THE PHYSIOLOGY OF STAPHYLOCOCCAL ENTEROTOXIN PRODUCTION

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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 extra-cellular 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 experi-
ment 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

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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, Hausler 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 (Sugiyama 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 C_1 (strain 137) and C_2 (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 entero-
toxins 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 super-
natant 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 staphylo-
coccal 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 entero-
toxin B have been reported (Hallander and Korlöf, 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 C_1, (Borja and Bergdoll, 1967),
and 7.0 for enterotoxin C_2 (Avena and Bergdoll, 1967). Their
molecular weights are reported as 34,700 (enterotoxin A),
28,366 (enterotoxin B), 34,100 (enterotoxin C_1) and 34,000
(enterotoxin C_2).

The marked differences in incidence of the various entero-
toxins have prompted several studies on possible underlying
reasons for these differences. Markus and Silverman (1969,
1970) reported that the mechanisms of production of entero-
toxins 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 entero-
toxin 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 entero-
toxin A and B differ in their production by L-forms of staphy-
lococci.   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 entero-
toxin 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 entero- toxin 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 investi- gations. 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 entero- toxin 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 desir-
able to monitor the concentration of dissolved oxygen in the
medium, and maintain a constant oxygen concentration through-
out growth.

1.4.4   Temperature

In recent years a number of reports have appeared in the
literature concerning the effect of temperature on entero-
toxin 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 tempera-
ture 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,

Hurst et al., 1973, Morse and Mah, 1973). The pH in these
studies was not strictly controlled, and there was a consider-
able 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 occur-
red. 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 repres-
sion 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-galactosi-
dase 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 produc-
tion 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 heme.   The
repression of eneterotoxin 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 sub-
sequently 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 extra-
cellular 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 character-
istic 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 produc-
          tion 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 STAPHYLO-
      COCCI.

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 entero-
toxins 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 pre-
cursor 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 entero-
toxin, 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 deter-
mine whether the effect of different conditions on entero-
toxin production was related specifically to enterotoxin, or
was a more general effect on the production of all extra-
cellular 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 PS 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 entero-
toxin 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_2HPO_4 1.0 g,
> KH_2PO_4 1.0 g, Mg SO_4 $7H_2O$ 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 auto-
> claved 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): $(NH_4)_2SO_4$ 1.0,
isodium citrate, 2 H_2O 0.5, $MgSO_4 7H_2O$ 0.1, $FeSO_4 .7H_2O$
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.

Media for non-replicating cells: The N-containing
medium for the resuspension of non-growing cells
contained Trypticase (BBL) 4%, K_2HPO_4 0.1%, glucose, 0.15%,
pH 7.1 (not adjusted). N-free medium contained K_2HPO_4,
3.5%, glucose 0.15%, pH 7.3 (adjusted with HCl). These
media differed from that of Markus and Silverman (1969)
in that glucose was reduced from 0.5% to 0.15%, and was
included in both media, in view of the reported glucose
repression of enterotoxin B (Morse et al., 1969). Glucose
was autoclaved separately at 10 psi for 10 min and added
to sterile medium.

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 spectro-
photometer 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 (μ) was calculated as follows:

$$\mu = \frac{(\log_{10} OD_{t2} - \log_{10} OD_{t1}) \, 2.303}{(t_2 - t_1)}$$

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 gyrotory 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
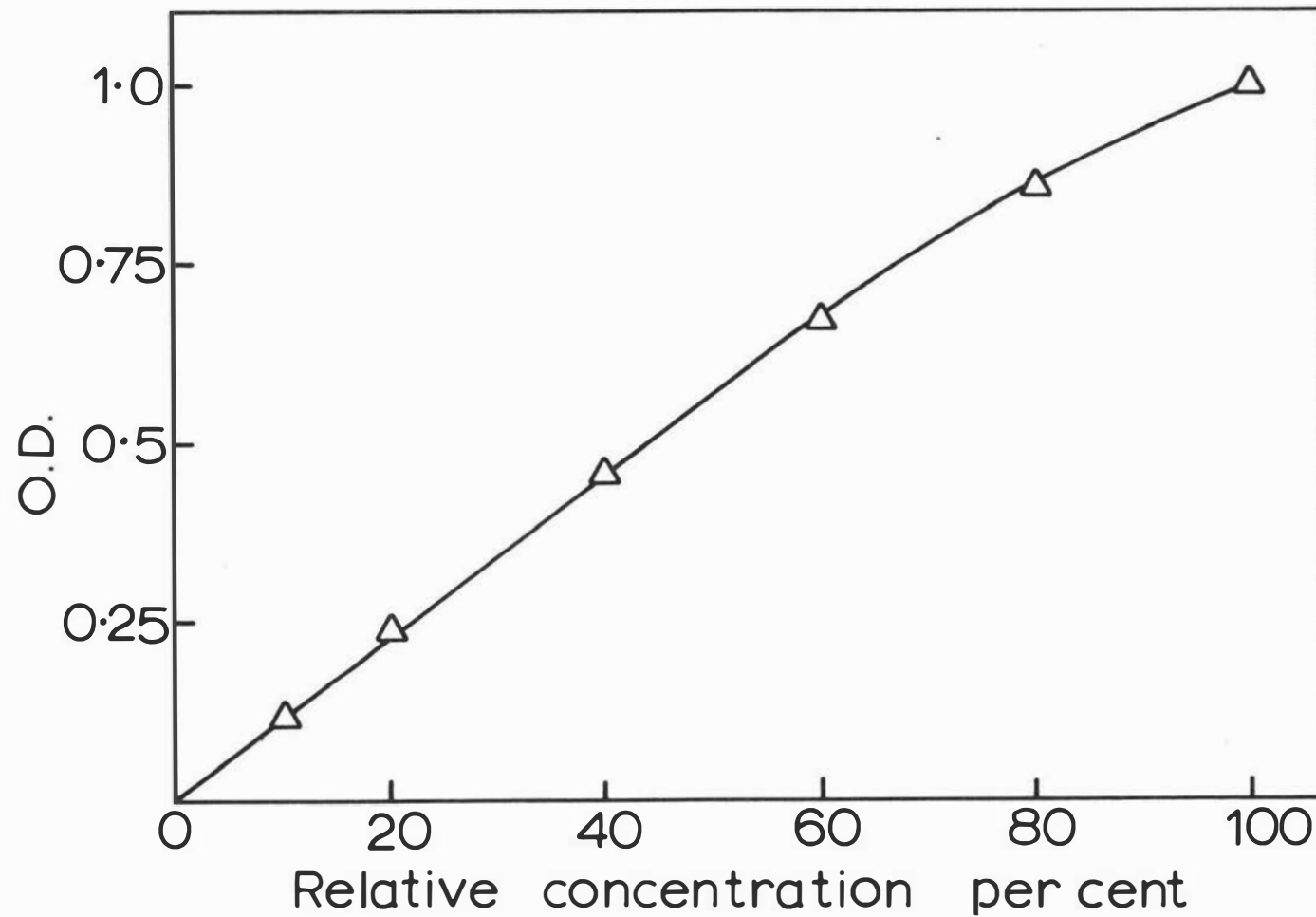
Figure 1   Relation between cell concentration and OD at 600 nm. 100 per cent
concentration corresponds to 0.25 mg/ml dry weight of cells.

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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 entero-toxin 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) micro-slide 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
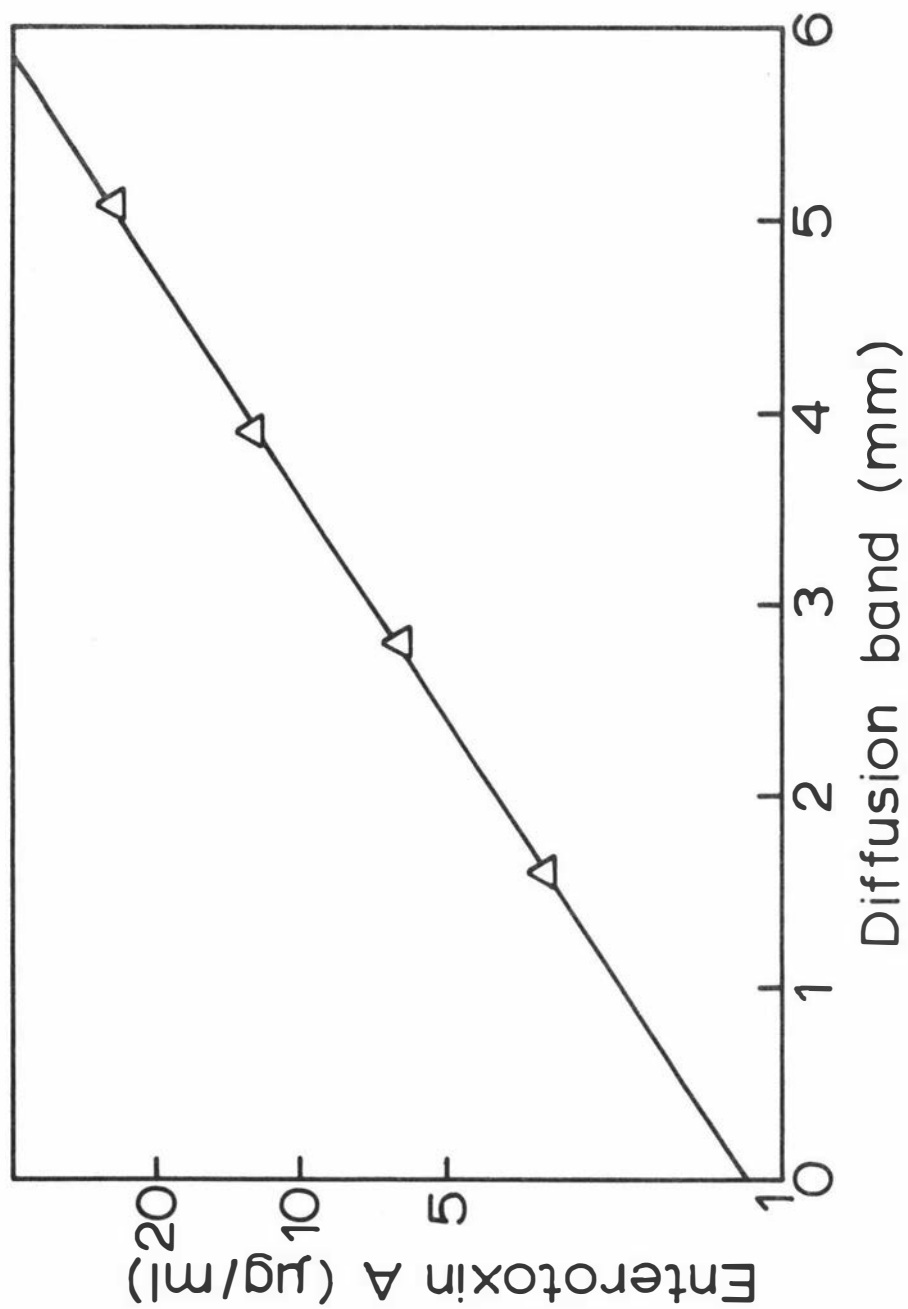
Figure 2   Standard curve for enterotoxin A determination.

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
arbitary 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°.   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° water-bath,
and recorded in units relative to the activity of dilutions
of egg-white lysozyme (Worthington (EWL) 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 measure-
ment of TEP a modification was made of the method of
Stadtman et al., (1951).   Thirty per cent trichloroacetic
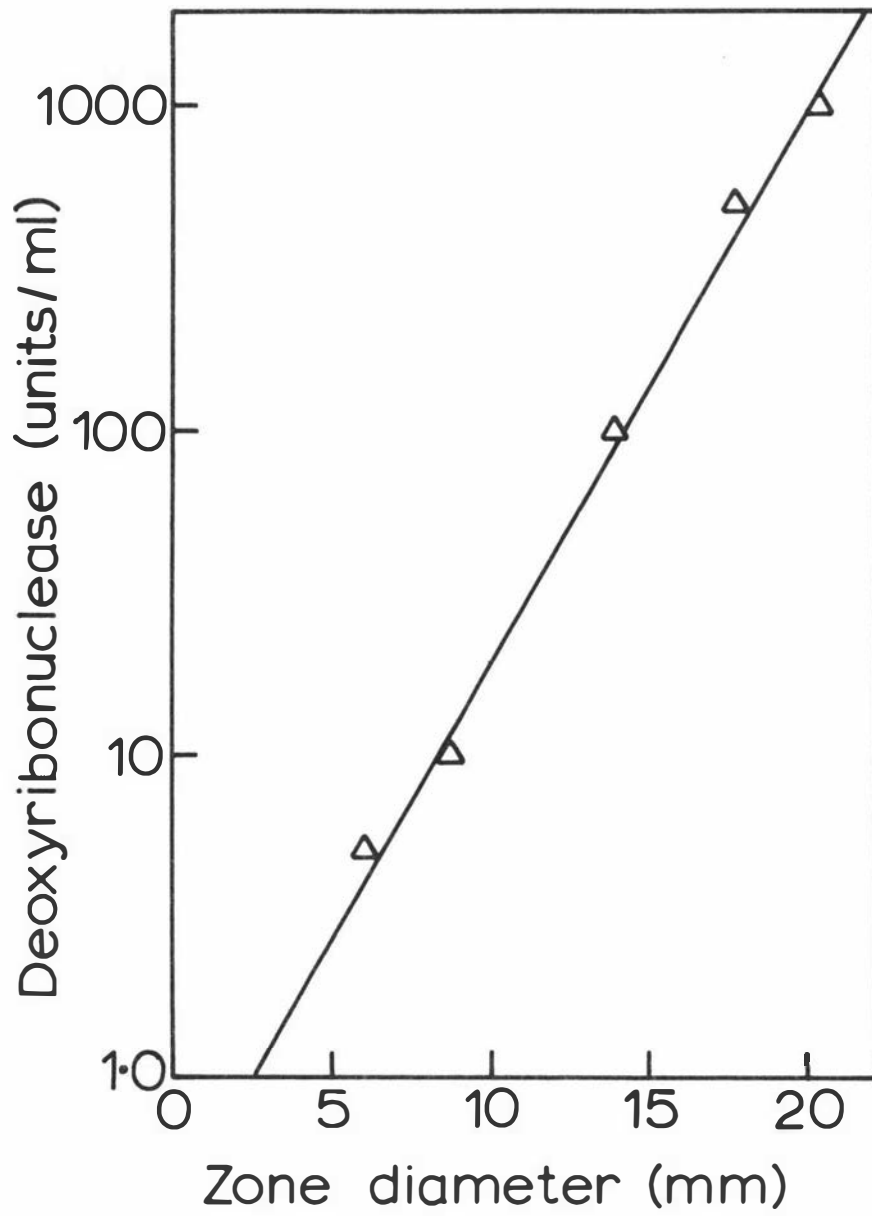
Figure 3   Standard curve for deoxyribonuclease determination.
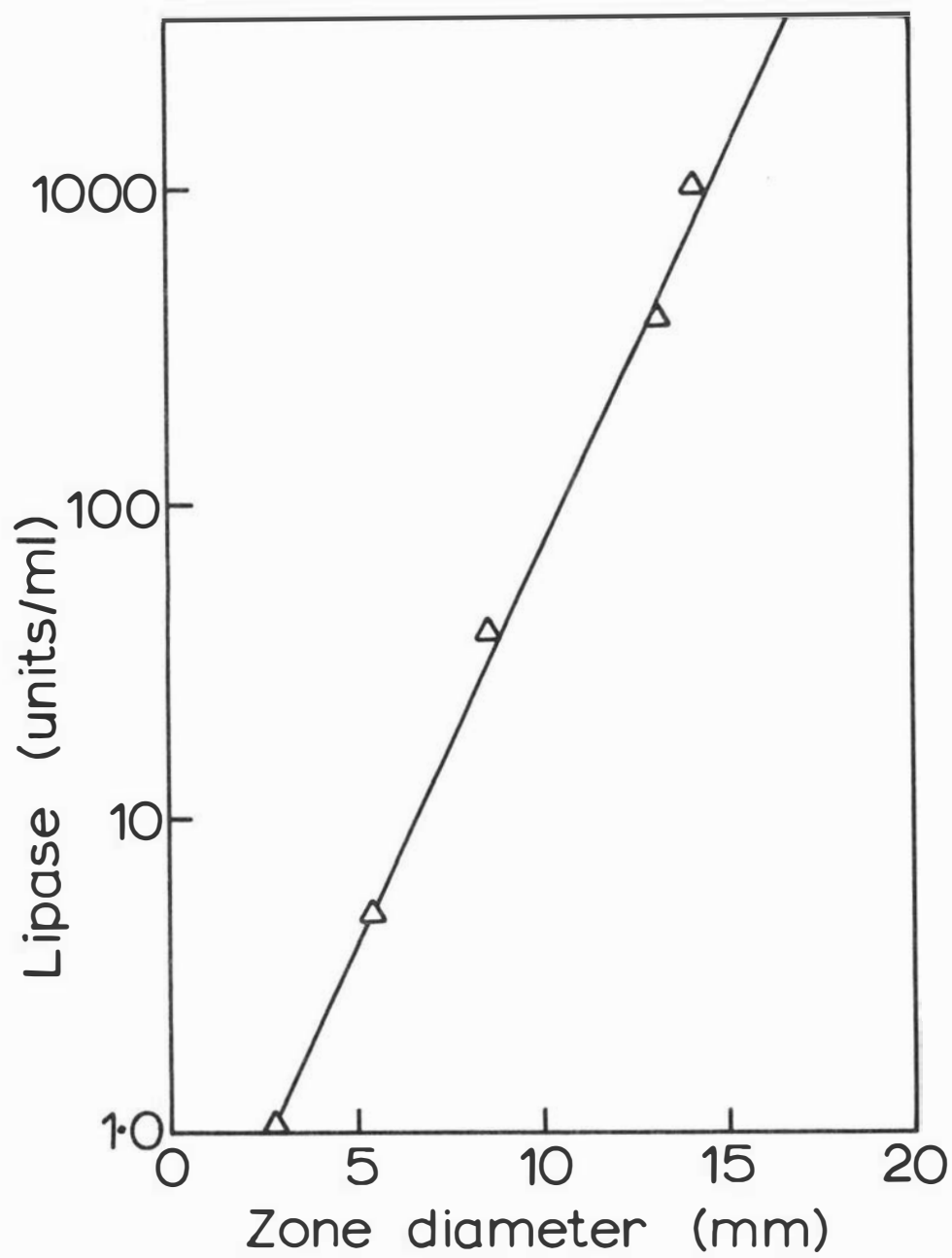
Figure 4   Standard curve for lipase determination.
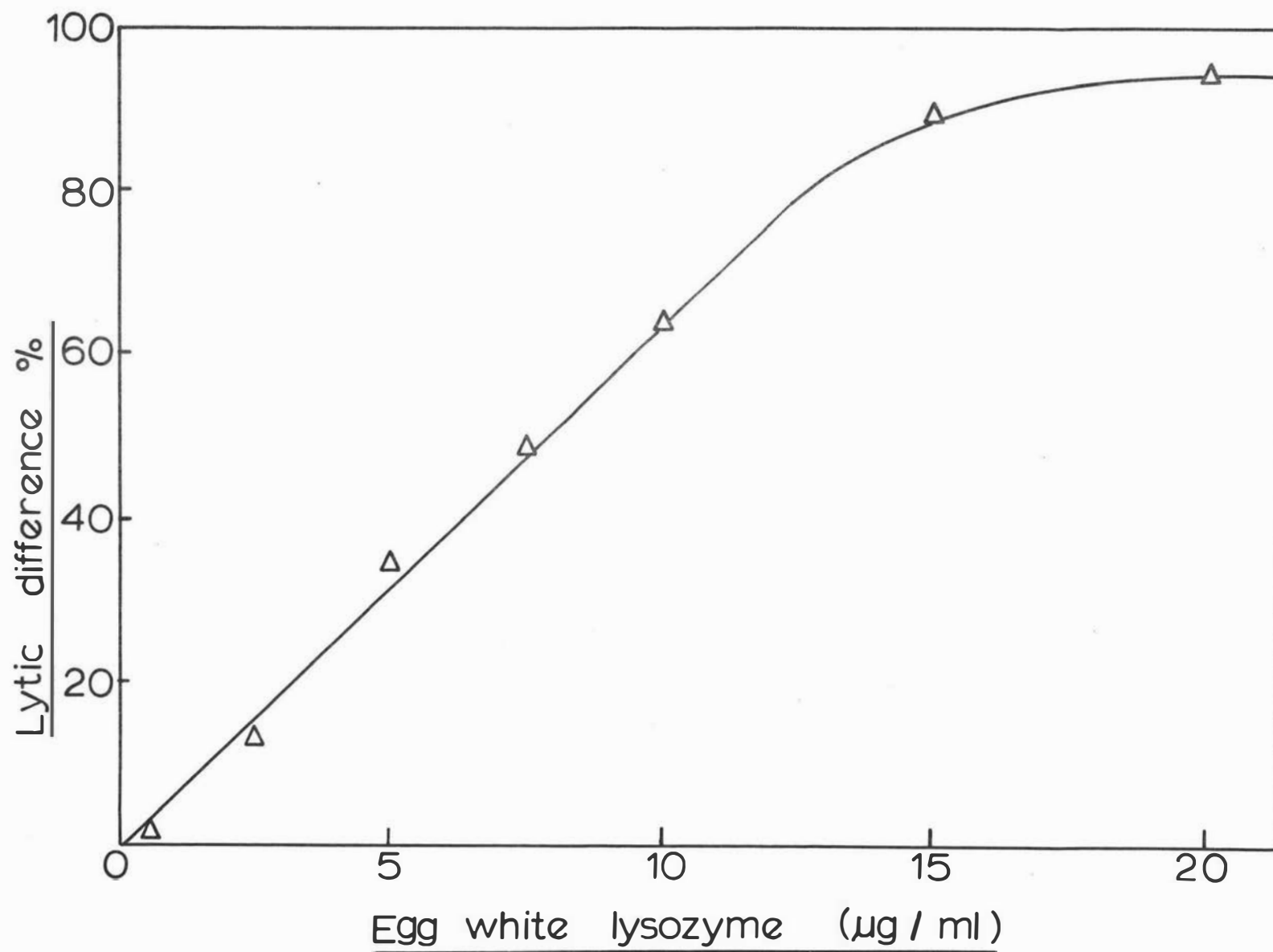
Figure 5   Standard curve for lysozyme determination.

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acid (0.5 ml) was added to a mixture of 1.0 ml supernatant
and 2.0 ml 0.85% saline, and the OD 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 entero-
toxin 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 (Pirt, 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 μ = specific growth rate and x_0 = initial cell mass

$$p = q_p \int_{t_0}^{t} x_0 e^{ut} \, dt + p_0$$
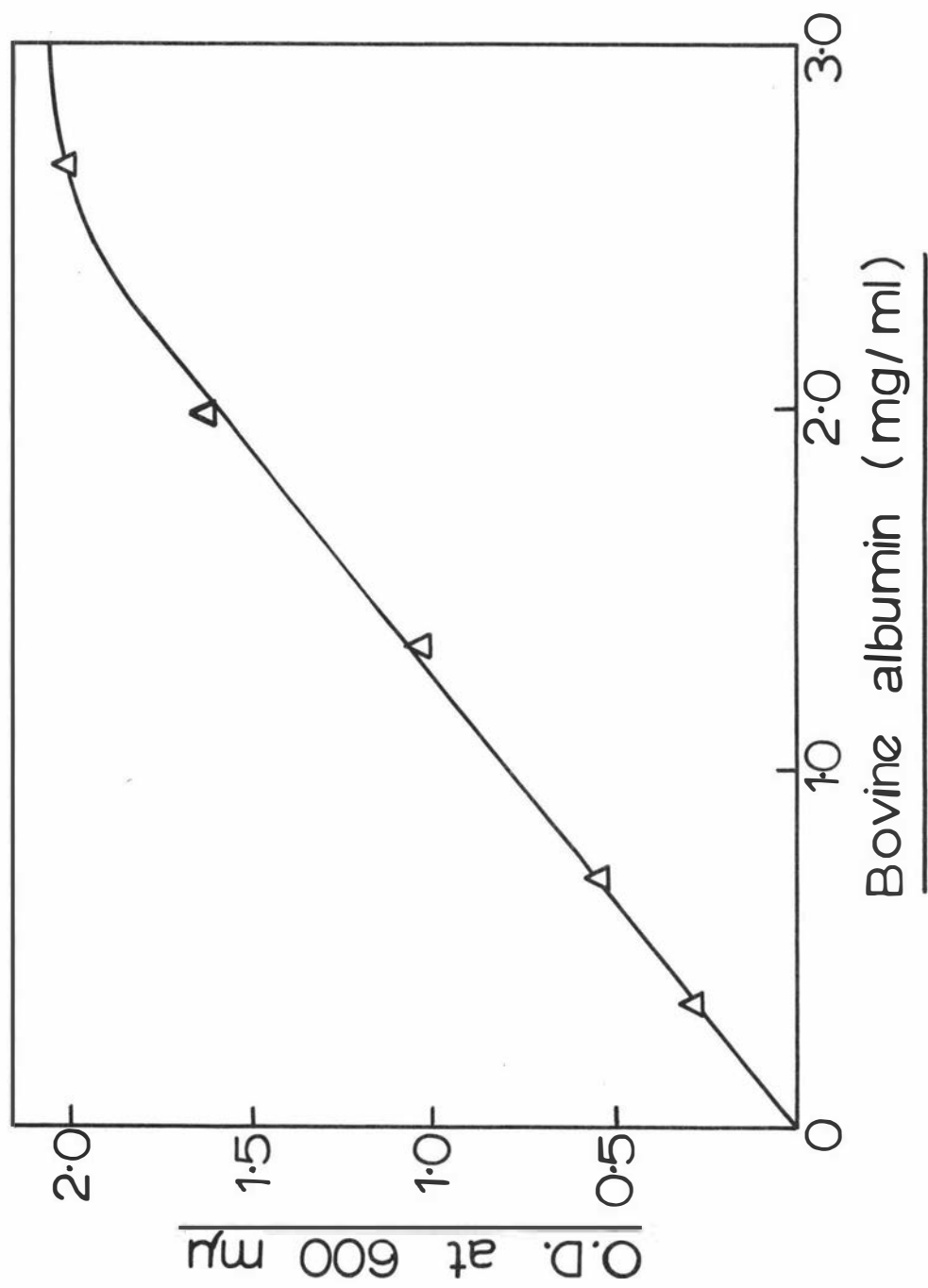
Figure 6   Standard curve for TEP determination.

$$\therefore \quad p - p_0 = q_p \, x_0 \, \frac{1}{\mu} \left[e^{\mu t} - e^{\mu t_0} \right]$$

$$\therefore \quad q_p = \frac{p - p_0}{x_0 e^{\mu t} - x_0 e^{\mu t_0}} \, \mu$$

i.e., the specific rate of product formation per unit of cell

mass $= \dfrac{\text{increase in product}}{\text{increase in cell mass}} \times$ 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:

$$q_p = \frac{p_{t_2} - p_{t_1}}{OD_{t_2} - OD_{t_1}} \, \mu$$

q_p units for batch cultures are units of product per unit
cell mass per hour, the unit of product being μg for entero-
toxins, 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 μg/
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
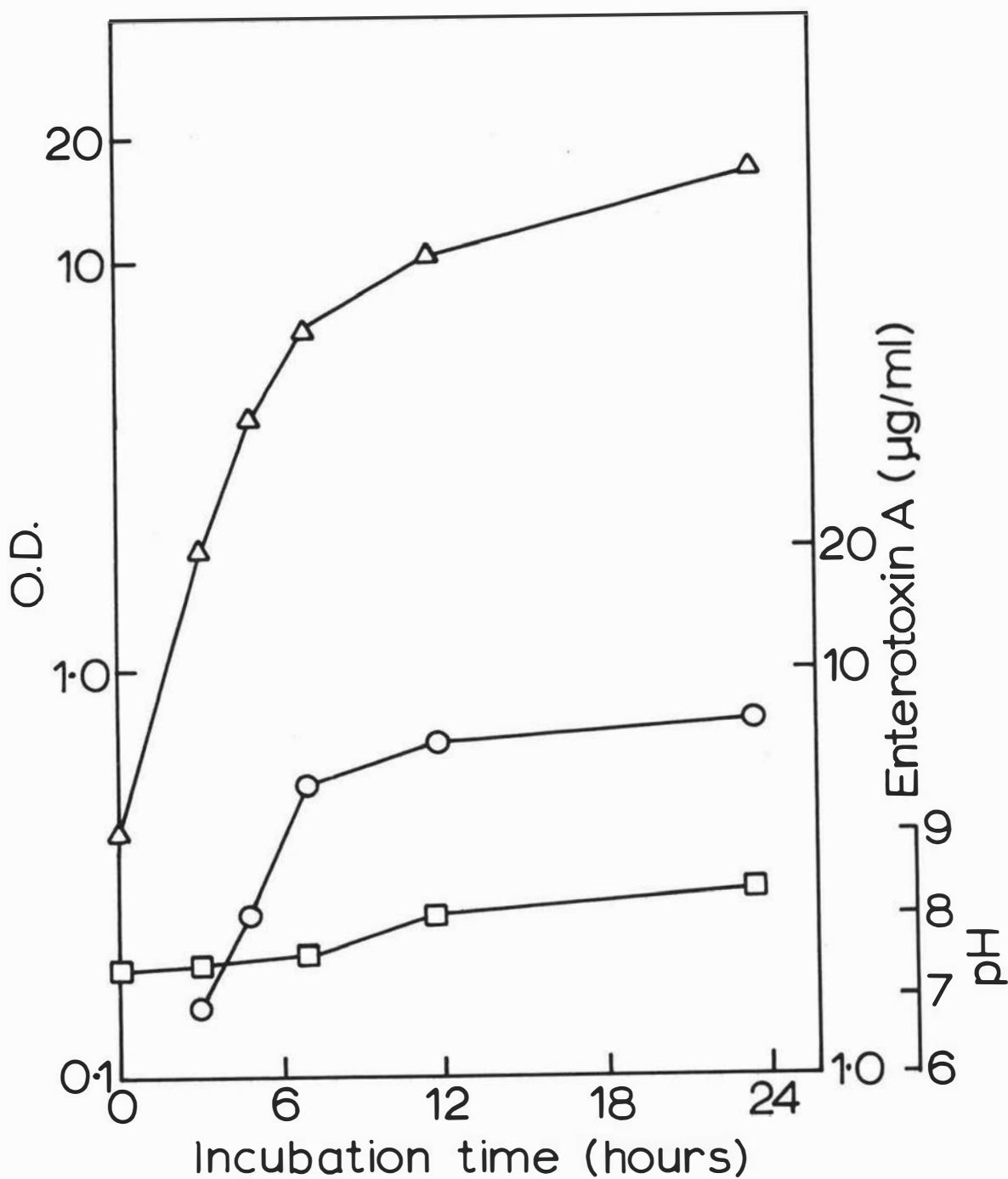
Figure 7   Growth and enterotoxin A production by strain 100 in a shake-flask in CH medium. OD, △; enterotoxin A, ○; pH, □.
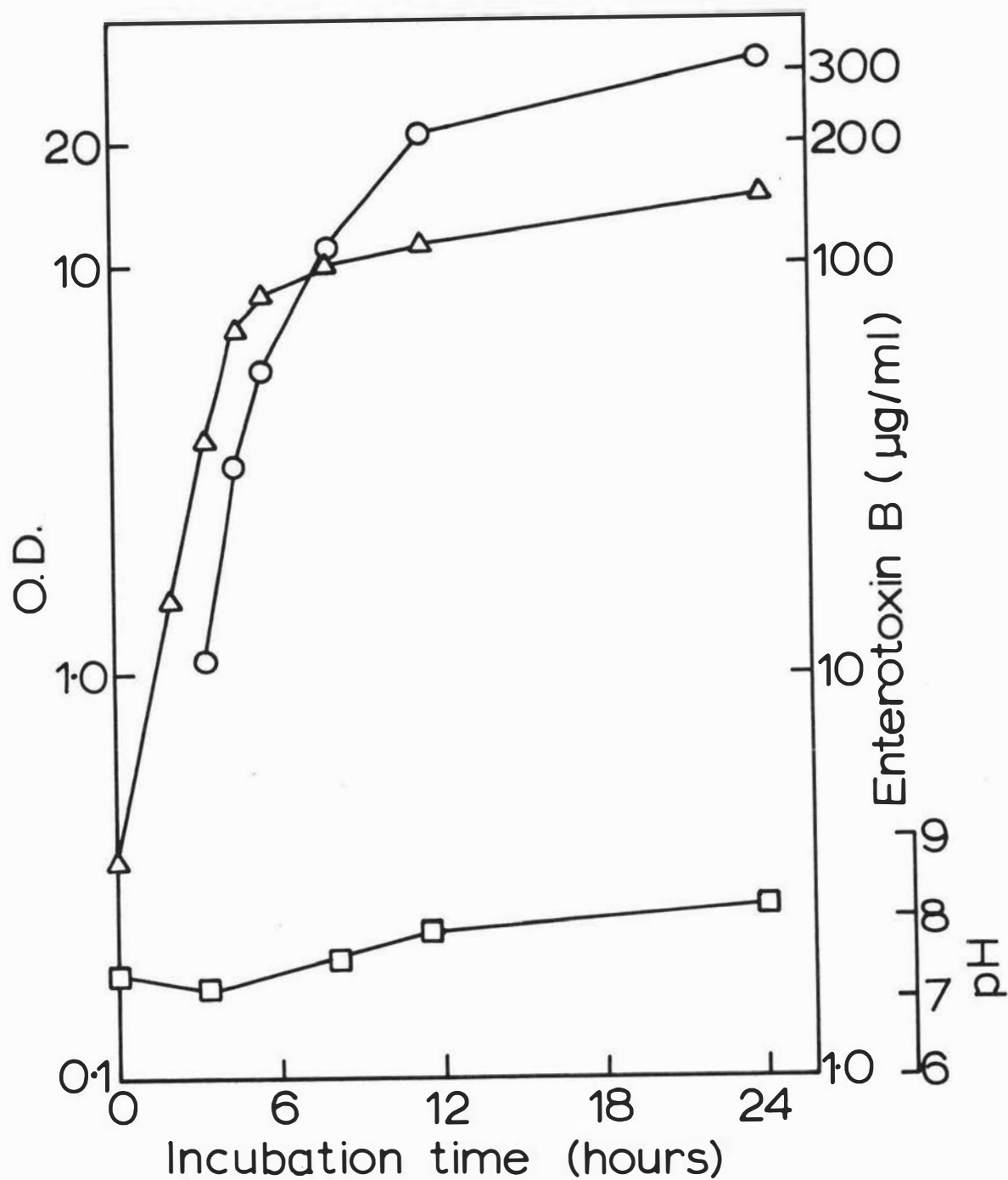
Figure 8   Growth and enterotoxin B production by strain S–6 in a shake-flask in
CH medium. OD, △; enterotoxin B, ○; pH, □.

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 S-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. Entero-toxin 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 entero-toxin 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, 361, 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
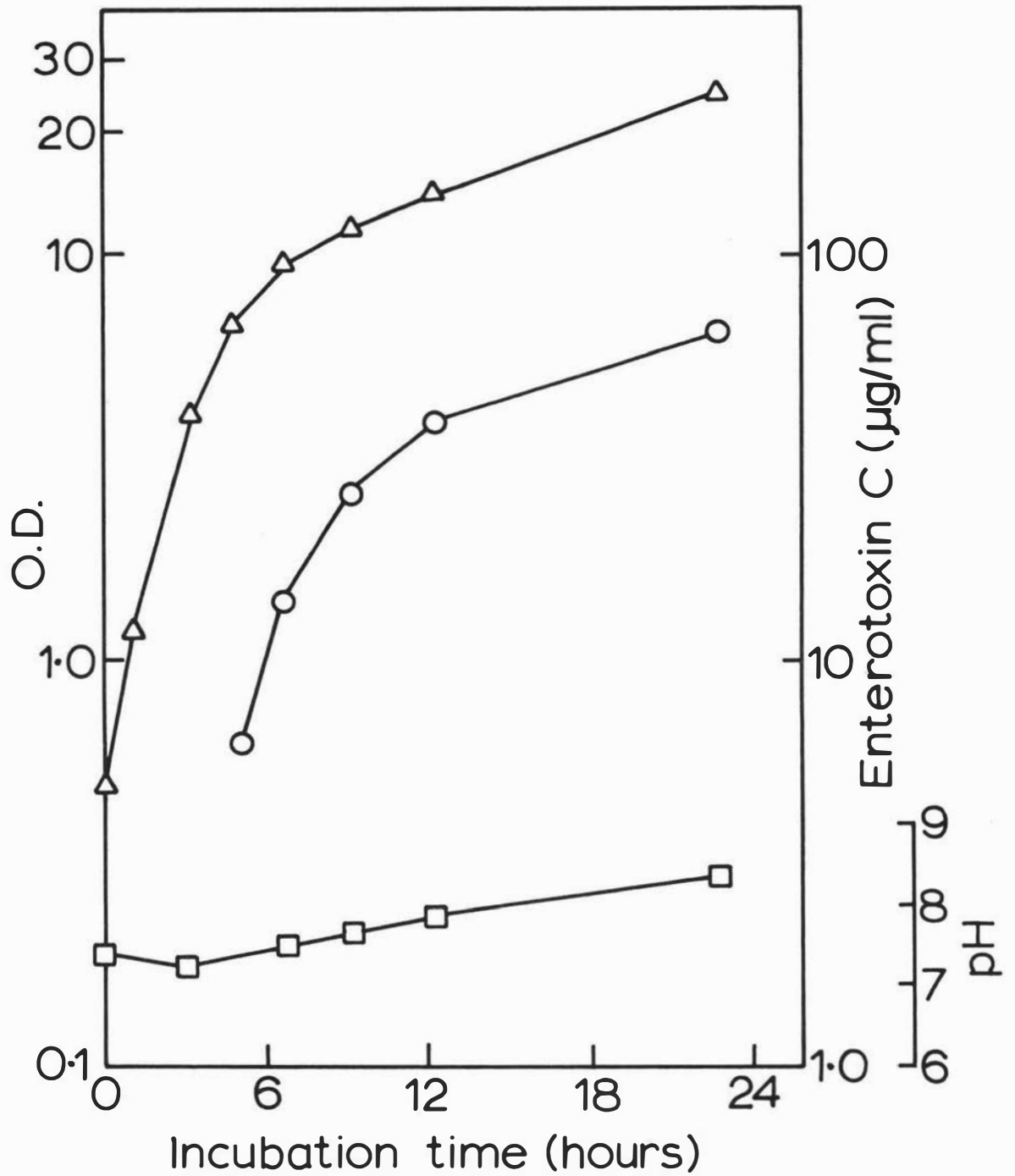
Figure 9   Growth and enterotoxin C production by strain 361 in a shake-flask in CH medium. OD, △; enterotoxin C, ○; pH, □.
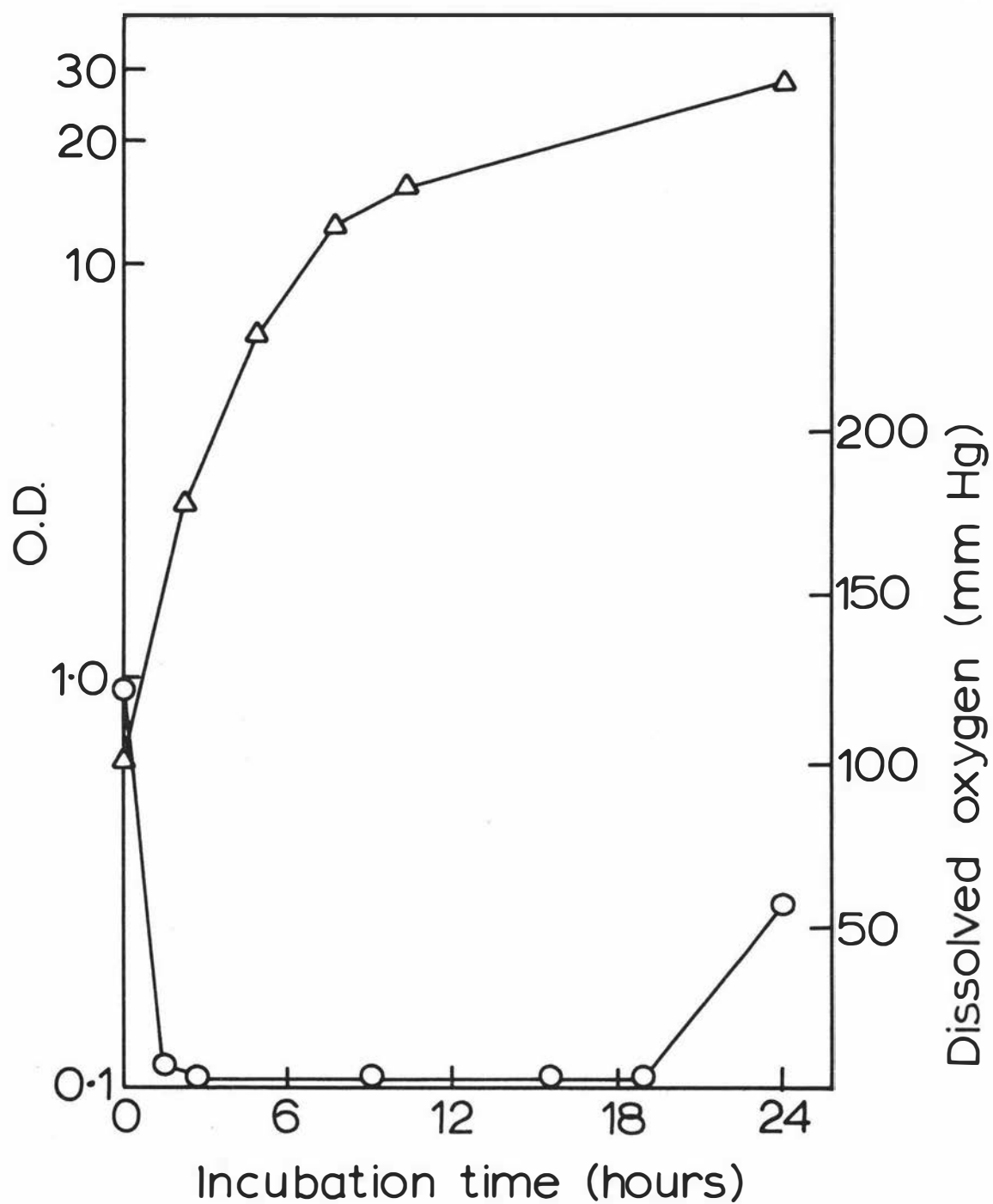
Figure 10   Oxygen utilization by strain 10 during 24 h incubation in a shake-flask.

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.

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

[a] ND indicates not detectable.
[b] Enterotoxin D reported as titre showing positive slide gel-diffusion test.

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

Strain and enterotoxin type	Medium	Specific growth rate (μ)	OD	Enterotoxin		TEP (mg/ml)	pH
				(μg/ml)	(q_p)		
100 A	CH	0.27	21	8	0.18	0.13	8.60
100 A	AA	0.49	18	7	0.07	0.14	7.25
S-6 B	CH	0.54	15	320	2.10	2.95	8.15
S-6 B	CH[a]	0.60	14	310	2.10	3.08	8.15
S-6 B	AA	0.63	17	190	1.0	0.66	7.95
361 C	CH	1.23	26	63	0.90	0.75	8.55
361 C	AA	0.59	25	100	1.50	0.64	8.45

[a]Initial pH 6.5 as compared with usual pH of 7.3 for CH medium.

was slightly decreased in AA medium.   No significant dif-
ferences 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 coincid-
ed 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 concentra-
tions 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 entero-
toxin 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 NH_4^+ ions in AA medium

AA medium contains 14.7 µmoles of K^+ ions as KH_2PO_4 and 7.6
µmoles NH_4^+ as $(NH_4)_2SO_4$.   As it had been reported (Friedman
1966, Kimble 1970) that 36.7 µmoles K^+ ions as K_2HPO_4 com-
pletely inhibited the production of enterotoxin B by strain
S-6, the effect of K^+ ions in AA medium was investigated.
Friedman also reported that NH_4^+ ions overcame this inhibi-
tion, and that the inhibition was partially dependent on pH.
Shake-flask cultures (200 ml AA medium in 1 litre flasks)
with and without $(NH_4)_2SO_4$, 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 pro-
duction of enterotoxin B, the specific growth rate (µ) and
the OD after 12 h incubation are shown in Table 3.   Increas-
ing 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 $(NH_4)_2SO_4$, at initial pH values of 6.5 and 7.7.

K^+ ions (μmoles/ml)	Initial pH	$(NH_4)_2SO_4$ (μmoles/ml)	Specific growth rate (μ)	Final OD	Enterotoxin B (μg/ml)
14.7	6.5	7.6	0.55	11.6	134
30.0	6.5	7.6	0.51	10.5	146
50.0	6.5	7.6	0.51	14.6	140
14.7	6.5	0	0.39	10.5	67
30.0	6.5	0	0.46	10.2	72
50.0	6.5	0	0.39	10.0	72
14.7	7.7	7.6	0.36	3.3	ND[a]
30.0	7.7	7.6	0.46	3.7	ND[a]
50.0	7.7	7.6	0.37	4.9	ND[a]
14.7	7.7	0	0.10	2.5	ND[a]
30.0	7.7	0	0.16	3.5	ND[a]
50.0	7.7	0	0.14	1.9	ND[a]

[a] ND indicates not detectable.

presence or absence of $(NH_4)_2 SO_4$. When the initial pH of
the medium was 6.5, the omission of $(NH_4)_2 SO_4$ 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
$(NH_4)_2 SO_4$ 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 dif-
ference 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 μg/ml enterotoxin B and 0.83 mg/ml TEP after 4 h
in N-medium, whereas in N-free medium only 5 μg/ml enterotoxin
B and 0.10 mg/ml TEP were produced. In the presence of
chloramphenicol, the production of enterotoxin B was reduced
to 1-2 μg/ml, and there was no detectable increase in TEP in
either medium. The metabolism of trypticase 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.

Medium	Cm[a] (μg/ml)	OD[b] Initial	Final	pH Initial	Final	Enterotoxin B (μg/ml)	TEP (mg/ml)
N-medium	0	0.16	0.19	7.1	8.1	63	0.83
N-medium	250	0.17	0.18	7.1	8.1	1	0
N-free medium	0	0.16	0.15	7.4	7.5	5	0.10
N-free medium	250	0.16	0.14	7.4	7.5	2	0

[a] Cm indicates chloramphenicol.

[b] 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 chlor-
amphenicol.   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.

Strain and enterotoxin type	Cm^a (µg/ml)	Enterotoxin (µg/ml)			
		10 h cells		20 h cells	
		N-medium	N-free medium	N-medium	N-free medium
100 A	0	8	Tr^a	11	Tr^a
100 A	250	ND^b	ND^b	Tr^a	Tr^a
22 A	0	6	3	6	ND^b
22 A	250	ND^b	ND^b	ND^b	ND^b
22 B	0	120	3	135	2
22 B	250	ND^b	1	1	1
S-6 B	0	270	5	63	5
S-6 B	250	ND^b	2	1	2
3 C	0	152	2	33	ND^b
3 C	250	ND^b	Tr^a	ND^b	ND^b
361 C	0	22	2	20	ND^b
361 C	250	ND^b	2	ND^b	ND^b
293 D	0			c^+	ND^b
293 D	250			ND^b	ND^b
485 D	0			$1/2^d$	ND^b
485 D	250			ND^b	ND^b

[a] Tr indicates trace.

[b] ND indicates not detectable.

[c] + indicates undiluted supernatant positive by slide-gel diffusion test.

[d] 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 entero-
toxin 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 condi-
tions 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 entero-
toxin 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 NH_4^+ 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 NH_4^+ 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 produc-
tion 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 (Metzger 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 Metzger 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 tempera-
ture was 65 C.

The inoculum for the fermenter consisted of cells grown for
16 h on a New Brunswick gyrotory shaker, washed and resuspended,
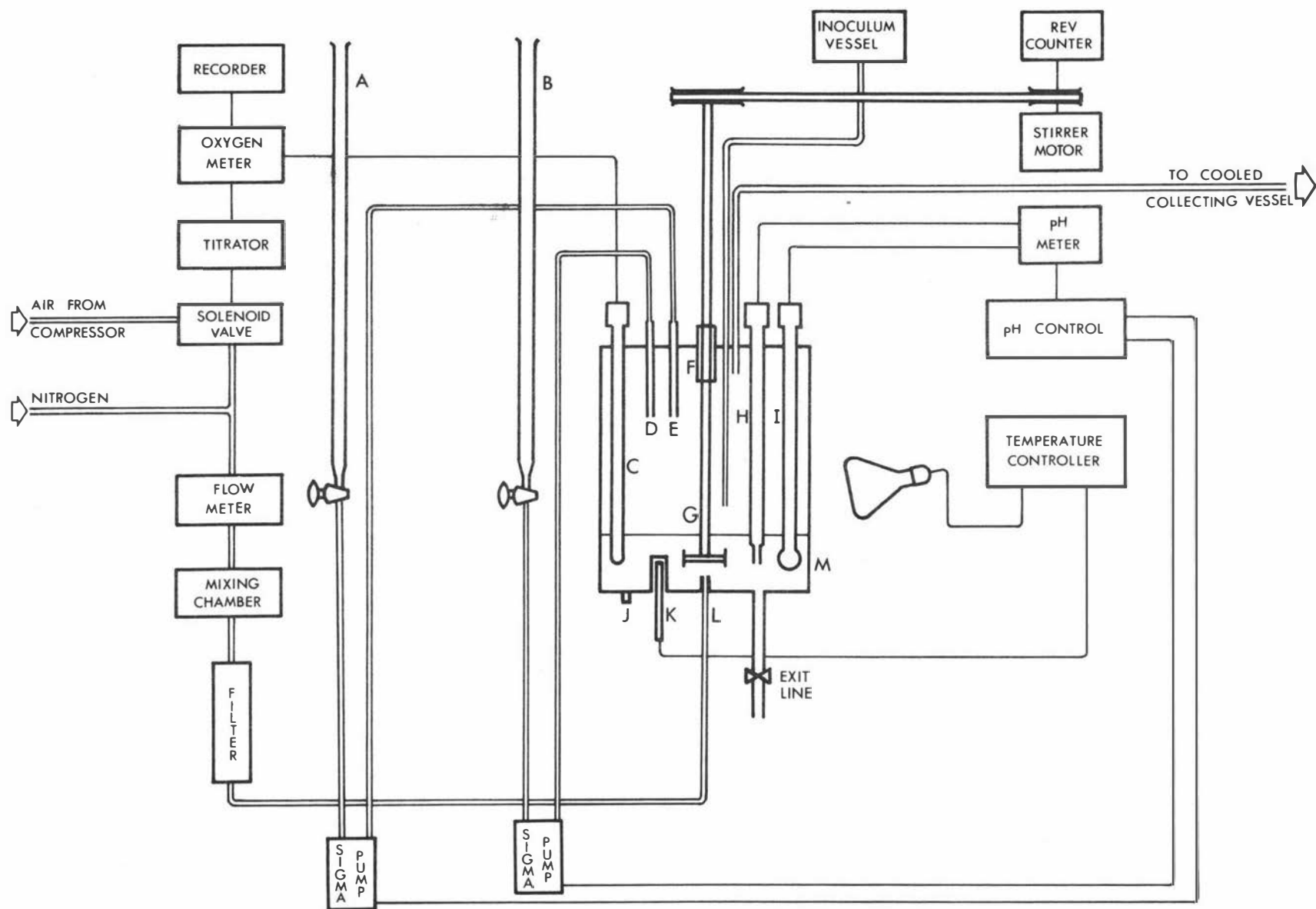
Figure 11 Diagram of fermenter. A, alkali reservoir; B, acid reservoir; C, oxygen sensor; D, acid inlet; E, alkali inlet; F, water-cooled stirrer gland; G, stirrer; H, KCL electrode; I, glass electrode; J, sampling port; K, thermometer; L, air inlet; M, 2 litre glass fermenter.

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 OD 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 N_2 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 dis-
cussed 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 N_2
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 OD determinations.   Samples were then centri-
fuged 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 TEP 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.

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

a 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 requir-
ing 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 extra-
cellular proteins, shake-flasks were inoculated with over-
night cultures of staphylococci, and additions of antifoam
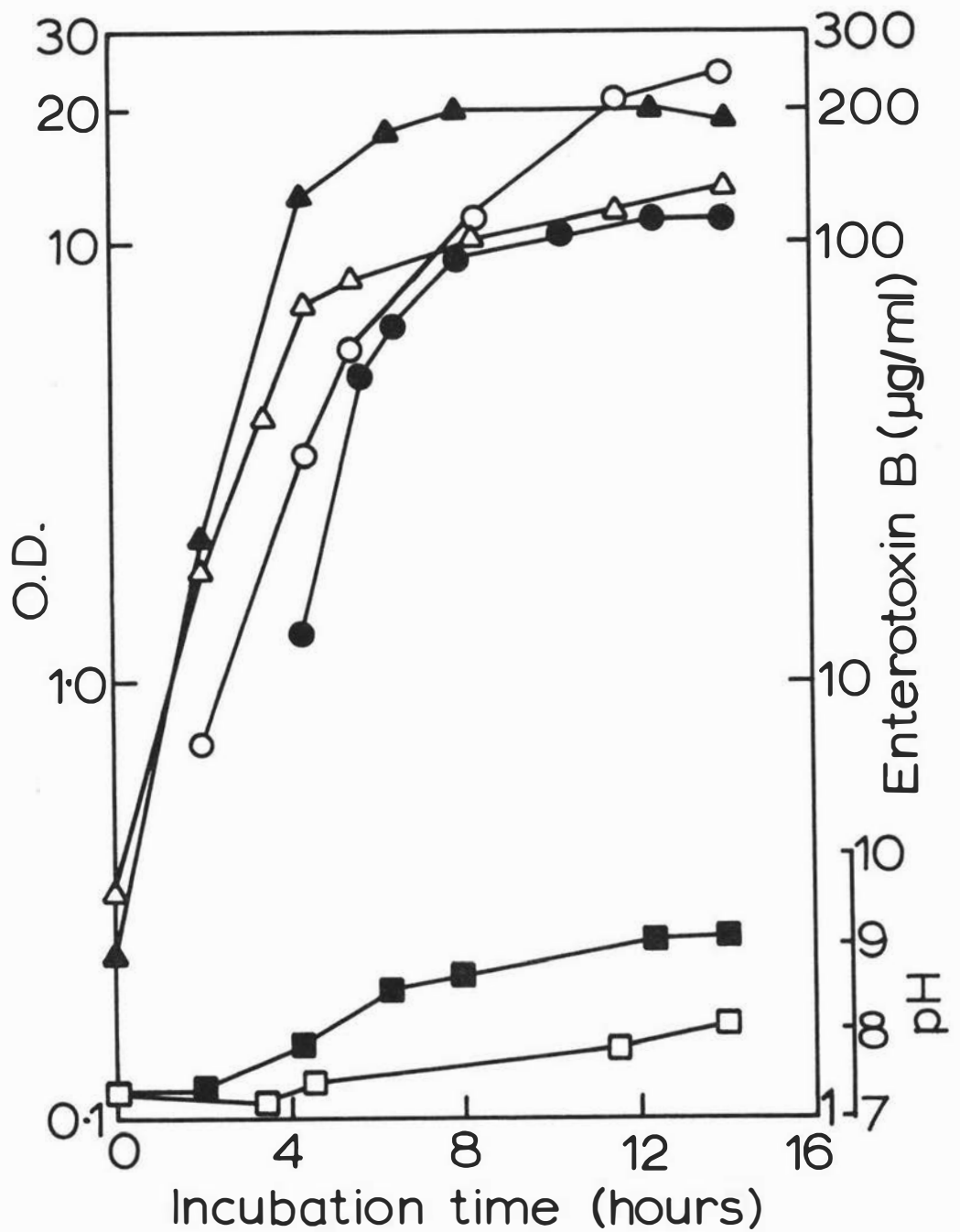
Figure 12   **Growth and enterotoxin B production by strain S—6 in a shake-flask (open symbols) and a fermenter (closed symbols) in CH medium. OD, △; enterotoxin B, ○; pH, □.**
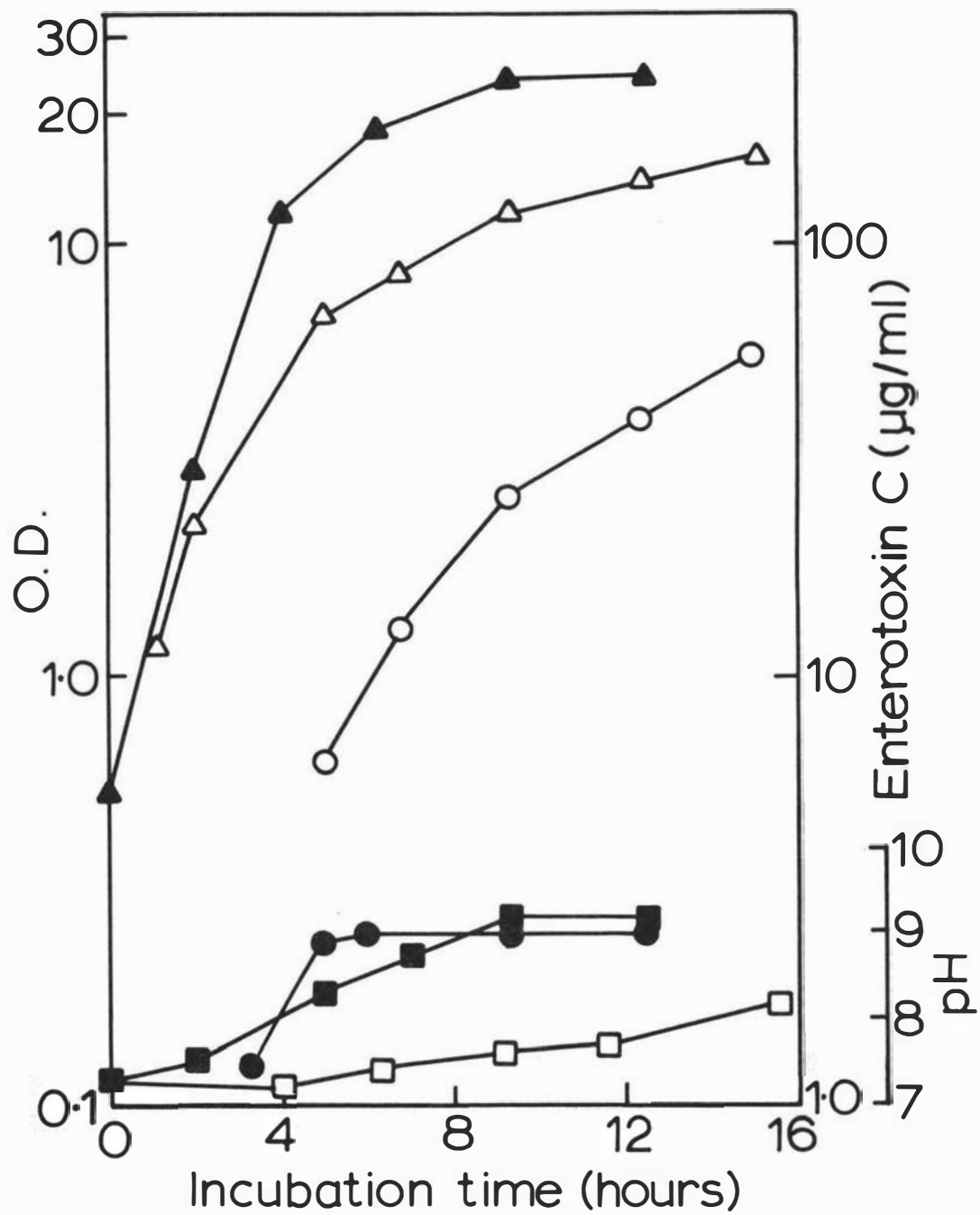
Figure 13   Growth and enterotoxin C production by strain 361 in a shake-flask
(open symbols) and a fermenter (closed symbols) in CH medium.
OD, △; enterotoxin C, ○; pH, □.

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 produc-
tion 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.

Strain and type	Antifoam (mg/ml)	Enterotoxin (µg/ml)	Lipase (units/ml)	Deoxyribonuclease (units/ml)	Lysozyme (units/ml)	TEP (mg/ml)
10 A	0	2	90	62	3.5	0.06
10 A	1.5	2	58	52	2.4	0.05
10 A	5.0	2	64	43	1.4	0.04
100 A	0	8	260	112	1.45	0.04
100 A	1.5	8	170	108	0.6	0.03
100 A	5.0	6	100	51	0	0.03
743 A	0	2	110	62	3.5	0.07
743 A	1.5	2	88	62	3.5	0.07
743 A	5.0	2	84	45	1.3	0.07
22 B	0	76	150	1200	1.0	0.07
22 B	1.5	72	140	640	0.3	0.07
22 B	5.0	40	70	560	0	0.06
30 B	0	106	16	290	2.35	0.09
30 B	1.5	45	14	140	1.40	0.07
30 B	5.0	15	12	82	0.70	0.05

Table 7 (Contd.)

Strain and type	Antifoam (mg/ml)	Enterotoxin (μg/ml)	Lipase (units/ml)	Deoxyribonuclease (units/ml)	Lysozyme (units/ml)	TEP (mg/ml)
S-6 B	0	256	280	700	0.95	0.20
S-6 B	1.5	214	210	440	0.40	0.16
S-6 B	5.0	72	140	240	0	0.12
3 C	0	42	140	62	3.5	0.20
3 C	1.5	23	116	62	1.3	0.20
3 C	5.0	19	84	62	0.4	0.12
37 C	0	44	130	72	3.0	0.23
37 C	1.5	30	92	82	1.6	0.19
37 C	5.0	26	90	70	0.65	0.10
361 C	0	38	280	44	3.5	0.35
361 C	1.5	28	280	36	3.2	0.21
361 C	5.0	13	170	35	1.95	0.19

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

Aeration	Strain and enterotoxin type	Final OD	Final pH	Enterotoxin (µg/ml)	TEP (mg/ml)
Control	10 A	21	8.10	2	0.22
Baffled	10 A	19	8.50	2	1.50
Control	100 A	17	8.40	6	0.03
Baffled	100 A	17	8.70	12	0.08
Control	743 A	18	8.15	ND	0.35
Baffled	743 A	24	8.60	1	1.55
Control	22 B	16	8.40	94	0.34
Baffled	22 B	16	8.90	105	0.61
Control	30 B	17	8.15	90	0.36
Baffled	30 B	20	8.50	116	1.34
Control	S-6 B	14	8.15	116	2.35
Baffled	S-6 B	14	8.80	196	2.32
Control	3 C	12	8.05	27	0.50
Baffled	3 C	21	8.45	40	1.03
Control	37 C	10	8.15	33	0.15
Baffled	37 C	19	8.60	55	0.60
Control	361 C	25	8.15	67	1.45
Baffled	361 C	23	8.70	28	5.0

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.

Aeration	Strain and type	Lipase (units/ml)	Deoxyribo- nuclease (units/ml)	Lysozyme (units/ml)
Control	10 A	185	94	4.15
Baffled	10 A	350	250	3.45
Control	100 A	210	150	1.0
Baffled	100 A	320	160	0.50
Control	743 A	37	43	4.5
Baffled	743 A	110	120	>6.0
Control	22 B	390	1700	1.20
Baffled	22 B	1250	4000	1.75
Control	30 B	7	110	2.40
Baffled	30 B	37	600	3.05
Control	S-6 B	780	700	4.0
Baffled	S-6 B	3120	2100	0.50
Control	3 C	58	36	4.0
Baffled	3 C	75	80	5.20
Control	37 C	140	28	4.25
Baffled	37 C	530	67	5.60
Control	361 C	700	59	>6.0
Baffled	361 C	1060	170	>6.0

TABLE 9.   Effect of dissolved oxygen concentra-
tion   on enterotoxin and TEP production in the
fermenter.

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

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 produc-
tion 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 limita-
tion, 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 - 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 µg/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 µg/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 10. Effect of pH on the production of enterotoxins A, B and C and other extracellular proteins in CH medium.

Strain and type	pH	Enterotoxin (µg/ml)	(q_p)	Lipase (units/ml)	Deoxyribo-nuclease (units/ml)	Lysozyme (units/ml)	TEP (mg/ml)	Final CD
100 A	SF[a]	8	0.21	190	165	0.8	0.13	21
100 A	NC[b]	8	0.39	7	90	0	0.04	16
100 A	6.0	11	0.11	236	22	0	0.04	11
100 A	6.5	22	0.27	196	38	0.9	0.14	26
100 A	7.3	15	0.21	2	96	1.0	0.04	17
100 A	8.0	7	0.16	2	75	1.0	0.04	11
S-6 B	SF[a]	290	3.60	1210	960	1.2	2.95	16
S-6 B	NC[b]	114	1.25	415	2600	0	0.97	20
S-6 B	6.5[c]	169	1.30	920	310	>6.0	0.73	25
S-6 B	7.3[c]	180	1.90	1180	710	4.4	1.67	24
S-6 B	8.0	124	1.20	415	970	2.1	1.14	25
361 C	SF[a]	63	0.83	1470	32	>6.0	0.75	26
361 C	NC[b]	3	0.09	214	32	1.25	0.09	25
361 C	6.5	4	0.07	168	17	4.0	0.27	25
361 C	7.3	5	0.05	214	36	4.8	0.70	30
361 C	8.0	7	0.13	115	37	1.55	0.40	24

[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 character-
istic 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 con-
trolled 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
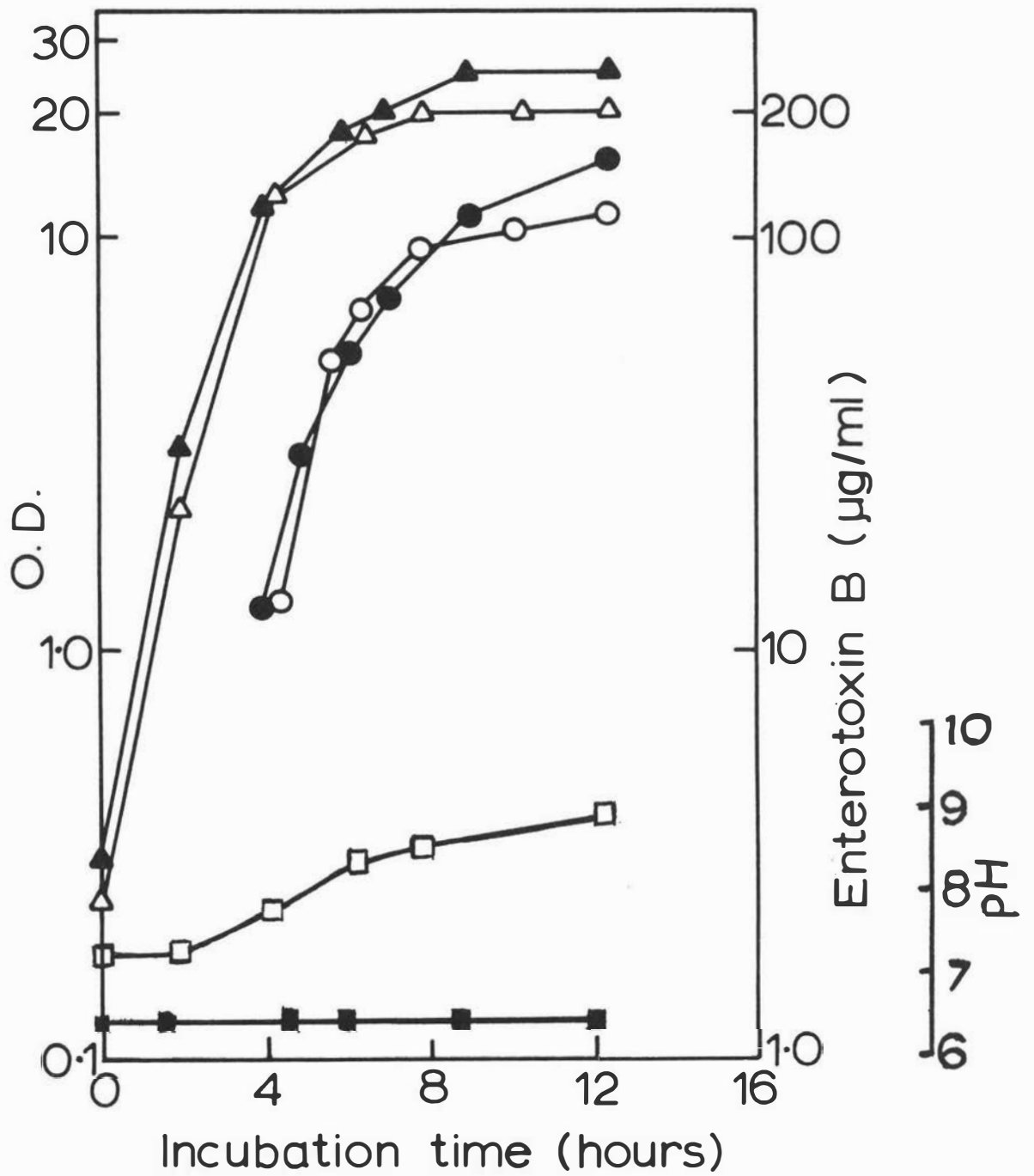
Figure 15   Growth and enterotoxin B production by strain S—6 in a fermenter in CH medium at uncontrolled pH (open symbols) and pH 6.5 (closed symbols). OD, △; enterotoxin B, ○; pH, □.

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 TEP 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 con-
siderably 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 entero-
toxin 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 experi-
ments.   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 pro-
duction.

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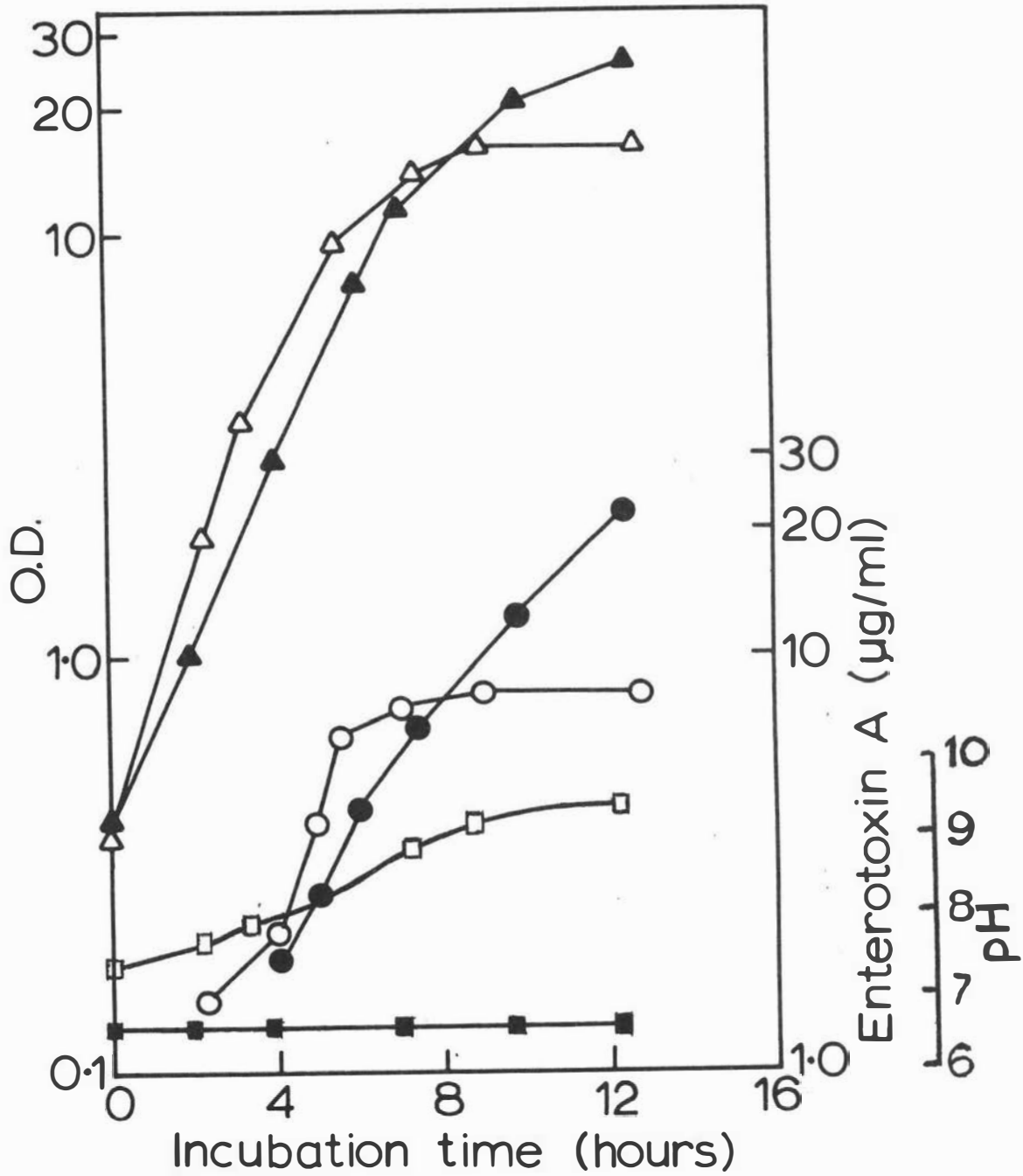generally higher under conditions of controlled pH than at
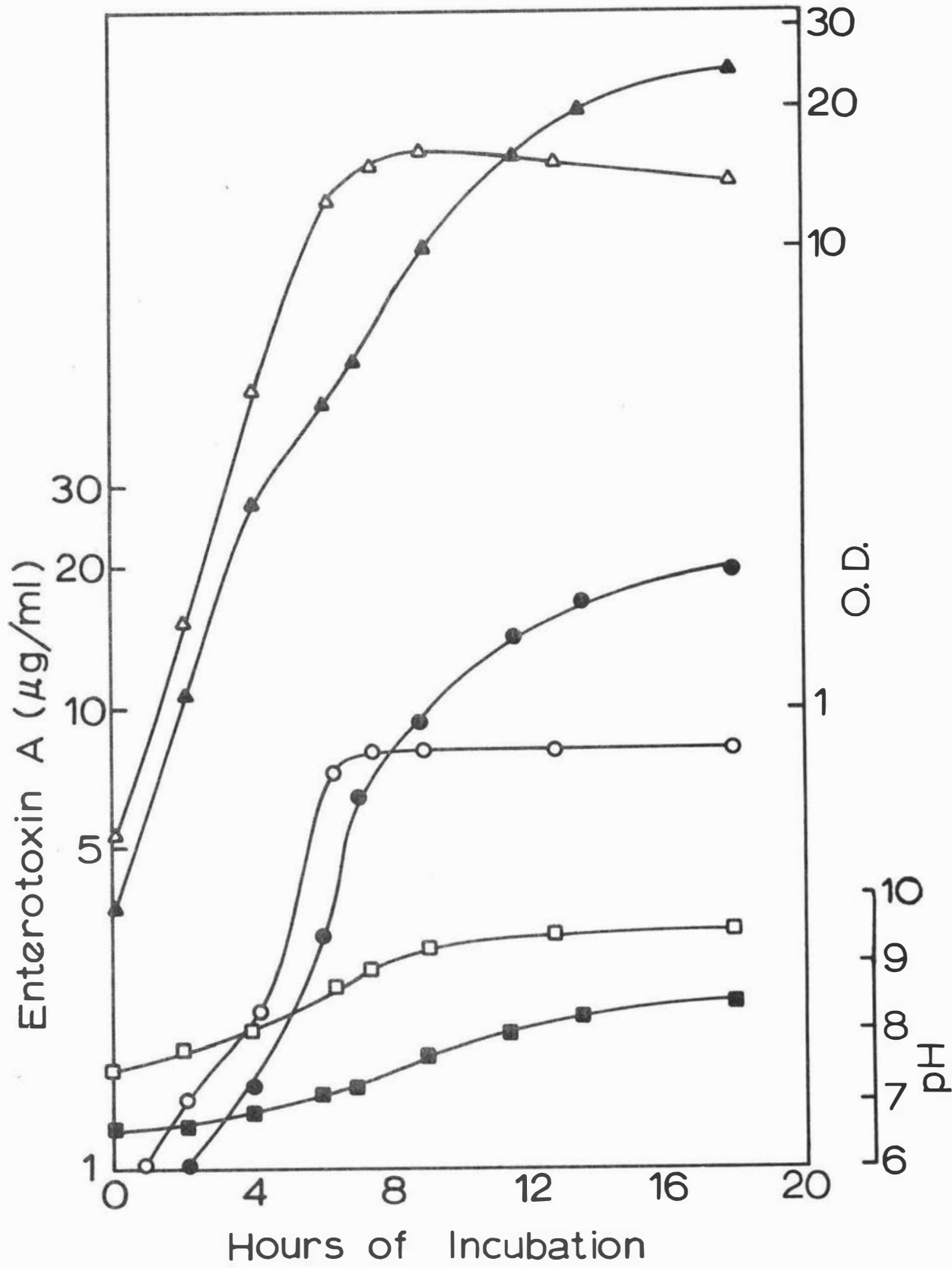
Figure 16   Growth and enterotoxin A production by strain 100 in a fermenter in CH
medium (open symbols) and AA medium (closed symbols).
OD, △; enterotoxin A, ○; pH, □.

TABLE 11.   Effect of pH on the productions of enterotoxins A, B and C and other extracellular proteins in AA medium.

Strain and enterotoxin type	pH	Enterotoxin (μg/ml)	(q_p)	Lipase (units/ml)	Deoxyribonuclease (units/ml)	Lysozyme (units/ml)	TEP (mg/ml)	Final OD
100 A	SF[a]	7	0.06	27	8	0.1	0.14	18
100 A	NC[b]	20	0.18	20	13	0	0.16	24
100 A	6.0	24	0.20	5	17	0	0.06	27
100 A	6.5	32	0.25	5	10	0.2	0.05	27
100 A	7.3	20	0.18	48	15	0	0.15	25
S-6 B	SF[a]	194	1.54	59	86	0.1	0.68	17
S-6 B	NC[b,c]	97	1.70	100	84	0.2	0.45	26
S-6 B	6.0[c]	96	1.83	38	28	0.2	0.46	35
S-6 B	6.5[c]	129	2.12	240	37	0.3	0.67	25
S-6 B	7.3[c]	131	1.91	110	47	0	1.12	23
S-6 B	8.0	78	1.02	114	125	0.1	0.46	17
361 C	SF[a]	100	1.20	67	10	1.1	0.64	24
361 C	NC[b]	35	0.55	90	19	1.1	0.53	28
361 C	6.0	25	0.34	48	12	0.9	0.21	17
361 C	6.5	53	0.71	320	8	3.1	1.02	23
361 C	7.3	30	0.37	320	19	3.0	0.90	26

[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 consider-
able 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 N_2 (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 approxi-
mately 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 repro-
ducible, and was not peculiar to strain 100. If strain S-6
was subject to an air and N_2 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 experi-
ments 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 CO_2.
The nitrogen in the air and N_2 mixture was therefore replaced
with CO_2, 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
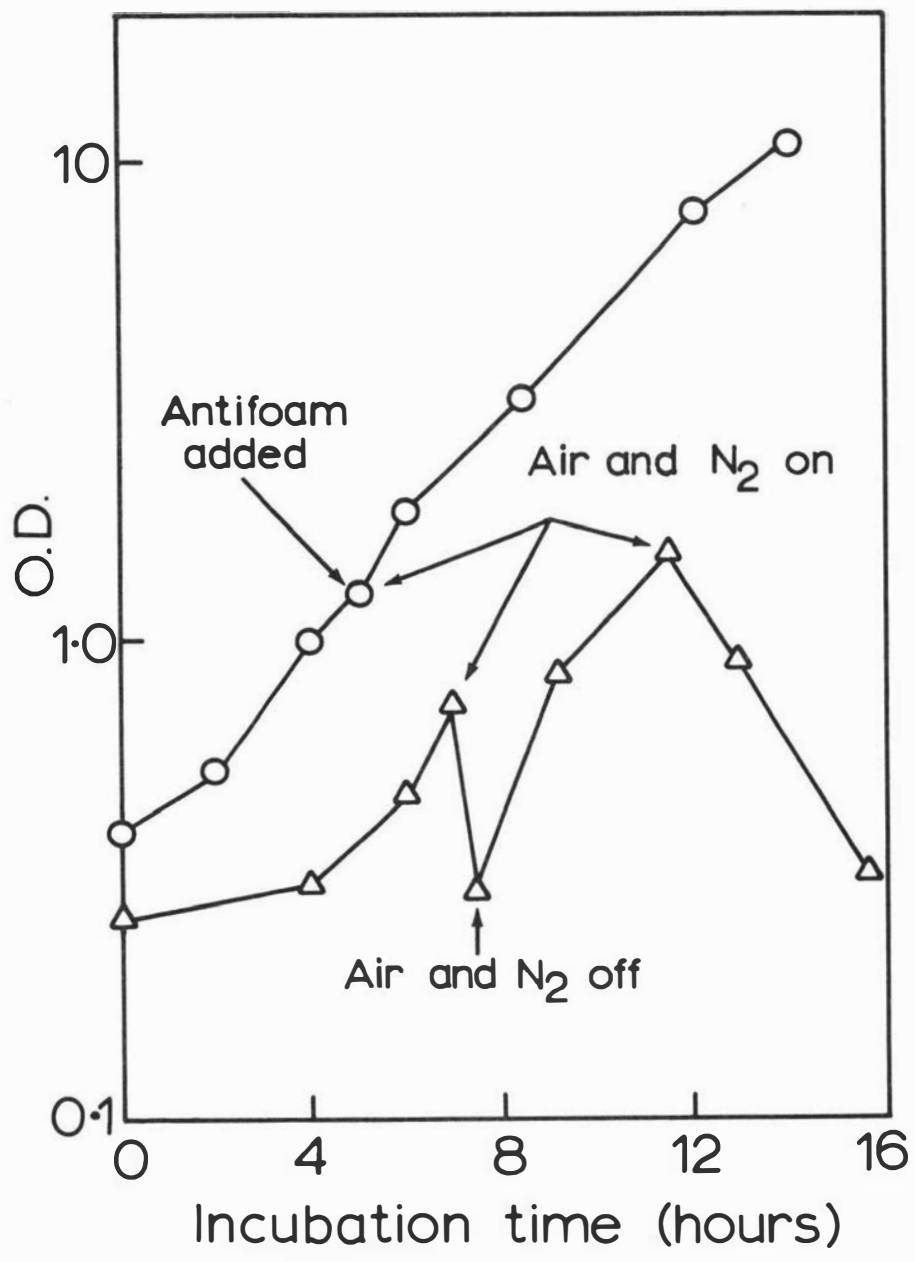
Figure 17 Effect of air and N_2 on OD of cells of strain 100 in a fermenter in AA medium when antifoam added (○) and in the absence of antifoam (△).

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 S-6.   This pro-
cedure 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 S-6 and 22 which produced both entero-
toxins A and B.   Thus the yield of both enterotoxins was
increased by growth of strain S-6 under controlled pH condi-
tions, 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 pro-
duction 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 entero-
toxin 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 Na Cl 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 extra-
cellular 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 aera-
tion 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 pro-
duction 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 produc-
tion 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 CH medium, it was found that the specific rate of entero-
toxin 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 fermenter 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 des-
cribed 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 Mah, 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 Pastan (1968) was used.   This consisted of 14.0 g of K_2HPO_4, 6.0 g of KH_2PO_4, 2.0 g of $(NH_4)_2 SO_4$, 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:

Glucose + ATP $\rightleftharpoons$ glucose-6-phosphate + ADP

glucose-6-phosphate + NADP $\rightleftharpoons$ NADH + H^+ +6-phosphogluconate

NADH 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 comple-
tion 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 β-galactosidase

Determinations of β-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-β-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 β-galactosidase was taken as that which hydrolysed 1 μmole ONPG in 3 h at 30 C. Although it has been reported (Morse et al., 1968) that staphylococcal β-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 (Morse 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
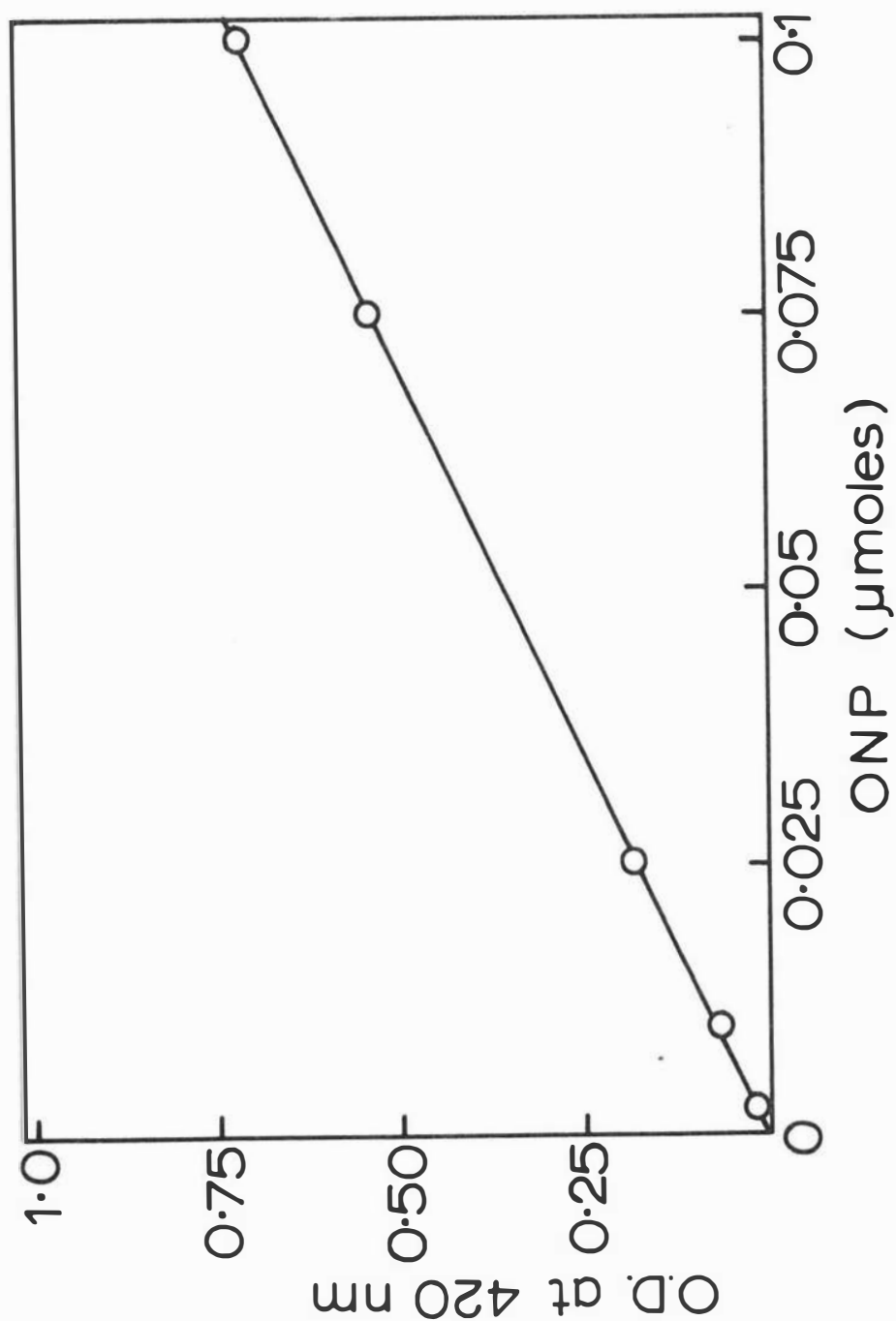
Figure 18   Standard curve for determination of β -galactosidase.

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.

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

4.3.2   Effect of added carbohydrates and related compounds
on the production of enterotoxins and other extra-
cellular 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 (μ) 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 S-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.

Strain and type	Carbon compound added	Initial[b] pH	Final[c] pH	Control[d] pH	Percentage[e] change in enterotoxin q_p
100 A	glucose	6.05	5.65	7.20	-88
100 A	glycerol	6.05	5.90	7.20	-76
100 A	pyruvate	6.65	6.80	7.20	-29
S-6 B	glucose	6.20	5.60	6.80	-65
S-6 B	glycerol	6.25	5.75	6.80	-74
S-6 B	pyruvate	6.50	6.65	6.80	+7
30 B	glucose	5.55	5.20	6.90	-96
30 B	glycerol	5.95	5.65	6.90	-75
30 B	pyruvate	6.50	6.60	6.90	-32
361 C	glucose	5.50	5.20	7.15	-100
361 C	glycerol	6.0	5.60	7.15	-100
361 C	pyruvate	6.65	6.80	7.15	+270
3 C	glucose	5.30	5.05	7.0	-100
3 C	glycerol	5.50	5.10	7.0	-100
3 C	pyruvate	6.65	7.10	7.0	0

[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 staphylococci in shake-flasks
in AA medium during 24 h incubation.

Strain and type	Carbon compound added	Percentage change[b] in q_p relative to controls			
		TEP	Lipase	Deoxyribo-nuclease	Lysozyme
100 A	glucose	-50	-100	-100	-100
100 A	glycerol	-100	-100	-100	-100
100 A	pyruvate	0	-65	+190	+300
S-6 B	glucose	-41	-100	-100	-100
S-6 B	glycerol	-42	-100	-100	-100
S-6 B	pyruvate	+55	+20	+55	+60
30 B	glucose	-100	-100	-100	-100
30 B	glycerol	-57	-100	-100	-100
30 B	pyruvate	-29	-81	+20	+65
361 C	glucose	-100	-100	-100	-50
361 C	glycerol	-100	-100	-100	-100
361 C	pyruvate	+200	+100	+170	+600
3 C	glucose	-94	-100	-100	-100
3 C	glycerol	-100	-100	-100	-100
3 C	pyruvate	+100	0	+28	+110

[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.

Extracellular protein	Percentage change[a] in q_p relative to controls[b]					
	100 A		S-6 B		30 B	
	Galactose	Lactose	Galactose	Lactose	Galactose	Lactose
Enterotoxin	-29	-35	+5	+16	-21	-11
Deoxyribonuclease	-20	+150	+40	-45	-100	-43
Lysozyme	0	+33	-70	0	-70	-30
Lipase	-100	-46	+21	+4	-100	-42
TEP	+100	0	+79	-10	+43	0
Initial pH[c]	6.35	6.73	6.45	6.55	6.35	6.75
Final pH[d]	6.10	6.90	6.45	6.76	5.55	6.12
[e]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

[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.

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.

strain 3i
3

Extracellular protein	Percentage change[a] in q_p relative to controls[b] Strain 36-C		Strain 3-C	
	Galactose	Lactose	Galactose	Lactose
Enterotoxin	+7	+70	−71	−17
Deoxyribonuclease	−21	−26	−72	−37
Lysozyme	+100	+200	+40	+160
Lipase	−67	−88	−100	−34
TEP	+100	+50	−23	0
Initial pH[c]	6.24	6.55	6.15	6.70
Final pH[d]	5.45	5.60	5.4	6.20
[e]pH of control flask	7.15	7.15	7.0	7.0
Time interval (hours)	6-9	6-9	6-9	6-9

[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%.   Entero-
toxin 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 B 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
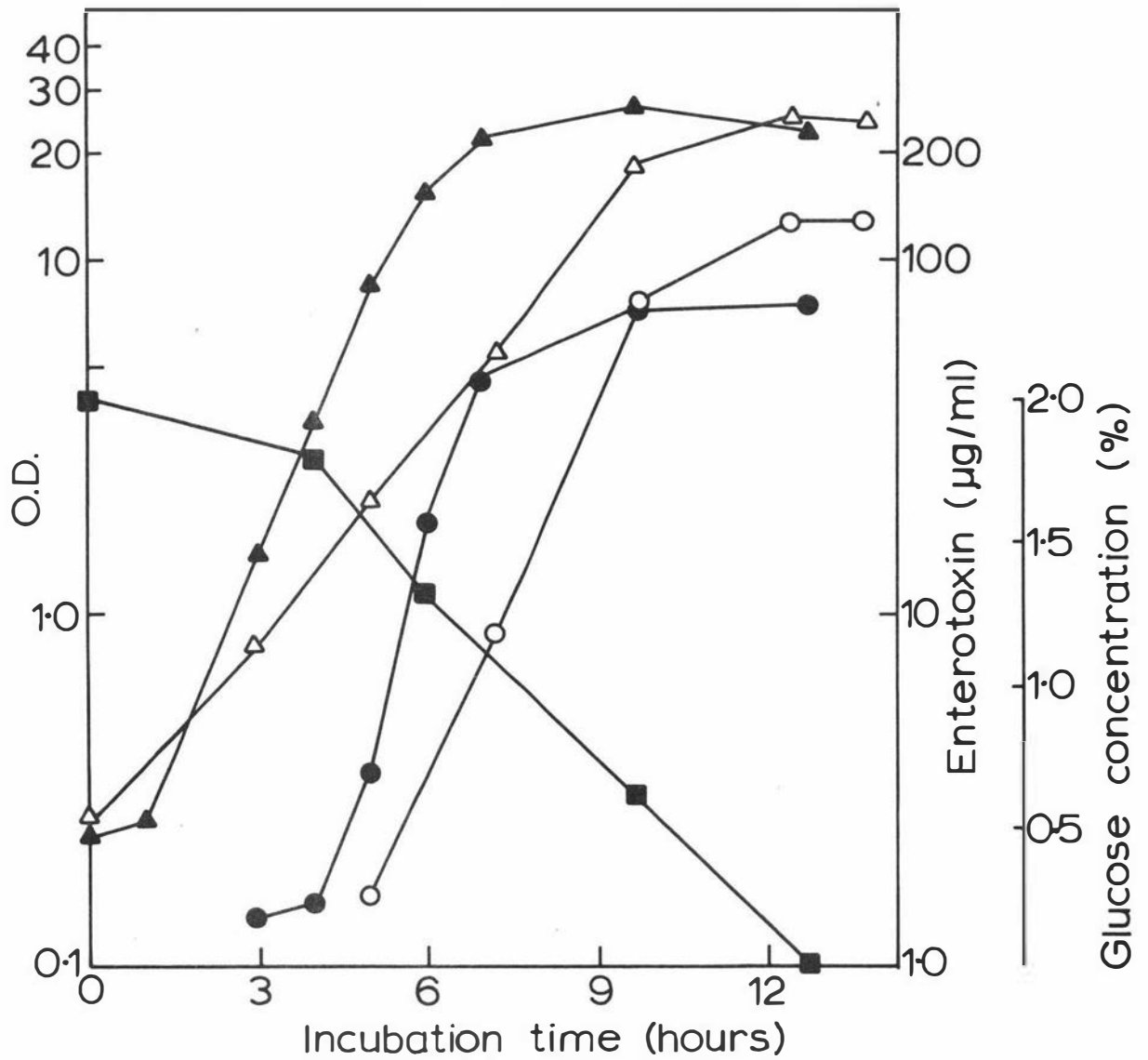
Figure 19   Growth and enterotoxin B production by strain S—6 in the fermenter in AA medium (open symbols), and in AA medium containing an initial concentration of 2% clucose (closed symbols).
OD, △, enterotoxin B, ○; glucose, ■.

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.

Strain and enterotoxin type	Initial glucose (per cent)	Final glucose (per cent)	Final OD	Specific growth rate (μ)	Enterotoxin (μg/ml)	TEP (mg/ml)
100 A	0	0	26.8	0.38	32	0.05
100 A	2	1.5	14.0	0.71	2	0.05
S-6 B	0	0	24.8	0.46	129	0.84
S-6 B	2	0.006	23.2	0.86	75	1.36
30 B	0	0	22.4	0.45	136	0.89
30 B	2	0.003	26.8	0.86	32	1.42
361 C	0	0	22.8	0.47	53	1.02
361 C	2	0.004	24.6	0.65	10	0.38

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.

	Strain and enterotoxin type	CD	Specific growth rate (μ)	Enterotoxin $(\mu g/ml)$	(q_p)	Lipase (units/ml)	Deoxyribo-nuclease (units/ml)	TEP (mg/ml)
Control	100 A	26.8	0.38	32	0.21	5	5	0.05
0.1 M glucose	100 A	14.2	0.71	3	0.12	0	8	0.05
Control	S-6 B	25.8	0.46	129	2.12	240	37	0.84
0.1 M glucose	S-6 B	36	0.91	51	1.34	147	500	0.46
0.1 M glycerol	S-6 B	35	0.82	29	1.00	34	450	0.55
Control	361 C	22.8	0.47	53	0.59	320	8	1.02
0.1 M glucose	361 C	24.6	0.80	15	0.16	201	14	0.36
Control	30 B	22.4	0.45	136	2.79	17	25	0.89
0.1 M glucose	30 B	28.8	0.82	26	0.99	7	125	0.70
0.1 M glycerol	30 B	26.4	1.18	35	0.94	13	246	0.68

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, deoxyribo-
nuclease 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 entero-
toxin 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 produc-
tion 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 produc-
tion 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
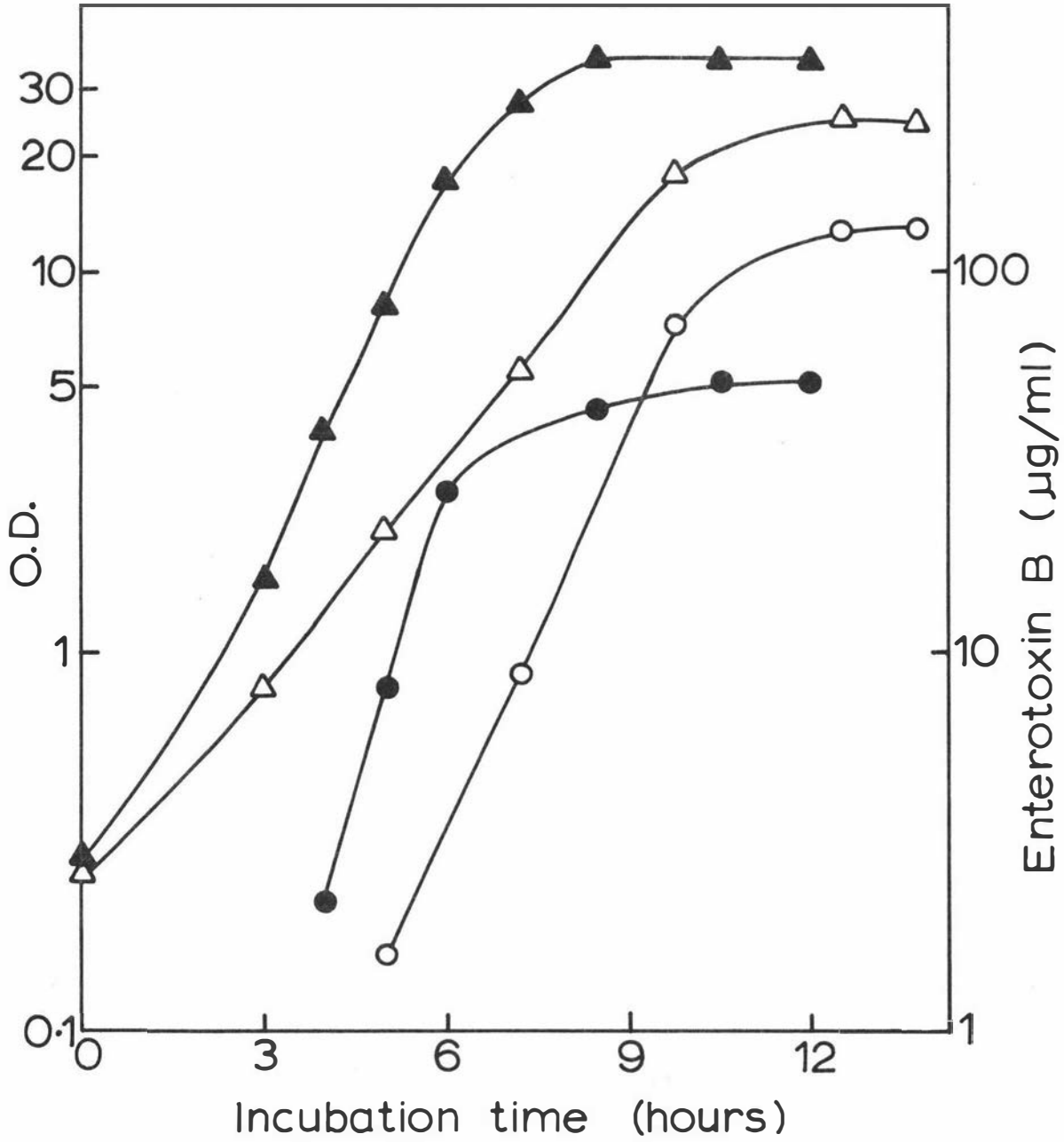
Figure 20 **Growth and enterotoxin B production by strain S–6 in AA medium (open symbols) and in AA medium with glucose concentration maintained at 0.1 M (closed symbols).**
OD, △; enterotoxin B, ○.

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 $5\frac{1}{2}$, $7\frac{1}{4}$ and 24 h.
In the flask from which thiamine was omitted, glucose concen-
trations 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 S.aureus S-6. Thiamine has been shown to
play a highly significant part in the metabolism of glucose
by staphylococci (Blumenthal, 1972). It is probable there-
fore that the results presented here indicate that in AA
medium, strain S-6 is able to synthesise its thiamine require-
ments. 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 produc-
tion 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-
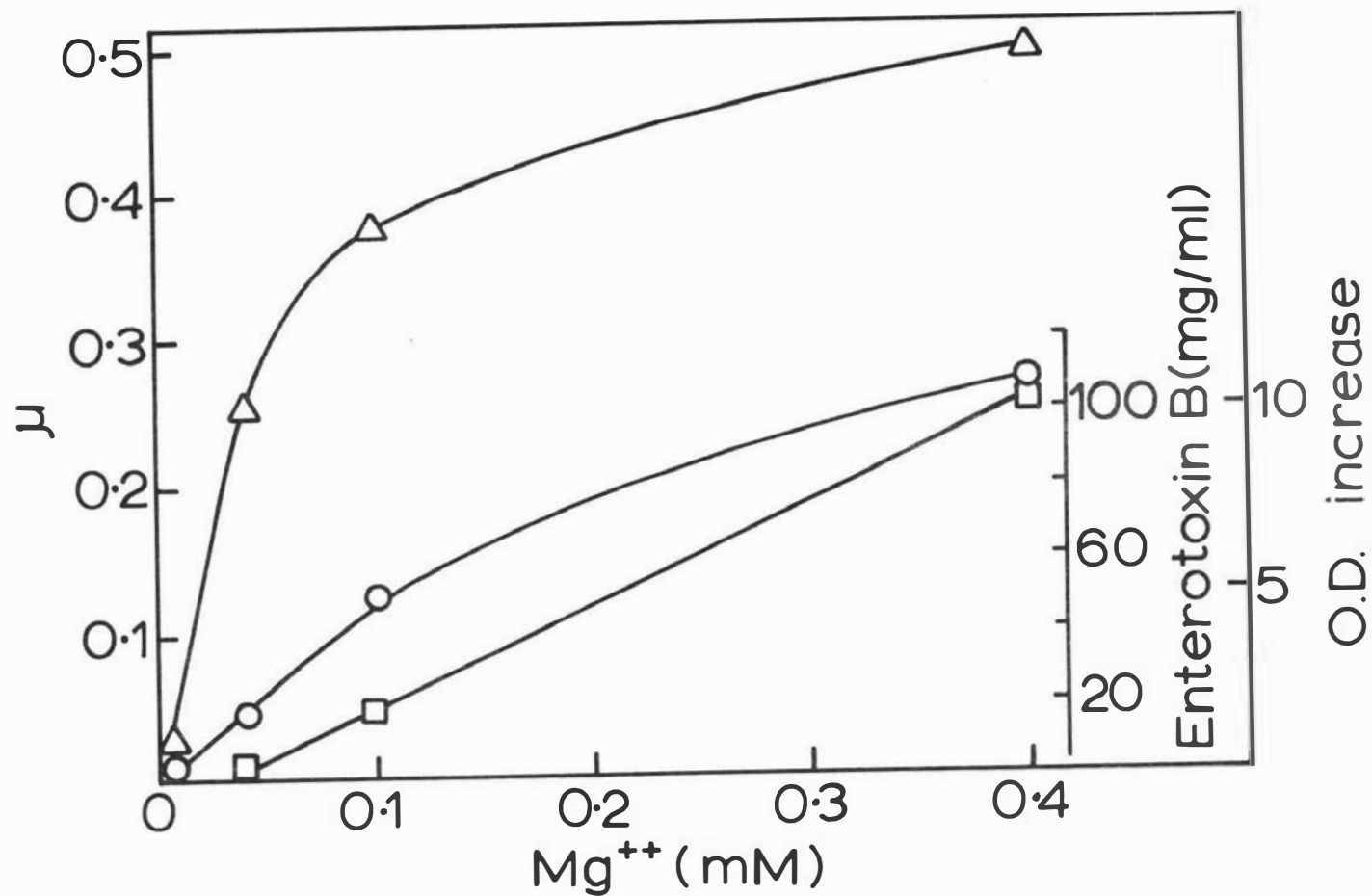
Figure 21   Specific growth rate (μ) △; increase in OD, ○; and production of enterotoxin B, □;
by strain S–6 during 12 h incubation in AA medium in shake-flasks with
different initial Mg++ concentrations.

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 38% 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
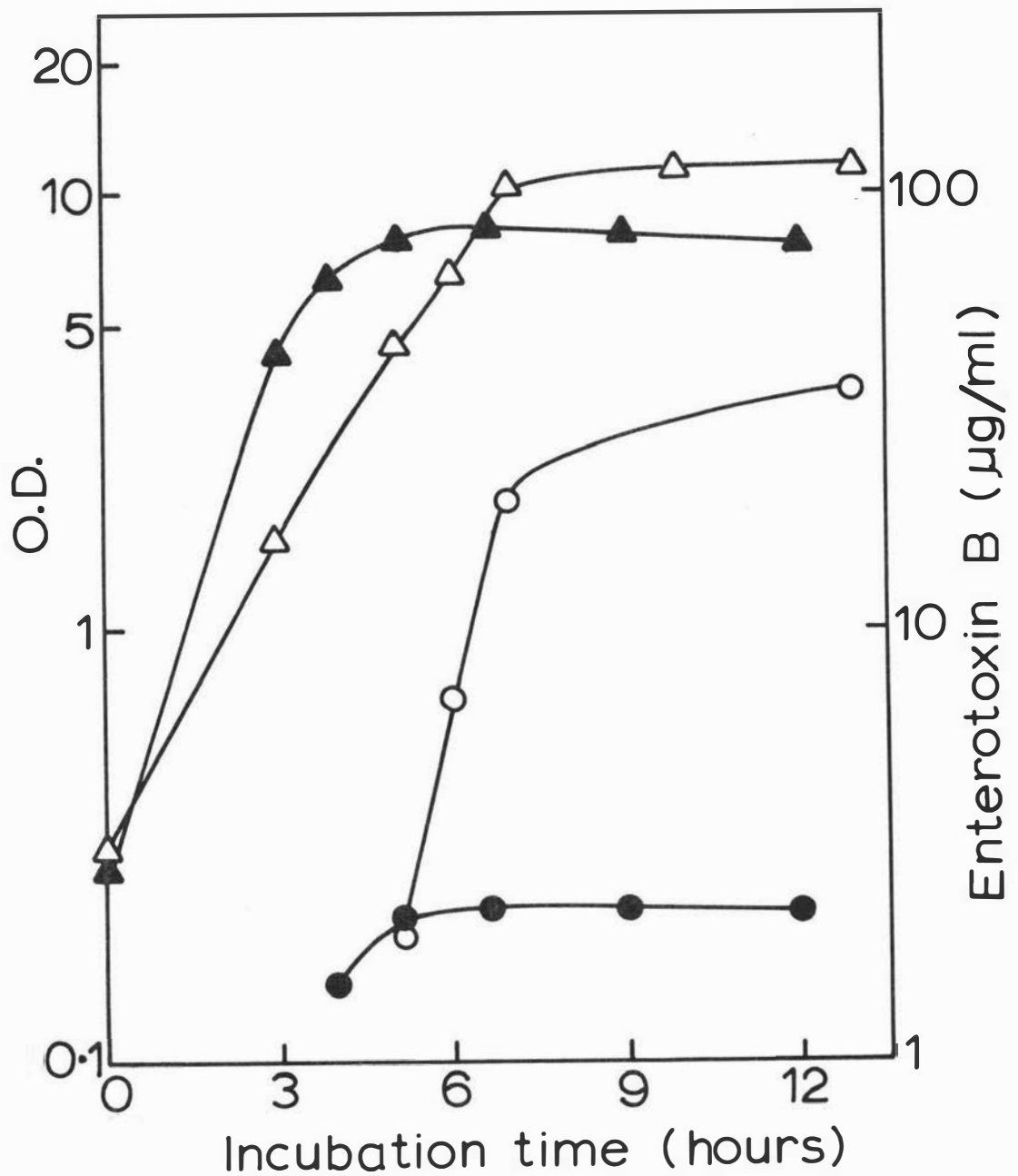
Figure 22   Growth and enterotoxin B production in AA medium containing a reduced concentration of Mg++ (0.2 mM) without glucose (open symbols) and with glucose maintained at 0.1 M (closed symbols).   OD, △; enterotoxin B, ○.

TABLE 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.

Strain and type	Glucose (molarity)	Final OD	Specific growth rate (μ)	Enterotoxin ($\mu g/ml$)	(q_p)	Lipase (units/ml)	Deoxyribo-nuclease (units/ml)	TEP (mg/ml)
100 A	0	3.5	0.34	Tr	—	3.6	0	0.08
100 A	0.1	3.3	0.33	Tr	—	2	0	0.04
S-6 B	0	11.5	0.41	35	1.19	70	17	0.28
S-6 B	0.1	8.3	0.98	2	0.11	8	15	0.08
361 C	0	9.0	0.56	5	—	7	3	0.01
361 C	0.1	8.4	0.62	ND	—	0	2	0.01

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 -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, S-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 phos-
phate 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.

Phosphate buffer (molarity)	Glucose (molarity)	pH at 2 h	pH at 4 h
0	0	7.8	8.5
0	0.1	6.2	5.4
0.2	0	7.2	7.4
0.2	0.1	6.7	6.4
0.4	0	7.0	7.0
0.4	0.1	6.8	6.7

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 1% galactose and two concentrations of phosphate
buffer on the production of enterotoxin B, lipase, deoxyribo-
nuclease and TEP by non-replicating cells of strain S-6 during
4 h incubation was then determined (Table 19).   The addition
of 1% 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.

TABLE 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.

Additions to AA medium	Final pH	Entero-toxin B µg/ml	TEP mg/ml	Lipase units	Deoxyribo-nuclease units.
Nil	7.95	224	0.84	450	86
1% galactose	7.47	224	0.87	560	103
0.2 M buffer	7.25	114	0.63	215	30
0.3 M buffer	7.16	86	0.56	137	14

The difference in glucose effect on enterotoxin B and
β-galactosidase production by non-replicating cells of strain
S-6 is shown in Fig.23. β-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 β-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 extra-
cellular 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 β-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.
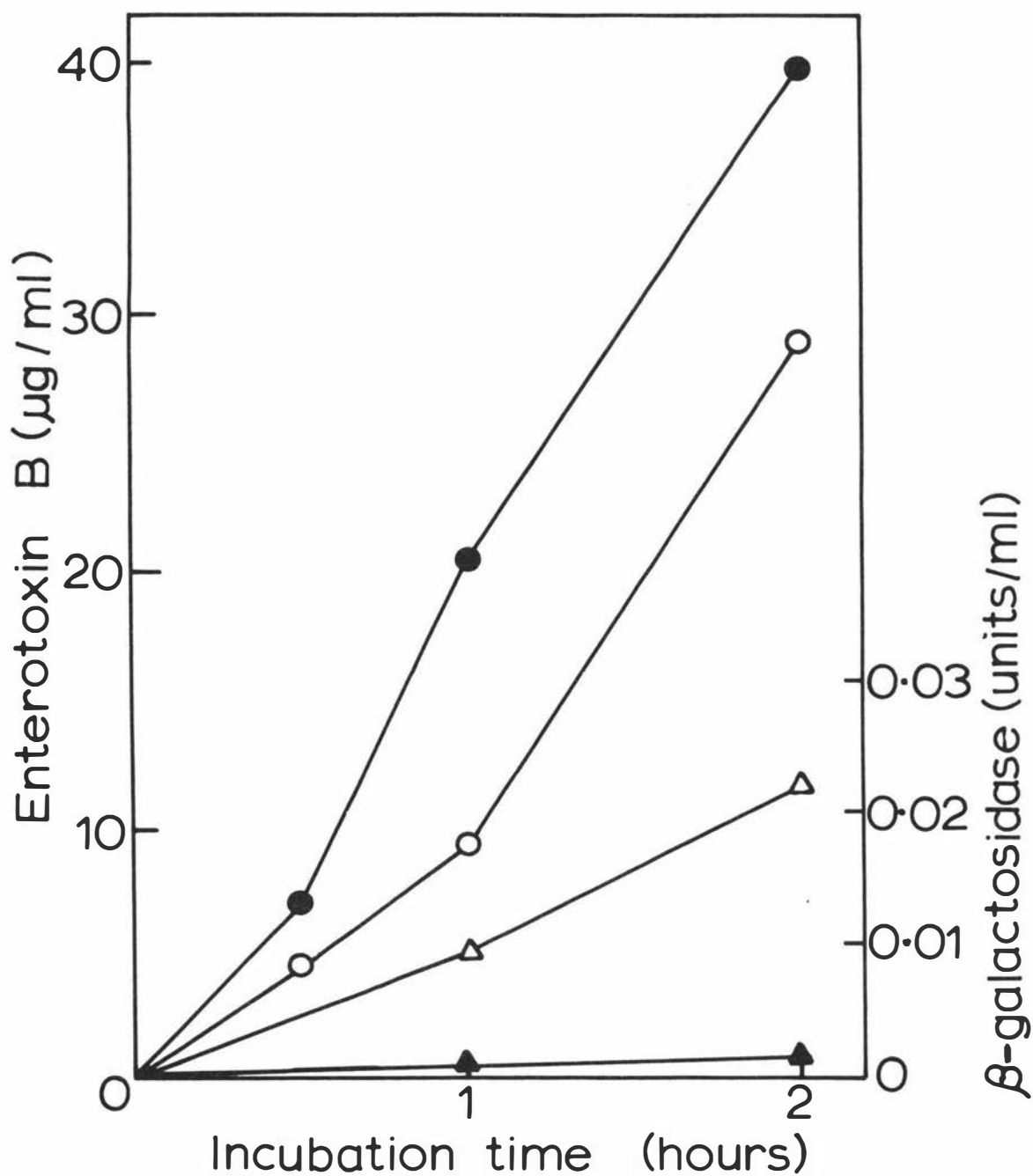
Figure 23 Effect of 0.1 M glucose on enterotoxin B and β -galactosidase production by non-replicating cells of strain S—6 without glucose (open symbols) and with 0.1 M glucose (closed symbols).
β -galactosidase, △; enterotoxin B, ○.

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[a] incubation.

	Strain and enterotoxin type	Enterotoxin (μg/ml)	β-galactosidase (units/ml)	TEP (mg/ml)	Lipase (units/ml)	Deoxyribonuclease (units/ml)	Final pH
Control	100 A	2	0.058	0.14	19	0	7.15
0.1 M glucose	100 A	2	0.003	0.12	7	0	6.30
0.1 M glycerol	100 A	1	0.005	0.10	7	0	6.85
Control	S-6 B	29	0.022	0.15	46	13	7.00
0.1 M glucose	S-6 B	40	0.001	0.20	57	10	6.65
0.1 M glycerol	S-6 B	35	0.005	0.21	46	10	6.40
Control	361 C	10	0.022	0.10	55	Trace	7.20
0.1 M glucose	361 C	12	0.005	0.11	55	Trace	6.70
0.1 M glycerol	361 C	10	0.007	0.11	55	Trace	7.15
Control	30 B	6	0.008	0.20	9	5	7.10
0.1 M glucose	30 B	11	0	0.18	12	3	6.55
0.1 M glycerol	30 B	5	0	0.18	10	4	6.90

[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 β-galactosidase.

4.3.9   Effect of chloramphenicol and actinomycin D on the production of enterotoxin B and β-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 µg/ml chloramphenicol, in agreement with the findings reported in Section 2.3.6.   The production of β-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 β-galactosidase followed (Fig.24).   A small amount (7 µg/ml) of enterotoxin was produced as compared with 59 µg/ml in the control, suggesting the presence of a small pool of mRNA.   However, β-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
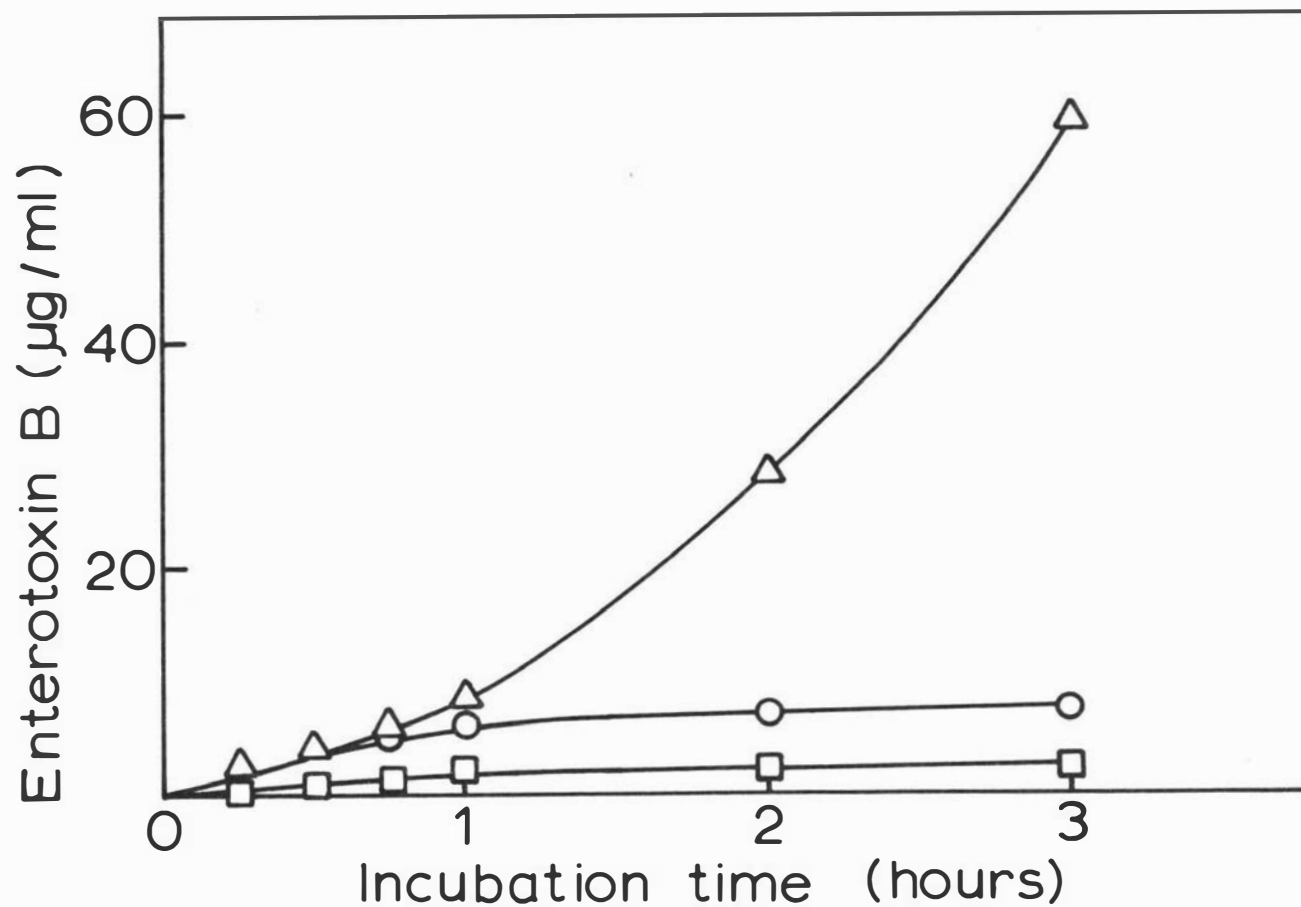
Figure 24 Effect of 100 µg/ml chloramphenicol and 10 µg/ml actinomycin B on enterotoxin B production by non-replicating cells of strain S—6.
control, △; control + 10 ug/ml actinomycin D, ○; control + 100 ug/ml chloramphenicol, □.

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 M 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) entero-
toxin 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 Pastan, 1968), to
overcome glucose repression of β-galactosidase, and to stimu-
late 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 Pastan.

E coli K12 FB240 (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 5×10^{-4} M isopropyl- -D-thiogalactoside (IPTG))(Sigma Chemical Co), to induce β-galactosidase. Flask 2 contained 5×10^{-4} M IPTG and 0.1 M glucose. Flask 3 contained 5×10^{-4} M IPTG, 0.1 M glucose and 5×10^{-3} M cAMP (Sigma Chemical Co). The flasks were incubated on the shaker at 37 C, and OD, pH and β-galactosidase determinations carried out after 10, 20, 40 and 80 min incubation. For β-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 β-galactosidase activity measured as the hydrolysis of ONPG after 20 min.

It was found (Fig.25) after 80 min incubation that the production of β-galactosidase in the presence of glucose was only 20% of that in the control flask. cAMP overcame glucose repression, and increased the production of β-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 β-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% galactose replaced IPTG as an inducer of β-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 β-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. β-galactosidase activity was measured as the hydrolysis of ONPG after 3 h incubation at 30 C. The production of β-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
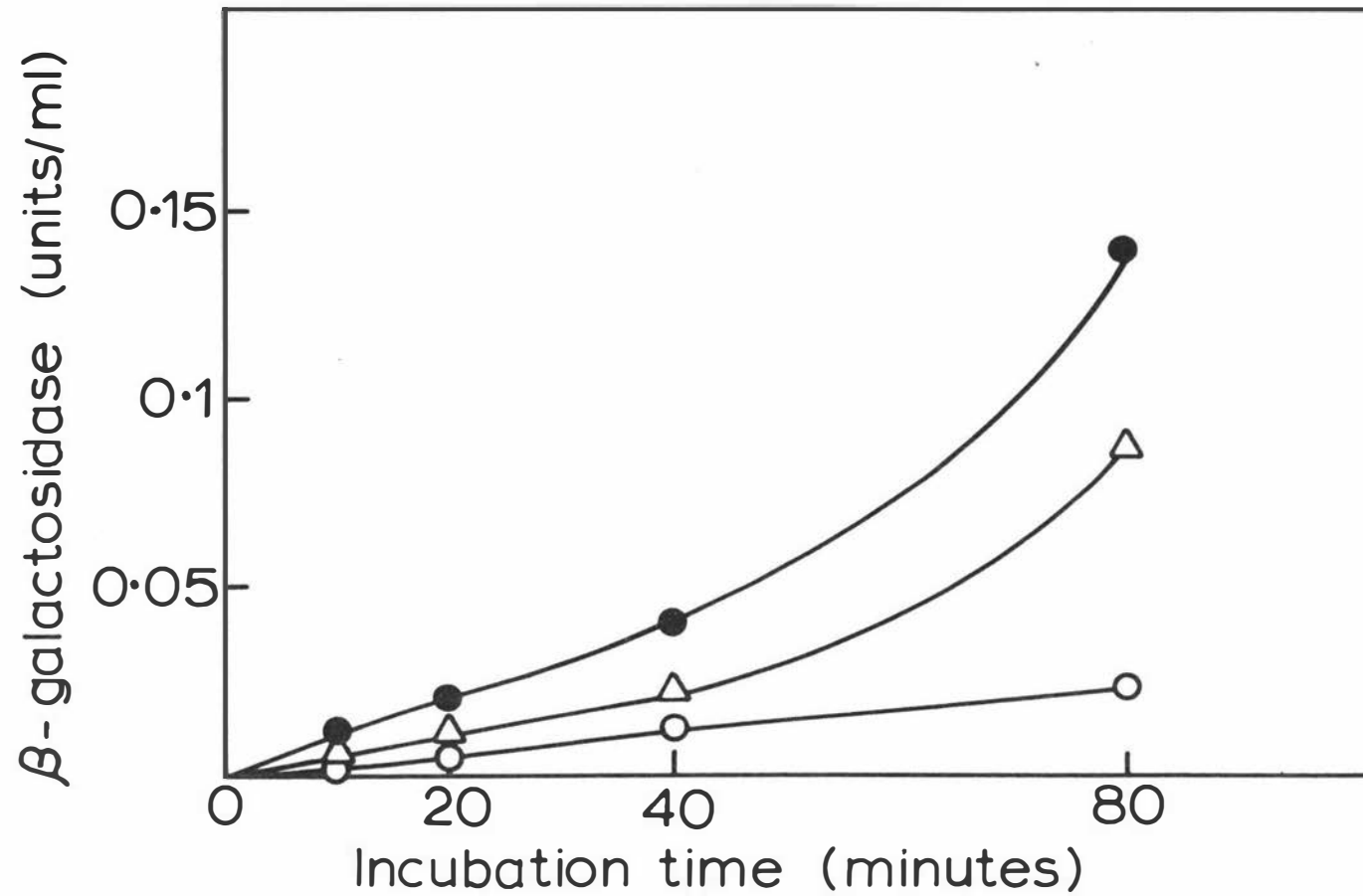
Figure 25   Effect of c—AMP on glucose repression of β-galactosidase production by <u>E. coli.</u> control $\triangle$; control + 0.1 M glucose, $\bigcirc$; control + 0.1 M glucose + 5 x 10^{3}M cAMP, ●.

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 <u>E.coli</u> and <u>S.aureus</u> was due to the difference in the medium in the two experiments. The work which had been carried out with <u>E.coli</u> was therefore repeated with 4% trypticase 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 x 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 Bergdoll (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
Metzger 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 signifi-
cant 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 deoxyribo-
nuclease 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 Mandelstam (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 (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 S-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 (Morse 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 β-galactosidase by non-replicating cells of staphylococci

Non-replicating cells of staphylococci produced enterotoxins and β-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 β-galactosidase production by resting cells of E.coli

(Magasanik, 1957), in which β-galactosidase was synthesised using endogenous sources of N, but again inhibition of protein synthesis prevented the production of β-galactosidase. The addition of 0.1 M glucose or glycerol resulted in an almost complete repression of β-galactosidase synthesis for four strains of staphylococci. However, the production of entero-toxin 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 TEP 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 β-galactosidase synthesis by resting cells of 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 repres-sion of β-galactosidase production in staphylococci than has been reported for E.coli (Neidhardt and Magasanik, 1956). These findings suggest that the mechanism for repression of enterotoxins by glucose is not the same as that for repres-sion of β-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, experi-
ments 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 resus-
pended 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 produc-
tion 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

S.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:

$$\frac{\text{flow rate (litres per hour)}}{\text{volume of culture (litres)}} \quad h^{-1}$$

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 approxi-
mately 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 mmoles of
glucose. Therefore the yield constant was approximately
50 mg dry weight of cells per mmole 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 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, i.e. 0.16 M glucose would be
required in the incoming medium in order to obtain a concentra-
tion 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.

5.2.5   Determinations of extracellular proteins

Determinations of enterotoxin B, lipase, deoxyribonuclease, lysozyme and TEP 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 MgSO$_4$. 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, $\dfrac{dp}{dt} = q_p x - Dp$

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 (Pirt, 1969).

In the steady state, $\dfrac{dp}{dt} = 0$

$$\therefore \quad q_p \, \bar{x} = D \, \bar{p}$$

$$\therefore \quad q_p = D \, \frac{\bar{p}}{\bar{x}}$$

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

$$q_p = \frac{\text{dilution rate x product concentration}}{\text{cell concentration}}$$

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 PMQII 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
$\bar{x} = Y (s_R - \bar{s})$, where s_R is the concentration of limiting
nutrient (Mg^{++}) being fed into the fermenter and $\bar{x}$ and $\bar{s}$ 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 investi-
gate 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

Dilution rate (h⁻¹)	Cells (mg/ml)	Enterotoxin B (μg/ml)	(q_p)	Mg^{++} (mM)	Yield constant (mg cells/mM Mg^{++})
0.07	3.24	123	2.66	0.04	20.2
0.14	2.30	88	5.36	0.06	16.4
0.24	1.66	80	11.50	0.07	12.7
0.24[a]	2.65	144	13.00	0.09	8.6

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

Dilution rate (h⁻¹)	TEP (mg/ml)	(q_p)	Lipase (units/ml)	(q_p)	Deoxyribonuclease (units/ml)	(q_p)	Lysozyme (units/ml)	(q_p)
0.07	1.64	0.035	161	3.45	56	1.21	0.55	0.012
0.14	0.72	0.043	100	6.10	26	1.59	0.10	0.006
0.24	0.39	0.056	123	17.82	23	3.33	0	0
0.24[a]	0.80	0.072	91	8.00	44	3.97	0.33	0.030

[a] Mg^{++} 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 concentra-
tions 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 (μ)
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 μg/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 mg/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 extra-
cellular 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 accord-
ance 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
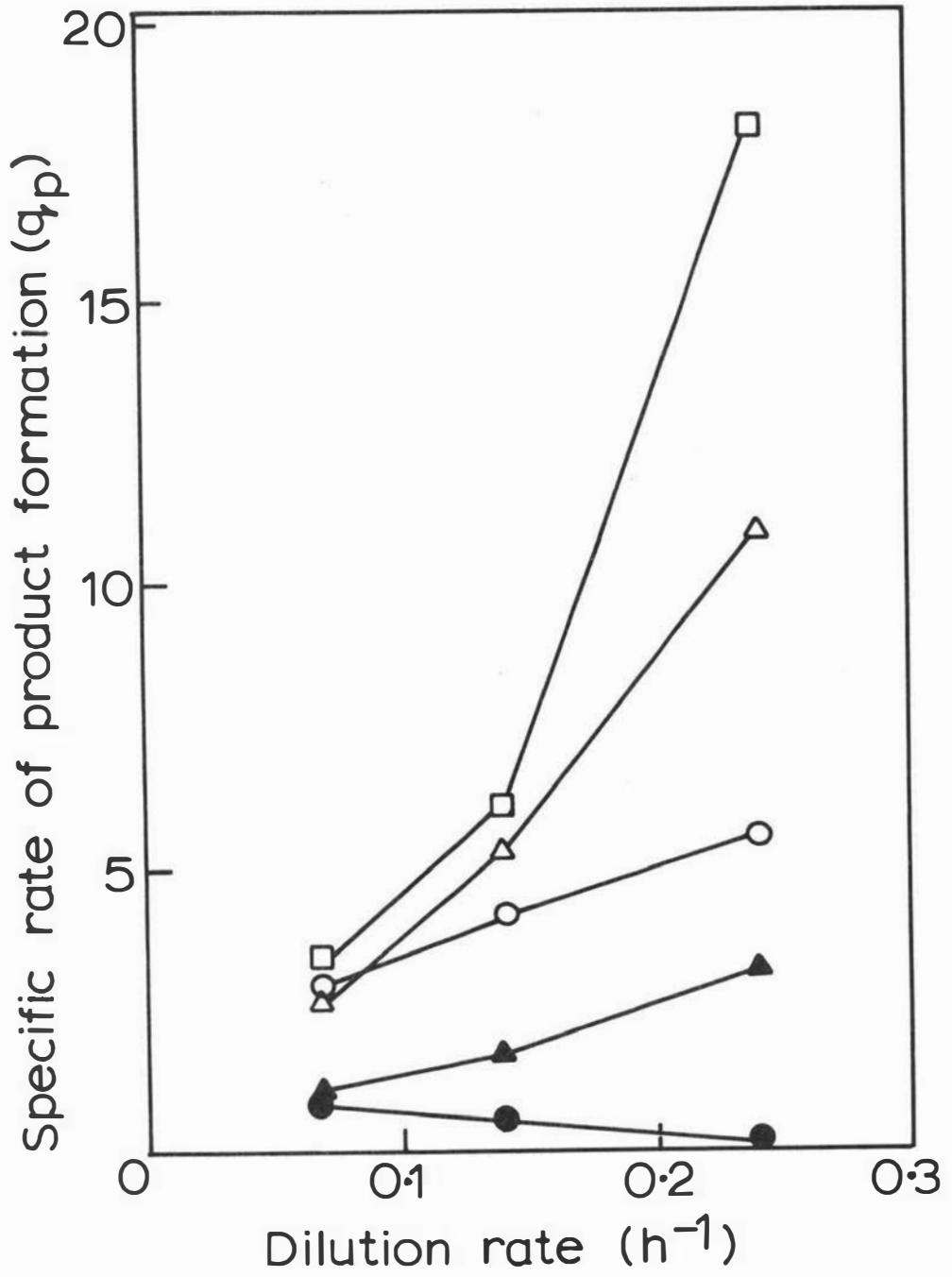
Figure 26   Effect of dilution rate on the specific rate of formation of the following proteins by strain S–6;
enterotoxin B, △; TEP (x 10), ○, lipase, □; deoxyribonuclease, (▲); lysozyme (x 100), ●.
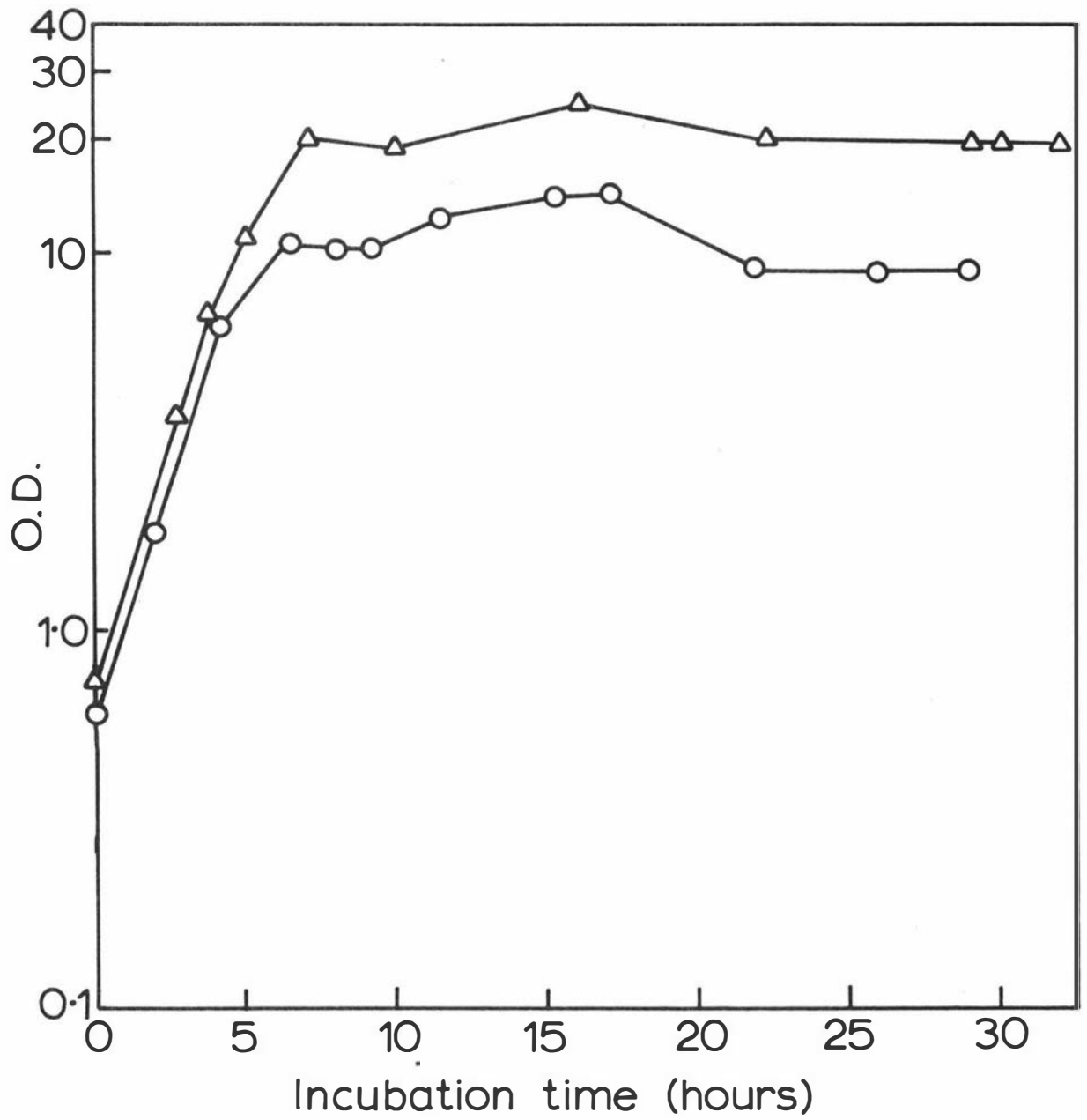
Figure 27 Effect of increasing Mg++ in incoming medium from 0.2 mM (○) to 0.4 mM (△) on OD in continuous culture of strain S–6.

specific rate of production of enterotoxin B, TEP, deoxyribo-
nuclease 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 micro-
organisms 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 OD, 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 fermenta-
tion 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).
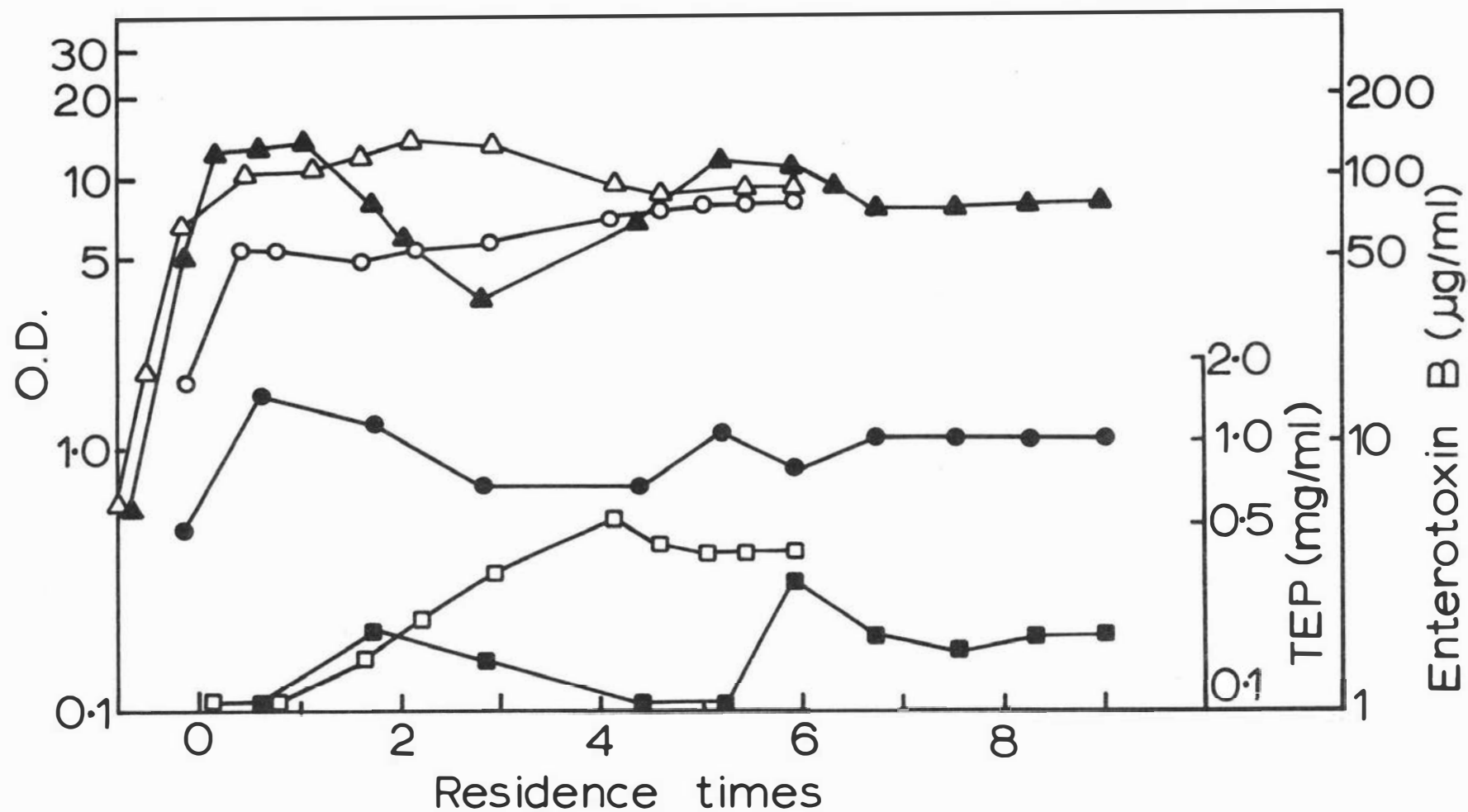
Figure 28   OD, enterotoxin B and TEP production by strain S—6 in continuous culture,
dilution rate 0.24 h−1, residence time 4.2 h, Mg++ in incoming medium 0.2 mM;
without glucose (open symbols) and with 0.1 M glucose (closed symbols).
OD, △; enterotoxin B, ○; TEP, □.
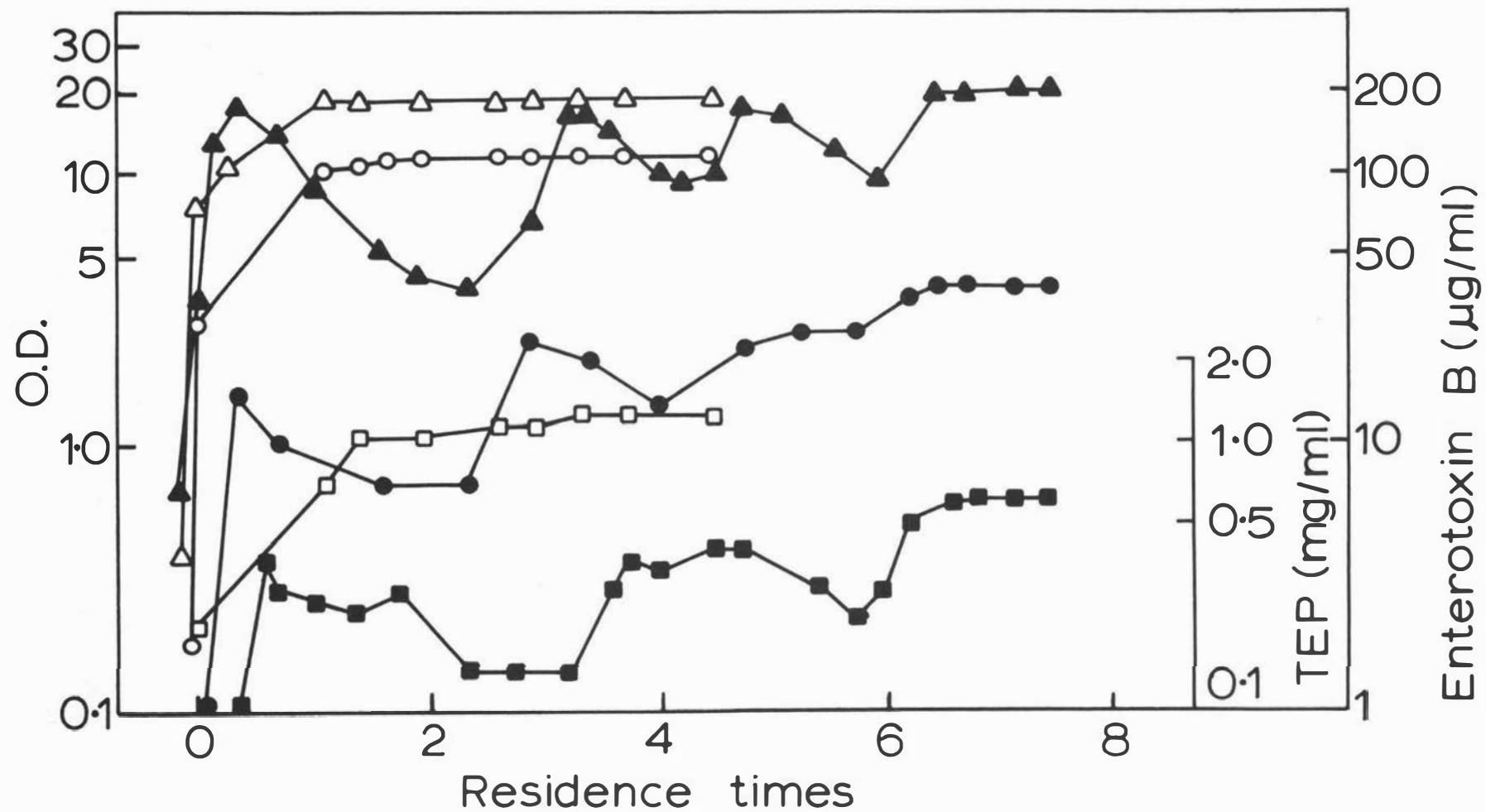
Figure 29   OD, enterotoxin B and TEP production by strain S—6 in continuous culture, dilution rate 0.07 h^{-1}, residence time 14.3 h, Mg++ in incoming medium 0.2 mM; without glucose (open symbols) with 0.1 M glucose (closed symbols), OD, △; enterotoxin B, ○; TEP, □.
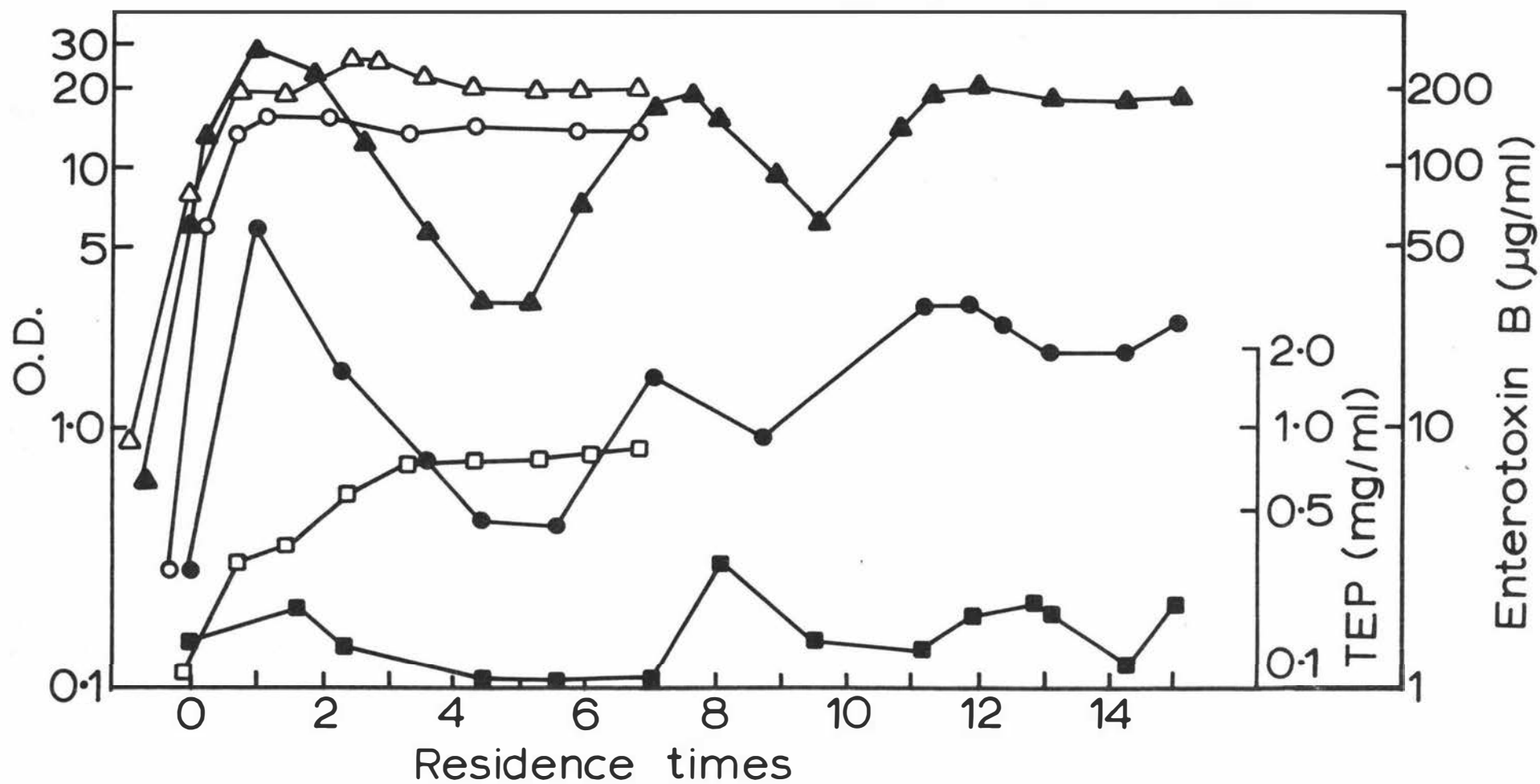
Figure 30   OD, enterotoxin B and TEP production by strain S—6 in continuous culture,
dilution rate 0.24 h^{-1}, residence time 4.2 h, Mg++ in incoming medium 0.4 mM;
OD, △; enterotoxin B, ○; TEP, □. without glucose (open symbols), with
0.1 M glucose (closed symbols).

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 S-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.

Dilution rate (h^{-1})	Glucose (M)	Cells (mg/ml)	Enterotoxin B (μg/ml)	(q_p)	TEP (mg/ml)	(q_p)	Mg^{++} (mM)	Yield constant (mg cells/mM Mg^{++})
0.07	0	3.24	123	2.66	1.64	0.035	0.04	20.2
0.07	0.09	3.49	39	0.77	0.65	0.013	0.04	22.0
0.24	0	1.66	80	11.50	0.39	0.056	0.07	12.7
0.24	0.10	1.50	11	1.75	0.18	0.029	0.09	13.5
0.24[a]	0	2.65	144	13.00	0.80	0.072	0.09	8.6
0.24[a]	0.10	3.0	18	1.44	0.22	0.017	0.10	10.0

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

Dilution rate (h^{-1})	Glucose (M)	Lipase (nits/ml)	(q_p)	Deoxyribonuclease (units/ml)	(q_p)	Lysozyme (units/ml)	(q_p)	β-hemolysin (titre)
0.07	0	161	3.46	56	1.21	0.55	0.012	1/64
0.07	0.09	76	1.52	56	1.12	0.35	0.006	1/4
0.24	0	123	17.82	23	3.33	0	0	1/32
0.24	0.10	51	8.20	26	4.16	0	0	0
0.24[a]	0	91	8.00	44	3.97	0.33	0.030	1/64
0.24[a]	0.10	53	4.24	137	11.00	0.13	0.010	1/4

[a] Mg^{++} 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 produc-
tion was decreased by 56%, and that of lysozyme production was
decreased by 50%. The β-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
β-hemolysin, as compared with a titre of 1/32 in the control
without glucose. By contrast, the production of deoxyribo-
nuclease was slightly stimulated by glucose.

At a dilution rate of 0.24 h^{-1} and with 0.4 mM Mg^{++} concentra-
tion 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 β-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 concentra-
tion 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 intra-
cellular 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 S-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 S-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 Mg^{++} in the incoming medium was 0.2 mM, and was increased more than three-fold by glucose when Mg^{++} 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 Mg^{++} was 0.4 mM, but was unaffected by glucose when the initial concentration of Mg^{++} was 0.2 mM.   It is likely that increasing the concentration of Mg^{++} to 0.4 mM in the incoming medium in continuous culture, increased the intracellular concentration of Mg^{++}.   A high intracellular concentration of Mg^{++} may therefore be necessary for the addition of glucose to result in increased synthesis of deoxyribonuclease.

5.4.2   Relation 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 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 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 AA medium over the period of maximum enterotoxin production.

The specific rate of enterotoxin production increased from ~~2.73~~ 2.66 to 11.5 μg/mg cells/h when the growth rate was increased from 0.07 to 0.24 h^{-1}. Lipase, deoxyribonuclease and TEP 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 TEP 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 amyloliquefaciens, 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 produc-
tion in strain S-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, Morse 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 S-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_p value)
for S-6 was 3.6 in the exponential phase (from 3 to $4\frac{1}{2}$ 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 TEP 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 produc-
tion is closely associated with growth. This is illustrated by
strain S-6, for which the specific rate of enterotoxin produc-
tion (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 A 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 (Morse et al., 1969, Markus and Silverman, 1969). However, the general definition of catabolite repression (Paigen 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 pre-requisite for enterotoxin production or release.

This difference in glucose effect on enterotoxin production by non-replicating cells grown in the presence of glucose, as com-pared 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

1. Arvidson, S. and Holme, T: 1971.
   Influence of pH on the formation of extracellular proteins
   by Staphylococcus aureus. Acta.Path.Microbiol.Scand.
   Section B 79: 604-413.

2. Arvidson, S., Holme, T. and Wadstrom, T: 1971.
   Influence of cultivation conditions on the production of
   extracellular proteins by Staphylococcus aureus.
   Acta.Path.Microbiol.Scand. Section B. 79: 399-405.

3. Avena, R.M. and Bergdoll, M.S: 1967.
   Purification and some physiochemical properties of enter-
   toxin C, Staphylococcus aureus strain 361. Biochemistry
   6: 1474-1480.

4. Bergdoll, M.S: 1962.
   Chemistry and detection of staphylococcal enterotoxin.
   Proc.of XIVth Research Conf.Circ.No.70.

5. Bergdoll, M.S: 1970.
   Enterotoxins, in Microbial Toxins, III. Ed.by Montie, T.C.,
   Kadis, S. and Ajli, S.J. Academic Press (London).

6. Bergdoll, M.S: 1972.
   The Enterotoxins in The Staphylococci. ed. Cohen, J.O.
   John Wiley and Sons, New York.

7. Bergdoll, M.S., Borja, C.R. and Avena, R.M: 1965.
   Identification of a new enterotoxin as enterotoxin C.
   J.Bacteriol. 90: 1481-1485.

8. Bergdoll, J.S., Borja, C.R., Robbins, R. and Weiss, K.F:
   1971.
   Identification of enterotoxin E. Infect.Immun. 4: 593-595.

9. Bergdoll, M.S., Surgalla, M.J. and Dack, G.M: 1959.
   Staphylococcal enterotoxin. Identification of a specific
   precipitating antibody with enterotoxin-neutralizing
   property. J.Immunol. 8: 334-338.

10. Bergdoll, M.S., Weiss, K.F. and Muster, M.J: 1967.
    Production of staphylococcal enterotoxin by a coagulase-
    negative micro-organism. Bacteriol.Proc. 1967, p.12.

11. Blumenthal, H.V: 1972.
    Glucose catabolism in staphylococci. p.111-135.
    In. J.A. Cohen (ed), The staphylococci. John Wiley &
    Sons Inc. NY.

12. Borja, C.R. and Bergdoll, M.S: 1967.
Purification and partial characterization of enterotoxin
C produced by Staphylococcus aureus strain 137.
Biochemistry 6: 1467-1473.

13. Both, G.W., McInnes, J.L., Hanlon, J.E., May, B.K. and
Elliot, W.H: 1972.
Evidence for an accumulation of messenger RNA specific
for extracellular protease and its relevance to the
mechanism of enzyme secretion in bacteria. J.Mol.Biol.
67: 199-217.

14. Carpenter, D.F. and Silverman, G.J: 1973.
Enterotoxin B production in a fermenter system.
ASM Proc. 1973.

15. Casman, E.P: 1958.
Serologic studies of staphylococcal enterotoxin.
Public Health Rept. US. 73: 599-609.

16. Casman, E.P: 1960.
Further serological studies of staphylococcal enterotoxin.
J.Bacteriol. 79: 849-856.

17. Casman, E.P., Bennett, R.W., Dorsey, A.E. and Issa, J.A:
1967.
Identification of a fourth staphylococcal enterotoxin,
enterotoxin D. J.Bacteriol., 94: 1875-1882.

18. Casman, E.P., Bergdoll, M.S. and Robinson, J: 1963.
Designation of staphylococcal enterotoxins.
J.Bacteriol. 85, 715-716.

19. Chesbro, W.R. and Auborn, K: 1967.
Enzymatic detection of the growth of Staphylococcus
aureus in foods. Appl.Microbiol. 15: 1150-1159.

20. Chesbro, W.R., Heydriek, F.P., Martineau, R. and
Perkins, G.N: 1965.
Purification of staphylococcal β -hemolysin and action on
staphylococcal and streptococcal cell walls.
J.Bacteriol. 89: 378-389.

21. Chou, G: 1971.
Medium for production of Clostridium perfringens toxin
in continuous culture. Appl.Microbiol. 21: 794-798.

22. Chu, F.S., Thadhani, K., Schantz, E. and Bergdoll, M.S:
1966.

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

23. Coleman, G: 1967.
Studies on the regulation of extracellular enzyme forma-
tion by Bacillus subtilis. J.Gen.Microbiol. 49, 421-431.

24. Coleman, G. and Elliot, W.H: 1962.
Studies on α-amylase formation by Bacillus subtilis.
Biochem.J. 83: 256-263.

25. Czop, J.K. and Bergdoll, M.S: 1970.
Synthesis of enterotoxin by L-forms of Staphylococcus
aureus. Infect.Immun. 1: 169-173.

26. Dale, W.M., Davies, J.V. and Russell, C: 1961.
Nitric oxide as a modifier of radiation effects on
Shigella flexneri. Intern.J.Radiation Biol. 4: 1-13.

27. De Crombrugghe, B., Perlman, R.L., Varmus, H.E. and
Pastan, I: 1969.
Regulation of inducible enzyme synthesis in Escherichia
coli by cyclic adenosine 3',5'-monophosphate. J.Biol.Chem.
244: 5828-5835.

28. Dietrich, G.G., Watson, R.J. and Silverman, G.J: 1972.
Effect of shaking speed on the secretion of enterotoxin
B by Staphylococcus aureus. Appl.Microbiol. 24: 561-566.

29. Dornbusch, k. and Hallander, H.O: 1973.
Transduction of penicillinase production and methicillin
resistance - enterotoxin B production in strains of
Staphylococcus aureus. J.Gen.Microbiol. 76: 1-11.

30. Drye, R.J. and Mah, R.A: 1969.
Stimulation of the differential rate of enterotoxin B
synthesis in Staphylococcus aureus S-6 by components in
casein hydrolysate. Bacteriol.Proc. 1969: p.10.

31. Duncan, J.L. and Cho, G.J: 1972.
Production of staphylococcal alpha toxin. Infect.Immun.
6: 689-694.

32. Eagon, R.G: 1968.
Advances in general microbiology methods.
Burgess Publishing Co. Minneapolis.

33. Epsom, J.E: 1964.
Staphylococcal food poisoning due to cheese.
Med.Offr. 112 (7) 105.

34. Evans, J.B. and Niven, C.F. Jr: 1950.
A comparative study of known food-poisoning staphylococci
and related varieties. J.Bacteriol., 59: 545-550.

35. Fildes, P., Richardson, G.M., Knight, B.C.J.G. and
Gladstone, G.P: 1936.
A nutrient mixture suitable for the growth of
Staphylococcus aureus. Brit.J.Exptl.Pathol. 17: 481-484.

36. Forsgren, A., Forsum, V. and Hallander, H.O: 1972.
Failure to detect cell-associated enterotoxin B in
S.aureus by immunofluorescence. Appl.Microbiol. 23:
559-564.

37. Freedman, D: 1969.
The shaker in bioengineering. Process Biochemistry,
4: (3) 35-40.

38. Friedman, M.E: 1966.
Inhibition of staphylococcal enterotoxin B formation in
broth cultures. J.Bacteriol. 92: 277-278.

39. Friedman, M.E: 1968.
Inhibition of staphylococcal enterotoxin B formation by
cell wall blocking agents and other compounds.
J.Bacteriol. 95: 1051-1055.

40. Friedman, M.E. and White, J.D: 1965.
Immunofluorescent demonstration of cell-associated
staphylococcal enterotoxin B. J.Bacteriol. 89: 1155.

41. Gallo, M. and Katz, E: 1973.
Regulation of secondary metabolite biosynthesis:
catabolite repression of phenoxazinone synthase and
actinomycin formation by glucose. J.Bacteriol. 109:
659-667.

42. Genigeorgis, C., Fonda, M.S., Mantis, A. and Sadler, W.W:
1971.
Effect of sodium chloride and pH on enterotoxin C
production. Appl.Microbiol. 21: 862-866.

43. Genigeorgis, C. and Sadler, W.W: 1966.
Effect of sodium chloride and pH on enterotoxin B
production. J.Bacteriol. 92: 1383-1387. (Erratum,
Vol.93, p.772.)

44. Gilbert, R.J., Wieneke, A.A., Lanser, J. and Šimkovičová, M
1972.

Serological detection of enterotoxin in foods implicated in staphylococcal food poisoning. J.Hyg., Camb. 70: 755-761.

45. Ginsberg, E.M. and Jagger, J: 1962.
Possible errors arising from the use of fritted glass filters for bubbling of cell suspensions especially in irradiation experiments. J.Bacteriol. 83: 1361-1362.

46. Gladstone, G.P: 1937.
The nutrition of Staphylococcus aureus: nitrogen require-ments. Brit.J.Exptl.Pathol. 18: 322-333.

47. Hallander, H.O: 1965.
Production of large quantities of enterotoxin B and other staphylococcal toxins on solid media. Acta Pathol. Microbiol.Scand. 63: 299-305.

48. Hallander, H.O. and Korlöf, B: 1967.
Enterotoxin-producing staphylococci. Acta.Path.Microbiol. Scand. 71: 359-375.

49. Hash, J.H: 1972.
Antibiotic mechanisms. Ann.Rev.Microbiol. 12: 35-56.

50. Hausler, Jr., W.J., Byers, E.J., Scarborough, Jr., L.C. and Hendricks, S.L: 1960.
Staphylococcal food intoxication due to Cheddar cheese. J.Milk Food Technol. 23: 1-6.

51. Hawiger, J: 1968.
Frequency of staphylococcal lysozyme produced by Staphylococcus aureus. J.Clin.Pathol. 21: 390-393.

52. Hengstenberg, W., Penberthy, W.K., Hill, K.L. and Morse, M.L: 1968.
Metabolism of lactose by Staphylococcus aureus. J.Bacteriol. 96: 2187-2188.

53. Herbert, D., Elsworth, R. and Telling, R.C: 1956.
The continuous culture of bacteria: a theoretical and experimental study. J.Gen.Microbiol. 14: 601-622.

54. Hobbs, B.C: 1955.
Public health problems associated with the manufacture of dried milk. I. Staphylococcal food poisoning. J.Appl.Bacteriol. 18: 484.

55. Hsie, A.W. and Rickenberg, H.V: 1967.
Catabolite repression in Escherichia coli. The role of glucose-6-phosphate. Biochem.Biophys.Res.Commun. 29: 303-310.

56.   Hurst, A., Collins-Thompson, E.L. and Kruse, H: 1973.
      Effect of glucose and pH on growth and enterotoxin B
      synthesis by Staphylococcus aureus strain S-6, after
      heat injury in sodium or potassium buffer.
      Canad.J.Microbiol. 19: 823-829.

57.   Idress, J.M. and Blumenthal, H.J: 1967.
      Effect of thiamine and niacin on the glucose catabolic
      pathways of staphylococci. Bacteriol.Proc. 1967: p.127.

58.   Jarvis, A.W. and Harding, S: 1972.
      Staphylococcal food-poisoning in New Zealand. NZ Med.J.
      76: 182-183.

59.   Jarvis, A.W. and Lawrence, R.C: 1969.
      A micro-method for the quantitative determination of
      deoxyribonuclease. Canad.J.Biochem. 47: 673-675.

60.   Jarvis, A.W. and Lawrence, R.C: 1970.
      Enterotoxigenic staphylococci in New Zealand. NZ.Med.J.
      72: 328-331.

61.   Jarvis, A.W. and Lawrence, R.C: 1971.
      Production of extracellular enzymes and enterotoxins A,
      B and C by Staphylococcus aureus. Infect.Immun. 4:
      110-115.

62.   Kato, E., Kahn, M., Kujovich, L. and Bergdoll, M.S: 1966.
      Production of enterotoxin A. Appl.Microbiol. 14: 966-972.

63.   Katsuno, W. and Kondo, M: 1973.
      Enterotoxin B synthesis by Staphylococcus aureus S-6
      715H in the presence of actinomycin D. Jap.J.Microbiol.
      17: 319-320.

64.   Kavanagh, F: 1963.
      Analytical Microbiology. Academic Press. New York and
      London.

65.   Kimble, C.E: 1970.
      Improved detection methods and physiological studies of
      staphylococcal enterotoxin. Ph.D thesis. Oregon State
      University.

66.   Klapper, B.F., Jameson, D.M. and Mayer, R.M: 1973.
      The purification and properties of an extracellular
      protease from Aspergillus oryzae. NRRL 2160.
      Biochem.Biophys.Acta. 304: 505-512.

67.   Kubitschek, H.E: 1970.
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
microbial lipases. Nature, London. 213: 1264-1265.

69.   Lowe, B: 1955.
Experimental cooking. pub. John Wiley and Sons Inc.
New York.

70.   Magasanik, B: 1957.
Nutrition of bacteria and fungi. Ann.Rev.Microbiol. 11:
221-252.

71.   Magasanik, B: 1961.
Catabolite repression. Cold Spring Harbor Symp.
Quart.Biol. 26: 249-256.

72.   Mah, R.A., Fung, D.Y.C. and Morse, S.A: 1967.
Nutritional requirements of Staphylococcus aureus S-6.
Appl.Microbiol. 15: 866-870.

73.   Mandelstam, J: 1961.
Induction and repression of β-galactosidase in non-
growing Escherichia coli. Biochem.J. 79: 489-496.

74.   Mandelstam, J: 1962.
The repression of constitutive β-galactosidase in
Escherichia coli by glucose and other carbon sources.
Biochem.J. 82: 489-493.

75.   Markus, A. and Silverman, G.J: 1969.
Enterotoxin B synthesis by replicating and non-replicating
cells of Staphylococcus aureus. J.Bacteriol. 97: 506-512.

76.   Markus, Z.A. and Silverman, G.J: 1970.
Factors affecting the secretion of staphylococcal
enterotoxin A. Appl.Microbiol. 20: 492-496.

77.   May, B.K. and Elliott, W.H: 1968.
Characteristics of extracellular protease formation by
Bacillus subtilis and its control by amino acid repression.
Biochim.Biophys.Acta. 157: 607-615.

78.   McClatchy, J.K. and Rosenblum, E.D: 1963.
Induction of lactose utilization in Staphylococcus aureus.
J.Bacteriol 86: 1211-1215.

79.   McLean, R.A., Lilly, H. and Alford, J.A: 1968.
Effects of meat-curing salts and temperature on the

production of staphylococcal enterotoxin B.
J.Bacteriol. 95: 1207-1211.

80. Metzger, J.F., Johnson, A.D., Collins II, W.S. and McGann, V: 1973.
Staphylococcus aureus enterotoxin B release (excretion) under controlled conditions of fermentation.
Appl.Microbiol. 25: 770-773.

81. Miller, R.D. and Fung, D.Y.C: 1973.
Amino-acid requirements for the production of entero-toxin B by Staphylococcus aureus. Appl.Microbiol. 25: 800-806.

82. Montiel, F. and Blumenthal, H.J: 1965.
Factors affecting the pathways of glucose catabolism and the tricarboxylic acid cycle in Staphylococcus aureus.
Bacteriol.Proc. 1965: p.77.

83. Morse, S.A. and Baldwin, J.N: 1971.
Regulation of staphylococcal enterotoxin B: effect of thiamine starvation. Appl.Microbiol. 22: 242-249.

84. Morse, S.A. and Baldwin, J.N: 1973.
Factors affecting the regulation of staphylococcal enterotoxin B. Infect.Immun. 7: 839-846.

85. Morse, M.L., Hill, K.L., Egan, J.B. and Hengstenberg, W: 1968.
Metabolism of lactose by Staphylococcus aureus and its genetic basis. J.Bacteriol. 95: 2270-2274.

86. Morse, S.A. and Mah, R.A: 1973.
Regulation of staphylococcal enterotoxin B: effect of anaerobic shock. Appl.Microbiol. 25: 553-557.

87. Morse, S.A., Mah, R.A. and Dobrogosz, W.J: 1969.
Regulation of staphylococcal enterotoxin B.
J.Bacteriol: 98: 4-9.

88. Neidhardt, F.C: 1960.
Mutant of Aerobacter aerogenes lacking glucose repression.
J.Bacteriol. 80: 536-543.

89. Neidhardt, F.C. and Magasanik, B: 1956.
The effect of glucose on the induced biosynthesis of bacterial enzymes in the presence and absence of inducer. Biochim.Biophys.Acta. 21: 324-334.

90.   Okinaka, R.T. and Dobrogosz, W.J: 1966.
      Enhahced catabolite repression in Escherichia coli
      by growth on combined substrates. J.Bacteriol. 92:
      526-527.

91.   Olson, Jr. J.C., Casman, E.P., Baer, E.F. and
      Stone, J.E.: 1970.
      Enterotoxigenicity of Staphylococcus aureus strains
      isolated from acute cases of bovine mastitis.
      Appl.Microbiol. 20: 605-607.

92.   Omori, G. and Kato, Y: 1959.
      A staphylococcal food-poisoning caused by a coagulase-
      negative strain. Biken's Journal. 2:   92.

93.   Paigen, K. and Williams, B: 1970.
      Catabolite repression and other control mechanisms in
      carbohydrate utilization. Adv.Microbiol.Physiol. 4:
      251-324.

94.   Palmer, I.S. and Malette, M.F: 1961.
      The effect of exogenous energy sources on the synthesis
      of β-galactosidase in resting-cell suspensions of
      Escherichia coli. J.Gen.Physiol. 45:   229-241.

95.   Perlman, R.L. and Pastan, I: 1968.
      Regulation of β-galactosidase synthesis in Escherichia
      coli by cyclic adenosine 3,'5'-monophosphate.
      J.Biol.Chem. 243:   5420-5427.

96.   Pirt, S.J: 1969.
      Microbial growth and product formation. p.199-221.
      In P.M.Meadow and S.J.Pirt (ed.), Microbial growth.
      19th Symp.Soc.Gen.Microbiol. University Press.
      Cambridge.

97.   Reiser, R.F. and Weiss, K.F: 1969.
      Production of staphylococcal enterotoxins A, B and C
      in various media. Appl.Microbiol. 18:   1041-1043.

98.   Richmond, M.H: 1959.
      Formation of a lytic enzyme by a strain of B.subtilis.
      Biochem.Biophys.Acta. 33: 78-92.

99.   Rotham-Denes, L.B., Hesse, J.E. and Epstein, H: 1973.
      Role of cyclic-adenosine 3'5'-monophosphate in the
      in vivo expression of the galactose operon of
      Escherichia coli. J.Bacteriol. 114:   1040-1044.

100. Scheusner, D.L., Hood, L.L., and Harmon, L.G: 1973.
Effect of temperature and pH on growth and enterotoxin
production by Staphylococcus aureus. J.Milk Food
Technol. 36: 249-252.

101. Simkovicova, J. and Gilbert, R.J: 1971.
Serological detection of enterotoxin from food-
poisoning strains of Staphylococcus aureus.
J.Med.Microbiol. 4: 19-30.

102. Stadtman, E.R., Novelli, G.D. and Lipmann, F: 1951.
Co-enzyme A function in acetyl transfer by the
phosphotransacetylase system. J.Biol.Chem. 191: 365-376.

103. Stanier, R.Y., Doudoroff, M. and Adelberg, E.A: 3rd ed.
1971.
General Microbiology. Macmillan and Co. Ltd, London.

104. Stark, R.L. and Middaugh, P.R: 1969.
Immunofluorescent detection of enterotoxin B in food
and a culture medium. Appl.Microbiol. 18: 631-635.

105. Stormonth, D.A. and Coleman, G: 1973.
Observations on the role of inorganic orthophosphate
in the regulation of the formation of Bacillus
amyloliquefaciens extracellular ribonuclease.
Biochem.Soc.Trans. 1: 392-394.

106. Strasters, K.C. and Winkler, K.C: 1963.
Carbohydrate metabolism of Staphylococcus aureus.
J.Gen.Microbiol. 33: 213-229.

107. Sugiyama, H., Bergdoll, M.S. and Dack, G.M: 1960.
In vitro studies on staphylococcal enterotoxin produc-
tion. J.Bacteriol 80:265-270.

108. Sulzer, F.T: 1964.
Nutritional requirements of enterotoxigenic strains
of Staphylococcus aureus. Annu.Progr.US Army Med.Res.
Dev.Command, contract no. DA-49-193-MD-2374.

109. Surgalla, M.J: 1947.
A study of the production of staphylococcal enterotoxin
in chemically defined mediums. J.Inf.Diseases. 81,
98-111.

110. Tanaka, S. and Iuchi, S: 1971.
Induction and repression of an extracellular proteinase
of Vibrio parahaemolyticus. Biken J. 14: 81-96.

111. Tatini, S.R., Cords, B.R., McKay, L.L. and Bennett, R.W: 1971a.
      Effects of growth temperature higher than optimal on
      production of staphylococcal enterotoxins and deoxyribo-
      nucleases. Bacteriol.Proc. 1971. p.17.

112. Tatini, S.R., Jezeski, J.J., Olson, Jr. J.C. and
      Casman, E.P: 1971b.
      Factors influencing the production of staphylococcal
      enterotoxin A in milk. J.Dairy.Sci. 54: 312-320.

113. Tempest, D.W., Hunter, J.R. and Sykes, J: 1965.
      Magnesium-limited growth of Aerobacter aerogenes in a
      chemostat. J.Gen.Microbiol. 39: 355-366.

114. Thatcher, F.S. and Simon, W: 1956.
      A comparative appraisal of the properties of
      'staphylococci' isolated from clinical sites and from
      dairy products. Canad.J.Microbiol. 2: 703-714.

115. Troller, J.A: 1971.
      Effect of water activity on enterotoxin B production
      and growth of Staphylococcus aureus. Appl.Microbiol.
      21: 435-439.

116. Troller, J.A: 1972.
      Effect of water activity on enterotoxin A production
      and growth of Staphylococcus aureus. Appl.Microbiol.
      24: 440-443.

117. Vandenbosch, L.L., Fung, D.Y.C. and Widomski, M: 1973.
      Optimum temperature for enterotoxin production by
      Staphylococcus aureus S-6 and 137 in liquid medium.
      Appl.Microbiol. 25: (3) 498-500.

118. Wadsworth, C: 1957.
      A slide microtechnique for the analysis of immune
      precipitates in gel. Intern.Arch.Allergy.Appl.
      Immunol. 10: 355.

119. Webb, R.B: 1963.
      Glycerol and water effects on x-ray sensitivity in
      Staphylococcus aureus. Radiation Res. 18: 607-619.

120. Weinberg, E.D: 1970.
      Biosynthesis of secondary metabolites: roles of trace
      metals. Adv.Microbiol.Physiol. 4: 1-44.

121.   Weirether, J.E., Lewis, E.E., Rosenwald, A.J. and
        Lincoln, R.E: 1966.
        Rapid quantitative serological assay of staphylococcal
        enterotoxin B.   Appl.Microbiol. 14:   284-291.

122.   Wise, W.S: 1951.
        The measurement of the aeration of culture media.
        J.Gen.Microbiol. 5: 167-177.

123.   World Health Organization. 1969.
        Surveillance summary of foodborne disease outbreaks:
        United States of America. 1968. Wkly.Epid.Rec., 44:
        349.

124.   Wu, C-H. and Bergdoll, M.S: 1971a.
        Stimulation of enterotoxin B production. I. Stimulation
        by fractions from a pancreatic digest of casein.
        Infect.Immun. 3:   777-783.

125.   Wu, C-H. and Bergdoll, M.S: 1971b.
        Stimulation of enterotoxin B production. II.
        Synthetic medium for staphylococcal growth and entero-
        toxin B production. Infect.Immun. 3: 784-792.

126.   Yano, T. and Koga, S: 1973.
        Dynamic behaviour of the chemostat subject to product
        inhibition. J.Gen.Appl. Microbiol. 19: 97-114.

127.   Zehren, V.L. and Zehren, V.F: 1968.
        Examination of large quantities of cheese for
        staphylococcal enterotoxin A. J.Dairy Sci. 51: 635-649.