The Assimilation of Amino Acids by Bacteria

18. THE INCORPORATION OF GLUTAMIC ACID INTO THE PROTEIN FRACTION OF *STAPHYLOCOCCUS AUREUS*

BY E. F. GALE AND JOAN P. FOLKES

*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry,
University of Cambridge*

(Received 4 May 1953)

Melchior, Mellody & Klotz (1948) and Melchior, Klioze & Klotz (1951) have shown that incubation of washed cells of *Escherichia coli* with ^{35}S -labelled methionine results in an incorporation of the isotope into the cell protein. This incorporation was abolished by heating the cells to 100° , was stimulated by the addition of glucose and ammonium ions, and was inhibited by sodium azide and 2:4-dinitrophenol. A number of workers have investigated the incorporation of labelled amino acids into the proteins of tissue slices, 'homogenates', bone marrow or reticulocytes (Melchior & Tarver, 1947; Zamecnik & Frantz, 1950; Winnick (1950); Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950, 1952; Kit & Greenberg, 1952; Siekevitz, 1952), and the question has arisen whether such incorporation involves protein synthesis, although net synthesis of protein has not been demonstrated and is improbable under many of the conditions used. The incorporation might be due to adsorption of labelled residues on to protein, but prolonged washing or dialysis has failed to remove the activity, and Borsook *et al.* (1952) have shown that labelled amino acids incorporated into the proteins of bone marrow or reticulocytes can be found in a variety of peptides obtained by partial hydrolysis of the proteins. Again, if carboxyl-labelled amino acids are used, treatment of the protein, after incorporation, with ninhydrin fails to remove the isotope, suggesting that the carboxyl group of the incorporated amino acid is no longer in the free state (Borsook *et al.* 1952). Holloway & Ripley (1952) have shown that the nucleic acid content of reticulocytes can be altered by injection of phenylhydrazine into rabbits some time before bleeding and that the incorporation of radioactive leucine into the protein of such reticulocytes can be correlated with their ribonucleic acid content. Doubt has been thrown on the biological significance of such studies by Brunish & Luck (1952), who have obtained incorporation of labelled amino acids into preparations of deoxyribonucleohistone and histone, and find that the rate of incorporation is increased by raising the temperature to 100° .

If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated with glucose and glutamic acid, free glutamic acid becomes concentrated within the cells, but there is no increase of cellular protein (Gale, 1947*a, b*). If the cells are incubated with glucose and a mixture of amino acids, which must include all those essential for growth, then the accumulation of free glutamic acid within the cells ceases, protein synthesis takes place and glutamic acid from inside and outside the cell is incorporated quantitatively into the cell protein (Gale, 1951; Gale & Folkes, 1953*a*). The rate of protein synthesis can be correlated with the nucleic acid content of the cells (Gale & Folkes, 1953*a*), and is increased by the addition of purines and pyrimidines to the incubation mixture. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis at bactericidal concentrations; bacitracin inhibits both protein and nucleic acid synthesis but at concentrations approximately 100 times that required to prevent growth, and penicillin at high concentrations prevents the stimulation of protein synthesis which is normally produced by the addition of purines and pyrimidines, while having little or no effect on the rate of protein synthesis occurring in the absence of such addition (Gale & Folkes, 1953*b*). It seemed that these experimental systems provided a means for the investigation of incorporation of labelled amino acids, and its relation to protein synthesis in *Staph. aureus* and the present paper presents results obtained with [^{14}C]glutamic acid.

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan, which has been used for previous studies in this series (Gale & Folkes, 1953*a, b*).

Growth and preparation of suspensions. The organism was grown for 15 hr. at 30° in the 'deficient' medium consisting of a salt mixture, glucose, marmite and arginine (Gale, 1951), dispensed in Roux bottles. The cells were harvested on the centrifuge, washed once with water and made into washed suspension in distilled water; suspension density = 25.0 mg. dry weight of cells/ml. Suspension densities were deter-

mined on a Hilger absorptiometer previously calibrated against the organism used.

Incubation mixtures. Solutions of amino acids and the mixture A thereof were prepared as previously described (Gale & Folkes, 1953a). The radioactive glutamic acid used for the majority of the experiments was synthetic DL-[α -carboxy- ^{14}C]glutamic acid of activity 0.9 mc/m-mole and was obtained from the Radiochemical Centre, Amersham. Some experiments, indicated in the text, were carried out with randomly labelled glutamic acid and other amino acids obtained biosynthetically. In general the radioactive amino acids were dissolved in buffered salt solution (Gale, 1947a) to a known strength or activity. Incubation mixtures were put up in 15 ml. centrifuge tubes; the following amounts of the various components were used and the final volume adjusted with water to 3.0 ml. in all cases: 0.2 ml. 20% (w/v) glucose, 0.3 ml. amino acid mixture A, 0.2 ml. washed suspension of *Staph. aureus* as above, 2.0 ml. buffered saline solution containing a known amount of radioactive amino acid, 0.2 ml. inhibitor solution; where any components were omitted an equivalent volume of water was substituted. The tubes were closed with rubber bungs and incubated in a water bath at 37°.

Preparation of protein fraction of cells for ^{14}C estimation. At the end of the incubation period, the reaction was stopped by the addition of 1.0 ml. 0.2M acetate buffer pH 4.5 to each tube, simultaneously immersing the tube in an ice bath. The tubes were then centrifuged at 1000 g for 10 min. and supernatant solutions withdrawn with a hypodermic syringe. The organisms were resuspended in approximately 5 ml. distilled water and again centrifuged; this washing procedure was repeated three times in all. After the third washing, the organisms were suspended in 2.0 ml. 5% (w/v) trichloroacetic acid (TCA) and placed in the ice chest for 2 hr. The precipitates were then centrifuged down at 1000 g for 5 min., washed once in 5% TCA, once in 5% acetic acid and once in 1% acetic acid. Each precipitate was suspended in 0.75 ml. 20% (v/v) aqueous acetone and 0.3 ml. samples, in duplicate, placed on polythene disks of surface area 2 sq.cm. One drop of 1% (w/v) polyvinyl alcohol was added to each sample and the preparations dried *in vacuo*. In this way a film of protein corresponding to 1.0 mg. dry weight of organism/sq.cm. was obtained on each disk.

Precipitates prepared in this way contain nucleic acid as well as protein. When any doubt existed concerning possible incorporation of isotope into nucleic acid as well as protein, the nucleic acid was removed from the sample by three consecutive extractions with 5% TCA at 90° for 10 min. (Gale & Folkes, 1953a) carried out after the first precipitation with TCA. When preparations were treated in this way, results are expressed in terms of the 'protein fraction'; when the extraction was omitted, results are expressed in terms of 'TCA precipitate'. 1.0 mg. dry weight of cells yields 0.65 mg. TCA precipitate or 0.49 mg. 'protein fraction'.

The ^{14}C content of the dried preparations was estimated by use of an end-window Geiger-Müller tube and scaler in the conventional manner. The apparatus was calibrated against known amounts of DL-[α -carboxy- ^{14}C]glutamic acid; 1.0 μmole gave 44 counts/min., and where the radioactivity of protein fractions was due solely to glutamic acid, activities have been expressed in terms of μmoles glutamic acid incorporated.

Estimation of radioactivity of free glutamic acid within the cells. Treatment of cell suspensions with TCA (as above) results in liberation of the free glutamic acid from the cells

into the supernatant liquid. Attempts were made to collect the supernatant and estimate the ^{14}C content after taking to dryness *in vacuo*. It was found that such films, even if treated with ether to remove the bulk of the TCA, tended to absorb water during the counting procedure with the result that counts were variable and subject to error. Salton (1951) showed that the free glutamic acid of *Staph. aureus* is released if the cells are treated with cetyltrimethylammonium bromide (CTAB). Accordingly, cells were suspended in water containing CTAB (100 $\mu\text{g.}/\text{mg.}$ dry weight of cells), incubated for 30 min. at 37° and the cells then removed on the centrifuge. The cells were washed in water (0.1 ml./5 mg. dry weight of cells) and the washings added to the CTAB extract. The CTAB extract was then evaporated to dryness, taken up in approx. 0.3 ml. water, transferred to a polythene disk and prepared for counting as above.

The total free glutamic acid content of the cells was estimated manometrically by use of the glutamic acid decarboxylase (Gale, 1945, 1947a).

Determination of the distribution of ^{14}C amongst the amino acids of the cell protein. Samples of the protein fraction corresponding to 6 mg. dry weight of cells were hydrolysed for 20 hr. in 0.4 ml. 6N-HCl in sealed tubes at 105°, evaporated to dryness *in vacuo*, taken up in approx. 0.5 ml. water and evaporated to dryness three successive times, taken up in 30 $\mu\text{l.}$ water and 10 $\mu\text{l.}$ used for spotting a paper chromatogram. Chromatograms were run in two dimensions using as solvents phenol:ammonia (80% phenol, 20% water and 0.3% ammonia solution (sp.gr. 0.880)) and *sec.*-butanol:formic acid:water in the proportions 70:10:20, v/v. After drying, the papers were placed in contact with X-ray-sensitive film (Ilford Ilfex) and the films developed, one after 3 days' contact, and a second after 14 days' contact to bring out any weakly radioactive spots. Active spots were located from the radioautographs, cut out of the paper, eluted and the eluate evaporated before counting as usual.

RESULTS

Incubation of washed suspensions of *Staph. aureus* with glucose and ^{14}C -labelled glutamic acid resulted in the appearance of radioactivity in the protein fraction of the cells whether other amino acids were present in the incubation mixture or not. The activity associated with the protein fraction was not significantly decreased by washing the protein precipitate three to five times with water, 5% TCA or 1% acetic acid, nor was there any loss of activity as a result of treatment of the precipitate at pH 2.7 in citrate buffer saturated with ninhydrin. There was no incorporation of activity if the cells were heated to 100° for 10 min. before incubation, and the incorporation was reduced to insignificant values if the incubation mixture contained 0.01M 2:4-dinitrophenol. Omission of glucose from the incubation mixture reduced the incorporation to less than 10% of that occurring in its presence. It appeared that an incorporation of ^{14}C into the protein fraction was occurring in these experiments and that the general properties of the process were similar to those previously reported by other workers for other cells (Melchior *et al.* 1948; Melchior *et al.* 1951).

Effect of amino acid mixture A on the rate of incorporation. Fig. 1 shows the rate of incorporation of ^{14}C into the TCA-precipitable fraction of washed *Staph. aureus* when the cells are incubated with glucose and (1) [^{14}C]glutamic acid or (2) a complete mixture of amino acids and [^{14}C]glutamic acid. In condition 2, net synthesis of cell protein occurs (Gale & Folkes, 1953a), and it can be seen that the rate and amount of ^{14}C incorporation are markedly greater than in condition 1 where net synthesis has not been demonstrated. If nucleic acid is not extracted from the TCA precipitates, the radioactivity is 8–11% higher under condition 1 and unchanged within experimental error under condition 2; this agrees with previous work which indicated that, when protein synthesis is taking place, glutamic acid is quantitatively taken up into the protein (Gale, 1951; Gale & Folkes, 1953a).

Distribution of ^{14}C amongst the amino acids of the cell protein. Samples of protein from cells which have been incubated with [α -carboxy- ^{14}C]glutamic acid under conditions 1 and 2 of Fig. 1 have been hydrolysed, run on paper chromatograms and radioautographed as described above. In one experiment the amount of radioactivity placed on the paper corresponded to 652 counts/min. from cells incubated under condition 1, and 5530 counts/min. from cells incubated under condition 2. After exposure of the chromatogram to X-ray-sensitive film for 3 days, the radioautographs showed, for both conditions, one spot only and that in a position corresponding to glutamic acid. After further exposure for 14 days, no further spots were visible on the paper corresponding to condition 1 but a faint spot in a position corresponding to leucine/isoleucine appeared on the paper for condition 2. After elution of the paper corresponding to the active glutamic acid spot, 83% of the activity initially added was recovered for condition 1, and 86% for condition 2, while recovery of control samples of [^{14}C]glutamic acid was 87 and 88%. Elution of the paper corresponding to the faintly active 'leucine' spot in condition 2 gave a further recovery of activity equal to 1.1% of the initial activity.

Effect of concentration of organism. Experiments were carried out in which the suspension density of the cells in the incubation mixture varied from 0.8 to 6.7 mg. dry weight/ml. Fig. 2 shows that the incorporation/mg. TCA precipitate falls if the suspension density exceeds 5.0 mg. dry weight/ml. under condition 1 or 2.0 mg. dry weight/ml. under condition 2. In all other experiments, the suspension density used was 1.7 mg. dry weight of cells/ml.

Effect of glutamic acid concentration. The rate of incorporation varies with the concentration of glutamic acid present for low values of the latter, but becomes independent of concentration when

this is greater than 0.9 $\mu\text{mole/ml.}$ under condition 1 or 3.3 $\mu\text{moles/ml.}$ under condition 2. The curves relating rate of incorporation to glutamic acid concentration resemble those obtained for the variation of enzyme velocity with substrate concentration

Fig. 1. Incorporation of radioactivity into the TCA-precipitable fraction of *Staphylococcus aureus* during incubation with ^{14}C -labelled glutamic acid. Washed suspension of *Staph. aureus* incubated at 37° and final suspension density = 1.7 mg. dry weight of cells/ml. in buffered saline solution containing 0.75% (w/v) glucose and 1.0 μmole [^{14}C]glutamic acid (specific activity 0.9 mc/m-mole)/ml. with the following additions. Condition 1, no addition; condition 2, addition of amino acid mixture A, final concentration of each component = 0.2 mg./ml.; condition 3, as condition 2 with omission of aspartic acid.

Fig. 2. Variation of incorporation of radioactivity with suspension density of *Staph. aureus* during incubation. Conditions as for Fig. 1 but final suspension density of washed cells varied as shown above; samples removed after 1 hr. at 37°.

and can be treated by the method of Lineweaver & Burk (1934). Fig. 3 shows the reciprocal of the rate of incorporation plotted against the reciprocal of glutamic acid concentration; calculation of the 'apparent Michaelis constant' for the incorporation process shows that the value of $0.21 \mu\text{mole}$ glutamic acid/ml. is obtained under both conditions 1 and 2.

Fig. 3. Variation of velocity of incorporation of radioactivity with concentration of $[^{14}\text{C}]$ glutamic acid; velocity and concentration plotted as reciprocals. Conditions as for Fig. 1, samples removed after 1 hr. at 37° . $S = \mu\text{mole } [^{14}\text{C}]$ glutamic acid (specific activity 0.9 mc/m-mole)/ml. $V = \text{counts/min. of TCA precipitate}$ corresponding to $2.0 \text{ mg. dry weight of cells}$, after 1 hr. incubation.

Table 1. *Effect of temperature on the incorporation of glutamic acid into the TCA precipitate from Staphylococcus aureus*

(Washed suspensions of *Staph. aureus* Duncan ($1.7 \text{ mg. dry wt. of cells/ml.}$) incubated at temperatures shown below for 60 min. in buffered saline solution containing 0.75% (w/v) glucose, $1.0 \mu\text{mole}$ glutamic acid (specific activity 0.2 mc/m-mole)/ml. with and without the addition of amino acid mixture A (final concentration of each component $= 0.2 \text{ mg./ml.}$). After incubation, cells washed three times in water, precipitated in the cold with 5% trichloroacetic acid; precipitates washed, dried and counted. Results expressed as μmole glutamic acid incorporated/TCA precipitate obtained from $2.0 \text{ mg. dry weight of cells.}$)

Temp. ($^\circ$)	Glutamic acid incorporated	
	Amino acid mixture A absent	Amino acid mixture A present
25	9.5	11.1
35	21.7	47.5
44	32.0	89.0
60	0.8	0.6
100	0.4	0.2

To economize in the use of isotope, experiments have usually been carried out with $0.2 \mu\text{mole } [^{14}\text{C}]$ glutamic acid/ml. diluted with $0.8 \mu\text{mole } [^{12}\text{C}]$ glutamic acid/ml. to saturate the incorporation mechanism.

Effect of temperature. Table 1 shows the rate of incorporation obtained under condition 1 or 2 over a range of temperatures. The rate increases as the temperature rises to $40\text{--}44^\circ$ but incorporation ceases at temperatures above 60° . The process is consequently different from that studied by Brunish & Luck (1952) in nucleohistone preparations where incorporation was greater at 100° than at 37° . It can be seen from Table 1 that the temperature coefficient for incorporation under condition 2 is greater than that under condition 1.

Fig. 4. Variation of rate of incorporation of radioactivity into the protein fraction of *Staphylococcus aureus* incubated with $[^{14}\text{C}]$ glutamic acid and mixtures of unlabelled amino acids. Washed suspension of *Staph. aureus* incubated for 1 hr. at 37° and final suspension density $= 1.7 \text{ mg. dry wt. of cells/ml.}$ in buffered saline containing 0.75% (w/v) glucose, $1.0 \mu\text{mole } [^{14}\text{C}]$ glutamic acid (specific activity 0.2 mc/m-mole)/ml. and amino acid mixtures as shown (+ = amino acid present; O = amino acid absent), each component at final concentration $= 0.2 \text{ mg./ml.}$ Basal mixture contains lysine, arginine, alanine, proline, cysteine, methionine, threonine, serine, at final concentration of L-isomer in each case $= 0.2 \text{ mg./ml.}$ Samples removed after 1 hr. incubation and protein fractions prepared for counting. 1 = Rate of incorporation when glutamic acid is the only amino acid present (condition 1); 2 = rate of incorporation when a complete mixture of amino-acids is present (condition 2).

Effect of other amino acids on glutamic acid incorporation. The incorporation studies so far described were carried out either with glutamic acid as the only amino acid present (condition 1) or with a complete mixture of amino acids present (condition 2). The effect was tried of adding amino acids singly and in mixtures to the incubation mixture containing glutamic acid. Most of the naturally occurring amino acids have little or no effect on glutamic acid incorporation when added singly although, as shown in Fig. 4, aspartic acid decreases while glycine increases the incorporation. The effect was next tested of adding the complete mixture A but omitting one essential amino acid; Fig. 4 shows that omission of phenylalanine or tyrosine has little effect, whereas omission of leucine, valine, tryptophan or histidine reduces the incorporation to that level obtained when glutamic acid is the only amino acid present. The effect of omitting aspartic acid is surprising in that the initial rate of glutamic acid incorporation is 230% of that obtained in the presence of the complete mixture, while the effects of omission of either glycine or histidine are largely overcome by the further omission of aspartic acid.

Fig. 1 shows that the effect of amino acid mixture A without aspartic acid is to increase the rate of glutamic acid incorporation during the initial stages of incubation only, and that the rate then decreases so that the amount of incorporation after 90 min. incubation is no greater than that obtained when glutamic acid is the only amino acid added. When the complete amino acid mixture A is present, the initial rate of incorporation is between that obtained for glutamic acid alone and that obtained for the mixture without aspartic acid, but this rate is maintained throughout the incubation period.

Actions of antibiotics on glutamic acid incorporation

Chloramphenicol. Fig. 5 shows the action of chloramphenicol on glutamic acid incorporation when incubation takes place in the absence (condition 1) and presence (condition 2) of amino acid mixture A. Under condition 2 the incorporation is highly sensitive to chloramphenicol, 85% inhibition occurring in the presence of that concentration of the antibiotic which prevents growth of the organism. The incorporation that takes place under condition 1 is much less sensitive, 30 μ g. chloramphenicol/ml. producing approximately 15% inhibition only. It can be seen from Fig. 5 that the rate of incorporation under condition 2 in the presence of high concentrations of chloramphenicol is less than that which occurs under condition 1 in the same antibiotic concentrations.

Aureomycin. Fig. 6 shows that aureomycin at low concentrations differentiates between glutamic

Fig. 5. Effect of chloramphenicol on rate of incorporation. Conditions as for Fig. 1 with addition of chloramphenicol as indicated; samples taken after 1 hr. at 37°. Cross-hatching indicates range of drug preventing growth in a complete medium inoculated with approx. 10^6 cells/5.0 ml.

Fig. 6. Effect of aureomycin on the incorporation of [14 C]glutamic acid in washed suspensions of *Staphylococcus aureus*. Conditions as for Fig. 1, with aureomycin added as indicated; inhibitions calculated as percentage of rate obtained in absence of aureomycin (= 952 counts/min./mg. protein fraction in condition 1, or 3034 counts/min./mg. protein fraction in condition 2). Cross-hatching indicates range of drug preventing growth in a complete medium inoculated with approx. 10^6 cells/5.0 ml.

acid incorporation under condition 1 and condition 2. At concentrations higher than 300 $\mu\text{g. aureomycin/ml.}$ incorporation is completely inhibited under both conditions.

Bacitracin. When glutamic acid is the only amino acid present, bacitracin at the growth-inhibitory concentration produces 50 % inhibition of the rate of incorporation. The shape of the inhibition curve, shown in Fig. 7, suggests that the process is a complex one involving at least two parts, one of which is completely inhibited at the bactericidal concentration of the antibiotic. At low concentrations, baci-

Fig. 7. Effect of bacitracin on the incorporation of [^{14}C]glutamic acid by washed suspensions of *Staphylococcus aureus*. Conditions as for Fig. 1 with bacitracin added as indicated and concentration of [^{14}C]glutamic acid = 1.0 μmole (specific activity 0.2 mc/m-mole)/ml. Inhibition calculated as percentage rate in absence of bacitracin (= 483 in condition 1 and 1099 in condition 2, counts/min./mg. protein fraction). Cross-hatching indicates range of bacitracin concentration preventing growth in a complete medium inoculated with approx. 10^6 cells/5.0 ml.

Fig. 8. Inhibition of [^{14}C]glutamic acid incorporation into the protein fraction of *Staphylococcus aureus* by penicillin. Details as for Fig. 1, condition 1. Samples taken after 60 min. incubation and inhibition determined as percentage of that incorporation obtained in the absence of penicillin. Points of different shape indicate different experiments with concentrations of [^{14}C]glutamic acid = 0.1 ($\times$, $\blacktriangle$) or 1.0 ($\circ$, $\bullet$) $\mu\text{mole/ml.}$

tracin has little effect on incorporation under condition 2 but the process, whether under condition 1 or 2, is completely inhibited at antibiotic concentrations approximately 100 times the bactericidal level.

Penicillin. Penicillin has no effect on glutamic acid incorporation under condition 2 until the antibiotic concentration is raised to approximately 10 000 times the growth inhibitory level. Fig. 8 shows, however, that bactericidal concentrations produce 45–50 % inhibition of the incorporation that occurs under condition 1, and that the curve shows an increase of only 10 % in the inhibition for an increase of penicillin from 0.1 to 100 international units/ml.

The actions of penicillin and bacitracin appear to be somewhat similar in that both give rise to approximately 50 % inhibition of glutamic acid incorporation under condition 1 when tested at the growth-inhibitory level. Table 2 shows that the two antibiotics have a synergic effect when added together at subbactericidal concentrations but not at concentrations corresponding to the plateaux in their inhibition curves.

Table 2. Actions of penicillin and bacitracin on the incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus*

(Conditions as for Table 1 with penicillin or bacitracin as below; temp. 37° . Protein fraction prepared after incubation, dried and counted.)

Penicillin (i.u./ml.)	Bacitracin ($\mu\text{g./ml.}$)	Glutamic acid incorporated ($\mu\text{mole/mg. protein}$)	Inhibition of glutamic acid incorporation (%)
0.0	0.0	26.1	—
0.03	0.0	21.4	18
0.0	3.0	26.4	0
0.03	3.0	17.7	32
1.0	0.0	15.8	39
0.0	30.0	12.8	51
1.0	30.0	10.8	58
10.0	0.0	14.9	43
0.0	100.0	12.0	54
10.0	100.0	11.15	57

Non-competitive nature of the inhibitions. In the following paper (Gale & Folk, 1953c) it is shown that the incorporation of phenylalanine into the protein of *Staph. aureus* is competitively inhibited by *p*-chlorophenylalanine, and non-competitively inhibited by aureomycin and chloramphenicol. Similar experiments have been carried out with the antibiotics and glutamic acid incorporation under the conditions described above; in all cases the inhibition proved to be non-competitive.

Radioactivity of free glutamic acid within *Staph. aureus* during incorporation into protein

Washed suspensions of *Staph. aureus* were incubated with [^{14}C]glutamic acid and glucose, samples withdrawn during a 90 min. incubation at 37° , and

the following estimations carried out: (1) free glutamic acid content of the cells, (2) radioactivity of free glutamic acid within the cells, and (3) radioactivity of the protein fraction obtained from the

cells. The relative specific activity of the free glutamic acid was calculated from (1) and (2). The experiment was repeated with the addition to the incubation mixture of either 1.0 mg. bacitracin/ml. or 0.3 international unit penicillin/ml. The presence of either penicillin or bacitracin has no effect on the accumulation of free glutamic acid within the cells, as previously shown (Gale & Taylor, 1947; Paine, 1951), but Fig. 9 shows that the antibiotics decrease the rate of glutamic acid incorporation into the protein fraction of the cells. In the absence of antibiotics, the specific activity of the free glutamic acid within the cells rises during the early stages of the incubation as the radioactive glutamic acid is taken into the cells; it then decreases as the activity of the protein increases. In the presence of either bacitracin or penicillin, the specific activity of the free glutamic acid rises to a slightly higher value than in the absence of antibiotics, and the decrease in specific activity during the later stages of incubation is reduced or, in the case of 1.0 mg. bacitracin/ml., abolished. The concentration of total free glutamic acid within the cells remains constant after the first 15 min. and is unaffected by the presence of either antibiotic.

Fig. 9. Effect of penicillin and bacitracin on the incorporation of [¹⁴C]glutamic acid into the protein fraction of *Staphylococcus aureus* (continuous lines) and on the specific activity of the free glutamic acid accumulated within the cells (broken lines). Washed suspension of *Staph. aureus* Duncan incubated in buffered saline solution containing 0.75% glucose, 0.1 μmole [¹⁴C]-glutamic acid (specific activity 0.9 mc/m-mole)/ml. and (C) no addition, (P) 0.3 i.u. of penicillin/ml., (B) 1.0 mg. bacitracin/ml. Samples taken after times shown. Incorporation measured on protein fractions of the cells; intact cells assayed for free glutamic acid by the decarboxylase method; free glutamic acid released from cells by treatment with CTAB, dried and counted. Specific activity of free glutamic acid calculated as counts/min./μg. free glutamic acid.

Actions of antibiotics on the incorporation of other amino acids

It is important to know to what extent the results described above for the incorporation of glutamic acid can be extended to other amino acids. Small amounts of amino acids randomly labelled with ¹⁴C were obtained by biosynthesis and the ability of washed suspensions of *Staph. aureus* to incorporate these amino acids was tested. Insufficient material

Table 3. *Action of antibiotics on the incorporation of amino acids into the protein fraction of Staphylococcus aureus Duncan*

(Washed suspensions of *Staph. aureus* (final suspension density = 1.7 mg. dry wt. of cells/ml.) incubated for 60 min. at 37° in buffered saline containing 0.75% (w/v) glucose and a radioactive amino acid at the concentration shown below, in the presence and absence of antibiotics as shown below. Amino acids other than cystine were randomly labelled with ¹⁴C; cystine used was ³⁵S-labelled. Protein fractions prepared after incubation, dried and counted.)

Amino acid	Radioactivity of incubation medium (counts/min./ml.)	Radioactivity of protein fraction (counts/min./mg.)	Inhibition of incorporation (%)				
			Penicillin (i.u./ml.)			Baci-tracin (100 μg./ml.)	Chloram-phenicol (100 μg./ml.)
			0.03	0.3	30.0		
Glutamic acid	2750	477	25	47	53	63	20
Glycine	5000	1213	8	22	37	26	12
Alanine	1960	175	6	12	15	20	21
Aspartic acid	2140	199	2	10	11	18	33
Isoleucine/leucine	5333	1277	—	4	15	17	60
Threonine	1400	127	—	2	15	15	33
Phenylalanine	4869	502	—	5	11	0	62
Lysine	1773	547	—	2	4	0	33
Arginine	1925	161	—	2	3	0	54
Methionine/valine	2400	300	—	0	1	0	57
Tyrosine	603	281	—	0	0	0	55
Proline	973	28	0	0	0	0	70
[³⁵ S]Cystine	1375	26	0	10	25	22	0

was available for the complete tests described for glutamic acid to be carried out but, in all cases tested, it was found that incubation of the cells with an amino acid and glucose resulted in radioactivity appearing in the protein fraction of the cells. The effect of penicillin, bacitracin and chloramphenicol was tested on this incorporation in each case, and the results are summarized in Table 3. Penicillin was tested at three concentrations, one below and two on the plateau obtained in the curve for inhibition of glutamic acid incorporation. Table 3 shows that there are marked differences in the sensitivity of the incorporation processes for various amino acids; some such as tyrosine, proline, lysine, arginine and the methionine/valine mixture being unaffected by penicillin. Glutamic acid is the most sensitive, followed by glycine and alanine, but the glycine incorporation is only approximately half as sensitive as glutamic acid incorporation. The activities of penicillin and bacitracin, at 'plateau concentrations', are very similar for all amino acids tested. The sensitivity of the processes towards chloramphenicol varies again, and there is a suggestion that those processes which are least sensitive to penicillin are most sensitive to chloramphenicol, and vice versa.

DISCUSSION

The results presented here demonstrate clearly that the processes whereby glutamic acid is incorporated into the protein fraction of *Staph. aureus* differ according to the conditions of incubation. It has been shown previously that washed suspensions of *Staph. aureus* will synthesize protein if they are incubated with glucose and a mixture of the amino acids essential for growth (Gale & Folkes, 1953*a*). Such protein synthesis is almost completely inhibited by concentrations of chloramphenicol or aureomycin which prevent growth (Gale & Folkes, 1953*b*). Under similar conditions (condition 2) radioactive glutamic acid is rapidly incorporated into the protein fraction of the cells and this incorporation is inhibited 85–90 % by chloramphenicol or aureomycin at the growth-inhibitory concentrations. So far it would appear that the incorporation of [^{14}C]glutamic acid can be taken as an indication of protein synthesis. However, if we alter the amino acid composition of the incubation mixture, we still obtain incorporation although net protein synthesis may no longer occur, and there would appear to be no correlation between the rate of glutamic acid incorporation, particularly during the early stages of incubation, and the rate of increase in cell protein.

There are very marked differences in the sensitivities of incorporation processes to antibiotics depending upon whether conditions are suitable for

protein synthesis or not. Thus incorporation under condition 1 is less sensitive to chloramphenicol and aureomycin, and more sensitive to bacitracin and penicillin, than incorporation under condition 2. Under condition 2 glutamic acid is taken up from the medium and added into the new protein directly and quantitatively (Gale, 1951; Gale & Folkes, 1953*a*); under condition 1 there is no increase in cell protein, but free glutamic acid accumulates within the cells (Gale, 1947*a*). Investigation of the specific activity of the free glutamic acid within the cells shows that, once the initial period of uptake is over, an increase in activity of the cell protein is accompanied by a decrease in activity of the free glutamic acid within the cell. Inhibition of the incorporation into the protein prevents the decrease in specific activity of the free glutamic acid. Since the concentration of total free glutamic acid within the cells remains constant during these changes, it would appear that the incorporation of radioactive glutamic acid into the cell protein occurs as a result of an exchange between the free glutamic acid molecules in the cell and glutamic acid residues in the protein. To prove that such an exchange does occur it would be necessary to show that active residues in the protein could be made to exchange with unlabelled free glutamic acid. Attempts to do this with intact cells have not been successful since the highly efficient concentration of free glutamic acid within the cells during the incorporation process has made it technically almost impossible to reverse the situation. However, such a reversal has been accomplished with cells damaged by supersonic vibration (Gale, 1953) and this will be reported later.

It would appear that glutamic acid incorporation can occur as a result of exchange reactions with cell proteins, and that the rate of such reactions can be greatly influenced by the presence of other amino acids. When, however, the medium contains all the amino acids necessary for protein synthesis to occur, there is a change in the reactions within the cell which results in glutamic acid (and other amino acids) being formed into new protein. Since high concentrations of chloramphenicol suppress incorporation during protein synthesis to a level well below that occurring during simple exchange, and since penicillin inhibits exchange incorporation but has no effect on incorporation during protein synthesis, it would seem that exchange reactions must be suppressed when protein synthesis begins. Whatever may be the final interpretation of these findings, it is clear that it is dangerous to use incorporation studies as measures of protein synthesis in living cells.

There have been many effects ascribed to penicillin in the past, but some of these can be demonstrated only if the cells are allowed to grow in the

presence of the antibiotic for a short time, e.g. inhibition of glutamic acid uptake (Gale & Taylor, 1947), or of guanosine oxidation (Gros, Beljanski & Macheboeuf, 1951), while others require very high concentrations of penicillin to show significant inhibitions, e.g. inhibition of ribonucleic acid breakdown (Krampitz & Werkman, 1947). The effect described here is the first that has been obtained with bactericidal concentrations of penicillin acting on washed suspensions of cells. At present it is difficult to interpret the effect. Penicillin appears to prevent exchange reactions which occur between cell proteins and certain amino acids in the amino acid pool of the cells. Of the amino acids affected, glutamic acid is the most sensitive; some amino acids such as proline, tyrosine and lysine are not affected at all. The shape of the curves obtained for the inhibition of glutamic acid incorporation by penicillin or bacitracin suggest that the reaction is a complex one; it may be that it is exchange with certain proteins only which is affected by penicillin. Such an action would presumably have no immediate effect on the enzymic activities of the non-growing cell but these proteins would be eliminated from the dynamic pool of the cell. Consequently when growth was resumed, enzymic activities due to these proteins would be lost, and this may be the explanation of the secondary effects mentioned above.

SUMMARY

1. When washed suspensions of *Staphylococcus aureus* are incubated with glucose and ^{14}C -labelled glutamic acid (condition 1), the protein fraction of the cells becomes progressively more radioactive.

The radioactivity is associated with the glutamic acid residues of the protein.

2. The rate of this glutamic acid incorporation is increased by the addition of a mixture of amino acids containing all those essential for growth (condition 2). Omission of certain essential amino acids, such as histidine or tryptophan, results in the rate of incorporation falling to that obtained in condition 1. Omission of aspartic acid results in a greatly increased rate of incorporation which lasts for 30–60 min. and then falls to that under condition 1.

3. The incorporation is abolished if the reaction temperature rises to 60° or over.

4. Incorporation under condition 1 is markedly less sensitive to inhibition by chloramphenicol or aureomycin, and markedly more sensitive to inhibition by penicillin or bacitracin, than incorporation under condition 2.

5. The incorporation of glutamic acid into cell protein under condition 1 is accompanied by a decrease in the specific activity but not the total amount of the free glutamic acid within the cells. Inhibition of incorporation by penicillin or bacitracin does not affect the total amount of free glutamic acid within the cells, but prevents the decrease in its specific activity.

6. The processes involved in the incorporation of a variety of radioactive amino acids show differing sensitivities to penicillin, bacitracin and chloramphenicol. The process most sensitive to penicillin is that involved in the incorporation of glutamic acid.

The authors are indebted to Dr K. McQuillen for preparing the radioautographs, and to Mr M. K. Gaitonde for a gift of ^{35}S -labelled cystine.

REFERENCES

- Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. (1950). *J. biol. Chem.* **186**, 297.
 Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. (1952). *J. biol. Chem.* **196**, 669.
 Brunish, R. & Luck, J. M. (1952). *J. biol. Chem.* **197**, 869.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Gale, E. F. (1947a). *J. gen. Microbiol.* **1**, 53.
 Gale, E. F. (1947b). *J. gen. Microbiol.* **1**, 327.
 Gale, E. F. (1951). *Biochem. J.* **48**, 290.
 Gale, E. F. (1953). 6th Int. Congr. Microbiology, Symp. on Bacterial Metabolism.
 Gale, E. F. & Folkes, J. P. (1953a). *Biochem. J.* **53**, 483.
 Gale, E. F. & Folkes, J. P. (1953b). *Biochem. J.* **53**, 493.
 Gale, E. F. & Folkes, J. P. (1953c). *Biochem. J.* **55**, 730.
 Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 314.
 Gros, F., Beljanski, M. & Macheboeuf, M. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1696.
 Holloway, B. W. & Ripley, S. H. (1952). *J. biol. Chem.* **196**, 695.
 Kit, S. & Greenberg, D. M. (1952). *J. biol. Chem.* **194**, 377.
 Krampitz, L. O. & Werkman, C. H. (1947). *Arch. Biochem.* **12**, 57.
 Lineweaver, H. & Burk, B. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Melchior, J. B., Klioze, O. & Klotz, I. M. (1951). *J. biol. Chem.* **189**, 411.
 Melchior, J. B., Mellody, M. & Klotz, I. M. (1948). *J. biol. Chem.* **174**, 81.
 Melchior, J. B. & Tarver, H. (1947). *Arch. Biochem.* **12**, 309.
 Paine, T. F. jun. (1951). *J. Bact.* **61**, 259.
 Salton, M. R. J. (1951). *J. gen. Microbiol.* **5**, 391.
 Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
 Winnick, T. (1950). *Arch. Biochem.* **27**, 65.
 Zamecnik, P. C. & Frantz, I. D. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 199.