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The Synthesis of Methionine from Homocysteine by Enzymic Extracts of *Escherichia coli*

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In earlier work from this Laboratory it was shown that washed suspensions of various strains of *Escherichia coli* synthesize methionine from homocysteine (Gibson, 1952; Gibson & Woods, 1952, 1960). With strains unable to synthesize serine evidence was obtained that this amino acid was the probable ultimate source of the C₁ fragment for the methylation. With strains requiring *p*-amino-benzoic acid for growth it was shown that this factor was essential for methionine formation and there was strong, though indirect, evidence that it must first be converted into some form of folic acid. The purpose of the present work was to explore the probable function of folic acid under the more closely defined conditions possible with cell-free enzymic extracts and to determine other cofactor requirements.

In exploratory experiments published briefly by

Cross & Woods (1954) it was found that enzymic extracts made by grinding the organisms with alumina synthesized methionine from homocysteine and serine in a reaction mixture containing also hexose diphosphate, pyridoxal phosphate, adenosine triphosphate and Mg²⁺ ions; preparations made by ultrasonic disintegration or by shaking with minute glass beads were less active. A heated extract of *E. coli* increased methionine synthesis by about half. It was later found (by Dr M. J. Cross and the present authors, unpublished work) that extracts of acetone-dried organisms were a more potent and reliable source of the enzyme systems concerned. The problem was therefore investigated in detail with this material. Part of this work has been the subject of a preliminary communication (Szulmajster & Woods, 1955).

METHODS AND MATERIALS

Growth of organisms

Escherichia coli PA15 was used throughout the work. It is an auxotrophic strain requiring either serine or glycine for growth and was originally obtained from Dr Barbara Wright. Stock cultures were maintained on slopes of tryptic meat-agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°.

For large-scale growth the basal medium contained (g./l.): K_2HPO_4 , 9.6; KH_2PO_4 , 4.4; $(NH_4)_2SO_4$, 1.0; NH_4Cl , 2.5; $MgSO_4 \cdot 7H_2O$, 0.1; glucose, 2.0; lactic acid, 10; glycine 0.75. After addition of Fe^{2+} ions (final concn. 4 μM), from a solution of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ in twice its molar concentration of sodium citrate, the pH was brought to pH 7.3 with 10N-NaOH. Basal medium (500 ml.) and powdered agar (15 g.) were autoclaved in enamelled trays (38 cm. $\times$ 25 cm. $\times$ 3 cm.) at 108° for 5 min. and then brought to 126° for 20 min. Each tray was inoculated with sufficient organisms (about 1 mg. dry wt.) to ensure confluent growth; the inoculum was derived from Roux bottles containing the same agar medium and incubated for 18 hr. The trays, usually in batches of 200–400, were incubated at 37° for 40 hr.

The harvested organisms (about 0.8–1.0 g. dry wt./tray) were made into a smooth suspension with a little water in a ball mill. After filtering through glass wool to remove particles of agar, the suspensions were stored at 0° in full screw-capped bottles.

Smaller batches of organisms which were occasionally required were grown in liquid culture in the above-described basal medium (500 ml.) with vigorous shaking for 16 hr. at 37°; the inoculum was 5 ml. of a culture (24 hr., 37°) on the same medium. The organisms were washed twice on the centrifuge with one-tenth of culture volume of 0.067M-phosphate buffer, pH 7.2.

Preparation of enzymic extract

Organisms harvested from 200 trays (about 170–200 g. dry wt.) were washed twice on a Sharples centrifuge with 5 l. quantities of 0.067M-phosphate buffer, pH 7.1. The final sediment was suspended with vigorous stirring in acetone (5 l.) at –20° and the suspension was filtered through a Büchner funnel. The operation was repeated with a further 5 l. of acetone and, after washing four times with acetone (400 ml.), the cake of organisms was placed in a desiccator over P_2O_5 and the desiccator was evacuated for several hours to remove residual acetone. The organisms were left in the closed desiccator overnight and finally stored at –15° in sealed containers. The acetone-dried powder has been kept for 8–10 months without loss of activity.

Samples of the acetone-dried organisms were intimately mixed for 20 min. (in a Potter–Elvehjem homogenizer) with ten times the powder volume of either water or 0.067M-phosphate buffer, pH 7.1, at 25°. The supernatant after centrifuging for 20 min. at 16 000 *g* was stored at 0° and will be referred to as crude enzymic extract.

For most experiments the crude extract was first dialysed for 18–24 hr. against running tap water; there was some loss of activity (up to 20%) even when necessary cofactors were added.

A unit of enzyme is defined as that quantity catalysing

the formation of 1 μM -mole of L-methionine in 3 hr. Specific activity is expressed as μM -moles of L-methionine formed/mg. of protein/hr.

Extract of heated organisms

Washed or acetone-dried organisms (each 1 g.) were suspended in 6 ml. of water, heated for 3 min. at 100° and centrifuged for 20 min. at 16 000 *g*. The supernatant will be referred to as extract of heated *E. coli*; it contained about 20 mg. dry wt./ml. In the final stages of the work the heated extract was diluted with an equal volume of acetone, the precipitate removed and the supernatant lyophilized. All three methods of preparation gave material with similar properties.

Reaction mixture for the synthesis of methionine

The complete reaction mixture in which synthesis of methionine was tested contained (in 2 ml. final volume in 125 mm. $\times$ 16 mm. tubes): enzyme preparation (4–5 mg. of protein); DL-serine (10 μ moles); DL-homocysteine (20 μ moles); diphosphopyridine nucleotide (DPN; 0.5 μ mole); adenosine triphosphate (ATP; 7 μ moles); $MgCl_2$ (5 μ moles); extract of heated *E. coli* (EHC; 0.2 ml.); phosphate buffer, 0.02M, pH 7.2. The tubes were normally incubated for 3 hr. at 37°. Any deviations from these standard conditions are noted in the Results section.

The reaction was stopped by placing the tubes in boiling water for 3 min.; the coagulated protein (which retained about 5–10% of the total methionine) was removed by centrifuging and the supernatant was used for assay of methionine.

A control in which the complete reaction mixture was heated to 100° for 5 min. at zero time was included in each experiment.

Assay of methionine

With *Leuconostoc mesenteroides* P60 the procedure of Gibson & Woods (1960) was followed exactly.

Assays with *Streptococcus faecalis* (which were used in the latter part of the work) have the advantage that incubation need be for only 16–17 hr., compared with 44–48 hr. with *L. mesenteroides*. L-Methionine must, however, be used as standard since the organism contains a racemase which converts the D- into the L-isomer (Shockman & Toennies, 1954). Results with the two organisms showed satisfactory agreement.

The strain used was American Type Culture Collection no. 9790. The basal medium of Toennies & Shockman (1953) was used, but the glucose concentration was raised to 4% (w/v) and one-tenth of the culture volume of peroxide-treated peptone was added to increase the growth rate. For preparation of the latter, Bactopeptone (Difco) (50 g.) was added to 460 ml. of water, 42 ml. of 10N-HCl and 5.1 ml. of H_2O_2 (100 vol.). After standing for 24 hr. at room temperature the solution was filtered, heated for 30 min. at 100°, adjusted to pH 6.7 with 10N-NaOH, diluted to 1 l. and stored at 2°.

The assay was carried out in final culture volumes of 2 ml.; sterilization was by autoclaving for 5 min. at 108°. The inoculum was 0.1 ml. of a 6 hr. culture on the same medium containing just the optimum concentration of methionine. After incubation at 37° (in a water bath) for 16–17 hr., growth was assessed and interpreted as in the *L. mesenteroides* assay (Gibson & Woods, 1960).

Other estimations

Glycine. This was determined by the method of Alexander, Landwehr & Seligman (1945); the accuracy in the present work was about ± 15 –20%.

Protein. For intact organisms the biuret method of Stickland (1951) was used. Protein in enzymic extracts was determined turbidimetrically after precipitation with trichloroacetic acid (Stadtman, Novelli & Lipmann, 1951); crystalline serum albumin was used as standard.

Chromatography

Ascending and descending chromatograms were run on Whatman no. 1 or no. 4 filter paper. Developing agents used were: (a) propanol–pyridine–water (1:1:1, by vol.); (b) butanol–propionic acid–water (prepared by mixing equal volumes of 84 ml. of water in 1246 ml. of butanol and 620 ml. of redistilled propionic acid in 730 ml. of water); (c) acetone–triethylamine–water (80:5:15, by vol.). The last-named solvent was used for the separation of glycine from serine (Wright & Stadtman, 1956). The detecting agent for amino acids was ninhydrin.

Chemicals

Folic acid derivatives. Tetrahydropteroylglutamic acid was prepared by catalytic hydrogenation of pteroylglutamic acid (O'Dell, Vandenbelt, Bloom & Pfiffner, 1947). A specimen of *N*-formylpteroyltriglutamic acid isolated from *Clostridium cylindrosporum* by Dr Barbara Wright was kindly provided by her. Teropterin (pteroyl- γ -glutamyl- γ -glutamylglutamic acid) was a gift from Dr T. H. Jukes. Other folic acid compounds were as described by Lascelles, Cross & Woods (1954).

Other chemicals. Pyridoxal phosphate was a gift from Dr K. Folkers and thiomethyladenosine from Dr F. Schlenk. ATP (potassium salt) was either prepared from rabbit skeletal muscle (LePage, 1949) or obtained commercially. Other chemicals were commercial products, recrystallized when necessary.

Potassium phosphate buffers were used throughout.

Isotope experiments

Estimates of radioactivity were made in an end-window Geiger–Müller counter with dry samples in flat-bottomed aluminium planchets. Reported values (net counts) have

been corrected for self-absorption by using a self-absorption curve for BaCO_3 . Sufficient counts were made to give a standard error of $\pm 5\%$.

Reaction products were brought to pH 2.0 and separated into anionic and cationic fractions by chromatography on ion-exchange resin (Dowex-50, H^+ form). Labelled methionine was recovered from the cationic fraction by addition of 10 μmoles of unlabelled L-methionine as carrier and isolation by paper chromatography. The methionine spot was eluted and counted. (The radioactive precursor added was serine; R_F values of methionine and serine are 0.58 and 0.36 respectively in propanol–pyridine–water.)

The methyl group was removed from methionine by the method of Vieböck & Brecher (1930) in a micro-Zeisel apparatus; the methyl iodide was trapped in cold acetone containing isothiurea. The methyl isothiuronium salt was detected on paper chromatograms by the method of Večeřa & Gasparič (1954) and, after elution, the radioactivity of the chromatographic spot was determined.

RESULTS

General properties of the crude enzyme extract

The activity of extracts of acetone-dried organisms made with phosphate buffer (0.067 M), pH 7.1, was twice that of those made with either water or sodium bicarbonate (0.067 M). With the crude enzymic extract a net synthesis of methionine occurs in phosphate buffer with homocysteine and serine only (Table 1); this was increased by 50–100% by addition of an extract of heated *E. coli* (EHC). The following had no effect either in the presence or in the absence of EHC: ATP, DPN, pyridoxal phosphate, pteroylglutamic acid and its N^5 -formyltetrahydro derivative (leucovorin).

The crude extracts contained about 15–17 mg. of protein/ml. and had a specific activity (no added EHC) of 20–30 μmoles of L-methionine/mg. of protein/hr. The cell debris remaining after extraction had no activity. No active material was sedimented from the extracts by centrifuging for 1 hr. at 80 000 g.

Table 1. Comparison of methionine synthesis by whole organisms and enzymic extracts

Whole organisms were incubated for 4 hr. at 37° in 2 ml. of a reaction mixture which contained (in addition to substances indicated): bacterial suspension (0.1 ml., equivalent to 0.4 mg. of protein); glucose (0.05 M); *p*-aminobenzoic acid (10 μM); pyridoxal phosphate (2.5 μM); cobalamin (10 $\mu\text{mg.}/\text{ml.}$) and phosphate buffer (0.01 M, pH 7.3). Crude enzymic extract was incubated for 3 hr. at 37° in 2 ml. of a reaction mixture which contained (in addition to substances indicated): enzymic extract (0.4 ml., equivalent to 5 mg. of protein) and phosphate buffer (5 mM, pH 7.3). EHC, Extract of heated *E. coli* (see Methods section). +, Present; –, absent.

Source of enzyme	Additions to reaction mixture			L-Methionine formed ($\mu\text{moles}/\text{mg.}$ of protein/hr.)
	Homocysteine (0.1 M)	Serine (0.1 M)	EHC (0.2 ml.)	
Intact organisms	–	–	–	25
	–	+	–	40
	+	+	–	438
Crude enzymic extract	–	–	–	0
	–	+	–	7
	+	+	–	23
	+	+	+	37

Relative activity compared with whole organisms. The specific activity of the crude extract, even with EHC added, was only about 7 % of that of the original organisms (Table 1). This may result from destruction of cellular organization or from the presence of some enzyme or cofactor in limiting quantities only.

Stability. The extract lost little or no activity if kept at 0° for 2 weeks or at -15° for several months. Lyophilization, however, led to a 70 % loss. It is relatively stable to heat, the loss being only 50 % in 30 min. at 45° (pH 7.1).

Other enzymic activities. The crude extract did not destroy methionine; the relative lack of activity compared with whole organisms cannot therefore be due to this. No evidence for homocysteine desulphhydrase activity was found by searching for its product (α -aminobutyric acid) on paper chromato-

grams. The extract contained a weak serine deaminase; however, only 8 % of serine present in the standard reaction mixture for methionine synthesis was converted into pyruvic acid.

Enzyme and substrate concentrations. The rate of methionine formation increased with enzyme concentration up to the equivalent of about 5 mg. of protein/ml. (Fig. 1). The approximate concentrations of serine and homocysteine for half-maximum rate of synthesis of methionine were 1 and 3 mM respectively (Fig. 2); the value for homocysteine is reduced to 1 mM if anaerobic conditions are used, suggesting that homocysteine is not metabolized.

pH. The optimum pH under the present conditions is not sharp; over 90 % of the optimum methionine synthesis was obtained in the range pH 7.0-8.5 (Fig. 3).

Dialysed enzyme extract

A requirement for several cofactors besides extract of heated *E. coli* (EHC) could be demonstrated if the crude extracts were dialysed 18-24 hr. against running tap water (Table 2); furthermore the effect of EHC was sharpened.

Inorganic phosphate. Replacement of phosphate buffer in the standard reaction mixture by tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer of the same pH and molarity led to a 75 % decrease in methionine synthesis (Table 3); the residual activity may be due to phosphate present in EHC. Activity was restored by the addition of phosphate (Table 3); it was shown that this was not due to the Na^+ and K^+ ions added consequentially.

Fig. 1. Effect of concentration of enzymic extract. The reaction mixture (2 ml.) contained DL-serine (20 μ moles) and DL-homocysteine (20 μ moles) in 0.02M-phosphate buffer, pH 7.2; crude enzymic extract was added as indicated. Incubation was for 3 hr. at 37°.

Fig. 2. Effect of concentration of substrates. The reaction mixture (2 ml.) contained 0.3 ml. of crude enzymic extract (equivalent to 4.5 mg. of protein) in 0.02M-phosphate buffer, pH 7.2, with either DL-serine or DL-homocysteine (both 20 μ moles). The other amino acid (DL-serine, ○; DL-homocysteine, ●) was added as indicated. Incubation was for 3 hr. at 37°.

Fig. 3. Effect of pH. The reaction mixture contained crude enzymic extract (equivalent to 5 mg. of protein), DL-serine (20 μ moles) and DL-homocysteine (20 μ moles) in 0.02M-buffer of the pH indicated. Nature of buffer: succinate (pH 4.0-6.0); phosphate (pH 6.5-7.5); tris (pH 8.0-9.2). Phosphate (40 μ moles, of corresponding pH) was added in each case to satisfy the requirement for this ion.

An attempt was made, with isotope technique, to determine if the phosphate was required for the initial phosphorylation of one of the main substrates (homocysteine and serine) participating in methionine formation; [^{32}P]orthophosphate of high specific activity (1.06×10^5 counts/min./ μmole) was added to the standard reaction mixture. The organic phosphate fraction contained about 40% of the original radioactivity. The cation fraction (which contains the amino acids) was separated on Dowex-50 resin but contained no radioactivity. With the limitations set by possible lability of the compounds it was provisionally concluded that phosphate is not concerned in a phosphorylation of serine or homocysteine; it may be required, among other things, for the regeneration of ATP, since adenosine pyrophosphatase is present in the extracts.

Diphosphopyridine nucleotide. Omission of DPN from the standard reaction mixture decreased methionine synthesis to about 40% (Table 2). Maximum activity was obtained with about 0.3 $\mu\text{mole/ml}$. When the enzymic extract was first incubated with nicotinamide (0.5 mM) the DPN requirement for subsequent methionine synthesis was decreased by 20–40%.

Adenosine triphosphate and magnesium ion. The joint omission of these substances reduced synthesis of methionine to about 40% in the experiment of Table 2; in another experiment (Table 4), in which dialysis was more exhaustive, a greater reduction was obtained. There was very little extra activity when either substance was added without the other. For maximum activity it was necessary to add them in a definite molar ratio (1.2 ATP : 1 Mg^{2+} ion); a greater or lesser ratio gave markedly less synthesis (Table 4). Adenosine diphosphate did not replace ATP.

A similar relationship between ATP and Mg^{2+} ions has been found by Kuby, Noda & Lardy (1954) for ATP-creatine phosphorylase. It is supposed that a complex of Mg^{2+} ions and ATP rather than free ATP is the metabolite and that competition between the enzyme and ATP for the Mg^{2+} ions may occur.

Pyridoxal phosphate. No effect of this coenzyme was found even after exhaustive dialysis of the crude enzymic extract. Some stimulation (20–40%) was occasionally obtained if both the enzyme and the EHC were first irradiated (sunlight) but the effect was not reproducible. An effect of pyridoxal phosphate had been observed by Cross & Woods (1954) with an enzyme preparation made by grinding with alumina. Absorption of the present dialysed extract with alumina led to a small but reproducible degree of resolution; in two experiments addition of pyridoxal phosphate (20 μM) to the supernatant after such treatment in-

creased methionine synthesis from 0.13 and 0.10 to 0.16 and 0.12 μmole respectively.

Extract of heated Escherichia coli. The effect of EHC observed with crude enzymic extracts (Table 1) was much more pronounced with well-

Table 2. *Requirements for the synthesis of methionine*

Substances were omitted as indicated from the standard reaction mixture (see Methods section). Source of enzyme: 0.4 ml. (4 mg. of protein) of dialysed enzymic extract. Incubation was for 3 hr. at 37°.

Substance omitted	L-Methionine formed ($\mu\text{m-moles}$)
None	190*
DL-Serine	20
DL-Homocysteine	10
EHC	40
DPN	65
ATP + Mg^{2+} ions	80
Phosphate	40

* With crude undialysed enzymic extract this figure was 205.

Table 3. *Effect of orthophosphate on methionine synthesis*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4 mg. of protein) was used except that phosphate buffer was omitted and replaced by phosphate or tris buffers of the same pH (7.2) and the molarities indicated. Incubation was for 3 hr. at 37°. +, Present; -, absent.

Phosphate buffer (mM)	Tris buffer (0.02M)	L-Methionine formed ($\mu\text{m-moles}$)
20	—	141*
—	+	31
5	+	65
10	+	103
20	+	138

* This figure is reduced to 33 if EHC is omitted.

Table 4. *Effect of adenosine triphosphate and Mg^{2+} ions on methionine synthesis*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4 mg. of protein) was used except that ATP and Mg^{2+} ions were added at the concentrations indicated. Incubation was for 3 hr. at 37°. Results (relative activity) are expressed as percentages of the highest value.

ATP (mM)	Mg^{2+} ions (mM)	Molar ratio ATP/ Mg^{2+} ions	Relative activity
0	0	—	5
3	0	—	16
0	2.5	—	18
3	2.5	1.2	100
3	0.5	6.0	15
3	1	3.0	23
20	2.5	8.0	71
6	10	0.6	54
6	5	1.2	96

dialysed extracts; methionine synthesis was reduced to 25% in its absence even with ATP, Mg^{2+} ions, DPN and PO_4^{3-} ions present (Table 2). The relationship of effect to concentration of EHC was not linear (Fig. 4); possibly inhibitory material is also present.

It appeared likely that at least one component of EHC would be a coenzyme form of folic acid. With whole organisms of another strain of *E. coli* Gibson & Woods (1960) found strong presumptive evidence that *p*-aminobenzoic acid (which is required for methionine synthesis) must first be converted into some form of folic acid. Tetrahydropteroylglutamic acid, albeit at relatively high concentrations, is an effective cofactor for transfers of C_1 units in the serine-glycine reaction and in purine formation (see review by Wright, 1958) and has recently been shown by Nakao & Greenberg (1958) to function in the overall methylation of homocysteine by serine or formaldehyde with sheep-liver homogenates. However, neither tetrahydropteroylglutamic acid nor its N^5 -formyl derivative (leucovorin) had any activity in the present system in replacing EHC. Pteroylglutamic acid and *p*-aminobenzoic acid were both also without effect, as were combinations of all the above substances with each other and with cobalamin or EHC. Similar results were obtained by Cross & Woods (1954) in this Laboratory with their extract of alumina-ground organisms. EHC could be replaced, however, by heated extracts of pigeon, pig or rat liver.

Properties and partial purification of extract of heated *Escherichia coli*

In a study of the conversion of serine into glycine by cell-free extracts of *Clostridium sticklandii*

(earlier called *Clostridium* HF) it was found that extracts of heated *Cl. cylindrosporum* had much greater cofactor activity than tetrahydropteroylglutamic acid; the new factor was called CoC (Wright, 1955*a, b*, 1956; Wright & Stadtman, 1956). A preparation of CoC kindly provided by Dr Barbara Wright proved to be active for methionine synthesis in the present test system; similarly, crude EHC was active for the conversion of serine into glycine by extracts of *C. sticklandii* (unpublished observations of Dr Barbara Wright). EHC, like CoC (Wright, 1955*a*), was stable in alkali but unstable to acid.

The purification of EHC was therefore attempted by the procedure used for the isolation of CoC. The crude EHC was chromatographed on paper with propanol-pyridine-water as solvent system. The active fraction (R_f 0.33–0.66) was eluted with aq. 10 mM- NH_3 soln. and lyophilized; on the basis of dry weight a fivefold purification was obtained. Over the range tested the effect on methionine synthesis increased linearly with concentration (Fig. 4). The absorption spectrum (after correction for nucleotides present) indicated the presence of a folic acid derivative but no further deductions could be made.

Effect of folic acid conjugates

Wright (1955*b*, 1956) found that CoC could be fractionated into several active compounds, all of them folic acid derivatives. There was evidence that one of them was an *N*-formylpteroyltrimethylglutamic acid, possibly the N^5 -compound. A specimen of this material was kindly given by Dr Barbara Wright. Although it increased methionine synthesis under the present conditions it was not as

Fig. 4. Effect of extract of heated *E. coli*. Standard reaction mixture containing dialysed enzymic extract (equivalent to 4 mg. of protein) was used with crude (●) or partly purified (○) extract of heated *E. coli* added as indicated. Incubation was for 3 hr. at 37°.

Table 5. Effect of folic acid derivatives on methionine synthesis

Standard reaction mixture containing dialysed enzymic extract equivalent to 3.2 mg. of protein was used, but with the extract of heated *E. coli* (EHC) replaced as shown. A final volume of 1 ml. only was used. Incubation was for 3 hr. at 37°.

Additions		L-Methionine formed (μm-moles)
Substance	Concn. (mg./ml.)	
None	—	60
EHC*	6	185
	(μM)	
<i>N</i> -Formylpteroyltrimethylglutamic acid	6	60
	20	84
	40	100
Pteroyltrimethylglutamic acid	6	64
	120	64

* Acetone-treated extract of heated *E. coli* (20 mg. dry wt./ml.) was used in this experiment (see Methods section).

Table 6. *Incorporation of [β - 14 C]serine into methionine*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 5 mg. of protein) was used except that [β - 14 C]serine was added to the DL-serine; DL-homocysteine was omitted, as indicated, in one experiment. Incubation was for 3 hr. at 37°.

	Expt. 1	Expt. 2*	
		With homocysteine	Without homocysteine
Specific activity of serine (counts/min./ μ mole)	9.2×10^3	2.3×10^4	2.3×10^4
L-Methionine formed			
By microbiological assay (μ m-moles)	250	280	40
By chromatographic isolation { (counts/min.) (μ m-moles)†	1660 177	5420 230	880 40
Activity of methylisothiuronium salt (counts/min.)	750	3189	—
Activity of residue after distillation of methyl iodide (counts/min.)	45	260	—

* A preparation from heated *C. cylindrosporum* replaced EHC in this experiment.

† Calculated from the activity of serine.

active as EHC on the basis of approximate folic acid content of the latter as judged from the absorption at 260 m μ (Table 5). Pteroyltriglutamic acid (teropterin) had no activity even at three times the concentration of the formyl derivative.

Incorporation of [β - 14 C]serine into methionine

Gibson & Woods (1960) found production of methionine by *E. coli* PA 15 (whole organisms) with serine as C₁-unit donor to be accompanied by the production of approximately equimolar quantities of glycine. It was probable therefore that the methyl group of methionine was derived from the β -C atom of serine; this occurs in animal tissues (Arnstein & Neuberger, 1953; Stekol, 1955). The point was investigated with enzymic extracts of *E. coli* PA 15 by including [β - 14 C]serine in the standard reaction mixture. Methionine was determined as usual by microbiological assay and, after addition of inactive carrier methionine, by determination of radioactivity after isolation by paper chromatography. The methionine was labelled but the quantity was about 25% smaller than that expected from the microbiological assay (Table 6), probably due to losses during the multiple manipulations with small quantities. The methionine was degraded and over 90% of the total radioactivity recovered (50–60%) was found in the methyl isothiuronium salt (Table 6); the low overall recovery is again probably due to experimental losses.

Fractionation of enzymic extract

The complexity of cofactors required for the methylation of homocysteine suggests that there may be several steps in the reaction; furthermore, the work of Emerson (1950) with *Neurospora* mutants tends to the same conclusion. A simple fractionation of the enzymic extract was therefore attempted to see if two or more defined fractions

Table 7. *Fractionation of enzymic extract*

Activity was estimated with the standard reaction mixture.

Fraction	Total protein (mg.)	Total activity (units*)	Specific activity*
Original extract	925	65 000	23
P1	150	2 730	6
P2	11	180	5
P3	145	6 760	16
P4	375	2 500	2
P5	98	1 220	4
P1 + P2; P1 + P4; P2 + P4; P2 + P5; P4 + P5	—	—	2–7
P3 + P4 + P5	618	24 500	51†

* For definition of these terms see Methods section.

† Expressed in terms of the protein content of fraction P3 only.

were required and if there were any evidence for an intermediate.

The crude enzymic extract was cooled to 0°, brought to pH 5.2 with N-acetic acid and centrifuged at 16 000 g for 10 min. The sediment (P1) was dissolved in minimal volume of 0.2M-phosphate buffer, pH 7.5.

The supernatant was brought to pH 6.0 with phosphate buffer and precipitated at 0° with increasing concentrations of ammonium sulphate. Fractions were collected successively at 0.35, 0.5, 0.6 and 0.9 saturation (P2–P5 respectively), dissolved in 0.2M-phosphate buffer and dialysed for 12–14 hr. at 0° against the same buffer.

With the exception of P3 the activity of all the fractions was low compared with that of dialysed crude extract (Table 7). Recombination of pairs of fractions led to no improvement. However, a mixture of fractions P3, P4 and P5 had about 2.5 times the total activity of the sum of the separate

fractions although only about 40 % of the original activity was recovered. The relatively high activity of P3 may therefore be due to some contamination with material precipitated in bulk with P4 and P5. The results obtained were not sufficiently clear-cut to offer any hope of detecting and isolating an intermediate.

Formation of glycine from serine

With whole organisms of the present strain Gibson & Woods (1960) found approximately equimolar formation of methionine and glycine from homocysteine and serine, as did also Cross & Woods (1954) with an enzyme preparation made by grinding with alumina. One component of the present enzymic extract might well be therefore an enzyme cleaving serine to glycine, the residual β -C-atom fragment being ultimately donated to

homocysteine. A similar enzyme found in animal tissues by Kisliuk & Sakami (1955) has been termed serine hydroxymethylase; it is also present in *C. sticklandii* (Wright & Stadtman, 1956). Gibson & Woods (1960) found some formation of glycine from serine in the absence of homocysteine by suspensions of *E. coli* PA15.

The products of typical experiments on methionine synthesis by dialysed enzymic extracts both in the presence and in the absence of homocysteine were assayed for glycine (Table 8). The results were irregular but showed definitely the formation of glycine from serine in the absence of homocysteine; in two out of three cases there was some increase of glycine when homocysteine was also present and methionine was formed. It was not expected that the results would have quantitative significance for the following reasons: (a) with the method of assay used the control values for glycine (serine absent) were of the same order as the additional glycine formed in the presence of serine; (b) a serine deaminase was also present (see beginning of Results section); (c) possible variability with different enzyme preparations in the extent of the competition between homocysteine and other endogenous acceptor systems for the C_1 -unit fragment released from serine. There was some evidence from isotope experiments that in the absence of homocysteine the other ultimate product was formate.

Cofactor requirements. The amount of glycine formed (homocysteine absent) was much reduced if phosphate, DPN or ATP + Mg^{2+} ions were omitted from the reaction mixture (Table 9). The extract of heated *E. coli* was also necessary; glycine formation was reduced two- to three-fold in its absence (Table 9). The cofactor requirements for glycine formation from serine are thus qualitatively similar to those for methionine formation from homocysteine and serine.

Table 8. *Production of glycine from serine*

Standard reaction mixture (containing dialysed enzymic extract equivalent to 4.5 mg. of protein) was used except that EHC was increased to 0.4 ml. and DL-homocysteine was omitted as indicated. Incubation was for 3 hr. at 37°.

	Expt.	Product (μ m-moles)	
		Glycine*	Methionine
Homocysteine absent	1	150	30
	2	120	35
	3	286	40
Homocysteine present	1	286	185
	2	170	170
	3	260	180

* Control values (serine absent) were 260, 170 and 370 for Expts. 1-3 respectively. These values have been deducted to obtain the figures given.

Table 9. *Cofactor requirements for the formation of glycine from serine*

Standard reaction mixture containing dialysed enzymic extract equivalent to 5 mg. of protein was used but with DL-homocysteine omitted and EHC added as shown; other omissions were as indicated. Incubation was for 3 hr. at 37°.

Vol. of extract of heated <i>E. coli</i> (ml.)	Other substances omitted	Glycine formed (μ m-moles)*	
		Expt. 1	Expt. 2
0	None	100	—
0.3	None	226	—
0.6	None	300	—
0.5	None	—	280
0.5	ATP + Mg^{2+} ions	—	130
0.5	DPN	—	140
0.5	PO_4^{3-} ions	—	160

* Control values without serine were deducted: these were 160 and 220 for Expts. 1 and 2 respectively.

DISCUSSION

The overall methylation of homocysteine by serine requires not only the transfer of the hydroxymethyl group from serine (at the formaldehyde level of oxidation), but also its reduction to a methyl group. Although DPN, which is shown to be required, may serve as intermediate there must be present some hydrogen-donor system. Since in the present work none was deliberately added (or appeared to be required) it must have been derived from the dialysed enzymic extract, from the extract of heated *E. coli* or from one of the substrates. The most probable source would appear to be pyruvate formed from serine by the serine deaminase known to be present; *E. coli* is known to contain a pyruvic-oxidase-enzyme system.

The work of Gibson & Woods (1960) with intact organisms coupled with the results of the present experiments make it reasonably certain that some coenzyme form of folic acid is required for the overall reaction. This would be expected for a C_1 -unit-transfer reaction and the extract of heated *E. coli* which was necessary had properties akin to the coenzyme required for glycine formation from serine by *C. sticklandii*; this is certainly a form of folic acid (Wright & Stadtman, 1956; Wright, 1956). Furthermore, a sample of an *N*-formylpteroyltriglutamic acid isolated from *C. cylindrosporum* had activity (though only at high concentrations) in the present system.

In all other C_1 -unit-transfer reactions so far examined tetrahydropteroylglutamic acid is active, though it may not be the natural coenzyme (see review by Wright, 1958). Moreover in work with sheep-liver homogenates Nakao & Greenberg (1958) gave evidence that it functions as cofactor in the methylation of homocysteine by serine and formaldehyde. Tetrahydropteroylglutamic acid was, however, completely inactive in the present experiments. It is possible that the bacterial extracts lack some enzyme(s) which convert it into the true coenzyme form or that some other factor is required for its utilization which is not required by the natural coenzyme. More recent work in this Laboratory described briefly by Kisluk & Woods (1957) and Helleiner, Kisluk & Woods (1957) has shown that if the organisms are grown originally on a medium containing added cobalamin the extracts of acetone-dried organisms are then able to use tetrahydropteroylglutamic acid as cofactor; furthermore, ultrasonic extracts of organisms from normal growth medium can also do so, but only if cobalamin is added to the extract.

It is clear that serine is degraded to glycine and that the residual β -C-atom of serine becomes the C atom of the methyl group of methionine. The formation of glycine from serine by the enzymic extracts also requires the extract of heated *E. coli* and, on the basis of the present results alone, this might be (though not of necessity) the only function of the coenzyme form of folic acid. Work in this Laboratory (Kisluk & Woods, 1959), however, has shown that tetrahydropteroylglutamic acid is an effective coenzyme for this reaction under normal test conditions whereas the synthesis of methionine requires the heated extract of the organism. The coenzyme form of folic acid must thus have a function in the acceptance as well as in the generation of the C_1 unit.

SUMMARY

1. Soluble extracts of acetone-dried *Escherichia coli* (an auxotrophic strain requiring serine or

glycine for growth) synthesize methionine from homocysteine and serine.

2. After dialysis of the enzymic extract there are requirements (not absolute) for phosphate and magnesium ions, adenosine triphosphate, diphosphopyridine nucleotide and a heated extract of *E. coli*.

3. The heated extract of *E. coli* probably contains a coenzyme form of folic acid. It is not replaced by tetrahydropteroylglutamic acid or its N^5 -formyl derivative. It is partially replaced by a *N*-formylpteroyltriglutamic acid extracted from *Clostridium cylindrosporum*.

4. Isotope technique showed that the β -carbon atom of serine became the methyl carbon atom of methionine.

5. The enzymic extracts also degraded serine to glycine; the cofactor requirements were qualitatively similar to those for methionine synthesis.

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Physical and Chemical Properties of Polysaccharides and Glycoproteins of the Yeast-Cell Wall

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The composition of the yeast-cell wall is known to be approximately glucan 29%, mannan 31%, protein 13%, lipid 8.5% and ash 3% (Northcote & Horne, 1952; Roelofsen, 1953). In addition, numerous investigators have reported the presence of 1–3% of glucosamine, which has been attributed to the occurrence of chitin in the wall (Roelofsen & Hoette, 1951; Houwink & Kreger, 1953; Falcone & Nickerson, 1956; Eddy, 1958). The existence of complexes between the polysaccharide and protein has often been inferred, and recently some evidence for a mannan-protein complex has been obtained (Falcone & Nickerson, 1956; Eddy, 1958). Usually strong alkali has been used to isolate the polysaccharide components and this treatment undoubtedly degrades any complexes between these molecules and protein. We therefore decided in these investigations to use milder extraction procedures. This has resulted in the isolation from the cell wall of several fractions which appear to be glycoproteins. As these fractions also contained glucosamine the distribution of this sugar among the cell-wall components was reinvestigated.

EXPERIMENTAL

Materials. The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers Co. Ltd.). Cell walls were prepared according to the method of Shockman, Kolb

& Toennies (1957). Each stainless-steel cup (internal measurements 12.5 cm. × 2.5 cm.) contained 20 ml. of a 10% (w/v) suspension of yeast and 20 g. of Ballotini no. 12 glass beads (Chance Bros. Ltd., Birmingham). Breakage was continued for 15 min. in an International refrigerated centrifuge at 1400 rev./min. at 4°. The walls were separated from any unbroken cells and cell contents by differential centrifuging according to Northcote & Horne (1952). The yield of freeze-dried walls was 1 g. from 100 g. of pressed yeast.

Analytical procedures

pH measurements. These were made with a Pye pH meter and a glass electrode at room temperature and by standardizing the apparatus with standard buffer tablets, pH values 4.01 and 6.99 (Burroughs Wellcome and Co.).

Boundary electrophoresis. The samples were examined as approximately 1.5% solutions in 0.05M-sodium borate buffer, pH 9.2, 0.1M-K₂HPO₄, pH 9.3, or 0.05M-sodium borate–8M-urea, pH 9.2. The Perkin-Elmer model 38 Tiselius electrophoresis apparatus was used.

Diffusion measurements. These measurements were made over a period of 3 days on 0.6% solutions in 0.1M-KH₂PO₄, pH 4.7. The Tiselius electrophoresis apparatus was used.

Sedimentation measurements. The samples were dissolved in 0.1M-KH₂PO₄ (0.05–2.5%) and sedimented at 2.69 × 10⁵g for 2.5 hr. in the Spinco Analytical ultracentrifuge model E (Specialized Instruments Co., Belmont, Calif., U.S.A.). Pictures were taken every 16 min.

Total nitrogen and phosphorus. The micro-Kjeldahl digestion method of Chibnall, Rees & Williams (1943) was used, followed by distillation and titration. Phosphorus was determined according to the method of Fiske & Subbarow (1925).

Neutral sugars. Samples were hydrolysed in 2N-H₂SO₄ and the sugars were identified chromatographically (Northcote, Goulding & Horne, 1958).

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