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FACTORS INFLUENCING THE AEROBIC RESPIRATION OF ESCHERICHIA COLI

by

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ABSTRACT

Facultative anaerobic bacteria such as Escherichia coli are capable of obtaining energy from glycolysis, aerobic respiration, and anaerobic respiration. Although a considerable amount of information is available on the production of ATP in E. coli by glycolysis, very little is known about the production of energy by aerobic or anaerobic respiration. Since the properties of aerobic respiration were experimentally more readily attacked, an investigation of the factors influencing the aerobic respiration and respiratory chain-linked energy production of E. coli was undertaken.

The principal technique utilized in these investigations was the polarographic determination of oxygen tension. Investigation of the properties of a commercially available, vibrating-reed, oxygen electrode revealed that silver ions were released from the silver anode of the uncoated oxygen electrode into the buffer solution surrounding the electrode. Loss of silver from the electrode was dependent on the buffer concentration and the type of buffer, and was relatively independent of the presence or absence of the polarizing voltage. It is postulated that the release of silver from the anode of the oxygen electrode involved chelation by the buffer ions. This problem was avoided subsequently by using a Clark oxygen electrode.

The pH, buffer ion and the buffer concentration of the assay medium were observed to influence the rate of respiration of E. coli. In addition the buffer ion and the pH influenced the linearity of oxygen consumption with time. Glycylglycine-KOH buffer, pH 7.0, at a concentration of 300 mM was determined as "optimal" according to the criteria of: (i) supporting a high respiratory rate; (ii) supporting a constant rate of oxygen utilization; and (iii) maintenance of these characteristics of the E. coli cell suspension

for a greater period of time than required to complete the experiment. The applicability of these criteria of classical enzyme kinetics to the determination of "optimal" conditions for the investigation of systems involved in energy conservation is questioned.

During the simultaneous measurement of acid production and oxygen consumption, a 15 to 30 second lag in acid production was observed to occur during the transition from aerobic to anaerobic glucose utilization. This observation is discussed with respect to the information currently available on the regulation of the amphibolic pathways of E. coli.

Silver ions inhibited the oxidation of endogenous substrates, glucose, glycerol, D- and L-lactate, acetate, succinate and fumarate by intact-cell suspensions of E. coli. The oxidation of formate was only slightly inhibited under the conditions which resulted in complete inhibition of respiration on the previously indicated substrates. The oxidation of glucose and glycerol was more sensitive to silver ions than that of D- or L-lactate, fumarate or succinate. This was attributed to inhibition of glyceraldehyde-3-phosphate dehydrogenase. Before the onset of the inhibition by silver ions there was a period when respiration was stimulated. This effect was similar to that given by 2,4-dibromophenol. With both compounds the degree of stimulation was larger in iron-sufficient than in iron-deficient cells. It is postulated that silver ions uncouple respiratory chain-linked energy production as well as inhibiting the respiratory chain and glyceraldehyde-3-phosphate dehydrogenase in E. coli.

Growth and cell respiration were affected when iron became limiting in batch cultures of E. coli growing on succinate. A decrease occurred in the efficiency with which succinate was converted to cell mass, in the

respiratory control ratio, and in the levels of nonheme iron, cytochrome $\underline{b}_1$, and NADH and succinate oxidase activities. On addition of ferric citrate to the iron-limited cultures the above components returned at different rates to the levels found in iron-sufficient cells. The concentration of nonheme iron, the respiratory control ratio and the efficiency of conversion of succinate to cell mass recovered more rapidly than the level of cytochrome $\underline{b}_1$ and the oxidase activities. Succinate oxidase activity recovered more rapidly than either succinate dehydrogenase or cytochrome $\underline{b}_1$ levels. It is postulated that nonheme iron is involved in respiratory chain-linked energy production in *E. coli*.

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ABBREVIATIONS

A.A. (or a.a.)	- amino acid
Acetyl-CoA (or acetyl-S-CoA)	- acetyl coenzyme A
Acetyl~P	- acetyl phosphate
ADP	- adenosine-5'-diphosphate
ALA	- δ-aminolevulinic acid
AMP	- adenosine-5'-monophosphate
cAMP	- 3',5'-adenosine monophosphate
atm	- atmosphere
ATP	- adenosine-5'-triphosphate
ATPase	- adenosine triphosphatase
C_2	- two carbon compound
C_4	- four carbon compound
C_4-dicarboxylic acids	- four carbon dicarboxylic acids
CCCP	- carbonyl cyanide m-chlorophenylhydrozone
CDP	- cytidine-5'-diphosphate
CoA	- coenzyme A
cyt.	- cytochrome
DBP	- 2,4-dibromophenol
DCCD	- dicyclohexylcarbodiimide
DCIP	- 2,6-dichlorophenolindophenol
DHAP	- dihydroxyacetonephosphate
DNA	- deoxyribonucleic acid
DNP	- 2,4-dinitrophenol
DTNB	- dithionitrobenzene
EDTA	- ethylenediamine tetraacetic acid

EDTH	- energy-dependent transhydrogenase
EPR	- electron paramagnetic resonance
FAD	- flavin adenine dinucleotide
FDP	- fructose-1,6-diphosphate
Fe	- nonheme iron
FMN	- flavin mononucleotide
F-6-P	- fructose-6-phosphate
fp (or Fp)	- flavoprotein
3-GAP (or GAP)	- glyceraldehyde-3-phosphate
GDP	- guanosine-5'-diphosphate
Glu	- glucose
L-α-glycerol-P	- L-α-glycerolphosphate
G-1-P	- glucose-1-phosphate
G-6-P	- glucose-6-phosphate
GSH	- glutathione, reduced
GTP	- guanosine-5'-triphosphate
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HQNO	- 2-heptyl-4-hydroxyquinoline-N-oxide
hexose-1-P	- hexose-1-phosphate
hexose-6-P	- hexose-6-phosphate
IA	- iodoacetic acid
IAA	- iodoacetamide
ITP	- inosine-5'-triphosphate
α-KG	- α-oxoglutarate (or α-ketoglutarate)
$Mg^{2+}(Ca^{2+})$-ATPase	- Mg^{2+}- or Ca^{2+}-stimulated adenosine triphosphatase

MOPS	- morpholinopropane sulfonic acid
NAD^+	- nicotinamide adenine dinucleotide
NADH	- nicotinamide adenine dinucleotide, reduced
$NADP^+$	- nicotinamde adenine dinucleotide phosphate
NADPH	- nicotinamide adenine dinucleotide phosphate, reduced
NAD(P)H	- pyridine nucleotides, reduced
NEM	- N-ethylmaleimide
OAA	- oxaloacetic acid
PBP	- pentabromophenol
PCMB	- p-chloromercuribenzoate
PEP	- phosphoenolpyruvate
2-PGA	- 2-phosphoglyceric acid
3-PGA	- 3-phosphoglyceric acid
6-P-gluconate	- 6-phosphogluconate
P-HPr	- phospho-HPr
P_i	- inorganic phosphate
PIPES	- piperazine-N,N'-bis(2-ethane sulfonic acid)
PMPS	- p-mercuriphenylsulfonate
PMS	- phenazine methosulfate
Pyr	- pyruvate
RCR	- respiratory control ratio
mRNA	- messenger ribonucleic acid
S_1	- $sugar_1$
S_1-P	- $sugar_1$ phosphate

SDS	- sodium dodecylsulfate
succinyl-CoA	- succinyl coenzyme A
TCA cycle	- tricarboxylic acid cycle
TES	- N-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid
TPP	- thiamine pyrophosphate
Tris	- tris(hydroxymethyl)aminomethane
Triose-P	- triose phosphate
TTFA	- thenoyltrifluoroacetone
TTFB	- 4,5,6,7-tetrachloro-2-trifluoromethyl benzimadozle
uv	- ultraviolet
UQ-8	- ubiquinone-8
UQ*-8	- ubisemiquinone-8
vit.K_2(40)	- vitamin K_2(40)

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"... For certain limited purposes all (these) things can be discussed in isolation and specific hypotheses made about their functioning. But it is only when they are assembled into a machine that they make complete sense, and when the assembly and its laws are ignored, unnecessary ad hoc hypotheses may appear needed to explain in terms of an isolated part of the machine facts which simply reflect the essential relation of this part to others."

- Dean & Hinshelwood, 1966.

1. INTRODUCTION

Although the regulation of mitochondrial respiration, and energy production linked to the respiratory chain, by adenine nucleotides and inorganic phosphate has been known for some time, the characteristics of these processes in bacterial systems have remained largely undefined. Consequently, efforts in Dr. Bragg's laboratory have been directed towards obtaining an understanding of the functioning of the respiratory chain of Escherichia coli (E. coli), and the regulation of respiratory chain-linked energy production in this organism.

Within the framework of this ultimate goal, the objectives of the research presented in this thesis were (i) to investigate the site(s) of silver ion inhibition of the aerobic respiration of E. coli with a view to the possible utilization of silver ions as a tool for the further investigation of respiratory chain function and regulation, and (ii) to investigate the involvement of iron in the function of the E. coli respiratory chain, with respect to the rate of electron transport, and to the coupling of energy conservation to the respiratory chain.

The principal analytical procedure that was utilized in the investigation of the factors influencing the aerobic respiration of E. coli was the measurement of the oxygen tension of the assay or culture medium. Since the aerobic respiration of an intact organism as measured by the disappearance of oxygen from the environment reflects the function and kinetic characteristics of all pathways and systems which provide reducing equivalents and oxygen to the terminal oxidase(s) of the electron transport chain, the introduction will include a discussion of: (i) the sequence and regulation of the

major systems in E. coli which are involved in providing substrates to the respiratory chain, and (ii) the characteristics of the respiratory chain of this organism.

1.1 Transport

Compounds with potential for providing reducing equivalents to the electron transport chain of E. coli are numerous (Figure 1.1). The prime requisite for metabolism of any of these nutrients by the cell is the penetration of the compound into the cell. This may occur by one or more of the following mechanisms: (i) group translocation; (ii) active transport; (iii) facilitated diffusion; or (iv) free diffusion. The distinguishing properties of these mechanisms have been outlined by Hayashi and Lin (1965), Kaback (1972) and Harold (1972). To date, research into the penetration of potential carbon and/or energy sources into E. coli has primarily been concerned with the following compounds or systems: (i) the phosphoenolpyruvate (PEP) phosphotransferase system; (ii) glycerol; (iii) L-α-glycerolphosphate; (iv) acetate; (v) formate; (vi) C_4-dicarboxylic acids; and (vii) the D-lactate oxidase coupled active transport system.

The phosphoenolpyruvate phosphotransferase system was first reported by Kundig et al., in 1964 and has become the classic example of uptake by group translocation, involving, in this instance, vectorial phosphorylation. The system in E. coli consists of three components; HPr, a heat stable, low molecular weight protein produced constitutively which functions as a phosphate carrier; Enzyme I which is a soluble, sugar nonspecific enzyme, also produced constitutively which catalyzes the phosphorylation of HPr by PEP; and Enzyme II, a group of membrane-bound enzymes of varying degrees of sugar specificity, some produced constitutively , some inducible, which catalyze

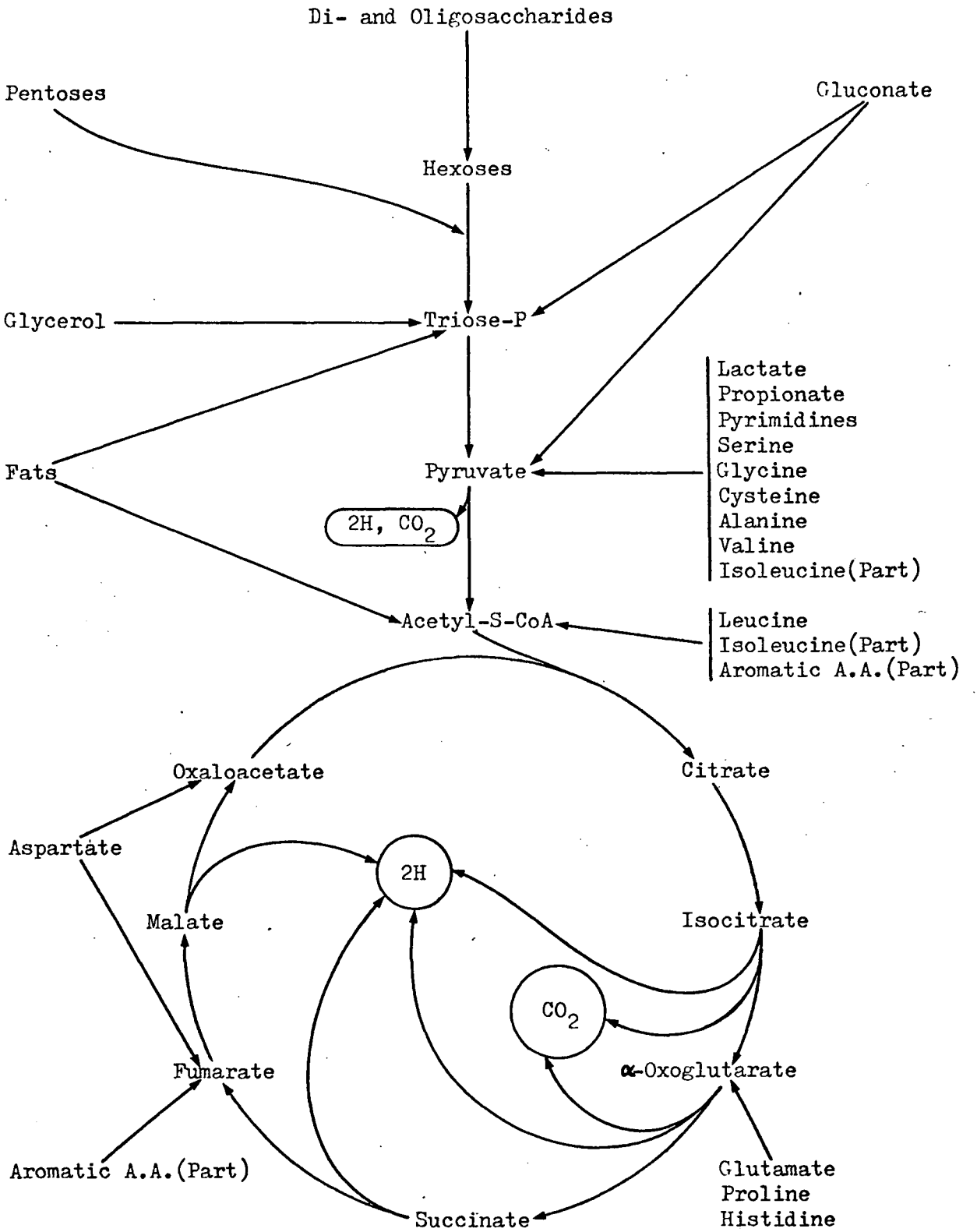


Fig. 1.1 Pathways for the catabolism by E. coli of common nutrients.

(Kornberg, 1970)

the transfer of the phosphoryl group from phosphorylated HPr to the sugar. This two-step process is summarized in Figure 1.2. Subsequently, Kundig and Roseman (1972a,b) have described the further resolution of Enzyme II into a sugar specific component, IIA, and a nonspecific component, IIB. In addition, the system has an absolute requirement for a divalent cation, and for phosphatidyl glycerol for optimal activity. There may be a fourth protein component of unknown function, and required for induced systems only. The sugars phosphorylated are all of the D-configuration and include glucose, mannose, fructose, their corresponding hexosamines and N-acetylhexosamines, α-methyl glucoside, galactose and thiomethylgalactoside. All, with the exception of fructose are phosphorylated at C-6. Fructose is phosphorylated at C-1. The requirement for PEP as phosphate donor is specific and cannot be replaced by any of the nucleoside 5'-mono-, di-, or triphosphates or any of a number of other potential phosphate donors (Kundig et al., 1964).

The PEP phosphotransferase system is present and functional in cytoplasmic membrane vesicles of E. coli. Kaback and co-workers have exploited the reduced complexity of the membrane vesicles to investigate the mechanism and regulation of the PEP translocation system (Kaback, 1970a,b). The data are consistent with the hypothesis that the PEP phosphotransferase system, in isolated membrane preparations, is subject to "product" inhibition by glucose-6-phosphate (G-6-P) and to "feedback" inhibition by glucose-1-phosphate (G-1-P). Inhibition by these sugar phosphates is noncompetitive, and the inhibitor sites are distinct, accessible from both sides of the membrane, possibly close physically and are under independent control.

In addition to inhibition by sugar phosphates, growth conditions appear to influence the properties of the PEP phosphotransferase system.

Enzyme I:

$$\text{Phosphoenolpyruvate} + \text{HPr} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{Phospho}\sim\text{HPr} + \text{Pyruvate}$$

Enzyme II:

$$\text{Phospho HPr} + \text{Sugar} \xrightarrow{Mg^{2+}} \text{Sugar-phosphate} + \text{HPr}$$

Net: (Enz I + Enz II):

$$\text{Phosphoenolpyruvate} + \text{Sugar} \xrightarrow[\text{HPr}]{Mg^{2+}} \text{Sugar-phosphate} + \text{Pyruvate}$$

Fig. 1.2 The phosphoenolpyruvate phosphotransferase system.

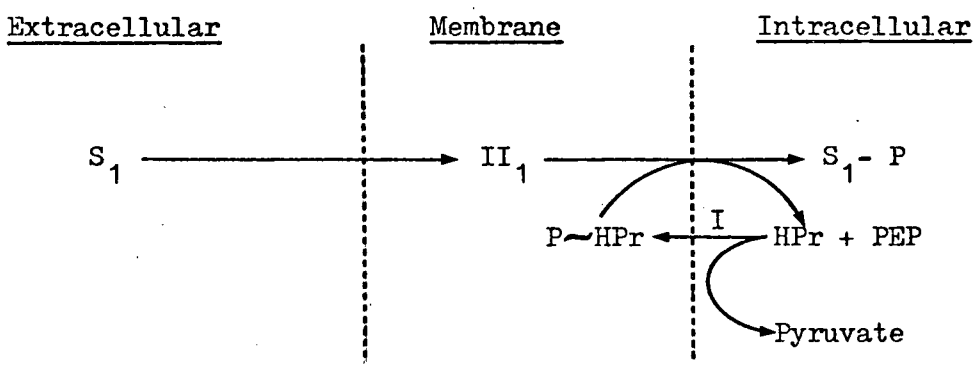


Fig. 1.3 The phosphoenolpyruvate phosphotransferase system.

(Roseman, 1969)

Kaback (1970b) reported that the carbon source on which the cells were grown — glucose, glycerol or succinate — affected (i) the rate and extent to which the sugar phosphate was accumulated, but not the amount of sugar phosphorylated, and (ii) the sensitivity of the initial rate of uptake to glucose-6-phosphate and to glucose-1-phosphate inhibition. Membrane vesicles prepared from cells in different phases of growth also showed differences in sugar phosphate transport and sensitivity to glucose-6-phosphate inhibition. Similarly, Kundig and Roseman (1971a) indicated that the amount of HPr decreased substantially when the cells reached the stationary phase of growth.

Present knowledge of the phosphoenolpyruvate phosphotransferase system may be presented schematically as in Figure 1.3.

Elucidation of the systems involved in glycerol uptake and L-α-glycerolphosphate transport in E. coli has been carried out entirely by Lin and associates (Hayashi et al., 1964; Hayashi and Lin, 1965; Zwaig and Lin, 1966; Cozzarelli et al., 1968; Sanno et al., 1968; Berman and Lin, 1971). The results of their investigations may be summarized as follows. The dissimilation of glycerol and L-α-glycerolphosphate by E. coli requires the induction of several gene products: (i) a specific protein postulated to mediate the facilitated diffusion of glycerol into the cell; (ii) glycerol kinase; (iii) L- α-glyerolphosphate dehydrogenase; and, (iv) the L- α-glycerolphosphate transport system. Synthesis of these gene products, coded by genes widely separated on the chromosome, is sensitive to catabolite repression and is negatively controlled by a single regulatory locus glp R, whose product is neutralized by the inducer L- α-glycerolphosphate.

L- α-glycerolphosphate is accumulated by the cells through the

mediation of active transport whereas glycerol uptake occurs *via* facilitated diffusion with the glycerol subsequently trapped through conversion to L-α-glycerolphosphate by the ATP-dependent glycerol kinase (Figure 1.4). The activity of the glycerol kinase is regulated by feedback inhibition by fructose-1,6-diphosphate. This provides an additional means of excluding the utilization of glycerol during glucose metabolism.

The following properties of acetate uptake by *E. coli* have been reported by Wagner *et al.*, (1972): (i) acetate uptake by cells grown on acetate was more rapid than by cells grown on glucose; (ii) was competitively inhibited by propionate but not by butyrate; and (iii) demonstrated saturation kinetics. Based on these preliminary results they concluded that a specific system exists for the uptake of acetate by *E. coli* but that it does not involve active transport as there was no detectable free acetate within the cell. By analogy with the mechanism proposed by Klein *et al.*, (1971) for fatty acid transport, Wagner *et al.*, suggested that the uptake of acetate may occur by vectorial acylation.

The failure of formate to sustain osmotic pressure across the cell membrane of *E. coli* was interpreted by Bovell *et al.*, (1963a,b) to indicate penetration of this compound into the cell by free diffusion. Measurement of the permeability of *E. coli* to sodium formate by a volume distribution technique supported this interpretation.

E. coli are capable of growing aerobically using C_4-dicarboxylic acids as the sole source of carbon. The penetration of these acids into the intact bacterial cell and membrane vesicles has been studied by Kay and Kornberg (1971) and Lo *et al.*, (1972a,b), and Rayman *et al.*, (1972a,b), respectively. The transport system was induced by C_4-dicarboxylic acids and was subject to

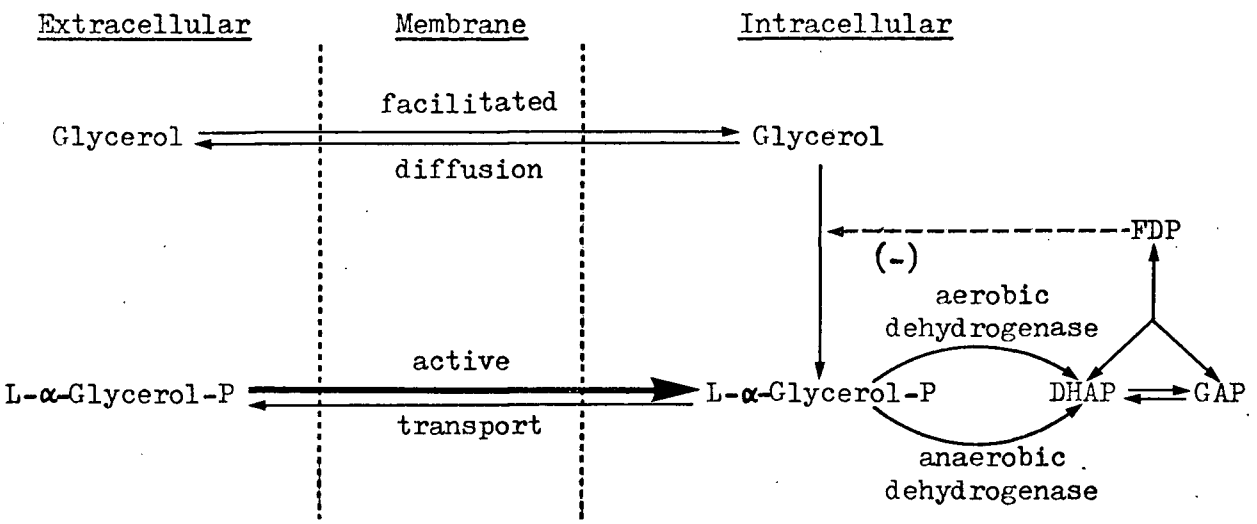


Fig. 1.4 Glycerol and L-α-glycerolphosphate dissimilation in *E. coli*.

(Berman and Lin, 1971)

catabolite repression. Kay and Kornberg failed to observe even a transiently raised intracellular concentration of C_4-dicarboxylic acid concomitant with their uptake and suggested that the energy required for the translocation was supplied by the rapid removal of the C_4-dicarboxylic acid through oxidative metabolism subsequent to entering the cell. However, Lo et al., (1972a,b) and Rayman et al., (1972a,b) reported succinate accumulation against a concentration gradient by intact E. coli and by membrane vesicles. The accumulation of succinate by active transport was suggested by: (i) recovery of succinate from the cell and membrane vesicles in an unaltered form, (ii) the high temperature coefficient and nonlinearity of the temperature dependence of the transport system, and (iii) the inhibition of succinate transport by inhibitors of respiration and uncouplers of energy conservation (Lo et al., 1972a,b; Rayman et al., 1972a,b).

The K_m and V_{max} values for fumarate and for succinate, competitive uptake studies and studies utilizing dicarboxylic acid transport negative mutants, demonstrated that a single transport system functioned in the transport of the C_4-dicarboxylic acids. The stereospecificity of the system was not absolute, but there was absolute specificity with respect to chain length and the requirement for the presence of two free carboxyl groups. (Kay and Kornberg, 1971).

Active accumulation of a compound requires energy expenditure. Lo et al., (1972b) clearly showed that the PEP phosphotransferase system was not involved in any way in the succinate uptake process and suggested that the inhibition of succinate transport by inhibitors of respiration and uncoupling agents indicated that the respiratory chain and possibly oxidative phosphorylation were required for succinate transport. Additional information on

this speculation has been provided by Rayman et al., (1972a,b) using membrane vesicles derived from a strain of E. coli lacking succinate dehydrogenase and fumarate reductase. They reported that succinate was accumulated in the presence of D-lactate, or ascorbate with phenazine methosulfate (ascorbate-PMS), but was not stimulated by the addition of adenosine triphosphate (ATP) or adenosine diphosphate (ADP). The characteristics of the transport system in the membrane vesicles were essentially identical with that of the intact cell. A notable difference, however, was the observation that compounds known to interfere with phosphorolytic and transphosphorylation reactions were without significant effect on succinate transport by the membrane vesicles. This result indicated that phosphorylated high energy intermediates were not involved in energizing succinate transport. In general, the characteristics of the D-lactate-linked, or ascorbate-PMS-linked succinate transport system as reported by Rayman et al., (1972b) were very similar to those of other D-lactate oxidase coupled transport systems previously described (Kaback, 1972). By analogy to the mechanism proposed by Kaback and Barnes (1971) for β-galactoside transport via E. coli membrane vesicles, Rayman et al., (1972b) proposed that succinate uptake was mediated by alternate reduction and oxidation of a succinate carrier in membranes. The characteristics of D-lactate oxidase coupled transport systems will be discussed in more detail below.

The D-lactate oxidase coupled transport systems of E. coli cytoplasmic membrane vesicles have been reported by Kaback and his colleagues to be responsible for the accumulation of amino acids, β-galactosides, galactose, glucuronic acid, arabinose, glucose-6-phosphate, manganous ions, and potassium ions in the presence of valinomycin, at rates comparable to those

of the intact cell. Oxidation of DL-α-hydroxybutyrate and succinate also stimulate the accumulation of these compounds but at a lower rate than observed with D-lactate. Although the rate of accumulation is slower, the transport efficiency (Konings and Freese, 1972) calculated for β-galactosides from the data of Barnes and Kaback (1971) indicated that the transport of β-galactosides linked to oxidation of DL-α-hydroxybutyrate was 2 to 3 times more efficient than that coupled to D-lactate oxidation although the authors suggest that DL-α-hydroxybutyrate is a substrate for the D-lactate oxidase system.

D-lactate and succinate were converted nearly stoichiometrically to pyruvate and fumarate respectively. There appeared to be no requirement for the generation of high energy phosphate compounds but there was an absolute requirement for electron transport. There was no evidence for any chemical transformation of the substrate during concentrative uptake driven by respiration (Kaback, 1972; Barnes and Kaback, 1970). Recently, Simoni and Shallenberger (1972) have presented evidence which indicated that the Mg^{2+} (Ca^{2+})-stimulated adenosine triphosphatase (Mg^{2+}(Ca^{2+})-ATPase) was required for the transport of amino acids linked to D-lactate oxidation.

The results of reduced-minus-oxidized difference spectra and the level of aerobic steady-state reduction of respiratory chain components of the membrane vesicles with D-lactate, succinate or reduced nicotinamide adenine dinucleotide (NADH) as electron donor was interpreted as indicating that the D-lactate, succinate and NADH oxidases utilized the same cytochrome system, and consequently, that the coupling between transport and D-lactate oxidase could not be related either to rates of electron flow to oxygen or to a unique cytochrome system coupled to D-lactate dehydrogenase. The

addition of increasing concentrations of NADH or succinate to the transport assay system in the presence of a fixed concentration of D-lactate failed to further stimulate transport. On the contrary, if the succinate dehydrogenase was more active than the D-lactate dehydrogenase the addition of succinate inhibited transport. Since succinate did not inhibit the partially purified D-lactate dehydrogenase nor the D-lactate-dichlorophenol-indophenol (DCIP) reductase, these results, and those obtained from the difference spectra suggested that the site of coupling of transport to D-lactate oxidation must occur prior to the entry of the electrons into the cytochrome system.

A comparison of the sensitivity of D-lactate-dependent respiration and D-lactate oxidation-coupled transport to inhibitors of electron transport and uncouplers of energy conservation demonstrated (i) the same degree of sensitivity to the respiratory chain inhibitors amytal, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and sodium cyanide, (ii) nearly complete inhibition of D-lactate coupled transport by azide or antimycin with no effect on D-lactate oxidation, and (iii) a marked inhibition of D-lactate oxidase coupled transport by the uncouplers of oxidative phosphorylation, 2,4-dinitrophenol (DNP), carboxyl cyanide m-chlorophenylhydrozone (CCCP) or valinomycin plus potassium ions, with no significant effect on D-lactate oxidation.

In addition to the above compounds, N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB) were found to be potent inhibitors of both the D-lactate oxidase-coupled transport and the D-lactate oxidase system. Barnes and Kaback (1971) concluded that since neither the primary D-lactate dehydrogenase nor the cytochrome system appeared to possess a sensitive

sulfhydryl site, the site of inhibition of D-lactate oxidation NEM and PCMB must occur between D-lactate dehydrogenase and the cytochromes, and probably this corresponded to the site at which transport was coupled to D-lactate oxidation.

The conclusions drawn must be considered with some reservation as other investigators have reported that the NADH oxidase (Bragg and Hou, 1967a), D-lactate-DCIP reductase and D-lactate oxidase (Bennett et al., 1966) activities of E. coli respiratory particles were significantly inhibited by PCMB. The failure of PCMB to inhibit the NADH oxidase of the membrane vesicles may be due to an inaccessibility of the PCMB-sensitive site of NADH oxidase in membrane vesicles. However, as Bennett et al., (1966) observed a similar degree of inhibition of D-lactate oxidase and D-lactate-DCIP-reductase, by PCMB, the inability of Barnes and Kaback (1971) to detect inhibition of the D-lactate-DCIP reductase as well as inhibition of the oxidase is hard to explain.

Extensive investigation of the temperature-dependent parameters of D-lactate oxidase and β-galactoside transport demonstrated the same activation energy for both processes. Determination of the kinetics of temperature induced, respiratory inhibitor-induced and uncoupler-induced efflux of β-galactoside, and the effect of sulfhydryl reagents on the kinetics of induced efflux, revealed that anaerobiosis, KCN, and HQNO stopped accumulation and also induced efflux of galactosides previously accumulated by the vesicles. By contrast, PCMB, and oxamate (which inhibits D-lactate dehydrogenase) were shown to block accumulation without inducing efflux.

These observations have prompted Kaback and Barnes (1971) to propose a model for the coupling of D-lactate oxidase to transport, in which the

"carriers" of the transport systems, in isolated membrane vesicles from E. coli, are electron transfer intermediates between D-lactate dehydrogenase and cytochrome b_1. These carriers possess sulfhydryl groups which undergo reversible oxidation-reduction. In the oxidized state the carrier has a high affinity site for the ligand which binds on the exterior surface of the membrane. Electrons coming ultimately from D-lactate reduce a critical disulfide in the carrier molecule resulting in a conformational change. Concomitantly the affinity of the carrier for its ligand is markedly decreased and the ligand is released on the interior surface of the membrane. The reduced form of the carrier is oxidized by the terminal portion of the respiratory chain and the conformation and affinity for ligand returns to that of the oxidized state.

As indicated by Harold (1972) the model proposed by Kaback and Barnes (1971) is open to criticism on a number of points. First, the ability of the membrane vesicles to oxidize NADH, synthesize phospholipids from ATP, and demonstrate ATPase activity, which the intact cells do not, suggest that a proportion of the vesicles are inside-out, are open, or are damaged in some way. Secondly, the failure of NADH or ATP to support transport may simply be due to an inability of these compounds to reach their proper site of action at the inner surface of competent vesicles. Thirdly, it is very difficult to reconcile the utilization of redox intermediates as transport carriers with the ability of E. coli to grow anaerobically at the expense of glycolysis alone. Finally, a major failure of the Kaback and Barnes model is its inability to account for the striking inhibition of transport by uncouplers, and by valinomycin in the presence of potassium. The fact that these compounds do not inhibit respiration, but only dissociate it

from transport, implies that cyclic oxidation and reduction of electron carriers is not by itself sufficient to drive active transport.

In this respect, the proposal by Simoni and Shallenberger (1972) that $Mg^{2+}(Ca^{2+})$-ATPase was required for active transport was of particular interest. However, it appears that the mutant employed by Simoni and Shallenberger, in addition to lacking the $Mg^{2+}(Ca^{2+})$ATPase, was deficient in the coupling factor(s) required to link energy-dependent systems directly to the respiratory chain (Bragg and Hou, 1973).

The possible implications of these recent results will be discussed in relation to energy coupling in E. coli (section 1.4).

1.2 Amphibolic pathways

Subsequent to gaining entry to the cell, all potential carbon and/or energy sources, directly or after a limited number of preliminary reactions, enter the amphibolic pathways of E. coli. The term 'amphibolic' was introduced by Davis (1961) to designate pathways that fulfill both an anabolic and a catabolic function, and includes glycolysis, glucogenesis, the phosphogluconate pathway, and the tricarboxylic acid cycle (TCA cycle).

Prior to discussing some of the controls of the amphibolic pathways of E. coli it is necessary to point out that investigations into the function and the control of the phosphogluconate pathway of E. coli have been very limited. Present evidence with respect to the metabolic function of the oxidative phosphogluconate pathway indicates that the primary role is probably the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for reductive biosynthesis, rather than the generation of ribose for nucleic acid synthesis (Caprioli and Rittenberg, 1969; Katz and Rognstad, 1967). As to regulation, the opinion generally held is that the

activity of the oxidative phosphogluconate pathway is regulated by the availability of nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Model and Rittenberg, 1967; Eagon, 1963). The demonstration by Sanwal (1970c) that the first enzyme of the oxidative phosphogluconate pathway, glucose-6-phosphate dehydrogenase, was allosterically inhibited by NADH and activated by spermidine, suggests that regulation of this pathway is probably more complex than originally thought.

Since our knowledge of the oxidative phosphogluconate pathway of E. coli is limited, it is of some importance to know the relative utilization of the Embden-Meyerhof pathway and the oxidative phosphogluconate pathway for glucose metabolism. Reports by Katz and Wood (1960, 1963) indicated that during logarithmic growth of E. coli between 20 to 30% of the glucose is metabolized through the pentose phosphate pathway. On entering the stationary growth phase this proportion may decrease to 5 to 10% (Model and Rittenberg, 1967).

The present state of our knowledge of the reaction sequences and regulatory mechanisms of the amphibolic pathways of E. coli, with the exception of the phosphogluconate pathway, has been summarized in Figure 1.5 and table 1.1. For a more comprehensive discussion of the regulation of the amphibolic pathways of bacteria, and its importance to bacterial physiology, the reader is referred to a review by Sanwal (1970a), and for regulation of the glyoxylate and the tricarboxylic acid cycles of E. coli in particular, to two articles by Kornberg (1966, 1970).

The controls of carbohydrate metabolism in E. coli tend to be different from, and more numerous than, those in eucaryotes. This is probably due to two characteristics of E. coli: (i) the lack of rigid compartmenta-

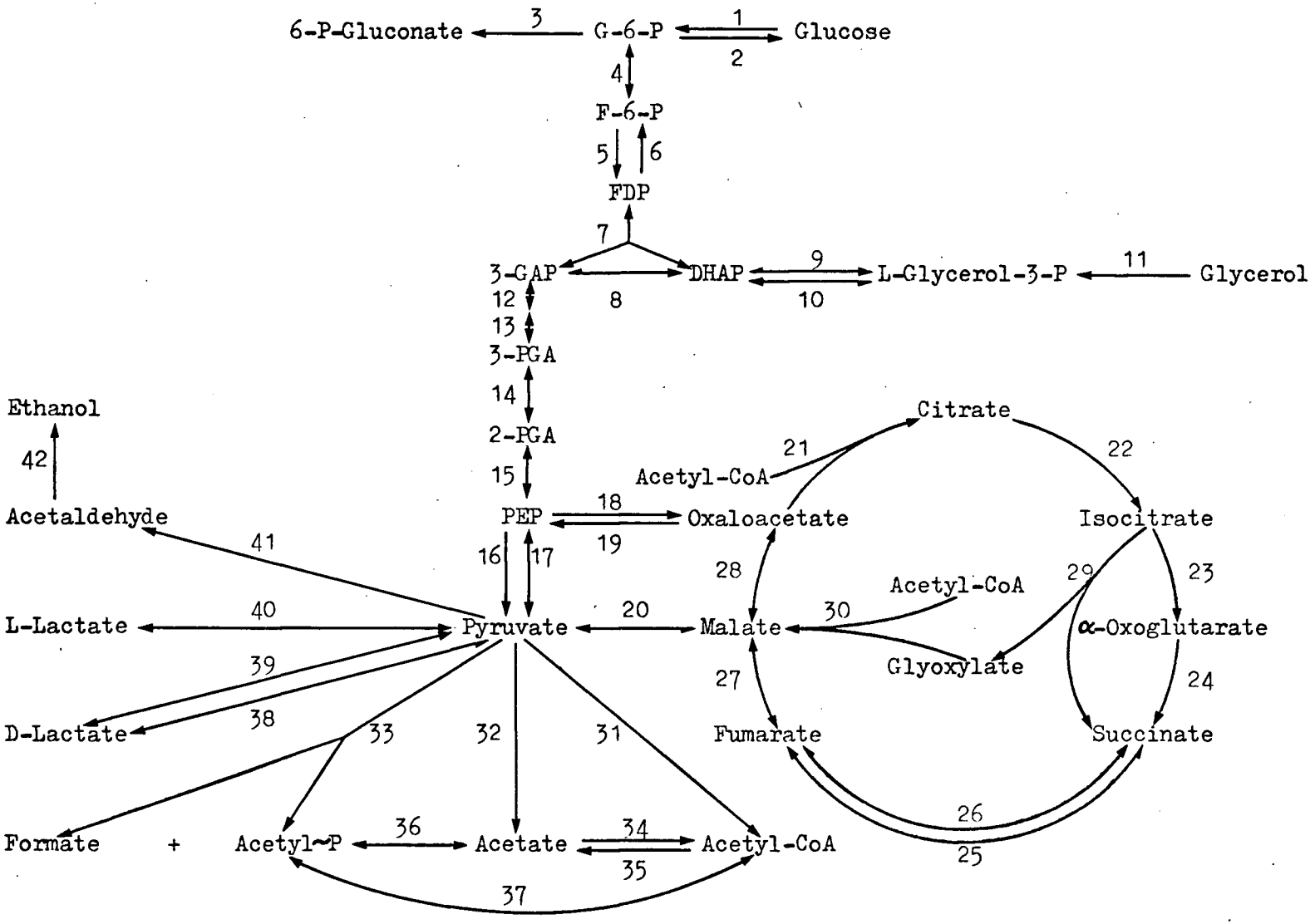


Fig. 1.5 Amphibolic pathways of E. coli.(Tarmy and Kaplan, 1968; Kornberg, 1970; Sanwal, 1970; Cunningham and Hager, 1970)

Table 1.1 Enzymes of the Amphibolic Pathways of *E. coli*

No.	Name	Nature	Cofactor	Inducer	Inhibitor	Activator	Reference
1	PEP phosphotransferase	P&S	Mg^{2+},	sugar,con.,	hexose-1-P, hexose-6-P		164,165,203, 205,289
2	Glucose-6-phosphatase						
3	Glucose-6-phosphate dehydrogenase	S	$NADP^{+}$,		NADH,	spermidine	277
4	Glucose phosphate isomerase						
5	Phosphofructokinase	S	Mg^{2+},ATP		PEP,ATP	FDP,ADP,GDP	5,20
6	Fructose diphosphatase	S	Mg^{2+},	con.,	ADP,AMP		97,99
7	Aldolase			con.,			275
8	Triosephosphate isomerase			con.,			275
9	L-Glycerol-3-phosphate dehydrogenase	S	NAD^{+},		L-glycerol-3-P		189
10a	L-Glycerol-3-phosphate dehydrogenase	P	FAD,	L-glycerol-3-P, R:glucose			196,319
10b	L-Glycerol-3-phosphate dehydrogenase	S	FAD,	L-glycerol-3-P, R:glucose			188
11	Glycerol kinase	S	Mg^{2+},ATP	L-glycerol-3-P, R:glucose			134,340,341
12	Glyceraldehydephosphate			con.,			275
13	Phosphoglycerate kinase			con.,			275

No.	Name	Nature	Cofactor	Inducer	Inhibitor	Activator	Reference
25	Succinate dehydrogenase	P		R:glucose			185
26	Fumarate reductase	P	FMN(?)	R:oxygen			146
27	Fumarate hydratase						
28a	Malate dehydrogenase	S	NAD^+,		NADH		140,274
28b	Malate oxidase	P	$^5NAD^+$,				69,71
29	Isocitrate lyase	S		R:PEP	PEP, pyruvate		148,198
30	Malate synthase	S		R:PEP,			90,148,198
31	Pyruvate dehydrogenase complex	S	TPP,NAD^+, lipoic acid	pyruvate	acetyl-CoA, NADH,GTP	PEP,AMP,GDP, pyruvate, energy charge	19,125,284, 285,290
32	Pyruvate oxidase	S	TPP,FAD			phospholipid	73,327
33	Phosphorclastic reaction						
34	Acetyl-CoA synthase						
35	Acetyl-CoA hydrolase						
36	Acetate kinase	S	Mg^{2+},ATP				269
37	Phosphate acetyl-transferase	S			NADH,ADP,ATP	pyruvate	300
38	D-Lactate dehydrogenase	S	NAD^+,			pyruvate, H^+	307

No.	Name	Nature	Cofactor	Inducer	Inhibitor	Activator	Reference
14	Phosphoglyceromutase			con.,			275
15	Phosphopyruvate hydratase			con.,			275
16a	Pyruvate kinase I	S	Mg^{2+},ADP	DR:glucose		FDP,PEP	224,225
16b	Pyruvate kinase II	S	Mg^{2+},ADP	con.,		AMP,	225,275
17	PEP synthase	S	Mg^{2+},ATP	$^{1}C_3$-cpd.,			64,275
18	PEP carboxylase	S	Mn^{2+},(Mg^{2+})	$^{1}C_3$-cpd.,	aspartate, malate	acetyl-CoA, PEP,^{2}(^{3}FDP, CDP,GTP)	47,65, ^{2}(278,279,280)
19	PEP carboxykinase	S	Mn^{2+},(Mg^{2+}), ATP	$^{4}C_4$-acids, R:glucose	NADH		150, 336
20a	Malate dehydrogenase (decarboxylating)	S	NAD^{+},	malate, R:glucose	CoA,ATP	aspartate	176,231,276
20b	Malate dehydrogenase (decarboxylating)	S	$NADP^{+}$,	con., CR:acetate & pyruvate R:glucose	NADH,NADPH, OAA,acetyl-CoA, c-AMP	NH_3	176,231,281, 282
21	Citrate synthase	S		R:glucose, R:anaerobiosis	NADH,α-KG, ATP,Mg^{2+}	acetyl-CoA, OAA	117,156,320-323, 335
22	Aconitase hydratase						
23a	Isocitrate dehydrogenase	S	Mn^{2+},$NADP^{+}$		ATP,ADP,GTP		^{2}(226),275
23b	Isocitrate dehydrogenase	S	Mn^{2+},$NADP^{+}$				^{2}(226),275
24	α-Oxoglutarate dehydrogenase	P	NAD^{+},	α-KG,			1,140

No.	Name	Nature	Cofactor	Inducer	Inhibitor	Activator	Reference
39	D-Lactate oxidase	P	(flavin)	con.,			14,131,191
40	L-Lactate oxidase	P	(flavin)	lactate			14,131,191
41	Pyruvate decarboxylase						
42	Alcohol dehydrogenase						

[1] [2] C_3-cpd,: lactate, pyruvate or alanine
[3] Salmonella typhimurium
[4] Cooperative activation by acetyl CoA and FDP, CDP or GTP
[5] C_4-acids: malate or succinate
Probably the fluorescence assays measured menaquinone (Newton et al., 1971)

Symbols:

P - particulate
S - soluble
con. - constitutive
R - repressed by
DR - derepressed by
CR - concerted repression by

tional controls of the type provided to eucaryotic cells by the presence of mitochondria, (ii) the fact that E. coli functions largely by anaerobic glycolysis even when grown aerobically on glucose. Control of the amphibolic pathways of E. coli is effected at two levels: (i) induction and repression of enzyme synthesis, and (ii) activation and inhibition of enzyme activity.

The importance of regulation of enzyme synthesis in the control of the amphibolic pathways of E. coli is readily apparent in the response of enzyme levels to growth conditions, particularly oxygen tension and carbon source. The level of the enzymes of the fermentative pathway of E. coli are not significantly influenced by the presence or absence of glucose or oxygen during growth, with the exception of one of the two pyruvate kinases which is derepressed during growth on glucose (Takahashi and Hino, 1968a; Sanwal, 1970a). However, this is definitely not the case with the enzymes of the tricarboxylic acid cycle. The lowest levels of tricarboxylic acid cycle enzymes are found in anaerobically grown cells (Gray et al., 1966a; Takahashi and Hino, 1968a). Amarasingham and Davis (1965) reported that α-oxoglutarate dehydrogenase is absent from anaerobically grown E. coli, and detectable in cells grown aerobically on glucose or lactate only after substantial accumulation of metabolites has occurred. These results prompted Amarasingham and Davis to propose that although the tricarboxylic acid pathway functions in a cyclic manner aerobic conditions, under conditions of anaerobic growth it is probably modified to a branched noncyclic pathway. An oxidative branch leads from citrate to α-oxoglutarate, serving a purely biosynthetic role. A reductive branch leads from oxaloacetate and malate to succinate via fumarate reductase, serving a biosynthetic role and providing, concurrently, a means for anaerobic electron transport (Hirsch et al., 1963). These

proposals have subsequently received support from investigations by Gray et al., (1966a) and Takahashi and Hino (1968a). The repression of TCA cycle enzymes during aerobic growth on glucose is generally considered to be due to the availability of energy from glycolysis with the result that the TCA cycle functions to generate intermediates for biosynthesis only (Gray et al., 1966a; Sanwal, 1970a).

The elevation of the tricarboxylic acid cycle enzymes during growth on intermediates of the tricarboxylic acid cycle is considered to be due to the simultaneous generation of energy and production of biosynthetic precursors by this pathway.

In addition to the control of the tricarboxylic acid cycle by repression and depression of enzyme synthesis as indicated above, a number of enzymes associated with the tricarboxylic acid cycle are induced in response to growth on specific carbon sources. These are the enzymes of the anaplerotic pathways of E. coli. It is apparent from Figure 1.1 that catabolic routes which do not give rise directly to intermediates of the tricarboxylic acid cycle enter the cycle as acetyl CoA. Each turn of the cycle results in the complete oxidation of one C_2-unit to two units of CO_2 and the continued operation of the cycle at an undiminished rate requires that the C_4-dicarboxylic acid that initiates each turn is regenerated in the course of the turn. However, intermediates of the cycle are continually withdrawn from it for biosynthetic purposes. Consequently, routes ancillary to the cycle must operate to ensure that the intermediates of the cycle are replenished. These are the anaplerotic pathways.

Elucidation of the anaplerotic reactions of E. coli has been largely due to Kornberg and co-workers (Kornberg, 1970). Their results and those of

others (References - Table 1.1) may be summarized as follows. Growth on carbon sources which are catabolized *via* acetyl CoA requires the replenishment of the C_4-dicarboxylic acid, oxaloacetate, for condensation with acetyl-CoA. This necessitates the induction of different enzymes dependent upon the carbon source used. Growth on glucose or glycerol induces the synthesis of phosphoenolpyruvate carboxylase (PEP carboxylase), while utilization of pyruvate, lactate or alanine as carbon source results in the induction of phosphoenolpyruvate synthase (PEP synthase) as well as phosphoenolpyruvate carboxylase. Growth on acetate, on the other hand, requires the induction of the glyoxylate bypass enzymes, isocitrate lyase and malate synthase with the subsequent oxidation of malate *via* malate dehydrogenase, to oxaloacetate. In addition to the induction of isocitrate lyase and malate synthase, Holms and Bennett (1971) reported that growth on acetate induced the synthesis of a protein which inhibited isocitrate dehydrogenase thus reducing the proportion of isocitrate metabolized by the tricarboxylic acid cycle.

Just as oxidation of acetyl-CoA *via* the tricarboxylic acid cycle requires an adequate supply of oxaloacetate to maintain initiation of the cycle, oxidation of TCA cycle intermediates requires an adequate means of generating acetyl-CoA, for the same purpose. This is provided by the induction, by C_4-dicarboxylic acids, of the enzymes phosphoenolpyruvate carboxykinase and NAD^+-specific malic enzyme which catalyze the formation of phosphoenolpyruvate from oxaloacetate, and pyruvate from malate, respectively. Both phosphoenolpyruvate and pyruvate are readily converted to acetyl-CoA.

Whereas the ability of a cell to synthesize new protein in response

to alterations in its environment appears to be inversely related to the ability of the organism to control its environment, the number and complexity of the allosteric controls of enzyme activity as indicated previously, appear to be related to the degree of compartmentation within the cell. Thus, although E. coli possesses controls identical to those of eucaryotes such as yeast; (i) end product inhibition, (ii) precursor activation, and (iii) energy-linked controls, the number and complexity of the controls (Table 1.1) particularly of the anaplerotic reactions is far greater in E. coli.

In addition to the preceding controls, a fourth type, at the level of enzyme activity, exists in E. coli. This is allosteric regulation by reduced pyridine nucleotides. Thus, while the tricarboxylic acid cycle of eucaryotes is regulated by ATP, or energy charge (Atkinson, 1965), NADH may be the prime control signal for the regulation of the tricarboxylic acid cycle in E. coli. Two crucial enzymes of the energy generating pathway, citrate synthase (Weitzman, 1966) and malic dehydrogenase (Sanwal, 1969) are allosterically inhibited by NADH. Weitzman (1966) and Kornberg (1970) consider NADH as an end product of the tricarboxylic acid cycle and propose that the action of NADH as an allosteric affector may be thought of as a type of end product inhibition. Sanwal (1970a) states that this is probably not the correct interpretation for the following reasons: (i) NADH is generated only at α-oxoglutarate dehydrogenase, and (ii) NADH allosterically inhibits several enzymes which have no connection whatsoever with the tricarboxylic acid cycle. It is necessary to question the validity of the initial point. If the TCA cycle is functioning in a cyclic manner, then NADH would be generated at both α-oxoglutarate and malate dehydrogenases,

as both are NAD-requiring enzymes. If during aerobic growth on glucose, the TCA cycle functions as a branched biosynthetic pathway with α-oxoglutarate dehydrogenase levels low or absent, until metabolites have accumulated (Amarasingham and Davis, 1965), one would expect initially no generation of NADH at α-oxoglutarate dehydrogenase. Subsequently, with the accumulation of metabolites, there would be generation of NADH by α-oxoglutarate dehydrogenase and possibly also by malate dehydrogenase, depending on the activity of the α-oxoglutarate dehydrogenase and the demand for succinate. Consequently, the initial argument is valid only under restricted conditions, unless the oxidation of malate to oxaloacetate occurs primarily *via* an NAD^+-independent malate oxidase (Cox *et al.*, 1968b; Newton *et al.*, 1971).

This does not detract from Sanwal's proposal (Sanwal, 1970a) that the regulation of amphibolic pathways in *E. coli*, by NADH, is related to the predominant utilization of glycolytically generated energy by this organism even during aerobic growth on glucose. *E. coli* possesses an NAD^+/NADH ratio of 0.5 when grown aerobically on glucose as compared to a ratio of 1.0 during aerobic growth on succinate. As a result, NADH inhibits citrate synthase and malate dehydrogenase to minimize the production of NADH by α-oxoglutarate and malate dehydrogenase under conditions in which the NADH level exceeds the capacity of the repressed electron transport chain.

Other enzymes which have been reported to be sensitive to allosteric inhibition by NADH include, $NADP^+$-specific malic enzyme (Murai *et al.*, 1972; Sanwal and Smando, 1969a,b; Katsuki *et al.*, 1967), glucose-6-phosphate dehydrogenase (Sanwal, 1970c), pyruvate dehydrogenase (Hanson and Henning, 1966; Shen and Atkinson, 1970), and phosphotransacetylase (Suzuki *et al.*,

1969). On the basis of this information the following statements can be made. First, NADH directly or indirectly regulates all NADPH generating enzymes in E. coli with the possible exception of the pyridine nucleotide transhydrogenase. Secondly, by regulating the enzymes indicated above, NADH indirectly regulates the availability of all biosynthetic precursors with the exception of those derived from glycolytic intermediates. The levels of NADPH, aspartate and glutamate may in turn influence the formation of biosynthetic precursors from glycolytic intermediates. Should this be the case, in E. coli, NADH could be a primary coordinator of cellular function in addition to, or replacing, ATP.

Another control mechanism which functions in the amphibolic pathways of E. coli is the existence of multiple enzymes catalyzing the same reaction. This mechanism is utilized when a reaction serves more than one purpose in the cell, and particularly when allosteric regulation with respect to one of these purposes would be detrimental to the remaining functions. The mechanism involves the utilization of induction, derepression or repression of enzyme synthesis in response to growth conditions. This is usually combined with differential allosteric regulation of the enzymes. The enzymes may possess quite distinct kinetic parameters such as K_m, V_{max}, and K_{eq}. The most thoroughly investigated examples of multiple enzymes in the amphibolic pathways of E. coli are the NAD^+-specific and $NADP^+$-specific malic enzymes (Sanwal, 1970b; Murai et al., 1972; Sanwal and Smando, 1969a,b; Katsuki et al., 1967), pyruvate kinases (Maeba and Sanwal, 1968; Malcovati and Kornberg, 1969; Sanwal, 1970a), and the particulate and soluble, FAD-linked, L-α-glycerolphosphate dehydrogenases (Koch et al., 1964; Weiner and Heppel, 1972; Kistler et al., 1969) and the NAD^+-specific, L-α-glycerolphosphate

dehydrogenase (Kito and Pizer, 1969).

In summary, the ability of E. coli to adapt to its environment is reflected in the diversity of controls possessed by this organism.

1.3 Respiratory chain

The respiratory chain of E. coli manifests the complexity one would expect of an organism possessing the diversity of metabolism indicated above, combined with the ability to respire aerobically or anaerobically. Anaerobically, E. coli can utilize nitrite, nitrate or fumarate in place of oxygen as electron acceptor. Electrons can enter the respiratory chain through various dehydrogenases such as formate dehydrogenase (Asnis et al., 1956; Taniguchi and Itagaki, 1960; Linnane and Wrigley, 1963; Gray et al., 1966b; Birdsell and Cota-Robles, 1970), L-α-glycerolphosphate dehydrogenase (Asnis et al., 1956; Koch et al., 1964; Weiner and Heppel, 1972), D- and L-lactate dehydrogenases (Asnis et al., 1956; Haugaard, 1959; Kline and Mahler, 1965; Kidwai and Murti, 1965; Bennett et al., 1966; Gutman et al., 1968), succinate dehydrogenase (Asnis et al., 1956; Kashket and Brodie, 1963a,b; Kidwai and Murti, 1965; Gray et al., 1966b;Gutman et al., 1968; Hendler et al., 1969; Birdsell and Cota-Robles, 1970; Kim and Bragg, 1971a; Hendler, 1971), malate dehydrogenase (Cox et al., 1968b;Gutman et al., 1968; Cox et al., 1970; Newton et al., 1971) and reduced nicotinamide adenine dinucleotide dehydrogenase (Asnis et al., 1956; Kashket and Brodie, 1960; 1963a,b; Bragg, 1965; Bragg and Hou, 1967a,b,c; Gutman et al., 1968; Hendler et al., 1969; Birdsell and Cota-Robles, 1970; Hendler, 1971). These enzymes are bound with varying degrees of firmness to the whole enzyme complex of the respiratory chain. Consequently, the first stage in the oxidation of substrates is not necessarily their dehydrogenation by cytoplasmic enzymes to produce

NADH. The process may begin with flavin enzymes which transfer an electron directly to the respiratory chain.

Information on the membrane bound dehydrogenases is fragmentary due to the difficulties associated with the solubilization and purification of membrane bound enzymes.

Linnane and Wrigley (1963) extracted a formate dehydrogenase - cytochrome b_1 complex from a particulate fraction of E. coli. The formate dehydrogenase was characterized as being a flavoprotein requiring sulfhydryl groups for activity. The nature of the flavin component was unknown. The possibility of metal ion involvement was suggested by the inhibition of the dehydrogenase activity of cyanide.

Glycerol-3-phosphate dehydrogenase was solubilized from an E. coli membrane fraction by Weiner and Heppel (1972). The results of polyacrylamide gel-electrophoresis in the presence of sodium dodecylsulphate (SDS) indicated that the enzyme was composed of two subunits. The prosthetic group was noncovalently bound FAD.

Although the membrane-bound, D-lactate dehydrogenase of E. coli has been purified approximately 250-300 fold (Barnes and Kaback, 1971; Kaback, 1972), there has been no report of the purification of the L-lactate dehydrogenase, or of the characteristics of the partially purified D-lactate dehydrogenase. Results of inhibitor studies reported by Bennett et al., (1966) were inconclusive as to the characteristics of these enzymes, although they did suggest that the D-lactate dehydrogenase was a flavoprotein requiring sulfhydryl groups for activity.

Solubilization of the succinate dehydrogenase of E. coli has been achieved by Kim and Bragg (1971a). The prosthetic group was not determined

but if it is flavin it must be tightly bound as is the case with the mitochondrial enzyme. The fact that the membrane bound enzyme was inhibited by PCMB suggested that sulfhydryl groups are probably required for activity.

Three distinct NADH dehydrogenase containing systems, designated menadione reductase I, menadione reductase II, and NADH oxidase, have been solubilized from the small particle fraction of the E. coli membrane by treatment with deoxycholate and ammonium sulfate (Bragg and Hou, 1967b). Mendione reductase I and II utilized both NADH and NADPH as electron donors and required the presence of menadione in order to utilize oxygen as an electron acceptor. Additional data suggest that the NAD(P)H dehydrogenase of menadione reductase I is a metalloflavoprotein. Results obtained with menadione reductase II do not permit deductions as to the nature of the prosthetic group of the NAD(P)H dehydrogenase component. However, the inhibitor data indicate that the dehydrogenase probably does not possess any metal ions essential for activity. The dehydrogenase component of the solubilized NADH oxidase appears to be a metalloflavoprotein.

Gutman et al., (1968) have investigated two NADH dehydrogenases of E. coli, (i) a soluble NADH diaphorase and (ii) an NADH dehydrogenase extracted from lyophilized small particles of E. coli with distilled water. Very little information was reported on the properties of the NADH diaphorase but the characteristics reported suggest that it might correspond to the menadione reductase I of Bragg and Hou (1967b). The NADH dehydrogenase extracted from the membrane fraction was characterized, without further purification, as a metalloflavoprotein containing FMN, FAD, nonheme iron and labile sulfide. These characteristics are similar to those reported by Bragg and Hou (1967b) for menadione reductase II, suggesting that these may

be the same enzyme. Gutman et al., (1968), due to the similarity of the properties of the solubilized NADH dehydrogenase and the membrane bound dehydrogenase of the NADH oxidase system, considered that they had solubilized the NADH dehydrogenase of the NADH oxidase system. However, their data indicated that extraction of 30 to 50% of the NADH dehydrogenase of the lyophilized membrane particles did not diminish the NADH oxidase activity of the extracted particles. This indicated either (i) that the NADH dehydrogenase extracted was not the dehydrogenase component of the NADH oxidase system, or (ii) the function of the NADH oxidase was rate limited at some site between the dehydrogenase and oxygen such that extraction of 30 to 50% of the dehydrogenase did not affect the rate of the system. As a result one cannot say with any certainty at this time whether the major NADH dehydrogenase component of the E. coli respiratory chain has been solubilized or what its characteristics are.

The cytochromes of E. coli have received more extensive investigation than the dehydrogenases associated with the electron transport chain, the first report appearing in 1934 (Keilin, 1934). Keilin demonstrated the presence in E. coli of cytochromes a_1, a_2, and b_1 but did not detect cytochrome c. The presence of cytochromes a_1, a_2, and b_1 in E. coli was confirmed by Yamagutchi (1937) who also detected low levels of cytochrome c. This was disputed by Keilin and Harpley who were unable to duplicate these results (Keilin and Harpley, 1941). The presence of cytochrome a_1, a_2 and b_1 was accepted without question. Subsequently Castor and Chance (1959) demonstrated the existence of a second b-type cytochrome in E. coli by the photochemical action spectra of cells in which respiration had been inhibited by carbon monoxide. This cytochrome, designated cytochrome o, was shown

to function as the terminal oxidase in logarithmic phase cells and shared this function with cytochrome $\underline{a}_2$ in the stationary phase.

It was not until the work of Wimpenny and coworkers (Gray et al., 1963; Wimpenny et al., 1963) that the discrepancy of the presence or absence of cytochrome c was clarified. Gray et al., (1963) discovered that anaerobic growth of E. coli, and related facultative anaerobes, on minimal salts medium resulted in the synthesis of a soluble c-type cytochrome, subsequently designated cytochrome c-552 (Fujita and Sato, 1963). Wimpenny et al., (1963) demonstrated that the synthesis of cytochrome c-552 was repressed by the presence of oxygen. Fujita and colleagues (Fujita, 1966a; Fujita and Sato, 1966a,b, 1967) and Cole (1968) have shown that the synthesis of cytochrome c-552 is stimulated under anaerobic conditions by the presence of nitrite in the medium and appears concurrently with the development of nitrite reductase activity. However, the exact relationship of cytochrome c-552 to nitrite reductase remains unclear.

Fujita and Sato (1963) also reported the existence in anaerobically grown E. coli of a second soluble c-type cytochrome, cytochrome c-550, and in anaerobically or aerobically grown E. coli, of a soluble b-type cytochrome, cytochrome b-562. Both of these cytochromes have been purified and characterized but their function is unknown (Fujita, 1966b; Itagaki and Hager, 1965; Hager and Itagaki, 1967).

Of the membrane bound cytochromes $\underline{a}_1$, $\underline{a}_2$, $\underline{b}_1$ and o, only cytochrome $\underline{b}_1$ has been isolated, purified and characterized (Fujita et al., 1963; Deeb and Hager, 1964). Generally, it has been considered that there was only one cytochrome $\underline{b}_1$ present. However, the results of Baillie et al., (1971) and Kim and Bragg (1971) suggest that there are probably two functionally,

if not chemically, distinct cytochrome b_1's in E. coli cells grown aerobically on glucose or succinate. De Moss and coworkers have reported the presence of two distinct cytochrome b_1's in anaerobically grow E. coli (Ruiz-Herrera et al., 1969). Anaerobic growth in the presence of nitrate resulted in the synthesis of a b-type cytochrome with an alpha band peak at 555 nm ($77^{o}K$) while anaerobic growth in the absence of nitrate resulted in a cytochrome b with an alpha band at 558 nm ($77^{o}K$). Subsequently, Ruiz-Herrera and De Moss (1969) reported that the cytochrome b-555 ($77^{o}K$), of nitrate induced E. coli consisted of two species differentiable on a kinetic basis. These researchers also reported that the nitrate-specific cytochrome b-555 ($77^{o}K$) components were distinct from the cytochrome b-555 ($77^{o}K$) components produced under aerobic conditions.

Thus, assuming that the measurement of reduced-minus-oxidized difference spectra at $77^{o}K$ results in a two to three nanometer shift of the absorption maximum to lower wavelength compared to those recorded at room temperature (Shipp, 1972a), it appeared, until recently, that the cytochrome complement of E. coli (λ_{max} at room temperature) consisted of: cytochromes a_1(590), a_2(630), b-557, b-562 and o(557) under aerobic growth conditions. Under anaerobic growth conditions there were low levels of cytochromes a_1 (590), a_2(630), o(557), b-562 and c-550 and depending on the terminal electron acceptor, substantial amounts of cytochrome b-560, or of two cytochrome b-557's, and cytochrome c-552. Recently, however, Shipp (1972a) has applied a fourth order finite difference analysis technique (Butler and Hopkins, 1970a,b) to low temperature reduced-minus-oxidized difference spectra of aerobically grown E. coli, whole cells and respiratory particles. His results indicated that the respiratory chain of E. coli may be considerably

more complex than anticipated. Shipp proposes that the alpha band previously attributed to cytochrome b_1 is a composite of the absorption bands of five or more pigments, cytochromes c-548, c-553, b-556, b-559 and b-565 (Shipp, 1972b).

Associated with the electron transport chain of E. coli is a second type of iron-containing respiration carrier, the iron-sulfur proteins or nonheme iron proteins. Very little is known about the function of respiratory chain-linked nonheme iron in E. coli. Investigations into this problem have largely been indirect, via the effect of metal chelators on (i) respiration rate; (ii) dehydrogenase activity and (iii) the steady state reduction of respiratory pigments. Direct measurement of nonheme iron by electron paramagnetic resonance (EPR) has been employed by Hamilton et al., (1970) and Hendler (1971).

Inhibition of NADH oxidase and succinate oxidase by the metal chelators salicylaldoxime, 8-hydroxyquinoline, 2,2'-dipyridyl, thenoyltrifluoroacetone (TTFA), and o-phenanthroline has been demonstrated (Bragg, in press). Evidence for the involvement of nonheme iron in the NADH dehydrogenase segment of the respiratory chain has been indicated by Bragg and Hou (1967a) and Gutman et al., (1968). Bragg and Hou (1967a) reported that 2,2'-dipyridyl inhibited NADH oxidation by E. coli respiratory particles and that this inhibition was competitive with the substrate. These results suggested that a metal ion, possibly nonheme iron, was close to the NADH binding site. Subsequently, Gutman et al., (1968) demonstrated that presence of nonheme iron in a solubilized NADH-ferricyanide reductase complex. However, the redox potential of this nonheme iron, which was reducible by ascorbate, must have been considerably more positive than would be expected for nonheme iron

associated with the NADH dehydrogenase substrate binding site. Consequently it must be located closer to oxygen. Although nonheme iron appears to be involved with NADH dehydrogenase, Kim and Bragg (1971a) reported that succinate-PMS reductase was not affected by levels of o-phenanthroline and thenoyltrifluoroacetone sufficient to inhibit the oxidase. It would appear that nonheme iron is not involved in the succinate diaphorase activity. Further investigations will be needed to establish whether this is also true for the succinate dehydrogenase associated with the electron transport chain.

Bragg (1970) and Kim and Bragg (1971b) have reported the existence, in E. coli respiratory particles, of a species of nonheme iron characterized by its rate of reaction with o-phenanthroline (reaction complete within one minute). This nonheme iron constituted approximately 20% of the total nonheme iron when the reducing equivalents were provided by NADH, ascorbate-PMS or dithionite (Bragg, 1970) but only about 7% of the total nonheme iron with succinate as electron donor (Kim and Bragg, 1971b). Investigating the location of the NADH-reducible nonheme iron, Bragg (1970) reported that the reduction of the nonheme iron by NADH was inhibited by HQNO and that although the rate of reduction was decreased, complete reduction of cytochrome b_1 could occur without reduction of nonheme iron. The conclusions drawn were: (i) that the NADH-reducible nonheme iron was located between ubiquinone and oxygen. However, since the nonheme iron was fully reducible by ascorbate-PMS and cytochrome b_1 was not, the site may be between cytochrome b_1 and oxygen. (ii) the NADH-reducible nonheme iron probably was not on the main respiratory pathway. Kim and Bragg (1971b) investigated the relationship of nonheme iron to the succinate oxidase system in a membrane fraction from E. coli. They reported that succinate was capable of reducing 55 to 70% of the cytochrome

b_1 and approximately 35% of the o-phenanthroline reacting nonheme iron reducible by either NADH or dithionite. However, in the presence of HQNO both cytochrome b_1 and the succinate-reducible nonheme iron reached anaerobic steady-state values which were equivalent to those given by NADH or dithionite. These results indicated that whereas HQNO inhibited the reduction of nonheme iron and cytochrome b_1 associated with the NADH oxidase system, it inhibits the oxidation of nonheme iron and cytochrome b_1 associated with succinate oxidase. It was not possible, on the basis of the data available, to ascertain the position of nonheme iron in the succinate oxidase system.

Hendler (1971) reported on the effect of TTFA on the generation of the EPR signal (g=1.94) associated with reduced nonheme iron. Thenoyltrifluoroacetone inhibited both the reduction by NADH of the nonheme iron species responsible for the electron paramagnetic resonance signal, and cytochrome b_1, but did not prevent the reoxidation of these respiratory chain components. From these results Hendler concluded that the nonheme iron reducible by NADH occurred between the NADH dehydrogenase and cytochrome b_1.

Although the results available indicate that nonheme iron may be linked to, or part of the respiratory chain, it is impossible to designate the role of nonheme iron in relation to the electron transport chain with any degree of certainty.

The function of the quinones of the cytoplasmic membrane, have been, and remain, one of the most baffling aspects of the respiratory chain of *E. coli*. The first information on the lipoquinones of *E. coli* appeared in 1959 when Lester and Crane reported that aerobically grown *E. coli* possessed a benzoquinone, ubiquinone-8, and a naphthoquinone, while the same organism

grown anaerobically contained only the naphthoquinone. Page et al., (1960) confirmed the identity of the major benzoquinone of E. coli as ubiquinone-8. Subsequently, Bishop et al., (1962) identified the principal naphthoquinone as vitamin $K_2(40)$. More recently, low levels of demethyl vitamin $K_2(40)$ (Dolin and Baum, 1965) and the ubiquinone-8 isoprenologues, ubiquinone-5, -6 and -7 have been detected in E. coli (Friis et al., 1966).

Commencing with the initial report by Lester and Crane (1959) there was considerable interest in the influence of growth conditions, particularly aeration rate, on the relative abundance of ubiquinone and menaquinone. Bishop et al., (1962) reported that they found no significant difference in the ubiquinone-8 and vitamin $K_2(40)$ levels of aerobically and anaerobically grown E. coli. However, subsequent reports by Polglase et al., (1966), Whistance and Threlfall (1968) and Whistance et al., (1969) supported the results of Lester and Crane (1959), that aerobic conditions favored the formation of ubiquinone-8 while anaerobic conditions favored the formation of vitamin $K_2(40)$.

The first indication that lipoquinones might be important in the respiratory chain and energy metabolism of E. coli was a report by Kashket and Brodie (1960) that ubiquinone stimulated respiration with NAD^+-linked substrates, whereas naphthoquinones stimulated both oxidation and phosphorylation when added to a near-ultraviolet irradiated system from E. coli.

Results from the same laboratory (Kashket and Brodie, 1962) subsequently demonstrated that cultures of E. coli, irradiated with light of 360 nm wavelength, grew aerobically on fermentable carbon sources, with a reduced cell yield, and, although retaining the ability to oxidize carbon sources such as succinate or malate, were unable to grow on them. These

results were interpreted as indicating a role for lipoquinones in the coupling of energy production by the respiratory chain.

To further elucidate the possible involvement of the quinones in oxidative phosphylation, Kashket and Brodie (1963a,b) fractionated sonicated E. coli, by differential centrifugation, into large membrane particles, small particles and supernate. Results from these systems led them to conclude that the naphthoquinone, vitamin $K_2(40)$, functioned in the respiratory chain for NADH oxidation at a position between flavoprotein and cytochrome b_1, while ubiquinone-8 was involved in the succinate oxidase pathway in an analogous position. NADH and succinate oxidases were considered to share a common electron transport chain from cytochrome b_1 to oxygen (Figure 1.6A).

The techniques of destruction of quinones by uv irradiation, such as used by Kashket and Brodie (1960, 1962, 1963a,b), or extraction of quinones with lipid solvents are subject to a number of criticisms. Brodie (1963) states that benzoquinones are much more slowly destroyed by light at 360 nm than menaquinones. Also, Bragg (1971) has shown that uv irradiation of E. coli respiratory particles results in a marked destruction of cytochrome a_2. The extraction of membranes with lipid solvents must result in damage to the membrane. Moreover, the subsequent addition of quinones in attempts to restore biological activity is subject to the point raised by Wagner and Folkers (1963), that is, are the effects of the compound when added to the system a true effect of that compound because it is a component of the system; or an effect because it is structurally analogous to a true component of the system?

The isolation of mutants unable to synthesize ubiquinone (ubi$^-$), or vitamin K_2(men$^-$) has provided a technique for investigating the location

and function of quinones with respect to the respiratory chain of E. coli. This technique is superior to either destruction of quinones by uv irradiation or their removal by solvent extraction. Jones (1967) was the first to exploit this technique, utilizing E. coli W and a mutant of this strain, isolated by Davis (1952) which was auxotrophic for 4-hydroxybenzoic acid. In the absence of 4-hydroxybenzoate the mutant was unable to synthesize ubiquinone-8, possessed a low respiration rate, and low NADH oxidase and NADH-cytochrome b_1 reductase activities. Preincubation of respiratory particles from the mutant with ubiquinone-6 largely restored NADH oxidase and NADH-cytochrome b_1 reductase activities. The succinate oxidase activity of the mutant respiratory particles was stimulated only about 50% as compared with a 20 to 50 fold increase in the NADH oxidase activity. Neither NADH dehydrogenase nor succinate dehydrogenase activities were affected by the absence of ubiquinone. These results strongly implicated ubiquinone in the NADH oxidase complex of the auxotroph and located the site of ubiquinone between NADH dehydrogenase and cytochrome b_1. The results with the succinate oxidase were equivocal. The applicability of these results to the wild type E. coli W is uncertain. The NADH oxidase activity of the auxotroph grown with 4-hydroxybenzoate supplementation was destroyed by uv irradiation and subsequently activity was partially restored by ubiquinone-6. There was no restoration of activity with vitamin K_2 isoprenologues. A similar preparation from E. coli W was not stimulated by ubiquinone-6 but activity was partially restored by preincubation with vitamin K_2(30), in agreement with Kashket and Brodie (1963b). The discrepancy between the results with the auxotroph and E. coli W may be related to the fact that the auxotroph even in the presence of 4-hydroxybenzoate was unable to synthesize normal levels

of vitamin K_2.

An extensive investigation of the role of quinones in E. coli respiration and growth had been carried out by Gibson and coworkers utilizing ubi⁻ and men⁻ mutants of E. coli K12. The ubi⁻ and men⁻ mutants grew aerobically on glucose. The ubi⁻ mutants had a growth yield similar to that for anaerobic growth (Cox et al., 1970), but neither mutant was able to grow on malate or succinate. However, a revertant of the men⁻ mutant was able to grow on malate, succinate and lactate although still unable to synthesize vitamin K_2 (Cox et al., 1968a). These results demonstrated that vitamin K_2 was not required for oxidation of, or growth on, malate, lactate or succinate and suggested that ubiquinone-8 was essential for electron transport or phosphorylation coupled to transport. Subsequently, Cox and Snoswell (1968) and Cox et al., (1968b) have shown that ubiquinone was involved in lactate and NADH oxidation, and approximately 50% of the malate oxidation, but was not involved in the oxidation of L-α-glycerolphosphate or dihydroorotate. Having demonstrated the involvement of ubiquinone in the respiratory chain, Cox et al., (1970) investigated the steady-state reduction levels of flavin, cytochrome b_1 and cytochrome a_2 of normal and ubi⁻ strains of E. coli K12. The effect of inhibitors on the steady-state reduction levels of these respiratory components of the normal strains was examined also in an attempt to elucidate the functional location of ubiquinone in the electron transport chain.

The levels of flavin and cytochrome a_2 in the ubi⁻ mutant were slightly higher and the concentration of cytochrome o slightly lower, than in the wild type revertant. Cytochrome b_1 levels were identical. The steady-state reduction levels of flavin and cytochrome b_1 in respiratory particles of the

ubi⁻ mutant, and the normal strain inhibited with piericidin A, were increased in both the NADH and lactate oxidase systems. It was also observed that the addition of NaCN to the respiratory particles of the normal strain, at a concentration which caused less inhibition of the NADH oxidase than ubiquinone deficiency, caused a greater increase in the level of steady-state reduction of cytochrome b_1.

Cox et al., (1970) interpreted these results to indicate that ubiquinone functions at two sites in the NADH oxidase portion of the respiratory chain. One site was placed before cytochrome b_1 and one site between cytochrome b_1 and oxygen. The results obtained with piericidin A also were consistent with inhibition at two sites, one before and one after cytochrome b_1. Ubiquinone appeared to function only after cytochrome b_1 in the lactate oxidase system since the steady-state reduction of cytochrome b_1 in membranes from the ubi⁻ strain was not markedly different from that in membranes from the normal strain in the presence of cyanide.

Subsequently, an investigation of the ubiquinone-8 present in the membranes of the normal strain indicated that approximately 50% of this quinone (concentration 25 times that of cytochrome b_1) was in the reduced form in the absence of added substrates whereas the other electron carriers were essentially fully oxidized. Cox et al., (1970) pointed out that if the high percentage of reduced ubiquinone was due to disproportionation of the semiquinone on extraction into the organic solvent, essentially all of the ubiquinone present should have been in the semiquinone form. However, electron paramagnetic resonance measurements (Hamilton et al., 1970) indicated that only 2% of the ubiquinone was in the semiquinone form, unless as suggested by Cox et al., (1970), the low signal might have been due to the

binding of the ubisemiquinone to a metal to give a chelate complex which was devoid of an electron paramagnetic signal (Beinert and Hemmerich, 1965). The addition of piericidin A to the membranes eliminated the EPR signal of the ubisemiquinone within 30 seconds and caused oxidation of the reduced ubiquinone within 10 minutes. However, the presence of piericidin A prevented the reduction of ubiquinone following the addition of substrate, although it increased the steady-state reduction of flavin and cytochrome b_1. These results led the authors to conclude that ubiquinone probably did not function as a direct electron carrier. This they considered particularly unlikely at two sites since the redox potential has not been demonstrated to be markedly changed by environment (Boyer, 1968).

As a possible explanation of the observed results, Cox et al., (1970) proposed the following. Ubiquinone-8 is complexed with an electron carrier. The electron carrier alone does not function as efficiently in electron transport as the carrier-ubiquinone complex, ubiquinone being in the semiquinone form. Inhibition of electron transport by piericidin A would occur by disruption of the complex. Nonheme iron was suggested as the most likely electron carrier to form a complex with ubiquinone since nonheme iron can function at different redox potentials (Boyer, 1968).

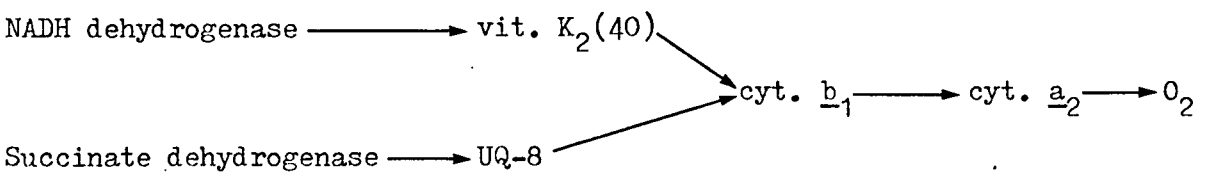
The functions of vitamin $K_2(40)$ are still unclear, however, Cox et al., (1970) state that preliminary investigations with men$^-$ mutants indicate that vitamin K_2 is not involved in aerobic respiration. Newton et al., (1971) have demonstrated that vitamin K_2 functions in the anaerobic oxidation of dihydroorotate utilizing fumarate as the electron acceptor.

A number of proposed models of the respiratory chain of E. coli have appeared in the literature (Kashket and Brodie, 1963b; Birdsell and Cota-

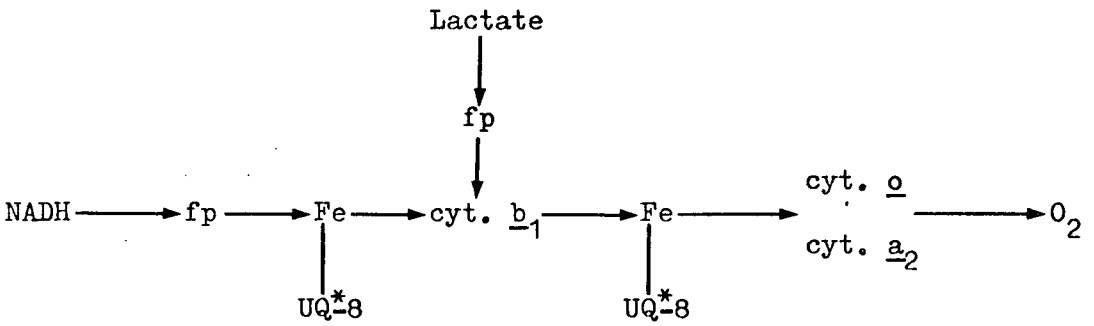
Robles, 1970; Cox et al., 1970; Hendler, 1971). The first model to appear. that of Kashket and Brodie, is presented in Figure 1.6A. It accounted for the experimental results available at the time but, as indicated above, subsequent research (Cox et al., 1968a; Cox et al., 1970) has raised considerable doubt as to the involvement of vitamin K_2 in the respiratory chain of E. coli. However, the results of Jones (1967) indicated that the possibility of a strain difference should not be overlooked. Figure 1.6B presents the model proposed by Cox et al., (1970), based on their investigations into the respiratory chain of E. coli K12. As stated by these authors, it represents a basis for further work taking into account their various observations. In this respect, their model is the simplest linear sequence of carriers that could account for their results. At least one of these observations requires qualification. No attempt was made to evaluate the increased contribution of cytochrome o to the cytochrome b_1 peak following the addition of NaCN to membranes from the normal strain of E. coli. Consequently, the validity of the statement that NaCN increased the steady-state reduction level of cytochrome b_1 above that observed with ubiquinone deficiency is open to question. However, the fact that reports by other investigators (Jones, 1967; Lightbown and Jackson, 1956; Bragg, 1970) have indicated inhibition by HQNO and piericidin A prior to cytochrome b_1 suggests that the conclusions that there are two sites of ubiquinone function is probably valid.

There remains a great number of unanswered questions, in addition to the necessity of verifying the proposals of Cox et al., (1970). Among these questions are the following: (i) What are the relative importances of the cytochromes a_2 and o as terminal oxidases? (ii) Where is cytochrome a_1

A.



B.



C.

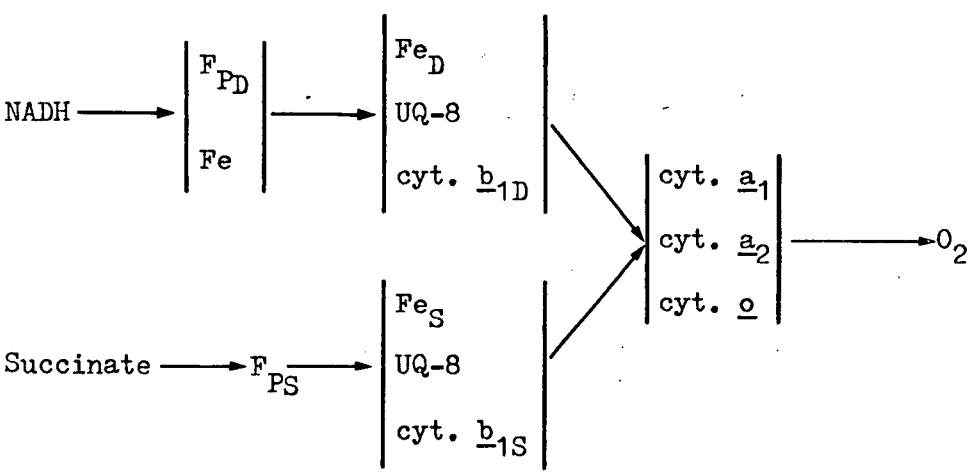


Fig. 1.6 Models of the electron transport systems of E. coli.

involved in the respiratory chain? (iii) Is there more than one functionally and/or chemically distinct cytochrome $\underline{b}_1$ as results presently suggest (Baillie et al., 1971; Kim and Bragg, 1971; Shipp, 1972a)? (iv) Are the electron transport chains utilizing either cytochrome $\underline{a}_2$ or cytochrome o as terminal oxidases identical with respect to all other components? (v) Is ubiquinone involved in the respiratory chain as an electron carrier, as a coupling component, or in a regulatory role? (vi) What is the function of nonheme iron?

With so many degrees of uncertainty in our knowledge of the respiratory chain of E. coli, perhaps the model proposed by Bragg (in press) (Figure 1.6C) is the most appropriate at present.

1.4 Oxidative phosphorylation

Historically, bacterial oxidative phosphorylation was first investigated in E. coli (Hersey and Ajl, 1951; Pinchot and Racker, 1951). Hersey and Ajl described the obligatory uptake of ^{32}P concurrent with succinate oxidation by cell-free extracts of E. coli. The uptake of ^{32}P was inhibited by 2,4-dinitrophenol and azide while NaF augmented the incorporation of ^{32}P into acid-labile phosphates. A maximum P:O ratio of 0.7 was obtained. Pinchot and Racker, also utilizing a cell-free system investigated phosphate uptake associated with NAD^+-dependent ethanol and acetaldehyde oxidation. Maximum P:O values of 1.0 and 1.5 respectively were reported. However, as Pinchot subsequently pointed out (1965), due to the presence of the glycolytic enzymes in the cell-free systems, the phosphorylation observed may have been due to substrate level phosphorylation.

Further reports on attempts to elucidate oxidative phosphorylation in E. coli did not appear until 1963. Bragg and Polglase (1963) reported

that phosphorylation coupled to NADH oxidation required both particulate and supernatant fractions from sonicated E. coli. They were able to obtain P:O values of 0.2-0.65. Kashket and Brodie (1963a) demonstrated that a slowly sedimenting membrane fraction (small particles) from sonicated E. coli, was capable of oxidizing succinate, α-oxoglutarate, malate, pyruvate and NADH, and of coupling the oxidation to phosphorylation, when supplemented with a 30 to 60% ammonium sulfate precipitated protein fraction from the supernate. Maximum P:O values of 0.5, 1.0, 1.1, 1.3 and 0.3 were obtained with succinate, α-oxoglutarate, malate, pyruvate and NADH respectively. Phosphorylation associated with malate and succinate oxidation was investigated in more detail particularly with respect to the action of uncouplers. Phosphorylation linked to NAD^+-dependent malate oxidation was uncoupled by PCMB, lapachol, benzoquinone-6, TTFA, malonate, usnic acid and low concentrations of dicumarol. Succinate-coupled phosphorylation was uncoupled only by dicumarol, lapachol and benzoquinone-6, while succinate oxidation was inhibited by PCMB and malonate. Neither pathway was sensitive to DNP. Nisman et al., (1963) investigated oxidative phosphorylation by membrane particles from E. coli spheroplasts lysed with digitonin. They reported the formation of ^{32}P-ATP and ^{32}P-ADP concurrently with NADH oxidation in the presence of AMP. Phosphate uptake and incorporation into ATP and ADP was inhibited by DNP, amytal and antimycin.

Investigating the component(s) present in the supernate which were essential for, or enhanced oxidative phosphorylation in E. coli membrane particles, Ota (1965) isolated three, apparently distinct, protein fractions that were essential for phosphorylation coupled to reduction of both oxygen and nitrate. Subsequently, Bragg and Hou (1968) demonstrated that a part-

iculate fraction of E. coli capable of NADH-dependent oxidative phosphorylation was stimulated by a coupling-factor preparation to yield a maximum P:O of 0.77.

These results are characteristic of those obtained for oxidative phosphorylation of bacteria. In general, the properties of fractionated bacterial systems differ from coupled phosphorylation in intact mitochondria in that the bacterial systems yield lower P:O ratios and fail to exhibit the type of respiratory control observed with mitochondria. These differences may be related to the more drastic procedure required to disrupt bacterial cells. In most bacterial systems soluble components which are necessary for the restoration of oxidation, phosphorylation, or both activities, of electron transport particles containing a structurally intact respiratory chain, are liberated during cell disruption. Bacterial systems also differ in their sensitivity to uncoupling agents. Some bacteria resemble mitochondrial systems in their response to low concentrations of these agents whereas others are totally insensitive or require higher concentrations than those normally used with mitochondria (Gel'man et al., 1967; Brodie and Gutnick, 1972).

Hempfling (1970a) appears to have clarified the uncertainty surrounding the P:O ratio of intact E. coli as compared to that of electron transport particles from this organism. He employed a technique whereby intact anaerobic E. coli were administered a pulse of oxygen and the concomitant changes in uptake of ^{32}P and NAD^+ levels were measureed. From this data values of $P:2e^-$ were calculated. Hempfling obtained apparent $P:2e^-$ values of 2.6-3.0.

The validity of this technique is based on the assumption that due to the rapidity of the terminal electron transport, the process of oxidative

phosphorylation is isolated kinetically rather than physically from contiguous interfering reactions. Hempfling also points out that energy-linked changes would contribute to an underestimate of the true P:$2e^-$ ratio. Energy-linked functions in E. coli will be discussed later.

Subsequently, Hempfling (1970b) investigated the influence of the carbon source and growth stage on the ability of intact aerobically grown E. coli to couple phosphate esterification to oxygen utilization. The results indicated (i) that a carbon source could be allocated to one of three categories according to the ability of the cells grown on the carbon source to carry out oxidative phosphorylation, and (ii) that with cells grown on glucose, the P:$2e^-$ ratio increased from 0.33 in the presence of glucose, to a value of 3.3 within 90 minutes of the exhaustion of glucose. The development of full oxidative phosphorylation, following release from catabolite repression required protein synthesis. There is considerable similarity between the growth dependence of development of oxidative phosphorylation as reported by Hempfling (1970b), and the development of oxidative phosphorylation associated with anaerobic to aerobic adaptation as described by Cavari et al., (1968), including the requirement for protein synthesis. Hempfling and Beeman (1971) demonstrated that the catabolite repression of oxidative phosphorylation could be relieved by the presence of 2-5 mM 3;5'-adenosine monophosphate (cAMP) in the growth medium. However, this appeared to alter some characteristic(s) of the system since oxidative phosphorylation by cells grown on glucose in the presence of cAMP was not sensitive to the uncoupler 2,4-dibromophenol (DBP).

The mechanism of oxidative phosphorylation is not known but the reaction responsible for the esterification of phosphate to ADP, to form

ATP, is generally considered to be the reversal of the membrane bound magnesium (or calcium) ion dependent, adenosine triphosphatase ($Mg^{2+}(Ca^{2+})$-ATPase) activity. Consequently, there is a great deal of interest in the properties of this enzyme. Recently, due to the efforts of researchers from three laboratories the membrane bound $Mg^{2+}(Ca^{2+})$-ATPase of E. coli has been solubilized and characterized in considerable detail (Evans 1969, 1970; Davies and Bragg, 1972; Kobayashi and Anraku, 1972). The reader should consult these papers for a discussion of the properties of this enzyme.

The first information on the properties of $Mg^{2+}(Ca^{2+})$-ATPase deficient mutants of E. coli was reported by Butlin et al., (1971). These mutants, designated uncA$^-$, were able to grow on glucose but were unable to grow with succinate or D-lactate as the sole source of carbon. All membrane components of the respiratory chain and the NADH and succinate oxidase levels of the mutant strain were identical with those of the normal strain. Neither $Mg^{2+}(Ca^{2+})$-ATPase nor oxidative phosphorylation was detectable in the uncA$^-$ strain. Kanner and Gutnick (1972a,b) and Simoni and Shallenberger (1972) have also described the isolation and characterization of $Mg^{2+}(Ca^{2+})$-ATPase deficient mutants of E. coli. Like the uncA$^-$ strains of Butlin et al., (1971), these mutants could not utilize TCA cycle intermediates or D-lactate as sole carbon sources and the aerobic growth yield of the mutants on glucose was intermediate between the aerobic and anaerobic growth yield of the corresponding wild type E. coli. However, whereas the anaerobic growth yield of the uncA$^-$ strain of Butlin et al., was approximately 64% of that for the normal strain, that of the mutant strain isolated by Simoni and Shallenberger was only 45% of the growth yield of the wild type.

The results obtained, particularly those of Butlin et al., suggest that the $Mg^{2+}(Ca^{2+})$-ATPase is essential for oxidative phosphorylation and is not required for growth when ATP is generated by substrate level phosphorylation. Since the aerobic growth yield of the ATPase deficient mutants was greater than the anaerobic growth yield of the wild type strain, it would appear, as suggested by Kanner and Gutnick (1972b) that the $Mg^{2+}(Ca^{2+})$-ATPase deficient mutants have retained the ability to couple glucose oxidation to the generation of some high energy state, or intermediate, which might be utilized to drive energy-requiring cellular processes. A possible explanation for the differences in the aerobic growth yields of the ATPase deficient mutants will be discussed later.

Interest in energy-linked reactions directly coupled to the electron transport chain of E. coli has increased markedly in the last half decade. Energy-linked systems, present in E. coli, capable of deriving energy from ATP and/or directly from the respiratory chain appear to include: (i) D-lactate oxidase-coupled transport systems (Kaback, 1972; Schairer and Haddock, 1972); (ii) the energy-dependent reduction of NAD^+ by succinate (Sweetman and Griffiths, 1971a); (iii) the energy-dependent pyridine nucleotide transhydrogenase (Murthy and Brodie, 1964); and possibly (iv) induced protein biosynthesis (Kováč and Kužela, 1966; Galotti, Kováč and Hess, 1968).

As the D-lactate oxidase-coupled transport systems have been discussed in section 1.1, they will not be discussed further at this point.

The only investigation on the energy-dependent reduction of NAD^+ by succinate, in E. coli has been by Sweetman and Griffiths (1971a). The reaction was dependent on Mg^{2+} and succinate, and ATP could only be partially replaced by ITP. ADP at a concentration equal to that of ATP caused a 50%

inhibition of the ATP-driven reduction of NAD^+ by succinate. The proposed stoichiometry for the reaction was one molecule of ATP hydrolyzed per molecule of NADH produced. The reaction was inhibited by the uncouplers 4,5,6,7-tetrachloro-2-trifluoromethyl benzimadazole (TTFB), pentabromophenol (PBP) and dicumarol but was insensitive to DNP or oligomycin. Low concentrations of piericidin A or HQNO stimulated the reduction of NAD^+ by succinate. Based on these results Sweetman and Griffith concluded that the reduction of NAD^+ by succinate involved NADH dehydrogenase, succinate dehydrogenase, and probably nonheme iron since the reaction was inhibited by TTFA. They proposed that high energy intermediates of oxidative phosphorylation provided the necessary energy supply.

The energy-dependent transhydrogenase (EDTH) first described by Murthy and Brodie (1964) has become a popular system for the investigation of the characteristics of energy-linked reactions in E. coli because of the ease and rapidity of the spectrophotometric assays. Initially, (Murthy and Brodie, 1964; Bragg and Hou, 1968) the system was investigated only as the ATP-driven reaction although Bragg and Hou (1968) suggested that since the energy-dependent transhydrogenase was more stable than oxidative phosphorylation to ultraviolet irradiation, that these processes probably involved different coupling factors. Fisher et al., (1970), Fisher and Sanadi (1971) and Kanner and Gutnick (1972b) subsequently demonstrated that the energy-dependent transhydrogenase could be driven via ATP or respiration-linked to energy production. Cox et al., (1971) and Kanner and Gutnick (1972b) have shown that mutants deficient in the $Mg^{2+}(Ca^{2+})$-ATPase lack the ATP-driven transhydrogenase but retain the respiration driven activity. The ATP-driven transhydrogenase did not require ubiquinone-8 or menaquinone-8 for

activity, and was inhibited by piericidin A at a site unrelated to the site of inhibition of the electron transport chain(Cox et al., 1971). Both the ATP-driven and respiration linked transhydrogenase were inhibited by TTFB, DNP, dicumarol, PBP and thyroxine while only the ATP-driven transhydrogenase was inhibited by dicyclohexylcarbodiimide (DCCD). Oligomycin had no effect on either the ATP-driven or the respiratory chain-linked energy-dependent transhydrogenase activities (Fisher and Sanadi, 1971; Sweetman and Griffiths, 1971b; Kanner and Gutnick, 1972b).

The information available suggests, as proposed by Slater (1953), that energy-linked reactions are driven by high-energy, nonphosphorylated intermediates of oxidative phosphorylation which can also be generated from, and are in equilibrium with the cellular ATP. The $Mg^{2+}(Ca^{2+})$-ATPase appears to be intimately involved in generating the high-energy intermediate(s) from ATP (Cox et al., 1971; Kanner and Gutnick, 1972b; Schairer and Haddock, 1972).

A coupling factor possessing $Mg^{2+}(Ca^{2+})$-ATPase activity but without energy-dependent transhydrogenase activity has been isolated and purified from E. coli (Bragg and Hou, 1972). The addition of the purified coupling factor to respiratory particles which had been "stripped", that is, were devoid of both the respiratory chain-linked and the ATP driven transhydrogenase activities, resulted in the recoupling of both of these activities. This system promises to provide further insight into the mechanism by which energy-linked systems are coupled to the respiratory chain and to ATP.

Simoni and Shallenberger (1972) have isolated an ATPase deficient mutant of E. coli which lacks the ability to transport alanine or proline with either ATP, or respiratory chain-linked D-lactate oxidation as the

source of energy. This is contrary to the results previously obtained with the energy-dependent transhydrogenase which, as indicated above, demonstrated that ATPase deficient mutants of E. coli lacked the ATP-driven EDTH but retained the electron transport system-linked EDTH (Cox et al., 1971; Kanner and Gutnick, 1972b). However, Bragg and Hou (1973), utilizing the energy-dependent transhydrogenase as a test system, have demonstrated that the ATPase deficient mutant of Simoni and Shallenberger lacked not only the ATPase, but also the coupling factor responsible for coupling the energy-dependent transhydrogenase directly to the respiratory chain. Thus, it appears that the same coupling factor could be involved in coupling amino acid transport and the energy-dependent, pyridine nucleotide transhydrogenase to energy generated via the electron transport chain. The lack of direct coupling of energy-linked reactions to the respiratory chain may explain the lower growth yield reported by Simoni and Shallenberger (1972) for the E. coli mutant, DL-54, as compared to the growth yield obtained by Butlin et al., (1971) for the uncA$^-$ mutants.

In summary, the preceding discussion of oxidation phosphorylation and energy-linked systems of E. coli indicates:

(i) E. coli is capable of a maximum P:O ratio of 3;

(ii) that the degree of coupling of phosphorylation to oxidation appears to be related to the ability of the substrate to maintain an adequate level of ATP by substrate-level phosphorylation;

(iii) that the energy-linked reactions are driven by nonphosphorylated, high-energy intermediates generated directly by the electron transport chain, or from ATP via the $Mg^{2+}(Ca^{2+})$-ATPase; and

(iv) that the energy-linked systems of E. coli include: (a) transport,

(b) NAD^+ reduction by succinate, (c) energy-dependent transhydrogenase, and possibly (d) protein synthesis.

The control of respiration in bacteria, like the control of other enzymatic pathways could be mediated at two levels: (i) molecular regulation of the enzymatic components of the pathway, and (ii) the regulation of the biosynthesis of the components of the pathway *via* induction of repression. The former, as usually expressed in the control of the electron transport system, is the stimulation of respiration by ADP and inorganic phosphate (P_i) followed by a return to the original rate on the exhaustion of either ADP or P_i. This is commonly referred to as respiratory control. As indicated previously, bacteria have been considered to lack respiratory control (Gel'man *et al.*, 1967; Brodie and Gutnick, 1972). However, increased rates of oxygen uptake on the addition of phosphate or phosphate-acceptor have been reported (Ishikawa and Lehninger, 1962; Revsin and Brodie, 1967; Scocco and Pinchot, 1968). The presence of respiratory control in intact bacteria has been indicated indirectly by the stimulation of the respiratory rate of whole cells on the addition of uncouplers of oxidative phosphorylation (Bovell and Packer, 1963; Bovell, Packer and Helgerson, 1963; Cavari *et al.*, 1967; Hempfling, 1970a,b).

Respiratory control has not been demonstrated conclusively in *E. coli*, either in intact cells or respiratory particles, nor has the addition of P_i and/or ADP to respiratory particles been shown to stimulate oxygen utilization. Oishi *et al.*, (1970) have reported the occurrence of respiratory control by phosphate, in intact *E. coli*, based on the observed stimulation of respiration by addition of phosphate to phosphate-depleted cells and the subsequent return to the non-stimulated rate on the depletion of P_i from

the assay medium. Unfortunately they were unable to eliminate the possibility that the stimulation of respiration was due to the coupling of phosphate transport to respiration and energy production. Cavari et al., (1967) have reported that respiration was stimulated and the incorporation of ^{32}P was inhibited on the addition of carboxylcyanide-m-chlorophenylhydrazone (CCCP) to suspensions of intact E. coli oxidizing glucose. However, identical levels of CCCP inhibited the oxidation of succinate, glutamate and pyruvate. Stimulation of formate and glucose oxidation by intact E. coli on the addition of DBP has been reported by Hempfling (1970a,b). However, although DBP abolished phosphorylation associated with the oxidation of endogenous NADH, it did not increase the rate of endogenous NADH oxidation (Hempfling, 1970a).

Although respiratory control has not been demonstrated in E. coli, it has been demonstrated in other bacteria. John and Hamilton (1970, 1971) have shown respiratory control in membrane particles of Micrococcus denitrificans and it has also been demonstrated in Azotobacter vinelandii membranes (Eilerman et al., 1970; Jones et al., 1971a,b).

Control of the respiration of E. coli by those environmental factors which more or less specifically cause alteration of the composition and/or function of the respiratory chain, usually via induction or repression of the synthesis of respiratory chain components, is of particular interest. The two factors which have been most thoroughly investigated in this respect are oxygen tension and the carbon source. The alterations in the levels of ubiquinone-8 and menaquinone-8 in response to aeration have been discussed. The influence of oxygen tension on the cytochrome components of the respiratory chain and respiratory activity of E. coli was first reported by Moss

(1952). His results demonstrated that an increase in the levels of cytochrome $\underline{a}_1$, $\underline{a}_2$ and $\underline{b}_1$ occurred on aeration. There was also an increase in the Qo_2. The Qo_2 was not closely related to the amount of cytochrome $\underline{a}_2$ present. Subsequently, Gray *et al.*, (1966b), Hino and Maeba (1966), Takahashi and Hino (1968a,b) and Cavari *et al.*, (1968) have investigated further the influence of oxygen on the development of the respiratory pathways. Gray *et al.*, (1966b) grew *E. coli* K12 under aerobic and anaerobic conditions and reported that higher levels of NADH oxidase, succinate oxidase, succinate dehydrogenase and cytochrome $\underline{b}_1$ were formed under aerobic than anaerobic conditions. Levels of cytochromes $\underline{a}_1$ and $\underline{a}_2$ were reported not to be influenced by these growth conditions, in contrast to the earlier reports of Moss (1952). Hino and Maeba (1966) observed that there was only a slight (2- to 3- fold) increase in the cytochrome content of anaerobic cells which had been aerated in a buffer containing casamino acids, while the respiratory activity had increased 20 to 30 fold. Inhibition of the development of increased respiratory activity by chlorophenicol demonstrated that the increase required protein synthesis, probably of some component other than cytochromes. Ishida and Hino (1972) suggested that the slight increase in the level of cytochromes was due to: (i) the increased formation of succinyl CoA; (ii) an increased level of an enzyme in a reaction between δ-aminolevulinic acid (ALA) and protoheme; (iii) the activation of preformed enzymes in a reaction between ALA and protoheme; and (iv) the induction of ALA synthase; as a result of the increased oxygen tension. Takahashi and Hino (1968a,b) demonstrated that the markedly higher respiratory activity of the aerated cells was due to: (i) the induction of transport systems for intermediates of the TCA cycle, and (ii) an approximately 10-fold increase

in the activity of the tricarboxylic acid cycle enzymes. The mechanism responsible for inducing the increased synthesis of the TCA cycle enzymes is unknown. However, Wimpenny (1969) has proposed that it may be related to the cellular NAD^+/NADH ratio.

Cavari et al., (1968) have made several interesting observations. First, NADH dehydrogenase and succinate dehydrogenase activities of E. coli B were not influenced by the anaerobic to aerobic transition. (Succinate dehydrogenase of E. coli K12 was found to increase as previously reported by Gray et al., 1966b). Secondly, the NADH oxidase activity of E. coli increased rapidly on exposure of the anaerobic cells to aeration, reaching the maximum aerobic level within 10 minutes. Thirdly, succinate oxidase and cytochrome levels increased more slowly and in parallel, and approached the levels found in aerobic cells only after one hour of aeration. Fourthly, the P:O ratio and CCCP sensitivity of cells grown on a carbon source of mannitol and fumarate, began to increase after one hour of aeration, when the succinate oxidase and cytochrome levels had nearly reached the aerobic level. The mechanism responsible for the rapid increase in NADH oxidase activity is not known, but due to the short time required it probably does not involve new protein synthesis. The lack of effect of the anaerobic to aerobic transition on the succinate dehydrogenase activity, and the parallel increase in the succinate oxidase activity and cytochrome level, suggests that the former is a result of the latter. As indicated previously, the increase in the level of cytochromes can probably be explained by the proposals of Ishida and Hino (1972). The development of oxidative phosphorylation and CCCP sensitivity was probably the same phenomenon reported to occur on depletion of glucose in cultures of aerobically grown E. coli (Hempfling, 1970b).

More recently, Wimpenny and Necklen (1971) and Harrison and Loveless (1971) have reported on the influence of oxygen tension on E. coli in continuous cultures. Wimpenny and Necklen observed that the levels of cytochrome a_2 and b_1 increased at low oxygen tension (1-5 mm Hg), while Harrision and Loveless reported increased respiration rates and decreased growth yield under similar conditions. The elevated levels of cytochrome a_2 and the increased respiration rate combined with a decreased growth yield, observed under conditions of low oxygen tension, may be due to a greater affinity of cytochrome a_2 than cytochrome o for oxygen (White, 1963), and the uncoupling of respiration from energy conservation (Harrison and Pirt, 1967), respectively.

In summary, the growth of E. coli under aerobic conditions in the absence of glucose, results in: (i) increased levels of TCA cycle enzymes; (ii) slightly increased levels of respiratory chain cytochromes; (iii) an increased level of ubiquinone-8; (iv) the activation of NADH oxidase; and (v) an increased capacity to perform oxidative phosphorylation; as compared to E. coli grown under anaerobic conditions.

The influence of the carbon source on the respiration rate is primarily via catabolite repression of the synthesis of TCA cycle enzymes. Halpern et al., (1964) demonstrated that aerobic growth on glucose repressed synthesis of the enzymes of the TCA cycle and of the transport systems required for the uptake of TCA cycle intermediates. This effect of glucose was subsequently confirmed, and extended to show that glucose had little effect on the cytochrome levels of aerobically grown cells (Hino and Maeda, 1966; Gray et al., 1966b; Takahashi and Hino, 1968a,b).

1.5 The influence of silver ions on growth and enzyme activity

Silver ions inhibit the glucose-dependent aerobic respiration of

E. coli (Rainnie and Bragg, 1971). The obvious question is - How?

The antibacterial activity of silver compounds has been known since 1869, and has been differentiated on a basis of concentration; (i) "poisoning" death (silver concentration $\geq 6 \times 10^{-5}$ g-ions per litre, and (ii) "oligodynamic" death (silver concentration $< 6 \times 10^{-5}$ g-ions per litre) (Romans, 1957a). The former is believed to occur as a result of the irreversible denaturation and precipitation of the proteins of the bacterial cell (Meyers *et al.*, 1970). The mechanism of the latter remains unclear although many proposals have been put forward (Romans, 1957a). The mechanism of the oligodynamic activity of silver probably differs from that of "poisoning" by silver only in that a smaller quantity of silver is bound to proteins with the result that catalytic proteins are reversibly inactivated rather than irreversibly denatured.

The reaction of silver with proteins occurs primarily at the available sulfhydryl groups forming insoluble mercaptides, a characteristic which has resulted in the use of silver salts for the amperometric determination of the sulfhydryl content of proteins (Cecil and McPhee, 1959; Benesch and Benesch, 1962; Leach, 1966). However, the stoichiometry of the silver ion-sulfhydryl reaction may not be 1:1, nor is the specificity absolute (Cecil and McPhee, 1959; Benesch and Benesch, 1962; Leach, 1966; Burton, 1958; Sluyterman, 1957; Kolthoff and Eisenstädter, 1961; Cole *et al.*, 1958) as silver has also been shown to form complexes with a histidine residue in invertase (Myrback, 1957) and with free riboflavin (Weber, 1950), and has been suggested to react with carboxyl groups (Dixon and Webb, 1964; Woratz and Thofern, 1955).

The reported effects of silver ions on eucaryotic systems include: (i) the inhibition of numerous enzymes and enzyme systems (Webb, 1966a,b); (ii) the stimulation of enzymes (Rapoport and Luebering, 1951; Chappell and Greville, 1954; Cooper, 1960); (iii) the stimulation and inhibition of respiration (Cook, 1926; Chappell and Greville, 1954; Grabske, 1966); (iv) alteration of membrane permeability (Romans, 1957a; Brierley et al., 1967); and (v) the inhibition of aerobic and anaerobic growth (Romans, 1957a; Takada and Joho, 1964).

Included among the enzymes inhibited by silver ions are a number of enzymes involved in the Embden-Meyerhof pathway, the tricarboxylic acid cycle, electron transport, and energy conservation.

Glycolytic enzymes from a number of different sources, yeast hexokinase (Titova, 1968), aldolase from rabbit skeletal muscle (Herbert et al., 1940), 3-phosphoglyceraldehyde dehydrogenase from rabbit and porcine skeletal muscle (Park et al., 1961; Boross, 1965; Boross and Keleti, 1965), and yeast pyruvate decarboxylase (Stoppani et al., 1952), have been demonstrated to be sensitive to inhibition by silver ions. Silver did not inhibit the catalytic activity of the yeast hexokinase but it prevented the inhibition of the enzyme by hydrocortisone. The inhibition of 3-phosphoglyceraldehyde dehydrogenase by silver ions resulted in a complete loss of dehydrogenase activity but had no effect on the transphosphorylation reaction (Park et al., 1961). Boross and colleagues have isolated the inhibited 3-phosphoglyceraldehyde dehydrogenase as a crystalline ternary complex consisting of one mole-equivalent of NAD^+ and four mole-equivalents of silver ions per monomer of of the enzyme. The concentration of silver ions required to bring about complete inhibition of these enzymes was in the order of 1×10^{-5} normal (N).

In addition to the preceding enzymes a number of glycolytic enzymes have been shown to be sensitive to thiol reagents other than silver ions and therefore may also be inhibited by silver ions (Webb, 1966b; Scott et al., 1970).

Of the TCA cycle enzymes, only isocitrate dehydrogenase (Kratochvil et al., 1967) glutamate dehydrogenase (Olson and Anfinsen, 1953) and fumarase (Laki, 1942), all from mammalian sources, have been shown to be inhibited by silver ions in the concentration range of 10^{-7} to 10^{-5} N. However, as in the Embden-Meyerhof pathway, a number of additional TCA cycle enzymes have been shown to be sensitive to thiol reagents such as p-chloromercuribenzoate (PCMB), p-mercuriphenylsulfonate (PMPS),iodoacetate (IA), iodoacetamide (IAM) and N-ethylmaleimide (NEM) and these may also be inhibited by silver ions (Barron and Singer, 1945; Webb, 1966b; Slater, 1949; Bernath and Singer, 1962).

The only information available on the affect of silver ions on electron transport is the inhibition of a partially purified NADH-cytochrome c reductase from pig liver microsomes, reported by Garfinkel (1957). The enzyme system retained 10% of its initial activity at a silver nitrate concentration of 3×10^{-3} N.

Slightly more information is available on the effects of silver ions on the enzyme systems involved in energy conservation. Chappell and Greville (1954) reported the stimulation of mitochondrial ATPase activity by silver ions (1×10^{-5} N). Subsequently, Chiga and Plaut (1959) reported the inhibition by silver ions (5×10^{-5} N), of the ADP$\rightleftharpoons$ATP and ATP$\rightleftharpoons P_i$ exchange reactions catalyzed by the enzyme purified from hog liver mitochondria. Cooper (1960) demonstrated that $AgNO_3$ at low concentrations mimicked the

action of dinitrophenol and stimulated the ATPase activity of digitonin particles prepared from rat-liver mitochondria. Silver nitrate levels greater than 4×10^{-8} moles per milligram of protein markedly inhibited the ATPase activity.

Related to the stimulation of ATPase activity at low concentrations of silver ions is the affect of silver ions on respiration. Cook (1926) reported that the respiration of Aspergillus niger, as measured by CO_2 production, showed an initial stimulation in rate, followed by inhibition (silver nitrate concentration of 1×10^{-4} N). Chappell and Greville (1954) demonstrated a two-fold increase in the respiration rate of rabbit brain mitochondria on the addition of silver nitrate to final concentration of 5×10^{-6} N. These results were confirmed by Grabske (1966) with rat-liver mitochondria at a silver nitrate concentration of 2.7×10^{-4} N. The results of Grabske, like those of Cook (1926), demonstrated an initial stimulation of respiration followed by inhibition. Thus, silver ions initially uncouple respiration of mitochondria in a manner analogous to dinitrophenol, followed by inhibition of respiration. Uncoupling of oxidative phosphorylation and energy transfer by other thiol reagents, has also been reported (Kielley, 1963; Boyer et al., 1966; Kurup and Sanadi, 1968).

Although, as indicated at the beginning of this section, silver ions have been recognized as bacteriocidal for more than a century, the physiological and biochemical affects of these ions on bacteria remain largely unknown.

The influence of silver ions on E. coli include (i) the inhibition of aerobic and anaerobic growth which the authors suggested was due to interference with the oxidation mechanism (Grumbach and Wehrli, 1948); (ii) the inhibition of succinate transport by E. coli membrane vesicles at a

silver nitrate concentration of 1 x 10^{-4} N (Rayman et al., 1972a,b); (iii) the inhibition of glucose, lactate, succinate and formate dependent reduction of methylene blue (Yudkin, 1937); (iv) the inhibition of citrate, isocitrate, succinate, fumarate and malate dependent reduction of triphenyltetrazolium chloride (Woratz and Thofern, 1955); and (v) the inhibition of hydrogenase (Yudkin, 1937; Joklik, 1950), formic hydrogenlyase (Yudkin, 1937) and catalase activities (Woratz and Thofern, 1955). Unfortunately, due to the techniques used for measuring the "dehydrogenase" activities (Yudkin, 1937; Woratz and Thofern, 1955) very little can be concluded as to the actual site of Ag^+ inhibition. Also, Yudkin observed that the concentration of silver ions required to inhibit the enzyme activities under his assay conditions was 10 to 100-fold greater than the lethal concentration.

Although inhibition of the above enzyme systems could certainly result in inhibition of respiration, no investigation has been reported on the influence of silver ions on the respiration of E. coli.

1.6 The influence of iron limitation on respiration and energy conservation

Waring and Werkman first reported, in 1942, on the iron requirement of bacterial growth. They observed that low levels of iron in the growth media of Aerobacter indologenes, Aerobacter aerogenes, Psuedomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli and Serratia marcescens resulted in reduced growth yields. This was subsequently confirmed with E. coli (Young et al., 1944; Ratledge and Winder, 1964) and has also been reported to occur with Corynebacterium diphtheriae (Pappenheimer and Hendee, 1947), Pseudomonas fluorescens (Lenhoff et al., 1956), Mycobacterium smegmatis (Winder and O'Hara, 1962), Neurospora crassa (Padmanaban and Sarma, 1965), Mycobacterium phlei (Antoine and Morrison, 1968) and Candida utilis (also classified as Torulopsis

utilis) (Clegg et al., 1969; Clegg and Garland, 1971; Clegg and Light, 1971).

The most obvious site for iron involvement in these organisms was cytochromes and consequently research was directed to the influence of iron limitation on enzyme systems associated with the respiratory chain. Effects were found. For example, Waring and Werkman (1944) reported decreased activities of lactate, pyruvate and acetate oxidases in A. indologenes grown under iron-deficient conditions. The succinate oxidase activity of C. diphtheriae was also found to be reduced under iron-deficient growth conditions (Pappenheimer and Hendee, 1947; Righelato, 1969) as was the cytochrome c oxidase of N. crassa (Nicholas and Commissiong, 1957). Growth of N. crassa and M. smegmatis under conditions of iron limitation resulted in reduced levels of NADH-cytochrome c reductase (Nicholas and Commissiong, 1957; Winder and O'Hara, 1964). Nicholas and colleagues have demonstrated lower NADH-nitrate reductase activity in N. crassa (Walker and Nicholas, 1961a; Nicholas and Wilson, 1964), P. aeruginosa (Fewson and Nicholas, 1961a) Micrococcus denitrificans (Fewson and Nicholas, 1961b) and Pseudomonas denitrificans (Radcliffe and Nicholas, 1970) due to growth under iron-limited conditions. The nitrite reductases of N. crassa (Nicholas et al., 1960) and P. aeruginosa (Walker and Nicholas, 1961b), and the nitric oxide reductase of the latter organism (Fewson and Nicholas, 1961c), also were present at reduced levels in iron-deficient cultures.

The reduced levels of many of these enzyme systems may be explained on the basis of reduced levels of cytochromes. Decreased levels of cytochromes, due to iron limitation, have been reported in cultures of P. fluorescens (Lenhoff et al., 1956) Spirillum itersonii (Clark-Walker et al., 1967), M. denitrificans (Imai et al., 1968), C. diphtheriae (Righelato and van Hemert,

1969; Righelato, 1969) and C. utilis (Light and Garland, 1971; Clegg and Garland, 1971; Ohnishi et al., 1969).

However, iron, as nonheme iron, has been shown to be a component of NADH dehydrogenase and succinate dehydrogenase (Hall and Evans, 1969). Decreased succinate dehydrogenase activity has been reported to occur in A. indologenes (Waring and Werkman, 1944), M. smegmatis (Winder and O'Hara, 1964), N. crassa (Padmanaban and Sarma, 1965) and C. diphtheriae (Righelato and van Hemert, 1969; Righelato, 1969) as a result of growth under iron-limited conditions.

Nonheme iron has been implicated as possibly functioning in oxidative phosphorylation (Hall and Evans, 1969; Bragg, 1973). Iron limitation has also been utilized to study the involvement of iron in oxidative phosphorylation in C. utilis (Clegg et al., 1969; Clegg and Garland, 1971; Ohnishi et al., 1969; 1971) and M. denitrificans (Imai et al., 1968)

Research by Garland's and Ohnishi's groups has demonstrated that the growth of C. utilis in batch or continuous culture under conditions of iron limitation resulted in the loss of energy conservation at site I as measured either by NADH-dependent P:O ratios or the energy-dependent reversal of electrons from glycerol-1-phosphate to endogenous pyridine nucleotide. At the concentration of iron at which site I phosphorylation was lost, there was also a loss of the g=1.94 EPR signal of the NADH dehydrogenase region of the respiratory chain. Clegg and Garland (1971) observed that incubating iron-deficient cells aerobically for several hours under non-growing conditions resulted in the recovery of site I energy conservation but not the g=1.94 EPR signal. Incubation in the presence of cycloheximide (360 μM) did not prevent the recovery of site I energy conservation. These

results suggested the dissociation of energy conservation at site I from the g=1.94 EPR signal and that de novo protein synthesis was not required for the recovery of site I phosphorylation.

Additional evidence for the involvement of nonheme iron proteins at site I has been obtained by Clegg and Garland (1971). Electron transport particles from iron-limited cells had lower nonheme iron and acid-labile sulfide content than those of iron-sufficient cells. The ratio of the decrease in the nonheme iron to the decrease in acid-labile sulfide on transition from iron-sufficient to iron-deficient conditions was 1.2. Since the ratio of iron to sulfide is usually 1.0 in iron-sulfur proteins (Hall and Evans, 1969), iron deficiency appeared to result in the loss of iron-sulfur proteins. Aerobic incubation of iron-limited cells in the presence of $FeSO_4$, under non-growing conditions, resulting in the recovery of site I phosphorylation, was accompanied by an increase in the content of both nonheme iron and acid-labile sulfide in the electron transport particles. Also supporting the idea that iron-sulfur proteins were involved in site I energy conservation was the finding by Haddock and Garland (1971) that sulfate limitation during the growth of C. utilis resulted in mitochondria lacking site I. In view of these results and having calculated that at least 90% of the nonheme iron in electron transport particles from C. utilis grown under iron-sufficient conditions was not required for site I energy conservation, Garland and coworkers have proposed that a small fraction of the mitochondrial nonheme iron proteins which does not show EPR signals may play a role in site I phosphorylation.

Ohnishi and colleagues have attempted to obtain more direct evidence for the involvement of nonheme iron proteins at site I. Ohnishi et al.,

(1972a) have restated and reinforced their initial proposal, that there is a close correlation between site I energy conservation and iron sulfur proteins responsible for the g=1.94 EPR signal in the NADH dehydrogenase region of the respiratory chain. In contrast to the results of Clegg and Garland (1971), their results indicated that there was recovery of site I phosphorylation, and the g=1.94 EPR signal on aeration of intact iron-deficient C. utilis under non-growing conditions. Incubation under the same conditions but in the presence of 100 μM cycloheximide prevented the recovery of both parameters. If the g=1.94 EPR signal is definitely associated with site I phosphorylation this would provide conclusive evidence for the involvement of nonheme iron in energy conservation at site I.

In contrast to the results with C. utilis, oxidative phosphorylation in respiratory particles from M. denitrificans was not affected by iron-deficiency although the EPR signal associated with the respiratory chain-linked nonheme iron disappeared (Imai et al., 1968). It should be noted however, that whereas Clegg and Garland (1971) obtained a 95% reduction in nonheme iron levels under conditions of iron limitation, Imai et al., reported a decrease of 85%. The decrease obtained by Imai et al., may have been inadequate to effect oxidative phosphorylation. Thus, it is not clear whether nonheme iron, as well as being a component of the respiratory chain, has a role in energy coupling in bacteria. It was for this reason that the influence of iron limitation on the respiration of E. coli was examined.

1.7 Objectives of the research reported in this thesis

It is apparent from the preceding discussion of the transport systems, amphibolic pathways, respiratory chain and oxidative phosphorylation of E. coli that there are major deficiencies in our understanding of each of

these systems. The lack of knowledge is most marked with respect to the sequence of the respiratory carriers, the regulation of the respiratory chain, and the characteristics of oxidative phosphorylation and/or energy-linked systems. Investigation of the respiratory chain of E. coli, and associated energy conservation, has been hampered by the following: (i) the absence of a discrete organelle possessing the respiratory chain and associated energy generating system(s), such that they can be isolated and investigated without complication from the energy utilizing systems of the intact cell, (ii) the lack of a sufficient range of inhibitors of the bacterial respiratory chain to permit an unequivocal determination of the sequence of the respiratory chain components, in contrast to the broad selection of inhibitors of the mitochondrial respiratory chain, and (iii) the conditions required to disrupt the bacterial cell may result in the loss or denaturation of factors required for respiration and/or oxidative phosphorylation.

As a result of the latter problem, cell-free systems from bacteria generally show much lower P:O values than those observed in tightly coupled mitochondria (Gel'man et al., 1967). Moreover, it was only recently that ADP-ATP medicated respiratory control has been demonstrated in bacterial cell-free systems (Jones et al., 1971b; John and Hamilton, 1971). Thus, cell-free systems were considered to be unsuitable for our studies. However, P:O values obtained with intact cells also may not accurately reflect the ability of the cell to generate energy via the respiratory chain. Energy-utilizing reactions, other than ATP formation, could compete for the respiratory chain-generated high energy states or intermediates (Harold, 1972). In mitochondria, that respiration which can be stimulated by ADP and which results in the conversion of ADP to ATP can also be stimulated by uncoupling

agents. This has also been shown with respiratory particles of M. denitrificans (John and Hamilton, 1971). The stimulation of respiration by uncoupling agents is probably due to the dissipation of a high energy state intermediate (Harold, 1972) and could be used as an indicator of the presence of the high energy state whether or not this could be converted to ATP. This concept has been used in the study of the influence of silver ions, and the effect of progressive iron limitation such as occurs during growth in an iron-limited batch culture, on the respiration and energy conservation of E. coli.

The observation that silver ions released from an oxygen electrode initially stimulated the respiration of E. coli prior to bringing about a complete inhibition of oxygen consumption, suggested that silver ions might function as an uncoupler as well as an inhibitor of the respiration and prompted an investigation of the site(s) at which silver ions influenced the aerobic respiration of E. coli in the hope that silver ions might be useable as a tool for further research into the sequence and energy coupling of the respiratory chain of E. coli.

In view of the observed influence of iron limitation on the site I energy-coupling of Candida utilis (Clegg et al., 1969; Clegg and Garland, 1971; Ohnishi et al., 1969, 1971), but apparent lack of effect on oxidative phosphorylation in respiratory particles from M. denitrificans (Imai et al., 1968), the role of iron in the respiratory chain, and in energy-coupling linked to the respiratory chain, in bacterial systems, is unclear. For this reason an investigation of the involvement of iron in the function of the E. coli respiratory chain and in the coupling of energy conservation to the respiratory chain was undertaken.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

The organism utilized for the majority of the experiments reported in this thesis was Escherichia coli, strain 482 of the National Research Council culture collection (E. coli NRC 482). This strain was obtained from the Department of Microbiology, University of British Columbia by Dr. W. J. Polglase of the Department of Biochemistry and was subsequently supplied to our laboratory by Dr. Polglase.

Additional strains of E. coli utilized were: (i) E. coli B-SG1, which was obtained from R. J. Harvey, Burroughs Wellcame Co., Research Triangle, North Carolina and which was originally isolated by J. Preiss of the Department of Biochemistry, University of California, Davis, California. E. coli B-SG1 lacks the enzyme adenosine diphosphate-glucose: α-4-glycosyl transferase and consequently is unable to synthesize glycogen. Nutritional requirements are the same as E. coli B wild type. (ii) E. coli ATCC 8739 (Crooke's strain) was obtained from the American type culture collection, Washington, D. C. The following strains of E. coli were obtained from Dr. Polglase's laboratory in 1969. The source from which Dr. Polglase obtained the strain is indicated in brackets. (iii) E. coli B, ATCC 11303 (American type culture collection); (iv) E. coli B/r, ATCC 12407 (American type culture collection); (v) E. coli K12 (Dr. Stock, Department of Microbiology, University of British Columbia); (vi) E. coli K12 met^-, a methionine requiring auxotroph of E. coli K12 (Dr. R. A. J. Warren, Department of Microbiology, University of British Columbia); (vii) E. coli var. communis D111 (Department of Bacteriology, Provincial Dairy School, St. Hyacinth,

P. Q.); (viii) E. coli UL 10.1 (Department of Bacteriology, University of Laval, Quebec, P. Q.); (ix) E. coli CRX-7(04863-P.D.) (Laboratory of Hygiene, Ottawa, Ontario); (x) E. coli W3110 (R. Sommerville, University of Michigan, Ann Arbor, Michigan); (xi) E. coli W1485 (R. Sommerville, University of Michigan, Ann Arbor, Michigan); (xii) E. coli B, streptomycin dependent (isolated in Dr. Polglase's laboratory from E. coli B, ATCC 11303); (xiii) E. coli UL 10.1, streptomycin dependent (isolated in Dr. Polglase's laboratory from E. coli UL 10.1) and (xiv) E. coli BRA (origin unknown).

2.1.2 Chemicals

The following chemicals were obtained from Calbiochem: N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), A grade; morpholinopropane sulfonic acid (MOPS), A grade; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), A grade; piperazine-N,N'-bis (2-ethane sulfonic acid), monosodium salt, monohydrate (PIPES), A grade; tris-(hydroxymethyl) aminomethane (Tris), A grade; triethanolamine hydrochloride, A grade; DL-glyceraldehyde-3-phosphate diethylacetal, barium salt, B grade; nicotinamide adenine dinucleotide, disodium salt, trihydrate, reduced (NADH), A grade; nicotinamide adenine dinucleotide, free acid, tetrahydrate (NAD^+), A grade; phenazine methosulfate; D- and L-lactate, lithium salts, A grade; and bovine serum albumin, crystalline, A grade.

Glycine hydrochloride, 2,4-dibromophenol (DBP) and 2,6-dichlorophenolindophenol monosodium salt (DCIP) were purchased from Eastman Organic Chemicals.

Fisher Scientific Co., supplied: glycine; monoethanolamine; o-phenanthroline; and silver nitrate, Fisher certified ACS grade.

Ultrapure grade tris-(hydroxymethyl) aminomethane, free base (Tris)

and ammonium sulfate were obtained from Schwarz-Mann Division of Becton, Dickinson and Company.

Glycylglycine, chloride free, was obtained from Nutritional Biochemicals and glutathione, reduced (GSH), was purchased from C. F. Boehringer and Soehne GmbH.

Heart infusion broth, dehydrated, was supplied by Fisher Scientific and Difco Laboratories, while the latter company also supplied special agar.

Nitrogen gas, L grade (99.97% N_2) was purchased from Canada Liquid Air Ltd.

The following reagents were prepared in the laboratory: 3-phosphoglyceraldehyde, free acid; monoethanolamine hydrochloride; monoethanolamine; triethanolamine; and iron-free sodium citrate. The procedures used are described in brief below.

The conversion of DL-glyceraldehyde-3-phosphate diethylacetal, barium salt, to DL-glyceraldehyde-3-phosphate was carried out by the technique of Meloche and Wood (1966), with a slight modification. Dowex AG-50 resin (H^+ form) was added to DL-glyceraldehyde-3-phosphate diethylacetal, barium salt until the latter dissolved and the pH of the solution was approximately 2.5. The solution was filtered to remove the AG-50 resin and the filtrate was incubated at 35°C for 48 hours to hydrolyze the acetal. The resulting solution was diluted to the required concentration.

Ethanolamine hydrochloride was prepared by the careful addition of a slight excess of concentrated hydrochloric acid to monoethanolamine. The resulting solution was allowed to cool and was then diluted with distilled water. The monoethanolamine hydrochloride was precipitated at 4°C by the addition of absolute ethanol to the aqueous solution. The crystals were

collected by filtration and purified by twice recrystallizing from aqueous ethanol. A melting point of 81-83°C was obtained.

Ethanolamine and triethanolamine solutions were prepared from solutions of ethanolamine hydrochloride and triethanolamine hydrochloride respectively, by passage through Dowex AG1-X4 (OH^- form) resin columns. The ethanolamine and triethanolamine solutions obtained were tested for the presence of chloride by the addition of silver nitrate.

Iron-free sodium citrate solutions were prepared by passage of the sodium citrate solutions through a Chelex 100 column (Na^+ form).

All other chemicals were reagent grade.

2.1.3 Equipment

The bacteria were harvested with either a Sorval centrifuge, model RC2-B, with GSA and SS-34 rotors, or a Beckman Spinco model J-21 centrifuge with J-10 and J-20 rotors.

The cell-free extract was prepared from disrupted *E. coli* by ultracentrifugation in a Beckman Spinco, model L2-65B, ultracentrifuge using a number 65 rotor.

The absorbance of bacterial cultures was measured with a Beckman model B, single beam spectrophotometer or a Coleman (Hitachi) model 124 double beam spectrophotometer. Quartz cuvettes of 10 mm path length were employed with the former instrument, while 10 mm path length quartz cuvettes with a 9 mm quartz path reduction bar, reducing the effective path length to 1 mm, were used with the latter instrument.

A Coleman model 124 spectophotometer coupled to a Sargent model SRG recorder was used for 3-phosphoglyceraldehyde dehydrogenase, succinate dehydrogenase and NADH oxidase assays, and for the determination of total

iron and protein. Quartz cuvettes of 10 mm path length were used in the enzyme assays and total iron determinations. Protein determinations were performed with optical glass cuvettes of 10 mm path length.

Dithionite-reduced minus hydrogen peroxide-oxidizing difference spectra were determined with a Cary model 15 spectrophotometer utilizing the zero to 0.1 absorbance scale and quartz cuvettes of 10 mm path length.

A Techtron IV atomic absorption spectrophotometer (Cary Instruments) with a hollow cathode lamp (serial no. AC761), and air-acetylene flame, was used for the determination of silver.

Bacterial cells were disrupted with either a Branson Sonifier, model W-185-C, or with an American Instruments Co., Inc., French pressure cell, 4-338 and French pressure cell press, 5-598A.

Oxygen consumption of E. coli cell suspension was monitored with: (i) an American Instrument Co., vibrating platinum electrode, C151-62081, coupled to a Varian strip chart recorder, model G-14A-2; (ii) a Gilson Medical Electronics Inc., Oxygraph model K-1C, with a Yellow Springs Instrument Co., Inc., model 4004 Clark-type oxygen electrode; or (iii) a Yellow Springs Instrument Co., oxygen monitor model 55, with a Yellow Springs Instrument Co., model 5533 Clark-type oxygen electrode, coupled to a Varian model G-14A-2 recorder. The latter instrument and electrode combination, without the coupled recorder, was used to monitor the oxygen level of growing cultures of E. coli (section 2.2.6.1).

Measurements of hydrogen ion concentration were made with a Fisher Scientific Accumet pH meter, model 310, in combination with one of the following electrodes. For routine buffer preparation and for monitoring the pH of the bacterial cultures, a Fisher Scientific standard combination

pH electrode, 13-639-90, was used. A Radiometer microcombination pH electrode, GK2321C, modified for use with the Fisher Accumet pH meter, or a Fisher Scientific microprobe combination pH electrode, 13-639-92, was used to adjust the pH during total iron determinations. The latter electrode was also employed to measure acid production concurrent with oxygen consumption in E. coli cell suspensions. The Accumet pH meter was coupled with a Varian model G-14A-2 strip chart recorder for these determinations.

The redox potential of growing cultures of E. coli was measured with a Photovolt pH meter, model 115, with a Beckman platinum redox electrode 39273. The $Ag^+/AgCl$ half-cell of the Fisher Scientific standard combination pH electrode, 13-639-90 was employed as the reference electrode.

The rate of aeration of the growing bacterial cultures was monitored with a Rogers Gilmont Instruments, size 4, fluid flowmeter. Aeration of the culture was provided via a Kimax 12C sintered glass sparger.

A Buchler Polystaltic pump R was employed to deliver 5 N HCl, or 5 N NaOH, to the culture vessel for the regulation of the pH of the bacterial culture. Circulation of the culture through the electrode vessel (Figure 2.1) was provided via a Gorman-Rupp Industries 60 cycle oscillating pump.

Media were filtered through 0.45 μ MF-Millipore membrane filters (47 mm diameter) and prefilters of glass fiber bound with a starch binder, in a pyrex Millipore filter holder.

Either a New Brunswick Scientific Co., Inc., reciprocal water bath shaker, model R76, or a Labline Inc., reciprocal water bath shaker 3581 was used for growth of the inoculating bacterial cultures.

2.1.4 Media

The following media were used during the research described in this

thesis.

Medium A corresponds to the medium of Davis and Mingioli (1950) and consists of 40.2 mM K_2HPO_4, 22.0 mM KH_2PO_4, 0.8 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$ and 1.7 mM sodium citrate.

Medium B differed from medium A in the deletion of sodium citrate.

Medium C differed from medium A in four respects: (i) sodium citrate was deleted; (ii) the $(NH_4)_2SO_4$ used in the medium was ultrapure grade; (iii) the concentration of $(NH_4)_2SO_4$ was 22.7 mM, and (iv) the medium was filtered through a 0.45 μ MF-Millipore membrane filter to reduce iron content, prior to the addition of $MgSO_4$. This medium will be referred to as iron-deficient medium.

Medium D corresponds to medium C to which ferric citrate was added to a final concentration of 6 μM. This medium will be referred to as iron-sufficient medium.

The media for growth of strains of E. coli K12 were supplemented with 500 μg of thiamine per litre, while E. coli K12 met$^-$ was grown with 2 mg methionine per litre. Streptomycin at a concentration of 1 g/litre was added to the media for growth of the streptomycin dependent strains of E. coli.

The pH of all media was adjusted to 6.9 to 7.1.

Heart infusion broth-agar for petri plates and slants consisted of 2.5% dehydrated heart infusion broth and 1.5% agar.

Minimal salts-glucose-agar slants were prepared from medium B with the addition of glucose (2 g/litre), and agar (15 g/litre).

2.2 Methods

2.2.1 Culturing procedures for bacteria

2.2.1.1 Maintenance of stock cultures

Stock cultures of E. coli were maintained at 4°C on minimal salts-glucose-agar slants, or heart infusion broth-agar slants in the case of E. coli K12 met⁻. Cultures were transferred on a monthly basis.

2.2.1.2 Growth of cultures for inoculation

Growth of an inoculum was commenced 48 hours prior to starting an experiment. Bacteria of the desired strain of E. coli were aseptically removed from a stock culture slant with a platinum or Nichrome wire inoculating loop, and were inoculated into a tube containing 10 ml of medium A or B (section 2.1.4) with a glucose concentration of 0.4% (w/v). The culture was incubated without shaking at 35-37°C for approximately 24 hours at which time 2.5 ml were inoculated into 25 ml of the desired medium (section 2.1.4), in a 100 ml Erlenmeyer flask, which contained the same carbon source, at the same concentration as was to be used in the final growth. The cultures were incubated in a reciprocating water bath shaker at 37°C for approximately 12 hours.

A sufficient number of 250 ml Erlenmeyer flasks containing 100 ml of sterile medium of the desired type and carbon source were inoculated with 2.5 ml of the immediately preceding culture, to provide a volume of inoculating culture equivalent to one-tenth to one-twentieth the volume of the final culture. The inoculating cultures were incubated at 37°C for 12 to 13 hours in a reciprocating water bath shaker.

2.2.1.3 Culture of the bacteria

Due to variations in the culturing conditions, they will be described in the appropriate sections.

2.2.2 Demonstration of silver release from the Aminco oxygen electrode

2.2.2.1 Growth and harvesting

Five hundred to 700 ml of freshly prepared, sterile medium A, containing glucose at a final concentration of 0.4% (w/v), in a two litre Erlenmeyer flask was inoculated with 50 to 70 ml of inoculating culture. The culture was grown at 37°C with an air flow rate of 6.7 l/min. Evaporation and cooling were minimized by bubbling the air through distilled water at 37°C prior to its entry into the culture. Growth of the culture was monitored *via* the absorbance at 420 nm and the cells were harvested in mid-logarithmic growth phase at an absorbance of 3.5 to 4.5.

Cells were harvested in stainless steel contrifuge bottles at 5,800 x *g* for 10 minutes at 4°C. The medium was decanted and the cells were suspended in 20 ml of 0.85% NaCl per centrifuge bottle. The resuspended cells were transferred to 50 ml cellulose nitrate centrifuge tubes and were centrifuged at 4,300 x *g* for 10 minutes at 4°C. The cells were resuspended in 10 ml of 0.85% NaCl per tube and were transferred to two weighed 50 ml cellulose nitrate centrifuge tubes. The cell suspensions were recentrifuged at 4,300 x *g* for 10 minutes at 4°C, the supernate was decanted, the tubes were drained, wiped dry and weighed. The wet weight of the bacteria was calculated by difference.

In order to reduce the endogenous respiration the cells were resuspended in 0.85% NaCl at a dilution of 1:80 (w/v) and incubated at 37°C with aeration for 15 to 60 minutes. At the end of this time the cell suspension was centrifuged at 4,300 x *g* for 10 minutes at 4°C, the supernatant fluid was decanted and the cell pellet was kept on crushed ice until required. When required, the cells were resuspended to the desired dilution in cold 300 mM glycylglycine-KOH buffer of pH 7.0.

2.2.2.2 Measurement of oxygen consumption

The oxygen consumption of the bacterial suspensions was measured with an Aminco oxygen electrode system consisting of a vibrating cathode of platinum wire sealed in a glass capillary, except for the tip. A bare silver wire was the stationary anode. The electrode was operated with a polarizing voltage of approximately 0.6 V. The current after amplification, was recorded continuously.

The assay system utilized for the investigation of the effect of cell concentration on respiration rate consisted of air-saturated, 270 mM glycylglycine-KOH buffer, pH 7.0, 1.8 mM, potassium phosphate buffer, pH 7.0, 0.5 mM KCl, 25 mM glucose and 200 µl of bacterial cell suspension, of increasing dilutions from 1:6 (w/v) to 1:26 (w/v), in a final volume of 4.00 ml.

The assay system used subsequently for the investigation of the non-linear relationship between cell concentration and respiration rate was composed of air-saturated, 281 mM glycylglycine-KOH buffer, pH 7.0, 1.8 mM potassium phosphate buffer, pH 7.0, 0.5 mM KCl, 26 mM glucose and 40-200 µl of bacterial cell suspension at a dilution of 1:10 (w/v). The final volume of the assay system was 3.8 ml to 4.00 ml.

All determinations were carried out at room temperature (22°C).

The Aminco oxygen electrode was found to be inadequately grounded when used separate from the Aminco-Chance dual wavelength spectrophotometer. Also, stirring in addition to the vibration of the platinum electrode was found to be essential in order to maintain a homogeneous suspension and a stable diffusion current when measuring the respiration of intact cells. Adequate stirring was accomplished by means of a Teflon-coated magnetic

flea and magnetic stirrer. The stirring rate was regulated *via* a Powerstat variable autotransformer. High stirring rates were avoided in order to minimize the back diffusion of oxygen into the system.

The oxygen level was expressed as a percentage of the oxygen content of the air-saturated buffer. Oxygen consumption rate was expressed as the decrease in percent oxygen saturation per minute per milligram of bacterial wet weight.

2.2.2.3 Determination of cell viability

The determination of the viability of *E. coli* was performed according to the "pour-plate" method as described by Harrigan and McCance (1966).

A 1.0 ml aliquot was removed at the desired time intervals after the addition of cell suspension to the assay system. The samples were serially diluted to a final cell concentration of 30 to 300 bacterial cells per millilitre in one-quarter strength Ringer's solution. One millilitre aliquots of the two highest dilutions were inoculated onto heart infusion broth-agar medium. Plates were prepared in triplicate. The sample was spread and then overlayed with molten 1% agar ($\sim$45°C). The petri plates were incubated at 35-37°C for 24 hours and the colonies marked and counted. The petri plates were re-incubated, under the same conditions for an additional 24 hours and were examined for the appearance of additional colonies. The number of viable cells was calculated for each sample, and these were analyzed for significant differences *via* the "unpaired t-test".

2.2.3 Measurement of silver release from the Aminco oxygen electrode

2.2.3.1 Sample preparation

Samples for silver analysis were collected under operational conditions identical to those used in measuring oxygen tension. Both the

anode and the cathode were partially immersed in 4.0 ml of buffer solution in a 5 ml vial which had previously been cleaned by soaking in 7.5 N HNO_3 and thoroughly rinsed with glass distilled water. The buffer solution consisted of air-equilibrated buffer, pH 7.0, and potassium chloride at a final concentration of 0.5 mM.

In the preparation of buffers, glycylglycine and HEPES were adjusted to the required pH with KOH. Phosphate buffer was prepared from potassium phosphates, and Tris was adjusted to the required pH with HCl.

Samples were obtained by removing the oxygen electrode from the buffer solution after a predetermined period of time and subsequently adding 10 µl of 7.5 N nitric acid (final concentration of 18.7 milliequivalents per litre) to reduce the tendency of silver ions to adhere to the glass vials. For the same reason the samples were assayed within 4 to 5 hours of their collection.

2.2.3.2 Atomic absorption spectrophotometry

The silver content of the samples was determined by atomic absorption spectroscopy at a wavelength of 3280.7 Å using a Techtron IV atomic absorption spectrophotometer. Standard solutions of silver nitrate were prepared in the appropriate buffers with nitric acid present at a final concentration of 18.7 milliequivalents per litre.

2.2.4 The influence of pH, buffer ion and buffer concentration on the respiration of E. coli

2.2.4.1 Growth and harvesting

E. coli NRC 482 was grown and harvested under conditions identical to those described in section 2.2.2.1. However, the procedure to reduce the endogenous respiration differed slightly from that described previously (section 2.2.2.1). The cell pellet was suspended 1:80 (w/v) in 0.85% NaCl

and the cell suspension was incubated at 37°C without aeration for 60 minutes. At the end of the incubation, 20 ml aliquots of cell suspension were transferred to weighed 50 ml cellulose nitrate centrifuge tubes and were centrifuged at 7,700 x g for 5 minutes at 4°C. The supernates were decanted, the tubes were drained, were wiped dry and then were weighed. The wet weight of the E. coli cell pellet was determined by difference.

The cell pellet was retained in crushed ice until needed and then was resuspended at a dilution of 1:10 (w/v) in the appropriate buffer.

2.2.4.2 Measurement of oxygen consumption

A Yellow Springs Instruments oxygen monitor, equipped with a Clark-type oxygen electrode and operating at a polarizing voltage of approximately 0.8 V, was employed to determine the oxygen consumption.

The assay system consisted of 5.0 ml of air-saturated buffer, 0.5 mM KCl, 9.6 mM glucose, and 50 µl of cell suspension in a total volume of 5.2 ml. The measurement of oxygen consumption was carried out at 37°C.

The isotonic buffers were prepared by mixing the isotonic solutions of the salt-form of a buffer with the isotonic solution of the corresponding free acid or free base, in the required proportions to obtain the desired pH. With the exception of the isotonic solutions of KH_2PO_4 and K_2HPO_4 whose concentrations were obtained from the Merck Index, the concentrations of the salt form, and the free-acid and free-base form of the buffer corresponding to isotonicity were calculated according to the Van't Hoff equation:

$$\pi = CRT$$

where π-is the osmotic pressure, C - the molar concentration, R - the gas constant and T - the absolute temperature.

The following assumptions or approximations were made in performing

these calculations: (i) the salt-form of the buffer was considered to be fully dissociated; (ii) the addition of a solution of the free-acid, or free-base form of the buffer to the salt-form was assumed to have an insignificant affect on the degree of dissociation of the salt-form of the buffer; and (iii) the influence of the hydrogen ion concentration on tonicity was considered negligible.

The acid-base pairs used in the investigation of the influence of pH and buffer ion on the respiration of E. coli were: citric acid (0.29 N)-sodium citrate (0.1 N); Tris hydrochloride (0.13 N)-Tris (0.26 N); sodium phosphate (monobasic) (0.18 N)-sodium phosphate (dibasic) (0.12 N); MOPS (0.26 N)-MOPS·Na^+ (0.13 N); HEPES (0.26 N)-HEPES·Na^+ (0.13 N); glycylglycine (0.26 N)-sodium glycylglycinate (0.13 N); triethanolamine hydrochloride (0.13 N)-triethanolamine (0.26 N); ethanolamine hydrochloride (0.13 N)-ethanolamine (0.26 N); and glycine (0.26 N)-sodium glycinate (0.13 N). The concentrations in brackets are those of the isotonic stock solutions used in the preparation of the isotonic buffers of desired pH. The buffers were prepared to give the specified pH at 37°C.

2.2.4.3 Protein Determination

Aliquots of 0.5 ml of cell suspension in buffer (1:10 w/v) were added to 4.5 ml of 1.1 N NaOH and were mixed thoroughly. Protein determinations were performed on 0.5 ml aliquots of this solution by the method of Lowry et al., (1951). Crystalline bovine serum was used as protein standard. The standard protein solutions were prepared in the appropriate buffer solutions as the buffer ion and concentration had a marked influence on the color development (Appendix B) (Turner and Manchester, 1970).

2.2.5 The influence of silver on the respiration of E. coli

2.2.5.1 Growth and harvesting

E. coli NRC 482 was grown and harvested according to the conditions described in section 2.2.2.1. The carbon sources used were 0.4% glucose, 0.4% glycerol, 0.8% DL-lactate, 0.4% succinate and 0.4% fumarate.

Two procedures for the reduction of endogenous respiration were used, dependent upon the parameters to be measured subsequently. When only oxygen consumption was to be measured the endogenous respiration was reduced by resuspending the cells 1:80 (w/v) in 0.85% NaCl and incubating at 37°C for 5 minutes with gentle aeration. Twenty millilitre aliquots were removed to weighed 50 ml cellulose nitrate centrifuge tubes. The cell suspension was centifuged at 4,300 x g for 10 minutes at 4°C, the supernates were decanted and the tubes were then drained, wiped dry and weighed. The weight of the cell pellet was calculated by difference.

The cell pellet was kept in crushed ice until required, at which time the cells were resuspended 1:10 (w/v) in 300 mM glycylglycine-KOH buffer, pH 7.0.

However, if acid production and oxygen consumption were to be measured simultaneously, the endogenous respiration was reduced by resuspending the cell pellet 1:80 (w/v) in medium B. The cell suspension was incubated at 37°C for 90 minutes with gentle aeration. On completion of the incubation, 40 ml aliquots of cell suspension were transferred to weighed 40 ml screw-cap, polycarbonate centrifuge tubes (Oak Ridge type, wide mouth) and were centrifuged immediately at 7,700 x g for 10 minutes at 4°C. The supernatant fluid was decanted, the tubes were drained, were wiped dry, and were weighed. The weight of the cell pellet was calculated by difference.

The cell pellets were maintained at room temperature, with the

centrifuge tube tightly capped to prevent dehydration of the pellet, until required. The duration at room temperature, prior to the assay of oxygen utilization and acid production was kept as short as possible (ca. 1 hour). The cell pellet was resuspended in 3 mM glycylglycine-KOH buffer, pH 7.5 to a final cell concentration of 0.1 g wet weight of E. coli per ml of cell suspension.

2.2.5.2 Measurement of oxygen consumption

The conditions used for the measurement of respiration only were the same as those specified in section 2.2.4.2. The buffer component of the assay system was 300 mM glycylglycine-KOH, pH 7.0. Substrates used in addition to glucose, were glycerol, D-lactate and L-lactate as lithium salts, potassium formate, potassium acetate, disodium succinate, and potassium fumarate, all at a final concentration of 9.6 mM. Silver nitrate was added to the "test" samples to yield a final concentration of 86 μM (450 nmoles of $AgNO_3$ were added to the assay system). Appropriate controls were performed.

2.2.5.3 Concurrent measurement of oxygen consumption and protein production

The oxygen consumption was measured with a Yellow Springs Instruments oxygen monitor, with Clark-type oxygen electrode. Hydrogen ion concentration was monitored with a Fisher Accumet pH meter, employing the expanded scale mode of operation, coupled with a Fisher microprobe combination pH electrode.

The concurrent measurement of oxygen utilization and acid production by E. coli NRC 482 was carried out in separate, identical assay systems. Electrical interaction of the two electrode systems necessitated this arrangement.

The components of the assay system were 2.9 mM glycylglycine-KOH

buffer, pH 7.5, 9.6 mM glucose, and 50 to 200 µl of cell suspension. The total volume was 5.2 ml. The "test" samples also received the addition of 125 nmoles of $AgNO_3$ (final concentration - 24 µM) subsequent to establishing the substrate-dependent rate of oxygen utilization and the rate of respiration-associated acid production. All determinations were performed at 37°C.

2.2.5.4 Assay for glyceraldehyde-3-phosphate dehydrogenase

E. coli NRC 482, grown and harvested as described in section 2.2.2.1, were resuspended 1:5 (w/v) in 30 mM Tris-HCl buffer, pH 8.0. The cell suspension was disrupted with a French press at a pressure differential of 20,000 p.s.i. The disrupted cells were centrifuged at 95,000 x g for two hours. The cell-free supernate was carefully removed with a Pasteur pipette and was kept in an ice bath.

The assay system was comprised of, in order of addition: 12.1 mM Tris-HCl buffer, pH 8.0, 1.7 mM glyceraldehyde-3-phosphate, 0.3 mM NAD^+, 4.9 mM cysteine, 20.6 mM sodium arsenate, 10 µl of enzyme source. The final volume was 2.56 ml. The assay was carried out at room temperature (22°C). The progress of the reaction was monitored via the increase in absorbance of the assay system at 340 nm.

Due to the insolubility of silver arsenate ($K_{sp} = 1 \times 10^{-22}$ moles^4/litre^4), silver could not be added directly to the assay system. The procedure adopted was to preincubate the cell-free supernate with an equal volume of 0.5 mM or 5 mM $AgNO_3$ solution. Ten microlitre aliquots were removed at recorded time intervals during the incubation and the glyceraldehyde-3-phosphate dehydrogenase activity was measured. The silver concentrations during the preincubation at room temperature were 0.25 mM and 2.5 mM. The corresponding concentrations in the final assay system were 1 µM and

10 μM, respectively.

2.2.6 The influence of iron limitation on respiration of *E. coli*

2.2.6.1 Growth and harvesting

The bacteria were grown on medium C or D, with either 0.6% succinate or 0.4% glucose as the carbon source. Additions of ferric citrate during growth, were of the required volume of a stock solution to yield a concentration of 6 μM in the culture medium.

The culture was grown in the apparatus shown in Figure 2.1. The culture vessel (12) was a brown glass jar with a capacity of 2500 ml. The culture was continuously circulated through a second sealed vessel (8) (50 ml capacity) at a rate of 200-250 ml/min by means of a 60 cycle oscillating pump (9). This vessel (8) contained a combination glass pH electrode (4), a platinum redox electrode (5), a Clark-type oxygen electrode (6), a small, Teflon-coated magnetic stirring bar (7) and was supported on a magnetic stirrer (10). The stirring rate was regulated *via* a Powerstat variable autotransformer.

Growth was initiated by inoculation of 1500-1800 ml of medium with 100 ml of inoculating culture (section 2.2.1.2). The culture was grown at 37°C with aeration through a sintered glass sparger (15) at an air flow rate of 4.4 l/min/l of culture. Growth was monitored by following the absorbance of the culture at 420 nm. The pH, oxygen tension and oxidation-reduction potential of the culture were determined at intervals of 15 or 30 min, depending on the rapidity of change in these parameters. The pH of the culture was maintained at pH 7.00 ± 0.15 by the addition of 5 N HCl, or 5 N NaOH *via* a peristaltic pump.

The cells were harvested through the sampling port (11) at specific

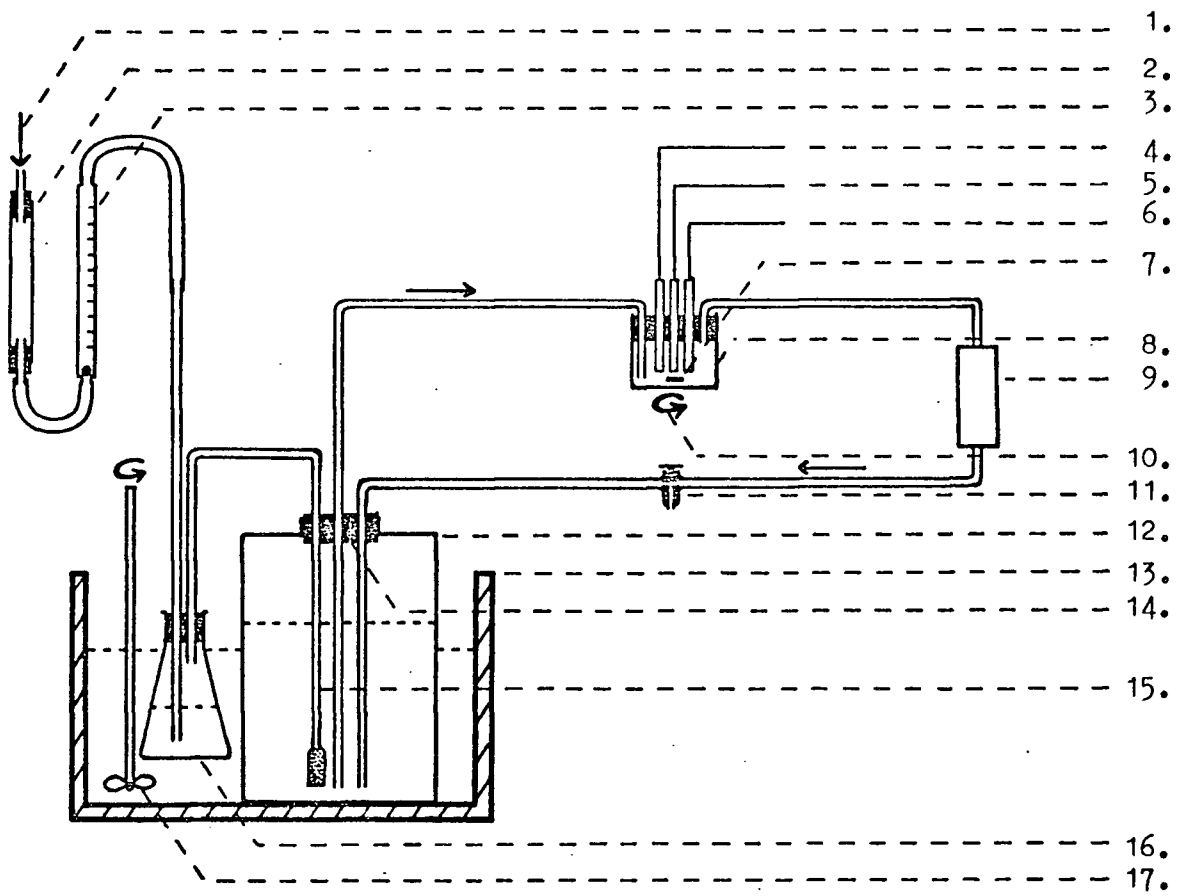


Fig. 2.1 Culture apparatus.

1. Air Input
2. Filter
3. Flow Meter
4. Combination pH Electrode
5. Platinum Redox Potential Electrode
6. Clark Oxygen Electrode
7. Magnetic Flea
8. Electrode Vessel
9. Oscillating Pump
10. Magnetic Stirrer
11. 3-Way Stopcock, Sampling Port
12. Culture Vessel (2.5 Litre)
13. Water Bath
14. Air Vent, and Addition Port
15. Sparger
16. Evaporation-Control Flask
17. Stirrer

times throughout growth and were sedimented immediately by centrifugation of the cell suspension at 12,000 x g for 10 min at 2°C. The cells were washed twice by sedimentation from a wash medium at 12,000 x g for 10 min at 2°C.

The wash medium used depended upon the assays to be performed subsequently. If only the cytochrome contents were to be measured, the cells were washed with medium C. However, if total iron was to be determined in addition to the cytochrome levels, the cells were washed with glass distilled water to minimize the retention of adsorbed iron by the cells. If, in addition, enzyme activities were to be assayed, then the cells were washed with 0.1 M Tris-HCl buffer, pH 7.5, prepared from ultrapure Tris.

The cell pellet obtained after washing was kept at 0°C until required.

2.2.6.2 Estimation of the efficiency of succinate conversion to cell mass

An approximate measurement of the efficiency with which the cell culture could convert disodium succinate to cell mass was obtained by calculating the increase in absorbancy of the culture at 420 nm over a specified time interval relative to the increase in pH, produced by the utilization of succinate, for the corresponding time period. Since the change in pH was small (0.04 to 0.15 pH units) and over the same pH range, pH 7.00 ± 0.15, it was proportional to the amount of succinate oxidized. The absorbance at 420 nm was strictly proportional to wet cell mass (Appendix A). "Efficiency" was defined as the change in the absorbance of the culture at 420 nm during a time interval (generally 30 min) divided by the change in the pH of the culture during the same time interval.

2.2.6.3 Measurement of the effect of dibromophenol and silver nitrate on oxygen consumption

Respiration of intact E. coli NRC 482, in the presence or absence of

dibromophenol (DBP) or silver nitrate, was determined at 22°C using an oxygraph equipped with a Clark-type oxygen electrode.

The assay procedure is as follows. Forty microlitres of cell suspension in 0.1 M Tris-HCl buffer, pH 7.5, (1:10 w/v) were added to 3.9 ml of air-saturated Tris-HCl buffer, pH 7.5, and the endogenous respiration was recorded for 3 minutes. At the end of this time 20 µl of M glucose was added (final concentration, 5 mM). When the glucose-dependent respiration rate was established, DBP or $AgNO_3$ were added to the desired final concentration.

2.2.6.4 Respiratory control ratio

Hempfling (1970b) has defined the ratio of the rate of oxygen utilization in the presence of DBP to the rate of oxygen utilization in the absence of DBP as the respiratory control ratio (RCR). This definition has been adopted in this thesis and its application extended to experiments in which DBP has been replaced by $AgNO_3$.

2.2.6.5 Determination of total iron and nonheme iron

The washed cell pellet was suspended 1:10 (w/v) in glass distilled water and a 2.0 or 3.0 ml sample was transferred to a labelled micro-Kjeldahl flask, calibrated to contain 10.0 ml. The sample was frozen in a dry ice-ethanol freezing mix and subsequently lyophilized. An appropriate blank of glass distilled water was carried through the entire procedure.

The total iron content of the lyophilized cell suspension was determined according to the method of King et al., (1964). The procedure was as follows. One millilitre of concentrated H_2SO_4 was added to each micro-Kjeldahl flask. The flasks were heated gently on Kjeldahl digestion heaters and rotated periodically until all cell material had gone into solution.

The temperature then was increased and heating was continued until the samples became viscous. The flasks were allowed to cool and 0.5 ml of 30% hydrogen peroxide was added dropwise to each sample. The procedure of heating, cooling and adding 30% hydrogen peroxide was repeated until a clear colorless solution was obtained.

After cooling the following reagents were added to the digested sample in the order indicated: 1.0 ml of 0.25% o-phenanthroline (aqueous solution), 0.5 ml of 1.0% hydroquinone (freshly prepared), 2.5 ml of 25% sodium citrate. The mixture was adjusted to pH 3.8 to 4.3 (pH meter) by the addition of 28% NH_4OH and made up to a volume of 10.0 ml with glass distilled water. The solutions were incubated at room temperature for 60 min, the absorbance at 510 nm was measured and the content of iron was calculated using an extinction coefficient of 11.1 $mM^{-1}cm^{-1}$.

Nonheme iron was calculated as the difference between the total iron and the heme iron. The value used for the level of heme iron was the cytochrome b_1 content unless the level of cytochrome a_2 in the cells was sufficiently high to be quantitated in which case the sum of the levels of cytochrome b_1 and cytochrome a_2 was used as the content of heme iron.

2.2.6.6 Preparation of cell extracts

The washed cell pellet was suspended 1:10 (w/v) in medium C, glass distilled water or 0.1 M Tris-HCl buffer, pH 7.5, depending upon the determinations to be performed (section 2.2.6.1). The cell suspension was disrupted at 0°C by two pulses of sonication, each of 30 sec duration, separated by a 30 sec period, unless the cell extract was to be used for assaying enzyme activity in which case a single period of sonication of 30 sec duration was used.

2.2.6.7 Determination of cytochromes a_2 and b_1

The levels of cytochrome $\underline{a}_2$ and $\underline{b}_1$ in cell extracts were determined from dithionite-reduced minus hydrogen peroxide-oxidized difference spectra. Three successive scans were carried out on each sample to ascertain that complete reduction and oxidation of the contents of the respective cuvettes had been obtained.

The content of cytochrome $\underline{a}_2$ was calculated from the height (in absorbance units) of the peak at 630 nm as measured along a vertical line drawn from the spectral recording at 630 nm to an extrapolation of the baseline at this point as shown in Figure 2.2. The level of cytochrome $\underline{b}_1$ was calculated in an analogous manner from the height (in absorbance units) of the peak at 560 nm. The peak height was measured along the vertical line drawn from the spectral trace at 560 nm to a point of intersection with a second line drawn through the spectral recording at 540 nm, and tangent to the spectral trace in the region of 580 nm as shown in Figure 2.2. The extinction coefficient used in the calculation of cytochrome $\underline{a}_2$ levels was 8.51 $mM^{-1}cm^{-1}$ (Jones and Redfearn, 1966) while the extinction coefficient used for cytochrome $\underline{b}_1$ was 16.0 $mM^{-1}cm^{-1}$ (Deeb and Hager, 1964).

2.2.6.8 Assay for succinate dehydrogenase

The succinate dehydrogenase present in the cell extract was "activated" by incubating 0.5 ml of cell extract and 0.5 ml of 0.1 M disodium succinate, pH 7.5, at 37°C for 10 min prior to assaying.

To a cuvette containing 1.0 ml of reaction mix, consisting of 2 mM KCN, 68 µM DCIP, 27.3 mM disodium succinate and 41 mM potassium phosphate buffer, pH 7.5, 5 µl of "activated" cell extract was added followed by the addition of 20 µl of 9 mM PMS. The succinate dehydrogenase activity was calculated from the rate of decrease in the absorbance at 600 nm. The assay

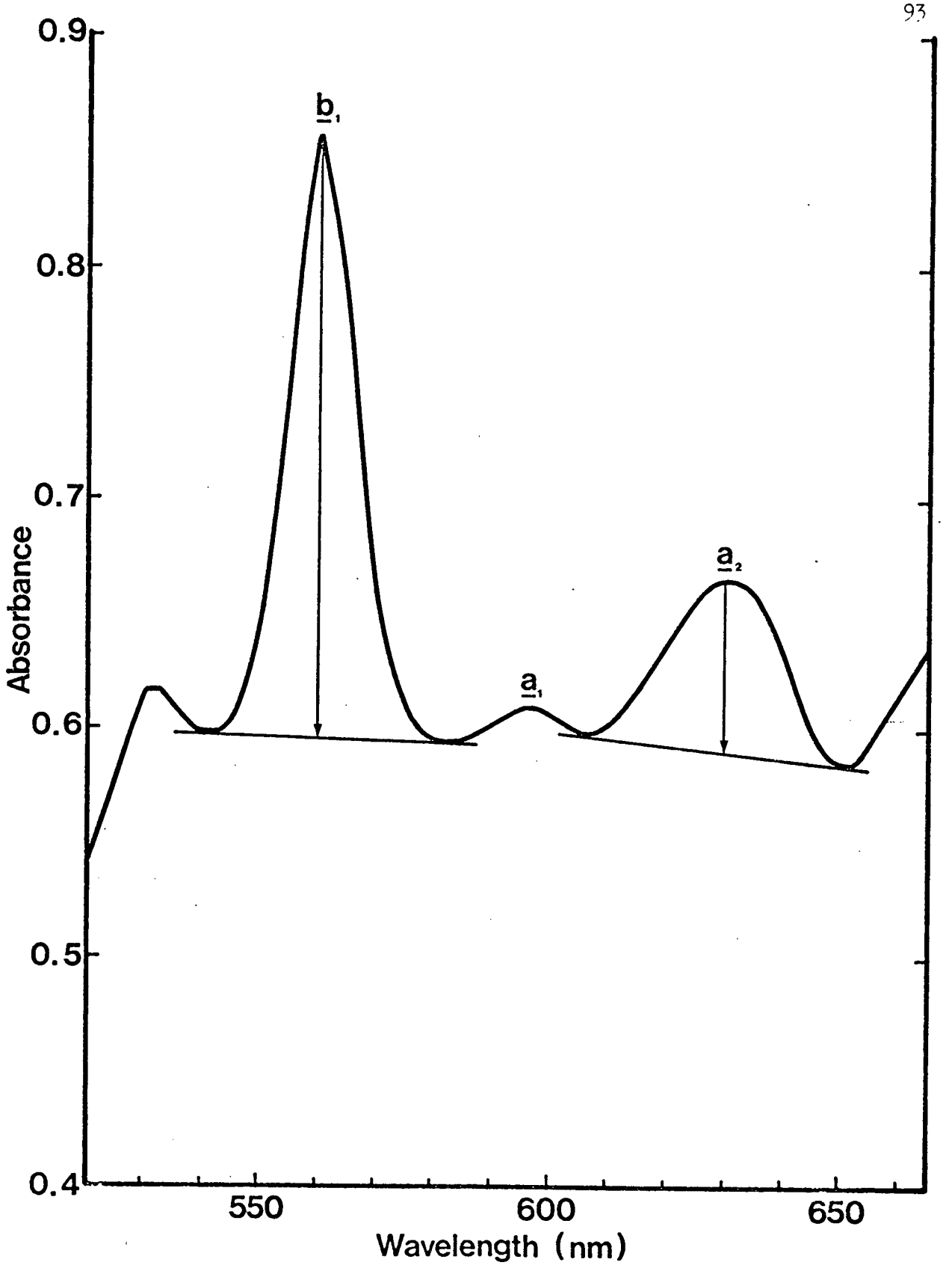


Fig. 2.2 Dithionite reduced-minus-oxidized difference spectrum. (method: sec. 2.2.6.7).

was performed at 22°C. Calculation were performed using an extinction coefficient for DCIP of 21.8 $mM^{-1}cm^{-1}$.

2.2.6.9 Assay for succinate oxidase

Succinate oxidase activity was determined from the rate of oxygen consumption on the addition of 200 µl of "activated" cell extract (sec.2.2.6.8) to 5.0 ml of 0.1 ml potassium phosphate buffer, pH 7.5, containing 19.6 mM disodium succinate. Oxygen consumption was measured at 37°C with a Clark-type oxygen electrode. The micromoles of oxygen consumed were calculated from an oxygen solubility of 0.199 µmoles/ml for air-equilibrated, triethanolamine buffered mitochondrial medium, pH 7.2, at 37°C (Lessler, M.A., 1972).

2.2.6.10 Assay for NADH oxidase

NADH oxidase was measured spectrophotometrically at 22°C by measuring the decrease in absorbance at 340 nm following the addition of 50 µl of cell extract to 2.0 ml of 0.1 ml potassium phosphate buffer, pH 7.5, containing 0.3 mM NADH. An extinction coefficient for NADH of 6.22 $mM^{-1}cm^{-1}$ was used for the calculations.

2.2.6.11 Glucose determination

Ten millilitre aliquots of cell suspension were removed as required and centrifuged at 0°C for 10 min at the maximum speed of a clinical centrifuge (approximately 2,000 x g). The supernate was removed carefully and used directly, or after dilution with 50 mM phosphate buffer, pH 7.0, for determination of the glucose content via the micro-Glucostat procedure.

3. PART I: SILVER IONS AND THE RESPIRATION AND ENERGY-COUPLING OF *E. COLI*

3.1 Results

3.1.1 Release of silver from the Aminco oxygen electrode

During preliminary experiments on factors influencing the respiration rate of *E. coli*, an investigation of the respiration rates of *E. coli* as a function of the quantity of cells in the assay system was undertaken (Figure 3.1). (The data has been replotted from continuous traces for convenience of presentation.) Curves A to E correspond to 33 mg, 18 mg, 12 mg, 8 mg, and 2 mg cells wet weight, respectively. Nearly linear oxygen utilization traces, and a linear relationship between the quantity of cells and the respiration rate had been expected. However, the results demonstrated: (i) a greater decrease than expected in the respiration rate with increasing dilution of the cell suspension; (ii) with decreasing cell concentration the shape of the oxygen utilization trace changed from linear (Curve A) to sigmoid (Curve D); and (iii) at a sufficiently low cell concentration there was a complete cessation of respiration prior to depletion of oxygen from the system (Curve E).

A number of alternative explanations of the observed results were apparent: (i) an increasing loss of cell viability, during the course of the assay, with increasing dilution of the cell suspension; (ii) the existence of respiratory control; or (iii) an inhibition of respiration. Of primary concern was the viability of the cells which had ceased to respire prior to the depletion of oxygen from the assay system. Viable cell counts as determined by the "pour-plate" technique (sec.2.2.2.3) (Table 3.1) indicated that there was no significant difference between the number of viable cells present in the assay system prior to the cessation of respiration, and the number

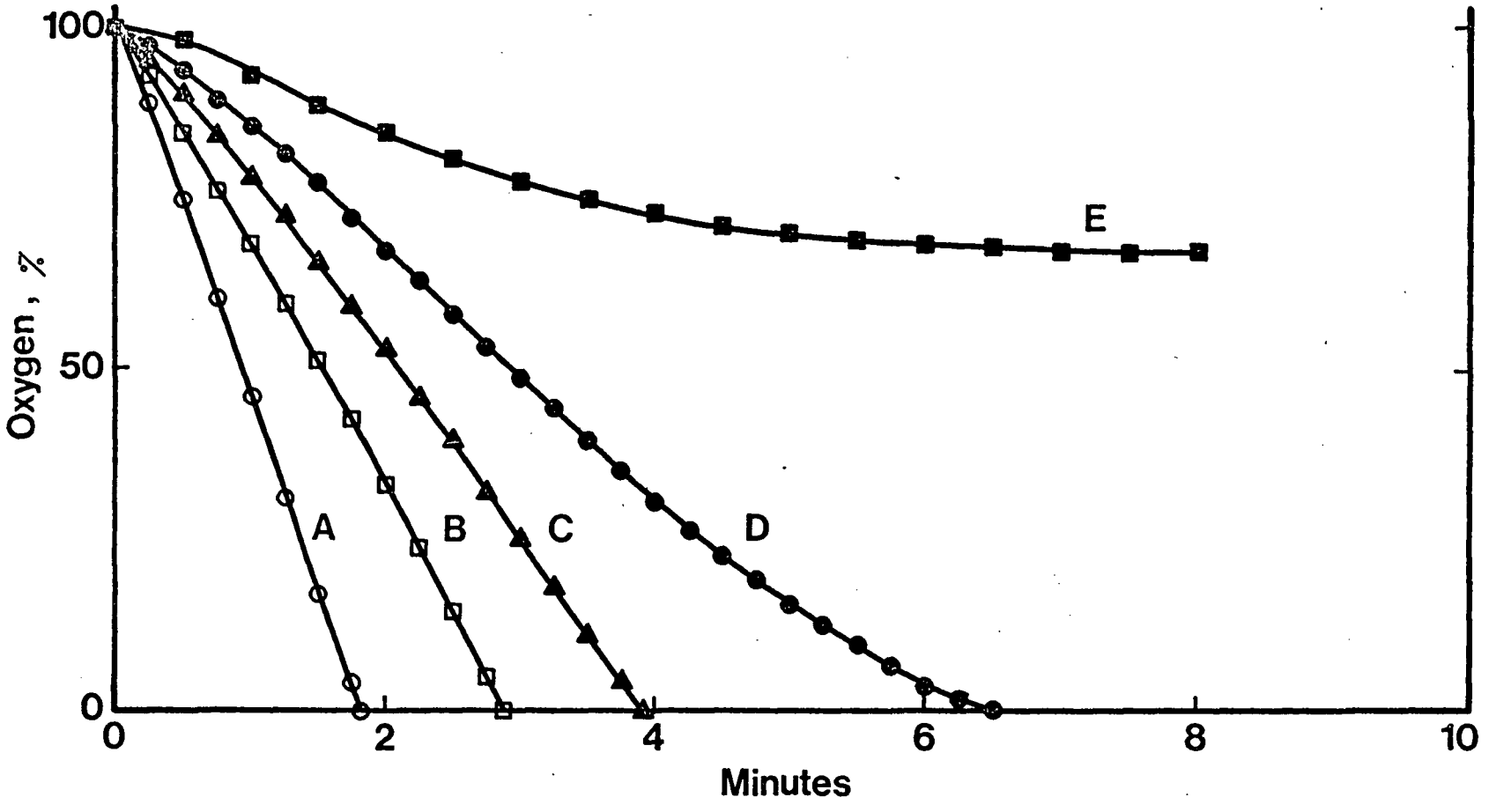


Fig. 3.1 Effect of cell concentration on oxygen consumption by E. coli cell suspensions as measured with the Aminco oxygen electrode. A: 33 mg; B: 18 mg; C: 12 mg; D: 8 mg; E: 2 mg; (method: sec.2.2.2.2).

Table 3.1

The viability of E. coli before and after the cessation of oxygen consumption.

Time[1]	Viable Cells (per ml)	"t" test value
Before	$(6.69 \pm 0.44)\times10^8$	
After	$(6.31 \pm 0.27)\times10^8$	1.19 ($\alpha = 0.05$, d.f. = 4)

[1] relative to the cessation of oxygen utilization (with reference to curve E of Figure 3.1, "before" corresponds to a time of about 1 minute while "after" would correspond to a time of approximately 8 minutes.)

present when respiration had ceased. These results suggested that the cessation of respiration prior to the depletion of oxygen from the assay system was due to either respiratory control or inhibition of respiration.

Prior to further examination of the cessation of respiration at low oxygen concentrations, the ubiquity of the phenomenon in strains of E. coli was examined. The following strains of E. coli: E. coli ATCC 8739, E. coli B (ATCC 11303), E. coli B str-D, E. coli B/r, E. coli BRA, E. coli K12, E. coli MK12, E. coli W1485, E. coli W3110, E. coli CRX, E. coli D111, E. coli UL 10.1, and E. coli UL 10.1 str-D ceased to respire prior to the depletion of oxygen from the system when assayed under conditions identical to those which gave rise to the cessation of respiration with E. coli NRC 482.

Due to the generally accepted usage of unshielded, platinum wire cathode-silver wire anode oxygen electrodes in the investigation of oxygen utilization by biological systems prior to the development of the Clark-type oxygen electrodes, metal ion inhibition of respiration was not suspected at first. However, the discovery that: (i) the addition of 1 umole of reduced glutathione to the assay system prior to the addition of the cell suspension prevented the cessation in oxygen consumption (Figure 3.2) and maintained a rate of oxygen consumption equal to that of the control (Figure 3.3); and (ii) that the addition of 2 umoles of reduced glutathione subsequent to the cessation of respiration resulted in a stimulation of respiration (Figure 3.4), prompted us to consider this possibility. To investigate the possibility that the Aminco oxygen electrode system was responsible for the observed cessation of the respiration of the E. coli cell suspensions, the measurement of oxygen consumption by E. coli cell suspensions was performed with the Clark-type oxygen electrode of a Gilson oxygraph under conditions iden-

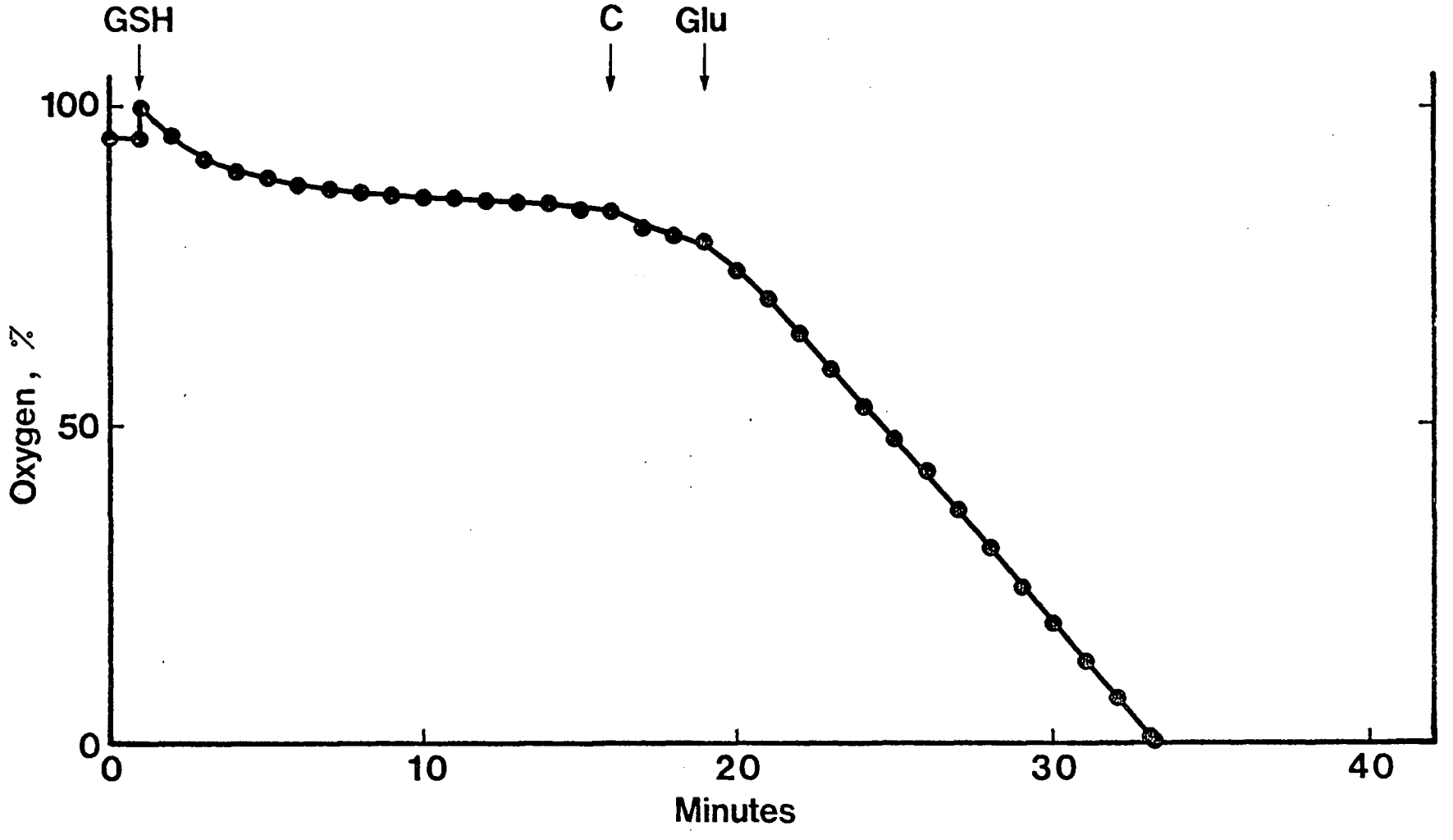


Fig. 3.2 Prevention of cessation of respiration of E. coli by the addition of reduced glutathione. O_2 uptake measured with Aminco electrode; GSH: 1 µmole GSH; C: 4 mg cells; Glu: 100 µmoles glucose; (method: sec. 2.2.2.2).

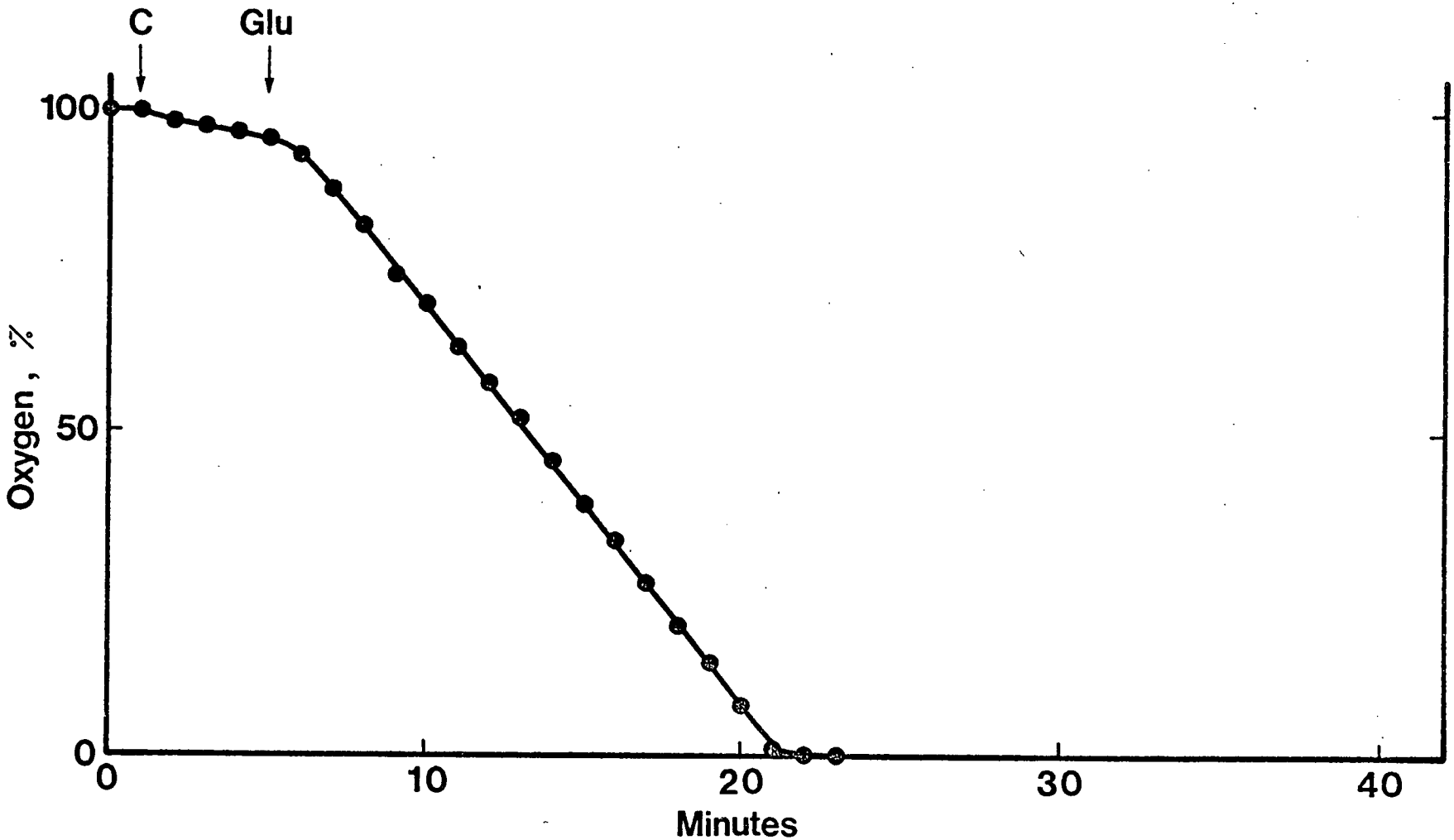


Fig. 3.3 Respiration of E. coli as measured with Clark-type oxygen electrode. C: 4 mg cells; Glu: 100 µmoles glucose; (method: sec. 2.2.2.2).

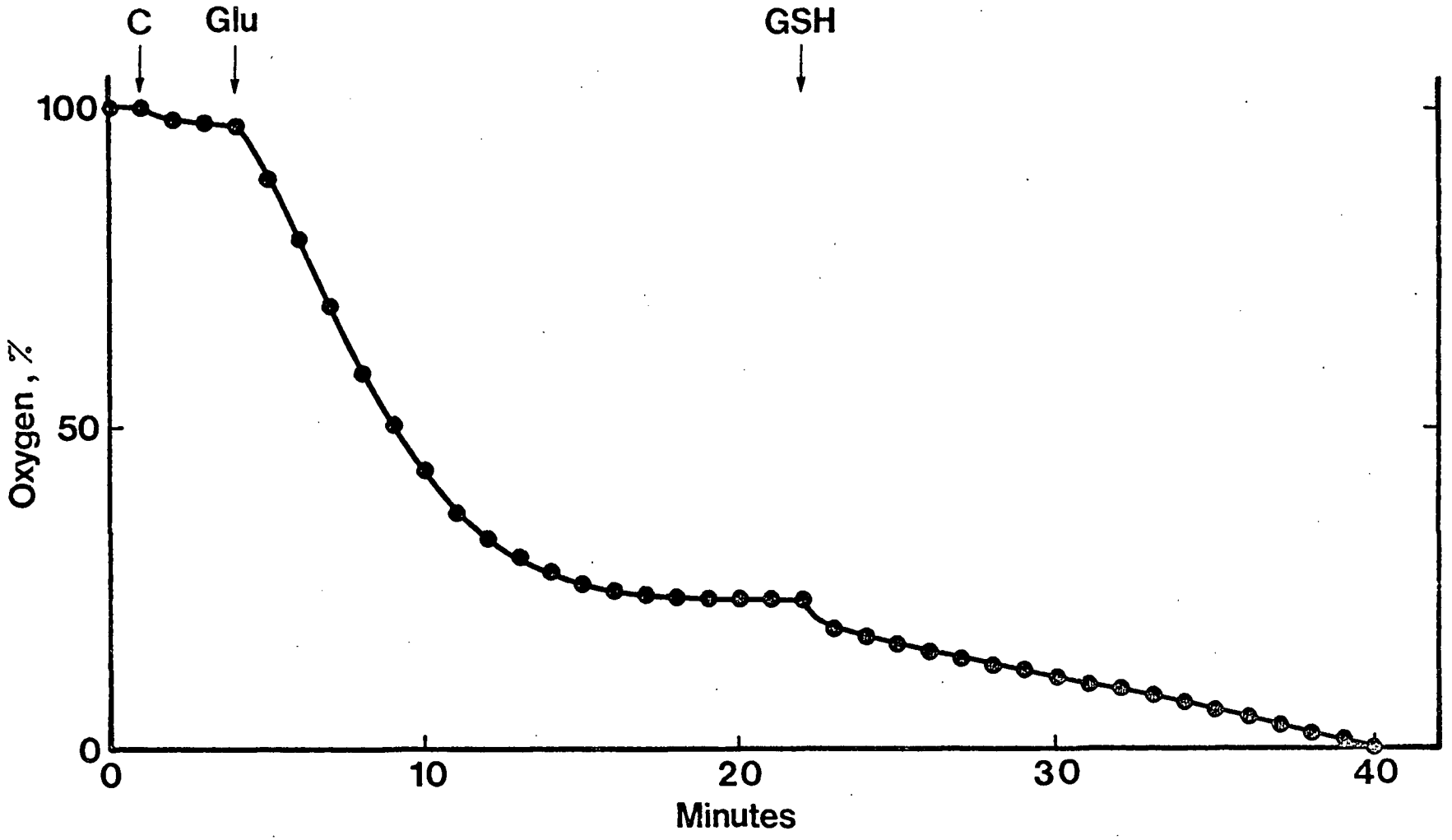


Fig. 3.4 Stimulation of respiration via the addition of reduced glutathione. O_2 uptake measured with Aminco electrode; C: 4 mg cells; Glu: 100 µmoles glucose; GSH: 2 µmoles GSH; (method: sec. 2.2.2.2).

tical to those under which a cessation of respiration was observed when oxygen consumption was measured with the Aminco oxygen electrode. The results obtained demonstrated a linear uptake of oxygen subsequent to the addition of glucose, and no cessation in oxygen consumption (Figure 3.3). This indicated that the Aminco oxygen electrode was responsible for the observed cessation of oxygen consumption but gave no indication of the mechanism involved.

During investigation of the mechanism by which the Aminco electrode system produced an inhibition of the respiration of E. coli it was observed that if the cessation of respiration was first obtained with the Aminco oxygen electrode, the Aminco electrode system removed, and the oxygen level subsequently monitored with a Clark-type oxygen electrode (Figure 3.5), the oxygen level did not alter. This indicated that the continued presence of the Aminco oxygen electrode was not required for maintenance of the cessation of respiration. These results suggested that an inhibitor of oxygen consumption was produced by, or released from, the Aminco oxygen electrode. This possibility was tested by performing a blank run with the Aminco oxygen electrode of approximately the same duration required to obtain the cessation of respiration but with no cells present. The Aminco oxygen electrode was then replaced with the Clark-type oxygen electrode and cells were added. The results (Figure 3.6) demonstrated that under these conditions cessation of respiration did occur in the same pattern as was characteristic of the Aminco oxygen electrode. This result demonstrated conclusively that the Aminco oxygen electrode system was responsible for the production or release of an inhibitor of the respiration of E. coli.

As indicated, there were two possible sources of an inhibitor in

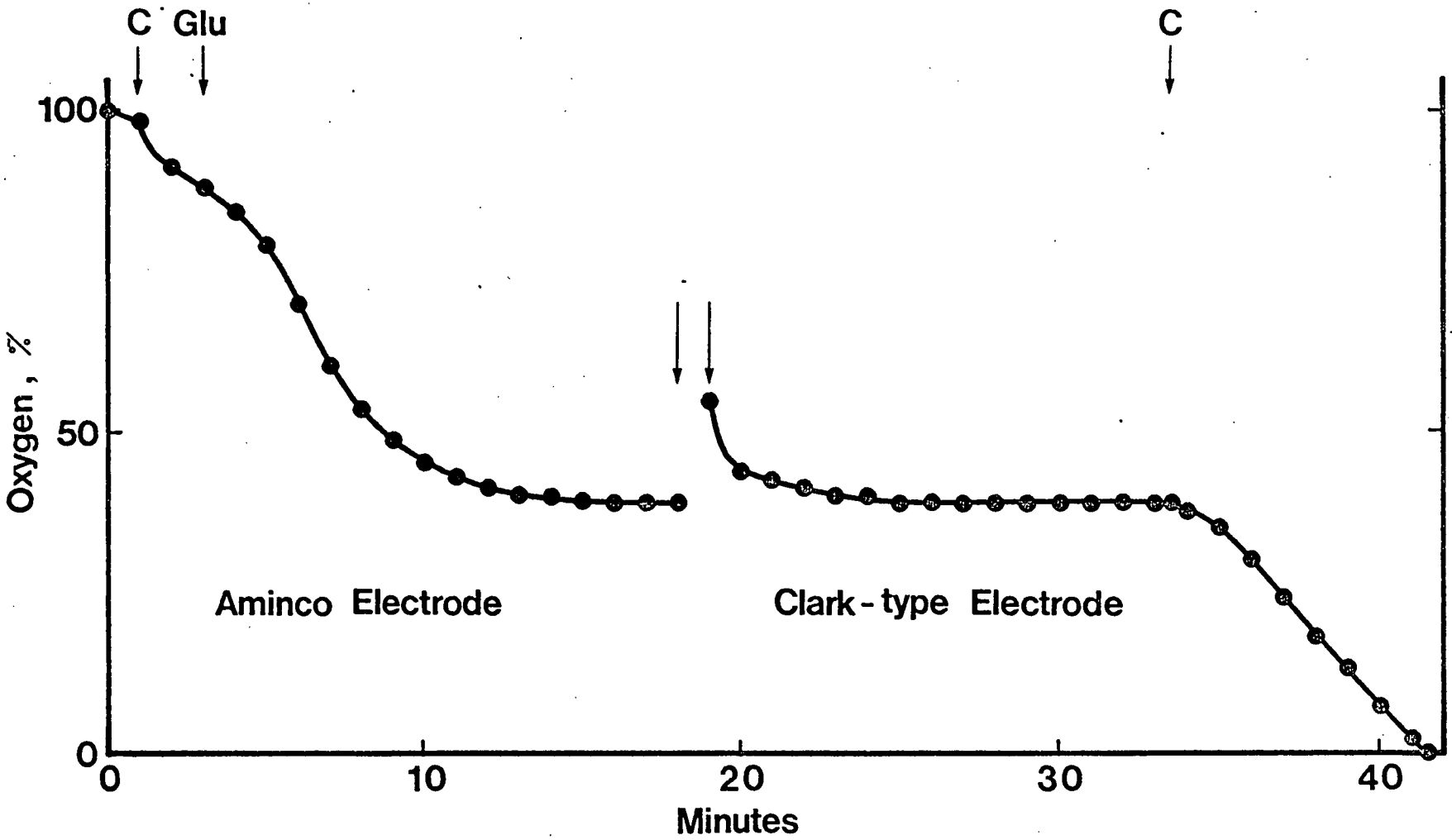


Fig. 3.5 Retention of the cessation of respiration following the replacement (arrows) of Aminco electrode by Clark-type electrode. C: 4 mg cells; Glu: 100 µmoles glucose; (method: sec. 2.2.2.2).

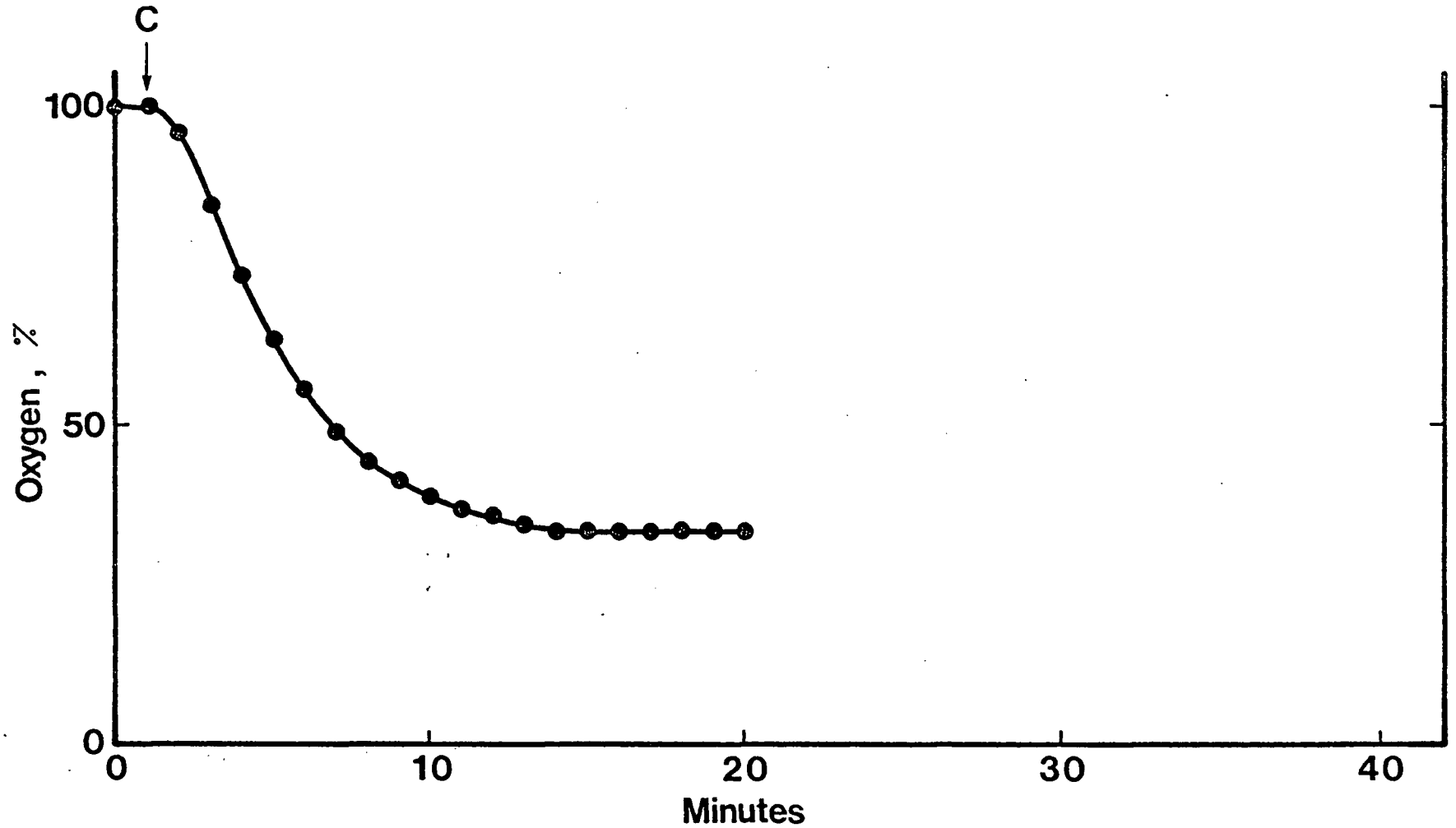


Fig. 3.6 Inhibition of the oxygen consumption of E. coli by a substance released by the Aminco electrode; O_2 uptake measured with Clark-type oxygen electrode. C: 4 mg cells; (method: sec. 2.2.2.2).

the Aminco oxygen electrode assay system: (i) an inhibitor produced from the medium components by the operation of the oxygen electrode; or (ii) an inhibitor released from the Aminco oxygen electrode system. The release of an inhibitor was favored as a result of the observed action of reduced glutathione in preventing and reversing the inhibition, suggesting the involvement of a metal ion.

There are only two major metal components of the Aminco oxygen electrode, the platinum of the cathode, and the silver of the anode. Since platinum is chemically very inert the most likely candidate for the inhibition of respiration was silver. Atomic absorption spectrophotometry of the assay medium from a blank run with the Aminco oxygen electrode indicated the presence of significant amounts of silver (sec.3.1.2). Further confirmation of the ability of silver to inhibit respiration was obtained when the addition of 125 nmoles of $AgNO_3$ to respiring suspensions of E. coli was shown to rapidly inhibit oxygen consumption (Figure 3.7).

3.1.2 Factors influencing the release of silver from the Aminco oxygen electrode

A further investigation of the release of silver from the anode of the Aminco oxygen electrode was decided upon for two reasons: (i) to determine why the inhibition of respiration by silver released from the naked anode of an oxygen electrode had not been cautioned against in the literature, although this type of oxygen electrode has been in use for some time, and (ii) the information would aid in evaluating the results reported by other researchers using this type of equipment.

Figure 3.8 demonstrates the influence of buffer ion on time dependent release of silver from the Aminco electrode system utilizing the four buffer systems: glycylglycine-KOH, HEPES-HCl, Tris-HCl, and potassium

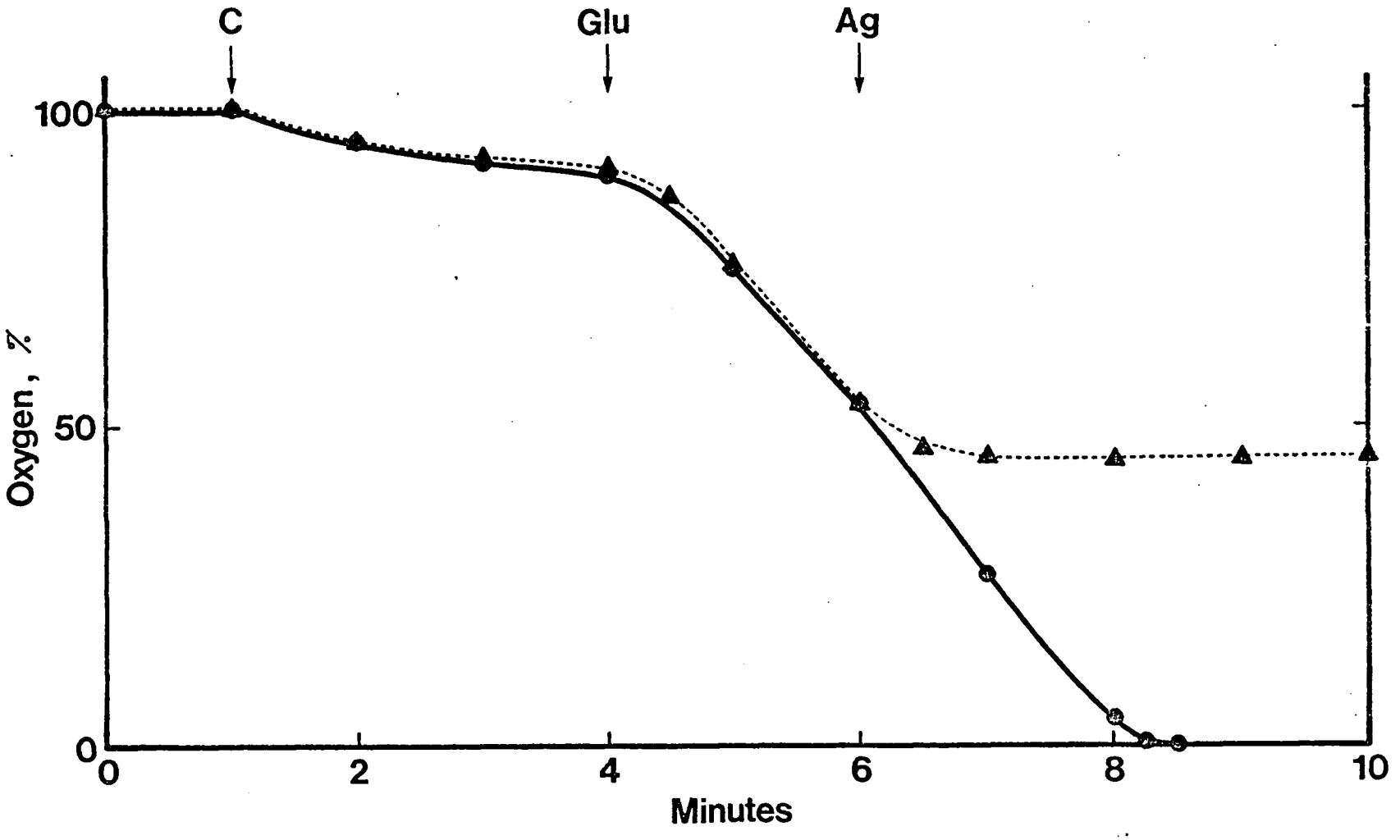


Fig. 3.7 Inhibition by silver of the respiration of E. coli as measured with Clark-type oxygen electrode. C: 10 mg cells; Glu: 50 µmoles glucose; Ag: 125 nmoles $AgNO_3$; ●: control; ▲: test; (method: sec. 2.2.5.2).

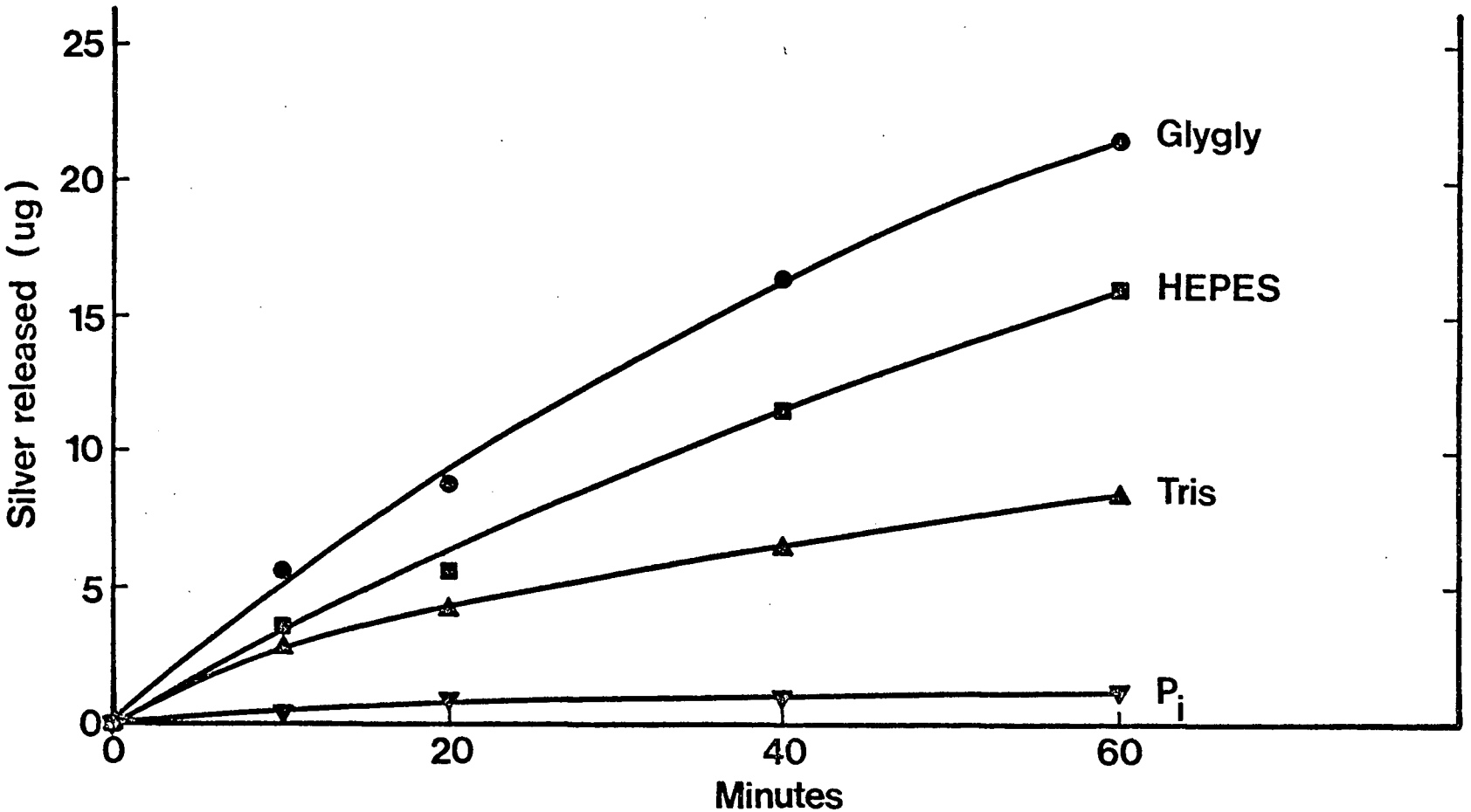


Fig. 3.8 Buffer dependence of the release of silver from the Aminco oxygen electrode. Glygly: 0.3 M glycylglycine-NaOH, pH 7.0; HEPES: 0.3 M HEPES-NaOH, pH 7.0; Tris: 0.3 M Tris-HCl, pH 7.0; P_i: 0.3 M potassium phosphate buffer, pH 7.0; (method: sec. 2.2.3).

phosphates, all 0.3 M with a pH of 7.0. The quantity of silver was both time- and buffer-dependent. The very minimal release in phosphate buffer was probably due to the insolubility of silver phosphate (Ksp = 1.6×10^{-18} $moles^4/litre^4$).

In order to obtain some insight into the mechanism of silver release, the time course of the release of silver from the anode in 0.3 M glycylglycine-KOH buffer, pH 7.0, in the presence or absence of the polarizing voltage was determined (Table 3.2). Although the presence of the polarizing voltage facilitated the release of silver from the anode it was not essential.

Since the release of silver from the anode was relatively independent of the presence or absence of the polarizing voltage, this suggested that the silver might be removed primarily by chelation in which case the amount released should be dependent upon the concentration of the chelating agent, presumably in this case the buffer. Table 3.3 demonstrates that the influence of buffer concentration on the release of silver from the oxygen electrode was quite marked.

3.1.3 The influence of pH, buffer ion and buffer concentration on the respiration of E. coli

Prior to proceeding with further investigations on the influence of silver on the respiration of E. coli, it was considered important to obtain a buffer system of a pH and concentration which consistently supported; (i) a high respiratory rate, (ii) a constant rate of oxygen utilization, and (iii) which would maintain these characteristics of the E. coli cell suspensions for a considerable period of time.

The results of the determination of the respiratory rates of E. coli cells suspended in nine isotonic buffers (sec.2.2.4.2), covering the pH

Table 3.2

Time course for the release of silver from the Aminco oxygen electrode, with presence or absence of polarizing voltage

Time (min.)	Silver Release, (μg) Polarizing Voltage Present	Absent	Difference
0	0	0	0
10	7.6	4.2	3.4
20	13.6	13.4	0.2
40	23.2	17.2	6.0
60	30.8	23.2	7.6

Table 3.3

Effect of concentration of buffer on the release of silver from the Aminco oxygen electrode

Buffer	Silver Released[1], (μg) 3 mM Buffer	30 mM Buffer	300 mM Buffer
Glycylglycine-KOH	0.52	0.72	30.8
Tris-HCl	0.44	0.88	6.8
Phosphate	0.48	0.72	1.8
HEPES-KOH	0.40	0.80	10.0

1 amount released in one hour.

range of 4 to 10, are presented in Figure 3.9. The respiration rate is expressed as the decrease in the percent oxygen saturation per min per mg wet cell weight. Determinations were performed in duplicate and the mean rate was plotted.

Of the buffers investigated high respiratory rates between pH 6 and 8 were obtained with glycylglycine, phosphate, Tris, citrate, MOPS and HEPES buffers. However, of these buffer systems only phosphate in the pH range 5.5 to 5.7, and glycylglycine over the pH range of 7.0 to 8.5 supported linear rates of oxygen consumption, as shown for glycylglycine buffer, pH 7.0, in Figure 3.10. The oxygen consumption traces of the other buffer systems indicated decreasing rates of oxygen utilization with decreasing oxygen level, as shown for HEPES buffer, pH 7.0, in Figure 3.10.

Of the two buffers, glycylglycine and phosphate, glycylglycine was the obvious choice for experiments involving the addition of silver due to the insolubility of silver phosphate.

The influence of the concentration of glycylglycine-KOH buffer, pH 7.0, on the respiration rate (Figure 3.11), and on the short term stability of the respiration rate of the cells suspended in the buffers (Table 3.4) was determined. Although the results presented in Figure 3.11 are from a single experiment they were reproducible. The respiration rate, expressed as the decrease in the percent oxygen saturation per min per mg of protein, was maximal with a buffer concentration of 100 to 300 mM (Figure 3.11), while the short term stability of the respiration rate was optimal with buffer concentrations in the range of 100 to 500 mM (Table 3.4).

The long term stability of respiration of cells suspended in 300 mM glycylglycine-KOH buffer, pH 7.0, (Table 3.5) demonstrated fluctuations in

Fig. 3.9 Buffer ion and pH dependence of the respiration of *E. coli*. Glygly: glycylglycine-NaOH; P_i: Na_2HPO_4-NaH_2PO_4; Tris: Tris-HCl; Trieth: triethanolamine-HCl; Cit: citrate-NaOH; MOPS: MOPS-NaOH; HEPES: HEPES-NaOH; Gly: glycine-NaOH; Eth: ethanolamine-HCl; all buffers were isotonic (method: sec 2.2.4.2); Rate: the decrease in the O_2 saturation/min/mg cell wet weight.

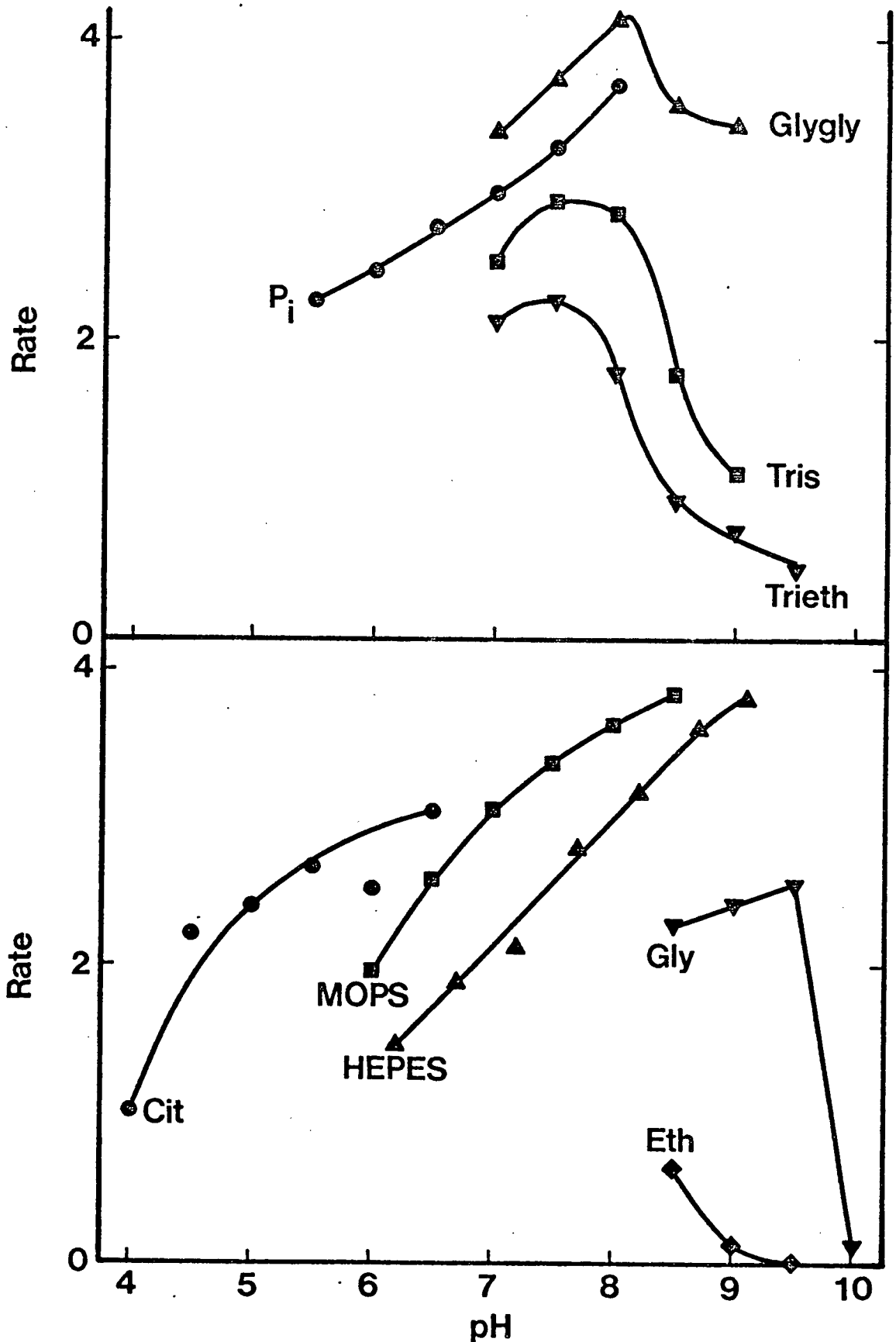

Rate
4
2
0
Glygly
P_i
Tris
Trieth
Rate
4
2
0
MOPS
HEPES
Cit
Gly
Eth
4
5
6
7
8
9
10
pH

Fig. 3.9

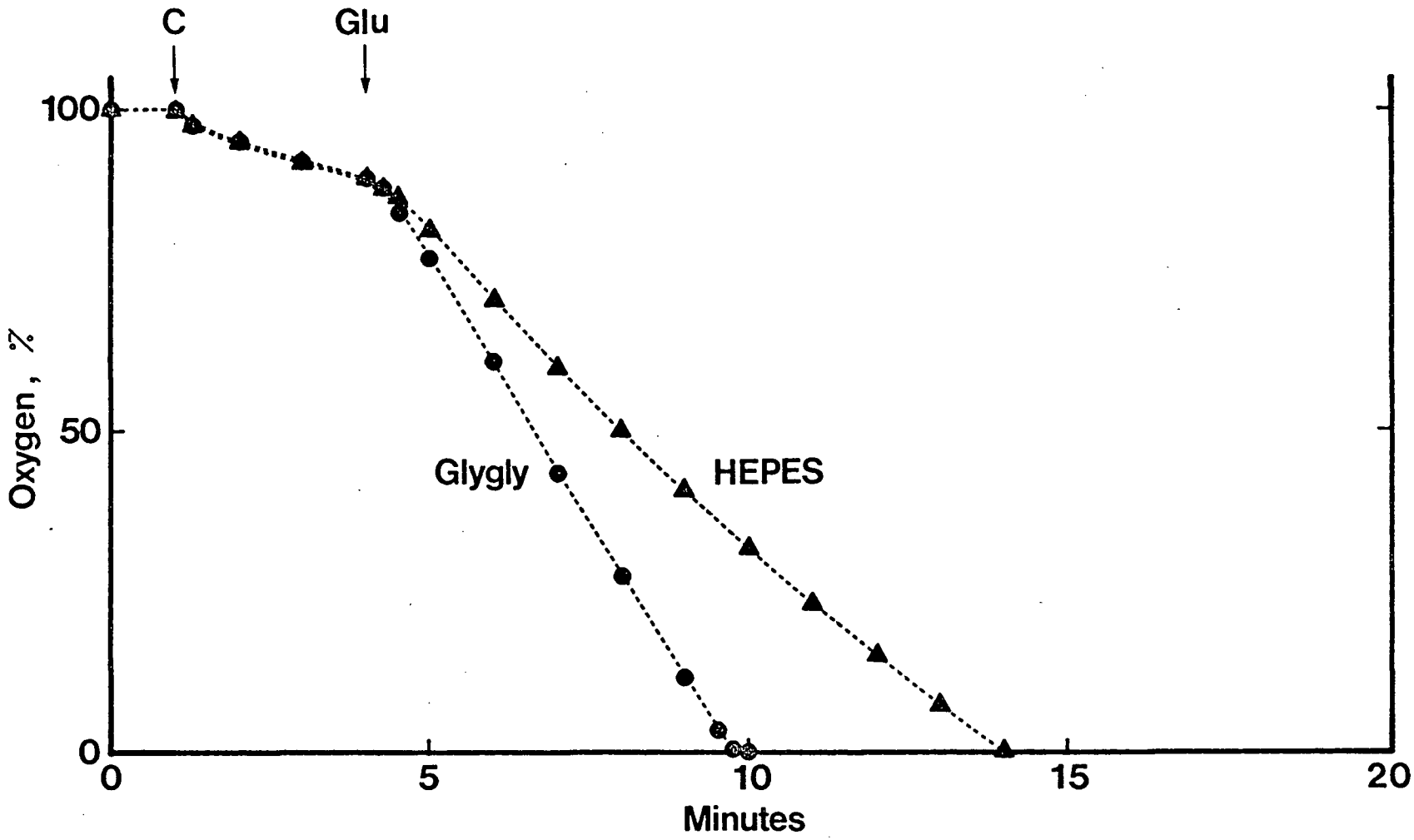


Fig. 3.10 The influence of buffer ions on the oxygen consumption traces of E. coli. Glygly: isotonic glycylglycine-NaOH, pH 7.0; HEPES: isotonic HEPES-NaOH, pH 7.0; C: 5 mg cells; Glu: 50 µmoles glucose; (method: sec. 2.2.4.2); (data from single determinations).

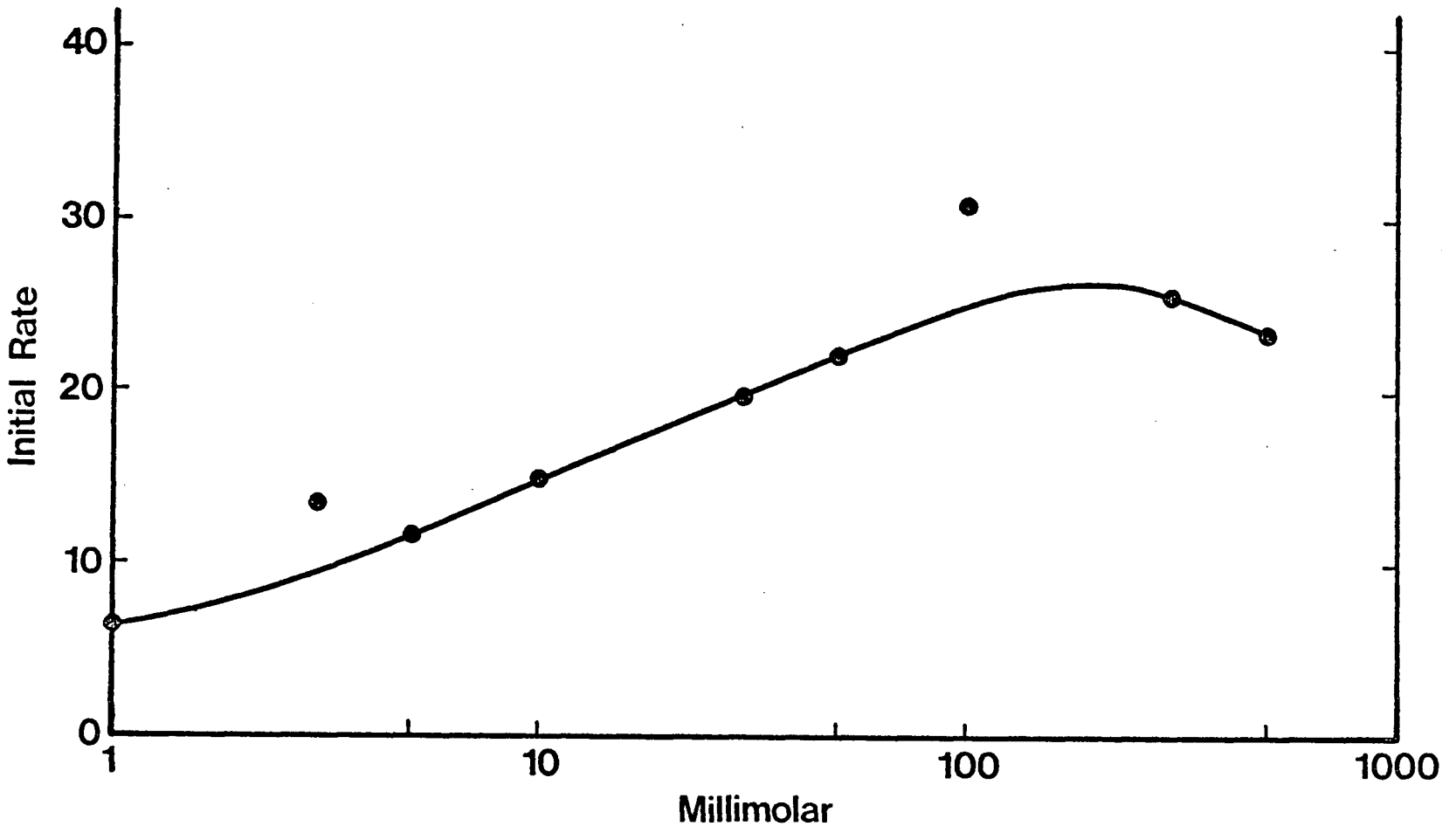


Fig. 3.11 The influence of the concentration of glycylglycine-KOH buffer, pH 7.0 on the respiration rate of E. coli. (method: sec. 2.2.4.2); Rate: the decrease in O_2 saturation/min/mg protein.

Table 3.4

The influence of the concentration of glycylglycine-KOH buffer, pH 7.0, on the respiration rate of E. coli

Concentration (mM)	Respiration Rate[1] (at times after suspension in buffer) 0	15'	30'
0	4.0	3.0	2.2
1	6.4	5.9	5.5
3	13.5	8.5	8.4
5	11.6	7.8	7.8
10	15.0	11.5	10.6
30	19.6	15.6	16.6
50	22.1	19.1	18.2
100	30.8	28.2	26.4
300	25.5	26.8	28.3
500	23.3	23.4	23.4

[1] respiration rate is expressed as: percent decrease in oxygen saturation per min per mg of protein.

Table 3.5

The influence of the duration of suspension in 300 mM glycylglycine-KOH buffer, pH 7.0, at 0°C, on the respiration of *E. coli*

Time (hr)	Rate of Respiration[1]
1	14.6
3	14.9
5	16.0
11	15.6
17	12.7
23	14.0
29	14.1
38	14.1
47	14.2

The regression line for:

$$y = 15.05 - 0.03\ (x)$$

where y: is the rate of respiration, and x: is the time

[1] respiration rate expressed as: percent decrease in oxygen saturation per min per mg protein.

values reported are the average of values obtained from duplicate experiments.

the respiration rate with a slight decrease in the rate of respiration over a 47 hour period. In this case the fluctuations were probably due to problems in stabilizing the temperature of the aluminum block incubation chamber of the Yellow Springs Instruments oxygen monitor.

Thus, 300 mM glycylglycine-KOH buffer, pH 7.0 (or pH 7.5) was selected as the most suitable buffer of those examined since it possessed the desired characteristics of supporting (i) a high respiratory rate, (ii) a constant rate of oxygen utilization, and (iii) which would maintain these characteristics of the E. coli cell suspensions for a considerable period of time.

3.1.4 Inhibition of the respiration of E. coli by added silver nitrate

Having established that silver metal (ie., nascent silver) or silver ions were responsible for the inhibition of the respiration of E. coli, the mechanism of their action was investigated.

In an attempt to localize the possible site(s) of inhibition by silver, E. coli were grown on different carbon sources to induce or repress specific enzyme systems of carbohydrate metabolism and/or transport (sec.1). The effect of $AgNO_3$ on substrate-dependent, or endogenous respiration then was tested (Figures 3.12 to 3.18 inclusive). In all figures the dotted line connecting the solid circles represents the control respiration of 5 mg of cells (wet weight) (C) plus 50 µmoles of the substrate indicated. The solid line connecting the solid triangles represents the results of the test system, identical to the control system with the exception that 0.5 to 2 minutes after the addition of 50 µmoles of substrate, 450 nmoles of $AgNO_3$ were added (Ag). The substrate dependent respiration in all cases is essentially linear. However, the addition of $AgNO_3$ rapidly inhibited the substrate dependent respiration in all cases with the exception of formate

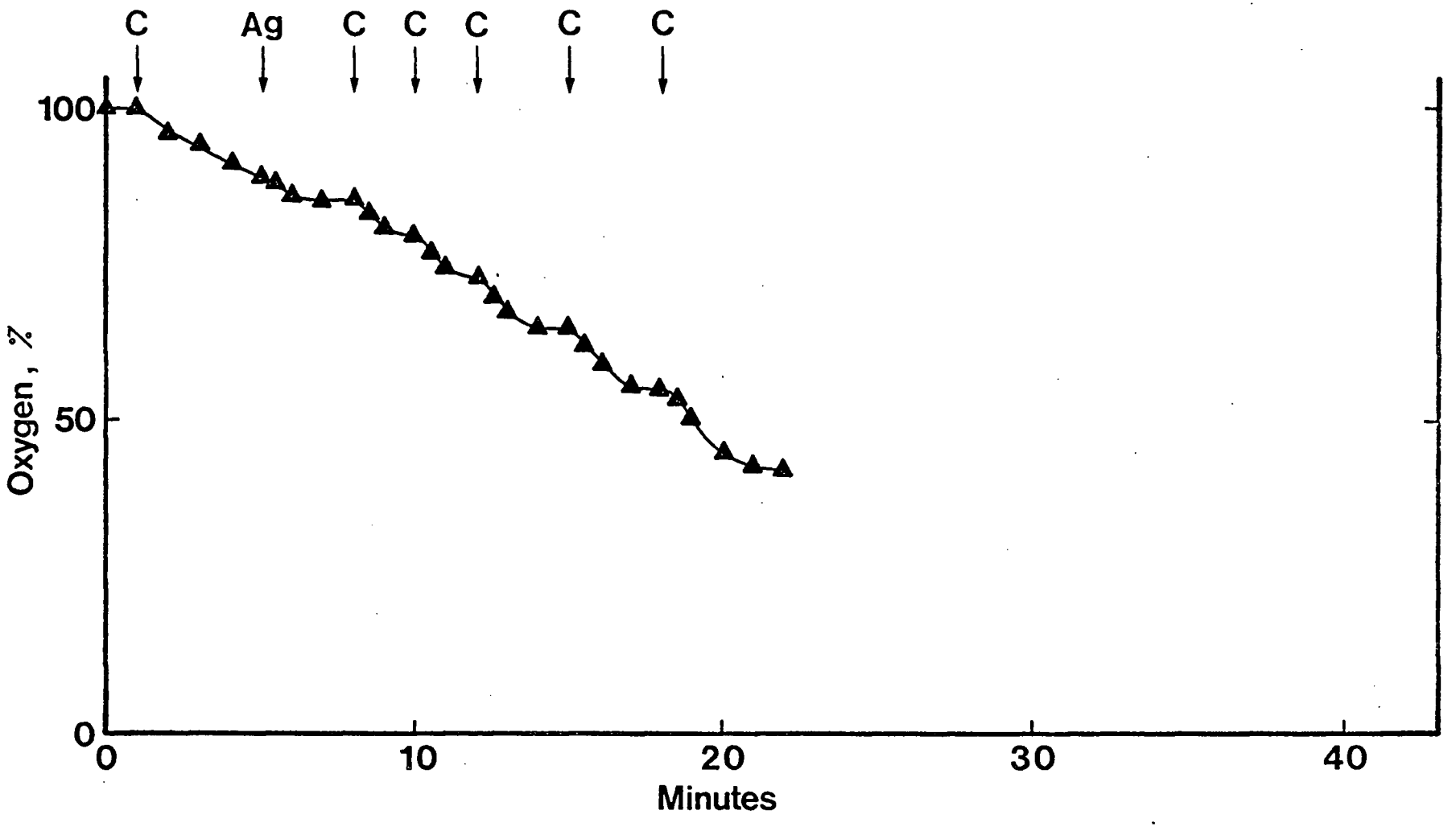


Fig. 3.12 Inhibition of the endogenous respiration of E. coli by silver. C: 5 mg cells; Ag: 450 nmoles $AgNO_3$; (method: sec. 2.2.5.2); carbon source: 0.4% glucose.

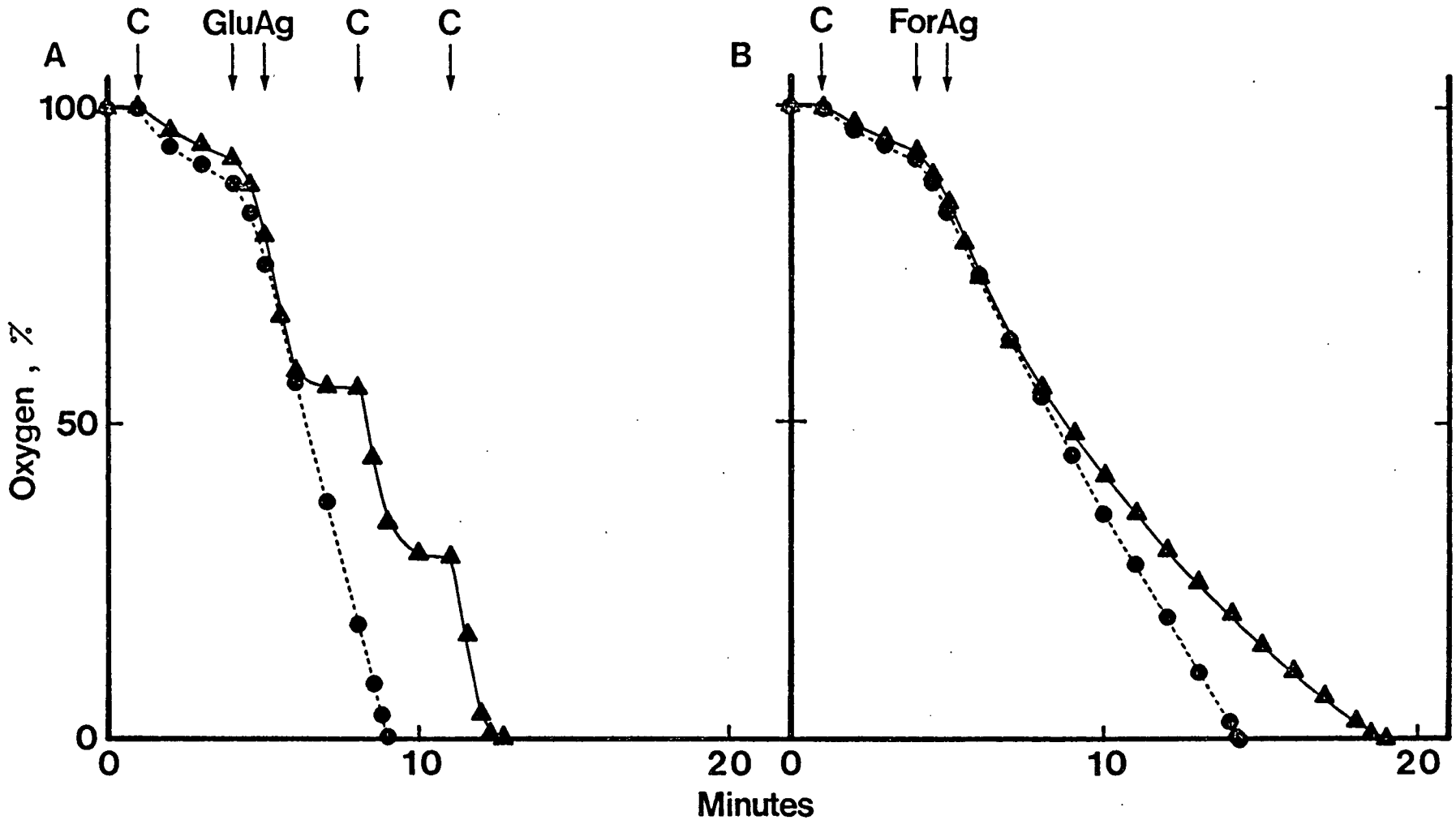


Fig 3.13 Inhibition of the glucose-dependent (A) and formate-dependent (B) respiration of E. coli by silver.
C: 5 mg cells; Glu: 50 µmoles glucose; For: 50 µmoles formate; Ag: 450 nmoles $AgNO_3$;
●:control; ▲:test; (method: sec.2.2.2.5); carbon source: 0.4% glucose.

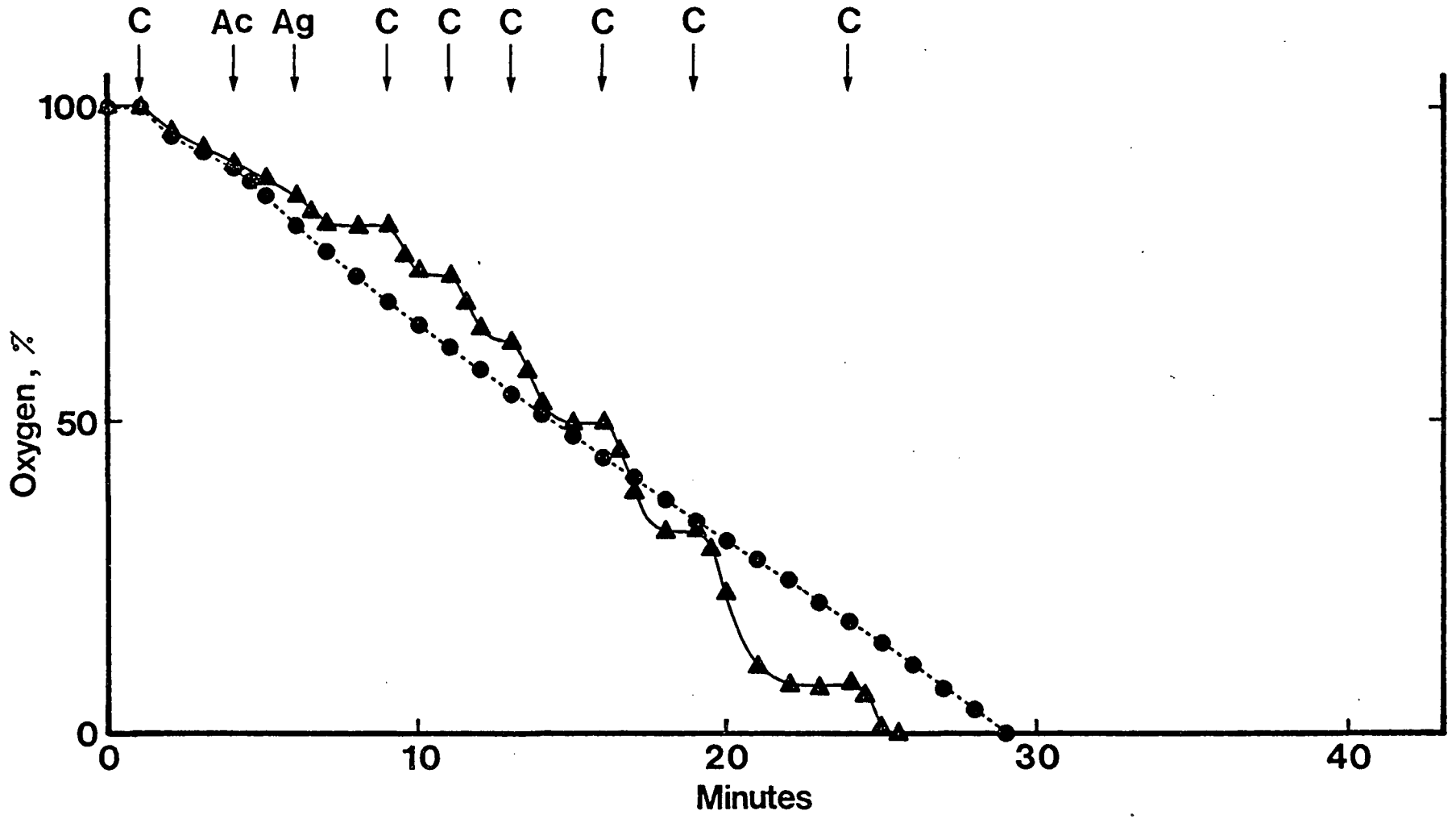


Fig. 3.14 Inhibition of the acetate-dependent respiration of E. coli by silver.
C: 5 mg cells; Ac: 50 µmoles acetate; Ag: 450 nmoles $AgNO_3$; ● : control; ▲ : test;
(method: sec.2.2.5.2); carbon source: 0.4% glucose.

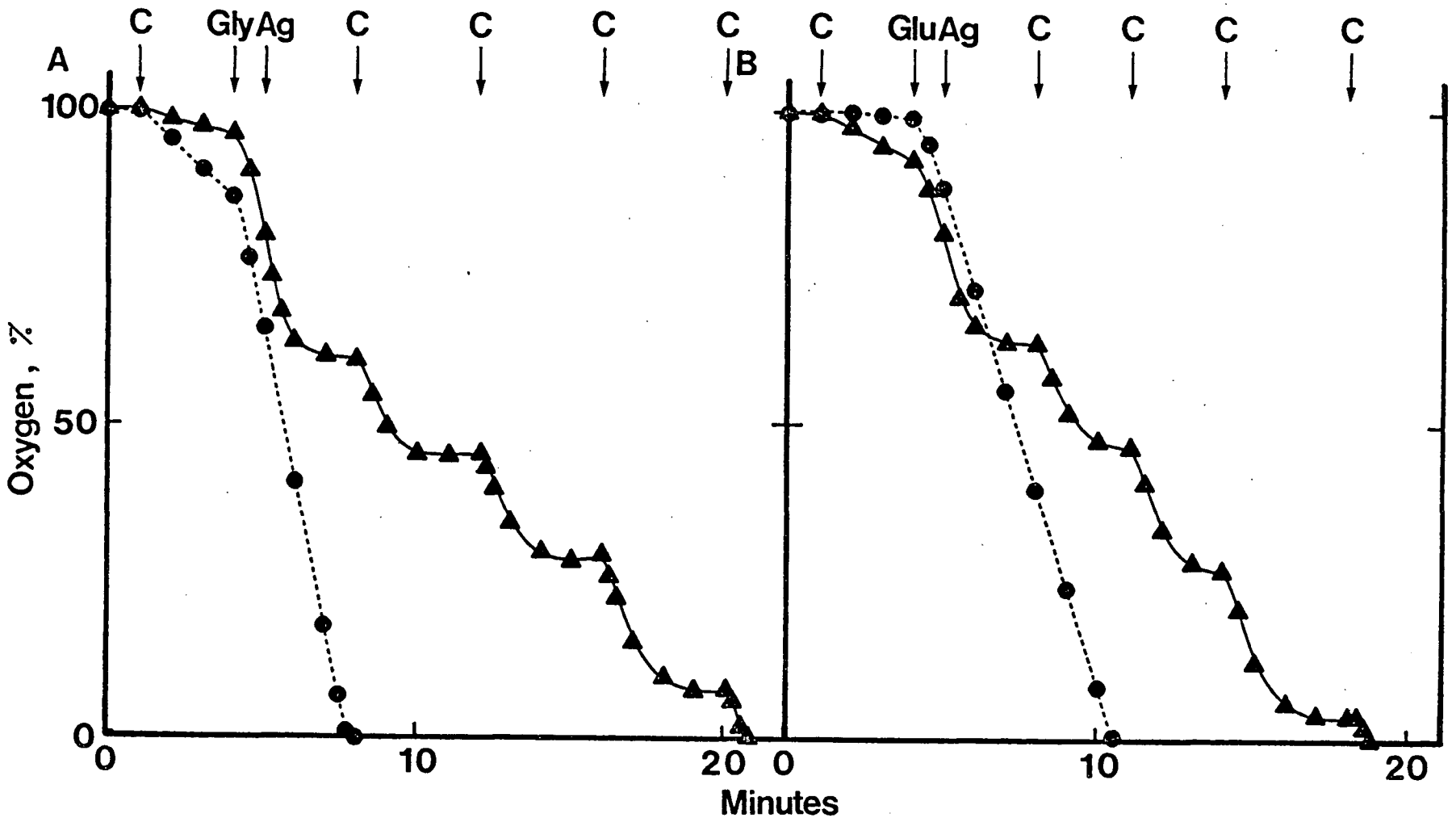


Fig. 3.15 Inhibition of the glycerol-dependent (A) and glucose-dependent (B) respiration of E. coli by silver.
C: 5 mg cells; Gly: 50 µmoles glycerol; Glu: 50 µmoles glucose; Ag: 450 nmoles $AgNO_3$;
● : control; ▲ : test; (method: sec.2.2.5.2); carbon source: 0.4% glycerol.

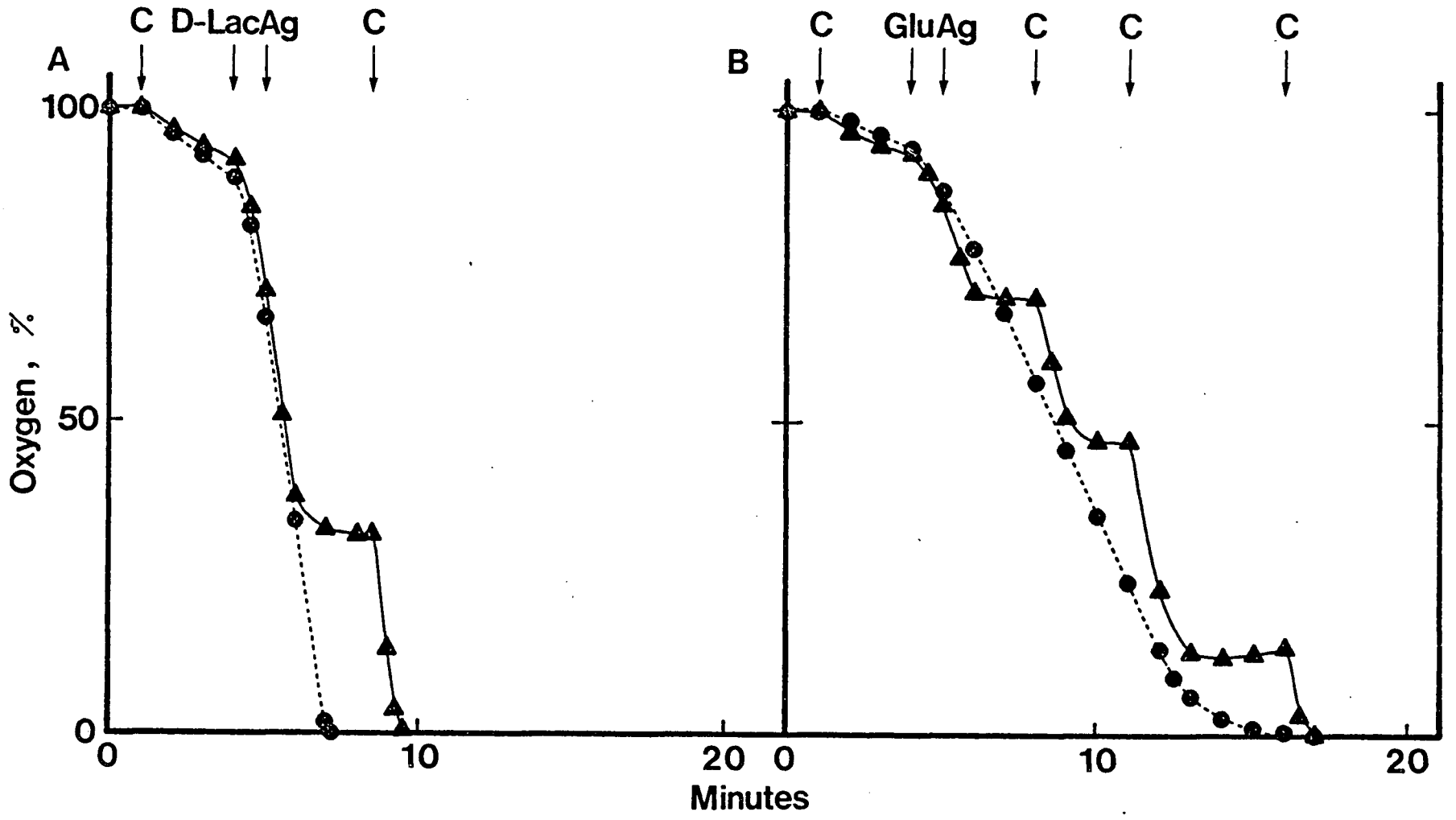


Fig. 3.16 Inhibition of the D-lactate-dependent (A) and glucose-dependent (B) respiration of E. coli by silver.
C: 5 mg cells; D-Lac: 50 µmoles D-lactate; Glu: 50 µmoles glucose; Ag: 450 nmoles $AgNO_3$;
● : control; ▲ : test; (method: sec.2.2.5.2); carbon source: 0.8% DL-lactate.

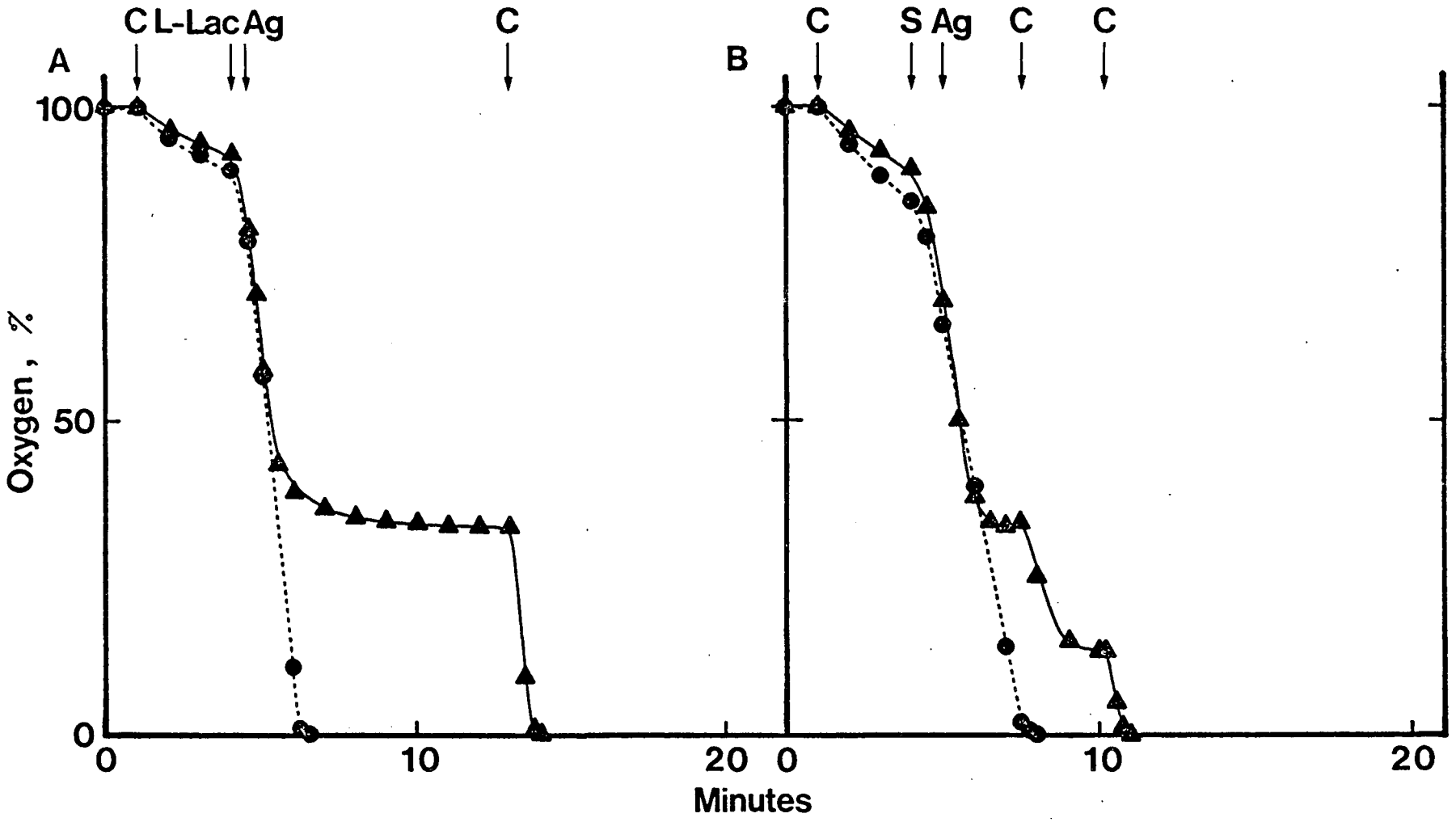


Fig. 3.17 Inhibition of the L-lactate-dependent (A) and succinate-dependent (B) respiration of E. coli by silver.
C: 5 mg cells; L-Lac: 50 µmoles L-lactate; S: 50 µmoles succinate; Ag: 450 nmoles $AgNO_3$; ● : control; ▲ : test; (method: sec.2.2.5.2); carbon source: A, 0.8% DL-lactate; B, 0.4% succinate.

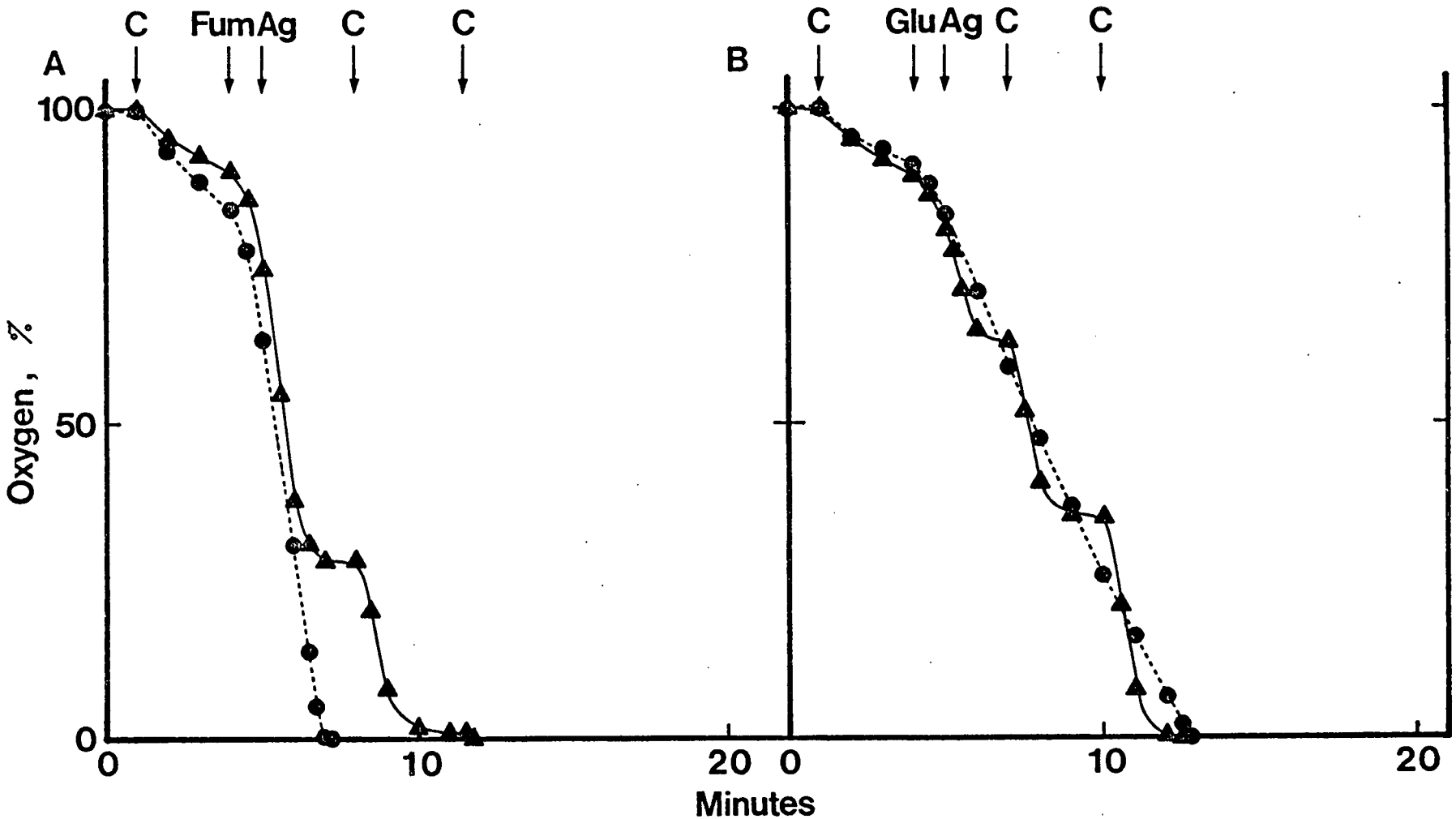


Fig. 3.18 Inhibition of the fumarate-dependent (A) and glucose-dependent (B) respiration of E. coli by silver.
C: 5 mg cells; Fum: 50 µmoles fumarate; Glu: 50 µmoles glucose; Ag: 450 nmoles $AgNO_3$;
● : control; ▲ : test; (method: sec.2.2.5.2); carbon source: 0.4% fumarate.

(Figure 3.13B).

The peculiar stepwise nature of the traces of the test system is due to the successive additions of 5 mg of cells. This experimental procedure was used in an attempt to titrate the amount of $AgNO_3$ added, and to routinely determine that the oxygen electrode response to zero oxygen corresponded to zero on the strip chart reading.

The response of endogenous respiration of E. coli to the addition of $AgNO_3$ is indicated in Figure 3.12. The results presented were obtained with E. coli grown on glucose as the carbon source, however, analogous results were obtained with E. coli grown on succinate. The response of the endogenous respiration of E. coli grown on other carbon sources to the addition of $AgNO_3$ was not determined.

The sensitivity of glucose-dependent or glycerol-dependent respiration to inhibition by silver is indicated by Figures 3.13A, 3.15A, 3.15B, 3.16B and 3.18B. By comparison, the respiration of E. coli utilizing D-lactate (Figure 3.16A), L-lactate (Figure 3.17A), succinate (Figure 3.17B) or fumarate (Figure 3.18A) as substrate appeared less sensitive to inhibition by silver than glycerol-dependent or glucose-dependent respiration. The responses of D-lactate, L-lactate, succinate and fumarate to inhibition by silver were very similar. Acetate-dependent oxygen consumption was very sensitive to inhibition by $AgNO_3$ (Figure 3.14). Formate oxidation (Figure 3.13B) was the least sensitive to inhibition of the substrates examined.

In general, the growth of E. coli on different carbon sources did not appear to have much influence on the response of any particular substrate-dependent respiration to silver inhibition.

In order to determine if glycolysis was more sensitive to inhibi-

tion than the combined function of the tricarboxylic acid cycle and the electron transport system as suggested by a comparison of the results of Figures 3.13A, 3.15A, 3.15B, 3.16B and 3.18B with those of Figures 3.16A, 3.17A, 3.17B and 3.18A, the effects of $AgNO_3$ on acid production and oxygen consumption with glucose as substrate were monitored concurrently, in separate but identical systems. Figure 3.19A indicates the results obtained with the control system of 20 mg wet weight of E. coli (C) and 50 µmoles of glucose (Glu). Oxygen consumption is indicated by the solid line and acid production, as pH, is plotted as the broken line. The results from the test system (Figure 3.19B) demonstrated that at a final siver nitrate concentration of 24 µM (125 nmoles of $AgNO_3$ added), acid production was inhibited immediately and completely, while oxygen consumption was only partially inhibited. These results suggested that there was at least one site in glycolysis which was more sensitive to inhibition by siver than the site(s) of the TCA cycle and the respiratory chain.

An indication of the location of the silver-sensitive site of glycolysis was provided by the similarity of the results obtained for silver inhibition of glucose-dependent, and glycerol-dependent respiration of cells grown on glycerol (Figure 3.15). The similarity of the pattern of response suggested that the site, in the glycolytic pathway, which possessed the high sensitvity to silver inhibition must occur in that portion of glycolysis which is common to the metabolism of both substrates, that is, between glyceraldehyde-3-phosphate and pyruvate. A possible candidate in this region of the glycolytic pathway was glyceraldehyde-3-phosphate dehydrogenase, an enzyme which is well known as possessing a thiol reagent sensitive site. As indicated in table 3.6, silver did inhibit glyceralde-

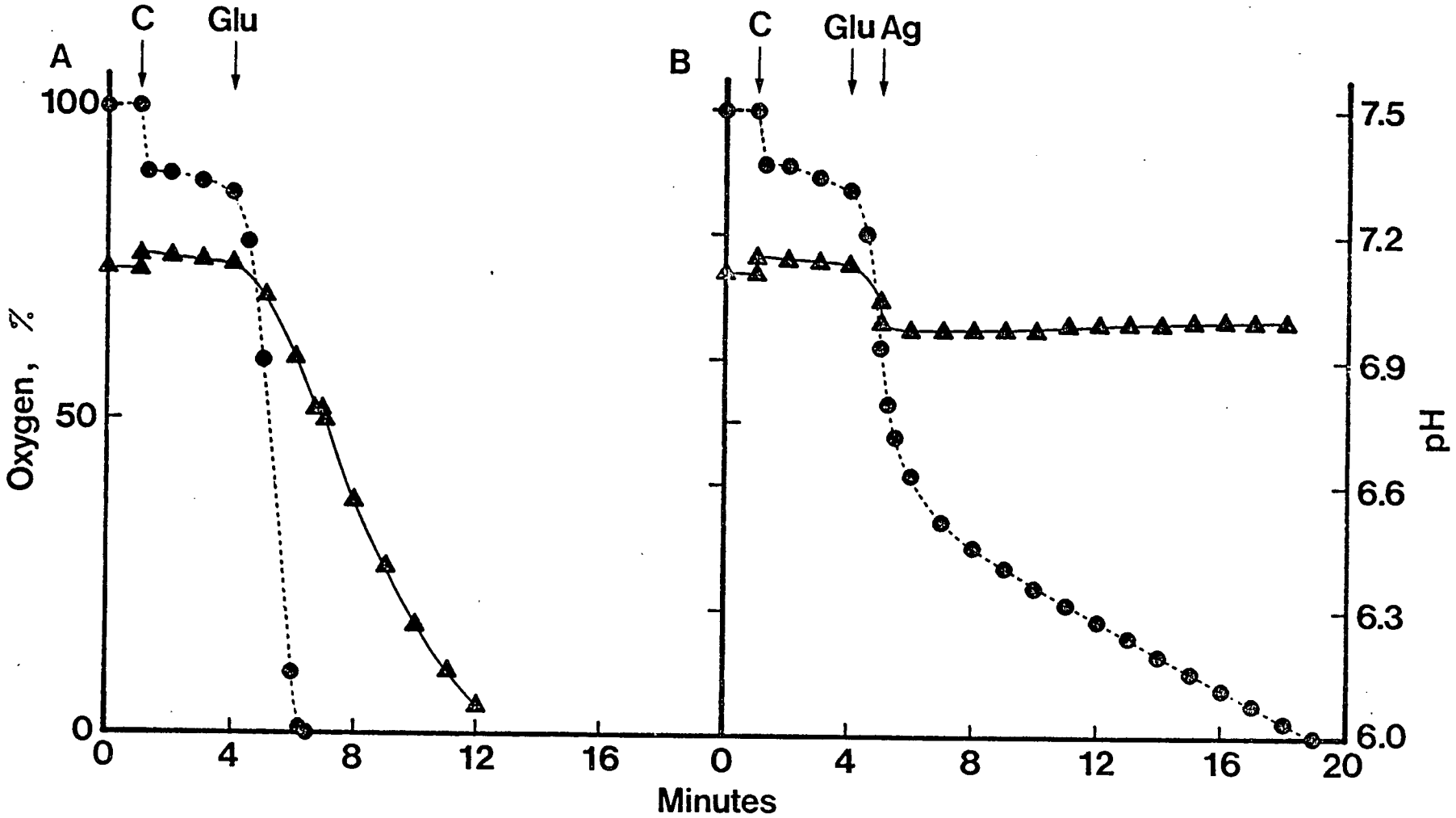


Fig. 3.19 Inhibition of the respiration and acid production of E. coli by silver. C: 20 mg cells; Glu: 50 µmoles glucose; Ag: 125 nmoles $AgNO_3$; ●: oxygen; ▲: pH; (method: sec. 2.2.5.3).

Table 3.6

The influence of silver nitrate and reduced glutathione on glyceraldehyde-3-phosphate dehydrogenase activity

Additions	Final Conc.	Duration of Preincubation (min) With Ag^+	With GSH	Activity 340/min
none		--	--	0.369
5 µl 0.50 mM $AgNO_3$	1 µM $AgNO_3$	5	--	0.169
		13	--	0.318
		20	--	0.224
5 µl 5.0 mM $AgNO_3$	10 µM $AgNO_3$	5	--	0.136
		13	--	0.050
		22	--	0.026
5 µl 5.0 mM $AgNO_3$ 100 µl 0.1 M GSH	10 µM $AgNO_3$ 4 mM GSH	5	2	0.124
		5	10	0.204
		5	18	0.142

values reported are from single determinations

hyde-3-phosphate dehydrogenase and the inhibition was partially reversed by reduced glutathione. However due to the requirement for arsenate in the assay medium, the assay for silver inhibition was somewhat less than satisfactory since silver arsenate is insoluble.

During the determination of the inhibition of the respiration of E. coli by silver (Figures 3.12 to 3.18 inclusive) it was observed that the addition of $AgNO_3$ stimulated the rate of oxygen consumption prior to inhibitin oxygen utilization. This is even more apparent in table 3.7 where the initial rates of oxygen consumption in the presence and absence of $AgNO_3$ are tabulated. This suggested the possibility that silver might function as an uncoupler prior to inhibiting respiration.

3.1.5 Selection of a carbon source for the growth of E. coli to be used for the investigation of the uncoupling of respiration by silver nitrate

In order to investigate the possible action of silver nitrate as an uncoupler of E. coli respiration it was essential to obtain E. coli cells which derived the majority of their energy requirement from the respiratory chain and which were "highly coupled". Of some interest to this problem was the report by Hempfling (1970b) that the levels of oxidative phosphorylation of E. coli, grown on a complex medium with glucose as the carbon source, was dependent upon the growth stage at which the cells were harvested. As a result it was decided to grow E. coli in batch cultures (sec. 2.2.6.1) using a number of different carbon sources in an attempt to evaluate the most suitable carbon source and growth phase to obtain the "highly coupled" cells required. The oxygen level of the growing culture was monitored to provide some information on the physiological character of the cell at any particular time during growth, while the redox potential

Table 3.7

The influence of the addition of silver nitrate on the initial rate of oxygen consumption.

Carbon Source	Substrate	Initial Rate of Oxygen Consumption[1] $AgNO_3$ Absent	$AgNO_3$ Present	RCR
Glucose	endog.	3.8	3.9	1.03
	glucose	18.9	30.0	1.59
	formate	9.9	13.5	1.36
	acetate	3.8	6.5	1.71
Glycerol	glycerol	24.0	31.0	1.29
	glucose	15.7	26.7	1.70
DL-Lactate	D-lactate	31.8	45.5	1.43
	L-lactate	46.0	49.6	1.08
	glucose	10.8	18.7	1.73
Succinate	succinate	25.3	40.5	1.60
Fumarate	fumarate	33.6	46.7	1.39
	glucose	12.4	24.4	1.97

[1] rate of oxygen consumption expressed as: the decrease in the percent oxygen saturation per min per mg cell (wet weight).

values reported are from single determinations.

was measured to investigate the correspondence between the measured redox potential and the oxygen level.

Prior to presenting the results of this section it is necessary to discuss a particular aspect of the design of the culture apparatus. The electrodes were separated from the bulk of the culture medium since the vigorous aeration resulted in unstable measurements. Consequently, the culture in the electrode vessel could be at a lower oxygen tension than the main vessel. However, as there was a dead space of only 2 ml between the main culture vessel and the electrode chamber, and since the liquid in the chamber was continually renewed at the rate of once every 15 sec, it is likely that the physical conditions in the electrode vessel were not greatly different from those in the main culture vessel. To check this point, the circulation of a mid-exponential phase culture between the two vessels was stopped. In 15 sec the oxygen tension in the electrode vessel dropped by 5%. This would probably be close to the maxium difference to be expected between the main culture and the electrode vessel.

Batch cultures of E. coli NRC 482 with 0.4% glucose as the carbon source, supplemented with ferric citrate (6 μM) (Figure 3.20) demonstrated three distinct phases of oxygen utilization. Two phases of growth were observed. During the first phase of oxygen utilization the level of oxygen saturation decreased at an accelerating rate from 0.5 to 4.75 hr, after inoculating the culture, at which time the culture became oxygen-limited and remained oxygen-limited until all the glucose had been exhausted at about 5.25 hr. The redox potential of the culture decreased essentially in parallel with the oxygen level, during the first phase of oxygen utilization, until the culture became oxygen-limited. Under the oxygen-limited conditions the

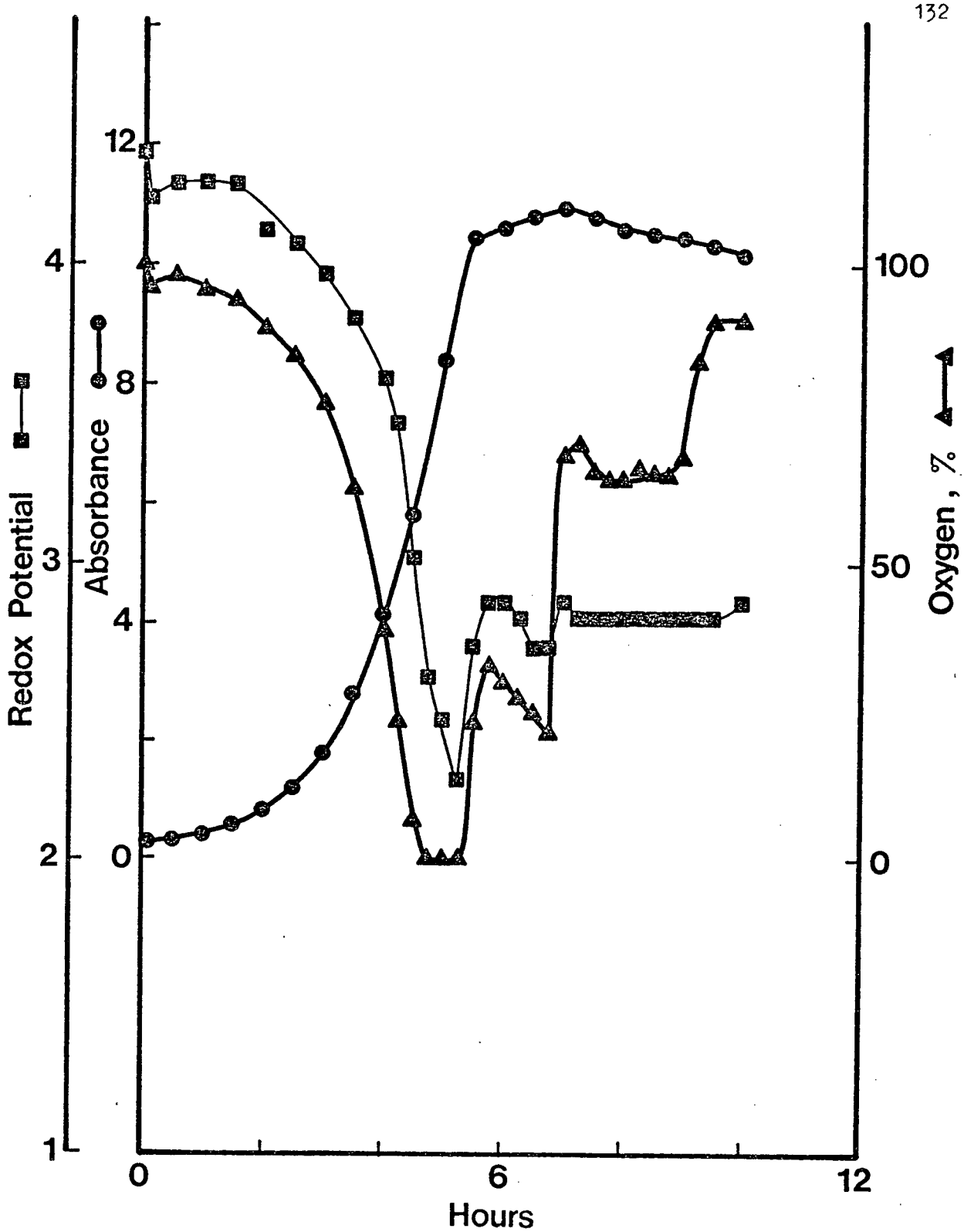


Fig. 3.20 Growth of E. coli on 0.4% glucose in medium containing 6 μM ferric citrate. Absorbance at 420 nm; units: redox potential, mV x 10^{-2} (methods: sec. 2.2.6.1).

redox potential continued to fall until the glucose content of the medium was depleted. The initial phase of growth was essentially exponential and corresponded to the first phase of oxygen utilization. There appeared to be little if any effect of the oxygen-limited conditions on the growth rate.

The relationship between the glucose concentration in the medium, redox potential and the growth of the culture, for a similar experiment is indicated in Figure 3.21. Measurement of the oxygen level was not performed during this experiment but the abrupt increase in the redox potential (corresponding to an increase in the level of oxygen saturation (Figure 3.20)), and cessation of growth corresponding to the depletion of glucose from the medium (8.5 hr) is demonstrated clearly.

On depletion of the glucose (Figure 3.20), the level of oxygen saturation increased for 30 min, and then proceeded to decrease at a linear rate for approximately one hour. This constituted the second phase of oxygen utilization and was accompanied by a low, essentially linear, growth rate. There was a close correspondence between the oxygen level and the redox potential of the culture during the second phase of oxygen utilization. Subsequent to the second phase of oxygen utilization, the oxygen level increased rapidly to approximately 70% of saturation, then dropped to 65% of saturation and remained constant at this value for 1.25 hr before returning to close to saturation. During this third phase, the cell mass of the culture declined slowly. The redox potential increased only slightly compared to the increase in oxygen level during the initial portion of the third phase of oxygen utilization, then remained constant at about 280 mV for the majority of this phase, failing to demonstrate an increase corresponding to the increase in oxygen content of the medium to close to the saturation level.

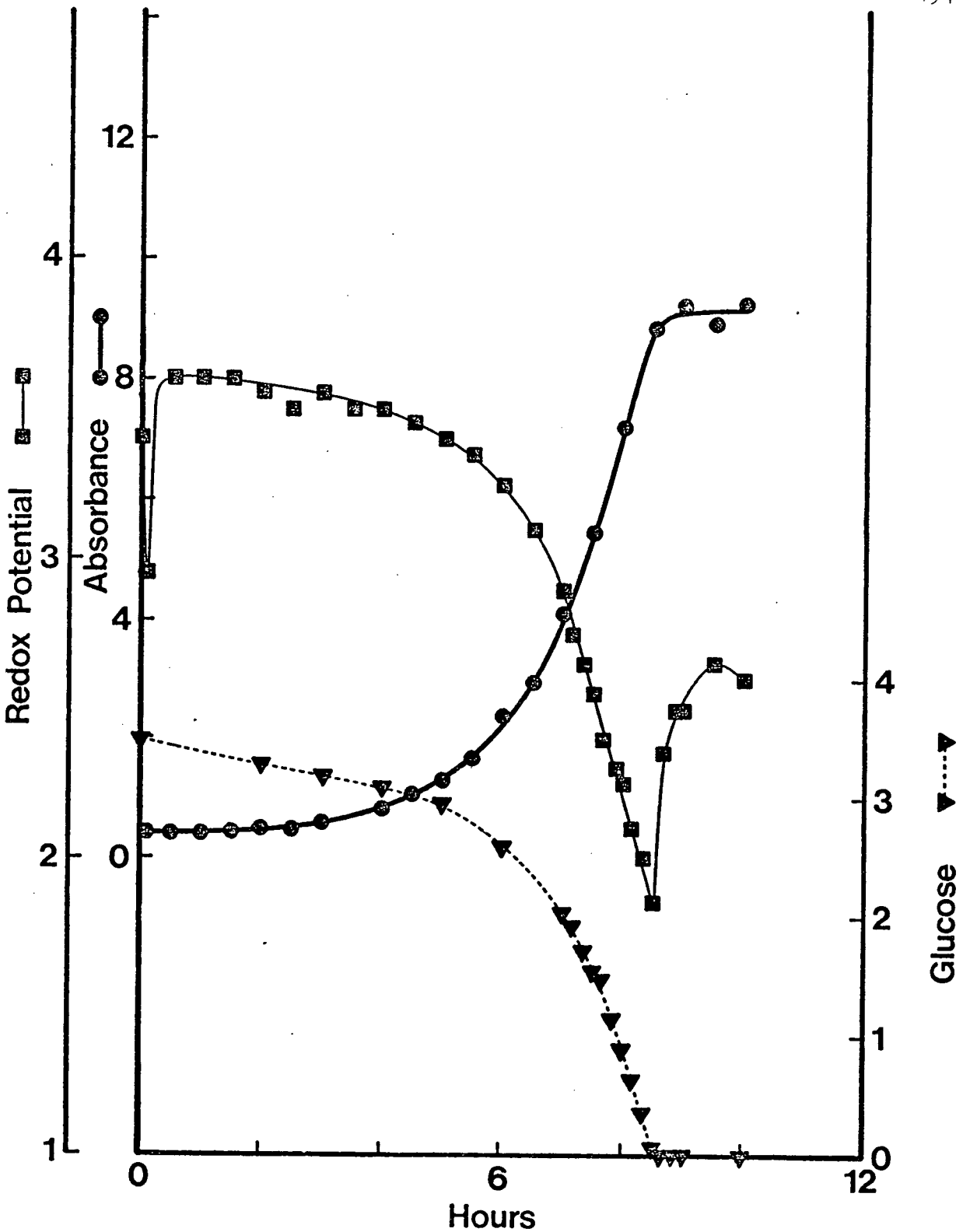


Fig. 3.21 Growth of E. coli on 0.4% glucose. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; glucose concentration, mg/ml. (methods: sec. 2.2.6.1 and 2.2.6.11)

The absolute values of the oxygen levels in Figures 3.20, 3.22, 3.23 and 3.24 are in some doubt due to an unexpected, large residual electrode current in the absence of oxygen. All data used in these figures were corrected using an "average" residual current value. However, the magnitude of the residual current as determined in subsequent experiments varied slightly, thus the uncertainty.

Growth of E. coli NRC 482 on 0.4% glycerol as carbon source (Figure 3.22) demonstrated a single distinct phase of oxygen utilization and growth comparable to the first phase of oxygen utilization of the culture grown with glucose as carbon source (Figure 3.20). The oxygen-limited period of growth was of greater duration in the glycerol grown culture than in the culture grown on glucose, and had a greater influence on the growth. The growth data show a definite decrease in growth rate corresponding to the oxygen-limited conditions. As in the case of the culture grown on glucose there was a close correlation between the level of oxygen saturation and the redox potential of the culture until the culture became oxygen-limited. Under the conditions of oxygen limitation the redox potential decreased markedly, reached a minimum, and increased rapidly prior to any large increase in the oxygen saturation. Subsequently, the level of oxygen saturation increased from 10 to 87% and remained essentially constant. During the same interval, however, the redox potential demonstrated a slight initial increase (7.25 to 7.75 hr), followed by a period of relatively rapid increase (7.75 to 8.5 hr) succeeded by a period of comparative constancy (9.0 to 11.0 hr). Thus, there was very poor correlation between changes in the oxygen level and the redox potential of a culture grown on glycerol, during and subsequent to the period of oxygen limitation.

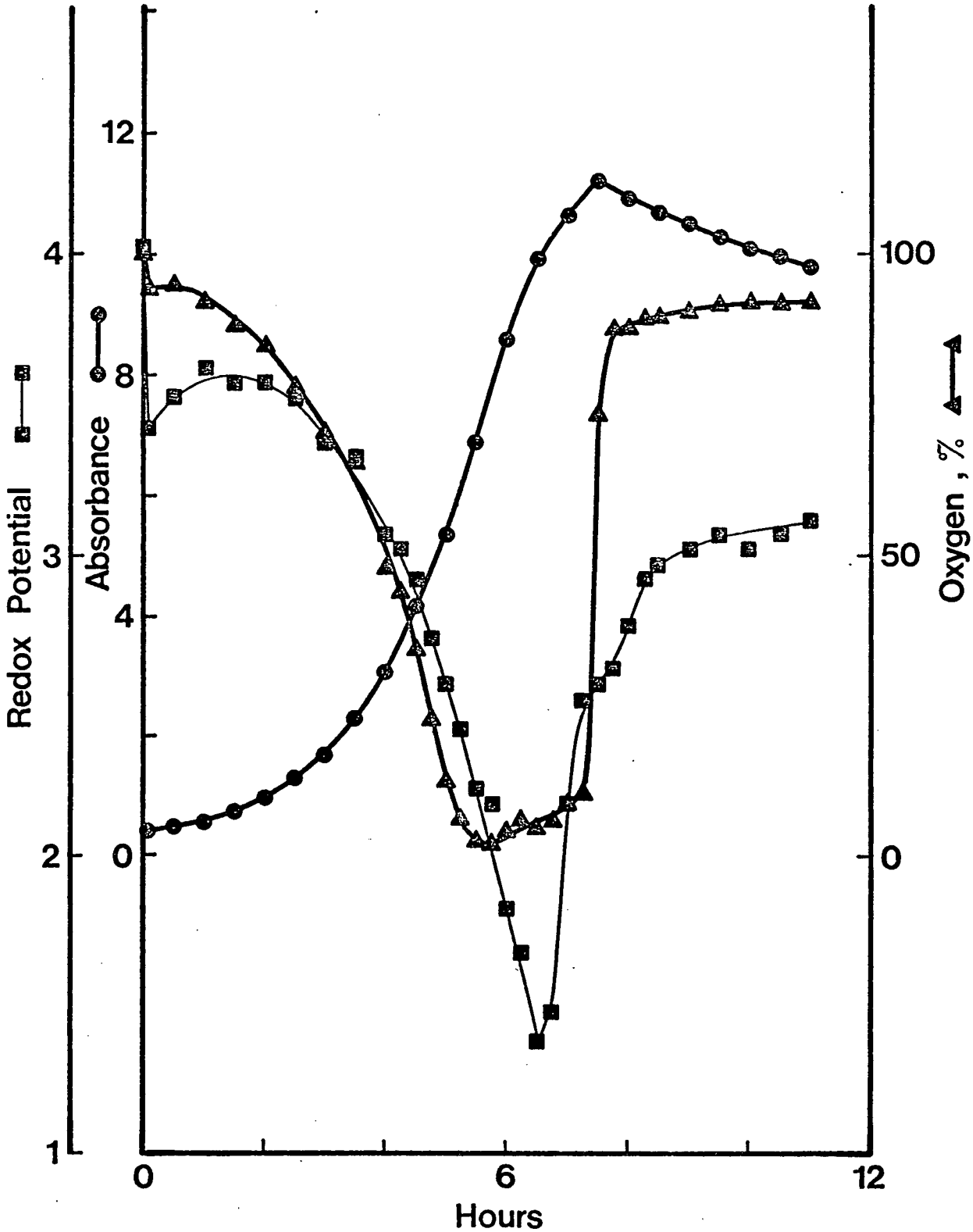


Fig. 3.22 Growth of E. coli on 0.4% glycerol. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; (methods: sec. 2.2.6.1)

With 0.8% DL-lactate as the carbon source, E. coli NRC 482 cultures demonstrated oxygen utilization and growth phases (0 to 7.5 hr, and 7.5 to 10.0 hr) (Figure 3.23) similar to the first two phases of oxygen utilization and the two growth phases of the E. coli culture utilizing glucose as the carbon source (Figure 3.20). The growth, however, showed the same sensitivity to oxygen limitation as was observed in cultures grown on glycerol as carbon source (Figure 3.22). The phases of redox potential and oxygen level show a reasonably close correspondence with the usual continued decrease in the redox potential during conditions of oxygen limitation. However, the second phase of oxygen utilization, consisting of a considerable decrease in the level of oxygen saturation, corresponded to a plateau in the redox potential.

A comparison of Figures 3.20, 3.22 and 3.24 indicates similarities in the redox potential measurements of cultures of E. coli grown with glucose, glycerol or DL-lactate as the carbon source. With each of these carbon sources the most predominant feature of the redox potential curve is the change in redox potential corresponding to the large first phase of oxygen utilization. However, glucose and DL-lactate grown cultures demonstrate distinct second phases of oxygen utilization with corresponding changes in redox potential. The slight shoulder (7.25 to 8.25 hr) during the rise in the redox potential, of a culture grown on glycerol (Figure 3.22), subsequent to the interval of oxygen limitation, possibly corresponds physiologically and biochemically to the more distinct second phases of the cultures grown on glucose (Figure 3.20) or DL-lactate (Figure 3.23).

Cultures of E. coli NRC 482 grown on 0.8% acetate (Figure 3.24) demonstrated a single phase of oxygen utilization and a smooth continuous

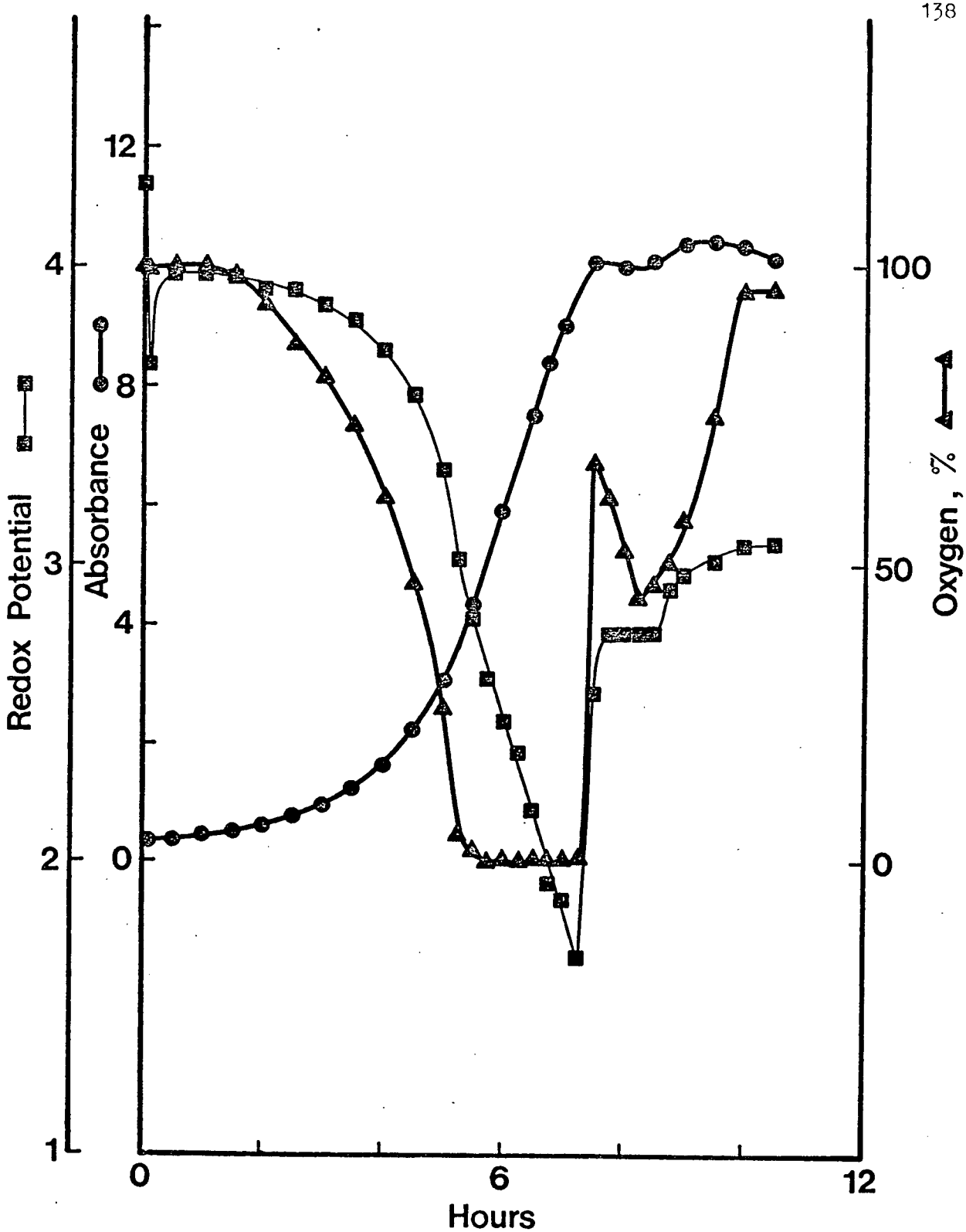


Fig. 3.23 Growth of E. coli on 0.8% DL-lactate. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; (methods: sec. 2.2.6.1).

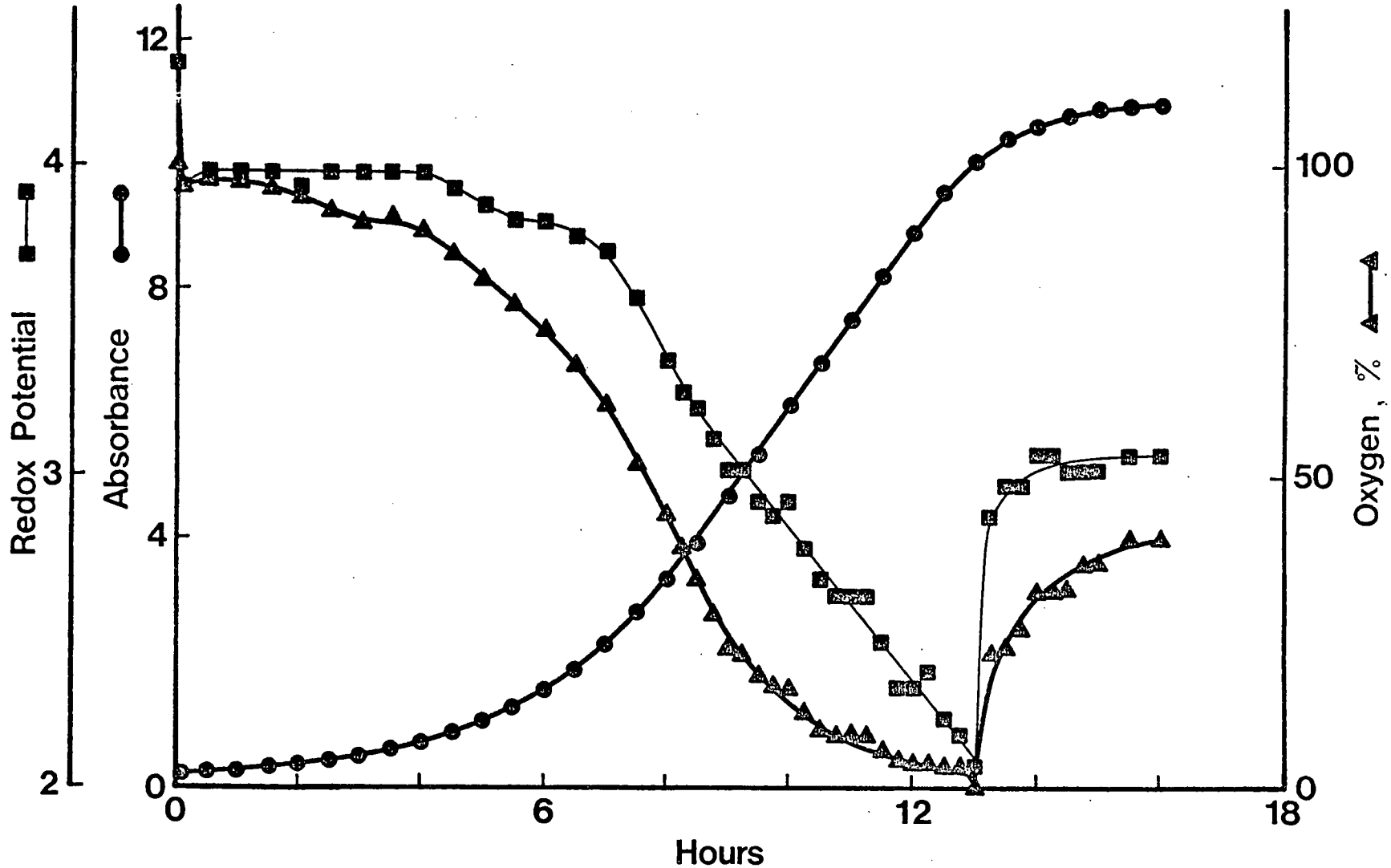


Fig. 3.24 Growth of E. coli on 0.8% acetate. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; (methods: sec. 2.2.6.1).

growth curve with no obvious discontinuities of the type apparent in the growth curve obtained from cultures grown on glucose (Figure 3.20) or DL-lactate (Figure 3.23). A considerable portion of the growth curve indicates a linear growth rate (8.5 to 12.0 hr) although there does not appear to be an obvious correlation with the level of oxygen saturation. The growth rate of E. coli NRC 482 with acetate as the carbon source was the slowest of the growth rate for the carbon sources investigated. The correspondence between the oxygen level and the redox potential was good with respect to general characteristics. The significance of the shoulders on the oxygen level and redox potential curves at 3.0 and 6.0 hr respectively is not known.

Growth of E. coli NRC 482 on 0.6% succinate in a medium supplemented with ferric citrate (6 μM) (Figure 3.25) demonstrated a single phase of oxygen utilization and growth. The oxygen level decreased rapidly and became limiting at approximately 5.5 hr at a level of 7 to 8% of saturation, considerably higher than expected. During the period of oxygen limitation growth was linear. Changes in the redox potential did not coincide completely with changes in the oxygen level and growth. The redox potential of the culture remained constant for the first 3.0 hr of growth by which time the oxygen saturation had decreased to 80%. The redox potential decreased linearly for the greater portion of the period of oxygen limitation but not during the initial 0.5 to 0.75 hr. No secondary phases of oxygen utilization or growth were observed in cultures which were allowed to proceed further into the stationary phase of growth.

Based on the results presented in Figures 3.20 to 3.25 inclusive,

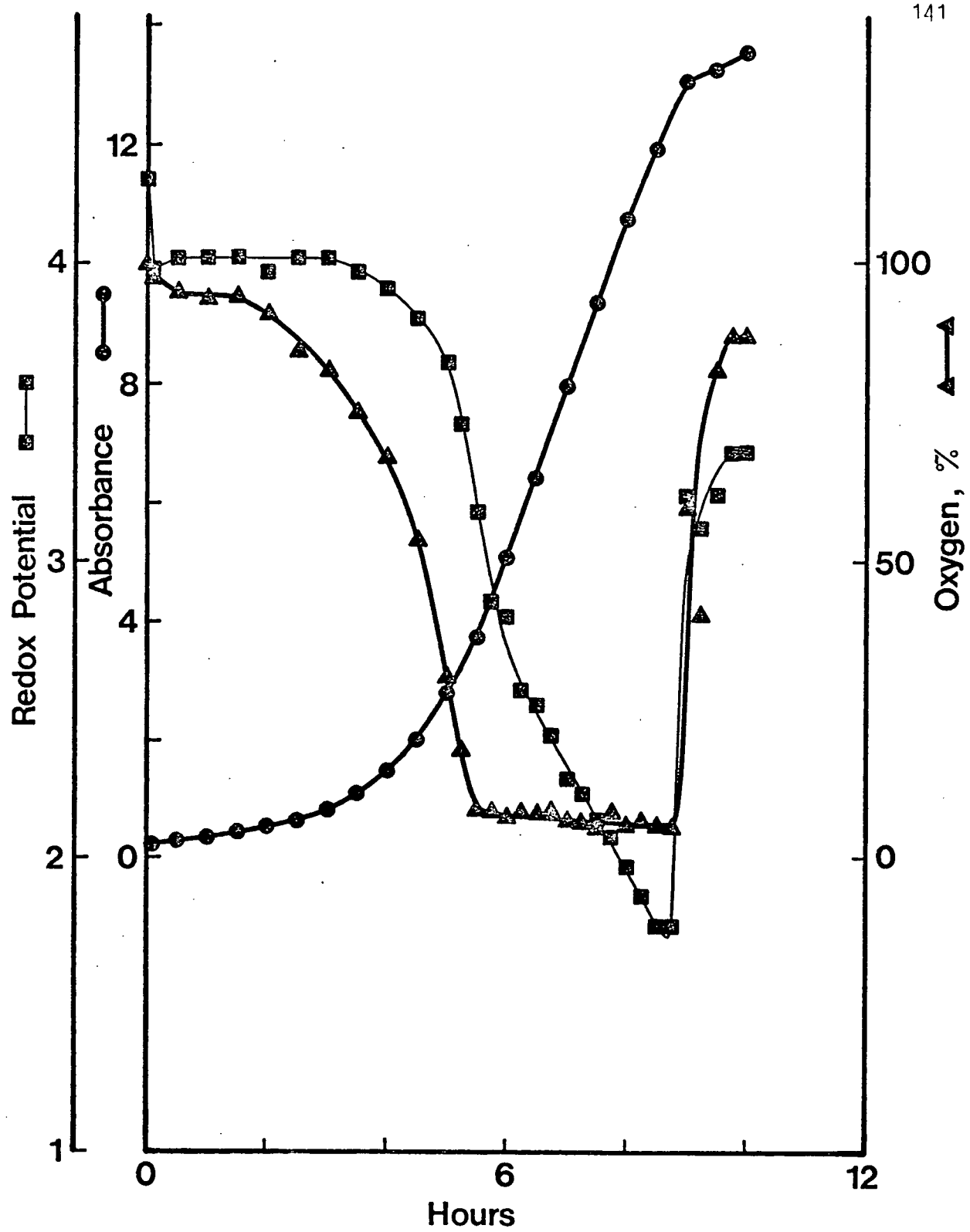


Fig. 3.25 Growth of E. coli on 0.6% succinate in medium containing 6 uM ferric citrate. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; (methods: sec. 2.2.6.1).

the data reported by Hempfling (1970b), the relative sensitivities of glycolysis and respiration to inhibition by silver nitrate (sec.3.1.4), and the requirement that the majority of the energy be obtained from the respiratory chain, succinate was chosen as the most suitable carbon source for the growth of cells for the investigation of the action of $AgNO_3$ as a uncoupler of the respiration of E. coli.

3.1.6 Uncoupling of the respiration of E. coli by added silver nitrate

Figures 3.26 and 3.27 demonstrate the uncoupling action of DBP and $AgNO_3$, respectively, on the respiration of E. coli grown under "iron-sufficient" conditions. The concentration dependence of DBP and $AgNO_3$ as uncouplers of the respiration of E. coli, and the importance of the presence of adequate iron in order to obtain "highly coupled" E. coli is further demonstrated in Figure 3.29 and table 3.8. Growth of E. coli under "iron-sufficient" conditions does not appear to alter the sensitivity of the respiration to uncoupling by $AgNO_3$, only the degree of uncoupling possible. On the other hand, E. coli grown under "iron-sufficient" conditions and "iron-limited" conditions appear to differ in sensitivity to the uncoupler DBP. A comparison of the concentration dependence of the RCR values obtained with the two uncouplers, DBP and $AgNO_3$ (Figure 3.29) indicates that $AgNO_3$ is the more effective uncoupler irrespective of whether the cells were "iron-sufficient" or "iron-limited".

3.2 Discussion

3.2.1 The measurement of oxygen tension with an oxygen electrode

Electrode measurements of oxygen consumption and evolution offer several advantages over manometric precedures. These are:

(i) complete determinations are usually of short duration;

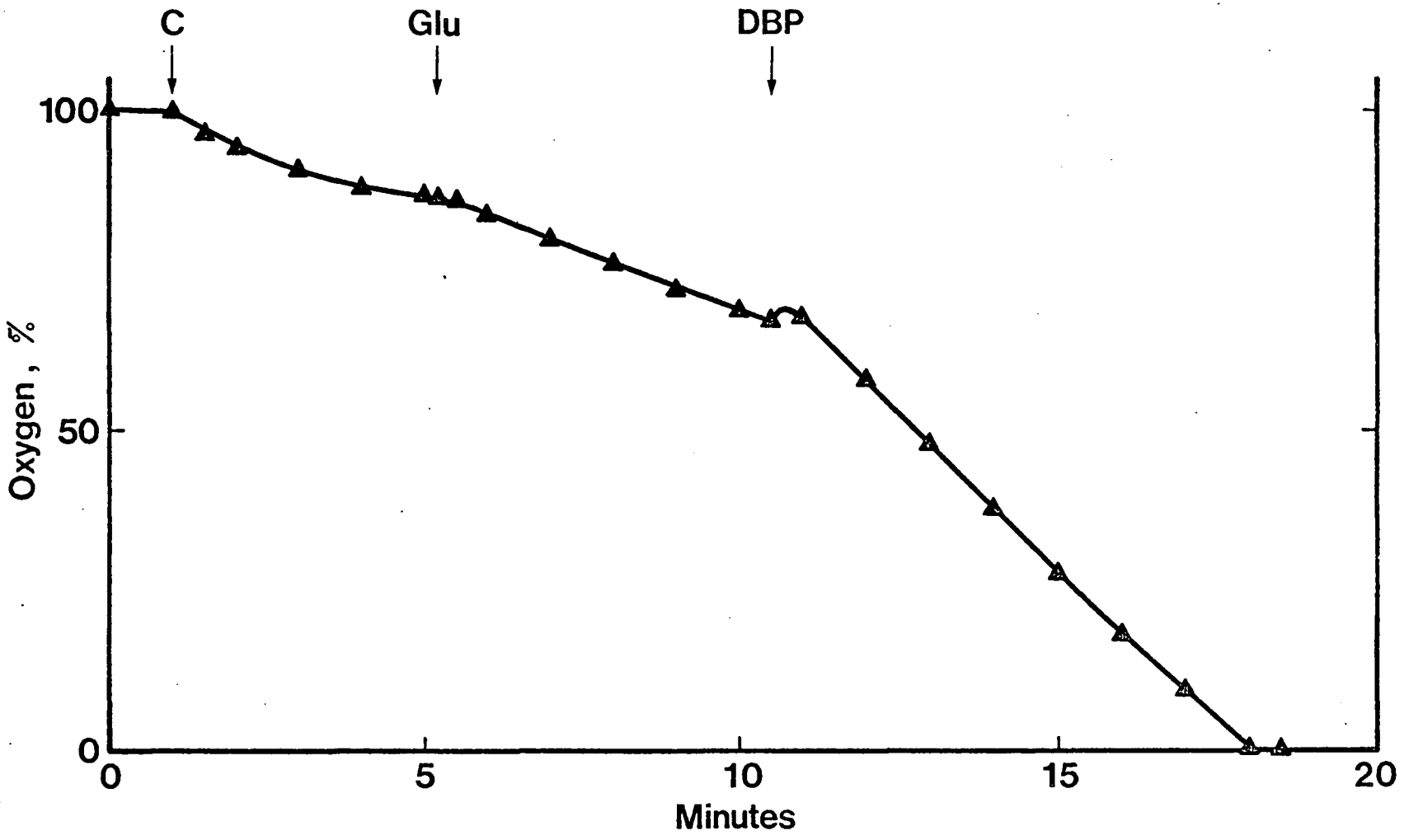


Fig. 3.26 Stimulation of the respiration of E. coli by 2,4-dibromophenol. C: 4 mg cells; Glu: 20 µmoles glucose; DBP: 50 µmoles of DBP; (method: sec. 2.2.6.3); temperature: 22°C.

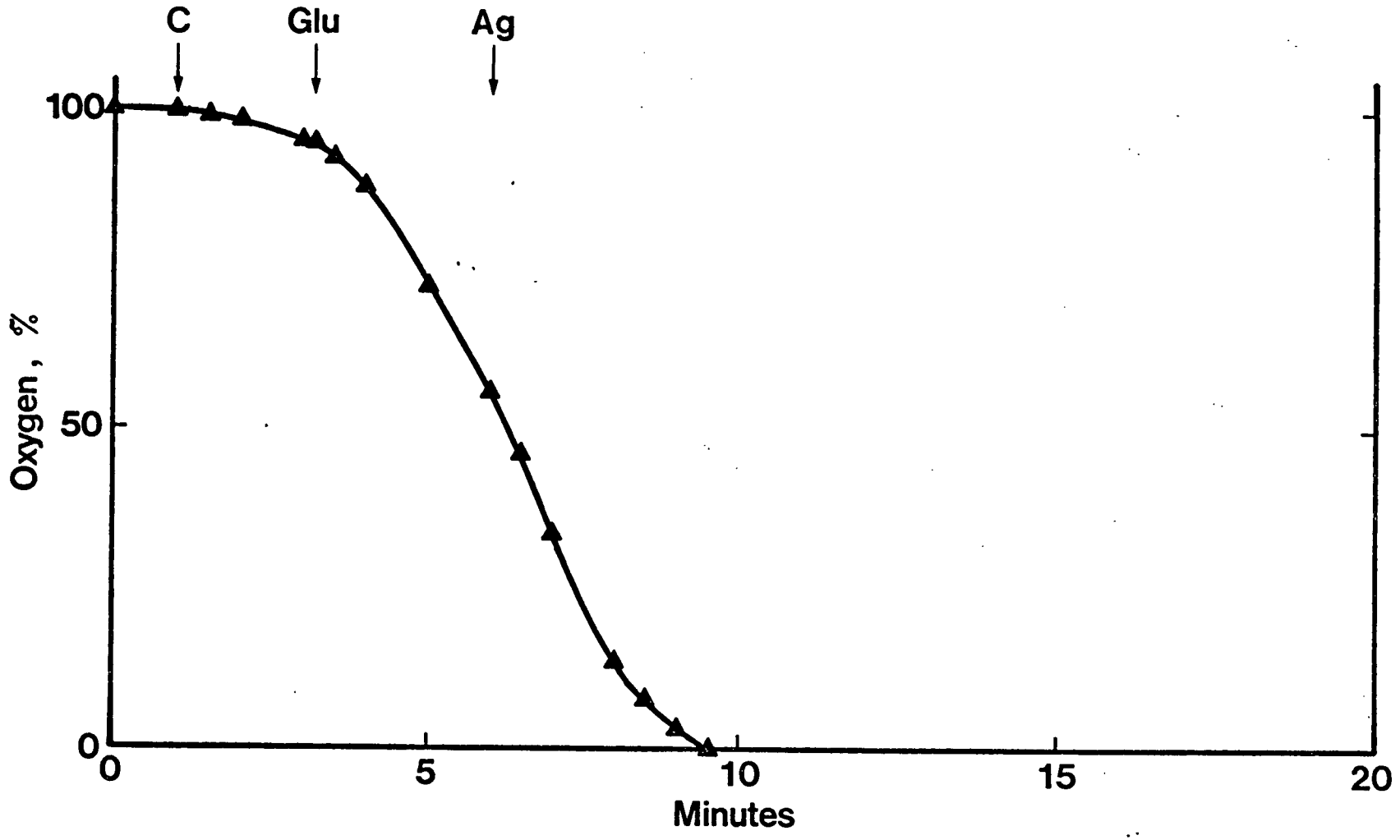


Fig. 3.27 Stimulation of the respiration of E. coli by silver nitrate. C: 20 mg cells; Glu: 20 umoles glucose; Ag: 1 umole $AgNO_3$; (method: sec 2.2.6.3); temperature: 22°C.

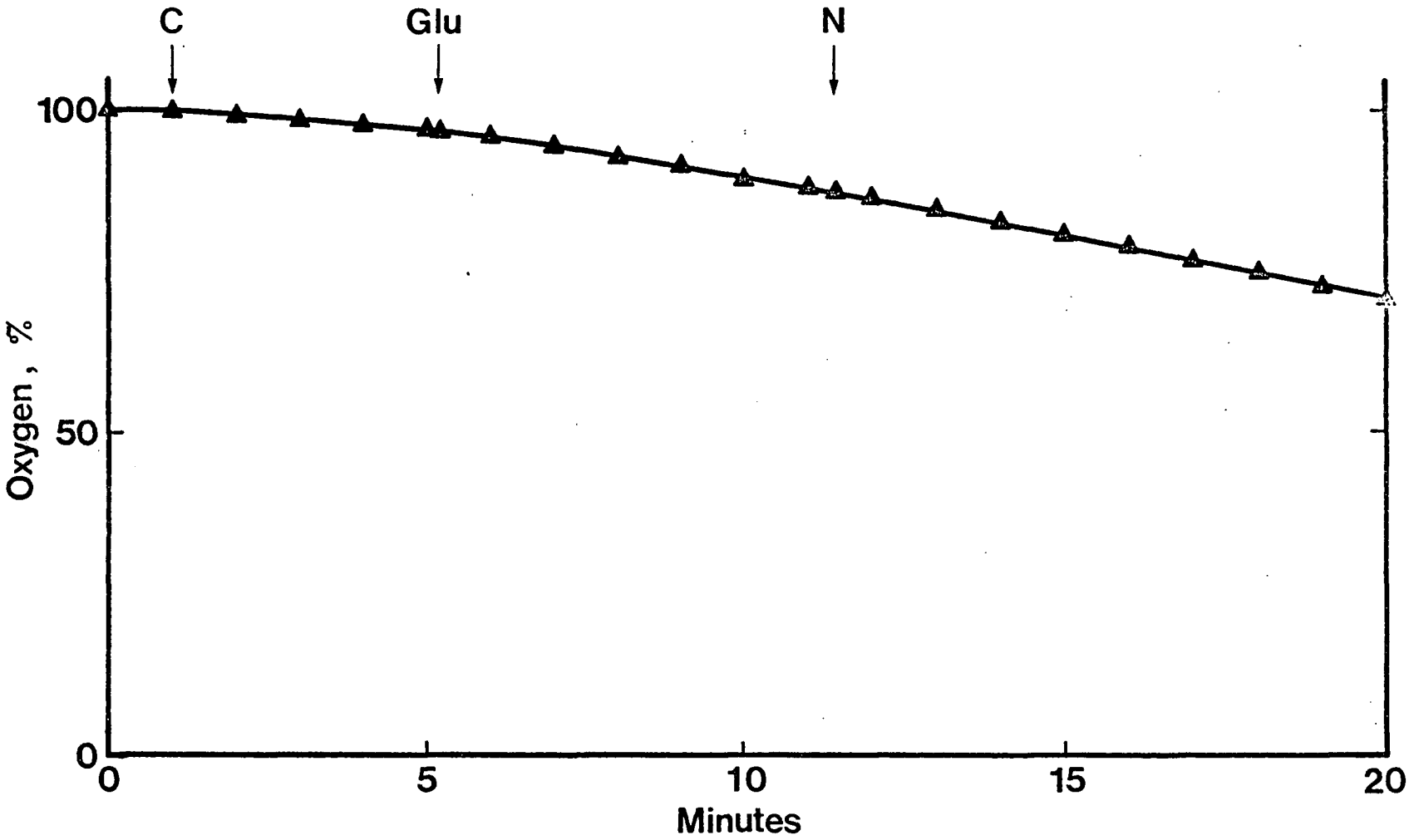


Fig. 3.28 The influence of potassium nitrate on the respiration of E. coli. C: 4 mg cells; Glu: 20 µmoles glucose; N: 200 nmoles of KNO_3; (method: sec. 2.2.6.3); temperature: 22°C.

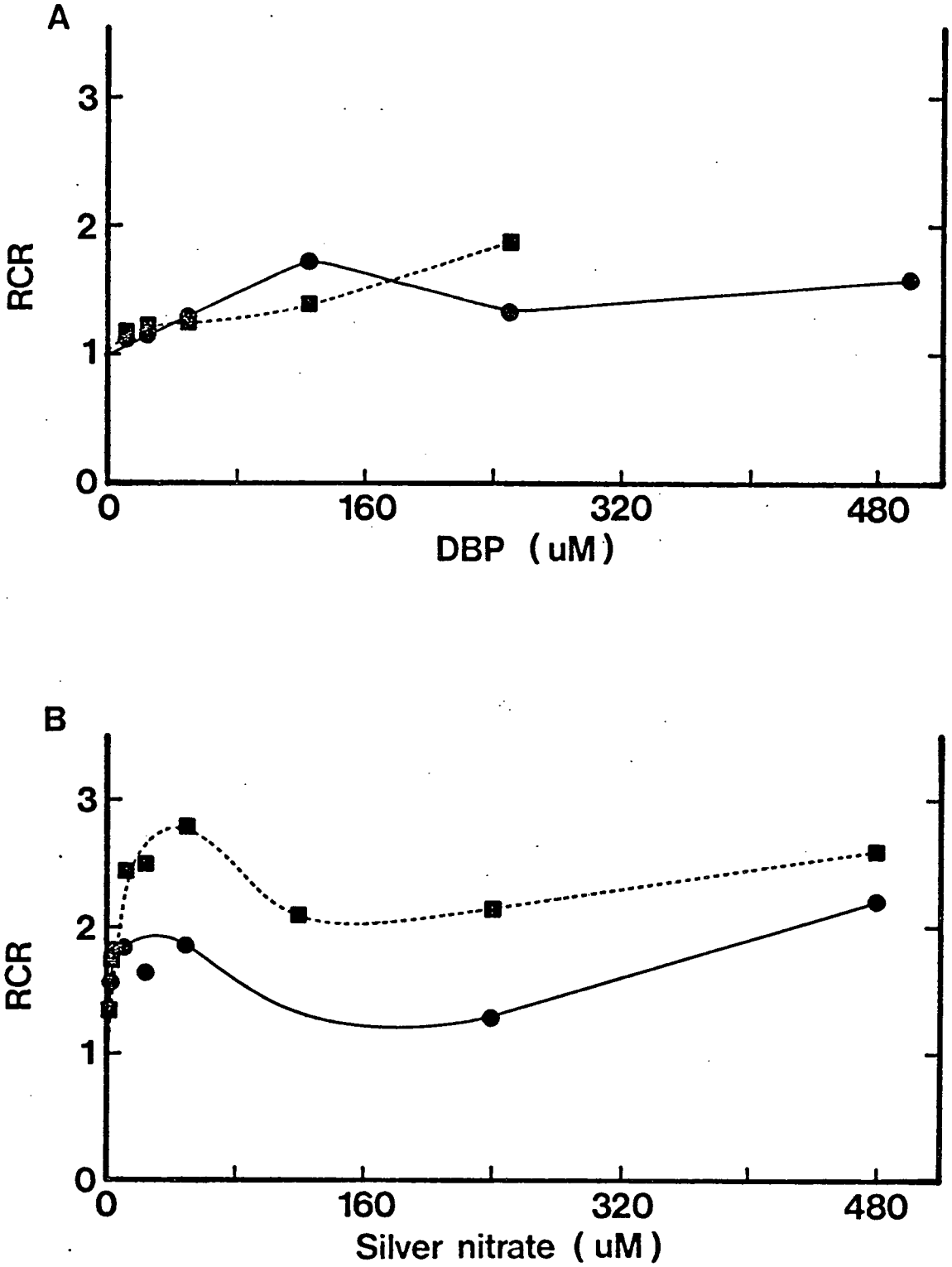


Fig. 3.29 The dependence of the respiratory control ratio (RCR) on the uncoupler concentration. A: DBP; B: $AgNO_3$; ●, iron-deficient; ■, iron-sufficient; (methods: sec.2.2.6.3 and 2.2.6.4).

Table 3.8

The stimulation of the respiration of E. coli by 2,4-dibromophenol and silver nitrate.

Uncoupling Agent (μM)		Rate of Oxygen Consumption[1] Uncoupler Absent	Uncoupler Present	RCR
Iron deficient cells:				
DBP	500	2.44	3.87	1.59
	250	3.21	4.28	1.33
	125	2.82	4.87	1.73
	50	2.96	3.81	1.29
	25	3.09	3.54	1.15
	12	3.30	3.73	1.13
Iron sufficient cells:				
DBP	500	2.94	--	--
	250	3.20	6.01	1.88
	125	3.04	4.22	1.39
	50	3.06	3.87	1.26
	25	3.01	3.66	1.22
	12	3.09	3.63	1.17
Iron deficient cells:				
$AgNO_3$	480	3.23	7.07	2.19
	240	2.76	3.55	1.28
	120	2.01	--	--
	50	2.90	5.37	1.86
	25	3.31	5.44	1.64
	12	3.10	5.70	1.84
	5	3.06	5.51	1.80
	2	3.17	4.90	1.55
Iron sufficient cells:				
$AgNO_3$	480	2.20	5.81	2.64
	240	2.62	5.64	2.15
	120	2.56	5.35	2.09
	50	1.99	5.57	2.80
	25	2.04	5.11	2.50
	12	1.98	4.86	2.45
	5	2.12	3.69	1.74
	2	2.45	3.25	1.33

[1] rate of oxygen consumption expressed as: the decrease in the present oxygen saturation per min per mg cell (wet weight).

values reported are single determinations.

(ii) some economy on the use of expensive substrate reagents is achieved since small reaction volumes are used;

(iii) multiple additions of substrates and/or inhibitors or effectors are readily made within a single experiment of short duration;

(iv) calculation of results and calibration procedures are much less time consuming;

(v) measurements made reflect oxygen exchange by chemical reaction and are not limited by the rate of equilibration of oxygen across a liquid-air interface;

(vi) measurements are made without the removal of carbon dioxide which in itself may be important to the cellular metabolism;

(vii) measurements are generally obtained as continuous recordings of changes in the level of oxygen saturation, permitting accurate determinations of changes in the rate of oxidation, and

(viii) circuits have been designed which permit the recording of the first derivative of the output from an oxygen electrode, a method which is often useful in detecting small changes in the rate of oxidation.

Two of the major disadvantages of oxygen electrode measurements are (i) the time of the reaction in an oxygen electrode chamber is limited by the amount of oxygen which can be dissolved in the incubation medium; and (ii) experiments are performed in a medium of constantly decreasing oxygen tension.

The former generally has not proven to be a disadvantage in investigations with mitochondrial suspensions, although the oxygen limitation may become a difficulty when estimation of changes in inorganic phosphate (P_i) or substrate concentration is necessary for direct calculations of P:O.

However, the number of energy-linked systems of the mitochondrion, a cell organelle which *in vivo* is buffered against major environmental fluctuations by the cytoplasmic membrane, are probably considerably fewer than those of the intact bacterium which must deal directly with all environmental stresses. Consequently, since researchers investigating the respiration of intact bacteria with oxygen electrodes generally utilize cell concentrations which are sufficient to deplete the assay medium of oxygen within a relatively short time ($\leq$ 10 min), in order to avoid errors due to the back diffusion of oxygen, the amount of oxygen available per cell may be insufficient to generate adequate energy to raise the bacterium to a high energy steady-state. This may be one reason why it has not been possible to demonstrate respiratory control in intact bacterial systems.

The fact that the measurement of respiration or oxidation is performed in a medium of constantly decreasing oxygen tension generally has not been considered a problem in the measurement of the respiration of mitochondrial or bacterial suspension where the "K_m" of the cytochrome oxidases for oxygen is usually of the order of 10^{-8} to 10^{-6} M (Longmuir, 1954). However, White (1963) has demonstrated that the sensitivity of the respiration of *Hemophilus parainfluenzae* to decreasing oxygen tension can vary considerably dependent upon the conditions used for growth of the organism. Clark and Sachs (1968) and Pietra and Cappelli (1970) have also reported on the sensitivity of the metabolism of cells to oxygen tension. Metabolic shifts encountered when cells are sensitive to changes in oxygen tension complicate the interpretation of data, and consequently limit the usefulness of the assay to those conditions under which the metabolism is independent of the oxygen concentration.

Both the problem of the limited amount of oxygen available and the problem of the constantly decreasing oxygen tension could be eliminated by the use of an "oxystat" procedure which would maintain the oxygen saturation of the electrode chamber at a predetermined level by the injection of small volumes of an oxygen-saturated solution. During the course of the assay the oxygen saturation of the system and the rate of addition of oxygen-saturated solution would be recorded simultaneously, permitting an accurate determination of the rate of oxygen utilization. Such oxystats have been designed for use with isolated tissues (Clark and Sachs, 1968; Garrison and Ford, 1970) but as yet have not been utilized with cell suspensions or subcellular particles.

No attempt will be made to discuss the operational sources of error of oxygen electrode measurements, such as back diffusion of oxygen (or air), temperature, agitation and electrode aging. The necessary precautions were taken in performing experiments to eliminate or minimize the introduction of errors due to these parameters. For a thorough discussion of these problems, the reader is referred to the following reviews on oxygen electrode measurements: Beechey and Ribbons, 1972; Lessler, 1972; and Lessler and Brierley, 1969.

3.2.2 A proposed mechanism for the release of silver from the anode of the Aminco oxygen electrode

During the presentation of the preceding sections of the thesis no indication has been given of the suspected mechanism by which the silver is released from the oxygen electrode, or of the form of the silver in solution.

If the silver was released electrolytically from the anode of the oxygen electrode: (i) the quantity released should have been independent of

the type of buffer (Figure 3.8), and the buffer concentration (Table 3.3) provided that the conductivity of the solutions were of the same order of magnitude; (ii) silver release into phosphate buffer should have been maximal due to the continuous removal of silver from solution as the insoluble silver phosphate (Figure 3.8 and Table 3.3); and (iii) the quantity of silver released should have been dependent upon the presence of the polarizing voltage (Table 3.2). None of these characteristics applied to the release of silver from the Aminco oxygen electrode.

The concentration dependence of the silver release from the anode of the oxygen electrode (Table 3.3) and the stability of the silver complex of Tris ($K_{assoc.} = 2.75 \times 10^{-7}$) (Benesch and Benesch, 1955), suggested that silver was probably chelated from the anode and existed in solution as a silver ion chelate of the buffer ion. This would explain the low quantity of silver released into the phosphate buffer. Although it is hard to reconcile the formation of a silver ion chelate of HEPES with the reported negligible metal binding constants of this buffer (Good et al., 1966), the data are consistent with the removal of silver from the anode as silver ion chelates.

As indicated previously (sec.3.1.2) one of the reasons for investigating the factors influencing the release of silver from the oxygen electrode was the apparent failure of previous researchers to be confronted with this problem. An examination of the data on the dependence of silver release on the type of buffer (Figure 3.8), and on buffer concentration (Table 3.3), indicates that the probable reason that the inhibition of respiration by silver released from the naked silver anode of an oxygen electrode had not been reported previously is that the conditions used by most researchers, 50 to 100 mM Tris buffers, would have resulted in a much lower level of

silver release than was obtained with 300 mM glycylglycine-KOH buffer. Unfortunately, 300 mM glycylglycine-KOH buffer, pH 7.0, which of the four buffer systems examined resulted in the maximal release of silver, was the buffer system that was chosen for the initial investigation of the respiration of E. coli using the Aminco oxygen electrode. Consequently a large amount of experimental data was unuseable in this thesis.

3.2.3 The influence of pH, buffer ion and buffer concentration on the respiration of E. coli

Although the volume of the bacterial cell and the bacterial protoplast, and the respiration rates of intact bacterial cells or spheroplasts have been demonstrated to be sensitive to the osmotic pressure and the pH of the suspending medium (Henneman and Umbreit, 1964a,b; Packer and Perry, 1961; Smith, 1962; Knowles and Smith, 1971a,b; Knowles, 1971) researchers primarily interested in the characteristics of the respiration of bacteria generally have not considered an investigation of the influence of the buffer ion, pH and concentration on the respiration to be important. However, the intact bacterium must deal directly with all environmental stresses, natural or experimental, to which it is exposed, and possesses the mechanism to do so. At present our knowledge is insufficient to even speculate as to what proportion of these mechanisms are directly or indirectly linked to energy production via the electron transport chain. Thus, the more closely the "optimum" conditions are approximated the less the likelihood that respiration will be influenced by unknown energy demands on the respiratory chain.

For this reason, an investigation of the influence of buffer ion, pH and concentration on the respiration of E. coli was performed. The major problem of such a project is the specification of the criteria one should employ to select an "optimum" buffer-pH combination. The criteria used in

the research reported in this thesis were the criteria of enzyme kinetics: (i) a high rate of oxygen consumption; (ii) a linear rate of oxygen utilization; and (iii) maintenance of these characteristics of the E. coli cell suspension over a greater period of time than required to complete an experiment.

During the initial investigation of the influence of buffer ion and pH on the respiration of E. coli, isotonic buffers were used to avoid the possibility of transport-linked energy demands or distortion of the cytoplasmic membrane resulting from differences in the osmotic pressure of the cell and the suspending medium. Data obtained subsequently (Figure 3.11 and Table 3.4) confirmed this concentration as "optimal" under the criteria used.

It is apparent from Figure 3.9 that high rates of glucose-dependent respiration can be obtained with several buffer ions, over a wide pH range. Due to the complexity of the system it is impossible to rationalize the rates of respiration obtained with the different buffer ions, or at the different pH values. Of the buffers investigated only phosphate and glycylglycine buffers fulfilled the second criterion of supporting a linear rate of oxygen utilization (Figure 3.10). Cells suspended in any of the remaining buffer systems demonstrated decreasing respiration rates with decreasing oxygen saturation (Figure 3.10). This has previously been attributed to the possession of a higher "K_m" for oxygen by the cells suspended in these buffers (Longmuir, 1954; White, 1963). However, since the cells utilized in these investigations were grown under identical conditions, this would require that the buffer ions were influencing the "K_m" of the cytochrome oxidase for oxygen. Although this would be a possibility in

cell extracts, it would appear unlikely to occur in the intact bacterial cell due to the impermeability of the latter to charged compounds.

An alternative explanation for the two classes of oxygen consumption traces obtained with the different buffers was suggested by the results obtained by Oishi and Aida (1970), during investigation of the control of bacterial respiration. These authors observed that the respiration of phosphate-starved bacteria was stimulated by the addition of inorganic phosphate and that this stimulated rate of respiration was maintained until either the inorganic phosphate was depleted from the medium or the level of the phosphate-pool of the cell was replenished. On the basis of the data presented the authors were unable to eliminate the possibility that the stimulation of respiration resulted from an energy demand for the active transport of phosphate rather than via the classical respiratory control of ADP, P_i and ATP. Thus, perhaps the linear rates of respiration obtained with cells suspended in phosphate or glycylglycine buffers were due to the stimulation of respiration as a result of the imposition of an energy demand on the cell, while the progressively decreasing respiration rates obtained with cells suspended in other buffer systems might indicate the approach of the cell to a high energy steady-state.

Irrespective of the cause(s) of the oxygen saturation independent, and the progressively decreasing respiration rates, the problem of minimizing unknown energy demands on the respiratory chain is one that must be dealt with before the characteristics of respiration, with associated respiratory control, can be investigated in the intact bacterial cell.

3.2.4 The inhibition of the respiration of E. coli by silver ions

One of the primary objectives of the research reported in this thesis

was to investigate the site(s) at which silver ions influenced the respiration of E. coli. As indicated in the introduction the aerobic respiration of an intact organism, as measured by oxygen consumption, reflects the function and kinetic characteristics of all pathways and systems which provide reducing equivalents and oxygen to the terminal oxidase(s) of the electron transport chain. Consequently, the inhibition of the respiration of E. coli could occur via:

(i) inhibition of transport of the substrate;

(ii) inhibition of one or more enzymes of the amphibolic pathways (Figure 1.5);

(iii) inhibition of respiratory chain enzymes; and/or

(iv) inhibition of energy coupling (i.e. the inhibition of the utilization of energy generated by the respiratory chain).

Irrespective of the number of sites of inhibition by silver in the systems indicated above, the demonstrated characteristics of the inhibition of respiration will be those of the site most sensitive to silver inhibition and which cannot be by-passed.

On examining the structure of the gram-negative cell (Costerton, 1970) it is possible to subdivide the interaction of silver ions with the cell into three stages: (i) at the cell wall; (ii) at the cytoplasmic membrane; and (iii) in the cytoplasm or cytosol. The cell wall is believed to function primarily as a molecular sieve, preventing the entrance of large molecules, and on this basis would be expected to have little if any effect on silver ions. However, Ca^{++}, Mg^{++}, K^{+} and phosphate are concentrated to high levels in the cell walls of gram-negative bacteria (Eagon, 1969). Consequently a large proportion of the silver ions interacting with an

E. coli cell might be precipitated or bound in the cell wall as silver phosphate. Under these circumstances the effective concentration that reaches the cytoplasmic membrane would be drastically reduced. However, due to the relatively nonspecific reactivity of silver with available sulfhydryl groups and possibly also histidine (sec.1.5), those enzyme systems in the cytoplasmic membrane should be among the most vulnerable.

The results obtained on the influence of silver ions on the respiration of E. coli can be roughly classified into four groups according to the apparent sensitivity to inhibition: (i) endogenous and acetate-dependent respiration (Figures 3.12 and 3.14, respectively); (ii) glucose- and glycerol-dependent respiration (Figures 3.13A, 3.15B, 3.16A, 3.18B, and 3.15A, respectively); (iii) D-lactate-, L-lactate-, fumarate- and succinate-dependent respiration (Figures 3.16A, 3.17A, 3.18A and 3.17B, respectively); (iv) formate-dependent respiration (Figure 3.13B).

Considering first of all the possibility that the transport of substrates (glucose, glycerol, D-lactate, L-lactate, fumarate, succinate and acetate) into E. coli is the site of silver inhibition of respiration, insufficient data are available to draw a conclusion as to whether this was the mechanism involved. With respect to this problem, however, Rayman et al., (1972a,b) have reported that the ascorbate-PMS-dependent, and the D-lactate-dependent active accumulation of succinate by membrane vesicles of E. coli were inhibited by silver. They did not indicate whether silver also inhibited the oxidation of D-lactate and ascorbate-PMS. Although their results indicated that D-lactate oxidation by the membrane vesicles was inhibited by the thiol reagents NEM and PCMB, ascorbate-PMS oxidation was not inhibited by NEM and was only slightly inhibited by PCMB. Succinate trans-

port, by the membrane vesicles, driven by either system was not inhibited by NEM (Rayman et al., 1972b), while D-lactate-dependent succinate uptake and D-lactate oxidation by intact E. coli K12 was inhibited by NEM (Lo et al., 1972b). The results obtained with respect to NEM inhibition of D-lactate oxidase but lack of NEM inhibition of the accumulation of succinate are difficult to comprehend and are in disagreement with the characteristics of D-lactate coupled transport systems as described by Kaback (1972). These discrepancies render it impossible to deduce whether the inhibition of succinate transport by silver was due to: (i) inhibition of the transport system; (ii) inhibition of the function of the respiratory chain; or (iii) the inhibition or uncoupling of the mechanism of energy coupling. The data presented in Figure 3.16A demonstrate that oxidation of D-lactate by intact E. coli was rapidly inhibited by silver nitrate at a concentration of 86 μM. This is comparable to the concentration (100 μM) reported by Rayman et al., (1972b) to cause 100% inhibition of succinate transport by membrane vesicles. This suggests that the observed inhibition of succinate transport may have occurred via the inhibition of D-lactate oxidation. Kinetic studies of the inhibition of succinate transport and D-lactate oxidation by silver ions are required to refute or verify this argument.

The action of silver ions as an uncoupling agent (sec.3.1.6; Figure 3.27; Table 3.8) could also inhibit the accumulation of succinate since this is presumably driven by a high energy intermediate generated by the respiration chain (Hong and Kaback, 1972; Simoni and Shallenberger, 1972; Kashket and Wilson, 1972; Bragg and Hou, 1973). The preceding discussion of the silver ion inhibition of succinate transport is equally applicable to fumarate transport (Kay and Kornberg, 1971; Lo et al., 1972b; Rayman et al., 1972b).

No experimental information is available on inhibition of glucose transport by silver ions. However, enzyme I of the PEP phosphotransferase system (sec.1.1) is sensitive to inhibition by the thiol reagents NEM, PCMB and dithionitrobenzene (DTNB) (Kundig and Roseman, 1971a). The inhibition by PCMB and DTNB can be reversed by reduced glutathione, cysteine and β-mercaptoethanol. Anraku (1968) demonstrated the inhibition of glucose uptake into E. coli by Zn^{++}, a heavy metal ion also believed to react with thiols (Kashara and Anraku, 1972), and similar results were reported by Eagon and Asbell (1969) with Pseudomonas aerugenosa. These results suggest that glucose transport is probably sensitive to inhibition by silver ions.

There is no information available on the inhibition by Ag^{+} of transport of glycerol, D-lactate, L-lactate and acetate into E. coli. These transport systems have been insufficiently characterized to permit speculations as to their sensitivity to thiol reagents.

It is questionable to attempt to deduce the site of action of silver ions from the action of other thiol-reacting agents. Brierley and co-workers investigated the influence of mercurical reagents and heavy metal ions on the permeability and ATPase activity of bovine heart mitochondria (Brierley et al., 1967, 1971; Scott et al., 1970, 1971). They have found that the extent of reaction of these reagents with the mitochondrial membrane depends on the polarity of the reagent, the anionic composition of the suspending medium, the pH, and to some degree on the metabolic status of the mitochondrion.

Thus, at present silver ions are only known to inhibit the accumulation of succinate and fumarate. Since the oxidation of formate (Figure 3.13B), a compound to which E. coli is freely permeable (Bovell et al.,

1963), was much less sensitive to inhibition by silver ions than respiration dependent on glucose, glycerol, D- or L-lactate, succinate, fumarate or acetate, the conclusion could be made that transport of the other substrates into E. coli was inhibited by silver and that this was the site at which respiration was inhibited. However, the fact that the endogenous respiration of cells grown on glucose (Figure 3.12) or succinate demonstrated stimulated and inhibited phases following the addition of $AgNO_3$, and a sensitivity to inhibition by Ag^+ equal to or greater than exogenous substrate-dependent respiration, suggests that it is unlikely that the observed silver ion inhibiton of respiration with exogenous substrates was due to the inhibition of transport of the substrate into the cell.

This suggested that silver inhibition at a site in the amphibolic pathways or the respiratory chain was probably responsible for the observed inhibition of respiration. In the subsequent discussion of the possible inhibition of the amphibolic pathways and the respiratory chain, the literature pertinent to the results will be dealt with concurrently with the discussion of the results.

Prior to this discussion a comment on the technique of successive additions of cells subsequent to the inhibition of respiration is in order. This unusual procedure was used initially in an attempt to titrate the number of silver ions present in solution to determine the approximate number of silver ions per cell required to completely inhibit the respiration. Yudkin (1937) had indicated that a single E. coli cell was capable of binding in the order of 5×10^8 silver ions, while approximately 1.3×10^7 and 1.7×10^7 silver ions per cell were required to bring about 50% inhibition of glucose and succinate diaphorase activities respectively. Although the desired values

could have been more accurately determined from the concentration dependence of the Ag^+ inhibition of respiration, or by reducing the amount of $AgNO_3$ originally added to the assay system, it was found that the pattern of response of oxygen consumption in the presence of silver ions to successive additions of E. coli provided a good indication of the sensitivity of substrate-dependent or endogenous respiration to inhibition by Ag^+. A lower concentration of $AgNO_3$ would have been insufficient to completely inhibit the D-lactate-, succinate-, and fumarate-dependent respirations (Figures 3.16A, 3.17A, 3.17B and 3.18A respectively).

The similarity of the response patterns obtained with the silver inhibition of endogenous respiration (Figure 3.12), and of the acetate-dependent respiration (Figure 3.14) of E. coli was unexpected. However, as the E. coli cells were grown on glucose as the carbon source and harvested during the late exponential phase of growth, the acetate transport system, and enzymes of the TCA and glyoxylate cycles would be repressed (sec.1.1 and 1.2 and Table 1.1). Consequently, the respiration, even with acetate present, would probably be largely endogenous and quite different from the response of cells which had been grown on acetate as carbon source. Figure 3.14 clearly demonstrates the influence of the titration (removal) of the silver ions on the rate and extent of oxygen utilization of each succeeding addition of E. coli. Due to the unknown nature of the endogenous substrates it is impossible to propose a specific site(s) of silver inhibition in the metabolism prior to the introduction of reducing equivalents into the respiratory chain.

Subsequent experiments indicated that the addition of $AgNO_3$ (24 μM) to cells metabolizing glucose immediately inhibited the decrease in pH while respiration continued at less than 10% of the rate prior to the addition of

silver nitrate (Figure 3.19). There are three possible explanations for these results.

The first possibility is that glycolysis was inhibited at a site between glucose and pyruvate. At a silver nitrate concentration of 24 μM, when acid production ceased, respiration continued due to the oxidation of accumulated acids (lactate, acetate and formate). This seems unlikely since the increase in pH subsequent to inhibition by Ag^+ does not appear adequate to account for the continued respiration on the basis of the oxidation of accumulated acids.

The second alternative is that the cessation in the decrease in pH does not represent a complete inhibition of acid production at a site between glucose and pyruvate, but that acid production was partially inhibited such that the rate of acid production was balanced by its rate of oxidation. The results (Figure 3.19) interpreted in terms of this proposal are consistent with glycolysis being more sensitive to inhibition by silver ions than the TCA cycle and/or the respiratory chain.

The third postulated mechanism also involves the inhibition of glycolysis between glucose and pyruvate. However, at a silver nitrate concentration of 24 μM the inhibition at this site was approximately 90%. Pyruvate production continued at 10% of the rate prior to the addition of the $AgNO_3$, accounting for the 10% respiration remaining. The complete inhibition of acid production could be accounted for by postulating the complete inhibition of a reaction between pyruvate and the excreted acid(s). The reactions involved could be the soluble D-lactate dehydrogenase catalyzed conversion of pyruvate to D-lactate (reaction 38, Figure 1.5) and/or the phosphorclastic reaction (reaction 33, Figure 1.5).

There is insufficient data available to make a choice between the second and third proposals. Neither of these proposals exclude the possibility of their being less sensitive sites of Ag^+ inhibition in the TCA cycle. Isocitrate dehydrogenase (Kratochvil et al., 1967) and fumarate (Laki, 1942) from mammalian sources have been shown to be inhibited by Ag^+ while aconitase (Krebs and Eggleston, 1944), α-oxoglutarate dehydrogenase (Baron and Singer, 1945; Gonda et al., 1957), and succinate dehydrogenase (Kim and Bragg, 1971a) have been shown to be inhibited by thiol-reacting reagents.

The similarity of the glucose-dependent and glycerol-dependent patterns of oxygen consumption (Figure 3.15) led to the conclusion that the glycolytic enzyme which possessed the high sensitivity to silver inhibition must occur between glyceraldehyde-3-phosphate and pyruvate as this portion of glycolysis is common to both substrates (Figure 1.5).

Based on this assumption the inhibition of glyceraldehyde-3-phosphate dehydrogenase by Ag^+ was examined (Table 3.6) as it is an enzyme in the desired portion of the Embden-Meyerhof pathway which is known to possess a thiol reagent-sensitive site. The glyceraldehyde-3-phosphate dehydrogenase from rabbit and porcine skeletal muscle have been shown to be inhibited by Ag^+ (Park et al., 1961; Boross, 1965; Boross and Keleti, 1965). A concentration of Ag^+ of 10 μM resulted in a 94% inhibition of the rabbit skeletal muscle enzyme which is comparable to the observed inhibition of glycolysis at a $AgNO_3$ concentration of 24 μM. Aldolase from rabbit skeletal muscle also is completely inhibited by a Ag^+ concentration of 20 μM. The results (Table 3.6) indicated that E. coli glyceraldehyde-3-phosphate dehydrogenase was inhibited by Ag^+. The observed variation in the activity of the enzyme at a final

assay concentration of 1 μM is probably due to the nonspecific binding and release of Ag^+ by other protein components of the 95,000 x g supernate and/or the precipitation of Ag^+ as silver arsenate. A higher concentration of $AgNO_3$ (10 μM) produced a progressive decrease in the enzyme activity. The observed increase in enzyme activity as a result of incubation of the inhibited enzyme with 0.1 M reduced glutathione may be due to the reversal of Ag^+ inhibition of the enzyme or to an activation of previously inactive but uninhibited enzyme. The presence of 4.9 mM cysteine in the assay medium suggests that the former explanation is more probable than the latter. Due to the problems of Ag^+ precipitation as Ag_3AsO_4, and the nonspecific binding of Ag^+ to proteins present in the supernate, it is impossible to evaluate silver ion concentration.

The specific site(s) of silver inhibition of glucose-dependent and glycerol-dependent respiration could be obtained by determining the levels of glycolytic and TCA cycle intermediates in the absence and presence of silver nitrate at concentrations which are insufficient to inhibit the respiratory chain but sufficient to inhibit glucose-dependent or glycerol-dependent respiration.

The similarity of the results obtained for silver inhibition of respiration with D-lactate, L-lactate, succinate and fumarate as substrates (Figures 3.16A, 3.17A, 3.17B and 3.18A respectively) suggests either that: the respective sites of silver inhibition have the same degree of sensitivity to Ag^+ or that there is a single site of silver inhibition common to the respiration of these four substrates. Of these substrates, the first three undergo an initial dehydrogenation *via* membrane bound dehydrogenases which introduce reducing equivalents directly into the respiratory chain (Figure

1.5 and Table 1.1). Fumarate is only one reaction removed from the introduction of reducing equivalents into the electron transport system *via* the membrane bound malate oxidate system, or two reactions removed *via* the soluble NAD^+-dependent malate dehydrogenase and the membrane bound NADH dehydrogenase of the respiratory chain. Consequently there are no, or at most two reactions prior to the entry of reducing equivalents from the substrate into the respiratory chain. Therefore, in all probability the site at which silver ions inhibit *E. coli* respiration on these substrates is in the electron transport chain itself.

Kasahara and Anraku (1972) have investigated the inhibition of the respiratory chain, in an *E. coli* membrane fraction, by zinc ions. They observed that the presence of Zn^{++} (1 mM) decreased the succinate-dependent aerobic steady-state reduction level of cytochrome b_1, and that Zn^{++} at a concentration of 100 μM inhibited succinate dehydrogenase completely and D-lactate dehydrogenase by 69%. However, Barnes and Kaback (1971) reported that neither the D-lactate-DCIP reductase of *E. coli* membrane vesicles nor the partially purified D-lactate dehydrogenase was sensitive to the thiol reagents NEM or PCMB. In agreement with the results of Kasahara and Anraku (1972), Bennett *et al.*, (1966) have reported that D-lactate-DCIP reductase of *E. coli* respiratory particles was inhibited by PCMB. L-lactate-DCIP reductase was only slightly inhibited. Barnes and Kaback (1971) also reported that NADH oxidation by membrane vesicles of *E. coli* was insensitive to inhibition by NEM and that the slight inhibition observed by PCMB was not reversed by dithiothreitol. However, Bragg and Hou (1967a) have demonstrated the inhibition of NADH oxidase by PCMB, and Kim and Bragg (1971a) found a 33% inhibition of *E. coli* succinate dehydrogenase by PCMB (0.5 mM) while at the

same concentration succinate oxidase activity was completely inhibited. Although it is impossible to conclude, from this conflicting information, whether the respiratory chain-linked dehydrogenases of E. coli are all sensitive to inhibition by thiol reagents it would appear safe to assume that they will not all have the same degree of sensitivity to silver inhibition. Reactive thiols have been demonstrated in the respiratory chain-linked dehydrogenases of mitochondria (Slater, 1949; Bernath and Singer, 1962; Singer and Gutman, 1971).

If one considers Ag^+ inhibition at a respiratory chain component present in each of the branches of the electron transport system prior to the convergence of these branches at cytochrome b_1 (Figure 1.6B or 1.6C) the same requirement of equal sensitivity to silver ion inhibition arises. Consequently, it seems more reasonable to propose that the site of silver inhibition of D-lactate-, L-lactate-, succinate- and fumarate-dependent respirations of E. coli is located in the respiratory chain between cytochrome b_1 and oxygen.

The site(s) at which Ag^+ inhibits the D-lactate-, L-lactate-, succinate- and fumarate-dependent respiration could be determined with a greater degree of certainty by determining the influence of increasing Ag^+ concentration on the level of the steady-state reduction of the respiratory chain components with each of these substrates. These determinations would be more readily performed using E. coli membrane vesicles or respiratory particles, in which case it would not be possible to examine the silver ion inhibition of the fumarate-dependent respiration.

The failure of formate-dependent respiration to be completely inhibited by a $AgNO_3$ concentration of 86 μM (Figure 3.13B) suggests that the

entire formate oxidase system was less sensitive to Ag^+ inhibition than the oxidation of any of the other substrates examined. This requires that the formate dehydrogenase be resistant to Ag^+ inhibition at the concentration employed and that the electron transport system of the formate oxidase must be distinct from the respiratory chain of the D-lactate, L-lactate, and succinate oxidases, at least in the region of silver ion inhibition of the latter oxidases.

No information is available on the sensitivity of the E. coli formate dehydrogenases to inhibition by silver ions. However, Yudkin (1937) reported that the formate diaphorase activity of E. coli was an order of magnitude less sensitive to inhibition by silver than the corresponding glucose and succinate diaphorase activities. This is in agreement with the results reported in this thesis.

With respect to the possibility that the electron transport system of the formate oxidase is distinct from the respiratory chain of the D-lactate, L-lactate and succinate oxidases, investigations by Birdsell and Cota-Robles (1970) have led them to propose a model of the respiratory chain of E. coli in which the formate oxidase system is distinct from the succinate and NADH oxidase systems except for the terminal oxidases, cytochromes a_2 and o.

The site at which formate oxidation is inhibited by Ag^+ could be determined by investigating the influence of higher concentrations of Ag^+ on the formate-dependent respiration and on the steady-state level of reduction of the respiratory chain components. To increase the sensitivity of the latter measurements it might be necessary to grow the cells in the presence of formate to induce higher levels of the formate oxidase system.

The author has described means by which the silver ion inhibition of

the various substrate dependent respirations could have been determined with a greater degree of accuracy. The reason that such experiments were not performed was that our primary interest in the influence of Ag^+ on the respiration of E. coli was its apparent uncoupling action.

Although silver ions have been shown to inhibit the regulation of yeast hexokinase (Titova, 1968), the author has not discussed the possible implications of silver ion inhibition of enzyme regulation on the results obtained as such influence would be essentially impossible to ascertain in an intact cell system.

3.2.5 Selection of a carbon source for the growth of E. coli to be used for the investigation of the uncoupling of respiration by silver nitrate

In evaluating the data presented in this section it is important to remember that the culture was aerated continuously. Therefore, the oxygen level at a particular time represents the balance between the rate of utilization of oxygen and the rate at which oxygen dissolves in the medium.

The investigation of the possible action of a substance as an uncoupler requires the utilization of a test system which generates the majority of its energy via the electron transport chain, and efficiently conserves this energy (i.e., a system which is "highly coupled"). These are essential prerequisites. If energy is generated by a system other than the respiratory chain, and/or if energy conservation is not "highly coupled", then the action of a substance as an uncoupler, causing a stimulation of respiration, at best will be minimal and at worst will be undetectable. Thus, as indicated in section 3.1.5, in order to investigate the action of silver nitrate as an uncoupler of E. coli respiration it was essential to obtain E. coli cells which derived the majority of their energy requirement from the respiratory chain, and which were "highly coupled".

In addition to the preceding requirements with respect to energy production and conservation, it was of practical importance to be able to harvest cells possessing uniform physiological and biochemical properties. A final consideration was the greater sensitivity of the glycolytic pathway than respiration to inhibition by $AgNO_3$ (sec.3.1.5 and 3.2.4). The inhibition of respiration by $AgNO_3$ when glycolytic intermediates are used as substrates might complicate the interpretation of the uncoupling data.

Growth of E. coli NRC 482 on glucose as carbon source resulted in three phases of oxygen utilization and two phases of growth (Figure 3.20) as described in section 3.1.5. The first phase was demonstrated to be due to glucose oxidation (Figure 3.21). The continued decrease in the redox potential during the interval when the oxygen electrode indicated no detectable oxygen is presumably due to the greater sensitivity of the redox electrode to low oxygen tensions but also could be due to the accumulation of redox active products (Jacob, 1970). The second phase of oxygen utilization and growth could correspond to the oxidation of lactate (Butlin et al., 1973) or pyruvate (Raunio, 1966) accumulated under these conditions. The third phase of oxygen utilization commencing approximately 2 hr after the depletion of glucose is believed to correspond to the induction of the glyoxylate cycle enzymes and the oxidation of acetate (Holms and Bennett, 1971). The second and third phases of oxygen utilization are more pronounced when ferric citrate has been added to the medium. The reason for the apparent decline in the absorbance during the period of acetate oxidation is unclear but may be related to a decrease in cell size and the dependence of light scattering on particle size and shape. There was no evidence to indicate that the decrease in absorbance was due to cell lysis (i.e., increased foaming). The

significance and the value of the redox measurements will be discussed later.

Glucose was considered undesireable as the carbon source for the growth of E. coli which were "highly coupled" to energy production via the electron transport chain for the following reasons:

(i) during exponential growth on glucose the major proportion of the energy utilized by E. coli is generated by glycolysis;

(ii) Hempfling's data (1970b) indicated that the coupling of energy production to the respiratory chain developed after approximately 90 min of incubation subsequent to the depletion of glucose from the medium;

(iii) the second and third phases of oxygen utilization would make it difficult to harvest cells with constant biochemical and physiological properties and;

(iv) glycolysis is more sensitive to inhibition by $AgNO_3$ than respiration.

E. coli can grow only aerobically with glycerol as carbon source. Consequently the development of oxygen limited conditions between 5.5 and 7.25 hr demonstrated a greater effect on growth (Figure 3.22) than was apparent with cultures grown on glucose (Figure 3.20), which can be oxidized fermentatively. The interpretation of the continued decrease in the redox potential under oxygen limited conditions is the same as that given for the glucose grown culture, that is, the greater sensitivity of the redox measurements at low oxygen tensions. The significance of the shoulder in the redox potential curve, between 7.25 and 8.25 hr is not known but may be related to the second phase of growth and oxygen utilization demonstrated by the cultures of E. coli grown on glucose (Figure 3.20), that is, oxidation of accumulated lactate or pyruvate, although there was no marked change in the oxygen

level during this interval of growth.

Although cells grown on glycerol obtain the majority of their energy from the respiratory chain and consequently could be harvested during the exponential phase of growth thus providing cells with constant characteristics, glycerol was considered to be unsuitable as the carbon source due to the greater sensitivity of glycolysis than respiration to inhibition by $AgNO_3$.

The cultures of E. coli NRC 482 grown with 0.8% DL-lactate as carbon source demonstrated two phases of oxygen utilization and growth (Figure 3.23) similar to the first two phases observed in the culture of E. coli grown on 0.4% glucose. The first phase of growth and oxygen utilization was interpreted as resulting from the oxidation of D-lactate and L-lactate, as E. coli has been shown to possess both D- and L-lactate oxidases when grown on DL-lactate (Bennett et al., 1966). D- and L-lactate, like glycerol, can not be oxidized fermentatively by E. coli. Consequently, there was a marked influence of the oxygen limited conditions on the growth rate of these cultures of E. coli. During the interval of oxygen limitation growth was essentially linear and the redox potential of the culture also decreased linearly. The substrate for the second phase of oxygen utilization is possibly excess pyruvate which would possibly accumulate under the conditions of oxygen limitation, as has been reported to occur with cultures grown on glucose (Raunio, 1966). However, as catabolite repression of the glyoxylate cycle enzymes would probably be much reduced with DL-lactate rather than glucose as the carbon source, the possibility that the second phase of oxygen utilization is due to the oxidation of acetate can not be eliminated.

Due to the second phase of oxygen utilization and the resulting

difficulty in harvesting cells with uniform biochemical and physiological properties, DL-lactate was considered to be unsuitable as the carbon source for the growth of "highly coupled" E. coli.

Growth of E. coli on 0.8% acetate was slow with a large proportion of the growth curve demonstrating a linear rate of growth (during the interval 8.0 to 12.0 hr) (Figure 3.24). The reason for the linear rate of growth is uncertain. It would not appear likely that it was due to oxygen limitation, since the oxygen level was 35 to 40% of saturation when the growth rate first became linear. Perhaps the respiratory chain of these cells have a low affinity for oxygen. This is a distinct possibility as the cells have a slow growth rate and were grown with an air flow rate of 4.4 l/min/l of medium and consequently may possess a cytochrome oxidase system with a high K_m for oxygen as has been reported for Hemophilus parainfluenzae and Azotobacter vinelandii (White, 1963; Nishizawa et al., 1971). As was observed in the culture of E. coli grown on 0.8% DL-lactate the decrease in the redox potential of the culture was linear during the period of linear growth. There are a number of other characteristics which are unusual. The smooth nature of the growth curve as it approaches the stationary phase, and the failure of the oxygen level to return to near saturation when growth has ceased suggests that the culture may have been limited by some component, other than the carbon source, possibly nitrogen, and that while growth has ceased metabolism of the carbon source has not. No significance has been attached to the shoulders in the oxygen level and redox potential curves at 3.0 and 6.0 hours respectively.

E. coli grown on succinate in the presence of 6 μM ferric citrate possessed the desired characteristics:

(i) of obtaining the majority of their energy requirements from the respiratory chain;

(ii) were "highly coupled" (sec.4.2.2);

(iii) of no secondary phases of oxygen utilization or growth so that cells of constant biochemical and physiological properties could be harvested during either the logarthmic or the stationary growth phase; and

(iv) interpretation of uncoupling data would not be complicated by the sensitivity of the glycolytic pathway to inhibition by $AgNO_3$.

Although the E. coli grown on acetate as carbon source also possessed these desired characteristics succinate was chosen in preference to acetate as the most suitable carbon source for the growth of E. coli "highly coupled" to energy production by the electron transport chain on the basis of the more rapid growth rate and the shorter adaptation procedure with succinate.

3.2.6 The uncoupling of the respiration of E. coli by added silver nitrate

While determining the characteristics of silver inhibition of the endogenous and substrate-dependent respiration of E. coli (Figures 3.12 to 3.18 inclusive) it was observed that the respiration immediately following the addition of silver nitrate, or immediately after the addition of E. coli to the assay medium containing substrate and $AgNO_3$, was greater than the control rate. The stimulation was particularly marked with cells grown on glucose as carbon source but utilizing acetate as the respiratory substrate, reaching a respiration rate 4-fold greater than that of the control (Figure 3.14). There are two alternative explanations for these observations: (i) that the rate of respiration was stimulated by the presence of the nitrate ion which was acting as an alternative electron acceptor; or (ii) that silver ions were acting as an uncoupling agent of E. coli respiration.

Figure 3.28 demonstrates that the addition of 40 μM KNO_3 to the assay system had little if any stimulatory effect on the respiration rate. This eliminated the first possibility. A comparison of the stimulation of the respiration of E. coli by $AgNO_3$ (Figure 3.27), and by a known uncoupler of mitochondrial respiration, 2,4-dibromophenol (Figure 3.26), supports the second interpretation. Additional support for the action of silver ions as uncouplers of E. coli energy conservation was obtained from Figure 3.29 and table 3.8. These results indicate that the respiration of highly coupled E. coli (grown under iron-sufficient conditions) (sec.4.2.2) was stimulated to a greater extent by $AgNO_3$ than the less highly coupled cells (grown under iron-limited conditions).

The uncoupling of mitochondrial respiration and energy conservation by silver ions has been reported by Chappell and Greville (1954) and Grabske (1966). Chappell and Greville were able to correlate the increased respiration rate with a five-fold increase in ATPase activity. Cooper (1960) has subsequently examined the influence of Ag^+ on the Mg^+-stimulated ATPase of submitochondrial particles prepared from rat liver mitochondria. A two-fold stimulation of ATPase activity was observed when approximately 50% of the total free, readily available, sulfydryl groups had reacted. Further addition of Ag^+, resulted in loss of activity. Similar results were obtained with $HgCl_2$ and PCMB as complexing agents. Thus, the observed stimulation of the respiration rate appears to be directly related to the stimulation of the ATPase by thiol reagents. Cooper (1960) proposed that the stimulating action of Ag^+ (Hg^{++} or PCMB) was due to the blockage of an inhibitory grouping at the active site.

Kielley (1963) has compared the loss of oxidative phosphorylation,

inhibition of the ATP - P_i exchange reaction and disappearance of DNP stimulation of the ATPase during titration with PCMB and suggests that these sensitivities are related to thiol groups of very similar reactivity, if not the same groups. These observations raise the possibility that the observed inhibition of the D-lactate, L-lactate-, succinate- and fumarate-dependent respirations by Ag^+, may be due to Ag^+ inhibition of the utilization of respiratory chain generated energy.

3.2.7 Redox potential as an indicator of the oxygen level of batch cultures of E. coli

During the experiment to select the most suitable carbon source, the usefulness of the redox potential as an indicator of the oxygen level and/or in providing additional information about the bacterial culture was evaluated. Jacob (1970) has discussed the measurement of the redox potential of microbial cultures in considerable detail. Compared to a limit of sensitivity of approximately 1 mm Hg for the measurement of the oxygen tension of the medium with an oxygen electrode, the dependence of the redox potential of the medium on the oxygen tension has been reported down to oxygen pressures of about 10^{-9} atm (Jacob, 1970). Thus, the redox potential of the culture is a much more sensitive indicator of the oxygen content of the medium at low oxygen tension, that is below oxygen tensions of approximately 10^{-3} atm. However, since the redox potential of the culture is a function of the log of the oxygen tension it is a less sensitive indicator of the changes in oxygen tension between 10^{-3} atm and 10^{-1} atm than the oxygen electrode which responds directly to the oxygen tension. In addition, the redox potential measured during the cultivation of microorganisms could be the result of metabolic products with redox character, and/or by the activity of different enzymes (oxidases, dehydrogenases) in the cell.

On closely examining Figure 3.20, and 3.22 to 3.25, inclusive, with respect to the relationship between the redox potential and the oxygen level of the bacterial culture, the following observations can be made. (i) Generally there is a considerable similarity in the shape of the redox potential curve and the oxygen saturation curve to the point at which the oxygen level becomes undetectable by the oxygen electrode. However, the redox potential changed much more rapidly than the log of the oxygen tension. (ii) During the interval when the oxygen tension was not detectable with the oxygen electrode the redox potential continued to decrease presumably due to the greater sensitivity of the redox electrode to low oxygen tension. However, during the period of oxygen-limited growth on carbon sources which had to be oxidized aerobically, the redox potential decreased linearly over essentially the same interval that the growth was linear (Figures 3.22, 3.23, 3.24 and 3.25). This suggested a closer relationship between the redox potential and the cell concentration or the accumulation of some product. (iii) The redox potential and oxygen saturation demonstrate the same phase particularly in the cultures grown on glucose and DL-lactate (Figures 3.20 and 3.23 respectively) with the redox potential demonstrating the lower sensitivity that one would expect of a logarithmic function of the oxygen concentration. However, the complete opposite was observed with E. coli cultures utilizing glycerol as the carbon source (Figure 3.22). Approaching the end of the interval of oxygen limitation the redox potential of the culture increased dramatically with little corresponding increase in the oxygen level. Considering these observations, the utilization of the redox potential of a culture as the sole indicator of the oxygen level seems undesirable, at least with aerobic or facultatively anaerobic organisms. The physiologically and

biochemically important concentrations of oxygen for cultures of E. coli as indicated by the influence of oxygen limitation on growth appears to be within the sensitivity range of the oxygen electrode and thus precludes the necessity of the sensitivity of the redox electrode. However, the work of Wimpenny and colleagues (Wimpenny, 1969; Wimpenny and Necklen, 1971) on the relationship between cytochrome synthesis and the redox potential of the culture demonstrates that redox potential measurements definitely have a place in the investigation of microbial processes.

4 PART II: IRON LIMITATION AND THE RESPIRATION AND ENERGY-COUPLING OF *E. COLI*

4.1 Results

4.1.1 The influence of iron limitation on the respiration and energy-coupling of *E. coli*

During a more detailed investigation of the growth of *E. coli* on succinate as carbon source, it was observed that the oxygen level of a culture of *E. coli*, grown in medium C, reached a plateau value well above zero oxygen saturation (Figure 4.1). The plateau was demonstrated to be real, and not an artifact of the electrode, by ceasing aeration and establishing the residual current of the electrode (the electrode current in the absence of oxygen) by flushing with nitrogen. During this period all growth ceased but recommenced immediately once aeration was restarted. The oxygen level returned slowly to the level of the plateau with reaeration of the culture.

Further investigation of this phenomenon (Figure 4.2) established that the plateau in the level of oxygen saturation of the culture medium continued with little change until all of the succinate had been utilized at which point the oxygen level returned to close to saturation. The onset of the plateau in oxygen level coincided with an initial change from an exponential to a linear growth rate which subsequently became a progressively decreasing rate. Associated with the plateau in oxygen level and the decrease in growth rate were a decrease in the efficiency of conversion of succinate to cell mass, and a decrease in the level of cytochrome b_1 per unit of wet cell mass.

Similar patterns of response were obtained with *E. coli* B and *E. coli* B-SG1.

During the plateau in oxygen level the cell mass increased 2 to 3 fold while the rate of oxygen utilization by the entire culture remained

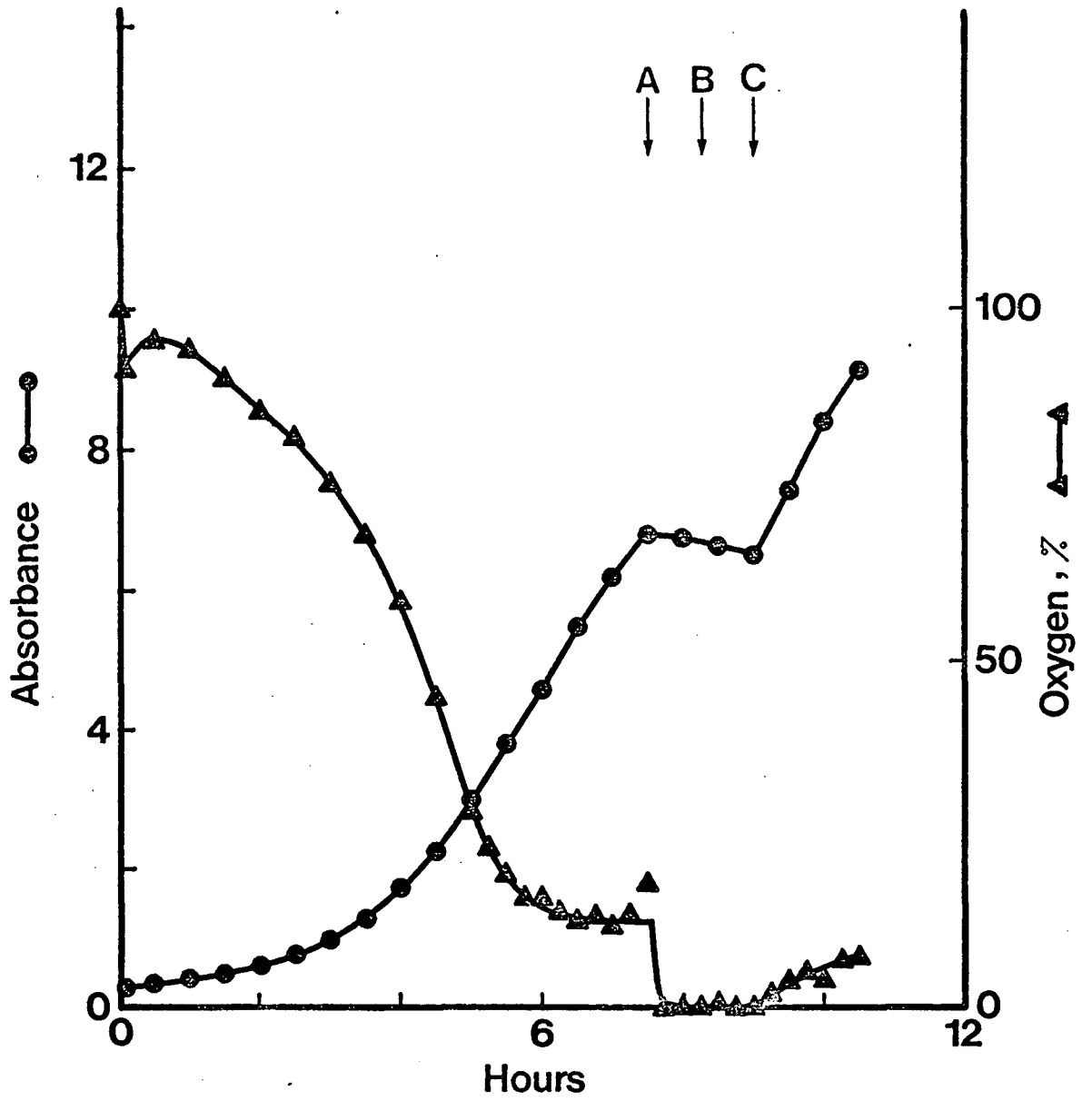


Fig. 4.1 Plateau in the oxygen level of a culture of E. coli growing on 0.6% succinate. A: aeration ceased; B: N_2 flushing commenced; C: aeration recommenced; Absorbance at 420 nm (methods: sec. 2.2.6.1).

Fig. 4.2 Growth and oxygen level (A), efficiency (B) and cytochrome $\underline{b}_1$ levels (C) of culture of *E. coli* growing on 0.6% succinate. Absorbance at 420 nm; units: cytochrome $\underline{b}_1$, nmoles/g cells (wet weight); (methods: sec. 2.2.6.1, 2.2.6.2 and 2.2.6.7).

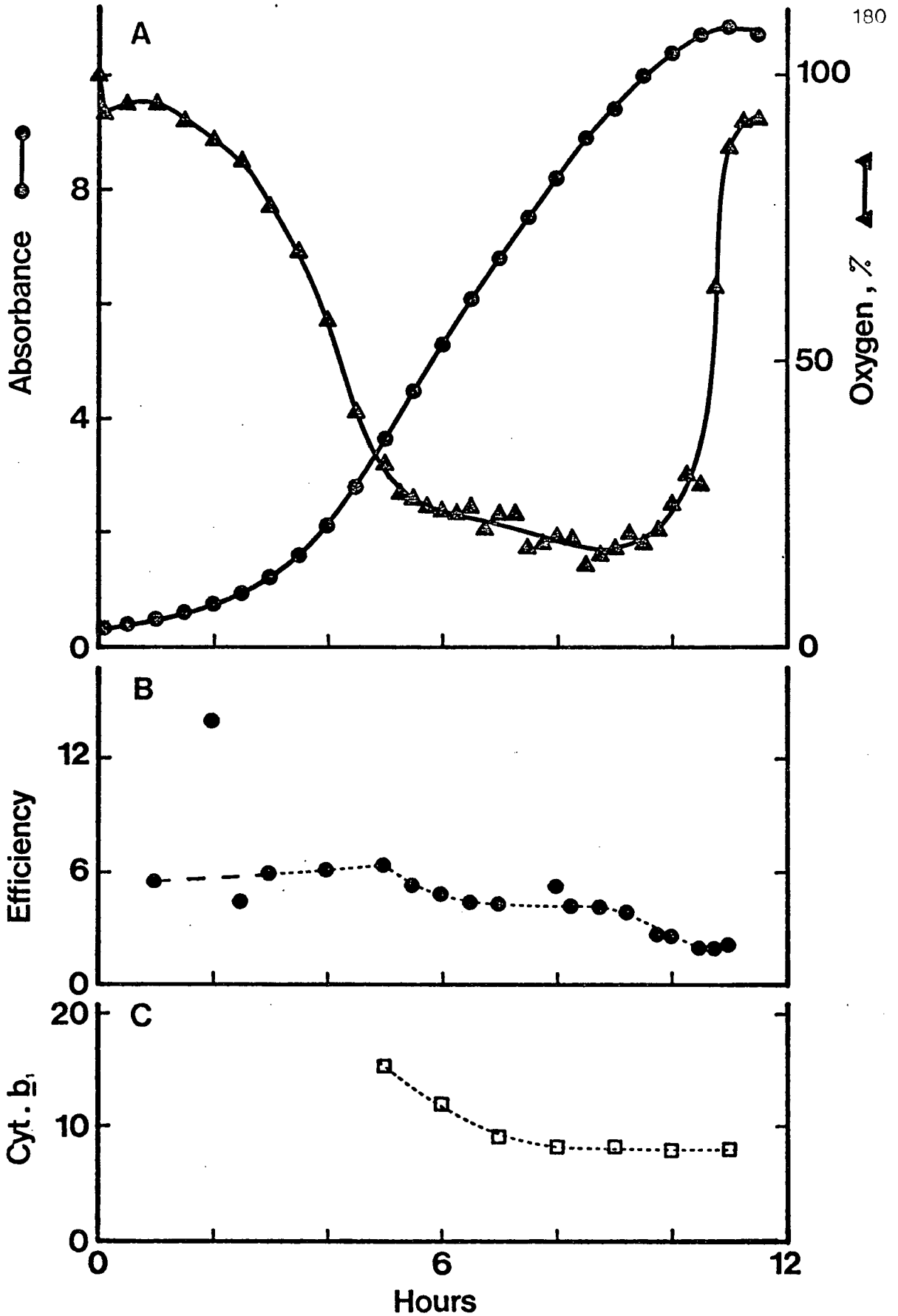

A
B
C
Absorbance
Oxygen, %
Efficiency
Cyt. b
Hours
0
4
8
50
100
6
10
12
20

Fig. 4.2

relatively constant. This would indicate that the rate of oxygen uptake per unit of cell mass decreased during the period of the plateau. This suggested that the respiration of the E. coli was limited by an inadequate level of some component. As a decrease in the level of cytochrome b_1 had been observed (Figure 4.2), iron appeared to be a likely candidate.

As shown in Figures 4.3 and 4.4, and table 4.1, the addition of ferric citrate to a final concentration of 6 μM resulted, after a 15 min lag, in a rapid drop in the level of oxygen saturation and an increase in the growth rate from 1.2 absorbance units/hr to 2.95 absorbance units/hr. However, the increased growth rate, subsequent to the addition of ferric citrate (7.5 hr), is less than the exponential growth rate prior to the onset of iron limitation (5.5 hr) (Figure 4.4). Measurement of the levels of nonheme iron and cytochrome b_1 prior to, and following the addition of ferric citrate indicated that during the two hours preceding the addition of ferric citrate the nonheme iron levels decreased by 70% while there was a corresponding decrease in cytochrome b_1 of 20%. Consequently the ratio of nonheme iron the cytochrome b_1 fell from 19.7:1 to 7.5:1 (Table 4.1). Subsequent to the addition of ferric citrate, and prior to any apparent changes in the growth rate or the rate of oxygen consumption (i.e., within the 0.25 hr following the addition of ferric citrate to the culture), the nonheme iron levels increased by greater than 290%. During the same period cytochrome b_1 levels increased by only 8%. Although both the rate and the amount of increase in the levels of nonheme iron were much greater, the increase in the level of cytochrome b_1 was considerable, being an increase of approximately 160%, in the 3 hr following the addition of ferric citrate.

With such marked changes in the levels of known respiratory chain

Fig. 4.3 The response of growth and oxygen level (A), and nonheme iron and cytochrome $\underline{b}_1$ levels (B) to the addition of ferric citrate (Fe) to a culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; units: cytochrome $\underline{b}_1$, nmoles/g cells (wet weight); nonheme iron, natoms/g cells (wet weight); (methods: sec. 2.2.6.1, 2.2.6.5 and 2.2.6.7).

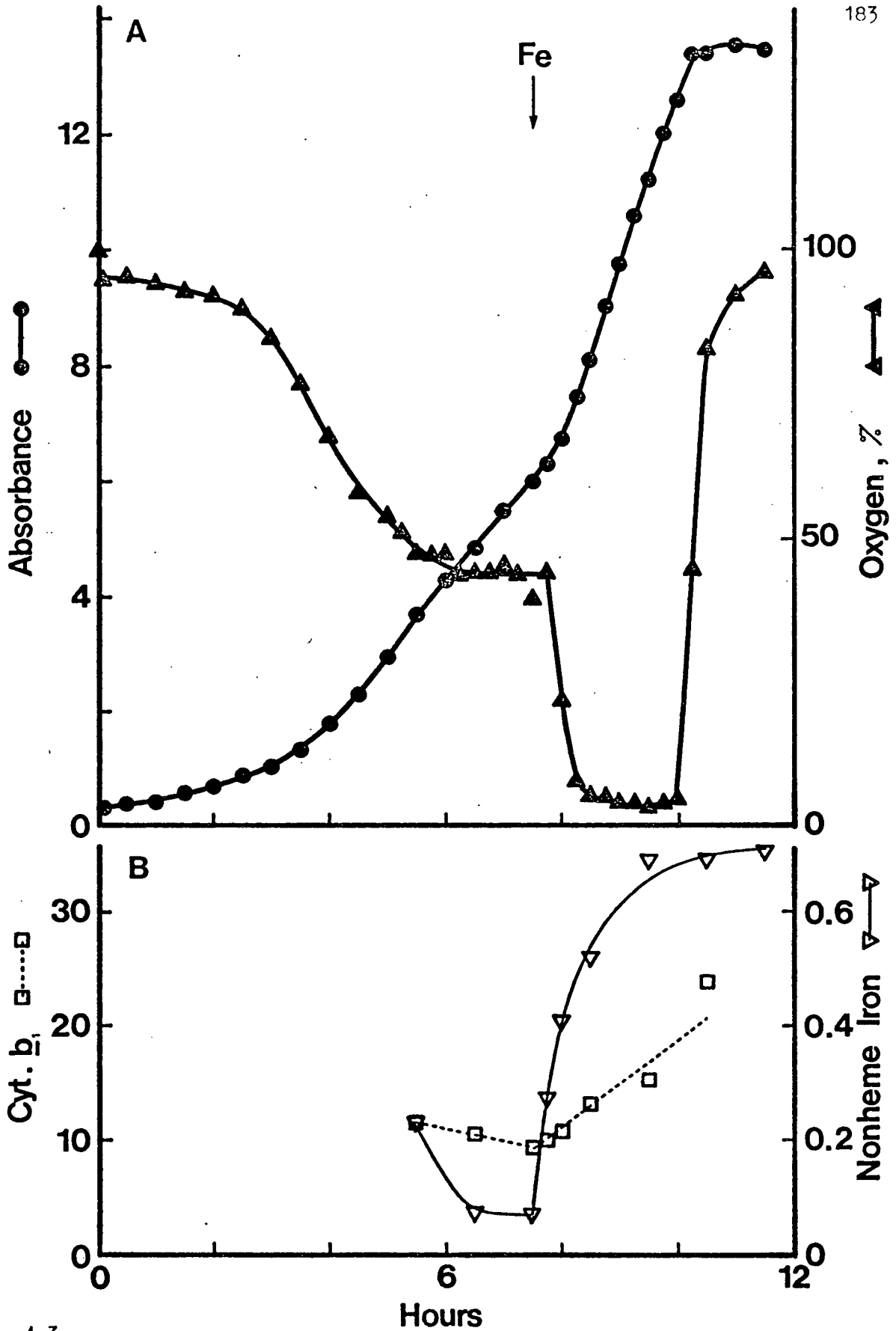

A
Fe
12
8
4
0
Absorbance
100
50
0
Oxygen, %
B
30
20
10
0
Cyt. b_i
0.6
0.4
0.2
0
Nonheme Iron
0
6
12
Hours

Fig. 4.3

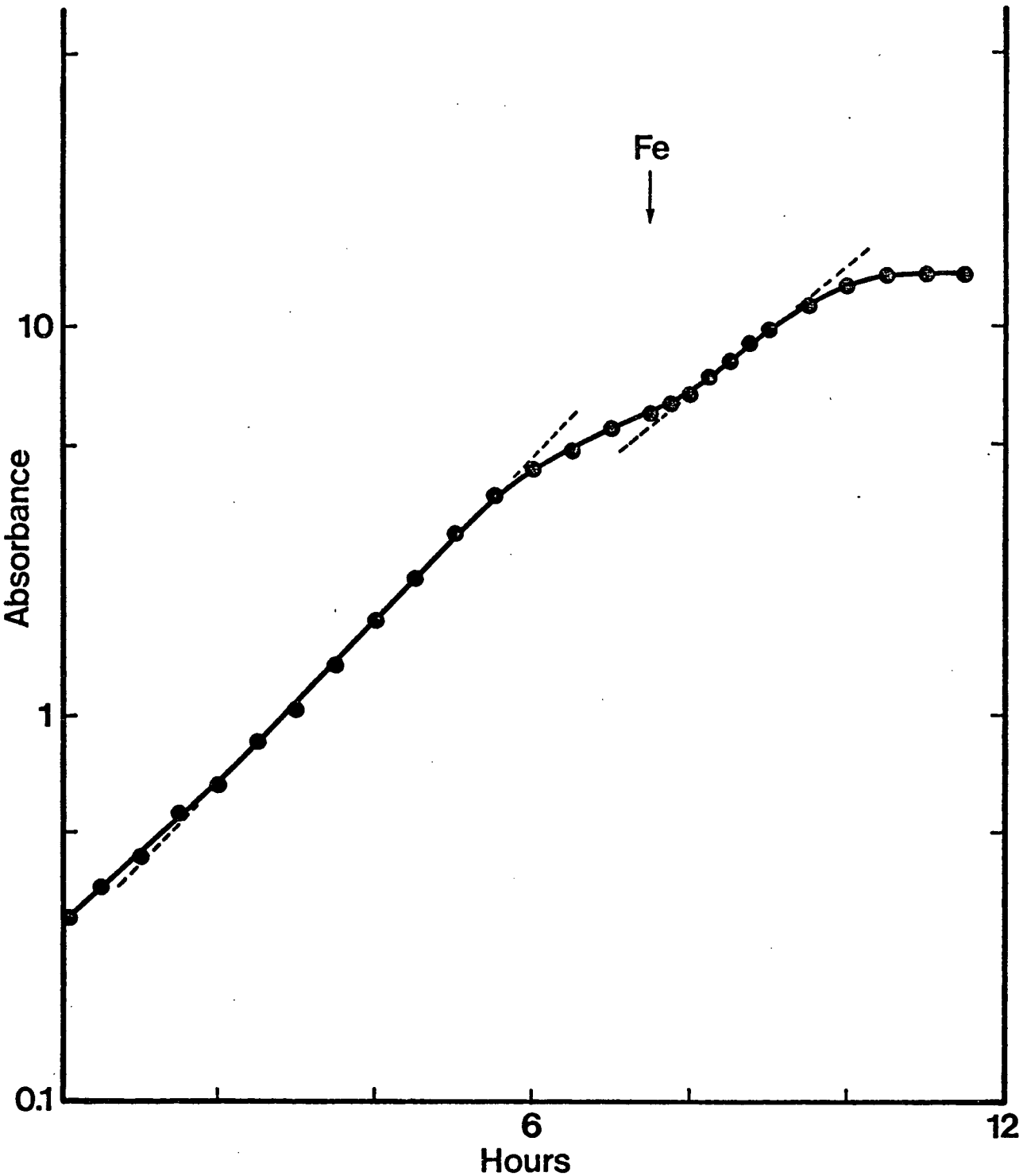


Fig. 4.4 A semi-log plot of the growth data from Fig. 4.3.

Table 4.1

The level of iron-containing respiratory chain components in cell extracts of iron-limited, succinate-grown, *E. coli* prior to, and following the addition of ferric citrate (final conc. 6 μM).

Time[1] (hr)	Nonheme Iron[2]	%	Cytochrome b_1[3]	%	Ratio[4]	A420	%
-2.0	227	329	11.5	125	19.7	3.68	
-1.0	73	106	10.4	113	7.0	4.83	
0	69	100	9.2	100	7.5	5.98	100
0.25	272	394	9.9	108	27.5	6.27	105
0.5	406	588	10.7	116	37.9	6.73	113
1.0	516	748	13.1	142	39.4	8.07	135
2.0	688	997	15.2	165	45.3	11.20	187
3.0	686	994	23.8	259	28.8	13.36	223
4.0	705	1022	--	--	--	13.44	225

[1] 0 corresponds to the time of addition of ferric citrate; negative time indicates time prior to the addition of ferric citrate.

[2] Values expressed as natoms per g cells (wet weight).

[3] Values expressed as nmoles per g cells (wet weight).

[4] Values expressed as natoms per nmoles.

components it was of considerable interest to investigate the influence of the addition of ferric citrate to an iron-limited culture on the level of respiratory chain associated enzymes. The results presented in Figure 4.5 and table 4.2 indicate the observed changes in the level of cytochrome $\underline{b}_1$ and in the activities of NADH oxidase, succinate oxidase and succinate dehydrogenase. The rate of increase in the enzyme activities and cytochrome $\underline{b}_1$ levels were comparable (Table 4.2). However, the level of succinate oxidase activity increased significantly faster than did the other three activities. The rate of increase in the levels of NADH oxidase, succinate oxidase, succinate dehydrogenase or cytochrome $\underline{b}_1$ (Figure 4.5) did not compare with the rapidity of the increase in nonheme iron levels (Figure 4.3).

To investigate the possibility that the observed changes in oxygen level, growth rate, cytochrome $\underline{b}_1$, nonheme iron, NADH oxidase, succinate oxidase and succinate dehydrogenase levels following the addition of ferric citrate might be due to the citrate ion rather than the ferric ion, sodium citrate was added to a culture of *E. coli* during the plateau in oxygen level (Figure 4.6). The addition of sodium citrate instead of ferric citrate to a final concentration of 6 μM did not result in marked changes in growth rate, the level of oxygen saturation, or cytochrome $\underline{b}_1$ levels. A comparison of the results obtained when no addition was made during the plateau in the oxygen saturation of the culture (Figure 4.2) and those obtained following the addition of sodium citrate (Figure 4.6) indicates that they are nearly identical, and distinctly different from the results obtained following the addition of ferric citrate during the plateau in oxygen saturation (Figure 4.3). Consequently, it can be concluded that the active component of the ferric citrate is the ferric ion.

Fig. 4.5 The response of growth and oxygen level (A) and, enzyme levels (B) to the addition of ferric citrate to a culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; units: specific activity, μmoles/min/g cells (wet weight); succinate oxidase, ●; succinate dehydrogenase, ▼; NADH oxidase, ■; (methods: sec. 2.2.6.1, 2.2.6.8, 2.2.6.9 and 2.2.6.10).

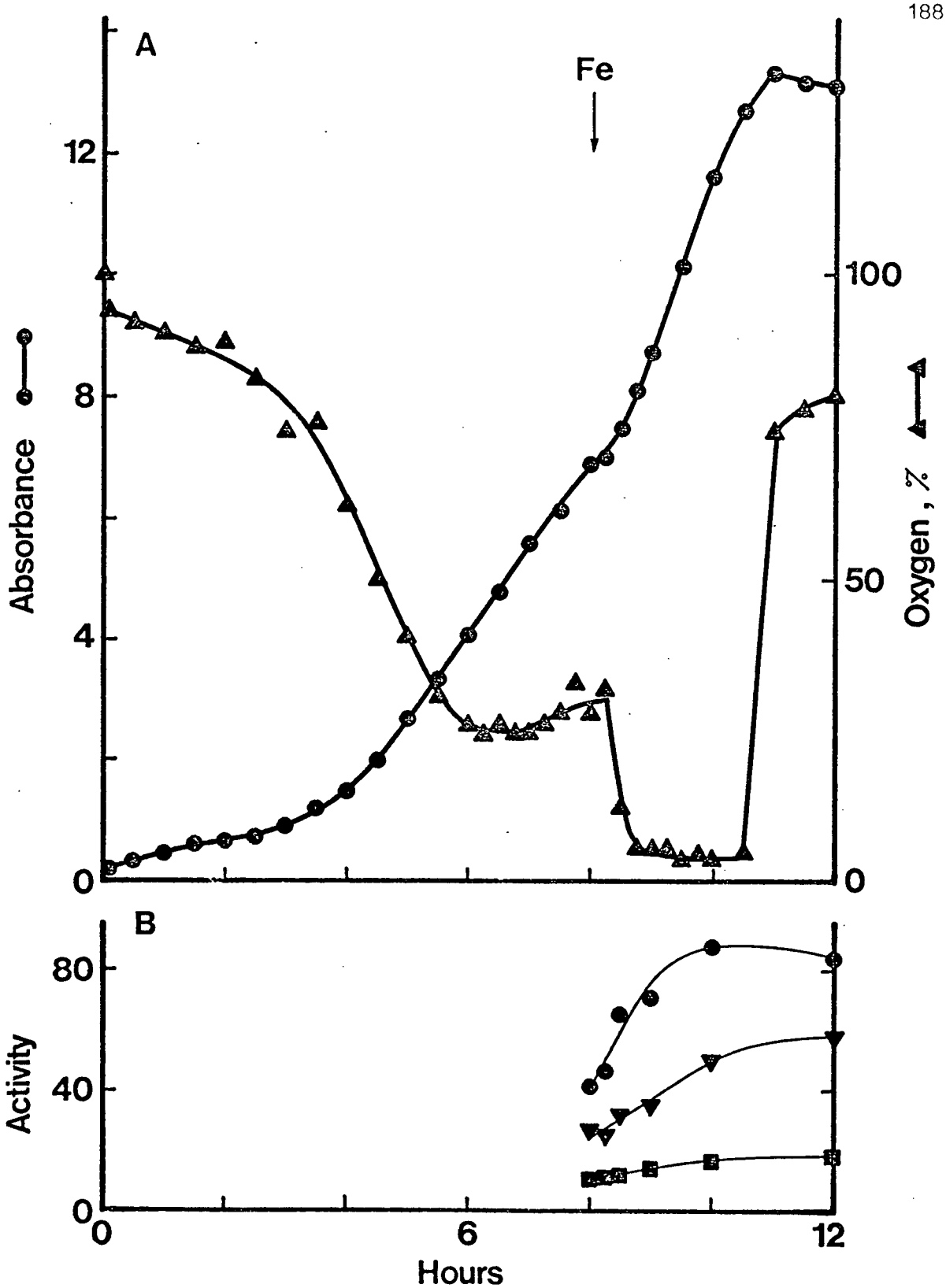

A
Fe
12
8
4
0
Absorbance
100
50
0
Oxygen, %
B
80
40
0
Activity
0
6
12
Hours

Fig. 4.5

Table 4.2

Enzyme activites in cell extracts of iron-limited, succinate-grown *E. coli* following the addition of ferric citrate (final conc. 6 μM).

Time (hr)	Cytochrome b_1 [1]	%	NADH Oxidase [2]	%	Succinate Oxidase [3]	%	Succinate Dehydrogenase [2]	%	A420	%
0	9.7	100	10.2	100	41.4	100	27.2	100	6.88	100
0.25	8.8	91	10.8	106	46.3	112	25.5	94	7.00	102
0.5	9.4	97	11.8	116	65.3	158	31.9	117	7.46	108
1.0	11.2	116	13.9	136	70.7	171	35.0	139	8.70	126
2.0	15.6	161	16.1	158	87.8	212	49.7	183	11.60	169
4.0	17.5	180	16.7	164	84.2	203	57.8	212	13.08	190

[1] Values expressed as nmoles per g cells (wet weight).

[2] Values expressed as μmoles substrate oxidized per min per g cells (wet weight). Temperature 22°C.

[3] Values expressed as μmoles substrate oxidized per min per g cells (wet weight). Temperature 37°C.

Fig. 4.6 The response of growth and oxygen level (A), efficiency (B) and cytochrome $\underline{b}_1$ levels (C) to the addition of sodium citrate (SC) to a culture of *E. coli* growing on 0.6% succinate. Absorbance at 420 nm; units: cytochrome $\underline{b}_1$, nmoles/g cells (wet weight); (methods: sec. 2.2.6.1, 2.2.6.2 and 2.2.6.7).

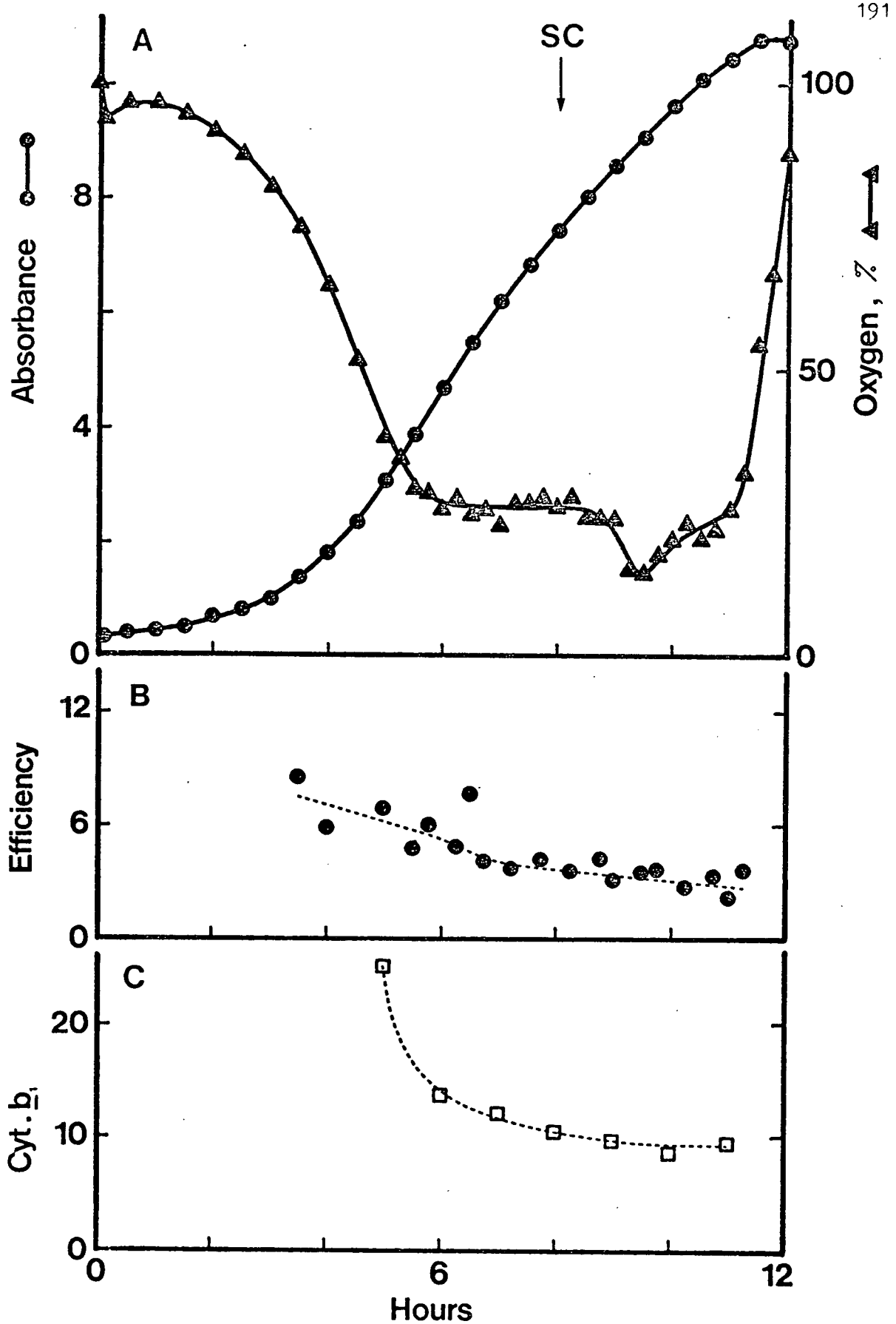

A
SC
Absorbance
Oxygen, %
0
4
8
50
100
B
Efficiency
0
6
12
C
Cyt. b
0
10
20
0
6
12
Hours

Fig. 4.6

However, it was observed that the addition of ferric citrate during the plateau in oxygen saturation of a nitrogen-limited culture of E. coli, failed to cause a decrease in the level of oxygen saturation (Figure 4.7). Subsequently addition of ammonium sulfate did result in a stimulation of respiration after a 15 min lag, suggesting that protein synthesis might be required for respiration to be stimulated by the addition of iron. Pertinent to this possibility was the observation that if E. coli were grown in the presence of trace elements (Holms et al., 1970) other than iron (Appendix C), the addition of ferric citrate during the plateau in oxygen saturation resulted in an immediate and rapid decline in the level of oxygen saturation (Figure 4.8). That is, growth of E. coli in the presence of trace elements other than iron abolished the 15 min lag in the response of oxygen saturation to the added ferric citrate. The response of growth to the added ferric citrate appeared to demonstrate the usual lag prior to attaining the new growth rate (Figure 4.8).

Having investigated an "iron-limited" culture (Figure 4.2), and the response of an "iron-limited" culture to the addition of ferric citrate (Figures 4.3 and 4.5, Tables 4.1 and 4.2), it was of considerable interest to investigate the growth, oxygen utilization, redox potential, cytochrome and nonheme iron levels, and the efficiency of conversion of carbon source to cell mass of an "iron-sufficient" culture of E. coli NRC 482. The results of this investigation are presented in Figures 4.9 to 4.12 inclusive. The data presented were obtained from the same experiment.

Growth and oxygen utilization were rapid with the level of oxygen saturation in the culture approaching, but not reaching zero. Preceding the period of oxygen limitation, growth of the culture demonstrated two

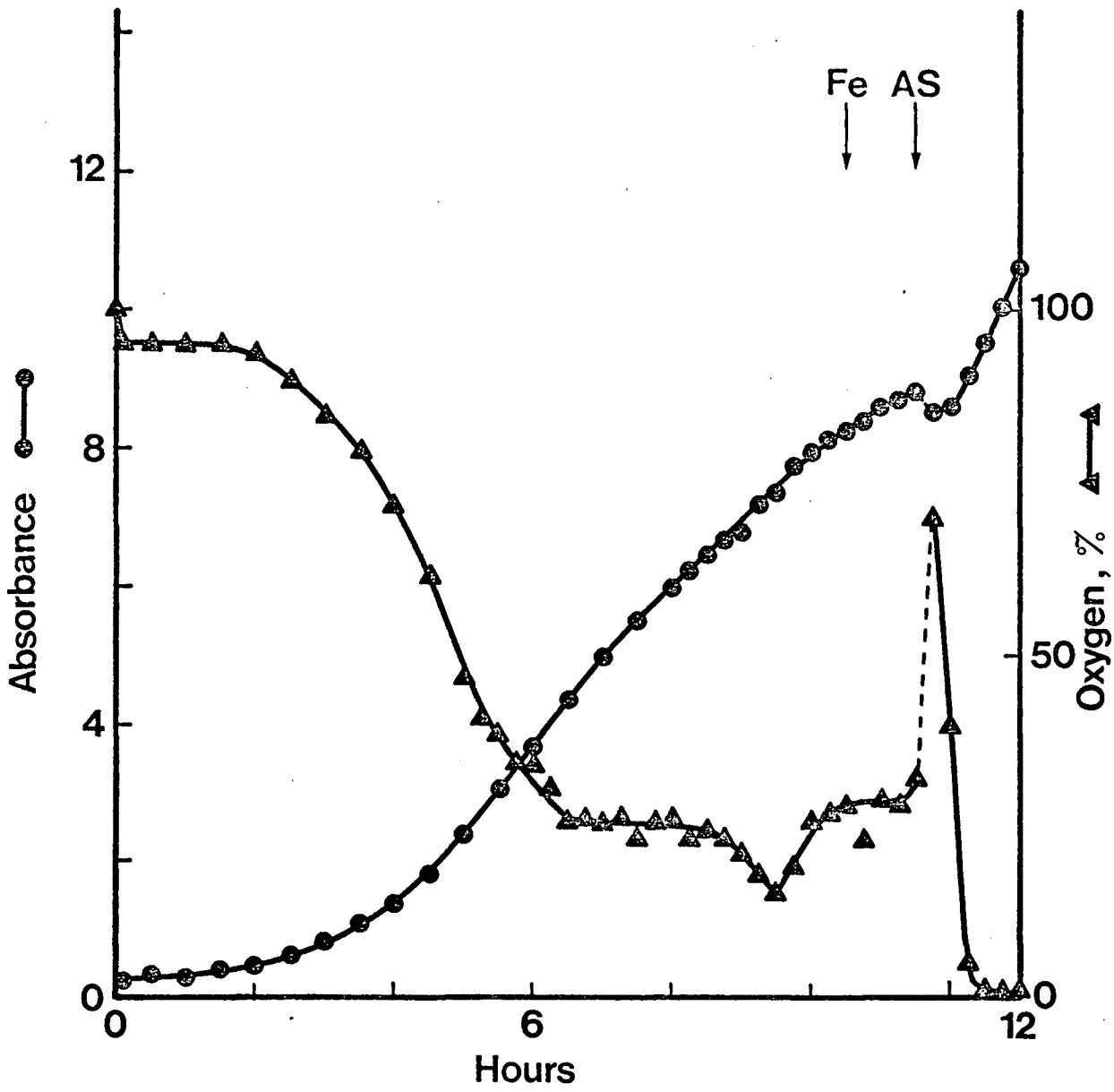


Fig. 4.7 The response of growth and oxygen level to the addition of ferric citrate (Fe) and ammonium sulfate (AS) to an iron-deficient, nitrogen-limited culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; (methods: sec. 2.2.6.1)

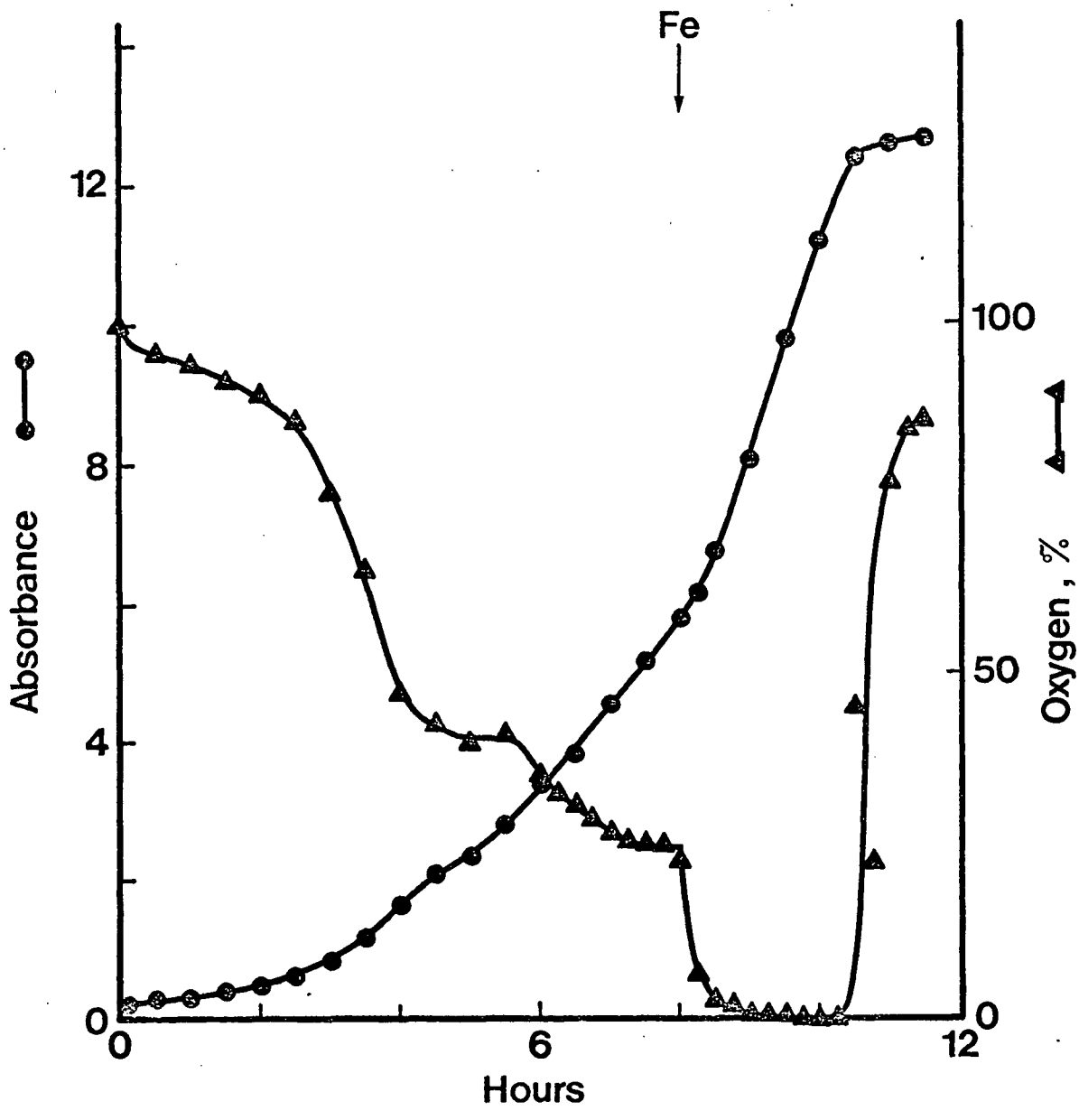


Fig. 4.8 The response of growth and oxygen level to the addition of ferric citrate (Fe) to a culture of E. coli growing on 0.6% succinate in a medium containing the trace metals Ca^{++}, Zn^{++}, Co^{++}, Mn^{++} and Cu^{++}. Absorbance at 420 nm, (methods: sec. 2.2.6.1).

Fig. 4.9 The growth and oxygen level (A) and cytochrome $\underline{a}_2$, cytochrome $\underline{b}_1$ and nonheme iron levels (B) of an iron-sufficient culture of *E. coli* growing on 0.6% succinate. Absorbance at 420 nm; units: cytochromes $\underline{a}_2$ and $\underline{b}_1$, nmoles/g cells (wet weight); nonheme iron, natoms/g cells (wet weight); (methods: sec. 2.2.6.1, 2.2.6.5 and 2.2.6.7).

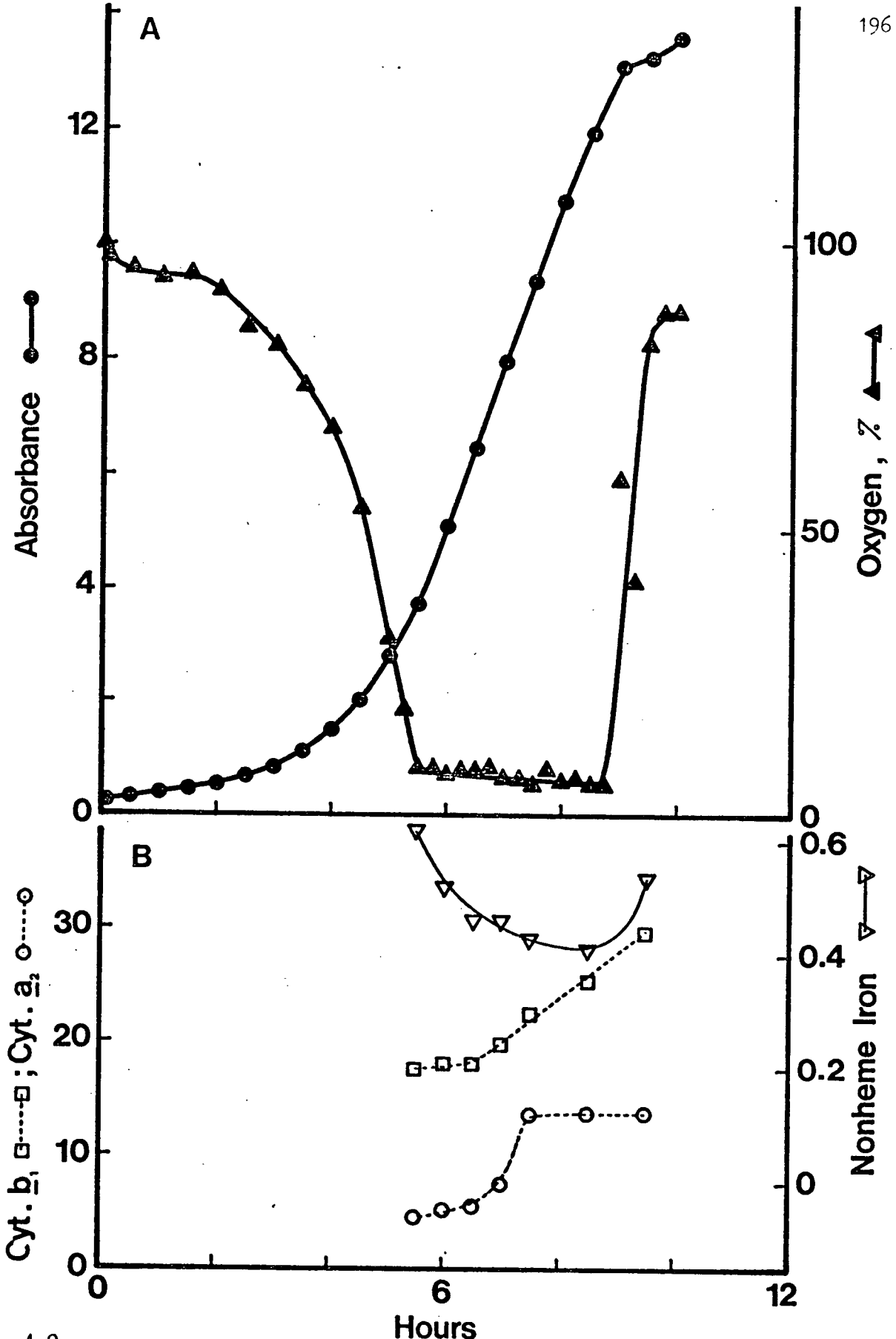

A
B
Absorbance
Oxygen, %
Cyt. b1 ; Cyt. a2
Nonheme Iron
Hours
0
4
8
12
50
100
10
20
30
0.2
0.4
0.6
6

Fig. 4.9

Fig. 4.10 The growth and oxygen level (A) and efficiency (B) of an iron-sufficient culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; (methods: sec. 2.2.6.1 and 2.2.6.2).

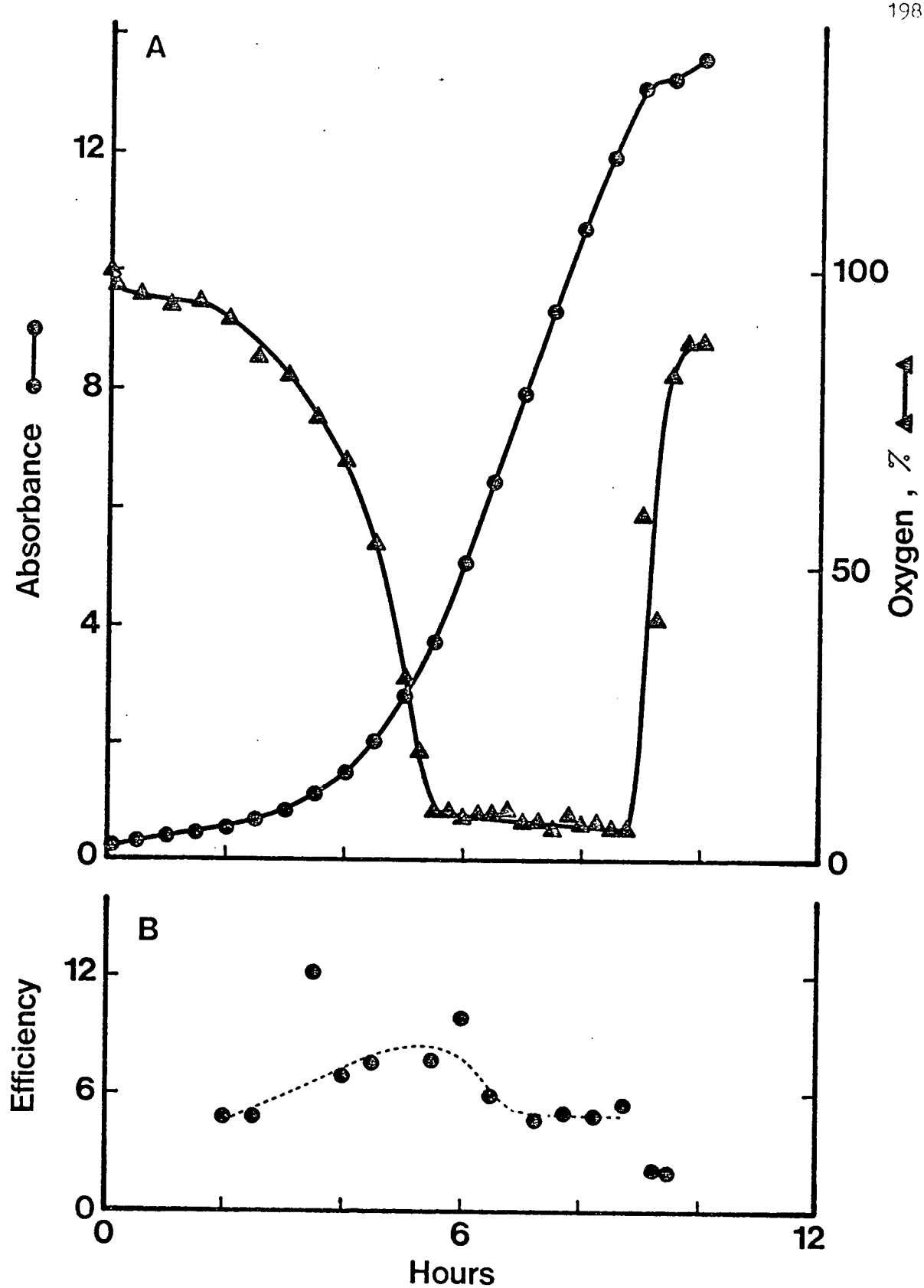

A
12
8
4
0
Absorbance
100
50
0
Oxygen , %
B
Efficiency
12
6
0
0
6
12
Hours

Fig. 4.10

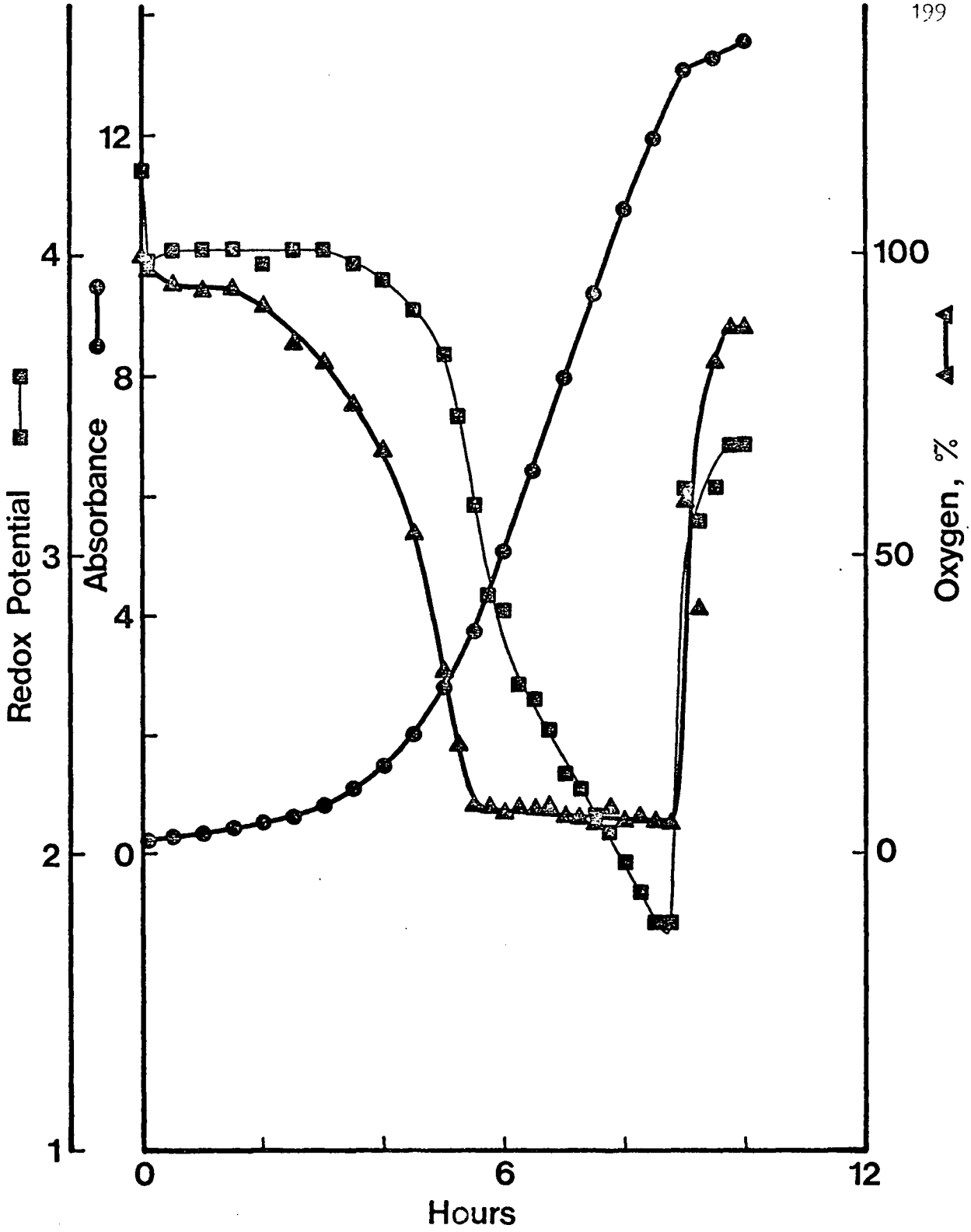


Fig. 4.11 The growth, oxygen level and redox potential of an iron-sufficient culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; units: redox potential, mV x 10^{-2}; (methods: sec. 2.2.6.1).

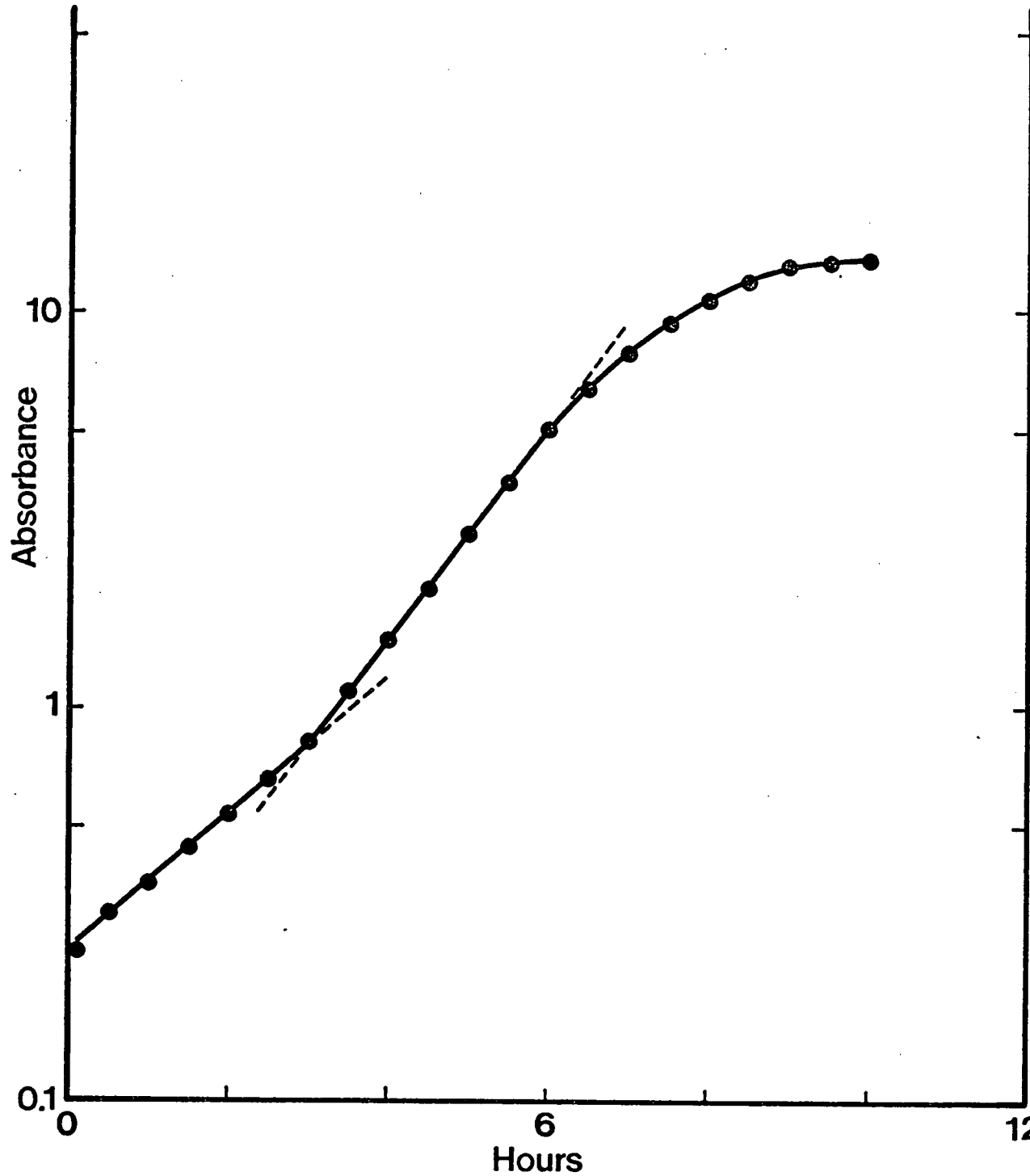


Fig. 4.12 A semi-log plot of the growth data from Fig. 4.9.

successive phases of exponential growth, (i) 0 to 3.0 hr, and (ii) 3.0 to 6.0 hr (Figure 4.12). During conditions of oxygen limitation the growth of the culture was linear with a rate of increase of 2.83 absorbance units/hr (Figure 4.9). During the same interval the nonheme iron level dropped by approximately 35%. Cytochrome $\underline{b}_1$ and $\underline{a}_2$ levels commenced to increase after 6.5 hr of growth. Cytochrome $\underline{a}_2$ demonstrated an increase in level of approximately 200% between 6.5 and 7.5 hr with no further increase. Cytochrome $\underline{b}_1$ levels increased linearly between 6.5 and 9.5 hr, increasing by a total of 70%. The redox potential of the culture was 230 mV (Figure 4.11) when the level of the cytochromes began to increase (Figure 4.9). The level of cytochrome $\underline{a}_2$ increased no further after the redox potential had reached 210 mV. The increase in the level of cytochrome $\underline{b}_1$ was continuing when the redox potential of the culture was 180 mV. The efficiency of conversion of succinate to cell mass increased up until between 5.0 and 6.0 hr. At approximately the same time as oxygen became limiting the efficiency began to decline (Figure 4.10).

For the purpose of comparing the response to iron limitation of cells grown on a fermentable carbon source with the response of cells grown on a nonfermentable carbon source, E. coli NRC 482 was grown on glucose as the carbon source under "iron-limited" conditions. As is apparent from Figure 4.13, although nonheme iron and cytochrome $\underline{b}_1$ levels were low and decreased during growth, the cells were capable of completely depleting the medium of oxygen with no apparent adverse effects on the growth rate (Figure 4.14).

The lack of effect of iron limitation on the growth of E. coli on glucose raised the possibility that iron was important in the coupling of energy production to growth when the carbon source was nonfermentable. This

Fig. 4.13 The growth and oxygen level (A) and cytochrome $\underline{b}_1$ and nonheme iron content (B) of an iron-limited culture of *E. coli* growing on 0.4% glucose. Absorbance at 420 nm; units: cytochrome $\underline{b}_1$ nmoles/g cells (wet weight); nonheme iron, natoms/g cells (wet weight); (methods: sec 2.2.6.2, 2.2.6.5 and 2.2.6.7).

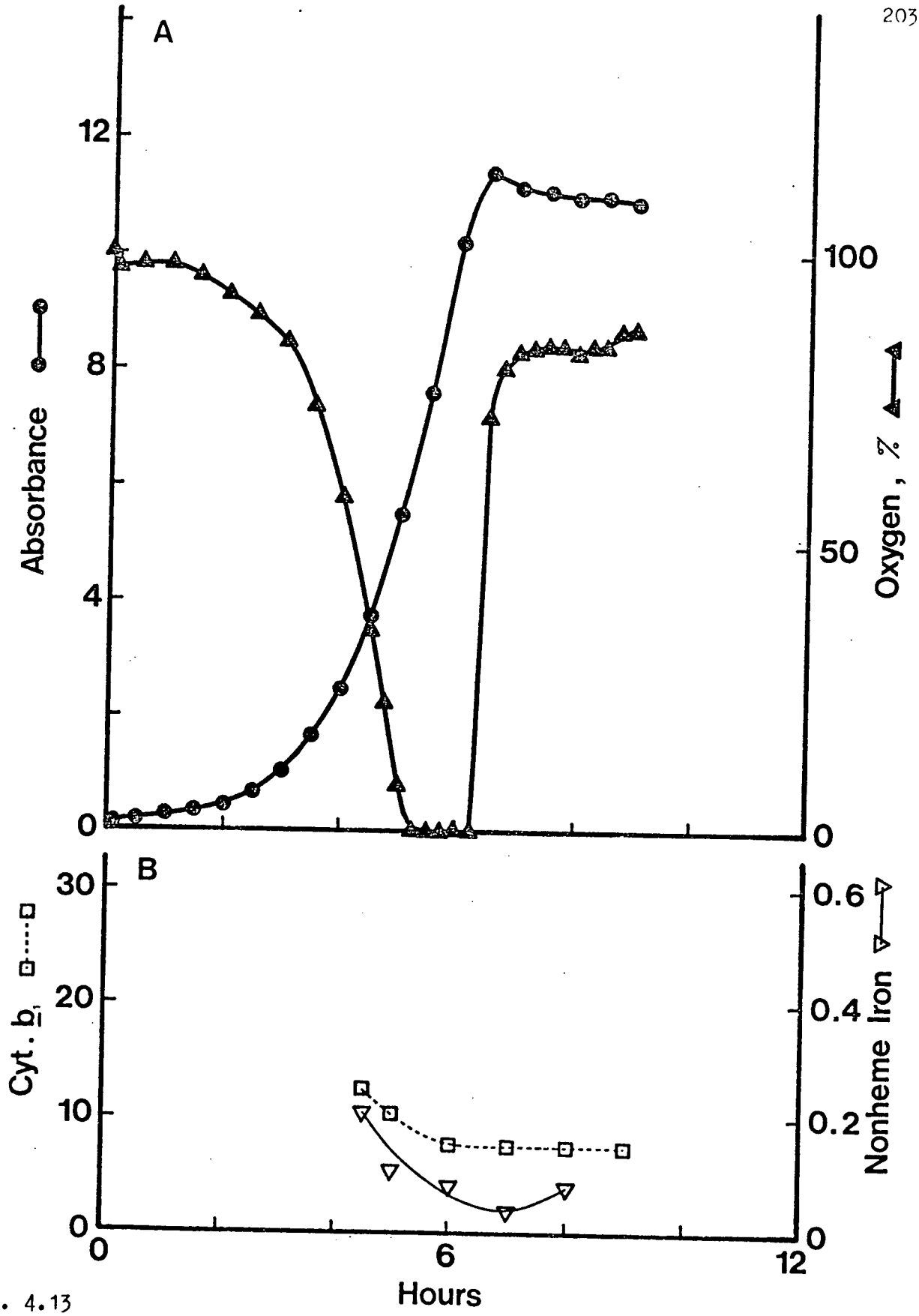

A
12
8
4
0
Absorbance
100
50
0
Oxygen, %
B
30
20
10
0
Cyt. b
0.6
0.4
0.2
0
Nonheme Iron
0
6
12
Hours

Fig. 4.13

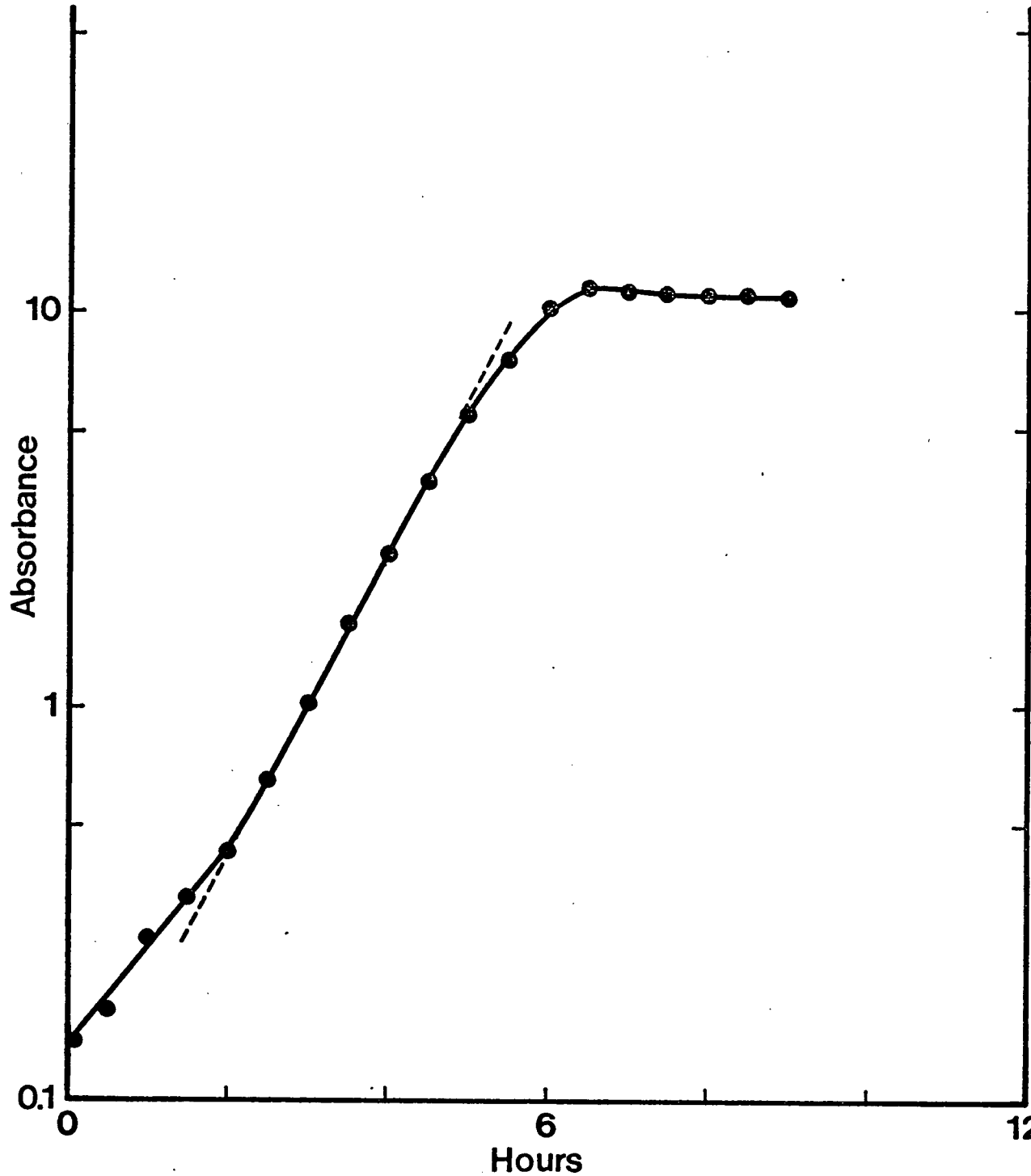


Fig. 4.14 A semi-log plot of the growth data from Fig. 4.13.

possibility was investigated by (i) calculating the conversion of succinate to cell mass prior to, and following the addition of ferric citrate, and (ii) determining the respiratory control ratio of the cells under "iron-limited" and "iron-supplemented" conditions as a measure of the degree of coupling of energy production to the respiratory chain. The results (Figure 4.15) demonstrate that the efficiency of the conversion of succinate to cell mass decreased until the plateau in the level of oxygen saturation was attained, at which point the efficiency became relatively constant. Following the addition of ferric citrate the efficiency increased for a period of 1 hr and then declined. The influence of iron limitation on the respiratory control ratio (RCR) was more dramatic. The RCR decreased progressively during the development of iron limitation. The addition of ferric citrate to the iron-limited culture resulted in a rapid increase in the value of the respiratory control ratio. The rapidity of the increase in the RCR, greater than 280% in the 15 min immediately following the addition of ferric citrate, was comparable to the rate of increase in the level of nonheme iron which increased by greater than 290% over the same time interval (Table 4.1).

These results supported the concept that iron was involved in the coupling of energy production to the respiratory chain and also provided the information required for obtaining "highly coupled" E. coli grown on succinate as the carbon source.

4.2 Discussion

4.2.1 Batch culture versus continuous culture

One of the primary considerations when commencing an investigation of this type is whether to utilize batch or chemostat culturing techniques. As pointed out by Clegg and Garland (1971) and Light and Garland (1971),

Fig. 4.15 The response of growth and oxygen level (A), efficiency (B) and the respiratory control ratio, RCR (C) to the addition of ferric citrate (Fe) to a culture of E. coli growing on 0.6% succinate. Absorbance at 420 nm; 2,4-dibromophenol concentration, ▼, 125 µM; ■, 50 µM; (methods: sec. 2.2.6.1, 2.2.6.2, 2.2.6.3 and 2.2.6.4).

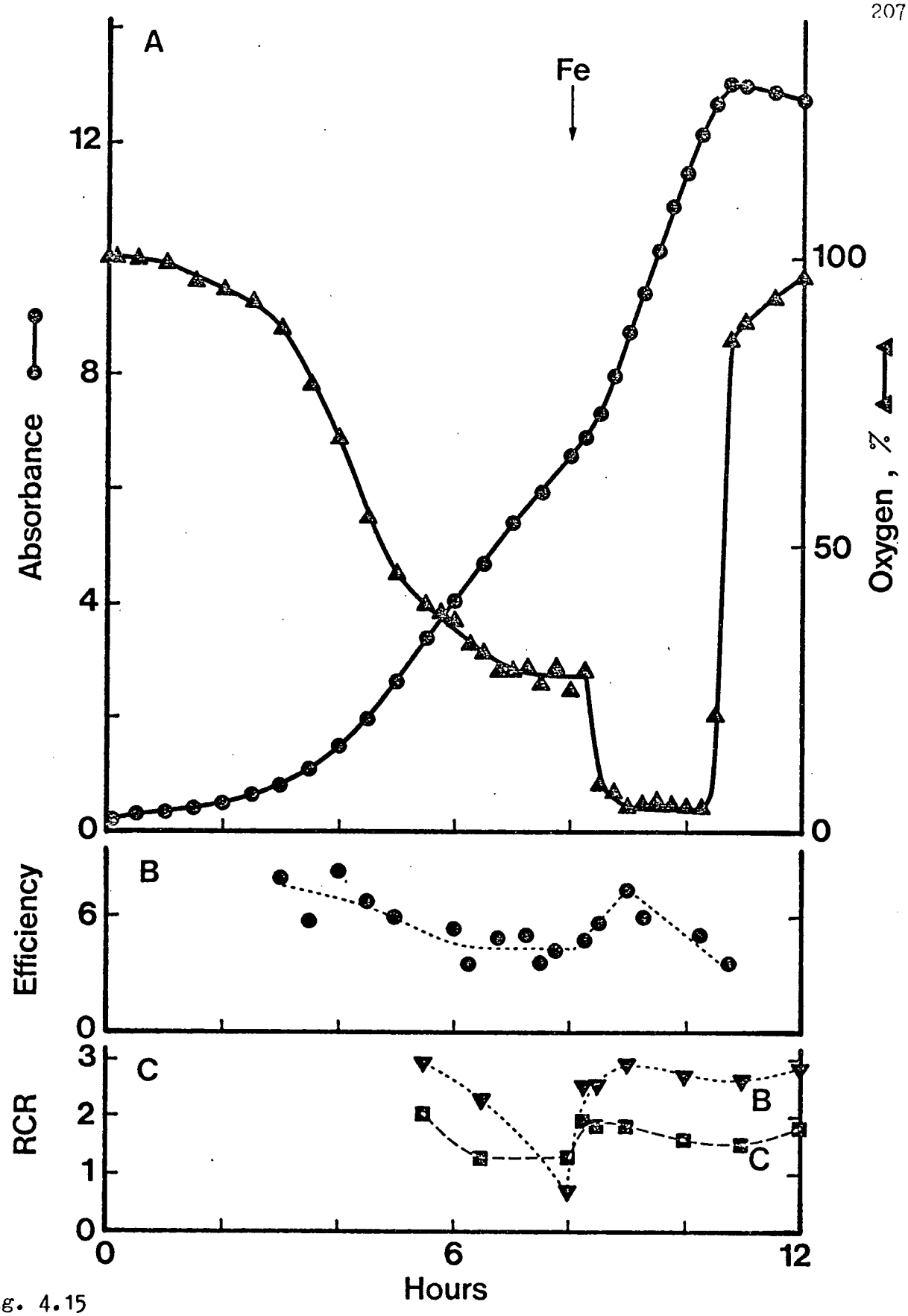

A
Fe
12
8
4
0
Absorbance
100
50
0
Oxygen , %
B
Efficiency
6
0
C
RCR
3
2
1
0
B
C
0
6
12
Hours

Fig. 4.15

the obvious attractions of the batch culture are its simplicity and lack of expense while those of the continuous culture are a reliable, convenient and reproducible supply of cells available at all times. More important, chemostats provide the ability to make accurate and repeatable alterations of the growth conditions leading to changes in respiratory or other functions. Pirt (see Demain and Cooney, 1972) has also pointed out that chemostats extend the range of conditions to be examined since they permit substrate limited growth for an indefinite period. Some of the additional beneficial features of the chemostat include the ability to vary the growth rate without otherwise changing the environment, the maintenance of a constant growth rate while varying other conditions such as temperature, oxygen tension and pH. However, as indicated by Maaløe and Kjeldgaard (1966) the completely undifferentiated behavior of a continuous culture is also a limitation in that the number of observable characters is reduced to a minimum. The relationship between two parameters may be more readily apparent as a result of the transition of conditions occurring in a batch culture. Consequently it is frequently advantageous to use batch cultures in establishing relationships between observed characteristics of the culture, and to utilize the more stringently controlled conditions of continuous culture to examine the relationship of these characteristics in greater detail. The reason that batch cultures were chosen was the ability to measure the change in cellular parameters as the cells depleted the iron content of the medium and the response of these parameters to the rapid re-establishment of iron sufficient growth conditions.

4.2.2 The influence of iron limitation on the respiration and energy-coupling of E. coli

The interpretation of the data presented in section 4.1.1 relies heavily on the results of investigations on the involvement of nonheme iron in site I oxidative phosphorylation of the yeast Candida utilis as reported by Garland and co-workers (Clegg et al., 1969; Light and Garland, 1971; Clegg and Garland, 1971; Haddock and Garland, 1971) and Ohnishi and colleagues (Ohnishi et al., 1969; Ohnishi et al., 1971; Ohnishi et al., 1972a), and on the validity of the respiratory control ratio as an indicator of the extent of coupling of energy conservation to energy generation via the respiratory chain (Hempfling, 1970b). These topics have been discussed in sections 1.6 and 1.4, respectively, and will not be dealt with further at this point.

There is little evidence available that iron is involved in energy-coupling in bacterial systems. Oxidative phosphorylation has been shown to be uncoupled by chelating agents in Azotobacter vinelandii (Jones et al., 1972), Streptococcus faecalis (Faust and Vandemark, 1970) E. coli (Kashket and Brodie, 1963a) and Mycobacterium phlei (Kurup and Brodie, 1967). The energy-dependent transhydrogenase of E. coli is also very sensitive to chelating agents (Bragg and Hou, unpublished data). The data presented in this thesis supports the hypothesis that nonheme iron is involved in energy-coupling in E. coli.

The greater sensitivity to iron deficiency of the growth and respiration of the culture grown on succinate (Figures 4.2, 4.3, 4.5 and 4.15) compared with that grown on glucose (Figure 4.13) is in accordance with the hypothesis that iron limitation affects the respiratory chain and/or coupled energy production since fermentation would provide energy during growth on

glucose.

Lower NADH oxidase, succinate oxidase, and succinate dehydrogenase activities (Figure 4.5, Table 4.2) and lower levels of cytochrome b_1 (Figure 4.3, Tables 4.1 and 4.2) in iron-deficient as compared to iron-supplemented cells, indicates that a direct effect on the respiratory chain must have occurred as has also been observed in iron-deficient Aerobacter indologenes (Waring and Werkman, 1944), Corynebacterium diphtheriae (Pappenheimer and Hendee, 1947; Righelato, 1969; Righelato and van Hemert, 1969), Neurospora crassa (Nicholas and Commissiong, 1957; Padmanaban and Sarna, 1965), Mycobacterium smegmatis (Winder and O'Hara, 1964), Pseudomonas fluorescens (Lenhoff et al., 1956), Spirillum itersonii (Clark-Walker et al., 1967), Micrococcus denitrificans (Imai et al., 1968) and Candida utilis (Light and Garland, 1971; Clegg and Garland, 1971; Ohnishi et al., 1969). Of the four respiratory chain associated parameters cytochrome b_1, NADH oxidase, succinate oxidase and succinate dehydrogenase, the succinate oxidase activity increased at a significantly faster rate than did the other three activities (Figure 4.5, Table 4.2). Since the level of succinate oxidase increased at a faster rate than that of both cytochrome b_1 and succinate dehydrogenase, it was unlikely that the latter components were rate limiting. Thus, since the rate of increase in the level of nonheme iron was more rapid than that of any other respiratory chain associated parameter measured, it is probable that the rate of the succinate oxidase was limited by a nonheme iron component. Such a component is known to be present in this system in E. coli (Kim and Bragg, 1971a). However, the possibility that iron may influence the synthesis of a component not measured, cannot be excluded. In addition to the preceding qualification, the reason that there was no

change in the rate of oxygen utilization during the 15 min immediately following the addition of 6 μM ferric citrate to the iron-limited culture, when the succinate oxidase activity increased by 12% during this time, remains unknown.

An effect of iron limitation on respiratory chain-linked energy production in addition to that on the respiratory chain was suggested by the following observations. During the development of iron limitation the level of nonheme iron per unit cell mass decreased by 70% to a nonheme iron: heme iron ratio of 7.5:1 (Figure 4.3, Table 4.1) and the respiratory control ratio decreased to close to 1.0 (Figure 4.15). The efficiency of conversion of succinate to cell mass decreased to the point at which the availability of oxygen became growth limiting and remained more or less constant at this level (Figure 4.15). Subsequent to the addition of ferric citrate the nonheme iron level (Figure 4.3, Table 4.1) and the respiratory control ratio (Figure 4.15) increased dramatically within the first 15 min. During this interval the increases in the levels of cytochrome $\underline{b}_1$ (Figure 4.3, Tables 4.1 and 4.2) and the respiratory chain associated enzymes (Figure 4.5, Table 4.2) were minimal with a slight decrease observed in the succinate dehydrogenase levels. There was no observable change in the efficiency (Figure 4.15).

Hempfling (1970b) has shown that stimulation of respiration of whole cells of E. coli oxidizing glucose, produced by the addition of 2,4-dibromophenol, occurs only when all three sites of oxidative phosphorylation are coupled. Thus, the similarity of the kinetics of the responses of the level of nonheme iron and the respiratory control ratio, particularly within the first 15 min of the addition of ferric citrate, compared with the much slower rate of increase in the oxidase activities and cytochrome $\underline{b}_1$ level,

is compatible with the hypothesis that nonheme iron has a role in energy conservation linked to the respiratory chain of E. coli. These experiments do not indicate if energy-coupling was effected at one or at all three sites in iron-limited cells of E. coli. At present there is no technique which will permit the measurement of the individual sites of oxidative phosphorylation in E. coli.

The rapidity of the increase in the nonheme iron levels and the RCR values suggests that protein synthesis was not required in the recovery of these parameters. This would agree with the results of Garland's group but not with the recent results reported by Ohnishi et al., (1972a).

If as proposed by Slater (1953) for mitochrondrial systems, the E. coli energy coupling mechanism involves the formation of a high energy intermediate, generated either via the electron transport system or by hydrolysis of ATP via ATPase, which is subsequently utilized to drive membrane associated cellular processes, then in order for iron limitation to have a minimal influence on the growth of E. coli on glucose, the involvement of iron in energy coupling would have to be between the respiratory chain and the high energy intermediate. This is in agreement with the results obtained with Candida utilis in which iron limitation results in the loss of the energy-dependent reversal of electrons from glycerol-1-phosphate to endogenous pyridine nucleotide, a process believed to be driven directly by a high energy intermediate.

The fact that the nonheme iron level continued to increase for an additional hour after, and to a final level 50% greater than its level when the RCR value had reached its maximum, was probably the same phenomenon as observed by Clegg and Garland (1971), that is, that only some 10% of the

total nonheme iron content of the mitochondria of C. utilis would be required to account for coupling at site I. This fact coupled with the observation that the cytochrome b_1 level of iron-limited E. coli, grown on either glucose or succinate, failed to decrease below a level of 7.5 to 8.0 nmoles per gram wet weight of cells while the nonheme iron levels in some of these experiments dropped to levels which were not detectable by the o-phenanthroline colorimetric assay used, suggests that a large portion of the nonheme iron may function as an iron source for heme synthesis. A second possibility that cannot be excluded at this time, is that the high nonheme iron levels may represent iron accumulated within the intact cell, in the process of being metabolized, but not associated with any particular system.

Returning to a consideration of the efficiency of conversion of succinate to cell mass during the development of iron-limited conditions, the decrease in the efficiency was probably due to the decrease in the coupling of energy conservation to the electron transport chain which in turn has been correlated with the decrease in the nonheme iron content of the cell. Following the addition of ferric citrate, however, the efficiency increased slowly, rather than demonstrating the rapid recovery shown by the nonheme iron levels and the degree of coupling of energy conservation to the electron transport system as indicated by the RCR values. The reasons for this remains unclear but may be due to the utilization of the increased energy available from the more "highly coupled" respiratory chain to drive processes such as the transport, the metabolism and/or the redistribution of the iron, or the synthesis of a minor cellular component(s) required before a detectable increase in cell mass could occur.

The subsequent decline in the efficiency of conversion of succinate

to cell mass (commencing at 9.0 hr) seems somewhat anomalous. Since these cells were iron-supplemented, the decrease in the "efficiency" was unexpected as the cells were expected to be well coupled. One would expect the efficiency of conversion of a nonfermentable carbon source to cell mass to be dependent upon the degree of coupling energy production to the respiratory chain. A possible explanation was suggested by the observation that the decline in the "efficiency" coincides with the onset of oxygen limited conditions, as indicated by the level of oxygen saturation and the growth of the culture. The relationship of these parameters as well as the redox potential of the culture is even more apparent in the iron sufficient culture (Figures 4.9 to 4.12).

The conditions of oxygen limitation appeared to influence both the "efficiency" and the cytochrome levels in iron-supplemented (Figure 4.3 and 4.15) and iron-sufficient cells (Figure 4.9 and 4.10). The decrease in growth yield at low oxygen tension has previously been reported with chemostat cultures of Aerobacter aerogenes (Harrison and Pirt, 1967; Harrison and Loveless, 1971) and E. coli (Dr. D. G. Kilburn, personal communication; Harrison and Loveless, 1971) and has been shown to occur concurrently with an increased rate of respiration. Harrison and Pirt (1967) have likened the effect of low oxygen tension on the growth yield and the respiration rate to that of an uncoupler of oxidative phosphorylation. Thus, the observed decrease in the efficiency of conversion of succinate to cell mass (Figure 4.10 and 4.15) is probably due to the "uncoupling" action of low oxygen tension.

Although apparent in both the iron-supplemented and the iron-sufficient cultures the influence of oxygen limitation was most apparent in

latter (Figure 4.9). Within half an hour of the onset of oxygen-limited growth the cytochrome $\underline{a}_2$ and cytochrome $\underline{b}_1$ levels began to increase markedly. Similar observation of increased levels of cytochromes at low oxygen tensions have been reported for Pseudomonas fluorescens (Lenhoff et al., 1956), Spirillum itersonii (Clark-Walker et al., 1967), Azotobacter vinelandii (Nishizawa et al., 1971), and Aerobacter aerogenes (Harrison, 1972). It is generally proposed that the reason for the increased level of cytochromes would be to make the small amounts of oxygen present at low oxygen tensions more readily available to the reducing systems of the cells. The induction of a cytochrome oxidase, such as cytochrome $\underline{a}_2$ at low oxygen tensions is generally assumed to be due to the possession of a lower K_m for oxygen. Thus the reduced efficiency of conversion of succinate to cell mass could occur either by an "uncoupling" mechanism as implied by Harrison and co-workers or via the utilization of an alternative respiratory chain sequence related to the induction of cytochromes $\underline{b}_1$ and $\underline{a}_2$.

With the equipment utilized it was not possible to determine the in situ respiration rates. Thus it is not known whether there was a corresponding increase in respiration with the decrease in "efficiency". However, since there was no apparent accumulation of intermediates of succinate oxidation, as indicated by a single phase of oxygen utilization, an increase in respiration rate probably did occur. Therefore, the decrease in efficiency of the iron-supplemented and iron-sufficient cultures under conditions of oxygen limitation was probably due to the "uncoupling" action of low oxygen tension. It is impossible to exclude the possibility that the efficiency of conversion of succinate to cell mass may have been influenced by iron at a level other than the generation and coupling of energy

conservation.

While there was a rapid recovery of nonheme iron levels and energy coupling (as indicated by the respiratory control ratio) within the first 15 min following the additon of ferric citrate, there was no detectable increase in either the rate of oxygen utilization or the rate of growth (Figure 4.3). The 15 to 30 min lag prior to the increase in rate of oxygen utilization suggested that the synthesis of some component, perhaps a protein, was required in order for respiration and/or growth to be stimulated by iron. Support for the concept of a requirement for protein synthesis was obtained when it was discovered that the addition of ferric citrate late in the growth of a nitrogen-limited culture (Figure 4.7) did not stimulate respiration or growth. The subsequent addition of $(NH_4)_2SO_4$ stimulated both growth and respiration following the usual 15 min lag period. However, it was subsequently discovered that if the iron-deficient medium was supplemented with mixture of trace metals other than iron (Ca^{++}, Zn^{++}, Cu^{++}, Mn^{++} and Co^{++}), the results obtained were strikingly different (Figure 4.8). There was an initial plateau in the oxygen level at approximately 40% of saturation. This was followed by a second plateau at an oxygen saturation of 25%. Addition of ferric citrate to the culture during the second plateau resulted in an immediate decrease in the level of oxygen saturation of the medium. There was no 15 min lag prior to the increase in the rate of oxygen consumption. However growth appeared to demonstrate the usual lag prior to attaining the new growth rate. The increase in the rate of oxygen utilization was far too rapid to have involved de novo protein synthesis. A possible explanation of the two plateau phases and the rapid response in oxygen utilization to the addition of ferric ion may involve a functional replacement of the ferric

ion under conditions of iron limitation, by one of the trace metals. Therefore, the first plateau probably represents a decreased respiration rate due to iron limitation at a particular site in the respiratory chain, as observed previously (Figures 4.3, 4.5 and 4.15). The second phase of oxygen consumption could be due to the substitution for iron of one of the other trace metals at the site(s) at which iron is limiting, resulting in the synthesis of a metal compound with a lower functional activity than the corresponding iron compound. The development of the plateau would then be due to the depletion of this trace metal from the medium. When ferric citrate was added to the medium there may have been a rapid exchange of ferric ion with the less functional cation resulting in an immediate increase in the rate of oxygen utilization.

When beginning the discussion of the influence of iron on the respiration of E. coli it was indicated that interpretation of the results relied heavily upon the report by Hempfling (1970b) that the stimulation of respiration of whole cells of E. coli oxidizing glucose produced by the addition of 2,4-dibromophenol occurred only when all three sites of oxidative phosphorylation were functional. Thus, the respiratory control ratio (RCR) defined as the rate of respiration in the presence of DBP relative to the rate of respiration in the absence of this compound, can be utilized as an indication of the degree of coupling of at least one site of energy conservation. Gutnick, (personal communication) has subsequently confirmed Hempfling's results.

However, during initial attempts to utilize this technique a number of anomalous observations were made. When glucose-dependent respiration rates, in the presence and absence of dibromophenol were determined at 37°C,

instead of room temperature as reported by Hempfing (1970b), although a transient stimulation of respiration was observed in a few instances, the majority of the samples showed no stimulation of respiration. Only a progressive inhibition of respiration was observed. A possible explanation for the decrease in the stimulation of respiration and the increase in the inhibition observed at 37°C may be that DBP has a biphasic action on electron transfer, a stimulation and an inhibition, with the latter possessing the greater temperature coefficient.

More difficult to understand was the observation that if glucose was replaced by succinate, a substrate which must generate the majority of its energy *via* the electron transport chain, and the respiration rates in the presence and absence of DBP were determined, then DBP at concentrations as high as 1 mM had no effect on the respiration rate 37°C. However, when the same measurements were performed at room temperature, the respiration rate was inhibited at DBP concentrations as low as 50 μM. This raises two questions: (i) Why is the succinate-dependent respiration of intact *E. coli* inhibited by DBP at room temperature but not at 37°C, when the reverse is true for glucose-dependent respiration? (ii) Why is the succinate-dependent respiration of *E. coli* not stimulated by DBP when the glucose-dependent respiration is stimulated? Cavari *et al.*, (1967) have reported similar observations with respect to the influence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) on glucose-, pyruvate-, succinate- and glutamate-dependent respiration of *E. coli*. Preincubation of *E. coli* with CCCP inhibited the succinate-, pyruvate- and glutamate-dependent respiration but stimulated the glucose-dependent respiration. Thus, the results appear to be characteristic of the interaction of uncouplers with *E. coli*. Cavari

et al., (1967) also observed that preincubation of E. coli with CCCP and a respiratory substrate prevented the inactivation of the respiratory chain. Of interest to this problem was the demonstration by Lo et al., (1972b) that the accumulation of succinate by E. coli was inhibited by uncoupling agents. This suggested that a possible explanation for the first question raised could be as follows. At 37°C sufficient succinate may have been accumulated prior to the addition of DBP to protect the electron transport chain from inhibition by DBP and also maintain the respiration rate. Whereas at room temperature the succinate accumulated prior to the addition of DBP may not have been sufficient to protect the electron transport chain and/or to maintain the respiration rate. Why the DBP does not stimulate the respiration at 37°C is unclear, unless the "protection" of the respiratory chain via succinate prevents either stimulation or inhibition.

Although the preceding is a possible explanation, considerably more information is required on the influence of uncouplers such as DBP on the respiration of intact bacteria inorder to clarify these problems and to validate the emphasis placed on the RCR values.

There are a number of questions which have arisen directly or indirectly from the research into the influence of iron on the respiration of E. coli. Detection of the location of the site responsible for the limitation of the respiration rate under conditions of iron deficiency should be possible through the determination of low temperature difference spectra of E. coli obtained by rapid sampling prior to, and immediately following the addition of ferric citrate to the iron-limited culture.

The function of the "excess" nonheme iron is of considerable interest. Location of the iron in the cell membrane, cell wall or cytoplasm is readily

determined by cell fractionation techniques. Localization of the nonheme iron would provide some indication as to its metabolic state and function. The sensitivity of the assay for nonheme iron could be increased by using the radioactive isotope of iron, ^{59}Fe (Clegg and Garland, 1971). The possibility that a considerable proportion of the nonheme iron functions as a precursor of cytochromes could be examined by adding a quantity of ^{59}Fe-labelled ferric citrate to the medium such that it would be taken up into the nonheme iron prior to the induction of significant cytochrome synthesis. The hemes of the cytochromes synthesized subsequently could be examined for the appearance of radioactivity due to ^{59}Fe. Also of considerable interest would be the determination of the EPR spectra of the E. coli membranes during the development of, and recovery from conditions of iron limitation. This would be of particular interest if determination of the redox potential of the signals were also performed by the technique of Ohnishi et al., (1972b). Thus, information on the relative order of the nonheme iron proteins involved with the respiratory chain of E. coli would be provided.

The question of the requirement of protein synthesis following the addition of ferric citrate and prior to the increased rates of oxygen consumption and growth could readily be tested via blocking protein synthesis with chloramphenicol. A failure to prevent the usual increase in respiration rate would indicate that protein synthesis was not required. Failure to obtain an increase in the rate of respiration in the presence of chloramphenicol could not be taken as conclusive evidence for a requirement for protein synthesis. Under these circumstances the rate of incorporation of a radioactivity labelled amino acid into the acid insoluble fraction of the cell, following the addition of ferric citrate to the culture, would have

to be used in order to provide a conclusive answer.

The role of the trace elements in the response of the growth and respiration of the iron-limited culture of E. coli is difficult to investigate. The trace metal involved should be identifiable by selective addition of trace metals to the culture medium. This may provide the necessary lead for further investigations.

On considering the investigation of the respiration of bacteria and its control, it becomes apparent that there has been no attempt to investigate the relationship between the biosynthetic energy demand and the respiration rate throughout the bacterial cell cycle. This would seem to be a logical starting point in any attempt to demonstrate the presence of respiratory control in the bacterial cell.

5 PART III: THE TRANSITION FROM AEROBIC TO ANAEROBIC GLUCOSE UTILIZATION

5.1 Results and discussion

5.1.1 The lag in acid production by E. coli associated with the transition from aerobic to anaerobic glucose utilization

During preliminary experiments on the simultaneous measurement of acid production and oxygen consumption (sec.2.2.5.3) by E. coli oxidizing glucose, it was observed that concurrent with the depletion of oxygen from the assay medium, there occurred a 15 to 30 sec lag in acid production. Following the "lag" period, acid production resumed at a rate nearly twice that observed during aerobic glucose utilization (Figures 5.1 and 5.2). These observations were not pursued further due to the necessity of completing other research in progress. Subsequently identical information, but of a more extensive nature, was retrieved from the literature (Hempfling et al., 1967). These researchers reported that the cessation in acid production occurred concurrently with:

(i) a cessation in the uptake of K^+ ions,

(ii) a cessation of glucose utilization,

(iii) a rapid decrease in the levels of glucose-6-phosphate and fructose-6-phosphate,

(iv) increased levels of dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and fructose-1,6-diphosphate,

(v) slight increases in the levels of 3-phosphoglyceric acid and 2-phosphoglyceric acid,

(vi) constant levels of PEP and pyruvate,

(vii) a marked decrease in citrate and isocitrate levels,

(viii) a slight increase in the level of malate,

(ix) a marked decrease in the level of NAD^+, presumably due to the form-

Fig. 5.1 Oxygen consumption and acid production during aerobic and anaerobic utilization of glucose by intact E. coli.

E. coli cells, grown and harvested as described in sec. 2.2.2.1 were resuspended 1:80 (w/v) in 0.85% NaCl and were incubated at 37°C for 10 min. The cells were sedimented by centrifugation for 5 min at approximately 2100 x g at 4°C. The supernate was removed and the cells were washed once with 3 mM glycylglycine-KOH buffer, pH 7.0. The cell pellet was resuspended in the same buffer at a cell concentration of 1:5 (w/v).

The assay system consisted of 2.8 mM glycylglycine-KOH buffer, pH 7.0, 25 mM glucose, and 0.5 mM KCl in a final volume of 4.0 ml. Oxygen tension was measured with the Aminco oxygen electrode. C: 40 mg cells; ●: oxygen; ▲: pH.

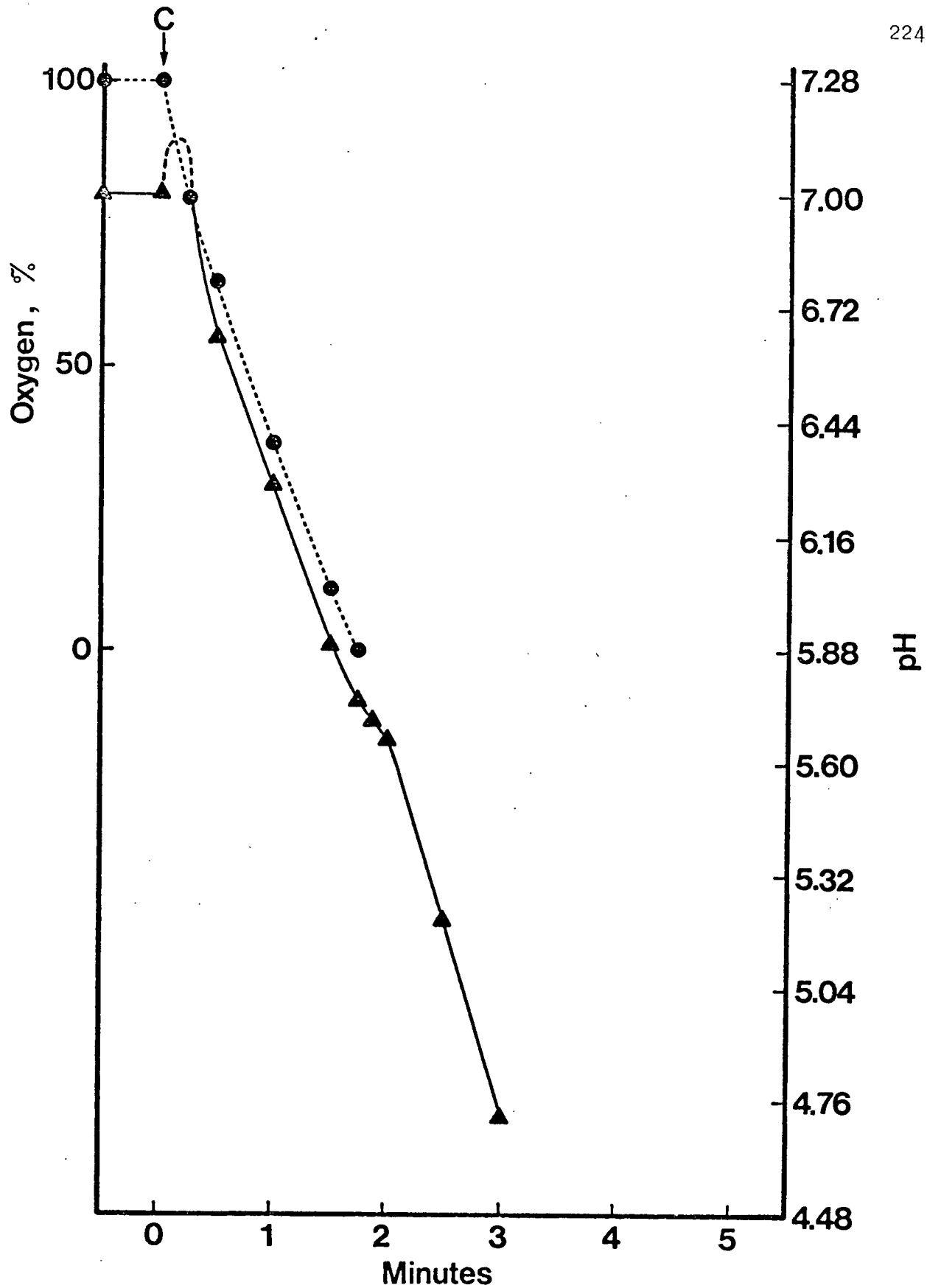

C
100
50
0
Oxygen, %
7.28
7.00
6.72
6.44
6.16
5.88
5.60
5.32
5.04
4.76
4.48
pH
0
1
2
3
4
5
Minutes

Fig. 5.1

Fig. 5.2 Oxygen consumption and acid production during aerobic and anaerobic utilization of glucose by Tris-EDTA permeabilized E. coli.

E. coli cells, grown and harvested as described in sec. 2.2.2.1, were suspended 1:80 (w/v) in 0.14 M Tris-HCl, pH 8.0 containing 0.1 mM EDTA and were incubated at 37°C for 10 min. The cells were sedimented by centrifugation for 5 min at approximately 2100 x g at 4°C. The supernate was removed and the cells were washed once with 3 mM glycylglycine-KOH buffer, pH 7.0. The cell pellet was resuspended in the same buffer at a cell concentration of 1:5 (w/v).

The assay system consisted of 2.8 mM glycylglycine-KOH buffer, pH 7.0, 25 mM glucose, and 0.5 mM KCl in a final volume of 4.0 ml. Oxygen saturation was measured with the Aminco oxygen electrode. C: 40 mg cells; ●: oxygen; ▲: pH.

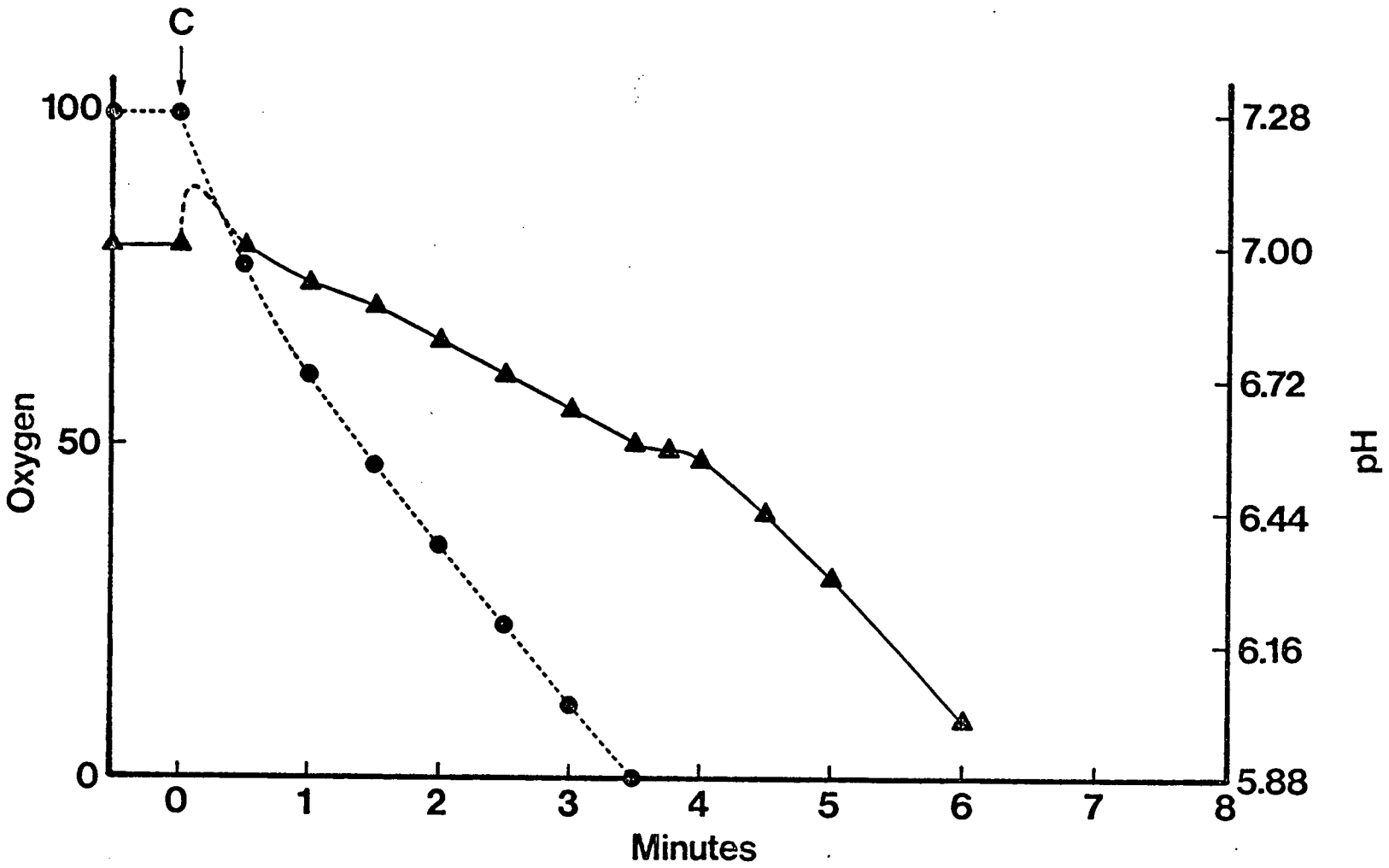


Fig. 5.2

ation of NADH (Estabrook et al., 1962), and

(x) a marked decrease in the level of ATP with a corresponding increase in ADP and a slight decrease in AMP levels.

Inorganic phosphate levels remained constant during this period. On the basis of these and other results Hempfling et al., (1967) proposed that the control of glycolysis in E. coli was mediated by changes in the relative activities of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase. At a given time, the identity of the particular enzymatic step determining the flux of glucose depends on the chemical environment (i.e., concentration of specific modifiers of enzymatic activity) within the cell. However, in attempting to explain their observations during the transition from aerobic to anaerobic glucose oxidation on the basis of this proposal, Hempfling et al., (1967) relied greatly upon the information available from the corresponding yeast system. Such an extrapolation is not valid (sec.1.2).

Re-examining the data of Hempfling et al., (1967) and Estabrook et al., (1962), in terms of present knowledge of the regulation of the amphibolic pathways of E. coli (sec.1.2), the most dramatic changes observed on the depletion of oxygen from the assay system were:

(i) the rapid reduction of NAD^+,

(ii) the marked decrease in the level of ATP,

(iii) the accumulation of fructose-1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, and

(iv) the decrease in the levels of glucose-6-phosphate and fructose-6-phosphate.

The high level of NADH possibly explains the accumulation of fructose-1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, due

to the fact that the NADH generated at glyceraldehyde-3-phosphate dehydrogenase was not oxidized. The decline in ATP levels may be due to the absence of oxidative phosphorylation under anaerobic conditions but is more probably due to the cessation of glycolysis resulting from the low levels of NAD^+. Decreased levels of ATP would in turn remove the inhibition from phosphofructokinase and explain the decrease in glucose-6-phosphate and fructose-6-phosphate, as well as contributing to the build up of fructose-1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The lack of build up in PEP and pyruvate as well as 2-phosphoglyceric acid and 3-phosphoglyceric acid would also be explained by the inhibition of glyceraldehyde-3-phosphate dehydrogenase as a result of the lack of NAD^+. The occurrence of the blockage of glycolysis at glyceraldehyde-3-phosphate dehydrogenase is also supported by the lack of phosphate uptake during the lag in acid production. Failure of glucose uptake probably resulted from low levels of PEP.

Thus in the "lag" all evidence supports the interpretation that the primary event is an inhibition of glycolysis at glyceraldehyde-3-phosphate dehydrogenase resulting from the unavailability of NAD^+. The results indicate that NADH is oxidized following the lag but by what system, and why wasn't there a smooth transition? How is the anaerobic NADH oxidation system initiated? The most likely candidate to initiate the oxidation of NADH would be the level of NADH. The results suggest an important role for the $NADH/NAD^+$ ratio (Sanwal, 1970a), or the NADH level as proposed by Wimpenny and Firth (1972), in the control of metabolism in E. coli. The involvement of the $NADH/NAD^+$ ratio in the regulation of the pyruvate dehydrogenase complex has been demonstrated by Shen and Atkinson (1970).

What system is responsible for the anaerobic oxidation of NADH? Considering, first of all, the acids produced aerobically and anaerobically, the acid accumulating aerobically is probably acetate (Holms and Bennett, 1971). As to the nature of the acid produced anaerobically, examination of Figure 1.5 and table 1.1 indicates that anaerobically there are but three possible reactions for the further metabolism of pyruvate which do not either generate NADH or directly require oxygen. These reactions are (i) the soluble, NAD^+-requiring, D-lactate dehydrogenase (reaction 38), (ii) the D-lactate dehydrogenase of the particulate D-lactate oxidase system (reaction 39), and (iii) the malic enzymes (reaction 20). Of these possibilities the equilibriums of the latter two favor the formation of pyruvate. Also the $NADP^+$-requiring malic enzyme is allosterically inhibited by NADH. As there was no increase in the level of pyruvate during the "lag", to shift the equilibrium toward the formation of either D-lactate *via* the reversal of the particulate D-lactate dehydrogenase, or to malate *via* the reversal of the NAD^+-requiring malic enzyme, this would suggest that, under the conditions of the assays, pyruvate is reduced to lactate *via* the soluble D-lactate dehydrogenase, the equilibrium of which favors the formation of D-lactate. Therefore, D-lactate acid would be the acid expected to accumulate anaerobically. Since the soluble D-lactate dehydrogenase of *E. coli* is an NAD^+-requiring enzyme, this would be the most probable system for the anaerobic oxidation of NADH. Tarmy and Kaplan (1968) investigated the kinetics of NADH oxidation *via* the soluble D-lactate dehydrogenase and concluded that they were of the Michaelis-Menten type with a K_m for NADH of 7×10^{-3} M. However, no attempt was made to evaluate the response of the enzyme to the $NADH/NAD^+$ ratio.

The reason(s) for the lack of smooth transition from aerobic acid production to anaerobic acid production is unclear. To investigate the possibility that it might be related to nucleotide levels, particularly pyridine and adenine nucleotides, E. coli cells were permeabilized with Tris-EDTA according to the technique of Leive (1965). Permeabilization of E. coli with Tris-EDTA has been demonstrated to cause the release of the nucleotide pool, and the breakdown of ribosomal RNA (Figure 5.3) (Leive and Kollin, 1967; Neu et al., 1966; 1967). The results were inconclusive. The cessation of acid production was of longer duration in the Tris-EDTA treated cells (Figure 5.2) than in those preincubated in 0.85% NaCl (Figure 5.1), but was not of an indefinite duration. This suggested that if pyridine nucleotides were involved as was presumed, they must not be completely released as a result of the permeabilization procedure. This raises the possibility of compartmentation.

A possible explanation for the increased duration of the "lag" in the Tris-EDTA treated cells is that the metabolism of these cells as indicated by the rate of oxygen consumption and acid production (Figure 5.2) is considerably slower than observed with the cells preincubated in 0.85% NaCl (Figure 5.1). Consequently, the extended duration of the "lag" may be due to the greater amount of time required to produce the concentration of NADH, or the NADH/NAD^+ ratio, required to initiate the anaerobic oxidation of NADH. Definite answers to the questions raised await further research.

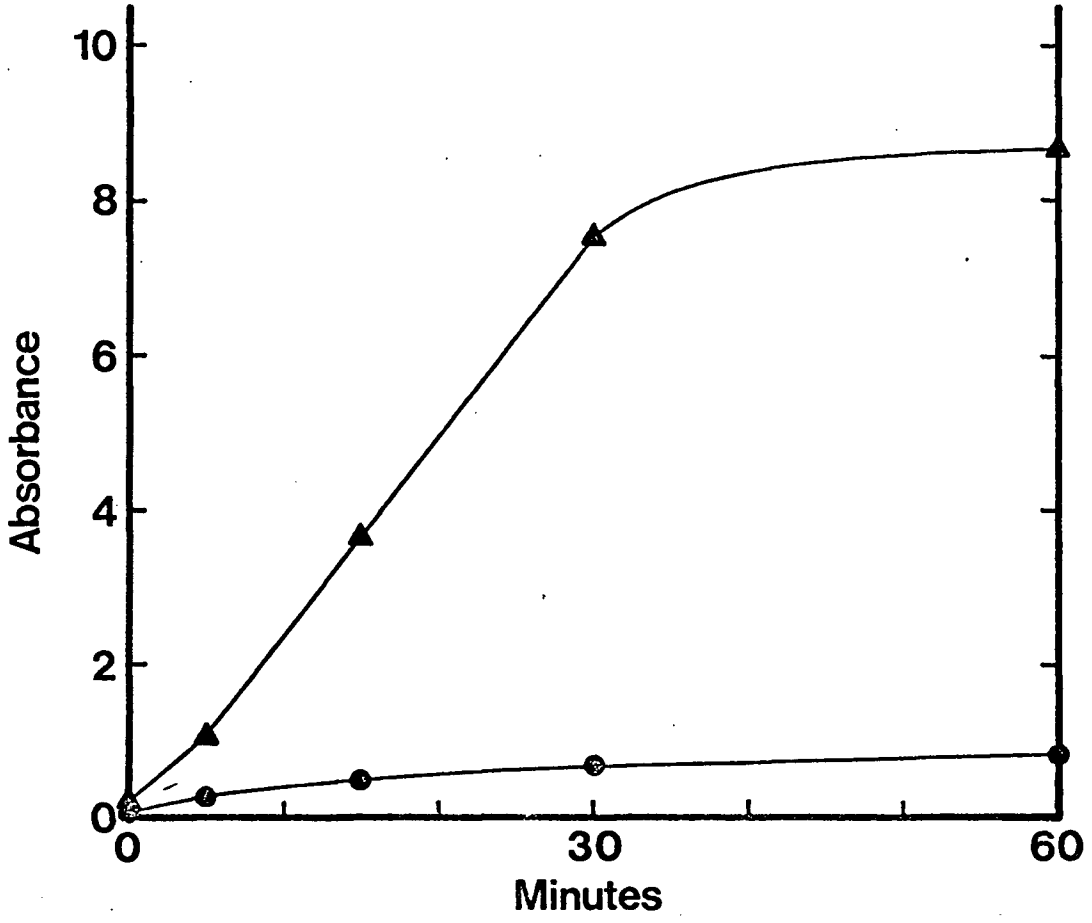


Fig. 5.3 The release of ultraviolet absorbing material from E. coli. E. coli cells, grown and harvested as described in sec. 2.2.2.1 were resuspended 1:80 (w/v) in 0.85% NaCl (●) or 0.14 M Tris-HCl, pH 8.0 containing 0.2 mM EDTA (▲) and were incubated at 37°C. Ten millilitre aliquots were removed at predetermined intervals, and the cells were immediately sedimented at 4,300 x g for 10 min at 4°C. The supernates were removed carefully and their absorbance was measured at 260 nm.

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APPENDIX A

The relationship between the absorbance, at 420 nm, of a culture of *E. coli* and the cell mass, measured as wet weight of cells (Figure A).

The cells were grown and harvested as described in sec. 2.2.6.1 with 0.6% succinate as carbon source. The data reported were obtained from duplicate experiments.

Absorbance (at 420 nm)	Wet Weight of Cells (per 100 ml)
3.08	0.20
4.69	0.38
6.22	0.55
7.44	0.68
8.57	0.80
9.64	0.90
10.44	0.95
3.65	0.32
5.27	0.48
6.78	0.65
8.17	0.80
9.38	0.90
10.36	1.00
10.84	1.02

The regression line for the combined data was

$$y = 0.103(x) - 0.081$$

where x is the absorbance at 420 nm, and y is the wet weight of cells per 100 ml expressed in grams.

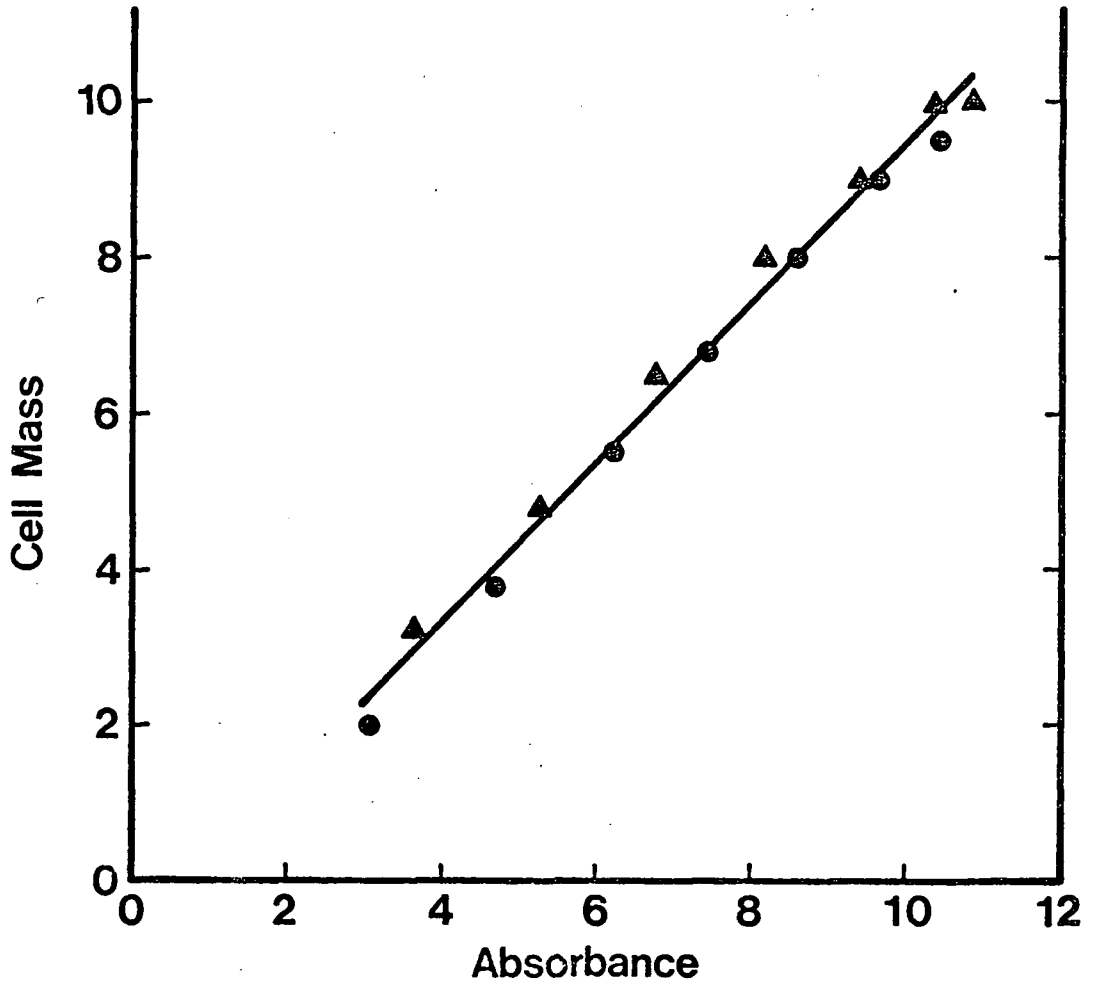

Fig. A The relationship between the absorbance, at 420 nm, of a culture of E. coli and the cell mass, measured as wet weight of cells. Units: cell mass, g/l of culture; ● : experiment 1; ▲ : experiment 2.

APPENDIX B

The influence of buffer concentration and buffer ion on the color development of the Lowry method of protein determination.

Standard protein solutions were prepared by dissolving weighed quantities of bovine serum albumin in the buffers indicated. Protein was assayed according to the standard procedure of Lowry et al., (1951). Absorbance was determined at 500 nm and/or 750 nm dependent upon the intensity of the color present. The appropriate buffer solution was assayed for color development concurrently with the protein standards prepared in that buffer, and was employed as the reference solution during the determination of the absorbance of the color developed by the protein standards.

Buffer	Concentration (mM)	Protein (ug)	A_{500}	A_{750}
Distilled water	---	7.5		0.028
		22.0		0.080
		37.0		0.134
Glycylglycine-KOH,pH7.0	1	7.5		0.030
		22.0		0.082
		37.0		0.137
	3	7.5		0.030
		22.0		0.083
		37.0		0.136
	5	7.5		0.030
		22.0		0.083
		37.0		0.138
	10	7.5		0.031
		22.0		0.084
		37.0		0.140
	30	9.0		0.037
		26.5		0.097
		44.5		0.160
	50	9.0		0.034
		26.5		0.092
		44.5		0.157
	100	9.0		0.034
		26.5		0.092
		44.5		0.153
	300	9.0		0.027
		26.5		0.066
		44.5		0.110
	500	9.0		0.013
		26.5		0.043
		44.5		0.076

Buffer	Concentration (mM)	Protein (ug)	A_{500}	A_{750}
HEPES-KOH,pH7.0	3	13.5	0.012	
		40.0	0.058	
		72.0	0.112	
	30	13.5	0.026	
		40.0	0.074	
		72.0	0.111	
	50	13.5	0.050	
		40.0	0.082	
		72.0	0.115	
	300	13.5	0.037	
		40.0	0.074	
		72.0	0.080	
PIPES-HCl,pH7.0	300	13.5	0.006	0.012
		40.0	0.012	0.028
		72.0	0.030	0.056
TES-KOH,pH7.0	300	13.5		0.060
		40.0		0.144
		72.0		0.258
Tris-HCl,pH7.0	300	13.5		0.024
		40.0		0.069
		72.0		0.125

Color development of buffer solutions (no protein present)

Buffer	Concentration(mM)	A_{500}	A_{750}
Water	---	0	0
HEPES-KOH,pH7.0	300	1.101	---
MOPS-KOH,pH7.0	300	0.057	---
PIPES-HCl,pH7.0	300	0.150	---
TES-KOH,pH7.0	300	---	0.073
Glycylglycine-KOH,pH7.0	300	---	0.028
Tris-HCl,pH7.0	300	---	0.109

Conclusions:

(i) all the buffers investigated gave significant color development in the absence of protein;

(ii) all the buffers, with the exception of TES-KOH, suppressed or reduced the color development of the protein standards.

APPENDIX C

Composition of the trace element solution (Figure 4.8).

Compound	Concentration (M)	
	Stock Solution	Medium
$CaCl_2 \cdot 6H_2O$	3.3×10^{-3}	3.3×10^{-6}
$ZnCl_2$	6.0×10^{-4}	6.0×10^{-7}
$CuSO_4 \cdot 5H_2O$	6.0×10^{-4}	6.0×10^{-7}
$MnCl_2 \cdot 4H_2O$	6.0×10^{-4}	6.0×10^{-7}
$CoCl_2 \cdot 6H_2O$	7.0×10^{-4}	7.0×10^{-7}
$Na_2 \cdot EDTA \cdot 2H_2O$	6.4×10^{-2}	6.4×10^{-5}