- Eggitt, P. W. R. & Norris, F. W. (1956). *J. Sci. Fd Agric.* **7**, 493.
- Eggitt, P. W. R. & Ward, L. D. (1953). *J. Sci. Fd Agric.* **4**, 569.
- Fieller, E. C. (1940). *Suppl. J. Roy. statist. Soc.* **7**, 50.
- Friedman, L., Weiss, W., Wherry, F. & Kline, O. L. (1958). *J. Nutr.* **65**, 143.
- Gitler, C., Sunde, M. L. & Boumann, C. A. (1958). *J. Nutr.* **65**, 397.
- Green, J., Edwin, E. E., Bunyan, J. & Diplock, A. T. (1960). *Biochem. J.* **75**, 456.
- Green, J., McHale, D., Marcinkiewicz, S., Mamalis, P. & Watt, P. R. (1959). *J. chem. Soc.* p. 3362.
- Green, J., Mamalis, P., Marcinkiewicz, S. & McHale, D. (1960). *Chem. & Ind.* p. 73.
- Green, J. & Marcinkiewicz, S. (1956). *Nature, Lond.*, **176**, 86.
- Green, J., Marcinkiewicz, S. & Watt, P. R. (1955). *J. Sci. Fd Agric.* **6**, 274.
- György, P. (1951). *Vitamin Methods*, vol. 2, p. 152, New York: Academic Press Inc.
- Muth, O. H., Oldfield, J. E., Remmert, L. G. & Schubert, J. R. (1958). *Science*, **128**, 1090.
- Nitowsky, H. M. & Tildon, J. T. (1956). *Amer. J. clin. Nutr.* **4**, 397.
- Rose, C. S. & György, P. (1950). *Blood*, **5**, 1062.
- Rose, C. S. & György, P. (1952). *Amer. J. Physiol.* **168**, 414.
- Roughton, F. J. W. & Booth, V. H. (1938). *Biochem. J.* **32**, 2049.
- Schwarz, K. & Foltz, C. M. (1957). *J. Amer. chem. Soc.* **79**, 3292.
- Smith, K. W., Marks, H. P., Fieller, E. C. & Broom, W. A. (1944). *Quart. J. Pharm.* **17**, 108.
- Vincent, P. C. & Blackburn, C. R. B. (1958). *Aust. J. exp. Biol. med. Sci.* **36**, 471.
- Voth, O. L. & Miller, R. C. (1958). *Arch. Biochem. Biophys.* **77**, 191.

Biochem. J. (1960) **75**, 467

Interrelationships between Folic Acid and Cobalamin in the Synthesis of Methionine by Extracts of *Escherichia coli*

BY R. L. KISLIUK*† AND D. D. WOODS

Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 28 September 1959)

Gibson & Woods (1960) showed that washed suspensions of various strains of *Escherichia coli* synthesize methionine from homocysteine; serine was an effective source of the methyl group. With *p*-aminobenzoic acid and cobalamin auxotrophs they also showed these vitamins to be essential for the reaction. Parallel inhibition by sulphathiazole of both the synthesis of methionine from homocysteine and of folic acid from *p*-aminobenzoic acid gave strong, though indirect, evidence that *p*-aminobenzoic acid must first be converted into some form of folic acid before it exerts its function. However, no folic acid derivative tested was able to replace *p*-aminobenzoic acid for methionine synthesis by the intact organisms.

Enzymic extracts of an auxotroph of *E. coli* requiring serine or glycine for growth were obtained by Cross & Woods (1954) by grinding with alumina and by Szulmajster & Woods (1955, 1960) by extracting acetone-dried organisms. Both preparations synthesized methionine from homocysteine and serine and there was, among other

factors, a partial requirement for an extract of heated *E. coli*. The last-named authors partly purified the heated extract and obtained evidence that it contained a folic acid derivative. With neither source of enzyme could the heated extract be replaced by either tetrahydropteroylglutamic acid or its *N*⁵-formyl derivative (leucovorin). This was surprising, because although tetrahydropteroylglutamic acid may not be the natural folic acid coenzyme (Wright, 1958) it is effective in replacing it in all other C₁-transfer reactions so far studied.

The object of the present work was to investigate this point further and to try to find conditions under which tetrahydropteroylglutamic acid could be used. Since cobalamin is known to be required for methionine synthesis in *E. coli* by growing cultures (Davis & Mingioli, 1950), washed suspensions (Gibson & Woods, 1952, 1960) and enzymic extracts (Helleiner & Woods, 1956) of cobalamin auxotrophs, special attention was paid to the effect of this vitamin. It was found that growth in the presence of added cobalamin permitted the utilization of tetrahydropteroylglutamic acid by enzymic extracts of acetone-dried organisms. Brief reports of some of the results have been given (Kisliuk & Woods, 1957, 1959).

* Fellow of the National Foundation for Infantile Paralysis, U.S.A.

† Present address: National Institutes of Health, Bethesda 14, Maryland, U.S.A.

MATERIALS AND METHODS

Organism. *Escherichia coli* PA15, an auxotrophic strain requiring serine or glycine for growth, was used throughout. It was obtained originally from Dr B. E. Wright and was maintained as described by Gibson & Woods (1960). The strain was chosen because when grown on glycine it synthesizes methionine more actively than other strains tested (Gibson & Woods, 1960); it was also used in these Laboratories by Cross & Woods (1954) and Szulmajster & Woods (1955, 1960) for the preparation of enzymic extracts which synthesize methionine.

Medium and growth. The glucose-inorganic salts medium of Davis & Mingioli (1950), supplemented with glycine (0.8 g./l.), was used except that the glucose was autoclaved with the other constituents. The organisms were grown semi-anaerobically in 5 l. culture volume in 5 l. flasks plugged with cotton wool for 48 hr. at 37°. The inoculum was 5 ml. of 18 hr. culture grown in the same medium. The yield of organisms was about 0.2 g. dry wt./l.; this was increased by about 20–25% when cobalamin (4 µg./l.) was added to the growth medium. The organisms were harvested in a continuous centrifuge and washed once with one-twentieth of the culture volume of water.

Preparation of enzymic extract. Organisms from 25 l. of medium were added to 2.5 l. of acetone at –15°, shaken vigorously and set aside at –15° for 20 min. The precipitate was collected on a sintered-glass funnel, washed three times with 100 ml. quantities of acetone at –15° and placed in a vacuum desiccator, which was exhausted for 3 hr. with a water pump. The powder (yield 0.16 g./l. of medium without cobalamin) retained full activity for at least 6 months when stored in a sealed container at –15°.

Acetone-dried organisms (1 g.) were extracted with 14 ml. of water by rubbing with a glass rod for 20 min. The extract was centrifuged (25 000 g.; 45 min.) at 0° and the supernatant dialysed for 14 hr. at 3° against 0.05 M-phosphate buffer, pH 7.8; it then contained 7–10 mg. of protein/ml. The ratio of absorption at 280 mµ to that at 260 mµ was 0.62–0.7, indicating a nucleic acid content of 10–17%.

From some experiments the dialysed extract was treated with Dowex-1 resin to remove residual folic acid coenzyme. The extract (10 ml.; 7.5 mg. of protein/ml.; 280 mµ/260 mµ ratio 0.62) was passed through a short column (1.5 cm. × 3 cm.) of Dowex-1 (chloride form, ×2, 50–100 mesh) at 3°; the column was washed with water until the original volume of solution was obtained (6.8 mg. of protein/ml.; 280 mµ/260 mµ ratio 0.7).

Bulk production of enzymic extracts. In the latter part of the work it was necessary to have a uniform source of enzymic extract for assaying the factor formed in the presence of cobalamin which permits the utilization of tetrahydropteroylglutamic acid. A large batch of organisms was grown on the surface of agar medium in trays and the product acetone-dried as described by Szulmajster & Woods (1960). We are greatly indebted to the Director and staff (particularly Mr F. C. Belton) of the Microbiological Research Establishment, Ministry of Supply, Porton Down, Wilts, for undertaking the growth of the organisms. The yield of organisms was higher than with the semi-anaerobic liquid cultures normally used but the activity of the enzymic extracts was 20–30% less.

Extract of heated *Escherichia coli*. Acetone-dried organisms (0.5 g.) were rubbed up with 3 ml. of water and heated at 100° for 3 min. The supernatant after centrifuging was stored at –15°.

Test for methionine synthesis. The standard reaction mixture used to test methionine synthesis by enzymic extracts was based on the work of Cross & Woods (1954) and Szulmajster & Woods (1960). It contained, in 2 ml. final volume of potassium phosphate buffer, pH 7.8 (containing 200 µmoles of phosphate ion): DL-homocysteine (20 µmoles); L-serine (10 µmoles); hexose diphosphate (15 µmoles); adenosine triphosphate (ATP; 10 µmoles); diphosphopyridine nucleotide (DPN; 1 µmole); pyridoxal phosphate (1 µmole); MgSO₄ (10 µmoles); the final pH was 7.4. Any changes from the standard mixture are noted for individual experiments; in particular the DL-homocysteine concentration was usually decreased when the formaldehyde derivative of tetrahydropteroylglutamic acid replaced serine as donor of C₁ units.

The reaction was carried out in a final volume of 2 ml. in Thunberg tubes filled with H₂ or N₂, which were incubated at 37°. The reaction was stopped by placing the tubes in a boiling-water bath for 3 min.; after centrifuging off the precipitate the supernatant fluid was assayed for methionine and other products.

Estimation and detection of methionine. The microbiological assay with *Leuconostoc mesenteroides* P60 described by Gibson & Woods (1960) was followed exactly. Standards included in each assay contained 20, 40, 80, 120 and 160 µm-moles of DL-methionine; each experimental sample was assayed in quadruplicate. The organism responds only to L-methionine. Apart from the specificity tests noted by Gibson & Woods (1960) it did not respond to cobalamin (2 µmg./ml.), DL-1,3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), L-cystathionine, DL-methionine methylsulphonium iodide (all at 50 µm-moles/ml.) or to the initial constituents of the standard reaction mixture; the latter mixture also did not inhibit the response to L-methionine.

In some experiments the production of methionine was verified by chromatography on Whatman no. 1 paper. The chromatograms were developed with butanol-formic acid-water (77:10:13, by vol.) (Strack, Friedel & Hamsch, 1956), which separates methionine (*R_F* 0.57) from 1,3-thiazane-4-carboxylic acid (*R_F* 0.40); the latter is formed spontaneously from homocysteine and formaldehyde at neutral pH. Typical reaction products in which methionine was indicated by microbiological assay showed a ninhydrin-positive spot with the same *R_F* as authentic L-methionine; furthermore, this was the only region of the chromatogram which satisfied the growth requirement of *L. mesenteroides* P60.

Estimation of serine. The method used was modified from that of Frisell, Meech & Mackenzie (1954). The reaction was stopped by adding 15% (w/v) trichloroacetic acid (0.8 ml.) and the precipitate removed by centrifuging. The supernatant (1.5 ml.) was pipetted onto a column of Dowex-50 resin (H⁺ form, ×8, 100–200 mesh, 0.5 cm. × 4 cm.) which was then washed with water (20 ml.) to remove any free formaldehyde. Serine was eluted with 4 N-HCl (20 ml.), collected in 25 ml. beakers and evaporated to dryness on a hot plate. The residue was taken up to 1 ml. with water plus a drop of methyl red (made up in a dilute acid), neutralized with 0.25 N-NaOH and again evaporated to dryness.

The residue was thoroughly mixed with 0.075 M-sodium metaperiodate (1.5 ml.) and set aside for 15 min. The solution was poured into a short column (1 cm. $\times$ 3 cm.) of Dowex-1 resin (chloride form, $\times$ 2, 50–100 mesh); this retains periodate but allows formaldehyde formed from the β -C of serine to pass through. A total volume of 10 ml. of eluate and washings was collected. Formaldehyde was assayed on appropriate samples with the acetylacetone reagent of Nash (1953).

When DL-homocysteine was present in the reaction mixture it was necessary to separate serine from 1:3-thiazane-4-carboxylic acid, which is also broken down by periodate to yield formaldehyde. This was achieved by substituting a column of Amberlite IR 120 resin (60–100 mesh, 0.8 cm. $\times$ 18 cm.) for the Dowex-50 used in the first treatment above. After washing the column with water the serine was eluted with N-HCl (15–40 ml.) and the estimation continued as described above. The thiazane-carboxylic acid (which is always present in the products) may arise by reaction of homocysteine either with the formaldehyde derivative of tetrahydropteroylglutamic acid or with free formaldehyde formed from the β -C of serine.

Serine formation was verified in typical experiments by chromatography on Whatman no. 1 paper. The developing agent, which separates serine (R_F 0.71) from glycine (R_F 0.56), was triethylamine-acetone-water (5:80:15, by vol.) (Korkes, S. cited by Wright & Stadtman, 1956).

Assay of cobalamin. The method used was modified from that of Hutner *et al.* (1949); the assay organism was *Euglena gracilis* var. *bacillaris*. Medium S of Lascelles (1956) was supplemented with (per l. final volume): (a) acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld, 1945) equivalent to 0.5 g. of casein and (b) 1.3 ml. of 4 mM-iron citrate (prepared as described by Lascelles, 1956). The pH was adjusted to 4.5 instead of 6.8.

The medium (5 ml. final volume in 16 mm. $\times$ 125 mm. tubes) was autoclaved (10 min. at 115°) after the addition of cobalamin or experimental samples. Standards covering the range 0–6 μ g. of cobalamin/ml. were included in each assay. The inoculum was derived from a 7-day culture on the same medium containing 4 μ g. of cobalamin/ml.; the organisms were centrifuged out, resuspended in the culture volume of 0.9% NaCl and 0.05 ml. of the suspension was added to each assay tube. Incubation was for 10 days at 30° and 1 ft. distant from two 60 W tungsten-filament lamps. Growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral-density filter.

Estimation of protein. This was determined spectrophotometrically according to Layne (1957). The ratio of absorption at 280 $m\mu$ and at 260 $m\mu$ also gave information on the relative amount of nucleic acid present.

Tetrahydropteroylglutamic acid. Pteroylglutamic acid (folic acid) was catalytically reduced by the method of O'Dell, Vandenbelt, Bloom & Pfiffner (1947). The product was assayed by iodine titration or by treatment with excess of formaldehyde and estimating the unbound formaldehyde with Nash (1953) reagent; 70% was capable of binding formaldehyde and was presumably tetrahydropteroylglutamic acid.

The formaldehyde derivative of tetrahydropteroylglutamic acid (tetrahydropteroylglutamic acid-formaldehyde) was prepared (in solution) by mixing equimolar solutions (usually 0.035 M) of the acid and formaldehyde in M-

phosphate buffer, pH 7.8, under an atmosphere of H_2 (Kisliuk, 1957).

Chemicals. The ATP, DPN and triphosphopyridine nucleotide (TPN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. Hexose diphosphate (barium salt) and cobalamin (Anacobin) were from British Drug Houses Ltd., London. Pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts), protamine sulphate (from herring roe) (L. Light and Co. Ltd., Colnbrook, Bucks) and DL-homocysteine (Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A.) were also commercial specimens.

RESULTS

It should be emphasized that positive results with cobalamin were obtained only when it was added to the medium on which the organisms were grown; it had no effect when added to the enzymic extracts of acetone-dried organisms harvested from a medium to which cobalamin had not been added.

Inhibition of methionine synthesis by tetrahydropteroylglutamic acid

With dialysed (though not Dowex-1-treated) enzymic extracts of organisms grown without cobalamin substantial synthesis of methionine occurs with L-serine as C_1 donor; there is presumably sufficient coenzyme form of folic acid remaining after dialysis to catalyse the transfer. This synthesis is, however, abolished if tetrahydropteroylglutamic acid is added (Fig. 1, no cobalamin in medium): 0.3 μ mole in 2 ml. of test solution completely inhibits and 50% reduction is given by 0.1 μ mole. The inhibition is not overcome by adding to the reaction mixture a heated extract of *E. coli* (presumed to contain the natural folic acid coenzyme) or cobalamin (37 μ m-moles). The mechanism of this inhibition is unknown and has not been further explored.

Influence of growth in the presence of cobalamin

Serine as one-carbon-atom donor. Methionine synthesis did occur from serine and homocysteine in the presence of tetrahydropteroylglutamic acid provided that the organisms were grown in a medium containing cobalamin; increasing concentration of cobalamin (up to about 1 μ mg./l. of medium) yielded extracts of increasing activity (Fig. 1). By contrast methionine synthesis in the absence of tetrahydropteroylglutamic acid (natural folic acid as coenzyme) was markedly diminished by low concentrations of cobalamin in the medium; at higher concentrations it was partially restored (Fig. 1). These results possibly suggest that a new pathway for methionine synthesis results from growth on cobalamin, a pathway in which tetrahydropteroylglutamic acid is used in place of the natural coenzyme.

Formaldehyde derivative of tetrahydropteroylglutamic acid as one-carbon-atom donor

Free formaldehyde will not serve as source of the methyl group of methionine with either intact organisms or enzymic extracts of *E. coli* PA15. It is probable that it is inactivated by spontaneous reaction with homocysteine to form 1:3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), which is biologically inert. Tetrahydropteroylglutamic acid-formaldehyde (Kisliuk, 1957) is also inactive with enzymic extracts from organisms grown without cobalamin. Growth with cobalamin, however, confers activity in this respect also (Fig. 1). The results of Fig. 1 give a falsely low impression of the activity of tetrahydropteroylglutamic acid-formaldehyde; the concentration of homocysteine used ($10\text{ }\mu\text{moles/ml.}$) was optimum for serine as donor whereas the optimum concentration for tetrahydropteroylglutamic acid-formaldehyde is only one-third of this and shows a sharp maximum (Fig. 2). It is possible that the tetrahydropteroylglutamic acid-formaldehyde is decomposed by the higher concentrations of homocysteine with formation of the thiazanecarboxylic acid. The relative rates of methionine synthesis from serine and tetrahydropteroylglutamic acid-

Fig. 1. Effect of varying concentrations of cobalamin in the growth medium on methionine synthesis by extracts of acetone-dried organisms. Standard reaction mixture was used, except that L-serine was omitted and hexose diphosphate was $10\text{ }\mu\text{moles}$. Source of enzyme: dialysed extracts of organisms grown in the presence of the stated concentrations of cobalamin and equivalent to 7 mg. of protein for the experiments with serine alone and to 9 mg. in other cases. Additions to reaction mixture: L-serine ($10\text{ }\mu\text{moles}$), tetrahydropteroylglutamic acid and tetrahydropteroylglutamic acid- CH_2O (each $3.6\text{ }\mu\text{moles}$). Incubated for 4 hr. ○, L-Serine; ●, L-serine plus tetrahydropteroylglutamic acid; ◐, tetrahydropteroylglutamic acid- CH_2O ; ●, tetrahydropteroylglutamic acid.

Fig. 2. Effect of concentration of DL-homocysteine on methionine synthesis. Standard reaction mixture was used, with the omission of L-serine, and, in the experiment with tetrahydropteroylglutamic acid- CH_2O , pyridoxal phosphate; DL-homocysteine was present as indicated. Source of enzyme: dialysed extract (equivalent to 10 mg. of protein) of organisms grown with cobalamin. Incubated for 3 hr. Additions to reaction mixture: ○, L-serine, $10\text{ }\mu\text{moles}$; ●, tetrahydropteroylglutamic acid- CH_2O , $3.3\text{ }\mu\text{moles}$.

Fig. 3. Rate of methionine synthesis with different C_1 -donor systems. Standard reaction mixture (L-serine omitted), but with DL-homocysteine 15 and $5\text{ }\mu\text{moles}$ for the experiments with L-serine and tetrahydropteroylglutamic acid-formaldehyde respectively, was used. Source of enzyme: dialysed extract (equivalent to 10 mg. of protein) of cobalamin-grown organisms. C_1 -donor systems: ○, serine ($10\text{ }\mu\text{moles}$) plus tetrahydropteroylglutamic acid ($1\text{ }\mu\text{mole}$); ●, tetrahydropteroylglutamic acid-formaldehyde ($3.3\text{ }\mu\text{moles}$).

formaldehyde respectively with appropriate concentrations of homocysteine gave a truer comparison (Fig. 3); the rate with tetrahydropteroylglutamic acid-formaldehyde is about one-half of that with serine.

Recovery of added methionine. Methionine (0.5 μ -mole) was quantitatively recovered if added to the standard reaction mixture (serine omitted) together with tetrahydropteroylglutamic acid-formaldehyde; this was true whether or not the original growth medium contained cobalamin. Breakdown of methionine (whether by reversal of the reaction under investigation or by some other mechanism) cannot therefore explain the results obtained.

Catalytic action of tetrahydropteroylglutamate

Residual folic acid coenzyme in dialysed enzymic extracts was removed by treatment with Dowex-1 resin. An extract of cobalamin-grown

organisms so treated can be reactivated (serine as C_1 donor) by tetrahydropteroylglutamic acid (Table 1). That tetrahydropteroylglutamic acid is acting catalytically is shown by the following quantitative considerations. At the lowest concentration of tetrahydropteroylglutamic acid added (0.3 μ mole) 1.2 μ moles of methionine was formed; the effective concentration of tetrahydropteroylglutamic acid would, however, be only 0.1 μ mole since it was only 70 % pure and it may be assumed that only one-half of this is the correct stereoisomer.

With serine as C_1 donor relatively high concentrations of tetrahydropteroylglutamic acid reduced methionine synthesis (20 % by 6 μ moles and 70 % by 12 μ moles, compared with the synthesis obtained with 1 μ mole). However, with tetrahydropteroylglutamic acid-formaldehyde as direct substrate methionine synthesis (compared with that given by 3.7 μ moles) decreased only 30 % on addition of 15 μ moles.

Other requirements for methionine synthesis

The effect of omitting the various components of the standard reaction mixture was tested with dialysed, Dowex-1-treated enzymic extracts of cobalamin-grown organisms with both serine and tetrahydropteroylglutamic acid-formaldehyde as C_1 donor (Table 2). Apart from the substrates and energy source (hexose diphosphate) there was strong evidence in both cases of requirements for ATP, DPN and Mg^{2+} ions; DPN could not be replaced by TPN, which was inhibitory when added as well as DPN. Similar requirements were found by Cross & Woods (1954) with their extract of

Table 1. *Activation of enzyme from cobalamin-grown organisms by tetrahydropteroylglutamic acid*

Standard reaction mixture (except that DL-homocysteine was 15 μ moles), containing dialysed, Dowex-1-treated extract of cobalamin-grown organisms equivalent to 9 mg. of protein, was used. L-Serine was C_1 donor. Incubated for 5 hr.

Tetrahydropteroylglutamic acid added (μ mole)	L-Methionine formed (μ mole)
0	0.06
0.3	1.36
0.4	1.84
0.6	2.30
1.0	2.80

Table 2. *Requirements for methionine synthesis by partly purified extracts of cobalamin-grown organisms*

Standard reaction mixture was used, omitting L-serine, and with DL-homocysteine present [15 μ moles (column *a*) and 10 μ moles (column *b*)]; other modifications were as indicated. When tetrahydropteroylglutamic acid and formaldehyde were both present (column *b*) they were added (3.3 μ moles) as a preformed equimolar mixture. For column *a* the amount of tetrahydropteroylglutamic acid was 1 μ mole. L-Serine (when present), 10 μ moles. Incubated for 2.5 hr.

C_1 donor	L-Methionine formed (μ mole)	
	L-Serine	Tetrahydropteroylglutamic acid-formaldehyde
Modifications to reaction mixtures	(<i>a</i>)	(<i>b</i>)
None	0.87	0.37
No L-serine	0.09	—
No formaldehyde	—	0.18
No tetrahydropteroylglutamic acid	0.08	0.06
No DL-homocysteine	0.08	0.11
No DPN	0.17	0.13
TPN (1 μ mole) instead of DPN	0.09	0.14
DPN plus TPN	0.23	0.24
No ATP	0.20	0.11
No hexose diphosphate	0.15	0.11
No pyridoxal phosphate	0.31	0.40
No Mg^{2+} ions	0.22	0.22
None	0.57	0.40
Plus cobalamin (50 μ mg.)	0.58	0.42

alumina-ground organisms and by Szulmajster & Woods (1955, 1960) with extracts of acetone-dried organisms of the present strain grown without cobalamin.

Omission of pyridoxal phosphate resulted in a sharp though variable drop in methionine synthesis (60–100% with different preparations) when serine was the C_1 donor (Table 2). In previous work (Cross & Woods, 1954; Szulmajster & Woods, 1960) the effect of this factor, though clear, was also somewhat variable. This may be due to the rapid rate of transformation of serine into glycine ($0.33 \mu\text{mole/min.}$; Fig. 4) compared with the rate of methionine formation ($0.006 \mu\text{mole/min.}$; Fig. 3). During the relatively long incubation required for methionine synthesis any trace of pyridoxal phosphate remaining in the extracts, or released during the incubation, might mediate the formation of some C_1 units from serine.

By contrast, when tetrahydropteroylglutamic acid-formaldehyde was C_1 donor, no requirement

for pyridoxal phosphate was found (Table 2); the possibility that it is concerned in a less easily dissociated form in the activation of homocysteine cannot be excluded. It is clear, however, that the carbon atom of formaldehyde need not be converted into the β -C of serine before its conversion into the methyl carbon atom of methionine. Furthermore, the addition of glycine ($1 \mu\text{mole}$) did not increase methionine synthesis with tetrahydropteroylglutamic acid-formaldehyde as C_1 donor as might be expected if the formation of serine was an obligatory step.

Effect of cobalamin. The enzymic extract used in the experiments of Table 2 was from organisms grown with cobalamin and this vitamin had, as might be expected, no effect on methionine synthesis. However, this was also the case with preparations from organisms grown without cobalamin (Table 4); the free vitamin promoted no synthesis of methionine with serine as C_1 donor and tetrahydropteroylglutamic acid as cofactor.

Degradation and synthesis of serine

Since it is probable that the reaction generating the C_1 unit is the conversion of serine into glycine it was necessary to determine whether growth on cobalamin affected this stage of the over-all synthesis of methionine. The present enzymic extracts catalysed both the breakdown of serine and its synthesis from glycine and tetrahydropteroylglutamic acid-formaldehyde. The assay of serine is technically more satisfactory than that of glycine, which was not attempted.

Fig. 4. Synthesis and breakdown of serine by enzymic extracts. Synthesis: Standard reaction mixture, with L-serine and DL-homocysteine omitted and glycine ($10 \mu\text{moles}$) and tetrahydropteroylglutamic acid-formaldehyde ($5 \mu\text{moles}$) added, was used. Dialysed enzymic extracts each contained 8.5 mg. of protein and were derived from organisms grown with (○) and without (○) cobalamin. Breakdown: Reaction mixture contained (in 2 ml.): L-serine ($5 \mu\text{moles}$), tetrahydropteroylglutamic acid ($5 \mu\text{moles}$), pyridoxal phosphate ($1 \mu\text{mole}$) and potassium phosphate, pH 7.8 ($200 \mu\text{moles}$). Dialysed enzymic extracts each contained 5 mg. of protein and were derived from organisms grown with (●) and without (●) cobalamin.

Table 3. Synthesis of serine by partly purified extracts

The reaction mixture contained (in 2 ml.): glycine, $10 \mu\text{moles}$; tetrahydropteroylglutamic acid-formaldehyde, $5 \mu\text{moles}$; potassium phosphate, pH 7.8, $200 \mu\text{moles}$; pyridoxal phosphate, $1 \mu\text{mole}$; dialysed, Dowex-1-treated extract of organisms grown either with or without cobalamin equivalent to 6 mg. of protein. Incubated for 6 min.

Modifications to reaction mixture	Serine formed (μmoles)*	
	Present	Absent
None	2.6	2.7
No glycine	0	0
No tetrahydropteroylglutamic acid-formaldehyde	0	0
No pyridoxal phosphate	0.1	0.15
Plus MnSO_4 ($1 \mu\text{mole}$)	1.3	1.0
Albumin† (6 mg.) instead of enzymic extract	0	—

* Values are corrected for a blank of $0.2 \mu\text{mole}$.

† Bovine serum albumin fraction V (The Armour Laboratories, Hampdon Park, Eastbourne).

The synthesis of serine by dialysed enzymic extracts (Dowex-1-treated) was rapid (Fig. 4) and dependent (Table 3) upon the presence of tetrahydropteroylglutamic acid (as tetrahydropteroylglutamic acid-formaldehyde), which was used equally well whether or not the organisms had been cultivated in the presence of cobalamin (Fig. 4). The degradation of serine also required tetrahydropteroylglutamic acid and again occurred irrespective of the presence of cobalamin in the original growth medium (Fig. 4). It was concluded that the function of tetrahydropteroylglutamic acid which is related to cobalamin concerns a later phase in the over-all synthesis of methionine.

The synthesis of serine also requires pyridoxal phosphate and is partly inhibited by Mn^{2+} ions (Table 3); DL-homocysteine (5 μ moles/ml.) reduced synthesis by 70 %, possibly due to the removal of C_1 units by the formation of 1:3-thiazane-4-carboxylic acid. Although the synthesis of serine from glycine and tetrahydropteroylglutamic acid-formaldehyde is very rapid it is certainly an enzymic reaction; there was no synthesis when albumin was substituted for the extract of organisms (Table 3). There was no requirement for ATP, DPN, hexose diphosphate or Mg^{2+} ions.

Factor present in cobalamin-grown organisms

The results so far described point to the presence in cobalamin-grown organisms of a factor which permits the utilization of tetrahydropteroylglutamic acid as coenzyme in the methylation of homocysteine. Further experiments were devoted to the simple properties of this factor.

Table 4. *Assay of the factor present in cobalamin-grown organisms*

Standard reaction mixture supplemented with tetrahydropteroylglutamic acid (2 μ moles) and containing dialysed extract (equivalent to 5 mg. of protein) of organisms grown without cobalamin (extract WC). Extract C (equivalent to 1.8 mg. of protein) was prepared in the usual way from organisms grown with cobalamin, and the protamine ppt. and supernatant (each 0.2 ml.) were derived from it (see text). Incubated for 3 hr.

Source of factor	Modifications to reaction mixture	L-Methionine formed (μ moles)
None	No tetrahydropteroylglutamic acid	0.55
None	None	0.08
Cobalamin (50 μ mg.)	None	0.10
Extract C	None	1.36
Extract C	No extract WC	0.11
Protamine ppt.	None	1.14
Protamine supernatant	None	0.13
Protamine ppt. plus protamine supernatant	None	1.16

Detection and assay. Amounts of enzymic extract of cobalamin-grown organisms insufficient alone to catalyse appreciable methionine synthesis enabled extracts of organisms grown without cobalamin to form methionine with serine as C_1 donor and tetrahydropteroylglutamic acid as cofactor (Table 4). Quantitative assay could be made in two ways: (a) by using a dialysed extract (organisms grown without cobalamin) treated with Dowex-1 resin to remove residual folic acid coenzyme or (b) by using extracts untreated with Dowex-1 and relying upon the fact that any effect of natural folic acid coenzyme is inhibited by the tetrahydropteroylglutamic acid which is added. The second method was simpler and proved reliable in practice; it was used as a routine in the work which follows. Samples of the material from cobalamin-grown organisms in amounts insufficient for methionine synthesis were added to the standard reaction mixture containing tetrahydropteroylglutamic acid and the normal quantity of enzymic extract from organisms grown without cobalamin used to demonstrate methionine synthesis (Table 4). Controls in which one or other of the two forms of extract was omitted were included in each experiment.

Properties of the factor. The experimental material was the initial aqueous extract of acetone-dried organisms harvested after growth on cobalamin. The factor was non-diffusible and was destroyed by heating to 65° for 3 min. It was stable to weak alkali (pH 10.4, 30 min. at 0°) but was labile in dilute acid; in 30 min. at 0° 30 % activity was lost at pH 4.2, and 45 % at pH 3.6. Freezing and thawing did not change the activity. When treated with Dowex-1 resin (as for the removal of residual folic acid coenzyme) about 40 % of the activity was lost.

Partial purification. The crude extract (8 ml.) was treated with 2 % (w/v) protamine sulphate (1.2 ml.). The supernatant after centrifuging contained no factor, but the precipitate (suspended in 8 ml. of 0.05M-potassium phosphate, pH 7.8) had 85 % of the original activity (Table 4). This was not increased by adding the supernatant as well. Controls in which protamine sulphate (0.5 mg./ml.) was added to the reaction mixture showed that this substance did not affect methionine synthesis.

The active material was extracted from the protamine precipitate by rubbing it up with 0.1M-potassium phosphate, pH 7.8 (5 ml.). After centrifuging to remove insoluble material the supernatant was dialysed for 12 hr. against more dilute (0.05M) potassium phosphate, pH 7.8; a fine white precipitate formed rapidly and this contained about 50 % of the total original activity (fraction I). The response of enzymic extracts of organisms grown without cobalamin to graded amounts of this

material (suspended in buffer) was not quite linear over the range tested (Table 5); fraction I itself, in the quantities used, did not promote methionine synthesis.

Fraction I was brought into solution by treating the suspensions with an equal volume of 0.1M-sodium pyrophosphate, pH 9.1, for 15 min. at 37°. The pyrophosphate was then removed by addition of two molar equivalents of magnesium sulphate and centrifuging. The resulting solution (fraction II) had about 75% of the activity of fraction I (Table 6) and this was not appreciably diminished by dialysis against distilled water for 14 hr. The amount of fraction II added in the experiments of Table 6 contained 0.2 mg. of protein (280 m μ /260 m μ ratio 1.3), some of which was probably protamine.

Cobalamin content. The soluble fraction II was assayed for cobalamin with *Euglena gracilis* and was found to contain 21 μ mg./mg. of protein. The value was not changed by dialysis (free cobalamin was diffusible under the conditions used) and it was concluded that the cobalamin present was in a bound state. The amount of the vitamin added with fraction II in the experiments of Table 6 was thus about 1.6 μ mm, which is of the same order of magnitude as that (7 μ mm) required for methionine synthesis by ultrasonic extracts of an auxotrophic strain of *E. coli* requiring cobalamin for growth (Helleiner, Kisliuk & Woods, 1957).

At the present stage of purification little significance can be attached to the presence of bound cobalamin in the active fraction; it may or may not be associated with a contaminating protein since the organisms had been grown originally with added vitamin.

Glycine as one-carbon-atom donor

Glycine replaced serine as C₁ donor but was only about one-third as active as the latter (Table 7). With a dialysed extract (Dowex-1-treated) of organisms grown without cobalamin the enzyme was activated either by a heated extract of the acetone-dried organisms or by tetrahydropteroyl-glutamic acid plus partly purified (fraction I) extract of organisms grown with cobalamin (Table 7). The position was therefore the same as with serine as donor.

DISCUSSION

Role of cobalamin

Growth on a medium containing cobalamin is essential if extracts of acetone-dried *Escherichia coli* are to synthesize methionine either with tetrahydropteroylglutamic acid as carrier of single-carbon-atom units from serine or with the formal-

Table 5. *Effect of partly purified extract of cobalamin-grown organisms on methionine synthesis by extracts of organisms grown without cobalamin*

Standard reaction mixture, with tetrahydropteroyl-glutamic acid (1 μ mole) added, was used. L-Serine was C₁ donor. Fraction I (see text) was derived from extract of cobalamin-grown organisms. Extract WC, dialysed extract of organisms grown without cobalamin (equivalent to 5.2 mg. of protein), was added as shown. Incubated for 3 hr. +, Present; -, absent.

Additions		L-Methionine formed (μ moles)
Extract WC	Fraction I (ml.)	
+	0	0.12
+	0.05	0.51
+	0.1	0.77
+	0.2	1.20
-	0.1	0.10

Table 6. *Assay of fractions derived from extracts of cobalamin-grown organisms*

Standard reaction mixture supplemented with tetrahydropteroylglutamic acid (1 μ mole), and containing dialysed extract (equivalent to 6 mg. of protein) of organisms grown without cobalamin, was used. Fraction I (0.1 ml.) and an equivalent volume of fraction II were derived from it as described in the text; dialysis was at 3° for 12 hr. against distilled water. Incubated for 3 hr.

Source of fraction	L-Methionine formed (μ mole)
None	0.10
Fraction I	0.88
Fraction II	0.64
Fraction II, dialysed	0.60

Table 7. *Comparison of glycine and serine as precursors of the methyl group of methionine*

Standard reaction mixture (without L-serine), containing dialysed, Dowex-1-treated extract of organisms grown without cobalamin (equivalent to 5 mg. of protein) was used. L-Serine or glycine (each 10 μ moles) was C₁ donor; tetrahydropteroylglutamic acid (when present), 1 μ mole. Fraction I (0.1 ml.) was derived (see text) from extract of cobalamin-grown organisms. For details of the heated extract of *E. coli* (0.1 ml.) see Materials and Methods section. Incubated for 3 hr.

Cofactors added	L-Methionine formed (μ mole)	
	With L-serine	With glycine
None	0.07	0.05
Tetrahydropteroylglutamic acid	0.05	0.05
Tetrahydropteroylglutamic acid plus fraction I	0.47	0.17
Heated extract of <i>E. coli</i>	0.36*	0.15*

* These values were reduced to 0.05 if serine and glycine respectively were omitted.

dehyde derivative of this compound (tetrahydropteroylglutamic acid-formaldehyde) as direct donor of C_1 units. Extracts from organisms grown without cobalamin are not activated by the free vitamin. It would appear likely that the treatment with acetone destroys an enzyme system(s) which converts cobalamin into a metabolically active form; ultrasonic extracts of both the present strain of *E. coli* and an auxotroph requiring cobalamin for growth respond to free cobalamin and in its presence they can use tetrahydropteroylglutamic acid as C_1 carrier or tetrahydropteroylglutamic acid-formaldehyde as direct C_1 donor (Helleiner & Woods, 1956; Helleiner *et al.* 1957; Woods, 1958).

Whilst this paper was in preparation there has been a brief report (Hatch, Takeyama & Buchanan, 1959) of the probable presence of a cobalamin-containing enzyme in a system synthesizing methionine isolated from a cobalamin auxotroph of *E. coli*.

The interconversion of serine and glycine by extracts of acetone-dried organisms is dependent on tetrahydropteroylglutamic acid, which is used whether or not the organisms have been grown with cobalamin. The effect of cobalamin must therefore be sought at some other stage of methionine synthesis than the initial activation of the C_1 donor. The nature of the effect is not made clear by the present experiments but a number of possibilities may be considered.

(1) *A derivative of cobalamin might convert tetrahydropteroylglutamic acid into a coenzyme form.* This seems unlikely because methionine synthesis by crude enzymic extracts (presumably containing a coenzyme form of folic acid) is completely inhibited by tetrahydropteroylglutamic acid; furthermore, a heated extract of *E. coli*, which also probably contains such a coenzyme form (Szulmajster & Woods, 1955, 1960), did not overcome the inhibition. Any coenzyme formed from tetrahydropteroylglutamic acid by the mediation of cobalamin would either have to be different from that found in normal cell extracts or it would have to be assumed that virtually all the added tetrahydropteroylglutamic acid was converted into a coenzyme form. However, heated extracts of cobalamin-grown organisms also did not overcome the inhibition by tetrahydropteroylglutamic acid. The alternative proposition is also unlikely since high concentrations of tetrahydropteroylglutamic acid do not markedly reduce methionine synthesis by extracts of cobalamin-grown organisms (i.e. when tetrahydropteroylglutamic acid is the carrier).

(2) *Cobalamin may be required for the synthesis of proteins concerned in methionine synthesis.* Certain of the properties of the material isolated from cobalamin-grown organisms and required for the

utilization of tetrahydropteroylglutamic acid in methionine synthesis resemble those of a protein (thermolability, non-diffusibility). It is possible therefore that cobalamin promotes the synthesis of a protein specifically required for the utilization of tetrahydropteroylglutamic acid. This would assume that the active factor is not itself a cobalamin derivative; the evidence on this point is so far inconclusive.

(3) *Cobalamin may act as a non-specific stimulator of a number of enzyme reactions.* Dubnoff & Bartron (1956) have given evidence for this as a general function of cobalamin. The following points illustrate important differences between the effect on methionine synthesis and such non-specific stimulations.

First, the metabolism of serine is unaffected by growth of the organism on cobalamin, whereas it is essential for methionine synthesis with tetrahydropteroylglutamic acid as cofactor.

Secondly, it is clear that accessory enzymes (such, for example, as are necessary for the formation of reduced DPN) are not limiting in extracts of organisms grown without cobalamin. For instance, crude enzymic extracts (containing natural folic acid coenzyme) synthesize methionine in the absence of tetrahydropteroylglutamic acid just as well as do extracts of cobalamin-grown organisms in its presence.

(4) *Possible breakdown of methionine.* It was possible that under the conditions imposed by the presence of tetrahydropteroylglutamic acid there was decomposition of methionine as rapidly as it was formed, and that growth on cobalamin in some way prevented this. However, it was found that added L-methionine could be completely recovered from systems containing enzymic extracts of both types.

(5) *A cobalamin derivative may act catalytically in the reduction of an intermediate at the hydroxymethyl level of oxidation to a methyl group.* This hypothesis presupposes that the active factor is a cobalamin derivative and this is not proved; such a role for cobalamin is favoured by its structural similarity to metalloporphyrins. It is suggested that homocysteine condenses with the ring-closed form of hydroxymethyltetrahydropteroylglutamic acid ($N^{5,10}$ -methylenetetrahydropteroylglutamic acid) to form the derivative (I) (Fig. 5). The cobalamin derivative might then transfer electrons from reduced DPN to (I), resulting in a reductive cleavage of the latter to methionine and tetrahydropteroylglutamic acid (Fig. 5). Such a reduction system might be considered analogous to the reverse of the electron transfer from flavoproteins to cytochrome; that is, a cobalamin derivative instead of cytochrome and a folic acid protein instead of flavoprotein.

Extracts of organisms (grown without cobalamin) carry out methionine synthesis by a pathway which is inhibited by tetrahydropteroylglutamic acid; the latter may therefore be acting as an inhibitory analogue of the naturally occurring folic acid coenzyme. Growth on a medium containing cobalamin may endow the extracts with an alternative mechanism for the reduction of the hydroxymethyl group in which tetrahydropteroylglutamic acid can act as the carrier of this group. Davis & Mingioli (1950) described mutants of *E. coli* that require either methionine or cobalamin for growth. It is possible that cobalamin acts by making possible an alternative pathway for the reduction of the hydroxymethyl group and not by providing the product of the reaction blocked by the mutation; in this case the metabolic lesion in these mutants would be at a step in the normal synthesis of methionine rather than (as is generally supposed) in the synthesis of cobalamin itself. The formation of the methyl group of methionine in prototrophic strains of *E. coli* may be independent of cobalamin, as it appears to be in higher plants for which cobalamin is not an essential metabolite.

(6) A cobalamin derivative may be required to activate amino acids. Wagle, Mehta & Johnson (1958*a, b*) have given evidence for this role of cobalamin in animal metabolism, but have later (Mehta, Wagle & Johnson, 1959) withdrawn the suggestion. Although it is possible to suppose that

in methionine synthesis cobalamin might be required for the formation of an adenosine monophosphate-homocysteine derivative which is the actual acceptor of the methyl group, it is difficult at present to reconcile such an hypothesis with the fact that cobalamin is required only when tetrahydropteroylglutamic acid is the carrier of C_1 units and not when natural folic acid coenzyme is available.

A second possibility is that the cobalamin derivative might be required for the activation of glutamate and thus for the formation of polyglutamyl derivatives of tetrahydropteroylglutamic acid, which may be more effective than the free compound as C_1 -unit carriers. Polyglutamyl derivatives of pteroylglutamic acid are more effective than tetrahydropteroylglutamic acid in catalysing the breakdown of serine by extracts of *Clostridium sticklandii* (Wright & Stadtman, 1956), and an *N*-formylpteroyltriglutamic acid partially replaces natural folic acid coenzyme in the present system (Szulmajster & Woods, 1960). The discussion of possibility (1) above also applies to this suggestion and militates against it.

Other considerations

Recent investigations of methionine synthesis in liver preparations (Nakao & Greenberg, 1958; Stevens & Sakami, 1958) show tetrahydropteroylglutamic acid to be an effective cofactor; no effect

Fig. 5. Hypothetical scheme of reaction. $R \cdot SH$ is homocysteine [$HS \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$]. PtH_4G and $PyAL \cdot PO_4$ represent tetrahydropteroylglutamic acid and pyridoxal phosphate respectively; (a) is a non-enzymic reaction. DPNH, Reduced DPN.

of cobalamin was reported. The other requirements were similar to those in the present work except that TPN was more active than DPN.

The mode of action of ATP is not known; it may react with homocysteine forming either *S*-adenosylhomocysteine or homocysteine-adenosine monophosphate, either of which might be the final acceptor of a reduced C_1 unit rather than free homocysteine. It probably also serves as a source of ADP for glycolysis. A requirement for inorganic phosphate for methionine synthesis was shown by Szulmajster & Woods (1960); phosphate was always added to the reaction mixture in the present work and the point was not further investigated.

The relative inactivity of glycine compared with serine as an ultimate source of the methyl group was also observed with suspensions of the organism (Gibson & Woods, 1960). However, conditions for optimum utilization of glycine by the enzymic extracts were not studied in detail. It is also possible that some of the C_1 units formed from glycine condense with unchanged glycine to form serine; glycine would thus be competing with the system synthesizing methionine for the available C_1 units.

SUMMARY

1. Aqueous extracts of acetone-dried *Escherichia coli* PA15 (a serine or glycine auxotroph) synthesize methionine from homocysteine and serine. Tetrahydropteroylglutamic acid appears to inhibit the function of the natural folic acid coenzyme present in the extracts.

2. Tetrahydropteroylglutamic acid will, however, itself serve as cofactor, but only provided that the organisms have been grown on a medium containing cobalamin. Addition of cobalamin to the enzymic extract has no effect.

3. A non-diffusible, heat-labile material which promotes methionine synthesis in the presence of tetrahydropteroylglutamic acid has been obtained from extracts of organisms grown with cobalamin and partly purified. The purified fraction still contains a significant amount of cobalamin.

4. The reversible transformation of serine into glycine is catalysed by tetrahydropteroylglutamic acid whether or not the organisms have been grown with cobalamin.

5. With L-serine as the C_1 donor the other requirements for methionine synthesis are homocysteine, hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, pyridoxal phosphate and Mg^{2+} ions. With the formaldehyde derivative of tetrahydropteroylglutamic acid as donor pyridoxal phosphate is not required.

6. Serine is not on the pathway of synthesis of the methyl group of methionine from formaldehyde.

7. Glycine has one-third of the activity of serine as C_1 donor when tested under the same conditions.

We are grateful to the Nuffield Foundation for a grant towards the expenses of this research. The work was also aided by a grant to the Department from the Rockefeller Foundation. Miss Jane Duncan gave skilled technical assistance.

REFERENCES

- Cross, M. J. & Woods, D. D. (1954). *Biochem. J.* **58**, xvi.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Dubnoff, J. W. & Bartron, E. (1956). *Arch. Biochem. Biophys.* **61**, 99.
- Frisell, W. R., Meech, L. A. & Mackenzie, C. G. (1954). *J. biol. Chem.* **207**, 709.
- Gibson, F. & Woods, D. D. (1952). *Biochem. J.* **51**, v.
- Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
- Hatch, F. T., Takeyama, S. & Buchanan, J. M. (1959). *Fed. Proc.* **18**, 243.
- Helleiner, C. W., Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
- Helleiner, C. W. & Woods, D. D. (1956). *Biochem. J.* **63**, 26r.
- Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L. & Jukes, T. H. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 118.
- Kisliuk, R. L. (1957). *J. biol. Chem.* **227**, 805.
- Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
- Kisliuk, R. L. & Woods, D. D. (1959). *Fed. Proc.* **18**, 261.
- Lascelles, J. (1956). *Biochem. J.* **62**, 78.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Mehta, R., Wagle, S. R. & Johnson, B. C. (1959). *Biochim. biophys. Acta*, **35**, 286.
- Nakao, A. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 603.
- Nash, T. (1953). *Biochem. J.* **55**, 416.
- O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S. & Pfiffner, J. J. (1947). *J. Amer. chem. Soc.* **69**, 250.
- Snell, E. E. & Rannefeld, A. N. (1945). *J. biol. Chem.* **157**, 475.
- Stevens, A. & Sakami, W. (1958). *Fed. Proc.* **17**, 316.
- Strack, E., Friedel, W. & Hamsch, K. (1956). *Hoppe-Seyl. Z.* **305**, 166.
- Szulmajster, J. & Woods, D. D. (1955). *Abstr. 3rd int. Congr. Biochem., Brussels*, p. 44.
- Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
- Wagle, S. R., Mehta, R. & Johnson, B. C. (1958a). *Biochim. biophys. Acta*, **28**, 215.
- Wagle, S. R., Mehta, R. & Johnson, B. C. (1958b). *J. biol. Chem.* **230**, 137.
- Woods, D. D. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, **11**, 302.
- Wright, B. E. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, **11**, 266.
- Wright, B. E. & Stadtman, T. C. (1956). *J. biol. Chem.* **219**, 863.
- Wriston, J. C. & Mackenzie, C. G. (1957). *J. biol. Chem.* **225**, 607.