The Assimilation of Amino-acids by Bacteria

8. Trace Metals in Glutamic Acid Assimilation and their inactivation by 8-Hydroxyquinoline

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With a Note on Relative Dissociation constants of some Metal-oxine Complexes

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SUMMARY: The assimilation of glutamic acid by washed suspensions of *Staphylococcus aureus* is inhibited by 8-hydroxyquinoline (oxine). Fermentation and respiration are also inhibited but only at higher concentrations of oxine than those required for the complete inhibition of assimilation. The inhibition of assimilation by oxine can be annulled by washing the cells in water or in salts of certain divalent metals; manganese salts are more effective than those of Co, Fe or Mg. The inhibition produced by high concentrations of oxine can be decreased by metals known to form chelate complexes with oxine; the addition of an equivalent of Mn completely abolishes the inhibition.

Glutamate assimilation in *Staph. aureus* is impaired by a deficiency during growth of either Mn or Mg, but is not abolished unless both metals are absent. Washed suspensions of Mn- and Mg-deficient cells are activated by the addition of either Mn or Mg; Mg is more effective than Mn in activating washed cells whereas Mn is more effective than Mg in activating growing cells.

Assimilation of glutamic acid is activated by either Mn or Mg, Mn being utilized preferentially during growth. In the absence of Mn, the organisms utilize Mg and the sensitivity of assimilation to inhibition by oxine decreases.

Certain amino-acids are taken up by Gram-positive bacteria and are concentrated in the free state inside the cell before taking part in the cell metabolism (Gale, 1947 *a*, 1947 *b*; Gale & Mitchell, 1947; Taylor, 1947). Amino-acids may pass across the cell-wall into the cell by diffusion, as in the case of lysine, or by an active cell process as in the case of glutamic acid. It is this latter energy-requiring transfer of amino-acids which is inhibited by penicillin (Gale & Taylor, 1947) and the sensitivity of a bacterial cell to penicillin is determined by the dependence of its growth on these assimilatory processes, since cells which can synthesize their essential amino-acids, and are consequently independent of assimilation of the preformed amino-acids, are resistant to penicillin (Gale & Rodwell, 1948, 1949). Complete understanding of the action of penicillin and of the assimilation of amino-acids awaits the elucidation of the mode of active transfer across the cell-wall. Studies of the action of inhibitors of the transfer might help in the elucidation. It seems probable that any inhibitor whose primary action is on assimilation would have an antibacterial 'spectrum' similar to that of penicillin.

The present study was prompted by the finding of Albert, Rubbo, Goldacre & Balfour (1947) that 8-hydroxyquinoline (oxine) has such a 'spectrum'. These workers found that of the seven possible isomers of hydroxyquinoline, only 8-hydroxyquinoline was antibacterial and, further, was the only isomer able to form chelate complexes with metals. The inhibition of the growth of Gram-positive bacteria could be annulled by the addition to the medium of four equivalents of cobalt whereas that of Gram-negative bacteria was annulled by four equivalents of zinc, iron or, in some species, copper, but not by cobalt at that concentration. They suggested that inhibitors of this nature 'function by disorganising the trace-metal mechanism essential for bacterial growth and that this effect depends upon combination between the drug and some metals in the bacterial surface'.

Mellor & Maley (1947, 1948) studied the stability of co-ordination complexes formed by metals and various chelating agents and found that complexes with the divalent metals fell into the following order of decreasing stability: Pd, Cu, Ni, Co, Zn, Cd, Fe, Mn, Mg. The order appears to be approximately the same for all organic compounds forming such complexes. The authors do not quote values for oxine but Mitchell (see Note, p. 385) has confirmed that the metals Co, Fe, Mn, Mg fall into the same order of stability for the complexes with oxine.

MATERIAL AND METHODS

The organism was the strain *Staph. aureus* D used for the investigations on the inhibition of glutamic acid assimilation by penicillin (Gale & Taylor, 1947).

General. The growth media, preparation of bacterial suspensions and methods for assay of glutamic acid within the cells were as previously described (Gale, 1947*a*). In general, *Staph. aureus* D was grown in a medium containing little free glutamic acid, the cells harvested after 6 hr. at 37° or 12 hr. at 30°, washed once and their internal free glutamic acid content measured; the cells were then suspended in buffered salt solution containing glutamic acid (200 μ l./ml.) and glucose (1.0 %) together with added test substances, and incubated at 37° in a final suspension strength corresponding to 1–2 mg. dry weight of organism/ml. After incubation for a given period, assimilation was stopped by rapid cooling to 0°, the cells centrifuged down, washed once in water and their internal glutamic acid content again measured. Concentrations of glutamic acid are expressed in μ l. equivalent to CO₂ liberated; thus 22.4 μ l. = 1 μ mol.

Metal-deprived medium. This medium was made up as follows: Na₂HPO₄, 4.0 g.; KH₂PO₄, 1.0 g.; NaCl, 1.0 g.; (NH₄)₂SO₄, 1.0 g.; arginine monohydrochloride, 1.0 g.; 10 % Marmite solution, 10.0 ml.; glass-distilled water, 980 ml. Glucose, 10.0 g. in 20 ml. water. The salts, arginine and Marmite were dissolved in 50 ml. glass-distilled water, and the pH adjusted to 8.5–9.0. The medium was then freed from polyvalent ions by alternate reaction with 8-hydroxyquinoline and extraction with chloroform by the method of Waring & Werkman (1942) except that about 50 mg. oxine were used for each treatment, the pH adjusted to an alkaline value and the process repeated five or six times. The first two or three chloroform extracts were green but the fifth was usually colourless. The efficiency of removal of ions such as Mn and Mg depends upon the

alkalinity of the medium and it is improbable that anything more than a partial removal of Mg is possible by this method. The glucose solution was similarly treated by oxine to remove metals; three treatments usually gave colourless chloroform extracts. The glucose was acidified with phosphoric acid and sterilized separately. The medium was made up to volume with glass-distilled water, the pH adjusted to 7.0 and solutions of trace elements added to replace those removed by the oxine treatment. These were used in the following final concentrations: Mg^{++} , $\text{m}/6 \times 10^3$; Fe^{++} , $\text{m}/6 \times 10^4$; Mn^{++} , $\text{m}/10^6$; Co^{++} and Zn^{++} , $\text{m}/6 \times 10^6$. The metals other than manganese were used as sulphates ('Analar' grade, British Drug Houses, Ltd.); the magnesium sulphate was treated with oxine at pH 7.0 to decrease possible traces of manganese. Mg-free manganese sulphate was prepared from potassium permanganate. All glass-ware was freed from metals by the method of Waring & Werkman (1942) and the media were sterilized in Pyrex flasks capped by Pyrex glass beakers.

The media were aerated during growth by bubbling filtered air through glass capillary tubes. The inoculum was prepared from the cells of an 18 hr. culture in medium A (Gale, 1947*a*) which were centrifuged down, washed twice in glass-distilled water and suspended in metal-deprived medium. An inoculum of *c.* 10^7 cells/l. medium was used and the cultures incubated at 30° for periods depending on the metal-deficiency of the medium (see below).

RESULTS

Sensitivity of Staph. aureus D to oxine

The threshold concentration of oxine inhibiting the growth of *Staph. aureus* D was determined by the method of serial dilution. It varied markedly with the nature of the medium. In medium A (casein digest, Marmite and glucose, Gale, 1947*a*) growth was inhibited for 48 hr. at 37° by 12–15 $\mu\text{g.}$ oxine/ml.; in medium B (salts, Marmite and glucose) growth was inhibited by 1.0–1.5 $\mu\text{g.}/\text{ml.}$ Since the two media differ mainly in their amino-acid concentration, the test was repeated in medium B to which vitamin-free acid-hydrolysate of casein (Ashe Laboratories) was added. With 0.1, 1.0 and 5.0 % casein hydrolysate, the threshold concentrations of oxine were 2.0, 4.0 and 10.0 $\mu\text{g.}$ oxine/ml. respectively.

Action of oxine on the accumulation of free glutamic acid within the cells

When *Staph. aureus* is grown in a medium rich in glutamic acid, the concentration of free glutamic acid in the cells rises throughout growth and becomes steady when cell-division ceases (Gale, 1947*b*). The concentration within the cell at any moment is determined by the balance between the rate at which the amino-acid enters the cell and the rate at which it is metabolized within the cell. The rate of assimilation is approximately constant throughout the growth period whereas the rate of protein formation is greatest during the early stages of growth and ceases when growth ceases; the altering balance between the two processes gives rise to the increasing concentration of free glutamic acid within the cell as growth continues. The action of penicillin in

inhibiting assimilation was first indicated by the fact that the free glutamic acid concentration within the cells decreased rapidly in penicillin-treated cultures (Gale & Taylor, 1947). Fig. 1 shows the effect on the internal concentration of glutamic acid of adding a bacteriostatic concentration of oxine to *Staph. aureus* D growing in medium A. The increase in cell-mass and in internal glutamic acid concentration is normal for about an hour after addition of the

Fig. 1 (a) Effect of addition of oxine to medium on growth of *Staph. aureus* D. Growth medium A; 50 μ g. oxine/ml. added after 4 hr. growth at 37°. (b) Effect of addition of oxine to growing culture on the internal accumulation of glutamic acid. Growth medium A; 50 μ g. oxine/ml. added after 4 hr. growth at 37°; conditions as (a).

oxine; after this, growth is slowed and the internal concentration falls. The concentration of oxine (50 μ g./ml.) was not sufficient to stop growth completely, because higher concentrations have complex actions on the general metabolism of the organism (see below). Four hours after the addition of oxine, growth starts again but the internal concentration of glutamic acid does not rise. This suggests that, as with penicillin, there has been interference with the passage of the amino-acid into the cell.

Action of oxine on glutamic acid assimilation in washed suspensions of Staphylococcus aureus D

The assimilation of glutamic acid can be measured in washed suspensions (Gale, 1947*a*). Penicillin does not affect assimilation by washed cells, it acts only on growing cells (Gale & Taylor, 1947). Oxine, on the other hand, inhibits glutamic acid assimilation in washed cells. Fig. 2 shows the increase in the internal concentration of free glutamic acid in the presence and absence of

oxine; concentrations of oxine which partially inhibit do so by diminishing the rate of passage of the amino-acid into the cell and not by decreasing the ability of the cell to accumulate glutamic acid. Fig. 3 shows the relation between the concentration of oxine and the inhibition of glutamate assimilation.

Fig. 2

Fig. 3

Fig. 2. Effect of oxine on the course of glutamic acid assimilation by washed suspensions of *Staph. aureus* D. Organism grown in medium B for 16 hr. at 30°; cells made into washed suspension and incubated in buffered salt solution containing 1.0 % glucose and 200 μ l. glutamic acid/ml. with oxine added as shown. Samples removed at intervals and assimilation stopped by rapid cooling to 0°.

Fig. 3. Inhibition of glutamic acid assimilation and of glucose fermentation in *Staph. aureus* D by addition of oxine to washed suspensions.

Action of oxine on general metabolism. The inhibition of assimilation by penicillin appears to be a specific effect as it occurs without affecting either the respiratory, fermentative or lysine-assimilation systems (Gale & Taylor, 1947). Table 1 shows the effect of 0.002 M oxine on certain metabolic activities of the

Table 1. *Metabolic activities of normal Staphylococcus aureus cells in the presence and absence of 0.02 M 8-hydroxyquinoline*

	Normal cells alone	Cells in presence of 0.002 M oxine
Respiration, Q_{O_2}	8	6
Glucose oxidation, Q_{O_2}	75	38
Glucose fermentation, $Q_{CO_2}^{acid}$	93	63
Lysine assimilation (μ l./100 mg. dry wt. cells)	44	36
Glutamic acid assimilation (μ l./100 mg. dry wt. cells)	440	30

organism, tested as described by Gale & Taylor (1947); at this concentration there is complete inhibition of glutamic acid assimilation and approximately 40 % inhibition of respiration and fermentation. Fig. 3 shows the relation

between oxine concentration and inhibition of fermentation; assimilation is markedly more sensitive than the glycolytic system; 0.0001 M oxine produces 60 % inhibition of the former while having no action on the latter, and the oxine concentration producing 50 % inhibition is 0.00008 M for assimilation and 0.0045 M for fermentation.

Reversal of oxine inhibition. The growth curves in Fig. 1 indicate that cells recover from oxine inhibition. To test whether the inhibition of assimilation is reversible, washed cells were incubated in 0.002 M oxine for 30 min. at 37°, centrifuged, and suspended in the usual buffer + glutamate + glucose medium. In control suspensions containing oxine there was complete inhibition of assimilation and 61 % inhibition of fermentation. With cells removed from the oxine solution, the inhibition of assimilation fell to 15 % and the inhibition of fermentation was completely abolished. Thus the inhibition can be reversed by removal of the oxine and resuspension of the cells in the usual salt mixture.

Reversal of oxine inhibition by metals. Albert *et al.* (1947) reversed growth-inhibition by oxine by adding to the medium four equivalents of cobalt. Since cobalt forms stable chelate complexes with oxine (Albert & Gledhill, 1947) this reversal might be due either to inactivation of the oxine by the cobalt or to replacement by cobalt of an essential metal inactivated or removed by oxine. If the former explanation were correct, then other complex-forming metals should be effective. This possibility was tested as follows: it was shown above that when cells were incubated in 0.002 M oxine for 30 min. at 37°, the inhibition was completely abolished by centrifuging the cells out of the oxine solution and resuspending them in buffered salt solution. When the preliminary incubation is carried out for 60 min. at 37° in 0.004 M oxine and the cells then washed once in water, neither assimilation nor fermentation recover completely but take place at rates *c.* 30–40 and 80 % respectively of the rates in untreated cells. The effect on assimilation and fermentation of washing these partially inhibited cells in solutions of various metallic sulphates can therefore be tested. In previous experiments, assimilation was tested in a buffered salt medium containing MgSO_4 (Gale, 1947*a*), but since magnesium forms weak complexes with oxine, it has been omitted from further experiments and the tests made in a medium containing 0.1 % KH_2PO_4 , 0.33 % Na_2HPO_4 and 0.1 % NaCl .

Table 2 shows the effect of washing the partially inhibited cells with solutions containing metals known to form complexes with oxine and to be of biological interest. The residual inhibition of assimilation is completely annulled by cobalt, ferrous iron and manganese and 70 % reversal is obtained with magnesium. The metals fall into the following order of decreasing efficiency: Mn, Co, Fe, Mg. The comparable results in column (c), Table 2, show that Mn is markedly more effective than Co at equal concentration (0.0004 M). This order resembles the order of stability of chelate complexes as determined by Mellor & Maley (1947, 1948; see above) except for the position of Mn, Cu and Zn. However, in parallel fermentation tests (Table 2) whereas Co, Mn, ferrous iron, and Mg all completely annulled the residual inhibition of fermentation, Cu and Zn in the concentrations tested abolished all fermentation. Attempts were made to use lower concentrations of these two metals but the glucose fermentation was inhibited

90 % by 0.00001 M-CuSO₄ or by 0.0002 M-ZnSO₄ so that concentrations of the order necessary to antagonize the oxine completely inhibit the fermentation. Since, under the conditions of test, fermentation provides the energy for the

Table 2. *Removal of oxine inhibition by washing with solutions of metallic ions*

Staph. aureus D was grown in medium B for 6 hr. at 37° and the cells made into washed suspension. The cells were incubated for 1 hr. at 37°, at a final suspension strength of 1–2 mg. dry weight/ml., in phosphate buffer pH 7.0 containing 0.004 M oxine and then centrifuged down. Samples of the partially inhibited cells were then washed once, either in distilled water or in metal solutions as below. The ability of the cells to assimilate glutamic acid was then tested in each case; the ability to ferment glucose was also tested by following the gas production in bicarbonate/CO₂ buffer system. If the glutamate assimilation of the untreated cells = X, that of the oxine-treated cells washed in water = Y, and that of the oxine-treated and metal-washed cells = Z, the percentage reversal of oxine inhibition as expressed below = 100 (Z – Y)/(X – Y). Comparable results are given in the same vertical column.

Metal	Concentration during washing (M)	Percentage removal of oxine inhibition			Inhibition of fermentation as percentage rate in control
		(a)	(b)	(c)	
Copper	0.001	0	—	—	100
	0.0002	0	—	—	100
Cobalt	0.01	57	—	—	28
	0.001	86	60	—	0
	0.0004	—	100	52	0
	0.0001	10	—	—	0
Zinc	0.001	0	—	—	100
Fe ⁺⁺	0.001	94	54	40	0
	0.0004	—	42	—	0
Fe ⁺⁺⁺	0.001	40	—	—	0
Manganese	0.0004	—	—	98	0
Magnesium	0.001	34	—	—	0
	0.004	51	—	67	0
	0.01	—	42	—	0

transfer of glutamic acid into the cells, there can be no recovery of assimilation in these cases. The inhibitory concentrations of Cu and Zn were not significantly lowered by the presence of oxine, so it appears that the fermentation enzymes in the cell have a higher affinity than oxine for these metals.

With the exception of Mn, the efficacy of the various metals in annulling oxine inhibition can be correlated with the stability of the chelate complexes; Mg forms the least stable complex and is the least effective. The data suggest that oxine combines reversibly with some substance in the bacterial cell and so inhibits glutamic acid assimilation; the presence of polyvalent metals in the environment results in withdrawal of the oxine from the complex within the cell, the efficiency of this action being determined by the relative stability of the chelate complexes inside and outside the cell.

Decrease of oxine inhibition by metals. The nature of the bacterial factor that reacts with oxine might be indicated by investigating the effect on assimilation of high concentrations of oxine in the presence of various metals with which it forms complexes. 0.004 M oxine was used and an equivalent concentration of various metals added. In most cases this produced heavy precipitates of

chelate compounds, but the precipitate did not interfere with the tests. Oxine alone completely inhibited assimilation, but the inhibition was diminished by the simultaneous presence of metals such as Co, Fe, Mn or Mg. The diminution was greatest when the metals were present in concentrations equivalent to the oxine. In some cases higher concentrations of metals gave inhibitions greater than that due to oxine alone, but these effects again corresponded to inhibition of the fermentation system. In concentrations equivalent to oxine, the metals

Table 3. *Diminution of oxine-inhibition of glutamic acid assimilation by the presence of metals*

Staph. aureus D was grown for 6 hr. at 37° in deficient medium B and the cells made into washed suspension. The ability to assimilate glutamic acid was then tested by incubating for 1 hr. in buffered salt solution (heavy-metal-free), glucose (1.0 %) and glutamic acid (200 μ l./ml.) and measuring the internal glutamic acid concentration before and after incubation. In parallel experiments, the assimilation was measured in the presence of 0.004 M oxine alone and in the presence of various metals, as their sulphates, as below. Results are expressed as percentage inhibition of assimilation, 0.004 M oxine causing 100 % inhibition. In each case, parallel determinations were made of the glucose fermentation of the organism.

Metal	Concentration (M)	Inhibition of glutamic acid assimilation (%)	Inhibition of glucose fermentation (%)
(Oxine alone)	0.004	100	40
Cobalt	0.001	83	—
	<i>0.002</i>	<i>25</i>	<i>32</i>
	0.004	55	—
	0.02	100	100
Fe ⁺⁺	0.001	76	—
	<i>0.002</i>	<i>48</i>	<i>15</i>
	0.004	94	92
Fe ⁺⁺⁺	0.002	79	75
Manganese	<i>0.002</i>	<i>0</i>	<i>17</i>
Magnesium	<i>0.002</i>	<i>87</i>	<i>20</i>

Values in italics represent those obtained when metallic-ion concentration was equivalent to oxine concentration.

decreased the inhibition in proportion to the stability of the corresponding oxine-metal complexes. Thus Co diminished the oxine inhibition by 75 % whereas Mg diminished it by c. 10 %, and iron was intermediate with diminution by 52 % (Table 3). However, Mn was again the most effective metal as, at a concentration equivalent to the oxine, it completely annulled inhibition of assimilation. Mn is thus better than Co and its activity cannot be correlated with its position in the stability series.

If the oxine is distributed between the bacterial factor and the metal in the environment in these experimental systems, the distribution is presumably determined by: (1) the relative affinities of the factor and of the metal for oxine; (2) the relative concentrations of the factor and the metal. Since the inhibition can be partly or completely removed by Mg at concentrations approximately the same as those effective in the case of Co, it is probable that the stability of the bacterial factor-oxine complex cannot be markedly greater than that of the magnesium-oxine complex. It is also probable that, when the oxine inhibition

is decreased by the presence of metal, if the metal added to the environment were identical with the bacterial factor, then inhibition would be completely abolished, since there would always be an excess of functional metal over inhibitor. This appears to be the case for Mn, which is the most effective metal in reversing inhibition (Table 2) and completely abolishes inhibition when present in amounts equivalent to the oxine (Table 3). It is interesting that, although Mn completely abolishes oxine inhibition of assimilation, it is less effective in decreasing the inhibition of fermentation.

Metals involved in glutamic acid assimilation

Effect of metal-deprivation of growth medium. The results described in the first section of this communication suggest that a metal is involved in the assimilation of glutamic acid by *Staph. aureus* and that this metal can combine reversibly with oxine. The identity of the metal may be established by removing metals one at a time from the medium and observing the effect on assimilation. Since the hypothetical metal combines with oxine, the method of Waring & Werkman (1942) for the removal of polyvalent metals from bacterial media is particularly suitable. The method was used by these authors for simple chemically defined media and is not so effective for complex media such as medium B used in these studies. However, when modified as described above, the method was sufficiently successful with medium B to give the following significant effects.

A large batch of medium was thoroughly 'stripped' of polyvalent metals, and divided into seven batches of 600 ml.; Cu^{++} , Co^{++} , Fe^{++} , Zn^{++} , Mn^{++} and Mg^{++} in the concentrations given on p. 371 were added, one different metal being omitted from each of six batches, the seventh batch containing all six metals. The batches were then inoculated and incubated; growth took place in all batches after a lag of 10–16 hr. The cells in each case were harvested when the turbidity was equal to that of a 12 hr. culture in medium B under normal conditions. The cells were washed once in glass-distilled water and their ability to assimilate glutamic acid tested by incubation for 45 min. in buffer solution (free from heavy metals) containing 1.0 % glucose and glutamic acid (200 $\mu\text{l.}/\text{ml.}$). The increase in the internal concentration of glutamic acid during incubation was not affected by the omission from the medium of Co, Cu, Zn; the increase in concentration was smaller in the Fe-deficient cells than in the control with all metals, and the increase was insignificant in the Mn- or Mg-deficient cells. The initial internal concentration before incubation with glutamic acid was higher in the Fe-deficient cells than in the other cells and it seemed probable that the smaller increase in the former on incubation was due to the fact that the initial concentration already approached saturation. To test this point, the rate of assimilation was determined in cells grown in media deficient in Fe, Mn, Mg, and no metals, respectively. Fig. 4 shows the results; the rate of glutamate assimilation is practically the same in the Fe-deficient as in the control cells, whereas it is markedly decreased in cells harvested from either Mn- or Mg-deficient media. The fermentation activity of the cells from the four cultures was determined; the rate was not significantly affected by either Fe- or Mn-deficiency whereas the rate in Mg-deficient cells was low.

Effect of manganese and magnesium deficiency on assimilation and fermentation

The results in Fig. 4 indicate that deficiency of either Mn or Mg impairs assimilation. The removal of Mg from the medium with oxine is unlikely to be highly efficient, since Mg forms chelate complexes of low stability. However,

Fig. 4. Rate of glutamic acid assimilation in metal-deprived cells. Organism grown in medium B deprived of metals by oxine treatment; the metals were then replaced with exceptions as below and the rate of glutamic acid assimilation measured for washed suspensions of cells harvested from the various cultures. Fermentation rates were determined on each suspension by measurement in Warburg manometers of the CO_2 evolution in the presence of glucose and CO_2 /bicarbonate buffer. ▲—▲ All metals replaced, $Q_{\text{CO}_2}^{\text{acid}}$ (glucose)=85. x—x Fe-deficient culture, $Q_{\text{CO}_2}^{\text{acid}}$ =87. ○—○ Mg-deficient culture, $Q_{\text{CO}_2}^{\text{acid}}$ =26. ●—● Mn-deficient culture, $Q_{\text{CO}_2}^{\text{acid}}$ =72.

the normal Mg requirements are high and by treating the medium with oxine at c. pH 9 it proved possible to lower the Mg concentration sufficiently to obtain significant effects, although these varied widely from batch to batch as judged by the length of the lag phase of growth and by the rate of fermentation of the harvested cells. In media deprived of both Mn and Mg, the lag phase varied from 5–20 hr. and the rate of fermentation was sometimes lowered to 30 % of that in the Mg-rich control. In two of 28 tests, no growth took place in the deprived medium, though it took place in portions of the same medium to which either Mn or Mg had been added. The Mn-tolerance of the organism

appeared to vary with the ionic balance in the medium because, although 40 $\mu\text{g. Mn/ml.}$ was tolerated in the full medium, concentrations of 0.5–1.0 $\mu\text{g./ml.}$ markedly decreased the rate of growth in Mg-deficient media.

Table 4. *Effect of Mn and Mg on rate of glutamic acid assimilation in deficient Staphylococcus aureus*

Staph. aureus D grown in medium B previously treated with oxine to remove metals and Mn or Mg then replaced as below. The deficient cells were made into washed suspension and the internal glutamic acid concentration determined. Samples were then incubated for 20 min. at 37° in buffered salt solution containing 1 % glucose, 200 $\mu\text{l. glutamate/ml.}$ and Mg or Mn as below. After 20 min. the samples were rapidly iced, centrifuged down and the new internal concentration assayed. The rate of assimilation is expressed as $\mu\text{l. glutamic acid increase in internal concentration/100 mg. cells/20 min.}$ Parallel determinations were made upon the fermentation rates of the cells.

Series	Concentration of metals added to growth medium		Rate of glutamate assimilation in presence of			Fermentation of glucose Q_{CO_2}
	Mn (M)	Mg (M)	No addition	0.001 M Mn	0.0001 M Mg	
1	0.00005	0.00017	224	—	—	—
	0.000042	0.00017	106	—	—	70
	0.000001	0.00017	156	220	200	66
	0.000001	0.00017	78	164	—	82
	Nil	0.00017	114	—	—	—
	Nil	0.00017	88	152	88	72
2	Nil	0.0001	20	108	74	66
	Nil	0.0001	21	52	—	60
	Nil	0.0001	14	93	93	—
	Nil	0.0001	0	30	0	56
3	0.000001	Nil	129	235	173	70
	0.000001	Nil	112	240	240	46
4	Nil	Nil	8	143	73	36
	Nil	Nil	0	150	150	48
5	Untreated medium B		280	302	240	106

Cells were grown in metal-free media to which various concentrations of Mn and Mg had been added. They were harvested early in the growth period and the rate of glutamic acid assimilation, and in most cases the rate of glucose fermentation, were determined; the results are shown in Table 4. The experiments were divided into four series: (i) the concentration of Mg was kept constant at $\text{M}/6000$ while that of Mn was decreased; (ii) Mg only was added, in sub-optimal concentrations; (iii) Mn was added but not Mg; (iv) neither Mn nor Mg was added. In the first series, the rate of assimilation decreased as the Mn content of the medium decreased but there was a significant activity even when no Mn was added. Assimilation was, however, further decreased and sometimes abolished when the Mg content was also decreased, as in the second series. The addition of Mn to $\text{M}/10^8$ to a medium without Mg gave an assimilation rate approximately half that attained in the normal untreated medium (series 3 and 5, Table 4), whereas there was little or no assimilation in cells harvested from media deficient in both Mn and Mg. It seems from these results that either Mn, or Mg, or both, are involved in the glutamic acid assimilation. However, the energy necessary for the transfer of glutamic acid into the cells

is derived from glycolysis, and Mg is known to be involved in several of the stages of the glycolytic cycle. It can be seen that Mg-deficiency is accompanied by a fall in the fermentation rate, and that assimilation is not completely stopped by Mn-deprivation alone. This suggests that Mg is involved in assimilation apart from its role in fermentation.

Reactivation of Mn- and Mg-deficient cells by metals. Tests were carried out on cells grown in media deprived of both Mn and Mg to determine whether assimilation was reactivated by metals added to the washed suspension. Co,

Fig. 5

Fig. 6

Fig. 5. Activation of washed suspensions of cells deficient in Mn and Mg by Mn and Mg. *Staph. aureus* D grown in medium B deficient in Mn and Mg; cells harvested and made into washed suspension and the rate of glutamic acid assimilation over 20 min. at 37° determined in the presence of either Mn or Mg as shown.

●—● Mn. ○—○ Mg.

Fig. 6. Activation of glutamic acid assimilation in Mn-deficient cells by Mn. *Staph. aureus* D grown in Mn-deficient medium B; cells harvested and made into washed suspension, incubated in buffered salt solution containing 1.0 % glucose, 200 μl. glutamic acid/ml. with and without 0.001 M-MnSO₄, samples taken at intervals as shown, cooled rapidly to 0° and internal glutamate concentration measured.

Fe and Zn had no effect on assimilation but 0.001 M-MgSO₄ or MnSO₄ markedly increased it (Fig. 5). Mg was more effective than Mn although both metals produced the same effect in optimal concentrations; Mn and Mg together in optimal concentrations had no greater effect than either alone. Table 4 shows the rates of assimilation obtained in washed deficient cells by the addition of m/1000 Mn or m/10⁴ Mg. In all cases Mn or Mg increased the rate, often to that of normal cells. In some cases Mg was less effective than Mn, but since the tests were made with the one concentration of metal only and since the sensitivity of the deficient cells to these metals is not constant from one batch

to another, it is probable that the smaller effect of Mg was due in some cases to its presence in suboptimal concentrations.

These results suggest that either Mn or Mg is involved in assimilation but that Mg is more effective than Mn in activating washed suspensions. The analysis of the inhibition of assimilation by oxine suggests strongly that in the normal cell Mn either is the metal concerned or can replace it. Further, since assimilation is a thousand times more sensitive than fermentation to oxine, and since oxine will not chelate to any extent with Mg at the experimental pH, the evidence points to Mn as the metal concerned in the normal cell. Mn certainly activates assimilation in deficient cells (Fig. 6) but Fig. 5 indicates that if both

Table 5. *Effect of washing on rates of assimilation*

Staph. aureus D was grown in medium B of controlled Mn or Mg content. In experiments *A*, the cells were harvested and their rate of glutamate assimilation (μ l. glutamic acid/100 mg. dry wt./20 min.) determined on a portion of the cells before any washing; a further portion was then washed twice in glass-distilled water at a final suspension strength of approximately 0.3 mg. dry wt. cells/ml. and the assimilation rate then determined. In experiments *B*, the cells were grown in media deficient in both Mn and Mg; after harvesting the cells were incubated for 15 min. at 37° in buffered salt solution containing 0.25% glucose and either 0.001 M-Mn or 0.0001 M-Mg; they were then centrifuged down and the assimilation rate determined on half of the cells without washing, the other half was then washed twice in glass-distilled water as above and the rate then determined.

Experiment	Metal content of growth medium		Pretreatment with metal	Rate of assimilation (μ l. glutamate/100 mg. dry wt. cells/20 min.)	
	Mn	Mg		Before washing	After washing
<i>A</i>	0.000001 M	Nil	None	139	147
	Nil	0.00017 M	None	129	104
<i>B</i>	Nil	Nil	0.001 M-Mn	206	206
	Nil	Nil	0.0001 M-Mg	206	148

Mg and Mn were present, Mg would be the more effective. Moreover Mn- and Mg-deficient cells that cannot assimilate are activated as well by Mg as by Mn (Table 4). It is possible that Mg diffuses in and out of the cell more readily than Mn and is more easily washed out of the cell. This was tested, firstly, by determining the effect of exhaustive washing on the assimilation rates of cells grown in Mn-without-Mg and in Mg-without-Mn. Secondly, cells were grown in media deficient in both Mn and Mg, incubated with optimal concentrations of either Mn or Mg, washed exhaustively in water, and their assimilation rates measured (Table 5). Washing had no effect on the cells grown in, or activated by, Mn and very little effect on those grown in, or activated by, Mg.

It is possible that the permeability of washed cells to metals may differ from that of growing cells, in which case the greater sensitivity to Mg shown in Fig. 5 might be due to the easier entrance of the Mg ion into the washed cell as compared with the larger Mn ion. The data in Table 4 suggest that Mn in the growth medium is effective at much lower concentrations than those needed to activate washed suspensions. To test this, organisms grown in a medium deficient in both Mn and Mg were incubated for 1 hr. at 37° in a modified

growth medium containing either Mn or Mg. The medium was composed of buffered salt solution with aneurin, nicotinamide, 0.1 % vitamin-free casein-hydrolysate lacking glutamic acid, and 0.5 % glucose, the whole being deprived of heavy metals by oxine treatment. To keep the internal concentration of glutamic acid low at this stage, the casein hydrolysate used was treated with glutamic decarboxylase until it was free from glutamic acid.

After the 1 hr. period of incubation, during which some growth, if not cell-division, might be expected to occur, the cells were centrifuged down and resuspended for the usual assimilation test. Under these conditions, the deficient cells again were activated by either Mn or Mg, but Mn was now much more effective than Mg (Fig. 7). The activation-concentration curve for Mg is approximately the same in washed or growing cells whereas Mn is *c.* 2500 times more effective in growing cells. It follows that if the cells grow in the presence of both Mn and Mg, the assimilation mechanism will be preferentially activated by Mn.

Oxine sensitivity of cells grown in the presence of Mn or Mg

The results so far obtained indicate that the assimilation of glutamic acid involves either Mn or Mg and that, although Mn is utilized preferentially during growth, its place can be taken by Mg. At pH 7 the stability of the Mg-oxine complex is much less than that of the Mn-oxine complex, so that it might be expected that the assimilation by cells grown in media containing Mg but deprived of Mn would be less sensitive to oxine than that of cells grown in media containing Mn but deficient in Mg. Fig. 8 shows the effect of oxine concentration on the rate of assimilation by cells grown (*a*) in normal untreated medium, (*b*) in medium deprived of Mg but supplied with Mn, and (*c*) in medium deprived of Mn but supplied with Mg. The sensitivity of the cells of culture (*c*) is significantly less than that of cells from either (*a*) or (*b*) which have substantially the same sensitivity. As Mg replaces Mn in the culture, the sensitivity to oxine of assimilation approaches that of fermentation, in which the principle metal is Mg.

DISCUSSION

The studies of Albert *et al.* (1947) on the antibacterial action of 8-hydroxyquinoline and related compounds strongly support the suggestion (Albert, 1944) that these substances act by combining with trace metals which are essential in bacterial metabolism and thereby prevent these metals performing their metabolic functions. The inhibition of glutamic acid assimilation in *Staph. aureus* by oxine and the partial or complete removal of this inhibition by certain metallic ions are consistent with the hypothesis that some metal which can combine with oxine forms an essential part of the assimilation mechanism and that this metal is inactivated by chelation with oxine. Two methods of inactivation seem possible: either the oxine combines with and inactivates the metal *in situ*, or the oxine combines with the metal and removes it from the organism. Since the metal-oxine complexes are more soluble in lipids than in water, it seems improbable that the complex would pass easily out of the organism into the medium. Further, the complete restoration of assimilation

by washing the oxine from the cells suggests that the oxine combines with the metal within the organism but, when washed away, leaves the metal behind. From an analysis of the effects of metals in annulling oxine inhibition of assimilation, it has been shown above that the metal concerned in the normal cell is probably manganese.

Fig. 7

Fig. 8

Fig. 7. Activation of glutamic acid assimilation in cells deficient in Mn and Mg by incubation in a modified growth medium containing Mn or Mg. *Staph. aureus* D grown in medium B deprived of both Mn and Mg; cells harvested and incubated for 1 hr. at 37° in the following medium with addition of either Mn or Mg as above: buffered salt solution; 0.1 % casein hydrolysate treated to remove vitamins, metals and glutamic acid; 0.5 % glucose; aneurin 1 μg./ml.; nicotinamide 1 μg./ml. After incubation the cells were centrifuged down and resuspended in buffered salt solution containing 1.0 % glucose and 200 μl. glutamic acid/ml. for 20 min. at 37°. At the end of this period, the cells were harvested, washed and the increase in internal glutamic acid concentration determined.

Fig. 8. Inhibition of glutamic acid assimilation by oxine; dependence on nature of metals present during growth. *Staph. aureus* D grown in: (a) medium B; (b) medium B deprived of Mg but containing $m/10^6$ -Mn; (c) medium B deprived of Mn but with $m/6000$ -Mg added. Assimilation inhibitions determined with washed suspensions in each case. Curve (d) shows oxine inhibition of glucose fermentation for cells of culture (c).

The results obtained with metal-deficient cultures indicate that either Mn or Mg is involved in assimilation. Mg is known to be necessary for the glucose fermentation system which provides energy for the transfer of glutamic acid across the cell-wall, but the results indicate that it can also play a part in the assimilation mechanism itself. Mg is known to activate many enzyme systems and is usually replaceable by Mn, although Pollock & Wainwright (1948) have recently obtained a growth effect on a Gram-negative organism which appears to be Mg-specific. When tests indicate that a system may be activated by either Mn or Mg, it is difficult to decide which metal is the natural activator *in vivo*. If the metals act in an ionic form then they are presumably freely interchangeable whether *in vivo* or *in vitro*, but if co-ordination complexes are involved within the organism, then the nature of the metal used 'naturally'

will depend upon the availability of the metal and the relative stabilities of the complexes formed. Mellor & Maley (1948) found that the order of stability of metal co-ordination complexes is approximately the same whatever the nature of the organic part of the complex; consequently if the formation of such complexes is involved, Mn will compete successfully with Mg and will be taken up preferentially. Both Mn and Mg can activate assimilation in washed cells, although Mg is more effective at low concentrations. This does not correlate with the argument developed above that the metal chelated with oxine in 'normal' cells is Mn, nor is it consistent with the marked difference in the oxine sensitivity of assimilation and fermentation in 'normal' cells. However, if activation of assimilation is carried out in a medium in which growth can occur, then Mn is 2500 times more effective than in a simple salt solution so that, in the growth medium, Mn is 30–50 times more effective than Mg. It seems probable that harvested and washed cells are less permeable to Mn than are cells growing and dividing.

It seems probable that the assimilation mechanism involves a metal whose function can be filled by either Mn or Mg and that, when the cell grows in a medium containing both Mn and Mg, Mn is preferentially co-ordinated. Mn forms a chelate complex with oxine which acts as a reversible inhibitor of assimilation. When growth takes place in a medium deficient only in Mn, then the mechanism becomes activated by Mg, but this results in a decrease in its sensitivity to oxine (see Fig. 8).

The present work was prompted by the finding of Albert *et al.* (1947) that oxine has an antibacterial 'spectrum' similar to that of penicillin and it has now been shown that both penicillin and oxine inhibit the transfer of glutamic acid into *Staph. aureus*. The mechanism of inhibition is almost certainly not the same for the two substances. This follows since (i) penicillin has no action on assimilation in washed cells where oxine causes complete inhibition, (ii) the inhibition of assimilation by growth in the presence of penicillin is irreversible, whereas the oxine inhibition is easily removed, (iii) oxine also has complex inhibitory actions on respiration and fermentation whereas penicillin has not at concentrations at least a thousand times greater than that required to inhibit assimilation, (iv) strains of *Staph. aureus* rendered resistant to penicillin are as sensitive to oxine as penicillin-sensitive strains, due perhaps to the non-specific nature of oxine as an inhibitor.