WIJESUNDERA, S., CROSS, M. J. & WOODS, D. D. (1960). *J. gen Microbiol.* **22**, 786–796

Vitamin B_6 and Glycine in the Synthesis of Methionine by Suspensions of *Escherichia coli*

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SUMMARY: Washed suspensions of two strains of *Escherichia coli* which require vitamin B_6 for growth form methionine in a reaction mixture containing homocysteine, serine, glucose, *p*-aminobenzoic acid and cobalamin only when pyridoxal or another member of the vitamin B_6 group (except pyridoxamine phosphate) is also added.

Serine is replaced by glycine as donor of the required one-carbon unit, but the activity of glycine is markedly increased when the organism is harvested from a medium containing glycine. Pyridoxal is not required for the utilization of glycine by such organisms, but is essential when glycine has not been present during growth. It is concluded that growth on glycine induces the formation of an alternative, and more efficient, mechanism for using glycine as one-carbon donor which is independent of pyridoxal. This mechanism was studied in suspensions of an auxotroph requiring serine or glycine for growth by isotopic technique; C-2 of glycine is incorporated into the methyl group of methionine at four times the activity at which it appears in a serine pool. Free serine is therefore not an intermediate in the utilization of glycine as a one-carbon donor. Cobalamin is required for maximal activity of both glycine and serine in the reaction.

Gibson & Woods (1960) showed that washed suspensions of *Escherichia coli* synthesize methionine from homocysteine. Using auxotrophic strains in which the presence of these vitamins could be kept under experimental control they obtained evidence that *p*-aminobenzoic acid and cobalamin were required for the reaction. Since derivatives of vitamin B_6 are so commonly required as coenzymes in amino acid metabolism, evidence was sought in the present work by methods similar to those of Gibson & Woods (1960) for their participation in the biosynthesis of methionine.

Using an auxotroph requiring either serine or glycine for growth Gibson & Woods (1960) also showed that serine acted as ultimate source of the methyl group of methionine; glycine was only active (and then less so) when the organism had been cultivated in the presence of this amino acid. In the present work some differences in the vitamin B_6 requirement were found according to whether serine or glycine was the donor of the one-carbon unit and the role of glycine was therefore further investigated. Johnson, Holdsworth, Parker & Kon (1957) have given evidence that with *Ochromonas malhamensis* glycine is the source of the methyl group of methionine.

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METHODS

Organisms. Escherichia coli, strains B 166 and D/W, are both auxotrophs which show a growth response to vitamin B_6; their growth requirements and other characteristics were fully analysed by Morris & Woods (1959). Strain D/W, a substrain of M 154-59L, was provided by Dr Elizabeth Work (Denman, Hoare & Work, 1955) and has an absolute requirement for vitamin B_6 when grown at 37°. Strain B 166 (obtained from Dr J. Gots) responds to vitamin B_6 or serine or glycine in a medium containing autoclaved glucose. *E. coli* PA 15, which requires serine or glycine for growth, was originally provided by Dr Barbara Wright. Stock cultures of all three organisms were maintained on tryptic digest meat agar slopes, subcultured monthly (18 hr. at 37°) and stored at 4°; they were stable during the period of the work.

Media. The basal growth medium for the preparation of washed suspensions was the glucose+salts medium of Davis & Mingioli (1950) except that the glucose was added to the rest of the medium before autoclaving at 115° for 10 min. The supplements used for obtaining organisms relatively deficient in vitamin B_6 were pyridoxal (10^{-8} M) for strain D/W, and a mixture of DL-serine, glycine and DL-alanine (each 10^{-3} M) for strain B 166. In the case of strain PA 15, glycine (10^{-2} M) was added.

General procedure for experiments on the synthesis of methionine

Organisms were grown and suspensions prepared as described by Wijesundera & Woods (1960). Methionine synthesis was studied in a solution (*MS*) which contained: DL-homocysteine (0·006 M), DL-serine (0·01 M), glucose (0·02 M), *p*-aminobenzoic acid (10^{-6} M), cobalamin (10 μmg./ml.) and 0·067 M-phosphate buffer (pH 7·4). The organisms were suspended in solution *MS* (2·5 ml.) at final concentrations equivalent to 0·25 mg. dry wt./ml. (strains B 166 and D/W) and 0·5 to 0·7 mg. dry wt./ml. (strain PA 15); incubation was in air for 6–16 hr. at 37°. After heating to 100° for 10 min. and centrifuging, the supernatant fluid was used for analysis of products. Other conditions and controls were as described by Wijesundera & Woods (1960).

Estimations. L-methionine and L-serine were assayed microbiologically with *Leuconostoc mesenteroides*, P 60, as described by Gibson & Woods (1960) and Lascelles, Cross & Woods (1954), respectively. The organism does not respond to the D-isomer of either amino acid.

Saccharomyces carlsbergensis (ATCC no. 4228) was used for the microbiological assay of vitamin B_6 by the procedure of Atkin, Schultz, Williams & Frey (1943); the organism responds to all known forms of the vitamin.

Experiments with radioactive substrates

Methionine synthesis was studied as above, except that incubation (in air) was in gas-tight fruit-preserving jars.

Isolation of serine and glycine. These amino acids were separated from the reaction mixture by chromatography on columns of Dowex-50 resin, with HCl solutions as eluants (Stein & Moore, 1949). Part of each fraction collected

was assayed for amino-nitrogen by the method of Moore & Stein (1948). Part of each serine fraction was also assayed microbiologically to determine the proportion of L-isomer present. It was never less than 95% in spite of the fact that DL-serine had been added originally; it is likely that the organism has a powerful D-serine deaminase. The remainder of the glycine and serine fractions were diluted about 1000-fold with known quantities of inactive glycine and DL-serine respectively and recrystallized from aqueous ethanol to constant count.

Isolation of methionine. This amino acid was separated from the reaction products by chromatography on Whatman no. 3 paper with 'butanol-acetic acid' (the upper layer of a mixture of *n*-butanol, glacial acetic acid and water in the proportion of 4:1:5). Chromatograms were developed from samples (1 ml.) and, after drying, a thin strip was cut from each side of the paper and treated with ninhydrin to determine the position of the methionine band. The main methionine area was cut out and extracted with water. The solution was concentrated and a portion assayed for methionine microbiologically; the usual over-all recovery was 70%. The remainder of the solution was diluted about 100-fold with a known quantity of inactive DL-methionine and recrystallized to constant count from water.

The methyl group of methionine was removed by treatment with hydriodic acid (Simmonds, Cohn, Chandler & du Vigneaud, 1943). The radioactivity of the tetramethylammonium iodide collected was measured and checked after conversion to the chloride.

Estimation of radioactivity. Samples were mounted at infinite thickness on polythene disks (1 cm.^{2}) and counted with an end window Geiger-Müller counter. Values are reported as counts/min./cm.^{2} at infinite thickness and have been corrected for background; they have a standard error of less than $\pm 1{\cdot}5\%$. These values are directly proportional to the specific activities of the substances counted and permit their activities to be compared in molecular terms (Popják, 1950).

Chemicals

Pyridoxal, pyridoxamine and their phosphate esters were kindly provided by Dr K. Folkers. δ-Aminolaevulic acid was a gift from Professor A. Neuberger. Isotopically-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks.

RESULTS

Requirement for vitamin B_6: serine as substrate

Escherichia coli, B 166, grows without pyridoxal in a medium containing autoclaved glucose provided that serine or glycine is present. Although some vitamin B_6 is synthesized under these conditions (Morris, 1959) the organisms may be expected to be at least relatively deficient in the vitamin. Growth with the supplement used (a mixture of DL-serine, glycine and DL-alanine) was only half that attained if pyridoxal were also present. Suspensions of such organisms synthesized no methionine with serine as one-carbon donor

unless pyridoxal was added to the reaction mixture (Fig. 1) and the amount formed showed a linear relationship to the logarithm of the concentration of the vitamin. Best synthesis was at 5×10^{-7} M, a concentration of the same order as that required for maximal growth.

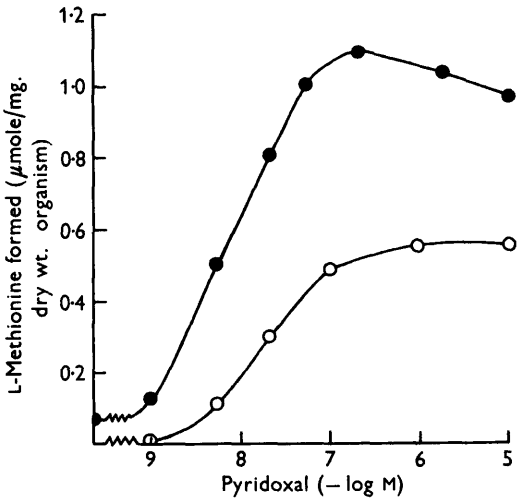

Fig. 1. Effect of the concentration of pyridoxal on the synthesis of methionine by suspensions of *Escherichia coli*, B 166 (○) and D/W (●). Organisms suspended in solution *MS* plus pyridoxal at the concentrations indicated. Incubated 16 hr.

Suspensions of strain D/W relatively deficient in vitamin B_6 were obtained from growth on suboptimal concentrations of pyridoxal (10^{-8} M; $3{\cdot}2 \times 10^{-8}$ M is required for half-maximal growth). Best synthesis of methionine was again dependent on pyridoxal (Fig. 1); the amount formed in its absence was usually low (as in the experiment quoted) but varied somewhat with different suspensions (e.g. that of Table 1) possibly due to carry over from the growth medium of traces of pyridoxal within the organisms.

Effect of various forms of vitamin B_6. Pyridoxine and the phosphate ester of pyridoxal were as effective as pyridoxal itself with both strains when tested at 10^{-6} M. Pyridoxamine, though not its phosphate ester, was active with the one strain tested (Table 1).

Requirement for vitamin B_6: glycine as substrate

Strain B 166. Suspensions of this organism, derived from growth as above in the absence of pyridoxal, did not synthesize methionine unless serine as well as the vitamin was added to the reaction mixture (Table 2); it may be assumed that under the conditions the organism cannot synthesize adequate amounts of either substance. Serine could be replaced by glycine as one-carbon donor, and in the presence of pyridoxal the yield of methionine was 80% of that with serine. However, in contrast to the results with serine, considerable methionine (about 60%) was formed without the addition of pyridoxal

Table 1. *Effect of vitamin B_6 derivatives on the synthesis of methionine by suspensions of* Escherichia coli, *B 166 and D/W*

Vitamin-deficient organisms suspended in solution *MS* plus vitamin B_6 derivatives (10^{-6} M) as indicated. Incubated 16 hr.

Form of vitamin B_6	L-Methionine formed (μmole/mg. dry wt. organism) with strain	
	B 166	D/W
None	< 0·02	0·50
Pyridoxin	0·51	1·60
Pyridoxal	0·50	1·50
Pyridoxal phosphate	0·60	1·55
Pyridoxamine	—	1·60
Pyridoxamine phosphate	—	0·60

—, Not tested.

(Table 2); this was further increased by the addition of serine as well as glycine. Comparison of the rates of formation of methionine under the three sets of conditions (glycine alone, glycine + serine, serine + pyridoxal) showed that when serine was present synthesis was relatively more rapid during the first part of the incubation (Fig. 2).

Table 2. *Effect of pyridoxal on the synthesis of methionine by* Escherichia coli, *B 166, with serine or glycine as source of the methyl group*

Vitamin-deficient organisms suspended in solution *MS* (serine omitted). DL-serine (10^{-2} M), glycine (10^{-2} M) and pyridoxal (10^{-6} M) when present. Incubated 16 hr.

Source of one-carbon unit	L-Methionine formed (μmole/mg. dry wt. organism) with pyridoxal	
	Absent	Present
None	< 0·02	0·02
Serine	0·02	0·50
Glycine	0·26	0·41
Serine + glycine	0·36	0·60

The possibility that these results were due to contamination of the glycine used with vitamin B_6 was tested by the use of four different samples, one of which had been successively treated with activated charcoal at pH 2 and 7; the results were the same as before. It was also conceivable that some synthesis of the vitamin had occurred in the course of the experiment itself in the presence of glycine. The reaction mixtures (including the organisms) were assayed microbiologically, but only trace amounts (5×10^{-10} M as pyridoxin) were found and there was no difference when serine instead of glycine was present during the incubation.

In the above experiments the organism had been grown on a medium containing glycine. With suspensions of *Escherichia coli*, PA 15, Gibson & Woods (1960) found only slight synthesis of methionine unless the organism had been grown with glycine. Strain B 166 was therefore grown with and without

glycine on a medium containing pyridoxal; synthesis of methionine by suspensions with glycine as the one-carbon donor was increased threefold by growth with glycine while that with serine as one-carbon donor was unchanged (Table 3).

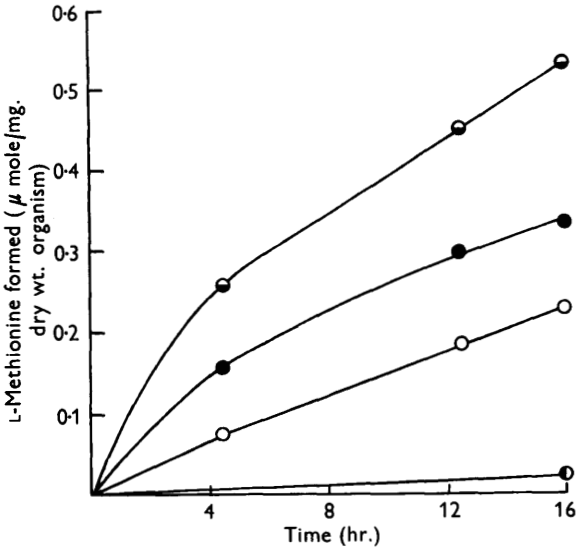

Fig. 2. Rate of formation of methionine by *Escherichia coli*, B 166, with various one-carbon donor systems. Organisms suspended in solution *MS* (serine omitted) supplemented with ◐, DL-serine (10^{-2} M); ○, glycine (10^{-2} M); ●, DL-serine (10^{-2} M) + glycine (10^{-2} M); ◒, DL-serine (10^{-2} M) + pyridoxal (10^{-6} M). Incubated 16 hr.

Table 3. *Effect of growth in the presence of glycine on the synthesis of methionine by* Escherichia coli, *B 166*

Organisms harvested after growth on basal medium supplemented with pyridoxal ($2{\cdot}5 \times 10^{-8}$ M) and glycine (2×10^{-3} M) as indicated. Organisms suspended in solution *MS* (serine omitted) plus pyridoxal (10^{-6} M) and DL-serine (10^{-2} M) or glycine (10^{-2} M) as shown. Incubated 16 hr.

Supplements to growth medium	L-Methionine formed (μmole/mg. dry wt. organism) when source of one-carbon unit is		
	Absent	DL-Serine	Glycine
Pyridoxal	< 0·01	0·09	0·10
Pyridoxal and glycine	< 0·01	0·10	0·32

Strain D/W. Vitamin B_6-deficient organisms of this strain were obtained by growth on a suboptimal concentration of vitamin. There was some synthesis of methionine (pyridoxal present) by suspensions without addition of either serine or glycine (Table 4); the organism is not in this case also a serine or glycine auxotroph and can presumably synthesize these amino acids from constituents of the reaction mixture. Synthesis of methionine was increased by either serine or glycine, the latter having about half the activity of the former, but the presence of pyridoxal was essential for the stimulation in both

cases (Table 4). The results with glycine (though not serine) as one-carbon donor were fundamentally changed by the incorporation of glycine into the growth medium. There was now considerable synthesis of methionine with glycine as donor in the absence of pyridoxal (Table 4) though the utilization of serine still required the vitamin.

Table 4. *Effect of growth in the presence of glycine on the synthesis of methionine by* Escherichia coli, *D/W*

Organisms harvested after growth on basal medium plus pyridoxal (10^{-8} M) with and without glycine (5×10^{-3} M). Organisms suspended in solution *MS* (serine omitted) supplemented with DL-serine (10^{-2} M), glycine (10^{-2} M) and pyridoxal (10^{-6} M) as indicated. Incubated 16 hr.

Supplements to reaction mixture	L-Methionine formed (μmole/mg. dry wt. organism) with organisms grown with glycine	
	Absent	Present
None	0·01	0·10
Pyridoxal	0·23	0·47
Serine	0·05	0·10
Serine + pyridoxal	0·70	0·90
Glycine	0·05	0·58
Glycine + pyridoxal	0·43	0·65

Synthesis of methionine by Escherichia coli *PA 15*

Incorporation of glycine into methionine. Direct evidence was sought by isotope technique as to whether the utilization of glycine as one-carbon donor proceeded by its intermediate conversion to serine. For these experiments strain PA 15 was used since suspensions synthesize methionine more actively than other strains (Gibson & Woods, 1960); glycine is used as one-carbon donor provided the amino acid has been present during growth.

Trial experiments showed that DL-serine was metabolized by the suspensions, probably by deamination to pyruvate since much alanine is found in the products (Gibson & Woods, 1960). The incorporation of C-2 of glycine was therefore studied by adding [^{14}C-2]glycine to the reaction mixture supplemented with excess DL-serine (2×10^{-2} M); sufficient serine remained after incubation for the desired period to make re-isolation possible.

C-2 of glycine was incorporated into the methyl group of methionine with a dilution of about 1 in 6 (Table 5). Since the activity found in methionine was higher than that in its methyl group it is probable that the C-2 of glycine also entered the homocysteine residue of methionine. The activity found in the re-isolated serine was only one quarter of that found in the methyl group of methionine (Table 5); it is clear therefore that C-2 of glycine was not incorporated into free serine before entering the methyl group of methionine. Alanine was not isolated from the products, but the fraction containing it was evaporated to dryness and counted directly; the activity was less than 3% of that of serine.

One possible source of error in the estimation of the activity of methionine and its methyl group may arise as the result of possible oxidation of

Table 5. *Incorporation of [^{14}C-2]glycine into methionine and serine by* Escherichia coli, *PA 15*

Organisms were suspended in solution *MS* (DL-serine raised to 2×10^{-2} M) supplemented with pyridoxal (10^{-6} M) and [^{14}C-2]glycine (10^{-2} M total glycine). Incubated 6·5 hr. Compounds isolated and counted as in Methods.

Compound	Activity (counts $\times 10^{-6}$/min./cm.^{2} at infinite thickness)
Glycine (initial)	145·0
Glycine (re-isolated after incubation)	99·3
Serine (remaining after incubation)	5·8
Methionine	35·7
Methionine (methyl group)	22·9

methionine (in the course of isolation) to methionine sulfoxide. *Leuconostoc mesenteroides*, P 60, responds to the sulphoxide (R. J. Rowbury, unpublished observations) and the values for methionine after elution from chromatograms may therefore be too high. If this is so the dilution factor calculated after the addition of carrier DL-methionine will be too low, as will also be the calculated activity of the methionine synthesized by the organism. This possible error does not affect the significance of the finding that the activity of the re-isolated serine is much lower than that of the methyl group of methionine.

When [^{14}C]formate (10^{-2} M) was added to the test system in place of glycine there was no significant incorporation of radioactive carbon into methionine, serine, glycine or alanine.

Effect of cobalamin. Cobalamin was required for the maximal synthesis of methionine from homocysteine whether glycine or serine was the source of the carbon of the methyl group (Table 6).

Table 6. *Effect of cobalamin on the synthesis of methionine by* Escherichia coli *PA 15*

Organisms suspended in solution *MS* (serine and cobalamin omitted) plus pyridoxal (10^{-6} M). Other supplements (when present): DL-serine (10^{-2} M), glycine (10^{-2} M) and cobalamin (10 μmg./ml.). Incubated 8 hr.

Supplements to reaction mixture			L-Methionine formed (μmole/mg. dry wt. organism)
DL-Serine	Glycine	Cobalamin	
−	+	−	0·17
−	+	+	0·99
+	−	−	0·39
+	−	+	1·62
−	−	+	0·04

+, Present; −, absent.

δ-Aminolaevulic acid. This substance is a precursor of porphyrins in animals and micro-organisms and is formed by the condensation of glycine and succinate (Shemin, 1955). At the concentration tested (10^{-2} M) it did not replace

serine for the synthesis of methionine either in the presence or absence of cobalamin. Porphyrin was detected spectroscopically, showing that the compound entered the organism.

DISCUSSION

Requirement for vitamin B_6: serine as substrate. Some form of vitamin B_6 was required for the methylation of homocysteine by serine in vitamin-deficient suspensions of the two auxotrophs which require the vitamin for growth. Pyridoxamine phosphate alone of the derivatives tested was inactive. The apparent inactivity of this compound in the transaminase reaction between glutamic acid and oxaloacetic acid has been explained on the basis of a slow combination between the apoenzyme and this form of the coenzyme (Meister, Sober & Peterson, 1952); this is unlikely to apply in the present case where a long incubation period was used. A possible explanation is that the organisms are impermeable to pyridoxamine phosphate.

Enzymic extracts of *Escherichia coli*, PA 15, prepared by a variety of methods, require pyridoxal phosphate for maximal formation of methionine from homocysteine and serine (Cross & Woods, 1954; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). It is probable that the reaction generating the one-carbon unit is the conversion of serine to glycine (Gibson & Woods, 1960; Cross & Woods, 1954; Szulmajster & Woods, 1960). Extracts of strain PA 15 also require pyridoxal phosphate for the synthesis of serine from glycine and the formaldehyde derivative of tetrahydropterolyglutamic acid: the addition of pyridoxal phosphate had, however, no effect on the formation of methionine from homocysteine and the formaldehyde derivative of tetrahydropteroylglutamic acid (Kisliuk & Woods, 1960). Pyridoxal phosphate probably therefore mediates only the transfer of the hydroxymethyl group from serine to a coezyme form of folic acid.

Glycine as donor of the one-carbon unit. The synthesis of methionine from glycine and homocysteine by strains B 166 and D/W was considerably increased when the organisms were grown originally with glycine present. Gibson & Woods (1960) made a similar observation with strain PA 15. In contrast to the results with serine considerable amounts of methionine were formed from glycine and homocysteine, even without addition of pyridoxal, by suspensions of strains B 166 and D/W (both grown in the presence of glycine). The smaller synthesis of methionine by suspensions of strain D/W (grown without glycine) was however dependent on pyridoxal.

Taken as a whole these results suggest that growth in the presence of glycine leads to the production of a mechanism by which this amino acid may be used as a donor of one-carbon units, and one moreover which is either independent of vitamin B_6 derivatives or requires much lower concentrations than does the mechanism whereby serine is utilized.

A complicating factor in the analysis of the vitamin B_6 requirement of strain B 166 for synthesis of methionine is that it forms more methionine when glycine and serine are both added than when glycine only is supplied, even though the organisms are pyridoxal-deficient as judged by their inability

to form methionine with serine as one-carbon donor. This finding remains unexplained. One possibility is that with serine and glycine present the organism is able to form sufficient vitamin B_6 to permit serine to be used significantly as one-carbon donor. However, Morris (1959) showed that suspensions of this strain synthesized only traces of vitamin B_6 in the presence of serine, glycine and glucose unless glycolaldehyde was also supplied.

The incorporation of C-2 of glycine into the methyl group of methionine was tested with suspensions of *Escherichia coli*, PA 15, harvested after growth with glycine in order to induce the mechanism of glycine utilization discussed above. The degree of incorporation was relatively high considering that serine, a more effective one-carbon donor, was present in the reaction mixture.

Johnson *et al.* (1957), discussing the formation of the methyl group of methionine from glycine by *Ochromonas malhamensis*, mentioned three possible pathways: (1) the formation of a serine hydroxymethyl group from C-2 of glycine (Arnstein, 1954) followed by its transfer to homocysteine; (2) oxidative deamination of glycine to glyoxylic acid which is decarboxylated to yield an 'active formaldehyde' which methylates homocysteine (Nakada, Friedmann & Weinhouse, 1955); (3) formation of δ-aminolaevulic acid and its conversion into α-ketoglutaraldehyde which in turn gives rise to succinate and 'active formaldehyde' (Shemin, 1955). With *Escherichia coli* in the present work, C-2 of glycine appears in the methyl group of methionine without appearing to the same degree in a serine pool; furthermore, the synthesis of methionine proceeds either without pyridoxal or with less than is required when serine is the substrate. This suggests that free serine is not an intermediate in the methylation of homocysteine by glycine via the pathway induced by growth on glycine. The possibility that serine is formed, but remains bound to the enzyme and does not exchange with free serine, cannot be excluded. δ-Aminolaevulic acid, though metabolized by *E. coli*, PA 15, does not replace glycine as methyl donor; this appears to rule out the third of the suggested pathways. The pathway via glyoxylic acid remains to be explored further, though this substance (10^{-2}M) also does not replace glycine (R. J. Rowbury, unpublished experiments in this laboratory). Whatever the mechanism of synthesis of methionine from glycine and homocysteine, the present results indicate that cobalamin is required, as it is also when the one-carbon donor is serine. Kisliuk & Woods (1960), using extracts of acetone powders of *E. coli* strain PA 15, obtained indirect evidence supporting the same conclusion.

One of us (S.W.) was indebted to the University of Ceylon for a post-graduate scholarship during the period of this work. The funds of the Guinness Research Fellowships (University of Oxford) contributed to the expense of this research.

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(*Received* 25 *January* 1960)