Amino Acid Metabolism and Protein Synthesis in a Pyrithiamine-Requiring *Staphylococcus aureus* Mutant

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1. Amino acid metabolism and protein synthesis in a *Staphylococcus aureus* mutant strain that requires pyrithiamine for optimum growth were studied and compared with those in the thiamine-requiring parent *S. aureus*. 2. The mutant strain utilized amino acids at a higher rate than did the parent strain. The utilization of glutamic acid, serine and glycine was much stimulated in the mutant strain. 3. The rate of oxidation of glutamic acid, aspartic acid, isoleucine and glycine was higher in the mutant strain. 4. The mutant strain contained serine, glycine, tyrosine, glutamic acid, aspartic acid, arginine and histidine as free amino acids, whereas the parent strain possessed lysine, arginine, histidine, aspartic acid and glutamic acid. 5. The mutant strain possessed slightly higher glutamate-oxaloacetate transaminase activity, whereas the activities of glutamate-pyruvate transaminase were similar in both strains. 6. The incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]$ -acetate into individual amino acids of the cell protein was greater in the mutant strain. 7. The incorporation of ^{14}C -labelled amino acids into the cell proteins of the mutant strain was not much different from that in the parent strain. 8. Induction of β -D-galactosidase in the mutant strain did not occur, whereas induction of this enzyme is possible in the parent strain. Thiamine or pyrithiamine has no direct effect on the induction of β -D-galactosidase.

Das & Chatterjee (1962*a,b*, 1963) reported that carbohydrate metabolism was altered in a pyrithiamine-requiring *Staphylococcus aureus* mutant (*S. aureus* A) compared with the parent strain. Glucose utilization was much depressed, with concomitant stimulation of the tricarboxylic acid cycle in the mutant strain. It was postulated that amino acid metabolism might also have changed so that the energy required for the growth of the mutant strain could be obtained through increased amino acid utilization.

In this paper amino acid metabolism and certain aspects of protein synthesis in the mutant strain are compared with those in the parent strain.

MATERIALS AND METHODS

Organism. A laboratory strain of thiamine-requiring *S. aureus* and a pyrithiamine-requiring mutant *S. aureus* A were used; the method of preparation and maintenance of the mutant were as described by Das & Chatterjee (1962*a*).

Growth of the organism in broth culture. *S. aureus* was grown in liquid medium (Das & Chatterjee, 1962*a*) containing thiamine, and *S. aureus* A in a medium containing pyrithiamine. Erlenmeyer flasks (250 ml.) containing medium (50 ml.) were inoculated with cell suspension (0.1 ml.) and incubated at 37° for 24 hr. unless otherwise stated. After growth, cells were harvested, washed and

finally resuspended in 0.85% NaCl (one-fifth of the original volume).

Preparation of samples for chromatography. Medium after bacterial growth was pretreated with Dowex 50 (H^+ form) by the method outlined by Plaisted (1958) before chromatography. For the estimation of free amino acids, cells grown for 18 hr. were extracted with aq. 80% (v/v) ethanol (Narasinha Rao & Venkataraman, 1952). The extract, after separation of cell debris, was evaporated to dryness and the residue was taken up in aq. 10% (v/v) propan-2-ol. Cell weight was determined by drying a portion of washed suspension of the cells to constant weight at 105°.

Chromatographic estimation of amino acids. The amino acid concentration of different samples was determined by two-dimensional descending paper chromatography with water-saturated phenol as the first solvent and butan-1-ol-acetic acid-water (4:1:1, by vol.) as the second solvent. After colour development with ninhydrin (0.5%, w/v, in acetone) the coloured spots were identified and separately eluted with 5 ml. of aq. 75% (v/v) ethanol containing 5 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /100 ml. (Giri, Radhakrishnan & Baidyanathan, 1952). The concentration of amino acids was determined colorimetrically.

Manometry. Aerobic oxidations with various amino acids as substrate were studied by the conventional manometric method (Umbreit, Burris & Stauffer, 1957) with suspensions of *S. aureus* and *S. aureus* A.

Enzyme assay. Glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase were assayed by the amount of pyruvate and oxaloacetate (Gunsalus & Stamer,

1955) formed respectively. Cell-free extracts of the organisms were prepared by the method of Das & Chatterjee (1963) and were used as the enzyme source.

Incubation with radioactive substrate. Labelling of amino acids in the cell protein during the utilization of [2-¹⁴C]-acetate was carried out in double-arm Warburg flasks. The incubation mixture contained cell suspension (6 mg. dry wt. of bacteria), acetate mixed with [2-¹⁴C]acetate (5 μ moles; this amount gives 9.85×10^5 counts/min.) and phosphate saline (Krebs & Eggleston, 1940) in a total volume of 2.5 ml. The labelled substrate was placed in one side arm of the flask, and the other arm contained 0.3 ml. of 30% (w/v) trichloroacetic acid. The central well contained filter paper soaked with 0.2 ml. of 20% (w/v) KOH. The incubation was carried out in a Warburg bath at 37° with constant shaking. After incubation for 60 min. the reaction was stopped by the addition of 6% (w/v) trichloroacetic acid. The precipitated protein was then purified by repeated washing with 6% (w/v) trichloroacetic acid, dissolving in 1.0N-NaOH and then by reprecipitation with 6% (w/v) trichloroacetic acid and washing with cold sodium thioglycollate, ethanol, ethanol-ether mixture and finally ether (Stachiewicz & Quastel, 1959; Bovernick, Schneider & Walter, 1959).

Dry protein (2 mg.) was then hydrolysed in a sealed tube with 6N-HCl at 110° for 24 hr. The HCl was removed from the hydrolysate over solid NaOH in a vacuum desiccator. The dried mass was then dissolved in 1 ml. of aq. 10% (v/v) propan-2-ol with about 1 mg. of Ag₂O to remove any trace of HCl present. Portions were chromatographed on Whatman no. 1 paper. Radioautography and radioassay were done by the method of Kornberg (1958). The papers were kept in contact with Ilford X-ray film (Red seal) for 2 weeks. The films were then developed and the radioactive amino acids were identified by comparison with X-ray films of a standard chromatogram treated similarly. The amount of radioactivity in each individual spot was measured with an end-window paper scanner (model 182 $\times$ Scaler; Nuclear-Chicago Corp.).

Protein synthesis. The incubation mixture contained 0.2 μ mole each of GTP, UTP, and CTP, 2.0 μ moles of ATP, 0.8 μ mole of mercaptoethanol, 30 μ moles of tris-HCl buffer, pH 7.6, amino acid mixture containing 0.5 μ mole of individual amino acids and cell-free extract equivalent to 4 mg. of protein in a final volume of 2.1 ml. Different ¹⁴C-labelled amino acids (0.02 μ mole of each) were added to separate incubation mixtures. The reaction mixture was incubated at 37° for 1 hr. and the reaction was stopped by the addition of 30% (w/v) trichloroacetic acid. The protein was redissolved in 1.0N-NaOH and was reprecipitated with 6% (w/v) trichloroacetic acid. The protein was purified as described above, transferred to planchets and dried under an infrared lamp. Radioactivity measurements were made in a windowless gas-flow counter with an M₅ semiautomatic sample changer (model 182 $\times$ Scaler; Nuclear-Chicago Corp.).

Induced enzyme synthesis. Both *S. aureus* and *S. aureus* A were grown in the medium described by Das & Chatterjee (1962a). After growth for 17 hr. at 37° the cells were collected by centrifugation at 0° and thoroughly washed with 0.85% NaCl solution. For induction of the enzyme the cells were then resuspended in the medium described by Creaser (1955). The medium for the mutant was supplemented with pyrithiamine (1 μ g./ml.) instead of the thiamine that was added to the medium for the parent *S. aureus* strain. Isopropyl β -D-thiogalactoside (0.2 mm) was added as the

inducer. After incubation for various times at 37° the cells were washed with cold 0.85% NaCl and collected by centrifugation at 0°. In a typical experiment the induction system contained 10 ml. of the Creaser medium (with added thiamine or pyrithiamine), 0.5 ml. of isopropyl β -D-thiogalactoside and 44 mg. wet wt. of cells.

Assay of β -D-galactosidase. β -D-Galactosidase activity was measured by the rate of hydrolysis of *o*-nitrophenyl β -D-galactoside. The assay mixture contained *o*-nitrophenyl β -D-galactoside (2 mm), Na₂HPO₄-KH₂PO₄ buffer, pH 7.4 (30 mm), cysteine (1.2 mm) and 8 mg. wet wt. of cells. The system was incubated at 37° for 30 min. The E_{420} value of the cell-free supernatant was measured in a Beckman model DU spectrophotometer. One unit of enzyme activity was taken as the amount of enzyme that hydrolyses 1 μ mole of *o*-nitrophenyl β -D-galactoside/min. at 37° in phosphate buffer, pH 7.4.

Chemicals. [2-¹⁴C]Acetate and ¹⁴C-labelled amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Isopropyl β -D-thiogalactoside and *o*-nitrophenyl β -D-galactoside were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Pyrithiamine was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

RESULTS

Utilization of amino acids. It has been found that *S. aureus* can utilize cysteine at a high rate. The rate of utilization by the parent strain of lysine, arginine, histidine, aspartic acid, serine, glycine, threonine, methionine, valine, alanine and also phenylalanine was also significantly high (Table 1), whereas glutamic acid, leucine, proline and hydroxyproline were utilized at a comparatively lower rate. Studies on the utilization of amino acids by the mutant *S. aureus* A indicated an appreciable increase in the utilization of lysine, arginine, histidine, phenylalanine and leucine when compared with the parent strain. The utilization of glutamic acid, serine and glycine was also stimulated. With the exception of aspartic acid, alanine and threonine, the utilization of other amino acids was stimulated, indicating an overall increased utilization of amino acids in the mutant organism.

Oxidation of amino acids by *S. aureus* and *S. aureus* A. Amino acids were oxidized at a higher rate by the mutant strain, particularly with respect to glutamic acid, isoleucine and glycine. The rate of oxidation of methionine, on the other hand, was much higher in the parent strain than in the mutant *S. aureus* A (Table 2).

Free amino acid content of *S. aureus* and *S. aureus* A. The parent strain contained arginine, histidine, lysine, aspartic acid and glutamic acid as free amino acids. The mutant strain contained these amino acids except for lysine, and serine, glycine and tyrosine in addition (Table 3). Concentrations of arginine, histidine and glutamic acid were increased in the mutant strain whereas the concentration of aspartic acid was decreased.

Table 1. *Utilization of amino acids by S. aureus and S. aureus A*

Bacterial growth was measured in a Klett–Summerson photoelectric colorimeter (filter no. 54) after incubation in the appropriate medium (see the Materials and Methods section) at 37°.

Incubation period (hr.)	Strain	Growth (Klett units)	Concn. of amino acids in fermentation broth (μ moles/100ml.)												
			Cys	Lys	Arg + His	Asp	Ser + Gly	Glu	Thr	Ala	Tyr	Met	Phe	Leu	Pro + Hyp
0	<i>S. aureus</i> <i>S. aureus A</i>	—	61	78	17	100	161	248	82	44	75	97	90	169	50
12	<i>S. aureus</i> <i>S. aureus A</i>	38 25	19 36	58 53	17 11	60 87	147 122	234 160	14 60	26 25	61 —	94 85	90 81	156 129	50 49
18	<i>S. aureus</i> <i>S. aureus</i>	80 82	13 19.5	22 46	16 8	47 67	135 78	154 115	10 51	22 15	43 —	79 56	82 74	124 80	44 45
24	<i>S. aureus</i> <i>S. aureus A</i>	156 161	13 7	41 38	16 —	21 48	123 67	142 102	— 49	4 20	32 —	47 35	69 32	158 56	36 42

Table 2. *Oxidation of different amino acids by S. aureus and S. aureus A*

Each Warburg flask contained 30 μ moles of substrate, cell suspension (6mg. dry wt. of bacteria) and phosphate saline (Krebs & Eggleston, 1940) in a total volume of 3ml. The central well contained filter paper soaked with 0.2ml. of 20% KOH. Incubation was at 37° for 60min.

Substrate	O ₂ consumed (μ l./mg. dry wt.)	
	<i>S. aureus</i>	<i>S. aureus A</i>
DL-Glutamic acid	15.1	24.5
L-Aspartic acid	2.7	2.8
L-Arginine	3.7	4.5
L-Histidine	9.2	9.6
DL-Isoleucine	5.6	13.6
DL-Alanine	16.8	20.2
DL-Valine	7.6	6.0
DL-Methionine	15.2	12.8
Glycine	3.2	10.6

Table 3. *Free amino acid content of S. aureus and S. aureus A*

Free amino acids were extracted from the cells with aq. 80% (v/v) ethanol. After separation of the debris the extracts were evaporated to dryness and taken up in aq. 10% (v/v) propan-2-ol. The amino acids were estimated by paper chromatography (see the Materials and Methods section).

Amino acid	Amount present (μ mole/mg. dry wt.)	
	<i>S. aureus</i>	<i>S. aureus A</i>
Arg + His	0.21	0.74
Lys	0.39	—
Asp	0.45	0.10
Glu	0.27	0.57
Ser + Gly	—	0.17
Tyr	—	0.25

Transaminase activity in cell-free extracts of S. aureus and S. aureus A. With cell-free extracts of both the strains, the glutamate-oxaloacetate transaminase activity was slightly higher in *S. aureus A*, but there was almost no change in the glutamate-pyruvate transaminase activity as a result of mutation.

Labelling of amino acids of the cell protein during the utilization of [2-¹⁴C]acetate. Incorporation of ¹⁴C from [2-¹⁴C]acetate into cell protein was greater in the mutant *S. aureus A* than in the parent strain (Table 4). Analysis of the percentage of the total radioactivity of cell protein in the individual amino acids showed that approx. 81% and 88%

could be accounted for in the parent and mutant strains respectively; of all the labelled amino acids detected by radioautography, incorporation into alanine was much greater in the mutant strain. The fact that almost all the amino acids in the protein of *S. aureus A* were labelled whereas many of the amino acids present in the cell protein of the parent strain were unlabelled may be explained by the overall net incorporation of [¹⁴C]acetate into cell protein, which was about four times as great in *S. aureus A* as in *S. aureus*.

Synthesis of protein. The synthesis of protein as determined by the incorporation of ¹⁴C-labelled amino acids remained almost the same with extracts of *S. aureus A* or *S. aureus* (Table 5).

Induced enzyme synthesis. As the induction of enzyme synthesis by the cell is another approach to

the study of aspects of protein synthesis, the induction of β -D-galactosidase in both *S. aureus* and *S. aureus* A was studied. Although in the presence of isopropyl β -D-thiogalactoside the parent strain could synthesize β -D-galactosidase, no synthesis occurred in the mutant strain (Table 6). To

determine whether pyrithiamine or thiamine had any effect on the process of induction of β -D-galactosidase, pyrithiamine was added to the induction medium containing thiamine for the parent strain and thiamine was added to the medium containing pyrithiamine for the mutant *S. aureus* A. These additions to the induction media had no effect on the induction of β -D-galactosidase.

Table 4. Incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{acetate}$ into amino acids of *S. aureus* and *S. aureus* A

The incubation mixture contained cell suspension (6mg. dry wt. of bacteria), acetate mixed with $[2\text{-}^{14}\text{C}]\text{acetate}$ ($5\mu\text{moles}$; this amount gave 9.85×10^5 counts/min.) and phosphate saline (Krebs & Eggleston, 1940) in a total volume of 2.5 ml. The mixture was incubated at 37° for 60 min. and the reaction was stopped by the addition of 6% (w/v) trichloroacetic acid. The purified isolated protein (2mg.) was hydrolysed in 6N-HCl in a sealed tube at 110° for 24 hr. After hydrolysis HCl was removed and a portion was chromatographed. Radioautography and radioassay were done by the method of Kornberg (1958).

Substance Protein	Radioactivity incorporated (counts/min./mg. dry wt.)	
	<i>S. aureus</i>	<i>S. aureus</i> A
	1.101×10^4	4.034×10^4
Cys	275	1260
Asp	1725	2280
Ser + Gly	1460	2425
Glu	4080	10310
Lys	—	3125
Arg + His	—	625
Thr	790	1715
Ala	620	4985
Tyr	—	780
Met + Val	—	1895
Phe	—	3335
Leu	—	2950

DISCUSSION

The above results indicate that in the mutant strain stimulated utilization of amino acids may account for the lower glucose assimilation by the strain and lead to growth equivalent to that of the parent organism, the growth medium used for either strain being identical with respect to carbon and nitrogen sources. In the pool of free amino acids in *S. aureus* A, histidine and glutamic acid were increased, whereas aspartic acid was decreased appreciably. The turnover of glutamic acid is higher in *S. aureus* A than in the parent strain. Studies on the incorporation of $[2\text{-}^{14}\text{C}]\text{acetate}$ into the constituent amino acids of the cell proteins of both strains indicate a marked stimulation in the protein metabolism of the organism after its mutation to the pyrithiamine-requiring strain. Overall stimulation of amino acid metabolism in *S. aureus* A cannot, of course, be confirmed conclusively by the results observed in the amino acid-incorporating studies. There is, however, a slight increase in the incorporation of $[^{14}\text{C}]\text{valine}$ and $[^{14}\text{C}]\text{phenylalanine}$ by the cell-free extracts of *S. aureus* A while the rate of incorporation of other labelled amino acids remained the same. It may therefore be assumed that the higher rate of metabolism of amino acids in the mutant strain is needed

Table 5. Incorporation of ^{14}C -labelled amino acids into protein of *S. aureus* and *S. aureus* A

The incubation mixture contained $0.2\mu\text{mole}$ each of GTP, UTP and CTP, $2.0\mu\text{moles}$ of ATP, $0.8\mu\text{mole}$ of mercaptoethanol, $30\mu\text{moles}$ of tris-HCl buffer, pH 7.6, amino acid mixture containing $0.5\mu\text{mole}$ of each individual amino acid and cell-free extract equivalent to 4 mg. of protein in a total volume of 2.1 ml. Different ^{14}C -labelled amino acids ($0.02\mu\text{mole}$ of each) were added to separate reaction mixtures, which were incubated at 37° for 1 hr. For the purification of the protein see the Materials and Methods section.

^{14}C -labelled amino acid	Initial radioactivity (counts/min.)	Radioactivity incorporated (counts/min./mg. of protein)	
		<i>S. aureus</i>	<i>S. aureus</i> A
DL- $[1\text{-}^{14}\text{C}]\text{Valine}$ ($0.03\mu\text{mole}$)	3.0×10^3	470	678
L- $[G\text{-}^{14}\text{C}]\text{Leucine}$ ($0.02\mu\text{mole}$)	3.2×10^3	582	560
DL-Phenyl $[1\text{-}^{14}\text{C}]\text{alanine}$ ($0.02\mu\text{mole}$)	3.4×10^3	558	750
L- $[Me\text{-}^{14}\text{C}]\text{Methionine}$ ($0.02\mu\text{mole}$)	3.3×10^3	670	680
L- $[2^{\text{m}}\text{-}^{14}\text{C}]\text{Histidine}$ ($0.02\mu\text{mole}$)	4.0×10^3	468	555

Table 6. *Induction of β -D-galactosidase by S. aureus and S. aureus A*

The induction mixture contained 44 mg. wet wt. of cells in 10 ml. of Creaser (1955) medium with different amounts of thiamine or pyrithiamine as shown and 0.5 ml. of isopropyl β -D-thiogalactoside (0.2 mM).

Incubation period (hr.)	Additions to induction medium		β -D-Galactosidase formed (units/mg. dry wt.)	
	For <i>S. aureus</i>	For <i>S. aureus</i> A	<i>S. aureus</i>	<i>S. aureus</i> A
0	Thiamine (1 μ g./ml.)	Pyrithiamine (1 μ g./ml.)	10	0
	Thiamine (1 μ g./ml.) + pyrithiamine (10–40 μ g./ml.)	Pyrithiamine (1 μ g./ml.) + thiamine (10–40 μ g./ml.)	10	0
2	Thiamine (1 μ g./ml.)	Pyrithiamine (1 μ g./ml.)	55	0
	Thiamine (1 μ g./ml.) + pyrithiamine (10–40 μ g./ml.)	Pyrithiamine (1 μ g./ml.) + thiamine (10–40 μ g./ml.)	55	0
3	Thiamine (1 μ g./ml.)	Pyrithiamine (1 μ g./ml.)	260	0
	Thiamine (1 μ g./ml.) + pyrithiamine (10–40 μ g./ml.)	Pyrithiamine (1 μ g./ml.) + thiamine (10–40 μ g./ml.)	260	0
4	Thiamine (1 μ g./ml.)	Pyrithiamine (1 μ g./ml.)	610	0
	Thiamine (1 μ g./ml.) + pyrithiamine (10–40 μ g./ml.)	Pyrithiamine (1 μ g./ml.) + thiamine (10–40 μ g./ml.)	610	0

for its normal maintenance, since there is dearrangement in the utilization of other metabolites by the strain. However, the rate of protein synthesis *in vitro* as indicated by the incorporation of ^{14}C -labelled amino acids is not much affected, and this is reflected by the apparently similar growth rates of both these strains. Though thiamine or pyrithiamine has no direct effect on the process of induction of β -D-galactosidase *in situ*, mutation of *S. aureus* to the pyrithiamine-requiring strain leads to an impairment of carbohydrate metabolism that could lead to a higher concentration of hexose in the pool of the mutant cells; consequently the inducibility of β -D-galactosidase would be inhibited. However, the loss in the inducibility of β -D-galactosidase might also be attributed to alterations in genetic loci of the organism as a result of the development of resistance towards pyrithiamine.

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