Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
THE PHYSIOLOGY OF STAPHYLOCOCCAL ENTEROTOXIN PRODUCTION

A thesis presented in partial fulfilment of the requirements for the degree of Ph.D in Microbiology at Massey University.

Audrey Winifred Jarvis
1974
ABSTRACT

The aims of this investigation were to study the environmental factors which determine enterotoxin production by staphylococci. Effects of pH, oxygen, medium composition, and in particular of added carbohydrates in a defined medium were investigated under controlled conditions, using several strains of each enterotoxin type.

The production of staphylococcal enterotoxins A, B and C in shake-flasks was studied throughout the growth cycle for nine strains of staphylococci. Enterotoxins were first detected in the exponential phase, and except for strain S-6, no appreciable increase in enterotoxin occurred during the stationary phase of growth. Enterotoxins were however produced in relatively small quantities by non-replicating cells from late exponential or stationary phases of growth. Chloramphenicol inhibited enterotoxin production by non-replicating cells. A small quantity of enterotoxin B was produced in the presence of actinomycin D, suggesting the accumulation of a small pool of mRNA for enterotoxin production.

The production of enterotoxins was investigated in a fermenter under controlled conditions of pH and aeration, using a casein hydrolysate medium and a defined amino acid medium. Enterotoxin production in the fermenter was considerably less than in shake-flasks for eight out of nine strains of staphylococci. The reasons for these differences between fermenter and shake-flask were investigated, particular attention being given to pH, aeration and antifoam.

Changes in the environment caused changes in the growth patterns of staphylococci as shown by alterations in the specific growth rate, the duration of the transition period from exponential to stationary phases of growth and the final cell yield. Attempts were made to determine how far the changes in the final yields of enterotoxin were due to effects on the specific rates of enterotoxin production, and which differences were consequences of altered growth patterns.
The optimum pH for enterotoxin A, B and C production in casein hydrolysate and amino acid media was determined. Growth of staphylococci under conditions of controlled pH resulted in a higher final yield of enterotoxin than growth without pH control. The implications of the effect of pH on enterotoxin A production in food is discussed.

The repression of enterotoxins when glucose or glycerol were added to the growth medium was studied under controlled conditions of pH, and with constantly maintained glucose or glycerol. Since enterotoxins A, B and C were repressed by both compounds in growing cells when the pH was held constant, repression was not due to the fall in pH which occurred when the pH was not controlled. The repression of enterotoxin by glucose or glycerol was always accompanied by an increase in growth rate. It has been suggested by other workers that there is an inverse relationship between growth rate and extracellular protein production (Coleman, 1967, Stormonth and Coleman, 1973). However, when *S. aureus* S-6 was grown in continuous culture, it was found that an increase in growth rate caused an increase in enterotoxin production. Also the addition of glucose to the medium repressed enterotoxin production when the growth rate was held constant.

Although glucose and glycerol repressed enterotoxin production by growing cells, neither compound inhibited enterotoxin production by non-replicating cells which had been grown without glucose. However, experiments with strain S-6 showed that cells grown in the presence of glucose and resuspended without glucose lacked the ability to produce enterotoxin B. This suggested that glucose inhibited the production of an essential pre-requisite for the synthesis or release of enterotoxin, such as an enzyme for the final conversion of an enterotoxin precursor to enterotoxin. Experiments with chloramphenicol had shown that there was no appreciable build-up of protein precursor for enterotoxin in non-replicating cells. However, very small accumulations of a precursor could repress enterotoxin synthesis by a feedback mechanism.
There were often marked differences in the effect of environmental changes on enterotoxin production by different strains of the same enterotoxin type. One of the aims of this investigation was to examine differences which have been reported to exist between mechanisms of production of the various enterotoxins, particularly enterotoxins A and B. The differences between strains were often greater than supposed differences between enterotoxins, and throughout this investigation several strains were used for each experiment wherever possible.

Staphylococci produce a large number of extracellular proteins. Throughout this study the production of lipase, deoxyribonuclease, lysozyme and total extracellular protein was followed to determine the extent to which the effects of environmental changes were specific to enterotoxin as distinct from a general effect on extracellular protein production.
ACKNOWLEDGEMENTS

I am grateful to Dr W.A. McGillivray, Director of the Dairy Research Institute for the opportunity to undertake this research.

I am also grateful to my supervisors, Drs G.G. Pritchard and R.C. Lawrence for their advice and encouragement throughout this investigation.

I am greatly indebted to Dr M.S. Bergdoll, University of Wisconsin, for supplying strains 100, S-6 and 361, and for the gift of enterotoxins A, B and C, and their corresponding antisera, also to Dr R.W. Bennett, for supplying strain 743.

My thanks are due to Mrs B. Hodren for excellent technical assistance, and to Dr Lawrence and Mrs Hodren for lipase, deoxyribonuclease and lysozyme assays. I would like to thank Dr K. Pearce for Mg^{++} determinations, and Dr K.R. Marshall for helpful discussion concerning the continuous culture experiments. I would also like to express my appreciation of the patience shown by my family during this study.

Part of the findings from Section 3 have been published in Infection and Immunity, 1973. 7: 874-854, under the title 'Production of staphylococcal enterotoxins A, B and C under conditions of controlled pH and aeration', by Audrey W. Jarvis, R.C. Lawrence and G.G. Pritchard.
## CONTENTS

1 INTRODUCTION  
1.1 Staphylococcal enterotoxins  
1.2 Incidence of enterotoxins  
1.3 Differences between enterotoxins  
1.4 Factors affecting the production of staphylococcal enterotoxins  
1.4.1 Medium composition  
1.4.2 pH  
1.4.3 Aeration  
1.4.4 Temperature  
1.5 Effect of glucose on enterotoxin production  
1.6 The control of extracellular protein production  
1.7 Aims of this investigation  

2 GENERAL CHARACTERISTICS OF ENTEROTOXIN A, B, C AND D PRODUCTION BY GROWING AND RESTING CELLS OF STAPHYLOCOCCI  
2.1 Introduction  
2.2 Materials and methods  
2.2.1 Staphylococcal strains  
2.2.2 Media  
2.2.3 Measurement of growth  
2.2.4 Cultural conditions  
2.2.5 Non-replicating cells  
2.2.6 Assays of extracellular proteins  
2.2.7 Specific rate of product formation in batch cultures  
2.3 Results  
2.3.1 Production of enterotoxins in shake-flasks  
2.3.2 Effect of temperature on growth and production of extracellular proteins
2.3.3 Comparison of CH and AA medium
2.3.4 Effect of arginine concentration in AA medium
2.3.5 Effect of K\(^+\) and NH\(_4\)\(^+\) ions in AA medium
2.3.6 Production of enterotoxins by non-replicating cells

2.4 Discussion
2.4.1 Relation between growth and enterotoxin production
2.4.2 Effect of environmental factors on enterotoxin production

3 PRODUCTION OF ENTEROTOXINS A, B AND C UNDER CONTROLLED CONDITIONS OF pH AND AERATION IN SYNTHETIC AND DEFINED MEDIA
3.1 Introduction
3.2 Materials and methods
3.2.1 Design and operation of fermenter

3.3 Results
3.3.1 Comparison of shaker-grown and fermenter-grown cultures
3.3.2 Effect of antifoam
3.3.3 Effect of increased aeration in shake-flasks
3.3.4 Effect of aeration on enterotoxin production in the fermenter
3.3.5 Effect of additions of HCl to the fermenter
3.3.6 Effect of pH on the production of enterotoxins and other extracellular proteins in CH medium
3.3.7 Effect of pH on the production of enterotoxins and other extracellular proteins in AA medium
3.4 Discussion

3.4.1 Differences between strains in response to conditions of growth
3.4.2 Differences in enterotoxin production in the fermenter and in shake-flasks
3.4.3 Effect of aeration on enterotoxin production
3.4.4 Effect of pH on enterotoxin production

4 EFFECT OF GLUCOSE AND GLYCEROL ON THE PRODUCTION OF ENTEROTOXINS AND OTHER EXTRACELLULAR PROTEINS

4.1 Introduction
4.2 Materials and methods
4.2.1 Strains
4.2.2 Media
4.2.3 Cultural conditions
4.2.4 Measurement of glucose concentration
4.2.5 Control of glucose and glycerol concentrations
4.2.6 Measurement of growth
4.2.7 Determination of extracellular proteins
4.2.8 Determination of β-galactosidase
4.2.9 Non-replicating cells

4.3 Results
4.3.1 Effect of glucose on enterotoxin B production in shake-flasks
4.3.2 Effect of added carbohydrates and related compounds on the production of enterotoxins and other extracellular proteins
4.3.3 Effect of glucose on enterotoxin and TEP production in the fermenter
4.3.4 Effect of constantly maintained 0.1 M glucose and 0.1 M glycerol on the production of extracellular proteins in the fermenter

4.3.5 Effect of adding glucose during exponential growth on the production of enterotoxin

4.3.6 Effect of omitting thiamine from AA medium

4.3.7 Effect of glucose on enterotoxin production under conditions of reduced Mg ++

4.3.8 Effect of glucose and glycerol on β-galactosidase and extracellular protein production by non-replicating cells of staphylococci

4.3.9 Effect of chloramphenicol and actinomycin D on the production of enterotoxin B and β-galactosidase by non-replicating cells

4.3.10 Production of enterotoxin B by non-replicating cells of S.aureus S-6 grown in the presence of glucose

4.3.11 Effect of cyclic adenosine 3',5' monophosphate (cAMP) on enterotoxin B production

4.4 Discussion

4.4.1 The production of enterotoxins at constant pH in the presence of 0.1 M glucose or 0.1 M glycerol

4.4.2 Glucose repression of enterotoxin in relation to the synthesis of other extracellular proteins

4.4.3 Relation of the change in the specific growth rate in the presence of glucose or glycerol to the repression of enterotoxin production

4.4.4 Effect of glucose on the production of enterotoxins under conditions of reduced Mg ++
5.4.5 Production of enterotoxins and 
β-galactosidase by non-replicating 
cells of staphylococci

5 THE PRODUCTION OF STAPHYLOCOCCAL ENTEROTOXIN BY BY 
S. AUREUS S-6 IN CONTINUOUS CULTURE: THE EFFECT OF 
GROWTH RATE AND GLUCOSE ON ENTEROTOXIN PRODUCTION

5.1 Introduction 114
5.2 Materials and methods 115

5.2.1 Strains 115
5.2.2 Medium and cultural conditions 116
5.2.3 Control of glucose concentration 117
5.2.4 Measurement of growth 117
5.2.5 Determinations of extracellular 
proteins 118
5.2.6 Specific rate of product formation in 
continuous culture 118
5.2.7 Mg++ determinations 119

5.3 Results 119

5.3.1 The effect of growth rate on the 
production of enterotoxin B and other 
extracellular protein 119
5.3.2 Effect of glucose on growth in con­ 
tinuous culture 124
5.3.3 Effect of glucose on the production 
of enterotoxin B and other extra­ 
cellular proteins 128

5.4 Discussion 131

5.4.1 Effect of glucose on the production 
of enterotoxin B and other extra­ 
cellular proteins at constant growth 
rate 131
5.4.2 Relation between growth rate and the 
production of enterotoxin B and other 
extracellular proteins 132
6 GENERAL DISCUSSION

6.1 General conclusions
6.2 Strain variation
6.3 Relation between enterotoxin production and growth
6.4 Enterotoxin production relative to the synthesis of other extracellular proteins in staphylococci
6.5 Glucose repression of enterotoxin production

7 REFERENCES
FIGURES

1. Relation between cell concentration and OD at 600 nm. 100 per cent concentration corresponds to 0.25 mg/ml dry weight of cells. 17
2. Standard curve for enterotoxin A determination. 20
3. Standard curve for deoxyribonuclease determination. 22
4. Standard curve for lipase determination. 23
5. Standard curve for lysozyme determination. 24
6. Standard curve for TEP determination. 26
7. Growth and enterotoxin A production by strain 100 in a shake-flask in CH medium. OD, Δ; enterotoxin A, O; pH, □. 28
8. Growth and enterotoxin B production by strain S-6 in a shake-flask in CH medium. OD, Δ; enterotoxin B, O; pH, □. 29
9. Growth and enterotoxin C production by strain 361 in a shake-flask in CH medium. OD, Δ; enterotoxin C, O; pH, □. 31
10. Oxygen utilization by strain 10 during 24 h incubation in a shake-flask. 32
11. Diagram of fermenter. A, alkali reservoir; B, acid reservoir; C, oxygen sensor; D, acid inlet; E, alkali inlet; F, water-cooled stirrer gland; G, stirrer; H, KCl electrode; I, glass electrode; J, sampling port; K, thermometer; L, air inlet; M, 2 litre glass fermenter. 47
12. Growth and enterotoxin B production by strain S-6 in a shake-flask (open symbols) and a fermenter (closed symbols) in CH medium. OD, Δ; enterotoxin B, O; pH, □. 51
13. Growth and enterotoxin C production by strain 361 in a shake-flask (open symbols) and a fermenter (closed symbols) in CH medium. OD, Δ; enterotoxin C, O; pH, □. 52
14. Growth and enterotoxin A production by strain 100 in a fermenter in CH medium at uncontrolled pH (open symbols) and at pH 6.5 (closed symbols). OD, Δ; enterotoxin A, O; pH, □. 61
15. Growth and enterotoxin B production by strain S-6 in a fermenter in CH medium at uncontrolled pH (open symbols) and pH 6.5 (closed symbols). OD, Δ; enterotoxin B, 0; pH, □.

16. Growth and enterotoxin A production by strain 100 in a fermenter in CH medium (open symbols) and AA medium (closed symbols). OD, Δ; enterotoxin A, 0; pH, □.

17. Effect of air and N₂ on OD of cells of strain 100 in a fermenter in AA medium when antifoam added (O) and in the absence of antifoam (Δ).


19. Growth and enterotoxin B production by strain S-6 in the fermenter in AA medium (open symbols), and in AA medium containing an initial concentration of 2% glucose (closed symbols). OD, Δ; enterotoxin B, 0; glucose, □.

20. Growth and enterotoxin B production by strain S-6 in AA medium (open symbols) and in AA medium with glucose concentration maintained at 0.1 M (closed symbols). OD, Δ; enterotoxin B, 0.

21. Specific growth rate (μ)Δ; increase in OD, 0; and production of enterotoxin B, □; by strain S-6 during 12 h incubation in AA medium in shake-flasks with different initial Mg²⁺ concentrations.

22. Growth and enterotoxin B production in AA medium containing a reduced concentration of Mg²⁺ (0.2 mM) without glucose (open symbols) and with glucose maintained at 0.1 M (closed symbols). OD, Δ; enterotoxin B, 0.

23. Effect of 0.1 M glucose on enterotoxin B and β-galactosidase production by non-replicating cells of strain S-6 without glucose (open symbols) and with 0.1 M glucose (closed symbols). β-galactosidase, Δ; enterotoxin B, 0.

24. Effect of 100 μg/ml chloramphenicol and 10 μg/ml actinomycin D on enterotoxin B production by non-replicating cells of strain S-6. control, Δ; control + 10 μg/ml actinomycin D, 0; control + 100 μg/ml chloramphenicol, □.
25. Effect of c-AMP on glucose repression of β-galactosidase production by E. coli. control Δ; control + 0.1 M glucose, 0; control + 0.1 M glucose + 5 x 10^{-3} M cAMP, ".

26. Effect of dilution rate on the specific rate of formation of the following proteins by strain S-6; enterotoxin B, Δ; TEP (x 10), 0; lipase, □; deoxyribonuclease, (△); lysozyme (x 100), 0.

27. Effect of increasing Mg^{2+} in incoming medium from 0.2 mM (○) to 0.4 mM (△) on OD in continuous culture of strain S-6.

28. OD, enterotoxin B and TEP production by strain S-6 in continuous culture, dilution rate 0.24 h^{-1}, residence time 4.2 h, Mg^{2+} in incoming medium 0.2 mM; without glucose (open symbols) and with 0.1 M glucose (closed symbols). OD, Δ; enterotoxin B, 0; TEP, □.

29. OD, enterotoxin B and TEP production by strain S-6 in continuous culture, dilution rate 0.07 h^{-1}, residence time 14.3 h, Mg^{2+} in incoming medium 0.2 mM; without glucose (open symbols) with 0.1 M glucose (closed symbols), OD, Δ; enterotoxin B, 0; TEP, □.

30. OD, enterotoxin B and TEP production by strain S-6 in continuous culture, dilution rate 0.24 h^{-1}, residence time 4.2 h, Mg^{2+} in incoming medium 0.4 mM; without glucose (open symbols), with 0.1 M glucose (closed symbols) OD, Δ; enterotoxin B, 0; TEP, □.
TABLES

1. The effect of temperature on the production of enterotoxin and other extracellular proteins by strains 100, S-6, 361 and 485 in CH medium in shake-flasks during 24 h incubation

2. Production of enterotoxins A, B and C in CH and AA media in shake-flasks during 24 h

3. Effect of K⁺ ions on the production of enterotoxin B by strain S-6 during 12 h incubation with and without (NH₄)₂SO₄, at initial pH values of 6.5 and 7.7

4. Enterotoxin B and TEP production by non-replicating cells of strain S-6 from stationary phase of growth during 4 h incubation

5. Production of enterotoxins in N-medium and N-free medium, with and without chloramphenicol (Cm) by non-replicating cells during 4 h incubation

6. Production of enterotoxins and TEP in shake-flask and fermenter in CH medium initial pH 7.3

7. Effect of silicone antifoam on the production of extracellular proteins by nine strains of staphylococci in CH medium in shake-flasks during 24 h incubation

8A. Effect of aeration on production of enterotoxins and TEP in CH medium in shake-flasks during 24 h incubation

8B. Effect of aeration on production of lipase, deoxyribonuclease and lysozyme in CH medium in shake-flasks during 24 h incubation

9. Effect of dissolved oxygen concentration on enterotoxin and TEP production in the fermenter

10. Effect of pH on the production of enterotoxins A, B and C and other extracellular proteins in CH medium

11. Effect of pH on the productions of enterotoxins A, B and C and other extracellular proteins in AA medium
12. Effect of 2% glucose on enterotoxin B and TEP production by six strains of *S. aureus* in shake-flasks in AA medium, initial pH 6.5 during 24 h incubation

13A. Effect of 0.1 M glucose, glycerol and pyruvate on the specific rate of formation (q_p) of enterotoxins by five strains of staphylococci in shake-flasks in AA medium during 24 h incubation

13B. Effect of 0.1 M glucose, glycerol and pyruvate on the specific rate of formation (q_p) of extracellular proteins by five strains of staphylococci in shake-flasks in AA medium during 24 h incubation

14A. Effect of 0.1 M galactose and lactose on the production of enterotoxins and other extracellular proteins by five strains of staphylococci in AA medium in shake-flasks during 24 h

14B. Effect of 0.1 M galactose and lactose on the production of enterotoxins and other extracellular proteins by five strains of staphylococci in AA medium in shake-flasks during 24 h

15. Effect of an initial concentration of 2% glucose on the production of enterotoxins and TEP by strains 100, S-6, 30 and 361 in the fermenter at pH 6.5, during 12 h incubation

16. Effect of 0.1 M glucose and 0.1 M glycerol on the production of enterotoxins A, B and C and other extracellular proteins by four strains of staphylococci during 12 h incubation at pH 6.5 in the fermenter

17. Effect of 0.1 M glucose on the production of enterotoxins and other extracellular proteins under conditions of reduced Mg^{++}, during 12 h incubation

18. Effect of 0.2 M and 0.4 M phosphate buffer pH 7.0 on pH changes in AA medium produced by non-replicating cells of *S. aureus* S-6 in the presence and absence of glucose
19. Effect of phosphate buffer pH 7.0 and 1% galactose on the production of extracellular proteins by non-replicating cells of S.aureus S-6 during 4 h incubation

20. Effect of 0.1 M glucose and glycerol on the production of enterotoxins A, B and C and other extracellular proteins, and of β-galactosidase, by non-replicating cells of staphylococci during 2 h incubation

21A. Effect of dilution rate on cell concentration and production of enterotoxin B by S.aureus S-6

21B. Effect of dilution rate on the production of TEP, lipase, deoxyribonuclease and lysozyme by S.aureus S-6

22A. Effect of 0.1 M glucose on the production of enterotoxin B and TEP by S.aureus S-6

22B. Effect of 0.1 M glucose on the production of lipase, deoxyribonuclease, lysozyme and β-hemolysin by S.aureus S-6.
1 INTRODUCTION

1.1 Staphylococcal enterotoxins

In common with other gram-positive organisms, staphylococci produce numerous extracellular proteins, including such products as hemolysins, coagulase, staphylokinase and leucocidins. Among the toxins produced are the staphylococcal enterotoxins, which when ingested are responsible for staphylococcal food-poisoning. Cases of food-poisoning due to staphylococcal enterotoxins have been reported in the literature for many years and a number of outbreaks have been traced to dairy products (Hobbs, 1955, Häusler et al., 1960, Epsom, 1964). Staphylococcal enterotoxins are the most common cause of food-poisoning in the USA (World Health Organization, 1969) and a frequent cause of food poisoning in Britain (Simkovicova and Gilbert, 1971). The enterotoxins are extracellular proteins, and attempts to demonstrate enterotoxin within washed cells have not been successful (Sugiymana et al., 1960.). Forsgren et al. (1972) failed to detect cell-bound enterotoxin, and it appears that earlier reports of cell-bound enterotoxin (Friedman and White, 1965, Stark and Middaugh, 1969, Morse and Baldwin, 1971) may have been due to the presence of protein A.

Enterotoxins consist of a series of proteins, which are differentiated on the basis of their different reactions with specific antibodies. In 1963 an alphabetical system of nomenclature was established (Casman et al., 1963) and to date five enterotoxins have been identified: A (Casman, 1960), B (Bergdoll et al., 1959), C (Bergdoll et al., 1965), D (Casman et al., 1967) and E (Bergdoll et al., 1971). Two different enterotoxin C's from different staphylococcal strains have been purified, and classified as C₁ (strain 137) and C₂ (strain 361) on the basis of their isoelectric points (Borja and Bergdoll, 1967, Avena and Bergdoll, 1967).

The enterotoxins are single-chain polypeptides, M.W. 30,000-34,000. They contain approximately 300 amino acid residues, with relatively large amounts of lysine, aspartic acid, glutamic acid and tyrosine.
1.2 Incidence of enterotoxins

The percentage of staphylococcal strains which produce enterotoxins has been reported as at least 50% (Casman et al., 1967, Bergdoll, 1972). However, the quantities of enterotoxin produced in cultures may be extremely low, and with some strains it may be necessary to concentrate the culture supernatant to detect enterotoxin. It is also possible that many strains of staphylococci produce enterotoxins that have not yet been identified and purified, and for which there is therefore no antiserum available.

It has generally been accepted that only coagulase-positive staphylococci produce enterotoxins (Evans and Niven, 1950). However, there have been a number of reports of the isolation of coagulase-negative strains which are enterotoxigenic (Omori and Kato, 1959, Bergdoll et al., 1967). Attempts have been made to relate enterotoxigenicity to the ability to produce other extracellular proteins, but no consistent correlations have been found (Thatcher and Simon, 1956, Chesbro and Auborn, 1967, Jarvis and Lawrence, 1971). Dornbusch and Hallander (1973) reported that the factors for the production of enterotoxin B and β-hemolysin, and for methicillin and cephalothin resistance, were eliminated and transduced together. They postulated that the genetic determinants for these characters were on one plasmid.

Food poisoning incidents are most commonly due to enterotoxins A or D (Casman et al., 1967, Gilbert et al., 1972, Jarvis and Harding, 1972). On the other hand, enterotoxin B has more commonly been associated with enteritis or clinical lesions. Hallander and Korlöf (1967) reported that 36% of staphylococcal strains isolated from clinical lesions produced enterotoxin B, as compared with 4% producing enterotoxin A. Jarvis and Lawrence (1970) also found that more clinical strains produced enterotoxin B than enterotoxin A. Casman et al., (1967), however, found that of 385 clinical strains, 28% produced enterotoxin A, as compared with 19% producing enterotoxin B. There is general agreement (Casman et al., 1967, Olson et al., 1970, Jarvis and Lawrence, 1970, 1971) that staphylococci of bovine origin rarely produce
enterotoxins A or B. However, 15% of bovine strains have been reported to produce enterotoxins C or D, or both C and D, (Olson et al., 1970).

1.3 Differences between enterotoxins

In addition to differences in the incidence of the production of the various enterotoxins, enterotoxins A, B and C differ markedly in the quantities in which each is generally produced. Enterotoxin A is usually produced at 5-10 µg/ml, (Bergdoll, 1970). Enterotoxin B is produced in much larger quantities, frequently 200-300 µg/ml, although there is considerable variation between strains, and strains producing less enterotoxin B have been reported (Hallander and Korlóf, 1967). Enterotoxin C is generally produced in quantities intermediate between A and B (Bergdoll, 1970). The isoelectric points differ for the enterotoxins, being 6.8 for enterotoxin A (Chu et al., 1966), 8.6 for enterotoxin B (Bergdoll, et al., 1965), 8.6 for enterotoxin C1, (Borja and Bergdoll, 1967), and 7.0 for enterotoxin C2 (Avena and Bergdoll, 1967). Their molecular weights are reported as 34,700 (enterotoxin A), 28,366 (enterotoxin B), 34,100 (enterotoxin C1) and 34,000 (enterotoxin C2).

The marked differences in incidence of the various enterotoxins have prompted several studies on possible underlying reasons for these differences. Markus and Silverman (1969, 1970) reported that the mechanisms of production of enterotoxins A and B differed in that enterotoxin A is a primary metabolite, and B is a secondary metabolite. (A primary metabolite has been defined as one which is produced in the exponential phase of growth, while a secondary metabolite is synthesised by cells that have stopped dividing (Weinberg, 1970)). The conclusions of Markus and Silverman were based on their work using one strain for enterotoxin A production, and one strain for enterotoxin B production. They found that enterotoxin A production by strain 100 occurred in the exponential phase of growth, while enterotoxin B production by strain S-6 occurred in the stationary phase. Enterotoxin A was shown to be produced by non-replicating cells only when harvested from the exponential phase of growth. Enterotoxin
B production was shown to occur in non-replicating cells harvested in the stationary phase of growth, but not those harvested in the exponential phase. From experiments in which chloramphenicol was used to inhibit protein synthesis, Markus and Silverman (1969, 1970) concluded that enterotoxin A production required new protein synthesis, but that enterotoxin B production occurred in the absence of new protein synthesis. These findings of Markus and Silverman suggested a fundamental difference in the mechanism of synthesis of enterotoxins A and B.

To account for the difference in the incidence of enterotoxins A and B in food poisoning, Troller (1971, 1972) investigated the effect of water activity on the production of enterotoxins A and B. Using one strain to investigate each enterotoxin, Troller found that reduction in water activity of the medium caused a much greater decrease in enterotoxin B production by strain C-243, than in enterotoxin A production by strain 196 E. From these results, he postulated that the lower incidence of enterotoxin B in food-poisoning outbreaks may be due to a greater sensitivity of enterotoxin B-producing strains to reductions in water activity levels.

It has been reported (Czop and Bergdoll, 1970), that enterotoxin A and B differ in their production by L-forms of staphylococci. Only L-forms from enterotoxin A-producing strains produced enterotoxin. It has been suggested that enterotoxin B production is related to the cell surface, whereas enterotoxin A synthesis is not (Bergdoll, 1970). Friedman (1968) showed that enterotoxin B formation by strain S-6 was inhibited by a number of cell wall blocking agents, and postulated that the cell surface may contain sites important to the synthesis of enterotoxin B.

1.4 Factors affecting the production of staphylococcal enterotoxins

1.4.1 Medium composition

The production of enterotoxins has in general been carried out in casein hydrolysate (CH) media, supplemented with
mineral salts and vitamins. Enzymatic digests of casein have been shown to be better for toxin production (Bergdoll, 1962) than acid-hydrolysed casein (Casman 1958, Kato et al., 1966). Drye and Mah (1969) investigated the production of enterotoxin B in four different casein hydrolysate media. Protein Hydrolysate Powder (PHP) gave the highest differential rate of enterotoxin synthesis as compared with total protein synthesis. A dialyzable component in PHP produced more enterotoxin than PHP itself. Wu and Bergdoll (1971a) investigated the fractions of PHP responsible for stimulation of enterotoxin B production. They found that the stimulatory effect of PHP on enterotoxin production was due to the presence of peptides in the medium.

Several workers have investigated the production of enterotoxin in defined media (Fildes et al., 1936, Gladstone, 1937, Surgalla, 1947, Sulzer, 1964, Mah et al., 1967, Wu and Bergdoll 1971b, Miller and Fung, 1973). Media used contained a selection of amino acids supplemented with mineral salts, niacin and thiamine. In general, these investigations have been carried out for enterotoxin B production by strain S-6. The medium of Wu and Bergdoll gave the highest enterotoxin B yield (125 µg/ml) of any of the defined media as compared with the usual yields of between 200 and 300 µg/ml in casein hydrolysate media.

1.4.2 pH

Almost all workers who have studied the effect of pH on enterotoxin production, have investigated only the effect of the initial pH of the medium. Reiser and Weiss (1969) investigated the effect of the initial pH of four different media on the production of enterotoxins A, B and C. It was found that an initial pH of 6.8 was optimal for enterotoxins B and C. Enterotoxin A production was less affected by changes in the initial pH, to as low as 5.3. Scheusner et al., (1973) studied the effect of pH on the production of enterotoxins A, B, C and D, using one strain for each enterotoxin. Growth occurred in the pH range between 4.96 and 9.02, and detectable production of all four enterotoxins occurred when the initial pH of the medium was between 5.15 and 9.02.
However, in the casein hydrolysate media generally used for enterotoxin production, the pH of the medium increases during growth to approximately 8.3-8.8. By contrast many foods are buffered in the range 6.0-6.5. Until 1973 no reports of the production of enterotoxin under controlled pH conditions had appeared in the literature. Metzger et al., (1973) studied the production of enterotoxin B by strain S-6 in CH medium in a fermenter. A comparison was made of the yield of enterotoxin B when the pH of the medium was not controlled, and at pH 6.0, 7.0 and 8.0. Maximum enterotoxin yield was found at pH 7.0. Carpenter and Silverman (1973) also grew strain S-6 in a fermenter under controlled pH. However, they found that growth under conditions where the pH was not held constant gave higher enterotoxin yields than growth at controlled pH. Maximum enterotoxin production with pH control occurred at pH 6.5 to 7.0. The production of several extracellular proteins by staphylococci under controlled conditions of pH has been reported by Arvidson et al., (1971) and Arvidson and Holme (1971), but enterotoxins were not included in their investigations. The optimum pH for the production of several enzymes was found to be between 6.5 and 7.5, depending on the enzyme and the strain under investigation.

1.4.3 Aeration

Aerated cultures have been shown to produce more enterotoxin B than static cultures (McLean et al., 1968). Studies on the production of staphylococcal enterotoxins have commonly been carried out in shake-flasks (Kato et al., 1966, McLean et al., 1968, Markus and Silverman, 1969, Reiser and Weiss, 1969). In shake-flasks the degree of aeration is dependent on the speed of shaking, the volume of medium, and the size and shape of flask (Freedman, 1969). Dietrich et al., (1972) investigated the effect of increased aeration on the production of enterotoxin B by five strains by altering the shaking rate. As shaking speed increased enterotoxin production increased up to an optimal speed of between 325 and 375 rpm. At higher speeds, enterotoxin production decreased. It was also found that altering other factors which affect oxygen transfer, such as flask size and the volume of medium, altered enterotoxin production.
In studies of enterotoxin B production in a fermenter, Metzger et al., (1973) maintained a constant air flow of 10 litres per min and a constant stirrer speed. However, even when a constant air flow is maintained, the dissolved oxygen content of the medium will decrease during fermentation, since the rate of consumption of oxygen will increase as the cell numbers increase. To adequately determine the effect of available oxygen on enterotoxin production it would be desirable to monitor the concentration of dissolved oxygen in the medium, and maintain a constant oxygen concentration throughout growth.

1.4.4 Temperature

In recent years a number of reports have appeared in the literature concerning the effect of temperature on enterotoxin B production, although very little work has been reported on the effect of temperature on the production of the other enterotoxins. It is evident that the optimum temperature depends on the medium and strain being used. Incubations for the production of enterotoxins have generally been carried out at 35-37°C. Bergdoll (1970) reported that enterotoxins can be produced at 25 and 30°C, but in lesser amounts than at 37°C. McLean et al., (1968) reported that detectable enterotoxin B was produced at 16°C in brain heart infusion medium (BHI) during prolonged incubation. Tatini et al., (1971a) found that in BHI the optimum temperature for the production of enterotoxins A, B, C and D was 45°C. By contrast, Dietrich et al., (1972) found the optimum temperature for enterotoxin B production by strain S-6 in a casein hydrolysate medium was 37°C and reported that there was neither growth nor toxin production at 45°C. Vandenbosch et al., (1973) using four different PHP media, found 40°C was the optimum temperature for enterotoxin B production by strains S-6 and 137.

1.5 Effect of glucose on enterotoxin production

The production of enterotoxin B by S. aureus S-6 has been reported to be subject to repression by glucose (Morse et al., 1969, Markus and Silverman, 1969, Morse and Baldwin, 1971,
Burst et al., 1973, Morse and Mah, 1973). The pH in these studies was not strictly controlled, and there was a considerable decrease in the pH of the medium due to glucose metabolism. Morse et al., (1969) found that the addition of glucose to a culture actively synthesising enterotoxin immediately repressed enterotoxin production without immediately affecting pH. In contrast, Metzger et al., (1973) postulated that pH rather than glucose was the limiting factor in enterotoxin B production. This conclusion was based on work with strain S-6, in which 0.2% glucose was added to a casein hydrolysate medium. However, the concentration of glucose was low, and the pH had begun to increase by the time at which enterotoxin B is usually first detected in the medium. This suggests that the glucose had been completely depleted before any significant enterotoxin production occurred. Miller and Fung (1973) used the completely defined 18 amino-acid medium devised by Wu and Bergdoll (1971b) and found that glucose repressed enterotoxin B production by strain S-6. However, the pH was not controlled and the concentration of glucose in the medium did not remain constant, decreasing from 1% to 0.06% during the experiment. They reported that repression was much less pronounced in the defined medium than that obtained with the complex medium of Morse et al., (1969). As the defined medium was increasingly simplified by the deletion of amino-acids, repression of enterotoxin B production by glucose correspondingly decreased.

It has generally been concluded that the inhibition of enterotoxin production by glucose is due to catabolite repression. Catabolite repression has been defined as a reduction in the rate of synthesis of certain enzymes, particularly those of degradative metabolism, in the presence of glucose or other rapidly metabolized carbon sources (Paigen and Williams, 1970). Transient repression has been defined as a period of more intense repression which occurs immediately after cells are exposed to glucose, and which may last for up to one generation time (Paigen and Williams, 1970). The production of α-toxin by staphylococci has been reported as subject to transient repression (Duncan and Cho, 1972). However, the concentration of glucose used by these workers was extremely low, and it is probable that glucose
had been depleted from the medium by the time the cells showed an apparent recovery from transient repression.

In general, carbon sources which support a rapid rate of growth are most effective in producing catabolite repression (Neidhardt and Magasanik, 1956, Neidhardt, 1960, Magasanik, 1961, Mandelstam, 1962, Gallo and Katz, 1973). Substrates which feed into the early reactions of glycolysis have been found to be the best repressors of enzyme synthesis (Okinaka and Dobrogosz, 1966, Hsie and Rickenberg, 1967). It is generally believed that both catabolite and transient repression are due to one or more intermediates in the metabolism of the carbon source exerting repression. Perlman and Pastan (1968) showed that glucose repression of β-galactosidase synthesis in E. coli was overcome by the addition of cyclic adenosine 3′5′-monophosphate (c-AMP) to the medium. De Crombugghe et al. (1969) showed that this was also true for a number of inducible enzymes in other organisms. A number of enzymes produced by staphylococci have been shown to be sensitive to catabolite repression including formimino-glutamic hydrolase and glutamic dehydrogenase, and several enzymes of the tricarboxylic acid cycle (Strasters and Winkler, 1963). However, it is not known whether the mechanism for catabolite repression in staphylococci is similar to that in E. coli.

Two factors which have been implicated in the mechanism of glucose repression of enterotoxin are thiamine, and the presence of a functional electron transport system. Morse and Baldwin (1971) studied the effect of glucose and pyruvate on enterotoxin B production at pH 6.0 and 7.7. It was shown that the elimination of thiamine from the medium prevented the oxidative decarboxylation of pyruvate, and partially or completely reversed the repression of enterotoxin synthesis by glucose or pyruvate. Morse and Mah (1973) found that a change from aerobic to anaerobic conditions during growth in glucose medium also reversed glucose repression of enterotoxin. It was postulated that the more complete oxidation of glucose in the presence of oxygen resulted in a higher energy yield per molecule of glucose, and that this increase in available energy was related to glucose repression. This was supported
in a subsequent paper by Morse and Baldwin (1973) in which they reported the effect of glucose on enterotoxin B production by *S. aureus* S-6 and a heme-requiring mutant, *S. aureus* S-6 H2. Strain S-6 H2 lacked a functional electron transport system unless the medium was supplemented with hemin. The repression of enterotoxin synthesis by glucose (at pH 6.0 to 7.7) or by pyruvate (at pH 7.7) occurred in the absence of a functional electron transport system, but was significantly enhanced in its presence. It was postulated that a functional electron transport system was involved in regulating the degree of glucose and pyruvate repression of enterotoxin B synthesis.

1.6 The control of extracellular protein production

The staphylococcal enterotoxins form part of a large group of extracellular proteins secreted by staphylococci. Although enterotoxins are not known to have any enzymic properties, the hypotheses which have been put forward to account for the secretion of exo-enzymes are likely to be relevant to the secretion of enterotoxins. May and Elliot (1968) postulated that bacterial extracellular enzymes are extruded through the cell membrane in a nascent form and are finally formed outside the cell membrane. Both et al. (1972) found evidence for a pool of mRNA for extracellular protease synthesis in *Bacillus amyloliquefaciens*. They proposed that mRNA for protease migrates to specific sites on the membrane where the enzyme is synthesised. The existence of a pool of mRNA was subsequently shown for the production of two other extracellular enzymes. Coleman (1967) studied the secretion of three extracellular enzymes by *Bacillus subtilis*. As a result of his investigations, he suggested a possible regulatory mechanism for the observed characteristics of extracellular enzyme secretion. When the rate of increase in cell mass was decreased the rate of enzyme secretion increased, and continued into the stationary phase of growth. Coleman postulated that there was competition for nucleic acid precursors between RNA for cell growth and that for extracellular protein synthesis. As growth rate decreased, ribosome synthesis also decreased. The nucleic acid precursor pool then increased in size, so that mRNA for
extracellular protein production could be formed at the maximum rate. It was suggested that all extracellular enzymes are thus under a common regulatory system in B. subtilis. It has been reported (Markus and Silverman, 1969) that enterotoxin B production by strain S-6 occurs in the stationary phase of growth. If this were a general characteristic of enterotoxin production, a similar control mechanism to that proposed for B. subtilis enzymes could apply.

It was therefore relevant in this study to investigate the effect of growth rate on enterotoxin production.

There have been no reports in the literature of the growth of staphylococci in continuous culture. The use of continuous culture would enable the effect of growth rate to be studied without making changes in the composition of the incoming medium. It would also enable the effect of changes in the medium composition to be studied without any alteration in growth rate taking place.

Staphylococci produce a large number of extracellular proteins. It was of interest to assay some of these in order to determine whether there was any evidence of a common regulatory control mechanism for the production of extracellular proteins in staphylococci. Lipase, deoxyribonuclease, lysozyme and total extracellular protein (TEP) were selected as being readily assayed in culture supernatants, and their production was followed in parallel to enterotoxin production throughout growth in most experiments.

1.7 Aims of this investigation

The aims of this investigation were four-fold:

(i) To study the characteristics of enterotoxin production throughout the growth cycle, with particular reference to differences between enterotoxins A, B and C. In order to distinguish between enterotoxin differences and variation between strains producing the same enterotoxin type, several strains were used for the production of each enterotoxin.
(ii) To examine the production of enterotoxins in a controlled environment, where pH, aeration and temperature were held constant, and to determine the effect of these parameters on enterotoxin production.

(iii) To study the effect of glucose and related compounds on enterotoxin production in a controlled environment, and independently of changes in growth rate.

(iv) To determine by the study of the production of other extracellular proteins, the extent to which the effects of environmental changes were specific to enterotoxin production only, or were more general effects on extracellular protein production.
2. GENERAL CHARACTERISTICS OF ENTEROTOXIN A, B, C AND D PRODUCTION BY GROWING AND RESTING CELLS OF STAPHYLOCOCCI.

2.1 Introduction

The relation between enterotoxin production by staphylococci and growth of these organisms is of fundamental importance in understanding the control of enterotoxin synthesis. It has been reported (Markus and Silverman, 1969, 1970) that enterotoxins A and B differ in that enterotoxin A is produced in the exponential phase of growth, while enterotoxin B is produced during the stationary phase. Therefore, in the initial stage of this study, experiments were undertaken in which the production of enterotoxins A, B and C by several strains of staphylococci was followed throughout the growth cycle.

Markus and Silverman also found evidence of a pool of precursor for enterotoxin B, but considered no such precursor was involved in the production of enterotoxin A. In order to determine whether the presence or absence of a precursor did in fact represent a difference between types of enterotoxin, the production of enterotoxins A, B, C and D by non-replicating cells was investigated. To separate differences in the production of the various enterotoxins from differences due to variation between strains, several strains producing each of the enterotoxins were used.

The effect of some environmental factors on the production of enterotoxins was also investigated. A comparison was made between enterotoxin production in a casein hydrolysate medium (Hallander, 1965) and a defined amino-acid medium (Wu and Bergdoll, 1971b) preparatory to work in a fermenter in a controlled environment (Section 3).

The production of lipase, deoxyribonuclease, lysozyme and total extracellular protein (TEP) was also studied to determine whether the effect of different conditions on enterotoxin production was related specifically to enterotoxin, or was a more general effect on the production of all extracellular proteins.
2.2 Materials and Methods

2.2.1 Staphylococcal Strains

Strains 100, S-6 and 361 were kindly supplied by M.S. Bergdoll (University of Wisconsin, Madison, Wis.), and are widely used for the production of enterotoxins A, B and C respectively. Strain S-6 also produces small amounts of enterotoxin A. Strains 10, 22, 28, 29 and 30 are from the International Phage Typing Set, strain 30 being reported in the literature as P5 53 (McClatchy and Rosenblum 1963). Strain 743 was kindly supplied by R.W. Bennett (FDA, Washington DC). Strains 10 and 743 produce enterotoxin A. Strains 22, 28, 29 and 30 produce enterotoxin B and a small amount of enterotoxin A, and it was hoped that use of these strains would enable comparisons of enterotoxin A and B production to be made within one strain. Strains 3 and 37 are clinical strains which produce large amounts of enterotoxin C.

2.2.2 Media

Cultures were maintained on Trypticase Soy Agar (BBL). Experiments with growing cells were carried out in casein hydrolysate medium as described by Hallander (1965) (referred to hereafter as CH Medium) or the defined amino-acid medium "4 (2.08%)" of Wu and Bergdoll (1971b) (referred to hereafter as AA medium). The composition of these media was as follows:

**CH medium:** Ferric citrate 0.0025 g, K$_2$HPO$_4$ 1.0 g, KH$_2$PO$_4$ 1.0 g, MgSO$_4$ 7H$_2$O 0.2 g, l-cystine 0.025 g, sodium acetate 0.025 g, l-tryptophan 0.075 g, calcium pantothenate 500 µg, thiamine hydrochloride 40 µg, nicotinic acid 1,200 µg, casamino acids (Difco) 20.0 g, protein hydrolysate (BBL trypticase) 20.0 g, distilled water one litre. The pH was adjusted to 7.3 with NaOH unless otherwise specified, and the medium autoclaved at 10 psi for 15 min.

**AA medium:** Amino-acids (L-forms) µg/ml: tryptophan 60, lysine 600, histidine 480, arginine 360, aspartic acid 2,400, threonine 2,400, serine 2,400, glutamic
acid 2,400, proline 2,400, glycine 2,400, alanine 2,400, cystine 240, valine 480, methionine 180, isoleucine 600, leucine 600, tyrosine 180, phenylalanine 200. Other compounds were added as follows: (mg/ml): (NH₄)₂SO₄ 1.0, isodium citrate, 2 H₂O 0.5, MgSO₄.7H₂O 0.1, FeSO₄.7H₂O 0.01, niacin 0.01, thiamine HCl 0.0005. Tryptophan and cystine were dissolved in 0.2 N HCl (50 ml per litre of medium), aspartic acid, glutamic acid and tyrosine were dissolved in 0.2 N NaOH (100 ml per litre of medium), and the remaining amino acids were dissolved in water. Niacin and thiamine-HCl were made up as concentrated stock solutions and stored at 4°C. The pH of the medium was adjusted to 6.5 or other specified pH with NaOH, and the medium autoclaved at 10 lb for 15 min.

2.2.3 Measurement of growth

Growth was followed by measuring the optical density (OD) of an appropriate dilution of culture on an SP 500 Unicam spectrophotometer at 600 nm. It was shown that there was a linear relationship between cell mass and OD, using the method described by Kavanagh (1963). Staphylococcal cells from the exponential phase of growth (4 h) were killed by steaming for 30 min., washed once and resuspended in chilled distilled water. One hundred μg/ml Merthiolate (Eli Lilly Co) was added as a preservative. The cells were allowed to stand for four hours and decanted. A suspension which had an OD of 1.0 at 600 nm was then prepared and this concentration of cells was termed 100%. Dilutions of 80, 60, 40, 20 and 10%
of cells in distilled water were then prepared and OD values read at 600 nm. The resultant curve (Fig. 1) showed that there was a linear relationship between OD and the cell mass from 0.0 to 0.7 OD units at 600 nm. All samples were diluted so that they fell within this range, and read against a medium blank that had been similarly diluted. OD readings were multiplied by the dilution for ease of comparison. Specific growth rate ($\mu$) was calculated as follows:

$$
\mu = \frac{\left(\log_{10} OD_{t_2} - \log_{10} OD_{t_1}\right)}{(t_2 - t_1)} \times 2.303
$$

for the time interval $t_1$ to $t_2$ (Stanier et al., 1971).

2.2.4 Cultural conditions

Shake flasks were incubated on a New Brunswick gyrorotary shaker at a shaking speed of approximately 300 rpm which was kept constant except as specified in the experiments with baffled flasks. Except as specified in the experiments in which temperatures were varied, all incubations were at 37°C.

The pH was recorded on a Radiometer pH meter.

A Beckman oxygen sensor was used to measure the concentration of dissolved oxygen where indicated. The oxygen sensor was calibrated in air so that a reading of 100% on a Beckman oxygen meter was equivalent to a partial pressure of oxygen of 152 mm Hg. The concentration of dissolved oxygen in the medium was then read on the oxygen meter, and was recorded on a Hitachi recorder.

2.2.5 Non-replicating cells

Non-replicating cells were prepared as described by Markus and Silverman (1969). Staphylococci were grown in CH medium (200 ml in 1 litre flask) on the shaker. At the required time cultures were harvested by centrifuging at 10,000 rpm for 15 min, then washed in 0.01 M phosphate buffer, pH 7.0, by resuspending and centrifuging at 10,000 rpm for 5 min. Tests showed that after two washings
Figure 1  Relation between cell concentration and OD at 600 nm.  100 per cent concentration corresponds to 0.25 mg/ml dry weight of cells.
small amounts of enterotoxin could still be detected in supernatants of resuspended cells of strains S-6 and 361. It was found that four washings with buffer for enterotoxin B and C-producing strains and one washing for enterotoxin A-producing strains ensured removal of enterotoxin to below the limit of detection and this procedure was adopted for all non-replicating cell experiments. The cells were resuspended in the appropriate medium so that a dilution of 1 in 200 gave an OD of 0.15–0.20 at 600 nm. An OD of 0.20 in the diluted suspension corresponded to approximately 10 mg/ml dry weight of cells in the undiluted suspension. Fifteen mls of this cell suspension were shaken in 100 ml flask at 37°C for specified times.

2.2.6 Assays of extracellular proteins

Samples were centrifuged at 10,000 rpm for 15 min to remove cells, and 100 µg/ml Merthiolate was added to supernatants which were then stored at 4°C. It was shown that neither the addition of Merthiolate, nor the storage of samples for at least 6 months affected the determination of any of the extracellular proteins which were assayed.

Enterotoxins. Enterotoxins A, B and C were assayed by the single-tube gel-diffusion method (Weirether et al., 1966). A solution of the specific enterotoxin antiserum in 0.02 M phosphate buffer, containing 0.85% NaCl, and 100 µg/ml Merthiolate, was added to 1% Difco Noble Agar solution at 48°C. Aliquots of the mixture (400 µl) were then placed in 4 mm diameter gel-diffusion tubes and allowed to solidify. Using a micropipette the agar was overlaid with 250 µl of a known or unknown enterotoxin solution in phosphate-buffered saline as described above containing an additional 2% NaCl. The pH and ionic strength of the enterotoxin solution have been shown to influence the rate of migration of the enterotoxin antiserum precipitin band (Weirether et al., 1966). All samples to be tested were therefore diluted with phosphate buffered saline at a concentration such that the samples contained the same final concentration of buffer and NaCl as the enterotoxin standards.
The length of each zone of precipitation was measured after the tubes had been incubated at 30°C for 20 h. The logarithms of known concentrations of enterotoxin were plotted against zone length, covering the range of 3 to 25 μg/ml enterotoxin. Samples to be tested were diluted with phosphate-buffered saline of the appropriate concentrations so that they fell within this range. A new standard curve was prepared with each test, a typical standard curve for an enterotoxin A determination being shown in Fig. 2. Enterotoxin B and C standard curves were similar to that shown for enterotoxin A. The solutions of enterotoxins and antisera were stored at -20°C. The same standard solutions of enterotoxin were used for any one set of experiments, and in most instances samples whose enterotoxin concentrations were to be compared with one another were tested at the same time. Under these conditions, the variation between duplicates was not greater than approximately 4%. Enterotoxin D was determined by the Wadsworth (1957) microslide gel-diffusion technique as described by Zehren and Zehren (1968), as antiserum supplies were inadequate for tube gel-diffusion tests. Samples to be tested were diluted 1/2, 1/4, 1/6, 1/8 and 1/10 in 0.85% saline. The concentration of enterotoxin D was recorded as the highest dilution which formed a line of precipitation with enterotoxin D antiserum. The line of precipitation was invariably concurrent with that produced by known enterotoxin D solutions.

Deoxyribonuclease. The estimation of deoxyribonuclease was carried out by a micro-slide gel-diffusion technique developed in this laboratory (Jarvis and Lawrence, 1969). A solution of agar (2% w/v) containing 1 mg/ml calf thymus DNA (Sigma Chemical Co.) was steamed for 15 min and added to an equal volume of 0.1 M Tris-HCl buffer pH 8.7, containing 0.02 M Ca++. One ml of this hot mixture was spread on a microscope slide over an area 2 in x 1 in. A hole 2.7 mm diameter was bored with a steel tube, and 0.004 ml of the deoxyribonuclease preparation added to the well. The slides were incubated in a moist chamber at 37°C for 20 h. The slides were then dipped in 1N-HCl for 15 sec., washed with
Figure 2 Standard curve for enterotoxin A determination.
water and the diameters of the zones of clearing read with vernier calipers. A standard curve was prepared using dilutions of purified staphylococcal deoxyribonuclease, an arbitrary value of 1000 units per ml being assigned to the purified preparation (Fig. 3). Relative enzyme activities in unknown samples were read from the standard curve. Samples from any one set of experiments were always tested together, and a standard deoxyribonuclease preparation was included with each test.

**Lipase.** Lipase activity was estimated by the size of the zone of clearing in tributyrin agar, using a microslide gel-diffusion technique (Lawrence et al., 1967). Five ml of 1.5% tributyrin emulsion were added to a hot solution of 0.5 g Ionagar (Oxoid) in 0.025 M phosphate buffer pH 8.0. This mixture was used to prepare slides and samples were added as for deoxyribonuclease determinations. The slides were incubated in a moist chamber at 37°C for 20 h, and the zones of clearing measured. Relative lipase activities of unknown samples were read from a standard curve prepared with purified micrococcal lipase (Fig. 4). Samples from any one set of experiments were always tested together, and a standard lipase preparation was included with each test.

**Lysozyme.** Lysozyme was determined essentially by the method used by Hawiger (1968). The enzyme preparation (0.1 ml) was added to 4 ml lysozyme substrate (Difco) (OD of 0.80-0.90) which had been previously warmed to 37°C. All OD readings were at 550 nm on an SP 500 spectrophotometer. Lysozyme activity was measured as the percentage decrease in OD after a further 30 min incubation in a 37°C water-bath, and recorded in units relative to the activity of dilutions of egg-white lysozyme (Worthington (EML) by measurement from a standard graph (Fig. 5). The assumption has been made that all staphylococcal lysozymes will have the same specificity in action toward *M. lysodeikticus* as egg-white lysozyme (Richmond, 1959).

**Total extracellular protein (TEP).** For the measurement of TEP a modification was made of the method of Stadtman et al., (1951). Thirty per cent trichloroacetic
Figure 3  Standard curve for deoxyribonuclease determination.
Figure 4  Standard curve for lipase determination.
Figure 5  Standard curve for lysozyme determination.
acid (0.5 ml) was added to a mixture of 1.0 ml supernatant and 2.0 ml 0.85% saline, and the CD at 600 nm read after 7 min. After the addition of TCA the OD increased rapidly, but had stabilized before 7 min. A standard curve was obtained using dilutions of bovine albumin, OD being a linear function of albumin concentration over the range 0.1-1.5 mg/ml (Fig.6).

2.2.7 Specific rate of product formation in batch cultures

The production of an extracellular protein such as enterotoxin could be expressed as a total yield attained when production has ceased, or as the amount produced during a specified time interval. However, changes in environment which resulted in changes in the production of enterotoxin were found to affect also such parameters as growth rate and cell yield. It therefore became necessary to express the rate of enterotoxin production in a way which would be independent of growth rate, cell yield and the length of time for which enterotoxin was being produced. This was done by the determination of the specific rate of product formation per unit of cell mass, for enterotoxin or other extracellular proteins, as follows:

\[ \frac{dp}{dt} = q_p x \]

where \( q_p \) = specific rate of product (p) formation per unit of cell mass (x) at time t (Firt, 1969).

During time interval \( t_0 \) to \( t_1 \)

\[ p = q_p \int_{t_0}^{t} x \, dt + p_0 \]

At time \( t \), \( x = x_0 \, e^{\mu t} \), where \( \mu \) = specific growth rate and \( x_0 \) = initial cell mass

\[ p = q_p \int_{t_0}^{t} x_0 \, e^{\mu t} \, dt + p_0 \]
Figure 6
Standard curve for TEP determination.
\[
\begin{align*}
\dot{p} - p_0 &= q_p x_0 \frac{1}{\mu} \left[ e^{\mu t} - e^{\mu t_0} \right] \\
q_p &= \frac{p - p_0}{x_0 e^{\mu t} - x_0 e^{\mu t_0}} \mu
\end{align*}
\]

i.e., the specific rate of product formation per unit of cell mass = \frac{\text{increase in product}}{\text{increase in cell mass}} \times \text{specific growth rate}. As it had been shown that OD was proportional to cell mass, \( q_p \) for time interval \( t_1 \) to \( t_2 \) was calculated as:

\[
q_p = \frac{p_{t_2} - p_{t_1}}{\text{OD}_{t_2} - \text{OD}_{t_1}} \mu
\]

\( q_p \) units for batch cultures are units of product per unit cell mass per hour, the unit of product being \( \mu g \) for enterotoxins, \( mg \) for TEP, and units as described in Section 2.2.6 for lipase, deoxyribonuclease and lysozyme.

2.3 Results

2.3.1 Production of enterotoxins in shake-flasks

The production of enterotoxin A by strains 100, 10 and 743, enterotoxin B by strains S-6, 22 and 30, and enterotoxin C by strains 361, 3 and 37 was followed throughout growth in 200 ml CH medium in 1 litre shake-flasks. Enterotoxin A production by strain 100 was first detected in the exponential phase when the OD was 2, and continued throughout growth (Fig.7). Enterotoxin A production decreased sharply as growth entered the stationary phase. A similar time course was found for the production of enterotoxin A for strains 10 and 743. However, the final yields of enterotoxin A were 8, 3 and 2 \( \mu g/\)ml for strains 100, 10 and 743 respectively.

Enterotoxin B production by strain S-6 is shown in Fig.8. Enterotoxin was first detected in the exponential phase when the OD was 3.7, and continued increasing throughout growth. There was a marked increase in enterotoxin B production in late exponential and early stationary phase, but it was found that this was not typical of enterotoxin B production. Enterotoxin B production by strains 22 and 30 was first
Figure 7  Growth and enterotoxin A production by strain 100 in a shake-flask in CH medium. OD, Δ; enterotoxin A, ○; pH, □.
Figure 8  Growth and enterotoxin B production by strain S-6 in a shake-flask in CH medium. OD, Δ; enterotoxin B, ○; pH, □.
detected in the exponential phase when OD was 3.4 and 5.0 respectively, and continued throughout growth. However, for these two strains the rate of enterotoxin B production decreased as growth rate decreased and there was no appreciable increase in enterotoxin concentration during the stationary phase. The final concentrations of enterotoxin B produced were 290, 104 and 150 µg/ml for strains 8-6, 22 and 30 respectively.

Enterotoxin C production by strain 361 is shown in Fig. 9. For strains 3 and 37, the time courses for enterotoxin C production were very similar to that for strain 361. Enterotoxin production was first detected in the exponential phase when OD readings were 5.6, 6.0 and 5.6 for strains 361, 3 and 37 respectively. Enterotoxin C production continued throughout growth, and the rate of enterotoxin production decreased as growth rate decreased. No increase in enterotoxin was evident after growth ceased.

Figures 7 to 9 also show the changes in pH that take place in shake-flask cultures. In CH medium the initial pH was 7.3, and the pH at 24 h was between 8.1 and 8.6. Strains 100, S-6, 22 and 361 showed a slight initial decrease in pH.

The changes in the concentration of dissolved oxygen in the medium in a shake-flask culture were studied. A Beckman oxygen sensor was fitted into a flask through a side port and the concentration of dissolved oxygen recorded during 24 h incubation. It was found with strain 10 (Fig. 10) that the oxygen concentration decreased rapidly from 122 to 2 mm Hg during the first 2 hr, and remained at almost zero reading until 19 h. The rate of growth had then decreased markedly, and the concentration of dissolved oxygen in the flask increased to 56 mm Hg by 24 h. An experiment carried out with strain 361 showed similar results.

2.3.2 Effect of temperature on growth and production of extracellular proteins.

Strains 100, S-6 and 485 were grown in 50 ml CH medium in 250 ml shake-flasks for 24 h, at temperatures of 30, 35, 40 and 45 C. The effect of temperature variation on specific
Figure 9  
Growth and enterotoxin C production by strain 361 in a shake-flask in CH medium. OD, Δ; enterotoxin C, ○; pH, □.
Figure 10  Oxygen utilization by strain 10 during 24 h incubation in a shake-flask.
growth rate, and the production of enterotoxins and other extracellular proteins during 24 h incubation is shown in Table 1. There was considerable variation between strains. For all strains the highest final OD occurred at 30 °C, the lowest growth rate and OD occurred at 45 °C. Enterotoxin production was maximum at 30-40, 40, 30-35 and 30 °C for strains 100, S-6, 361 and 485 respectively.

The enterotoxin concentrations are in some instances less than that reported earlier for shake-flasks. This variation is probably due to the differences in aeration and agitation in 50 ml medium in 250 ml flask as compared with 200 ml in 1-l flask used for growth curves. The specific growth rate was highest at 35 and 30 °C for strains 100 and S-6 and 30-40 °C for strains 361 and 485. Optimum temperatures for TEP production were 40 °C for strains 100 and S-6, and 45 and 35 for strains 361 and 485 respectively. Lysozyme production was maximum at 30 °C for all strains and deoxyribonuclease production was maximum at 30 °C for strains 100, 361 and 485, and at 40 °C for strain S-6.

2.3.3 Comparison of CH and AA media

It became evident during experiments in the fermenter (Section 3) that there would be advantages in using a completely defined medium, rather than the casein hydrolysate medium. It was hoped that growth in such a medium might cause less foaming in the fermenter than CH medium. A defined medium was also desirable in view of the anticipated work on the effect of glucose and related compounds on enterotoxin production. Since a defined amino-acid medium, developed for the production of enterotoxin B using strain S-6, had recently been reported in the literature (Wu and Bergdoll, 1971b), a comparison was made of the production of enterotoxins A, B and C in this medium (AA medium) and in the CH medium previously used.

The production of enterotoxin A and TEP by strain 100 was similar in both media (Table 2). The specific growth rate was less and the 24 h OD and pH were greater in CH medium. Strain S-6 produced less enterotoxin and TEP in AA medium, while showing a small increase in growth rate and OD. pH
TABLE 1. The effect of temperature on the production of enterotoxin and other extracellular proteins by strains 100, S-6, 361 and 485 in CH medium in shake-flasks during 24 h incubation.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Temperature</th>
<th>Specific growth rate (μ)</th>
<th>Final OD</th>
<th>Enterotoxin (μg/ml)</th>
<th>TEF (mg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>30 C</td>
<td>0.45</td>
<td>20</td>
<td>7</td>
<td>0.08</td>
<td>90</td>
<td>66</td>
<td>3.7</td>
</tr>
<tr>
<td>100 A</td>
<td>35 C</td>
<td>0.57</td>
<td>20</td>
<td>6</td>
<td>0.05</td>
<td>92</td>
<td>36</td>
<td>2.1</td>
</tr>
<tr>
<td>100 A</td>
<td>40 C</td>
<td>0.55</td>
<td>12</td>
<td>6</td>
<td>0.13</td>
<td>84</td>
<td>38</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>45 C</td>
<td>0.29</td>
<td>6</td>
<td>ND^a</td>
<td>0</td>
<td>44</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>S-6 B</td>
<td>30 C</td>
<td>0.66</td>
<td>18</td>
<td>123</td>
<td>0.90</td>
<td>410</td>
<td>230</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>35 C</td>
<td>0.36</td>
<td>15</td>
<td>152</td>
<td>0.95</td>
<td>180</td>
<td>155</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>40 C</td>
<td>0.37</td>
<td>13</td>
<td>208</td>
<td>1.36</td>
<td>290</td>
<td>270</td>
<td>0.6</td>
</tr>
<tr>
<td>S-6 B</td>
<td>45 C</td>
<td>0.20</td>
<td>12</td>
<td>85</td>
<td>0.48</td>
<td>260</td>
<td>245</td>
<td>0</td>
</tr>
<tr>
<td>361 C</td>
<td>30 C</td>
<td>0.50</td>
<td>28</td>
<td>37</td>
<td>0.37</td>
<td>690</td>
<td>39</td>
<td>&gt;6</td>
</tr>
<tr>
<td></td>
<td>35 C</td>
<td>0.45</td>
<td>26</td>
<td>43</td>
<td>0.30</td>
<td>500</td>
<td>34</td>
<td>&gt;6</td>
</tr>
<tr>
<td></td>
<td>40 C</td>
<td>0.51</td>
<td>28</td>
<td>30</td>
<td>0.56</td>
<td>810</td>
<td>29</td>
<td>5.2</td>
</tr>
<tr>
<td>S-6 B</td>
<td>45 C</td>
<td>0.34</td>
<td>16</td>
<td>29</td>
<td>1.06</td>
<td>510</td>
<td>30</td>
<td>1.4</td>
</tr>
<tr>
<td>361 C</td>
<td>485 D</td>
<td>0.49</td>
<td>28</td>
<td>1/6^b</td>
<td>0.07</td>
<td>23</td>
<td>42</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>485 D</td>
<td>0.45</td>
<td>25</td>
<td>1/2^b</td>
<td>0.10</td>
<td>200</td>
<td>17</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>485 D</td>
<td>0.48</td>
<td>22</td>
<td>1/2^b</td>
<td>0.05</td>
<td>340</td>
<td>17</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>485 D</td>
<td>0.31</td>
<td>17</td>
<td>ND^a</td>
<td>0.08</td>
<td>490</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

^a ND indicates not detectable.
^b Enteroxin D reported as titre showing positive slide gel-diffusion test.
TABLE 2. Production of enterotoxins A, B and C in CH and AA media in shake-flasks during 24 h.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Medium</th>
<th>Specific growth rate (µ)</th>
<th>OD</th>
<th>Enterotoxin (µg/ml)</th>
<th>TEP (µg/ml)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>CH</td>
<td>0.27</td>
<td>21</td>
<td>8</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>100 A</td>
<td>AA</td>
<td>0.49</td>
<td>18</td>
<td>7</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>S-6 B</td>
<td>CH</td>
<td>0.54</td>
<td>15</td>
<td>320</td>
<td>2.10</td>
<td>2.95</td>
</tr>
<tr>
<td>S-6 B</td>
<td>CH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60</td>
<td>14</td>
<td>310</td>
<td>2.10</td>
<td>3.08</td>
</tr>
<tr>
<td>S-6 B</td>
<td>AA</td>
<td>0.63</td>
<td>17</td>
<td>190</td>
<td>1.0</td>
<td>0.66</td>
</tr>
<tr>
<td>361 C</td>
<td>CH</td>
<td>1.23</td>
<td>26</td>
<td>63</td>
<td>0.90</td>
<td>0.75</td>
</tr>
<tr>
<td>361 C</td>
<td>AA</td>
<td>0.59</td>
<td>25</td>
<td>100</td>
<td>1.50</td>
<td>0.64</td>
</tr>
</tbody>
</table>

<sup>a</sup>Initial pH 6.5 as compared with usual pH of 7.3 for CH medium.
was slightly decreased in AA medium. No significant differences were produced by adjusting the initial pH of CH medium to 6.5 for strain S-6. Strain 361 showed a 60% increase in enterotoxin C production in AA medium. At the same time there was a marked decrease in growth rate but little change in TEP, OD or pH as compared with CH medium.

2.3.4 Effect of arginine concentration in AA medium

It appeared from the findings of Wu and Bergdoll (1971b) that the decline in enterotoxin B production by strain S-6 coincided with the depletion of arginine from the medium. In an attempt to increase enterotoxin B production by strain S-6 shake-cultures were incubated for 24 h with varying concentrations of arginine in the medium. Increasing the concentration of arginine from 360 μg/ml to 1440 μg/ml did not increase the specific growth rate, or the production of enterotoxin B. Adding 360 μg/ml arginine at 4, 8 and 12 hours similarly caused no change in growth rate or enterotoxin B production. The omission of arginine from the medium did decrease enterotoxin levels from 191 μg/ml in the AA control flask to 129 μg/ml, and the specific growth rate was reduced from 0.39 to 0.20.

2.3.5 The effect of K⁺ and NH₄⁺ ions in AA medium

AA medium contains 14.7 μmoles of K⁺ ions as KH₂PO₄ and 7.6 μmoles NH₄⁺ as (NH₄)₂SO₄. As it had been reported (Friedman 1966, Kimble 1970) that 36.7 μmoles K⁺ ions as K₂HPO₄ completely inhibited the production of enterotoxin B by strain S-6, the effect of K⁺ ions in AA medium was investigated. Friedman also reported that NH₄⁺ ions overcame this inhibition, and that the inhibition was partially dependent on pH. Shake-flask cultures (200 ml AA medium in 1 litre flasks) with and without (NH₄)₂SO₄, at initial pH values of pH 6.5 and 7.7, were inoculated with strain S-6. The concentration of K⁺ ions was increased by the addition of KCl. The production of enterotoxin B, the specific growth rate (μ) and the OD after 12 h incubation are shown in Table 3. Increasing the concentration of K⁺ ions to 50 μmoles/ml did not inhibit the production of enterotoxin B at either pH, in the
TABLE 3. Effect of $K^+$ ions on the production of enterotoxin B by strain S-6 during 12 h incubation with and without $(\text{NH}_4)_2\text{SO}_4$, at initial pH values of 6.5 and 7.7.

<table>
<thead>
<tr>
<th>$K^+$ ions (µmoles/ml)</th>
<th>Initial pH</th>
<th>$(\text{NH}_4)_2\text{SO}_4$ (µmoles/ml)</th>
<th>Specific growth rate (µ)</th>
<th>Final OD</th>
<th>Enterotoxin B (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.7</td>
<td>6.5</td>
<td>7.6</td>
<td>0.55</td>
<td>11.6</td>
<td>134</td>
</tr>
<tr>
<td>30.0</td>
<td>6.5</td>
<td>7.6</td>
<td>0.51</td>
<td>10.5</td>
<td>146</td>
</tr>
<tr>
<td>50.0</td>
<td>6.5</td>
<td>7.6</td>
<td>0.51</td>
<td>14.6</td>
<td>140</td>
</tr>
<tr>
<td>14.7</td>
<td>7.7</td>
<td>7.6</td>
<td>0.39</td>
<td>10.5</td>
<td>67</td>
</tr>
<tr>
<td>30.0</td>
<td>7.7</td>
<td>7.6</td>
<td>0.46</td>
<td>10.2</td>
<td>72</td>
</tr>
<tr>
<td>50.0</td>
<td>7.7</td>
<td>7.6</td>
<td>0.39</td>
<td>10.0</td>
<td>72</td>
</tr>
<tr>
<td>14.7</td>
<td>7.7</td>
<td>7.6</td>
<td>0.36</td>
<td>3.3</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30.0</td>
<td>7.7</td>
<td>7.6</td>
<td>0.46</td>
<td>3.7</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50.0</td>
<td>7.7</td>
<td>7.6</td>
<td>0.37</td>
<td>4.9</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>14.7</td>
<td>7.7</td>
<td>0</td>
<td>0.10</td>
<td>2.5</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30.0</td>
<td>7.7</td>
<td>0</td>
<td>0.16</td>
<td>3.5</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50.0</td>
<td>7.7</td>
<td>0</td>
<td>0.14</td>
<td>1.9</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND indicates not detectable.
presence or absence of \((\text{NH}_4)_2\text{SO}_4\). When the initial pH of the medium was 6.5, the omission of \((\text{NH}_4)_2\text{SO}_4\) caused a 50% decrease in the production of enterotoxin B, and a lesser decrease in growth rate (20%) and OD (10%). With an initial pH of 7.7, OD and growth rate were reduced, and enterotoxin was not detectable after 12 h incubation. The omission of \((\text{NH}_4)_2\text{SO}_4\) from the medium at this pH caused a much greater decrease in the rate of growth than at the lower initial pH.

2.3.6 Production of enterotoxins by non-replicating cells

Markus and Silverman (1969) reported that enterotoxin B production by non-replicating cells from the stationary phase of growth occurred in the absence of new protein synthesis, but that enterotoxin A production by non-replicating cells harvested at any stage of growth did not occur if protein synthesis was inhibited (Markus and Silverman, 1970). In order to determine whether this did in fact indicate a difference between the mechanism of synthesis of different enterotoxins, the production of enterotoxins A, B, C and D by non-replicating cells of staphylococci was investigated using strains 100 and 22 for the production of enterotoxin A, strains S-6 and 22 for the production of enterotoxin B, strains 3 and 361 for the production of enterotoxin C, and strains 293 and 485 for the production of enterotoxin D.

Table 4 shows results for strain S-6. Stationary phase cells produced 63 \(\mu\text{g}/\text{ml}\) enterotoxin B and 0.83 \(\text{mg}/\text{ml}\) TEP after 4 h in N-medium, whereas in N-free medium only 5 \(\mu\text{g}/\text{ml}\) enterotoxin B and 0.10 \(\text{mg}/\text{ml}\) TEP were produced. In the presence of chloramphenicol, the production of enterotoxin B was reduced to 1-2 \(\mu\text{g}/\text{ml}\), and there was no detectable increase in TEP in either medium. The metabolism of tryptase resulted in a rise in pH, even in the absence of TEP increase. There was also a small increase in OD in N-medium. However, as this increase was less than 10% in 4 hours, the term non-replicating has been retained to describe these cells throughout this study. In the N-free medium there was a small increase in pH, and a small decrease in OD. A summary of the production of enterotoxins by non-replicating cells of seven strains of staphylococci during 4 h incubation in N-medium and N-free
TABLE 4. Enterotoxin B and TEP production by non-replicating cells of strain S-6 from stationary phase of growth during 4 h incubation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cm&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>OD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pH</th>
<th>Enterotoxin B (µg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>N-medium</td>
<td>0</td>
<td>0.16</td>
<td>0.19</td>
<td>7.1</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.17</td>
<td>0.18</td>
<td>7.1</td>
<td>8.1</td>
</tr>
<tr>
<td>N-free medium</td>
<td>0</td>
<td>0.16</td>
<td>0.15</td>
<td>7.4</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.16</td>
<td>0.14</td>
<td>7.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cm indicates chloramphenicol.

<sup>b</sup> OD is that of a 1/200 dilution of cell suspension.
medium, with or without 250 µg/ml chloramphenicol, is given in Table 5. As Markus and Silverman (1969) had stated that the production of enterotoxin B by resting cells varied according to age, cells were harvested in late exponential phase (10 h) and in stationary phase (20-24 h). For strains 100, 22, S-6, 361 and 3, it was found that cells from either phase of growth produced enterotoxin in N-medium. Little or no enterotoxin was produced in N-free medium, or in the presence of chloramphenicol. For strains 293 and 485, only stationary-phase cells were tested. Both strains produced enterotoxin D in N-medium, but no enterotoxin was detected in the absence of N, or in the presence of chloramphenicol.

The medium used in this investigation differed from that of Markus and Silverman (1969) in containing 0.15% glucose as compared with 0.5% glucose. In order to determine whether the lack of enterotoxin B production in our N-free medium was due to the low concentration of glucose used, the experiment was repeated with strain S-6, using 0.5% glucose. In the N-containing medium, 300 µg/ml enterotoxin B was produced, and this was reduced to 2 µg/ml in the presence of chloramphenicol. In the N-free medium, 20 µg/ml of enterotoxin B was produced. However, in the presence of chloramphenicol this was reduced to 2 µg/ml, indicating that the production of enterotoxin in N-free medium was due to new protein synthesis, resulting from the metabolism of endogenous N.

2.4 Discussion

2.4.1 Relation between growth and enterotoxin production

Markus and Silverman (1969, 1970) reported that enterotoxins A and B differed in that enterotoxin A is a primary metabolite and enterotoxin B is a secondary metabolite. (A secondary metabolite has been defined as one which is synthesised by cells which have stopped dividing as distinct from a primary metabolite, which is one formed during cell multiplication (Weinberg, 1970).) These conclusions of Markus and Silverman were based on work carried out with strains 100 and S-6 for the production of enterotoxins A and B respectively. In the present study, 3 strains were used to study each of the three
TABLE 5. Production of enterotoxins in N-medium and N-free medium, with and without chloramphenicol (Cm) by non-replicating cells during 4 h incubation.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Cm&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>Enterotoxin (µg/ml)</th>
<th>10 h cells</th>
<th>20 h cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N-medium</td>
<td>N-free medium</td>
<td>N-medium</td>
</tr>
<tr>
<td>100 A</td>
<td>0</td>
<td>8</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>100 A</td>
<td>250</td>
<td>10</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>22 A</td>
<td>0</td>
<td>6</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>22 A</td>
<td>250</td>
<td>6</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>22 B</td>
<td>0</td>
<td>120</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135</td>
</tr>
<tr>
<td>22 B</td>
<td>250</td>
<td>120</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135</td>
</tr>
<tr>
<td>S-6 B</td>
<td>0</td>
<td>270</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63</td>
</tr>
<tr>
<td>S-6 B</td>
<td>250</td>
<td>270</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63</td>
</tr>
<tr>
<td>3 C</td>
<td>0</td>
<td>152</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33</td>
</tr>
<tr>
<td>3 C</td>
<td>250</td>
<td>152</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33</td>
</tr>
<tr>
<td>361 C</td>
<td>0</td>
<td>22</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>361 C</td>
<td>250</td>
<td>22</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>293 D</td>
<td>0</td>
<td></td>
<td></td>
<td>c&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>293 D</td>
<td>250</td>
<td></td>
<td></td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>485 D</td>
<td>0</td>
<td></td>
<td></td>
<td>1/2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>485 D</td>
<td>250</td>
<td></td>
<td></td>
<td>1/2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tr indicates trace.

<sup>b</sup> ND indicates not detectable.

<sup>c</sup> + indicates undiluted supernatant positive by slide-gel diffusion test.

<sup>d</sup> 1/2 indicates titre of 1/2 by slide-gel diffusion test.
enterotoxins A, B and C, throughout the growth cycle. It was found that the time course for enterotoxin B production by strain S-6, in CH medium, was not typical of enterotoxin B production. With all nine strains, enterotoxins were first detected in the exponential phase of growth when the OD of the cultures had reached between 3.6 and 6.0. Only for strain S-6 was there any appreciable production of enterotoxin during the stationary phase of growth and this was less in AA medium than in CH medium. It was later shown (Section 3.3) that in the fermenter enterotoxin B production by strain S-6 followed growth in AA medium, but there was still significant enterotoxin production in the stationary phase in CH medium. This finding is in agreement with that of Wu and Bergdoll (1971b) that enterotoxin production is related to the nutrient conditions and not necessarily restricted to any particular phase of growth. Klapper et al., (1973) found that the stage of growth at which protease was produced by Aspergillus oryzae was dependent on the medium in which it was grown. Protease produced in shake cultures was secreted in the stationary phase when cells were grown on nutrient broth, and in early growth in a defined salts medium. Similar findings were reported by Coleman and Elliot (1962) for the production of α-amylase by Bacillus subtilis.

The production of enterotoxins by non-replicating cells of staphylococci was studied, using two strains for the production of each of the enterotoxins A, B, C and D. It was found that all four enterotoxins were produced by non-replicating cells harvested in the late exponential or stationary phase, and resuspended in a N-containing medium. Only very small amounts of enterotoxin were produced in the absence of N. In the presence of chloramphenicol enterotoxin production was inhibited in both N-containing and N-free media indicating that the small amount of enterotoxin produced in N-free medium was due to the metabolism of endogenous N. This dependence of enterotoxin A, B, C and D production on new protein synthesis for all strains showed that there was no appreciable pool of protein precursor for enterotoxin.

There are some characters that are consistent for one enterotoxin type, e.g. in shake-flasks enterotoxin A was always
produced in small amounts (2-9 µg/ml), as compared to 100-300 µg/ml for enterotoxin B, and 60-90 µg/ml for enterotoxin C. However, it is evident that because of the variation that exists between strains, more than one strain should be used to determine the characteristics of a particular enterotoxin type. Conclusions based on the use of one strain for one enterotoxin type may well lead to erroneous conclusions with regard to supposed differences between enterotoxin types.

2.4.2 Effect of environmental factors on enterotoxin production

It was found that the 18 amino-acid medium of Wu and Bergdoll (1971b) (AA medium) was suitable for the production of enterotoxins A, B and C by strains 100, S-6 and 361 respectively. The specific rate of enterotoxin production was less in AA medium for strains 100 and S-6, and considerably higher in AA medium for enterotoxin C production by strain 361, as compared with CH medium. However, yields of all three enterotoxins indicated that it would be possible to use AA medium for further work.

Friedman (1966) and Kimble (1970) reported that K⁺ inhibited the production of enterotoxin B by strain S-6. The inhibition was reported to be overcome by NH₄⁺ and to be greater at pH 7.7 than 7.0. Results presented here show that in AA medium (initial pH 6.5) increasing the K⁺ concentration to 50 µmoles/ml had no adverse effect on enterotoxin production, but that the omission of NH₄⁺ from the medium decreased enterotoxin production considerably more than cell yield. Increasing the initial pH to 7.7 decreased growth so markedly that no enterotoxin could be detected, and it was not possible therefore to determine the effect of K⁺ on enterotoxin production at this higher pH. The medium used in any particular investigation thus has a considerable effect on the results obtained.

The optimum temperature for enterotoxin production varied between 30 and 40 C for the four strains studied. The optimum temperature of 40 C for enterotoxin B production by strain S-6 was in agreement with the findings of Vandenbosch
et al., (1973) using strains S-6 and 137 in a number of PHP media. However, Tatini et al., (1971a) reported for three strains, producing enterotoxin AB, ABC and ABD respectively, that the optimum temperature for enterotoxin production was 45°C. The present study shows that the optimum temperatures for the production of enterotoxins, lipase, deoxyribonuclease, lysozyme and TEP showed considerable variation between strains. For any one strain, the temperature which gave maximum cell yield or highest growth rate was not necessarily that which gave maximum production of extracellular proteins. The yields of lipase, deoxyribonuclease, lysozyme and TEP reported here also indicate that the optimum temperature is not the same for the production of all extracellular proteins for any one strain.

In the absence of one temperature at which enterotoxin production was maximum for all strains the conventional temperature of 37°C was adhered to as approximating to optimum temperature, and enabling comparisons to be made with other investigations.

The pH of the medium increased during growth, the final pH showing considerable variation between strains. This increase in pH has suggested to several workers (Bergdoll, 1970, Hurst et al., 1973) that an increase in pH may be necessary for the production of enterotoxins. However, it was evident that growth under conditions of controlled pH would be necessary to determine the effect of pH on enterotoxin production.

The concentration of dissolved oxygen in the medium was shown to decrease rapidly during the exponential phase in shake-flask cultures, and to increase during the stationary phase. Therefore, if the control of enterotoxin synthesis were to be studied independently of environmental variation it would be necessary to use a system which incorporated control of dissolved oxygen in the medium.

Studies of the production of enterotoxins under controlled conditions of pH and aeration are described in Section 3.
3 PRODUCTION OF ENTEROTOXINS A, B AND C UNDER CONTROLLED CONDITIONS OF PH AND AERATION IN SYNTHETIC AND DEFINED MEDIA

3.1 Introduction

The effect of the initial pH of the medium on enterotoxin production has been studied (Genigeorgis and Sadler, 1966, Reiser and Weiss, 1969, Genigeorgis et al., 1971, Tatini et al., 1971b, Scheusner et al, 1973). However, except for two recent investigations on enterotoxin B production (Netzger et al., 1973, Carpenter and Silverman, 1973) no work has been reported on the production of enterotoxins under controlled pH.

Aeration has been shown to greatly affect the production of enterotoxin B (McLean et al., 1968, Dietrich et al., 1972). Although in the fermenter experiments of Netzger et al., (1973) and Carpenter and Silverman (1973) the flow of air was constant, no studies have been reported where the dissolved oxygen in the medium has been measured, and maintained at a constant concentration. It was shown in Section 2 that the pH and the concentrations of dissolved oxygen in the medium altered during growth of staphylococci in shake-flasks. Therefore, in order to study the effect of pH and aeration on enterotoxin production, staphylococci were grown in a fermenter with the pH and the concentration of dissolved oxygen constantly maintained. In this section the production of enterotoxins A, B and C by nine strains of staphylococci in the fermenter is compared with that in shake-flasks. Enterotoxin production when the pH was not held constant, and at controlled pH over a range between 6.0 and 8.0, in two different media is described. The effect of aeration on the production of enterotoxins A, B and C in shake-flasks and in the fermenter is reported.

As in the work described in Section 2, the production of other extracellular proteins was determined to find out the extent to which changes in pH or aeration affected enterotoxin specifically, as distinct from an effect on extracellular protein production generally.
3.2 Materials and methods

Organisms, media and the methods for the determination of enterotoxins and other extracellular proteins were as described in Section 2.2.

3.2.1 Design and operation of fermenter

The fermenter is illustrated diagrammatically in Fig. 11. Two litres of medium were maintained at 37°C in a 5-litre glass fermenter. The pH was automatically controlled by the addition of 1 N HCl or NaOH by Sigma pumps as required, and was continually recorded. In preliminary comparisons of fermenter and shake-flask cultures in CH medium, air was supplied to the fermenter at the rate of 4 litres per min throughout the experiments. In all other experiments the fermenter was aerated by means of a mixture of air and nitrogen maintained at a constant flow rate. The concentration of dissolved oxygen in the medium was controlled by intermittent additions of air into the nitrogen stream through a solenoid valve actuated by a Beckman oxygen sensor and oxygen concentrations were continually recorded. The culture was agitated by an impeller with 2 in. blades, run at 1,000 rpm unless otherwise specified. Samples were taken with a sterile syringe through a sampling port in the base of the fermenter. Additions of sterilized silicone antifoam (Dow-Corning) were made through the sampling port with a sterilized syringe as required.

The fermenter was autoclaved at 30 psi for 30 min, then secured in place and steamed for 30 min with all lines closed except the lower exit line. During steaming a check was made for leaks and the acid and alkali inlet lines were connected. The pH electrodes were sterilized in 2% hypochlorite for 15 min and inserted into the fermenter just before steaming was completed. Air flow (0.5 litres per min) was then turned on and the oxygen sensor was sterilized for 15 min in 1% HCl 70% ethanol, and inserted into the fermenter when the temperature was 65°C.

The inoculum for the fermenter consisted of cells grown for 16 h on a New Brunswick gyrotry shaker, washed and resuspended,
Figure 11  Diagram of fermenter.  A, alkali reservoir; B, acid reservoir; C, oxygen sensor; D, acid inlet; E, alkali inlet; F, water-cooled stirrer gland; G, stirrer; H, KCL electrode; I, glass electrode; J, sampling port; K, thermometer; L, air inlet; M, 2 litre glass fermenter.
all these operations being carried out in the medium to be used in the fermenter. The cells were added to 2 litres of medium prewarmed to 37°C, and the CD adjusted if necessary, so that experiments which were to be compared began with similar inoculum.

The inoculated medium was then run into the fermenter through the medium inlet, and the stirrer set at 1,000 rpm. In CH medium a mixture of air and N₂ was then turned on at the required flow rate. As there was some foaming immediately, antifoam (0.3 mg/ml) was added. The oxygen titrator was adjusted until the required concentration of dissolved oxygen was obtained. In AA medium it was found that if no antifoam were added to the fermenter, the cells were extremely sensitive to the initial flow of gas and lysis occurred. This was particularly so for strain 100 and this will be further discussed in Section 3.3.8. The following standard operating procedure was therefore adopted. When growth was established in the exponential phase and OD had reached approximately 1.0, 0.3 mg of antifoam per ml of culture was added, and air and N₂ were turned on and maintained at 0.3 litres per min. No further additions of antifoam were made.

Samples were taken at approximately hourly intervals. Growth was followed by CD determinations. Samples were then centrifuged and enterotoxin and other determinations carried out as described in Section 2.2.6. At 4 hourly intervals, samples were taken from the fermenter and plated to nutrient agar, to check for the presence of contaminants in the fermenter.

3.3 Results

3.3.1 Comparison of shaker-grown and fermenter-grown cultures

Nine strains of staphylococci were grown in 200 ml of CH medium in 1 litre shake-flasks. The same nine strains were grown in CH medium in the fermenter with the pH not controlled and with an air flow of 4 litres per min. The production of enterotoxins and TEF was followed throughout growth, and is shown for nine strains under both sets of conditions in Table 6. Shake-flask figures are those at 24 h, fermenter figures are those at 12-14 h, but in every case the fermentation was
TABLE 6. Production of enterotoxins and TEP in shake-flask and fermenter in CH medium initial pH 7.3.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Strain and enterotoxin type</th>
<th>Final CD</th>
<th>Final pH</th>
<th>Enterotoxin (µg/ml)</th>
<th>(µg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake-flask</td>
<td>10 A</td>
<td>29</td>
<td>8.1</td>
<td>3</td>
<td>0.02</td>
<td>0.98</td>
</tr>
<tr>
<td>Fermenter</td>
<td>10 A</td>
<td>24</td>
<td>8.9</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>0.22</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>100 A</td>
<td>21</td>
<td>8.4</td>
<td>8</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>Fermenter</td>
<td>100 A</td>
<td>16</td>
<td>9.3</td>
<td>8</td>
<td>0.39</td>
<td>0.04</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>743 A</td>
<td>28</td>
<td>8.4</td>
<td>2</td>
<td>0.19</td>
<td>1.10</td>
</tr>
<tr>
<td>Fermenter</td>
<td>743 A</td>
<td>25</td>
<td>8.8</td>
<td>1</td>
<td>0.01</td>
<td>0.38</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>22 B</td>
<td>22</td>
<td>8.4</td>
<td>104</td>
<td>1.20</td>
<td>0.28</td>
</tr>
<tr>
<td>Fermenter</td>
<td>22 B</td>
<td>23</td>
<td>8.9</td>
<td>66</td>
<td>0.63</td>
<td>0.17</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>30 B</td>
<td>24</td>
<td>8.4</td>
<td>150</td>
<td>1.60</td>
<td>0.55</td>
</tr>
<tr>
<td>Fermenter</td>
<td>30 B</td>
<td>25</td>
<td>8.9</td>
<td>52</td>
<td>0.46</td>
<td>0.15</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>S-6 B</td>
<td>16</td>
<td>8.2</td>
<td>290</td>
<td>3.60</td>
<td>2.95</td>
</tr>
<tr>
<td>Fermenter</td>
<td>S-6 B</td>
<td>20</td>
<td>9.0</td>
<td>114</td>
<td>1.25</td>
<td>0.97</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>3 C</td>
<td>24</td>
<td>8.4</td>
<td>62</td>
<td>0.41</td>
<td>0.59</td>
</tr>
<tr>
<td>Fermenter</td>
<td>3 C</td>
<td>22</td>
<td>8.9</td>
<td>11</td>
<td>0.03</td>
<td>0.27</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>37 C</td>
<td>27</td>
<td>8.3</td>
<td>86</td>
<td>0.68</td>
<td>0.22</td>
</tr>
<tr>
<td>Fermenter</td>
<td>37 C</td>
<td>24</td>
<td>8.9</td>
<td>3</td>
<td>0.04</td>
<td>0.71</td>
</tr>
<tr>
<td>Shake-flask</td>
<td>361 C</td>
<td>26</td>
<td>8.2</td>
<td>63</td>
<td>0.83</td>
<td>0.75</td>
</tr>
<tr>
<td>Fermenter</td>
<td>361 C</td>
<td>25</td>
<td>9.0</td>
<td>3</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND indicates not detectable.
continued until the OD had become stable, and subsequent tests showed that enterotoxin levels had also stabilised. For eight strains, the production of enterotoxin in the fermenter was markedly less than in shake-flasks, the exception being strain 100, which produced similar amounts of enterotoxin under both sets of conditions.

In these preliminary experiments, it was observed that the increased yields of enterotoxins in shake-flasks were generally associated with a longer transition period from exponential to stationary phase and a significantly lower rate of increase in pH in the cultures during growth as shown for strains S-6 and 361 in Fig. 12 and 13 respectively. The specific rates of enterotoxin production ($q_p$ in Table 6) were calculated for the period of maximum enterotoxin production. It was found that for the eight strains which produced a lower yield of enterotoxin in the fermenter, there was also a lower specific rate of enterotoxin production in the fermenter than in shake-flasks.

It was also found that the yields of lipase, lysozyme, deoxyribonuclease and TEP were almost invariably higher in shake-flasks than in the fermenter (compare first two rows for each strain in Table 10).

Factors which could account for these differences between the shaker and fermenter-grown cultures were therefore examined, particular attention being given to the effects of aeration, pH and the addition of antifoam during growth in the fermenter.

### 3.3.2 Effect of antifoam

In the fermenter, the amount of antifoam required depended on the strain, those such as S-6 and 361 with a high TEP requiring a total of 3–4 mg/ml in CH medium. Additions of antifoam caused an immediate decrease in oxygen in solution as recorded by the oxygen sensor, which was compensated for rapidly by increased additions of air. To determine the effect of antifoam on the production of enterotoxins and other extracellular proteins, shake-flasks were inoculated with overnight cultures of staphylococci, and additions of antifoam
Figure 12  Growth and enterotoxin B production by strain S—6 in a shake-flask (open symbols) and a fermenter (closed symbols) in CH medium. OD, △; enterotoxin B, ○; pH, □.
Figure 13  Growth and enterotoxin C production by strain 361 in a shake-flask (open symbols) and a fermenter (closed symbols) in CH medium. OD, △; enterotoxin C, ○; pH, □.
were made at times corresponding to additions on the fermenter (0, 4, 5, 6, 7 and 10 hours) to give final concentrations of 0, 1.5 and 5 mg/ml silicone antifoam. Three strains producing enterotoxin A, three producing B and three producing C were tested, and results with these nine strains are presented in Table 7. The additions of antifoam did not affect the rate of growth or the yield of cells in shake-flasks. The production of enterotoxins B and C was considerably reduced, but enterotoxin A production appeared to be less affected by the presence of antifoam, being unaffected by 1.5 mg/ml in the three strains tested, and 25% reduced by 5 mg/ml for one strain only. With all nine strains, antifoam caused a general depression of the production of extracellular protein, causing reductions in lipase, lysozyme, deoxyribonuclease and TEP.

3.3.3 Effect of increased aeration in shake-flasks

The cultures in the fermenter were grown under conditions of considerably higher aeration than that in shake-flasks. Therefore, preliminary experiments were carried out in shake-flasks to determine the effect of increased aeration for nine strains, using 1 litre flasks with and without side baffles. Side baffles have been reported to increase oxygen absorption rates approximately threefold (Freedman 1969). Baffled and non-baffled control flasks for experiments with any one strain were inoculated with aliquots of the same cell suspension, and incubated on the shaker together. To avoid excessive foaming in the baffled flasks it was necessary to reduce shaker speed considerably. The exponential growth rate was almost doubled, and the final pH was higher in the more aerated flasks. Table 8A shows the effect of increased aeration on the levels of enterotoxins and TEP produced during 24 h incubation in CH medium. Enterotoxin production was greater in baffled flasks for seven out of the nine strains, and was similar in both flasks for strain 10. However, enterotoxin C production by strain 361 was reduced in the baffled flask from 67 to 28 µg/ml. This was evidently not a characteristic of enterotoxin C production, as strains 3 and 37 showed an increase in growth and a corresponding increase in enterotoxin C production in the baffled flasks. It was also noticeable that strain S-6
TABLE 7. Effect of silicone antifoam on the production of extracellular proteins by nine strains of staphylococci in CH medium in shake-flasks during 24 h incubation.

<table>
<thead>
<tr>
<th>Strain and type</th>
<th>Antifoam (mg/ml)</th>
<th>Enterotoxin (μg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 A</td>
<td>0</td>
<td>2</td>
<td>90</td>
<td>62</td>
<td>3.5</td>
<td>0.06</td>
</tr>
<tr>
<td>10 A</td>
<td>1.5</td>
<td>2</td>
<td>58</td>
<td>52</td>
<td>2.4</td>
<td>0.05</td>
</tr>
<tr>
<td>10 A</td>
<td>5.0</td>
<td>2</td>
<td>64</td>
<td>43</td>
<td>1.4</td>
<td>0.04</td>
</tr>
<tr>
<td>100 A</td>
<td>0</td>
<td>8</td>
<td>260</td>
<td>112</td>
<td>1.45</td>
<td>0.04</td>
</tr>
<tr>
<td>100 A</td>
<td>1.5</td>
<td>8</td>
<td>170</td>
<td>108</td>
<td>0.6</td>
<td>0.03</td>
</tr>
<tr>
<td>100 A</td>
<td>5.0</td>
<td>6</td>
<td>100</td>
<td>51</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>743 A</td>
<td>0</td>
<td>2</td>
<td>110</td>
<td>62</td>
<td>3.5</td>
<td>0.07</td>
</tr>
<tr>
<td>743 A</td>
<td>1.5</td>
<td>2</td>
<td>88</td>
<td>62</td>
<td>3.5</td>
<td>0.07</td>
</tr>
<tr>
<td>743 A</td>
<td>5.0</td>
<td>2</td>
<td>84</td>
<td>45</td>
<td>1.3</td>
<td>0.07</td>
</tr>
<tr>
<td>22 B</td>
<td>0</td>
<td>76</td>
<td>150</td>
<td>1200</td>
<td>1.0</td>
<td>0.07</td>
</tr>
<tr>
<td>22 B</td>
<td>1.5</td>
<td>72</td>
<td>140</td>
<td>640</td>
<td>0.3</td>
<td>0.07</td>
</tr>
<tr>
<td>22 B</td>
<td>5.0</td>
<td>40</td>
<td>70</td>
<td>560</td>
<td>0</td>
<td>0.06</td>
</tr>
<tr>
<td>30 B</td>
<td>0</td>
<td>106</td>
<td>16</td>
<td>290</td>
<td>2.35</td>
<td>0.09</td>
</tr>
<tr>
<td>30 B</td>
<td>1.5</td>
<td>45</td>
<td>14</td>
<td>140</td>
<td>1.40</td>
<td>0.07</td>
</tr>
<tr>
<td>30 B</td>
<td>5.0</td>
<td>15</td>
<td>12</td>
<td>82</td>
<td>0.70</td>
<td>0.05</td>
</tr>
<tr>
<td>Strain and type</td>
<td>Antifoam (mg/ml)</td>
<td>Enterotoxin (µg/ml)</td>
<td>Lipase (units/ml)</td>
<td>Deoxyribonuclease (units/ml)</td>
<td>Lysozyme (units/ml)</td>
<td>TEP (mg/ml)</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>----------------------------</td>
<td>---------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S-6 B</td>
<td>0</td>
<td>256</td>
<td>280</td>
<td>700</td>
<td>0.95</td>
<td>0.20</td>
</tr>
<tr>
<td>S-6 B</td>
<td>1.5</td>
<td>214</td>
<td>210</td>
<td>440</td>
<td>0.40</td>
<td>0.16</td>
</tr>
<tr>
<td>S-6 B</td>
<td>5.0</td>
<td>72</td>
<td>140</td>
<td>240</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>3 C</td>
<td>0</td>
<td>42</td>
<td>140</td>
<td>62</td>
<td>3.5</td>
<td>0.20</td>
</tr>
<tr>
<td>3 C</td>
<td>1.5</td>
<td>23</td>
<td>116</td>
<td>62</td>
<td>1.3</td>
<td>0.20</td>
</tr>
<tr>
<td>3 C</td>
<td>5.0</td>
<td>19</td>
<td>84</td>
<td>62</td>
<td>0.4</td>
<td>0.12</td>
</tr>
<tr>
<td>37 C</td>
<td>0</td>
<td>44</td>
<td>130</td>
<td>72</td>
<td>3.0</td>
<td>0.23</td>
</tr>
<tr>
<td>37 C</td>
<td>1.5</td>
<td>30</td>
<td>92</td>
<td>82</td>
<td>1.6</td>
<td>0.19</td>
</tr>
<tr>
<td>37 C</td>
<td>5.0</td>
<td>26</td>
<td>90</td>
<td>70</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>361 C</td>
<td>0</td>
<td>38</td>
<td>280</td>
<td>44</td>
<td>3.5</td>
<td>0.35</td>
</tr>
<tr>
<td>361 C</td>
<td>1.5</td>
<td>28</td>
<td>280</td>
<td>36</td>
<td>3.2</td>
<td>0.21</td>
</tr>
<tr>
<td>361 C</td>
<td>5.0</td>
<td>13</td>
<td>170</td>
<td>35</td>
<td>1.95</td>
<td>0.19</td>
</tr>
</tbody>
</table>
TABLE 8A. Effect of aeration on production of enterotoxins and TEP in CH medium in shake-flasks during 24 h incubation.

<table>
<thead>
<tr>
<th>Aeration</th>
<th>Strain and enterotoxin type</th>
<th>Final OD</th>
<th>Final pH</th>
<th>Enterotoxin (µg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 A</td>
<td>21</td>
<td>8.10</td>
<td>2</td>
<td>0.22</td>
</tr>
<tr>
<td>Baffled</td>
<td>10 A</td>
<td>19</td>
<td>8.50</td>
<td>2</td>
<td>1.50</td>
</tr>
<tr>
<td>Control</td>
<td>100 A</td>
<td>17</td>
<td>8.40</td>
<td>6</td>
<td>0.03</td>
</tr>
<tr>
<td>Baffled</td>
<td>100 A</td>
<td>17</td>
<td>8.70</td>
<td>12</td>
<td>0.08</td>
</tr>
<tr>
<td>Control</td>
<td>743 A</td>
<td>18</td>
<td>8.15</td>
<td>ND</td>
<td>0.35</td>
</tr>
<tr>
<td>Baffled</td>
<td>743 A</td>
<td>24</td>
<td>8.60</td>
<td>1</td>
<td>1.55</td>
</tr>
<tr>
<td>Control</td>
<td>22 B</td>
<td>16</td>
<td>8.40</td>
<td>94</td>
<td>0.34</td>
</tr>
<tr>
<td>Baffled</td>
<td>22 B</td>
<td>16</td>
<td>8.90</td>
<td>105</td>
<td>0.61</td>
</tr>
<tr>
<td>Control</td>
<td>30 B</td>
<td>17</td>
<td>8.15</td>
<td>90</td>
<td>0.36</td>
</tr>
<tr>
<td>Baffled</td>
<td>30 B</td>
<td>20</td>
<td>8.50</td>
<td>116</td>
<td>1.34</td>
</tr>
<tr>
<td>Control</td>
<td>8-6 B</td>
<td>14</td>
<td>8.15</td>
<td>116</td>
<td>2.35</td>
</tr>
<tr>
<td>Baffled</td>
<td>8-6 B</td>
<td>14</td>
<td>8.80</td>
<td>196</td>
<td>2.32</td>
</tr>
<tr>
<td>Control</td>
<td>3 C</td>
<td>12</td>
<td>8.05</td>
<td>27</td>
<td>0.50</td>
</tr>
<tr>
<td>Baffled</td>
<td>3 C</td>
<td>21</td>
<td>8.45</td>
<td>40</td>
<td>1.03</td>
</tr>
<tr>
<td>Control</td>
<td>37 C</td>
<td>10</td>
<td>8.15</td>
<td>33</td>
<td>0.15</td>
</tr>
<tr>
<td>Baffled</td>
<td>37 C</td>
<td>19</td>
<td>8.60</td>
<td>55</td>
<td>0.60</td>
</tr>
<tr>
<td>Control</td>
<td>361 C</td>
<td>25</td>
<td>8.15</td>
<td>67</td>
<td>1.45</td>
</tr>
<tr>
<td>Baffled</td>
<td>361 C</td>
<td>23</td>
<td>8.70</td>
<td>28</td>
<td>5.0</td>
</tr>
</tbody>
</table>
produced considerably less enterotoxin B (196 µg/ml), even in the baffled flask, at the reduced shaking speed used in this experiment than was generally obtained in shake-flasks at higher shaking speeds (300 µg/ml).

As pH 6.5 was commonly used in fermenter experiments, experiments with baffled and non-baffled flasks were carried out in medium at an initial pH of 6.5. Similar results to those at pH 7.3 were obtained at this lower pH.

Increased aeration caused an increase in TEP for all strains when grown in baffled flasks (Table 8A). Production of lipase and deoxyribonuclease was also higher for all nine strains, and lysozyme production higher for five strains in baffled flasks (Table 8B). These results, in general, agree with the data from antifoam experiments which indicate that high yields of enterotoxins were associated with high yields of other extracellular proteins. The major exception was the production of enterotoxin C by strain 361, where a decrease in enterotoxin production was associated with an increased TEP in the baffled flask (Table 8A).

3.3.4 Effect of aeration on enterotoxin production in the fermenter

Strains 100, S-6 and 361 were grown in the fermenter at a constant pH of 6.5, and with dissolved oxygen concentration maintained at either 5-7 mm Hg or 30-40 mm Hg. Antifoam additions were such that for any one strain, similar additions were made at similar times at both levels of aeration. The effect of oxygen concentration on the production of enterotoxins and TEP is shown in Table 9. The production of enterotoxin A by strain 100 was similar at both concentrations of oxygen while OD and TEP were slightly increased at higher aeration. Strain S-6 produced considerably more enterotoxin B at the higher oxygen concentration, and at the same time showed a smaller increase in OD and TEP.
TABLE 8B. Effect of aeration on production of lipase, deoxyribonuclease and lysozyme in CH medium in shake-flasks during 24 h incubation.

<table>
<thead>
<tr>
<th>Aeration</th>
<th>Strain and type</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 A</td>
<td>185</td>
<td>94</td>
<td>4.15</td>
</tr>
<tr>
<td>Baffled</td>
<td>10 A</td>
<td>350</td>
<td>250</td>
<td>3.45</td>
</tr>
<tr>
<td>Control</td>
<td>100 A</td>
<td>210</td>
<td>150</td>
<td>1.0</td>
</tr>
<tr>
<td>Baffled</td>
<td>100 A</td>
<td>320</td>
<td>160</td>
<td>0.50</td>
</tr>
<tr>
<td>Control</td>
<td>743 A</td>
<td>37</td>
<td>43</td>
<td>4.5</td>
</tr>
<tr>
<td>Baffled</td>
<td>743 A</td>
<td>110</td>
<td>120</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>Control</td>
<td>22 B</td>
<td>390</td>
<td>1700</td>
<td>1.20</td>
</tr>
<tr>
<td>Baffled</td>
<td>22 B</td>
<td>1250</td>
<td>4000</td>
<td>1.75</td>
</tr>
<tr>
<td>Control</td>
<td>30 B</td>
<td>7</td>
<td>110</td>
<td>2.40</td>
</tr>
<tr>
<td>Baffled</td>
<td>30 B</td>
<td>37</td>
<td>600</td>
<td>3.05</td>
</tr>
<tr>
<td>Control</td>
<td>S-6 B</td>
<td>780</td>
<td>700</td>
<td>4.0</td>
</tr>
<tr>
<td>Baffled</td>
<td>S-6 B</td>
<td>3120</td>
<td>2100</td>
<td>0.50</td>
</tr>
<tr>
<td>Control</td>
<td>3 C</td>
<td>58</td>
<td>36</td>
<td>4.0</td>
</tr>
<tr>
<td>Baffled</td>
<td>3 C</td>
<td>75</td>
<td>80</td>
<td>5.20</td>
</tr>
<tr>
<td>Control</td>
<td>37 C</td>
<td>140</td>
<td>28</td>
<td>4.25</td>
</tr>
<tr>
<td>Baffled</td>
<td>37 C</td>
<td>530</td>
<td>67</td>
<td>5.60</td>
</tr>
<tr>
<td>Control</td>
<td>361 C</td>
<td>700</td>
<td>59</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>Baffled</td>
<td>361 C</td>
<td>1060</td>
<td>170</td>
<td>&gt;6.0</td>
</tr>
</tbody>
</table>
TABLE 9. Effect of dissolved oxygen concentration on enterotoxin and TEP production in the fermenter.

<table>
<thead>
<tr>
<th>Strain and type</th>
<th>Oxygen (mm Hg)</th>
<th>Final OD</th>
<th>Enterotoxin (µg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>5-7</td>
<td>22</td>
<td>21</td>
<td>0.12</td>
</tr>
<tr>
<td>100 A</td>
<td>30-40</td>
<td>26</td>
<td>21</td>
<td>0.14</td>
</tr>
<tr>
<td>S-6 B</td>
<td>5-7</td>
<td>21</td>
<td>122</td>
<td>0.69</td>
</tr>
<tr>
<td>S-6 B</td>
<td>30-40</td>
<td>25</td>
<td>169</td>
<td>0.73</td>
</tr>
<tr>
<td>361 C</td>
<td>5-7</td>
<td>27</td>
<td>5</td>
<td>0.12</td>
</tr>
<tr>
<td>361 C</td>
<td>30-40</td>
<td>25</td>
<td>4</td>
<td>0.27</td>
</tr>
</tbody>
</table>

The production of enterotoxin C was extremely low (4 µg/ml) compared to that in shake-flasks (approximately 60 µg/ml), and was similar at both levels of aeration. This was similar to the finding (Table 6) that strain 361 produced only 3 µg/ml in the fermenter at uncontrolled pH as against 63 µg/ml in shake-flask.

In an attempt to find an enterotoxin C-producing strain that would give adequate enterotoxin C production in the fermenter, strains 3 and 37 were grown in the fermenter with dissolved oxygen levels of 5-7 mm Hg, and at uncontrolled pH. Shake-flask cultures (200 ml CH medium in 1 litre flasks) were grown at the same time with the same batch of medium and using the same inoculum. Strain 3 produced 62 µg/ml enterotoxin C in the shake-flask, but only 11 µg/ml in the fermenter, and strain 37 produced 86 µg/ml in the shake-flask and only 3 µg/ml in the fermenter. Strain 361 was then grown in the fermenter with no antifoam additions, with the stirrer speed reduced to 200 rpm, and the concentration of dissolved oxygen reduced to 4 mm Hg. No significant increase in enterotoxin C production was however obtained. When the flow of gas was reduced from 2 litres per min to 0.03 litres per min, enterotoxin C production was increased to 21 µg/ml. Further decrease in gas flow or stirrer speed evidently produced an oxygen limitation, and growth and enterotoxin production were reduced.
3.3.5 Effect of additions of HCl to the fermenter

To maintain constant pH conditions during a fermenter experiment, HCl giving a final concentration of up to 0.1 M in the medium was required. To determine whether the Cl\(^{-}\) concentration affected the production of enterotoxins, the effect of similar concentrations of NaCl on enterotoxin production was determined for strains 100, S-6 and 361. NaCl was added to shake-flask cultures at intervals when the OD of each culture corresponded to OD in fermenter experiments at which maximum HCl additions were required. This reduced the production of enterotoxins A, B and C by 7, 25 and 10\% respectively, and TEP by 0, 29 and 12\% in these three strains.

3.3.6 Effect of pH on the production of enterotoxins and other extracellular proteins in CH medium

The production of enterotoxins and other extracellular proteins by strains 100, S-6 and 361 was studied in the fermenter over a pH range of 6.0 to 8.0, by using CH medium, standardized additions of antifoam, air - \(\text{N}_2\) flow of 2 litres per min and a partial pressure of dissolved oxygen of between 30 and 40 mm Hg.

**Effect of pH on enterotoxin A production.** Enterotoxin production was followed throughout growth in all experiments. Enterotoxin A production and OD for strain 100 are shown in Fig. 14 at pH 6.5, and when the pH was not controlled. Controlling pH at 6.0, 6.5 or 7.3 caused a marked increase in enterotoxin A production by strain 100, with an optimal pH of 6.5 (Table 10). Strain S-6, which did not produce detectable quantities of enterotoxin A in the shake-flask, or in the fermenter without pH control, produced 3 \(\mu\)g/ml in the fermenter at pH 6.5. Three A-producing strains, 10, 22 and 743, were then grown in the fermenter at pH 6.5 and without pH control. Strain 743 showed a slight increase in enterotoxin A production but strain 10 did not show any increase in enterotoxin A production under controlled pH conditions. Strain 22, which produced 2 to 3 \(\mu\)g/ml enterotoxin A in the shake-flask, did not produce detectable enterotoxin A in the fermenter under any combination of aeration and pH. The effect of varying
Figure 14  Growth and enterotoxin A production by strain 100 in a fermenter in CH medium at uncontrolled pH (open symbols) and at pH 6.5 (closed symbols). OD, △; enterotoxin A, ○; pH, ▲.

<table>
<thead>
<tr>
<th>Strain and type</th>
<th>pH</th>
<th>Enterotoxin (µg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
<th>TEP (mg/ml)</th>
<th>Final CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>6.0</td>
<td>11</td>
<td>236</td>
<td>22</td>
<td>0</td>
<td>0.04</td>
<td>11</td>
</tr>
<tr>
<td>100 A</td>
<td>6.5</td>
<td>22</td>
<td>196</td>
<td>38</td>
<td>0.9</td>
<td>0.14</td>
<td>26</td>
</tr>
<tr>
<td>100 A</td>
<td>7.3</td>
<td>15</td>
<td>2</td>
<td>96</td>
<td>1.0</td>
<td>0.04</td>
<td>17</td>
</tr>
<tr>
<td>100 A</td>
<td>8.0</td>
<td>7</td>
<td>2</td>
<td>75</td>
<td>1.0</td>
<td>0.04</td>
<td>11</td>
</tr>
<tr>
<td>S-6 B</td>
<td>6.5</td>
<td>169</td>
<td>1.30</td>
<td>920</td>
<td>310</td>
<td>&gt;6.0</td>
<td>25</td>
</tr>
<tr>
<td>S-6 B</td>
<td>7.3</td>
<td>180</td>
<td>1.90</td>
<td>1180</td>
<td>710</td>
<td>4.4</td>
<td>24</td>
</tr>
<tr>
<td>S-6 B</td>
<td>8.0</td>
<td>124</td>
<td>1.20</td>
<td>415</td>
<td>970</td>
<td>2.1</td>
<td>24</td>
</tr>
<tr>
<td>361 C</td>
<td>SF</td>
<td>63</td>
<td>0.83</td>
<td>1470</td>
<td>32</td>
<td>&gt;6.0</td>
<td>26</td>
</tr>
<tr>
<td>361 C</td>
<td>NC</td>
<td>3</td>
<td>0.09</td>
<td>214</td>
<td>32</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>361 C</td>
<td>6.5</td>
<td>4</td>
<td>0.07</td>
<td>168</td>
<td>17</td>
<td>4.0</td>
<td>25</td>
</tr>
<tr>
<td>361 C</td>
<td>7.3</td>
<td>5</td>
<td>0.05</td>
<td>214</td>
<td>36</td>
<td>4.8</td>
<td>25</td>
</tr>
<tr>
<td>361 C</td>
<td>8.0</td>
<td>7</td>
<td>0.13</td>
<td>115</td>
<td>37</td>
<td>1.55</td>
<td>24</td>
</tr>
</tbody>
</table>

a SF indicates shake-flask cultures. All other experiments carried out in the fermenter.
b NC indicates pH was not controlled.
c Strain S-6 also produced enterotoxin A at pH 6.5 (3 µg/ml) and pH 7.3 (2 µg/ml).
pH on enterotoxin A production was therefore a characteristic of the strain under examination.

The increase in enterotoxin A production at pH 6.5 was associated with a longer transition period from exponential to stationary phase for strains 100 and 743, as shown for strain 100 in Fig. 14. The specific rate of enterotoxin A production was determined for strain 100 ($q_p$ in Table 10). It was found that $q_p$ was actually highest when the pH was not controlled in the fermenter, and that the increase in enterotoxin yield during 12 h incubation at pH 6.5 was apparently due to the change in growth pattern, rather than to an increase in the specific rate of enterotoxin production at a controlled pH. A comparison of $q_p$ at pH 6.0, 6.5, 7.3 and 8.0 showed that when pH was controlled, pH 6.5 gave the highest specific rate of enterotoxin A production for strain 100.

**Effect of pH on enterotoxin B production.** Enterotoxin B production by strain S-6 was higher under conditions of controlled pH than without pH control and showed an optimal pH of 6.5 to 7.3 (Table 10). Enterotoxin B production by strain 22 was not, however, higher under controlled pH conditions. This result for strain 22 was in agreement with the finding that the production of enterotoxin A by this strain also did not increase under controlled pH.

The increase in enterotoxin B production by strain S-6 was associated with a slightly longer transition period from exponential to stationary phase and a higher cell yield (Fig. 15). Determination of the specific rate of enterotoxin B production showed that this was highest at controlled pH, and the increase in enterotoxin yield as compared with that without pH control was not due solely to the change in growth pattern. It was also found that at controlled pH, the optimum pH for enterotoxin B production was 7.3.

**Effect of pH on enterotoxin C production.** Enterotoxin C production by strain 361 in CH medium was extremely low in the fermenter under all conditions of pH, as compared with that in shake-flasks (Table 10). Enterotoxin C yield was
Figure 15  Growth and enterotoxin B production by strain S–6 in a fermenter in CH medium at uncontrolled pH (open symbols) and pH 6.5 (closed symbols). OD, ◇; enterotoxin B, ○; pH, □.
slightly higher at controlled pH as compared with production when pH was not controlled in the fermenter, with an optimum pH of 8.0.

The specific rate of enterotoxin C production under any conditions of pH in the fermenter was extremely low (between 0.05 and 0.13 µg/unit cell mass/h) as compared with shake-flasks (0.83), with an optimum pH of 8.0 in the fermenter. There was very little difference in growth pattern between that when the pH was not controlled, and at pH 6.5, 7.3 and 8.0.

Effect of pH on other extracellular proteins. The production of lipase, deoxyribonuclease, lysozyme and TEF during 12 h incubation in the fermenter was higher at controlled pH than at uncontrolled pH, except for deoxyribonuclease production by strain S-6 (Table 10). Maximum yields were generally obtained between 6.5 and 7.3, but the optimum pH varied considerably between strains.

3.3.7 Effect of pH on the production of enterotoxins and other extracellular proteins in AA medium

It had been found in preliminary shake-flask experiments (Section 2.3.3) that in AA medium, the production of enterotoxin A by strain 100 was similar to that in CH medium. Enterotoxin B production in AA medium was less than in CH medium, but still appeared to be adequate for further experiments. The production of enterotoxin C, which in the fermenter in CH medium had been extremely low, was higher in AA medium than in CH medium in shake-flasks. Growing the staphylococcal strains in AA medium in the fermenter also had the great advantage over CH medium that very little foaming occurred and consequently antifoam was not needed, apart from an initial 0.3 mg/ml as described in Section 3.2.1. The production of enterotoxin in AA medium was therefore determined at varying pH values, with the partial pressure of dissolved oxygen at between 30 and 40 mm Hg.

Effect of pH on enterotoxin A production. When the pH was not held constant, strain 100 grew more slowly in AA than in CH medium with a corresponding lesser increase in pH, and
and the final yield of enterotoxin A was considerably higher in the AA medium (Fig. 16). However, the specific rate of enterotoxin production in the fermenter without pH control was 0.18 µg/unit cell mass/h in AA medium, as compared with 0.39 in CH medium, indicating that the higher final yield of enterotoxin A in AA medium was due to the change in growth pattern resulting in continued enterotoxin production.

The production of enterotoxin A by strain 100 was highest under controlled pH conditions in AA medium, with an optimum pH of 6.5 in agreement with the findings in CH medium. The pattern of growth was similar in the fermenter under all pH conditions. The specific rate of enterotoxin production (q_p in Table 11) indicates that the variation in yields of enterotoxin A under different pH conditions in AA medium was due to a difference in the specific rate of enterotoxin production.

**Effect of pH on enterotoxin B production.** The production of enterotoxin B by strain 3-6 during 12 h in the fermenter was lower in AA medium under all conditions of pH. However, the specific rates of enterotoxin production were higher in AA medium (q_p in Table 11), than in CH medium (q_p in Table 10). In AA medium, the yield and specific rates of enterotoxin production were both highest at controlled pH as compared with uncontrolled pH. The highest yield during 12 h was obtained at a pH of between 6.5 and 7.3 while the optimum pH for the specific rate of enterotoxin production was 6.5.

**Effect of pH on enterotoxin C production.** The yield and specific rates of enterotoxin C production by strain 361 were much higher in AA medium in the shake-flask and fermenter, under all conditions of pH. In AA medium, the enterotoxin yield was highest under controlled conditions of pH, with an optimum pH of 7.3. The specific rates of enterotoxin C production (q_p in Table 11) were also higher at controlled pH, with an optimum pH of 6.5.

**Effect of pH on other extracellular proteins.** The production of lipase, deoxyribonuclease, lysozyme and TEP was generally higher under conditions of controlled pH than at
Figure 16  Growth and enterotoxin A production by strain 100 in a fermenter in CH medium (open symbols) and AA medium (closed symbols).
OD, △; enterotoxin A, ○; pH, □.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>pH</th>
<th>Enterotoxin (µg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
<th>TEP (mg/ml)</th>
<th>Final OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>SF</td>
<td>7</td>
<td>0.06</td>
<td>27</td>
<td>8</td>
<td>0.1</td>
<td>0.14</td>
</tr>
<tr>
<td>100 A</td>
<td>NC</td>
<td>20</td>
<td>0.18</td>
<td>20</td>
<td>13</td>
<td>0</td>
<td>0.16</td>
</tr>
<tr>
<td>100 A</td>
<td>6.0</td>
<td>24</td>
<td>0.20</td>
<td>5</td>
<td>17</td>
<td>0</td>
<td>0.06</td>
</tr>
<tr>
<td>100 A</td>
<td>6.5</td>
<td>32</td>
<td>0.25</td>
<td>5</td>
<td>10</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>100 A</td>
<td>7.3</td>
<td>20</td>
<td>0.18</td>
<td>48</td>
<td>15</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>S-6 B</td>
<td>SF</td>
<td>194</td>
<td>1.54</td>
<td>59</td>
<td>86</td>
<td>0.1</td>
<td>0.68</td>
</tr>
<tr>
<td>S-6 B</td>
<td>NC</td>
<td>97</td>
<td>1.70</td>
<td>100</td>
<td>84</td>
<td>0.2</td>
<td>0.45</td>
</tr>
<tr>
<td>S-6 B</td>
<td>6.0</td>
<td>96</td>
<td>1.83</td>
<td>38</td>
<td>37</td>
<td>0.3</td>
<td>0.67</td>
</tr>
<tr>
<td>S-6 B</td>
<td>6.5</td>
<td>129</td>
<td>2.12</td>
<td>240</td>
<td>37</td>
<td>0.3</td>
<td>0.67</td>
</tr>
<tr>
<td>S-6 B</td>
<td>7.3</td>
<td>131</td>
<td>1.91</td>
<td>110</td>
<td>47</td>
<td>0</td>
<td>1.12</td>
</tr>
<tr>
<td>S-6 B</td>
<td>8.0</td>
<td>78</td>
<td>1.02</td>
<td>114</td>
<td>125</td>
<td>0.1</td>
<td>0.46</td>
</tr>
<tr>
<td>361 C</td>
<td>SF</td>
<td>100</td>
<td>1.20</td>
<td>67</td>
<td>10</td>
<td>1.1</td>
<td>0.64</td>
</tr>
<tr>
<td>361 C</td>
<td>NC</td>
<td>35</td>
<td>0.55</td>
<td>90</td>
<td>19</td>
<td>1.1</td>
<td>0.53</td>
</tr>
<tr>
<td>361 C</td>
<td>6.0</td>
<td>25</td>
<td>0.34</td>
<td>48</td>
<td>12</td>
<td>0.9</td>
<td>0.21</td>
</tr>
<tr>
<td>361 C</td>
<td>6.5</td>
<td>53</td>
<td>0.71</td>
<td>320</td>
<td>8</td>
<td>3.1</td>
<td>1.02</td>
</tr>
<tr>
<td>361 C</td>
<td>7.3</td>
<td>30</td>
<td>0.37</td>
<td>320</td>
<td>19</td>
<td>3.0</td>
<td>0.90</td>
</tr>
</tbody>
</table>

a SF indicates shake-flask cultures. All other experiments carried out in the fermenter.
b NC indicates pH was not controlled.
c Strain S-6 also produced enterotoxin A at uncontrolled pH (2 μg/ml), pH 6.0 (3 μg/ml); pH 6.5 (2 μg/ml) and pH 7.3 (2 μg/ml).
uncontrolled pH (Table 11). Although there was a considerable strain variation, maximum yields of these extracellular proteins were obtained between pH 6.5 and 7.3 in most cases.

3.3.8 Effect of gas flow on staphylococcal cells in AA medium

In initial experiments with AA medium in the fermenter, the same aeration procedure was adopted as for CH medium, i.e. air and N₂ (2 litres per min) were turned on and the oxygen titrator adjusted so that the partial pressure of dissolved oxygen was maintained at 30-40 mm Hg. However, as little foaming occurred in AA medium, no antifoam was added, whereas 0.3 mg/ml antifoam was added initially to CH medium. It was found with strain S-6 that there was a lag period of approximately 10 h. Kubitschek (1970) reported that a culture may fail to grow in a chemostat if it is aerated immediately. Therefore, aeration was delayed until growth was established and OD reached approximately 1.0, and this greatly reduced the lag period for strain S-6.

However, when strain 100 was grown in AA medium in the fermenter, there was immediate lysis of cells, and the OD rapidly decreased (Fig.17). This effect was extremely reproducible, and was not peculiar to strain 100. If strain S-6 was subject to an air and N₂ flow of 4 litres per min as soon as the fermenter had been inoculated, a culture with an initial OD of 0.20 was reduced to 0.01 in 4 h. Shake-flask experiments showed that cells taken from the fermenter were capable of normal growth, and that the medium did not contain an inhibitor. It has been reported (Kubitschek, 1970) that in order to adapt to chemostat culture some cells require CO₂. The nitrogen in the air and N₂ mixture was therefore replaced with CO₂, but this did not prevent lysis of the staphylococcal cells. Lysis was not decreased by using an inoculum of younger cells or of higher cell density.

Experiments with strains 100 and S-6 in which air (0.3 litres per min) was bubbled through cultures in boiling tubes showed that it was possible to lyse cells which had been grown in either AA or CH medium. This suggested that the significant
Figure 17  Effect of air and N₂ on OD of cells of strain 100 in a fermenter in AA medium when antifoam added (○) and in the absence of antifoam (△).
difference between the effect of aeration on cultures grown in CH medium, and those grown in AA medium in the fermenter might be that silicone antifoam was added to former as soon as the air and $N_2$ flow was begun. It was shown that if 0.3 mg/ml of antifoam were added to the culture in AA medium one minute before air and $N_2$ were turned on no lysis occurred with either strain 100 (Fig.17) or strain 5-6. This procedure was therefore adopted for all fermenter experiments in AA medium, as described in Section 3.2.1.

Webb (1963) found that there was a substantial drop in cell count of staphylococcal suspensions through which oxygen, nitrogen, or helium were bubbled. Inactivation of cells by bubbling has also been reported by Dale et al. (1961) and Ginsberg and Jagger (1962). It has been suggested (Webb 1963) that severe forces of surface tension are associated with bubbling. It is possible that the effect of antifoam in preventing cell lysis is due to the ability of antifoam to reduce surface tension.

3.4 Discussion

3.4.1 Differences between strains in response to conditions of growth

The pattern of production of enterotoxins from nine different enterotoxigenic strains of staphylococci in response to a wide range of growth conditions varied considerably. The strain to strain differences between staphylococci producing a specific enterotoxin were very marked. This was particularly well demonstrated by strains 5-6 and 22 which produced both enterotoxins A and B. Thus the yield of both enterotoxins was increased by growth of strain 5-6 under controlled pH conditions, whereas the production of neither enterotoxin A nor B was increased when strain 22 was grown at controlled pH. There have been a number of reports in the literature suggesting differences in mechanisms of synthesis of enterotoxins A and B (Markus and Silverman 1969, 1970; Troller 1971, 1972). However, the variation between strains reported here throws some doubt on the validity of conclusions based on results obtained from one strain producing one enterotoxin only.
3.4.2 Differences in enterotoxin production in the fermenter and in shake-flasks

The higher production of enterotoxins A, B and C by eight out of nine strains in shake-flasks as compared with the fermenter may be attributed to several factors. Growth in the fermenter was frequently associated with an exponential phase of shorter duration and a shortened transition period from exponential to stationary phase. This generally resulted in a decrease in the time for which enterotoxins were produced. However, determination of the specific rate of enterotoxin production showed that this change in growth pattern alone did not account for the decrease in enterotoxin production in the fermenter as compared with that in shake-flasks.

It was shown in shake-flasks that the addition of antifoam to the medium decreased the production of all extracellular proteins, although the extent to which this occurred depended on the strain. Antifoam decreased the diffusion of oxygen into the medium, and may have affected extracellular protein production by lowering the rate of diffusion of oxygen into the cell. The production of enterotoxin A by three strains was less sensitive to the presence of antifoam than the production of enterotoxins B and C from six other strains. Production of the other extracellular proteins measured however were similarly reduced for all nine strains. It is therefore probable that the addition of antifoam to the fermenter was a factor contributing to the decrease in enterotoxin production in CH medium. In AA medium, much smaller additions of antifoam were required, and it is less likely that these affected enterotoxin production.

The addition of 0.1 M NaCl decreased the production of all three enterotoxins, and the other extracellular proteins measured. This is lower than the concentration reported by other workers to reduce enterotoxin yield (Genigeorgis and Sadler, 1966; MacLean et al., 1968). If this effect is attributable to the chloride ion, then similar additions of HCl may have lowered yields of enterotoxins and other extracellular proteins in experiments where a constant pH was maintained.
3.4.3 Effect of aeration on enterotoxin production

The finding that cultures in baffled flasks produce more enterotoxin than their controls is in general agreement with the findings of Dietrich et al., (1972) that increasing aeration increases enterotoxin production. These workers found that beyond an optimal shaking speed there was a decrease in enterotoxin production. It may be that the reduction in enterotoxin C production by strain 361 in baffled flasks occurred because this strain has a low optimal aeration. However, the increases in enterotoxin production observed in baffled flasks are significantly less than increases which have been reported to be obtained by increasing shaking speeds (Dietrich et al., 1972). It is possible that baffles have some effect on enterotoxin production other than that due to increased aeration.

In the fermenter, aeration did not affect the production of enterotoxin A by strain 100, provided the partial pressure of oxygen was above approximately 5 mm Hg. Enterotoxin C production by strain 361 similarly was not improved by increasing dissolved oxygen above a minimum level of 5-7 mm Hg. However, enterotoxin C production was slightly increased by reducing the gas flow from 2 litres per min to 0.3 litres per min. Enterotoxin B production by strain S-6 was significantly higher when the oxygen was increased from 5-7 mm Hg to 30-40 mm Hg, and so did seem to be more affected by the concentration of dissolved oxygen than enterotoxin A and C production by strains 100 and 361 respectively.

3.4.4 Effect of pH on enterotoxin production

For the three strains studied in detail (100, S-6 and 361) the yield of enterotoxin in the fermenter during 12 h incubation was higher under controlled conditions, compared with production when pH was not controlled. For strain 100 in AA medium, and strains S-6 and 361 in both media, the specific rates of enterotoxin production in the fermenter were also highest under conditions of controlled pH. However, for strain 100 in GH medium, it was found that the specific rate of enterotoxin production was highest in the fermenter when the pH was not controlled, indicating that the high yields obtained at
pH 6.5 and 7.3 in the fermentor had occurred because of
the changes in growth pattern.

The increase in the final yield of enterotoxin A which
occurred when some strains were grown at controlled pH may
be of considerable significance. For strain 100, the yield
was increased from 8 µg/ml in the conventional laboratory
shake-flask to 32 µg/ml at pH 6.5 in AA medium. Enterotoxin
A was not detected in shake-flask cultures of strain S-6, but
3 µg/ml of enterotoxin A was produced at controlled pH in the
fermenter. As many foods are buffered at pH 6.0-6.5 (Lowe,
1955), strains of staphylococci which produce little or no
enterotoxin A under the usual conditions of laboratory tests
may in food produce sufficient enterotoxin to cause food­
poisoning. The low yields of enterotoxin A (1-6 µg/ml)
produced in various synthetic media, even at different initial
pH values (Reiser and Weiss, 1969) and hence the relative
unavailability of both the toxin and its antiserum, have
greatly limited studies on enterotoxin A. The use of the
chemically defined amino-acid medium in a fermenter as de­
scribed in this study would be a suitable means of producing
large quantities of enterotoxin A.

In studying the effects of pH, aeration, antifoam, NaCl and
change in growth pattern on enterotoxin production, the yields
of lipase, deoxyribonuclease, lysozyme and TEP have also been
determined. It appeared that some effects, such as the
decrease in enterotoxin production in the fermenter as com­
pared with in shake-flasks, or the decrease resulting from
presence of antifoam or NaCl, were general effects on the
production of extracellular proteins. The higher enterotoxin
yields obtained in baffled flasks also was associated with a
general increase in extracellular protein production for most
strains. However, there were a number of instances where a
decrease in enterotoxin production was accompanied by an
increase in other extracellular proteins. The experiments
at controlled pH showed that the pH which gave maximum
enterotoxin yield was not always that which gave the highest
yield of other extracellular proteins.
4. EFFECT OF GLUCOSE AND GLYCEROL ON THE PRODUCTION OF ENTEROTOXINS AND OTHER EXTRACELLULAR PROTEINS

4.1 Introduction

Enterotoxin B production by strain S-6 has been reported to be subject to glucose repression (Morse et al., 1969, Markus and Silverman, 1969, Morse and Baldwin 1971, Morse and Nah, 1973). No studies of the effect of glucose on the production of enterotoxins A or C have been described in the literature. When staphylococci are grown in casein hydrolysate media containing glucose, there is a rapid decrease in pH, and after 24 h incubation the pH may be as low as 5.1. On the other hand, in casein hydrolysate medium without glucose, the pH increases during growth to approximately 8.5. In order to satisfactorily differentiate between the effect of pH and the effect of glucose per se on enterotoxin production, it was necessary to grow staphylococci under controlled conditions. Such studies are described for enterotoxins A, B and C in this section. During growth in shake-flasks, the glucose in the medium is rapidly utilized. To prevent this change in glucose concentration during growth, experiments were carried out in which the concentration of glucose in the fermenter was constantly maintained at 0.1 M. The effect of other carbohydrates and related compounds, such as glycerol and pyruvate, on enterotoxin production was also investigated.

It was found that repression of enterotoxin production by glucose or glycerol was accompanied by an increase in growth rate. Experiments with non-replicating cells were carried out in an attempt to separate the effect of glucose and glycerol on enterotoxin production from a change in enterotoxin production resulting from an altered growth rate.

4.2 Materials and methods

4.2.1 Strains

Staphylococcal strains were as described in Section 2.2.1. Escherichia coli K 12 PB 240 (λ⁺) was supplied by the Department of Microbiology and Genetics, Massey University.
4.2.2 Media

The defined amino acid medium (AA medium) described in Section 2.2.2, was used in the majority of experiments. For experiments with non-replicating cells, 0.2 M phosphate buffer (pH 7.0) was included in AA medium. In specified experiments, the concentration of Mg^{++} was reduced from 0.4 mM to 0.2 mM. For experiments including cyclic adenosine 3':5' monophosphate (cAMP), medium A as described by Perlman and Fastan (1968) was used. This consisted of 14.0 g of K_2HPO_4, 6.0 g of KH_2PO_4, 2.0 g of (NH_4)_2SO_4, 0.2 g of MgSO_4·7 H_2O and 5 g glycerol per litre. The pH was 7.0 without adjustment. Modifications were made to this medium as indicated. Glucose, galactose, glycerol and lactose were autoclaved separately, and pyruvate was sterilised using a millipore filter, and added to sterile media as required.

4.2.3 Cultural conditions

The inoculum for the fermenter consisted of cells grown for 16 h in shake-flasks, washed and resuspended in AA medium. The fermenter and its operating procedure have been described in Section 3.2.1. For experiments including glucose or glycerol the following modification was made. Since with these substrates in the medium, there was no increase in pH during the fermentation, only NaOH was required for pH adjustment. The burette containing HCl was replaced by one containing sterile glucose or glycerol, which was connected to the alkali inlet without going through the Sigma pump. The required additions of glucose or glycerol were then made manually by opening the burette.

4.2.4 Measurement of glucose concentration

Glucose was determined enzymatically by the glucostat method (Calbiochem, Los Angeles, California). This depends on the following reactions:

\[
\text{Glucose} + \text{ATP} \rightleftharpoons \text{glucose-6-phosphate} + \text{ADP}
\]
\[
\text{glucose-6-phosphate} + \text{NADP} \rightleftharpoons \text{NADPH} + \text{H}^+ + \text{6-phosphogluconate}
\]

NADPH was measured by the change in the absorbance at 340 nm in
in the enzyme-substrate solution after the glucose solution has been added, and the reaction allowed to proceed to completion at room temperature. Glucose concentration was calculated from the relationship that the absorbance of one micromole per ml of NADH at 340 nm in a 1 cm light path is 6.22. This method measures concentrations of glucose between 0.002 M and 0.01 M. Dilutions of samples required to bring glucose concentrations into this range were made with AA medium. Initially, known concentrations of glucose in AA medium were determined to check the validity and specificity of the assay in this medium.

4.2.5 Control of glucose and glycerol concentrations

Glucose determinations were carried out on samples taken throughout a fermenter experiment where the medium contained an initial glucose concentration of 2%. At the same time, the additions of N NaOH required to maintain pH at 6.5 were recorded. It was found that the utilization of 0.294 g glucose required the addition of 1 ml 2N NaOH. Therefore by the manual addition of 2 ml of 0.441 g/ml glucose solution for each 3 ml NaOH added automatically to maintain pH, it was possible to maintain the concentration of glucose between 0.09 and 0.11 M.

The concentration of glycerol was maintained at approximately 0.1 M by making the assumption that the utilization of 2 moles glycerol and 1 mole glucose required the same volume of NaOH to keep the pH constant.

4.2.6 Measurement of growth

Growth in shake-flask and fermenter experiments was followed by measuring OD, as described in Section 2.2.3.

4.2.7 Determination of extracellular proteins

Assays of enterotoxins, lipase, deoxyribonuclease, lysozyme and TEP were carried out as described in Section 2.2.6.
4.2.8 Determination of \( \beta \)-galactosidase

Determinations of \( \beta \)-galactosidase were carried out by a modification of the method described by Eagon (1968). Five ml staphylococcal cell suspension were centrifuged at 10,000 rpm for 5 min, all centrifugations being carried out in a refrigerated centrifuge. The cells were then washed in cold distilled water, and resuspended in 1 ml cold distilled water. Using a micropipette, 0.2 ml cells was added to 7.8 ml 0.2 M sodium phosphate buffer pH 7.25, and 2 ml 0.01 M \( o \)-nitrophenyl-\( \beta \)-d-galactopyranoside (ONPG) (Calbiochem, L.A.) A blank was prepared in which 2 ml water replaced ONPG. Both tubes were incubated 3 h at 30 C. The cells were then centrifuged at 10,000 rpm for 5 min, and 1 ml supernatant added to 3 ml 0.3 M sodium carbonate to prevent further reaction and intensify the color. The color developed was read at 420 nm. A standard curve was prepared of ONP against OD at 420 nm (Fig.18), and one unit of \( \beta \)-galactosidase was taken as that which hydrolysed 1 \( \mu \)mole ONPG in 3 h at 30 C. Although it has been reported (Norse et al., 1968) that staphylococcal \( \beta \)-galactosidase is active only against phosphorylated ONPG, it was found that ONPG was a satisfactory substrate when intact cells were used, presumably due to the phosphorylation of ONPG by the cells in the process of accumulating the substrate (Norse et al., 1968).

4.2.9 Non-replicating cells

These were prepared from cells grown in AA medium in shake-flasks, as described in Section 2.2.5. Washed cells were resuspended in the appropriate medium so that a dilution of 1 in 200 gave an OD of approximately 0.20. Forty ml of cell suspension per 150 ml flask were then shaken at 37 C for specified times.

4.3 Results

4.3.1 Effect of glucose on enterotoxin B production in shake-flasks

In preliminary experiments, six enterotoxin B-producing strains of staphylococci were incubated in 50 ml AA medium in 250 ml
Figure 18. Standard curve for determination of \( \beta \)-galactosidase.
shake-flasks, with and without 2% glucose in the medium. The effect of glucose on enterotoxin B and TEP production during 24 h incubation is shown in Table 12. For all six strains, the production of enterotoxin B was almost completely repressed and TEP production was reduced by between 25 and 80% in the presence of glucose. In the control flasks without glucose, the pH increased from 6.5 to between 8.3 and 8.8, while in the glucose medium the pH decreased to between 5.0 and 6.0.

**TABLE 12. Effect of 2% glucose on enterotoxin B and TEP production by six strains of S. aureus in shake-flasks in AA medium, initial pH 6.5 during 24 h incubation.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose (per cent)</th>
<th>Final OD</th>
<th>Final pH</th>
<th>Enteroxin B (µg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-6</td>
<td>0</td>
<td>18</td>
<td>8.3</td>
<td>116</td>
<td>0.54</td>
</tr>
<tr>
<td>S-6</td>
<td>2</td>
<td>14</td>
<td>5.3</td>
<td>5</td>
<td>0.10</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>26</td>
<td>8.5</td>
<td>93</td>
<td>0.56</td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>21</td>
<td>6.0</td>
<td>10</td>
<td>0.20</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>21</td>
<td>8.4</td>
<td>61</td>
<td>0.65</td>
</tr>
<tr>
<td>28</td>
<td>2</td>
<td>22</td>
<td>5.4</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>20</td>
<td>8.5</td>
<td>93</td>
<td>0.47</td>
</tr>
<tr>
<td>29</td>
<td>2</td>
<td>26</td>
<td>5.6</td>
<td>4</td>
<td>0.35</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>20</td>
<td>8.5</td>
<td>98</td>
<td>0.47</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>22</td>
<td>5.2</td>
<td>3</td>
<td>0.10</td>
</tr>
<tr>
<td>39</td>
<td>0</td>
<td>20</td>
<td>8.8</td>
<td>116</td>
<td>0.80</td>
</tr>
<tr>
<td>39</td>
<td>2</td>
<td>21</td>
<td>5.0</td>
<td>9</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**4.3.2 Effect of added carbohydrates and related compounds on the production of enterotoxins and other extracellular proteins**

In further preliminary experiments the effect of 0.1 M glucose, pyruvate, glycerol, galactose and lactose on the production of extracellular proteins was determined for five strains of staphylococci in AA medium in shake-flasks. The addition of all five compounds caused an increase in the specific growth rate ($\mu$) in the five strains. The specific rate of product
formation \( q_p \) was calculated for the time interval which gave adequate enterotoxin levels with least change in pH. For convenience of presentation the results have been expressed as the percentage changes in \( q_p \) of the extracellular proteins examined for the five strains when grown in the presence of glucose, glycerol or pyruvate, (Tables 13A and 13B), and galactose or lactose (Tables 14A and 14B), as compared with control flasks where the strains were grown in AA medium only.

Glucose greatly decreased the production of all extracellular proteins investigated for all five strains. At the same time there was a marked drop in pH of the medium. The addition of glycerol produced a similar effect to that of glucose for all strains.

In the presence of pyruvate, there was a slight increase in pH similar to that in the control flasks. Pyruvate stimulated the production of deoxyribonuclease and lysozyme for all strains but the effect on other extracellular proteins varied greatly between strains. Strains 5-6 and 361 showed increases also in enterotoxin and lipase production, strains 100 and 30 showed a decrease in enterotoxin and lipase production, and strain 3 showed no change in enterotoxin or lipase production. The effects of galactose and lactose (Tables 14A and 14B) were intermediate between those of glucose and pyruvate, both compounds causing a decrease in the production of enterotoxin in three out of five strains and of lipase in four out of five strains.

It was possible that the differences in effects of various compounds, particularly the differences between pyruvate and glucose in their effects on enterotoxin and deoxyribonuclease, were simply a result of the differences in pH developed in the medium. Therefore the effect of glucose and glycerol, which had given the greatest repression of extracellular proteins and the most marked drop in pH of the medium, was investigated in the fermenter under conditions of controlled pH.
TABLE 13A. Effect of 0.1 M glucose, glycerol and pyruvate on the specific rate of formation \( q_p \)^a of enterotoxins by five strains of staphylococci in shake-flasks in AA medium during 24 h incubation.

<table>
<thead>
<tr>
<th>Strain and type</th>
<th>Carbon compound added</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Control pH</th>
<th>Percentage change in enterotoxin ( q_p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>glucose</td>
<td>6.05</td>
<td>5.65</td>
<td>7.20</td>
<td>-88</td>
</tr>
<tr>
<td>100 A</td>
<td>glycerol</td>
<td>6.05</td>
<td>5.90</td>
<td>7.20</td>
<td>-76</td>
</tr>
<tr>
<td>100 A</td>
<td>pyruvate</td>
<td>6.65</td>
<td>6.80</td>
<td>7.20</td>
<td>-29</td>
</tr>
<tr>
<td>S-6 B</td>
<td>glucose</td>
<td>6.20</td>
<td>5.60</td>
<td>6.80</td>
<td>-65</td>
</tr>
<tr>
<td>S-6 B</td>
<td>glycerol</td>
<td>6.25</td>
<td>5.75</td>
<td>6.80</td>
<td>-74</td>
</tr>
<tr>
<td>S-6 B</td>
<td>pyruvate</td>
<td>6.50</td>
<td>6.65</td>
<td>6.80</td>
<td>+7</td>
</tr>
<tr>
<td>30 B</td>
<td>glucose</td>
<td>5.55</td>
<td>5.20</td>
<td>6.90</td>
<td>-96</td>
</tr>
<tr>
<td>30 B</td>
<td>glycerol</td>
<td>5.95</td>
<td>5.65</td>
<td>6.90</td>
<td>-75</td>
</tr>
<tr>
<td>30 B</td>
<td>pyruvate</td>
<td>6.50</td>
<td>6.60</td>
<td>6.90</td>
<td>-32</td>
</tr>
<tr>
<td>361 C</td>
<td>glucose</td>
<td>5.50</td>
<td>5.20</td>
<td>7.15</td>
<td>-100</td>
</tr>
<tr>
<td>361 C</td>
<td>glycerol</td>
<td>6.0</td>
<td>5.60</td>
<td>7.15</td>
<td>-100</td>
</tr>
<tr>
<td>361 C</td>
<td>pyruvate</td>
<td>6.65</td>
<td>6.80</td>
<td>7.15</td>
<td>+270</td>
</tr>
<tr>
<td>3 C</td>
<td>glucose</td>
<td>5.30</td>
<td>5.05</td>
<td>7.0</td>
<td>-100</td>
</tr>
<tr>
<td>3 C</td>
<td>glycerol</td>
<td>5.50</td>
<td>5.10</td>
<td>7.0</td>
<td>-100</td>
</tr>
<tr>
<td>3 C</td>
<td>pyruvate</td>
<td>6.65</td>
<td>7.10</td>
<td>7.0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Time intervals for which figures are reported are 7-12 h (strain 100), 4-6 h (strains S-6 and 30), 6-9 h (strains 361 and 3).

^b pH at the beginning, and ^c pH at the end of the specified time interval for which \( q_p \) values are reported.

^d pH in the control flask at the end of the specified time interval.

^e Percentage change is relative to control containing no glucose, glycerol or pyruvate. -100 indicates complete repression.
### TABLE 13B. Effect of 0.1 M glucose, glycerol and pyruvate on the specific rate of formation ($q_p$)\(^a\) of extracellular proteins by five strains of staphylococcci in shake-flasks in AA medium during 24 h incubation.

<table>
<thead>
<tr>
<th>Strain and type</th>
<th>Carbon compound added</th>
<th>Percentage change(^b) in $q_p$ relative to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>THP</td>
</tr>
<tr>
<td>100 A glucose</td>
<td>-50</td>
<td>-100</td>
</tr>
<tr>
<td>100 A glycerol</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>100 A pyruvate</td>
<td>0</td>
<td>-65</td>
</tr>
<tr>
<td>S-6 B glucose</td>
<td>-41</td>
<td>-100</td>
</tr>
<tr>
<td>S-6 B glycerol</td>
<td>-42</td>
<td>-100</td>
</tr>
<tr>
<td>S-6 B pyruvate</td>
<td>+55</td>
<td>+20</td>
</tr>
<tr>
<td>30 B glucose</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>30 B glycerol</td>
<td>-57</td>
<td>-100</td>
</tr>
<tr>
<td>30 B pyruvate</td>
<td>-29</td>
<td>-81</td>
</tr>
<tr>
<td>361 C glucose</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>361 C glycerol</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>361 C pyruvate</td>
<td>+200</td>
<td>+100</td>
</tr>
<tr>
<td>3 C glucose</td>
<td>-94</td>
<td>-100</td>
</tr>
<tr>
<td>3 C glycerol</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>3 C pyruvate</td>
<td>+100</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Time intervals as in note 'a' Table 14 A.

\(^b\) See note 'e' Table 13A.
TABLE 14A. Effect of 0.1 M galactose and lactose on the production of enterotoxins and other extracellular proteins by five strains of staphylococci in AA medium in shake-flasks during 24 h.

<table>
<thead>
<tr>
<th>Extracellular protein</th>
<th>Percentage change(^{\text{a}}) in q(_p) relative to controls(^{\text{b}})</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 A</td>
<td>5-6 B</td>
<td>30 B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>Lactose</td>
<td>Galactose</td>
<td>Lactose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>-29</td>
<td>-35</td>
<td>+5</td>
<td>+16</td>
<td>-21</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>-20</td>
<td>+150</td>
<td>+40</td>
<td>-45</td>
<td>-100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0</td>
<td>+33</td>
<td>-70</td>
<td>0</td>
<td>-70</td>
</tr>
<tr>
<td>Lipase</td>
<td>-100</td>
<td>-46</td>
<td>+21</td>
<td>+4</td>
<td>-100</td>
</tr>
<tr>
<td>TEP</td>
<td>+100</td>
<td>0</td>
<td>+79</td>
<td>-10</td>
<td>+43</td>
</tr>
</tbody>
</table>

| Initial pH\(^{\text{c}}\) | 6.35 | 6.73 | 6.45 | 6.55 | 6.35 | 6.75 |
| Final pH\(^{\text{d}}\)  | 6.10 | 6.90 | 6.45 | 6.76 | 5.55 | 6.12 |
| pH of control flask     | 7.20 | 7.20 | 6.80 | 6.80 | 6.90 | 6.90 |
| Time interval hours     | 7-12 | 7-12 | 4-6  | 4-6  | 4-6  | 4-6  |

\(^{\text{a}}\) -100 indicates complete repression.

\(^{\text{b}}\) Control flasks contained no galactose or lactose.

\(^{\text{c}}\) pH at the beginning, and \(^{\text{d}}\) pH at the end of the specified time interval for which q\(_p\) values are reported.

\(^{\text{e}}\) pH given is that in control flask at end of specified time interval.
**Table 14B. Effect of 0.1 M galactose and lactose on the production of enterotoxins and other extracellular proteins by five strains of staphylococci in AA medium in shake-flasks during 24 h.**

<table>
<thead>
<tr>
<th>Extracellular protein</th>
<th>Percentage change(^a) in (q_p) relative to controls</th>
<th>Strain J - C</th>
<th>Strain J - C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galactose</td>
<td>Lactose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>+7</td>
<td>+70</td>
<td>-71</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>-21</td>
<td>-26</td>
<td>-72</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>+100</td>
<td>+200</td>
<td>+40</td>
</tr>
<tr>
<td>Lipase</td>
<td>-67</td>
<td>-88</td>
<td>-100</td>
</tr>
<tr>
<td>TEP</td>
<td>+100</td>
<td>+50</td>
<td>-23</td>
</tr>
<tr>
<td>Initial pH(^c)</td>
<td>6.24</td>
<td>6.55</td>
<td>6.15</td>
</tr>
<tr>
<td>Final pH(^d)</td>
<td>5.45</td>
<td>5.60</td>
<td>5.4</td>
</tr>
<tr>
<td>pH of control flask</td>
<td>7.15</td>
<td>7.15</td>
<td>7.0</td>
</tr>
<tr>
<td>Time interval (hours)</td>
<td>6-9</td>
<td>6-9</td>
<td>6-9</td>
</tr>
</tbody>
</table>

\(^a\) -100 indicates complete repression.
\(^b\) Control flasks contained no galactose or lactose.
\(^c\) pH at the beginning, and \(^d\) pH at the end of the specified time interval for which \(q_p\) values are reported.
\(^e\) pH given is that in control flask at end of specified time interval.
4.3.3  **Effect of glucose on enterotoxin and TEP production in the fermenter**

Figure 19 shows the effect of an initial concentration of 2% glucose on the production of enterotoxin B by strain S-6 during 12 h, and the utilization of glucose during that period. By 12 h, glucose concentration had dropped to 0.006%. Enterotoxin B concentration reached 50 μg/ml at a time when 1% glucose still remained in the medium. However, the final concentration of enterotoxin was 74% less than in the control experiment without glucose. At the same time the specific growth rate was 85% greater in the glucose experiment. Similar experiments were carried out with strains 100, 30 and 361, and the production of enterotoxins and TEP followed throughout growth. The effect of an initial concentration of 2% glucose for these strains during 12 h incubation is shown in Table 15.

The production of enterotoxin was markedly reduced for all four strains, although the TEP production was reduced only for strain 361. At the same time there was an increase in the specific growth rate of between 28 and 48%. During growth, the concentration of glucose in the medium decreased rapidly for strains S-6, 30 and 361. The slow utilization of glucose by strain 100 is indicative of the slower growth characteristic of this strain, particularly in AA medium.

4.3.4  **Effect of constantly maintained 0.1 M glucose and 0.1 M glycerol on the production of extracellular proteins in the fermenter**

As the final concentrations of glucose in the fermenter were extremely low, the experiments with strains 100, S-6, 30 and 361 were repeated with the concentration of glucose in the fermenter constantly maintained at 0.1 M. The production of enterotoxins, lipase, deoxyribonuclease and TEP were followed throughout growth and 12 h figures are given in Table 16. The reduction in enterotoxin production by strains 100, 30 and 361 was similar to that in the previous experiment. For strain S-6, the production of enterotoxin B was repressed by 60%, as compared to 43% when the glucose concentration was not
Figure 19  Growth and enterotoxin B production by strain S-6 in the fermenter in AA medium (open symbols), and in AA medium containing an initial concentration of 2% glucose (closed symbols).
OD, △, enterotoxin B, ○; glucose, ■.
TABLE 15. Effect of an initial concentration of 2% glucose on the production of enterotoxins and TEP by strains 100, S-6, 30 and 361 in the fermenter at pH 6.5, during 12 h incubation.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Initial glucose (per cent)</th>
<th>Final glucose (per cent)</th>
<th>Final OD</th>
<th>Specific growth rate (μ)</th>
<th>Enteroxin (μg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>0</td>
<td>0</td>
<td>26.8</td>
<td>0.38</td>
<td>32</td>
<td>0.05</td>
</tr>
<tr>
<td>100 A</td>
<td>2</td>
<td>1.5</td>
<td>14.0</td>
<td>0.71</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>S-6 B</td>
<td>0</td>
<td>0</td>
<td>24.8</td>
<td>0.46</td>
<td>129</td>
<td>0.84</td>
</tr>
<tr>
<td>S-6 B</td>
<td>2</td>
<td>0.006</td>
<td>23.2</td>
<td>0.86</td>
<td>75</td>
<td>1.36</td>
</tr>
<tr>
<td>30 B</td>
<td>0</td>
<td>0</td>
<td>22.4</td>
<td>0.45</td>
<td>136</td>
<td>0.89</td>
</tr>
<tr>
<td>30 B</td>
<td>2</td>
<td>0.003</td>
<td>26.8</td>
<td>0.86</td>
<td>32</td>
<td>1.42</td>
</tr>
<tr>
<td>361 C</td>
<td>0</td>
<td>0</td>
<td>22.8</td>
<td>0.47</td>
<td>53</td>
<td>1.02</td>
</tr>
<tr>
<td>361 C</td>
<td>2</td>
<td>0.004</td>
<td>24.6</td>
<td>0.65</td>
<td>10</td>
<td>0.38</td>
</tr>
</tbody>
</table>
### TABLE 16. Effect of 0.1 M glucose and 0.1 M glycerol on the production of enterotoxins A, B and C and other extracellular proteins by four strains of staphylococci during 12 h incubation at pH 6.5 in the fermenter.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Strain and enterotoxin type</th>
<th>CD</th>
<th>Specific growth rate (µl)</th>
<th>Enterotoxin (µg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 A</td>
<td>26.8</td>
<td>0.38</td>
<td>32</td>
<td>5</td>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>100 A</td>
<td>14.2</td>
<td>0.71</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>S-6 B</td>
<td>25.8</td>
<td>0.46</td>
<td>129</td>
<td>240</td>
<td>37</td>
<td>0.84</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>S-6 B</td>
<td>36</td>
<td>0.91</td>
<td>51</td>
<td>147</td>
<td>500</td>
<td>0.46</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>S-6 B</td>
<td>35</td>
<td>0.82</td>
<td>29</td>
<td>34</td>
<td>450</td>
<td>0.55</td>
</tr>
<tr>
<td>Control</td>
<td>361 C</td>
<td>22.8</td>
<td>0.47</td>
<td>53</td>
<td>320</td>
<td>8</td>
<td>1.02</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>361 C</td>
<td>24.6</td>
<td>0.80</td>
<td>15</td>
<td>201</td>
<td>14</td>
<td>0.36</td>
</tr>
<tr>
<td>Control</td>
<td>30 B</td>
<td>22.4</td>
<td>0.45</td>
<td>136</td>
<td>17</td>
<td>25</td>
<td>0.89</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>30 B</td>
<td>28.8</td>
<td>0.82</td>
<td>26</td>
<td>7</td>
<td>125</td>
<td>0.70</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>30 B</td>
<td>26.4</td>
<td>1.18</td>
<td>35</td>
<td>13</td>
<td>246</td>
<td>0.68</td>
</tr>
</tbody>
</table>
maintained. All four strains showed a marked decrease in lipase production, and three out of four a decrease in TEP production in the presence of glucose. At the same time the specific growth rate increased for all four strains, with a shorter transition period from exponential to stationary phase of growth, and a reduction in the length of time for which enterotoxin is produced. This is shown for strain S-6 in Fig. 20.

The effect of 0.1 M glycerol on enterotoxin, lipase, deoxyribonuclease and TEP production was determined for strains S-6 and 30 (Table 16). As with glucose, there was an increase in growth rate, and a decrease in the production of enterotoxin B, lipase and TEP.

In an attempt to determine the effect of glucose on enterotoxin production independently of growth rate and the time for which enterotoxin was produced, the specific rate of enterotoxin \( (q_p) \) was calculated for the period of maximum enterotoxin production as described in Section 2.2.7 and is shown in Table 16. For all four strains, \( q_p \) shows a considerable decrease in the presence of 0.1 M glucose or 0.1 M glycerol.

Deoxyribonuclease production showed a different response to the presence of 0.1 M glucose or glycerol from that of the other proteins assayed. (Table 16). Deoxyribonuclease production was increased for all four strains, showing a 13- and 5-fold increase in the presence of glucose and a 12- and 10-fold increase in the presence of glycerol for strains S-6 and 30 respectively.

4.3.5 Effect of adding glucose during exponential growth on the production of enterotoxin

In a further experiment 2% glucose was added to a culture of strain S-6 growing in the fermenter at pH 6.5, when the culture had been incubated for 4 h. At this point the culture was in the exponential phase of growth and enterotoxin production had just begun. The specific rate of enterotoxin production \( (q_p) \) value under these conditions was 1.52, as compared with 1.34 when 2% glucose was added with the inoculum and 2.12 when no glucose was in the medium at all. The total
Figure 20  Growth and enterotoxin B production by strain S-6 in AA medium (open
symbols) and in AA medium with glucose concentration maintained at 0.1 M
(closed symbols).
OD, △; enterotoxin B, ○.
yield of enterotoxin was 72 µg/ml when glucose was added at 4 h, as compared with 75 µg/ml when glucose was added with the inoculum and 129 µg/ml when no glucose was added to the medium.

4.3.6 Effect of omitting thiamine from AA medium

It has been reported (Morse and Baldwin 1971) that for S. aureus S-6, elimination of thiamine from a casein hydrolysate medium partially or completely reversed the repression of enterotoxin B synthesis by glucose. As the AA medium used throughout this investigation contained 0.5 µg/ml thiamine, it was decided to investigate the effect on glucose repression of omitting thiamine from the medium. Strain S-6 was grown in AA medium for 16 h, the cells were then washed four times in 0.01 M phosphate buffer, pH 7.0, to remove traces of thiamine, and used to inoculate 200 ml medium, with and without thiamine, in 1 litre shake-flasks. Growth, pH, TEP and enterotoxin B production were followed for 24 h.

In the flask without thiamine, the specific growth rate was 0.50, as compared with 0.51 in the usual AA medium. In both flasks, enterotoxin B production was 150 µg/ml, and TEP production 0.48 mg/ml after 24 h incubation. The final OD and pH were 15.6 and 7.9 respectively in the control flask as compared with 17.0 and 8.0 in the absence of thiamine.

An experiment was then carried out to determine the effect of glucose on enterotoxin production in the presence and absence of thiamine. In order to prevent any carry-over of thiamine with the inoculum, cells were grown in AA medium without thiamine and washed four times in buffer before being used to inoculate two shake-flasks. Both flasks contained 0.1 M glucose. One flask contained the usual concentration of thiamine in AA medium, and one contained no thiamine. Growth, pH, TEP and enterotoxin B production, and glucose utilization were followed throughout growth. Again, it was found that the omission of thiamine from AA medium made no significant differences to any of these parameters. In both flasks enterotoxin B production was reduced to 10 µg/ml and the
final pH was 5.05. The utilization of glucose was very similar in both flasks. In the control flask, glucose concentrations were 0.081, 0.068 and 0.045 M at 5.5, 7.7 and 24 h. In the flask from which thiamine was omitted, glucose concentrations were 0.084, 0.063 and 0.057 M at these times.

It is evident that the omission of thiamine from AA medium had no effect on growth, enterotoxin B production or glucose utilization by **E. aerogen S-6**. Thiamine has been shown to play a highly significant part in the metabolism of glucose by staphylococci (Blumenthal, 1972). It is probable therefore that the results presented here indicate that in AA medium, strain S-6 is able to synthesize its thiamine requirements. This finding would be in agreement with the reports of Idriss and Blumenthal (1967) and Mah et al. (1967) that the vitamin requirements of staphylococci depended partly on the growth medium.

4.3.7 Effect of glucose on enterotoxin production under conditions of reduced Mg^{++}

Enterotoxin synthesis in the fermenter experiment at controlled pH was never completely repressed by glucose. It has been shown (Mandelstam 1961, Palmer and Mallette, 1961) that in **E.coli** the repression of β-galactosidase by glucose was increased when the supply of other nutrients was limited. To demonstrate whether or not this also applied to the apparent catabolite repression of enterotoxin synthesis by glucose, the effect of glucose was studied under conditions of reduced Mg^{++} concentration.

The concentrations of Mg^{++} that would limit growth were determined in shake-flasks for strains 100, S-6 and 361 as a preliminary to fermenter experiments designed to determine whether such an increase in catabolite repression could be obtained with enterotoxin production. The relationship between Mg^{++} concentration and specific growth rate (μ), cell yield as measured by increase in OD, and enterotoxin B production during 12 h is shown for strain S-6 in Fig. 21. A decrease in Mg^{++} from 0.4 mM to 0.1 mM reduced μ from 0.49 to 0.375, the cell yield from 11.2 to 5.0, and enterotoxin B production from 104 µg/ml to 16 µg/ml. The large reduction in entero-
Figure 21  Specific growth rate ($\mu$) $\triangle$; increase in OD, $\circ$; and production of enterotoxin B, $\square$; by strain S–6 during 12 h incubation in AA medium in shake-flasks with different initial Mg++ concentrations.
toxin B production as compared with the decrease in cell yield suggested that enterotoxin B production was affected by the concentration of Mg^{++} independently of the effect on growth. It was therefore decided to use 0.2 mM Mg^{++} which could be expected to limit growth, but still give adequate yields of enterotoxin B.

For strains 100 and 361 it was found in shake-flasks that growth rates and cell yields of these strains were less sensitive than strain S-6 to a reduction in the concentration of Mg^{++}. It was decided to use 0.04 and 0.08 mM Mg^{++} for strains 100 and 361, respectively, in fermenter experiments.

These three strains were then used to determine the effect of 0.1 M glucose on the production of enterotoxin and other extracellular proteins at pH 6.5 in the fermenter, with the reduced concentration of Mg^{++} described above. Although the reduction in Mg^{++} from 0.4 mM to 0.2 mM was not sufficient to reduce the specific growth rate for strain S-6 in the fermenter, the cell yield was decreased by 55\% without glucose, and by 77\% in the presence of glucose (Fig. 22), by the reduction in Mg^{++} concentration (compare Tables 16 and 17). In the control experiments without glucose, the specific rate of production of enterotoxin was reduced by 43\% when the Mg^{++} concentration was reduced by 50\%.

The total yield of enterotoxin B was reduced by 94\% and the specific rate of enterotoxin production was reduced by 91\% in the presence of glucose and 0.2 mM Mg^{++} (Table 17). This can be compared with a 60\% reduction in the yield of enterotoxin B, and a 36\% reduction in the specific rate of enterotoxin B production in the presence of glucose with the higher level of 0.4 mM Mg (Table 16). Similarly, the reduction in lipase and TEP production in 0.2 mM Mg^{++} in the presence of glucose was also greater than the reduction due to glucose in 0.4 mM Mg^{++}. However, at the lower concentration of Mg^{++}, the stimulation of deoxyribonuclease production by glucose was not observed.

For strains 100 and 361, reducing the concentration of Mg^{++} to 0.04 and 0.08 mM respectively caused a reduction in the
Figure 22 Growth and enterotoxin B production in AA medium containing a reduced concentration of Mg++ (0.2 mM) without glucose (open symbols) and with glucose maintained at 0.1 M (closed symbols). OD, Δ; enterotoxin B, ○.
<table>
<thead>
<tr>
<th>Strain and type</th>
<th>Glucose (molarity)</th>
<th>Final OD</th>
<th>Specific growth rate (µ)</th>
<th>Enterotoxin (µg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 A</td>
<td>0</td>
<td>3.5</td>
<td>0.34</td>
<td>Tr</td>
<td>3.6</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>100 A</td>
<td>0.1</td>
<td>3.3</td>
<td>0.33</td>
<td>Tr</td>
<td>2</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>S-6 B</td>
<td>0</td>
<td>11.5</td>
<td>0.41</td>
<td>35</td>
<td>70</td>
<td>17</td>
<td>0.28</td>
</tr>
<tr>
<td>S-6 B</td>
<td>0.1</td>
<td>8.3</td>
<td>0.98</td>
<td>2</td>
<td>8</td>
<td>15</td>
<td>0.08</td>
</tr>
<tr>
<td>361 C</td>
<td>0</td>
<td>9.0</td>
<td>0.56</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>361 C</td>
<td>0.1</td>
<td>8.4</td>
<td>0.62</td>
<td>ND</td>
<td>0</td>
<td>2</td>
<td>0.01</td>
</tr>
</tbody>
</table>
specific growth rate and cell yield (Table 17) as compared with 0.4 mM Mg++ (Table 16). The production of enterotoxin C and lipase by strain 361 was completely repressed, and the production of TEP by strain 100 50% repressed in the presence of glucose. However, there was such a large decrease in all extracellular proteins measured at these reduced concentrations of Mg++ that it was not possible from these experiments to determine conclusively the effect of reduced Mg++ on the repression of these enterotoxins by glucose.

4.3.8 Effect of glucose and glycerol on β-galactosidase and extracellular protein production by non-replicating cells of staphylococci.

In fermenter and shake-flask experiments, it was found that reduced enterotoxin production in the presence of glucose or glycerol was always accompanied by an increase in the specific growth rate. In order to determine whether the effect of glucose and glycerol on enterotoxin production occurred independently of the change in growth rate, experiments were carried out in which the production of enterotoxins by non-replicating cells in the presence and absence of glucose and glycerol was investigated.

Staphylococcal cells are known to produce β-galactosidase in the presence of a suitable inducer (McClatchy and Rosenblum, 1963). It was therefore decided to add an inducer of β-galactosidase to non-replicating cells of staphylococci, as this would allow comparisons to be made with an enzyme known to be subject to catabolite repression (Blumenthal, 1972). Non-replicating cells were grown in AA medium in shake-flasks harvested at 16 h and washed in buffer as described in Section 2.2.5. Preliminary experiments showed that if cells of strain 100, ε-6, 30 or 361 were resuspended in AA medium containing 1% galactose, and incubated in shake-flasks at 37 C, β-galactosidase was induced. Neither IPTG nor melibiose was found to be a suitable inducer for staphylococcal β-galactosidase, in agreement with reports of Hengstenberg et al., (1968). The incubation of non-replicating cells with glucose or glycerol produced a considerable decrease in pH.

Further preliminary experiments were therefore carried out
with strain S-6 to determine the concentration of phosphate buffer which would prevent a large decrease in pH (Table 18).

**TABLE 18.** Effect of 0.2 M and 0.4 M phosphate buffer pH 7.0 on pH changes in AA medium produced by non-replicating cells of *S. aureus* S-6 in the presence and absence of glucose.

<table>
<thead>
<tr>
<th>Phosphate buffer (molarity)</th>
<th>Glucose (molarity)</th>
<th>pH at 2 h</th>
<th>pH at 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7.8</td>
<td>8.5</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
<td>6.2</td>
<td>5.4</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>7.2</td>
<td>7.4</td>
</tr>
<tr>
<td>0.2</td>
<td>0.1</td>
<td>6.7</td>
<td>6.4</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>0.4</td>
<td>0.1</td>
<td>6.8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

It was found that while in the absence of buffer, glucose caused a fall in pH to 5.4; in 0.2 and 0.4 M buffer, the pH decreased to 6.4 and 6.7 respectively from an initial pH of 7.0.

The effect of 1½% galactose and two concentrations of phosphate buffer on the production of enterotoxin B, lipase, deoxyribonuclease and TEP by non-replicating cells of strain S-6 during 4 h incubation was then determined (Table 19). The addition of 1½% galactose had no effect on the production of enterotoxin B or TEP and caused an increase in the production of lipase and deoxyribonuclease. This concentration of galactose was therefore used in subsequent experiments where β-galactosidase induction was required. However, 0.2 M buffer decreased the production of enterotoxin, lipase and deoxyribonuclease, and TEP by 50, 52, 65 and 25% respectively. The production of these extracellular proteins was further decreased in the presence of 0.3 M buffer. It was therefore decided to use 0.2 M phosphate buffer, as giving satisfactory pH control, and allowing enterotoxin production which though partially reduced, was still adequate to detect differences produced by changes in environment.
The difference in glucose effect on enterotoxin B and \( \beta \)-galactosidase production by non-replicating cells of strain S-6 is shown in Fig. 23. \( \beta \)-galactosidase synthesis was almost completely inhibited by the presence of glucose. By contrast, glucose stimulated the production of enterotoxin up to 2 h. After 3 h incubation, pH had decreased to 6.0 in glucose, as compared with 7.0 in the control flask, and there had been a corresponding small decrease in enterotoxin production.

Table 20 shows the effects of 0.1 M glucose and 0.1 M glycerol on the production of enterotoxins, lipase, deoxyribonuclease, TEP and \( \beta \)-galactosidase by non-replicating cells of strains 100, S-6, 30 and 361. Figures for strains S-6, 30 and 361 are those after 2 h incubation, when the decrease in pH was not sufficient to affect enterotoxin production. For strain 100, 4 h figures are given because of the low yields of extracellular proteins from this strain. Enterotoxin production was similar to or greater in the presence of glucose compared to that in the control flasks, where glucose was absent. However, at the same time the synthesis of \( \beta \)-galactosidase was almost completely inhibited by 0.1 M glucose. Lipase and TEP production showed no significant decreases in the presence of glucose except for strain 100, which showed a decrease in lipase production. Deoxyribonuclease production after only 2 h incubation was low for all four strains, but there was a small decrease in the presence of glucose for strain S-6.
Figure 23  Effect of 0.1 M glucose on enterotoxin B and β-galactosidase production by non-replicating cells of strain S-6 without glucose (open symbols) and with 0.1 M glucose (closed symbols).
β-galactosidase, △; enterotoxin B, ○.
TABLE 20. Effect of 0.1 M glucose and glycerol on the production of enterotoxins A, B and C and other extracellular proteins, and of β-galactosidase, by non-replicating cells of staphylococci during 2 h\(^\text{a}\) incubation.

<table>
<thead>
<tr>
<th>Strain and enterotoxin type</th>
<th>Enterotoxin (µg/ml)</th>
<th>β-galactosidase (units/ml)</th>
<th>TEP (mg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 A</td>
<td>2</td>
<td>0.058</td>
<td>0.14</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>100 A</td>
<td>2</td>
<td>0.003</td>
<td>0.12</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>100 A</td>
<td>1</td>
<td>0.005</td>
<td>0.10</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>S-6 B</td>
<td>29</td>
<td>0.022</td>
<td>0.15</td>
<td>46</td>
<td>13</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>S-6 B</td>
<td>40</td>
<td>0.001</td>
<td>0.20</td>
<td>57</td>
<td>10</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>S-6 B</td>
<td>35</td>
<td>0.005</td>
<td>0.21</td>
<td>46</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>361 C</td>
<td>10</td>
<td>0.022</td>
<td>0.10</td>
<td>55</td>
<td>Trace</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>361 C</td>
<td>12</td>
<td>0.005</td>
<td>0.11</td>
<td>55</td>
<td>Trace</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>361 C</td>
<td>10</td>
<td>0.007</td>
<td>0.11</td>
<td>55</td>
<td>Trace</td>
</tr>
<tr>
<td>Control</td>
<td>30 B</td>
<td>6</td>
<td>0.008</td>
<td>0.20</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>0.1 M glucose</td>
<td>30 B</td>
<td>11</td>
<td>0</td>
<td>0.18</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>0.1 M glycerol</td>
<td>30 B</td>
<td>5</td>
<td>0</td>
<td>0.18</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Strain 100 figures at 4 h.
Glycerol produced a similar effect to glucose for all strains, except that glycerol exerted a slightly less inhibitory effect than glucose on $\beta$-galactosidase.

4.3.9 Effect of chloramphenicol and actinomycin D on the production of enterotoxin B and $\beta$-galactosidase by non-replicating cells

It was possible that the production of enterotoxins by non-replicating cells in glucose might be due to the presence of a precursor (Markus and Silverman, 1969) or of a pool of mRNA, (Coleman, 1967) which enabled enterotoxin production to continue. However, it was found (Fig. 24) that enterotoxin B production by non-replicating cells of strain S-6 was completely inhibited by 100 $\mu$g/ml chloramphenicol, in agreement with the findings reported in Section 2.3.6. The production of $\beta$-galactosidase was similarly inhibited by chloramphenicol.

Actinomycin D is reported to inhibit the production of mRNA without inhibiting protein synthesis (Hash, 1972). Non-replicating cells of strain S-6 were incubated for 3 h with 10 units/ml of actinomycin D and the production of enterotoxin B and $\beta$-galactosidase followed (Fig. 24). A small amount (7 $\mu$g/ml) of enterotoxin was produced as compared with 59 $\mu$g/ml in the control, suggesting the presence of a small pool of mRNA. However, $\beta$-galactosidase was completely and immediately repressed, showing that the delay in the repression of enterotoxin B was not due to a delay in the penetration of the cells by actinomycin D.

4.3.10 Production of enterotoxin B by non-replicating cells of S. aureus S-6 grown in the presence of glucose

Experiments had shown that the production of enterotoxin by non-replicating cells was inhibited by chloramphenicol, and therefore required new protein synthesis. However, it was possible that cells grown in AA medium without glucose and resuspended in the presence of glucose already contained some necessary prerequisite, possibly an enzyme involved in enterotoxin synthesis. If the production of this were inhibited
Figure 24  Effect of 100 μg/ml chloramphenicol and 10 μg/ml actinomycin B on enterotoxin B production by non-replicating cells of strain S-6.
control, △; control + 10 μg/ml actinomycin D, ○; control + 100 μg/ml chloramphenicol, □.
by glucose in growing cells, this would explain the decrease in enterotoxin production in growing cells, and the absence of glucose repression in non-replicating cells. To test this hypothesis the following experiment was carried out:

Strain S-6 was grown in AA medium in shake-flasks containing 0.1 h glucose and harvested at 16 h. The cells were washed four times in phosphate buffer and resuspended in the presence and absence of glucose in AA medium containing phosphate buffer. The cells were resuspended so that a dilution of 1/200 gave an OD of approximately 0.20, and incubated on the shaker as described in Section 2.2.5. During 2 h incubation, only 2 µg/ml enterotoxin B was produced in each flask. Growing cells in the presence of glucose had therefore almost completely inhibited the production of enterotoxin when the cells were resuspended with or without glucose. At the same time, cells grown in the absence of glucose produced 90 µg/ml of enterotoxin when resuspended in glucose-containing medium, and in agreement with earlier results (Section 4.3.9) enterotoxin production was completely repressed by addition of chloramphenicol to the medium.

4.3.11 Effect of cyclic adenosine 3',5'-monophosphate (cAMP) on enterotoxin B production

Cyclic AMP has been reported (Perlman and Fastan, 1968), to overcome glucose repression of β-galactosidase, and to stimulate the production of β-galactosidase in the absence of glucose. Before attempting to investigate the effect of cAMP on glucose repression in staphylococci, experiments were carried out to duplicate the work of Perlman and Fastan.

E coli K12 B240 (λ+) was grown for 16 h in medium A (Section 4.2.2). Twenty-five mls of this culture were added to 200 ml fresh medium A. After 4 h incubation, the culture was actively growing in the exponential phase of growth. The cells were then centrifuged at 10,000 rpm for 15 min, washed in 0.01 M phosphate buffer (pH 7.0), and resuspended in medium A so that the OD of a 1/10 dilution was approximately 0.50. Twenty-five ml of cell suspension were then incubated in each of three 100 ml shake-flasks as follows: Flask 1
(control flask) contained $5 \times 10^{-4}$ M isopropyl- -D-thiogalactoside (IPTG) (Sigma Chemical Co), to induce $\beta$-galactosidase. Flask 2 contained $5 \times 10^{-4}$ M IPTG and 0.1 M glucose. Flask 3 contained $5 \times 10^{-4}$ M IPTG, 0.1 M glucose and $5 \times 10^{-3}$ M cAMP (Sigma Chemical Co). The flasks were incubated on the shaker at 37°C, and OD, pH and $\beta$-galactosidase determinations carried out after 10, 20, 40 and 80 min incubation. For $\beta$-galactosidase determinations, 2.5 ml cells were centrifuged, washed in cold distilled water and resuspended in 1 ml cold distilled water. 0.2 ml cell suspension was incubated at 30°C with ONPG-buffer mixture, and $\beta$-galactosidase activity measured as the hydrolysis of ONPG after 20 min.

It was found (Fig. 25) after 80 min incubation that the production of $\beta$-galactosidase in the presence of glucose was only 20% of that in the control flask. cAMP overcame glucose repression, and increased the production of $\beta$-galactosidase to 60% above that in the control flask. During this time there was an approximately three-fold increase in OD, and a decrease in pH from 7.0 to 6.9 in all three flasks.

Similar experiments were then carried out with S. aureus strain 30, which produces higher yields of $\beta$-galactosidase than strain S-6. Since staphylococci require an organic N source, medium A was supplemented with 4% trypticase (medium AT). Also, in the staphylococcal experiments $1/3$ galactose replaced IPTG as an inducer of $\beta$-galactosidase. Cells were grown in AA medium, harvested at 16 h and washed in phosphate buffer. The cells were resuspended in medium AT so that the OD of 1/10 dilution was approximately 0.5, and 25 ml per 100 ml shake-flask incubated for 3 h. After 1, 2 and 3 h, OD and pH were measured, and $\beta$-galactosidase determinations were carried out. Five ml cells were washed in cold distilled water and resuspended in 1 ml cold distilled water, 0.2 ml of resuspended cells being incubated with ONPG and buffer. $\beta$-galactosidase activity was measured as the hydrolysis of ONPG after 3 h incubation at 30°C. The production of $\beta$-galactosidase during 3 h incubation in the presence of glucose was 0.001 units/ml as compared with 0.026 units/ml in the control flask without glucose. The addition of cAMP did not overcome the
Figure 25  Effect of c-AMP on glucose repression of β-galactosidase production by E. coli.
control Δ; control + 0.1 M glucose, ○; control + 0.1 M glucose + 5 x 10^{-3}M cAMP, •.
repression of β-galactosidase by glucose. The experiment was repeated with the concentration of cAMP increased to $10^{-1}$ M. This concentration reduced the pH of the medium to 5.1 and it was readjusted to 7.0 with 2 N NaOH. However, this higher concentration of cAMP still did not overcome glucose repression of β-galactosidase. During 3 h incubation, the OD of the cell suspension increased approximately two-fold in the control flask, and three-fold in the flasks containing glucose. The pH remained at 7.0 in the control flask and decreased to 6.4 in the presence of glucose.

Enterotoxin production during 3 h incubation of these cells growing in the presence of glucose was reduced from 32 to 21 μg/ml. In the presence of glucose and cAMP, 19 μg/ml of enterotoxin was produced, indicating that cAMP did not overcome the repression of enterotoxin B by glucose for strain 30.

It was possible that the difference in the effect of cAMP obtained with E.coli and S.aureus was due to the difference in the medium in the two experiments. The work which had been carried out with E.coli was therefore repeated with 4.5 trypsin added to the medium, and 1% galactose replacing IPTG as an inducer of β-galactosidase. Although galactose was a less effective inducer than IPTG, it was again found that the repression of β-galactosidase production by glucose was overcome by $5 \times 10^{-3}$ M cAMP.

4.4 Discussion

4.4.1 The production of enterotoxins at constant pH in the presence of 0.1 M glucose or 0.1 M glycerol

Growth of staphylococci in the presence of glucose or glycerol in shake-flasks resulted in an almost complete repression of enterotoxins and other extracellular proteins studied. Since metabolism of glucose or glycerol under these conditions leads to a large decrease in pH, the very low extracellular protein production could be due to the low pH, rather than to repression by glucose (Metzger et al., 1973). However, when strains 100, S-6 and 361 were grown in the fermenter under controlled conditions of pH and aeration, the production of
enterotoxins A, B and C was still greatly reduced, although not completely repressed. Thus it is clear that glucose inhibits enterotoxin production independently of its effect on pH. This finding is in agreement with the conclusions of Morse et al., (1969) and Morse and Mah (1973) for enterotoxin B, although their studies were carried out in complex media and without highly controlled conditions of pH. Miller and Fung (1973) similarly reported that in shake-flasks, using the defined AA medium of Wu and Börgdoff (1971b), enterotoxin B production was repressed by glucose. However, in their experiments during 24 h incubation the concentration of glucose decreased from 1% to 0.06%, and the pH decreased from 6.0 to 5.0. In the same medium without glucose, the pH increased from 6.0 to 7.5 during 24 h.

The findings presented here are in contrast to those of Netzger et al., (1973). These workers grew strain S-6 at pH 7 in a fermenter in a protein hydrolysate medium, and concluded that pH rather than glucose appeared to be the limiting factor in the release of enterotoxin B. However, the initial glucose concentration in the medium was only 0.2%, and this was not maintained throughout the fermentation. There was no significant enterotoxin production during the first three hours incubation. In the fermenter without pH control, the pH began to increase at that point, and it is probable that the glucose in the medium had been depleted.

4.4.2 Glucose repression of enterotoxin in relation to the synthesis of other extracellular proteins

The production of enterotoxins, lipase and TEP was repressed by glucose or glycerol, with or without pH control. However, when the pH was held at 6.5, the production of deoxyribonuclease was stimulated. Production of deoxyribonuclease by strain S-6 was increased 13-fold in the presence of glucose and 12-fold in the presence of glycerol. Production by strain 30 increased 5-fold in the presence of glucose, and 10-fold in the presence of glycerol.

Stormonth and Coleman (1973) studied the production of three extracellular enzymes in *Bacillus amyloliquefaciens* and
postulated that all extracellular enzymes are under a common regulatory system. The data presented here indicate that in staphylococci there is more than one control mechanism for the production of extracellular proteins.

4.4.3. Relation of the change in the specific growth rate in the presence of glucose or glycerol to the repression of enterotoxin production

The decrease in the production of enterotoxins and other extracellular proteins by glucose or glycerol was invariably accompanied by an increase in the specific growth rate. This was so in the fermenter or shake-flasks, although in the fermenter where the pH was controlled the increase in growth rate was much greater, and the final cell yield generally higher, than in shake-flasks. This is in agreement with the finding of Handelstam (1962) and Gallo and Katz (1973) that the carbon sources which support a rapid growth rate were most effective in producing catabolite repression. However, an increase in growth rate due to an added substrate was not always accompanied by repression of extracellular proteins, since pyruvate which produced an increase in growth rate similar to that caused by glucose in shake-flasks, increased the production of enterotoxin and lipase for strains S-6 and 361. Neidhardt and Magazanik (1956) reported that for E. coli, glucose and glycerol produced a similar increase in growth rate, but glucose caused much greater repression of β-galactosidase than glycerol.

The increase in specific growth rate in the fermenter in the presence of glucose or glycerol was associated with an exponential phase of shorter duration, and a corresponding decrease in the length of time for which enterotoxins were produced. However, by calculating the specific growth rate of enterotoxin production per unit of cell mass \( \dot{q}_p \) it was possible to obtain a figure for enterotoxin production which was independent of the rate of growth, and the length of time during which enterotoxin was being produced. There was a considerable decrease in the specific rate of enterotoxin production in the presence of glucose or glycerol. However, for three out of four strains, the decrease in the final
concentration of enterotoxin caused by glucose was greater than the decrease in the specific rate of enterotoxin production. This indicates that the decrease in enterotoxin concentration at 12 h resulted partly from a decrease in the rate of production, and partly from a change in growth pattern in the presence of glucose.

4.4.4 Effect of glucose on the production of enterotoxins under conditions of reduced Mg++

*S. aureus* 3-6 was grown at pH 6.5, in the presence and absence of glucose, with the concentration of Mg++ in the medium reduced from 0.4 mM to 0.2 mM. This reduction in the concentration of Mg++ resulted in a marked decrease in the cell yield. At the same time, there was a considerable increase in the degree of repression of enterotoxin B, as measured by the specific rate of enterotoxin production ($q_p$). The degree of repression by glucose of lipase and TEP production was also greater when the concentration of Mg++ was reduced. Reduction in Mg++ concentration thus had the converse effect on glucose repression to that reported when thiamine was omitted from the medium (Horse and Baldwin, 1971). It has been postulated (Paigen and Williams (1970)), that the concentration in a bacterial cell of the intermediates in the metabolism of glucose which are responsible for glucose repression will depend both on the rate at which these compounds are formed, and the rate at which they are further metabolised. It is possible that the decrease in Mg++ concentration in these experiments affected the metabolism of glucose in such a way that there was an accumulation of some intermediate in the metabolism of glucose which was responsible for glucose repression.

4.4.5 Production of enterotoxins and $\beta$-galactosidase by non-replicating cells of staphylococci

Non-replicating cells of staphylococci produced enterotoxins and $\beta$-galactosidase in the presence of an exogenous source of N. The production of both these proteins was dependent on new protein synthesis. There have been similar reports of $\beta$-galactosidase production by resting cells of *E. coli*
(Magasanik, 1957), in which \( \beta \)-galactosidase was synthesised using endogenous sources of N, but again inhibition of protein synthesis prevented the production of \( \beta \)-galactosidase. The addition of 0.1 M glucose or glycerol resulted in an almost complete repression of \( \beta \)-galactosidase synthesis for four strains of staphylococci. However, the production of enterotoxin was not significantly decreased in the presence of glucose or glycerol, glucose even increasing the production of enterotoxins for three out of the four strains. At the same time, there were no significant decreases in TEF or lipase production by non-replicating cells in the presence of glucose or glycerol for three out of four strains. These findings are similar to those of Mandelstam (1961), that \( \beta \)-galactosidase synthesis by resting cells of \textit{E. coli} was inhibited by added carbon sources, but at the same time there was a slight increase in the production of total extracellular protein. However, it is noticeable that glycerol causes greater repression of \( \beta \)-galactosidase production in staphylococci than has been reported for \textit{E. coli} (Reidhardt and Magasanik, 1956). These findings suggest that the mechanism for repression of enterotoxins by glucose is not the same as that for repression of \( \beta \)-galactosidase in staphylococci. Such conclusions are in agreement with other reports that catabolite repression of protein synthesis may be mediated in different ways in the same organism (Tanaka and Iuchi, 1971, Rotham-denes et al., 1973).

Experiments with chloramphenicol indicated that the production of enterotoxin in the presence of glucose or glycerol was not due to the presence of a protein precursor in the resting cells. The production of a small amount of enterotoxin B in the presence of actinomycin D was also reported by Katsuno and Kondo (1973). These results are similar to the findings of Both et al., (1972) who showed that continued production of protease occurs in the presence of actinomycin D, but not in the presence of chloramphenicol. They presented evidence which indicated that there was present in harvested cells an accumulated pool of mRNA which supported enzyme synthesis for a period after an inhibitor of RNA synthesis had been added to the cell suspension.
However, the amount of enterotoxin B produced in the presence of actinomycin D indicated a very much smaller mRNA pool than was found by these workers in *B. subtilis*.

Although it was shown that new protein synthesis was required for enterotoxin production by non-replicating cells, experiments with strain S-6 indicated that cells grown in the absence of glucose contained some prerequisite for enterotoxin B production that was lacking in cells grown in the presence of glucose. Thus, cells grown in glucose and harvested at 16 h were not capable of producing enterotoxin B when resuspended in a non-replicating state, either with or without glucose.

The experiments with resting cells were undertaken in an attempt to determine whether glucose and glycerol repressed the production of enterotoxins under conditions where there was no increase in growth rate associated with the added compound. However, the difference between the effect of glucose on enterotoxin production by non-replicating cells as compared with growing cells was shown to be due to the presence in non-replicating cells of some prerequisite of enterotoxin production, rather than to a difference in the effect of glucose on growth rate under the two sets of conditions. Therefore, to determine the effect of glucose on enterotoxin production by growing cells without any change in growth rate occurring, it would be necessary to study enterotoxin production under conditions of continuous culture. Such studies are described in Section 5.
5. **THE PRODUCTION OF STAPHYLOCOCCAL ENTEROTOXIN B BY S. AUREUS S-6 IN CONTINUOUS CULTURE: THE EFFECT OF GROWTH RATE AND GLUCOSE ON ENTEROTOXIN PRODUCTION**

### 5.1 Introduction

In studying the effect of glucose and glycerol on enterotoxin production under controlled conditions (Section 4) it was found that repression of enterotoxin was invariably accompanied by an increase in growth rate. It has been reported (Mandelstam, 1962, Gallo and Katz, 1973) that carbon sources which support a rapid rate of growth are most effective in producing catabolite repression. It has also been found that for *B. subtilis* growth rate appears to be associated with a regulatory mechanism for secretion of extracellular enzymes. It was postulated that when the growth rate was increased, the secretion of all extracellular enzymes was decreased (Coleman, 1967). It was therefore desirable to separate the effect of a change in the rate of growth on enterotoxin production and the effect of glucose *per se* in repressing enterotoxin production.

An attempt had been made (Section 4.3.8) to do this with non-replicating cells, but there appeared to be differences between non-replicating and growing cells which were not due solely to the difference in growth rate. Studies of the production of enterotoxin B by *S. aureus* S-6 under conditions of continuous culture were therefore undertaken in order to answer the following three specific questions:

1. Does glucose repress the production of enterotoxin B independently of any change in growth rate, i.e. does glucose repression occur if the growth rate is kept constant?

2. Does a change in growth rate without any change in the composition of the incoming medium alter the production of enterotoxin?
3. Do the rates of production per unit cell mass of enterotoxin B, lipase, deoxyribonuclease, lysozyme, 
β-hemolysin and TEP all alter in the same way when glucose is added to the medium at constant growth 
rate, or when the growth rate is changed without change in the medium, as would be expected if all 
extracellular proteins of staphylococci were under one common regulatory mechanism?

The results obtained from an investigation of enterotoxin B production in continuous culture are described in Section 5.

5.2 Materials and methods

5.2.1 Strains

*Staphylococcus aureus* S-6 was used in all continuous culture experiments.

5.2.2 Medium and cultural conditions

The inoculum for the fermenter consisted of cells grown in shake-flasks for 16 h, washed and resuspended, all these 
operations being carried out in AA medium.

AA medium was also used in the fermenter. It had been shown in batch culture experiments that 0.2 mM Mg ++ as compared with 
the usual concentrations of 0.4 mM would reduce the cell yield of strain S-6, and except where otherwise stated this concentra-
tion was used. Mg ++ was thus the limiting nutrient. The medium contained 0.3 mg/ml silicone antifoam (*Dow Corning*). 
The fermenter was as described in Section 3.2.1 with the 

following modifications made for continuous culture. An over-
flow exit line was put in so that the vessel held 1870 mls. 
Medium was fed in through an air-jacketed port by a Sigma 
pump set at the required speed. To prevent excessive splash-
over into the exit line, the stirrer speed was reduced to 
800 rpm, as compared with batch cultures (1000 rpm). The pH 
of the medium was maintained at 6.5 by the addition of N HCl 
in the absence of glucose or 2 N NaOH in glucose experiments. 
Glucose solutions were autoclaved separately and added to 
sterile medium. Dissolved oxygen concentration was maintained 
at 30 mm Hg as described in Section 3.2.1.
The fermenter was inoculated with washed cells from 16-hour shake-cultures which were then grown up in batch culture, using the same medium as that which would be added during continuous culture. When the OD was near the expected final OD for continuous culture, the exit line was opened, and the addition of medium commenced. Samples were collected from the exit line into a graduated cylinder for measured time intervals to determine the flow rate. The speed of the pump was adjusted until the required flow rate had been obtained and no further alterations to the pump speed were made during an experiment. The dilution rate (D) was calculated as follows:

\[
\frac{\text{flow rate (litres per hour)}}{\text{volume of culture (litres)}}\, h^{-1}
\]

Samples were taken at 2-3 h intervals through the sampling port, and the supernatants used for the determination of extracellular proteins. At less frequent intervals, 60 ml samples were collected via the exit line, for determination of cell dry weights and \( Mg^{++} \) determinations on supernatants.

5.2.3 Control of glucose concentration

Glucose concentrations were determined by the glucostat method as described in Section 4.2.4.

If glucose was to be added to the medium, 0.1 M glucose was added to the fermenter initially, and the concentration of glucose maintained throughout the batch culture stage by manual additions of glucose as described in Section 4.2.5. When continuous culture was initiated, the glucose inlet from the burette was disconnected. The concentration of glucose in the fermenter was then maintained automatically, since glucose was present in the medium being fed into the fermenter.

The yield constant \((Y)\) for a particular nutrient is defined as the cell mass produced per unit of substrate utilized (Kubitschek 1970). In continuous culture, the yield constant \((Y)\) for any nutrient is given by the equation:

\[
\bar{X} = Y (s_R - \bar{S}),
\]
where $s_R$ is the concentration of nutrient entering the fermenter, and $\bar{x}$ and $\bar{s}$ are the steady state values for the concentrations of cells and nutrient respectively in the fermenter. The concentration of glucose in the medium required to give a steady state concentration in the culture of approximately 0.1 M glucose was calculated as follows: It was found experimentally in batch culture that for strain S-6, an increase in cell mass of 1.06 mg utilized 0.02 moles of glucose. Therefore the yield constant was approximately 50 mg dry weight of cells per mole of glucose utilized. The approximate cell concentration expected in the steady state in a particular glucose experiment could be predicted from the corresponding cell concentration in a control experiment carried out at the same dilution rate, in the absence of glucose. Using the above equation, and the yield constant of 50 mg, it was then possible to calculate the glucose concentration required in the medium entering the fermenter to result in a steady state concentration of 0.1 M glucose. Thus, the cell concentration without glucose at $D = 0.07 \text{ h}^{-1}$ was 3.2 mg/ml. Substituting this in the above equation, 

$$3.2 = 50 (s_R - 0.10),$$

$$\therefore s_R = 0.16, \text{ i.e. } 0.16 \text{ M glucose would be}$$

required in the incoming medium in order to obtain a concentration of 0.1 M in the medium in fermenter in the steady state. The concentration of glucose in the medium was measured at intervals during the steady state, and it was confirmed that by this method the concentration of glucose was maintained at between 0.09 and 0.11 M.

### 5.2.4 Measurement of growth

Growth was followed by determination of OD of suitably diluted samples as described in Section 2.2.3. OD values were multiplied by the dilution for ease of comparison. Dry weights were determined by centrifuging samples at 12,000 rpm for 10 min to concentrate the cells. Cells were washed and resuspended in cold distilled water, and duplicate aliquots dried in tared aluminium dishes at 105°C for 3 h.
Determinations of extracellular proteins

Determinations of enterotoxin B, lipase, deoxyribonuclease, lysozyme and TAL were carried out as described in Section 2.2.6.

β-hemolysin titres were determined as described by Chesbro et al., (1965). Sheep erythrocytes were washed and resuspended to a 1% suspension in 0.01 M phosphate buffer pH 6.8, containing 0.85% NaCl and 0.01 M H₃PO₄. 0.5 ml of this cell suspension was added to 0.5 ml of serial two-fold dilutions of each sample, and the mixtures were incubated for 1 h at 37 °C, followed by 4 h at 4 °C. The titre was the highest dilution which produced 50% lysis as measured against a control suspension of mechanically lysed cells.

5.2.6 Specific rate of product formation in continuous culture

Under conditions of continuous culture, the specific rate of increase in the concentration of a product in the medium will depend on the rate of product formation, and the rate at which the product is being washed out of the fermenter, i.e. the dilution rate.

Thus, \( \frac{dp}{dt} = q_p x - Dp \)

where \( q_p = \) specific rate of product formation per unit of cell mass, at time \( t \), \( D \) is the dilution rate, \( x \) is the concentration of cells and \( p \) the concentration of product (Firt, 1969).

In the steady state, \( \frac{dp}{dt} = 0 \)

\[ . . . \quad q_p \bar{x} = D \bar{p} \]

\[ . . . \quad q_p = D \frac{\bar{p}}{\bar{x}} \]

i.e. in the steady state, the specific rate of product formation per unit of cell mass,

\[ q_p = \frac{\text{dilution rate} \times \text{product concentration}}{\text{cell concentration}} \]
In this section on continuous culture, the cell concentration is measured as dry weight of cells per ml, and this figure is used to calculate $q_p$ values. The specific rate of product formation in batch cultures was calculated using an increase in OD to measure an increase in cell mass (Section 2.2.7).

5.2.7 Mg$^{++}$ determinations

The concentration of Mg$^{++}$ was determined by flame absorption photometry, using a Zeiss PMZII spectrophotometer fitted with an FAZ flame attachment. An acetylene air flame and a modulated Perkin-Elmer magnesium hollow cathode lamp were used. Standards with a concentration of 0.02, 0.04 and 0.08 mM Mg$^{++}$ were read, interspersed with samples which had been diluted so that they fell within this range.

The yield constant ($Y$) for Mg$^{++}$ was calculated as described for glucose in Section 5.2.3 according to the equation

$$X = Y (s_R - S),$$

where $s_R$ is the concentration of limiting nutrient (Mg$^{++}$) being fed into the fermenter and $X$ and $S$ are the steady state values of dry weight of cells per ml and Mg$^{++}$ concentration respectively.

5.3 Results

5.3.1 The effect of growth rate on the production of enterotoxin B and other extracellular protein

The production of enterotoxin B and other extracellular proteins was determined in continuous culture using AA medium in the absence of glucose at three different growth rates to investigate whether a change of growth rate per se was important in determining the production of enterotoxin. The three dilution rates used were 0.07, 0.14 and 0.24 h$^{-1}$, corresponding to residence times of 14.3, 7.1 and 4.2 h respectively. At each dilution rate, the fermentation was continued for at least two residence times after the steady state had been established. Determinations of dry weight of cells, Mg$^{++}$ concentration, enterotoxin B, lipase, deoxyribonuclease and lysozyme were carried out on samples taken at intervals throughout the fermentations. The averages of three determinations for each of these in the steady state of growth are reported in Tables 21A and 21B.
TABLE 21A. Effect of dilution rate on cell concentration and production of enterotoxin B by S. aureus S-6

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Cells (mg/ml)</th>
<th>Enterotoxin B (µg/ml)</th>
<th>Mg²⁺ (mM)</th>
<th>Yield constant (mg cells/mM Mg²⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>3.24</td>
<td>123</td>
<td>2.66</td>
<td>0.04</td>
</tr>
<tr>
<td>0.14</td>
<td>2.30</td>
<td>88</td>
<td>5.36</td>
<td>0.06</td>
</tr>
<tr>
<td>0.24</td>
<td>1.66</td>
<td>80</td>
<td>11.50</td>
<td>0.07</td>
</tr>
<tr>
<td>0.24a</td>
<td>2.65</td>
<td>144</td>
<td>13.00</td>
<td>0.09</td>
</tr>
</tbody>
</table>

TABLE 21B. Effect of dilution rate on the production of TEP, lipase, deoxyribonuclease and lysozyme by S. aureus S-6

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>TEP (mg/ml)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>1.64</td>
<td>161</td>
<td>56</td>
<td>1.21</td>
</tr>
<tr>
<td>0.14</td>
<td>0.72</td>
<td>100</td>
<td>26</td>
<td>1.59</td>
</tr>
<tr>
<td>0.24</td>
<td>0.39</td>
<td>123</td>
<td>23</td>
<td>3.33</td>
</tr>
<tr>
<td>0.24a</td>
<td>0.80</td>
<td>91</td>
<td>44</td>
<td>3.97</td>
</tr>
</tbody>
</table>

aMg²⁺ in incoming medium 0.4 mM, as compared with usual concentration of 0.2 mM.
The steady state concentrations of extracellular proteins are not by themselves a valid measure of extracellular protein production, since the concentration in the medium depends both on the rate of production and the rate of removal, i.e. the dilution rate (D). Therefore, both the steady state concentrations per ml and the specific rates of production \( q_p \) per unit cell mass per hour are given for enterotoxin and other extracellular proteins.

In the steady state in continuous culture, the growth rate \( (\mu) \) equals the dilution rate \( (D) \). When the growth rate was increased from 0.07 to 0.24, there was an increase from 2.66 to 11.5 \( \mu \)g/mg cells/h in the specific rate of enterotoxin production (Table 21A). The specific rate of TEP production showed a much smaller increase from 0.035 to 0.056 \( \mu \)g/mg cells/h (Table 21B). The specific rate of production of lipase increased from 3.46 to 17.82, and of deoxyribonuclease from 1.21 to 3.33 units/mg cells/h. Lysozyme was the only extracellular protein assayed which showed a decrease in the specific rate of production as growth rate increased. At \( D = 0.07 \), 0.012 units of lysozyme/mg cells/h were produced, whereas no lysozyme activity was detected at \( D = 0.24 \) at this concentration of \( Mg^{++} \). The relationship between growth rate and the production of these extracellular proteins is shown in Fig. 26. The concentration of cells in the steady state decreased from 3.24 to 1.66 \( mg \) dry weight/ml as the growth rate was increased from 0.07 to 0.24 \( h^{-1} \). This is in accordance with predictions for continuous culture (Herbert et al., 1956).

Table 21A shows the steady state concentration of \( Mg^{++} \) in the medium, and the yield constant for \( Mg^{++} \). When the growth rate was increased from 0.07 to 0.24 \( h^{-1} \), the yield constant decreased from 20.2 to 12.7 \( mg \) cells per mM \( Mg^{++} \). This suggests that the more rapidly growing cells contain a higher intracellular concentration of \( Mg^{++} \).

Tables 21A and 21B also show the effect of increasing the concentration of \( Mg^{++} \) in the incoming medium from 0.2 to 0.4 mM. This resulted in an increase in cell concentration (Fig. 27) as would be expected of a limiting nutrient. The
Figure 26  Effect of dilution rate on the specific rate of formation of the following proteins by strain S-6; enterotoxin B, Δ; TEP (x 10), ○, lipase, □; deoxyribonuclease, (▲); lysozyme (x 100), ●.
Figure 27  Effect of increasing Mg++ in incoming medium from 0.2 mM (○) to 0.4 mM (△) on OD in continuous culture of strain S–6.
specific rate of production of enterotoxin B, TEP, deoxyribonuclease and lysozyme was higher at the higher concentration of Mg\(^{++}\). An unexpected finding was that lipase production was reduced at the higher Mg\(^{++}\) concentration. The yield constant was reduced from 12.7 to 8.6 mg cells per mM Mg\(^{++}\) when the Mg\(^{++}\) in the incoming nutrient was increased to 0.4 mM, suggesting that a higher intracellular concentration of Mg\(^{++}\) had resulted. The change in yield constant reported here has also been found with other organisms. Tempest (1970) states that the Mg\(^{++}\), K\(^{+}\) and P contents of microorganisms are known to vary with growth rate, and that therefore the yield may be expected to vary with dilution rate when the availability of any of these substances is made to limit growth.

5.3.2 Effect of glucose on growth in continuous culture

When the medium contained 0.1 M glucose, there were marked transient oscillations in CD, enterotoxin B and TEP. These oscillations were highly reproducible. When the dilution rate was 0.24 h\(^{-1}\), a steady state was only achieved after approximately seven residence times (28 h) in the presence of glucose, as compared with approximately four residence times (17 h) in the control experiment without glucose (Fig. 28). When the dilution rate was 0.07 h\(^{-1}\), a steady state was only achieved after approximately six residence times (87 h) when glucose was in the medium, as compared with one residence time (14 h) in the control without glucose (Fig. 29). The addition of glucose also resulted in transient oscillations when the Mg\(^{++}\) concentration in the incoming medium was increased to 0.4 mM (Fig. 30).

The more prolonged oscillations at the lower dilution rate suggested that there may be an inhibitory product of fermentation which was produced at the end of the batch culture phase of growth. Such an inhibitor would be less rapidly washed out at the lower dilution rate. Such end product inhibition was considered responsible for damped oscillations observed in continuous culture by Yano and Koga (1973).
Figure 28  OD, enterotoxin B and TEP production by strain S–6 in continuous culture, dilution rate 0.24 h⁻¹, residence time 4.2 h, Mg++ in incoming medium 0.2 mM; without glucose (open symbols) and with 0.1 M glucose (closed symbols).

OD, △; enterotoxin B, ○; TEP, □.
Figure 29  OD, enterotoxin B and TEP production by strain S–6 in continuous culture, dilution rate 0.07 h$^{-1}$, residence time 14.3 h, Mg$^{++}$ in incoming medium 0.2 mM; without glucose (open symbols) with 0.1 M glucose (closed symbols), OD, \( \blacktriangle \); enterotoxin B, \( \bigcirc \); TEP, \( \bigcirc \).
Figure 30  OD, enterotoxin B and TEP production by strain S-6 in continuous culture, dilution rate 0.24 h⁻¹, residence time 4.2 h, Mg²⁺ in incoming medium 0.4 mM; OD, Δ; enterotoxin B, ○; TEP, □. without glucose (open symbols); with 0.1 M glucose (closed symbols).
Preliminary experiments in shake-flasks indicated that an inhibitor of growth might be present. Samples of medium from two fermenter experiments, one with and one without glucose, were collected 2 h after the addition of medium to the batch culture was begun. Glucose and Mg++ determinations were carried out and the concentrations of these in both samples adjusted to 0.065 M glucose and 0.4 mM Mg++. The samples were then millipore filtered, and 25 ml of each added to 25 ml of fresh AA medium in each of two 250 ml flasks. The flasks were inoculated with washed cells of strain 5-6 and incubated on the shaker, and growth followed for 12 h. In the flask containing medium from the glucose experiment the maximum growth rate was 0.46, as compared with 0.66 in the control flask. This suggested that a growth inhibitor may have been produced when staphylococci were grown in AA medium in the presence of glucose.

5.3.3 Effect of glucose on the production of enterotoxin B and other extracellular proteins

The effect of glucose on the production of enterotoxin B and other extracellular proteins at constant growth rate was determined at two different dilution rates (D = 0.07 and D = 0.24). At the higher dilution rate, the effect of glucose was determined for two different concentrations of Mg++ in the incoming medium (0.2 mM and 0.4 mM Mg++). The production of enterotoxin B and TEP was followed throughout growth, and is shown for these three sets of conditions in Fig. 28, 29 and 30. In each graph growth in batch culture is also shown, and zero residence time indicates when the addition of medium was begun. Lipase, deoxyribonuclease, lysozyme, β-hemolysin and TEP production were also followed throughout growth and the averages of three steady-state values of each of these are given in Tables 22A and 22B. The determination of β-hemolysin was included because the genetic determinants for enterotoxin B and β-hemolysin have recently been reported to be on the same plasmid (Dornbusch and Hallander, 1973). It was considered possible therefore that the production of these two proteins might respond similarly to the presence of glucose.
**TABLE 22A.** Effect of 0.1 M glucose on the production of enterotoxin B and TEP by *S. aureus* S-6.

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Glucose (M)</th>
<th>Cells (mg/ml)</th>
<th>Enterotoxin B (µg/ml)</th>
<th>TEP (mg/ml)</th>
<th>Mg²⁺ (mM)</th>
<th>Yield constant (mg cells/ml Mg²⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0</td>
<td>3.24</td>
<td>123</td>
<td>2.66</td>
<td>0.035</td>
<td>0.04</td>
</tr>
<tr>
<td>0.07</td>
<td>0.09</td>
<td>3.49</td>
<td>39</td>
<td>0.77</td>
<td>0.013</td>
<td>0.04</td>
</tr>
<tr>
<td>0.24</td>
<td>0</td>
<td>1.66</td>
<td>80</td>
<td>11.50</td>
<td>0.056</td>
<td>0.07</td>
</tr>
<tr>
<td>0.24</td>
<td>0.10</td>
<td>1.50</td>
<td>11</td>
<td>1.75</td>
<td>0.026</td>
<td>0.09</td>
</tr>
<tr>
<td>0.24ᵃ</td>
<td>0</td>
<td>2.65</td>
<td>144</td>
<td>13.00</td>
<td>0.072</td>
<td>0.09</td>
</tr>
<tr>
<td>0.24ᵃ</td>
<td>0.10</td>
<td>3.0</td>
<td>18</td>
<td>1.44</td>
<td>0.017</td>
<td>0.10</td>
</tr>
</tbody>
</table>

**TABLE 22B.** Effect of 0.1 M glucose on the production of lipase, deoxyribonuclease, lysozyme and β-hemolysin by *S. aureus* S-6.

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>Glucose (M)</th>
<th>Lipase (units/ml)</th>
<th>Deoxyribonuclease (units/ml)</th>
<th>Lysozyme (units/ml)</th>
<th>β-hemolysin (titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0</td>
<td>161</td>
<td>56</td>
<td>0.55</td>
<td>1/64</td>
</tr>
<tr>
<td>0.07</td>
<td>0.09</td>
<td>76</td>
<td>56</td>
<td>0.35</td>
<td>1/4</td>
</tr>
<tr>
<td>0.24</td>
<td>0</td>
<td>123</td>
<td>23</td>
<td>0</td>
<td>1/32</td>
</tr>
<tr>
<td>0.24</td>
<td>0.10</td>
<td>51</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.24ᵃ</td>
<td>0</td>
<td>91</td>
<td>44</td>
<td>0.33</td>
<td>1/64</td>
</tr>
<tr>
<td>0.24ᵃ</td>
<td>0.10</td>
<td>53</td>
<td>137</td>
<td>0.13</td>
<td>1/4</td>
</tr>
</tbody>
</table>

ᵃ Mg²⁺ in incoming medium 0.4 mM, as compared with usual concentration of 0.2 mM.
At the lower dilution rate, the addition of glucose to the medium reduced the specific rates of production of enterotoxin B by 70% and TEP by 60%. The specific rate of lipase production was decreased by 56%, and that of lysozyme production was decreased by 50%. The $\beta$-hemolysin titre was reduced 16-fold in the presence of glucose. In contrast, glucose had no significant effect on deoxyribonuclease production.

At the higher dilution rate of 0.24 h$^{-1}$ and with 0.2 mM Mg$^{++}$ concentration in the incoming nutrient, glucose in the medium reduced the specific rate of enterotoxin production by 85% (Table 22A). The specific rates of production of TEP and lipase were reduced by 50% and 55% respectively (Tables 22A and 22B). No lysozyme was detected with or without glucose. The presence of glucose completely inhibited the production of $\beta$-hemolysin, as compared with a titre of 1/32 in the control without glucose. By contrast, the production of deoxyribonuclease was slightly stimulated by glucose.

At a dilution rate of 0.24 h$^{-1}$ and with 0.4 mM Mg$^{++}$ concentration in the incoming nutrient, 0.1 M glucose in the medium similarly caused decreases in the specific rates of production of enterotoxin (90%), of TEP (75%), of lipase (48%), of lysozyme (70%), and of $\beta$-hemolysin (16-fold). However, at this Mg$^{++}$ concentration there was more than a three-fold stimulation of deoxyribonuclease production in the presence of glucose.

The concentration of limiting nutrient in the fermenter during the steady state is theoretically independent of the concentration of nutrient in the incoming medium (Section 5.2.7). In the presence of glucose, the steady state concentrations of Mg$^{++}$ were very similar, whether the incoming medium contained 0.2 mM Mg or 0.4 mM Mg$^{++}$ (Table 22A). However, with 0.4 mM Mg$^{++}$ the yield constant was 10.0, as compared with 13.5 mg cells per mM Mg$^{++}$ at the lower initial Mg$^{++}$ concentration. This suggests that with the higher initial concentration of Mg$^{++}$, the intracellular concentration of Mg$^{++}$ in the steady state was increased.
5.4 Discussion

5.4.1 Effect of glucose on the production of enterotoxin B and other extracellular proteins at constant growth rate

Enterotoxin B production by S. aureus 5-6 was reduced by between 70 and 90% by the addition of 0.1 M glucose to the medium without any change in growth rate taking place. Glucose repression of enterotoxin production in batch cultures in the fermenter or in shake-flasks was invariably accompanied by an increase in growth rate (Section 4.4.3). Other workers have reported that carbon sources which support a rapid rate of growth are most effective in producing catabolite repression (Mandelstam, 1962, Gallo and Katz, 1973). However, it is shown by the results presented here that an increase in growth rate is not necessary for glucose repression to occur. Indeed, glucose repression of the specific rate of enterotoxin production by S. aureus 5-6 at constant growth rate in continuous culture was greater (85-90%) than in batch culture (38%) where there was an increase in growth rate in the presence of glucose.

The inhibitory effect of glucose was not specific to the production of enterotoxin B. Lipase, lysozyme, β-hemolysin and TEP production was also considerably reduced in the presence of glucose. However, the production of deoxyribonuclease was not decreased in the presence of glucose when the concentration of Mg²⁺ in the incoming medium was 0.2 mM, and was increased more than three-fold by glucose when Mg²⁺ was 0.4 mM. These results are similar to those obtained in batch culture (Section 4.3.4 and 4.3.7) where deoxyribonuclease production was greatly stimulated by glucose when the initial concentration of Mg²⁺ was 0.4 mM, but was unaffected by glucose when the initial concentration of Mg²⁺ was 0.2 mM. It is likely that increasing the concentration of Mg²⁺ to 0.4 mM in the incoming medium in continuous culture, increased the intracellular concentration of Mg²⁺. A high intracellular concentration of Mg²⁺ may therefore be necessary for the addition of glucose to result in increased synthesis of deoxyribonuclease.
5.4.2 Relationship between growth rate and the production of enterotoxin B and other extracellular proteins

The growth of staphylococci in continuous culture has not previously been reported. In this study, enterotoxin B has been produced in continuous culture at three different dilution rates. The highest specific rate of enterotoxin production \((q_p)\) was \(13.0 \text{ µg/mg cells/h}\). If cell mass is measured as OD, instead of cell dry weight, then this specific rate of enterotoxin production becomes \(1.73 \text{ µg/cell unit mass/h}\). This figure can be compared with a specific rate of enterotoxin production of 2.12 in batch culture (Section 4.3.5) by strain S-6, in LA medium over the period of maximum enterotoxin production.

The specific rate of enterotoxin production increased from \(22 \text{ µg/mg cells/h}\) to \(11.5 \text{ µg/mg cells/h}\) when the growth rate was increased from 0.07 to 0.24 h\(^{-1}\). Lipase, deoxyribonuclease and TAP production also increased with the increase in growth rate, but except for lipase the increases were much less marked than with enterotoxin. Coleman (1967) studied the secretion of three extracellular enzymes by Bacillus subtilis. He showed that there was an inverse relationship between growth rate and synthesis of all three extracellular enzymes. However, the increases reported here in the specific rates of production of enterotoxin B, lipase, deoxyribonuclease and TAP as growth rate increased indicate that this was not so for these extracellular proteins of staphylococci. Lysozyme was the only extracellular protein studied for which production decreased as the growth rate increased.

The synthesis of all extracellular proteins was not repressed by glucose in the same way. Indeed, in some experiments, glucose caused a marked stimulation of deoxyribonuclease production. It was also found that changes in growth rate affected different extracellular proteins in different ways. Enterotoxin B production was increased at the same time that lysozyme production was decreased. Stormonth and Coleman (1973) studied the production of three enzymes by Bacillus amylobacter faciens, and suggested that all extracellular enzymes were under a common regulatory system. However, from the findings presented here, it is evident that not all extracellular proteins produced by staphylococci are under a common regulatory system.
6. GENERAL DISCUSSION

6.1 General conclusions

In addition to the conclusions presented in the discussion following each section, the following general conclusions are drawn from the overall findings of this investigation.

1. That strains producing a particular enterotoxin type do not always respond to growth conditions in the same way. It is therefore inadvisable to draw conclusions regarding the physiological characteristics of a particular enterotoxin type from studies carried out with one strain only.

2. That production of the enterotoxins is closely related to growth, and is greatest for actively growing cells.

3. That while in most experiments there was a close similarity in the way in which enterotoxin and lipase production responded to changes in environment, other extracellular proteins were differently affected. Therefore, control of enterotoxin production appears in certain respects, at least, to be independent of the control of general extracellular protein production.

4. That glucose inhibited enterotoxin production independently of its effect on pH and growth. While this appears to be due to catabolite repression, it is unlikely that it is a direct repression of the synthesis of mRNA for enterotoxin.

6.2 Strain variation

Strains of the same enterotoxin type frequently showed considerable differences in their responses to environmental changes. This was well illustrated by the effect of pH control on the production of enterotoxins A and B in CH medium in the fermenter. Thus for strain S-6 the production of both enterotoxins A and B was increased when grown at a constant pH of 6.5 compared with production under conditions where pH was not controlled. For strain 22, on the other hand, there was
no increase in either enterotoxin A or B at any controlled pH. The production of enterotoxin C in baffled shake-flasks was less than that in control flasks without baffles. However, experiments with strains 3 and 37 showed that this was a characteristic of strain 361, and not true of other strains producing enterotoxin C. In investigating the effects of added carbohydrates on enterotoxin production, the addition of galactose and lactose to cultures of two enterotoxin B-producing strains caused an increase in enterotoxin B production in strain 5-6 and a decrease in strain 30. Other workers have frequently reported findings which they consider indicate general differences in the mode of production of the different enterotoxins, particularly in comparisons between enterotoxin A and enterotoxin B production (Markus and Silverman, 1969, 1970; Troller 1971, 1972). Results presented here suggest such apparent differences may well have been due to the use of one strain only in these experiments, and therefore due to a difference between strains, rather than between enterotoxin types.

6.3 Relation between enterotoxin production and growth

It has been generally accepted that enterotoxin B production occurs only in the stationary phase of growth (Markus and Silverman, 1969, 1970; Horse and Baldwin, 1973). However, it has been shown in this investigation that for each of the three enterotoxins studied in nine different strains, the amount of enterotoxin produced is closely related to the growth pattern, and that the rate of enterotoxin production is greatest in actively growing cells. In batch cultures, all three enterotoxins were first detected during the exponential phase of growth. Only strain 5-6 showed any significant increase in enterotoxin during stationary phase, and this was evident only in shake-flasks, and not in the fermenter. Even in shake-flasks, the specific rate of enterotoxin production (q value) for 5-6 was 3.6 in the exponential phase (from 3 to 4/2 h in Fig.8), as compared with 2.8 in the early stationary phase (from 6 to 7 h).
The finding that enterotoxin B was produced by actively growing cells was further supported by continuous culture experiments. In actively growing cells in a balanced state, the rate of enterotoxin production at a dilution rate of 0.24 was 1.74 (q_p value per unit OD), which was only slightly less than the q_p value of 2.12 in batch culture in the fermenter. The specific rate of enterotoxin production actually increased when the dilution rate was increased from 0.07 to 0.24. Therefore, it could be expected that if the dilution rate had been further increased to be more nearly that of the growth rate obtained in batch culture (0.46), enterotoxin production in continuous culture would have been at least as high as that in batch culture.

It has been suggested from studies in *B. subtilis* (Coleman, 1967) that extracellular enzymes are under a common control mechanism related to growth rate. As the growth rate decreased the production of extracellular enzymes increased, and it was concluded that this was due to an increase in the availability of nucleotide precursors for extracellular protein mRNA synthesis. Evidence was found in the present investigation for a small pool of mRNA for enterotoxin production such as was reported for protease production in *B. subtilis* (Both et al., 1972). However, it is clear from the increases in enterotoxin B, lipase, deoxyribonuclease and TEF production obtained when growth rate was increased in continuous culture of strain S-6, that a control mechanism whereby extracellular protein production is inversely related to growth rate is not present in staphylococci.

All three enterotoxins studied were produced by non-replicating cells. However, enterotoxin production per unit cell mass was considerably less for non-replicating cells than for growing cells, again supporting the conclusion that enterotoxin production is closely associated with growth. This is illustrated by strain S-6, for which the specific rate of enterotoxin production (per mg dry weight of cells per unit time) was 1.6 for non-replicating cells in a N-containing medium as compared with 13.00 for cells actively growing in continuous culture with a growth rate of 0.24 h⁻¹.
In assessing the effect of changes in the environment on enterotoxin production, it is necessary to separate those changes in enterotoxin yield which reflect an alteration in the growth pattern, from those which are due to a change in the production of enterotoxin per unit cell mass per unit time. A change in environment may increase the final concentration of enterotoxin in the medium reached during a total incubation period by increasing the duration of exponential growth, the cell yield, or growth rate. For example, growth of staphylococci under controlled pH conditions in two different media increased the total production of enterotoxin by three strains. In all cases but one, there was also an increase in the specific rate of enterotoxin production. However, for strain 100 the increase in enterotoxin yield due to controlled pH was due to an extended transition period from exponential to stationary phases of growth, not to an increase in the specific rate of enterotoxin production. The increase in enterotoxin yield which was obtained for three out of five strains when grown under conditions of controlled pH could be highly significant in food, where pH is frequently buffered at pH 6.0-6.5. Strains which do not produce detectable enterotoxin A in the conventional laboratory shake-flask may produce sufficient enterotoxin under the buffered conditions which frequently exist in food to be responsible for food-poisoning.

6.4 Enterotoxin production relative to the synthesis of other extracellular proteins in staphylococci

Throughout this investigation the production of lipase, deoxyribonuclease, lysozyme and TEP has been studied in parallel with enterotoxin production. Some changes in the environment, such as the addition of antifoam to the medium, appeared to exert an overall effect on extracellular protein production. In general, the response of lipase production to environmental change was very similar to that of enterotoxin, and changes in enterotoxin production in response to environment were therefore not entirely specific to enterotoxin. However, instances such as the glucose stimulation of deoxyribonuclease production showed clearly that the effects on enterotoxin production of changes in growth condition were not always general effects on total extracellular production.
6.5 Glucose repression of enterotoxin production

Glucose repressed the production of enterotoxins A, B and C by growing cells. Experiments in which staphylococci were grown in a fermenter under conditions of controlled pH showed that this was independent of pH changes produced in shake-flasks. Glucose in the medium resulted in a shorter exponential phase and a more rapid rate of growth. However, the specific rate of enterotoxin production was reduced by glucose, showing that the effect of glucose was not due to a change in growth pattern. Experiments with strain S-6 in continuous culture showed that glucose repressed enterotoxin B production when the growth rate was held constant, i.e. glucose repression was independent of changes in growth rate. Enterotoxin repression was not specifically due to glucose, since other readily fermentable compounds such as glycerol produced the same effect. In fact, glycerol caused an even greater degree of enterotoxin repression than glucose.

Since the inhibitory effect of glucose on enterotoxin production is not due to changes in pH or rate of growth, it seems reasonable to invoke catabolite repression to account for the glucose effect, as has been done by several authors (Norse et al., 1969, Markus and Silverman, 1969). However, the general definition of catabolite repression (Faigen and Williams, 1970) merely describes a phenomenon, but does not necessarily imply a specific mechanism. One of the few systems where the mechanism is understood in some detail is the c-AMP-mediated repression of β-galactosidase in E. coli. Here, the presence of glucose reduces the intracellular concentration of c-AMP, and since this compound is required for the initiation of transcription, enzyme synthesis is repressed. Clearly glucose repression of enterotoxin synthesis is not controlled directly in the same way. Although glucose repressed enterotoxin production by growing cells, it did not repress production of enterotoxin by non-replicating cells. Experiments with chloramphenicol and actinomycin D showed that enterotoxin production by non-replicating cells required new protein synthesis and that only a small part of the enterotoxin synthesis could be accounted for by a pool of mRNA. However, although in non-replicating
cells enterotoxin production was not inhibited by glucose, at the same time β-galactosidase synthesis was almost completely repressed. This indicates that the mechanisms by which glucose decreases the production of enterotoxins is different from that by which β-galactosidase is inhibited. Attempts to overcome repression of β-galactosidase in staphylococci by c-AMP were unsuccessful, but this may have been due to lack of uptake of c-AMP by the cells. The inhibition by chloramphenicol of enterotoxin production in non-replicating cells, and lack of repression by glucose, indicates that the sites at which these two compounds act in inhibiting enterotoxin synthesis are not the same.

If cells of *S. aureus* S-6 were first grown in glucose and then resuspended with or without glucose, they were no longer able to produce enterotoxin. It is evident that cells which had been grown in the presence of glucose lacked some essential prerequisite for enterotoxin production or release.

This difference in glucose effect on enterotoxin production by non-replicating cells grown in the presence of glucose, as compared with cells grown without glucose may be related to the differences in the glucose metabolism of such cells. Strasters and Winkler (1963) found that if cells were grown without glucose and resuspended in glucose, 64% of the glucose was oxidized via the hexose monophosphate pathway (HMP). If the cells were grown in glucose and resuspended in glucose, only 34% of glucose was oxidized via the HMP pathway. Montiel and Blumenthal (1965) similarly found a 50% reduction in HMP activity in resting cells which had been grown in glucose. The tricarboxylic acid cycle was almost completely repressed in cells which were grown in glucose, as compared with an active TCA cycle in cells grown without glucose (Strasters and Winkler, 1963).

It is also possible that glucose represses an enzyme concerned with the release of enterotoxin from the cell. A small accumulation of enterotoxin may then be responsible for the inhibition of further enterotoxin production by a feed-back mechanism.
Yet another possibility is that there is a polypeptide precursor of enterotoxin which requires a modification by an enzyme to produce the final immunologically-identifiable form. Such an enzyme might be present in cells harvested at 16 h if grown without glucose, but absent in cells grown in the presence of glucose. This hypothesis would suggest that glucose repression acts on an enzyme concerned in enterotoxin production, rather than acting directly on the synthesis of mRNA for enterotoxin.
7. **REFERENCES**

   Section B 29: 604-413.

   Influence of cultivation conditions on the production of extracellular proteins by *Staphylococcus aureus*.  

   Purification and some physiochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. Biochemistry  
   6: 1474-1480.

   Chemistry and detection of staphylococcal enterotoxin.  
   Proc.of XIVth Research Conf.Circ.No.70.

   Enterotoxins, in Microbial Toxins, III. Ed.by Montie, T.C.,  

   The Enterotoxins in The Staphylococci. ed. Cohen, J.O.  
   John Wiley and Sons, New York.

   Identification of a new enterotoxin as enterotoxin C.  

   Identification of enterotoxin E. Infect.Immun. 4: 593-595.

   Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin-neutralizing  


Furification and partial characterization of enterotoxin C produced by Staphylococcus aureus strain 137.
Biochemistry 6: 1467-1473.


Enterotoxin B production in a fermenter system.

Serologic studies of staphylococcal enterotoxin.
Public Health Rept. US. 72: 599-609.

Further serological studies of staphylococcal enterotoxin.


Designation of staphylococcal enterotoxins.


Purification of staphylococcal β-hemolysin and action on staphylococcal and streptococcal cell walls.


Purification and characterisation of staphylococcal enterotoxin A. Biochemistry: 5: 3281-3289.


Regulation of inducible enzyme synthesis in Escherichia coli by cyclic adenosine 3',5'-monophosphate. J.Biol.Chem. 244: 5828-5835.


Staphylococcal food poisoning due to cheese. Med.Offr. 112 (7) 105.
The shaker in bioengineering. Process Biochemistry, 4: (3) 35-40.
Inhibition of staphylococcal enterotoxin B formation by cell wall blocking agents and other compounds.


Introduction to research with continuous cultures.
Prentice-Hall Inc., Englewood Cliffs, NJ.

68. Lawrence, R.C., Fryer, T.F. and Reiter, B: 1967.
A rapid method for the quantitative estimation of

Experimental cooking. pub. John Wiley and Sons Inc.
New York.

70. Magasanik, B: 1957.
221-252.

Quart.Biol. 26: 249-256.

Nutritional requirements of Staphylococcus aureus 5-6.

Induction and repression of β-galactosidase in non-

The repression of constitutive β-galactosidase in
Escherichia coli by glucose and other carbon sources.

Enterotoxin B synthesis by replicating and non-replicating

Factors affecting the secretion of staphylococcal
enterotoxin A. Appl.Microbiol. 20: 492-496.

Characteristics of extracellular protease formation by
Bacillus subtilis and its control by amino acid repression.

Induction of lactose utilization in Staphylococcus aureus.
J.Bacteriol 86: 1211-1215.

Effects of meat-curing salts and temperature on the


Enterotoxigenicity of _Staphylococcus aureus_ strains isolated from acute cases of bovine mastitis.

A staphylococcal food-poisoning caused by a coagulase-negative strain. _Biken's Journal._ 2: 92.


Regulation of β-galactosidase synthesis in _Escherichia coli_ by cyclic adenosine 3',5'-monophosphate.

Microbial growth and product formation. p.199-221.


Co-enzyme A function in acetyl transfer by the phosphotransacetylase system. J.Biol.Chem. 191: 365-376.


In vitro studies on staphylococcal enterotoxin production. J.Bacteriology 265-270.


Examination of large quantities of cheese for staphylococcal enterotoxin A. J. Dairy Sci. 51: 635-649.