and γ -glutamylhydrazides effectively. The pure hydrazide was converted into the azide in chloroform solution and coupled in one experiment with L-alanine methyl ester and in another with diethyl L-glutamate, according to the procedure of Le Quesne & Young (1950*b*). The products were saponified with *N*-NaOH, and after acidification the acids were extracted into ethyl acetate, which, after drying (Na_2SO_4), was removed under reduced pressure. The crude residues were hydrogenated in the normal fashion, with palladium black as catalyst, in the presence of acetic acid. The crude products (10% solutions in water) were examined by paper chromatography, with butanol-acetic acid-water as solvent; in both cases α -peptide was detected. A further portion of the pure hydrazide was coupled with L-alanine benzyl ester under the conditions of Sachs & Brand (1954); hydrogenation of the crude product gave material which, without purification, was examined by paper chromatography as above, and again α -isomer was detected.

SUMMARY

1. For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands *et al.* 1957), the following new peptides have been synthesized: L-valyl-L-glutamic acid, L-valyl- α -L-glutamyl-L-valine, L-leucyl- α -L-glutamyl-L-leucine, α -L-glutamyl- α -L-glutamyl-L-glutamic acid. α -L-Glutamyl-L-alanine has been prepared by an alternative route.

2. When γ -glutamyl peptides are synthesized through benzyloxycarbonyl- γ -L-glutamyl azide some α products may occur in the crude material (as found by Sachs & Brand, 1954), but subsequent recrystallization will usually eliminate the α -isomer.

Analyses were by Weiler and Strauss, Oxford. We are grateful to the Medical Research Council for a grant for scientific assistance.

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The Assimilation of Amino Acids by Bacteria

23. ACCUMULATION OF FREE GLUTAMIC ACID WITHIN *STAPHYLOCOCCUS AUREUS* INCUBATED WITH DERIVATIVES OF GLUTAMIC ACID*

By D. A. ROWLANDS

The Dyson Perrins Laboratory, University of Oxford

E. F. GALE, JOAN P. FOLKES

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

AND D. H. MARRIAN

Department of Radiotherapeutics, University of Cambridge

(Received 10 July 1956)

Gram-positive bacteria concentrate certain amino acids in the free state in the internal medium of the cells (Gale, 1947, 1953; Taylor, 1947). The passage of amino acids across the cell wall and associated structures may be by a process of diffusion, as with lysine (Gale, 1947; Najjar & Gale, 1950), or by an active process involving exergonic metabolism on the part of the cell, as with glutamic acid (Gale, 1947). If washed suspensions of *Staphylococcus*

aureus (*Micrococcus pyogenes* var. *aureus*) are incubated with glucose and glutamic acid, the amino acid enters the cell and becomes concentrated therein, the ratio of internal to external glutamic acid becoming as high as 350–400:1 when the external concentration is small. The accumulation is prevented by the presence of inhibitors of fermentation, 'uncoupling agents', 8-hydroxyquinoline, or by growth of the cells in the presence of penicillin for a short time before harvesting (Gale, 1949, 1951; Gale & Taylor, 1947). Free amino acids accumulate

* Part 22: Creaser (1956).

within the cells when protein synthesis is inhibited or when cells are incubated in media containing single amino acids or simple mixtures which are unable to support protein synthesis (Gale, 1953).

Understanding of the relation between amino acid accumulation and protein synthesis, and of the importance of the accumulation process to the life of the cell, will involve elucidation of the energy-linked process concerned in the active systems. It may be that there is a membrane which lies in the surface of *Staphylococcus* and is impermeable to glutamic acid, passage of this amino acid into the cell occurring by active transport. Alternatively, it may be that the surface structures are permeable to glutamic acid and that the substance measured within the cell as 'free glutamic acid' is actually a labile derivative thereof which cannot be distinguished from the free amino acid by present methods of detection and liberation from the cell. Britten (1952) investigated the passage of ^{14}C -labelled glutamic acid into *Staph. aureus* and distinguished two processes: a temperature-dependent exchange between added glutamic acid and glutamic acid 'stores' within the cells, and a rapid temperature-independent exchange between added amino acid and glutamic acid in a 'water space' corresponding to 30–40% of the packed cell (pellet) volume. This latter finding appeared to support the hypothesis that glutamic acid diffuses freely into a substantial portion of the staphylococcal cell, but further investigation (Britten, 1955) showed that the 'water space' included intercellular water corresponding to 26–30% of the pellet volume and that, consequently, the staphylococcal cell itself was impermeable to free glutamic acid. This fact has been confirmed by Mitchell & Moyle (1956), who have established the existence of an osmotic barrier in this organism. The present communication deals with the accumulation of free glutamic acid within staphylococci incubated in media containing various derivatives of glutamic acid.

ORGANISM AND METHODS

Organism. The organism used throughout this work was the strain of *Staph. aureus* Duncan used for earlier studies of amino acid assimilation (Gale, 1947, 1951) and also used by Britten (1952). Cultures were grown for 14 hr. at 25° in Roux bottles, each containing 150 ml. of the deficient medium previously described (salt solution containing arginine, Marmite and glucose; Gale, 1947). Cells were harvested on the centrifuge, washed once and suspended in water; the suspension density was determined turbidimetrically in a Hilger absorptiometer previously calibrated against the organism used.

Incubation conditions. Centrifuge tubes (100 ml.) were incubated in a water bath at 37° (or other temperature as indicated); each tube contained 20 ml. of buffered saline solution, pH 7.0 (Gale, 1947), 1.0 ml. of 25% (w/v) glucose,

1.5 ml. of solution of glutamic acid derivative under test and 2.5 ml. of suspension of washed *Staph. aureus* adjusted to a suspension density of 20 mg. dry wt. of organisms/ml. In controls in which components of the incubation mixture were missing, the volume was adjusted to 25 ml. with water. Incubation was stopped when desired by addition to each tube of 10 ml. of 0.2 M acetate buffer, pH 4.5, and about 15 g. of crushed ice. Organisms were then centrifuged down, washed twice in 0.01 M acetate buffer, pH 4.5, made up to 2.5 ml. in water, and the free glutamic acid within the cells was determined by the specific decarboxylase method (Gale, 1945, 1947).

Rate of accumulation of free glutamic acid within cells incubated with derivatives. The rate of accumulation of free glutamic acid within the cells is dependent upon the external concentration of amino acid for low values of the latter, but independent once the external concentration has exceeded a saturation value (Gale, 1947). It was desirable in these experiments to work at concentrations of derivatives exceeding the saturation level, although the limited supply of some of the substances made it impossible to use these in concentrations well in excess of the saturation value. Consequently, preliminary experiments were carried out with each derivative in order to determine the saturation concentration; with glutamyl peptides this concentration was approx. 1.0 $\mu\text{mole/ml.}$, and later experiments were carried out in a final concentration of 1.5–2.0 $\mu\text{moles of peptide/ml.}$ Experiments were then performed in which the rate of accumulation of free glutamic acid within the cells was determined during incubation with the derivative in the presence and absence of glucose; in all cases the results were compared with the rate of accumulation occurring in cells incubated in the presence of glutamic acid and glucose. In some studies the effect of temperature on the process was investigated by carrying out incubations simultaneously at 20°, 25° and 37°.

Determination of rate of hydrolysis of peptides within cells. As Zamecnik & Stephenson (1947) have shown, the rate of hydrolysis of a peptide can be followed in the presence of the specific amino acid decarboxylase which attacks one of the hydrolysis products. Glutamic acid decarboxylase does not attack peptides containing glutamic acid (Gale, 1946) and can be used to study the rate of liberation of glutamic acid from peptides. To obtain a measure of the rates of hydrolysis, within *Staph. aureus* cells, of the peptides used in these studies, the following procedure was adopted: a thick suspension of cells in 0.1 M phosphate buffer, pH 5.5, was disintegrated by exposure to supersonic vibration at 25 kcy./sec. for 30 min. in a Mullard 500 w magnetostrictor oscillator, and 1.0 ml. of the disintegrated suspension (approx. 30 mg. dry wt. of cells) was shaken in a Warburg vessel with 1.0 ml. of L-glutamic acid decarboxylase preparation (Gale, 1945), 1.0 ml. of 0.2 M acetate buffer, pH 5.5, and 0.5 ml. of 0.035 M peptide, the peptide and decarboxylase preparations being made up in acetate buffer and adjusted to pH 5.5. The steady rate of CO_2 evolution was taken as a measure of the liberation of glutamic acid from the peptide; the amount of decarboxylase preparation used was such that the rate of decarboxylation of glutamic acid was 2–3 times greater than the fastest liberation of CO_2 from the system containing peptide preparations.

Determination of partition coefficients. A quantity (10 μmoles) of each peptide was weighed out and dissolved in 1.0 ml. of water saturated with *n*-butanol. The peptide

solution was placed in a 10 ml. tube and 5.0 ml. of *n*-butanol saturated with water added; the tube was sealed and shaken overnight at room temperature (20–22°) and then left at 25° for 48 hr. For estimation, 0.1 ml. of the aqueous phase and 1.0 ml. of the butanol phase were withdrawn, run into small beakers and evaporated to dryness over CaCl_2 . The remaining mixture was transferred to a new tube, washed in with 0.1–0.3 ml. of water, saturated with butanol, resealed and equilibrated as above. Further samples were then withdrawn for estimation and the whole operation was repeated so that three consecutive estimations of the partition coefficient were obtained. The amount of peptide in each phase was estimated by development of colour with ninhydrin according to the procedure of Troll & Cannan (1953); the material in the evaporated butanol phase was dissolved in 0.17 *M* sodium acetate solution (0.5 ml.), 0.2 ml. of ninhydrin and 1.0 ml. each of the phenol and KCN-pyridine solutions were added and the solution was washed into a heating tube with two 0.5 ml. quantities of 60% ethanol, heated for 4 min. at 100° and finally made up to 10.0 ml. with 60% ethanol. The equivalent material from the aqueous phase was treated with twice these quantities of the various reagents and the final volume adjusted to 20.0 ml. The optical density of the solutions was compared at 570 μ . and estimated against standard peptide solutions.

Sources of glutamic acid derivatives

We are indebted to Dr J. S. Fruton for samples of α -L-glutamyl-L-glutamic acid, to Dr A. C. Chibnall for β -hydroxyglutamic acid, 5-oxo-L-pyrrolidine-2-carboxylic acid and α -aminobutyric acid, and to Dr D. W. Clayton for diethyl L-glutamate, α -L-glutamyl-L-glutamic acid and α -L-glutamyl-L-glutamic acid.

N-Phosphoryl-L-glutamic acid was prepared as the dimagnesium salt by the method of Winnick & Scott (1947). Its glutamic acid content was determined by estimation of the free glutamic acid liberated after 10 min. hydrolysis at 100° in *N*-HCl. Incubation of *N*-phosphoryl-L-glutamic acid with glutamic acid decarboxylase resulted in a slow evolution of CO_2 corresponding to the rate of liberation of free glutamic acid by hydrolysis.

N-Acetyl-L-glutamic acid was prepared by the method of Cahill & Burton (1940). This substance was not attacked by glutamic acid decarboxylase.

Diethyl L-glutamate hydrochloride was prepared by the method of Chiles & Noyes (1922); it was not attacked by glutamic acid decarboxylase.

Diethyl *N*-dibenzylphosphoryl-L-glutamate was prepared by the general procedure reported by Atherton, Openshaw & Todd (1945). Diethyl L-glutamate (5.54 g.) and 7.15 g. of dibenzyl phosphite were dissolved in CCl_4 (25 ml.) and drops of a solution containing 2.8 ml. of *N*-methylmorpholine in 5 ml. of CCl_4 added. A precipitate formed and the whole was left overnight at room temperature. An equal volume of water was then added and the organic layer separated. The aqueous phase was extracted three times with 10 ml. of CCl_4 and the combined extracts were added to the original layer and dried over anhydrous Na_2SO_4 . Removal of the solvent *in vacuo* left an almost colourless residue which rapidly crystallized. The solid was dissolved in CCl_4 (15 ml.), filtered and treated with 25 ml. of dry hexane. On standing, colourless needles of diethyl *N*-dibenzylphosphoryl-L-glutamate separated, m.p. 66–67°, yield 60.5%. (Found:

C, 59.6; H, 6.69; N, 2.72; calc. for $\text{C}_{23}\text{H}_{30}\text{O}_7\text{NP}$: C, 59.6; H, 6.53; N, 3.02%.)

Diethyl *N*-phosphoryl-L-glutamate. Diethyl *N*-dibenzylphosphoryl-L-glutamate (3.0 g.) was dissolved in 100 ml. of 95% ethanol and hydrogenated over reduced palladium oxide (0.3 g.) at room temperature and pressure. When 318 ml. of H_2 had been absorbed, uptake ceased (removal of two benzyl groups requires 290 ml. at s.t.p.). The catalyst was filtered off and the solvent removed *in vacuo* at 30°, leaving a clear resin (1.85 g.; yield 100%). (Found: C, 38.6; H, 6.49; N, 4.66; calculated for $\text{C}_9\text{H}_{18}\text{O}_7\text{NP}$: C, 38.1; H, 6.35; N, 4.95%.) The compound was neutralized before testing.

Glutamyl peptides. The preparation of peptides, other than those mentioned above, is described in the preceding paper (Rowlands & Young, 1957).

RESULTS

Substances giving rise to free glutamic acid within cells incubated in the absence of glucose

N-Phosphoryl-L-glutamic acid. Fig. 1 shows the time course of the accumulation of free glutamic acid within *Staph. aureus* incubated with *N*-phosphorylglutamic acid in the presence and absence of glucose. In the presence of glucose, the accumulation within the cell is the same whether

Fig. 1. Accumulation of free glutamic acid within *Staph. aureus* incubated with various external sources of glutamic acid. Cells were incubated at a final suspension density of 2.0 mg. dry wt./ml. in buffered saline at pH 7.0 (glutamic acid, diethyl glutamate) or 5.5 (*N*-phosphorylglutamate), at 37° in the presence of the following: curve 1, 9 μ moles of diethyl L-glutamate/ml.; curve 2, 9 μ moles of *N*-phosphoryl-L-glutamate/ml. added at time 0; curve 3, as for curve 2 with a further 9 μ moles of *N*-phosphorylglutamate/ml. added at time 40 min. as indicated by arrow; curve 4, as for curve 2, with addition of 1% (w/v) of glucose; curve 5, 5 μ moles of L-glutamate and 1% of glucose.

glutamic acid itself or the *N*-phosphoryl derivative is the external source. In the absence of glucose, no accumulation occurs when glutamic acid is the external source but a significant accumulation occurs when *N*-phosphorylglutamic acid is present in the external medium. The rate of accumulation in the latter case varied widely in different experiments but has reached values 60–70 % of that obtained when glucose and glutamic acid are present. The figure shows that the accumulation with *N*-phosphorylglutamic acid as source rapidly decreases in rate. The phosphoryl derivative is unstable and it seemed probable that the rapid cessation of glutamic acid accumulation might be due to decomposition of the derivative in the medium during incubation; that this is the explanation is shown by the fact that accumulation is resumed if a further supply of phosphoryl derivative is added to the incubation medium, as shown in Fig. 1. The nature of the progress curve after a single addition makes it impossible to carry out an accurate study of the kinetics of the process, and conclusions have to be based upon rates measured within 20 min. of the addition. Determination of the rate of accumulation over a range of temperatures gives a value of approx. 2.4 for the temperature coefficient. Fig. 2 shows that the optimum pH for glutamic acid accumulation in this case is approx. 5.5.

Fig. 2. Effect of pH of medium on rate of accumulation of free glutamic acid within *Staph. aureus* incubated with either *N*-phosphoryl-L-glutamate or diethyl L-glutamate. Conditions were as for curve 1 or 2 of Fig. 1.

Diethyl L-glutamate. Fig. 1 shows that a slow accumulation of free glutamic acid takes place in *Staph. aureus* incubated in the presence of diethyl glutamate. The further addition of glucose was found usually to have little effect; in some experiments the rate of accumulation was increased by approx. 50 % in the presence of glucose, whereas in the majority of tests the enhancement was within experimental error. Fig. 2 shows that the optimum pH for the accumulation was 8.5, although the effect of pH was not marked over the range tested. The temperature coefficient for the process over the range 20–37° was 1.8; this is the lowest coefficient measured in the present experiments.

The accumulation of free glutamic acid within cells incubated with diethyl glutamate must follow upon two processes: passage of the ester or its hydrolysis products into the cell, and hydrolysis of the ester to liberate free glutamic acid. It seemed possible that the latter process might be limiting. To test this, cells were incubated with ester in the usual way, centrifuged down and the cell batch divided into two; the free glutamic acid content of one portion was determined immediately while the other portion was washed and then incubated for 1 hr. at 37° in the absence of added ester before determination of the free glutamic acid content. Table 1 shows that there is a marked increase in the free glutamic acid content during the second period of incubation, presumably due to hydrolysis of ester accumulated (but not hydrolysed) within the cell during the first incubation period. It would appear, therefore, that the rate of penetration of ester into the cells is greater than its rate of hydrolysis to the free acid within the cells.

Table 1. Accumulation of free glutamic acid within *Staphylococcus aureus* incubated with diethyl glutamate

Washed suspension of *Staphylococcus aureus* Duncan was incubated at a final density of 2.2 mg. dry wt. of cells/ml. in buffered saline, pH 7.5, containing 9.0 μmoles of diethyl glutamate/ml. at 37°; samples were withdrawn at 30 and 60 min. and divided into two: one portion was analysed immediately for free glutamic acid, and the second portion was washed once in water, suspended in buffered saline at 2.2 mg. dry wt./ml. and incubated for 1 hr. at 37° before analysis for free glutamic acid.

Time of first incubation (min.)	Increase in internal free glutamic acid (μmoles/100 mg. dry wt. of cells)	
	Estimated on first harvesting	Estimated after second incubation
30	2.3	9.25
60	6.9	22.1

Diethyl N-phosphoryl-L-glutamate. *Staph. aureus* cells were incubated with a range of concentrations of a preparation of diethyl N-phosphorylglutamate in the presence and absence of glucose over the pH range 5.0–9.0. In no case was there any significant accumulation of free glutamic acid within the cells.

Substances giving rise to free glutamic acid within cells incubated in the presence of glucose

Glutamine. Fig. 3 shows the course of accumulation of free glutamic acid within *Staph. aureus* incubated with glutamine and glucose. The rate of accumulation is slower than that obtained when free glutamic acid itself is present in the external medium. No accumulation takes place if glucose is omitted from the medium.

Peptides containing a glutamic acid residue. Table 2 lists the glutamyl peptides tested which give rise to free glutamic acid within cells when incubation takes place in the presence of glucose; no peptide has yet been obtained which will give rise to free glutamic acid within cells in the absence of glucose in the incubation medium. The rate at which free glutamic acid accumulates (expressed in all cases in terms of the rate with glutamic acid and

glucose present as 100) within the cells varies widely with the peptide used as source. In general, α -glutamyl peptides are markedly better sources than the corresponding γ -glutamyl peptides. Fig. 3 shows the time course of the accumulation that occurs when various polyglutamic acid peptides are used as source; the substances have the following order of decreasing efficiency as source: glutamic acid, α -glutamylglutamic acid, α -glutamylglutamic acid, γ -glutamylglutamic acid, α -glutamyl- α -glutamylglutamic acid. In all cases the sources used consisted of L-isomers only. α -Glutamylglycine is almost as effective a source as glutamic acid itself, and both α -glutamylvaline and α -glutamylleucine give rise to glutamic acid within the cell at rates markedly greater than that obtained with glutamic acid itself as external source.

Factors affecting the rate of accumulation. Inspection of the data in Table 2 suggests that two factors might influence the rate of accumulation: (1) since peptides containing valine or leucine are more effective sources than those containing less lipid-soluble residues, it seems possible that penetration of a lipid-like barrier affects the rate of accumulation within the cell; (2) since α -glutamyl peptides are more effective sources than γ -glutamyl peptides, it seems possible that the action of an α -peptidase is limiting in some cases. As a measure of the lipid solubility of the derivatives, the partition coefficient between *n*-butanol and water was determined, and the peptidase action of a broken cell preparation (broken cells being used to avoid complications due to differences in penetration) towards various peptides was measured as described above. The results are shown in Table 2, and it can be seen that there are wide variations both in the rates of hydrolysis and in the partition coefficients of the peptides tested. In certain cases (e.g. α -glutamylglutamic acid, α -glutamylglutamic acid, α -glutamyl-tyrosine or -leucine) there appears to be correspondence between high rates of accumulation and high partition coefficients, but there is no such correlation for the whole range of peptides studied. Likewise there are some examples, such as alanylglutamic acid and α -glutamyl-valine or -leucine, where high rates of accumulation correspond with rapid rates of hydrolysis, but this correspondence does not extend to the tripeptides studied. However, there is a highly significant correlation between the rate of accumulation and the product of the partition coefficient and the rate of hydrolysis.

The rates of hydrolysis of γ -glutamyl peptides are within the experimental error of the method used and, although such peptides are poor sources of internal free glutamic acid, there would nevertheless appear to be a correlation between their activity in this sense and their partition coefficient.

Fig. 3. Rate of accumulation of free glutamic acid within *Staph. aureus* incubated with various external sources of glutamic acid. Cells were incubated at a final suspension density of 2.0 mg. dry wt./ml. in buffered saline at pH 7.0 containing 1% (w/v) of glucose and the following sources of glutamic acid at a final concentration of 1.5 μ moles/ml.: curve 1, sodium L-glutamate; curve 2, α -L-glutamyl-L-glutamic acid; curve 3, L-glutamine; curve 4, α -L-glutamyl-L-glutamic acid; curve 5, γ -L-glutamyl-L-glutamic acid; curve 6, α -L-glutamyl- α -L-glutamyl-L-glutamic acid.

Table 2. *Rate of accumulation of free glutamic acid within Staphylococcus aureus incubated in the presence of glucose and peptides containing glutamic acid*

Rate of accumulation was determined in washed suspensions of cells incubated at a final density of 2.0 mg. dry wt./ml. in buffered saline, pH 6.5, containing 1% of glucose and peptide at final concn. of 1.5 μ moles/ml. Temperature, 37°. Rates are expressed on basis of rate obtained with L-glutamic acid as source = 100. Temperature coefficients are calculated as increase in rate on raising temperature from 20 to 30°. Partition coefficients are between *n*-butanol and water, and rates of hydrolysis are determined as in text; rate of hydrolysis is expressed as μ l. of CO₂ evolved/hr. from system containing acetate buffer, pH 5.5, 0.005M peptide, disintegrated *Staph. aureus* and L-glutamic acid decarboxylase preparation.

Peptide	Rate of accumulation of free glutamic acid (a)	Temp. coefficient (b)	Partition coefficient (butanol-water) (c)	Rate of hydrolysis (d)	(c $\times$ d)
α -L-Glutamyl-L-leucine	180	2.33	0.08	1500	120
α -L-Glutamyl-L-valine	140	2.25	0.046	1300	60
L-Leucyl- α -L-glutamyl-L-leucine	115	—	0.18	288	52
L-Alanyl-L-glutamic acid	110	—	0.024	1110	27
L-Valyl-L-glutamic acid	106	—	0.044	1100	49
(L-Glutamic acid)	100	2.8	0.027	—	—
α -L-Glutamyl-L-alanine	100	—	0.013	1010	13
L-Valyl- α -L-glutamyl-L-valine	94	—	0.076	377	29
α -L-Glutamyl-L-tyrosine	89	—	0.06	920	55
α -L-Glutamylglycine	88	—	—	—	—
α -L-Glutaminyl-L-glutamic acid	69	2.5	0.035	565	20
(L-Glutamine)	60	2.5	0.021	—	—
α -L-Glutamyl-L-glutamic acid	45	2.0	0.013	720	9.4
α -L-Glutamyl-L-aspartic acid	35	—	0.017	813	14
Glycyl-L-glutamic acid	22	—	—	—	—
γ -L-Glutamyl-L-valine	20	—	0.11	—	—
γ -L-Glutamyl-L-leucine	16	—	0.21	—	—
Glutathione	15	2.5	—	—	—
γ -L-Glutamyl-L-glutamic acid	13	2.4	0.025	3	—
α -L-Glutamyl- α -L-glutamyl-L-glutamic acid	7	—	0.015	435	6.5
γ -L-Glutamyl-L-aspartic acid	5	—	0.018	0	—
γ -L-Glutamylglycine	3	—	0.021	—	—

Statistical data

 α -Glutamyl peptides

Correlation between first and last columns:

$$(c \times d) = 0.57(a) - 14,$$

 γ -Glutamyl peptides

$N = 12$, correlation coeff. = 0.845, $P < 0.01$.

Correlation between first and third columns:

$$c = 0.0102(a) - 0.040,$$

$N = 5$, correlation coeff. = 0.944, $P = 0.02$.

Table 3. *Concentration of inhibitors producing 50% inhibition of the rate of accumulation of free glutamic acid within Staphylococcus aureus incubated with glutamic acid derivatives*

Conditions as in Fig. 1 and Table 2.

Derivative	Sodium azide (mM)	2:4-Dinitrophenol (mM)	8-Hydroxyquinoline (mM)
A. In the presence of glucose			
L-Glutamic acid	6.4	0.45	0.08
L-Glutamine	9.0	0.40	0.08
α -L-Glutamyl-L-glutamic acid	3.5	0.24	—
α -L-Glutamyl-L-valine	—	0.20	—
Glutathione	2.0	0.08	1.2
(Glucose fermentation)	10	10	5.0
B. In the absence of glucose			
<i>N</i> -Phosphoryl-L-glutamic acid	3.2	0.3	2.0
Diethyl L-glutamate	6.4	0.3	5.0

Actions of inhibitors on the accumulation of glutamic acid within cells incubated with various external sources

Table 3 shows the concentration of certain metabolic inhibitors which decreases by 50 % the rate of accumulation of glutamic acid within cells incubated with various external sources of the amino acid. The inhibition obtained when glutamic acid is the external source has been discussed in previous papers (Gale, 1949, 1951). There are no marked differences in the sensitivities of the accumulation processes with the various sources in the presence of either sodium azide or 2:4-dinitrophenol, although the accumulation occurring in the presence of *N*-phosphorylglutamic acid or of diethyl glutamate (in the absence of glucose) is much less sensitive to the action of 8-hydroxyquinoline than that which occurs when glutamic acid, in the presence of glucose, is the external source.

DISCUSSION

Argument concerning the nature of the active concentration of free glutamic acid within *Staph. aureus* centres on the question whether energy is required to transport glutamic acid across an otherwise impermeable membrane, or whether it is required for some metabolic modification or binding after diffusion of the molecule into the cell interior. When a derivative of glutamic acid is used as the external source, accumulation of the free amino acid within the cell will depend also upon liberation of glutamic acid from the derivative. From a study of the factors involved in controlling the rate of accumulation of free glutamic acid within cells incubated with various derivatives thereof, it should be possible to decide whether there is any barrier to free penetration of any of these derivatives, since such a barrier would impose a limitation on their accumulation and metabolism within the cell.

When glutamyl peptides are supplied as external source, the rate of accumulation of free glutamic acid within the cell can be correlated with the product of the partition coefficient between *n*-butanol and water, and the rate of hydrolysis of the peptide by cell extracts. If no penetration factor were involved, a correlation would be expected with the rate of hydrolysis alone. The penetration factor might relate either to the peptide itself or to the products of hydrolysis, assuming this to take place before penetration. Since the same product of hydrolysis, glutamic acid, is estimated in all cases, it follows that the penetration factor must relate to the peptide and not to the products. It seems probable, therefore, that the various peptides

which give rise to free glutamic acid within the cells have to penetrate some barrier before they are broken down to liberate free glutamic acid. That external glutamic acid itself has to pass some barrier is indicated by the fact that certain peptides, such as α -glutamyl-valine and -leucine, give rise to a more rapid accumulation of glutamic acid within the cell than is obtained with glutamic acid itself as external source. This confirms the demonstration by Mitchell & Moyle (1956) that the staphylococcal cell is impermeable to free glutamic acid. The presence of a barrier is further indicated with diethyl glutamate, where evidence has been obtained of accumulation of the ester within the cell, the ester remaining within the cell despite washing with water.

The only substances which have been found to yield glutamic acid within the cell when incubation has taken place in the absence of glucose have been the diethyl ester and the *N*-phosphoryl derivative of glutamic acid. If the temperature coefficient can be taken as any indication of the energy of activation in a system as complex as this, the ester passes into the cell more readily than the phosphorylated derivative. In all the other cases investigated, a source of energy is necessary for the accumulation of free glutamic acid, which suggests that a process of active transport across the barrier is involved. It is possible that the breakdown of some molecules of *N*-phosphorylglutamic acid yield energy for the transport of other molecules or of the liberated glutamic acid; this would be in accord with the high temperature coefficient for the overall process and also with its sensitivity to uncoupling agents such as sodium azide and dinitrophenol. It seems probable that the penetration of the ester occurs by passive transport, although the overall process is again sensitive to sodium azide and dinitrophenol. Cohen & Rickenberg (1956) have recently demonstrated that the passage of amino acids into *Escherichia coli* is an energy-linked reaction and have suggested that enzyme-like 'permeases' in the cell membrane are involved.

Kihara, McCullough & Snell (1952) have shown that D-alanine prevents the growth of *Lactobacillus arabinosus* by inhibiting the assimilation of L-alanine, but that D-alanine has no inhibitory action in the presence of alanyl peptides. The authors suggest that the D-amino acid inhibits the passage of L-alanine across the cell membranes, whereas peptides pass independently and are converted into the free amino acids within the cell. A similar hypothesis has been developed to explain other instances where the inhibitory action of amino acid analogues is overcome by the presence of peptides (Kihara & Snell, 1955). The results presented in this communication are consistent with such hypotheses.

SUMMARY

1. Free glutamic acid accumulates within *Staphylococcus aureus* incubated in the presence of *N*-phosphoryl-L-glutamic acid or diethyl glutamate.

2. Free glutamic acid does not accumulate within cells incubated with peptides containing glutamic acid residues unless glucose is also present. The rate of accumulation is higher when incubation takes place with α -glutamyl peptides than with γ -glutamyl peptides and is affected by the rate of hydrolysis of the peptide within the cell and also by the partition coefficient of the peptide between *n*-butanol and water.

3. When *Staphylococcus aureus* is incubated with glucose and di- or tri-peptides containing α -glutamyl residues, the rate of accumulation of free glutamic acid within the cells can be correlated with the product of the rate of hydrolysis and the partition coefficient between butanol and water.

4. Accumulation of free glutamic acid in all cases studied is inhibited by sodium azide and 2:4-dinitrophenol.

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Phospholipids

4. ON THE COMPOSITION OF HEN'S EGG PHOSPHOLIPIDS*

BY D. N. RHODES AND C. H. LEA

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

(Received 10 August 1954)

Despite the long-established use of egg yolk as a convenient source of phospholipids, data available in the literature on the composition of this important fraction of the yolk (over 20% of the total solids) are incomplete and contradictory. Wittecoff (1951), in his recent monograph, was able to quote only three analyses, the earliest of which (30% lecithin, 70% kephalin) is clearly in conflict with the later data of Nottbohm & Mayer (1933) (72% lecithin, 28% kephalin) and of Kaucher, Galbraith, Button & Williams (1943) (72% lecithin, 25% kephalin, 2.5% sphingomyelin).

The contents of lecithin obtained by these later workers were based upon estimation of choline after

hydrolysis, as were the recently published figures of 80% (van Handel, 1954), 82% (Sulser, 1954), 76% (Thiele, 1955) and 78% (Tsuji, Brin & Williams, 1955).

The remainder of the lipid nitrogen is usually reported as 'kephalin', but direct determination of the amino nitrogen has indicated considerably higher values, e.g. 42% (Chargaff, Ziff & Rittenberg, 1942), 38% (Kline & Johnson, 1946), 35% (Kline, Gegg & Sonoda, 1951) and 9.4, 20 and 32% (Sulser, 1954). Chargaff *et al.* (1942) used a direct van Slyke determination on the intact phospholipid, a procedure known to be subject to interference by the unsaturated acids present (Lea & Rhodes, 1954). Kline *et al.* (1951) also used this method, but stated that the chosen reaction time of 5 min. gave a

* Part 3: Lea, Rhodes & Stoll (1955).