Inter-relationships of Serine, Glycine and Vitamin B₆ in the Growth of Mutants of *Escherichia coli*

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SUMMARY: Five mutants of *Escherichia coli* which grow to a greater or less extent when the minimal medium is supplemented with vitamin B₆ have been studied. They fall into two groups according to the further effect of serine + glycine on growth. (1) Two strains showed full and rapid growth with pyridoxine alone and serine + glycine neither replaced this vitamin nor enhanced growth in its presence. (2) Three strains showed delayed and suboptimal growth with pyridoxine alone. With these serine + glycine had two effects: (a) it decreased the lag and enhanced the final extent of growth when added with pyridoxine; (b) it permitted suboptimal growth in the absence of pyridoxine. The second effect was dependent on (two strains), or greatly increased by (one strain), the concurrent addition of glycolaldehyde.

Nothing is known of the pathway of synthesis of vitamin B₆ by organisms not requiring an external source of it for growth. Mutant strains of *Escherichia coli* which multiply only when vitamin B₆ is supplied are more rare than those which respond to vitamin B₆ or serine or glycine. The simplest explanations of the apparent replacement of the vitamin by these amino acids are: (a) the amino acids are end products of reactions catalysed by vitamin B₆ derivatives; consequently, when provided preformed, they decrease or abolish the need for the vitamin; (b) the synthesis of serine and glycine by the organism is limited, and one or other is a reactant at some step in the biosynthesis of vitamin B₆.

A detailed study was made of the properties of mutants of this type in an attempt to discover an immediate biochemical lesion resulting from the mutation and possibly to obtain some conception of the route of synthesis of vitamin B₆ and its derivatives by normal strains of *Escherichia coli*.

METHODS

Escherichia coli. Stock cultures were maintained on tryptic meat agar slopes and were subcultured monthly (16 hr. at 37°). Strains B and 15 (American Type Culture Collection, ATCC no. 9723) were prototrophs. Strains B166 and B167 (obtained from Dr J. Gots and derived from strain B), and 22-99 (obtained from Professor B. D. Davis) required vitamin B₆ or serine or glycine for growth. Two strains responded only to vitamin B₆. These were: strain M2, which required the vitamin at all temperatures and was isolated by us by the technique of Adelberg & Myers (1953) after u.v. irradiation of strain 15; strain DW, a substrain of M154-59L, provided by Dr Elizabeth Work (Denman, Hoare & Work, 1955) which required vitamin B₆ for growth at 37° but responded also to certain mixtures of amino acids at lower temperatures.

Strain B166 was used for most of the work to be described.

Media

Based on glucose. Medium G1 was that of Davis & Mingioli (1950) and medium G2 the same but with the glucose concentration increased to 1% (w/v). The glucose when not autoclaved separately in aqueous solution (as in media G1 and G2) was sterilized as indicated in individual experiments.

Based on lactate. Medium L contained (g./l.): KH₂PO₄, 2; Na₂HPO₄, 12H₂O, 8; (NH₄)₂SO₄, 2.5; NH₄Cl, 2.5; lactic acid, 10; MgSO₄·7H₂O, 0.05. After addition of Fe²⁺ (final conc. 4×10^{-6} M) from a solution of FeSO₄·(NH₄)₂SO₄·6H₂O in twice its molar concentration of sodium citrate, the pH value was brought to 7.4 with 10 N-NaOH.

All media were prepared at double strength and brought to the desired volume with water and other experimental additions before autoclaving for 7 min. at 115° (unless otherwise stated).

Supplements. Amino acid mixture AA-SG contained the following amino acids at 5×10^{-3} M: DL-alanine, L-arginine, DL-aspartic acid, L-cysteine HCl, DL-glutamic acid, DL-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-lysine diHCl, DL-methionine, DL-phenylalanine, DL-proline, DL-threonine, DL-tryptophan, DL-tyrosine, DL-valine.

The purines + pyrimidines mixture contained 2×10^{-4} M adenine, and 10^{-4} M each of guanine, uracil, thymine and hypoxanthine. Addition of a little N-HCl facilitated solution (later neutralized).

Vitamin mixture V contained (μg./ml.): riboflavin, 50; thiamine, 60; calcium pantothenate, 60; nicotinic acid, 100; biotin, 0.1; *p*-aminobenzoic acid, 1.4; cobalamin, 0.1.

Growth conditions

Static culture. Inoculated medium (5 ml.) in 150 × 19 mm. hard glass tubes were incubated at 37° in racks sloped at 5° to the horizontal. The inoculum was 0.1 ml. of aqueous suspension of organisms (about 2.5×10^7 /ml.) derived from a 24 hr. culture on tryptic meat agar.

Shaken culture. Medium (5 ml.) inoculated as above was contained in optically matched 150 × 150 × 16 mm. L-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) which were clamped to a rocking device (similar to that of van Heyningen & Gladstone, 1958) and shaken in a 37° water bath at 36 oscillations/min., of excursion 10 cm.

When larger quantities of organisms were required, Erlenmeyer flasks (1 l.) containing 500 ml. medium were inoculated with about 2.5×10^8 organisms and shaken at 37° (circular motion, 220 rotations/min. of radius 2 cm.).

Assessment of growth

The extent of growth was measured with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex, England) with a neutral density filter; the uninoculated medium was used to give the zero setting. The cross arms of the 16 mm. L-tubes were constructed to fit the instrument; samples of other cultures were assessed in 6 mm. tubes. A curve relating the readings in the two types of tubes was constructed, and the growth achieved by the

organisms under all conditions of incubation expressed in terms of the EEL reading which would have been obtained in 1-tubes. The relation between instrument reading and dry weight was determined for each strain used and was found to be linear up to a reading of 35–40; a reading of 20 was equivalent to 0.6 mg. dry wt./ml.

Estimation of glycolaldehyde

The colorimetric method of Dische & Borenfreund (1949) was used. Trichloroacetic and glacial acetic acids were of AR grade and the diphenylamine recrystallized twice from 70% (v/v) ethanol before immediate use. The colour developed was measured by reading optical densities at 660 and 580 m μ . The method was stated to permit the determination of glycolaldehyde in the presence of a fifty-fold excess of trioses and other aldehydes and sugars. In the present work, no aldehyde or other chemically related compound tested at concentrations within this range was found to interfere appreciably with the assay of glycolaldehyde.

A standard curve relating D_{660} – D_{580} to glycolaldehyde concentration over the range 2×10^{-4} to 10^{-3} M was made in each assay. The relationship was always linear.

Chemicals

Glycolaldehyde. Commercial preparations were normally used, but for critical tests one sample was recrystallized from absolute ethanol. A further pure specimen (m.p. 94.5–95°) was prepared from tartaric acid via dihydroxyfumaric acid (Powers, Tabakoglu & Sable, 1955) and recrystallized from dry acetone. Glycolaldehyde-2-phosphate (barium salt) was kindly provided by Dr E. Racker and converted to the sodium salt as described below.

Other chemicals. A solution of β -hydroxypyruvic acid was obtained by the controlled alkaline hydrolysis of β -bromopyruvic acid (Sprinson & Chargaff, 1946). Methylglyoxal in solution was prepared from dihydroxyacetone (Umbreit, Burris & Stauffer, 1945) and assayed by Friedemann's (1927) method. Glucose reductone (*enoltartronaldehyde*) was made from glucose (von Euler & Martius, 1933).

Xylulose-5-phosphate, sedoheptulose-7-phosphate (as barium salts) and the *o*-nitrophenylhydrazone of L-erythrulose were gifts from Dr B. L. Horecker. Sodium salts (in solution) were obtained by batch treatment with Amberlite IR 50 resin in the Na⁺ form, while a solution of free L-erythrulose was prepared by a small-scale adaptation of the method of Müller, Montigel & Reichstein (1937). A solution of D-erythrose-4-phosphate was obtained (Ballou, Fischer & McDonald, 1955) from a specimen of the cyclohexylamine salt of the acetal donated by Dr C. E. Ballou. δ -Aminolaevulinic acid was given by Professor A. Neuberger. All other chemicals were commercial specimens.

RESULTS

Except in the final section, the experiments were all performed with *Escherichia coli* B166 which grows on certain media with added glycine or serine or vitamin B₆. For reasons explained later the amino acids were used, unless

otherwise stated, as an equimolar mixture of DL-serine and glycine; this will be referred to as serine + glycine, the molar concentration given being in terms of the total amino-nitrogen. Serine and glycine are known to be metabolically very closely related in *E. coli* (Cutinelli *et al.* 1951; Abelson, 1954; Meinhart & Simmonds, 1955), and as they were essentially interchangeable in the present study, their use in admixture introduces no difficulties of interpretation. Vitamin B₆ is used in general reference to the group; individual members will be specifically named.

General characteristics of growth

Comparison of glucose and lactate as main carbon source. The response of strain B166 to pyridoxine (10^{-6} M), serine + glycine (10^{-3} M) and mixtures, was tested on the simple glucose-containing medium G1, and the ammonium lactate medium L (Figs. 1 and 2). The main carbon source was added to each of the media before autoclaving.

Fig. 1

Fig. 2

Fig. 1. Growth of *Escherichia coli* B166 on medium G1 (with glucose autoclaved in the medium). Basal medium was supplemented with serine + glycine, 10^{-3} M (○), pyridoxine, 10^{-6} M (●), serine + glycine and pyridoxine (●); growth on unsupplemented medium of prototrophic parent strain *Escherichia coli* B (—x—). Incubated in shaken culture at 37°.

Fig. 2. Growth of *Escherichia coli* B166 on lactate medium L. Basal medium was supplemented with serine + glycine, 10^{-3} M (○), pyridoxine, 10^{-6} M (●), serine + glycine and pyridoxine (●). Incubated in shaken culture at 37°.

A notable difference was that serine + glycine did not support growth on the lactate medium. In both media, however, most rapid and extensive growth occurred with pyridoxine plus serine + glycine, the rate then approaching that of the parent strain growing on unsupplemented medium. With pyridoxine

alone, growth was delayed in both media but the final mass was 80 to 90 % of that obtained with the mixture. In all cases where growth occurred on lactate medium it was slightly delayed, but finally 10–20 % greater, than with the same supplement in the glucose medium.

In glucose medium supplemented only with serine + glycine, growth was delayed less than with pyridoxine alone, but the final mass was only 50–60 % of that found with both supplements (Fig. 1). The mixture of serine and glycine had therefore two apparently distinct effects on growth; (a) it supported growth in the absence of pyridoxine but only on the glucose medium, while (b) on both media, and with pyridoxine present, it decreased the delay in onset of growth by 20–25 hr. and slightly increased the final density.

Response to vitamin B₆. With the five forms of vitamin B₆ tested on medium G1 (Table 1) growth was most rapid with pyridoxine; the concentration for half-maximal growth was 2×10^{-7} M. Pyridoxal was intrinsically more active (10^{-7} M gave half-maximal response) but growth was delayed a further 12 hr. Similar results were obtained on medium L. Pyridoxine was subsequently used routinely.

Table 1. *Response of Escherichia coli B166 to various forms of vitamin B₆*

Basal medium G1 with glucose autoclaved in the medium at 115°/7 min. Incubation in static culture at 37°; tubes containing serine + glycine incubated for 30 hr. only, the remainder for 80 hr.

Supplement	Conc. (M)	Growth (EEL reading) with serine + glycine (10^{-3} M)	
		Absent	Present
Pyridoxine	10^{-6}	14	18
	3×10^{-7}	11	18
	10^{-7}	4	10
	3×10^{-8}	0	6
	10^{-8}	0	0
Pyridoxal	10^{-6}	12	17
Pyridoxamine	10^{-6}	6	14
Pyridoxal-5'-phosphate	10^{-6}	5	—
Pyridoxamine-5'-phosphate	10^{-6}	0	—

With serine + glycine also present (Table 1) the amount of pyridoxine required was decreased to about one-third (6×10^{-8} M for half-maximal growth); in these experiments results were read at 30 hr. since on longer incubation there was of course growth in the absence of added pyridoxine.

Response to serine, glycine and related compounds. The concentration of serine + glycine required on medium G1 (glucose added before autoclaving and no added pyridoxine) was 10^{-3} and 2.5×10^{-4} M for maximal and half-maximal growth respectively (Table 2). Similar concentrations were required for the other effect of serine + glycine, i.e. to promote earlier growth when pyridoxine was present. Glycine and DL-serine were individually active in both the above senses, but the mixture gave somewhat more rapid and heavier growth. In addition, results were erratic with suboptimal concentrations of the separate amino acids. This appeared to be due to partial reversion of the

Table 2. *Amino acid growth requirements of Escherichia coli B166 in the absence of added vitamin B₆*

Incubation for 70 hr. at 37°. Other conditions as in Table 1.

Amino acid	Conc. (M)	Growth (EEL reading)
Nil	—	0
DL-Serine + glycine	4×10^{-3}	12
	10^{-3}	12
	5×10^{-4}	9
	2.5×10^{-4}	6
	1.25×10^{-4}	4
	6.25×10^{-5}	3
L-Serine	5×10^{-4}	7
D-Serine	5×10^{-4}	0
Glycine	5×10^{-4}	8
DL-Homoserine	10^{-3}	0
DL-Threonine	10^{-3}	Trace*

* 5 after longer incubation (110 hr.).

mutant to prototroph-like character. Random individual tubes grew heavily after 48 hr. incubation and subcultures into basal medium gave growth (though less rapidly than the parent strain) without the addition of either pyridoxine or serine + glycine. The properties of these substrains have not been further pursued. These changes were less frequent with mixtures of glycine and serine at suboptimal concentration and did not occur when the concentration of the individual amino acids exceeded 4×10^{-4} M. With serine alone (at optimal concentration) only the L-isomer was active, either in the presence or absence of pyridoxine.

There is evidence that *Escherichia coli* can form glycine from both threonine and homoserine (Gibson, 1952; Abelson, 1954). DL-Threonine at high concentration supported detectable growth in the absence of pyridoxine but DL-homoserine was inactive (Table 2).

Other amino acids or substances metabolically related to serine and glycine and found to be inactive (over a concentration range of 10^{-6} to 5×10^{-3} M) were: formiminoglycine, glycollic, glyoxylic, pyruvic, β -hydroxypyruvic and δ -aminolaevulinic acids, ethanolamine, DL-alanine, L-arginine, DL-asparagine, DL-aspartic acid, DL-glutamic acid, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, DL-tryptophan, DL-tyrosine and DL-valine.

Effect of autoclaved glucose. The fact that serine + glycine supported growth of strain B166 in the absence of pyridoxine, but only on the glucose-containing medium, suggested that the activity of the amino acids was dependent on the concurrent presence of some substance formed as a result either of glucose metabolism by the organism or of the heat-sterilization of the medium. In the above experiments, the glucose had been added to the other constituents of the medium before autoclaving. When the glucose was separately sterilized by Seitz-filtration, and then added to the remainder of the medium (which had been autoclaved as usual), serine + glycine no longer supported growth. This

was also the case when the glucose was separately sterilized by autoclaving in aqueous solution (in glass-distilled water when the pH was 5.4; Fig. 3). Autoclaving in 0.04M-phosphate buffers of various pH values showed that only at about pH 7 and above was a solution of glucose obtained which permitted growth on serine + glycine (Fig. 3).

Fig. 3. Comparison of growth promoting activity of heated glucose solutions (block diagram) with their content of substance assaying as glycolaldehyde (—x—). Activated glucose samples were prepared by autoclaving glucose solutions (10 mg./ml.) in 0.04M-phosphate buffers. Activity measured as extent of growth of *Escherichia coli* B166 in static culture on medium G2 supplemented with sample plus serine + glycine (10^{-3} M).

For present purposes, the term 'activated glucose' will be used in reference to glucose solutions which permit growth of the organism on serine + glycine without added pyridoxine. 'Inactive glucose' will be used in the opposite sense. Untreated glucose was inactive, and remained so after sterilization by Seitz-filtration or by autoclaving at pH 6.5 and below.

The action of activated glucose

Conditions for formation. Glucose (10 mg./ml.) was autoclaved at various combinations of temperature and time, at pH values ranging from 6 to 8. After neutralizing, the solution was added to previously sterilized basal medium supplemented only with serine + glycine. Best activation occurred when autoclaving was carried out at 10 lb. sq. in./7 min. at pH 7.4 (Fig. 3). At higher pH values or with more severe autoclaving, there was obvious caramelization and a decrease in activity, probably due rather to the concurrent production of inhibitory substances than to decreased formation of the active

factor. Onset of growth in the presence of serine + glycine plus pyridoxine was delayed 10, 10 and 30 hr., respectively, when the glucose was treated at pH 7.8 (10 lb. sq. in./7 min.) or at pH 7.4 for 15 min. at 10 and 15 lb. sq. in.

Circumstances when required. As shown above both serine + glycine and activated glucose were required for growth in the absence of pyridoxine. Experiments with mixtures of inactive and activated glucose showed that the maximum effect was obtained when at least 80 % of the glucose in the medium was provided in the activated form.

When pyridoxine was present (either with or without serine + glycine), activated glucose was not required, nor did it increase the rate or final extent of growth (Fig. 4). The only effect was a slight delay in the onset of growth, possibly again due to the presence of inhibitory substances. Activated glucose was therefore not required for the second action of serine + glycine (i.e. decreasing the lag when pyridoxine is present), but only for the total replacement of pyridoxine.

Table 3. *Reducing agents and the growth of Escherichia coli B166 on serine + glycine*

The reducing agents were sterilized by Seitz-filtration. Incubated in static culture 64 hr. at 37°.

Supplement	Conc. (M)	Growth (EEL reading) with serine + glycine (10 ⁻³ M)	
		Alone	+ Pyridoxine (10 ⁻⁶ M)
Nil	—	0	15
Ascorbic acid	2×10^{-3}	0	0
	4×10^{-4}	0	(15)
	8×10^{-5}	0	15
Glucose reductone	10^{-5}	0	(15)
*Activated glucose	5×10^{-2}	10	15

() Signifies growth delayed 10 hr. compared with control.

* Control in which glucose was added to basal medium G1 before autoclaving.

Effect of reducing agents. The activation of glucose might simply have been due to the development of non-specific reducing conditions in the autoclaved mixtures. One powerful reducing agent known to be formed when alkaline solutions of glucose are heated is glucose reductone (*enoltartronaldehyde*; Pigmann & Goepp, 1948). This compound (10^{-10} to 10^{-5} M) did not replace activated glucose for growth of strain B166 on serine + glycine; at concentrations above 10^{-5} M it partly inhibited growth in the controls (Table 3). It may be partially responsible for the slight delay in growth obtained when activated glucose was present in the medium. Ascorbic acid was chosen as a reducing agent of carbohydrate-like nature, though one which is not formed from glucose on autoclaving. It, too, failed to replace activated glucose at the concentrations tested (10^{-8} to 5×10^{-3} M) and was inhibitory at the higher concentrations (Table 3).

Activated glucose was also necessary for growth on serine + glycine under anaerobic conditions (Table 8). It was concluded that the activation of glucose is due to the formation of some specific compound which together with serine + glycine permits growth of strain B166 in the absence of added vitamin B₆.

Fig. 4

Fig. 5

Fig. 4. Effects of (a) inactive glucose (—) and (b) activated glucose (---) on growth of *Escherichia coli* B166 in medium G1 supplemented with pyridoxine, 10^{-6} M, alone (●) and with serine + glycine 10^{-3} M (○). Incubated in shaken culture at 37° .

Fig. 5. Growth of *Escherichia coli* B166 on medium G2 supplemented with serine + glycine 10^{-3} M and indicated concentrations of glycolaldehyde. Incubated in shaken culture at 37° .

Experiments with other sugars and derivatives. Material active in supporting growth in a medium supplemented only with serine + glycine was also formed on autoclaving certain other hexoses and pentoses at slightly alkaline pH values.

Solutions (20 mg./ml.) were (a) Seitz-filtered, (b) autoclaved at the existing pH (5.4–5.7), (c) autoclaved in 0.04 M phosphate buffer pH 7.4, and (d) added to the supplemented basal medium (pH 7) before autoclaving. The treated solutions were tested at a final concentration of 4 mg./ml. The additional carbohydrate did not affect the rate or extent of growth in control media supplemented with pyridoxine (with or without serine + glycine).

None of the sugars permitted growth with serine + glycine unless they had first been autoclaved at pH 7 or 7.4 (Table 4). The three sugar alcohols and 3-O-methylglucose remained inactive even after such treatment. Fructose gave rise most easily to inhibitory substances, and the growth which did occur on serine + glycine with autoclaved preparations of fructose was delayed 24–48 hr. as compared with the other sugars.

Several of the carbohydrates are fermented by *Escherichia coli* and could replace glucose as the main carbon source for growth of the mutant in the

presence of pyridoxine. In these cases, development of activity was also assayed by adding the carbohydrate to the medium (i.e. G1 without glucose) as the sole carbon source either before autoclaving or as a Seitz-filtered solution after autoclaving. The sugar alcohol mannitol was inactive in both cases; the sugars, as before, were active only after autoclaving (Table 5).

Table 4. *Ability of variously treated sugars and their derivatives to replace activated glucose*

Criterion of activity was growth of *Escherichia coli* B166 on medium G2 supplemented with serine + glycine (10^{-3} M) plus the test compound. All compounds under test were used at a final concn. of 4 mg./ml. and autoclaved ($115^{\circ}/7$ min.) as shown at 20 mg./ml.

Compound	Seitz-filtered	Autoclaved in		
		Aqueous soln. (pH 5.4-5.6)	Medium (pH 7)	PO ₄ buffer (pH 7.4)
Galactose	—	—	+	+
Mannose	—	—	+	+
Fructose*	—	—	+	+
Sorbose	—	—	+	+
Arabinose	—	—	+	+
Xylose	—	—	+	+
Ribose	—	—	+	+
Mannitol	—	—	—	—
Sorbitol	—	—	—	—
Erythritol	—	—	—	—
3-O-Methyl-glucose	—	—	—	—

+ = Activation; — = no activation.

* Activated fructose was somewhat inhibitory (delayed growth).

Table 5. *Growth of Escherichia coli B166 in the presence of serine + glycine plus variously treated sugars*

Basal medium G1, but with compound specified replacing glucose, plus serine + glycine (10^{-3} M); all autoclaving was at $115^{\circ}/7$ min. Incubation in static culture for 80 hr. at 37° .

Compound	Seitz-filtered	Autoclaved in		
		Aqueous soln. (pH 5.4-5.6)	Medium (pH 7)	PO ₄ buffer (pH 7.4)
Galactose	—	—	+	+
Mannose	—	—	+	+
Fructose*	—	—	+	+
Arabinose	—	—	+	+
Xylose	—	—	+	+
Mannitol	—	—	—	—

+ = Growth; — = no growth.

All the above substrates supported growth in the presence of pyridoxine.

* Activated fructose was somewhat inhibitory (delayed growth even in the presence of pyridoxine).

With the relatively small number of carbohydrates tested it would appear that the active substance was formed only when there was an actual or potential aldehyde group at C-1 and an unsubstituted hydroxyl group on C-3.

Behaviour of known degradation products of glucose. Concurrently with the above experiments, a number of compounds reported to be amongst the products of alkaline degradation of glucose (Evans, 1942; Pigmann & Goepp, 1948) were assayed for activity. These were added in Seitz-filtered solution over a range of concentrations (10^{-6} to 5×10^{-3} M), to medium G2 containing serine + glycine.

No growth was obtained with the following substances: lactic, pyruvic, acetic, formic and tartronic acids, methylglyoxal, dihydroxyacetone, DL-glyceraldehyde and formaldehyde. Also tested and found to be inactive was 5-hydroxymethylfurfural (which in any event is predominantly a product of the acidic degradation of glucose).

Glycolaldehyde (5×10^{-5} to 10^{-3} M) supported good growth in the presence of serine + glycine and absence of added pyridoxine. A recrystallized commercial specimen and material prepared from dihydroxyfumaric acid synthesized in this laboratory and recrystallized to m.p. $94.5-95^\circ$, were both quantitatively as active as the commercial product which was used in all subsequent experiments.

Further experiments with glycolaldehyde

Media containing glucose. The ability of glycolaldehyde to support growth of *Escherichia coli* B166 on medium G2 supplemented with serine + glycine was studied in aerobic, shaken culture (Fig. 5). Delayed and suboptimal growth was obtained with a glycolaldehyde concentration of 10^{-5} M; 5×10^{-4} M supported greatest growth. Above these concentrations, progressive retardation and diminution of growth was noted until at 5×10^{-3} M it was completely inhibited (even in the presence of pyridoxine). Glycolaldehyde (5×10^{-4} M) was more effective than activated glucose in supporting growth on serine + glycine (Fig. 5, compare Fig. 1), the delay in growth being decreased from 30 to 15 hr. The presence of glycolaldehyde, however, had no effect on growth of the organism in the presence of pyridoxine, altering neither the lag period nor the final yield of organisms (Fig. 6, compare Fig. 4).

Although final growth was better in shaken than in stationary culture under all conditions, the effect of aeration was relatively greater for growth on serine + glycine. Thus the final mass on serine + glycine and glycolaldehyde in shaken tubes was 65–70 % of that with pyridoxine also present but only 50–55 % in stationary tubes (from data of Fig. 6 and Table 7). The optimal glycolaldehyde requirement for growth in stationary culture was somewhat higher (10^{-3} M) than in shaken culture.

Lactate medium. *Escherichia coli* B166 did not respond to serine + glycine alone in medium L (Fig. 2). Addition of activated glucose permitted limited growth; the concentration of activated glucose required being approximately the same as with the wholly glucose-containing basal medium. Glycolaldehyde (5×10^{-4} M) again replaced activated glucose, allowing suboptimal growth to occur with no greater delay than when pyridoxine was also present (Fig. 7). The final growth was not improved by increasing the concentration of glycolaldehyde; indeed, this was somewhat toxic at a concentration (10^{-3} M)

which gave good growth on medium G2. The response in lactate medium to pyridoxine alone, or together with serine + glycine, was not affected by glycolaldehyde (Fig. 7, compare Fig. 2).

Whilst 5×10^{-4} M-glycolaldehyde bettered the action of activated glucose in the glucose medium, it was less effective in that based on lactate (Fig. 7). When inactive glucose (10 mg./ml.) was added with the glycolaldehyde, there was no effect on the ultimate mass achieved in the presence of pyridoxine, but the suboptimal growth obtained in the absence of added vitamin was

Fig. 6. Growth of *Escherichia coli* B166 on medium G2 supplemented with glycolaldehyde (5×10^{-4} M). Other additions were: serine + glycine 10^{-3} M (○), pyridoxine, 10^{-6} M (●), serine + glycine plus pyridoxine (⊙). Incubated in shaken culture at 37°.

Fig. 7. Growth of *Escherichia coli* B166 on lactate medium L in the presence (—) or absence (---) of glycolaldehyde, 5×10^{-4} M. Other supplements were: serine + glycine, 10^{-3} M (○); pyridoxine, 10^{-6} M (●); serine + glycine plus pyridoxine, (⊙); serine + glycine plus activated glucose (□). Incubated in shaken culture at 37°.

Fig. 8. Growth of *Escherichia coli* B166 on lactate medium L containing glycolaldehyde (5×10^{-4} M) and inactive glucose (10 mg./ml.). Other supplements were: serine + glycine, 10^{-3} M (○); pyridoxine, 10^{-6} M (●); serine + glycine plus pyridoxine (⊙). Incubated in shaken culture at 37°.

doubled (Fig. 8, compare Fig. 7). Galactose, gluconate and fructose could be substituted for glucose, but other possible carbon sources, e.g. arabinose, ribose, pyruvate, glyceraldehyde and acetate, were inactive or much less active. Addition of glycolaldehyde alone to either glucose or lactate medium did not allow of growth of the organism or reduce its pyridoxine requirement.

Apparent glycolaldehyde content of activated glucose. It was calculated from the results of Figs. 1 and 5 that activated glucose (10 mg./ml.) might contain from 1 to 4×10^{-4} M glycolaldehyde. A glucose solution of strength and pH value identical with that of medium G2 (10 mg./ml.; pH 7) was autoclaved under the standard conditions of 7 min. at 115° (10 lb./sq.in.) and the glycolaldehyde content of the product estimated chemically. About 2×10^{-4} M was found to be present (using an unautoclaved solution as blank). The dose-response curve with increasing concentrations of activated glucose could be superimposed upon the standard curve. Glycolaldehyde formation under other conditions of autoclaving was also determined (Fig. 3). Increasing ability to support growth on serine + glycine was matched by increasing

glycolaldehyde content. Conditions which decreased biological activity (greater degree of heating or of alkalinity) still produced the maximum glycolaldehyde concentration; this may be a further indication that the diminished activity was due to concurrent formation of inhibitory substances.

Specificity of requirement for glycolaldehyde. A number of substances either related structurally to glycolaldehyde, or known to function under other circumstances as donors or acceptors of 'active glycolaldehyde' (Horecker & Mehler, 1955), did not replace glycolaldehyde in promoting growth of *Escherichia coli* B166 on serine + glycine. The compounds tested over a range of concentrations (10^{-6} to 2×10^{-3} M) in Seitz-filtered solution, included: ethanolamine, ethyleneglycol, glycollic, glyoxylic and oxalic acids, glycolaldehyde-2-phosphate, D-erythrose-4-phosphate, L-xylulose-5-phosphate, sedoheptulose-7-phosphate and L-erythrulose. It is possible that the phosphate esters do not permeate the organism.

Table 6. *Dihydroxyfumaric acid and β -hydroxypyruvic acid-supported growth of Escherichia coli B166 on medium G2 with serine + glycine*

All compounds were added aseptically as Seitz-filtered solutions. Basal medium was G2 with serine + glycine (10^{-3} M). Static cultures were incubated for 80 hr. at 37° .

Final conc. (M)	Growth (EEL reading) with		
	Glycol- aldehyde	Dihydroxy- fumaric acid	β -Hydroxy- pyruvic acid
4×10^{-3}	0	—	7
10^{-3}	14	12	10
5×10^{-4}	13.5	10	8.5
2×10^{-4}	11	5	5
5×10^{-5}	9	0	0
10^{-5}	7	0	0
10^{-6}	0	0	0

Dihydroxyfumaric acid and β -hydroxypyruvic acid were active (Table 6), but since the required concentration was about ten times that of glycolaldehyde this might have been due to spontaneous or metabolic conversion to glycolaldehyde. The spontaneous decomposition of dihydroxyfumaric acid is utilized for the chemical preparation of glycolaldehyde (Powers *et al.* 1955) and a solution of the acid kept at 60° for 30 min., assayed with organism B166 as if it had in fact been converted to its molar equivalent of glycolaldehyde. Difficulty was met in the colorimetric assay of glycolaldehyde in solutions of β -hydroxypyruvic acid, but maximum and minimum values for glycolaldehyde in a 5×10^{-3} M solution were 5×10^{-4} and 3×10^{-4} M respectively (see also Milhaud, Benson & Calvin, 1956).

Specificity of the amino acid requirement in the presence of glycolaldehyde. The response of strain B166 to amino acid mixtures was studied at various temperatures since mutant DW will grow at 25° in the absence of added vitamin B₆ when provided with a comprehensive mixture of amino acids (see below). When incubated at either 25 or 37° , growth of organism B166 took place on medium G2 supplemented with casein hydrolysate only when glycolaldehyde

(10^{-3} M) was also added. With a mixture of amino acids not including serine or glycine, growth in the presence of glycolaldehyde was less than with serine + glycine plus glycolaldehyde. The growth obtained in the absence of serine + glycine was due entirely to the DL-threonine content of the amino acid mixture (Table 7). Addition of the amino acid mixture without serine or glycine (AA-SG) decreased the pyridoxine requirement of the mutant only very slightly.

The quantitative requirements of organism B166 for serine + glycine were unchanged when 10^{-3} M-glycolaldehyde was used in place of activated glucose in medium G2 with or without pyridoxine.

Table 7. *Growth of Escherichia coli B166 in the presence of amino acids*

Supplements: glycolaldehyde, 10^{-3} M; pyridoxine, 10^{-6} M; serine + glycine, 10^{-3} M; DL-threonine, 10^{-3} M. AA-SG; amino acid mixture (each at 5×10^{-3} M) not containing serine + glycine used in 1/10 dilution. AA + PP + V; amino acids plus purines, pyrimidines and vitamins used in 1/10 dilution. Basal medium was G2 and incubation was in static culture for 84 hr. at 37°.

	Growth (EEL reading)
Glycolaldehyde	0
+ serine + glycine	12
+ AA-SG	7
+ DL-threonine	8
Pyridoxine	21
+ serine + glycine	24
+ AA + PP + V	22

Effect of changed conditions of culture. Under the usual conditions of aerobic culture at 37° and pH 7 there was an evident difference between the response of the organism to vitamin B₆ and to glycolaldehyde plus serine + glycine (Fig. 6). Although anaerobic growth was overall less good than that obtained aerobically the response of strain B166 to the compounds of interest remained relatively unchanged. The superiority of 10^{-3} M-glycolaldehyde to activated glucose in medium G2 was a little more marked (Table 8).

At 25°, aerobic growth in the presence of added pyridoxine alone was much delayed (110 hr.), but the decrease in incubation temperature had only a slight effect upon growth in the absence of added vitamin B₆ (Table 8). Growth on pyridoxine was again more adversely affected than that obtained on serine + glycine plus glycolaldehyde when the pH value of the medium was decreased to 6.5 (Table 8).

Escherichia coli B166 was most sensitive to inhibition by 4-deoxypyridoxine when growing in the presence of pyridoxine. Addition of serine + glycine decreased the sensitivity, while growth in the absence of vitamin B₆ was least affected. The organism was also most susceptible to inhibition by isoniazid when growing on vitamin B₆. The addition of serine + glycine to the pyridoxine-containing medium decreased sensitivity to the greatest extent. Growth on serine + glycine plus glycolaldehyde showed an intermediate degree of resistance.

Table 8. *Effect of changes in cultural conditions on growth of Escherichia coli B166 in the presence and absence of pyridoxine*

Basal medium G2 with supplements as shown of DL-serine + glycine, 10^{-3}M ; activated glucose, 10 mg./ml.; glycolaldehyde, 10^{-3}M ; pyridoxine, 10^{-6}M . Incubation was for 80 hr. at the temperature and under the conditions shown. Aerobic cultivation in sloped tubes in air. Anaerobic cultivation in upright tubes in modified Kilner jar containing 5% (v/v) CO_2 in N_2 .

				Growth (EEL readings)			
Atmosphere				Anaerobic	Aerobic		
Temperature				37°	37°	25°	37°
pH				7.0	7.0	7.0	6.5
Supplement							
Nil				0	0	0	0
Pyridoxine				8	13	0*	8.5
Serine + glycine +							
activated glucose				2.5	10.5	—	—
glycolaldehyde				4.5	11	9	10.5
pyridoxine				11	20	15	15

* Some growth on prolonged incubation.

Characteristics of other vitamin B₆ auxotrophs of Escherichia coli

Mutants showing an effect with serine + glycine. Strain B167 closely resembled B166 under all conditions tested, i.e. in its ability to grow in the absence of added vitamin B₆ only when both serine + glycine and glycolaldehyde were provided, and in the enhancement and acceleration of its growth on pyridoxine by serine + glycine. There was no growth on media G2 or L with serine + glycine or glycolaldehyde as sole supplements.

Strain 22-29 differed from the other two members of this group in being able to grow on serine + glycine alone, and eventually, though only slightly, when glycolaldehyde was the sole supplement to medium G2. Growth on pyridoxine alone was again belated and suboptimal, but best growth occurred with a mixture of serine + glycine plus either pyridoxine or glycolaldehyde (Fig. 9).

Mutants showing no serine + glycine effect. Vitamin B₆ was required by strain DW for growth at 37°. At this temperature, it grew well with only short delay on medium G2 supplemented with 10^{-6}M -pyridoxine. The final extent of growth was increased by the concurrent addition of a comprehensive amino acid mixture, but in this serine and glycine played no special role. Glycolaldehyde (10^{-3}M) alone, or in admixture with serine + glycine (10^{-3}M), was without effect.

At 25° growth was possible without added vitamin B₆ when an acid-hydrolysate of casein, or a comprehensive amino acid mixture was supplied (Wijesundera, 1954). Serine and glycine seemed to have no part in this replacement action. Fairly good growth was obtained in medium G2 supplemented with DL-alanine plus DL-methionine ($5 \times 10^{-4}\text{M}$) (Table 9). Glycolaldehyde at concentrations below 10^{-3}M had no observable effect in the presence or absence of added vitamin B₆.

Strain M2 was isolated in the course of a specific search for pyridoxine-requiring mutants amongst the products of u.v. irradiation of wild type *Escherichia coli*. It showed an absolute requirement for vitamin B₆ over a temperature range of 25–37°, in media based either on glucose or lactate;

Fig. 9

Fig. 9. Growth of *Escherichia coli* 22-99 on medium G2 with and without glycolaldehyde (10^{-3} M). No further supplement ($\times$); serine + glycine, 10^{-3} M ($\circ$); pyridoxine, 10^{-6} M ($\bullet$); serine + glycine plus pyridoxine ($\circ$). Incubated in shaken culture at 37°.

Fig. 10

Fig. 10. Growth of auxotroph *Escherichia coli* M2 in medium G2 containing pyridoxine, 10^{-7} M ($\bullet$), compared with growth of parent organism *Escherichia coli* strain 15 in unsupplemented medium G2 ($\times$). Both incubated in shaken culture at 37°.

Table 9. Growth of *Escherichia coli* DW at 25° on supplemented medium G2

Supplements: pyridoxine, 10^{-6} M; amino acid mixtures used in 1/10 dilution to give final amino acid concs. of 5×10^{-4} M (each); DL-alanine, 10^{-3} M; DL-methionine, 5×10^{-4} M. Incubated in static culture 86 hr. at 25°.

Supplement	Growth (EEL reading)
Nil	0
Pyridoxine	17
Casein hydrolysate	16
Amino acid mixture AA	17
Amino acid mixture AA-SG	20
DL-Alanine + DL-methionine	15
DL-Alanine	< 1
DL-Methionine	0

growth comparable with that of the parent strain was obtained with 10^{-7} M-pyridoxine (Fig. 10). Occasionally, heavy growth appeared in a tube containing a casein hydrolysate supplement, but whenever this took place, examination proved that reversion had occurred. In the usual media the organism was stable even at very suboptimal concentrations of pyridoxine. Serine + glycine (10^{-3} M) had no effect either alone, or in admixture with 10^{-3} M-glycolaldehyde (Table 10).

When growth of strain M2 was limited by suboptimal concentrations of pyridoxine (4×10^{-8} M) the organisms clumped together producing an abnormal

appearance in shaken culture; the culture medium was found to contain abnormally high concentrations of keto acids.

The results obtained with all the mutants used are summarized and compared in Table 11.

Table 10. *The pyridoxine requirement of Escherichia coli M2*

Basal medium G2 was supplemented as shown with serine + glycine, 10^{-3} M, and glycolaldehyde, 10^{-3} M. Incubated in static culture 42 hr. at 37° .

Pyridoxine conc. (M)	Growth (EEL reading) with			
	No addition	Serine + glycine	Glycolaldehyde	Serine + glycine + glycolaldehyde
10^{-6}	27	27	27	27
10^{-7}	27	27	27	27
5×10^{-8}	22	22	8	< 1
2×10^{-8}	0	0	0	0

Table 11. *Summary of characteristics of vitamin B₆ mutants of Escherichia coli*

Growth response indicated by + + +, + +, $\pm$ +, etc., obtained in medium G2 supplemented as shown with serine + glycine 10^{-3} M; glycolaldehyde 10^{-3} M; pyridoxine 10^{-6} M.

Mutant	Supplement					
	None	Pyridoxine	Serine + glycine + pyridoxine	Serine + glycine + glycolaldehyde	Serine + glycine	Glycolaldehyde
B166	0	(+ +)	+ + +	$\pm$ +	0	0
B167	0	(+ +)	+ + +	$\pm$ +	0	0
22-99	0	(+ +)	+ + +	+ + +	(+ +)	($\pm$)
DW	0	+ +	+ +	0	0	0
M2	0	+ + +	+ + +	0	0	0

() denotes appreciable delay before beginning of growth.

DISCUSSION

Glycolaldehyde and activated glucose. Glycolaldehyde was able to reproduce in every particular the biological activity of heated glucose solutions with *Escherichia coli* strain B166; an optimal concentration consistently improved upon their action. Even the observation that glycolaldehyde could at no concentration satisfactorily substitute for activated glucose in the lactate medium was fully explained by the concurrent action of unchanged glucose. The assay of the glycolaldehyde content of activated glucose samples by the Dische-Borenfreund method is sufficiently accurate for it to be concluded that (a) sufficient glycolaldehyde is present to account for the biological activity of the samples, and (b) the glycolaldehyde is formed only during the specific autoclaving procedure necessary for the production of activated glucose. Glycolaldehyde formation from glucose during alkaline autoclaving would probably proceed by way of scission of the 1:2-enediol intermediate into C_2

and C₄ fragments (Fig. 11). This accords with the finding that the sugar alcohols were not activated by autoclaving, for these compounds would be unable to form the enediolate ion. Again, scission at this position apparently requires migration of the hydrogen atom from the C-3 hydroxyl group; this could not occur in 3-O-methylglucose, which was found not to form the active factor.

Fig. 11

Several instances are known of the development of biological activity during heat sterilization of glucose-containing media (Orla-Jensen, 1933; Rose & Peterson, 1949; Cheldelin, 1954; Hachisuka, Kato, Asano & Kuno, 1955; Ramsey & Lankford, 1956; Field & Lichstein, 1957; Sergeant, Lankford & Traxler, 1957). When the medium contains amino acids, Maillard-type, or other, interactions between these and the hexoses have been described. For example, N-D-glucosylglycine has been found to possess the same growth stimulating activity for *Lactobacillus gayonii* as medium-autoclaved glucose (Rogers, King & Cheldelin, 1953). Scission products have occasionally been held responsible for the growth stimulatory or inhibitory effects of glucose autoclaved in the absence of amino compounds; pyruvate, for example, replaced autoclaved glucose for *Streptococcus salivarius* and *Lactobacillus bulgaricus* (Smiley, Niven & Sherman, 1943; Snell, Kitay & Hoff-Jørgensen, 1948). However, the present study appears to be the first to implicate glycolaldehyde as a component of alkaline autoclaved glucose of possible biological significance.

Specificity of requirement for free glycolaldehyde. It is noteworthy that of all the vitamin B₆-requiring mutants of *Escherichia coli* examined, only with those whose growth on pyridoxine was accelerated and enhanced by serine + glycine could any action of glycolaldehyde be demonstrated. Although ethanolamine has been suggested as a possible precursor of free glycolaldehyde in animal tissues (Weissbach & Sprinson, 1953) this compound could not substitute for glycolaldehyde with *E. coli* B166, nor would several reported products of glycolaldehyde metabolism, e.g. glycollic acid, glyoxylic acid, glycine (Kun, Dechary & Pitot, 1954; Friedmann, Levin & Weinhouse, 1956), ethylene glycol (Sable, 1952; Buyze, 1955), and erythrulose (Horecker & Mehler, 1955). The activated two carbon fragment transferred in transketolase-catalysed reactions has tentatively been identified with 'active glycolaldehyde' or glycolaldehyde-thiamine pyrophosphate (Horecker, Smyrniotis & Klenow, 1953). Whether free glycolaldehyde can enter into equilibrium with the 'active

glycolaldehyde' fragment is not known. The isotopic data of Friedmann *et al.* (1956) suggest that in animal tissues it cannot. This would explain why in the present study none of the 'active glycolaldehyde' donors tested was capable of substituting for free glycolaldehyde (though it is further possible that the phosphate esters were not available to the intact organism).

The high optimal concentration of glycolaldehyde (5×10^{-4} to 10^{-3} M) suggests that if its sole action is in the replacement of vitamin B₆ it is either very rapidly destroyed by the organism or acts indirectly. Free glycolaldehyde is in fact metabolized extensively and rapidly by growing cultures and washed suspensions of *Escherichia coli* B166 (Mr C. Mulder, personal communication). In contrast to serine + glycine, glycolaldehyde seems to have no specific action on the organism other than in its role in vitamin B₆ replacement; under no circumstances did glycolaldehyde exercise any effect in the presence of pyridoxine.

The vitamin B₆ auxotrophs of Escherichia coli. These are divisible into two groups differentiated by their response to serine + glycine and glycolaldehyde (Table 11). It is difficult to believe that those strains (B166, B167 and 22-99) for which these compounds can substitute for vitamin B₆ are true vitamin B₆ auxotrophs, for no concentration of any form of vitamin B₆ is able to evoke maximal growth in the absence of serine + glycine. The amino acids seemingly serve a double function, for besides their growth-enhancing effect in the presence of pyridoxine they must be concerned with glycolaldehyde in replacing vitamin B₆; if this is not so glycolaldehyde alone would be expected to evoke at least the suboptimal growth given by pyridoxine. The organisms are thus subject to a multiple metabolic block and must be at least considered as serine + glycine plus vitamin B₆ auxotrophs. A further complication is introduced by the finding that the magnitude of the growth response of strain B166 to serine + glycine plus glycolaldehyde was dependent upon the basal medium used, and that (unlike 22-99) maximal growth is never obtained in the absence of added vitamin B₆. A further substance may be concerned in the vitamin B₆-replacement action which is more readily formed from glucose than lactate. Could this be provided preformed, growth on serine + glycine plus glycolaldehyde might at least attain that degree found with serine + glycine plus pyridoxine. If the same substance had still further functions in these mutants, their growth in its presence together with serine + glycine and either pyridoxine or glycolaldehyde might reach the maximum attained by the prototroph. A fuller appraisal of the growth characteristics of these mutants has been attempted elsewhere (Morris, 1958).

It is considered that *Escherichia coli* M2 alone of all the mutants studied is a true vitamin B₆ auxotroph in that its requirement for the vitamin is absolute and its growth response to pyridoxine maximal. The ability of strain DW to grow in the absence of added vitamin B₆ at 25° is probably due to its metabolic block being incomplete at this temperature.

It has not been possible from the growth properties to establish with certainty the nature of the biochemical lesion or lesions in the mutants studied. In particular, it cannot be decided whether serine + glycine and

glycolaldehyde decrease the need for vitamin B₆ or take part in its synthesis. An attempt to discriminate between these two possibilities by a direct study of vitamin B₆ synthesis in the light of the present results will be described in the following paper (Morris, 1959).

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