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THE INTRACELLULAR AMINO ACIDS OF *STAPHYLOCOCCUS AUREUS*: RELEASE AND ANALYSIS

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The investigations of GALE[1] and TAYLOR[2] showed that cells of a number of gram-positive bacteria (14 species) and yeasts (3 species) contain certain free amino acids, which were detected and estimated by enzymic methods after liberation by treatment of washed cells with boiling water or detergent substances. *Staphylococcus aureus* was shown to contain free glutamic acid and lysine; washed suspensions of cells of this organism were able to establish concentration gradients of these amino acids across the cell surface, and further results showed that a number of other amino acids could be concentrated within the cells (GALE[3]). Chromatographic investigations of the free internal amino acids have been made with *Corynebacterium diptheriae*[4], *Neurospora crassa*[5,6], *Aspergillus nidulans*[7] and *Saccharomyces cerivisiae*[8].

No evidence was found in early studies for the existence of free amino acids within cells of gram-negative bacteria; however MANDELSTAM[9] demonstrated chromatographically the presence of certain free amino acids within cells of *Escherichia coli*, and accumulation of radioactive amino acids in cells of this organism has been observed[10,11]. Loss of internal amino acids from cells of *E. coli* on washing with media of low osmotic pressure was observed by BRITTEN[12] and osmotic sensitivity of this type may have been responsible for the failure of earlier attempts to detect free amino acids in cells of gram-negative organisms.

The objects of the work described here were to determine the conditions under which the free internal amino acids of *S. aureus* are released, and to investigate the nature of the amino acids present in the internal pool.

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MATERIALS AND METHODS

Organism and media

Staphylococcus aureus strain Duncan (GALE AND TAYLOR[13]) was grown in Roux bottles in the medium B (GALE[1]). For experiments in which concentration gradients of amino acids across the cell surface were to be determined, it was necessary to know the concentration of amino acids in the growth medium and a synthetic medium was used, containing glucose (1%, w/v), thiamine, nicotinic acid and pyridoxin (2 μg each/ml), L-arginine-HCl, L-cysteine, L-histidine-HCl, DL-isoleucine, L-leucine, DL-methionine, L-lysine-HCl, L-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and L-valine (0.5 μmole each L-isomer/ml) and L-alanine, L-aspartic acid, L-glutamic acid and glycine (1.25 μmole each/ml). Cells were harvested from cultures in the exponential phase of growth at 37° at a culture density corresponding to *c* 0.3 mg dry weight of cells/ml. They were washed twice with distilled water by centrifugation, and were suspended in distilled water at *c* 10 mg/ml; when the effect of the osmotic pressure of the washing medium was being investigated, appropriate concentrations of sucrose or of NaCl were used in place of distilled water.

Measurement of rates of release of soluble constituents

A sample of a washed cell suspension was added to a solution of the extracting agent to give a final cell density of 1–1.5 mg dry weight/ml. The suspension was rapidly mixed and incubated in a water bath maintained at either 5° or 37°; samples were withdrawn by pipette and the cells rapidly separated on membrane filters. At zero time a control sample was taken from an identical suspension in distilled water; small quantities of amino acids, inorganic phosphate and 260 mμ-absorbing material were found in the filtrate, probably due to lysis of some cells during preparation of the suspension, and these values were substracted from experimental estimations. Aliquots of the filtrates were taken for estimation of substances released.

"Oxoid" membrane filters (3 cm diameter) were used, mounted in a metal assembly allowing rapid changing of filters. Using 0.5 mg cells/sample, complete separation of cell residues was obtained in 10–20 sec. Recoveries of extracted substances by this method were identical with those obtained by centrifugal separation, and no contamination from the filters by amino acids, phosphate or 260 mμ-absorbing material was experienced. For the preparation of larger samples for chromatography, cells were centrifuged after extraction; the supernatant was removed by decanting, lyophilised, and taken up in a small volume of water for application to paper chromatograms or in *c* 2 ml for column chromatography. Samples which contained trichloroacetic acid were extracted with ether.

Amino acids were estimated by the method of COCKING AND YEMM[14]; it was later found that over 90% of the ninhydrin-reacting material in the extracted fractions could be identified as amino acids, and the results of ninhydrin estimations are expressed as concentrations of amino acids by reference to a glutamic acid standard.

Proline was estimated separately by the method of TROLL AND LINDSLEY[15].

Inorganic phosphate was estimated according to FISKE AND SUBBAROW[16].

Absorption spectra

Absorption spectra of extracted material were determined in the Beckman model DU spectrophotometer against a blank of the relevant extracting agent; estimations of 260 mμ-absorbing material were made similarly.

Paper chromatography

Samples were applied to squares of Whatman No. 4 paper (25 cm × 25 cm), which were developed by the ascending technique with *sec.*-butanol–formic acid–water (70:10:20) (solvent A) followed by aqueous phenol (80%, w/v) containing 0.3% (v/v) ammonia (sp.gr. 0.88) (solvent B) in the second dimension. After drying, chromatograms were sprayed either with ninhydrin or, for the detection of proline, with isatin (both reagents 0.2%, w/v, in acetone), and developed by heating at 105° for 3–5 min. Components present were identified by comparison with the position of authentic amino acid markers.

Chromatography on Dowex-50 × 4. The columns, operation and analyses of effluent fractions were as described by MOORE AND STEIN[17]. Fractions constituting a single peak were pooled and lyophilised and after desalting by ion exchange[18], the components present were identified by one-dimensional paper chromatography in the solvent systems A and B, using authentic amino acids as markers.

Identification of the unknown substance U on paper chromatograms. To minimise the possibility that U might be a product of autolytic breakdown of some cell component, a rapid procedure was used for harvesting and extracting cells. Cells were harvested from an exponentially-growing culture (250 ml) on a membrane filter (6 cm diameter) in *c* 3 min at 5°. The cells (*c* 100 mg dry weight) were removed from the filter by agitating it in a small volume of ice-cold water, and

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ethanol was immediately added to the cell suspension to give a final concentration of 50% (v/v). After centrifuging the sample at 3000 *g* for 30 min at 5°, the supernatant was removed and lyophilised. The residue was taken up in a small volume of water and applied on a line near to one edge of a sheet of Whatman No. 3 paper; the paper was developed in chromatographic solvent B. Marker strips cut from the side of the paper were sprayed with ninhydrin and used to locate the band containing U; this band was cut out and eluted with water. The eluate was lyophilised and applied to a second similar paper; after development of the paper with solvent A the band containing U was again detected, eluted and the eluate lyophilised. Such preparations of U contained a small quantity of proline, but this did not interfere with subsequent tests. Preparations were hydrolysed with 6 *N* HCl for 16 h at 105° in a sealed tube; HCl was removed in an air stream at 45°, and the sample was re-evaporated from small volumes of water. A small quantity of water was added and the sample was centrifuged to remove solid material before application to paper chromatograms.

Suspension densities

Suspension densities were determined by weighing samples of a cell suspension after drying in an air oven at 105° for 8 h.

RESULTS

Effect of wash medium on recovery of free amino acids

There is little difference between the quantity of free amino acids extractable from cells of *S. aureus* when sucrose or NaCl at concentrations ranging from 0.01 *M* to 1.0 *M* are used for washing in place of distilled water (Table I). This is in contrast to the behaviour of cells of *E. coli* strain B (BRITTEN[12] and unpublished results of the author). In all other experiments reported here cells were washed in distilled water.

TABLE I

EFFECT OF WASHING MEDIUM ON FREE AMINO ACID CONTENT OF *S. aureus*

Molarity of medium used for washing cells	*Amino acid content (extracted by 5% TCA, 15 min, 5°) of cells washed in*	
	NaCl	*Sucrose*
0*	487	487
0.01	475	500
0.2	505	540
0.4	518	475
0.6	543	—
0.8	525	510
1.0	497	521

* Cells washed in distilled water.

Comparison of extracting agents

Progress curves for the release of amino acids from cells of *S. aureus* by various extracting agents are presented in Fig. 1. Parallel release of inorganic phosphate and 260 mμ-absorbing material occurred in all the procedures investigated; the release of these substances by boiling water and by trichloroacetic acid (TCA) is also shown in Fig. 1. Absorption spectra of material released showed a single peak at approximately 260 mμ (Fig. 2); SALTON[19] has shown that the 260 mμ-absorbing substances released from *S. aureus* by cetyltrimethylammonium bromide (CTAB) are purines and pyrimidines and the corresponding nucleosides and nucleotides.

The same quantity of amino acids were released from cells by all the extraction procedures used (Table II); the times in which release is complete are also shown in

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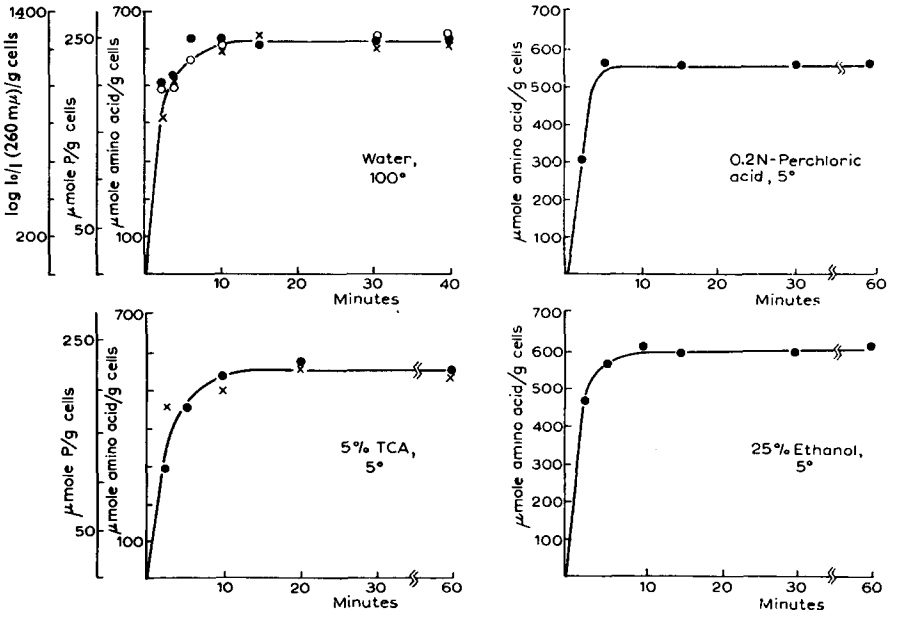

Fig. 1. Release of amino acids (●—●), inorganic phosphate (P) (×—×) and 260 mμ-absorbing material (○—○) from cells of *S. aureus* by various extracting agents. Cell density 1.5 mg/ml.

TABLE II

RELEASE OF FREE AMINO ACIDS FROM *S. aureus* BY VARIOUS EXTRACTING AGENTS

Extracting agent	*Temperature of extraction, °*	*Time for complete release (min)*	*Total quantity of amino acid released (μmole/g)**
Water	100	10	430
Cetyltrimethylammonium bromide (100 μg/ml)	37	30	409
Trichloroacetic acid (5% w/v)	5	15	474
Perchloric acid (0.2 *N*)	5	5	422
Ethanol (25% v/v)	5	10	401
n-Butanol (10% v/v)	5	10	420
N,N-Dimethylformamide	5	20**	431
HCl, 0.1 *N*	5	5	448
NaOH, 0.1 *N*	5	5	467

* Mean values of duplicate samples.
** Followed by dilution to 50% (v/v) and incubation for a further 5 min at 5°.

Table II (in the case of CTAB-treated *S. aureus*, a secondary autolytic release occurs more slowly[19]). N,N-Dimethylformamide (DMF) was found particularly useful for the preparation of relatively salt-free fractions for examination by paper chromatography; complete extraction required treatment of cells at 5° with 100% DMF for 20 min followed by dilution to 50% (v/v) and incubation for a further 5 min. Lower concentrations of DMF probably do not completely break the osmotic barrier of the cells, while the solubility of amino acids is limited in non-aqueous DMF. Lipid was removed from DMF extracts by the method of DOROUGH AND SEATON[20].

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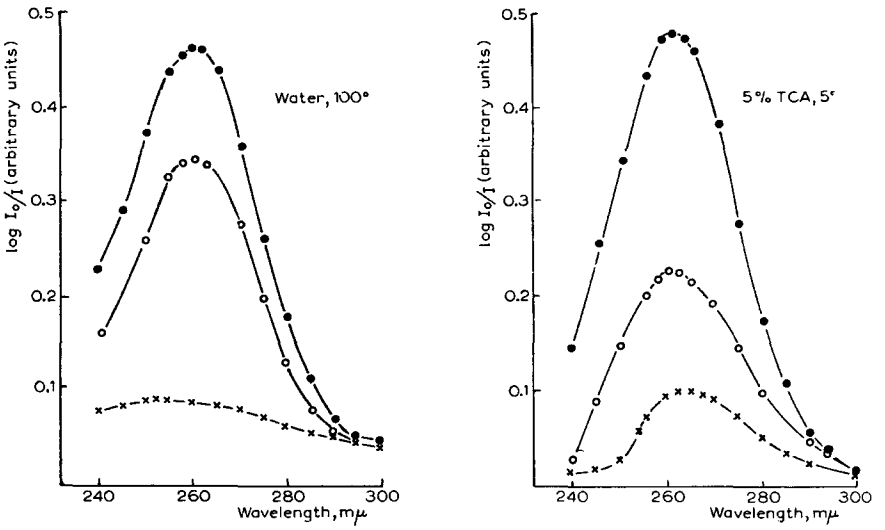

Fig. 2. Absorption spectra of material released from *S. aureus* by boiling water and TCA. Cell density 1.2 mg/ml; samples taken at 0 min (×—×), 2 min (○—○) and 10 min (●—●).

Amino acids present in the internal pool

Examination of extracted material by paper chromatography shows that almost all the amino acids found in the cell protein are present in the internal pool (Fig. 3). Cysteine and tryptophan do not appear on the chromatograms illustrated, although cysteine was occasionally detected; analyses by ion-exchange chromatography showed that cystine is present but that the amount of tryptophan is small (Fig. 4). No amino sugars were detected on paper chromatograms sprayed according to the modified ELSON-MORGAN procedure[21].

For more detailed quantitative analysis of the internal amino acids, columns of 4 % cross-linked Dowex-50 resin were used. Fig. 4 shows the elution pattern obtained when the TCA-soluble fraction from *S. aureus* (70 mg dry weight) was chromatographed on such a column. Substances in the effluent peaks were identified by their elution volume, and also by paper chromatography in 2 solvent systems after desalting by ion-exchange. In addition to the amino acids identified by paper chromatography, a number of other components were observed in column eluates. The peak eluted at 95 ml correspond in its position to urea*; that eluted at 315 ml correspond to oxidised glutathione, but identification was not carried further on the small amount of material available. Various slow-moving peaks were also detected; these may have contained peptide components. Of the material loaded onto the column (12.2 μM glutamic acid equivalents), 96.7 % was recovered as amino acids and ammonia so that the quantity of TCA-soluble peptides, if present, must be less than *c* 6 μM (glutamic acid equivalents)/g cells.

* *Note added in proof.* Ammonia is liberated on treatment of the material in peak A with crystalline urease; quantitative estimations using this method show a urea content in the whole TCA-soluble fraction of *c* 5 μM/g cells, approximately equal to the quantity in peak A, which has been calculated in Fig. 4 from the ninhydrin colour yield for urea.

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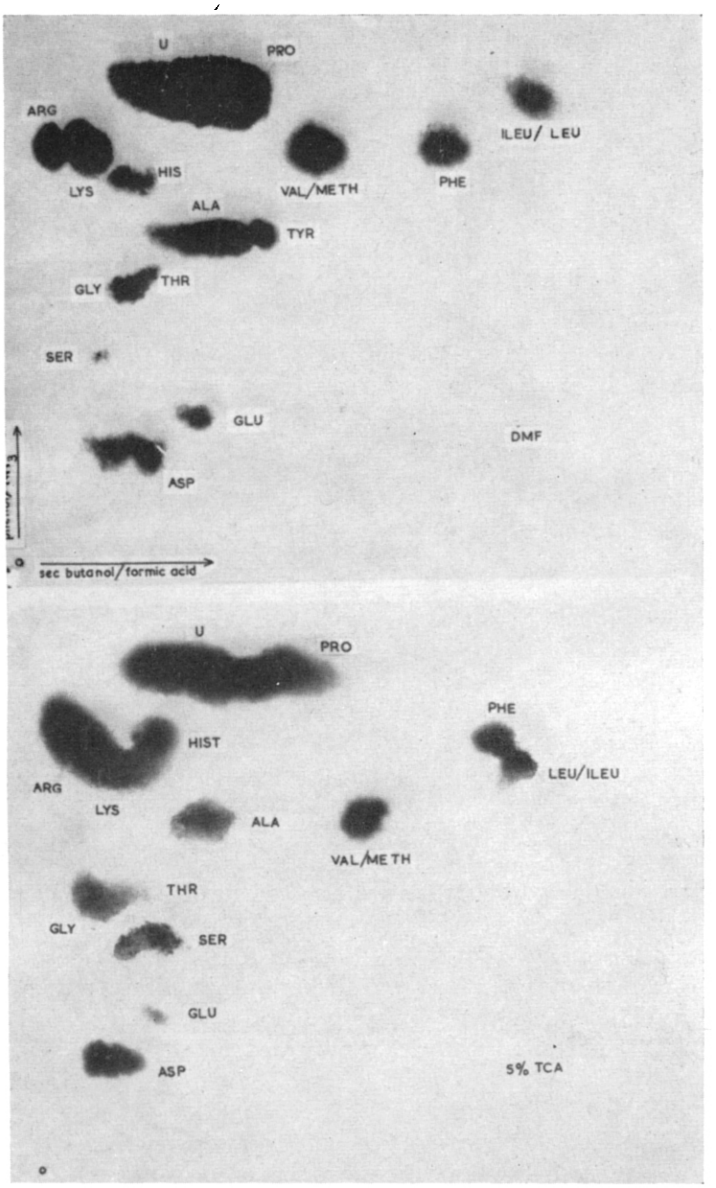

Fig. 3. Chromatograms of free amino acids of *S. aureus*, liberated by TCA and by DMF. The proline region sprayed with isatin and the remainder with ninhydrin. Identification of the unknown component U described in the test. Fractions from *c* 10 mg dry weight of cells.

Concentration of amino acids by growing cells of S. aureus

From the results of the analysis shown in Fig. 4 the internal concentration of individual amino acids in the cell was calculated, using a value of 1.7 ml/g cells for the intracellular water space of *S. aureus*[22]; these concentrations are shown in Table III. Since *S. aureus* has very little ability to break down amino acids (HILLS[23]), the

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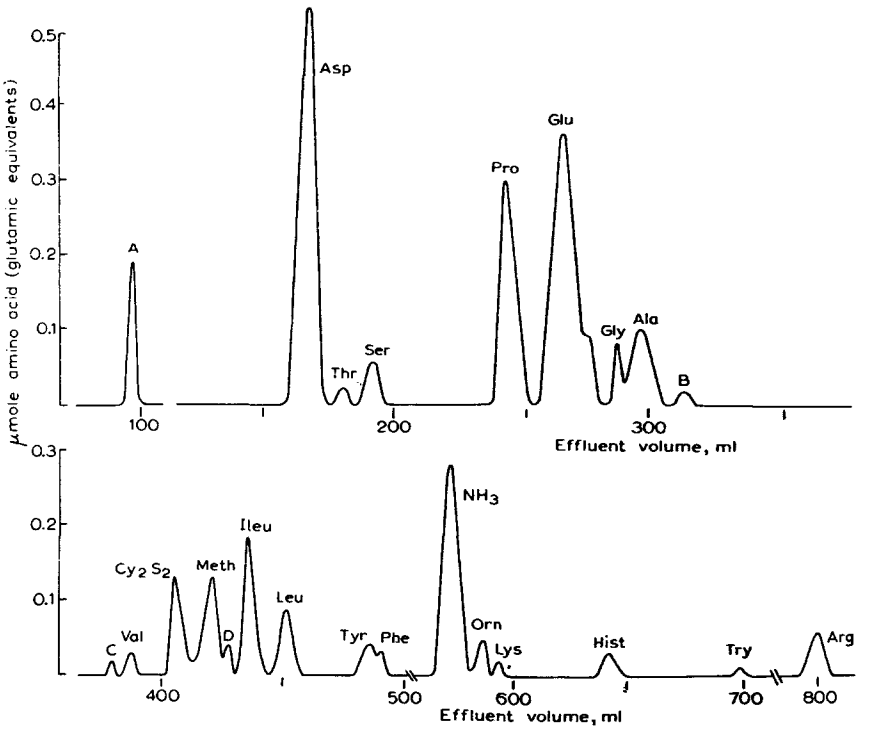

Fig. 4. Separation of free amino acids, released by TCA, on Dowex-50 × 4 (150 × 0.9 cm). Elution with 0.2 *N* Na-citrate–acetate buffer pH 3.1 at 40°; temperature raised to 50° at 270 ml, and after 350 ml buffer continuously changing to pH 5.1 and 1.4 *N* [Na^+]. Fraction size 1.5 ml; TCA extract from 70 mg dry weight of cells, growing in synthetic medium. A, B, C and D are unidentified components.

concentrations of amino acids in the growth medium at the time of harvesting were calculated from the initial concentration present, the quantity of cells and the amino acid composition of the cell-protein fraction (unpublished results of the author). If any metabolism of an amino acid occurred, other than its incorporation into protein these values will be upper limits. Consequently, the ratios of internal to external concentrations for individual amino acids, presented in Table IV, are minimal values.

Variation of free amino acid content during growth

Fig. 5 shows the total quantity of free amino acids, extracted by TCA, present in cells of *S. aureus* at successive times during growth.

Nature of the substance U on paper chromatograms

A spot due to an unidentified substance U, having R_F values similar to those of proline in the solvent systems used, was observed on paper chromatograms of fractions prepared by all extraction methods. This component, after elution from paper, was homogeneous on rechromatography in 4 solvent systems and also on electrophoresis at pH 9.7 (0.05 *M* carbonate buffer), at which pH its mobility was slightly less than that of arginine. It also passed through dialysis tubing. Paper chromatograms of hydrolysates of this substance showed the presence of glutamic

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TABLE III

FREE AMINO ACIDS IN EXPONENTIALLY-GROWING *S. aureus*

Cells growing in synthetic medium.

Amino acid	*Quantity (μmole/g) in amino acid pool*	
	(1)	*(2)**
Glutamic acid	39.6	49.2
Aspartic acid	38	41.7
Proline	16.8	—
Isoleucine	8.5	41.8
Leucine	2.6	
Methionine	6.7	
Alanine	8.1	10.6
Cystine**	5.5	—
Serine	3.4	—
Glycine	2.8	8.0
Tyrosine	2.4	—
Lysine	2.2	6.8
Arginine	2.2	—
Histidine	1.7	—
Phenylalanine	1.3	—
Threonine	1.0	3.9
Tryptophan	0.3	—

* Column 1 refers to cells harvested under the conditions described in "METHODS"; column 2 to an incomplete analysis of cells harvested earlier in the exponential phase of growth.

** Including cysteine which was oxidised during chromatography.

TABLE IV

CONCENTRATION OF AMINO ACIDS BY EXPONENTIALLY-GROWING *S. aureus*

Amino acid	*Ratio of internal to external concentration*	
	(1)	*(2)**
Glutamic acid	25.4	36.6
Proline	23.2	—
Aspartic acid	22.6	28.2
Isoleucine	13.3	—
Methionine	8.3	—
Alanine	5.4	6.7
Serine	5.4	—
Lysine	4.6	12.1
Leucine	4.5	—
Tyrosine	3.1	—
Arginine	3.0	—
Glycine	2.3	5.6
Histidine	2.2	—
Phenylalanine	1.8	—
Threonine	1.6	6.0

* Column 1 refers to cells harvested under the conditions described in "METHODS"; column 2 to an incomplete analysis of cells harvested earlier in the exponential phase of growth. (Tryptophan and cysteine were not estimated in the protein fraction so no gradients were calculated for these amino acids).

acid, alanine, glycine and lysine (Fig. 6). These components were further identified by co-chromatography with radioactive markers and by electrophoresis at pH 9.7 (0.05 *M* carbonate buffer). No amino sugars were detectable on paper chromatograms

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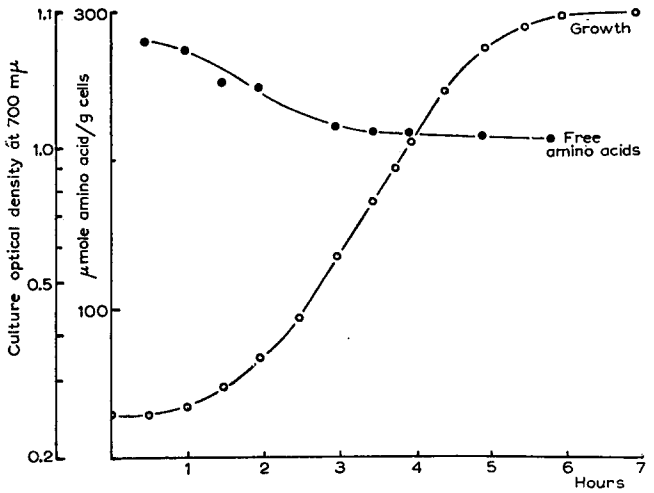

Fig. 5. Variation of free amino acid content (●—●), extracted by TCA, during growth (○—○) of *S. aureus*.

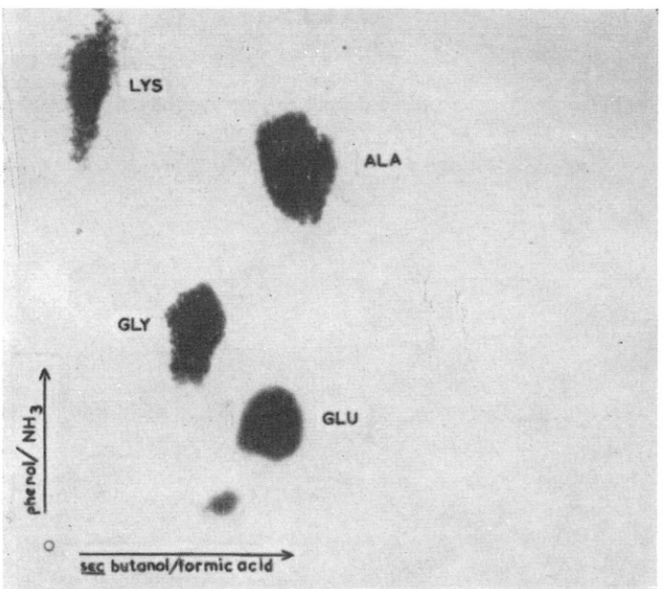

Fig. 6. Separation of hydrolysate of substance U (Fig. 3) by paper chromatography. In addition to glutamic acid, alanine, glycine and lysine, a trace of aspartic acid is present in this preparation; this component was found only occasionally and in variable amount.

of hydrolysates using the modified ELSON-MORGAN procedure[21]; papers sprayed for the detection of phosphate[24] gave negative results, and preparations separated on paper freed of ultraviolet-absorbing material[25] showed no ultraviolet absorption. It is of interest that the amino acids present in this peptide are the same as those present in the cell wall of *S. aureus*.

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DISCUSSION

The selection of the most satisfactory procedure for extracting the soluble constituents of bacterial cells depends largely on the type of analysis to be carried out. Detergent substances, for example polymyxin[26] and CTAB[19] may not, however, inhibit the secondary release of larger quantities of cell material due to autolytic breakdown, and extraction by such methods may not give a valid estimation of the normal internal constituents of the cell. For certain soluble constituents the degree of extraction varies with different extracting agents; for example ethanol extracts considerable quantities of peptides from certain microbial cells[27,28] which have not been reported in extracts obtained by other methods. For estimations of internal amino acids, all the procedures investigated here extract similar quantities; for examination by paper chromatography the most satisfactory extracting agents appear to be ethanol and DMF. Although membrane filters have been found satisfactory for the separation of cell residues of *S. aureus* it is possible that filters of the type and pore size used here would not be as satisfactory for the separation of cells of other organisms.

The amino acid pool of *S. aureus* contains all the amino acids found in the cell protein and cell wall, although it is not yet known if any of the internal amino acids are in the D-configuration, as are certain of the amino acid residues of the cell wall[29,30]. Under the conditions of growth used here, the relative concentrations of amino acids in the pool appear to bear no relation to their relative concentrations in the cell protein; proline, for instance, is a major component of the pool but a minor component of the protein. It would seem possible that a compensatory mechanism exists by which the relative concentrations of amino acids in the internal pool are so maintained that the amino acids for which the protein-synthesising systems of the cell have a lower affinity are maintained at a higher concentration, and *vice versa*. Results presented elsewhere[31] suggest that in *S. aureus*, as in other microorganisms which have been investigated[8,11], the amino acid pool supplies amino acids for protein synthesis.

The results of the present analysis show that growing cells of *S. aureus* are able to maintain concentration gradients of most amino acids across the cell surface; these gradients are highest for glutamic and aspartic acids and proline, and somewhat lower for isoleucine and methionine. The relative magnitudes of the concentration gradients observed here are similar to those which GALE[3] measured in washed suspensions of cells using ^{14}C-labelled amino acids, but their absolute magnitudes are much smaller; for instance cells in washed suspension are able to maintain a 300-fold concentration gradient of glutamic acid compared with the 30-fold gradient observed here. It would seem likely that, under optimal conditions, much higher gradients could be similarly attained for the other amino acids which are concentrated by growing cells.

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SUMMARY

The quantity of free amino acids within exponentially-growing cells of *Staphylococcus aureus* is not affected by the osmotic pressure of the external medium, in contrast to that of *Escherichia coli* B. All the extraction procedures investigated, including most commonly-used techniques, liberate similar quantities of amino acids (of the order of 200–500 μmole/g cells); progress curves of extraction have been determined.

Chromatographic examination shows the presence in this internal pool of all amino acids found in the cell protein; major components are proline, glutamic and aspartic acids, isoleucine, methionine and alanine. Concentration gradients exist of most amino acids across the cell surface; these gradients are greatest for proline, glutamic and aspartic acids, isoleucine and methionine.

An upper limit has been estimated to the quantity of TCA-soluble peptides which are present in exponentially-growing cells; one dialysable peptide, containing glutamic acid, glycine, alanine and lysine, has been detected in TCA extracts.

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