REGULATION

of

ISOCITRATE DEHYDROGENASE

in

ESCHERICHIA COLI

by

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Finally I wish to thank my wife, Wilma, who, throughout the research involved in, and especially the writing of, this thesis, has shown great patience and understanding.

ABBREVIATIONS

All abbreviations are those recommended by The Biochemical Journal in "Policy of the Journal and Instructions to Author", with the exception of,

IC de H	-	isocitrate dehydrogenase
ICL	-	isocitrate lyase
CS	-	citrate synthase
2 - O de H	-	2 - oxoglutarate dehydrogenase.

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INTRODUCTION

RATIONALE FOR THESIS.

In recent years a great deal of scientific interest has centred on the mechanisms which control the activity of enzymes in the cell. However, most attention has been focused on those enzymes whose activities have been shown to vary over wide ranges, depending on the nutritional environment of the cell, and relatively little attention has been devoted to those enzymes whose activities remain virtually constant regardless of nutritional conditions. A much fuller discussion of this problem has been given by Pardee and Beckwith (1963).

Holms (1966) reported that synthesis of β-galactosidase, in a mutant of E.coli in which synthesis of the enzymes of the lactose operon is permanently derepressed (because of a faulty repressor), is nevertheless still subject to some form of control. This worker showed that synthesis of β-galactosidase during growth on glycerol ammonium salts proceeded at four times the rate achieved by cells during growth on glucose ammonium salts. Hence synthesis of this enzyme, although no longer subject to the control mechanism described by Jacob and Monod (1961), is still subject to some form of control, possibly that described by Magasanik (1961) as catabolite repression.

Leggate and Holms (1968) reported that, when maximally induced, the synthesis of β-lactamase in Staphylococcus aureus proceeded at a constant differential rate which was independent of the nutritional composition of the growth medium.

In the first system (Holms, 1966), synthesis of β-galactosidase is considered to be constitutive, in that the rate of synthesis no

longer responds to the presence of an inducer, but is, nonetheless, still subject to variation depending on the nutritional composition of the growth medium, whereas, in the second system (Leggate and Holme, 1968), although β-lactamase is an inducible enzyme, once maximally induced, its synthesis appears to proceed at a constant rate which is independent of the composition of the growth media. In both these systems, the enzyme studied was gratuitous under most of the conditions investigated, in that, once synthesized, it was of no use to the cell. These results led us to consider what mechanisms might operate to control those enzymes which were both constitutive and essential for the cell during aerobic growth in simple defined media.

During aerobic growth in defined media synthesis of several of the enzymes of the tricarboxylic acid cycle proceeds at a more or less constant rate (Gray, Wimpenny and Mossman, 1966). We therefore decided to investigate the means by which E.coli controls the activity of its tricarboxylic acid cycle enzymes during aerobic growth in defined media.

THE TRICARBOXYLIC ACID CYCLE IN BACTERIA -- HISTORICAL

It is now generally accepted that the tricarboxylic acid cycle occupies a central position in the metabolism of most organisms (Krebs and Lowenstein, 1960). The cycle was first conceived to explain the terminal stages of carbohydrate metabolism in pigeon breast muscle and pigeon liver (Krebs, 1937, Krebs and Johnson, 1937). The data which culminated in the postulation of the cycle have been dealt with by Krebs (1943), and will not be dealt with here.

The existence of the tricarboxylic acid cycle and its role in the terminal stages of carbohydrate metabolism in mammalian cells quickly gained acceptance, but a considerable period of time elapsed before the cycle was shown to play a similar role in bacterial metabolism. Krebs himself originally expressed the view that he considered it improbable that the cycle played a major role in the metabolism of microorganisms (Krebs and Johnson, 1937, Krebs, 1943). This view was apparently confirmed some years later when Karlsson and Barker, (1948), using cell suspensions of Azotobacter agilis and measuring oxygen uptake in the Warburg apparatus demonstrated that cells grown on, and able to metabolize, succinate, fumarate or malate, gave only a very low initial oxygen uptake when confronted with cis-aconitate or 2-oxoglutarate. Oxygen uptake increased markedly after a period, and this lag was taken as evidence that adaptation to metabolize these compounds was occurring. These workers could show no oxygen uptake whatsoever when citrate was used as substrate. Subsequent studies by Ajl (1950) gave similar results with Escherichia coli and Aerobacter aerogenes. These workers suggested, on the basis

of the above results, that it was unlikely that the tricarboxylic acid cycle constituted a major pathway of metabolism in these cells.

This conclusion was based on the technique of simultaneous adaptation outlined by Stanier (1947). This was, that if a substrate was metabolised via a sequence of compounds, the cells automatically adapted to metabolize the individual compounds of the pathway and would metabolise these immediately when added, providing they were able to enter the cell. Therefore, those workers reasoned, if metabolism of glucose, acetate, succinate was achieved via the tricarboxylic acid cycle, the enzymes for the metabolism of all the intermediates, constituting the cycle, should be present. Their experimental results apparently contradicted this. It was very soon shown, however, that whereas whole cell suspensions would not oxidise various intermediates of the cycle, corresponding cell free extracts would (Stone and Wilson, 1952; Barrett, Larson and Kallio, 1953; Kogut and Podoski, 1953). Kogut and Podoski (1953) also reported the presence of all the tricarboxylic acid cycle enzymes in their extracts, barring citrate synthase, which had previously been shown to be present by Ochoa, Stern and Schneidor (1951). Subsequent studies on the metabolism of radio-labelled acetate showed that label was incorporated into tricarboxylic acid cycle intermediates as would be predicted if metabolism occurred via the cycle (Sas and Krampitz, 1954; Swim and Krampitz, 1954).

Thus by the mid-fifties the presence of the tricarboxylic acid cycle in bacteria was firmly established. This has been further consolidated by the demonstration of all the cycle enzymes in a number

of bacterial genera. (Murthy, Sirsi and Ramakrishnan, 1962; Williams and Rainbow, 1964; Truper 1965; Gray, Wimpenny and Mossman, 1966). However this does not prove that all bacteria possess a functional tricarboxylic acid cycle (Williams and Rainbow, 1964).

Once the role of the cycle as a pathway of terminal oxidation became accepted, it very soon became obvious that this was not its only role. Roberts, Cowie, Britten, Bolton and Abelson (1953), Abelson, Bolton, Britten, Cowie and Roberts (1953) and McQuillen and Roberts (1954) conclusively established that the cycle played a major role in the biosynthesis of amino acids, and therefore indirectly in the synthesis of the major cell polymers. That this was indeed the case was confirmed by Gilvarg and Davis (1956), who showed that a mutant of Escherichia coli, lacking citrate synthase activity, would only grow on glucose providing the medium was supplemented with glutamate or, to a lesser extent, with 2-oxoglutarate. These workers also showed that acetate was metabolized at a very greatly reduced rate in the mutant, when compared with its wild type parent. These results suggested that the tricarboxylic acid cycle was the major, if not the only, pathway for the terminal stages of glucose metabolism, and for the metabolism of acetate.

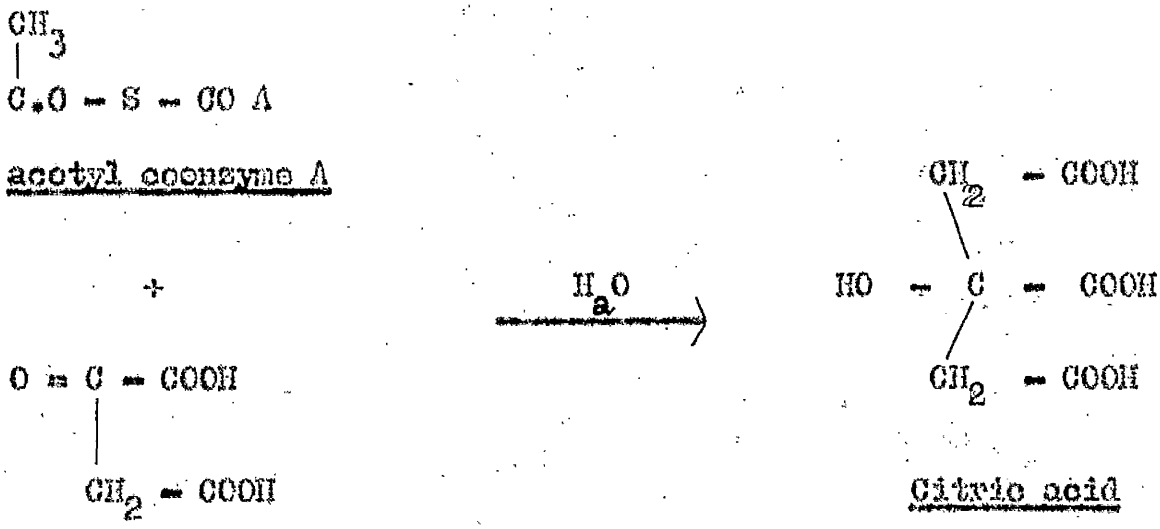
The problems originally encountered with whole cells, and overcome by using cell extracts, argued that a permeability barrier exists to limit entry of the intermediates of the cycle. The ability of cells to adapt themselves to oxidise these compounds, was shown to be inhibited by prior irradiation of the cells or by inhibition of

protein synthesis, (Barrett, Larson and Kallio, 1953; Kogut and Podoski, 1953), strongly implying that the synthesis of specific transport systems were required before entry and subsequent oxidation could be achieved. These observations have recently been substantiated. (Takahashi and Hino, 1968; Kay and Kornberg, 1969).

THE TRICARBOXYLIC ACID CYCLE

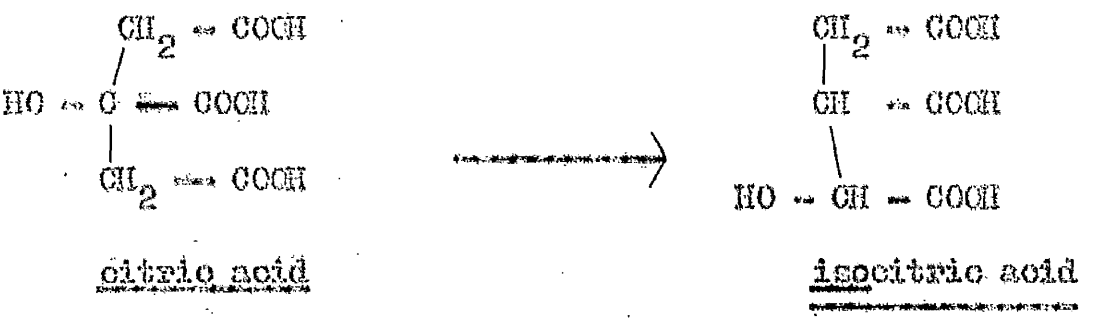
As the following work is concerned with the control of the activity of enzymes of the tricarboxylic acid cycle, it is as well at this point to outline the reactions involved. The cycle serves the function of oxidising acetate to carbon dioxide and water, preserving some of the energy released during the metabolism.

The first reaction of the cycle involves the condensation of acetyl-coenzyme A with oxaloacetate to form the tricarboxylic acid, citric acid. The reaction is catalysed by the enzyme citrate oxaloacetate - lyase (coenzyme A-acetylating), EC 4.1.3.7, more commonly known as citrate synthase. (Stern, 1961).



Citric acid is then converted to its isomer isocitric acid, by the enzyme citrate (isocitrate) hydro-lyase, EC 4.2.1.3. or aconitate

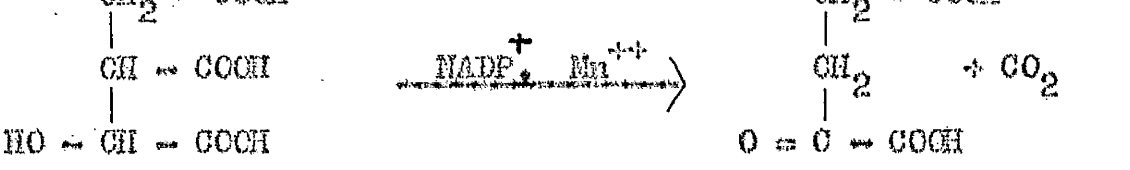
hydratase (Dickman, 1961).



citric acid → isocitric acid

The conversion of citric acid to isocitric acid may or may not, involve the intermediate formation of the common dehydration product cis-aconitic acid. (See Krebs and Lowenstein, 1960, p. 143-5).

Isocitric acid is then dehydrogenated and decarboxylated to yield a five carbon dicarboxylic acid, 2-oxoglutaric acid. The enzyme catalysing the reaction is L_s - isocitrate : $NADP^+$ oxidoreductase (decarboxylating) EC 1.1.1.42 or isocitrate dehydrogenase. (Plaut, 1963).



isocitric acid → 2 - oxoglutaric acid

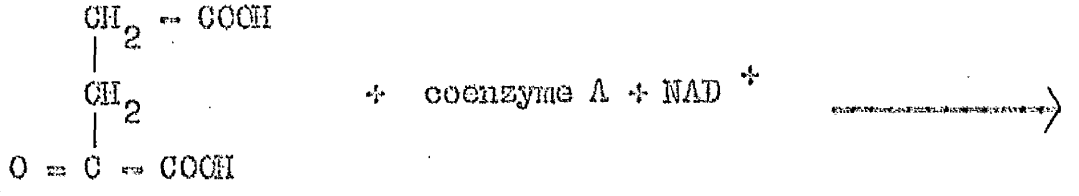
A second enzyme, L_s - isocitrate : NAD^+ oxidoreductase (decarboxylating), E.C. 1.1.1.41, is also known to catalyse the above reaction.

It has been suggested that this reaction proceeds in two stages, with the intermediate formation of oxalosuccinic acid. However, this compound has not been isolated from the reaction medium and it is presumed that, if formed, it remains enzyme bound (See Krebs and Lowenstein, 1960, p. 145-6).

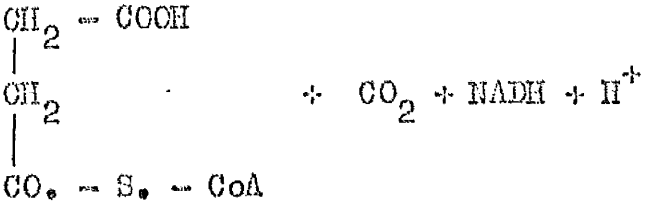
The next stage in the cycle is the decarboxylation of

2-oxoglutarate to yield an activated form of succinic acid, namely succinyl-coenzyme A, a reaction catalysed by a complex of enzymes under the collective name of the 2-oxoglutarate dehydrogenase complex or 2-oxoglutarate dehydrogenase for short (Reed and Cox, 1966). The complex requires thiamine pyrophosphate, coenzyme A, lipoic acid, FAD and NAD^+ as co-factors.

The overall reaction catalysed is,



2 - oxoglutaric acid



succinyl-coenzyme A

Succinic acid is formed from succinyl-coenzyme A by cleavage of the thioacyl bond. The cleavage is coupled to the formation of a nucleoside triphosphate, which for the bacterial enzyme is ATP. An enzyme from mammalian sources phosphorylates either GDP or IDP. The enzymes catalysing the reaction are

succinate : coenzyme A ligase (ADP) E.C. 6.2.1.5. or

succinate : coenzyme A ligase (GDP) E.C. 6.2.1.4.

(Hager, 1962).

A third enzyme has been found in mammalian tissues which does not involve the formation of a nucleoside triphosphate, namely,

succinyl-coenzyme A hydrolase, E.C. 3.1.2.3. (Gergely, Hele, Ramakrishnan, 1952). The reaction occurring in bacteria may be represented thus,

$CH_2 - COOH$
$|$
$CH_2 - C.O. - S. CoA$ + ADP + $PO_4^{\equiv}$

succinyl - coenzyme A

$\longrightarrow$ $CH_2 - COOH$ / $CH_2 - COOH$ + coenzyme A + ATP

succinate

Succinate is then dehydrogenated to give fumaric acid, a reaction catalysed by succinate : (acceptor) oxidoreductase, E.C. 1.3.99.1, or succinate dehydrogenase. The enzyme can be coupled to dyes such as phenazine alkylsulphate as an acceptor, but the physiological acceptor is probably a cytochrome. (Singer and Kearney, 1963).

The reaction catalysed is

$CH_2 - COOH$ / $CH_2 - COOH$ $\longrightarrow$ $CH. - COOH$ ‖ $CH. - COOH$

succinic acid fumaric acid

Fumaric acid is then hydrated to yield malic acid. This reaction proceeds under the action of L-malate hydro-lyase, E.C.4.2.1.2, or fumarate hydratase. (Alberty, 1961).

The reaction is

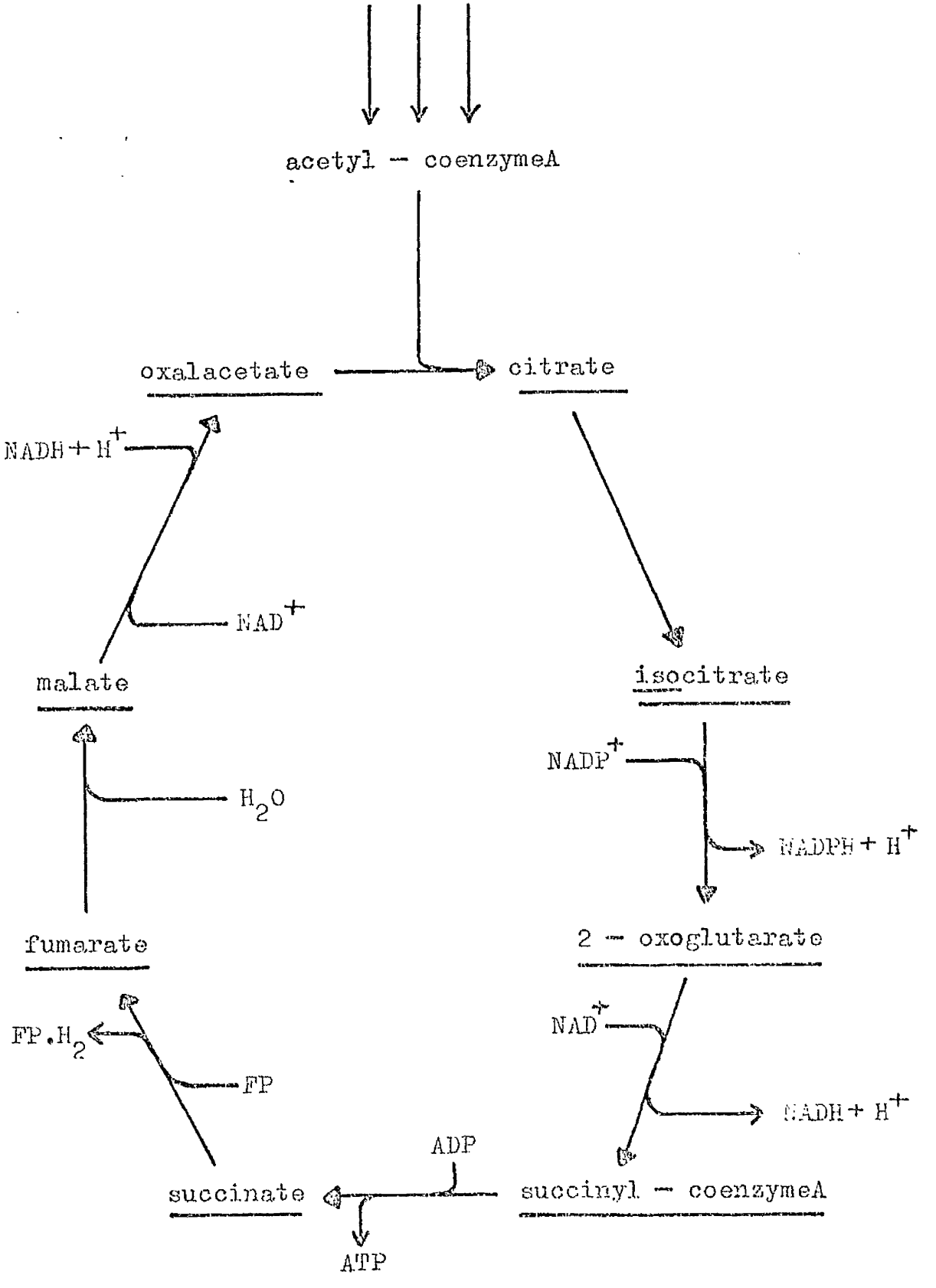
$CH. - COOH$ ‖ $CH. - COOH$ + H_2O $\longrightarrow$ $HO - CH. - COOH$ / $CH_2 - COOH$

fumaric acid malic acid

FIGURE 1

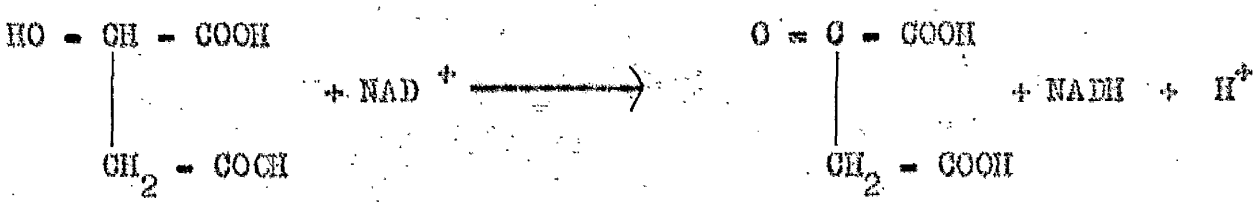
The tricarboxylic acid cycle

FIGURE 1


acetyl — coenzymeA
oxalacetate
citrate
NADH + H+
NAD+
malate
isocitrate
NADP+
NADPH + H+
H2O
fumarate
2 — oxoglutarate
FP.H2
FP
NAD+
NADH + H+
ADP
succinate
succinyl — coenzymeA
ATP

Malic acid is then dehydrogenated to give oxaloacetic acid, thus regenerating one of the components of the initial reaction of the cycle. The enzyme catalysing this last step is L-malate : NAD^+ oxidoreductase, E.C. 1.1.1.37, or malate dehydrogenase.

The reaction catalysed is,



malic acid — oxaloacetic acid

Thus one turn of the cycle converts a molecule of acetate to carbon dioxide and water, produces reduced pyridine nucleotides, which can be oxidized via the electron transport chain with the concomitant formation of ATP, and regenerates the initial acceptor molecule. A diagrammatic representation of the tricarboxylic acid cycle can be seen in Figure 1.

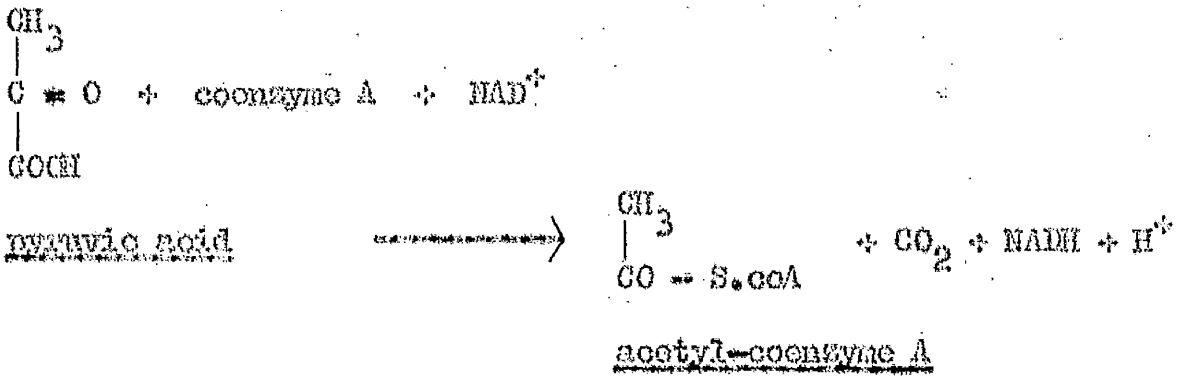
A much fuller discussion of the cycle and its individual reactions has been given by Krebs and Lowenstein (1960).

FORMATION OF ACETYL-COENZYME A.

The initial reactants of the tricarboxylic acid cycle are acetyl-coenzyme A and oxaloacetic acid and an adequate supply of both compounds must be maintained if the cycle is to function.

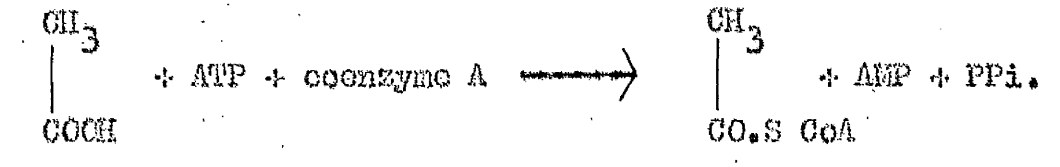
Acetyl-coenzyme A is produced during the metabolism of a variety of compounds, including sugars, amino acids and simple mono-, di-, and tricarboxylic acids. These compounds are either metabolized directly to acetyl-coenzyme A or to compounds which can be metabolized to acetyl-coenzyme A.

Many sugars are metabolized to intermediates of the glycolytic pathway, which ultimately yields pyruvic acid, an immediate precursor of acetyl-coenzyme A. The reaction is catalysed by the pyruvate dehydrogenase complex (Reed and Cox, 1966), and is analogous to the reaction catalysed by the 2-oxoglutarate dehydrogenase complex previously described. The same multiple cofactor requirement is also shown by this enzyme complex. The reaction can be summarized thus,



pyruvic acid — acetyl-coenzyme A

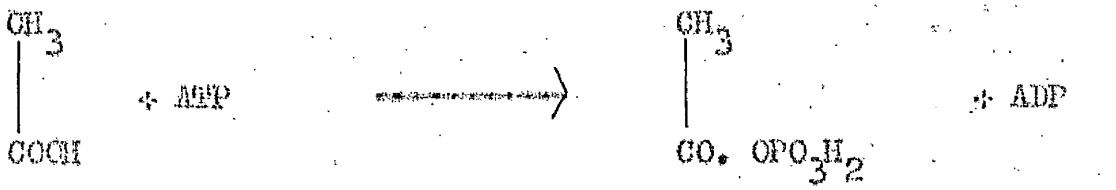
Acetate itself can be directly converted to acetyl-coenzyme A by the enzyme acetate : coenzyme A ligase (AMP) (EC 6.2.1.1.) or acetyl-coenzyme A synthetase. Jones, Lipmann, Hilz and Lynen (1953) showed that the conversion of acetate to the coenzyme A ester was accomplished at the expense of a pyrophosphate cleavage of adenosine triphosphate, i.e.



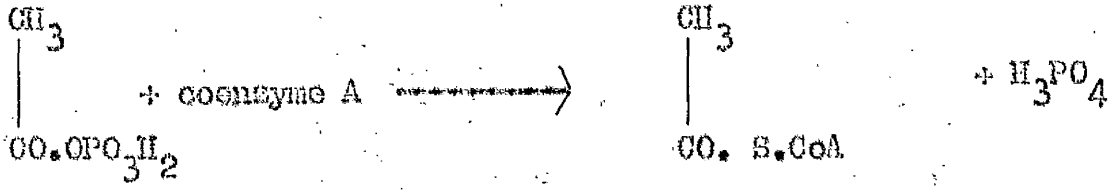
acetic acid — acetyl-coenzyme A

Alternatively the joint action of acetate kinase (ATP : acetate phosphotransferase, EC 2.7.2.1) and phosphate acetyltransferase: EC. 2.3.1.8.) can achieve the net synthesis of acetyl-coenzyme A from acetate and ATP. That these enzymes can indeed work in this

way was shown by Rose, Grunberg-Manago, Korey and Ochoa (1955) working with enzymes from E.coli. The reactions involved may be written



acetic acid — acetyl-phosphate



acetyl-phosphate — acetyl-coenzyme A.

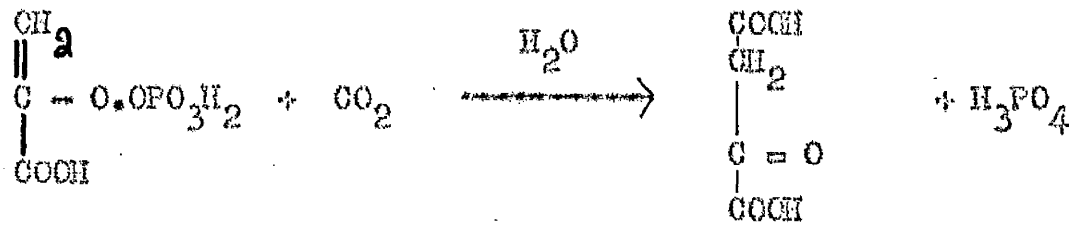
PATHWAYS INTIMATELY ASSOCIATED WITH AN ACTIVE TRICARBOXYLIC ACID CYCLE.

The action of the tricarboxylic acid cycle can satisfactorily account for the complete combustion of glucose or acetate to carbon dioxide and water, one turn of the cycle regenerating oxaloacetic acid, thus allowing entry of a further acetate unit as acetyl-coenzyme A. However, it has been conclusively shown that intermediates of the cycle are utilised in the synthesis of many cell components. Thus if an intermediate of the cycle is withdrawn, regeneration of oxaloacetate cannot occur. If this loss is not compensated for by adding back some other intermediate which can give rise to oxaloacetate, the cycle will cease to function. This problem, and its solution, has been discussed at length in a number of reviews. (Kornberg, 1959; Kornberg and Elsden, 1961; Kornberg, 1966(a)). I propose here to deal only with those aspects which I feel are pertinent to the following work. A discussion of similar problems and solutions facing organisms growing on various other carbon sources can be found in the above mentioned reviews.

ANAPLEROTIC PATHWAYS DURING GROWTH ON GLUCOSE.

Cells growing on glucose utilize the tricarboxylic acid cycle, not only to combust acetyl-coenzyme A formed via glycolysis and the pyruvate dehydrogenase complex to give energy, but also to provide precursors for biosynthesis. However, the sequential operation of glycolysis and the tricarboxylic acid cycle does not allow for removal of intermediates from the cycle for biosynthesis. Therefore some mechanism for replenishing the cycle must exist. That such a

pathway does exist, at least in Escherichia coli, was established by Kornberg and his co-workers. They showed that they were able to obtain mutants of E.coli which were unable to grow on glucose or pyruvate unless the medium was supplemented with small amounts of dicarboxylic acids. Those workers showed that the mutants lacked the enzyme phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) (Canovas and Kornberg, 1965; Canovas and Kornberg, 1966; Ashworth and Kornberg, 1966), whereas the wild type parent organism contained substantial quantities of this enzyme. Phosphoenolpyruvate carboxylase catalyses the formation of oxaloacetic acid from phosphoenolpyruvate, by incorporation of a molecule of carbon dioxide into the C_3 - acid.



phosphoenolpyruvate oxaloacetate

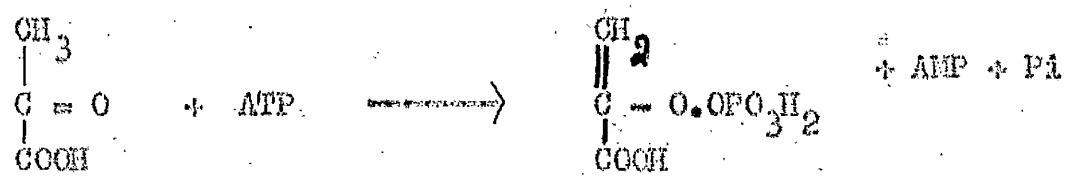
The action of this enzyme ensures a supply of 4 carbon, dicarboxylic acids for use as acceptor in the tricarboxylic acid cycle.

Canovas and Kornberg (1966) showed that the enzyme was greatly activated, over 30 fold, by acetyl-coenzyme A. They suggested that such activation was a control mechanism which would ensure an adequate supply of oxaloacetate from sugar metabolism, for condensation with acetyl-coenzyme A and therefore maintain a functional tricarboxylic acidcycle. This type of replenishment pathway has been termed an

anaplerotic pathway (Kornberg, 1965).

ANAPLEROTIC SEQUENCES DURING GROWTH ON PYRUVATE.

Metabolism of pyruvate presents a slightly more complex situation than growth on glucose. For metabolism of pyruvate to proceed via the tricarboxylic acid cycle, a supply of oxaloacetic acid must be provided. Mechanisms are known which are potentially capable of converting pyruvate to 4 carbon dicarboxylic acids (Kornberg, 1966 (b)). However, it has been shown that in E.coli a different mechanism operates. Cooper and Kornberg (1967) and Brice and Kornberg (1967) have shown that the ability to utilise pyruvate as a growth compound is dependent on the ability to synthesize the enzyme phosphoenolpyruvate synthase. These workers showed that this enzyme was sufficient and necessary for growth on pyruvate, providing the cells could grow on glucose. They showed that the reaction catalysed is,



pyruvic acid — phosphoenolpyruvic acid

Thus the coupling of phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase activities can synthesise oxaloacetic acid from pyruvic acid and provide the necessary entry for acetyl-coenzyme A, also formed from pyruvic acid, into the tricarboxylic acid cycle. The formation of phosphoenolpyruvate from pyruvate, (via phosphoenolpyruvate synthase), is also the first stage in the reversal of glycolysis, a process essential for carbohydrate synthesis.

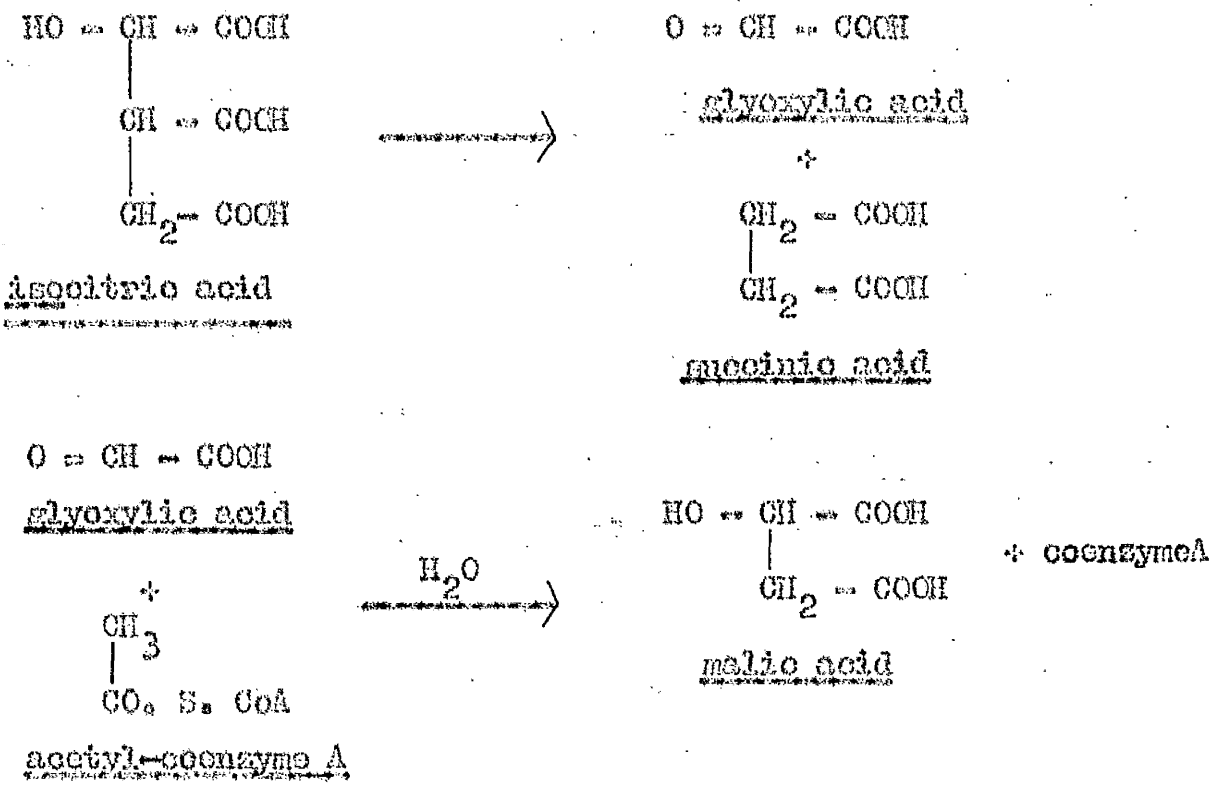
Thus the organism can use the tricarboxylic acid cycle for the provision of energy and biosynthetic precursors when the cells are growing on sugars, e.g. glucose, pyruvate or compounds metabolized directly to these compounds.

ANAPLEROTIC SEQUENCES DURING GROWTH ON ACETATE.

The problem appears much more difficult when organisms grow solely at the expense of acetate. As has been stated, the tricarboxylic acid cycle can combust acetate to carbon dioxide and water, but again, no provision is made in this scheme for the supply of biosynthetic precursors by the cycle.

Early workers had demonstrated that the initial reactions of the tricarboxylic acid cycle are used in the metabolism of acetate (Gilvarg and Davis, 1956), and concluded that the cycle was the major pathway of acetate metabolism. However, the cycle alone cannot support growth. The discovery of two enzymes, which when working in conjunction, achieve the synthesis of one molecule each of succinic and malic acids from one molecule of oxaloacetic acid and two molecules of acetyl-coenzyme A, solved the dilemma (Campbell, Smith and Eagles, 1953; Wong and Ajl, 1956). That the two enzymes, isocitrate lyase (L_s = isocitrate glyoxylate-lyase, E.C. 4.1.3.1.) and malate synthase (L - malate glyoxylate-lyase, (coenzyme A-acetylating), E.C. 4.1.3.2.) work in a sequential manner was demonstrated by Kornberg and Madsen (1958) using extracts of *Pseudomonas fluorescens*, and Reeves and Ajl (1960) showed that both enzymes are formed adaptively in *E.coli* when switched to acetate medium.

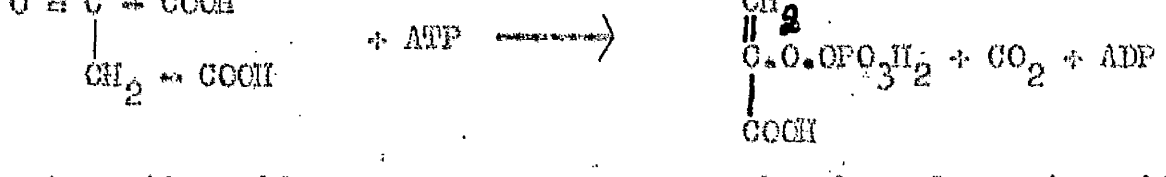
The two reactions can be represented as follows,



These reactions combine to bypass the decarboxylating steps of the tricarboxylic acid cycle, and hence acetate carbon is conserved. One dicarboxylic acid can be used to continue acetate incorporation into the system, while the other can be used for biosynthesis.

FORMATION OF 3-CARBON ACIDS DURING GROWTH ON ACETATE.

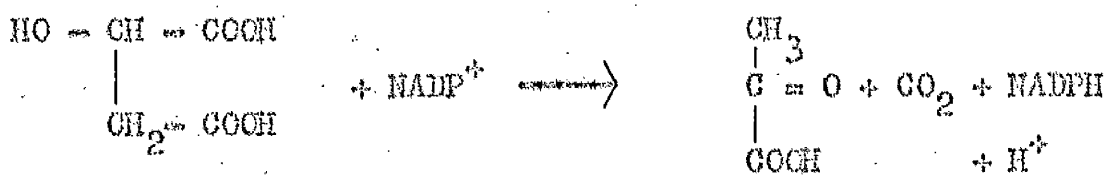
The formation of 3 carbon acids during growth on acetate is also necessary. Kornberg (1965, 1966(b)) has suggested that this is achieved in E.coli by the enzyme phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32) which catalyses the reaction,



The action of malic enzyme (E.C. 1.1.1.40) can also achieve the synthesis of a 3 carbon acid from a 4 carbon dicarboxylic acid.

mbine to bypass the decarboxylating stage of

The reaction utilizes malic acid and $NADP^+$ and can be represented thus,



malic acid — pyruvic acid

This reaction may also have a role in the formation of 3 carbon acids during growth of *E.coli* on acetate (Kornberg, 1966(b)).

MECHANISMS REGULATING ENZYME ACTIVITY.

Once the existence of the tricarboxylic acid cycle in bacteria had been established various investigators turned to study how its activity is controlled. However, before I deal with the available evidence on control of the cycle in bacteria, I would like to briefly outline some known mechanisms by which control could be exerted.

(a) Enzyme formation in response to substrate presence -- Induction.

It has been known for a number of years now that some organisms produce certain enzymes only when they are required. Thus E.coli will only produce the enzymes of the lactose operon when lactose, or an analogue of lactose, is present in the growth medium. This phenomenon is called enzyme induction (Jacob and Monod, 1961) and many examples are known. (Sheppard and Englesberg, 1967; Bilozikian, Kaempfer and Magasanik, 1967; Cozzarelli, Freedberg and Lin, 1968; Clarke, Houldsworth and Lilley, 1968; Kennedy and Fewson, 1968).

Jacob and Monod (1961), working with the enzymes of lactose metabolism in E.coli, put forward a scheme which explained their observations. The basic Jacob and Monod theory of induction-repression of enzyme synthesis has been retained as a working model to the present day, with one or two additions. The scheme can be considered as a logical extension of the one gene -- one enzyme hypothesis suggested by Beadle and Tatum (1941), in that discrete portions of the bacterial genome, called genes, were considered to contain the information for the construction of one enzyme protein. To this basic postulate, Jacob and Monod added the idea of genes whose product performed solely a regulatory function. The basic outline

of the scheme suggested can be seen in Figure 2.

The scheme envisages the existence of two distinct segments of the genome which cooperatively control the expression of the structural genes. One of these control genes is thought to direct the synthesis of a molecule which is released into the cytoplasm of the cell, and is then capable of interacting with the second control gene.

The gene which gives rise to the cytoplasmic product was called the regulator gene, or "R" gene, and the product of this gene was termed the repressor. The second control gene, called the operator or "O" gene is thought to be adjacent to the structural genes. In the uninduced system, the "R" gene produces repressor which combines with the "O" gene. When this occurs expression of the structural genes is blocked. However, when an inducer is added to the system, it binds to the repressor, thus antagonising the interaction of the repressor with the "O" gene. Once the block at the "O" gene has been removed, expression of the structural genes as m-RNA and then as enzymes, can proceed.

To account for the kinetics of enzyme synthesis after removal of inducer, Jacob and Monod (1961) were led to postulate the existence of a short-lived species of molecule which intervened between the information coded in the structural genes and its final expression in terms of protein. This species was very soon discovered and identified as a form of ribonucleic acid and called messenger - RNA (m-RNA) (Brenner, Jacob and Meselson, 1961). The mechanism of protein synthesis has recently been reviewed by Lengyel and Soll (1969).

FIGURE 2

Diagrammatic representation of the mechanism of enzyme induction.

"R" = regulator gene

"O" = operator gene

"STRUCTURAL" = structural genes coding for inducible enzymes.

(a) system uninduced

(b) system induced

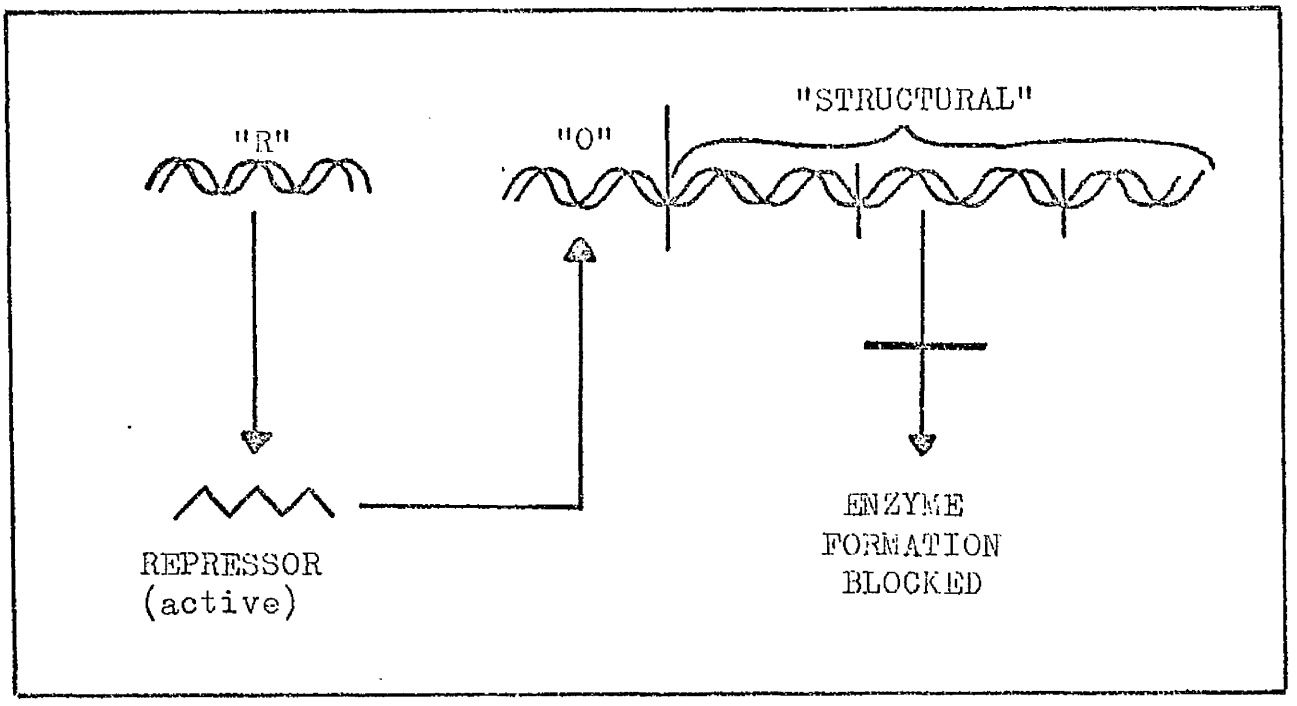


FIGURE 2(a)

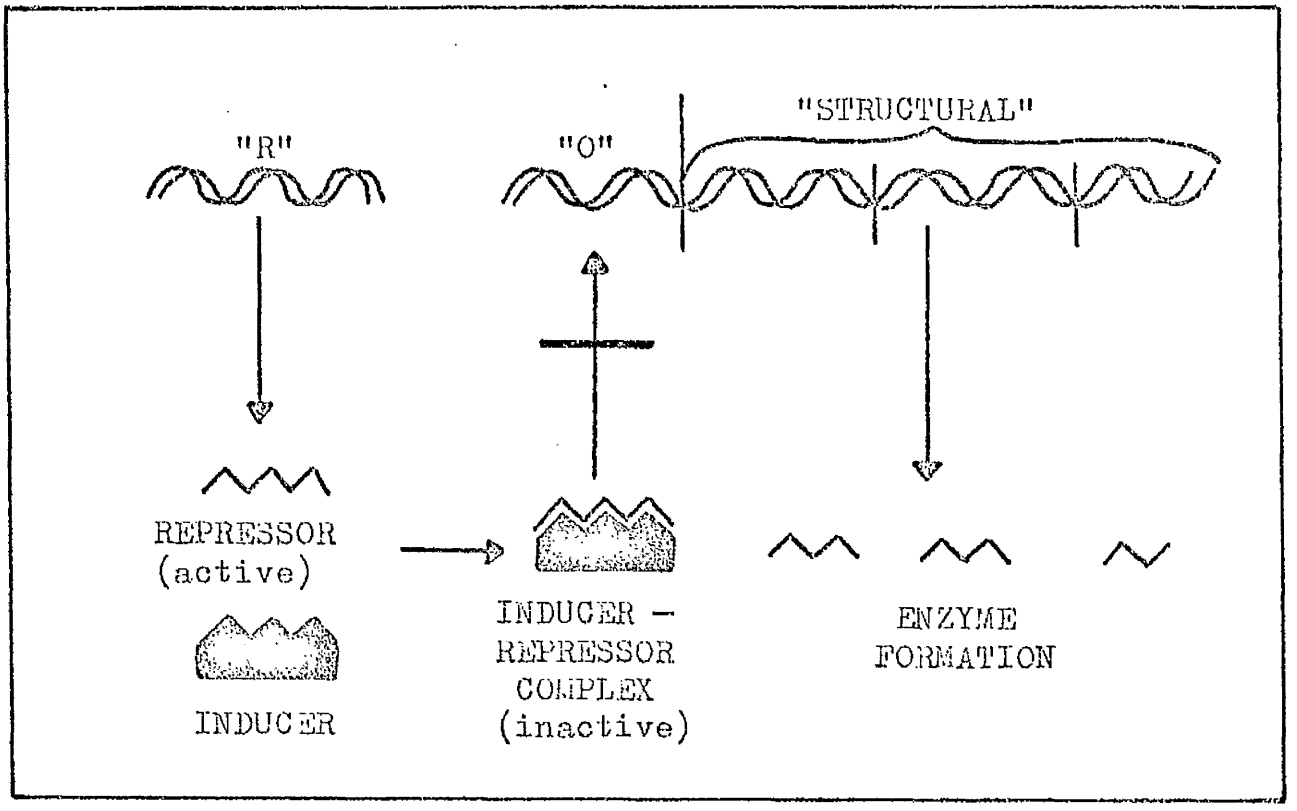


FIGURE 2(b)

(b) Cessation of enzyme synthesis in presence of product — Repression.

It has also been known for some time that the synthesis of certain enzymes is curtailed if the product of the enzyme, or pathway to which the enzyme belongs, is added to the growth medium. (Vogel, 1957; Yates and Pardee, 1957; Jacoby and Gorini, 1969; Kano, Matsushiro and Shimura, 1968; Datta, 1969). This phenomenon has been termed enzyme repression (Vogel, 1957).

Jacob and Monod (1961) observed that with a minor modification, the scheme proposed to explain enzyme induction would also explain enzyme repression. The scheme is outlined in Figure 3.

As can be seen, in most aspects the scheme corresponds to that shown in Figure 2. The only difference is in the role of the regulator gene product. In this case the product is inactive and termed an aporepressor. The aporepressor is activated when it binds the ultimate product of the biosynthetic sequence, called a corepressor. The aporepressor-corepressor complex, which is active, binds to the operator gene, and so curtails enzyme synthesis. In the absence of corepressor, i.e. product of enzyme or pathway, no active repressor is formed and protein synthesis proceeds.

Recent work by Gilbert and Muller-Hill (1967) has provided very strong confirmatory evidence for the postulated regulator gene product, and has shown that it binds to a specific segment of the bacterial chromosome. More recent work by Beckwith and his colleagues has provided further insight into the mechanism of regulation operating at the C-gene. (Miller, Beckwith and Muller-Hill, 1968).

FIGURE 3

Diagrammatic representation of the mechanism of enzyme repression.

"R" = regulator gene

"O" = operator gene

"STRUCTURAL" = structural genes coding for repressible enzymes.

(a) non-repressed system

(b) repressed system

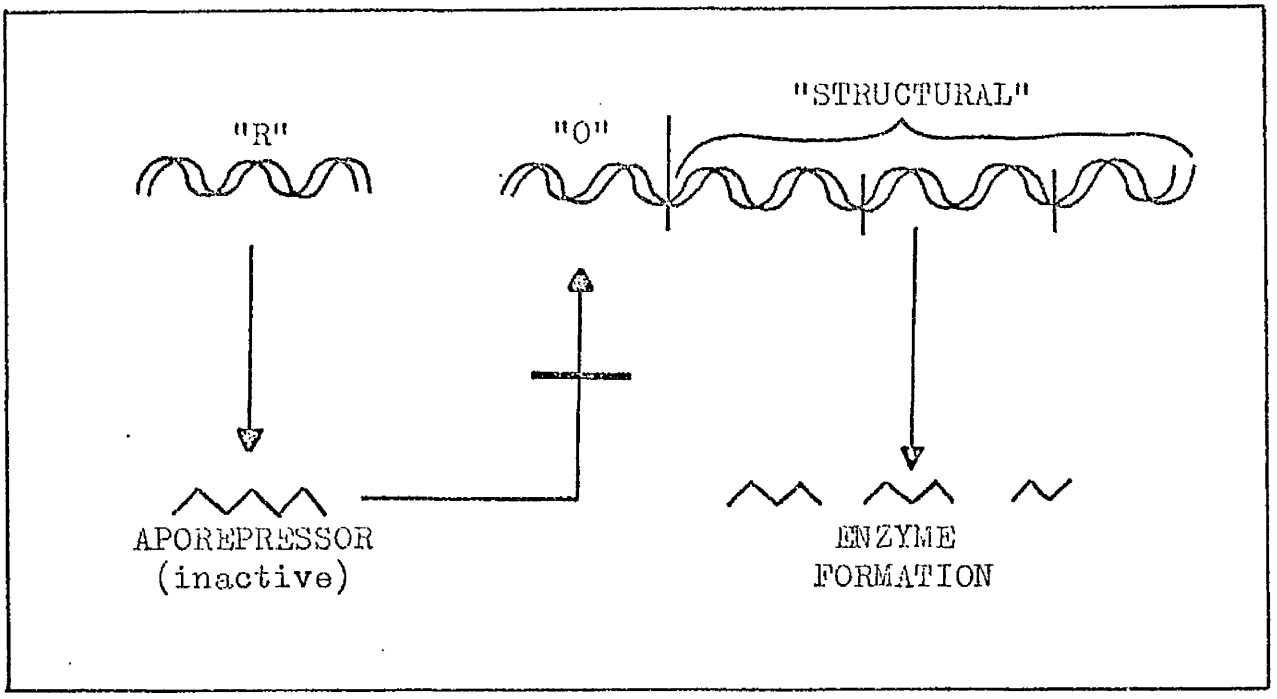


FIGURE 3(a)

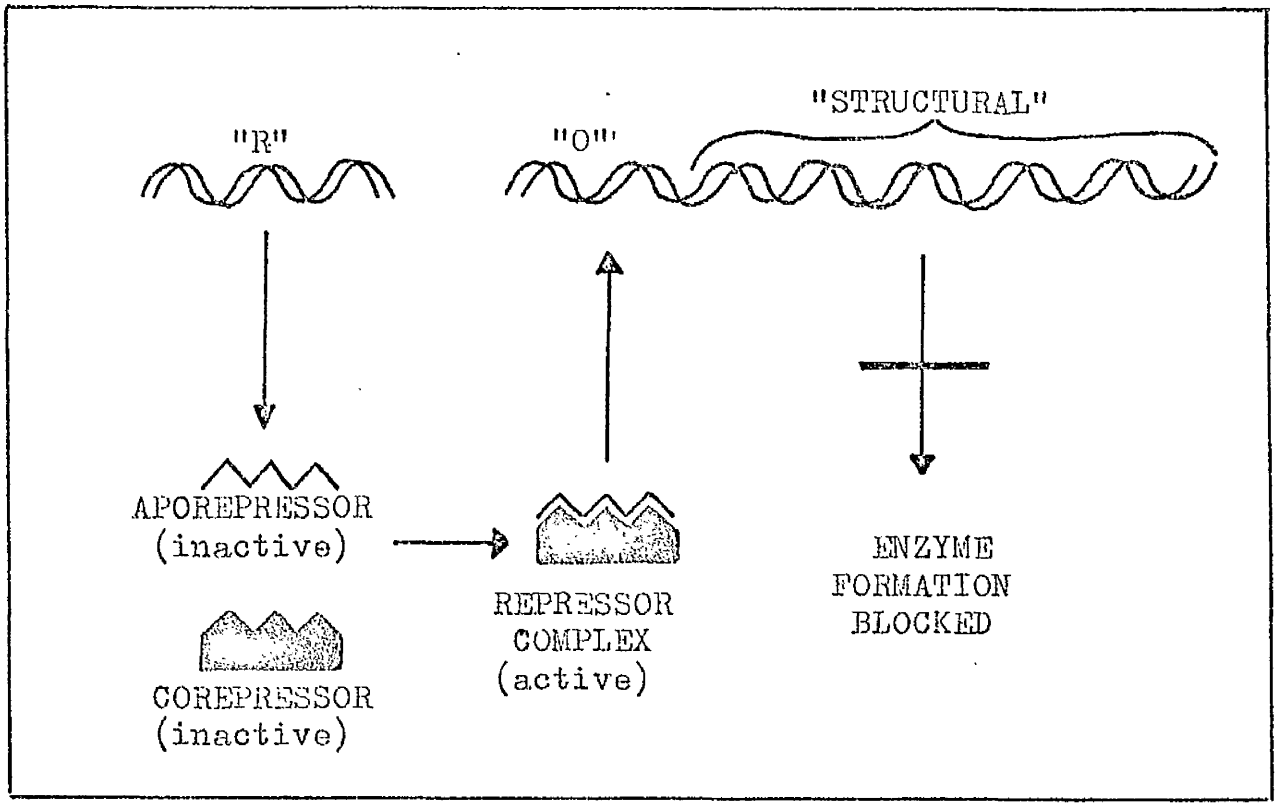


FIGURE 3(b)

The schemes envisaged for enzyme induction and enzyme repression are still not entirely proven, though proof for specific systems is steadily accumulating. These schemes however may require modification to explain results observed in other systems. A review of this field, covering objections to the proposed schemes and divergences from them exhibited by other enzyme systems, has recently been made by Richmond (1968).

(c) Catabolite Repression of enzyme synthesis.

Epps and Gale (1942) first observed that the synthesis of certain enzymes was repressed by the presence of glucose in the medium. These observations were extended by Monod (1947) who discovered that glucose prevented the formation of catabolic enzymes essential, and unique, to the metabolism of certain other sugars. The effect was therefore called the "glucose effect". It was later shown (Neidhardt and Magasanik, 1956, 1957; Mandelstam, 1961) that compounds other than glucose would produce a similar effect. This led Magasanik (1961) to apply the term "catabolite repression" to the phenomenon.

Most of the work concerned with catabolite repression has centred round the search for the so-called catabolite repressor and compounds such as ATP, NADPH, glucose-6-phosphate and acetyl-glucosamine have been implicated. (Prevost and Moses, 1967; Dobrogosz, 1968; Hsie and Rickenberg, 1967).

Recent studies by Ullmann and Monod (1968), and Pastan and Perlman (1969), Perlman and Pastan (1968) have indicated that catabolite repression can be relieved by the addition of cyclic-3', 5' - AMP. More recently, Perlman and Pastan (1969) have shown that a mutant of

E.coli lacking the enzyme adenyl cyclase, which forms cyclic-AMP from ATP, has great difficulty in using a wide range of carbon sources. Addition of cyclic-AMP restores utilization of all the compounds to normal. Thus it is difficult to escape the conclusion that cyclic-AMP may be intimately concerned in regulating the synthesis of a number of enzyme systems and that catabolite repression is more due to a lack of something rather than an overproduction.

(d) Feedback Inhibition.

Enzyme activity may be subject to inhibition, and it has been found that a number of enzymes are inhibited by compounds bearing little resemblance to the enzyme substrate. Such inhibition has been described by Monod, Changeux and Jacob (1963) as allosteric inhibition. This form of control is seen to operate in a number of biosynthetic pathways where the product of the pathway inhibits the first enzyme unique to its formation. (Yates and Pardee, 1956; Woitzman and Wilson, 1966; Jensen, 1969; Datta, 1969).

Allosteric inhibition is thought to be effected by the enzyme binding the inhibitor at a site distinct from the active site and that such attachment induces structural changes in the enzyme protein making it unsuitable for its role as a catalyst. Removal of the inhibitor allows the enzyme protein to revert to its catalytically active form.

(e) Other forms of control.

Enzyme activity can be altered by the presence of simple competitive inhibitors, such as the inhibition of succinate dehydrogenase by malonate (Quastel and Wooldridge, 1928). The relevance of this form

of inhibition in a metabolic situation is unclear. Changes in enzyme activity can also be achieved by either polymerization or depolymerization mechanisms. The best known and studied example of this form of control is that of the enzyme phosphorylase (Hales, 1967), where two phosphorylase b units are converted to one phosphorylase a unit, with considerably enhanced activity, through the mediation of an enzyme, phosphorylase b kinase, at the expense of 4 molecules of ATP. Phosphorylase a can be converted back to phosphorylase b, releasing four molecules of inorganic phosphate, by another enzyme, phosphorylase phosphatase.

Enzyme inactivation by the attachment of an organic group is also known. Glutamine synthetase can be inactivated in an enzyme mediated reaction by the attachment of an adenyl residue to the enzyme protein. A second enzyme can remove the adenyl residue and so reactivate the enzyme (Shapiro and Stadtman, 1968).

Just how widespread the last two activation-inactivation mechanisms are remains to be seen.

CONTROL OF THE TRICARBOXYLIC ACID CYCLE

How do the control mechanisms described in the previous section apply to the enzymes of the tricarboxylic acid cycle? In bacteria, little work has been performed on control of the enzymes of the cycle. Collins and Lascelles (1962) showed that when Staphylococcus aureus was grown with glucose in the medium, synthesis of two of the dehydrogenases of the cycle, those for isocitrate and succinate, was repressed. This repression of cycle enzymes by glucose was also noted in Bacillus subtilis (Hanson, Blicharska, Arnaud and Saulmajster, 1964). In both cases repression of synthesis appeared to be severe. A similar phenomenon was observed by Gray, Wimpenny and Mossman (1966) working with E.coli. When glucose was added to cultures growing in a complex medium severe repression of some cycle enzymes resulted. Even when growing in synthetic medium, when glucose was the carbon source, some enzymes, specifically citrate synthase and fumarase, were produced in much lower amounts than when glycerol was the carbon source. These results suggest that some form of catabolite repression may be operative on this system.

Amarasingham and Davis, (1965) reported the results of various experiments which they interpreted as showing that 2-oxoglutarate dehydrogenase, in E.coli, is induced by acetate or possibly 2-oxoglutarate.

More recently Rowe and Weitzman (1969) reported that citrate synthase from E.coli is allosterically inhibited by NADH and Weitzman and Dunmore (1969) reported that the same enzyme is also inhibited by 2-oxoglutarate. These results, coupled to that of Amarasingham, suggest a mechanism of the feedback or end-product

inhibition type.

Not directly concerned with the cycle, but related to it, Schwartz, Old and Reed (1968) showed that acetyl-coenzyme A can inhibit the pyruvate dehydrogenase complex which is also activated by phosphoenolpyruvate, suggesting mechanisms which control the formation of acetyl-coenzyme A.

Hanson and Cox (1967) reported that the addition of glutamate or yeast extract to cultures of E.coli growing in a glucose salts medium, caused a severe repression of aconitate hydratase and isocitrate dehydrogenase synthesis. A similar effect was noted in Bacillus subtilis. Maximal repression required both a rapidly catabolized carbon source and 2-oxoglutarate or L-glutamate. Recent work by Flechtner and Hanson (1969) showed that similar conditions also promoted a repression of citrate synthase synthesis in B. subtilis. These workers suggest that in B.subtilis citrate synthase and aconitate hydratase are coordinately controlled.

Isocitrate dehydrogenase from various organisms has been shown to be inhibited by a mixture of glyoxylic acid and oxaloacetic acid, and such inhibition has been related to the operation of the glyoxylate, and tricarboxylic acid cycles, (Shiio and Ozaki, 1968; Marx and Weber, 1969). Reeves, Brehmeyer and Ajl (1968) have purported to show the existence of two $NADP^+$-specific isocitrate dehydrogenases in E.coli when growing in glucose medium and that a third enzyme, electrophoretically distinct from the two demonstrated during growth on glucose, was formed during growth on acetate. Self and Weitzman (1970) have demonstrated the presence of a single $NADP^+$- specific isocitrate dehydrogenase

in cells of E.coli grown on acetate using the technique of zonal centrifugation. The relevance of possibly three distinct isocitrate dehydrogenases being synthesized by E.coli is obscure. However, the possibility of polymeric forms of a single enzyme is not ruled out.

Thus, mechanisms involving induction, repression, catabolite repression, allosteric inhibition and possibly multiple enzyme forms are implicated in the control of activity of the tricarboxylic acid cycle.

PROPOSED METHOD OF STUDY.

We wished to examine the means by which enzyme activity, specifically that of the tricarboxylic acid cycle enzymes in E.coli, is controlled. We proposed to initiate the study by altering the growth medium in a known manner and observing the effect that this change produced on enzyme activity. To facilitate the correlation of changes in growth conditions with the possible change in enzyme activity, we decided to adopt the following procedures.

Cells would be inoculated into a constant initial concentration of a primary carbon and energy source, to which the cells were trained, and at a predetermined turbidity i.e. after a fixed amount of the carbon and energy source had been utilized, the compound whose effect was to be tested would be added to the culture.

It was hoped that the technique described above would standardize the various manipulations and permit a high degree of reproducibility to be attained. Comparison of enzyme activity in the culture to which the addition had been made with that in an untreated culture could then be made, and the effect of the addition assessed.

A second proposed line of attack was to be as follows. Cells would be grown on a limiting concentration of a known, single carbon and energy source until the stationary phase was reached. A predetermined time after growth on the primary carbon and energy source ceased, more of the same carbon and energy source, enough to permit one further generation of growth, would be added, plus a second compound, and the effect of this second compound on enzyme activity

would be observed. Enzyme activity would be followed throughout the single generation and compared to the activity in cultures to which a further supply of the original carbon source, by itself, had been added.

Again it was hoped that by adopting such a procedure, a reproducible state, prior to the addition of the challenge compound, would be achieved. However, before this technique could be used, it had to be shown that the enzyme activities being studied were stable once the stationary phase was reached.

Ideally it was hoped that both methods would give the same answer so that the second technique, which is the easier to manage technically, could be used routinely.

In the event, our expectation of a steady state was not achieved and the work developed into a study of the mechanism whereby isocitrate dehydrogenase activity was regulated after exhaustion of the primary carbon source.

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM

The organism used in the studies reported in this thesis was Escherichia coli ML308, obtained from the American Type Culture Collection, catalogue number 15224. Owing to a mutation in the gene coding for the repressor of the lactose operon, the organism synthesizes the enzymes of the lactose operon constitutively. In other respects the strain is regarded as wild type.

STORAGE OF ORGANISM

The organism was obtained in the form of a lyophilized pellet. The pellet was suspended in 10 ml sterile nutrient broth in a universal container, and incubated for 24 hours at 37°C. This culture was then streaked on to nutrient agar plates, which were incubated for 48 hours at 37°C. A typical colony was picked off one of the nutrient agar plates and used to inoculate 10 ml sterile nutrient broth, which was then incubated for 24 hours at 37°C. The resulting culture was checked for homogeneity by microscopic examination, using gram stain and phase contrast techniques, and by restreaking on to nutrient agar plates and screening, after incubation, for contaminants.

The identity of the organism was verified using fermentation tests, comparing the results with those produced by known strains of Escherichia coli and Aerobacter aerogenes. A further check could be made by assaying for β-galactosidase, which, as it is produced constitutively in the strain of E.coli under investigation, is present during growth on all media.

Once the identity of the organism had been verified, the nutrient

broth culture was used to inoculate a series of 10 ml volumes of sterile cooked meat medium in universal containers, which were then incubated for 24 hours at 37°C. These cooked meat cultures were used for long term storage of the organism at 4°C.

Every three months, 10 ml volumes of sterile nutrient broth were inoculated from one of the cooked meat cultures and were then incubated for 24 hours at 37°C. These nutrient broth cultures were stored at 4°C and were the source of organism for the preparation of inocula. This procedure, and that following for the preparation of inocula, ensured that the culture used to inoculate experimental flasks was always a fixed number of generations from the starting material, and the complete history of the culture known.

All incubations in complex media (10 ml) were carried out using stationary culture conditions.

PREPARATION OF INOCULA

100 ml of defined medium in a 500 ml conical flask was inoculated with 3 drops, using a pasteur pipette, from a nutrient broth culture and incubated at 37°C with shaking on an Orbital Shaker (L.H. Engineering Co. Ltd.,) for 15 hours. 1 ml of this culture was then inoculated into a further 100 ml of sterile, homologous definedmedium, which was incubated at 37°C for 7 hours on the shaker. This second passage culture in defined medium was then stored at 4°C and used as the source of organism trained to the particular carbon source contained in the defined medium. 24 hour prior to an experiment, 1 ml of a second passage culture was inoculated into a further 100 ml of the same sterile defined medium, which was incubated at 37°C on the shaker for 7 hours. This

third passage culture was stored overnight at 4°C and used as the source of organism for the inoculation of growth flasks.

Occasionally it proved necessary to alter the time of one of the serial passages, depending upon the carbon source used for growth. When dicarboxylic acids were used, the second and third passages were lengthened to 8 hours and when acetate was the growth substrate, a 24 hour first passage was necessary and the second and third passages were lengthened to 15 hours.

MEDIA

Cooked meat medium

Nutrient broth medium

Nutrient agar

These media were prepared using Oxoid dehydrated media. They were prepared following the instructions supplied, and were sterilised by autoclaving at 15 p.s.i. The sterile media were stored at 4°C till required.

Defined media

Defined media consisted of a basic mineral salts medium (basal salts medium) supplemented with a known carbon source. The concentration of the mineral salts in the final growth media were,

KH_2PO_4	40mM
$(NH_4)_2SO_4$	10mM
$MgSO_4 \cdot 7H_2O$	2mM
$FeSO_4 \cdot 7H_2O$	10µM

When the media were used for growth of inocula, the carbon source was present at a concentration equivalent to 60 matoms of carbon per litre in the final growth medium. When the media were used for growth experiments, carbon source present at a final concentration equivalent to 12 matoms of carbon per litre growth medium was used, unless otherwise stated.

DEFINED MEDIA VARIATIONS

When the carbon source in the defined medium was an organic acid, such as acetate or one of the compounds constituting the tricarboxylic acid cycle, the concentration of $MgSO_4.7H_2O$ in the medium was lowered to 0.5mM, to prevent precipitation of magnesium phosphate which tended to occur, owing to a rise in the pH of the culture medium as utilization of the organic acid proceeded. Lowering the magnesium concentration produced no observable changes in the system under study.

PREPARATION OF DEFINED MEDIA

(a) Inocula media

Potassium dihydrogen phosphate and ammonium sulphate were prepared as a single solution, the pH of which was adjusted to 7.1 with 5N NaOH. 60 ml volumes were autoclaved in 500 ml conical flasks at 15 p.s.i. The carbon source and magnesium sulphate were prepared as a second solution. 40 ml volumes of these solutions were autoclaved in medicine flats at 5 p.s.i. The complete growth medium was constituted by adding the 40 ml carbon and magnesium solution to the phosphate and ammonium sulphate solution, using aseptic transfer. The medium was completed by the addition of 1.25 ml of sterile ferrous sulphate solution. Final concentration of all media components was as

previously stated for defined media.

(b) Growth media

The various components of the defined media were prepared as separate solutions. The complete growth medium was constituted immediately before inoculation.

Potassium dihydrogen phosphate solution was prepared at a concentration of 66.7mM and adjusted to pH 7.1 with 5N NaOH. 480 ml volumes of this solution were measured into one litre side arm flasks and the volume made up to 760 ml with glass distilled water. The flasks were then sterilised by autoclaving at 15 p.s.i.

Solutions of the following mineral salts were prepared:

800 mM $(NH_4)_2 SO_4$

160 mM $MgSO_4 \cdot 7H_2O$

800 μM $FeSO_4 \cdot 7H_2O$, adjusted to pH 2.0 with N HCl

The mineral salt solutions were sterilised by autoclaving at 15 p.s.i.

Carbon compounds required as growth substrates were prepared as solutions at 400 times the concentration required in the final growth medium, the pH being adjusted to 7.0, if necessary, with either NaOH or HCl. Sterilisation was achieved by autoclaving at 5 p.s.i. or by millipore filtration if the heat stability of the compound was in doubt.

All mineral salt solutions were stored at room temperature, as were solutions of carbon compounds, unless storage over a long period was contemplated, when sterile solutions were stored at $4^{\circ}C$.

STERILIZATION

(a) Autoclaving

Solutions were autoclaved in a Manlove-Alliot autoclave supplied with steam from a Speedlec-electrode boiler. Times for autoclaving various volumes at 5 p.s.i. or 15 p.s.i. to achieve sterilization were worked out by a previous worker (Fewson, unpublished results). The efficacy of autoclaving was routinely checked using Browne sterilizer control tubes (type one - black spot).

(b) Filtration

Filtration was performed using Sterifil filter holders, capacity 250 ml, fitted with membrane filters of pore size 0.22 μ. All components were obtained from Millipore.

The filter unit was sterilized by autoclaving at 15 p.s.i. After the filtration, the sterilized solutions were aseptically transferred to 5 oz round bottles, also sterilized by autoclaving at 15 p.s.i.

GROWTH

A batch culture technique was used for growing the organism. 800 ml volumes of sterile defined medium at 37°C, in one litre side arm flasks, were inoculated with 10 ml of a third passage culture, grown in the same defined medium. The cultures were maintained at 37°C in the apparatus described by Harvey, Fewson and Holms (1968). The culture was stirred by a rotating bar magnet within the flask, magnetically linked to a horse shoe magnet under the flask. The speed of rotation was such that a breaking vortex was achieved in the flask, creating a large number of small bubbles, as can be seen in the

PHOTOGRAPH 1

Individual components of a growth flask

(A) Stainless steel cap for side-port of flask

(B) Bar magnet

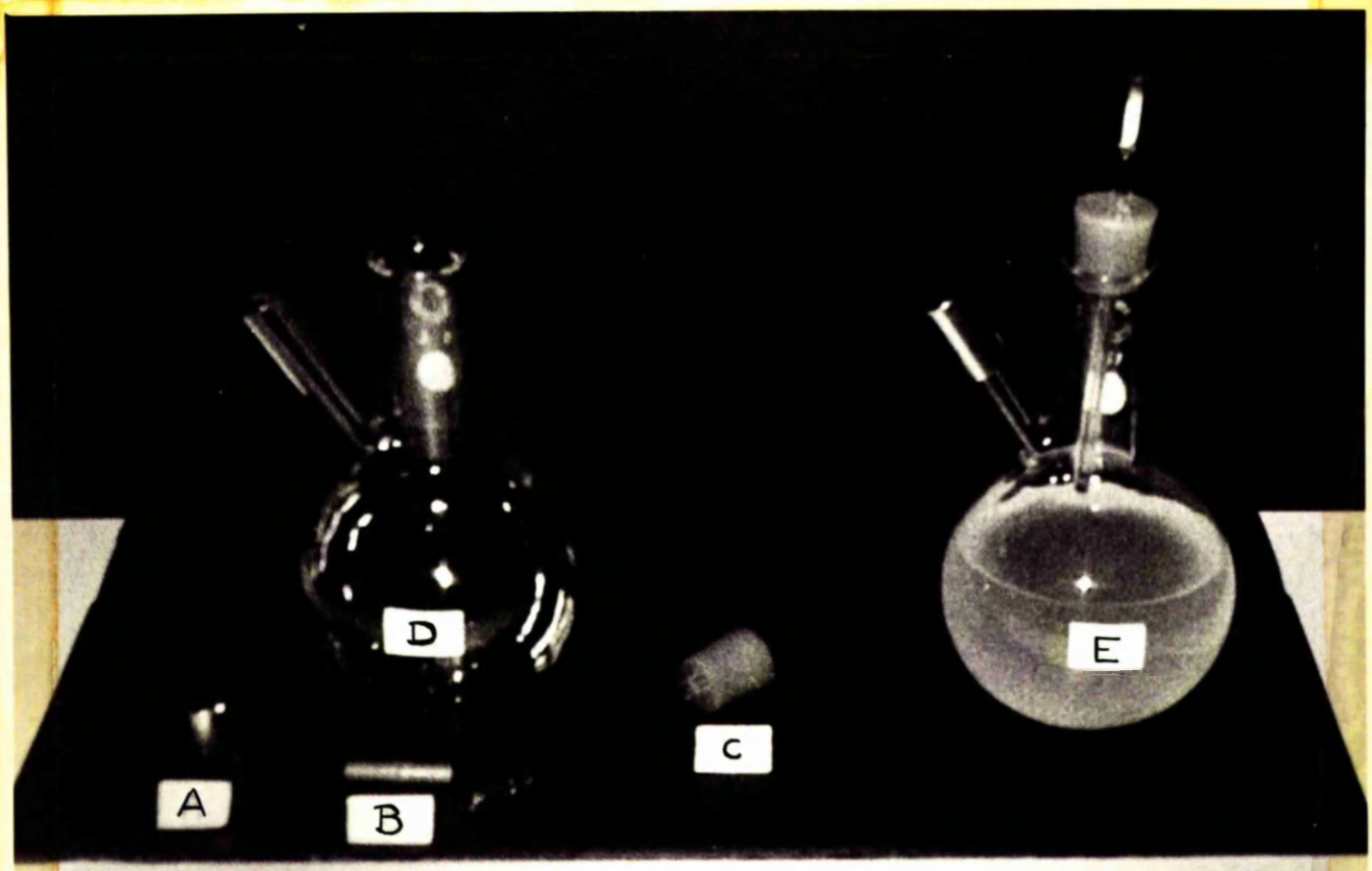
(C) Silicone bung with fitted aerator tube

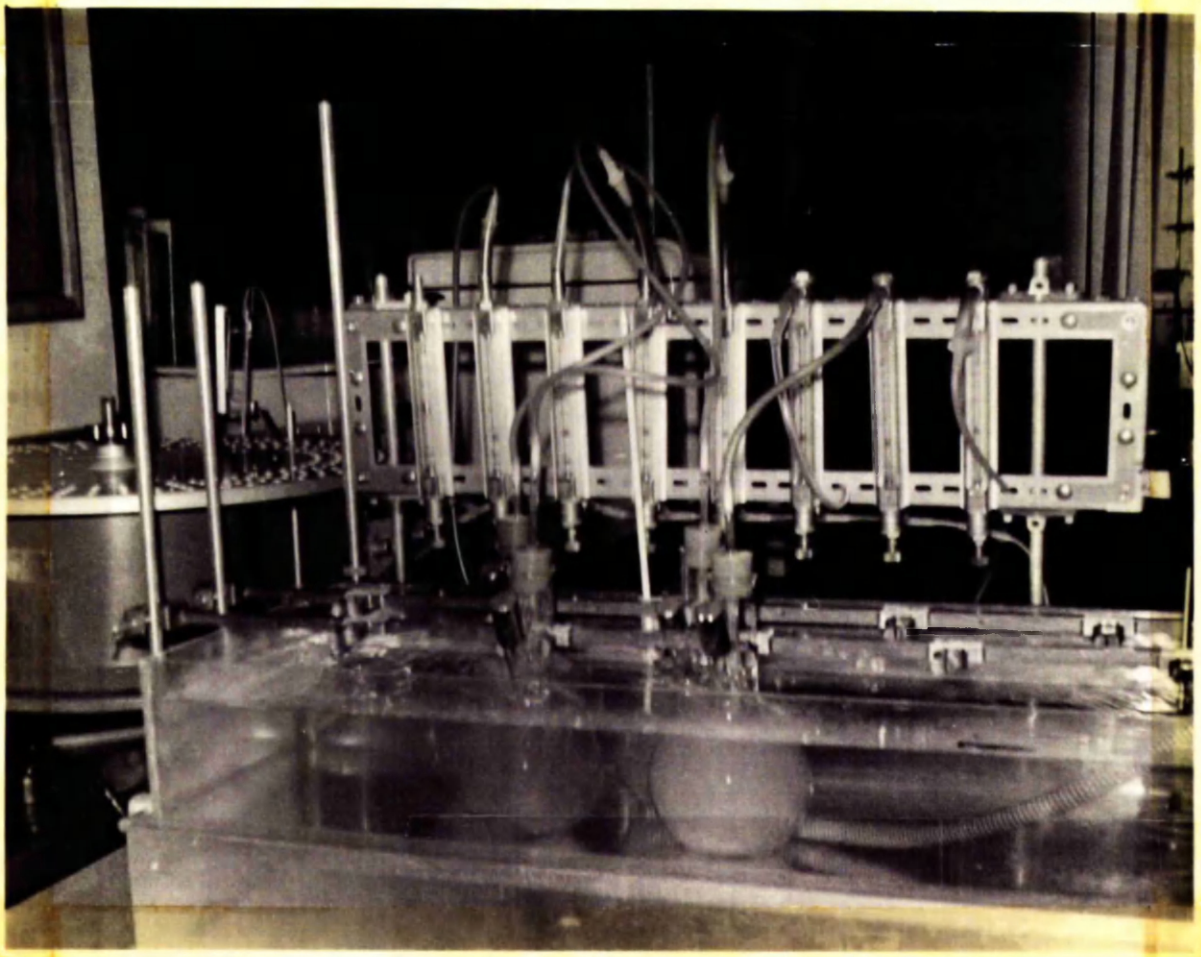
(D) 1 litre side-arm flask

(E) Assembled system containing 800ml of culture

PHOTOGRAPH 2

Growth apparatus in use.


A
B
C
D
E



accompanying photograph. Compressed air was passed through a charcoal filter and then into the flasks at a flow rate of 100 ml per min. The gas flow rate was monitored using gas flow gauges supplied by G. A. Platon Ltd. These procedures ensured that truly aerobic conditions existed within the flask (Leggat, 1967).

The components and assembled system can be seen in the accompanying photographs.

MEASUREMENT OF GROWTH

Growth was followed turbidimetrically by periodically measuring the optical density of the culture at a wavelength of 420 nm. Turbidity measurements were made using a double beam spectrophotometer (Unicam SP800) fitted to an external recorder, a desk type servoscribe potentiometric recorder (Smiths Industries Ltd.). Optical density response was linear with respect to culture turbidity up to a value of 0.3. Thereafter any increase in culture turbidity gave rise to a turbidity reading lower than the true value. Using dilutions, a correction curve of true turbidity against measured turbidity was constructed, and periodically checked. All turbidity measurements above an optical density of 0.3 were corrected using the correction curve.

SAMPLING FOR MEASUREMENT OF CULTURE TURBIDITY

Samples for turbidity measurements were obtained via the side-port of the flask and 4 ml samples of the growing culture were pipetted on to one drop of 40% formaldehyde solution to stop growth. The culture turbidity was then read in 1 cm light path Spectrosil quartz cuvettes against air as reference. The actual culture turbidity was obtained by subtracting a blank value of the reading given by a 4 ml sample of

uninoculated medium sampled on to one drop of 40% formaldehyde solution. All readings from the same flask were made in the same quartz cuvette. The corrected turbidity readings were plotted on semi-logarithmic graph paper against time.

ENZYME ASSAYS

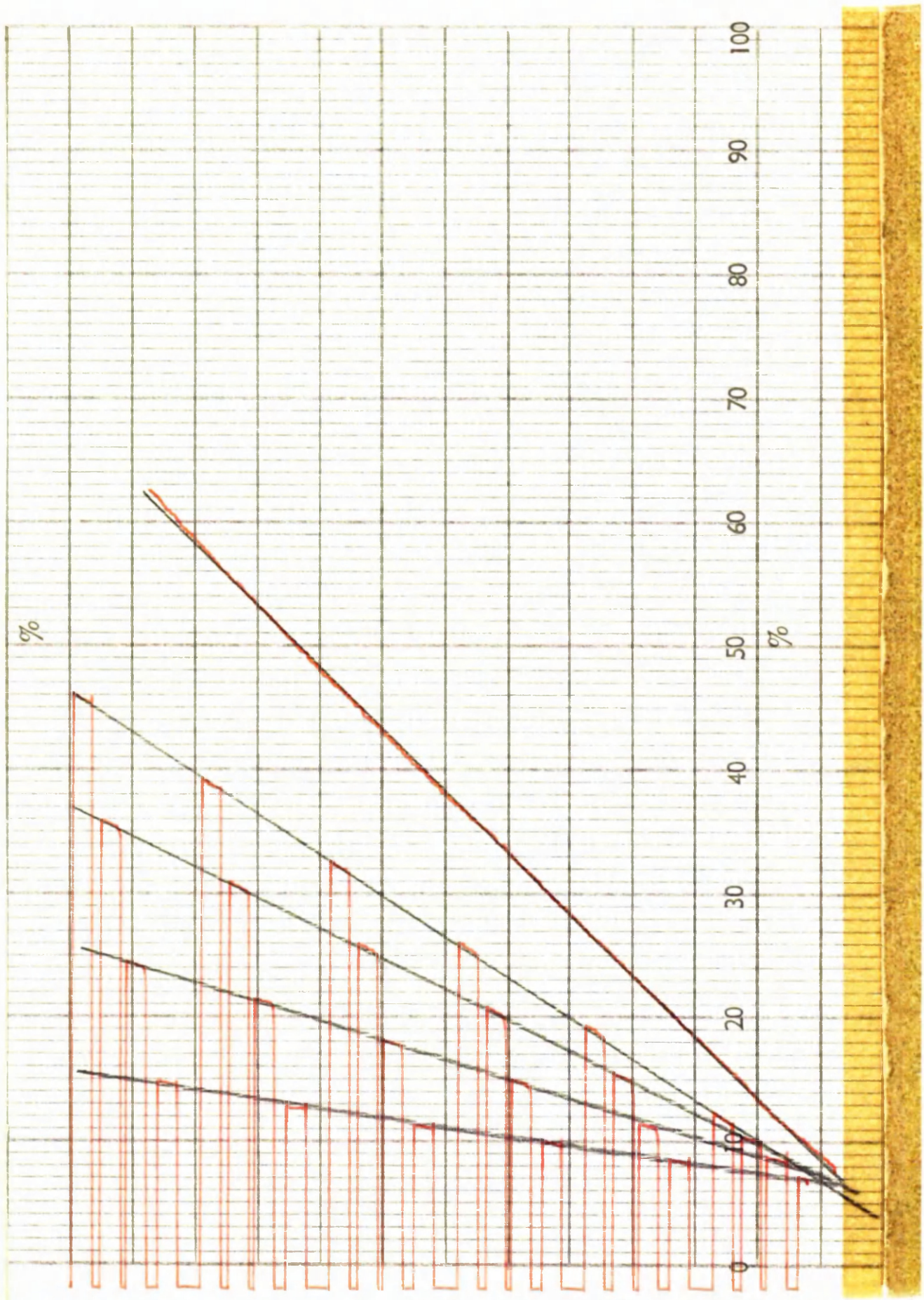
All enzyme assays were measured kinetically in a double beam spectrophotometer (Unicam SP800). All assays were performed in 1 cm light path Spectrosil quartz cuvettes, under strictly controlled temperature conditions. The internal cuvette holder of the SP800 was maintained at 27°C by passing water from an external supply, the temperature of which was maintained by a Circotherm IIa Constant Temperature Unit (Shandon Scientific Co.Ltd.)

A second cuvette holder, also maintained at 27°C, was used outside the SP800 to hold the cuvettes while assay reagents were being pipetted into them. The cuvettes were transferred to the internal cuvette holder prior to the addition of the reagent required to initiate the reaction. Mixing after this addition was achieved by use of a small plastic paddle. The course of the reaction was recorded using an external servoscribe recorder (Smiths Industries Ltd.), attached to the SP800. An example of the traces obtained is shown in the accompanying photograph. The paper travels through the recorder at a constant speed, which is known. The vertical divisions on the recorder chart paper are at intervals of 1 cm. For enzyme assays the chart paper travelled through the recorder at 3 cm per min.

When the enzyme being assayed was measured by the oxidation or

PHOTOGRAPH 3

Servoscribe recorder traces showing examples of four cuvettes read simultaneously and one cuvette read continuously.


%
100
90
80
70
60
50
%
40
30
20
10
0

reduction of a pyridine nucleotide, the extinction co-efficient was taken as 6.22×10^6.

ENZYME ACTIVITIES

One unit of enzyme is defined as that amount of enzyme which catalyses the conversion of 1 nmole of substrate per min. at 27°C. In practice, for the pyridine nucleotide linked reactions, this means the reduction or oxidation of 1 nmole of pyridine nucleotide per min. For the isocitrate lyase reaction it means the formation of 1 nmole of glyoxylic acid phenylhydrazone per min.

ISOCITRATE DEHYDROGENASE

The enzyme was assayed as follows:-

1.8 ml isocitrate substrate, pH 7.5
0.1 ml 6mM $MnCl_2$
1.0 ml cell extract containing enzyme

Components were mixed by inversion over parafilm.
0.1 ml 24mM $NADP^+$ was added to start the reaction.

It is important that the reagents be added to cuvettes in the order shown. When cell extract was added to isocitrate substrate before the $MnCl_2$ solution and the reaction initiated by $NADP^+$ addition, a maximum rate of reaction was not achieved.

Isocitrate substrate contains:

1.67mM D,L - isocitric acid, trisodium salt,
in 54mM tris buffer, adjusted to pH 7.5 with N HCl.

The reaction was followed by measuring the reduction of $NADP^+$ at 340nm.

Isocitrate substrate and manganese chloride solutions were preincubated at 27°C ~~for~~ and the $NADP^+$ solution was kept on ice. The cell extract was preincubated at 27°C for 2 to 3 min. prior to assay.

2-OXOGLUTARATE DEHYDROGENASE

The enzyme was assayed using a modification of the assay used by Mukherjee, Matthews, Horney and Reed (1965) to assay the purified enzyme.

1.0 ml 0.5M tris buffer adjusted to pH 7.5 with N HCl

0.1 ml 100mM L-Cysteine HCl, pH 7.0

0.1 ml 10mM coenzymeA

0.1 ml 10mM thiamine pyrophosphate

0.5 ml 30mM KCN, adjusted to pH 7.5 with N HCl

Components were mixed

0.1 ml cell extract containing enzyme

Components were mixed by inversion over parafilm and the reaction was initiated by the addition of,

0.1 ml 35mM NAD^+

0.1 ml 200mM 2-oxoglutaric acid, pH 7.5.

The reaction was followed by measuring the reduction of NAD^+ at 340nm. The tris buffer and potassium cyanide solutions were preincubated at 27°C, while enzyme substrate and all co-factors were stored on ice. The cell extract was preincubated at 27°C for 2 min. prior to assay. The reaction was read against a blank reaction with water substituted for coenzyme A.

Initiation of the reaction with NAD^+ plus 2-oxoglutarate produces a faster rate of reaction than initiating the reaction with addition of cell extract.

MALATE DEHYDROGENASE

The enzyme was assayed using a modification of the procedure used by Mehler, Kornberg, Grisolia and Ochoa (1948).

1.5 ml 50mM glycylglycine, pH9.0

0.1 ml 4.5mM NADH

1.1 ml water

0.2 ml cell extract containing enzyme

Components were mixed by inversion over parafilm and the reaction started by the addition of

0.1ml 6.0mM oxaloacetate acid.

The reaction was followed by measuring the oxidation of NADH at 340nm.

Glycylglycine buffer and water were preincubated at 27°C and enzyme substrate and co-factor were kept on ice. The cell extract was also kept on ice till required.

ISOCITRATE LYASE

The enzyme was assayed using a modification of the procedure described by Dixon and Kornberg (1959).

1.0 ml 0.2M KH_2PO_4, pH6.8

0.1 ml 150mM $MgSO_4$ $7H_2O$

0.1 ml 100 mM Phenylhydrazine HCl

0.1 ml 60mM L-cysteine HCl

0.6 ml water

1.0 ml cell extract containing enzyme.

Components were mixed by inversion overparafilmand the reaction started by the addition of

0.1 ml 200mM isocitric acid lactone (allo free)

The reaction was followed by measuring at 324nm the production of the phenylhydrazone derivative of the glyoxylic acid formed in the cleavage of isocitric acid. The extinction co-efficient for glyoxylic acid phenylhydrazone was taken as 1.7×10^4 (Dixon and Kornberg, 1959).

The phosphate buffer, water and magnesium sulphate solution were preincubated at 27°C while all other reagents were kept on ice. The cell extract was preincubated for 2-3 min. at 27°C prior to assay.

EXTRACTION OF ENZYMES

The tricarboxylic acid cycle enzymes

When isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and malate dehydrogenase activities were being measured in the same sample the enzymes were extracted as follows. A sample of culture was harvested by centrifugation at 10,000g for 15 min. at 4°C. The cell pellet was resuspended in ice cold glass distilled water to an estimated turbidity of 8.0, equivalent to a bacterial protein concentration of 1.0 mg per ml. The actual turbidity of resuspension was measured at 420nm.

0.75 ml of the water resuspension was diluted with a further 0.75 ml water and 1.5 ml ice cold 0.3M sodium chloride containing 10 mg per ml bovine plasma albumin. The 3.0 ml diluted resuspension was then disrupted by continuous sonication for 75 sec. at 2.6-2.8 amps. 0.5 ml of the sonicated extract was assayed for isocitrate dehydrogenase activity adjusting the volume to 1.0 ml with 0.15M sodium chloride solution containing 5 mg per ml bovine plasma albumin.

1.5 ml of the water resuspension was diluted to 3.0 ml with ice cold 0.1M tris(hydroxymethyl)aminomethane, pH7.5.

The 3.0 ml of cell suspension were disrupted using continuous sonication for 60 sec. at 1.6-1.8 amps. 1.0 ml of the sonicated extract was assayed for 2-oxoglutarate dehydrogenase activity. A portion of the remainder of the sonicated extract was diluted 1 in 20 or 1 in 40 with chilled 0.05M tris buffer, pH7.5 containing 5 mg per ml bovine plasma albumin. 0.2 ml of the tris buffer diluted extract was assayed for malate dehydrogenase activity.

Isocitrate dehydrogenase alone

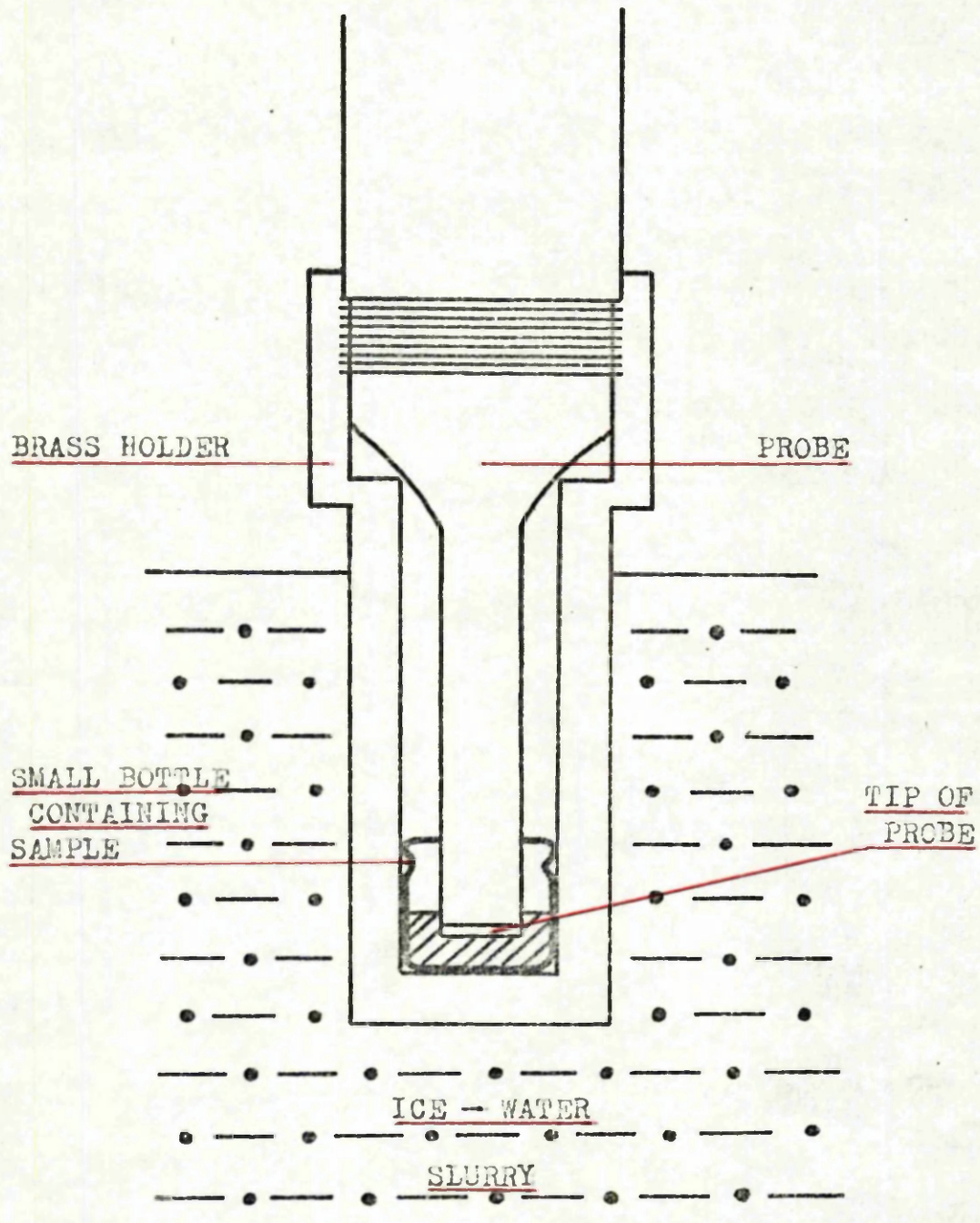
When isocitrate dehydrogenase was the only tricarboxylic acid enzyme assayed, the enzyme was extracted as follows. 2.0 ml of the growing culture were pipetted on to 2.0 ml ice cold 0.3M sodium chloride solution containing 10 mg per ml bovine plasma albumin and mixed. 3.0 ml of the diluted culture were sonicated as described previously for release of isocitrate dehydrogenase activity, and 1.0 ml of the sonicated extract was assayed for enzyme activity, after prior incubation at 27°C.

Isocitrate lyase

The extraction procedure for isocitrate lyase was identical to that given above for the extraction of isocitrate dehydrogenase by itself. 1.0 ml of the sonicated extract was assayed after prior incubation at 27°C.

PREPARATION OF WASHED CELL EXTRACTS

The preparation of cell extracts for inhibition and heat treatment studies of isocitrate dehydrogenase was as follows. Cells, grown under the required conditions, were harvested by centrifugation at 10,000 *g*



DIAGRAMMATIC REPRESENTATION OF APPARATUS USED FOR SONICATION

for 10 min. at 4°C. The resultant cells were resuspended in ice-cold 0.15M sodium chloride solution and recentrifuged. The washed cells were resuspended to the required cell density in 0.15M sodium chloride containing 5 mg/ml bovine plasma albumin. 3 ml volumes of this resuspension were sonicated and combined until sufficient volume for the proposed studies was obtained.

A minor variation of this procedure was adopted for the heat studies performed on isocitrate dehydrogenase and this is noted when it arises.

BOVINE PLASMA ALBUMIN IN CELL EXTRACTS

Bovine plasma albumin was added to cell suspensions to stabilise enzymes after release from cells for assay. This could also be achieved if cells were resuspended to a density which was sufficiently high to provide bacterial protein for stabilisation, i.e. resuspension to a turbidity of 40 prior to sonication.

SONICATION

Disruption by ultrasonics was achieved using a soniprobe, type 1130A (Dawe Instruments Ltd.). The sample was contained in a small bottle which was placed in a brass holder. The brass holder was screwed on to the probe and this procedure automatically reproduced the distance the tip of the probe protruded into the suspension. The whole assembly was supported in an ice-water slurry during the sonication proper and the brass holder served as a very efficient heat trap, throughout the times of sonication used. A diagrammatic representation of this apparatus can be seen in figure opposite.

CENTRIFUGATION

Procedure for harvesting cultures

(a) Small volumes of culture were harvested by centrifugation using an MSE13 or an MSE18 refrigerated centrifuge, maintained at 4°C, using an 8 x 50 ml rotor, spun at 10,000 r.p.m. for 15 min. (12,000g).

(b) Large volumes of culture were harvested by centrifugation using an MSE18 refrigerated centrifuge, maintained at 4°C, with a 6 x 250 ml rotor, spun at 9,000 r.p.m. for 15 min. (10,000g).

High Speed Centrifugation

A Beckman Spinco Model L2, maintained at 4°C was used for high speed centrifugation. Antimonium 50, fixed angle rotor, spun at 50,000 r.p.m. for 60 min. was used (144,000g).

MEASUREMENT OF CARBON DIOXIDE

Carbon dioxide was measured using an MSA infrared analyser, Lira Model 300 (Mine Safety Appliances Co. Ltd.), by the method described by Hamilton and Holms(1970).

MEASUREMENT OF ACETATE

Acetate was assayed according to the method of the Boehringer Co. (London) Ltd.

The assay contained the following components:

1.0 ml acetate sample

1.5 ml 0.125M triethanolamine, pH7.4

0.1 ml ATP, sodium salt, 100 mg per ml.

0.1 ml phosphoenolpyruvate, 15 mg per ml

0.1 ml NADH$_2$ sodium salt, 5 mg per ml

0.1 ml 0.2M $MgCl_2$

20 µl pyruvate kinase, 2 mg per ml

20 µl lactate dehydrogenase, 5 mg per ml

20 µl myokinase, 2 mg per ml

40 µl acetate kinase, 1 mg per ml

The reaction was started by the addition of acetate kinase.

All the enzymes were obtained from the Boehringer Corporation (London) Ltd. in the form of ammonium sulphate suspensions, which were used directly.

A standard curve covering the range 0-400 nmoles acetate per assay was constructed each time acetate assays were performed. An example of such a standard curve can be seen in figure 4. The amount of acetate measured is **directly** proportional to the degree of oxidation of NADH occurring over 90 min. at 27°C. The reaction was carried out in 3" x $\frac{3}{8}$" test tubes and read in 1 cm light path Spectrosil quartz cuvettes at 340 nm in a Unicam SP800 spectrophotometer.

MEASUREMENT OF GLUCOSE

Glucose was assayed in the following manner, according to the method of Bergmeyer (1963). The assay contained the following components;

1.0 ml glucose sample

1.0 ml 0.1M tris buffer, pH8.0 (adjusted with N HCl)

0.4 ml 50mM $MgCl_2 . 6H_2O$

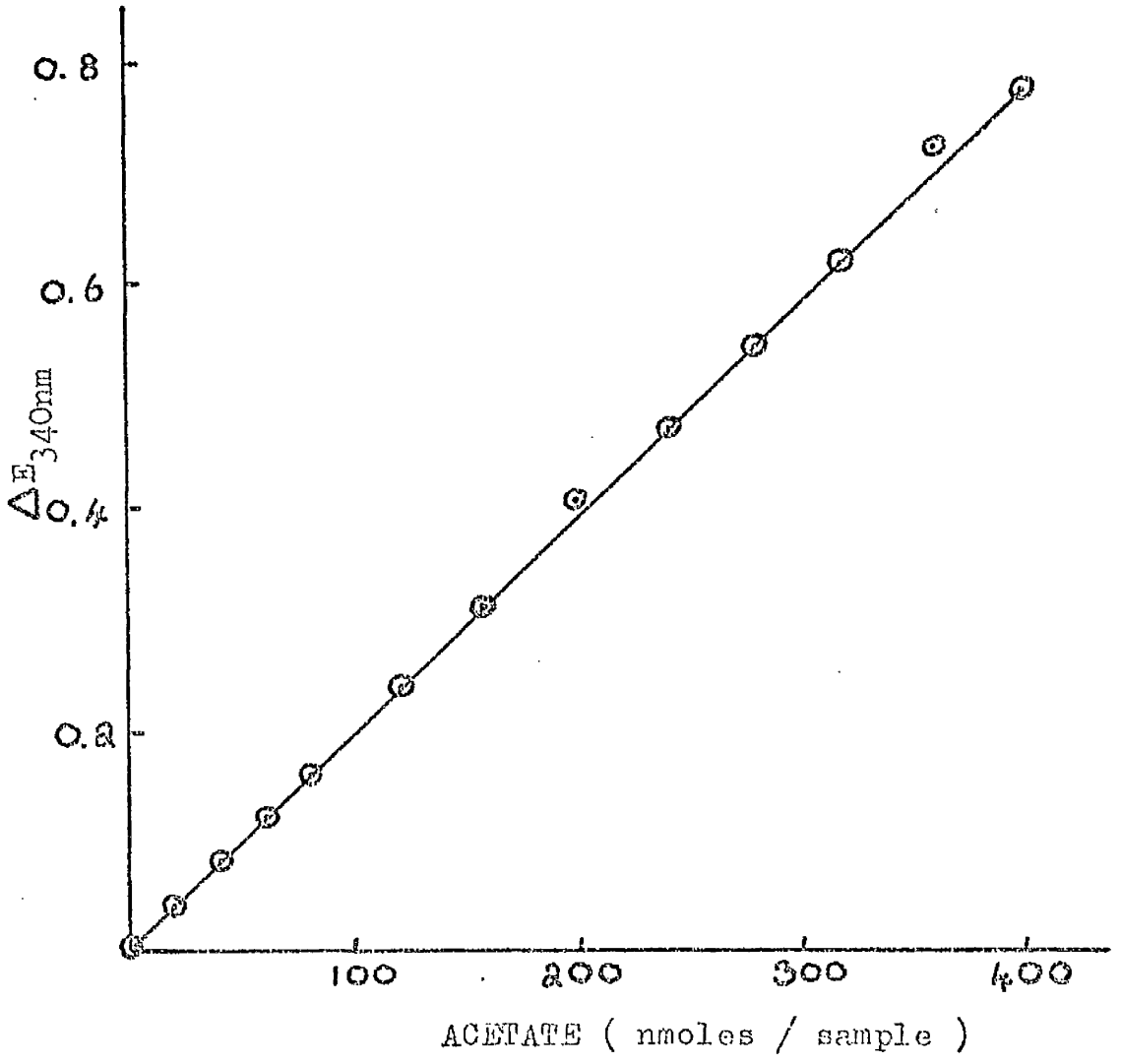
0.1 ml bovine plasma albumin solution, 4 mg per ml

0.1 ml 10mM ATP, sodium salt

FIGURE 4

Standard acetate calibration curve.

FIGURE 4


0.8
0.6
0.4
0.2
ΔE_{340nm}
100
200
300
400
ACETATE (nmoles / sample)

These components were mixed by inversion over parafilm.

0.1 ml hexokinase

0.1 ml glucose-6-phosphate dehydrogenase

0.2 ml 5mM $NADP^+$

were added to start the reaction and components again mixed by inversion over parafilm. Hexokinase (Sigma, Type C-130, 170 units per mg) was diluted 1 in 100 with a solution of bovine plasma albumin, 100 ug per ml, before use.

Glucose-6-phosphate dehydrogenase (Sigma, Type X, 300-330 units per mg - now unobtainable) was diluted 1 in 25 with glass distilled water before use.

Standard curves of glucose concentration, covering the range 0-400 nmoles glucose per assay, were constructed each time a series of assays was performed. An example of such a standard curve can be seen in figure 5. The amount of glucose measured is directly proportional to the degree of reduction of $NADP^+$, occurring over 30 min. at 27°C.

The reaction was carried out in 3" x ½" test tubes and read in 1 cm light path Spectrosil quartz curvettes at 340nm, in a Unicam SP800 spectrophotometer.

PREPARATION OF SAMPLES FOR GLUCOSE, GLYCEROL OR ACETATE ESTIMATIONS

(a) Culture sampling

Reagent :- tris - magnesium buffer, pH8.5 prepared as follows:-

0.1M tris

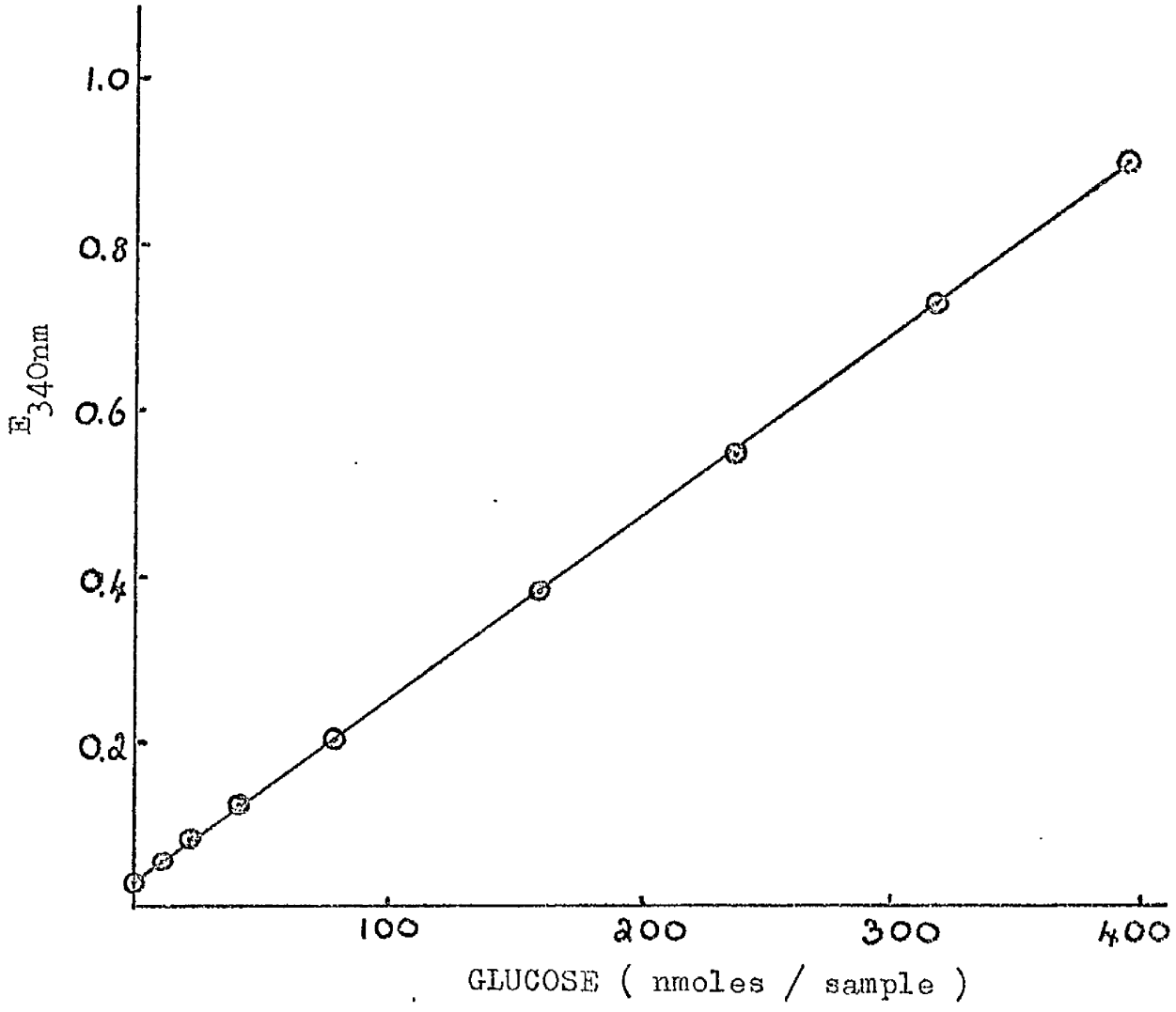
0.1M $MgCl_2 . 6H_2O$

brought to pH8.5 with HCl.

FIGURE 5

Standard glucose calibration curve

FIGURE 5



2.0 ml volumes of tris-magnesium buffer were dispensed into 6" x $\frac{5}{8}$" test tubes and frozen. 4 ml of the growing culture was pipetted on to a frozen pellet and the test tube agitated, using a Vortex Jr. Mixer, until the frozen pellet had completely melted. Whole cells and the precipitate of magnesium phosphate, which resulted when the pellet melted, were removed by centrifugation. The supernatant fluid was decanted, frozen and stored at -10°C, till required.

(b) Preparation of samples for assay

When samples were required for assay the frozen samples were thawed and any precipitate which had formed on freezing and thawing was removed by centrifugation. The samples were then assayed for the required component. The volume of sample assayed was varied (10-1000 µl), depending on the concentration of the component in the sample.

In the glucose and acetate assays, sample volumes less than 1.0 ml w made up to this volume using the following solution.

40 ml basal salts medium (growth medium lacking a carbon source) were mixed with 20 ml tris-magnesium buffer. The combined solution was chilled on ice and the resultant precipitate removed by filtration. The clear solution obtained was used to dilute glucose and acetate samples prior to assay.

Samples assayed for glycerol were adjusted to 1.0 ml using glass distilled water.

Samples for glucose, glycerol or acetate assays were dispensed into 3" x $\frac{5}{8}$" test tubes using eppendorf pipettes with disposable tips.

The required volume of dilution medium was then added, followed by the other reaction mixture components.

MEASUREMENT OF PROTEIN

Protein was measured according to the method of Lowry, Rosebrough, Farr and Randall (1951). The bacterial cell protein was solubilized for assay according to the procedure of Fowson (unpublished results). A sample of culture was centrifuged at 10,000 *g* for 15 min. at 4°C. The pellet was resuspended to approximately 1 mg wet wt per ml in 0.67 N NaOH and was digested overnight at 30°C. Samples of standard protein, bovine plasma albumin, were also digested overnight in 0.67 N NaOH, at 30°C. The digested protein samples were then assayed as indicated.

CHEMICALS

With the exception of the ones listed below, chemicals were of AnalaR quality from British Drug Houses Ltd., Poole, Dorset.

Chemicals obtained from Sigma London Chemical Co.Ltd., London were:

S-acetyl coenzymeA, sodium salt, grade II

Adenosine - 5 - monophosphoric acid, sodium salt (AMP)

Adenosine - 5 - diphosphate, sodium salt (ADP)

Adenosine - 5 - triphosphate, disodium salt (ATP)

Adenosine 3', 5' cyclic monophosphoric acid (cyclic 3', 5' AMP)

Cocarboxylase (thiamine pyrophosphate chloride)

Coenzymed (from yeast), grade I

β-diphosphopyridine nucleotide (NAD^+)

β-diphosphopyridine nucleotide, reduced form (NADH)

D(+) Galactose

Glycylglycine (free base)

DL-Isocitrate acid, trisodium salt, type I

cis-Oxaloacetic acid (oxaloacetic acid), grade I

Trizma Base, reagent grade [Tris (hydroxymethyl) aminomethane]

Chemicals obtained from The Boehringer Corporation (London) Ltd., Ealing, London:

nicotinamid-adenin-dinucleotidphosphat, di-natrium salz ($NADP^+$)

phosphoenol-pyruvat, mono-kalium salz, (phosphoenol-pyruvate)

triethanolamin-hydrochlorid (triethanolamine hydrochloride)

Chemicals obtained from L. Light & Co. Ltd., Poyle, Colnbrook, Bucks were:

cis - Aconitic acid anhydride

Sodium pyruvate

Chemicals obtained from Fluka AG, Chemische Fabrik, Buchs SG were:

Aepfelsaure (L-malic acid)

2-oxo-glutarsaure (2-oxoglutaric acid)

Chemicals obtained from T. J. Sax & Son Ltd., Holborn, London, were:

All amino acids used,

(L configuration)

Sources of the following chemicals were:

Bovine Albumin Powder (fraction V from bovine plasma)
Armour Pharmaceutical Co.Ltd., Eastbourne.

Chloromycetin (Chloramphenicol)
Parke, Davis & Co., Hounslow, London.

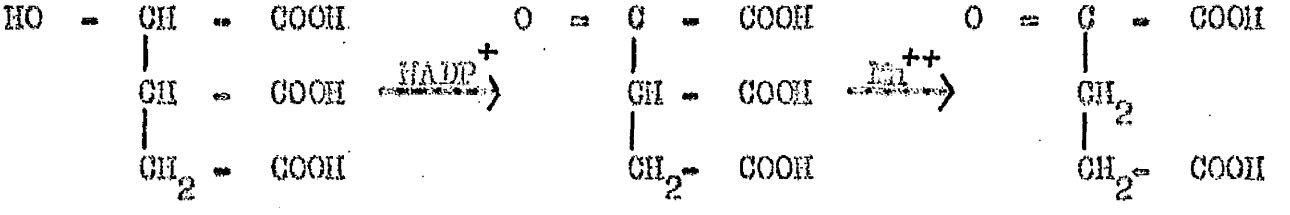
Potassium cyanide
Hopkin & Williams Ltd., Chadwell Heath, Essex.

Puromycin dihydrochloride
Nutritional Biochemicals Co., Cleveland, Ohio.

DEVELOPMENT AND EVALUATION OF ENZYME ASSAYS

ISOCITRATE DEHYDROGENASE ASSAY.

L_D- isocitrate:$NADP^+$ oxidoreductase (decarboxylating) or as designated from now on, isocitrate dehydrogenase, catalyses the conversion of L_D-isocitric acid to 2 - oxoglutaric acid. The reaction involves a dehydrogenation and a decarboxylation. The dehydrogenation is specific for $NADP^+$, NAD^+ being completely inactive. The decarboxylation is a β - keto decarboxylation and requires a metal ion, specifically manganese, although magnesium will substitute but is much less effective. The reaction is thought to proceed via the formation of oxalosuccinic acid, but attempts to isolate this compound during the reaction have been unsuccessful. It is therefore thought that if this compound is formed, it remains firmly bound to the enzyme. The reaction may be written,



L_D-isocitric acid — oxalosuccinic acid — 2-oxoglutaric acid

REPRODUCIBILITY OF ASSAY.

Isocitrate dehydrogenase activity remains directly proportional to enzyme protein up to 110 units of enzyme per ml sample assayed i.e. producing an optical density change of 0.25 per minute at 340 nm (Figure 6).

Release of enzyme activity was directly proportional to the turbidity of the cell suspension sonicated, up to a turbidity of

FIGURE 6

Relation of isocitrate dehydrogenase activity to enzyme protein.

FIGURE 6

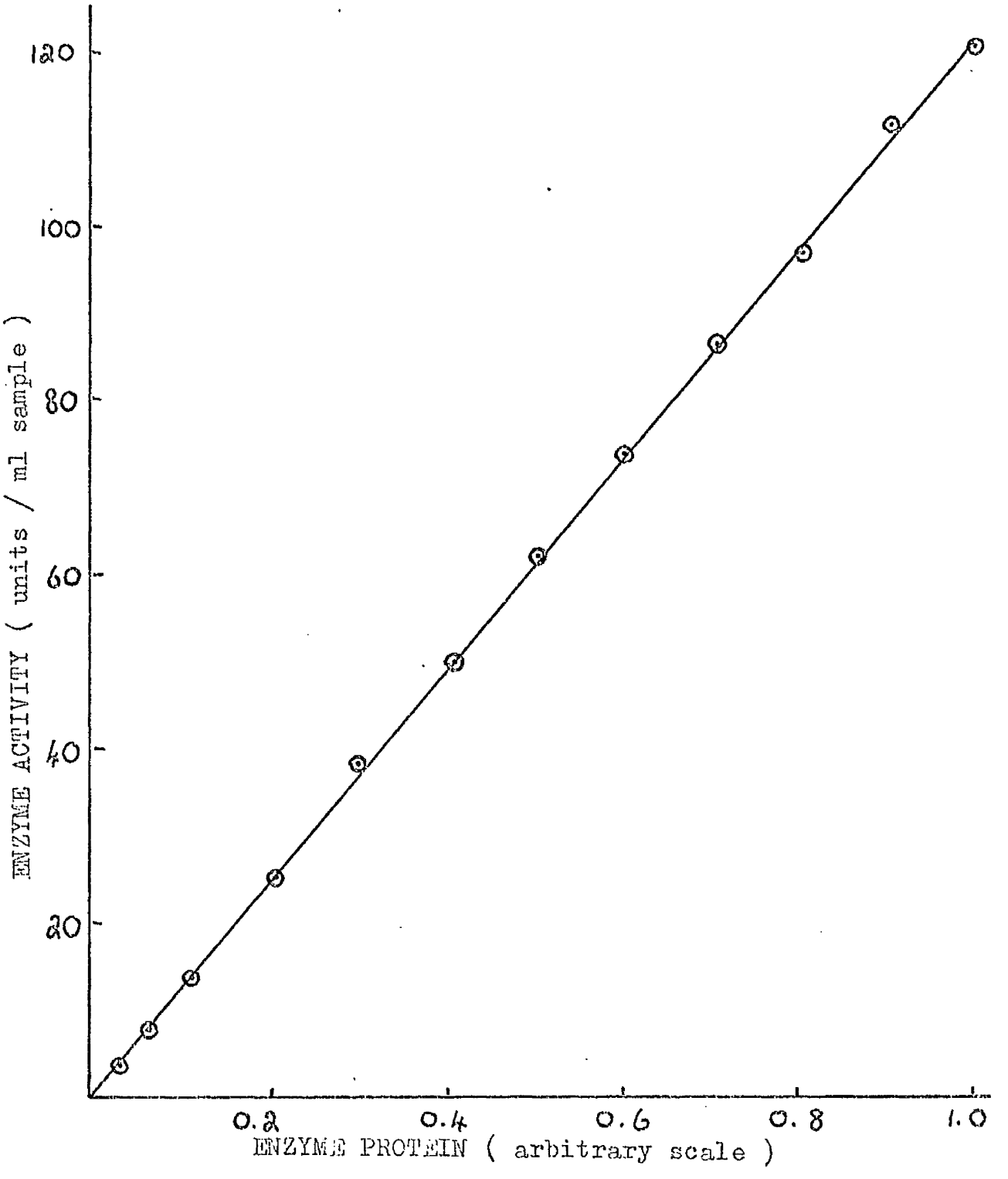
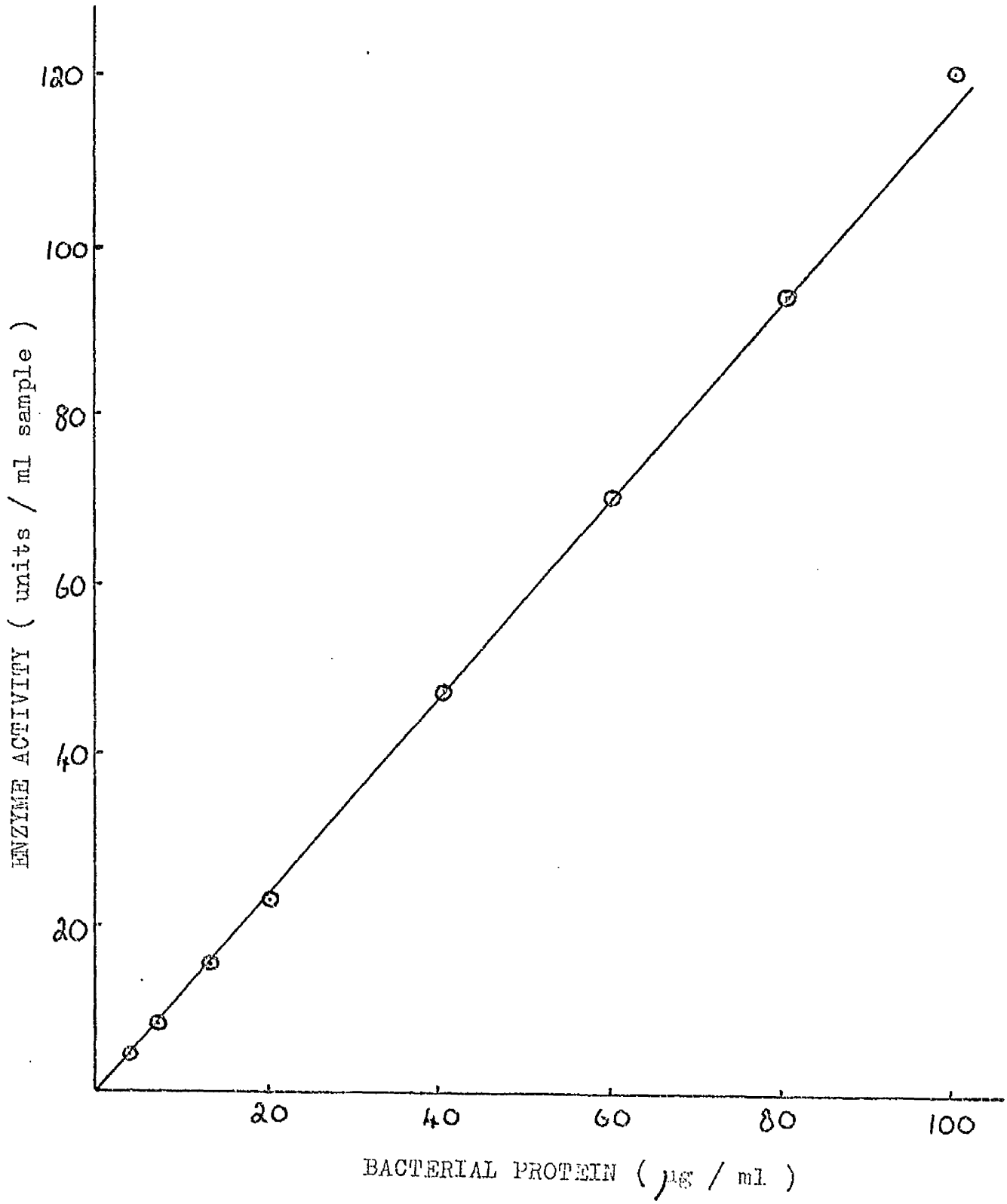

120
100
80
60
40
20
ENZYME ACTIVITY (units / ml sample)
0.2
0.4
0.6
0.8
1.0
ENZYME PROTEIN (arbitrary scale)

FIGURE 7.

Relation of isocitric dehydrogenase release to density of cell suspension at sonication.

FIGURE 7


120
100
80
60
40
20
ENZYME ACTIVITY (units / ml sample)
20
40
60
80
100
BACTERIAL PROTEIN (µg / ml)

0.8 at 420 nm corresponding to a bacterial protein concentration of 100µg per ml. (Figure 7).

For these particular studies on the reproducibility of sonication and assay of isocitrate dehydrogenase, the cells were first harvested and then resuspended in ice cold 0.15M sodium chloride containing 5mg per ml Bovine Plasma Albumin , Fr5, (BPA - saline), prior to sonication, and all dilutions were performed using BPA - saline. The results show that the level of enzyme activity released from the cells is directly proportional to the number of cells present.

When a number of assays were performed on the same cell extract, all activities fell within a range of ± 0.5% of the mean.

When a number of batches from the same cell suspension were sonicated, all activities released fell within a range of ± 2.5% of the mean.

STORAGE OF SAMPLES

When all three tricarboxylic acid cycle enzyme activities were assayed in the same sample of cells, it was necessary to store some of the samples for up to 8 hours. Cells were stored on ice in the form of pellets, obtained after initial harvesting by centrifugation, as described in the methods section. Enzyme stability was checked throughout the 8 hour storage period, and isocitrate dehydrogenase activity was found to be stable over this time of storage.

ASSAY OF ISOCITRATE DEHYDROGENASE WITHOUT HARVESTING CELLS.

It very soon became apparent that it would be of enormous benefit to be able to measure isocitrate dehydrogenase activity in cell samples without first having to harvest the cells. The time saved in bypassing

this step would allow a greater freedom of manoeuvre when planning experiments.

It was already apparent that it was possible to measure isocitrate dehydrogenase activity in low turbidity cell suspensions (Figure 7), therefore it only remained to determine if the presence of culture medium exerted an effect, inhibitory or otherwise, on the release or assay of the enzyme.

Cells were grown on glucose, glycerol or casamino acids growth medium and the cultures harvested immediately after inoculation, during logarithmic phase growth and at the end of growth. The harvested cells were discarded and the remaining growth medium, freed of cells, was stored on ice. Cells, harvested after growth on glucose - salts, were resuspended in the different growth media at various stages of nutrient depletion, obtained as described above, as well as in 0.15M sodium chloride solution containing 5mg per ml BPA. The resuspensions were diluted 1 in 2 with BPA - saline and 3ml volumes sonicated. 1ml volumes of the sonicated suspensions were assayed for isocitrate dehydrogenase activity and the results obtained can be seen in Table 1. The concentration of carbon sources in the growth media used to obtain the various growth phase type conditions for subsequent resuspension media was higher than that used in most of the experiments subsequently reported in this thesis, glucose and glycerol being present at a concentration equivalent to 400 matoms of carbon per litre. Casamino acids were present at a concentration of 1.4g per 100ml.

As can be seen from the results, the presence of the growth medium, even with the carbon source present at a high concentration,

TABLE 1

Effect of growth medium on release and assay of lipoamide dehydrogenase.

RESUSPENSION MEDIUM	ISOCITRATE DEHYDROGENASE ACTIVITY (expressed as a percentage of that obtained using saline for resuspension)		
	Fresh Growth Medium	Log – phase Growth Medium	Stationary – phase Growth Medium
Glucose Salts	95.8	94.7	92.7
Glycerol Salts	97.2	98.7	98.7
Casamino Acids Salts	103.8	108.8	108.1
Saline	100		

TABLE 1

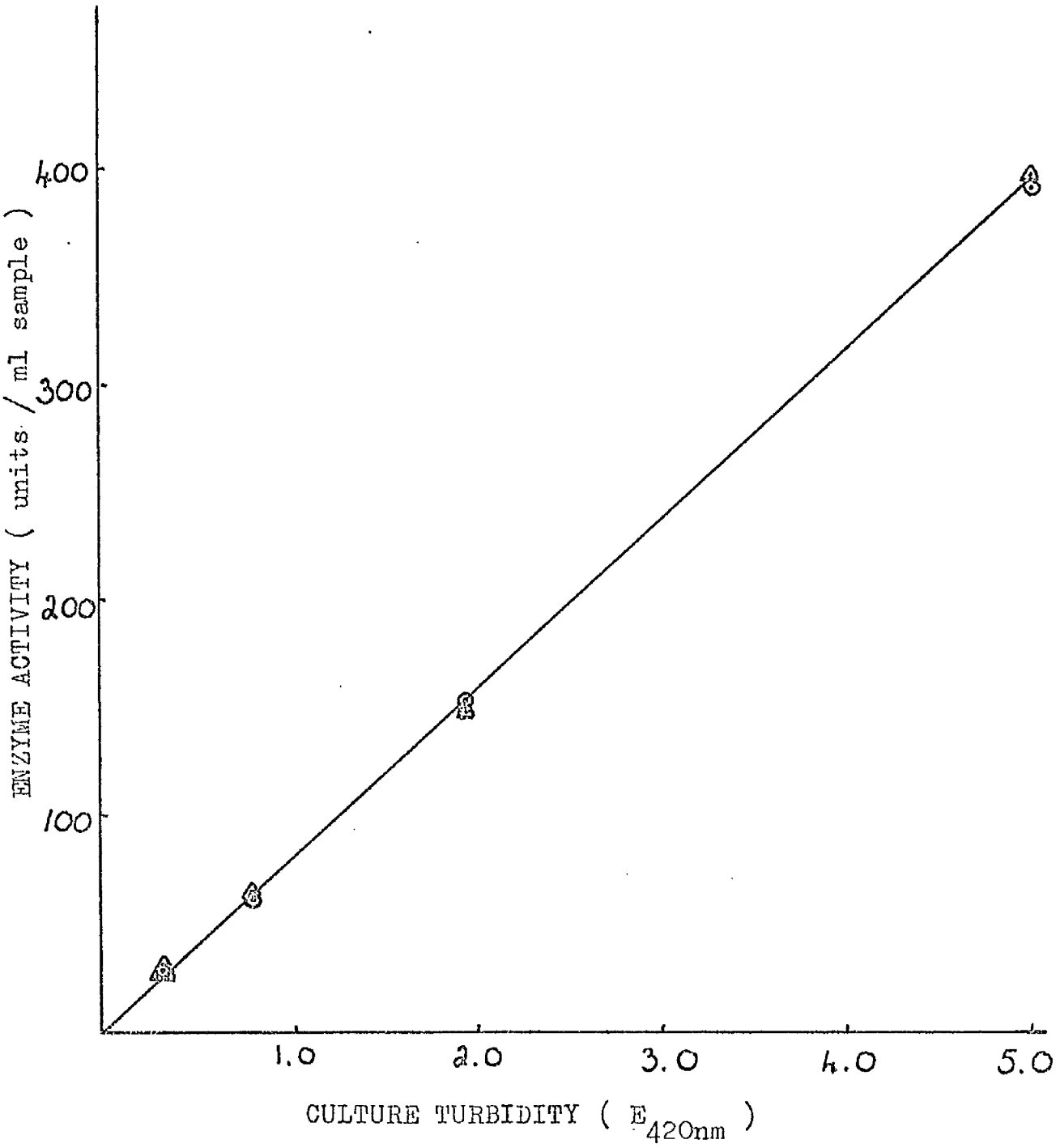
FIGURE 8

Effect of harvesting cells prior to sonication on release of isocitrate dehydrogenase activity from E.Coli grown on 10mM glucose.

⊙—— cells not harvested before sonication

△—— cells harvested and resuspended before sonication.

FIGURE 8


400
300
200
100
ENZYME ACTIVITY (units / ml sample)
1.0
2.0
3.0
4.0
5.0
CULTURE TURBIDITY (E_{420nm})

or when a large number of compounds are present i.e. casamino acids, has little detectable effect on enzyme activity. Therefore it was concluded that harvesting prior to sonication was not necessary when the enzyme being assayed was isocitrate dehydrogenase.

In order to check that this conclusion was valid, E.coli was grown on 10.0mM glucose and the culture was periodically sampled in order to measure isocitrate dehydrogenase activity and culture turbidity. Cells were prepared for release of isocitrate dehydrogenase activity by sonication in the following ways.

(a) No harvesting.

2ml of the growing culture was pipetted onto 2ml of chilled 0.3M sodium chloride solution containing 10mg/ml bovine plasma albumin, and 3ml of the resultant suspension was sonicated to release isocitrate dehydrogenase activity for assay. 1.0 ml of the sonicated extract was assayed after prior incubation at 27°C.

(b) Cells harvested.

At the same time as the 2ml sample was taken for immediate assay of isocitrate dehydrogenase activity, a sample of culture was harvested by centrifugation at 10,000g for 15 minutes at 4°C. The cell pellet was resuspended in 0.15M sodium chloride solution containing 5mg/ml bovine plasma albumin to a turbidity (E 420nm) of approximately 1.0. The turbidity of the resuspension was measured accurately. 3ml of the sodium chloride-bovine plasma albumin suspension was sonicated to release isocitrate dehydrogenase activity and 1.0ml of the sonicated extract was assayed for enzyme activity after prior incubation at 27°C. The enzyme activities calculated from both techniques were

plotted as enzyme activity (units/ml culture) against culture turbidity (E 420nm). The results obtained can be seen in Figure 8, which confirm the observation that harvesting the culture prior to sonication was not necessary when assaying for isocitrate dehydrogenase activity.

EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON THE ENZYME ACTIVITY OF ISOCITRATE DEHYDROGENASE.

E.coli was grown on both glucose (10mM) -, and glycerol (20mM) - minimal salts medium. During growth samples were taken, harvested, washed and suspended in 0.15M sodium chloride solution containing 5mg/ml bovine plasma albumin. The samples were sonicated to release isocitrate dehydrogenase activity. The effect of chloramphenicol and puromycin on this activity was then tested. The results can be seen in Table 2.

As can be seen, little or no effect is observed in the presence of chloramphenicol or puromycin over the concentration range 3×10^{-3} mM - 3×10^{-1}mM.

TWO ISOCITRATE DEHYDROGENASES?

It is known that two isocitrate dehydrogenases are present in mammalian cells, one specific for $NADP^+$ and the other specific for NAD^+, the latter also being allosterically activated by ADP (Chen and Plaut, 1962, 1963; Stein, Kirkman and Stein, 1967).

It has also been shown that two enzymes are present in yeast, the NAD^+ specific enzyme being associated with the mitochondria and activated by AMP rather than ADP, while the $NADP^+$ specific enzyme is found in the cytoplasm and is not affected by adenosine phosphates

TABLE 2

Effect of chloramphenicol and puromycin on isocitric dehydrogenase activity in cell extracts of E.coli.

FOR GLUCOSE GROWN CELLS 100% = 24 units enzyme/assay

FOR GLYCEROL GROWN CELLS 100% = 51 units enzyme/assay

MEDIUM ON WHICH CELLS GROWN	NO DRUG ADDED	DRUG ADDED TO ASSAY					
		CHLORAMPHENICOL			PUROMYCIN		
		3×10^{-3} (mM)	3×10^{-2} (mM)	3×10^{-1} (mM)	3×10^{-3} (mM)	3×10^{-2} (mM)	3×10^{-1} (mM)
	ENZYME ACTIVITY (%)	ENZYME ACTIVITY REMAINING (%)			ENZYME ACTIVITY REMAINING (%)		
GLUCOSE (10mM)	100	100	100	99	100	104	104
GLYCEROL (20mM)	100	101	104	108	101	102	104

TABLE 2

(Kornberg and Pricer, 1951; Hogeboom and Schneider, 1950; Plaut and Plaut, 1952; Bernofsky and Utter, 1966).

Only one enzyme, the $NADP^+$ enzyme, has been reported in E.coli and our attempts to detect an NAD^+ specific enzyme in E.coli failed.

There remained, however, the possibility that E.coli synthesized more than one $NADP^+$ specific isocitrate dehydrogenase. These enzymes, if they existed, might be synthesized differentially depending on the carbon source available for growth, e.g. the cells might synthesize a specific enzyme when growing on carbohydrates like glucose, and a second, distinct enzyme when growing on a different type of compound such as casamino acids or glycerol. In an attempt to clarify this issue, we decided to look at the enzyme's requirements. The enzyme was obtained from cells of E.coli after growth on glucose, glycerol or casamino acids. The enzyme's requirements were studied by calculating the K_m values for substrate, cofactor and ion. The enzyme was not purified in any way for these studies, apart from the cells being harvested and washed in ice cold saline prior to resuspension in EPA - saline and sonication. The results obtained are shown in Table 3.

It can be seen that the K_m's for substrate, cofactor and ion do not vary greatly, regardless of the source of enzyme. There is some scatter of values, particularly with the K_m's obtained for $NADP^+$, but it must be remembered that crude enzyme extracts were used. It was therefore considered that the K_m values obtained showed reasonable agreement and that if more than one isocitrate dehydrogenase was formed by E.coli then their requirements were almost identical and

PAGE 3

Substrate, cofactor and ion K_m's for isocitrate dehydrogenase in crude extracts of *E. coli*.

CARBON SOURCE USED FOR GROWTH	K_m (x M)		
	ISOCITRIC ACID	$NADP^+$	Mn^{++}
GLUCOSE	6.8×10^{-6} 6.0×10^{-6}	9.7×10^{-6} 8.3×10^{-6}	8.1×10^{-6} 7.9×10^{-6}
GLYCEROL	6.3×10^{-6} 6.0×10^{-6}	6.8×10^{-6} 7.5×10^{-6}	8.0×10^{-6} 7.7×10^{-6}
CASAMINO ACIDS	7.2×10^{-6} 6.8×10^{-6}	12.3×10^{-6} 9.8×10^{-6}	6.7×10^{-6} 7.6×10^{-6}
AVERAGE K_m FOR ALL CARBON SOURCES	6.5×10^{-6}	9.1×10^{-6}	7.7×10^{-6}

TABLE 3

very similar assay systems would be required to detect them.

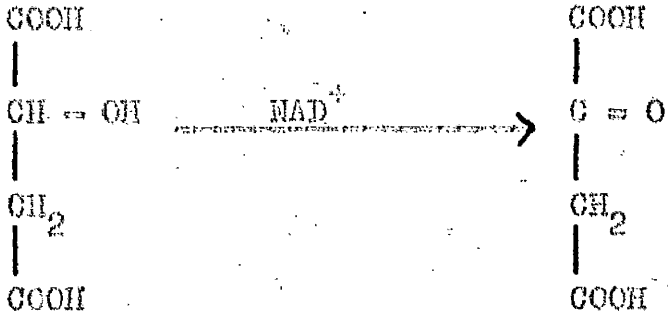
We also attempted to distinguish two or more enzymes, if formed, using heat inactivation. We were unable to detect any difference in the heat inactivation pattern of enzymes extracted from cells grown on different carbon sources. Therefore if E.coli synthesizes more than one isocitrate dehydrogenase, they possess similar heat inactivation patterns or a second enzyme is synthesized outside the reference of the experiments performed.

The Lineweaver - Burk plots, used to calculate the K_m's of the enzyme, extracted from cells grown on different carbon sources, were linear, indicating that the presence of more than one enzyme in each of the extracts was unlikely.

Therefore, as we could obtain no evidence to the contrary, we assumed that only one isocitrate dehydrogenase was present using our conditions of culture, i.e. the $NADP^+$ specific isocitrate dehydrogenase previously described.

MALATE DEHYDROGENASE ASSAY

Malate: NAD^+ oxidoreductase, otherwise known as malate dehydrogenase and henceforth referred to as such, catalyses the conversion of malic acid to oxaloacetic acid. The reaction is reversible with the equilibrium for the reaction firmly in favour of malic acid formation. The reaction proceeds in vivo in the direction of oxaloacetic acid formation, as this product is continuously metabolized in other reactions thus disturbing the equilibrium and permitting the reaction to proceed in this direction. The reaction may be written:



Malic acid — Oxaloacetic acid

NAD^+ is the only cofactor required by the enzyme. The reaction is measured in the reverse direction to that proposed as the physiologically important reaction, namely, measuring the rate of conversion of oxaloacetic acid to malic acid by following the associated oxidation of reduced pyridine nucleotide at 340mm.

The concentration of oxaloacetic acid must not exceed 0.2mM in the assay, as higher levels are inhibitory (Hamilton, unpublished results).

REPRODUCIBILITY OF SONICATION AND ASSAY

Malate dehydrogenase activity is directly proportional to the amount of enzyme protein, or bacterial cell protein, up to an activity of 80 units of enzyme per assay, corresponding to a rate of change of optical density, at 340 nm., of 0.166 per minute (Figure 9).

Cells were always harvested and resuspended to a turbidity of 4.0, prior to sonication. No data were compiled on the effect of varying the density of cell suspension which was sonicated, on the release of enzyme activity.

When a number of assay were performed on the same cell extract, all activities fell within a range of $\pm$ 2.5% of the mean.

When a number of batches from the same cell suspension were sonicated, all activities released fell within a range of $\pm$ 1.5% of the mean.

ENZYME STORAGE

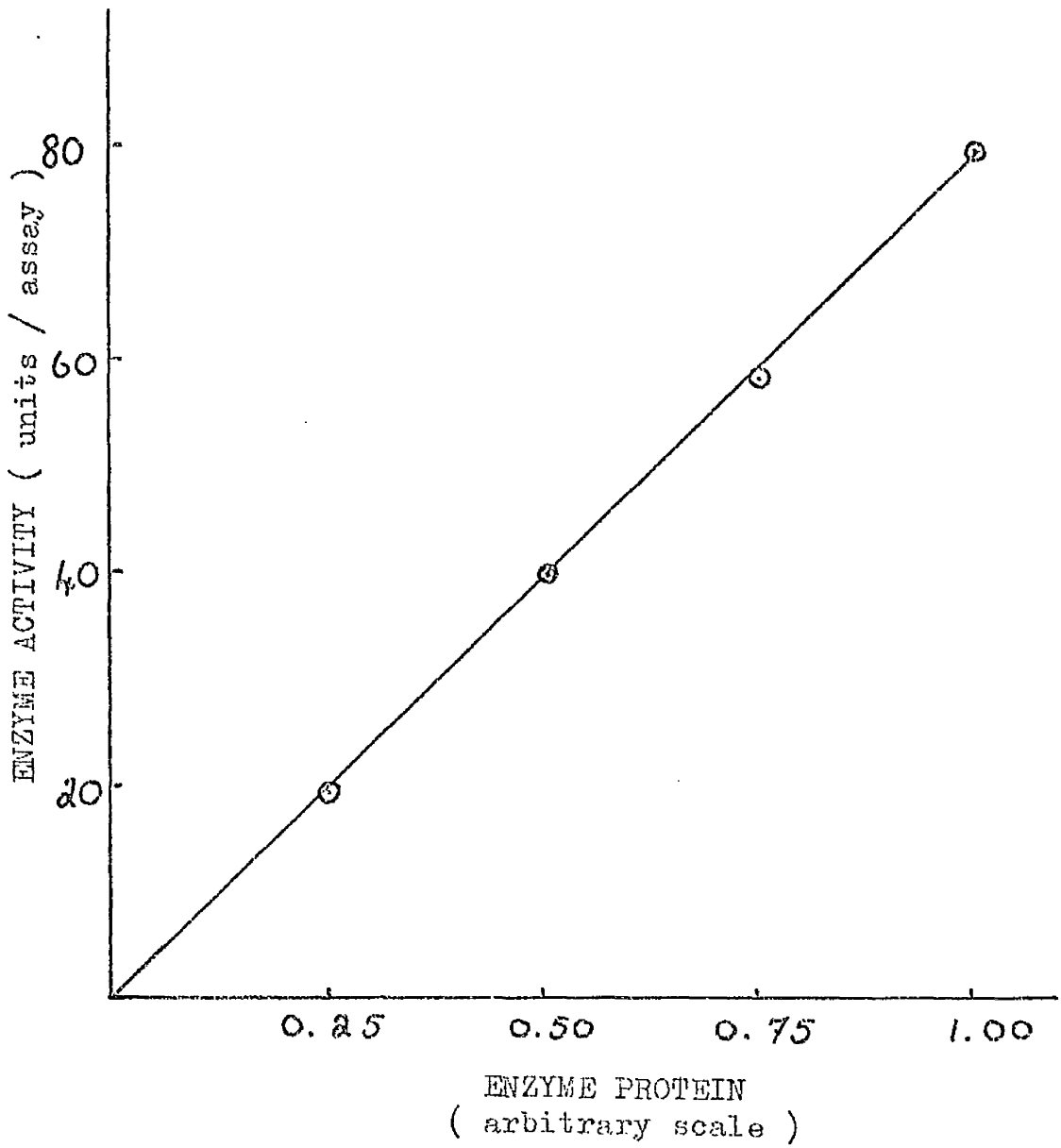
The stability of malate dehydrogenase in cell pellets, obtained after harvesting, when stored for up to 8 hours on ice, was investigated. It was found that the enzyme was stable over this time period.

FIGURE 2)

Relation of malate dehydrogenase activity

to enzyme protein.

FIGURE 9


ENZYME ACTIVITY (units / assay)
80
60
40
20
0.25
0.50
0.75
1.00
ENZYME PROTEIN
(arbitrary scale)

2-OXOGLUTARATE DEHYDROGENASE ASSAY

The term 2-oxoglutarate dehydrogenase, as used in this thesis, designates the structurally integrated unit of enzyme activities, which catalyses the conversion of 2-oxoglutarate to succinyl-coenzyme A with release of CO_2 and the associated reduction of NAD^+. The enzyme complex requires a number of cofactors, including NAD^+, thiamine pyrophosphate, coenzyme A. Although no requirement for lipoic acid in the assay for the enzyme could be shown, this coenzyme is known to be required by the enzyme complex, probably so tightly bound to enzyme protein that interchange with added lipoic acid is precluded. The reaction is complex but can be divided into a number of simpler reactions (Reed & Cox, 1966).

$$R.CO.COOH + TPP\text{-}Enz_I \longrightarrow R.CH(OH)\text{-}TPP\text{-}Enz_I + CO_2 \quad (1)$$

$$R.CH(OH)\text{-}TPP\text{-}Enz_I + Lip.S_2\text{-}Enz_2 \longrightarrow R.CO\text{-}S\text{-}Lip.SH\text{-}Enz_2 + TPP\text{-}Enz_I \quad (2)$$

$$R.CO\text{-}S\text{-}Lip.SH\text{-}Enz_2 + HS\text{-}Co.A \longrightarrow Lip(SH)_2\text{-}Enz_2 + R.CO.S\text{-}Co.A \quad (3)$$

$$Lip(SH)_2\text{-}Enz_2 + Enz_3\text{-}FAD \longrightarrow Lip.S_2\text{-}Enz_2 + Enz_3\text{-}FADH_2 \quad (4)$$

$$Enz_3\text{-}FADH_2 + NAD^+ \longrightarrow Enz_3\text{-}FAD + NADH + H^+ \quad (5)$$

where R.— $\equiv$ $HOOC.CH_2.CH_2$.—

Enzyme1 (Enz_1) is a decarboxylase enzyme requiring thiamine pyrophosphate as cofactor and is called 2-oxoglutarate decarboxylase. Enzyme2 (Enz_2) binds, very firmly, a residue of lipoic acid, and is called lipoyl reductase transacetylase. Enzyme3 (Enz_3) is a

flavoprotein enzyme, with a flavoprotein moiety very firmly bound, and is called dihydrolipoyl dehydrogenase.

The separate enzyme activities have been demonstrated, and the complex can be split into its separate components and the individual enzyme activities assayed (Reed & Cox, 1966).

The activity of the enzyme complex is measured in the following studies by utilising the activity of the last reaction in the sequence i.e. reaction 5.

An absolute requirement in the assay for coenzymes and NAD^+ has been shown, while addition of thiamine pyrophosphate enhances enzyme activity by 40%. No requirement could be shown for lipoic acid or FAD.

The enzyme activity of the complex is measured by estimating the rate at which NAD^+ is reduced in the presence of excess of all components required by the enzyme complex. Assay of enzyme activity is hampered by the presence of an NADH dehydrogenase activity in crude enzyme extracts which oxidizes the NADH formed by 2-oxoglutarate dehydrogenase. It was found that the NADH dehydrogenase activity could be inactivated sufficiently to permit assay of the 2-oxoglutarate dehydrogenase activity present in the extract, by the addition of potassium cyanide to the assay at a concentration of 5mM.

2-OXOGLUTARATE DEHYDROGENASE ACTIVITY

It was found that, using the assay outlined in the materials and methods section, 2-oxoglutarate dehydrogenase activity was not directly proportional to enzyme protein concentration, the activity being lower

than expected when the enzyme protein was diluted. However it was discovered that dilution of enzyme protein did yield a characteristic and highly reproducible result (Figure 10). As can be seen the curve consists of two distinct sections, a curved section at low enzyme activity and a linear section once enzyme activity reaches a sufficiently high threshhold level. It was therefore possible to extrapolate all enzyme activities obtained on to the linear portion of the curve and hence obtain relative specific activities. These specific activities, however, are not to be confused with the true specific activities obtained for the other enzymes. In effect, a specific activity calculated from the dilution curve is that which would be obtained if the linear portion of the curve was displaced to the left and extrapolated through the origin. This means that for each calculation the turbidity axis must be recalibrated, using as a basis the turbidity of the original sample assayed.

When a number of assays were performed on the same cell extract, all activities fell within a range of ± 3.5% of the mean.

When a number of batches from the same cell suspension were sonicated, all activities released fell within a range of ± 1.5% of the mean.

ENZYME STORAGE

Again, samples were stored after harvesting in the form of cell pellets, on ice, for up to 8 hours. The stability of the enzyme over this time period was checked, and the results indicated that the enzyme was fairly stable, though a slow, progressive loss of enzyme

FIGURE 10

Relation of 2-oxoglutarate dehydrogenase activity to enzyme protein.

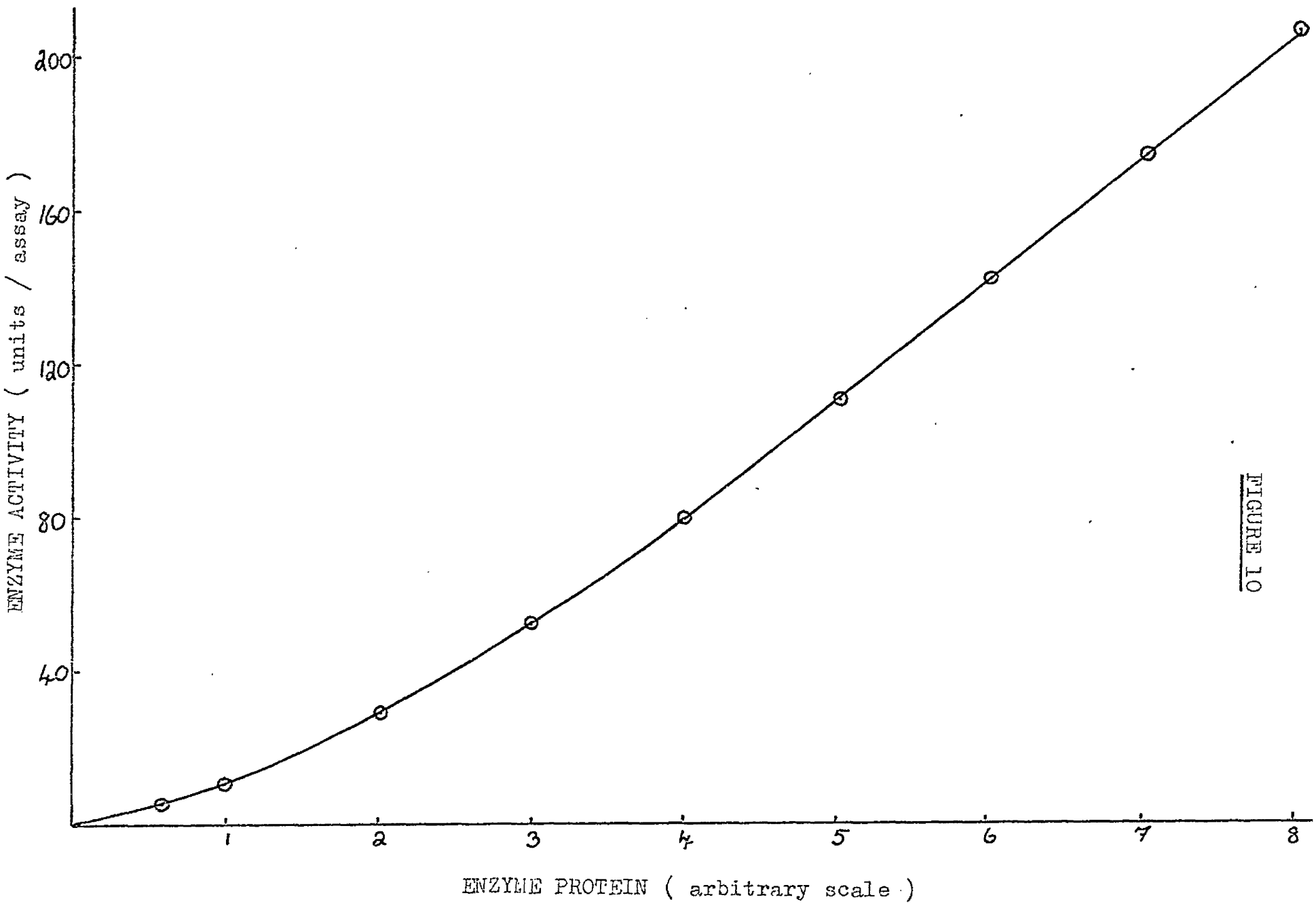
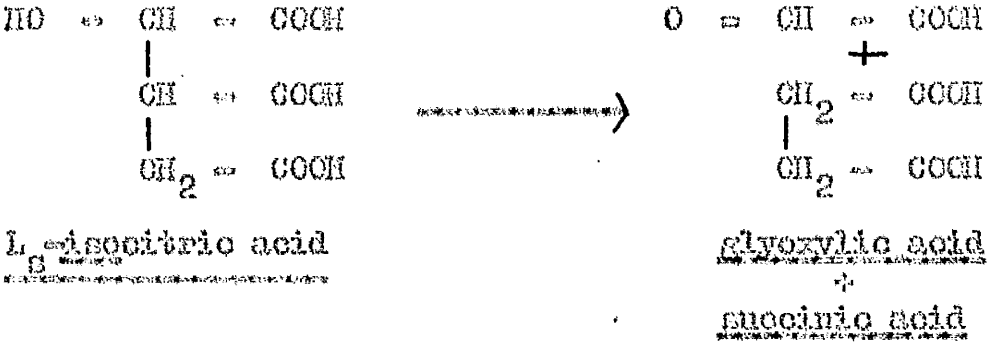

ENZYME ACTIVITY (units / assay)
200
160
120
80
40
1
2
3
4
5
6
7
8
ENZYME PROTEIN (arbitrary scale)

FIGURE 10

activity over the 8 hours was observed. The loss was not more than 10% over the 8 hours and this was considered to be acceptable.

ISOCITRATE LYASE ASSAY.

L_s-isocitrate: glyoxylate lyase, otherwise known as isocitrate lyase, catalyses the cleavage of isocitric acid to glyoxylic acid and succinic acid. The reaction may be written,



L_s-isocitric acid

glyoxylic acid
+
succinic acid

The enzyme requires magnesium ions and L-cysteine, to function.

This particular assay was not worked out in the same detail as were the assays for the tricarboxylic acid cycle enzymes. The reproducibility of both sonication and assay over a small number of samples was checked and found to be reliable.

No quantative importance is placed on the isocitrate lyase assays reported in this thesis, but the qualitative importance is not impaired.

(N.B. No attempt was made to actively hydrolyse the isocitric acid lactone to isocitric acid proper for use in the assay, as it was found that enzyme activity could be detected using a solution of the lactone. As the enzyme has been reported to be inactive towards the lactone (Dixon and Kornberg, 1959), enough free isocitric acid must exist under the conditions of assay to permit the reaction to proceed. No reaction could be detected in the absence of enzyme).

MODE OF COMPARISON OF ENZYME ACTIVITIES.

MODE OF COMPARISON OF ENZYME ACTIVITIES.When a comparison of enzyme activities grown under different conditions is to be made, to make any comparison meaningful, the enzyme activities in the different systems must be expressed in a common manner. This is achieved by expressing the enzyme activities as functions of some standard parameter. One accepted method is to express enzyme activity as a function of total cell protein, and another is to express enzyme activity as a function of total cell mass. In practice, enzyme activity can be expressed as a function of the turbidity of the bacterial culture, as turbidity measurements are proportional to the total cell mass of the culture. From this it is clear that if total cell protein is directly proportional to total cell mass, then measurement of culture turbidity will also be a measure of total cell protein. Figure 11 shows the relationship between turbidity and total cell protein in two cultures of E.coli growing on different carbon sources. It can be seen that the relationship is a linear one. Therefore, when comparing enzyme activities over a number of different conditions, the comparison is achieved using culture turbidity as the standard parameter.

In order to obtain a rate of enzyme synthesis, the data were graphed as suggested by Monod, Pappenheimer, Cohen-Bazire, (1952). This involved plotting, on arithmetic scales, the enzyme activity measured at a number of times during growth against the turbidity of the culture at those same times. When the rate of synthesis was constant, a straight line resulted, the slope of which gave the differential rate of enzyme synthesis with respect to the standard

FIGURE 1

Relationship between total bacterial cell protein and turbidity in growing cultures of *E. coli*.

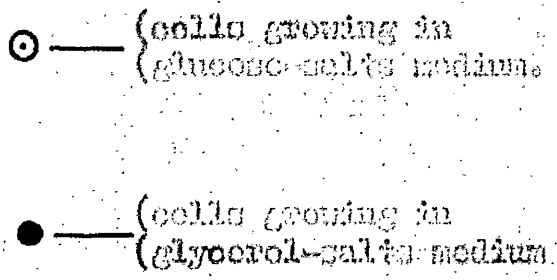
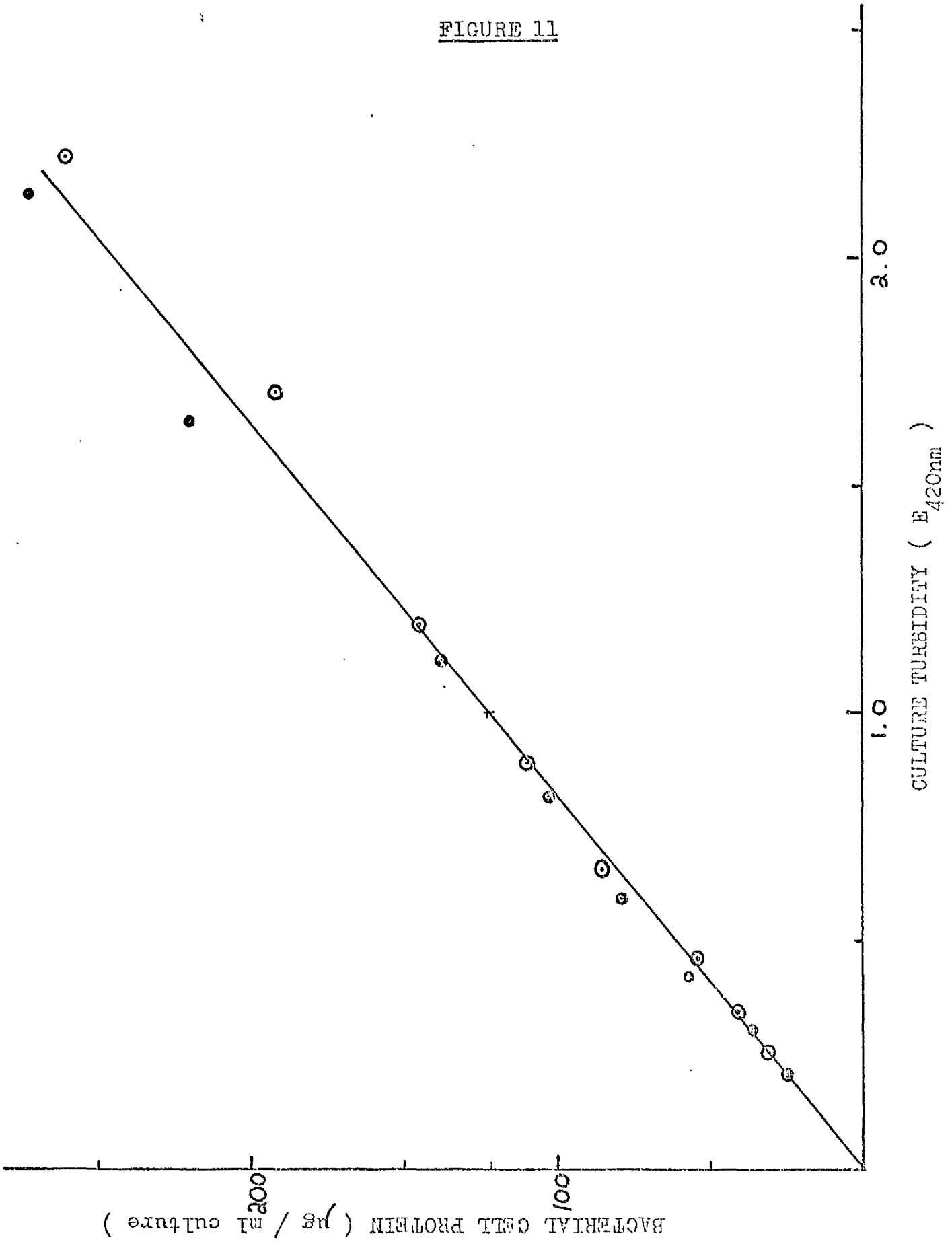


FIGURE 11


BACTERIAL CELL PROTEIN (µg / ml culture)
200
100
1.0
2.0
CULTURE TURBIDITY (E_{420nm})

parameter. The value obtained is referred to as the P - value of the enzyme.

When rates of synthesis could not be calculated, the specific activity of the enzyme of the sample was determined. The specific activity was calculated by expressing the enzyme activity of the sample relative to the turbidity of the sample. Both P - values and specific activities are expressed in the same units. Namely,

$$\frac{\text{Units enzyme / ml culture}}{\text{Culture turbidity } (E_{420nm})}$$

These values can easily be converted to a protein based standard, as a culture turbidity of 1.00 was equivalent to 125µg total bacterial protein per ml culture. Therefore multiplication of the specific activity or P - value by $\frac{1000}{125}$ or 8 will express either value in the units.

$$\frac{\text{Units enzyme / Ml. culture}}{\text{Total bacterial protein (mg) / Ml. culture}}$$

or

$$\frac{\text{Units}}{\text{mg Total bacterial protein}}$$

RESULTS

STUDY OF REPRESSION OF

ISOCITRATE DEHYDROGENASE SYNTHESIS

Effect of glucose concentration on growth and production of isocitrate dehydrogenase in E.coli

Cells, previously trained through three serial subcultures on glucose, were inoculated into fresh growth medium containing different concentrations of glucose.

The concentration of glucose, over the range tested, effected neither the growth rate nor the rate of production of isocitrate dehydrogenase (Table 4).

Effect of varying carbon and energy source on growth and on production of isocitrate dehydrogenase by E.coli

Cells, trained through three serial subcultures on a variety of carbon and energy sources, were inoculated into fresh, homologous medium.

The results indicate that the rate of synthesis of isocitrate dehydrogenase depended on the carbon and energy source utilized, as did growth rate (Table 5).

Effect of inoculum training on isocitrate dehydrogenase in E.coli growing on glucose after the addition of L-glutamate or 2-oxoglutarate

E.coli, trained through three serial subcultures to grow on glucose, 2-oxoglutarate or L-glutamate as sole source of carbon and energy, were inoculated into glucose (2.0mM) media. After one generation's growth, 2-oxoglutarate (1.0mM), L-glutamate (1.0mM) or both were added to the cultures.

TABLE 4

Effect of glucose concentration on growth and enzyme production by *E.coli*.

INITIAL CONCENTRATION OF GLUCOSE IN GROWTH MEDIUM (mM)	SPECIFIC GROWTH RATE	ISOCITRATE DEHYDROGENASE P — VALUE
1.0	0.925	76.5
2.0	0.925	76.5
4.0	0.946	77.0
10.0	0.946	78.0
20.0	0.946	78.0

TABLE 4

TABLE 5

Effect of carbon and energy source on growth rate and isocitrate dehydrogenase synthesis by E. coli.

CARBON SOURCE USED FOR GROWTH	INITIAL CONCENTRATION OF CARBON SOURCE (mM)	SPECIFIC GROWTH RATE	ISOCITRATE DEHYDROGENASE P – VALUE
LACTOSE	1.0	0.906	53.0
GALACTOSE	2.0	0.906	79.0
GLUCOSE	2.0	0.925	76.5
FRUCTOSE	2.0	0.742	62.0
GLYCEROL	4.0	0.719	93.0
2 – OXO – GLUTARATE	3.2	0.520	90.0
SUCCINATE	4.0	0.579	96.0
MALATE	4.0	0.661	92.0

TABLE 5

The addition of 2-oxoglutarate or L-glutamate to cultures inoculated with glucose trained cells produced little alteration in the differential rate of synthesis of isocitrate dehydrogenase (Table 6), while addition of both compounds produced a slight repression of enzyme synthesis.

When cells, originally trained to grow on 2-oxoglutarate or L-glutamate, were growing on glucose, the addition of the compound to which the cells were trained produced a noticeable repression of enzyme synthesis, while addition of the alternative compound, either by itself or plus training compound, produced little effect.

Cells were grown on L-glutamate for one passage and then on 2-oxoglutarate for one passage. These L-glutamate-2-oxoglutarate trained cells were inoculated into glucose (2.0mM) media, which were then treated as described above. In this system the addition of either compound produced substantial repression which was not increased by the addition of both compounds simultaneously.

Effect of substituting L-glutamate as nitrogen source on isocitrate dehydrogenase in E.coli growing on glucose

Glucose trained cells were inoculated into glucose (2.0mM) medium containing sufficient ammonium ion for one further generation of growth. 1 hour after growth ceased L-glutamate (2.0mM) was added to the medium.

L-glutamate supported further growth (Figure 12b) but synthesis

TABLE 6

Effect of previous growth training on the cells' response to the addition of 2-oxoglutarate and/or glutamate. Effect of the additions on the P-value of isocitrate dehydrogenase.

COMPOUND ADDED TO CULTURES DURING GROWTH ON 2.0mM GLUCOSE	ISOCITRATE DEHYDROGENASE							
	CELL TRAINING PRIOR TO GROWTH IN GLUCOSE SALTS MEDIUM							
	3 PASSAGES IN GLUCOSE SALTS MEDIUM		3 PASSAGES IN 2 — OXO — GLUTARATE SALTS MEDIUM		3 PASSAGES IN GLUTAMATE SALTS MEDIUM		1 PASSAGE IN GLUTAMATE SALTS 1 PASSAGE IN 2 — OXOGLUTARAT SALTS MEDIUM	
	P — value	% decrease	P — value	% decrease	P — value	% decrease	P — value	% decrease
none	83	0	125	0	84	0	87.5	0
2 — oxo — glutarate (1.0mM)	76	8	95	24	80	5	50	43
glutamate (1.0mM)	76	8	122.5	2	51	39	50	43
2 — oxo — glutarate (1.0mM) glutamate (1.0mM)	70	16	95	24	51	39	50	43

TABLE 6

FIGURES 12a & b

Effect of changing the nitrogen source used for growth on subsequent growth of culture and activity of Isocitrate dehydrogenase.

⊙ —— Culture turbidity

● —— Isocitrate dehydrogenase

FIGURE 12a -- ammonium sulphate (1.0mM) added to culture (↑)

FIGURE 12b -- L-glutamate (2.0mM) added to culture (↑)

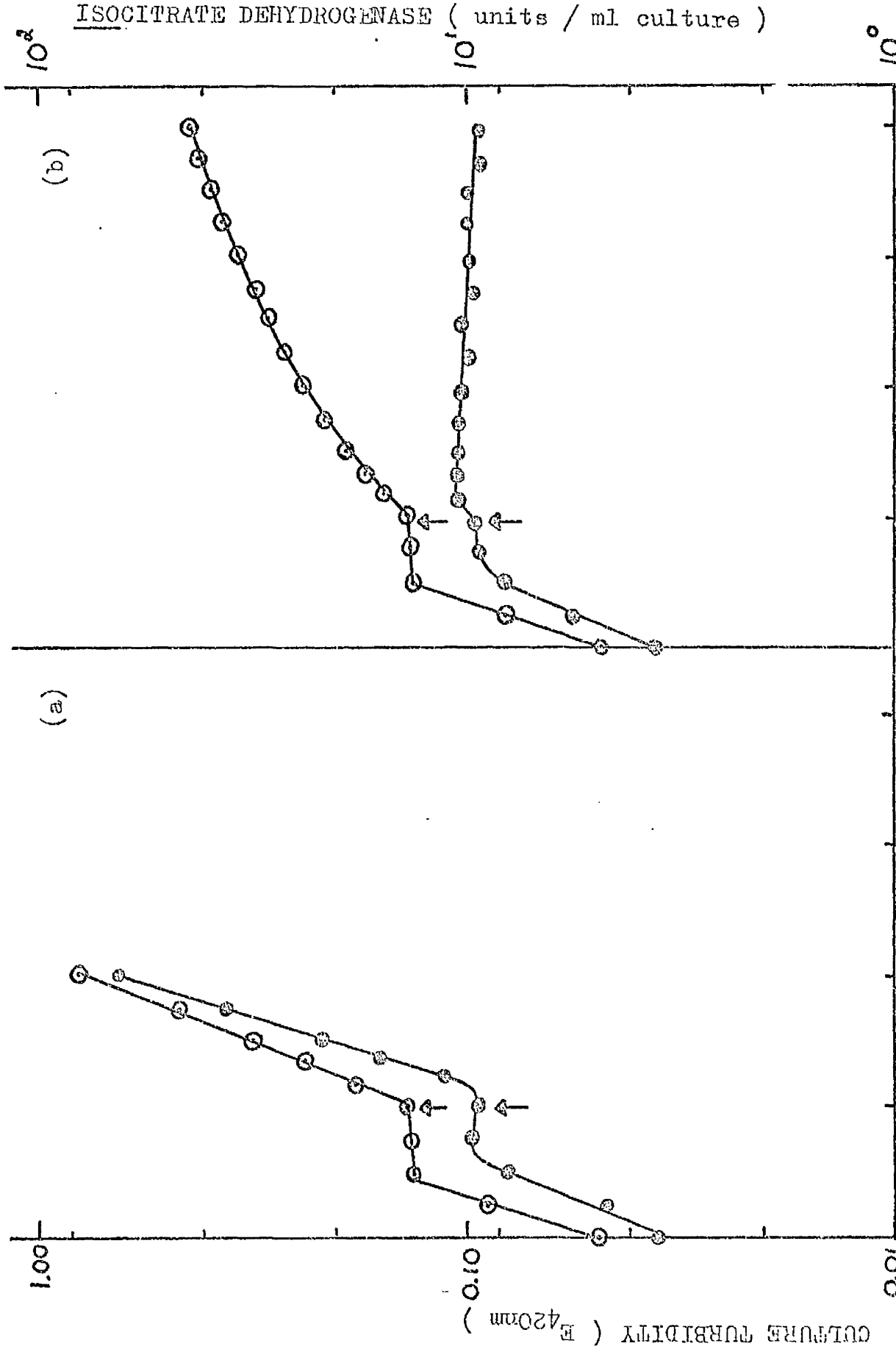

ISOCITRATE DEHYDROGENASE (units / ml culture)
10^2
10^1
10^0
(b)
(a)
TIME (hr)
2
4
6
8
CULTURE TURBIDITY (E_{420nm})
1.00
0.10
0.01

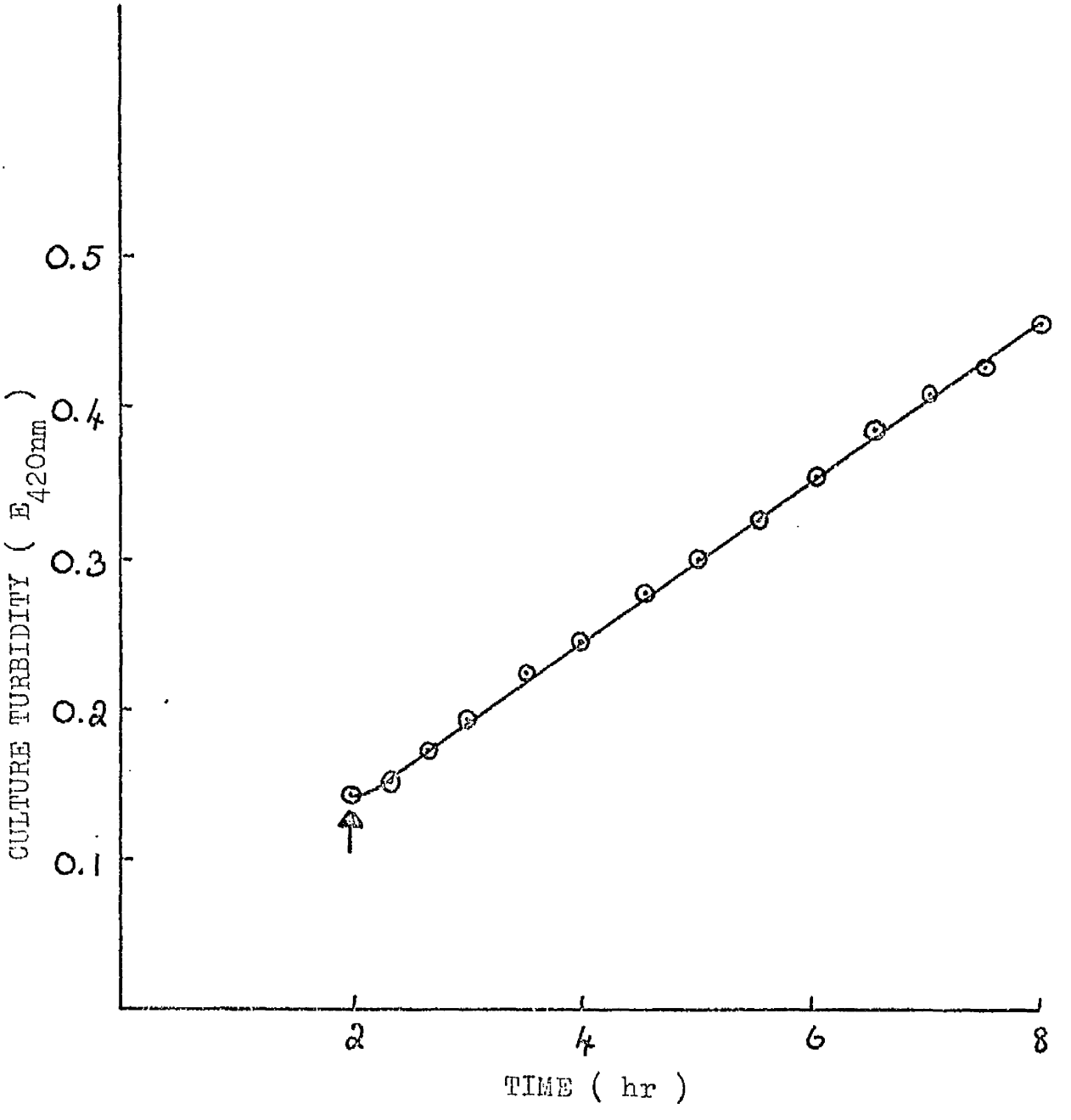
FIGURE 12

FIGURE 13

Effect on growth of *E. coli* of substituting L-glutamate for ammonium ion as nitrogen source.

L-glutamate added to culture at (↑)

FIGURE 13



of isocitrate dehydrogenase was completely repressed. This result can be compared with that in Figure 12a, where, after ammonium ion was added back, growth resumed as did synthesis of isocitrate dehydrogenase, both at rates associated with growth on glucose-salts.

An interesting result emerges if growth on glucose-glutamate is replotted on an arithmetic scale, as this growth is seen to be arithmetic (Figure 13).

STUDY OF ISOCITRATE DEHYDROGENASE ACTIVITY UNDER CONDITIONS OF LIMITED GROWTH

Enzyme stability after the cessation of growth.

Cells of E.coli were grown on either glucose (2.0mM) or glycerol (4.0mM). Culture turbidity was measured throughout logarithmic growth and well into the stationary phase (Figure 14). Immediately before growth ceased, and at intervals thereafter, samples of culture were harvested by centrifugation at 10,000g for 15 minutes at 4°C and the activities of isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and malate dehydrogenase measured.

The results (Figure 15) show that after growth on glycerol, the activities of all three enzymes remained fairly constant throughout a 4 hour period following the cessation of growth, as did the activities of oxoglutarate dehydrogenase and malate dehydrogenase after growth on glucose. However, after growth on glucose, the activity of isocitrate dehydrogenase began to fall, and so continued during the following 60 to 90 minutes. Approximately 80% of the original activity was lost during this time. After a further 60 to 90 minutes, enzyme activity began to rise again and reached 75% of the maximal value 4 hours after the end of growth.

The technique of using stationary phase cultures to study enzyme synthesis, suggested in the introduction ("Proposed method of study"), depends upon enzyme activity remaining stable after growth ceases. If this condition is not fulfilled, the hope that this approach would ensure reproducible conditions at challenge will not be achieved and thus invalidate the technique. Thus for the two substrates used, it appeared that glycerol could be used in the manner proposed, but

FIGURE 14

Growth of *E.coli* on a limiting concentration of carbon and energy source.

△ — cells grown on 2.0ml glucose

⊙ — cells grown on 4.0ml glycerol

FIGURE 14

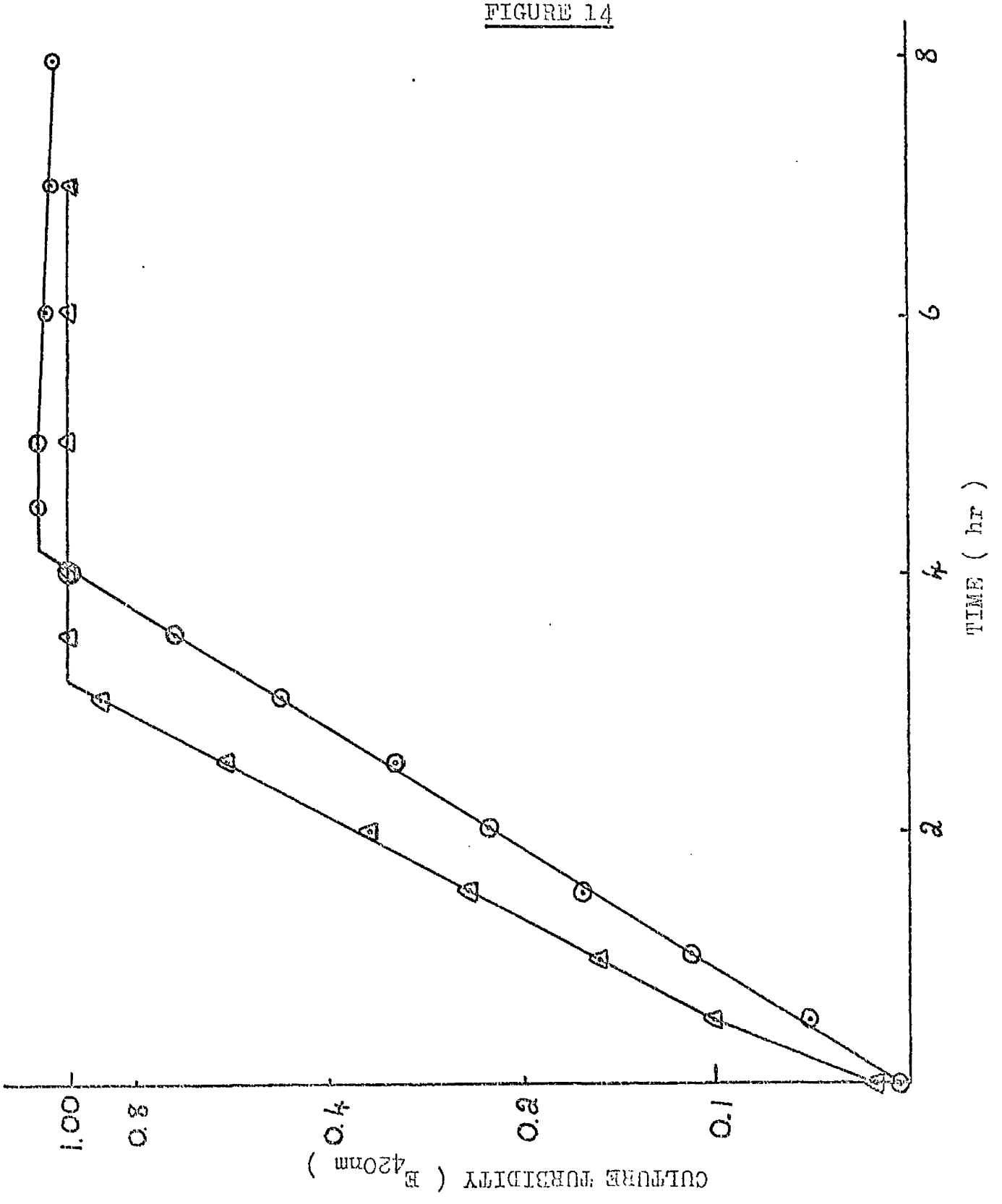


FIGURE 15

Stability of three tricarboxylic acid cycle enzymes in *E.coli* after growth on limiting concentrations of carbon and energy source.

(a) cells grown on 4.0mM glycerol

(b) cells grown on 2.0mM glucose

⊙ — isocitrate dehydrogenase

⊡ — 2-oxoglutarate dehydrogenase

△ — malate dehydrogenase

SPECIFIC ACTIVITY MALATE DEHYDROGENASE
1500
1000
500
(b)
150
100
50
SPECIFIC ACTIVITY ISOCITRATE DEHYDROGENASE
(a)
1
2
3
4
TIME AFTER CESSATION OF GROWTH (hr)
15
10
5
SPECIFIC ACTIVITY 2 -- OXOGLUTARATE DEHYDROGENASE

FIGURE 15

glucose could not.

It was of interest, however, to discover why a loss of isocitrate dehydrogenase activity occurred after growth on glucose. I decided to further investigate this phenomenon.

Isocitrate dehydrogenase activity in, and carbon dioxide production by, E.coli during and after growth on limiting glucose or glycerol

During logarithmic growth on either glucose (2.0mM) or glycerol (4.0mM), the activity of isocitrate dehydrogenase and the production of carbon dioxide rose exponentially (Figure 16). At the end of growth on glycerol, enzyme activity remained constant and carbon dioxide production declined sharply and continued to fall throughout the 2-2½ hour period following the cessation of growth over which it was monitored (Figure 16a).

After growth on glucose, enzyme activity fell and then recovered, as already described. When growth ceased, carbon dioxide production declined, partially recovered 30 minutes later and increased throughout the following 2 hours, whereupon it again fell, this time showing no recovery (Figure 16b). Enzyme activity was restored when carbon dioxide production fell for the second time.

Acetate production and utilisation and isocitrate dehydrogenase activity during and after growth on glucose or glycerol

On glycerol (4.0mM), cell growth and isocitrate dehydrogenase activity gave the profiles previously described (Figure 17a).

FIGURE 16

Isocitrate dehydrogenase activity and carbon dioxide production by *E.coli* during and after growth on a limiting concentration of carbon and energy source.

(a) cells grown on 4.0mM glycerol

(b) cells grown on 2.0mM glucose

⊙ — Isocitrate dehydrogenase

● — carbon dioxide

CARBON DIOXIDE PRODUCTION (ul / ml culture / min)
1.00
0.10
0.01
(b)
GROWTH
(a)
GROWTH
ISOCITRATE DEHYDROGENASE (units / ml culture)
1.0
0.0
0.00
2
4
6
8
TIME (hr)

FIGURE 16

Growth ceased due to glycerol exhaustion, while during growth a low level of acetate, which did not exceed 0.05mM, was maintained in the medium (Figure 17b). No acetate, however, could be detected in the medium after growth ceased.

On glucose (2.0mM) growth and enzyme activity also behaved as previously described (Figure 18a). Growth ceased once glucose had been completely utilised, while acetate accumulated in the medium throughout growth, reaching a level of 1.0mM at the end of growth (Figure 18b). The acetate was utilized after a 30 minute lag and was exhausted 2½-3 hours following the cessation of growth.

A small, but reproducible, increase in culture turbidity could be detected approximately 2½ hours after growth on glucose ceased.

Effect of acetate on isocitrate dehydrogenase activity and carbon dioxide production in cultures of E.coli when added after growth on glycerol

When acetate (0.75mM) was added to a culture at the end of growth on glycerol (4.0mM), isocitrate dehydrogenase activity declined and recovered, while carbon dioxide production was maintained at a high level before finally decreasing (Figure 19 ; cf., Figure 16a). Enzyme activity recovered once carbon dioxide output fell.

FIGURE 17

Glycerol and acetate levels and isocitrate dehydrogenase activity during and after growth of E.coli.

(a) ⊙ — isocitrate dehydrogenase

● — culture turbidity

(b) ⊡ — glycerol

△ - - acetate

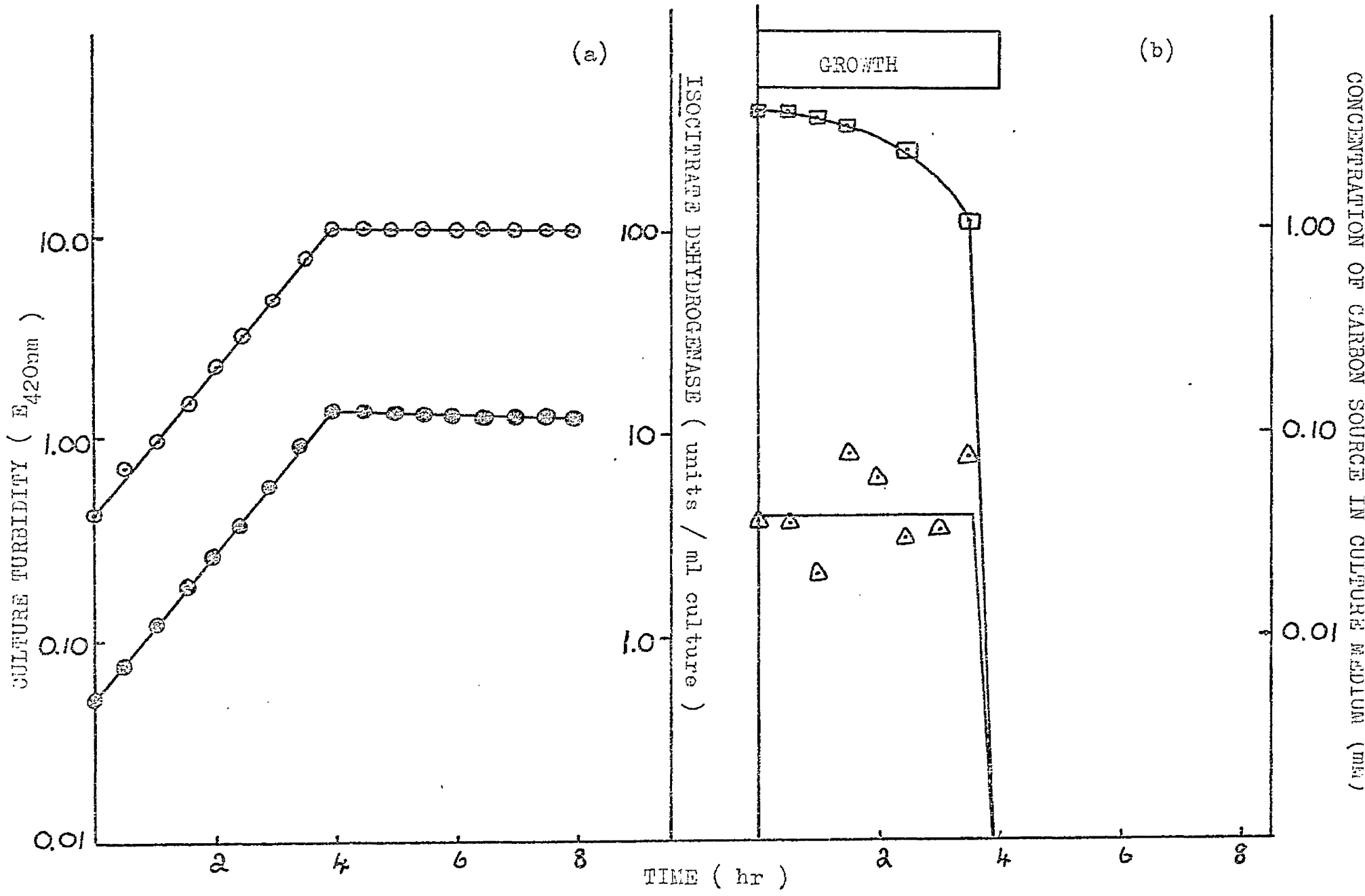

(a)
(b)
GROWTH
CULTURE TURBIDITY (E_{420nm})
ISOCITRATE DEHYDROGENASE (units / ml culture)
CONCENTRATION OF CARBON SOURCE IN CULTURE MEDIUM (m
TIME (hr)
10.0
1.00
0.10
0.01
100
10
1.0
2
4
6
8

FIGURE 17

FIGURE 10

Glucose and acetate levels and isocitrate dehydrogenase activity during and after growth of *E. coli*.

isocitrate dehydrogenase

culture turbidity

glucose

acetate

CONCENTRATION OF CARBON SOURCE IN CULTURE MEDIUM (mM)
1.00
0.10
0.01
(b)
GROWTH
2
4
6
8
TIME (hr)
ISOCITRATE DEHYDROGENASE (units / ml culture)
(a)
100
10
1.0
CULTURE TURBIDITY (E_{420nm})
10.0
1.00
0.10
0.01

FIGURE 18

FIGURE 19

Effect of acetate on isocitrate dehydrogenase and carbon dioxide production when added to a culture of E.coli at the end of growth on glycerol (4.0mM).

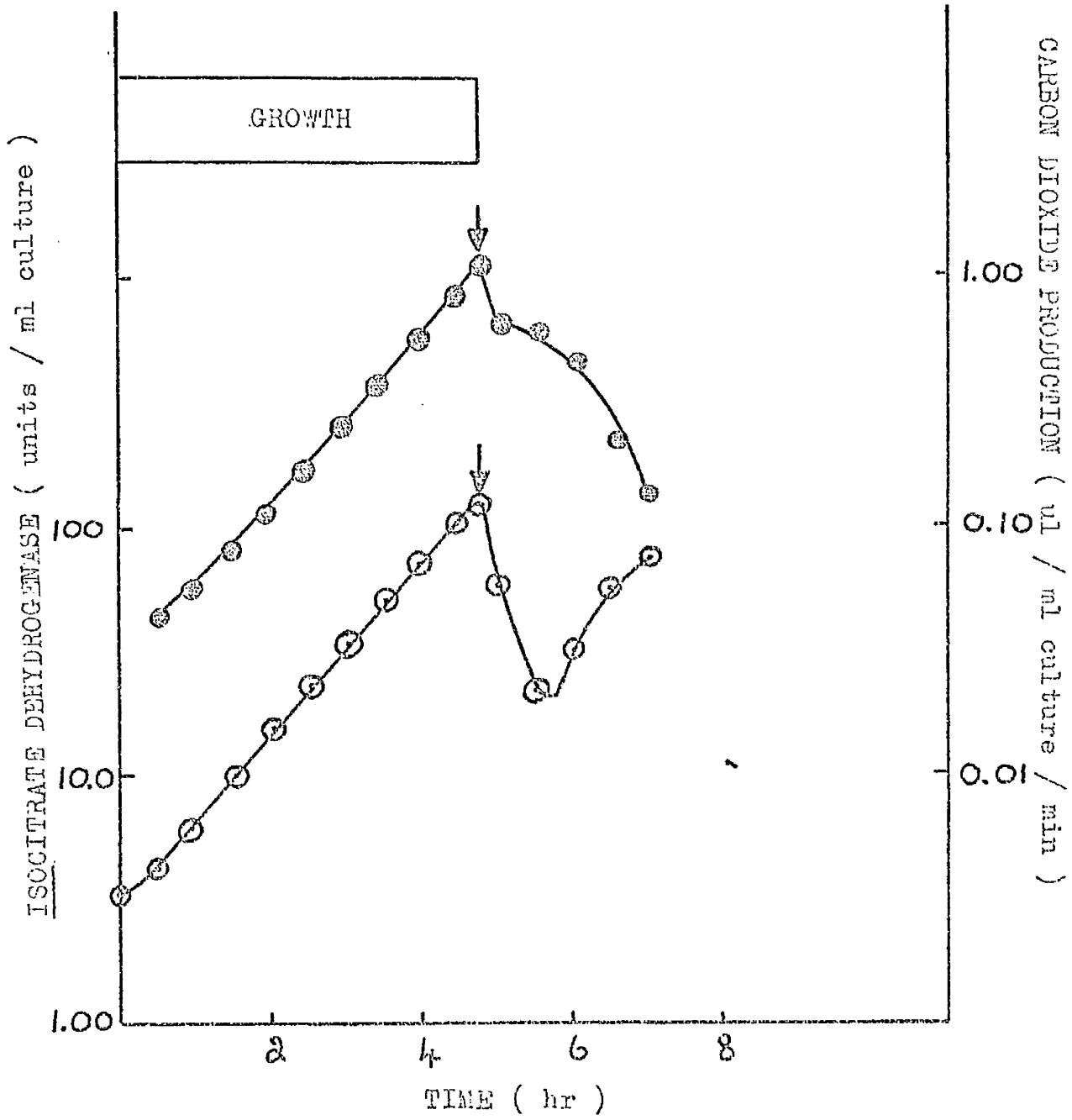
(↓) acetate added

(↓) to 0.75mM

⊙ ⟷ isocitrate dehydrogenase

● ⟷ carbon dioxide

FIGURE 19


GROWTH
ISOCITRATE DEHYDROGENASE (units / ml culture)
CARBON DIOXIDE PRODUCTION (ul / ml culture / min)
1.00
10.0
100
0.01
0.10
1.00
2
4
6
8
TIME (hr)

Isocitrate dehydrogenase activity at the end of growth on a variety of carbon and energy sources

E.coli was grown on several single carbon and energy sources. The growth media were inoculated with cells trained through three serial subcultures in homologous media. Duplicate cultures were set up, and at the end of growth, acetate (1.0mM) was added to one culture of each pair.

Growth on glucose, lactose or pyruvate was followed by a loss of isocitrate dehydrogenase activity, which was later partially restored (Table 7), while no loss of enzyme activity was observed after growth on the other carbon sources tested. A loss of enzyme activity in these latter cultures was induced by the addition of acetate (1.0mM) at the end of growth. Enzyme activity was later restored.

Effect of different carbon source during inoculum training on isocitrate dehydrogenase in E.coli during and after subsequent growth on glucose

Cells, trained through three serial subcultures on various carbon and energy sources, were harvested, washed in chilled phosphate buffer (.40mM) and inoculated into glucose (2.0mM) medium.

The results (Table 8) show that previous growth on compounds other than glucose did not prevent decay of isocitrate dehydrogenase activity at the end of growth on glucose. However, previous growth history did influence the growth rate on glucose and, in these experiments, led to a fractional increase in the differential rate of isocitrate dehydrogenase synthesis.

TABLE 7

Behaviour of isocitrate dehydrogenase activity following growth on limiting concentrations of various carbon and energy sources, with or without the addition of acetate at the end of growth.

CARBON AND ENERGY SOURCE	ISOCITRATE DEHYDROGENASE ACTIVITY FOLLOWING GROWTH ON A LIMITING CONCENTRATION OF CARBON AND ENERGY SOURCE			
	NO ADDITION OF ACETATE AT THE END OF GROWTH		ACETATE ADDED TO 1.0mM AT THE END OF GROWTH	
	fall	recovery	fall	recovery
lactose(1.0mM)	+	+	/	/
galactose(2.0mM)	—	—	+	+
glucose(2.0mM)	+	+	+	+
fructose(2.0mM)	—	—	+	+
glycerol(4.0mM)	—	—	+	+
pyruvate(5.0mM)	+	+	/	/
2 - oxoglutarate(2.4mM)	—	—	+	+
succinate(3.0mM)	—	—	+	+
malate(3.0mM)	—	—	+	+

TABLE 7

TABLE 8

Effect of previous growth on dehydrogenase doubling and effect growth of E.coli on glucose (2.0%).

CARBON SOURCE USED DURING INOCULUM TRAINING	SPECIFIC GROWTH RATE ON 2.0mM GLUCOSE	ISOCITRATE DEHYDROGENASE	
		P — VALUE	LOSS OF ACTIVITY AT THE END OF GROWTH
glucose	0.906	77.5	+
galactose	0.869	86.0	+
glycerol	0.816	84.0	+
pyruvate	0.742	76.5	+
malate	0.816	81.0	+

TABLE 8

Adaptation to acetate after growth on glucose or glycerol

E.coli was grown on glucose (2.0mM) and glycerol (4.0mM). The activity of isocitrate lyase once stationary phase was reached was measured. After growth on glucose, but not glycerol, there was a considerable increase in enzyme activity (Figure 20).

The addition of acetate (6.0mM) to cultures 2½ hours or more after growth on glucose (2.0mM) ceased resulted in an almost immediate resumption of growth (Figure 21). Addition of acetate at any time up to 2½ hours after the end of growth produced no further growth until 2½ hours after growth initially ceased, when in all cases growth resumed.

When acetate (6.0mM) was added after growth on glycerol (4.0mM), resumption of growth depended on when acetate was added. The longer the addition of acetate was delayed, the longer the lag after the addition before growth resumed and the slower the initial growth rate (Figure 22).

Effect of inhibitors of protein synthesis on various parameters when added after growth on glucose or glycerol

After growth on glucose (2.0mM), chloramphenicol (0.3mM) or puromycin (0.3mM) was added to the medium. The addition of either drug prevented loss of isocitrate dehydrogenase activity (Figure 23), while addition of chloramphenicol 2 hours after growth ceased did not prevent restoration of enzyme activity (Figure 24).

When a similar experiment was performed using cells grown on

FIGURE 20

Synthesis of isocitrate lyase after growth on glucose (2.0ml) and glycerol (4.0ml).

⊙—— Enzyme production after growth on glucose.

●—— Enzyme production after growth on glycerol.

FIGURE 20

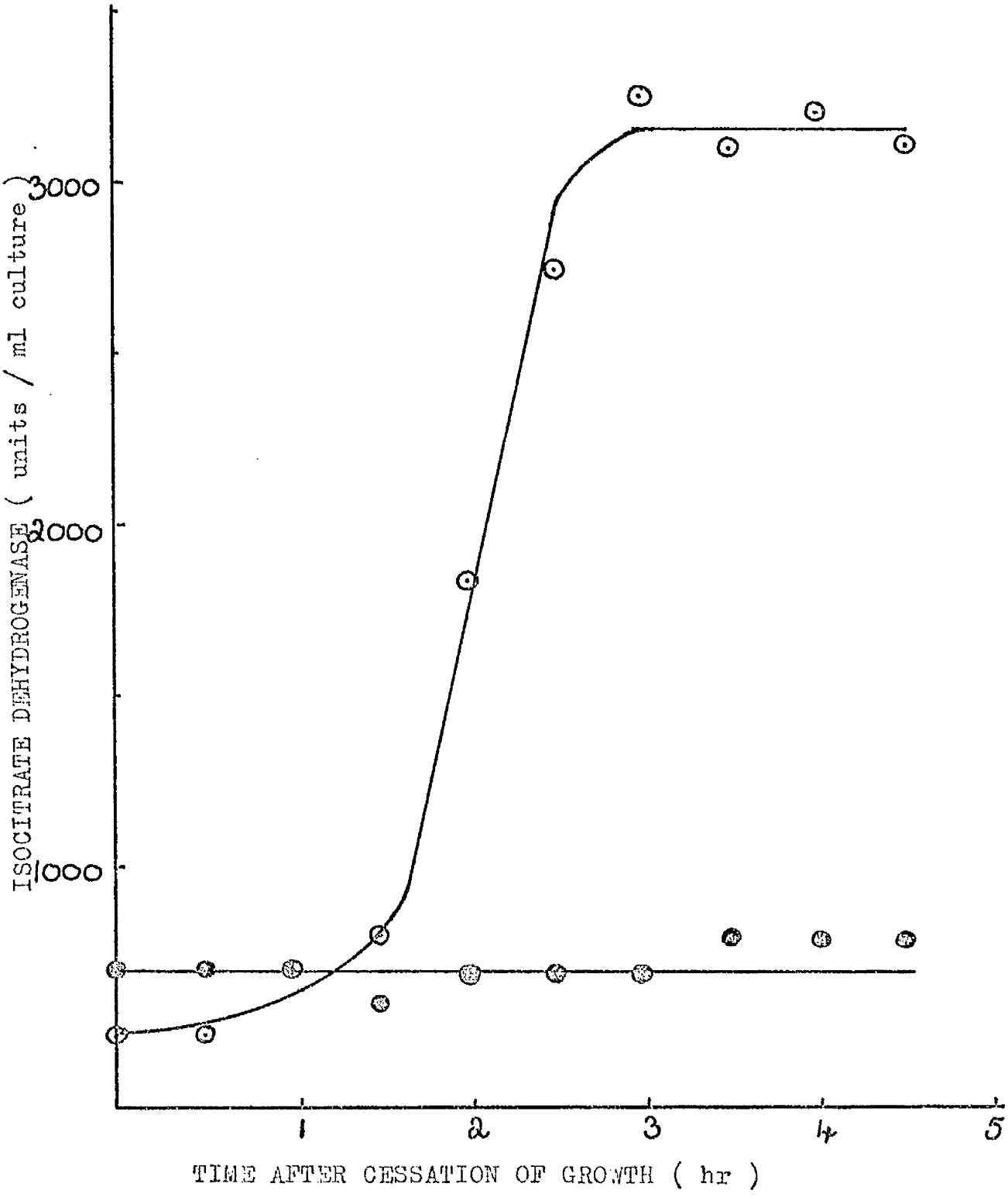

ISOCITRATE DEHYDROGENASE (units / ml culture)
3000
2000
1000
1
2
3
4
5
TIME AFTER CESSATION OF GROWTH (hr)

FIGURE 21

Effect of delay before addition of acetate on subsequent growth of *E. coli*, after initial growth on 2.0% glucose.

Acetate added to a final concentration of 6.0% (▲).

FIGURE 21

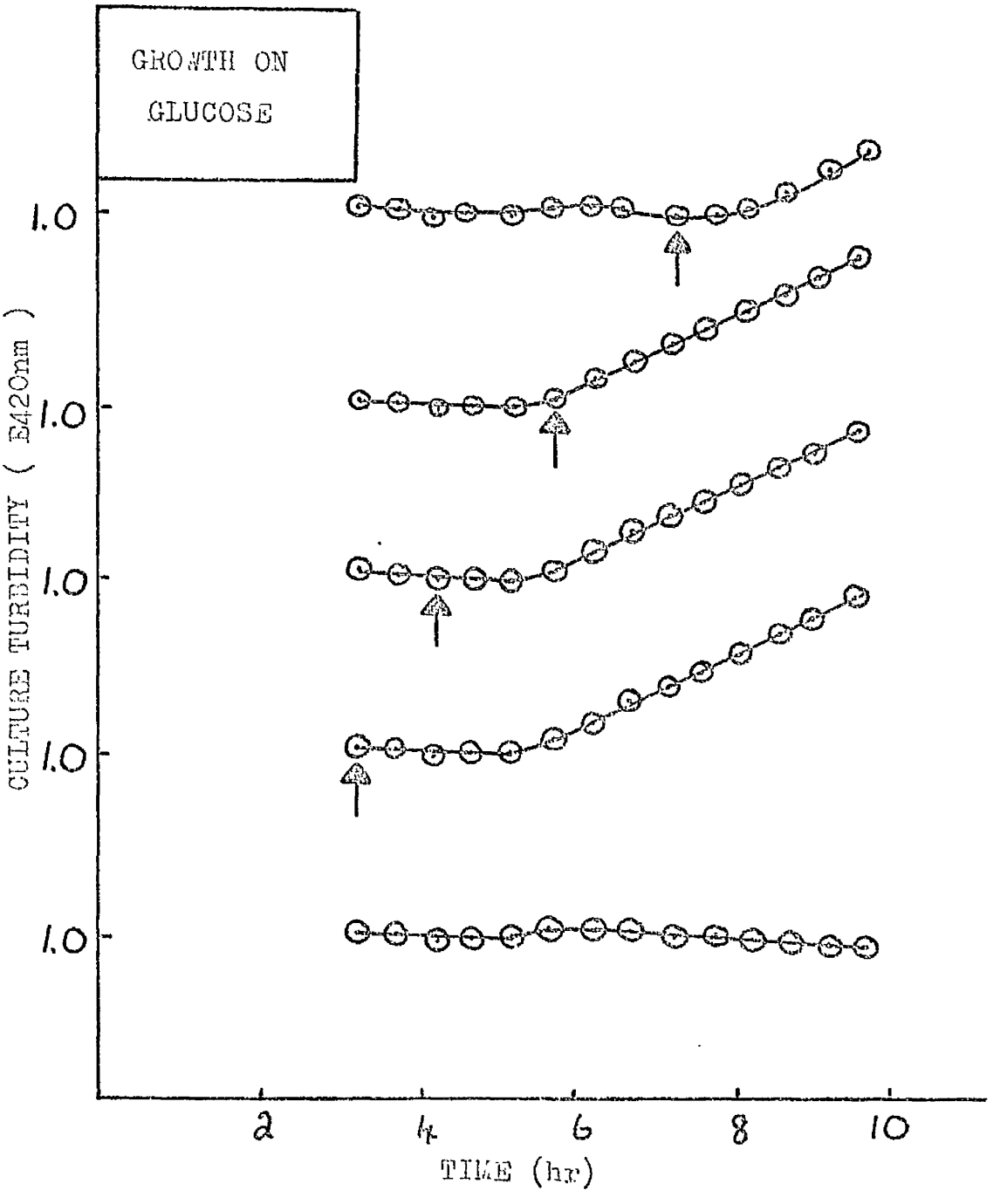
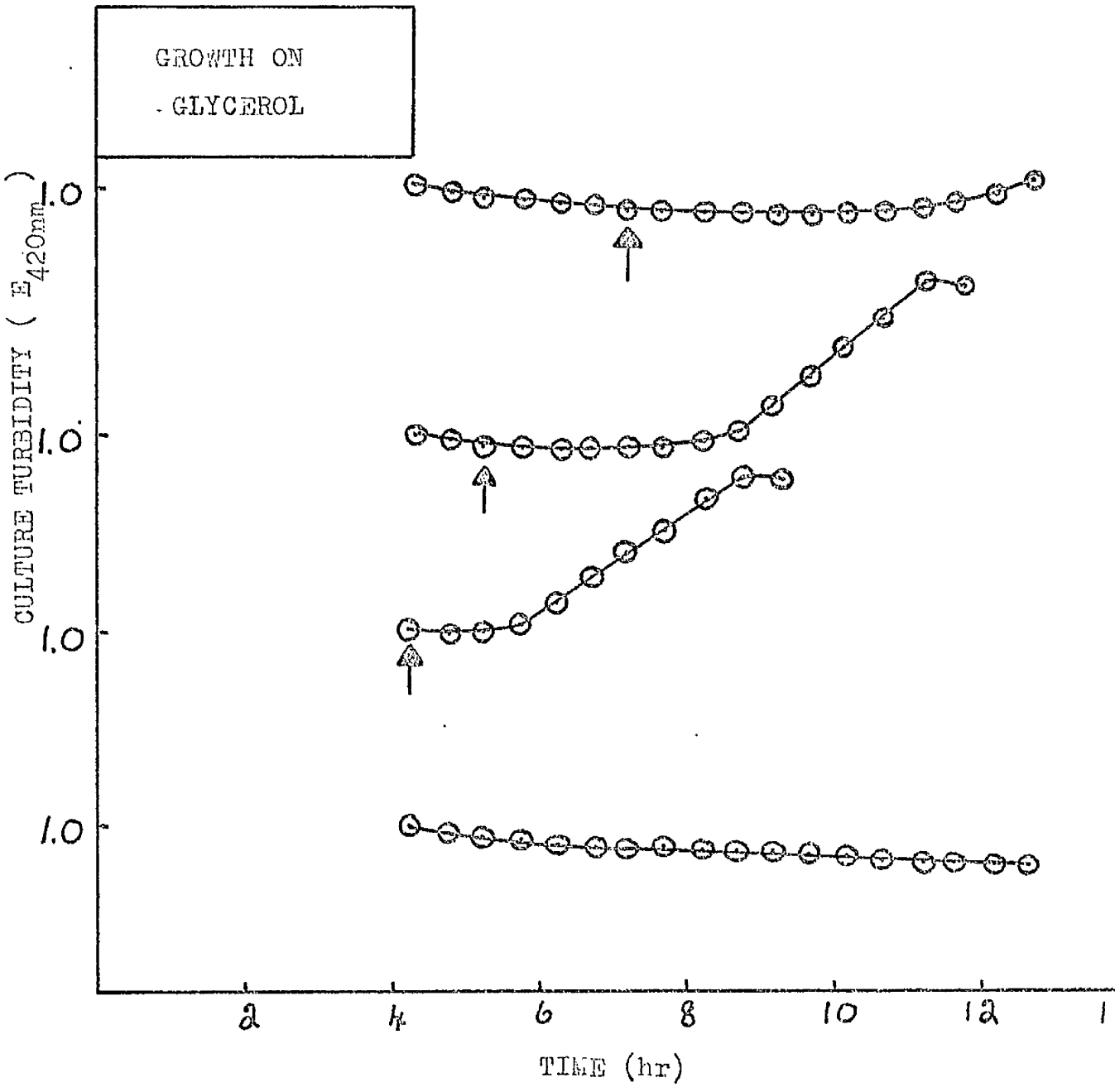

GROWTH ON
GLUCOSE
CULTURE TURBIDITY (E420nm)
1.0
1.0
1.0
1.0
2
4
6
8
10
TIME (hr)

Figure 22

Effect of delay before addition of acetate on subsequent growth of *E. coli*, after initial growth on 4.0mM glycerol.

Acetate added to a final concentration of 6.0mM (↑)

FIGURE 22


GROWTH ON
GLYCEROL
CULTURE TURBIDITY (E_{420nm})
1.0
1.0
1.0
1.0
4
6
8
10
12
TIME (hr)

glycerol (4.0mM), and a mixture of acetate (1.0mM) and chloramphenicol (0.3mM) was added at the end of growth, no loss of enzyme activity occurred (cf. Figure 19).

The addition of chloramphenicol at the end of growth on glucose abolished the secondary increase in carbon dioxide production (Figure 25), which instead, steadily declined at the end of growth, and was then maintained at a very low level, which was, nonetheless, higher than the basal rate registered in the control culture.

A similar experiment confirmed the effect of chloramphenicol on isocitrate dehydrogenase (Figure 26a) and further showed that addition of the drug also prevented the increase in isocitrate lyase activity (Figure 26b). Acetate utilization continued in the presence of drug, albeit at a much reduced rate (Figure 26c); cf. Figure 25).

Effect of various carbon compounds on isocitrate dehydrogenase activity when added at the end of growth on glucose.

Various compounds, tricarboxylic acid cycle intermediates or closely related compounds, were added to cultures of E.coli at the end of growth on glucose (2.0mM), and the effect on isocitrate dehydrogenase observed. The results are presented in Table 9 and individual examples illustrating the variation in response are shown in Figure 27.

Basically, three types of result were obtained, symbolized A, B and C. Compounds which failed to prevent loss of enzyme activity were assigned to Class A, which included glycine, glutamate and 2-oxoglutarate.

FIGURE 23

Effect of inhibitors of protein synthesis on the loss of Isocitrate dehydrogenase activity after growth of E.coli on 2.0mM glucose.

● — culture to which no drug was added

⊙ — culture to which chloramphenicol was added to a concentration of 0.3mM

△ — culture to which puromycin was added to a concentration of 0.3mM

Drugs added at point indicated by arrow (↓)

FIGURE 23

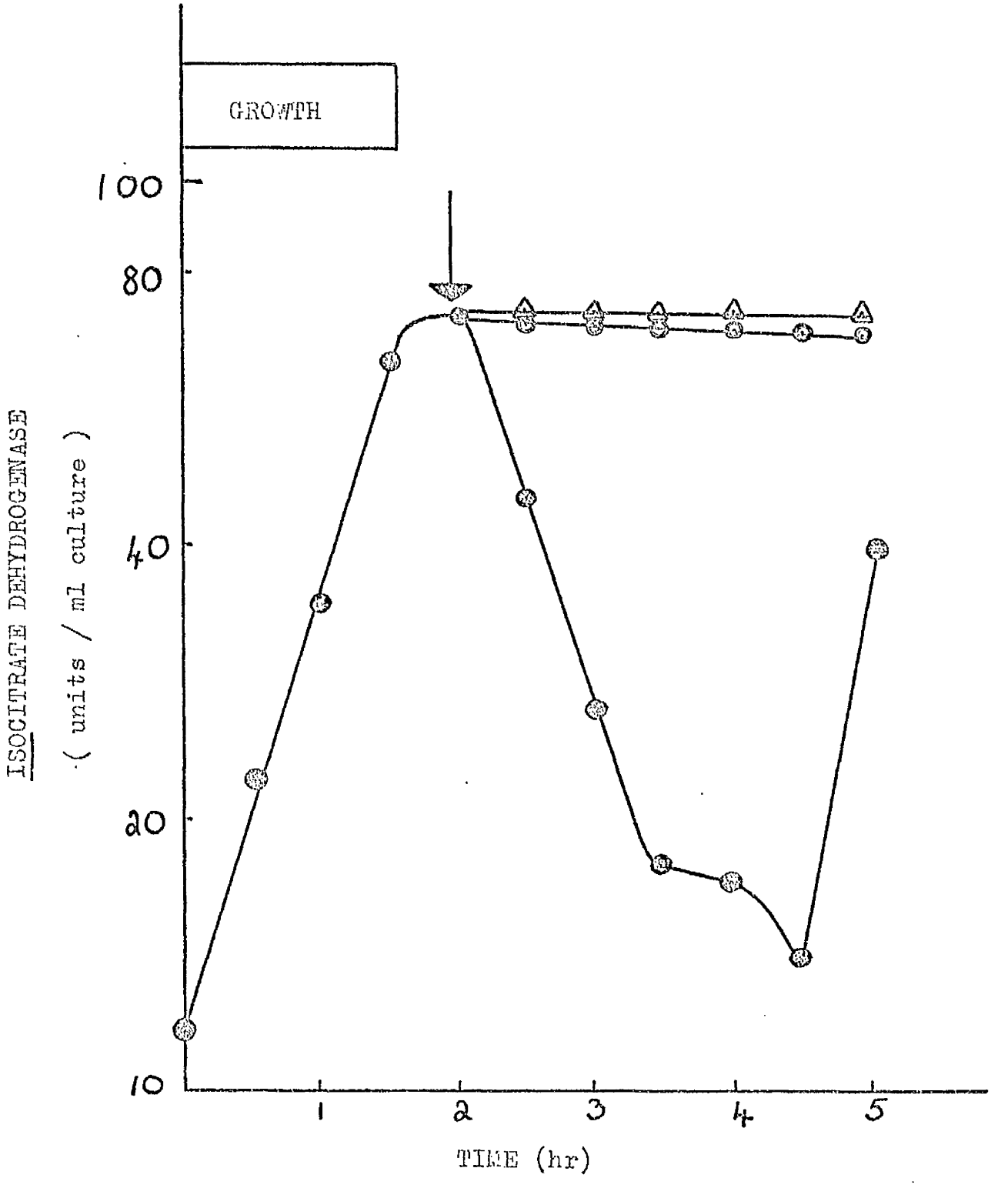

GROWTH
100
80
40
20
10
ISOCITRATE DEHYDROGENASE
(units / ml culture)
1
2
3
4
5
TIME (hr)

FIGURE 24

Effect on isocitrate dehydrogenase of adding chloramphenicol to a culture of E.coli grown on 2.0% glucose, 2 hours after growth ceased.

⊙ —— culture to which no addition was made

● —— culture to which chloramphenicol was added to 0.3% at point indicated by arrow (↑).

FIGURE 24

GROWTH
ISOCITRATE DEHYDROGENASE (units / ml culture)
100
10
1.0
2
4
6
8
TIME (hr)

FIGURE 25

Effect of chloramphenicol on carbon dioxide production by *E.coli*.

⊙ —— control culture; no addition

● —— chloramphenicol added to 0.3m*M* (↓)

FIGURE 25

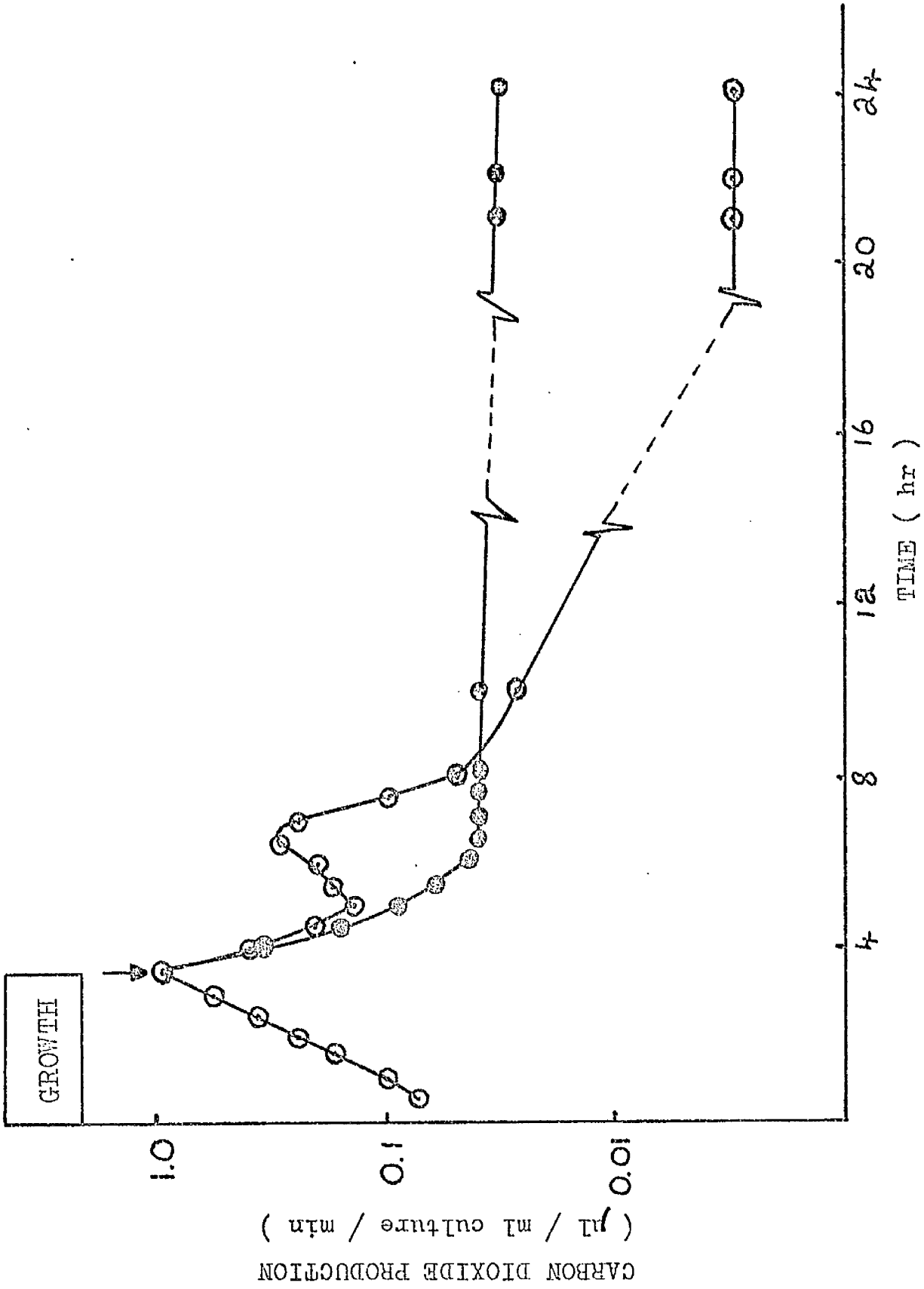

CARBON DIOXIDE PRODUCTION
(µl / ml culture / min)
1.0
0.1
0.01
GROWTH
4
8
12
16
20
24
TIME (hr)

FIGURE 26

Effect of chloramphenicol when added to a culture of *E.coli* at the end of growth on 2.0ml glucose, to a final concentration of 0.3ml.

(a) The effect of the addition of chloramphenicol on the activity of isocitrate dehydrogenase.

⊙ —— culture to which no drug was added.

● —— culture to which drug was added at point indicated by arrow (↓)

(b) The effect of the addition of chloramphenicol on the activity of isocitrate lyase.

⊙ ---- culture to which no drug was added

● —— culture to which drug was added at point indicated by arrow (↓)

(N.B. Times at which no enzyme values are plotted gave values below 10^2 units/ml culture.)

(c) The effect of the addition of chloramphenicol on the utilization of acetate accumulated during growth on glucose.

⊙ —— culture to which no drug was added

● —— culture to which drug was added at point indicated by arrow (↓)

FIGURE 26a

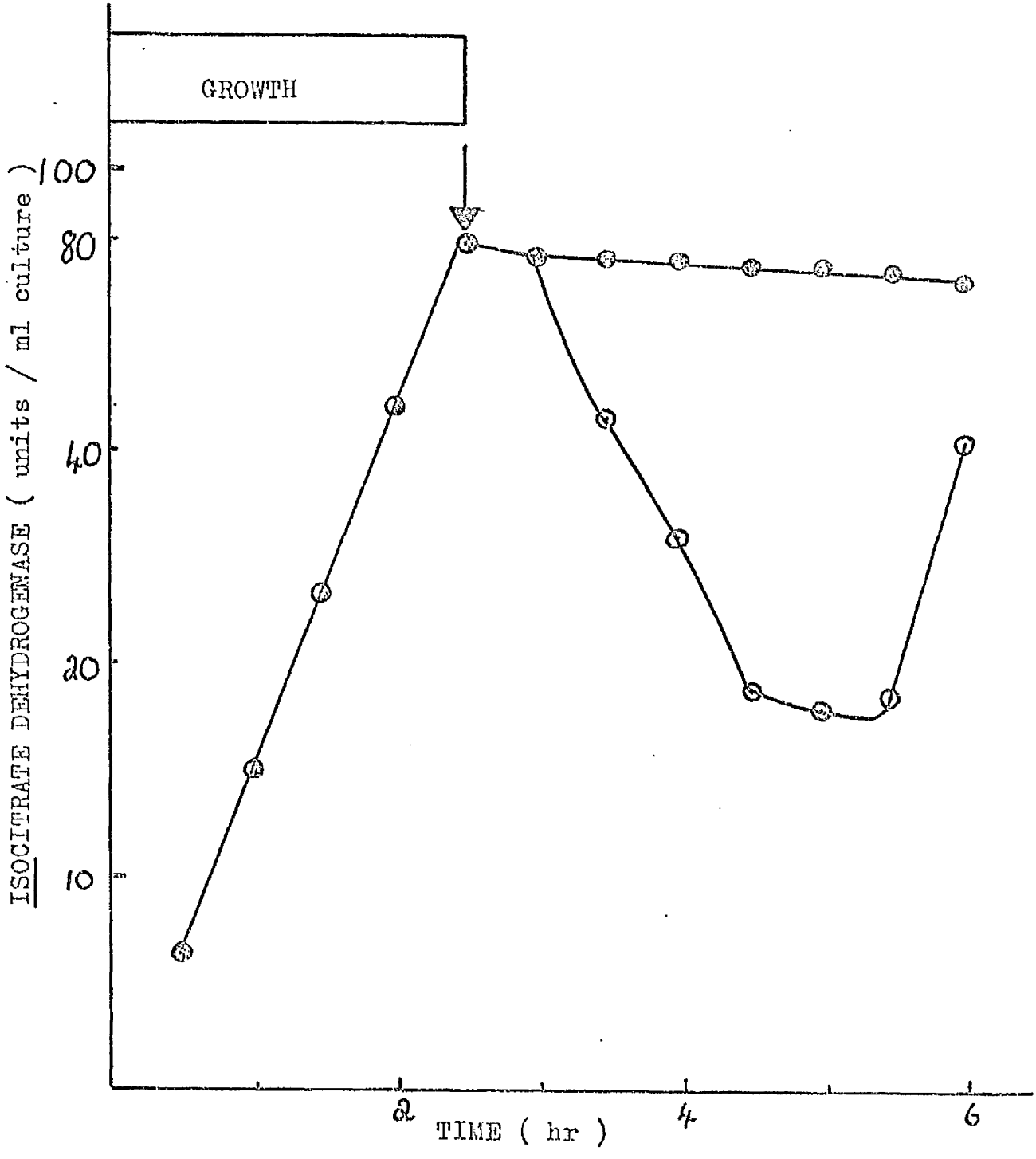

GROWTH
ISOCITRATE DEHYDROGENASE (units / ml culture)
100
80
40
20
10
2
4
6
TIME (hr)

FIGURE 26b

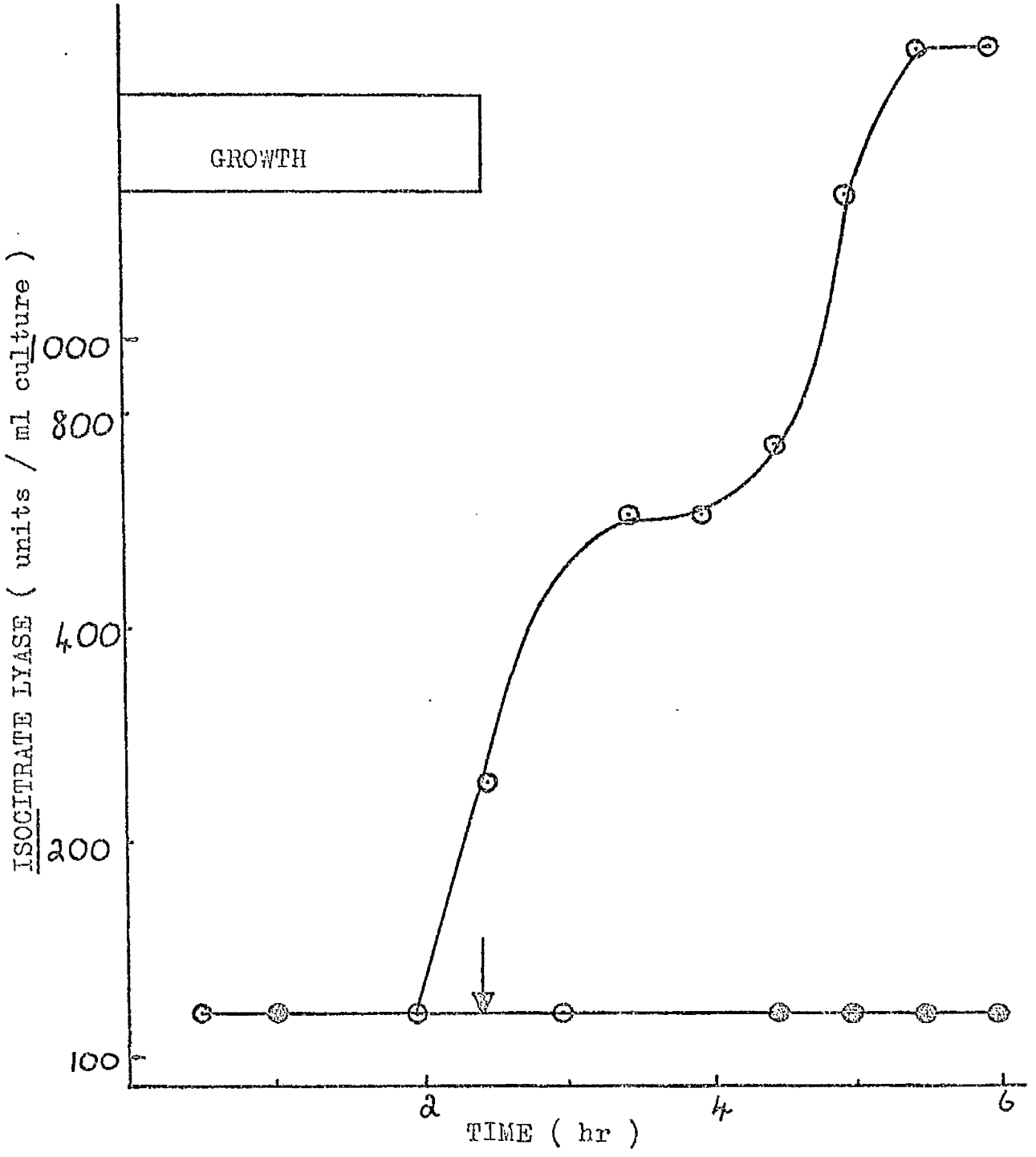
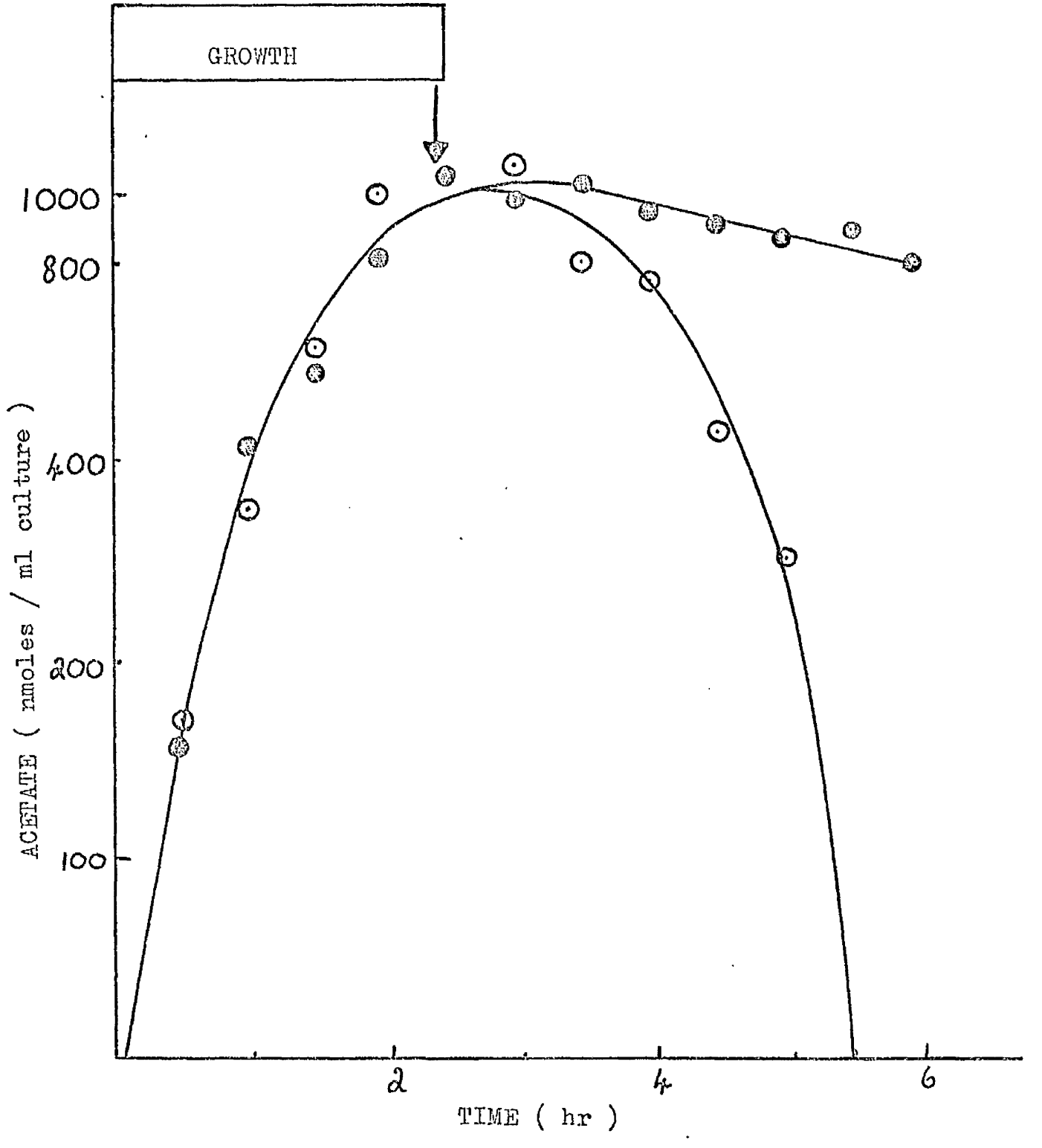

GROWTH
ISOCITRATE LYASE (units / ml culture)
1000
800
400
200
100
2
4
6
TIME (hr)

FIGURE 26c


GROWTH
1000
800
400
200
100
ACETATE (nmoles / ml culture)
2
4
6
TIME (hr)

A second group of compounds profoundly altered the loss and recovery profile, all but preventing loss of enzyme activity. However, a small fall in enzyme activity did occur and was later reversed. Those compounds, designated Class B, were oxaloacetate, aspartate and glyoxylate. All other compounds tested completely prevented loss of enzyme activity (Class C) and included succinate, fumarate, malate, alanine and serine.

The addition of pyruvate at the end of growth on glucose produced a response differing from those noted above. This result is presented in detail in Figure 27(a).

Effect of various compounds on isocitrate dehydrogenase when added at the end of growth on glycerol

E.coli was grown on glycerol (4.0mM). At the end of growth, those compounds described in the previous experiment were added and the effect on isocitrate dehydrogenase was again noted.

Again the results could be classified into the 3 categories A, B and C (Table 10), the letters retaining the meaning assigned in the previous experiment. All additions, with the exception of pyruvate and acetate, produced a C type response, i.e. no loss of enzyme activity. Acetate addition produced an A type response while addition of pyruvate induced a B type profile.

When acetate was added simultaneously with the various compounds, different responses were obtained, which nevertheless could be designated A, B or C. Thus simultaneous addition of acetate with

FIGURE 27

Effect of the addition of different carbon compounds to cultures of E. coli at the end of growth on 2.0ml glucose.

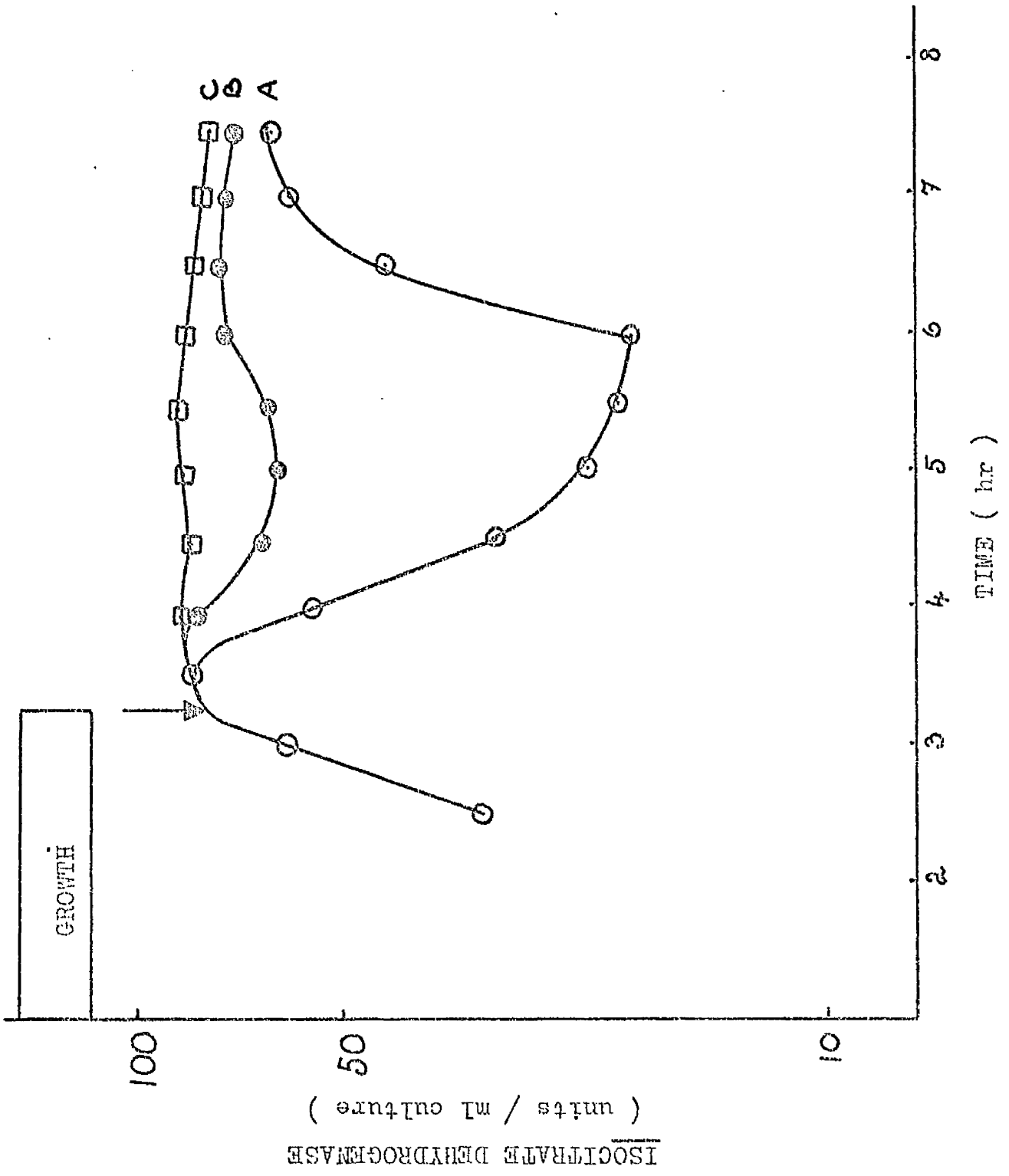
⊙ ---- culture to which no addition was made.

⊡ ---- culture to which fumarate was added to a final concentration of 1.0M at point indicated by arrow (↓).

● ---- culture to which glyoxylate was added to a final concentration of 1.0M at point indicated by arrow (↓).

The three types of curve obtained are designated A, B, C, as shown in the figure, and these symbols are used in Table 9 to indicate the type of curve obtained after each addition.

FIGURE 27


C
B
A
GROWTH
100
50
10
ISOCITRATE DEHYDROGENASE
(units / ml culture)
2
3
4
5
6
7
8
TIME (hr)

PAGE 9

Effect of adding various carbon compounds to cultures of E.coli at the end of growth on 2.0% glucose.

(N.B. the result obtained after the addition of pyruvate was unusual, and is dealt with more fully in Figure 27(a)).

CARBON SOURCE ADDED AT THE END OF GROWTH ON LIMITING (2.OmM) GLUCOSE ALL ADDITIONS TO 1.OmM	ISOCITRATE DEHYDROGENASE ACTIVITY PROFILE OBTAINED AFTER GROWTH ON LIMITING (2.OmM) GLUCOSE
no addition	A
pyruvate	A
acetate	A
2 — oxoglutarate	A
succinate	C
fumarate	C
malate	C
oxaloacetate	B
glyoxylate	B
alanine	C
serine	C
glutamate	A
aspartate	B
glycine	A

TABLE 9

FIGURE 27(a)

Effect of adding pyruvate to a culture of E.coli at the end of growth on 2.0ml glucose.

⊙— culture to which no addition was made.

●— culture to which pyruvate was added to a final concentration of 1.0Ml at point indicated by arrow (↓).

The addition of pyruvate at the end of growth on limiting glucose delayed the fall in isocitrate dehydrogenase activity by approximately 30 minutes, but did not prevent it. Once initiated, the whole effect was greatly exaggerated.

FIGURE 27a

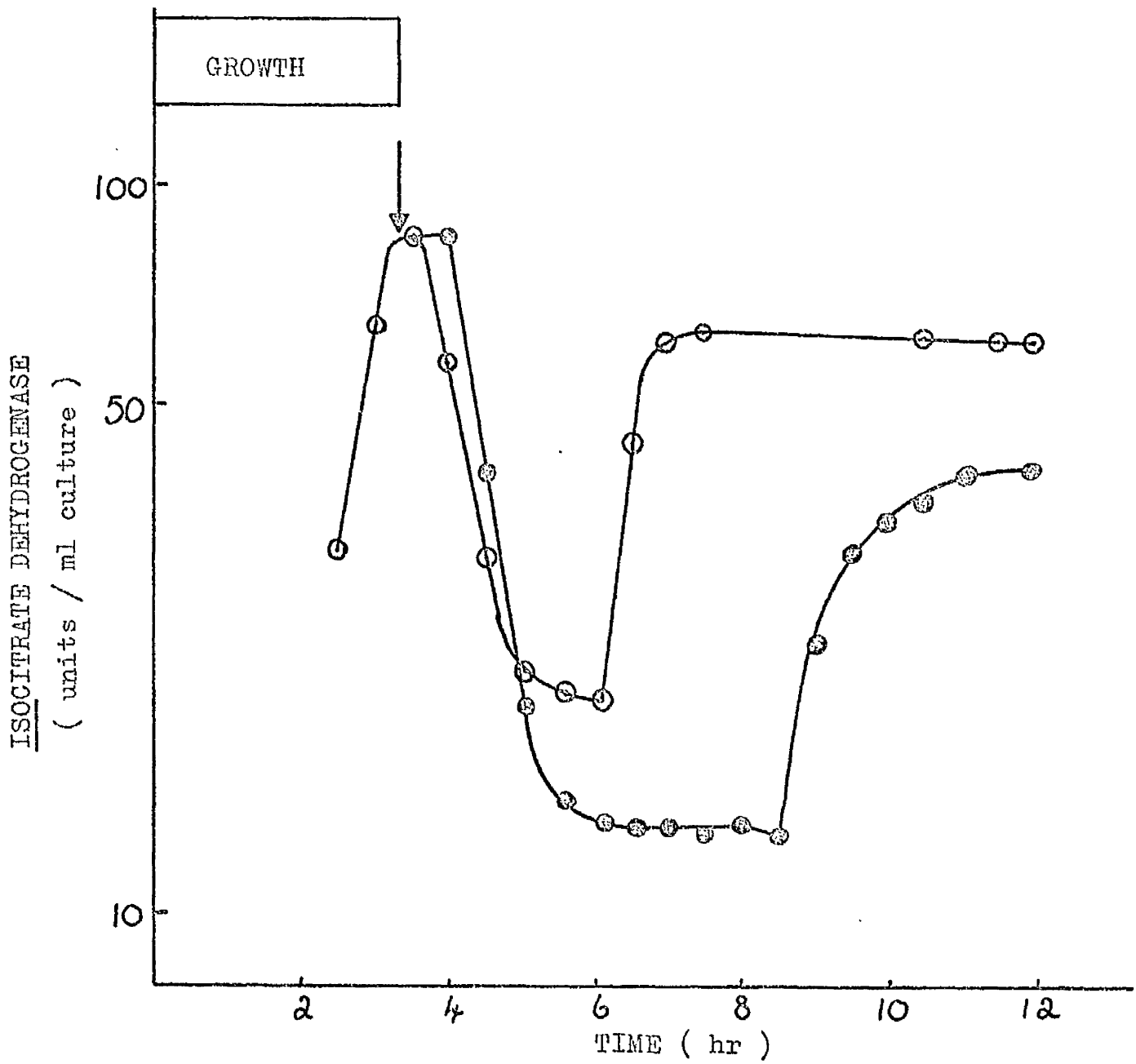

GROWTH
100
50
10
ISOCITRATE DEHYDROGENASE
(units / ml culture)
2
4
6
8
10
12
TIME (hr)

TABLE 10

Effect on isocitrate dehydrogenase of adding various carbon compounds, with or without acetate, to cultures of *E.coli* at the end of growth on 4.0ml glycerol.

Symbols A,B,C retain the meaning designated to them in legend to Figure 27.

* Again the addition of pyruvate, in the presence of acetate, initially delayed loss of enzyme activity and an exaggerated effect was obtained when loss of activity did occur, as has been reported following the addition of pyruvate at the end of growth on 2.0ml glucose.

CARBON SOURCE ADDED AT THE END OF GROWTH ON LIMITING (4.0mM) GLYCEROL ALL ADDITIONS TO 1.0mM	ISOCITRATE DEHYDROGENASE ACTIVITY PROFILE OBTAINED AFTER GROWTH ON LIMITING (4.0mM) GLYCEROL	
	no acetate added	1.0mM acetate added
no addition	C	A
* pyruvate	B	A
acetate	A	—
2 — oxoglutarate	C	A
succinate	C	B
fumarate	C	B
malate	C	C
oxaloacetate	C	B
glyoxylate	C	B
alanine	C	B
serine	C	C
glutamate	C	A
aspartate	C	B
glycine	C	A

TABLE 10

pyruvate, 2-oxoglutarate, glutamate or glycine produced a loss of enzyme activity while malate or serine completely prevented loss. The remaining compounds all antagonised, but did not completely prevent, decay of enzyme activity (response B) induced by acetate alone (response C). These compounds were succinate, fumarate, oxaloacetate, glyoxylate, alanine and aspartate.

Effect of acetate on isocitrate dehydrogenase when added after the cycle of loss and recovery of activity after growth on glucose

The addition of a small amount of acetate (1.0ml) to a culture of E. coli which had undergone an initial cycle of loss and recovery of isocitrate dehydrogenase activity, resulted in further loss, this time at a much faster rate than the loss occurring immediately after growth on glucose. Enzyme activity was again restored some time later (Figure 28).

Similar experiments were then performed, adding acetate, plus or minus various carbon compounds, and enzyme activity observed. Figure 29 shows the four types of result obtained, exemplified by the addition of acetate alone (response A), acetate + pyruvate (response C), acetate + glyoxylate (response B) and acetate + aspartate (response D). The change in time scale, hours to minutes, should be noted.

Table 11 records all the results obtained. Addition of acetate induced an immediate loss of isocitrate dehydrogenase activity which reached a minimal value 15-20 minutes after the addition (response A),

FIGURE 23

Effect on isocitrate dehydrogenase of adding acetate to a culture of E.coli 4 hours after growth on glucose ceased.

⊙ ····· culture to which no addition was made.

● —— culture to which acetate was added to 1.0ml (↓).

FIGURE 28

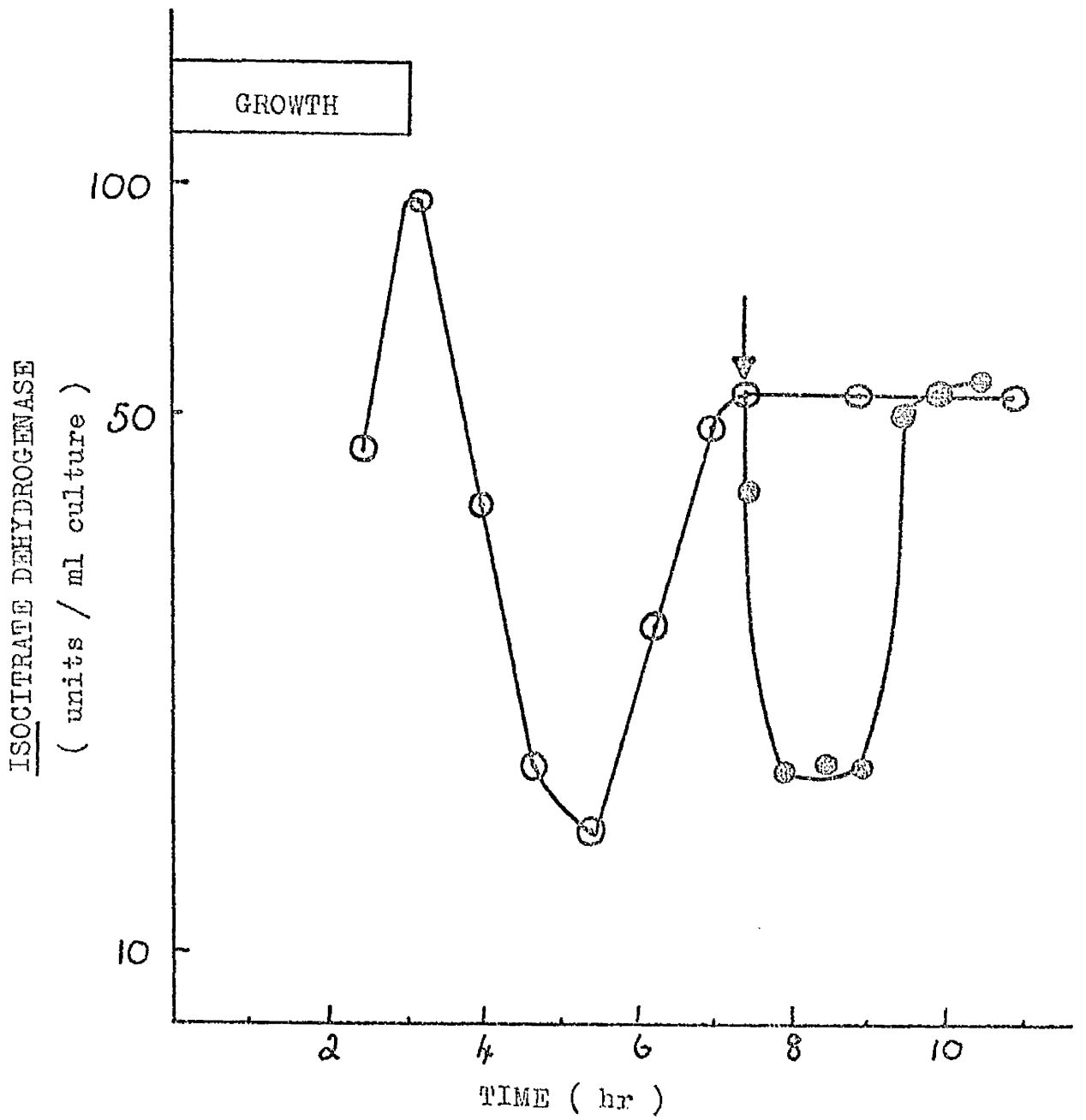

GROWTH
100
50
10
ISOCITRATE DEHYDROGENASE
(units / ml culture)
2
4
6
8
10
TIME (hr)

FIGURE 29

Effect of different carbon compounds, when added with acetate to cultures of *E. coli* 4 hours after growth on limiting glucose (2.0mM), on isocitrate dehydrogenase.

⊙ —— culture to which acetate alone was added (↓)

● ---- culture to which acetate plus pyruvate to 1.0mM was added at point indicated by arrow (↓).

⊡ —— culture to which acetate plus glyoxylate to 1.0mM was added at point indicated by arrow (↓)

△ ···· culture to which acetate plus L - aspartate to 1.0mM was added at point indicated by arrow (↓).

The four types of curve, obtained are designated A, C, D, E, as shown in the figure, and these symbols are used in Table 11 to indicate the type of curve obtained after each set of additions.

FIGURE 29

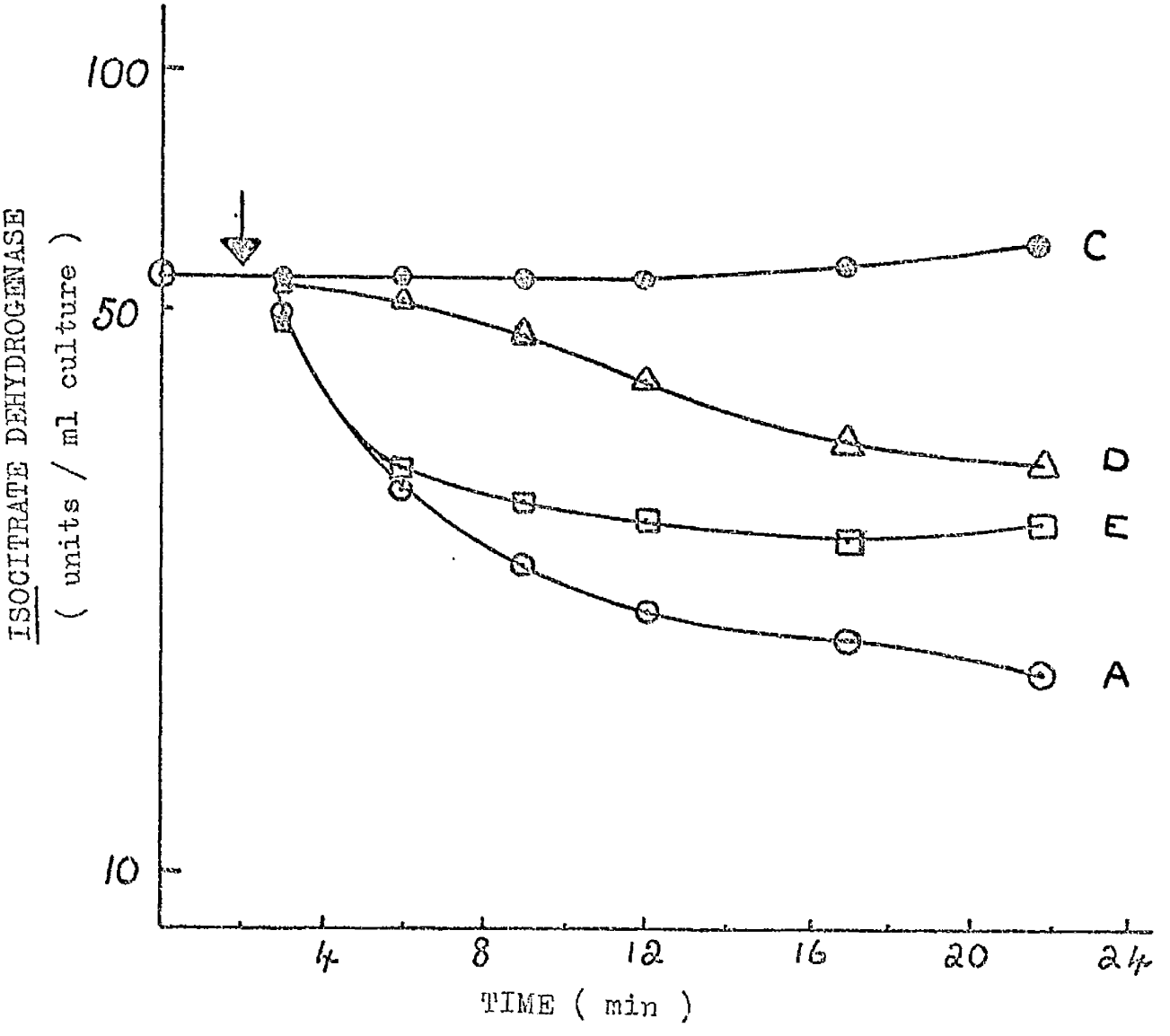

100
50
10
ISOCITRATE DEHYDROGENASE
(units / ml culture)
C
D
E
A
4
8
12
16
20
24
TIME (min)

TABLE 11

Effect of various compounds on the loss of isocitrate dehydrogenase induced by acetate in cells of E.coli 4 hours after growth on glucose ceased.

Symbols used in the Table, namely A, C, D, E are used according to the meaning assigned to each in Figures 27 and 29.

CARBON SOURCE ADDED TO 1.0mM ALONG WITH ACETATE TO 1.0mM AFTER ENZYME RECOVERY	ISOCITRATE DEHYDROGENASE ACTIVITY PROFILE OBTAINED AFTER ADDITIONS
no addition	A
pyruvate	C
2 — oxoglutarate	A
succinate	E
fumarate	E
malate	E
oxaloacetate	C
glyoxylate	E
alanine	D
serine	C
glutamate	A
aspartate	D
glycine	D

TABLE 11

and this result was unaffected by the simultaneous addition of 2-oxoglutarate or glutamate.

Addition of pyruvate, oxaloacetate or serine with the acetate completely abolished loss of enzyme activity (response C). Addition of other compounds, along with acetate, did not completely prevent decay of enzyme activity, but some antagonism was apparent. Two basic antagonistic effects were evident. The first of these alternatives was obtained after the addition of acetate plus succinate, fumarate, malate or glyoxylate. Enzyme activity was initially lost at the same rate as after the addition of acetate alone, but loss ceased 4 minutes after the addition. No further loss occurred and after a further 10-15 minutes enzyme activity began to rise (response E).

The second alternative was obtained following the addition of acetat plus alanine, asparate or glycine. These additions markedly slowed the rate at which enzyme activity decayed, but a minimal value approximating to the minimal level registered with response E was attained (response D)

Effect of sugars on isocitrate dehydrogenase when added simultaneously with acetate after the cycle of loss and recovery of enzyme activity after growth on glucose

The strain of E.coli used in these studies carries a mutation in the regulator gene of the lactose operon which results in the constitutive synthesis of the enzymes required for lactose metabolism.

When glucose, lactose or galactose were added simultaneously with acetate, as in the previous experiment, the results shown in Figure 30 were obtained. Glucose and lactose complete prevented the

FIGURE 30

Behaviour of isocitrate dehydrogenase activity when various carbohydrates plus acetate were added to cultures of E.coli 4 hours after growth on 2.0ml glucose ceased.

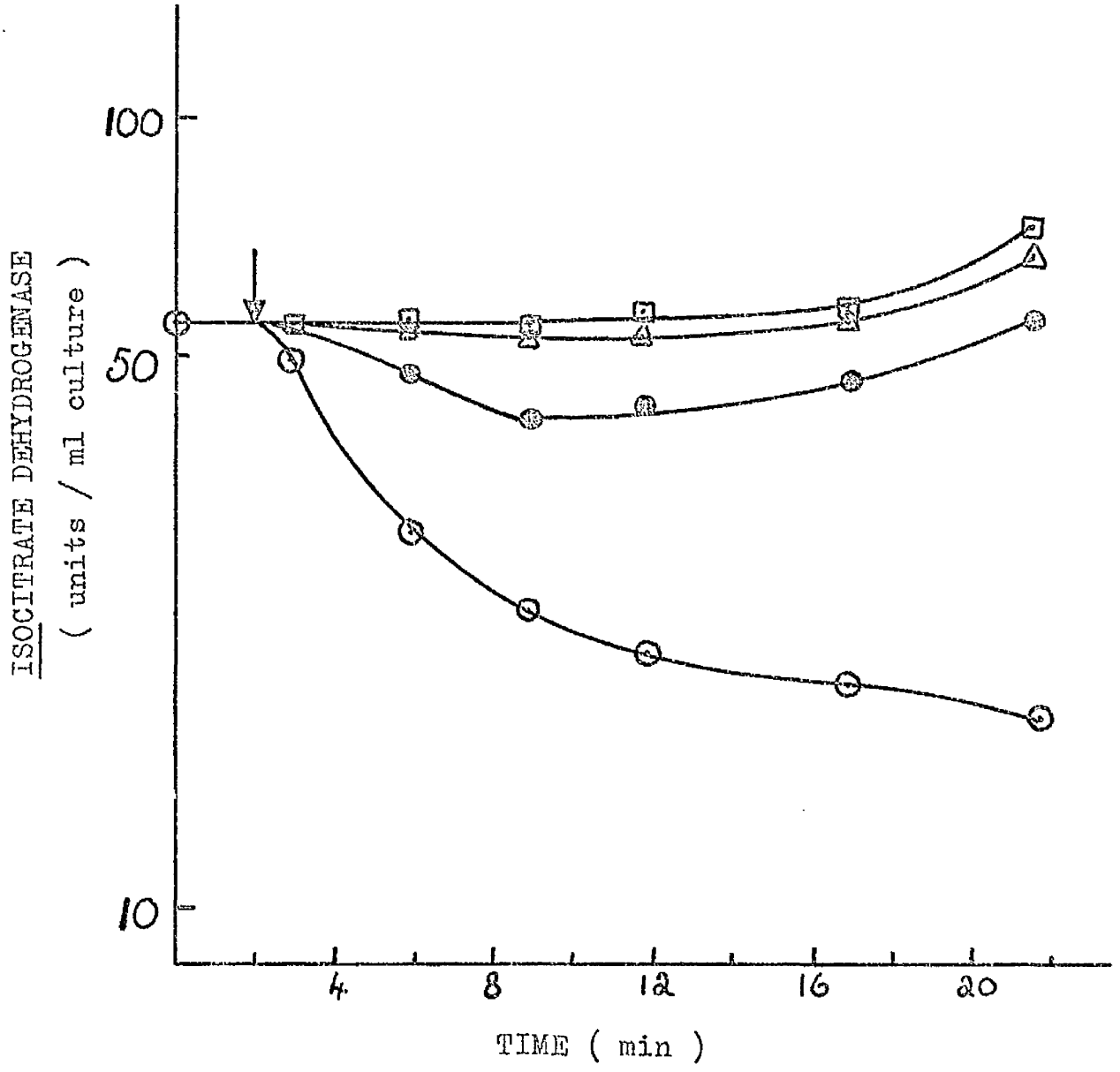
⊙ ---- culture to which acetate alone was added (↓)

⊡ · · · culture to which acetate plus glucose to 1.0ml was added at point indicated by arrow (↓).

△ - - - culture to which acetate plus lactose to 1.0ml was added at point indicated by arrow (↓).

● -·-· culture to which acetate plus galactose to 1.0ml was added at point indicated by arrow (↓).

FIGURE 30


ISOCITRATE DEHYDROGENASE
(units / ml culture)
100
50
10
4
8
12
16
20
TIME (min)

loss of enzyme activity induced by acetate. The addition of galactose simultaneously with acetate did not initially prevent, but did, however, slow down the rate at which activity was lost. 5 minutes after addition, loss of isocitrate dehydrogenase activity was halted and the lost enzyme activity was restored.

STUDY OF ISOCITRATE DEHYDROGENASE ACTIVITY DURING GROWTH ON ACETATE

Isocitrate dehydrogenase activity during growth on glucose or acetate

E.coli, trained through 3 serial subcultures on glucose or acetate, was inoculated into fresh homologous media. The activity of isocitrate dehydrogenase under either growth condition can be seen in Table 12.

Effect of glucose and pyruvic acid on isocitrate dehydrogenase in E.coli growing on acetate

The addition of glucose (2.0mM) or pyruvate (1.0mM) to cells growing on acetate (30.0mM) elicited an immediate increase in isocitrate dehydrogenase activity (Figure 31). Pyruvate produced the greater effect and after the addition, enzyme specific activity rose from 24.0 to 88.0 and then fell to the level registered before the addition.

After glucose was added, enzyme specific activity swiftly rose to 55.0, and thereafter continued to rise, but more slowly.

Addition of pyruvate (1.0mM) produced little change in growth rate, whereas addition of glucose led to a faster growth, which was maintained till glucose was exhausted.

Effect of chloramphenicol on isocitrate dehydrogenase when added simultaneously with pyruvate or glucose during growth on acetate

Figure 32 shows that when pyruvate, plus or minus chloramphenicol, was added to a culture of E.coli growing on acetate (30.0mM), enzyme activity increased, initially at the same rate, in both systems.

TABLE 12

Differential rates of synthesis of isocitric dehydrogenase activity in cells of E. coli growing on glucose or acetate.

CARBON AND ENERGY SOURCE	INITIAL CONCENTRATION OF CARBON SOURCE IN GROWTH MEDIUM (mM)	P – VALUE ISOCITRATE DEHYDROGENASE
GLUCOSE	2	85
ACETATE	30	28

TABLE 12

FIGURE 31.

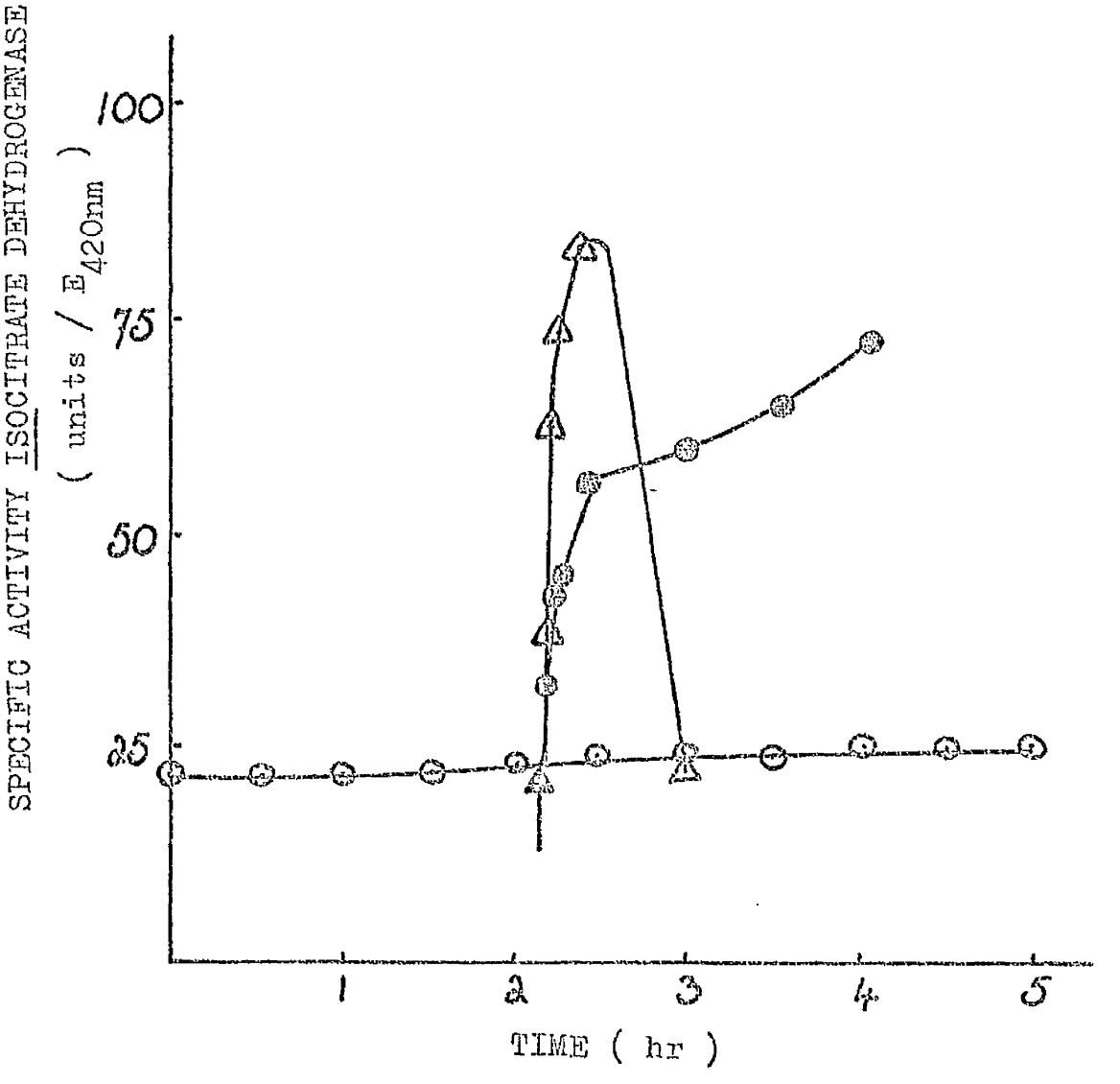
Effect of adding glucose or pyruvate, to cultures of *E.coli*, growing on 30.0ml acetate, on the activity of isocitrate dehydrogenase.

⊙ —— culture to which no addition was made.

● —— culture to which glucose was added to 2.0ml at point indicated by arrow (↑).

△ —— culture to which pyruvate was added to 1.0ml at point indicated by arrow (↑).

FIGURE 31


SPECIFIC ACTIVITY ISOCITRATE DEHYDROGENASE
(units / E_{420nm})
100
75
50
25
1
2
3
4
5
TIME (hr)

Addition of drug, however, lowered the maximum specific activity achieved. After reaching a maximum, enzyme activity in both cases fell to the level recorded before the additions.

When glucose alone was added, the result described in the last experiment was obtained. When chloramphenicol was also added, increase in enzyme activity was slight and transient (Figure 33).

Addition of chloramphenicol considerably slowed down, but did not immediately halt, increase in culture turbidity.

Addition of drug 2 hours before the addition of pyruvate or glucose did not alter the response from that obtained after simultaneous addition.

Effect of glucose, plus or minus chloramphenicol, on isocitrate dehydrogenase when added during growth on acetate, the cells having been previously grown on glucose

Glucose trained E.coli were inoculated into fresh glucose (2.0mM) medium and isocitrate dehydrogenase activity followed. When enzyme activity reached a minimum value after growth ceased, acetate (30.0mM) was added. Growth resumed, at the expense of acetate and enzyme activity was maintained at a low level. After one generation, glucose, plus or minus chloramphenicol, was added, either addition resulting in a swift rise in enzyme activity (Figure 34 cf., Figure 33).

Addition of drug 2 hours before glucose did not significantly alter the response from that obtained after simultaneous addition of glucose and drug.

FIGURE 32

Behaviour of the activity of isocitrate dehydrogenase when pyruvate, plus or minus chloramphenicol, was added to cultures of *E. coli* growing on 30.0 mM acetate.

⊙ ---- culture to which no addition was made.

△ —— culture to which pyruvate was added to 1.0 ml at point indicated by arrow (↑).

● ---- culture to which pyruvate was added to 1.0 ml, plus chloramphenicol to 0.3 mM, at point indicated by arrow (↑).

FIGURE 32

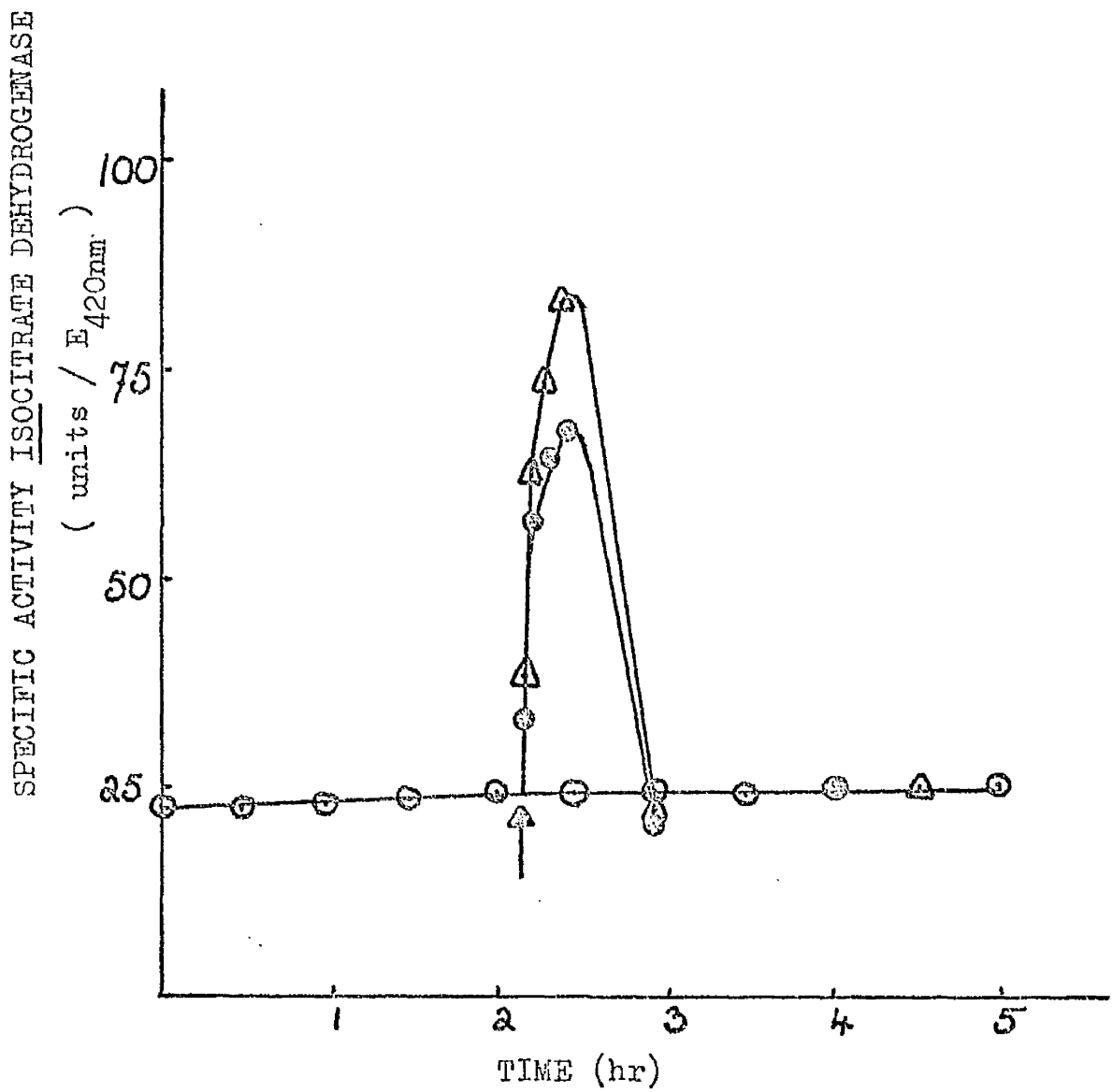

SPECIFIC ACTIVITY ISOCITRATE DEHYDROGENASE
(units / E_{420nm})
100
75
50
25
1
2
3
4
5
TIME (hr)

FIGURE 33

Behaviour of the activity of _in vivo_ to dehydrogenase when glucose, plus or minus chloramphenicol, was added to cultures of E.coli growing on 30 ml medium.

⊙ ..... culture to which no addition was made.

△ ..... culture to which glucose was added to 2 ml at point indicated by arrow (↑).

● ..... culture to which glucose was added to 2 ml, plus chloramphenicol to 0.3 ml, at point indicated by arrow (↑).

FIGURE 33

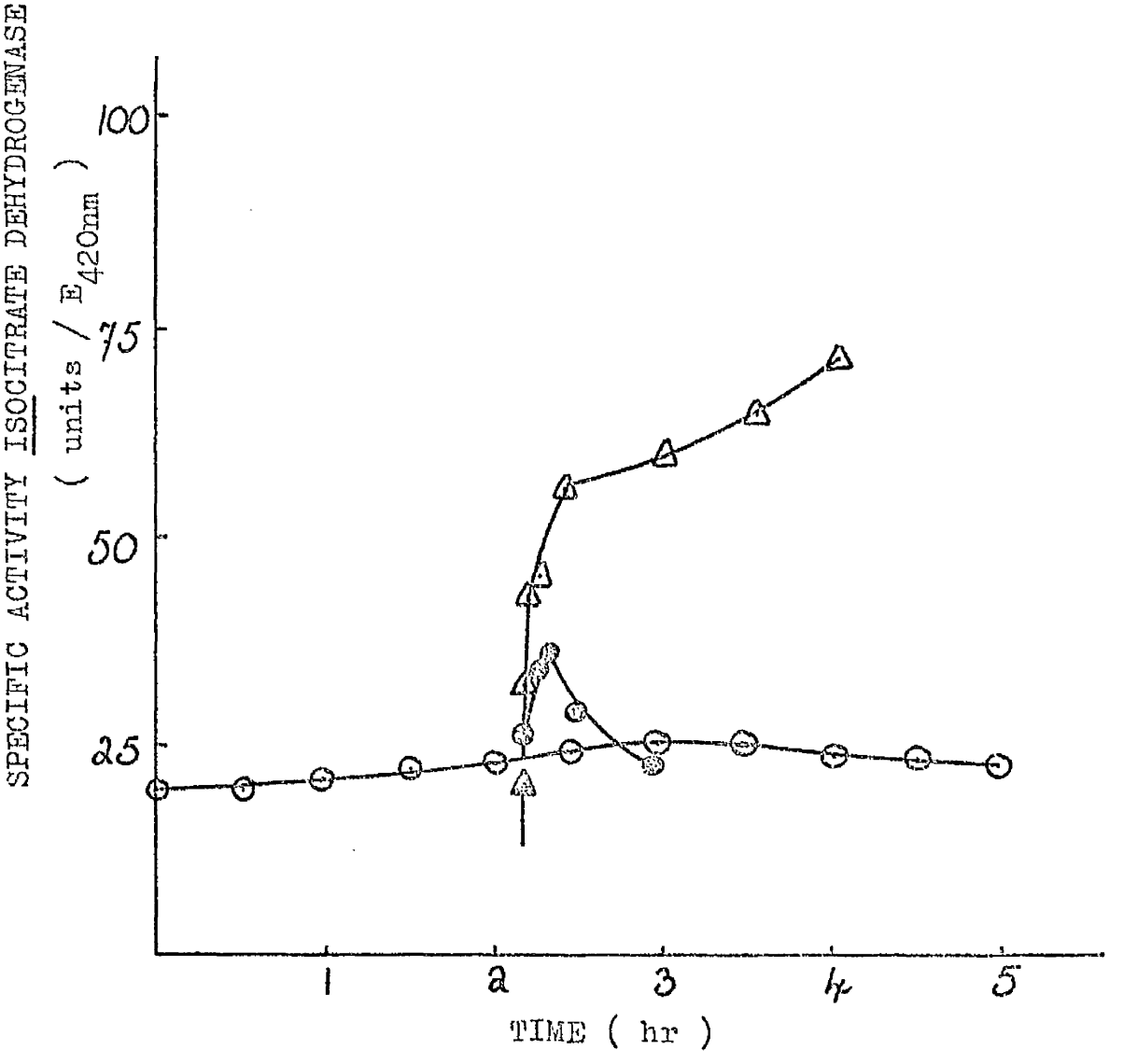

SPECIFIC ACTIVITY ISOCITRATE DEHYDROGENASE
(units / E_{420nm})
100
75
50
25
1
2
3
4
5
TIME (hr)

FIGURE 24

Effect on isocitrate dehydrogenase activity of adding glucose, plus or minus chloramphenicol, to cultures of E.coli growing on 30.0mM acetate, but previously grown on glucose.

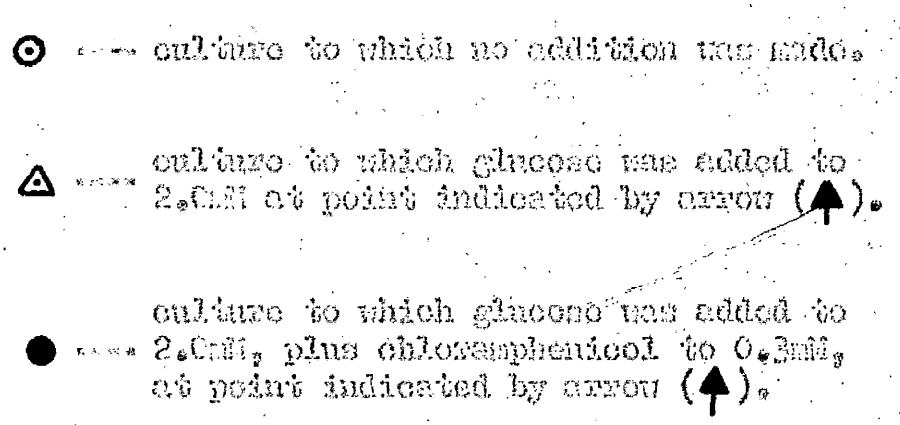
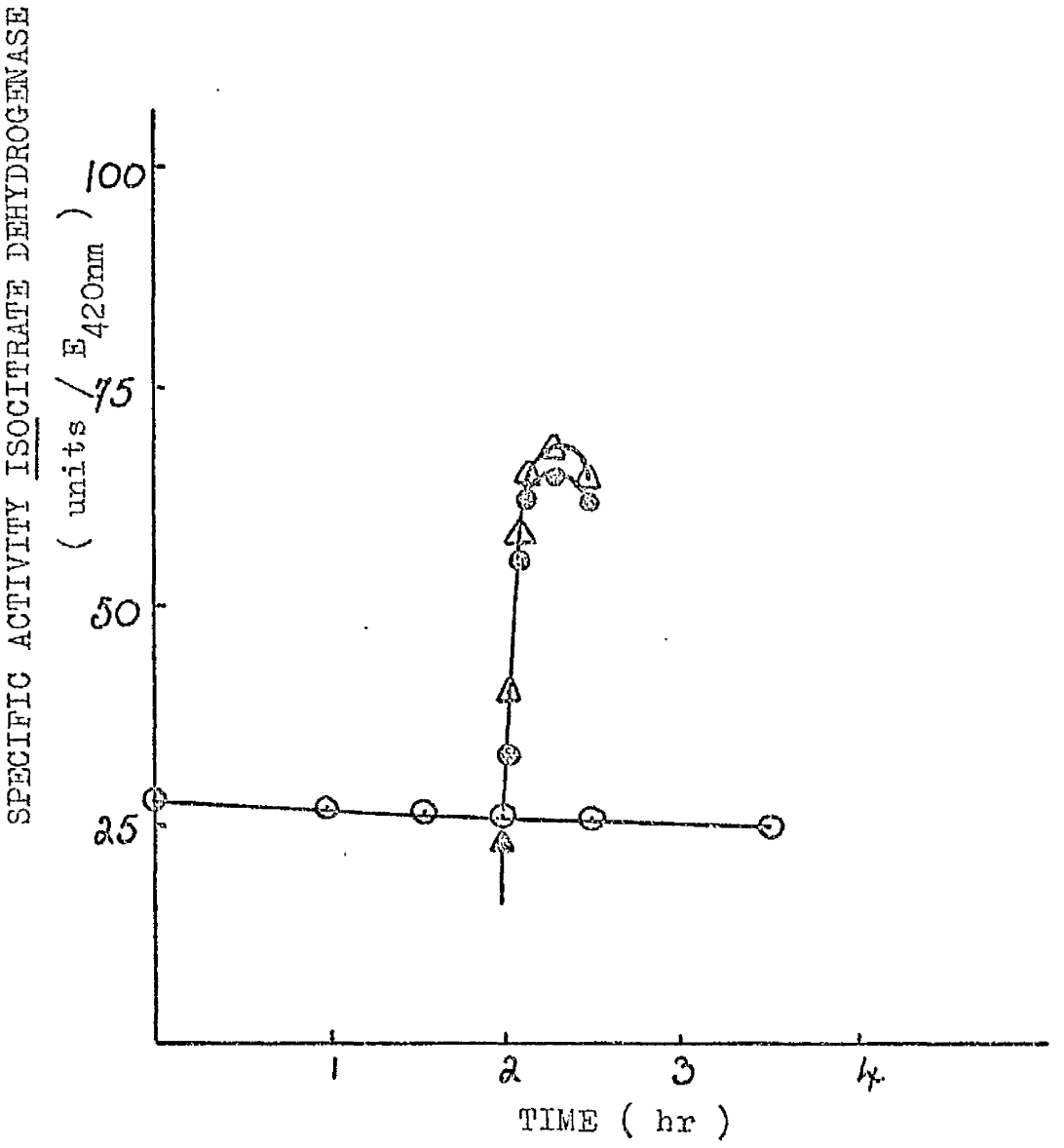


FIGURE 34


SPECIFIC ACTIVITY ISOCITRATE DEHYDROGENASE
(units / E_{420nm})
100
75
50
25
1
2
3
4
TIME (hr)

Effect of resuspending acetate grown E.coli in a basal salts medium on isocitrate dehydrogenase

Cells, grown on 30mM acetate from an acetate trained inoculum, were harvested at a turbidity of 1.00, after 4 generations' growth, by centrifuging at 10,000g for 15 minutes at 4°C. Isocitrate dehydrogenase, followed throughout growth, had a P-value of 28.0. The specific activity of the enzyme at time of harvesting, but prior to centrifugation was calculated. After centrifugation, the resultant cell pellet was resuspended in chilled basal salts medium (growth medium lacking a carbon source), to a turbidity of 50.0. Enzyme specific activity in the resuspended cells was estimated. The resuspended cells were inoculated into fresh acetate (30.0mM) growth medium or basal salts medium, maintained at 37°C. The inoculated media were vigorously agitated and aerated from the time of inoculation.

The specific activity of isocitrate dehydrogenase at harvesting was 27.5 (cf., P-value = 28.0). After resuspension this value had risen to 58.0 (Table 13). On reinoculation into fresh acetate medium, enzyme specific activity quickly fell to the value registered at harvesting. Inoculation into basal salts medium produced a completely different result. Enzyme activity continued to rise and reached a value of 104, 40 minutes after reinoculation, which was maintained.

Increases in enzyme specific activity occurred in the absence of a carbon and energy source, and in this system no increase in turbidity was observed over the 60 minutes following inoculation into basal salts medium.

TABLE 13

Effect of removing, and then adding back, acetate to cultures of E.coli, grown on 30.0mM acetate, on the activity of isocitrate dehydrogenase.

STATE OF CELLS EXAMINED FOR ENZYME ACTIVITY	SPECIFIC ACTIVITY OF ISOCITRATE DEHYDROGENASE	
IMMEDIATELY PRIOR TO HARVESTING	27.5	
AFTER HARVESTING AND RESUSPENSION IN CHILLED BASAL SALTS MEDIUM BUT PRIOR TO REINOCULATION	58.0	
TIME AFTER REINOCULATION (min)	medium into which cells were reinoculated	
	basal salts medium	basal salts medium + 30mM acetate
0.5	59.0	38.0
20	92.0	30.0
40	104.0	22.5
60	106.0	23.0

TABLE 13

TABLE 14

Effect of removing acetate, in the presence of chloramphenicol, from cultures of *E. coli*, grown on 30 mM acetate, on the activity of isocitrate dehydrogenase.

STATE OF CELLS EXAMINED FOR ENZYME ACTIVITY	SPECIFIC ACTIVITY OF ISOCITRATE DEHYDROGENASE	
IMMEDIATELY PRIOR TO HARVESTING	21.8	
AFTER HARVESTING AND RESUSPENSION IN CHILLED BASAL SALTS MEDIUM BUT PRIOR TO REINOCULATION	37.0	
TIME AFTER REINOCULATION (min)	medium into which cells were reinoculated	
	basal salts medium	basal salts medium + 30mM acetate
0.25	41.1	38.8
15	74.4	30.8
33	91.1	31.9
48	89.3	29.3

TABLE 14

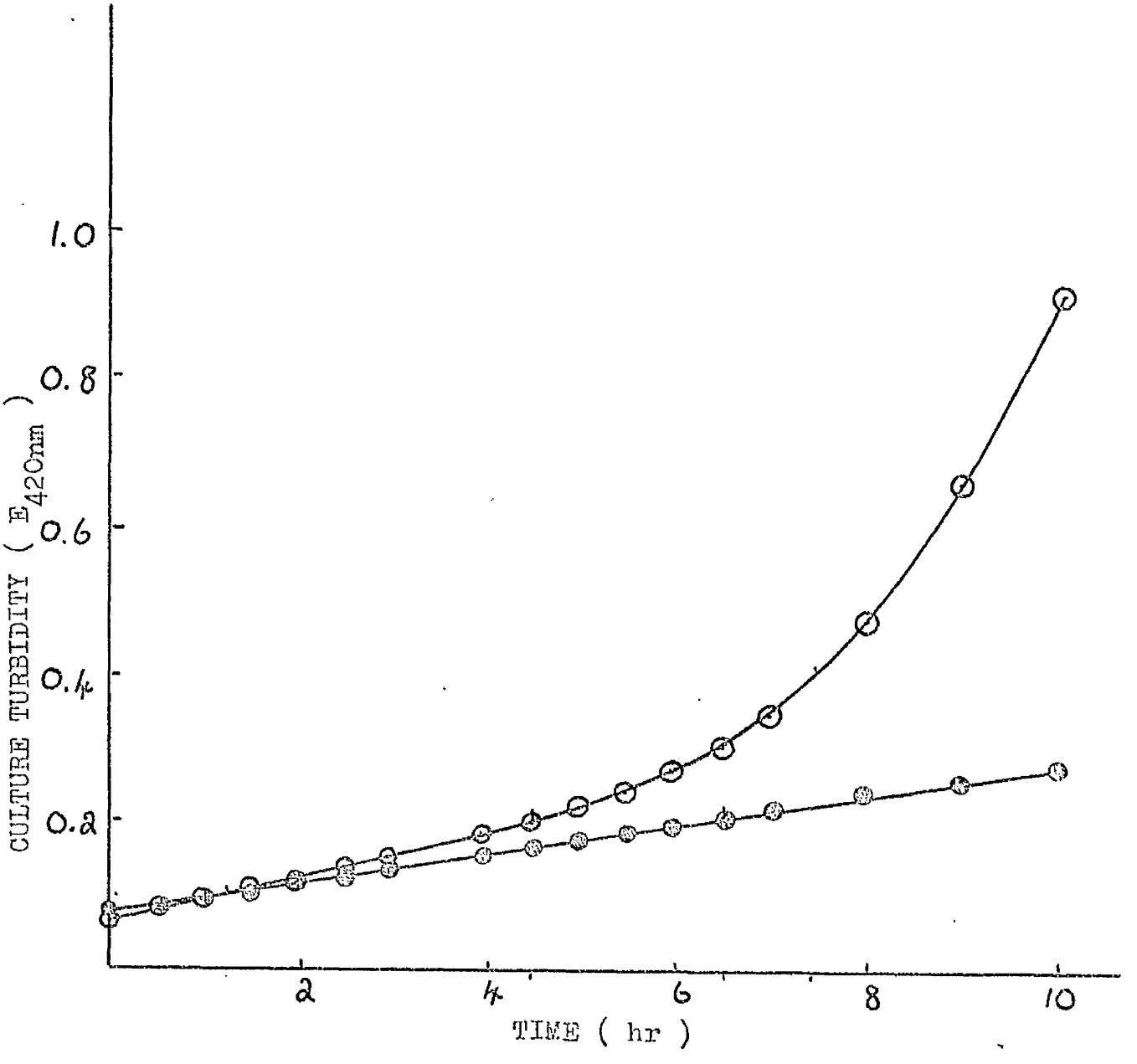
FIGURE 35

Growth of E.coli, previously trained to grow on 30.0mM acetate, after inoculation into either 6.0mM or 30.0mM acetate.

⊙ —— cells growing in medium which initially contained 30.0mM acetate.

● —— cells growing in medium which initially contained 6.0mM acetate.

FIGURE 35



The experiment was repeated, but one hour before the cells were harvested chloramphenicol (0.3mM) was added to the culture. After harvesting the cells were resuspended in chilled basal salts medium containing chloramphenicol (0.3mM) and reinoculated into basal salts medium, with or without acetate (30.0mM), but containing chloramphenicol (0.3mM) and maintained at 37°C. The specific activity of isocitrate dehydrogenase was calculated at each step.

The results (Table 14) show that the addition of chloramphenicol did not alter the result obtained in its absence (cf., Table 13).

Growth of E.coli on limiting acetate

An interesting result emerged during studies of growth on different concentrations of acetate. Cells previously grown on 30mM acetate, were inoculated into media containing 30mM or 6.0mM acetate.

Cells inoculated into the higher acetate concentration grew logarithmically, while cells inoculated into the lower acetate concentration grew arithmetically (Figure 35).

INHIBITION AND PHYSICAL STUDIES ON ISOCITRATE DEHYDROGENASE

Isocitrate dehydrogenase in extracts of glucose grown E.coli

Cells from a culture grown on glucose (2.0mM) were periodically harvested by centrifugation at 10,000g for 15 minutes at 4°C. The culture was sampled:-

(1) immediately before growth on glucose ceased, when isocitrate dehydrogenase activity was high.

(2) 2½ hours after growth ceased, when enzyme activity was at a minimum.

(3) 4 hours after growth ceased, when enzyme activity had been restored.

Equal volumes of cell extracts containing high and low enzyme activities were mixed and the activity of isocitrate dehydrogenase in the combined extract measured. This was found to equal the sum of the activities in the individual components of the mixture. A similar result was obtained on mixing cell extracts with low and recovered activity or with high and recovered activity.

activities were mixed and the activity of isocitrate dehydrogenase in

Effect of various intermediary metabolites on the activity of isocitrate dehydrogenase

A culture of E.coli was grown on glucose (2.0mM) and cells harvested, by centrifugation at 10,000g for 15 minutes at 4°C, during growth and 2½ hours after growth ceased. The cells, washed by resuspending in chilled 0.15M NaCl and recentrifuging, were resuspended in ice cold 0.15M NaCl containing 5 mg/ml bovine plasma albumin. 3ml volumes of these resuspensions were sonicated to release enzyme activity

and homologous sonicated suspensions combined till the required volume of extract was achieved.

Enzyme assay reaction mixtures were set up containing enzyme but not $NADP^+$. To these were added the various compounds to be tested, and the mixtures were left at 27°C for 5 minutes. The cuvettes were then transferred to the Unicam SP800 and the reaction started by addition of $NADP^+$. All compounds tested were at a final concentration of 1.0mM, with the exception of acetyl-coenzymeA which was added to 0.1mM. The results can be seen in Tables 15 and 16.

When the compounds were added singly, only 2-oxoglutarate and oxaloacetate affected enzyme activity. However, when pairs of compounds were added it was found that a mixture of oxaloacetate and glyoxylate produced severe inhibition of enzyme activity. No other pair of compounds significantly changed enzyme activity, but a mixture of 2-oxoglutarate and oxaloacetate did produce a greater inhibition than either individual compound. Enzyme activity was affected to the same degree regardless of which extract was used.

Effect of high energy compounds on isocitrate dehydrogenase

Enzyme assays were prepared containing high or low isocitrate dehydrogenase activity cell extracts (from glucose-grown cells), but lacking $NADP^+$. The compound to be tested was added to a final concentration of 1.0mM, mixed and mixtures left at 27°C for 5 minutes. The enzyme reaction was initiated by addition of $NADP^+$.

TABLE 15

Effect of various intermediary metabolites on the activity of isocitrate dehydrogenase from E.coli.

All carbon compounds with the exception of acetyl-coenzymeA (0.1mM), were present in the assay at a final concentration of 1.0mM.

FOR HIGH ENZYME ACTIVITY EXTRACTS

0% inhibition = 68.5 units enzyme / assay.

FOR LOW ENZYME ACTIVITY EXTRACTS

0% inhibition = 14.2 units enzyme / assay.

CARBON SOURCE ADDED IN CONJUNCTION WITH PRIMARY COMPOUND	PERCENTAGE INHIBITION REGISTERED IN THE PRESENCE OF CARBON SOURCES									
	acetate	acetyl – coenzymeA	citrate	cis – aconitate	2 – OXO – glutarate	succinate	fumarate	malate	oxaloacetate	glyoxylate
no addition	0	0	0	2	10	2	0	0	13	5
acetate	—	0	0	0	8	2	2	0	13	4
acetyl – coenzymeA	—	—	0	3	11	5	—	4	17	4
citrate	—	—	—	2	9	0	0	2.5	9	0
cis – aconitate	—	—	—	—	8	3.5	2	2	13	0
2 – oxo – glutarate	—	—	—	—	—	8.5	11	8	18.5	12.5
succinate	—	—	—	—	—	—	0	0	13.5	4
fumarate	—	—	—	—	—	—	—	2	15	6
malate	—	—	—	—	—	—	—	—	7	0
oxalo – acetate	—	—	—	—	—	—	—	—	—	88

TABLE 15

page 16

Effect of various intermediary metabolites, plus or minus either pyruvate or phosphoenol-pyruvate, on the activity of isocitrate dehydrogenase from cells of E. coli, after growth on 2.0mM glucose, and possessing a lowered isocitrate dehydrogenase activity.

0% inhibition ≡ 35 units enzyme / assay

Specific activity isocitrate dehydrogenase in extract = 26 units / optical density unit (E_{420nm}).

ADDITION TO ASSAY (1.0mM)	PERCENTAGE INHIBITION OF ISOCITRATE DEHYDROGENASE		
	NO ADDITION	PYRUVATE TO 1.0mM	PHOSPHOENOL – PYRUVATE TO 1.0mM
pyruvate	−1.0	—	0
phosphoenolpyruvate	1.0	0	—
acetate	0	−2.0	3.5
citrate	−2.5	−2.0	1.0
cis – aconitate	−3.0	−2.0	1.0
2 – oxoglutarate	12.5	9.0	12.0
succinate	−2.0	−4.0	2.0
fumarate	−2.0	−4.0	0
malate	−3.0	−4.0	0
oxaloacetate	30.0	11.5	20.5
glyoxylate	0	1.5	5.0

TABLE 16

TABLE 17

Effect of various high energy compounds on the activity of isocitrate dehydrogenase from cells of E.coli

(a) harvested just before the cessation of growth on 2.0ml glucose, and containing high enzyme activity

and

(b) harvested when isocitrate dehydrogenase activity had reached its lowest value.

(a) control - 0% inhibition ≡ 80 units enzyme / assay

Specific activity isocitrate dehydrogenase = 80

(b) control - 0% inhibition ≡ 34 units enzyme / assay

Specific activity isocitrate dehydrogenase = 24.

ADDITION TO ASSAY (1.0mM)	PERCENTAGE INHIBITION OF ISOCITRATE DEHYDROGENASE	
	EXTRACT WITH HIGH ISOCITRATE DEHYDROGENASE ACTIVITY	EXTRACT WITH LOW ISOCITRATE DEHYDROGENASE ACTIVITY
no addition	0	0
ATP	40	40
ADP	15	21
AMP	4	4
cyclic – 3',5' – AMP	8	11
acetyl – phosphate	0	0
phosphoenolpyruvate	10	15
coenzymeA	10	14

TABLE 17

The presence of ATP lowered the rate of reaction by 40%. Both extracts tested gave similar results (Table 17).

Effect of dialysis of cell extracts in which isocitrate dehydrogenase was inhibited by the combined effect of oxaloacetate and glyoxylate

Oxaloacetate (3.0mM) and glyoxylate (3.0mM) were added to cell extracts of glucose (2.0mM) grown E.coli possessing either high or low isocitrate dehydrogenase activity. On assay, inhibitors were diluted to 1.0mM. Inhibited and non-inhibited cell extracts were dialysed for 15 hours against 1000 volumes of 0.15M NaCl at 4°C. The results are presented in Table 18.

Addition of both oxaloacetate and glyoxylate to either extract produced severe inhibition of isocitrate dehydrogenase activity. Dialysis significantly relieved inhibition and 50% activity was recovered in both extracts. Dialysis of non-inhibited cell extracts did not significantly affect enzyme activity, nor did storage at 4°C for 15 hours.

Effect of heat on isocitrate dehydrogenase activity

Effect of heat on isocitrate dehydrogenase activity cells harvested, by centrifugation at 10,000g for 15 minutes at 4°C, during logarithmic growth, 2½ hours and 4 hours after growth ceased. Cell extracts, prepared as described in the legends to figures 36(a) and (b), were subjected to a variety of heat treatments.

Effect of dialysis of cell extracts containing isocitrate dehydrogenase inhibited by the presence of oxaloacetate and glyoxylate.

TREATMENT TO WHICH ENZYME WAS SUBJECTED	ISOCITRATE DEHYDROGENASE			
	MAXIMUM ACTIVITY EXTRACT		MINIMUM ACTIVITY EXTRACT	
	units per ml culture	% initial activity	units per ml culture	% initial activity
—	50.4	100	16.4	100
dialysis	44.0	87.0	14.9	91.0
(3.0mM glyoxylate + 3.0mM oxaloacetate)	0.8	1.6	0	0
(3.0mM glyoxylate + 3.0mM oxaloacetate) + dialysis	24.6	50.5	8.1	49.5

TABLE 18

FIGURE 36(a)

Effect of increasing temperature on the activity of isocitrate dehydrogenase in extracts of *E. coli* NL308.

Cells of *E. coli* were grown on 2.0% glucose and harvested at various times. The harvested cells were washed with 0.15M NaCl and recentrifuged. The resultant cell pellet was resuspended in 0.15M NaCl to a bacterial protein concentration of 5 mg/ml. 3.0ml volumes were disrupted using ultrasonics, and the activity of isocitrate dehydrogenase was assayed. The remainder of the extract was then maintained at the required temperature for 10 minutes and the temperature of incubation was noted. The enzyme activity after heating was then assayed.

⊙ - - - enzyme from cells actively growing on 2.0% glucose, harvested just before the cessation of growth and possessing high enzyme activity.

△ - - - enzyme from cells after growth on 2.0% glucose, harvested 2½ hours after growth ceased and possessing low enzyme activity.

● - - - enzyme from cells after growth on 2.0% glucose, harvested 4 hours after growth ceased and possessing recovered enzyme activity.

FIGURE 36a

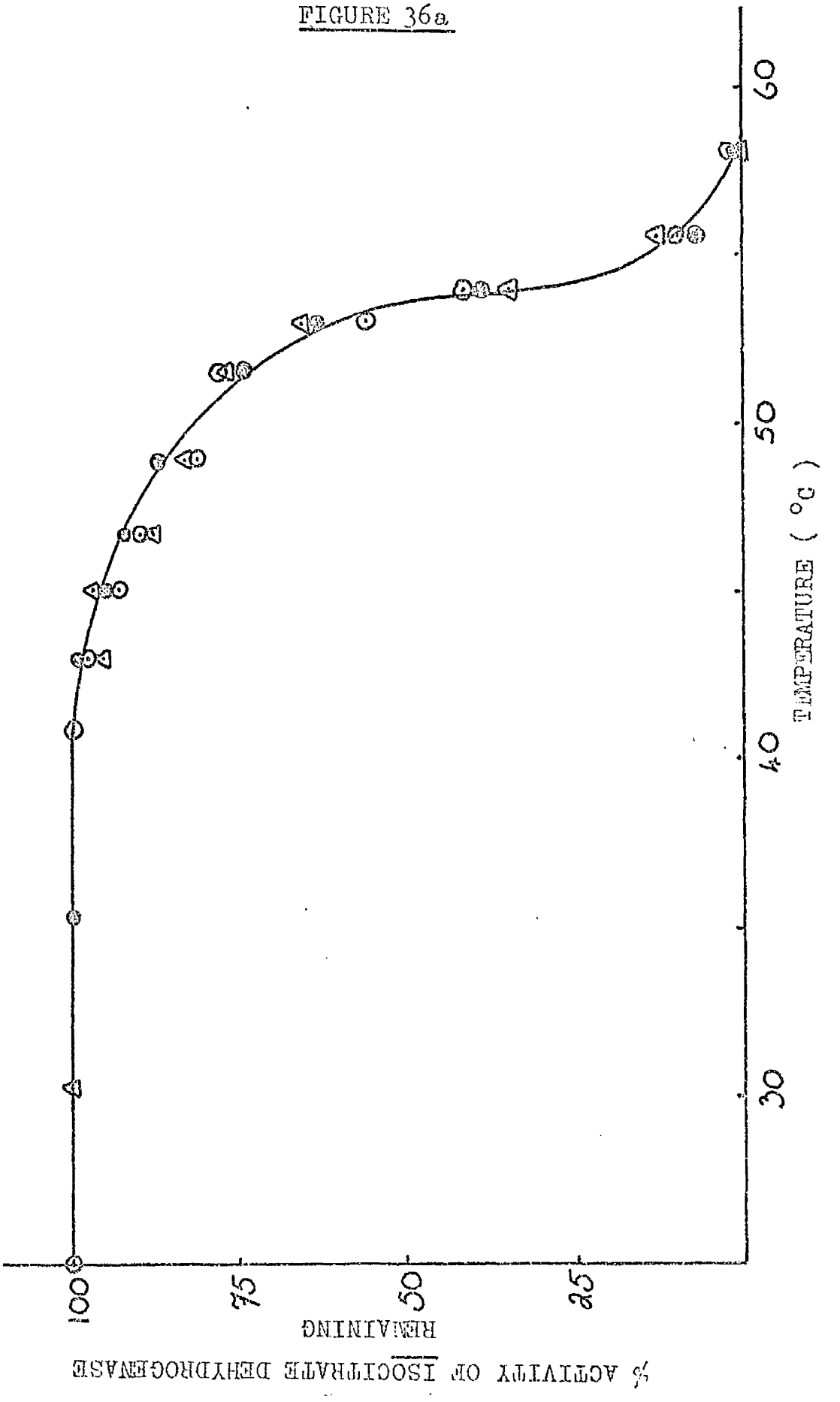

60
50
40
30
TEMPERATURE (°C)
100
75
50
25
REMAINING
% ACTIVITY OF ISOCITRATE DEHYDROGENASE

FIGURE 36(b)

Effect of maintaining isocitrate dehydrogenase, from *E. coli*, grown on 2.0mM glucose, at 52°C.

The enzyme extract was prepared as described in the legend to Figure 36(a), except that after centrifugation several cell extracts were combined. The initial enzyme activity of the extract was measured and then periodically measured while the extract was maintained at 52°C.

⊙ ——— enzyme from cells actively growing on 2.0mM glucose, harvested just before the cessation of growth and possessing high enzyme activity.

△ - - - enzyme from cells after growth on 2.0mM glucose, harvested 2½ hours after growth ceased and possessing low enzyme activity.

● ——— enzyme from cells after growth on 2.0mM glucose, harvested 4 hours after growth ceased and possessing recovered enzyme activity.

FIGURE 36b

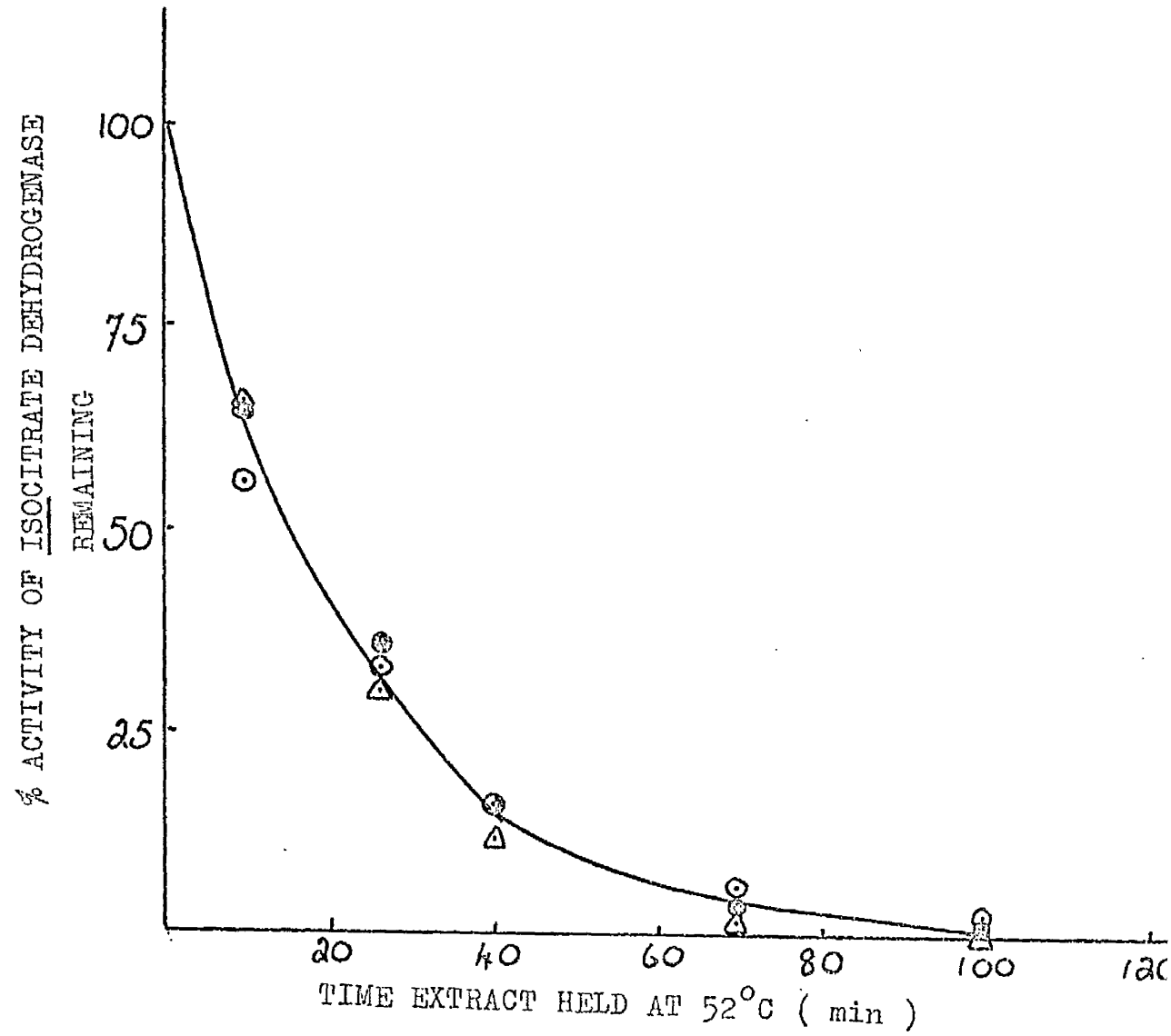

% ACTIVITY OF ISOCITRATE DEHYDROGENASE REMAINING
100
75
50
25
20
40
60
80
100
120
TIME EXTRACT HELD AT 52°C (min)

Figure 36(a) shows the effect of maintaining cell extracts at different temperatures for a fixed time. It can be seen that loss of isocitrate dehydrogenase activity occurred at comparable rates in all three extracts.

Figure 36(b) shows the effect of maintaining cell extracts at 52°C for varying periods of time. Similar profiles for loss of enzyme activity were obtained for the three extracts.

High-speed centrifugation of cell extracts

Cell extracts of glucose grown E.coli possessing high or low enzyme activity were centrifuged at 144,000g for 60 minutes at 4°C. The resultant pellets and supernatant fluids were tested for isocitrate dehydrogenase activity, which was found exclusively in the supernatant fluid. All enzyme activity centrifuged was recovered.

DISCUSSION.

The aim of this thesis is to examine some of the mechanisms which control the activity of isocitrate dehydrogenase in E.coli. I propose to split the discussion into three parts. First I will discuss the data presented in the results section of this thesis, referring to literature reports only when they significantly influenced the line of research. Second, I will present a model which explains the results obtained and will attempt to justify it on the basis of my own and other researchers results. Finally, I will discuss how the model might be subjected to further experimental test.

(A) EXPERIMENTAL RESULTS.

REPRESSION OF SYNTHESIS OF ISOCITRATE DEHYDROGENASE.

The first experiments I performed were simply designed to investigate the effect of different carbon sources and varying carbon source concentration on the synthesis of isocitrate dehydrogenase by E.coli. Table 5 (p.66b) shows that a change of carbon source may alter the differential rate of enzyme synthesis, as well as the growth rate, while varying glucose concentration over a 20-fold range had no effect (Table 4, p.66a). The former result is reminiscent of catabolite repression (Magasanik, 1961).

Hanson and Cox (1967) reported that the addition of glutamate to cultures of E.coli growing on glucose ammonium salts resulted in a severe repression of isocitrate dehydrogenase synthesis. Attempting to confirm this finding I could show no repression of enzyme synthesis in cultures of our organism after the addition of glutamate during growth on glucose (Table 6, p.67a). However, earlier experimenters had commented on the existence of a permeability barrier to glutamate and that a high rate of uptake was achieved only after selection of mutants with this specific property (Halpern and Umbarger, 1960).

Cells were inoculated into a medium containing glutamate or 2-oxoglutarate as sole source of carbon and energy. After 3-4 days the cultures became turbid. These cells would then grow, without a lag, when inoculated into fresh homologous medium. After 3 serial subcultures the cells were inoculated into glucose ammonium salts and subsequently either glutamate or 2-oxoglutarate was added to the medium. Isocitrate dehydrogenase synthesis was repressed after the

addition of either glutamate or 2-oxoglutarate, providing the cells had been previously trained to the compound, otherwise no effect was noted (Table 6, p.67a). Addition of glutamate and 2-oxoglutarate simultaneously gave a similar response to the addition of the effective compound by itself.

Halpern and Umbarger (1960) state that the ability to grow on glutamate and 2-oxoglutarate involve distinct mutational events. Hence, cells trained to grow on glutamate were inoculated into fresh medium containing 2-oxoglutarate as carbon source. When this culture was fully grown the cells were inoculated into glucose ammonium salts and glutamate, 2-oxoglutarate or both were added to the growing culture. This time, the addition of either compound repressed enzyme synthesis but the addition of both compounds simultaneously produced no further repression (Table 6, p.67a).

A second approach to the problem of forcing glutamate into the cell was used. Cells, trained to glucose ammonium salts, were inoculated into fresh glucose medium containing glutamate as nitrogen source instead of ammonium ion. Such a change permitted growth, but synthesis of isocitrate dehydrogenase was completely repressed (Figure 12(b), p.67b).

These results confirm those reported by Hanson and Cox (1967) that the addition of glutamate to cultures of E.coli results in a repression of synthesis of isocitrate dehydrogenase.

ACETATE ACCUMULATION DURING GROWTH ON GLUCOSE.

At the beginning of the discussion I hinted that synthesis of isocitrate dehydrogenase may be subject to a catabolite repression-like phenomenon. I decided to look more closely at this, and, to tackle it, decided to use the limiting carbon source technique outlined in "Proposed Methods of Study" (p.29).

If this technique was to operate successfully, isocitrate dehydrogenase activity had to remain stable between exhaustion of the primary carbon source and addition of the test compound either with or without further primary carbon source. I therefore investigated the stability of isocitrate dehydrogenase, malate dehydrogenase and 2-oxoglutarate dehydrogenase, after growth on limiting glucose and glycerol. The activity of all three enzymes was stable after growth on glycerol (Figure 15a, p.69b) as was that of malate dehydrogenase and 2-oxoglutarate dehydrogenase after growth on glucose (Figure 15b, p.69b). The activity of isocitrate dehydrogenase was not stable after growth on glucose. As no loss of activity of the other two enzymes occurred and activity was stable after growth on glycerol, the effect was apparently specific to isocitrate dehydrogenase and the stationary phase after growth on glucose.

This result cast doubt on the validity of the suggested approach and indicated that conditions at the end of growth would have to be further investigated before I could confidently use the limited nutrient technique. On the other hand, a loss of isocitrate dehydrogenase activity at the end of growth had not previously been

reported and we considered that it might indicate a hitherto unsuspected form of control. It therefore appeared an ideal phenomenon for further investigation and within the scope of the thesis.

I started by considering the state of the culture as it entered stationary phase. It has been known for a long time that utilization of many carbon compounds by micro-organisms can result in the production of various intermediary metabolites which accumulate in the medium. The best documented examples of these are the products of fermentation (Wood, 1961). However, during aerobic growth, partially oxidized intermediates may accumulate in the medium.

Lodge and Hinshelwood (1943) reported that when Bacterium lactis aerogenes (Aerobacter aerogenes) in the early stages of logarithmic growth were reinoculated into fresh homologous medium, they lagged before growth resumed. The lag could be abolished if growth medium, freed of cells, was also added. These workers concluded that the cell-free medium contained something, produced by the cells, which was essential for growth. These observations were extended by Dagley, Dawes and Morrison (1950a), also working with Aerobacter aerogenes, who further showed that the lag was appreciably shortened when glutamate or succinate were added at the time of inoculation. These workers also concluded that the cells excreted some compound into the medium during growth, and subsequently showed the presence of various amino acids in the culture media of A.aerogenes and E.coli during growth on glucose. Histidine, aspartate, glutamate and alanine were identified (Dagley, Dawes and Morrison, 1950b).

before growth resumed. The leg could be amputated if growth would

Dagley, Dawes and Morrison (1950 b, 1951) reported the accumulation of pyruvate in the medium during growth of A.aerogenes on various carbon sources and during growth of E.coli on glucose.

The experiments of Dagley and his associates were performed to a large extent with unaerated cultures, and these workers found that gentle aeration considerably lowered the concentration of products detected (Dagley, Dawes and Morrison, 1950b). Britton (1954) reported the presence of various compounds in the medium after growth of E.coli in a highly aerated glucose medium. The major product excreted was acetate. Trace amounts of amino acids were also detected, glutamate being the major component. Work by Hadjipetrou, Gerrits and Stouthamer (1964) showed that A.aerogenes grown aerobically on glucose produced acetate, which accumulated in the medium, and was utilized once the glucose had been exhausted. Accumulation of acetate has also been reported during growth, on glucose supplemented media, of Bacillus subtilis (Hanson, Srinivasan and Halvorson, 1963) and Staphylococcus aureus (Gardner and Lascelles, 1962).

These reports, plus my own observations, suggested that growth on glucose, but probably not glycerol, resulted in the production of some compound which accumulated in the medium and was possibly utilized once glucose was exhausted. To test this supposition I examined the metabolic activity of cultures of E.coli after growth on either glucose or glycerol, by measuring the production of carbon dioxide by the cultures. I found that the metabolic activity of cells grown on glycerol declined sharply once growth ceased, whereas after growth on glucose metabolic activity initially fell but after

30 minutes was restored (Figure 16, p.70a). 2 hours later the metabolic activity of these cells fell sharply once more. These results supported the previous conclusion that growth on glucose, but not glycerol, resulted in the production of some compound which was oxidised once glucose was exhausted.

Following the literature, I decided to look for acetate or pyruvate in the medium during growth and found that acetate accumulated continuously during growth on glucose and was utilised after glucose was exhausted (Figure 18, p.71b). No pyruvate was detected in the medium.

When the cells were grown on glycerol, traces of acetate were detected in the medium during growth, but no accumulation occurred (Figure 17, p.71c). No acetate was detected in the medium once growth ceased and again no pyruvate was detected during or after growth.

FATE OF ACCUMULATED ACETATE.

When the profiles of carbon dioxide evolution, acetate utilization and isocitrate dehydrogenase (Figures 16(b) and 18, p. 70a and 71b), obtained after growth on glucose, are compared, it is seen that enzyme activity fell once acetate utilization had started and was restored only after all the acetate had been used. These results suggested that the utilization of the accumulated acetate and loss of isocitrate dehydrogenase activity were linked in some way. This idea was put to the test by adding acetate to a culture, at the end of growth on glycerol, in which enzyme activity is normally stable.

This induced loss of enzyme activity (Figure 19, p.71c), which was subsequently restored, once acetate oxidation ceased. These results confirmed the view that metabolism of acetate results, in this strain of E.coli, in an inactivation of isocitrate dehydrogenase. These conclusions were further confirmed by the observation that when no loss of enzyme activity occurred after growth on several carbon sources, the addition of acetate induced a loss (Table 7, p.72a). In all cases enzyme activity was recovered after 2-3 hours. In addition, training the cells to various carbon sources before putting them into glucose medium did not abolish loss of isocitrate dehydrogenase after growth on glucose (Table 8, p.72b), indicating, presumably, the production of acetate during growth. Hence the cells completely adapt to growth on glucose within the four generations over which growth occurred, if they were not already so adapted.

When cells, not trained to acetate, are confronted with it as sole source of carbon and energy, two choices confront the organisms. They can oxidise the acetate to carbon dioxide and water using the tricarboxylic acid cycle, a course which does not permit growth, due to a lack of biosynthetic intermediates, or the cells can adapt to acetate as sole source of carbon and energy. Kornberg and Madsen (1958) and Reeves and Ajl (1960) showed that this latter process results in the appearance of enzymes of the "glyoxylate bypass" and Ashworth and Kornberg (1964) showed that operation of this pathway is essential for growth on acetate. The question therefore arises as to which alternative the cell adopts after growth on glucose.

If the latter course is adopted this should be accompanied by the production of enzymes of the "glyoxylate cycle".

I found that after growth on glucose, the activity of isocitrate lyase was greatly increased (Figure 20, p.73a). After growth on glycerol, no increase in the activity of this enzyme was observed. These results show that after growth on glucose, the cells start to adapt to growth on acetate. The capacity to grow on acetate is attained after growth on glucose regardless of whether acetate is added to the culture or not (Figure 21, p.73b). In contrast, cells grown on glycerol only develop the capacity to grow on acetate if this compound is added to the culture (Figure 22, p.73c). The evidence, therefore, supported the view that, after growth on glucose the cells adapt to growth on acetate rather than simply oxidize the compound via the tricarboxylic acid cycle.

ACETATE METABOLISM AND ISOCITRATE DEHYDROGENASE.

The question remained as to whether adaptation is a necessary condition for loss of isocitrate dehydrogenase activity. A very simple experiment was therefore performed. Cells were grown on glucose and at the end of growth chloramphenicol or puromycin was added to the culture. The addition of either of these drugs prevented loss of isocitrate dehydrogenase activity (Figure 23, p.74a). Further investigation revealed that addition of chloramphenicol prevented the increase in isocitrate lyase activity (Figure 26(b), p.74e), whereas utilization of acetate continued (Figure 26c, p.74f;

figure 25, p.74c), albeit at a greatly reduced rate.

These results suggested that loss of isocitrate dehydrogenase activity occurs only if adaptation to growth on acetate takes place. However, the possibility that other compounds can induce loss of isocitrate dehydrogenase activity had not so far been investigated. Therefore, the ability of various compounds, other than acetate, to induce a loss of enzyme activity after growth on glycerol was investigated. Pyruvate was the only compound whose addition resulted in a loss of enzyme activity (Table 10, p.75d), but unlike acetate, loss did not follow immediately on addition of the compound but rather after a lag of about 30 minutes.

With the exception of pyruvate, the evidence supported the hypothesis that loss of isocitrate dehydrogenase activity only results from a change in metabolism to utilisation of acetate as sole source of carbon and energy. The anomalous result obtained after the addition of pyruvate should be considered with the result in Table 7 (p.72a) which shows that cells grown on pyruvate lose isocitrate dehydrogenase activity at the end of growth, indicating that pyruvate itself does not induce a loss of enzyme activity but more probably that metabolism of pyruvate results in the production of acetate, which can then induce the decay of enzyme activity. This latter possibility has not so far been further investigated.

The addition of drugs which inhibit protein synthesis prevents loss of isocitrate dehydrogenase activity. This result has been attributed to the prevention of adaptation to acetate as sole source

of carbon and energy. If this conclusion was correct, the addition of a second carbon source to cultures grown on glucose might produce a similar result. Certain compounds are capable of antagonising and completely preventing loss of enzyme activity (Table 9, p.75b), and this was further confirmed by the simultaneous addition of the test compound with acetate after growth on glycerol, when basically the same results were obtained (Table 10, p.75d).

Such results add support to the hypothesis that adaptation to acetate as sole source of carbon and energy is a necessary precondition for loss of isocitrate dehydrogenase activity.

The results in Figure 15 (p.69b) show that enzyme activity recovered some time after the initial fall, and I showed that recovery occurred after acetate was exhausted (Figures 26 a & b, p.74d & f). This suggested that not only was adaptation to acetate necessary, but that utilisation of acetate was also required, a conclusion supported by the observation that isocitrate dehydrogenase was induced to fall a second time, after recovery had occurred after growth on glucose, by the addition of acetate to the culture (Figure 28, p.76a). What is more, loss of enzyme activity occurred much more rapidly.

A similar experiment was then performed, but this time a second carbon source was added simultaneously with acetate after recovery of enzyme activity had occurred. The addition of pyruvate, oxaloacetate or serine completely blocked the loss of enzyme activity normally induced by acetate (Table 11, p.76c), while various other compounds, including malate and succinate, antagonised loss of enzyme activity after a short lag. (The antagonistic effect exerted by oxaloacetate

may be due to the presence of pyruvate, as it has been shown that although solutions of this compound are stable at $0^{\circ}C$, in a buffered medium at pH 7.4 at room temperature, oxalacetate rapidly undergoes spontaneous decarboxylation to yield pyruvate /Hamilton, unpublished results/. The rate of breakdown would be expected to be even more rapid at $37^{\circ}C$, the temperature of the growth medium.) This set of results showed, that not only is adaptation to acetate necessary before a loss of isocitrate dehydrogenase can occur, but that utilization of acetate in a certain way is also necessary.

An interesting extension of the above work is presented in Figure 30 (p.77a). Here it can be seen that addition of glucose or lactose complete blocked loss of enzyme activity, when added simultaneously with acetate after recovery of enzyme activity, while, initially, galactose only partially blocked the loss. The cells used in this experiment had originally been grown on glucose and thus will contain the enzymatic machinery for glucose metabolism and the enzymes unique to lactose METABOLISM are produced constitutively by this strain of E.coli. Hence glucose and lactose could be metabolized immediately after addition. Such metabolism prevents loss of isocitrate dehydrogenase activity. On the other hand, the enzymes unique to galactose metabolism are inducible in this strain of E.coli, and were not induced prior to galactose challenge. Hence, when the mixture of acetate and galactose were added, two process would occur. Metabolism of acetate as sole carbon and energy source would start, resulting in the initiation of isocitrate dehydrogenase inactivation.

The cells would also, however, adapt to galactose as a carbon and energy source which does not result in inactivation of enzyme activity (Table 7, p.72a). The results show that galactose metabolism is established very quickly with the result that loss of isocitrate dehydrogenase is halted, and indeed reversed.

The evidence so far accumulated suggested that cells utilising solely acetate for growth require a lower level of isocitrate dehydrogenase activity than cells growingunder a variety of other conditions. This conclusion is confirmed directly by the results presented in Table 12 (p.79a), which show that the differential rate of increase in isocitrate dehydrogenase activity in cells growing on acetate was approximately one third of that in cells growing on glucose.

As enzyme activity in acetate growing cells is low and that in cells growing on a different carbon source is high, it was decided to test the effect of certain carbon sources when added to cultures growing on acetate. The addition of pyruvate or glucose resulted in an immediate increase in isocitrate dehydrogenase activity (Figure 31, p.79b). Furthermore, when the cells were removed from acetate growth medium and placed in a medium with no carbon source (a situation similar to the end of growth on limiting carbon source when the carbon source is completely utilized), isocitrate dehydrogenase activity showed a four-fold increase (Table 13, p.81a).

EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON RECOVERY OF ISOCITRATE DEHYDROGENASE ACTIVITY.

Isocitrate dehydrogenase undergoes two distinct changes in

activity after growth on glucose (Figure 15, p.69b). We demonstrated that inhibitors of protein synthesis prevented loss of enzyme activity, so it seemed logical to test the effect of these compounds on the recovery of activity. The results of such an experiment showed that recovery of enzyme activity occurred despite the presence of chloramphenicol (Figure 24, p.74b). This suggested that recovery of enzyme activity was independent of protein synthesis, and therefore could not have occurred by de novo synthesis of enzyme.

Cells growing on acetate and challenged with pyruvate and chloramphenicol immediately increased isocitrate dehydrogenase activity (Figure 32, p.80a) as if no drug had been added. On the other hand, the simultaneous addition of chloramphenicol and glucose did not result in an increased enzyme activity (Figure 33, p.80b), as did the addition of glucose alone.

The cells used in these experiments were trained and grown on acetate. Therefore, cells were trained to glucose and grown on acetate. Under these conditions, cells growing on acetate and challenged with glucose, plus or minus chloramphenicol, increased their isocitrate dehydrogenase activity to the same extent (Figure 34, p.80c). This suggests that cells trained to acetate are not fully adapted to growth on glucose, for if they were the addition of glucose plus chloramphenicol would have produced an increase in isocitrate dehydrogenase activity.

These results supported the idea that recovery of enzyme activity was not achieved by de novo enzyme synthesis. However, this was

tested under more rigorous conditions, the results of which are presented in Table 14 (p.81b). This experiment shows that reactivation of enzyme can occur, in the absence of a carbon and energy source, but in the presence of chloramphenicol, even when growth was stopped by addition of the drug and all further manipulations were performed in its presence. Further, addition of the drug resulted in a cessation of synthesis of β-galactosidase (Bennett, unpublished results). Since this enzyme is synthesized constitutively, cessation of its synthesis is taken to indicate a total cessation of protein synthesis. Thus, this experiment confirmed that reactivation of isocitrate dehydrogenase can occur in the absence of protein synthesis.

The need to harvest cells growing on acetate and resuspend them in a medium lacking acetate, rather than grow the cells in limiting acetate and follow growth till acetate was exhausted was necessary because of the peculiar growth characteristics of the cells on limiting acetate. Acetate trained cells were found to grow logarithmically in a medium with a high acetate concentration but growth became arithmetic when cells were inoculated into a medium with a low acetate concentration (Figure 35, p.81c). Thus, experiments using limiting acetate for growth were abandoned.

The reason for the switch to an arithmetic growth rate in a medium containing a low acetate concentration has not been further investigated.

INHIBITION OF, AND PHYSICAL STUDIES ON, ISOCITRATE DEHYDROGENASE.

Shiio and Ozaki (1968) reported that the $NADP^+$- specific

isocitrate dehydrogenase of E.coli was inhibited by a mixture of oxaloacetate and glyoxylate, although individually neither compound caused inhibition. It has also been known for some time that the NAD^+-specific isocitrate dehydrogenase of mammalian tissues requires ATP for full activity, although this compound does not participate in the reaction catalysed (Plaut, 1963). I therefore decided to investigate the effect of a number of compounds on isocitrate dehydrogenase extracted from cells at various stages in the cycle of loss and recovery of activity. Severe inhibition by a mixture of oxaloacetate and glyoxylate was observed, while oxaloacetate exhibited slight inhibitory powers by itself (Table 15, p.84a). Of the other compounds tested, 2-oxoglutarate inhibited to a slight degree while the others had no effect. A mixture of 2-oxoglutarate and oxaloacetate produced a greater degree of inhibition than the individual compounds, but no other pairing produced inhibition. The same degree of inhibition was observed, regardless as to whether an extract containing high enzyme activity or one containing low enzyme activity was used. None of the additions increased enzyme activity in the low activity extract. Further, attempts to activate the inactivated enzyme with pyruvate or phosphoenolpyruvate, either alone or plus other compounds, failed (Table 16, p.84b), as did attempts to reactivate the enzyme using high energy compounds (Table 17, p.84c). In fact, the addition of ATP inhibited the enzyme in both high and low activity extracts. This may have been due, however, to chelation of the Mn^{++} required by the enzyme

(Hampton and Hanson, 1969), but the point was not further investigated.

When extracts containing high and low *isocitrate* dehydrogenase activity were mixed, the activity of the mixture was the sum of the individual enzyme activities of the mixture components (p.83). We concluded that this result ruled out the possibility of a diffusible inhibitor being present in the low activity extract. This point was further tested by attempting to reactivate the enzyme in low activity extracts by dialysis (Table 18, p.85a). No increase in enzyme activity was achieved, whereas dialysis of extracts inhibited with oxaloacetate and glyoxylate did relieve the inhibition to a significant extent (Table 18, p.85a), and to the same degree in both high and low activity extracts. These results indicate that loss of activity after growth on glucose and inhibition of *isocitrate* dehydrogenase by oxaloacetate and glyoxylate are distinct from one another, though not necessarily independent.

I compared the properties of the enzyme in high and low activity extracts using heat treatment (Figure 36a, p.85b; Figure 36b, p.85c) and high speed centrifugation (p.86) but neither technique could differentiate between the enzymes, indicating either that the enzymes are identical or that the techniques are too insensitive for the purpose intended.

(B) THE MODEL

FIGURE 37

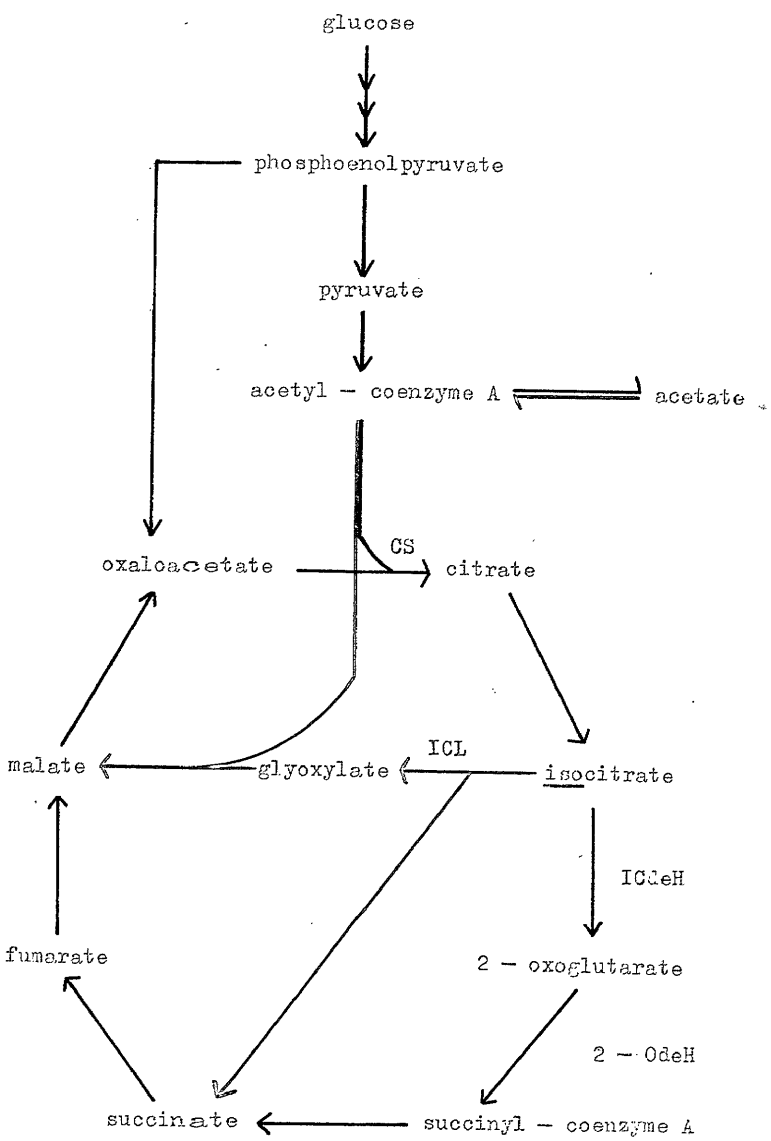


FIGURE 37

The relation between the intermediary metabolism of glucose and acetate.

Black arrows indicate glycolysis and tricarboxylic acid cycle.

Red arrows indicate glyoxylate bypass.

ENZYMES

CS	–	citrate synthase
ICdeH	–	isocitrate dehydrogenase
2-OdeH	–	2-oxoglutarate dehydrogenase
ICL	–	isocitrate lyase

THE MODEL

We propose the following model to account for the results described in the previous section. The metabolic framework within which it operates can be seen in Figure 37.

Cells growing aerobically catabolize glucose, via glycolysis, to provide energy for growth. The end products are acetyl-coenzymeA and, indirectly, oxaloacetate which are required for the production of intermediary metabolites, via the tricarboxylic acid cycle. During this catabolic process more acetyl-coenzymeA is produced than can be utilised and, as a build up in the concentration of this compound might inhibit glucose metabolism, it is degraded to acetate, which is released into the growth medium.

Once glucose is exhausted, the cells adapt to utilize acetate as sole source of carbon and energy. Adaptation creates a metabolic conflict in that the "glyoxylate bypass", essential for growth on acetate, requires isocitrate as its initial substrate, a compound which can already be further metabolized by operation of the tricarboxylic acid cycle, also essential for growth on acetate.

During growth on acetate, the flow of acetate, via isocitrate, into the "glyoxylate bypass" (red arrows in Figure 37) regulates the quantity of acetate which is metabolised via the tricarboxylic acid cycle (black arrows in Figure 37). An increase in the flow of isocitrate through the "glyoxylate bypass" (red arrows in Figure 37), via isocitrate lyase (ICL in Figure 37), causes a decrease in the flow of isocitrate round the tricarboxylic acid cycle by inactivating

isocitrate dehydrogenase (ICdeH in Figure 37). Conversely, when the flow of isocitrate through the "glyoxylate bypass" decreases, isocitrate dehydrogenase is reactivated, thus enabling the tricarboxylic acid cycle to cope with the potential increase in isocitrate concentration.

The mechanism can be seen as one which favours metabolism via the "glyoxylate bypass", when operation of this pathway is essential for growth.

In addition, isocitrate dehydrogenase is subject to several other control mechanisms, which are listed below.

(a) Product inhibition by 2-oxoglutarate

(b) Concerted inhibition by oxaloacetate plus glyoxylate

(c) Feedback repression of enzyme synthesis by either 2-oxoglutarate or L-glutamate

(d) Catabolite repression.

JUSTIFICATION AND EVALUATION OF THE MODEL

(1) PRODUCTION OF ACETATE FROM GLUCOSE

My results confirm those of Britten (1954) and show that when E.coli grows aerobically at the expense of glucose, a considerable quantity of acetate, accounting for one sixth of the carbon originally present as glucose, accumulates in the medium. This production of acetate appears, at first sight, to be very wasteful, especially as aerobically growing E.coli can use acetate to provide energy and carbon skeletons for biosynthesis. Thus acetate can be metabolized via the

tricarboxylic acid cycle, so long as a source of oxaloacetate is maintained (Figure 37), which would be expected to be the case during growth on glucose (Canovas and Kornberg, 1965). Therefore, in the absence of evidence to the contrary, it is reasonable to suppose that it is to the cell's advantage to produce acetate from glucose, which it then discards. What is this advantage?

It is well known, that in the presence of glucose the synthesis of many enzymes is repressed, a phenomenon termed catabolite repression (Magasanik, 1961). Gray, Wimpenny and Hossman (1966) reported that during growth in glucose ammonium salts, the activity of citrate synthase in E.coli K12 was approximately 50% of that in cells grown in glycerol ammonium salts and I have found that in the strain of E.coli used in our work, glucose grown cells possessed only 40% of the citrate synthase activity observed in glycerol grown cells (Bennett, unpublished results). At its simplest, this means that, potentially, glycerol grown cells can convert acetyl-coenzymeA to citrate twice as fast glucose grown cells. Hence, if production of acetyl-coenzymeA were more rapid than its utilisation it would accumulate, which might necessitate its removal by conversion to acetate. Therefore in cells growing on glucose, because of the lowered citrate synthase activity, production of acetyl-coenzymeA may be more rapid than its utilisation.

However, questions still arise as to why acetyl-coenzymeA does not limit its own production if there is a danger of overproduction. It is known that acetyl-coenzymeA inhibits pyruvate dehydrogenase, the first enzyme of the complex which converts pyruvate to acetyl-coenzymeA

(Schwartz, Old and Reed, 1968), and it has been suggested that acetyl-coenzymeA can also inhibit glucose uptake (Morgan and Kornberg, 1969). Therefore an accumulation of acetyl-coenzymeA should limit further production. Consequently if acetyl-coenzymeA is able to control its own metabolism, the production of acetate may be indicative of a form of metabolism which is of advantage during growth on glucose. Again, what is this advantage?

It has been suggested that when growing at the expense of glucose, enteric organisms obtain most of the energy they require from glycolysis, using the tricarboxylic acid cycle as a biosynthetic pathway (Roberts, Cowie, Britten, Bolton and Abelson, 1953; Gray, Wimpenny and Mossman, 1966). Richmond and Haaland (1961) found that Salmonella typhimurium, growing on glucose, had a relatively low cytochrome and inorganic iron content, in comparison with the same organism growing on tricarboxylic acid cycle intermediates, when ATP formation is solely via oxidative phosphorylation, using the reduced pyridine nucleotides produced by operation of the tricarboxylic acid cycle. These workers also found that organisms grown on tricarboxylic acid cycle substrates did not immediately grow on glucose and suggested that adaptation was first required, possibly indicating a lack of glycolytic capacity during growth on the former compounds. When E.coli was used in place of Salmonella typhimurium, similar, but less dramatic, results were obtained.

Weitzman (1966) has shown that the citrate synthase of E.coli is subject to a powerful inhibition by NADH, which is allosteric in nature (Rowe and Weitzman, 1969). It has been suggested (Sanwal, 1970)

that the reason for this inhibition is that E.coli is a facultative anaerobe and probably uses glycolysis for energy production during aerobic growth on glucose. Hence, when glycolysis supplies the energy required by the cell, further energy production, using NADH formed during the operation of the tricarboxylic acid cycle, is unnecessary. Moreover, if the rate of NADH production during aerobic glycolysis is fast enough to saturate the oxidative phosphorylation pathway, further production of NADH will be a liability to efficient growth. Thus, inhibition of citrate synthase by NADH can limit the production of unwanted NADH by the tricarboxylic acid cycle. Such a mechanism is obviously of great value during anaerobic growth, when oxidation of NADH can no longer be coupled to molecular oxygen but is instead coupled to oxidized fragments of the original substrate, and where overproduction of NADH must be avoided.

Amarasingham and Davis (1965) reported that during the initial stages of aerobic growth on glucose there was no detectable activity of 2-oxoglutarate dehydrogenase in cells of E.coli K12. This enzyme was formed towards the end of growth on glucose, and these workers attributed this to induction by a product of glucose metabolism. To use their own terms, they suggested that acetate is the "nutritional inducer", while 2-oxoglutarate may be the "physiological inducer". Such a block in the tricarboxylic acid cycle during growth on glucose would preclude its use as an energy generating pathway (Figure 1). In addition, Wright, Hsoba and Sanwal (1967) have shown that 2-oxoglutarate inhibits the citrate synthase of E.coli, an observation confirmed by Weitzman and

Dunnoro (1969). Hence, if production of 2-oxoglutarate exceeds utilisation it can inhibit further production by a feedback inhibition of citrate synthase. Such a possibility might arise during growth on glucose due to a low level of 2-oxoglutarate dehydrogenase. Inhibition of citrate synthase by compounds such as NADH or 2-oxoglutarate will exacerbate what may already be a metabolic bottleneck.

Sufficient evidence exists, therefore, to support the contention that during growth on glucose energy is obtained from glycolysis, while the tricarboxylic acid cycle, or such of its enzymes as are present, operates as a biosynthetic pathway. This may afford an explanation for the production of acetate during growth on glucose.

Energy is obtained from glucose by rapid catabolism via the Embden-Meyerhof pathway, resulting in the production of acetyl-coenzymeA. Some of this compound is used for biosynthesis, mainly via the tricarboxyli acid cycle, but also by pathways such as that for fatty acid synthesis. However, as glucose is broken down to fulfil the cells' energy requirements, more acetyl-coenzymeA is produced than can be used for biosynthesis. If the acetyl-coenzymeA were permitted to accumulate, it would eventually halt further glucose utilization with a resultant drop in energy formation and, finally, cessation of growth. To prevent this eventuality, the level of acetyl-coenzymeA is kept low by converting the excess to acetate, with a possible bonus of further energy formation, as ATP, if acetate is formed by sequential operation of phosphate acetyltransferase and acetate kinase (p.12, by reversing the direction of the reactions as there presented).

(2) ACETATE METABOLISM AFTER GROWTH ON GLUCOSE

When the cells exhaust their supply of glucose, they turn to the accumulated acetate (Figure 18(b) p.71b). In order to use this compound for growth, Ashworth and Kornberg (1964) have shown that the "glyoxylate bypass" is an essential pathway. I have shown that following growth on glucose, the cells synthesise isocitrate lyase, the first enzyme of the "glyoxylate bypass" sequence (Figure 20, p.73c). Moreover, after growth on glucose the cells automatically adapt to growth on acetate, no further acetate being required (Figure 21, p.73b). It is concluded that after growth on glucose cells adapt to growth on the accumulated acetate rather than simply oxidize it by pathways already present in the cells.

Therefore, it is within the context of growth on acetate that the loss of activity of isocitrate dehydrogenase must be considered.

ISOCITRATE DEHYDROGENASE - WHEN IS IT INACTIVATED?

Inactivation of isocitrate dehydrogenase occurs when intermediary metabolism requires the operation of the "glyoxylate bypass", as for example during growth on acetate. This statement implies two requirements for loss of enzyme activity. First, the cell must possess the enzymatic machinery of the "glyoxylate bypass" and second, this machinery must be actively working. What evidence is there to support these claims?

First, the requirement for the "glyoxylate bypass". When cells, grown on glucose, are confronted with acetate, they start to synthesise isocitrate lyase (Figure 20, p.73a) (and presumably malate synthase since

the two enzymes are co-ordinately DEREPRESSED /Reeves and Ajl, 1960; Kornberg, 1966a/ during a period of adaptation lasting approximately 2½ hours, after which time growth resumes providing there is enough acetate to support it (Figure 21, 73b). During this period of adaptation acetate is utilized (Figure 18b, p.71b) and loss of isocitrate dehydrogenase activity occurs (Figure 18a, p.71b). When synthesis of isocitrate lyase, and hence adaptation, is prevented by adding chloramphenicol to the culture medium (Figure 26b, p.74b), no loss of isocitrate dehydrogenase activity occurs (Figure 26a, p.74d). Hence adaptation, and not just acetate utilization, is required before loss of enzyme activity is initiated. This is further substantiated by the observation of Kornberg, Collins and Bigley (1960) that the addition of succinate to cultures of Micrococcus denitrificans resulted in the repression of synthesis of isocitrate lyase and I have shown that addition of malate or succinate to cultures of our organism growing on acetate stops synthesis of this enzyme (Dennett, unpublished results). Thus, when cells are challenged with acetate plus one of a number of compounds, including malate or succinate, isocitrate dehydrogenase is no longer inactivated as when acetate itself was the challenge (Tables 9 & 10, p. 75b & 75d). In the light of the evidence above, it is reasonable to conclude that these compounds prevent adaptation to acetate by blocking synthesis of the enzymes of the "glyoxylate bypass". In so doing they also prevent loss of isocitrate dehydrogenase activity. Therefore, it is a reasonable deduction that loss of isocitrate dehydrogenase activity occurs only when the enzymes of the

"glyoxylate bypass" are present in the cell.

Second, the requirement for an operating "glyoxylate bypass". It was observed at the start of this project that isocitrate dehydrogenase activity showed two changes after growth on glucose. Activity first declined and then recovered (Figure 15, p.69b). I was able to demonstrate that enzyme activity could be induced to fall once again by adding more acetate to the culture (Figure 28, p.76a). This result suggests that adaptation to acetate is not the sole requirement for inactivation of isocitrate dehydrogenase, but that acetate metabolism is also necessary, as when metabolism of acetate ceases, isocitrate dehydrogenase activity is restored (Figure 18, p.71b). This conclusion is supported by the results in Table 13 (p.81a), which show that cells growing on acetate and possessing low isocitrate dehydrogenase activity greatly increase the activity of this enzyme when transferred to an environment lacking acetate, even though no alternative carbon source is provided. Moreover, the addition of pyruvate or glucose to cultures growing on acetate as sole carbon and energy source results in an immediate activation of isocitrate dehydrogenase (Figure 31, p.79b).

The results presented above support the deduction that inactivation of isocitrate dehydrogenase occurs only when the cells' intermediary metabolism is operating as if acetate is the sole source of carbon and energy.

ISOCITRATE DEHYDROGENASE - WHY IS IT INACTIVATED?

At this stage, an obvious question comes to the fore. What is

it about growth on acetate that necessitates the inactivation of isocitrate dehydrogenase?

It has been observed that adaptation to acetate involves synthesis of isocitrate lyase, an enzyme essential during growth on acetate (Ashworth and Kornberg, 1964). This enzyme requires the same substrate as the one inactivated during growth on acetate, namely, isocitrate dehydrogenase. Hence a competition between the "glyoxylate bypass" (red arrows in Figure 37) and the tricarboxylic acid cycle (black arrows in Figure 37) for their common intermediate, isocitrate, is set up, as metabolism by both pathways is essential during growth on acetate. In such a situation it seems reasonable that one or other or both enzyme activities should be subject to regulation, so that the cell retains control over the flux of intermediary metabolites.

The "glyoxylate bypass" maintains a supply of four carbon dicarboxylic acids which are used for biosynthesis, either directly or after conversion to three carbon acids, and the "bypass" also guarantees an adequate supply of oxaloacetate to enable acetate metabolism to continue. Inactivation of isocitrate dehydrogenase has been shown to be most rapid and severe when the cells are adapted to acetate and this compound constitutes the sole source of carbon and energy (Figure 28, p.76a). We therefore considered situations when the requirement for the "glyoxylate bypass" was not absolute and in some cases, not necessary.

It has been shown that four carbon dicarboxylic acids and three carbon acids repress synthesis of isocitrate lyase and also inhibit its

activity (Kornberg, Collins and Bigley, 1960; Ashworth and Kornberg, 1963). Therefore, the addition of those compounds, or ones easily metabolized to them, which make the "glyoxylate bypass" redundant, would be expected to result in restricted metabolism through the "glyoxylate bypass", due to inhibition of isocitrate lyase. If inactivation of isocitrate dehydrogenase is a mechanism associated with the level of "glyoxylate bypass" activity, such additions might result in less isocitrate dehydrogenase inactivation. In practice, this proves to be true, as seen from the results presented in Table 11 (p.76c) and Figure 30 (p.77a). These show that several compounds antagonize loss of enzyme activity, even though acetate is present and the cells are fully adapted to it. These compounds would all be expected to render the "glyoxylate cycle" wholly, or partially, redundant. Interesting variations in the results of this series of experiments were obtained and are worthy of further comment.

The effect of some of the compounds added, notably succinate and malate was not immediate (Table 11, p.76c). However, in experiments of this type, the cells were trained and grown on glucose. Kay and Kornberg (1969) have shown a requirement for an active transport mechanism for four carbon dicarboxylic acids, and the experience of early investigators (see "Introduction - Historical", p. 3-6) suggest that this system is not present during growth on glucose or during the subsequent utilization of acetate. The time lapse between addition of the dicarboxylic acid to the culture and its effect becoming manifest can therefore be explained if, during this time, a transport mechanism for dicarboxylic acids is either synthesized or

activated.

Some compounds do not completely prevent loss of isocitrate dehydrogenase activity but do antagonize the degree of inactivation and the rate at which activity is lost. Examples of this type of inactivation were obtained after the simultaneous addition of acetate and various amino acids (Table 11, p.76c). One explanation for this effect is, that although the provision of one of these compounds lowers the requirement for "glyoxylate bypass" activity, it does not abolish it completely. Hence competition between isocitrate lyase and isocitrate dehydrogenase, although less intense, is still present. Thus inactivation of the dehydrogenase occurs at a reduced rate and to a lesser degree.

These results convincingly support our previous deductions, namely that growth on acetate requires an operating "glyoxylate bypass" which results in the inactivation of isocitrate dehydrogenase. This conclusion can be enlarged in that it is possible that the rate of decay of isocitrate dehydrogenase activity depends on the intracellular metabolic activity of isocitrate lyase, a deduction wholly consistent with the results presented in Figure 20 (p.76a) which shows that enzyme activity falls faster in response to the presence of acetate when cells are adapted to acetate and hence contain isocitrate lyase than when cells are in the process of adapting and contain considerably lower levels of this enzyme. Reactivation of isocitrate dehydrogenase proceeds when isocitrate lyase becomes inactive, either through inhibition when "glyoxylate bypass" activity is redundant or through lack of substrate by exhaustion or removal of the acetate from the

medium. The former point is supported by the results presented in Figure 31 (p.79b) which show that the addition of pyruvate or glucose to cells growing on acetate resulted in an immediate increase in isocitrate dehydrogenase activity. The metabolism of either compound produces phosphoenolpyruvate, a potent inhibitor of isocitrate lyase, in addition to which pyruvate is itself an inhibitor of this enzyme. Addition of pyruvate would therefore be expected to inhibit the operation of the "glyoxylate bypass" and such an addition results in a rapid increase in isocitrate dehydrogenase activity. The effect of glucose, although qualitatively similar, is quantitatively less dramatic. This can probably be attributed to reasons already discussed, namely, that acetate trained cells are not fully adapted to glucose metabolism (p. 100).

Hence the reason for inactivation of isocitrate dehydrogenase, is, that during growth on acetate, a flow of carbon through the "glyoxylate bypass" (red arrows in Figure 37) is essential while, although some metabolism by the tricarboxylic acid cycle is necessary, excess would be wasteful. Inactivation of isocitrate dehydrogenase (ICdeH, Figure 37) is seen therefore as a mechanism whereby the flow of carbon round the tricarboxylic acid cycle is restricted when metabolism by way of the "glyoxylate bypass" is essential for growth. The degree and rate of inactivation appear to be controlled by the level of "glyoxylate bypass" activity, i.e. by the flow of carbon into the bypass, via isocitrate lyase (ICL, Figure 37).

ISOCITRATE DEHYDROGENASE - HOW IS IT INACTIVATED?

This has been dealt with to some extent, earlier in the discussion. However I will briefly summarise my findings.

(1) Inhibitors of protein synthesis prevent loss of isocitrate dehydrogenase activity (Figure 23, p.74a) in cells not adapted to acetate.

(2) Inhibitors of protein synthesis do not prevent recovery of enzyme activity when acetate is exhausted or removed (Figure 24, p.74b; Table 14, p.81b).

(3) Isocitrate dehydrogenase is inhibited by a mixture of oxaloacetate and glyoxylate (Table 15, p.84a), but activity is largely regained on dialysis (Table 18, p.85a).

(4) Inactivation of enzyme can be distinguished from inhibition by oxaloacetate and glyoxylate because activity is not regained on dialysis and the inactivated enzyme is still susceptible to inhibition by those two compounds (Table 18, p.85a).

(5) Attempts to stimulate the inactivated enzyme using various intermediary metabolites failed (Tables 15, 16 and 17, p. 84a, 84b and 84c).

As enzyme activity increases in the absence of protein synthesis (point 2), the increased activity must be associated with pre-existing protein, inferring that loss and recovery of activity involve inactivation and reactivation of the same enzyme protein. This point has not so far been subject to critical experimental analysis.

However, the behaviour of the enzyme, before and after loss of activity and on recovery, when subjected to various heat treatments was identical (Figures 36 (a) and (b), p. 85b and 85c), as was its behaviour under high speed centrifugation.

I found that normal and inactivated enzymes (point 3) were inhibited to the same degree by a mixture of oxaloacetate and glyoxylate. Shiio and Ozaki (1968) observed that isocitrate dehydrogenase from Brevibacterium flavum is reversibly inhibited by glyoxylate and oxaloacetate but that the inhibition can be relieved by dialysis. When dialysis of the inhibited enzyme from our organism was tried (point 3), it was shown that inhibition by glyoxylate and oxaloacetate could be, in part, relieved. This was in contrast to the inactivation which occurs after growth on glucose which was not relieved by dialysis (point 4).

A 25% relief of the inhibition imposed by 1.0mM glyoxylate and 1.0mM oxaloacetate was achieved by doubling the concentration of isocitrate in the assay, while such a procedure led to no rise in the activity of the inactivated enzyme (Bennett, unpublished results). Any attempt to stimulate enzyme activity was also singularly unsuccessful.

This evidence tells us more about how the enzyme is not inactivated than how it is. Hence, it is not inactivated by destroying the enzyme protein, nor is activity lost after growth on glucose through inhibition by glyoxylate and oxaloacetate.

Loss of activity requires a flow of carbon through the "glyoxylate

bypass" i.e. an active isocitrate lyase. Therefore, although inhibition by glyoxylate and oxaloacetate has been ruled out, those compounds may play some role in the mechanism. It is of interest in this context to note that dialysis of the inhibited enzyme did not result in recovery of 100% of the original enzyme activity (Table 18, p.85a).

I conclude that inactivation and reactivation are metabolic processes of the cell, possibly requiring specific enzyme systems for both operations. Such mechanisms are not unknown in biochemistry (Hales, 1967; Leisinger, Vogel and Vogel, 1969; Shapiro, Kingdon and Stadtman, 1967; Verricchio and Holzer, 1969).

Results which may have some bearing on this aspect of the problem are those of Reeves and his colleagues (Reeves, Drohmeyer and Ajl, 1968; Reeves and Drohmeyer, 1969; Reeves and Houston, 1970), which report the existence of two $NADP^+$ - specific isocitrate dehydrogenase isoenzymes in glucose grown E.coli, and a third enzyme, distinct from the two found during growth on glucose, in acetate grown cells. The relevance of these observations is, at present, obscure. They may indicate the ability of one enzyme protein to alternate among various stable forms with varying activity, due possibly to polymerization reactions. Such a mechanism would explain my results and, if enzyme mediated reactions were necessary, would explain the difficulty encountered when trying to reactivate the enzyme.

ISOCITRATE DEHYDROGENASE -- OTHER MECHANISMS OF CONTROL.

(a) PRODUCT INHIBITION BY 2-OXOGLUTARATE.

Isocitrate dehydrogenase is inhibited by 2-oxoglutarate (Table 11, p.76c), which in effect means that if production of this compound exceeds utilization, a build-up in concentration will tend to inhibit further production. This may be of some relevance during growth on glucose, viewed in the light of the results reported by Amarasingham and Davis (1965), that E.coli lacks 2-oxoglutarate dehydrogenase during growth on glucose.

(b) CONCERTED INHIBITION BY OXALOACETATE AND GLYOXYLATE.

Reported by Shiio and Ozaki (1968), they suggested that this form of inhibition would favour metabolism of isocitrate via the "glyoxylate cycle" when this pathway is necessary for growth. From the point of view of my results such inhibition is seen as control over and above that of inactivation, but performing essentially the same task. Inactivation can be envisaged as a "coarse" control, while inhibition by oxaloacetate and glyoxylate is a "fine" control.

(c) FEEDBACK REPRESSION OF ENZYME SYNTHESIS.

This has been dealt with at the start of the discussion. Synthesis of isocitrate dehydrogenase can be repressed by an external supply of 2-oxoglutarate or L-glutamate, providing the cells are permeable to these compounds. At present the effective compound of the two cannot be decided. The benefit from such a control mechanism is apparent if one looks at the integration of the tricarboxylic acid cycle and glutamate biosynthesis. The first three steps of the cycle produce

2-oxoglutarate (Figure 37) for glutamate biosynthesis, and in some cases for further metabolism round the cycle to yield energy. However, during growth on glucose this latter function is probably minimal. Therefore if one adds 2-oxoglutarate or L-glutamate to the culture medium and these are used, there is then no need to synthesise them. Hence a feedback mechanism, possibly effected by 2-oxoglutarate, as in some cases this compound would be required for purposes other than synthesis of glutamate, is an efficient means of preventing wastage of metabolic resources in synthesising and using enzymes of a pathway which is not required. If this argument is correct, one would expect both citrate synthase and aconitate hydratase to be subject to repression under the same conditions (Henson and Cox, 1967; Flechtner and Henson, 1969). In addition it is of interest to note that citrate synthase is subject to feedback inhibition by 2-oxoglutarate (Wright, Meeba and Samuel, 1967). Such inhibition - repression mechanisms are reminiscent of control mechanisms operating in wholly biosynthetic pathways.

(d) CATABOLITE REPRESSION.

On the basis of the results in Table 4 (p.66a) it is suggested that isocitrate dehydrogenase may be subject to some form of catabolite repression but further investigation is necessary before any attempt can be made to expand this statement.

(c) EXTENSION OF THE WORK

SUGGESTIONS

THE MODEL. - ASPECTS SUSCEPTIBLE TO FURTHER EXPERIMENTATION.

The model postulated accounts for all the experimental data accumulated to date, and although much of it is still no more than hypothesis, it does provide a framework for further experimentation. Points peripheral to the model raise some interesting facts for speculation. Thus, do the varying differential rates of synthesis of isocitrate dehydrogenase obtained during growth on several carbon sources indicate that this enzyme is subject to some form of catabolite repression? This is a point worthy of study, possibly including also other tricarboxylic acid cycle enzymes.

Another point of interest, but again peripheral to the central model, is that the first three enzymes of the tricarboxylic acid cycle may be coordinately regulated (though they need not necessarily form an operon) by a feedback repression mechanism. Here, the approach used to show that isocitrate dehydrogenase synthesis can be repressed by 2-oxoglutarate and L-glutamate, could be used in an attempt to show that citrate synthase and aconitate hydratase are also repressed.

Much more central to the postulated model is the accumulation of acetate. Indeed, why does acetate accumulate during growth on glucose, but not many related compounds or compounds metabolised in a similar manner?

This could be initially tackled from two directions. We have suggested that during growth on glucose, glycolysis is so rapid that it can satisfy the cells' energy requirements. Hence, slowing the

rate of glycolysis, without affecting the potential activity of other pathways, might result in the utilization of acetyl-coenzyme A for energy production, with a resultant drop, or even cessation, of acetate production. This end might be achieved by growing cells on glucose in the presence of methyl-α-glucoside, a substrate for the glucose permease but which is not metabolized once inside the cell. This may result in a lowered rate of uptake of glucose and hence a lowered rate of glycolysis, but is obviously dependent on the relative affinity of glucose and methyl-α-glucoside for the permease system. It remains to be seen, if, under such growth conditions, the accumulation of acetate can be lowered.

A second factor suggested as being responsible for the accumulation of acetate is the lowered tricarboxylic acid cycle activity, as exemplified by the low level of citrate synthase activity and possible lack of 2-oxoglutarate dehydrogenase activity. Hence, if cells containing considerably increased levels of these enzymes are inoculated into glucose ammonium salts, the accumulation of acetate may be considerably lowered or even, initially, abolished. This might be achieved by using cells originally trained to a variety of carbon sources other than glucose and which have been shown to possess varying, but higher, levels of activity of the tricarboxylic acid cycle enzymes. Such training may also yield cells capable of metabolising glucose (via glycolysis) at different rates, which would be of interest from the point of view of the first line of attack suggested. A combination of both lines of approach might provide the answer to the question, "Why does acetate accumulate during growth on glucose?"

However the major question which is still unanswered is, "How is isocitrate dehydrogenase inactivated and reactivated?" So far all we can say is how inactivation and reactivation do not occur, and little guidance is available from my results as to how it does occur. My own opinion is that the mechanism involves changes in the three dimensional structure of the enzyme and that these changes are enzyme mediated. Such mechanisms are known to operate with other enzymes. Hence the activation and inactivation of phosphorylase requires specific enzymes for each process, (Hales, 1967), as does the inactivation and reactivation of glutamine synthetase (Shapiro, Kingdon and Stadtman, 1967).

Any attack on this problem will inevitably mean purification of isocitrate dehydrogenase at various stages of inactivation and reactivation and a comparison of their properties. Any further comment would be premature.

SUMMARY

After aerobic growth on limiting glucose the activity of isocitrate dehydrogenase in Escherichia coli ML308 falls by approximately 80% but largely recovers 3 hours later. During logarithmic growth acetate accumulates in the medium and once the sugar is exhausted the cells adapt to the acetate. Adaptation depends on the synthesis of isocitrate lyase and malate synthase which together constitute the "glyoxylate bypass".

Growth on acetate demands both isocitrate lyase and isocitrate dehydrogenase activities but these compete for their common substrate, isocitrate. Under these conditions isocitrate dehydrogenase is inactivated and the degree of inactivation depends on the cells requirements for "glyoxylate bypass" activity. This control is seen in cells growing on acetate which synthesise isocitrate dehydrogenase protein but maintain it in a largely inactivated state. When acetate is exhausted or removed from the culture, isocitrate dehydrogenase activity increases rapidly. Addition, to cells growing on acetate, of compounds which make the "glyoxylate bypass" redundant also cause a rapid increase in enzyme activity.

We conclude that inactivation of isocitrate dehydrogenase is a mechanism whereby the flow of isocitrate into the "glyoxylate bypass" restricts isocitrate metabolism via the tricarboxylic acid cycle.

Isocitrate dehydrogenase can be further regulated by repression of synthesis. In addition the enzyme can be inhibited by 2-oxoglutarate or a mixture of glyoxylate and oxaloacetate. This latter inhibition may be superimposed on the inactivation of enzyme which occurs during growth on acetate.

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