL-tryptophan, 40 mg.; DL-tyrosine, 200 mg.; DL-valine, 240 mg.; glucose, 25 g.; sodium acetate trihydrate, 20 g.; NH_4Cl , 3 g.; KH_2PO_4 , 0.6 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.6 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 20 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg.; xanthine, 12 mg.; adenine, guanine and uracil, each 10 mg.; riboflavin, 0.5 mg.; nicotinic acid, 1 mg.; calcium pantothenate, 0.6 mg.; thiamine hydrochloride, 0.6 mg.; biotin, 1 $\mu\text{g.}$; *p*-aminobenzoic acid, 0.14 mg.; pH adjusted to 6.8. Pyridoxal (Seitz-filtered) was added with the inoculum to a final concentration of 0.2 μM . The basal medium was prepared as described for the medium L of Lascelles *et al.* (1954). Glycine (75 mg./l.) or DL-methionine (75 mg./l.) was added for the assay of methionine and glycine respectively.

Preparation of suspensions of *Escherichia coli*

Medium A (2 ml. in 125 mm. $\times$ 16 mm. tubes), containing the appropriate growth factor, was inoculated from a fresh slope culture and incubated for 24 hr. (48 hr. with *E. coli*

55/5) at the temperature shown in Table 1. Roux bottles containing the same medium (100 ml.) were sown with 0.1 ml. of a dilution of this culture (Table 1) chosen to yield growth equivalent to 0.05–0.1 mg. dry wt. of organisms/ml. after incubation for about 16 hr. in the horizontal position. The organisms were centrifuged, washed once on the centrifuge with half the culture volume of 6.7 mM-phosphate buffer, pH 7.4, and finally suspended in 0.133 M-buffer at twice the concentration desired in the solution used for testing methionine synthesis.

In some earlier experiments, the organisms were incubated (0.1 mg. dry wt./ml.) for 5 hr. at 37° in a mixture of glucose (0.01 M) and phosphate buffer (0.067 M; pH 7.4) before testing for methionine synthesis; they were then centrifuged and suspended as above. Such organisms will be referred to as 'preincubated'; under certain conditions they showed increased ability (variable and never more than twofold) to synthesize methionine compared with untreated organisms.

Assessment of growth and mass of organisms

The extent of growth of cultures and the dry weight of suspensions of organisms was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) in 6 mm. tubes and a neutral density filter. The relationship between galvanometer reading and dry weight was determined for each of the organisms used. With the different strains of *E. coli* the dry wt./ml. corresponding to a reading of 10 varied only from 0.29 to 0.36 mg.

Throughout this paper the quantity of organisms expressed as milligrams refers to the dry weight.

General procedure for study of methionine synthesis

Reaction mixture. The organisms were suspended at a concentration of 0.1–0.4 mg. dry wt./ml. in solution *M* (2.5 ml.) which contained basically: DL-homocysteine (6 mM), glucose (0.02 M) and phosphate buffer (0.067 M, pH 7.4). The tubes (125 mm. × 16 mm.) were incubated upright in air at 37° unless otherwise stated; anaerobic conditions did not improve methionine synthesis.

In each experiment there was a control in which the complete reaction mixture was heated for 5 min. at 100° at zero time.

Preparation of material for analysis. The reaction was stopped, and any free methionine within the organisms liberated, by heating to 100° for 10 min. Samples of the supernatant fluid after centrifuging were used for microbiological assay and chromatography.

In some experiments to confirm the identity of methionine the products were acid-hydrolysed or oxidized. For the former, the sample (10 ml.) was mixed with 10 N-HCl (10 ml.) and refluxed for 4 hr. Excess of acid was removed by evaporation to dryness under reduced pressure; this was twice repeated, 10 ml. of water being added between each distillation. The final residue was taken up in a little water, neutralized with 0.1 N-NaOH and diluted to the original volume. For oxidation, the sample (10 ml.) was treated with an equal volume of 30% (w/v) H_2O_2 for 1 hr. at room temperature and the solution evaporated to dryness on a boiling-water bath; the destruction of H_2O_2 was completed by two further evaporations after adding water (20 ml.). The residue was dissolved in 10 ml. of water.

Microbiological assay of methionine and glycine

Leuconostoc mesenteroides P 60. This organism has been reported as suitable for the assay of methionine and glycine (Riesen, Schweigert & Elvehjem, 1946; Dunn, Camien, Shankman & Block, 1946). This was confirmed in the present work and a linear and reproducible growth response was obtained for quantities of both DL-methionine and glycine in the range 0.01–0.1 μ mole (Fig. 1).

Procedure. The technical details were as described for the assay of serine with the same organism (Lascelles *et al.*

1954; Lascelles & Woods, 1954), except that (a) the modified media described above were used, (b) the organisms of the inoculum were washed once on the centrifuge with twice the culture volume of water and resuspended in the culture volume of water, (c) incubation was always in air and for 44–48 hr.

The samples under test (or appropriate dilutions) at two different concentrations were added in quantities of 0.1–0.8 ml.; standards containing three different quantities of DL-methionine (usually 0.01, 0.04 and 0.1 μ mole) or glycine (0.02, 0.08 and 0.2 μ mole) were set up in each complete assay.

Growth response to experimental samples. It was possible to match the dose/response curve for typical samples from experiments with suspensions of organisms to that for pure methionine or glycine over the whole of the useful assay range (Fig. 1). In methionine assays on samples containing less than 0.01 μ mole/ml. (and which had therefore to be added undiluted to the assay tubes in up to 0.8 ml. amounts) the growth attained with larger amounts was occasionally less than would have been expected from the growth given by half the quantity; in such cases the higher value for methionine was accepted since it appeared that material inhibitory to the assay organism was present. Values for methionine of less than 0.2 μ mole/mg. dry wt. of organisms in the terminology below were therefore subject to greater than the usual error.

Specificity. The organism responded only to the L-isomer of methionine; the racemic mixture was used as standard since the presence of D-isomer did not affect the results.

The samples under assay were likely to contain relatively high concentrations (with respect to biological activity) of components of solution *M* and experimental supplements. Various mixtures of homocysteine, serine, cobalamin, pteroylglutamic acid, *N*⁵-formyltetrahydropteroylglutamic acid, choline and betaine neither replaced methionine for *Leuconostoc mesenteroides* P 60 nor affected growth in optimum methionine concentrations. The heated control (see above) also served as a check in this respect. Glycine was not replaced by glycolic acid or betaine.

Assessment of results. The methionine or glycine content of samples was obtained by direct comparison of the responses obtained at two concentrations with the dose/response curve given by the standards. Results are expressed as μ moles of L-methionine or glycine formed/mg. dry wt. of *E. coli*. Variation in replicate experiments, and the recovery values for methionine or glycine added to typical samples, showed that the over-all accuracy of the method was about $\pm 10\%$.

Microbiological assay of folic acid

Lactobacillus casei factor. The assay described by Lascelles & Woods (1952) was used. Results are expressed as concentration of the factor in the samples in terms of pteroylglutamic acid, which was used as standard.

Leuconostoc citrovorum factor. The details of the procedure given by Lascelles & Woods (1954) were followed; the standard was *N*⁵-formyltetrahydropteroylglutamic acid (leucovorin) and results are expressed in terms of the equivalent concentration of this substance.

Chromatography

Paper. Descending chromatograms were developed on Whatman no. 1 paper with either (a) 'butan-1-ol-acetic acid' [the upper layer of butan-1-ol-acetic acid-water

Fig. 1. Microbiological assay of (a) methionine and (b) glycine with *L. mesenteroides* P 60. ●, Pure methionine and glycine; ○, experimental samples from a test with suspensions of *E. coli* 55/5 incubated in solution *M* plus serine (10 mM) and cobalamin (10 μ g./ml.). Growth is recorded as EEL photoelectric colorimeter readings.

Table 2. *Synthesis of methionine by suspensions deficient in p-aminobenzoic acid or cobalamin*

Organisms (0.1 mg./ml., strain 273/384; 0.18 mg./ml., strain 200) were incubated for 16 hr. and 8 hr. respectively in solution *M* modified as indicated. *p*-Aminobenzoic acid, μM ; cobalamin, 10 $\mu\text{mg./ml.}$ 'Preincubated' cells were incubated at 37° for 5 hr. in glucose (0.01 *M*) and phosphate buffer (0.067 *M*, pH 7.4) before testing for methionine synthesis.

Strain	Substances added (+) or omitted (-)				L-Methionine formed ($\mu\text{moles/mg. of organisms}$)	
	DL-Homo- cysteine	Glucose	<i>p</i> -Amino- benzoate	Cobalamin	Untreated organisms	'Preincubated' organisms
273/384 (deficient in <i>p</i> -aminobenzoic acid)	+	+	+	-	.	0.35
	+	+	+	+	.	0.60
	+	+	-	+	.	<0.05
	+	-	+	+	.	0.07
	-	+	+	+	.	<0.05
200 (deficient in cobalamin)	+	+	-	+	0.60	0.86
	+	+	-	-	<0.05	<0.05
	+	-	-	+	<0.05	0.17
	-	+	-	+	<0.05	<0.05
	+	+	+	-	<0.05	.
	+	+	+	+	0.58	.

(4:1:5, by vol.)] or (b) 'phenol-water' (a saturated solution of water in A.R. phenol; the atmosphere of the tank was saturated with acetic acid vapour). The chromatograms were run until the solvent front had advanced 30–40 cm. (18–24 hr. at room temperature). The detecting agent was ninhydrin.

Resin. Columns of Dowex-50 resin were used for the separation of glycine from certain reaction mixtures. The procedure of Moore & Stein (1951) was used with the details given by Lascelles & Woods (1954).

Chemicals

DL-Homocysteine. Commercial DL-homocysteine was reduced with metallic sodium in liquid ammonia (du Vigneaud, Audrieth & Loring, 1930); excess of ammonia was removed under vacuum and the product dissolved in 0.5 *N*-HCl and stored at 0° under N_2 (' O_2 -free'). It was neutralized with 2 *N*-NaOH just before use. The stock solution was 0.1 *M* with respect to DL-homocysteine, but iodometric titration (Lavine, 1935) showed that some homocystine (up to 0.02 *M*) was also present. To minimize auto-oxidation the homocysteine was always added last to reaction mixtures.

Vitamins. The cobalamin used was Anacobin (British Drug Houses Ltd., London). Members of the folic acid group were as described by Lascelles *et al.* (1954).

Other chemicals. Amino acids and other constituents of the chemically defined media were commercial products recrystallized only if suspected of containing traces of the substances under investigation.

Phosphate buffers were prepared from stock solutions of Na_2HPO_4 , $12\text{H}_2\text{O}$ and KH_2PO_4 .

RESULTS

Suspensions of organisms deficient in p-aminobenzoic acid

Preparation of suspensions. *E. coli* 273/384, which requires *p*-aminobenzoic acid for growth on the glucose-ammonium salts medium (*A*), can be grown on a medium devoid of this factor provided

that thymine, purines and a mixture of certain amino acids are present (Lampen *et al.* 1949). In the present work these amino acids were supplied either as the pure DL-compounds (supplement *F*) or, with other amino acids, as casein hydrolysate (supplement *E*). In each case $\mu\text{MM-p-aminobenzoic acid}$ was also added; this is only one-tenth of the concentration required for just visible growth in the basal medium but led to more consistent and somewhat heavier growth with the supplements. It did not affect the requirement for *p*-aminobenzoic acid for methionine synthesis by the harvested organisms.

Requirements for the synthesis of methionine. Such organisms formed methionine when incubated in solution *M*, which contained only buffered glucose, homocysteine and *p*-aminobenzoic acid. There was little or no synthesis in the absence of any of these compounds, though it was just detectable when glucose only was omitted (Table 2). Cobalamin further increased methionine synthesis, but to a variable extent (see also Table 5); it was normally added in all further tests.

The amount of methionine formed was proportional to the concentration of *p*-aminobenzoic acid over the range 0.01–0.1 μM (Fig. 2*a*); 0.01 μM is also about the limiting concentration for growth on the basal medium *A*.

Neither pteroylglutamic acid nor its N^5 -formyl-tetrahydro-derivative (leucovorin) replaces *p*-aminobenzoic acid for the growth of this mutant (Lampen *et al.* 1949; Woods, 1952); they were also inactive (at μM) for the production of methionine by suspensions.

Concurrent synthesis of methionine and folic acid. Although the two synthetic forms of folic acid were inactive, it was possible that other forms were produced by the organism itself from *p*-aminobenzoic

acid and promoted methionine synthesis. Lascelles & Woods (1952; and unpublished work) found suspensions of this strain of *E. coli* to synthesize from *p*-aminobenzoic acid a form of folic acid active for the growth of *Lactobacillus casei* but giving a dose/response curve somewhat different from that for either pteroylglutamic acid or leucovorin.

The conditions necessary for the formation of

methionine in solution *M* were also sufficient for the concurrent production of material(s) active for the growth of both *Lactobacillus casei* and *Leuconostoc citrovorum* (Table 3); they were not formed in the absence of *p*-aminobenzoic acid.

Inhibition of synthesis of methionine by sulphathiazole. The utilization of *p*-aminobenzoic acid by strain 273/384 both for growth and for synthesis of folic acid by suspensions is inhibited competitively by sulphonamides (Lascelles & Woods, 1952). The effect of sulphathiazole on methionine production by suspensions of organisms rich and poor in pre-formed folic acid was tested as follows. Organisms harvested from the medium deficient in *p*-aminobenzoic acid were divided into four portions; one of these (I) was held at 0° while the other parts were further treated. The second portion (II) was incubated with glucose, glutamate and *p*-aminobenzoic acid in order to load the organisms with folic acid (Lascelles & Woods, 1952). The other two portions were similarly incubated except that in one *p*-aminobenzoic acid was omitted (III) and in the other sulphathiazole was added (IV); in both (III) and (IV) the organisms should remain low in folic acid (Lascelles & Woods, 1952). Each batch of treated organisms was re-harvested and washed once on the centrifuge. The effect of *p*-aminobenzoic acid and sulphathiazole on methionine synthesis in solution *M* was then tested (Table 4).

Fig. 2. Effect of the concentration of (a) *p*-aminobenzoic acid and (b) cobalamin on the synthesis of methionine by suspensions of *E. coli*: (a) strain 273/384 and (b) strain 200. Organisms (0.33 mg./ml.) were incubated in solution *M* for 16 hr.

Table 3. Concurrent formation of methionine and folic acid

Organisms (strain 273/384) were incubated for 18 hr. in solution *M* plus cobalamin (10 μmg./ml.) with or without *p*-aminobenzoic acid (μM).

Expt. no.	Concn. of organisms (mg./ml.)	<i>p</i> -Amino-benzoic acid	Product		
			L-Methionine (mm)	<i>Lactobacillus casei</i> factor (μmm)	<i>Leuconostoc citrovorum</i> factor (μmm)
1	0.23	-	0.02	0.1	0.3
		+	0.36	6.4	8.0
2	0.28	-	0.02	<0.1	0.1
		+	0.24	8.0	10.3

Table 4. Effect of sulphathiazole on synthesis of methionine

Treatment: *E. coli* 273/384 (0.25 mg./ml.) were incubated for 5 hr. at 37° in 0.067 M-phosphate buffer containing the substances indicated [glucose, 10 mM; L-glutamate, 10 mM; *p*-aminobenzoic acid (*p*-AB), 10 μM; sulphathiazole, mM]. Test for methionine synthesis: Treated organisms (0.25 mg./ml.) were incubated for 13 hr. at 37° in solution *M* plus cobalamin (10 μmg./ml.) with and without sulphathiazole and *p*-aminobenzoic acid as shown.

Additions for test of methionine synthesis		L-Methionine formed (μmole/mg. of organisms) by organisms previously treated with			
		Nothing (control) (I)	Glucose, glutamate, <i>p</i> -AB (II)	Glucose, glutamate (III)	Glucose, glutamate, <i>p</i> -AB, sulphathiazole (IV)
Sulphathiazole (mM)	-	<0.05	0.06	0.05	<0.05
	-	0.74	0.78	0.60	0.56
	+	<0.05	0.63	0.05	<0.05
	+	<0.05	0.68	0.05	<0.05
	+	0.64	.	.	0.68
<i>p</i> -AB					
	.				
	μM				
	.				
	μM				
	2 mM				

Synthesis of methionine by the untreated organisms (I) in the presence of *p*-aminobenzoic acid was inhibited by sulphathiazole and the inhibition was overcome by increasing the concentration of *p*-aminobenzoic acid. The organisms (II) containing preformed folic acid produced, even in the absence of added *p*-aminobenzoic acid, 80 % as much methionine as the untreated batch plus this vitamin. This is not due to carry-over of free *p*-aminobenzoic acid from the treatment system since methionine formation was not in this case inhibited by sulphathiazole. The control batches of treated organisms (III and IV) behaved in essentially the same way as the untreated one; the treatment would not have led to the formation of intracellular folic acid.

These experiments provide support for the idea that the function of *p*-aminobenzoic acid in methionine synthesis is exerted after its conversion into a folic acid-like compound; they do not, however, exclude the possibility that the active substance is not a folic acid but is formed from *p*-aminobenzoic acid by a sulphonamide-sensitive reaction.

Other strains of Escherichia coli. Methionine synthesis by *E. coli* 518 (the parent strain of the mutant used above) and by *E. coli* 200 (a cobalamin auxotroph) was neither dependent on nor stimulated by *p*-aminobenzoic acid; neither strain requires the vitamin for growth and they can presumably synthesize it. A double mutant (Y44) requiring both *p*-aminobenzoic acid and histidine for growth was again dependent on *p*-aminobenzoic acid for methionine synthesis by harvested suspensions (S. Wijesundera & D. D. Woods, unpublished experiments).

Suspensions of organisms deficient in cobalamin

Preparation of suspensions. Certain mutant strains of *E. coli* (Davis & Mingioli, 1950) will grow on medium *A* only if provided either with cobalamin or methionine. It was likely therefore that

growth with methionine would produce organisms at least relatively deficient in vitamin B₁₂ compared with other strains. There is so far no satisfactory evidence whether cobalamin auxotrophs can or cannot synthesize the vitamin in quantities insufficient to support growth. *E. coli* 200 harvested from medium *A* containing 0.13 mm-methionine was used for most of the work reported.

Requirements for methionine synthesis. Methionine was formed by organisms incubated in solution *M* only if cobalamin was added (Table 2). The amount synthesized bore a linear relation to the concentration of cobalamin from about 0.1–1.0 µmg./ml. (Fig. 2*b*); 0.1 µmg./ml. supports about 80 % of maximal growth of the organism on medium *A*.

Of the other constituents of solution *M*, homocysteine was essential, but there was 20 % methionine formation (with 'preincubated' organisms only) in the absence of glucose (Table 2).

Effect of anaerobic conditions on the requirement for cobalamin. Dubnoff (1950) has suggested that cobalamin is required for the reduction of homocystine to homocysteine. Control suspensions incubated in solution *M* without cobalamin gave a strongly positive nitroprusside reaction, showing that free thiol groups were still present and that the homocysteine had not become completely oxidized.

Incubation in N₂ or H₂ instead of air did not affect the absolute requirement for cobalamin for methionine production by strain 200. Similarly, it did not increase methionine synthesis by strain PA 15 in the absence of added cobalamin; the synthesis by this organism is increased about five-fold by the vitamin (see below).

Other strains of Escherichia coli. Methionine formation by suspensions of strain 113/3 (another mutant requiring methionine or cobalamin for growth) was also negligible unless cobalamin were added (Table 5).

With five other strains two- to five-fold increases in synthesis were obtained on adding cobalamin to

Table 5. *Effect of cobalamin on synthesis of methionine by various strains of Escherichia coli*

Organisms at the stated concentration were incubated at 37° in solution *M* supplemented as required with µM-*p*-aminobenzoic acid (strain 273/384) or 10 mM-DL-serine (strains PA 15 and 55/5). Cobalamin concentration was 10 µmg./ml., when present.

Strain of <i>E. coli</i>	Growth factor required	Concn. of organisms (mg./ml.)	Time of incubation (hr.)	L-Methionine formed (µmoles/mg. of organisms)	
				Without cobalamin	With cobalamin
113/3	Cobalamin or methionine	0.21	16	<0.05	0.67
86	None	0.21	15	0.59	1.80
518	None	0.37*	17	0.10	0.54
273/384	<i>p</i> -Aminobenzoic acid	0.25*	13	0.23	0.72
PA 15	Serine	0.32	8	0.57	4.00
55/5	Serine	0.32	8	1.40	2.50

* Preincubated organisms (see Table 2).

solution *M* (Table 5). With the one strain (273/384) tested extensively the increase also varied within these limits with five different batches of organisms. Since the growth of these strains on medium *A* is neither dependent on nor increased by cobalamin it was not expected that it would affect methionine formation by suspensions; it has not been determined whether the organisms are relatively deficient in the vitamin at the time of harvesting or whether losses occur during the preparation of the suspensions.

Serine as source of the methyl group in methionine synthesis

In the experiments so far described no substance was added to the reaction mixture specifically as source of the one carbon atom unit; the carbon atom of the methyl group of methionine must therefore have been derived from glucose, homocysteine or endogenous cell constituents.

Effect of serine and other one-carbon-atom donors. In early experiments with strains requiring *p*-aminobenzoic acid or cobalamin a number of substances (e.g. formate, betaine, choline, glycine), known to act as precursors or sources of methyl groups in animal metabolism, failed to increase methionine synthesis in solution *M*. In nine

experiments serine alone gave some increment, though to a highly variable extent (sometimes none at all), with different batches of either organism (Table 6). When active, serine increased both the rate and extent of methionine formation.

Inhibition of methionine synthesis by carbon dioxide. Lascelles *et al.* (1954) found CO₂ plus glycine to replace serine for the growth of *Leuconostoc mesenteroides* P60, and it was at one time thought possible that CO₂ might serve as ultimate source of a one-carbon-atom unit for the formation of serine from glycine by this organism (Lascelles, Cross & Woods, 1951). Methionine synthesis by suspensions of strain 200 was not increased by an atmosphere enriched with CO₂; on the contrary it was reduced to about at least a half and in some cases was completely suppressed (Table 7). Similar results were obtained with two normal strains (86 and 518), synthesis being reduced by 86 and 41% respectively. The slight fall in pH in the presence of CO₂ did not account for these results.

Since one possible explanation of the effect of CO₂ was that it prevented the formation of a one-carbon-atom donor, a number of such compounds (and others) were tested for ability to overcome the inhibition of methionine synthesis. DL-Serine (10 mM) restored synthesis almost completely

Table 6. *Effect of serine on synthesis of methionine by Escherichia coli strains 200 and 273/384*

Organisms were incubated in solution *M* plus (strain 200) 10 µmg. of cobalamin/ml. for 8 hr. or (strain 273/384) µM-*p*-aminobenzoic acid for 8–16 hr.

Strain of <i>E. coli</i>	Number of experiments	Concn. of organisms (mg./ml.)	Percentage increase in L-methionine formed (µmoles/mg. of organisms) with serine (10 mM) added
200	6	0.18–0.31	0–55
273/384	3	0.15–0.26	33–154

Table 7. *Inhibition of the synthesis of methionine by carbon dioxide*

E. coli 200 was incubated in solution *M* plus cobalamin (10 µmg./ml.) and other additions, as stated, in open tubes (controls) or in Thunberg tubes evacuated and filled with 20% (v/v) CO₂ in air.

	Expt. no.				
	1	2	3	4	5
Concn. of organisms (mg./ml.)	0.28	0.28	0.32	0.24	0.21
Time of incubation (hr.)	16	16	16	9	8
L-Methionine formed in control (µmoles/mg. of organisms)	1.29	2.40	0.44	0.95	0.65
Percentage suppression of methionine formation by CO ₂					
Addition (10 mM)	84	76	100	49	41
None		3	13	1	3
DL-Serine	.	.	.	49	.
Choline	83	.	.	51	.
Formate	83	.	.	47	.
Betaine	.	.	.	50	43
Glycine	82	.	.	.	40
DL-Threonine	.	.	.	.	38
DL-Homoserine	.	.	.	.	.

(Table 7), but no other substance, including a number of amino acids not listed, had any detectable effect. DL-Serine still had pronounced activity at 1.0 mM, but none at 0.1 mM.

Strains requiring either serine or glycine for growth. The ability of serine both to increase methionine synthesis (albeit to a variable extent) and to overcome the inhibition of such synthesis by CO₂ suggested that it might be the main source of the one-carbon-atom unit for condensation with homocysteine. The strains of *E. coli* so far used did not require added serine for growth and were presumably able to synthesize it from constituents of the culture medium. Since a source of both carbon (glucose) and nitrogen (homocysteine) was present, serine may also have been formed by suspensions in solution *M*. Together with the known ability of *E. coli* to catabolize serine (Chargaff & Sprinson, 1943), this may well account for the erratic results noted above.

The best way of determining the absolute effect of serine on methionine formation appeared to be the use of mutants requiring serine for growth and presumably unable to synthesize it. Two strains (PA 15 and 55/5) were available which required either serine or glycine for growth on medium *A* at

37°; synthesis of serine by suspensions was therefore likely to be limited unless glycine was present. Strain 55/5 grew at 25° without these amino acids and at this temperature must be considered potentially able to synthesize them.

Suspensions of strain PA 15 formed no detectable methionine in solution *M*, even with cobalamin added, unless serine was present (Table 8). Similar results were obtained with strain 55/5 at 37°; at 25°, however, although good methionine synthesis was not obtained unless serine was present, there was a small but reproducible synthesis in the absence of added serine (Table 8). This result is in accord with the growth requirements of the strain, which suggest that it can make some serine at 25°.

The following amino acids and possible one-carbon-atom donors (each tested at both 10 and 1 mM) failed to replace serine for methionine synthesis by either strain PA 15 or 55/5 or both: each of the amino acids listed in the *Leuconostoc mesenteroides* basal medium (except threonine), L-hydroxyproline, DL- α -aminobutyric acid, formate, choline, betaine, xanthine, ethanol and glycollic acid. Glycine and, to a lesser extent, DL-threonine and DL-homoserine had activity under certain conditions to be described later.

The results with both strains strongly support the view that serine is the main one-carbon-atom donor for methionine formation by *E. coli* and that strains able to achieve synthesis of serine in growing cultures can also do so to some extent under the conditions of incubation in solution *M*.

Requirement for vitamins in the presence of serine. If serine is a normal reactant in methionine formation it is possible that the requirements for *p*-aminobenzoic acid and cobalamin demonstrated with strains 273/384 and 200 were for the synthesis of serine rather than its condensation with homocysteine. There is evidence (reviewed by Woods, 1952) that members of the folic acid group are

Table 8. *Effect of serine on synthesis of methionine by serine/glycine auxotrophs*

Organisms harvested from medium *A* with (PA 15) or without (55/5) 10 mM-serine were incubated (0.31 mg./ml.) in solution *M* in the presence or absence of serine (mM).

Strain of <i>E. coli</i>	Incubation		L-Methionine formed (μ moles/mg. of organisms)	
	Temp.	Time (hr.)	Serine absent	Serine present
PA 15	37°	8	<0.05	1.85
55/5	37	16	<0.05	3.70
	25	16	0.13	3.00

Table 9. *Dependence of synthesis of methionine on p-aminobenzoic acid and cobalamin when serine is present*

Vitamin-deficient organisms (0.21 mg./ml.) were incubated for 17 hr. in solution *M* with the additions shown.

Strain of <i>E. coli</i>	Additions			L-Methionine formed (μ moles/mg. of organisms)
	Cobalamin (10 μ mg./ml.)	<i>p</i> -Amino- benzoic acid (μ M)	Serine (10 mM)	
273/384 (deficient in <i>p</i> -aminobenzoic acid)	+	+	+	2.00
	+	—	+	<0.05
	+	+	—	0.89
	+	—	—	<0.05
200 (deficient in vitamin B ₁₂)	+	—	+	0.89
	—	—	+	<0.05
	+	—	—	0.65
	—	—	—	<0.05

* Cobalamin was added in all cases with strain 273/384 since it is known (Table 5) markedly to increase methionine formation.

Table 10. *Concurrent formation of methionine and glycine*

Organisms at the stated concentration were incubated for 8 hr. at 37° in solution *M* supplemented with cobalamin (10 µmg./ml.) and, where indicated, with serine (10 mm).

<i>E. coli</i> strain	Growth medium	Concn. of organisms (mg./ml.)	Addition to solution <i>M</i>	Product (µmoles/mg. of organisms)	
				L-Methionine	Glycine
PA 15	<i>A</i> + serine	0.34	.	<0.05	0.15
			Serine	1.40	1.21
PA 15	<i>A</i> + glycine	0.27	.	<0.05	0.15
			Serine	2.85	0.96
55/5	<i>A</i> *	0.24	.	<0.05	<0.10
			Serine	2.34	1.74

* Growth temperature 25°.

required for the formation of serine from glycine by several bacteria, and it was at one time thought that cobalamin had a function in serine-glycine interconversion in animal tissues (see discussion by Arnstein, 1955).

There was still an absolute requirement for *p*-aminobenzoic acid and cobalamin for synthesis of methionine by suspensions of strains 273/384 and 200 respectively, even though preformed serine was present (Table 9); it is clear therefore that both factors are required for the condensation reaction apart from any function they may or may not have in serine formation.

Production of glycine. There is good evidence that glycine is the other main product of the reaction between homocysteine and serine. With strain 200 (a cobalamin auxotroph) methionine formation is not dependent on added serine, which it can presumably synthesize. Methionine and glycine were produced in approximately equimolar quantities whether or not serine was added, but no glycine was formed from serine when cobalamin was omitted and methionine synthesis could not occur (Table 12). Either the formation of glycine is linked to the methylation of homocysteine or the formation of glycine from serine itself requires cobalamin.

With strains (PA 15, 55/5) which cannot synthesize serine at the temperature of the test, methionine formation required added serine and, in a number of experiments, 0.75–0.9 mole of glycine was found for each mole of methionine (Table 10). With strain 55/5 a ratio near unity was maintained almost throughout the period of methionine production (Fig. 3); with strain PA 15 glycine formation decreased in rate more sharply and at an earlier stage than did that of methionine.

A strict molecular equivalence of methionine and glycine would not be expected, and would be of doubtful significance for two reasons. First, the organisms cause slight, though unequal, apparent destruction of both amino acids in conditions comparable with the synthesis tests. Thus suspensions of strain PA 15 removed (per mg. of organism in 4 hr.) 0.3 and 0.55 µmole of methionine and glycine

Fig. 3. Formation of methionine and glycine by suspensions of *E. coli* 55/5. Organisms (0.23 mg./ml.) were incubated in solution *M* plus DL-serine (10 mm) and cobalamin (10 µmg./ml.). ●, Methionine; ○, glycine; —, homocysteine present; ---, homocysteine omitted from solution *M*.

respectively. Secondly, there is some production of glycine from serine in the absence of homocysteine (Fig. 3); it is not known to what extent, if any, this is suppressed when methionine synthesis can occur.

When strain PA 15 was grown originally on glycine instead of serine, the yield of glycine was only one-third of that of methionine (Table 10); possible reasons for this have not been investigated.

Further experiments with mutants requiring serine or glycine for growth

Growth on serine or glycine in relation to methionine synthesis by suspensions. *E. coli* PA 15 will grow on medium *A* supplemented with either serine or glycine and can presumably therefore

form either amino acid from the other. Organisms harvested from a serine-containing medium utilized this amino acid, presumably as a one-carbon-atom donor, for methionine formation from homocysteine; glycine, however, showed only just detectable activity in this respect (Fig. 4). When the organisms were grown with glycine the activity of this amino acid was greatly increased, and after incubation for 8 hr. the suspensions had formed about 75 % as much methionine as with serine as

donor (Fig. 4). The results suggest that an adaptive process, involving the presence of glycine itself during growth, is required to produce the enzyme systems concerned in the activation of glycine as one-carbon-atom donor. This is supported by the fact that even with glycine-grown cells there is an increasing rate of methionine synthesis with time (Fig. 4).

Surprisingly, growth on glycine in place of serine also more than doubled both the rate of production and final yields of methionine when serine was used as one-carbon-atom source (Fig. 4); this result was consistently obtained and will be discussed later.

Threonine and homoserine as one-carbon-atom donors; conversion into glycine. Suspensions of strain PA 15 cannot synthesize serine or glycine from constituents of solution *M*; the ability of related compounds to replace these amino acids as source of the single-carbon-atom unit may thus be conveniently tested with this strain.

A large number of amino acids and other compounds were tested, but only threonine and homoserine were effective (Table 11) and then only with organisms harvested from growth on glycine; serine-grown organisms were inactive. Since growth on glycine is essential for the utilization of glycine itself, and since neither threonine nor homoserine was as effective as glycine, it was possible that the two hydroxyamino acids were first converted into glycine before acting as one-carbon-atom donors. This was tested directly by assaying glycine as well as methionine (Table 11); glycine was found to be produced from both threonine and homoserine whether or not the conditions (presence or absence of homocysteine) permitted methionine synthesis. Formation of glycine from the hydroxyamino acids was further confirmed with suspensions of strain 200 (a cobalamin auxotroph); here methionine synthesis can be controlled by the presence or absence of cobalamin.

Fig. 4. Effect of growth in serine or glycine on the utilization of these amino acids as one-carbon-atom donors by *E. coli* PA 15. Organisms (0.3 mg./ml.) were incubated in solution *M* plus cobalamin (10 μ mg./ml.) and with either DL-serine (10 mM) or glycine (10 mM). ●, Serine; ○, glycine as one-carbon-atom donor. Organisms were grown on serine (---) or glycine (—).

Table 11. *Formation of methionine and glycine from various one-carbon-atom donors by a serine/glycine auxotroph*

Suspensions of *E. coli* PA 15 harvested from growth on medium *A* plus either serine or glycine were incubated for 8 hr. at 37° in solution *M* plus cobalamin (10 μ mg./ml.), with homocysteine omitted as shown. Source of single-carbon-atom unit (10 mM-DL-) was added as indicated. Concn. of organisms: serine-grown, 0.34 mg./ml.; glycine-grown, 0.27 mg./ml.

Grown with	Homocysteine in solution <i>M</i>	Product formed (μ moles/mg. of organisms)				
		No addition	With serine	With glycine	With threonine	With homoserine
Methionine formed						
Serine	Absent	<0.05	<0.05	<0.05	<0.05	<0.05
Serine	Present	<0.05	1.40	0.11	<0.05	<0.05
Glycine	Present	<0.05	2.85	1.95	0.84	0.37
Glycine formed						
Serine	Absent	0.15	0.43	—	0.82	0.88
Serine	Present	0.15	1.21	—	0.59	0.59
Glycine	Present	0.15	0.96	—	—	—

Table 12. *Methionine and glycine formation from various one-carbon-atom donors by a cobalamin auxotroph*

E. coli 200 (0.26 mg./ml.) was incubated in solution *M* for 8 hr. at 30° with cobalamin (10 µmg./ml.) added as shown. Amino acid supplements (10 mM-DL-) were added as indicated.

Cobalamin	Product formed (µmoles/mg. of organisms)				
	No addition	With serine	With glycine	With threonine	With homoserine
Methionine formed					
Absent	<0.05	<0.05	<0.05	<0.05	<0.05
Present	0.60	0.69	0.53	0.73	0.67
Glycine formed					
Absent	0.20	<0.10	—	1.72	0.96
Present	0.40	0.67	—	1.04	0.82

In the absence of methionine formation glycine was formed from homoserine and threonine, but not from serine (Table 12). When methionine was synthesized there was a slight decrease in the amount of glycine formed from threonine and homoserine. With this strain (as opposed to PA 15) it was noted that the utilization of glycine was not dependent on previous growth on glycine since the growth medium did not contain this amino acid.

It is concluded that the apparent activity of homoserine and threonine as sources of the one-carbon-atom unit may be accounted for by their prior conversion into glycine.

Methionine synthesis by a normal strain

The results described in preceding sections were obtained with a variety of mutant strains of *E. coli*, not all derived from the same parent. It was thought advisable to attempt to obtain similar evidence with a single prototrophic strain, even though the effects could not be expected to be absolute because of the ability of the prototroph to synthesize the metabolites in question. Strain 518 (the parent strain of the *p*-aminobenzoic acid auxotroph 273/384) was chosen for this work; the complete system used for synthesis was solution *M* plus serine (10 mM) and cobalamin (10 µmg./ml.).

Serine as one-carbon-atom donor. The presence of serine increased methionine synthesis by 40%; furthermore, an atmosphere of 20% (v/v) of CO₂ decreased synthesis by about 40%, the inhibition being overcome completely by serine.

Effect of cobalamin. The inclusion of cobalamin increased methionine formation by a factor of 2.5–5.0, both in the presence and absence of serine (see also Table 5). As with all other strains not requiring cobalamin for growth, the effect of this vitamin was clear-cut but variable.

Folic acid. *p*-Aminobenzoic acid had no effect on methionine synthesis by this strain, which is known to synthesize both *p*-aminobenzoic acid and folic acid (Lascelles & Woods, 1952); the organisms have

presumably a full complement of folic acid at the time of harvesting. Attempts were made to obtain suspensions relatively deficient in folic acid by growing the cultures in sub-inhibitory concentrations of sulphathiazole (0.5 µM) followed by incubation of the harvested organisms in buffered glucose for 6 hr. This reduces the folic acid content of the organisms (Lascelles & Woods, 1952) but not apparently to a sufficient extent, since there was still no reduction of methionine synthesis, compared with normal controls, and no effect of *p*-aminobenzoic acid was found.

Confirmatory tests for methionine and glycine

The identification of methionine and glycine as products of the reaction between homocysteine and serine rests on the specificity of the assay organism (*Leuconostoc mesenteroides* P 60). Although this was entirely satisfactory within the limits of the checks made, certain ancillary tests were carried out. For these experiments the two serine/glycine mutants (PA 15 and 55/5) were used since they produced the largest quantities of methionine and glycine. For controls serine was omitted from the synthesis system.

Glycine. Organisms were removed by centrifuging from the heated products of synthesis experiments and the supernatant was chromatographed on columns of Dowex 50 resin (see the Methods section). A peak corresponding to glycine was obtained. The concurrent formation of alanine made a satisfactory quantitative estimation of glycine impossible with the quantities formed. There was no positive correlation between the formation of alanine and methionine; it was shown that at least one source of alanine was a transamination reaction between homocysteine and pyruvate, the latter being presumably derived from serine or glucose.

Methionine. Supernatants from synthesis experiments were chromatographed on paper with butanol–acetic acid and phenol–water as solvents. A ninhydrin-positive spot with the same *R_f* as

synthetic methionine was detected by microbiological assay. Acid-hydrolysis of the samples before chromatography did not affect the result, but peroxide treatment destroyed this ninhydrin-positive material. Similarly, peroxide destroyed the activity of the samples for growth of *L. mesenteroides* P 60.

In a further experiment with the products formed by strain PA 15, two samples, one from the control without serine and the other from a complete synthesis system, were chromatographed in duplicate for 18 hr. with butanol-acetic acid; a solution of methionine was similarly treated. The papers were dried at room temperature and one of each set of duplicates was sprayed with ninhydrin to locate the spots. The other strips were cut into 2 cm. squares and each section was folded and placed in methionine assay medium (2 ml.). After autoclaving and inoculation with *L. mesenteroides* P 60 the tubes were incubated as for a methionine assay. Growth occurred only in those tubes corresponding to the spot given by synthetic methionine and the spot with the same R_f obtained from the complete synthesis system.

Samples from experiments with strain 200 (cobalamin auxotroph) and strain 273/384 (*p*-aminobenzoic acid auxotroph) had to be added in relatively large amounts to detect a spot corresponding to methionine. The experiment was satisfactory with phenol-water as solvent but with butanol-acetic acid ninhydrin-positive material with R_f only slightly greater than that of methionine was detected in controls from which the vitamins had been omitted and in which no methionine was detectable by microbiological assay. The nature of this substance was not further investigated in the present work.

DISCUSSION

It is not proposed to discuss the present results extensively since they have formed the basis of further experiments at the cell-free enzyme level on the role of folic acid and cobalamin; preliminary reports of some of these studies have been published (Cross & Woods, 1954; Szulmajster & Woods, 1955; Helleiner & Woods, 1956; Kisliuk & Woods, 1957; Helleiner, Kisliuk & Woods, 1957).

It is likely that the serine $\rightarrow$ glycine reaction is the ultimate main source of single-carbon-atom units for the methylation of homocysteine by suspensions of *E. coli*. Not only is serine an obligatory requirement for methionine synthesis by strains which cannot synthesize serine, but glycine always accompanies methionine as a product with those strains (e.g. the prototrophs) which do not require serine as a reactant. The only other substances found to act (and less efficiently) as one-

carbon-atom donors were glycine and amino acids metabolically convertible into it (homoserine, threonine). Formaldehyde, a likely possible donor, could not be expected to act as it combines spontaneously with homocysteine to yield 1:3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), which is metabolically inert in the present system (unpublished experiments of I. Maclean in this Laboratory).

The greatly increased efficacy of serine as donor when strain PA 15 is harvested from growth on glycine rather than serine is puzzling and has not so far been explained. The parallel observation with the same strain that growth on glycine is essential for the activity of glycine as donor could be explained by some type of adaptive enzyme formation. The nature of the other product formed from glycine when it acts as donor has not been investigated. Unpublished experiments with [14 C]glycine by M. J. Cross in this Laboratory showed that serine was not formed as an intermediate. Kizer, Speck & Aurand (1955*a, b*) have obtained indirect evidence that serine (perhaps in the form of phosphoserine) is required for methionine synthesis from homocysteine by growing cultures of *Streptococcus lactis*.

The experiments on the parallel inhibition by sulphonamide of the synthesis of both methionine and of folic acid derivatives gives strong, though indirect, evidence that *p*-aminobenzoic acid is converted into some form of folic acid before it exerts its function, presumably as a one-carbon-atom carrier, in the synthesis of methionine. In extension of this work Szulmajster & Woods (1955) have briefly reported that a dialysed cell-free preparation from acetone-dried organisms required a cofactor whose properties were akin to those of a natural coenzyme form of folic acid (Wright, 1955). Tetrahydropteroylglutamic acid cannot replace this factor (Cross & Woods, 1954) but can do so if the organisms are originally grown on a medium containing added cobalamin (Kisliuk & Woods, 1957). A preparation made by sonic disintegration can also use tetrahydrofolic acid, but only if cobalamin is also added (Helleiner *et al.* 1957). In recent work with sheep-liver homogenates Nakao & Greenberg (1958) give evidence that tetrahydropteroylglutamic acid functions as a cofactor in the overall methylation of homocysteine by serine or formaldehyde; cobalamin, however, had no effect.

Cobalamin was required absolutely for methionine synthesis by suspensions of mutants requiring this factor (or methionine) for growth. It has since been briefly reported (Helleiner & Woods, 1956) that sonic disintegrates show the same requirement. Suspensions of all strains (whether cobalamin-requiring or not) showed marked, though variable, increases in methionine synthesis when cobalamin was added. A possible explanation is

tha. the washed suspensions are partially deficient in cobalamin, owing either to inherent lack of synthetic ability or to losses during the preparation of suspensions. Alternatively, it is possible that the normal mechanism of methionine synthesis does not require cobalamin, but that this vitamin permits an alternative and more efficient mechanism of conversion of homocysteine into methionine.

SUMMARY

1. Washed suspensions of a number of normal and mutant strains of *Escherichia coli* converted homocysteine into methionine; the latter was determined by microbiological assay with *Leuconostoc mesenteroides* P 60.

2. By the use of auxotrophic mutants unable to synthesize *p*-aminobenzoic acid and cobalamin respectively, it was shown that both these vitamins are essential for the reaction.

3. Synthetic forms of folic acid did not replace *p*-aminobenzoic acid. Sulphathiazole, however, inhibited both the synthesis of methionine and the conversion of *p*-aminobenzoic acid into folic acid.

4. Serine is the probable ultimate source of the methyl group. Methionine is not formed by mutants requiring serine or glycine for growth unless serine (or, under limited conditions, glycine) is added.

5. Glycine is formed concurrently with methionine whether or not it is essential to add serine.

6. Glycine, threonine and homoserine also act as one-carbon-atom donors, though less efficiently than serine. There is evidence that threonine and homoserine must first be converted into glycine.

One of us (F.G.) is indebted to the Australian National University for a scholarship during the period of this work. We are grateful to Mrs E. MacPhee for valuable technical assistance and to the Rockefeller Foundation for a grant to the Laboratory.

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