The Assimilation of Amino-acids by Bacteria

7. The Nature of Resistance to Penicillin in *Staphylococcus aureus*

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SUMMARY: Penicillin blocks the assimilation of glutamic acid by *Staphylococcus aureus*; the effective concentration of penicillin is of the same order as that required to inhibit growth of the organism whether the culture is sensitive or resistant to penicillin. Serial subcultivation in increasing concentrations of penicillin results in the selection of resistant mutants; as resistance increases, the ability of the cells to assimilate glutamic acid decreases. The efficiency of the assimilation process in highly resistant cells is poor, but they can synthesize all their amino-acid requirements from ammonia and glucose.

The decrease in assimilatory efficiency as resistance to penicillin increases is correlated with an increase in the ability of the cells to synthesize amino-acids. Reverse mutations, having decreased ability to synthesize certain amino-acids, were obtained from highly resistant strains and had increased sensitivity to penicillin. Strains of *Staph. aureus* requiring several amino-acids as nutrients have been 'trained', by subcultivation in media progressively more deficient in amino-acids, to dispense with the addition to the medium of all amino-acids other than cystine and histidine; the increase in synthetic ability was accompanied by a marked increase in penicillin resistance.

It is suggested that penicillin interferes with the mechanism whereby certain amino-acids are taken into the cell, and that the sensitivity of the cell to penicillin is then determined by the degree to which its growth processes are dependent upon assimilation of preformed amino-acids rather than upon their synthesis.

Previous papers in this series have shown that Gram-positive bacteria are able to assimilate certain amino-acids and to concentrate them in the free state within the cells prior to utilization (Gale, 1947*a*; Taylor, 1947). In parenthesis it may be noted that the word 'assimilation' as used in the present and previous papers of this series denotes transfer from the external environment into the cell, and does not connote incorporation, for example, into protein. The free amino-acids thus accumulated within the cells provide a reservoir which is drawn upon for anabolic processes. The concentration of free amino-acid attained within the cell is determined by the balance between the rate at which the amino-acid enters the cell and the rate at which it is there utilized (Gale & Mitchell, 1947; Gale, 1947*b*). The passage of amino-acids across the cell wall may be by diffusion as in the case of lysine, or it may be by a process requiring energy supplied by some exergonic metabolism. When penicillin is added to a growing culture of *Staph. aureus*, the cells become progressively less able to assimilate glutamic acid until eventually this amino-acid does not enter the cell at all. Since the utilization of glutamic acid within the cell is unaffected by penicillin, the internal reservoir is steadily depleted until protein synthesis ceases (Gale & Taylor, 1947). Penicillin thus prevents the passage of glutamic acid, and probably other amino-acids, into the cell.

If the antibiotic properties of penicillin are due primarily to its capacity to prevent the passage of amino-acids into the cell, it should be possible to correlate this effect with the acquisition of resistance when organisms are subjected to serial subcultivation in increasing concentrations of penicillin.

Demerec (1945) has studied the acquisition of resistance to penicillin under these conditions and has shown that cultures of *Staph. aureus* contain a small number of mutants whose resistance is greater than that of the majority of the cells. When such cultures are subjected to a concentration of penicillin which limits the growth of most of the cells, a selective growth of the resistant mutants takes place. Repetition of the process results in the progressive selection of mutants of steadily increasing resistance. The resistant cells arise by spontaneous mutation and not as a result of the action of penicillin on the bacterial cells; the mutation rate is of the order of 1 in 10^7 – 10^8 generations. A preliminary note (Gale, 1947*c*) reported that resistant mutants selected by cultivation in penicillin are less efficient in assimilating glutamic acid than the sensitive parent strains. Bellamy & Klimek (1948*b*) trained a strain of *Staph. aureus* to a resistance 60,000 times that of the parent culture and have selected mutants which are Gram-negative, pleomorphic and strict aerobes. Investigations of the amino-acid metabolism of these highly resistant organisms (Gale & Rodwell, 1948) show that they are able to synthesize all their amino-acid requirements from ammonia and glucose, although their catabolic activities are significantly the same as those of the parent strains.

In this communication we show that the penicillin resistance of *Staph. aureus* is determined by the degree to which its growth processes are independent of the assimilation of preformed amino-acids. Penicillin prevents the passage of such amino-acids across the cell wall, and consequently protein synthesis within the cell is stopped unless the cell is able to synthesize its constituent amino-acids instead of taking them preformed from the external environment.

Organisms and methods

The greater part of the work described below was carried out with two strains of *Staph. aureus*: (i) *Staph. aureus* 6773: a strain isolated from a nasal swab of a patient treated with penicillin and having a penicillin sensitivity on isolation of 5–9 units/ml.; isolated and given to us by Dr B. Topley; (ii) *Staph. aureus* 209: obtained from the American Type Culture Collection and sent to us by Dr W. D. Bellamy; penicillin sensitivity = 0.05–0.06 units/ml. Other strains of *Staph. aureus* mentioned below were isolated by members of the staff of the Cambridge Pathology Department.

Preparation of resistant strains. These were prepared in the usual manner by serial subcultivation in the presence of increasing concentrations of penicillin. The highly resistant organism 209 ($P_{60}T_{35}$) was prepared by Bellamy & Klimek (1948*b*), who kindly gave us cultures for these experiments. These highly resistant organisms will grow in the presence of 4 mg. crystalline penicillin (*c.* 7000 units)/ml. medium. Commercial crystalline penicillin was used throughout.

Investigation of nutrition of organisms. The nitrogen requirements of staphylococci were investigated by Gladstone (1937); his methods were followed in this work. In general, a complete nutrient medium consisting of salts, nicotinamide, aneurin and pure amino-acids (Gladstone, 1937) was prepared and components then omitted one at a time to determine the effect upon the growth of the organism. Gladstone also showed that staphylococci can be trained to dispense with added amino-acids if these are withdrawn progressively from the medium and their nitrogen equivalent supplied by ammonium ions. The same procedure was used to obtain the non-exacting cultures of *Staph. aureus* 209 and 6773 described later in this paper.

Investigation of glutamic acid assimilation. The passage of glutamic acid from the external environment into the cells and its accumulation there as the free amino-acid was investigated as described previously (Gale, 1947*a*; Gale & Mitchell, 1947; Gale & Taylor, 1947). Quantities of glutamic acid are expressed in terms of μ l.; 22.4 μ l. glutamic acid = 1 μ mol.

Preliminary investigations

Effect of increased resistance on the blocking of glutamic acid assimilation

The investigations on the effect of penicillin on glutamic acid assimilation (Gale & Taylor, 1947) were carried out with a strain of *Staph. aureus* sensitive to 0.08 unit penicillin/ml. With this organism the assimilation of glutamic acid was completely prevented in 90 min. by the addition of 5 units penicillin/ml. and in 180 min. by 0.1 unit/ml. In order to determine whether the effective concentration of penicillin varied with the resistance of the organism, the experiment was repeated with *Staph. aureus* 6773 trained to a resistance of 60 units/ml. Penicillin was added to cultures after 3½ hr. growth and the cultures then left in the incubator for a further period of 3 hr.—a period equivalent to that required in former tests for the limiting concentration of penicillin to exert its full effect. The organisms were then harvested and their ability to take up glutamic acid determined as previously described (Gale & Taylor, 1947). In this case the assimilation was unaffected by 1.0 unit penicillin/ml., decreased by 44 % in the presence of 10 units/ml., and completely prevented by 100 units/ml. It is clear that an increase in the resistance of the culture as tested by the growth inhibition was accompanied by an increase in the amount of penicillin needed to prevent the passage of glutamic acid into the cell.

Inhibition of internal metabolism

Previous studies have shown that penicillin interferes with the passage of glutamic acid across the cell wall. This process was studied by investigating alterations in the level of free glutamic acid within the cell. This level is affected by the rate of passage of the amino-acid into the cell and by the rate of its utilization within the cell (Gale & Mitchell, 1947). Consequently if the passage of glutamic acid into the cell is to be studied by this method, it is necessary to inhibit its utilization within the cell. Utilization involves at least two processes: condensation of the amino-acid, with others, into protein (Gale, 1947*b*),

and entrance into the 'metabolic pool' of the cell, without protein formation (Gale & Mitchell, 1947). The protein synthesis takes place only in growing cells and can be eliminated by working with well-washed suspensions of cells. The remaining metabolism (transfer of glutamic acid to the metabolic pool), which takes place in resting and growing cells, can be inhibited by suitable concentrations of certain triphenylmethane dyes. The rates of these two processes vary with the age of the culture from which the cells are harvested. This can be shown (Gale, 1947*b*) by harvesting cells at various times during the growth period and estimating: (1) the internal level of free glutamic acid directly after harvesting; (2) the level attained within the cell after it has been allowed to come into equilibrium, in washed suspension, with an external glutamic acid concentration equal to that in the medium during growth; (3) the level attained within the cell treated as in (2) but in the presence of a concentration of crystal violet which inhibits the transfer of glutamic acid to the metabolic pool. Each level determined represents the balance between the rates of assimilation and utilization under the conditions used. Previous experiments of this nature were carried out with *Streptococcus faecalis* where it was shown that: (a) the rate of protein formation (represented by level 2 – level 1) was greatest during the early stages of growth, fell off during growth and ceased when growth ceased; (b) the rate of the dye-inhibitable metabolism (transfer into the metabolic pool, represented by level 3 – level 2), was greatest during the later stages of growth but fell rapidly after growth had ceased; (c) the rate of assimilation (represented by level 3) was approximately constant throughout the growth period.

In *Strep. faecalis* the rate of dye-inhibitable metabolism may approach half the rate at which the amino-acid enters the cell. When similar experiments were carried out on *Staph. aureus* it was found that the rate of the dye-inhibitable part of the glutamic acid utilization was much smaller, in comparison with the rate of assimilation, in these cells than in *Strep. faecalis*. *Staph. aureus* is about ten times as sensitive to crystal violet as *Strep. faecalis* when judged by the growth inhibition test; investigations on the assimilation of glutamic acid in the presence of crystal violet in experiments similar to those previously described (Gale & Mitchell, 1947) showed that a similar internal inhibition took place with a consequent increase in the level of free glutamic acid attained within the cell, but that optimal effects were obtained with a concentration of dye only 1/50–1/100 of that required for *Strep. faecalis* cells. In the latter the presence of the dye may result in an increase in the internal level of glutamic acid to 300–400 % of that attained in the absence of dye; in *Staph. aureus* the increase in level is 5–15 % of that attained in the absence of dye.

Fig. 1 shows the curves obtained for internal glutamic acid levels, determined under the three conditions described above, for *Strep. faecalis* and *Staph. aureus*. The picture of glutamic acid utilization differs in the two organisms. In *Staph. aureus* the rate which it is built into protein is nearly equal to the rate of uptake of glutamic acid in young cultures, so that little free glutamic acid accumulates in such cells; this rate decreases as growth continues, so that the amount of free glutamic acid in the cell increases and reaches a maximum level

at the end of growth when protein formation ceases. At all times during the growth phase the rate of the utilization process which is inhibitable by dye, i.e. transfer to the metabolic pool, is small in comparison with the rate of uptake of glutamic acid.

Fig. 1. Utilization of glutamic acid by *Strep. faecalis* and *Staph. aureus*. Curves represent internal level of glutamic acid (μl./100 mg. cells) in cells: (1) $\times$ — $\times$ harvested from growth medium containing 200 μl. glutamic acid/ml. (2) $\circ$ — $\circ$ incubated for 1 hr. at 37° in washed suspensions in salt solution containing 1 % glucose and 200 μl. glutamic acid/ml. (3) $\bullet$ — $\bullet$ incubated for 1 hr. at 37° in washed suspension in salt solution containing 1 % glucose, 200 μl. glutamic acid/ml. and crystal violet at a final concentration $1/10^4$ for *Strep. faecalis* and $1/10^6$ for *Staph. aureus*. Rate of 'condensation' of glutamic acid into protein in comparison with rate of assimilation is given by (2-1). Rate of entry of glutamic acid into metabolic pool, in comparison with rate of assimilation, given by (3-2).

In order, therefore, to study the process whereby glutamic acid passes across the cell wall, preparations of cells were made by harvesting cells from 6-7 hr. cultures, washing them in water and then treating the washed cells, in a suspension containing c. 1-2 mg. dry weight of cells/ml., with crystal violet in final dilution $1/10^6$. In such cells glutamic acid enters the cell until a steady state is obtained, and the level attained within the cell is determined by the ability of the cell to maintain a difference in concentration across the cell wall and is not affected by internal metabolic processes.

The assimilation process in cells of differing resistance to penicillin

Concentration gradient across cell wall

Taylor (1947) showed that the ability to concentrate glutamic acid inside the cells differs in different micro-organisms. All the Gram-positive bacteria examined have exhibited this ability, and, of the species studied, strains of *Staph. aureus* effected the highest internal concentration for a given external

concentration. It was thought that the sensitivity to penicillin might be correlated with this ability to concentrate glutamic acid across the cell wall. To test this, a number of bacteria of differing penicillin sensitivities were grown, and washed suspensions of cells treated with crystal violet were prepared as described above. These cells were then incubated for 1 hr. at 37° in a suitable buffer mixture containing 200 μ l. glutamic acid/ml. and 1 % glucose as energy source; after incubation the internal level of free glutamic acid was determined as previously described (Gale, 1947*a*). Table 1 shows

Table 1. *Penicillin resistance and internal concentration of glutamic acid*

Organisms grown for 6 hr. in medium B (Gale, 1947*a*) harvested and washed in water; suspended for 1 hr. at 37° at a suspension strength of approx. 1 mg. dry weight of cells/ml. in buffer-salt solution containing 200 μ l. glutamic acid/ml., 1 % glucose and crystal violet to a final dilution of 1/1,000,000 for *Staph. aureus* and *B. subtilis* or 1/10,000 for *Strep. faecalis*; cells then washed in water and internal glutamate concentration assayed.

Organism	Strain	Growth inhibition test (units penicillin/ml.)		Internal concentration of glutamic acid (μ l./100 mg. cells)
		Growth	No growth	
<i>Staph. aureus</i>	563	0.02	0.04	1165
<i>B. subtilis</i>	St.	0.04	0.06	26
<i>Staph. aureus</i>	209	0.05	0.08	560
<i>Staph. aureus</i>	D	0.06	0.08	660
<i>Strep. faecalis</i>	ST	6.0	8.0	534
<i>Staph. aureus</i>	6773	5.0	10.0	880
<i>Staph. aureus</i>	6773	15	20	880
<i>Staph. aureus</i>	6773	60	70	750
<i>Staph. aureus</i>	6773	250	300	740
<i>Staph. aureus</i>	6773	2000	4000	705
<i>Staph. aureus</i>	6773	6000	—	0
<i>Staph. aureus</i>	209 (P ₆₀ T ₃₅)	7000	—	0

that there is no correlation between the internal levels attained and the penicillin sensitivity as determined in the growth inhibition test. The organism 6773 was trained to a final resistance of 6000 units penicillin/ml. and tested at various stages during the training process. It can be seen that the internal level does decrease slowly as resistance increases, but this decrease bears little relation to the increase in penicillin resistance. It will be seen below that the ability to assimilate glutamic acid decreases as resistance increases and that the falling internal levels shown in Table 1 may be a reflexion of the fact that the internal environment is not saturated in the cells of high resistance. At the highest penicillin level (6000 units/ml.) the cells became Gram-negative, and it was no longer possible to determine any free glutamic acid within the cells. These results accord with the findings of Taylor (1947) that free amino-acids are not concentrated within Gram-negative cells.

Dependence of internal concentration on external concentration

In earlier studies on the assimilation of glutamic acid by *Strep. faecalis*, curves were given showing the dependence of the internal concentration on the external concentration (Gale, 1947*a*). These curves were, however, determined

with washed cell suspensions, and the internal levels found were affected by internal metabolic processes. When the curves are redetermined using dye-treated washed cells, they then assume the shape shown in Fig. 2. The internal concentration of glutamic acid is independent of the external concentration except for very low values of the latter. Fig. 2 shows curves obtained for *Strep. faecalis* (cf. Gale, 1947*a*, Fig. 8) and for *Staph. aureus*. The values attained at saturation are different in the two species of cell, but it is improbable, as shown above, that this is a factor in determining penicillin sensitivity.

Fig. 2. Relation between internal and external glutamate concentration. Cells harvested from deficient medium, washed and treated with crystal violet to inhibit internal metabolism, incubated for 1 hr. in presence of 1% glucose and glutamic acid at concentrations shown, and internal level of free glutamic acid then assayed.

Further, the slope of the curve relating internal concentration to external concentration for low values of the latter is steeper for the *Staph. aureus* than for the *Strep. faecalis* strain used. This means that *Staph. aureus* effected a more efficient concentration of glutamic acid at low external concentrations than *Strep. faecalis*. The *Staph. aureus* strain used is sensitive to 0.08 unit penicillin/ml., while the *Strep. faecalis* strain is resistant to 5 units penicillin/ml. As a measure of the slope of the concentration curve, we have defined the 'assimilation constant' as that external concentration which gives rise to an internal concentration equal to half that attained at saturation. The reciprocal of the assimilation constant is thus a measure of the ability of the cell to assimilate glutamic acid.

Variation of 'assimilation constant' with penicillin resistance

Fig. 3 shows the internal concentration curves, expressed as percentage internal saturation, for organisms of differing penicillin sensitivity. The organisms were grown as usual for 6 hr., harvested, washed, treated with

crystal-violet and the usual suspensions made. The internal level of glutamic acid was determined over a range of external concentrations. When the external concentration is small, assimilation may result in a lowering of the external concentration; to avoid significant alterations of this nature, the cells were suspended in large volumes of solution such that the suspension contained 0.2–0.5 mg. dry weight bacteria/ml.

Fig. 3. Variation of assimilation constant with penicillin resistance. Penicillin resistance as determined by growth inhibition test is shown in units/ml. at the top of each curve. All curves—except the broken one—refer to *Staph. aureus* strains.

Determinations were first carried out with four strains of *Staph. aureus* of differing penicillin resistances. Fig. 3 shows that the value of the assimilation constant increases with the penicillin resistance. In order to determine whether this was a true correlation, the organism 6773 of resistance 15 units penicillin/ml. was trained by the usual method to an ultimate resistance of 6000 units/ml., and the assimilation constant was determined on cells having levels of resistance of 15, 60, 250, 2000 and 4000 units/ml. respectively. Fig. 3 shows that the assimilation constant increased markedly as the resistance increased. Fig. 4 shows the correlation between the value of the assimilation constant and the log of the penicillin resistance for the various organisms tested. Most of the points were determined with *Staph. aureus*, but values obtained for a sensitive *Bacillus subtilis* and a moderately resistant *Strep. faecalis* appear to fall on the curve obtained with the staphylococci. It is clear that the assimilation constant increases rapidly with penicillin resistance. Consequently the ability of the cell to assimilate glutamic acid decreases as the resistance increases; serial subcultivation in penicillin selects resistant cells from the culture, and these cells have less efficient assimilatory mechanisms than the sensitive parent cells.

Nature of the highly resistant cells

When the resistance of *Staph. aureus* 6773 is increased from 2000 to 6000 units/ml., the cells undergo changes in morphological, cultural and staining characteristics as described by Bellamy & Klimek (1948*b*) for *Staph. aureus* 209.

The resistant organisms obtained from both strains are highly pleomorphic, strictly aerobic and Gram-negative (see Pl. 1). The general properties and amino-acid metabolism of these organisms have been described by Bellamy & Klimek (1948*b*) and by Gale & Rodwell (1948), respectively. The amino-acid

Fig. 4. Relation between value of assimilation constant and log of penicillin resistance expressed in units/100 ml. ● *Staph. aureus*. ○ *B. subtilis*. ◐ *Strep. faecalis*. (Ordinate = assimilation constant expressed in μl. glutamic acid/ml.).

breakdown by the two resistant organisms is significantly the same as that accomplished by the parent strains; all four organisms possess arginine dihydrolase and urease to approximately the same degree of activity.

Relation between amino-acid synthesis and penicillin resistance

Synthetic abilities of Staphylococcus aureus mutants obtained by penicillin-training procedures

The nutritional requirements of the various strains of *Staph. aureus* obtained in this work were studied by the method of Gladstone (1937). Gale & Rodwell (1948) found that, while the parent penicillin-sensitive strains of *Staph. aureus*

6773 and 209 were exacting towards nicotinamide, aneurin and a range of amino-acids, the highly resistant organisms obtained therefrom could grow in a medium containing salts, ammonium ions, glucose and aneurin.

Table 2. *Relation between synthetic abilities and penicillin resistance in Staphylococcus aureus*

Mutants were selected by training in the presence of penicillin.

Nutritional requirements investigated as described by Gladstone (1937)

+ = presence essential for growth in same time as in complete medium; no growth takes place in absence.

— = presence not essential for growth in same time as in complete medium.

(88) etc. = in absence of the given amino-acid, growth equal to that in complete medium took place after a delay of 88 etc. hr.

Organism	...	6773	6773	6773	209	209	209 (reverse mutants)	209
Gram reaction	...	+	+	—	+	—	+	+
Penicillin resistance:								
after 36 hr.	...	5	2000	6000	0.05	7000	400	1000
after 88 hr.	...	9	2000	6000	0.05	7000	8000	1700
Nutrients:								
Nicotinamide		+	+	—	+	—	—	—
Aneurin		+	+	+	+	+	+	+
Proline	(88)	(88)	—	—	+	—	(88)	(64)
Histidine	+	+	—	—	+	—	(64)	(64)
Valine	+	+	—	—	+	—	(88)	(88)
Glycine	+	(88)	—	—	+	—	(160)	+
Glutamic acid	(40)	—	—	—	—	—	(64)	—
Aspartic acid	+	(64)	—	—	+	—	(160)	(64)
Leucine	(40)	(40)	—	—	+	—	—	—
Cystine	+	+	—	—	+	—	+	+
Phenylalanine	(40)	(64)	—	—	—	—	—	—
Arginine	+	+	—	—	—	—	+	+

Table 2 shows the nutritional requirements of these organisms and of other mutants obtained therefrom. The organism selected from *Staph. aureus* 6773 at a resistance level of 2000 units/ml. is a pigmented, Gram-positive *Staph. aureus* with nutritional requirements intermediate between those of the sensitive parent strain and those of the highly resistant non-exacting organism. Thus the organism resistant to 2000 units is completely independent of pre-formed glutamic acid and will adapt to the absence of glycine and aspartic acid in 88 and 64 hr. respectively, while the parent organism cannot dispense with either glycine or aspartic acid and adapts to the absence of glutamic acid in 40 hr.

Table 2 also sets out the nutritional requirements of two organisms obtained by reverse mutation from the highly resistant 209 (P₆₀T₃₅). These organisms were isolated by the procedure described by Bellamy & Klimek (1948*b*) and are pigmented Gram-positive *Staph. aureus*. When tested for penicillin resistance by the usual method involving incubation for 36 hr. the two organisms are resistant to 400 and 1000 units/ml. respectively. Likewise their nutritional requirements are wide on initial incubation. On continued incubation, how-

ever, they adapt to the absence of several amino-acids: presumably the experimental procedure results in the selection of less exacting mutants (i.e. better amino-acid synthesizers). On initial test both organisms required proline, histidine, valine, aspartic acid, cystine, glycine and arginine while the organism resistant to 400 units/ml. also required glutamic acid. After 88 hr. incubation growth of the same organism occurred in the absence of proline, histidine, valine and glutamic acid, whereas the 1000-unit organism no longer required preformed proline, histidine, valine and aspartic acid. At the same time, the resistance of both organisms had approximately doubled.

The highly resistant 209 (P₈₀T₃₅) organism can synthesize nicotinamide, and this ability is retained by the reverse (i.e. amino-acid requiring) mutants. It is interesting that whereas the initial parent 209 does not require added arginine, both reverse mutants tested have lost the power of synthesizing arginine.

It appears from the data in Table 2 that the penicillin-resistant organisms have high synthetic abilities; that penicillin training results in the selection of synthetically competent cells; and that reverse mutation to a more sensitive form is accompanied by loss of synthetic ability, the sensitivity of the reverse mutants depending upon the degree of synthetic competence. These results correlate well with the previous findings that increase in penicillin resistance is accompanied by decreased efficiency in amino-acid assimilation.

Penicillin resistance of non-exacting mutants of Staphylococcus aureus

Gladstone (1937) showed that nutritionally non-exacting strains of *Staph. aureus* can be obtained from exacting strains when the organisms are serially subcultivated in medium from which essential amino-acids are progressively withdrawn. Using the technique described by Gladstone we have endeavoured to train the strains 6773 and 209 to synthesize their amino-acid requirements from ammonia and glucose. It has been possible to train both strains to dispense with the addition of all amino-acids other than cysteine and histidine. Gladstone recorded that some strains can dispense with cysteine if provided with thiolacetic acid, but we have not been able to accomplish this change with the two organisms under investigation. The penicillin resistance of the comparatively non-exacting cultures was determined as usual and compared with that of the exacting parent cultures. Table 3 shows that the non-exacting mutants selected by the nutritional restriction procedure have much higher resistance than the parent strains; in the case of *Staph. aureus* 209, the parent strain requires seven amino-acids and is sensitive to 0.05–0.06 unit penicillin/ml., while the mutant, which requires only cysteine and histidine as amino-acid nutrients, is resistant to 250 units/ml.

DISCUSSION

The early studies on assimilation of glutamic acid by *Staph. aureus* showed that the addition of penicillin to the medium during growth was followed by an impairment of the assimilation process which eventually led to a complete cessation of the passage of the amino-acid into the cell (Gale & Taylor, 1947).

The amino-acid which accumulates within the cell acts as a reservoir which is drawn upon for anabolic processes, so that, when the entry of the amino-acid is prevented, the reservoir is depleted and anabolic processes cease. When the culture is serially subcultivated in increasing concentrations of penicillin, resistant mutants are selected, and the higher the resistance of these mutants, the less efficient is their assimilation of glutamic acid. Growth of a cell involves synthesis of protein and consequently the provision of its constituent amino-

Table 3. *Relation between synthetic abilities and penicillin-resistance in Staphylococcus aureus*

Mutants obtained by training in depleted media (Gladstone, 1937)

Organism ...	6773	6773	209	209
Penicillin resistance (units/ml.) 36 hr. test	5	100	0.06	250
Nutrients required:				
Nicotinamide	+	+	+	+
Aneurin	+	+	+	+
Proline	+	—	+	—
Histidine	+	+	+	+
Valine	+	—	+	—
Glycine	+	—	+	—
Glutamic acid	+	—	—	—
Aspartic acid	+	—	+	—
Leucine	+	—	+	—
Cystine	+	+	+	+
Arginine	+	—	—	—

acids; such provision can be made either by synthesis or by assimilation of preformed amino-acids from the external environment. If a cell loses the ability to synthesize a given amino-acid, then the growth of that cell becomes dependent upon its ability to assimilate that amino-acid preformed. The resistant mutants are less able to assimilate glutamic acid than the sensitive ones, and cells selected for high levels of penicillin resistance are those with small assimilation efficiency. If these cells are to grow, they must be able to synthesize the amino-acids which they are unable to take from the environment; if the penicillin resistance is pushed to its limit, then it follows that the cells then selected must be able to synthesize all those amino-acids whose assimilation is prevented by penicillin.

The assimilation studies have been restricted to glutamic acid, since this is the only amino-acid which can be effectively studied by the present technique (Gale, 1947*a*). It has always been considered possible that the effects studied were symptomatic of general changes in assimilatory processes rather than specific for glutamic acid. The results given in the latter part of this paper support this. The increase in penicillin resistance is correlated with a decrease in the ability to assimilate glutamic acid, but when we examine the synthetic abilities which enable the organism to overcome the assimilation impairment, we find that there is a general gain in the ability to synthesize amino-acids, while glutamic acid itself appears to be comparatively unimportant. For

example, glutamic acid is not an essential nutrient for *Staph. aureus* 209 at any stage. Glutamic acid is taken into the cell by an active process requiring energy, and the information at our disposal suggests that many other amino-acids are assimilated in a like manner, e.g. aspartic acid and histidine (Gale, 1947*a*). The passage of lysine into the cell is a physical process not requiring a concomitant energy-yielding process, and may be exceptional; the assimilation of lysine is not prevented by penicillin (Gale & Taylor, 1947), and lysine is not an essential nutrient for the organisms studied here.

It seems probable that penicillin acts by preventing the passage across the cell wall of those amino-acids whose migration involves an active process. Resistant mutants are those whose growth is less dependent upon this kind of assimilation and whose ability to make protein escapes from the restriction imposed by penicillin, by synthesizing amino-acids instead of assimilating them preformed from the environment. Knight (1936) and Lwoff (1943) have both put forward the hypothesis that organisms become nutritionally exacting as a result of prolonged growth in media rich in the essential growth factors and amino-acids; growth is supposed to proceed more effectively by the assimilation of preformed protoplasmic components rather than by synthesis, and in due course, the synthetic abilities are lost by disuse. The action of penicillin is to reverse this process.

The facts presented in this communication have been obtained with mutants of *Staph. aureus*, and it is important to determine whether the results are applicable to other organisms, to explain differences in penicillin resistance between genera and species. Table 4 presents data, gathered from the literature, concerning the amino-acid requirements and penicillin sensitivity of a variety of organisms. It can be seen that there is general agreement with the hypothesis that penicillin sensitivity can be correlated with the dependence of the growth process on preformed amino-acid assimilation, i.e. that the more synthetically competent the organism, the greater its resistance to penicillin. The assimilation studies so far described have been carried out with Gram-positive organisms, since the ability of these organisms to concentrate amino-acids within the cell has provided the point of experimental attack.

The nutritional requirements of certain Gram-negative organisms indicate that these must also be able to assimilate some preformed amino-acids, although such assimilation is not accompanied by an internal concentration of the free amino-acid prior to further utilization. The Gram-positive group of bacteria is, in general, highly exacting towards amino-acids and sensitive to penicillin; but within the Gram-negative group we also find degrees of disability in amino-acid synthesis, and there is again a correlation between the degree of synthetic disability and sensitivity to penicillin.

The question arises, to what extent is this prevention of assimilation processes the primary point of action of penicillin? Penicillin only kills cells that are growing in its presence; the assimilation process is blocked only when the cell grows in its presence. This suggests that the primary action of penicillin is to inhibit the formation of a substance whose synthesis is essential for the assimilation process to occur. If nutritionally exacting cells are to grow—in

Table 4. Nutritional requirements and penicillin resistance of certain bacteria: collected data

	<i>Escherichia coli</i>	<i>Shig. dysenteriae</i>	<i>Proteus vulgaris</i>	<i>Eberthella typhosa</i>	<i>Lacto-bacillus casei</i>	<i>C. diph-theriae</i>	<i>Staph. aureus</i>	<i>Bacillus anthracis</i>	<i>Strep. haemolyticus</i>	<i>Neisseria intracellularis</i>	<i>Neisseria gonorrhoeae</i>
Gram reaction	—	—	—	—	+	+	+	+	+	—	—
Penicillin sensitivity range in units/ml. (Duguid, 1946) ...	30–300	30–100	3–30	10–30	1–10	0.1–1	0.01–0.1	0.01–0.1	0.01–0.03	0.003–0.03	0.003–0.01
Glycine	—	—	—	—	•	•	+	+	•	—	+
Alanine	—	—	—	—	•	•	•	•	+	—	—
Serine	—	—	—	—	•	•	+	+	+	+	+
Cystine	—	—	—	—	•	•	•	•	+	—	—
Phenylalanine	—	—	—	—	•	•	+	+	+	—	—
Tyrosine	—	—	—	—	•	•	+	+	+	—	—
Tryptophan	—	—	—	+	•	•	•	•	+	—	—
Threonine	—	—	—	—	•	•	•	•	+	—	—
Valine	—	—	—	—	•	•	•	•	+	—	—
Leucine	—	—	—	—	•	•	•	•	+	—	—
Isoleucine	—	—	—	—	•	•	•	•	+	—	—
Glutamic acid	—	—	—	—	•	•	•	•	+	—	—
Aspartic acid	—	—	—	—	•	•	•	•	+	—	—
Histidine	—	—	—	—	•	•	•	•	+	—	—
Lysine	—	—	—	—	•	•	•	•	+	—	—
Arginine	—	—	—	—	•	•	•	•	+	—	—
Methionine	—	—	—	—	•	•	•	•	+	—	—
Proline	—	—	—	—	•	•	•	•	+	—	—
Hydroxyproline	—	—	—	—	•	•	•	•	•	—	—
Nicotinic acid	—	+	+	—	•	•	•	•	•	—	—
Aneurin	—	—	—	—	•	•	•	•	•	—	—
Riboflavin	—	—	—	—	•	•	•	•	•	—	—
Pantothenate	—	•	—	—	•	•	•	•	•	—	—
Pyridoxal	—	—	—	—	•	•	•	•	•	—	—
Biotin	—	—	—	—	•	•	•	•	•	—	—
Folic acid	—	—	—	—	•	•	•	•	•	—	—
Purines, etc.	—	—	—	—	•	•	•	•	•	—	—
Nutrition references:	1, 2	3	4	5	6	7, 8	9	10	11, 12	13, 14	

(See References)

the absence of mutational changes—they must synthesize this mechanism, necessary for the assimilation of preformed amino-acids, as new cells are formed. Inhibition of the synthesis of a part of this mechanism would impair the assimilation process, and the sensitivity of the cell to penicillin would then be determined by the dependence of its growth processes on the assimilation mechanism. Present indications are that the assimilation mechanism involves ribonucleic acid synthesis which is the primary point of penicillin attack. It is hoped to deal with this point in a later publication.

REFERENCES

Figures in brackets preceding some references below, refer to figures indicating references in Table 4.

- BELLAMY, W. D. & KLIMEK, J. W. (1948*a*). The relation between induced resistance to penicillin and oxygen utilization. *J. Bact.* **55**, 147.
- BELLAMY, W. D. & KLIMEK, J. W. (1948*b*). Some properties of penicillin-resistant staphylococci. *J. Bact.* **55**, 153.
- DEMEREK, M. (1945). Genetic changes in *Staphylococcus aureus* producing strains resistant to various concentrations of penicillin. *Ann. Mo. bot. Gdn.* **32**, 131.
- (1) DORFMAN, A., KOSER, S. A., REAMES, H. R., SWINGLE, K. F. & SAUNDERS, F. (1939). Nicotinamide and related compounds as essential growth substances for dysentery bacilli. *J. infect. Dis.* **65**, 163.
- DUGUID, J. P. (1946). The sensitivity of bacteria to the action of penicillin. *Edin. med. J.* **53**, 401.
- (5) DUNN, M. S., SHANKMAN, S., CAMIEN, M. N. & BLOCK, H. (1947). The amino-acid requirements of twenty-three lactic acid bacteria. *J. biol. Chem.* **168**, 1.
- (3) FILDES, P. (1938). The growth of *Proteus* on ammonium lactate plus nicotinic acid. *Brit. J. exp. Path.* **19**, 239.
- (4) FILDES, P., GLADSTONE, G. P. & KNIGHT, B. C. J. G. (1933). The nitrogen and vitamin requirements of *B. typhosus*. *Brit. J. exp. Path.* **14**, 189.
- (11) FRANTZ, I. D. (1942). Growth requirements of *Meningococcus*. *J. Bact.* **43**, 757.
- GALE, E. F. (1947*a*). The assimilation of amino-acids by bacteria. 1. The passage of certain amino-acids across the cell-wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.
- GALE, E. F. (1947*b*). The assimilation of amino-acids by bacteria. 6. The effect of protein synthesis on glutamic acid accumulation and the action thereon of sulphathiazole. *J. gen. Microbiol.* **1**, 327.
- GALE, E. F. (1947*c*). Correlation between penicillin resistance and assimilation affinity in *Staphylococcus aureus*. *Nature, Lond.*, **160**, 407.
- GALE, E. F. & MITCHELL, P. D. (1947). The assimilation of amino-acids by bacteria. 4. The action of triphenylmethane dyes on glutamic acid assimilation. *J. gen. Microbiol.* **1**, 299.
- (8) GALE, E. F. & RODWELL, A. W. (1948). Amino-acid metabolism of penicillin resistant staphylococci. *J. Bact.* **55**, 161.
- GALE, E. F. & TAYLOR, E. S. (1947). The assimilation of amino-acids by bacteria. 5. The action of penicillin in preventing the assimilation of glutamic acid by *Staphylococcus aureus*. *J. gen. Microbiol.* **1**, 314.
- (7) GLADSTONE, G. P. (1937). The nutrition of *Staph. aureus*: nitrogen requirements. *Brit. J. exp. Path.* **18**, 322.
- (9) GLADSTONE, G. P. (1939). Interrelationships between amino-acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

- (12) GROSSOWICZ, N. (1945). Growth requirements of *Neisseria intracellularis*. *J. Bact.* **50**, 109.
- (13) KANE, L. W. & MUELLER, J. H. (1944). Growth requirements of *Neisseria gonorrhoeae*. *J. Bact.* **47**, 287.
- KNIGHT, B. C. J. G. (1936). Bacterial nutrition. *Spec. Rep. Ser. med. Res. Coun., Lond.* (London, H.M. Stat. Office).
- (2) KOSER, S. A. & WRIGHT, M. H. (1943). Experimental variation of nicotinamide requirement of dysentery bacilli. *J. Bact.* **46**, 239.
- LWOFF, A. (1943). *L'Evolution physiologique*. Paris: Hermann et Cie.
- (10) McILWAIN, H. (1940). The nutrition of *Streptococcus haemolyticus*. Growth in a chemically defined medium: need for vitamin B₆. *Brit. J. exp. Path.* **21**, 25.
- (6) MUELLER, J. H. (1940). Nutrition of diphtheria bacillus. *Bact. Rev.* **4**, 97.
- TAYLOR, E. S. (1947). The assimilation of amino-acids by bacteria. 3. Concentration of free amino-acids in the internal environment of various bacteria and yeasts. *J. gen. Microbiol.* **1**, 86.
- (14) WELTON, J. P., STOKINGER, H. E. & CARPENTER, C. M. (1944). A chemically defined medium for the cultivation of the *Gonococcus*. *Science*, **99**, 372.

EXPLANATION OF PLATE

Fig. 1. *Staph. aureus* 6773, parent strain; Gram stain; photographed through green filter. Magnification $\times 4800$.

Fig. 2. Highly resistant organism derived from *Staph. aureus* 6773; Gram stain; photographed through green filter. Magnification $\times 4800$.

Photomicrographs by V. C. Norfield, Strangeways Research Laboratory, Cambridge.

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Fig. 1

Fig. 2

E. F. GALE & A. W. RODWELL—THE NATURE OF PENICILLIN RESISTANCE IN
STAPH. AUREUS. PLATE 1