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The Synthesis of Serine and *Leuconostoc citrovorum* Factor by Cell Suspensions of *Streptococcus faecalis* R

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The evidence that growth factors of the folic acid group are required for some step in the biosynthesis of serine by various micro-organisms has been reviewed by Shive (1951), Woods (1952) and Lascelles, Cross & Woods (1954). It is of an indirect nature and springs from observations with growing cultures that serine is a component of certain mixtures of amino acids and nucleic acid derivatives which can replace the requirement for *p*-aminobenzoic acid or folic acid† either for growth or for overcoming inhibition of growth by sulphonamides (Winkler & de Haan, 1948; Lampen, Jones & Roepke, 1949; Holland & Meinke, 1949; Snell, 1951; Lascelles *et al.* 1954).

Glycine replaces serine for the growth of mutant strains of *Neurospora* and *Escherichia coli* (Tatum, 1949; Roepke, Libby & Small, 1944; Wright, 1951) and, under certain conditions, of *Leuconostoc mesenteroides* P 60 (*Streptococcus equinus* P 60) (Lascelles *et al.* 1954); glycine may therefore be an intermediate in serine formation, as it may be also in animal tissues (see below). After growth of *Torulopsis utilis* on a medium containing [*carboxy*-^{14}C]-glycine the bulk of the isotope was found in the glycine, serine and proline fractions of the hydrolysed cell-protein (Ehrensvärd, Sperber, Saluste, Reio & Stjernholm, 1947).

Growth experiments with micro-organisms also provide evidence, again indirect, that folic acid is required for the conversion of glycine into serine. With *Ln. mesenteroides* P 60 there was an increased requirement for *p*-aminobenzoic acid or N^5-formyl-5:6:7:8-tetrahydropteroylglutamic acid (leucovorin) when the organism grew with high concentrations of glycine in the absence of serine (Lascelles *et al.* 1954). Folic acid would not replace serine for the growth of *Lactobacillus bifidus* unless glycine were present (Nepple, Wright & Skeggs, 1951) while, with the protozoon *Tetrahymena pyriformis* W, Kidder & Dewey (1953) found that growth occurred on a medium containing glycine but no serine only if high concentrations of folic acid were added; increased thioctic acid (protogen) was also required.

Pyridoxal is required for growth of *Ln. mesenteroides* P 60 on media containing glycine but no serine (Lascelles *et al.* 1954); it may therefore also take part in the synthesis of the latter amino acid from the former. A function of vitamin B_6 at some stage of serine formation had also been indicated by replacement experiments with growing cultures of *Streptococcus faecalis*, *Lactobacillus arabinosus* and *Ln. mesenteroides* (Lyman, Moseley, Wood, Butler & Hale, 1947; Steele, Sauberlich, Reynolds & Baumann, 1949).

In the experiments with growing cultures summarized above, with few exceptions, synthesis of serine was not actually demonstrated but inferred from the production of new cell material (assumed to contain serine) on media not containing this amino acid. Lascelles *et al.* (1954), however, showed that cells of *Ln. mesenteroides* grown with glycine contained a normal content of serine, and Ehrensvärd *et al.* (1947) also have this confirmation with *Torulopsis utilis*. In most cases the growth media contained many other substances besides those discussed; some of these or their metabolic

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† The term folic acid will be used throughout in general reference to the group as a whole; individual members will be named specifically.

products may also have been necessary for serine formation. The object of the present work was to determine the conditions necessary for serine formation under the more controlled conditions possible with cell suspensions; some of the results have already been reported briefly (Lascelles & Woods, 1950; Lascelles, Cross & Woods, 1951). *Strep. faecalis* R was chosen because this organism does not require serine for growth under appropriate conditions and can presumably synthesize it. Furthermore, cells deficient in folic acid may be obtained by growth on a medium containing certain amino acids, purines and thymine; folic acid is not then required (Stokes, 1944; Lampen & Jones, 1946; Snell, 1951). Addition of D-alanine to the normal medium permits growth in the absence of vitamin B_6, and cells devoid of this vitamin may thus be obtained (Holden, Furman & Snell, 1949).

The growth experiments with micro-organisms give no clue as to the nature or source of the one-carbon residue also required if serine is formed directly from glycine. Help here comes from experiments with animal tissues using isotopic tracer technique though no actual increase in serine was shown. Sakami (1948) fed [*carboxy*-^{13}C]glycine and [*carboxy*-^{14}C]formate to rats and found the liver-serine to contain ^{13}C in C-1 and ^{14}C in C-3; this is consistent with the formation of serine from glycine by condensation with a one-carbon unit derived from formate. Other compounds found to provide C-3 of serine in similar work with intact animals, liver slices and tissue preparations include formaldehyde, acetone, methionine, choline, sarcosine and glycine itself (Sakami, 1949*a*, *b*; Siekevitz & Greenberg, 1949, 1950; Siegel & Lafaye, 1950; Kruhøffer, 1951; Mitoma & Greenberg, 1952). Árnstein & Neuberger (1953*a*, *b*) have found, however, that C-2 of glycine is not a significant source of one-carbon residues in whole animals unless glycine is given in abnormally large amounts. Decreased ability to incorporate [*carboxy*-^{14}C]-glycine into serine has been shown both with rats deficient in folic acid and with liver extracts of pyridoxin-deficient chicks (Plaut, Betheil & Lardy, 1950; Deodhar & Sakami, 1953); an effect of *Leuconostoc citrovorum* factor on the incorporation of C-2 of glycine into C-3 of serine by chick liver preparations has also been reported (Kelley, 1951). Two important brief reports on these aspects of serine synthesis (Blakley, 1954; Kisliuk & Sakami, 1954) which appeared after this paper had been prepared are considered in the Discussion.

ORGANISMS AND METHODS

Preparation of cell suspensions of Strep. faecalis *R*

Organism. Stock cultures of *Strep. faecalis* Rogers (American Type Culture Collection no. 8043) were maintained in stab culture on medium B1 of Nimmo-Smith, Lascelles & Woods (1948), transferred every fortnight and stored at 4°.

Medium. Cells deficient in both folic acid and vitamin B_6 were obtained by growth on medium *G*, which was based on that of Bellamy & Gunsalus (1945). It contained (in each litre of final medium): acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld, 1945) equivalent to 5 g. original casein; DL-tryptophan, 100 mg.; DL-cysteine, 200 mg.; DL-alanine, 200 mg.; glucose, 10 g.; glacial acetic acid, 0·8 ml.; mercaptoacetic acid (redistilled), 0·08 ml.; KH_2PO_4, 8 g.; $MgSO_4$, $7H_2O$, 0·4 g.; $MnSO_4$, $4H_2O$, $FeSO_4$, $7H_2O$ and NaCl, 20 mg. each; adenine, guanine and uracil, 10 mg. each; nicotinic acid, 5 mg.; riboflavin and calcium pantothenate, 1 mg. each; biotin, 1 μg. Thymine and pteroylglutamic acid were added to give final concentrations of 2×10^{-5} M and 10^{-9} M respectively; the pH was adjusted to 7·2.

Even though thymine and purines were present (Stokes, 1944) growth was slow and irregular if pteroylglutamic acid was omitted altogether; it was therefore added but only at one-tenth the concentration required for optimum growth, in order to restrict the folic acid content of the harvested cells. Under these conditions thymine improved growth slightly.

Growth. Medium *G* (200 ml. quantities in 250 ml. conical flasks) was autoclaved 10 min. at 10 lb./sq.in. and warmed to 37° just before inoculating with 0·4 ml. (about 4×10^8 cells) from a 16 hr. culture (37°) on the same medium plus 10^{-7} M pteroylglutamic acid. This culture had been sown (1 loop) from a 24 hr. culture (37°) on a complex medium (B2, Nimmo-Smith *et al.* 1948), which had been inoculated in turn from a fresh 24 hr. stab culture. The flasks were incubated for 8 hr. at 37°; by this time the maximum cell density attained on this medium (about 0·5 mg. dry wt. cells/ml.) had been reached. This was about one-third of that achieved in the presence of optimum concentrations of pyridoxal and pteroylglutamic acid.

The cells were centrifuged out, resuspended in the culture volume of 0·02 M phosphate buffer pH 6·9, and again centrifuged. The cell paste was stored overnight at 4°; it lost no activity in this time.

The phosphate buffers used throughout were prepared from stock solutions of Na_2HPO_4, $12H_2O$ and KH_2PO_4.

General procedure for study of serine synthesis

Reaction mixture. The washed cells were suspended at a concentration of 0·5–1·0 mg. dry wt./ml. in 5 ml. quantities (in $5 \times \frac{5}{8}$ in. Pyrex tubes) of solution *S*, which normally contained: glycine, sodium formate and glucose, each 0·02 M; pyridoxal, 5×10^{-7} M; pteroylglutamic acid, 2×10^{-6} M; phosphate buffer pH 6·9, 0·1 M. Modifications in particular experiments are noted in the text. The tubes were usually incubated for 8 hr. at 37°. In each experiment there was a control containing cells suspended in the complete mixture and heated 5 min. at 100°.

In some experiments the test for synthetic ability was preceded by treatment of the cells with various members of the folic acid group. A mixture of cells, glucose, buffer (concentrations as above) and the test substance (total volume 5 ml.) was incubated for 1–2 hr. at 37°. The cells were centrifuged out, washed twice with 5 ml. quantities of 0·85% (w/v) NaCl and tested for ability to synthesize serine in solution *S* with and without added folic acid.

Preparation of material for assay. The reaction was stopped, and any free serine within the cells liberated, by immersing the tubes in boiling water for 10 min.; the cells were then centrifuged out. Samples of the supernatant fluid were brought to pH 1·5–2 with N-HCl, autoclaved at 10 lb./sq.in. pressure for 10 min., cooled and neutralized with N-NaOH. The acid treatment was required to destroy *Ln. citrovorum* factor which interferes with the serine assay (Lascelles *et al.* 1954).

Assay of serine

The microbiological assay of serine with *Ln. mesenteroides* P 60, as adapted for this work, has been described elsewhere (Lascelles *et al.* 1954; medium L_8 used). Incubation was in air or N_2 for 42–46 hr. The growth response to DL-serine and to typical samples corresponded over a wide range of concentration (Fig. 1*a*). Standards containing 2, 4, 8 and 16×10^{-5}M DL-serine were set up in each assay. The acid-treated samples under test were added in quantities within the range 0·1–0·8 ml.; their content of serine was obtained by direct comparison of the responses obtained at two concentrations with the dose/response curve given by the standards.

Results are expressed as μmoles L-serine formed/mg. dry wt. cells of *Strep. faecalis*; the assay organism responds only to L-serine. The overall accuracy of the method was about ±10% for 0·02–0·1 μmole L-serine.

Assay of Leuconostoc citrovorum *factor*

The test organism, hitherto known as *Leuconostoc citrovorum* (American Type Culture Collection no. 8081), has been reported by Felton & Niven (1953) to be a typical strain of *Pediococcus cerevisiae*; it was maintained as described by Lascelles & Woods (1952).

The assay procedure followed that of Sauberlich & Baumann (1948) as slightly modified by Lascelles & Woods (1952), except that the amino acids of the basal medium were replaced by a mixture of DL-tryptophan, DL-cysteine

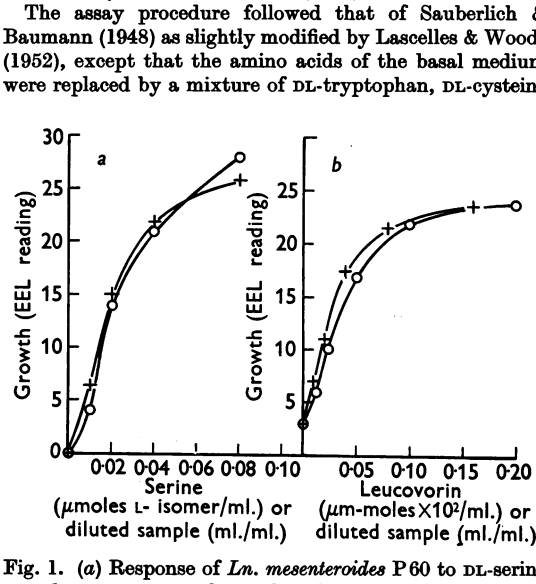

Fig. 1. (*a*) Response of *Ln. mesenteroides* P 60 to DL-serine and to experimental samples. Serine, ○; sample (acid-treated), +. (*b*) Response of *Ln. citrovorum* to leucovorin and to experimental samples. Leucovorin, ○; sample, +. (The samples were obtained from typical experiments in which cells suspended in complete solution *S* had synthesized serine.)

(each 100 mg./l.) and vitamin-free acid-hydrolysed casein prepared as by Snell & Rannefeld (1945) and added in amount equivalent to 5 g. original casein/l.

Samples of reaction mixtures were prepared for assay as for serine except that the acid-treatment step was omitted; they had usually to be diluted before adding to the assay medium. Leucovorin (synthetic *Ln. citrovorum* factor) was used as standard and gave a dose/response curve similar to that of the samples under test (Fig. 1*b*). The effective range of the assay was from 0·2 to 1·5 μμmoles of leucovorin with an accuracy of about ±10%; results are expressed in terms of μm-moles leucovorin formed/mg. dry wt. cells *Strep. faecalis* R.

Estimation of amino-nitrogen

Fractions obtained from chromatography of the products of synthesis reactions were assayed for amino-N by the photometric ninhydrin method of Moore & Stein (1948), but modified to conserve ninhydrin. The quantity of ninhydrin reagent used for each 1 ml. sample was reduced to 0·25 ml. and the tubes were heated for 15 min. at 115° in an autoclave instead of in a boiling-water bath. After dilution with *n*-propanol the colour developed was measured in a Spekker absorptiometer (Hilger and Watts Ltd., London) using no. 606 filter (peak transmission 577 mμ.) and 1 cm. cells. For specific assay of the serine fraction, DL-serine was used as standard; the relationship between instrument reading and serine concentration was linear between 1 and 8×10^{-4}M serine.

Growth experiments with Streptococcus faecalis *R*

The basal medium (*F*) used was that of Henderson & Snell (1948) modified for the present purpose by omitting glycine, serine, pteroylglutamic acid, *p*-aminobenzoic acid and all forms of vitamin B_6, and by adding thymine (2×10^{-5}M). The medium was used in a final volume, including experimental additions and inoculum, of 2 ml. in $5 \times \frac{5}{8}$ in. tubes and was incubated in air at 37°. Each tube was inoculated with 0·1 ml. of a 1/500 dilution of a 24 hr. culture in medium *B* 2 (see above); this had been sown in turn from a stock 24 hr. stab culture. General procedures were as described for *Ln. mesenteroides* P 60 by Lascelles *et al.* (1954).

Extent of growth is recorded as galvanometer reading of an EEL photoelectric colorimeter (Evans Electroselenium, Harlow, Essex); the relation between dry weight of cells and the reading was linear over the range used; 0·49 mg./ml. gave a reading of 10.

Chemicals

The source of the materials and the preparation and storage of stock solutions of pyridoxal and members of the folic acid group have been described by Lascelles *et al.* (1954). N^{10}-Methylpteroic acid was a gift from Dr T. H. Jukes (Lederle Laboratories).

RESULTS

Substrates required for the synthesis of serine

Cell suspensions of *Strep. faecalis* R deficient in folic acid and pyridoxal synthesized serine when incubated in solution *S*, which contained these substances together with glycine, formate and glucose (Fig. 2, Table 1). Serine formation increased with

Table 1. *Substances required for the synthesis of serine and* Leuconostoc citrovorum *factor*

Cells (0·7 mg. dry wt./ml.) incubated 8 hr. in 0·1 M phosphate buffer with the additions shown.

Additions					Product	
Glycine (0·02 M)	Formate (0·02 M)	Glucose (0·02 M)	Pteroyl-glutamate (2×10^{-6} M)	Pyridoxal (5×10^{-7} M)	L-Serine (μmoles/mg. cells)	*Ln. citrovorum* factor (μm-moles/mg. cells)
+	+	+	+	+	[0·06	<0·0001]*
+	+	+	+	+	0·52	0·114
−	+	+	+	+	<0·05	0·183
+	−	+	+	+	0·07	0·030
+	+	−	+	+	0·06	0·003
+	+	+	−	+	0·07	<0·001
+	+	+	+	−	0·08	0·330
−	+	+	+	−	.	0·228
−	−	+	+	−	.	0·027
−	+	−	+	−	.	<0·001
−	+	+	−	−	.	<0·001

* Control with heated cells (see Methods).

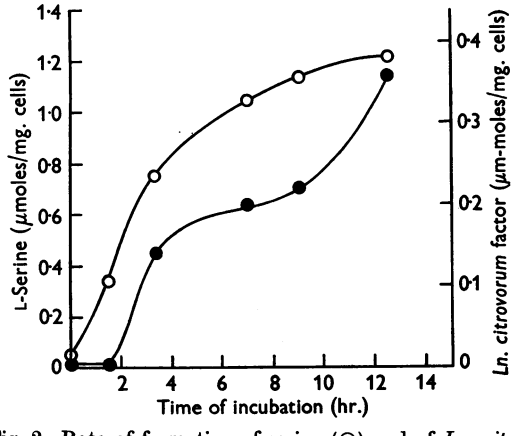

Fig. 2. Rate of formation of serine (○) and of *Ln. citrovorum* factor (●). Tubes, each containing 2·5 mg. cells in 5 ml. complete solution *S*, were incubated for times shown; controls with heated cells contained <0·05 μmole L-serine and <0·001 μm-mole *Ln. citrovorum* factor/mg. cells.

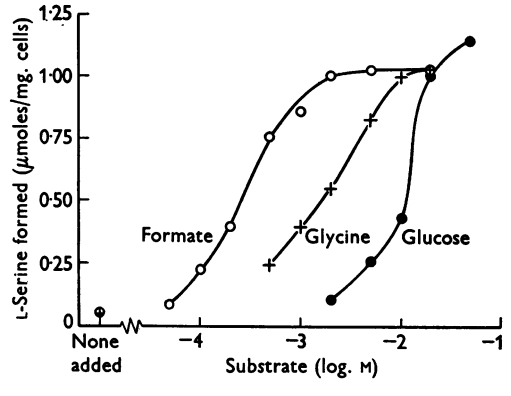

Fig. 3. Effect on serine synthesis of the concentration of formate (○), glycine (+) and glucose (●). Cells (0·67 mg./ml.) were incubated 8 hr. in solution *S* containing variable amounts of the test substance and normal amounts of the other two. Controls with heated cells showed <0·05 μmole L-serine/mg. cells.

time of incubation up to about 12 hr. (Fig. 2). There was some variation, with different batches of cells, in the amount of serine formed in the standard incubation time of 8 hr., but it was rarely outside the range 0·5–1·0 μmole/mg. dry wt. cells.

The single omission of either glycine, formate or glucose from solution *S* reduced synthesis to about one-tenth of that with the complete mixture (Table 1). Less formate (about 0·002 M) than glycine (about 0·02 M) was required for optimum serine formation (Fig. 3). Synthesis fell sharply when glucose was reduced below 0·02 M and increased only slightly with higher concentrations (Fig. 3); such high concentrations were not used as a routine in order to avoid complications in certain analytical methods.

Source of the single-carbon unit. No substance tested was found adequately to replace formate for serine synthesis in the present system. Methionine, choline, betaine and sarcosine (all tested at 0·01 M) were inactive. Formaldehyde (10^{-3} M) also failed to promote serine production in the absence of formate; at higher concentrations it inhibited synthesis with formate present. No serine was formed without formate even when glycine concentration was raised to 0·05 M; it is unlikely, therefore, that this amino acid can also provide the single-carbon unit.

Pyruvate had some activity, but at optimum concentration (10^{-2} M) gave only 40 % of the serine obtained with optimum formate (2×10^{-3} M); with pyruvate also at the latter concentration the yield of serine was reduced to 20 % of that with formate.

Growing cultures of *Ln. mesenteroides* P 60 synthesize serine from glycine if incubated in an atmosphere enriched with CO_2 or if leucovorin is present (Lascelles *et al.* 1954). Incubation of suspensions of *Strep. faecalis* R in solution *S* under atmospheres containing 5 % (v/v) CO_2 did not affect serine synthesis or abolish the need for formate. The presence of leucovorin (2×10^{-6} M) did not reduce the amount of formate required.

Effect of vitamin B_6

The presence of D-alanine in medium *G* permits growth of *Strep. faecalis* R in the absence of vitamin B_6, and the harvested cells are devoid of the vitamin (Holden *et al.* 1949). They formed little or no serine in solution *S* when pyridoxal was omitted (Table 1). Synthesis became detectable with 10^{-9} M pyridoxal and reached an optimum at about 5×10^{-8} M (Fig. 4). Pyridoxal phosphate had only about one-fiftieth of the activity of pyridoxal; it may not penetrate the cells as easily.

In experiments reported earlier (Lascelles & Woods, 1950) the effect of pyridoxal, though marked, was not absolute. These experiments were done before the discovery that *Ln. citrovorum* factor, when present with high concentrations of glycine, could induce growth of the assay organism in the absence of serine (Lascelles *et al.* 1954); the samples for assay were not, at that time, acid-treated. It is shown later that *Ln. citrovorum* factor is formed by cell suspensions in solution *S* concurrently with serine.

Effect of folic acid

The cells of *Strep. faecalis* R were harvested from a medium containing initially only suboptimum quantities of pteroylglutamic acid (see Methods); they contained no folic acid detectable by assay with *Lactobacillus casei* by the method of Lascelles & Woods (1952). Such cells synthesized no serine when pteroylglutamic acid was omitted from the reaction mixture (Table 1); the amount of serine formed increased with increasing pteroylglutamic acid concentration up to 2×10^{-7} M (Fig. 4).

Replacement of pteroylglutamic acid. Members of the folic acid group which support growth of *Strep. faecalis* R were also active in replacing pteroylglutamic acid for serine synthesis by cell suspensions (Table 2). Of the compounds tested only N^{10}-formylpteroylglutamic acid was as active as pteroylglutamic acid itself; it is also equally effective for growth (Gordon, Ravel, Eakin & Shive, 1948). Rhizopterin (N^{10}-formylpteroic acid), even with L-glutamate added, was less active than pteroylglutamic acid for serine synthesis though it is

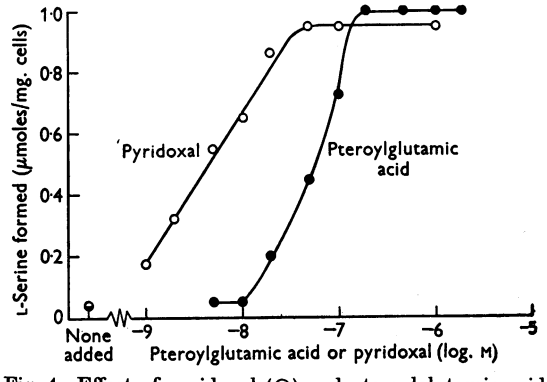

Fig. 4. Effect of pyridoxal (○) and pteroylglutamic acid (●) on the synthesis of serine. Cells (○, 0·8 mg./ml.; ●, 0·7 mg./ml.) were incubated 8 hr. in solution *S* containing variable concn. of the factor under test and normal concn. of the other. Controls with heated cells showed 0·05 μmole L-serine/mg. cells.

Table 2. *Effect of various members of the folic acid group on serine synthesis*

Cells (0·8 mg. dry wt./ml.) incubated 8 hr. in solution *S* containing either pteroylglutamic acid or the other substances shown.

Addition	Concn. (M)	L-Serine formed (μmoles/mg. cells) in Expt. 1	Expt. 2
None	—	<0·05	<0·05
Pteroylglutamic acid	2×10^{-6}	[<0·05	<0·05]*
		1·15	0·82
	2×10^{-7}	1·10	.
N^{10}-Formylpteroylglutamic acid	2×10^{-6}	1·15	0·85
	2×10^{-7}	1·15	.
Leucovorin	2×10^{-6}	0·85	0·55
	2×10^{-7}	0·80	.
Rhizopterin + L-Glutamic acid	2×10^{-6} + 10^{-2}	0·95	0·65
	2×10^{-7} + 10^{-2}	0·80	.
L-Glutamic acid	10^{-2}	0·09	.

* Control with heated cells.

10 times more active for growth (Rickes, Chaiet & Keresztesy, 1947).

Leucovorin (N^5-formyl - 5:6:7:8 - tetrahydropteroylglutamic acid, calcium salt pentahydrate; synthetic *Ln. citrovorum* factor) is a mixture of the (+)L- and (−)L- isomers, where L refers to the stereochemical configuration of the glutamic acid residue and (+) or (−) to the optical rotation attributable to the asymmetric carbon atom (C-6) in the pteridine ring. The (−)L- form has been isolated and found to have twice the activity of the racemic compound for the growth of *Strep. faecalis* R (Cosulich, Smith & Broquist, 1952). Since the latter has in turn about half the activity of pteroylglutamic acid, it is likely that the (−)L- isomer has potency equal to that of pteroylglutamic acid (Broquist, Brockman, Fahrenbach, Stokstad & Jukes, 1952). Leucovorin was rather less active than pteroylglutamic acid for serine synthesis by cell suspensions; this cannot be attributed to the presence of only 50% active isomer, since a tenfold increase in concentration did not increase the amount of serine formed (Table 2).

p-Aminobenzoic acid was inactive both for growth and for serine synthesis by cell suspensions.

Activity of cells previously incubated with members of the folic acid group

It seemed possible that pteroylglutamic acid took part in serine synthesis after conversion into some more complex coenzyme-like form. Experiments similar in principle to those of Novelli & Lipmann (1950) with pantothenate were carried out. These workers found that previous incubation of pantothenate-deficient yeast cells with this vitamin and glucose resulted in a greatly increased rate of acetate oxidation: during the first incubation the pantothenate was partly converted into coenzyme A, which was inactive in stimulating acetate oxidation when supplied exogenously.

Cells of *Strep. faecalis* R deficient in folic acid were incubated in buffered glucose with and without members of the folic acid group (see Methods). After washing, their ability to synthesize serine was tested in solution *S* in the presence and absence of added pteroylglutamic acid.

Cells first incubated in glucose plus pteroylglutamic acid no longer required the latter for serine synthesis; they were, however, no more active than untreated cells tested in complete solution *S* (Fig. 5). Incubation in buffer or buffered glucose alone gave cells with reduced synthetic ability compared with cells treated with pteroylglutamic acid when both were finally tested in solution *S* containing the vitamin (Table 3). With formate as well as pteroyl-

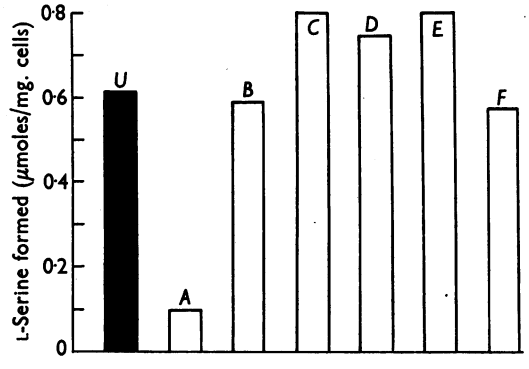

Fig. 5. Synthesis of serine by cells first incubated with pteridine derivatives. Cells (0·5 mg./ml.) first treated 1 hr. in buffered glucose containing: *A*, formate or no addition; *B*, pteroylglutamic acid; *C*, pteroylglutamic acid plus formate; *D*, leucovorin; *E*, N^{10}-formylpteroylglutamic acid; *F*, rhizopterin plus glutamate. Cells then separated, washed and incubated 6 hr. in solution *S* without pteroylglutamic acid. *U*, untreated cells incubated 6 hr. in solution *S* containing pteroylglutamic acid. Concn. used: pteridine derivatives, 2×10^{-6}M; formate, 0·02M; L-glutamate, 0·01M.

Table 3. *Effect on synthesis of serine of previous incubation of the cells with glucose, formate and pteroylglutamic acid derivatives*

Cells (3·7 mg.) were first incubated 1 hr. in 5 ml. phosphate buffer pH 6·9 (0·1M) containing the substances shown. They were then washed twice with 5 ml. 0·85% (w/v) NaCl, suspended in 5 ml. solution *S* with and without pteroylglutamate (2×10^{-6}M), and incubated 6·5 hr. (Expt. 1) or 7·5 hr. (Expt. 2).

Additions for first incubation			L-Serine (μmoles/mg. cells) formed during second incubation in the presence or absence of pteroylglutamate			
			Expt. 1		Expt. 2	
Glucose (0·02M)	Formate (0·02M)	Pteridine derivative (2×10^{-6}M)	Absent	Present	Absent	Present
−	−	None	<0·05	0·29	0·05	0·45
+	−	None	<0·05	0·37	0·06	0·53
−	−	Pteroylglutamate	0·09	0·25	0·17	0·59
+	−	Pteroylglutamate	0·44	0·45	0·63	0·72
−	+	Pteroylglutamate	0·09	0·31	.	.
+	+	Pteroylglutamate	0·51	0·50	.	.
−	−	Leucovorin	0·30	0·40	0·27	0·53
+	−	Leucovorin	0·54	0·54	0·72	0·75

glutamic acid present during the first incubation the cells synthesized 15–40 % more serine than those treated only with pteroylglutamic acid (Fig. 5). The increased activity, though small, was obtained (within the range stated) in twenty-five experiments in which different crops of cells were used. A similar increase in synthesis occurred when leucovorin or N^{10}-formylpteroylglutamic acid (both formyl derivatives) replaced pteroylglutamic acid plus formate for the first incubation (Fig. 5). Serine formation by all these types of treated cells was only increased slightly, if at all, by the inclusion of pteroylglutamic acid in solution *S* for the second incubation (Table 3). Cells first treated with N^{10}-formylpteroic acid (rhizopterin) plus glutamate, although synthesizing serine in solution *S* without added folic acid, did not show greater activity than cells treated with pteroylglutamic acid.

Omission of glucose during the treatment with pteroylglutamic acid reduced the ability of the cells to synthesize serine in solution *S* without this vitamin by 80–100 % (Table 3); in similar experiments with leucovorin activity was about halved. It is possible that glucose may be needed as an energy source both for the passage of these compounds into the cell and for their conversion into some more complex form.

Inhibition by N^{10}*-methylpteroic acid.* The fact that growth of *Strep. faecalis* R is inhibited by N^{10}-methylpteroic acid and that the inhibition is overcome competitively by pteroylglutamic acid (Cosulich & Smith, 1948) suggests that it may prevent the conversion of the latter into a more complex form. Serine formation was reduced to less than half when the analogue was added at equimolar concentration to pteroylglutamic acid; at higher concentrations inhibition was almost complete (Table 4). The inhibition was overcome competitively by pteroylglutamic acid over a hundred-fold range of inhibitor concentration; the data of Table 4 show also that approximately 40 % maximal synthesis occurred when the molar ratio N^{10}-methylpteroic acid/pteroylglutamic acid was unity for the range 2×10^{-4} to 2×10^{-6} M.

Serine synthesis with leucovorin in place of pteroylglutamic acid was at least 100 times less sensitive to the analogue (Table 4), although inhibition did occur at the highest concentration it was possible to test (2×10^{-4} M). The limits set by the activity of leucovorin and the solubility of the inhibitor made it impossible to establish whether the relationship was competitive over a significant range of concentration. The greater activity of leucovorin compared with pteroylglutamic acid in overcoming inhibition suggests that the action of the methylpteroic acid on serine synthesis is in part due to inhibition of the conversion of pteroylglutamic acid into a form of folic acid similar to, if not identical with, leucovorin. The analogue may also inhibit the further utilization of leucovorin.

N^{10}-Methylpteroic acid was also used in experiments similar in principle to those described in the previous subsection. First, cells were treated in buffered glucose with various concentrations of the analogue present and then tested for serine synthesis in solution *S* with either pteroylglutamic acid or leucovorin. Secondly, cells were first treated with pteroylglutamic acid or leucovorin and finally tested for serine synthesis in the absence of folic acid but with various amounts of the methylpteroic acid present. The results (Table 5) show that previous treatment with the analogue reduced the ability of cells to synthesize serine to less than a half with pteroylglutamic acid as source of folic acid, but that with leucovorin synthesis was normal. The synthesis of serine by cells previously treated with either form of folic acid was not inhibited by the methylpteroic acid. These facts again are in accord with the view

Table 4. *Effect of* N^{10}*-methylpteroic acid on synthesis of serine and of* Leuconostoc citrovorum *factor*

Cells at a final concn. of 0·8 mg./ml. incubated 8 hr. in solution *S* containing pteroylglutamic acid (or leucovorin) and N^{10}-methylpteroic acid at the concentrations shown.

	Serine (*a*, μmoles/mg. cells) or leucovorin (*b*, μm-moles/mg. cells) formed in presence of methylpteroic acid							
Pteroylglutamic acid (M)	0		2×10^{-6} M		2×10^{-5} M		2×10^{-4} M	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0	<0·05	0·001	.	.	.	.	.	.
[2×10^{-6}]*	[0·05	0·001]*	.	.	.	.	.	.
2×10^{-6}	0·70	0·16	0·30	0·05	0·15	0·01	0·15	0·01
2×10^{-5}	0·70	0·16	0·45	0·10	0·25	0·02	0·16	0·01
2×10^{-4}	0·71	0·18	0·60	0·21	0·60	0·12	0·30	0·15
Leucovorin								
2×10^{-6} M	0·61	.	0·59	.	0·60	.	0·25	.

* Control with heated cells.

that pteroylglutamic acid is converted into a substance with similar properties to leucovorin before it functions in serine synthesis, and that the analogue inhibits such conversion.

N^{10}-Methylpteroic acid, at the concentrations carried over, did not affect the serine assay, since *Ln. mesenteroides* P 60 is relatively insensitive to this analogue. This is not the case with aminopterin (4-aminopteroylglutamic acid), and the effect of this analogue in serine synthesis in the present system could not be tested; it is a more potent inhibitor of the growth of *Strep. faecalis* R than the methylpteroic acid.

Effect of cobalamin

There is some relationship between the functions of cobalamin and folic acid in metabolism. Both are required, for example, for the conversion of homocysteine into methionine by *Esch. coli* and for the synthesis of nucleic acid derivatives by certain Lactobacilli (see review by Woods, 1952). It has been suggested on the basis of experiments with cobalamin-deficient animals that it may also be concerned with serine formation, though probably only in an indirect manner; there is however little evidence for this (Arnstein & Neuberger, 1953*a*; Stekol, Hsu, Weiss & Smith, 1953).

Cobalamin (0·1–0·5 μg./ml.) had no effect on serine synthesis by cells of *Strep. faecalis* R in complete solution *S*, and it neither replaced nor reduced the requirement for either formate or pteroylglutamic acid. Cells incubated in buffered glucose with the vitamin had no greater synthetic ability. No definite conclusions can be drawn from these experiments, since the organism can presumably synthesize cobalamin (it does not require it for growth) and no attempt had been made to render the cells deficient.

Table 5. *Synthesis of serine by cells previously incubated with methylpteroic acid, pteroylglutamic acid or leucovorin*

Expt. 1: cells (3·5 mg./5 ml.) first incubated 1·5 hr. in phosphate buffer, pH 6·9 (0·1 M), glucose (0·02 M) and N^{10}-methylpteroic acid (as stated). Cells centrifuged out, washed twice with 5 ml. 0·85% (w/v) NaCl and incubated 7 hr. in 5 ml. solution *S* containing either pteroylglutamate or leucovorin (2×10^{-6} M).

Expt. 2: cells (3·7 mg./5 ml.) first incubated 1·5 hr. in buffered glucose (as Expt. 1) containing pteroylglutamate or leucovorin (2×10^{-6} M). Cells washed as in Expt. 1 and incubated 8 hr. in 5 ml. solution *S* containing the stated concn. of N^{10}-methylpteroic acid, but no pteroylglutamate.

Expt. no.	N^{10}-Methylpteroic acid (M) present during:	L-Serine (μmoles/mg. cells) formed in second incubation: With (during second incubation)	
		Pteroyl-glutamate	Leucovorin
1	First incubation		
	0	0·52	0·51
	2×10^{-6}	0·25	0·49
	10^{-5}	0·16	0·51
	10^{-4}	0·15	0·48
2	Second incubation	With (during first incubation)	
		Pteroyl-glutamate	Leucovorin
	0	0·54	0·71
	2×10^{-6}	0·55	0·70
	10^{-5}	0·50	0·63
	10^{-4}	0·51	0·73

Synthesis of Leuconostoc citrovorum *factor*

Work described in previous sections supports the view that pteroylglutamic acid does not function directly in serine synthesis but must first be transformed to some leucovorin-like compound. It was now found (Fig. 2) that serine synthesis by washed cells in solution *S* was accompanied by conversion of part of the pteroylglutamic acid into material supporting the growth of *Ln. citrovorum*, and therefore presumably at least closely related to leucovorin.

Substrates required. Ln. citrovorum factor, like serine, was not formed when either pteroylglutamic acid, formate or glucose was absent from the reaction mixture (Table 1). Glycine and pyridoxal (essential for serine synthesis) were not required, and were omitted from solution *S* for the experiments of Fig. 6 in which the requirement for formate and pteroylglutamic acid was explored quantitatively. Maximum synthesis of *Ln. citrovorum* factor was obtained with about one-fifth of the concentration of formate required for maximum serine synthesis (compare Fig. 6 and Fig. 3): the concen-

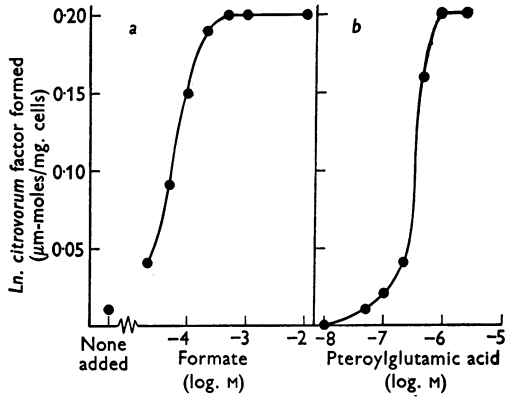

Fig. 6. Effect of formate (*a*) and pteroylglutamic acid (*b*) on the synthesis of *Ln. citrovorum* factor. Cells (0·7 mg./ml.) were incubated 8 hr. in buffered glucose (as Table 5) containing (*a*) 2×10^{-6} M pteroylglutamic acid and formate as shown, and (*b*) 0·02 M formate and pteroylglutamic acid as shown.

tration of pteroylglutamic acid required was however about 5 times greater than for maximum serine formation (compare Fig. 6 and Fig. 4).

Effect of N^{10}*-methylpteroic acid.* Inhibition of the synthesis of *Ln. citrovorum* factor occurred at the same order of concentration as with serine and was overcome competitively by pteroylglutamic acid (Table 4). The results were similar when the simplified reaction mixture containing neither glycine nor pyridoxal was used.

Further identification of serine

The microbiological assay of serine used (Lascelles *et al.* 1954) was designed to obviate interference by either *Ln. citrovorum* factor or high concentrations of glycine possibly present in the samples assayed. It was desirable, however, to identify serine as the product of synthesis by cell suspensions by an alternative method. Methods based on periodate oxidation were unsatisfactory, since recovery of serine added to concentrated samples of the products was low and erratic. Chromatography on columns of Dowex-50 resin (Moore & Stein, 1951) was successful.

Columns (0·9 × 15 cm.) were packed with Dowex-50 (mesh 250–500) buffered with 0·1 M sodium citrate pH 3·42; elution was with the same buffer and at room temperature. Cell suspensions of *Strep. faecalis* R were incubated in solution *S* with ('experimental') and without ('control') pteroylglutamic acid. Only traces of serine (by microbiological assay) were formed under the latter condition, but the major reactants and metabolic products (e.g. of glucose) were present. After incubation, samples were prepared as usual, concentrated tenfold under reduced pressure and brought to pH 2·8–3·0 before placing on the columns (Moore & Stein, 1951). The distribution of amino-N in successive 1 ml. fractions of the eluate was determined.

With 'experimental' samples the amino-N pattern (Fig. 7*a*) corresponded to that given by a mixture of pure serine and glycine at the expected concentrations (Fig. 7*b*). 'Control' samples showed only glycine (Fig. 7*a*). When DL-serine was added to 'control' samples the distribution of amino-N corresponded to that given by 'experimental' samples (Fig. 7*c*). Microbiological assays were carried out with the serine fractions obtained from the columns; the values for serine were in close agreement with those given by the ninhydrin method.

Quantitative recovery of serine from the columns was not, however, achieved; this was so both for pure serine and that present in samples. The total serine found in the combined serine fractions from 'experimental' samples was 45–50 % of that added as determined by microbiological assay. The percentage recovery of pure serine added to 'control' samples was the same (Fig. 7). The recovery of serine from mixtures of pure serine and glycine was slightly higher (70 %). The reason for these incomplete recoveries is not known, but it is at least certain that the major part of material estimated as serine by the microbiological assay is in fact serine.

Growth experiments

A basal medium (*F*) was chosen which supported growth of *Strep. faecalis* R in the absence of pteroylglutamic acid and pyridoxal when both serine and glycine (each 10^{-4} M) were added; growth was, however, improved by pteroylglutamic acid. When serine was omitted there was little or no growth unless the glycine concentration was increased tenfold; furthermore, such growth was absolutely dependent on both pteroylglutamic acid and pyridoxal (Table 6). Addition of formate to the

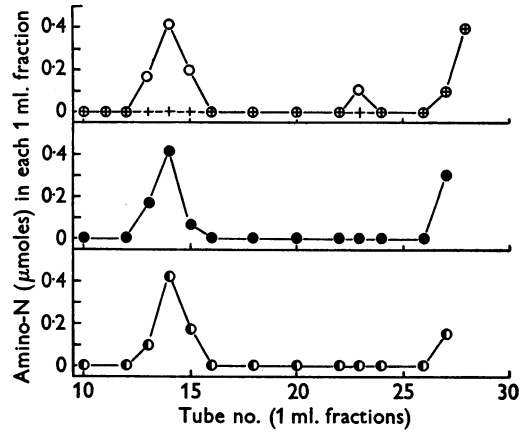

Fig. 7. Identification of serine by chromatography on Dowex-50 resin. (*a*) 'Experimental' (○) and 'control' (+) samples containing respectively 1·6 and 0·08 μmoles L-serine by microbiological assay. (*b*) Mixture (●) of DL-serine (1 μmole) and glycine (10 μmoles). (*c*) Mixture (◐) of 'control' sample and DL-serine (1·4 μmoles).

Table 6. *Replacement of serine by glycine for the growth of* Streptococcus faecalis *R*

Medium *F* was supplemented as shown. Incubation, 46 hr.

Additions to medium				
Glycine (M)	DL-Serine (10^{-4} M)	Pteroylglutamate (10^{-7} M)	Pyridoxal (2×10^{-7} M)	Growth (EEL reading)
10^{-4}	−	+	+	2
	+	+	+	21
	+	−	−	12
	+	−	+	13
	+	+	−	20
10^{-3}	−	+	+	19
	−	−	+	0
	−	+	−	0
	−	−	−	0

medium neither permitted growth in the absence of serine (glycine present) nor reduced the amount of serine required.

Assuming that the protein of the cells contains serine, these experiments show that growing cultures are also able to synthesize serine from glycine by a reaction dependent upon pteroylglutamic acid and pyridoxal; they give no indication of the source of the one-carbon residue, which must be derived from glucose or some other constituent of the medium.

DISCUSSION

Substrates. A high concentration of glycine (0·02 M) was required for optimum synthesis of serine by cell suspensions of *Strep. faecalis* R. Only about 5% of the glycine-N was converted into serine-N; since the remainder of the glycine could be recovered (microbiological assay with *Ln. mesenteroides* P 60) the need for this excess was not due to destruction by the organism. It is possible that some derivative or metabolic product rather than glycine itself condenses with a one-carbon residue to yield a three-carbon precursor of serine; the function of pyridoxal may be at this step (see below).

If formate is presumed to be the only source of the one-carbon unit, then approximately 50% is converted into serine. Available sources of the one-carbon unit appear to be more limited with *Strep. faecalis* R than with animal tissues, though it must be remembered that actual increment in serine was not shown with the latter. Formaldehyde, glycine and other one-carbon donors active in isotope exhange experiments with animal tissues were inactive in the present system. On the grounds of the greater requirement for 6:8-dimercaptooctanoic acid (as well as pteroylglutamic acid) for growth of *Tetrahymena pyriformis* W in the absence of serine, Kidder (1953) has suggested that pyruvate may give rise directly to an active one-carbon residue (formyl CoF) as well as to acetyl CoA; pyruvate was however less active than formate in our experiments with *Strep. faecalis* R.

Formate is also required for the synthesis of *Ln. citrovorum* factor from pteroylglutamic acid by the test organism. If this is a necessary step for the function of the latter compound in serine synthesis (see below), then formate may be required only for this reaction and not as the bulk source of one-carbon residues. Against this it was found (*a*) that a higher concentration of formate was required for the synthesis of serine than of *Ln. citrovorum* factor, and (*b*) that formate was still required when leucovorin replaced pteroylglutamic acid as source of folic acid.

With growing cultures of *Strep. faecalis* R exogenous formate is not required for the synthesis of serine from glycine; the one-carbon residue must come here either from some constituent of the growth medium other than glucose, or result from a changed glucose metabolism (compared with cell suspensions) caused by such a constituent: this matter is being pursued. The utilization of formate as one-carbon donor for serine synthesis by cell suspensions would require the presence of a hydrogen donor system. This may account for the requirement for glucose, the metabolism of which may also act as source of energy for the condensation.

Vitamin B_6. Both cell suspensions and growing cultures of *Strep. faecalis* R require pyridoxal absolutely for the formation of serine from glycine. The experiments of Deodhar & Sakami (1953) indicate a similar function in chicken liver, but in recent work with partially purified extracts of pigeon liver, Kisliuk & Sakami (1954) and Blakley (1954) have found no stimulation by pyridoxal phosphate; the former authors suggest that this coenzyme may be strongly bound to the protein.

Metzler, Longenecker & Snell (1954) have obtained a non-enzymic synthesis of serine by heating together glycine, formaldehyde, pyridoxal and metal ions (e.g. Al^{3+}); they propose an initial combination of glycine and pyridoxal with formation of a Schiff base which then reacts further with formaldehyde. If, as they also suggest, the enzymic mechanism is similar—an attractive hypothesis—the high concentration of glycine required may be due to a relatively low affinity of glycine for prosthetic groups containing pyridoxal or its phosphate ester. With organisms which do not require exogenous glycine for growth the Schiff base may be available as an intermediate formed in glycine biosynthesis.

Folic acid. The experiments with cell suspensions of *Strep. faecalis* R confirm the less direct evidence from work with growing cultures of various micro-organisms that some form of folic acid is required for serine synthesis at a stage at which a one-carbon unit is added. Similar observations have been made in this laboratory with cell suspensions of *Ln. mesenteroides* P 60 and *Saccharomyces cerevisiae* Y 47 (M. J. Cross and P. M. Meadow, unpublished work).

Although pteroylglutamic acid is as active as any other pteridine derivative tested with untreated cells, several other results point to a reduced and formylated compound (akin to leucovorin) as the substance with coenzyme-like activity: (1) Cells first treated with pteroylglutamic acid plus formate or with leucovorin synthesize serine rather more actively than those treated with pteroylglutamic acid alone. (2) Synthesis of serine is accompanied by conversion of part of the pteroylglutamic acid into *Ln. citrovorum* factor and both processes are inhibited by N^{10}-methylpteroic acid. (3) Synthesis

of serine is much less sensitive to inhibition by this analogue when leucovorin is used in place of pteroylglutamic acid. (4) Synthesis by cells first treated as in (1) is unaffected by the analogue. (5) With cells first treated with the analogue synthesis is normal with leucovorin but reduced with pteroylglutamic acid.

There is no evidence, however, from our experiments that the active form of folic acid is leucovorin itself, although it has a number of similar properties. Indeed leucovorin has been consistently less active than pteroylglutamic acid (with or without formate) under comparable conditions (e.g. Table 2, Fig. 5). The material synthesized concurrently with serine gave an almost identical growth response with *Ln. citrovorum* (Fig. 1*b*), but unknown higher forms of folic acid may behave similarly. Tetrahydropteroylglutamic acid has 2·5 % of the activity of leucovorin for this organism (Broquist, Fahrenbach, Brockman, Stokstad & Jukes, 1951), but the stability of the material in the present samples to storage without anaerobic precautions and to heat (they were autoclaved in the assay medium) makes it unlikely that it was this substance.

Synthesis of *Ln. citrovorum* factor from pteroylglutamic acid and formate has also been shown recently by Broquist, Kohler, Hutchison & Burchenal (1953) with cell suspensions of *Strep. faecalis* R and an amethopterin-resistant strain derived from it, and by Nichol (1954) with cell suspensions and cell-free extracts of the same organisms. In this work serine was found to replace formate as one-carbon donor. As in the present work, Nichol (1954) found the synthesis to be much increased by glucose and inhibited by a folic acid analogue (aminopterin). We also find that formate is still required for synthesis by the normal strain from N^{10}-formylpteroylglutamic acid and N^{10}-formylpteroic acid; with their resistant organism Broquist *et al.* (1953) report the same thing with the former compound and with tetrahydropteroylglutamic acid. It is clear that formate has some function besides the provision of the formyl group.

Very recently Blakley (1954) and Kisliuk & Sakami (1954) have published brief accounts of important work in which it is shown that tetrahydropteroylglutamic acid activates the interconversion of glycine and serine by partially purified cell-free extracts of pigeon liver in conditions where both pteroylglutamic acid and leucovorin are inactive or much less active. Kisliuk & Sakami suggest that N^5-hydroxymethyltetrahydropteroylglutamic acid, or a derivative, rather than leucovorin, is the immediate source of the one-carbon unit in serine synthesis. Blakley envisages the possibility that tetrahydropteroylglutamic acid combines reversibly with formaldehyde with ring formation to an imidazolidine derivative with a methyl group linking N^5 to N^{10}. Either of these two possibilities is certainly compatible with our experiments, which indicate that leucovorin is unlikely to be the actual coenzyme for serine synthesis by *Strep. faecalis* R. Another recent brief report (Greenberg, 1954) is also of great importance in relation to the form of folic acid active in cell metabolism. Leucovorin was much less effective than yeast or liver extract for the activation of a purified pigeon liver extract which catalysed the formation of inosine 5-phosphate from formate and 5 - aminoglyoxaline - 4 - carbonamide - 5′ - phosphoribitide. Incubation of the enzyme with adenosine triphosphate and either leucovorin or tetrahydropteroylglutamic acid plus formate gave a substance which was more active than yeast extract and which donated a one-carbon residue directly to the carbonamide; the chemical nature of this substance is not yet known.

SUMMARY

1. Cells of *Streptococcus faecalis* R, harvested from a medium deficient in folic acid and vitamin B_6, synthesized serine when incubated in a buffered mixture of glycine, formate, glucose, pyridoxal and pteroylglutamic acid. The omission of any one of these substances reduced serine formation to one-tenth or less.

2. Serine was estimated by specific microbiological assay; it was further identified in typical experiments by chromatography on Dowex resin.

3. Formate was not replaced by other substances active as one-carbon donors with animal tissues.

4. N^5 - Formyl - 5:6:7:8 - tetrahydropteroylglutamic acid (leucovorin) and N^{10}-formylpteroylglutamic acid replaced pteroylglutamic acid, though the former was somewhat less active.

5. Cells first treated in buffered glucose with leucovorin or with pteroylglutamic acid plus formate (and then washed) had rather greater synthetic ability than cells similarly treated with pteroylglutamic acid alone; such synthesis was no longer dependent on added folic acid.

6. A *Leuconostoc citrovorum* factor was formed concurrently with serine in the complete system. Glycine and pyridoxal were not required for this synthesis.

7. Synthesis of both serine and *Ln. citrovorum* factor was inhibited (competitively with pteroylglutamic acid) by N^{10}-methylpteroic acid. Serine formation by cells first treated with leucovorin or pteroylglutamic acid plus formate was insensitive to the analogue as was also that of untreated cells when leucovorin replaced pteroylglutamic acid in the test system.

8. It is concluded that pteroylglutamic acid is transformed to a form of folic acid similar to but

probably not identical with leucovorin before it is active in serine synthesis by this organism.

9. Serine is required for growth of *Strep. faecalis* R on a medium not containing folic acid or vitamin B_6; it can be replaced by glycine (at high concentration) only if sources of these vitamins are added, but formate is not required.

We are grateful to Sir Rudolph Peters for his interest in this work and to our colleague Mr M. J. Cross for helpful discussion and for carrying out the tests on the replacement of formate by pyruvate.

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