Biochem. J. (1962) **83**, 430

The Assimilation of Amino Acids by Bacteria

27. THE RELATIONSHIP BETWEEN GLYCEROL AND 'INCORPORATION FACTOR'*

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(*Received* 6 *September* 1961)

Studies on the incorporation of amino acids, purines and pyrimidines into the trichloroacetic acid-insoluble fraction of disrupted *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) cells have shown that these incorporation processes depend on the presence of a trace component or components of nucleic acid preparations, called the 'incorporation factor(s)' (Gale & Folkes, 1958*a*, *b*). A method for the purification of incorporation factor from bacterial nucleic acid preparations has been described by Gale & Folkes (1958*a*) but the amounts obtained until recently were too small for identification. The active material was unstable on keeping, destroyed by acid hydrolysis, resistant to treatment with periodate and showed no significant absorption in the ultraviolet at 260 mμ (Gale, 1959). In 1959 an incorporation-factor preparation was worked up from 1 kg. of dried *Bacillus megaterium* and a few milligrams of the product were investigated, after storage for 5 months, by Kuehl, Demain & Rickes (1960), who found that the preparation consisted mainly of glycerol. Demain, Newkirk, Barnes & Hendlin (1962) showed that the incorporation-factor preparation stimulated the incorporation of glycine by suspensions of *B. megaterium* that had been 'starved' by preincubation in buffer for 1 hr. at 37°, and that a similar stimulation could be obtained by addition of a number of substances of which glycerol was the most effective. It is improbable that glycerol itself is the incorporation factor as they possess different stabilities and other properties. Further, as shown in this paper, glycerol does not replace incorporation factor in the disrupted staphylococcal system. In the present investigation an intact-cell system has been obtained, by modification of the method of Demain *et al.* (1962), which distinguishes between the incorporation-factor preparation and glycerol and provides evidence that incorporation factor may be a derivative of glycerol. The intact-cell system has proved less specific than the disrupted staphylococcal preparation; no substance active in the latter has proved inactive in the former, but the reverse situation does not hold true.

* Part 26: Gale & Folkes (1958*b*).

METHODS

Organism. A variety of organisms was surveyed for ability to show an effect of incorporation factor similar to that obtained by Demain *et al.* (1962). Best results were obtained with a laboratory strain of a coliform organism, *Escherichia coli* KI, originally isolated as a contaminant of other cultures. This organism was used for all the investigations described in this paper other than those for which a preparation of disrupted staphylococcal cells was used, as previously described (Gale & Folkes, 1955, 1958*a*, *b*).

Growth medium. The organism was grown in a liquid medium containing salts, glucose, arginine and Marmite (Gale, 1951) dispensed in 150 ml. quantities in Roux bottles which were incubated at 25° on their sides.

Preparation of suspensions. After growth under conditions described below, the organisms were centrifuged down, washed once with water, and suspended in water. The suspension density was determined turbidimetrically on a Hilger spectrophotometer previously calibrated in terms of dry weight of suspension against turbidity. Suspensions were used on the day of preparation.

Determination of incorporation of radioactivity from [^{14}C]*glycine.* Cells were suspended at a final density of 0·1 mg. dry wt./ml. in 3·0 ml. of incubation mixture containing 1·5 ml. of buffered saline, pH 6·5 (22 mM-KH_2PO_4, 70 mM-Na_2HPO_4, 8·5 mM-$MgSO_4$, 50 mM-NaCl), 0·2 μc (54 μm-moles) of [^{14}C]glycine (generally labelled), test solution and water to 3·0 ml. [^{14}C]Glycine (The Radiochemical Centre, Amersham, Bucks.) was used at a specific activity of 3·7 μc/μmole. Incubation was carried out in 15 ml. centrifuge tubes standing in a water bath at 37° and was stopped at the required time by addition of 3·0 ml. of 10% (w/v) trichloroacetic acid. The tubes were cooled, and the precipitate was centrifuged down, washed once in 3 ml. of 5% (w/v) trichloroacetic acid, once in 5% (v/v) acetic acid, and then once in 1% acetic acid. The pellet was then suspended in about 0·3 ml. of aq. 20% (v/v) acetone and transferred to a planchet, surface area 3 cm.^{2}, where it was distributed over a disk of lens tissue. Vinyl alcohol (1 drop, aq. 1%) was added to fix the tissue and the planchet then dried under a lamp before counting in a conventional end-window Geiger–Müller scaler equipment (1 μm-mole of glycine gave 703 counts/min.).

Distribution of radioactivity in cell fractions. The distribution of radioactivity from [^{14}C]glycine was investigated according to the procedure described by Roberts, Abelson, Cowie, Bolton & Britten (1955). Their method was used to separate the cell pellet into lipid, ethanol-soluble protein, hot 5% trichloroacetic acid-soluble and insoluble fractions. The protein-containing fractions were then hydro-

lysed for 20 hr. at 105° with 6N-HCl in a sealed tube, the excess of HCl was removed *in vacuo*, and the hydrolysate spotted on Whatman no. 1 paper for two-dimensional chromatography. The chromatogram was developed in one direction with butan-2-ol–formic acid–water (7:1:2, by vol.) and in the second direction with phenol–ammonia (sp.gr. 0·88)–water (800:3:200). After drying, the chromatogram was exposed for 10 days to Ilford Industrial X-ray film and the distribution of radioactivity determined by inspection of the radioautograph. Quantitative assessment of the distribution was obtained by cutting out radioactive areas from the chromatogram, eluting material from these areas with water, and counting the dried eluates in the usual manner.

Preincubation of suspensions. Addition of 1 unit of incorporation factor produced a variable stimulation of incorporation of glycine. As found by Demain *et al.* (1962), this stimulation could be increased by preincubation of the cells. Table 1 lists some of the variables that were tested in an attempt to obtain maximal stimulation. At first, cells were suspended in buffered saline (diluted with 2 vol. of water) at a final suspension density of 2·0 mg./ml. Incorporation into untreated cells was increased by 37% in the presence of incorporation factor, and this stimulation was approximately doubled by preincubation in the salt medium for 1 hr. at 37°; when the cells were centrifuged out of the preincubation medium and resuspended before the test, the degree of stimulation was increased. Incubation in the salt medium was more effective than incubation in water for the same time. Reduction of the suspension density during preincubation increased the subsequent effect of incorporation factor, and the best results were obtained by preincubation for 30 min. in salt medium with the cells at a density of 0·6 mg. dry wt./ml. It was thought that the concentration of phosphate or Mg^{2+} ions during preincubation might affect the subsequent effect of incorporation factor, but Table 1 shows that buffered saline is as effective for preincubation purposes as any of the phosphate–magnesium mixtures tested; consequently the buffered saline was used for the remainder of the experiments.

The greater effect of incorporation factor after preincubation procedures is due not to enhanced incorporation but to decreased incorporation in the absence of incorporation factor. The degree of stimulation attained in the presence of incorporation factor, after preincubation, was found to vary widely from culture to culture, and this was traced to an effect of 'age of culture' (Fig. 1). Cells were harvested at different times during their growth at 30°; each batch was made into a washed suspension, preincubated for 30 min. in buffered saline at a suspension density of 0·6 mg. dry wt. of cells/ml., centrifuged down, and resuspended at 0·1 mg. dry wt./ml. for determination of incorporation in the presence and absence of incorporation factor. Fig. 1 shows that the stimulation obtained is greatest for cells harvested in the early stages of growth,

Table 1. *Effect of preincubation conditions on the subsequent stimulation of glycine incorporation by incorporation factor*

Escherichia coli KI cells were harvested during early exponential growth; they were washed with water and preincubated under conditions shown below, then either added directly (Spin −) or centrifuged down and resuspended (Spin +) at a final density of 0·1 mg. dry weight of cells/ml. in buffered saline containing [^{14}C]glycine with and without incorporation factor; they were incubated for 60 min. at 37° and reaction was stopped with trichloroacetic acid. Incorporation of radioactivity was determined on the washed precipitate as described in the text.

Preincubation conditions				Radioactivity incorporated (counts/min./0·3 mg. dry wt.)		Increased radioactivity due to factor (%)
Medium	Suspension density (mg./ml.)	Time (min.)	Spin	Factor absent	Factor present	
Buffered saline	2·0	0	−	3507	4797	37
	2·0	30	−	3040	5231	72
	2·0	60	−	2574	4612	79
	2·0	60	+	2565	5183	100
	0·6	60	+	3437	8515	148
	2·0	60	+	4136	7751	88
	4·0	60	+	5318	7565	43
Water	2·0	60	+	4673	7019	53
Buffered saline	0·6	0	+	3507	4001	13
	0·6	15	+	1511	3680	143
	0·6	30	+	1193	4087	243
	0·6	60	+	1179	4020	240
Phosphate (0·1 M)	0·6	60	+	928	4828	420
(0·05 M)	0·6	60	+	730	3493	380
(0·01 M)	0·6	60	+	732	5143	600
Phosphate (0·05 M) + Mg^{2+} (10 mM)	0·6	60	+	805	4772	495
(3 mM)	0·6	60	+	843	4971	390
(1 mM)	0·6	60	+	812	5683	600
Buffered saline	0·6	60	+	848	5646	570
Tris (0·05 M)	0·6	60	+	737	4461	505

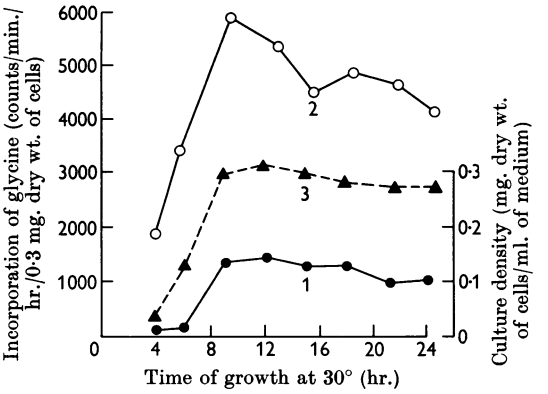

Fig. 1. Effect of 'age of culture' on the stimulation by incorporation factor of glycine incorporation by preincubated *Escherichia coli* cells. *E. coli* KI cells were grown at 30° and harvested in batches at the times indicated. The cells were preincubated in buffered saline, suspension density 0·6 mg. dry weight/ml., for 30 min. at 37°, then centrifuged down and resuspended at a density of 0·1 mg./ml. in buffered saline containing [^{14}C]glycine alone (curve 1) and in the presence of 1 unit of incorporation factor/3·0 ml. (curve 2). After incubation at 37° for 1 hr., the radioactivity was determined on the trichloroacetic acid-precipitable fraction. Curve 3 shows the changes in culture density occurring during the growth period.

falling from 1300% for '6 hr. cells' to 245% for '24 hr. cells'. All further experiments were therefore carried out with cells harvested as early as feasible and at a culture density of about 0·1 mg. dry wt. of cells/ml. This corresponded to growth for about 6 hr. at 30° or 9 hr. at 25°, neither time being particularly convenient for carrying out the tests on the same day as harvesting. The following procedure was eventually adopted and has worked successfully throughout the investigation.

E. coli KI was maintained by daily subculture in 1% peptone broth incubated at 30°. Roux bottles containing the growth medium were refrigerated for at least 24 hr., removed at about 11 p.m. and each bottle was inoculated with 3 drops of 24 hr. peptone culture; bottles were then placed on their sides in an air incubator at 25° and organisms harvested at 9 a.m. the next morning. This procedure regularly provided cells in which the incorporation, measured as above, of glycine was stimulated 10- to 13-fold by the presence of incorporation factor.

A number of experiments were carried out to determine whether the stimulation produced by incorporation factor could be made even greater by modifications during the preincubation stage. The effect of addition to the preincubation medium of glucose, amino acids, ammonium chloride, purines, pyrimidines and nucleotides was tested. Stimulation was increased by addition of glucose and amino acids to the preincubation medium, but the effect was variable and not greater than 130% of that obtained in buffered salts alone. Consequently the buffered salts solution was chosen as the routine medium for preincubation.

Final procedure adopted for preparation of cells. Cells were grown as described above and harvested in the early exponential phase of growth. After harvesting, the washed cells were preincubated in buffered saline at a suspension density of 0·6 mg. dry wt./ml. for 30 min. at 37°; the cells were then centrifuged down, resuspended in buffered saline and added to the test system to give a final suspension density of 0·1 mg. dry wt. of cells/ml.

Incorporation-factor preparation. Incorporation factor was prepared from dried *B. megaterium* cells as described by Gale & Folkes (1958*a*), and used at stage 6 of the published procedure (paper chromatography with ether–ethanol–ammonia). The preparation at this stage has a faint yellow colour which can be removed by passing the aqueous solution through a column of ECTEOLA cellulose; the active material is not adsorbed, and this procedure does not alter the activity or nature of the response to incorporation factor described in the intact-cell system below.

Incorporation-factor preparations are standardized by their activity in promoting incorporation of amino acid (e.g. glycine or glutamic acid) into the trichloroacetic acid precipitate obtained from the nucleic acid-depleted, disrupted staphylococcal-cell preparation as previously described (Gale & Folkes, 1958*a*); 1 unit of incorporation factor is that amount of preparation which, in a volume of 3·0 ml., promotes the incorporation of glycine (or glutamic acid) to the same extent as excess of staphylococcal nucleic acid. Preparations vary in their degree of purity and 1 unit of incorporation factor corresponds in different preparations to 1–4 μg. dry wt.

Glycerol. Standard solutions were prepared from glycerol dried to constant weight in a tared weighing bottle over sulphuric acid *in vacuo*.

Preparation of 'formin'. The general directions of Koehler (1913) and Delaby & Dubois (1928) were followed. Glycerol (46 g.) and formic acid (26 g.) were heated in a stoppered flask at 80° for 2 hr. and the reaction mixture was then distilled at low pressure. Material distilling below 140° (/6 mm. Hg) was discarded, and three fractions were then collected by distillation at: (*a*) 145–153°/7 mm.; (*b*) 145–155°/10 mm.; (*c*) 155–160°/10 mm. Saponification of fraction (*a*) for 1 hr. at 100° in 0·1 N-NaOH, followed by back-titration of excess of alkali, showed that the fraction contained approximately equimolar amounts of glycerol and formic acid. Similar treatment of fraction (*c*) gave a composition of approximately 1·8 moles of formic acid/mole of glycerol. Gas chromatography of fraction (*a*) showed that it contained at least six components; we are indebted to Dr R. S. Airs of the Thornton Research Centre for providing samples of the six components after separation on a preparative gas chromatogram.

Other materials. Methylidene glycerols were prepared according to the method of Hibbert & Carter (1928).

Triacetin was prepared according to the method of Perkin & Simonsen (1905) and 1-monoacetin by the method of Schuette & Hale (1930).

β-Hydroxypyruvic aldehyde was prepared by the method of Evans (1938).

Reductone was prepared by the method of Euler & Martius (1933).

Pyruvic aldehyde was prepared by distillation of commercial 'Methyl glyoxal' and collection of the fraction boiling at 25–26°/6 mm. in a receiver cooled in acetone–solid CO_2.

We are indebted to Dr E. Webb for samples of tributyrin, triolein and 1-monobutyrin, and to Dr Letters for

samples of glycidol (2,3-epoxypropan-1-ol) and glycerol 1,2-phosphate. Other substances mentioned below were commercial preparations of AnalaR grade.

Periodate treatment. For the experiments described below on the treatment of glycerol with periodate, glycerol was dissolved in 0·05 M-ammonium acetate buffer, pH 5·5, and appropriate amounts of sodium periodate solution were added. The mixture was incubated at 37° for 60 min. and then evaporated to dryness *in vacuo*. The dry material was then taken up in a few drops of 0·05 M-ammonium acetate, pH 5·5, and run in a paper-electrophoresis apparatus for 1 hr. at 10 v/cm. The paper was then dried and the material remaining at the origin eluted with water. We are indebted to Dr R. Markham, F.R.S., for advice on the use of this method.

RESULTS

Incorporation of glycine carbon by Escherichia coli *KI after preincubation in buffered saline*

Effect of incorporation-factor preparation. Fig. 2 shows the effect of incorporation factor on the incorporation of radioactivity into trichloroacetic acid-insoluble material by *E. coli* KI after preincubation as described above. Incorporation in the presence of incorporation factor followed a linear time course, and the degree of stimulation of incorporation (over that of the control without incorporation factor) was proportional to the amount of incorporation factor added up to a concentration of 1 unit/3·0 ml. Higher concentrations of incorporation factor did not give significantly higher rates of incorporation.

The value of the control has been deducted from experimental rates of incorporation in subsequent Figures.

Effect of glycerol. Fig. 3 shows the effect of glycerol in the same system; glycerol had little initial activity compared with incorporation factor but stimulation increased during incubation and a concentration of 0·1 mM-glycerol gave rise, after incubation for 60 min., to stimulation equal to that produced by 1 unit of incorporation factor. Increasing the concentration of glycerol did not appreciably decrease the lag period or alter the degree of stimulation eventually attained. Initial concentrations of less than 0·1 mM-glycerol gave rise to smaller degrees of stimulation; the activity obtained, after the lag period, in the presence of an initial concentration of 0·01 mM-glycerol was approximately equivalent to that produced by 0·5 unit of incorporation factor.

The effect of glycerol in Fig. 3 might be due to the formation, during the lag period, of metabolites of glycerol which then stimulate glycine incorporation. Fig. 4 shows the results of an experiment designed to test this hypothesis. Glycerol added at zero time, together with glycine, showed the usual stimulatory effect after a lag period of 60 min. (curve *A*).

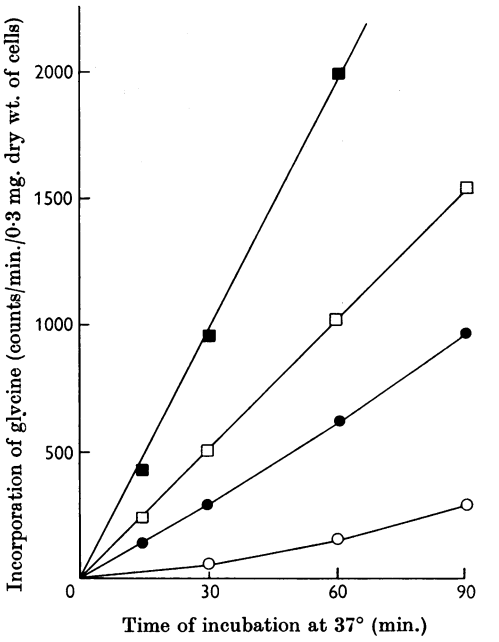

Fig. 2. Effect of incorporation-factor preparation on incorporation of glycine by preincubated *Escherichia coli* cells. *E. coli* KI cells were harvested in the early exponential phase of growth, preincubated in buffered saline, centrifuged down and resuspended in buffered saline and [^{14}C]glycine as for Fig. 1, with the addition of the following amounts of incorporation factor/3·0 ml.: ○, none (control); ●, 0·25 unit; □, 0·5 unit; ■, 1 unit.

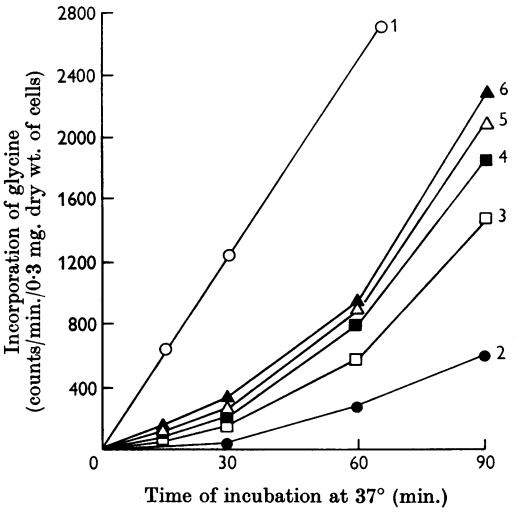

Fig. 3. Effect of glycerol on incorporation of glycine by preincubated *Escherichia coli* cells. Conditions were as for Fig. 2 with the following additions: curve 1, 1 unit of incorporation factor; for other curves, glycerol at concentrations of 0·01 (2), 0·03 (3), 0·1 (4), 0·3 (5), and 1·0 mM (6). Control without addition deducted in all cases.

When the addition of glycerol was delayed until 60 min. after the addition of glycine, then the stimulation still took place only after a lag period (curve *B*). When glycerol was added 60 min. before addition of glycine, incorporation of the latter was immediately stimulated to the same extent as by incorporation factor (curve *C*). This demonstrates that incubation of glycerol with the suspension of organisms for 60 min. yields material that is immediately as effective in stimulating glycine incorporation as the incorporation-factor preparation. Incubation of the organisms in the absence of either glycerol or glycine did not result in the production of stimulatory material within the experimental period.

Effect of glucose. Fig. 5 shows the effect of glucose over the same range of concentration as that of glycerol in Fig. 3. The effect was completely different; glucose gave an initial stimulation of incorporation equivalent to that produced by incorporation factor, but this effect decreased rapidly and stimulation almost ceased after a time proportional to the initial glucose concentration.

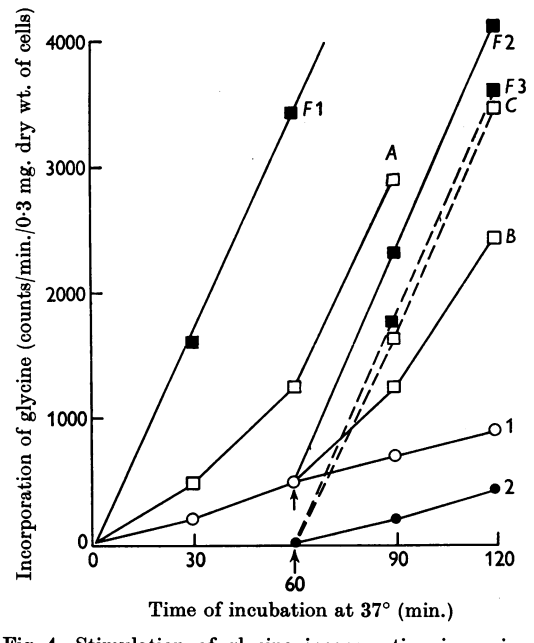

Fig. 4. Stimulation of glycine incorporation in preincubated *Escherichia coli* cells: development of glycerol effect. General conditions were as for Fig. 2. Controls: glycine added at zero time (curve 1) or at 60 min. (curve 2). Curves *F*: 1 unit of incorporation factor/3·0 ml. added at zero time (curve *F*1) or at 60 min. (curves *F*2 and *F*3). Curves *A*, *B*, *C*: 0·1 mM-glycerol added at zero time (curves *A* and *C*) or at 60 min. (curve *B*). In curve *A* glycine and glycerol were added together; in curve *B*, glycine was added 60 min. before glycerol; in curve *C*, glycerol was added 60 min. before glycine.

Breakdown products and other derivatives of glycerol. Fig. 6 shows that dihydroxyacetone and glyceraldehyde were markedly less effective than glycerol and that the stimulation produced in their presence did not significantly increase during the incubation period used. α-Glycerophosphate had an activity about that of glyceraldehyde, but β-glycerophosphate and glycerol 1,2-phosphate were inactive. The following were inactive over the range 1·0–0·01 mM: ethylene glycol, glyoxal, glycidol, glycol aldehyde, glycollic acid, glyoxylic acid, glyceric acid, propylene glycol, erythritol, reductone, hydroxypyruvic acid, hydroxypyruvic aldehyde, tartronic aldehyde, tetronic acid lactone, L-2-hydroxybutyrolactone, lactic acid, oxalic acid, inositol, erythrose, rhamnose, L-fucose.

Pyruvic aldehyde. Fig. 7 shows the effect of pyruvic aldehyde which, at concentrations between 0·01 and 0·1 mM, gave a linear stimulation of glycine incorporation. At a concentration of 0·1 mM pyruvic aldehyde had an effect equivalent to 0·5 unit of incorporation factor, but higher concentrations did not give a proportional increase in stimulatory effect and no concentration has yet been found which is as effective as 1 unit of incorporation factor. The highest stimulation recorded, with 0·2 mM-pyruvic aldehyde, was equal to that of 0·75 unit of incorporation factor; increasing the concentration to 0·3 mM reduced the stimulation to the equivalent of 0·6 unit of incorporation factor. Pyruvic aldehyde had no

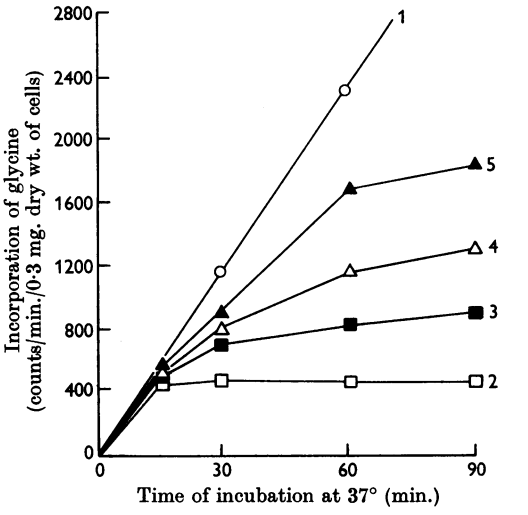

Fig. 5. Effect of glucose on incorporation of glycine by preincubated *Escherichia coli* cells. Conditions were as for Fig. 2 with the following additions: curve 1, 1 unit of incorporation factor/3·0 ml.; for other curves, glucose at concentrations of 0·03 (2), 0·1 (3), 0·3 (4), and 1·0 mM (5). Control without addition deducted in all cases.

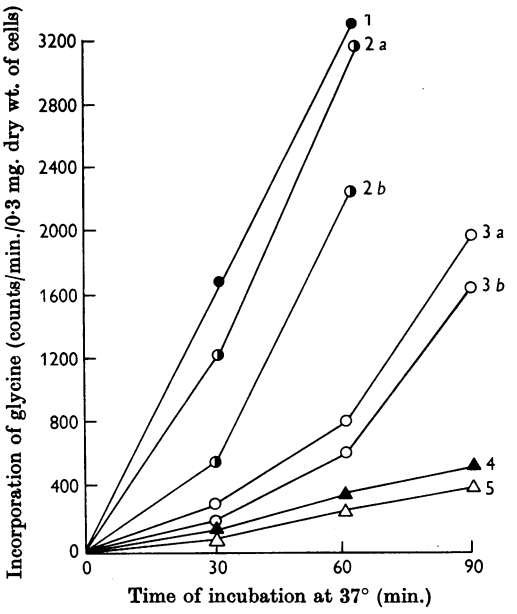

Fig. 6. Effect of glycerol derivatives on incorporation of glycine by preincubated *Escherichia coli* cells. Conditions were as for Fig. 2 with the following additions: curve 1, 1 unit of incorporation factor/3·0 ml.; curves 2, formin at (*a*) 0·3 and (*b*) 0·1 mM; curves 3, glycerol at (*a*) 0·3 and (*b*) 0·1 mM; curve 4, 0·1 mM-dihydroxyacetone; curve 5, 0·1 mM-glyceraldehyde.

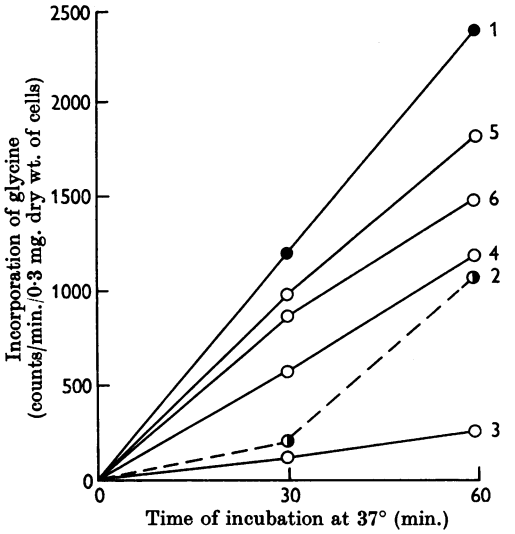

Fig. 7. Effect of pyruvic aldehyde on incorporation of glycine by preincubated *Escherichia coli* cells. Conditions were as for Fig. 2 with the following additions: curve 1, 1 unit of incorporation factor/3·0 ml.; curve 2, 0·1 mM-glycerol; curves 3–6, pyruvic aldehyde at concentrations of 0·01 (3), 0·1 (4), 0·2 (5), and 0·3 mM (6).

effect on incorporation processes in the disrupted staphylococcal-cell preparation (see below) and has not been further investigated.

Effect of periodate treatment of glycerol. The activity of the incorporation-factor preparation is not affected by treatment with periodate (Gale, 1959), whereas glycerol is destroyed by such treatment. Glycerol was treated with periodate and the reaction products separated as described above; material remaining at the origin on electrophoresis was eluted and tested in the glycine incorporation system. Treatment of glycerol with 2 or 3 molar equivalents of sodium periodate led to complete loss of activity; treatment with 0·5 molar equivalent gave material which was approximately three times as active as the equivalent amount of glycerol and which gave a linear stimulation of glycine incorporation. It seemed probable that the response could be due to oxidation products of glycerol (formaldehyde and formic acid) or substances produced by interaction of these products with residual glycerol.

Fig. 8 shows the effect on glycine incorporation of formic acid and formaldehyde; both substances stimulate incorporation, the aldehyde becoming inhibitory at concentrations greater than 0·3 mM. The effect of glycerol and sodium formate (or formaldehyde) added together is greater than the

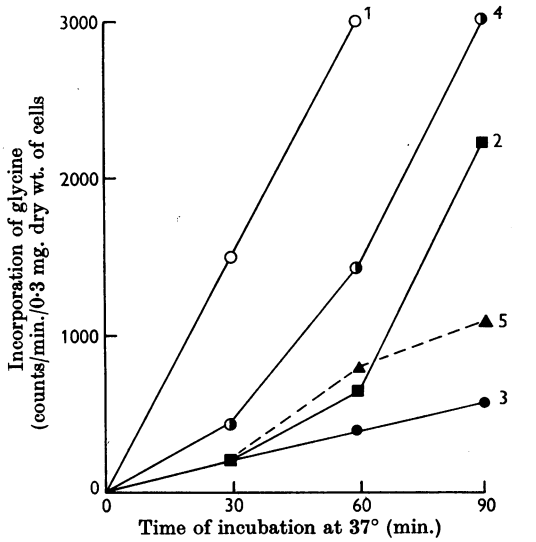

Fig. 8. Incorporation of glycine by preincubated *Escherichia coli* cells in the presence of glycerol, sodium formate and formaldehyde. Conditions were as for Fig. 2 with the following additions: curve 1, 1 unit of incorporation factor/3·0 ml.; curve 2, 0·1 mM-glycerol; curve 3, 0·1 mM-sodium formate; curve 4, 0·1 mM-glycerol + 0·1 mM-sodium formate; curve 5, 0·1 mM-formaldehyde. Control without addition deducted in all cases.

sum of their separate effects. It is improbable that the effect of periodate-treated glycerol was due to simple mixtures of glycerol, formate and formaldehyde, as the electrophoresis procedure, followed by drying of the paper, would be expected to remove both formate and formaldehyde from the preparation. The activity of the periodate-treated material was not reduced by drying *in vacuo*, nor did repetition of the process in the absence of glycerol yield active material. Further, neither formate nor formaldehyde stimulated incorporation in the disrupted staphylococcal-cell preparation, although the periodate-treated glycerol was more active than glycerol itself. Attention was therefore directed to compounds of formic acid or formaldehyde and glycerol.

Effect of 'formin'. Fig. 6 shows the effect on glycine incorporation of formin prepared as described in the Methods section. On the assumption that the composition of formin approximates to the monoformic ester, 0·3 mM-formin stimulated incorporation to an extent approaching that of 1 unit of incorporation factor. Results were somewhat variable: 0·3 mM-formin produced a stimulation which varied between 60 and 90% of that of 1 unit of incorporation factor, and the response was sometimes linear and sometimes displayed a lag period less marked than that obtained with glycerol. As described above, the formin preparation can be resolved by gas chromatography into at least six components. Six fractions have been obtained by quantitative chromatography; two of the fractions had an activity and lag similar to that obtained with glycerol whereas the other four were all more active than glycerol and showed a smaller lag in the onset of stimulation. No one fraction was more effective, on a weight basis, than the parent formin and so the activity of this preparation could not be attributed to one highly active component. Formaldehyde reacts with glycerol to produce methylidene glycerol (Hibbert & Carter, 1928); a mixture of the 1,2- and 1,3-methylidene glycerols was found to have an activity less than that of glycerol.

Other glycerides. To determine whether the activity of formin was a non-specific effect of glycerides in general, the action of 1-monoacetin, triacetin, 1-monobutyrin, tributyrin and triolein was tested. The triglycerides were inactive. Monoacetin and monobutyrin had a small stimulatory effect, about 30% of that of glycerol itself.

Distribution of glycine carbon after incorporation. Cells (18·0 mg. dry wt.) were preincubated and then incubated, under the conditions of Fig. 3, with [^{14}C]glycine in three equal portions: (*a*) alone; (*b*) with incorporation factor; (*c*) in the presence of 0·3 mM-glycerol. Incubation was continued for 90 min., the tubes were then cooled rapidly and cold-trichloroacetic acid was added to give a final concentration of 5% (w/v). After 1 hr. in the cold, the precipitate was centrifuged down, washed once with cold 5% trichloroacetic acid, and the pellet was then fractionated according to the procedure of Roberts *et al.* (1955). The mean results (with ranges in parentheses) from four complete experiments with different cultures were as follows. Of the radioactivity in the pellet from cells (*a*) incubated alone with glycine, 5·6% (4·5–6·3%) was found in the lipid fraction, 14·1% (13·0–15·5%) in the hot-trichloroacetic acid-soluble fraction ('nucleic acid'), and 80% (78–81%) in the protein-and-wall fractions of which 37·1% (25–48%) was ethanol-soluble and the remainder was precipitated in the hot-trichloroacetic acid-insoluble fraction. The presence of incorporation factor or glycerol during incubation had little effect on the proportion of the radioactivity in the protein–wall fraction which was reduced from 80 to 73% (70–75%); there was, however, a significant reduction in the proportion in the 'nucleic acid' fraction and an increase in that in the lipid fraction. Thus incorporation factor reduced the proportion in the 'nucleic acid' fraction from 14·1 to 9·2% (8·3–11·0%) and increased that in the lipid fraction from 5·6 to 16·2% (14·4–19·0%). The corresponding values for cells incubated in the presence of glycerol were: 'nucleic acid' 10·5% (9·1–12·5%); lipid 14·7% (12·0–16·2%). The presence of glycerol therefore has the same general effect as incorporation factor on the distribution of glycine carbon.

The protein-containing fractions were hydrolysed and the distribution of radioactivity among the amino acids was examined by radioautography as described in the Methods section. For the three conditions of incubation, the greater part of the radioactivity (*a*, 65; *b*, 70; *c*, 76%) was associated with the glycine–serine area of the chromatogram, the remainder being distributed over areas corresponding to at least 10 other amino acids. The nature of the labelled material in the lipid and 'nucleic acid' fractions will be dealt with in a later paper. The overall incorporation was 75% inhibited by the addition of 30 μg. of chloramphenicol/ml. to the incubation medium whether the cells were incubated alone or with incorporation factor or glycerol.

Stimulation of amino acid incorporation in the disrupted staphylococcal-cell preparation

Since the incorporation factor was originally discovered during studies with the disrupted staphylococcal-cell preparation and is normally assayed with that preparation (Gale & Folkes, 1958*a*), it is of interest to see what effect glycerol, and some of the other substances described above, have in the staphylococcal assay system.

Fig. 9 shows the action of glycerol and formin on the incorporation of glutamic acid (condition 2, Gale & Folkes, 1958*a*) into the trichloroacetic acid-precipitable fraction of nucleic acid-depleted disrupted staphylococcal cells. Glycerol was markedly less effective than incorporation-factor preparation; the optimum concentration of glycerol is 0·1 mM, higher concentrations being less effective. As in the experiments with *E. coli* KI, described above, the stimulation in the presence of glycerol increased with time, but the degree of stimulation obtained after 60–90 min. varied from preparation to preparation and only rarely reached that given by incorporation factor. Formin was again some three times as effective as glycerol, and could replace incorporation factor although only at a much higher concentration than that of incorporation-factor preparation. Results with the fractions separated from formin were again variable, but were in general agreement with those obtained with *E. coli* KI in that three of the fractions were more effective than glycerol, but no one component was more effective on a weight basis than the parent formin mixture.

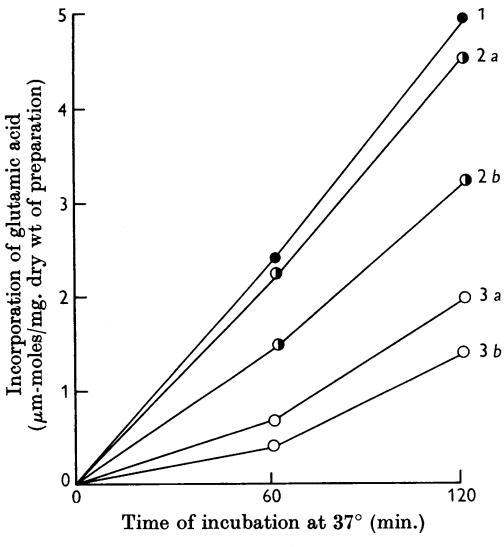

Fig. 9. Effect of incorporation factor, glycerol and formin on the incorporation of glutamic acid under conditions of protein synthesis by disrupted staphylococcal cells. The nucleic acid-depleted disrupted staphylococcal-cell preparation was incubated in the presence of ATP, hexose diphosphate, 18 amino acids, purines, pyrimidines, ribose and [^{14}C]glutamic acid (condition 2 of Gale & Folkes, 1955, 1958*a*, *b*) in the presence of: curve 1, 1 unit of incorporation factor/3·0 ml.; curves 2, formin at concentrations of 0·1 (*a*) and 0·03 mM (*b*); curves 3, glycerol at concentrations of 0·1 (*a*) and 0·03 mM (*b*). Radioactivity was determined on the trichloroacetic acid-precipitable fraction. Values for control without addition were deducted in all cases.

Pyruvic aldehyde, sodium formate and formaldehyde were inactive in the staphylococcal system. α-Glycerophosphate, β-glycerophosphate and glycerol 1,2-phosphate had an activity 30–40% of that of glycerol; dihydroxyacetone, glyceraldehyde and their phosphates are inactive.

DISCUSSION

The responses of glycine incorporation in preincubated *E. coli* KI cell preparations to the presence of incorporation factor and glycerol indicate clearly that incorporation factor is not glycerol. Since investigation of an incorporation-factor preparation by Kuehl *et al.* (1960) showed that the main component of that preparation was glycerol, the question now arises whether incorporation factor itself is a minor component of that preparation or whether glycerol is a breakdown product of incorporation factor. The latter seems probable since incorporation factor is known to be unstable and the responses to glycerol shown in Fig. 4 suggest that glycerol, during metabolism, can give rise to material which has the same effect as incorporation factor. Demain *et al.* (1962) find that glycerol will replace incorporation factor in their *B. megaterium* system but they did not attempt the type of modification described here which gives differing responses to glycerol and incorporation factor. The possibility also arises that glycerol acts as a source of energy in cells 'starved' by preincubation procedures, but comparison of the responses to incorporation factor, glycerol and glucose in Figs. 2, 3 and 5 indicates that this is not the only function of either incorporation factor or glycerol in promoting glycine incorporation under the experimental conditions used. In the disrupted staphylococcal-cell preparation, ATP and hexose diphosphate are supplied as energy source (Gale & Folkes, 1955).

If we accept, as a reasonable working hypothesis, the suggestion that glycerol is metabolized to give incorporation factor, then we must next look for the type of derivative involved. The evidence obtained above shows that breakdown products of glycerol are not involved. Since the activity of incorporation factor is not destroyed by treatment with periodate, the 2-position, at least, of glycerol must be substituted. Investigation of the effect of treating glycerol with less than equimolar amounts of periodate showed that formin is more effective than glycerol as a replacement for incorporation factor. It is improbable that a formic ester of glycerol is the actual incorporation factor since three or four fractions of formin proved equally effective, and about 100 μg. was required to give an effect equal to that obtained with 1–4 μg. of incorporation factor preparation. It may be

that formic esters of glycerol are metabolized to incorporation factor-like material more readily than glycerol itself; this could be due to substitution of other residues for the formyl groups, or to protection of glycerol from metabolic breakdown.

There is no point in attempting, at present, to interpret in detail what is occurring during the stimulation of incorporation of glycine carbon by incorporation factor or glycerol. The next stage is to follow the metabolism of glycerol under the experimental conditions described here and in previous work (Gale & Folkes, 1958*a*, *b*). We are now studying the incorporation of radioactivity from DL-[1-^{14}C]glycerol into the 'nucleic acid' fraction (hot-ethanol insoluble, cold-trichloroacetic acid insoluble, hot-trichloroacetic acid soluble) of both *E. coli* KI and disrupted staphylococcal cells, and evidence has been obtained for the presence in this 'nucleic acid' fraction of labelled components which readily break down to yield free glycerol.

SUMMARY

1. The incorporation of glycine carbon into the trichloroacetic acid-precipitable fraction of *Escherichia coli* KI, harvested in the early exponential stage of growth and preincubated for 30 min. in buffered saline, is stimulated 10- to 13-fold by 'incorporation factor' preparation. The response is linear with time for at least 2 hr.

2. Glycerol has little or no initial effect under these conditions, but stimulation develops during incubation and, after a lag period of about 60 min., an initial concentration of 0·1 mM-glycerol gives a stimulation equivalent to that obtained with incorporation factor. Higher concentrations of glycerol are not more effective.

3. When the washed cells are incubated with glycerol for 60 min. before the addition of glycine, incorporation of the latter is stimulated immediately on addition and the response is linear.

4. Glucose gives an immediate stimulation of glycine incorporation but the effect decreases rapidly with time.

5. No breakdown product of glycerol has been found which has significant stimulatory action. A 'formin' preparation containing formic acid esters of glycerol is approximately three times as active as glycerol itself. Other glycerides are inactive.

6. Glycerol is markedly less effective than incorporation-factor preparation in promoting glutamic acid incorporation, under conditions of protein synthesis, in disrupted staphylococcal cells. Formin is again more effective than glycerol and, at a relatively high concentration, can replace incorporation-factor preparation.

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Inhibition of Glycolysis in Rat Skeletal Muscle by Malonate

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(*Received* 15 *August* 1961)

Fawaz, Tutunji & Fawaz (1958) studied the effect of sodium malonate on the isolated dog heart (heart–lung preparation). Although 0·03 M-malonate almost completely inhibited the Krebs cycle in pigeon-breast or sheep-heart mince, it had no deleterious effect on the performance of the heart; however, a substantial amount of succinate accumulated in the cardiac tissue. When the concentration of malonate was raised to 0·06 M, rapid failure ensued, accompanied by a decreased heart rate and