Biosynthesis of β-Lactamase in *Staphylococcus aureus*

by

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
CTP	Cytidine triphosphate
CBAP	2(2'-Carboxyphenyl) benzoyl-6-amino penicillanic acid
IPTG	Iso propylthiogalactoside
ATP	Adenosine Triphosphate
p.s.i.	pounds per square inch
ONPG	o-nitrophenyl-β -galactoside

Abbreviations for amino acids were those recommended by the Biochemical Society.

INTRODUCTION

β-lactamase in Staphylococcus aureus became important when early clinical experience with penicillin demonstrated that not all staphylococcal infections were amenable to therapy. Gots (1940) suggested a correlation between resistance to penicillin and the occurrence of β-lactamase in S. aureus. Although some laboratory strains of S. aureus can be trained to be resistant to high concentrations of penicillin without having β-lactamase (Barber, 1953), the correlation between content of β-lactamase and resistance to penicillin has been amply confirmed and extended. It has been shown that the effectiveness of various penicillins varies inversely with their ease of hydrolysis by the enzyme. (Depue, Moat & Bondi, 1964; Knox & Smith, 1962; Swallow & Sneath, 1962; Novick, 1962). β-lactamase is unique among the inducible enzymes. As far as is known, β-lactamase synthesis is gratuitous in the sense that its synthesis does not contribute to the metabolism of compounds necessary for the production of cell material or energy. Because of this, it permits the study of enzyme synthesis in the absence of complicating factors introduced by the requirement of the enzyme for growth of the organism. The β-galactosidase of Escherichia coli is another system where this is possible, since it has been shown that the formation of this enzyme can be induced by compounds other than substrates of the enzyme, and under conditions of growth where the enzyme is not required for the metabolic breakdown of β-galactosides. This system has been considerably exploited and much information about the mechanism of synthesis of this enzyme in E. coli has accrued over the last twenty years.

The phenomenon of enzyme induction

It has been known for over 60 years that certain enzymes of micro organisms are formed only in the presence of their

specific substrate. (Duclaux, 1899; Dienert, 1900; West, 1901). This effect, later named "enzymatic adaptation" by Karstrom (1938), has been the subject of a great deal of experimentation and speculation. For a long time, "enzymatic adaptation" was not clearly distinguished from the selection of spontaneous variants in growing populations. It was suggested that enzymatic adaptation and selection represented alternative mechanisms for the acquisition of a new enzymatic property. In 1946, adaptive enzyme synthesis was shown to be controlled in bacteria by discrete, specific, stable (i.e. genetic), determinants (Monod, Andureau, 1946). A large number of inducible systems has been discovered in bacteria. In fact, enzymes which attack exogenous substrates are, as a general rule, inducible in these organisms. The phenomenon does exist in cells of higher organisms, but is more difficult to study. Often, the presence of substrate induces the formation of several enzymes whose synthesis is coordinately controlled as in the "lac" operon of E. coli which codes for three enzymes (Jacob & Monod 1961). In sequential induction (Stanier, 1947), it is now realised that the enzymes are synthesised in coordinate groups, in response firstly to the primary inducer and subsequently to the product of the first group of enzymes. (Stevenson & Mandelstam, 1965).

Most of the fundamental characteristics of the induction effect have been established in the study of the lactose system of E. coli and the β-lactamase of B. cereus.

The lactose system of E. coli

Lactose and other β-galactosides are metabolised by the hydrolytic transglycosylase β-galactosidase. This enzyme is active almost exclusively on β-galactosides unsubstituted on the galactose ring. (Monod, Cohen-Bazire & Cohn, 1951). Possession of β-galactosidase activity, however, is not sufficient to allow the most efficient utilisation of lactose by intact E. coli cells. Another component, distinct from

β-galactosidase, is required to allow penetration of the substrate into the cell (Monod, 1956; Richenberg, Cohen, Buttin & Monod, 1956; Cohen & Monod, 1957; Pardee, 1957; Kepes, 1960). Analysis of this active permeation process shows that it obeys the classical laws of enzyme kinetics allowing determination of Km and Vmax. The specificity is high since the system is active only with galactosides (α or β), or thiogalactosides. The spectrum of affinities (1/Km) is very different from that of β-galactosidase. The permeation system is inducible (Jacob & Monod, 1961) and its formation has been shown to be invariably associated with protein synthesis. There appears to be little doubt that this specific permeation system involves a specific protein, formed upon induction, which has been called galactoside permease (Jacob, & Monod, 1961). Zabin, Kepes & Monod (1959) demonstrated a third activity induced by growth in the presence of galactosides. This enzyme catalyses the transfer of an acetyl group from acetyl-coenzyme A to a thiogalactoside. The function of the enzyme remains obscure but it has been suggested that it may be involved in the permeation process (Jacob & Monod, 1961). However, mutants have been isolated which do not possess transacetylase but rely on the permease system.

A primary problem to which much experimental work has been devoted, is whether the considerable increase in specific activity of the enzyme, on growth in the presence of galactosides, is de novo synthesis of enzyme protein or activation or conversion of pre-existing proteins. Cohn & Torriani, (1952) showed that β-galactosidase as an antigen was distinct from all other proteins in uninduced cells. Later work demonstrated a protein Fz, which cross reacted with β-galactosidase antiserum. This protein was subsequently shown not to be a precursor of β-galactosidase and not to be a part of the molecule.

During synthesis, the enzyme does not derive any of

its sulphur (Monod & Cohn, 1953; Hogness, Cohn & Monod, 1955) or carbon (Rotman & Spiegelman, 1954) from pre-existing proteins.

The inducer, therefore brings about the complete de novo synthesis of enzyme molecules. This principle has now been confirmed with other enzyme systems (Pollock & Kramer, 1958), and, indeed, is now a part of the definition of the phenomenon.

Kinetics of induction

The kinetics of β-galactosidase induction have been considered by Monod, Pappenheimer & Cohen-Bazire, (1952). They demonstrated that the rate of synthesis of the enzyme was proportional to the increase in total protein i.e. a linear relationship is obtained when total enzyme activity was plotted against mass of the culture. The slope of the line:- $P=\Delta z/\Delta M$ where z is β-galactosidase and M is the total protein of the cell is the "differential rate of synthesis." Herzenberg (1959) demonstrated that the increase in enzyme was directly proportional to increase in total protein, from the moment of addition of inducer, in a strain of E. coli, lacking the permease, and in which β-galactosidase is induced under gratuitous conditions. Pardee and Prestidge (1961) extended these observations to the initial events taking place on addition of an inducer to growing cells of E. coli. They showed that enzyme formation begins about 3 minutes (at 37°C) after addition of inducer. Removal of inducer causes cessation of enzyme synthesis after the same short time. The differential rate of enzyme synthesis varies with the concentration of inducer giving a different saturation value for different inducers. (Monod, 1956). The inducer therefore acts in a manner which is (kinetically) similar to that of a dissociable activator in an enzyme system; activation and inactivation follow very rapidly upon addition or removal of the activator.

Studies on the specificity of induction have shown that only galactosides will induce. However, there is no quantitative correlation between inducing capacity and the substrate activity or affinity of the various galactosides tested. (Jacob & Monod, 1961; Cohen-Bazire, John N 1951). Considering the evidence, Jacob & Monod, (1961) conclude that there is no correlation between the molecular structure of the inducer and the structure of the binding site of the enzyme. Consideration of the inducing capacity has brought to light a remarkable correlation between the amounts of β-galactosidase and thiogalactoside transacetylase induced. The proportions of these two enzymes are always the same with different inducers and with different concentrations of the same inducer (Jacob & Monod, 1961). This suggests that the synthesis of these two enzymes is regulated by the same controlling mechanism.

Genetic regulatory mechanisms

Genetic regulatory mechanisms controlling β-galactosidase synthesis have been discovered in E. coli by considering the properties of mutants of this organism and their ability to synthesise β-galactosidase. It is possible to isolate constitutive mutants of E. coli which synthesise β-galactosidase and acetylase in the absence of inducer (Monod, 1956; Rickenberg, Cohen, Buttin & Monod, 1956; Pardee, Jacob & Monod, 1959). Mutations of the structural genes are also found which have no β-galactosidase (z^-) or no acetylase (a^-). β-Galactosidaseless strains have normal amounts of acetylase (z^-a^+) and acetylaseless strains have normal amounts of β-galactosidase (z^+a^-). Constitutive mutations always affect both β-galactosidase and acetylase. This suggests that there is a region of the genetic material which controls the inducibility of β-galactosidase and thiogalactoside transacetylase (Jacob & Monod, 1961). This region is referred to as the regulatory gene (i^+). A study of

diploids constructed from an inducible strain lacking β-galactosidase and a constitutive strain with galactosidase ($i^+z^-/F\ i^-z^+$) and the corresponding mutants for the acetylase ($i^-a^+/F\ i^+a^-$) shows that inducibility is dominant over constitutivity. Jacob and Monod (1961) conclude that the regulatory gene determines the synthesis of a repressor which is inactive or absent in the i^- mutants.

In experiments with 5 - methyltryptophan, Pardee and Prestidge (1959) showed that repressor accumulates when protein synthesis is blocked by the amino acid analogue. They conclude that the repressor is not a protein. However, consideration of the specificity requirements of such a molecule makes it difficult to hypothesise a molecule other than one containing at least some protein since no other molecules have been shown to have the specificity of proteins. Miller & Sobell (1966) have put forward a model for gene repression which involves a ribonucleoprotein containing a stretch of pyrimidines complementary to a stretch of purines on the operator. In this model ten pyrimidines could code for one thousand repressors which meets the requirement for great specificity. Recently Gilbert & Müller-Hill (1966) have purified the repressor of β-galactosidase. It is uninducible and has a molecular weight of 150,000 - 200,000. It has an unusually high affinity for isopropylthiogalactoside, an inducer of β-galactosidase, and probably has two sites for IPTG per molecule. This agrees with the theoretical prediction of Boezie and Cowie (1961) who predicted two binding sites on the basis of the kinetics of induction of the enzyme with different concentrations of inducer. Gilbert & Müller-Hill's studies show that the part of the repressor which reacts with inducer is a protein because pronase and/or heat destroy it; RNase and DNase have no effect. According to these authors, the repressor occurs in a small number of copies (about ten) per cell. These findings

do not rule out RNA as part of the molecule since the experiments measured only the ability to bind with inducer. It is quite possible that another part of the repressor is RNA which could then bind to DNA.

The point of action of the repressor has been called the operator gene and has been shown to be separate from the structural gene. (Jacob & Monod, 1961). Richmond (1966) states that it has been postulated that the operator has two distinct sections.

The experiments described on the lactose system of E. coli have led Jacob and Monod (1961) to propose the scheme outlined in figla &lb. The regulator gene (i^+) produces a repressor which, in the absence of inducer, reacts with the operator region blocking expression of the structural genes. Inducer combines with the repressor and thus stops it from reacting with the operator region. The derepressed operator then allows the expression of the structural gene and β-galactosidase is synthesised from amino acids according to the scheme outlined below.

Protein synthesis

Much work has been done in recent years on the mechanism of protein synthesis. The following account of the modern conception of the processes involved is compounded from several recent reviews (Chantrenne, 1961 ; Wiseman, 1965; Watson, 1964; Schweet and Heintz, 1966).

Protein molecules are derived from the condensation of many amino acids. Protein chains can consist of well over one hundred amino acid residues and enzymes can consist of more than one chain. The specificity of action of enzyme proteins resides in the three dimensional structure of the protein and in the sequence of amino acids in the polypeptide chain.

Fig. 1. Summary of the control of enzyme synthesis according to the theory of Jacob and Monod (1961). (a) In uninduced cells the regulatory gene (i) produces a repressor which reacts with the operator gene (o) and blocks its action on the structural genes (z and y). (b) In induced cells the inducer (I) reacts with the repressor and stops it blocking the operator gene. The operator gene is then free to activate the structural genes and enzymes are synthesised.

The mechanism of induction of β-galactosidase

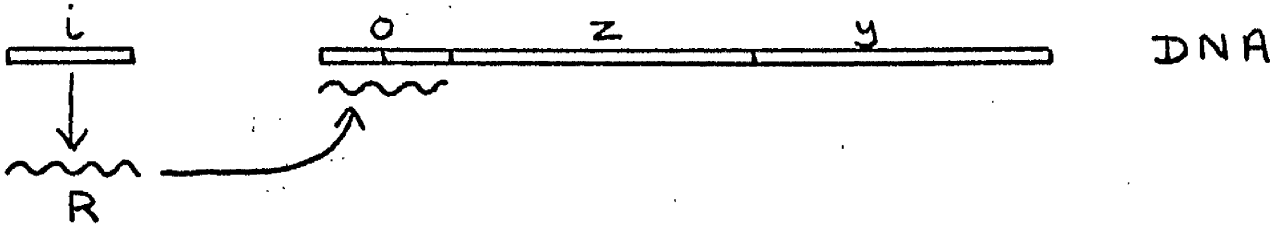


Fig. 1a Cells without inducer

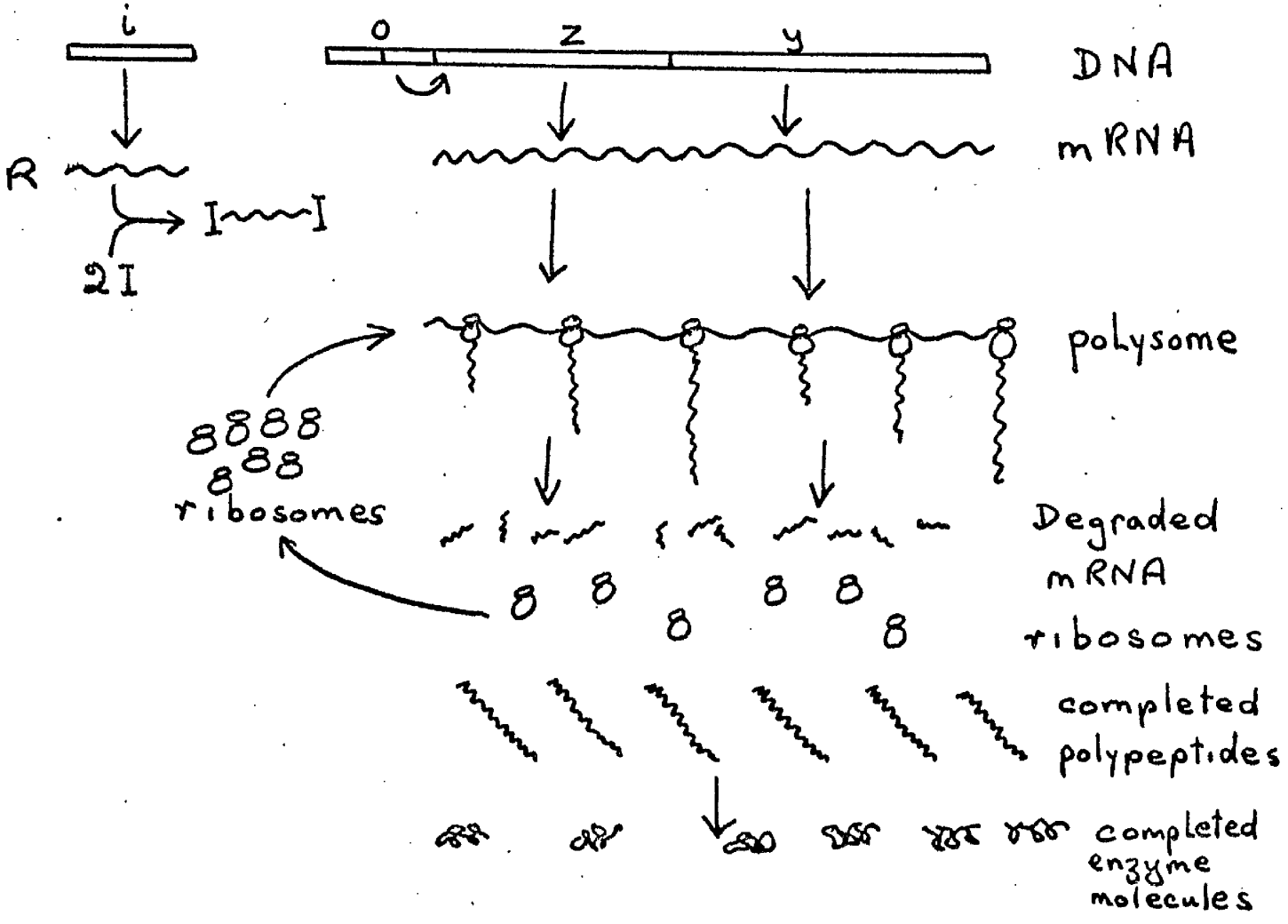


Fig 1 b Cells in presence of inducer

The first step in the synthesis of a protein from amino acids is the activation of the amino acids. This was first demonstrated in animal tissues by Hoagland (1955) and in microbial cells by De Moss and Novelli (1955), who found that labelled inorganic pyrophosphate could transfer labelled phosphorous to ATP by a reaction which required amino acids. It was soon confirmed that an amino acid adenylate was formed and that the reaction was catalysed by enzymes specific for each amino acid. (Davie, Konigsberger & Lipmann, 1956). The specificity of these activating enzymes is not absolute since the leucine activating enzyme from E. coli can also activate valine. (Bergmann, Berg & Diekmann, 1961).

By labelling the amino acid, it was found that the label is transferred through the amino acid adenylate to a RNA fraction which remains soluble on centrifugation at 100,000 g. This RNA fraction is now called transfer RNA and has been purified and the primary structure of one species determined (Holley et. al., 1965). There is a highly specific transfer RNA for each amino acid (Loftfield, Hecht & Eigner, 1963). Transfer RNA reacts with the corresponding amino acid adenylate held on its activating enzyme to form a transfer RNA - amino acid.

The transfer RNA - amino acid is then transferred to the ribosomes under the influence of transfer enzymes which are specific for the transfer RNA - amino acid. The protein is synthesised on polysomes (Warner, Knopf & Rich, 1963) which are groups of ribosomes held together by a strand of messenger RNA containing the information for the sequence of the amino acids in the protein.

This information is stored in the cell as the sequence of bases on the DNA i.e. the genes. Each gene carries the information for the synthesis of one polypeptide chain.

DNA is a double-chain polymer, each chain comprised of

alternating deoxyribose-sugar and phosphate molecules. To each sugar is attached a nitrogenous base of which there are two types; the two purines, adenine and guanine, and the two pyrimidines, cytosine and thymine. The units of base-sugar-phosphate, i.e. nucleotides, are joined together through the sugar phosphate backbone to produce a single polynucleotide chain. The DNA contains two such chains, joined by hydrogen bonding between the purine and pyrimidine bases which face inward, the sugar-phosphate chains forming an outside backbone, and which are wound helically around each other. The bases are specifically paired; adenine bonds with thymine, and guanine bonds with cytosine. (Watson & Crick, 1953).

The property of specific hydrogen bonding between bases makes it possible for a specific RNA to be synthesised on one strand of the DNA as template. Thus the coding information is transferred to a specific RNA molecule, called messenger RNA. This messenger RNA now combines with ribosomes to form polysomes on which the protein is synthesised.

The processes taking place on the polysome are postulated to be as follows (Watson, 1963). Transfer RNA has a sequence of three bases which are complementary to the code for the amino acid it carries. These three bases align themselves along the mRNA so that they link by hydrogen bonds to the three bases on the mRNA which code for the amino acid. The transfer RNA which carries the amino acid coded for by the next three bases now aligns itself next to the first molecule of transfer RNA. A reaction now takes place between the two amino acids so that a peptide bond is formed between the carboxyl group of the first amino acid and the amino group of the second. In this process the first transfer RNA is ejected leaving the second transfer RNA attached to a dipeptide. A third molecule of transfer RNA with its amino acid attached now aligns itself

along the next three bases in the mRNA and the process is repeated leaving the third transfer RNA attached to a tripeptide. Thus the protein grows one amino acid a time from the N - terminal end until it is complete. Nothing is known about the formation of the tertiary structure of the protein. Chantrenne (1961) has postulated that it forms spontaneously as the polypeptide grows.

This scheme of protein synthesis implies that the sequence of bases on the gene should be colinear with the sequence of amino acids in the protein.

It has been demonstrated by Yanofsky, Henning, Helinski & Carlton (1963) that two mutations, in the tryptophan synthetase A gene in E. coli, which map within one or two bases of each other effect a substitution of the same amino acid. Recently, a number of mutations on the tryptophan synthetase A gene have been identified and shown to be colinear with the amino acid changes on the tryptophan synthetase A protein (Yanofsky, Drapeau, Guest & Carlton, 1967).

The coding ratio has been shown to be 3 (or less likely 6 or 9) (Crick, Barnett, Brenner & Watts-Tobin, 1961). Comparison of the length of the gene with the number of amino acids in the protein supports a triplet code. (Yanofsky et.al., 1963).

A "dictionary" of "code words" for each amino acid has been compiled from studies with artificial mRNA molecules of known sequence. The sequence of three bases which codes for each amino acid is now known. There is some duplication in that same three bases can sometimes stimulate the incorporation of more than one amino acid (ambiguity) and sometimes the incorporation of a particular amino acid is stimulated by more than one sequence of three bases (degeneracy). Degeneracy in the code appears to be limited mainly to the third base in the codon. Crick (1966) has suggested that the first two bases

may pair with the codon on the mRNA and the third can pair with several bases. Much of the ambiguity first encountered has been eliminated by more detailed study of the local environment of coding in the ribosome and adjustment of conditions in the in vitro assay.

Variations in enzyme activity during growth of bacteria

The control processes discussed thus far, account for synthesis or lack of synthesis of an enzyme; i.e. an all or none phenomenon. The quantity of an enzyme can be controlled by the amount of inducer present below a certain concentration less inducer gives less enzyme (Boezie & Cowie, 1961). This is a very specific form of control affecting the one enzyme or group of enzymes. Another form of control is that of end product repression. The synthesis of many enzymes has been shown to be specifically repressed under the action of certain products of the enzyme reaction. Often the repressor is not the product of the enzyme in question but the product of another enzyme further down a metabolic pathway. For example, the synthesis of aspartate transcarbamylase is specifically repressed by orotic acid and CTP. Both these compounds are products of reactions which are relatively distant, in the metabolic sequence of reactions initiated by aspartate transcarbamylase. The overall effect, however, is that if sufficient quantities of an end product are present, enzyme synthesis is repressed and production of the end product ceases. Often, the end product of the reaction inhibits the activity of the enzyme as well as repressing its synthesis. It is thought that this process of end product repression is mediated through repressors of gene function.

Control of a metabolic pathway has been studied in detail by Stevenson and Mandelstam (1965) who examined the degradation of mandelate and p -hydroxymandelate in Pseudomonas fluorescens.

The first three enzymes involved are common to both pathways and are induced by either substrate. They are repressed by the immediate products of the reactions, benzoate and p-hydroxybenzoate. The three enzymes are co-ordinately induced as are the enzymes further down the pathway. A group of enzymes which are co-ordinately induced and repressed is called a regulon. In this system, Stevenson & Mandelstam have shown that the induction of the enzymes of each regulon is specific but the repression of each regulon is mediated either by the end-product of the regulon or the end-product of any of the succeeding regulons. This phenomenon they have termed multi-sensitive repression.

The principles involved in the control of this pathway show how a pathway can be controlled with the minimum expenditure of energy for synthesis of the enzymes involved. Measurement of these enzymes during a growth cycle of the organisms would, if the control were fine enough, show no variations in the level of enzyme present. However, if the synthesis of the enzymes is not very finely controlled one might expect cyclic changes in enzyme synthesis to be evident, since there would be a certain amount of overshoot in the induction and repression each time they were applied. Cyclic changes in enzyme activity have been observed (Boddy, Clarke, Dilly, 1966) thus supporting the view that in some cases, at least, the control is not very fine.

Many studies have been made of synchronous populations of bacteria to see how enzymes behave during growth. Pardee (1965) has shown that several enzymes can be synthesised in a discontinuous manner and suggests that this is due to variations in concentration of repressor during a division cycle. It has been suggested (Halvorson et. al.,1966) that the gene has a period of accessibility to transcription. This is supported

by the work of Masters, Kuempel & Pardee (1964) who showed that the synthesis of several enzymes in synchronously growing B. subtilis was periodic. However it has been shown that the synthesis of ATC ase in E. coli was periodic when the synthesis of β-galactosidase was not. In several cases the periodic synthesis of enzymes has been shown to be ordered in the division cycle, suggesting sequential transcription of the DNA. It is clear that the synthesis of enzymes during the division cycle is controlled by processes other than induction or repression since inducer is present (or repressor absent) in all these cases.

Control in systems other than the "lac" region of E. coli

The alkaline phosphatase system in E. coli K12 is a repressible enzyme which appears to be controlled by two regulatory genes (Echols, Garen, Garen, & Torriani, 1961). It is proposed that the function of the first gene is to synthesise an endogenous inducer and that the second gene synthesises a substance which modifies the inducer to form a repressor at high concentrations of orthophosphate.

The arginine biosynthetic pathway has been studied in several coliform bacteria and at least seven enzymes are recognised. Arginine represses these enzymes. A single regulator gene has been shown to control the constitutivity of at least five of these enzymes. However, genetic mapping procedures have shown that the structural genes for these enzymes are not closely linked but are scattered over several regions of the genetic map. (Buttin G., 1961)

In the biosynthesis of histidine, in Salmonella species, which requires at least seven enzymes, histidine represses all seven enzymes in a co-ordinate manner. It is suggested that the seven enzymes constitute one operon. (Ames, Hartman, Jacob 1963).

Galactose fermentation is mediated by three enzymes, a kinase, a transferase and an epimerase which act sequentially

to produce glucose from galactose. These enzymes are inducible, (Buttin, 1961) and constitutive mutants have been isolated which have mutations mapping in one small region. This suggests that these enzymes form an operon and are co-ordinately controlled by a regulator gene.

From studies with these systems it would seem that the scheme outlined by Jacob and Monod (1961) for the control of inducible β-galactosidase and associated permease and acetylase may be widely applicable at least in general terms.

β-Lactamase

This enzyme was previously referred to as penicillinase (E.C.3.5.2.6.). β-Lactamase was discovered by Abraham and Chain (1940) in several bacteria including E. coli, S. aureus and a gram negative rod contaminating their penicillium fermentation from which they prepared their penicillin. The reaction catalysed by the enzyme was defined by an international group of workers during the years of the second world war (See Chemistry of Penicillin, 1949). Penicillins are hydrolysed at the amide bond of the β-lactam ring to form the corresponding penicilloic acid; cephalosporins are hydrolysed at the same position (Sabath, Jago & Abraham, 1965). Many derivatives of penicillanic acid and cephalosporanic acid are susceptible to hydrolysis by this enzyme. Studies on the action of enzyme preparations on these derivatives lead to the conclusion that a single broad spectrum β-lactamase is synthesised rather than several enzymes (Richmond 1963).

Gots (1945) first associated the resistance of S. aureus to penicillin with the presence of β-lactamase. Suggestions that penicillin induced a mutation which caused β-lactamase synthesis were refuted by the work of Demerec (1948) who showed that a mutation independent of penicillin was responsible for the selection of resistant variants. This was confirmed using a replicate plate technique (Sneath, 1955). Nevertheless,

it had been suggested that penicillin induced the formation of the enzyme (Bellamy & Klimek, 1948). This was shown to be correct in S. aureus (Geronimus and Cohen, 1956; Geronimus & Cohen 1958) and B. cereus (Pollock, 1950).

Pollock has made an intensive study of the process of induction in B. cereus. Since the inducer, penicillin, is an antibiotic, Pollock (1950) devised a system of inducing B. cereus to form β-lactamase in the absence of free penicillin. He showed that pretreatment of the cells at 0°C with penicillin induced them to form β-lactamase during subsequent growth in medium containing no penicillin. This pretreatment resulted in linear production of enzyme during logarithmic growth. Pollock and Perret (1951) showed that S^{35} is fixed from penicillin in B. cereus during this cold pretreatment and that maximum fixation occurs at the same concentration of penicillin which gives maximum induction of β-lactamase. This is consistent with the hypothesis that B. cereus binds penicillin irreversibly and the bound penicillin is responsible for the subsequent induction of β-lactamase in penicillin free medium. Suerkson (1964) has shown that protoplasts do not bind S^{35} penicillin and cannot be induced to form β-lactamase. He concludes that the binding site for penicillin lies between the cell wall and membrane and that it is connected closely with the site of inducibility of the cells.

The process of induction of β-lactamase in growing cells has been divided into three phases by Pollock (1952). The first phase is a primary interaction between penicillin and cells. This is 50% complete in 60 seconds. The second phase is a latent phase, lasting about 15 minutes during which no enzyme is formed. The metabolic processes underlying this phase are inhibited by lack of oxygen. Lastly, there is an active phase during which β-lactamase is formed at a constant rate determined by the concentration of penicillin. In these

experiments penicillin was added to logarithmically growing cells and destroyed a short time later by adding β-lactamase. Consequently, we probably have the same situation as obtains in the cold pretreatment method where penicillin is bound irreversibly and linear production of β-lactamase ensues during logarithmic growth. Kramer (1957) agreed that anaerobiosis is inhibitory during the latency period if applied in the absence of penicillin but showed that in the presence of penicillin, β-lactamase is synthesised anaerobically. Kramer and Straub (1957) have shown that the latency period is the time required for a new specific RNA to be synthesised. This is in agreement with studies on the β-galactosidase system in E. coli where it has been shown that the lag is the time required for a new RNA to be formed (Kepes, 1963).

In an effort to find out if induced enzyme was different from constitutive enzyme or basal enzyme, Pollock (1956) studied the properties of enzyme extracted from induced and uninduced cells. No differences were detected. However, B. cereus and B. subtilis have enzymes which are antigenically different and have different Km values (Manson, Pollock & Tridgell, 1954). During studies on the enzymes from five different organisms, Manson & Pollock (1953) showed that the enzymes could be protected from inactivation at 100°C by 1% gelatin, 1% gum acacia, infusion broth tryptic digest broth or versene.

Although the broad spectrum β-lactamases undoubtedly exist, Pollock (1956) demonstrated the existence of two enzymes (α & γ) in B. cereus which react differently to anti serum of exo-β-lactamase. Both are inducible and the proportions remain constant during induction. γ-Type is sensitive to iodine and is apparently cell bound. α-Type is insensitive to iodine. Urea can cause the conformation of the enzyme to change so that γ-type is converted to α-type (Citri & Garber,

1958) and sodium hydroxide can cause a $\alpha \rightarrow \gamma$ shift (Citri, 1958). Further studies on the isolated enzyme have shown that these changes are in the conformation of the active site of the enzyme (Citri, Garber 1960; Citri, Garber & Cole, 1960). Citri and Garber (1961) have shown that the active site of the molecule can be altered by dimethoxybenzamido-penicillanic acid so that it now becomes sensitive to iodine. It seems then that the α and γ enzymes are two interchangeable forms of the same enzyme.

The extracellular nature of β-lactamase in *B. cereus* is well established (Pollock, 1956). In *B. subtilis* the enzyme is partly released into the medium and it has been shown that the liberation is controlled by enzymatic reactions involving detachment of the enzyme from the cell envelope. (Pollock, 1961). Recently, Coles and Gross (1967) working with *S. aureus* constitutive for β-lactamase have shown that the extracellular nature of the enzyme is greatly dependant on the concentration of ions in the medium. By suitable modifications of CY medium, they were able to increase the cell bound β-lactamase from 60% to 98% of the total.

To demonstrate that induction of β-lactamase in *B. cereus* resulted in *de novo* synthesis of protein, Pollock and Kramer (1958) grew cells in labelled medium (either with C^{14} or S^{35}), transferred the cells to unlabelled medium and induced the enzyme. The extracted enzyme contained no label. The converse experiment, growing in unlabelled medium and inducing in labelled medium gave enzyme with a great deal of radioactivity. They conclude that induction of β-lactamase results in *de novo* synthesis of enzyme.

In an effort to gain more information on the synthesis of β-lactamase, several workers have used inhibitors of protein and RNA synthesis. Chantrenne and Devreux (1960a) have shown that azaguanine inhibits the synthesis of RNA, catalase and

β-lactamase in B. cereus. The inhibition can be reversed by guanosine. The restoration of catalase activity is achieved more readily than the restoration of β-lactamase synthesis (Chantrenne, Devreux, 1960b). During this restoration of β-lactamase synthesis the differential rate is reduced. (Chantrenne, Leclerq-Calingaert, 1963). Richmond (1960) showed that amino acid analogues were incorporated into B. cereus β-lactamase, and that there is a differential effect on β-lactamase before growth ceases completely. It would seem then that synthesis of all proteins is not affected equally by these inhibitors. This fact is supported by the action of actinomycin D on B. cereus and B. subtilis (Pollock 1963). In B. subtilis a concentration of actinomycin D sufficient to give 30% inhibition of growth will abolish α-gluconidase activity but not affect β-lactamase. In B. cereus, the effect is dependent on the stage of induction; inducible cells continue to synthesise β-lactamase for up to 40 mins whereas constitutive cells synthesise enzyme for up to 2 hours. This suggests a long half-life of mRNA for β-lactamase. This finding has been confirmed in B. licheniformis (Yudkin 1966) where actinomycin D allowed synthesis to continue for a longer time in constitutive cells than in inducible cells. Again it is suggested that mRNA for β-lactamase has a long half-life and further that mRNA constitutive strains have a longer half-life than that from inducible strains. Yip, Shah and Day (1964) have studied the inducible β-lactamase in B. cereus. They find that cephalosporin C stimulates the specific activity obtained after pretreatment at 0°C with penicillin and this increase is repressed by 6-amino-penicillanic acid. On the basis of the effect of various inhibitors of protein and RNA synthesis, they postulate two stages of control of synthesis of the enzyme 1. at the level of protein synthesis and 2. at the level of mRNA synthesis. I have

Most of the work on β-lactamase has been done with gram

positive organisms but gram negative organisms do contain the enzyme. For example, A. aerogenes has a β-lactamase which is not inducible and it has been shown that sonication increases the activity of the enzyme (Hamilton-Miller, 1963). This is taken as an indication of a permeability barrier to penicillin in these cells. Further evidence for the existence of a permeability barrier comes from studies with A. cloacae where it has been shown that the increase of activity on sonication is different for quinacillin than for benzylpenicillin. This is best explained by a permeability barrier which can discriminate between the two penicillins (Smith, Hamilton-Miller & Knox, 1964). There is no evidence for a permeability barrier in B. cereus and B. licheniformis (Pollock, 1956). Most evidence shows that there is no permeability barrier in S. aureus but the work of Pepue et.al., (1964) suggests there might be.

To sum up, then, β-lactamase is found in many bacteria but not all bacteria. (Chain, et.al., 1949). There is no evidence for its occurrence in organisms other than bacteria. It is inducible in many organisms and has been used as a system to study enzyme induction. These studies have mainly been carried out in gram positive organisms but the enzyme does exist in gram negative organisms.

β-Lactamase in Staphylococcus aureus

β-lactamase in S. aureus was originally thought to be uninducible. Geronimus and Cohen (1956) demonstrated that S. aureus increased β-lactamase activity on shaking with penicillin in a Warburg apparatus. The increase was prevented by chloramphenicol, sodium azide, or 8-hydroxyquinoline and they concluded that the enzyme was inducible. Later it was demonstrated that cells grown in the presence of penicillin contained much more enzyme than cells grown in the absence of penicillin (Geronimus and Cohen, 1958) whether the enzyme was

assayed
~~assay~~ as whole cells or sonicated extracts. Propanol, toluene and other alcohols have been shown to stimulate penicillinase activity in whole cells but Saz, Lowery & Jackson (1961) have shown that cell free extracts are also stimulated. This phenomenon, then, is distinct from induction.

Induction of β-lactamase in S. aureus can be accomplished using benzylpenicillin or many substituted penicillanic acids. (Depue, Moat & Bondi 1964; Knox & Smith 1962; Swallow & Sneath 1962). The specificity of induction is very broad when considering the acyl side-chain. Cephalosporin C has a modified penicillanic acid nucleus and this has also been shown to be an inducer (Swallow & Sneath, 1962). Some rather unusual inducers have been discovered by Saz and Lowery (1964). They have shown that homogramicidin S and a cyclic hexa-peptide act as inducers in S. aureus.

One of the most effective antistaphylococcal penicillins, 2, 6-dimethoxy-phenylpenicillin (methicillin) is a good inducer and is hydrolysed only slowly by the enzyme (Novick 1962). This substance, then, could be useful as a gratuitous inducer.

In contrast to the B. cereus system (Pollock 1950) in which β-lactamase is induced after a short exposure of the cells to penicillin, S. aureus requires the continuous presence of inducer to maintain enzyme synthesis (Novick, 1962; Swallow & Sneath 1962) and since methicillin is not hydrolysed significantly by β-lactamase it is useful for following induction during growth. Unfortunately its antibiotic activity prevents its use at anything but very low concentrations.

Experiments designed to show that staphylococcal β-lactamase is inducible (Geronimus and Cohen, 1958) also showed that ultrasonic disruption of the cells did not increase the activity of the enzyme. It was concluded that there was no permeability barrier in S. aureus. Similar observations rule out a

permeability barrier to penicillin in *B. cereus* (Pollock, 1956). However, Depue *et. al.* (1964) demonstrated a 2.5 fold increase in β-lactamase activity on mechanical disruption of staphylococcal cells. Apart from this work, permeability barriers to penicillin have, so far, only been demonstrated in gram negative organisms (see p.19).

Saz *et. al.*(1961) found that the enzyme was particulate in nature in *S. aureus* but Chaikovskaya (1964) in common with other workers found that a large proportion of the total β-lactamase is extracellular. However, the work of Coles and Cross (1967) mentioned earlier shows that the conditions of growth can affect the binding of the enzyme. It would seem, then, that it is important to compare different strains of *S. aureus* under the same growth conditions.

The induction of β-lactamase has been studied under different conditions by several workers. Steinman (1961) showed that induction was less effective at high concentrations of cells and did not occur at all at very high concentrations of cells using benzylpenicillin as inducer. The rate of formation of β-lactamase was optimal in growing cultures derived from physiologically young cells. Chaikovskaya (1964) found that high densities of cells, above 5×10^8 cells/ml, were induced to a lesser extent than lower densities and that the degree of induction decreased as the density of cells increased, above 5×10^8 cells/ml. She also showed that the order of effectiveness as inducers of β-lactamase was oxacillin > methicillin > 6-amino penicillanic acid > benzylpenicillin. Leitner, Sweeney, Martin and Cohen (1963) studied the induction of β-lactamase in *S. aureus* under different growth conditions by exposure to benzylpenicillin for 10 minutes and subsequent removal by β-lactamase addition or filtration and washing. They showed that lowering the p H of the growth medium from 7.4 to 5.4 allowed synthesis of β-lactamase to continue for a longer time. Ferrous ions also

allowed synthesis to continue for longer periods. Variation in pH did not affect the lag in induction which was found to be 3-4 minutes. The degree of induction is approximately proportional to the length of exposure of the cells to free inducer and varies with the initial concentration of inducer. They conclude that β-lactamase synthesising capabilities deteriorate more rapidly during growth at neutral pH than at acidic pH.

Kaminski, Bondi, St. Phalle & Hoat (1959) have studied the synthesis of β-lactamase in a constitutive mutant of S. aureus. In a defined medium, constitutive β-lactamase synthesis was stimulated by certain amino acids and by maltose as carbon source.

The genetics of β-lactamase formation in S. aureus

Fairbrother, Parker & Eaton (1954) showed that penicillin sensitive variants could be isolated from cultures of resistant Staphylococcus aureus. The process is accelerated at 44°C but some strains did not yield any sensitive variants even after prolonged incubation. These observations have been extended by Novick (1963) who studied the genetics of β-lactamase formation in S. aureus. He noted that the capacity for synthesis of β-lactamase was lost at a frequency of 1:1000. The data are most easily explained on the basis of a plasmid containing the β-lactamase gene. Harmon (1964) confirmed this frequency of loss and showed that acridine orange accelerated it and suggested that the genetic determinant for β-lactamase was a cytoplasmic particle. Novick and Richmond (1965) studied the β-lactamase plasmids and showed that they contain linkage groups which undergo mutation, segregation, and recombination. There is no cell to cell transfer of plasmids and they obtained no evidence for stable incorporation of the plasmid into the chromosome. They also showed that the plasmid contains the markers for enzyme secretion and inducibility. Although the plasmid character of the genetic determinant for β-lactamase is well established,

Foster (1966) has shown that in her strain of S. aureus the gene is stably incorporated in the chromosome. Ashenhov (1966) demonstrated that strains of S. aureus fall into three groups. The first has the β-lactamase gene located on a plasmid, the second has the gene stably incorporated into the chromosome and the third behaves neither as a plasmid nor as a stably incorporated gene but exists in some more complex state.

Ritz and Baldwin (1961) demonstrated transduction of the capacity to synthesise β-lactamase among strains of S. aureus. In a study of many strains of S. aureus Richmond (1965) was able to class the enzymes from these strains into three types. Type A has a higher specific activity than type B but similar affinity for anti-A serum. Types A and C have similar specific activity but type C has a lower affinity for anti-A serum. By transduction of the capacity for inductive synthesis of type C to a strain constitutive for type A, a heterodiploid is formed.

Inducibility is dominant over constitutivity in the heterodiploid and the induced enzyme consists of equal quantities of type A and type C. This elegantly demonstrates that the mechanism of control in S. aureus is analogous to that in E. coli for the β-galactosidase system. (Jacob & Monod, 1961) i.e. the i^+ gene (or regulator gene) produces a product which inhibits the function of the structural gene.

In extension of this work Richmond (1966 a&b) has isolated strains with widely varying activities and degrees of inducibility. Among these is an unusual class of mutant which he calls microconstitutive. These strains are constitutive but have very low levels of enzyme. He has shown that the mutation in these strains is not on the regulator gene. Since it is unlikely to be on the structural gene it is most likely an operator gene mutation. However, this does not agree with current views that the operator gene may be in two sections.

(Richmond 1966b). Further genetic studies on the β-lactamase plasmid (Richmond, 1967) have revealed that plasmids associate and segregate together and can be transduced together. Consideration of the properties of these associated diploids leads Richmond (1967) to suggest that the plasmid is circular.

In summary, β-lactamase synthesis in S. aureus is controlled by mechanisms fundamentally the same as those operating in the control of β-galactosidase in E. coli. The kinetics of the process are not so well established in S. aureus and, in particular, much of the work which has been done relied on inducers which were both antibacterial agents and frequently β-lactamase substrates.

The intention of the present work was to take advantage of the availability of an inducer which possesses unique properties. It was felt that the combination of high inductive power, β-lactamase insensitivity and minimal antibacterial potency which is found in this inducer (CBAP) would permit a more definitive description of β-lactamase induction in S. aureus. Reports of interaction of nutritional factors and β-lactamase synthesis suggested that it would be worth considering the possibility of competition among inducible enzyme systems. In the event, competition could not be detected and the bulk of the work is a study of the kinetics of β-lactamase synthesis under various conditions of change and challenge.

MATERIALS AND METHODS

Organism.

The organism used in these studies was Staphylococcus aureus 023/19. This strain was isolated from a fatal case of staphylococcal pneumonia in Manchester Royal Infirmary and came to us from the research laboratories of I.C.I. at Alderley Park in Cheshire. It is resistant to several antibiotics including penicillins but excluding methicillin. Its β-lactamase is inducible i.e. enzyme concentration increases on addition of penicillin to growing cultures.

Growth media.

Two growth media have been used in these experiments (a) nutrient broth supplemented with glucose and (b) a completely defined medium. Other media used for the maintainence of cultures were nutrient agar, nutrient broth, and Robertsons cooked meat medium.

(a) Nutrient broth was made up from Oxoid dehydrated material and sterilised at 15 p.s.i.. Glucose (10% w/v) was sterilised separately at 5 p.s.i. and added to the complex part of the medium when cool. The complete medium contained:-

Peptone	5g.
Sodium chloride	5g.
'Lab-lemco' Beef Extract	1g.
Yeast Extract	2g.
Glucose	2g.

Glass distilled water to 1 litre.

(b) The defined medium was developed from those of Fildes, Richardson, Knight & Gladstone (1936) and Bondeci (1956) and contained

L-cystine, L-tryptophan (M/10,000)

L-arginine, L-histidine, L-phenylalanine (M/4,000)

L-valine, L-leucine, glycine, L-glutamic acid (M/1,500)
L-aspartic acid, L-proline (M/1,500)
Potassium dihydrogen phosphate (M/20) adjusted to pH 7.4 with normal sodium hydroxide.
Thiamine HCl, nicotinamide (400 µg/litre)
Biotin (2 µg/litre)
Glucose (12mM)
Magnesium sulphate (0.2mM)
Ferrous ammonium sulphate (0.08mM)

All the amino acids except cystine and aspartic acid were dissolved in water at 100x the final concentration and stored at -10°C. Cystine was dissolved in N/10 hydrochloric acid and aspartic acid in 500mls water containing 3.7mls 5N. sodium hydroxide and both stored at -10°C.

Thiamine and nicotinamide were dissolved in water in the same solution at 250x final concentration and stored at -10°C.

Magnesium sulphate was stored at 250x final concentration in water at -10°C.

Biotin, at 250x final concentration in water, was stored at -10°C.

Ferrous ammonium sulphate was made up freshly as required.

1 litre of medium was prepared by adding 10ml. of each amino acid solution to 100ml. of 0.5M phosphate buffer pH 7.4 and 40ml. water. This solution was sterilised at 5 p.s.i. in the autoclave.

To 698ml. of water was added 4ml. biotin solution, 4ml nicotinamide/thiamine solution, 4ml. magnesium sulphate solution, 4ml freshly prepared 2mM ferrous ammonium sulphate and 40ml. of freshly prepared 0.3M glucose. This solution was sterilised at 5 p.s.i. in the autoclave.

The complete medium was made by mixing the two solutions once they had cooled.

Nutrient Broth.

Nutrient broth used for maintainence of cultures was made up from Oxoid dehydrated material and sterilised at 15 p.s.i.. The medium contains in 1 litre:-

"Lab-lemco" Beef Extract	1g.
Yeast Extract	2g.
Sodium Chloride	5g.
Peptone	5g.

The medium was stored at 4°C.

Nutrient agar.

Nutrient agar was made up from Oxoid dehydrated material. It was soaked for 15 mins in distilled water and sterilised at 15 p.s.i.. While still molten, the medium was distributed into petri dishes and, when solidified, stored at 4°C. The medium contains in 1 litre the same components as nutrient broth with the addition of 15gm. agar.

Cooked meat medium.

This was made up from Oxoid dehydrated medium. One tablet was soaked in 10ml water for 15 mins and sterilised at 15 p.s.i. This modification of Robertson's medium has the following composition:-

Peptone	10g.
Lab-lemco beef extract	10g.
Neutral heart tissue	30g.
Sodium Chloride	5g.
Glass distilled water to 1 litre	

The complete medium had a pH of 7.6 and was stored at 4°C.

Sterilisation.

A Manlove-Alliot autoclave supplied with steam from a Speedylec electrode boiler was used. The efficacy of sterilisation at 5, 10 and 15 p.s.i. was assessed by following temperature change with thermocouples placed in various sizes of container

containing various quantities of water. In this way the length of time required for sterilisation was related to volume of medium and pressure of steam employed. Routinely, the efficacy of the process was checked by the colour change of Browne's tubes.

Storage of organism.

The culture was obtained as a freeze-dried sample. This was grown overnight at 37°C in a tablet bottle (16ml capacity) containing 8ml nutrient broth, and then streaked on nutrient agar. After 48 hrs growth at 37°C, a typical clone was picked off into nutrient broth and grown overnight. This culture was checked for homogeneity (microscopically and by streaking) and was used to inoculate a number of cooked meat medium (10/ml medium in 16ml tablet bottles). After 18 hrs growth at 37°C the cooked meat cultures were stored at 4°C.

Each month, a nutrient broth culture (8ml in 16ml tablet bottle) was grown overnight from the cooked meat cultures and this nutrient broth used as a working stock. Any inoculum required for an experiment was derived from this nutrient broth. This procedure ensured that the inoculum was never more than a few passages from the original culture, thus minimising the risk of variation of the strain by selection.

Preparation of inocula.

When a nutrient broth inoculum was required, 0.5ml of working stock culture was inoculated into 100ml of nutrient broth + glucose in a 500ml conical flask. This was grown for 7 hours on an orbital shaker (E.H. Engineering, Mark 1) at 37°C. After storage overnight at 4°C., this culture was used to inoculate the growth flasks for the experiment.

When a defined medium inoculum was required, 0.5ml of working stock was inoculated into 100ml of defined medium in a 500ml conical flask, and grown on the orbital shaker at 37°C for 20 hrs. After two further subcultures overnight at 37°C,

the third passage was used directly, without storage, as inoculum.

Measurement of growth.

Measurement of extinction at 500 mμ for nutrient broth cultures and 350 mμ for cultures in defined medium was taken as a measure of growth. Extinction was measured on a Spectronic 20 spectrophotometer, in optically matched 105mm.x 13mm tubes.

Glassware. All glassware used in growth experiments was boiled in a 1% solution of haemosol and thoroughly rinsed in tap water, followed by glass distilled water.

Assessment of defined medium.

Amino acids were omitted singly and in combination from the defined medium and these media tested for capacity to support growth. A universal container containing 10ml of medium was inoculated with four drops of culture from a pasteur pipette and incubated overnight at 37°C without shaking. A medium was taken to support growth if growth was sustained for 3 passages.

Growth experiments.

I wanted to follow β-lactamase synthesis during growth. To ensure that growth conditions were the same in each experiment, it was necessary to grow the cultures under rigidly controlled conditions. The cultures were grown in one litre flasks containing 800ml. medium in water bath at 37°C. Controlled aeration was achieved by making use of the principle developed by Schlegel, Kaltwasser and Gottschalk (1961) for growing cultures in a controlled atmosphere. The cultures were stirred by a high speed magnetic stirrer so that the vortex produced broke into many small bubbles as shown in fig. 2 (p 29.). The speed of stirring was controlled by a rheostat on the electric motor and this was set to the same mark for each experiment. Side-arms fitted to the growth flasks facilitated sampling and made it possible to follow growth and β-lactamase synthesis relatively easily.

Fig. 2. Flask being stirred by a magnetic stirrer so that the vortex breaks into many small bubbles. Cultures were grown in flasks like this.

Fig. 2

Measurement of rate of oxygen transfer.

The method used depends on the catalysis of the oxidation of sulphite by copper ions (Cooper, 1944). The procedure used was as follows. 800ml of 0.1N sodium sulphite was placed in a growth flask (1 litre). The aeration was set up and the oxidation of sulphite started by adding 2 x 10^{-3}M copper sulphate. The rate of oxidation of sulphite was measured by taking 5ml samples at several times into 10ml of 0.1N iodine in 2% (w/v) potassium iodide. The unreacted iodine was titrated with 0.1N sodium thiosulphate. In this way several condition of aeration were measured. The effect of variation in partial pressure of oxygen was assesed by use of gas mixtures. Oxygen and oxygen free nitrogen were mixed as shown schematically in fig. 3 (p 30.). The mixtures were then used to supply the atmosphere in the growth flasks at the rate of 1.5 volume changes per minute.

Protein estimation.

Samples for protein determination were collected from growth flasks and pipetted into an equal volume of ice cold 10% trichloracetic acid and stored in ice until the end of the experiment. The precipitated cells were centrifuged down and resuspended in 0.66N NaOH so that the concentration of cells was approximately 1mg dry wt./ml. and digested overnight at 30°C. This procedure was found to solubilise the protein of this organism (Fewson, unpublished observations). After centrifugation at 5000 r.p.m. for 15 mins, the protein in the supernatant fluid was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

β-Lactamase assay.

β-Lactamase was assayed in samples of whole cells in which growth and enzyme synthesis was stopped with hibitane (chlorhexidine digluconate). The rate of hydrolysis of penicillin V to penicilloic acid was taken as a measure of the β-lactamase

Fig. 3. Preparation of mixtures of oxygen and nitrogen for experiments on aeration. Each rectangle represents a flow meter (Gapmeter) and the figure inside is the flow of gas. Starting at the bottom left hand corner, it can be seen that mising a flow of 1000 ml oxygen / min with a flow of 1000 ml nitrogen / min gives a mixture of 50% oxygen in nitrogen. Some of this is tapped off through the flowmeter at the top; the remainder is channelled through the next flowmeter to mix with another flow of nitrogen to give a final mixture of 30% oxygen. This process can be repeated and the flows adjusted to give a variety of concentrations of oxygen in nitrogen. Screw clips, marked K, were used to control the flow of gas.

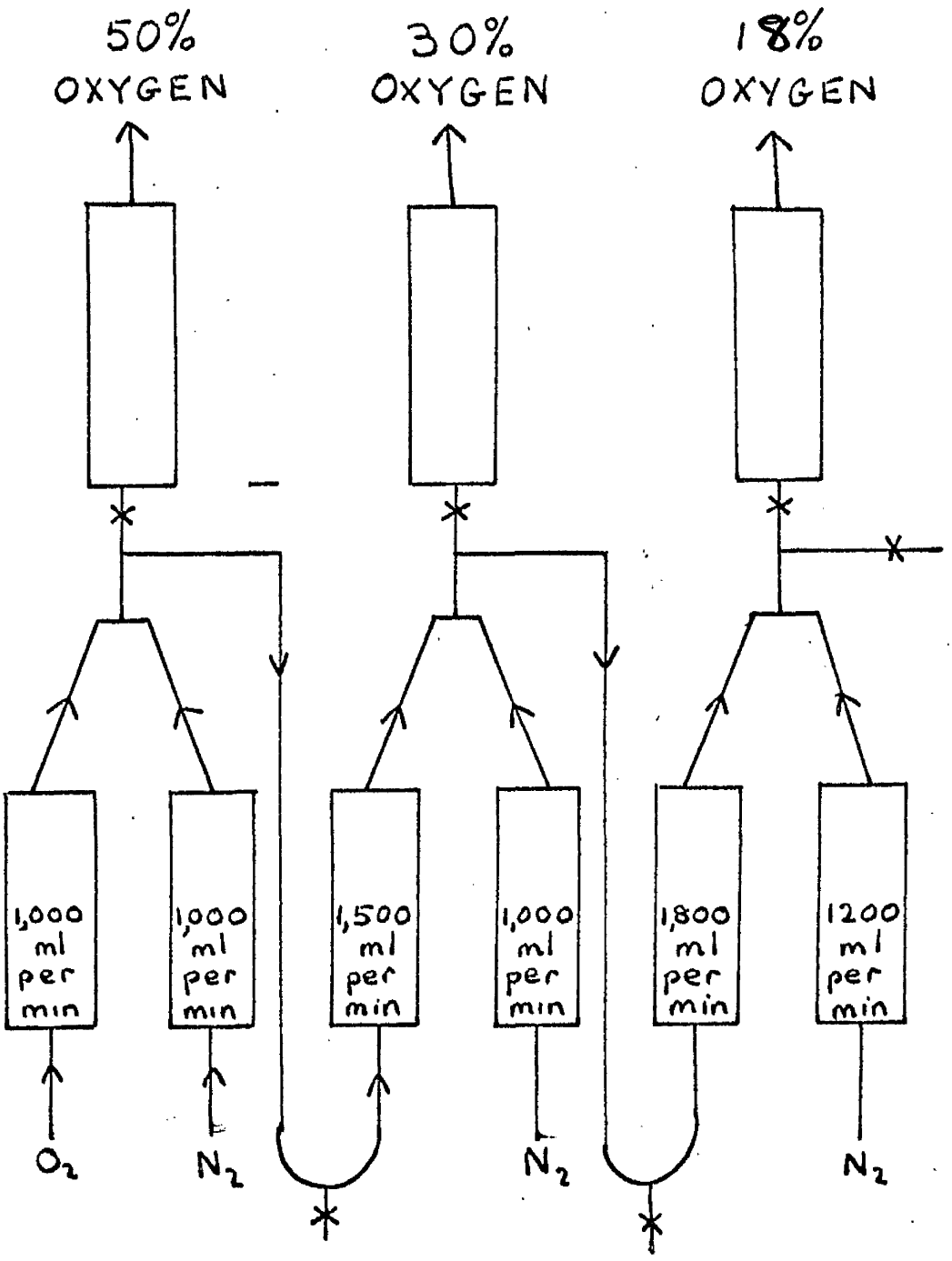

50%
OXYGEN
30%
OXYGEN
18%
OXYGEN
1,000 ml per min
1,000 ml per min
1,500 ml per min
1,000 ml per min
1,800 ml per min
1200 ml per min
O2
N2
N2
N2

Fig. 3

content of the cells. The unit of activity was defined by Pollock (1953). The unit used here is that amount of enzyme which catalyses the hydrolysis of 1 μ mole of penicillin V in 1 hour at 37°C and pH 7.0. The assay procedure was as follows:-

To 10ml of 20mM penicillin V in 0.02M phosphate buffer pH 7.0 in a 50ml conical flask
add 0.2ml chlorhexidine digluconate (1mg/ml)
and 5.0ml phosphate buffer (0.02M, pH 7.0).
Allow to come to 37°C in a water bath.
Add 5.0ml of culture containing 10 μg/ml chlorhexidine digluconate.
Incubate at 37°C with shaking and measure the rate of hydrolysis of penicillin V.

In early experiments several samples were taken from each assay flask and assayed for penicilloic acid but in later experiments one sample only was taken after 20 minutes incubation and the blank value, obtained by incubating the flask with reagents and growth medium but no cells subtracted.

Assay of penicilloic acid.

β-lactamase in this strain of Staphylococcus aureus is an intracellular enzyme. It was therefore convenient to stop the enzyme reaction by filtering out the cells. In earlier experiments sintered glass filters of porosity N° 5, were used. These were effective but very difficult to clean and trouble was encountered from the occasional filter which for some reason hydrolysed the substrate penicillin, to give false high values. This part of the assay was improved in later experiments by the use of disposable membrane filters. Those with pore size 0.20 u were found to be effective in removing the cells.

Penicilloic acid was then measured on suitable dilutions of the filtrate in 0.05M acetate buffer pH 4.6. This reduced the pH of the filtrate to 5.0 which also helped to stop the reaction

if any enzyme came through the filter. Penicilloic acid was measured by its capacity to decolorise a starch/iodine solution according to the method of Goodhall & Davies (1961) using the Technicon autoanalyser. (fig. 4 p.32a).

The Technicon Autonanalyser.

This instrument was used to estimate penicilloic acid as shown schematically in (fig. 5 p. 32b). In principle, starch/iodine reagent is pumped continuously through the apparatus and samples are introduced intermittently into the flow.

Samples are placed in small polystyrene cups which sit on the sampler plate. The sampler automatically places the sample line into a cup for a given time and then into water for a given time. Meanwhile the sample plate is turned automatically so that the next cup is brought into position for sampling. The sample line, the reagent (starch/iodine) line and the air line are continually pumped by a proportioning pump. The air breaks up the reagent line into short segments thus minimising mixing of samples. The sample mixes with the starch/iodine reagent and decolourised it to an extent dependent on the concentration of penicilloic acid. After passing through the coils, which allow time for the reaction to go to completion, the change in absorbance is measured on the colorimeter using a 550 filter, and recorded on a chart. A typical chart recording is shown in (fig. p. 32c). The manifold system was duplicated with a sample line which was approximately one third the diameter of the first one. This second system was fed through a separate colorimeter and a separate recorder, thus allowing a greater range of concentrations to be measured without further dilution.

Standard penicilloic acid solutions were run through the system for each experiment. These were made from a 10mM solution of penicilloic acid in 0.5M acetate buffer pH 4.6.

Fig. 4. The technicon autoanalyser used in this work to measure penicilloic acid.

Fig. 4

F.2

Fig. 5. Flow diagram for the technicon autoanalyser.

This scheme is described in the text.

The diameters of the tubing were.

(1) 0·040 in.; colour code:- White/white

(2) 0·065 in.; colour code:- blue/blue

(3) 0·020 in.; colour code:- orange/yellow

(4) 0·056 in.; colour code:- yellow/yellow

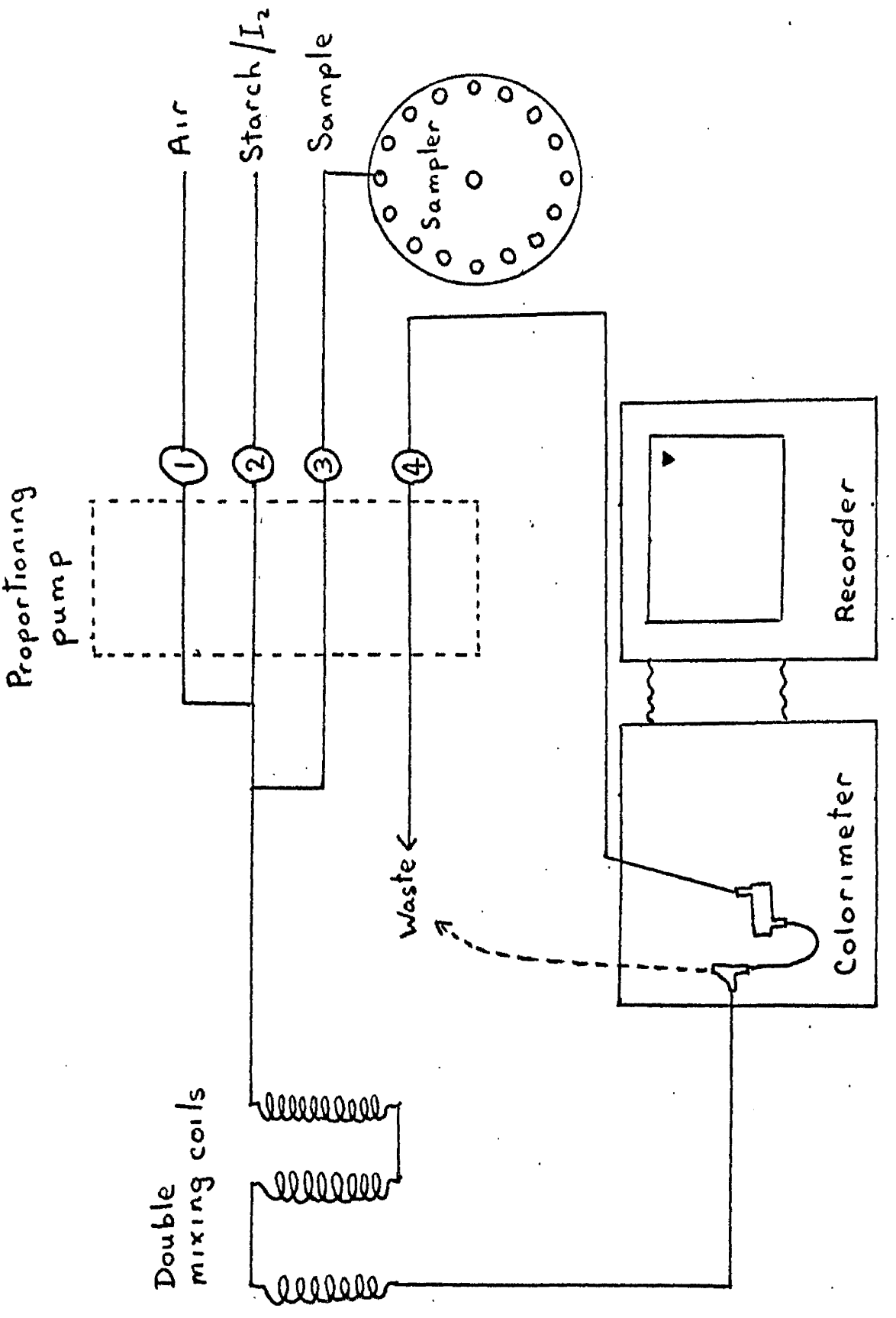


Fig. 5. Flow diagram for determination of penicilloic acid

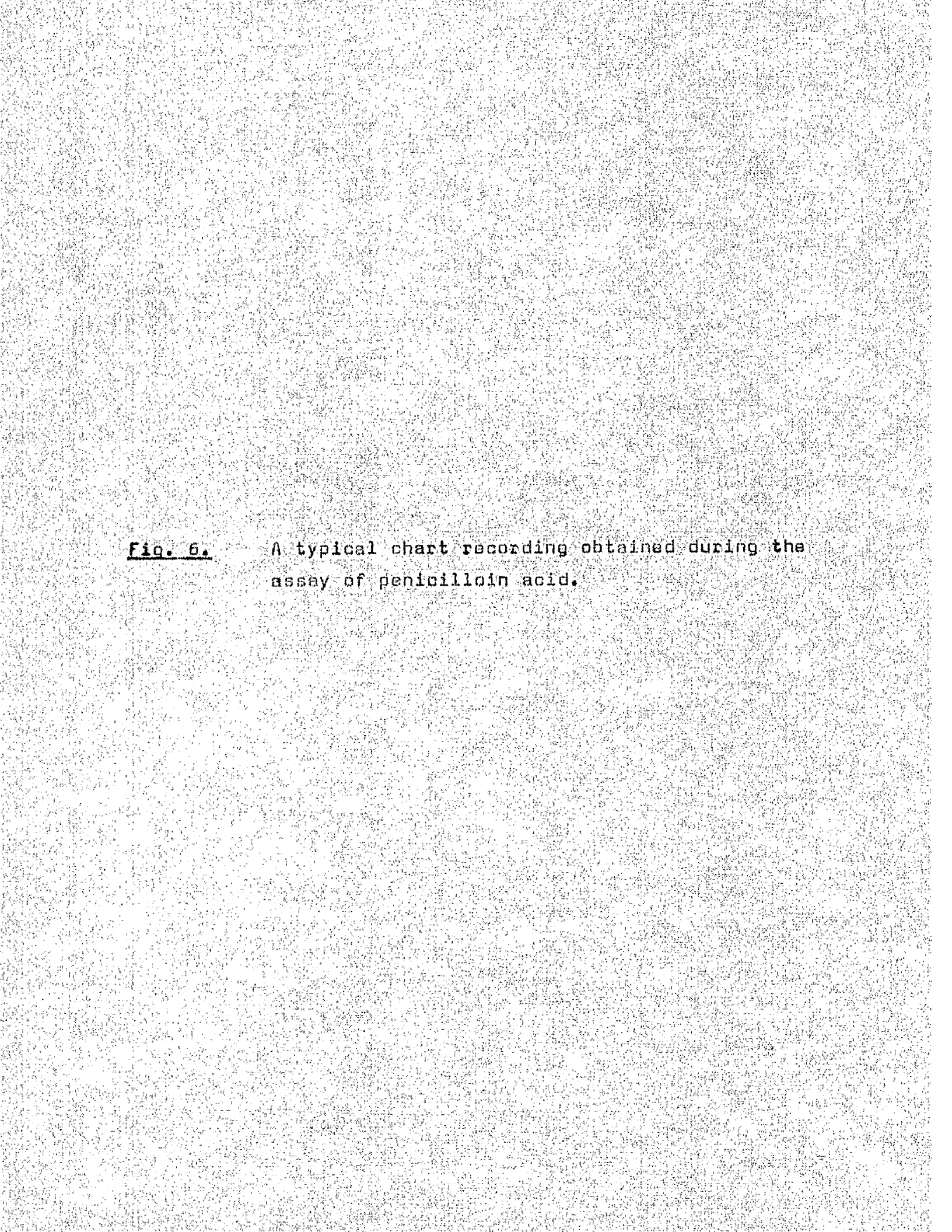


Fig. 6. A typical chart recording obtained during the assay of penicilloin acid.

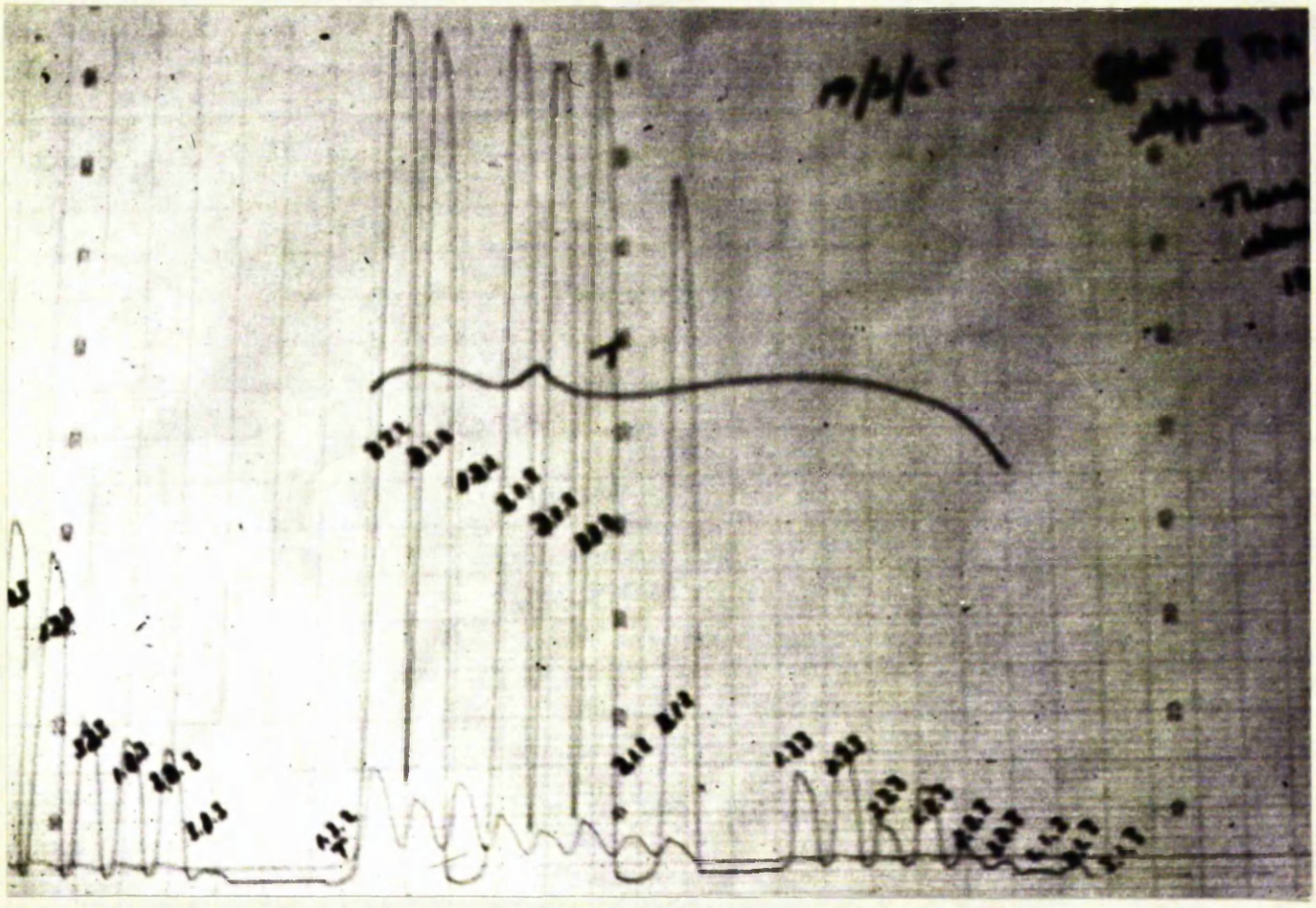


Fig. 6

Penicillin V (0.097gm.) was dissolved in 1.25ml.1N sodium hydroxide, allowed to stand for twenty minutes, 2.5ml 1N acetic acid added and the volume made up to 25ml. with glass distilled water. This results in a 10mM solution of penicilloic acid since the β-lactam ring of penicillin V is known to be hydrolysed by alkali. Concentrations of penicilloic acid from 20 μM to 80 μM were measured in the first system and concentrations from 80 μM to 280 μM were measured in the second system where a smaller sample was taken.

Starch/iodine reagent.

This reagent was made up as follows.

Solution A. 50ml N. sodium hydroxide
and 100ml N. acetic acid
was added to approximately 1 litre with distilled water.
1.5ml 0.1N iodine in 2% w/v potassium iodide was added

Solution B. 3gms hydrolysed starch was suspended in 20ml water and added to 200ml boiling water.
1.5gms potassium iodide was added.
When the solutions had cooled,
solution B was added to solution A and made up to 2 litres with distilled water.

Sonic disruption of the cells.

A Dawe soniprobe type 1130 was used at 6 amps for varying periods of time as shown in table 1. The volume of culture used was 15ml in a 50ml pear shaped flask fitted with four cooling vanes. For the experiments with β-lactamase, cells were grown in nutrient broth supplemented with glucose (66.7mM), phosphate buffer (39.7mM) and magnesium sulphate (1.6mM) and in the presence of 2.5 μM methicillin.

Detection of penicillinase in colonies.

The method of Novick and Richmond (1965) was used. This is as follows:-

The organisms were grown on an agar petri plate, to which inducer had been added, for 48 hours. The plates were dried at 37°C with the lids off. The surface was flooded with 1.5ml of 0.25% (w/v) N-phenyl-1-naphthylamine-azo-o-carboxybenzene in N, N-dimethyl formamide with 6% N. sodium hydroxide.

After drying in the hood with the lids off, the stain was developed by flooding with 1.5ml of aqueous penicillin G. (I used penicillin V). The acid released changed the indicator from yellow to purple.

β-galactosidase assay.

The method followed was that of Creaser (1955) which is as follows:-

1ml cysteine (20mg/ml)
1ml buffer (0.5M phosphate pH 7.1)
2ml culture
1ml ONPG

Incubation at 37°C was allowed to continue for 60 mins when 4ml sodium carbonate (0.4M) was added. The yellow colour was read at 420 mμ on the Spectronic 20 after centrifugation at 5000 rpm for 20 mins.

Nitrate reductase assay.

The method was as described by Chang and Lascelles (1963). Cells were centrifuged in the cold at 9000 rpm/25 mins., washed in 0.5M phosphate buffer pH 7.2 and resuspended in phosphate buffer.

To 1ml cell suspension in a Thunberg tube,

1ml 0.15M lactate
0.3ml 0.5M phosphate buffer pH 7.2.
and 0.4ml water were added.
0.3ml 1M potassium nitrate were placed in the side arm.

The tube was evacuated and filled with nitrogen. The evacuation was repeated three times. The contents of the side-

arm and main tube were mixed and incubated for 15 mins. The reaction was stopped with Fewson & Nicholas reagent (1961)

i.e. 0.1ml 1M Zinc Acetate
1.9ml 95% ethanol

After centrifugation, nitrite in the supernatant fluid was assayed as follows. (Fewson and Nicholas 1961)

to 2.5ml water
1.0ml supernatant fluid
and 0.5ml 1% (w/v) sulphanilamide in 2N H_2SO_4
were added.

The contents were mixed and allowed to stand for 2 minutes. 1.0ml 0.02% (w/v) N-1-naphthylethylenediamine dihydrochloride was added.

The contents of the tube were again mixed and allowed to stand for 10 minutes.

The extinction was read at 550 mμ.

Experiments where growth and β-lactamase synthesis were measured.

This type of experiment forms the bulk of this thesis. The procedure followed was usually to add inducer to the flask at the same time as the inoculum and measure β-lactamase once the culture was growing. Thus many of the graphs show growth and enzyme from four hours in defined medium and from 1 hour in nutrient broth. In defined medium there was often a lag of four hours before logarithmic growth started, so that by measuring the growth and β-lactamase from this time, the greater part of the growth curve is covered. The lag in nutrient broth did not usually exceed half and hour. The synthesis of enzyme on addition of inducer to the culture once growing, is treated in a seperate section. Unless stated otherwise, inducer was added at the time of inoculation.

Enzyme assays were performed immediately the sample was taken from the growth flask. The turbidity of the culture was measured soon afterwards (within half an hour), because it was

found that the samples underwent slight lysis if kept for several hours.

Removal of inducer from growing cultures.

A sample of the culture was filtered through a 1" diameter metrical filter (Gelman) of 0.20 u pore size by suction until the filter was not quite dry. 10ml of the medium into which the cells were being transferred was then blown onto the surface of the filter in two 5ml aliquots and sucked not quite dry. The filter was then put into the flask of fresh medium and growth and enzyme followed in this flask.

Materials.

All chemicals used were of Analar grade and obtained from B.D.H. Poole, England, except the following:-

Amino acids were of Japanese biosynthetic origin obtained from T.J. Sas and Son Ltd, Victoria House, Vernon Place, London W.C.1.

Penicillin V (phenoxypenicillin) and other penicillin derivatives were gifts from Imperial Chemical Industries, Alderley Park, Macclesfield, Cheshire.

2 (2' -carboxyphenyl) -benzoyl-6-aminopenicillanic acid (CBAP) was synthesised by organic chemists of the research laboratories of I.C.I., Cheshire and gifted to us by I.C.I.

Hydrolysed starch was obtained from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

Chlorhexidine digluconate (20% w/v) (Hibitane) -obtained from Imperial Chemical Industries, Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire.

Membrane filters were Gelman metrical filters obtained from Camlab, Cambridge, England. Gelman filter holders, 1" diameter, were also obtained from Camlab, Cambridge, England and modified in this department.

Nutrient broth was obtained from Oxoid Ltd. Southwark Bridge Rd., London S.C.1.

Oxygen and oxygen free nitrogen were obtained from British Oxygen Co Ltd., Glasgow.

Chloramphenicol was obtained from Parke Davis & Co Ltd., Hounslow, Nr. London.

Actinomycin D was a gift from Prof. R.M.S. Smellie.

Haemosol - obtained from Meinecke & Company Inc. P.O. Box 6862, Baltimore, Maryland, U.S.A.

RESULTS

1 (a) β-lactamase assay.

Fig. 7 (p.38a) shows that production of penicilloic acid in the β-lactamase assay is linear with time, for the four concentration of cells used. This was confirmed in many experiments for concentrations of cells ranging up to a fully grown culture in nutrient broth. The rate of production of penicilloic acid, i.e. activity of β-lactamase, is proportional to the concentration of cells, i.e. enzyme. (Fig. 8 p.38b).

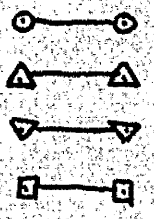
Attempts were made to detect extracellular enzyme by removal of the cells with a membrane filter (0.20 u pore size, Gelman metricel) and assay of the enzyme in the filtrate. In both induced and uninduced cultures, the extracellular enzyme was found to be less than 4% of the total enzyme present. (See table 2 p.38c). Holms (unpublished data) has shown that the extracellular enzyme in this strain, measured on the supernatant fluid obtained by centrifugation, is normally very much less than 4% but varies with the age of the culture.

Table 1 p.38d shows the effect of sonic disruption on cells grown in nutrient broth supplemented with phosphate buffer (39.7mM), glucose (66.7mM) magnesium (1.6mM) and induced with methicillin (2.5 μm). Sonication releases protein into the supernatant fluid. The total enzyme activity increases but remains predominantly particulate. The zero time figures confirm that the extracellular enzyme is less than 4%.

1 (b) β-galactosidase.

Growth on succinate (12mM) with melibiose or phenyl- β -D-thiogalactoside in concentrations ranging from 0.3mM to 12mM gave no β -galactosidase activity. Even after incubation of the assay for over two hours at 37 °C., no increase in o-nitrophenol could be detected. Concentrations of lactose from 2.0mM to

Fig. 7. Production of penicilloic acid during assay of lactamase in whole cell suspensions of *S. aureus* C23/19. Cells were grown in nutrient broth containing 2·5 uM methicillin. Each assay flask contained the following in a total volume of 20 ml. at 37°C :- phosphate buffer pH 7·0 (20 mM); penicillin V (20 mM); hibitane (10 ug/ml) and 5 ml of cell suspension.



○—○ fully grown culture diluted 1/2.
△—△ " " " " 5/8.
▽—▽ " " " " 3/4.
□—□ " " " " 7/8.

Production of penicilloic acid during assay of β-Lactamase

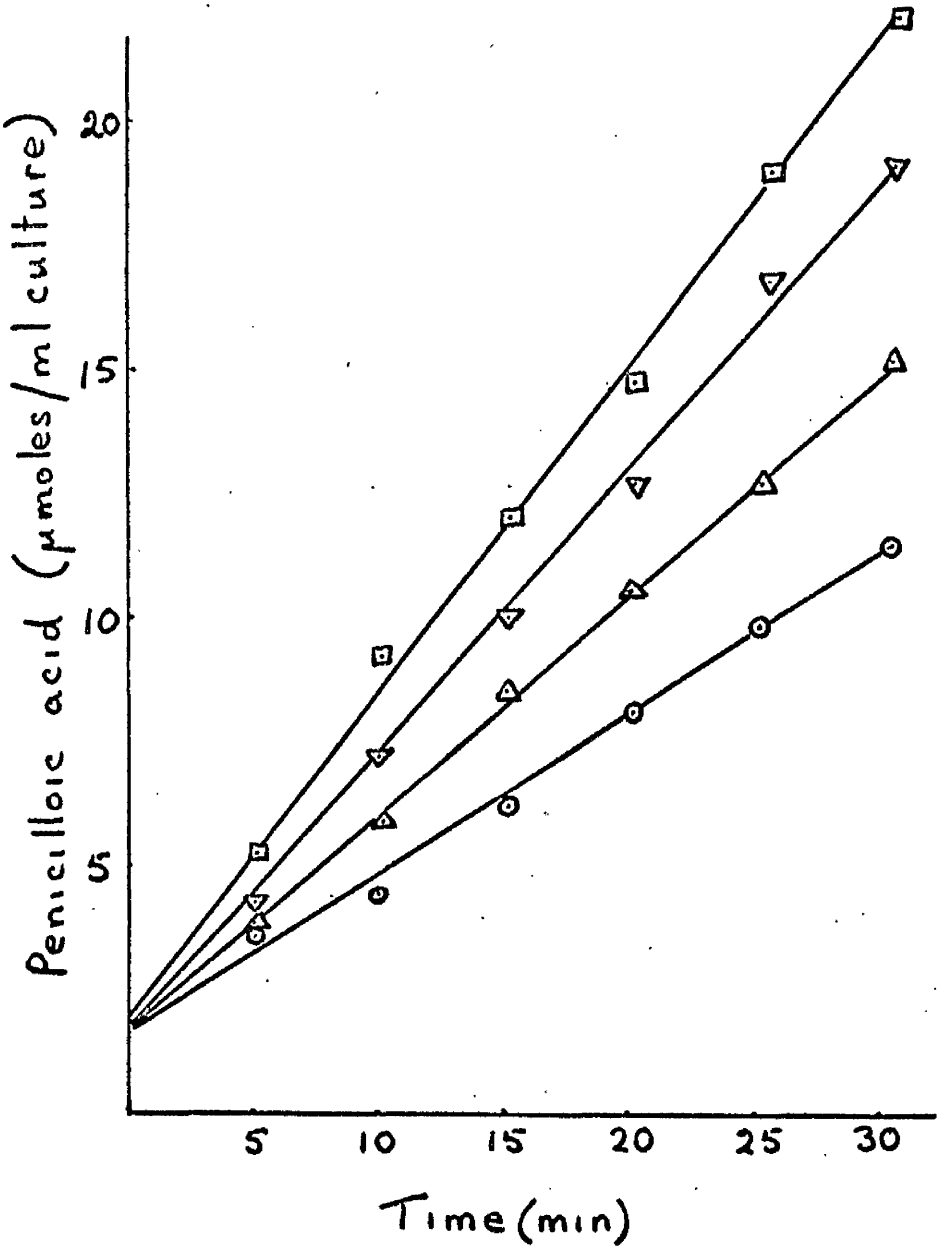


Fig 7

Fig. 8. β-Lactamase as a function of the concentration of cells in the assay. The contents of each assay flask are as described in fig.7. Cells were grown in nutrient broth containing 2·5 μM methicillin.

β-Lactamase activity as a function of the concentration of cells.

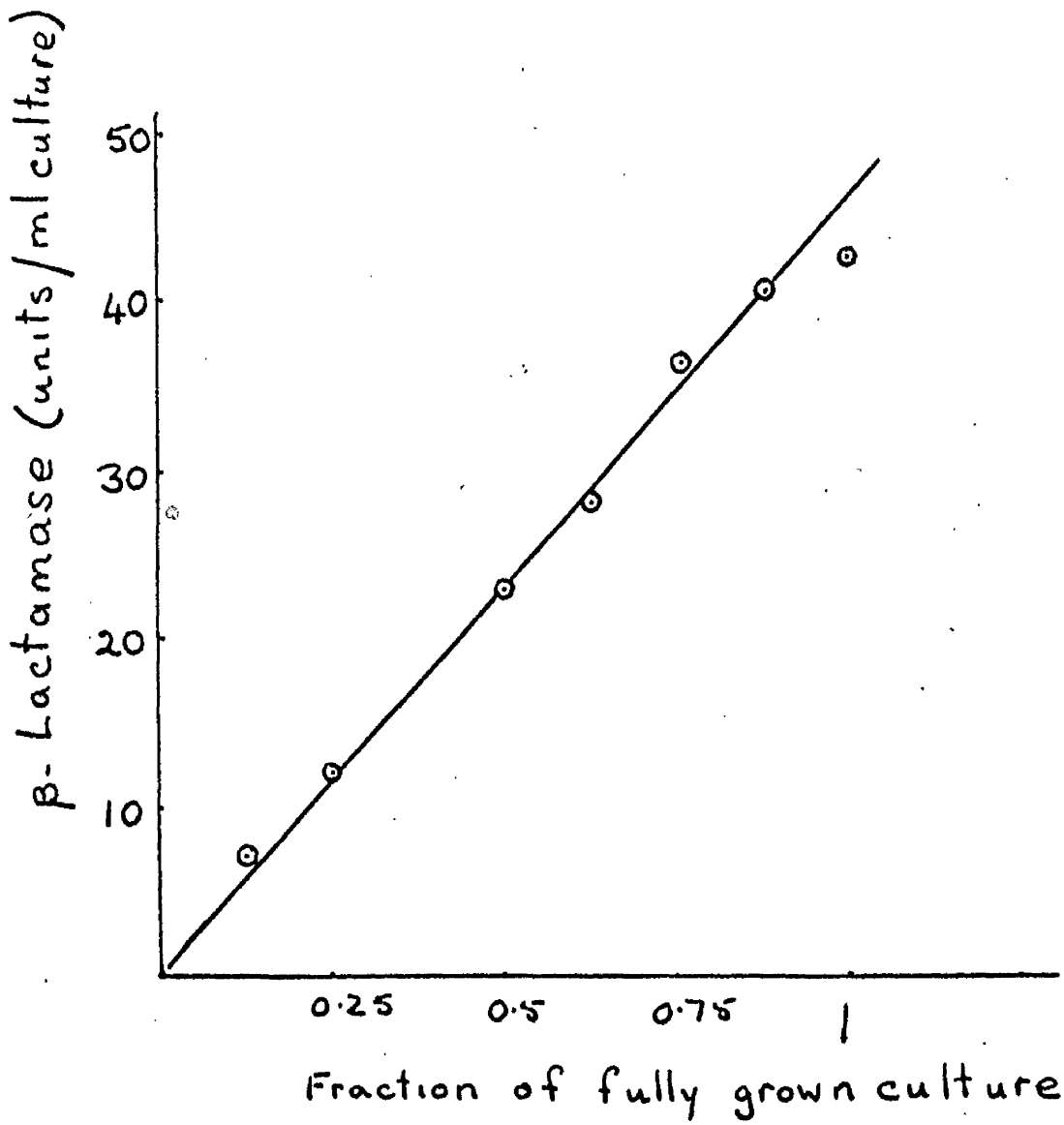


Fig. 8

Table 2. Percentage of β lactamase which is extracellular in S. aureus C23/19 growing in nutrient broth.

concentration of CBAP in growth medium μM	β - lactamase				
	units/ml		specific activity		% extra-cellular enzyme
	Total	Extra-cellular	Total	Extra-cellular	
50	142.7	4.7	100.9	3.32	3.29
Nil	0.75	Nil	0.75	Nil	Nil

Table 1. Ultrasonic disruption of S.aureus C23/19 growing in nutrient broth + magnesium sulphate (1.6 mM), phosphate (40 mM), glucose (67 mM) and induced with 2.5 μM methicillin.

Time of sonication	β-lactamase units/ 0.1 ml		Protein μg/mg of wet wt.	
	Supernate	Total	Supernate	Total
0 min	13	504	18	85
2 "	91	1024	40	80
4 "	92	1359	47	85
8 "	52	2404	64	80
16 "	122	1439	77	95

8.0mM gave no enzyme but lower concentrations did (see table 3 p.39a). Galactose at concentrations from 3mM to 12mM gave the amounts of enzyme shown in table 3 p.39a. Lower concentrations gave no detectable enzyme. The induction of β-galactosidase is optimal at 30mM galactose (fig. 9 p.39b). Addition of glucose (12mM) to the medium abolishes induction of the enzyme and the presence of cysteine in the assay does not increase activity of induced cultures.

1 (c) Nitrate reductase assay.

(i) Conditions of induction of nitrate reductase.

Cells grown aerobically in the presence of nitrate, harvested, washed and resuspended in buffer, did not reduce nitrate to nitrite. However, cells grown anaerobically in the presence of nitrate did reduce nitrate. On changing a culture from aerobic to anaerobic conditions in the presence of nitrate, normal growth rate was regained after a short lag. Nitrate reductase was synthesised in this lag as shown by the accumulation of nitrite in the medium.

(ii) Effect of toluene.

Oxygen inhibits the assay of nitrate reductase (Fewson and Nicholas, 1961). Under the anaerobic conditions of the assay, toluene increased the activity by 100%. Assay of the enzyme in the presence of oxygen (i.e. in 6" x 5/8" test tubes open to the atmosphere) reduced the activity to very low levels. Addition of toluene could increase the aerobic activity to 60% of the anaerobic activity. However, this technique could not be made sufficiently reproducible for use (Table 4 p.39c). The activities obtained are not proportional to the concentration of cells employed.

Effect of cyanide.

Cyanide inhibited both the aerobic and anaerobic activity of the enzyme.

Table 3. β-Galactosidase (m.units/mg wet wt.) in cells of S.aureus C23/19 grown in defined medium with the concentrations of lactose or galactose shown.

Carbon source	Concentration (mM)					
	12	9	6	3	1	0.5
Galactose	0.39	0.20	0.17	0.04	Nil	Nil
lactose	Nil	Nil	Nil	Nil	0.07	0.06

fig 9. Optimum concentration of galactose for induction of β-galactosidase in S. aureus 23/19. Cells were grown overnight at 37°C with shaking in nutrient broth containing the concentrations of galactose indicated, harvested, washed in 0.5 M phosphate buffer pH 7.1. Each assay contained o-nitrophenyl - β- D- galactopyranoside (2.5mM); phosphate buffer pH 7.1 (0.1M); whole cell suspension (2 ml.). Reaction was allowed to proceed at 37°C for 1 hour. 4 ml. bicarbonate (0.4M) was added and extinction read at 420 mμ.

(o——o) Cells grown in absence of glucose

(Δ——Δ) Cells grown in absence of glucose Assay contained cysteine (5 mg/ml)

(●——●) Cells grown in presence of glucose (10 mg/ml) as well as galactose

(▲——▲) Cells grown in presence of glucose (10 mg/ml) and cysteine (5 mg/ml)

Optimum concentration of galactose for induction of β-galactosidase

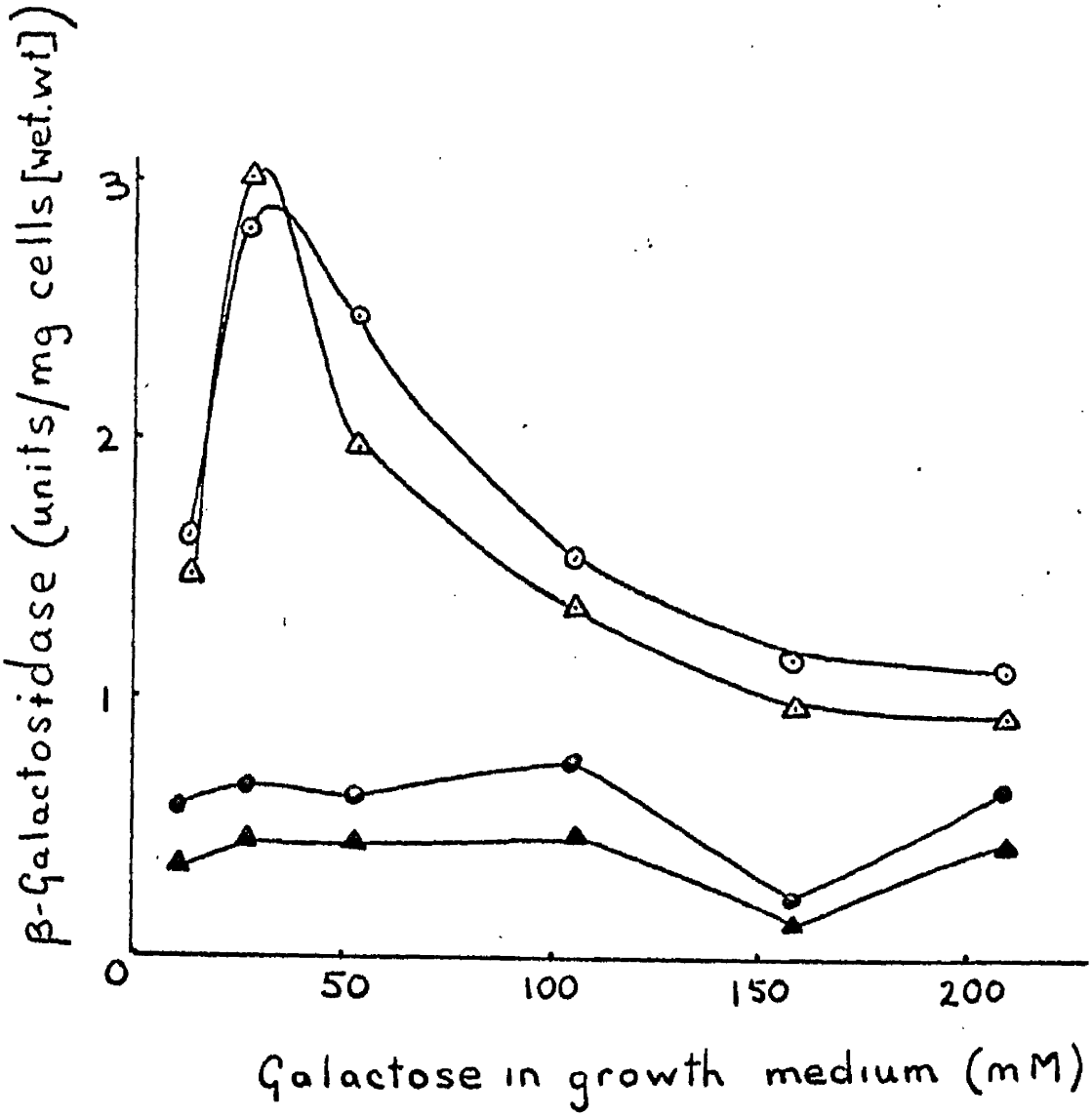


Fig 9

Table 4. Nitrate reductase activity and the effect of toluene in *S. aureus* C23/19 growing in defined medium.

Relative concentration of cells	μ moles nitrate reduced per hour			
	aerobic assay		anaerobic assay	
	+ Toluene	No Toluene	+ Toluene	No Toluene
1	34	4	53	41
0.8	21	5	26	8
0.6	10	8	17	1
0.4	3	1	7	3
0.2	0	0	0	0

1(d) Aeration in growth flasks.

The dggree of aeration under different conditions of stirring was determined by measuring the rate of oxidation of sulphite (Cooper, 1944). The degree of stirring was not measured quantitatively but is assessed as "vortex half way down the flask", "vortex breaking" and "very vigourous stirring". Table 5a (p.40c) shows the rate of oxidation of sulphite under these conditions with and without change of the air phase in the flask. It can be seen that the rate of oxidation increases as the rate of stirring increases but changing the air phase by pumping air at 4000ml/min does not alter the rate of oxidation of sulphite during very vigourous stirring. Table 5b (p.40d) shows the effect of partial pressure of oxygen on the rate of oxidation of sulphite. As expected, the rate of oxidation depends on the partial pressure of oxygen. These data were obtained using an apparatus designed to stir five flasks simultaneously and the degree of stirring cannot be increased to the "very vigourous stirring" of table 5a (p.40c). The degree of stirring is "vortex breaking" and this corresponds to the speed of stirring during growth experiments. Note that the 20% oxygen gives the same rate of oxidation as that obtained in table 5a (p40c) with air (without change of gas phase).

Change in composition of the gas phase produces an almost instantaneous alteration in the rate of oxidation of sulphite (fig. 10 p.40e).

(11) Growth of Staphylococcus aureus C23/19.

(a) Growth in nutrient broth.

Nutrient broth supports a typical logarthmic response but the approach to stationary phase is rather slow. (fig. 11 p.40b). Addition of glucose to a final concentration of 0.2% (w/v) lengthens the logarithmic phase and sharpens the approach to stationary phase (fig. 11 p.40b). Addition of phosphate buffer (0.05M) rdduces both the rate of growth and the yield

Fig.10. Effect of changing the atmosphere in the growth flask on the rate of oxidation of sodium sulphite. Each flask contained :- sodium sulphite (1M); copper sulphate (2×10^{-3} M). The atmosphere was changed at the time indicated by the arrows.

(△——△) Oxygen free nitrogen - changed to 20% Oxygen at 115 min.

(⊙——⊙) 20% oxygen - changed to oxygen free nitrogen at 115 min.

Change of gas phase in growth flasks and rate of oxidation of sulphite

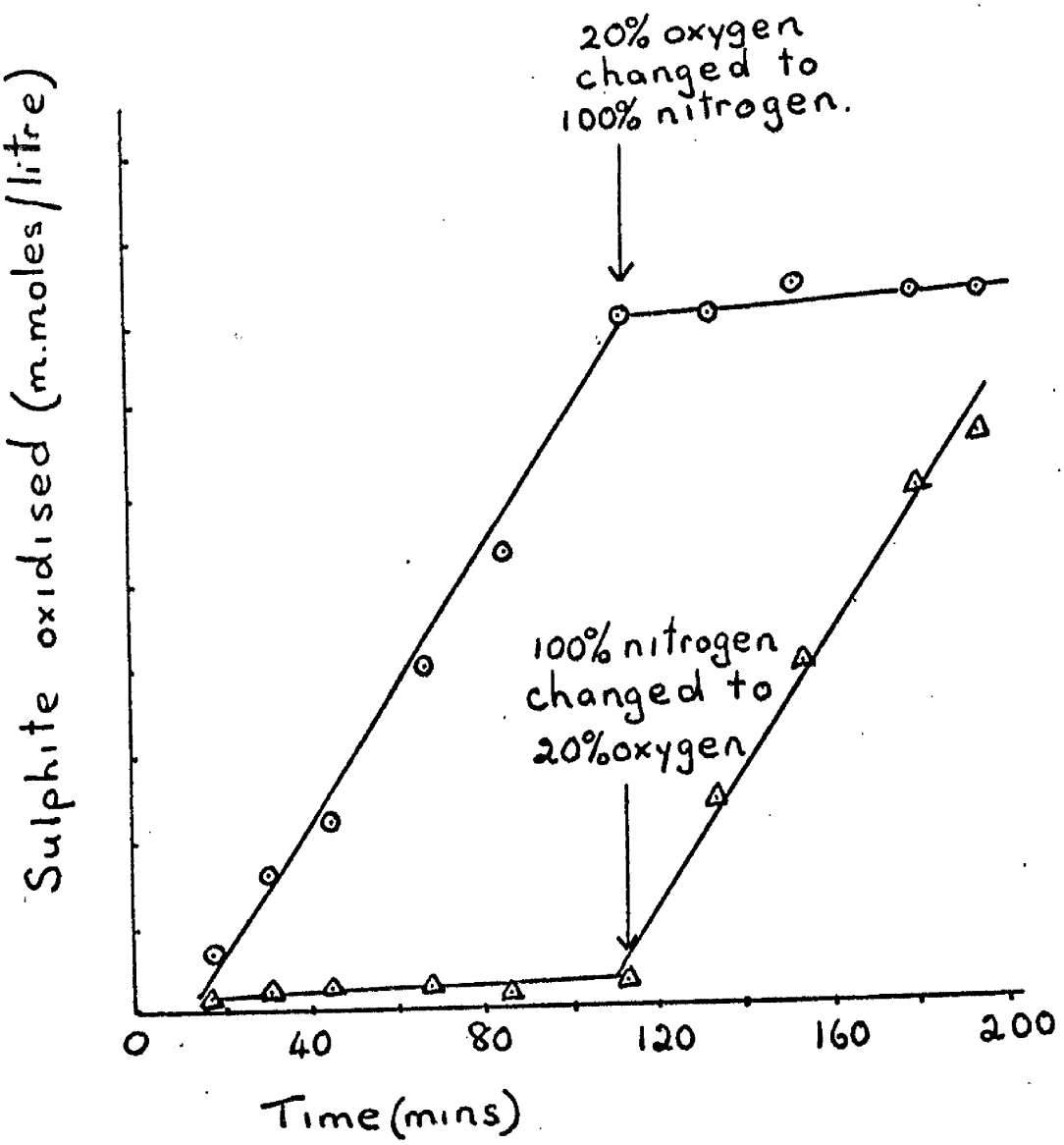
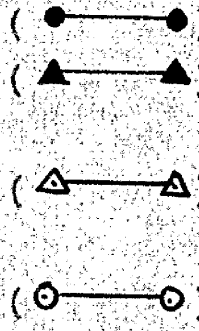


Fig 10

Fig.11. Growth of *S. aureus* C23/19 in nutrient broth with additions



(●——●) nutrient broth alone

(▲——▲) nutrient broth + phosphate buffer pH 7.4 (0.05 M)

(△——△) nutrient broth + glucose (0.2% W/V) + phoephate buffer pH 7.4 (0.05 M)

(⊙——⊙) nutrient broth + glucose (0.2% W/V)

Growth of S. aureus C23/19 in nutrient broth with various additions

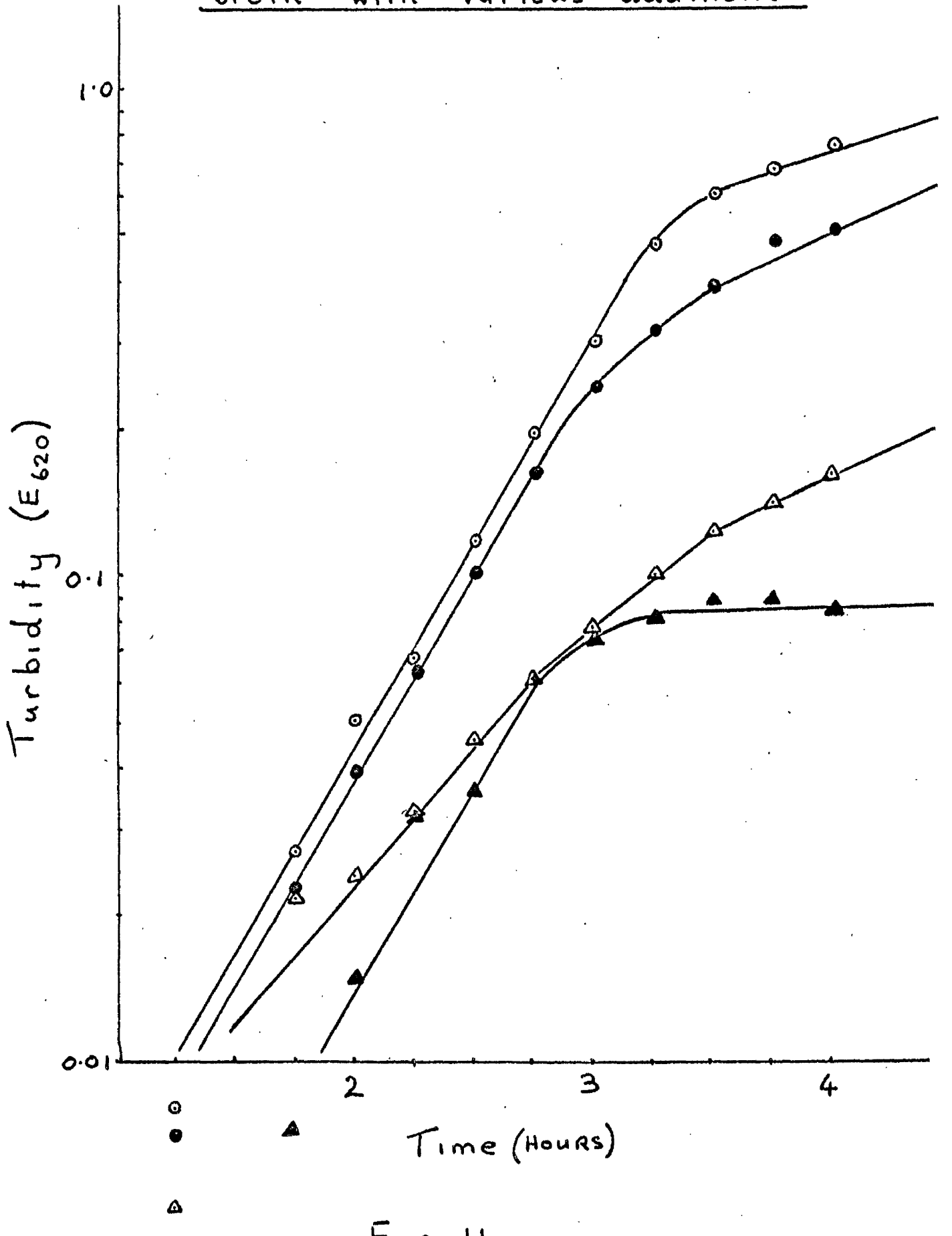


Fig. 11

Table 5a Effect of stirring on rate of oxidation of sulphite in growth flasks.

Speed of stirring	Gas phase change ml/min	Sulphite Oxidation ml oxygen/min
Vortex halfway down	Nil	0.4
Vortex touching bottom	Nil	0.7
Vortex breaking	Nil	3.9
Fast stirring	Nil	9.0
Very vigourous stirring	Nil	29.0
Very vigourous stirring	4000	27.8
Very vigourous stirring	oxygen 1000	112.0

Table 5b. Effect of partial pressure of oxygen on the rate of oxidation of sulphite in growth flasks stirred at the rate of "vortex breaking"

percentage of oxygen in nitrogen	Change of gas phase ml/min	Sulphite oxidation ml oxygen per min.
0.0	500	0·1
1·0	500	0·2
10·0	500	1·9
20·0	500	4·4
100·0	500	25·0

obtained (fig. 11 p.40b). Glucose partially relieves this inhibition. This rather unusual effect was found even with freshly prepared phosphate buffer. The effect of phosphate concentration on the yield and growth rate, is shown in fig. 12 (p. 41a). The yield and rate of growth are directly related to the concentration of phosphate.

Fig. 13 (p.41b) shows growth and β-lactamase synthesis during a growth cycle in nutrient broth alone and in nutrient broth plus glucose. The curve shown is that obtained with 0.2% (w/v) glucose. Identical curves were obtained with 0.5% (w/v) and 1.0% (w/v) glucose. β-lactamase synthesis is directly related to growth.

(b) Growth in defined medium.

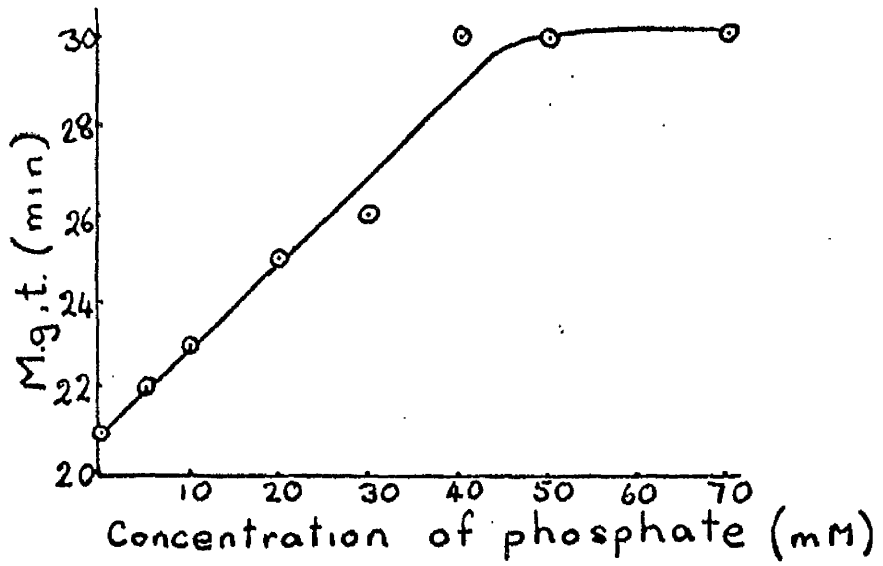
Tables 6 (p41d) show the effect of ommission of amino acids singly and in combination from the medium of Wildes et.al. (1936). Arginine, cystine, glycine, histidine and proline are essential for growth. Omitting pairs of amino acids does not inhibit growth unless one of the amino acids is an essential one. Omitting three amino acids at a time did not inhibit growth unless one of the amino acids was an essential one except in one case, where omission of tryptophan, phenylalanine and leucine at the same time did not permit growth. Omission of any four amino acids prevents growth.

Fig. 14 (p. 41e) shows growth of S. aureus C23/19 in defined medium containing eleven amino acids. The rate of growth and the lag are almost identical for three different sizes of the same inoculum. It was difficult to reproduce exactly the same pattern of growth in defined medium. Growing the inoculum for longer or shorter times did not make the growth pattern any more reproducible; nor did growing the inoculum through different numbers of passages. Autoclaving the growth flasks in dilute haemosol and rinsing thoroughly in glass distilled water improved

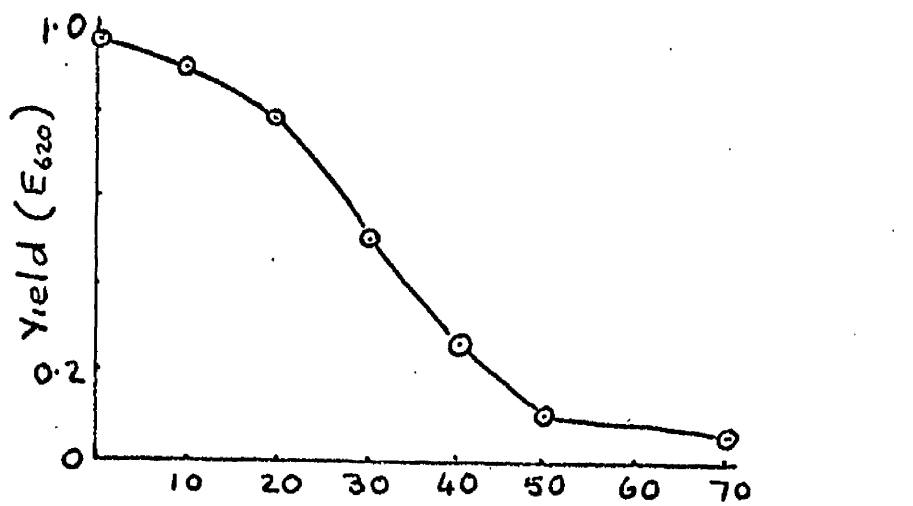
Fig. 12 (a) Effect of concentration of phosphate buffer pH 7.4 on the mean generation time (m.g.t.) of *S.aureus* C23/19 growing in nutrient broth

(b) Effect of concentration of phosphate buffer pH 7.4 on the yield of *S.aureus* 23/19 growing in nutrient broth.

Effect of phosphate on yield and growth rate of S. aureus C23/19



(a)



(b)

Fig. 12

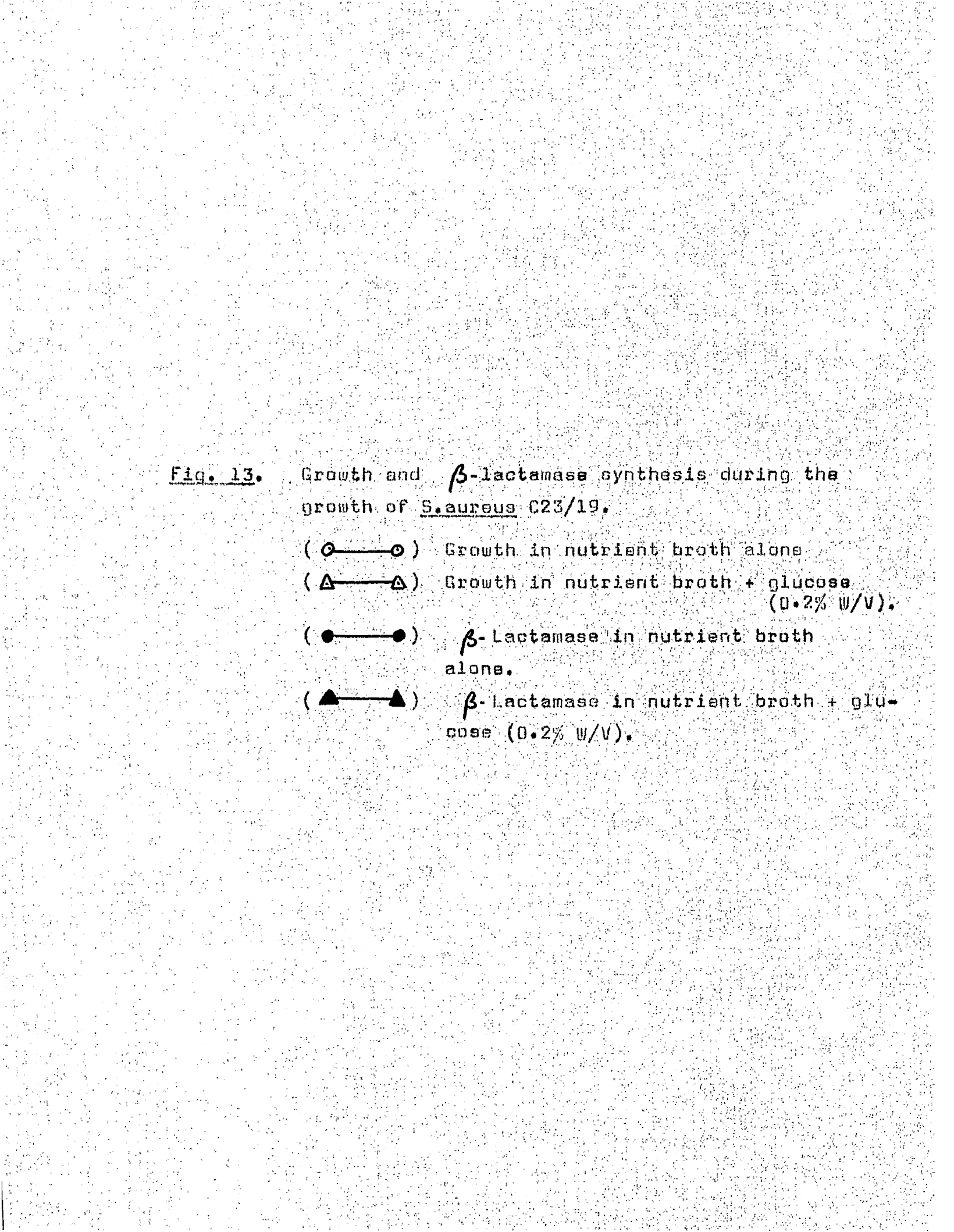


Fig. 13. Growth and β-lactamase synthesis during the growth of S.aureus C23/19.
(○——○) Growth in nutrient broth alone
(△——△) Growth in nutrient broth + glucose (0.2% W/V).
(●——●) β-Lactamase in nutrient broth alone.
(▲——▲) β-Lactamase in nutrient broth + glucose (0.2% W/V).

Growth and β-lactamase synthesis in S. aureus C23/19 growing in nutrient broth with and without glucose

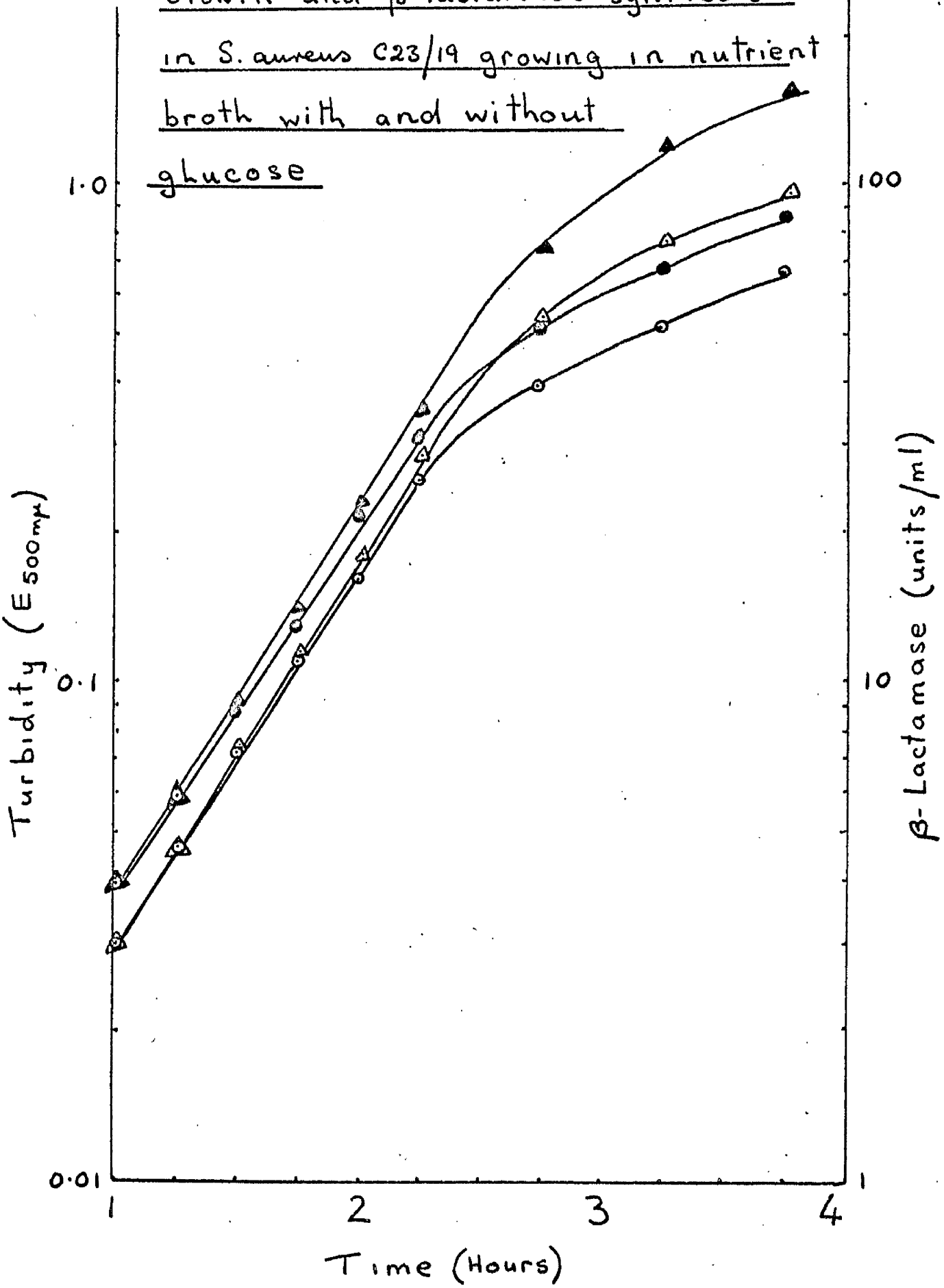


Fig 13

Table 6. Growth in defined medium with the omission of amino acids singly and in combination.

Amino acid omitted	Growth	Amino acids omitted	Growth	Amino acids omitted	Growth
Arg.	-	His,Trp	-	His,Trp,Leu	-
Asp	+	His,Phe	-	His,Phe,Leu	-
Cys-Cys	-	His,Glu	-	His,Glu,Leu	-
Glu	+	Trp,Phe	+	His,Phe,Trp	-
Gly	-	Trp,Glu	+	His,Trp,Glu	-
His	-	Phe,Glu	+	Trp,Phe,Glu	-
Leu	+	Leu,Phe	+	Leu,Phe,Glu	+
Pro	-	Leu,Glu	+	Leu,Trp,Glu	+
Phe	+	Leu,Trp	+	Leu,Trp,phe	+
Trp	+			His,Phe,Glu	-
Val	+				

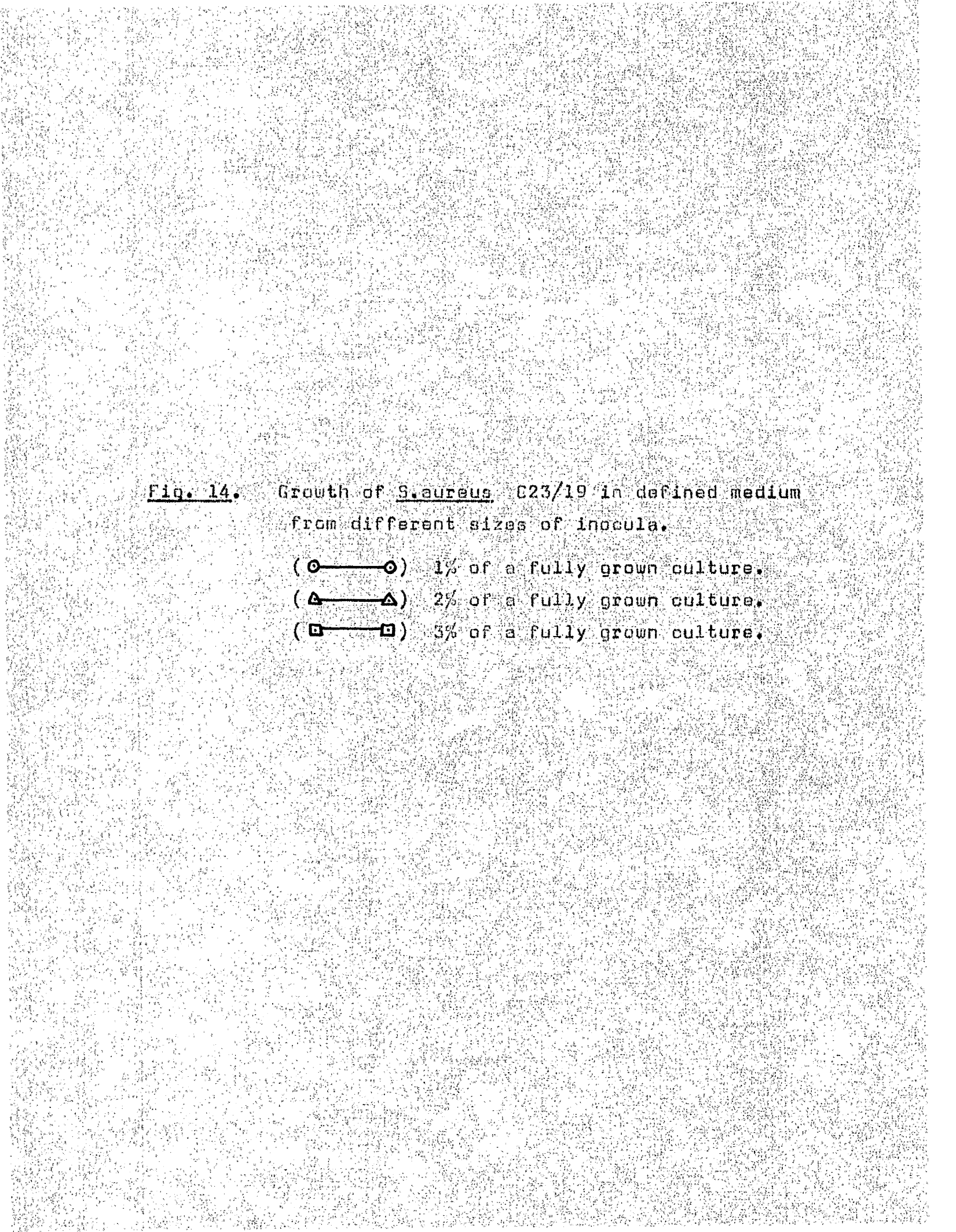


Fig. 14. Growth of S.aureus C23/19 in defined medium from different sizes of inocula.

(⊙——⊙) 1% of a fully grown culture.

(△——△) 2% of a fully grown culture.

(□——□) 3% of a fully grown culture.

Growth of S. aureus C23/19 in defined medium with glucose

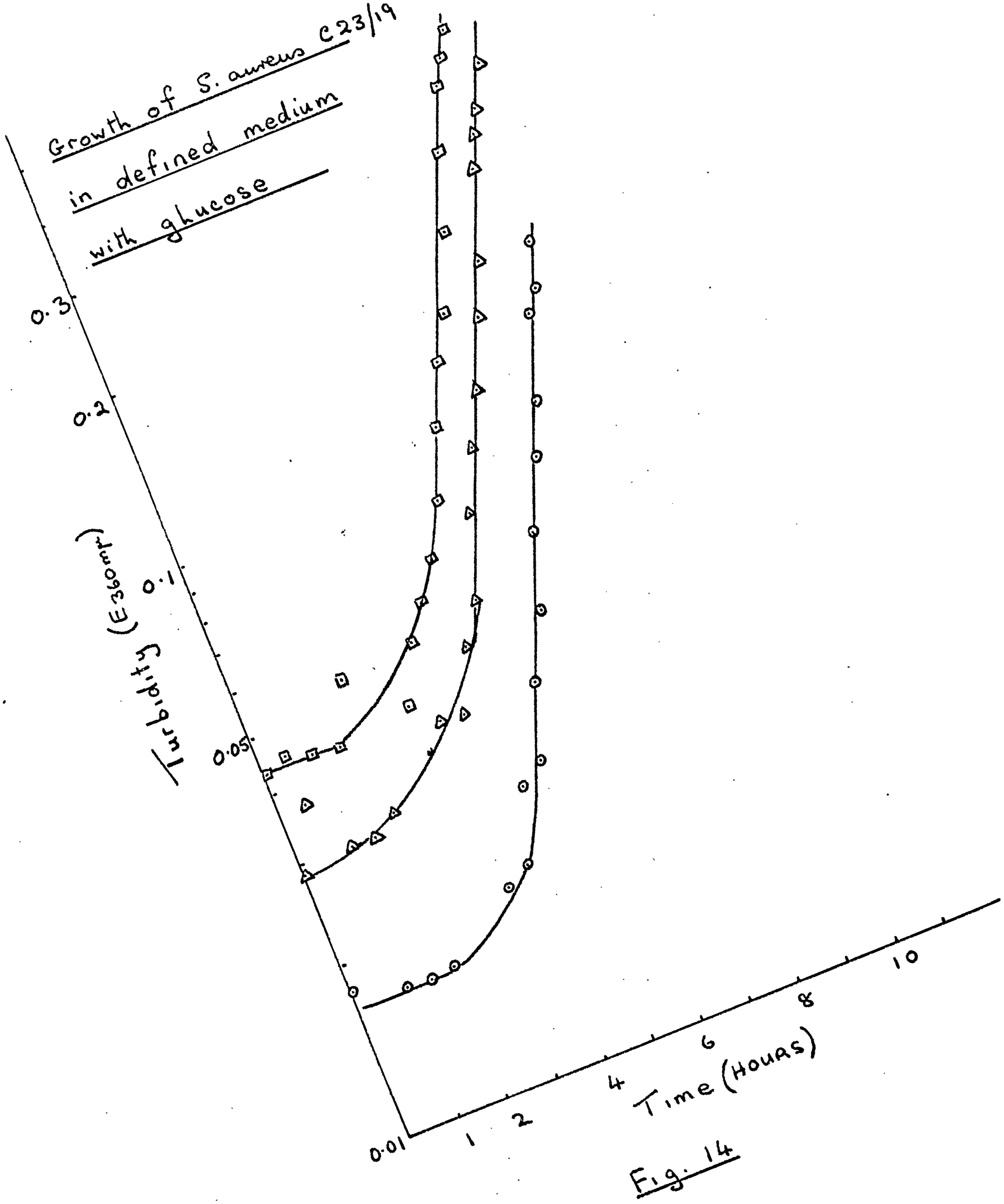


Fig. 14

matters a bit but did not completely eliminate the variability. Reducing the concentration of phosphate in the medium and sterilisation by Seitz filtration instead of autoclaving did not eliminate the variability. Mean generation times could be as long as 120 minutes and as short as 60 minutes with most being 90-100 minutes. Addition of yeast extract did not reduce this mean generation time unless added in substrate amounts (0.5% (w/v)). The lag time in this medium was usually four hours and seldom varied from this, but occasionally it was reduced to about two hours for no apparent reason.

(iii) Competition between enzyme systems.

(a) β-lactamase and β-galactosidase.

S. aureus C23/19 has only a small amount of β-galactosidase. (Section 1b). Table 7 (p.42a) shows the differential rates obtained in several experiments in defined medium with glucose and galactose as carbon sources. There is no difference between the differential rate in glucose and that in galactose.

(b) β-lactamase in defined medium with other carbon sources.

Table 8 (p.42b) lists the specific activities obtained with 19 different carbon sources in defined medium. The specific activity falls within a narrow range for most of the compounds. Arabinose, and sucrose have β-lactamase levels considerably higher than that in glucose. The turbidities of these cultures were very different and therefore they were not at the same stage of growth.

(c) β-lactamase in nutrient broth supplemented with different carbon sources.

Table 9 (p.42c) shows the differential rate of β-lactamase synthesis in nutrient broth supplemented with different carbon sources. The five carbon sources arabinose, sucrose, glycerol, glucose, and lactose, tested in this way, all support the same differential rate of β-lactamase synthesis.

Table 7. Differential rate of β-lactamase synthesis in defined medium with glucose or galactose as source of carbon.

Differential rate (units/unit E_{350})	
Glucose	Galactose
107	104
95	102
96	100
84	98

Table 8. Specific activity of β-lactamase in *S.aureus* growing in defined medium with different carbon sources

Carbon source	β-lactamase units/unit E_{350}
Arabinose	83
Asparagine	65
Fructose	57
Fumarate	64
Galactose	58
Glucose	48
Glucosamine	55
Gluconate	60
Glycerol	46
Lactate	57
Lactose	54
Mannitol	53
Malate	71
Pyruvate	59
Ribose	51
Sucrose	91
Succinate	66
Sorbitol	64

Table 9. Differential rate of β-lactamase synthesis in S.aureus C23/19 growing in nutrient broth supplemented with different carbon sources

Carbon source	Differential rate(units/unit E_{500})
Arabinose	96
Sucrose	108
Glycerol	100
Glucose	108
Lactose	100

(d) β-lactamase and nitrate reductase.

Fig. 15 (p. 43a) shows β-lactamase and turbidity of a culture initially grown aerobically in the presence of nitrate. At 8 hours (in the middle of logarithmic phase) the gas phase was changed from air to oxygen free nitrogen. It can be seen that change to anaerobic conditions (induced by the arrow) causes a lag in growth and enzyme synthesis for about one hour. They then continue at a slightly reduced rate. During this change, nitrate reductase is induced (see section 1(c)(1).). The differential rate of β-lactamase synthesis remains constant throughout the growth aerobically and anaerobically (fig. 16 p. 43b). Thus induction of a new enzyme, nitrate reductase, does not affect the synthesis of β-lactamase in S. aureus 623/19.

(e) Induction of β-lactamase in presence of nitrate reductase.

The initial kinetics of induction during aerobic growth in defined medium containing nitrate were compared with the kinetics during anaerobic growth in the same medium. Fig. 17 (p.43c) shows the growth of the two cultures. At the arrow (↓) the gas phase of one was changed to oxygen free nitrogen. Once growth was established again inducer (CBAP) was added to both flasks at the arrows (↑) and β-lactamase measured from that time. Fig. 18 (p.43d) shows the plot of the logarithm of β-lactamase *vs* time for the two flasks from the time of addition of inducer. The β-lactamase content of the cultures rises very quickly in both flasks. The doubling time of enzyme in the culture growing anaerobically in the presence of nitrate appears faster than in the culture growing aerobically. The differential rate of β-lactamase synthesis is the same in both cultures. (Fig. 19 p.43e).

(IV) Effect of inhibitors on growth and β-lactamase synthesis.

(a) Effect of Hibitane (chlorhexidine digluconate)

Hibitane (10 μg/ml) stops growth and β-lactamase synthesis

Fig. 15. Growth(o——o) and β lactamase synthesis (▲——▲) during growth indefined medium containing nitrate (10 mM) and CBAP (50 μM) At the arrow, oxygen free nitrogen (500 ml/min.) was passed into the flask.

Growth and β-lactamase synthesis in S. aureus growing aerobically and changed to anaerobic growth

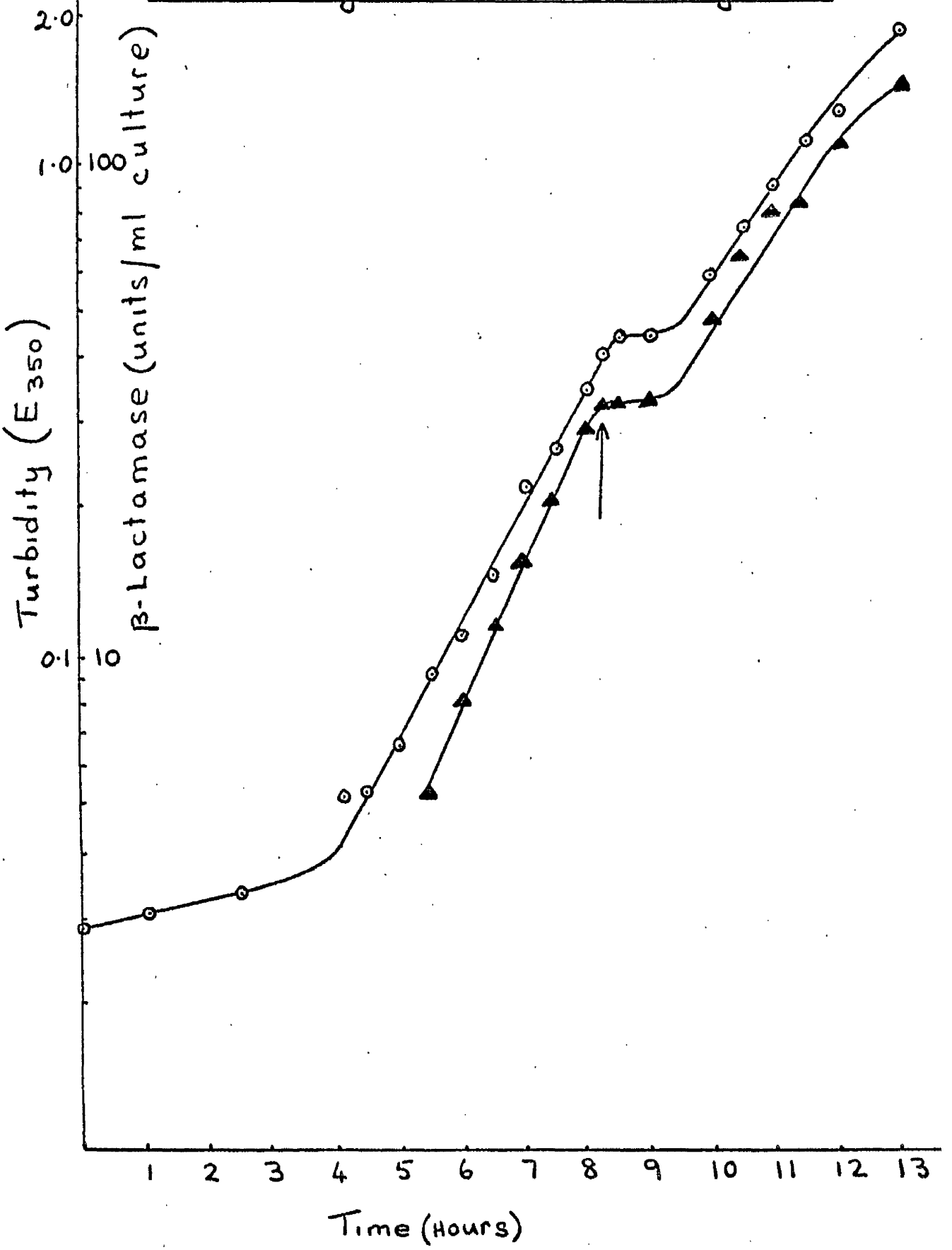


Fig 15

Fig. 16. Differential rate of β-lactamase in the culture of fig. 15. The point where nitrogen was passed into the flask is indicated by the arrow. The differential rate is 80 units/ unit E_{350}

Differential rate of β-lactamase synthesis during aerobic growth followed by anaerobic growth

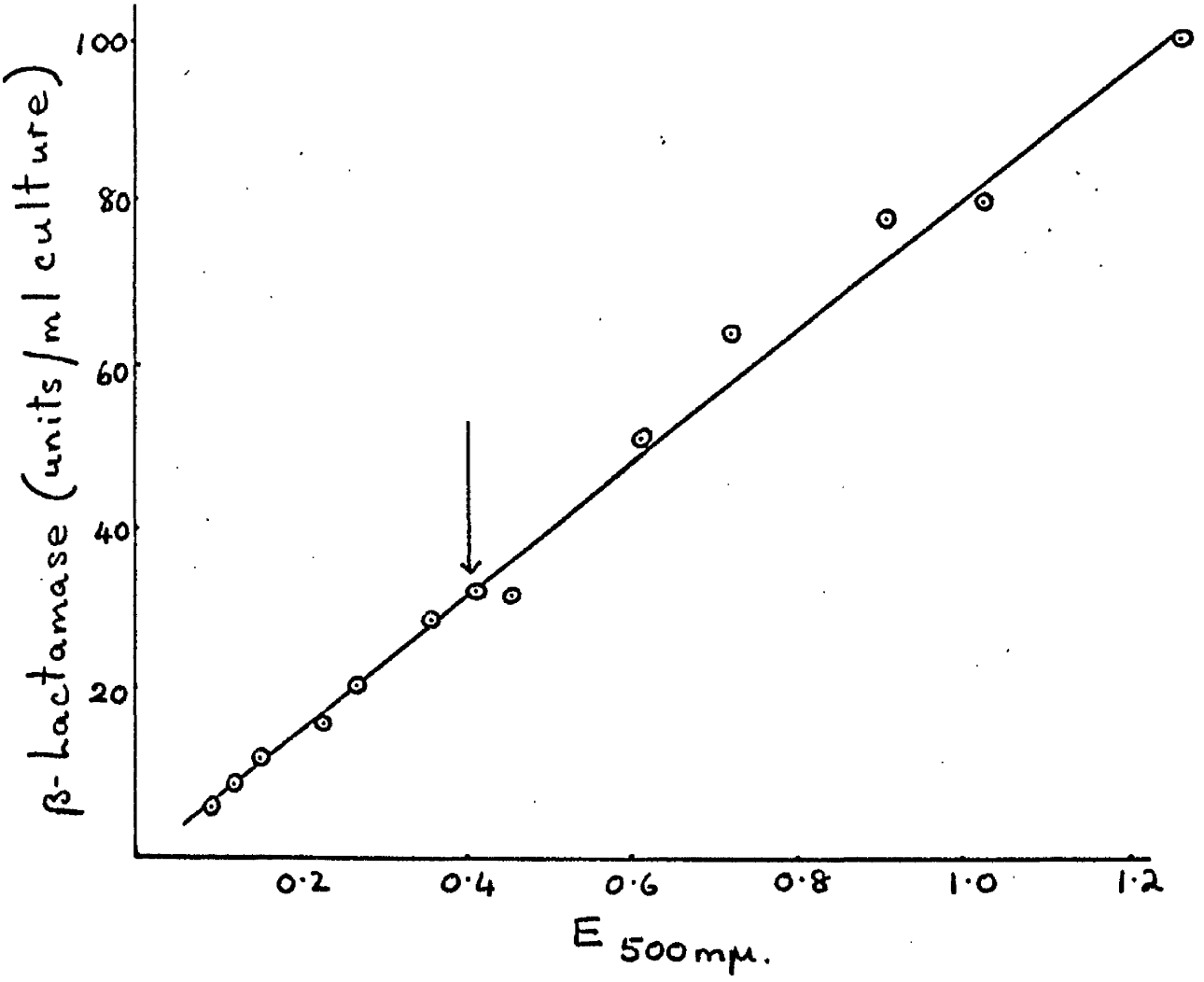


Fig 16

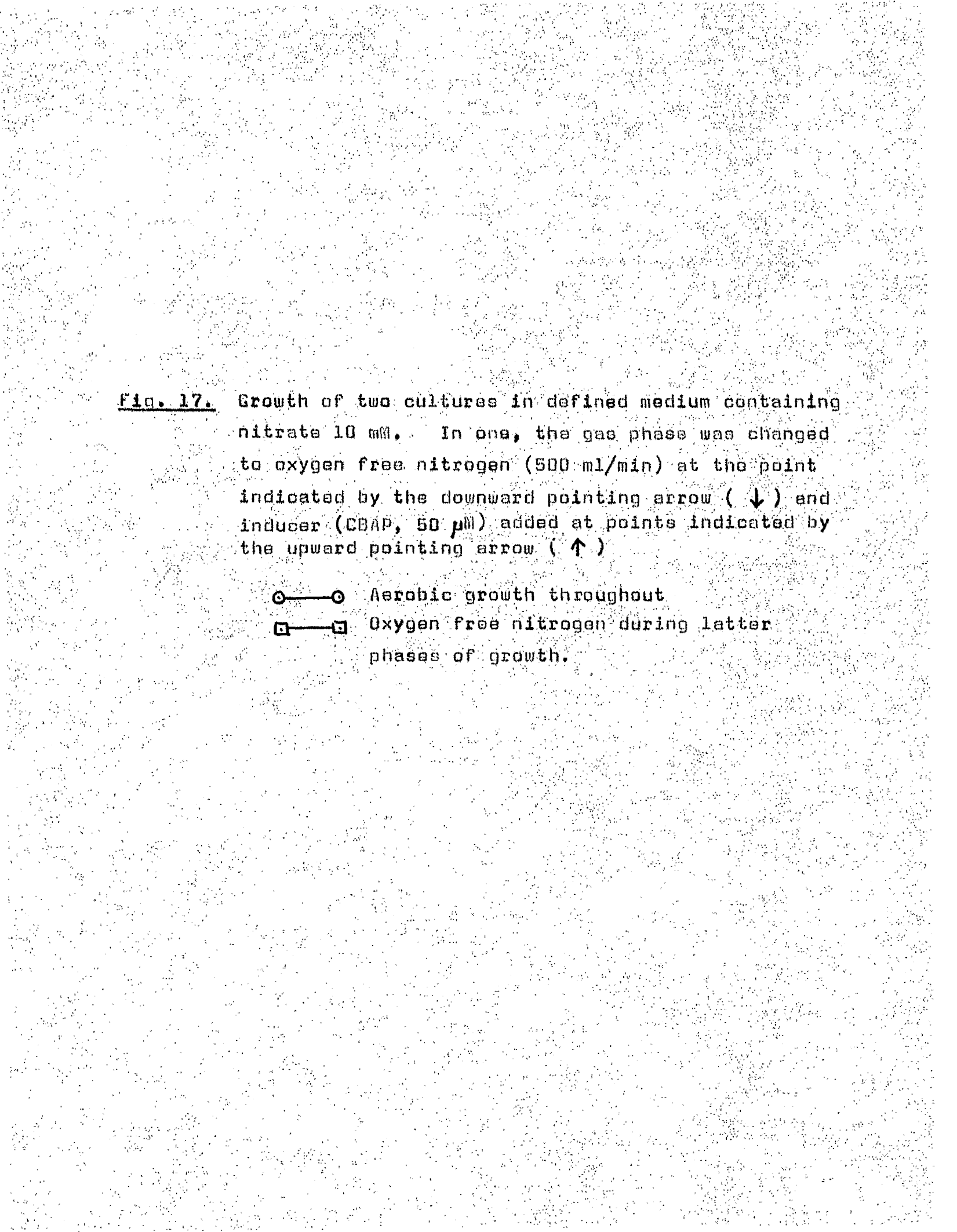


Fig. 17. Growth of two cultures in defined medium containing nitrate 10 mM. In one, the gas phase was changed to oxygen free nitrogen (500 ml/min) at the point indicated by the downward pointing arrow (↓) and inducer (CDAP, 50 μM) added at points indicated by the upward pointing arrow (↑)

o——o Aerobic growth throughout

□——□ Oxygen free nitrogen during latter phases of growth.

Growth of S. aureus aerobically and when changed to anaerobic growth

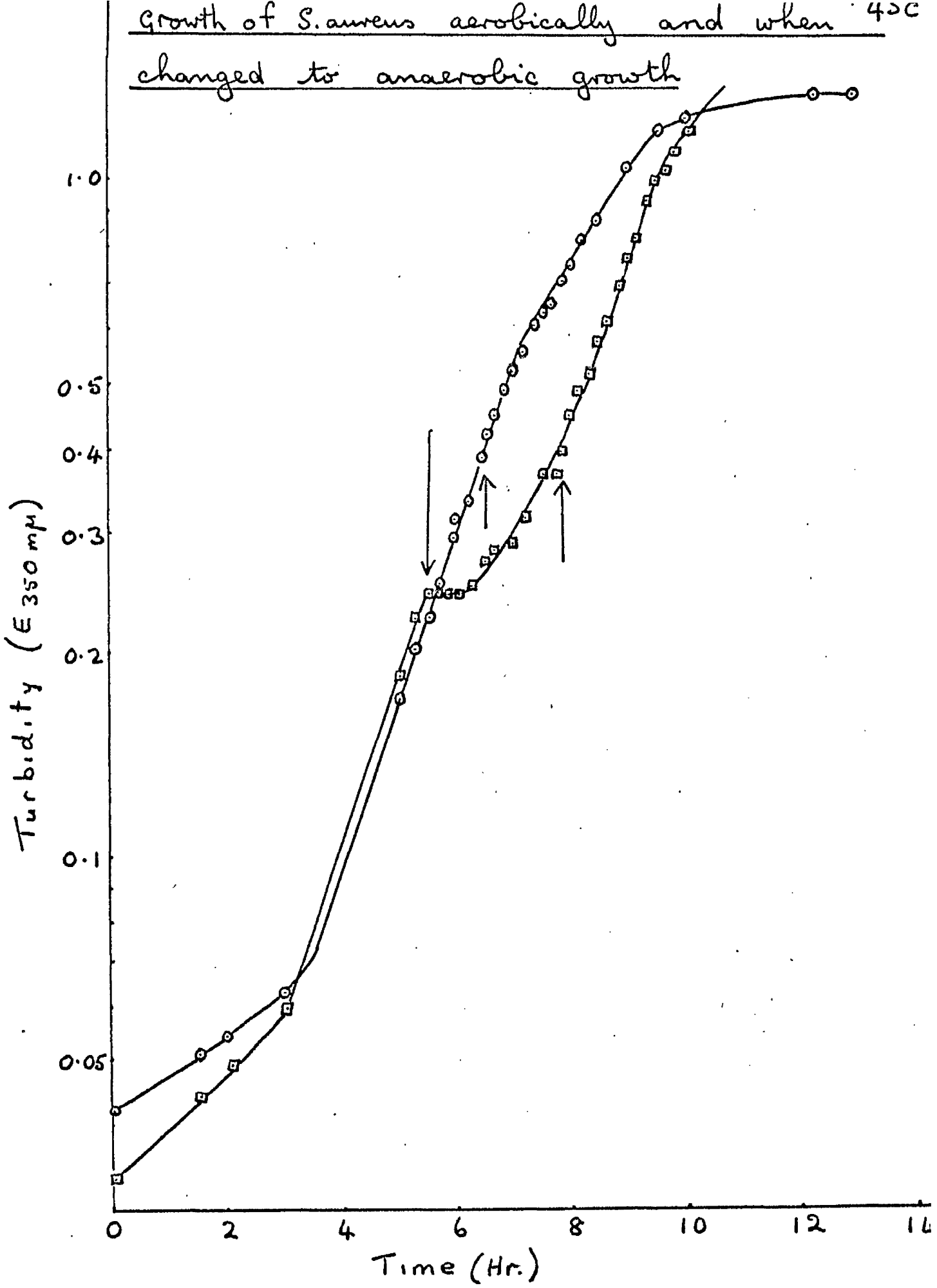


Fig 17

Fig. 18. β-Lactamase synthesis from the moment of addition of inducer in the cultures of Fig.17.
(●——●) β-lactamase in culture growing aerobically in presence of nitrate
(■——■) β-lactamase in culture growing anaerobically in presence of nitrate.

β-Lactamase synthesis on addition of inducer to cultures growing aerobically and anaerobically

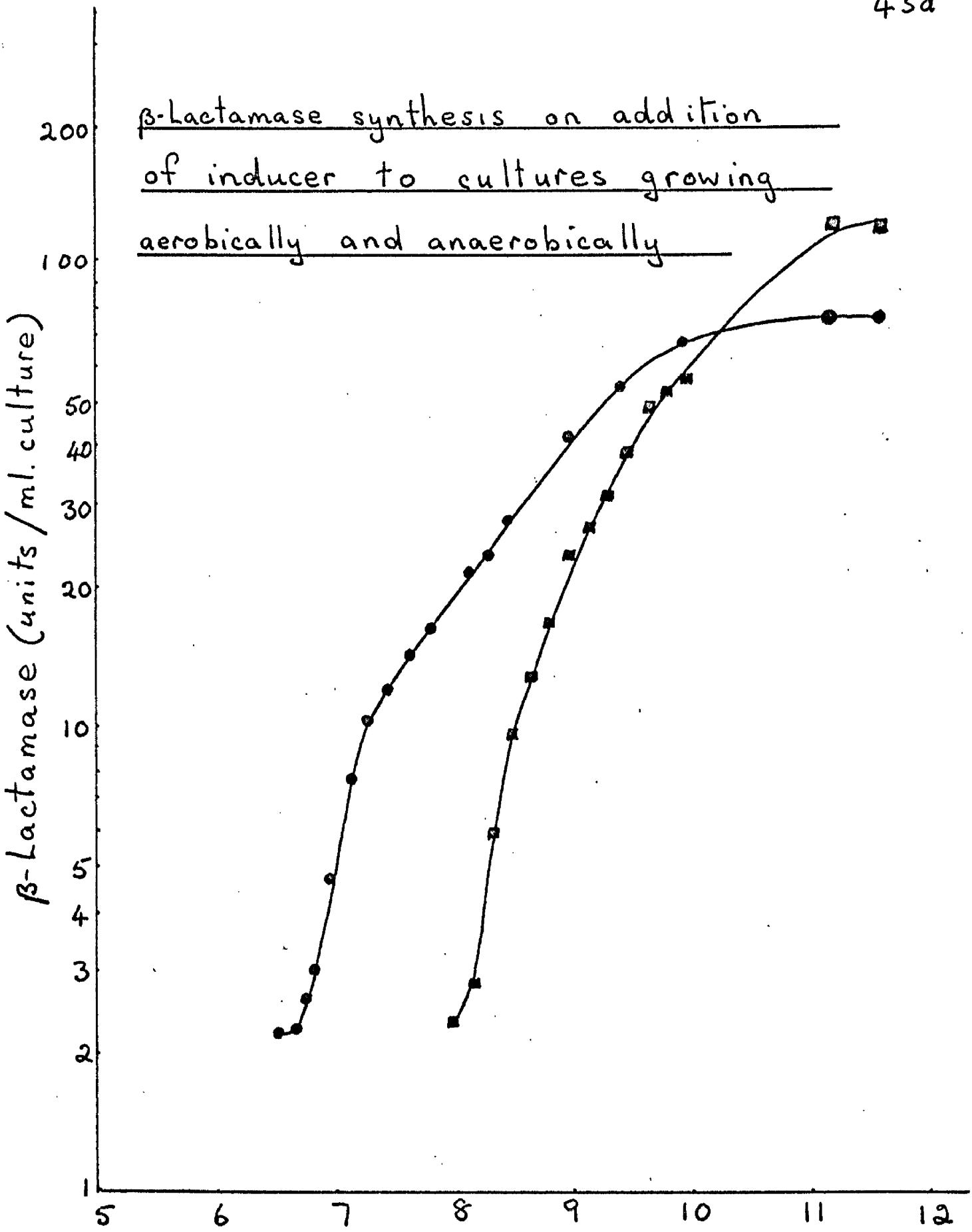


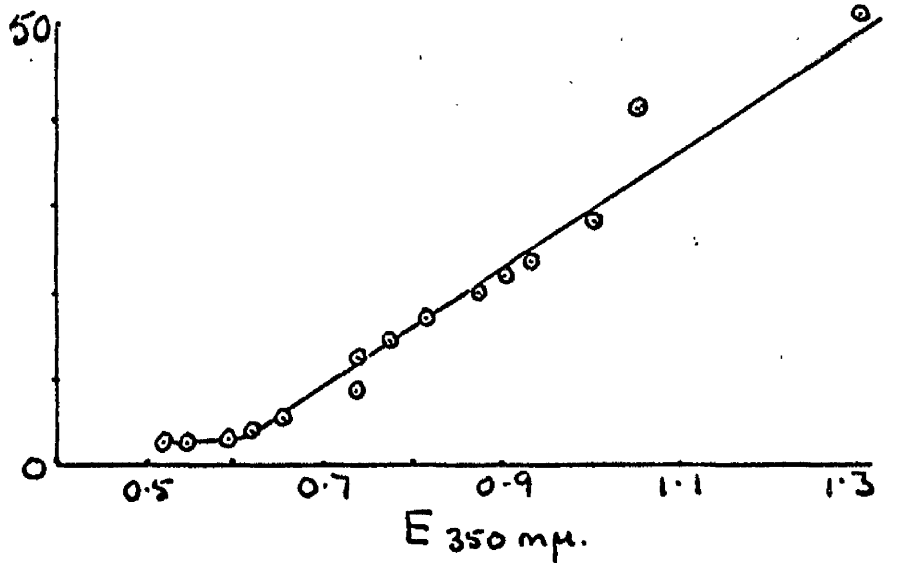
Fig 18

Fig. 19. ^{D}Differential rate of β-lactamase synthesis in

(a) culture of Fig.17. growing aerobically in the presence of nitrate. Differential rate = 75

(b) culture of Fig.17 growing anaerobically in the presence of nitrate. Differential rate = 80.

Differential rate of β-lactamase synthesis in S. aureus growing aerobically and anaerobically



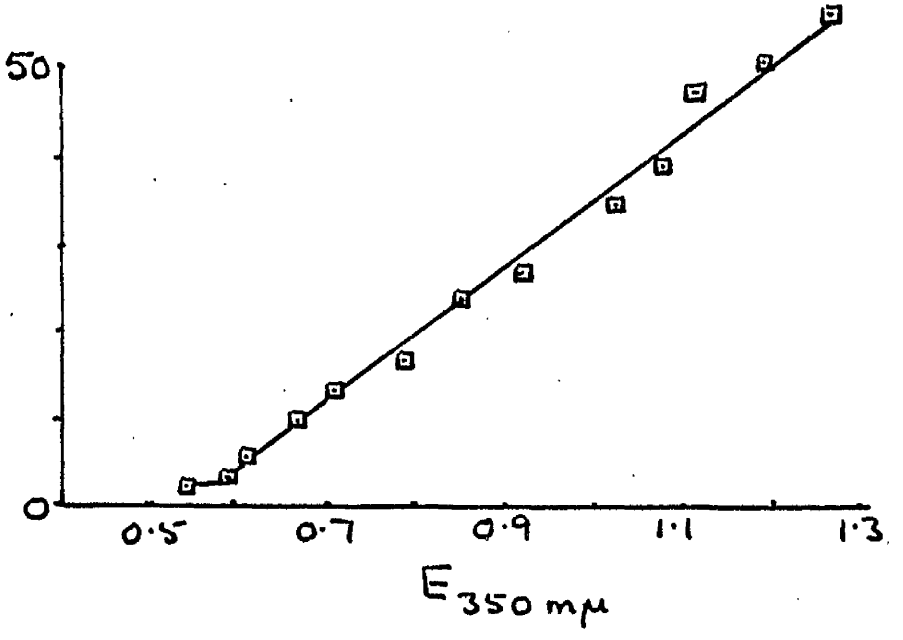


Fig 19

in cultures growing logarithmically on nutrient broth + glucose (0.2% w/v) with CBAP (50 μm) as inducer. (Fig. 20 p.44a). Growth ceases immediately and after 15 mins turbidity begins to fall slowly. Enzyme synthesis stops immediately and remains constant thereafter for at least one hour and a half.

(b) Effect of chloramphenicol.

Chloramphenicol (15 μg/ml) stops β-lactamase synthesis and causes an immediate fall in turbidity of cultures growing in nutrient broth + glucose (0.2% w/v) with CBAP (50 μm) as inducer (fig. 21 p.44b). After ten minutes the turbidity rises again to a value above that obtaining when chloramphenicol was added. β-lactamase remains constant for well over an hour.

(c) Effect of actinomycin D.

Actinomycin D (5 μg/ml) stops growth and β-lactamase synthesis in S. aureus 023/19 growing in nutrient broth + glucose (0.2% w/v) almost immediately and remains constant for over an hour. Turbidity continues to rise for a short time, then falls and finally rises again to reach a turbidity of about twice that obtaining when actinomycin D was added. (Fig. 22 p.44c).

(V) β-lactamase synthesis under different conditions of growth.

(a) Effect of type and concentration of inducer

The efficacy of several inducers of β-lactamase was tested in both nutrient broth + glucose and in defined medium. Of the three inducers, methicillin, cephalosporin C and 2 (2' carboxyphenyl)- benzoylaminopenicillanic acid (CBAP) (fig. 23 p.44d), CBAP gave the highest specific activity in both media (table 10 p.44e). The effect of concentration of inducer on the specific activity obtained in nutrient broth + glucose is shown in fig. 24 (p.44f) for methicillin and CBAP. At concentrations above 2.5 μm, methicillin reduces the yield of cells obtained and the rate of growth, and stops growth completely at concentrations above 10 μm. CBAP, on the other hand, does

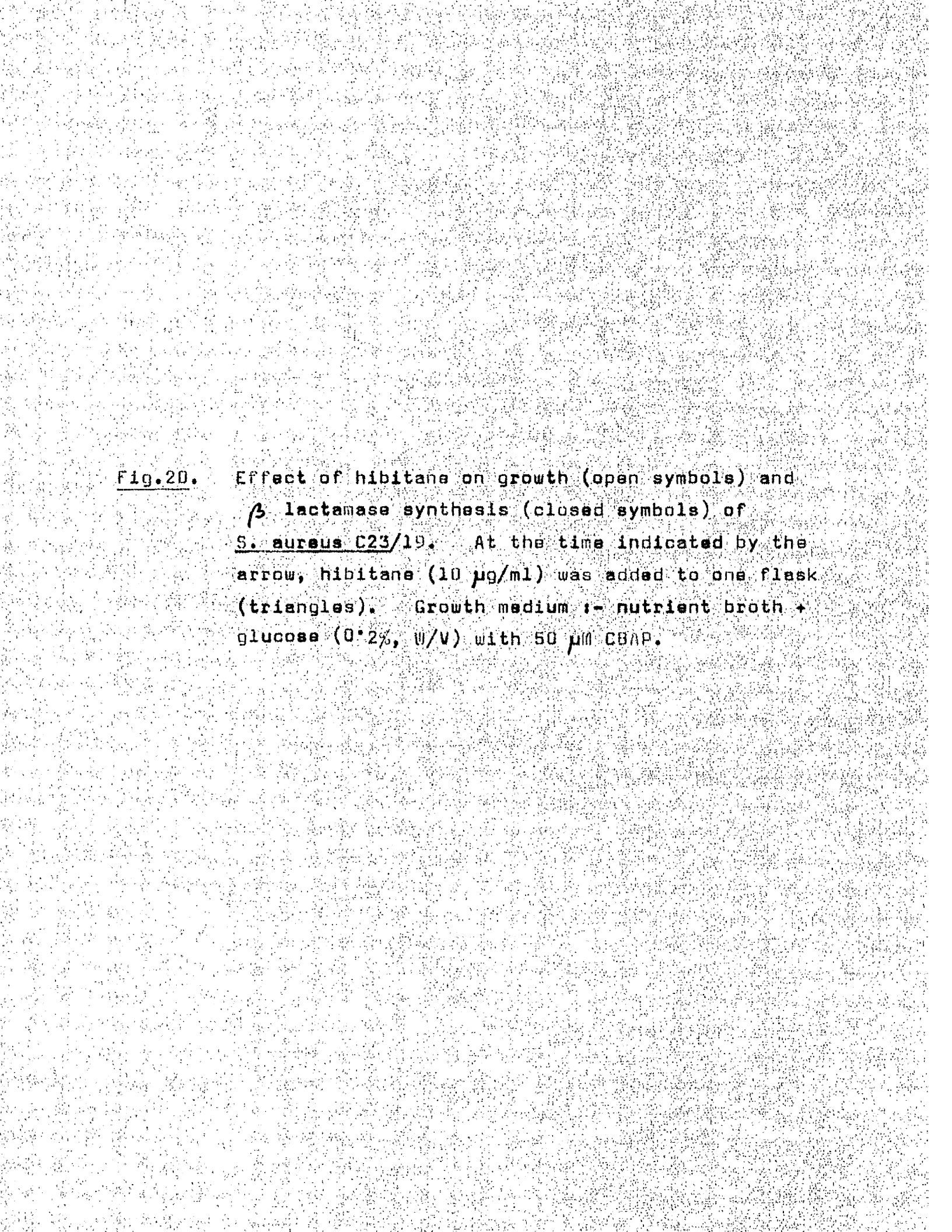


Fig.20. Effect of hibitane on growth (open symbols) and β lactamase synthesis (closed symbols) of S. aureus C23/19. At the time indicated by the arrow, hibitane (10 µg/ml) was added to one flask (triangles). Growth medium :- nutrient broth + glucose (0·2%, W/V) with 50 µM CBAP.

EFFECT OF HIBITANE ON GROWTH AND β-LACTAMASE SYNTHESIS IN S. AUREUS

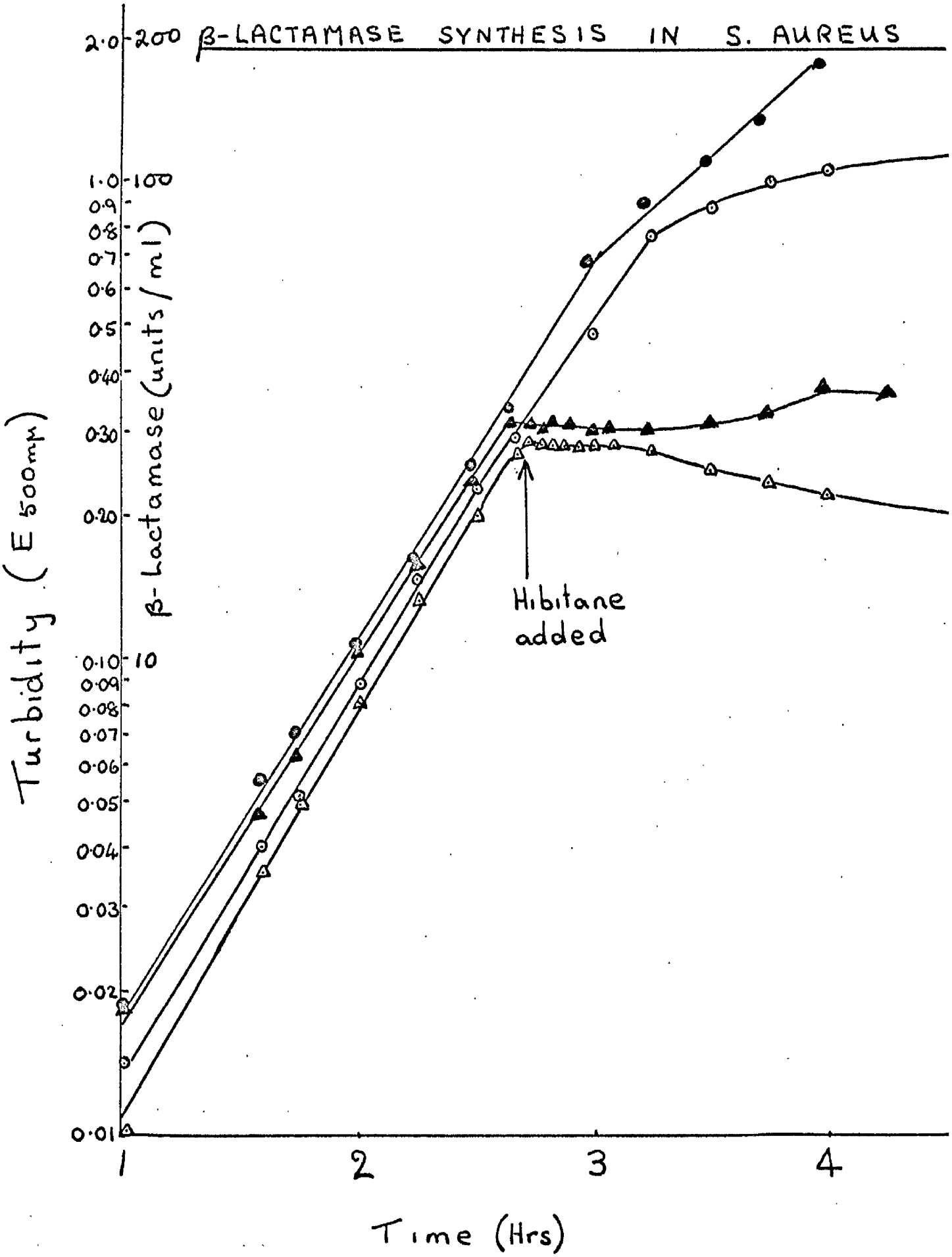


Fig 20

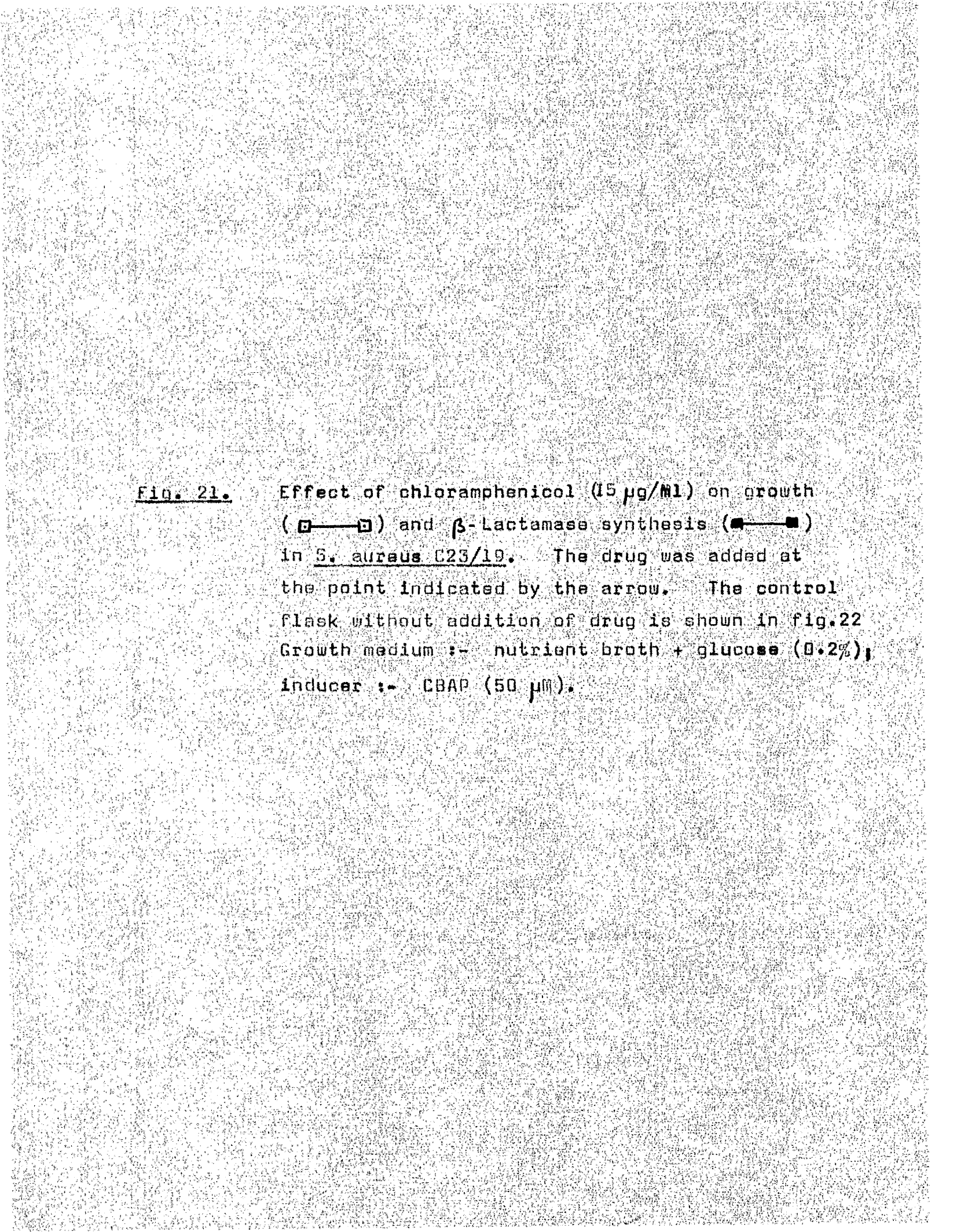


Fig. 21. Effect of chloramphenicol (15 μg/Ml) on growth (□——□) and β-Lactamase synthesis (■——■) in S. aureus C23/19. The drug was added at the point indicated by the arrow. The control flask without addition of drug is shown in fig.22 Growth medium :- nutrient broth + glucose (0.2%); inducer :- CBAP (50 μM).

EFFECT OF CHLORAMPHENICOL ON GROWTH AND β-LACTAMASE SYNTHESIS IN *S. aureus*

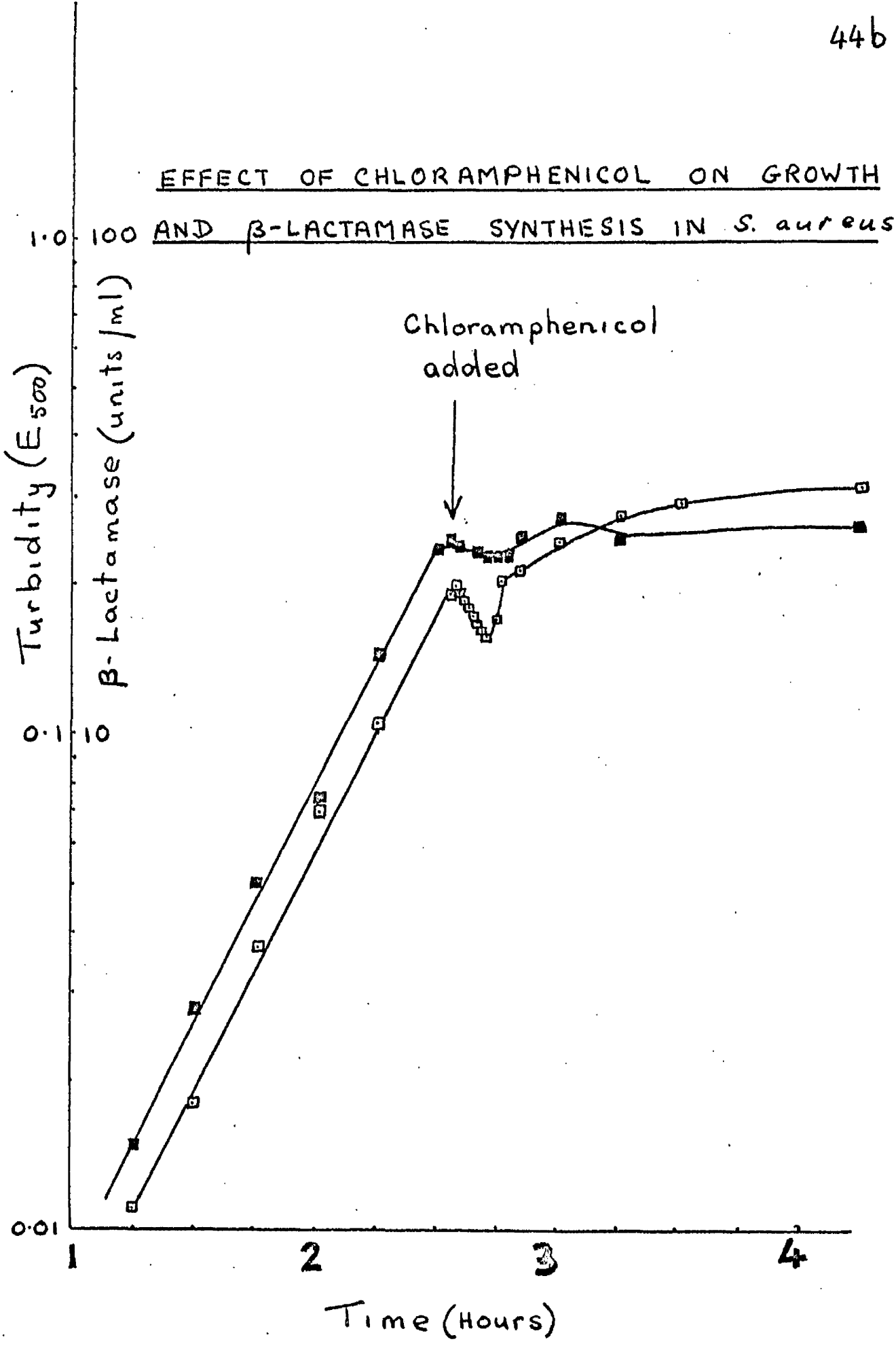


Fig 21

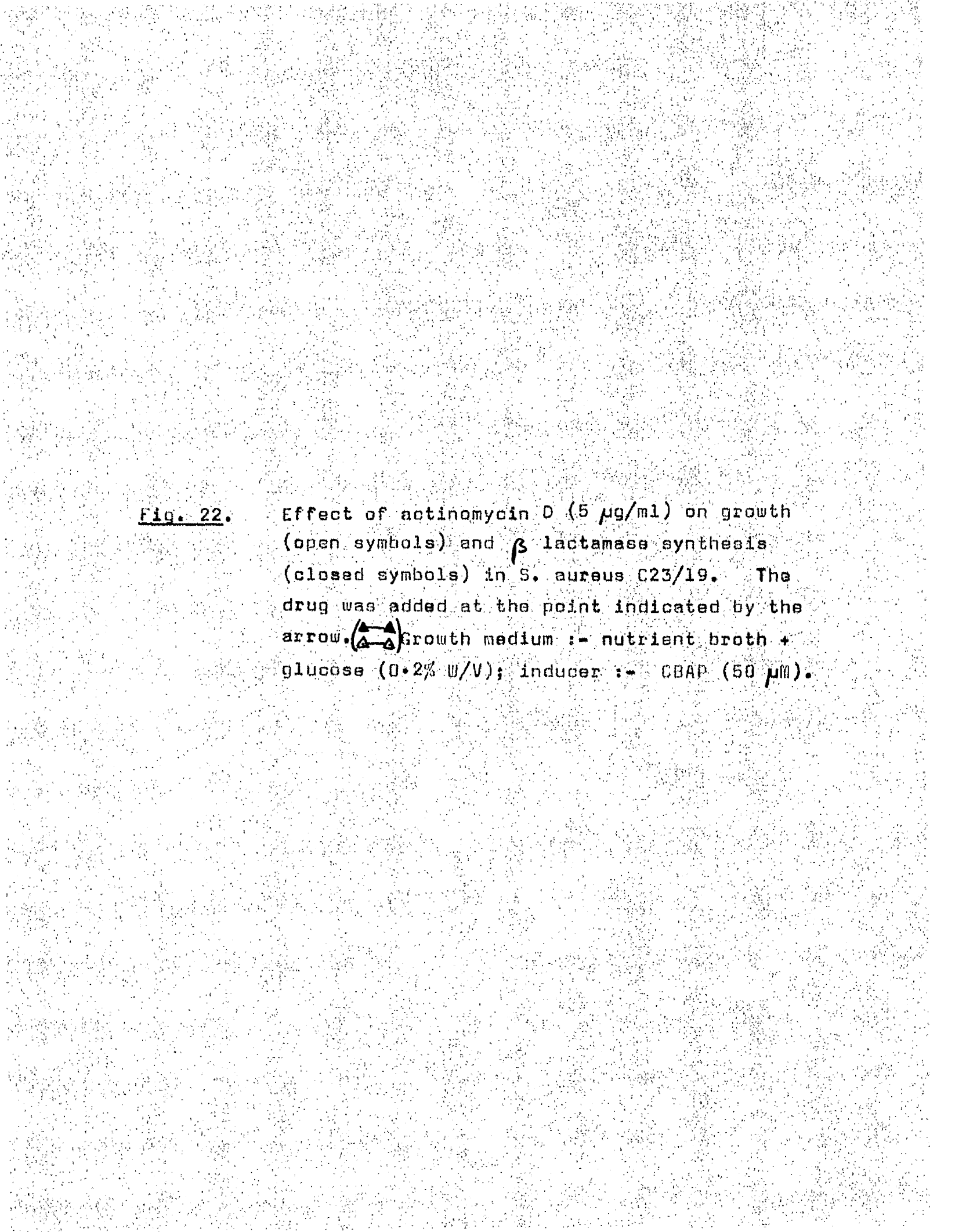


Fig. 22. Effect of actinomycin D (5 μg/ml) on growth (open symbols) and β lactamase synthesis (closed symbols) in S. aureus C23/19. The drug was added at the point indicated by the arrow. (▲—▲ ▲—▲) Growth medium :- nutrient broth + glucose (0·2% W/V); inducer :- CBAP (50 μM).

EFFECT OF ACTINOMYCIN D ON GROWTH AND β-LACTAMASE SYNTHESIS

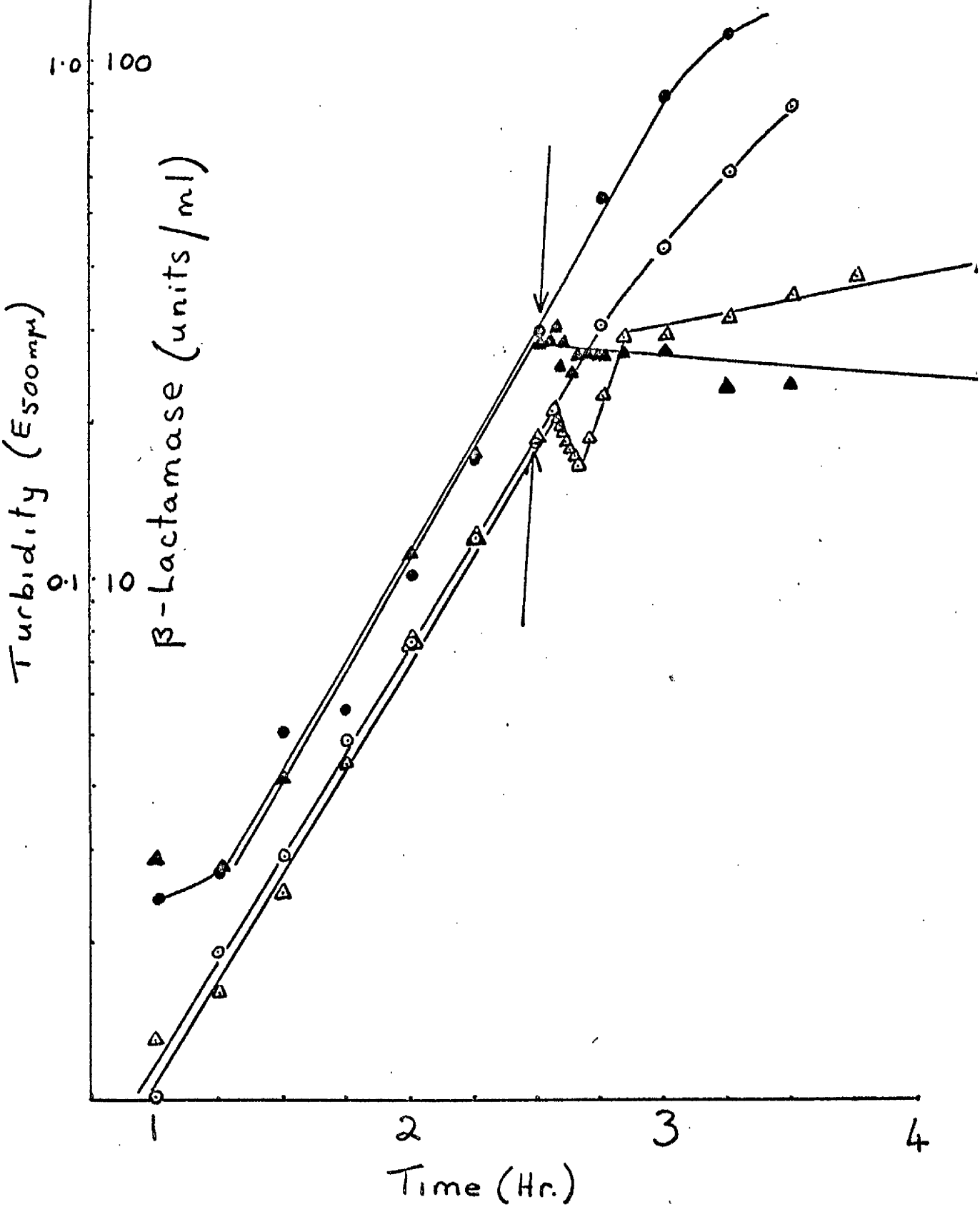
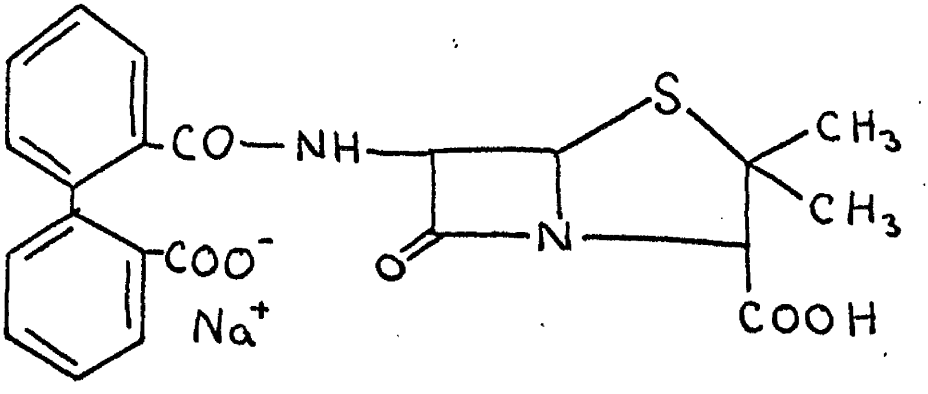


Fig 22

Fig. 23 The structure of the inducer CBAP. This compound is a gratuitous inducer of β-lactamase in *S. aureus* C23/19; it has no antibiotic activity.



2-(2'-CARBOXYPHENYL)-BENZOYL-6-AMINO-PENICILLANIC ACID.

Fig. 23

Table 10. Specific activity of β-lactamase in S.aureus C23/19 growing in nutrient broth and defined medium with different inducers.

Inducer	Specific activity (units/unit E_{350})	
	Nutrient broth	Defined medium
Methicillin	30	30
Cephalosporin C	Not tested	20
CDAP	50	50

Fig. 24. Specific activity of β-lactamase obtained with different concentrations of CBAP (●——●) and methicillin (⊙——⊙). The cultures were grown in nutrient broth + glucose (0.2% W/v)

β-Lactamase induced by different concentrations of inducer

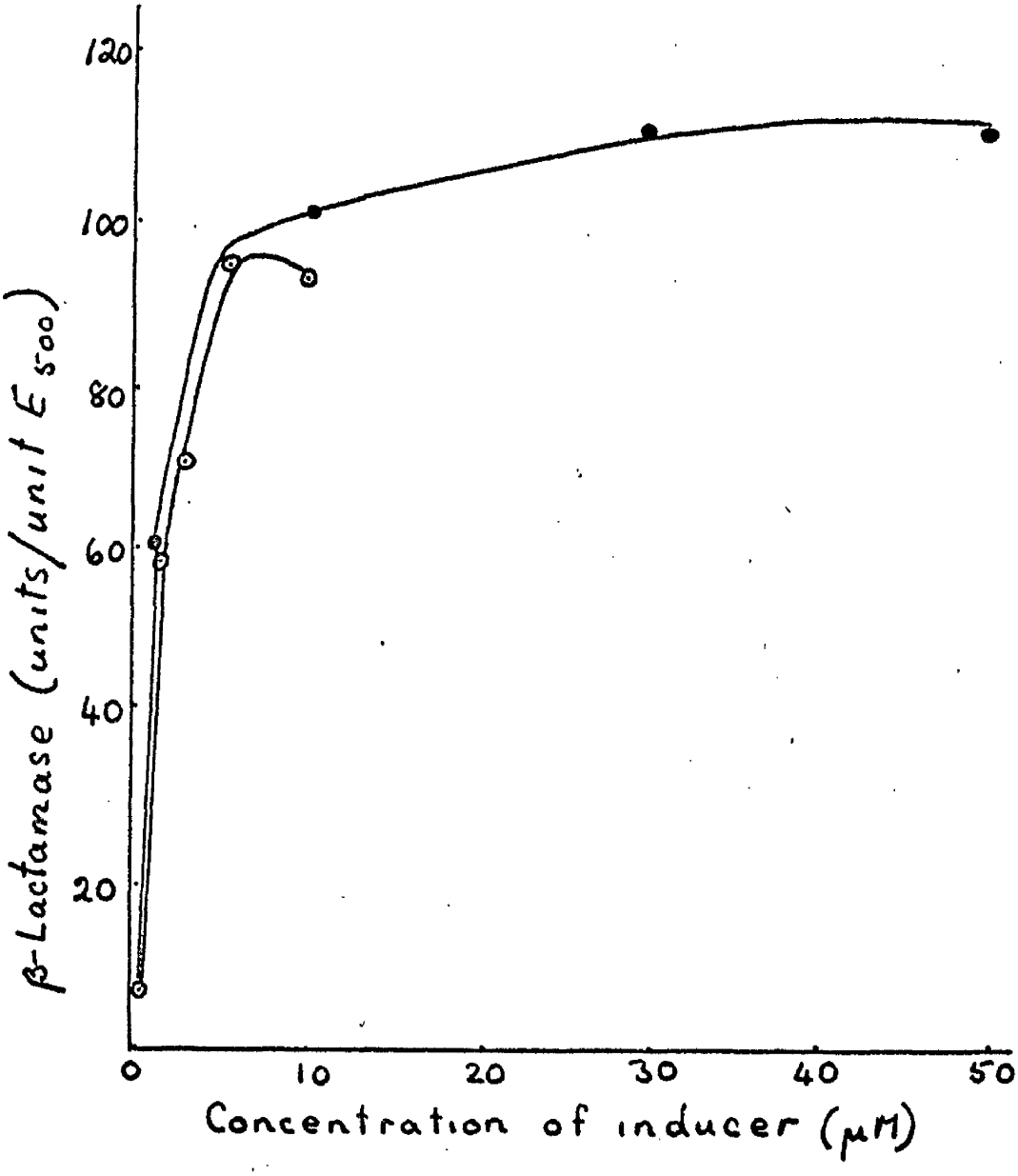


Fig 24

not stop growth even when used at very high concentrations (500 μm). It does, however, have a slight effect on growth (fig. 25 p. 45a). The lag is increased with increasing concentration of inducer and the yield is correspondingly decreased. There is no effect on the rate of growth in the logarithmic phase. The specific activity of β-lactamase remains constant during logarithmic phase in all these concentrations of CBAP and rises as the culture enters stationary phase. (fig. 26 p.45b). In some experiments, the specific activity did not remain constant during logarithmic phase but fluctuated in a fairly regular manner (see fig. 27 p. 45c and fig. 53 p.52£). From these experiments, CBAP was chosen as the most suitable inducer since at a concentration of 50 μm which saturated the induction system (fig. 24 p.44f) no gross antibiotic effects are apparent.

(b) Growth and β-lactamase synthesis under different aeration conditions.

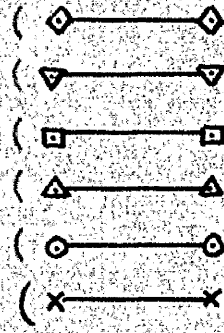
Different volumes of culture in the growth flask (200-800ml) do not alter the growth rate nor the differential rate of enzyme synthesis. This is true whether the air phase in the flask is changed or not. (Table 11 p.45d).

Table 12 (p.45e) shows the mean generation times of cultures growing with different concentrations of oxygen in the gas phase. The growth rate is reduced by a high concentration of oxygen. This is most markdd in the flask containing 400ml culture. Although the mean generation time is markedly increased, the differential rate of enzyme synthesis is the same in all cases.

Fig. 27 (p 45c) shows the specific activity of β-lactamase during aerobic and anaerobic growth in nutrient broth. The specific activity attained aerobically is four times that attained anaerobically. During growth, the specific activity rises in a series of waves reaching a maximum at the onset of stationary phase (3.5 hours).

A differential plot of the data (fig. 28 p.45d) shows that

Fig. 25. Effect of CBAP on the growth of *S. aureus* C23/19 growing in nutrient broth + glucose (0·2% W/V)



Growth of S. aureus in presence of different concentrations of CBAP

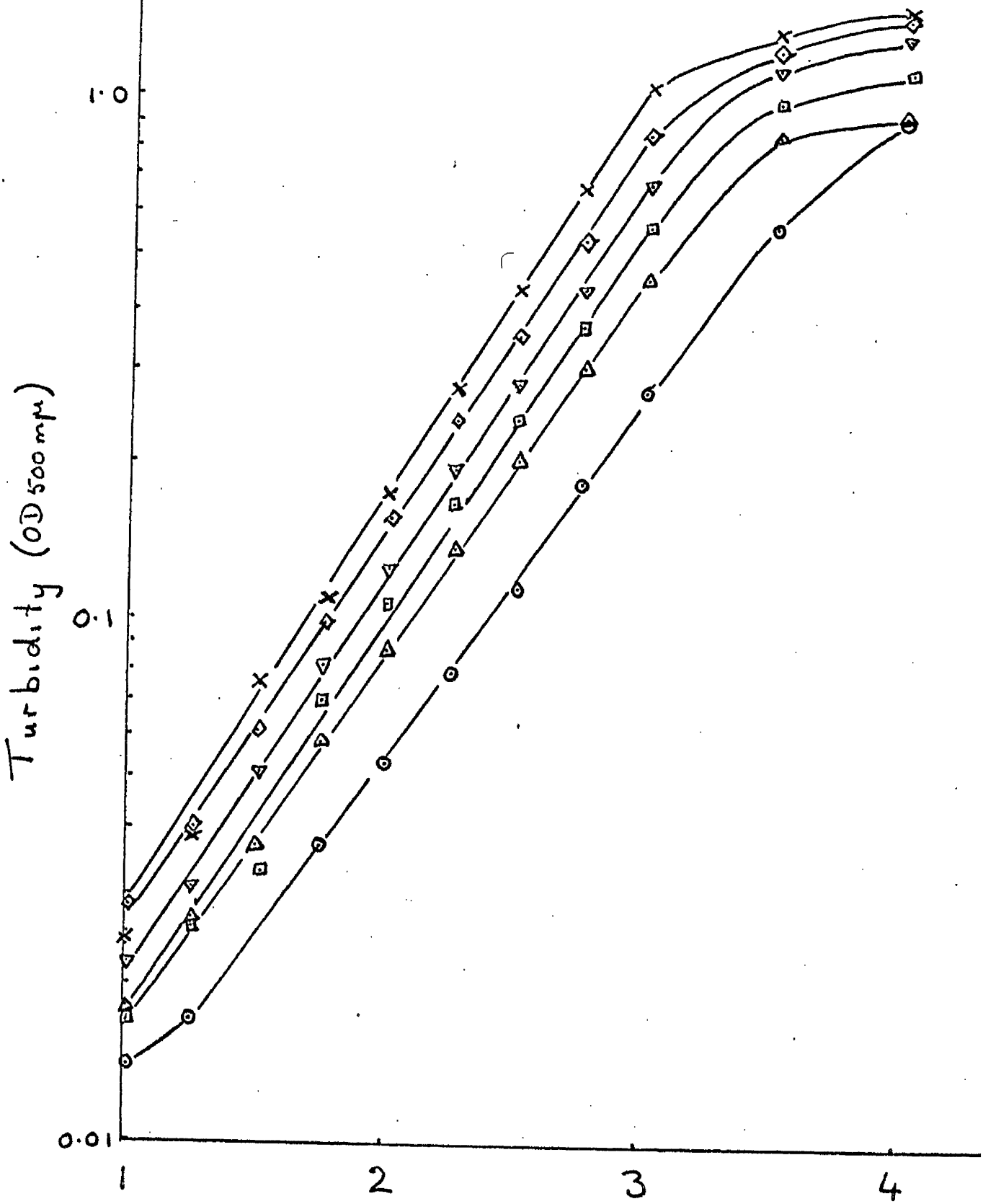
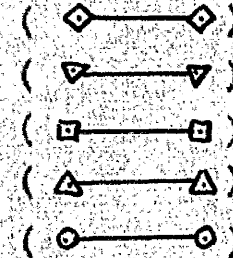


Fig 25

Fig. 26. Specific activity of β lactamase in S. aureus C23/19 during growth in nutrient broth + glucose (0·2% W/V) containing different concentrations of CBAP.



Specific activity of β-Lactamase in S. aureus growing in the presence of different concentrations of CBAP

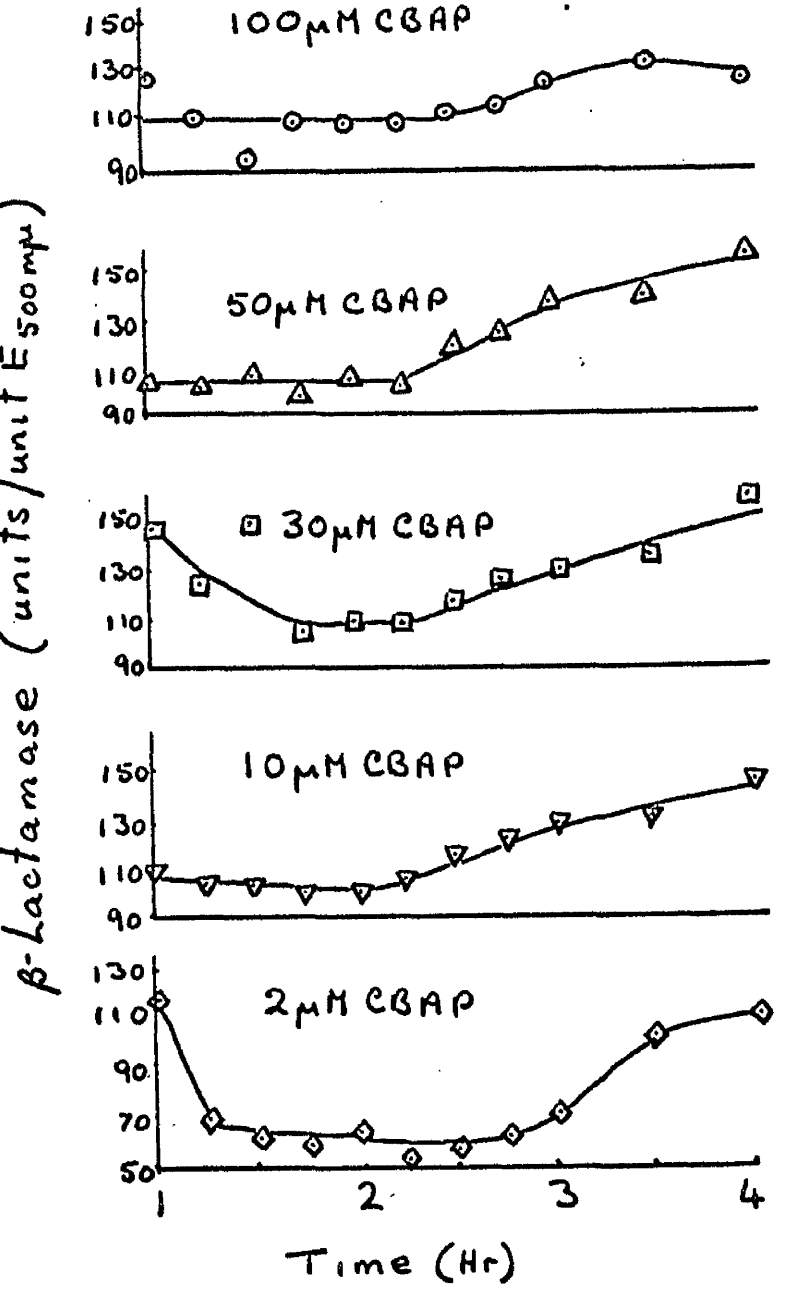


Fig 26

Fig. 27. The Specific activity of β-lactamase in S. aureus C23/19 growing in nutrient broth+ glucose (0.2%) containing COAP (50 μM)

Aerobic

Anaerobic

Anaerobic

Specific activity of β-lactamase in S. aureus growing aerobically and anaerobically in nutrient broth

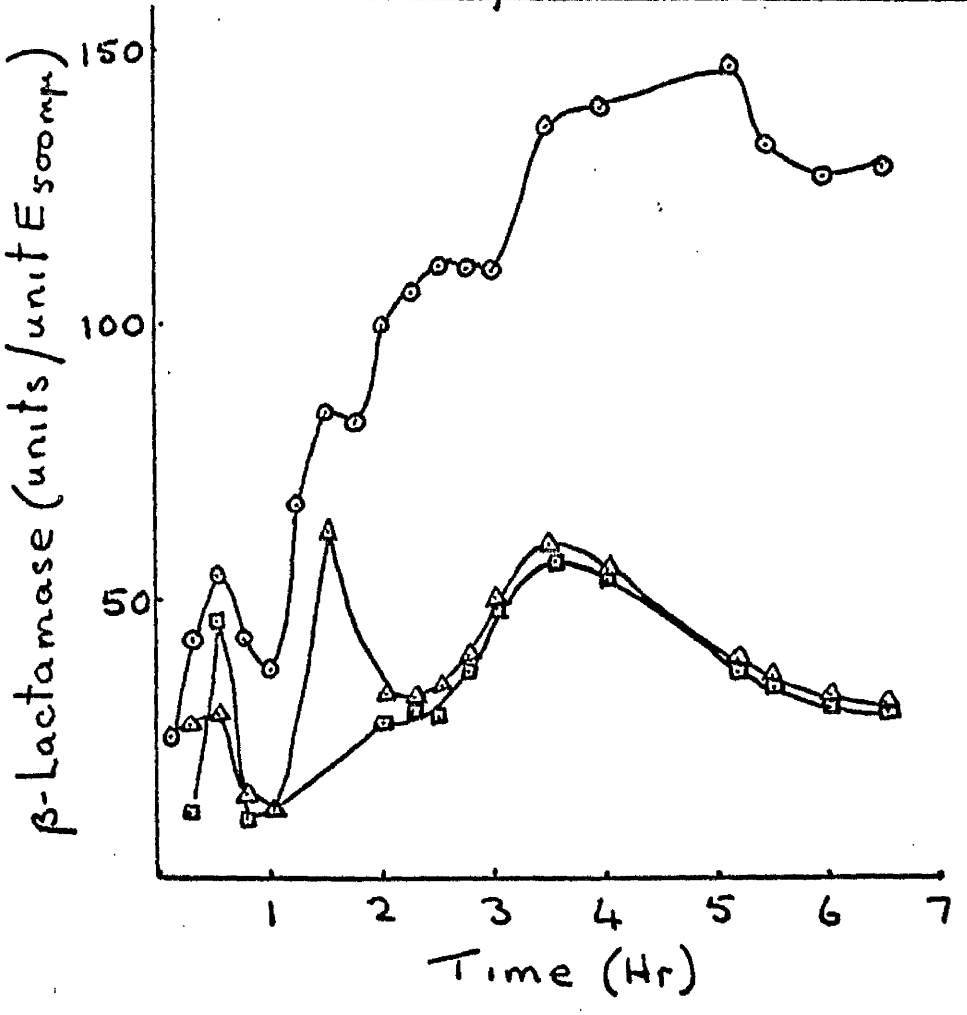


Fig 27

Fig. 20. Differential rate of β-lactamase synthesis in the cultures of fig.27. The dotted line is a differential rate of 72 units/unit E_{500}

(⊙——⊙) aerobic

(▲——▲) Anaerobic

Inset is the anaerobic culture on a different scale. The differential rate during aerobic growth is 100 changing to 200 at an optical density of 1.0. During anaerobic growth the differential rate is 72.

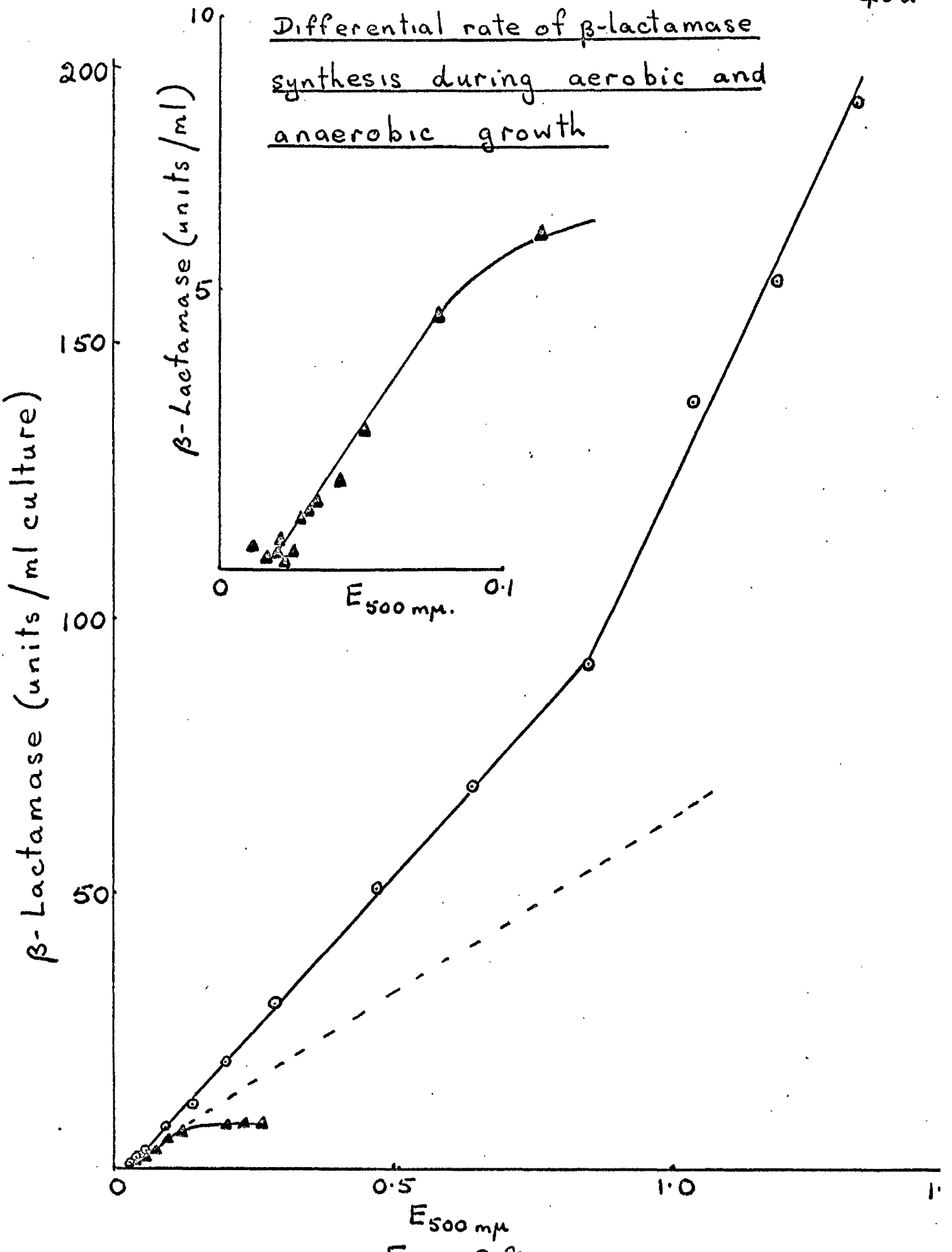

Differential rate of β-lactamase synthesis during aerobic and anaerobic growth
β-Lactamase (units/ml culture)
200
150
100
50
0
0·5
1·0
E 500 mμ
β-Lactamase (units/ml)
10
5
0
0·1
E 500 mμ.

Fig 28

Table 11. Effect of different volumes of culture in the growth flask on growth rate and differential rate of β- lactamase synthesis in S.aureus C23/19 growing in defined medium.

Culture Volume (ml)	Growth m.g.t (min)		Differential rate of lactamase synthesis units/unit E_{350}	
	Air phase change		Air phase change	
	250 ml/min	Nil	250 ml/min	Nil
800	75	69	122	130
600	60	75	91	114
400	70	75	112	122
200	78	85	110	106

Table 12. Effect of partial pressure of oxygen on growth and β-lactamase synthesis in S.aureus C23/19 growing in nutrient broth.

Gas phase		Growth m.g.t.(min)		β-lactamase differential rate	
% oxygen	rate of change	800 ml culture	400 ml culture	800 ml culture	400 ml culture
50	300 ml/min	96	190	94	102
30	" " "	74	63	94	109
17	" " "	78	69	102	96
Air	" " "	78	72	108	112
air	Nil	82	84	105	110

the rate of enzyme synthesis is lower in the culture growing anaerobically. However this lower differential rate is not the only reason for the final specific activity being four times as great in the culture growing aerobically. The β-lactamase synthesis stops before growth stops in the anaerobic culture. Thus the specific activity of β-lactamase drops at the end of the experiment. Note that, in the culture growing aerobically, the differential rate increases as the culture enters stationary phase.

Fig. 29 (p.46a) shows the specific activity of β-lactamase during growth of S. aureus in defined medium and the effect of nitrogen for short periods of time. The specific activity increases throughout growth in a stepwise manner. These steps do not correspond to generations. Nitrogen passed into the flasks for 2 mins, 4 mins and 8 mins had no effect on the specific activity. Nitrogen passed into the flask for 16 mins causes a slight drop in specific activity which is subsequently corrected during the later phases of growth. This drop in specific activity was the result of growth continuing while enzyme synthesis stopped.

(c) Effect of temperature.

Fig. 30 (p.46b) shows the doubling time of turbidity and β-lactamase in defined medium as a function of temperature. The lower the temperature the greater is the doubling time. Arrhenius plots give straight lines from which one can derive an activation energy of 17,000 cals. Analagous results were obtained with growth in nutrient broth. A combination of medium and temperature allowed consideration of doubling times varying from 30 mins to 190 mins. Over the range 30-150 mins, the doubling time of enzyme is directly related to the mean generation time (fig. 31 p.46c). Comparison of the doubling times of enzyme and turbidity, suggests that the rate of enzyme synthesis is much faster than the rate of synthesis of turbidity at 27°C in

Fig. 29. Specific activity of β-lactamase during growth of S. aureus in defined medium containing CDAP(50 nM) At the points indicated by the arrows, oxygen free nitrogen was passed into the flask for 2 mins.(△—△) 4 mins (□—□), 8 mins (●—●) and 16 mins.(▲—▲) One flask (⊙—⊙) had no nitrogen passed in.

Effect of short periods of nitrogen on specific activity of β-lactamase

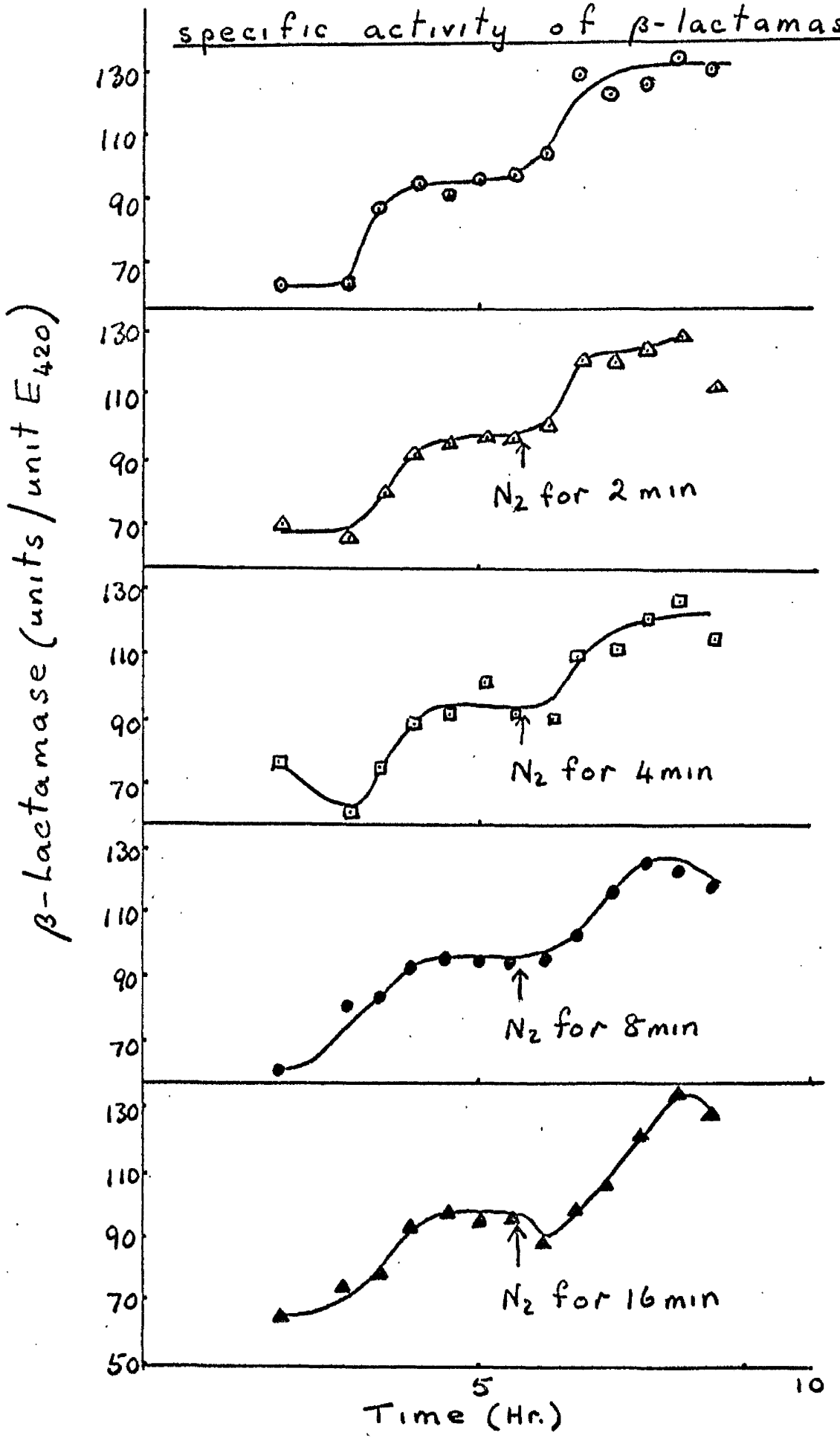


Fig. 29

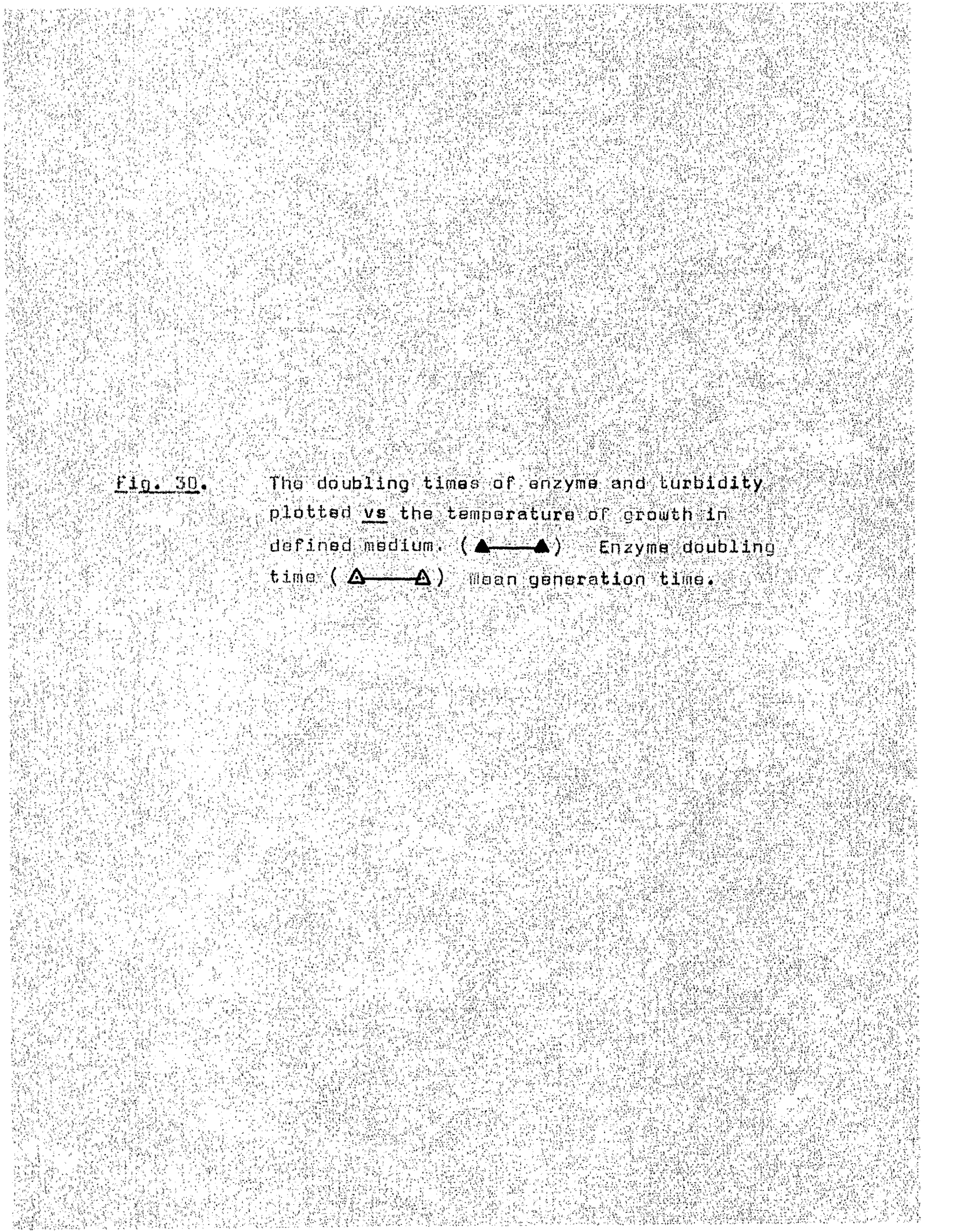


Fig. 30. The doubling times of enzyme and turbidity plotted *vs* the temperature of growth in defined medium. (▲——▲) Enzyme doubling time (△——△) Mean generation time.

Variation of doubling time of turbidity and β-lactamase with temperature during growth

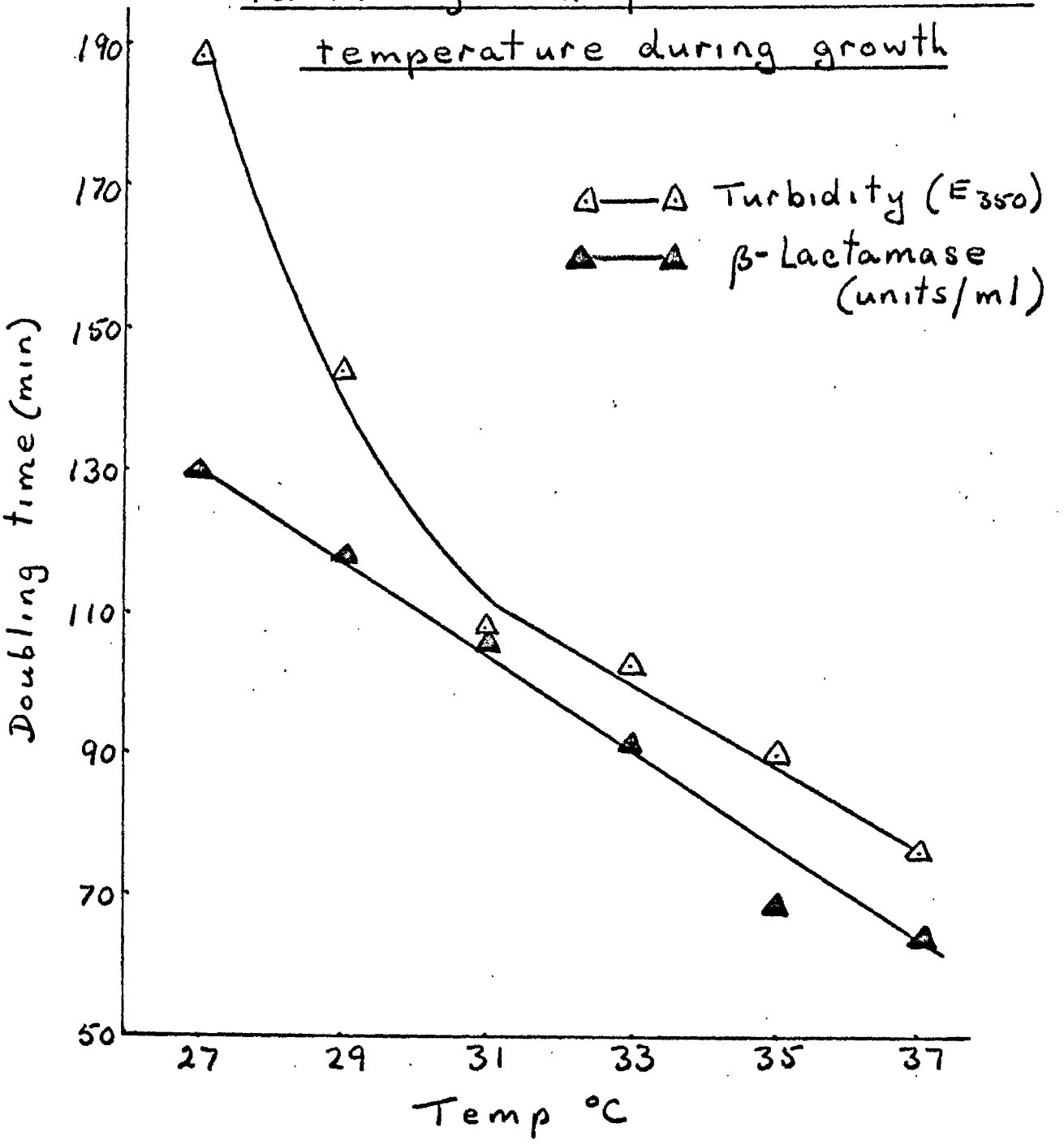


Fig. 30

Fig. 31. The doubling time of enzyme is plotted *vs* the doubling time of turbidity. The dotted line is the expected line if enzyme and turbidity double atthe same rate. The mean generation times were varied by growing *S. aureus* C23/19 in defined medium and nutrient broth at different temperatures. In nutrient broth the mean generation time varied from 30 mins to 70 mins. In defined medium the mean generation times varied from 80 to 190 mins. The inducer was CBAP (50 μM).

Relationship between doubling time of turbidity and doubling time of β-lactamase

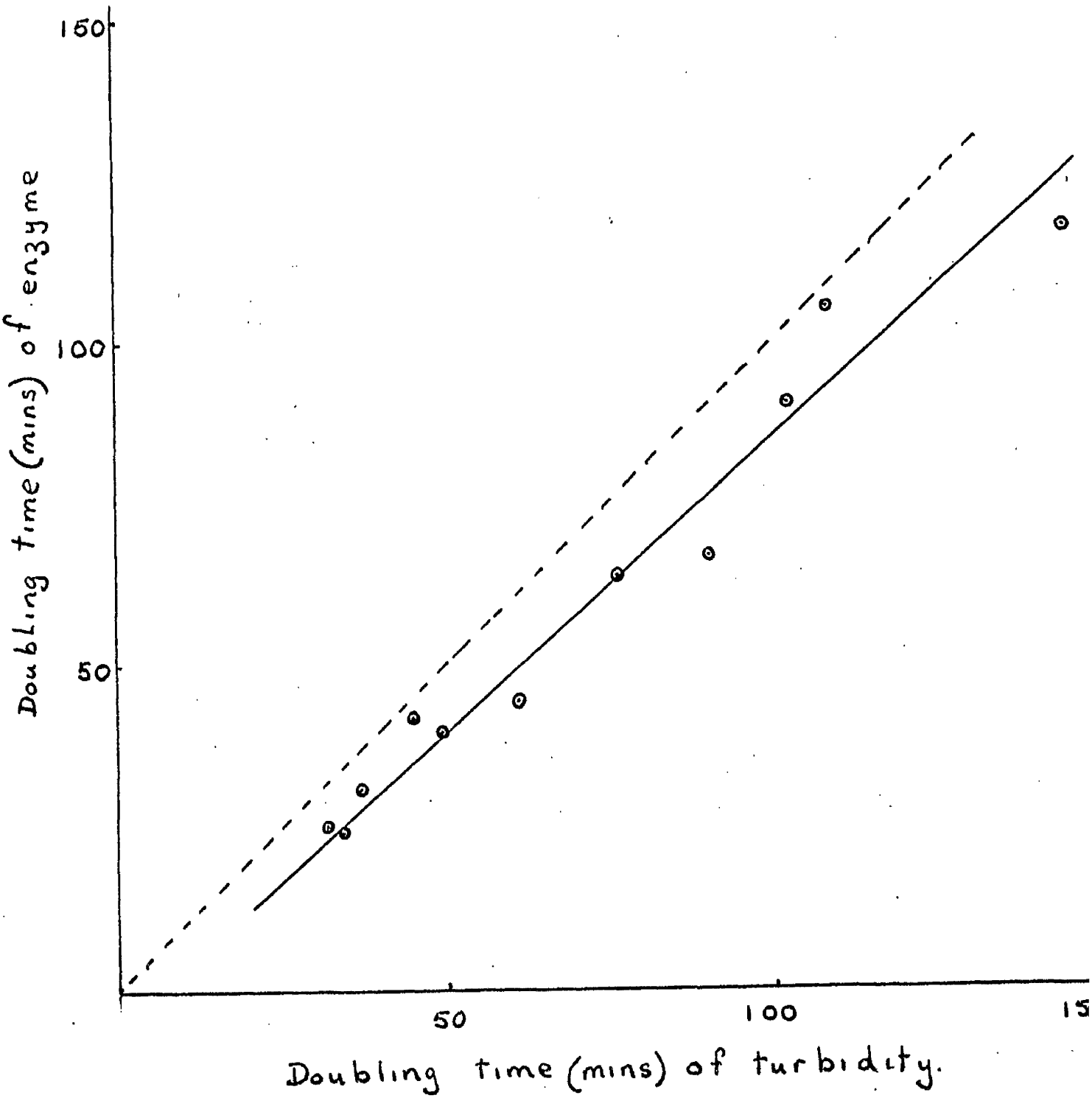


Fig 31

defined medium (fig. 30 p.46b). However if we consider the differential rate of enzyme synthesis, obtained by plotting β-lactamase vs turbidity, for the different temperatures in both media (table 13 p.47a), it is immediately apparent that the differential rate of enzyme synthesis is not altered by temperature. This is true even at 27°C in defined medium where the doubling times of enzyme and turbidity are so different. It is concluded that the mean generation time of the organism has no effect on β-lactamase synthesis. The turbidity was measured at 500 mμ for nutrient broth and 350 mμ for defined medium. The turbidity at 350 mμ is 1.9 times greater than that at 500 mμ. When the differential rates in nutrient broth are corrected for turbidity at 350 mμ, we see that there is little difference in the differential rate of β-lactamase synthesis in the two media.

(d) Discontinuous synthesis of β-lactamase.

Fig. 32 (p.47a) shows the logaritha of β-lactamase, turbidity and protein vs time for a culture growing in defined medium. In this experiment β-lactamase synthesis is discontinuous and this synchronous behaviour is also seen in the turbidity. Total protein also shows evidence of stepwise increase but the increments are not in phase with those of β-lactamase or turbidity.

Fig. 33 (p.47b) shows a culture in which β-lactamase synthesis was synchronised to a considerable extent during growth in defined medium. Attempts were made to reproduce discontinuity of growth and enzyme synthesis but none of the methods used were successful in reproducibly inducing synchrony. Best results were obtained when the inoculum was allowed to grow well into the stationary phase repeatedly. It seemed at first that passage from a fast growing medium to a slow growing medium aided in producing synchrony but this could not be definitely established.

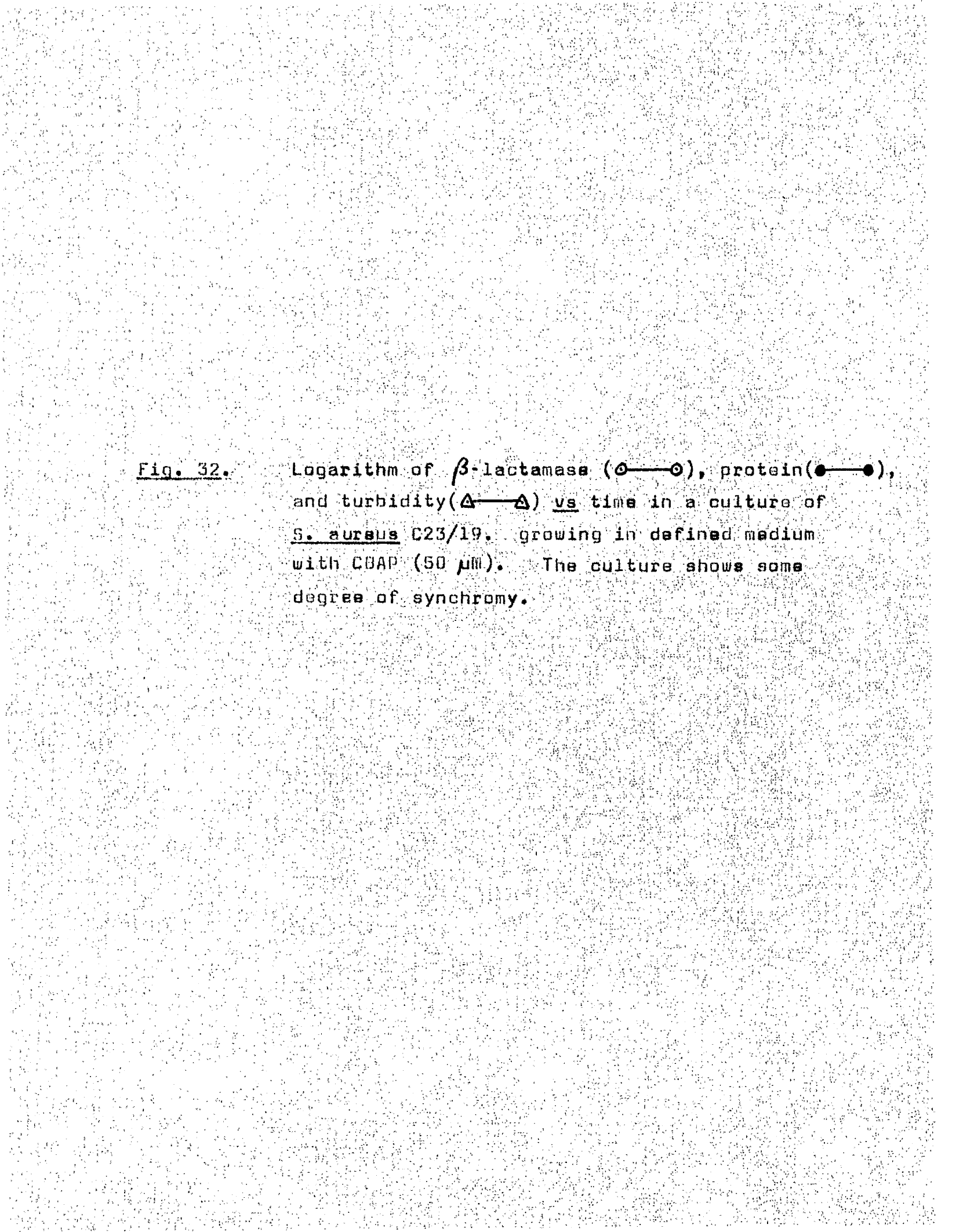


Fig. 32. Logarithm of β-lactamase (⊙——⊙), protein(●——●), and turbidity(△——△) vs time in a culture of S. aureus C23/19, growing in defined medium with CDAP (50 μM). The culture shows some degree of synchromy.

Discontinuous synthesis of β-lactamase during growth in defined medium

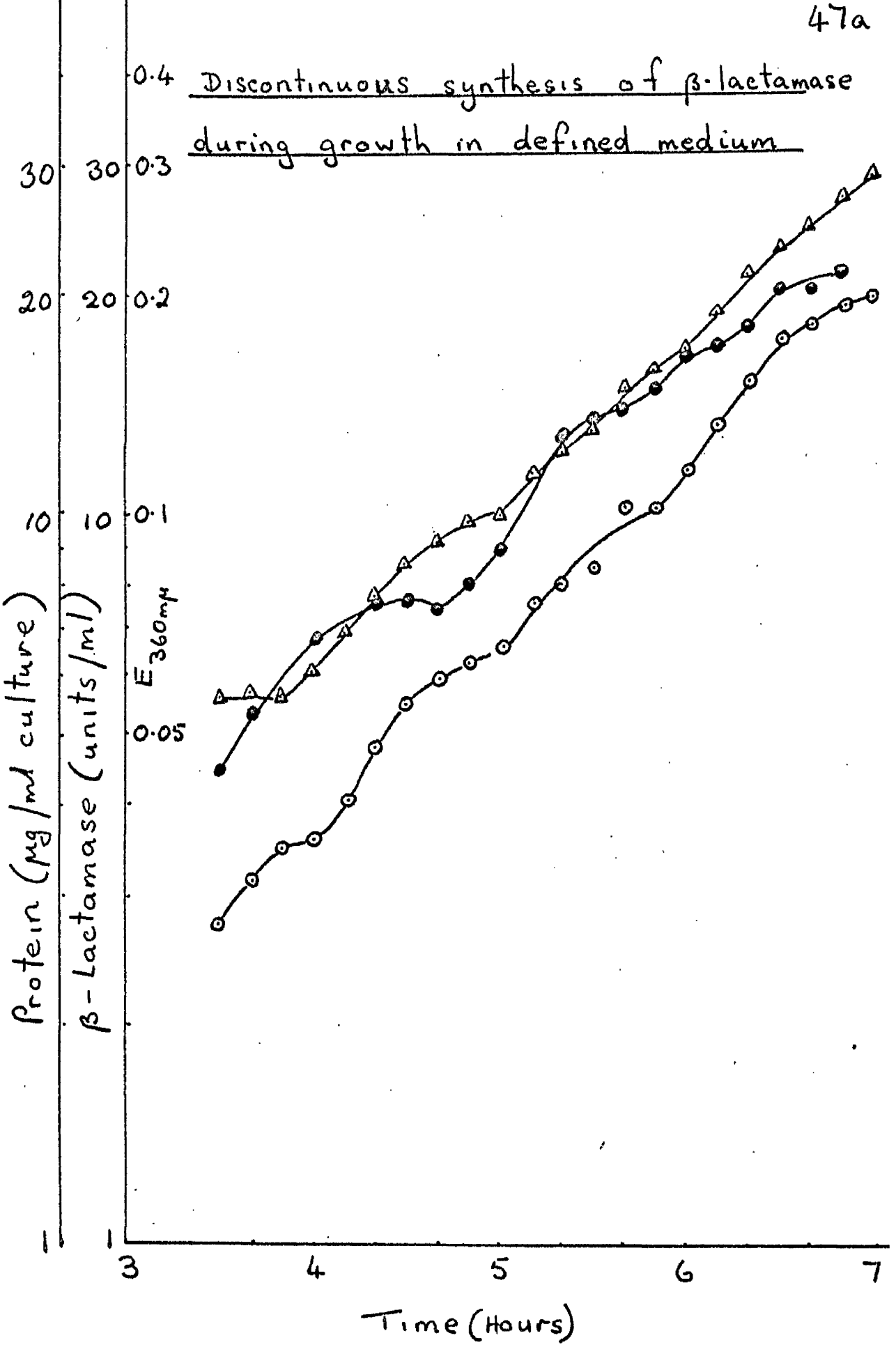


Fig 32

Fig. 33. Logarithm of β-lactamase and turbidity *vs* time, in a culture of *S.aureus* C23/19, growing indefined medium with 50 μM CDAP. This culture shows a considerable degree of synchrony in enzyme production but little or none in turbidity.

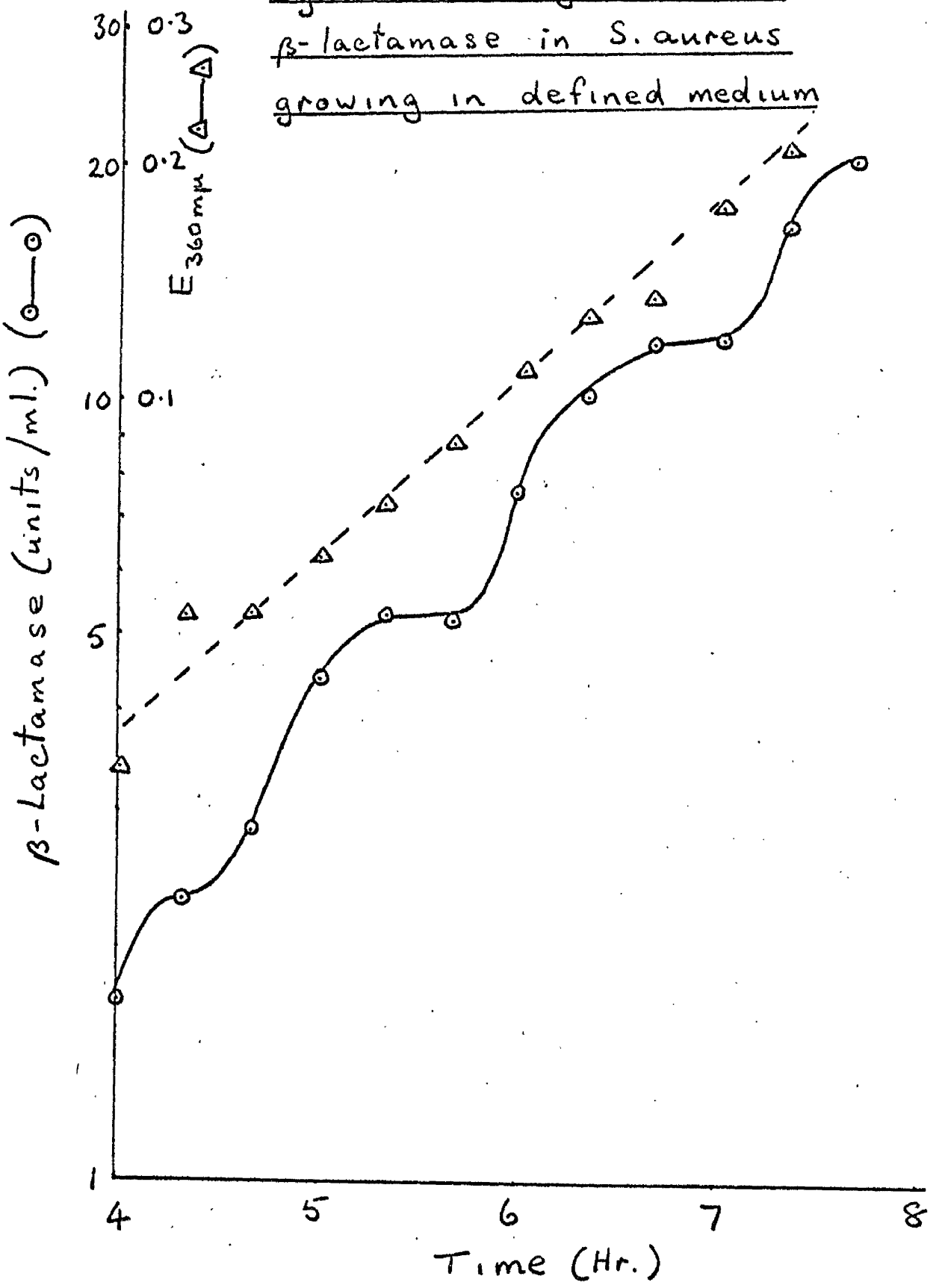

Synchronous synthesis of
β-lactamase in S. aureus
growing in defined medium
β-Lactamase (units/ml.) (o—o)
E360mμ (Δ—Δ)
30
20
10
5
1
0·3
0·2
0·1
4
5
6
7
8
Time (Hr.)

Fig. 33.

Table 13. Differential rate of β-lactamase synthesis in S.aureus C23/19 growing at different temperatures in defined medium and nutrient broth.

Temperature	Nutrient broth		defined medium
	units/E_{500}	units/E_{350}	units/E_{350}
27°C	122	64	77
29°C	120	63	82
31°C	120	63	78
33°C	138	73	80
35°C	122	64	80
37°C	120	63	80

The turbidity of the cultures growing in nutrient broth was measured at 500 mu but the differential rates are shown as both units/E_{500} and units/E_{350}. The values at E_{350} were obtained by dividing those at E_{500} by 1.9 since the turbidity at 350 mμ is greater than that at 500 mμ by this factor.

(c) Stability of capacity to form β-lactamase.

No loss of capacity to synthesise β-lactamase was detected in colonies grown on nutrient agar containing CBAP, when tested by the method of Novick and Richmond (1965).

(VI) Induction of β-lactamase.

(a) Effect of different sizes of inocula in defined medium.

Increased size of inoculum in defined medium results in decreased rate of growth. This effect is not the result of toxic products carried over in the medium since a small inoculum with a large amount of spent medium gives the rate of growth expected from the size of inoculum. Fig. 34 (p.48a) shows growth and β-lactamase in four cultures inoculated with different sizes of inoculum, 2%, 5%, 10% and 20% of fully grown culture. The enzyme shows an initial rise which last for approximately the same time in each flask although the total enzyme at the end of this time is greater the greater the inoculum. After this initial rise the enzyme doubling rate is close to the doubling rate of turbidity in each case. In this experiment, as in all the others so far described, inducer was added at the same time as the flasks were inoculated. It seemed that this initial rise in enzyme was the process of induction of the enzyme. This rise is not so noticeable in thefflask inoculated with 2% of fully grown culture.

(b) Induction of β-lactamase at different stages of growth.

Addition of inducer at different stages of growth of S. aureus in defined medium (fig. 35 p.48b) and in nutrient broth (fig. 36 p.48c) causes an initial very fast rise in enzyme level. Thereafter the enzyme doubles at a rate which is approximately the same as the doubling rate of turbidity. The enzyme rises just as quickly initially when inducer is added in stationary phase as when it is added in logarithmic phase. The term stationary phase is not strictly accurate here since the culture was still growing slowly with a doubling time of about 3 hours in nutrient broth and about 8 hours in defined medium. Nevertheless, the

Fig. 34. Growth (on the left) and β-lactamase (on the right) during growth of S. aureus C23/19. in defined medium from different sizes of inoculum. The inducer (CBAP, 50 μM) was added at time of inoculation.

(o——o) 2% of fully grown culture as inoculum.
(▽——▽) 5% " " " " " "
(△——△) 10% " " " " " "
(□——□) 20% " " " " " "

Synthesis of β-lactamase in S. aureus growing in defined medium from different sizes of inoculum.

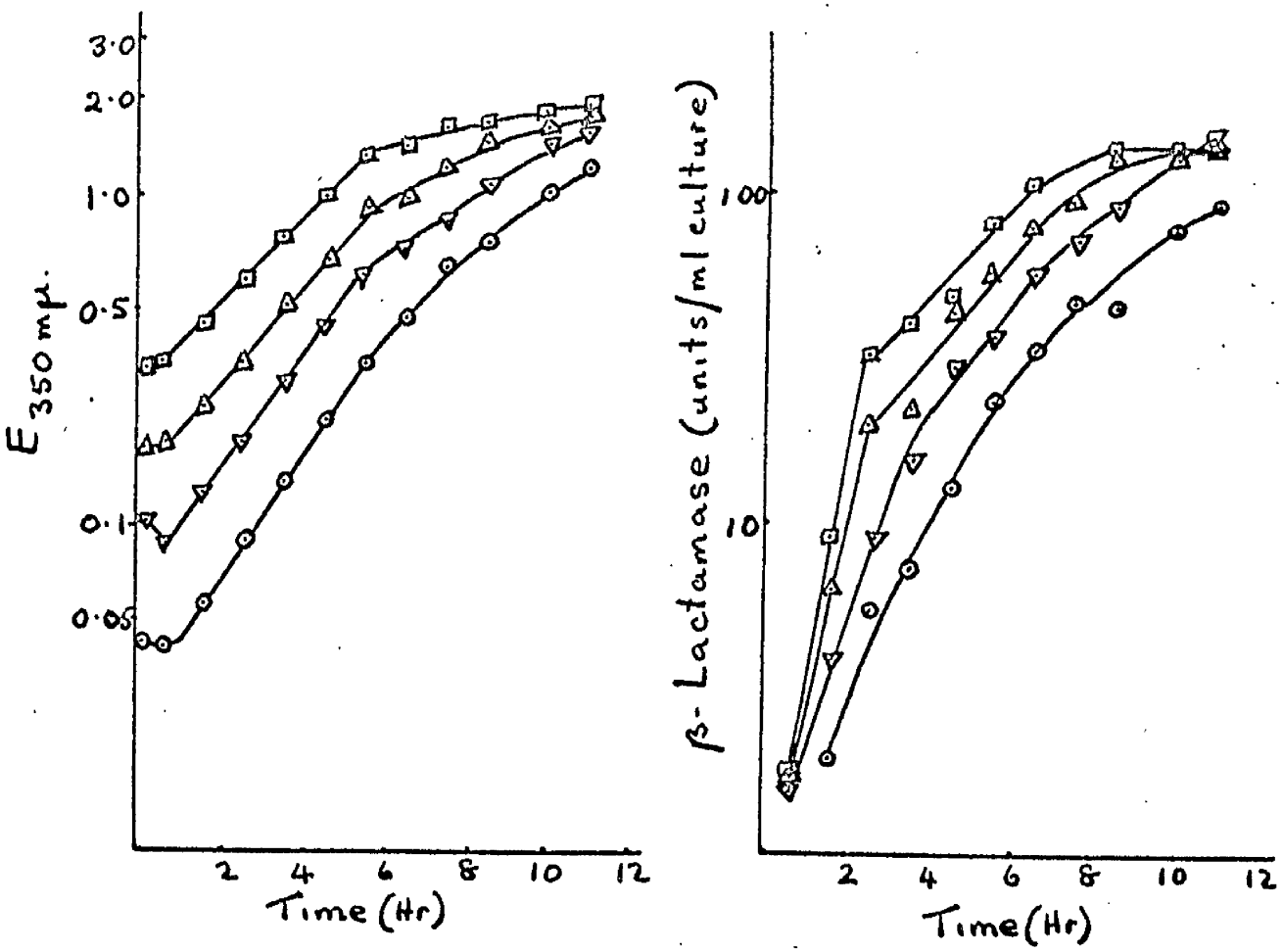


Fig 34

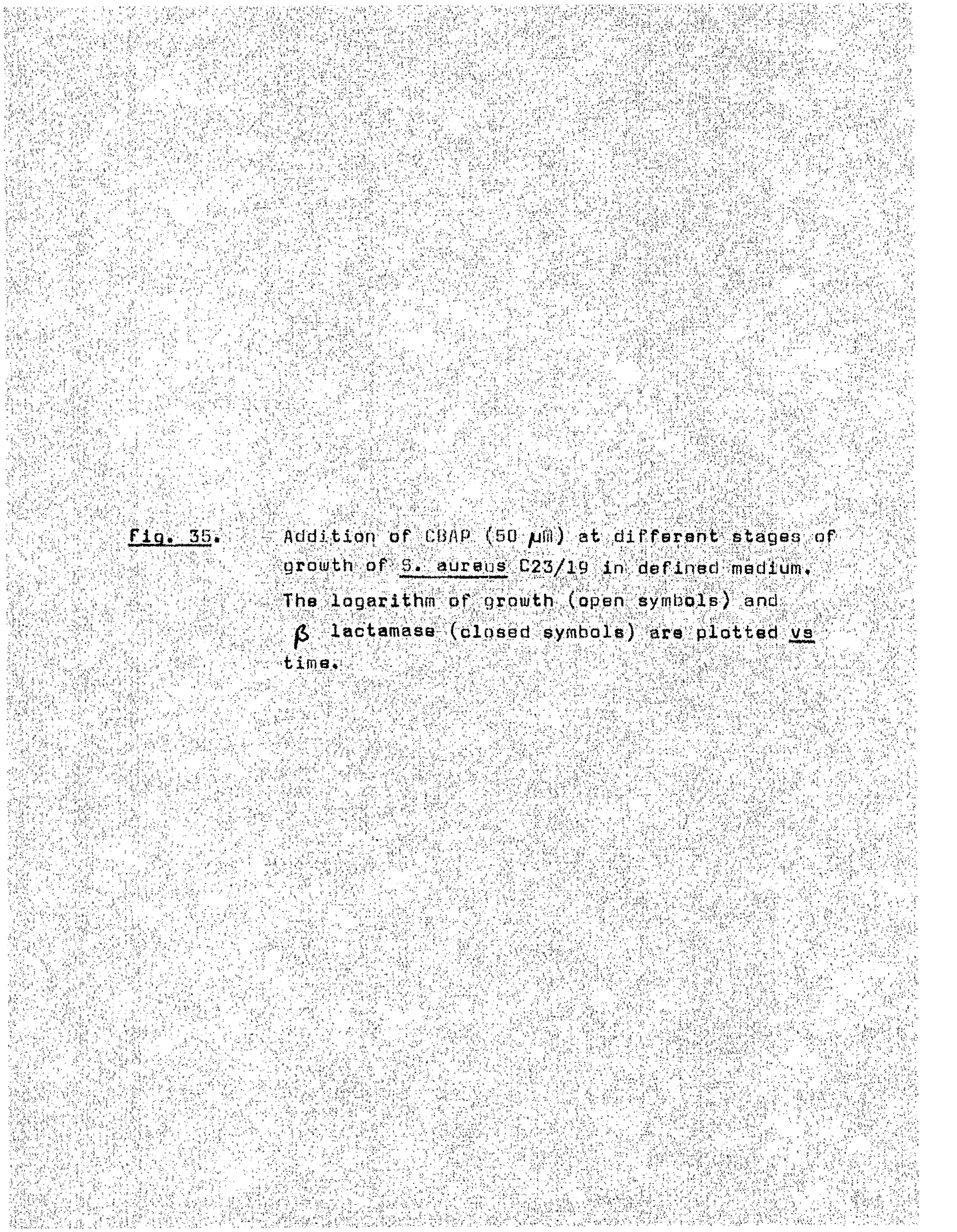


Fig. 35. Addition of CBAP (50 μM) at different stages of growth of S. aureus C23/19 in defined medium. The logarithm of growth (open symbols) and β lactamase (closed symbols) are plotted vs time.

Addition of inducer at different stages of growth of S. aureus in defined medium

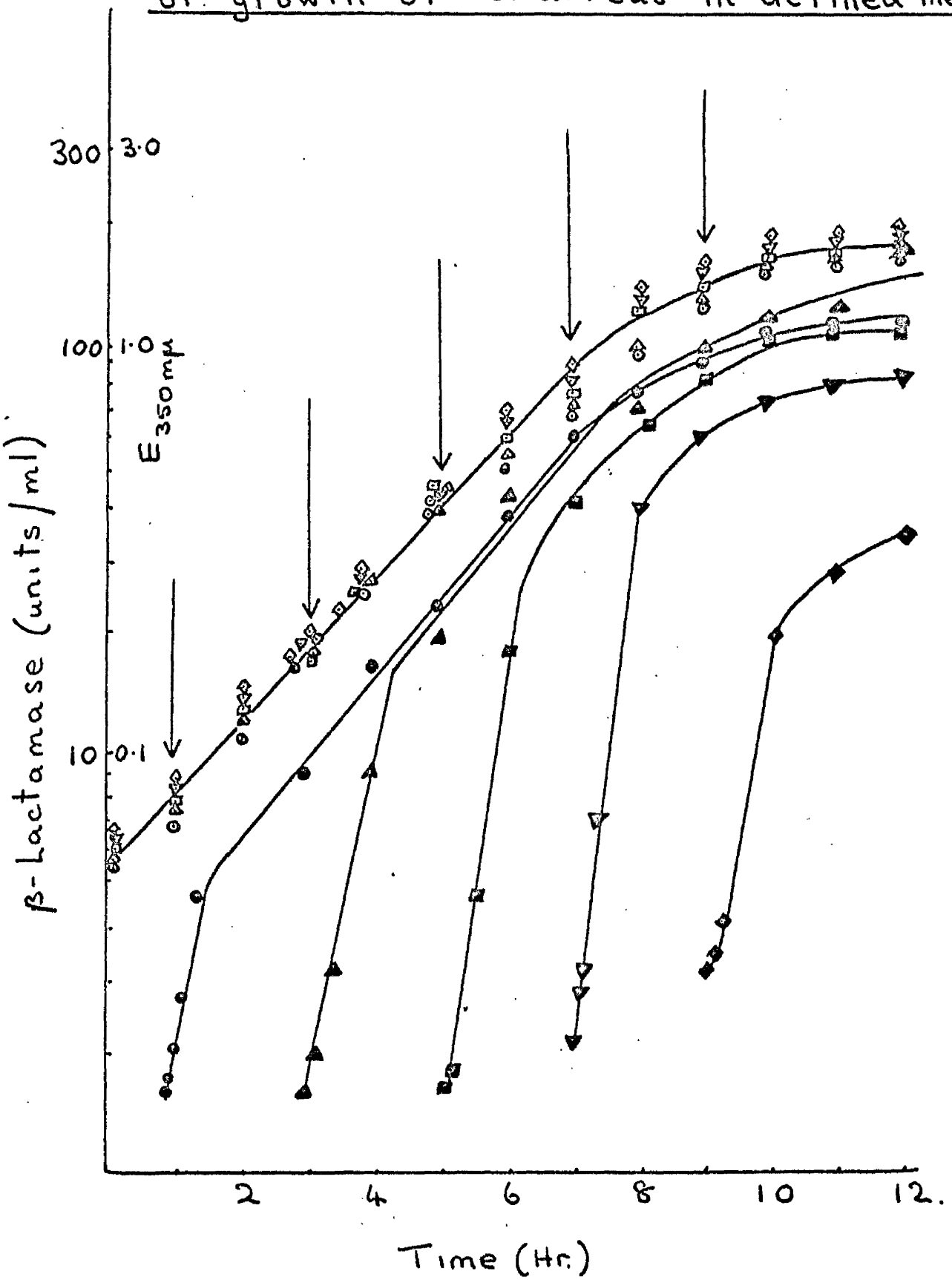


Fig 35

Fig. 36. Addition of CBAP (50 μM) at different stages of growth of S. aureus C23/19 in nutrient broth + glucose (0·2% W/V). The logarithm of growth (open symbols) and β-lactamase (closed symbols) are plotted vs time.

Addition of inducer at different stages of growth of S. aureus in nutrient broth

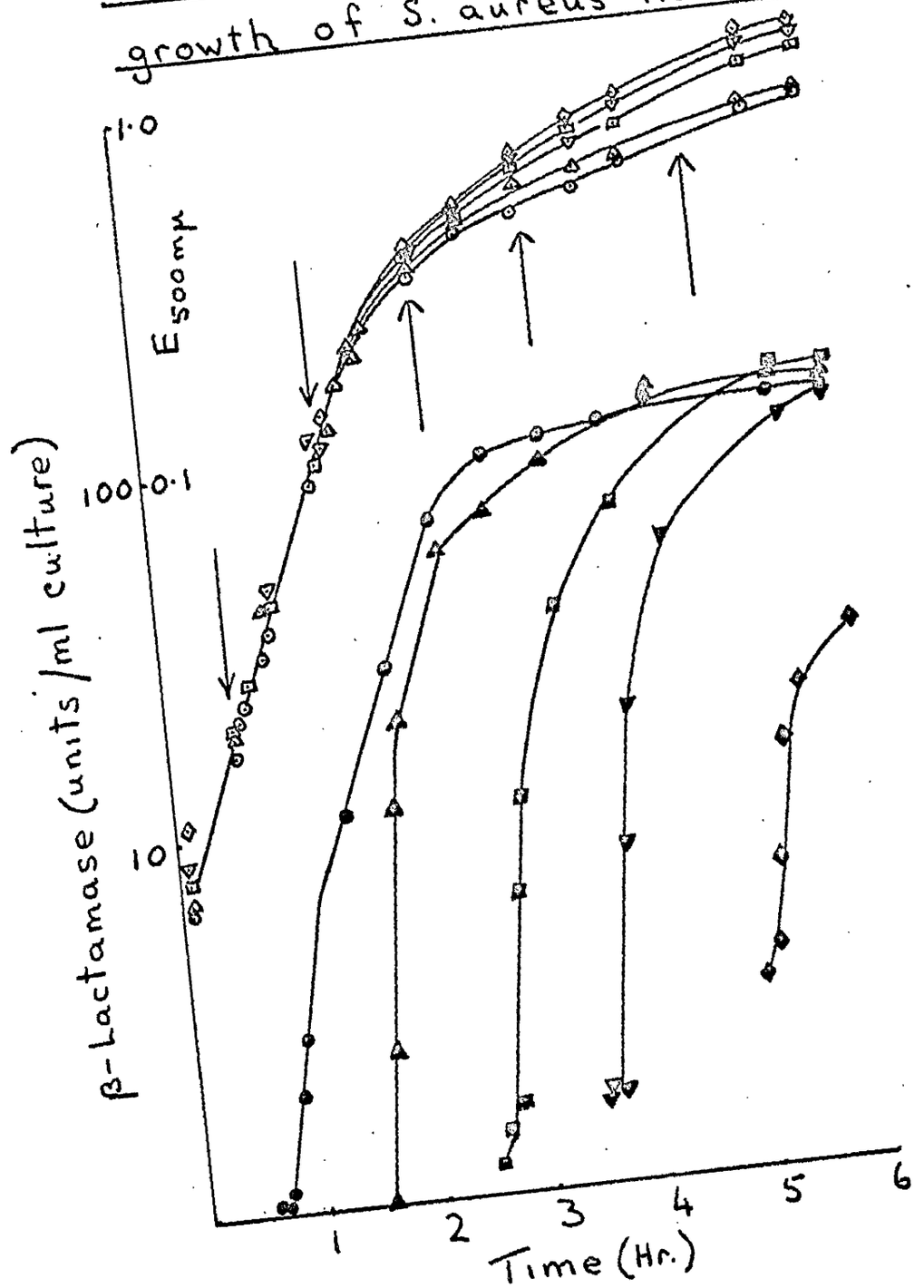


Fig 36

doubling time of enzyme was about 10 mins in nutrient broth and about 15 minutes in defined medium. In nutrient broth, the duration of this initial rapid rise is about half an hour. This is close to the mean generation time of the culture. In defined medium, the duration of the initial rise is again about one generation time (in this case 60 minutes).

A more detailed examination of the initial rise in enzyme supports the hypothesis that the duration of this phase is one generation time in nutrient broth (fig. 37 p.49a) but in defined medium the rise in enzyme approaches the steady state doubling time in a smooth curve and the initial rise does not appear to last for one generation time (fig. 38 p.49b).

(c) Initial kinetics of induction of β-lactamase.

The routine method of measurement of β-lactamase (penicilloic acid present after a given time of assay) was not sufficiently accurate to determine very low levels of enzyme. In order to determine (a) the basal level of enzyme, and (b) the kinetics of induction from the basal level to fully induced level the method of assay was modified. In each assay, samples were taken at various time intervals and assayed for penicilloic acid. The sensitivity of the penicilloic acid estimation was increased by decreasing the dilution in acetate buffer from 1/10 to 1/4. The results were expressed graphically and the slope of the line determined. Using this modified technique enzyme was determined at frequent intervals before and after addition of inducer to cells growing logarithmically in nutrient broth. The log. of β-lactamase is plotted against time in fig. 39 (p.49c). The basal level of enzyme doubles at a rate which is very close to the rate of doubling of turbidity. (All the points for the basal level of enzyme are not shown in fig. 39 p.49c). It can be seen that the rise of enzyme on addition of inducer to a culture growing in nutrient broth is a smooth curve and does not last

Fig. 37. The logarithm of β-lactamase *vs* time in a culture to which CBAP (50 μM) was added in the middle of logarithmic phase (△——△) compared with a culture to which CBAP (50 μM) was added at the time of inoculation (⊙——⊙) The mean generation time of the culture was 27 minutes.

Induction of β-lactamase during growth of S. aureus in nutrient broth.

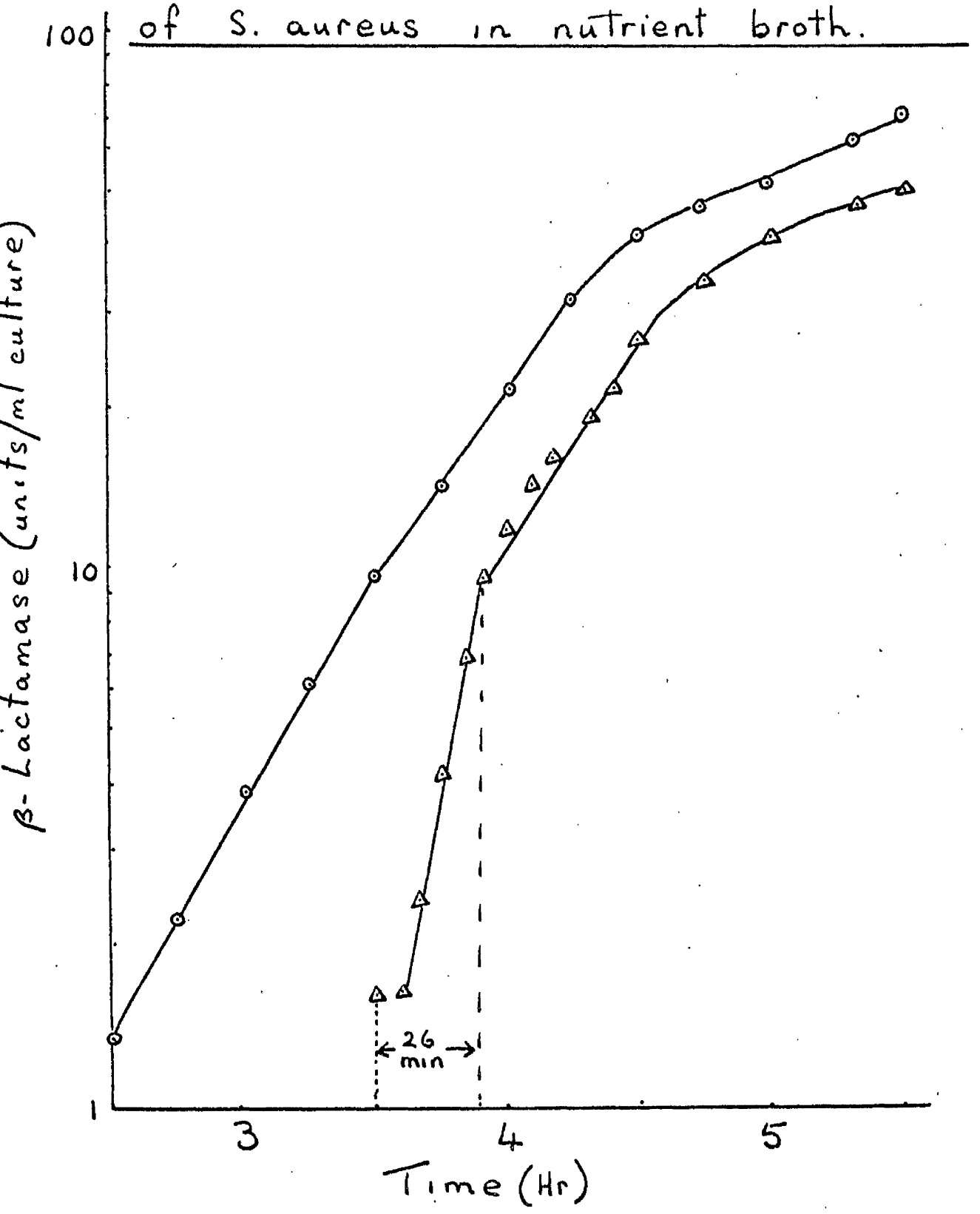


Fig 37

Fig. 38. Initial kinetics of induction of lactamase in defined medium. CBAP (50 μM) was added to a logarithmically growing culture of S. aureus C23/19 and β-lactamase measured at frequent time intervals thereafter. This graph shows the logarithm of β-lactamase vs time.

Initial kinetics of induction of β-Lactamase in S. aureus growing in defined medium

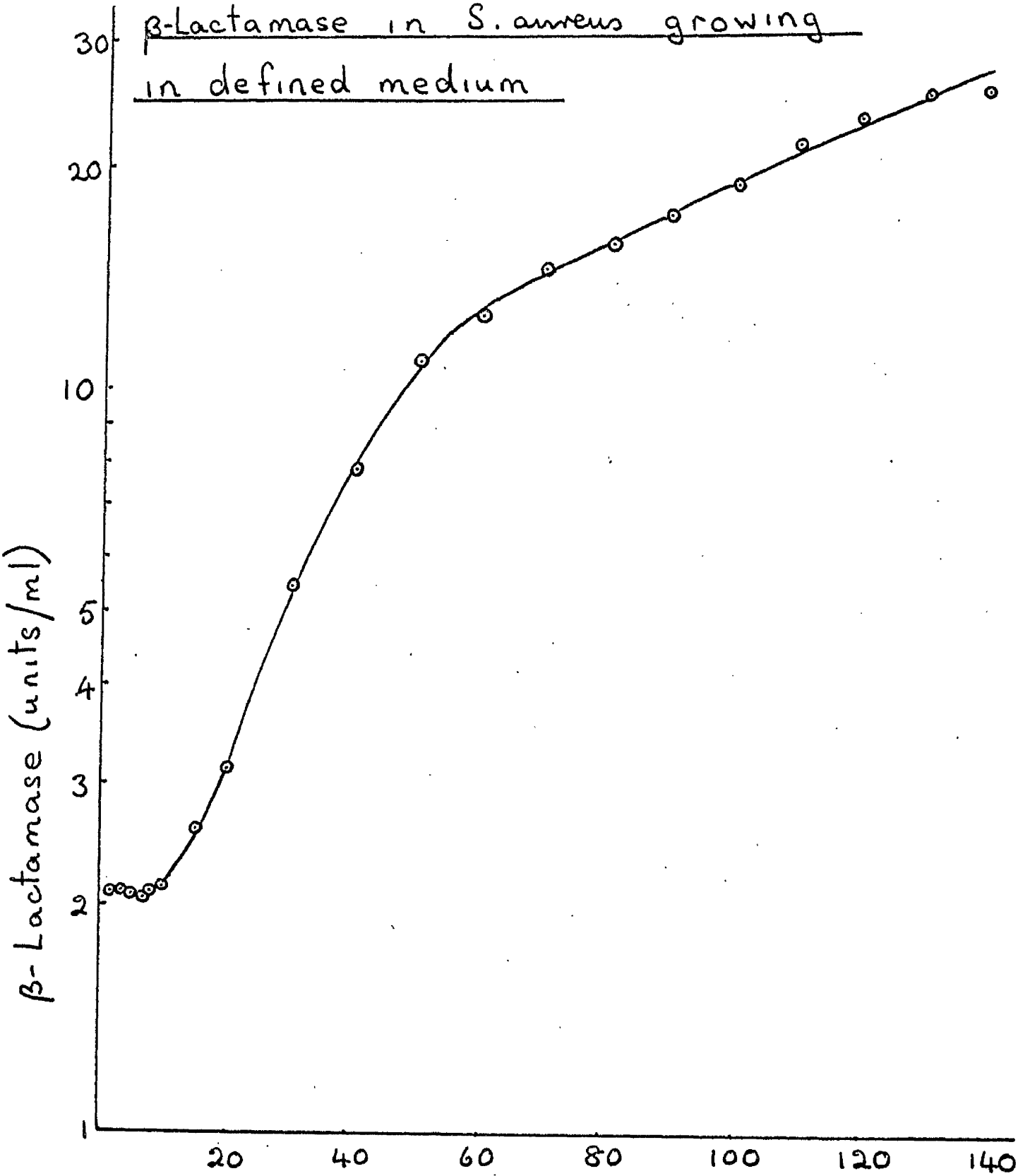


Fig. 38

Fig. 39. The logarithm of β-lactamase *vs* time in a culture of *S. aureus* C23/19 growing in nutrient broth + glucose (0.2% W/V). Inducer (CBAP, 50 μM) of β-lactamase was added at the time indicated by the arrow.

Initial Kinetics of induction of β-Lactamase in S. aureus growing in nutrient broth

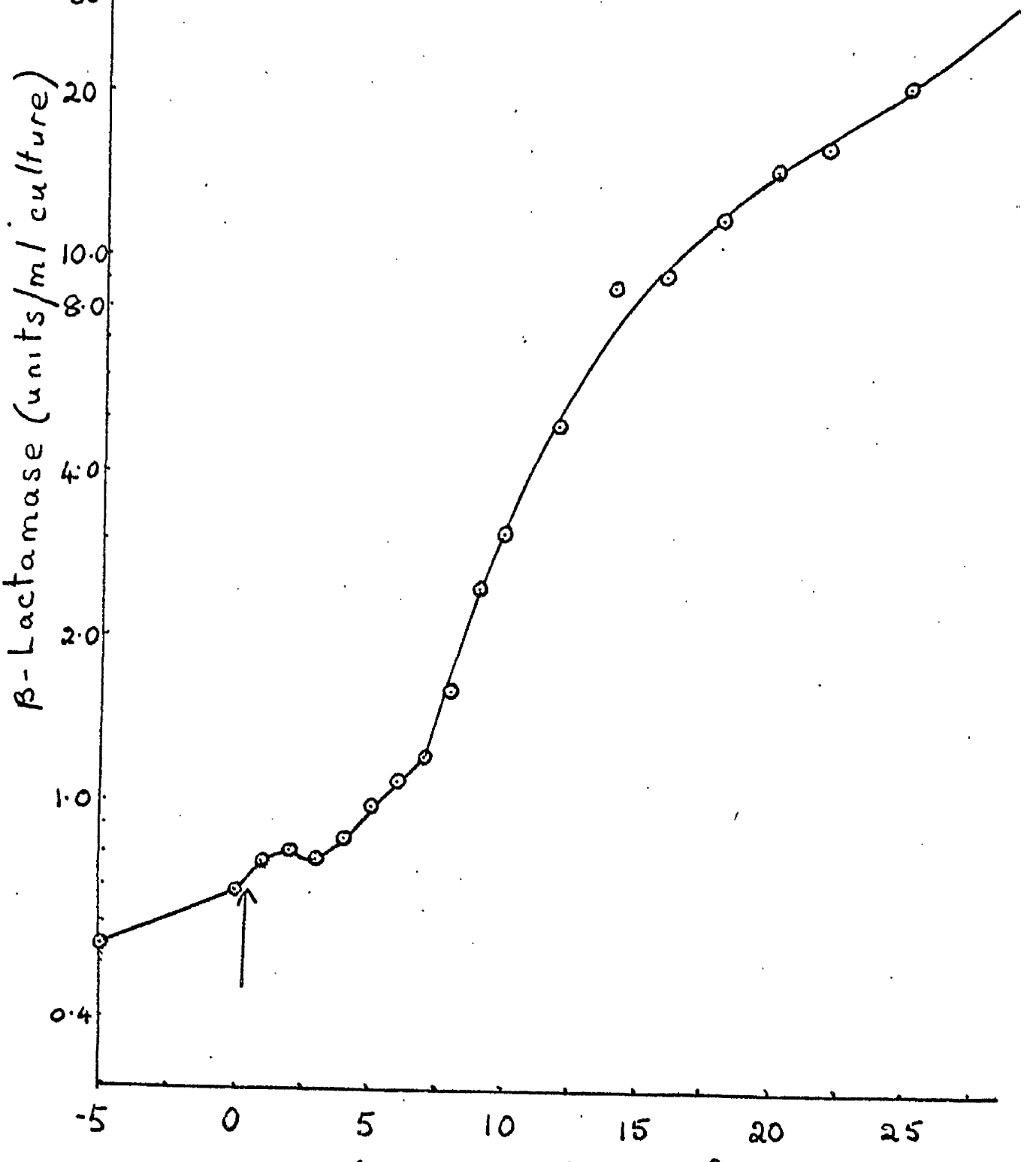


Fig 39.

for one generation time. The ratio of induced to uninduced level is 130.

The data for the initial kinetics of induction in nutrient broth (fig. 39 p.49c) and defined medium (fig. 37 p.49a) plotted on an arithmetic scale (fig. 40 p.50a and fig. 41 p.50b) show that there is a lag of 3 minutes in nutrient broth before any enzyme is synthesised and a period of four minutes before the full rate of synthesis is reached. In defined medium the lag is 8 minutes and the acceleration period 0 mins. Measurement of enzyme at frequent time intervals after the addition of inducer at three different stages of growth in defined medium showed (fig. 42 p.50c) that the approach to the steady state doubling time was a smooth curve. The specific activity of these cultures plotted vs time and compared with a culture to which inducer was added at the time of inoculation, (fig. 43 p.50d), shows that these cultures do not reach the same specific activity until well into the stationary phase. The earlier in the growth curve inducer is added, the higher the specific activity reached. In the culture to which inducer was added at time of inoculation, the specific activity falls in the end because increase of turbidity overtook increase of enzyme. The culture to which inducer was added at 5 hours shows a fall in specific activity which parallels that in the latter flask. The other two flasks do not show this fall in specific activity.

(d) The differential rate of β-lactamase synthesis on addition of inducer to growing cultures of S. aureus.

If the data for addition of inducer at different stages of growth in defined medium (see fig. 35 p.48b) are plotted as β-lactamase vs turbidity to obtain a value for the differential rate of β-lactamase synthesis (Monod, et.al. 1952) the graphs shown in fig. 44 (p.50e) are obtained. It can be seen that as inducer is added later in the growth cycle, there is a lag in

Fig.40. Initial kineties of induction of β-lactamase in nutrient broth + glucose (0.2% W/V). This graph is drawn from the data of fig.39.

Initial kinetics of induction of β-lactamase in S. aureus growing in nutrient broth

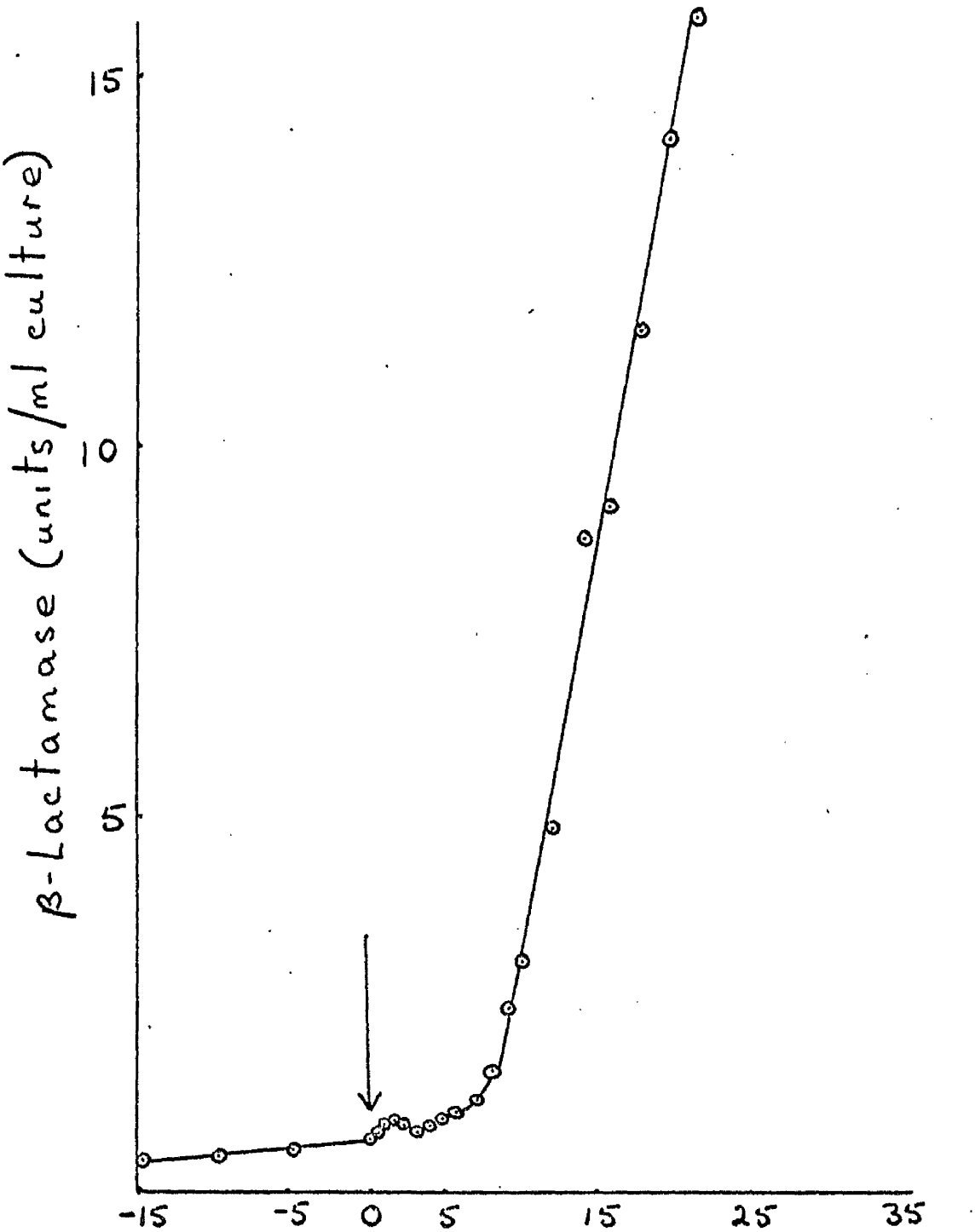


Fig 40

Fig. 41. Initial kinetics of induction of β-lactamase in defined medium. This graph is drawn from the data of fig. 38.

Initial kinetics of induction of β-lactamase in S. aureus growing in defined medium

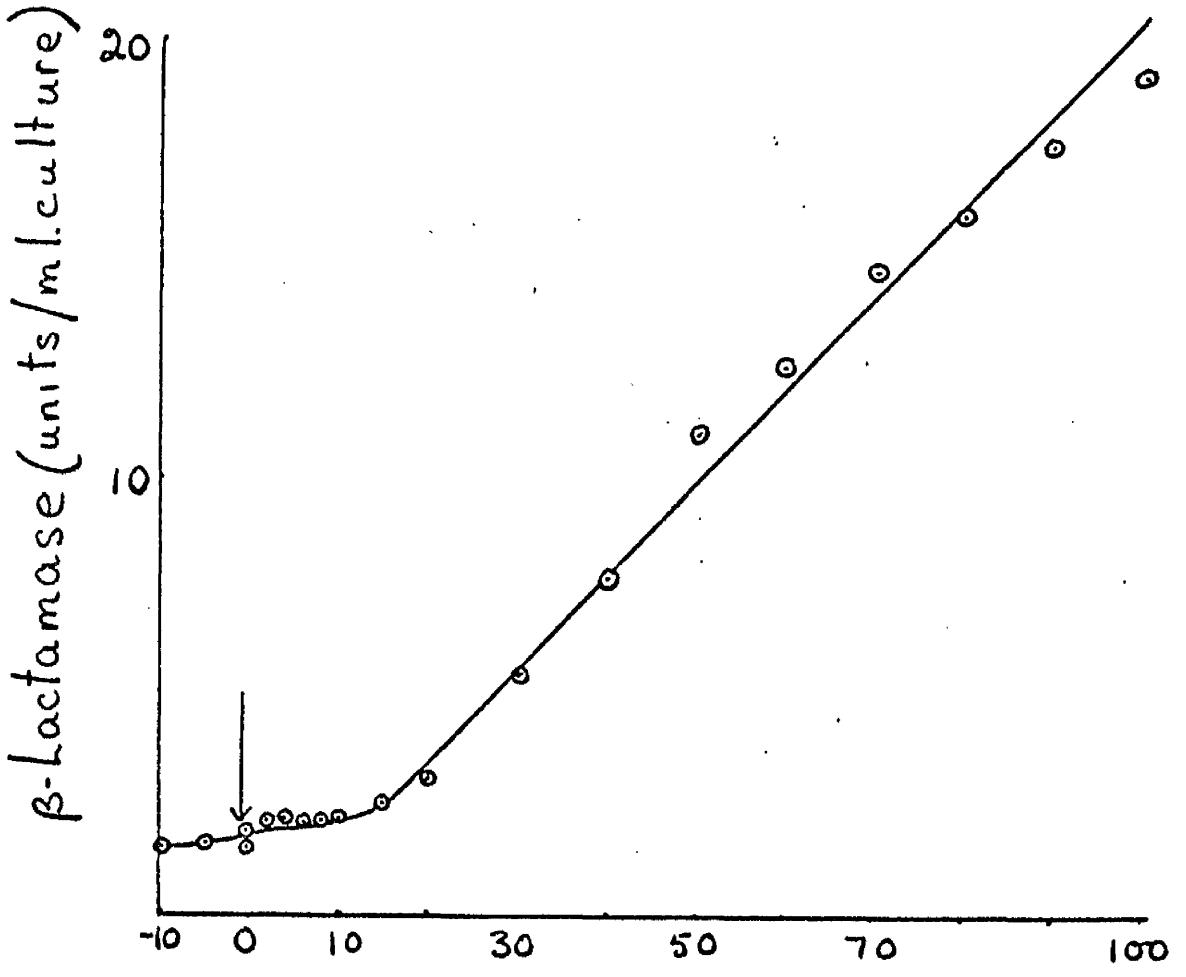


Fig 41

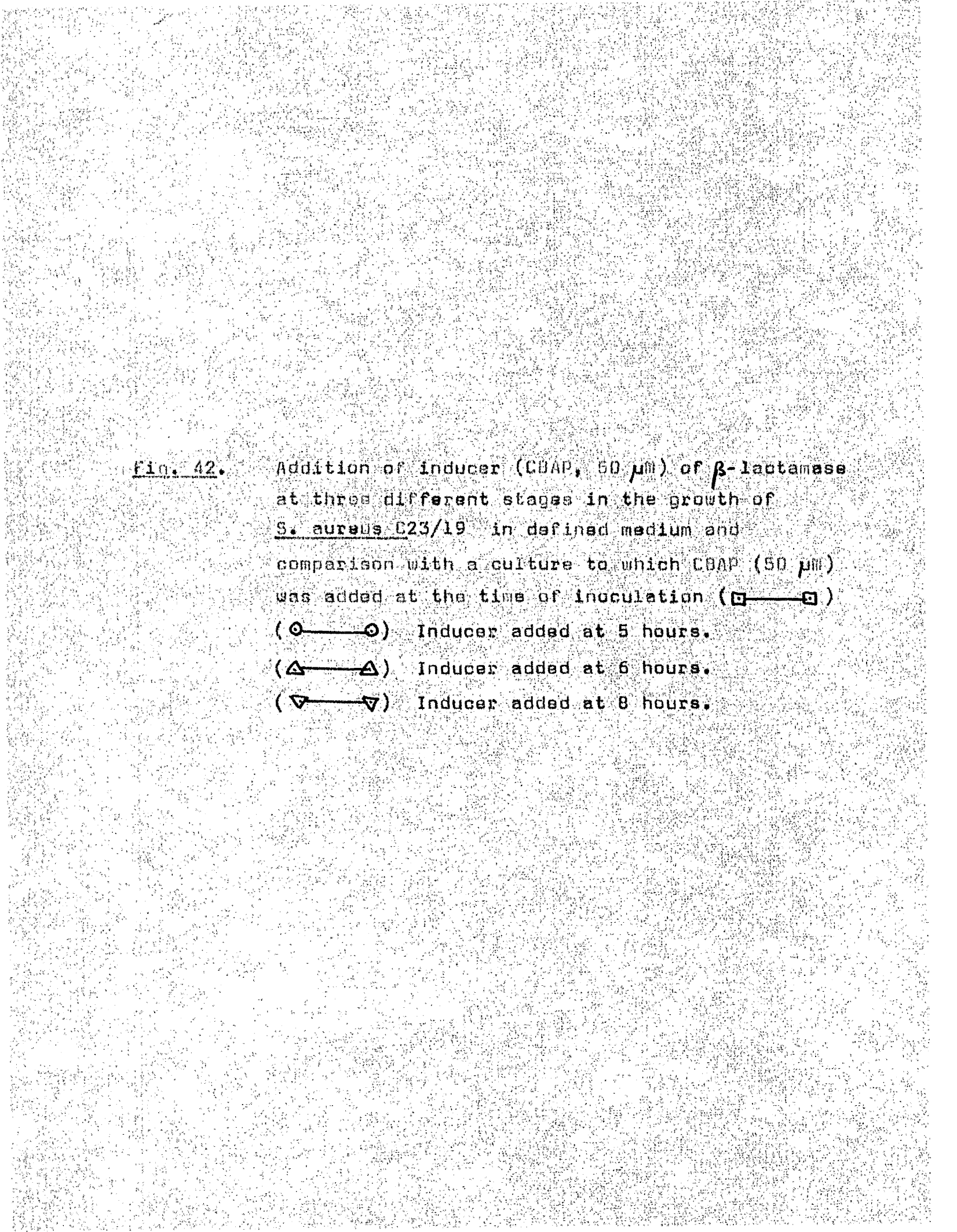


fig. 42. Addition of inducer (CDAP, 50 μM) of β-lactamase at three different stages in the growth of S. aureus C23/19 in defined medium and comparison with a culture to which CDAP (50 μM) was added at the time of inoculation (□——□)

(○——○) Inducer added at 5 hours.

(△——△) Inducer added at 6 hours.

(▽——▽) Inducer added at 8 hours.

Addition of inducer at different stages of growth of S. aureus in defined medium

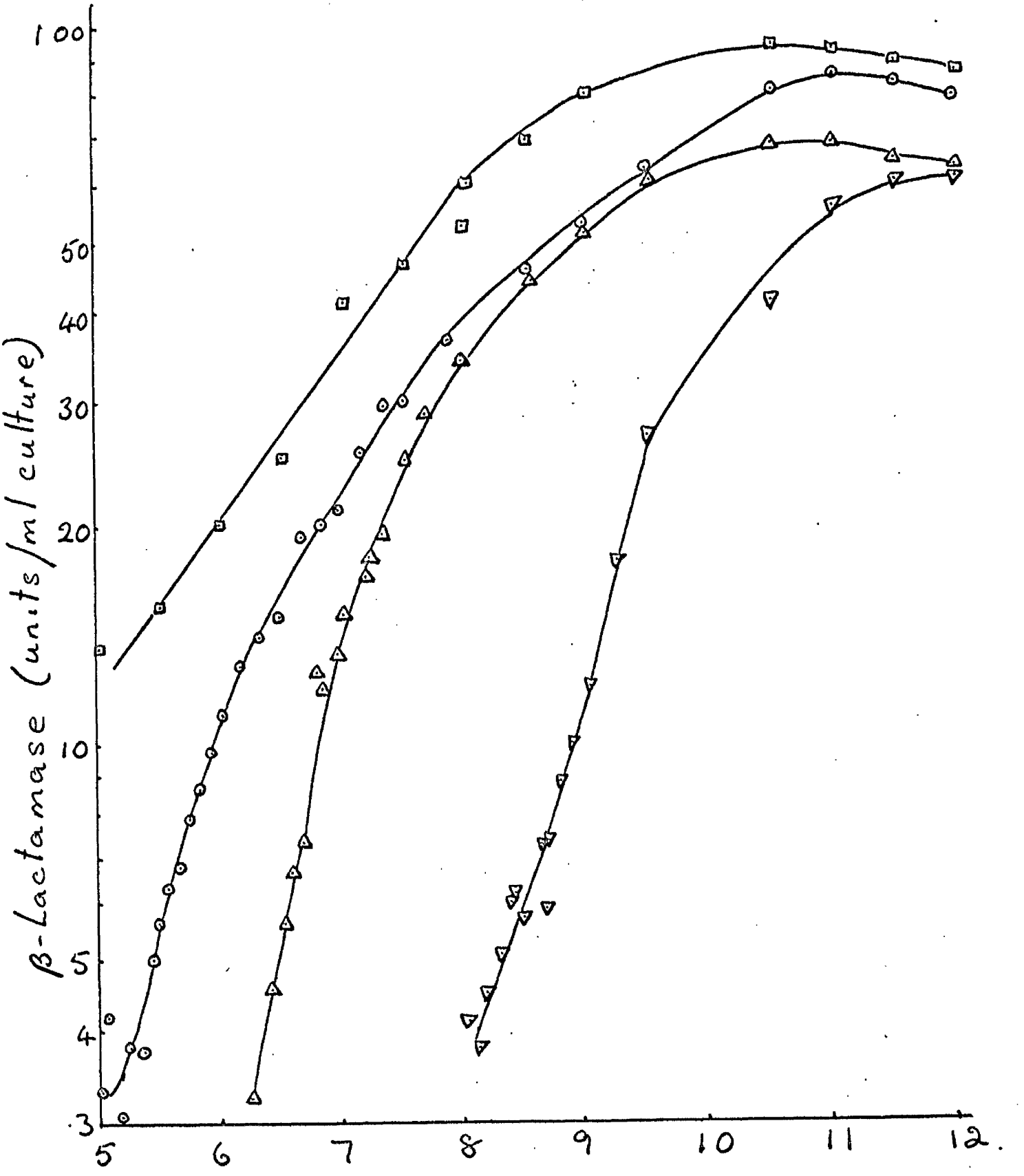


Fig 42

Fig. 43. Specific activity of β-lactamase for the cultures in fig. 42.

(□—□) CDAP (50 μM) added at 0 hrs.

(○—○) CDAP " " " 5 hrs.

(△—△) " " " " 6 hrs.

(▽—▽) " " " " 8 hrs.

Specific activity of β-lactamase on addition of inducer to S.aureus at different stages of growth in defined medium

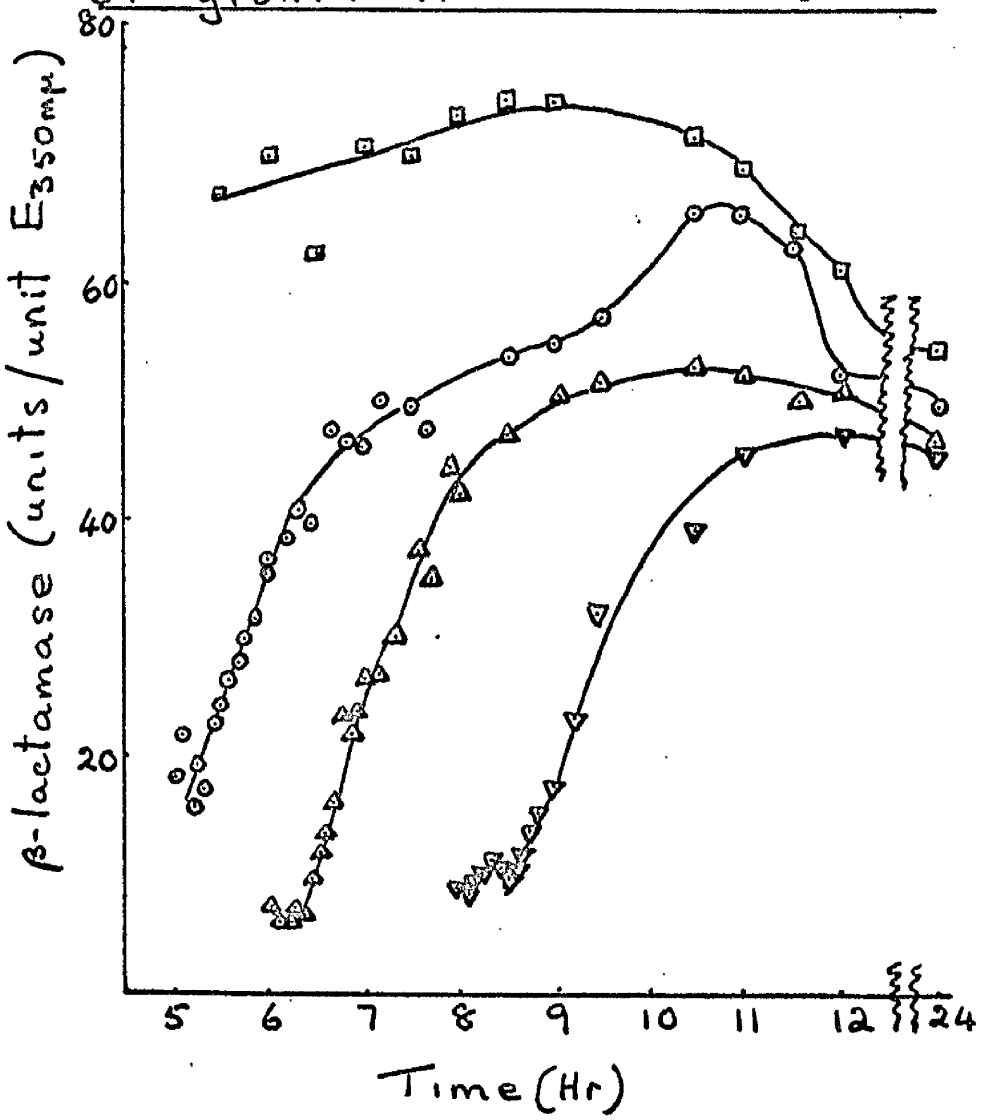


Fig 43

Fig. 44. Differential rate of β-lactamase in the cultures of fig.35 where COAP (50 μM) was added to cultures of *S. aureus* C23/19 growing in defined medium

Differential rate of β-lactamase synthesis on addition of inducer to S. aureus growing in defined medium

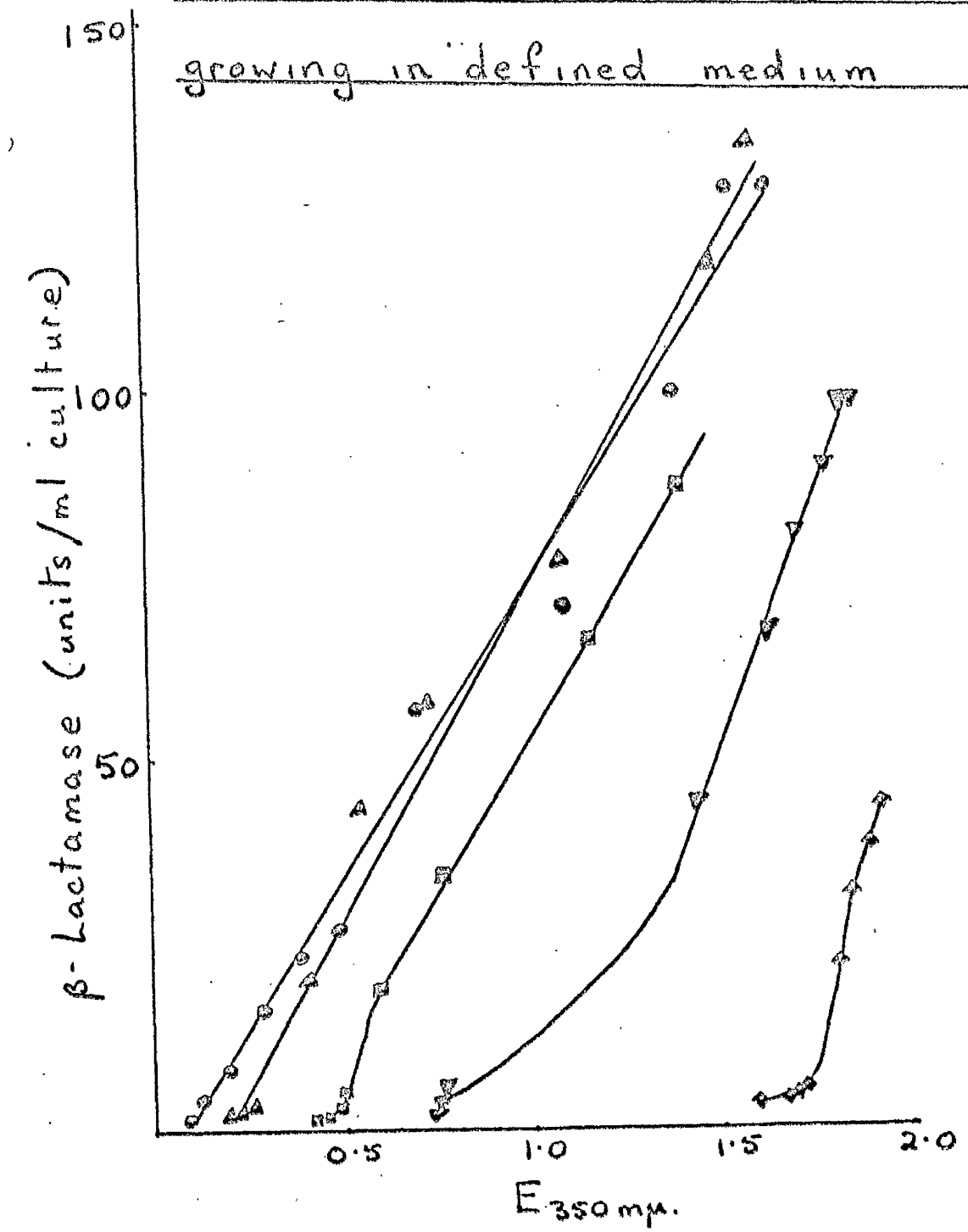


Fig 44

the rate of synthesis of β-lactamase relative to turbidity. This is also observed when the data from fig. 42 (p.50c) where inducer was added at three points in the growth cycle as well as at the time of inoculation, are plotted in the same way (fig. 45 p.51a). In the case where inducer was added at a turbidity of about 0.2, the differential rate of β-lactamase synthesis is constant from the moment of addition of inducer despite the fact that the initial rate of doubling is very much greater than the steady state rate of doubling (see fig. 42 p.50c). After the initial lag observed at higher cell densities the differential rate is constant. The differential rate of β-lactamase synthesis is about the same in all cases once it is established.

The data from experiments where inducer was added at different stages of growth in nutrient broth (see fig. 36 p.48c) give a similar series of graphs when plotted in this way. (Fig. 46 p. 51b). The later in the growth cycle inducer is added, the longer is the lag in establishing a constant differential rate of β-lactamase synthesis. As in defined medium, the differential rate of β-lactamase synthesis in nutrient broth is constant from the moment of addition of inducer when inducer was added early in the growth curve despite the fact that the initial doubling time is so very much greater than the steady state doubling time (see fig. 36 p.48c).

The lag in establishing a constant differential rate of enzyme synthesis is sometimes a true lag, i.e. the differential rate of enzyme synthesis is close to zero as in fig. 45 (p.51a) and sometimes there is simply a somewhat lower differential rate of enzyme synthesis as in fig. 46 (p.51b). This pattern of a lower differential rate of β-lactamase followed by a higher one is seen in the plot of β-lactamase vs turbidity for the initial kinetics of β-lactamase induction in nutrient broth (fig. 47 p.51c).

Fig. 45. Differential rate of β-lactamase synthesis in the cultures of fig.42 where CBAP(50 μM) was added, to cultures growing in defined medium, at different stages of growth.

Differential rate of β-lactamase synthesis on addition of inducer to S. aureus growing in defined medium

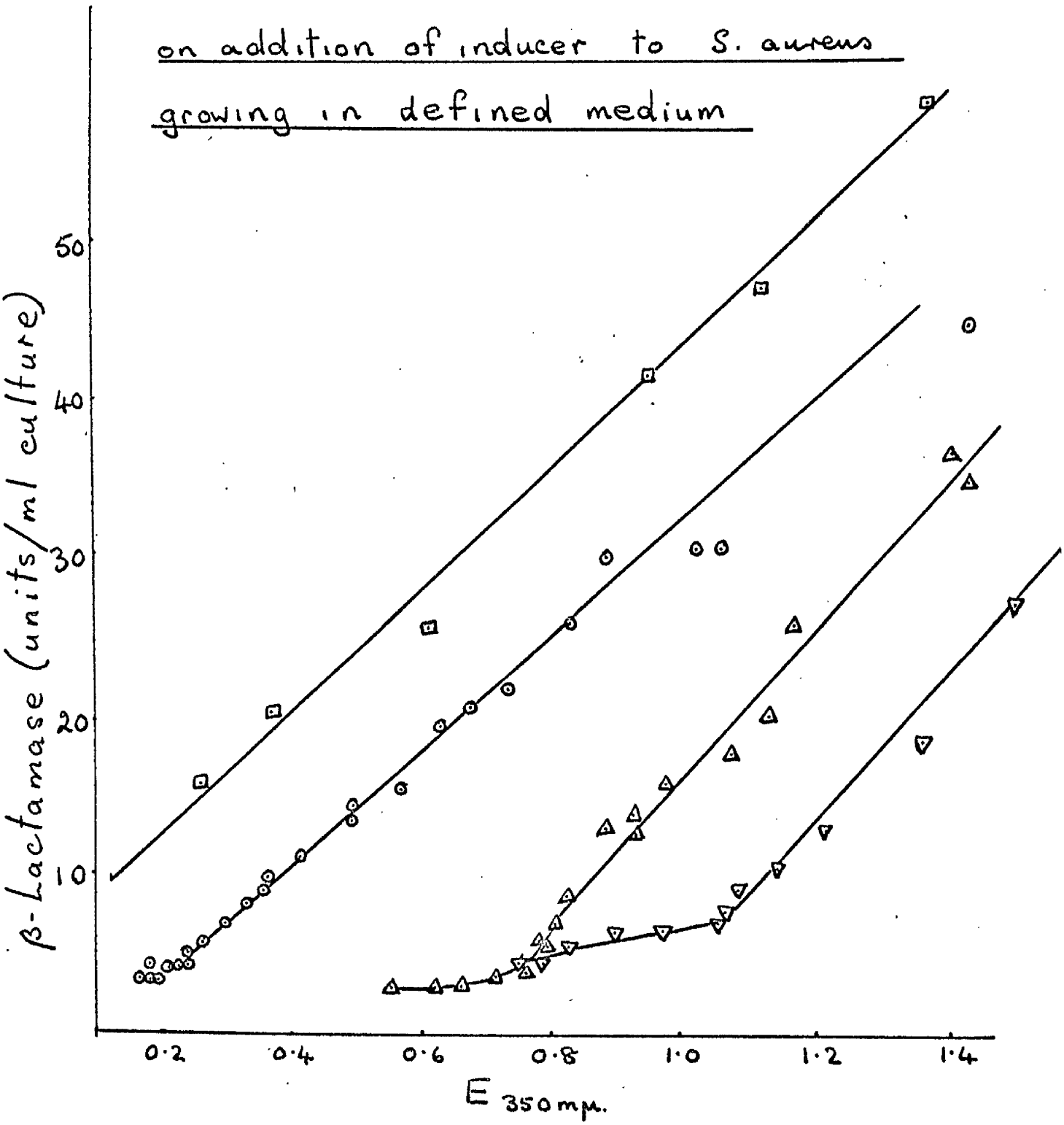


Fig 45

Fig. 46. Differential rate of β- lactamase synthesis in the cultures of fig.36 where CBAP (50μM) was added to cultures growing logarithmically in nutrient broth + glucose (0·2% W/V)

Differential rate of β-lactamase synthesis on addition of inducer to S. aureus growing in nutrient broth

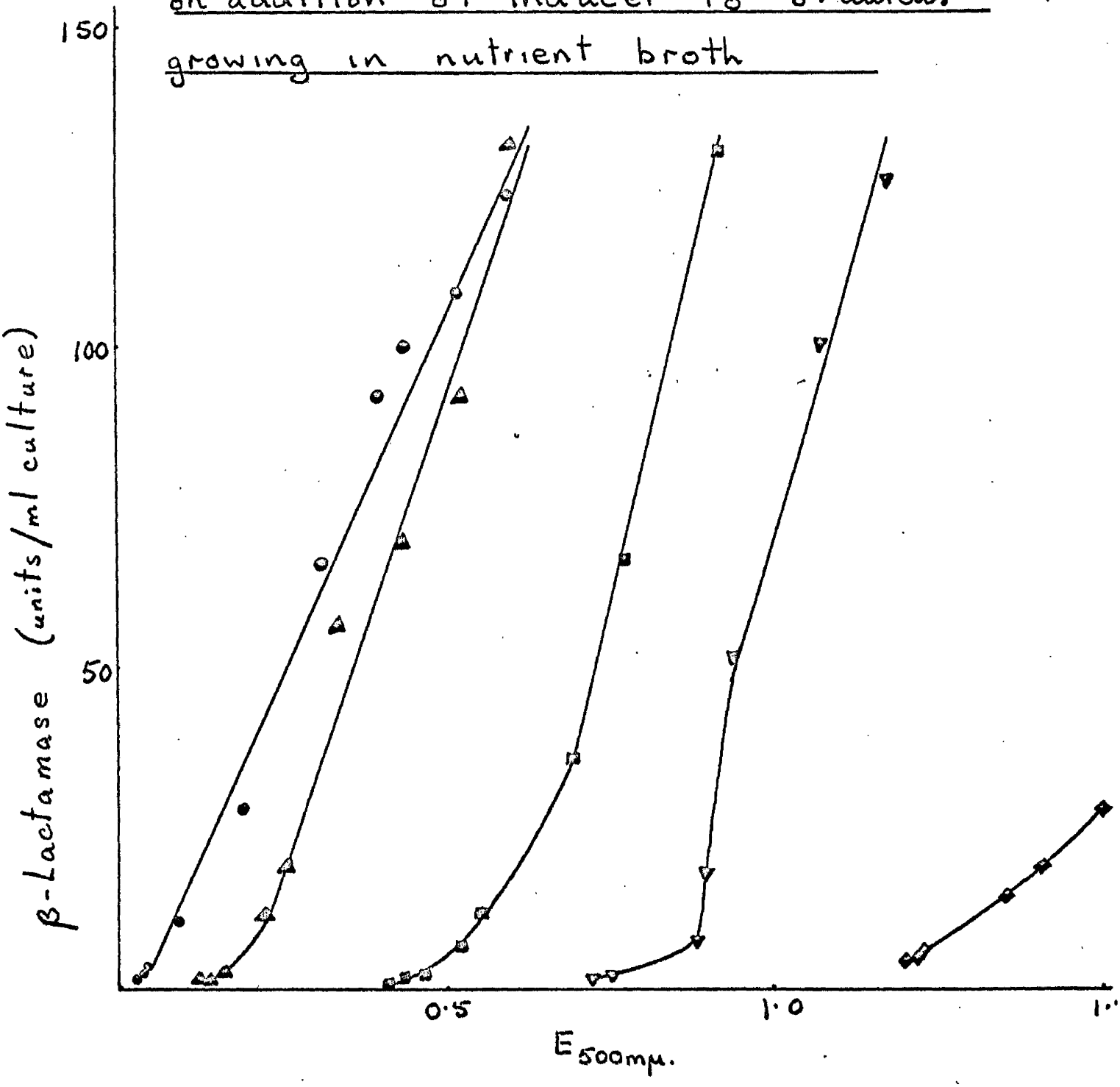


Fig 46

Fig. 47. Differential rate of β-lactamase synthesis in a culture of S.aureus C23/I9 growing in nutrient broth + glucose (0.2% W/V) to which inducer (CBAP, 50 μM) was added during logarithmic phase. These data are from the experiment described in Fig. 39. The differential rate is 46 initially and changes to 70, which is close to the value usually obtained in this medium.

Differential rate of β-Lactamase synthesis in nutrient broth.

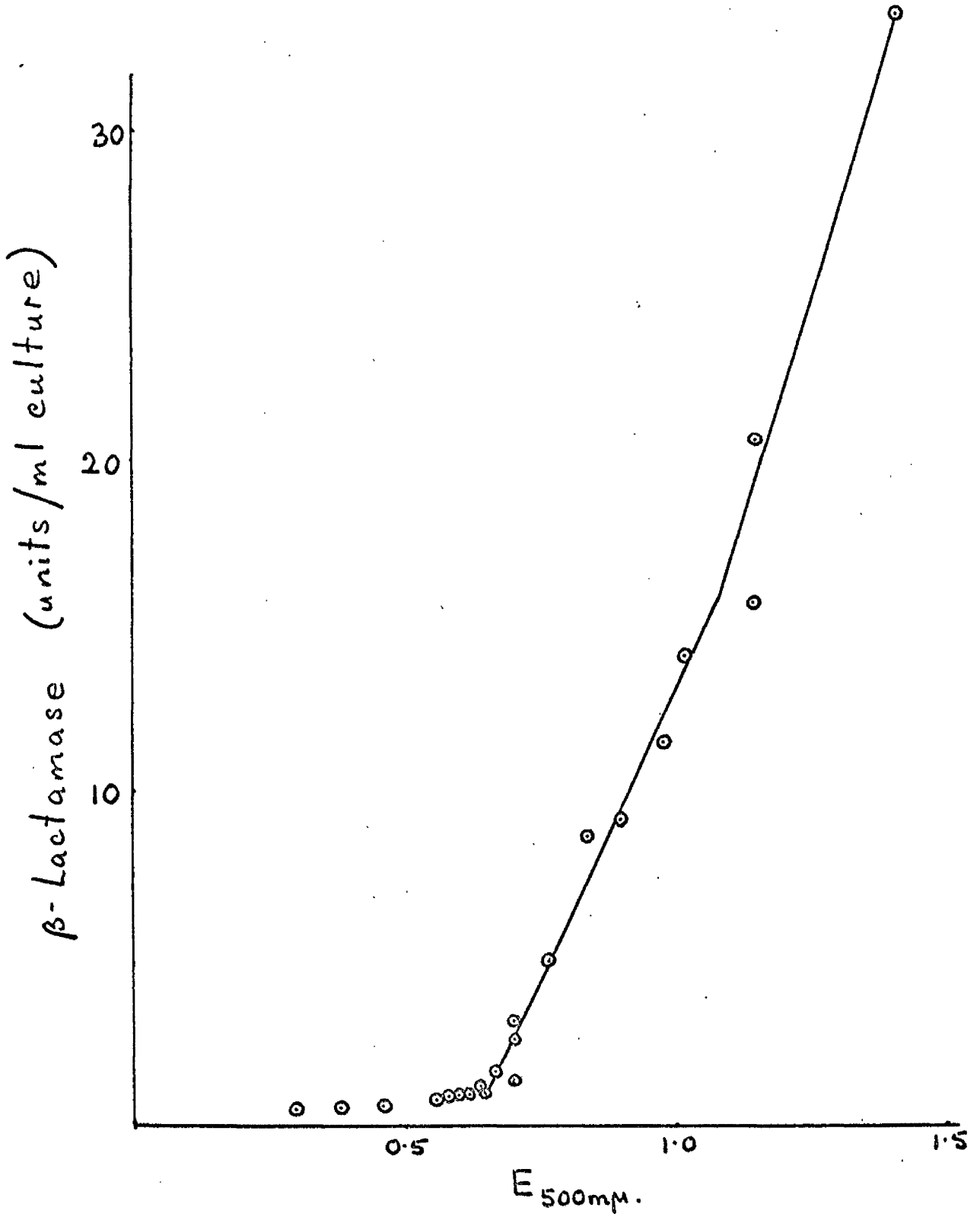


Fig 47

In defined medium, the same pattern is observed with either glucose or galactose as carbon source. There is a lag in establishing a constant rate of differential synthesis. (Fig. 48 p.52a).

(e) Effect of temperature on induction of β-lactamase in S.aureus

Addition of inducer to cells growing at different temperatures in nutrient broth, causes the level of enzyme to rise and approach the steady state doubling time in a smooth curve at each temperature tested, 29°C, 31°C, 35°C, 37°C. (fig. 49 p.52b). The differential rate is about the same in each case and at each temperature there is a lag. (Fig. 50 p.52c).

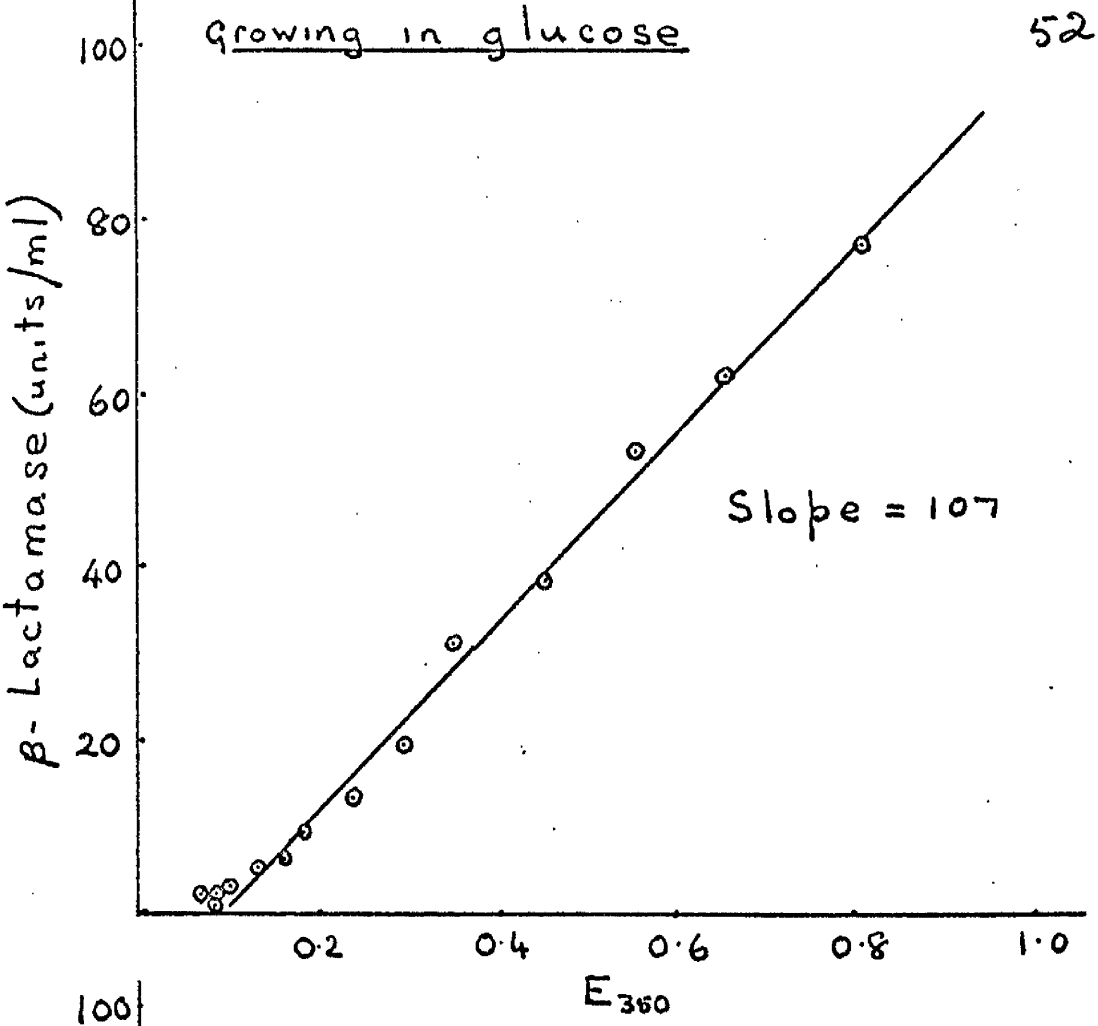
(f) Induction at low concentrations of inducer.

Fig. 51 (p.52d) shows the logarithm of growth and β-lactamase plotted *vs* time with 50 μM CBAP and 0.5 μM CBAP. There is a lag in the synthesis of enzyme on addition of 0.50 μM inducer compared with the addition of 50 μM inducer. When synthesis of enzyme does start in 0.5 μM CBAP, it does so at a time when growth is slowing down. Enzyme doubles at a rate of one doubling in 10 minutes whereas turbidity at the same time doubles every hour. A plot of β-lactamase *vs* turbidity for these data and for a culture induced with 2 μM CBAP (fig. 52 p.52e) shows that the differential rate accelerates in the early stages of growth and the acceleration is slower the lower the concentration of inducer. A constant differential rate (133 units/unit E_{500}) is reached during the very slow growth after logarithmic phase in presence of 0.5 μM inducer. Although growth is very much slower at this stage this value for the differential rate is about the same as that obtained when measured during logarithmic phase with 50 μM CBAP (see *e.g.* table 13).

(g) Change of concentration or inducer.

Fig. 53 (p.52f) shows the specific activity of β-lactamase in three cultures inoculated with washed cells of a fully induced

Fig. 48. Differential rate of β-lactamase in defined medium with glucose or galactose as carbon source. CBAP (50 μM) was added at the time of inoculation of the culture and β-lactamase and turbidity measured during subsequent growth.


Growing in glucose
β-Lactamase (units/ml)
100
80
60
40
20
Slope = 107
0·2
0·4
0·6
0·8
1·0
E350

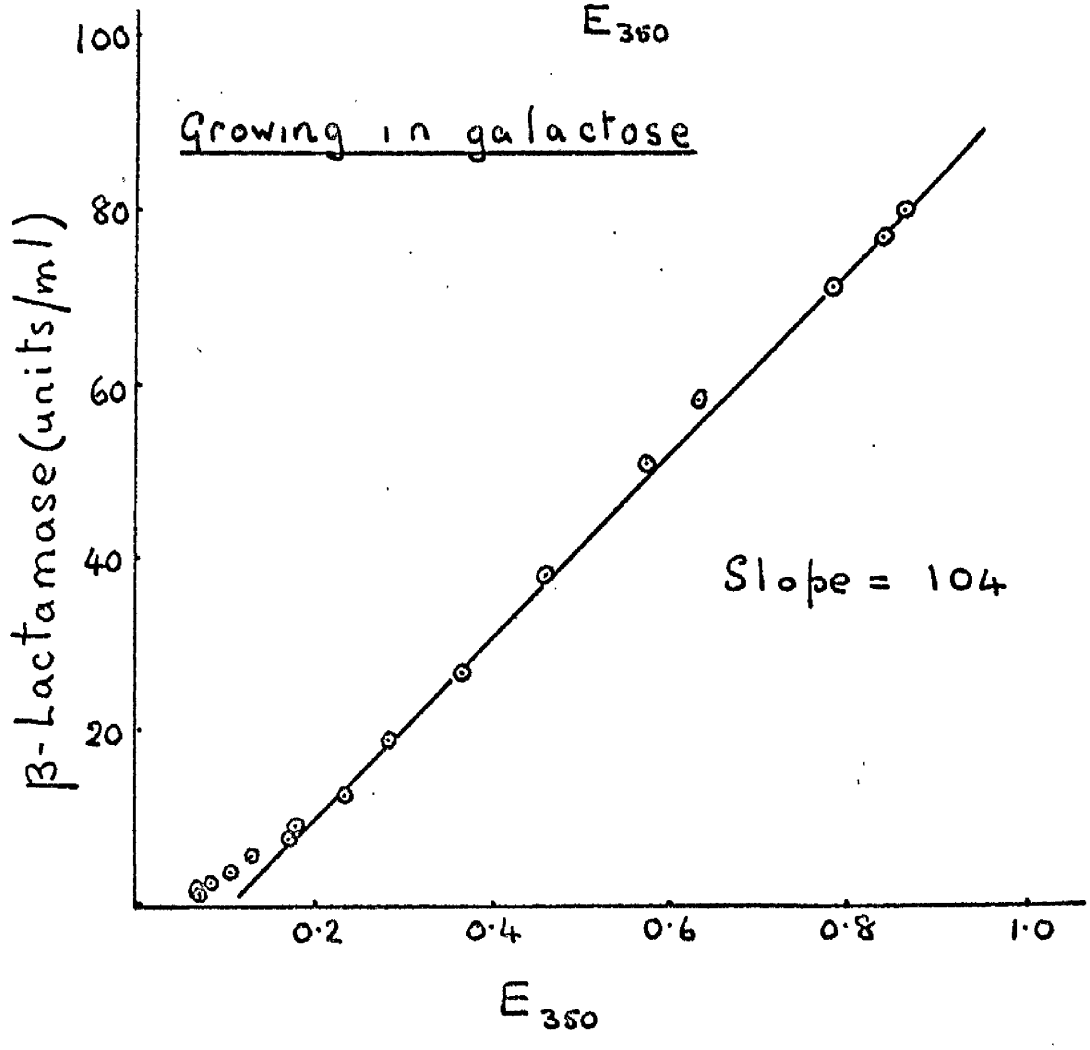

Growing in galactose
β-Lactamase (units/ml)
100
80
60
40
20
Slope = 104
0·2
0·4
0·6
0·8
1·0
E350

Fig 48

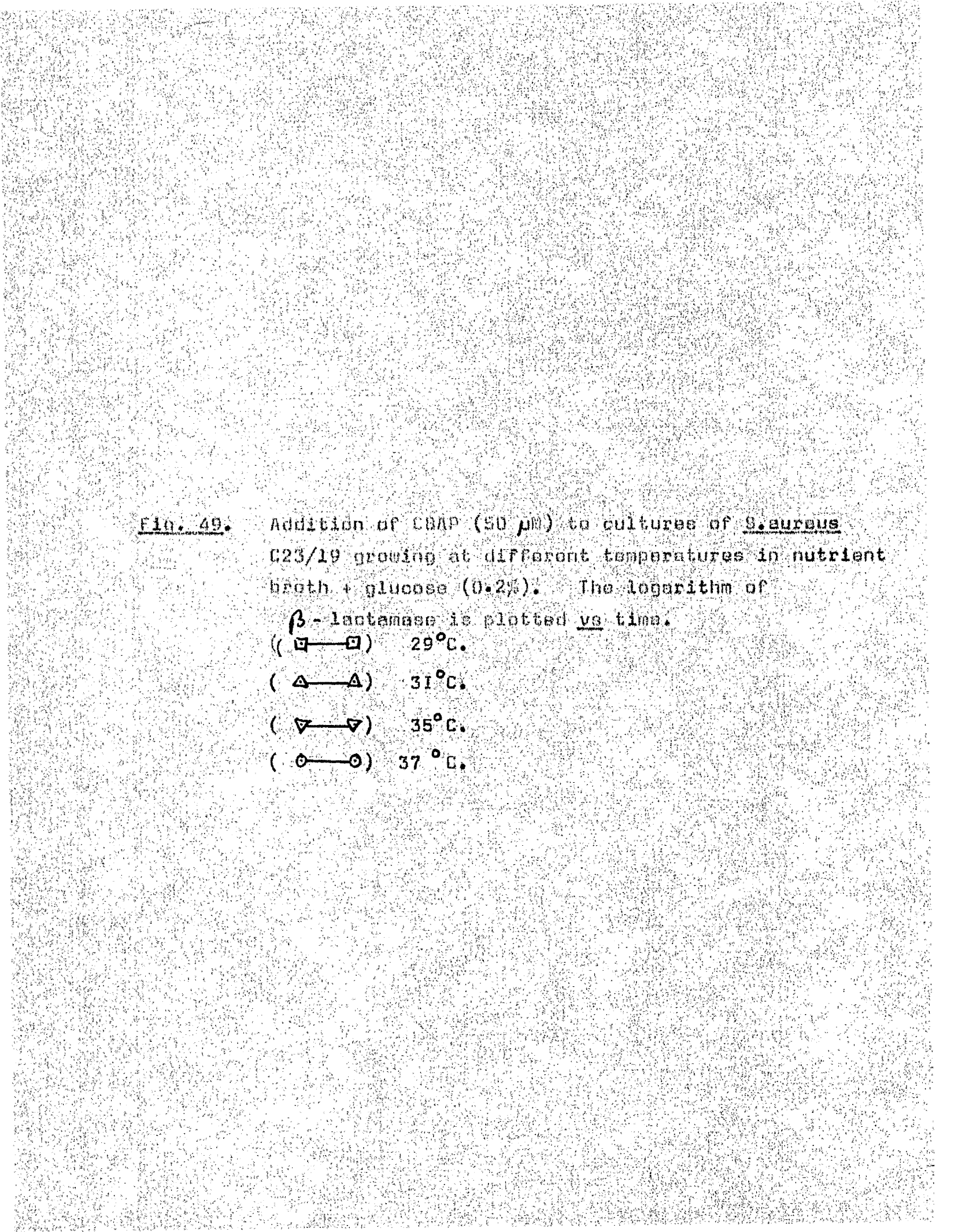


Fig. 49. Addition of CDAP (50 μM) to cultures of S.aureus C23/19 growing at different temperatures in nutrient broth + glucose (0.2%). The logarithm of β-lactamase is plotted vs time.

(□—□) 29°C.

(△—△) 31°C.

(▽—▽) 35°C.

(○—○) 37°C.

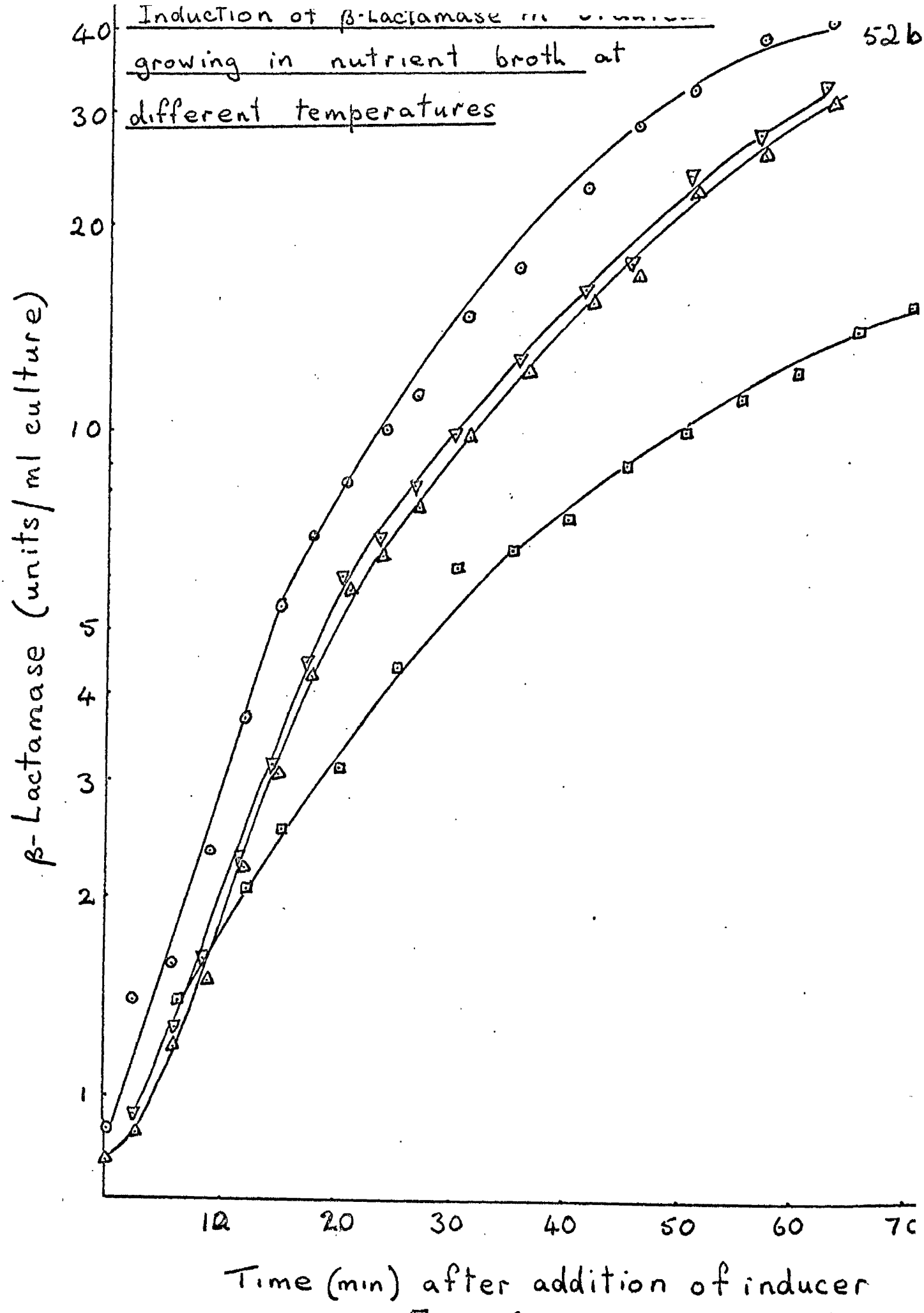

Induction of β-Lactamase in ... growing in nutrient broth at different temperatures
β-Lactamase (units/ml culture)
40
30
20
10
5
4
3
2
1
10
20
30
40
50
60
70
Time (min) after addition of inducer

Fig. 49.

Fig. 50. Differential rate of β-lactamase synthesis in the cultures of Fig. 49. where *S.aureus* C23/19 is growing in nutrient broth + glucose at different temperatures.

Differential rate of β-Lactamase synthesis in S. aureus growing in nutrient broth.

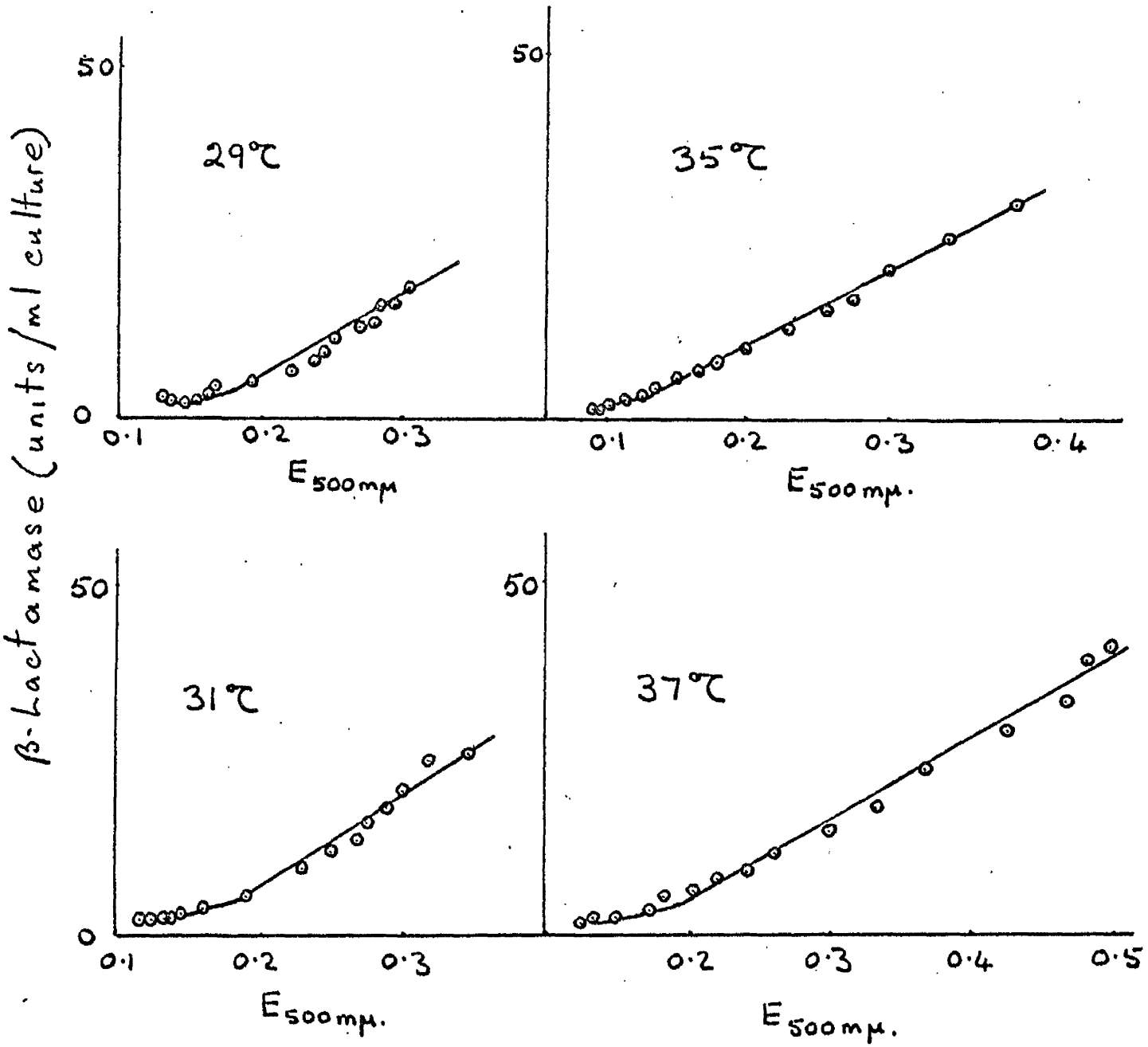


Fig. 50

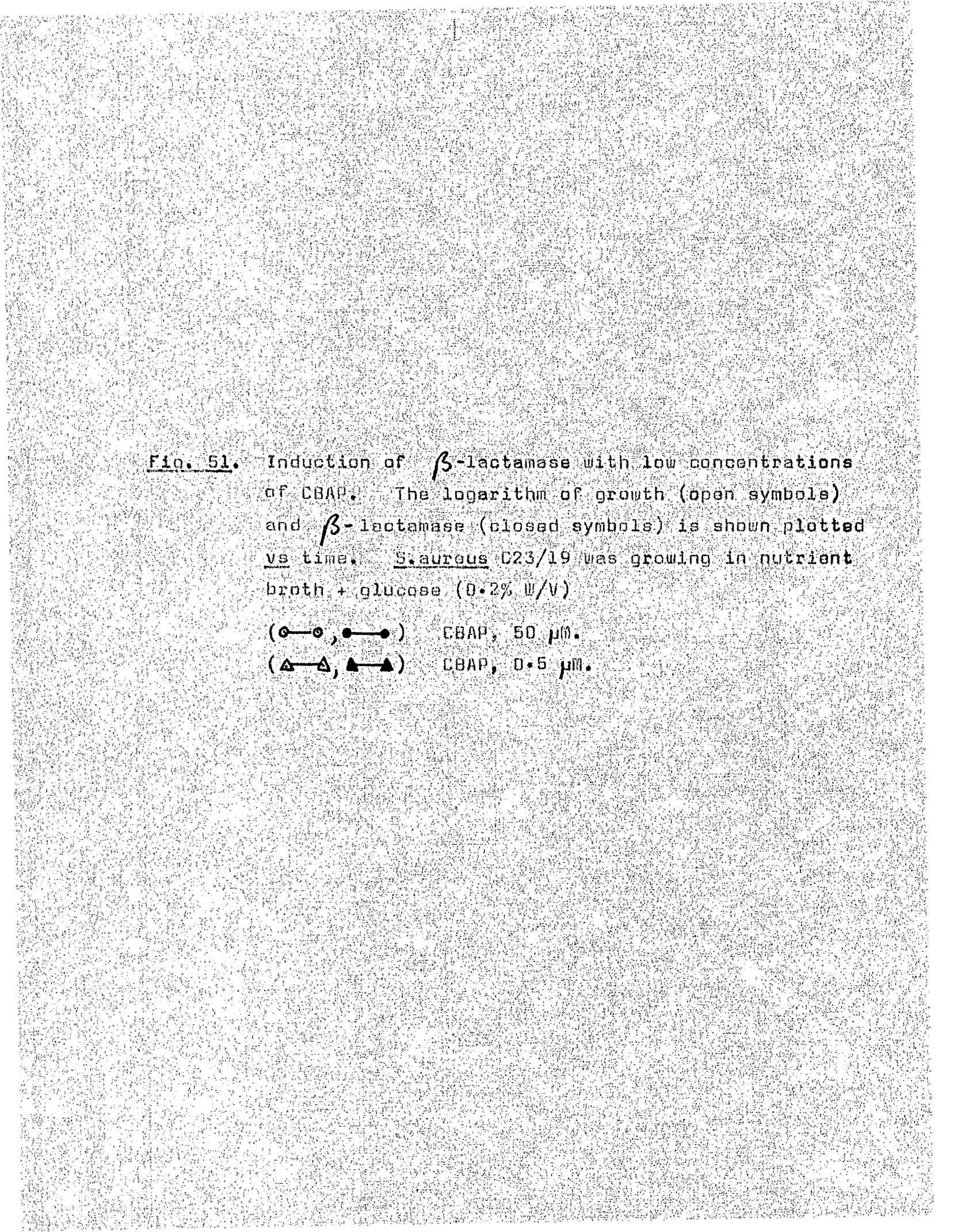


Fig. 51. Induction of β-lactamase with low concentrations of CBAP. The logarithm of growth (open symbols) and β-lactamase (closed symbols) is shown plotted vs time. S.aureus C23/19 was growing in nutrient broth + glucose (0.2% W/V)

(○—○, ●—●) CBAP, 50 μM.

(△—△, ▲—▲) CBAP, 0.5 μM.

Induction of β-lactamase with different concentrations of inducer.

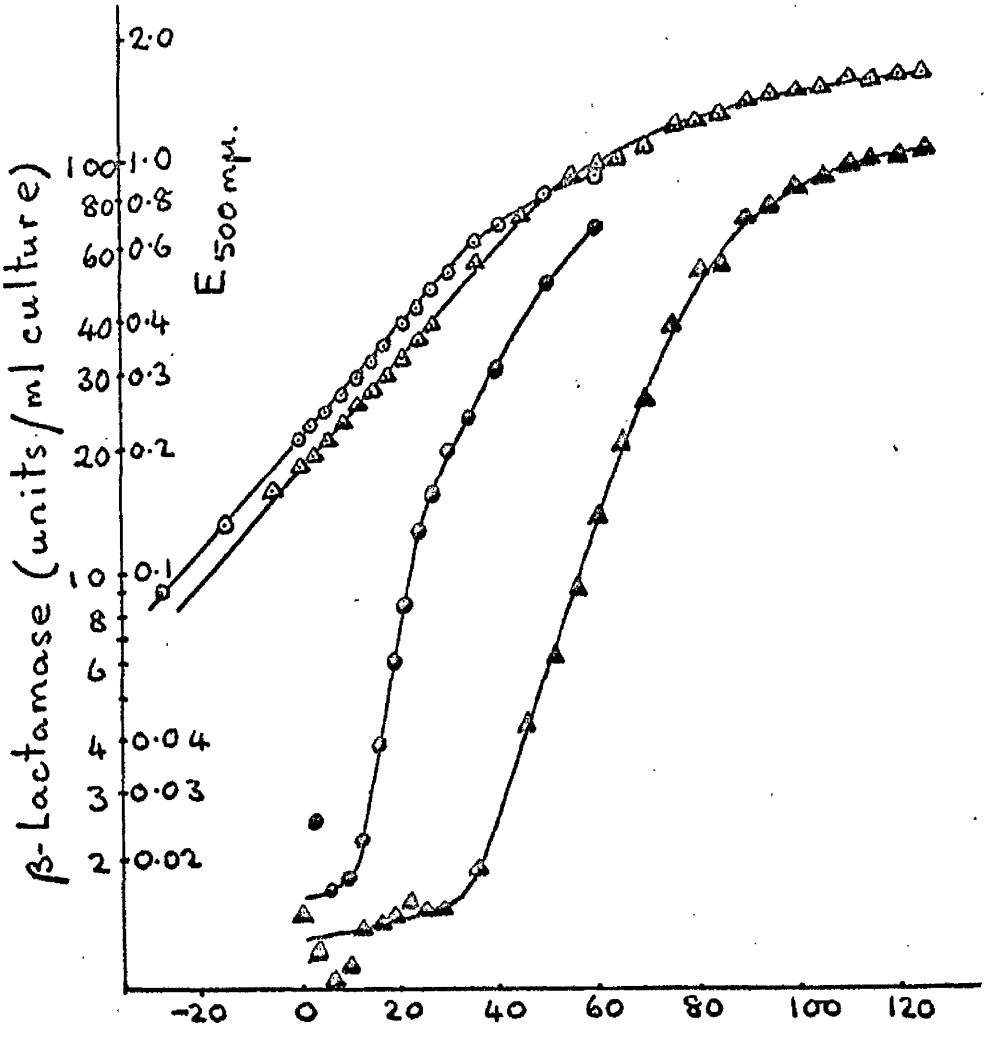
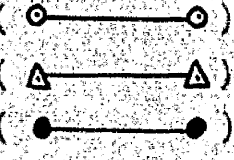


Fig 51

Fig.52. Differantial rate of β-lactamase synthesis in cultures of S.aureus C23/19 growing in nutrient broth + glucose (0.2% W/V) with different concentrations of inducer. The data for 50 μM and 0.5 μM CBAP are from fig.51. The 2 μM CBAP is from a separate experiment



50 μM CBAP

2 μM CBAP

0.5 μM CBAP.

Differential rate of β-lactamase synthesis on induction with different concentrations of inducer

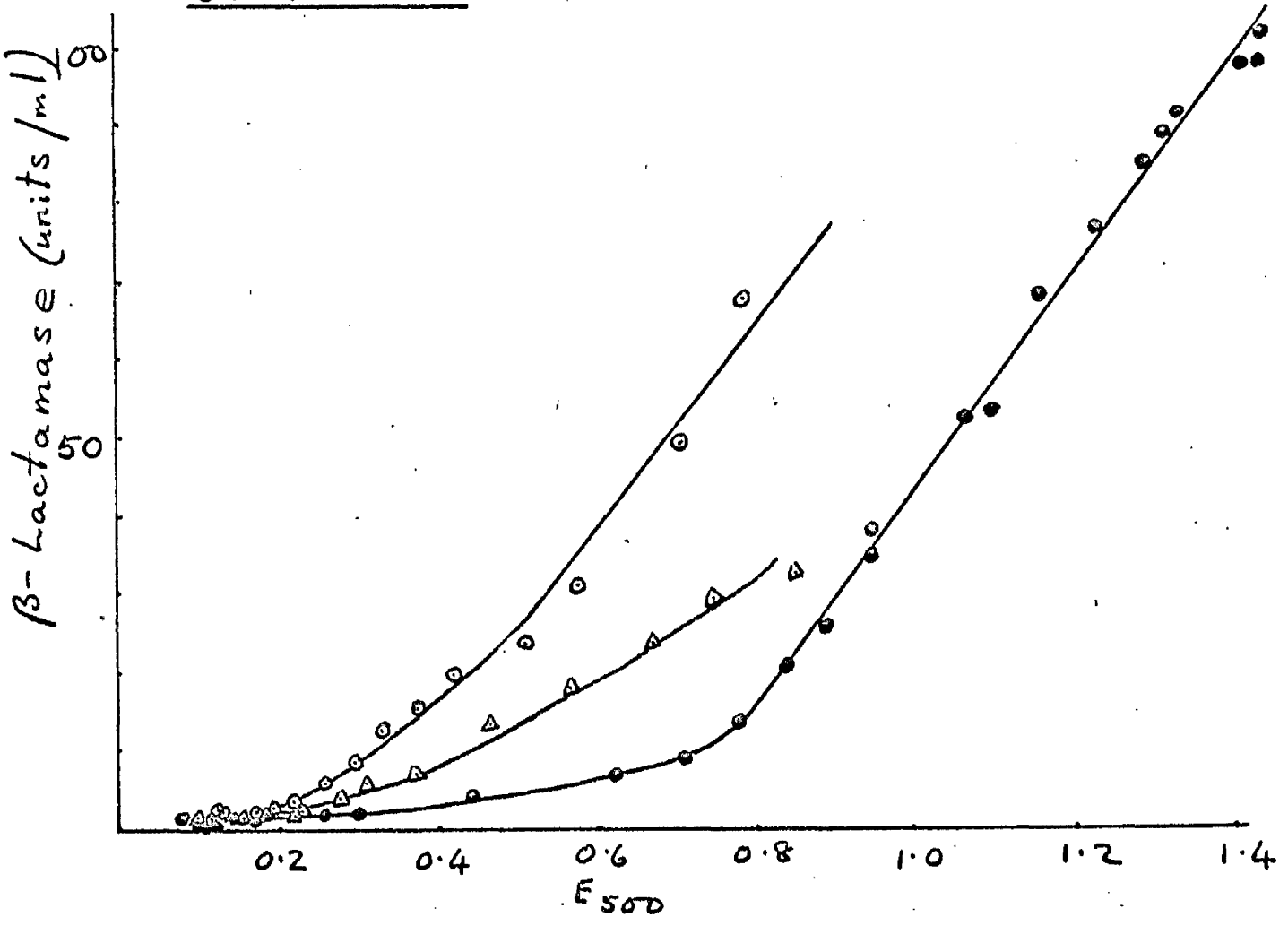
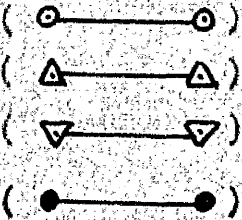


Fig. 52

Fig.53. Specific activity of β-lactamase during growth of cultures of S.aureus C23/19. in different concentrations of CBAP. The inoculum was grown in the presence of 50 μM CBAP, filtered washed and inoculated into the flasks containing different concentrations of CBAP.



(⊙——⊙) 50 μM CBAP

(△——△) 0·5 μM CBAP

(▽——▽) No CBAP.

(●——●) Growth of all these flasks.

Specific activity of β-lactamase on changing the concentration of inducer

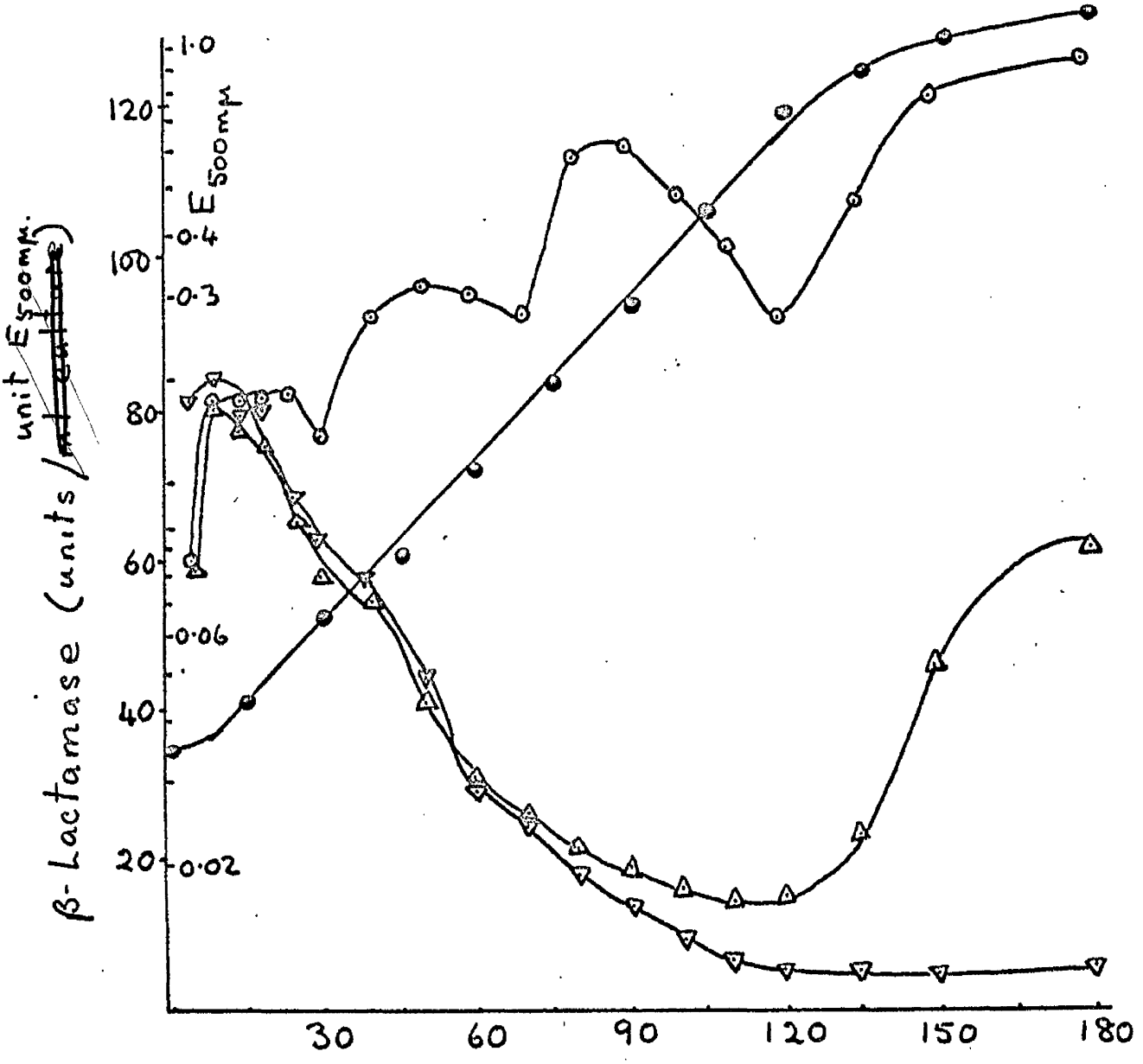


Fig 53

culture. The concentration of inducer in these three flasks was 50 μM, 0.5 μM and nil respectively. In the flask containing 50 μM inducer, the specific activity starts at a higher value and rises somewhat during growth in a series of waves. Specific activity in the other two flasks falls from the initial high value. In the culture with no inducer, specific activity continues to fall throughout growth. Howevever, the specific activity in the culture with 0.5 μM inducer, after falling for a considerable part of the growth, rises again towards the end. The cultures grew logarithmically from the beginning to 120 mins when they entered the very slow growth phase which I have called stationary phase. The differential rate of β-lactamase synthesis in 50 μM CBAP remains at a high value, (109 units/unit E_{500}) but is immediately reduced to a very low value (11 units/unit E_{500}) in 0.5 μM inducer, and nil in the culture containing no inducer. However at a turbidity of about 1.0 when the culture is entering stationary phase the differential rate in 0.5 uM accelerates to a very high value (170 units/unit E_{500}). (Fig. 54 p.53a).

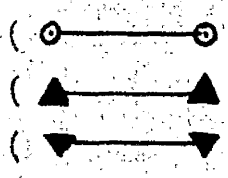
(h) Absorption of inducer.

Inducer was measured by hydrolysis in alkali to the corresponding penicilloic acid and subsequent reaction with starch/iodine solution. Although it proved possible to do this the difference between the amount measured before and after addition of cells was too small to be significant by this technique. To measure the absorption of inducer it would be necessary to resort to radioactive techniques.

(V11) Removal of inducer.

Fig. 55 (p.55b) shows the synthesis of β-lactamase on removal of inducer by filtration from a culture growing in defined medium. For some time β-lactamase increases at the same rate as in an induced culture and then synthesis falls off and stops.

Fig.54. Differential rate of β-lactamase synthesis in the cultures of fig. 53.



Differential rate of β-lactamase synthesis on changing the concentration of inducer

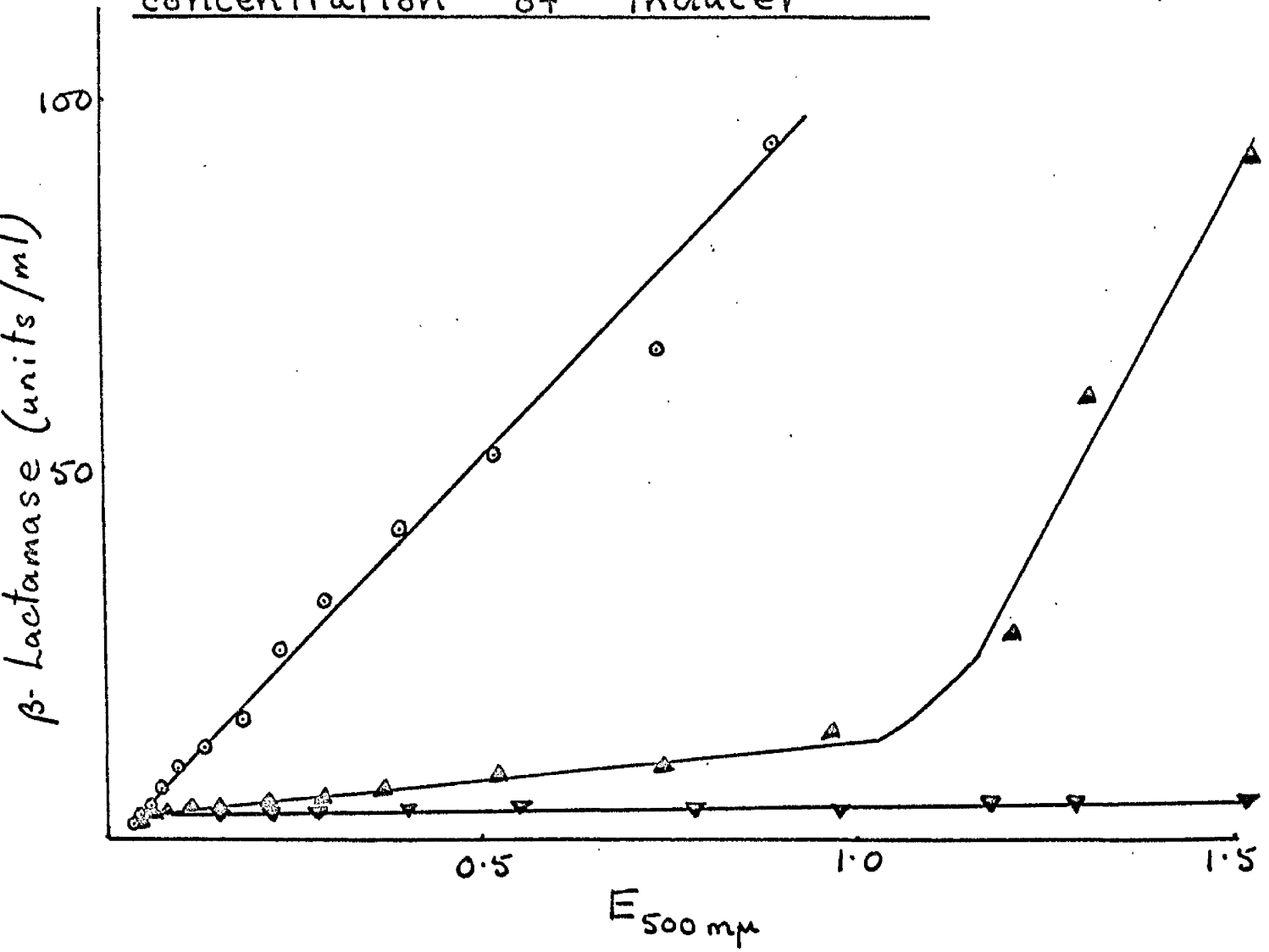
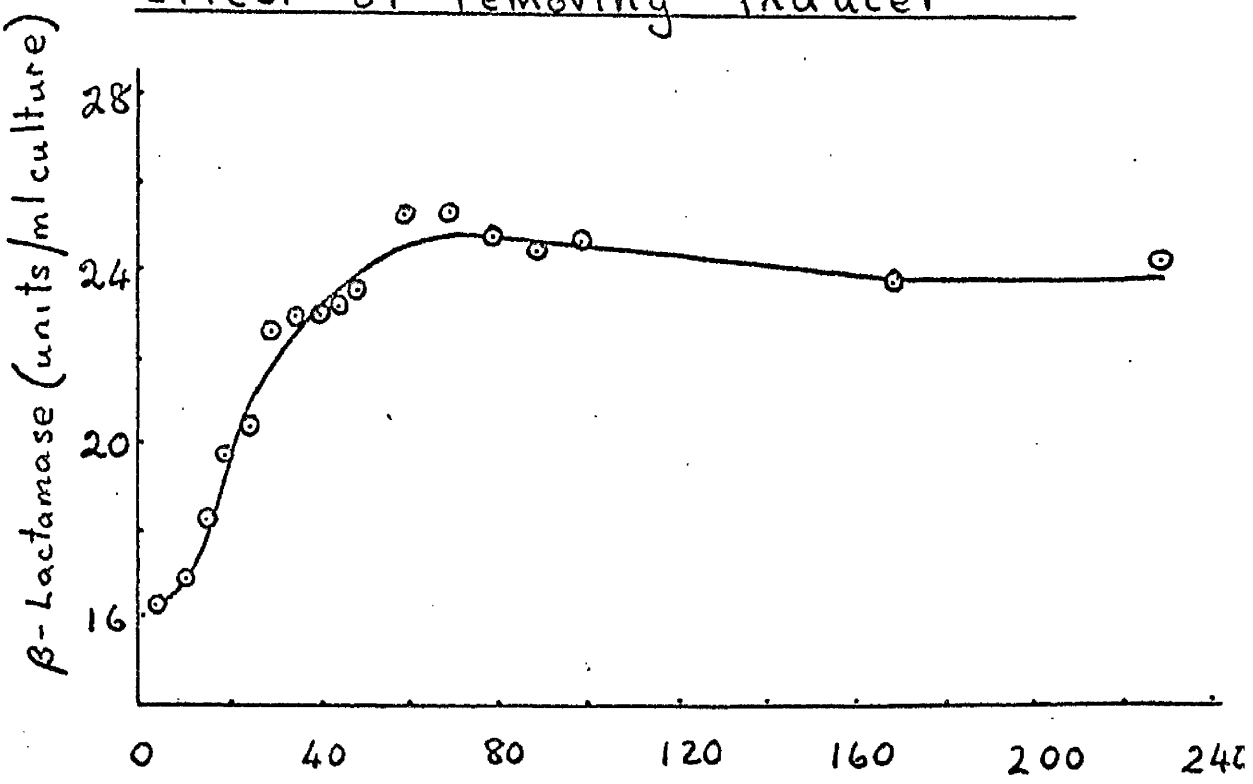


Fig. 54

Fig. 55. Removal of inducer from a culture of *S.aureus* C23/19 growing in defined medium containing 50 μM CBAP. The top curve shows enzyme *vs* time after removal of the inducer by filtering and washing the cells. The bottom curve is the logarithm of the decay of β-lactamase synthesis *vs* time. From this graph the half-life of β-lactamse synthesising ability is 12.5 min.

Effect of removing inducer



Time (min) after resuspension in inducer-free medium.

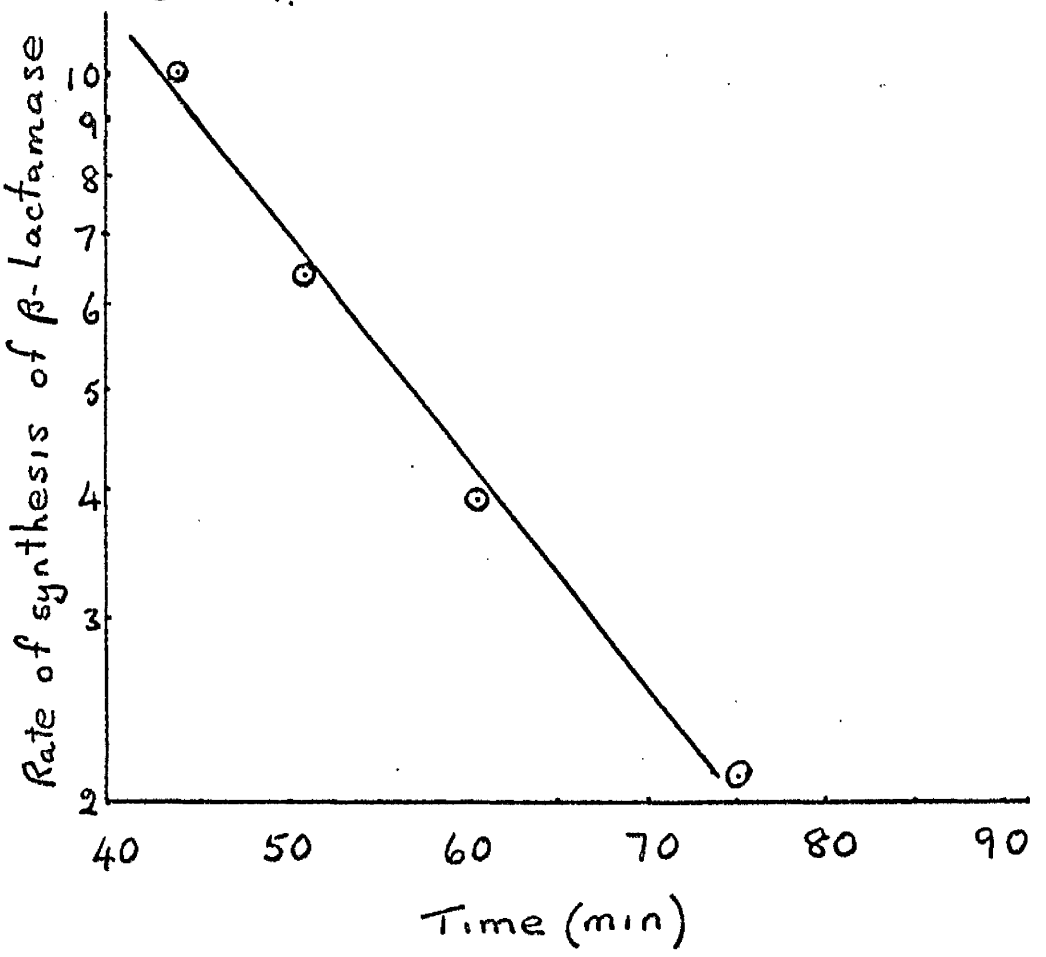


Fig. 55

The rate of decay of β-lactamase synthesising ability can be measured by plotting against time the logarithm of the enzyme synthesised in consecutive five minute periods. A straight line is obtained showing the fall off in enzyme synthesising capacity to be exponential with a half life of 12.5 mins. Similar plots are shown in fig. 56 (p.54a) for a culture growing in nutrient broth. The half-life of β-lactamase synthesising ability in this case is 5 mins.

The ratio of mean generation time in nutrient broth to that in defined medium is 23 mins./60 mins. = 0.383.

The ratio of the half-life of β-lactamase synthesising ability in nutrient broth to that in defined medium is 5/12.5 = 0.399. Therefore the half-life varies inversely with the rate of growth.

Fig. 56. Removal of inducer from a culture of *S.aureus* C23/19 growing in nutrient broth + glucose (0.2% W/V) containing 50 μM CGAP. The top curve shows enzyme *vs* time after removal of the inducer by filtering and washing the cells. The bottom curve is the logarithm of the decay of β-lactamase systhesis *vs* time. From this graph the half life of β-lactamase synthesising ability is 5 min.

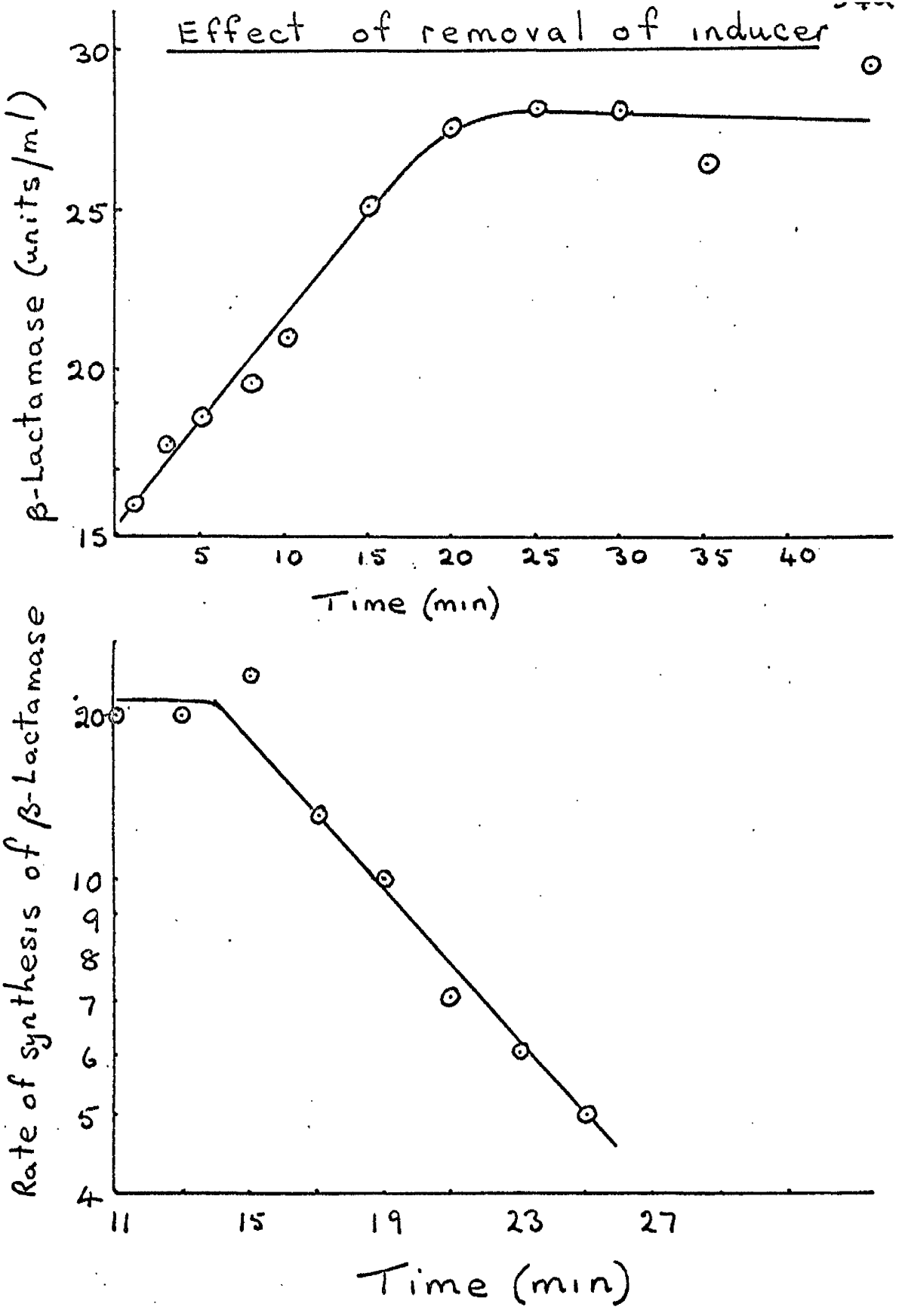

Effect of removal of inducer
β-Lactamase (units/ml)
30
25
20
15
5
10
15
20
25
30
35
40
Time (min)
Rate of synthesis of β-Lactamase
20
10
9
8
7
6
5
4
11
15
19
23
27
Time (min)

Fig 56

DISCUSSION

Assay of enzymes.

All enzymes were assayed using whole cells. The β-lactamase activity measured in whole cells is less than that obtained with a sonicated sample of cells (table 1 p.38d), but it has been assumed that the proportion of activity measured is constant in all circumstances. To measure the activity on sonicated samples might introduce errors, since the degree of stimulation may vary according to the physiological state of the cell. It would be difficult to assess the degree of stimulation as this does not depend on release of enzyme into the surrounding liquid. The enzyme remains particulate. The observed increase of activity could be due to the disruption of a permeability barrier. On the other hand, disruption of the cell could change the local environment of the enzyme, thus changing its activity. The implication that the local environment of the enzyme within the whole cell is not optimal for activity may well be true and could be a basis for control of activity. Propanol has been shown to stimulate activity of β-lactamase both in whole cells and in isolated enzyme preparations. (Saz et.al., 1964). This effect on the enzyme is reversible. (Holms, unpublished data). Thus it is conceivable that the activity could be controlled by changing the configuration of the enzyme. Thus, it becomes clear that we must be careful in interpretation of data such as obtained by sonic disruption of the cells. A stimulation of activity does not necessarily mean there is a permeability barrier which controls entry of substrate.

The particulate nature of the enzyme is unusual. Most strains of S. aureus have extracellular enzyme. Coles and Cross (1967) have shown that the extracellular nature of the enzyme is dependant on the ionic complement of the medium. It could be

that the medium used here is conducive to an intracellular binding of the enzyme.

To measure enzyme during growth, hibitane was used to stop enzyme synthesis at the time of sampling. This antibacterial acts by a general blockage of transport of materials into the cell. (Hugo and Longworth 1964 a & b). Hibitane stops growth and synthesis of enzyme just as quickly as chloramphenicol. (fig.20 p.44a and fig. 21 p.44b).

The stimulation of nitrate reductase activity by toluene (table 4 p. 39c) would support the argument that there is an accessibility barrier to some substrates in this organism. However, although toluene has been shown to destroy the β-galactoside permeability barrier in E. coli, the same reservations must be borne in mind here as were applied to the results of sonic disruption of the cell. The stimulation with toluene was not easily reproducible. The conditions of treatment with toluene were probably the variable factor here since this has been shown to be difficult to standardise in the treatment of E. coli for β-galactosidase assay. (Holms, unpublished observations). The assay of nitrate reductase must be carried out under anaerobic conditions because oxygen acts as an inhibitor competing for the electrons on the reduced cytochromes. Pewson and Nicholas (1961) showed that carbon monoxide could inhibit the action of oxygen and allow the assay to be performed in the presence of oxygen. In view of the similarity of action of cyanide and carbon monoxide on cytochromes, we decided to see if cyanide would allow the assay to be carried out under aerobic conditions. It did not. All nitrate reductase assays were therefore done under anaerobic conditions.

The very low content of β-galactosidase in this strain was unexpected. Creaser (1955) had shown the inducibility of β-galactosidase in S. aureus. He obtained activities 10 fold

greater than obtained in S. aureus C23/19. The low content of β-galactosidase made it less likely that any competitive effect on the synthesis of β-lactamase would be observed.

Induction of β-lactamase.

Addition of inducer to our strain of S. aureus increases the specific activity by a factor of 130 (p 50). The inducer used in this work is unique among available derivatives of penicillin in that the antibiotic activity is negligible while the inducing activity remains high. 2-(2'-Carboxyphenyl) benzoyl-6-aminopenicillanic acid (CBAP) is not a substrate for the enzyme. Thus for the first time, it has been possible to study the induction of β-lactamase over relatively long periods of time in S. aureus, an organism which requires the constant presence of inducer for induction. It can be used at much higher concentrations than methicillin (fig. 24 p.44f) without killing the cells. However it does have a small effect on the growth of S. aureus C23/19 (fig. 25 p.45a). It induces a greater specific activity of β-lactamase than either methicillin or cephalosporin C, which is a competitive inhibitor of β-lactamase. (Abraham & Newton, 1956). The use of CBAP as inducer has permitted the gratuitous synthesis of β-lactamase to be followed during growth under a variety of conditions.

Growth and β-lactamase synthesis.

Nutrient broth + glucose has been used as growth medium because this gave the longest logarithmic phase of growth. Comparisons of enzyme synthesis between different cultures are valid only if taken from the same stage of growth. For this reason, it was preferable to make measurements during logarithmic phase for as long as possible. Phosphate buffer added to the nutrient broth shortened the logarithmic phase and reduced the yield of cells in stationary phase. This effect is completely unprecedented as far as I know. Fresh phosphate solutions had the same effect.

The same batch of chemicals was added to make up media for other organisms in this laboratory and no inhibition was noted. Further investigation, however, is outside the scope of this work and nutrient broth + glucose was chosen as the most suitable medium.

The pattern of growth of this organism in defined medium was very difficult to reproduce. There was usually a lag of four hours which could not be reduced by addition of amino acids, removal of phosphate, or different regimens of inoculum training. Cultures were inoculated from a fresh inoculum without washing the cells. Washing the inoculum cells did not improve growth. Growth, once started, was often erratic and did not give a smooth exponential increase. All the experiments with defined medium described here, however, are experiments where growth was smooth and regular. The mean generation times obtained in defined medium varied a great deal for no apparent reason. The requirements of the organism as incorporated in this medium were determined by measurement of growth yield (Fildes et.al., 1936). More information may be obtained about the requirements if these were studied under conditions where growth rate and lag could be measured.

Growth and β-lactamase have been measured under various conditions. The specific activity of β-lactamase has been shown to vary somewhat during growth. In the experiments with different concentrations of CBAP (fig. 26 p.45b), the specific activity is constant during logarithmic phase but rises as the cultures enter stationary phase. In other experiments (fig. 29 p.46a fig. 53 p.52f) the specific activity varies during logarithmic phase. These periodic variations do not correspond to generations of growth. Variations of this kind throw doubt on the interpretation of earlier data (table 8, p. 42b) which measured the specific activity at one point in a growth curve. Nineteen different

carbon sources support specific activities within a narrow range with the exception of arabinose and sucrose which give somewhat higher values. In view of the variations noted in specific activity during growth and the differences in turbidity of these cultures at the time of measurement, I hesitate to say that more enzyme is synthesised in these cases. When these two carbon sources in nutrient broth are compared with glycerol, glucose and lactose, the differential rates of β-lactamase are indistinguishable. The differential rate of enzyme synthesis is a much more meaningful measurement in this case and we must conclude that these carbon sources support the same differential rate of β-lactamase synthesis.

These periodic variations in β-lactamase synthesis may be associated with some degree of synchrony in the growth of the culture but no other indications of synchrony have been observed. If a sufficient number of samples is taken, degrees of synchrony can be detected by turbidity measurements but in most of the experiments an insufficient number of samples was taken for this to be noticed. Similar variations in specific activity have been observed during studies on a derepressed β-galactosidase strain of E. coli in this laboratory (Holme, unpublished observations). They have also been demonstrated in continuous culture of Pseudomonas aeruginosa (Boddy, Clarke & Lilly, 1966) in studies with an inducible amidase. Presumably this is an indication that the mechanisms controlling enzyme synthesis are not finely adjusted and there is a degree of overshoot when they are applied and released. One can envisage such a control mechanism operating in the case of amidase where presumably some product of the reaction represses the formation of the enzyme. Amidase level would be controlled by end product repression, a well known phenomenon. However, application of this model to β-lactamase synthesis requires that there be a corepressor of β-lactamase

synthesis. To date there has been only one report of catabolite repression of β-lactamase synthesis. (Yip et.al., 1964). These authors claim that 6-amino-penicillanic acid represses the formation of β-lactamase in B. cereus. Nevertheless, even if a corespressor of β-lactamase does exist, the inducer used in these experiments (CBAP) is not hydrolysed by the enzyme (Holms, unpublished data). Thus no corespressor would be formed. Similar arguments apply to the derepressed β-galactosidase in E. coli under gratuitous conditions.

Other possibilities can be considered. Firstly, β-lactamase could act on an endogenous substrate to give a product which is a corepressor of enzyme synthesis. Tipper and Strominger (1965), studying the mechanism of action of penicillin, have suggested that penicillin inhibits the final cross-linking of peptide chains in the formation of the cell wall. Since certain peptides (Saz et.al., 1964) have been shown to be inducers of β-lactamase, it is tempting to suggest that β-lactamase has a natural role to play in the synthesis of the bacterial cell wall. It would then not to be too great a step to suggest that cyclic changes were controlled by the production of a corepressor from the natural substrate. It has always been a mystery why bacteria should possess β-lactamase since penicillins are not widespread in nature. Although organisms which possess the enzyme would have a selective advantage in an environment containing penicillin, there must be very few such environments. One has been found in hedgehogs in New Zealand. These animals are heavily infected with the dermatophyte Trichophyton mentagrophytes which produces a penicillin-like antibiotic. The S. aureus strains isolated from the skin and nose of these animals are predominantly β-lactamase producers. (Smith & Marples, 1964). However, if there is an internal substrate for β-lactamase one would expect that this substance would also act as an inducer and it does not do so in this organism.

On the other hand, if the substrate is mucopeptide the spacial separation of this from the site of induction might prevent the induction.

The second possibility depends on the work of Yip et.al. (1964) who suggested that there are two points of control in the synthesis of β-lactamase; one at the level of transcription of the DNA, and the other at the level of translation of the mRNA. If there is a mechanism controlling protein synthesis which is distinct from the genetic regulatory mechanisms already known, these periodic changes could be explained by invoking this mechanism. However, it is difficult to envisage a mechanism of control which is at the same time specific and does not operate through the gene. Any such mechanism, in order to be specific, must act on the mRNA since ribosomes are non specific in their action. There is little evidence for such a mechanism.

In considering periodic changes in specific activity, it is important to realise what I define as specific activity. This is the number of units of enzyme per unit of turbidity. Ideally one should express this as units/mg of protein. It has been shown many times in this laboratory and others that protein is directly related to turbidity during growth of a culture. I have not converted my turbidity measurements to protein so that it may be clear that protein was not actually measured in most cases. It is possible that, under some circumstances, turbidity and protein are not directly related. This is illustrated in fig. 32 (p.47a) where a partially synchronous culture was sampled at frequent intervals for turbidity, protein, and β-lactamase. It can be seen that turbidity and protein do not exactly correspond during growth. The ratio of turbidity to protein ranges from 0.010 to 0.012 in this experiment. Periodic changes in enzyme activity could result from variation in the relationship of turbidity to protein.

Although cyclic changes in specific activity may not reflect changes in enzyme relative to total protein, discontinuous β-lactamase synthesis occurs in synchronous cultures (fig. 34 p.48a). The turbidity in this case shows little sign of synchrony but since turbidity is probably a measure of the mass of the culture rather than number of cells this is not surprising. The increments in level of enzyme are doublings. Such periodic enzyme synthesis has been explained on the basis of gene repression by Pardee (1965). He suggests that the burst of enzyme synthesis comes at a time when the gene is being replicated and that repression is applied soon afterwards. Several workers (Masters & Donachie, 1966; Masters, Kusmpel & Pardee, 1964) have shown that the timing of enzyme synthesis in the division cycle of bacteria is always the same. The order of synthesis of several enzymes corresponds to their linear order in the gene. This suggests that translation of the mRNA is closely associated with replication of the genes. Such a mechanism would explain the discontinuous synthesis of β-lactamase in the absence of any known corepressor formation. There is no evidence on how such a mechanism would work. There is a lot of evidence which suggests that DNA is replicated sequentially. When one considers the tangle of DNA which probably exists inside the cell, one realises that it is a major problem to organise not only its replication but also the separation of the two strands. Pollock (1966) in a lecture given in Glasgow University drew attention to this problem and suggested that the DNA might be moved into position so that only the parts on the perimeter of the mass of DNA are replicated. This would facilitate the separation of the two chromosomes. If such a mechanism exists, it is a small step to postulate that genes are only transcribed while at the perimeter of the mass. Discontinuous enzyme synthesis would follow.

β-Lactamase was measured during growth in defined medium and nutrient broth + glucose at different temperatures. The Differential rate of enzyme synthesis was independent of temperature. (fig. 30 p.46b). If one considers the rate of doubling as the rate of synthesis of enzyme, it would appear that at 37°C in defined medium (fig. 30 p.46b) the rate of synthesis of enzyme is greater than the rate of synthesis of mass. However, the differential rate shows that the increase in enzyme is a constant proportion of the increase in mass at all temperatures including 27°C in defined medium. This apparent anomaly is discussed further in the section on induction of β-lactamase

Aeration of cultures.

The degree of aeration was measured in growth flasks as the rate of oxidation of sulphite (Cooper, 1944). To equate the rate of sulphite oxidation with the degree of aeration actually taking place in the culture is a very different matter. The literature on this subject is vast and complex and a discussion would be out of place here. Essentially the data on the rate of oxidation of sulphite show that the magnetic stirrers used for growth of the cultures do give a reproducible degree of aeration (table 5 a&b p. 40 c&d). For the purposes of comparing enzyme synthesis under different growth conditions this was what mattered. It could be guaranteed that each flask, being stirred at the same rate, was being aerated at the same rate. The rate of oxidation of sulphite was proportional to the partial pressure of oxygen in the atmosphere thus showing that the system for mixing oxygen and nitrogen was giving mixtures with the calculated composition. The rate of oxidation of sulphite also showed that the gas phase in the flasks could be changed almost instantaneously (fig 10 p. 40a).

In the last analysis, the only way to measure the efficiency of aeration is to grow the culture and observe the result.

Various volumes of culture in the growth flask did not alter the rate of growth or the differential rate of β-lactamase synthesis (Table 11 p.45d). However, a flask with 400 ml of culture was more susceptible to inhibition by high oxygen tension than a flask with 800 ml of culture. There must, therefore, be greater access to the cells in a flask containing 400 ml of culture. Although growth varied, the differential rate of β-lactamase synthesis remained constant with different partial pressures of oxygen and different volumes of culture in the flask (table 12, p.45e). Again the use of the differential rate as the best parameter for comparison is illustrated.

In defined medium, S. aureus C23/19 does not grow anaerobically. In nutrient broth + glucose there is only a small amount of growth anaerobically. The specific activity in nutrient broth + glucose is four times higher when the culture is grown aerobically than when grown anaerobically. The plot of the differential rate (fig. 28 p45d) of β-lactamase synthesis shows that during the early stages of growth the differential rate is not very different from that in the aerobic culture. However, β-lactamase synthesis ceases before growth ceases and thus the specific activity falls (fig. 27 p.45c). A four fold difference in specific activity on the basis of turbidity may not be so great on a protein basis. This would be especially true if turbidity did not bear the same relationship to protein aerobically as it does anaerobically. If, for example, a capsule was formed anaerobically, turbidity would rise but protein would not. It would be desirable to make protein measurements if this type of experiment were pursued further.

In defined medium, S. aureus grows anaerobically in the presence of nitrate. Changing a culture from aerobic to anaerobic growth in the presence of nitrate induces the enzyme nitrate reductase (p.39). The differential rate of β-lactamase

synthesis does not vary throughout this process (fig. 16 p.43b). In a similar culture, addition of CBAP during the anaerobic growth in presence of nitrate in nutrient broth gives synthesis of β-lactamase which appears to be different from that in an aerobic culture (fig. 18 p.43d) until the differential rates of synthesis are examined. (fig. 19 p.43e). It is then clear that there is no difference between these cultures in capacity to induce β-lactamase.

During some experiments in defined medium, enzyme levels appeared to fluctuate erratically. For technical reasons, the stirrers had to be stopped occasionally for short periods (about 15-30 seconds). Since aeration in the cultures depends on stirring, short periods of anaerobiosis were deliberately created to see if this affected enzyme levels. No effect was observed on the specific activity in growth flasks which had nitrogen passed in for periods up to 8 minutes. The observed fluctuations, therefore, were not due to short periods of anaerobiosis. Simply stopping the stirring for short periods had no effect. In the flask which was made anaerobic for 16 minutes, the specific activity falls and is apparently brought back to normal during subsequent growth. Pollock (1953) has shown that the processes underlying the latent period of induction of β-lactamase in B. cereus are inhibited by anaerobiosis. It is possible that during this short period of anaerobiosis, these processes were inhibited in S. aureus and later enzyme synthesis was induced. This possibility is worth further investigation.

Competition among enzyme systems.

The rate of synthesis of β-lactamase gratuitously induced in S. aureus 623/19 has been measured under a variety of conditions of growth. It has emerged that the differential rate of β-lactamase synthesis remains surprisingly constant during wide variations in growth.

There is no substantial change in differential rate of β-lactamase synthesis when a culture in nutrient broth is grown aerobically or anaerobically. Variations in the partial pressure of oxygen during growth in nutrient broth does not affect the differential rate of enzyme synthesis. Even oxygen poisoning does not alter it.

Concurrent induction of β-galactosidase and β-lactamase, as well as the enzymes probably induced for the metabolism of galactose, does not change the differential rate of β-lactamase synthesis. It again remains unchanged when β-lactamase and nitrate reductase are concurrently induced. During growth in nutrient broth supplemented with five different carbon sources, the differential rate is again unchanged. Specific activity measurements indicate that, in defined medium, the differential rate of synthesis is unchanged during growth in nineteen different carbon sources. Even growth in the two widely different media, nutrient broth and defined medium, produces no substantial change in the differential rate of enzyme synthesis.

The constancy of the differential rate of β-lactamase synthesis when widely different demands are made on the metabolic capacity of the cells may be a function of the fact that the enzyme is gratuitously induced. In a non gratuitous system one would expect variations in the synthesis of the enzyme due to mechanisms such as end product inhibition and repression. Measurements with a gratuitously induced enzyme would only show competitive effects which acted on mechanisms of protein synthesis common to several enzyme systems. The mechanisms concerned with induction itself are thought to be very specific. The constancy of the differential rate of a completely gratuitous enzyme, β-lactamase, under widely different growth conditions would support this high specificity. The supply of common intermediates such as amino acids is probably adequate in these media.

Therefore no competition is evident due to lack of supplies of these intermediates or energy.

Induction of β-lactamase.

We thought the sharp rise in level of enzyme on addition of CBAP reflected the time required for the processes of induction to be completed. This was apparently a lengthy process. It seemed that the initial rapid rise took one generation and was followed by the steady state rate of doubling (fig. 37, p.49a). The sharp rise was first noticed in defined medium with CBAP when cultures were grown from different sizes of inocula (fig. 34, p. 48a) and β-lactamase measured from the time of inoculation. In the culture grown from 2% inoculum, the initial rise is not noticeable. Only at the higher cell densities is it evident. Thus, this phase was missed in other experiments which usually started from a 1% inoculum.

On examination of this initial phase in more detail (fig. 38, p.49b and fig. 39, p.49c) we see that a plot of logarithm of enzyme *vs* time shows a rise in level of enzyme which is a smooth curve, not two distinct phases. Plotting these same data on an arithmetic scale gives fig. 41 (p.50b) and fig. 40 (p.50a) which is the conventional way to plot the data for the initial kinetics of induction. This plot reveals a lag in enzyme synthesis followed firstly by a short acceleration period and then an apparently linear synthesis of enzyme. In nutrient broth the lag is 3 minutes and the acceleration four minutes. In defined medium these two phases together take about 15 minutes. The latter figure is in agreement with similar experiments in *B. cereus* (Pollock, 1952; Leitner *et.al.*, 1963). These values are related to the rate of growth supported by the medium since they are shorter in nutrient broth where the mean generation time is shorter. This could, of course, simply mean that nutrient broth supplies the necessary intermediates more readily. The fact that synthesis

of enzyme appears to be linear was not expected since the culture is growing logarithmically. Another surprising fact emerges on further consideration of these kinetics (fig. 42 p.50c and fig. 43, p.50d). The specific activity attained depends primarily on the stage of growth reached when inducer is added. This is in agreement with several workers who have shown that increasing cell density gives less enzyme on induction (Chaikovskaya, 1964; Leitner et.al., 1964). The specific activity in our cultures does eventually reach the same value but only when the culture has ceased to grow logarithmically (at about 9 hours). The phase of growth immediately after the logarithmic phase in these cultures is not stationary phase. Growth continues for some time but at a much reduced rate.

The kinetics of induction observed are most easily explained by consideration of a hypothetical culture growing logarithmically at a rate which is close to that of the experimental cultures. If we choose a culture which is doubling at a rate of once every 24 mins and draw a straight line on semi-log paper to represent this growth, we can read from the graph the number of cells at any given time. Table 14 (p.68a) shows the results obtained for such a theoretical culture. The number of cells at each time is shown in column 3. Consider now a constitutive enzyme. The hypothetical culture will synthesise enzyme at the same rate as all other components of the cell. Enzyme will, therefore, double at the same rate as cell number. Therefore, the figures for cell number can be used to represent the amount of enzyme at these times. These are shown in column 4. The amount of enzyme synthesised in each interval can be found by difference. These are shown as Δ enzyme in column 5. Now consider an inducible enzyme in the same culture. Inducer is added to the culture at 12 minutes. Assume that, from the moment of addition of inducer, this culture synthesises induced enzyme at the same rate as the

Table 14. Growth and enzyme synthesis in a theoretical culture in which enzyme is synthesised at the same rate in both induced and constitutive strains.

Time	Number of generations	Number of cells	constitutive enzyme	Δ enzyme	Induced enzyme
0	0	100	100		0
				12	
4	1/6	112	112		0
				14	
8	2/6	126	126		0
				15	
12	3/6	141	141		0
				18	
16	4/6	159	159		18
				19	
20	5/6	178	178		37
				22	
24	1	200	200		59
				24	
28	1.1/6	224	224		83
				27	
32	1.2/6	251	251		110
				30	
36	1.3/6	281	281		140
				35	
40	1.4/6	316	316		175
				39	
44	1.5/6	355	355		214
				45	
48	2	400	400		259
				49	
52	2.1/6	449	449		308
				51	
56	2.2/6	500	500		359
				60	
60	2.3/6	560	560		419

constitutive enzyme. Since we know how much constitutive enzyme is synthesised in successive time intervals, we can calculate the total induced enzyme present from the moment of addition of inducer. These values are shown in column 6. A graph of logarithm of enzyme vs time from the moment of addition of inducer for this hypothetical culture is shown in fig. 57 (p.69a). The enzyme level rises in a smooth curve and approaches the steady state doubling time (represented by the dotted line) asymptotically. Thus we have an explanation for the sharp rise in enzyme synthesis noted in our cultures on addition of inducer. Fig. 58 (p.69b) shows the plot of theoretically induced enzyme vs time. The level of enzyme rises in a shallow curve. With actual experimental data, it is not possible to differentiate between such a shallow curve and a straight line and most workers simply draw a straight line. However, it is important to keep in mind that this is not a linear synthesis of enzyme. The theoretical graph of enzyme vs number of cells is shown in fig. 59 (p. 69c) where we see that the differential rate of enzyme synthesis is constant from the moment of addition of inducer. This is analagous to the result found by Herzenberg (1959) for β-galactosidase in E. coli.

The observed kinetics of β-lactamase synthesis are consistent then with the operation of a constant differential rate of enzyme synthesis from soon after addition of inducer. However, the consideration of the data presented here has brought to light to light some apparent anomalies which should be emphasised. If we look at doubling times as a measure of the rate of synthesis of enzymes we are liable to be misled since a graph which shows a very fast doubling time followed by a slower doubling time (fig. 57 p. 69a) in fact has the same differential rate of synthesis throughout these changes. In considering the data for the effect of temperature on synthesis of β-lactamase

Fig. 57. Induced enzyme synthesis in a theoretical culture. The dotted line is the steady state rate of doubling.

Induction of an enzyme in a hypothetical culture

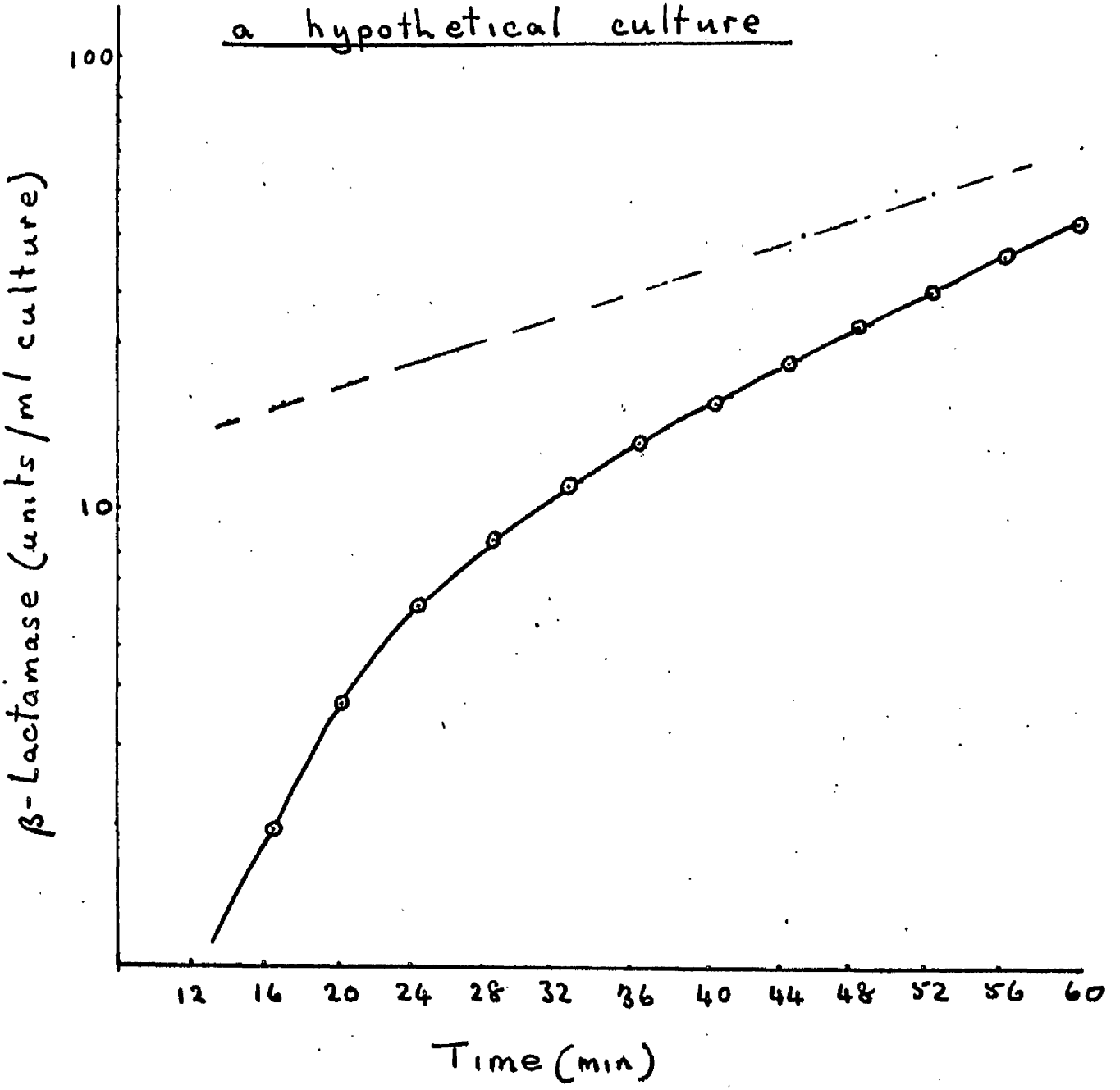


Fig. 57

Fig. 58. Arithmetic plot of the data from Fig. 57.

Induction of an enzyme in a hypothetical culture

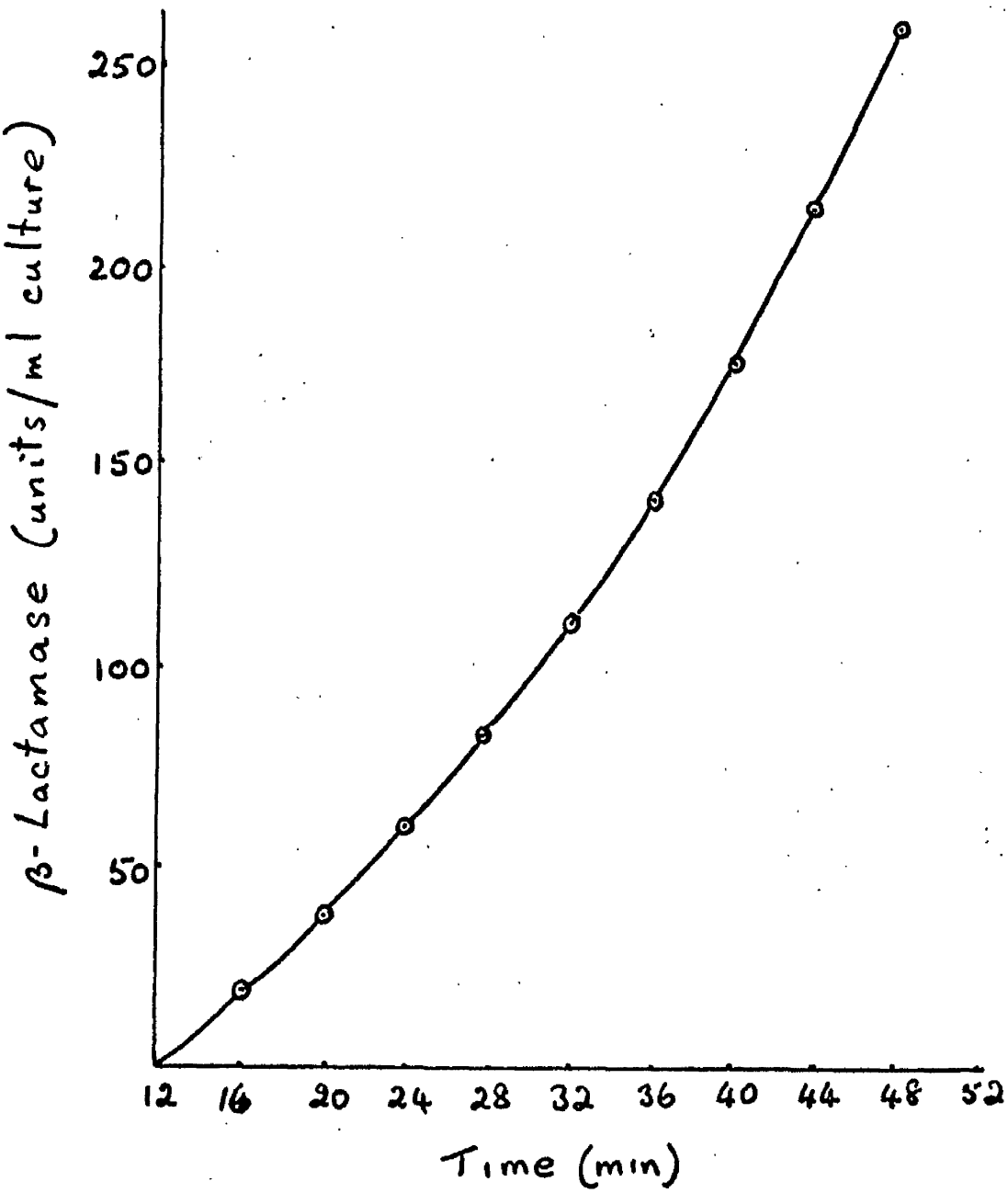


Fig. 58

Fig. 59. Differential rate of enzyme synthesis in the theoretical culture.

Induction of an enzyme in a hypothetical culture

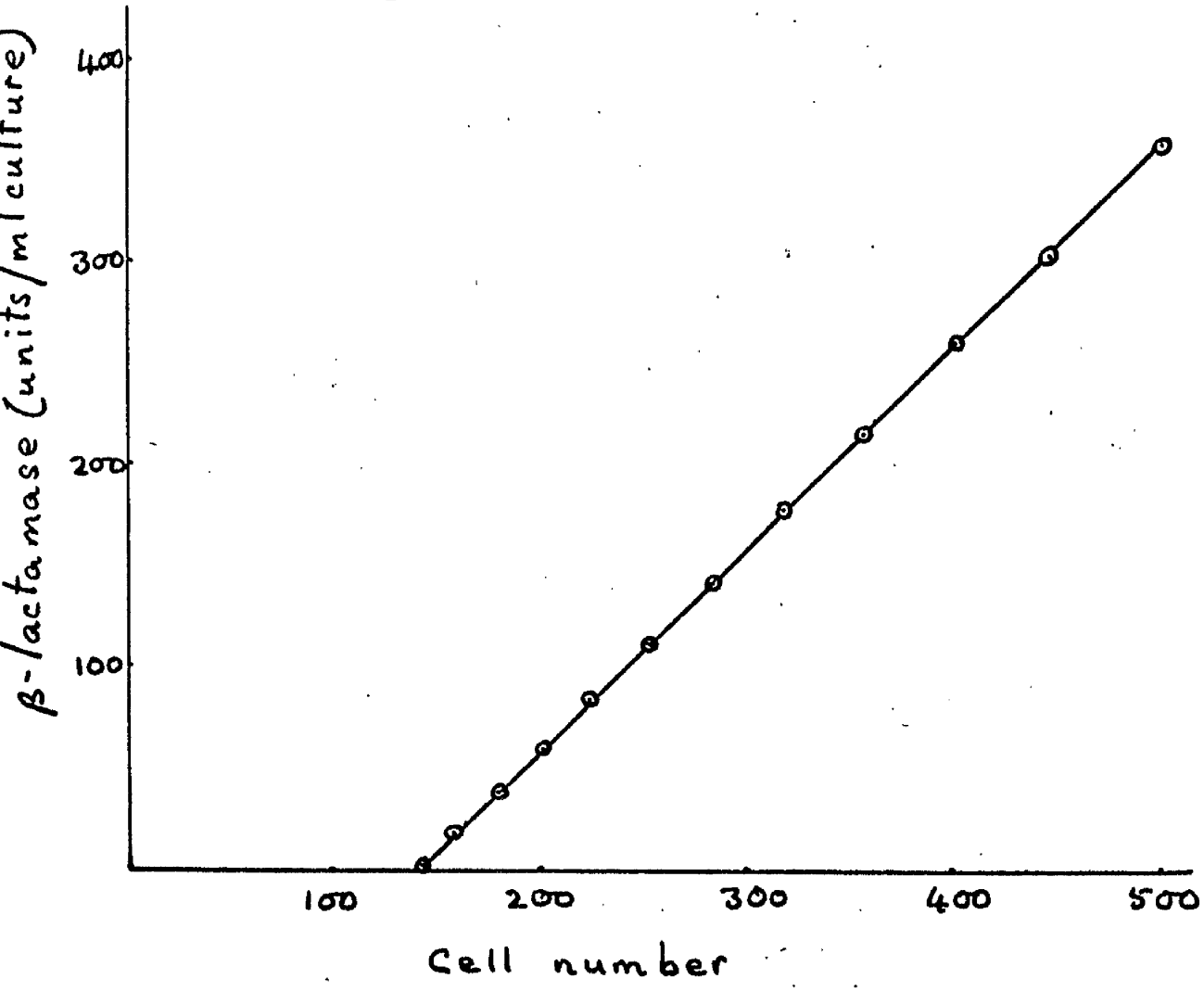


Fig. 59

(figs 30 & 31 pp 46b&c) it was noted that invariably the doubling time of enzyme was greater than that of turbidity. When we consider that the level of enzyme approaches the steady state doubling time asymptotically, it becomes clear that in these cultures we are observing the latter part of the theoretical curve whown in fig. 57 (p 69a). Thus it isnot valid to consider doubling times of enzyme as a measure of the rate of synthesis. The theoretical culture also explains why the specific activity varies with the stage of growth when inducer is added. The asymptotic approach to the steady state doubling time shows that the specific activity cannot be expected to be the same until a much longer time has passed than is available in one growth cycle. Although specific activity measurements in the first growth cycle are likely to give misleading results when considering enzyme synthesis, this is the parameter which matters when considering the organism's ability to destroy penicillins. These kinetics show that the organism's ability to destroy penicillins increases markedly diring the first generation time and more slowly thereafter and does not reach its maximum until well after the first growth cycle. The implications of this for therapy are that it would probably be better to apply a large dose of penicillin initially rather than smaller doses repeatedly.

The differential rate of β-lactamase synthesis.

Plots of β-lactamase vs turbidity for the experiments where inducer was added at different stages of growth (figs. 44-46 pp 50e-51b) show that the differential rate is constant from shortly after addition of inducer. They also show that the later in growth inducer is added the more pronounced becomes the lag before a constant differential rate of β-lactamase synthesis is established. A lower differential rate initially, changing to a higher rate after a short time is seen in many experiments. (e.g. fig. 47, p.51c; fig 40, p.50a; & fig 48, p.52a). This

is now recognised to be related to the cell density. The specific activity when inducer is added at different stages of growth will be affected by this lag. Thus the reasons for different specific activities are two fold; the asymptotic approach to the steady state doubling time and the lag in establishment of a constant differential rate in later stages of growth.

Experiments with low concentrations of CBAP demonstrate a lag before establishment of the constant differential rate. (fig. 52, p.52e). These kinetics at low concentration of inducer are similar to those obtained with β-galactosidase in E. coli containing a permease (Cohn, 1957). Does S. aureus 023/19 possess a permease for CBAP? The data for addition of inducer at different stages of inducer would be consistent with this since increasing the cell density is effectively the same as reducing the concentration of inducer. Combine this with the experiments on sonic disruption of the cells and there is strong evidence for a permease activity in this organism. However, examination of the β-lactamase of a fully induced culture switched to a lower concentration of CBAP, still reveals a lag in establishing the constant differential rate of enzyme synthesis. This lag cannot be attributed to a permease. One would expect the permease to be fully induced after growth in 50 μM CBAP and there should be no lag on switching to 0.5 μM inducer.

These data can be considered in another way. Induction with low concentrations of inducer always shows a low differential rate initially and as the culture enters stationary phase, the differential rate changes to a much higher value. An increase in differential rate is seen in other experiments when the culture enters stationary phase. (see e.g. fig 28, p.45d). Although the data of fig. 52 (p.52e) can be considered as showing an acceleration at low concentrations of inducer, a low differential rate initially would also be consistent with the data. In

view of the data from experiments on switching from high to low concentration of CBAP, this is probably the correct view.

The increase in differential rate of β-lactamase synthesis in the slow growth after logarithmic phase is dramatic in the case of 0.5 μM CBAP. The increase with 50 μM CBAP (fig 28, p.45d) is not so marked but is nevertheless, definite.

I have mentioned earlier (p. 61) that in some circumstances, turbidity measurements may not be an accurate reflection of protein content. Such a discrepancy could explain the rise (or occasionally fall) of specific activity as the culture enters the very slow phase of growth after logarithmic phase (see e.g. fig 26, p.45b). However, the discordance between protein and turbidity would need to be very large indeed, to account for the increase in differential rate observed with low concentration of CBAP in the last phase of growth.

A possible explanation of this phenomenon presents itself by consideration of the half-life of mRNA for β-lactamase. Many reports have suggested that β-lactamase has a mRNA with a relatively long half-life. (Pollock, 1963; Yudkin & Davis, 1965) and, further, that constitutive strains have β-lactamase mRNA with a longer half-life than that in inducible strains. (Yudkin, 1966). I have measured the half-life of β-lactamase synthesising ability in this organism on removal of inducer by filtration. (figs. 55 & 56 pp53b & 54a). It is assumed that withdrawal of inducer causes cessation of synthesis of mRNA and the subsequent synthesis is due to the mRNA already synthesised up to this time. The resulting fall in ability to synthesise β-lactamase is a measure of the half-life of mRNA. In practice, there was always a short period of time during which enzyme was synthesised linearly before the rate of synthesis declined. This is presumably

due to small quantities of inducer remaining in the cell or to the release of mRNA, whose synthesis was already initiated at the time of inducer withdrawal. However, when synthesis of enzyme finally stops it does so rather slowly and the derived curve shows that the decay in rate of enzyme synthesis is exponential both in defined medium and complex medium. On the assumption that enzyme synthesis stops because inducer has been diluted out to a concentration which no longer stimulates the synthesis of mRNA, the latter part of these curves gives a measure of the half-life of mRNA. These were found to be 12.5 minutes in defined medium and 5 minutes in nutrient broth. Either of these figures is much longer than the usually quoted figure of 2 minutes for mRNA in general in bacteria.

Actinomycin D inhibits DNA primed RNA synthesis (Levinthal, Keynan & Higa, 1962). It has been shown to act on S. aureus. (Kirk, 1960). Its use to measure the half-life of mRNA is well established. I attempted to confirm the half-life values by the use of this antibiotic, but found that actinomycin D had a variable effect on S. aureus. In the graph shown (fig. 22 p.44c) actinomycin D terminates β-lactamase synthesis almost immediately but turbidity increased for some time. Another experiment showed the same effect on growth but for some reason enzyme assays in the flask containing antibiotic was very erratic. Unfortunately, there was insufficient actinomycin D available to follow this up. The result is that I have not yet confirmed these half-life values using this method.

Kepes, (1963) has calculated the half-life of mRNA for β-galactosidase on the assumption that the approach to steady state of synthesis is exponential and depends on the half-life of mRNA. Attempts to calculate the half-life of β-lactamase mRNA from such data reveal that the approach to steady state is not exponential in S. aureus but linear. This is the result

expected from a mRNA with a long half-life since decay would be negligible during this phase. The half-life, then, of β-lactamase mRNA in S. aureus is relatively long.

In the last phase of growth, the environment has considerably changed. The pH has dropped, toxic products have probably accumulated and other changes have no doubt taken place. These changes have slowed down growth by inhibition of one or more vital enzymes. This will result in accumulation of some materials and disappearence of others with the result that some enzymes will be repressed by end-products and others will not be induced because their inducers have been used up. In general, this phase will be a phase of reorganisation of the enzyme complement of the organism. However, β-lactamase is a truly gratuitous enzyme in this system. It takes no part in the metabolism of the cell and its inducer is not used up. It can then continue to be synthesised as long as β-lactamase mRNA remains.

Since mRNA decay appears to be related to the growth rate, β-lactamase mRNA would decay much more slowly in this last phase of growth. Therefore, the synthesis of the enzyme can continue, probably at the same rate as before and, since growth is much less, the differential rate increases. Leitner et.al. (1963) have shown that β-lactamase synthesis, in the absence of inducer in S. aureus, continues for much longer at pH 5.4 than at pH 7.4. Since pH has dropped in our cultures at the last phase of growth there could be a similar mechanism acting in both cases. Pinsky and Stokes (1952) showed that adaptability increased in late phases of growth. Presumably some similar mechanisms are operative here. The slow decay of mRNA could also explain the fact that it is possible to induce formation of enzymes in washed cell suspensions in the absence of a source of energy. In these circumstances only a small amount of mRNA synthesis would be required to give detectable amounts of enzyme since the mRNA

would not be broken down. This is supported by the work of Pardee (1955) who showed that in cell suspensions of pyrimidineless mutant of E. coli, enzyme was induced to a much greater extent in the presence of uracil and absence of energy source than in the presence of uracil and presence of energy source.

This last phase of growth is probably closer to the natural condition of bacteria, since their environment is probably that of slow growth in an exhausted medium. In any given environment a given complement of enzymes would be required. On changing to a new environment, as probably happens frequently in nature, a new complement of enzymes will be required. Obviously the organisms which can adapt more quickly will be selected out. To conserve the use of energy and materials, it would be essential that synthesis of useless enzymes be shut off quickly. Hence, there would be a tendency towards organisms which have a mRNA with a short half-life. Why does β-lactamase mRNA then, have a relatively long half-life? To answer this question one would need to know much more about the natural role of β-lactamase. However, the fact that it plays no direct role in metabolism of compounds for energy may be of some significance.

If the synthesis of β-lactamase continues in the last phase of growth because mRNA is broken down much more slowly, it raises the possibility that enzyme synthesis can be controlled by variation in the stability of mRNA. Yudkin (1966) has suggested that the decay of mRNA for β-lactamase is accelerated by the product of the regulator gene, the repressor. Because of the change observed in β-lactamase synthesis in going from logarithmic phase to the last slow phase of growth, this phase may provide a useful environment for the study of the control of mRNA decay.

All this is conjecture, however. The rate of decay of enzyme synthesis suggests that β-lactamase has a mRNA with a relatively long half-life. At

low concentrations of inducer, there is a marked increase in the differential rate of β-lactamase synthesis on going from logarithmic phase of growth to the last slow phase of growth. This increase occurs at higher concentrations of inducer but is less marked. Wide variations in growth conditions have demonstrated no other situation in which the differential rate of β-lactamase synthesis varies provided the concentration of this completely gratuitous inducer is constant.

Conclusions.

A completely gratuitous inducer, CBAP, allows synthesis of β-lactamase to continue at the same differential rate during logarithmic growth regardless of the conditions of growth. The other metabolic activities of the cell do not influence the differential rate of synthesis of β-lactamase.

Growth at several different temperatures in two different media does not alter the differential rate. Neither does growth from different sizes of inocula where, in defined medium, the growth rates are widely different. Growth anaerobically gives much the same differential rate as growth aerobically. Different partial pressure of oxygen during growth again gives the same differential rate of β-lactamase synthesis. Even conditions of oxygen poisoning give the same differential rate.

Induction of other enzymes does not affect the differential rate. For example induction of β-galactosidase or nitrate reductase does not alter it; neither does the induction, which must occur, of the enzymes for metabolism of nineteen different carbon sources. Even growth in defined medium produces no substantial change from that obtained in a complex medium.

From all this we must conclude that induction of a completely gratuitous enzyme by a completely gratuitous inducer does indeed give gratuitous synthesis unaffected by the other metabolic activities of the cell.

SUMMARY

β-Lactamase is an inducible enzyme in Staphylococcus aureus C23/19. Until recently, the only available inducers were powerful antibacterials which were hydrolysed by the enzyme. Those which were not hydrolysed by the enzyme were either weak inducers or had antibacterial activity. CBAP, 2 (2'-carboxy - phenyl) benzoyl-6-aminopenicillanic acid, combines insensitivity to β-lactamase with minimal antibacterial activity while still retaining activity as an inducer.

Using CBAP, the differential rate of β-lactamase synthesis has been determined on cells growing under accurately reproducible conditions. The rate is affected by a wide variety of growth conditions. Among these were growth at different temperatures in both a defined medium and a complex medium, which produced a wide variation in growth rate; aeration by mixtures of oxygen and nitrogen where the percentage of oxygen varied up to 50%; even when growth was slowed down by high oxygen tension, the differential rate remained the same. Very little variation is seen in the differential rate when the culture is grown anaerobically in nutrient broth compared to an aerobic culture. Growth from different sizes of inocula in defined medium results in different growth rates but the differential rate of β-lactamase synthesis is unaltered. Concurrent induction of other enzymes does not affect the differential rate of enzyme synthesis. Those tested were β-galactosidase, nitrate reductase, and those which are required for the metabolism of nineteen different carbon sources utilised by this organism.

Addition of CBAP to growing cells causes an initial rapid rise in the level of β-lactamase before the steady state rate of doubling is reached. It is demonstrated, by consideration of a hypothetical culture synthesising an inducible enzyme, that these kinetics are consistent with a constant differential rate

of enzyme synthesis from soon after addition of inducer. These theoretical considerations demonstrate that the doubling time of enzyme and specific activity measurements lead to erroneous conclusions if they are used to describe the rate of synthesis of an inducible enzyme. The kinetics of induction are consistent with a constant differential rate, after a lag, which is dependent on the concentration of CBAP. However, the lag and acceleration period are dependent on the growth medium.

Discontinuous β-lactamase synthesis has been observed in this strain of S. aureus. Since the synthesis of the enzyme is completely gratuitous, it cannot be subject to control by catabolite repression. It is suggested that the translation of the gene is dependent on DNA replication.

Examination of the differential rate of β-lactamase synthesis on addition of inducer at different stages of growth reveals that there is an initial lag in the synthesis of enzyme relative to turbidity at high cell densities. A similar lag occurs at low inducer concentrations. Both observations could be consistent with the presence of a permease controlling entry of CBAP to the cells. The increase of activity obtained when the cells are disrupted by ultrasound, supports this hypothesis.

However, on transfer of growing cells from high to low inducer concentration, a lag in enzyme synthesis is still evident. It is reasonable to assume that growth on high CBAP concentration would result in permease induction. On this evidence, therefore, it is concluded that the β-lactamase system does not depend on an induced permease.

The kinetics of induction at low CBAP levels are consistent with a very low differential rate of synthesis from the moment of addition of inducer. After the logarithmic growth phase is complete, the differential rate increases. The increase in differential rate when growth slows occurs in several other

circumstances but is most marked in low concentration of inducer.

It is shown that the half life of β-lactamase mRNA is relatively long in this organism and is dependent on the growth medium. It is suggested that, as growth slows, mRNA decay slows and therefore the capacity to synthesise enzyme remains. Since the inducer, CBAP, is present at the same concentration at all times, enzyme synthesis continues unabated and the differential rate increases by virtue of diminution in growth rate.

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Biosynthesis of β-Lactamase in Staphylococcus aureus

by

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SUMMARY

β-Lactamase is an inducible enzyme in Staphylococcus aureus 623/19. Until recently, the only available inducers were powerful antibacterials which were hydrolysed by the enzyme. Those which were not hydrolysed by the enzyme were either weak inducers or had antibacterial activity. CBAP, 2 (2'-carboxyphenyl) benzoyl-6-amino-penicillanic acid, combines insensitivity to β-lactamase with minimal antibacterial activity while still retaining activity as an inducer.

Using CBAP, the differential rate of β-lactamase synthesis has been determined on cells growing under accurately reproducible conditions. The rate is affected by a wide variety of growth conditions. Among these were growth at different temperatures in both a defined medium and a complex medium, which produced a wide variation in growth rate; aeration by mixtures of oxygen and nitrogen where the percentage of oxygen varied up to 50%; even when growth was slowed down by the high oxygen tension, the differential rate remained the same. Very little variation is seen in the differential rate when the culture is grown anaerobically in nutrient broth compared to an aerobic culture. Growth from different sizes of inocula in defined medium results in different growth rates but the differential rate of

β-lactamase synthesis is unaltered.

Concurrent induction of other enzymes does not affect the differential rate of enzyme synthesis. Those tested were β-galactosidase, nitrate reductase, and those which are required for the metabolism of nineteen different carbon sources utilised by this organism.

Addition of CBAP to growing cells causes an initial rapid rise in the level of β-lactamase before the steady state rate of doubling is reached. It is demonstrated, by consideration of a hypothetical culture synthesising an inducible enzyme, that these kinetics are consistent with a constant differential rate of enzyme synthesis from soon after addition of inducer. These theoretical considerations demonstrate that the doubling time of enzyme and specific activity measurements lead to erroneous conclusions if they are used to describe the rate of synthesis of an inducible enzyme. The kinetics of induction are consistent with a constant differential rate, after a lag, which is dependent on the concentration of CBAP. However, the lag and acceleration period are dependent on the growth medium.

Discontinuous β-lactamase synthesis has been observed in this strain of S. aureus. Since the synthesis of the enzyme is completely gratuitous, it cannot be

subject to control by catabolite repression. It is suggested that the translation of the gene is dependent on DNA replication.

Examination of the differential rate of β-lactamase synthesis on addition of inducer at different stages of growth reveals that there is an initial lag in the synthesis of enzyme relative to turbidity at high cell densities. A similar lag occurs at low inducer concentrations. Both observations could be consistent with the presence of a permease controlling entry of CBAP to the cells. The increase of activity obtained when the cells are disrupted by ultrasound, supports this hypothesis.

However, on transfer of growing cells from high to low inducer concentration, a lag in enzyme synthesis is still evident. It is reasonable to assume that growth on high CBAP concentration would result in permease induction. On this evidence, therefore, it is concluded that the β-lactamase system does not depend on an induced permease.

The kinetics of induction at low CBAP levels are consistent with a very low differential rate of synthesis from the moment of addition of inducer. After the logarithmic growth phase is complete, the differential

rate increases. The increase in differential rate when growth slows occurs in several other circumstances but is most marked in low concentration of inducer.

It is shown that the half life of β-lactamase mRNA is relatively long in this organism and is dependent on the growth medium. It is suggested that, as growth slows, mRNA decay slows and therefore the capacity to synthesise enzyme remains. Since the inducer, CBAP, is present at the same concentration at all times, enzyme synthesis continues unabated and the differential rate increases by virtue of diminution in growth rate.