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The Folic Acid and Serine Nutrition of *Leuconostoc mesenteroides* P 60 (*Streptococcus equinus* P 60)

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SUMMARY: *Leuconostoc mesenteroides* P 60 (*Streptococcus equinus* P 60) requires serine for growth under normal conditions. On a medium containing relatively high concentrations of glycine it can dispense with serine provided that (a) pyridoxal is present, and (b) either Leucovorin (folinic acid) is added or the atmosphere is enriched with CO₂. Increasing concentrations of CO₂ or Leucovorin decrease the concentration of glycine required to support growth without added serine. Pyridoxal is not necessary for optimal growth with added serine, but the required concentration of the latter is twice as great.

Higher concentrations of *p*-aminobenzoic acid or Leucovorin are required for growth on glycine than on serine. Leucovorin replaces *p*-aminobenzoic acid for growth on serine, and both this factor and CO₂ for growth on glycine. With the basal medium used (containing purines and all other amino-acids) thymidine supports growth in the absence of *p*-aminobenzoic acid only when serine is added.

Growth is not inhibited by sulphonamide when Leucovorin is present; with *p*-aminobenzoic acid there is the usual competition. Pteroylglutamic acid is inactive with this organism.

A valid assay for serine is possible, even with high concentrations of glycine present, when precautions are taken to destroy either Leucovorin or pyridoxal in the samples, and to prevent the atmosphere becoming enriched with CO₂.

In connexion with work on the synthesis of serine by cell suspensions of *Streptococcus faecalis* R (Lascelles & Woods, to be published) it was necessary to devise an assay for small quantities of serine in the presence of relatively high concentrations of glycine and of members of the folic acid group of growth factors. Attention was given to microbiological assay since chemical methods proved insufficiently sensitive or otherwise unsatisfactory with the type of material to be analysed. Amongst micro-organisms reported to require serine for growth, *Leuconostoc mesenteroides* P 60 has been frequently used or suggested for assay of serine (Dunn, 1949; Steele, Sauberlich, Reynolds & Baumann, 1949; Barton-Wright, 1952). The nutritional requirement of this organism for serine was therefore investigated in detail, particularly in relation to the effect of various folic acid derivatives and of varying amounts of glycine. Some of these results have already been briefly reported (Lascelles & Woods, 1950; Lascelles, Cross & Woods, 1951).

It was found that, under certain conditions of growth, *Ln. mesenteroides* P 60 could synthesize serine for itself. Nevertheless, it was possible to devise a valid assay by taking steps to ensure that such conditions did not exist. The growth experiments upon which these conclusions are based are reported in detail since they also yielded considerable information concerning the final stage of serine synthesis by this organism. The literature bearing on this matter will be considered in the Discussion in relation to the present results.

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MATERIALS AND METHODS

The organism. *Leuconostoc mesenteroides* P 60 (American Type Culture Collection, no. 7881) was maintained in stab culture on medium B1 of Nimmo-Smith, Lascelles & Woods (1948). Stock cultures were subcultivated fortnightly and incubated 24 hr. at 37°; they were stored at 2°. It has recently been reported (McCleskey, 1952) that strain P 60 of this organism is improperly placed in the genus *Leuconostoc* and is closely related to *Streptococcus equinus*. The original name is retained here (even if incorrect) to avoid confusion in reference to earlier work.

Media. The chemically-defined basal medium (medium L) was a modification of that used by Sauberlich & Baumann (1948) for the assay of *Ln. citrovorum* factor with that organism. In particular, the concentration of DL-alanine was increased since this overcame the inhibition of growth which occurred in the absence of pyridoxal when the serine concentration exceeded 2×10^{-4} M.

Medium L contained (per litre final volume): DL-alanine, 450 mg.; L-arginine hydrochloride, 200 mg.; DL-aspartic acid, 200 mg.; L-asparagine, 400 mg.; DL-cysteine hydrochloride, 130 mg.; DL-glutamic acid, 600 mg.; L-histidine hydrochloride, 60 mg.; DL-isoleucine, 240 mg.; DL-leucine, 240 mg.; DL-lysine hydrochloride, 400 mg.; DL-methionine, 100 mg.; DL-phenylalanine, 100 mg.; DL-proline, 200 mg.; DL-threonine, 200 mg.; DL-tryptophan, 40 mg.; DL-tyrosine, 200 mg.; DL-valine, 240 mg.; glucose, 20 g.; sodium acetate trihydrate, 20 g.; NH_4Cl , 3 g.; KH_2PO_4 , 0.6 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.6 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 20 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg.; adenine, guanine, xanthine and uracil, each 10 mg.; riboflavin, 0.5 mg.; nicotinic acid, 1 mg.; calcium pantothenate, 0.6 mg.; thiamine hydrochloride, 0.6 mg.; biotin, 1 μg . Before the addition of the other constituents the amino-acids were dissolved in 40 ml. N-NaOH, the solution diluted to 200 ml. and brought to pH 6-7 with 10N-HCl. The complete medium (quantities for 1 l.) was adjusted to pH 6.8, boiled gently for 3 min., filtered and diluted to 500 ml. (double strength medium L).

In tests designed to show the effect of *p*-aminobenzoic acid and related substances it was necessary to treat the amino acids with charcoal. After dissolving in N-NaOH as above the solution was brought to pH 3 with 10N-HCl; 2 g. charcoal (Norit) was added, the mixture held at 100° for 45 min. and filtered.

Medium L_s was used for the assay of serine. It was prepared from medium L by adding (final concn.) 5×10^{-4} M-glycine; 10^{-6} M-*p*-aminobenzoic acid, 10^{-7} M-pteroylglutamic acid and, when desired, 2×10^{-7} M-pyridoxal.

Procedure. The double strength medium (L or L_s) was distributed in 1 ml. quantities in hard glass tubes ($5 \times \frac{5}{8}$ in.). After adding test materials and water to a total volume of 1.9 ml., the tubes were autoclaved 7 min. at 10 lb./sq.in. Leucovorin (synthetic *Ln. citrovorum* factor) and pyridoxal were added when required as sterile solutions after the autoclaving.

The inoculum was derived from a fresh stab culture in medium B1 which

was subcultured into tryptose-glucose broth (medium B 2, Nimmo-Smith *et al.* 1948) and incubated 24 hr. at 37°. The cells were centrifuged out, suspended in the original culture volume of water and diluted 1/50; each tube of test medium was inoculated with 0.1 ml. (about 4×10^6 cells/ml. final medium). For experiments on the effect of *p*-aminobenzoic acid and derivatives it was necessary first to wash the cells three times with the culture volume of 0.85 % (w/v) NaCl.

Incubation was at 37° for 42–46 hr. For atmospheres other than air the adapted 'Kilner' fruit-preserving jars referred to by Nimmo-Smith *et al.* (1948) were used.

The extent of growth is recorded as the galvanometer reading given by an EEL photoelectric colorimeter (Evans Electroselenium Co.) using 0.25 in. tubes and a neutral density filter; the uninoculated medium was used to obtain the zero setting. A culture containing 0.29 mg. dry weight cells/ml. gave a reading of 10; there was a linear relationship between reading and dry weight of cells up to a reading of 35.

Special chemicals. 'Leucovorin' (Ca salt pentahydrate, 99 % purity) was a gift from Dr H. P. Broquist (Lederle Laboratories); a stock solution (10^{-3} M) was prepared in sterile water; it was not further sterilized. 'Aldehyde-free' pteroylglutamic acid was kindly supplied by Dr T. H. Jukes of the same Laboratories; a 10^{-3} M stock solution was prepared by dissolving the solid in 10^{-2} M-NaHCO₃. Pyridoxal hydrochloride was a gift from Dr K. Folkers (Merck Inc.); the stock solution in water (2×10^{-3} M) was sterilized by Seitz-filtration. All three solutions were stored at -20° in the dark. Thymidine was a gift from Dr E. E. Snell and L-serine from Dr A. Neuberger.

RESULTS

Effect of carbon dioxide on the ability of glycine to replace serine

When cultures were incubated in air, N₂ or H₂, serine was essential for growth even with high concentrations of glycine present. In atmospheres containing 5 % (v/v) CO₂, however, serine was not required provided there was a sufficiently high concentration of glycine (Table 1).

The concentration of glycine required to give a given growth response in the absence of serine was a function of the partial pressure of CO₂ (Fig. 1*a*); as the latter was increased the necessary amount of glycine decreased. Maximum growth in the presence of 5 % CO₂ was reached with 0.01 M-glycine.

With an optimal concentration of serine present added CO₂ was not needed (Table 1). When, however, the serine concentration was suboptimal, the presence of CO₂ increased growth markedly (Fig. 1*b*); under these conditions it is probable, therefore, that the available serine is supplemented by synthesis from glycine.

Replacement of CO₂ by dicarboxylic acids. Citrate, oxaloacetate and α -keto-glutarate, but only at concentrations of 0.01 M and above, partially replaced the need for added CO₂ for growth with glycine (Table 1, Exp. 2). Other

carboxylic acids, including formate, were without effect. Certain dicarboxylic acids have been shown to replace CO_2 for the growth of *Escherichia coli* (Lwoff & Monod, 1947; Ajl & Werkman, 1948).

Table 1. *Effect of carbon dioxide and certain carboxylic acids on growth in the presence of serine or glycine*

Medium L was supplemented with *p*-aminobenzoic acid (10^{-4}M), pteroylglutamic acid (10^{-7}M), pyridoxal ($2 \times 10^{-7}\text{M}$) and the other substances shown. The carboxylic acids were added as the sodium salts (pH 6.8); oxaloacetate and α -ketoglutarate were sterilized by filtration. When citrate was added the medium was further supplemented with ($\mu\text{g./ml.}$) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 400; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 80; $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 30. Incubation 43 hr. in the atmosphere shown.

Exp.	Atmosphere (5% (v/v) CO_2 when present)	Carboxylic acid ($2.5 \times 10^{-2}\text{M}$)	Growth (EEL reading) with added	
			Glycine (10^{-2}M)	Serine* (10^{-3}M)
1	Air	—	0	31
	Air/ CO_2	—	26	30
	H_2	—	2	29
	H_2/CO_2	—	28	30
2	N_2	—	0	28
	N_2/CO_2	—	28	30
	N_2	Formate	4	—
	N_2	Malate	5	—
	N_2	Fumarate	5	—
	N_2	Succinate	3	—
	N_2	Citrate	13†	—
	N_2	Oxaloacetate	15†	—
	N_2	α -Ketoglutarate	9†	—

* Glycine ($5 \times 10^{-4}\text{M}$) was also present (see text).

† No growth occurred when glycine was omitted.

Relative activity of serine and glycine. In the presence of added CO_2 the concentration of glycine required for optimal growth was about 50 times that of serine (cf. Fig. 1*a, b*). With serine present, and whether or not incubation was in an atmosphere enriched with CO_2 , glycine was still essential, though the molar concentration was diminished to 0.02–0.05 of that required with serine absent (Fig. 2). This requirement for glycine when serine is present in excess shows that the organism cannot convert serine to glycine (if it does so at all) at a rate sufficient to support growth; it can, however, carry out the converse reaction when the atmosphere is enriched with CO_2 . Certain animal tissues are able actively to convert serine to glycine (Shemin, 1946).

Requirement for pyridoxal. When serine was present in optimal concentration pyridoxal increased growth only slightly; it was essential for growth when glycine plus CO_2 replaced serine (Table 2, Exp. 1). This was still true at the limiting effective concentrations of both glycine and CO_2 (Table 2, Exp. 2). Pyridoxal is therefore required, directly or indirectly, for the conversion of glycine to serine.

When serine was present, but in suboptimal concentration, pyridoxal markedly increased growth: about twice as much serine was required for

Fig. 1a. Effect of concentration of glycine and of carbon dioxide on growth without serine. Medium L was supplemented with $10^{-6} M$ -*p*-aminobenzoic acid, $10^{-6} M$ -pteroylglutamic acid and $2 \times 10^{-7} M$ -pyridoxal. Incubation 44 hr. in atmospheres of N_2 containing the % (v/v) CO_2 shown.

Fig. 1b. Effect of CO_2 and Leucovorin on growth with various concentrations of serine. Medium L contained supplements as in Fig. 1a and $5 \times 10^{-4} M$ -glycine. Incubation 44 hr. in N_2 (●—●), 5% CO_2 in N_2 (○—○), N_2 with medium containing $10^{-7} M$ -Leucovorin (+—+).

Fig. 2. Relative requirement of glycine growth in the presence and absence of serine. Medium L was supplemented as in Fig. 1a. Incubation 44 hr. in H_2 (containing CO_2 where indicated). Serine absent: 5% CO_2 (●—●) or $10^{-7} M$ -Leucovorin (●—●). Serine present (○—○).

a given growth response in the absence of pyridoxal as in its presence (Fig. 3). Similar results were obtained with both DL- and L-serine, though the latter was twice as active as the racemic form whether or not pyridoxal was present.

Table 2. *Effect of pyridoxal on growth in the presence of glycine and serine*

Medium L was supplemented with *p*-aminobenzoic acid (10^{-6} M), pteroylglutamic acid (10^{-7} M) and the other substances shown. Incubation 44 hr. in atmosphere shown.

Exp.	Atmosphere	Glycine (M)	Serine (M)	Growth (EEL reading) with pyridoxal (2×10^{-7} M)	
				Absent	Present
1	5 % CO ₂ in H ₂	5×10^{-4}	—	0	2
		10^{-2}	—	0	26
		5×10^{-4}	10^{-3}	24	29
2	0.06 % CO ₂ in N ₂	2×10^{-2}	—	1	11
	20 % CO ₂ in N ₂	10^{-3}	—	0	17

Fig. 3. Response of *Ln. mesenteroides* P60 to L- and DL-serine in the presence and absence of pyridoxal (B₆). Medium L was supplemented with 10^{-6} M-*p*-aminobenzoic acid, 10^{-7} M-pteroylglutamic acid and 5×10^{-4} M-glycine. Pyridoxal (2×10^{-7} M) present (---) or absent (—); L-serine (○), DL-serine (●). Incubation 44 hr. in air.

It appears that this organism cannot use the D-isomer. The increased stimulation of growth by pyridoxal at limiting serine concentrations again suggests that under these conditions the supply of serine is supplemented by synthesis from glycine.

Requirement for members of the folic acid group

Pennington (1946) first reported *p*-aminobenzoic acid to be essential for the growth of *Ln. mesenteroides* P60; other strains of this organism which do not respond to *p*-aminobenzoic acid have been found to need pteroylglutamic

acid (Whiteside-Carlson & Carlson, 1949). The requirement of the present strain for members of the folic acid group was tested on medium L, based on charcoal-treated amino acids (p. 268). Best growth on this medium was about 70 % of that on the medium containing untreated amino acids.

p-Aminobenzoic acid. This substance was essential for growth either on a medium containing serine or on one in which serine was replaced (as above) by high concentrations of glycine and a CO₂-enriched atmosphere. Under the latter conditions, however, the quantitative requirement for *p*-aminobenzoic acid was increased to about 5 times that when serine was supplied (Fig. 4*a*). An atmosphere containing added CO₂ did not influence the concentration of

Fig. 4*a* and *b*. Response of *Ln. mesenteroides* P60 to *p*-aminobenzoic acid (*a*) and Leucovorin (*b*) for growth in serine or high concentrations of glycine. Medium L was supplemented with 2×10^{-7} M-pyridoxal and 10^{-3} M-DL-serine plus 5×10^{-4} M-glycine (●—●) or 10^{-2} M-glycine (○—○, +—+). Incubation 44 hr. in 5 % CO₂ in N₂ (○—○, ●—●) or N₂ only (+—+).

p-aminobenzoic acid required for growth with serine present. The requirement for *p*-aminobenzoic acid was maintained over the whole range of effective concentrations of glycine and CO₂; thus in the limiting conditions of 2×10^{-2} M-glycine with 0.065 % CO₂ and 10^{-3} M-glycine with 20 % CO₂ (each giving only half-optimal growth) the need was still absolute. The increased need for *p*-aminobenzoic acid for growth in the absence of serine provides evidence that the factor (or some derivative) is concerned in the conversion of glycine to serine as well as in other reactions essential for growth.

Other members of the group. Neither pteroylglutamic acid nor its N¹⁰-formyl derivative supported growth at concentrations up to a thousand times that effective with *p*-aminobenzoic acid; this was true both for growth with serine and with glycine plus CO₂ (Table 3). Pterotic acid and rhizopterin

(*N*¹⁰-formylpteroic acid) were also inactive. It is probable, therefore, either that these substances do not permeate the cell or that the organism cannot convert them (as it presumably does *p*-aminobenzoic acid) to the form in which folic acid ultimately has function in metabolism. Experiments with the *N*⁵-formyltetrahydro derivative of pteroylglutamic acid (Leucovorin), which did replace *p*-aminobenzoic acid for growth, are discussed later.

Table 3. *Replacement of p-aminobenzoic acid by members of the folic acid group and by thymidine*

Medium L was based on charcoal-treated amino acids and was supplemented with 2×10^{-7} M-pyridoxal and the substances shown. Incubation 44 hr. in 5% (v/v) CO₂ in H₂.

Addition	Concn. (M)	Growth (EEL reading) in presence of	
		Glycine (10^{-2} M)	Serine* (10^{-3} M)
None		0	2
<i>p</i> -Aminobenzoic acid	10 ⁻⁷	18	20
Pteroylglutamic acid		0	3
<i>N</i> ¹⁰ -Formylpteroylglutamic acid		0	3
Leucovorin		18	18
Thymine	5 × 10 ⁻⁶	0	4
Thymidine		0	20

* Glycine (5×10^{-4} M) was also added.

Replacement of p-aminobenzoic acid by thymidine. Thymine or thymidine has frequently been found to be a component of mixtures which will support the growth of organisms requiring members of the folic acid group in the absence of these factors. Shive, Eakin, Harding, Ravel & Sutherland (1948) found the inhibition of growth of a strain of *Ln. mesenteroides* by a methylpteroylglutamic acid to be overcome by thymidine, but not thymine, as well as by pteroylglutamic acid.

With the present strain thymidine supported growth in the absence of *p*-aminobenzoic acid when the medium contained serine, but not when the latter was replaced by glycine plus CO₂ (Table 3); thymine was inactive, which suggests that the organism cannot convert it to the deoxyriboside. When the medium (which already contains purine and an amino acid mixture) is supplemented with serine and thymidine it apparently contains all metabolites in the synthesis of which *p*-aminobenzoic acid is concerned. When, however, the supply of serine is dependent on formation from glycine, *p*-aminobenzoic acid is still required; this confirms the specific function of the growth factor in the conversion of glycine to serine. With *Strep. faecalis* and a similar type of medium, Broquist & Snell (see Snell, 1951) found the growth requirement for folic acid to be abolished when thymine and serine were added; without thymine, folic acid was again required and the need was increased tenfold when serine was also omitted.

Experiments with Leucovorin

There is now very strong evidence that the naturally occurring factor required for the growth of *Ln. citrovorum* (ATCC 8081)* is $(-)_L$ -5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. The synthetic material (Leucovorin, folinic acid-SF) is a mixture of the $(+)_L$ - and $(-)_L$ -isomers of this compound and has half the activity of the crystalline natural factor (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad & Jukes, 1952; Cosulich, Smith & Broquist, 1952; Sauberlich, 1952; Pohland, Flynn, Jones & Shive, 1951; Bond, Bardos, Sibley & Shive, 1949; Keresztesy & Silverman, 1951). Leucovorin also replaces pteroylglutamate for the growth of *Strep. faecalis* R and *Lactobacillus casei*, but its ability to promote growth of organisms responding to *p*-aminobenzoic acid has not been reported.

Table 4. *Growth with high concentration of glycine: replacement of CO₂ and p-aminobenzoic acid by Leucovorin*

Medium L (based on charcoal-treated amino acids) was supplemented with 10^{-2} M-glycine and the substances shown. Incubation: 44 hr. in atmosphere stated.

'Acid-treated' Leucovorin was prepared by bringing an aqueous solution to pH 2 with N-HCl, autoclaving at 10 lb. pressure/sq.in. for 7 min. and neutralizing (pH 7) with sterile N-NaOH; it was added to the sterile basal medium.

Exp.	Atmosphere	Additions				Growth (EEL reading)
		<i>p</i> -Amino- benzoate (10^{-2} M)	Leucovorin (10^{-7} M)	Leucovorin 'acid-treated' (10^{-7} M)	Pyridoxal (2×10^{-7} M)	
	N ₂	—	—	—	+	0
		+	—	—	+	1
		—	+	—	+	18
		—	+	—	—	0
	5 % CO ₂ in N ₂	—	—	—	+	0
		+	—	—	+	20
		+	—	—	—	0
		—	+	—	+	21
2	N ₂	—	+	—	—	0
		+	—	—	+	1
		—	+	—	+	18
		—	—	+	+	2
	5 % CO ₂ in N ₂	+	—	+	+	3
		—	—	—	+	0
		+	—	—	+	18
		—	+	—	+	19
		—	—	+	+	16

Replacement of p-aminobenzoic acid and of CO₂. Leucovorin had a dual effect with *Ln. mesenteroides* P60. First, it replaced *p*-aminobenzoic acid both for growth with added serine and for growth with glycine plus CO₂ (serine absent). Secondly, it abolished the need for added CO₂ under the latter conditions; with optimal concentrations of Leucovorin (10^{-8} M and above) growth with 0.01 M-glycine in an atmosphere of N₂ was equal to that found with *p*-aminobenzoic acid or Leucovorin in an atmosphere enriched with CO₂ (Fig. 4b, Table 4).

* It has recently been reported (Felton & Niven, 1953) that this organism is incorrectly identified as *Ln. citrovorum*; it is a typical strain of *Pediococcus cerevisiae*.

In a series of experiments Leucovorin was 2–5 times less active on a molar basis than *p*-aminobenzoic acid in supporting growth either with serine or high concentrations of glycine (cf. Fig. 4*a* with 4*b*); thus the concentrations required for half-maximal growth were (Leucovorin first): 5.4×10^{-10} M and 3.6×10^{-10} M with serine, and 6.4×10^{-9} M and 1.4×10^{-9} M with glycine. These figures are taken from the experiments of Fig. 4 with a gas phase containing CO₂ in each case. The presence of CO₂ had no significant effect on the quantitative requirement for either growth factor when serine was present, or on that for Leucovorin for growth on glycine (Fig. 4*b*); it was as usual required for growth on glycine with *p*-aminobenzoic acid.

A difference of a factor of two between the intrinsic activities of Leucovorin and *p*-aminobenzoic acid is possible since the former contains only 50 % of the natural isomeride.

The growth factor activity of Leucovorin for *Ln. citrovorum* is lost by autoclaving it at pH 2. After such treatment (Table 4, Exp. 2) it no longer replaced CO₂ for growth on the glycine medium (serine absent), but it retained the property of replacing *p*-aminobenzoic acid under the conditions where the latter was active (presence of serine or of glycine plus CO₂). Acid treatment of Leucovorin is known to result in a mixture of products (Cosulich, Roth, Smith, Hultquist & Parker, 1951), but further interpretation of the present results is not possible in view of the complexity of the chemical changes occurring during such treatment.

Effect of glycine concentration on Leucovorin requirement. The concentration of glycine required for growth (serine absent) with Leucovorin was almost identical with that required when growth was promoted by *p*-aminobenzoic acid plus CO₂ (Fig. 2); it was again about 50 times greater than that when serine was present. The concentration of glycine needed bore a reciprocal relation to the concentration of Leucovorin added (Fig. 5); a reduced quantity of glycine could be compensated (within limits) by increased Leucovorin. This effect is essentially similar to that observed earlier with CO₂ (Fig. 1*a*).

Requirement for pyridoxal for growth on glycine. The replacement of *p*-aminobenzoic acid by Leucovorin did not modify the absolute requirement for pyridoxal for growth (serine absent) with high concentrations of glycine (Table 4).

Effect of Leucovorin on inhibition of growth by sulphathiazole. The fact that Leucovorin replaces *p*-aminobenzoic acid for the growth of *Ln. mesenteroides* P 60 suggests that the latter substance plays its part in cell metabolism through intermediate conversion to Leucovorin or a closely similar folic acid derivative. Since, however, the concentration of Leucovorin required was slightly higher than *p*-aminobenzoic acid, other interpretations were possible, and confirmation was sought by comparing the effects of the two factors on inhibition of growth by sulphathiazole on media containing either serine or high concentrations of glycine. There was, as anticipated, a strictly competitive relationship between *p*-aminobenzoic acid and the sulphonamide over a 1000-fold range of concentration of the latter (Fig. 6). With Leucovorin present (at just optimal concentration for growth), the organism was almost completely

insensitive to sulphathiazole, even at the highest concentration (Fig. 6). This would be expected if Leucovorin is the product of utilization of *p*-aminobenzoic acid whose formation is prevented by sulphonamide.

Fig. 5. Effect of concentration of Leucovorin on the requirement for glycine (serine absent). Medium L plus 2×10^{-7} M-pyridoxal, 10^{-6} M-*p*-aminobenzoic acid and the concentration (M) of Leucovorin shown on each curve. Incubation, 45 hr. in N_2 .

Fig. 6. Ability of *p*-aminobenzoic acid and Leucovorin to overcome inhibition of growth by sulphathiazole. Medium L (charcoal-treated amino-acids) plus 2×10^{-7} M-pyridoxal and supplemented with either (a) 10^{-2} M-serine + 5×10^{-4} M-glycine and incubated in N_2 (○, ●), or (b) 10^{-2} M-glycine and incubated in 5% CO_2 in N_2 (●, ○). Leucovorin (Lv), ----; *p*-aminobenzoic acid (*p*-AB), —.

The basal medium used in this work contained an amino acid mixture (except serine and glycine) and purines; the deduction that *p*-aminobenzoic acid functions through conversion to a Leucovorin-like substance can therefore only strictly be applied to its role in the synthesis of thymidine (results on serine medium) and in the conversion of glycine to serine (results on the high glycine medium).

Serine content of organisms grown in serine-free medium

It has been assumed in previous sections that when the organism grew in a serine-free medium containing high concentrations of glycine (with added CO₂ or Leucovorin) it was able to do so by synthesizing the required serine from glycine. This is a reasonable assumption in view of the likelihood that bacterial protein must contain serine in all circumstances. However, the matter was tested experimentally in the case of cells grown in glycine plus CO₂ by comparing their serine content with that of normal cells.

Medium L (no serine) was supplemented with 0.01M-glycine and the required amounts of *p*-aminobenzoic acid and pyridoxal for growth under these conditions. After inoculation the culture was incubated in 5% CO₂ in N₂. To avoid any possible carry-over of serine, the inoculum was derived from the twelfth successive subculture of the organism on the same medium. The cells were harvested at full growth by centrifuging, and washed once in phosphate buffer. A sample of the final cell suspension was hydrolysed by heating in 6N-HCl in a sealed tube for 17 hr. at 105°. The hydrolysate was neutralized and assayed for serine by the microbiological assay described below. Control cells were derived from culture on a medium based on acid-hydrolysed casein (which contains serine) and were treated in the same way. The serine contents were (% serine of dry wt.): 0.9 for cells grown in glycine plus CO₂; 1.0 for the control cells. These are minimum values since some serine is known to be lost during acid hydrolysis. The interest here however is in the comparative rather than absolute contents.

The presence of serine in the hydrolysate was confirmed chromatographically after conversion to the dinitrophenyl derivative (Campbell & Work, 1952). There is no doubt therefore that cells grown in the presence of glycine and CO₂ do, in fact, synthesize serine.

The assay of serine

It is clear from Fig. 3 that medium L is a suitable one for the assay of serine; thus with excess pyridoxal present the dose-response relationship is linear for the range 0.5 to 4×10^{-5} M-L-serine. The preceding work has shown, however, that the presence of pyridoxal, glycine or Leucovorin-like substances in the material under test, or of CO₂ in the atmosphere, might seriously affect the validity of the assay.

Effect of pyridoxal. A valid assay would be obtained either with pyridoxal totally absent or present in excess. If the basal medium contains no pyridoxal (which is not essential for growth), any contribution of pyridoxal from the material under test would lead to erroneously high figures for serine (Fig. 3).

Excess pyridoxal (see Methods) should therefore be added for routine assays; it must be omitted, however, for special tests described later.

Effect of glycine. Even at the low concentration at which it is itself required for optimal growth, glycine increases growth with suboptimal serine, but only when the atmosphere contains CO₂ or when Leucovorin is present (Fig. 1*b*). If further glycine is added with the material assayed, this effect becomes more serious since the required concentration of CO₂ (Fig. 1*a*) or Leucovorin (Fig. 5) is then reduced, and growth may occur even in the absence of serine. For a valid assay it is therefore essential to remove Leucovorin or like substances from the test material and to make sure that the atmosphere of incubation does not become enriched with CO₂.

Control of CO₂. *Leuconostoc mesenteroides* P60 produces only traces of CO₂ during growth on medium L_s and gives a homolactic fermentation of glucose (McCleskey, 1952; and present work). A sufficiently low concentration of CO₂ in the gas phase is attained by incubation of the tubes in a closed vessel filled with N₂ or H₂; the vessel should not however contain too many tubes. Incubation in air has sometimes been found to lead to erratic results. Although the normal CO₂ content of fresh air (0.03 %) would not interfere, the slightly higher content to be expected in a closed incubator containing other cultures may approach the critical level of 0.06 % effective with 10⁻²M-glycine (Table 2). Citrate, oxaloacetate and α -ketoglutarate are not normally likely to be present in material to be assayed at the high concentrations at which they replace CO₂. It is clear that a basal medium containing citrate cannot be used if the test material is likely to contain relatively large amounts of glycine.

*Removal of *Ln. citrovorum* factor.* Treatment of Leucovorin with dilute acid destroys its activity both for *Ln. citrovorum* (Broquist, Stokstad & Jukes, 1950) and in the present sense (Table 4). All material for assay should therefore be treated as in Exp. 2 of Table 4. It has been found, however, that complete removal is not certain when Leucovorin is added to bacterial extracts at concentrations exceeding 10⁻⁶M. With each new type of material it is necessary to check (by specific assay with *Ln. citrovorum*) that this factor has been completely destroyed.

Assay in the absence of pyridoxal. When serine is absent, pyridoxal is essential for growth at any concentration of glycine even when CO₂ or Leucovorin is also present (Tables 2 and 4); serine, however, supports growth in the absence of pyridoxal. An assay in the absence of pyridoxal, even though less sensitive (Fig. 3), should always be used as a further check on the validity of results obtained as above. Pyridoxal is omitted from medium L_s and the samples for assay treated to destroy members of the vitamin B₆ group. Steele *et al.* (1949), who had found these substances to stimulate growth of *Ln. mesenteroides* in the absence of serine, destroyed them by submitting materials for assay to ultraviolet irradiation. Pyridoxal can also be inactivated by autoclaving with tryptophan (Snell, 1945), and this method was used here. The experiment of Table 5 shows that the treatment is efficient and also results in no loss of serine. A solution of serine autoclaved with pyridoxal and tryptophan gave the same growth response as an untreated solution; the removal of

pyridoxal is shown by the fact that growth is halved when pyridoxal is absent from the assay medium (Table 5, cf. Fig. 3).

Table 5. *Destruction of pyridoxal by autoclaving with tryptophan*

A solution containing DL-serine (10^{-3}M), DL-tryptophan (10^{-2}M) and pyridoxal (10^{-6}M) was autoclaved at a pressure of 15 lb./sq.in. for 30 min. The treated solution was compared with DL-serine of equivalent molarity for its ability to support growth on medium L_8 with and without pyridoxal. Incubation: 44 hr. in air.

Supplement (ml.)*		Growth (EEL reading) with pyridoxal ($2 \times 10^{-7}\text{M}$)	
Treated solution	DL-serine (10^{-3}M)	Absent	Present
0	0	0	0
0.1	0	8	16
0.2	0	17	23
0.4	0	24	26
0	0.1	9	16
0	0.2	16	24
0	0.4	25	28

* Final volume of each culture was 2 ml.

DISCUSSION

Glycine as precursor of serine. The fact that glycine (albeit at rather high concentrations) is able to replace serine under certain conditions for the growth of *Ln. mesenteroides* P60, and that cells grown in this way have a normal content of serine, provides strong evidence that this organism is able to convert glycine to serine. The possibility that glycine may be a normal intermediate in serine synthesis by micro-organisms has previously been suggested by observations that certain induced mutants of *Neurospora* and *E. coli* will grow if given either serine or glycine (Tatum, 1949; Roepke, Libby & Small, 1944; Wright, 1951). Further support for a direct origin of serine from glycine comes from a finding that, when *Torulopsis utilis* is grown on a medium containing [*carboxy*- ^{14}C] glycine as sole source of carbon, the bulk of the isotope is found in the serine fraction of the hydrolysed cell-protein (Ehrensward, Sperber, Saluste, Reio & Stjernholm, 1947). In animal tissues Sakami (1948) has shown by isotope technique that C-2 of glycine is incorporated into C-2 of serine; C-3 of the latter may be derived from several methyl-group donors including formate and glycine itself (Sakami, 1948, 1949; Siegel & Lafaye, 1950).

Function of folic acid in the conversion of glycine to serine. The increased quantitative requirement for *p*-aminobenzoic acid (or Leucovorin) in growth under conditions where serine must be derived from glycine clearly indicates a specific function of folic acid at this step in serine synthesis. The ability of certain mixtures of amino acids and nucleic acid derivatives to replace the growth factor function of *p*-aminobenzoic acid and pteroylglutamic acid (and the anti-sulphonamide function of the former) had earlier suggested that

folic acid was required at some stage in serine formation (see reviews by Shive, 1951; Woods, 1952); serine was specifically implicated by Lampen, Jones & Roepke (1949) and Winkler & de Haan (1948) in the case of *p*-aminobenzoic acid and by Broquist & Snell (see Snell, 1951) in the case of pteroylglutamic acid. Wold & Sirny (1953) briefly reported that Leucovorin will replace serine for the growth of *Ln. mesenteroides* P60. Their basal medium contained glycine and citrate, and, as would be expected from the work reported here, supported some growth without added serine; the need for the latter was made absolute in their tests by increasing the concentration of L-alanine present. It was also mentioned that a relatively high glycine content increased growth in the serine-free medium.

A function of folic acid in the conversion of glycine to serine has received further indirect support from experiments with growing cultures of *Lactobacillus bifidus* (Nepple, Wright & Skeggs, 1951) and *Tetrahymena pyriformis* (*geleii*) (Kidder & Dewey, 1953). With the former organism folic acid would not replace serine unless glycine were present, while with the latter glycine replaced serine only when relatively high concentrations of folic acid were added. Evidence that the reaction promoted here by folic acid is one in which a one-carbon residue is transferred comes from Plaut, Bethel & Lardy (1950) who found that the incorporation of ^{14}C from formate into C-3 of serine was markedly less in folic acid-deficient rats than in normal animals.

Metabolic relationship of p-aminobenzoic acid and Leucovorin. It has been seen that Leucovorin can replace *p*-aminobenzoic acid for the growth of *Ln. mesenteroides* P60; slightly higher molar concentrations of the former are required than would be expected simply on a basis of a 50% content of an inactive isomeride and a molecular equivalence with *p*-aminobenzoic acid. Nevertheless, the behaviour of the two growth factors in the presence of sulphonamide (Fig. 6) is in accord with that expected if Leucovorin (or a similar substance) is the sole product of the metabolism of *p*-aminobenzoic acid by the cell. Pteroylglutamic acid, however, is without activity either in replacing *p*-aminobenzoic acid or overcoming sulphonamide inhibition. Unless it be supposed that pteroylglutamate (though not the closely related Leucovorin) is unable to permeate the cell, a reasonable explanation of these results would be that this organism is able to convert either *p*-aminobenzoic acid or Leucovorin, but not pteroylglutamic acid, to the ultimate coenzyme form of folic acid; i.e. that pteroylglutamate is not a direct intermediate in the synthesis of the coenzyme.

Leuconostoc mesenteroides P60 is unique among organisms so far tested which require *p*-aminobenzoic acid, in that Leucovorin is active when pteroylglutamate is not. There is considerable variation among such organisms in the ability of pteroylglutamic acid to replace *p*-aminobenzoic acid and to render the organism insensitive to sulphonamides (Woods, 1950); but in all other cases the two forms of folic acid have both been either inactive or active for a given test organism (unpublished observations, see Woods, 1952). It would not be justifiable therefore to deduce from the present work either that Leucovorin is a normal intermediate in the synthesis of a coenzyme form of folic

acid, or that it is itself that coenzyme. These matters are discussed more fully elsewhere (Woods, 1952, 1953).

Requirement for vitamin B₆ in serine synthesis. Pyridoxal was found to be essential for growth whenever the organism was obliged to synthesize serine from glycine (Tables 2 and 4), though not when exogenous serine was supplied. It is probable therefore that this factor, presumably as pyridoxal phosphate, is also required for the glycine → serine transformation. Deodhar & Sakami (1953) briefly reported evidence that incorporation of [¹⁴C] formate into serine by chick tissues is dependent on vitamin B₆. Earlier work had also suggested that vitamin B₆ was needed at some stage in serine synthesis. *Strep. faecalis* R was found to require serine for growth (in an otherwise complete amino acid medium) only when pyridoxin was lacking, while growth of both *Lactobacillus arabinosus* and *Ln. mesenteroides* in the absence of this amino acid was stimulated by members of the vitamin B₆ group (Lyman, Moseley, Wood, Butler & Hale, 1947; Steele *et al.* 1949).

Although *Ln. mesenteroides* P60 grew fully without addition of pyridoxal when serine was present, the concentration of this amino acid then required was double that needed when pyridoxal was also added (Fig. 3). No explanation has been found for this; since the same result was obtained with both DL- and L-serine the possibility that pyridoxal permitted the organism to use the D-isomer appears to be excluded. Vitamin B₆ derivatives are known to have such action with other amino acids, and pyridoxal phosphate has been shown to be the coenzyme of an alanine racemase enzyme system (Lyman & Kuiken, 1948; Wood & Gunsalus, 1951).

Effect of carbon dioxide. A more detailed analysis of the need for CO₂ or Leucovorin for growth with glycine (serine absent) will be published elsewhere and has been reported briefly (Cross, 1953). The inverse quantitative relationship between the required concentrations of glycine and CO₂ (Fig. 1*a*) led to the suggestion that CO₂ might serve, with growing cultures, as a source of the necessary one-carbon residue for condensation with glycine (Lascelles *et al.* 1951); later experiments with ¹⁴CO₂ have shown however that this is not the case. Leucovorin replaces CO₂ in this effect and there is again an inverse quantitative relationship with glycine. The quantity of serine found in the cells after growth in such conditions showed that it was impossible that the formyl group of Leucovorin itself was the ultimate source of the C-3 of serine; at least 200 molecules of serine were formed for each molecule of Leucovorin present at the time of inoculation.

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REFERENCES

- AJL, S. J. & WERKMAN, C. H. (1948). Replacement of CO₂ in heterotrophic metabolism. *Arch. Biochem.* **19**, 483.
- BARTON-WRIGHT, E. C. (1952). *The Microbiological Assay of the Vitamin B Complex and Amino-acids*, p. 130. London: Pitman and Sons, Ltd.
- BOND, T. J., BARDOS, T. J., SIBLEY, M. & SHIVE, W. (1949). The folinic acid group, a series of new vitamins related to folic acid. *J. Amer. chem. Soc.* **71**, 3852.
- BROQUIST, H. P., STOKSTAD, E. L. R. & JUKES, T. H. (1950). Some biological and chemical properties of the citrovorum factor. *J. biol. Chem.* **185**, 399.
- CAMPBELL, P. N. & WORK, T. S. (1952). The biosynthesis of protein. 1. Uptake of glycine, serine, valine and lysine by the mammary gland of the rabbit. *Biochem. J.* **52**, 217.
- COSULICH, D. B., ROTH, B., SMITH, J. M., HULTQUIST, M. E. & PARKER, R. P. (1951). Acid transformation products of leucovorin. *J. Amer. chem. Soc.* **73**, 5006.
- COSULICH, D. B., SMITH, J. M. & BROQUIST, H. P. (1952). Diastereoisomers of leucovorin. *J. Amer. chem. Soc.* **74**, 4215.
- CROSS, M. J. (1953). *Leuconostoc citrovorum* factor and the synthesis of serine by micro-organisms. *6th Int. Congr. Microbiol. Abstr.* **1**, 121, Rome.
- DEODHAR, S. & SAKAMI, W. (1953). Biosynthesis of serine. *Fed. Proc.* **12**, 195.
- DUNN, M. (1949). Determination of amino acids by microbiological assay. *Physiol. Rev.* **29**, 219.
- EHRENSVÄRD, G., SPERBER, E., SALUSTE, E., REIO, L. & STJERNHOLM, R. (1947). Metabolic connection between proline and glycine in the amino acid utilisation of *Torulopsis utilis*. *J. biol. Chem.* **169**, 759.
- FELTON, E. A. & NIVEN, C. F. (1953). The identity of '*Leuconostoc citrovorum*, Strain 8081'. *J. Bact.* **65**, 482.
- KERESZTESY, J. C. & SILVERMAN, M. (1951). Crystalline citrovorum factor from liver. *J. Amer. chem. Soc.* **73**, 5510.
- KIDDER, G. W. & DEWEY, V. C. (1953). Influence of thioctic acid and folic acid on amino-acid synthesis in *Tetrahymena*. *Fed. Proc.* **12**, 230.
- LAMPEN, J. O., JONES, M. J. & ROEPKE, R. R. (1949). Mutant strains of *Escherichia coli* unable to synthesize *p*-aminobenzoic acid. *J. biol. Chem.* **180**, 423.
- LASCELLES, J. & WOODS, D. D. (1950). Synthesis of serine by micro-organisms. *Nature, Lond.* **166**, 649.
- LASCELLES, J., CROSS, M. J. & WOODS, D. D. (1951). *Leuconostoc citrovorum* factor and the synthesis of serine by micro-organisms. *Biochem. J.* **49**, lxvi.
- LWOFF, A. & MONOD, J. (1947). Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.
- LYMAN, C. M. & KUIKEN, K. A. (1948). Effect of vitamin B₆ on the utilisation of D amino acids by lactic acid bacteria. *Fed. Proc.* **7**, 170.
- LYMAN, C. M., MOSELEY, D., WOOD, S., BUTLER, B. & HALE, F. (1947). Some chemical factors which influence the amino acid requirements of the lactic acid bacteria. *J. biol. Chem.* **167**, 177.
- MCCLESKEY, C. S. (1952). The identity of strain P-60 of *Leuconostoc mesenteroides*. *J. Bact.* **64**, 140.
- NEPPE, H. M., WRIGHT, L. D. & SKEGGS, H. R. (1951). The role of folic acid in the nutrition of *Lactobacillus bifidus*. *Bact. Proc.* p. 143.
- NIMMO-SMITH, R. H., LASCELLES, J. & WOODS, D. D. (1948). The synthesis of 'folic acid' by *Streptobacterium plantarum* and its inhibition by sulphonamides. *Brit. J. exp. Path.* **29**, 264.
- PENNINGTON, D. (1946). Assay of *p*-aminobenzoic acid. *Science*, **103**, 397.
- PLAUT, G. W. E., BETHEIL, J. J. & LARDY, H. A. (1950). The relationship of folic acid to formate metabolism in the rat. *J. biol. Chem.* **184**, 795.

- POHLAND, A., FLYNN, E. H., JONES, R. G. & SHIVE, W. (1951). A proposed structure for folinic acid-SF, a growth factor derived from pteroylglutamic acid. *J. Amer. chem. Soc.* **73**, 3247.
- ROEPKE, R. R., LIBBY, R. L. & SMALL, M. H. (1944). Mutation or variation of *Escherichia coli* with respect to growth requirements. *J. Bact.* **48**, 401.
- ROTH, B., HULTQUIST, M. E., FAHRENBACH, M. J., COSULICH, D. B., BROQUIST, H. P., BROCKMAN, J. A., SMITH, J. M., PARKER, R. P., STOKSTAD, E. L. R. & JUKES, T. H. (1952). Synthesis of Leucovorin. *J. Amer. chem. Soc.* **74**, 3247.
- SAKAMI, W. (1948). The conversion of formate and glycine to serine and glycogen in the intact rat. *J. biol. Chem.* **176**, 995.
- SAKAMI, W. (1949). The conversion of glycine into serine in the intact rat. *J. biol. Chem.* **178**, 519.
- SAUBERLICH, H. E. (1952). Comparative studies with the natural and synthetic citrovorum factor. *J. biol. Chem.* **195**, 337.
- SAUBERLICH, H. E. & BAUMANN, C. A. (1948). A factor required for the growth of *Leuconostoc citrovorum*. *J. biol. Chem.* **176**, 165.
- SHEMIN, D. (1946). The biological conversion of L-serine to glycine. *J. biol. Chem.* **162**, 297.
- SHIVE, W. (1951). The functions of B-vitamins in the biosynthesis of purines and pyrimidines. *Vitam. & Horm.* **9**, 75.
- SHIVE, W., EAKIN, R. E., HARDING, W. M., RAVEL, J. M. & SUTHERLAND, J. E. (1948). A crystalline factor functionally related to folic acid. *J. Amer. chem. Soc.* **70**, 2299.
- SIEGEL, I. & LAFAYE, J. (1950). Formation of the β -carbon of serine from formaldehyde. *Proc. Soc. exp. Biol., N.Y.* **74**, 620.
- SNELL, E. E. (1945). The vitamin B₆ group. V. The reversible interconversion of pyridoxal and pyridoxamine by transamination reactions. *J. Amer. chem. Soc.* **67**, 194.
- SNELL, E. E. (1951). Growth factors in bacterial nutrition. Chapter in *Plant Growth Substances*, p. 431. Ed. Skoog, F. Wisconsin University Press.
- STEELE, B. F., SAUBERLICH, H. E., REYNOLDS, M. S. & BAUMANN, C. A. (1949). Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. *J. biol. Chem.* **177**, 533.
- TATUM, E. L. (1949). Amino acid metabolism in mutant strains of micro-organisms. *Fed. Proc.* **8**, 511.
- WHITESIDE-CARLSON, V. & CARLSON, W. W. (1949). The vitamin requirements of *Leuconostoc* for dextran synthesis. *J. Bact.* **58**, 135.
- WINKLER, K. C. & DE HAAN, P. G. (1948). On the action of sulfanilamide. XII. A set of non-competitive sulfanilamide antagonists for *Escherichia coli*. *Arch. Biochem.* **18**, 97.
- WOLD, F. & SIRNY, R. J. (1953). Effect of folinic acid on serine synthesis in *Leuconostoc mesenteroides* P-60. *Fed. Proc.* **12**, 292.
- WOOD, W. A. & GUNSALUS, I. C. (1951). D-Alanine formation: a racemase in *Streptococcus faecalis*. *J. biol. Chem.* **190**, 403.
- WOODS, D. D. (1950). Biochemical significance of the competition between *p*-amino-benzoic acid and the sulphonamides. *Ann. N.Y. Acad. Sci.* **52**, 1199.
- WOODS, D. D. (1952). The function of folic acid and related growth factors in the metabolism of micro-organisms. *Symposium sur le métabolisme microbien*, p. 86. *Int. Congr. Biochem.* **2**. Paris.
- WOODS, D. D. (1953). Folic acid and related compounds in the metabolism of micro-organisms. *Brit. med. Bull.* **9**, 122.
- WRIGHT, B. E. (1951). Enhancement of the normal reversion-rate of a serineless *Escherichia coli* mutant by certain organic acids. *Nature, Lond.* **168**, 1087.