REFERENCES

Baker, D. & Nelson, J. M. (1943). *J. gen. Physiol.* **26**, 269.
Bortels, H. (1930). *Arch. Mikrobiol.* **1**, 333.
Boswell, J. G. & Whiting, G. C. (1938). *Ann. Bot.*, N.S., **11**, 847.
Chance, B. (1949). *Science*, **109**, 204.
Dixon, M. & Zerfas, L. G. (1940). *Biochem. J.* **34**, 371.
Gregg, D. C. & Miller, W. H. (1940). *J. Amer. chem. Soc.* **62**, 1374.
Haas, E. (1937). *Biochem. Z.* **291**, 79.
Hewitt, E. J., Agarwala, S. C. & Jones, E. W. (1950). *Nature, Lond.*, **166**, 1119.
Hewitt, E. J. & Jones, E. W. (1947). *J. Pomol.* **23**, 254.
Hewitt, E. J., Jones, E. W. & Williams, A. H. (1949). *Nature, Lond.*, **163**, 681.
Horner, C. K., Burk, D., Allison, F. E. & Sherman, M. S. (1942). *J. agric. Res.* **65**, 173.
Jahr, K. F. (1941). *Chem. Zbl.* **1**, 184.
Keilin, D. & Mann, T. (1937). *Proc. roy. Soc.* B, **122**, 119.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.
Mulder, E. G. (1948). *Plant & Soil*, **1**, 94.
Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
Rosenheim, A., Hakki, M. & Krause, O. (1932). *Z. anorg. Chem.* **209**, 175.
Watt, G. W., Achorn, S. L. & Marley, J. L. (1950). *J. Amer. chem. Soc.* **72**, 3341.

The Assimilation of Amino-Acids by Bacteria

13. THE EFFECT OF CERTAIN AMINO-ACIDS ON THE ACCUMULATION OF FREE GLUTAMIC ACID BY *STAPHYLOCOCCUS AUREUS*: EXTRACELLULAR PEPTIDE FORMATION

BY E. F. GALE AND MARGUERITE B. VAN HALTEREN
Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge

(*Received* 27 *March* 1951)

If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated in buffered salt solution containing glutamic acid and glucose, the amino-acid enters and accumulates in the free state within the cells (Gale, 1947). The passage of glutamic acid into the cells is energy-linked and can be uncoupled from glycolysis by inhibitors such as sodium azide, 2:4-dinitrophenol, aureomycin and 8-hydroxyquinoline (Gale, 1949, 1951*a*; Gale & Paine, 1951). If a complete mixture of natural amino-acids is added to the external medium, the accumulation of free glutamic acid within the cells ceases, and glutamic acid becomes incorporated within the cellular material in a combined form from which it can be liberated by acid hydrolysis (Gale, 1951*b*). The addition of incomplete mixtures of amino-acids to the external medium results in a reduction in the rate of accumulation of free glutamic acid within the cells (Gale, 1951*b*), and the present communication deals with the mechanism of this inhibition.

EXPERIMENTAL

Organism. The organism used throughout this work was *Staphylococcus aureus* Duncan (Gale & Taylor, 1947).

Conditions of growth. The organism was grown for 16 hr. at 30° in the 'deficient medium B' consisting of a salt-mixture solution containing 1% glucose, 0·1% arginine and 0·2% marmite (Gale, 1947). The medium was distributed in 150 ml. quantities in Roux bottles.

Determination of glutamic and aspartic acids. Glutamic acid was determined manometrically by use of the specific glutamic acid decarboxylase preparation (Gale, 1945). Aspartic acid was determined by the manometric method of Krebs (1950) using glutamic acid decarboxylase in conjunction with glutamic-aspartic transaminase. The accumulation of these acids within the cells was studied by the methods previously described (Gale, 1947, 1951*b*).

Determination of combined glutamic acid. For the determination of total glutamic acid, thick suspensions of cells were hydrolysed for 18 hr. in boiling 6N-HCl, the acid removed by evaporation to dryness *in vacuo*, the hydrolysate dissolved in water and the pH adjusted to 4·5 before estimation with the decarboxylase preparation. The total glutamic acid was then corrected for the free glutamic acid content to give combined glutamate. Solutions obtained after removal of cells by centrifuging were also examined for combined glutamate in a similar fashion.

Chromatographic investigation of solutions. Incubation mixtures were freed from bacteria by centrifuging and then reduced to one-tenth of their original volume by evaporation under reduced pressure at a temperature below 40°; after neutralization they were deproteinized by the addition of 1 vol. ethanol and 6 vol. $CHCl_3$, the samples centrifuged and the aqueous layer used for chromatographic examination.

The general procedures used for paper chromatography were those described by Consden, Gordon & Martin (1944).

Whatman filter papers nos. 1 or 4 were used, except for the separation of larger amounts of material or to increase the concentration of components present in small amount in which case Whatman paper no. 3 was used. Two solvents were used: butanol-acetic acid-water mixture (Woiwod, 1949) and a propanol-ammonia (0·3%) mixture containing 80% (v/v) propanol. Chromatograms were coloured with 0·1% ninhydrin solution in chloroform containing 0·1% collidine (Woiwod, 1949).

When new spots, possibly representing peptide material, were found, large-scale chromatograms were run; strips were cut from the papers and sprayed to locate the spots or streaks; horizontal strips were then cut out of the paper as indicated by the guide strips and the material eluted with water. Samples of eluted materials were run again to test the accuracy of the separation. Other samples were hydrolysed for 18 hr. at 105° in 6N-HCl and the hydrolysates taken to dryness at room temperature under reduced pressure; excess HCl was removed by addition of distilled water followed by evaporation to dryness, the process being carried out three times. Examination of the peptides for identification of amino-acids carrying a free amino group was carried out by the deamination method of Consden, Gordon & Martin (1947) and by preparation of dinitrophenyl derivatives according to Sanger (1945, 1946).

When the incubation mixtures contained cysteine, chromatograms showed a number of spots corresponding to partial oxidation products and the spots showed 'tailing'. Treatment of the solutions with bromine water (Consden & Gordon, 1950) oxidizes cysteine to cysteic acid which gives one clearly defined spot. However, during the course of this work it was found that treatment of cysteine with bromine water in the presence of glutamic acid gave rise to a new spot, running between glutamic acid and cysteic acid in butanol-acetic acid, which behaved as though it were a simple dipeptide of cysteic acid and glutamic acid (Van Halteren, 1951). An alternative treatment of cysteine-containing solutions was to allow them to autoxidize by standing in an open vessel for several days. Solutions so treated were preserved by addition of $CHCl_3$. Chromatograms prepared from such solutions showed that cysteine was largely converted to cysteic acid and no new spots were observed as a result of such treatment.

Staph. aureus cells contain high concentrations of certain amino-acids in the free state within the cells (Gale, 1947; Taylor, 1947) and in some experiments small amounts of lysine, glutamic acid or alanine leaked out of the cells in the course of incubation. When this occurred, the spots concerned were also evident in the various control mixtures. Woiwod & Proom (1950) have pointed out that γ-aminobutyric acid may sometimes leak out of the cells. This type of leakage of soluble cell constituents is most marked with staphylococci when incubation takes place in the absence of glucose.

Synthetic peptides. We are indebted to Sir Charles Harington, F.R.S., for a sample of γ-glutamylcysteine; to Dr G. T. Young for α- and γ-glutamylglycine; to Dr R. Consden for α-glutamylalanine, and to Dr W. T. J. Morgan for samples of di-, tri-, and tetra-glycylglycine.

RESULTS

Preliminary work. During investigations of the effect of amino-acid mixtures on the accumulation of free glutamic acid inside *Staph. aureus* (Gale, 1951*b*) it was found that mixtures of three or four amino-acids reduced the rate of accumulation, and that the degree of this reduction was increased by increasing the number of amino-acids in the external mixture. It was decided to test the action of amino-acids, other than glutamic acid, when added one at a time to the experimental mixture of buffered salt, glucose and glutamic acid. In the first place the other amino-acids were added in a concentration equal to twice that of the glutamic acid and the rates of accumulation of free glutamic acid within the cells compared, in each case, with the rate when glutamic acid was the only amino-acid added to the external medium. Table 1 shows the results obtained; the amino-acids

Table 1. *Effect of amino-acids on the rate of accumulation of internal glutamic acid in* Staphylococcus aureus

(Washed suspensions of *Staph. aureus* Duncan, grown for 16 hr. at 30° in deficient medium, incubated in buffered salt solution containing 1% glucose and 1·35 μmol. sodium glutamate/ml. Effect on rate of accumulation of internal free glutamic acid of addition of other amino-acids at concentration of 2·7 μmol./ml. Results expressed as percentage rate in absence of added amino-acids; figures in brackets give range where number of tests was greater than 5.)

Amino-acid added	Rate as percentage control
(1)	
L-Aspartic acid	30 (25–60)
L-Cysteine	40 (10–54)
L-Cystine (sat. soln.)	54 (30–75)
L-Alanine	55 (30–80)
Glycine	65 (30–80)
L-Serine	75
L-Asparagine	82
(2)	
L-Ornithine	97
L-Proline	98
D-Alanine	100
L-Phenylalanine	103
L-Methionine	103
L-Lysine	104
L-Tyrosine	107
L-Tryptophan	107
L-Arginine	107
L-Threonine	110
L-Histidine	110
(3)	
L-Isoleucine	112 (105–118)
L-Valine	117 (106–145)
L-Leucine	118 (105–133)

fall into three groups: (1) those such as aspartic acid, cysteine, alanine and glycine, which cause a marked decrease in the rate of free glutamic acid accumulation; (2) those which have no marked effect, and (3) those such as valine and leucine which appear to accelerate the rate of free glutamic acid accumulation. L-Alanine has a marked inhibitory action whereas D-alanine is without effect. Aspartic acid is inhibitory whereas asparagine has little effect.

In the course of more detailed investigations it became clear that the action of aspartic acid was different from that of the other amino-acids in group 1 and, accordingly, this amino-acid will be dealt with separately.

Inhibition of free glutamic acid accumulation by aspartic acid

Effect of concentration. The addition of aspartic acid to the buffered salt solution containing glucose and glutamic acid results in a decrease in the rate of accumulation of free glutamic acid within the cells; if the concentration of aspartic acid is twice that of glutamic acid, then this rate is approximately halved. Fig. 1 shows that the inhibition increases as the concentration of aspartic acid, relative to that of

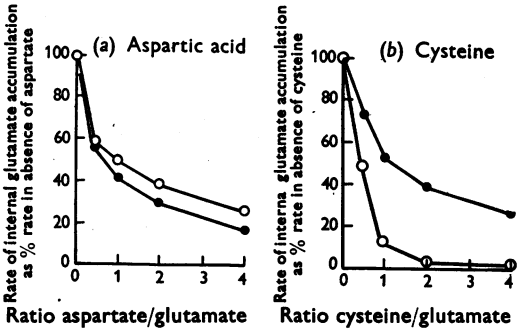

Fig. 1. (*a*) Effect of relative concentrations of aspartic and glutamic acids on rate of accumulation of free glutamic acid within *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated in buffered salt solution containing 1% glucose, sodium glutamate at final concentration 1·34 (●) or 10·7 (○) μmol./ml. and sodium aspartate to give ratios indicated by abscissae. Rate of free glutamic acid accumulation within the cells determined over 60 min. at 30°. (*b*) Effect of relative concentrations of cysteine and glutamic acid on rate of accumulation of free glutamic acid within *Staph. aureus*. Conditions as for Fig. 1*a* with substitution of cysteine for sodium aspartate.

glutamic acid, increases. The percentage inhibition was determined for ratios of aspartic to glutamic acid ranging from 0·5 to 4·0 for glutamic acid concentrations of 1·34 and 10·7 μmol./ml.; Fig. 1 shows that the degree of inhibition of the accumulation of free glutamic acid is determined by the ratio of the two amino-acids and not by the absolute concentration of either.

Course of the accumulation of internal free aspartic and glutamic acids. In the first paper of this series (Gale, 1947) it was shown by a non-specific method that aspartic acid could not enter *Streptococcus faecalis* unless glucose was being metabolized; the passage of aspartic acid into the Gram-positive bacterial cell thus occurred under conditions similar to those required for the entry of glutamic acid. This has now been confirmed in the case of *Staph. aureus* by the use of a method specific for aspartic acid (Krebs, 1950). By this method it is possible to determine both aspartic and glutamic acids on the same sample of cells and Fig. 2 shows the rates of appearance of these acids in the free state within *Staph. aureus* cells incubated in the presence of glucose and glutamic acid (curve *A*); aspartic acid (curve *D*); and glutamic acid and one or two equivalents of aspartic acid (curves *B* and *C*). It can be seen that not only does the presence of aspartic acid inhibit the accumulation of free glutamic acid, but that the presence of glutamic acid also inhibits the

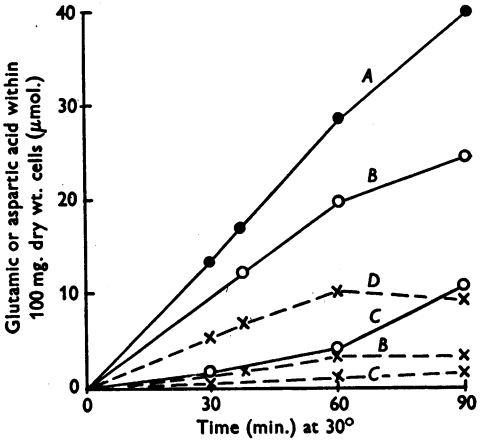

Fig. 2. Accumulation of glutamic and aspartic acids within *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* incubated, at final suspension density = 2·0 mg. dry wt. of cells/ml., in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. (curve *A*), 1·34 μmol. sodium glutamate + 1·34 μmol. sodium aspartate/ml. (curve *B*), 1·34 μmol. sodium glutamate + 2·7 μmol. sodium aspartate/ml. (curve *C*), 1·34 μmol. sodium aspartate/ml. (curve *D*). Incubation at 30° and samples removed at intervals for estimation of internal concentration of free glutamic acid (continuous lines) and free aspartic acid (broken lines).

accumulation of aspartic acid. When the external concentration of aspartic acid is twice that of the glutamic acid, the sum of the concentrations of the two acids within the cell is markedly less than the concentration of glutamic acid when that amino-acid is the only one present. Consequently each amino-acid must exert some inhibitory action on the accumulation of the other.

Changes in the distribution of free and combined glutamate during incubation with glutamic and aspartic acids. When *Staph. aureus* is incubated with glutamic acid and glucose, free glutamic acid accumulates within the cells and this is drawn from the external medium; the amount of free glutamic acid removed from the medium is invariably greater than the amount which appears within the cells and,

Table 2. *Changes in distribution of free and combined glutamate during incubation of* Staphylococcus aureus *with aspartic acid*

(Washed suspensions of *Staph. aureus*, suspension density = 2·0 mg. dry wt. of cells/ml., incubated for 1 hr. at 37° in buffered salt medium containing 1% glucose, 1·34 μmol. sodium glutamate/ml. with and without 2·7 μmol. sodium aspartate/ml. Results expressed as μmol. glutamic acid/100 mg. dry wt. of cells.)

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + aspartate	Glucose + glutamate + aspartate	Initial value
Change in internal free glutamate	−4·0	+33·3	+0·2	+10·5	15·6
Change in external free glutamate	+1·7	−53·9	+9·2	−24·7	67·1
Change in combined glutamate of cells	−0·6	− 4·4	−2·9	− 3·6	33·1
Change in combined glutamate of medium	+0·8	+ 0·8	+1·7	+ 1·9	0·0
Balance	−2·1	−24·2	+8·2	−15·9	—

since there is no increase in the combined glutamate of either the cells or the supernatant medium, the glutamic acid which disappears on balance must presumably have undergone metabolism (Gale, 1951*b*; Gale & Mitchell, 1947). In Table 2 the changes in free and combined glutamate of cells and medium which occur when *Staph. aureus* is incubated with glucose and glutamic acid are compared with those that occur when aspartic acid is also present. The effect of the addition of aspartic acid is to reduce, by approximately the same proportion, (1) the rate of accumulation of free internal glutamic acid within the cells, (2) the rate of removal of free glutamic acid from the external medium and (3) the rate of disappearance or metabolism of glutamic acid. There is no significant appearance of combined glutamate in the supernatant medium or increase in the combined glutamate of the cells.

When the cells are incubated with glucose and aspartic acid there is no significant increase in the free internal glutamic acid of the cells, but some glutamic acid appears in the external medium; this must presumably arise as the result of transamination (Lichstein & Cohen, 1944).

Chromatographic examination of supernatant media. In all the cases discussed above (Table 2) the supernatant solutions, obtained by centrifuging down the cells at the end of the incubation, were concentrated *in vacuo* and examined by paper chromatography. In no case was it possible to demonstrate the presence of any ninhydrin-positive material other than free glutamic and aspartic acids.

Inhibition by cysteine, cystine, alanine or glycine of accumulation of free glutamic acid

Concentration effects. Fig. 1*b* shows the degree of inhibition of the rate of free glutamic acid accumulation produced by the presence of cysteine at concentrations from one to four times that of the glutamic acid in the external medium. The inhibition increases as the ratio of cysteine to glutamic acid increases. In the case of aspartic acid the inhibition was not affected by the absolute concentration of the amino-acids, but Fig. 1*b* shows that any given ratio of cysteine to glutamic acid has a higher inhibitory action when the concentration of glutamic acid is 10·7 μmol./ml. than when it is 1·34 μmol./ml. Similar effects of absolute concentration were obtained in the cases of inhibition by alanine or glycine.

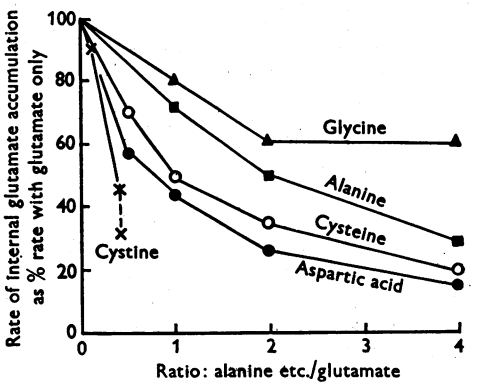

Fig. 3. Effect of amino-acids on rate of accumulation of free glutamic acid in *Staph. aureus*. Washed suspensions of 'deficient' *Staph. aureus* incubated at 30° and final suspension density = 2·0 mg. dry wt./ml. in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml. and one of the above amino-acids at concentrations relative to glutamic acid as indicated by the abscissae. Rate of accumulation of free glutamic acid within the cells determined over 0–60 min. and expressed, in each case, as percentage of the rate when glutamic acid is the only external amino-acid present.

Fig. 3 shows the effect of increasing the concentration of each of the inhibitory amino-acids while keeping the glutamic acid concentration constant. Cystine at low concentrations proves markedly more effective as an inhibitor than cysteine, but solubility considerations prevented its investigation at higher concentrations. The inhibitory effects obtained with aspartic acid and cysteine were reasonably consistent throughout the period covered by these experiments, but the effects of alanine and glycine varied widely from time to time. In the case of

glycine, the concentration necessary to produce 50% inhibition of the rate of accumulation of free glutamic acid varied from three to twelve times that of the glutamic acid.

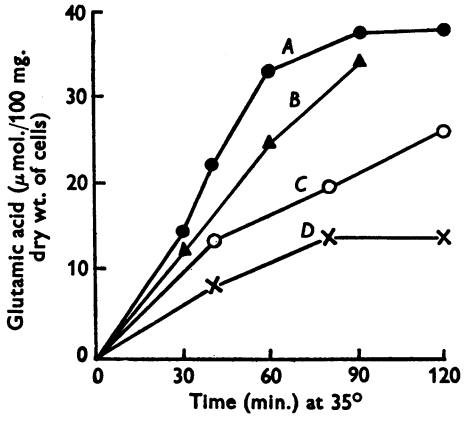

Fig. 4. Effect of glycine, cysteine or cystine on rate of accumulation of free glutamic acid in *Staph. aureus*. Rate of accumulation of free glutamic acid within deficient *Staph. aureus* incubated at 35° in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. with (*A*) no addition, (*B*) the addition of 2·7 μmol. glycine/ml., (*C*) 2·7 μmol. cysteine/ml., (*D*) cystine to give saturated solution.

Course of the inhibition. Fig. 4 shows the effect of glycine, cysteine and cystine on the rate of the accumulation of internal free glutamic acid. Cysteine and glycine appear to inhibit this rate, but do not decrease the final internal concentration attained. In the case of cystine the inhibition increases with time until, after 2 hr. at 37°, accumulation of free glutamic acid within the cells appears to cease.

Experiments were performed in which the cells were incubated for 1–2 hr. at 37° with glucose and one of the inhibitory amino-acids prior to exposure to glutamic acid and glucose in the absence of other amino-acids. Such pretreatment with cystine for 2 hr. resulted in 30–50% decrease in the subsequent rate of accumulation of free glutamic acid; in no other case was any significant effect of pretreatment observed. It seems possible that the inhibition produced by cystine may be more complex than that due to the other amino-acids investigated.

Changes in distribution of free and combined glutamate. Table 3 shows the changes that occur in the distribution of free and combined glutamate of cells and supernatant medium when *Staph. aureus* is incubated with glucose, glutamic acid and either cysteine or alanine. The presence of either of these amino-acids results in a decrease in the rate of accumulation of free glutamic acid inside the cells, whereas the rate of its removal from the external medium increases. This effect on the external glutamic acid is the opposite of that obtained when aspartic acid is added (Table 2). The amount of glutamate that disappears on balance is slightly increased when cysteine or alanine is present; there is no significant increase in the combined glutamate of the cells, but a significant amount of combined glutamate appears in the external medium. The amount of combined glutamate appearing in the medium is approximately equal to the decrease in the amount of free glutamic acid accumulating within the cells.

Table 3. *Changes in distribution of free and combined glutamate during incubation of* Staphylococcus aureus *with glutamic acid and either cysteine or alanine*

(Washed suspensions of *Staph. aureus*, suspension density = 2·0 mg. dry wt./ml., incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml. with 2·7 μmol. cysteine or alanine/ml. as below. Results expressed as μmol. glutamic acid/100 mg. dry wt. of cells.)

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + cysteine	Glucose + glutamate + cysteine	Initial value
Change in internal free glutamate	−1·0	+28·0	+1·0	+14·7	24·2
Change in external free glutamate	+0·8	−42·2	+0·4	−51·0	67·0
Change in combined glutamate of cells	−2·0	− 3·7	−2·8	− 2·6	35·0
Change in combined glutamate of medium	+0·2	+ 0·8	+1·8	+16·3	0·0
Balance	−2·0	−17·1	−0·4	−23·6	—

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + alanine	Glucose + glutamate + alanine	Initial value
Change in internal free glutamate	−4·0	+27·1	−1·7	+18·1	30·4
Change in external free glutamate	+3·8	−42·5	+2·7	−58·0	67·1
Change in combined glutamate of cells	−0·6	− 4·2	−4·0	− 4·1	37·2
Change in combined glutamate of medium	+0·8	0·0	+1·6	+15·8	0·0
Balance	0	−19·6	+1·4	−28·2	—

Similar results were obtained when either cystine or glycine was added to the glutamic acid and glucose incubation mixture, although the amounts of combined glutamate formed under these conditions were smaller than when cysteine or alanine was added.

Effect of glucose. The accumulation of internal free glutamic acid does not take place, under the experimental conditions used, unless glucose is present as energy source. The accumulation of combined glutamate occurs, in these experiments, in the external medium and it was therefore of interest to

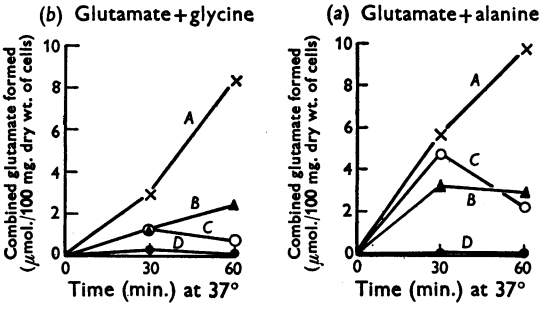

Fig. 5. Effect of glucose on the formation of combined glutamate in the medium during incubation of *Staph. aureus* with glutamic acid and (*a*) alanine or (*b*) glycine. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated at 37°, final suspension density = 2·0 mg./ml., in buffered salt solution containing 1·34 μmol. sodium glutamate/ml., 1·34 μmol. alanine (*a*) or 4·0 μmol. glycine/ml. (*b*) and glucose as below. Supernatants collected at 30 and 60 min., evaporated *in vacuo* to one-tenth volume and glutamic acid content determined before and after hydrolysis in 6 N-HCl for 18 hr. Increase in glutamic acid during hydrolysis = combined glutamate and expressed as increase in combined glutamate/100 mg. dry wt. of cells. Initial glucose content of media: (*A*) 0·5, (*B*) 0·2, (*C*) 0·1, (*D*) 0·0 % (w/v).

determine whether glucose was necessary for its production. Experiments were first performed with organisms washed twice in distilled water and then incubated with glutamic acid and one of the inhibitory amino-acids; in no case could the formation of combined glutamate in the external medium or the accumulation of free glutamic acid within the cells be demonstrated. The cells were then incubated with glutamic acid, either alanine or glycine, and amounts of glucose to give initial concentrations of 0, 0·1, 0·2 and 0·5 % (w/v). Fig. 5 shows that a linear formation of combined glutamate occurs throughout 60 min. in the presence of 0·5 % glucose, whereas in the presence of 0·1 % glucose there is an initial formation of combined glutamate which decreases again after the glucose is exhausted in approximately 30 min. It is clear that the presence of glucose is essential for the extracellular formation of combined glutamate in these experiments.

Effect of internal free glutamic acid concentration. When *Staph. aureus* is incubated with glucose and a complete mixture of amino-acids, combined glutamate becomes incorporated in the cellular material and the rate of formation of cellular combined glutamate is dependent on the concentration of free glutamic acid within the cells (Gale, 1951*b*). The question arises whether the internal concentration of free glutamic acid plays any part in the formation of extracellular combined glutamate when the cells are incubated with glucose and either cysteine or alanine. To test this point a batch of cells was prepared in the usual way and divided into two portions; one portion was incubated for 30 min. with glucose and glutamic acid to build up a high internal concentration of free glutamic acid; the other portion was incubated with glucose only. The two suspensions were then washed free from glutamic acid and each was incubated with glucose and either cysteine, glycine or alanine. Table 4 shows that the presence of either

Table 4. *Effect on concentration of internal free glutamic acid of incubation in presence of 'inhibitory' amino-acids*

(Washed suspension of 'deficient' *Staph. aureus* Duncan divided into two portions: each incubated for 30 min. at 37° in buffered salt solution containing (*A*) 1 % glucose, (*B*) 1 % glucose + 1·34 μmol. sodium glutamate/ml. Each then washed twice in distilled water and incubated, at suspension density = 2·0 mg. dry wt. of cells/ml., for 30 min. at 37° in buffered salt solution containing 1 % glucose and other amino-acids as below. Change in concentration of internal free glutamic acid determined during second incubation.)

First incubation in presence of	Second incubation in presence of (μmol./ml.)		Change in internal free glutamic acid (μmol./100 mg. dry wt. of cells)
(*A*) Glucose	Glutamic acid	(1·34)	+35·2
	Glutamic acid + cysteine	(1·34) (2·7)	+28·1
	Glutamic acid + alanine	(1·34) (4·0)	+26·1
	Glutamic acid + glycine	(1·34) (4·0)	+20·6
	(Glucose only)		− 0·9
	Cysteine	(2·7)	− 2·1
	Alanine	(4·0)	− 3·3
	Glycine	(4·0)	− 0·9
(*B*) Glucose + glutamic acid	(Glucose only)		− 9·4
	Cysteine	(2·7)	− 9·4
	Alanine	(4·0)	− 7·3
	Glycine	(4·0)	− 9·1

of these amino-acids in the external medium had little or no significant effect in removing free glutamic acid from within the cells; consequently the free glutamic acid accumulating within the cells cannot readily enter into combination with either cysteine, glycine or alanine outside the cell.

Nature of extracellular peptides formed

Supernatant media obtained in each of the cases discussed above were examined by paper chromatographic methods. The results for each incubation mixture will be discussed separately.

Incubation mixture: glutamic acid + cysteine. Fig. 6 shows the chromatograms given by the supernatant media obtained after incubation of *Staph. aureus* with buffered salt solution, glucose and (II) glutamic acid, (III) cysteine, and (IV) glutamic acid and cysteine. The solutions were treated with bromine water to oxidize cysteine to cysteic acid and the papers run in butanol-acetic acid solvent. In IV a new spot appears, running with an R_F of about 0·10 between glutamic acid and cysteic acid. This new substance does not appear in the supernatant after

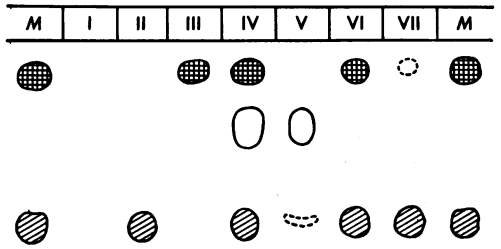

Fig. 6. Diagrammatic representation of paper chromatograms obtained from supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamate and cysteine. Conditions as for Table 3. Concentrated supernatants treated with bromine water and chromatograms run in butanol-acetic acid solvent. *M*, Markers: cysteic acid, ⊞; glutamic acid (bottom), ▨; I, supernatant from incubation mixture containing glucose only; II, supernatant from incubation mixture containing glutamic acid and glucose; III, supernatant from incubation mixture containing glucose and cysteine; IV, supernatant from incubation mixture containing glucose, glutamic acid and cysteine; V, material eluted from new intermediate spot in IV; VI, material from V after hydrolysis for 18 hr. at 105° in 6N-HCl; VII, material from V treated with nitrous fumes before hydrolysis for 18 hr. at 105° in 6N-HCl.

incubation with glutamic acid only (II) and occasionally appears in trace amounts in the control incubation mixture containing cysteine only (III). (*Staph. aureus* contains high concentrations of free glutamic acid within the cells.) The material corresponding to the new spot has been eluted (V) and hydrolysed, when it gives rise to cysteic and glutamic acids only (VI). The trace of free glutamic acid appearing in V may be due to incomplete separation of the peptide from glutamic acid during the primary separation or to some degree of hydrolysis occurring during the elution and running in acid solvent. If the eluted material is deaminated before hydrolysis, the cysteic acid spot disappears completely or, occasionally, remains as a ghost spot (VII). These results indicate that the new material is a simple peptide of glutamic acid and cysteine, the cysteine carrying a free amino group. This was confirmed by application of Sanger's terminal group method (Sanger, 1945, 1946); treatment of the eluted peptide with fluorodinitrobenzene gave a single spot of the dinitrophenyl derivative with R_F value about 0·6 when run in butanol-acetic acid solvent; on hydrolysis the cysteic acid spot disappeared while the glutamic acid spot remained and gave the usual ninhydrin reaction.

The peptide was run alongside γ-glutamylcysteine and was not identical with the synthetic peptide.

Some doubt was felt concerning the interpretation of these results in view of the artifacts found by Van Halteren (1951) to follow bromine treatment of cysteine in the presence of other amino-acids. However, controlled experiments showed that the amount of material obtained by elution of the new spot in IV was greatly in excess of the amount obtained from a similar position after the 'artifact reaction' only. To confirm the true nature of the biological material, all procedures were repeated using supernatant solutions that had been allowed to autoxidize instead of being treated with bromine water. Separation of the spots was not so clean, but it was possible to repeat and confirm the observations outlined above.

No other new ninhydrin-positive material has been found in the incubation mixture containing glucose, glutamic acid and cysteine.

Incubation mixture: glutamic acid + cystine. Supernatant solutions obtained after incubation of *Staph. aureus* with glucose, glutamic acid and cystine showed, after treatment with bromine water or after autoxidation, a new spot running between cysteic acid and glutamic acid. The new material ran in the same position as that obtained from the incubation mixture containing glutamic acid and cysteine. The yield of material was small, owing to the low solubility of cystine, and no detailed investigations were carried out.

Incubation mixture: glutamic acid + alanine. The chromatograms of supernatants obtained after incubation of *Staph. aureus* with glucose, glutamic acid and alanine showed two new spots running between those of glutamic acid and alanine in either solvent employed (Fig. 7, I: *Y* and *Z*). One of these spots (*Y*) runs with an R_F value slightly less than that of glycine. The material (*Y*) was eluted, run again to ensure that no free glycine was carried with it, and hydrolysed when it split to give glutamic acid, glycine and alanine (Fig. 7, V). None of the components disappeared after deamination or treatment of *Y* with fluorodinitrobenzene before hydrolysis. The second new spot (Fig. 7, I: *Z*) runs slightly more slowly than alanine; the material was eluted (Fig. 7, VI) and split on hydrolysis to alanine and

glutamic acid only (Fig. 7, VII). After deamination and hydrolysis, the alanine spot disappeared (Fig. 7, VIII) suggesting that (*Z*) is a simple peptide of glutamic acid and alanine, the alanine carrying the free amino group.

On one occasion a third new spot, running slower than glutamic acid, was obtained (Fig. 7, I: *X*) in propanol-ammonia solvent. On hydrolysis the material obtained from this spot (Fig. 7, II) split to give a well marked glutamic acid spot and a trace of alanine (Fig. 7, III).

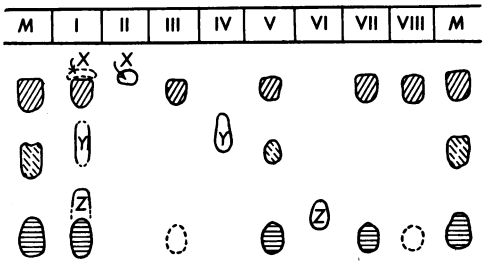

Fig. 7. Diagrammatic representation of paper chromatograms obtained from supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamic acid and alanine. Washed suspensions of 'deficient' *Staph. aureus* Duncan, incubated for 1 hr. at 37° with glucose, sodium glutamate and alanine (conditions as for Table 3). Organisms removed by centrifuging and supernatants concentrated to dryness at room temperature. Chromatograms run in propanol-ammonia solvent. *M*, Markers: glutamic acid, ▨; glycine, ⊠; alanine, ⊟. I, supernatant from incubation mixture containing glucose, glutamate and alanine; II, material eluted from position I, *X*; III, *X* after hydrolysis for 18 hr. at 105° in 6N-HCl; IV, material eluted from position *Y* in I; V, *Y* after hydrolysis for 18 hr. at 105° in 6N-HCl; VI, material eluted from position I, *Z*; VII, *Z* after hydrolysis for 18 hr. at 105° in 6N-HCl; VIII, *Z* deaminated by treatment with nitrous fumes before hydrolysis and then hydrolysed for 18 hr. at 105° in 6N-HCl.

None of the components *X*, *Y* or *Z* corresponded on the chromatogram with the position of α-glutamylalanine. None of the new spots appeared in control incubation mixtures containing glutamic acid and glucose only, although faint spots were obtained in the positions of *Y* and *Z* when incubation took place with alanine and glucose only.

Incubation mixture: glutamic acid + glycine. Supernatants obtained from incubation mixtures containing glutamic acid, glycine and glucose showed a strong new spot running more slowly than glycine in butanol-acetic acid solvent; in some samples there was evidence of other material running between this spot (Fig. 8, I: *A*) and that of glycine. On elution (Fig. 8, II) and hydrolysis, the material corresponding to the new spot gave rise to glycine only (Fig. 8, III). The spot gave an initial yellow colour when treated with ninhydrin and the colour turned the normal purple on standing. The material of spot *A* was compared, in butanol-acetic acid solvent, with tetra-, tri- and di-glycylglycine. The R_F value of the polyglycine preparations decreased with increasing molecular weight and the material (*A*) had the same R_F value as tetraglycylglycine (Fig. 8, IV–VI). The polyglycine spot appeared whenever *Staph. aureus* was incubated with glucose and glycine. When glutamic acid was also present, the amount of polyglycine decreased when judged by the strength of the ninhydrin reaction on the paper. It has been

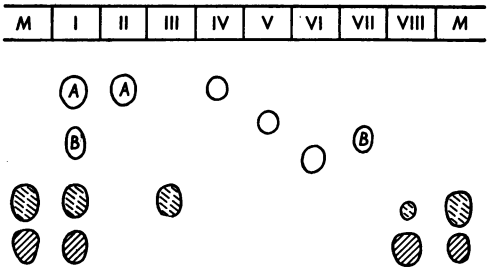

Fig. 8. Diagrammatic representation of paper chromatograms of supernatant media obtained by incubation of *Staph. aureus* with glucose, glutamic acid and glycine. Conditions as for Fig. 7 with glycine substituted for alanine; chromatograms run in butanol-acetic acid solvent. *M*, Markers: glutamic acid, ▨; glycine, ⊠. I, supernatant from incubation mixture containing glucose, glutamate and glycine; II, material eluted from position I, *A*; III, *A* after hydrolysis for 18 hr. at 105° in 6N-HCl; IV, tetraglycylglycine; V, triglycylglycine; VI, diglycylglycine; VII, material eluted from position I, *B*; VIII, *B* after hydrolysis for 18 hr. at 105° in 6N-HCl.

mentioned above that the action of glycine on glutamic acid uptake is erratic and the chromatographic results were also inconsistent. A peptide has been isolated from supernatant solutions run in butanol-acetic acid solvent; it runs between the polyglycine and glycine (Fig. 8, I: *B*) and complete separation has not been achieved. On hydrolysis the material eluted from position (*B*) (Fig. 8, VII) liberated glutamic acid and glycine, the increase in the glutamic acid spot being markedly greater than that of the glycine spot (Fig. 8, VIII).

Effects of simple mixtures of amino-acids

Table 5 shows the effect, on the rate of accumulation of free glutamic acid within the cells, of the presence of certain other amino-acids. The inhibition produced by the presence of cysteine is increased by the further addition of cystine, glutathione, alanine or glycine. In general, the inhibitions produced by any of the 'inhibitory group' of amino-acids appear to be additive when these amino-acids are present together. On the other hand, the inhibition produced by either cysteine or alanine is largely released by the further addition of valine or leucine.

This can be correlated with the fact, noted above, that valine and leucine produce an acceleration of the rate of accumulation of glutamic acid within the cell.

Table 5. *Effect of simple mixtures of amino-acids on the accumulation of free glutamic acid within* Staphylococcus aureus

(Washed suspensions of 'deficient' *Staph. aureus* Duncan incubated in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml. with other additions as below. Rate of accumulation of free glutamic acid within the cells determined and expressed as percentage rate occurring in absence of amino-acids other than glutamic acid.)

Amino-acids present in addition to glutamic acid (concentrations in μmol./ml.)	Rate of accumulation of internal free glutamic acid as percentage of that in control without added amino-acids
None	100
Cysteine (1·34)	70
Cystine (sat. soln.)	60
Glycine (4·0)	79
Glutathione (2·7)	91
Cysteine (1·34) + cystine (sat.)	9
Cysteine (1·34) + glycine (4·0)	8
Cysteine (1·34) + glutathione (2·7)	7
Cystine (sat.) + glutathione (2·7)	0
Cystine (sat.) + glycine (4·0)	0
Cysteine (2·7)	54
Alanine (4·0)	72
Valine (2·7)	106
Leucine (2·7)	108
Cysteine (2·7) + valine (2·7)	92
Cysteine (2·7) + leucine (2·7)	94
Alanine (4·0) + valine (2·7)	87
Alanine (4·0) + leucine (2·7)	96

DISCUSSION

When *Staph. aureus* is incubated with glutamic acid in the presence of glucose, the amino-acid enters and accumulates in the free state within the cells. If certain other amino-acids are present, the accumulation of the free glutamic acid within the cells is reduced, but peptides containing glutamic acid accumulate in the external medium. Quantitative analysis of the changes occurring within a given time shows that the amount of combined glutamate accumulating outside the cells as peptide is approximately equal to the reduction in the amount of free glutamic acid accumulating within the cells. Both accumulation of internal free glutamic acid and of external combined glutamate require the presence of glucose. There would appear to be a connexion between the processes involved in the transfer of glutamic acid into the cell on the one hand and in extracellular peptide formation on the other. The fact that the transfer of glutamic acid can be dissociated from glucose fermentation by the action of uncoupling agents (Gale, 1951*a*) has led to the suggestion that some energy-linked metabolism of glutamic acid must take place before or during its transfer across the osmotic barrier of the cell wall. Since the process can be shunted, by the presence of certain other amino-acids outside the cell, to form extracellular peptides, it is possible that peptide-bond formation, or a transpeptidation reaction, may be part of this 'transferring metabolism'. The action of valine and leucine in accelerating the accumulation of glutamic acid, and in releasing the inhibition due to cysteine or alanine, might likewise depend upon the formation of peptides which could penetrate the cell wall more rapidly than the glutamic acid metabolite itself. Experiments are now being carried out with synthetic peptides in order to test this hypothesis.

Identification of the various peptides formed between glutamic acid and cysteine, alanine or glycine has not been completed. In the cases of alanine and glycine, the chromatographic results indicate that a complex system is involved. One of the peptides formed from alanine and glutamic acid appears to be a simple peptide, and the properties of the peptide obtained from glutamic acid and cysteine are consistent with a dipeptide structure; in both cases, deamination and terminal-group tests indicate that the free amino group belongs to the amino-acid other than glutamic acid. Consequently the peptide bond involves the amino group of glutamic acid.

The action of aspartic acid is clearly different from that of cysteine, alanine and glycine. No evidence can be obtained for the accumulation of a peptide of glutamic and aspartic acids. The transfer of aspartic acid into the cell also occurs only in the presence of glucose and it may be that aspartic acid and glutamic acid enter the cell by processes involving a common step so that each appears to compete with the other.

SUMMARY

1. The accumulation of free glutamic acid within *Staphylococcus aureus*, which occurs when the cells are incubated with glutamic acid and glucose, is reduced if aspartic acid, cysteine, alanine or glycine is also present. The presence of either valine or leucine produces an acceleration of the rate of accumulation of free glutamic acid.

2. The inhibition produced by aspartic acid is dependent upon the relative concentrations of aspartic and glutamic acids present. The presence of aspartic acid also reduces the rate at which glutamic acid is withdrawn from the medium. The accumulation of aspartic acid within the cells also requires glucose and is inhibited by the presence of glutamic acid.

3. The inhibition produced by (i) cysteine, (ii) alanine, or (iii) glycine increases as the ratio of each to glutamic acid increases; the inhibition produced

by any given ratio depends also on the absolute concentrations.

4. The decrease in the rate of accumulation of internal free glutamic acid in these cases is accompanied by the formation of combined glutamate in the medium. Chromatographic examination shows that peptide formation has occurred. In the three cases, the extracellular peptides have been hydrolysed and then give glutamic acid and (i) cysteine, (ii) either alanine or alanine and glycine (two peptides), (iii) glycine; a polyglycine is also formed in (iii), but its formation is independent of the presence of glutamic acid.

5. The presence of glucose is essential for the synthesis of these extracellular peptides.

One of the authors (M.B.V.H.) is indebted to the British Council for a scholarship, during the tenure of which part of this work was carried out. Both authors wish to express their thanks to Dr F. A. Isherwood and to the Director of the Low Temperature Research Station for facilities and advice on chromatographic procedures, and to Dr F. Sanger for the gift of fluorodinitrobenzene and assistance in its use.

REFERENCES

Consden, R. & Gordon, A. H. (1950). *Biochem. J.* **46**, 8.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. (1949). *J. gen. Microbiol.* **3**, 369.
Gale, E. F. (1951*a*). *Biochem. J.* **48**, 286.
Gale, E. F. (1951*b*). *Biochem. J.* **48**, 290.
Gale, E. F. & Mitchell, P. D. (1947). *J. gen. Microbiol.* **1**, 299.
Gale, E. F. & Paine, T. F. (1951). *Biochem. J.* **48**, 298.
Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 77.
Krebs, H. A. (1950). *Biochem. J.* **47**, 605.
Lichstein, H. C. & Cohen, P. P. (1944). *J. biol. Chem.* **157**, 85.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1946). *Biochem. J.* **40**, 261.
Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 86.
Van Halteren, M. B. (1951). *Nature, Lond.* (in the Press).
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
Woiwod, A. J. & Proom, H. (1950). *J. gen. Microbiol.* **4**, 501.

Studies in Vitamin A

16. PREPARATION OF NEOVITAMIN A ESTERS AND NEORETINENE$_1$

By P. D. DALVI and R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 7 *February* 1951)

From the time that the structure of vitamin A was established the possibility had to be admitted that fish-liver oils might contain isomers other than the all-*trans* form, although steric considerations might restrict the number. The most convincing evidence for the existence of *cis-trans* isomers depends: (*a*) on the low yield of crystalline all-*trans*-vitamin A from solutions of the richest concentrates, and (*b*) on the evidence of Robeson & Baxter (1945) that some of the vitamin A in oils and concentrates is slow to react with maleic anhydride. The portion which reacts readily is the all-*trans* form which also crystallizes first. The remainder is neovitamin A, a *cis*-isomer in which only the double bond nearest to the $-CH_2OH$ or $-CH_2OCOR$ group possesses the *cis* configuration. Robeson & Baxter obtained crystalline derivatives of neovitamin A differing from the corresponding derivatives of all-*trans*-vitamin A. They also devised an analytical procedure for determining neovitamin A in fish-liver oils. Their results and those of Meunier & Jouanneteau (1948) indicate that neovitamin A makes up a substantial but variable proportion of the total vitamin A in liver oils. The existence of neovitamin A makes it desirable to know whether the biological activity of fish-liver oils varies with the proportion of the total vitamin A present in the neo form. It is not easy to establish quantitatively a difference between the biological potency of all-*trans*- and neo-vitamin A. Robeson & Baxter failed to find any significant difference, but their experiments may not have been on a scale sufficiently large to establish a small difference.

The existence of neovitamin A also makes it necessary to compare very closely the ultraviolet absorption shown by all-*trans*- and neo-vitamin A. The precise form of the absorption curve is important in the analysis of liver oils, when, as is the rule, irrelevant absorption needs to be allowed for. The simpler procedures for 'correcting' for irrelevant absorption are based on a reference curve for pure all-*trans*-vitamin A and if the vitamin A present in