The Differential Effect of Arginine and Canavanine on Growth and Enzyme Formation in *Staphylococcus aureus* 524 SC

By M. H. RICHMOND

National Institute for Medical Research, Mill Hill, London, N.W. 7

It has been known for many years that bacteria, incubated in the absence of amino acids essential for growth, are incapable of net synthesis of cell protein. Furthermore, Pardee & Prestidge (1956), Yčas & Brawerman (1957) and Gros & Gros (1958) have reported that cultures of *Escherichia coli*, incubated under similar conditions, are unable to synthesize ribonucleic acid or deoxyribonucleic acid. Rogers & Mandelstam (1958), Mandelstam & Rogers (1959) and Hancock & Park (1958) have shown that *Staphylococcus aureus* can synthesize the mucopeptide component of the bacterial cell wall when no protein synthesis is possible through lack of amino acids essential for exponential growth. Prolonged incubation in such media therefore leads to profound changes in cell composition. Little other work, however, has been reported on the effect of low concentrations of essential amino acids on the formation of cell components. Under such conditions, it seemed possible that the synthetic processes dependent on a supply of amino acid might be impaired to different extents. It was of particular interest to see whether the synthesis of nucleic acids and of the various enzyme proteins was affected to the same extent, or whether some processes were more sensitive than others.

The organism chosen for these studies was *S. aureus* strain 524 SC (Rogers, 1953) because it is fully exacting to arginine and is known to synthesize a constitutive extracellular hyaluronidase (Rogers, 1954, 1957), a constitutive extracellular enzyme which can lyse *Micrococcus lysodeikticus* (Richmond, 1959a), an intracellular phosphatase and an inducible intracellular β-galactosidase. The hyaluronidase and the lytic enzyme were particularly interesting because they appear in synthetic medium at an increasing differential rate during exponential growth of the organism. It seemed possible that the synthesis of these enzyme proteins might be less closely coupled with the synthesis of other cell proteins and therefore particularly sensitive to a lowered amino acid supply. Further advantages are that cell-wall synthesis in this strain has been studied extensively and protein 'turnover' is very low (about 1%/hr.; Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959).

The first part of this paper reports experiments carried out to study the formation of various cell components and enzymes when cultures of *S. aureus* 524 were incubated in a fully synthetic medium containing low concentrations of arginine. The second part of the paper describes the effect on the formation of various cell components and enzymes in *S. aureus* 524 of replacing arginine by a structural analogue which could be metabolized by the organisms. L-Canavanine was chosen for this purpose as this amino acid has been reported by a number of workers to compete effectively with arginine (Volcani & Snell, 1948; Horowitz & Srb, 1948; Walker, 1955), although it has not been reported to replace arginine in protein. Previous work on the response of organisms to analogues of essential amino acids has produced no very clear picture. *Escherichia coli* grown in the presence of selenomethionine (a methionine analogue) can synthesize an enzymically active β-galactosidase (Cowie & Cohen, 1957); whereas Gros & Gros (1958) have shown that a phenylalanine-requiring mutant in the presence of *p*-fluorophenylalanine can synthesize a certain amount of nucleic acid but no protein. Brawerman & Yčas (1957) have reported that a tryptophan-requiring mutant of *E. coli* can double in protein and nucleic acid content when grown on azatryptophan (a tryptophan analogue); under these conditions these organisms can synthesize active β-galactosidase (Pardee, Shore & Prestidge, 1956; Pardee & Prestidge, 1958).

MATERIALS

Organisms. Staphylococcus aureus 524 SC (Rogers, 1953; Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959) was normally maintained in ampoules in the dried state, a new ampoule being opened for each experiment. Dried cultures were opened at intervals to check viability, purity and enzyme production. *Micrococcus lysodeikticus* (NCTC 2665) was used in the preparation of the substrate for lytic-enzyme estimations. The organisms were maintained as described previously (Richmond, 1959b).

Media. S. aureus 524 was found to grow well in Hedley–Wright broth containing 2·5% (w/v) of β-glycerophosphate or in casein hydrolysate medium containing 0·5% of glucose, nicotinic acid (1 μg./ml.) and thiamine (1 μg./ml.) (Rogers, 1945).

The fully defined media used were based upon medium 18AA (Table 1). The mixture of amino acids (Roche Biochemicals Ltd.) and nicotinic acid was dissolved in half the final volume of 0·05M-Na₂HPO₄ and the pH value brought to 6·8 with N-HCl. This solution was autoclaved at 10 lb./in.² for 20 min. The histidine, tryptophan and thiamine (each at ten times the final concentration) were sterilized separately by Seitz filtration. D-Glucose and the 'oligodynamic salt solution' (Pollock & Kramer, 1958) were autoclaved separately. The solution of the amino acid mixture and nicotinic acid was cooled to about 35°, and histidine, tryptophan, glucose and salts were added under sterile conditions together with enough sterile water to bring the medium to the required final volume. Tween 80 (0·04%, v/v) was added to the medium (if required) to minimize surface denaturation of extracellular enzymes (Richmond, 1958). For specific purposes, 18AA medium was modified by omitting arginine [17AA(-Arg) medium]; or arginine and glucose [17AA(-Arg-Glucose) medium].

Amino acids. L-Arginine HCl (Roche Biochemicals Ltd.) was used throughout these studies. I am indebted to Dr J. Mandelstam for a generous gift of L-canavanine, which had been prepared from jack-beans by the method of Kitagawa & Yamada (1932). Ionophoresis of arginine and canavanine in 2% (w/v) (NH₄)₂CO₃ buffer, pH 9·0, completely separated the two amino acids, which migrate in opposite directions. No arginine could be detected in a sample of 200 μg. of canavanine thus examined. The amino acids were detected with diacetyl and alkaline α-naphthol by the method of Szafir & Bennett (1953).

Table 1. *Composition of* 18AA *medium (see text)*

Amino acid mixture 'A'	Wt. (g.)
DL-Alanine	0·4
L-Aspartic acid	0·4
L-Arginine HCl	0·15
L-Glutamic acid	0·6
Glycine	0·6
DL-*iso*Leucine	0·4
DL-Leucine	0·4
L-Lysine HCl	0·2
DL-Methionine	0·6
DL-Phenylalanine	0·4
L-Proline	0·25
DL-Serine	0·15
DL-Threonine	0·1
L-Tyrosine	0·2
L-Valine	0·4
Nicotinic acid	0·005

Amino acid mixture 'A' was dissolved in 500 ml. of 0·05M-Na₂HPO₄ and adjusted to pH 6·8 with N-HCl. This was autoclaved at 10 lb./in.² for 20 min., cooled and the following additions were made under sterile conditions:

	Vol. (ml.)
Thiamine HCl (2 mg./ml.)	1·0
L-Tryptophan (2 mg./ml.)	100·0
L-Cystine (2 mg./ml.)	100·0
L-Histidine (2 mg./ml.)	100·0
D-Glucose (4%, w/v)	100·0
'Oligodynamic' solution of salts*	0·1
Sterile water to 1 l.	

* Pollock & Kramer, 1958.

The L-[*carboxy*-¹⁴C]leucine was obtained from The Radiochemical Centre, Amersham, Bucks; it was used at a specific activity of 0·05 μc/μmole unless otherwise stated.

Other compounds. I am indebted to Dr H. J. Rogers for a gift of potassium hyaluronate and to Dr J. Mandelstam for o-nitrophenyl-β-D-galactoside.

METHODS

Preparation of bacteria for experimental purposes

Dried organisms from ampoules were inoculated into Hedley–Wright broth (12 ml.) containing 2·5% of β-glycerophosphate. This culture was incubated at 35° overnight on a graded shaker (1 foot throw, 86 cyc./min.) (Kantorowicz, 1951). After 16 hr. growth, the culture was diluted into 100 ml. of Hedley–Wright broth and incubation continued until growth was exponential. The organisms were removed from the culture by centrifuging, washed twice with water (15 ml.) and resuspended in a convenient volume of 0·9% NaCl soln. for inoculation. The experimental media were contained in conical flasks of at least five times the culture volume to ensure maximum aeration and were brought to the required temperature and degree of aeration by incubation for 30 min. under experimental conditions before inoculation. Organisms were suspended to an initial culture density of about 150 μg. dry wt. of bacterial cells/ml. and incubated (Rogers, 1954) under conditions described above.

Growth was followed turbidimetrically (Rogers, 1954) and samples of convenient size were removed aseptically as required for analysis or estimation of enzyme activity.

Preparation of samples for enzyme estimations

Samples (containing about 2–3 mg. dry wt. of bacterial cells/ml.) were pipetted into 0·1 vol. of chloramphenicol (100 μg./ml., final concn.). The organisms were centrifuged for 10 min. at 3000 g, resuspended in 0·01M-Na₂HPO₄–KH₂PO₄ buffer, pH 7·0, and assayed for intracellular enzymes (β-galactosidase and phosphatase) and extracellular enzymes (lytic enzyme and hyaluronidase).

Enzyme estimations

β-Galactosidase. A modification of the method of Rickenberg & Lester (1955) was used. Samples of cells (prepared as described above) were incubated for 3 min. at 37° and 1 ml. of o-nitrophenyl-β-D-galactoside (1 mg./ml. in 0·01M-KH₂PO₄–Na₂HPO₄ buffer, pH 7·0) was added. Incubation was continued for 15 min. and the reaction stopped by addition of 1 ml. of M-K₂CO₃ soln. The tubes were transferred to room temperature for 1 hr. to let the colour develop fully, the organisms were removed by centrifuging (3000 g, 10 min.) and the concentration of o-nitrophenol was estimated by specific absorption at 420 mμ in the Unicam Spectrophotometer, model SP. 500. Enzyme was measured as μmoles of o-nitrophenol liberated/mg. dry wt. of bacterial cells/hr. at 37°.

Phosphatase. Phosphatase was estimated by following the release of o-nitrophenol from o-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 mg./ml. in 0·01M-phosphate buffer, pH 7·0) under exactly the same conditions as were used for estimation of β-galactosidase. Enzyme activity was expressed in the same units.

Lytic enzyme. Lytic enzyme was estimated by its ability to solubilize cell suspensions of *Micrococcus lysodeikticus* (Richmond, 1959*a*). Details of the estimation together with method of preparation of the substrate and definition of the enzyme unit were exactly as described for estimation of the *Bacillus subtilis* R lytic enzyme (Richmond, 1959*b*).

Hyaluronidase. Preparations of hyaluronidase were made by growing *Staphylococcus aureus* 524 in casein hydrolysate media under the conditions described above until the culture density reached about 1·0–1·2 mg. dry wt. of bacteria/ml. The culture medium containing the enzyme was cleared of organisms by centrifuging, dialysed for 24 hr. against 100 vol. of 0·001 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, and concentrated by freeze-drying. Estimation of this enzyme was carried out as described by Rogers (1954) and expressed as turbidity reducing units/ml. (TRU/ml.).

Preparation of samples for chemical fractionation

Samples (containing approx. 1 mg. dry wt. of organisms) were removed from the culture, pipetted into an equal volume of 10 % (w/v) trichloroacetic acid at 4° and stored at that temperature for 1 hr. The precipitate was removed by centrifuging and fractionated by a modification of the procedures of Ogur & Rosen (1950) and McQuillen & Roberts (1954).

(1) *Preparation of lipid and 'alcohol-soluble' protein fractions.* The precipitated material was washed with 5 % (w/v) trichloroacetic acid (3 ml.) at 4° and then extracted twice at 75° for 30 min. with 2 ml. of ethanol–water (3:1, v/v). The extracts were removed on the centrifuge, combined and split into an ether-soluble (lipid) and water-soluble (alcohol-soluble protein) fraction by addition of 2 vol. of water and 2 vol. of ether. The two layers obtained in this way were separated and taken to dryness *in vacuo*.

2*a. Simultaneous extraction of ribonucleic acid and deoxyribonucleic acid.* The insoluble residue from (1) was suspended in 5 % trichloroacetic acid (3 ml.) and heated on a water bath at 95° for 30 min. The supernatant was removed on the centrifuge, and the extraction with trichloroacetic acid and centrifuging were repeated. The acid was removed from the combined extracts by shaking five times with an equal volume of ether. The ether layer was discarded and the aqueous layer taken to dryness at 100° in a stream of air.

2*b. Separate extraction of ribonucleic acid and deoxyribonucleic acid.* Ribonucleic acid (RNA) was extracted from the residue from (1) by treatment with *N*-perchloric acid overnight at 2° (Ogur & Rosen, 1950). The perchloric acid (containing the RNA) was removed by centrifuging. Deoxyribonucleic acid (DNA) was estimated directly on the residue.

3. *Preparation of 'protein + cell-wall fraction'.* The protein + cell-wall fraction was prepared by washing the insoluble residue from (1) + (2*a*) successively with aqueous ethanol and ether. The washings were discarded.

Estimation of chemical components

Nucleic acids. RNA was estimated by its specific absorption at 260 mμ. A 1 mg./ml. solution of pure RNA was assumed to have a specific absorption of 28 extinction units (Gale & Folkes, 1953). DNA was estimated as deoxyribose by the method of Burton (1955).

Bacterial cell wall. Cell wall was estimated by the concentration of material reacting as amino sugar in hydrolysates of the protein + cell-wall fraction. About 1·5 mg. of protein + cell wall was hydrolysed (4 N-HCl; 4 hr., 105°) in a sealed tube. This process liberates free glucosamine and muramic acid with negligible destruction (Perkins & Rogers, 1959). The HCl was removed at 100° in a stream of air, residual traces of acid being removed by successive addition of several volumes of water and evaporation to dryness. The residue was dissolved in a known volume of water and the concentration of amino sugar present estimated by the method of Elson & Morgan (1933).

Radioactivity determination. Material to be estimated for radioactivity was taken to dryness at 105° (if necessary) and dissolved or suspended as finely as possible in aqueous 75 % (v/v) acetone (1 ml.). Samples (0·3 ml.), containing less than 0·5 mg. dry wt. of material, were transferred to stainless-steel planchets (1 cm.²), dried at 50° and radioactivity was measured by use of an end-window Geiger–Müller tube and scaler. Samples were counted until at least 10³ counts above background had been registered and it was assumed that the samples counted here were infinitely thin. Background radioactivity was measured before and after counting; 5·0 μmc of L-[*carboxy*-¹⁴C]leucine gave an average of 614 counts/min. in the apparatus used.

Terminology

Bacterial growth rate is defined as the increase in bacterial cell dry wt./unit time: $\Delta G/\Delta T$. Differential rate of enzyme formation is defined as the increase in enzyme activity in a given time/increase in bacterial dry wt. in the same period: $\Delta E/\Delta G$ (Monod, Pappenheimer & Cohen-Bazire, 1952).

RESULTS

Growth and enzyme formation in a fully defined medium

Staphylococcus aureus 524 grew well in the fully synthetic 18AA medium under the conditions described above (see Methods section). The generation time of the culture in this medium was about 50–55 min.; organisms cultured in casein hydrolysate medium or Hedley–Wright broth (Rogers, 1945) have a generation time of 40 and 35 min. respectively. In 18AA medium, growth continued to a final density of about 3·5–4·0 mg. dry wt. of bacterial cells/ml., although at densities greater than about 1·4 mg./ml. growth ceased to be truly exponential; this should not affect the experiments described in this paper, which were normally carried out over a range in bacterial-cell density from 100 to 800 μg./ml. During exponential growth over this range of cell densities, the rate of total protein, RNA, DNA and cell-wall formation remained constant.

Phosphatase. No phosphatase activity was released into the growth medium during exponential growth in 18AA medium and the differential rate of formation of cell-bound enzyme under these conditions remained constant, provided that the concentration of inorganic phosphate in the medium did not change markedly. Concentrations

of phosphate greater than 0·04M caused suppression of enzyme formation and no enzyme was detectable in phosphate concentrations greater than 0·15M.

Lytic enzyme and hyaluronidase. Both these enzymes are liberated into the growth medium during exponential growth in Hedley–Wright broth, casein hydrolysate medium or 18AA medium (Rogers, 1954, 1957; Richmond, 1959a). At any instant, the extracellular enzyme activity of either enzyme represented more than 80% of the total activity present in the culture after disruption in the Mickle disintegrator. No hyaluronidase activity was detected (by methods used in these studies) at culture densities less than about 150 μg. dry wt. of bacterial cells/ml., and no lytic enzyme below about 70 μg./ml. Subsequently, the differential rate of formation of both enzymes increased rapidly for the duration of exponential growth (Richmond, 1959a).

β-Galactosidase. Galactose (optimum concentration: 2%, w/v) was found to be the best inducer of β-galactosidase formation; lactose induced poorly (1–2% of the effect of galactose at equivalent molar concentrations) and thiomethyl-β-D-galactoside (Hogness, Cohn & Monod, 1955; Creaser, 1955; Monod, 1956) was inactive. The induction by galactose was completely suppressed by the presence of glucose. When organisms were incubated at very high suspension densities (about 1·5 mg. dry wt. of bacterial cells/ml.) in media containing mixtures of glucose and galactose, induction was delayed by an amount proportional to the initial glucose concentration; presumably induction was suppressed while any glucose remained in the culture medium. Cultures grown in a mixture of the two sugars showed a typical 'diauxie' growth curve (Monod, 1942). Attempts to liberate the enzyme from the organisms by treatment with toluene (Monod, Cohen-Bazire & Cohn, 1951) or cetyltrimethylammonium bromide (Salton, 1951), by disruption in the ultrasonic disintegrator (Gale & Folkes, 1955) or the Mickle disintegrator (Mickle, 1948), or in the press designed by Hughes (1951), resulted in complete loss of activity (cf. Creaser, 1955).

Growth and changes in enzyme components on incubation in the absence of arginine

Exponential growth was not possible when arginine was omitted from the growth medium. Fig. 1 shows that culture inoculated to an initial density of about 200 μg. dry wt. of bacterial cells/ml. and incubated in 17AA(-Arg) medium, increased in turbidity about 85% in the course of incubation for 3 hr., after which there was no further change. Changes occurring in composition

of *S. aureus* 524 during incubation under these conditions were studied as follows.

Formation of ribonucleic acid and deoxyribonucleic acid. There was a net increase of about 13% in the RNA content of cultures grown in the absence of arginine; there was no significant increase in the content of DNA in the same experiment. Measurement of the accumulation of material with a specific absorption at 260 mμ, (a) in the cold 5% trichloroacetic acid-soluble 'pool' or (b) in the culture medium, showed that these results could not be due to synthesis at the control rate accompanied by the rapid breakdown and release of RNA from the organisms. The rate of RNA synthesis was about half that reported by Mandelstam & Rogers (1959) for organisms of the same strain incubated in a buffered salts medium containing glucose and glycine.

Incorporation of L-[carboxy-^{14}C]leucine into the protein and lipid. S. aureus 524 was found to incorporate radioactivity from [carboxy-^{14}C]leucine predominantly into the lipid, protein + cell-wall fraction and alcohol-soluble protein fraction of the cells. Organisms were inoculated into 18AA medium containing mM-[carboxy-^{14}C]leucine to an initial density of about 200 μg. dry wt. of organisms/ml. and incubated for 1 hr. Growth and incorporation were stopped by the addition of an equal
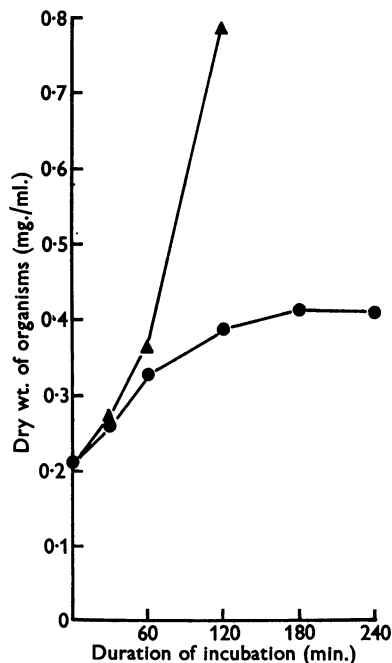
Fig. 1. Growth of organisms in fully synthetic amino acid media. ▲, 18AA medium; ●, 17AA(-Arg) medium. Initial culture density: 215 μg. dry wt. of organisms/ml.

volume of cold 10% trichloroacetic acid containing 1% (w/v) of L-[¹²C]leucine and the resulting precipitate was fractionated to produce lipid, alcohol-soluble protein, RNA, DNA and protein + cell-wall fractions as described above. In such an experiment, 41% of the radioactivity appeared in the lipid, 45% in the protein and 4·3% in the alcohol-soluble protein. As the cell wall of *S. aureus* 524 contains less than 2% (by wt.) of leucine (J. Mandelstam & H. R. Perkins, personal communication) the incorporation of radioactivity into the protein + cell-wall fraction is a valid measure of protein synthesis provided that the fraction is free from contaminating lipid. Fig. 2 shows the time course of incorporation of [¹⁴C]leucine in a similar experiment.

When organisms were incubated in 17AA(-Arg) medium in the presence of [*carboxy*-¹⁴C]leucine the rate of incorporation into lipid was initially about 60% of that in the control culture in 18AA medium, but fell to about 35% in the course of incubation f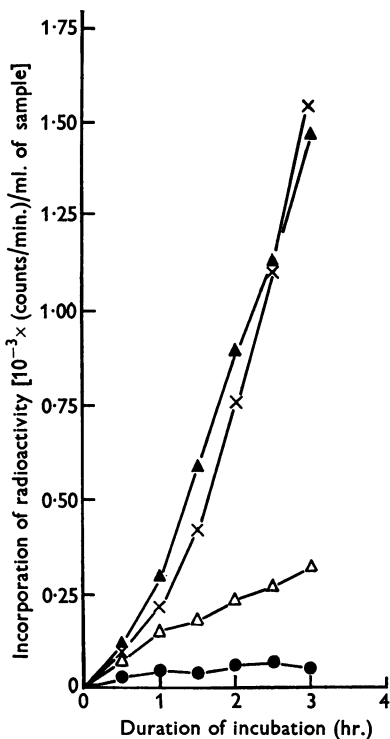or 60 min. On the other hand, practically no incorporation of radioactivity into the protein + cell wall occurred during incubation for 150 min. in the 17AA medium. The incorporation of radioactivity into the alcohol-soluble protein fraction continued at a reduced rate under these conditions; accurate measurements were not possible because of the low levels of incorporation and the high inherent risk of contamination with lipid.

Cell-wall synthesis. The mucopeptide component of *S. aureus* 524 cell wall contains the amino acids lysine, glycine, alanine and glutamic acid together with the amino sugar derivatives *N*-acetylglucosamine and *N*-acetylmuramic acid (Mandelstam & Rogers, 1959; Perkins & Rogers, 1959). As most of the mucopeptide component of the wall is insoluble in hot 5% trichloroacetic acid (Hancock & Park, 1958), mucopeptide synthesis was measured by following the amount of amino sugar liberated from the protein + cell-wall fraction on hydrolysis (see Methods section). Fig. 3 shows that the quantity of trichloroacetic acid-precipitable amino sugar increased about 85% by weight in the course of incubation for 3 hr. This corresponds to an increase in cell-wall content of about 170 μg./mg. initial dry wt. of organisms. Mandelstam & Rogers (1959) have reported that omission of glucose from their incubation mixtures resulted in loss of ability to synthesize cell wall. Similarly, in these experiments, omission of glucose from 17AA(-Arg) medium prevented increase in turbidity and synthesis of the mucopeptide component of the cell wall.

Fig. 2. Time course of incorporation of radioactivity from [*carboxy*-¹⁴C]leucine into the lipid fraction (×, △) and the protein + cell-wall fraction (▲, ●) of organisms incubated in media with and without arginine. Initial suspension density: 210 μg. dry wt. of organisms/ml. [¹⁴C]Leucine concentration: mM at 0·05 μc/μmole. Media: 18AA (×, ▲); 17AA(-Arg) (△, ●).
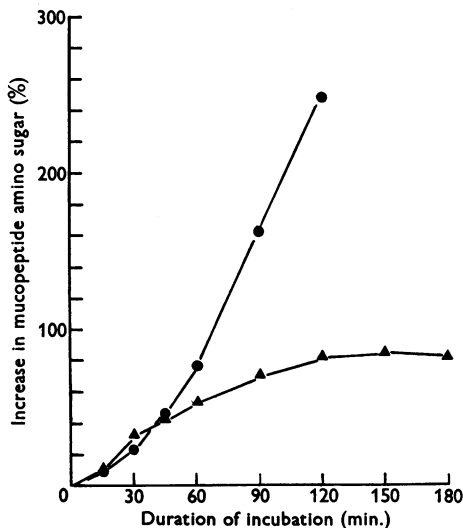

Fig. 3. Formation of 'mucopeptide' amino sugar during incubation of organisms in 18AA medium (●), and in 17AA(-Arg) medium (▲). Initial culture density: 200 μg. dry wt. of organisms/ml. Mucopeptide amino sugar was estimated as described in the Methods section.

Enzyme formation. No formation of hyaluronidase, lytic enzyme or phosphatase could be detected during incubation of cultures in 17AA(-Arg) medium. Furthermore, it was not possible to induce cultures to form β-galactosidase although this enzyme was formed abundantly in control cultures after induction.

Growth in limited concentrations of arginine

The effect of low concentrations of arginine was studied by augmenting the basal 17AA(-Arg) medium with small quantities of arginine. Organisms were inoculated into batches of 17AA(-Arg) medium to which were added 2, 4, 5, 10 and 100 μg. of arginine/ml.; a further culture incubated in the absence of arginine acted as control. Fig 4 shows that the initial growth rate was the same in the various concentrations of added arginine but that the total rapid growth obtained depended (up to about 5 μg./ml.) on the amount of this amino acid added. The total growth obtained on 2, 4 and 5 μg./ml. indicated that 1 μg. of arginine supported the formation of about 60 μg. of cell material. Addition of higher concentrations of arginine did not lead to increased total growth, presumably because some other factor became limiting before the arginine was exhausted. At low concentrations, the rapid phase of growth was followed by a period of slow increase in turbidity. Changes in cell components during this period were essentially similar to those occurring in the absence of added arginine.

Nucleic acid synthesis. The formation of RNA and DNA was studied in cultures incubated in basal medium 17AA(-Arg) containing 2, 4 and 100 μg. of arginine/ml. A net increase in RNA and DNA continued throughout the period of rapid growth and there was no change in the differential rates of synthesis of either nucleic acid at low amino acid concentrations.
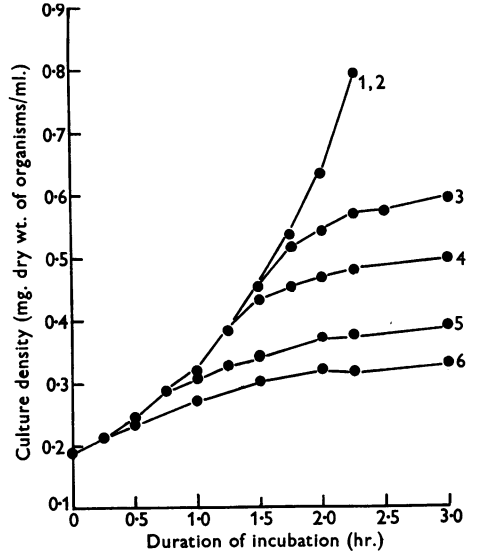

Fig. 4. Growth curves obtained by incubating organisms in the basal 17AA(-Arg) medium containing various added amounts of arginine. Arginine concentrations (μg./ml.): 1, 100; 2, 10; 3, 5; 4, 4; 5, 2; 6, nil. Initial culture density: 180 μg. dry wt. of organisms/ml.
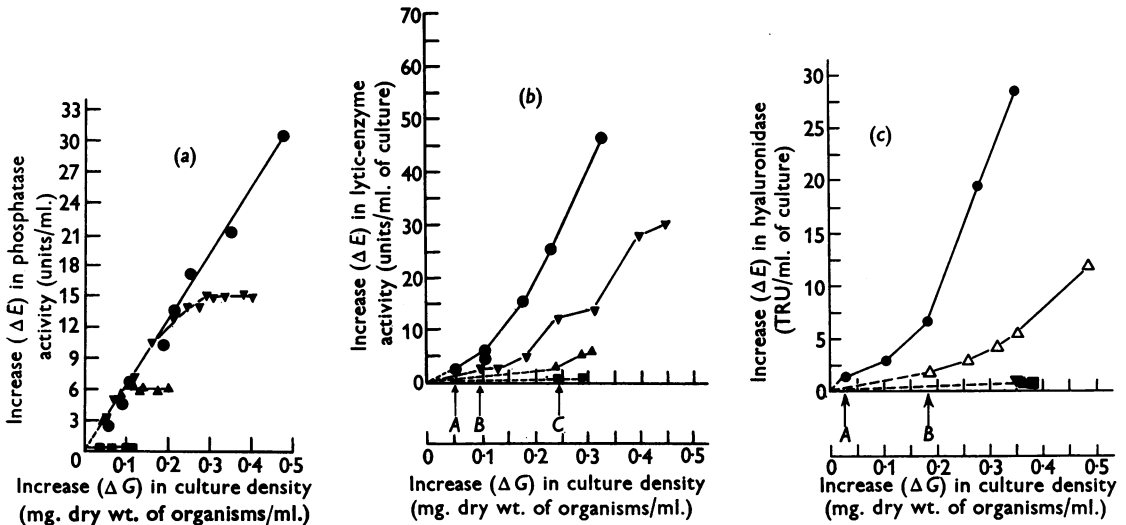

Fig. 5. Differential plots (ΔE/ΔG) of phosphatase (a), lytic enzyme (b) and hyaluronidase (c) formation in cultures grown on basal 17AA(-Arg) medium supplemented with arginine (μg./ml.); (●), 100; (▼), 4; (▲), 2; (△), 10 (c only); (■), nil. Initial culture density: 205 μg. dry wt. of organisms/ml. Points A, B and C (b), A and B (c) are referred to in the text.

Phosphatase. Variations in arginine concentration had no significant effect on the differential rate of phosphatase synthesis (Fig. 5a). The total amount of enzyme formed in the presence of 4 μg. of arginine/ml. was almost exactly twice that formed in 2 μg./ml.; higher concentrations did not lead to a proportional increase in the amount of enzyme formed.

Lytic enzyme and hyaluronidase. The formation of both these enzymes was impaired at low concentrations of arginine. There was a lag in all cultures before lytic enzyme (Fig. 5b) appeared. In 100 μg. of arginine/ml., lytic enzyme was detectable after incubation for about 15 min. (*A*, Fig. 5b) and thereafter it accumulated at an increasing differential rate for at least 60 min. In the presence of 4 μg./ml., however, although growth was as rapid as in 100 μg./ml., enzyme formation did not commence for about 40 min. (*B*, Fig. 5b) and then occurred at a lower differential rate. In 2 μg./ml. the lag period was about 90 min. (*C*, Fig. 5b) and the differential rate about one-quarter of that in 4 μg./ml. Parallel experiments confirmed that, under these conditions, the extracellular enzyme activity was closely related to the total activity detectable after disruption of the organisms in the Mickle disintegrator. Fig. 5c shows the appearance of hyaluronidase in the same experiment. After a lag of about 20 min. (*A*, Fig. 5c) enzyme formation occurred in the culture containing 100 μg. of arginine/ml.; a similar culture incubated in 10 μg. of arginine/ml. showed some enzyme formation after incubation for 85 min. (*B*, Fig. 5c). No enzyme was detected at any time in the other cultures. To check that these results were not due to enzyme inactivation under these conditions, crude *S. aureus* 524 hyaluronidase was added to each culture at the beginning of a similar experiment (final concentration: 30 TRU/ml.; prepared as described in the Methods section). After incubation for 150 min. (Table 2) enzyme activity in the culture containing 100 μg. of arginine/ml. increased about twofold; that in 4 and 2 μg./ml. and in the control culture remained approximately constant.

β-Galactosidase. The differential rate of β-galactosidase synthesis was studied in a similar way to that of phosphatase except that 17 AA(-Arg-Glucose) medium plus galactose was used in place of 17 AA(-Arg) medium. All the cultures incubated in the presence of arginine underwent a lag before growth and enzyme formation occurred; the duration of the lag was slightly increased in lower arginine concentrations. Subsequently, the rate of enzyme production increased either until halted by lack of arginine or until a maximum rate was achieved after about 90 min. incubation. A differential plot of β-galactosidase formation (Fig. 6) shows that, even in the presence of 100 μg. of

Table 2. *Stability of hyaluronidase under various conditions of incubation*

Cultures were incubated in the 17 AA(-Arg) medium with addition of arginine and crude staphylococcal hyaluronidase, as shown.

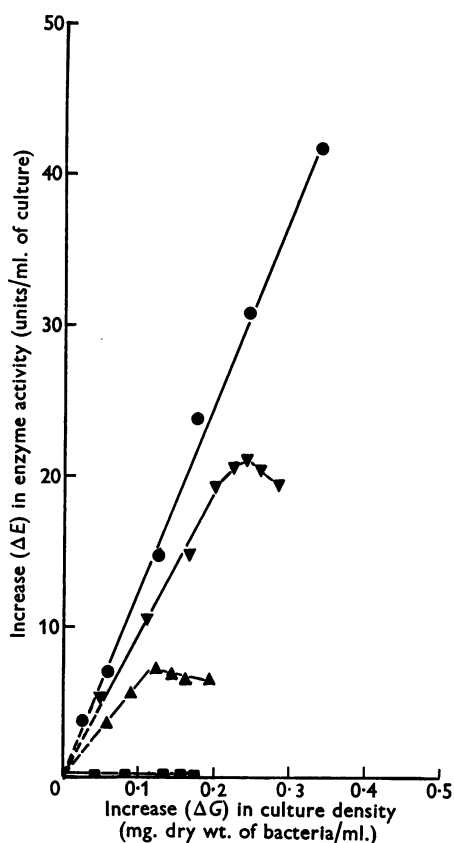| Additions		Hyaluronidase activity (TRU/ml.) after incubation	
Arginine (μg./ml.)	Hyaluronidase (TRU/ml.)	30 min.	150 min.
100	30	38	64
4·0	30	28	31
2·0	30	28	32
Nil	30	27	28

Fig. 6. Differential plot (Δ*E*/Δ*G*) of β-galactosidase formation in cultures incubated in media containing various concentrations of arginine. Media: basal 17 AA(-Arg-Glucose) medium containing 2 % of galactose and arginine (μg./ml.) as follows: (●), 100; (▼), 4; (▲), 2; (■), nil. The mean generation time of organisms grown in 17 AA(-Arg-Glucose) augmented with arginine and galactose was approx. the same as that achieved in 18 AA medium.

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arginine/ml., the differential rate was approximately linear. Possible reasons for this are discussed below (see Discussion). Fig. 6 also shows that the differential rate of β-galactosidase formation varied with the concentration of the arginine in the medium. In 4 and 2 μg. of arginine/ml. the differential rates are about 70 and 50 % of the rate achieved in 100 μg./ml.

To see whether the cessation of β-galactosidase formation at low concentrations of arginine was due to limitation by the amino acid, a further 2 μg./ml. was added to a culture which had been incubated in 2 μg./ml. and had ceased to grow and to form enzyme. Growth and enzyme formation recommenced immediately and continued until the second batch of arginine was exhausted; addition of more arginine elicited a further burst of growth and enzyme formation. The differential rate of enzyme formation increased with successive batches of arginine until it reached the rate characteristic of organisms grown in 100 μg./ml.

Effect of replacement of L-arginine by L-canavanine

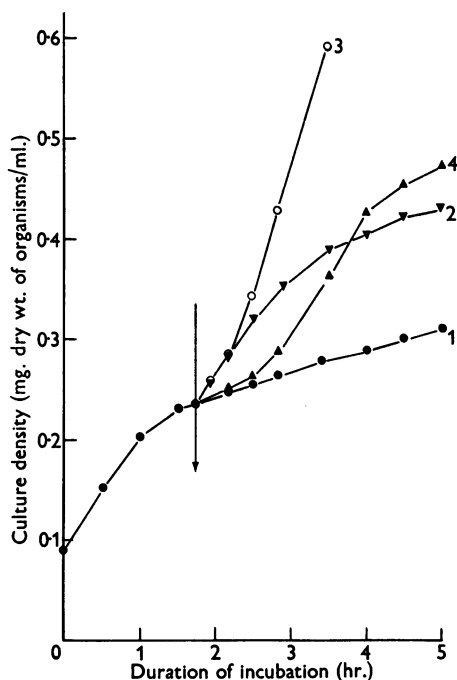The effect of canavanine was studied on organisms which had been grown in 17AA(-Arg) medium

Fig. 7. Effect of arginine and canavanine on the growth of S. aureus 524. Media: phase I; basal 17AA(-Arg) medium +2 μg. of arginine/ml. Culture was divided at the arrow into four parts and additions were made as follows: 1 (●), nil; 2 (▼), 2 μg. of arginine/ml.; 3 (○), 100 μg. of arginine/ml.; 4 (▲), 50 μg. of canavanine/ml.

containing 2 μg. of arginine/ml. A culture growing exponentially in Hedley–Wright broth was centrifuged, washed twice with 17AA(-Arg) medium and resuspended in the basal 17AA(-Arg) medium to which was added 2 μg. of arginine/ml. Incubation was continued until rapid growth ceased (phase 1), and the culture was then divided into four parts (Fig. 7) to which additions were made as follows: phase II, culture 1, nil; culture 2, 2 μg. of arginine/ml.; culture 3, 100 μg. of arginine/ml.; culture 4, 50 μg. of canavanine/ml. Fig. 7 shows that growth occurred immediately in cultures 2 and 3, and that growth in culture 2 slowed markedly after about 80 min. when the supply of arginine was exhausted. Culture 4, on the other hand, increased in turbidity slowly for the first 40 min. but thereafter more rapidly, reaching a maximum rate after about 75 min. Growth slowed after about 150 min., at which point the culture had increased in turbidity about 80 %. Under the conditions of this experiment, canavanine can be detected in the protein + cell-wall fraction of the organisms (M. H. Richmond, unpublished results) and the effect of this phenomenon on enzyme formation and changes in cell composition was studied as follows.

Nucleic acid synthesis. Organisms of this strain can synthesize RNA and DNA in media containing 1–2 μg. of arginine/ml. When the arginine in such an experiment was replaced by 50 μg. of canavanine/ml., no significant increases in RNA and DNA were observed over the control values obtained in the absence of either amino acid.

Cell-wall synthesis. The increase in trichloroacetic acid-precipitable amino sugar was studied in organisms grown in the basal 17AA(-Arg) medium (culture 1), in similar medium augmented with 2 μg. of arginine/ml. (culture 2), with 100 μg. of arginine/ml. (culture 3) and with 50 μg. of canavanine/ml. (culture 4). The progress curves (Fig. 8) show that organisms incubated in the presence of canavanine were capable of forming cell-wall material at about the same rate as cultures incubated in 2 μg. of arginine/ml. The total quantities of wall synthesized in the presence of 2 μg. of arginine/ml. or 50 μg. of canavanine/ml. are about 100 and 140 μg./mg. initial dry wt. of organisms respectively. Cell-wall preparations of S. aureus 524 contain less than 1 % (by wt.) of arginine (H. R. Perkins & J. Mandelstam, personal communication) and attempts to demonstrate the incorporation of canavanine into cell-wall preparations prepared by the method of Hancock & Park (1958) were unsuccessful.

Enzyme formation and protein synthesis: phosphatase, hyaluronidase and lytic enzyme. No increase in the level of phosphatase activity, or appearance of hyaluronidase or lytic enzyme, could be detected in cultures incubated for 3 hr. in the basal

17AA(-Arg) medium containing 50 μg. of cana-
vanine/ml. Addition of 50 μg. of canavanine/ml. to
cultures growing in the basal 17AA(-Arg) medium
containing 2, 10 or 100 μg. of arginine/ml. had no
effect on the differential rates of enzyme formation
regardless of the time of addition of the analogue.

β-Galactosidase. The effect of canavanine on β-
galactosidase synthesis was studied in cultures
partly induced by a period of growth (phase I,
Fig. 9) in 17AA(-Arg-Glucose) medium to which
had been added 2% of galactose and 2 μg. of
arginine/ml. Fig. 9 (phase II) shows that the
addition of 50 μg. of canavanine/ml. when the
arginine was exhausted did not result in any
further formation of β-galactosidase, despite the
fact that the turbidity of the culture increased.
Addition of 2 μg. of arginine/ml. in place of cana-
vanine led to immediate formation of β-galacto-
sidase.

Incorporation of [carboxy-^{14}C]*leucine.* The results
reported above show that the increase in turbidity
which occurred when cultures were incubated in
the presence of canavanine was not associated with
the formation of active forms of any of the enzymes
tested. To decide whether protein synthesis occurred

under these conditions, the uptake of [carboxy-^{14}C]-
leucine into the protein + cell-wall fraction of the
organisms was followed in the presence of an
inhibitory concentration of canavanine. A culture
was grown in the basal 17AA(-Arg) medium con-
taining 2 μg. of arginine/ml. until the arginine was
exhausted. The culture was then divided into four
parts and the following additions were made: nil
(culture 1); 2 μg. of arginine/ml. (culture 2);
100 μg. of arginine/ml. (culture 3); 50 μg. of
canavanine/ml. (culture 4); [carboxy-^{14}C]leucine
(final concn. mM) was added to each culture im-
mediately after the addition of the amino acids.
The cultures were incubated as described pre-
viously and measured samples, removed at inter-
vals, were transferred to an equal volume of cold
10% trichloroacetic acid containing 1% (w/v) of
[^{12}C]leucine to stop growth and incorporation. The
rate of incorporation in the presence of the ana-
logue into the protein + cell-wall fraction (Fig. 10a)
was slower than that observed in arginine. The
total amount of radioactivity incorporated in the
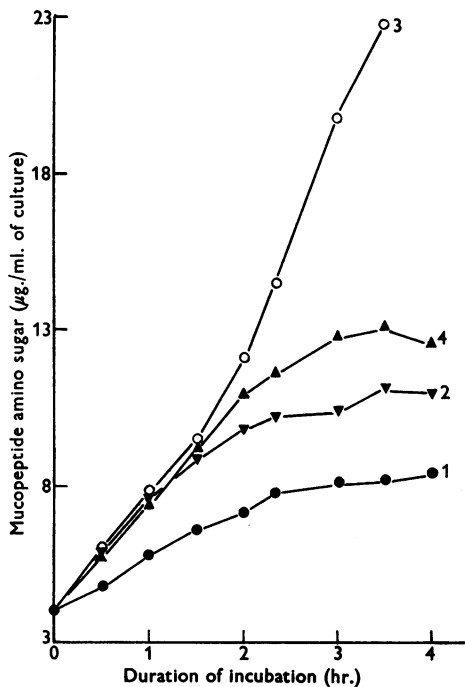canavanine experiment corresponds to about 5 μg.

Fig. 8. Increase of 'mucopeptide' amino sugar in cultures
incubated in the basal 17AA(-Arg) medium containing
the following additions/ml.: 1 (●), nil; 2 (▼), 2 μg. of
arginine; 3 (○), 100 μg. of arginine; 4 (▲), 50 μg. of
canavanine. Initial culture density: 210 μg. dry wt. of
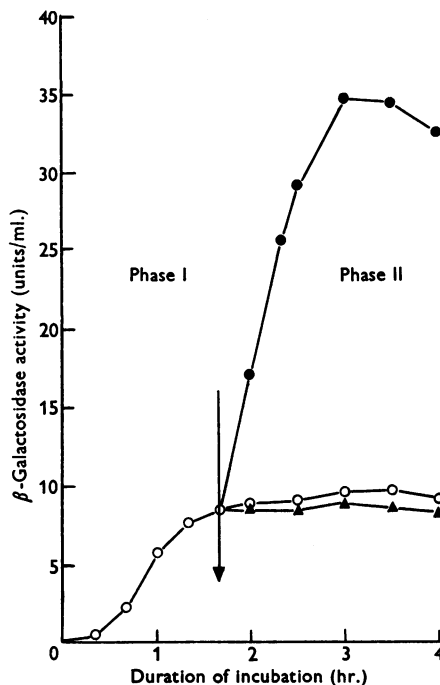organisms/ml.

Fig. 9. Effect of arginine and canavanine on β-galacto-
sidase formation in cultures partially induced by pre-
vious incubation in low concentrations of arginine.
Media: phase I; 17AA(-Arg-Glucose) medium containing
2% of galactose and 2 μg. of arginine/ml. Further
additions made at the arrow (phase II) were as follows:
(●), 2 μg. of arginine/ml.; (○), nil; (▲), 50 μg. of
canavanine/ml.

of leucine/mg. initial dry wt. of organisms. Addition of 30 μg. of chloramphenicol/ml. at the same time as the addition of the leucine resulted in a decrease of about 80% in the radioactivity incorporated into the protein + cell-wall fraction in the first 60 min. of incubation. Incorporation of radioactivity into the lipid fraction of the organisms occurred in the presence of canavanine (Fig. 10b). The rate of incorporation in the presence of canavanine was about the same as that occurring in the presence of arginine during the first 60 min. of incubation, and was unaffected by the presence of chloramphenicol.

DISCUSSION

Cultures of *Staphylococcus aureus* 524, incubated in media lacking arginine, are incapable of synthesizing RNA, DNA or protein. These changes are similar to those found by earlier workers (Sands & Roberts, 1952; Pardee & Prestidge, 1956; Yčas &

Brawerman, 1957; Gros & Gros, 1958) with amino acid auxotrophs of *Escherichia coli* incubated in the absence of the required amino acid.

Despite the absence of protein synthesis the staphylococci were able to synthesize both lipid and the mucopeptide component of the cell wall, and it appears therefore that the omission from the growth medium of a single essential amino acid resulted in changes in cell biosynthesis very similar to those found when this strain was incubated in a buffered medium containing glucose and glycine (Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959). The use of trichloroacetic acid-precipitable amino sugar to estimate the level of the mucopeptide component of the cell wall is not vitiated by the recent findings of Baddiley and his co-workers (Armstrong, Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958) that the cell wall of several species of *S. aureus* contains a complex polymer of ribitol, *N*-acetyl glucosamine, D-alanine and orthophosphate, as this
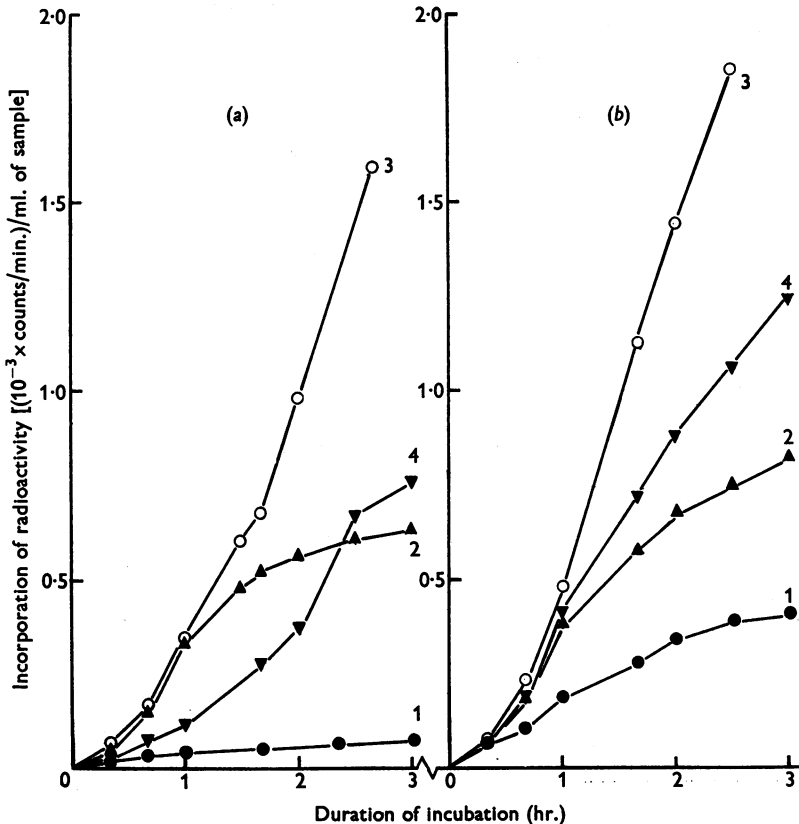

Fig. 10. Comparison of the incorporation of [*carboxy*-^{14}C]leucine into the protein + cell-wall fraction (a) and lipid fraction (b) of organisms incubated in the basal 17 AA(-Arg) medium containing various concentrations of arginine or canavanine, as follows: 1 (●), nil; 2 (▲), 2 μg. of arginine/ml.; 3 (○), 100 μg. of arginine/ml.; 4 (▼), 50 μg. of canavanine/ml [^{14}C]Leucine concentration: mM, at 0·05 μc/μmole, in all cultures.

component may be extracted from cells with cold 5% trichloroacetic acid. Gros & Gros (1958), working with amino acid auxotrophs of *E. coli*, have shown that as little as 2 µg./ml. of the required amino acid allowed immediate synthesis of RNA. In the experiments reported above it was never possible to show an altered differential rate of RNA or DNA synthesis (ΔRNA/ΔG or ΔDNA/ΔG) on addition of low concentrations of arginine. These conditions did, however, have an effect on the synthesis of some of the enzymes studied. Although the differential rate of phosphatase synthesis was unaffected, no hyaluronidase was formed when cultures were incubated in the presence of 2 or 4 µg. of arginine/ml., and lytic-enzyme synthesis was delayed and occurred at a lower differential rate (see Fig. 5). These results show a range of sensitivity of the synthetic mechanism to low arginine concentration in this order: hyaluronidase > lytic enzyme > phosphatase, RNA, DNA > cell-wall mucopeptide. It was not possible to show any effect on the differential rates Δphosphatase/ΔRNA or Δphosphatase/ΔDNA, provided that the inorganic phosphate content of the growth medium was not altered. Similar experiments to study the differential rate of β-galactosidase formation showed a marked effect of arginine (Fig. 6), but these results are difficult to interpret for the following reasons. First, the estimation of β-galactosidase activity was carried out on intact organisms because disruption of the cells by any of the methods available led to complete loss of enzyme activity (cf. Creaser, 1955). The activity of the enzyme measured may not therefore reflect the amount of β-galactosidase protein synthesized but rather the activity of the system responsible for accumulating *o*-nitrophenyl-β-D-galactoside within the organisms (see Cohn, 1957).

Secondly, galactose is not a 'gratuitous' inducer (Monod & Cohn, 1952) of β-galactosidase for this strain of *S. aureus*, which seems to require carbohydrate in the culture medium for active growth and enzyme formation. Organisms grown in glucose did not contain an active 'galactozymase' system and could not therefore metabolize galactose in large quantities to produce energy. Furthermore, glucose could not be added with the galactose as it completely suppresses the formation of both the 'galactozymase' system and β-galactosidase. On transfer from glucose- to galactose-containing medium the 'galactozymase' system was induced (Creaser, 1955; M. H. Richmond, unpublished work), and this breaks down the galactose to produce the energy necessary for rapid growth and β-galactosidase synthesis. It follows therefore that the addition of galactose to cultures resulted in several changes in the metabolism of the organisms

and these may secondarily affect the synthesis of β-galactosidase.

If this difficulty is ignored, the sequence of decreasing sensitivity of synthetic processes to low arginine concentration becomes: hyaluronidase > lytic enzyme > β-galactosidase > phosphatase, RNA, DNA > cell-wall synthesis.

The studies reported by Virtanen (1948; Virtanen & De Ley, 1948) on enzyme formation in *E. coli* grown on very low concentrations of ammonium ion as sole nitrogen source, could be consistent with these results. However, these authors studied total enzyme content of the organisms and not the rates of enzyme formation.

Both hyaluronidase and the lytic enzyme are predominantly extracellular and this may be related to their particular sensitivity to arginine deprival. Gale (1953) has reported that *S. aureus* is capable of accumulating amino acids within the osmotic membrane of the cell. In this way the concentration of a limiting amino acid within the cell may be about 10–20 times its concentration in the growth medium. Experiments with protoplasts of Gram-positive bacteria have led to the view that the osmotic barrier of the Gram-positive cell is in the cytoplasmic membrane (Weibull, 1956; McQuillen, 1956, 1958) and, as more than 85% of the total extractable amounts of both hyaluronidase and the lytic enzyme may be detected in the growth medium, it seems likely that these enzymes are synthesized in this membrane. If this is so, the synthetic sites of these enzymes may not be supplied with arginine that has passed through the osmotic barrier/cytoplasmic membrane, but directly from the growth medium. In this way, when a limited quantity of arginine is available, the level of amino acid reaching the hyaluronidase- and lytic enzyme-synthesizing sites may be appreciably lower than that reaching the enzyme-forming sites within the organisms. In non-limiting concentrations of arginine this effect would be lessened, and this is supported by the finding that synthesis of both extracellular enzymes is rapid in the presence of 100 µg. of arginine/ml. This mechanism cannot, however, be the direct cause of the increased differential rate of hyaluronidase and lytic-enzyme synthesis observed as exponential growth proceeds (Rogers, 1954, 1957; Richmond, 1959a).

Organisms grown in media in which arginine was replaced by canavanine were able to incorporate radioactive leucine into the protein fraction of the organisms, but formed no active phosphatase, β-galactosidase, hyaluronidase or lytic enzyme. The incorporation was inhibited more than 80% by concentrations of chloramphenicol inhibitory to growth, and these results imply that protein synthesis continued in these cultures for about 2–3 hr. but without leading to the formation of active

versions of the enzyme tested. Munier & Cohen (1956) have shown in similar experiments, involving p-fluorophenylalanine, that the analogue was incorporated into the cell protein in place of phenylalanine. Canavanine has been reported to replace arginine in a number of biosynthetic reactions (Nakatsu, 1956; Walker, 1956; Kihara & Snell, 1957), and it seemed likely that the protein synthesis occurring in cultures grown in the presence of the analogue was associated with incorporation of canavanine into protein in the place of arginine. The detection of canavanine in the protein fraction of the organisms (M. H. Richmond, unpublished work) supports this hypothesis. It is not possible to say at this juncture whether canavanine inhibits the formation of phosphatase, β-galactosidase, hyaluronidase and the lytic enzyme completely or whether the formation of inactive enzyme 'analogues' occurs. If modified enzyme molecules are made, the effect of the amino acid analogues on their activity would depend, chiefly, on two factors. First, whether the analogue completely replaces the normal amino acid. Secondly, whether the replacement of a given amino acid residue results in an enzyme with a modified activity, or an inactive protein.

In the experiments reported above, the organisms are completely exacting to arginine and therefore, if enzyme 'analogues' are formed, their arginine residues would probably be completely replaced with canavanine and be inactive. Experiments to decide whether phosphatase, β-galactosidase, hyaluronidase or lytic-enzyme 'analogues' are formed under the conditions of these experiments must await methods of purification of the enzymes and the preparation of specific antisera.

SUMMARY

1. Cultures of *Staphylococcus areus* 524, incubated in a fully synthetic amino acid medium lacking arginine, synthesize some lipid and the mucopeptide component of the bacterial cell wall but no ribonucleic acid, deoxyribonucleic acid and general cell protein. Similarly, such cultures do not form active extracellular hyaluronidase, lytic enzyme and intracellular phosphatase; nor can they be induced to form intracellular β-galactosidase.

2. Addition of 2 μg. of arginine/ml. to such cultures fully restores the ability of the organisms to synthesize ribonucleic acid, deoxyribonucleic acid and phosphatase. Under these conditions, however, no hyaluronidase formation can be detected, the synthesis of lytic enzyme is markedly impaired and the differential rate of β-galactosidase formation is depressed to about half the rate achieved in 100 μg. of arginine/ml.

3. As a result of these experiments, the following sequence of decreasing sensitivity to limiting arginine concentrations is suggested: hyaluronidase > lytic enzyme > β-galactosidase > phosphatase, ribonucleic acid and deoxyribonucleic acid > cell-wall mucopeptide.

4. Cultures incubated in a fully synthetic amino acid medium containing canavanine in the place of arginine increase in turbidity, synthesize cell-wall mucopeptide, ribonucleic acid, deoxyribonucleic acid and general cell protein but do not form active versions of any of the enzymes tested.

5. A possible mechanism of these changes is discussed.

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The Spectrophotometric Determination of Protein at 210 mμ

By M. P. TOMBS, FELICITY SOUTER AND N. F. MACLAGAN

Department of Chemical Pathology, Westminster Medical School, London, S.W. 1

The absorption spectra of proteins in the region above 240 mμ are well known and extensively used in analytical procedures. The peak in the region of 280 mμ may be used for the analysis of tyrosine and tryptophan (Beaven & Holiday, 1952), and measurement of this peak provides, for relatively homogeneous proteins, a simple and accurate method of determining relative concentrations (e.g. Tombs, 1957). With more complex mixtures such as serum the various proteins will contain widely varying amounts of tyrosine, tryptophan and phenylalanine, and measurements at 280 mμ can no longer be used as a reliable index of total protein content, even where a comparison between one serum and another is all that is required.

Below 230 mμ the absorption of both proteins and peptides rises steeply. However, Goldfarb, Saidel & Mosovich (1951) have shown that there is a definite maximum near 190 mμ which is due mainly to the specific absorption of peptide bonds. If this is so one might expect that all proteins would have a similar specific absorption in this region, since they all have similar peptide-bond contents. Goldfarb *et al.* (1951) report figures which suggest that this is indeed the case for human and bovine albumin and γ-globulin, egg albumin and gelatin. It seemed possible therefore that measurement in the neighbourhood of this peak might offer a rapid and simple method for the estimation of proteins.

A method involving measurement at 215 and 225 mμ has recently been proposed by Waddell (1956) and Bendixen (1957). The choice of a suitable wavelength in this region depends partly on instrumental limitations and partly on the possible contribution of the aromatic amino acids to the specific absorption of individual proteins. Results described here and briefly reported elsewhere (Tombs, Souter & Maclagan, 1959) suggest that 210 mμ is the optimum wavelength for our conditions of measurement.

EXPERIMENTAL AND RESULTS

Nitrogen estimations. Total protein from 0·1 ml. of serum was precipitated with aluminium tungstate (Lorant, 1957), and nitrogen in the precipitate was determined by the micro-Kjeldahl method. A factor of 6·25 was used to convert nitrogen content into protein content.

Absorption measurements. These were made with a standard Hilger Uvispek spectrophotometer, but similar results have also been obtained with a Unicam SP. 500 spectrophotometer. Paired 1 cm. silica cells were used with solvent as blank. A slit width of 1·4 mm. was used at 210 mμ. Cells were cleaned in conc. HNO_3 and cell-matching was checked at frequent intervals.