The Assimilation of Amino Acids by Bacteria

19. THE INHIBITION OF PHENYLALANINE INCORPORATION IN *STAPHYLOCOCCUS AUREUS* BY CHLORAMPHENICOL AND *p*-CHLOROPHENYLALANINE

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In the previous paper of this series (Gale & Folk, 1953c) it was shown that incubation of washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) with glucose and [14 C]-glutamic acid resulted in incorporation of the labelled glutamic acid into the protein fraction of the cells. This process occurred in the absence of demonstrable protein synthesis and underwent considerable modification in properties when a complete mixture of amino acids was added. Under these conditions net synthesis of protein took place and was inhibited by bactericidal concentrations of chloramphenicol (Gale & Folk, 1953a, b). The incorporation of labelled glutamic acid was equally sensitive to the antibiotic as long as a complete mixture of amino acids was present but became markedly less sensitive when glutamic acid was the only amino acid present in the incubation mixture.

Woolley (1951) found that phenylalanine would decrease the antibacterial action of chloramphenicol when this was present in concentrations which partially inhibited growth, although the amino acid was ineffective as antagonist when growth inhibition was complete. He pointed out that there are structural similarities between chloramphenicol and phenylalanine, and suggested that the antibiotic acts as an inhibitor of phenylalanine metabolism and may prevent the incorporation of phenylalanine into proteins. Halvorson & Spiegelman (1952) investigated the inhibition of growth and enzyme formation in yeast by amino acid analogues, and found that certain derivatives of phenylalanine, *o*- and *p*-fluorophenylalanine in particular, were potent inhibitors whose action could be competitively antagonized by the corresponding amino acid only. It was thought that a study of the actions of chloramphenicol and an analogue of phenylalanine on the incorporation of that amino acid into the protein fraction of *Staph. aureus* might throw light on the relationship between amino acid incorporation and protein synthesis, and also on the mode of action of chloramphenicol.

METHODS

The organism, methods of growth, preparation of suspensions, incubation mixtures and conditions, preparation and counting of radioactive protein fractions were the same as those described in the previous paper (Gale & Folk, 1953c). The radioactive phenylalanine, obtained from the Radiochemical Centre, Amersham, was DL- $[\beta\text{-}^{14}\text{C}]$ phenylalanine of specific activity 1.19 mc/m-mole. The amino acid mixture used was that described as mixture A by Gale & Folk (1953a) with the omission of glutamic acid and phenylalanine; these were added in labelled or non-labelled form as described below. Gale & Folk (1953c) showed that the rate of incorporation of glutamic acid into the protein of *Staph. aureus* varied with the concentration of glutamic acid in a manner similar to that obtained for the variation of the rate of an enzyme-catalysed reaction with substrate concentration, and that a straight line was obtained if the reciprocal of the rate was plotted against the reciprocal of the glutamic acid concentration. A similar situation has been found in the present studies and it has been possible to study the inhibition of incorporation by application of the methods of analysis devised by Lineweaver & Burk (1934).

RESULTS

Incorporation of phenylalanine into the protein fraction of Staph. aureus. Incubation of washed suspensions of *Staph. aureus* with glucose and ^{14}C -labelled phenylalanine (condition 1) results in a progressive incorporation of ^{14}C into the protein fraction of the cells in a manner similar to that previously described for glutamic acid (Gale & Folk, 1953c). The addition of a complete mixture of amino acids (condition 2) markedly increases the rate of incorporation. Under both conditions the incorporation is prevented by prior heating of the cells to 100° for 10 min.

Effect of phenylalanine concentration on the rate of incorporation. Fig. 1 shows the effect of plotting the reciprocal of the rate of ^{14}C incorporation into the protein fraction against the reciprocal of the concentration of [^{14}C]phenylalanine present during incubation under conditions 1 and 2. The 'apparent Michaelis constant' for incorporation under con-

Fig. 1. Relation between rate of incorporation and phenylalanine concentration: incorporation of phenylalanine by *Staph. aureus*. $S = \mu\text{mole DL-}^{14}\text{C}$ phenylalanine/ml. $V = \text{rate of incorporation into protein fraction expressed in counts/min./mg. protein fraction of cells after 1 hr. incubation at } 37^\circ$. Washed suspension of *Staph. aureus* incubated at final suspension density 1.7 mg. dry wt. of cells/ml. in buffered saline containing 0.75% (w/v) glucose and ^{14}C phenylalanine with (condition 2) and without (condition 1) complete mixture of amino acids at final concentration of each component (other than phenylalanine) = 0.2 mg./ml.

dition 2 (0.45 $\mu\text{mole/ml.}$) is greater than that obtained under condition 1 (0.015 $\mu\text{mole/ml.}$). In the case of glutamic acid incorporation there was no difference in the values of the 'apparent Michaelis constant' under the two conditions (Gale & Folkes, 1953c).

Action of chloramphenicol on phenylalanine incorporation. Fig. 2 shows the effect of chloramphenicol on the incorporation of either phenylalanine or glutamic acid in the presence and absence of a complete amino acid mixture. It can be seen that the incorporation of phenylalanine is more sensitive to inhibition by chloramphenicol than is incorporation of glutamic acid whether other amino acids are present or not, and that both incorporations are more sensitive under condition 2 than under condition 1. Figs. 3a and 3b show the effect of two or three concentrations of chloramphenicol on the relation between phenylalanine concentration and rate of incorporation. Whether studied under condition 1 or 2, the lines obtained show a typical non-competitive inhibition of incorporation by chloramphenicol (see Lineweaver & Burk, 1934). Table 1 shows that there is a small increase in the calculated 'apparent Michaelis constant' when chloramphenicol inhibits phenylalanine incorporation under condition 1, whereas there is a marked decrease in the calculated value when inhibition occurs under condition 2. Similar experiments have been carried out with aureomycin and terramycin, and in these cases the inhibition

Fig. 2. Inhibition of incorporation of phenylalanine (○) and glutamic acid (●) by chloramphenicol in the presence (condition 2) and absence (condition 1) of a mixture of amino acids. Washed suspension of *Staph. aureus* incubated at final suspension density 1.7 mg. dry wt. of cells/ml. at 37° in buffered saline containing 0.75% (w/v) glucose and either 1.0 $\mu\text{mole } ^{14}\text{C}$ glutamic acid (specific activity 0.2 mc/m-mole) or 0.5 $\mu\text{mole } ^{14}\text{C}$ phenylalanine (specific activity 1.19 mc/m-mole)/ml. with (condition 2) and without (condition 1) amino acid mixture A (less either glutamic acid or phenylalanine, respectively) at a final concentration of each component = 0.2 mg./ml. Samples taken at 30 and 60 min., protein fraction prepared, dried and counted. Inhibition calculated as percentage rate of incorporation in the absence of chloramphenicol. Cross-hatched area shows range of antibiotic preventing growth of an inoculum of approx. 10^6 cells/ml. in fully nutrient medium.

is non-competitive and no change in the calculated Michaelis constants occurs, within experimental error, under condition 1 (see Fig. 3a and Table 1).

Action of p-chlorophenylalanine on phenylalanine incorporation. Figs. 3a and 3b also show the inhibitory action of two concentrations of p-chlorophenylalanine on phenylalanine incorporation under conditions 1 and 2. Under both conditions, the lines obtained are typically those given by competitive inhibition of incorporation (Lineweaver & Burk, 1934). Table 1 shows that there is a marked increase in the calculated 'Michaelis constants' under either condition 1 or 2 in the presence of p-chlorophenylalanine.

Action of p-chlorophenylalanine on the incorporation of glutamic acid. Table 2 shows that p-chlorophenylalanine has little inhibitory action on glutamic acid incorporation under either condition 1 or 2. In the experiments described so far, under condition 2 the only amino acid present in limiting concentration has been the labelled amino acid

whose incorporation is being studied. It seemed possible that the effect of both chloramphenicol and *p*-chlorophenylalanine on glutamic acid incorporation under condition 2 might be increased if the concentration of phenylalanine in the amino acid

Fig. 3a. Effect of chloramphenicol (CAP), *p*-chlorophenylalanine (CQ) and aureomycin (A) on the incorporation of phenylalanine into the protein fraction of *Staphylococcus aureus* incubated with [14 C]phenylalanine and glucose. Details as for Fig. 1 (condition 1).

Fig. 3b. Effect of chloramphenicol (CAP) and *p*-chlorophenylalanine (CQ) on the incorporation of phenylalanine into the protein fraction of *Staphylococcus aureus* incubated with [14 C]phenylalanine, glucose and a complete mixture of amino acids. Details as for Fig. 1 (condition 2).

mixture were also made limiting. Table 2 shows that although the action of *p*-chlorophenylalanine is affected to some extent by the phenylalanine concentration, the analogue has little inhibitory action on glutamic acid incorporation under condition 2 even when the phenylalanine concentration is reduced to such a level that the incorporation of phenylalanine is almost completely abolished by the analogue. This conclusion was confirmed (Table 2, Expt. 4) by carrying out parallel experiments in which either the phenylalanine or the glutamic acid was labelled. Table 3 shows that the action of chloramphenicol on the incorporation of glutamic acid under condition 2 is not affected by the phenylalanine concentration.

Relation between inhibition of protein synthesis and inhibition of incorporation. Experiments were carried out in parallel which enabled the action of *p*-chlorophenylalanine to be tested on net protein synthesis, determined as described by Gale & Folkes (1953a), and on the incorporation of either glutamic acid or phenylalanine. Table 4 shows that the inhibition of phenylalanine incorporation approximately parallels the inhibition of net protein synthesis, whereas the inhibition of glutamic acid incorporation bears no relation to that of net protein synthesis.

Table 1. Effect of inhibitors on the 'apparent Michaelis constant' for the incorporation of phenylalanine into the protein fraction of *Staphylococcus aureus*

(Washed suspension of *Staph. aureus* incubated at final suspension density 1.7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline solution containing glucose 0.75% (w/v) and [14 C]phenylalanine (specific activity 1.19 mc/m-mole) at concentrations 0.01, 0.02 and 0.1 μ mole/ml. with and without amino acid mixture A + glutamic acid at a final concentration of each component = 0.2 mg./ml. Inhibitors added as below. 'Apparent Michaelis constants' calculated according to Lineweaver & Burk (1934) in each case and expressed as μ mole/ml.)

Inhibitor	Concn. (μ mole/ml.)	Apparent Michaelis constant for phenyl- alanine incorporation	
		Amino acids present	Amino acids absent
None	—	0.45	0.012
Chloramphenicol	0.012	0.15	—
	0.04	0.07	—
	0.12	—	0.0165
	0.4	—	0.0205
	1.2	—	0.025
Terramycin	0.0064	—	0.012
	0.00064	0.22	—
Aureomycin	0.002	—	0.016
	0.00062	0.37	—
<i>p</i> -Chlorophenylalanine	5.0	0.54	0.091

Table 2. *Effect of p-chlorophenylalanine on the incorporation of phenylalanine or glutamic acid into the protein fraction of Staphylococcus aureus*

(Washed suspension of *Staph. aureus* incubated at final suspension density 1.7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline solution containing 0.75% (w/v) glucose and unlabelled or ¹⁴C-labelled glutamic acid (specific activity 0.9 mc/m-mole), and unlabelled or labelled [¹⁴C]phenylalanine (specific activity 1.19 mc/m-mole) as below. Amino acid mixture is complete mixture A less glutamic acid and phenylalanine, added where shown at a final concentration of each component = 0.2 mg./ml. Results expressed as % inhibition of incorporation of radioactive amino acid occurring in absence of inhibitor.)

Expt.	Amino acid mixture	[¹² C]-Glutamic acid (μmole/ml.)	[¹² C]-Phenylalanine (μmole/ml.)	[¹⁴ C]-Glutamic acid (μmole/ml.)	[¹⁴ C]-Phenylalanine (μmole/ml.)	Radio-activity protein fraction no inhibitor (counts/min./mg.)	p-Chloro-phenylalanine (μmole/ml.)	% Inhibition of incorporation of	
								[¹⁴ C]-Glutamic acid	[¹⁴ C]-Phenylalanine
1	0	—	—	—	0.1	483	0.5	—	11
	0	—	—	—	0.1	483	5.0	—	57
	0	—	—	0.1	—	1816	5.0	0	—
2	+	1.3	—	—	0.2	1494	0.5	—	38
	+	1.3	—	—	0.2	1494	5.0	—	70
	+	1.3	—	—	0.1	858	5.0	—	88
3	+	—	13.0	0.1	—	2129	5.0	0	—
	+	—	1.3	0.1	—	2020	5.0	5	—
	+	—	0.13	0.1	—	2050	5.0	3	—
	+	—	0.013	0.1	—	2322	5.0	18	—
4	+	—	0.1	0.1	—	2000	5.0	4	—
	+	0.1	—	—	0.1	823	5.0	—	60

Table 3. *Effect of chloramphenicol on the incorporation of phenylalanine or glutamic acid into the protein fraction of Staphylococcus aureus*

(Details as for Table 2.)

Expt.	Amino acid mixture	[¹² C]-Glutamic acid (μmole/ml.)	[¹² C]-Phenylalanine (μmole/ml.)	[¹⁴ C]-Glutamic acid (μmole/ml.)	[¹⁴ C]-Phenylalanine (μmole/ml.)	Radio-activity protein fraction no inhibitor (counts/min./mg.)	Chloramphenicol (μmole/ml.)	% Inhibition of incorporation of	
								[¹⁴ C]-Glutamic acid	[¹⁴ C]-Phenylalanine
1	0	—	—	—	0.3	622	0.4	—	62
	0	0.9	—	0.1	—	532	0.4	26	—
2	+	1.0	—	—	0.3	6653	0.04	—	88
	+	1.0	1.3	0.1	—	1603	0.04	36	—
3	+	0.9	13.0	0.1	—	1040	0.04	48.5	—
	+	0.9	1.3	0.1	—	1136	0.04	54.5	—
	+	0.9	0.13	0.1	—	1027	0.04	41.5	—
	+	0.9	0.013	0.1	—	1014	0.04	46.0	—
4	+	—	0.1	0.1	—	2000	0.015	4.0	—
	+	0.1	—	—	0.1	823	0.015	—	37

Actions of other antibiotics on phenylalanine incorporation. Table 5 shows the inhibition of phenylalanine incorporation under conditions 1 and 2 by a number of antibiotics. The inhibition of glutamic acid incorporation in parallel experiments is also given for comparison. The incorporation of phenylalanine, whether under condition 1 or 2, is more sensitive to the action of chloramphenicol, aureomycin or terramycin than is the incorporation of glutamic acid, but whereas the latter is sensitive to inhibition by penicillin or bacitracin under condition 1, these antibiotics have no effect on phenylalanine incorporation under the conditions used.

DISCUSSION

Woolley (1951) has suggested that the action of chloramphenicol is due to its relationship to phenylalanine. The incorporation of phenylalanine into the protein fraction of *Staph. aureus* is certainly more sensitive to inhibition by chloramphenicol than is the incorporation of glutamic acid but not more so than the incorporation of proline, leucine or tyrosine (Gale & Folkes, 1953c). Moreover, the difference in sensitivity is also shown with aureomycin and terramycin. Analysis of the nature of the inhibition

Table 4. *Action of p-chlorophenylalanine on net protein synthesis and on the incorporation of either phenylalanine or glutamic acid into the protein fraction of Staphylococcus aureus*

(Washed suspension of *Staph. aureus* incubated at final suspension density 2.0 mg. dry wt. of cells/ml. for 1 hr. at 37° with 0.75% (w/v) glucose and amino-acid mixture A less glutamic acid and phenylalanine (final concentration of each component = 0.2 mg./ml.) with additions of glutamic acid and phenylalanine as follows: (a) Unlabelled glutamic acid and phenylalanine; 100 mg. dry weight of cells; protein precipitated at end of incubation and in initial sample with 5% (w/v) cold trichloroacetic acid and protein-glutamate estimated after hydrolysis (Gale & Folkes, 1953a). (b) Unlabelled glutamic acid and ¹⁴C-labelled phenylalanine (specific activity 1.19 mc/m-mole); 5.0 mg. dry weight of cells; protein fraction prepared and counted. (c) Unlabelled phenylalanine and ¹⁴C-labelled glutamic acid (specific activity 0.1 mc/m-mole); otherwise as (b).)

Incubation mixture			Increase in protein-glutamate (mg./100 mg. dry wt. of cells)	Incorporation into protein fraction, no inhibitor (counts/min./5 mg. dry wt. of cells)		% Inhibition by 5.0 μmoles p-chlorophenylalanine/ml.		
Glucose amino acid mixture	Glutamic acid (μmole/ml.)	Phenylalanine (μmole/ml.)		[¹⁴ C]-Phenylalanine (b)	[¹⁴ C]-Glutamic acid (c)	(a)	(b)	(c)
+	1.0	0.01	0.17	672	909	100	89	20
+	1.0	0.1	0.92	5054	942	58	62	4
+	1.0	1.0	1.27	11805	915	2	18	2

Table 5. *Effect of antibiotics on the incorporation of glutamic acid or phenylalanine into the protein fraction of Staphylococcus aureus*

(Washed suspension of *Staph. aureus* incubated at final suspension density 1.7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline containing 0.75% (w/v) glucose and either 1.0 μmole [¹⁴C]glutamic acid (specific activity 0.2 mc/m-mole) or 0.1 μmole [¹⁴C]phenylalanine (specific activity 1.19 mc/m-mole) with or without a complete amino acid mixture at final concentration of each component = 0.2 mg./ml. Results expressed as percentage rate of incorporation obtained in absence of inhibitor in each case.)

Antibiotics	Concentration (μg./ml.)	% Inhibition of incorporation			
		Amino acid mixture present		Amino acid mixture absent	
		Glutamic acid	Phenylalanine	Glutamic acid	Phenylalanine
Chloramphenicol	100	—	—	26	62
	10	70	88	6	21
Aureomycin	1	—	—	32	69
	0.3	51	85	—	—
Terramycin	3.0	—	—	34	55
	0.3	26	53	—	—
Penicillin	0.3	0	0	40	5
Bacitracin	100	0	0	55	0

of phenylalanine incorporation by chloramphenicol shows that it is non-competitive, and very different from the competitive inhibition shown by p-chlorophenylalanine. Clearly the action of chloramphenicol on protein synthesis cannot be ascribed to a specific inhibition of phenylalanine incorporation, although the fact that the 'apparent Michaelis constant' for the latter process shows some increase in the presence of chloramphenicol but not of aureomycin, may indicate that the structural relationship may influence the inhibitory action of the former antibiotic. Under condition 2 chloramphenicol produces a marked decrease in the

'apparent Michaelis constant' and this may be a reflection of the difference in the 'apparent Michaelis constants' under conditions 1 and 2, and of the fact that incorporation under condition 2 is more sensitive to chloramphenicol than under condition 1.

Under condition 2 the cells are able to accomplish a net synthesis of protein (Gale & Folkes, 1953a), and it might be thought that the incorporation of any labelled amino acid would provide a measure of protein synthesis under these conditions. If this were the case, an inhibitor which prevented protein synthesis should affect the incorporation of all

amino acids to the same extent. This is not the case; it has been shown above that chloramphenicol and *p*-chlorophenylalanine affect the incorporation of glutamic acid and phenylalanine (under condition 2) to different extents. In the case of *p*-chlorophenylalanine it is possible to inhibit the incorporation of phenylalanine almost completely without producing significant effect on the incorporation of glutamic acid, and the inhibition of protein synthesis correlates with the effect on phenylalanine. It would seem that inhibition of protein synthesis follows the inhibition of incorporation of that amino acid most sensitive to the inhibitor used. Prevention of protein synthesis by abolishing the uptake of phenylalanine has little or no effect on the rate of glutamic acid incorporation under condition 2, and this confirms the previous studies on glutamic acid incorporation (Gale & Folkes, 1953c) in which it was shown that addition of a complete amino acid mixture increased the rate of glutamic acid incorporation but that omission of phenylalanine from that mixture had little effect. It is clear that the rate of incorporation of a labelled amino acid into the protein fraction of a living cell cannot necessarily be taken as a measure of the rate of protein synthesis within that cell.

SUMMARY

1. Incubation of washed suspensions of *Staphylococcus aureus* with glucose and ¹⁴C-labelled phenylalanine results in incorporation of the isotope into the protein fraction of the cells. The rate of incorporation can be markedly increased by the addition of a complete mixture of amino acids to the incubation mixture.

2. The incorporation of phenylalanine, whether in the presence or absence of the amino acid mixture, is competitively inhibited by *p*-chlorophenylalanine, and non-competitively inhibited by chloramphenicol, aureomycin or terramycin.

3. The incorporation of phenylalanine is more sensitive to the action of chloramphenicol, aureomycin or terramycin than is the incorporation of glutamic acid.

4. When a complete mixture of amino acids is present, an inhibitor may affect net protein synthesis, the incorporation of phenylalanine and the incorporation of glutamic acid to different extents. *p*-Chlorophenylalanine completely inhibits net protein synthesis and phenylalanine incorporation while being without significant effect on glutamic acid incorporation.

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The Reaction of Fluorodinitrobenzene with the α - and ϵ -Amino Groups of Collagen

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The technique developed by Sanger (1945) for the determination of free amino groups in proteins has been applied to collagen and procollagen with the object of gaining information about the form and size of the molecule. So far this technique has only been applied to a few of the insoluble proteins: wool keratin, myosin and tropomyosin. In keratin a comparatively large number of terminal amino groups were detected suggesting that the molecule consists of a relatively large number of polypeptide chains (Middlebrook, 1951; Blackburn, 1949). With myosin and tropomyosin, however, Bailey (1951) was unable to detect the presence of any terminal

amino groups and suggested the possibility of a cyclic molecule.

The evidence available on the structure of collagen from X-ray and electron microscope observations has recently been reviewed by Bear (1952). The fibrils are considered to be formed of regularly coiled polypeptide chains arranged roughly parallel to one another, with regions of similar chemical structure matching transversely. Collagen contains no cystine and there is no evidence for any covalent cross-linkages between the chains, suggesting that, in contrast to keratin, the molecule may consist of a single polypeptide chain. Pauling & Corey (1951),