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A BIOCHEMICAL STUDY OF THE DIAUXIC GROWTH OF PSEUDOMONAS AERUGINOSA

Thesis

presented for the

Degree of Doctor of Philosophy

in the

University of Glasgow

by

William Allan Hamilton

Department of Biochemistry

April, 1961

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A BIOCHEMICAL STUDY OF THE DIAUXIC GROWTH OF *PSEUDOMONAS AERUGINOSA*.

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2. The Nature of the Diauxic Effect with Glucose and Organic Acids in *Pseudomonas aeruginosa*.
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Introduction.

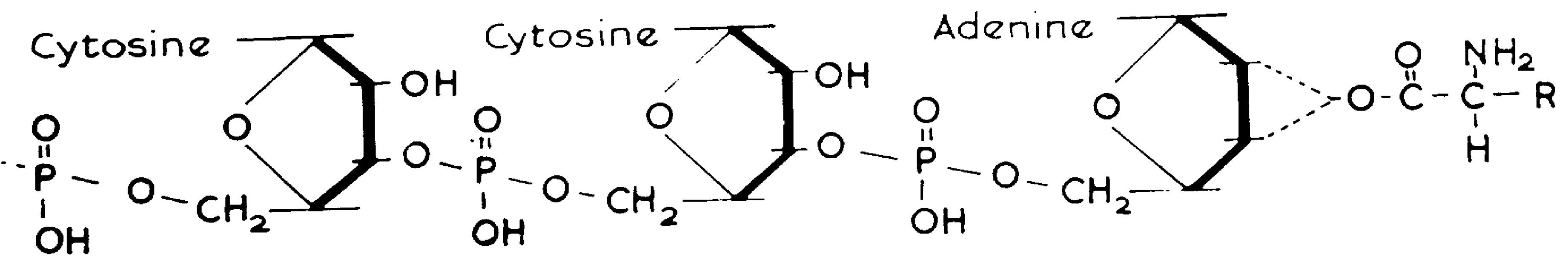
For ease of reference, the subjects discussed in this introduction have been introduced under appropriate section headings. As it has been attempted to write this introduction as a composite whole, it is hoped that these divisions will not appear to intrude too artificially on the essential unity of the discussion.

Protein Synthesis.

In a study of control mechanisms in bacteria and in particular of induced enzyme synthesis, it is necessary first to consider the general method of protein synthesis. Several reviews on this subject have been published recently, among which may be cited those of Crick (1958), Simkin (1959), Pollock (1959) and Halvorson (1960).

Accumulating evidence from many laboratories shows that the initial step in protein synthesis is the formation of an adenosine monophosphate (AMP) - acyl amino acid complex from adenosine triphosphate (ATP) and the amino acid. This activation is catalysed by an activating enzyme, there being a specific enzyme for each amino acid. These enzymes are also believed to catalyse the next step in the sequence of synthesis which is the transfer of the activated amino acid to a low molecular weight ribonucleic acid (RNA) existing in the cytoplasm. The amino acid is believed to be linked to this RNA at the 2'- or 3'-carbon atom in the ribose moiety of the terminal adenine nucleotide (Hecht, Stephenson & Zamecnik, 1959). These workers also concluded that a specific RNA exists for each amino acid and that each of these has the common terminal base sequence of adenine-cytosine-cytosine (Fig.1).

Figure 1.



Terminal structure of soluble RNA with attached amino acid

From this point the theory becomes hypothetical in character since, although there is a wealth of circumstantial evidence available, there is little or no direct experimental proof for the existence of the various intermediate structures proposed, or for their supposed mode of action. The hypothesis generally held at present is known as the template hypothesis. It is envisaged that the amino acids are arranged on a template in a definite sequence prior to peptide bond formation and the synthesis of the protein molecule. It is held that RNA is one, if not the sole component of this template and that the base sequence in the RNA determines the sequence in which the amino acids will become attached to it. It is believed that the information determining this sequence is originally held in the deoxyribonucleic acid (DNA) of the genetic material and that this information is passed on in some way to the RNA of the template.

Ribosomes are ribonucleoprotein particles attached to the endoplasmic reticulum in mammalian cells (Petermann & Hamilton, 1957), and also found in bacteria (Schachman, Pardee & Stanier, 1952), probably in close association with the plasma membrane. Although the results with bacteria are variable, ribosomes have been implicated as a major site of protein synthesis in mammalian systems (Cohn & Butler, 1957; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin & Work, 1957). It is believed that the RNA of the cytoplasm, or

soluble RNA (sRNA) as it is called, transports its attached amino acids to the RNA template in the ribosomes where the sRNA and amino acids are attached in the order determined by the base code of the template RNA. Peptide bond formation takes place and the protein molecule is released from the template, taking up its characteristic structure with cross-linking between the amino acid chains and folding of these chains as determined by its own intramolecular forces. The sRNA is also released from the template and is then free to continue the transport of activated amino acids to the template. Although the data with bacteria are as yet incomplete, it seems reasonable at present to assume that the plasma membrane and its associated particles may play the role of the endoplasmic reticulum and ribosomes in the cells of higher forms (Butler, Crathorn & Hunter, 1958; Spiegelman, 1959).

Induced Enzyme Synthesis

a) General

It will be seen that this hypothesis states that the spectrum of proteins, and of enzymes in particular, to be found in a cell is determined by the code carried and transmitted to succeeding generations in the genetic material of that cell, that is, the cell is of a specific genotype. There do exist, however, certain secondary control mechanisms which can influence which of the properties determined by the genetic material are manifest by the cell under a given set

of conditions, that is, different phenotypes can exist within the one genotype. One such phenomenon is induced enzyme synthesis. Induced enzyme synthesis can be defined formally as the increase in ratio of the rate of synthesis of a given enzyme to the rate of synthesis of total cell protein resulting from exposure of the cells to compounds (known as inducers) which are identical with or structurally related to the substrates of the given enzyme. Constitutive enzymes, on the other hand, are enzymes whose synthesis is not dependent on the presence of an exogenous inducer. A closely related phenomenon is that of repression of enzyme synthesis, in which the synthesis of an apparently constitutive enzyme can be repressed by a substance known as a repressor, which is generally a product of the action of the enzyme or series of enzymes of which it is a member.

b) β-Galactosidase of *Escherichia coli*

In a study of induced enzyme synthesis, or induction, many systems have been examined, but the most complete study has been made of the metabolism of β-galactosides by *Escherichia coli* by Monod and co-workers. The physical and chemical properties of purified β-galactosidase of *E.coli* have been extensively studied and characterised (Cohn, 1957). β-Galactosidases isolated from constitutive strains and from a number of inducible strains have been identified as identical

protein structures. This identity has been established by studies of antigenicity, turnover number, kinetic constants for several substrates, sodium and potassium ion activation, thermal inactivation, and relative affinity of various competitive inhibitors in the presence of different ion activators (Monod & Cohn, 1952; Cohn, 1957; Hogness, 1959). Immunological studies (Cohn & Torriani, 1952; Cohn & Torriani, 1953) and experiments in which the cellular proteins were labelled with ^{35}S or ^{14}C prior to a period of starvation followed by induction in the presence of unlabelled sulphur or carbon (Hogness, Cohn & Monod, 1955; Rotman & Spiegelman, 1954; Hogness, 1959), have shown that appearance of enzymic activity during induction is paralleled by a corresponding quantitative increase in an antigenic component identifiable as β-galactosidase, and that these appearances are the result of *de novo* protein synthesis. If a precursor of the protein β-galactosidase does exist in the cells, then it can do so only in extremely small amounts, sufficient in fact to give approximately the same number of molecules of precursor per cell as the number of templates one might expect to find in the cell (Hogness, 1959). Similar immunological data to those cited above have been obtained by Pollock (1950) in a study of the induced penicillinase of *Bacillus cereus*. The radioactive data of Hogness *et al.* (1955) and of Rotman &

Spiegelman (1954) also show that the synthesis of β-galactosidase and other proteins of E.coli is essentially an irreversible process. This stability of the β-galactosidase once it has been formed in a growing population due to the presence of an inducer is also demonstrated by the experiments of Monod & Cohn (1952), Rickenberg, Yanofsky & Bonner (1953) and Benzer (1953). These workers showed that when the inducer is eliminated from the medium or its action stopped by the addition of an inhibitor, synthesis of β-galactosidase ceases but the existing activity of the culture remains constant or, expressed as per mg. dry bacterial weight, it is "diluted out" by the continued growth of the culture.

c) Differential Rate of Synthesis and Gratuity

In the study of induction two techniques are recommended, namely, the expression of induction as the differential rate of synthesis of the enzyme (Monod, Pappenheimer & Cohen-Bazire, 1952) and the use of conditions of gratuity during induction (Monod & Cohn, 1952). The differential rate of synthesis is the ratio of the rate of synthesis of the induced enzyme to the rate of increase in bacterial mass, and is a direct measure of the specific effect of the inducer on the rate of enzyme synthesis. In studying induction in a cell population one can only extrapolate the data to the cellular level if one makes the assumption that all the cells

are participating equally and simultaneously in the induced synthesis. There are two known types of condition that can cause a heterogeneous response in a bacterial population when exposed to the action of an inducer. Firstly, if the inducer is the sole source of carbon and energy in the medium, then any cell with a residual amount of the induced enzyme involved in the metabolism of the inducer will be able to grow and divide rapidly and so increase its initial advantage and establish heterogeneity in the population. This has been shown experimentally by Benzer (1953). Heterogeneity is overcome by using the conditions of gratuity as outlined by Monod & Cohn (1953) in which a non-metabolised inducer is used in conjunction with a non-inducing source of carbon and energy. Secondly, it has been shown by Rickenberg, Cohen, Buttin & Monod (1956) that β-galactosidase induction can be divided into two separate processes. The first of these is the accumulation of the inducer within the cell by the action of a unit called galactoside permease which has the characteristics of an enzyme and whose synthesis can also be induced by inducers of β-galactosidase. It is the internal inducer which is responsible for the induction of both the permease and β-galactosidase. Consequently, any residual activity of the permease in certain cells of a population would increase markedly the internal accumulation of the inducer and so increase the synthesis of both the permease and

β-galactosidase thus giving rise once again to a heterogeneous response of the population. Therefore, in order to obtain a homogeneous population with respect to induction, not only must conditions of gratuity be employed but also high enough concentrations of the inducer must be present to saturate the internal site of induction by the process of diffusion through the bacterial membrane without the necessity of permease action.

d) Specificity of Inducer

Before discussing the kinetics and mechanism of induction, it is necessary first to consider the inducer itself and also the action of the permease. Hogness (1959) has described the work which shows that the process of enzyme induction is independent of enzyme action, since being a substrate for β-galactosidase is neither a sufficient nor necessary condition for a compound to function as an inducer. Thus, phenyl β-galactoside is an excellent substrate of β-galactosidase, but is not functional as an inducer. Methyl β-thiogalactoside, on the other hand, is not a substrate of the enzyme, but is an excellent inducer. In addition to this qualitative data as to the independence of substrate and inducer functions, quantitative measurements of the ability of many thiogalactosides to induce β-galactosidase synthesis and the ability to complex with the active site of this

enzyme, as either substrate or competitive inhibitor, show no correlation. Thus phenyl ethyl β-thiogalactoside is an extremely good competitive inhibitor of β-galactosidase action ($K_I = 1.5 \times 10^{-5}M$), but can induce synthesis of β-galactosidase to a rate that is only one-eighthieth that obtainable with methyl β-thiogalactoside, although this latter compound is about 800-fold less efficient as a competitive inhibitor ($K_I = 1.2 \times 10^{-2}M$). On the other hand, melibiose, an α-galactoside which is neither a substrate nor an effective competitive inhibitor of β-galactosidase, is quite effective as an inducer of this enzyme. Thus, it would appear that, in activating the enzyme forming units within the cell, the inducer reacts with a site which is structurally similar but not identical to the active site of β-galactosidase. Halvorson (1960) quotes similar evidence from β-glucosidase synthesis in Saccharomyces cereviseae (Duerksen & Halvorson, 1958 and 1959) and he also concludes that the induction process is independent of enzyme action and that all inducers appear to have the property of complexing with the enzyme, although this property of itself cannot confer the properties of an inducer. In studying penicillinase induction of B. cereus, Pollock has shown that the inducer acts as a catalyst in the sense that one molecule of inducer may cause the formation of more than one molecule of enzyme (for review, see Pollock, 1959).

Permease.

a) General

The properties and function of the β-galactoside permease in *E.coli* have been reviewed by Cohen & Monod (1957). That a cellular osmotic barrier does exist in bacteria has been shown by the work of Mitchell & Moyle (1956a, 1956b, 1957). These workers were able to measure the actual intracellular osmotic pressures of certain bacterial cells, and found a value of 20-25 atmospheres in the case of *Staphylococcus aureus*; a value which indicates that most of the low molecular weight intracellular compounds must be free and in solution within the osmotic barrier. They have also shown that the rate of equilibration of internal and external osmotic pressures is extremely low and in some cases never reached. These results, using various carbohydrates and polyhydric alcohols, illustrate the low permeability of bacterial cells to these substances. The bacterial cell is enclosed by two structures, the cell wall which is responsible for maintaining the structural integrity of the cell and withstanding the osmotic pressure of the contents of the cell (Weibull, 1956), and a finer membrane lying immediately beneath the wall; this structure has been called the plasma membrane. The cell wall can be dissolved away by various techniques using lysozyme or its synthesis prevented by growing

the cells in the presence of penicillin, leaving the cell material surrounded only by the plasma membrane. If the suspension is in an isotonic suspending medium these structures are fairly stable and have been given the name of protoplasts. Work with these protoplast structures has shown that the site of the permeability barrier is the plasma membrane. It is believed that the impermeability of this barrier is not absolute but that a slow rate of diffusion may occur in either direction, tending to equilibrate the outside and inside concentrations.

Early evidence that there might be specific mechanisms involved in the transport of metabolites into the bacterial cell across the osmotic barrier came from the studies of amino acid accumulation in *Streptococcus faecalis* by Gale

amino acid accumulation in *Streptococcus faecalis* by Gale

metabolise a substrate although the enzymes necessary for this metabolism could be demonstrated in cell-free systems. Such cells were said to be cryptic and crypticity was shown to be extremely stereospecific in character in that substrates closely related to that which was not metabolised could be readily metabolised by the cells (Deere, Dulaney & Michelson, 1939; Liebowitz & Hestrin, 1945; Doudoroff, 1951; Doudoroff, Hassid, Putnam, Potter & Lederberg, 1949; and Doudoroff, Palleroni, MacGee & Ohara, 1956).

b) *β-Galactoside Permease in Escherichia coli*

A demonstration of the existence of a specific permeation reaction for galactosides in *E. coli* was given by Cohen & Monod (1957). A suspension of cells, previously induced by growth in the presence of a galactoside, was shaken for a few moments with ^{35}S labelled thiomethyl β-galactoside. When the cells were rapidly separated from the suspending fluid they were found to retain an amount of radioactivity corresponding to an intracellular concentration of galactoside which may exceed by 100-fold or more its concentration in the external medium. Non-induced cells did not accumulate any significant amount of radioactivity. The galactoside accumulation is fairly rapid (reaching a stable maximum in 5-20 mins., depending on the galactoside), reversible, strictly stereospecific and the accumulated radioactivity, which can be quantitatively extracted with boiling water, has been shown by chromatographic analysis to be associated with free unchanged galactoside.

A consideration of several factors allowed Cohen & Monod (1957) to decide in favour of the stereospecific functioning of the penetration mechanism being the result of the action of a catalytic reaction rather than a stoichiometric combination with a specific receptor site. In the first plac

it is almost impossible to imagine that the bacterial cell could possess enough receptor sites (probably protein or associated with protein) to accommodate not only the large internal accumulations of galactoside (up to 5% of the dry weight) but also the accumulation of other carbohydrates, amino acids etc. which have been demonstrated. Mandelstam (1958), however, from his studies of amino acid accumulation in _E.coli_, disagrees with this conclusion. A detailed consideration of the kinetics of accumulation is also found to be incompatible with the stoichaiometric model. There still remain, however, two possibilities as regards the mechanism of the permease reaction. So far we have only sought to correlate the galactoside accumulation with the permease activity and have not considered the forces which "bind" the internal galactoside to the cell and allow its accumulation. Once again there are two possibilities, either the membrane is completely permeable and the accumulation is the result of the specifically catalysed loose binding to some non-specific, non-diffusible cell constituent, or the membrane is impermeable to the galactosides whose accumulation is the result of a specifically catalysed entry reaction, the galactoside existing in a free state within the cell. That we must consider the permease action and resulting accumulation in the latter manner is shown by the osmotic data of Mitchell & Moyle (1956a) and Sistrom (1958).

Cohen & Rickenberg (1955) and Rickenberg, Cohen, Buttin & Monod (1956) have shown that the metabolic uncoupling agents 2,4-dinitrophenol and sodium azide inhibit the accumulation process, and Kepes (1957) has demonstrated that energy, derived from the oxidation of endogenous reserves, is expended in the accumulation process. There is, however, no evidence that energy-coupling of this nature is required when the permease is functioning *in vivo* as the first stage in the hydrolysis of galactosides, as opposed to its action as a "pump" catalysing the accumulation of non-metabolized galactosides against a concentration gradient.

The induction of the galactoside permease reveals a strict stereospecificity of inducer, closely related to that for the β-galactosidase. Consideration of this induction and its kinetics together with the specificity and kinetics of the permease action, plus the fact that *de novo* protein synthesis has been shown to accompany the induction, leaves no doubt that a specific inducible protein is responsible for the activity of this system. The complete system may however also involve non-inducible and non-specific components. Cohen & Monod (1957) therefore suggest that the term "permease" should be used to designate only the specific, inducible protein component of the system.

c) Mutations Involving Permease

The existence of cryptic strains of microorganisms has already been mentioned. These strains are mutants of the wild type in which both the permease and the β-galactosidase are inducible. Other mutations which have been studied have an inducible permease but no β-galactosidase, a constitutive permease but no β-galactosidase and a permease and β-galactosidase which are both constitutive (Lederberg, Lederberg, Zinder & Lively, 1951; Cohen-Bazire & Jolit, 1953; Rickenberg, Cohen, Buttin & Monod, 1956). The existence of these mutant types proves that the permease and β-galactosidase are genetically and functionally distinct. Experimental data obtained from studying different mutations have shown that the permease and the β-galactosidase form a metabolic sequence *in vivo* and that in the normal induced wild type, the permease is the controlling factor in hydrolysis of galactosides. A further interesting feature of this action of the permease results from the fact that the intracellular concentration of the galactoside, controlled by the permease, is not only the substrate of the β-galactosidase but also its inducer and also the inducer of the permease. The permease therefore not only controls the activity but also the induction of the β-galactosidase and of itself. Cohen & Monod (1957) have used this fact to explain the autocatalytic

kinetics of β-galactosidase induction at low inducer concentrations, as opposed to the linear kinetics Herzenberg (1959) demonstrated in cryptic strains where high enough concentrations were employed to overcome the osmotic barrier.

The genetic control of β-galactosidase system in *E.coli* has been studied by Pardee, Jacob & Monod (1959). These workers have shown that at least three related cistron regions are involved. The "z" region controls not only the presence or absence of the enzyme β-galactosidase but also its structure; the "y" region controls the permease in a like manner, and the "i" region controls whether the two enzymes are constitutive or inducible.

d) Other Permeases

In their review, Cohen & Monod (1957) discuss a number of other bacterial systems closely related to the β-galactoside permease of *E.coli*. Among these are the inducible glucoside permease of *E.coli* (Stoeber, 1957); the accumulation by *E.coli* of α-methyl glucoside by a system apparently identical with or closely related to the glucose permease; inducible permeation factors manifest in the oxidation of intermediates of the tricarboxylic acid (TCA) cycle in *Pseudomonas fluorescens* (Barrett, Larson & Kallio, 1953; Kogut & Podoski, 1953), in *Aerobacter aerogenes* (Davis, 1956), and in *E.coli* (Gilvarg & Davis, 1956); the possible involvement of a stereospecific

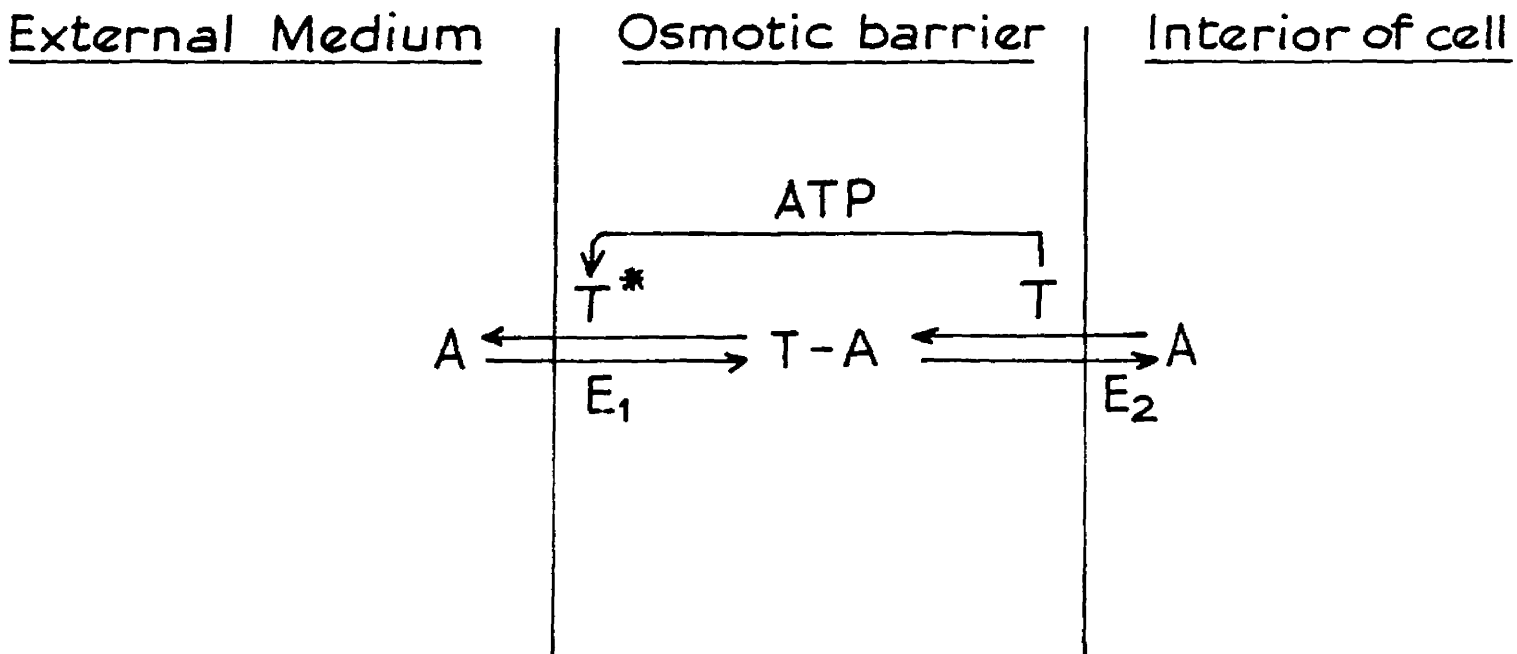
penetration factor in the metabolism of tartaric acid isomers by Pseudomonas strains (Shilo & Stanier, 1957); the accumulation of amino acids by E.coli (Britten, Roberts & French, 1955), and by other microorganisms (Gale, 1954; Mathieson & Catcheside, 1955). Cohen & Monod also give an explanation of physiological interactions between amino acids in E.coli in terms of the permease theory. It is obvious from these data that in any bacterial study the entry of metabolites into the cell must be considered if a true evaluation of the cell's activities is to be achieved.

e) Model of Permease Action

It has already been stated that the exit of accumulated substrates from the cell was believed to be by free diffusion, but more recent work by Kepes (1960) on the galactoside permease of E.coli and by Horecker, Thomas & Monod (1960) on galactose transport in E.coli has shown that the exit reaction is not free diffusion but rather an inducible carrier-dependent system.

Consideration of the evidence discussed above permits a theoretical model of permease action to be envisaged (Horecker, Thomas & Monod, 1960), Fig.2. In presenting this model, it must be made clear that its purpose is solely to give a diagramatic representation of the experimental results without making any further assumptions regarding, for example,

Figure 2.



Model of Permease Action

After Horecker, Thomas & Monod (1960)

A is substrate.
T is non-specific carrier, used in all permease systems.
T^* is "activated" carrier.
E_1 & E_2 are enzymes of A-permease system.

the mechanism of energy-coupling during accumulation of a non-metabolised substrate. Horecker *et al.* (1960) have used such a model to discuss the entry and exit reactions in the transport of galactose by *E.coli*. For the entry of galactose, the first step would be reaction of external galactose with the activated transporter substance (T^*) present in the osmotic barrier. This reaction is catalyzed by an enzyme E_1, assumed also to be present in the osmotic barrier. The second step would require dissociation of the transporter-galactose complex, catalysed by an enzyme E_2, found inside the cell. This would regenerate the transporter and liberate free galactose within the cell, the transporter being re-activated by the hydrolysis of 1 mole of ATP for every mole of galactose transported into the cell. Once the galactose is in the interior of the cell, enzyme E_1 would become un-available to it. The galactose would accumulate until the reaction catalysed by E_2 became appreciably reversible, owing to the high concentration of internal galactose. Eventually this reaction would become equal in rate to that catalysed by E_1, and a steady state would be reached. ATP has also been implicated in membrane transport by the work of Abrams, McNamara & Johnson (1960) on the metabolic lysis of protoplasts of *Streptococcus faecalis*, and of Post, Merrit, Kinsolving & Albright (1960) on sodium and potassium transport in human erythrocytes. Without doubt models of this

type will prove extremely valuable, particularly in suggesting future experiments designed to elucidate the mechanism of membrane transport, both in bacterial and mammalian systems.

Large differences in enzymic activity are often found between intact cells and extracts and it has frequently been assumed that the penetration of the substrate is the rate limiting step in the intact cells, as in the case of so-called cryptic strains described previously. Rotman (1956) has studied such a difference in β-galactosidase activities of E.coli. He found his data to be incompatible with hypotheses based on passive diffusion, product inhibition and inhibitors compounded with the enzyme, and concluded that an inducible penetration mechanism was involved in β-galactoside metabolism. Rotman (1956) found, however, that there were small differences between his system and that of Cohen & Monod (1957), but these could be resolved using an assumption made by Monod (1956) that competition exists between inducer and inhibitor for a site or substance which remains constant in amount per cell during induction and is therefore distinct from both the permease and β-galactosidase. Rotman suggests that this site, "s", corresponds to the accumulation system which, although requiring energy, need not be inducible or highly specific. It seems reasonable to consider this site, "s", as being possibly identical with the common transporter, "T",

of the model proposed by Horecker, Thomas & Monod (1960).

Other explanations of apparent "permeability" effects

On the other hand, studies of the oxidation of citrate by E.coli (MacDonald & Gerhardt, 1958) and by baker's yeast (Foulkes, 1954) led the authors to conclude that the low oxidation of citrate by whole cells, as compared to cell-free extracts, was not due to surface impermeability of the cells.

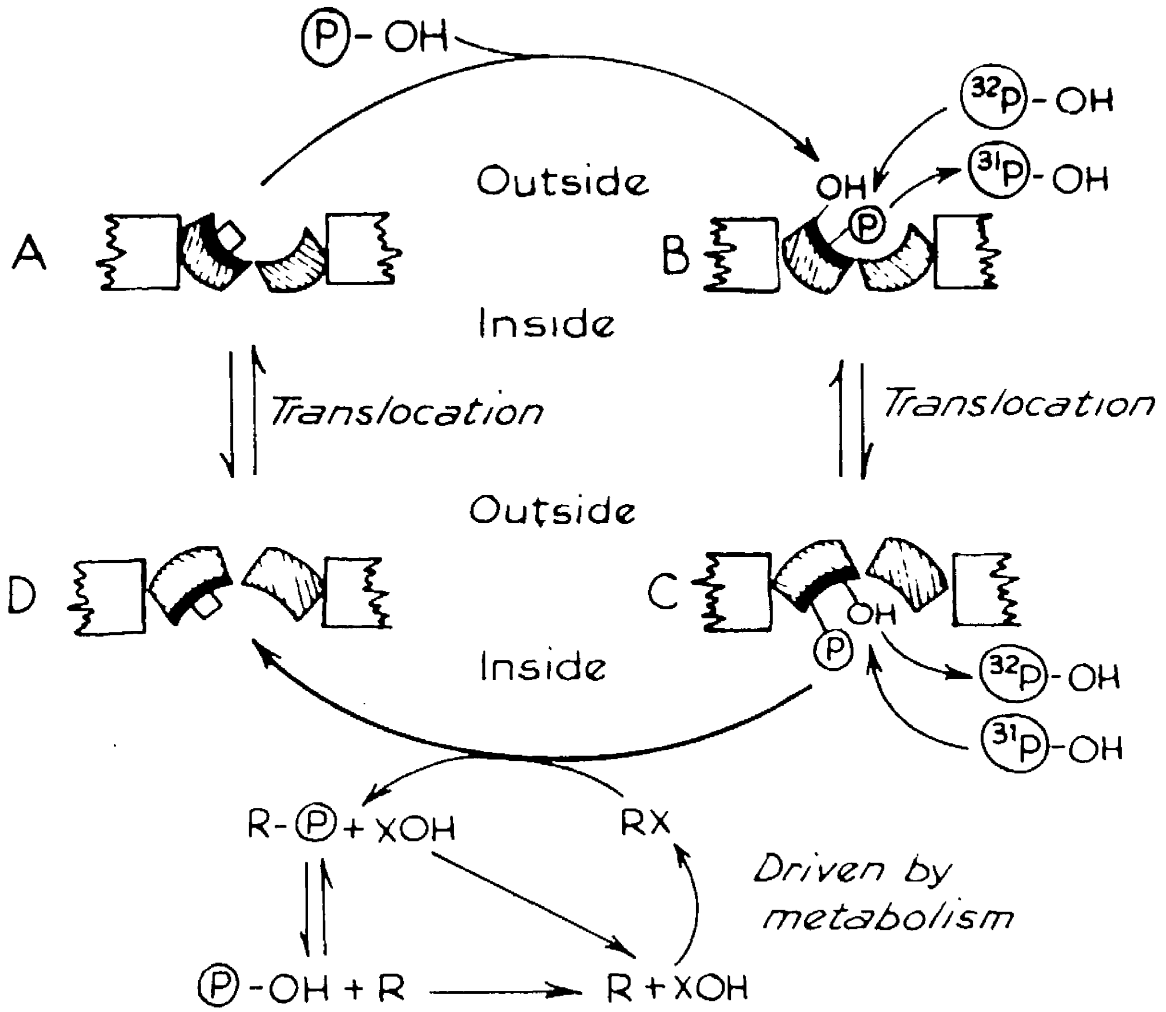
In studies of the increase in activity of yeast catalase on lysis of the cells, Kaplan & Paik (1956) claimed that this and other changes in the properties of the enzyme after lysis were due not to the removal of a permeability barrier, but to what they called "enzyme alteration". The authors' hypothesis to explain enzyme alteration is that it consists in the desorption of the enzyme from some intra-cellular interface at which the catalase normally exists in a partially disordered condition within the intact cell, this desorption being accompanied by an increase in activity and altered enzymic properties.

Translocases

The work of Mitchell & Moyle (1956a, 1956b, 1957) on the osmotic barrier in bacteria has already been mentioned. These workers have described (1956c) how the plasma membrane

acts not only as an osmotic barrier but also as an osmotic linkage between the external and internal media for the transport of nutrient and waste materials between the cell and its environment. Such a transport of phosphate is visualized in Fig.3. The specificity and kinetics of phosphate transport in *Staphylococcus aureus* resemble those of enzyme linked reactions and the temperature characteristics suggest a complex movement of components of the plasma membrane during the passage of phosphate from one side to the other. The material of the plasma-membrane is a complex lipoprotein, of which there is sufficient to form about one monolayer of protein and one of lipid. The protein component has been shown to include a high proportion of the cytochromes of the cell and also certain enzymes, for example acid phosphatase, succinic dehydrogenase and lactic dehydrogenase. Similar activities have been found in membrane fraction of *Bacillus megaterium* (Storck & Wachsman, 1957) and of *Proteus vulgaris* (Feldman & O'Kane, 1960). Mitchell & Moyle (1958) hold that the penetration of metabolites through the plasma membrane is brought about by the action of these normal metabolic enzymes which by virtue of their peculiar spatial configuration within the membrane are able to catalyse the transport of their substrates across the osmotic barrier. In this way metabolic energy is converted to osmotic work by the formation and opening of covalent links between enzymes or coenzymes in the

Figure 3.



Model of Phosphate Transport *After Mitchell, 1957*

= plasma membrane, = translocase.

= active site or translocation, Ⓟ-OH = H_3PO_4.

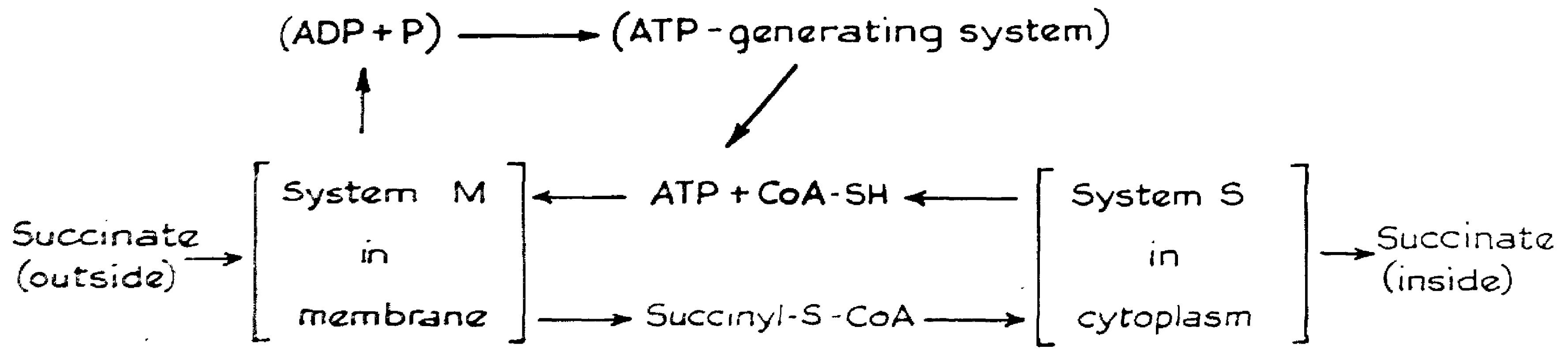
membrane and the transported "molecules" that are carried as chemical groups exactly as in enzyme-catalysed group-transfer reactions. The name of translocase has been given to these enzymes catalysing transport. Mitchell & Moyle (1959) have recently described a possible scheme of succinate transport in *Micrococcus lysodeikticus* (Fig.4).

in *Micrococcus lysodeikticus* (Fig.4).

Mitchell (1959) has explained crypticity in terms of the translocase hypothesis. He points out that both the correct location and orientation of the metabolic enzyme within the plasma membrane are necessary in order that it may function as a translocase, and that crypticity may be the result of a mutation affecting the site of the enzyme within the cell rather than its synthesis. It is, however, difficult to understand how this hypothesis could adequately explain the short lag period found in the oxidation of various intermediates of the TCA cycle by *Ps. fluorescens* and shown by Kogut & Podoski (1953) and Barrett, Larson & Kallio (1953) to be due to the initial impermeability of the cell to the substrate.

Although it is not yet possible then to discuss every aspect of membrane transport in bacteria in terms of a single theory, a great deal is now known concerning these mechanisms, their sites, specificity and kinetics. As most work has been done with the β-galactoside permease by Monod and coworkers, particularly in connexion with induced enzyme synthesis, the

Figure 4.



After Mitchell & Moyle (1959)

<u>Scheme for Succinate Transport</u>
involving ATP and Coenzyme A (CoA-SH)

term permease will be used throughout this work to designate a stereospecific catalytic mechanism of transport.

Kinetics of Induced Enzyme Synthesis.

An explanation in terms of the β-galactoside permease of the apparent autocatalytic induction of β-galactosidase in E.coli when growing in the presence of low concentrations of inducer, has already been discussed. A careful study of these kinetics by Cohn & Horibata (1959b) has shown that they are the result of an heterogeneous response of the population to inducer at the cellular level.

Campbell & Spiegelman (1956), from studies of the long-term adaptation to galactose fermentation by yeast (a phenomenon possessing exponential kinetics, similar to β-galactosidase induction in E.coli, at low levels of inducer) have proposed that it is possible the templates are initially inactive and are autocatalytically activated during the course of induction. Cohn & Horibata (1959b), however, claim that it is more reasonable to explain the exponential kinetics in terms of a permease, or other early enzyme in the pathway of galactose metabolism whose activity controls its induction in the same way as does the β-galactoside permease of E.coli. What may be termed the "pre-induction effects" are also the result of the action of the permease in increasing the concentration not only of its own inducer but also that of β-galactosidase. Monod (1956) showed that when cells were

grown on β-methyl galactoside prior to experimental study, the induction of β-galactosidase was more sensitive to lower concentrations of inducer, as compared to cells grown in the absence of galactoside, and the kinetics of the induction were linear. The work of Cohn & Horibata (1959a) and Novick & Weiner (1957) has shown that cells which have been pre-induced with galactoside will continue to synthesise both β-galactosidase and the permease even when growth occurs in the presence of glucose or another inhibitor, so long as inducer remains present in the medium. This is known as the phenomenon of _maintenance_, and it is not found with non-induced cells.

The linear kinetics of induction of β-galactosidase and the exceedingly short time lag between the addition of the inducer and the establishment of this rate of synthesis, make it most unlikely that the induction process involves the synthesis of new RNA templates (Hogness, 1959). Neidhardt & Magasanik (1960) have also found that RNA synthesis does not occur during the period of induction of _Aerobacter aerogenes_ to growth on _myo_-inositol.

Mechanism of Induced Enzyme Synthesis.

It is now possible to consider the mechanism of induced enzyme synthesis. Cohn & Monod (1953) proposed the _unitary hypothesis_, which states that the mechanisms of synthesis of inducible and constitutive enzymes are the same,

the latter being controlled by endogenously synthesized inducers. The conditions of this hypothesis are not by the β-galactosidase of E.coli, since the enzymes produced by inducible and constitutive strains have been shown to be identical, and since the genetic data of Pardee, Jacob & Monod (1959) have shown that the change from constitutivity to inducibility is determined by a cistron unit different from that determining the structure and function of the β-galactosidase molecule itself. Within the unitary hypothesis there are three possible mechanisms of induced enzyme synthesis.

1. The generalized induction hypothesis. Inducers are necessary components of enzyme synthesis both in constitutive and in inducible cells. Endogenous inducers are synthesized by constitutive cells but not by inducible cells, which must therefore, receive exogenous inducers for enzyme synthesis.

2. The different-template hypothesis. Two different types of template exist for enzyme synthesis. In constitutive cells, the templates function without the aid of inducers. In inducible cells, the inducers are necessary to activate the template. No endogenous inducer or repressor is assumed. It is assumed that the amino acid ordering function of each template is the same.

3. The repressor hypothesis. The templates in inducible and constitutive cells are the same and do not require activation

by inducers. Substances specifically inhibiting template function (repressors) are synthesized by inducible cells but not by constitutive cells. Exogenous inducers function by destroying this inhibition of enzyme synthesis.

In the models of induced enzyme synthesis proposed by Hogness (1959) and Halvorson (1960) the inducer is shown to be effective in aiding the removal of the protein molecule from its template. Presumably the same site of action is envisaged in the functioning of the different-template and repressor hypotheses.

The genetic experiments of Pardee, Jacob & Monod (1959) using different zygotes of E.coli have shown that the i^+ is dominant over the i^- cistron; that is, the i^+ or "inducible" cistron is associated with the positive function. As Hogness (1959) points out, although this is in agreement only with the repressor hypothesis in which the inducible strains have the positive function of synthesis of the repressor, the data can still be made to fit the generalised-induction hypothesis if one assumes that the positive function is the synthesis in the inducible strains of an enzyme capable of destruction of the endogenous inducer. It is not possible therefore at this time to describe completely the action of the inducer in the control of induced enzyme synthesis. It seems extremely likely nonetheless that a very close connection will be found to exist among the mechanisms of repression, induction and

inhibition of induction.

Repression of Enzyme Synthesis

A number of cases of inhibition of enzyme synthesis by a product of the enzyme's action have been reported. Among these are the inhibition of synthesis of tryptophan synthetase by tryptophan in *E.coli* (Monod & Cohen-Bazire, 1953), the inhibition of synthesis of methionine synthetase by methionine in *E.coli* (Cohn, Cohen & Monod, 1955) and the inhibition by arginine of the synthesis of acetylornithase, ornithine transcarbamylase and arginosuccinase, all intermediate enzymes on the biosynthetic pathway of arginine in *E.coli* (Vogel, 1957; Gorini & Maas, 1958; Gorini, 1960). This type of regulatory mechanism has been given the name of *enzyme repression*. Vogel (1958) and Gorini (1960) have suggested that repression and induction are analogous phenomena in terms of function and mechanism and that there is a close analogy between the repressor and the inducer. Further evidence for the close connection between induction and repression is found in recent work of Pardee & Prestidge (1959). These workers report experiments which suggest that β-galactosidase inducible *E.coli* differ from constitutive organisms in that they synthesize a repressor.

Inhibition of Induced Enzyme Synthesis

The observation has been made repeatedly that the induced synthesis of many different enzymes can be inhibited by a wide variety of carbohydrates (Happold & Hoyle, 1936; Epps & Gale, 1942; Gale, 1943; Monod, 1947, 1956; Bonner, 1955; Cohn, 1951; Monod & Cohn, 1952; Neidhardt & Magasanik, 1956a; Herzenberg, 1958). One of the most characteristic and potent of these carbohydrate inhibitors is glucose. As a result the general phenomenon has been called the "glucose effect" (Cohn, 1956; Cohn & Monod, 1953).

Diauxie

The most striking manifestation of the glucose effect is the phenomenon of diauxie, first discovered by Monod (1942). He found that when E.coli were inoculated into a defined medium containing glucose and, say, fructose as sources of carbon and energy, a continuous growth response was obtained. When, however, the medium contained galactose as the second sugar together with the glucose, growth occurred in two phases which were separated by a lag phase during which no growth took place. It was shown that the first phase of growth corresponded to the complete and exclusive utilization of the glucose as the substrate of growth, the lag phase was the period during which the induced enzymes required for galactose metabolism were synthesized, and the second growth

phase corresponded to the utilization of the galactose as substrate of growth. Monod (1942) called these peculiar growth kinetics diauxie, i.e., double growth. Pavin (1952), Bagley & Dawes (1953) and Green, quoted by Davis (1956) showed that in _Aerobacter aerogenes_ these growth kinetics extended to mixtures of glucose and various organic acids. It is apparent that the explanation of diauxie must be sought in terms of the mechanism of the inhibition of induced enzyme synthesis by glucose.

Mechanism of Inhibition of Induced Enzyme Synthesis

Neidhardt & Magasanik (1956a, 1956b, 1957) have studied the inhibition by glucose of the induced synthesis of histidase, glycerol dehydrogenase and myoinositol dehydrogenase in _A. aerogenes_. They have shown that the inhibition is not reversed by supplementing the growth medium with amino acid mixtures, purine and pyrimidine bases, or vitamin B_{12}. Cells growing on glucose, far from being deficient in the substances necessary for protein synthesis and cell growth, amino acids, etc., were actually shown to possess these substances in excess. These authors (1956b, 1957) came to the conclusion that the value to the cell of the inducible enzymes studied lay in their ability to produce these intermediate substrates of protein and nucleic acid synthesis, and that glucose, in producing an excess of the same substrates, caused the

inhibition of synthesis by virtue of one of these common metabolic intermediates acting as a repressor.

Cohn & Horibata (1959c) have also studied the mode of action of the glucose effect in the β-galactosidase of E.coli. They have come to the conclusion that the mechanism is complex, comprising two actions: a weak direct one, shown at high concentrations and noncompetitive in nature; and a strong indirect one, caused by a metabolic derivative, apparently competitive in nature and shown at low concentrations of glucose. The fact that glucose does not inhibit β-galactosidase synthesis in constitutive strains is explained by the fact that if the cells are unable to metabolise their endogenous reserves with the production of repressor, then presumably neither can they form repressor from added glucose. Constitutive synthesis of β-glucosidase in yeast has however been shown to be sensitive to glucose inhibition (MacQuillen, Winderman & Halvorson, 1960).

In mutant studies with Salmonella typhimurium, Englesberg (1959) has shown that a mutation involving relief of the sensitivity of the wild type to inhibitory effects of glucose, as manifest in diauxie during growth in media with glucose and, say, citrate as sources of carbon and energy, also involves a decreased rate of growth on glucose and a two- to threefold increase in the acid phosphatase activity of the cell. Englesberg concludes that the increased phosphatase

acts in partially short circuiting glucose utilisation at the phosphorylated stage and so lowering the rate of growth on glucose. These results suggest two explanations of the glucose diauxie phenomenon in this organism.

1) The phosphorylated and non-phosphorylated intermediates in glucose metabolism up to, but not including, TCA cycle intermediates are the repressors of induced enzyme synthesis in this system.

2) Inorganic phosphate is limiting during glucose metabolism, i.e., inorganic phosphate may be necessary for some aspect of induced enzyme synthesis and may only be present in an adequate concentration for this function in the presence of an increased level of acid phosphatase.

Racker & Wu (1959) have shown that the Pasteur effect in ascites tumour cells can be released to a considerable extent by a high extracellular concentration of inorganic phosphate. These workers demonstrated that ATP produced by oxidative phosphorylation is not as efficient in glucose phosphorylation reactions as is ATP produced in glycolysis, and they have concluded that the Pasteur effect may be visualised to be due to the low availability of intracellular inorganic phosphate under aerobic conditions which limits glycolytic synthesis of ATP and therefore limits efficient glucose uptake. A similar explanation of the Pasteur effect is proposed by Lynen, Hartmann, Netter & Schuegraf (1959)

from their studies with yeast.

The phenomena of induction and repression are concerned solely with the control of the synthesis of enzymes and in no way affect the activity of the enzyme once it has been released from its template in an active form. The irreversibility of synthesis has already been referred to in the discussion of the _de novo_ synthesis involved in induction. Several authors, among them Rickenberg, Yanofsky & Bonner (1953) and Davis (1956) have shown that when induced enzyme synthesis is inhibited by glucose, the existing activity of the culture remains constant and, expressed as per mg. dry weight of cells, is diluted out by further growth. Although glucose inhibits induction to galactose in _A. aerogenes_ Neidhardt & Magasanik (1956) have shown that cells which have been induced to galactose metabolise galactose in washed cell suspension and that this metabolism is not inhibited by glucose. On the other hand, Kleinzeller, Málek, Fraus & Skoda (1952) found that even when the yeast _Rhodotorula gracilis_ had been induced to xylose, the metabolism of xylose by washed suspensions was inhibited by glucose.

Negative Feed Back Inhibition

Pardee (1959) has discussed a mechanism in which the activity of the enzyme is affected, and not the synthesis. This is the mechanism of _negative feed back inhibition_, in

which the product of a series of enzymic reactions can inhibit the action of an early enzyme in the series. In one particular case that he describes (Yates & Pardee, 1956) cytidylic acid inhibits the action of an early enzyme in the sequence of pyrimidine biosynthesis in E.coli catalysing the condensation of aspartate and carbamyl phosphate.

When one considers the very many different conditions in which bacteria and other microorganisms are found to exist and multiply, it is obvious that such mechanisms as induction and repression of enzyme synthesis and feed back inhibition of enzyme action, must be of the utmost importance in the control and regulation of cell metabolism.

Pseudomonas aeruginosa

a) Initial Observation

The possibility that Psuedomonas aeruginosa might display some interesting and unusual variations on these control mechanisms occurred to us in view of an observation by Liu (1952) that the organism failed to produce acid from carbohydrate when growing in a peptone-carbohydrate broth. Apparently the peptone was used as the preferential source of carbon as well as of nitrogen. Liu (1952) also showed that carbohydrate utilisation in synthetic media could be inhibited by simple carbon compounds such as sodium citrate and sodium succinate. These results seem to constitute an

example of a mechanism acting in the opposite manner to the glucose effect described previously.

b) Description of Organism

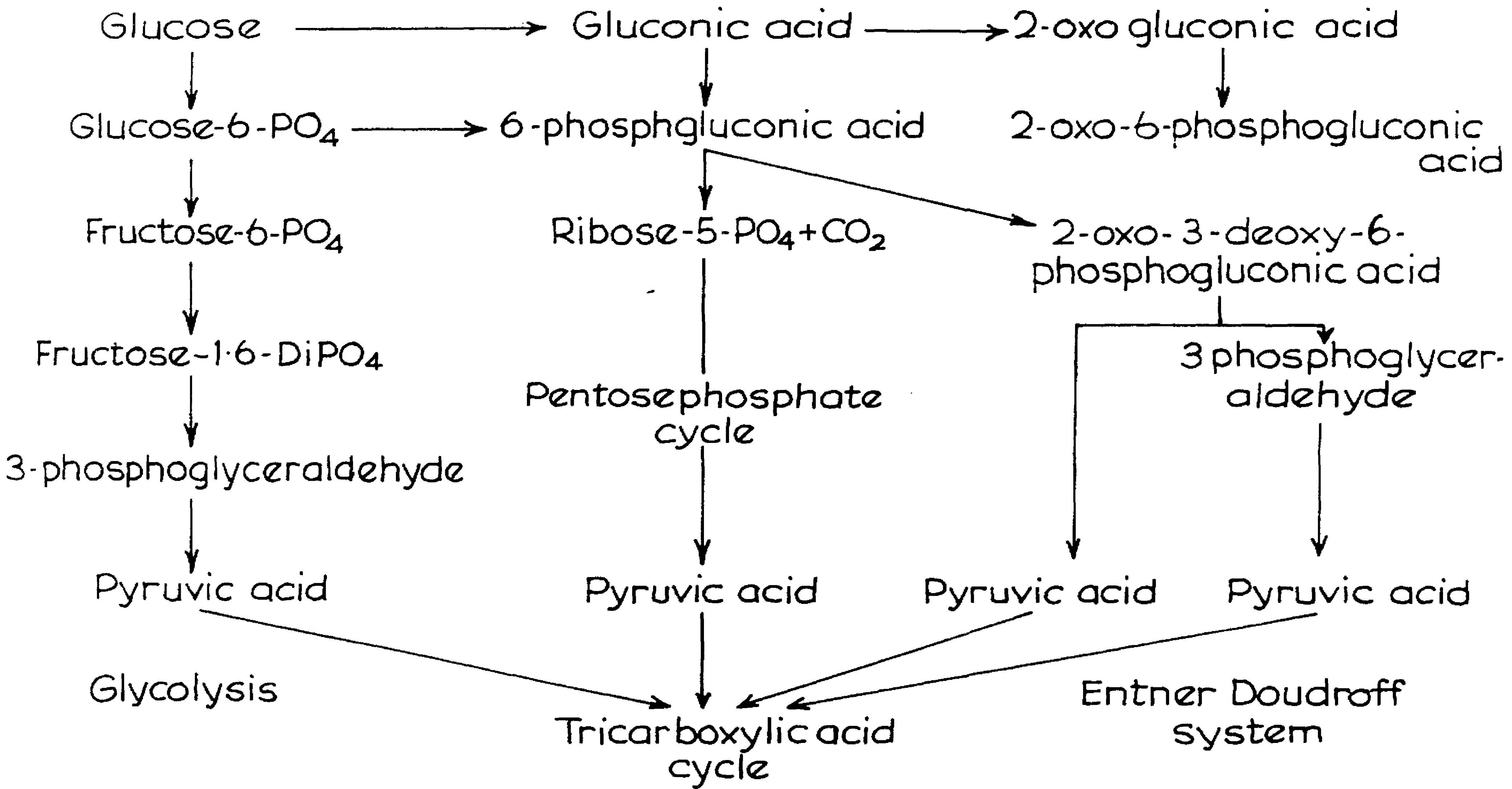
Pseudomonas aeruginosa is described in Bergey's *Manual of Determinative Bacteriology*, Seventh Edition (1957) as a Gram negative facultative aerobe. It exists in bacillary form, the rods being about 0.5 by 1.5 microns and occurring, singly, in pairs, or in short chains. The rods are motile, possessing one to three polar flagella. Nitrates can be reduced to nitrites and nitrogen. Glucose, fructose, galactose, arabinose, maltose, lactose, sucrose, dextrin, inulin, glycerol, mannitol and dulcitol are not fermented. Glucose is oxidised, however, to gluconic and 2-oxogluconic acids and other intermediates. Previously the organism has been known as *Bacillus pyocyaneus* and *Pseudomonas pyocyanea* owing to the distinctive production of the respiratory pigment pyocyanine by the organism. This has, however, been shown not to be typical of all strains and Haynes (1951) has proposed a scheme of identification based on three properties; a) ability to grow well at 42°C, b) ability to oxidise gluconic acid to 2-oxogluconic acid, and c) ability to produce a slime in static culture in a medium containing potassium gluconate as the principal carbon source. The organism is found in polluted water and sewage and in certain

animal and human lesions, in particular in pus which has gathered in a burn wound; a colloquial name for the organism is the blue puss organism. Polymyxin is the only antibiotic to which *Ps. aeruginosa* shows any sensitivity (Newton, 1956).

c) Pathways of Glucose Metabolism

The study of the pathways of carbohydrate metabolism in *Ps. aeruginosa* has been made chiefly by Campbell and his coworkers and by Claridge & Werkman. The possible pathways of glucose metabolism to the stage of pyruvate are described in Fig.5. Warburton, Eagles & Campbell (1951) showed that although pyruvate was an intermediate in glucose metabolism, fluoride did not impair the conversion of glucose to pyruvate; this indicates that the enzyme enolase is not necessary for the production of pyruvate and that therefore the Embden-Meyerhoff glycolytic pathway (EMP) if operative cannot be the sole pathway of glucose metabolism in this organism. Campbell & Norris (1950) also showed an apparent lack of glucose 1-phosphate (G-1-P), glucose 6-phosphate (G-6-P), fructose 6-phosphate (F-6-P) and fructose 1,6-diphosphate (F-1,6-diP), all normal intermediates of glycolysis, in the acid-soluble phosphorus fraction of cells of *Ps. aeruginosa* harvested from a glucose medium. Claridge & Werkman (1953 and 1954) have, however, demonstrated the presence of

Figure 5.



hexokinase, gluconokinase, aldolase and 3-phosphoglyceraldehyde dehydrogenase in cell-free extracts of _Ps. aeruginosa_. These workers (1954) also showed that extracts contained the enzymes necessary to reduce di- and triphosphopyridine nucleotide (DPN and TPN) in the presence of G-6-P and F-6-P and DPN only in the presence of ribose 5-phosphate (R-5-P); these reactions are indicative of the hexose monophosphate oxidative pathway (HMP).

A number of workers have described the importance of a non-phosphorylated direct oxidation of glucose to gluconic and 2-oxogluconic acids, with the oxygen uptake of one half and one mole of oxygen respectively per mole of glucose oxidised (Campbell, Ramakrishnan, Linnes & Eagles, 1956; Campbell & Norris, 1949; Stokes & Campbell, 1951; Claridge & Werkman, 1953; Campbell, Linnes & Eagles, 1954; Newton, 1953). These oxidations were found not to require either DPN or TPN as cofactors (Campbell, Ramakrishnan, Linnes & Eagles, 1956; Campbell, Linnes & Eagles, 1954) and the most active hydrogen acceptor was pyocyanine (Ramakrishnan & Campbell, 1955). De Ley & Stouthamer (1959) have shown a gluconic acid dehydrogenase to exist in _Acetobacter suboxydans_; this enzyme is found in extracts in association with the particulate matter which is probably derived from the plasma membrane. The enzyme is probably linked directly to the cytochromes. This direct oxidative pathway of glucose to 2-oxogluconic acid is

also found in *Ps. fluorescens* (Entner & Stanier, 1951; Wood & Schwerdt, 1953; Wood, 1955). A related direct oxidation of disaccharides by certain *Pseudomonads* without prior hydrolysis or phosphorylation has been described by Bentley & Slechta (1960).

Claridge & Werkman (1954a) showed that the oxidation of radioactively labelled 2-oxogluconate by *Ps. aeruginosa* led to the appearance of the label in a number of amino acids, intermediates of the Krebs cycle, dihydroxyacetone, glucose phosphate and a nucleic acid. De Ley & Vandamme (1955) have demonstrated the presence of 2-oxogluconokinase in *Ps. aeruginosa*. There is no evidence for the further oxidation of 2-oxogluconic acid to 2,5-dioxogluconic acid in this organism; such an oxidation has been found in *Acetobacter melanogenum* (Katznelson, Tenenbaum, Tatum, 1953). De Ley & Defloor (1959) have described a TPNH-linked 2-oxogluconoreductase in *Corynebacterium helvolum* and De Ley & Verhofstede (1957) a TPNH-linked 2-oxo-6-phosphogluconoreductase in *Aerobacter cloacae*. The metabolism of 2-oxogluconate in *Leuconostoc mesenteroides* has been shown by Ciferri & Blakley (1959) to proceed via 2-oxo-6-phosphogluconate, 6-phosphogluconate, ribulose 5-phosphate + CO_2, xylulose 5-phosphate, glyceraldehyde 3-phosphate and acetyl phosphate. However, the exact route of further metabolism of 2-oxogluconate in *Ps. aeruginosa* is not yet known.

Stern, Wang & Gilmour (1960), by use of a radio-respirometric technique, claim that 71% of glucose is catabolised in *Ps. aeruginosa* by way of the Entner Doudoroff (EDS) pathway (Entner & Doudoroff, 1952). Wood (1955) has described the production of glyceraldehyde 3-phosphate (G-3-P) and pyruvate from 6-phosphogluconate (6-PG) by the EDS pathway in *Ps. fluorescens* and also of sedoheptulose 7-phosphate (S-7-P) by the operation of the HMP pathway in the same organism.

Recently it has been shown that the metabolism of fructose and mannose by *Ps. aeruginosa* involves specific inducible permeases for both these substrates (Eagon & Williams, 1960).

d) Tricarboxylic Acid Cycle

Campbell & Smith (1956) demonstrated that *Ps. aeruginosa* possesses all the enzymes of the TCA cycle and also the enzyme isocitratase. The presence of both isocitratase and malate synthetase and the operation of the glyoxylate cycle has been demonstrated in *Pseudomonas* KB1 by Kornberg & Madsen (1957). Although the TCA cycle operates in *Ps. aeruginosa*, it is found that cells exhibit a lag period before oxidising certain of the intermediates of the cycle unless they have been grown in the presence of the specific intermediate. Clarke & Meadow (1959) and Campbell & Stokes (1951)

have shown that cell-free extracts of these cells will oxidize all the intermediates without a lag period, and that the behaviour of whole cells is the result of the necessity of synthesising specific inducible permeases to allow the entry of the substrates to the cells.

c) Endogenous Respiration

Warren, Ells & Campbell (1960) have recorded evidence that the endogenous reserves of Ps. aeruginosa might take the form of amino acid containing polymers rather than the usual polysaccharides. Previous to this, Norris, Campbell & Ney (1949) had discussed certain peculiarities of the endogenous respiration in this organism. They pointed out that it was not diminished by either vigorous aeration or storage, that 2,4-dinitrophenol did not inhibit oxidative assimilation, and that endogenous and substrate oxidation could proceed side by side. They claimed that in Ps. aeruginosa the system for glucose oxidation is inducible and that, in consequence, the endogenous reserves cannot be oxidized by way of glucose and therefore are probably not of normal polysaccharide-type composition. The evidence that the system for glucose oxidation in Ps. aeruginosa is not constitutive is not however unequivocal. A similar lack of oxidation of substrate by whole cells has been shown in the case of TCA cycle intermediates (Clarke & Meadow, 1959; Campbell & Stokes, 1951), fructose

(Eagon & Williams, 1960) and acetate (Campbell, Norris & Norris, 1949) to be due to the lack of a permease and not to any dearth of intracellular oxidizing enzymes. In the case of glucose metabolism this possibility was not considered. The fact that sodium citrate and sodium succinate can, however, inhibit glucose utilisation (Lin, 1962) does suggest some peculiarity in the system for glucose oxidation in *Ps. aeruginosa*.

Aims of Study

The purpose of the work described in this thesis has been to study the system for glucose oxidation in *Ps. aeruginosa*. Such a study can be considered under three main headings:

1. Are the enzymes of glucose oxidation inducible or constitutive?
2. Is there a glucose permease and if so what is its role in glucose oxidation?
3. How does the presence of organic acids affect glucose oxidation?

The results obtained from this study will be discussed in terms of the current theories of enzyme induction and repression.

METHODS

1) Organism.

The organism used throughout this work was donated by Dr. R.W. Kinney of the Department of Bacteriology of Iowa State College, Iowa, U.S.A. The strain was one used by Dr. R.J. Beers and designated 2F32.

2) Methods of Culture.

The organism was maintained of slopes of nutrient agar with subculturing at intervals of about one month.

For experimental study the organism was grown in a liquid synthetic medium. The composition of this medium was:

3 parts of a solution consisting of 9 g. KH_2PO_4 and 2 g. $(NH_4)_2SO_4$ per litre glass distilled water, pH adjusted to 7.1 with NaOH. - Solution A

2 parts of a solution consisting of 20 g. carbon source (carbohydrate or sodium salt of an organic acid) plus 10 ml. 10% w/v solution of $MgSO_4$ per litre glass distilled water. Where necessary pH was adjusted to 7.1. - Solution B.

A large number of carbon sources, including glucose, fructose, gluconate, glycerol, acetate, lactate, citrate, fumarate, succinate, malate and malonate, was found to support growth in this medium.

Solution A was autoclaved at 15 lb. per in.^{2} for 15 min.; Solution B was sterilised by boiling and, when both solutions were cool, added aseptically to Solution A.

Volumes (25 ml.) of medium, in 100 ml. conical flasks, with varying carbon sources, were inoculated from a slope culture and grown overnight at 37^0 on a rotary shaker, the rate of shaking being 48 cycles/min. These cultures were then used as inocula for further subcultures of the organism in media with the same defined carbon source. The inoculum was 0.1 ml. of a stationary phase culture into 25 ml. medium. The organism was taken through at least six subcultures with a particular carbon source before being inoculated into a larger volume of medium, containing the same carbon source, for experimental study of the organism. At the same time another 25 ml. culture was inoculated so as to be available as inoculum for the growth of the next culture. The larger cultures were grown with forced aeration.

The conditions of inoculation and growth, about 17 hr. incubation, resulted in the cells being in the stationary phase for some hours prior to harvesting, with a final population of the order of 1.0 mg. dry bacterial weight/ml. Although the metabolic activity of these cells was rather lower than that of cells harvested during the logarithmic phase of growth, this was compensated by the increased

yield of cells obtained under the conditions employed.

In this manner all the cells studied have been fully trained to grow in a synthetic medium containing one defined carbon source, say glucose. These cells are therefore referred to as glucose-grown cells.

3) Characteristics of Liquid Cultures.

On reaching the stationary phase, liquid cultures produced a green fluorescent pigment. This pigment has not yet been identified. Pigment production was particularly marked in cultures containing citrate or succinate as the sole carbon source and the colour of these cultures appeared to be connected with their final alkaline pH. Addition of a few drops of acid completely destroyed the green colour which could be restored by further addition of alkali. On standing for about 2 weeks at 4° this green colour had a tendency to turn brown.

All the cultures became extremely viscous and "ropy" in appearance on standing at 4°, this phenomenon again being most marked with cells grown on organic acids. Consequently, cells used as inocula for larger cultures for metabolic studies were always taken from a freshly grown culture.

Harvested cells had a red colour, which was particularly marked in the case of gluconate-grown cells. Citrate grown cells were comparatively pale in colour. The colour

is probably due to the cytochromes of the cells, and this matter will be discussed further under the heading of "Cytochrome Pigments in Cell-free Extracts" (Section 11f, below).

4) Production of Pyocyanine.

In their studies of the glucose and gluconic dehydrogenases of *Ps. fluorescens*, Wood & Schwerdt (1953) concluded that although cytochromes with absorption maxima at 550 and 565 mμ were coupled with these enzymes, the lack of cyanide inhibition below a concentration of 10^{-3}M and the absence of a cytochrome oxidase spectrum suggested that a typical cytochrome oxidase is not involved as the terminal hydrogen carrier for these enzymes. Ramakrishnan & Campbell (1955) showed that a concentration of 10^{-3}M cyanide actually activated the gluconic dehydrogenase of *Ps. aeruginosa* and that pyocyanine was the most active hydrogen acceptor for the purified enzyme. The authors concluded that these results cast doubt on the involvement of a normal cytochrome system as hydrogen carriers in this organism and suggested that at least part of this role may be taken *in vivo* by the pigment pyocyanine.

Burton, Campbell & Eagles (1948) have described a growth medium for the production of pyocyanine by *Ps. aeruginosa*

The concentrations listed below are in percent weight to volume :

Glycerol	1.0
Glycine	0.6
L-Leucine	0.8
$MgSO_4.7H_2O$	8.0
K_2HPO_4	0.04
KH_2PO_4	0.01
KCl	0.004
$FeSO_4.7H_2O$	0.001

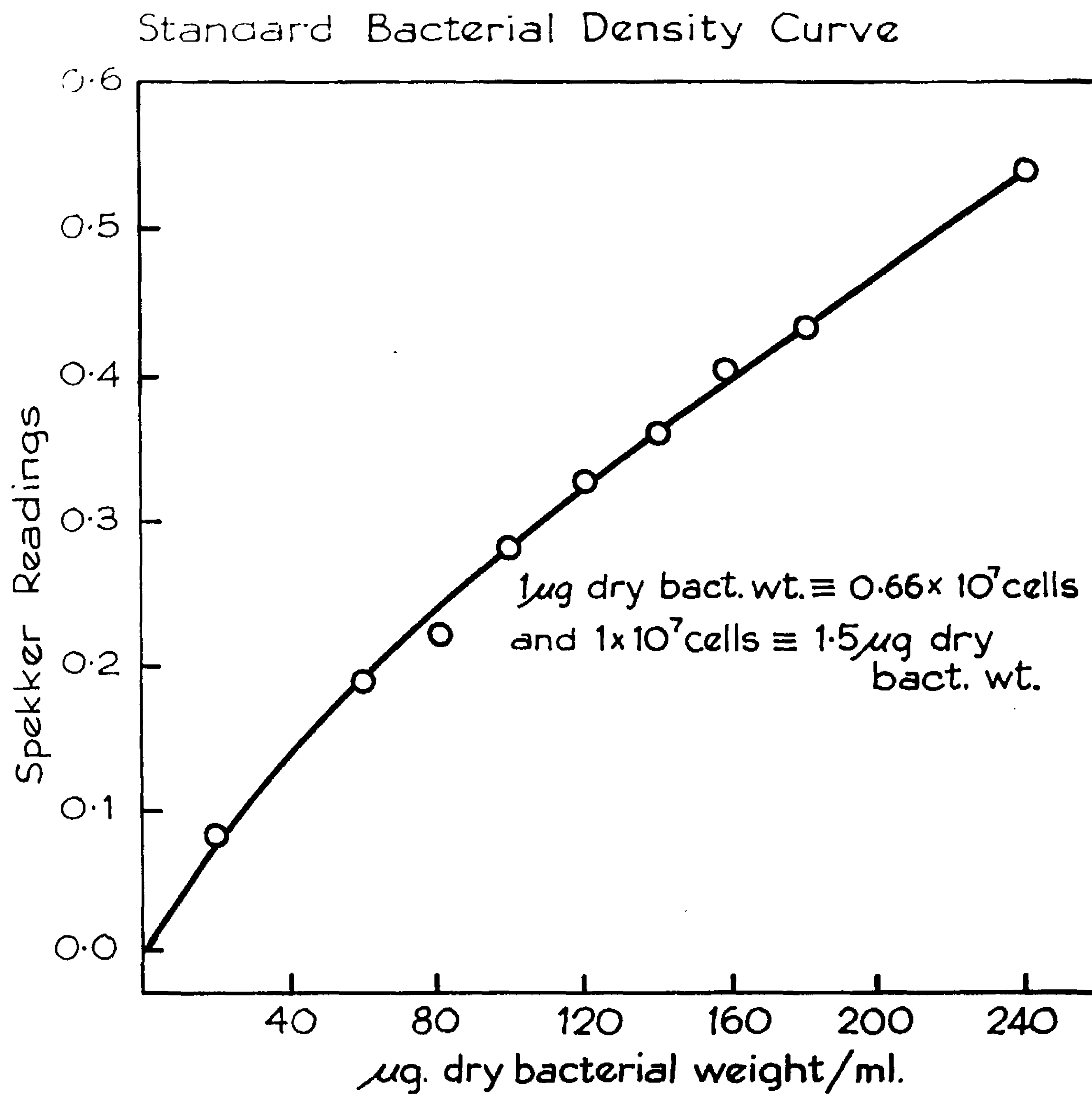
5 litres of this medium were inoculated with a fresh 25 ml. culture of glucose-grown cells and grown with forced aeration for 6 days. The cells were then harvested and the pyocyanine extracted from the medium into 500 ml. chloroform by shaking with four successive volumes of chloroform. The chloroform-insoluble water-soluble red coloured acid form of pyocyanine was extracted from the chloroform with 200 ml. 0.1N HCl and the aqueous solution neutralised with an equal volume of 0.1N NaOH. The blue solution of the neutral pyocyanine was evaporated to dryness, the main volume of the liquid being evaporated at 50° on a rotary evaporator and the remainder by P_2O_5 in a vacuum desiccator, and the pyocyanine dissolved in 10 ml. of water. Prepared in this way the pyocyanine solution contained a large amount of NaCl.

Attempts were made to desalt the preparation by shaking with the Amberlite resins IRC 50 and IRA 401, mixed in the proportions, by weight, 1 to 2.5, but the method was unsuccessful since the pyocyanine was also extracted from the solution by the resins.

5) Measurement of Growth.

A calibration curve was drawn relating dry bacterial weight and cell numbers to extinction, as measured in the Hilger Spekker absorptiometer using micro-cells, of light path 1 cm., and Chance glass filters H 503 and OB 2 (Fig. 6). The calibration curve was prepared as follows. Cells were harvested, washed twice with water and finally taken up as a thick suspension in water. Two 1 ml. samples of this suspension were pipetted onto weighed watchglasses and dried to constant weight at 105°. Care was taken to allow the watchglasses to cool in a desiccator prior to weighing. In this way the dry bacterial weight of the cell suspension was obtained. Dilutions of the suspension were then made and their extinction measured in the Spekker. The cell population was obtained by counting the most dilute suspension in a bacterial counting chamber of depth 0.02 mm. and area $\frac{1}{400}$ mm^2.

Figure 6.



6) Harvesting of Cells.

The cells were harvested by centrifugation on an M.S.E. High Speed Centrifuge at 10,000 rev. per minute for 4 minutes. The cells were normally washed once in 9 g. per litre KH_2PO_4 buffer pH 7.1 by taking up the cell pellets to a smooth suspension in the buffer, transferring all the cells to one tube (c. 45 ml.) and centrifuging again. The cell pellet was then taken up in the required volume of buffer. Where the cells were otherwise treated, by washing in water for instance, any alterations in the above method are specially mentioned in the relevant parts of the Methods and Results sections.

7) Induction Techniques.

Induction of acid-grown cells to glucose was carried out in a buffered solution without the addition of a nitrogen source. In one experiment, Solution A was substituted for buffer without any increase being obtained in the level of induction. The contents of the induction flask were in the same relative concentrations to those used in the manometric experiments, as described in Section 9) below. The contents of the induction flask were:

40 ml. phosphate buffer, pH 7.1 (9 g. per litre),

10 ml. Solution B containing glucose or methyl α-glucoside (Me α-G) as carbon source,

10 ml. cell suspension, prepared by harvesting the cells from a 250 ml. stationary phase culture.

These were mixed in a 250 ml. conical flask and incubated at 37° on the rotary shaker for a period of usually 3 hr. In experiments in which a larger number of cells was being induced, several such induction flasks were set up. At the end of the induction period the cells were harvested, washed once in buffer and taken up as a suspension in buffer.

Glucose-grown cells were induced to organic acids in two ways:

i) Induction to acetate was carried out as above with acetate being substituted as the carbon source in Solution B.

ii) Cells were induced to citrate by growth in a glucose-citrate mixed medium for 2.5 hr.

8) Growth Experiments.

Inoculation with acid-grown cells was achieved by adding a small volume of an overnight culture to the fresh medium. In the experiments with glucose-grown cells as inocula, the overnight culture was harvested, the cells washed and taken up as a suspension in buffer and a portion of this suspension used as inoculum.

The growth media were made up of 3 parts of Solution A and 2 parts of a carbon source solution containing $MgSO_4$ in the same concentration as in Solution B.

For experiments with inoculum cells grown on an organic acid, the carbon sources were 3.55 mM glucose plus either 2.7 mM or 10.0 mM organic acid. These last two figures are approximate and varied with the actual organic acid employed. The relationship between the concentration of the sole carbon source in the medium and the stationary population achieved had already been established. Media (25 ml. volumes) were made up in 100 ml. conical flasks containing 15 ml. Solution A, 0.1 ml. 10% $MgSO_4$ and varying amounts of a 2% solution of glucose or a 2.2% solution of citrate, the volume being made up to 25 ml. with distilled water. Growth was carried out at 37° on the rotary shaker and the final stationary populations of the cultures recorded and plotted against the concentration of either glucose or citrate in the media. The glucose and lower organic acid concentrations used in the experiments described in this section were limiting for growth. The higher concentration of organic acid was usually just in excess of that required to support the highest population possible in this synthetic medium, growing under conditions where aeration of the culture was achieved by shaking.

In the experiments with glucose-grown inocula the carbon sources were 5 mM glucose and 3 mM citrate, the only organic acid used in these experiments.

Experiments with acid-grown inocula were carried out with 100 ml. shaken cultures, those with glucose-grown inocula with 2.5 litre cultures, grown with forced aeration.

Samples (5 ml.) were pipetted off at intervals for the estimation of growth and, after removing the cells by centrifugation, the supernatants were analysed to follow the disappearance of the carbon sources from the medium.

In the experiments with glucose-grown inocula, 100 ml. samples were withdrawn at intervals, the cells harvested, washed and taken up in buffer and their oxidative activity towards glucose and citrate estimated manometrically.

9) Manometric Techniques.

Manometric estimations of oxygen uptake were made by the direct method as described by Umbreit, Burris & Stauffer (1949). The normal flask contents were:

	Additions	ml.
Side arm	Substrate	0.5
Centre well	KOH (20%)	0.2
Flask	Phosphate buffer, (9 g. per litre)	1.8
	Cell suspension or extract	0.5

(The flasks were shaken at 37° in an atmosphere of air. The rate of shaking was 120 cycles/min.).

Mg^{++} was present in the flask contents in all Warburg experiments. Normally the substrate was Solution B, but where the substrate solution did not contain Mg^{++} this was added by using buffer containing 0.67 ml. 10% $MgSO_4$ solution/100 ml.

Where measured, the density of cell suspensions used varied between 0.5 and 3.0 mg. dry bacterial weight/ml. and the protein content of extracts between 10.0 and 20.0 mg. protein/ml. Where the contents differed from these, as for example in experiments with cell-free extracts in which cofactors were added, attention is drawn to this in the discussion of the methods used in these experiments. Rates of oxidation were measured as the Q_{O_2} for the particular substrate, i.e., $\mu l. O_2$/hr./mg. dry bacterial weight.

Rectangular and circular tank Moslab models were used.

10) Experiments with Washed Cell Suspensions.

The metabolic activity of non-proliferating suspensions was assayed in two ways.

(1) By measurement of oxygen uptake in the Warburg apparatus with a single substrate. In all such studies endogenous oxygen uptake was measured with water replacing

the substrate and this value was subtracted from the uptake observed with the added substrates. Where the effect of the addition of chloramphenicol (CAP) was studied, 0.2 ml. of a solution containing 0.24 mg./ml. was added, giving a final concentration in the flask of 60 μg./ml.

(ii) By measurement of the substrate disappearance from an incubation mixture containing more than one substrate.

In this second case, cells treated in a number of ways were studied.

a) Citrate-grown cells, induced by incubation with glucose for 2 hr.
   Simultaneous metabolism of equimolar amounts of citrate and glucose was studied.

b) Acetate-grown cells, induced by incubation with glucose for 2 hr.
   Simultaneous metabolism of equimolar amounts of acetate and glucose was studied.

c) Glucose-grown cells, uninduced to organic acids.
   Effect of the presence of equimolar amounts of organic acids on the metabolism of glucose was studied.

d) Glucose-grown cells, induced by incubation with acetate for 2 hr.
   Simultaneous metabolism of equimolar amounts of acetate and glucose was studied.

e) Glucose-grown cells, induced to citrate by growth for

2.5 hr. in a glucose-citrate mixed medium.

Simultaneous metabolism of equimolar amounts of citrate and glucose was studied.

For these experiments each reaction vessel was a 50 ml. conical flask containing 4 ml. washed cell suspension, 4 ml. 9 g./litre phosphate buffer and 1 ml. of each of the two substrates being studied, to give a final substrate concentration of either 10 mM or 20 mM. In each case a control flask was set up containing only glucose as substrate. The flasks were incubated with reciprocal shaking at 37° and 1 ml. samples withdrawn at intervals, pipetted into 4 ml. ice cold water, centrifuged and suitable dilutions of the supernatants made for assay of the substrates.

ii) Cell-free Preparations.

a) Methods of Lysis.

(i) Penicillin treatment.

A 25 ml. overnight culture of citrate-grown cells was inoculated into the following growth medium:

30 ml. Solution B, with citrate as carbon source,

1.5 ml. 10% $MgSO_4$,

45 ml. Solution A

1 ml. penicillin solution (containing 12 mg. penicillin)

15 g. sucrose (to give a final concentration of 20% w/v sucrose).

The sucrose acts as an osmotic stabiliser to prevent the lysis of any protoplasts formed from growth of the cells in this penicillin containing medium. The high concentration of Mg^{++} in the medium is employed since it has also been shown to increase the stability of protoplast structures (Weibull, 1956).

Even after 20 hr. growth there was little or no evidence of protoplast formation or of lysis when a sample of the culture was added to water, thus diluting the sucrose concentration and thereby decreasing the osmotic pressure of the medium; such a decrease would be expected to cause the lysis of any cell structures which were devoid of a complete cell wall.

It would appear therefore that growth in the presence of penicillin has no effect on the cell wall of Ps. aeruginosa, although Park & Strominger (1957) have suggested that growth in the presence of penicillin inhibits cell wall synthesis in Staphylococcus aureus.

(ii) Lysozyme treatment.

Repaske (1958) has described a method, employing the combined action of 2-amino-2-hydroxymethylpropane 1:3-diol (Tris), ethylene diaminotetra-acetic acid (EDTA) and lysozyme, for the lysis of Ps. aeruginosa and other Gram negative bacteria. Two methods, modifications of the method of

Repaske, were used in this work.

a) Initially the cells were harvested, washed once in 0.005M Tris buffer, pH 8, then taken up as a fairly thick suspension in 0.1 M Tris buffer, pH 8. A volume of this suspension was incubated, without aeration, for 1 hour at 37° with equal volumes of 5 mM EDTA and 100 µg./ml. lysozyme. The incubation mixture contained sucrose to give a final concentration of 20% w/v. The cells were harvested, washed once with 20% sucrose in 0.1 M Tris buffer then taken up in 0.1 M Tris. This suspension was incubated with a little deoxyribonuclease to decrease the high viscosity and this allowed the material to be brought into a smooth suspension. Where lysis, due to the absence of sucrose as an osmotic stabiliser, was not complete, the suspension was subjected to treatment for 60 seconds with a Mullard 50 watt Ultrasonic Drill (Type E 7680), in order to obtain a cell-free preparation. As assayed manometrically, however, these preparations had lost all oxidative activity. This destruction of activity was shown to be due to the EDTA. Whole cells, momentarily exposed to EDTA and then immediately centrifuged, were found to have undergone some lysis and to have lost all oxidative activity.

b) In studies of the effectiveness of various substances as osmotic stabilisers, a further modification of the

method of obtaining protoplasts was employed. Cells were harvested, washed twice with water and then taken up in 0.1 M Tris. This suspension was diluted to about 1.5 mg. dry bacterial weight/ml. for use in the lytic medium. Once again equal volumes of cell suspension, 5 mM EDTA and 100 µg./ml. lysozyme were mixed and in this case a high degree of lysis was obtained within 5 minutes. Lysis was measured by the decrease in extinction at 660 mµ as measured in Hilger Uvispek spectrophotometer using glass cuvettes with a light path of 1 cm.

In order that a substance should be effective as an osmotic stabilizer of protoplast structures, the protoplast must be impermeable to that substance otherwise the concentration of the substance within the cells will be the same as that of the suspending medium and therefore the substance will not increase the osmotic pressure of the medium relative to the cytoplasm of the protoplast. Citrate-grown cells which had been induced with glucose for 3 hours were compared with cells incubated with water for 3 hours; glucose at varying concentrations was added to the lysis medium and its effectiveness as an osmotic stabilizer of the protoplasts formed studied by its ability to inhibit the decrease in extinction of the suspension.

b) Polymyxin Treatment.

Polymyxin is the only antibiotic to which Ps. aeruginosa shows any sensitivity. Newton (1956) has reviewed the work relating to the action of polymyxin on certain Gram negative organisms, in particular, Ps. aeruginosa. Polymyxin is known to effect a disorganisation of the plasma membrane, thus leading to altered permeability properties of the cell. The effectiveness of polymyxin treatment was assayed by measuring the release of material with absorption maxima at 260 mμ (purines, pyrimidines and nucleotides) from the cells into the medium. The following protocol was found to be the most effective. Cells were harvested, washed in 1% saline solution and taken up in this solution to give a final concentration of 1.5 mg. dry bacterial weight/ml. Volumes of this suspension were incubated without aeration for 2 hr. at 37° in the presence of polymyxin at a final concentration of 100 μg./ml. During the incubation the suspension flocculated but could be resuspended evenly by shaking the tube. The cells were harvested, washed in 9 g. per litre phosphate buffer and taken up in this buffer. The cells were difficult to get into a smooth suspension and the suspension then obtained appeared very thin.

Although activity of glucose 6-phosphate and isocitrate dehydrogenases could not be demonstrated by the spectrophotometric assay (Section 11, d(i) below), in untreated

glucose-grown cells, they could be demonstrated in polymyxin treated cells. When, however, these cells were assayed manometrically for oxygen uptake with glucose, glucose 6-phosphate and iso-citrate as substrates, they were found to have no activity.

c) Cell Disruption Techniques.

Various methods of disrupting the osmotic barrier of the cells were attempted and the activity of these preparations assayed by oxygen uptake measured on the Warburg apparatus.

(i) Mickle Tissue Disintegrator. 5 ml. of Ballotini
No.12 (i) Mickle Tissue Disintegrator. 5 ml. of Ballotini
at 4° with 10 ml. of bacterial suspension in the Mickle (1948) Disintegrator for periods of 1 hr. and 3 hr. The extracts showed no activity.

(ii) Toluene Treatment. 3 ml. of cell suspension was shaken with 1 ml. toluene for 5 min. at room temperature. The suspension showed no activity.

(iii) Hughes' Press. Cell paste was cooled to -15° and crushed in a Hughes' (1951) press. The paste was thawed out and the debris separated at 15,000 r.p.m. on the M.S.E. High Speed centrifuge for 60 min. The cell-free extract had no activity. Later work however showed that oxidative activity was dependent on the particulate matter of the extract, and

under the conditions used here this fraction would be separated from the cell-free extract by the centrifugation.

(iv) Vacuum Drying. A suspension of cells in water was evaporated to dryness over P_2O_5 in a vacuum desiccator. The cells were then taken up in 9 g. per litre phosphate buffer and assayed but found to be inactive.

(v) Alumina and Powdered Glass Grinding. A cell paste was ground in a glass mortar with alumina powder (grade 3/50, Griffin & George, Glasgow) or Pyrex powdered glass (200 mesh), the whole operation being carried out at 4°. The cell debris and powder were centrifuged and the resulting cell-free extract assayed. Neither method gave extracts with activity in the Warburg assay, but an extract from glucose-grown cells made with glass powder was found by spectrophotometric assay (Section 11.d(i) below) to have high glucose 6-phosphate and iso-citrate dehydrogenase activities and moderate 6-phosphogluconate (6-PG) dehydrogenase activity.

(vi) Nelco Homogeniser. The method used was that of Lamanna & Mallette (1954). A thick cell suspension (6 ml.) was homogenised with 4 ml. Ballotini No.12 glass beads for a total of 25 min. in an M.S.E. homogeniser (Nelco) fitted with a masticator attachment and a 25 ml. Universal container which was surrounded by ice. The treatment was conducted in 5 min. periods. The glass beads and cell debris were separated by centrifugation but the extract showed no activity.

(vii) <u>Milner, Lawrence & French Press.</u> Milner et al. (1950) have described a method for disruption of micro-organisms. A thick cell suspension was subjected to a pressure of about 60 tons and, by adjustment of a needle valve, forced slowly through a narrow orifice. Cell debris was separated by centrifugation at 10,000 r.p.m. for 1 min. in the M.S.E. High Speed centrifuge and the cell-free extract was found to have slight oxidative activity.

(viii) <u>Freeze Drying.</u> A thick cell suspension was freeze dried and the cells stored at -18°. For assay a suspension was made in 9 g. per litre phosphate buffer. These preparations had only slight oxidative activity in the Warburg.

Of the above, methods (i), (ii), (iii), (iv), (v), (vi) all gave preparations which failed to demonstrate any oxidative activity even towards the substrate on which the cells had been grown. Methods (ii), (iv), (v), (vi) were all carried out on succinate grown cells and addition of 0.2 ml. of 340 mg./100 ml. solution of phenazine methosulphate (a respiratory carrier related to pyocyanine) did not affect the oxygen uptake with either succinate or glucose as substrate. Method (viii) gave preparations from glucose-grown cells which although they showed a slow rate of oxidation of glucose did not have the permeability barrier destroyed and did not oxidise iso-citrate (Campbell & Stokes, 1951). Extracts prepared from glucose-grown cells by method (vii) did,

however, have this barrier removed and had oxidative activity to both glucose and succinate. These activities were, however, comparatively low.

(ix) Ultrasonic Disintegration. The method finally adopted for the production of cell-free extracts was to subject a thick cell suspension (about 5 ml.) to the action of the Mullard 50 watt Ultrasonic Drill (Type E7680) for periods of 5 minutes. The cells were contained in a straight walled glass vessel which was surrounded by ice. This method did not give complete destruction of the cells and to obtain a true cell-free extract the preparation was centrifuged for 15 minutes on a Baird and Tatlock semi-micro angle-head centrifuge at an unspecified speed. The conditions of extract formation and activity assay were varied by using a number of buffer systems and also by adding various cofactors.

A solution of heat stable cofactors extracted from succinate-grown cells was obtained by harvesting 400 ml. of a culture, taking the cells up in 10 ml. of water and placing in a boiling water bath for 25 min. The cellular material was separated by centrifugation and the cofactor solution stored at -18°. Other cofactors used were:-

phenazine methosulphate (340 mg./100 ml.),

pyocyanine (concentration of solution obtained in extraction unknown),

2,6-dichlorophenol indophenol (DCPIP) (50 mg./100 ml.),

triphosphopyridine nucleotide (TPN) (2 mM),

diphosphopyridine nucleotide (DPN) (2 mM),
flavin adenine dinucleotide (FAD) (1 mg./ml.),
cytochrome c (cyt c) (3 mg./ml.), and
methylene blue (0.5 mM).

The efficiency of these cofactors in increasing the rate of oxygen uptake in the Warburg was studied. Phenazine possibly had a slight effect on glucose oxidation. Pyocyanine slightly increased endogenous oxidation and also the oxidation of glucose and iso-citrate. TPN caused a slight raising of the rate of oxidation of the endogenous reserves and a marked raising with iso-citrate as substrate, which rate was not further affected by addition of FAD, cyt c or DPN, but was slightly increased by the further addition of methylene blue. The oxidation of glucose was not affected by the addition of any of these cofactors.

In the experiments in which the oxidative activity towards glucose of whole cells and extracts from succinate-grown cells induced by incubation with glucose for varying periods of time was studied, the protocol was as follows. 1 litre of culture was harvested and the cells taken up in 40 ml. 9 g. per litre phosphate buffer. Volumes (10 ml.) of this suspension were induced by incubation with glucose for periods of 0, 15, 30 and 60 min., the cells harvested and made up to 5 ml. in 0.04 M $NaHCO_3$ pH 7.1 which contained reduced glutathione at a final concentration of 0.01 M. Volumes

(5 ml.) of these suspensions plus 1 ml. of heat stable co-factor solution were treated for 5 min. with the ultrasonic drill to obtain disrupted cell preparations. These preparations were assayed in the Warburg with 0.4 ml. of the buffer replaced with 0.2 ml. phenazine and 0.2 ml. heat stable co-factor solutions. Although these preparations were not centrifuged, the linearity of the rate of oxygen uptake argues against the activity being due to whole cells in the preparation. The nitrogen contents of these preparations were not measured but they were prepared from suspensions of equal cell density (11.0 mg./ml.).

Due to the low and irreproducible levels of activity that were demonstrable in the Warburg, extracts were assayed for the activity of individual enzymes. For these experiments the following protocol was developed. Cells from a 500 ml. culture were harvested, washed in 0.1 M Tris buffer pH 7.1 and then taken up as a thick suspension in the same buffer. Approximately 5 ml. of the suspension was treated in the ultrasonic drill for 5 min. and the preparation then centrifuged for 15 min. Initially the 9 g. per litre phosphate buffer was employed, but this was found to inhibit both glucose 6-phosphate and 6-phosphogluconate dehydrogenases. Wood & Schwerdt (1953) have also reported that phosphate buffer inhibits oxygen uptake of sonic extracts of _Ps. fluorescens_ with glucose 6-phosphate or 6-phosphogluconate as substrate.

A number of experiments were designed to study the effect of the simultaneous metabolism of citrate on the metabolism of glucose by extracts from citrate-grown glucose-induced cells. The cells from a 1 litre culture were induced by incubation with glucose for 3 hr. then harvested, washed once in 9 g. per litre phosphate buffer containing 0.67 ml. 10% $MgSO_4$/100 ml. and then taken up as a thick suspension in this Mg^{++} supplemented buffer. Two 5 ml. portions of this suspension were treated in the ultrasonic drill for 5 min. and the combined extract centrifuged for 5 min. Two 50 ml. conical flasks were set up with the following contents:-

	Additions (ml.)	
	Test	Control
Extract	4.0	4.0
Chloramphenicol (0.6 mg./ml.)	3.0	3.0
TPN (2 mM)	0.4	0.4
Methylene blue (0.5 mM)	0.6	0.6
Glucose (50 mM)	1.0	1.0
Sodium citrate (50 mM)	1.0	-
Water	-	1.0

and incubated with reciprocal shaking at 37°.

Samples (1 ml.) were withdrawn at intervals, placed in a boiling water bath for 5 min., 4 ml. water was added, the denatured protein material centrifuged down and the supernatant decanted off, filtered and stored at -18°. This method effectively removed sufficient protein for glucose estimations to be carried out on 1 ml. portions of the filtrate without further treatment. For estimation of citrate, 1 ml. of filtrate was added to 2 ml. of 40% trichloroacetic acid, the precipitated protein centrifuged and the estimation carried out on 1 ml. of the supernatant. For chromatography 0.2 ml. of the untreated filtrate was spotted on the paper.

In one of these experiments estimations of keto acids were carried out on samples from the test flask. Trichloroacetic acid (3 ml. of 40%) was added to 1 ml. of the filtrate, the protein precipitate centrifuged down and 3 ml. of the supernatant assayed for total keto acid by ethyl acetate extraction procedure.

To compare oxygen uptake with substrate disappearance, parallel Warburg flasks were set up to assay oxidation with water, glucose and citrate as sole substrates. The volumes of reactants were 0.23 of those of the reaction flasks for the study of substrate disappearance.

Since these extracts had only been centrifuged for 5 min., they contained a number of whole cells. Extracts which were centrifuged for 15 min. were found to have lost

almost all of their activity, presumably due to the separation of the particulate matter. The concentration of chloramphenicol employed, although not affecting the rate of oxidation, prevented growth of these cells during the period of incubation. The number of whole cells in the incubation flasks was determined by doing viable counts. Using a series of test-tubes of 10 ml. volumes of sterile water, a 1 in 50,000 dilution of the contents of the incubation flasks was obtained. This dilution (0.1 ml.) was plated on nutrient agar in a petri dish, incubated at 37° overnight and the colonies counted.

d) Assays of Individual Enzymes in Cell-free Extracts.

The following enzymes were assayed, all at 37°:-

(i) Glucose 6-phosphate, 6-phosphogluconate and Iso-citrate Dehydrogenases.

These enzymes were assayed by following the rate of reduction of TPN as measured by the increase in extinction at 340 mμ in the Hilger Uvispek spectrophotometer using quartz cells of light path 1 cm. This instrument had a specially designed cell housing permitting the circulation of water from a thermostat to maintain the temperature at 37°. The contents of the cuvettes were:

	ml.
Glycylglycine, pH 7.1 (0.04 M)	2.0
Substrate (10 mM)	0.5
Extract	0.1, 0.2 or 0.3
TPN (3 mM)	0.1 or 0.2

The contents of the cuvette, minus the TPN, were equilibrated at 37° for 10 min., then the TPN was added, the cuvette contents mixed and readings taken every 15 sec. A control cuvette was set up with water replacing the substrate and the increase in extinction was subtracted from that observed with added substrates. The activity, based on the initial rate as ΔE/min., can be expressed as µmoles substrate metabolised/hr./mg. protein using the molar extinction coefficient of TPNH at pH 7 and at 340 mµ, which is 6.22×10^3 (Kornberg & Horecker, 1953).

(ii) Hexokinase, Gluconokinase and 2-oxogluconokinase.

Hexokinase could not be assayed by measuring the increase in extinction at 340 mµ in the presence of excess glucose, ATP and TPN, i.e., by measuring the conversion of glucose 6-phosphate, formed from glucose and ATP by the action of hexokinase, to 6-phosphogluconate, since ATP was found to inhibit both the glucose 6-phosphate dehydrogenase of the

extract and commercially prepared enzyme. Wood & Schwerdt (1953) reported a similar inhibition of the glucose 6-phosphate dehydrogenase of Ps. fluorescens.

The method employed for the assay of these three kinases was the manometric procedure of Colowick & Kalckar (1943). The contents of the Warburg flasks were:-

	Additions	ml.
Side arm	$NaHCO_3$ (0.04 M)	0.2
	ATP (0.05 M)	0.2
Flask	Extract	0.2
	$MgCl_2$ (0.04 M)	0.2
	Substrate (10 mM)	1.0
	KF (0.5 M)	0.25
	$NaHCO_3$ (0.04 M)	0.95

The reaction was carried out at 37° in an atmosphere of 95% N_2/5% CO_2.

The substrate was water, as a control, or 10 mM glucose, sodium gluconate or sodium 2-oxogluconate. Potassium fluoride was used to inhibit the adenosine triphosphatase activity of the extract. The activity of the kinases, based on the initial rate in µl. CO_2 evolved/min., was expressed as µmoles substrate metabolised/hr./mg. protein.

(iii) Combined Action of 6-phosphogluconate Dehydrase and 2-oxo-3-deoxy-6-phosphogluconate Aldolase of the Entner-Doudoroff Scheme, and the Enzymes for the Conversion of 3-phosphoglyceraldehyde to Pyruvate.

For the assay of the enzymes of the Entner-Doudoroff scheme the protocol was as follows. An incubation at 37° was carried out in a 6 in. by 1 in. tube with the following reactants.

Additions	ml.
Glycylglycine, pH 7.1 (0.04 M)	2.4
Hydrazine (0.56 M)	1.0
Sodium arsenite (0.1 M)	0.1
Extract	0.5
6-phosphogluconate (10 mM)	1.0

The reactants, minus the 6-phosphogluconate, were equilibrated to temperature for 10 min. and the 6-phosphogluconate added at zero time. An endogenous control flask with water as substrate was also set up. Samples (1 ml.) were withdrawn at 0.25, 2, 4 and 8 min., 0.3 ml. 40% trichloroacetic acid added, the precipitated matter centrifuged and pyruvate estimated by the toluene extraction method of Friedmann & Haugen (1943) on 1 ml. of the supernatant. (It is not

possible to use the direct method of these authors in this system as the reagents were precipitated.) The hydrazine reacts with the 3-phosphoglyceraldehyde formed from one half of the 2-oxo-3-deoxy-6-phosphogluconate molecule by the action of the aldolase and the arsenite prevents the further metabolism of the pyruvate formed from the other half. Under these conditions, therefore, 1 μmole of 6-phosphogluconate gives 1 μmole of pyruvate. The activity, based on the initial rate in μmoles pyruvate formed/min., was expressed as μmoles 6-phosphogluconate metabolised/hr./mg. protein.

To test the activity of enzymes converting 3-phosphoglyceraldehyde to pyruvate, hydrazine was replaced by water in the above reaction and under these conditions, if the enzymes are present, 1 μmole 6-phosphogluconate should yield 2 μmoles pyruvate.

(iv) Enzymes from the Conversion of 3-phosphoglyceric acid to Pyruvate.

The assay was carried out at 37° in a 6 in. by 1 in. tube with the following reactants:

Additions	ml.
Glycylglycine, pH 7.1 (0.04 M)	3.3
3-phosphoglyceric acid (10 mM)	2.0
ADP (20 mM)	0.5
Sodium arsenite (0.1 M)	0.2
$MgCl_2$ (0.04 M)	0.1
Water	0.5
Extract	0.5

Samples (1 ml.) were withdrawn at intervals over a 60 min. period, 0.2 ml. 40% trichloroacetic acid added, the precipitated protein centrifuged down and pyruvate estimated by the direct method on 0.5 ml. of the supernatant.

(v) Glucose and Gluconic Dehydrogenases.

The method of assay was a modification of that due to Ghiretti & Barron (1954) in which 2,6-dichlorophenol indophenol (DCPIP) is added as hydrogen acceptor and the activity of the enzyme assayed by the rate of reduction of the DCPIP measured by the decrease in extinction at 607 mμ. When this method was preformed in an atmosphere of air considerable re-oxidation of the reduced DCPIP was observed. Consequently the following method of Peel (1960) was adopted. Pyrex test-

tubes, 5 in. by 0.63 in., were fitted with side arms of approximately 1 ml. volume. The reaction mixture was:-

	Additions	ml.
Tube	Glycylglycine, pH 7.1 (0.04 M)	4.5
	Substrate (10 mM)	1.0
	2,6-dichlorophenol indophenol (50 mg./100 ml.)	0.2
Side arm	Extract	0.3

The tubes were kept in a water bath at 37° until immediately prior to the beginning of the assay. A stream of nitrogen was passed through the contents of the tube for 2 min. This was achieved by using a Pasteur pipette fixed in a rubber stopper. When the stopper sealed the mouth of the tube, the tip of the pipette just reached the bottom of the tube and when the pipette was connected to the nitrogen cylinder the stream of gas passed through the reactants in the tube and out by way of the side arm. At the end of the gassing period the side arm outlet was sealed with a rubber cap and the pipette and stopper withdrawn and the mouth of the tube rapidly sealed with a Subaseal cap (Turnover type, size 17; Subaseal Ltd., Barnsley).

At zero time the extract was tipped from the side arm into the tube and the reactants thoroughly mixed. At 15 sec. intervals over a 5 min. period the extinction was read at 607 mμ in a Unicam SP 350 Model D.G. Spectrophotometer. The substrates used were glucose and sodium gluconate, with water as an endogenous control. Owing to the variable and often high rate of reduction of DCPIP in the control tube, particularly with extracts from citrate-grown cells, it was not possible to express activities in a quantitative manner. This high activity of the control was not diminished by dialysis for 5 hr. against tap water.

With glucose or gluconate as substrate there is no reduction of TPN by the extract.

(vi) Fructose 1,6-diphosphate Aldolase.

In this assay a 6 in. by 1 in. tube contained the following reactants:

Additions	ml.
Glycylglycine, pH 7.1 (0.04 M)	1.0
Tris buffer, pH 7.1 (0.1 M)	1.0
Sodium arsenate (0.05 M)	1.0
Fructose 1,6-diphosphate (10 mM)	2.0
DPN (2 mM)	0.5
Extract	0.5

The tube was incubated at 37° and 1 ml. samples withdrawn at intervals over a 20 min. period. Trichloroacetic acid (2 ml. of 10%) was added to each, the precipitate centrifuged down and fructose estimations carried out on 0.5 ml. of the supernatant.

(vii) Phosphatases.

The phosphatase activity of extracts was assayed by estimating the release of inorganic phosphate from the following substrates: glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, α-glycerophosphate and ATP. The inorganic phosphate of the extract was also estimated. The assay was carried out both at pH 5.0 and pH 8.1 in small test-tubes with the following contents:

	Additions	ml.
pH 5.0	Phthalate buffer, pH 5.0	1.6
	Substrate (10 mM)	1.0
	$MgCl_2$ (0.012 M)	0.1
	Extract	0.3
pH 8.1	$NaHCO_3$, pH 8.1 (0.04 M)	1.6
	Substrate (10 mM)	1.0
	$MgCl_2$ (0.012 M)	0.1
	Extract	0.3

The phthalate buffer, pH 5.0, was made up by the method of Gomori (1955). The tubes were incubated at 37° and 0.5 ml. samples withdrawn at intervals over a 20 min. period. Trichloroacetic acid (2.5 ml. of 5%) was added, the protein precipitate centrifuged down and 1.0 ml. portions of the supernatant used in inorganic phosphate estimations.

e) Measurement of Protein Content of Extracts.

The nitrogen content of the extracts was obtained by the biuret method of Stickland (1951). The results, expressed as mg. protein nitrogen, were multiplied by the factor 6.25 for approximate conversion to mg. protein.

f) Cytochrome Pigments in Cell-free Extracts.

Horio (1958a, b), Yamanaka (1959) and Higashi (1960) have studied the cytochrome content of *Ps. aeruginosa* and the manner in which the various cytochromes are linked in the electron transport pathway. These workers have identified the following cytochromes in *Ps. aeruginosa*:

P-cytochrome$_{551}$ with absorption maxima in the reduced state at 551, 521 and 416 mμ;

P-cytochrome$_{554}$ with absorption maxima in the reduced state at 554, 525 and 416 mμ;

P-cytochrome$_{560}$ with absorption maxima in the reduced state in the region of 560 and 530 mμ;

P-cytochrome oxidase with absorption maxima in the reduced state at 620-630, 551, 548, 521-523 and 418 mμ;

and a compound, P-blue protein with an absorption maximum in the oxidised state at 695 mμ.

The cytochrome content of extracts was studied by obtaining the absorption spectrum between 400 and 650 mμ in the Cary Recording Spectrophotometer Model 11. The cuvette, (light path 1 cm.), contents for study of the spectrum of the extract in the oxidised state were as follows:

Additions	ml.
Extract	1.0
Phosphate buffer, pH 7.1 (9 g. per litre)	1.3
Glycerol (50% v/v)	1.0
Potassium ferricyanide (0.1% w/v)	0.05

To obtain the reduced spectrum, solid sodium hydrosulphite was added. In practice it was found that only one spectrum was obtained with maxima at 552-551, 523-521 and 418-416 mμ, corresponding closely to the spectrum of reduced P-cytochrome$_{551}$ or mammalian cytochrome c. Absence of a cytochrome oxidase was indicated both by the absence of a characteristic peak in the region 630-620 mμ and by the inability to obtain the cytochrome in the oxidised state. This might possibly be due to the cytochrome oxidase remaining

attached to the particulate matter of the cells and being centrifuged down in the preparation of these extracts. Two methods were attempted in order to obtain cytochrome oxidase in solution. A bacterial suspension (5 ml.) was treated with the ultrasonic drill for 5 min. with 80 mg. sodium deoxycholate and another 5 ml. portion treated for 15 min., both extracts being centrifuged for 5 mins. The supernatants, however, showed only the 521, 552 and 417 mμ peaks and the sediments had no absorption maxima in the 400 to 600 mμ ranges.

13) <u>Studies with Methyl α -Glucoside.</u>

a) <u>Synthesis.</u>

Radioactive Me α - G was synthesized from [U-^{14}C] glucose by the method of Cadotte, Smith & Spriestersbach (1952). [U-^{14}C] glucose (4.78 mg., 0.1 mC) was transferred quantitatively into the reaction tube with 15 ml. methanol, 0.9952 g. unlabelled A.R. glucose and 1.0 g. Amberlite IR 120 resin added and the tube then sealed and heated to 112° for 2 hr. After cooling, the tube was opened and the resin filtered off and extracted twice with boiling methanol. The combined filtrate and washings were reduced to small bulk (about 5 ml.) and Me α - G crystallised out. Me α - G was recrystallised from hot ethanol and its purity verified by determinations of melting point (m.p. 166°) and mixed

melting point with recrystallized commercial Me α-G (m.p. 166°), and by descending chromatography. Spots on the chromatogram were identified both by a specific staining technique (Section 14, below) and by autoradiography. The synthesised [^{14}C] Me α-G gave one large spot with the same R_f as a marker of commercial Me α-G, with a slight contamination of glucose, as shown by the staining technique. The autoradiograph showed that all the radioactivity had moved with the Me α-G spot. The yield of Me α-G was 216.2 mg. with a specific activity of 0.1 µC/mg. To this was added 17.8 mg. unlabelled Me α-G and the combined Me α-G dissolved in 34 ml. water to give a 36 mM solution of specific activity 0.64 µC/ml.

b) Accumulation of Methyl α-glucoside.

The accumulation within the cells of high concentrations of Me α-G was studied with cells treated in the following manners:

(i) Citrate-grown cells, induced by incubation with Me α-G for 3 hr.

Subsequent incubation with [^{14}C] Me α-G was for a period of 20 min.

(ii) Glucose-grown cells. Incubated with [^{14}C] Me α-G for 60 min.

(iii) Glycerol-grown cells. Incubated with [^{14}C] Me α-G for 135 min.

(iv) Glycerol-grown cells, grown in the presence of Me α-G
Also incubated with [^{14}C] Me α-G for 135 min.

(v) Glycerol-grown cells. Incubated with [^{14}C] Me α-G for 60 min. in the presence of added Solution B with glycerol as carbon and energy source.

The protocol for these accumulation studies was as follows:

An overnight culture (250 ml.) was harvested, induced by incubation with Me α-G for 5 hr. in experiment (i) only, and the cells taken up in 10 ml. 9 g. per litre phosphate buffer. The contents of the incubation flask were:

Additions	ml.
Cell suspension	6.0
[^{14}C] Methyl-α-glucoside 36 mM, in Experiments (i) & (ii) 3.6 mM, in Experiments (iii), (iv) & (v)	1.0
Water, in Experiments (i), (ii), (iii) & (iv) Solution B, in Experiment (v)	2.0

The incubation flasks, 50 ml. conical flasks, were incubated at 37° with reciprocal shaking. Samples (2 ml.) were pipetted off at zero time and after the period of

incubation noted in each experiment. These samples were centrifuged rapidly and 0.1 ml. of the supernatant plated for assay of radioactivity. The centrifuge tube was drained, the cells taken up in 4 ml. of ice cold water and 0.1 ml. of this suspension was also plated. The cells were again centrifuged, 0.1 ml. of the supernatant plated, the cells again taken up in 4 ml. of ice cold water and 0.1 ml. of the resulting suspension was plated.

c) Effect of Incubation with Methyl α -glucoside on the Metabolism of /U-^{14}C/ Glucose.

A culture (250 ml.) of citrate-grown cells was harvested, induced by incubation with Me α - G for 3 hr., the cells harvested, washed and made up to 15 ml. with 9 g. per litre phosphate buffer. Seven Warburg flasks were made up containing 9 g. per litre phosphate buffer supplemented with 0.67 ml. 10% $MgSO_4$/100 ml. One flask served as an endogenous control for oxygen uptake measurements and had 0.5 ml. water in the side arm, and in the other six the substrate was 0.5 ml. 18 mM /U-^{14}C/ glucose of specific activity 0.64 μC/ml. After 0, 15, 60, 90, 150 and 195 min. from the start of the incubation, a glucose flask was taken off and 2 ml. of the contents pipetted into 2 ml. of ice cold water and centrifuged. Portions (0.1 ml.) of the

supernatant were plated for radioactive assay, the cell pellet was drained and then taken up in 4 ml. of ice cold water and 0.1 ml. of the resulting suspension was plated. Glucose was also estimated by the Nelson (1944) method on 1 ml. of a 1 in 2 dilution of the supernatant.

Thus the disappearance of glucose and of radioactivity from the medium, the radioactivity taken up by the cells and the oxygen uptake were all measured and, using the specific activity of the glucose and assuming complete oxidation of the glucose to carbon dioxide and water, these were all expressed as umoles glucose. Since the radioactivity of the cells was not lost on washing, it was assumed that this represented material assimilated into the cellular protoplasm.

Portions (0.05 ml.) of the supernatants were spotted on a chromatogram and the spots identified after development by staining and autoradiography.

13) Methods of Assaying Radioactivity.

a) Counting Techniques.

Counting was done by a combination of a thin end-window Geiger-Müller tube mounted in a lead castle and a Panax scaling unit. The samples were plated directly on to metal planchettes with an effective raised area of 1.5 cm^2. and these were mounted on the uppermost stage of the castle. Replicate samples were plated and counted for 10 min. As samples were plated at infinite thinness, no correction for

self-absorption was applied. With the more active samples, coincidence corrections were applied using a standard curve for the counting system relating counts/min. to coincidence correction.

The planchettes were cleaned by rubbing the surface with water and detergent powder, using the tip of the finger. They were then washed with tap and distilled water and dried by pressing between sheets of filter paper. Planchettes were used only once. To ensure a uniform distribution of material on the planchettes, two drops of ethanol were first placed on the surface of the planchette then 0.1 ml. of the radioactive sample added from an 0.2 ml. E-Mil Gold Line graduated pipette; the tip of the pipette touched the surface of the ethanol throughout the period of delivery. The planchette was then dried under an infra-red lamp.

b) Autoradiography.

Identification of radioactive materials on chromatograms was carried out by placing the developed chromatogram in contact with a Kodak Industrex Type D X-ray film for a period of two to three weeks. Development of the film with Kodak D.19b developer gave dark spots corresponding to the radioactive spots on the chromatogram.

14) Chromatography.

Chromatographic separation was obtained by descending chromatography on Whatman No.1 paper. Two solvent systems were used. For the study of the purity of Me α-G, the system was ethyl acetate (40 ml.), pyridine (10 ml.) and water (40 ml.). For the separation of glucose, 2-oxo-gluconic acid and pyruvic acid, the system was ethanol (45 ml.) methanol (45 ml.) and water (10 ml.). R_F values with this solvent system were extremely variable and identification of spots was always achieved by comparison with a marker spot run on the same chromatogram. Gluconate and 2-oxogluconate ran very close together in this system, and this situation was further complicated by the fact that, in the solutions chromatographed, these solutes were always dissolved in 9 g. per litre phosphate buffer which causes marked streaking in the region between the origin and the gluconate and 2-oxo-gluconate spots. As a result of this, it was impossible to decide unequivocally whether spots obtained in this region were gluconate, 2-oxogluconate or a mixture of both. Fortunately, in a number of cases, oxygen uptake studies with glucose as substrate aided identification.

The staining technique used in both cases was that of Trevelyan, Proctor & Harrison (1950) using silver nitrate in aqueous acetone and sodium hydroxide in aqueous ethanol as the two sprays and washing the papers in 6N ammonium

hydroxide and then several times in water.

15) Chemical Methods of Estimation.

a) Glucose was estimated by the method of Nelson (1944).

b) Citrate, as citric acid, was estimated by the method of McArdle (1955).

c) Pyruvate and keto-acids of the tricarboxylic acid cycle were estimated by the toluene and ethyl acetate extraction techniques of Friedemann & Haugen (1943).

d) Fructose was estimated by the method of Heyrovský (1956).

e) Phosphate was estimated by the method of Allen (1940).

f) Acetate was steam distilled in a Markham (1942) still after acidification to approximately pH 3.5 with H_2SO_4, and estimated by titration with 0.01N NaOH using a Conway micro-burette. The indicator was phenol red. The 0.01N NaOH was kept in a CO_2-free atmosphere and a stream of CO_2-free air was bubbled through the liquid during titration.

As shown by Lewis & Elsden (1955), carrying out the steam distillation at pH 3.5 minimised the possibility of high readings as a result of the distillation of pyruvate along with the acetate.

16) Sources of Chemicals.

Wherever possible, chemicals of analytical or laboratory reagent quality were used. Special chemicals used were

(supplier's name in brackets):

Chloramphenicol B.P. was a gift from Parke, Davis & Co.Ltd.

Polymyxin B sulphate (potency 7812 u/mg.) was a gift from Pfizer Ltd.

Lysozyme, crystallized from egg white (Armour Laboratories).

Glutathione, reduced (Light & Co.Ltd.).

Phenazine (Light & Co.Ltd.)

2-Oxogluconic acid, as calcium salt (Light & Co.Ltd.).

Gluconic acid lactone (Light & Co.Ltd.).

Iso-citric acid lactone (Light & Co.Ltd.).

Glucose 6-phosphate, as barium salt heptahydrate (Sigma Chem.Co

6-Phosphogluconate, as sodium salt (Sigma Chem.Co.).

Adenosine triphosphate, as disodium salt trihydrate (Sigma Chem.Co.)

Flavin adenine dinucleotide, approximate assay 80% (Sigma Chem. Co.)

Diphosphopyridine nucleotide (Boehringer & Soehne).

Triphosphopyridine nucleotide, as monosodium salt tetrahydrate, 98-100% pure (Sigma Chem.Soc.).

/U-^{14}C/ Glucose (Radiochemical Centre, Amersham).

Penicillin (Glaxo Laboratories Ltd.).

Deoxyribonuclease (Nutritional Biochemicals Corp.).

Cytochrome c, prepared from horse heart, was a gift from Dr. G. Leaf of this Department.

Glucose 6-phosphate dehydrogenase (Sigma Chem.Co.).

Fructose 1,6-diphosphate, as calcium salt (Light & Co.Ltd.).

<u>Amberlite resins IRC 50, IRA 401 and IR 120</u> (British Drug Houses).

<u>Adenosine diphosphate</u>, as trisodium salt monohydrate (Boehringer & Soehne).

<u>3-phosphoglyceric acid</u>, as barium salt (Boehringer & Soehne).

<u>α-glycerophosphate (DL)</u>, sodium salt (Sigma Chem. Co.).

<u>Glucose 1-phosphate</u>, dipotassium salt (British Drug Houses).

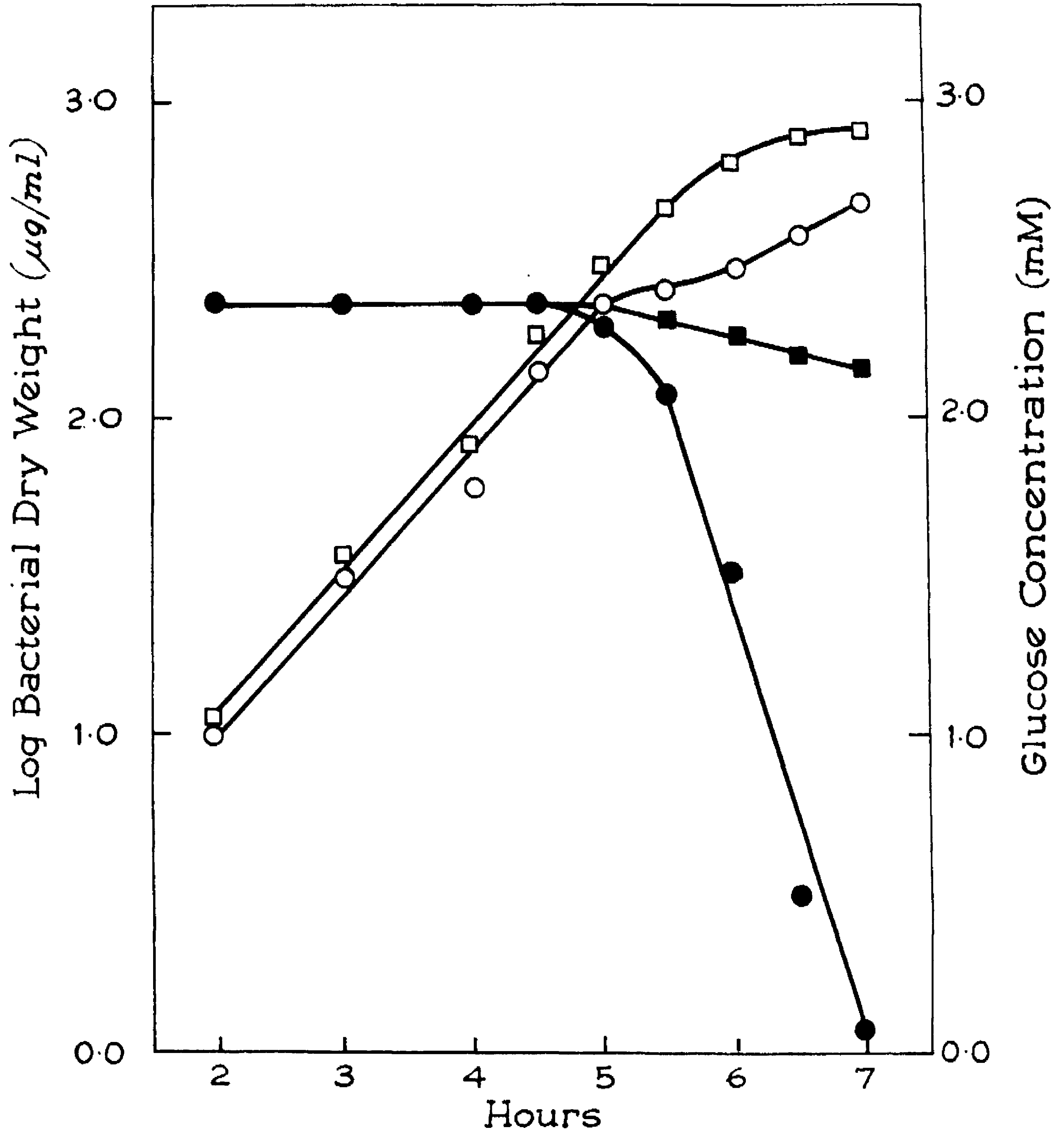
RESULTS

1). Studies of Diauxic Growth.

As a result of Liu's (1952) report that sodium citrate and sodium succinate inhibited the production of acid from glucose by *Ps. aeruginosa*, our first experiments were designed to study the kinetics of growth in an ammonium salt medium containing glucose and an organic acid as the sources of carbon and energy. The protocol of these experiments is given in Section 8 of the Methods. The organic acids studied in these experiments were succinate, citrate, acetate, fumarate and malate, and the inocula were portions of overnight cultures grown on the same organic acids. The results with succinate and citrate are typical of these experiments and are recorded in Figs. 7 and 8 respectively.

The growth kinetics show the characteristics of diauxie, but differ from all previously reported cases in that glucose is utilised as carbon and energy source during the second logarithmic phase of growth and not the first. Both concentrations of citrate were limiting for growth (Fig. 8), and in the presence of the lower concentration there is a slight utilisation of glucose during the first logarithmic phase of growth, although the rate of utilisation increases dramatically with the onset of the second phase. There may, therefore, be a critical threshold concentration

Figure 7.



Diauxic Growth of Citrate-Grown Cells in Glucose Media containing 2·69 and 17·93 mM Citrate

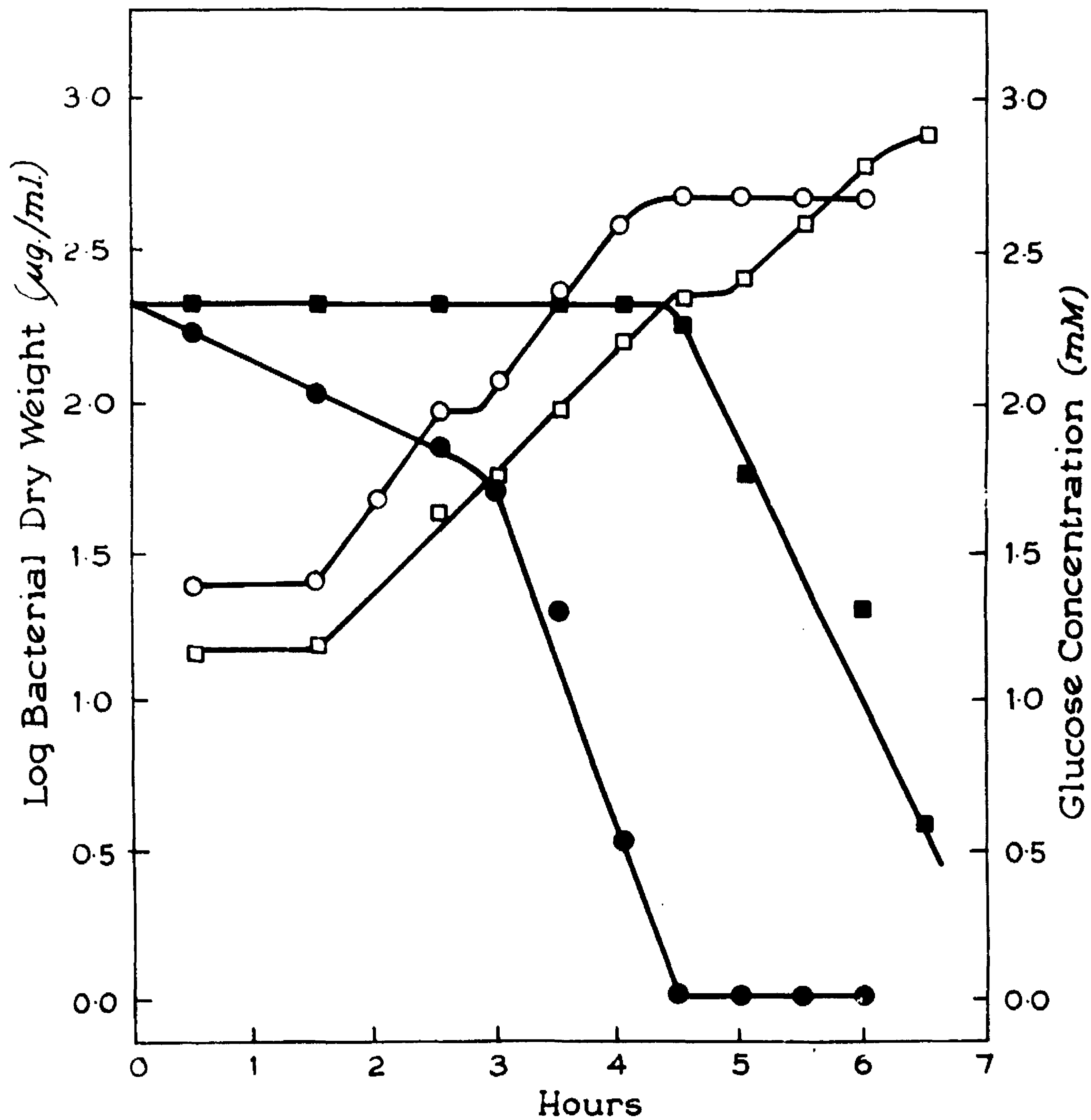


Figure 8.

Figs. 7 & 8.

O————O, Growth of culture, in presence of the lower concentration of organic acid.

□————□, Growth of culture, in presence of the higher concentration of organic acid.

●————●, Glucose concentration in the medium, in presence of the lower concentration of organic acid.

■————■, Glucose concentration in the medium, in presence of the higher concentration of organic acid.

of citrate necessary to suppress glucose metabolism completely. This small initial utilization of glucose was not found in the diauxic studies with succinate, acetate, fumarate or malate as the organic acid.

Several other systems which exhibit diauxie have been studied in an effort to elucidate the mechanism of this effect. In all cases it has been shown that at least one of the components of the system of metabolism of the secondary substrate is inducible. In the glucose-lactose diauxie in E.coli (Monod, 1942), the first step in the metabolism of lactose is the inducible enzyme β-galactosidase; in the glucose-citrate diauxie in A. aerogenes, under anaerobic conditions (Dagley & Dawes, 1953), the inducible enzyme is citridesmolase or citratase; and in the glucose-citrate diauxie in A. aerogenes, under aerobic conditions (Green, reported by Davis, 1956), the metabolism of citrate is controlled by the inducible citrate permease. It is believed that diauxie results from the inhibition by glucose of the synthesis of the inducible components necessary for the metabolism of the secondary substrate.

It has been the main purpose of the work reported in this thesis to study the unusual diauxie manifest by Ps. aeruginosa and to seek an explanation of it in terms of the factors controlling glucose metabolism in this organism.

2). Studies of Oxidation.

a) Kinetics of Oxidation.

The experiments on the oxidation of various substrates by *Ps. aeruginosa* reported below were carried out as described in Section 9 of the Methods.

The rates of oxidation of various organic acid substrates by glucose-grown cells are depicted in Fig.9. It will be noted that there is a lag phase, with an acceleration in the rate of oxygen uptake, before the final linear rate of oxidation is established. The length of the lag phase and the rate of oxidation finally attained are characteristic of the particular acid. The oxidation of glucose by these cells is included for comparison.

The oxidation of glucose by cells grown on various organic acids, and also by cells grown in a medium in which a peptone solution (2% w/v) replaced solution B, is shown in Fig.10. The graphs of oxygen uptake are labelled according to the acid on which the cells are grown. The oxidation of glucose by glucose-grown cells is included for comparison. Once again a lag period is observed before the establishment of a linear rate of oxidation and the length of this lag varies with the carbon source on which the cells have been grown. This variation in the length of the lag phase is also seen in Fig.11, where the oxidation of glucose by cells

Figure 9.

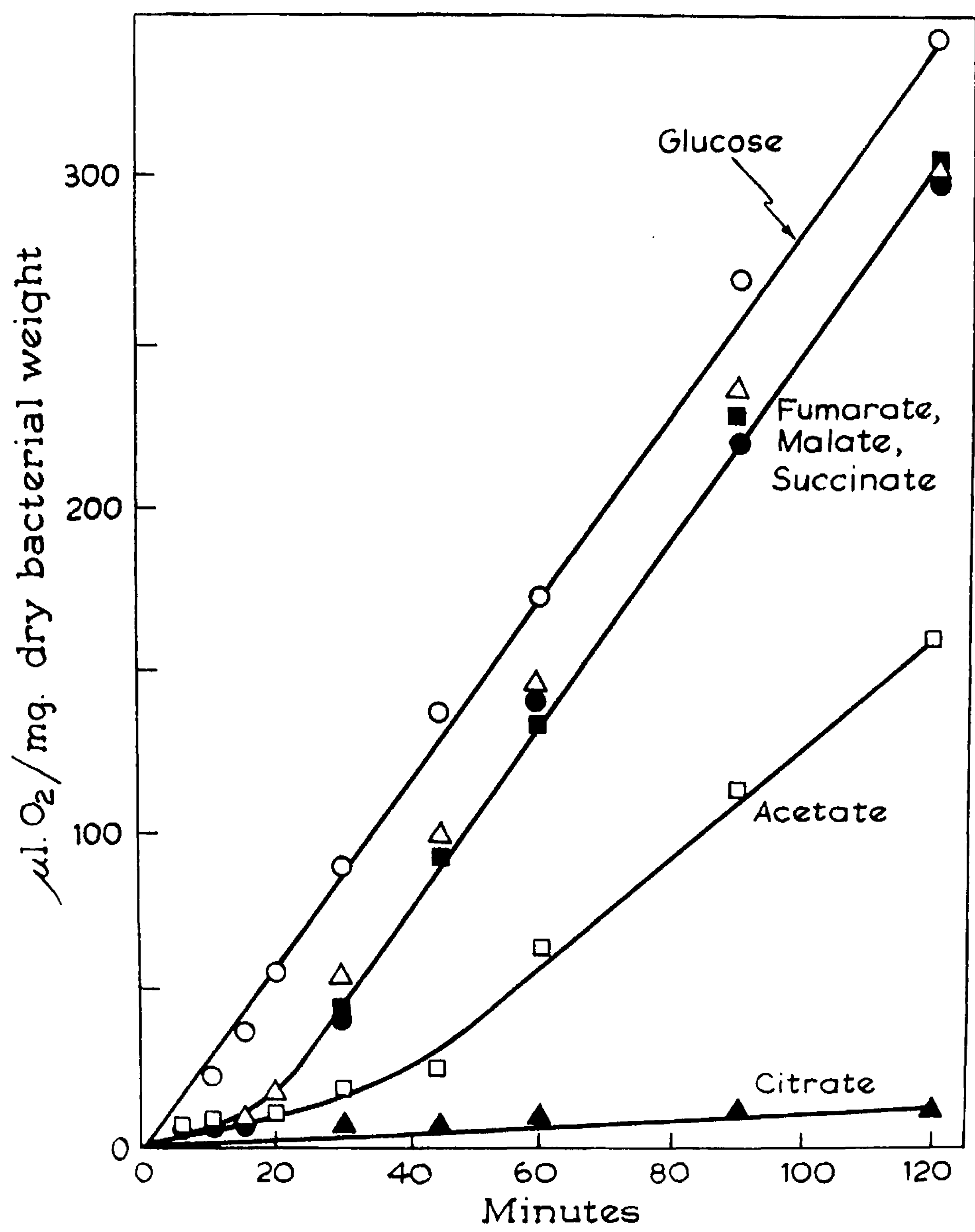


Figure 10.

Oxidation of Glucose by Cells Grown on Different Carbon Sources

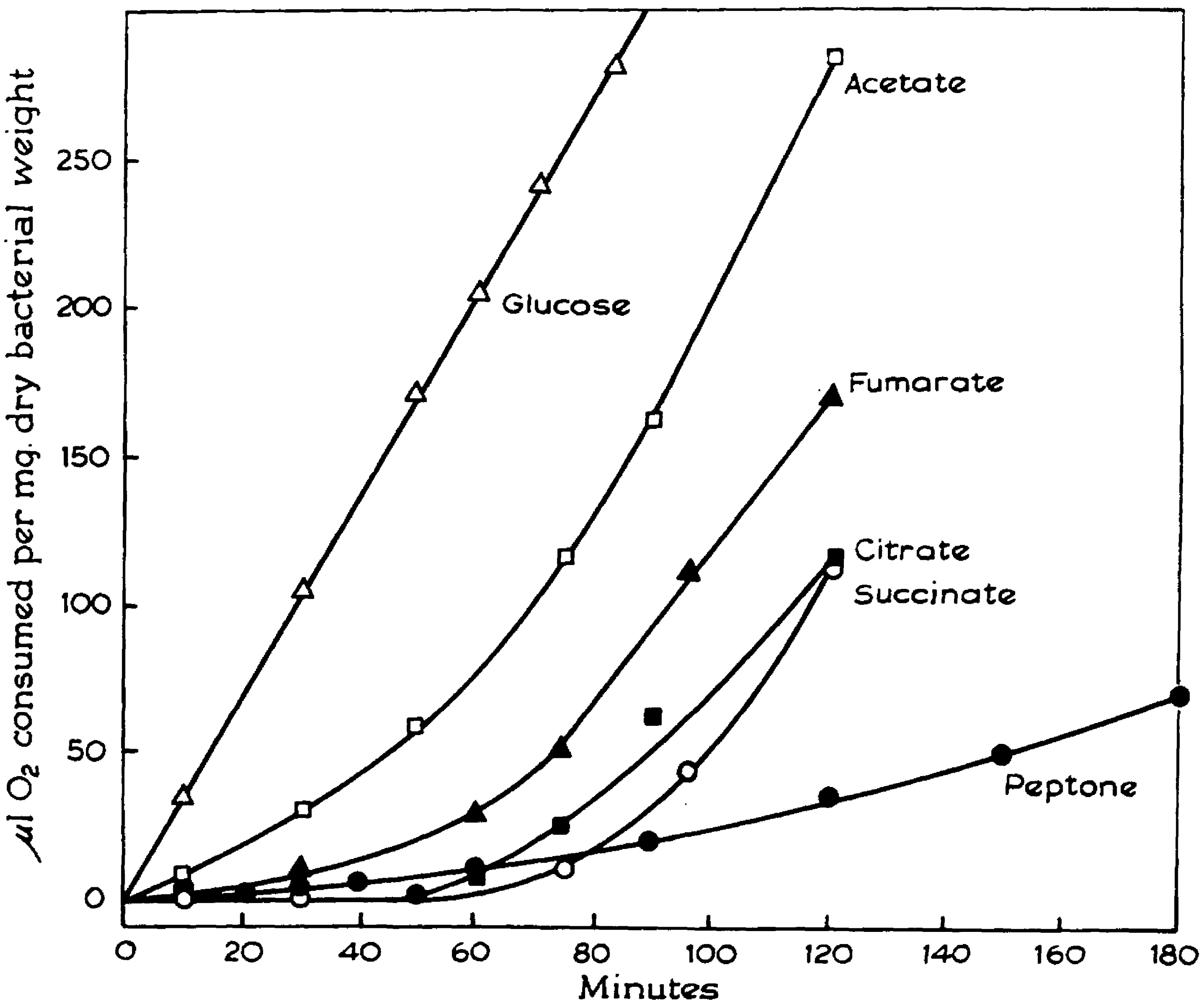
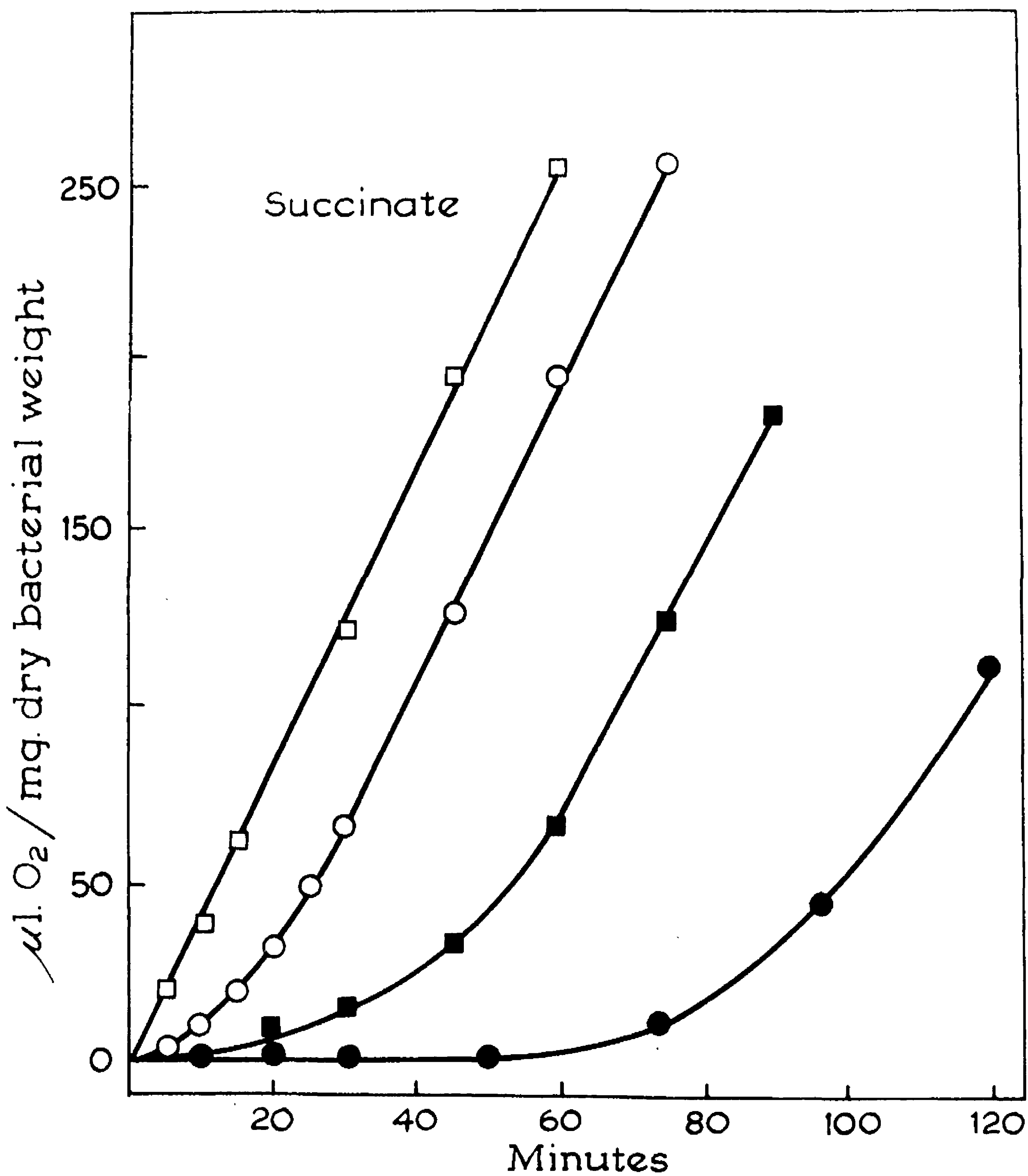


Figure 11.



Figs. 9, 10 & 11.

Contents of Warburg flasks

Centre well:-	KOH (20%)	0.2 ml.
Flask:-	Phosphate buffer, pH 7.1 (9 g. per litre)	1.8 ml.
	Cell suspension	0.5 ml.
Side arm:-	Substrate (as 2% solution containing 0.1% (w/v) $MgSO_4.7H_2O$)	0.5 ml.

Endogenous control flask contained 0.5 ml. water in the side arm.

from different succinate cultures is recorded. The oxidation of succinate is included for comparison.

It was found that for any one batch of cells, the length of the lag phase was not influenced by "ageing", i.e., storing the cells as a pellet at 4°. The activity of the cells was measured on successive days over a period of four days.

b) Chloramphenicol and Mg^{++} Effects

That the lag and acceleration phases observed are the result of protein synthesis is indicated by studies with chloramphenicol. In the experiment recorded in Fig.12, chloramphenicol was added to the contents of the Warburg flask. It can be seen that, under these conditions of arrested protein synthesis, the oxidation of succinate by glucose-grown cells is unable to proceed at a rate faster than the low rate found at the beginning of the lag phase in the absence of chloramphenicol. It should be noted that chloramphenicol has no effect on the rate of glucose oxidation.

The effect of Mg^{++} is also shown in Fig.13, where the oxidation, by glucose-grown cells, of acetate and the effect on this oxidation of the addition of Mg^{++} and chloramphenicol, is recorded. In this experiment, the deficiency of Mg^{++} was obtained by using a 2% solution of acetate without

Figure 12.

Glucose-grown cells

Oxidation of Glucose and Succinate in presence and absence of Chloramphenicol

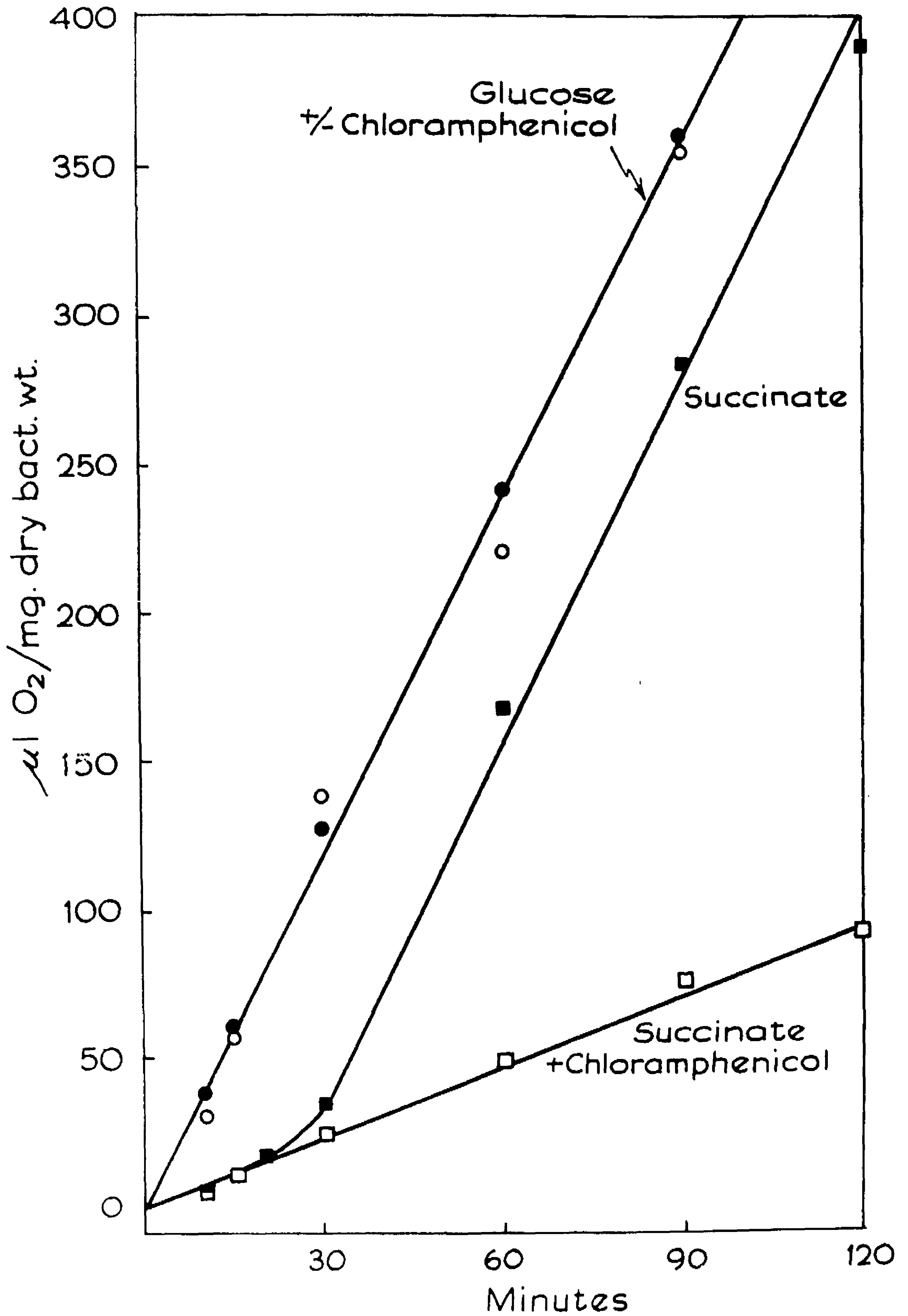


Fig. 12.

The protocol was the same as for Figures 9, 10 and 11.

In the flasks containing chloramphenicol, the main compartment contained 1.6 ml. 9 g. per litre phosphate buffer and 0.2 ml. chloramphenicol solution (0.84 mg./ml.) instead of 1.8 ml. buffer.

$MgSO_4$, in place of Solution B, as substrate. Although Mg^{++} itself has no effect on the rate of oxidation of acetate in the absence of Mg^{++}, it does considerably minimise the effect of chloramphenicol. The reason for this is obscure. In Fig.14, however, it is seen that not only does Mg^{++} decrease the effect of chloramphenicol on the oxidation of glucose by acetate-grown cells, but it also decreases markedly the length of the lag phase encountered in the absence of both chloramphenicol and Mg^{++}.

From their studies of the tricarboxylic acid cycle in Ps. aeruginosa, Campbell & Stokes (1951) and Clarke & Meadow (1959) have concluded that in this strict aerobe the enzymes of the cycle are constitutive but the metabolism of exogenous intermediates of the cycle is controlled by inducible permeases. Thus the lag phase and chloramphenicol effect in the oxidation of the organic acids, by glucose-grown cells, can be explained in terms of these inducible permeases. It is noteworthy that the synthesis of the permease for citrate appears to take longer than the synthesis of those for the dicarboxylic acids fumarate, malate and succinate.

The explanation of the longer lag phase encountered in the oxidation of acetate might well be that not only is the permease being synthesised, but also the inducible

Figure 13.

Effect of Chloramphenicol (CAP) and Mg^{++} on the Oxidation of Acetate by Glucose-grown Cells.

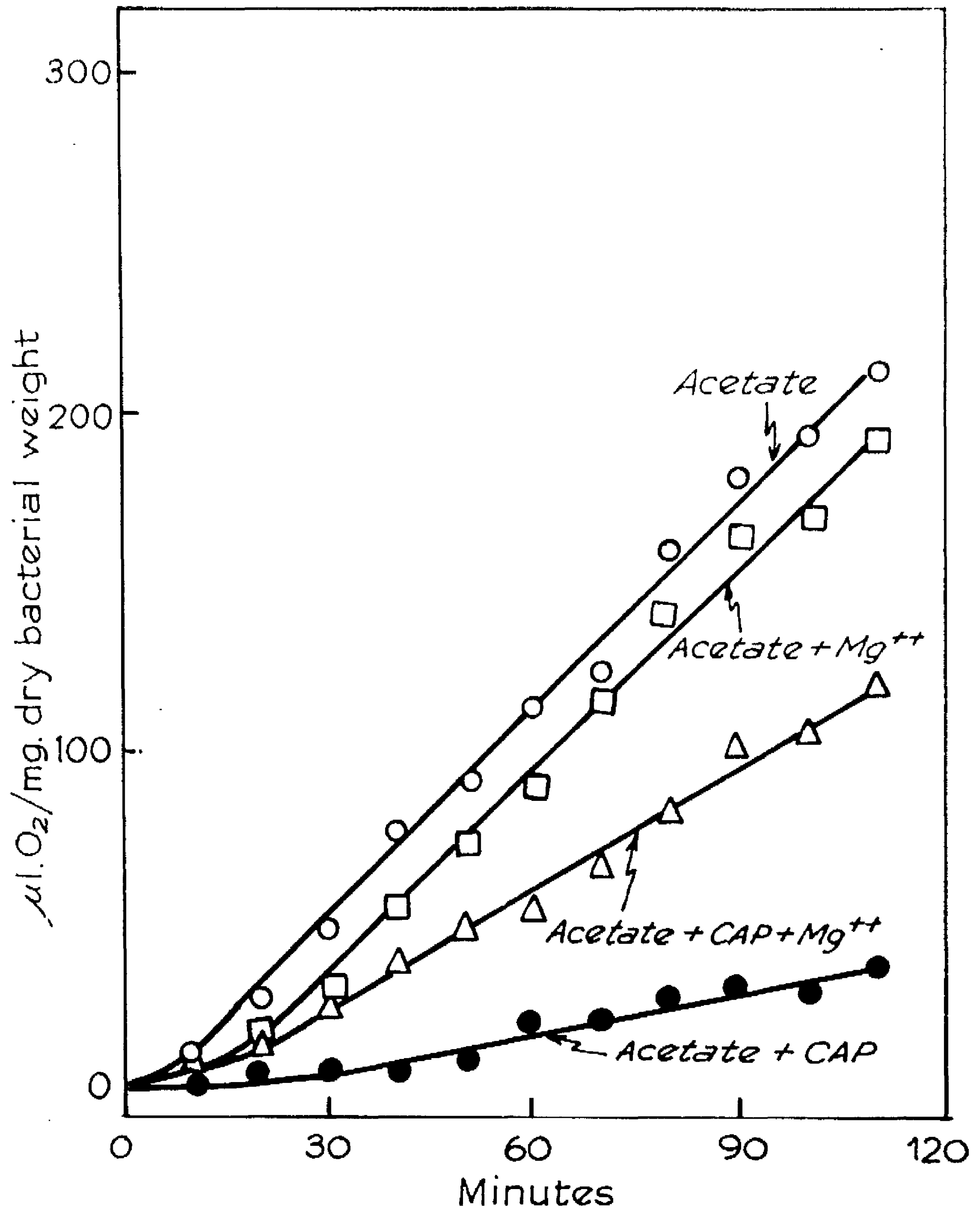
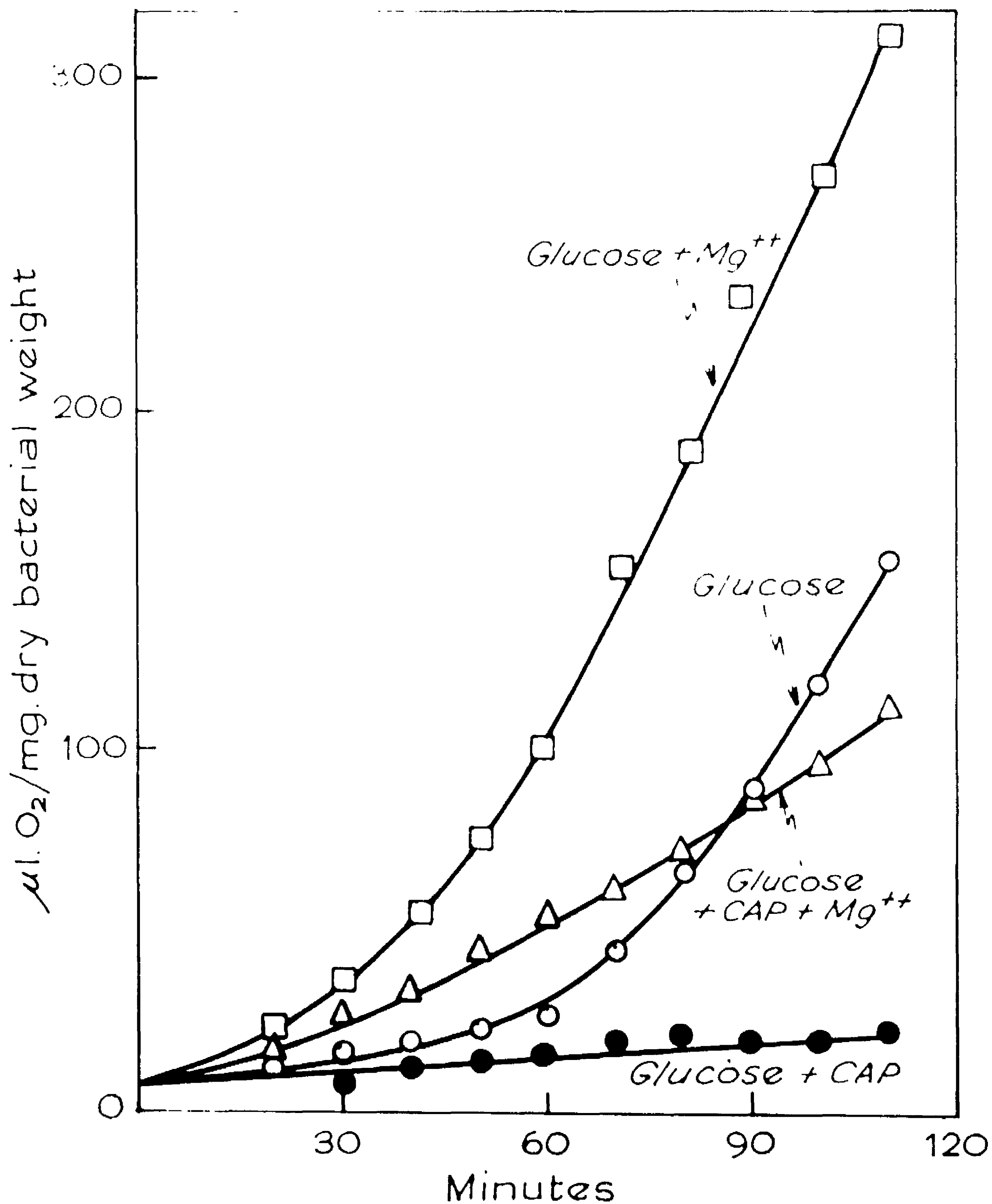


Figure 14.

Effect of Chloramphenicol (CAP) and Mg^{++} on the Oxidation of Glucose by Acetate-grown Cells.



Figs.13 & 14.

The protocol was the same as for Fig.12. Mg^{++} was omitted from the substrate solution in the side arm and added specially in the cases recorded in the Figures where the presence of Mg^{++} was required.

enzymes iso-citratase and malate synthetase. Although these enzymes are not required for the complete ______ion of acetate to carbon dioxide and water, if any of the acetate is being metabolized by pathways of assimilation involving intermediates of the tricarboxylic acid cycle, then the presence of these two inducible enzymes is necessary for the oxidative metabolism of acetate.

The explanation of the lag phase and chloramphenicol and Mg^{++} effects in the oxidation of glucose by acid-grown cells must therefore be sought in terms of the possible inducible character of both the intracellular enzymes of metabolism and, if one should exist, a permease for glucose. The fact that Mg^{++} decreases the length of the lag phase in glucose oxidation is the first indication that a system is being induced, different from the permeases for the oxidation of the organic acids.

3). Studies with Disrupted Cell Preparations and Cell-free Extracts.

a) Oxidation of Glucose by Preparations from Succinate-grown Cells.

The results of an experiment in which the oxidation of glucose by succinate-grown cells, previously incubated with glucose for periods of 0, 15, 30 and 60 min., was studied

are recorded in Fig.15. These results are to be compared with the oxidation of glucose by preparations obtained from these cells by treatment with the ultrasonic drill, and also recorded in the same Figure. The protocol for this experiment is given in Section 11.c(ix) of the Methods. The period of exposure of the suspensions to glucose is recorded beside the corresponding graph of oxygen uptake. The oxidation of succinate is recorded in the whole cell graph for comparison. The results shown here are typical of several experiments with succinate and citrate-grown cells.

The disrupted preparations from cells which had not been exposed to glucose show a low activity towards glucose and this activity increases with increasing time of exposure to glucose. The whole cells, on the other hand, only show the effect of induction, as manifest in the shortening of the lag phase, after a period of over 30 min. exposure to glucose. It may be inferred from this that succinate-grown cells have a low basal level of intracellular glucose metabolizing enzymes and that this level increases with increasing periods of exposure to glucose, i.e., the enzymes are being induced, and also that a glucose permease is being induced. The fact that the whole cells, in contrast to disrupted preparations, only show the effect of induction

Figure 15.

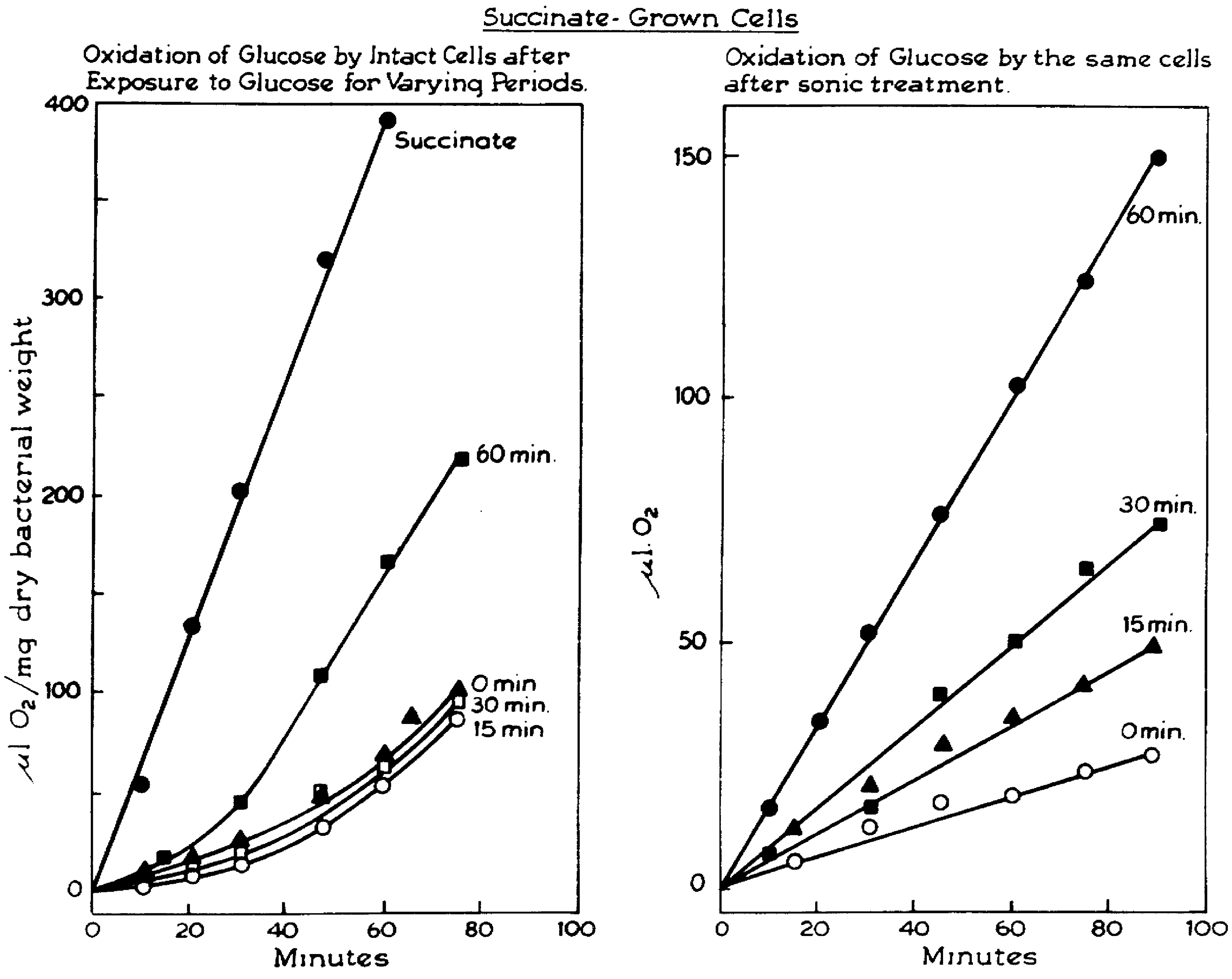


Fig. 15.

Contents of Warburg flasks.

Experiment with Whole Cells.

Centre well:-	KOH (20%)	0.2 ml.
Flask:-	Phosphate buffer, pH 7.1 (9 g. per litre)	1.8 ml.
	Cell suspension (1.1 mg./ml.)	0.5 ml.
Side arm:-	Substrate	0.5 ml.

(as 2% solution containing 0.1% (w/v) $MgSO_4.7H_2O$)

Experiment with Disrupted Cells.

Centre well:-	KOH (20%)	0.2 ml.
Flask:-	Phosphate buffer, pH 7.1 (9 g. per litre)	1.4 ml.
	Phenazine methosulphate (3.4 mg./ml.)	0.2 ml.
	Heat stable cofactor solution	0.2 ml.
	Disrupted cell preparation (from suspension of density 11.0 mg./ml.)	0.5 ml.
Side arm:-	Substrate	0.5 ml.

(as 2% solution containing 0.1% (w/v) $MgSO_4.7H_2O$)

Endogenous control flasks contained 0.5 ml. water in the side arm.

after periods of over 30 min., suggests that the induction of the permease proceeds more slowly than that of the intra-cellular enzymes and that it is the entry of glucose to the cells which is the rate limiting step in the oxidation of glucose by intact cells. Presumably sufficient glucose can enter the cell by the process of free diffusion to permit some induction of the intracellular enzymes of glucose metabolism.

b) Enzymes of Glucose Metabolism.

The possible pathways of metabolism of glucose to the stage of pyruvate have already been discussed in the Introduction and shown in Fig.5. Several enzymes involved in these pathways have been assayed in cell-free extracts as described in Section II.d of the Methods. The activities of some of these enzymes in extracts from glucose, citrate and succinate-grown cells are recorded in Table I. Although not recorded in the Table, the activities of glucose and gluconic dehydrogenases followed the pattern clearly established with the other enzymes, i.e., the activities of these enzymes were high in extracts from glucose-grown cells and extremely low in extracts from citrate and succinate-grown cells.

The omission of hydrazine from the assay system for 6-phosphogluconate dehydrase and 2-oxo-3-deoxy-6-phospho-

Table 1.

Activities of Enzymes in Extracts

Expressed as μmole substract metabolized/hr./mg. protein (initial rates)

	Glucose-grown cells	Citrate-grown cells	Succinate-grown cells
Hexokinase	1·47	0·18	0·20
Gluconokinase	0·58	0·05	0·00
2-oxogluconokinase	0·22	0·00	0·00
Glucose-6-phosphate dehydrogenase	2·88	0·06	0·28
6-phosphogluconate dehydrogenase	2·04	0·00	0·00
6-phosphogluconate dehydrase; 2-oxo-3-deoxy-6-phospho-gluconate aldolase	2·70	0·00	0·05

Table 2.

Activities of Enzymes in Extracts

Expressed as μmole substrate metabolized/hr./mg. protein *(initial rates)*.

Enzyme	*Citrate-grown cells*		*Succinate-grown cells*	
	Uninduced	*Induced with α-Methylglucoside*	*Uninduced*	*Induced with Glucose*
Hexokinase	0·18	0·56	0·20	0·37
Gluconokinase	0·05	0·19	0·00	–
2-oxogluconokinase	0·00	0·00	0·00	–
Glucose 6-phosphate dehydrogenase	0·06	0·22	0·28	1·84
6-phosphogluconate dehydrogenase	0·00	0·00	0·00	0·72
6-phosphogluconate dehydrase } 2-oxy-3-deoxy-6-phosphogluconate aldolase }	0·00	–	0·05	–

gluconate aldolase allows the further metabolism of 3-phosphoglyceraldehyde to pyruvate if the enzymes necessary for this conversion are present in the extract (Methods, Section II,d(iii)). The presence of these enzymes was demonstrated in extracts of glucose-grown cells. The rate of production of pyruvate in the absence of hydrazine was almost double the rate in its presence, thus demonstrating that in the overall conversion of 6-phosphogluconate to pyruvate the rate limiting step, under these conditions, is either the dehydrase or the aldolase catalysed reaction.

The enzymes converting 3-phosphoglyceric acid to pyruvate were also assayed in extracts prepared from glucose and citrate-grown cells. It was found that the overall activity of these enzymes was extremely low and approximately the same in the two extracts.

The activity of fructose 1,6-diphosphate aldolase, one of the three enzymes peculiar to the glycolytic pathway, was assayed in extracts of cells grown on glucose, citrate and acetate. Negligible activity was found in all three cases.

The activity of iso-citrate dehydrogenase, which might be expected to be independent of the conditions of growth of the cells, was assayed in a number of extracts from glucose, gluconate and citrate-grown cells, and found to be relatively

constant with a value of approximately 8 umoles of substrate metabolized/hr./mg. protein. The differences in the levels of activity of the enzymes recorded in Table 1 would not therefore be expected to be due to differences in the efficiency of the extraction of enzymes from cells grown on various substrates and it seems reasonable to suppose that they reflect the *in vivo* levels of activity of these enzymes.

That the levels of activity in extracts of succinate-grown cells of the enzymes hexokinase and glucose 6-phosphate and 6-phosphogluconate dehydrogenases (and glucose and gluconic dehydrogenases) can be increased by incubation of the cells with glucose for 3 hr. is demonstrated in Table 2. The effect on citrate-grown cells of incubation with methyl α-glucoside, also recorded in this table, will be discussed below.

From these results the following conclusions can be drawn. Firstly, there is evidence for the operation of three pathways of metabolism of glucose as far as pyruvate. These are:

a) the direct non-phosphorylative pathway of oxidation of glucose to gluconic and 2-oxogluconic acids;

b) the hexose monophosphate oxidative pathway with its characteristic enzyme, 6-phosphogluconate dehydrogenase,

c) the Entner-Doudoroff scheme with the enzymes peculiar to this pathway, 6-phosphogluconate dehydrase and 2-oxo-3-deoxy-6-phosphogluconate aldolase.

There is no evidence for the operation of fructose 1,6-diphosphate aldolase, and it is unlikely therefore that glycolysis is of significance in this organism, a conclusion which was also reached by Stern, Wang & Gilmour (1960) on the basis of isotopic experiments. Secondly, these intra-cellular enzymes of glucose metabolism are inducible.

c) Phosphatase Activity in Extracts.

The work of Englesberg (1959), as discussed in the Introduction, has shown that in *Salmonella typhimurium* there is a close connection between resistance to the glucose dieuxic effect and the level of the acid phosphatase activity in the cells. However, with *Ps. aeruginosa* it was found that when glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, α -glycerophosphate and ATP were used as substrates, extracts of glucose, succinate and citrate-grown cells had no phosphatase activity. The protocol of these assays is given in Section 11.d(vii) of the Methods.

4). A Permease for Glucose.

The work of Campbell & Stokes (1951) and Clarke & Meadow (1959) has established the existence of permeases

for intermediates of the tricarboxylic acid cycle in *Ps. aeruginosa* and Eagon & Williams (1960) have also demonstrated the existence of permeases for the hexoses fructose and mannose. The results already reported in Section 3.a indicate that an inducible permease may also be involved in the metabolism of glucose.

The purpose of the experiments described below has been threefold; namely, (1) to endeavour to prove whether or not a permease for glucose does exist in *Ps. aeruginosa*, (2) if it does, whether it is inducible or constitutive, and (3) to examine the part it might play in the mechanism of diauxie in this organism.

a) *Studies with Methyl α -glucoside.*

Methyl α -glucoside was found neither to support growth when supplied to the organism as the sole source of carbon and energy, nor to be metabolised, as measured by oxygen uptake in the Warburg. However, incubation of citrate-grown cells with methyl α -glucoside for a period of 3 hr. caused a considerable induction of the glucose oxidising system (Fig.16), as manifest by the shortening of the lag phase and the high level of rate of oxidation attained in the presence of chloramphenicol. These rates are to be compared with those recorded in the same Figure obtained

with another batch of cells, from the same culture, which had been incubated with water for a period of 3 hr. This induction is demonstrated at enzyme level in Table 2. Methyl α -glucoside is therefore a non-metabolised inducer of the glucose oxidising system in Ps. aeruginosa.

In their review of permeases, Cohen & Monod (1957) have reported that in E. coli the permease for glucose also appears to be responsible for the accumulation of up to 100 μmoles of methyl α -glucoside per g. of cells. If the glucose and methyl α -glucoside permeases were also identical in Ps. aeruginosa, this would afford a means of studying the glucose permease in this organism. Since methyl α -glucoside is not metabolized by the cells, the possibility of sequential induction is ruled out and, at the most, the glucoside should be capable of inducing only the permease and the enzymes which normally have glucose as their substrate, i.e., hexokinase and glucose dehydrogenase. However, as shown in Table 2, there is also an increase in the activity of gluconokinase and glucose 6-phosphate dehydrogenase following induction of citrate-grown cells by incubation with methyl α -glucoside.

Two methods were used to examine the relationship between the glucose and methyl α -glucoside permeases, if they exist in this organism, and the mechanism of induction

by methyl α -glucoside.

(i) Accumulation of Methyl α -glucoside.

Glucose-grown cells and glycerol-grown cells, which oxidise glucose without a lag, should contain a permease for glucose; and citrate-grown, methyl α -glucoside induced cells and glycerol-grown cells, either grown in the presence of methyl α -glucoside or induced by incubation with it, should contain a permease for methyl α -glucoside, assuming, of course, that both these permeases do exist.

As described in Section 12.b of the Methods, these cells were tested for their ability to accumulate [^{14}C] methyl α -glucoside intracellularly. Surprisingly, with none of these cells of different origin was there any evidence whatsoever of accumulation of the glucoside. The results of the experiment with citrate-grown, methyl α -glucoside induced cells which were incubated with [^{14}C] methyl α -glucoside for 20 min. are recorded below. The radioactivity associated with each fraction is recorded as counts/min./flask.

	Counts/min./flask
Cells at zero time	4,770
Supernatant at zero time	134,010
Cells after 20 min.	6,048
Supernatant after 20 min.	128,013.

The radioactivity associated with the cells at zero time is probably largely a measure of the volume of the suspending medium trapped in the centrifuged cell pellet. The slight increase in the radioactivity of the cells after 20 min. cannot be compared with the rapid and dramatic intracellular accumulation of, for example, thiomethyl β-galactoside by E. coli (Cohen & Monod, 1957).

These authors have drawn attention to the fact that the accumulation of non-metabolised compounds by bacterial cells is an energy dependent reaction. In an experiment with glycerol-grown cells, glycerol was added as an energy source during the period of incubation with $[^{14}C]$ methyl α-glucoside, but once again no accumulation could be demonstrated.

(ii) Metabolism of $[U-^{14}C]$ Glucose by Citrate-grown Cells Induced by Incubation with Methyl α-glucoside.

If the explanation of the induction of glucose oxidation by incubation with methyl α-glucoside is that both a common permease and the intracellular enzymes hexokinase and glucose dehydrogenase are being induced, then one might expect, with cells induced in this way, an initial accumulation of glucose within the cells. This would result from the action of the permease, prior to the induction of the complete intracellular enzyme system and consequent

metabolism of the glucose.

The protocol for experiments designed to examine this possibility is described in Section 12.c of the Methods. The results of one such experiment are recorded in Fig.17. It will be seen that here again there is no evidence of a rapid accumulation of the [U-^{14}C] glucose within the cells, but rather a steady rate of assimilation of the radioactivity into the cellular material.

One very interesting feature of these experiments is the difference between the disappearance from the supernatant of glucose, as measured by the method of Nelson (1944), and of radioactivity. During the period between the 15 and 60 min. samples, although there has been a marked drop in the glucose concentration, this has not been paralleled by an equal drop in the radioactivity of these supernatants. Presumably, therefore, during this period an intermediate, or intermediates, of glucose metabolism is accumulating in the supernatant prior to the induction of the complete system for glucose oxidation and consequent metabolism of the intermediate to assimilated carbon, carbon dioxide and water. A closer consideration of this period allows us to identify this intermediate. Between 15 and 60 min. the metabolism of glucose can be described as follows:-

Figure 17.

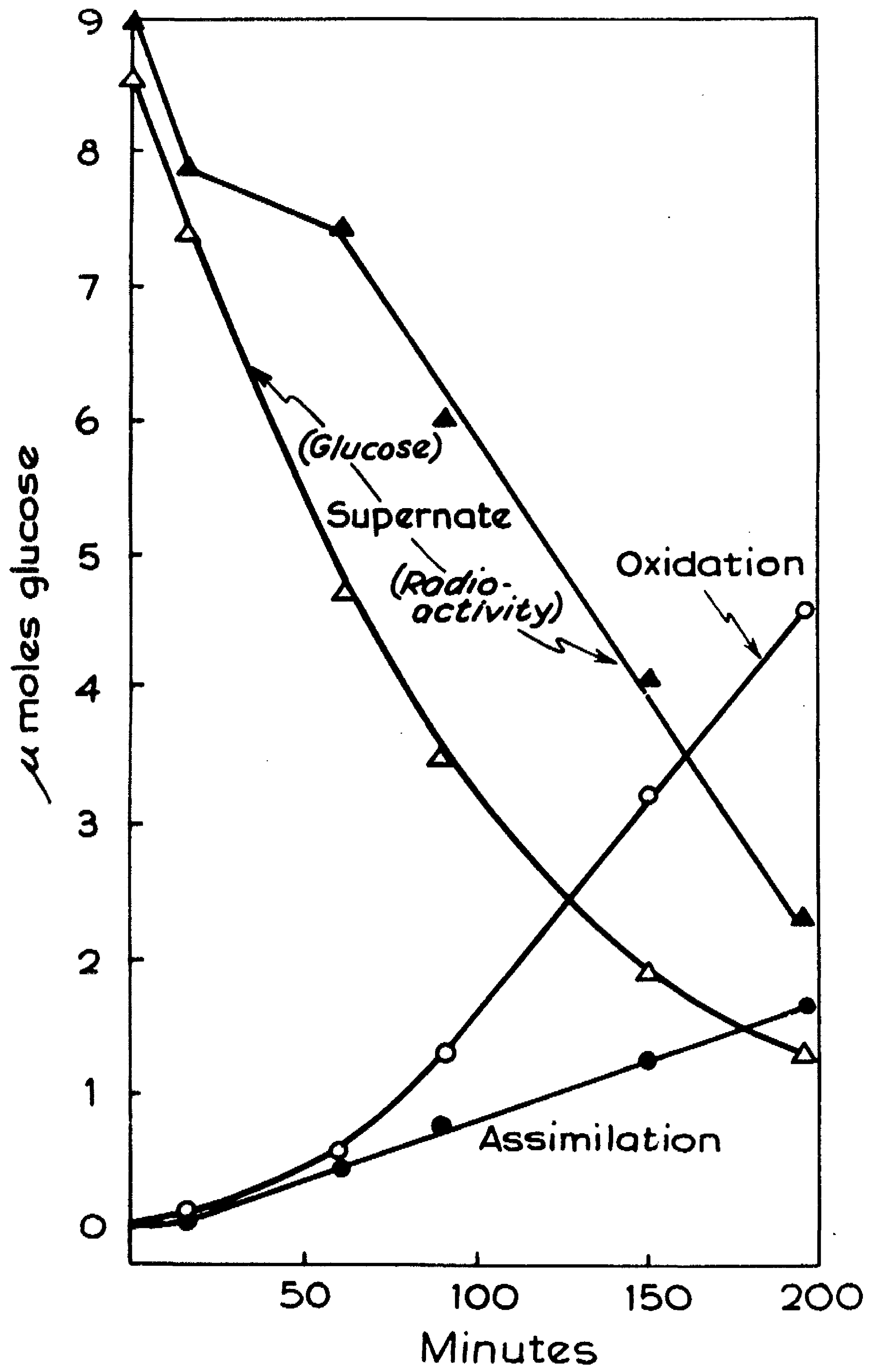


Fig.17.

Contents of Warburg flasks.

Centre well:-	KOH (20%)	0.2 ml.
Flask:-	Phosphate buffer, pH 7.1 (9 g. per litre containing 0.67 ml. 10% $MgSO_4$ per 100 ml.)	1.8 ml.
	Cell suspension	0.5 ml.
Side arm:-	[U-^{14}C] Glucose (18 mM)	0.5 ml.

Endogenous control flask contained 0.5 ml. water in the side arm.

Glucose assimilated into cells 0.4 μmoles.

Oxygen consumption 2.5 μmoles.

Glucose disappearance from supernatant;

by chemical assay,	by radioactive assay,
2.7 μmoles.	0.4 μmoles.

i.e., 2.7 μmoles glucose have been metabolised by two routes,

a) 0.4 μmole has been assimilated by the cells and therefore its associated radioactivity has disappeared from the supernatant;

b) the remaining 2.6 μmoles have been oxidised, with the consumption of 2.5 μmoles O_2, to an intermediate which, with its associated radioactivity, has accumulated in the supernatant.

Within the limits of experimental error, this corresponds to an oxygen consumption of 1 μmole O_2 per μmole glucose for the conversion of glucose to its intermediate. This is the oxygen requirement for the oxidation of glucose to 2-oxogluconate. Identification of 2-oxogluconate as the intermediate accumulating in the supernatants of the incubation flasks during the period of 15 to 60 min. has been achieved by paper chromatography and autoradiography, bearing in mind the limitations discussed in Section 14 of the Methods.

As discussed in Section 12.c of the Methods, the graph of oxygen uptake in Fig.17 was drawn, using the axes

of μmoles glucose (in this case oxidised) and time, by making the assumption that glucose was oxidized completely to carbon dioxide and water. This assumption is now seen to be invalid for two reasons:-

a) Throughout the period of the experiment, a portion of the carbon from glucose is being assimilated into the cellular protoplasm.

b) At least between 15 min. and 60 min. glucose is only being oxidized to the stage of 2-oxogluconate.

The ordinate for this graph should therefore be μmoles O_2 consumed rather than μmoles glucose oxidised, but the shape of the curve would not be altered. Indeed, the smoothness of this curve throughout the period of incubation, despite the two stages to which glucose is oxidised, is rather remarkable, although the end of the lag phase and establishment of a linear rate of oxidation does correspond approximately to the establishment of the linear rate of disappearance of radioactivity from the supernatant.

(iii) Conclusions.

These experiments with methyl α-glucoside have therefore given no indication of mechanisms for the concentration of glucose or methyl α-glucoside within the cells. Incubation of citrate-grown cells with methyl α-glucoside, however, does cause induction of glucose dehydrogenase and hexokinase

and, surprisingly, gluconic and glucose 6-phosphate dehydrogenases and gluconokinase. Apparently the synthesis of the enzymes of the direct pathway of glucose oxidation is more efficiently induced by methyl α-glucoside than is the synthesis of hexokinase.

b) Studies of Effectiveness of Glucose as an Osmotic Stabiliser of Protoplast Structures.

Ps. aeruginosa may be lysed by the technique described in Section 11.a,(ii)b of the Methods. Protoplast structures may be obtained in the presence of an osmotic stabiliser and the efficiency of a substrate in acting as such a stabiliser may be used as a measure of the permeability of the cells to that substrate. Since the formation of protoplasts considerably decreases the fall in extinction at 660 mμ of suspensions during lysis, the efficiency of a substrate at a given concentration in the medium, as a stabiliser is proportional to the difference between the extinction of the suspension in the presence of that concentration of substrate and the extinction in the complete absence of substrate. In Fig.18 $\Delta E_{660\ m\mu}$ is this difference measured 4 min. after the addition of the cell suspension to the lysis medium. Citrate-grown cells incubated with glucose (induced cells) and with water (uninduced cells) for 5 hr. are compared in

Fig.18 for the stability of their protoplasts in the presence of increasing concentrations of glucose in the lysis medium. It will be seen that at all concentrations studied, excluding 0.3 M, the protoplasts from induced cells are less stable than those from uninduced cells, i.e., the protoplasts, and therefore the cells, have become more permeable to glucose as a result of incubation of the cells with glucose prior to protoplast formation. These experiments therefore indicate the existence of an inducible glucose permease in Ps. aeruginosa.

In discussing these experiments, two other points are worthy of comment. Firstly, the apparent difference between the permeability of induced and uninduced cells is not very striking. As described in Section 11a,(ii)a of the Methods however, the high EDTA concentration used in the lysis medium completely inhibited the oxidative activity of the lysates. It may be, therefore, that although the glucose permease has been induced, its activity is severely decreased in the protoplasts by the EDTA in the medium. Secondly, when the concentration of glucose in the medium is less than 0.2 M, there is a dramatic increase in the lysis of the protoplasts from uninduced cells, due, presumably, to the fact that the osmotic pressure within the protoplasts now exceeds that of the suspending medium. This allows us

Figure 18.

Stability in Glucose Media of "Protoplasts" from Citrate-grown Cells, uninduced and induced to Glucose.

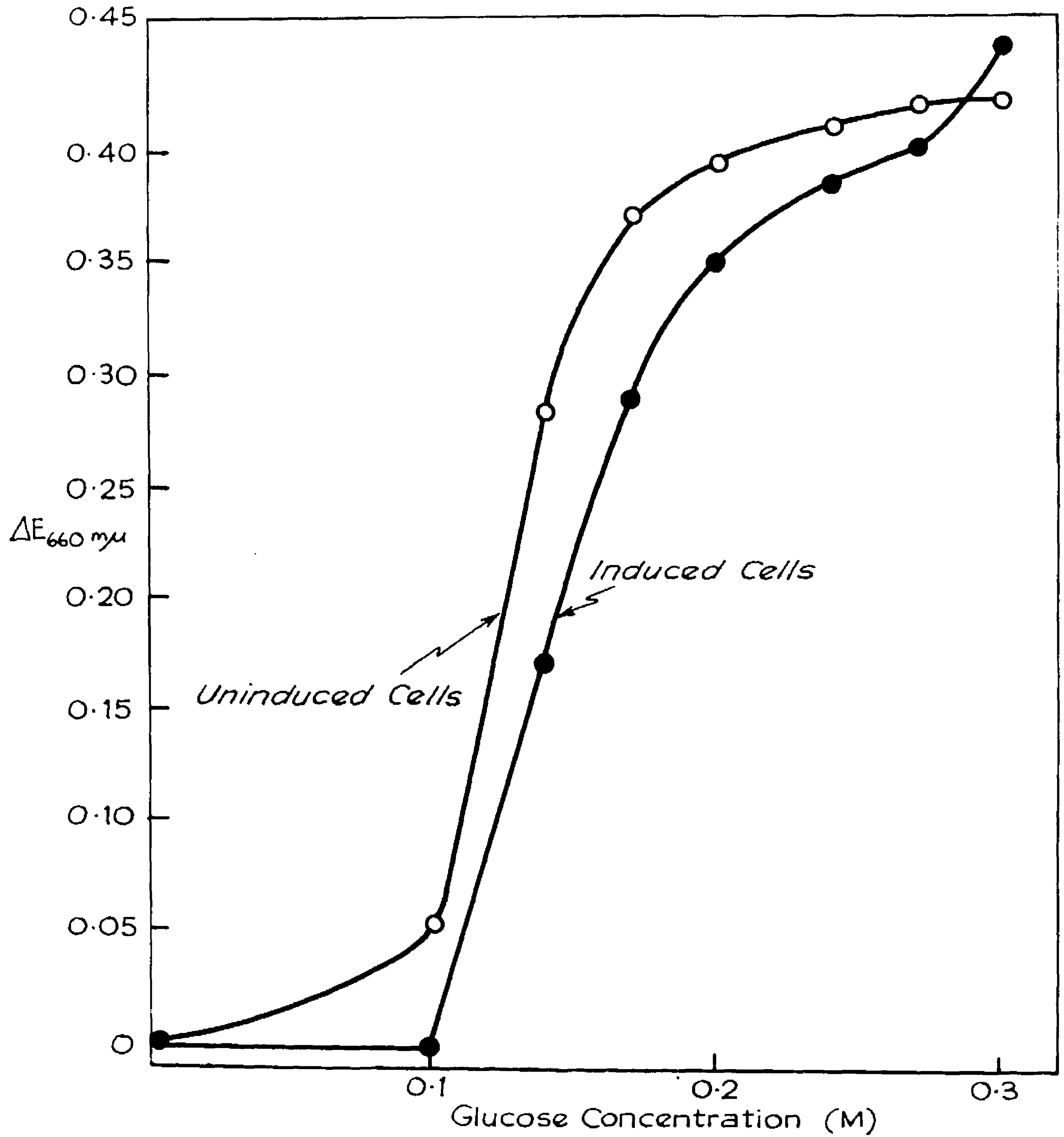


Fig. 19.

Lysis medium

Cell suspension (1.25 mg./ml.)	1.67 ml.
Lysozyme (100 μg./ml.)	1.67 ml.
EDTA (5 mM)	1.67 ml.

Solid glucose was added to give required concentration in the medium.

$\Delta E_{660\ m\mu}$ is the difference between the extinction of the suspension in the presence of a given concentration of glucose and the extinction of the suspension in the complete absence of glucose.

to put a value on the internal osmotic pressure of the protoplasts, and therefore of the whole cells, of approximately 5 atmospheres. This agrees well with values for other Gram negative organisms, as quoted by Mitchell & Moyle (1956b).

c) Growth Studies in Glucose-Citrate Media, with Glucose-Grown Cells as Inocula.

A number of growth experiments using glucose-grown cells as inocula were carried out as described in Section 6 of the Methods. The results of one such experiment are recorded in Fig.19. Although one might argue that different growth rates are evident between 1 hr. and 2 hr., between 2 hr. and 3.25 hr., and between 3.25 hr. and 6 hr., there is no evidence for a characteristic diauxic phasing of either the growth or the substrate metabolism, as measured by its disappearance from the medium. Both glucose and citrate are seen to be metabolized throughout the complete growth cycle and in Table 5 are recorded the activities towards these substrates of cells harvested from the medium at intervals during growth. The system contained chloramphenicol in order to eliminate any induction during the period of assay. The glucose-grown inoculum cells have no oxidative activity to citrate over the period of a 1 hr.

Fig.19.

○————○, Growth of culture.

□————□, Glucose concentration in the medium.

△————△, Citrate concentration in the medium.

Figure 19.

Growth of Glucose-grown Cells in Medium with Concentrations:- *Glucose 5mM; Citrate 3mM.*

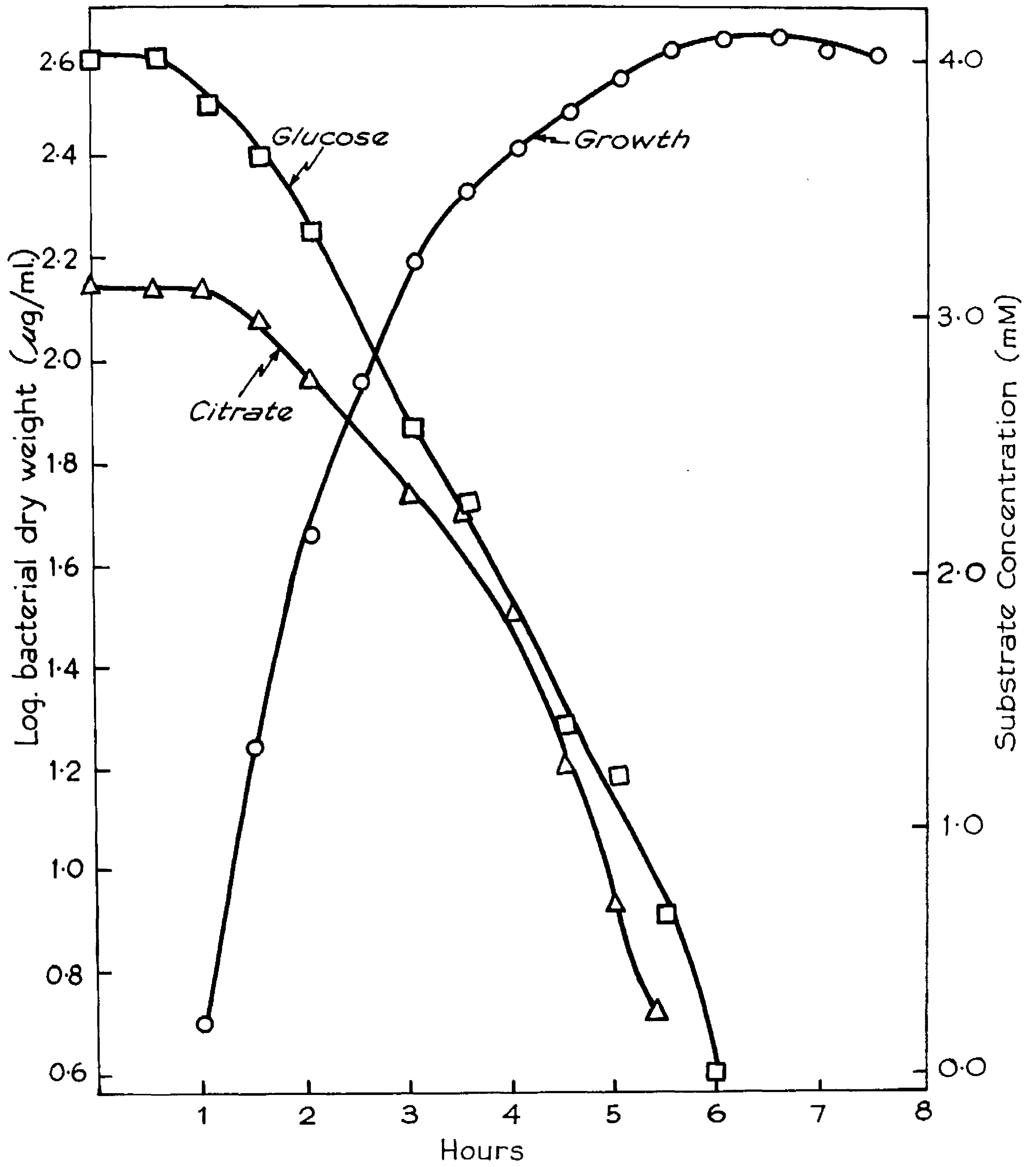


Table 3.

Activity of Cells Harvested from Diauxic Growth Experiment.

Time of Harvesting (hrs)	Q_{O_2} Citrate	Q_{O_2} Glucose
Inoculum Cells	0	133
2	117	196
3	183	236
4	190	198
5	210	240
7	203	290

Table 3.

Contents of Warburg flasks used in activity assay.

Centre well:-	KOH (20%)	0.2 ml.
Flask:-	Phosphate buffer, pH 7.1 (9 g. per litre)	1.6 ml.
	Chloramphenicol (0.84 mg./ml.)	0.2 ml.
	Cell suspension	0.5 ml.
Side arm:-	Substrate	0.5 ml.

(as 2% solution containing 0.1% (w/v) $MgSO_4.7H_2O$)

Endogenous control flasks contained 0.5 ml. water in side arm.

The Q_{O_2} value for a substrate is the μl. O_2 consumed/hr./mg. dry bacterial weight when the cells are oxidising that substrate.

assay but as growth proceeds, the inoculum cells and their progeny are induced to the citrate permease and so the Q_{O_2} value for citrate of cells harvested from the medium rises to a value which then remains constant. The Q_{O_2} value for glucose, although rather variable, remains relatively constant throughout the growth cycle, and certainly does not decrease as a result of inhibition of synthesis of the inducible enzymes of glucose metabolism. The low Q_{O_2} value of the inoculum cells is a result of their having been harvested during the stationary phase, as already mentioned in Section 2 of the Methods. These results constitute an example of the phenomenon of "maintenance" in which the synthesis of an inducible system, once established, as, for example, by growth on the inducer, continues at constant rate even in the presence of a substance which otherwise inhibits the synthesis of the inducible system. Cohn & Horibata (1959a) have explained the mechanism of maintenance in terms of an inducible permease being the first step both in the induction pathway and the pathway of metabolism of the substrate.

These growth experiments with mixed medium therefore provide some supporting evidence for the existence of an inducible glucose permease in _Ps. aeruginosa_.

5). Studies with Non-proliferating Coli Suspensions.

In a previously reported case of diauxie (Neidhardt & Magasanik, 1956a) it has been shown to be the synthesis of the induced enzymes of the secondary substrate which is inhibited and the activity of any preformed enzyme is not affected. Using the protocol described in Section 10 of the Methods, the effect of organic acids on the metabolism of glucose was studied with suspensions of cells treated in a number of different ways.

a) Acid-grown Cells.

The simultaneous metabolism of citrate and glucose and of acetate and glucose by citrate and acetate-grown cells, respectively, which have been induced by incubation with glucose is depicted in Figs.20 and 21. In both cases, in the system containing the acid and glucose, the metabolism of glucose is inhibited until the acid concentration in the medium has fallen to a low level. However, in Fig.21, there is a lag of 30 min. before glucose is metabolized in the absence of any acetate and this might still represent induced enzyme synthesis. The explanation of the non-metabolism of glucose in the presence of acetate would then simply be that the induction was being inhibited, as in the diauxie experiments; one would, however, expect a 30 min. period of induction after the final disappearance of the acetate before

Effect of citrate on oxidation of glucose by citrate-grown cells after 2 hours exposure to glucose.

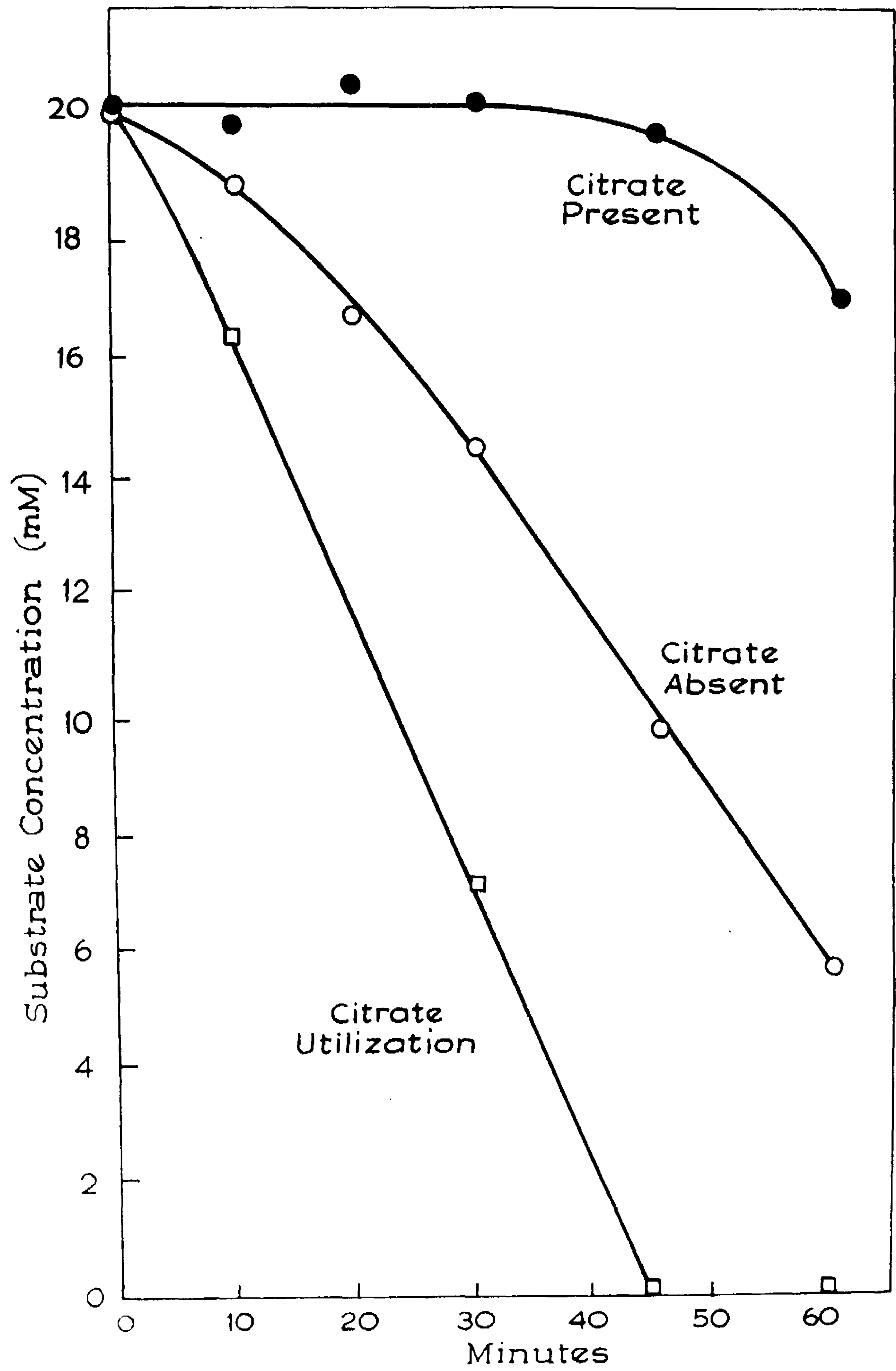


Fig.20.

Contents of incubation flasks.

	Control	Test
Phosphate buffer, pH 7.1, (9 g. per litre)	4 ml.	4 ml.
Cell suspension	4 ml.	4 ml.
Glucose (200 mM)	1 ml.	1 ml.
Citrate (200 mM)	-	1 ml.
Water	1 ml.	-

1 ml. samples were withdrawn at intervals, pipetted into 4 ml. of ice cold water and centrifuged. 1 ml. of a 1 in 4 dilution of supernatants was used for glucose estimations and 1 ml. of a 1 in 12 dilution for citrate estimations.

Figure 21.

Effect of Acetate on Oxidation of Glucose by Acetate-grown cells after 2 hours exposure to Glucose.

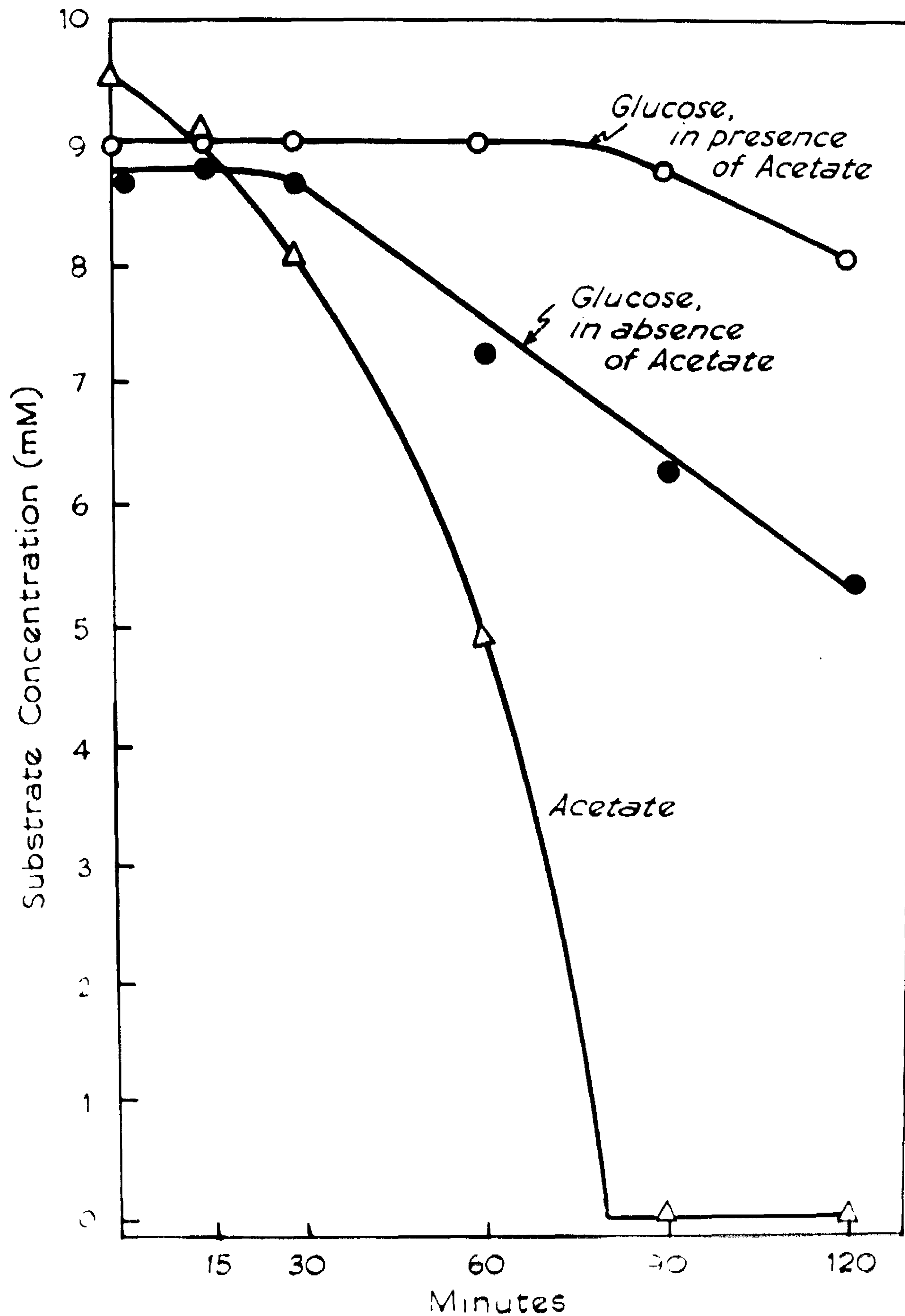


Fig.21.

Contents of incubation flasks.

	Control	Test
Phosphate buffer, pH 7.1 (9 g. per litre)	4 ml.	4 ml.
Cell suspension	4 ml.	4 ml.
Glucose (100 mM)	1 ml.	1 ml.
Acetate (100 mM)	-	1 ml.
Water	1 ml.	-

1 ml. samples were withdrawn at intervals, pipetted into 4 ml. of ice cold water and centrifuged. 1 ml. of a 1 in 2 dilution of supernatants was used for glucose and acetate estimations.

the glucose would be metabolized and this is not found.

However, the lack of any lag and the essential linearity of the rate of glucose metabolism in the absence of citrate in Fig.20 argues against there being any possibility that the inhibition is of synthesis rather than of activity.

b) Glucose-grown Cells.

This inhibition of activity was not, however, found with cells which had been grown on glucose, rather than merely incubated with that substrate after growth on an organic acid.

(1) Uninduced Cells.

The effect of the simultaneous presence of equimolar amounts of cis-aconitate, citrate, fumarate, iso-citrate, α-oxoglutarate, malate, succinate or acetate on the metabolism of glucose by cells which had not been induced to the particular acid was studied. The results with some of these is recorded in Fig.22. None of these acids affects the rate of glucose metabolism, but as they are all dependent upon inducible permeases, which have not been induced in these cells, for entry to the cells, it is, perhaps, not surprising that they have no effect on the metabolism of glucose. This experiment does, however, rule out the possibility that the organic acids inhibit glucose metabolism by

Figure 22.

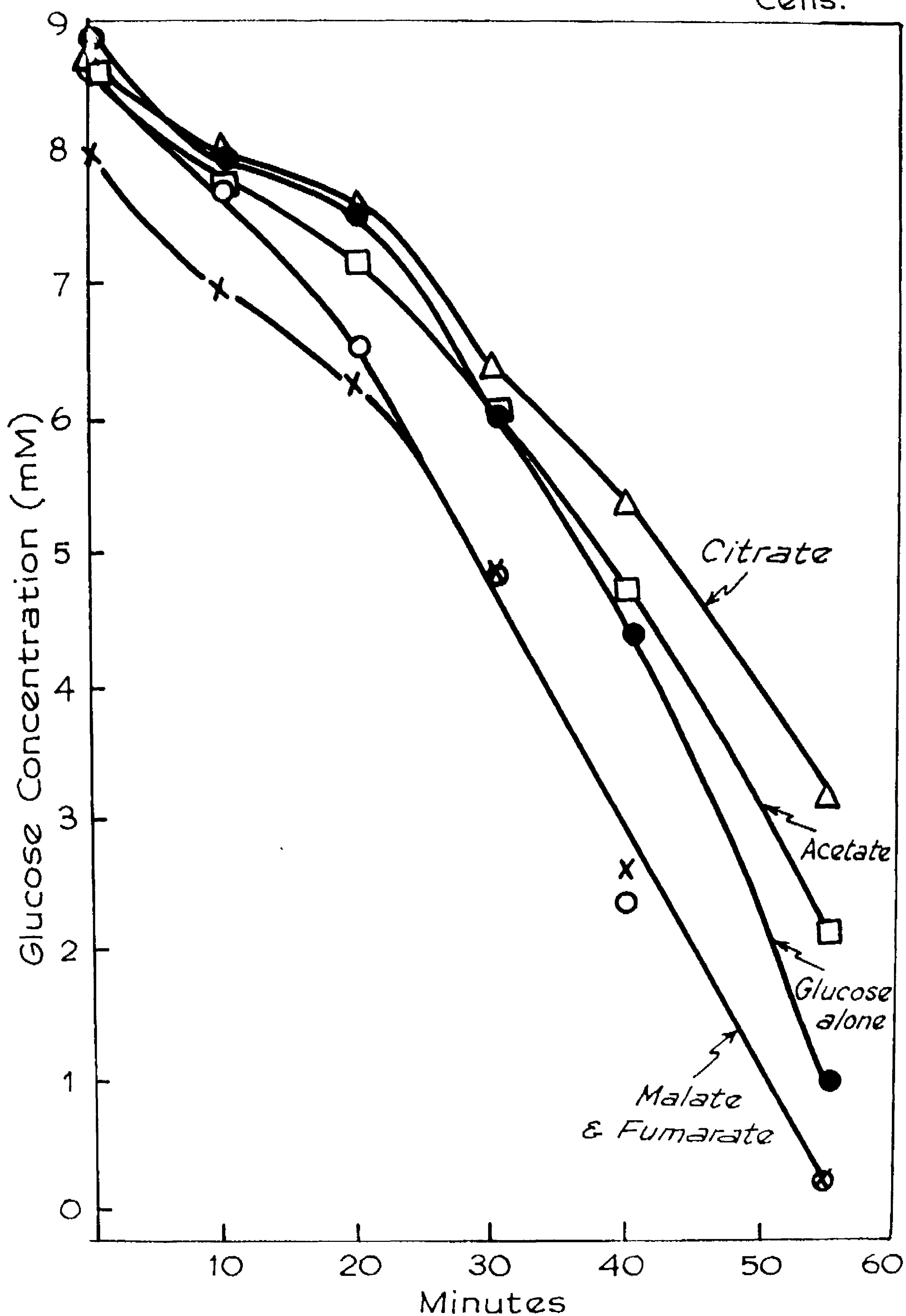


Fig.22.

The protocol was the same as for Fig.21, except that the organic acids citrate, acetate, malate and fumarate were all added to separate test flasks.

Estimations were made of glucose only.

a simple external "blocking" of glucose entry to the cell.

(ii) Cells Induced by Incubation with Acetate.

Although the cells used in the experiment recorded in Fig.23 were induced to acetate (Methods, Section 7), it was still found that acetate had no effect on glucose metabolism.

(iii) Cells Induced by Growth in the Presence of Citrate.

Even after 5 hr. incubation with citrate in the absence of a nitrogen source, glucose-grown cells were only slightly induced to citrate. Glucose-grown cells which were capable of metabolising both glucose and citrate were obtained by harvesting cells from the glucose-citrate mixed growth medium described in Section 8 of the Methods. Once again, however, as shown in Fig.24, the organic acid did not affect the rate of glucose metabolism.

These results were obtained from a medium which is only lacking a nitrogen source in order to be a complete growth medium, i.e., buffer supplemented with Mg^{++} was used. The experiment was also carried out in a full growth medium using Solution A plus 0.67 ml. 10% $MgSO_4$/10 ml. in place of the buffer and the rates of metabolism of glucose and citrate were the same as those recorded in Fig.24.

To summarise the results obtained with non-proliferating cell suspensions, we can say that two types of

Figure 23.

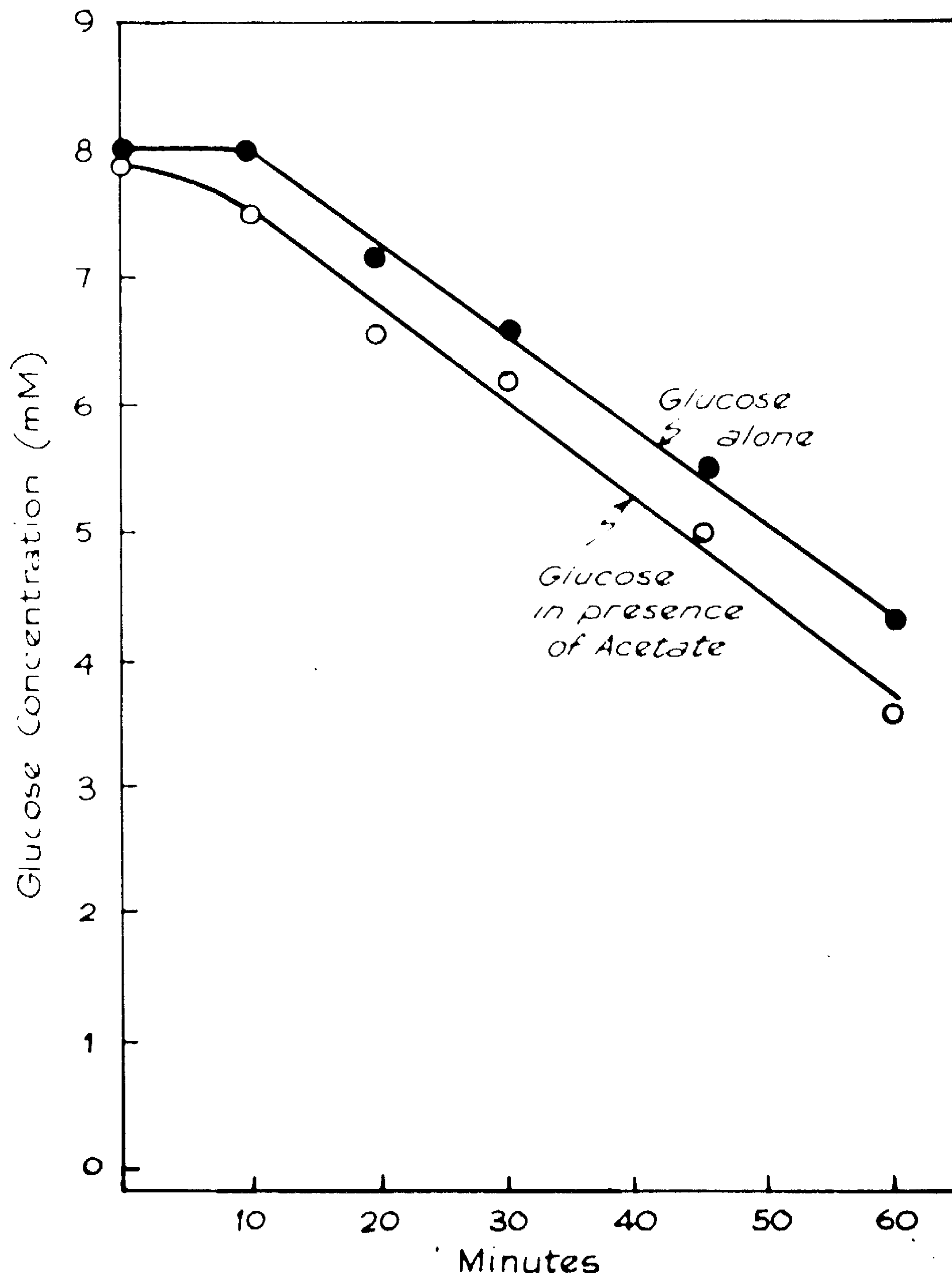


Fig.23.

The protocol was the same as for Fig.21.

Figure 24.

Effect of Citrate on Oxidation of Glucose by Glucose-grown Cells after 2·5 hr. growth in Glucose-Citrate Diauxie growth medium.

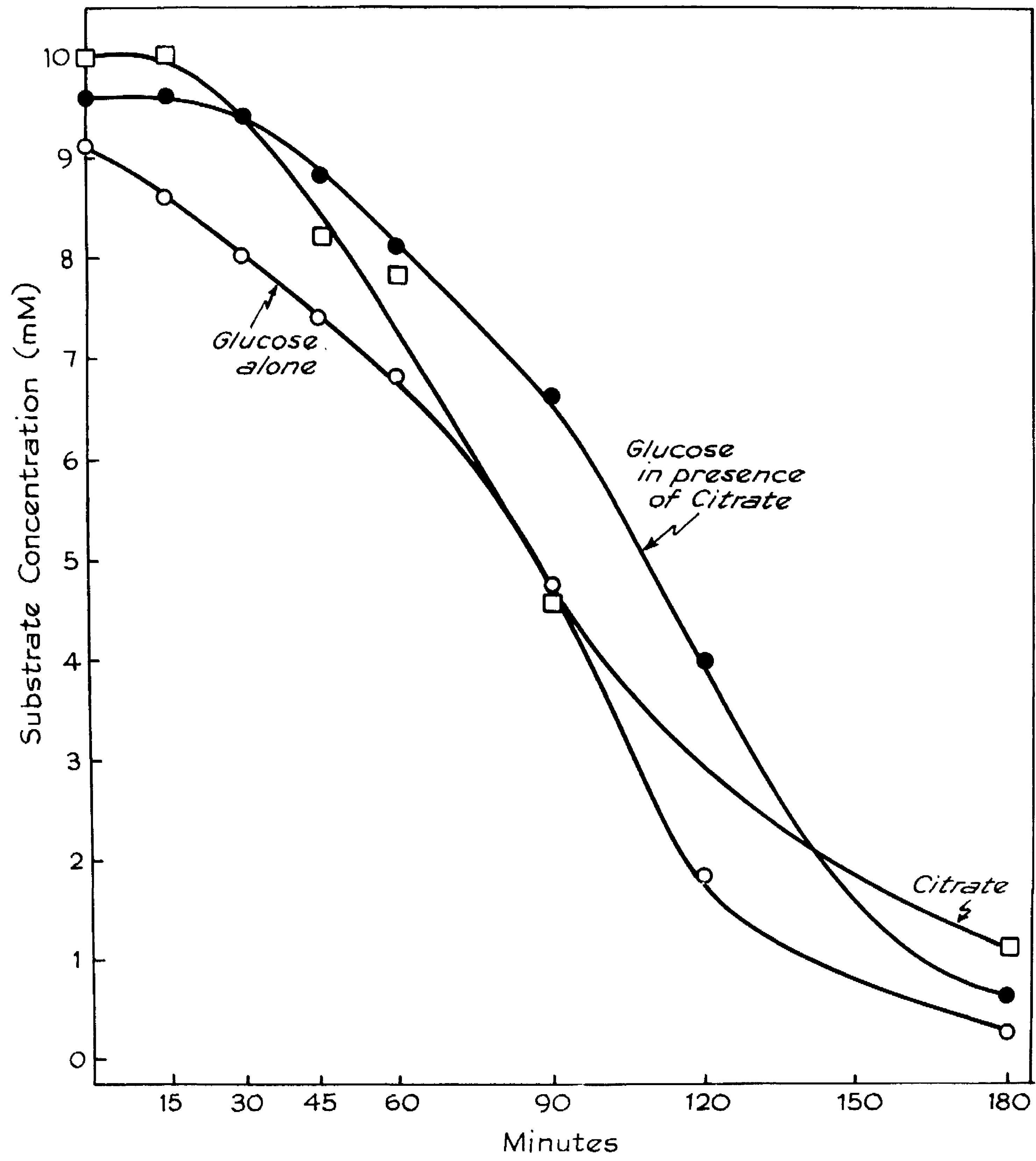


Fig.24.

Contents of Incubation flasks

	Control	Test
Phosphate buffer, pH 7.1 (9 g. per litre, containing 0.67 ml. 10% $MgSO_4$/100 ml.)	6 ml.	6 ml.
Cell suspension	2 ml.	2 ml.
Glucose (100 mM)	1 ml.	1 ml.
Citrate (100 mM)	-	1 ml.
Water	1 ml.	-

1 ml. samples were withdrawn at intervals, pipetted into 4 ml. of ice cold water and centrifuged. 1 ml. of a 1 in 2 dilution of supernatants was used for glucose estimations and 1 ml. of a 1 in 6 dilution for citrate estimations.

behaviour are evident. Firstly, the metabolism of glucose by cells that have been grown on glucose, whether subsequently induced with an organic acid or not, is unaffected by the simultaneous presence of the organic acid. Secondly, the metabolism of glucose by cells grown on an organic acid and then induced with glucose, is markedly inhibited by the simultaneous presence of the organic acid. It is not possible at this point to say what the explanation of this difference might be, but presumably it is connected in some unknown way with a difference, either quantitative or qualitative, in the enzymes of glucose metabolism from cells grown on glucose as opposed to those merely induced by a short period of incubation with glucose in the absence of a nitrogen source.

6). Studies of the Inhibition of Glucose Metabolism in Extract Preparations.

The experiments described below were conducted as discussed in Section II.c(ix) of the Methods. The results from one of these experiments are recorded in Fig.25. The cells had been grown on citrate and induced by incubation with glucose.

It is interesting to note that the inhibition of glucose metabolism in the test extends over a period of 1 hr.

Figure 25.

Effect of Citrate on Oxidation of Glucose by Cell-free Extracts of Citrate-grown Cells induced to Glucose for 3 hours.

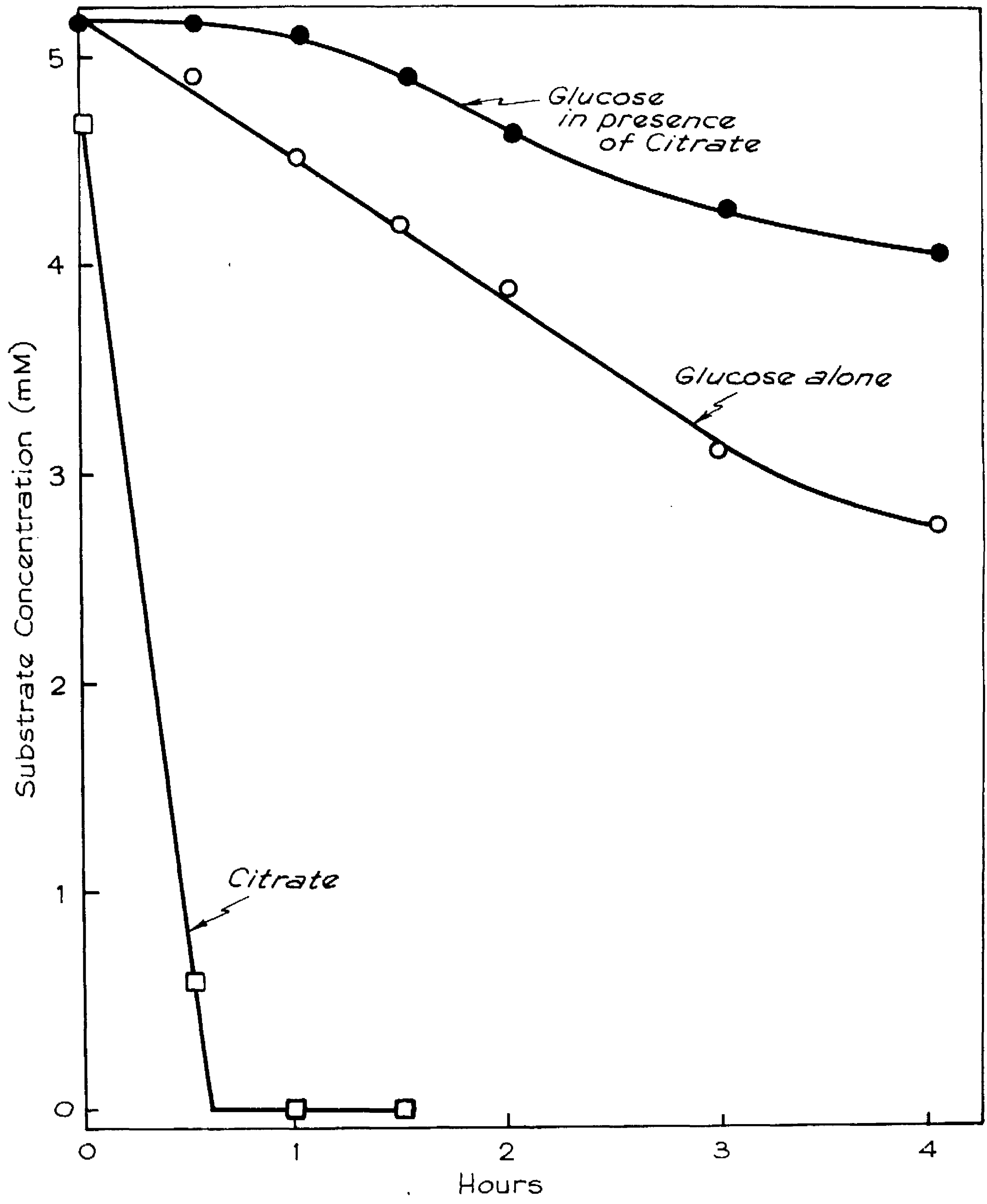


Fig.25.

Contents of Incubation flasks.

	Control	Test
Extract	4 ml.	4 ml.
Chloramphenicol (0.6 mg./ml.)	3 ml.	3 ml.
TPN (2 mM)	0.4 ml.	0.4 ml.
Methylene Blue (0.5 mM)	0.6 ml.	0.6 ml.
Glucose (50 mM)	1 ml.	1 ml.
Citrate (50 mM)	-	1 ml.
Water	1 ml.	-

1 ml. samples were withdrawn at intervals, heated for 5 min. on a boiling water bath, 4 ml. water added and the samples centrifuged.
1 ml. of supernatants was used for glucose estimations.
1 ml. of supernatants was pipetted into 2 ml. trichloro-acetic acid (40%), centrifuged and 1 ml. of these super-natants was used for citrate estimations.

although citrate itself has completely disappeared from the medium in just over 30 min. The parallel manometric assay of the extracts shows that a high linear rate of oxygen uptake with citrate as substrate continues for a period of 2 hr. These studies of oxidation also showed a total oxygen uptake of approximately 2.5 μmoles O_2/μmole citrate which agrees closely with results with whole cells and demonstrates that non-oxidative reactions, presumably synthesis, involving the carbon from citrate are occurring normally in these preparations. The supernatants from the test flasks in one experiment were assayed for keto acids to determine if there was an accumulation of any of those which are intermediates of the tricarboxylic acid cycle, but no keto acids were found in any of the supernatants. These considerations reveal that the inhibition of glucose metabolism is more likely to be the result of some aspect of normal oxidation and metabolism of citrate than to be caused by the build up of a high concentration of citrate or any of its intermediates.

The disappearance of glucose in the control, i.e., in the absence of citrate, is linear, as also is the rate of oxygen uptake in the parallel manometric assay. Comparing these, we can say that the rate of oxygen uptake is 1 μmole O_2/μmole glucose disappeared, which once again is exactly

the requirements for the conversion of glucose to 2-oxo-gluconate. Chromatography of the supernatants from the control revealed an accumulation of 2-oxogluconate throughout the period of incubation.

As discussed under Methods (Section 11c(2m)), the extracts used in these experiments contained a large number of whole cells, since extracts which had been centrifuged for longer periods, in order to separate whole cells, were also found to have lost oxidative activity, and it was therefore important to determine that the inhibition was not simply due to the activity of the whole cells and not of the extract. Viable counts were carried out on the incubation flasks at the beginning and end of each experiment. In every case there were fewer cells at the end of the experiment than at the beginning, indicating a possible bacteriocidal action of chloramphenicol. In the experiment shown in Fig.25, the viable counts on the contents of the flasks at the beginning of the incubation showed them to contain about 8.8×10^{9} cells per flask. An identical experiment to that being discussed was carried out using a whole cell suspension which gave the incubation flasks a population of 1.06×10^{10} cells per flask (this figure was verified by a viable count; in this case there was no evidence of a bacteriocidal action of chloramphenicol). Comparison with

this whole-cell experiment showed that not more than 1 µmole of the 12.5 µmoles of glucose disappearing from the control during the first 2 hr. in the experiment of Fig.25 could be the result of the activity of the whole cells in the extract. This conclusion is strengthened by the fact that chromatographic analysis of the control flask in the whole cell experiment shows no accumulation of 2-oxogluconate, in contrast to the marked accumulation found in the extract experiments.

The strict linearity of the glucose disappearance from the control flask and of the oxygen uptake with both glucose and citrate as substrate and also the high concentration of chloramphenicol, argue against any possibility of induction occurring during the incubation and being the cause of the effects recorded. There seems little doubt that in this system we are dealing with an inhibition of function of enzymes, or a single enzyme, of glucose metabolism by the simultaneous metabolism of citrate in a cell-free system.

Although this result appears well established, it may not reflect accurately the situation within the intact cell. Extracts prepared in the manner of those used in the extract experiments did not have glucose 6-phosphate or 6-phosphogluconate dehydrogenase or hexokinase activity. The inhibition of the dehydrogenase activities by phosphate

on the activity of the dehydrogenases for glucose, glucose 6-phosphate, and 6-phosphogluconate nor on hexokinase, and the dehydrase and aldolase of the Entner-Doudoroff scheme. Time has not yet permitted a similar study with extracts of citrate-grown glucose-induced cells, i.e., cells which do exhibit the inhibitory effect, both with whole cells and extracts, and it may be possible to demonstrate an effect of citrate on the individual enzymes in these preparations comparable to its effect on the overall activity of the extracts.

It is realised that the experiments reported above are still very much exploratory in character. Several very obvious improvements will be required to be made in the techniques employed in order that this most interesting effect may be more accurately and thoroughly investigated.

Firstly, it is imperative that true cell-free extracts, which still possess complete oxidative activity, be obtained. Secondly, by the addition of ATP and the use of tris buffer instead of phosphate, it should be possible to obtain extracts with full hexokinase and glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities, thus giving conditions approximating more closely to those within the intact cell. Thirdly, by either increasing the rate of glucose metabolism, or decreasing the rate of citrate

metabolism, it should be possible to make the two rates more nearly equal, thus allowing a more exact characterization of the inhibitory effect.

buffer has already been mentioned, and when the assays were carried out in glycylglycine buffer both enzymes were found to be quite active. The hexokinase assay was simply based on the measure of TPNH formation, which would result from the combined action of hexokinase, glucose 6-phosphate dehydrogenase and TPN on glucose, without the addition of ATP. The conditions of this assay are those under which the enzyme would have to function in the extract experiments. No hexokinase activity could be demonstrated however, even when the phosphate buffer was replaced by glycylglycine buffer.

The lack of these enzyme activities under the conditions of the experiment, and the oxidative and chromatographic data already recorded, all indicate that glucose is metabolized in the control by the enzymes glucose and gluconic dehydrogenase with the accumulation of 2-oxogluconate. The inhibition of glucose metabolism, due to the metabolism of citrate in the test, seems most likely therefore to be the result of an inhibition of the function of glucose dehydrogenase.

The effect of citrate on the activity of each enzyme in the extracts must be determined by the methods described in Section IId of the Methods. With extracts of glucose-grown cells, citrate has been shown to have no effect

The complexity of the problem of the non-genetic mechanisms for the control of enzyme synthesis and action in bacteria has been discussed and a description given of some of the important work carried out in this field, almost entirely during the last decade. More detailed discussion of this work is to be found in the reviews of Magasanik (1957), Hogness (1959) and Halvorson (1960).

It will be the purpose of this Discussion, where possible, to elaborate on the relevance of the results recorded in this thesis to the current ideas on control mechanisms in bacteria. It is, however, realised that much of the interest of this work lies not so much in the results which have been obtained as in its value for future research on the unusual and interesting system which has been discovered and characterised. Consequently, every attempt will be made to suggest improvements in the methods used and experiments carried out, and, in particular, attention will be drawn to ideas for future research which seem likely to be of considerable interest and importance.

The discovery of the unusual diauxie phenomenon in this organism immediately focuses attention on several questions, to some of which we have been able to give an answer, while others must be the subject of future studies.

DISCUSSION.

The inhibition of synthesis of many induced enzymes has been found to be a property of certain carbohydrates and in particular of glucose, so much so that the phenomenon has been called the "glucose effect" (Cohn & Monod, 1953; Cohn, 1956). In all cases of diauxie recorded to date, the metabolism of the second substrate for growth is under inductive control and the synthesis of the necessary component has been inhibited by the primary substrate, usually glucose. In *Ps. aeruginosa*, however, glucose has been found to be the secondary substrate for growth in media containing glucose and any one of a number of organic acids.

It has been shown that when glucose is oxidised by cells grown on any one of these organic acids as the sole source of carbon and energy, there is a period of increasing rate of oxygen uptake. Studies with chloramphenicol, an antibiotic known to inhibit protein synthesis, indicate that this period represents the synthesis of some protein component, or components, necessary for the oxidation of glucose. This synthesis appears to occur only in the presence of glucose and is therefore an example of induced enzyme synthesis or, more correctly, of induced protein synthesis.

A similar period of induced protein synthesis was shown to be necessary for the oxidation of organic acids by glucose-grown cells, and this was found by Campbell & Stokes

(1951) and Clarke & Meadow (1959) to represent the synthesis of the inducible permeases for these organic acids, the intracellular metabolising enzymes of the tricarboxylic acid cycle being constitutive in this strict aerobe. Eagon & Williams (1960) have also demonstrated the existence of inducible permeases for the hexoses fructose and mannose in *Ps. aeruginosa.* Consequently, the induction occurring during the oxidation of glucose by cells grown on an organic acid may represent the synthesis of an intracellular enzyme, or enzymes, or of a permease, or of both these entities.

The data recorded on the levels of activity of certain of the enzymes of glucose metabolism in extracts of cells, which had been subjected to varying conditions of growth and incubation with glucose and methyl α -glucoside, show quite clearly that all the intracellular enzymes of glucose metabolism examined, except perhaps those converting 3-phosphoglycerate to pyruvate, are inducible in *Ps. aeruginosa* (Tables 1 & 2).

These enzymic data also give some confirmation of the pathways of glucose metabolism in this organism as worked out in various studies by Campbell and his colleagues, and by Claridge and Workman, mostly by identification of intermediates, and by Wang, Stern & Gilmour (1959), using their radiorespirometric technique. These pathways are the direct

oxidation to gluconic and 2-oxogluconic acids, the Entner-Doudoroff system and a 1C-5C cleavage of hexose monophosphate, possibly as the first step in the hexose monophosphate oxidative pathway. Although Claridge & Werkman (1953) have obtained evidence of fructose 1,6-diphosphate aldolase in extracts of Ps. aeruginosa, in the work described here, no activity was found in the assay for this enzyme. Stern, Wang & Gilmour (1960) and Campbell & Norris (1950) also were unable to find evidence for the operation of the glycolytic pathway in this organism.

The inducible character of the enzymes of glucose catabolism studied in this work raises interesting speculation regarding the role of carbohydrate material in oxidative assimilation and growth, and its route, or routes, of synthesis in cells grown on organic acids. Although only the rates of oxygen uptake have been studied in the oxidation of substrates, it has been noted on a number of occasions with glucose and citrate that only about half the theoretical oxygen uptake for complete oxidation of the substrate is actually obtained. Similar results were obtained by Norris, Campbell & Ney (1949). Presumably a proportion of the substrate has been assimilated by the cells, although a study of oxidative assimilation in this organism has yet to be carried out. A steady rate of assimilation of radioactivity

into the cells has been found during the metabolism of [U-^{14}C] glucose. Warren, Ells & Campbell (1960) have recently published data indicating that the endogenous reserves of _Ps. aeruginosa_ are nitrogenous and probably protein in nature. It is hoped in the near future to study the carbohydrate content of the cells and carry out a more complete investigation of the enzymes of carbohydrate metabolism.

It is important that a satisfactory quantitative analysis for the glucose and gluconate dehydrogenases be obtained. The significance of this direct oxidative pathway for glucose to 2-oxogluconate in _Pseudomonas_ and related species is uncertain. Our results on the metabolism of glucose by extracts and by citrate-grown cells after incubation with methyl α-glucoside, and the work of Campbell and his colleagues, also on _Ps. aeruginosa_, and of De Ley & Stouthamer (1959) on _Acetobacter suboxydans_, show that the pathway is of some importance in these organisms. There is some doubt regarding the energy gain in the operation of this pathway. Campbell, Ramakrishnan, Linnes & Eagles (1956) have shown that with extracts oxidising glucose by the direct pathway there is no formation of "high energy" phosphate nor reduction of either DPN or TPN and that the same molar growth yield is obtained with cells growing on glucose,

gluconate or 2-oxogluconate as the sole carbon and energy source. Stradins, Campbell, Rogenkamp & Campbell (1961) have recently shown, however, that resting cell suspensions of _Ps. aeruginosa_ exhibit substrate-dependent phosphorylation and with equivalent amounts of glucose, gluconate and 2-oxogluconate it is found that the more oxidised substrates support appreciably less incorporation of ^{32}P.

The further metabolism of 2-oxogluconate has been studied in a number of organisms but in this work only a weak 2-oxogluconokinase has been demonstrated; this enzyme has also been found in _Ps. aeruginosa_ by De Ley & Vandamme (1955).

The extracts we have prepared carried out the oxidation of glucose only by the direct pathway to 2-oxogluconate, and even this activity was lost if more intensive centrifugation was employed. This suggests that the system is particulate, and De Ley & Stouthamer (1959) have shown that gluconic dehydrogenase in _Acetobacter suboxydans_ is a particulate enzyme, probably linked directly to the cytochromes. With longer periods of sonication, therefore, it might be possible to disrupt this particulate matter further and so allow a better separation of any remaining intact cells without sedimenting the oxidative activity. Preliminary experiments in this direction have not, however, been very successful.

Consideration of the many data on bacterial permease systems (Cohen & Monod, 1957) and in particular on the permeases for intermediates of the tricarboxylic acid cycle in _Ps. aeruginosa_ (Campbell & Stokes, 1951; Clarke & Meadow, 1959) and on the permeases for the hexoses fructose and mannose, also in _Ps. aeruginosa_ (Eagon & Williams, 1960), makes it extremely likely that the metabolism of exogenous glucose by _Ps. aeruginosa_ is also dependent on an inducible permease.

Unfortunately the results recorded in this Thesis do not give a definite answer to this important question, although they do suggest strongly that an inducible permease for glucose does in fact exist in this organism.

The experiment in which the effect of induction to glucose on the oxidation of whole cells and disrupted cell preparations from a succinate-grown culture was studied (Fig.16), provides evidence for the existence of an inducible permease for glucose. The activity of this permease appears to be the rate limiting step in the oxidation of glucose by whole cells. The disrupted cell preparations used undoubtedly contained a large number of intact cells; however, the linearity of the rates of oxygen uptake displayed and the fact that exposure to glucose for 15 and 30 minutes increased the level of activity above that found in uninduced cells,

i.e., results in opposition to the whole cell activities, argue strongly that the effects recorded are real and the conclusions justified.

The experiments to test the stability of protoplast structures in varying concentrations of glucose (Fig.18) also suggest the presence of an inducible permease. The system used in these studies is, however, relatively crude since the protoplasts were not isolated prior to assay of their permeability. The presence of EDTA in the lysis medium has been shown to destroy the oxidative activity of the lysate and therefore might possibly also affect the activity of the permease. If a more satisfactory method of preparing active protoplasts from this Gram negative organism can be obtained, the method of Sistrom (1958) might afford a more sensitive assay of their permeability. In this technique, the protoplasts are suspended in a medium made isotonic with saline, for example, and small amounts of the solute being tested are added to the medium. If the protoplasts are permeable to this solute, their internal osmotic pressure rises with consequent lysis of the protoplasts.

Although methyl α-glucoside has been shown to be a non-metabolised inducer of the system for glucose metabolism in this organism, the intracellular accumulation of [^{14}C] methyl α-glucoside or [U-^{14}C] glucose could not be

demonstrated after treatment of the cells in ways that might have been expected to ensure the presence of the permeases for these substances. It is not possible therefore to confirm that the permeases for glucose and methyl α-glucoside are one and the same, as has been found in *E. coli* (Cohen & Monod, 1957), although the fact that methyl α-glucoside is an inducer of the system for glucose metabolism does suggest that they might be, assuming of course that these permeases do exist.

In their studies of the accumulation of methyl β-thiogalactoside by the β-galactoside permease in *E. coli*, Cohen & Monod (1957) used a substrate concentration in the medium of 0.05 mM. In the experiments recorded here, the concentration of [^{14}C] methyl α-glucoside was 4 mM. Use of a lower concentration would make any intracellular accumulation against the concentration gradient much more easily demonstrable.

The action of the permease as a "pump" accumulating its "substrate" within the cell is believed to be dependent on a coupling of the permease to energy yielding reactions of metabolism (Cohen & Monod, 1957), and therefore inability to demonstrate an accumulating mechanism does not necessarily disprove the existence of the permease itself.

The effect of prior incubation with methyl α-glucoside on the metabolism of glucose by citrate-grown cells (Table 8 and Fig.17) seems worthy of further consideration. It seemed reasonable to expect the non-metabolised methyl α-glucoside only to be capable of inducing the permease for glucose and the enzymes of initial attack on this substrate, i.e., hexokinase and glucose dehydrogenase. In fact, evidence was obtained for the induction of hexokinase and glucose, gluconic, glucose 6-phosphate and 6-phosphogluconate dehydrogenases, but no direct evidence for the induction of a permease. It is therefore desirable that a more complete study of the effect of induction with methyl α-glucoside should be made at the enzyme level. This might help in an understanding of the significance of the accumulation of 2-oxogluconate in the medium in the metabolism of whole cells (Fig.17). A further aid to the study of [^{14}C] glucose metabolism in this experiment would be an analysis of the rate of $^{14}CO_2$ evolution and a comparison of this with the oxygen uptake data.

The linear rate of assimilation of radioactivity into the cells found under these conditions is rather surprising in view of the increasing rate of oxidation, and therefore of energy production, during the first 90 minutes. If it is assumed that this represents true synthesis of cellular

material from the metabolised glucose, then it is difficult to understand where the cell gets the necessary energy for the endergonic reactions, prior to the establishment of the oxidation of glucose beyond the stage of 2-oxogluconate, unless the simultaneous metabolism of endogenous reserves is occurring. In this connection it is noteworthy that Norris, Campbell & Ney (1949) came to the conclusion that endogenous metabolism in *Ps. aeruginosa* is not suppressed by the metabolism of exogenous glucose. On the other hand, until cells have been fractionated following "assimilation" of [^{14}C] glucose, to investigate the nature of the radioactivity accumulated intracellularly, it might be misleading to presume that linear accumulation of radioactivity represents a linear deposition of the same substance, or substances, throughout the period of observation.

Perhaps the most convincing indirect evidence for the existence of an inducible permease for glucose in *Ps. aeruginosa* has come from studies of the growth of glucose-grown cells inoculated into a medium containing both glucose and citrate as carbon sources (Fig.19 and Table 3). Citrate inhibits the induction of the enzymes of glucose metabolism when citrate-grown cells are inoculated into a growth medium containing both glucose and citrate as carbon sources. However, with an inoculum of glucose-grown cells in this same

mixed medium, the synthesis of the enzymes of glucose metabolism continued throughout the growth cycle at a rate not less than that observed in a medium containing glucose as the sole source of carbon (Table 3). These enzymes also possessed activity during growth, as assayed by the disappearance of glucose from the medium (Fig.19). This phenomenon had previously been discovered with the β-galactoside system in E. coli (Novick & Weiner, 1957; Cohn & Horibata, 1959a) and given the name "maintenance".

These authors have explained maintenance in terms of an inducible permease. It is believed that inhibitors of enzyme induction act by giving rise to a substance, the repressor, which competes with the inducer at the enzyme forming site and inhibits the action of this site, i.e., the process of induction is inhibited. The effectiveness of the inhibition is dependent therefore on the relative concentrations of the substrate, or inducer, and the repressor within the cell. If entrance of the inducer into the cell is mediated by an inducible permease, whose induction is also dependent on the intracellular concentration of inducer, then the following situation arises. Uninduced cells contain no permease, no intracellular enzymes and no internal inducer; induction occurs when a few molecules of inducer enter the cell by passive diffusion and induce a small

amount of permease and intracellular enzyme, the permease immediately catalyses the entry of larger amounts of inducer into the cell and, as a result, the induction of the permease and intracellular system is complete. This whole process takes place extremely rapidly and its importance in establishing two separate phenotypes, (i.e., cells which possess the completely induced system and those which possess none, existing side by side in a growing population) is discussed by Cohn & Horibata (1959b). In the presence of an inhibitor, the intracellular concentration of inducer, on account of diffusion, is never great enough to overcome the effect of the repressor and, consequently, induction is inhibited. With induced cells, on the other hand, the presence of the permease ensures that there will always be a high enough concentration of inducer within the cells to combat the action of repressor and so, even in the presence of an inhibitor, induction of both the permease and the intracellular system will continue. This is, of course, the condition of maintenance.

Cohn & Horibata (1959a) have also shown that the presence of a permease, which is induced by the product of its own action, or any similar self-inducing enzyme in the metabolism of galactose by yeast, could also explain the long term adaptation of these organisms (Spiegelman & De Lorenzo,

1953). The presence of a permease for galactose has been demonstrated in yeast by Douglas & Condie (1954).

The results obtained with Ps. aeruginosa are, therefore, strongly indicative of the presence of an inducible permease for glucose.

The current hypothesis of the mechanism of the inhibition of induced enzyme synthesis, and therefore of diauxie, has been outlined by Cohn & Horibata (1959c) and is called the "generalised repression hypothesis" (Hogness, 1959; Vogel, 1957). This hypothesis envisages that inhibitors are metabolised with the production of a repressor and the strength of the inhibition is dependent on the amount of repressor formed from the inhibitory substrate. Glucose has been shown to be the most powerful inhibitor of induced enzyme synthesis in many systems and Neidhardt & Magasanik (1956b) have shown that in A. aerogenes glucose is metabolised aerobically at a rate which is at least double that commensurate with the growth rate and total crop of cells. There is a high level of "high-energy" phosphate, presumably ATP, in the cells and, in the presence of inorganic nitrogen, a plentiful supply of nitrogenous organic compounds. The induced synthesis of histidase is inhibited by glucose except in cases where, as a result of mutation for instance, its production is necessary for growth of the organism.

These authors conclude that the metabolism of glucose occurs so rapidly in the cells, with the accumulation of its intermediary products (energy, carbon intermediates and, in the presence of inorganic nitrogen, amino acids etc.) that the further production of these intermediates by the action of inducible enzymes on other carbohydrates etc., is unnecessary and the synthesis of these enzymes is therefore repressed. This repression of the synthesis of degradative enzymes as a result of the build up of the products of their action is parallel to the repression of the synthesis of synthetic enzymes (Monod & Cohen-Bazire, 1953; Cohn, Cohen & Monod, 1953; Vogel, 1957; Gorini & Maas, 1958; Gorini, 1960).

Englesberg (1959) has shown that the resistance to the inhibition by glucose of induced protein synthesis in mutants of Salmonella typhimurium is associated with a reduced rate of growth on glucose and an increase in the acid phosphatase content of the cells, as compared with the glucose sensitive wild type. He concludes that a possible mechanism of glucose inhibition is the high rate of production of intermediates of its metabolism which can act as repressors. Another interpretation is that the concentration of inorganic phosphate may be critical for induced enzyme synthesis. The evidence obtained on the level of activity of the phosphatase enzymes would appear to rule out this possibility,

however, in *Ps. aeruginosa*.

It appears likely, therefore, that the explanation of the peculiar diauxic effect in *Ps. aeruginosa* could lie in the fact that the intermediates for growth might be obtained more rapidly and efficiently from the metabolism of the organic acids, intermediates of the tricarboxylic acid cycle, rather than of glucose. The only value to the organism of the enzymes of glucose metabolism would therefore be in the conversion of glucose to these intermediates of the tri-carboxylic acid cycle, which is the terminal oxidation path-way for glucose in this organism. These enzymes would there-fore only be synthesized in the presence of exogenous glucose, and under conditions where the concentration of the inter-mediates for growth, i.e., of the repressor or repressors, was not sufficiently high to inhibit this induced enzyme synthesis. The particularly long lag phase found in the oxidation of glucose by peptone-grown cells (Fig.10) is in line with this idea, since one would expect a large intra-cellular accumulation of essential intermediates from growth on such a rich medium. However, the molar growth yields on citrate and glucose have been found to be the same and the rates of growth on citrate and glucose are equal (Fig.5). Experiments are, however, planned in which it is hoped to assay the "high energy" phosphate content of cells during

exponential growth on different carbon sources.

The work of Rotman & Spiegelman (1954) and Hogness, Cohn & Monod (1955) has shown that β -galactosidase and the other proteins of *E. coli* once formed are stable in a growing population, i.e., the proteins are in static not dynamic equilibrium. This stability of β -galactosidase during growth on glucose as sole source of carbon has been demonstrated by Rickenberg, Yanofsky & Bonner (1953), Cohn (1956) and Bonner (1958). The stability of citrate permease in *A. aerogenes* (Green, quoted by Davis, 1956) and galactozymase in *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* (Spiegelman & Reiner, 1947) under similar circumstances has also been demonstrated. Neidhardt & Magasanik (1956a) have also shown that although the synthesis of the inducible enzymes of galactose metabolism in *A. aerogenes* is inhibited by glucose, washed cell suspensions of induced cells are capable of metabolising glucose and galactose simultaneously. These results indicate that although the synthesis of induced enzymes can be inhibited by repression, there is no evidence of an inhibition of function of the enzyme once it has been formed. This latter would be an example of negative feed back inhibition (Umbarger, 1958; Pardee, 1959) since galactose, after its initial phosphorylation and transformation to glucose 1-phosphate, is metabol-

ized by the same pathways as glucose and to the same intermediates.

Such an inhibition has been found in Ps. aeruginosa (Figs.20 & 21). The only other known cases are with the yeast Rhodotorula gracilis in which the metabolism of xylose by washed suspensions was inhibited by glucose (Kleinzeller, Málek, Praus & Skoda, 1952) and with the bacterium Sarcina lutea in which the metabolism of pyruvic acid by washed suspensions was inhibited by glucose (E.A. Dawes & W.H. Holms, unpublished). Nothing is known of the mechanism of these two inhibitions. In Sarcina lutea, however, glucose is oxidised by way of pyruvate, and a conceivable explanation of the effect would be for intracellular pyruvate to inhibit the pyruvate permease, although there is no information on the existence of such a permease in Sarcina lutea. This would constitute a special case of negative feed back inhibition of enzyme action in which the "enzyme" is a permease, and would be comparable with the inhibition by glucose of the synthesis of citrate permease in E. coli (Green, quoted by Davis, 1956).

In cell extracts of Ps. aeruginosa the inhibition of function is shown to act on the pathway of direct oxidation of glucose to 2-oxogluconate at the enzyme glucose dehydrogenase (Fig.25). These extracts, however, were capable of

oxidising glucose only by the direct pathway, and it is hoped to complete this study with extracts that possess full oxidative activity towards glucose.

The inhibition of glucose metabolism by organic acids was not, however, manifest with cells which had been grown on glucose as the sole source of carbon (Figs.22, 23 & 24). At present it is not possible to explain this difference between cells in which the glucose enzymes have been induced by growth and by incubation in the absence of a nitrogen source. Presumably the explanation lies in a difference in the levels of the glucose enzymes resulting from the two different methods of induction.

Although bacterial proteins are generally considered to be stable under growing conditions, cases have been recorded in which enzyme activity is lost during growth on an inhibitory substrate (Spiegelman & Dunn, 1947; Hamilton, 1953) and turnover of protein at a rate of approximately 5% per hour has been found in non-proliferating suspensions of bacteria (Mandelstam, 1960). Under the conditions of induction used in the work described in this Thesis, i.e., in the absence of an added nitrogen source, presumably many of the amino acids required for the synthesis of the glucose enzymes are derived from such a turnover, although Warren, Ells & Campbell (1960) have shown that the endogenous

reserves of Ps. aeruginosa are nitrogenous in nature, and they might conceivably supply the amino acids required for induced enzyme synthesis. The explanation of the inhibition of glucose metabolism by the metabolism of citrate and acetate cannot however be due to a breakdown of the glucose enzymes since, on the exhaustion of the organic acid from the medium, glucose metabolism begins immediately at a linear rate, without the lag that would occur if the enzymes were being reinduced. These results with washed cell suspensions and extracts do seem, therefore, to be true cases of negative feed back inhibition.

Further kinetic studies of induction to glucose, both under conditions of growth and in the absence of a nitrogen source, are planned. It is intended also to study in detail the effect of the simultaneous presence of an organic acid during induction by incubation with glucose in the absence of a nitrogen source, although preliminary experiments indicate that induction is inhibited, as in a growing population demonstrating diauxie. A study of the effect of growth and incubation, in the presence and absence of organic acids, on preformed glucose enzymes should yield much useful information on the mechanism of deadaptation. Exploratory work indicates that during growth of glucose-grown cells in a citrate medium, even in the absence of an

inducer, the synthesis of the glucose system continues, although at a reduced rate.

It is hoped that further knowledge of the processes of induction and deadaptation of the enzymes of glucose metabolism obtained from such studies will make possible a more complete understanding of the mechanisms of induction and repression of enzyme synthesis and of negative feed back inhibition of enzyme action in Ps. aeruginosa.

The peculiarities of these effects in this organism make the system particularly interesting and valuable as a tool for the study of the general problem of non-genetic control mechanisms in living tissues. Consideration of the biochemical reactions in this organism might also be of some value to the bacteriologist whose interests lie in the fields of taxonomy or evolution.

SUMMARY.

1. The growth of Pseudomonas aeruginosa in an ammonium salts medium containing an organic acid and glucose as the sole sources of carbon and energy results in the phenomenon of diauxie, i.e., double growth. The organic acid is utilised during the first growth cycle and glucose during the second.
2. The oxidation of organic acids by glucose-grown cells and of glucose by organic acid-grown cells involves an initial period of induced protein synthesis.
3. In the case of the oxidation of organic acids the permeases, which facilitate entry of the compounds into the cell, are inducible; the intracellular enzymes for organic acid oxidation are constitutive.
4. The following enzymes of glucose metabolism have been detected and assayed in sonic extracts of the organism:

   Glucose and gluconic dehydrogenases;

   Hexokinase, gluconokinase and 2-oxogluconokinase;

   Glucose 6-phosphate and 6-phosphogluconate dehydrogenases;

   6-Phosphogluconate dehydrase and 2-oxo-3-deoxy-6-phosphogluconate aldolase.
5. The occurrence of these enzymes is consistent with the operation of three pathways of glucose oxidation,

(a) the direct non-phosphorylative oxidation of glucose to gluconic and 2-oxogluconic acids.

(b) The hexose monophosphate oxidative pathway.

(c) The Entner-Doudoroff pathway.

6. All the enzymes of glucose metabolism which were assayed are inducible.

7. Methyl α-glucoside has been shown to be a non-metabolized inducer of the system for glucose metabolism in this organism. No evidence has been obtained, however, for a single mechanism causing the accumulation of either [^{14}C] methyl α-glucoside or [U-^{14}C] glucose within the cells.

8. Several results obtained are indicative of the presence of an inducible permease for glucose, namely:-

(a) The difference observed between the oxidation of glucose by whole cells and disrupted cell preparations of succinate-grown cells subsequent to induction with glucose.

(b) A comparison of the stability in glucose media of protoplasts prepared from citrate-grown cells before and after induction with glucose.

(c) The "maintenance" effect (as described by Cohn & Horibata) observed with glucose-grown cells when they are inoculated into a growth medium

containing both glucose and citrate as carbon and energy sources.

9. The metabolism of an organic acid does not affect the metabolism of glucose by non proliferating cell suspensions which have been,
   (a) grown on glucose,
   (b) grown on glucose and subsequently induced by induction with the organic acid,
   or (c) grown on glucose and subsequently induced by growth in the presence of both glucose and the organic acid.

10. The metabolism of an organic acid does inhibit the metabolism of glucose by non-proliferating cell suspensions which have been grown on the organic acid and subsequently induced by incubation with glucose.
    This inhibition has been shown, in experiments with cell extracts, to be of the enzyme glucose dehydrogenase.

11. The relevance of these results to the current hypotheses of the mechanisms of non-genetic control of metabolism in bacteria has been discussed and some ideas for future research outlined.

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A Biochemical Study of the Diauxic Growth

of *Pseudomonas aeruginosa*.

By William Allan Hamilton, B.Sc.

The growth of *Pseudomonas aeruginosa* in an ammonium salts medium containing an organic acid and glucose as the sole sources of carbon and energy, results in the phenomenon of diauxie, i.e., double growth. Contrary to all previously recorded examples of this phenomenon in other organisms, the organic acid is utilized during the first growth cycle and glucose during the second.

The oxidation of organic acids by cells grown on glucose as the sole source of carbon and energy, i.e., glucose-grown cells, and the oxidation of glucose by organic acid-grown cells, involves an initial period of induced protein synthesis. In the case of the oxidation of organic acids the permeases, protein components of the plasma membrane which facilitate the entry of the compounds into the cell, are inducible; the intracellular enzymes for organic acids are constitutive in this organism.

A number of enzymes of glucose metabolism have been found in cell extracts. These are:- glucose and gluconic dehydrogenases; hexokinase, gluconokinase and 2-oxogluconokinase; glucose 6-phosphate and 6-phosphogluconate dehydro-

genases; and 6-phosphogluconate dehydrase and 2-oxo-3-deoxy-6-phosphogluconate aldolase. These data are consistent with three pathways of glucose metabolism; the direct non-phosphorylative oxidation to 2-oxogluconate; the Entner-Doudoroff pathway; and the hexose monophosphate oxidative pathway. The enzymes of glucose metabolism studied are inducible in this organism.

Methyl α -glucoside has been shown to be a non-metabolized inducer of the system for glucose metabolism, but no evidence was obtained for a single mechanism causing the accumulation of either [^{14}C] methyl α -glucoside or [U-^{14}C] glucose within the cells.

Several results obtained, however, are indicative of the presence of an inducible permease for glucose, namely; (a) the difference observed between the oxidation of glucose by whole cells and disrupted cell preparations of cells grown on succinate and subsequently induced with glucose; (b) a comparison of the stability in glucose medi of protoplasts prepared from citrate-grown cells before and after induction with glucose; (c) the continued synthesis of the enzymes of glucose metabolism when glucose-grown cells are inoculated into a growth medium containing glucos and citrate as carbon and energy sources.

The metabolism of an organic acid does not affect metabolism of glucose by non-proliferating cell suspensions

which have been, (a) grown on glucose, (b) grown on glucose and subsequently induced by incubation with the organic acid, or (c) grown on glucose and subsequently induced by growth in the presence of both glucose and the organic acid. However, the metabolism of an organic acid does inhibit the metabolism of glucose by non-proliferating cell suspensions which have been grown on the organic acid and subsequently induced by incubation with glucose. This inhibition has been shown, in experiments with cell extracts, to be of the enzyme glucose dehydrogenase.

The relevance of these results to the current hypotheses of the mechanisms of non-genetic control of metabolism is discussed.

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