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Factors influencing the action of glycine on *Escherichia coli* in nutrient broth and in hypertonic medium

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The effect of various glycine concentrations in nutrient broth and in a hypertonic nutrient medium against *E. coli* at various temperatures has been examined. Optimum spheroplast formation was found to occur in bacteria which were in the logarithmic growth phase. Glycine has no action on the viability of non-growing bacteria.

IT has been known for many years that glycine exerts an antibacterial effect against *Escherichia coli* (Gordon & McLeod, 1926). More recently, it has been shown that glycine induced lysis of washed suspensions of this organism (Cowles, 1947; Maculla & Cowles, 1948; Gordon, Hall & Stickland, 1951).

Jeynes (1957) found that, in nutrient medium supplemented with high concentrations of sucrose, glycine "removed the cell walls" of certain bacteria to produce protoplasts which were stable in this medium. Jeynes (1961) later produced evidence to substantiate his statement that the spherical bodies produced as a result of glycine treatment did not contain any of the original cell wall constituents. This is in contrast to the findings of Cota-Robles & Duncan (1962) that the osmotically labile spherical forms of *E. coli* B, obtained in the presence of certain D-amino-acids, possess cell wall material.

D-Amino-acids have also been found to induce spheroplasts in *Alcaligenes fecalis* (Lark & Lark, 1959) and in *Rhodospirillum rubrum* (Tuttle & Gest, 1960). However, apart from the discovery (Cota-Robles & Duncan, 1962) that optimum conversion of bacteria to spheroplasts is obtainable only with cells in the early logarithmic phase of growth, little information is available on the factors influencing spheroplast induction. The present investigation has been concerned with a study of the effect of temperature of incubation, age of bacterial cells and glycine concentration on the effect of this amino-acid against *E. coli* in both nutrient broth and a hypertonic nutrient medium.

Experimental and results

MATERIALS

The organism was a laboratory strain of *E. coli* Type I, which has previously been used in this laboratory (John & Russell, 1963). Nutrient broth and nutrient agar were prepared from the respective Oxoid granules. Hypertonic medium consisted of nutrient broth containing 0·25% w/v magnesium sulphate, $MgSO_4,7H_2O$, and either 0·33M or 0·66M sucrose. The pH of all media was 7·4.

Sucrose, glycine, magnesium sulphate, disodium hydrogen phosphate and potassium dihydrogen phosphate, were of analytical reagent quality.

Water was obtained from an all-glass still.

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METHODS

Viable counts were made by serial dilution in sterile water, followed by plating into nutrient agar. Plates were incubated at 37° and colony counts were made after 48 hr.

Measurement of bacterial growth was made with the Spekker absorptiometer, using Standard 20 mm cells, and a standard Kodak filter No. 6. The cells, with specially prepared metal caps, were sterilised by dry heat for 30 min at not above 150°. In all experiments, control media (bacteria absent) were used.

Examination of spheroplasts was made by means of a Cook-Troughton microscope, under oil-immersion, × 100 objective, and by phase contrast.

Effect of glycine on E. coli *at various temperatures.* In this series of experiments, 0·2 ml of an overnight 37° broth culture of *E. coli* was added to 20 ml of nutrient broth or of hypertonic medium, containing varying concentrations of glycine. Incubation was carried out at the required temperature, and extinction measurements made at intervals.

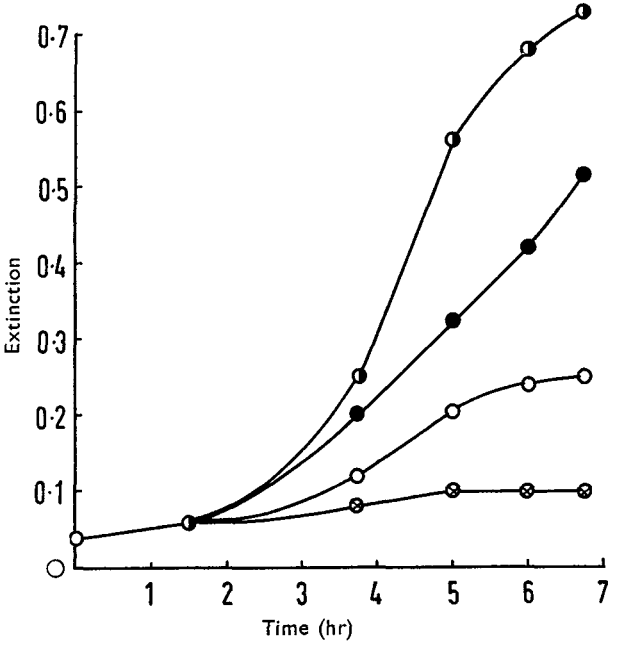

FIG. 1. Effect of glycine on *E. coli* in nutrient broth containing 0·33 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. ◑—◑ Glycine absent. ●—● Glycine 10 mg/ml. ○—○ Glycine 15 mg/ml. ⊗—⊗ Glycine 20 mg/ml.

Typical examples of the effect of glycine on *E. coli* are provided in Figs 1 and 2. Results of the other experiments can be summarised as follows: Extinction at 44° showed the greatest increase with time when glycine was absent, and the least when the highest concentration of glycine (15 mg/ml) was present. Extinction was greater when 0·33M sucrose was included than with 0·66M sucrose. At 20°, extinction

increased slowly after a 2 hr lag phase in 0·33M sucrose Mg^{2+} broth (glycine absent) and to a still slower extent in the presence of 10 mg/ml glycine. With glycine absent, a 6 hr lag phase was apparent in 0·66M sucrose Mg^{2+} broth; no increase in opacity was evident even after 24 hr when 10/ml glycine was included.

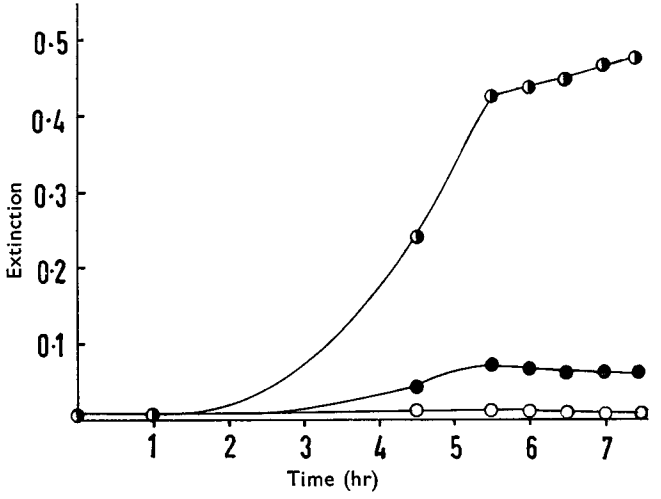

FIG. 2. Effect of glycine on *E. coli* in nutrient broth containing 0·66 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. Key: as in Fig 1.

To determine whether an increase in extinction when the hypertonic medium was employed could be correlated with spheroplast formation, samples were removed and examined microscopically (Table 1).

Effect of glycine on the viability of washed suspensions of E. coli. An overnight 100 ml 37° broth culture of the organism was centrifuged, the residue washed twice with 40 ml of sterile water, and finally resuspended in 12 ml of sterile water. From this, 3 × 3 ml volumes were reserved, and made up to 20 ml with buffer solution and glycine to give final concentrations of glycine of 0, 10 and 20 mg/ml. The pH in each case was 7·3. Opacity measurements during subsequent incubation at 37° gave erratic results; however, the fact that there is no loss in viability after treatment for 5 hr (Table 2) indicates that the organisms have not suffered lysis during this period.

Effect of glycine on the viability of E. coli *at* 4°. The non-lethal effect of glycine against "resting" cells of *E. coli* was confirmed by treatment of the organism with the amino-acid in 0·33M sucrose-Mg^{2+}-broth at 4° (Table 3). Spheroplasts were not induced by any glycine concentration.

Effect of glycine on logarithmic phase of E. coli Glycine induces spheroplast formation at growth temperatures but not at 4°, and has no lethal effect on washed suspensions (Table 2). This points to the action of this substance being directed, at least in part, towards an inhibition of cell wall synthesis. This being so, it would be expected that the

TABLE 1. MICROSCOPICAL EXAMINATION OF THE EFFECT OF GLYCINE ON *E. coli* AT VARIOUS TEMPERATURES

Nutrient broth containing 0·25% Mg^{2+} and	Concn. of glycine* (mg/ml)	Temp. of incubation (°C)	Microscopical examination at 5 hr
0·33M sucrose	10, 15 and 20	4	No spheroplasts induced
	10	20	Spheroplasts; small
	15		Optimum number of spheroplasts; small
	20		Spheroplasts; small
	10	37	Morphological variants
	15		Spheroplasts
	20		Optimum number of spheroplasts
	10	44	Spheroplasts
	15		Spheroplasts
	20		Optimum number of spheroplasts†
0·66M sucrose	10, 15 and 20	4	No spheroplasts induced
	10, 15 and 20	20	Spheroplasts poorly developed
	10	37	Optimum number of spheroplasts
	15		Few spheroplasts
	20		No spheroplasts observed
	10	44	Few spheroplasts at 5 hr
	15		(Small, numerous spheroplasts
	20		after 24 hr)

* No spheroplasts formed in the absence of glycine.
† Variety of spheroplasts forms, including "crescents," observed.

antibacterial, and hence the spheroplasting, activity of glycine would be enhanced if tested against young, logarithmic phase bacteria as opposed to non-growing or stationary phase cells.

TABLE 2. EFFECT OF GLYCINE ON WASHED SUSPENSIONS OF *E. coli* AT pH 7·3

Glycine conc. (mg/ml)	Viable count/ml at 0 hr	Viable count/ml after 5 hr at 37°
0	$1·4 \times 10^8$	$1·4 \times 10^8$
10	$1·4 \times 10^8$	$1·5 \times 10^8$
20	$1·4 \times 10^8$	$1·5 \times 10^8$

A 10 ml overnight 37° broth culture of *E. coli* was centrifuged, and the residue washed twice with 2 ml of sterile water, and finally resuspended in 1 ml of broth. This suspension was added to 19 ml of broth in a mixing tube and the mixture then transferred to a Spekker cell which was incubated at 37°. Readings against a control nutrient broth (bacteria absent) were made at intervals, and when it was observed that the organism had entered the logarithmic phase of growth, 1 ml samples were removed

TABLE 3. EFFECT OF GLYCINE ON *E. coli* AT 4°

Glycine conc. (mg/ml)	Viable count/ml at 0 hr	Viable count/ml after 5 hr
0	$4·6 \times 10^6$	$4·6 \times 10^6$
10	$4·6 \times 10^6$	$3·9 \times 10^6$
15	$4·6 \times 10^6$	$3·7 \times 10^6$
20	$4·6 \times 10^6$	$4·8 \times 10^6$

and added to 0·33M sucrose - Mg^{2+} - broth containing final concentrations of glycine of 0, 10, 15 and 20 mg/ml. The final volume in each case was 20 ml. Incubation was carried out at 37°, and extinction measurements made at intervals (Fig. 3).

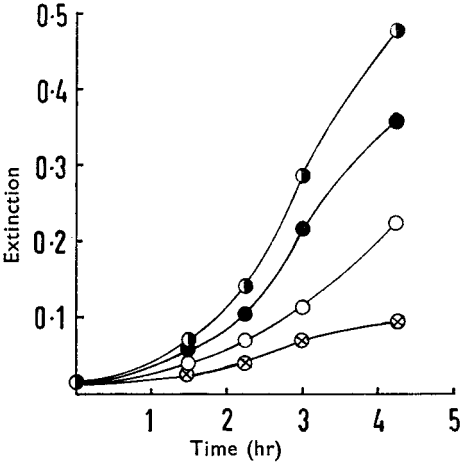

FIG. 3. Effect of glycine on logarithmic phase *E. coli* in nutrient broth containing 0·33 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. Key: as in Fig. 1.

Microscopical examination revealed that spheroplasts induced by this method were larger (7·5–9·5 μ) than those induced in the earlier experiments (4–4·5 μ average).

Discussion

The finding that glycine induces spheroplast formation (McQuillen, 1960) has been amply confirmed in the present investigation. The rate at which multiplication is taking place at the time of treatment with glycine is important, since optimum conversion of rods to spheroplasts occurred when the amino-acid was added to logarithmic phase cells (Fig. 3, Table 1). Further, since the spheroplasts had an average diameter much greater than that of those induced from stationary phase cultures, it can be inferred that the spheroplasts were probably produced at an earlier period in the former instance. Additional evidence that glycine is most active against rapidly-growing bacteria is obtained from the data obtained on spheroplast formation at different temperatures (results described briefly in the text; also Figs 1 and 2, and Table 1). Spheroplasts are best induced at the optimum temperature for growth (Table 1).

Glycine has no effect on non-growing bacteria, since (a) it does not kill *E. coli* at 4° (Table 3), and does not induce spheroplasts at this temperature, and (b) the highest concentration of glycine used, 20 mg/ml, has no effect on the viability of washed suspensions during treatment of the bacteria for 5 hr at 37° (Table 2). This latter finding is in contrast to the results obtained by Gordon and colleagues (Gordon & McLeod, 1926;

Gordon & Gordon, 1947; Gordon & others (1951) and by Maculla & Cowles (1948). Both groups of investigators measured the amount of protein released into the supernatant fluid after the glycine-treated cells had been removed by centrifugation. The period of contact of the amino-acid and bacteria was of longer duration (ca. 16 hr at 37°) and the glycine concentrations were higher, than employed in the present work.

After formation, spheroplasts continue to increase in size, with a consequent increased opacity (Hugo & Russell, 1960). However, although a change in opacity in hypertonic medium is a rapid and convenient method of following spheroplast induction, it should always be accompanied by microscopical examination, since an increased extinction could also be caused as a result of (i) ordinary bacterial multiplication or (ii) the induction of aberrant forms of the type described by Hugo & Russell (1961) in a study of the effect of penicillin on *Aerobacter cloacae*. Spheroplasts were stable in media containing either 0·33M or 0·66M sucrose. No spherical forms were observed in ordinary nutrient broth containing glycine.

Osmotically stable rod forms, which, after removal of spheroplasts by dilution into water, gave rise to colony formation when plated into nutrient agar (John & Russell, 1963) were frequently encountered.

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