

STUDIES ON THE EFFECT OF ASCORBIC ACID ON ESCHERICHIA COLI AND ITS
BACTERIOPHAGE, T7

BY

HOLLY ELIZABETH RICHTER

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ABSTRACT

Ascorbic acid has been implicated as an antiviral agent by several reports and the effect of ascorbic acid on a simple Escherichia coli bacteriophage, T7, was reviewed in this report in an attempt to extend and corroborate the literature data. A modified plating procedure incorporating a liquid incubation of the bacterial-phage mixture prior to plating revealed, contrary to earlier reports, that ascorbate treatment of the doubled stranded DNA containing phage, T7, was not lethal except after extended treatment times or after treatment with high concentrations of ascorbic acid. Only single strand nicks were observed in the phage DNA after ascorbate treatment except after heat treatment and then double strand nicks accumulated. Following ascorbate treatment there was a delay, proportional to the extent of nicking, in the replicative cycle of the phage, manifested by a delay in the formation of the large molecular weight concatemeric DNA intermediate. This delay in the replicative cycle allowed time for repair of the nicks and subsequent production of viable phage from DNA with as many as 20 or more nicks, but required a period of liquid incubation. Catalase inhibited the ascorbate induced nicking of DNA except after longer treatments. Therefore the use of ascorbic acid as an antiviral agent against viruses containing double stranded nucleic acid is questionable although it could be effective against viruses containing single stranded nucleic acid.

When one considers the current widespread use of ascorbic acid by humans and the importance of the balance of resident bacteria populations to the human body, the lack of information regarding the effect of ascorbic acid on bacteria represents a striking gap in the literature on ascorbate. Therefore, its effect on the metabolism of E.coli, a common inhabitant of the human digestive system, was investigated. When ascorbic acid was added to the growth medium of Escherichia coli the cellular response was complex and depended upon the carbon source. In all media there was a substantial induction of catalase synthesis ranging from a two-fold increase in succinate-salts medium to a seven-fold increase in LB medium. This induction was inhibited by chloramphenicol showing that protein synthesis was necessary. Hydrogen peroxide, a product of ascorbate oxidation, caused a similar induction, implicating it as the active species in catalase induction by ascorbate. The presence or absence of glucose or cAMP in the growth medium did not affect either the basal level or the extent of catalase induction even in an adenylate cyclase mutant, leading to the conclusion that classical catabolite repression was not involved in catalase gene expression.

Immediately following ascorbate addition, there was a rapid drop in the basal levels of catalase in all media which was reversed more quickly in media containing amino acids. Histidine acting as a metal chelator slowed the oxidation of ascorbate and thereby reduced the amount of inhibition. Because hydrogen peroxide did not elicit the same response, an ascorbate oxidation product was the inhibitory species and it was most active in a membrane mediated mechanism.

In aerobic media, ascorbate caused a pause in growth whereas in the presence of cyanide, the growth rate was stimulated and in anaerobic LB and anaerobic glycerol-fumarate media the extent of growth was enhanced. These phenomena were attributed to the reducing ability of ascorbate which, depending on growth conditions could either stimulate or inhibit cellular respiration. Respiration in the presence of cyanide was stimulated by ascorbate and normal respiration was inhibited although the carbon source affected the degree of the effect.

Cultures of E. coli growing in glucose-salts, lactose-salts and maltose-salts media exhibited a greater sensitivity to ascorbate than did cultures growing on any other carbon sources and it was found that the apparent uptake of glucose, but not of glycerol, by whole cells was inhibited by ascorbate. Ascorbate itself was not taken up by the cells.

Ascorbic acid did not affect the levels of superoxide dismutase in E. coli.

DEDICATION

To Mom, Dad, Pat, Chris, Jay, Liz and Mary and to Bruce.

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INTRODUCTION

The effects of chemicals on all aspects of life is common reality. Of particular interest currently is the effect of acid rain on lakes, streams, soils and all living things growing in them. The effects of toxic chemical wastes in our drinking water and soil can become a part of the food we eat and be introduced into our bodies. Therefore, studies on how various chemicals do effect living (and non-living) systems are important.

Ascorbic acid is a necessary part of our diet. It is the active ingredient in fruit which was eaten to prevent scurvy many years ago and it is thought that megadoses of vitamin C are able to cure the common cold and cancer (Pauling, 1970).

Due to its chemistry, a strong reducing ability and highly ionic and free radical properties, ascorbate would be expected to have a multiplicity of effects on living systems. In the presence of divalent cations ascorbate is able to be oxidized to dehydroascorbate and H_2O_2 (Weissberger and Luvalle, 1944). Hydrogen peroxide can subsequently be oxidized to the hydroxyl radical (Haber and Weiss, 1934). It is the hydroxyl radical which is known to be able to interact with the DNA backbone to cause nicking (Morgan et al, 1976) and Murata and coworkers concluded that this was the lethal or inactivating mechanism of ascorbate (Murata et al, 1971; Murata and Uike, 1973; Murata, 1975). However, this conclusion conflicted with other reports showing that single strand nicking was not lethal to bacteria and bacteriophage (Yoakum, 1975; Freifelder,

1965; Amanthaswamy et al., 1979; Stephan, 1973; Pao and Speyer, 1973; Amanthaswamy and Eisenstark, 1976).

Ascorbate also inhibits catalase (Orr, 1966, 1967a and b) but since the oxidation of ascorbate results in the production of H_2O_2 it was thought that ascorbate may be able to induce catalase synthesis in cultures of Escherichia coli. This was found to be the case and resulted in a good system in which to study and partially characterize catalase gene expression.

The subject of this thesis was to first study the effect of ascorbic acid on DNA. This was done conveniently using T7 bacteriophage DNA. Further studies were carried out on the interaction of ascorbate treated phage and its host E.coli B23. The effect of ascorbate on E.coli itself was then conducted and an interesting system was found in which to study catalase gene expression.

ABBREVIATIONS

A600	absorbance at 600 nanometers
AA	ascorbic acid
AFR	ascorbic free radical
ATP	adenosine triphosphate
bis	N,N'-methylene bisacrylamide
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
cyt	cytochrome
DNA	deoxyribonucleic acid
dNp	deoxy 3' monophosphate nucleoside
dpN	deoxy 5' monophosphate nucleoside
E. coli	Escherichia coli
Eo'	redox potential
EDTA	ethylene diamine tetra-acetic acid
emf	electromotive force
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HCR	host cell reactivation
HRP	horseradish peroxidase
IPTG	isopropyl- β -D-thiogalactoside
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide

NADP nicotinamide adenine dinucleotide phosphate
NMR nuclear magnetic resonance
O.D.260 optical density at 260 nanometers
ONPG ortho-nitrophenyl- -D-galactoside
POPOP 1,4-bis-{2(5-phenyl oxazolyl)}-benzene
recA recombination, A locus
RNA ribonucleic acid
S30 supernatant obtained after centrifugation at 30,000xg
SCE sister chromatid exchange
SDS sodium dodecyl sulphate
SOD superoxide dismutase
TBHP tertiary butyl hydroperoxide
TBP tertiary butyl peroxide
TCA trichloroacetic acid
TEMED N,N,N',N'-tetramethylenediamine
Ti titanium
Tris trihydroxymethylaminomethane
UV ultraviolet

Chapter I

LITERATURE REVIEW

Ascorbic acid, commonly known as vitamin C, was first isolated in 1928 by Albert Szent-Györgyi. Up to that point, it had been recognized that there was a substance contained in citrus fruit and other fresh fruits and vegetables that prevented the occurrence of scurvy, but little success had been achieved in isolating it. Recently, vitamin C has again received attention in the prevention of the common cold, in obtaining general well being and in anticancer therapy, primarily through the efforts of Pauling (1970).

There is extensive literature regarding the role of ascorbic acid in many biochemical and physiological processes, some of which will be discussed here. The chemistry of ascorbic acid will also be discussed in order to explain: (1) its possible participation in bacterial respiration; (2) its involvement in oxygen byproduct metabolism; and (3) its effect on DNA. It will be seen that the role played by ascorbic acid in such biological processes is due to its characteristic physicochemical properties and oxidation/reduction potential.

1.1 ASCORBIC ACID

1.1.1 Chemistry of Ascorbic Acid

The tremendous reducing power of ascorbic acid was recognized very soon after its isolation by Szent-Györgyi. His early studies characterized "hexuronic acid" by its strong reducing power and reversible oxidizability (Szent-Györgyi, 1931), leading to his suggestion that ascorbic acid could be involved as a catalyst in cellular respiration. In order to understand how ascorbic acid could be involved in so many biochemical processes, it is necessary to gain a greater understanding of the chemistry involved.

L-ascorbic acid, (Figure 1), is a γ -lactone (or an α keto lactone) with the molecular formula $C_6H_8O_6$ and has a molecular weight of 176.13, containing an enediol structure at carbon atoms 2(α C) and 3(α C), and an acid ionizing group in water (Lewin, 1976). The optical absorbance spectrum of the ascorbate anion has been determined in many laboratories (Lewin, 1974; Hewitt and Dicks, 1961). It has an absorption maximum between 265 and 266 nm with a molar absorptivity ranging from 7500 to 16,500, depending on the experimental conditions under which the measurements were made. Another absorption maximum occurs at 244 nm with the molar absorbtivity reported variously as 10,500 (Lewin, 1976) and 11,900 to 12,200 in the presence of sorbitol (Lawendal, 1956,1957). Dehydroascorbic acid does not absorb in the region 230 - 280 nm but does have a weak absorption at 300 nm with a molar absorbtivity of 720 (Mattock, 1965).

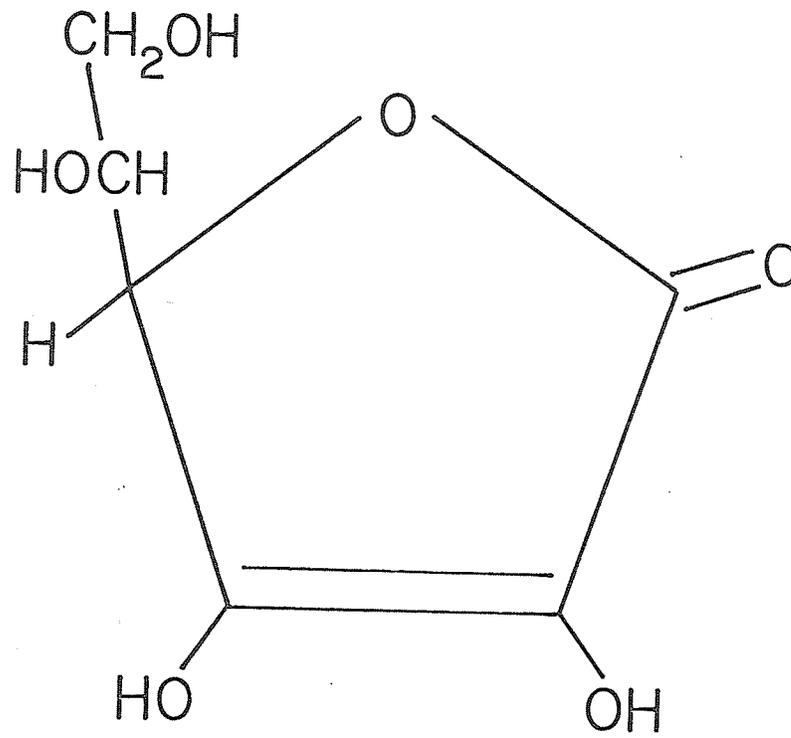


Figure 1: L-ascorbic acid.

Ascorbic acid is fairly stable in powder or crystalline form but once in solution it is quite unstable and readily undergoes oxidation. In aerobic conditions both the rate and extent of oxidative deactivation of ascorbate is increased (Morton, 1942). Even under strict anaerobic conditions, a continuing drop in the absorbance of aqueous solutions of ascorbate occurs (Lewin, 1974) which can be attributed to (i) auto-oxidation catalyzed by traces of multivalent cations such as Cu^{+2} and Fe^{+2} and (ii) a photochemical reaction resulting from the presence of light during preparation of the solution (Hendricks and Demoor, 1964).

Ascorbic acid ionizes in two stages as the pH of its aqueous solution is raised by the addition of base. At 37°C the first pK value is 4.18 and pK_2 is 11.6 (Borsook et al., 1937). These ionizations take place at C-2-OH and C-3-OH.

1.1.2 Products Obtained in the Oxidation of Ascorbic Acid

1.1.2.1 Ascorbate Free Radical

The oxidation/reduction path of ascorbate to dehydroascorbate is shown in Figure 2. When ascorbic acid is oxidized, it generally follows the Michaelis concept (Michaelis, 1932) of a two step oxidation involving a free radical intermediate. The existence of an intermediate oxidation compound, molecular formula $\text{C}_6\text{H}_7\text{O}_6$, with semiquinone properties between ascorbate and dehydroascorbate was established very early (Bezssonoff and Wolosyn, 1938; Weissberger et al., 1943). This compound has been given many names, including monodehydroascorbate or semidehydroascorbate, but because of its free radical nature it is often called ascorbate free radical (Lagercrantz, 1964).

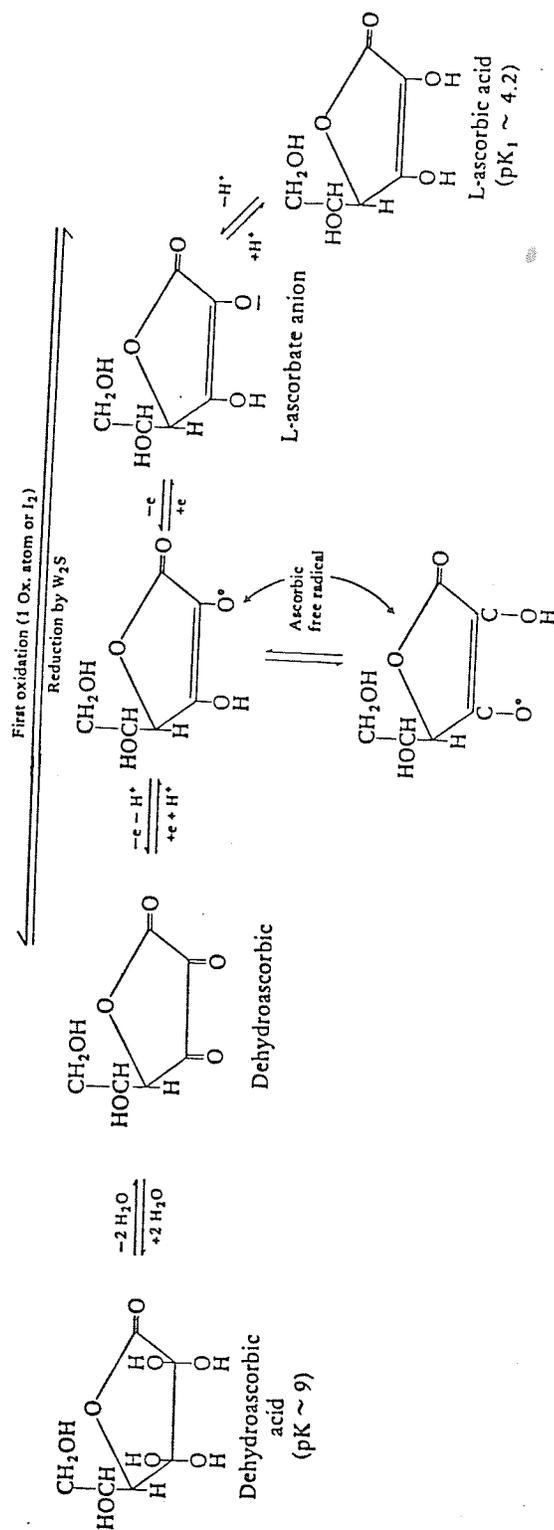


Figure 2: The oxidation/reduction pathway of ascorbic acid to dehydroascorbic acid (Lewin, -1976).

Ascorbate free radical can be formed in several types of reactions:

1. Mixing ascorbic acid with dehydroascorbic acid at acid pH resulted in the formation of ascorbate free radical (Forester et al., 1965).
2. The oxidation of ascorbic acid by molecular oxygen (Lagercrantz, 1964) also produced ascorbate free radical which was detected in the pH range 4.0 to 9.6 in solutions with dissolved oxygen. The electron spin resonance (ESR) spectrum of the free radical was observed for several hours when the tubes containing the solutions were stoppered.
3. Irradiation of ascorbate with light (Lewin, 1976) also produced the free radical.
4. Hydroxide radicals generated in Fenton's reagent can also react further with ascorbic acid generating ascorbate free radical (Weissberger and LuValle, 1944).

Spectroscopic studies of the ascorbate free radical have shown that the species has two absorption maxima, one at 360 nm and one between 285 and 310 nm (Bielski and Richter, 1975). Detailed studies of the electronic structure of the ascorbate free radical and model compounds by electron paramagnetic spectroscopy (Laroff et al., 1972) suggested that the predominant form present over the entire pH range (0 to 14) was that shown in Figure 3 (Bielski and Richter, 1975). One problem in studying the ascorbate free radical was that it was difficult to generate without introducing interfering species such as iron in the Fenton reagent system.

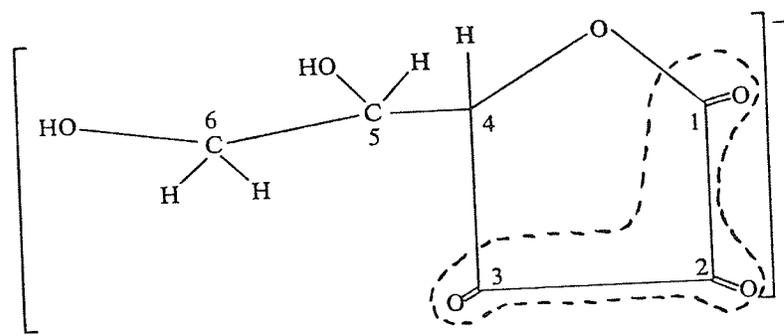
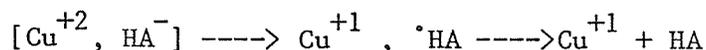


Figure 3: The ascorbate free radical (Bielski and Richter, 1975).

1.1.2.2 Dehydroascorbate

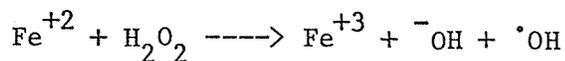
As well as yielding an ascorbate free radical intermediate during the course of oxidation, the total oxidation of one mole of ascorbic acid in the presence of molecular oxygen leads to the formation of one mole of dehydroascorbic acid and hydrogen peroxide (Weissberger and LuValle, 1944). The oxidation is catalyzed by divalent copper cations, and occurs slowly in the presence of cyanide, thiocyanate or EDTA, which, as chelators, would suppress the catalytic action of metals. A complex is formed between the copper ion and ascorbate ion within which an electron transfer occurs resulting in the conversion of the monovalent ascorbate ion to a semiquinone-like radical as Cu^{+2} is reduced to Cu^{+1} .



The unbracketed complex is probably unstable while the liberated Cu^{+1} undergoes rapid oxidation to Cu^{+2} .

Gamma-irradiation has also been shown to cause the oxidation of ascorbic acid in the presence of ferrous (Fe^{+2}) ion (Ban and King, 1956) with the production of dehydroascorbate and hydrogen peroxide.

Under conditions where hydrogen peroxide is produced through the oxidation of ascorbic acid, the presence of ferrous ion as in the Fenton reagent, (Haber and Weiss, 1934) results in the production of the hydroxyl radical, a highly reactive entity that can attack DNA (Morgan et al., 1976), membrane lipids and other essential cell components (Fridovich, 1978):



1.1.3 Oxidation-Reduction Reactions of Ascorbic Acid

The tendency of a substance to donate or accept electrons (to undergo oxidation or reduction) can be described quantitatively in terms of its electrode potential or oxidation/reduction potential. Table 1 contains standard reduction potentials at pH 7.0, the E'_0 values, for various half cell reactions involved in substrate dehydrogenations and electron transport reactions. Carriers in a respiratory electron transport chain are organized in a series with gradually increasing E'_0 values interposed between the initial substrate dehydrogenation which has a negative E'_0 value and the terminal reduction of oxygen which has a positive E'_0 value (Figure 4).

The dehydroascorbic acid/ascorbic acid half cell reaction exhibits a redox potential of +0.058 V (Ball, 1937) which has been redetermined to be as high as +0.08 V (White et al., 1978). The E'_0 for the ascorbate free radical/ascorbate half cell reaction is more positive at +0.32 V (Weiss, 1975) making it a stronger oxidizing agent.

It is possible to calculate the free energy involved in the oxidation of ascorbic acid to dehydroascorbic acid and hydrogen peroxide as being -35 kcal/mol, suggesting a highly favorable reaction.

Table 1. Some biological half cell reactions and redox potentials.

Redox System	E'_0 (pH 7.0)
$\frac{1}{2}O_2 + 2H^+ + 2e \rightarrow H_2O$	+0.816
$Fe^{3+} + e \rightarrow Fe^{2+}$	+0.771
AFR + e \rightarrow AA	+0.34
$\frac{1}{2}O_2 + H_2O + 2e \rightarrow H_2O_2$	+0.30
$Cu^{++} \rightarrow Cu$	+0.345
Cytochrome a $Fe^{3+} + e \rightarrow$ cytochrome a Fe^{2+}	+0.29
Cytochrome c $Fe^{3+} + e \rightarrow$ cytochrome c Fe^{2+}	+0.25
2:6-Dichlorophenolindophenol(ox) + $2H^+ + 2e \rightarrow$ \rightarrow 2:6-Dichlorophenolindophenol (red)	+0.22
Cytochrome c_1 $Fe^{3+} + e \rightarrow$ cytochrome c_1 Fe^{2+}	+0.22
Cytochrome b_2 $Fe^{3+} + e \rightarrow$ cytochrome b_2 Fe^{2+}	+0.12 (pH 7.4)
Ubiquinone + $2H^+ + 2e \rightarrow$ dihydroubiquinone	+0.10
Cytochrome b_5 $Fe^{3+} + e \rightarrow$ cytochrome b_5 Fe^{2+}	+0.03
Cytochrome b $Fe^{3+} + e \rightarrow$ cytochrome b Fe^{2+}	
Dehydroascorbate + $2H^+ + 2e \rightarrow$ ascorbate	+0.058
FAD + $2H \rightarrow$ FADH + H	-0.05
Glutathione(ox) \rightarrow glutathione (red)	-0.33
$NAD^+ \rightarrow$ NADH	-0.33
$H^+ \rightarrow \frac{1}{2}H_2$	-0.42

(Lewin, 1976)

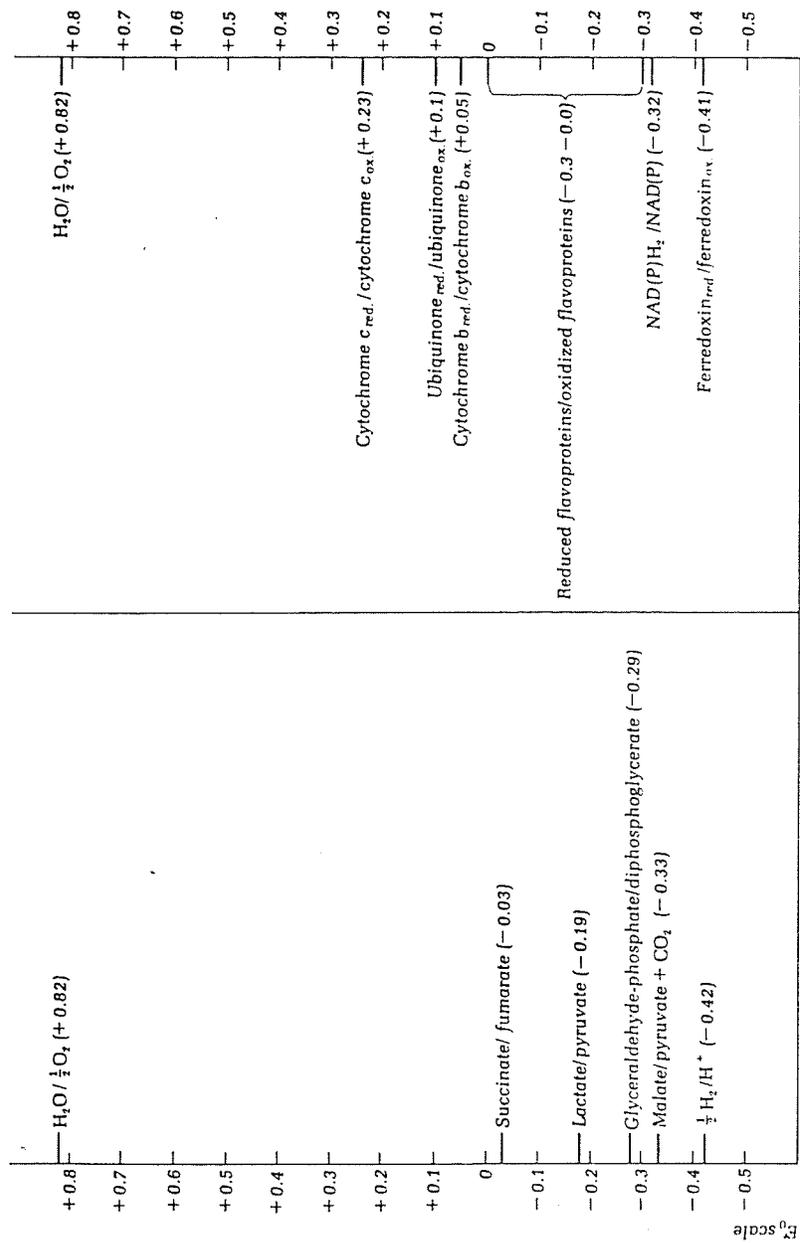
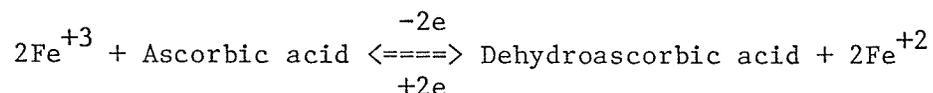


Figure 4: Relationships between some biological half cell reactions and redox potentials (Stanier et al., 1970).

1.1.4 Ascorbate and Enzyme Activity

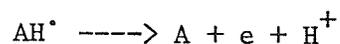
Significant catalysis has been observed when ascorbate is added to a number of enzymatic systems especially those associated with hydroxylations involving $\text{NADH}_2^+/\text{NAD}^+$ (Lewin, 1976). For example, the hydroxylation of the lysine and proline, constituents of collagen, involves ascorbate or the ascorbate free radical (Barnes and Kodicek, 1972) and both electron transfer and energy transfer are involved. The ability of the ascorbate free radical to participate in reactions to a greater extent than ascorbic acid or dehydroascorbic acid is ascribed primarily to its participation in one electron transfer reactions to which most active enzyme systems are restricted. For instance, the process:



would require a three body collision to transfer two electrons in two one-electron transfer steps, compared to a two body collision which would be required for a single one electron transfer from ascorbate free radical to Fe^{3+} . The former situation would be less likely to occur and therefore would be less favorable. Furthermore, ascorbate free radical would be expected to exist at a higher energy level, a result of the unpaired electron, making the energy of activation for electron transfer lower than for ascorbic acid.

Kern and Racker (1954) showed that NADH-oxidase was stimulated by the addition of ascorbic acid and that the ascorbate free radical was implicated. Schneider and Staudinger (1965) examined the reduction of an

'oxidation product' of ascorbic acid with NADH_2^+ serving as electron donor using animal microsomal enzymes. They found that in the presence of the enzyme fraction and NADH_2^+ , ascorbic acid remained in the reduced state even when ascorbic acid oxidase was added. They concluded that in this case only the free radical, and not dehydroascorbate acted as an electron acceptor. Matthews (1951) and Nason et al. (1954) have suggested that the ascorbate free radical is involved in the enzyme catalyzed oxidation of NADH_2^+ . They also suggested that the free radical produced during the oxidation of ascorbic acid by ascorbic acid oxidase and the Cu^{+2} oxygen systems (Nason et al., 1953) was the active species in cyt c reduction. The relatively high activity of the ascorbate free radical can be explained by its highly unstable electronic configuration and the expected low oxidation-reduction potential of the free radical reaction itself:



(Michaelis and Schubert, 1938).

On this basis Yamazaki (1962) suggested that ascorbic acid can reduce systems which have lower oxidation/reduction potentials if the ascorbate free radical is formed either by ascorbic acid oxidase or by chemical means.

From the table of emf values and because of the necessity of a 3 body collision, it would be predicted that ascorbic acid should reduce cyt c

very slowly in a non-enzymatic reaction, whereas the ascorbate free radical should reduce cytochrome c much more rapidly. The presence of ascorbic acid oxidase in the ascorbic acid-cytochrome c reaction stimulated the reaction considerably and since ESR studies (Yamazaki and Piette, 1961) have shown that the ascorbate free radical was present during the reaction, it was concluded that the free radical was the reducing species.

1.1.5 Other Biological Systems Involving Ascorbic Acid

The ascorbic acid oxidation/reduction system consists of three parts: ascorbic acid as a reducing agent, ascorbate free radical as both a reducing agent and an oxidizing agent, and dehydroascorbic acid as an oxidizing agent as well as a molecule capable of combining with groups containing labile hydrogen. The ability of this system to participate in several biochemical oxidation/reduction reactions has been recognized. However, biochemical systems are complex and it is interesting to note the number of different types of reactions in which ascorbic acid is involved.

1.1.5.1 cAMP and cGMP Hydrolysis

Moffat et al. (1972) established chromatographically that ascorbate was one of many substances which exerted an inhibitory action on the hydrolysis of cAMP by cAMP phosphodiesterase. Both 3',5'-cAMP and ascorbate possess ring structures formed as a result of dehydration and both have a negatively charged oxygen atom attached to the ring. Therefore

it has been suggested that ascorbate is actually a competitor for active sites in phosphodiesterase causing decreased hydrolysis of cAMP.

1.1.5.2 Hormone Production

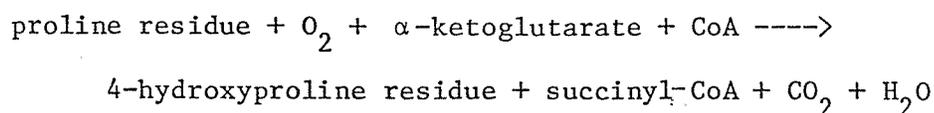
Ascorbate is involved in the production of adrenaline, noradrenaline and serotonin with the common feature being the hydroxylating activity of ascorbate (Blumberg et al., 1965; Borg, 1965).

1.1.5.3 Antihistamine Activity

Histamine is found stored in the mammalian body in tissue mast cells or basophilic leukocytes (Eisen, 1974). It is released by various drugs and antigens and cAMP inhibits this release (Shimizu et al., 1969). Businco (1949) concluded that ascorbic acid and nicotinamide were very potent anti-histamine substances in guinea pigs. This is very useful in preventing the production of excessive quantities of histamine which are formed during biochemical stress.

1.1.5.4 Tissue Repair

Ascorbate is involved in the formation of collagen required for healing damaged tissues. The 4-hydroxyproline residues present in collagen and a few other fibrous proteins are formed by post translational modification of proline involving proline 4-monooxygenase. This mixed function oxygenase utilizes α -ketoglutarate as co-reductant and Fe^{+3} and ascorbate as cofactors:



(Barnes and Kodicek, 1972). Active hydroxyl radicals may arise from the intervention of ascorbic free radical (Gould, 1970).

1.1.5.5 Inhibition of DNA Synthesis

In a DNA synthesis inhibition test with HeLa cells (Galloway et al., 1979) ascorbate gave results typical of DNA damaging chemicals. Another test of DNA damage, the in vivo sister chromatid exchange test (SCE) (Nakanishi and Schreider, 1979) also proved to be positive for ascorbate. Catalase was shown to reduce this effect in both cases.

1.1.5.6 Effect of Ascorbate on Bacteria

Apparently some bacteria need vitamin C and are able to synthesize it (Bourne and Allen, 1935). This question has been studied most extensively in lactic acid bacteria where it was found that the growth of some strains was increased in response to vitamin C added to the culture medium while others were retarded and some did not respond either way. No explanations were given for these observations at the time but it was proposed later that ascorbate may be required in some bacteria to maintain the activity of enzymes with thiol groups (Doelle, 1969).

1.1.5.7 Chemical Reactions of Ascorbate with Amines

Ascorbic acid prevents the in vivo and in vitro formation of toxic nitrosamines from a variety of amines with sodium nitrite (Kamm et al., 1975). Vitamin C also forms salts with numerous metallic ions and with organic bases such as cinchona alkaloids, quinine, quinidine, hydroquinine, and hydroquinidine. Bailey et al. (1945) concluded that a cova-

lent link was formed between the ring nitrogen of nicotinamide and some part of ascorbate but Najer and Guepet (1954) later concluded that the isolated product was primarily an ascorbate-nicotinamide salt, with some association between the amide group and a secondary hydroxyl group of ascorbate.

1.1.5.8 Inactivation of Viruses

Ascorbate is known to cause the inactivation of a variety of bacteriophages and viruses (Murata et al., 1975; Murata et al., 1976; Murata et al., 1973; Bissell et al., 1980; Murata et al., 1971; Murata, 1975). Free radicals produced in the oxidation of ascorbate in aqueous medium are responsible because of their destructive attack on phage DNA. This will be discussed in greater detail later.

1.1.5.9 Ascorbate and Cell Elongation

It has been shown that fully elongated plant cells show a four to five fold increase in ascorbic acid content as compared to that of meristematic cells (Dhar et al., 1981). Accumulation of ascorbic acid precedes and initiates cell elongation. Thus it seems that the promotion of stem tissue growth induced by auxin is associated with an increase in ascorbic acid or a more reduced state, while the inhibition of leaf tissue growth is associated with a decrease in ascorbic acid or a more oxidized state.

1.1.5.10 Ascorbate and Electron Transport

Due to its reducing powers, ascorbate has the capability of donating electrons to many components involved in electron transport. This will be discussed in greater detail later.

When the preceding ten sections are considered, it is apparent that ascorbate does not have a single simple mode of action. Rather, it participates in a diverse group of biochemical reactions perhaps reflecting its diverse chemistry, and it would not be surprising to find still more biochemical roles for ascorbate.

1.2 CLASSICAL CATABOLITE REPRESSION

The differential rate of β -galactosidase (E.C.3.2.1.23) synthesis as well as other catabolic enzymes is reduced by the presence of certain sugars such as glucose (Tyler et al., 1969). This repression was thought not to be due to glucose itself, but to catabolites derived from the rapid metabolism of glucose (Magasanik, 1961). Apparently, if the rate of anabolism is limited then any source of carbon can severely repress the synthesis of β -galactosidase (Mandelstam, 1962). This phenomenon has been termed the glucose effect (Epps and Gale, 1942), metabolic repression (McFall and Mandelstam, 1963), or catabolite repression (Magasanik, 1961). The mechanism of catabolite repression of β -galactosidase has been thoroughly investigated. For example, Nakada and Magasanik (1964) found that glucose repressed the synthesis of mRNA specific for β -galactosidase and Perlman and Pastan (1968a,b) showed that this repression by glucose was overcome in the presence of cAMP. Several in-

investigators have studied the mechanism by which glucose lowered the intracellular levels of cAMP. It was not the result of a more rapid degradation of the cyclic nucleotide (Nielsen et al., 1973), but rather, the result of glucose reducing the synthesis of cAMP without affecting the release of cAMP into the medium (Peterkofsky and Gazdar, 1973). Studies with several mutants of the phosphotransferase carbohydrate transport system (PTS) indicated that the ability of glucose to inhibit adenyl cyclase activity was mediated by one or more components of the PTS system (Pastan and Perlman, 1969; Peterkofsky and Gazdar, 1975), either by the phosphorylation-dephosphorylation reaction itself (Peterkofsky and Gazdar, 1975) or by the interaction of a membrane component with adenyl cyclase (Saier and Feucht, 1975).

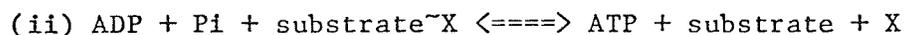
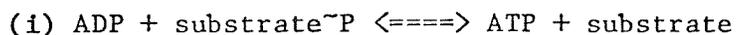
This cAMP effect is further mediated by a cAMP receptor protein (Emmer et al., 1970), and the generally accepted model is that the CRP-cAMP complex exerts positive control at the level of transcription initiation of catabolite sensitive operons. However, recent studies (Guidi-Rontani et al., 1980), suggested that catabolite repression could occur in the absence of cAMP receptor protein (CAP, CRP), casting doubt on the universality of the mechanism.

1.3 RESPIRATION AND ELECTRON TRANSPORT

1.3.1 Bacterial Respiration

Bacteria are able to obtain energy for growth from a variety of reactions. The particular pathway which is utilized is dependent upon growth conditions, that is, whether they are growing aerobically or

anaerobically and which carbon source is available. There are two general methods which a cell utilizes for the conservation of energy. The first is the formation of ATP through substrate level phosphorylation of which there are two basic reactions: (Haddock, 1977):



The second is the production of ATP through oxidative phosphorylation. Photophosphorylation also produces ATP but will not be considered. In oxidative phosphorylation ATP synthesis is coupled to a series of discrete electron transfers from compounds with a more negative E'_0 to acceptors with a more positive E'_0 . The cytoplasmic membrane and the membranous structures connected with it contain the components of the respiratory chain and oxidative phosphorylation (Gelman et al., 1967), (Figure 5).

There are three physicochemical models explaining the way in which redox reactions are coupled to ATP production: the chemical theory, the chemiosmotic theory and the conformational theory (for reviews see Baltscheffsky, 1974; and Boyer, 1974).

The bacteria used in this work was Escherichia coli and the review will be simplified by concentrating on respiration in that species. Escherichia coli is a gram negative facultative anaerobe able to derive energy for growth both fermentatively via glycolysis and oxidatively using molecular oxygen as the terminal electron acceptor under aerobic

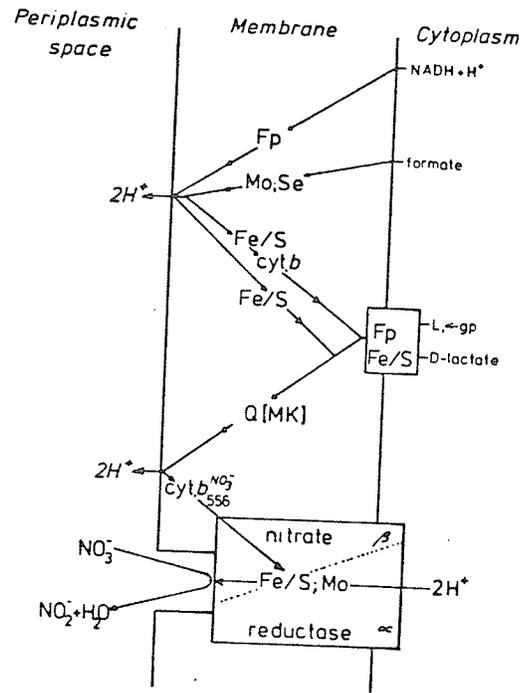


Figure 5: Proposed functional organization of the redox carriers responsible for aerobic electron transport in *E. coli* (Haddock and Jones, 1977).

conditions, and nitrate or fumarate as the terminal electron acceptors under anaerobic growth conditions. The redox carriers present at a particular time depend upon the growth phase, the terminal electron acceptor, the carbon source and the strain of cell.

1.3.2 Redox Carriers

Three classes of redox carriers are involved in the electron transport process. Two classes consist of enzymes with firmly bound prosthetic groups capable of undergoing oxidation and reduction; the flavoproteins and the cytochromes. The third class consists of non protein carriers of low molecular weight, the quinones.

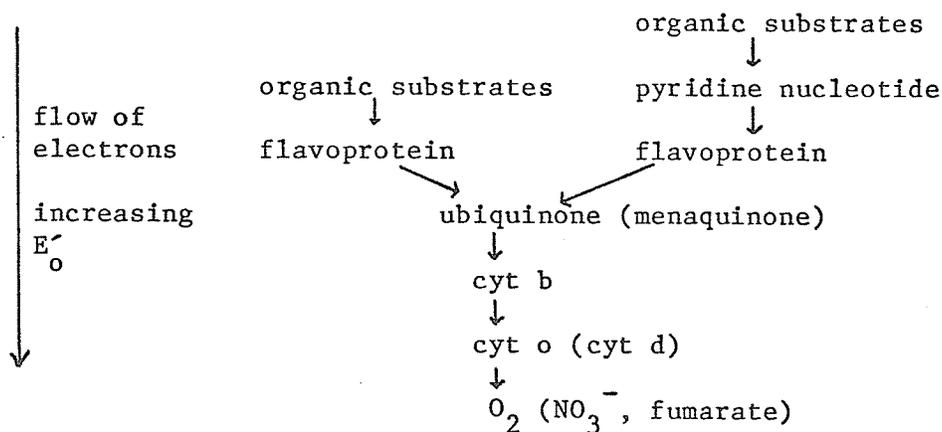
Flavoproteins contain the prosthetic groups flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) which are derived biosynthetically from riboflavin. Both flavin groups possess the same active site capable of undergoing reversible oxidation and reduction but the flavoproteins differ widely in their E'_0 values and are involved in many oxidation-reduction reactions. Some are active in the primary dehydrogenation of organic substrates while others act by accepting electrons from NADH_2^+ formed in a primary substrate dehydrogenation.

The acceptors for the reoxidation of these enzymes vary too. Some reduced flavoproteins are autoxidizable by molecular oxygen, a reaction accompanied by the formation of hydrogen peroxide but usually the flavoproteins do not transfer electrons directly to oxygen. Their reoxidation is mediated by subsequent carriers of the chain, either quinones or cytochromes. Escherichia coli synthesizes both ubiquinone-8, a benzo-

quinone, and menaquinone-8, a naphthoquinone (Pandya and King, 1966). In general ubiquinone is present in larger amounts in E.coli growing aerobically while menaquinone predominates in cells growing anaerobically (Bishop et al., 1962). High concentrations of menaquinone are also found in E.coli cells grown aerobically in the presence of potassium cyanide (Ashcroft and Haddock, 1975). Both ubiquinone and menaquinone can accept electrons directly from NADH_2 (Pandya and King, 1966) both in the absence and presence of cyanide (4mM).

The cytochromes are proteins which contain the heme prosthetic group, a cyclic tetrapyrrole with an atom of iron chelated within the ring system. Electron transfer by cytochromes involves a reversible oxidation or reduction of this iron atom.

The respiratory electron transport system of E.coli is outlined generally as shown below although many variations of this scheme can exist:



(derived from Stanier, 1970)

The membrane bound redox carriers present when E.coli is grown under

vigorous aeration and in the presence of a non-fermentable carbon source include ubiquinone, cytochromes b_{556} , b_{562} and o. Cytochrome o is classified as a type b cytochrome and serves as the terminal oxidase (Haddock et al., 1976). One distinguishing feature of cyt o is its high affinity for cyanide (Pudek et al., 1974) and when E.coli is grown in the presence of cyanide, alternate redox carriers, cyt b_{558} and cyt d, are synthesized (Ashcroft and Haddock, 1975). These cytochromes are also coordinately synthesized under other growth conditions:

1. during late exponential or stationary phase of aerobic cultures growing on non fermentable carbon sources (Pudek and Bragg, 1975).
2. during aerobic growth in the presence of glucose (Haddock and Schairer, 1973).
3. during anaerobic growth on either fermentable substrates or on glycerol with fumarate (Haddock et al., 1976).

It has also been shown that under all these conditions there are increased levels of menaquinone.

1.3.3 Catabolite Repression and Aerobic Electron Transport

Aerobic growth on glucose as a carbon source has been shown to result in decreased levels of cyt b, cyt o and protoheme as well as decreased efficiency in oxidative phosphorylation (Hempfling, 1970). These studies showed that the efficiency of oxidative phosphorylation in E.coli B grown in glucose containing medium was less than 10% of that of organisms grown in medium containing no carbohydrate substrates. Based on

these results it was concluded that part of the enzymatic or structural apparatus of oxidative phosphorylation was subject to catabolite repression. No cAMP studies were done at that time but further studies (Hempfling and Beeman, 1971) showed that cAMP was involved in cytochrome production. An adenylate cyclase lacking strain, C57, was shown to have a 2.5 to 4 fold reduced level of cytochromes when grown in the absence of cAMP and the addition of cAMP resulted in increased cytochrome production. The components required for normal cytochrome levels which were dependent on cAMP were not determined.

1.3.4 Anaerobic Electron Transport

Under anaerobic conditions E.coli can derive energy for growth from either sugar fermentation or from oxidative phosphorylation using fumarate or nitrate as terminal electron acceptors. The characterization of the redox carriers responsible for nitrate reduction has shown that the minimal components of electron transport are two formate dehydrogenases, a quinone (either ubiquinone or menaquinone), a b type cytochrome (b_1) synthesized only in the presence of nitrate under anaerobic conditions (Ruiz-Herrera et al., 1971) and nitrate reductase (Enoch and Lester, 1974).

Studies of the components involved in fumarate reduction are not as extensive. Menaquinone (Newton et al., 1971) and fumarate reductase (Miki et al., 1973) are known to be required, but the role of cytochromes is not clear and the process may be cytochrome independent (Singh and Bragg, 1975).

1.3.5 Role of Ascorbic Acid in Electron Transfer

Ascorbic acid satisfies the main criterion of a respiratory carrier intermediate, the capability of reversible oxidation and reduction. Consideration of electrode potentials of other oxidation-reduction systems shows that the dehydroascorbic acid/ascorbic acid system may be reduced, in theory, by a large number of systems including NADH_2^+ and FADH_2 . Ascorbic acid can be oxidized by an even greater number of molecules including ubiquinone, cyt c, cyt b₂, and cyt a, (Table 1).

As already noted the ascorbate free radical has been implicated as a participant in oxidation-reduction reactions (Iyanagi, 1969) and in various enzymatic reaction. Therefore, it is theoretically possible that ascorbic acid could participate in energy producing oxidation-reduction reactions. The earliest studies which suggested that hexuronic acid (ascorbic acid) was involved as a catalyst in the respiration of the cell were done by Szent-Gyorgi (1930). He also found that in the presence of ascorbic acid, respiration in cabbage leaves was inhibited only 6% by 0.01% cyanide whereas, in the absence of ascorbate, respiration was inhibited 60 to 75% by cyanide. This effect was found to be enzyme mediated (Szent-Györgyi, 1931).

Ascorbic acid is thought to play a specific respiratory function as part of a reduced NADH oxidizing system based on experiments using juice of barley seedlings (James et al., 1944). A similar reaction in the presence of a yeast fraction requiring both ascorbic acid and ascorbic acid oxidase has also been reported (Matthews, 1951). Dehydroascorbic acid failed to serve as an oxidant of NADH in both systems. Both results are consistent with the idea that ascorbic acid, or an ascorbic

acid oxidation product is capable of feeding electrons into some type of respiratory chain accelerating ATP or energy production.

Another respiratory system where ascorbic acid is involved is that of germinating pea seeds. Reduced glutathione and ascorbic acid act as respiratory carriers in an enzyme system which transfers hydrogen to molecular oxygen from substrates of NADP^+ -linked dehydrogenases (Mapson and Moustafu, 1956). Ascorbic acid has been implicated as an agent in barley respiration linking the oxidation and glycolysis stages of respiration (James and Cragg, 1943; James et al., 1944). Ascorbic acid has also been used as a tool in the determination of phosphorylation sites in the electron transport systems in mitochondria (Racker, 1965) where the third phosphorylation site is usually measured with a system using ascorbate coupled with either tetramethyl phenylene diamine or phenazine methosulfate (Jacobs, 1960) to reduce cytochrome c.

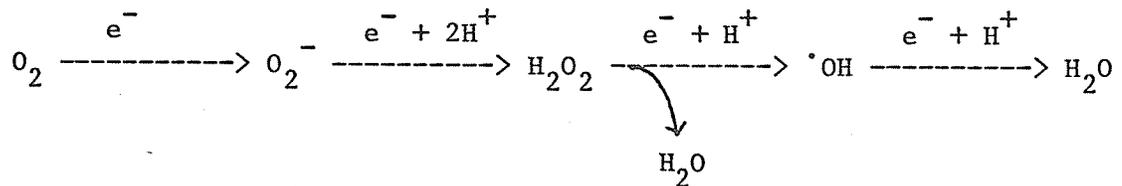
Ascorbic acid does not seem to play a crucial role in bacterial respiration, with exception of certain lactic acid bacteria. Most information regarding the interaction of ascorbic acid and bacterial respiration deals with the ability of individual respiratory carriers to accept electrons from ascorbate. For example, quinone has proven to be an excellent electron acceptor (Dixon, 1971). In reactions stimulated by traces of catalytic metals, FADH_2 can reduce dehydroascorbate and ascorbate can reduce cytochrome c. Bragg and Hou (1974) have shown that ascorbate can drive the energy dependent transhydrogenation of NAD^+ by NADPH when NADH reduction of cytochromes was inhibited by ammonia. This was used as indirect evidence pointing to the existence of a second en-

ergy conservation site in addition to the one in the NADH dehydrogenase region of the respiratory chain previously demonstrated by Sweetman and Griffiths (1971).

1.4 OXYGEN AND OXYGEN BYPRODUCTS

Oxygen in the ground state is quite inactive as an oxidizing agent because of a spin restriction imposed by its unpaired electrons having parallel spins. This prohibits direct entry of paired electrons to ground state molecular oxygen and requires that the addition of spin paired electrons occur such that one electron spin is inverted to avoid the placement of two parallel spins in the same orbit, a situation prohibited by the Pauli exclusion principle.

Complete reduction of molecular oxygen to water requires four electrons. If these electrons were added in a sequential univalent process, several intermediates are possible. These are superoxide anion radical, hydrogen peroxide and the hydroxyl radical (Fridovich, 1978):



1.4.1 Superoxide Anion

The superoxide anion is produced by a one-electron or univalent reduction of ground state oxygen. This anion can dismutate spontaneously to singlet oxygen (Pashen and Weser, 1973). Excited or energized oxygen has two metastable singlet states, Σ^1 , and Δ^1 (Table 2). The Δ^1 singlet state is relatively long-lived and is most likely the species formed in biological systems (Kellogg and Fridovich, 1975). Singlet oxygen has been implicated in oxidative destruction of histidine and, as a result, is a major cause of inactivation of many enzymes (Spikes and Straight, 1967).

Superoxide anion is produced by many chemical systems including the reduction of oxygen by reduced dyes or flavins (Nishihimi et al., 1972) and by ferredoxins (Misra, et al., 1971). Many biological systems also produce superoxide anion including phagocytosis (Patricia et al., 1975), tryptophan dioxygenase (Hirata, et al., 1971), and oxidative enzymes such as xanthine oxidase (Fridovich, 1970). Superoxide was also thought to arise from the oxidation of ascorbic acid (Puget and Michelson, 1974), but this was found not be true (Halliwell and Foyer, 1976; Morgan, 1976).

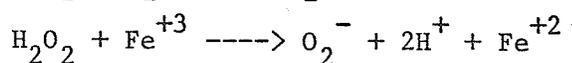
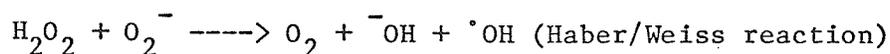
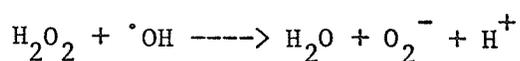
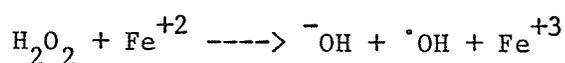
TABLE 2
OXYGEN STATES

State of Oxygen Molecule	Symbol	Energy Above Ground State	Orbitals
2nd excited	1_{Σ}	37 K. cal	\uparrow \downarrow
1st excited	1_{Δ}	22 K. cal	\uparrow \downarrow —
Ground	3_{Σ}		\uparrow \uparrow

(Table according to Foote (1968)).

1.4.2 Hydroxyl Radical

The hydroxyl radical is a weak acid with a pKa of 4.8 (Behar et al., 1970) and can be produced by univalent oxidation of hydrogen peroxide. Haber and Weiss (1934) have attributed the reactivity of a mixture of iron salts and water, Fenton's reagent, as a hydroxylating agent to the formation of the hydroxyl radical:



The hydroxyl radical is the most potent oxidant known and attacks every type of organic molecule (Neta et al., 1968).

1.4.3 Hydrogen Peroxide

Hydrogen peroxide is the most stable of the intermediates produced by the reduction of molecular oxygen. It is also the least reactive and most easily detected intermediate. Hydrogen peroxide can be generated directly by the divalent reduction of oxygen or indirectly by the univalent reduction of molecular oxygen by dismutation of the superoxide produced. Many oxidases such as uricase and D-amino acid oxidase produce hydrogen peroxide during the reduction of oxygen. The production of hydrogen peroxide has been observed in a number of other systems including mitochondria (Boveris et al., 1972), liver slices (Portwich and Aebi,

1960), tissue homogenates (Portwich and Aebi, 1960), illuminated chloroplasts (Halliwell, 1974), microsomal suspensions (Thurman et al., 1972), phagocytosing granulocytes (Paul and Sbarra, 1968), aerobically growing microorganisms (Zobell and Little, 1967), reactions resulting in the oxidation of flavins (Dixon, 1971) and the oxidation of ascorbate (Weissberger et al., 1943; Morgan, et al., 1976; Richter and Loewen, 1981).

The toxicity of hydrogen peroxide is well established. Oxygen tolerances of aerobes and obligate anaerobes have been based on the toxicity of hydrogen peroxide. Aerobes were thought to contain the enzyme catalase as a defense mechanism whereas anaerobes did not contain catalase and were killed on exposure to molecular oxygen because of H_2O_2 evolution (Gordon et al., 1953).

1.4.4 Oxygen Enhanced Toxicity

It is well known that oxygen enhances the lethality of many compounds and, conversely, the presence of these compounds increases the toxicity of oxygen to bacterial systems. This is accomplished indirectly by the production of O_2^- or H_2O_2 from such compounds as streptonigrin (Hassan and Fridovich, 1977d), paraquat (Hassan and Fridovich, 1978a and 1979) and pyocyanine (Hassan and Fridovich, 1980). In the case of streptonigrin the oxygen enhanced toxicity is due to a cyclic reduction and reoxidation which produces O_2^- . In the case of paraquat, it is reduced within the cell and then reoxidized by molecular oxygen to produce O_2^- . Pyocyanine is able to cause the oxidation of reduced nicotinamide adenine dinucleotide, producing O_2^- in the absence of enzymatic catalysis.

It is also capable of diverting electron flow and thus increases the production of O_2^- and H_2O_2 . The antibiotic action of pyocyanine is thus largely a reflection of the toxicity of H_2O_2 and O_2^- and can be overcome by catalase and superoxide dismutase.

1.4.5 Effects of Oxygen Intermediates on Biological Systems

The biological effects of oxygen reduction intermediates are attributable to their free radical characteristics. Many of the reactions reported for other free radicals have been documented to occur with one or more of the reactive products from molecular oxygen. The incubation of tissue homogenates or suspensions of subcellular particles in the presence of air or oxygen produced peroxides as a result of the reactions initiated and/or propagated by free radical species (Barber and Wilbur, 1959).

Hydrogen peroxide has a variety of effects in biological systems. For example, it lowers ATP levels in platelets (Homes and Robkin, 1977) and bacteria (Swedes et al., 1975). Hydrogen peroxide toxicity has been associated with lipid peroxidation in the endoplasmic reticulum (Slater and Sawyer, 1971), and has been shown to cause the destruction of many membrane components and cytoplasmic constituents such as enzymes (Bernheim et al., 1952) and oxidizable small molecules. Hydrogen peroxide causes alterations in isolated DNA resulting in the liberation of all four bases as well as disruption of the DNA sugar-phosphate backbone (Freese et al., 1967; Rhaese and Freese, 1968; Uchida et al., 1965; Yamafugi and Uchida, 1966). Hydrogen peroxide damage to DNA in human fi-

broblasts is mediated by a metal-macromolecule complex giving rise to the hydroxyl radical which is the DNA damaging agent (Mereghini and Hoffman, 1980). Amanthaswamy and Eisenstark (1977) have attributed an increased sensitivity of E.coli mutants to hydrogen peroxide to a reduced capacity for repairing single strand breaks caused by hydrogen peroxide.

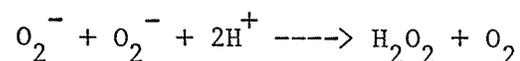
The superoxide anion has been implicated as a mediator in drug induced oxidative hemolysis (Golberg, 1976) and also as a source of DNA defects where failure of defense mechanisms leads to spontaneous cancer in individuals (Totter, 1980). The superoxide anion may cause depolymerisation of acid polysaccharides (McCord, 1974), peroxidation of liposomes and lysis of erythrocytes by direct oxidative attack on cell stroma (Kellogg and Fridovich, 1977). Lipid peroxidation can also result from O_2^- and H_2O_2 generated in a xanthine oxidase system (Kellogg and Fridovich, 1975). Goldstein and Weisman (1977) have suggested that O_2^- and related reactive molecules were capable of perturbing lipid bilayers thereby allowing leakage of normally impermeant anions, an observation which fit the model for oxidative damage to membranes as proposed by Suwa et al. (1977).

It is quite obvious that the various intermediates involved in oxygen reduction are potentially quite lethal and can have drastic effects on many biological processes. It is not surprising that multiple defenses have arisen to scavenge these intermediates in order to negate their toxic effects.

1.5 PROTECTIVE MECHANISMS OF OXYGEN METABOLISM

The toxicity of molecular oxygen in a biological system is a result of the formation of the highly reactive intermediates superoxide, hydroxyl radicals and hydrogen peroxide. Since oxygen is required in the normal respiration of aerobic microorganisms, enzymatic defenses are necessary to protect against the deleterious actions of these toxic by-products. The most common of the protective enzymes and the reactions which they catalyze are:

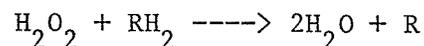
1. superoxide dismutase



2. catalases

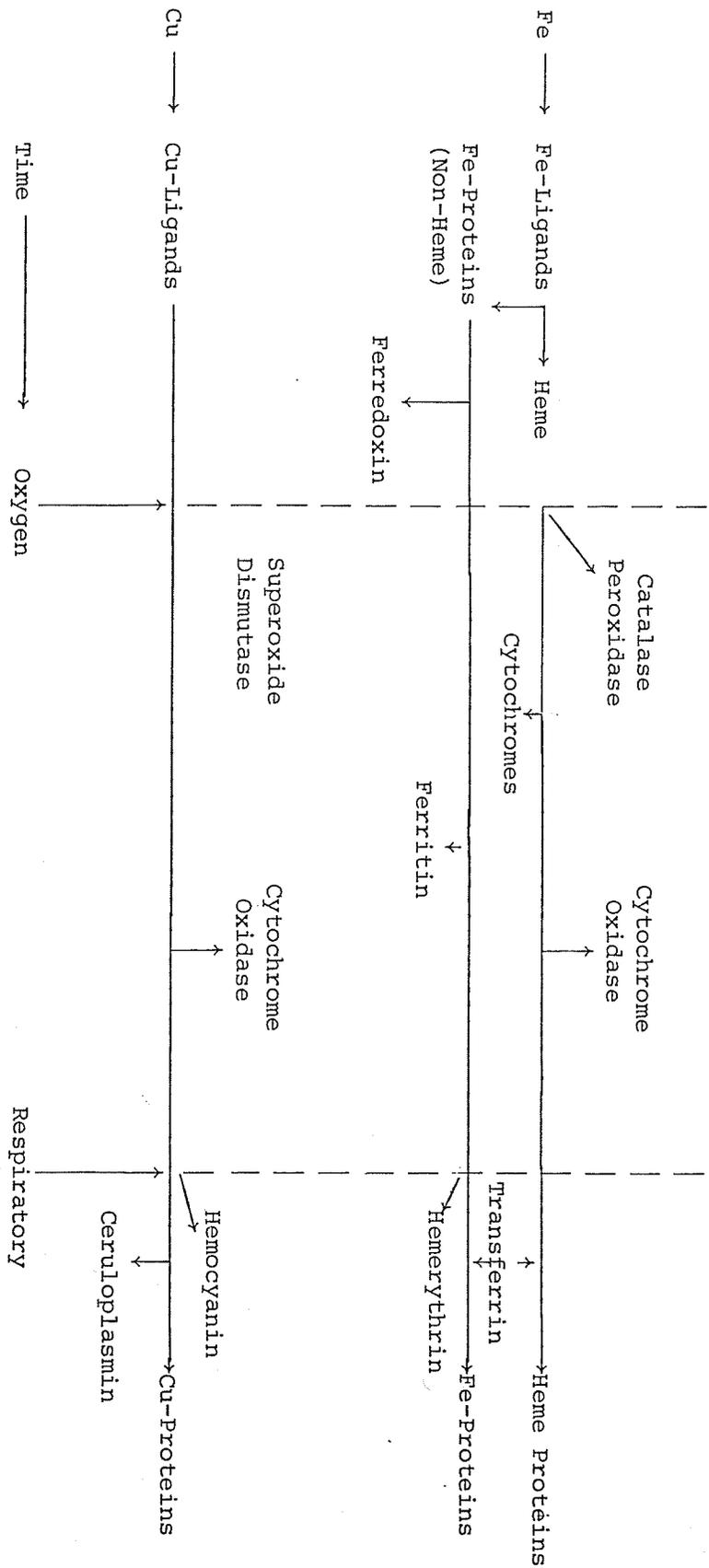


3. peroxidases



The biochemical association among catalases, peroxidases and superoxide dismutase is reflected in their common defense against toxic oxygen byproducts. This is reflected by a common evolutionary structural as well as functional development (Frieden, 1976). This relationship and those of other iron and copper proteins is shown in Figure 6.

Figure 6. The evolutionary sequence and development of Fe and Cu metalloproteins.

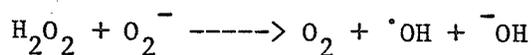


Reference: Frieden (1976)
 Taken from A. Buchanan Ph.D. Thesis

1.5.1 Superoxide Dismutase

The superoxide dismutases generate molecular oxygen and hydrogen peroxide from the superoxide anion preventing spontaneous dismutation of this anion to singlet oxygen (Pashen and Weser, 1973).

Because superoxide dismutase acts to reduce the intracellular levels of O_2^- it also prevents the generation of singlet oxygen by the Haber-Weiss reaction:



(Krinsky, 1977).

The interaction of superoxide dismutase and catalase is important in preventing the occurrence of the Haber-Weiss reaction. There are four main groups of superoxide dismutase: copper and zinc containing found in eukaryotic cells (Beem et al., 1974), manganese containing found in prokaryotes (Keele, et al, 1970), manganese containing found in eukaryotic mitochondria (Weisiger and Fridovich, 1973) and iron containing (Gregory and Fridovich, 1973 and 1974b). E.coli has three forms of SOD (Hassan and Fridovich, 1977a,b,c,and d): an iron superoxide dismutase (FeSOD) (Yost and Fridovich, 1973), a manganese SOD-MnSOD (Keele et al., 1970) and a hybrid composed of one subunit from FeSOD and one subunit from MnSOD (Dougherty, et al., 1978). The FeSOD is constitutive being present even in anaerobically growing cells whereas MnSOD is under repression control and is made in response to oxygen (Hassan and Fridovich, 1977b) as well as to O_2^- produced from streptonigrin and paraquat (Has-

san and Fridovich, 1977d and 1978a). The enzymatic mechanism of SOD from E.coli has been extensively described (Fridovich, 1975 and 1978).

Catabolite repression is not involved in the regulation of superoxide dismutase (Hassan and Fridovich, 1977a,b,and c) but there is a distinct effect of glucose on its synthesis. This effect is thought to be due to the low generation of O_2^- when cells are fermenting glucose and relief from this glucose effect was seen immediately when paraquat (which stimulates O_2^- production) was added to the medium (Hassan and Fridovich, 1978a).

1.5.2 Peroxidases

Peroxidases catalyze the oxidation of many organic compounds using hydrogen peroxide as the electron acceptor. The enzymes usually combine only with hydrogen peroxide and methyl and ethyl peroxides as substrates although there has been a probable



hydrogen donor

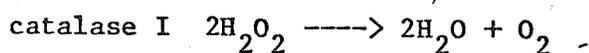
role suggested for the liver catalase/peroxidase activity in the oxidation of formic acid (Aebi et al., 1957) and nitrite (Heppel and Porterfield, 1949).

Peroxidases act at low levels of H_2O_2 and are widely distributed in many cell types (Mills, 1959; Cohen and Hochstein, 1963). The mode of action of peroxidase differs from catalase in that catalase is involved in electron pair transfers while single electron transfers are charac-

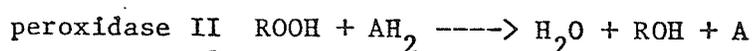
teristic of the peroxidatic mechanism (Yamazaki, 1974). In addition peroxidases do not evolve molecular oxygen. Gregory and Fridovich (1974) noted distinct catalase and peroxidase bands on polyacrylamide gels of E.coli extracts and it was later shown that of the two activity bands separable on gels, one was a general peroxidase which was subsequently purified and characterized (Claiborne, 1978; Claiborne and Fridovich, 1979).

1.5.3 Catalase

The enzyme catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase (E.C.1.11.1.6) from bovine liver has a molecular weight of 240,000 daltons and contains four ferriprotoporphyrin groups per molecule (Aebi, 1974). Catalase has an absorption maximum at 405 nm and a millimolar extinction coefficient of $38-40 \times 10^3$. The enzyme is quite widespread (Sumner and Dounce, 1937; Bonnischen, 1947; Herbert and Pinsent, 1948; Laskowski and Sumner, 1941; Rao et al., 1964; Sulebelle and Rege, 1967) and has two activities, either as a catalase or as a peroxidase:



Decomposition of H_2O_2



Oxidation of hydrogen donors.

The reaction which occurs is determined by the concentration of hydrogen donor present. When the concentration of hydrogen peroxide is low and in the presence of electron donors, the enzyme acts as a peroxi-

dase but if the hydrogen peroxide concentration is high, the enzyme acts as a catalase. Initially, a catalase-hydrogen peroxide complex I is formed and in catalactic reactions a second molecule of hydrogen peroxide serves as hydrogen donor for complex I (Aebi, 1974) Figure 7. The decomposition of hydrogen peroxide is very fast with a rate constant of approximately $10^7 \text{ sec}^{-1} \text{ mol}^{-1}$. The peroxidatic reactions have rate constants of the order of 10^2 - $10^3 \text{ sec}^{-1} \text{ mol}^{-1}$ (Aebi, 1974). The kinetics of the catalase reaction are quite incongruous in that saturation by the substrate does not occur at concentrations up to $5\text{M H}_2\text{O}_2$, but that concentrations above $0.1\text{M H}_2\text{O}_2$ inactivate the enzyme (Aebi, 1974). Adler (1963) found no correlation between catalase activity and the sensitivity of E.coli to H_2O_2 and suggested that the inability of catalase to protect E.coli from exogenous H_2O_2 could be attributed to the inactivation of catalase by H_2O_2 in high concentrations. Catalase has been found to be inactivated or inhibited by a variety of other agents including the free radical products $\cdot\text{OH}$ and $\cdot\text{O}_2\text{H}$ from ascorbate oxidation (Orr, 1966, 1967a,b). This inhibition was shown to be due to an actual degradative change in the catalase molecule caused by ascorbate both alone and in the presence of Cu^{+2} . Catalase has also been found to be inactivated anaerobically in solutions containing ascorbate free radical (Vogt and Weiss, 1976). A very different sort of catalase inhibitor, a protein which was heat-labile and was destroyed by trypsin, has been purified from maize (Sorenson and Scandalios, 1980). The activity of the inhibitor varied inversely with catalase activity in the scutellum of the germinating seed (Sorenson and Scandalios, 1976) and constituted one of several mechanisms regulating catalase activity in that tissue.

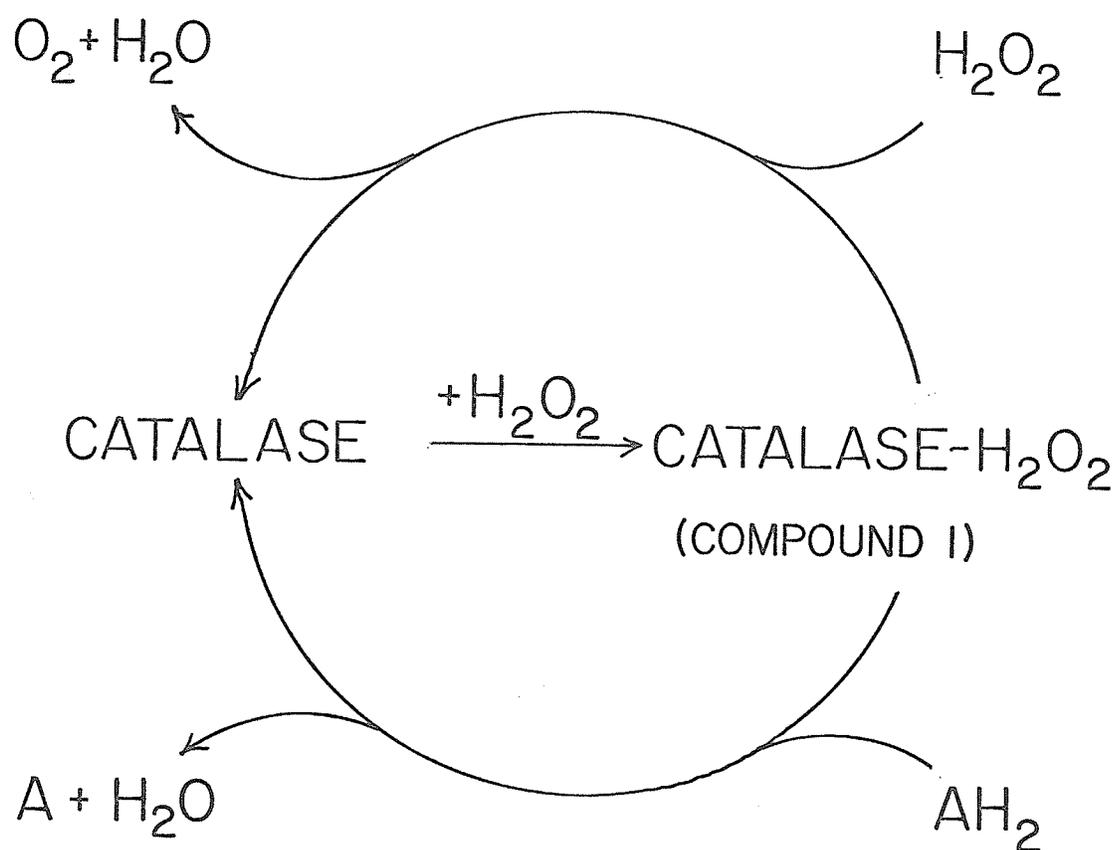


Figure 7: Mechanism of catalase-hydrogen peroxide interaction (Masters and Holmes, 1979).



Flavonoid compounds including silymarin, a drug used in preventing damage to liver cell membranes by a variety of hepatotoxic agents, significantly inhibited catalase in vitro (Koch et al., 1980). From structure-activity relationships it was apparent that the o-diphenolic group of silymarin was responsible for its inhibitory activity toward catalase.

As well as inhibiting catalase at high concentrations, hydrogen peroxide is known to stimulate catalase production in microorganisms (Yoshpe-Purer et al., 1977; Hassan and Fridovich, 1980; Richter and Loewen, 1981) and in rabbit eyes (Bhuyan and Bhuyan, 1977). The importance of a balance between catalase and H_2O_2 levels was illustrated in the latter case where the inhibition of catalase allowed H_2O_2 levels to rise which in some instances promoted cataract development on the eye.

In another mammalian system, rats were fed a dilute aqueous solution of H_2O_2 for a prolonged period causing a significant increase in the activity of peroxide metabolizing enzymes of most tissues. Superoxide dismutase, peroxidase and catalase activities all increased with the greatest increase occurring in catalase (Matkovics and Novac, 1977).

An instance where the activity of catalase results in an inhibition of a biochemical function is in pea chloroplasts (Brennan and Anderson, 1980). Dark activation of light inactivated glucose-6-phosphate dehydrogenase was inhibited by catalase in a broken pea chloroplast system. These results suggested that the dark activation of glucose 6-phosphate dehydrogenase may involve oxidation by H_2O_2 of thiol groups on the enzyme which were reduced in the light by the light effect mediator system.

Catalase is not the only protection cells have against hydrogen peroxide. In red blood cells various reducing molecules such as glutathione, NADH and NADPH can react directly with H_2O_2 . In this respect, the activity of the hexose monophosphate shunt is important since it produces NADPH, the necessary cofactor for glutathione regeneration by glutathione reductase (Sullivan and Stone, 1980). A different view of the importance of catalase to the cell has been expressed by Carlsson and Carpenter (1980). They suggested that the $recA^+$ gene product was more important than catalase in protecting the cell against the toxic effect of hydrogen peroxide. Various deoxyribonucleic acid repair deficient strains of E.coli were exposed to H_2O_2 and the killing of the strains observed. It was found that $recA^-$ strains were most rapidly killed and that there was no correlation between the killing rate of the strains and the capacity of intact cells to decompose H_2O_2 or the level of catalase and superoxide dismutase in cell free extracts. It is known that the $recA$ protein catalyzes initiation of strand exchange during genetic recombination (McEntee et al., 1979) and therefore is most critical to the survival of E.coli. It can be argued then that a $recA^-$ strain of E.coli would be more sensitive to any type of chemical reagent that could have an effect on the DNA.

1.6 REGULATION OF CATALASE ACTIVITY

1.6.1 Catalase Isoenzymes

E.coli responded to both extra and intracellular H_2O_2 with the production of catalase (Yoshpe-Purer et al., 1977; Hassan and Fridovich, 1980; Richter and Loewen, 1981). Originally, Gregory and Fridovich (1974) reported one catalase-hydroperoxidase activity in E.coli extracts but this was later resolved into two isoenzyme bands the slower of which was an active general peroxidase as shown by its o-dianisidine peroxidase activity (Claiborne and Fridovich, 1979). This slower moving catalase band, hydroperoxidase I, was produced constitutively under both anaerobic and aerobic conditions while the faster band appeared in aerobic medium and in the presence of anaerobic medium with NO_3^- present.

1.6.2 Catabolite Repression of Catalase

A glucose effect has been observed in the synthesis of catalase in E.coli (Hassan and Fridovich, 1978; Yoshpe-Purer et al., 1977), yeast (Sulebele and Rege, 1967 and 1968), and Bacteroides fragilis (Gregory et al., 1977) but only in the case of E.coli (Hassan and Fridovich, 1978b) has cAMP been implicated in catalase gene expression. When cAMP was added to a rich medium there was no effect on the basal level of catalase during logarithmic growth but the diauxic response was accelerated after glucose depletion. At the same time that the switch in metabolism occurred, catalase activity increased and this was interpreted to mean that cAMP facilitated catalase gene expression. This was a rather indirect illustration of cAMP involvement and an alternate explanation is

possible. The diauxic shift indicates that other metabolic pathways are being turned on upon the depletion of glucose and H_2O_2 may be produced in these steps. For example, flavoproteins such as succinate dehydrogenase may be reduced and in turn oxidized by molecular oxygen resulting in the formation of hydrogen peroxide. This H_2O_2 could then induce catalase synthesis. With regards to the other reports of catabolite repression, the growth phase of the cells was not rigorously controlled and many extracts were of stationary phase cells where cellular metabolism and hence H_2O_2 production is not clearly defined.

Curiously, Hassan and Fridovich (1980) have reported up to a 10 fold induction of catalase by pyocyanine in cells growing in 0.5% glucose. This is equivalent to the induction in the absence of glucose and the presence of cAMP. One could argue that the glucose effect is a result of changes in metabolism rather than of catabolite repression involving cAMP and CRP.

1.6.3 Effect of Oxygenation on Catalase Activity

Growth in the presence of either oxygen or nitrate as the terminal electron acceptor resulted in higher levels of catalase in cells grown into stationary phase (Hassan and Fridovich, 1978b). Rather than a direct effect of oxygen or nitrate on catalase synthesis, this was attributed to a coordinate relationship between the synthesis of electron transport intermediates and the synthesis of catalase. The actual levels of electron transport intermediates in anaerobic, aerobic or anaerobic- NO_3^- cells have not been determined in such a way that a comparison

can be made, but the synthesis of different chains in aerobic and anaerobic nitrate systems would require that catalase synthesis be coordinately controlled with two separate groups of electron transport intermediates. Complicating the picture further is the observation of Yoshpe-Purer et al. (1977), that catalase levels in anaerobic stationary phase cells could be higher than in aerobic stationary phase cells. They concluded that catalase induction occurred as an indirect response to oxygen. In cultures induced with H_2O_2 , catalase synthesis declined when the addition of inducer was discontinued despite continuous aeration. Unlike cultures induced with H_2O_2 where catalase activity rose despite poor growth both aerobic and anaerobic cultures exhibited a parallel increase in catalase activity to protein synthesis.

Considerable information has accumulated concerning catalase gene regulation in higher organisms such as corn (Quail and Scandalios, 1971; Scandalios, 1974; Sorenson and Scandalios, 1976; Scandalios et al., 1980). This includes the identification of two structural genes and a temporal regulatory gene (Scandalios et al., 1980). In microorganisms it is surprising to find that this work has been the subject of less extensive investigation. In fact, the gene or genes for catalase have not even been mapped in E.coli. With the ease of identifying the catalase gene product on polyacrylamide gels (Gregory and Fridovich, 1974a), of assaying for catalase activity (Rorth and Jensen, 1967) and of inducing catalase mRNA synthesis, it is surprising that this is the case.

1.7 MODIFICATIONS WHICH INACTIVATE DNA

Certain chemicals such as ascorbic acid may also have a direct effect on the DNA of an organism. If DNA modification occurs in a dynamic system, such as during phage DNA replication, it can have a profound effect on the viability of the phage. DNA repair systems may allow the phage to complete its life cycle providing the damage is not too extensive. Unfortunately not all repair is free of errors and mutations may arise in this way.

Nucleic acid modification can be divided into two basic types, primary and final alterations (Freese and Freese, 1966). Primary alterations of nucleic acids are classified into nonhereditary, mutagenic, and inactivating alterations. Unless there is enzymatic repair of the modified nucleic acid, primary alterations are of three types: (i) nonhereditary alterations which neither block DNA synthesis nor induce mutations; (ii) mutagenic alterations caused by agents such as nitrous acid, ethyl methane sulfonate, and hydroxylamine which do not retard nucleic acid synthesis but which induce mutations; (iii) inactivating alterations, so named because they block or inactivate nucleic acid synthesis, which only occasionally induce mutations. Inactivating alterations will be discussed in greater detail below.

Final nucleic acid alterations are the result of the primary alterations modified by several rounds of nucleic acid replication and can take three forms: (i) they may have no phenotypic effect on an organism; (ii) they may be mutagenic causing a phenotypic effect or (iii) they may be lethal (Table 3). Final alterations can be divided into a

class of large deletions which involves the loss of genetic material or its exchange by translocation, inversion, duplication or insertion, and a class of point mutations which involves base substitutions by transitions or transversion, and small deletions.

The study of nucleic acid modification involves a variety of techniques including the use of UV and NMR spectroscopy, chromatography, radioactively labelled DNA, and basic chemical analysis. Backbone breakage can be measured by decreases in sedimentation constant on alkaline sucrose gradients (Litwin et al., 1969) and light scattering techniques. The methods of determining the actual phenotypic consequences of nucleic acid alterations are also varied. Cytological studies can be used to determine chromosomal breaks when there are large chromosomal changes. Genetic fine structure measurements as well as the extent of mutation reversion can be used to analyze the extent and specificity of small nucleic acid alterations. These studies frequently make use of viruses, in particular the use of the rII system of T_4 (Hayes, 1970) and transforming DNA (Freese and Strack, 1962). In yet another system, the Ames test (Ames, 1979) uses bacteria in determining the extent of mutation. From these studies it has been found that many of the reactions involving nucleic acid modification are more complicated than was originally thought. For example:

1. Some chemicals do not directly interact with nucleic acids but must first be altered in the cell before they exert an effect.

Mitomycin has no effect on naked DNA, but after conversion to an alkylating agent inside the cell it becomes quite mutagenic (Iyer and Szybalski, 1963).

Table 3. Classification of primary nucleic acid alterations and their possible genetic and phenotypic consequences.

Type of primary nucleic acid alteration	Representative type of nucleic acid change	In absence of other enzyme reactions: prevents nucleic acid duplication across altered site	Consequences for information content of nucleic acid	Known phenotypic effects
Nonhereditary	Methylation or glucosylation of bases or covering of DNA regions by basic peptides	No	None Reduced or lost (DNAase)	None Host induced modification, other control mechanisms
Mutagenic	Modifications of isolated bases, not preventing base pairing but changing its specificity	No	None Altered sequence of nucleotides Reduced or lost (DNAase)	None Mutation Lethal (vital genes)
Inactivating	Alterations of one or more bases, preventing base pairing; cross-linking; backbone breakage	Yes	None (repair of DNA) Altered sequence of nucleotides (large alteration; or small alteration if nucleic acid duplicates) Reduced or lost (chromosomal breakage)	None Mutation Lethal

(From Freese and Freese, 1966)

2. Experimental conditions such as pH, concentration and oxygen supply may determine the type or extent of modification.
3. Primary nucleic acid alterations may give rise to secondary alterations as when the alkylation of purines causes spontaneous depurination and strand breakage.

In the absence of repair systems, inactivating alterations usually block replication. They are usually lethal and only seldom do mutations result. In fact the frequency of mutation caused by mutagenic agents is 10^2 to 10^3 greater than the frequency caused by inactivating agents (Freese and Freese, 1966).

Free radicals act principally as inactivating agents. Superoxide and hydroxyl radicals can be produced by ionizing radiation in oxygen or water (Kanazir, 1969) and can subsequently attack nucleic acids, particularly the sugar/phosphate backbone. In addition, H_2O_2 (Butler, 1953), hydroxylamine (Freese et al., 1961) and ascorbic acid (Murata and Kitagawa, 1973; Murata and Uike, 1976; Murata et al., 1975) are all known to produce free radicals which can inactivate phage.

Ascorbate also seems to enhance the DNA damaging effect of other molecules. Freshly mixed N-methyl-N'-nitro-N-nitrosoguanidine and ascorbate enhanced DNA fragmentation as measured by shifts in alkaline sucrose gradient sedimentation profiles (Koropatnik and Stich, 1980). Chartreusin, an antitumor glycoside antibiotic, has been found to induce single strand scission in PM2 phage DNA (Yagi et al., 1981). This effect was enhanced in the presence of reducing agents such as ascorbic acid, dithiothreitol, or $NaBH_4$. However, controls relating the effect

of the reducing agents alone on PM2 phage were not done and Morgan et al. (1976) have noted the danger of such reagents. This reaction of reducing reagents with DNA is thought to be mediated by H_2O_2 production since the presence of catalase inhibits the nicking effect.

The primary effect of radical producing agents seems to be the alteration of DNA either through DNA strand cleavage or through base modification or removal which stop DNA replication. In this way transforming DNA and viruses are inactivated with a minor mutagenic effect.

Chapter II

MATERIALS AND METHODS

2.1 MATERIALS

The following chemicals and enzymes were obtained from:

Sigma: agarose, ethidium bromide, chloramphenicol, catalase-bovine liver, t-butylhydroperoxide, cAMP, piperidine, [^{14}C] glucose, [^3H] glycerol, cytochrome c-Type III, DNaseI from bovine pancreas.

Fisher Scientific Co: L-ascorbic acid, carbon decolorizing alkaline

Norite A, 30% hydrogen peroxide, ammonium persulfate, urea.

Miles Chemical Co.: all restriction enzymes.

New England Nuclear: [^{32}P] orthophosphate in water, [^{14}C] ascorbic acid.

Schwartz Chemical: CsCl.

Nutritional Biochemical Corp.: xanthine.

Eastman Co.: acrylamide.

J. T. Baker: N,N,N',N'-tetramethylethylenediamine, acrylamide.

CANALCO: N,N'-methylenebisacrylamide.

Difco: yeast extract, bactotryptone.

Brinkmann: 1.5 mL Eppendorf tubes, Model 5412 Eppendorf centrifuge

All other chemicals were reagent grade or better.

2.2 ORGANISMS AND GROWTH

2.2.1 Bacterial Strains

The most commonly used strain was E. coli B23, a prototroph. The recA strain used was E. coli B5975 obtained from the Yale stock culture collection, the parental strain was E. coli B5974. The adenylate cyclase mutant was kindly supplied by Dr. Mary Lynn Duckworth, MP259-K12 cya^- , HfrH, the parental strain was MP180-K12, wild type, HfrH.

2.2.2 Media

E. coli was grown in the following media:

(a) Minimal salts medium:

Tris-HCl	0.1M pH 7.6
sodium sulfate	0.16mM
magnesium sulfate	1.0mM
ammoniumsulfate	7.5mM
calcium chloride	0.1mM
ferric chloride	3 μ m
potassium phosphate	0.64mM added after autoclaving (KH_2PO_4)
trace elements	0.1mL/100 mL added after autoclaving

This media was supplemented with 16.7mM glucose, 21.7mM glycerol, 67.7mM succinate, 6.7mM histidine and 0.1% casamino acids as specified in individual experiments.

Trace elements:

$FeSO_4 \cdot H_2O$ 2.5g

H_3BO_3	2.9g
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	1.2g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.09g
conc. H_2SO_4	5.0mL
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	2.5g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2.1g into 1 liter

(b) LB Medium:

(Miller, 1974) yeast extract	5g
Bacto tryptone	10g
sodium chloride	5g
distilled water	1000mL

2.2.3 Growth Conditions

Cultures of E. coli were grown at 37°C on a shaker bed. Growth was followed with a Klett Summerson colorimeter using a blue filter. Anaerobic cultures were grown in flasks sealed with Subaseals (Gallenkamp) and flushed with nitrogen gas for one hour prior to use. Subsequently added solutions were treated similarly and added using a syringe. Anaerobic samples were taken with a syringe, placed in a test tube, and immediately frozen in a dry ice-ethanol bath. Aerobically grown E. coli samples were pipetted into a test tube and placed on ice for approximately 30 minutes until further analysis was done.

Ascorbic acid was freshly prepared prior to use, flushed with nitrogen for anaerobic experiments and added to cultures at a cell density of about 70 Klett units or 0.115 mg dry cell weight per mL in LB medium

and 0.106 mg dry cell weight per mL in minimal media. Effectors were added to cultures as specified in individual experiments.

2.2.4 Bacteria Titer

Bacterial titers were determined by plating appropriately diluted solutions (in SM buffer) on LB or glucose minimal media plates containing 1.5% agar.

2.2.5 Phage Stock Preparation

T7 bacteriophage stock cultures were prepared as specified in Studier (1969). A single plaque of T7 bacteriophage was added to 5 mL of a fresh overnight culture of E.coli B23 plus 15 mL of LB medium in a 100 mL flask. The flask was shaken at 37°C until lysis was apparent, usually within 2-4 hours. As soon as lysis appeared complete, 1 g of NaCl was dissolved in the lysate, which was then centrifuged for 10 minutes at 10,000 rpm at 4°C in a Sorvall centrifuge using an SS34 rotor. The supernatant was decanted into a screw-capped culture tube and stored at 4°C. Such lysates were stable for several months, and the phage retained the ability to adsorb well in liquid culture. Titers were generally between 10^{10} and 2×10^{11} /mL.

2.2.6 [³²P] T7 Bacteriophage Preparation

Uniformly labelled [³²P] T7 phage was prepared as described by McDonnell et al. (1977) with slight modifications. E. coli B23 was grown in LB medium to 5×10^8 /mL after which the culture was centrifuged, washed

two times in SM buffer and diluted three fold into glucose minimal medium. The label, 1.0m Ci [32 P] (Sp.Act. 0.3mCi/mgP) was added to the medium and the bacteria grown at 37°C on a shaker bed to 5×10^8 /mL. This culture was infected with T7 bacteriophage at a MOI of 5 with phage diluted to 2 mL in SM buffer prior to addition. Growth was allowed to proceed for 1 hour at 37°C on a shaker bed after which visible lysis was evident. One milliliter of CHCl_3 was added and the incubation continued for 10 minutes. The lysate was decanted from the CHCl_3 and 20 μ L of DNase (10mg/mL) was added followed by a further 15 minute incubation at 37°C. The cell debris was removed by centrifugation for 10 min. at 10,000 rpm in a Sorvall centrifuge in an SS34 rotor at 4°C and the supernatant was centrifuged for 1 hour at 110,000xg. The pellet was re-suspended in 10.0mL of SM buffer and centrifuged again at 110,000xg for 45 minutes at 4°C. Following a third centrifugation the pellet was re-suspended in 2.0mL of SM buffer to which 2.0mL of saturated CsCl was added with mixing. The CsCl solutions were centrifuged at 28,000 rpm for 17 hours in an SW50.1 rotor using Beckman nitrocellulose tubes. The solution was collected through a hole pierced in the bottom of the tube or by a syringe passed through the side of the tube when the phage band was visible. The phage solution was dialyzed for 3 hours against two changes of SM buffer or two changes of 10mM Tris-HCl pH 7.6.

Ultracentrifugations were carried out in Beckman centrifuges models L, L2-65B, or L3-50. Radioactive counting was carried out in a Beckman Model LS230 liquid scintillation counter. [32 P] phage DNA was detected by Cerénkov counting (Kobayashi and Maudsley, 1974).

2.2.7 Phage Titer

Appropriately diluted solutions of T7 phage were titered using the double layer technique of Adams (1959). One tenth of one milliliter of T7 phage dilution in SM buffer plus 0.2mL of an overnight culture of B23 were mixed and 2.5mL of top agar composed of LB medium and 0.7% agar was added, mixed and poured onto an LB agar plate (1.5% agar). Incubation was at 37°C for 3-4 hours or overnight at room temperature.

2.2.8 Cellular Dry Weight Determination

Duplicate samples of 10mL volume were taken from growing cultures at various Klett readings and centrifuged at 10,000 rpm for 10 minutes in a Sorvall centrifuge. The cells were washed 3 times in SM buffer and re-suspended in the same buffer. Aliquots were pipetted into pre-weighed aluminum plates and stored at 105°C until constant weight was obtained. An aliquot of buffer alone was dried to account for buffer weight. In LB medium 100 Klett units represents 0.165mg/mL and in minimal media 100 Klett units represents 0.152mg/mL.

2.2.9 Phage Inactivation by Ascorbate

Phage inactivation experiments were carried out as described by Murata and Kitagawa (1973). Following ascorbate treatment as specified in individual experiments, 1mM EDTA was added to the reaction mixture which was then diluted 100 fold in SM buffer and chilled on ice. The surviving phage were titered as described above or the phage dilution and culture were allowed to incubate for 30 minutes at 25°C first and then top

agar cooled to 30°C was added and the mixture poured onto an agar LB media plate.

2.2.10 Delayed Lysis Experiments

All infections were carried out at a MOI equal to one. Two minutes after infection with untreated phage or phage pretreated with ascorbate, the culture was centrifuged, the supernatant containing unadsorbed phage removed and the bacterial sediment was resuspended at the same optical density in the same media warmed to 37°C. Where specified, samples were taken after lysis and immediately placed on ice. For bacterial survivors, an aliquot was taken, diluted in SM buffer and plated on LB plates. For phage survivors, an aliquot was taken and centrifuged to remove debris. The aliquots were diluted and plated as described above.

2.3 METHODS

2.3.1 Preparation of Buffers and Reagents

2.3.1.1 Gel electrophoresis 10x buffer:

108g Tris

55g boric acid

8.5g Na₃EDTA up to 1 liter with deionized water, pH 8.3.

2.3.1.2 SM buffer:

20mM Tris-HCl pH 7.6

0.1M NaCl

1.0mM MgSO₄ · 7H₂O

0.01% gelatin - for use with phage

2.3.1.3 Z buffer:

16.1g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

5.5g $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$

0.75g KCl

0.246g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

2.6mL mercaptoethanol to one liter, pH 7.0

2.3.1.4 Cesium chloride solution

Concentrated cesium chloride was purified to remove any ultraviolet absorbing material by boiling with Norite. The solution was filtered while hot and upon cooling, a portion of the CsCl was allowed to crystallize to give a saturated solution.

2.3.1.5 Scintillant

Scintillation fluid was prepared by adding 15.2g PPO and 0.38g POPOP to one gallon of toluene.

2.3.1.6 Silylating solution

All 1.5mL Eppendorf tubes used for phage DNA experiments were silylated by soaking in 5% dimethyldichlorosilane in carbon tetrachloride for 2 hours. After drying, the tubes were rinsed thoroughly in distilled water and dried at 65°C overnight.

2.3.1.7 Dialysis tubing

Dialysis tubing was prepared by boiling in 5% Na_2CO_3 , 50mM EDTA followed by a thorough rinse in deionized water and a subsequent boiling in deionized water. The dialysis tubing was stored at 4°C in 0.5mM EDTA pH 8.0.

2.3.2 Enzyme Assays

2.3.2.1 Superoxide dismutase

Superoxide dismutase activity in sonicated cell extracts was determined by the method of Buchanan and Lees (1976). The 2mL cell extracts were prepared by centrifuging culture aliquots three times from SM buffer followed by resuspension in SM buffer and sonication with intermittent cooling on ice for a total of 3 minutes at 4°C . The resulting suspension was centrifuged and aliquots of the supernatant used directly for the assay.

Superoxide anion was generated by the reaction between xanthine and xanthine oxidase. It was able to reduce acetylated cytochrome c at a measurable rate and the presence of superoxide dismutase inhibited this reduction by removing superoxide anion. Therefore the inhibition of the reduction of acetylated cytochrome c was used as a measure for superoxide dismutase activity. One hundred microliters of acetylated cytochrome c (prepared according to Azzi et al., 1975) was added to a 1mL cuvette containing 0.5mL of 10^{-2}M borate and 10^{-3}M EDTA (pH 9.0), .1mM xanthine and varying amounts of extracts to be assayed for SOD in a final volume of 1.0mL. The reaction was starting by added $40\mu\text{g}$ of xanthine oxidase. A unit of SOD activity is defined as being that amount

of enzyme which produces a 50% inhibition in the rate of reduction of acetylated cytochrome c at pH 9.0 and 25°C. Assays were carried out using a Gilford Model 2400 recording spectrophotometer at 550 nm.

2.3.2.2 Catalase assay

Catalase activity was determined both in whole cells taken directly from a growing culture and chilled on ice and in sonicated cell extracts. The sonicated cell extracts were prepared as described for the superoxide dismutase assay extracts. The S30 extract was prepared as described by Hassan and Fridovich (1977). Cells were grown to a Klett reading of 60 and centrifuged 15 minutes at 10,000 rpm in a Sorvall centrifuge. They were washed 2 times in 0.1M potassium phosphate buffer pH 7.0. The pellets were resuspended in 0.05M potassium phosphate buffer pH 7.0, 10^{-4} M EDTA and sonicated in 30 second pulses for a total of 3 minutes at 4°C. The resulting suspension was centrifuged at 30,000xg in a 60 Ti Beckman rotor for 60 minutes. The S30 supernatant was used directly for catalase enzyme assay at different ascorbate concentrations.

Exogenous catalase, where added, was removed before the assay for endogenous catalase by washing the cell pellet three times in SM buffer. The intracellular catalase activity quantitation was not affected by this washing.

Catalase activity was determined using a Gilson oxygraph Model KM equipped with a Clarke type oxygen electrode to quantitate the production of oxygen produced in the decomposition of H_2O_2 at 37°C (Rorth and Jensen, 1967). Fractions to be assayed were diluted an appropriate

amount in a volume of 1.9mL and the reaction was initiated by the addition of 100 μ L of 42mM H₂O₂. One unit of catalase is defined as the amount of enzyme decomposing 1 μ mole of H₂O₂ per minute. Enzyme activities are expressed as units per mg dry cell weight where the dry cell weight was determined from the Klett-Summerson colorimeter reading of the culture as described above.

2.3.2.3

β -Galactosidase assay

The assay for β -galactosidase was carried out as described by Miller (1974). β -Galactosidase hydrolyzes β -D-galactosides and it can be measured with chromogenic substrates, colorless substrates which are hydrolyzed to yield colored products. For example, ONPG (o-nitrophenyl- β -D-galactoside) is hydrolyzed by β -galactosidase to galactose and the intensely yellow o-nitrophenol with an absorption maximum at 420 nm and a corresponding molar absorptivity of 21.3×10^3 at pH 10.2. In order for the generated o-nitrophenol to be proportional to the amount of enzyme present and to the time of reaction, the ONPG must be present in excess. Optimum results were obtained with an amount of enzyme which took between 15 minutes and 6 hours to develop a faint yellow color. The reaction was stopped by adding 1M Na₂CO₃ which shifted the pH to 11 and inactivated the enzyme.

At -2 minutes 1.0mL of culture was pipetted into an equal volume of Z buffer. Two drops of 0.1% SDS and 4 drops of CHCl₃ were added, the solution was vortexed for 10 seconds and allowed to settle. At -1 minute another aliquot was taken and treated similarly. Upon being assayed,

these aliquots provided the basal level of β -galactosidase. At zero time 2.5mL of 1×10^{-2} M IPTG in water (isopropyl- β -D-thiogalactoside) was added to the E. coli culture and mixed well. After adding IPTG, time points were taken at various times up to 1 hour and treated with Z buffer, SDS and CHCl_3 as just described. For each time point a mL aliquot was removed and chilled for an A_{600} reading.

After all samples had been treated, they were decanted from the chloroform globule and incubated at 28°C for 5 minutes. The assay was initiated by the addition of 0.4mL of ONPG solution (4mg/mL in 0.1M potassium phosphate buffer pH 7.0) and was shaken. Each sample was incubated for 30 minutes at 28°C and the reaction was stopped by adding 1.0mL of 1M Na_2CO_3 .

The samples were centrifuged to remove cell debris and the A_{420} was determined. The following relationship was used to calculate the activity of β -galactosidase:

$$\text{Units of } \beta\text{-galactosidase activity} = \frac{1000 \times \text{OD}_{420}}{\text{time of assay} \times \text{vol.} \times \text{OD}_{600}}$$

in which 'volume' is culture aliquot volume. Specific activity was determined from the relationship of Klett reading to dry cell weight.

2.3.3 Other Assays

2.3.3.1 H_2O_2

The procedure of Hildebrandt (1975) was used to determine H_2O_2 concentration. The assay depended on the formation of $\text{Fe}(\text{SCN})_3$ from ferro-

ammonium sulfate and potassium thiocyanate (KSCN) after the oxidation of Fe^{+2} to Fe^{+3} . An intense color developed that could be measured at 480nm.

Two and one half mL of sample were transferred into glass test tubes to which 0.5mL of 10mM ferroammonium sulfate and 0.2mL of 2.5M KSCN were added. The samples were allowed to stand for 10 minutes at 25°C before reading. Because of its reducing property unreacted ascorbate interfered with the assay. To remove the unreacted ascorbate, three hundred mg of Norite were added to each sample prior to the addition of ferroammonium sulfate and KSCN. After vortexing, the samples were allowed to sit 30 minutes and the Norite was removed by centrifugation. The assay was then continued normally.

2.3.3.2 cAMP quantitation

The quantitation of cAMP was carried out using a kit purchased from Amersham following published procedures (Brown et al., 1971; Gilman, 1970). The method is based upon the competition between unlabelled cAMP and a fixed quantity of tritium labelled cyclic AMP for binding to a protein which has a high affinity for cAMP. The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay. The concentration of cAMP in the unknown is determined by comparison with a linear standard curve. There was less than 1.0 nM cAMP in LB medium.

2.3.3.3 Glucose quantitation

Glucose quantitation was carried out using a Glucostat kit purchased from Boehringer Mannheim and following the instructions provided. There was less than 8 μ M glucose in LB medium.

2.3.3.4 Assay of protein synthesis

Two 25mL cultures of E. coli B23 were grown in glucose minimal medium supplemented with 0.1% casamino acids. At a Klett reading of 70, a [¹⁴C] protein hydrolysate was added to both flasks along with 0.57mM ascorbic acid to one of the flasks (specific activity of the protein hydrolysate was 250 μ Ci/5mL, and approximately 1.0 μ Ci was added). One mL samples were taken at specified times, mixed with 1mL of 10% TCA on ice and filtered as described for the uptake studies. The filters were washed with 1% TCA, dried and counted as described for the uptake assays.

2.3.3.5 Respiration studies

Rates of respiration were determined in whole cells prepared as specified in the individual experiment. A Gilson oxygraph Model KM equipped with a Clarke type oxygen electrode to quantitate oxygen uptake was used. Respiration rates are expressed as nmoles oxygen/min per mg dry cell weight. Effectors were added as described in the individual experiments.

2.3.5 Uptake Assays

2.3.5.1 Assay procedure

E. coli B23 was grown in glucose or glycerol minimal media at 37°C on a shaker bed to a Klett reading of 150 (0.227mg/mL dry cell weight). They were collected by centrifugation, washed 2 times and resuspended in minimal medium to the cell density above minus the carbon source. The resuspended cells were preincubated for 10 minutes at 37°C after which chloramphenicol (.1mM) was added to minimize the effect of macromolecular synthesis on uptake studies. This was followed by a further 5 minute incubation period before the uptake experiments were initiated by the addition of a solution containing the labelled substrate and any other required components. The solution was mixed rapidly and incubated for specified times at room temperature. The reaction was terminated by filtering the incubation mixture through 47mm HAWP Millipore filters (0.45 µm pore size) which were washed immediately with 15mL of cold 20mM potassium phosphate buffer buffer pH 7.6. The filters were allowed to dry, dissolved in scintillant and counted in a Beckman LS-230 liquid scintillation counter. The entire filtration and washing procedure never exceeded 20 seconds. The net accumulation of a substrate was obtained by subtracting any background counts arising from non specific binding from the total radioactivity on the filters.

To study the inhibition of D-glucose or glycerol uptake by ascorbate or H₂O₂, these components were normally added to the reaction mixture along with the [¹⁴C] labelled D-glucose or [³H] glycerol mix at concentrations specified in individual experiments.

Specific activities: [^{14}C] glucose 3.1mCi/mmol, labelled at C-1.
[^{14}C] ascorbate 9mCi/mmol, labelled at C-1.
[^3H] glycerol 500mCi/mmol, labelled at C-2.

2.3.4.2 Incubation time

To determine the critical reaction velocity of various substrates in the transport studies, a time dependent uptake experiment was performed first. The linear portion of the curve of substrate accumulated versus time was assumed to represent the critical reaction rate. The incubation times for the transport assays were chosen to fall within the linear portion of the curve. The incubation time for sugar uptake ([^{14}C] glucose, [^3H] glycerol) was 1 minute at a concentration of 50 μM . For ascorbate, incubation times from 1 to 5 minutes at concentrations ranging from 10 μM to 1.0mM did not result in uptake.

2.3.5 Gel Electrophoresis

2.3.5.1 Polyacrylamide gels for catalase and peroxidase

Catalase and peroxidase activities were visualized as described by Gregory and Fridovich (1974) on 12% polyacrylamide gels run as described by Davis (1964). Extracts for gel electrophoresis were prepared by sonication as described for the SOD assay extracts, with 25mL of cell culture being used. After the addition of H_2O_2 or ascorbate, growth was allowed to continue for 45 minutes to 1 hour as specified in the indi-

vidual experiment. The cells were cooled on ice, centrifuged and washed 2 times in SM buffer. After the final washing the cells were resuspended in a volume of 1mL of SM buffer and sonicated at 4°C for a total of 3 minutes in 30 second pulses. The resulting suspension was then centrifuged and the protein concentration of the supernatant was determined by the Lowry method (Lowry, 1951) using bovine serum albumin as the standard. Appropriate volumes of the extract were mixed 1:1 with a solution of 40% sucrose, 0.1% bromphenol blue dye.

After electrophoresis at 4ma per gel for 2 1/2 hours, using Tris(5mM)-glycine(38mM) electrophoresis buffer, which moved the blue dye to within 0.5cm of the bottom of the 6.5cm gels, the gels were stained for catalase by soaking for 45 minutes in 50mM potassium phosphate pH 7.0 containing 0.4mg/mL of diaminobenzidine hydrochloride (DAB hydrochloride) and 0.05mg/mL of horseradish peroxidase (HRP). In order to stain for peroxidase, HRP was omitted from the staining mixture. After rinsing with distilled water, the gels were soaked in 3mM H₂O₂ - 50mM potassium phosphate pH 7.6 until staining was complete (2-4 hours), and the H₂O₂ solution was replaced with distilled water. Gels stained for peroxidase could be stored overnight in the H₂O₂ solution causing a uniform staining of the gel except where there was a band of catalase activity lacking peroxidase activity. The staining in this case was the result of the gradual chemical peroxidation of the diaminobenzidine. In this way, bovine catalase which lacked a peroxidase activity and an E. coli catalase activity, (which migrated more slowly than the predominant isoenzyme pair), could be identified on the gels stained for peroxidase.

The catalase staining procedure described by Gregory and Fridovich (1974) which used 20mM H_2O_2 created a background stain which was too strong and more effective staining was achieved with 3mM H_2O_2 .

A band of catalase activity was observed as an achromatic band against a medium brown background while a band of peroxidase was observed as a dark brown band against a clear or light brown background.

2.3.6.2 Denaturing polyacrylamide gels for DNA

Denaturing polyacrylamide gels were made using 20cm x 20cm slab gels prepared in an apparatus described for agarose gels. They were prepared by mixing appropriate amounts of a 30%(w/v) acrylamide/1%(w/v) N,N'-methylene bisacrylamide stock solution with urea (final concentration of urea 7M) and a one tenth volume of 10 x concentrated gel electrophoresis buffer. This mixture was incubated at 37°C for 15 minutes and degased under vacuum. Ammonium persulfate, 0.1% wt/vol., was added and a 20mL volume was removed and mixed with 10 μ L of TEMED (N,N,N',N'-tetramethylethyl-enediamine). This was poured into the gel mold to make a 1-2cm plug at the bottom of the mold to prevent leakage. After the plug was polymerized, the remaining acrylamide-urea solution was mixed with TEMED in the proportion 40 μ L TEMED to 80mL of gel solution and poured into the mold. A sample well mold was installed and the gel allowed to polymerize. Following polymerization, the well mold was removed and the sample wells rinsed. Samples were mixed with an equal volume of 80% formamide, 10mM NaOH, 1mM EDTA, 0.05% bromphenol blue, heated at 90°C for 1 minute, quick chilled in ice water and loaded.

Samples for denaturing polyacrylamide gel were prepared according to the following protocol. Whole [^{32}P] T7 phage was taken up in 20mM Tris/HCl pH 7.6, 0.1M NaCl, 1mM MgSO_4 , 200 $\mu\text{g}/\text{mL}$ catalase and treated with 5.5mM ascorbate at room temperature for time periods as specified in the individual experiment. The reaction was stopped by adding 1mM EDTA. To release the DNA from the phage, the solution was heated at 65 $^{\circ}\text{C}$ for 2 minutes. The DNA was ethanol (final conc. 75% v/v) precipitated 3 times, the last two times from 0.3M NaOAc (0 $^{\circ}\text{C}$). In each precipitation step, the mixture was chilled in a dry ice-ethanol bath for 10 minutes and centrifuged in a Brinkmann Eppendorf centrifuge for 5 minutes. The DNA pellet was washed with 1 mL of 95% ethanol, centrifuged and dried under vacuum. The pellet was redissolved in freshly diluted 0.1M piperidine and the cap of the Eppendorf sealed. The reaction mixture was heated at 90 $^{\circ}\text{C}$ in a water bath for 30 minutes after which it was then frozen and lyophilized. Ten microliters of water was added to dissolve the pellet after which the solution was frozen and lyophilized. This was repeated 3 times. The sample was then treated as described above and run at 500`V for 3 hours.

2.3.6.3 Neutral agarose gel electrophoresis

Agarose was dissolved in boiling 1X gel electrophoresis buffer (1.2g per 100mL). The solution was allowed to cool to 55-60 $^{\circ}\text{C}$ and poured into a slab gel. The slab gel molds were constructed from two 20cm x 20cm glass plates held 2mm apart by plexiglass strips. The edges were sealed with paraffin oil and the plates held in place using clamps. The bottom opening was sealed with plasticene. The agarose solution was poured to

the top of the glass plates and a plexiglass sample well mold was placed in the top opening when the apparatus was full. The gel was allowed to solidify for approximately 1 hour and the well mold and plasticene were removed. The sample wells were rinsed and samples were mixed with an equal volume of 5% sodium dodecyl sulfate, 25% glycerol, 0.05% bromphenol blue, heated at 65°C for 5-10 minutes and loaded on the agarose gel in a volume of 25-40 μ L. The gel was run at 100-110V or 1.5ma overnight.

2.3.5.4 Denaturing agarose gel electrophoresis

The procedure followed was essentially as above with specific changes as described by McDonnell et al. (1977). Agarose gels, 1.2%, were prepared in 30mM NaOH, 2mM EDTA and were run in 30mM NaOH, 2mM EDTA. Samples were mixed with an equal volume of 0.1M NaOH, 1mM EDTA, 25% glycerol, 0.05% bromphenol blue, heated at 90°C for 15 seconds and loaded.

2.3.6 Analysis of Ascorbate-treated DNA

2.3.6.1 Preparation of T7 DNA for Restriction Nuclease Analysis

T7 DNA for restriction nuclease analysis was prepared as described by Rosenberg et al. (1979). A 10 μ L aliquot of purified phage particles with an A_{260} of 6.0 after dialysis in 0.2M NaCl, 2mM Tris-HCl pH 7.6, 0.2mM EDTA was added to 30 μ L of 10mM Na₃EDTA and heated for 2 minutes at 65°C. This released the DNA which was precipitated with two volumes of 95% ethanol at -20°C in a dry ice-ethanol bath. The suspension was centrifuged for 5 to 10 minutes in an Eppendorf centrifuge, the supernatant removed and the pellet of DNA was dried under vacuum. Restriction nu-

lease treatments were carried out at 37°C for 1-2 hours in a volume of 50µL in the following buffers:

Xba I

6mM Tris-HCl pH 7.6
0.1M NaCl
6.0mM MgCl₂

Hae III

50mM Tris-HCl pH 7.4
5mM MgCl₂
10mM mercaptoethanol

Hpa I

20mM Tris-HCl pH 7.4
10mM MgCl₂
1mM dithiothreitol
20mM KCl

The reactions were stopped with the addition of 20µL of 5% sodium dodecyl sulfate, 25% glycerol, 0.05% bromphenol blue and heated at 65°C for 10 minutes. 25µL were loaded on a neutral agarose gel and run at 100-110V or 1.5 ma overnight at room temperature except where otherwise specified. Following agarose gel electrophoresis DNA bands were localized by soaking the gel in 0.4µg/mL ethidium bromide in gel electrophoresis buffer for about 30 minutes and viewing under a UV light. Labeled DNA was detected by autoradiography*. Pictures were taken by using a Polaroid camera with a Kodak red filter and illumination of the gel with a UV light for 1-2 minutes. UV illumination was at 254 nm.

* The gel was wrapped in Saran wrap, a Kodak X-ray film placed on top and the gel and film were placed in a lead covered folder and exposure was for 24-48 hours.

2.3.6.2 Effect of Ascorbate on T7 DNA as Viewed on Agarose Gels

Both unlabelled and [^{32}P] T7 phage in SM buffer were treated with ascorbate as specified in individual experiments. The reaction was stopped by adding EDTA to a concentration of 1mM and chilling on ice. The sample was then heated at 65°C for 2 minutes to release the DNA, mixed with an equal volume of 5% SDS, 25% glycerol, 0.05% bromphenol blue, heated again for 10 minutes at 65°C and loaded on an agarose gel in a volume of 25-40 μL and run as described above.

2.3.6.3 Effect of Ascorbate on Restriction Nuclease Cleavage

T7 phage DNA was isolated as described above. The DNA was dissolved in SM buffer and treated with ascorbate as specified in the individual experiment. The reaction was stopped by adding 1mM EDTA and 0.1M NaCl. The DNA was reprecipitated with ethanol and dried under vacuum. Restriction nuclease treatments were carried out as described above. The reactions were initiated with the addition of 1 μL of enzyme.

2.3.7 Other Separation Techniques

2.3.7.1 Alkaline Sucrose Gradient Analysis of T7 DNA

[^{32}P] T7 phage in SM buffer was treated with ascorbate as specified in individual experiments. The reaction was quenched by adding EDTA to a concentration of 1mM and chilling on ice. The total volume loaded on each gradient was 200 μL and contained approximately 100,000 cpm. Each sample was treated with 0.2M NaOH, heated at 37°C for 5 minutes and loaded on a linear 5% to 20% alkaline sucrose gradient (0.25M NaOH, 0.1M

NaCl, 1mM EDTA). The gradients were run in polyallomar tubes in an SW50.1 rotor at 45,000 rpm for 2.5 hours at 4°C. To collect gradient fractions, the bottom of each tube was pierced and fractions collected into 1.5mL Eppendorf tubes. Radioactive peaks were detected by Cerénkov counting (Kobayashi and Maudsly, 1974). The number of nicks in the DNA were analyzed as described by Litwin et al. (1969), where D2/D1 values were analyzed. D2 equals the distance from the top of the gradient sedimented by an unknown sample and D1 equals the distance from the top of the gradient sedimented by the DNA molecule of unit length.

2.3.8.2 Neutral Sucrose Gradient Analysis of T7 DNA

[³²P] T7 phage was treated as described above. Prior to loading, each sample was heated at 65°C for 2 minutes to release the DNA and loaded on a linear 5% to 20% neutral sucrose gradient in 0.1M NaCl, 0.05 M EDTA, 20mM Tris-HCl pH 7.0 prepared in polyallomar tubes and were run as described above.

2.3.8.3 Neutral Sucrose Gradient Analysis of T7 DNA Following Infection

The experiment was performed essentially as described by Frankel (1966) and Hausmann and LaRue (1969). E. coli B23 was grown in a 10mL volume to a density of 2×10^8 /mL, when it was infected at a MOI of 5 with [³²P] T7 phage that had been treated with 2.8mM ascorbate in LB media for 5 minutes. Aliquots of 0.5mL were removed from the infected culture at various times and mixed with 0.5mL of lysis buffer containing 0.1M EDTA pH 8.5, 0.1M NaCN and 100µg/mL lysozyme. The mixture was incubated at 65°C for 1 minute when SDS was added to a concentration of 0.1%. Af-

ter a further 10 minute incubation at 65°C the samples were cooled to room temperature and 0.5mL was loaded onto a linear 5% to 20% sucrose gradient in 0.1M NaCl, 0.05M EDTA, 20mM Tris-HCl pH 7.0 prepared in polyallomar tubes. The gradients were run in a Beckman SW41 rotor at 35,000 rpm for 2.5 hours at 4°C. The bottoms of the tubes were pierced and 40-45 fractions were collected in 1.5mL Eppendorf tubes. The radioactivity was detected by Cerénkov counting (Kobayashi and Maudsley, 1974).

All studies described above were carried out using E.coli strain B23 unless specified otherwise.

2.3.8.4 Thin layer chromatography

Thin layer chromatography of free bases, nucleosides and nucleotides was carried out on Brinkmann Polygram CEL300 UV254 cellulose plates using either solvent A: isopropanol:conc. $\text{NH}_4\text{OH}:\text{H}_2\text{O}$, (7:1:2, by vol), or solvent B: 1-butanol-isobutyric acid-conc. ammonium hydroxide-water (75:37.5:2.5:25, by vol) in one dimension and either solvent A or B and Pabst (0.1M sodium phosphate buffer, 6M ammonium sulphate, 0.5M n-PrOH) for two dimensional chromatography.

Reaction mixtures were composed of 0.10mM nucleoside or nucleotide in SM buffer, 200µg/mL of catalase and 5.7mM ascorbic acid where specified. The total reaction volume was 50µL. After the reaction was allowed to proceed for the specified time, the reaction was stopped by adding EDTA to a concentration of 1.0mM. Any further treatment was as described in the individual experiment. Five to 10 µL were spotted on cellulose plates and run as described in the individual experiment.

Chapter III

PART I: EFFECT OF ASCORBIC ACID ON T7 BACTERIOPHAGE

3.1 INTRODUCTION

Bacteriophage T7 has been used in many different laboratories for a wide variety of biochemical, genetic and physical chemical studies. It is an ideal bacteriophage to work with because it is easy to grow and purify and has been very well characterized physicochemically, physiologically and genetically (Studier, 1969 and 1972). The phage has a unit weight of 40×10^6 and contains a double stranded DNA molecule with a molecular weight of 26×10^6 (Studier, 1969). The DNA has non circularly permuted terminally redundant ends (Ritchie et al., 1967) which have recently been sequenced (Dreiseiklmann et al., 1980). The main advantage of the use of T7 in biological studies is that of its size: it contains one fifth the information content of the T-even phages, T2 and T4, and has none of the functions associated with the formation and glucosylation of hydroxymethylcytosine. T7 is a virulent phage and has no functions necessary for lysogeny. It codes primarily for those functions related to phage replication and assembly.

Ascorbic acid, or free radical products formed from its oxidation, are known to have an "inactivating effect" on many bacteriophages, including J1, A, MS2, as well as on avian Rous sarcoma virus (Murata and Kitagawa, 1973; Murata et al., 1975; Murata and Uike, 1976; Bissell et al., 1980). The purpose of this study was to investigate the nature of

the chemical lesions in T7 DNA caused by ascorbic acid and their effect on the bacteriophage replication cycle.

3.2 RESULTS

3.2.1 Ascorbic Acid and in vitro Inactivation of Bacteriophage T7

The rate at which various concentrations of ascorbic acid inactivated T7 was studied using the standard double layer technique. Bacteriophage T7 was diluted to approximately 5×10^8 per mL in SM buffer and incubated with different concentrations of ascorbic acid. Aliquots were removed at various times, and diluted 100 fold in SM buffer to stop any further reaction. These samples were then titered after appropriate dilution by mixing with either a wild type strain B23 (Figure 8) or a recA strain B5975 (Figure 9) at 45°C in top agar and immediately pouring onto the bottom layer. The rate at which T7 phage was inactivated was similar to the rate at which other double stranded DNA containing phage were inactivated (Murata and Kitagawa, 1973). The same experiment was repeated but with the procedural modification that the phage-bacteria mixture was allowed to incubate at 25°C for 30 minutes prior to being poured onto the bottom layer. The effect of the 25°C incubation was to reduce the amount of inactivation in both the wild type strain (Figure 10) and the recA strain (Figure 11).

While the rates of inactivation were qualitatively the same in the two strains, two subtle differences were evident at the higher ascorbate concentrations suggesting a role for the recA gene product in the repair of the ascorbate induced lesions. First there was a slightly faster rate

Figure 8. Inactivation of bacteriophage T7 by ascorbic acid in SM buffer (I).

The standard double layer titering procedure using wild type E. coli B23 as host was used as described in the Methods. The concentrations of ascorbic acid used were 0 mM, (○); 0.057 mM, (●); 0.285 mM, (□); 0.57 mM, (■); 2.85 mM, (△); 5.7 mM, (▲); 28.5 mM, (▽). The initial phage titer was 5×10^8 /mL.

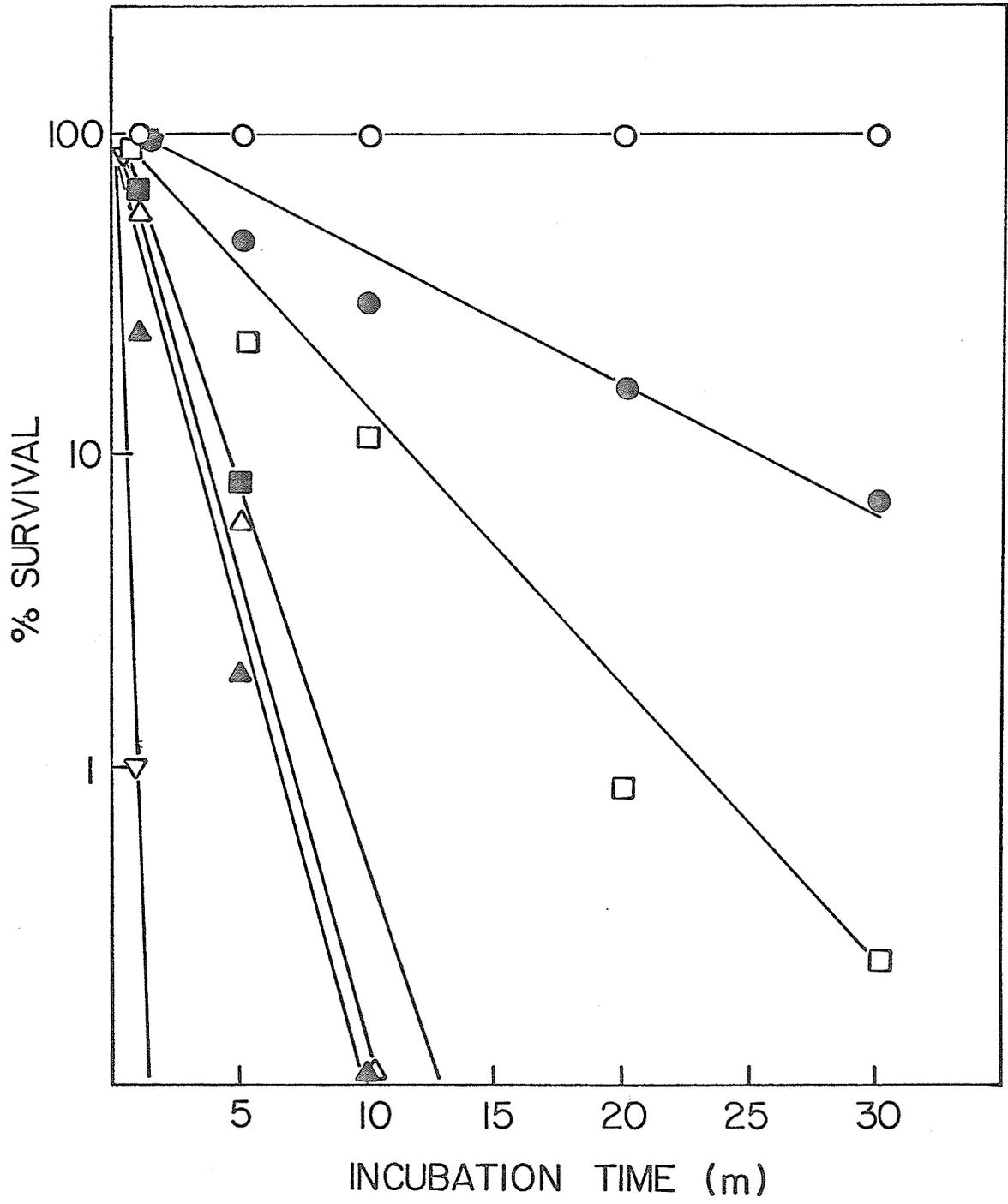


Figure 9. Inactivation of bacteriophage T7 by ascorbic acid in SM buffer. (II).

The standard double layer titering procedure using a rec A⁻ mutant E. coli B 5975 as host was carried out as described in Methods. The symbols are as in Figure 8.

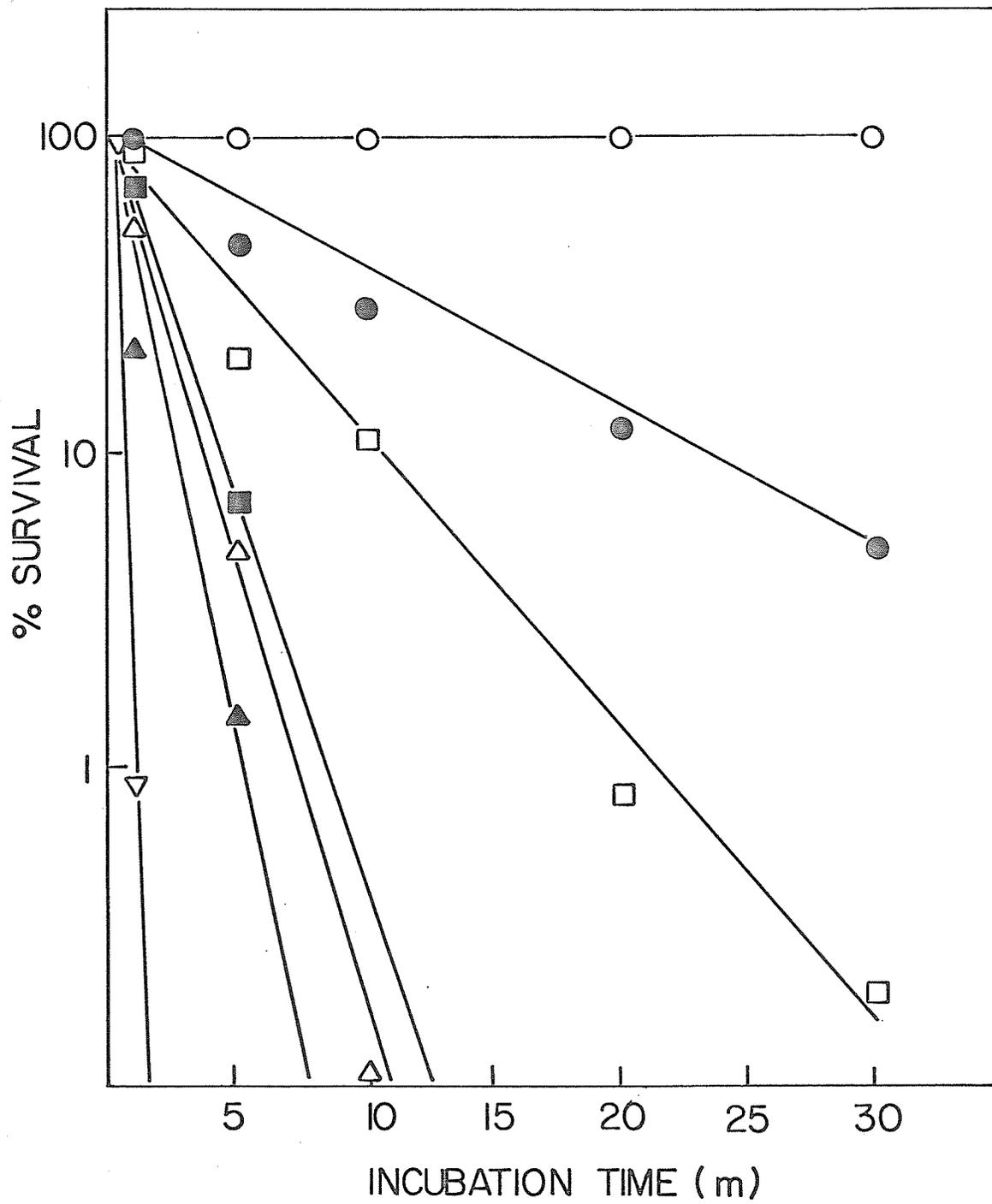


Figure 10. Inactivation of bacteriophage T7 by ascorbic acid in SM buffer (III).

The modified double layer titering procedure and E. coli B23 were used. As described in Methods the phage and bacteria mixture was incubated for 30 minutes before plating. The symbols are as described in Figure 8.

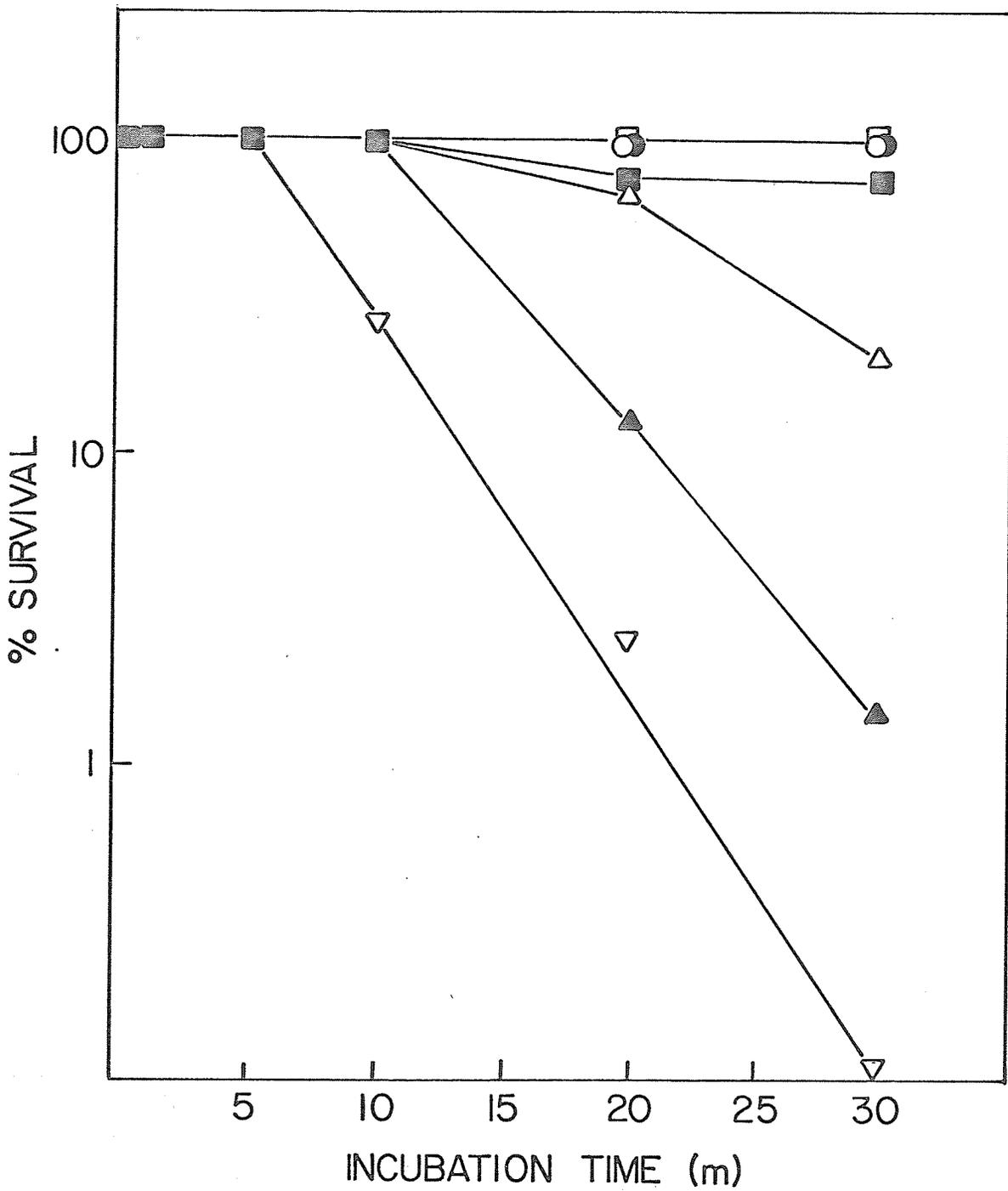
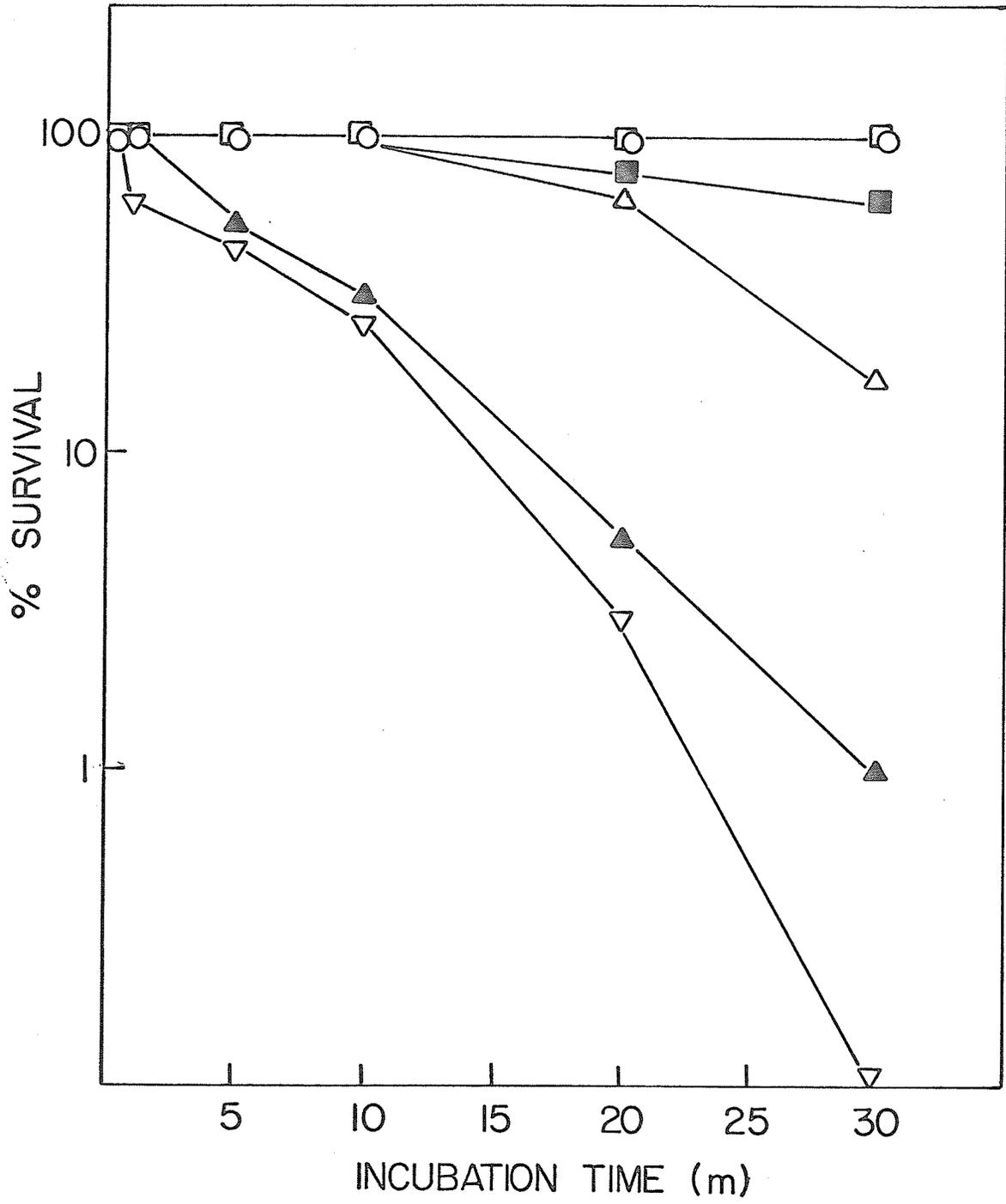


Figure 11. Inactivation of bacteriophage T7 by ascorbic acid in SM buffer (IV).

All details are described in Figure 10, except that the host was E. coli B 5975, a rec A⁻ strain.



of inactivation when the *recA* strain was the plating host and second, the repair efficiency or recovery rate during the supplementary incubation was lower in the *recA* strain. For example, after treatment with 5.7 mM ascorbate, there was 100% survival among phage plated on the wild type strain but only 30% survival among phage plated in the *recA* strain. Therefore the *recA* gene product plays a role in allowing the phage to recover from the ascorbate treatment but this role is relatively minor.

This data also illustrates the importance of titering conditions in phage inactivation experiments. The rates of inactivation resulting from ascorbate treatment or any other treatment must be reported in terms of the degree of recovery time allowed prior to plating.

3.2.2 Delayed Lysis Effect

Another manifestation of the effect of ascorbate on bacteriophage T7 was observed when *E. coli* B23 growing in the presence of ascorbate was infected with T7. The burst size was the same as in the absence of ascorbate, but the time of lysis was delayed and the length of the delay was proportional to the ascorbate concentration (Table 4).

A similar effect was observed when the phage was pretreated with ascorbate and then added to a culture of *E. coli* in the absence of ascorbate (Table 5 and Figure 12). Five minutes after infection, at a MOI of 1, the infected culture was centrifuged; the supernatant containing any unadsorbed phage was removed; and the bacterial pellet resuspended at the same cell density in fresh media warmed to 37°C. In the case of untreated phage, cell lysis was detectable as a sharp decline in optical

TABLE 4

Effect of exogenous ascorbic acid on cell lysis by T7.

Ascorbic Acid Concentration (mM)	Time of Lysis (min)	Burst size ($\times 10^2$)
control	35	1.6
5.7	60	1.6
19.25	135	1.5
28.5	>290	1.6
57.0	no lysis	—
92.75	no lysis	—

Infection was carried out at a MOI of 1 in the presence of the indicated concentrations of ascorbic acid. Infection was allowed to proceed for 5 minutes in the presence of ascorbic acid. Any unabsorbed phage were removed by centrifugation and the cells were resuspended in fresh medium warmed to 37°C containing ascorbic acid. Phage titer was determined as described in Materials and Methods.

density starting about 30 minutes after infection and was usually complete by 90 minutes. This is evident in Figure 12. In contrast, cells infected with ascorbic acid treated phage did not lyse immediately. The onset of lysis was delayed but the duration of the lysis period itself was not increased significantly. During the period after infection when lysis was delayed, the bacteria continued to grow quite well. Even though the final optical density after infection was higher than that of the control, the extent of lysis was the same. The bacterial survival rate after lysis was less than 0.01% and the phage burst size in all cases was the same as the control (Table 5). If the lysed cultures, including that lysed with untreated phage, were incubated overnight, phage resistant bacterial cultures were obtained. The range of ascorbate concentrations, pretreatment times, times of lysis and phage titers immediately after lysis are compiled in Table 5. Only at the very high concentrations of ascorbic acid and after longer pretreatment times was there a total inhibition of lysis and phage production.

In order to characterize the progeny phage, the lysates resulting from cultures infected with phage pretreated for various times with 5.7 mM ascorbate were analyzed in CsCl equilibrium gradients. A UV absorbing peak corresponding to whole phage of normal size was evident for all lysates except when phage treated for 30 minutes was used (Figure 13). No lysis had occurred in this latter case, and no phage were produced. The identity of the heavier fraction at the bottom of the gradient was not determined.

TABLE 5

Effect of pretreatment of T7 phage with ascorbic acid on time of lysis.

Ascorbic acid Concentration (mM)	Pretreatment Time (min)	Time of Lysis (min)	Phage titer after Lysis ($\times 10^{10}$)
B23			
0.057	0	30	2.0
	1	45	2.0
	5	60	2.3
	10	60	3.0
	20	150	2.0
	30	no lysis	---
	2.8	0	40
1		60	3.0
5		90	2.3
10		105	2.8
20		160	3.0
30		no lysis	---
5.7		0	40
	1	60	2.0
	5	90	2.0
	10	120	1.8
	20	165	1.6
	30	no lysis	---
	28.5	0	40
1		50	1.0
5		90	0.8
10		---	---
20		---	---
30		---	---
B5975			
2.8	0	50	2.0
	1	60	1.7
	5	90	2.0
	10	115	0.02
	20	no lysis	---
	30	no lysis	---

E. coli B5975 (recA^-) and B23 were grown to 1.0×10^8 per mL in LB medium and infected at a MOI = 1.0.

Figure 12. Delayed lysis effect.

E. coli B23 was infected at a MOI of one with T7 bacteriophage that had been pretreated for specified times with 2.85 mM ascorbic acid in LB medium. Growth was carried out in LB medium. The times of ascorbic acid pretreatment were 0 minutes, (●); 1 minute, (○); 5 minutes, (■); 10 minutes, (▲); 20 minutes, (□); 30 minutes, (△). Infection was carried out at $t=1.5h$.

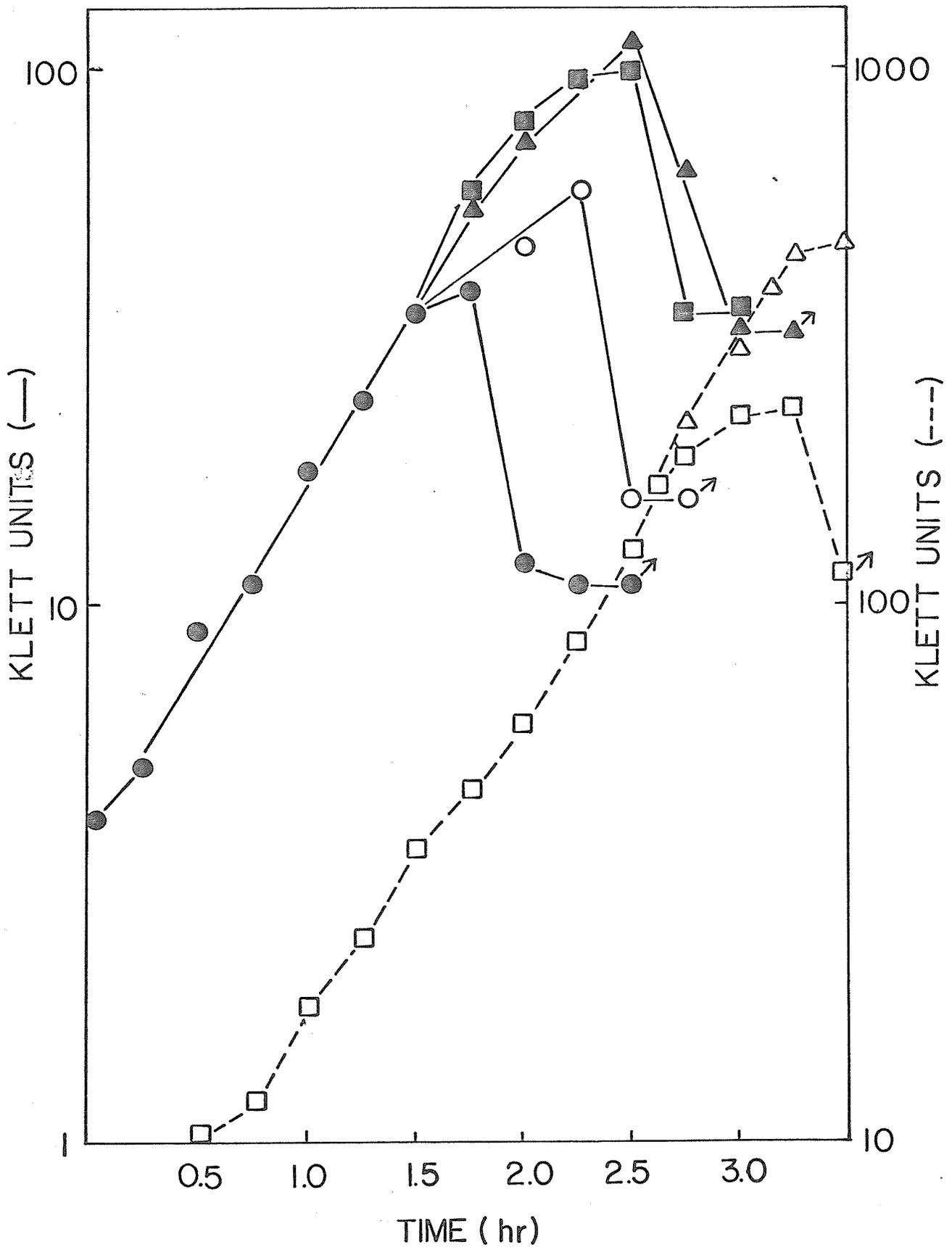
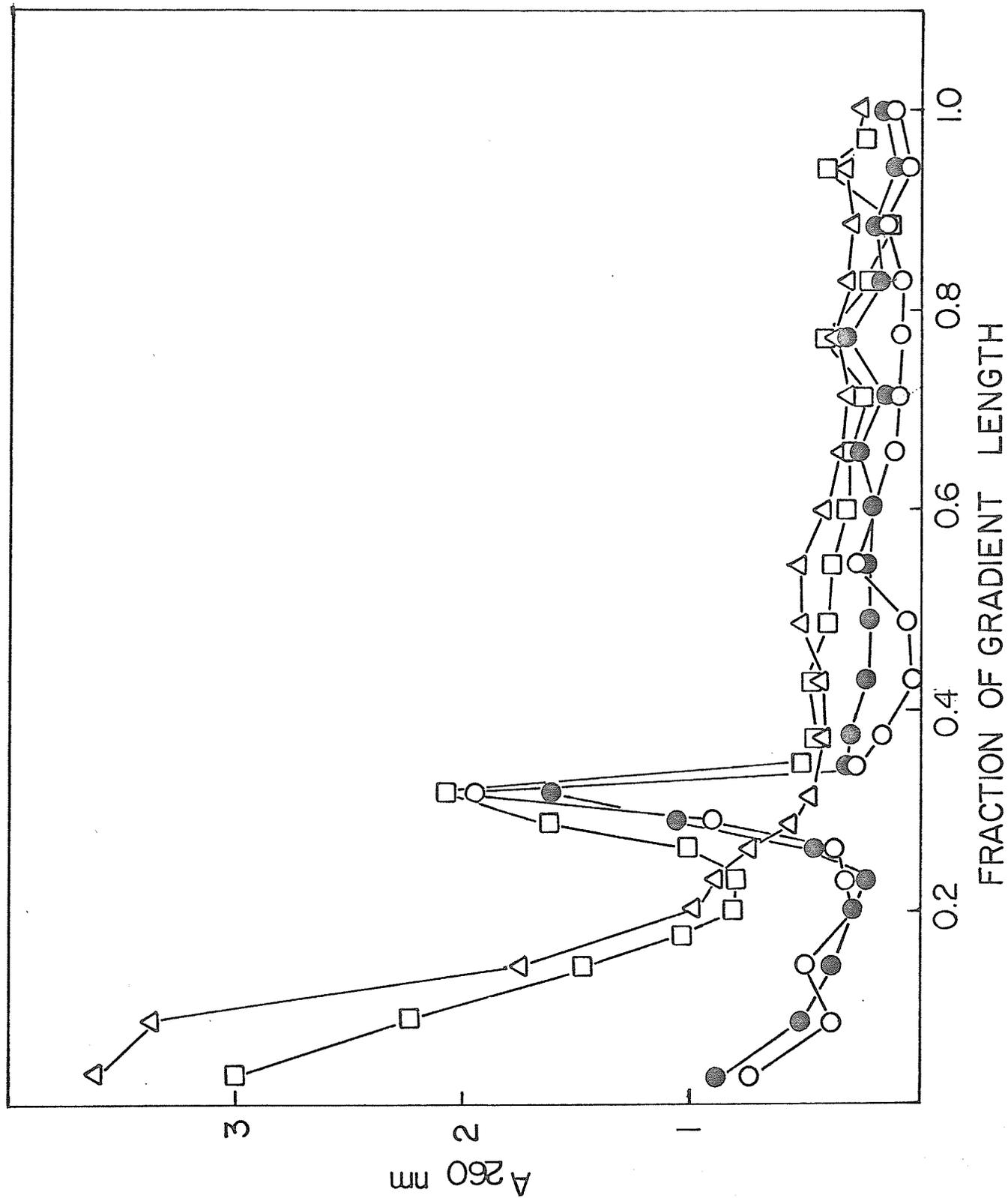


Figure 13. Analysis of the lysates of cultures infected with T7 phage treated for various times with 5.7 mM ascorbate.

Lysate aliquots of 2 mL were clarified by centrifugation and dialyzed against 6 changes of SM buffer for 48 hours. The lysate was then mixed with an equal volume of saturated CsCl and centrifuged as described in Methods. The lysates used were from cultures infected with phage pretreated with ascorbate for the following times: 0 minutes, (●); 1 minute, (○); 20 minutes, (□); and 30 minutes, (Δ).



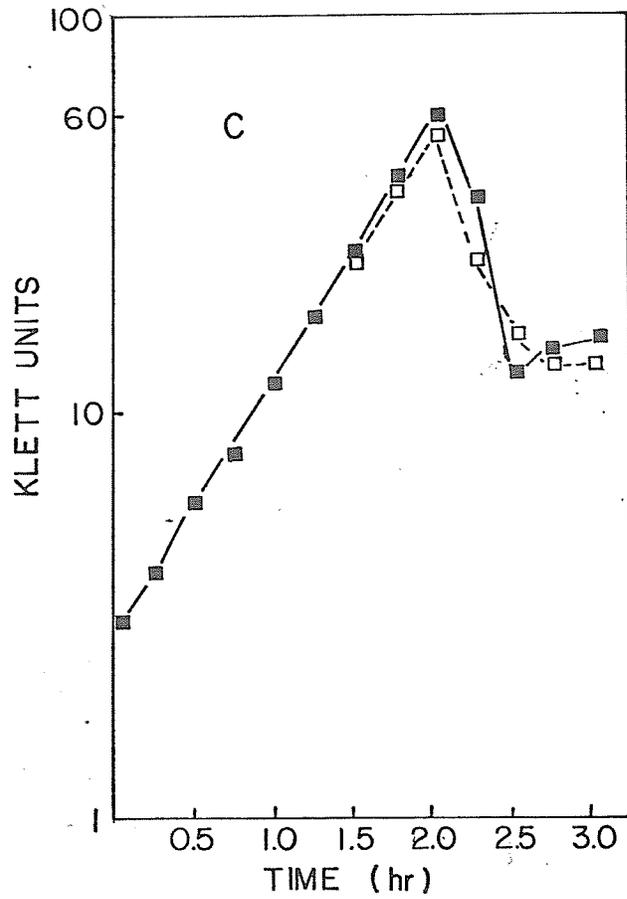
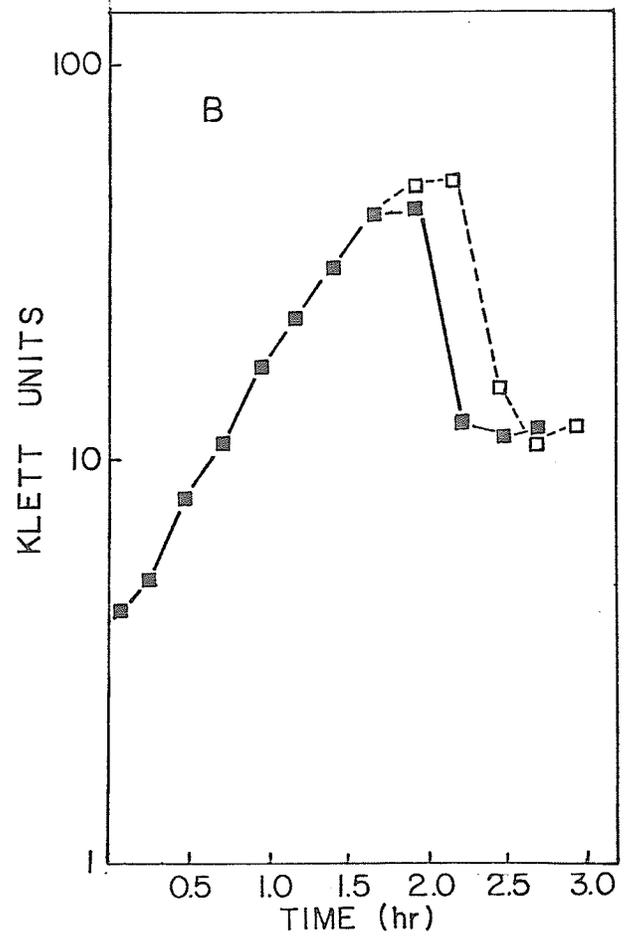
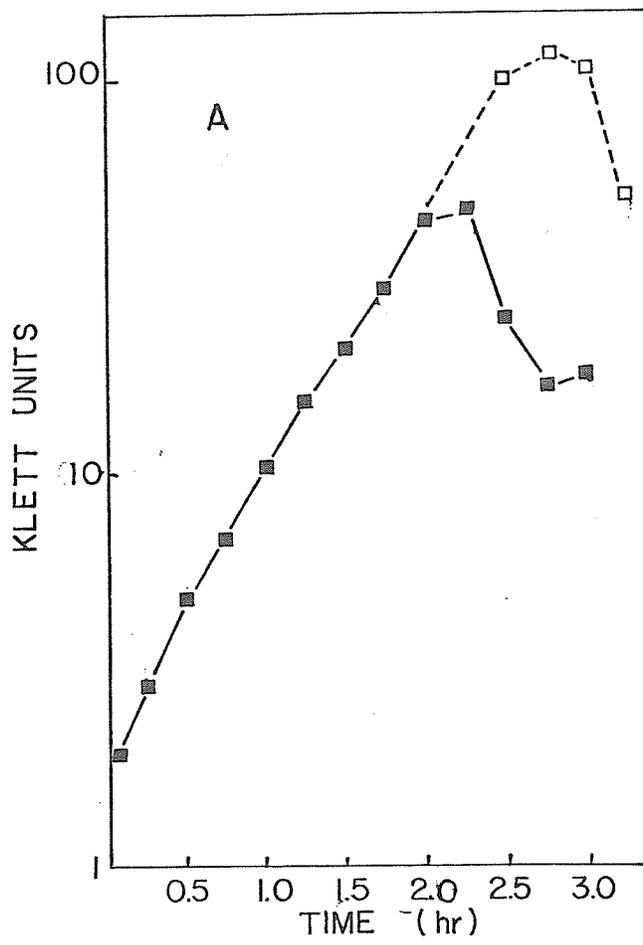
As another means of characterizing the progeny phage the lysates from cultures infected with phage pretreated with ascorbate were used to re-infect E. coli. All cultures were lysed normally within 40 minutes except for the culture reinfected with the 30 minute lysate where as noted above, lysis had not occurred.

The multiplicity of infection influenced the length of delay in lysis after infection with ascorbate treated phage (Figure 14 A,B,C,). When the MOI was 0.1 there was a pronounced delay in lysis of up to 1.5 hours probably the result of multiple lysés and reinfections necessary to infect all cells present. At a MOI of 1 the culture lysed after a 15 minute delay and at a MOI of 10, the culture lysed with the control. At a MOI of 10, lysis could have been from without. In all the experiments described in this report a MOI of one phage per bacterium was used as the standard infection procedure.

The fact that the phage titer obtained even after a delayed lysis was normal and that the progeny phage infected normally indicated that the inactivating event as described by Murata and Kitagawa (1973) was being repaired in an actively growing E. coli culture. This was consistent with the observation in Part A that incubation prior to plating increased phage viability. This will be discussed in greater detail after the nature of the effect of ascorbate on T7 is described.

Figure 14. Effect of multiplicity of infection on delay of lysis and growth.

E. coli B23 was infected with T7 bacteriophage that had been treated for 2 minutes with 5.7 mM ascorbic acid. Growth was followed as described in Methods. No ascorbic acid treatment, (■); ascorbic acid treatment, (□). Infection was carried out at a MOI of 0.1 (A), 1.0 (B), and 10.0 (C).



3.2.3 Effect of Ascorbic Acid on T7 Bacteriophage DNA

In aqueous media especially in the presence of the divalent cation Fe^{+2} and Cu^{+2} , ascorbic acid can undergo oxidation to form dehydroascorbic acid and H_2O_2 (Morgan et al., 1976; Weisberger and LuValle, 1934). This oxidation is also accompanied by the formation of free radicals (Weisberger et al., 1943; Weisberger and LuValle, 1944; Orr, 1966) which are known to interact with bacteriophage DNA (Murata and Kitagawa, 1973; Murata et al., 1975; Murata and Uike, 1976; Morgan et al., 1976). The end result is that ascorbic acid is able to cause nicking of most phage DNA even when the DNA is encapsulated.

Following treatment of whole phage with ascorbate, the integrity of the DNA was analyzed using alkaline sucrose gradients, neutral sucrose gradients and agarose gel electrophoresis. Whether the nicks were single or double stranded and the degree of nicking could be determined using these techniques.

3.2.3.1 Alkaline Sucrose Gradients

T7 phage that had been treated for specified time periods at various concentrations of ascorbic acid and then with NaOH to release the DNA, were analyzed using alkaline sucrose gradients. As shown in Figure 15, treatment of T7 phage with 0.57 mM ascorbic acid in SM buffer caused a very rapid accumulation of nicks in the DNA. The abrupt shifts of the DNA peak up the gradient indicated that the nicking was random along the DNA. The number of nicks was estimated as described by Litwin (1969)

and the data are compiled in Table 6. Greater than 20 nicks per molecule cannot be estimated by this method and with the ascorbate concentration above 0.285 mM, less than 10 minutes was required to accumulate that many nicks. Approximately the same rate of nick formation was observed at 5.7, 2.85 and 0.285 mM ascorbate suggesting that the active species was not present in proportion to the amount of ascorbate. Rather, there must have been a limit to the rate at which the active species was formed independent of the ascorbate concentration above a certain level, probably determined by the rate of oxidation.

Freifelder (1965) observed that the effect of X-rays on T7 bacteriophage was much greater in buffer than it was in media broth and a comparison of Table 6 and Table 7 reveals that ascorbate induced cleavage of DNA was more effective in SM buffer than in LB medium. The rate of nick formation in LB medium by 5.7 mM ascorbic acid was approximately half of the rate in SM buffer. The reduced nicking in LB medium may have been a result of the reduced availability of metal ions because of the high concentration of metal chelators such as histidine. This would result in a smaller amount of the nick forming species being formed because ascorbate oxidation would be slowed.

3.2.3.2 Neutral Sucrose Gradient Analysis

In order to differentiate between single and double strand nicks introduced into the DNA, the DNA was also analyzed on neutral sucrose gradients. In neutral sucrose gradients the secondary structure provides sufficient stability that single strand nicks do not fragment the DNA. Only double strand nicks or two single strand nicks on opposite strands

Table 6. Nick analysis of T7 phage DNA.

Rate of nick formation in [^{32}P] T7 phage DNA during treatment of whole phage with ascorbic acid in SM buffer as analyzed on alkaline sucrose gradients. The procedure is described in Methods.

TABLE 6

Gradient analysis of T7 DNA - treatment in SM buffer.

	Ascorbate Concentration (mM)	Treatment Time (min)	D2/D1	Nicks
A.	5.7	0	-----	0
		1	0.710	5
		5	0.436	16
		10	0.282	>20
		20	0.154	>20
		30	0.103	>20
		60	0.080	>20
B.	2.8	0	-----	0
		1	0.722	5
		5	0.443	15
		10	0.317	>20
		20	0.264	>20
		30	0.080	>20
		60	0.050	>20
C.	0.57	0	-----	0
		1	0.752	4
		5	0.430	15
		10	0.330	>20
		20	0.231	>20
		30	0.165	>20
		60	0.099	>20
D.	0.285	0	-----	0
		1	0.824	3
		5	0.486	9
		10	0.395	20
		20	0.260	>20
		30	0.219	>20
		60	0.094	>20
E.	0.057	0	-----	0
		1	0.830	2
		5	0.690	4
		10	0.550	8
		20	0.491	12
		30	0.380	18
		60	0.340	>20

very close together will result in fragmentation of the DNA being evident on the gradient. Unlike ascorbate treated DNA analyzed on alkaline sucrose gradients, ascorbate treated DNA analyzed by neutral sucrose gradients showed little evidence of nicking except at longer times. In LB medium smaller material appeared after a 20 minute treatment with 5.7 mM ascorbate and after a 30 minute treatment a majority of the phage DNA had been cleaved by double strand breaks (Figure 16). In SM buffer a 30 minute treatment with 2.8 and 5.7 mM ascorbate resulted in the formation of even smaller fragments than were observed in LB medium (Figure 17). It is also relevant that a 30 minute treatment of infecting phage with 5.7 mM ascorbate in LB prevented culture lysis suggesting a link between double strand breaks and phage inactivation.

3.2.3.3 Agarose Gel Electrophoresis

T7 phage DNA was also analyzed on agarose gels following ascorbate treatment. Treatment of T7 bacteriophage with ascorbate in SM buffer for 5 minutes had no effect on the appearance of the DNA band after electrophoresis (Figure 18 a). However, after heat treatment at 90°C for 10 minutes there was extensive fragmentation which increased in proportion to the ascorbate concentration (Figure 18 b). A comparison of the fragments obtained from ascorbate treatment, with HpaI and HaeIII digest patterns of T7 DNA (McDonnell et al., 1977), showed the fragment range was between 6400 and 1800 base pairs in size. A similar one min-

Figure 15. Alkaline sucrose gradient analysis of T7 DNA.

Alkaline sucrose gradient analysis of [^{32}P] T7 DNA after whole phage was treated with 0.57 mM ascorbic acid in SM buffer for various times. The procedure is described in Methods. The phage were treated for 0 min, (●); 1 minute, (○); 5 minutes, (△); 10 minutes, (▲); 20 minutes, (□); 30 minutes, (■); 1 hour, (▽). Radioactivity was determined by the Cerénkov method.

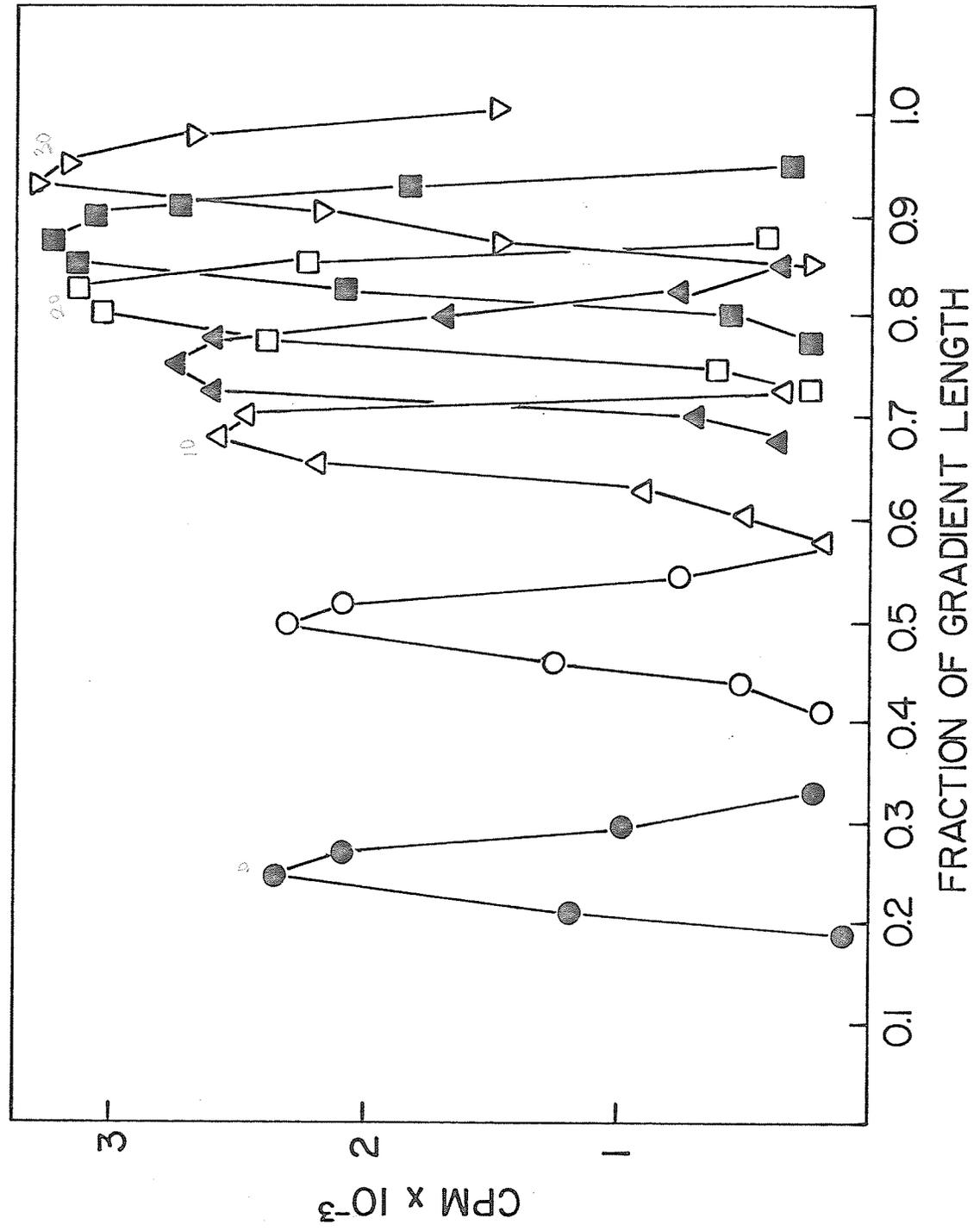


TABLE 7

Gradient analysis of T7 DNA - treatment in LB buffer.

Treatment Time (min)	D2/D1	Nicks
0	-----	0
1	0.971	1
5	0.565	9
10	0.374	19
20	0.218	>20
30	0.182	>20
60	0.159	>20

Whole phage were treated with ascorbic acid (5.7mM) in LB medium and run on alkaline sucrose gradients as described in the Methods.

Figure 16. Neutral sucrose gradient analysis of T7 phage in LB media.

Neutral sucrose gradient analysis of [^{32}P] T7 DNA following treatment of the whole phage with 5.7 mM ascorbic acid in LB medium. The procedure is described in Methods. The times of ascorbate treatment were 0 minutes, (■); 5 minutes, (○); 10 minutes, (△); 20 minutes, (□); 30 minutes, (▽). Radioactivity was determined by the Cerénkov method.

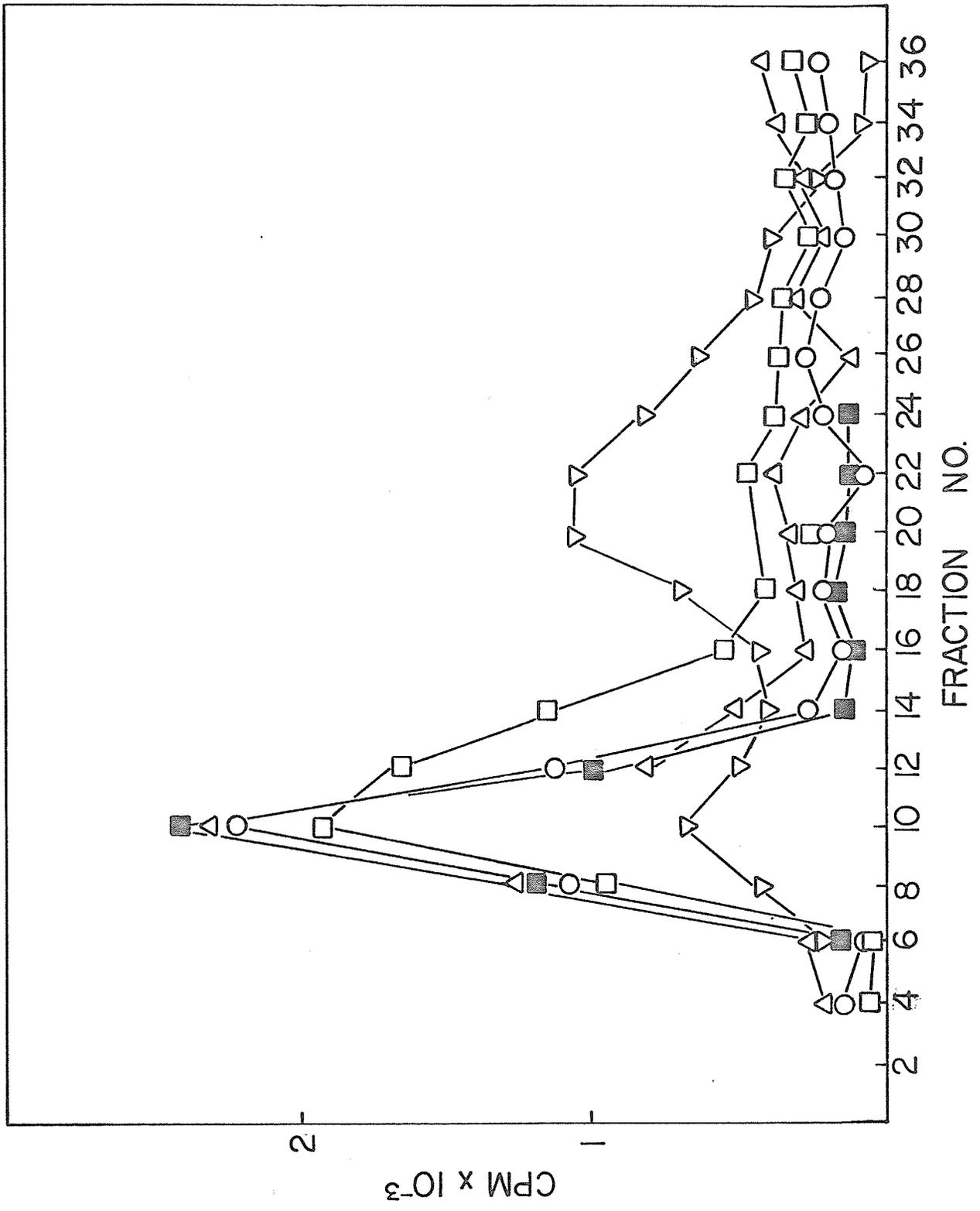
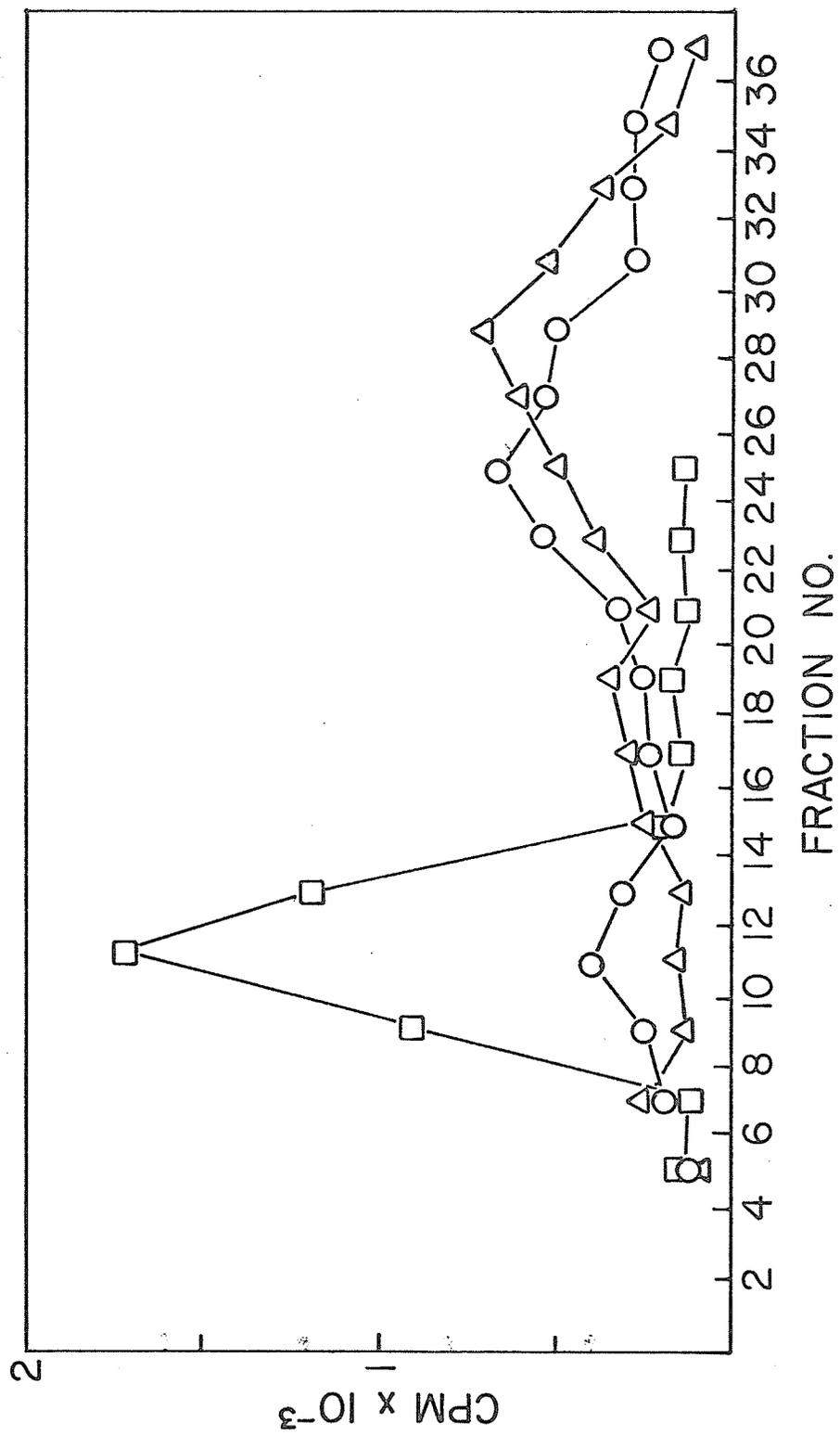


Figure 17. Neutral sucrose gradient analysis of T7 phage in SM buffer.

Neutral sucrose gradient analysis of [^{32}P] T7 DNA following treatment of whole phage with ascorbic acid for 30 minutes in SM buffer. The procedure is described in Methods. The ascorbic acid concentrations were 0 mM, (\square); 2.8 mM, (\circ); and 5.7 mM, (Δ). Radioactivity was determined by the Cerénkov method.



ute ascorbate treatment resulted in only limited fragmentation while a ten minute ascorbate treatment resulted in more extensive fragmentation. In both cases heat treatment was necessary for fragment formation. The lack of fragmentation without heat treatment was consistent with only single strand nicks being introduced during the short ascorbate treatments.

Following the 5 minute treatment of bacteriophage T7 with ascorbic acid at various concentrations, the DNA was also analyzed on a denaturing agarose gel by comparison with a HaeIII digest of untreated T7 DNA. No further fragmentation was made evident under the denaturing conditions.

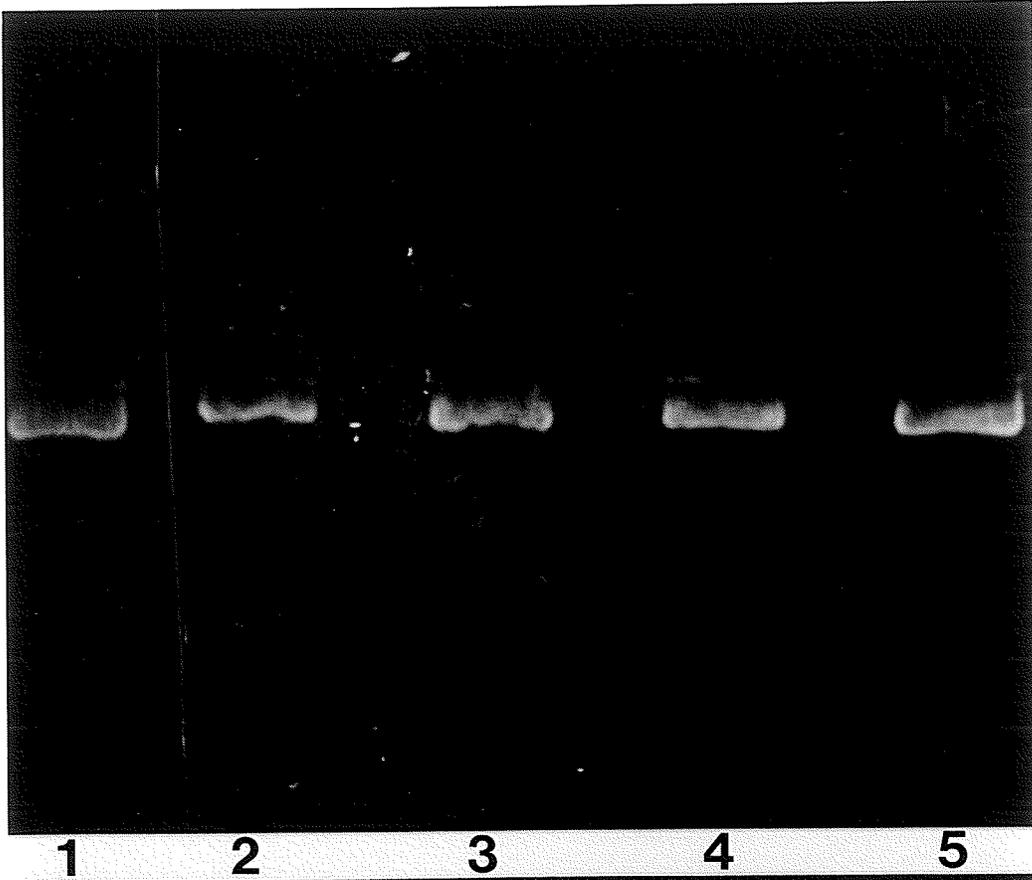
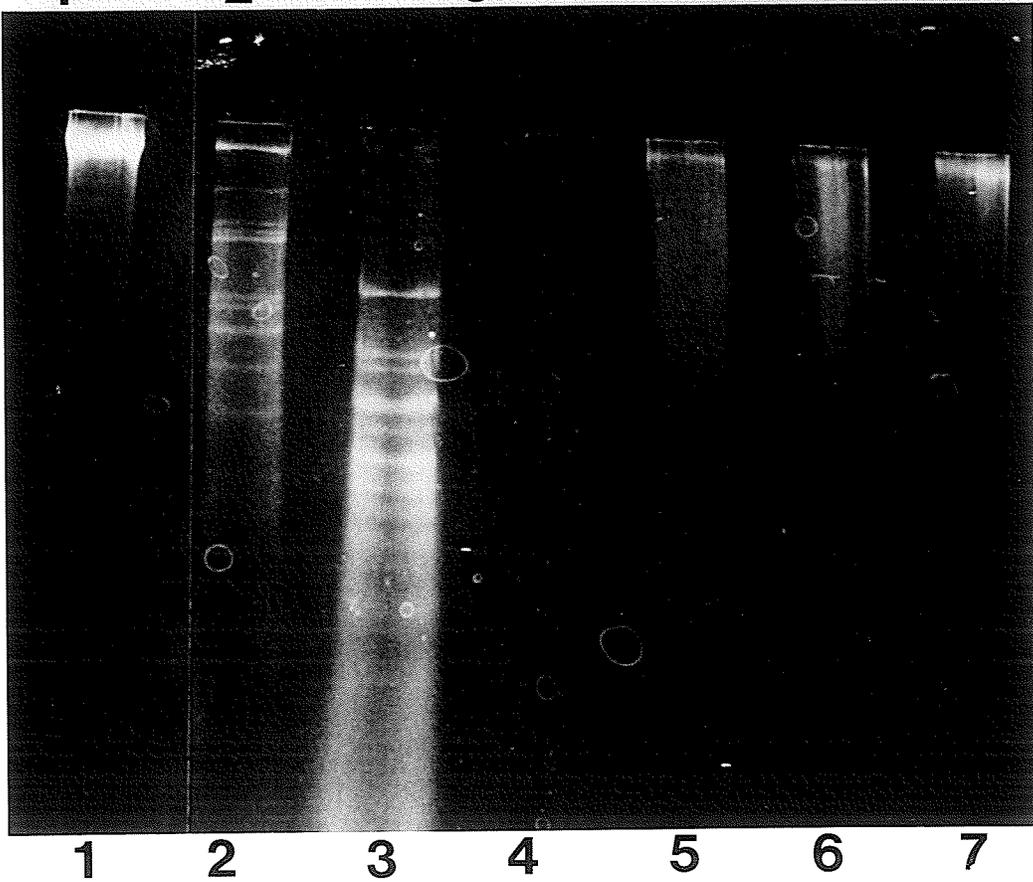
3.2.3.4 Effect of Ascorbate Cleavage on Restriction Nuclease Cleavage

One possible procedure for investigating the sequence specificity of the ascorbate induced cleavage of DNA was to study the effect of ascorbate cleavage on the availability of restriction nuclease sites. T7 bacteriophage DNA was isolated as described in Methods and treated with 28.5 mM, 5.7 mM and 2.8 mM ascorbic acid for 3 minutes in SM buffer. After treatment the DNA was reprecipitated with ethanol, washed in ethanol, dried under vacuum and then dissolved in the appropriate buffer for the particular restriction enzyme. As shown in Figure 19, there was no effect on restriction cleavage pattern of either the HaeIII digest with the recognition sequence GGCC or the HpaI digest with the recognition sequence GTTAAC. The reduced amounts of DNA in lanes 7 and 8 are probably the result of inefficient ethanol precipitation of smaller frag-

Figure 18. Effect of ascorbate on phage DNA.

T7 bacteriophage was treated in SM buffer with the specified concentrations of ascorbic acid for 5 minutes. The phage was lysed to release the DNA as described in Methods. Heat treatment where specified was carried out in SM buffer as described in Methods. DNA was viewed after ethidium bromide intercalation by fluorescence. Figure 18b phage samples were heated as specified.

- a.
 1. T7 control
 2. T7 + 28.5 mM ascorbic acid
 3. T7 + 5.7 mM ascorbic acid
 4. T7 + 2.8 mM ascorbic acid
 5. T7 + 0.57 mM ascorbic acid
- b.
 1. T7 control
 2. T7, Hpa I digest
 3. T7, Hae III digest
 4. T7 + 28.5 mM ascorbic acid
 5. T7 + 5.7 mM ascorbic acid
 6. T7 + 2.8 mM ascorbic acid
 7. T7 + 0.57 mM ascorbic acid.

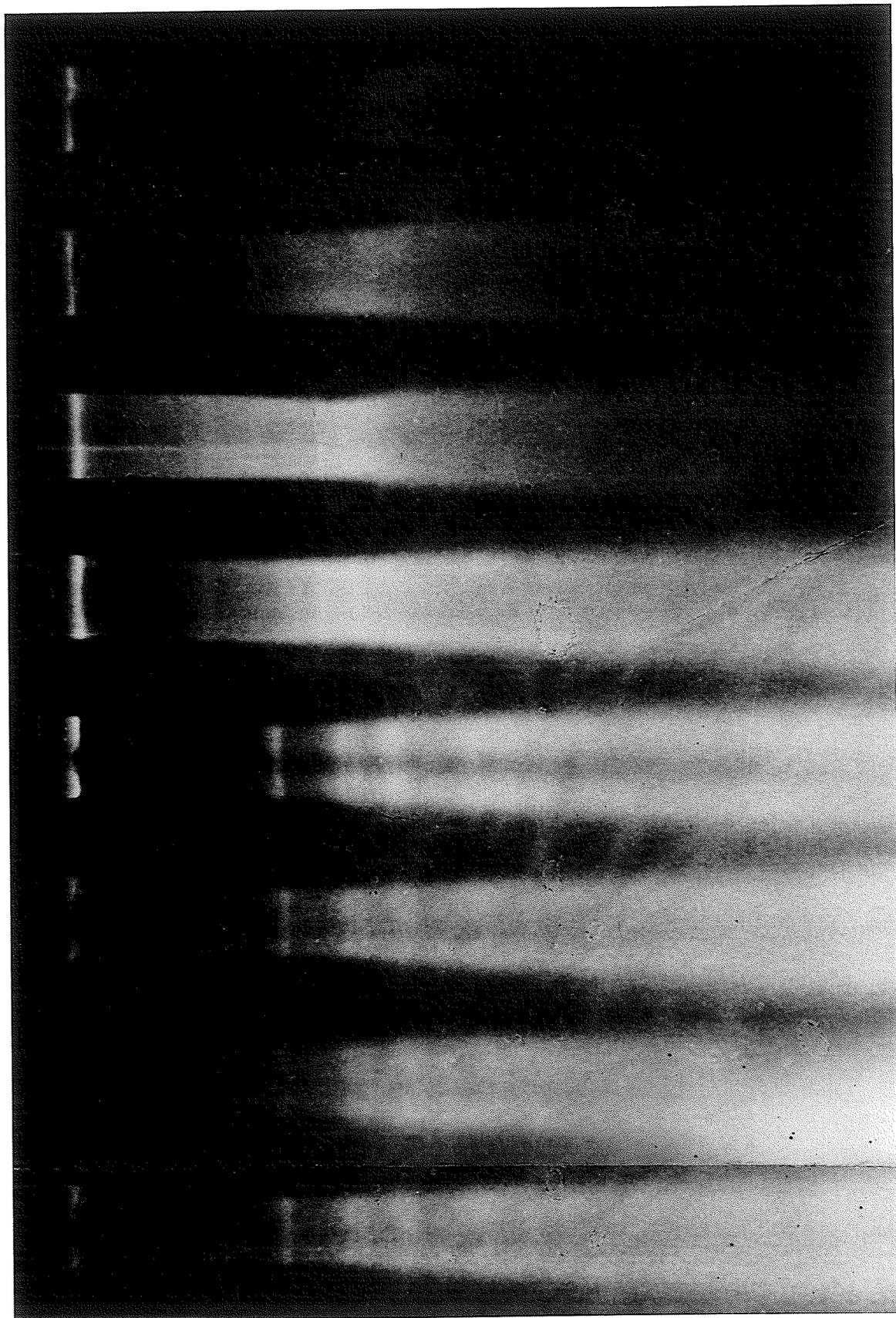
a**b**

ments. A ten minute ascorbate treatment preceding HaeIII nuclease cleavage also failed to have any specific effect on the nuclease cleavage. Therefore the ascorbate induced cleavage of DNA did not affect specific sequences preferentially, at least not those involved with HpaI and HaeIII recognition.

Figure 19. Effect of ascorbic acid on restriction nuclease cleavage of T7 DNA.

T7 DNA was isolated as described in the Methods and treated with the specified concentration of ascorbic acid for 3 minutes. Preparation of the sample for loading and running on a neutral agarose gel was as described in Methods. The control was treated like the other samples but without the treatment with ascorbic acid. DNA was viewed after ethidium bromide intercalation by fluorescence.

1. T7 control DNA, Hae III digest
2. T7 DNA + 28.5 mM ascorbic acid + Hae III
3. T7 DNA + 5.7 mM ascorbic acid + Hae III
4. T7 DNA + 2.8 mM ascorbic acid + Hae III
5. T7 control DNA, Hpa I digest
6. T7 DNA + 28.5 mM ascorbic acid + Hpa I
7. T7 DNA + 5.7 mM ascorbic acid + Hpa I
8. T7 DNA + 2.8 mM ascorbic acid + Hpa I.



8

7

6

5

4

3

2

1

3.2.4 Effect of Ascorbic Acid on Bacteriophage T7 DNA Replication

The DNA replication process following T7 phage infection was investigated using neutral sucrose gradients as described by Hausmann and LaRue (1969) and Frankel (1966). Following the infection of *E. coli* with uniformly labelled [32 P] T7 phage, aliquots were taken at various times and sedimented through neutral sucrose gradients. Within two minutes after infection with untreated phage, a high molecular weight intermediate, characteristic of T7 DNA replication (Studier, 1969), was formed (Figure 20). When the phage had been pretreated for 5 minutes with 2.8mM ascorbic acid, the DNA was injected into the bacteria, but a longer time was required for the appearance of the high molecular weight intermediate (Figure 21). Therefore the injected DNA was biologically active but a longer time was required for DNA replication to initiate, presumably to allow for nick repair. Freifelder (1965,1966) and Murata and Kitagawa (1973) have also observed that damaged phage DNA can be injected into the bacterium, but in the latter reference it had been concluded that the DNA was not biologically active.

Figure 20. T7 replication cycle.

Isolation and characterization of replication intermediates in T7 bacteriophage DNA replication. This experiment was carried out as described in the Methods section. Samples were removed and analyzed at the following times after infection:

▽ - control T7 phage, no infection

○ + 2 minutes after infection

△ + 5 minutes after infection

□ + 15 minutes after infection

● + 30 minutes after infection

▲ + 40 minutes after infection, lysis had occurred.

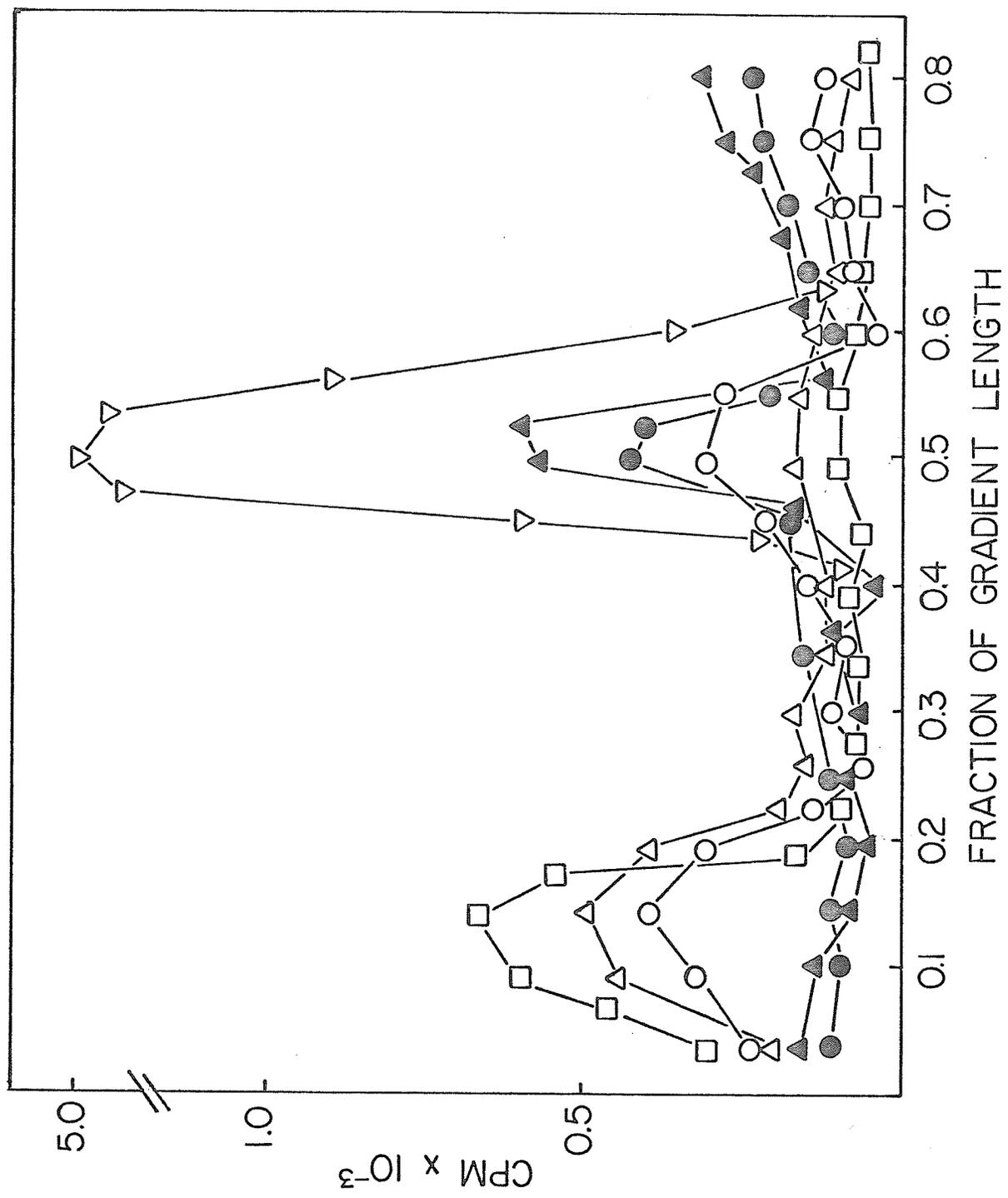


Figure 21. Effect of ascorbic acid pretreatment of T7 phage on the DNA replication cycle.

This experiment was performed as described in the Methods. The phage had been pretreated for 5 minutes with 2.8 mM ascorbic acid prior to infection. Samples were removed and analyzed at the following times after infection:

- ▽ control T7 phage, no infection
- + 2 minutes after infection
- + 30 minutes after infection
- ▲ + 60 minutes after infection
- + 85 minutes after infection.

3.2.5 Effect of Catalase on Ascorbic Acid Nicking of T7 DNA

Using an ethidium bromide intercalation assay, Morgan et al. (1976) have shown that the ascorbate induced nicking of PM2 DNA was suppressed by 1.25 $\mu\text{g}/\text{mL}$ of catalase. The work of Morgan et al. has been extended to include T7 phage but with alkaline sucrose gradients being used as the analytical tool, rather than ethidium bromide intercalation. Whole phage were treated with ascorbic acid in the presence of two concentrations of catalase after which the reaction was stopped and the phage lysed with 1mM EDTA and 0.2M NaOH. Prior to the 5 minute treatment with 5.7 mM ascorbate, catalase at 100 $\mu\text{g}/\text{mL}$ and 200 $\mu\text{g}/\text{mL}$ were added to the mixture. Only the 200 $\mu\text{g}/\text{mL}$ concentration eliminated the nick formation (Figure 22) and longer ascorbate treatments in both LB medium and SM buffer even caused nicking in the presence of 200 $\mu\text{g}/\text{mL}$ of catalase (Table 8 A). Controls were run with only catalase present and no nicking was evident even after one hour (Table 8 B). It is not clear why there was more nicking in LB medium than SM buffer, a fact which was inconsistent with the data in Tables 6 and 7. These results suggest that another ascorbate mediated effect may be responsible for the nicking observed on alkaline sucrose gradients at the later time points. This effect may be a more specific ascorbate effect on bases or may be the result of an organic peroxide.

Table 8. The effect of catalase on ascorbic acid treatment of T7 DNA.

Nick analyses were carried out as described in the Methods and previous tables, except that in this case treatment was in the presence of 200 $\mu\text{g}/\text{ml}$ of catalase (A). In (B) no ascorbate was present.

TABLE 8

Effect of catalase on ascorbic acid treatment of T7 DNA.

A.

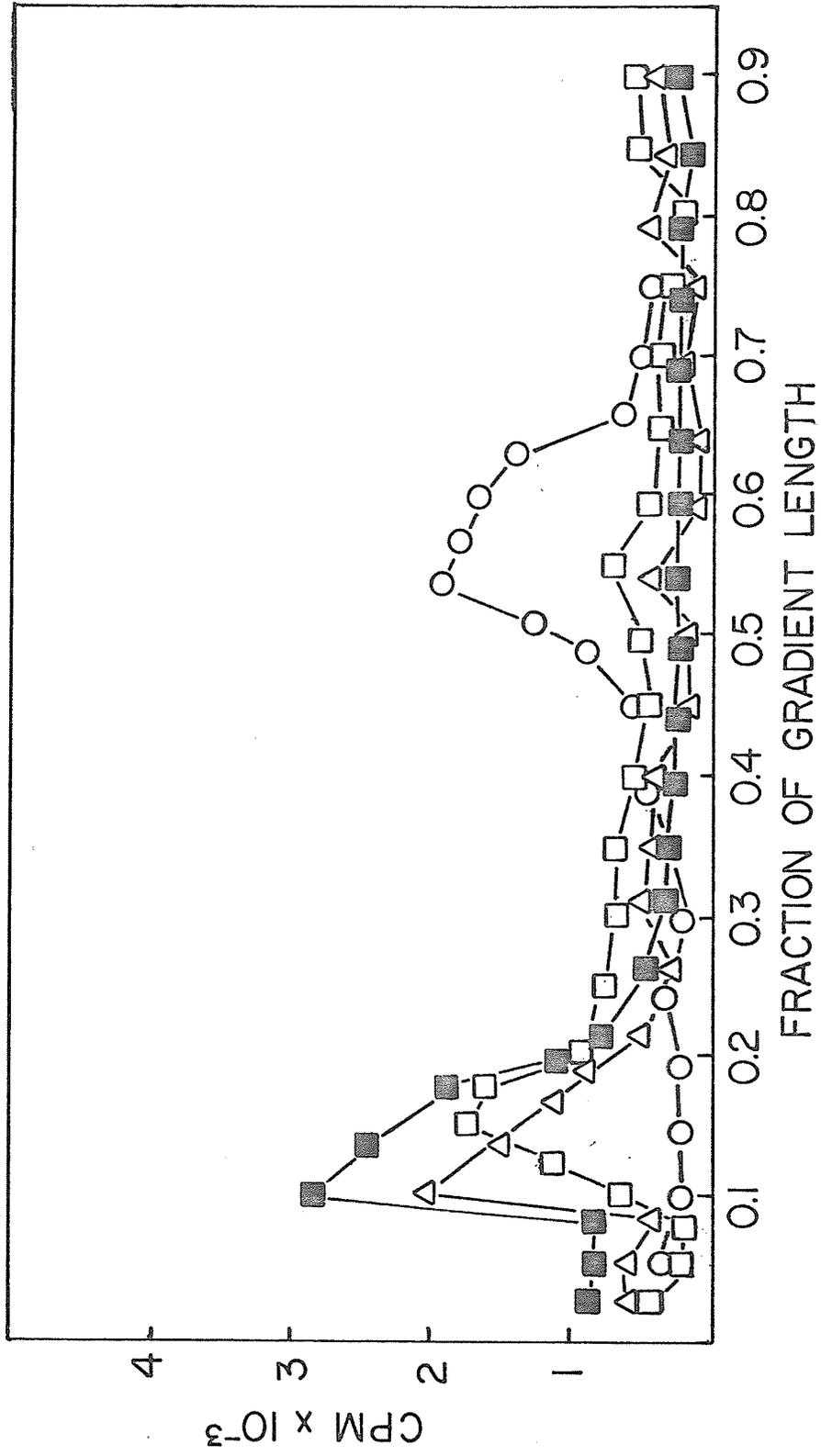
Ascorbate Treatment (min)	Medium	D2/D1	Nicks
	LB		
control		-----	---
5		1	0
10		1	0
20		0.870	2.0
30		0.720	3.8
60		0.400	18
	SM buffer		
control		-----	---
5		1	0
10		1	0
20		1	0
30		0.870	2.0
60		0.620	6.2

B.

Catalase Treatment (min)	Medium	D2/D1	Nicks
	LB		
5		1	0
10		1	0
20		1	0
30		1	0
60		1	0
	SM buffer		
5		1	0
10		1	0
20		1	0
30		1	0
60		1	0

Figure 22. The effect of catalase on ascorbate induced nicking of T7 DNA.

T7 bacteriophage were treated with 5.7 mM ascorbic acid for 5 minutes in the presence of specified concentrations of catalase. The reaction was stopped by the addition of EDTA to a final concentration of 1 mM and NaOH to a final concentration of 0.2 M. Sedimentation analyses were performed on alkaline sucrose gradients as described in the Methods section. T7 bacteriophage were treated with the following additions: (■), no addition; (○), 5.7 mM ascorbate; (□), 5.7 mM ascorbate and 100 $\mu\text{g}/\text{mL}$ catalase; (Δ), 5.7 mM ascorbate +200 $\mu\text{g}/\text{mL}$ catalase. The D_2/D_1 values obtained for the above gradients were: -, 0.492, 0.952, and -, respectively. This corresponds to 0, 9, 1.5, and 0 nicks respectively.



3.2.6 Effect of Ascorbic Acid on Deoxyribonucleotides

As described in the last section, catalase did not completely eliminate the nicking effect of ascorbate on T7 phage DNA at the longer time points. In order to determine if ascorbate was having a more specific base modification effect, the individual deoxyribonucleotides and deoxyribonucleosides were treated with ascorbate. The 5'deoxyribonucleotide monophosphates (dpN), 3'deoxyribonucleotide monophosphates (dNp) and deoxyribonucleotides (dN) were studied but there were no observable differences in the results obtained with either set of nucleotides or nucleosides. Both one dimensional chromatography and the two dimensional chromatography system of Rogg et al. (1976) were used in an attempt to separate any nucleotides with modified bases from the starting materials in experiments performed as described in the Methods. After the reaction, aliquots were spotted on cellulose plates which were developed either in one dimension in solvent A, solvent B or Pabst, or in two dimensions by running in either solvent A or B in the first dimension and Pabst in the second dimension.

After examination under UV light, there was no ascorbate-induced change in migration of any of the nucleotides. From the results obtained here it was apparent that if there were a specific ascorbate effect, it was not seen under these conditions. Alternatively, the effect seen with the phage DNA may have been due to the fact that 200µg/mL of catalase was not enough to eliminate the nicking effect caused by hydroxyl radicals formed from the breakdown of hydrogen peroxide derived from the oxidation of ascorbate.

3.3 DISCUSSION

The experiments in this section were directed toward clarifying the contradictions underlying reports describing the effect of ascorbic acid on bacteriophage. It had been well documented (Murata and Kitagawa, 1973; Murata et al., 1975) that ascorbic acid inactivated both single and double stranded DNA containing phage and that the mode of action was through the introduction of single strand nicks. It is clear how single strand nicks would inactivate single strand DNA containing phage but several reports (Yoakum, 1975; Freifelder, 1965; Amanthaswamy et al., 1979; Stephan, 1973; Pao and Speyer, 1973; Amanthaswamy and Eisenstark, 1976) have shown that single strand nicks in both bacterial and bacteriophage DNA are non lethal. How then was ascorbic acid having such a pronounced inactivating effect on double stranded DNA containing bacteriophage?

3.3.1 Recovery of "Inactivated" T7 Phage

A comparison of Figures 8 and 9 and Figures 10 and 11 reveals that the standard double layer plating technique resulted in inactivation rates similar to those of Murata and Kitagawa (1973) but that an incubation period prior to plating the liquid culture significantly reduced the inactivation rate. In other words the rapid rates of inactivation were partially an artifact of an efficient plating procedure. This phenomenon can be explained in terms of liquid holding recovery which allows an enhancement of host cell reactivation (HCR). Host cell reactivation is a well documented phenomenon of repair of bacteriophage DNA

within the host cell that has been damaged by such things as UV irradiation or chemicals. This effect is usually characterized by either delayed intracellular growth of the phage or delayed prophage induction in lysogenic cells (Harm, 1966). Host cell reactivation is responsible for repair of a number of lesions, both non-lethal and lethal, that affect DNA: thymine dimer (Harm, 1965), lethal damage by mitomycin C (Harm, 1965), single strand breaks (Stephan, 1973), damage caused by ethyl methane sulfonate (Ray et al., 1970). In fact it has been suggested that with the exception of double strand breaks, all radiation induced lesions including base and sugar modifications, can be partly or fully repaired in some strains of E. coli by host cell reactivation (Stephan, 1973). E. coli B wild type strains are HCR⁺.

Cultures of UV irradiated E. coli B have higher survival rates if held for several hours in a non nutrient liquid prior to plating (Harm, 1966). Jagger et al. (1964) suggested that liquid holding increased UV survival by delaying cellular growth after plating thus allowing more time for the repair processes to occur. It was suggested that bacteria perform host cell reactivation under starvation conditions when DNA synthesis is not occurring. Therefore the repair occurs in the liquid minimal medium rather than afterwards on the plate when active growth occurs. The effect was first noted by Sauerbier (1962) for both E. coli DNA repair and phage DNA repair.

Clearly host cell reactivation would explain the difference seen in percentage survival between the two plating procedures. Furthermore the rapid inactivation observed by Murata et al. (1973 and 1976) was the re-

sult of an omission of the liquid holding step to allow repair of the nicked DNA. Normally single strand nicks in double stranded DNA are not lethal but growth on agar plates does not allow time for nick repair to occur efficiently producing a system where single strand nicks are lethal.

Only small differences in the recovery of ascorbate treated phage were observed between wild type and *recA* strains and they were observed at the higher concentrations of ascorbate. The *recA* function would appear to play some role in the HCR process but not a major role.

3.3.2 Delayed Lysis Effect

The process of host cell reactivation gives rise to the phenomenon of delayed lysis. The damaged phage DNA is injected into the bacterium and must be repaired prior to undergoing replication. The extent of the damage determines the length of time needed for repair, creating a direct relationship between the length of the ascorbate treatment and the length of the delay before lysis. Consistent with this interpretation is the fact that DNA replication, as indicated by the appearance of concatemeric DNA, was delayed by ascorbate treatment. Once the DNA was repaired normal replication ensued and normal progeny phage, as determined by infectivity and sedimentation analysis, were formed. Only at very high ascorbate concentrations or after long treatment times were the phage sufficiently damaged so that no progeny phage were produced. Either double strand nicks were formed from the close proximity of two single strand nicks or some other low frequency inactivating lesion had occurred.

The first report by Luria of the phenomenon of a growth delaying effect on bacterial viruses appeared in 1944. In the growth experiments using UV irradiated phage, adsorption to sensitized bacteria was normal; the phage burst size was normal; but the constant period, the minimal interval between infection and lysis (latent period), and the rise period, the spread in the values of this interval for individual cell, increased with increasing amounts of irradiation. This effect was also reported to be non-hereditary; that is, the offspring of the irradiated virus were quite normal in their replication cycle. More recently the alkylation of T7 phage DNA was shown to delay phage development (Karska-Wysocki and Mamet-Bradley, 1981). With up to 700 alkylations per DNA molecule, the latent period was longer but the burst size was equivalent to that of untreated phage. A significant delay in phage DNA replication was observed which would have allowed ample time for repair.

An example of true phage inactivation occurred with near UV irradiation in the presence of hydrogen peroxide (Ananthaswamy et al., 1979; Hartman et al., 1979) causing protein-DNA crosslinking which impeded DNA injection.

3.3.3 Effect of Ascorbic Acid on Encapsulated T7

The principal lesion in DNA caused by ascorbic acid is the introduction of single strand nicks (Murata and Kitagawa 1973; Morgan et al., 1976). A comparison of the alkaline and neutral sucrose gradient profiles verifies these earlier reports by showing that ascorbate introduced single strand nicks into T7 DNA. The analytical procedure of Lit-

win cannot estimate more than 20 nicks per molecule but phage with far in excess of 20 nicks per DNA were capable of injecting their DNA. Once injected, host cell reactivation repaired the lesions and DNA replication commenced. Only when double strand nicks became evident was the phage actually inactivated.

Another means of estimating the extent of nicking caused by ascorbate on T7 DNA was to compare the size of DNA fragments separated on agarose gels with the size of known restriction fragments. Consistent with the presence of single strand nicks, there was no evidence of fragmentation of T7 DNA treated with 5.7 mM ascorbic acid using agarose gel electrophoresis unless the secondary structure was disrupted by heating to 90°C. After the heat treatment fragments in the range of 1800 to 6400 bases in length were observed consistent with there being 7 - 20 nicks per molecule. The longer the ascorbate treatment, the smaller the fragments. The requirement for heat to fragment the DNA illustrates the importance of secondary and tertiary structure in maintaining a unit molecule for injection, repair and replication.

One means of analyzing for chemical or biological modification of DNA is to monitor changes in restriction cleavage patterns. The action of restriction enzymes is mediated by base specificity and presumably some secondary structure inherent within this base specificity. Ascorbic acid did not appear to have a very great effect in disrupting the action of the enzyme with the DNA substrate.

3.3.4 Relation of the Repair Process and DNA Replication

As discussed earlier, the extent of damage to the DNA molecule determined the length of time needed for repair. The delay in the appearance of the high molecular weight concatemeric form of T7 DNA indicated that DNA repair had to occur prior to the onset of DNA replication. Once repair had occurred normal phage development ensued. The repair of single strand nicks would most probably involve a DNA ligase and/or DNA polymerase I activity of E. coli and the T7 phage encoded ligase activity (Yoakum, 1975; Ray et al., 1970). The ligase is coded for by one of the early genes of T7 bacteriophage, gene 1.3 and the host RNA polymerase can transcribe T7 DNA up to and including the ligase gene (Studier, 1972). Therefore it is quite probable that a combination of both host and viral enzymes are involved in the repair of single strand nicks resulting in normal transcription, translation and replication. The recA gene product seems to play only a minor role in the repair process. Characteristics of the recA protein include the formation of duplex DNA, the aggregation of complementary single strands of DNA and DNA dependent nucleoside triphosphatase activity (Lehman, 1980). The formation of duplex DNA and the aggregation of complementary single strands of DNA could conceivably be involved in the nick repair, but does not appear to be critical to the process.

When E. coli is subject to certain DNA damaging agents, the result is the induction of SOS pathways (Hanawalt et al., 1976). Accompanying DNA repair enzymes induction is also a large induction of the rec A gene product, all a manifestation of SOS function. From data obtained here it

appears that the rec A protein is not involved to a large extent in the repair of DNA damage that could be caused by ascorbic acid. Recently it has been reported that damage of T7 phage by γ -irradiation, which includes the nicking of the DNA, is not inhibited by the influence of the SOS pathways (Bleichrodt and Roos-Verheij, 1980).

3.3.5 Catalase and Ascorbic Acid

Nicking of T7 DNA by hydroxyl radicals formed in the oxidation of ascorbic acid may not be the only effect of ascorbate on DNA. Breakdown of H_2O_2 , formed in the oxidation of ascorbic acid, by catalase did not completely inhibit the nicking effect. This could mean either that the catalase did not completely remove H_2O_2 allowing the formation of some hydroxyl radicals or that an organic radical was formed which was able to cause nicking directly or indirectly. The fact that catalase has such a high specific activity and that extremely high concentrations were used should rule out the first alternative. DNA damage induced by ascorbic acid at the longer time points in the presence of catalase, as reflected in the changes in sedimentation on alkaline sucrose gradients, could have been due to an alteration of the base in such a way that the N-glycosidic bond became alkali and heat labile or to removal of the base from the sugar-phosphate backbone. Both possibilities would give rise to alkali labile phosphodiester bonds since apurinic and apyrimidinic acids are highly alkali sensitive.

Morgan et al. (1976) showed that catalase inhibited the DNA nicking caused by a hydroxyl radical producing system. Dimethylsulfoxide is able to prevent 80% of the single strand DNA breaks induced by gamma ir-

radiation and inhibits completely the DNA nicking of hydroxyl radicals produced in an iron/H₂O₂ system (Repine et al., 1981). These investigators concluded that the hydroxyl radical was responsible for 80% of the DNA single strand breaks after gamma irradiation and that the remaining 20% of the nicks were due to interactions not involving the hydroxyl radical. Such a conclusion could also apply to the ascorbate system where hydroxyl radicals are involved as the principal cause of DNA nicking but where nicking still occurs under conditions where hydroxyl radicals should be eliminated.

Chapter IV

PART II: EFFECT OF ASCORBIC ACID ON ESCHERICHIA COLI

4.1 INTRODUCTION

As already noted, the addition of ascorbate to cultures infected with T7 caused a delay in the time of lysis although ascorbate had no apparent effect on the bacteria under these conditions. Therefore the bacteria contained a system which prevented any of the deleterious effects by ascorbate on E. coli and which allowed repair and replication of T7 bacteriophage to occur. An explanation for this phenomenon was investigated.

Catalase and superoxide dismutase appear to be essential for the protection of aerobically growing organisms against the toxic effects of hydrogen peroxide and superoxide anion formed in many cellular reactions and by certain antibiotics. Some aspects of the control of synthesis of these enzymes have been studied both without (Hassan and Fridovich, 1977b; Hassan and Fridovich, 1978b; McCord and Fridovich, 1969; Yoshpe-Purer et al., 1977) and with pyocyanine and paraquat present (Hassan and Fridovich, 1978a; Hassan and Fridovich, 1979; Hassan and Fridovich, 1980). Details as to the regulation of expression of these genes are few. Catabolite repression was concluded to affect catalase but not superoxide dismutase (Gregory et al., 1977; Hassan and Fridovich, 1977c; Hassan and Fridovich, 1978b; Sulebele and Rege, 1967 and 1968; Yoshpe-

Purer et al., 1977) although much of the data presented was inconclusive.

Ascorbic acid has been shown to react with oxygen in the presence of metal ions to produce H_2O_2 (Weissberger and Luvalle, 1943; Morgan et al., 1976; Richter and Loewen, 1981), and free radical intermediates from the oxidation of ascorbate have been implicated in the inhibition of catalase by ascorbate (Orr, 1966; 1967a and b). Because ascorbic acid oxidation yields H_2O_2 but no superoxide anion (Halliwell and Foyer, 1976) it was expected that catalase but not superoxide dismutase would be induced by the addition of ascorbic acid to the oxygenated cultures of E. coli. The effect of ascorbate on catalase levels has been investigated and a useful system for studying catalase gene expression has been developed using ascorbate. In addition the effect of ascorbate on sugar transport and cellular respiration has been investigated.

4.2 EFFECT OF ASCORBATE ON CATALASE LEVELS IN E. COLI

4.2.1 Catalase Induction by Ascorbate in LB Medium

E. coli responded to the addition of 5.7 mM ascorbic acid with a seven to eight fold increase in catalase activity within thirty minutes (Figure 23). Initially there was a rapid drop in the catalase activity from which it took three or four minutes to recover and this drop was coincident with a slight pause in growth rate. Subsequent growth rates with and without ascorbic acid were identical. Because no catalase activity was found in the medium, this drop in activity was not the result of release into the medium. The addition of chloramphenicol to the cul-

ture at the same time as ascorbic acid prevented the induction of catalase indicating that protein synthesis was required for the increase in catalase activity. As can be seen in Figure 23, the catalase level of the control culture increased slightly as the cells approached stationary phase.

The extent of catalase induction in whole cells over a wide range of ascorbic acid concentrations is included in Table 9. Very little induction occurred below 0.057 mM ascorbic acid and maximal induction occurred between 0.57 and 5.7 mM. The drop in catalase activity observed at 57 mM ascorbic acid coincided with cell death and a severe inhibition of endogenous catalase.

A cell free S30 system was prepared as described in the Methods. Following the addition of various concentrations of ascorbic acid the mixtures were allowed to incubate for 15 minutes after which catalase activity was assayed (Table 10). As in the whole cell assays, the greatest activity was found after induction with 5.7 mM ascorbic acid. The protein concentration in the S30 was very low, (0.2 mg/mL) which is perhaps one reason for the inefficient induction, which allowed only a 100% increase over the basal level.

One of the two catalase activities in E. coli has been shown to be an effective general peroxidase as well (Claiborne and Fridovich, 1979) and the two activities can be separated by electrophoresis on polyacrylamide gels. The catalase and peroxidase activities in sonicated cell extracts were visualized on 12.0% polyacrylamide gels before and after ascorbate addition. As shown in Figure 24, there was an identical increase in the

Figure 23. Effect of ascorbic acid on catalase activity in E. coli growing in LB medium.

In parallel cultures the following additions of ascorbic acid were made at time 0: none (○), 5.7 mM (●) and 5.7 mM ascorbic acid with 0.1 mM chloramphenicol (Δ). Cell growth was followed in the control culture (□) and the ascorbic acid supplemented culture (■). Catalase activity was expressed as units per mg dry cell weight.

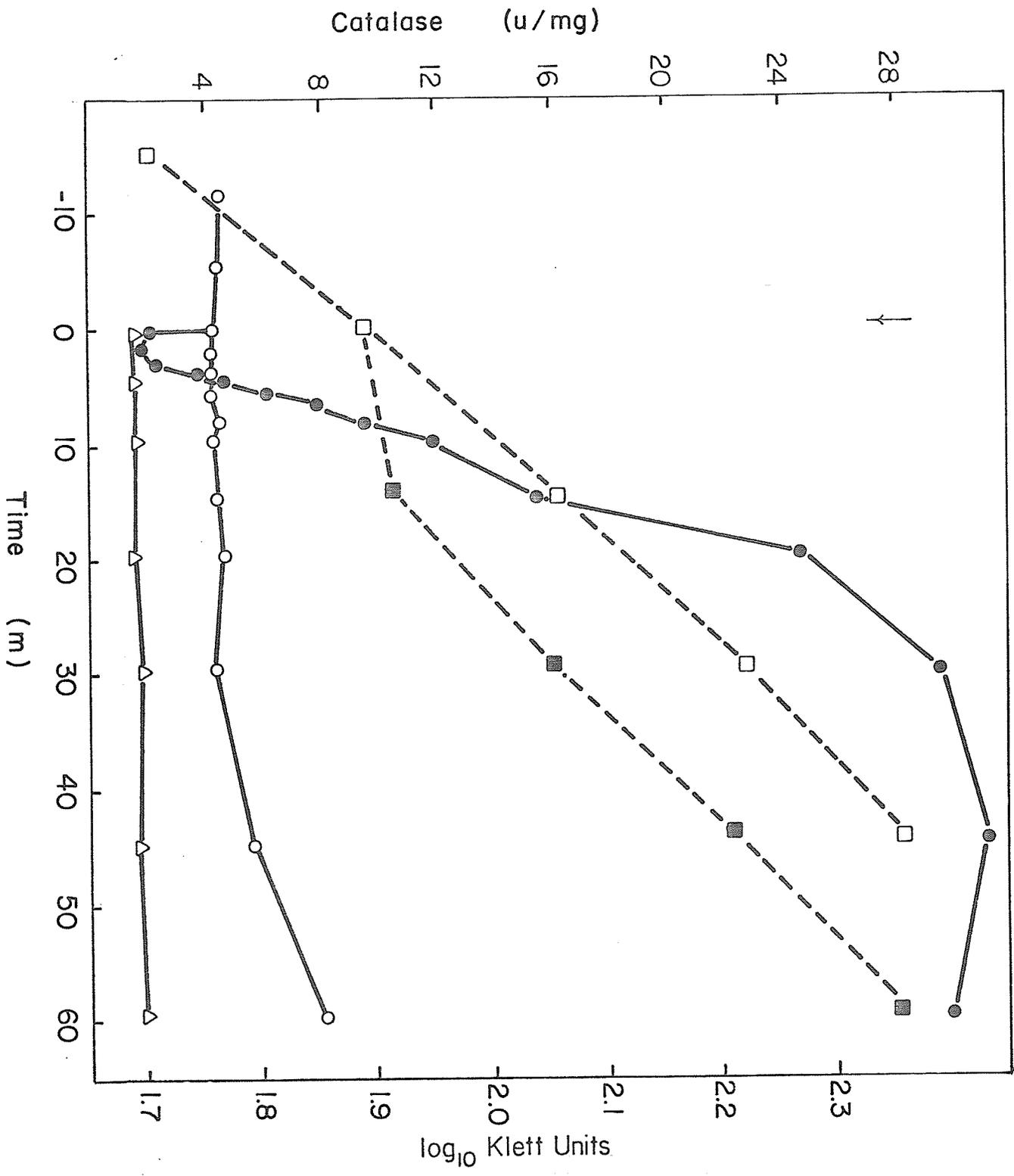


Table 9. Effect of ascorbic acid concentration on fully induced catalase levels one hour after ascorbic acid addition to Escherichia coli growing in LB medium.

Ascorbic acid Concentration (mM)	Catalase (u/mg dry cell weight)
5.7×10^{-5}	4.0
5.7×10^{-4}	4.8
5.7×10^{-3}	5.6
5.7×10^{-2}	12.0
5.7×10^{-1}	32.0
5.7	28.8
57	0
0	4.1

Table 10. Effect of ascorbic acid concentration on catalase levels in cell free extracts.

Ascorbic acid concentration (mM)	Catalase u/mg protein
5.7×10^{-3}	0.0063
5.7×10^{-2}	0.0066
5.7×10^{-1}	0.0074
5.7	0.0097
57	-
0	0.0049

predominant isoenzyme pair possessing both catalase and peroxidase activity following the addition of ascorbic acid to a culture in LB medium. A minor catalase activity not possessing peroxidase activity could also be seen to increase near the top of the gel although it is not as evident in the photograph. This catalase activity band was most effectively visualized on gels stained for peroxidase activity after soaking in H_2O_2 overnight. Catalase activities including bovine catalase which lack the peroxidase activity showed up as a clear band against a pale brown background (Figure 24). The minor peroxidase activity below the two main catalase-peroxidase bands decreased. Hassan and Fridovich (1980) have also observed that both of the main catalase-peroxidase activities or isoenzymes were increased during aerobic growth of E. coli in the presence of pyocyanine.

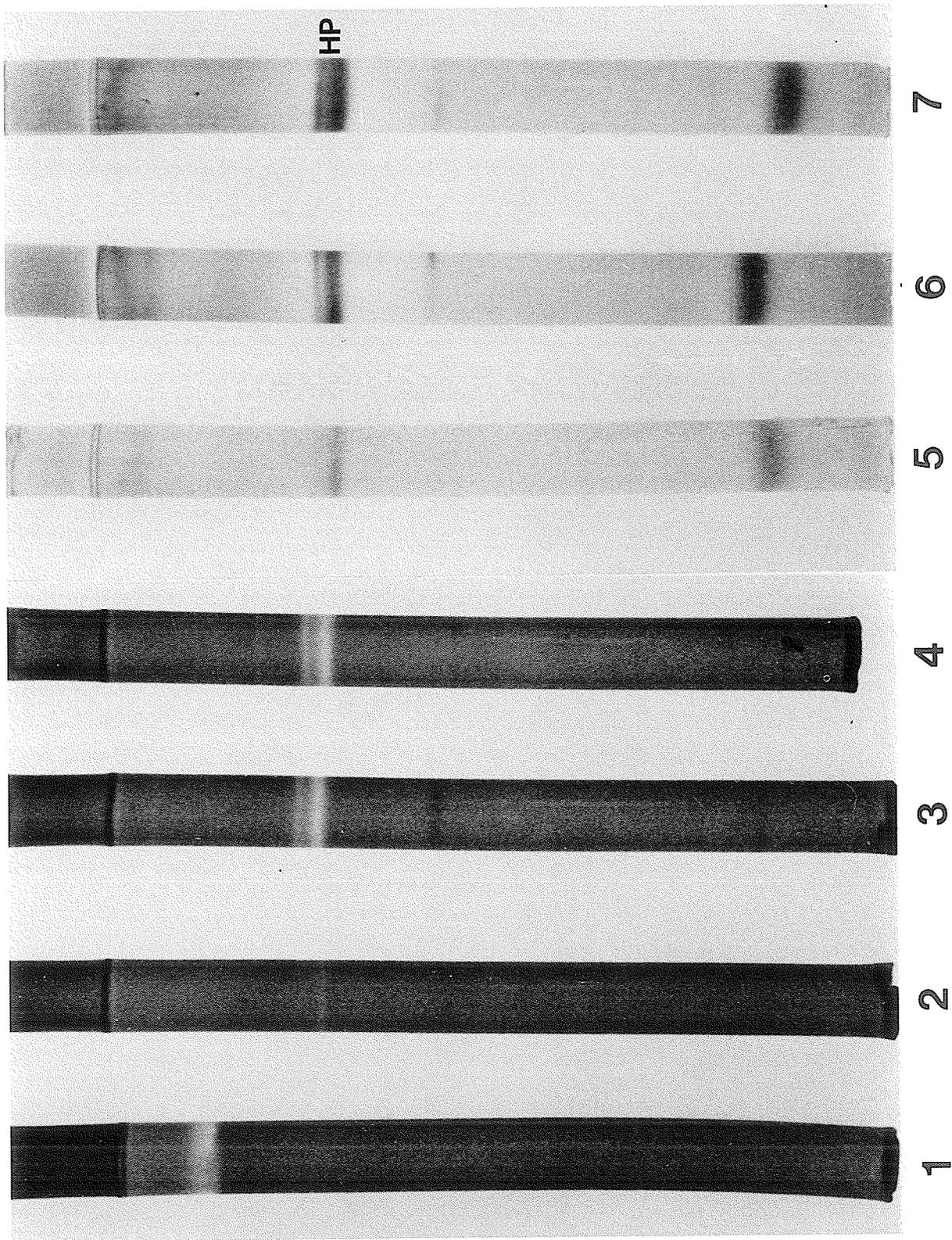
Attempts were made to separate the isoenzymes on 4% (Claiborne and Fridovich, 1979) and 7.5% (Gregory and Fridovich, 1974) polyacrylamide gels but the catalase activity band migrated coincident with the bromphenol blue dye. Only 12% gels gave satisfactory separation. Despite the difference in gel concentration between this work and earlier reports, the gel patterns in Figure 24 are similar to those in earlier reports.

Figure 24. Visualization of catalase and peroxidase activities from sonicated cell extracts.

The cells were extracted 30 minutes after the addition of 5.7 mM ascorbic acid or 0.75 mM H₂O₂ to aerated cultures of E. coli in LB medium. Polyacrylamide (12%) gels 1-4 were stained for catalase and gels 5-7 were stained for peroxidase.

Gel 1: 20 µg bovine liver catalase; Gels 2 and 5: 400 µg of protein from a culture without ascorbate or H₂O₂ added; Gels 3 and 6: 400 µg of protein from a culture with ascorbate added; Gels 4 and 7: 400 µg of protein from a culture with H₂O₂ added.

HP: hydroperoxidase. The lower most band is blue dye.



4.2.2 Catalase Induction by Ascorbate in Glucose Salts Medium

When ascorbic acid was added to a culture of *E. coli* growing aerobically in a glucose-salts medium, the lethal dose was much lower than that in LB medium. As shown in Figure 25, both 2.8 mM and 5.7 mM ascorbic acid caused an immediate disappearance of catalase activity and a cessation of growth (Figure 26). There were fewer than 0.1% survivors and as determined by plating 15 minutes after the addition of 5.7 mM ascorbic acid. Growth in 2.8 mM ascorbic acid restarted after 4 to 5 hours but only following the recovery in catalase activity. Lower concentrations of ascorbic acid (0.57 mM) induced a two fold increase in catalase without the initial drop in activity or any disruption of growth. Unlike the cellular response in LB medium, the basal level of catalase did not rise as cells reached stationary phase in glucose salts medium.

In Figure 27 A, it can be seen that the upper catalase-peroxidase band, HP-I, was induced to a greater extent than the lower band HP-II. The minor peroxidase band below the two catalase-peroxidase bands was also increased but the minor catalase band near the top of the gel observed in LB grown cells, was not visible.

When glucose-salts medium was supplemented with casamino acids, cells grown in this medium responded to the addition of 0.57 mM and 2.8 mM ascorbic acid with a six fold increase in catalase activity (Figure 28). The disruptive effect of the higher ascorbic acid concentrations was ameliorated somewhat and growth commenced sooner than in unsupplement-

Figure 25. Effect of ascorbic acid on catalase activity of E. coli in glucose-salts medium.

The following concentrations of ascorbic acid were added at time 0: none (○), 0.57 mM (●), 2.8 mM (△), 5.7 mM (▲). Catalase activity is expressed as units per mg dry cell weight.

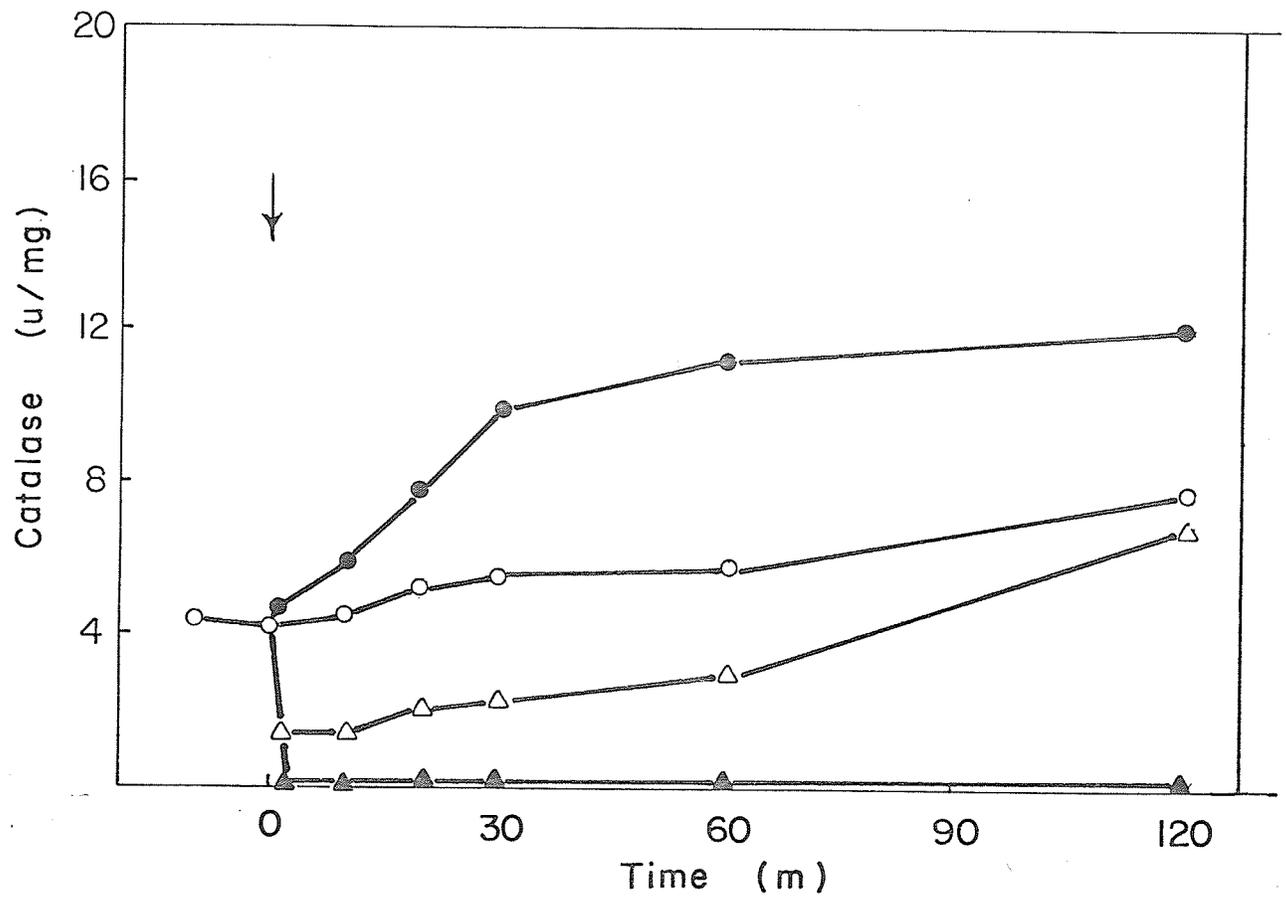


Figure 26. Effect of ascorbic acid on the growth rate of E. coli B growing in glucose-salts medium.

The following concentrations of ascorbic acid were added at time 0 to glucose-salts medium: none (○), 0.57 mM (●), 2.8 mM (△), 5.7 mM (▲). The dotted lines represent those cultures supplemented with 0.1% casamino acids. Catalase activity was expressed as units per mg dry cell weight.

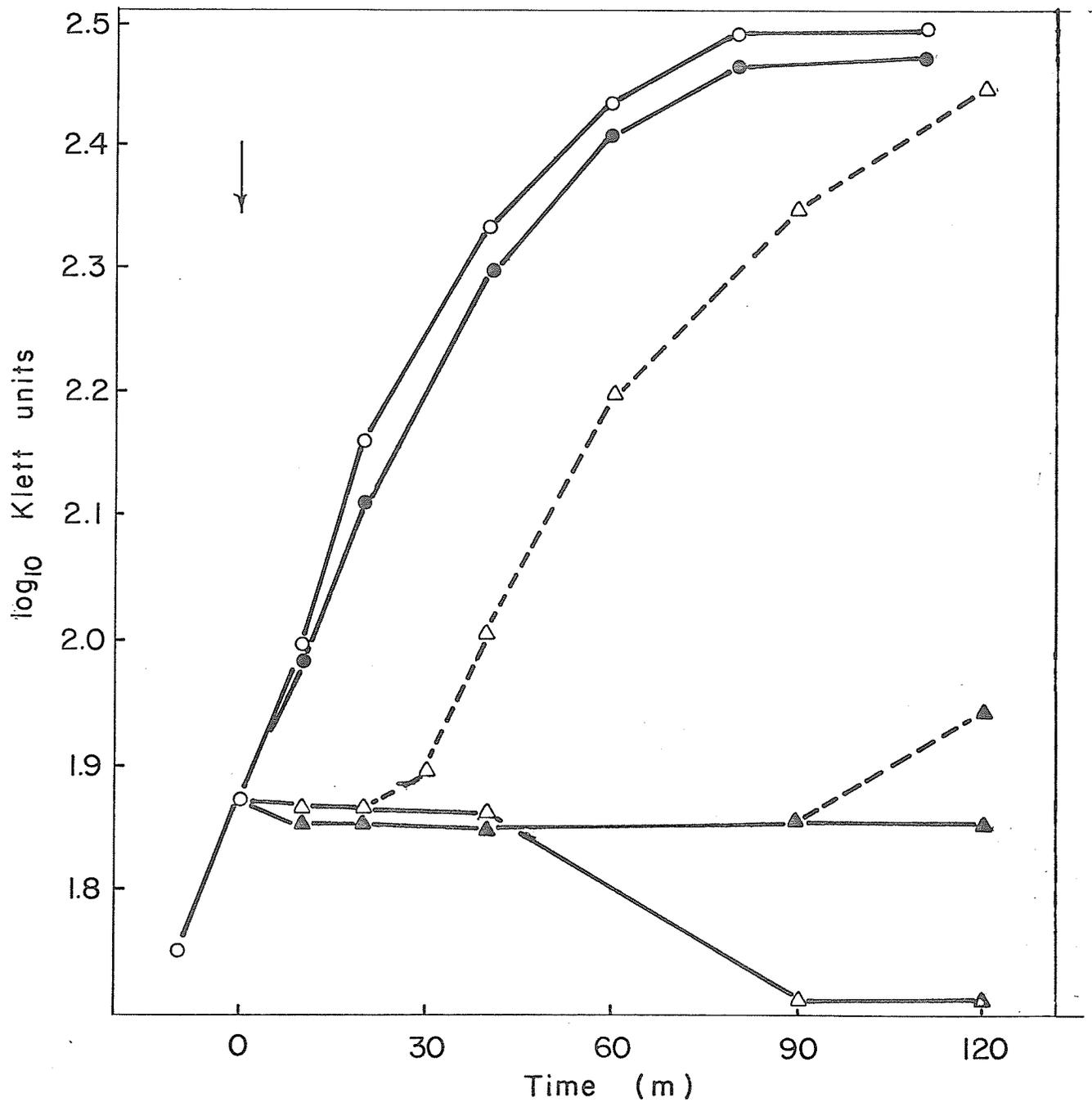
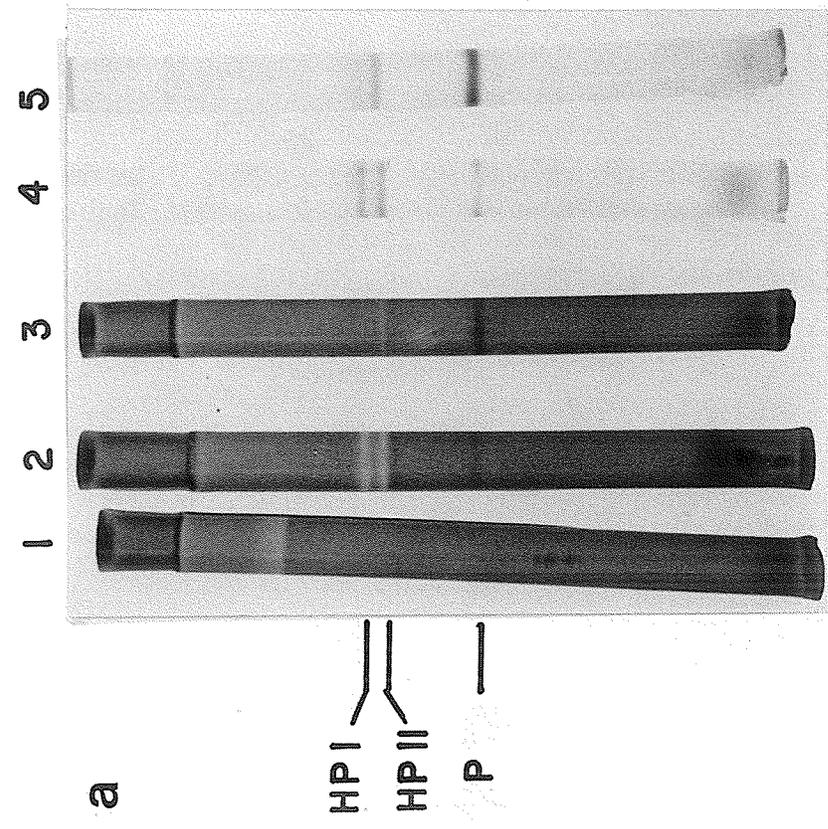
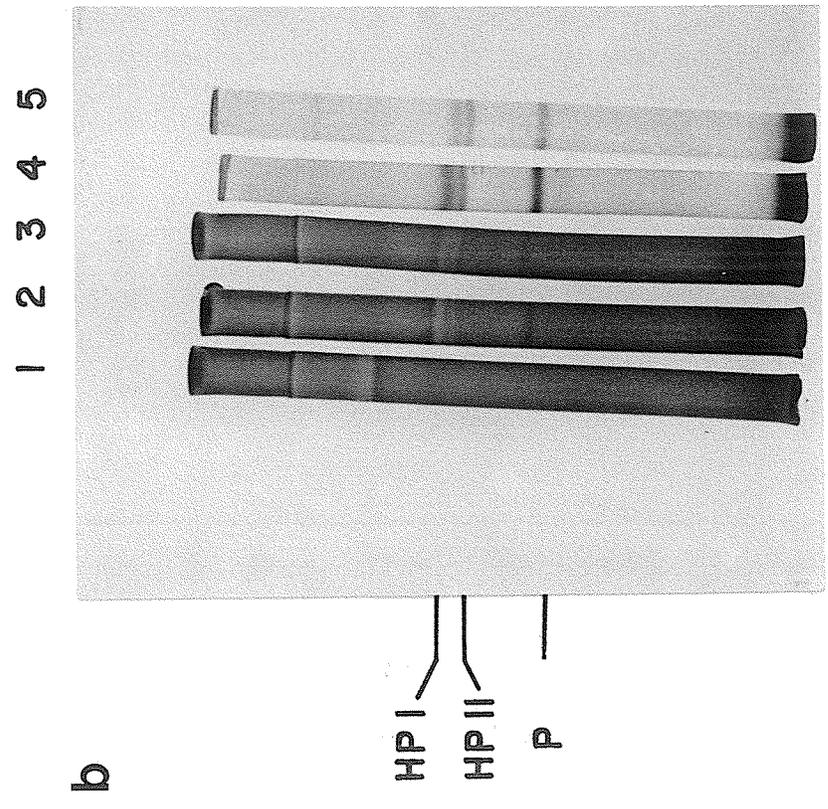


Figure 27. Visualization of catalase and peroxidase activities in extracts from E. coli growing in glucose salts medium (A) and from glucose salts medium supplemented with 0.1% casamino acids (B). A. (1) bovine catalase, (2) and (4) 85 μ g protein from a culture treated with ascorbate, (3) and (5) 210 μ g of protein from a culture without ascorbate treatment. B. (1) bovine catalase, (2) and (4) 100 μ g of protein from a culture treated with ascorbate, (3) and (5) 250 μ g of protein from a culture without ascorbate treatment. Polyacrylamide (12%) gels 1-3 were stained for catalase and gels 4 and 5 were stained for peroxidase as described in the Methods. HP-hydroperoxidase, P-peroxidase.



ed medium after the addition of 2.8 mM and 5.7 mM ascorbic acid (Figure 28). It can be seen more clearly here, that the induced level of catalase drops toward basal level as growth progresses. In LB grown cells in which catalase was induced with ascorbate, the catalase levels did not return to the basal levels of 5 u/mg dry cell weight and the levels of catalase in the control culture increased with time.

Both of the main catalase-peroxidase bands, HPI and HPII (Figure 27 B) were induced by ascorbate while the lower peroxidase activity decreased. This is very similar to the situation observed in extracts from cells grown in LB medium except for the absence of the upper catalase band seen in LB medium.

[¹⁴C] amino acid incorporation was followed in cells grown in glucose-salts medium supplemented with 0.1% casamino acids. As can be seen from the data in Table 11, there was no significant increase in the rate of general protein synthesis in those cells where ascorbate was added as compared to the control.

Figure 28. Effect of ascorbic acid on catalase activity in E. coli B growing in glucose salts medium supplemented with 0.1% casamino acids.

The following concentrations of ascorbic acid were added at time 0 to glucose-salts medium with casamino acids: none (O), 0.57 mM (●), 2.8 mM (Δ) and 5.7 mM (▲). Catalase activity was expressed as units per mg dry cell weight.

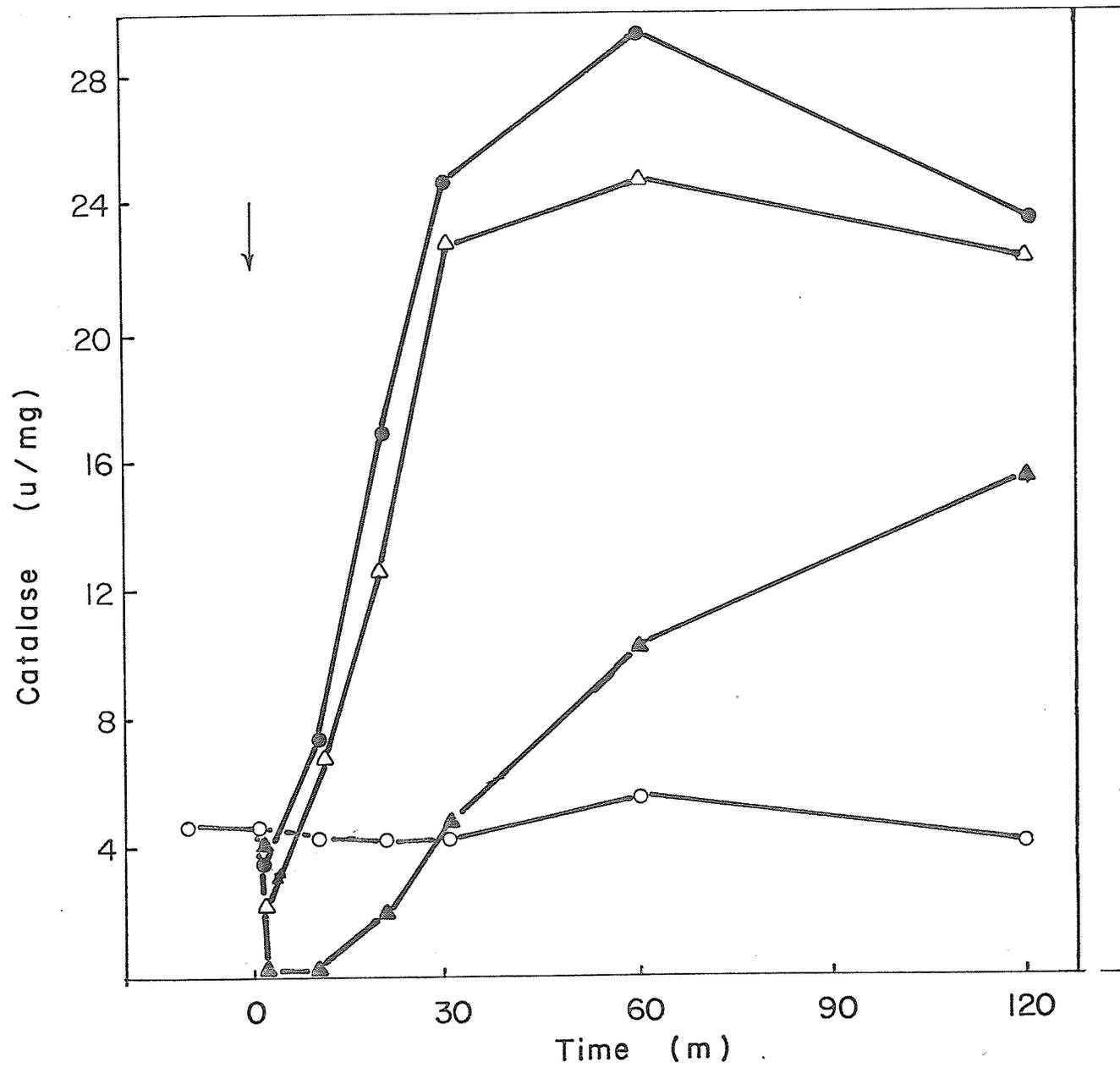


Table 11. The effect of 2.8 mM ascorbic acid on the incorporation of a ^{14}C -labelled amino acid hydrolysate. The procedure followed was as described in the Methods. Units employed are cpm per mg dry cell weight.

Time	- ascorbic acid cpm/mg	+ ascorbic acid
-10	3920	4620
- 5	7203	6943
+ 1	8139	8079
+ 3	8263	8336
+ 5	9625	11577
+10	11473	13868
+20	15105	13364
+30	14406	15613
+45	18500	20095
+60	17592	21311

Ascorbic acid was added at time 0.

4.2.3 Catalase Induction by Ascorbate in Glycerol-Salts Medium

Cells growing in glycerol-salts medium also responded to ascorbic acid by synthesizing catalase but the response differed somewhat from that of cells growing in glucose-salts medium. As shown in Figure 29, there was only a brief drop in catalase activity even with 5.7 mM ascorbic acid and there was little effect on growth.

Supplementing the medium with casamino acids allowed a six fold increase in catalase activity (Figure 30) similar to the increases in both LB medium and casamino acid supplemented glucose-salts medium.

Figure 29. Effect of ascorbic acid on catalase activity in E. coli B growing in glycerol-salts medium.

The following concentrations of ascorbic acid were added at time 0: none (○), 0.57 mM (●), 2.8 mM (△) and 5.7 mM (▲). A short pause of 10 minutes in the growth rate occurred after 2.8 mM and 5.7 mM ascorbic acid addition but no effect was noted after 0.57 mM ascorbate addition. Catalase activity is expressed as units per mg dry cell weight.

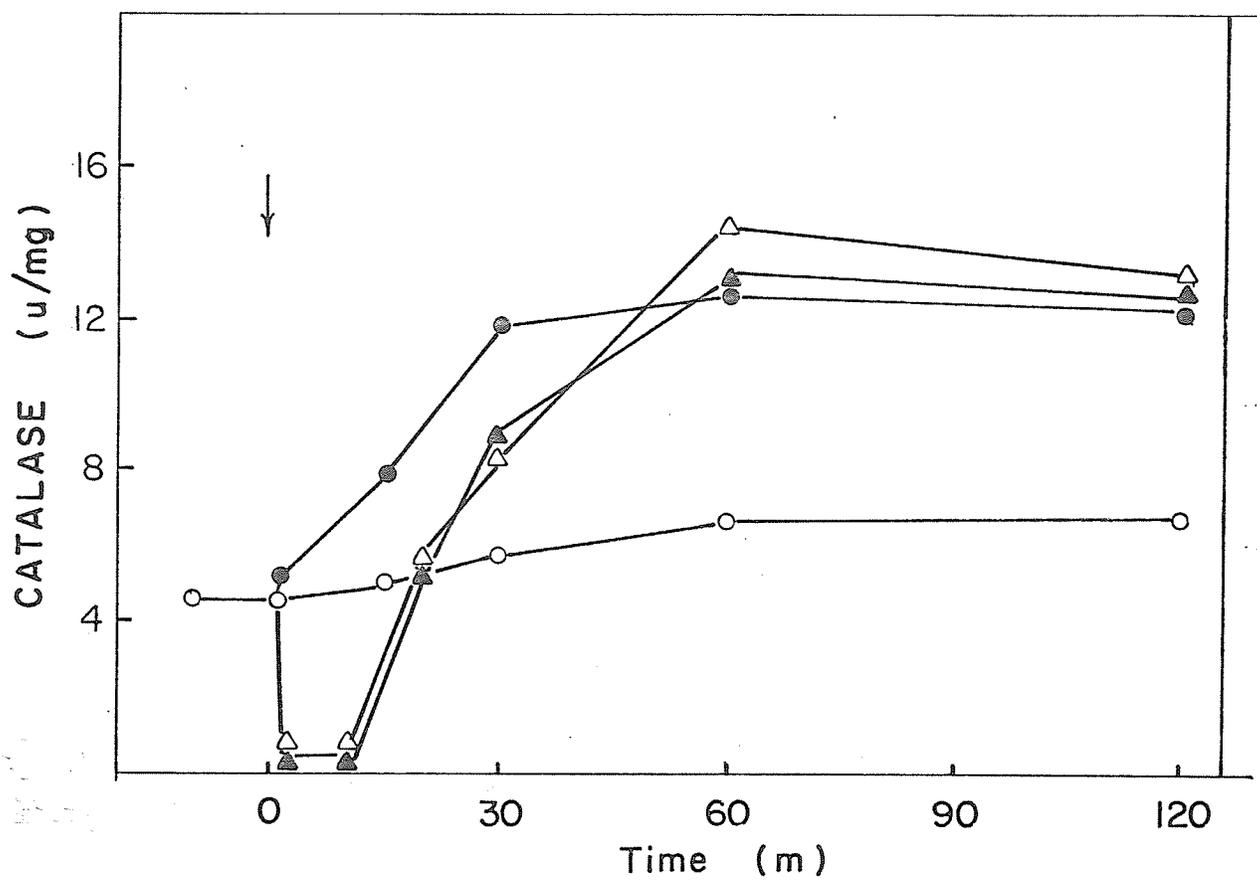
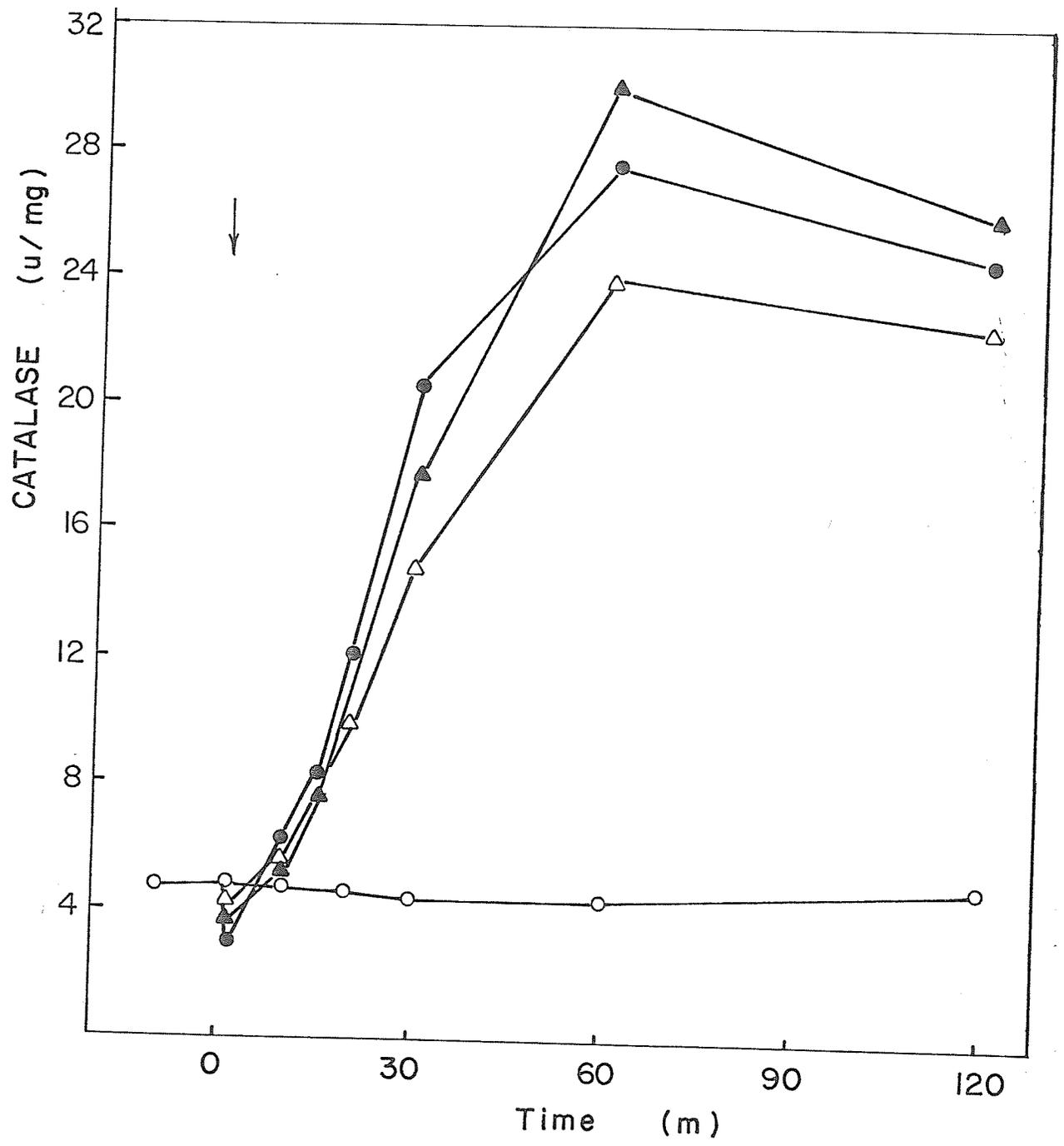


Figure 30. Effect of ascorbic acid on catalase activity in E. coli
B growing in glycerol salts medium supplemented with 0.1% casamino acids.

The following concentrations of ascorbic acid were added at
time 0: none (○), 0.57 mM (●), 2.8 mM (△) and 5.7 mM (▲).

Catalase activity is expressed as units per mg dry cell weight.



4.2.4 Catalase Induction by Ascorbate in Succinate-Salts Medium

Cells in succinate-salts medium grew much more slowly but responded to ascorbic acid qualitatively the same as cells in glycerol-salts medium (Figure 31). The basal level of catalase in the succinate-salts grown cells was 40% higher than in either glycerol-salts, glucose-salts or LB medium, but the induction was about to the same level observed in glucose-salts and glycerol-salts grown cells, approximately 13.0 units per mg dry cell weight.

Catalase induction in succinate-salts casamino acid medium (Figure 32) grown cells was to approximately 24 units per mg dry cell weight, slightly lower than in the other two media.

Figure 31. Effect of ascorbic acid on catalase activity in E. coli B growing in succinate-salts medium.

The following concentrations of ascorbic acid were added at time 0: none (○), 0.57 mM (●), 2.8 mM (△) and 5.7 mM (▲). There was no visible effect on cell growth. Catalase activity is expressed as units per mg dry cell weight.

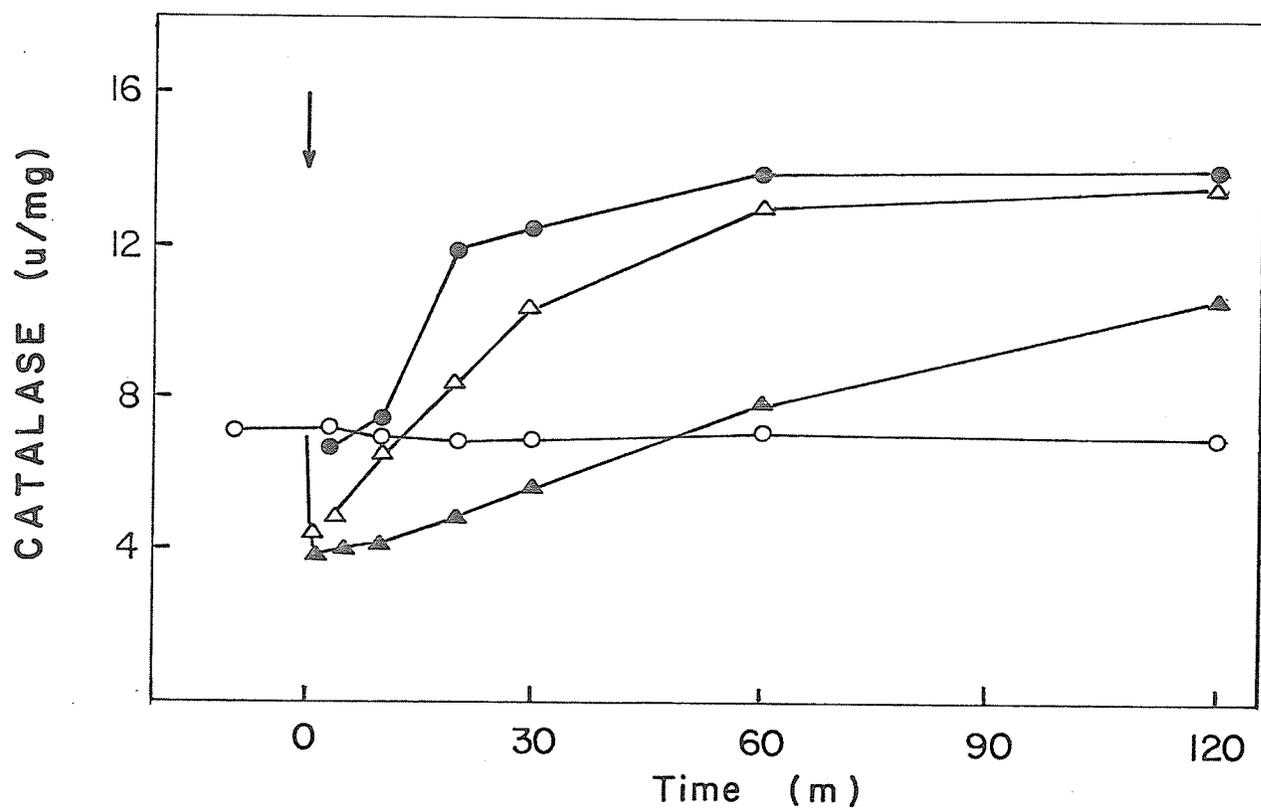
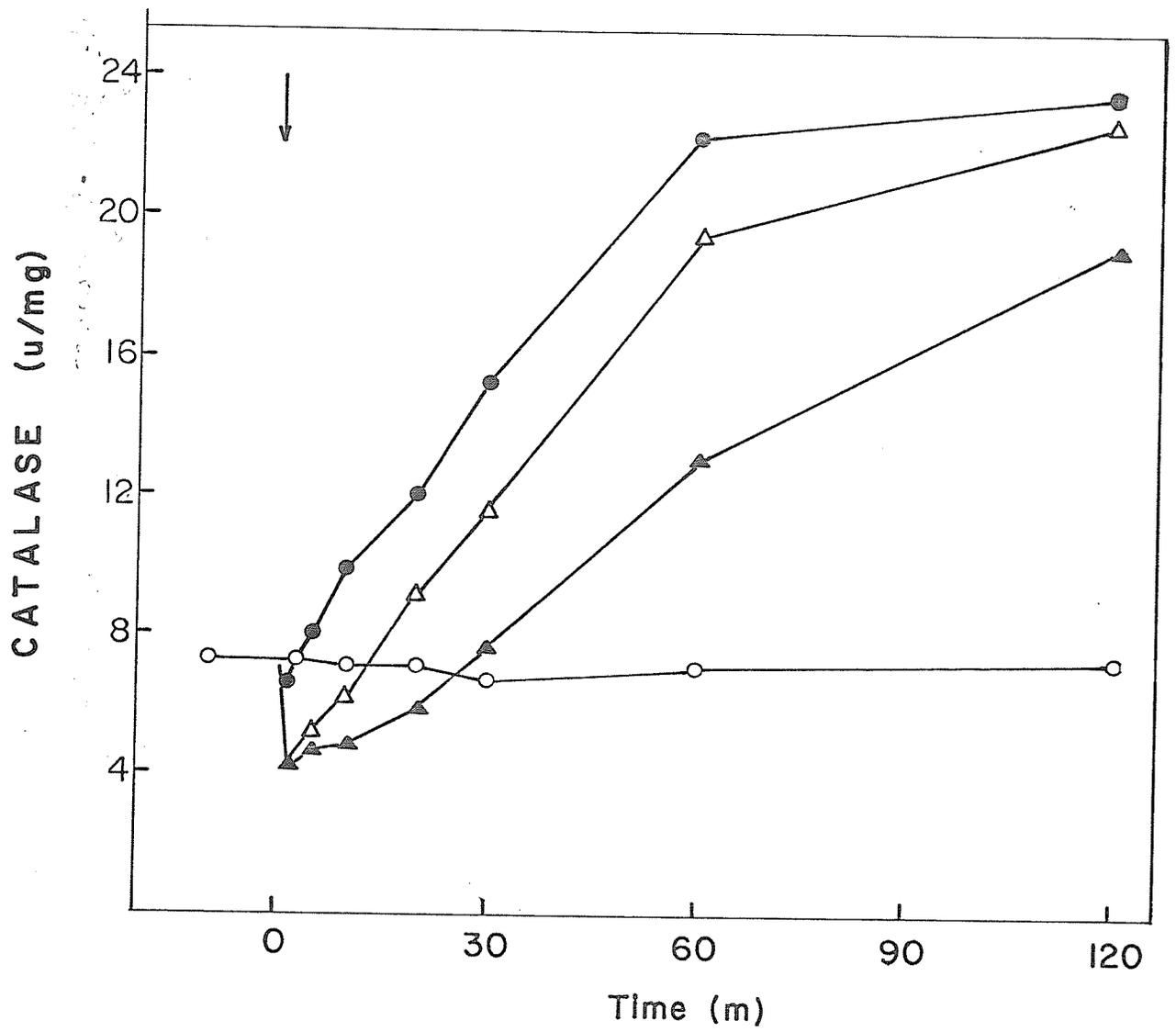


Figure 32. Effect of ascorbic acid on catalase activity in E. coli B growing in succinate-salts medium supplemented with 0.1% casamino acids.

The following concentrations of ascorbic acid were added at time 0: none (○), 0.57 mM (●), 2.8 mM (△), and 5.7 mM (▲). Catalase activity is expressed as units per mg dry cell weight.



4.3 EFFECT OF H₂O₂ ON CATALASE LEVELS IN E. COLI

4.3.1 Quantitation of H₂O₂ Resulting from Ascorbate Oxidation in Aerated Medium

It has been shown that catalase synthesis in E. coli increased in response to H₂O₂ generated in situ from pyocyanine (Hassan and Fridovich, 1980) and to H₂O₂ added directly to the medium (Yoshpe-Purer et al., 1977). Therefore the well characterized oxidation of ascorbate to yield dehydroascorbate and H₂O₂ provided a clear rationale for the induction of catalase by ascorbate. This has been confirmed by the quantitation of H₂O₂ produced from ascorbate in aerated minimal salts medium. The H₂O₂ concentrations were found to be significantly lower than the ascorbate concentrations (Table 12) suggesting a slow but constant oxidation of ascorbate in the growth medium.

Table 12. Hydrogen peroxide quantitation. Hydrogen peroxide concentration five minutes after the addition of ascorbic acid to minimal salts medium (A) and LB medium (B).

Ascorbic acid mM	H ₂ O ₂ mM
A	
0.057	0.019
0.28	0.037
0.57	0.052
2.8	0.118
5.7	0.122
B	
0.57	0.045
2.8	0.060
5.7	0.065

4.3.2 Catalase Induction by H₂O₂ in LB Medium

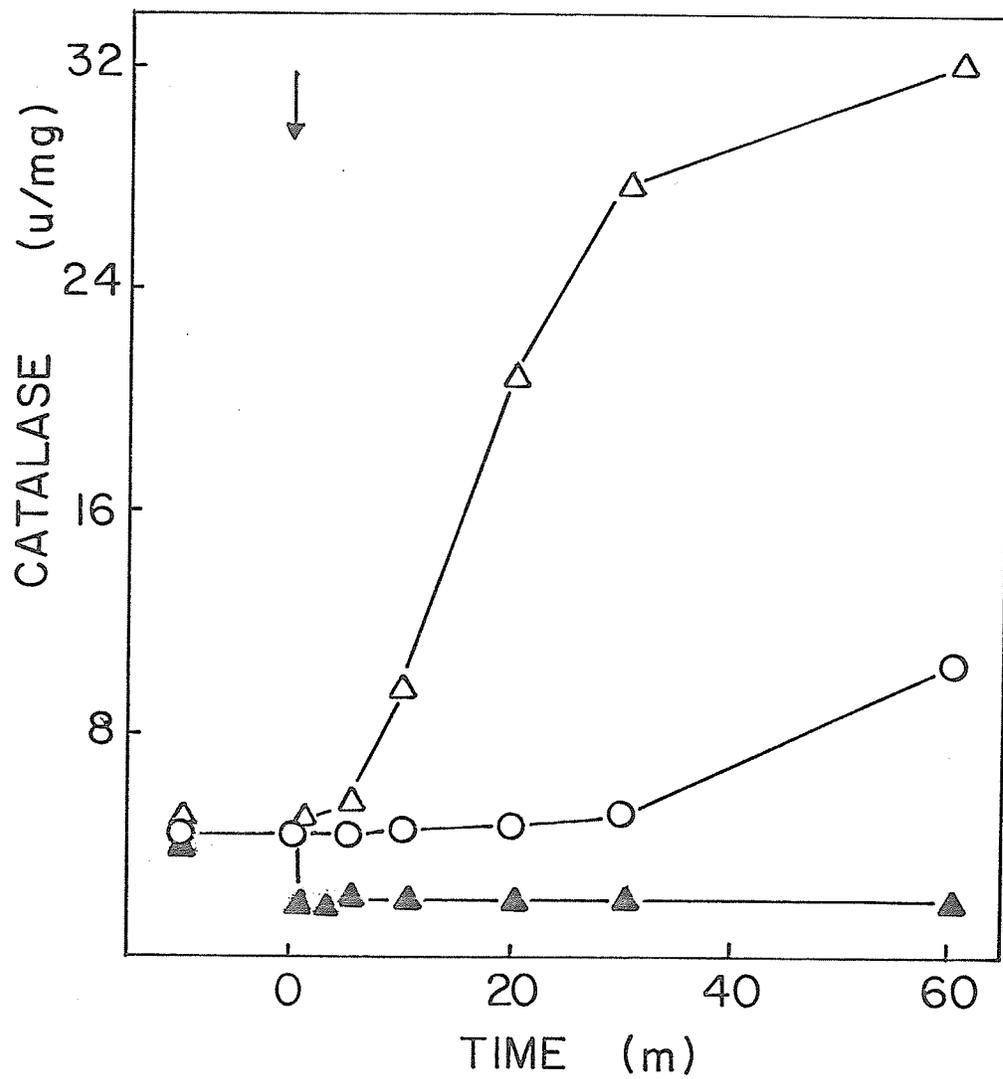
Despite an earlier report (Yoshpe-Purer et al., 1977) which indicated that it was difficult to achieve direct H₂O₂ induction of catalase, a single addition of either 0.15 mM H₂O₂, 0.75 mM H₂O₂ (Figure 33) or 3.3 mM H₂O₂ was sufficient to cause an induction of catalase synthesis. There was no inhibition of endogenous catalase observed in the first five minutes, nor was there any change in the growth rate.

Chloramphenicol prevented the increase in catalase caused by H₂O₂ addition. Before initiating the actual assay on individual culture aliquots, it was necessary to allow samples containing the higher concentrations of H₂O₂ to sit at 30°C for several minutes until all of this H₂O₂ was degraded and O₂ evolution stopped. The reason for the difficulties in observing catalase induction after H₂O₂ addition that were experienced by earlier workers (Yoshpe-Purer et al., 1977) is unknown.

As shown in Figure 24, there was an identical increase in the predominant isoenzyme pair possessing both catalase and peroxidase activity following the addition of either ascorbate or H₂O₂.

Figure 33. Effect of H_2O_2 on catalase levels in E. coli growing in aerated LB medium.

At time 0 the following supplements were added to separate cultures: nothing (○), 0.75 mM H_2O_2 (△), 0.1 mM chloramphenicol with 0.75 mM H_2O_2 (▲). Catalase activity was expressed as units per mg dry cell weight.



4.3.3 Catalase Induction by H_2O_2 in Minimal Salts Media

Addition of H_2O_2 to cells growing in glucose-salts medium resulted in the induction of catalase synthesis. Hydrogen peroxide at 0.15 mM and 0.75 mM (Figure 34) affected catalase levels in E. coli similar to the lower concentration of ascorbic acid. Even 3.3 mM H_2O_2 caused a prompt increase in catalase levels without the initial inhibition of catalase activity or killing of the cells, but there was a pause in growth lasting 30 minutes. These concentrations of H_2O_2 were all significantly higher than those assayed following ascorbic acid addition (Table 12) to growth media and therefore H_2O_2 obtained from the oxidation of ascorbic acid, could not be responsible for the disappearance of catalase activity and disruption of growth. Rather, this appeared to be due to a direct effect of ascorbic acid or its oxidation products.

The effect of H_2O_2 on catalase synthesis in cells growing in glycerol-salts medium was similar to the effect on catalase in cells growing in glucose-salts medium. There was no effect on growth and the final levels reached were 16 u/mg dry cell weight or 3 times the basal level.

The final level of catalase in a H_2O_2 induced culture grown in succinate-salts medium was about the same as the levels in cultures grown in glycerol-salts and glucose-salts media (Figure 35), although the basal level was higher in succinate salts medium.

The induced levels of catalase were not maintained as long following H_2O_2 induction as following ascorbate induction in any of the media investigated.

Figure 34. Effect of H_2O_2 on catalase induction in E. coli B growing in glucose salts medium.

In parallel cultures there was no H_2O_2 added (\circ), 0.75 mM H_2O_2 added (\bullet) at time 0 or both 0.75 mM H_2O_2 and 0.1 mM chloramphenicol added (Δ) at time 0. Cell growth was unaffected. Catalase activity is expressed as units per mg dry cell weight.

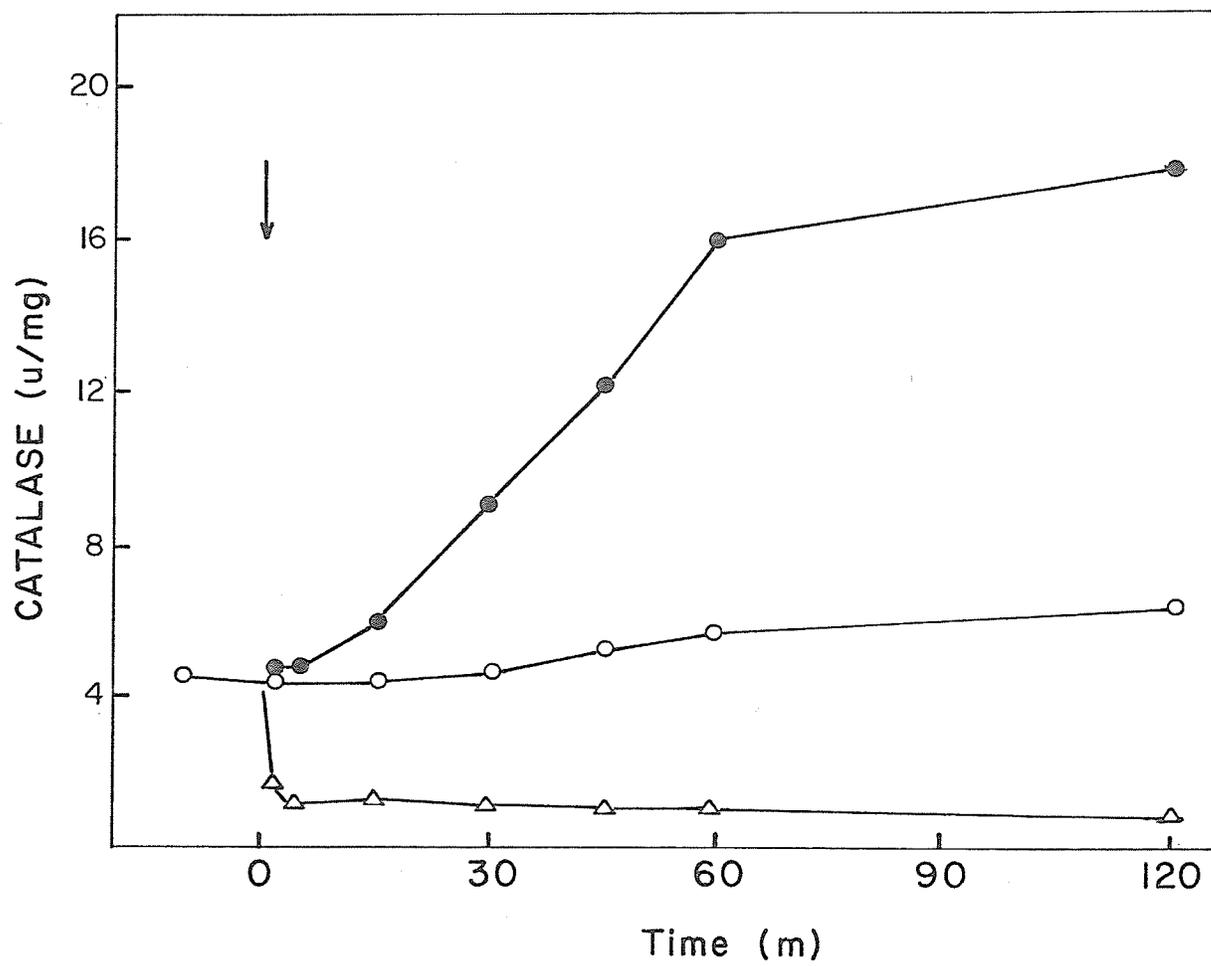
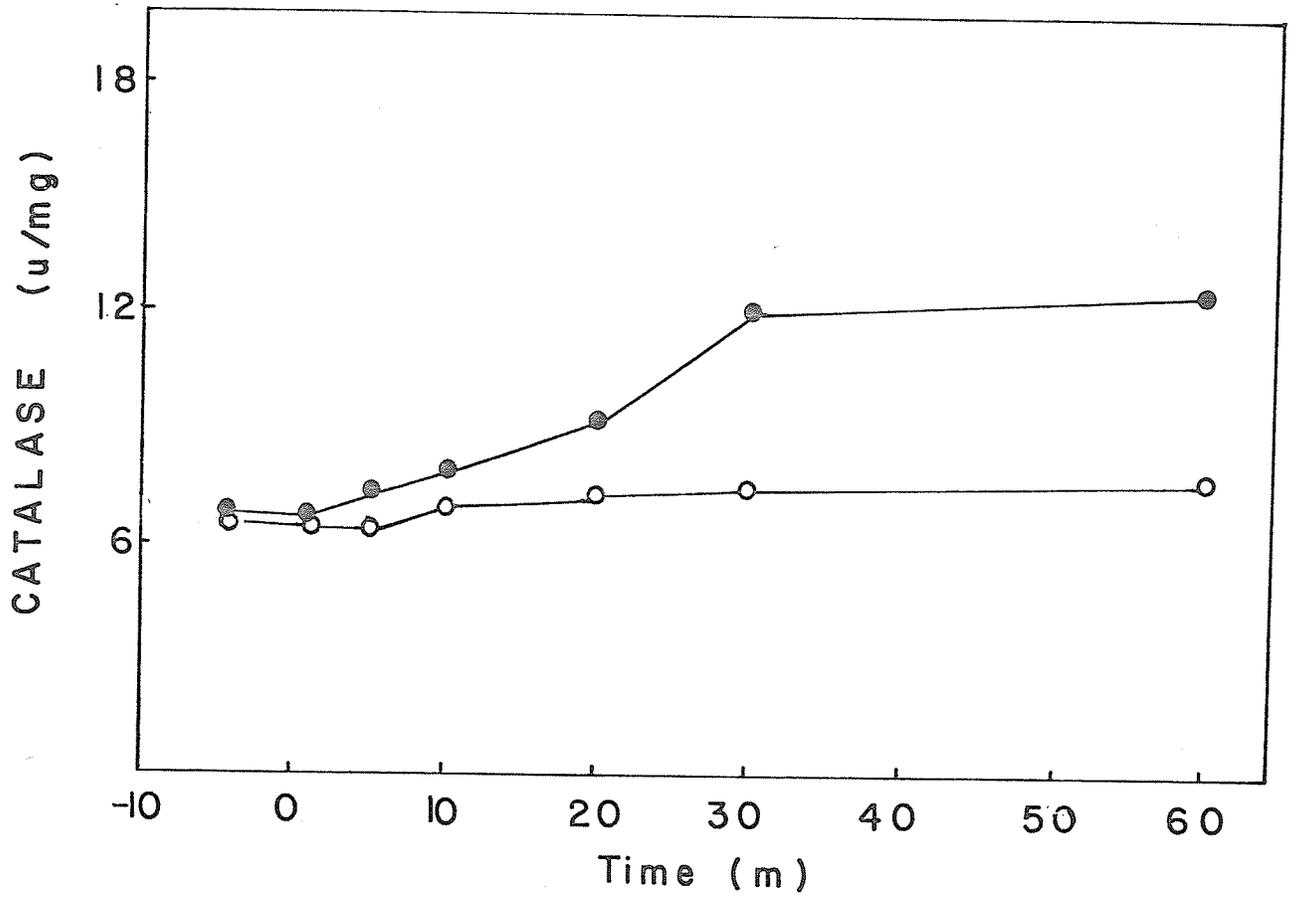


Figure 35. Effect of 0.75 mM H₂O₂ on catalase activity in E. coli B growing in succinate salts medium.

The following additions were made at time 0: none, (O), 0.75 mM H₂O₂, (●). Catalase activity was expressed as units per mg dry cell weight.



4.3.4 Effect of Exogenous Catalase and EDTA on Catalase Induction

Hassan and Fridovich (1980) have shown that the addition of exogenous catalase to growth medium reduced the effectiveness of pyocyanine as an antibiotic. Exogenous catalase would be expected to remove H_2O_2 from the medium before it could induce cellular catalase synthesis. Increasing concentrations of exogenous catalase in rich medium progressively reduced catalase induction to a point where 50 $\mu\text{g}/\text{mL}$ of bovine catalase, in LB medium prevented catalase induction (Figure 36). The presence of exogenous catalase also prevented the induction of catalase by ascorbate in glucose salts medium (Figure 37 A) and protected the cells from the killing effect of 5.7 mM ascorbic acid. The drop in catalase immediately following ascorbate addition was reduced.

The chelating agent EDTA would be expected to remove metal ions from the medium and reduce the rate of oxidation of ascorbate. As shown in Figure 37 B, the presence of 1.25 mM EDTA in glucose salts medium eliminated all response of catalase to ascorbic acid although there was a slight reduction in growth rate. This effect was also observed in LB medium.

Figure 36. Effect of exogenous catalase on catalase induction in E. coli B.

Catalase activity was assayed one half hour after the addition of 5.7 mM ascorbic acid to cultures in LB medium containing different concentrations of exogenous bovine liver catalase. Before assaying, the culture aliquots were centrifuged and washed 3 times in SM buffer, and resuspended in LB medium. Catalase activity was expressed as units per mg dry cell weight.

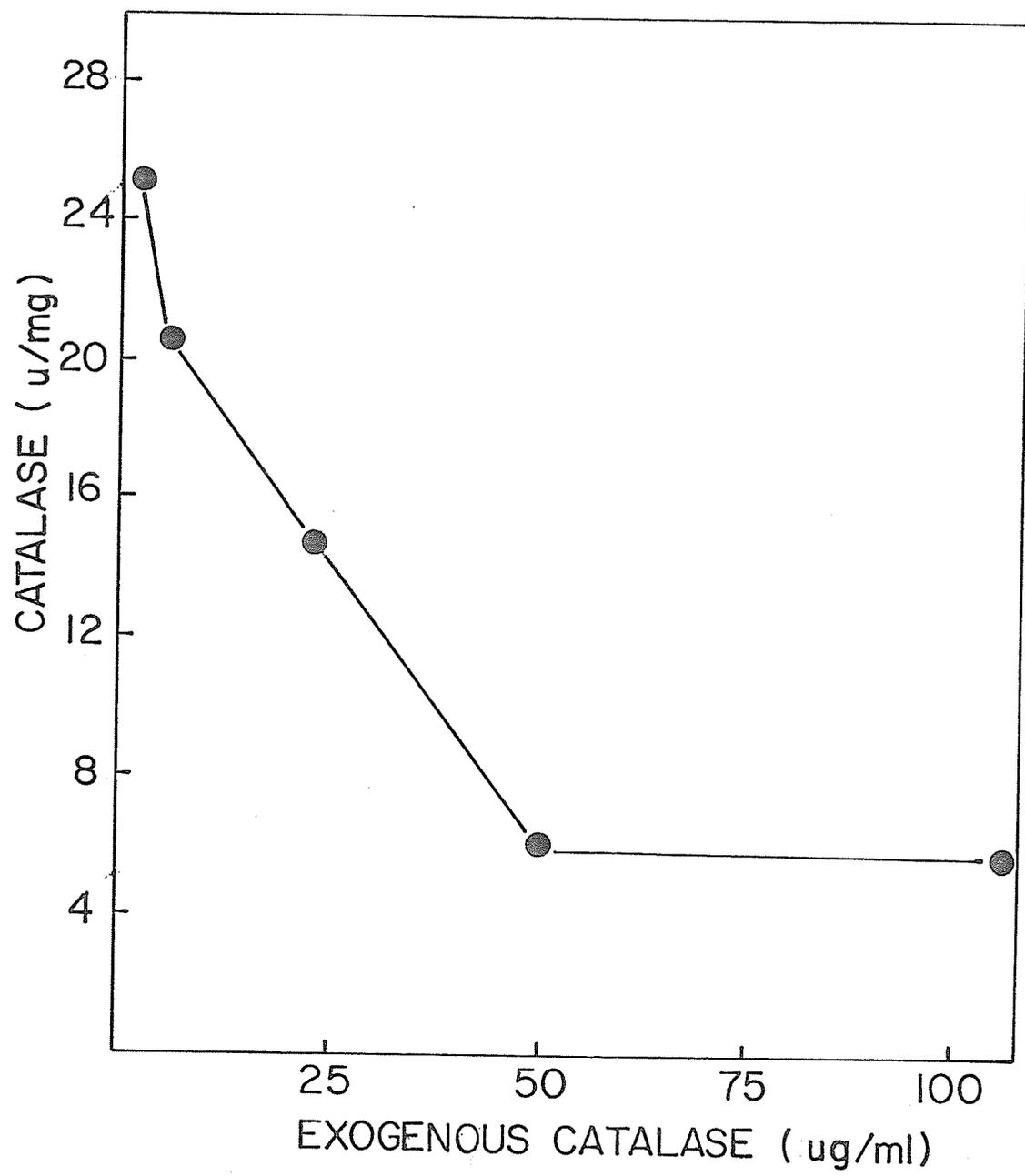
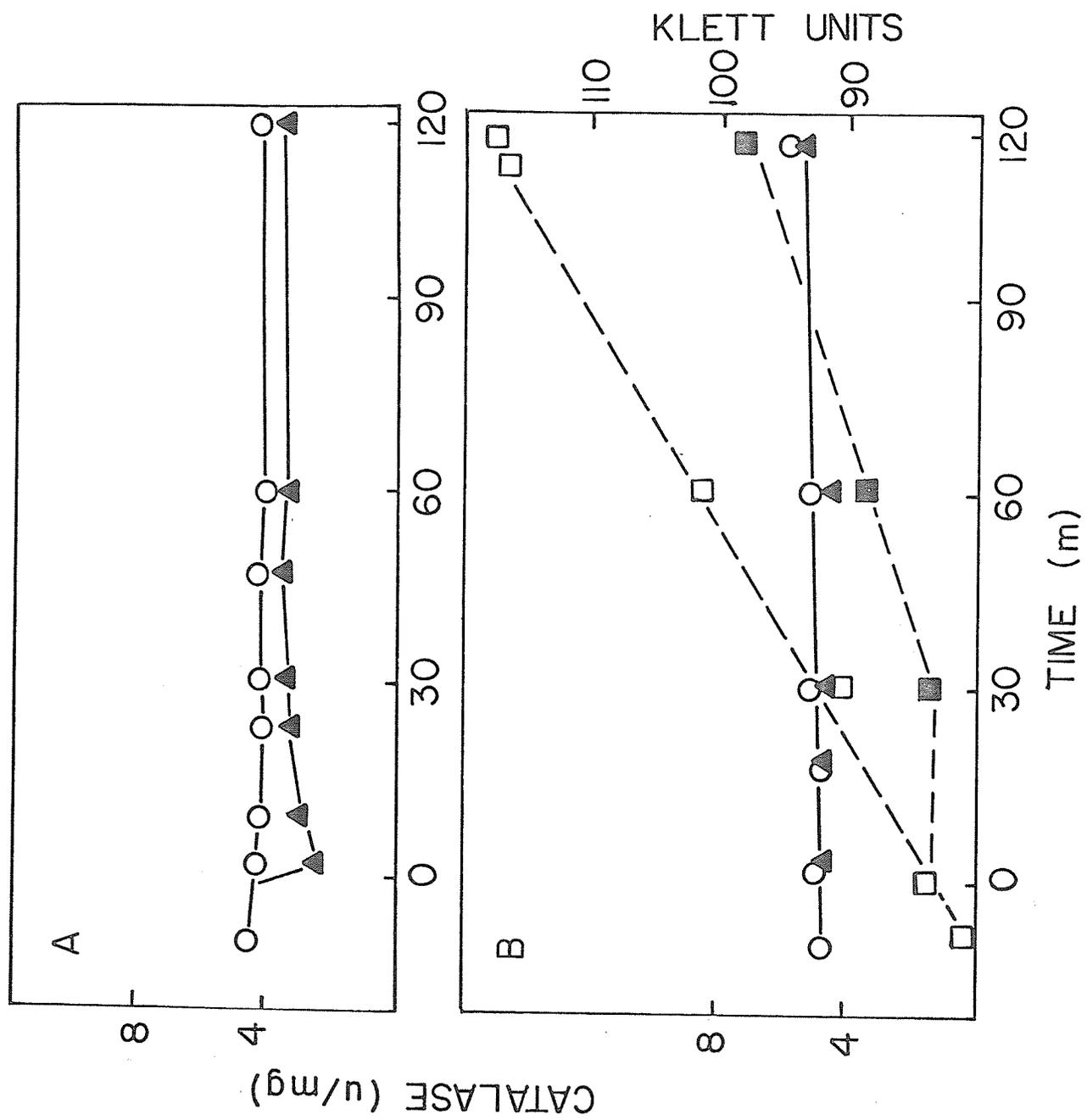


Figure 37. Effect of exogenous catalase and EDTA on catalase activity in E. coli B.

Catalase activity was assayed in aerobic cultures grown in glucose-salts medium containing 50 $\mu\text{g}/\text{mL}$ bovine catalase after the addition of no (\circ) and 5.7 mM (\blacktriangle) ascorbic acid (A). The aliquots were treated as described in the previous figure.

Catalase activity was assayed in aerobic cultures growing in LB medium containing 1.25 mM EDTA after the addition of no (\circ) and 5.7 mM (\blacktriangle) ascorbic acid. Growth was followed without (\square) and with (\blacksquare) ascorbic acid (B). Catalase activity was expressed as units per mg dry cell weight.



4.4 EFFECT OF AMINO ACIDS ON THE INHIBITION OF CATALASE BY ASCORBATE

As noted above, there was a rapid drop in catalase activity after addition of 5.7 mM ascorbic acid to a culture of E. coli growing in LB medium from which it took three to four minutes to recover. In glucose-salts medium the inhibition of catalase was absolute and only at ascorbate concentrations of 2.8 mM and below was there recovery of activity within 4 hours. Cells in glycerol and succinate-salts media did not appear to be as sensitive as cells in glucose-salts medium. The drop in activity did not occur after the addition of H_2O_2 to any medium. An earlier report (Orr, 1966) had indicated that ascorbic acid or free radical products resulting from its oxidation could inhibit catalase and how such products could have different effects in different media formed the subject of further study.

In Table 13 the percentages of inhibition of catalase in cell free extracts by various growth medium components are compiled. When the extract was prepared in LB medium, ascorbate had no effect on catalase activity even if glucose was present. When the extract was prepared in minimal salts medium, 0.28 mM ascorbate completely inhibited the catalase. Several supplements were included to study their effect on the inhibition of catalase by ascorbate and only LB medium or casamino acid relieved the inhibition of catalase. When each of the individual amino acids was tested, only histidine relieved the inhibition implicating it as the active component in LB medium and casamino acids which prevented the inhibition of catalase by ascorbic acid. Furthermore, histidine was

effective both in preventing inhibition when it was added prior to ascorbic acid and in relieving inhibition when it was added after ascorbic acid had already inhibited the enzyme. Histidine is an effective chelator of certain metal ions required for ascorbic acid oxidation, particularly copper (O'Sullivan, 1969) and the protective effect was presumably the result of slower ascorbic acid oxidation producing lower levels of free radical intermediates which were responsible for the inhibition of catalase. Unfortunately, histidine interfered with the assay for H_2O_2 possibly through chelation of required metal ions, and it was not possible to quantitate H_2O_2 in media containing high levels of histidine.

When 6.7 mM histidine was used as the amino acid supplement in glucose salts medium there was only a small inhibition of catalase activity by 2.8 mM ascorbic acid (Figure 38) but, unlike growth in the casamino acid supplemented culture, subsequent catalase synthesis and growth (Figure 38) occurred very slowly indicating that ascorbate was affecting another aspect of cellular metabolism, possibly glucose transport (see below). Histidine could act to prevent the inhibition of catalase activity presumably by chelation as in cell free extracts, but a more complete amino acid supplement and metal ions were required to facilitate protein synthesis for the production of the catalase necessary for protection of the cell against H_2O_2 and alone to act as a carbon source when glucose transport was inhibited (see below). In Table 14, it can be seen that groups of five amino acids added to glucose-salts medium supported catalase synthesis to levels intermediate between the unsupplemented and casamino acid supplemented media. Clearly, the availabil-

Table 13. Inhibition of cell free catalase activity by ascorbic acid in the presence of various medium components.

Origin of cell extract ¹	Component added ²	% Inhibition
A. LB medium	asc + Fe ²⁺	0
	glu + Fe ²⁺	0
	glu + asc + Fe ²⁺	0
B. Minimal Salts	asc + Fe ²⁺	100
	glu + Fe ²⁺	0
	glu + asc + Fe ²⁺	100
	asc	100
	glu	0
	asc + glu	100
	BSA (100 µg/mL) + asc + Fe ²⁺	100
	cytochrome <u>c</u> (10 µM) + asc	100
	LB medium (1:1), (v/v) + asc	34
	casamino acids (0.1% w/v) + asc	20
	ala + asc	100
	pro + asc	100
	gly + asc	100
	tyr + asc	100
	gln + asc	100
	arg + asc	100
	trp + asc	92
	asp + asc	100
	lys + asc	100
	asn + asc	100
	val + asc	100
	phe + asc	100
	ser + asc	100
glu + asc	100	
his + asc	6	
cys + asc ³	100	
leu + asc	100	
ile + asc	100	
met + asc	100	
thr + asc	100	
his + added after asc	29	

¹Exponential phase cultures were chilled, sonicated and centrifuged. The supernatant was used directly in the assay.

²The final concentrations were as follows: asc, 0.28 mM, FeSO₄, 10 µM; glucose, 5.5 mM; and individual amino acids, 5 mM.

³Cysteine at 5 mM inhibited catalase.

ity of amino acids was a key factor in determining the amount of catalase synthesized as well as the degree of inhibition of catalase by ascorbate.

In sonicated extracts free radical products from ascorbate oxidation could clearly interact with catalase. The situation in whole cells was not as clear. For example, treatment of whole cells growing in LB medium with ascorbate caused the rapid but transient inhibition of catalase. If the same culture aliquots were sonicated prior to the catalase assay no inhibition was observed (Figure 39). Therefore, LB medium could protect cell free catalase but not intracellular catalase from ascorbate inhibition. Because ascorbate and its oxidation products do not enter the cell (see below) the cell wall or membrane must be mediating the inhibitory effect on whole cells. The mechanism is not understood.

For cells growing in glucose-salts medium, sonication did not relieve ascorbate inhibition (Figure 39) presumably because the salts medium unlike LB medium did not contain histidine to prevent further ascorbate oxidation. Glucose- H_2O_2 mixtures incubated at $37^\circ C$ did not inhibit catalase (Table 13) eliminating the possibility of a glucose oxidation product (Pigman and Goepf, 1948) being the inhibitor.

Figure 38. The effect of ascorbic acid on E. coli B growing in glucose-salts media supplemented with histidine.

At time zero 2.8 mM ascorbic acid was added (O) and catalase activity determined. Growth was also followed (□). The concentration of histidine was 6.7 mM. Catalase activity was expressed as units per mg dry cell weight.

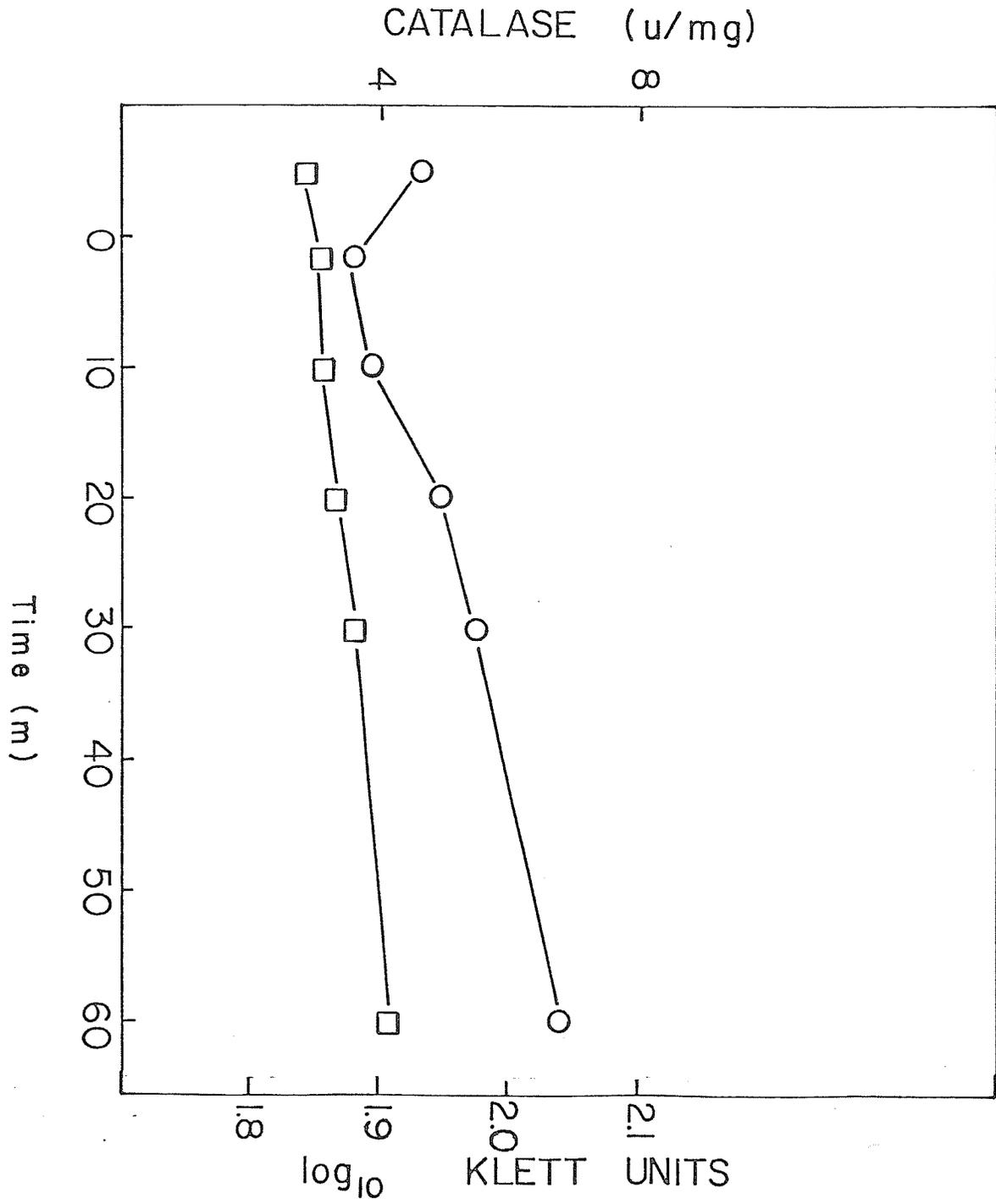


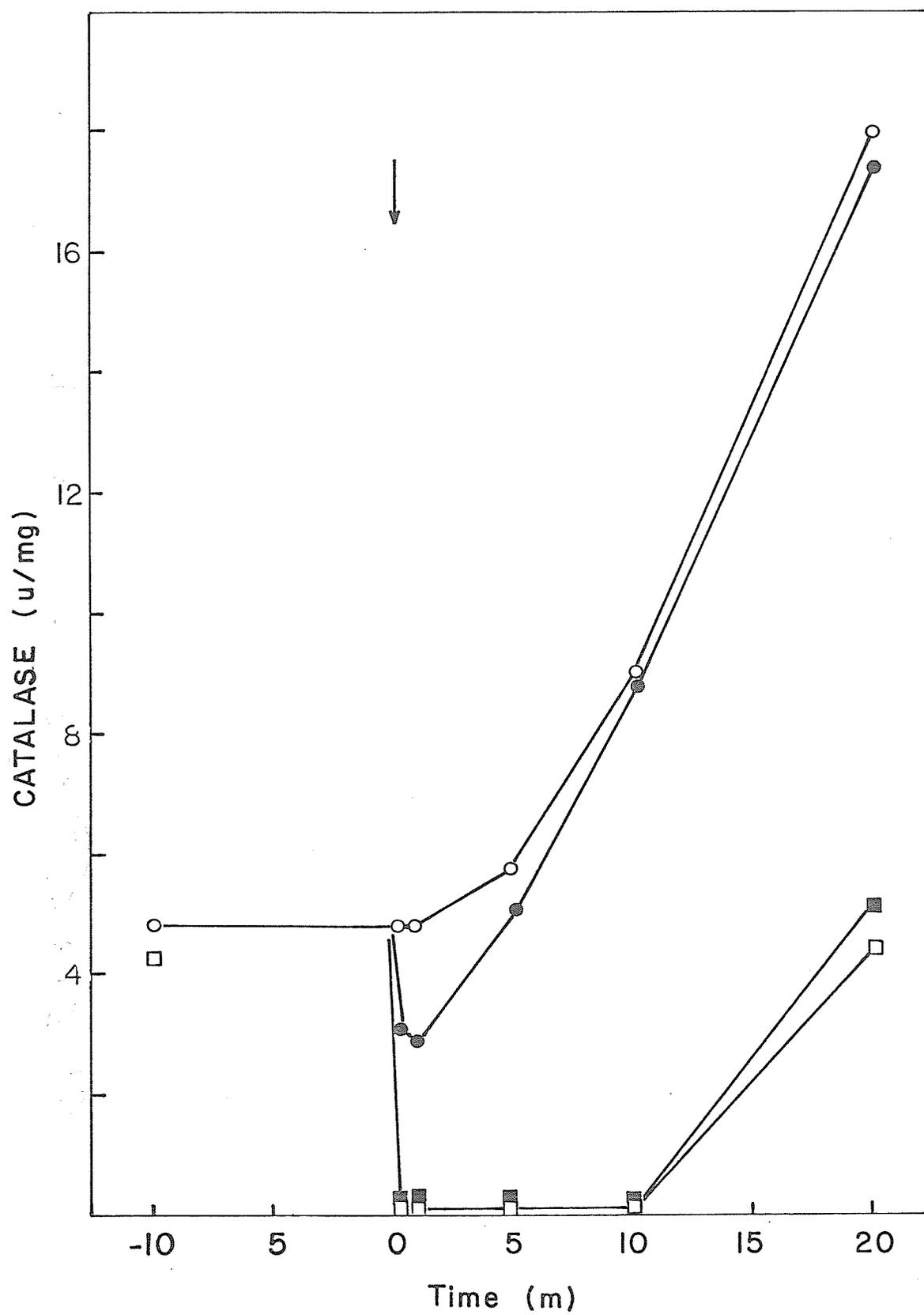
Table 14. Effect of amino acid supplements on the induction of catalase one hour after the addition of 0.57 mM ascorbic acid to E. coli growing in glucose-salts medium.

Supplement*	Catalase (u/mg dry cell weight)
none	6.2
ile, met, his, asp, cys	19.0
leu, trp, thr, gln, arg	20.2
ala, pro, gly, tyr, glu	22.4
val, phe, ser, asn, lys	24.0
casamino acids	32.0
none (no ascorbic acid)	6.6

*Individual amino acid concentrations were 5 mM while the casamino acid supplement was 0.1% w/v or approximately 0.3 mM of each amino acid.

Figure 39. Effect of sonication on the catalase activity in sonicated and whole cell culture aliquots.

The addition of 5.7 mM ascorbic acid to cells growing in LB medium was carried out as described. (○, sonicated aliquot) (●, unsonicated aliquot) and of 2.8 mM ascorbic acid to glucose salts medium (□, sonicated aliquot) (■, unsonicated aliquot). Catalase is expressed as units per mg dry cell weight.



4.5 EFFECT OF REC A ON CATALASE INDUCTION BY ASCORBATE

When E. coli is subject to certain conditions which interfere with DNA synthesis or cause DNA damage, there is induction of the SOS functions (Hanawalt et al., 1976). The most prominent of the responses is the induction of the rec A gene product. If catalase was involved in the SOS functions, perhaps its induction in a rec A mutant compared to the wild type E. coli would be different. This was not found to be the case. In both E. coli B5974 and B5975 rec A the levels of catalase induced by ascorbic acid were the same and there did not appear to be any relationship between catalase induction and rec A protein function in the response to ascorbic acid.

4.6 EFFECT OF T-BUTYL PEROXIDES ON CATALASE LEVELS IN E. COLI

The addition of 5.7 mM tert-butyl peroxide to E. coli growing in LB medium did not affect cell growth but also had no effect on catalase levels (Table 15). There are three possible explanations: 1. The tert-butyl peroxide did not break down to the reactive peroxide radicals under the growth conditions used. 2. The tert-butyl peroxide or tert-butyl peroxide radicals could not enter the cell. 3. The tert-butyl peroxide radical did not induce catalase. The only higher concentration of peroxide which was tested was 57 mM which resulted in cell death.

Unlike tert-butyl peroxide, 5.7 mM tert-butyl hydroperoxide in LB medium did induce catalase in E. coli although not to the same extent as either ascorbate or H_2O_2 (Table 16). Exogenous catalase (100 μ g/mL) did

Table 15. Effect of t-butyl peroxide on catalase induction in LB medium.

Time (m)	Catalase Activity (units per mg dry cell weight)	
	-TBP	+TBP
-10	5.4	5.6
0	5.2	5.2
1	5.2	6.6
10	5.2	6.2
20	5.6	5.8
30	5.6	6.2
60	10.0	9.0
120	12.2	11.8

At time 0, nothing or 5.7 mM tert-butylperoxide was added to a culture of *E. coli* growing in LB medium. Aliquots were taken and assayed for catalase activity.

not prevent this induction indicating that it was not H_2O_2 formed from tert-butyl hydroperoxide in the medium which was causing the induction. Whether it was the hydroperoxide itself once inside the cell, the tert-butyl peroxide radical or the hydroxide radical formed inside the cell which was responsible for the induction was not determined.

Table 16. Effect of t-butylhydroperoxide on catalase induction in LB medium.

Time (m)	Catalase Activity (units per mg dry cell weight)		
	-TBHP	+TBHP	+TBHP + catalase
-10	4.8	4.8	4.4
0	4.8	5.0	4.8
+ 1	5.2	5.4	6.4
+ 5	4.8	6.2	8.2
+10	5.2	7.8	9.6
+20	4.8	13.2	17.0
+30	5.8	16.0	18.2
+60	11.2	18.4	19.2

At time 0, nothing, 5.7 mM t-butyl hydroperoxide or 5.7 mM t-butylhydroperoxide and 100 $\mu\text{g}/\text{mL}$ catalase were added to a culture of E. coli in LB medium. Aliquots were taken and assayed for catalase activity.

4.7 EFFECT ON ASCORBIC ACID AND H_2O_2 ON CATALASE LEVELS IN ANAEROBIC CULTURES OF E. COLI

4.7.1 Catalase Induction in Anaerobic LB and Minimal Salts Media

Since oxygen is required for the production of H_2O_2 from ascorbic acid, no catalase should be induced in response to ascorbic acid addition to anaerobic cultures of E. coli and, as shown in Figure 40, there was no induction of catalase even after 5.7 mM ascorbic acid was added to a culture growing anaerobically in LB medium. There was also no pause in growth and no inhibition of catalase evident following the addition of ascorbic acid to the medium. There was, however, a diauxie effect observed in the growth curve (Figure 41) and increasing concentrations of ascorbic acid allowed the anaerobic cultures to grow to successively greater cell densities. Either ascorbate was being used as a carbon source (which seemed unlikely since it did not enter the cell, see below) or ascorbate was acting as an electron source to allow further cell growth in the absence of further sugar metabolism.

A similar effect was observed in cells growing anaerobically in glycerol-fumarate-salts medium and glycerol-aspartate-salts medium (Figure 42 A and B). Although a diauxie effect was not evident, those cultures containing ascorbic acid grew faster to a final cell density 33% higher than in those cultures without ascorbate.

In Figure 43, it can be seen that the lower catalase-peroxidase band in anaerobically growing cells was predominant and this did not change following the addition of ascorbic acid. The peroxidase activity below

Figure 40. Effect of ascorbic acid on catalase activity in E. coli B growing anaerobically in LB media.

The concentration of ascorbic acid added at time zero was none (○), and 5.7 mM (▲). Catalase activity was expressed as units per mg dry cell weight.

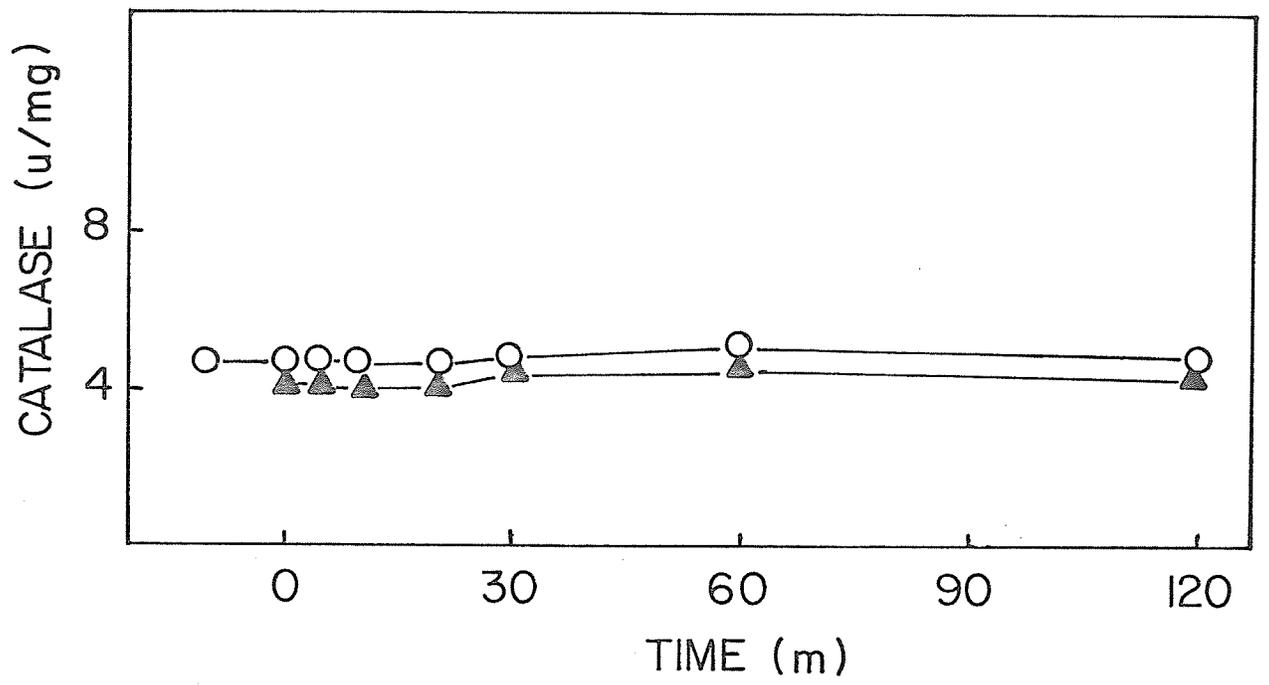


Figure 41. Effect of ascorbic acid on the growth rate of E. coli B growing anaerobically in LB medium.

At time 0, 2.8 mM (●) and 5.7 mM (▲) ascorbate was added to a culture of E. coli growing anaerobically in LB medium. Parallel cultures were allowed to grow anaerobically (○) and aerobically (Δ) without supplements.

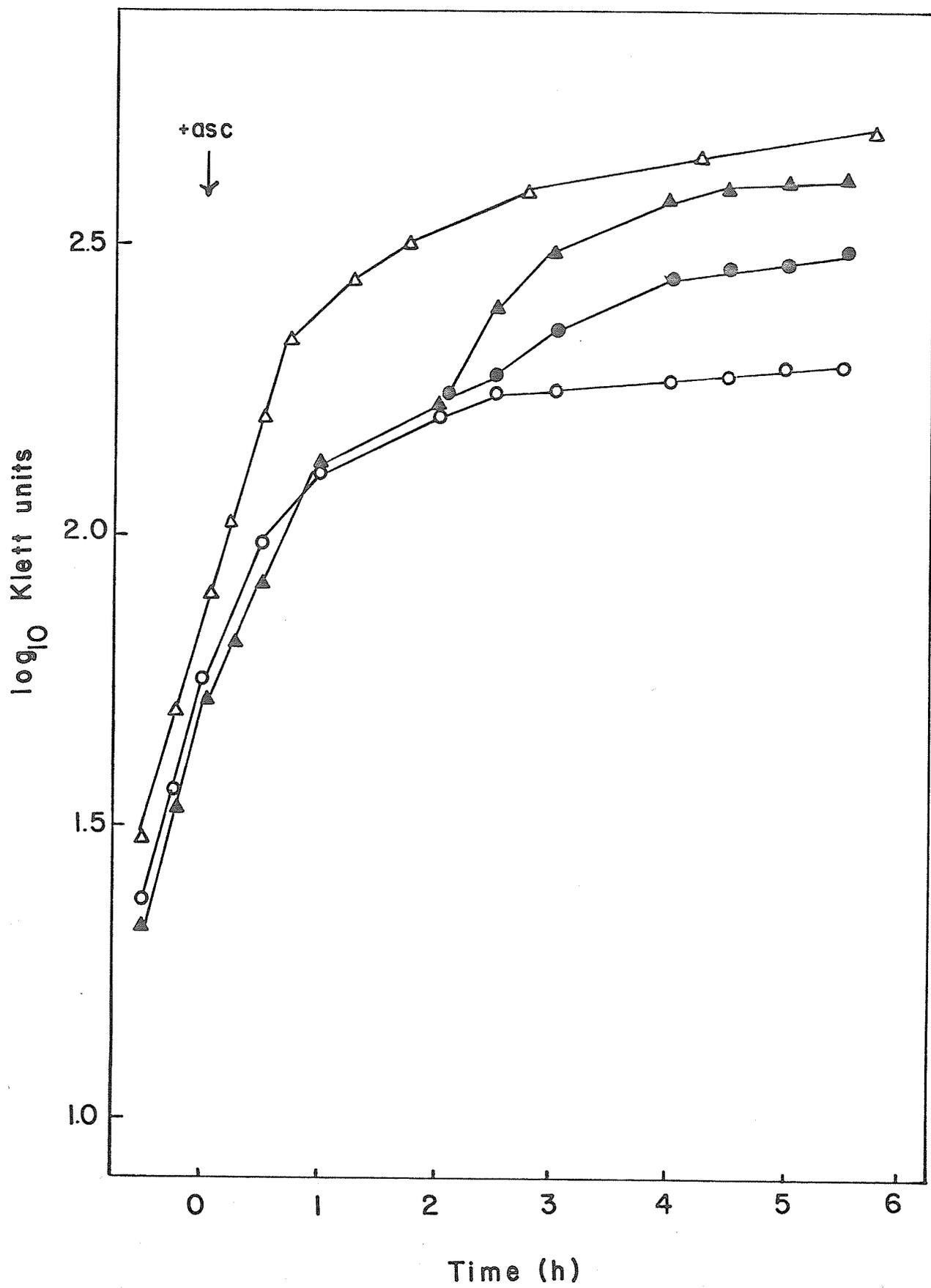
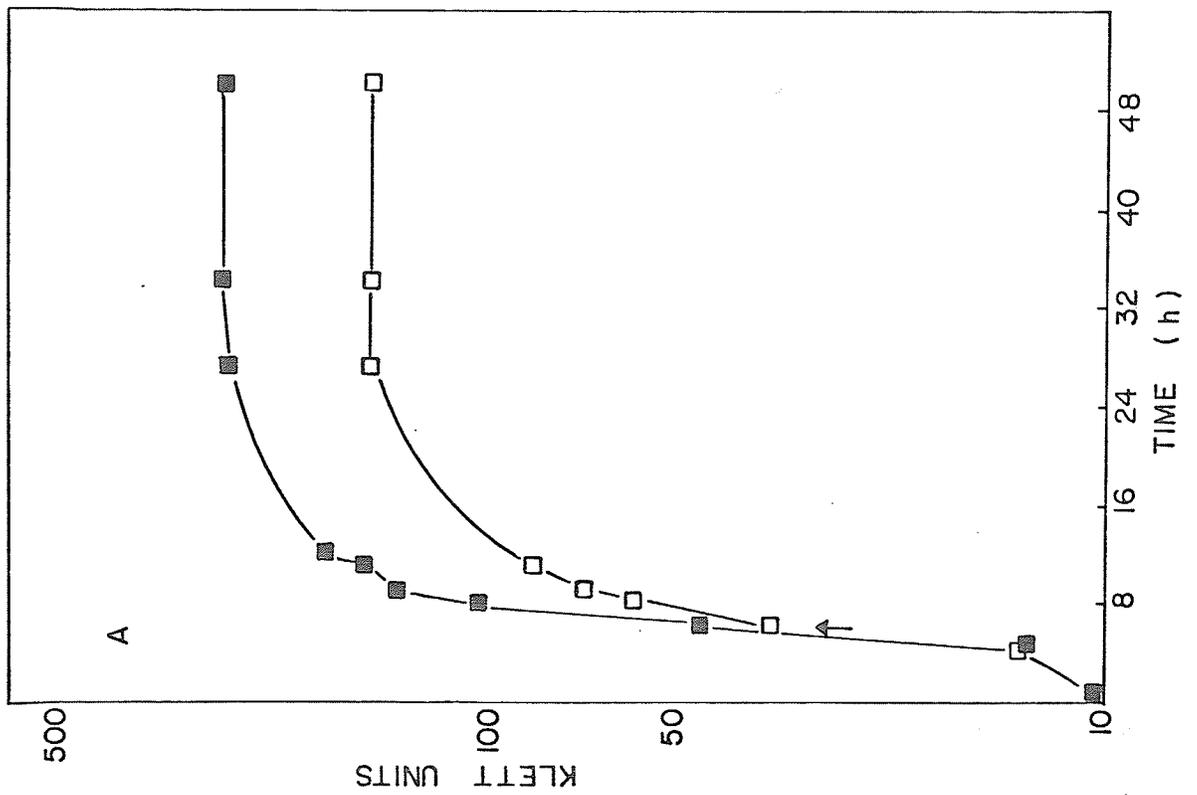
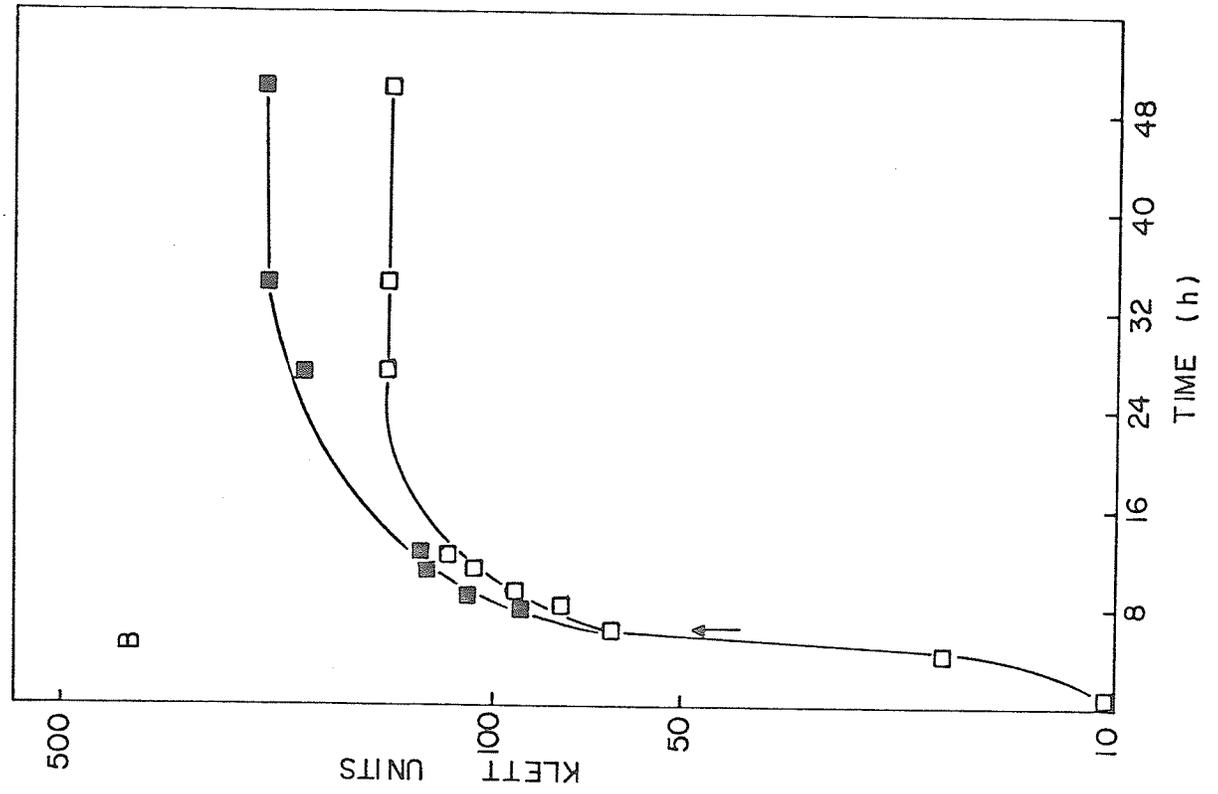


Figure 42. Effect of 2.8 mM ascorbic acid on cells growing anaerobically in a minimal salts medium.

(A) is supplemented with 20 mM glycerol and 20mM fumarate and (B) contains 20mM glycerol and 20 mM aspartate. At the specified time, indicated by the arrow, nothing (□) or 2.8 mM ascorbic acid (■) was added.



the two main isoenzyme bands did not appear to be as dominant in cells growing in anaerobic LB medium as compared to aerobic LB medium.

In order to confirm that catalase could be induced in anaerobic medium, 0.75 mM H_2O_2 was added to a culture in anaerobic LB medium where it was found to cause a four fold increase in catalase activity (Figure 44).

As shown in Figure 44, there was no induction of catalase after 5.7 mM ascorbic acid was added to either anaerobic glucose salts medium, (Figure 45 A), or anaerobic glycerol salts medium, (Figure 45 B). Ascorbic acid did cause a short pause in the growth of both cultures and, unexpectedly, a transient drop in catalase activity. This inhibition of catalase was of longer duration in glycerol-salts medium than in glucose-salts medium which was the reverse of what had occurred in aerated media. The fact that the pause in growth was of much shorter duration than the drop in catalase activity indicated that anaerobic cultures of E. coli could grow in the absence of catalase.

To confirm that catalase could be induced in anaerobic minimal medium as well as LB medium, 0.15 mM H_2O_2 was added to a culture in glucose-salts-casamino acids media. A single addition caused a small increase in catalase synthesis while two sequential additions 25 minutes apart doubled the amount of induction (Table 17).

Figure 43. Visualization of catalase and peroxidase activities in sonicated extracts of E. coli grown in anaerobic LB medium.

2.8 mM ascorbic acid was added where specified. Gel 1: contains 20 μ g (40 u) of bovine catalase. Gels 2 and 4: 200 μ g or protein from a culture with ascorbate added. Gels 3 and 5: 200 μ g protein from a culture without ascorbate added. Polyacrylamide gels (12%) 1-3 were stained for catalase, gels 4 and 5 were stained for peroxidase activity as described in Methods.

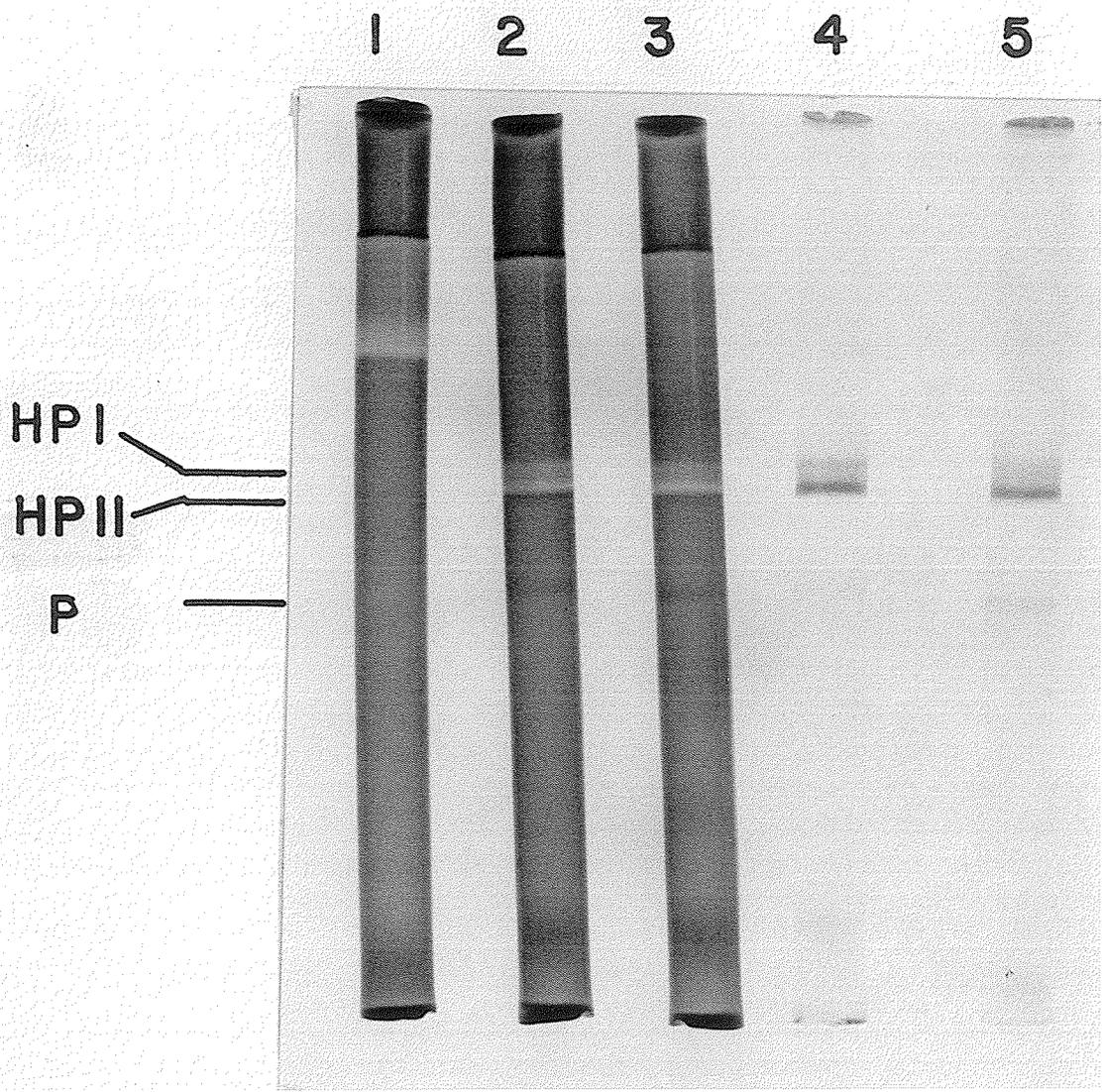


Figure 44. Effect of ascorbic acid and H₂O₂ on catalase levels in E. coli growing in anaerobic LB medium.

At time 0, the following supplements were added to separate cultures: nothing (O), 5.7 mM ascorbic acid (□), and 0.75 mM H₂O₂ (Δ). Catalase activity was expressed as units per mg dry cell weight.

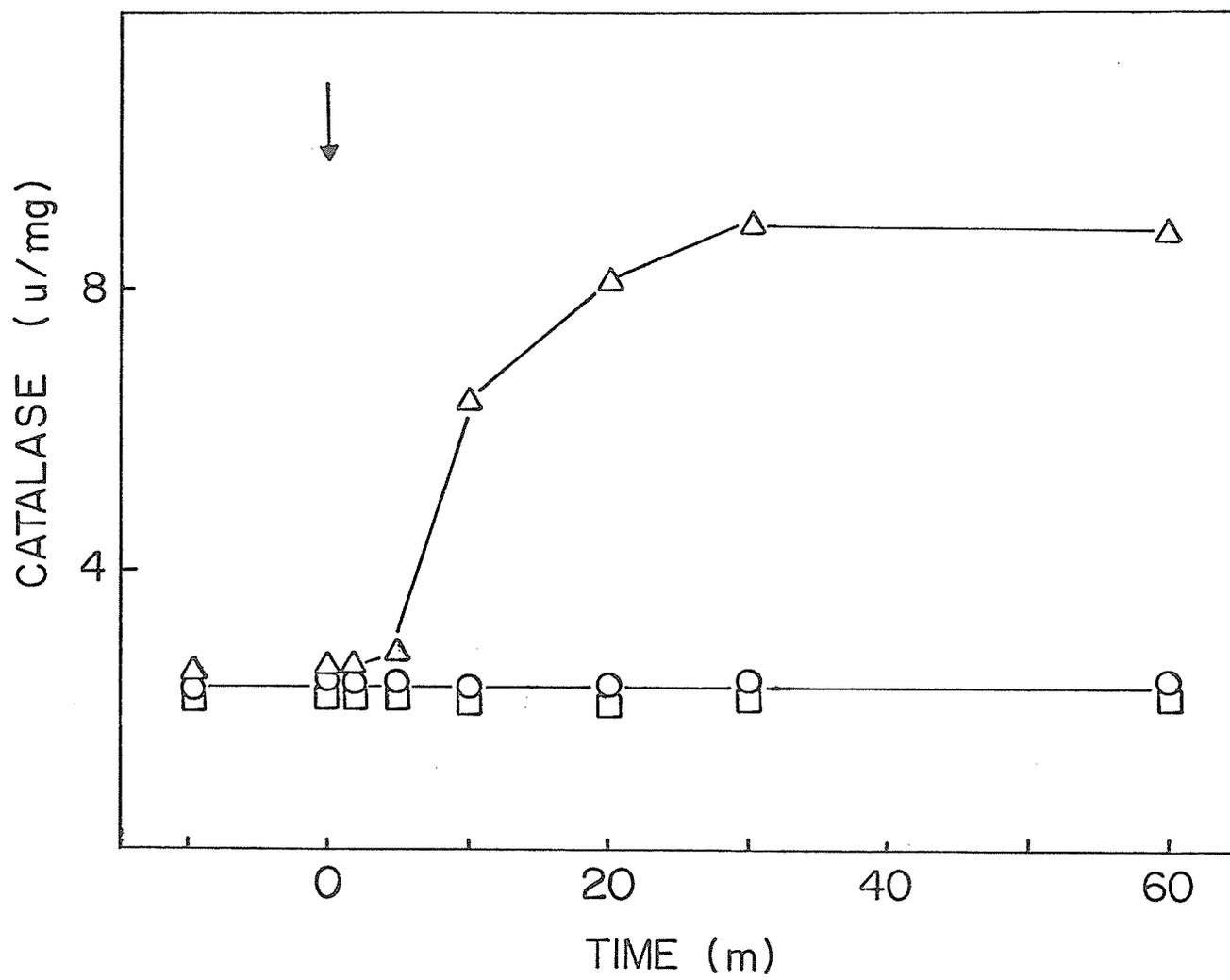


Figure 45. Effect of ascorbic acid on catalase activity in E. coli B growing anaerobically in glucose-salts medium (A) and glycerol salts medium (B).

The following concentrations of ascorbic acid were added at time 0, none (○), 0.57 mM (●), 2.8 mM (△), and 5.7 mM (▲). Catalase activity was expressed as units per mg dry cell weight.

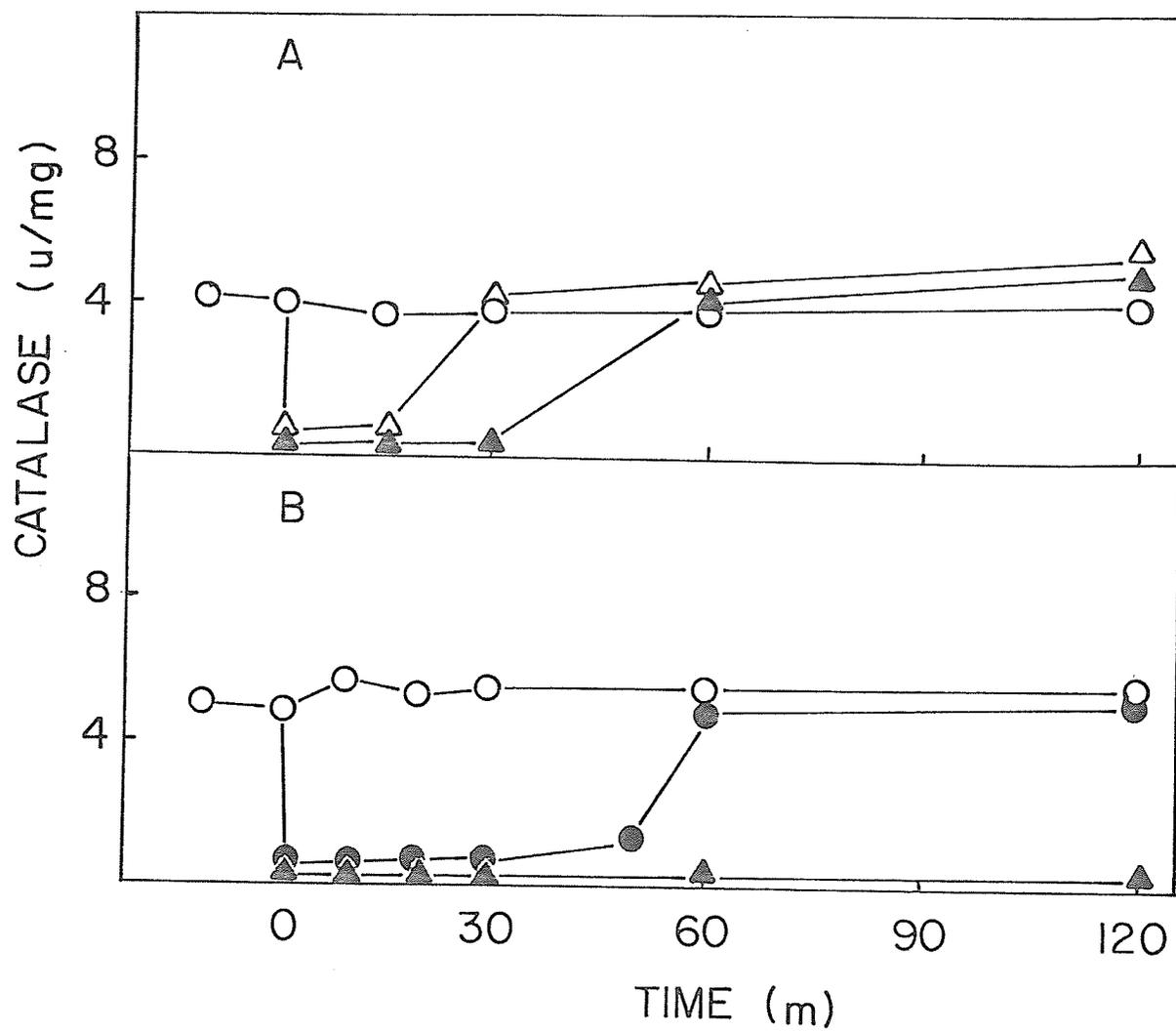


Table 17. Induction of catalase in E. coli B23 grown anaerobically in glucose salts medium supplemented with 0.1% casamino acids.

Time (m)	Catalase μ /mg dry cell weight	
	-H ₂ O ₂	+H ₂ O ₂
A.		
- 10	4.1	4.4
0 (+H ₂ O ₂)	4.0	4.4
+ 1	4.0	4.2
+ 5	4.0	4.2
+ 10	4.2	5.0
+ 20	4.0	5.4
+ 30	4.0	6.4
+ 60	3.6	6.8
+120	3.2	4.6
B.		
- 10	3.8	4.0
0 (+H ₂ O ₂)	4.0	4.2
+ 1	4.0	4.2
+ 5	4.0	4.4
+ 10	4.0	5.6
+ 20	4.2	6.6
+ 25 (+H ₂ O ₂)	-	-
+ 30	4.4	7.8
+ 60	4.4	10.8
+120	2.6	10.0

Hydrogen peroxide was added to a final concentration of 0.15 mM at zero time. Where indicated (B) a second addition of H₂O₂ was made. Aliquots were taken and assayed as described in the Methods.

4.7.2 Catalase Induction in LB-Nitrate and Minimal-Salts-Nitrate Media

The presence of nitrate in the medium of an anaerobic culture of E. coli grown into stationary phase has been found to cause a two fold increase in the level of catalase (Hassan and Fridovich, 1978b). As shown in Figure 45, nitrate did not affect the basal level of catalase, 4 units per mg dry cell weight. When ascorbic acid was added to unsupplemented anaerobic glucose-salts medium, a 2 to 3 fold increase in catalase occurred (Figure 46 A) and a casamino acids supplement allowed a 4 to 5 fold increase (Figure 46 B). There was no visible effect on the growth rate in either case. Both catalase-peroxidase activities visualized on polyacrylamide gels were induced following addition of 2.8 mM ascorbic acid (Figure 47).

The presence of exogenous catalase, even at 100 $\mu\text{g}/\text{mL}$ which was enough to completely prevent catalase induction in aerobic cells, did not prevent catalase induction in anaerobic cells growing in the presence of nitrate (Table 18). It is not clear how H_2O_2 could be formed under these conditions but another hydroperoxide might possibly be formed as a result of electron donation from ascorbate to an unknown intermediate in nitrate reduction. The fact that t-butyl hydroperoxide could induce catalase synthesis in a process unaffected by exogenous catalase suggested that this may be possible.

The effect of ascorbate on catalase induction in a culture growing anaerobically in LB medium containing nitrate was investigated (Table 19 and Figure 48). Unlike induction observed in nitrate containing salts

Figure 46. Effect of ascorbic acid on catalase activity in anaerobic cultures of E. coli B.

E. coli was grown in glucose-salts medium with 20 mM potassium nitrate (A) and 0.1% casamino acid supplemented glucose-salts medium also with 20 mM potassium nitrate (B). The following concentrations of ascorbic acid were added at time zero: none (○), 0.57 mM (●), 2.8 mM (Δ). No effect was observed on the growth rate following ascorbic acid addition.

Catalase activity was expressed as units per mg dry cell weight.

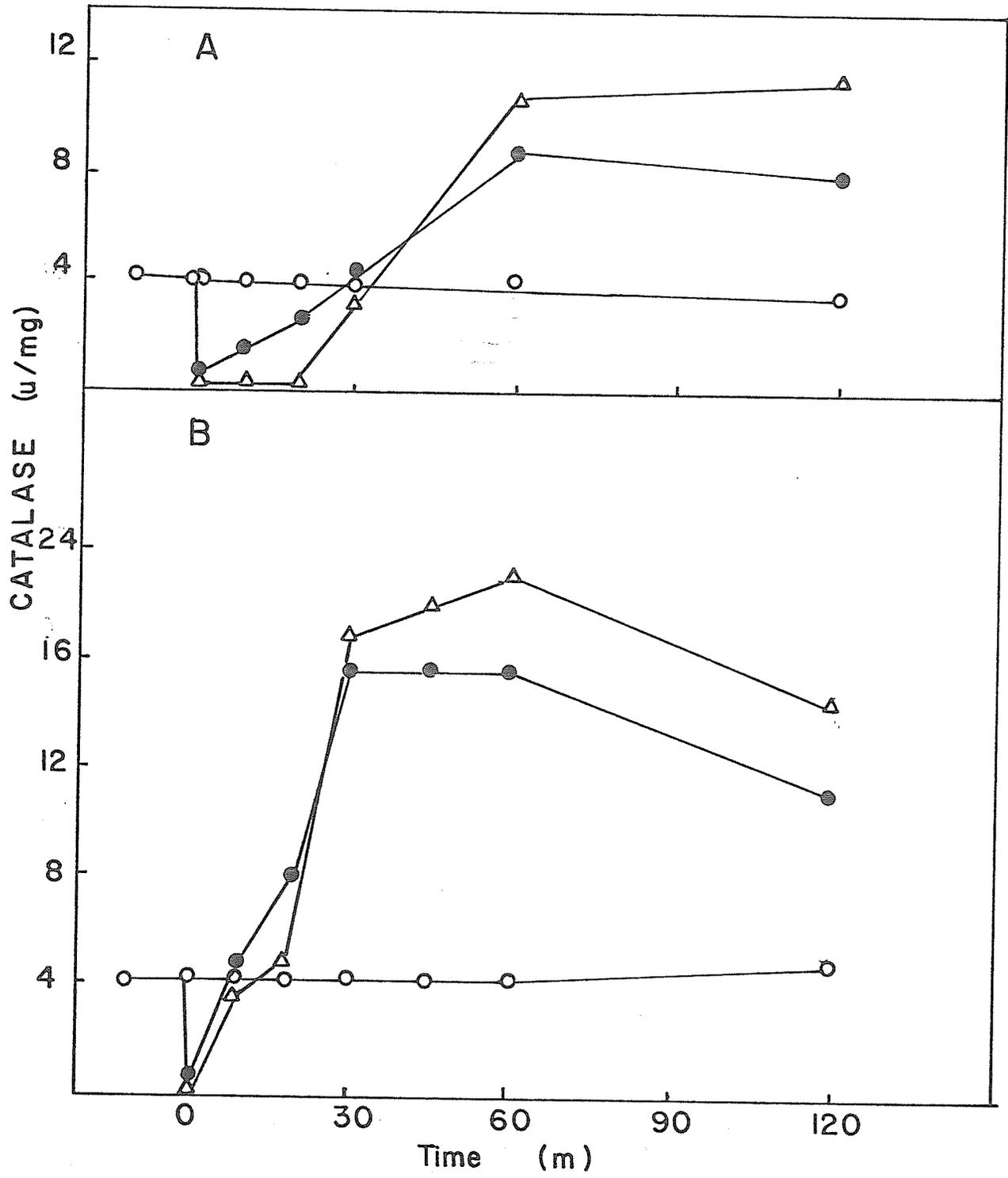


Figure 47. Visualization of catalase and peroxidase activities in extracts of E. coli grown anaerobically in glucose-salts medium in the presence of 20 mM potassium nitrate with and without ascorbic acid.

Gel 1: 20 μ g (40 u) bovine catalase. Gels 2 and 4: 280 μ g of protein from a culture treated with ascorbate. Gels 3 and 5: 280 μ g of protein from a culture not treated with ascorbate.

Polyacrylamide (12%) gels 1-3 were stained for catalase and gels 4 and 5 were stained for peroxidase activity as described in the Methods.

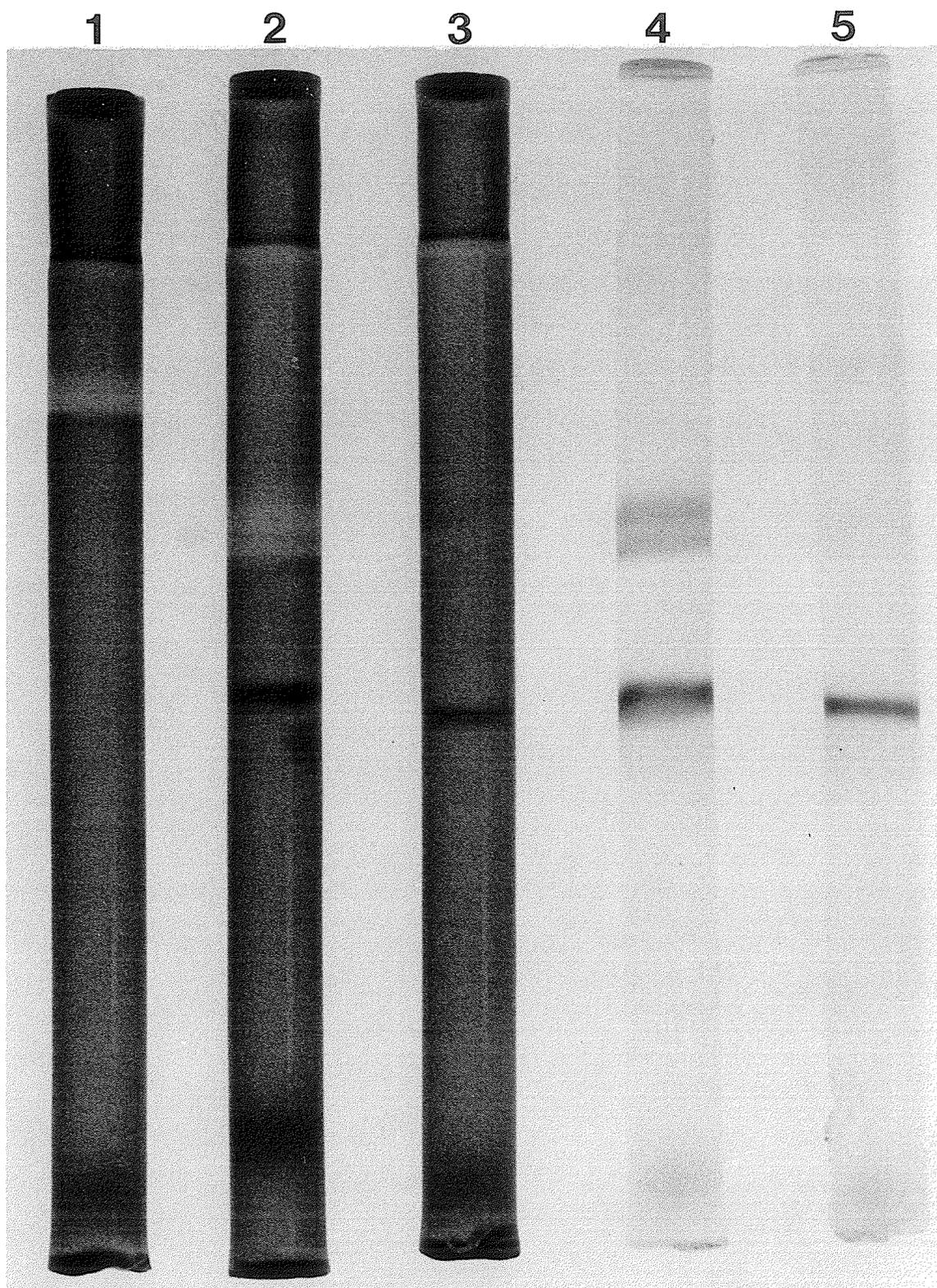


Table 18. Effect of 2.8 mM ascorbic acid on catalase activity on E. coli growing on glucose-minimal salts medium supplemented with 0.1% casamino acids, 20 mM potassium nitrate, and 100 $\mu\text{g}/\text{mL}$ exogenous catalase.

Time (m)	Endogenous Catalase (u/mg dry cell weight)	
	-ascorbate	+ascorbate
-10	4.4	4.4
+ 1	4.8	-
+15	4.8	5.4
+30	5.2	12.8
+60	6.8	16.4

medium, no induction was observed even when 0.3% glucose was added to the nitrate-LB medium. Because a component of the LB medium appeared to prevent catalase induction when nitrate was present, anaerobic glucose-salts-nitrate medium was supplemented with LB medium and the individual components of LB medium, bactotryptone and yeast extract separately and together, and the ascorbate induction of catalase was followed (Table 20). Only when the two components of LB medium were mixed was the induction prevented.

Table 19. Effect of potassium nitrate (20mM) on catalase induction.

E. coli was grown anaerobically in LB medium in the presence of 20 mM nitrate without (A) or with 16.7 mM glucose (B).

A. Time (m)	+ NO ₃ ⁻ - ascorbate	+ NO ₃ ⁻ + ascorbate	- NO ₃ ⁻ - ascorbate
- 10	4.2	3.8	4.0
+ 1	4.0	2.9	4.0
+ 5	4.0	2.6	3.8
+ 10	4.0	2.4	4.0
+ 20	3.8	2.2	3.8
+ 30	4.0	2.8	4.0
+ 60	4.4	4.4	4.4
+120	6.2	6.6	8.6
B. - 10	4.0	4.0	4.0
+ 1	4.0	1.4	4.2
+ 5	3.6	1.6	4.6
+ 10	4.0	1.6	4.4
+ 20	3.8	2.2	3.8
+ 30	3.8	2.0	4.0
+ 60	3.4	1.2	4.2
+120	2.4	1.0	4.8

Induction was at time 0, by 2.8 mM ascorbate.

Figure 48. Visualization of catalase and peroxidase activities in extracts of E. coli grown anaerobically in LB medium in the presence of 20 mM potassium nitrate with and without ascorbate added.

Gel 1: 20 μ g (40 units) bovine catalase. Gels 2 and 4: 200 μ g protein from a culture treated with ascorbate. Gels 3 and 5: 200 μ g protein from a culture not treated with ascorbate. Polyacrylamide (12%) gels 1-3 were stained for catalase and gels 4 and 5 were stained for peroxidase activity.

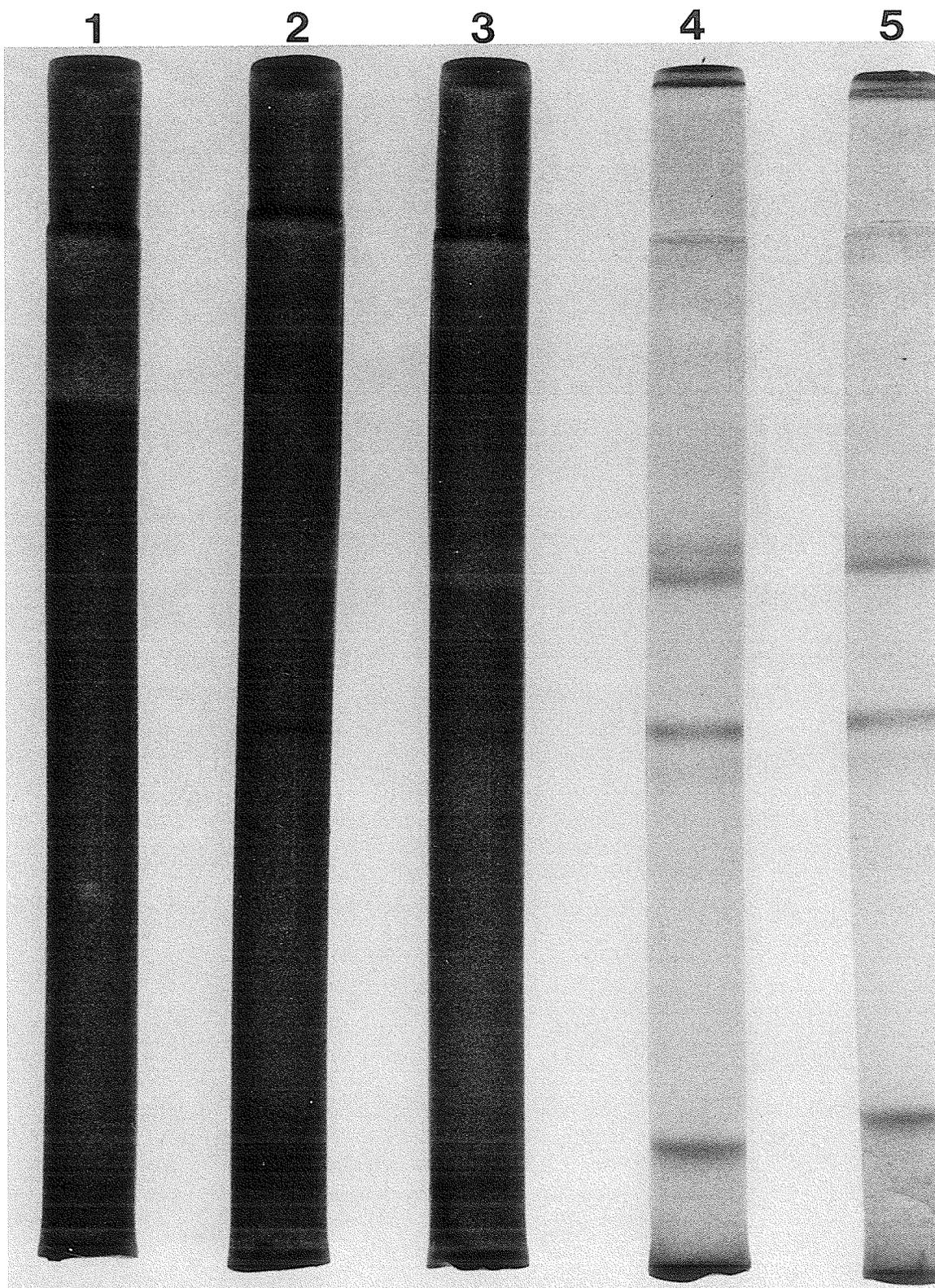


Table 20. Effect of various media components on the ascorbic acid induction of catalase in *E. coli* growing anaerobically in glucose-salts medium supplemented with 20 mM potassium nitrate and 0.1% casamino acids.

Time (m)	Catalase Activity (units/mg dry cell weight)				
	A	B	C	D	E
-10	4.4	4.0	4.2	4.0	4.0
+ 1	4.6	-	4.0	3.0	4.0
+ 5	4.8	2.0	4.4	2.8	4.0
+10	4.6	3.0	4.0	3.6	4.2
+20	4.8	3.8	5.0	4.4	4.4
+30	5.2	9.4	10.8	12.2	5.2
+60	6.8	11.4	12.6	11.8	6.6

At time 0, 2.8 mM ascorbate was added to cultures containing: B no supplements; C 0.5% yeast extract; D 1% tryptone; and E 0.5% yeast extract and 1% tryptone. Ascorbic acid was not added to culture A containing no supplements. Aliquots were removed and assayed for catalase.

4.7.3 Catalase Induction in Anaerobic Glycerol-Fumarate-Salts Medium

Anaerobic electron transport and oxidative phosphorylation can also occur in cells growing on glycerol and fumarate. The effect of ascorbic acid on catalase levels in such cells was investigated and it was found that no increase in catalase occurred in response to ascorbic acid (Table 21). Supplementing the medium with either casamino acids to facilitate protein synthesis (Table 21 B) or nitrate (Table 21 C) did not result in any increase in catalase synthesis. The lack of catalase induction in anaerobic LB and glycerol-fumarate media with or without nitrate suggested a similarity in metabolism between the two media. For example, growth in anaerobic LB medium could involve a process similar to that in glycerol-fumarate medium with aspartate being deaminated as a source of fumarate.

Table 21. Effect of ascorbate on *E. coli* growing anaerobically in glycerol - fumarate-salts medium.

A. Time (m)	Catalase Activity (units/mg dry cell weight)	
	- ascorbate	+ ascorbate
- 10	4.2	4.0
+ 1	4.2	-
+ 5	4.2	-
+ 10	4.0	-
+ 20	4.2	2.0
+ 30	4.0	1.8
+ 60	3.0	2.4
+120	2.6	3.8
B. - 10	3.8	3.4
+ 1	4.0	2.8
+ 5	3.6	3.0
+ 10	3.8	3.0
+ 20	3.4	3.4
+ 30	4.0	3.8
+ 60	2.6	3.8
+120	2.2	4.0
C. - 10	5.0	5.2
+ 1	4.6	2.0
+ 5	4.6	3.2
+ 10	4.4	3.8
+ 20	4.0	4.4
+ 30	4.0	4.6
+ 60	4.6	6.0
+120	6.4	6.6

Ascorbic acid (2.8 mM) was added at time 0 to cultures growing anaerobically in minimal salts medium containing 20 mM glycerol and 20 mM fumarate as well as either 0.1% casamino acids (B) or 20 mM nitrate (C).

4.8 EFFECT OF ASCORBATE ON CELLULAR RESPIRATION

The presence of ascorbate in anaerobic LB medium (Figure 41), anaerobic glycerol-fumarate medium (Figure 42 A) and anaerobic glycerol-aspartate medium (Figure 42 B) resulted in supplementary growth of the cells in approximate proportion to the amount of ascorbate present. Because ascorbate was not transported into the cell (see below) it was concluded that ascorbate was having its effect by in some way donating electrons to a component of the membrane which could then be used for further cellular metabolism either by stimulating respiration or by causing the formation of a metabolizable substrate to further growth.

Electron flow in E. coli normally occurs via cytochrome o oxidase but the enzyme has a high affinity for cyanide and is inhibited by low concentrations of cyanide allowing cytochrome d oxidase to become the main pathway. This suggested a simple system for investigating the effect of ascorbate on two different pathways of electron flow. Initial studies were conducted by growing E. coli B23 in the presence of a range of cyanide concentrations causing a progressively longer generation time:

Concentration KCN (mM)	Generation Time (h)
0	0.3
0.001	0.3
0.01	0.3
0.1	0.5
1.0	22
2.0	26

The effects of ascorbate on cells growing aerobically in the presence of 1 mM and 0.5 mM cyanide was investigated and a significant increase in the rate of growth of cells in LB, succinate-salts, and glycerol-salts media, but not in glucose-salts medium was observed (Figures 49, 50, 51, 52 respectively), which was proportional to the amount of ascorbate added (Figure 49). The final cell density was the same regardless of the ascorbate concentration indicating that it was the respiration rate alone which was affected and that supplementary growth was not being promoted as had occurred in anaerobic LB medium.

The rates of respiration were studied with whole cells that had been grown in LB and minimal salts media with or without ascorbate treatment prior to isolation. As can be seen from Table 22, the presence of EDTA did not affect oxygen uptake by the cells but it did prevent the fast oxidation of ascorbate under the assay conditions. Cyanide also prevented the fast oxidation of ascorbate but did have a significant effect on the respiration rate. Those cells that had been pretreated with ascorbate in glucose-salts medium and glycerol-salts medium had an approximately 50% lower rate of respiration than the untreated cells, whereas cells that had been pretreated with ascorbate in succinate-salts medium and LB medium had a respiration rate unchanged from the untreated cells. In all cases, cyanide had a large effect on respiration although those cells that had been pretreated with ascorbate were less sensitive. Addition of ascorbate after cyanide addition resulted in a stimulation of respiration in all cases except for those cells growing in succinate-salts medium and those cells pretreated with ascorbate in glucose-salts and succinate-salts media. The greatest amount of stimulation occurred

Figure 49. The effect of ascorbic acid on cyanide resistant growth of E. coli B23 in LB medium.

At the specified optical density no ascorbic acid (□), 0.57 mM ascorbic acid (△), and 5.7 mM ascorbic acid (○) were added and growth was followed. The concentration of cyanide was 1 mM.

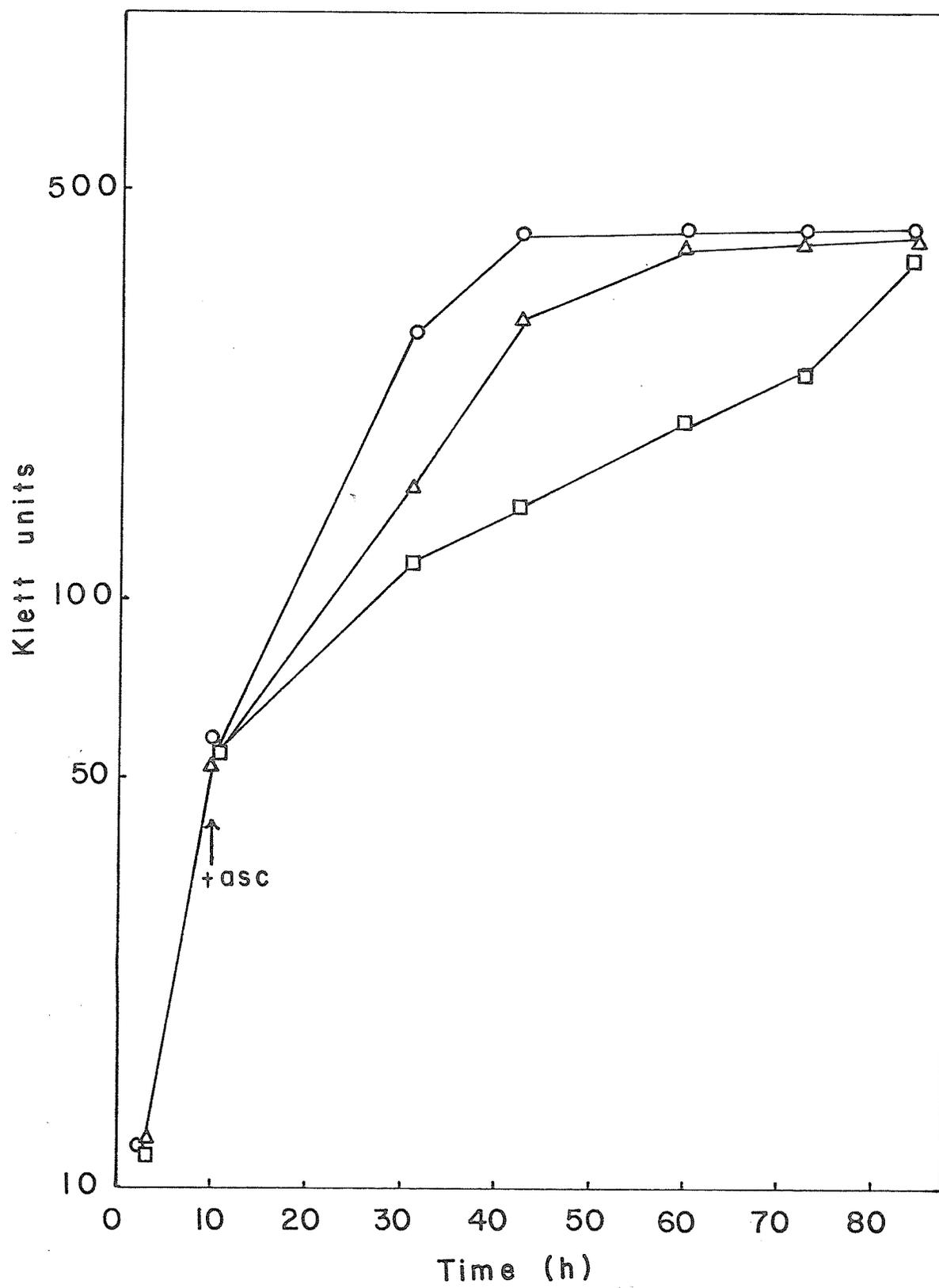


Figure 50. Effect of ascorbic acid on E. coli B23 growing in succinate-salts medium supplemented with 0.1% casamino acids and 0.5 mM potassium cyanide.

At the indicated cell density 2.8 mM ascorbic acid was either not added (O) or added (●) to the culture and growth was followed.

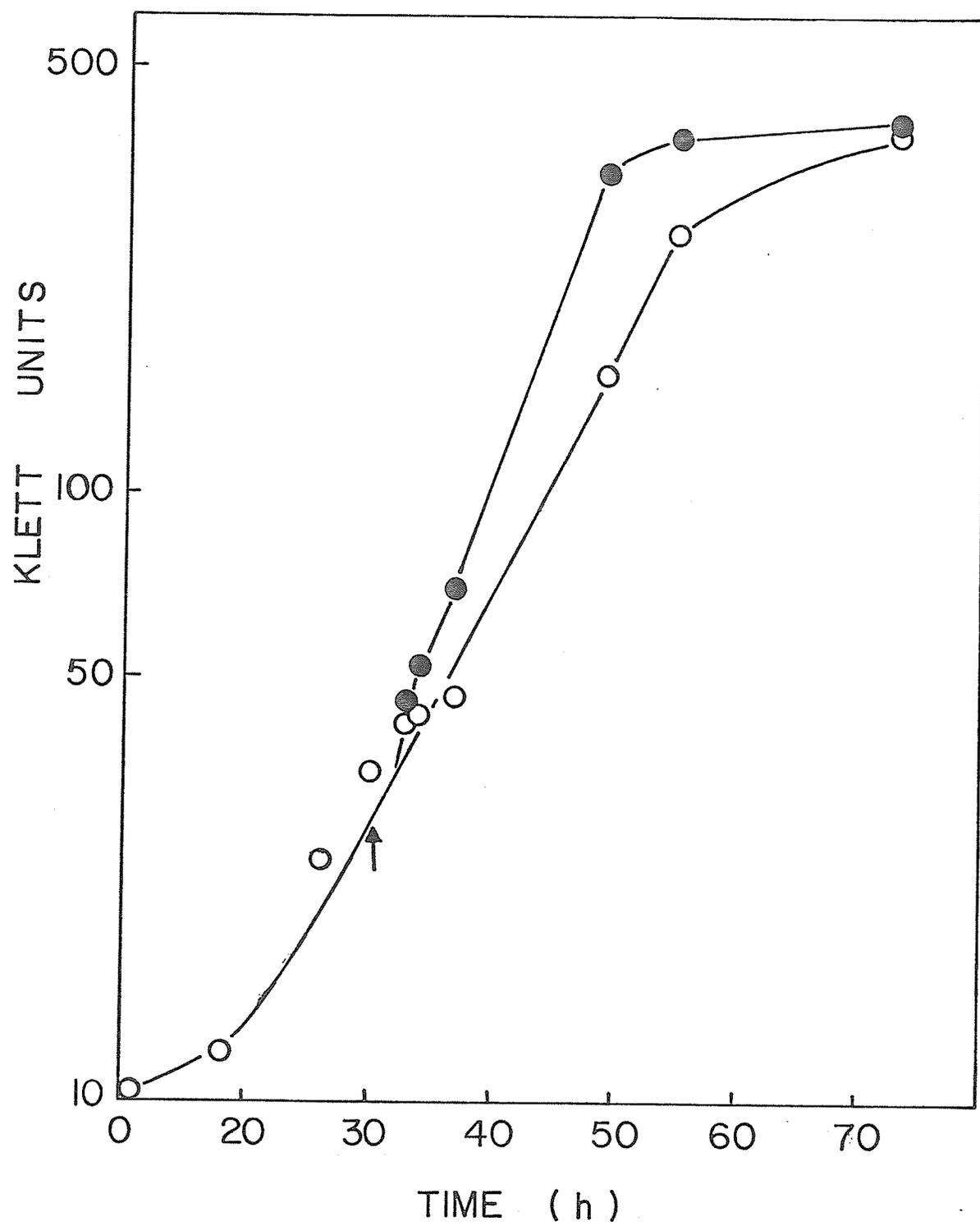


Figure 51. Effect of ascorbic acid on E. coli B23 growing in glycerol-salts medium supplemented with 0.5 mM cyanide.

At the indicated time, 2.8 mM ascorbic acid was either not added (O) or added (●) to the culture and growth was followed.

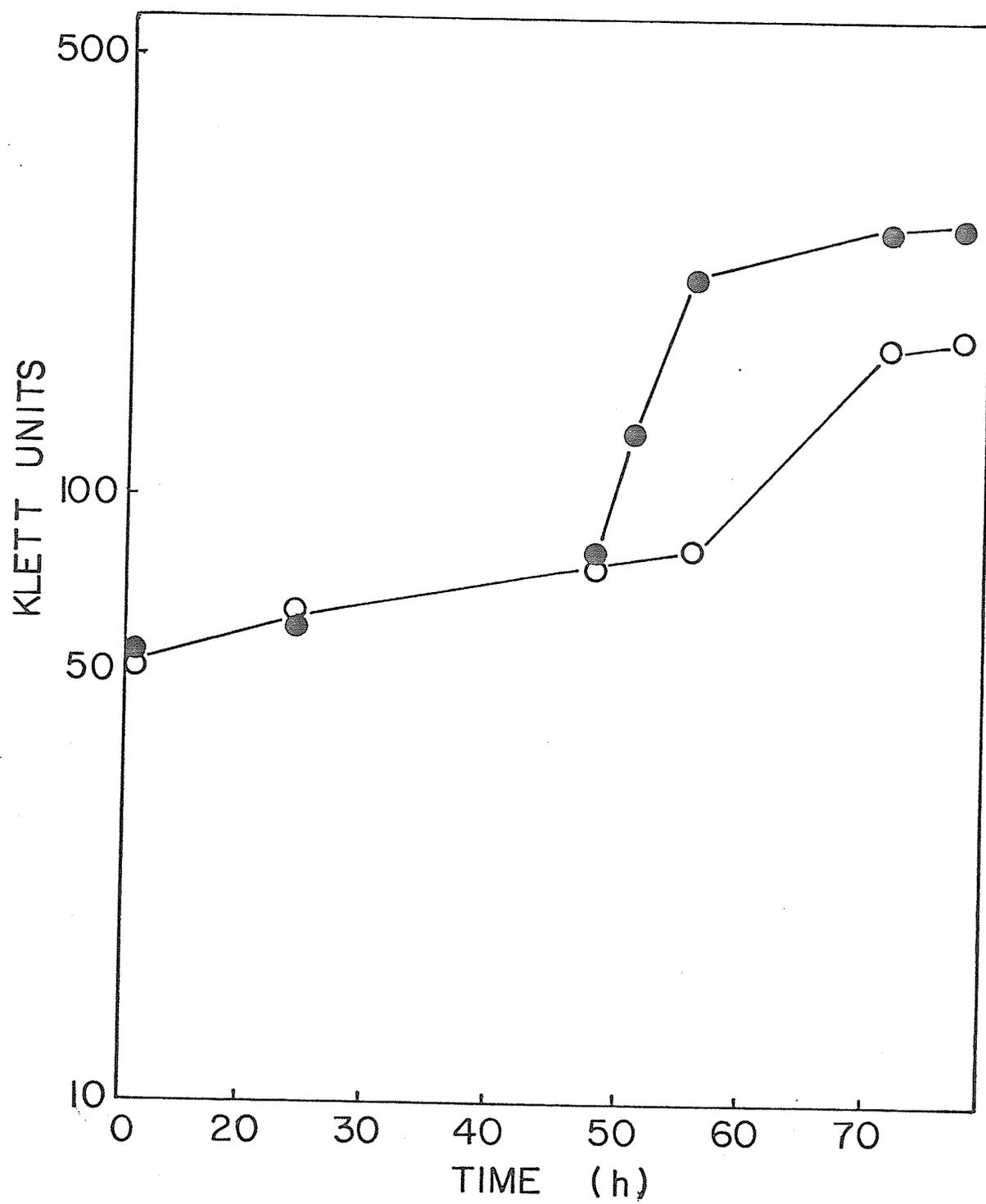
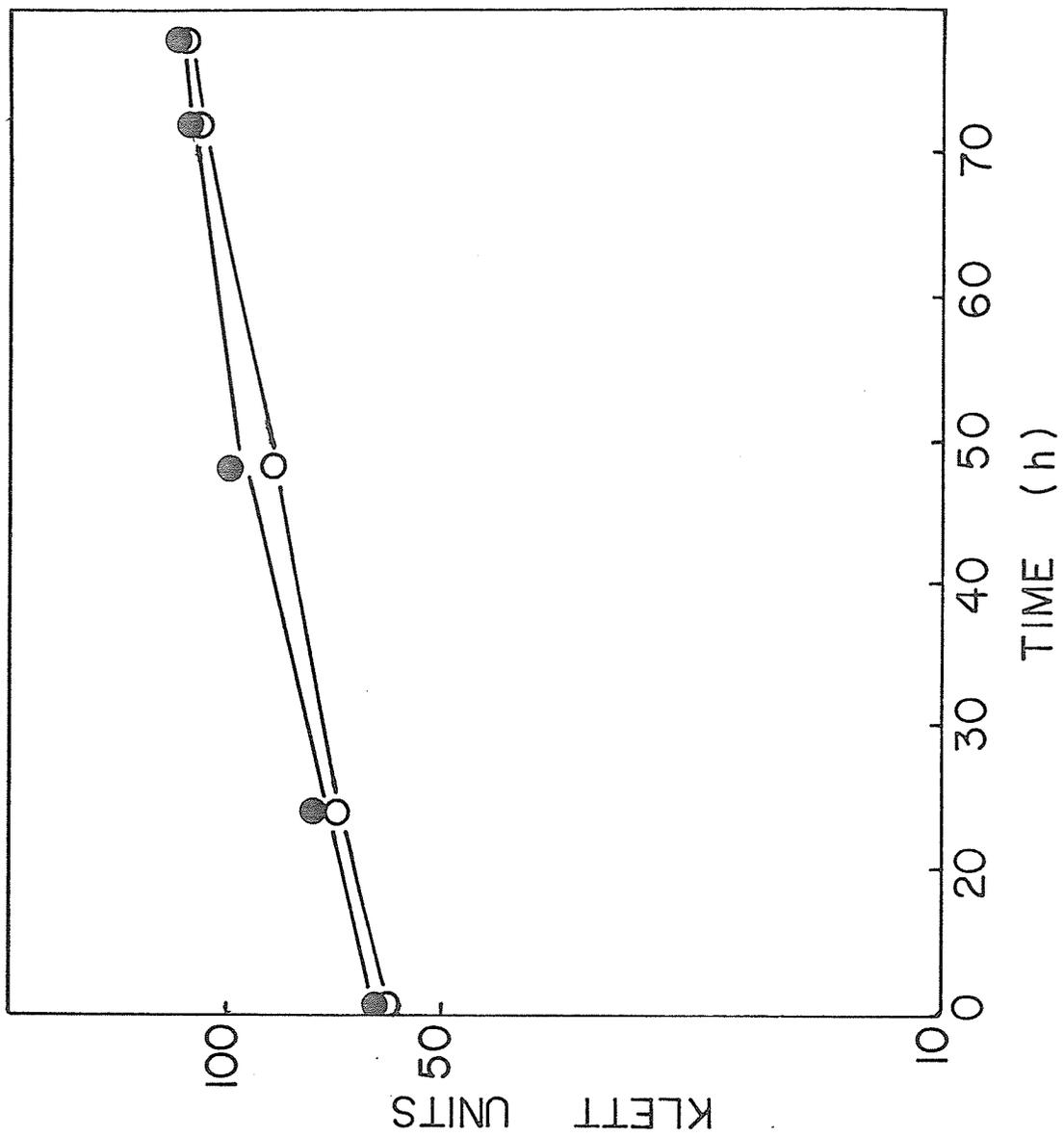


Figure 52. Effect of ascorbic acid on E. coli B23 growing in glucose-salts medium supplemented with 0.5 mM cyanide.

At the indicated time 2.8 mM ascorbic acid was either not added (○) or added (●) to the culture and growth was followed.



in LB grown cells not pretreated with ascorbate, where the rate of respiration increased 45 times. With the exception of the succinate-salts cultures, the respiration of E. coli in the presence of cyanide was stimulated by ascorbate in these whole cell respiration studies.

Ascorbate was also added to cells in the presence of EDTA which would parallel normal respiration conditions except that EDTA would prevent ascorbate oxidation in the medium. In all cases except LB grown cells there was an inhibition of respiration. Curiously, ascorbic acid and EDTA stimulated respiration in all cells growing in LB medium. The presence of metal chelators such as histidine probably slow down the oxidation rate of ascorbate as compared to the other media, resulting in ascorbate stimulation of respiration by electron donation.

The respiration rates in cells isolated from minimal salts media supplemented with casamino acids (Table 23) were qualitatively the same as those in Table 22 using cells from unsupplemented media, except that ascorbate stimulated cyanide resistant respiration only in glucose grown cells. If the casamino acid mixture was omitted from the assay mixture, the respiration rates were the same in succinate grown cells but lower in glucose-grown cells indicating an adaptation to the amino acid supplement.

The effect of H_2O_2 on cellular respiration of E. coli grown in glucose salts media was also investigated. Cells pretreated with H_2O_2 had the same basal rate of respiration as those not pretreated, approximately 180 nmoles O_2 /min mg dry cell weight. The inhibitory effect of ascorbate on the respiration rate in the presence of EDTA was the same

Table 22. Effect of ascorbic acid on respiration in E. coli B23 grown in various media.

Medium	± EDTA	Rate of Oxygen Uptake (nmol/min·mg)			
		+ NaCN	NaCN +asc	EDTA +asc (1)	EDTA +asc (2)
glc	207.5	12.5	41.3	158.2	104.1
glc + asc	100.3	50.4	50.4	5.38	32.9
gly	363.1	13.0	33.5	220.3	162.8
gly + asc	139.7	40.7	83.8	50.2	50.2
succ	211.5	5.4	5.4	195.3	156.3
succ + asc	205.1	8.7	8.9	173.6	141.1
LB	997.2	2.1	91.1	1236.9	1236.9
LB + asc	1006.9	3.2	71.2	1758.7	1709.4

E. coli was grown to a Klett reading of 70 and, where specified, ascorbic acid was added to the cultures after which they were allowed to grow a further 15 minutes. The cells were then centrifuged, washed 2 times in SM buffer and resuspended in 5 mL of minimal-salts medium supplemented with various carbon sources corresponding to the original growth medium. Respiration assays were carried out as described in Methods. The respiration units are nmol O₂/min mg dry cell weight.

The final concentration of EDTA and NaCN were 1.25 mM. The concentration of ascorbic acid was 2.75 mM after addition (1) and 5.5 mM after addition (2).

Table 23. Effect of ascorbic acid on respiration in *E. coli* growth in various casamino acid supplemented media. The cells were grown up and assayed as described in Table 22.

Medium	Rate of Oxygen Uptake (nmol/min·mg)				
	± EDTA	+ NaCN	NaCN +asc	EDTA +asc (1)	EDTA +asc (2)
glc	209.5	7.5	31.7	193.7	177.8
glc + asc	165.8	6.3	31.7	136.5	146.0
gly	372.5	8.8	8.8	286.6	273.3
gly + asc	244.7	6.6	6.6	209.4	180.8
succ	267.4	2.1	2.1	242.3	196.3
succ + asc	229.7	2.1	2.1	210.9	196.3

The final concentrations of EDTA and NaCN were 1.25 mM. The concentration of ascorbic acid was 2.75 mM after addition (1) and 5.5 mM after addition (2). Respiration units are nmol O₂/min mg dry cell weight.

(approximately 30%) whether the cells had been pretreated with H_2O_2 or not. It was concluded that unlike ascorbate, H_2O_2 had no effect on cellular respiration.

4.9 EFFECT OF ASCORBATE ON SUGAR UPTAKE

The extent of the inhibition of respiration by ascorbate was similar in all three minimal media and yet cells growing in glucose-salts medium were much more sensitive to ascorbate than cells growing in either glycerol-salts or succinate-salts media. Some aspect of cellular metabolism unique to glucose cultures was affected by ascorbate with drastic consequences to the cell. As compiled in Table 24, ascorbic acid reduced the rate of [^{14}C] glucose uptake by 50%, but had no effect on the rate of [3H] glycerol uptake (Table 25). Hydrogen peroxide, a product of ascorbate oxidation, did not affect the rate of glucose uptake (Table 26) indicating that it was the ascorbate itself that was affecting glucose uptake or some other aspect of glucose metabolism which was reflected in apparent inhibition of uptake. Ascorbate itself was not taken up by the cell over a concentration range from 10 μM to 1 mM (Table 27). Therefore, the striking effect of ascorbate on the growth of cells in glucose-salts medium may be attributed to the concerted action of ascorbate in inhibiting both respiration and some aspect of glucose metabolism, possibly glucose transport.

Since different transport mechanisms are involved in the transport of glucose and glycerol there appeared to be some specificity as to the type of transport process affected by ascorbic acid. Because some other

Table 24. Effect of ascorbate on [^{14}C] glucose uptake.

Condition	Rate of [^{14}C] glucose ¹ uptake (pmol/min mg dry cell weight)
control	350 \pm 24
ascorbate ² added at same time as glucose	176 \pm 21
ascorbate added at 30 seconds after glucose	295 \pm 21
cell suspension incubated 5 minutes with ascorbate before addition of ^{14}C glu	172 \pm 15

¹Final concentration of glucose was 50 μM .

²Final concentration of ascorbate was 5.7 mM.

Experiments performed as described in Methods.

Table 25. Effect of ascorbate on [³H] glycerol uptake.

Condition	Rate of [³ H] glycerol ¹ uptake (pmol/min mg dry cell weight)
control	1.50 ± .13
ascorbate ² added at same time as glycerol	1.42 ± .10
ascorbate ² added 15 seconds after glycerol	1.42 ± .11

¹Final concentration of glycerol was 50 μM.

²Final concentration of ascorbate was 5.7 mM.

Experiments performed as described in Methods.

Table 26. Effect of H₂O₂ on [¹⁴C] glucose uptake.

Condition	Rate of ¹⁴ C glucose ¹ uptake (pmol/min mg dry cell weight)
control	316 ± 16
0.1 mM H ₂ O ₂ added at same time as glucose	379 ± 21
0.5 mM H ₂ O ₂ added at same time as glucose	339 ± 20
1.6 mM H ₂ O ₂ added at same time as glucose	336 ± 17
0.1 mM H ₂ O ₂ added 15 seconds after glucose	386 ± 22
0.5 mM H ₂ O ₂ added 15 seconds after glucose	387 ± 24

¹Final concentration of glucose, 50 μM.
Experiments performed as described in Methods.

Table 27. [^{14}C] ascorbate uptake in E. coli B.

Ascorbate concentration μM	CPM/mL
Background	12
10	17
50	19
100	20
200	44
300	47
1000	58

[^{14}C] ascorbate, was allowed to incubate with cells for 2 minutes before filtering. No uptake was seen above background.

S.A. of [^{14}C asc] equals 10^6 cpm/ μmol .

sugars of interest were difficult to obtain radioactively labelled, the effect of ascorbate on the growth of cells using these other sugars was used as a crude criteria for whether or not ascorbate affected other transport systems. As shown in Table 28, the cultures using melibiose or lactose had their growth inhibited by ascorbate for the same length of time as experienced by glucose cultures. By contrast cultures growing on galactose, arabinose and maltose were not affected by ascorbate.

While this is not conclusive evidence that sugar transport was being affected in the glucose, melibiose and lactose cultures it may be significant that the transport systems involved are all dependent on a type of membrane energization (lactose and melibiose in the form of an ionic gradient) or deenergization (glucose) (Reider et al., 1979) which could be affected by ascorbate. In fact, Reider et al. have reported that the uptake of α -methyl glucoside was inhibited by ascorbate. Transport systems involving other mechanisms are not affected as much by ascorbate. Further transport studies are required to confirm such a hypothesis.

Table 28. Effect of 2.8 mM ascorbic acid on growth in minimal-salts medium supplemented with various carbon sources.

Supplement	Growth delay (min)
glucose	180
lactose	210
melibiose	>120
galactose	0
arabinose	0
maltose + fructose	0
casamino acids	30

The final concentration of glucose and galactose was 16.7 mM; of lactose, melibiose and maltose was 3.0 mM; of arabinose 15.0 mM; of fructose 0.83 mM; and of casamino acids, 0.1%. The delay in growth is defined as the amount of time taken before active growth resumed as seen in Figure 26.

4.10 ROLE OF CATABOLITE REPRESSION IN CONTROL OF CATALASE GENE EXPRESSION

A link between the synthesis of catalase and the synthesis of components of the respiratory chain in E. coli was suggested and the presence of two electrophoretically distinct catalases, one which was constitutive and the other repressed by glucose or the lack of oxygen was noted by Hassan and Fridovich (1978b). The key role of glucose or catabolite repression in suppressing the synthesis of catalase was noted in yeast (Sulebele and Rege, 1967 and 1968), Bacteroides fragilis (Gregory et al., 1977) and E. coli (Yoshpe-Purer et al., 1977; Hassan and Fridovich, 1978b) where the more rapid turn on of catalase synthesis in the diauxic response in the presence of cAMP was interpreted to implicate catabolite repression in catalase gene expression (Hassan and Fridovich, 1978b).

An additional level of control was suggested when H_2O_2 (Yoshpe-Purer et al., 1977) and H_2O_2 generated from pyocyanine (Hassan and Fridovich, 1980) and ascorbate (see above and Richter and Loewen, 1981) caused an increase in catalase synthesis. Analogous to the IPTG inactivation of a repressor protein to turn on β -galactosidase synthesis, H_2O_2 or a reaction product could have been serving to inactivate a repressor protein which was blocking catalase gene expression. The facile nature of ascorbate or direct H_2O_2 induction of catalase synthesis (see above and Richter and Loewen, 1981) suggested a possible tool to be used in the study of catalase gene expression.

The results already reported above do not support the idea of catabolite repression being involved in catalase gene expression. The basal intracellular levels of catalase during exponential cell growth were the same, approximately 5.0 units per mg dry cell weight in LB medium, glucose-salts medium and glycerol-salts medium with or without a casamino acid supplement. If catabolite repression was involved, the intracellular levels of catalase in the cells growing in glucose minimal medium would be expected to be lower than the level in cells growing on glycerol. The complex composition of LB medium made it difficult to predict what should be expected but the level of glucose, less than 8 μ M, was very low. Only cells growing in succinate-salts medium exhibited a slightly higher basal level, approximately 7.0 units per mg dry cell weight. Furthermore, the fully induced level of catalase was the same, 26.0 units per mg dry cell weight in cells growing in glucose-salts, glycerol-salts and succinate-salts medium. Whether or not glucose was present in the growth medium did not affect either the basal level or fully induced levels of catalase.

The effect of glucose in various media was investigated further. The presence of glucose in LB medium (Figure 53), glycerol-salts medium and succinate-salts medium (Figure 54) had no significant effect on the induction of catalase by H_2O_2 or ascorbate. In LB medium (Figure 55) and succinate-salts medium (Figure 54) whether the glucose was added at inoculation, 10 minutes before addition of H_2O_2 or 2 minutes after the addition of H_2O_2 had no effect on the induction of catalase.

Figure 53. The effect of glucose in LB medium on catalase induction by 0.42 mM H₂O₂ and 5.7 mM ascorbic acid.

The following concentrations of glucose were present:
none (○), 24 mM (●), (■), (□) and 48 mM (△). Ascorbic acid was added at zero time to one culture (■), while 0.42 mM H₂O₂ was added at zero time to cultures (□) and (△). Glucose was added at a Klett reading of approximately 25, while ascorbate and H₂O₂ were added at a Klett reading of approximately 70. Catalase activity is expressed as units per mg dry cell weight.

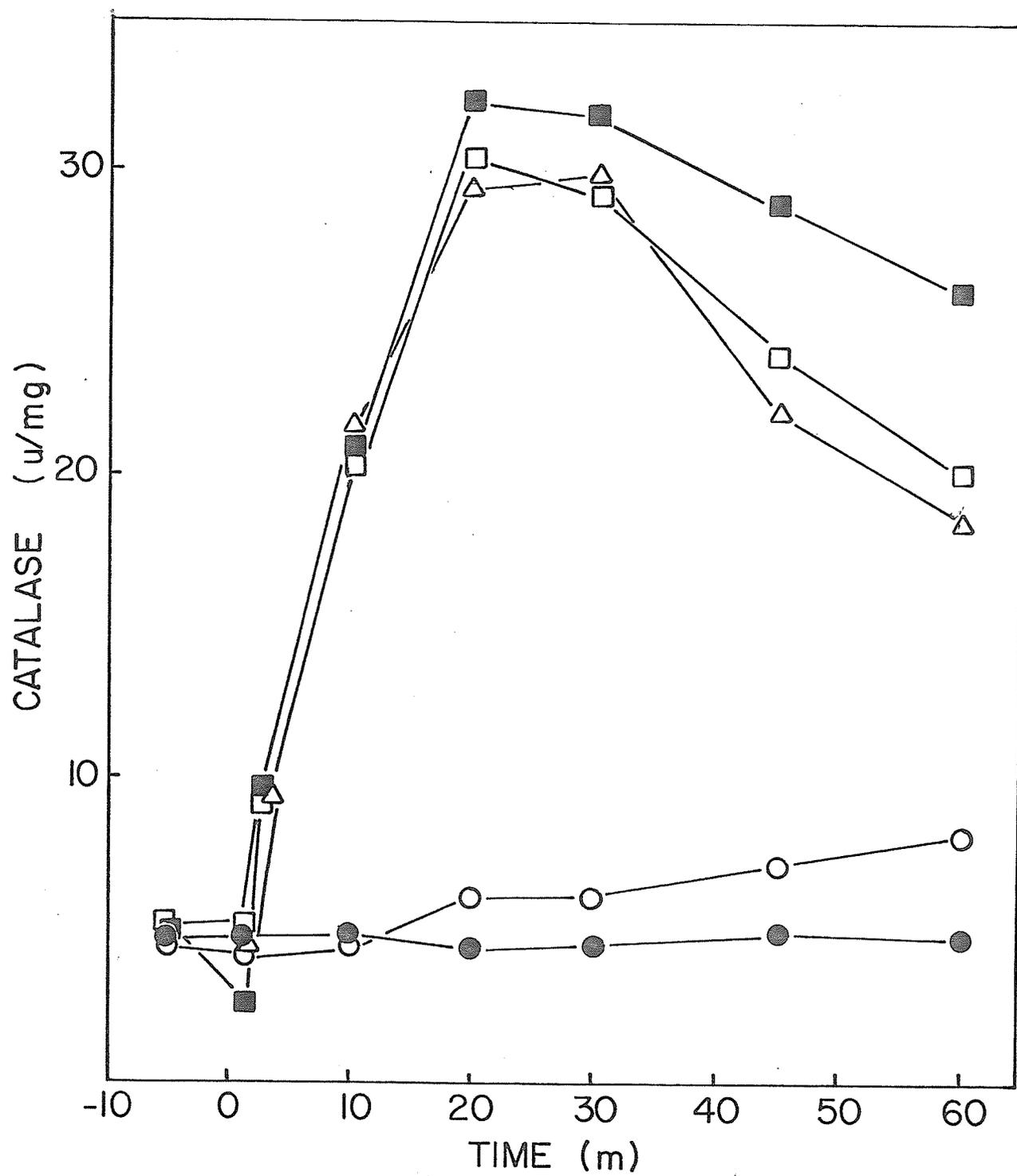


Figure 54. Effect of glucose on the induction of catalase by H_2O_2 in E. coli growing in succinate-salts medium.

Glucose (12 mM), was added at a Klett reading of 25 (\square \blacksquare) and 0.75 mM H_2O_2 was added at zero time (\bullet \blacksquare). No glucose or H_2O_2 was added to the control cultures (\circ \square). Catalase activity is expressed as units per mg dry cell weight.

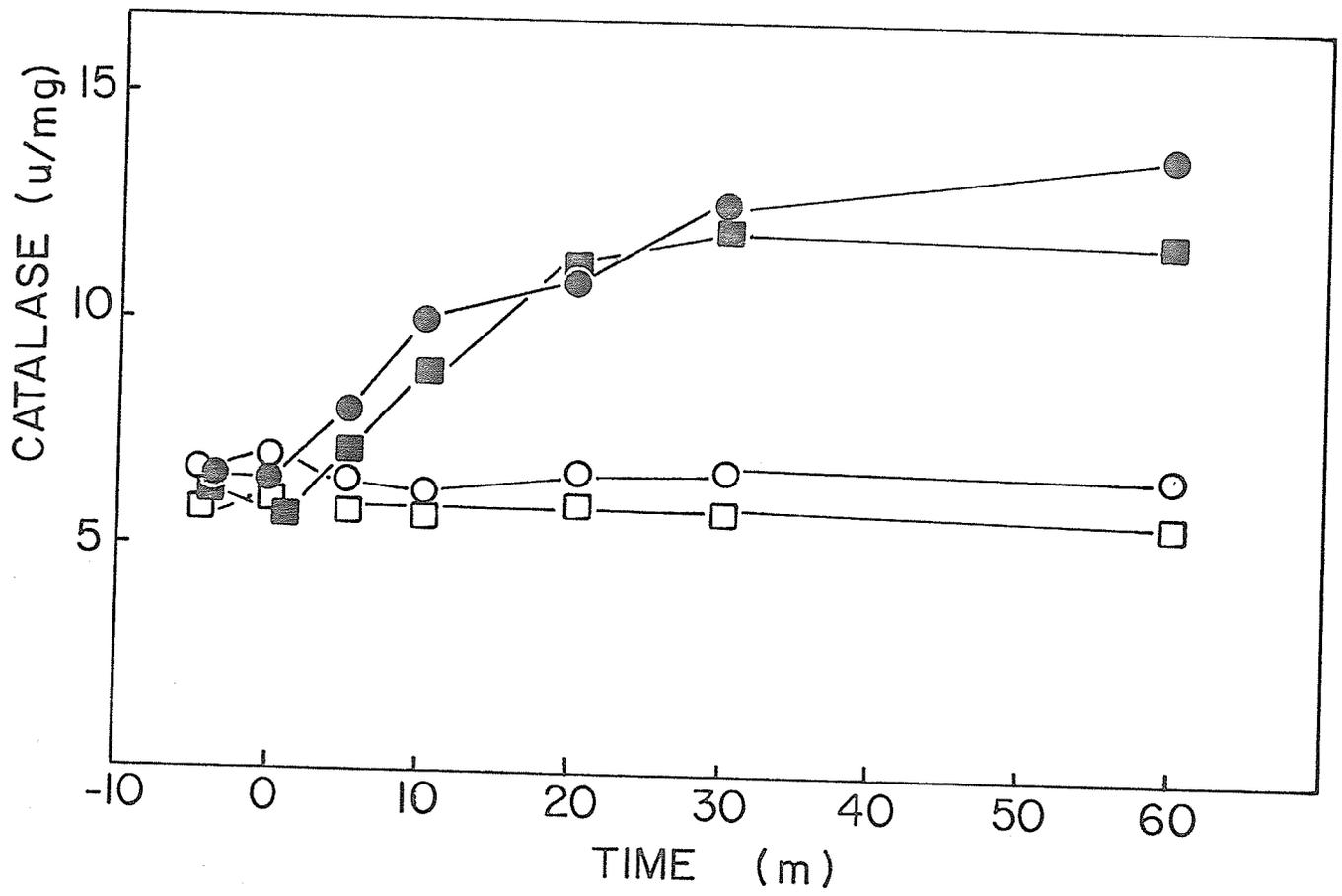
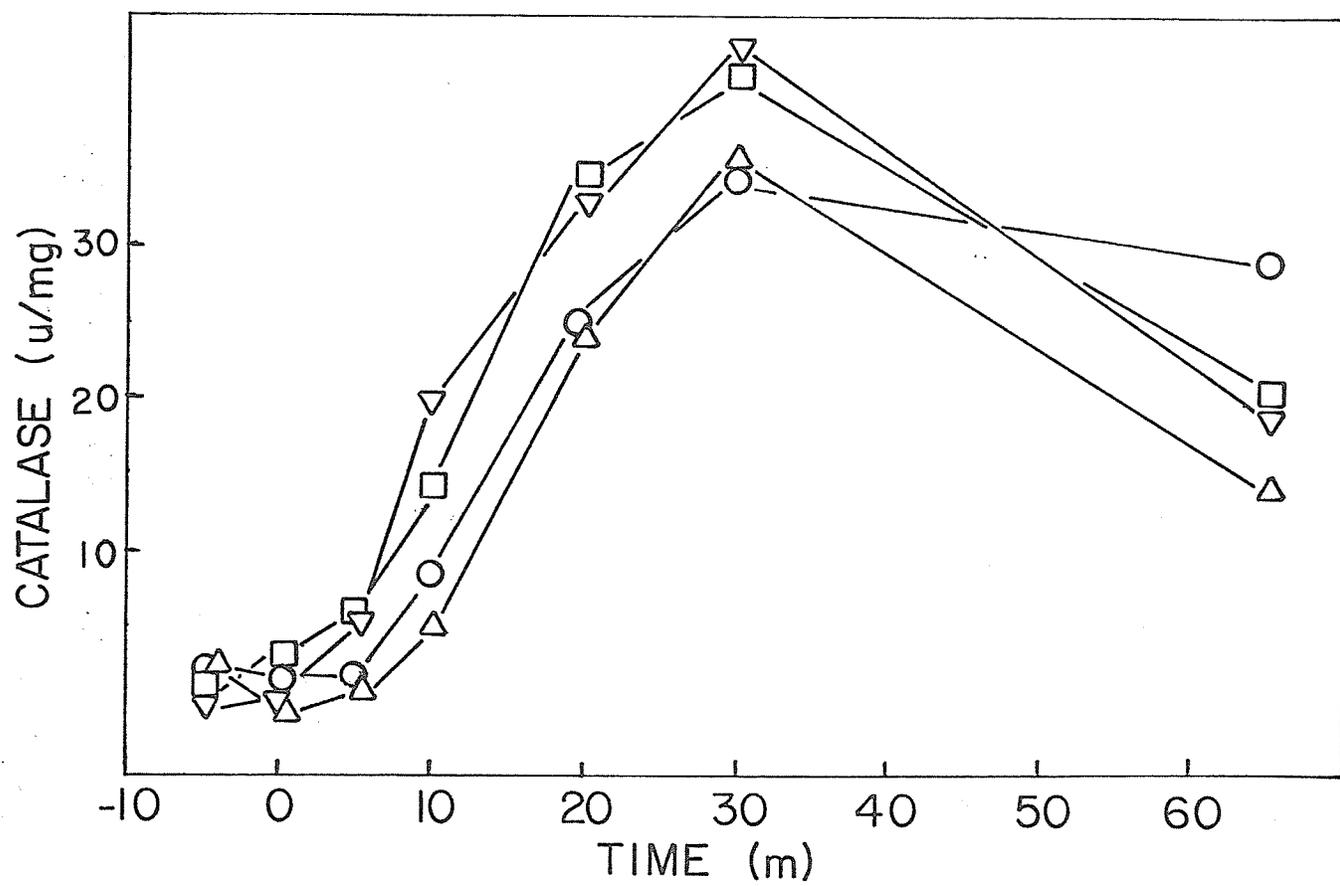


Figure 55. Effect of the time of glucose addition on catalase induction in LB medium.

Glucose (50 mM) was not added (○), added at inoculation (□), added at 10 minutes prior to H₂O₂ induction (Δ) and added 2 minutes after H₂O₂ induction (∇). Catalase was induced at time 0 by 0.62 mM H₂O₂. Catalase activity is expressed as units per mg dry cell weight.



Two other sugars, fructose and glycerol were added to LB medium in order to study whether or not they would affect the level of catalase induction. As shown in Figure 56, neither had any effect.

In order to further investigate the discrepancy between these data and the data in earlier reports in regards to catabolite repression, the role of cAMP in catalase induction was studied directly. As shown in Figures 57, 58 and 59, the addition of cAMP to either LB media or glucose salts medium did not cause any increase in either the basal or fully induced levels of catalase relative to a culture lacking cAMP. Even 5 mM cAMP had no effect on the basal and fully induced levels in glucose containing culture.

In order to confirm that cAMP was indeed effective under these growth conditions, the changes in the levels of β -galactosidase in response to the addition of cAMP was investigated. This response was also looked at in an *E. coli* adenylate cyclase mutant. As shown in Table 29, β -galactosidase was not induced by IPTG added to the adenylate cyclase mutant in either LB medium or glycerol-salts medium in the absence of cAMP. The addition of IPTG to the wild type strain in either medium did induce galactosidase and the inclusion of 1 mM cAMP in the adenylate cyclase mutant cultures promoted β -galactosidase in response to IPTG (Table 29). Therefore the cAMP, added to both rich and minimal media was effective in modulating β -galactosidase synthesis and should have been effective in modulating catalase synthesis if it was involved.

When the induction of catalase in LB cultures of the adenylate cyclase mutant and its wild type parental strain was assayed following as-

Figure 56. Effect of various sugars on catalase induction in LB medium.

At a Klett reading of 35, 12 mM glucose (□), 12 mM fructose (○), 20 mM glycerol (Δ) and nothing (●) was added to LB medium. Hydrogen peroxide (0.62 mM) was added at zero time. Catalase activity is expressed as units per mg dry cell weight.

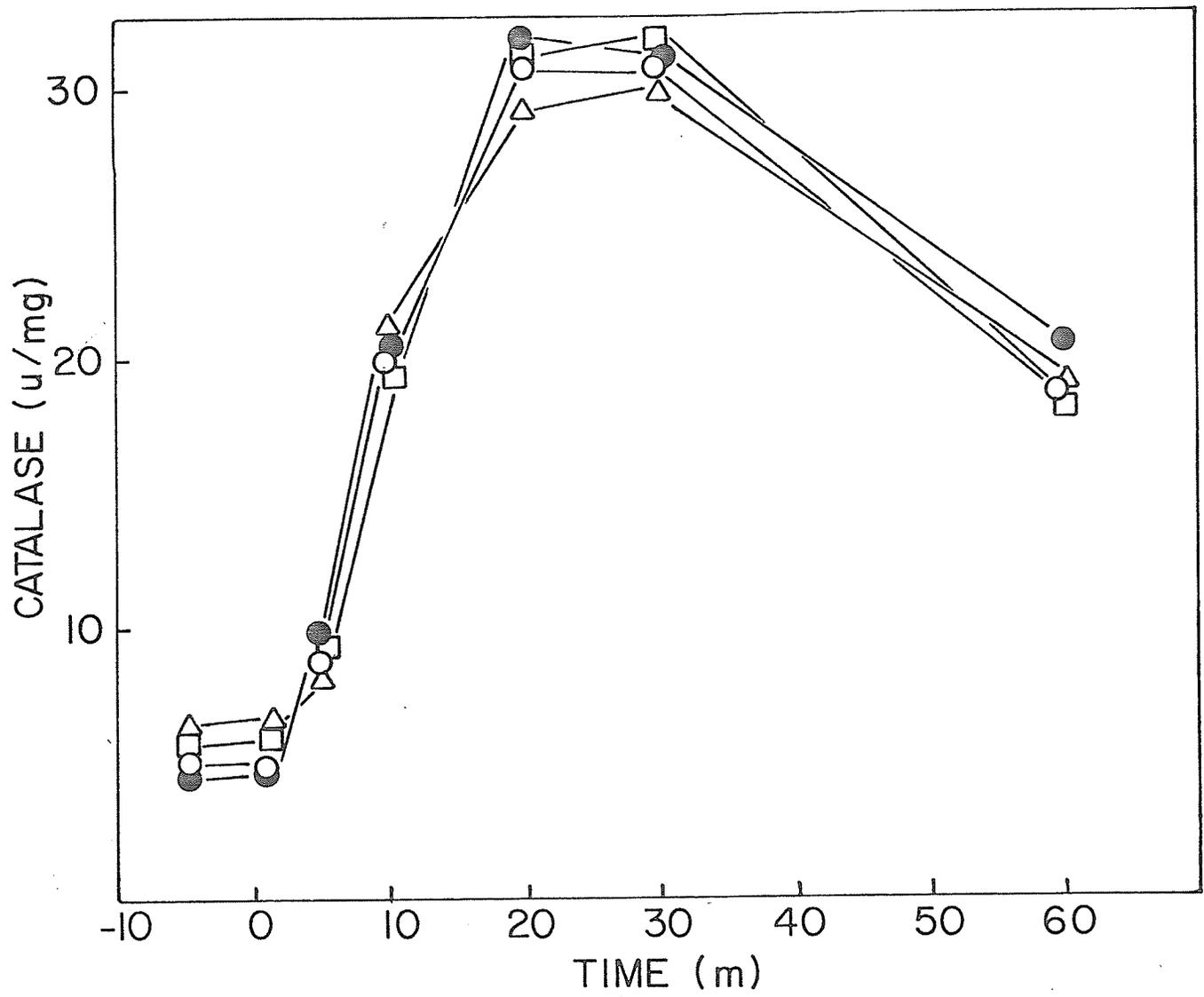


Figure 57. Effect of glucose and cAMP in LB medium on induction of catalase.

Induction was with 5.7 mM ascorbic acid added at zero time. LB medium was supplemented with the following: nothing (○); 4 mM cAMP (□); 24 mM glucose (●); 4 mM cAMP and 24 mM glucose (■). Catalase activity is expressed as units per mg dry cell weight.

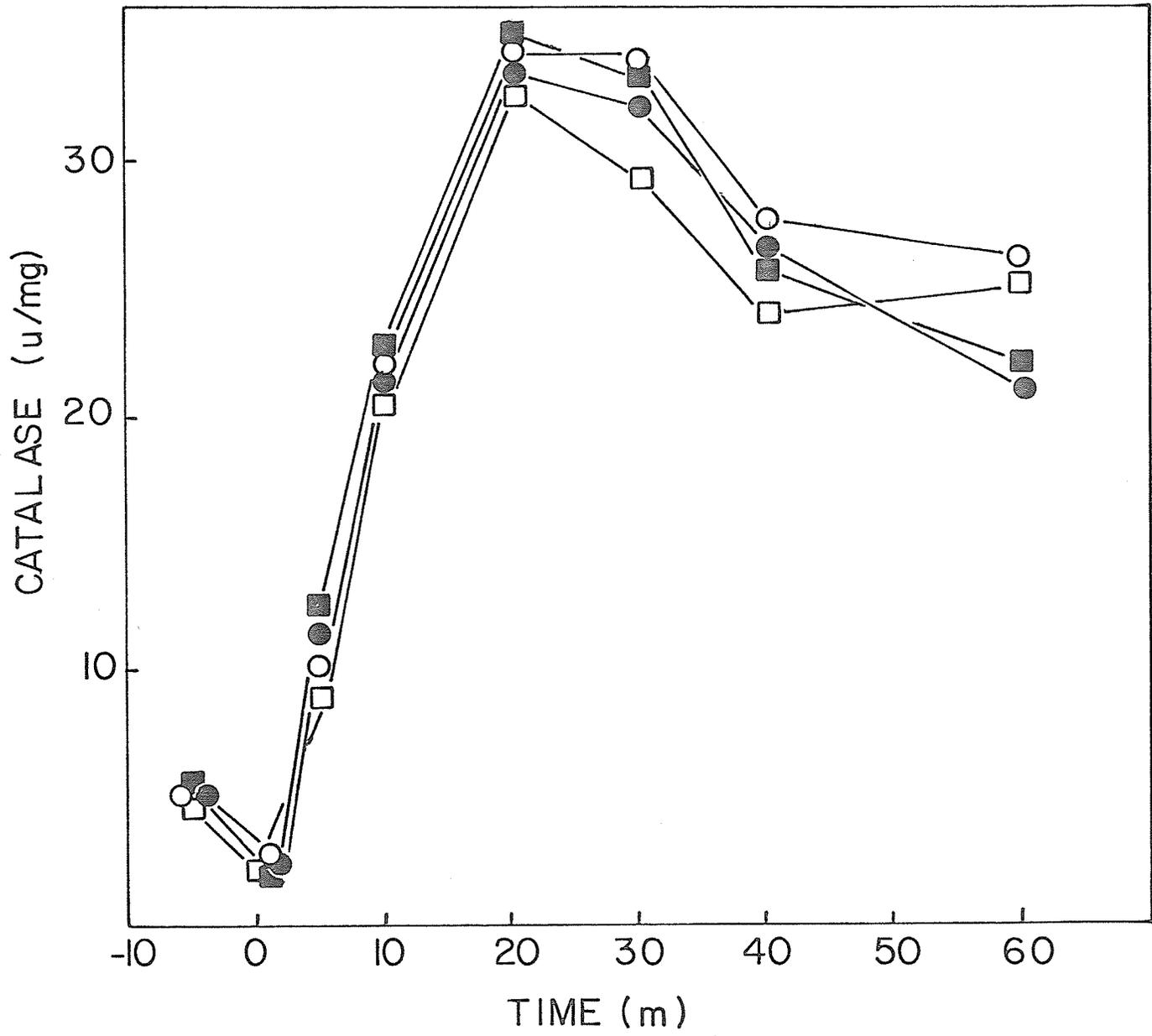


Figure 58. Effect of cAMP in glucose salts medium on catalase synthesis in E. coli B induced by ascorbic acid.

Catalase activity was assayed in aerobic cultures of E. coli B growing in unsupplemented glucose-salts medium without (○) and with (●) 2.8 mM ascorbic acid added at time 0 and growing in glucose-salts medium supplemented with 1 mM cAMP without (□) and with (■) 2.8 mM ascorbic acid added at time 0. No cAMP was present in culture (△) and (▲). Ascorbic acid was added at zero time to (▲). Catalase activity is expressed as units per mg/dry cell weight.

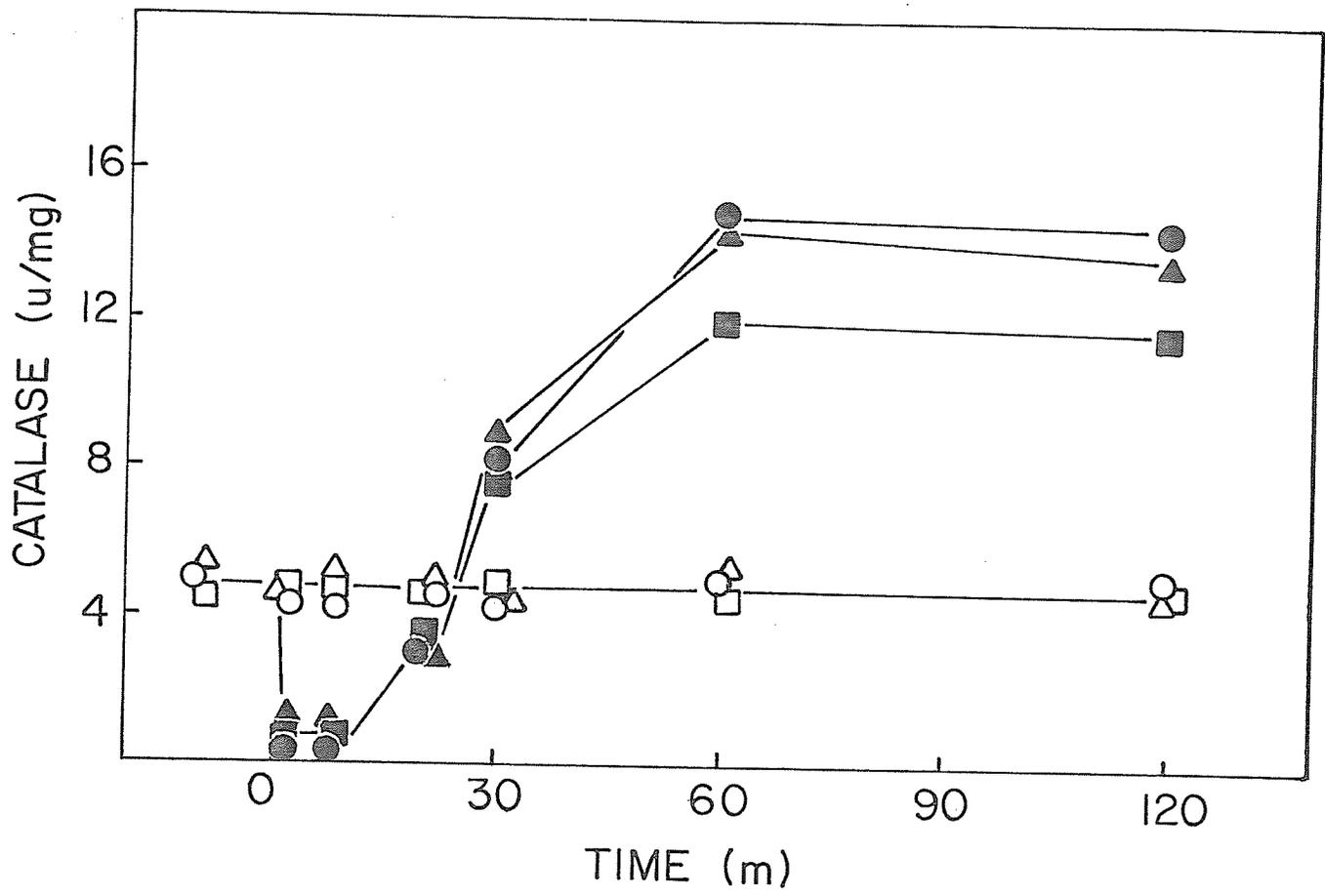


Figure 59. Effect of cAMP in glucose salts medium on catalase synthesis in E. coli induced by H₂O₂.

Glucose-salts medium was supplemented with nothing (○, ●) or 5 mM cAMP (△, ▲) and the cultures were induced with 0.62 mM H₂O₂ (●, ▲). Catalase activity is expressed as units per mg dry cell weight.

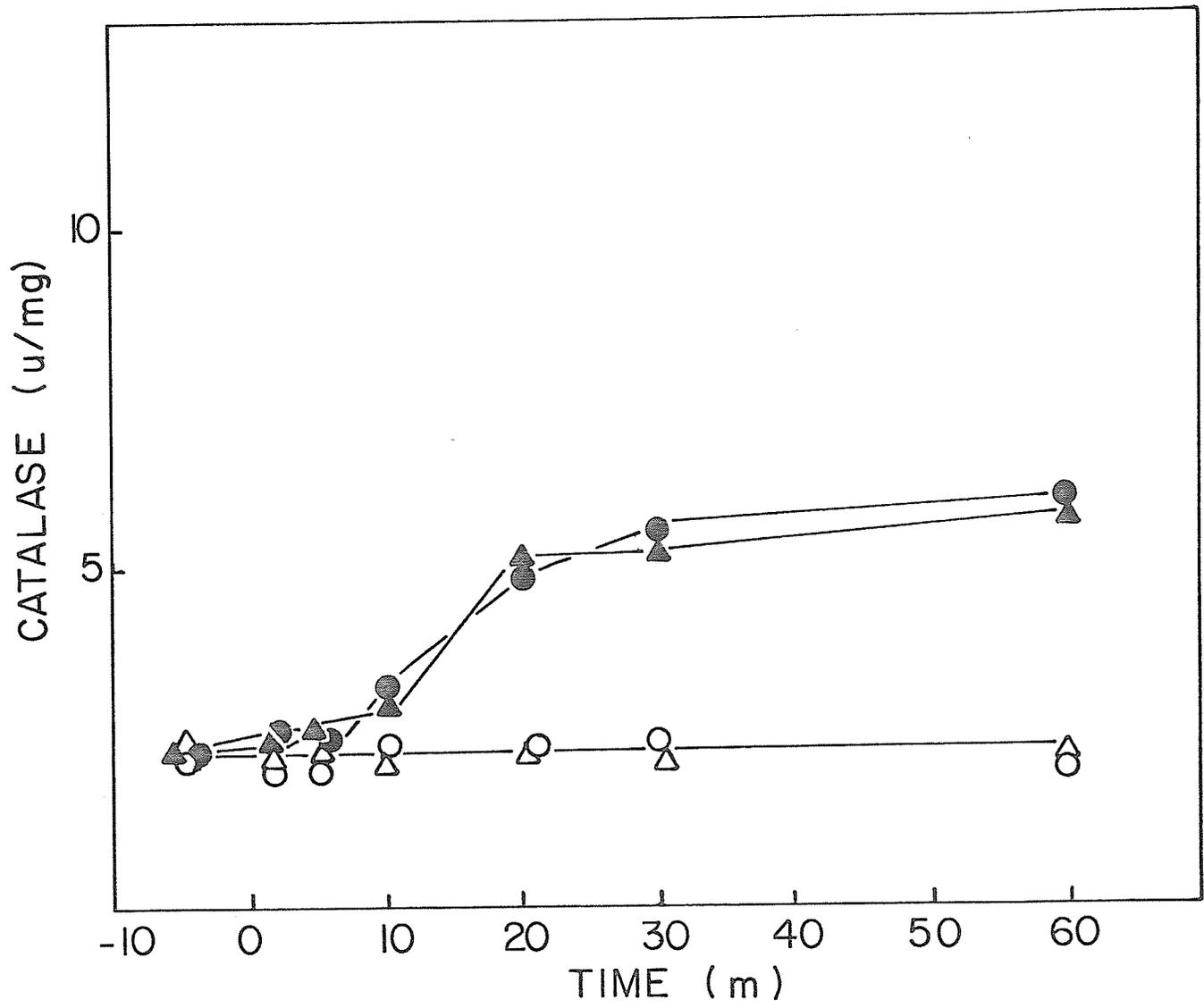


Table 29. Induction of β -galactosidase in E. coli strains MP180 (wild type) and MP 259 (cya) in LB medium and glycerol-salts medium with and without cAMP. The procedure is described in the Methods.

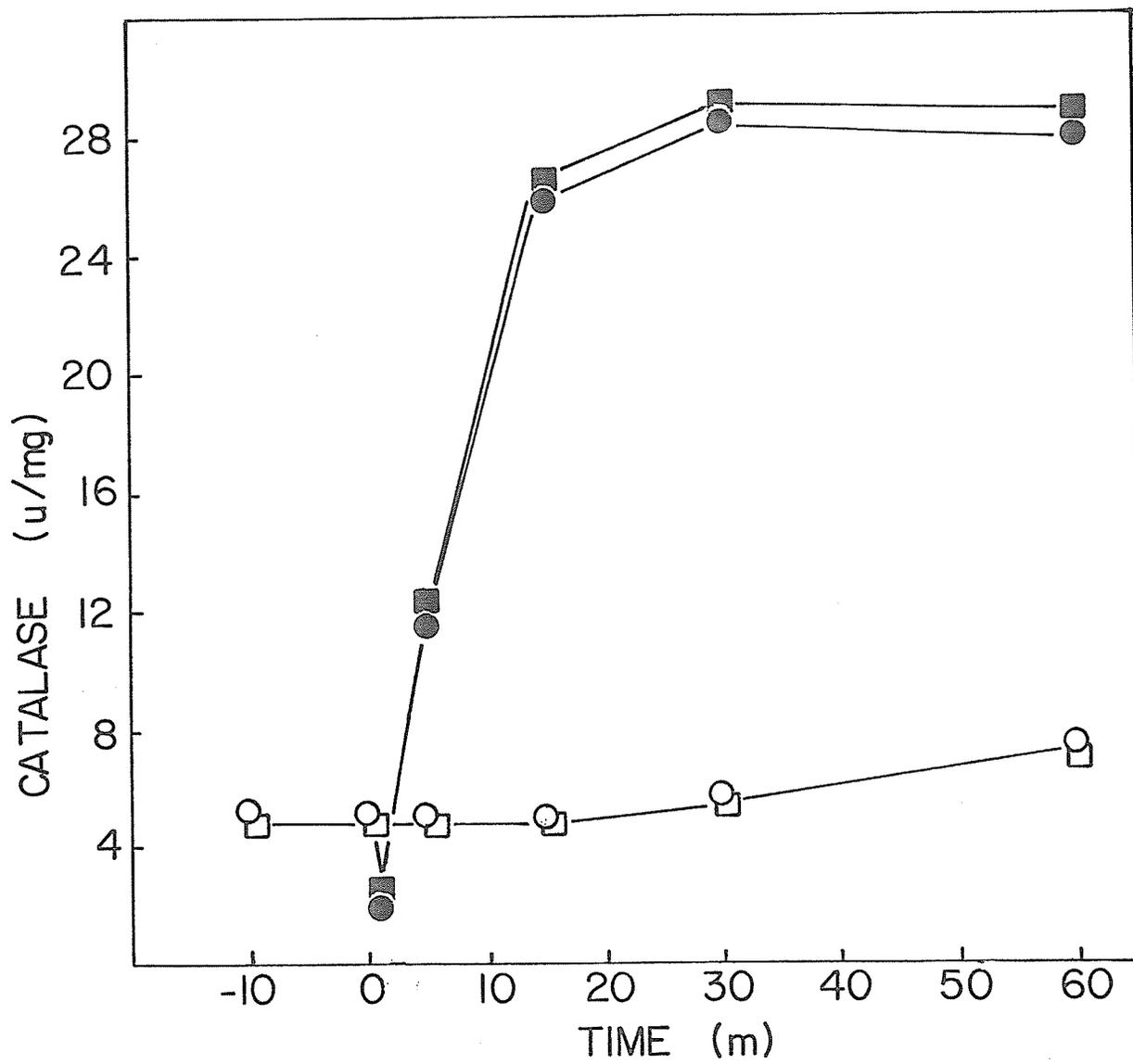
Strain	Medium	Time after IPTG added (min)	β -galactosidase (Units/mg dry cell weight)	
			-cAMP	+cAMP
MP 180	LB	- 2	96	96
		- 1	101	97
		+ 1	216	161
		+ 5	310	262
		+10	580	529
		+20	623	640
		+30	721	709
MP 259	LB	- 2	42	87
		- 1	38	90
		+ 1	38	152
		+ 5	41	247
		+10	44	530
		+20	38	622
		+30	22	696
MP 180	glycerol-salts	- 2	25	33
		- 1	26	36
		+ 1	41	47
		+ 5	153	147
		+10	247	261
		+20	330	344
		+30	393	389
MP 259	glycerol-salts	- 2	15	34
		- 1	16	27
		+ 1	15	44
		+ 5	9	150
		+10	2	252
		+20	11	348
		+30	14	394

corbate addition, (Figure 60) there was virtually no difference in the rate or extent of catalase induction even though there was not sufficient cAMP in LB medium ($<1nM$) to affect catabolite sensitive genes.

From the above results it is apparent that the induction of catalase was not dependent on cAMP nor was it promoted by cAMP. This should lead to the conclusion that cAMP and, therefore, classical catabolite repression involving the cAMP-CAP complex is not a factor in catalase gene expression.

Figure 60. Catalase induction in an adenylate cyclase mutant.

Catalase activity was assayed in E. coli MP 180 (K12 wild type) in LB medium without (○) and with (●) 2.8 mM ascorbic acid added at time 0. Catalase activity was also assayed in E. coli MP 259 (K12, cya) in LB medium without (□) and with (■) 2.8 mM ascorbic acid added at time 0. Catalase activity is expressed as units per mg dry cell weight.



4.11 EFFECT OF ASCORBIC ACID ON SUPEROXIDE DISMUTASE LEVELS IN E. COLI

The activity of superoxide dismutase assayed following the addition of 5.7 mM ascorbic acid to aerated cultures of E. coli did not significantly change (Table 30). Since superoxide dismutase levels in the cell respond to the presence of superoxide anion (Hassan and Fridovich, 1977d), the lack of response after ascorbic acid addition suggested that superoxide anion was not formed during the oxidation of ascorbic acid which would agree with the conclusion of Halliwell and Foyer (1976). However, because superoxide anion cannot pass through the cell membrane (Hassan and Fridovich, 1979) and because the oxidation of ascorbate occurred in the medium, changes in superoxide dismutase could not be expected.

Table 30. Superoxide dismutase activity in E. coli with and without 5.7 mM ascorbic added at time 0.

Time (m)	Superoxide dismutase (u/mg dry cell weight)	
	- ascorbate	+ ascorbate
-10	14.8	14.8
0	14.7	14.7
1	14.7	13.5
10	13.8	13.4
20	13.7	12.5
30	13.5	12.4
60	14.0	12.2

4.12 DISCUSSION

The interaction of ascorbic acid with E. coli is a very complex process. The main aspect of the cellular response to ascorbic acid described is the induction of catalase activity. Within this context several related phenomena have also been described, these include, the effect of ascorbate on respiration, growth, and transport of glucose molecules into E. coli. Earlier reports had implicated exogenous hydrogen peroxide in the induction of catalase synthesis in E. coli and this work clearly shows: (1) that H_2O_2 is formed from ascorbic acid in oxygenated medium and (2) that H_2O_2 as well as other hydroperoxides or their breakdown products, can induce catalase synthesis in E. coli regardless of the carbon source. As well as supporting these conclusions, the data also provide considerable information regarding catalase gene expression some of which is not entirely consistent with conclusions drawn in earlier reports.

4.12.1 Aerobic Catalase Induction

Modifications in growth conditions as simple as anaerobiosis or reduced amino acid availability have a significant effect on the extent of catalase induction and on the species of catalase which is produced. In cells growing in minimal-salts medium, catalase activity was increased from the basal level of 5.7 u/mg dry cell weight to 13.0 units per mg dry cell weight in response to ascorbic acid regardless of the carbon source. This level was less than half of what the cell was capable of

synthesizing given an ample supply of amino acids to facilitate protein synthesis. The predominant pair of hydroperoxidase activities (HPI and HPII) increased equally following the addition of ascorbic acid to LB medium and amino acid supplemented medium. In unsupplemented minimal medium, one of the hydroperoxidase activities, HPI, was induced preferentially, but in anaerobic cultures it was the other activity, HPII, which was produced preferentially both at the basal and induced levels. Whether one or more genes is involved in catalase production and regulation is not known. Clearly the regulation of the hydroperoxidase and peroxidase genes is a very complex process and one aspect, that of catabolite repression, will be discussed below. A complete explanation for these observations must await a more detailed genetic analysis of the hydroperoxidase loci.

There is still the question of what actually is the inducing species involved in catalase gene expression. Most reports take it for granted that it is H_2O_2 which is the inducing species although it has not been stated as such. Hassan and Fridovich (1978b) observed elevated catalase levels in stationary phase cells grown anaerobically in the presence of nitrate but attributed this to a link between the synthesis of electron transport intermediates and the synthesis of catalase. The experiments performed with tert-butyl hydroperoxide may indicate otherwise. When tert-butyl hydroperoxide (TBHP) is hydrolyzed it breaks down to the hydroxylradical ($\cdot OH$) and the t-butyl oxide radical ($\cdot OR$, where R is the t-butyl group). Hydrogen peroxide would not be expected to be formed (Noller, 1966). The presence of TBHP in solution caused significant

catalase induction which was not inhibited by exogenous catalase, implicating either the hydroxyl radical or the organic oxide radical as the actual inducer. Unfortunately it is not possible to distinguish between a specific hydroxide radical effect and a general radical effect using this limited data.

Another possible explanation for the catalase induction may lie in the catalase-peroxidase (hydroperoxidase) relationship. The usual substrates for peroxidases are hydrogen peroxide and methyl and ethyl peroxides but there has been a probable role suggested for the liver hydroperoxidase activity in the oxidation of formic acid (Hebi et al., 1957) and nitrite (Heppel and Porterfield, 1949). It seems possible, therefore, that TBHP may be a substrate for and an inducer of the peroxidase activity, indirectly affecting the catalase activity as well. This would make both H_2O_2 and organic hydroperoxides or their radical products the common inducers of the catalase and hydroperoxidase activities combined in one protein.

Both ascorbate and H_2O_2 induced catalase activity at the same rate but the increased levels of catalase resulting from H_2O_2 induction did not last as long. The production of H_2O_2 from ascorbic acid, is dependent upon the oxidation process which may continue to produce H_2O_2 for longer times providing an overall greater inducing capacity. The H_2O_2 added directly would not be replenished after degradation by the catalase and its effect would be of shorter duration.

Ascorbic acid had its most striking effect on cells using glucose as the sole carbon source, an effect which can be discussed in two parts.

First, ascorbic acid inhibited cellular respiration independent of the carbon source. Second, ascorbic acid inhibited glucose uptake but not glycerol uptake. These two observations will be addressed later in the discussion.

The rapid disappearance of catalase activity following ascorbate addition to unsupplemented minimal media was the result of inhibition caused by free radical products from the oxidation of ascorbic acid (Orr, 1966 and 1967a, b). Orr concluded that it was either the hydroxyl radical or perhydroxyl radical which was involved in this inhibition, but the data presented here does not support this conclusion. The addition of H_2O_2 to minimal media where divalent cations would catalyze its breakdown to the hydroxyl radical, did not elicit this inhibitory response. Furthermore, the presence of TBHP which would yield the hydroxyl radical in aerobic LB medium did not cause any inhibition of catalase. In anaerobic glucose-salts medium, ascorbate could not be oxidized to form H_2O_2 and yet this same inhibitory response was observed implicating an ascorbate radical or metabolite as the inhibitor of catalase.

Both minimal salts medium supplemented with casamino acids and LB medium prevented the inhibition of catalase by ascorbate because they contained histidine which could chelate the metal ions necessary for ascorbic acid oxidation and thereby prevent the formation of the inhibitory species. In the case of anaerobic medium where H_2O_2 production would not occur but where catalase was inhibited by ascorbate, there would still be enough metal ions present to catalyze the loss of an electron

from ascorbic acid resulting in the formation of the inhibitory species but no H_2O_2 .

The importance of using well defined conditions to study gene expression could be seen in the experiments employing LB medium composed of tryptone and yeast extract. Because bacteria are often grown in such undefined media, the possibility of unexpected interference from an unknown medium component must always be considered. For example, in this study, histidine was the likely component which interfered with the oxidation of ascorbic acid but a more detailed analysis of the medium would be necessary to confirm this conclusion.

4.12.2 Anaerobic Catalase Induction

Catalase was not induced by ascorbic acid in cells growing anaerobically in LB medium or glucose-salts medium. This was the expected response because H_2O_2 could not be formed from ascorbate in the absence of oxygen. However ascorbate did affect the extent of culture growth in LB medium. In the later stages of growth, the presence of ascorbic acid caused a diauxic effect or essentially a turn on of further growth. This was probably not due to the metabolism of ascorbic acid since it did not enter the cell. Rather, a more likely explanation of the increased amount of growth is that ascorbate, acting as an electron donor, allowed further metabolism of certain metabolic intermediates or a turn on of respiration. For example, ascorbate may provide electrons for the reduction of fumarate to succinate. Alternatively there may have been a stimulation of respiration such as occurred in the presence of EDTA and

cyanide ion although it is not clear if the respiratory chain of anaerobic cells is sufficiently intact to function (Singh and Bragg, 1976).

Hassan and Fridovich (1978b) have reported that in anaerobic cells it was the slow moving hydroperoxidase activity which predominated, whereas the opposite was observed in this study where the fast moving species predominated in anaerobic LB grown cells even after the addition of ascorbic acid. The fact that exponential phase cells were used in the current study whereas stationary phase cells were used in the earlier report may explain the discrepancy but also raises the question of whether or not metabolite variations affect hydroperoxidase gene expression in anaerobic cells as well as in aerobic cells.

The addition of H_2O_2 to anaerobic LB medium did result in catalase production. This would not be expected to occur if catalase induction was strictly linked to the synthesis of components of the aerobic electron transport chain (Hassan and Fridovich, 1978b) for which there appears to be reduced synthetic capability under anaerobic conditions (Haddock and Jones, 1977). Similarly H_2O_2 but not ascorbic acid caused the induction of catalase in anaerobic salts medium. Significantly, there was no difference in the basal level of catalase in cultures grown anaerobically and aerobically showing that the presence of oxygen does not necessitate higher catalase levels for protection.

It has been reported (Hassan and Fridovich, 1978b) that nitrate induced catalase synthesis in anaerobic cultures possibly in conjunction with other respiratory chain components. Unfortunately only late log or stationary phase cultures were employed in this earlier report. The

current study showed that the basal levels of catalase in anaerobic cultures were unaffected by nitrate. By combining results from the earlier report and the current study it would seem that anaerobic metabolism with nitrate mimics aerobic metabolism in that there were low basal levels of catalase during log phase and elevated levels in stationary phase. Furthermore, the addition of ascorbic acid to anaerobic nitrate medium also mimicked the aerobic situation with a 5 fold induction of catalase. H_2O_2 could not be formed directly from ascorbic acid anaerobically but since the enzyme with catalase activity is also a general peroxidase (Claiborne and Fridovich, 1979), it is possible that an as yet unidentified peroxide formed in the reaction may be the inducer of the enzyme. The fact that t-butyl hydroperoxide could induce catalase synthesis lends credibility to this hypothesis. Furthermore since H_2O_2 could induce catalase in cells growing anaerobically without nitrate, the link between catalase synthesis and the synthesis of other respiratory chain components (Hassan and Fridovich, 1978b) cannot be very tight. This was also illustrated by the fact that ascorbic acid did not induce catalase synthesis in anaerobic glycerol-fumarate medium where electron transport occurs. Although the role of cytochromes in transferring electrons to fumarate from glycerol is unknown, it has been shown that functional cytochromes are required for anaerobic active transport (Singh and Bragg, 1976). The most important factor in the induction of catalase synthesis, therefore, would seem to be the presence of a hydroperoxide inducer rather than a link with electron transport intermediate synthesis. That is, that the intermediates produced by

whatever metabolic route is active were the determining factors in the level of cellular catalase.

Interestingly, stimulation of growth by ascorbate occurred only when cells were gaining energy from electron transport and oxidative phosphorylation in glycerol-fumarate, glycerol-aspartate and by analogy, LB medium. Since ascorbate could not enter the cell it would seem that electrons from ascorbate stimulated additional electron transport and perhaps the production of a metabolite such as succinate which could be metabolized further.

4.12.3 Ascorbic Acid and Respiration

In 1961 Mitchell proposed the chemiosmotic coupling hypothesis for conservation of energy across membranes. In this theory, energy derived from biological oxidations can be stored across a membrane in the form of a proton electrochemical potential difference (Mitchell, 1961). This potential is the energy source used to drive several membrane related cellular processes including oxidative phosphorylation (respiration) (Wilson et al., 1976) and nutrient transport (Wilson et al., 1978). Redox proteins of the electron transport chain are functionally organized in the membrane so as to generate some form of charge separation. This charge separation is used to generate ATP by discrete steps that occur in oxidative phosphorylation. This charge separation can also be used to establish a proton motive force across the membrane that is responsible for energy dependant transport of solutes across the coupling membrane by many pathways. There is a very close connection between cellular respiration and solute transport.

In studies conducted with whole cells grown in various media it was found that ascorbate had a significant inhibitory effect on cellular respiration in glucose-salts medium and glycerol-salts medium, but a smaller effect on cells in succinate salts medium and no effect on cells in LB medium. A casamino acid supplement was found to reduce this inhibitory effect of ascorbic acid on cellular respiration in glucose-salts and glycerol-salts media to a level similar to that which occurred in succinate-salts medium. This may be a reflection of similarities in oxidative metabolism in the presence of succinate and casamino acids which differs from oxidative metabolism in the presence of glucose and glycerol. One major effect of oxidative phosphorylation (respiration) inhibitors is to collapse proton and/or ion gradients that normally exist across the cytoplasmic membrane. When the great reducing power and ionic properties of ascorbate are considered, it is not clear how ascorbate would collapse such gradients but its presence could conceivably disrupt electron flow, perhaps by hydroxyl radical modification of a membrane component, thereby inhibiting respiration. The differing amounts of inhibition observed in different media could be due to a number of things such as differences in the membrane constitution of cells grown in different media, or alternatively, to certain media components which modulate the inhibitory effect of ascorbate. For example, the respiration of glycerol grown cells was inhibited more than glucose or succinate grown cells while cells grown in LB medium actually had their respiration rate enhanced. Cyanide resistant respiration was stimulated in all media except where succinate was the carbon source. Unfortunately

there was not a direct relationship between the effect of ascorbate on respiration and its effect on growth. For example, cyanide resistant respiration was stimulated in glucose grown cells but the growth rate was unaffected, whereas in succinate grown cells, respiration was unaffected but the growth rate was increased. In LB medium both cyanide resistant respiration and growth were stimulated by ascorbate. Clearly the overall effect of ascorbate is complex and a single manifestation of the ascorbate interaction is not sufficient to fully explain its effect.

When cyanide was present in the medium, the amount that the growth rate was stimulated was proportional to the amount of ascorbate present except for cells growing in glucose medium. The metal chelating properties of cyanide ion result in a slower rate of ascorbate oxidation (Weissberger et al., 1943) which could be compared to a more controlled rate of oxidation. The cyanide effect was also mediated by the degree of oxygenation. Compounds such as phenylenediamine methanesulfonate (Jacobs, 1960) also stabilize ascorbate and prevent certain disruptive ionic interactions which otherwise would be caused by a molecule with such high reducing powers and ionic properties. Under more controlled conditions it might be possible for ascorbate to introduce electrons into the respiratory chain stimulating respiration, and, except where metabolite transport is also affected as in glucose medium, growth as well.

A scheme is presented in Figure 61 that illustrates where ascorbic acid or ascorbate free radical could possibly donate electrons and thus stimulate respiration. The presence of cyanide causes the synthesis of

the alternative redox carriers cyt b₅₅₈ and cyt d in E. coli (Ashcroft and Haddock, 1975). Increased levels of menaquinone also result (Haddock and Jones, 1977). The fact that quinones can easily accept electrons from ascorbic acid (Dixon, 1971) suggests the logical series of electron transfers shown in Figure 61, from ascorbate to quinones to cytochromes. Whether it is the enhanced quinone levels or the different cytochromes which are responsible for the enhancement of respiration by ascorbate wasn't determined.

These observations should prompt further studies to clarify the role of ascorbate in metabolism and may be useful in an industrial application. For instance, growth of a particularly useful biochemical producing strain of E. coli could be promoted by ascorbate in the presence of cyanide which would prevent contaminating bacteria from growing.

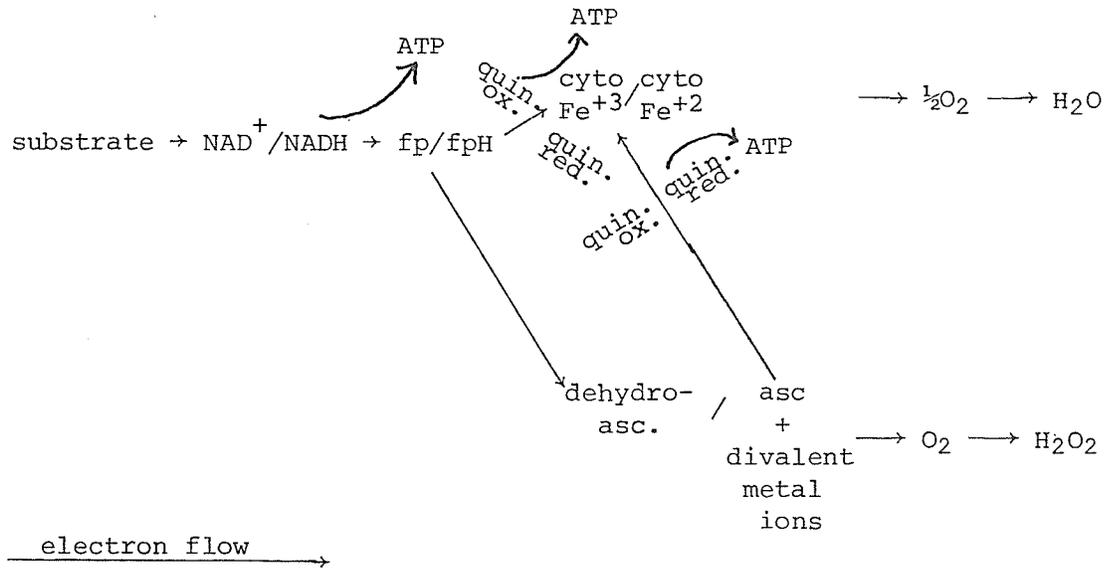


Figure 61. Proposed scheme for electron donating ability of ascorbic acid in electron transport chain of *E. coli*.

4.12.4 Uptake

Because the results relating to glucose uptake by whole cells could also be interpreted in terms of ascorbate affecting other aspects of glucose metabolism which are reflected in glucose uptake, they do not prove that ascorbate inhibits glucose transport. Indeed further experiments are necessary to clarify the precise nature of the ascorbate effect on glucose uptake. However, it remains that ascorbate does have a significant effect on the growth of cells in glucose medium and a consideration of other data leads one to speculate that glucose transport is affected by ascorbate.

The glucose transport system is a PEP dependent sugar phosphotransferase system involving both soluble and membrane bound carriers. The fact that glucose transport is dependent upon the membrane being in a de-energized state (Reider et al., 1979) suggests a mechanism by which ascorbate, with a significant reducing power, could affect this form of active transport. Ascorbate does interfere with cellular respiration indicating that it may be interfering with some aspect of the membrane potential. The uptake of both lactose and melibiose involve an electrochemical gradient and ascorbate also inhibits the growth of cells on these carbohydrates. On the other hand, glycerol is transported by facilitated diffusion not involving any sort of membrane potential and neither the uptake of glycerol nor growth on glycerol was affected by ascorbate. Similarly, succinate uptake can occur independent of a proton gradient (Ramos and Kaback, 1977) and the uptake of galactose, ara-

binose and maltose involves shock sensitive systems (Dills et al., 1980); and the growth of cells on these carbon sources is not significantly affected by ascorbate.

Such speculation will help to direct further experimentation to determine whether the glucose transport system is directly affected by ascorbate, whether ascorbate acts principally by modifying the membrane potential or whether there is some other aspect of glucose metabolism being affected.

4.12.5 Catabolite Repression

When there is both glucose and lactose in a medium in which E. coli is growing, the glucose is metabolized preferentially and synthesis of the enzymes for lactose utilization is inhibited. This phenomenon is referred to as catabolite repression. This has been attributed to a decrease in intracellular cAMP levels, that is, repression of catabolic enzyme synthesis by glucose is mediated by low intracellular cAMP levels. Addition of cAMP overcomes glucose repression of these enzymes.

Catabolite repression is usually identified with the action of a cAMP-cAMP receptor protein complex, although such a mechanism has not been identified in all organisms exhibiting the phenomenon. Very recently, however, catabolite repression has been observed in E. coli in the absence of cAMP receptor protein casting doubt on the universality of this mechanism (Guidi-Rontani et al., 1980). Therefore it is possible that some of the catabolite repression effects described in the literature are unrelated to cAMP effects and instead may be the result of other more complex metabolic changes or interactions. Catabolite repression has been implicated in the control of catalase synthesis in Bacteroides fragilis (Gregory et al., 1977), S. cerevisiae (Sulebele and Rege, 1967 and 1968), and E. coli (Yoshpe-Purer et al., 1977; Hassan and Fridovich, 1978b) but only the latter report actually implicated cAMP in the repression mechanism. It was observed that cAMP without affecting basal catalase levels, caused a more rapid turn-on of catalase synthesis in cells approaching stationary phase. At the same time, cAMP caused a speed-up in the diauxic response and if catalase was being produced in

response to metabolites (eg., hydroperoxides) produced in the secondary metabolism, then the cAMP effect on catalase turn-on could be indirect. The other reports describing catabolite repression in catalase gene expression (Gregory et al., 1977; Sulebele and Rege, 1967 and 1968; Yoshpe-Purer et al., 1977) based their conclusions on catalase levels observed during growth on different carbon sources and during growth into stationary phase without directly studying cAMP. There could very well have been significant variations in the intracellular metabolite concentrations depending on the stage of growth, carbon source availability, or pH, some of which could result in peroxide formation causing catalase synthesis. The results here have shown that glucose in the growth medium of E. coli did not affect catalase basal levels or induction differently from the non-fermentable carbon sources glycerol and succinate. Furthermore, the presence of cAMP in glucose salts medium did not enhance catalase synthesis and the lack of cAMP in the growth medium of an adenylate cyclase mutant did not reduce catalase synthesis. The classical catabolite repression mechanism involving the cAMP-cAMP receptor protein complex was therefore eliminated as a possible element of control in catalase synthesis. Other metabolite changes clearly affect catalase synthesis in E. coli and may explain the glucose effects observed by others. This work has not attempted to discern whether a catabolite repression mechanism involving cAMP is involved in other species where gene expression may be quite different from E. coli.

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