The results of these dialyses are consistent with the view that the zinc is mainly bound to imidazole groups of the proteins.

5. About four-fifths of the ^{65}Zn present in both the liver and the tumour is freely exchangeable with Zn^{2+} ions at pH 7.4 and can be removed by dialysing the mash against buffers containing excess of exchangeable zinc or the zinc-binding ethylenediaminetetra-acetic acid.

6. About one-sixth of the ^{65}Zn in these tissues could not be removed by exchange or by combination with ethylenediaminetetra-acetic acid, and this fraction probably represents ^{65}Zn in zinc bound to protein.

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Incorporation of Canavanine by *Staphylococcus aureus* 524 SC

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Previous experiments (Richmond, 1959) have shown that *Staphylococcus aureus* 524, which is normally exacting for arginine, can, when incubated in a fully synthetic amino acid medium containing canavanine in place of arginine, increase in turbidity and synthesize cell protein. Under these conditions, however, the cultures do not synthesize active forms of β -galactosidase, hyaluronidase, phosphatase or the lytic enzyme. It seemed possible therefore that canavanine was incorporated into cell protein in place of arginine, resulting in the formation of inactive enzymes.

A number of workers have reported that certain amino acid analogues can be incorporated into protein of micro-organisms in place of normal amino acids and that this process is associated with variable effects on the enzyme-forming ability of the organisms (Munier & Cohen, 1956, 1959; Cowie & Cohen, 1957; Pardee & Prestidge, 1958; Cohen & Munier, 1959).

This paper describes the detection of canavanine in the protein of *S. aureus* which has been grown in its presence.

MATERIALS AND METHODS

Organism. *Staphylococcus aureus* 524 SC (Rogers, 1953) has been used throughout these studies. The organism was maintained as described by the author (Richmond, 1959).

Media. Hedley-Wright broth (Rogers, 1954) and the fully synthetic amino acid media 18AA and 17AA(-Arg) have been described previously (Richmond, 1959). Experiments involving the use of radioactive lysine were carried out in 18AA medium from which the lysine had been omitted (17AA-Lys medium).

Amino acids and derivatives. The compounds used in these studies and their sources were as follows. L-Canavanine (Dr J. Mandelstam and Dr R. Davies); dinitrophenyl (DNP)-arginine (Dr F. Sanger); DNP- ϵ -lysine (Dr R. R. Porter). The [^{14}C]lysine was obtained from The Radiochemical Centre, Amersham, Bucks. It was used at a specific activity of 0.1 $\mu\text{C}/\text{m-mole}$.

Preparation of organisms for experimental purposes. Unless otherwise stated, organisms were prepared as described previously (Richmond, 1959) and inoculated into 17AA(-Arg) medium containing 50 μg . of canavanine/ml. The culture was incubated at 35° and shaken at the maximum rate (87 cye./min., 1 ft. throw) of a graded shaker (Kantorowicz, 1951); growth was followed turbidimetrically

(Rogers, 1954) and samples were removed for fractionation when required.

Cell fractionation. Samples were precipitated by the addition of sufficient cold trichloroacetic acid (20%, w/v) to bring the final concentration to 5%. After standing for 2 hr. at 2°, the precipitate was removed on a centrifuge and fractionated to yield the ethanol-soluble protein and protein + cell wall fraction (Richmond, 1959). Cell wall preparations were made by the method devised by Cummins & Harris (1956) and modified by Mandelstam & Rogers (1959).

Preparation of dinitrophenyl-protein (DNP-protein). About 20 mg. of protein + cell wall fraction was suspended in 0.4M-trimethylamine carbonate buffer, pH 9.0, and treated with 1-fluoro-2:4-dinitrobenzene (FDNB) as described by Sanger & Thompson (1953). Excess of reagent and dinitrophenol were removed as described by those authors.

Hydrolysis of normal and dinitrophenyl-protein + cell wall fractions. Samples were hydrolysed overnight in 6N-HCl at 105° in sealed tubes to liberate free amino acids. At the end of this period, the hydrolysates were evaporated to dryness at 100° on a water bath in a stream of air and traces of HCl removed by repeated evaporation of three to four batches (2 ml.) of water at 100°. A sample of 200 µg. of L-canavanine treated in this way was recovered in greater than 80% yield.

Chromatography. Descending chromatography of free amino acids and their DNP derivatives was carried out in either butanol-acetic acid-water (60:10:27, by vol.) or 2-methylbutan-2-ol-0.1M-phthalate buffer, pH 6.0 (Blackburn & Lowther, 1951). Whatman no. 1 or no. 3 paper was used, depending on the quantity of material to be separated. The detection of the position of bands to be eluted and the method of elution were carried out as described by Mandelstam (1958).

Ionophoresis. Amino acids were separated by ionophoresis on Whatman no. 3 paper in buffers of the following composition: ammonium carbonate (2%, w/v) adjusted to pH 9.1 with aq. NH₃ soln. (sp.gr. 0.880); pyridine-acetic acid-water (10:0.4:90, by vol.), pH 6.5 (Ryle, Sanger, Smith & Kitai, 1955), and pyridine-acetic acid-water (1:10:89, by vol.), pH 3.6 (Ryle *et al.* 1955). All these buffers were removed by heating papers in an incubator at 50° for 30 min.

Most experiments were carried out by the 'closed-strip method' (Block, Durrum & Zweig, 1958), in an apparatus similar to that designed by Kunkel (1954), with a potential

gradient of 15 v/cm. The preliminary experiments on the separation of canavanine from the protein of *S. aureus* were done in the apparatus of Ryle *et al.* (1955) with a potential gradient of 40 v/cm. The positions of amino acids on chromatograms and ionophoresis papers were determined with ninhydrin in the normal manner; arginine and canavanine were also detected by the alkaline α -naphthol-diacetyl reagent of Szafr & Bennett (1953).

RESULTS

A culture of *S. aureus* prepared as described above was inoculated to an initial density of about 0.15 mg. dry wt. of bacterial cells/ml. in 17 AA(-Arg) medium containing 50 µg. of L-canavanine/ml. and incubated on a shaker at 35°. A similar culture incubated in 17 AA(-Arg) medium to which 4 µg. of L-arginine/ml. had been added acted as a control. After incubation for 3 hr., the cultures were precipitated with cold trichloroacetic acid and fractionated to yield the protein + cell wall fraction (see Methods section). This fraction, which usually constituted about 50% of the total dry weight of material precipitated, was washed in aqueous 75% ethanol and hydrolysed in 6N-HCl to liberate free amino acids.

The arginine and canavanine were separated from the other amino acids in the hydrolysates by chromatography with butanol-acetic acid-water with arginine and canavanine markers. Under these conditions, arginine and canavanine run slowly ($R_F \sim 0.1$) and do not separate. After 24 hr. in this solvent the chromatograms were dried and the position of the arginine-canavanine band was detected by spraying the markers with ninhydrin. The area containing these amino acids was cut from the chromatogram and eluted into 4 ml. of water. Material separated in this way was usually contaminated with variable quantities of glutamic acid, lysine, proline, histidine and aspartic acid. The eluate was taken to dryness at 100° and transferred to paper for ionophoresis at pH 9.1, 6.5 or 3.5 in the appropriate buffers. A distinct spot corre-

Table 1. *Mobilities of arginine and canavanine during paper electrophoresis in buffers at pH 9.1, 6.5 and 3.5*

+ Migration towards cathode; -, migration towards anode.

Buffer	pH value	Distance moved/2 hr. at 40 v/cm. (cm.)	
		Arginine	Canavanine
Ammonium carbonate (2%, w/v)	9.1	+ 5.3	- 6.7
Pyridine-acetic acid-water (10:0.4:90, by vol.)	6.5	+ 31	+ 27.5
Pyridine-acetic acid-water (1:10:89, by vol.)	3.5	+ 43	+ 43

sponding to canavanine was obtained in the hydrolysate from organisms grown with canavanine at both pH 9.1 and 6.5; no canavanine was detected in the control. At pH 3.5 the arginine and canavanine markers did not separate and no additional spot was detected in hydrolysates of organisms grown in the presence of the analogue. The mobilities of arginine and canavanine on ionophoresis at pH 9.1, 6.5 and 3.5 are shown in Table 1.

Although these results showed that canavanine could be detected in the protein+cell wall fraction of *S. aureus* grown in the presence of the analogue it was possible that the canavanine was adsorbed on to the protein+cell wall fraction rather than incorporated into the protein with formation of covalent bonds. To reduce the possibility that the presence of canavanine was due to adsorption, the following control experiments were carried out.

(1) The efficiency of the fractionation procedure in freeing the protein+cell wall fraction of uncombined basic amino acids was tested as follows. A culture of *S. aureus*, prepared in the normal manner, was suspended to an initial density of 200 $\mu\text{g./ml.}$ in 17AA(-Lys) medium to which [^{14}C]lysine had been added. This amino acid was chosen as [^{14}C]arginine was not available. The culture was immediately precipitated with 20% trichloroacetic acid. The precipitate obtained in this way was fractionated by the normal procedure and the contamination of the protein+cell wall fraction with lysine estimated by determining the radioactivity in the sample. The maximum level of radioactivity detected in such experiments showed the contamination of the protein+cell wall fraction to be less than 10 $\mu\text{g.}$ of lysine/g. dry wt. of organisms. A contamination of this magnitude in 20 mg. of protein+cell wall fraction would not be detected by the methods which have been used to detect canavanine.

(2) A culture grown with arginine was precipitated with trichloroacetic acid immediately after the addition of 100 $\mu\text{g.}$ of canavanine/ml. and fractionated as described above. No canavanine could be detected in the protein+cell wall fraction.

(3) A culture was incubated for 3 hr. in 17AA(-Arg) medium to which 50 $\mu\text{g.}$ of canavanine and 50 $\mu\text{g.}$ of arginine/ml. had been added; a culture in 18AA medium acted as control. Under these conditions the organisms grew exponentially at the same rate as the control and no canavanine could be detected in the protein+cell wall fraction of either culture.

(4) Samples of 20 mg. of protein+cell wall fraction from cultures grown in canavanine were subjected to ionophoresis at pH 6.5 and 9.1 before hydrolysis. No canavanine could be detected. Similar samples, separated after hydrolysis, were found to contain canavanine.

(5) Samples of 20 mg. of protein+cell wall fraction from organisms grown with canavanine were converted into the DNP derivative by the action of FDNB. DNP-Protein+cell wall fraction was subjected to ionophoresis at pH 6.5 and 9.1 before hydrolysis. If free canavanine were adsorbed on to the protein the detection of DNP-canavanine would be expected. Dinitrophenol and a little DNP- ϵ -lysine were the only compounds detected. A further sample from the same batch of material, hydrolysed with acid and then separated by ionophoresis, was shown to contain free canavanine. It seems likely therefore that the canavanine found in the protein+cell wall fraction under these conditions is not adsorbed non-specifically but is combined in such a manner that the $\alpha\text{-NH}_2$ group of the molecule is not available to react with FDNB.

In an attempt to obtain an estimate of the quantity of canavanine incorporated into the protein+cell wall fraction during incubation for 3 hr., the arginine and canavanine spots after treatment with ninhydrin were cut from ionophoresis papers run at pH 9.1 and eluted into water. The marker quantities of arginine and canavanine were treated similarly and the quantity of the amino acid was measured by determining the absorption at 570 $\text{m}\mu$ due to the ninhydrin colour in the eluates. The level of canavanine in the protein+cell wall fraction was found to be about one-tenth of the arginine on a molar basis.

It was not possible to detect either arginine or canavanine in hydrolysates of 50 mg. of cell wall prepared from organisms grown in the presence of the analogue. The canavanine isolated from the protein+cell wall fraction must therefore have originated in the protein part of the fraction.

DISCUSSION

The results reported in this paper suggest that canavanine is incorporated into the protein fraction of *S. aureus* 524 which had been grown in its presence. This strain of *S. aureus* increased in turbidity by about 80% during incubation for 2-3 hr. in a medium containing canavanine but no arginine (Richmond, 1959). Volcani & Snell (1948) and Muraoka & Takadi (1954) have reported that certain *Lactobacillus* species and strains of *Streptococcus faecalis*, which are exacting for arginine, grew to a limited extent when incubated in a medium containing canavanine in place of arginine. A growth response of this kind may be characteristic of bacteria which are capable of incorporating canavanine into their protein.

Despite the incorporation of canavanine into the protein fraction of *S. aureus* 524, it is unlikely that canavanine is incorporated into the protein of all

species of micro-organisms. Pardee & Prestidge (1958) have reported that canavanine is not capable of supporting growth of an arginine-requiring mutant of *Escherichia coli*. This is, perhaps, because some strains of *E. coli* are reported to decarboxylate canavanine to yield an amine containing an *O*-guanidyl group (presumably, $\text{H}_2\text{N}\cdot\text{C}(\text{:NH})\cdot\text{NH}\cdot\text{O}\cdot[\text{CH}_2]_3\cdot\text{NH}_2$) (Hagihara, 1956). Furthermore, Kihara, Prescott & Snell (1955) have shown that canavanine can be degraded to homoserine + guanidine by some strains of *Strep. faecalis* and that these strains are therefore resistant to canavanine. Other actions of bacteria on canavanine are summarized by Kalyankar, Ikawa & Snell (1958).

As canavanine is so similar in structure to arginine and active in so many enzyme systems which are normally active on that amino acid (see Kihara *et al.* 1955; Meister, 1957) it is not, perhaps, surprising that it should be incorporated into the protein fraction of organisms. The results reported in this paper give no indication whether canavanine replaces arginine residues in the protein. If such replacement does occur, however, the marked difference between the pH value of the isoelectric point of the two amino acids (arginine, 10.7; canavanine 7.9; Spector, 1956) gives rise to the hope that the 'analogous' proteins may be separated readily by physicochemical methods.

SUMMARY

1. The presence of canavanine was detected in the protein + cell wall fraction of *Staphylococcus aureus* 524 grown in its presence.

2. No canavanine could be detected in cell-wall preparations from cells grown in the presence of arginine under similar conditions.

3. The canavanine was found to be incorporated in the protein + cell wall fraction in such a way that the amino group of the amino acid was not free to react with 1-fluoro-2:4-dinitrobenzene.

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Permeability of *Escherichia coli* to Ribose and Ribose Nucleotides

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Lutwak-Mann (1936) and Stephenson & Trim (1938) have shown that washed suspensions of *Escherichia coli* are able to degrade adenine nucleotides to form hypoxanthine, inorganic phosphate, ammonia and ribose. Stephenson & Trim also found that ribose did not accumulate but was fer-

mented with the liberation of a gas, and that the rate of gas production from adenosine was much greater than that from free ribose.

In previous experiments on the effect of nucleotides on various enzyme reactions in *E. coli* (Eggleson, 1957, 1958; Krebs, Jensen & Eggleson, 1958),