THE NITRATE-REDUCING SYSTEM OF BACILLUS STEAROTHERMOPHILUS

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SUMMARY

Bacillus stearothermophilus is a thermophilic organism with an optimum temperature for growth of 60°. The role of nitrate in the anaerobic metabolism of the organism, the nature of the nitrate reducing system and the enzymic properties and heat stability of the isolated enzyme were studied.

The organism grew well under anaerobic conditions on glucose, sucrose, and starch with the production of acid. Nitrate slightly stimulated the anaerobic growth rate on these carbohydrates, however the production of acid showed that glycolysis was the main route of carbohydrate catabolism even though nitrate was acting as an electron acceptor.

The organism would grow anaerobically on glycerol only in the presence of nitrate and the fact that acid was not produced under these conditions indicated that glycolysis was not the sole route of glycerol metabolism.

The levels of nitrate reductase activity and the activities of tricarboxylic acid cycle enzymes; isocitric dehydrogenase, aconitase, succinic dehydrogenase, fumarase, and malic dehydrogenase were determined under various conditions of growth in the presence and absence of nitrate.

Nitrate reductase was found in extracts from cells grown anaerobically on glucose and its production was stimulated 4-fold in the presence of nitrate.

Low but consistent levels of tricarboxylic acid cycle enzymes were found in cells grown anaerobically on glucose and these levels increased slightly under aerobic conditions (1.3-fold to 3.8-fold increases). The highest levels were found in cells grown aerobically on glycerol.

The levels of isocitric dehydrogenase, succinic dehydrogenase and malic dehydrogenase increased slightly, and fumarase and aconitase decreased, when the cells were grown anaerobically on glucose in the presence of nitrate. On the other hand, with the exception of aconitase, the levels of all these enzymes in cells grown anaerobically on glycerol plus nitrate were intermediate between those found in cells grown aerobically on glucose and aerobically on glycerol.

Both nitrate, under anaerobic conditions, and oxygen stimulated the synthesis of cytochrome c_1. The concentration of cytochrome b_1 only increased under anaerobic conditions in the presence of nitrate. The highest concentrations of both cytochrome c_1 and cytochrome b_1 occurred in cells grown anaerobically on glycerol plus nitrate.

The $NADH_2$-dependent nitrate reducing system was found to reside in protoplast membranes that also contained cytochromes b_1 and c_1. Spectral studies showed conclusively that cytochrome b_1 served as an electron transferring component of the nitrate reductase system whereas cytochrome c_1 was a component of an associated aerobic pathway. A scheme for both pathways based upon spectral studies and inhibitor studies is presented.

Treatment of the protoplast membranes with cholate and ammonium sulphate resulted in the solubilization of nitrate reductase free from both cytochrome b_1 and flavin. Reduced benzyl viologen, but not $NADH_2$, served as an electron donor for nitrate reduction. Benzyl viologen-nitrate reductase activity was inhibited by metal chelating agents indicating that a metal was necessary for its activity.

The soluble enzyme had an optimum pH between 6.1 and 6.3 in 0.1M phosphate buffer. The K_m values for nitrate at 40^o and 60^o were 1.52×10^{-4}M and 2.03×10^{-4}M respectively. The activation energy for nitrate reduction was -9,250 cal. between 35^o and 65^o. Heating the enzyme at 60^o for 2 hr. resulted in only 13.7% inactivation indicating that the enzyme was intrinsically heat stable.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text.

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ABBREVIATIONS

ATP	Adenosine 5'-triphosphate.
cal.	Gram-calorie.
DEAE-cellulose	Diethylaminoethycellulose.
DNA	Deoxyribonucleic acid.
E	Extinction.
	Molar extinction coefficient: the extinction of a molar solution in a 1 cm. light path.
EDTA	Ethylenediaminetetra-acetate.
FAD	Flavine-adenine dinucleotide.
FMN	Flavine mononucleotide.
g	Acceleration due to gravity.
NAD, $NADH_2$	Oxidized and reduced forms respectively of nicotinamide-adenine dinucleotide.
NADP, $NADPH_2$	Oxidized and reduced forms respectively of nicotinamide-adenine dinucleotide phosphate.
RNA	Ribonucleic acid.
t-RNA	Transfer-RNA.
m-RNA	Messenger-RNA.
tris	2-Amino-2-hydroxymethylpropane-1,3-diol.

CHAPTER I

LITERATURE SURVEY

PART 1

THERMOPHILIC BACTERIA

A. DEFINITION OF THERMOPHILIC BACTERIA

Poikilothermic organisms capable of living at elevated temperatures have interested bacteriologists and biochemists for many decades. A lot of confusion exists about the classification of microorganisms which grow optimally at temperatures from 40° to 90° (Morrison & Tanner, 1924; Cameron & Esty, 1926; Eckford, 1927; Prickett, 1928).

The majority of investigators have defined three broad groups:

(i) Strict thermophiles: bacteria that grow at 55° but not at room temperature

(ii) Facultative thermophiles: bacteria that have a maximum temperature for growth between 50° and 55° but are capable also of growing at room temperature

(iii) Thermotolerant bacteria: bacteria having a maximum temperature for growth at 40° to 50° and are capable of growing at room temperature.

Imsenecki & Solnzeva (1945) have defined true thermophiles as organisms whose optimal temperature range for growth lies between 55° and 60°. They divided this group further into:

(i) Stenothermophiles: organisms which grow at 60° and only after many days at 28° - 30°

(ii) Eurithermophiles: organisms that are capable of growing at 60° and exhibit slow to abundant growth at 28° to 30°.

Any classification of microorganisms based upon optimum growth temperature and slight variations in physiology obviously has its limitations. The confusion which has existed in this field is evident when the aerobic spore-forming Bacilli are considered (Bergey 1948, 1957). The sixth edition of Bergey (1948) describes fourteen species of Bacilli which are classified as thermophiles, that is, growing optimally above 50°; whereas the seventh edition of Bergey (1957) describes only one of the Bacilli as a thermophile, namely Bacillus stearothermophilus with an optimum temperature for growth from 50° to 65°.

Allen (1953) studied 105 isolates of thermophilic spore-forming Bacilli which she divided into four groups on the basis of morphology and physiology, the type species of which are similar to B. circulans, B. coagulans, B. subtilis and B. sphaericus. Allen presented evidence for the selection of thermophilic mutants from cultures of mesophilic Bacilli and she concluded that there was no justification for a separate class of thermophilic Bacilli; on the basis of morphology and physiology she considered B. stearothermophilus to be a thermophilic variant of B. circulans.

Bausum & Matney (1965) have provided evidence for the existence of a temperature boundary between mesophilic bacterial growth and thermophilic bacterial growth and placed the limits of this boundary at 44° and 52°. Eurithermophilic Bacilli grown at 37° required a period of adaptation at an intermediate temperature (49°) before they were capable of growing at 55°. Raising the temperature of growing cultures from 37° to 55° without holding them at the intermediate

temperature resulted in the death of the organisms, however cultures of stenothermophilic Bacilli previously grown at their minimum growth temperature of 44° were not killed and showed no lag in growth when exposed to a temperature of 65°. Spores formed at 37° by a eurithermal organism, B. licheniformis Allen, produced identical numbers of colonies when quantitatively plated and grown at 37° and 55° respectively. Since the minimum temperatures for growth of Allen's thermophilic variants overlap the maximum temperatures for the growth of the parent mesophilic Bacilli, it is possible that the mesophilic Bacilli which she used were in fact, eurithermophiles, and the mechanism employed for obtaining thermophilic cultures was adaptation rather than selection of heat resistant mutants.

B. GROWTH REQUIREMENTS INDUCED BY HIGH TEMPERATURE

Thermophilic organisms have been isolated from a variety of sources; indeed the early phases of the investigations of thermophiles was wholly concerned with ecology and few attempts were made to obtain pure cultures. During the last 20 years the growth requirements of various strains of the thermophilic Bacilli have been investigated.

Bonner (1957) has introduced the term 'temperature lesion' to describe the high-temperature-induced growth deficiencies which are repairable with specific substances. Growth substances needed to repair temperature lesions in any one organism vary with the temperature at which the lesion is induced. Generally, a single substance may repair a lesion induced by a given temperature but a further increase in temperature may bring about a requirement for another substance to support

growth. These lesions do not appear to be due to gene mutations since the normal growth of the organisms occurs upon lowering the temperature.

Substances capable of repairing temperature lesions which become manifest at temperatures between 45° and 80° in various strains of Bacilli, are compounds such as biotin, desthiobiotin, histidine, folic acid, methionine, tryptophan, pyridoxal, thiamine, glutamic acid and complex mixtures such as yeast extract, beef extract, peptone and proteose (Campbell & Williams, 1953a, 1953b; Baker, Hutner & Sabotka, 1955; Sie, Sabotka & Baker, 1961; Sie & Sabotka, 1961).

Campbell & Williams (1953a, 1953b) demonstrated that the requirement for biotin to support growth at 55° by various strains of B. coagulans and B. stearothermophilus, was due to the apparent inactivation of the enzyme systems required to synthesize pimelic acid or to transform pimelic acid into desthiobiotin or to convert desthiobiotin into biotin.

There are numerous examples of temperature lesions occurring in mesophilic and eurithermophilic bacteria which can be alleviated by growth substances. The requirement of Lactobacillus arabinosis for phenylalanine and tyrosine to support growth at 37°, and for aspartic acid to support growth at 39°, was abolished if the CO_2 tension was increased (Borek & Waelsch, 1951). The bacterium also had these growth requirements at 26° if the CO_2 tension was decreased to very low levels; the apparent temperature lesion therefore appeared to be due to the decreased solubility of CO_2 at the higher growth temperatures

When the growth temperature of a microorganism is raised the

increased synthesis of metabolites can produce temperature lesions. Bird & Gots (1958) found that a strain of E. coli requiring methionine or p-aminobenzoic acid for growth at 37° had the same requirement when grown at room temperature in the presence of homocysteine. The authors suggested that homocysteine prevented the formation of methionine by interfering with a co-factor derived from p-aminobenzoic acid and that the temperature lesion was actually due to the increased synthesis of homocysteine at the higher temperatures.

When grown at low riboflavin concentrations a mutant of Neurospora crassa, requiring riboflavin at 30° but not at 25°, synthesized increased amounts of cytochromes with a concomitant increased requirement for iron. If sufficient riboflavin was present the organism had a requirement for molybdenum due to the increased production of the molybdo-flavoprotein enzyme nitrate reductase (Nicholas, 1956).

C. THERMOBIOSIS

The mechanisms which enable thermophilic bacteria to grow and carry on active metabolism at temperatures which produce lethal lesions in mesophilic bacteria are not obvious.

Renewed interest in thermophilic bacteria in recent years, resulting in work on intracellular protein, extracellular proteins and protein synthesis, is providing a basic picture but no single factor can be cited as responsible for the heat stability of these organisms.

It is likely that a variety of factors are accountable for the ability of thermophilic bacteria to grow at high temperatures and these will be considered in the following sections.

1. Heat Stability of Intracellular Proteins

a. Soluble Proteins.

The criterion used throughout this thesis for differentiating soluble and particulate proteins is that a protein which is not sedimented after being subjected to an average centrifugal field of 100,000 x g for at least 60 min. is considered soluble unless additional data indicate otherwise.

Very few truly soluble intracellular enzymes or proteins have been examined for heat stability in vitro. Koffler & Gale (1957) have shown that the coagulation temperature of total cytoplasmic protein from thermophiles is higher than the coagulation temperature of similar protein from mesophiles.

Thompson & Thompson (1962), using a partially purified aldolase (fructose-1, 6-diphosphate D-glyceraldehyde-3-phosphate-lyase E.C. 4.1.2.13) from B. stearothermophilus, stated that the enzyme lost only 5% of its original activity after being heated at 70° for 60 min. Identical results were obtained whether the enzyme was extracted from cells grown at 45° or 65°.

A pyrophosphatase (pyrophosphate phosphohydrolase E.C. 3.6.1.1.), which was heat stable, was obtained from B. stearothermophilus (strain 10) grown at both 60° and 70° (Brown, Militzer & Georgi, 1957). When the same organism was grown at 40° it produced a pyrophosphatase

which was less stable to heat. A puzzling feature of this enzyme was that its optimum temperature for activity was 60° and this was independent of the temperature at which the organism was grown.

Nakamura (1960) investigated the heat stability of catalase found in the supernatent obtained by centrifuging lysed cells of a thermophilic bacterium at 100,000 x g for 20 min. The crude enzyme lost 10% of its activity after it had been heated at 65° for 10 min.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidored uctase (phosphorylating), E.C. 1.2.1.12) from B. stearothermophilus has been crystallised and some of its chemical and physical properties have been studied (Amelunxen, 1966, 1967). The enzyme retained 95% of its activity after it had been kept at 90° for 10 min.; whereas glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle was almost completely inactivated after 10 min. at 70°. The optical rotation of the enzyme from B. stearothermophilus did not change in the presence of 8M urea but the muscle enzyme produced a large increase in negative rotation when incubated in 8M urea. The specific activity of the thermophilic enzyme decreased by only 11% when it was kept in 8M urea for several hours whereas the muscle enzyme was completely inactivated under the same conditions. The optical rotation studies and the specific activity determinations in urea, indicate that little, if any, change in the tertiary structure of the thermophile enzyme occurs with this denaturing agent.

b. Membrane-bound Proteins

The majority of thermophilic enzymes which have been investigated have been bound to particulate matter; probably derived from the limiting cell membrane and the intracytoplasmic membranes.

Militzer et al (1951) obtained an apyrase system from B. stearothermophilus the activity of which decreased slightly after being heated at 65° for 2 hr. The authors considered the 'apyrase' system to be a soluble enzyme; however, the speed and time of centrifugation used to obtain the enzymically active supernatant from lysed cells (40,000 x g for 15 min.) preclude using the term 'soluble' to describe the physical state of the enzyme. Subsequent attempts to purify the 'apyrase' indicated that it was in fact bound to a particulate structure (Militzer & Tuttle, 1952).

Militzer & Tuttle (1952) have partly purified an adenosine triphosphatase by treating the apyrase-containing particles with acetone. They stated that the preparation was stable after heating for 2 hr. at 65° but was slowly inactivated at 75°.

Malic dehydrogenase (L-malate: NAD oxidoreductase E.C. 1.1.1.37) has been studied by Allen (1953) and by Militzer, Sonderegger, Tuttle & Georgi (1949). Allen investigated the thermal stability of malic dehydrogenase in dried cells of a thermophile of the B. circulans group (Allen's thermophilic B. circulans probably is similar to B. stearothermophilus). She found that the malic dehydrogenase activity decreased slightly after being subjected to a temperature of 65° for 30 min.

Militzer et al (1949) treated B. stearothermophilus with lysozyme and from the lysed cells obtained a 'red fraction' which contained malic dehydrogenase. The particulate enzyme retained 72% and 60% of its initial activity after being heated at 65° for 30 min. and 120 min. respectively.

The malate dehydrogenase from B. stearothermophilus has been solubilized by sonic irradiation of whole cells and purified by heat treatment, ammonium sulphate fractionation and DEAE cellulose chromatography (Murphy, Barnaby, Lin & Kaplan, 1967). The heat stability of the purified enzyme was compared with that of crystalline malate dehydrogenase from B. subtilis. The activity of the B. subtilis enzyme remained constant when it was heated at temperatures from 0° to 45° for 20 min.; between 45° and 55° the enzyme was completely inactivated. The activity of the thermophilic enzyme decreased only 10% after it had been heated at 70° for 20 min. but was completely inactivated between 70° and 80°. The optimum temperature of the malate dehydrogenase from B. subtilis was 50° whereas the enzyme from B. stearothermophilus had an optimum temperature of 65°. Murphy et al could not find any particle-bound enzyme after sonic disintegration of the cells and only one electrophoretic form of the soluble enzyme was detected, therefore they concluded that the soluble malic dehydrogenase had been bound to particulate matter and was released during the sonic treatment of the cells.

The activity of a cytochrome oxidase system in a particulate fraction from B. stearothermophilus decreased by 50% after being kept at a temperature of 65° for 90 mins. (Militzer, Sonderegger, Tuttle

& Georgi, 1950).

In contrast to the thermostability of both the particulate and the soluble malic dehydrogenase, the particulate pyruvic oxidase system from the same organism was shown to be heat labile when released from the cell (Militzer & Burns, 1954). The pyruvic oxidase activity of whole cells remained constant over a period of 45 min. at 60^{o} - whereas the pyruvic oxidase activity of the cell-free particulate fraction decreased by almost 90% when heated at 80^{o} for 15 min. The authors showed that Mg^{++}, oxygen and pyruvate partly prevented inactivation by heat. The pyruvic oxidase system is complex, with several enzyme components, and any experimental work in which the inactivation by heat of particulate pyruvate oxidase is estimated by measuring oxygen uptake cannot be regarded as conclusive evidence for the heat inactivation of the component enzymes.

The reduced nicotinamide adenine dinucleotide-tetrazolium oxidoreductase activity of the cytoplasmic membranes from B. stearothermophilus was shown to be stable to heat while it was bound to the membranes but was heat labile after solubilization and partial purification (Holdsworth, personal communication).

Heat stable enzymes have been demonstrated in the spores of mesophilic bacilli and are of interest because the mode of stabilization of mesophilic spore enzymes and thermophilic enzymes may be similar. Stewart & Halvorson (1954) investigated the heat stability of alanine racemase (E.C. 5.1.1.1.) in spores and vegetative cells of B. cereus. The initial activity of the alanine racemase of whole

spores decreased by less than 10% after heating at 80° for 2 hr. whereas the alanine racemase of whole cells was rapidly inactivated. Sonic oscillation of the spores followed by differential centrifugation produced particle-bound and soluble alanine racemase. The particulate enzyme exhibited similar heat stability to that of the enzyme present in whole spores but the soluble enzyme was extremely heat labile. Further sonic treatment of the particles resulted in the solubilization of more heat labile alanine racemase. Thus heat stability is a property of this enzyme only when it is bound to particulate matter.

The catalase present in spores of B. cereus was shown to be quite stable to heat treatment whereas the catalase of whole cells was completely inactivated in less than 5 min. at 80°, (Lawrence & Halvorson, 1954). Unlike the alanine racemase activity of B. cereus spores, mentioned above, heat stability was not a property peculiar to the particle-bound catalase since the supernatant, obtained after centrifuging disrupted spores at 140,000 x g for 4 hr., contained both heat-stable and heat-labile catalase. The authors suggested that two species of catalase may have been present; one heat stable and the other heat labile and not detectable until released from whole spores.

2. The Heat Stability of Extracellular Proteins and Enzymes

The extracellular proteins and enzymes which have been obtained from bacteria grown at high temperatures have been heat stable. Campbell (1955) showed that eurithermal Bacilli produced heat-stable

α-amylase (α-1, 4-glucan 4-glucanohydrolase, E.C. 3.2.1.1.) at temperatures above 50° whereas heat-labile α-amylase was produced by the same organism below 48°. Campbell (1954) crystallized α-amylase produced by B. coagulans (43 P-4) (a eurithermal Bacillus) when the organism was grown at 55° and 35° respectively. The α-amylase produced at 55° retained 88% of its initial activity after being heated at 90° for 1 hr. whereas the α-amylase produced at 35° retained only 6% of its initial activity under identical conditions. Crystalline α-amylase from the stenothermophilic bacterium B. stearothermophilus is extremely heat stable. Manning & Campbell (1961) found that it retained 100% of its initial activity after being heated at 70° for 24 hr. and lost only 29% of its initial activity after 20 hr. at 85°.

Another exoenzyme which has been studied is a protease from B. thermoproteolyticus (Endo, 1962; Ohta, Ogura & Wada, 1966; Ohta, 1967). The enzyme was first isolated by Endo (1962), who showed that its activity decreased by less than 50% after 1 hr. at 80°. The logarithm of the Michaelis constant and the logarithm of the maximum velocity were both linear functions of the reciprocal of the absolute temperature at temperatures between 25° and 80°. (Ohta et al, 1966). When the enzyme had been heated at 80° for 1 hr. the Michaelis constant was found to be higher than that obtained using untreated enzyme. The maximum velocities, however, were the same for both the untreated enzyme and the heat-treated enzyme - indicating that none of the enzyme molecules were completely denatured (V max constant). Some conformational change had occurred, since the affinity of the enzyme for its substrate decreased after heat treatment.

Flagella, while not wholly extracellular nevertheless exist mainly in the external environment. Koffler and co-workers have carried out a number of experiments on flagella and the protein derived from flagella - flagellin. The decrease in viscosity of suspensions of purified flagella was used by Koffler, Mallet & Adye (1957) as a measure of the stability of flagella under various conditions. The flagella from mesophilic bacteria disintegrated when the temperature was raised above 50^{o}, whereas the flagella from thermophilic bacteria remained intact at temperatures as high as 70^{o}. A strain of B. subtilis which would not grow above 52^{o} (a temperature between the maximum temperatures for growth of the mesophilic bacteria and the thermophilic bacteria) produced flagella whose viscosity decreased when the temperature was raised above 60^{o}. Thus the temperatures at which the different flagella preparations started to disintegrate mirrored the temperatures at which the respective bacteria ceased to grow. Thermophile flagella were stable when incubated in 6M urea, 10M acetamide and 3.5mM sodium dodecyl sulphate whereas mesophilic flagella disintegrated under these conditions.

Abram & Koffler (1965) reported that flagellin, which was formed when flagella from B. stearothermophilus 10 were subjected to pH2.3, reaggregated to form flagella-like filaments at temperatures which caused denaturation of mesophile flagellin. The thermophile filaments resembled native flagella in their morphology and in their stability to heat and to phosphotungstic acid. Abram and Koffler concluded that the heat stability of thermophilic flagella resided in the flagellin monomers.

3. The Heat Stability of the Protein Synthesizing Systems of Thermophilic Bacteria

When the many possible factors which contribute to the growth of thermophilic organisms at high temperatures are considered it becomes apparent that the continued synthesis of enzymes is of great importance. The mechanism for replacing enzymes, denatured by heat, needs to be efficient and relatively unaffected by the high temperature for growth. Several groups of workers have investigated the protein synthesizing systems in various strains of thermophilic Bacilli and their findings will be discussed in this section.

The heat stability of DNA isolated from thermophilic bacteria has been compared with DNA from mesophilic bacteria (Mamur, 1960). The Tm values (the temperature at the midpoint of the hyperchromic transition curve) did not differ significantly from those obtained using mesophile DNA.

The ribosomes from thermophilic bacteria have been examined, over a wide range of temperatures, both for their ability to support amino acid incorporation at high temperatures, and for their structural stability.

The thermal denaturation profiles for ribosomes both from E. coli and from B. stearothermophilus have been determined (Saunders & Campbell, 1966). E. coli ribosomes started to dissociate at 58° and exhibited a large hyperchromic shift at 70°, with a Tm of 72°. On the other hand there was no rise in extinction until a temperature of 72° was reached when B. stearothermophilus ribosomes were heated. The Tm of the thermal denaturation profile for the ribosomes from the thermophile was 78°.

Thermal denaturation curves of the 4S, 16S and 23S RNA from both organisms were similar and there was no marked difference in the amino acid composition of their ribosomal proteins. The authors suggested that the heat stability of the thermophile ribosomes might be due to an unusual packing arrangement of the ribosomal protein to the RNA or to differences in the primary structure of the protein.

Mangiantini, Tecce, Toschi & Trentalance, (1965), found that the ribosomes from B. stearothermophilus were stable up to a temperature of 65°; above this temperature there was a sudden rise in extinction. The extinction of E. coli ribosomes increased at a temperature between 50° and 55°; the hyperchromic effect being much larger than that obtained with B. stearothermophilus ribosomes.

Friedman & Weinstein (1965) heated ribosomes from B. stearothermophilus for 10 min. at 65° and then tested them for polyuracil-directed phenylalanine incorporation at 37°. The heat-treated ribosomes retained 85% of the activity of unheated ribosomes, whereas E. coli ribosomes tested under the same conditions retained less than 5% of the control activity.

Stenesh & Holazo (1967) determined the thermal denaturation profiles and the purine and pyrimidine base ratios of the ribosomal RNA from three thermophilic strains and from three mesophilic strains of the genus Bacillus. In contrast to the results of Saunders and Campbell (1966), mentioned above, the Tm values for the ribosomal RNA from the three thermophiles were 68.6°, 70.0° and 70.4°, whereas the Tm values for the RNA from the three mesophiles were 64.5°, 64.3°,

and 65.1°. The RNA from the thermophiles had a higher guanine + cytosine (G + C) content and a lower adenine + uracil (A + U) content than did the mesophile RNA. This would account for the higher Tm values of the thermophile RNA.

Friedman & Weinstein (1965, 1966) and Arca, Calvori, Frontali & Tecce (1964) have reported that the thermal denaturation profiles of the total transfer RNA (t-RNA) isolated from B. stearothermophilus and E. coli respectively were not significantly different. The t-RNA from E. coli, when incubated with aminoacyl synthetases from B. stearothermophilus, formed L-isoleucyl-t-RNA complex at temperatures up to 80° whereas when using E. coli aminoacyl synthetases, formation of the complex ceased at 60° (Arca et al, 1964).

The ability of aminoacyl synthetases from B. stearothermophilus to form aminoacyl adenylates ('activated amino acids') and to catalyse the formation of aminoacyl-t-RNA complexes at high temperatures has been studied. Arca et al (1964) showed that extracts of B. stearothermophilus catalyzed the formation of L-leucyl-, and L-isoleucyl-, adenylates at temperatures up to 70° and 80°, respectively, before a decrease in activity occurred. However the amounts of L-leucyl-, and L-isoleucyl-, t-RNA complexes formed decreased at 70°, and the maximum rate was at 40°. Pre-incubation of the extracts at 80° for 10 min. did not appreciably effect the amount of L-isoleucyl-t-RNA formed at 50°. The non-enzymic cleavage of L-isoleucyl-t-RNA at 70° and 80° resulted in a loss of 52% and 72% respectively when compared with the amount remaining at 40° after the same length of time. Bubela & Holdsworth (1966a) have shown

that the non-enzymic cleavage of total aminoacyl-t-RNA complexes results in only 30% of the original complex remaining after 40 min. at 63°. This destruction of aminoacyl-t-RNA complexes at temperatures above 40° may have been responsible for the occurrence of a maximum rate of formation of both leucyl-, and isoleucyl-, t-RNA at 40° reported by Arca et al (1964).

Friedman & Weinstein (1966) reported that the maximum rate of formation of phenylalanyl-, and leucyl-, t-RNA complexes was obtained at 65° when extracts from B. stearothermophilus were used with t-RNA from either B. stearothermophilus or E. coli. Their results did not provide any proof that the aminoacyl synthetase from B. stearothermophilus were heat-stable since the incubation time used was only one minute.

In contrast to the results obtained by Arca et al (1964), Bubela and Holdsworth (1966a), have shown that the rate of activation of amino acids by extracts of B. stearothermophilus was slow below 40° and reached a maximum at 60° using incubation times of ten minutes. Similar results were obtained for the formation of aminoacyl-t-RNA complexes. Furthermore they showed that the activity of aminoacyl synthetases decreased when the enzymes were incubated at temperatures above 40° so that after 10 mins. at 60° the activity was only 50% of the initial activity obtained at 20°.

Friedman & Weinstein (1966) have studied the incorporation of ^{14}C labelled lysine, phenylalanine and proline into a subcellular preparation from B. stearothermophilus containing ribosomes, t-RNA,

aminoacyl synthetases and presumably endogenous m-RNA. The optimum temperature for the incorporation of lysine and phenylalanine was 55^{o} and that for proline was 60^{o}. A similar cell-free preparation was used to measure the poly-uracil (poly-U)-directed incorporation of phenylalanine. With this system the poly-U stimulation of phenylalanine incorporation was greater at 37^{o} than at 65^{o}. To achieve a greater stimulation at 65^{o} than at 37^{o}, the concentration of Mg^{++} in the incubation mixture had to be raised from 0.01M to 0.018M and the amount of added poly-U had to be increased to 500 µg. of poly-U per incubation mixture. This high level of polynucleotide may have helped stabilize a part of the system.

Bubela & Holdsworth (1966a) showed that the 20,000 g supernatant from a cell-free extract of B. stearothermophilus incorporated ^{14}C-labelled amino acids at 63^{o}; however the amount of incorporation at that temperature was only twice that found at 0^{o} and was very much less than the amount of incorporation into whole cells at 63^{o}. When poly-U was added to the 20,000 x g supernatant and phenylalanine incorporation was measured over a range of temperatures, maximum incorporation took place at 45^{o}. This result agrees with that obtained by Friedman & Weinstein (1966) which was discussed above.

Examination of fractions obtained from whole cells and from protoplasts of B. stearothermophilus, which had previously been incubated with ^{14}C-labelled amino acids at 63^{o}, revealed that the most active fraction was the one containing cytoplasmic membranes (Bubela & Holdsworth, 1966a). When membranes that had been washed three times were incubated with radioactive amino acids at 60^{o} the major

part of the radioactivity was associated with RNA in the membranes. Supplementation of the incubation mixture with crude RNA extracted from B. stearothermophilus resulted in an increased incorporation of the amino acids into protein. The maximum amount of incorporation took place at 60°. Only 20% decrease in activity occurred after the membranes had been heated at 65° for 10 min. Membranes that were treated with high frequency sound, released a soluble fraction which could activate amino acids; however the soluble system was less stable than the membrane-bound system and there was a 45% loss in activity when this preparation was heated at 63° for 10 min.

4. The Role of Stabilizing Factors and of the Primary, Secondary and Tertiary Structures in the Maintenance of Heat Stability in Proteins and Nucleic Acids.

So far only three enzymes from thermophilic bacteria have been purified to the state where they could be analyzed for the existence of heat-stabilizing factors: α-amylase (Manning & Campbell, 1961), glyceraldehyde-3-phosphate dehydrogenase (Amelunxen, 1966), and protease (Endo, 1963). Manning & Campbell (1961) showed that the α-amylase from B. stearothermophilus required calcium for maximum activity, however in this respect the enzyme did not differ from other α-amylases. Two atoms of calcium were firmly bound per molecule of the thermophile enzyme and were not removed by repeated dialysis against EDTA; hence there was no evidence to show that bound calcium was involved in the heat stability of the enzyme. Amino-acid analysis (Campbell & Manning, 1961) revealed that the enzyme had a high content of proline and glutamic acid, no

tryptophane, and a low content of tyrosine. The amount of glutamic acid plus aspartic acid was 2 to 2½ times-, and the amount of proline 4½ times-, greater than that found in α-amylase from other sources. Four half-cystine residues were found per mole of the heat-stable enzyme and it was assumed that they existed as cystine in the native enzyme since no sulphydryl groups could be detected. The enzyme had a large negative optical rotation (characteristic of unfolded proteins) which was not affected by 8M urea, 4M guanidine or high temperatures. The authors suggested that the enzyme existed as a semi-random - or random-coiled, hydrated molecule and that this lack of tertiary structure was responsible for its resistance to inactivation by solvents that break hydrogen bonds.

Unlike the α-amylase discussed above the thermostable protease from B. thermoproteolyticus existed in solution as a compact globular protein (Ohta et al 1966; Ohta, 1967). Amino acid analysis of the enzyme revealed that it contained no measurable cystine or cysteine and had a high content of tyrosine and an abundance of aromatic amino acids with hydrophobic side chains. Nineteen of the twenty nine tyrosine residues present in the enzyme behaved abnormally when the enzyme was titrated. The absence of a Cotton effect, in enzyme which had been modified by heating at 80° for 20 min., or by treatment with 8M urea, and the fact that the enzyme was stable at 80° only at a pH where the tyrosine did not ionize indicated that the tyrosine was involved in some way in the stability to heat of this enzyme (Ohta, 1967). The abnormal ionization of the tyrosine residues may be due to hydrogen bonding of the phenolic groups or to their being closely

associated with other non-polar side chains and Ohta concluded that the tyrosine residues stabilized the protein through hydrogen bonding of the phenolic groups and hydrophobic bonding involving the aromatic ring. Ohta stated that removal of calcium ion from the enzyme by dialysis against EDTA caused a loss in its activity and a decrease in its helical conformation; however no experimental evidence was given to support this statement.

Although the thermostable glyceraldehyde-3-phosphate dehydrogenase from B. stearothermophilus has been isolated, there is little information to explain its heat stability. Amelunxen (1967) showed that the partial specific volume of the enzyme was similar to that found for glycoproteins. The author reported that preliminary chemical analyses of the enzyme showed that a carbohydrate was firmly bound to the enzyme. The significance of the carbohydrate is not known.

Koffler et al (1957) could not find a stabilizing factor in the flagella of thermophilic Bacilli. When mixtures of flagella from thermophilic and mesophilic Bacilli were tested for heat stability at 60^{o} the mesophile flagella disintegrated while the thermophile flagella remained intact. If a thermostabilizing factor was present on the thermophilic flagella it was not transferable by simple diffusion. Extracts prepared from the cytoplasm or from the flagella of thermophilic organisms did not exert any stabilizing effect on the mesophile flagella. Thermophile flagella treated with ion-exchange resins remained heat-stable. The amino acid composition of flagella from thermophilic Bacilli was different from that found for the

flagella of mesophilic Bacilli (Abram & Koffler, 1962). Five of six strains of thermophilic bacteria tested had flagella containing more acidic amino acids than the flagella from seven mesophilic strains. The thermophilic flagella contained more serine than threonine whereas the mesophile flagella contained more threonine than serine. The amount of threonine in the thermophilic flagella was less than that found in the mesophile flagella. The effect of these differences in amino acid composition upon the heat stability of the respective flagella has not been determined. Abram & Koffler (1963) reported that flagella from strains 10 and 39 of B. stearothermophilus contained firmly-bound carbohydrate.

Mathemeier & Morita (1964) investigated the effect of magnesium (Mg^{++}) and cobalt (Co^{++}) on the activity of pyrophosphatase from B. stearothermophilus. They stated that when Co^{++} was used as a co-factor at 60^{o} the activity was half that obtained using Mg^{++} as co-factor. In contrast the activity of the pyrophosphatase at 80^{o} when Co^{++} was present was 20 times that obtained with Mg^{++} present. Examination of their data shows that the activity with Mg^{++} as co-factor decreased by 97% between 60^{o} and 80^{o}, whereas the activity with Co^{++} was approximately the same at both temperatures. The decrease in the activity of the Mg^{++}-activated enzyme was probably due to precipitation of magnesium pyrophosphate which they observed at temperatures above 70^{o}.

The thermal stabilities of t-RNA and a mixture of poly-U + poly-A were dependent on the Mg^{++} concentration (Friedman & Weinstein, 1966). When the t-RNA from B. stearothermophilus and from E. coli

was heated in the absence of magnesium, broad heat denaturation profiles were obtained with Tm values at about 60°. In the presence of 0.01M Mg^{++} the t-RNA from both species melted at higher temperatures and the Tm values were raised by 20° to approximately 80°. A mixture of equal amounts of poly-U and poly-A had a Tm of approximately 53° in the absence of Mg^{++}, whereas the Tm at a concentration of 0.005M Mg^{++} was approximately 80°. It is not apparent whether these findings have any bearing on the differences between thermophiles and mesophiles, since the t-RNA of both species was stabilized by magnesium.

5. The Dynamic Theory of Thermophilic Growth

The theory that thermophiles were capable of growing at high temperatures because they were able to rapidly repair vital cell constituents which had been destroyed by heat has been put forward by Gaughran (1947) and Allen (1950, 1953). Allen (1950) demonstrated that, in the absence of nutrients, resting cells of thermophilic bacteria were no more heat resistant than mesophilic bacteria. Allen presented 'killing' curves for mesophiles and thermophiles suspended in bicarbonate buffer at 55°. The rate of death was the same order of magnitude for both types of organism. Addition of glucose had little effect but the addition of nitrogenous nutrients allowed the thermophile to grow while the mesophile continued to die. Allen (1953) estimated the number of viable cells present at 60° in a culture of a thermophile, B. circulans, and showed that when active growth had ceased the viable count decreased rapidly. The total

number of cells, throughout the logarithmic phase of growth, was always greater than the number of viable cells. Allen suggested that provided the overall rate of the anabolic processes exceeded that of the catabolic processes, in the cell, then the total viable population increased; but when the catabolic processes became dominant a rapid decline in the viable cell population occurred.

The turnover of proteins and nucleic acids in whole cells of B. stearothermophilus was investigated by Bubela & Holdsworth (1966). The turnover of protein (these authors used the time taken for half the previously ^{14}C-labelled protein to breakdown) at 63^{o} was 1 to 2 min. Even at 40^{o} the turnover of protein was 10 min. and this was more rapid than that for E. coli which, in similar experiments, was found to be 24 mins. The turnover time of labile RNA in the thermophile was 1 min. at both 40^{o} and 63^{o}. The rapid turnover of protein and labile RNA (presumed to be m-RNA) was consistent with the theory that the thermophilic organism had a mechanism which could synthesize proteins at a rapid rate.

D. DISCUSSION

A search of the literature, pertaining to thermophilic bacteria, reveals that of the few cell-free soluble or particulate enzymes examined from bacteria grown above 50^{o} most were heat stable.

One of the prerequisites for the continued activity of an enzyme at high temperatures is that the three-dimensional configuration of

its active centre must remain constant. This situation may arise in one of three ways:

(i) The intramolecular bonding, in the enzyme, which is responsible for the spatial relationship of the essential chemical groups in the active centre, must be resistant to breakage at the high temperatures. This type of heat stability is defined as intrinsic heat stability.

(ii) The enzyme may be attached to, or incorporated into, a cellular structure, such as a lipoprotein membrane, which provides a semi-rigid support and prevents the unfolding of the polypeptide chain(s) of the enzyme.

(iii) A specific heat stabilizing factor, other than a cell organelle, may prevent heat denaturation by its mode of binding to the enzyme.

The soluble enzymes, and flagella, studied so far appear to be intrinsically heat-stable. It must be pointed out that the association of the soluble enzymes with heat-stabilizing factors has not been disproved. The absence of such factors can only be proved by further analyses of the relevant pure enzymes.

The particulate enzymes, reviewed in the previous sections, fall into all three categories with regard to their resistance to heat denaturation. The enzymes that were heat-stable both in their particulate and solubilized forms were intrinsically heat-stable. One enzyme that was heat stable in its particulate form became heat-labile after

it had been solubilized and partly purified. This enzyme was apparently stabilized by its association with lipoprotein membranes. The remaining example is that of a complex particulate enzyme system which was stable to heat only in the presence of an inorganic ion, oxygen and its substrate.

Although detailed studies by various workers have shown that the DNA, ribosomes, ribosomal RNA and t-RNA from thermophilic bacteria were heat-stable, the reconstituted protein synthesizing systems and the protein synthesizing activity of crude extracts were not stable to heat. The low activity of these systems was probably due to the instability, at high temperatures, of the aminoacyl synthetases, aminoacyl t-RNA complexes and the m-RNAs; the latter having a short half-life even in mesophilic organisms. The presence of nucleotidases in cell-free extracts would also lead to a decrease in amino acid incorporation. Protein synthesis and m-RNA synthesis was found to proceed so rapidly in vivo that long-lived aminoacyl t-RNA complexes and m-RNA are probably not necessary in the whole cell.

The membrane-bound protein-synthesizing system, which was heat-stable, is interesting since it may be a major pathway for the synthesis of proteins in vivo. This system became heat-labile after it was released from the cell membranes, and is a further example of the stabilization of enzymes, at high temperatures, by their close association with cellular structures.

The evidence presented previously suggests that thermophilic bacteria continue to grow and metabolise at temperatures which cause

the death of mesophilic bacteria because of the following factors:

(i) Thermophilic bacteria contain a high proportion of heat-stable enzymes which are of two general types:

a. Intrinsically heat-stable enzymes.

b. Enzymes which are stable to heat because of their association with cellular structures or heat stabilizing factors.

(ii) The high rate both of metabolism and protein synthesis, in thermophilic bacteria, result in the rapid replacement of those enzymes which are denatured by heat.

PART 2

NITRATE REDUCTASE IN MICROORGANISMS

A. DEFINITION OF NITRATE REDUCTASE

The reduction of nitrate (in which nitrogen has an oxidation level of 5+) to a series of nitrogen compounds with oxidation levels from 4+ (nitrite) to 3- (ammonium) is catalyzed, in living organisms, by a number of enzymes. Nitrate reductase, the initial enzyme in the series, catalyzes the reduction of nitrate to nitrite.

Two general types of nitrate reduction have been recognized:

(i) Nitrate assimilation or assimilatory nitrate reduction in which the nitrate is converted to the ammonium or amino level and is ultimately used for the synthesis of nitrogen-containing compounds. The nitrate reductase responsible for the formation of nitrite in this process is called assimilatory nitrate reductase.

(ii) Nitrate respiration or dissimilatory nitrate reduction in which nitrate is used as a terminal electron acceptor in place of oxygen. The reduction products are not used further and are excreted. If the products of nitrate respiration are gaseous the process is known as denitrification. The enzyme responsible for the reduction of nitrate to nitrite in this process is called respiratory nitrate reductase.

The two types of nitrate reductase will be discussed separately in the following sections.

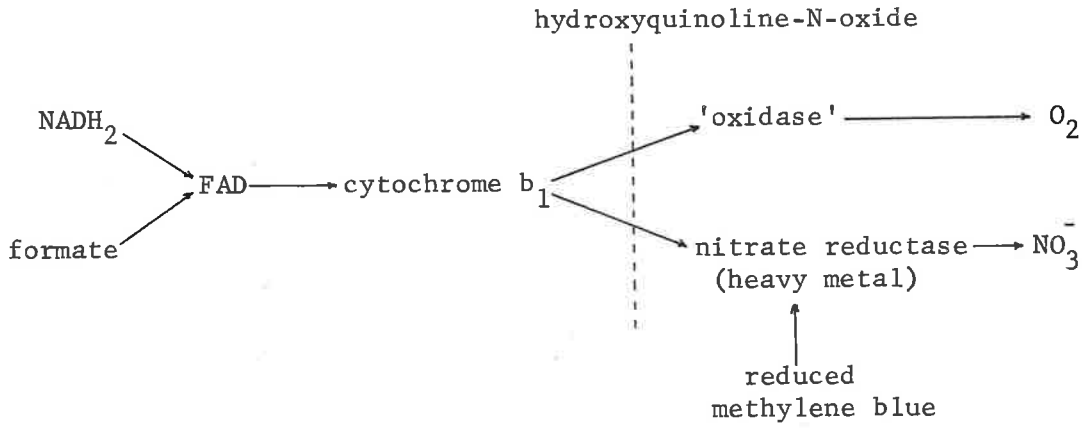
B. RESPIRATORY NITRATE REDUCTASE

Quastel, Stephenson & Wetham (1925) demonstrated that a strain of E. coli was unable to grow anaerobically with lactate as a sole carbon source unless nitrate was present.

Subsequently it was shown that the reduction of nitrate was mediated by an enzyme (Quastel & Woolridge, 1929). The earliest report concerning the reduction of nitrate by cell-free extracts from E. coli was made by Green, Stickland & Tarr (1934). This was followed by the work of Yamagata (1938a; 1938b) in which he showed that cell-free extracts, obtained from autolyzed E. coli, reduced nitrate with leuco-methylene blue as a hydrogen donor.

Sato & Egami (1949) reported that the particulate nitrate-reducing system from E. coli (Yamaguchi strain), grown aerobically on complex medium containing nitrate, was inhibited by carbon monoxide and that light reversed the inhibition. These authors believed that the nitrate reductase was an iron-enzyme; probably cytochrome b_1. Later observations showed that, unlike the cytochrome oxidase activity of the particles, the nitrate reductase activity was inhibited by thiourea (Sato & Niwa, 1952). Since cytochrome b_1 was common to both the cytochrome oxidase system and the nitrate reductase system, the nitrate reductase could not have been identical to the cytochrome. The role of cytochrome b_1 in the particulate nitrate reducing system from aerobically grown E. coli was finally established on the basis of spectral and inhibitor studies made by Taniguchi, Sato & Egami (1956). They found that the reduced spectrum of cytochrome b_1 disappeared after

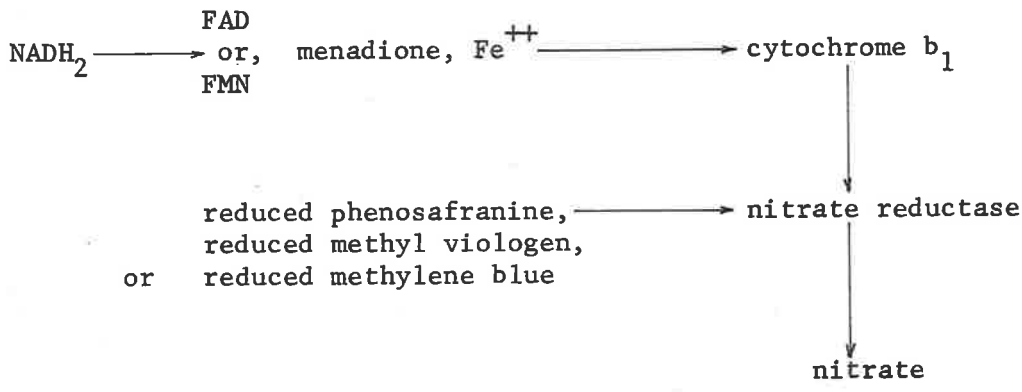
the anaerobic addition of nitrate. Furthermore 2-n-heptyl-4-hydroxyquinoline-N-oxide, which is an inhibitor of the oxidation of reduced cytochrome b_1 (Lightbown & Jackson, 1956), inhibited the transfer of electrons from $NADH_2$ or formate to nitrate reductase. The above observations and the stimulation of $NADH_2$-nitrate reductase activity by FAD, led Taniguchi et al. to propose the following scheme for nitrate reduction:



Iida & Taniguchi (1959) confirmed the electron transport sequence proposed by Taniguchi et al. (1956) and extended it to include a heat-stable, acid-labile, dialyzable factor which was only partly replaceable by Fe^{++}, menadione or FAD. They further showed that inhibition by Dicumarol was partly reversed by menadione. The inhibition of reduced methylene blue-nitrate reductase activity by metal-binding agents indicated that an unknown metal component was associated with the terminal nitrate reductase. Treatment of the particulate system with isobutanol resulted in the cleavage of the respiratory chain between $NADH_2$, formate and cytochrome b_1.

A non-particulate nitrate reducing system was isolated from

E. coli (Yamaguchi strain) grown aerobically on a synthetic medium (Itagaki & Taniguchi, 1959). $NADH_2$, but not formate, could be used as a hydrogen donor for the reduction of nitrate. The addition of FAD, FMN, menadione and ferrous ions to dialyzed preparations stimulated this $NADH_2$-nitrate reductase activity. Fe^{++} alone could be used as an electron donor for nitrate reduction via cytochrome b_1 and both this activity and that of $NADH_2$-nitrate reductase were inhibited by 2-n-heptyl-4-hydroxyquinoline-N-oxide. Dicumarol inhibition of $NADH_2$-nitrate reductase activity was reversed by menadione which indicated that a naphthoquinone derivative was a member of the electron transport chain. A heavy metal component was implicated in the terminal portion of the chain by the fact that leuco-methylene blue-nitrate reductase activity was inhibited by both cyanide and azide. Itagaki and Taniguchi proposed the following sequence for the components of the soluble $NADH_2$-nitrate reductase system:



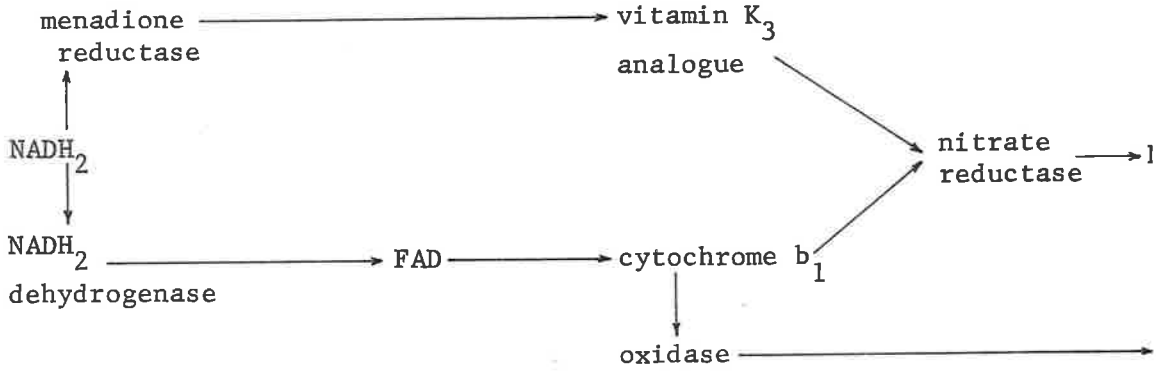
The particulate nitrate reductase obtained from E. coli (Yamaguchi strain) which had been grown anaerobically on complex

medium in the presence of nitrate (Taniguchi & Itagaki, 1960) was found to be similar to the particulate system extracted from aerobically-grown E. coli (Taniguchi et al., 1956). When the particulate system from anaerobically-grown cells was treated with deoxycholate and snake venom it yielded a soluble formate-nitrate reductase system (Itagaki, Fujita & Sato, 1961, 1963). Spectroscopic evidence showed conclusively that cytochrome b_1 was involved in the electron transfer from formate to nitrate reductase. The cytochrome was autoxidizable and the oxidation was not prevented by hydroxyquinoline-N-oxide whereas oxidation of cytochrome b_1 by nitrate was (Itagaki, Fujita & Sato, 1963). The terminal nitrate reductase was released from the particulate system by heat-treatment and incubation with Steapsin (Taniguchi & Itagaki, 1959). The soluble enzyme would accept electrons from reduced dyestuffs but not from $NADH_2$ or formate. The authors produced evidence that the enzyme contained flavin but no cytochrome. In a later communication Taniguchi & Itagaki (1960) presented a modified extraction procedure in which the particulate nitrate reductase was subjected to heat treatment in sodium phosphate pH 8.3 followed by incubation in the same buffer for 20 hr. at 4°. After purification, by adsorption onto calcium phosphate gel and by ammonium sulphate fractionation, soluble nitrate reductase was obtained which differed from the Steapsin-solubilized enzyme in that the flavin content had decreased to insignificant levels. The purified enzyme had a molecular weight of 10^6 and contained one atom of molybdenum and about 40 non-haem iron atoms per molecule.

Ferricyanide, ferrous sulphate, ascorbate, reduced mammalian cytochrome c, reduced cytochrome c_3 and $NADH_2$ were inactive as electron donors for nitrate reduction. However slight activity was found when reduced FAD, reduced FMN or reduced riboflavin were used.

Reduced methyl viologen and reduced benzyl viologen were the most effective of the artificial electron donors tested.

Medina & Heredia (1958) and Heredia & Medina (1960) obtained a particulate fraction from aerobically grown E. coli (strain 86) which they claimed contained two nitrate reducing systems. Their scheme is reproduced below.



They based this scheme on the following results:

(i) Nitrate reductase activity was not detected aerobically unless menadione was added to the extract.

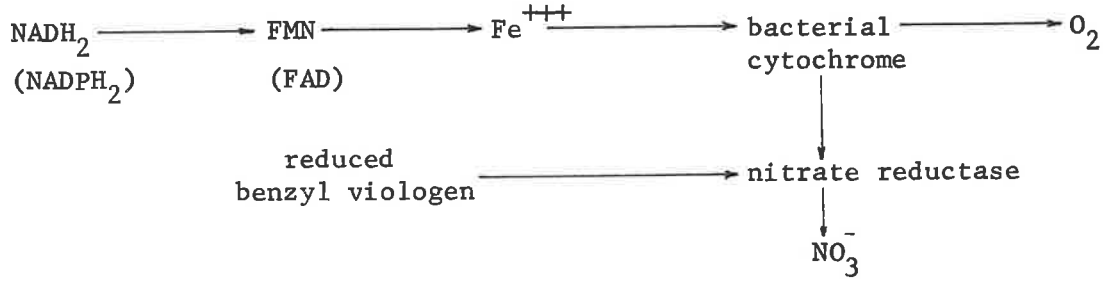
(ii) The inhibition of $NADH_2$-nitrate reductase activity by atebrine and by 2-n-heptyl-4-hydroxyquinoline-N-oxide was reversed by menadione; FAD did not reverse the inhibition caused by atebrine.

(iii) The anaerobic reduction of nitrate by $NADH_2$ was stimulated by FAD and did not require menadione.

A close examination of their results reveals that the aerobic oxidation of $NADH_2$ was stimulated 10-fold upon the addition of menadione. This suggests that a naphthoquinone derivative should be included in the pathway from $NADH_2$ to oxygen.

The formate-nitrate reductase system, solubilized by Itagaki et al. (1963) required menadione for maximum activity. 2n-heptyl-4-hydroxyquinoline-N-oxide inhibited formate nitrate reductase activity both in the presence and absence of menadione. The response of the hydroxyquinoline-N-oxide-inhibited nitrate-reductase activity to increasing concentrations of menadione was non-competitive in contrast to the results of Heredia & Medina (1960).

A particulate nitrate-reducing system was obtained from Achromobacter Fischeri by osmotic lysis of the cells (Sadana & McElroy, 1957). The system was separated into a soluble $NADH_2$-cytochrome c reductase fraction and a soluble nitrate-reductase contaminated with autoxidizable cytochrome c. Sadana & McElroy proposed the following scheme for reduced pyridine nucleotide-nitrate reductase:

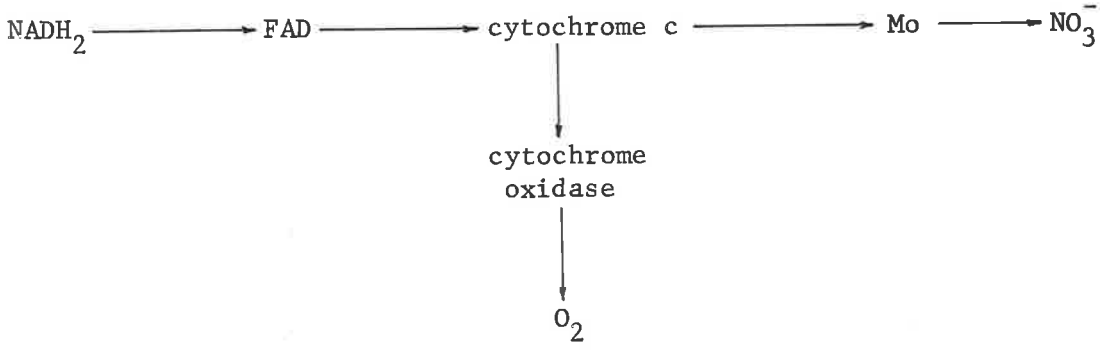


The $NADH_2$-cytochrome c reductase portion of the chain required FMN or FAD for full activity. Carbon monoxide inhibited the $NADH_2$-nitrate reductase activity and this inhibition was reversed by light. On the other hand the reduced benzyl viologen-nitrate reductase activity was unaffected by CO - indicating that the terminal nitrate reductase was not a cytochrome.

In a later communication Sadana, Rao & Joshi (1963) described a purification procedure for the nitrate reductase which resulted in the removal of the cytochrome impurities. The purified enzyme would not oxidize $NADH_2$ or $NADPH_2$ with either oxygen, dystuffs, or cytochrome c as electron acceptors.

A number of metal ions including Fe^{++}, Fe^{+++} and molybdenum as well as FMN or FAD failed to stimulate the reduced benzyl viologen-nitrate reductase activity. In the ultracentrifuge two components were observed for the soluble enzyme, with S_{20w} values of 1.5 and 4.5 respectively. These low values indicated that the enzyme from A. fischeri was a much smaller molecule than the E. coli nitrate reductase (Taniguchi & Itagaki, 1960).

Fewson & Nicholas (1961a) have described a particulate nitrate-reducing system from Pseudomonas aeruginosa. The proposed sequence of electron transfer in the system is shown below:



The spectra reproduced by Fewson & Nicholas showed that the nitrate reducing system contained a b- type and a c-type cytochrome; both of these cytochromes became oxidized upon the addition of nitrate. However the authors stated that cytochrome b did not appear to be involved because Antimycin A (50 μM) did not inhibit the $NADH_2$-nitrate reductase activity. This assumption may not be correct since Antimycin A has been shown to be inaffective as an inhibitor in many bacterial systems unless extremely high concentrations are used.

Fewson & Nicholas (1961b) have also stated that cytochrome c was involved in the electron transfer pathway from hydrogen and $NADH_2$ to nitrate in the bacterium Micrococcus denitrificans. These observations were contrary to those of Vernon (1956a) who demonstrated that the cytochromes b_1 from both Ps. denitrificans and Micrococcus denitrificans were oxidized immediately when nitrate was added anaerobically to the respective purified b_1 cytochromes plus cell extracts. On the other hand the reduced purified Pseudomonas cytochrome c plus extracts from Ps. denitrificans remained unoxidized after the addition of nitrate.

A nitrate reducing system from Neurospora crassa has been purified by Nicholas & Wilson (1964). The authors claimed that the enzyme was of the dissimilatory type and they based this claim on the fact that an increase occurred in both the amounts of b- and c-type cytochromes and nitrate reductase at low oxygen tensions. Nutritional studies showed that when the iron concentration of the medium was decreased to low levels the specific activity of the nitrate reductase decreased (Walker & Nicholas, 1961; Nicholas & Wilson, 1964). Nowhere in their communication (Nicholas & Wilson, 1964) did the authors show conclusively that the enzyme was of the dissimilatory type. Further work will need to be done in order to characterize the enzyme. This enzyme will be considered further in the section on assimilatory nitrate reductase.

Cheniae & Evans (1959) isolated a particulate fraction with nitrate reductase activity from Rhizobium japonicum. Nitrate was reduced by the particles with either $NADH_2$ or succinate as substrate and both of these activities were inhibited by Antimycin A. The terminal nitrate reductase enzyme accepted electrons from either reduced benzyl viologen or reduced methyl viologen. The involvement of a naphthoquinone derivative in the electron transfer pathway from $NADH_2$ to nitrate reductase was indicated by the inhibition of $NADH_2$-nitrate reductase activity by Dicumarol. Because of the difficulties encountered in measuring the spectrum of the particle suspension direct evidence for the role of a cytochrome in the particulate nitrate

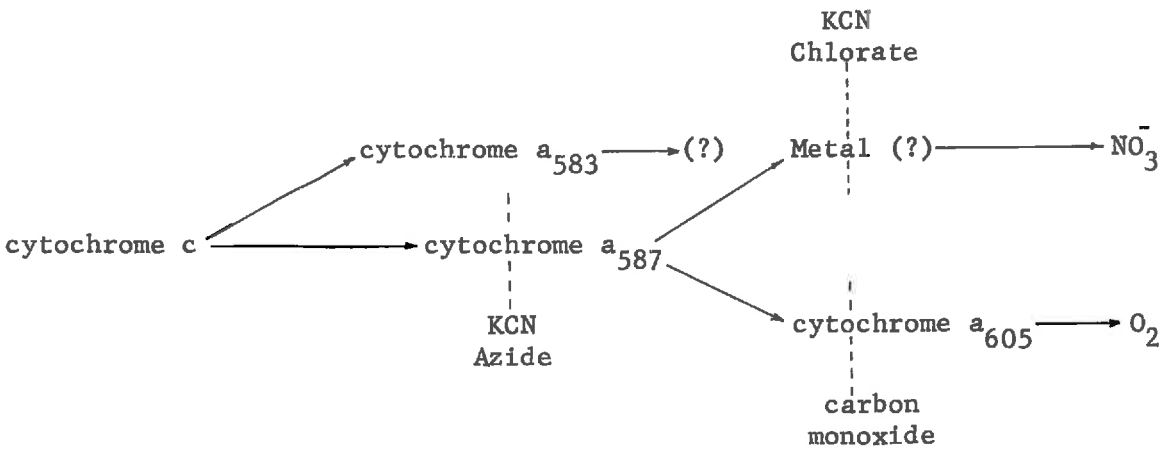
reducing system was inconclusive.

Solubilization of the particulate nitrate reductase was accomplished by grinding and alternately freezing and thawing the bacteria in the presence of Steapsin (Lowe & Evans, 1964). The purification and characterization of the enzyme was complicated by the loss of activity which occurred unless benzyl viologen and sodium dithionite ($Na_2S_2O_4$) were present. The 11-fold purified enzyme was inactive when $NADH_2$, $NADPH_2$, succinate, reduced ferredoxin or reduced molybdate were used as donors. Reduced benzyl viologen and reduced methyl viologen were the only effective electron donors amongst those tested. The reduced benzyl viologen-nitrate reductase activity was insensitive to most metal chelating agents with the exception of thiourea and diethyl-dithiocarbamate. The loss of activity which occurred after dialysis against cyanide could not be restored by a number of metals including iron and molybdenum.

The role of nitrate reductase in the symbiotic nitrogen fixation mediated by R. japonicum is not clear but Cheniae & Evans (1957) have found a positive correlation between nitrate reductase activity and the nitrogen fixing capacity of various strains in symbiosis with the host plant.

A respiratory nitrate reducing system from the chemoautotroph Nitrobacter agilis has been studied by Straat & Nason (1965). They found that the partly purified system contained cytochromes c, b, a, and a_1. The cytochrome a_1 spectrum at liquid nitrogen temperatures

was found to consist of two components with α bands at 583mμ and 587mμ respectively. Reduced horse heart cytochrome c would serve as the electron donor for nitrate reduction. The reduced Nitrobacter cytochrome c and one component of the cytochrome a_1 (α peak at 587mμ ; referred to hereafter as cytochrome a_{587}) were involved in the electron transfer to nitrate. The cytochrome c oxidase system contained these same cytochromes plus cytochrome a (cytochrome a_{605}) which acted as the terminal electron acceptor. Carbon monoxide combined with cytochrome a_{605} and inhibited the cytochrome c oxidase activity but cytochrome c-nitrate reductase activity was not affected by this inhibitor. Studies with metal chelating agents revealed that a heavy metal was involved in the electron transport chain from cyt a_{587} to nitrate. Cyanide inhibited both cytochrome c oxidase and cytochrome c-nitrate reductase activity. The inhibition of nitrate reductase by cyanide exhibited competitive kinetics with respect to nitrate if the nitrate was present before the addition of cyanide. Chlorate also was found to be a competitive inhibitor of nitrate reductase. The following sequence of electron transport was proposed:

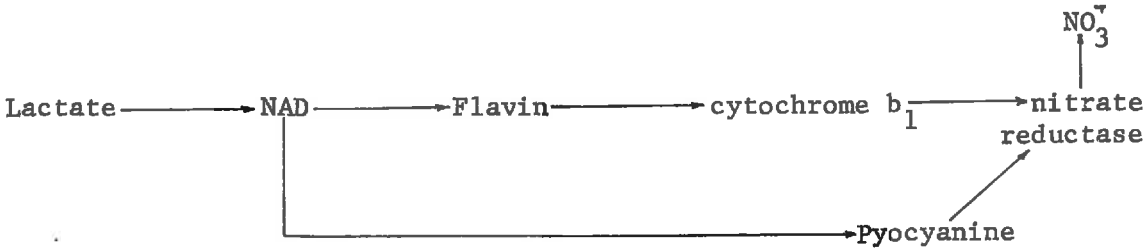


The nitrate reductase system of anaerobically-grown Bacillus cereus has been prepared in a cell-free state by disruption of the cells followed by ammonium sulphate fractionation (Hackenthal & Hackenthal, 1965). 70% to 96% of the initial activity remained in the supernatant after centrifugation of the partly purified enzyme system at 100,000 x g for 3 hr. The nitrate reductase system accepted electrons from $NADH_2$ (or $NADPH_2$) and required FMN (or FAD) for maximum activity. 2n-heptyl-4-hydroxyquinoline-N-oxide inhibited $NADH_2$-nitrate reductase activity although when exogenous FMN was present inhibition did not occur. Menadione stimulated $NADH_2$-nitrate reductase activity in the absence of added FMN. Both reduced FMN and reduced FAD were utilized as electron donors for nitrate reduction. Reduced methyl viologen and reduced benzyl viologen were the most active electron donors respectively. No inhibition was observed with atebrine and the authors assumed that the nitrate reductase system did not contain flavoprotein.

The by-passing of the site of hydroxyquinoline-N-oxide inhibition by FMN is interesting in view of the lack of inhibition by atebrine. The authors suggested that the nitrate reductase system consisted of menadione reductase, cytochrome b_1 and nitrate reductase and that the activity with FMN or FAD was brought about by an associated $NADH_2$ dehydrogenase. It is possible that the two types of nitrate reductase were present i.e. a dissimilatory nitrate reductase and an assimilatory nitrate reductase, with the latter utilizing reduced flavin nucleotides for the reduction of nitrate.

Chang & Lascelles (1963) studied a strain of Staphylococcus aureus (strain SG511 Var) which was unable to reduce nitrate to nitrite unless haemin was present. The cell-free nitrate reductase system from organisms grown on nitrate-medium without haemin could utilize lactate as a hydrogen donor provided haemin was added to the reaction mixture. Haemin was not required if pyocyanine was used as an artificial electron donor in conjunction with lactate and lactic dehydrogenase.

Chang & Lascelles suggested that the path of electron transfer to nitrate was as follows:



This scheme was based upon the following observations:

(i) NAD and FMN or FAD were necessary for the maximum activity of the haemin dependent system.

(ii) The haemin dependent reduction of nitrate was inhibited by the cytochrome b inhibitor 2-n-heptyl-4-hydroxy-quinoline-N-oxide and by amytal, whereas electron transfer mediated by pyocyanine was not affected by either of these inhibitors.

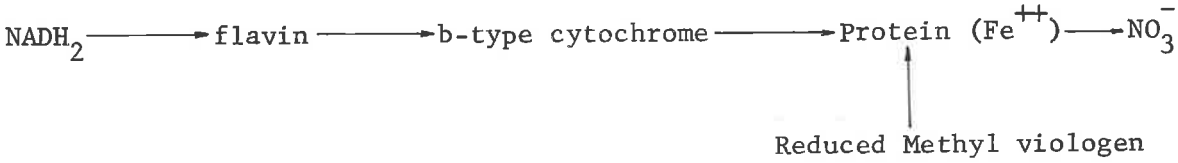
(iii) Cytochrome absorption bands were not detected in cell-free

extracts from organisms grown without haemin and the level of lactate-nitrate reductase activity was not significant. However the washed particulate fraction obtained from crude extracts, which had been pre-incubated with haemin, exhibited the absorption spectrum of cytochrome b_1. The same particles reduced nitrate with lactate as a donor, in the absence of added haemin.

Inhibition of the haemin-dependent nitrate reductase system by metal chelators and cyanide or azide implicated heavy metals as components of the system. Iron, as iron citrate, stimulated the production of nitrite.

The nitrate reducing system from Rhodospirillum rubrum has been solubilized and purified by Katoh (1963). The purified complex was active with either $NADH_2$ or reduced methyl viologen as electron donors. Antimycin A almost completely inhibited $NADH_2$-nitrate reductase activity but did not affect methyl viologen-nitrate reductase activity. Neither FMN, FAD nor menadione caused any marked stimulation of the $NADH_2$-nitrate reductase activity. Cyanide, 8-hydroxyquinoline and O-phenanthroline inhibited both the $NADH_2$-nitrate reductase system and the benzyl viologen-nitrate reductase system. 75% of the reduced benzyl viologen-nitrate reductase activity was lost after dialysis against cyanide; ferrous ions caused a slight reactivation of the enzyme but molybdate had no effect. The participation of a b-type cytochrome and flavin in electron transfer from $NADH_2$ to nitrate was demonstrated by spectral

studies. Katoh suggested the following scheme for Rsp. rubrum nitrate reductase:



Downey (1966) has investigated the effects of nitrate upon the anaerobic growth of Bacillus stearothermophilus (strain 2184). The anaerobic growth rate of the organism increased approximately ten-fold when nitrate was added to complex medium containing sucrose; however this growth rate was very much less than that found under aerobic conditions. Nitrate did not stimulate anaerobic growth on minimal medium with or without casein hydrolysate.

Particles prepared from cells grown anaerobically on complex mediums in the presence of nitrate, reduced nitrate to nitrite with $NADH_2$, malate, succinate or reduced benzyl viologen as donors. The same particles contained cytochrome b_1 and c_1; cytochrome a_3, which was present in similar preparations obtained from aerobically grown cells, was missing.

The addition of nitrate to a particulate preparation in which the cytochromes had been reduced with malate resulted in the oxidation of the cytochrome b_1; cytochrome c_1 remained reduced but became oxidized when oxygen was bubbled through the preparation. 2n-heptyl-4-hydroxyquinoline-N-oxide, and its 2-n-nonyl analogue,

caused only slight inhibition of the malate-nitrate reductase activity and had no affect on either reduced benzyl viologen-, or succinate-, nitrate reductase activity. Cyanide, azide, 8-hydroxyquinoline and EDTA inhibited nitrate reductase activity with either malate, succinate or reduced benzyl viologen as hydrogen donors. Malate- and succinate-, nitrate reductase were both inhibited by mepacrine. Downey did not postulate a scheme for electron transfer from the various donor substances to nitrate but it is obvious from his data that cytochrome b_1, and not cytochrome c_1, is the cytochrome which mediates electron transfer to nitrate reductase in B. stearothermophilus.

C. ASSIMILATORY NITRATE REDUCTASE

The assimilatory nitrate reductase from Neurospora crassa was purified 60- to 70-fold by Nason & Evans (1953). The enzyme showed a specificity for $NADPH_2$ as hydrogen donor; the maximal rate obtained with $NADPH_2$ was 20 times that obtained using $NADH_2$. Enzyme inactivated during ammonium sulphate fractionation was reactivated in full by FAD and in part by FMN. Further evidence for the presence of FAD in the enzyme was provided by fluorescence studies and by enzymic studies using the apo-enzyme of D-amino acid oxidase.

The inhibition of $NADPH_2$-nitrate reductase activity by p-chloromercuribenzoate was reversed by both cysteine and glutathione indicating that -SH groups on the enzyme were necessary for full activity.

The enzyme was inhibited by cyanide, azide and a number of metal chelators suggesting that a heavy metal component was necessary for activity. However nitrate reductase activity was not stimulated by a number of metals including molybdenum. Iron, magnesium, zinc and manganese were apparently not necessary for maximum enzyme activity since the specific activity of nitrate reductase was high in mycelia deficient in these metals. Nutrient studies showed that mycelia from both Neurospora crassa and Aspergillus niger contained less nitrate reductase when grown on media deficient in molybdenum than mycelia grown on molybdenum-rich media (Nicholas, Nason & McElroy, 1954). That molybdenum was part of the nitrate reductase enzyme was demonstrated by Nicholas & Nason (1954a). They showed that purified enzyme was partly inactivated after dialysis against cyanide with a concomitant decrease in the molybdenum content of the enzyme. When molybdenum salts were added to cyanide-free enzyme, that had been inactivated by dialysis against cyanide, reactivation of the enzyme was obtained. However the specific activity of nitrate reductase in extracts from mycelia grown on molybdenum-deficient medium was not increased by the addition of molybdenum. This suggested that molybdenum was also necessary for the de novo synthesis of the enzyme.

The sequence of the components of the enzyme was found to be:

$$NADPH_2 \longrightarrow FAD(\text{or } FMN) \longrightarrow Mo \longrightarrow NO_3^-$$

(Nicholas & Nason, 1954b). This sequence was based on the following observations:

(i) Flavin (FMN) was reduced by $NADPH_2$ in the absence of nitrate under anaerobic conditions. Molybdenum was not essential for this step since the amount of $FMNH_2$ formed by both molybdenum-free enzyme and cyanide-inhibited enzyme was the same as that formed by 'native' enzyme.

(ii) $FMNH_2$ served as a donor for the reduction, by nitrate reductase, of added molybdate. Of the other metals tested only iron (Fe^{+++}) oxidized $FMNH_2$ enzymically. The rate of oxidation with Fe^{+++} was one-fifth of the rate with molybdate.

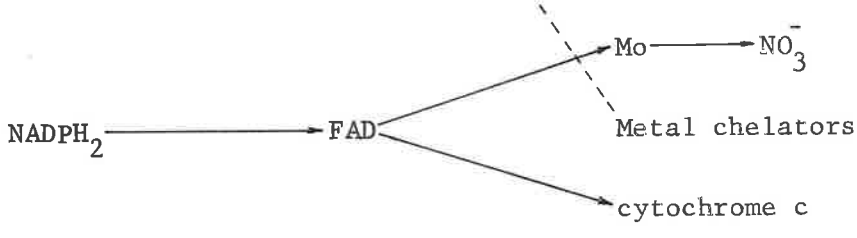
(iii) Dithionite-reduced-molybdate acted as an electron donor for the enzymic reduction of nitrate. Neither FMN nor FAD stimulated the molybdate-nitrate reductase activity. The bound molybdenum of the enzyme was not readily reduced by dithionite since low activity was observed when dithionite alone was used as a donor.

The possibility exists that the enzyme consisted of one protein with two catalytic activities, namely; $NADPH_2$-flavin : oxido-reductase activity; and reduced flavin -nitrate: oxidoreductase activity or that two proteins, firmly bound together, catalyzed these two activities. However Nicholas & Nason were unable to demonstrate

the presence of more than one protein by fractionation techniques, although electrophoresis showed that the protein was not homogeneous.

Experiments conducted by Kinsky & McElroy (1958) revealed that $NADPH_2$-nitrate reductase from Neurospora crassa was closely associated with $NADPH_2$-cytochrome c reductase. The latter activity increased in parallel with $NADPH_2$-nitrate reductase activity during purification. The increase in $NADPH_2$-cytochrome c reductase activity was concurrent with the increase in $NADPH_2$-nitrate reductase activity when Neurospora crassa was grown on media containing increasing amounts of nitrate. However when ammonia was the sole source of nitrogen, the mycelia lacked nitrate reductase activity whereas cytochrome c reductase activity was present but its specific activity was only 50% of that found in nitrate-grown mycelia.

Experiments with cell-free nitrate reductase showed that, unlike the $NADPH_2$-cytochrome c reductase, the $NADPH_2$-nitrate reductase was inhibited by metal chelating agents. The following relationship between the two activities was proposed:



This scheme supports the suggestion that at least two distinct enzymes may be involved in the reduction of nitrate by $NADPH_2$.

Sorger (1965) has investigated the relationship between $NADPH_2$-nitrate reductase and $NADPH_2$-cytochrome c reductase in Neurospora crassa. He showed that both activities were induced by nitrate and both were repressed by α methyl alanine. Both enzyme activities were inactivated to the same degree when subjected to heat treatment. In a later communication Sorger (1966) reported the fractionation, using sucrose gradient centrifugation, of cell-free extracts of nitrate-induced wild-type Neurospora crassa. He obtained two fractions: one heavy fraction with $NADPH_2$-cytochrome c reductase activity and another lighter fraction with both $NADPH_2$-cytochrome c reductase and $NADPH_2$-nitrate reductase activities. When extracts were treated with Steapsin before centrifugation on sucrose gradients, the light $NADPH_2$-nitrate reductase was apparently converted to a form which retained $NADPH_2$-cytochrome c reductase activity but had lost most of its nitrate reductase activity. The heavy fraction, exhibiting $NADPH_2$-cytochrome c reductase activity, was unaffected by Steapsin treatment. The approximate S_{20w} values of the two light fractions were: 6.8 for the untreated $NADPH_2$-nitrate reductase and 3.8 for the Steapsin treated fraction. Extracts from non-induced wild-type Neurospora contained two fractions, a major heavy fraction and a minor fraction corresponding to the fraction with an S_{20w} value of 6.8, found in induced extracts. Sorger suggested that the heavy fraction, with $NADPH_2$-cytochrome c reductase activity, found in extracts from both non-induced-, and nitrate-induced-, wild-type neurospora was a constitutive enzyme system

whereas the fraction with the S_{20w} value of 6.8, found mainly in nitrate-induced Neurospora extracts was an inducible $NADPH_2$- nitrate reductase associated with nitrate-inducible $NADPH_2$-cytochrome c reductase.

An investigation of the sucrose gradient distribution of constitutive $NADPH_2$-cytochrome c reductase, nitrate inducible $NADPH_2$-cytochrome c reductase and benzyl viologen nitrate reductase activity of extracts from mutants of Neurospora crassa, which were deficient in inducible $NADPH_2$-nitrate reductase activity provided the following information:

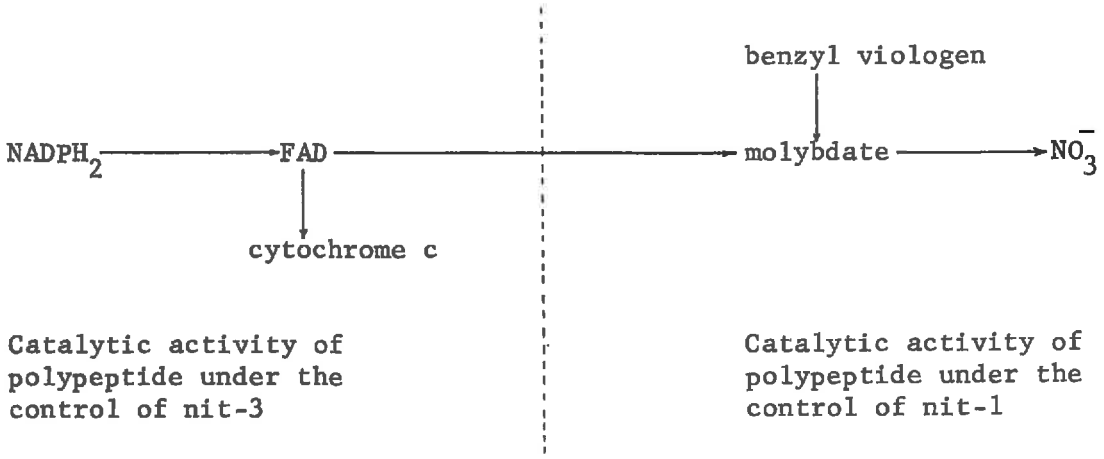
(i) Mutant nit-1 could synthesize constitutive and nitrate-inducible $NADPH_2$-cytochrome c reductase but not benzyl viologen-nitrate reductase.

(ii) Mutants nit-2 and nit-5 could synthesize only constitutive $NADPH_2$-cytochrome c reductase.

(iii) Mutant nit-3 synthesized constitutive $NADPH_2$-cytochrome c reductase and benzyl viologen-nitrate reductase.

It must be emphasized that none of the above mutants contained $NADPH_2$-nitrate reductase.

On the basis of the above observations Sorger proposed the following scheme:



In view of the above observations by Sorger the classification of the benzyl viologen-nitrate reductase purified by Nicholas & Wilson (1964), which was briefly discussed in the section on respiratory nitrate reductase, requires careful consideration. According to the scheme proposed by Sorger the terminal nitrate reductase should accept electrons from reduced $FADH_2$, however Sorger did not test $FADH_2$ alone as a donor for nitrate reductase. On the other hand Nicholas & Wilson (1964) demonstrated that their partly purified enzyme did not use $FMNH_2$ as a donor. Until the physiological donor for the nitrate reductase isolated by Nicholas & Wilson is determined the classification of the enzyme remains uncertain.

The $NADPH_2$-nitrate reductase of Aspergillus nidulans was found to be similar to the Neurospora crassa enzyme in that it had $NADPH_2$-cytochrome c reductase activity (Pateman, Cove, Reever & Roberts, 1964). These authors investigated the induction and repression of $NADPH_2$-nitrate reductase and $NADPH_2$-cytochrome c reductase in a number of mutants that showed only slight growth with nitrate as a sole

nitrogen source. On the basis of their results they postulated that:

(i) a co-factor, probably containing molybdenum, was necessary for nitrate reductase activity and that some of the mutants were unable to synthesize this co-factor,

(ii) the nitrate reductase protein possessed $NADPH_2$-cytochrome c reductase activity that was not dependent upon the co-factor for activity,

(iii) the co-factor acted as a repressor of nitrate reductase protein synthesis in the absence of nitrate, and nitrate acted as a depressor by interacting with the co-factor.

Pateman et al. suggested that both nitrate inducible $NADPH_2$-cytochrome c reductase activity and $NADPH_2$-nitrate reductase activity were catalyzed by the same protein. All the mutants had $NADPH_2$-cytochrome c reductase activity when grown without nitrate. This enzyme was probably constitutive and not the same as the nitrate inducible enzyme.

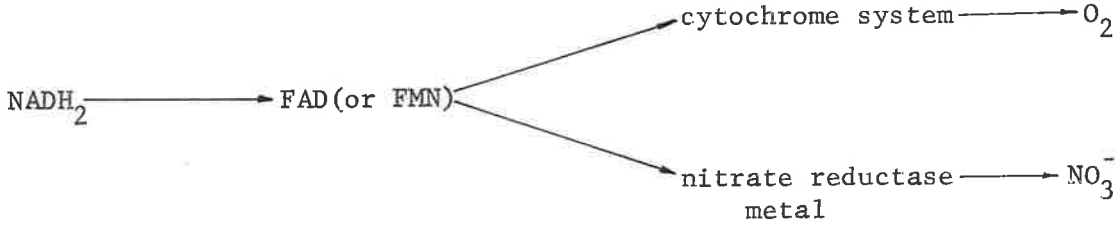
Cove & Coddington (1965) have purified the $NADPH_2$-nitrate reductase, from Aspergillus nidulans. The 300-fold purified enzyme also had $NADPH_2$-cytochrome c reductase activity. The ratio: $NADPH_2$-nitrate reductase activity to $NADPH_2$-cytochrome c reductase activity was 1:3.2. This ratio was similar to that found during each of the purification steps with the exception of an initial ammonium sulphate fraction that had a ratio of 1:14.5. The ratio in crude extracts was

1:6.5. Cove and Coddington suggested that the large amount of cytochrome c reductase activity found in the first ammonium sulphate fraction was due to enzyme not associated with $NADPH_2$-nitrate reductase. The $NADPH_2$-nitrate reductase activity of the purified enzyme was stimulated by FAD and to a lesser extent by FMN. The enzyme was inactive with $NADH_2$ as a hydrogen donor.

Assimilatory nitrate reductase from E. coli has been purified 15-fold by Nicholas & Nason (1955). The enzyme was specific for $NADH_2$ as a hydrogen donor; slight activity was obtained with $NADPH_2$. Both FAD and FMN stimulated $NADH_2$-nitrate reductase activity and fluorometric and enzymic analyses revealed that 90% of the flavin in the enzyme was FAD. The participation of sulphydryl groups in the enzymic reaction was demonstrated by the inhibitory effect of p-chloromercuribenzoate. This inhibition was reversed by glutathione. A metal component was implicated by the inhibition that occurred in the presence of cyanide, azide and 8-hydroxyquinoline. The authors stated that the specific activity of the nitrate reductase in extracts from molybdenum deficient cells was one third of that found in normal cells. Iron deficiency had no effect upon nitrate reductase activity. Molybdenum caused reactivation of enzyme that had been inactivated by dialysis against cyanide. Nicholas & Nason suggested that the sequence of electron transport was as follows:

$$NADH_2 + FAD(FMN) \longrightarrow Mo \longrightarrow NO_3^-$$

A rather unusual assimilatory nitrate reductase has been found in Azotobacter vinelandii (Taniguchi & Ohmachi, 1960). Unlike the Neurospora, Aspergillus and E. coli enzymes, which were soluble, the Azotobacter enzyme was found in particles, presumably fragmented cell membranes. The same particles contained a $NADH_2$-oxidase system. Although spectrophotometric observations revealed that cytochromes were involved in the aerobic oxidation of $NADH_2$, the cytochromes remained in the reduced state during the anaerobic oxidation of $NADH_2$ by nitrate. The activity of the enzyme with $NADPH_2$ was 50% of that obtained with $NADH_2$. The involvement of flavin in the $NADH_2$ -nitrate reductase system was indicated by both the stimulatory effect of FAD or FMN and the inhibition of nitrate reduction by amytal. The nitrate reductase activity, using an artificial electron donor, was inhibited by cyanide and azide suggesting that a heavy metal was part of the terminal enzyme. Taniguchi & Ohmachi proposed the following scheme:



A soluble nitrate reductase has been extracted from nitrate-grown cells of the yeast Hansenula anomala, (Silver, 1957). The enzyme was a pyridine nucleotide-dependent metalloflavoprotein.

Spectrophotometric studies showed that cytochromes were not involved in the reduction of nitrate.

D. THE REDUCTION OF NITRITE

Nitrite, the product of nitrate reduction may be reduced to a number of inorganic nitrogen compounds. The enzymic steps and the intermediate compounds between nitrite and ammonium are a matter of some controversy at the present time. A number of intermediate compounds have been postulated, some of which are toxic and others which are gaseous in their free state.

The reduction of nitrite has been reviewed by Nicholas (1961); Taniguchi (1961); Nason (1962); Takahashi, Taniguchi & Egami (1963), and Kessler (1964). Nitrite reduction will not be discussed further in this literature survey.

E. DISCUSSION

The respiratory nitrate reductase systems surveyed here were characterized by their dependence upon cytochromes as electron donors. The type of cytochrome involved in nitrate respiration varied with the species studied. Cytochromes of the b-type were members of the respiratory nitrate reductase systems in some bacteria and cytochromes of the c-type were found in similar systems from other bacteria. In one organism a c-type cytochrome and an a-type cytochrome were sequentially involved in electron transfer to nitrate reductase. With the

exception of the latter system only one cytochrome component was found in each of the nitrate reducing systems studied. Generally the respiratory nitrate reducing systems contained fewer components than the aerobic oxidase systems. However many of the components such as $NAD(P)H_2$ dehydrogenases, flavins and some of the cytochromes were apparently common to both types of respiratory chain.

The assimilatory nitrate reductases reduced nitrate without the intervention of cytochromes, although in some cases cytochrome c reductase was associated with the purified assimilatory nitrate reductase. The assimilatory nitrate reductases from different species did not show much variation in the components involved in the transfer of reducing power from reduced pyridine nucleotides to nitrate. The main difference between species was the type of pyridine nucleotide required for nitrate reduction.

One common factor occurred in both the respiratory nitrate reductases and the assimilatory nitrate reductases, and that was the essential role that molybdenum played in both types of nitrate reduction. Unfortunately too few of the terminal nitrate reductases have been separated from their respective electron transport chains to enable a comparison to be made between them. Apparently no attempts have been made to determine whether the isolated assimilatory nitrate reductases were single proteins with several different catalytic centres or whether the 'enzymes' were composed of sub-units, each catalyzing a different step in the electron transfer sequence.

Evidence suggests that at least one assimilatory nitrate reducing system is composed of two polypeptide chains, one of which is the terminal nitrate reductase. It would be interesting to determine whether controlled treatment of purified assimilatory Neurospora crassa or Aspergillus nidulans assimilatory nitrate reductase with enzymes such as lipases, phospholipases or proteinases would result in a fraction devoid of $NADPH_2$-nitrate reductase activity but with benzyl viologen-nitrate reductase activity. The presence of benzyl viologen-nitrate reductase activity in extracts lacking $NADPH_2$-nitrate reductase activity from Neurospora crassa supports the existence of a terminal nitrate reductase analogous to that solubilized from the respiratory nitrate reducing systems of both E. coli and Achromobacter fischeri. A comparison between the terminal nitrate reductase enzymes solubilized from both types of nitrate reducing system, in the one organism (e.g. E. coli), may reveal that the terminal enzymes are identical and that the two types of systems differ only in the sequence of electron-transferring components that donate electrons to nitrate reductase.

THE SCOPE OF THIS THESIS

Wilson (1965) reported that B. stearothermophilus reduced nitrate to nitrite under anaerobic conditions. This thesis is concerned with an investigation of the role of nitrate in the anaerobic metabolism of the organism and the characterization of the nitrate reducing system. Furthermore the heat stability and a number of enzymic properties and kinetic parameters of the isolated enzyme will be determined.

CHAPTER II

GENERAL METHODS

1. ORGANISM

Bacillus stearothermophilus sp. unclassified, was obtained from the University of Melbourne, Parkville, Victoria.

2. MEDIA

(a) Growth Medium

The medium used for growth on a large scale contained: 0.5% (w/v) polypeptone (Baltimore Biological Laboratories, U.S.A.), 0.3% (w/v) Basamine (autolysed yeast extract, Anhauser-Busch Inc., U.S.A.), 0.05% (w/v) KH_2PO_4, 0.15% (w/v) K_2HPO_4, 0.00025% (w/v) Ca^{++}, 0.015% (w/v) Mg^{++} and 1ml. per litre of final medium of 'metals' solution. The 'metals' solution had the following composition:

di-sodium ethylenediaminetetra-acetate (EDTA)	0.25g.
$ZnSO_4.7H_2O$	1.5g.
$FeSO_4.7H_2O$	0.25g.
$MnSO_4.H_2O$	0.18g.
$CoCl_2.6H_2O$	0.005g.
$Na_2MoO_4.2H_2O$	0.08g.
$CuSO_4.5H_2O$	0.7mg.

The salts were dissolved in 90ml. of distilled water and the pH of the solution was adjusted to 7.0 with NaOH before it was made up to 100ml. with distilled water.

Throughout the thesis this complete medium is referred to as polypeptone-basamine medium. The final pH of the medium after auto-

claving at 15 lb. per squ.in. for 30 min. was 6.8. Sterile solutions of sugars and other carbon sources were added as noted under the relevant experiments.

(b) Stock Culture Medium

The medium for maintaining stock cultures was similar to the polypeptone-basamine medium except that Ca^{++}, Mg^{++} and 'metals' solution were omitted and 2% (w/v) agar was included. The agar medium was dispensed into 1 oz. screw-capped bottles and sterilized by steaming for 1 hr. on each of three successive days. Agar medium sterilized in this way did not liquify as readily during growth of the organism at 60^{o} as did similar medium that had been autoclaved. Unopened slopes incubated at 35^{o} or 60^{o} for 14 hr. did not show any signs of microbial growth and sterile slopes were routinely stored at room temperature.

3. MAINTENANCE OF STOCK CULTURES

The organism was usually inoculated onto four slopes which were incubated in an upright position for 14 hr. at 60^{o}. One slope was left unopened and was used, after 3 weeks to a month, to inoculate a further four slopes. At approximately six-monthly intervals all existing cultures were killed and a new series of slopes was started from a freshly-opened vial of freeze-dried organisms.

4. CULTURE VESSEL FOR LARGE-SCALE GROWTHS

The vessel consisted of a 10 litre pyrex flask which had a 100mm. flat flange neck (Quickfit & Quartz, U.K., Cat. No. FV10L) accommodating a 100mm. multi socket/flat flange adapter lid (Quickfit & Quartz, U.K., Cat. No. MAF3/52). The lid had a vertical B24 centre socket and 4 side sockets. The B24 socket was used, in conjunction with a B24 cone, to hold a 28 in.-long, 6mm. diam. solid stainless steel rod. The rod was attached to the cone by means of a piece of gum rubber tubing. The vertical B19 side socket held a 15 in. combined glass-calomel electrode (Titron Instruments, Victoria, Cat. No. 123BR (Special)) which was cemented into a B19 cone with silicone rubber cement (Dow-Corning 'Silastic' 731RTV). A 7mm. internal diameter steel tube, that had a porous 'aquarium-bubbler' glued to one end with 'Araldite', was fixed to a B19 cone with rubber pressure tubing. The steel tubing had been bent to follow the contours of the flask so that the porous sparger was situated centrally about 3cm. above the base of the flask. Two tubes were introduced through the remaining 5^{o} B19 socket. One tube was used for adding alkali and the other for removing samples. A double surface B34 condenser was fitted into the B34 socket.

Before the lid was placed on the flask a perforated 'Vibro-Mixer' disk (Vibro-Mixer, A.G. für Chemie-Apparatebau, Zürich) was attached to the end of the rod with a screw and the rod height was adjusted so that the disk was positioned about 3cm. above the porous

sparger. When the rod was vibrated in a vertical direction by the 'Vibro-Mixer' liquid was expelled through the perforations of the disk onto the sparger. This dispersed the gas escaping from the sparger and prevented the formation of large bubbles as well as thoroughly mixing the liquid in the flask.

5. STERILIZATION OF pH ELECTRODE

The combined electrode was exposed to a closed atmosphere of ethylene oxide (5% ethylene oxide/95% CO_2, C.I.G. Ltd., Alexandria, N.S.W.) for 20 hr. The sterile electrode was placed in sterile distilled water 12 hr. before it was used.

6. STANDARDIZATION OF pH METER

The electrode was placed in the flask containing 8 litres of sterile polypeptone-basamine medium at 60°. A sample of the medium was withdrawn and its pH was measured at 60° with an external electrode which had been standardized against 0.05M potassium hydrogen phthalate at 60° (pH = 4.1, Lange, 1961). The internal electrode was then connected to a 'Radiometer 25' pH meter and the meter was adjusted to the pH of the medium as determined by the external electrode.

7. GROWTH OF B. STEAROTHERMOPHILUS

8 litres of polypeptone-basamine medium at 60° was inoculated with 800ml. of a 14 hr. still culture grown in the same medium at 60°.

The sterile carbon source and sterile potassium nitrate were then added. The pH was kept at 6.8 by the addition of 2N-KOH with a 'Radiometer Titrator 11'. Gas (O_2 or N_2) was filtered through a membrane filter before it was passed into the medium. The 'Vibro-Mixer' was set at an amplitude of 1 to 1.5mm. When high flow rates of gas were used 0.5ml. of Dow-Corning 'Antifoam A' was added to prevent excessive frothing. When the extinction at 700m_μ, of the culture reached 0.5 the contents of the flask were poured onto 4Kg. of crushed ice. This decreased the temperature and diluted any further acid produced by the organism. The cells were harvested at 2,000 x g for 40 min. in a Mistral 6L centrifuge (M.S.E., London, England). Sedimented cells were resuspended in ice-cold 10^{-5}M EDTA, pH 7.4, and were centrifuged at 12,000 x g for 10 min. at 2^{o} (M.S.E., HS18 centrifuge). This procedure was repeated twice with distilled water in place of EDTA. The average yield of cells was 1.2g. wet weight per litre of growth medium.

8. PREPARATION OF PROTOPLASTS

Washed cells were suspended in 0.3M sucrose-0.1M potassium phosphate buffer, pH 7.4, so that the final concentration of cells was 10% (wet weight per vol.). Egg-white lysozyme (Sigma, 3 x crystallized) was added to give a final concentration of 0.05% (w/v) and the cell suspension was incubated at 35^{o}. After 1 hr. the suspension of protoplasts was cooled and centrifuged at 7,000 x g for

30 min. at 2°, (M.S.E., HS18, head No. 69179) and the supernatant was carefully removed. An amount of sucrose-phosphate buffer equal to one tenth the original volume was added to the loosely packed protoplasts which were then gently homogenised, by hand, in a loosely-fitting teflon homogeniser.

9. PREPARATION OF MEMBRANES

Protoplasts, prepared as above, were disrupted in 10 volumes of vigorously stirred water containing 0.2 μg. of deoxyribonuclease (Worthington, type II) per ml. At this stage the suspension became viscous and was left at room temperature for 30 min. before it was centrifuged at 23,000 x g for 60 min. at 2°. All subsequent centrifugations were done at 2°. The supernatant was discarded and the membrane pellet was resuspended in 200ml. of ice-cold water and was centrifuged at 23,000 x g for 60 min. The pellet, resuspended in 35ml. of cold 0.1M potassium phosphate buffer, pH 7.4, was centrifuged at 6,000 x g for 5 min. (M.S.E. HS18, head No. 69181). The 6,000 x g precipitate, consisting of unlysed cells, was discarded and the supernatant was centrifuged at 38,000 x g for 1 hr. The sedimented material is referred to as protoplast membranes. If the protoplast membranes were to be used for experiments involving spectral studies, $NADH_2$ oxidase, or $NADH_2$-nitrate reductase measurements they were kept at 4° and used within 24 hr. Membranes to be used for benzyl viologen-nitrate reductase measurements were stored at -10°.

10. NITRATE REDUCTASE ASSAYS

(a) $NADH_2$-Nitrate Reductase Assay

The assay system contained in 0.9ml.; 0.1ml. of cell-extract (5 to 10 µg. of protein); potassium phosphate buffer, pH7.4, 50 µmoles; potassium nitrate, 20 µmoles. The tubes (15ml. Martin Christ centrifuge tubes) were flushed with nitrogen, capped and pre-incubated for 4 min. The reaction was started by the addition of 1 µmole of $NADH_2$ (in 0.1ml.) and the tubes were incubated for a further 2 min. 0.1ml. of 1M zinc acetate was added to terminate the reaction and the tubes were plunged into crushed ice. 0.9ml. of ice-cold ethanol was added and after thorough mixing the tubes were centrifuged at 1,500 x g for 10 min. Nitrite was estimated in the ethanolic supernatant.

(b) Reduced Benzyl Viologen-Nitrate Reductase Assay

A modification of the method of Lowe & Evans (1964) was used. The reaction mixture contained in 0.9ml.: 0.1ml. of extract (5 to 10 µg. of protein); potassium phosphate buffer, pH 7.4, 50 µmoles; potassium nitrate, 20 µmoles; benzyl viologen, 0.4 µmoles. The tubes were gassed with nitrogen, capped and pre-incubated for 4 min. The reaction was started by adding 0.1ml. of 0.5% (w/v) sodium dithionite (approx. 3 µmoles) in 0.25M potassium phosphate buffer, pH 7.4, and the tubes were re-capped. After 2 min. the cap was removed to admit air and the dithionite and reduced benzyl viologen

were oxidized by mixing the contents of the tube on a 'Whirlimixer' (Scientific Industries (U.K.) Ltd.). The tubes were cooled in ice and 0.1ml. of ice-cold 0.1M lead acetate was added to each tube and the contents of the tubes were mixed. 0.9ml. of ice-cold ethanol was added to each of the tubes, which were then shaken and centrifuged at 1,500 x g for 10 min. Nitrite was estimated on the ethanolic supernatant.

The amount of cell-extract used in both systems was such that the nitrite produced per tube did not exceed 100mμ moles per 2 min. The reduced benzyl viologen method described above differed from the original method in several respects:

(i) Sodium dithionite was dissolved in 0.25M potassium phosphate, pH 7.4, whereas in the method of Lowe & Evans (1964), dithionite was added as a one to one mixture of $Na_2S_2O_4$ and $NaHCO_3$.

(ii) Lowe & Evans did not deproteinize their reaction mixture nor did they remove any of the oxidation products of dithionite before adding the diazotizing reagent. Lead acetate and ethanol were used in the modified method to precipitate the protein and to remove the oxidation products of dithionite, such as bisulphite and thiosulphate which react with nitrite in acid conditions.

(c) Estimation of Nitrite

Nitrite standards, with a range from 25mμ moles to 100mμ moles, were prepared in the appropriate reaction mixture, without the enzyme, and were carried through the same procedure as the tubes containing enzyme except that the incubation steps were omitted. 1ml. of each ethanolic supernatant was placed in a 'Spectronic 20' tube (Bausch & Lomb Inc., Rochester, N.Y., U.S.A.). 1ml. of 1%(w/v) sulphanilamide in 3N-HCl was added and the contents were mixed. 1ml. of 0.04% (w/v) aqueous 1-naphthyl-ethylenediamine dihydrochloride was added and, after mixing, the tubes were left for 10 min. at room temperature. The extinction at 540mμ was determined against a reagent blank in a 'Spectronic 20' spectrophotometer.

Diazotization and coupling were carried out in dim light because of the instability of the diazo compound and of the azo dye to bright light (Lascelles, personal communication).

10A SODIUM DITHIONITE; STORAGE AND SOLUTION

(a) Storage

Because of the instability of sodium dithionite and the variability of different batches the following procedure was adopted for storing the reducing agent. The top third of a freshly opened bottle of sodium dithionite (Laboratory Reagent, British Drug Houses, Poole, England) was discarded and the remaining solid was dispensed into ½ oz. screw-capped bottles and stored over silica gel. In some cases larger

quantities were placed in 'Clinbritic' bottles, a serum stopper was fitted and the bottle was evacuated and flushed with oxygen-free nitrogen. Dithionite, stored in both ways, remained free-flowing and gave consistent results over a period of three years.

(b) Solution

A 0.5% (w/v) solution of sodium dithionite was prepared by weighing 50mg. of dithionite into a 15ml. glass centrifuge tube and adding 10ml. of 0.25M potassium phosphate, pH 7.4. A serum stopper was fitted and the tube was evacuuated and flushed several times with deoxygenated 'oxygen-free' nitrogen. The dithionite was dissolved by inverting the tube and was kept unopened in ice.

11. PURIFICATION OF ETHANOL

Variable and low yields of nitrite were obtained using the above methods if the ethanol contained much aldehyde. Spectroscopic ethanol (95%, British Drug Houses) was satisfactory but because of its high cost was not used. S.V.R. ethanol (spiritus vinus rectificatus) was purified by the method of Leighton, Crary & Schipp (1931) except that the final step involving the use of activated aluminium amalgum was omitted.

12. DEOXYGENATION OF NITROGEN

The last trace of oxygen was removed from 'oxygen-free' nitrogen (Commonwealth and Industrial Gases, Australia)with vanadous sulphate

as described by Umbreit, Burris & Stauffer (1957). A safety device was included to prevent the pressure of the gas at the end of the gas train from exceeding atmospheric pressure. It consisted of a T tube with a drawn-out tip which was immersed in distilled water to a depth of about 5mm. A two-way burette tap was attached to the end of the train. One outlet of the tap was connected to a water pump, another to the gas train and to the third was attached a piece of pressure tubing. By attaching an anaerobic cuvette to the pressure tubing and turning the tap through 360° the cuvette could be evacuuated and refilled with deoxygenated nitrogen.

13. ESTIMATION OF PROTEIN

(a) Biuret Method

The biuret method described by Layne (1957) was used for protein concentrations greater than 1mg. per ml.

(b) Micro-Biuret Method

The micro-biuret method of Ellman (1962) was used for estimating small amounts of protein (up to 300 µg. per ml.).

Standard protein solutions were prepared from crystalline bovine serum albumin (Sigma, St. Louis, U.S.A.).

CHAPTER III

THE ANAEROBIC GROWTH OF B. STEAROTHERMOPHILUS ON VARIOUS CARBON SOURCES IN THE PRESENCE AND ABSENCE OF NITRATE

A. METHODS

1. The measurement of the anaerobic growth rate of B. stearothermophilus in small tubes

7.4ml. of sterile polypeptone-basamine medium was asceptically pipetted into sterile 'Spectronic 20' tubes. The carbon source and potassium nitrate were each added as sterile solutions in a volume of 0.2ml. to give the appropriate final concentrations. Where the carbon source or potassium nitrate was omitted each was replaced by the same volume of sterile distilled water. Each tube was inoculated with 0.2ml. of a suspension of B. stearothermophilus produced by washing down a 16 hr. slope with 3ml. of polypeptone-basamine medium. After inoculation, the tubes were capped with 'Suba-seal' stoppers (Suba-seal No. 21) and two sterile 20 gauge needles were introduced into each tube. The tubes were gassed for 3 min. with oxygen-free nitrogen that had been passed through a sterile 'Milipore' filter, and the needles were removed. The tubes were incubated at 60° in a bath of distilled water and the extinction of the media, at 700 mμ , was measured every 10 min. in a 'Spectronic 20' spectrophotometer. The growth curves were drawn on graph paper and the linear portion of the exponential phase of growth was used to calculate the growth rate, k, which was expressed as $\Delta E_{700m\mu}$ per hr.

B. RESULTS

Fig. 1 & fig. 2 are examples of the growth curves obtained using the above method. There was no detectable growth on polypeptone-

Fig. 1

The Effect of Nitrate on the Anaerobic Growth of *B. stearothermophilus* on a Medium containing Fructose.

The polypeptone-basamine medium was used with addition of KNO_3, or fructose, or both substances.

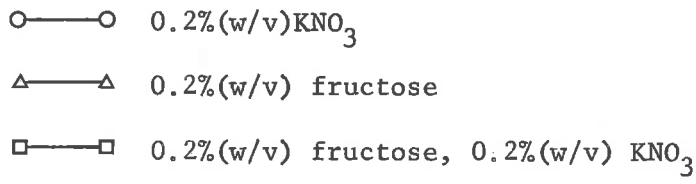


Fig.1

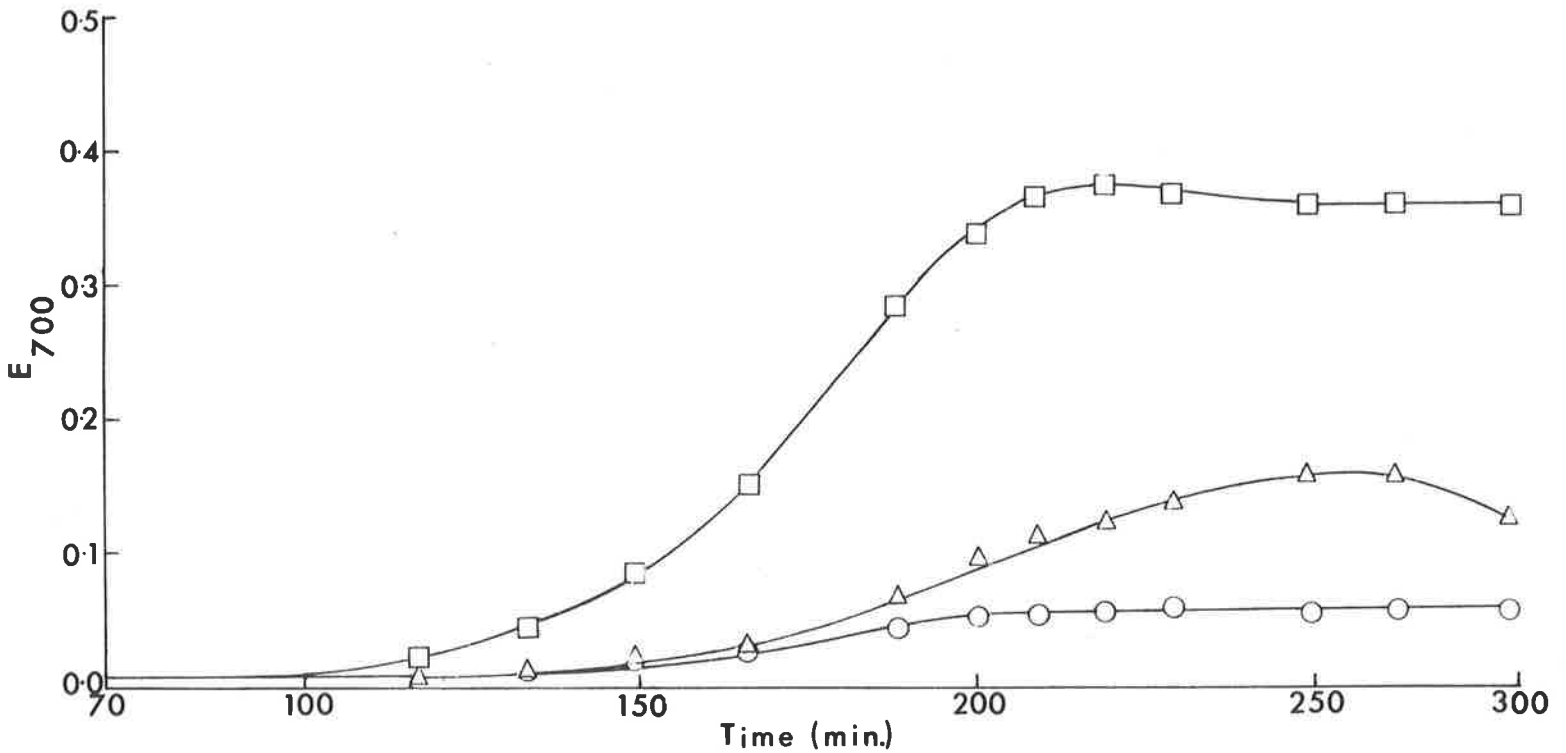


Fig. 2

The Effect of Nitrate on the Anaerobic Growth of *B. stearothermophilus* on a Starch-Containing Medium.

The polypeptone-basamine medium was used with additions of KNO_3, or starch, or both substances.


0.2%(w/v) KNO_3


0.2%(w/v) starch


0.2%(w/v) starch, 0.2%(w/v) KNO_3

Fig. 2

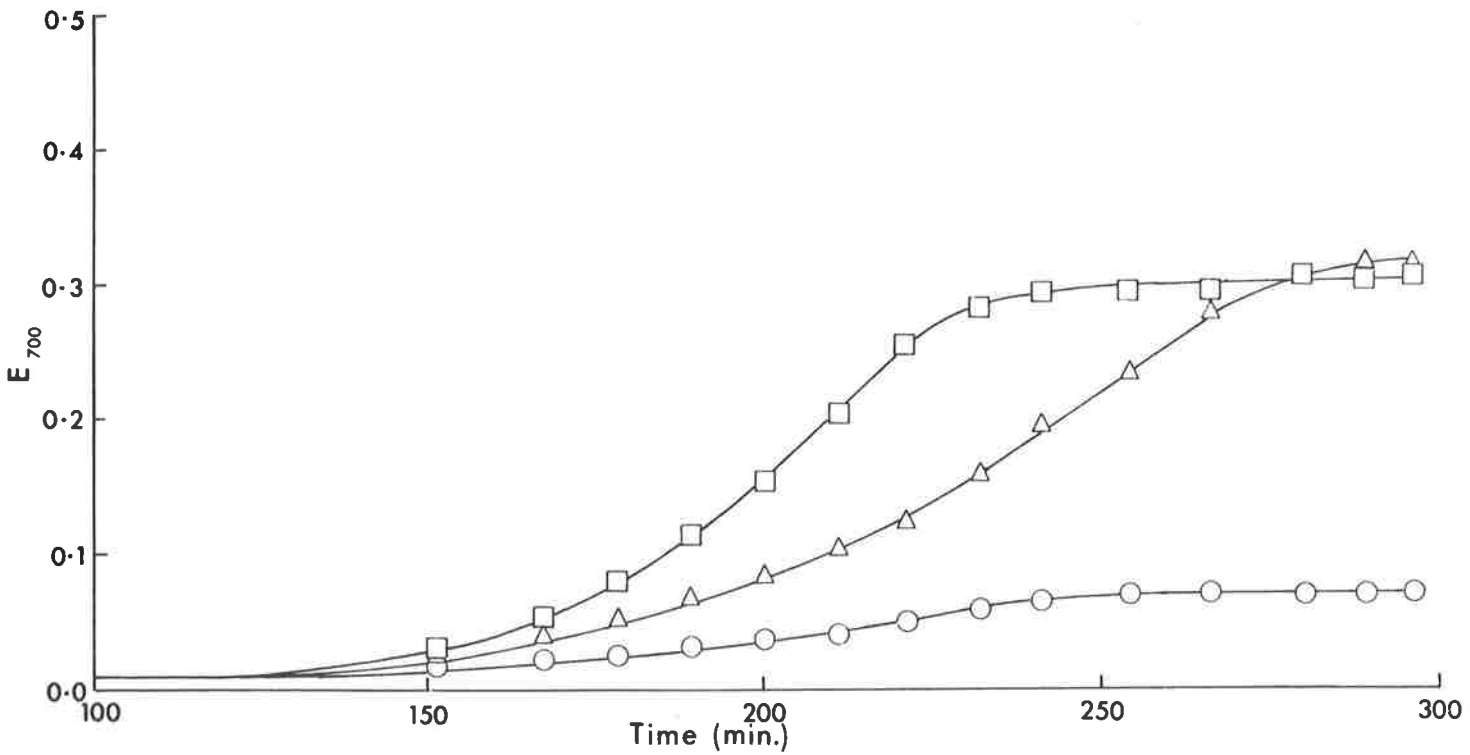


Table 1

Growth Rate, Cell Density and pH of Cultures Grown on Different Carbon Sources in the Presence and Absence of Nitrate.

The growth rates ($E_{700m\mu}$ per hr.) were determined in media containing polypeptone-basamine and various carbohydrates with and without nitrate.

The maximum extinction of the cultures (E_{max}) was used as a measure of the final cell density.

pH values were determined with a combined glass-calomel electrode in conjunction with a Radiometer 22 pH meter.

Table 1

	minus nitrate			plus nitrate		
	10^2k	E_{max}	Final pH	10^2k	E_{max}	Final pH
Glucose	26	0.355	5.7	38	0.405	5.85
Starch	21	0.305	5.75	29	0.290	5.8
Sucrose	25	0.350	5.7	36	0.375	5.75
Fructose	11	0.155	6.02	36	0.37	5.8
Glycerol	0	-	-	8	0.26	6.55

basamine medium in the absence of both a carbon source and nitrate (not shown in figs. 1 & 2). When 0.2% (w/v) potassium nitrate was present slight growth was observed, apparently due to the utilization of a substrate present in a very small amount (see figs. 1 & 2).

Table 1 lists the rates of growth, the maximum extinctions at 700 mμ (E max) and the final pH values of anaerobic cultures that contained glucose, starch, sucrose, fructose and glycerol, respectively with and without nitrate.

The organism grew anaerobically, in the absence of nitrate, on glucose, starch, sucrose and fructose but growth was not observed on glycerol medium.

The presence of 0.2% potassium nitrate increased the growth rate approximately 1.5-fold in glucose medium, approximately 1.4-fold in starch medium and sucrose medium, and approximately 3-fold in fructose medium. Nitrate was essential for anaerobic growth on glycerol medium.

Lactate, proline, glutamate and succinate each at a final concentration of 0.2% (w/v) and acetate at a final concentration of 0.4% (w/v) were tested separately for their ability to support growth under anaerobic conditions in the presence of nitrate. The growths obtained were found to be identical to that observed in a control consisting of polypeptone-basamine containing the same concentration of nitrate.

The growth rate on polypeptone-basamine medium containing 0.2%

glucose, 0.2% lactate and 0.2% nitrate was the same as that found in the absence of lactate.

Since B. stearothermophilus would not grow anaerobically on glycerol in the absence of nitrate this carbon source was used to test the effects of increasing amounts of nitrate. A series of tubes were prepared containing 0.4% (w/v) glycerol and 0.05%, 0.1% 0.2%, 0.4% and 0.8% (w/v) potassium nitrate, respectively in polypeptone-basamine medium. Controls were included containing the same amounts of nitrate but lacking glycerol. Fig. 3 shows the growth curves obtained with the different amounts of nitrate.

Slight growth obtained on the polypeptone-basamine-nitrate controls was the same for all concentrations of nitrate. Growth curves for these controls were not included in the figure. Increasing the concentration of nitrate did not increase the growth rate on glycerol. However the length of the exponential phase of growth was increased by increasing concentrations of nitrate up to 0.4%. At a concentration of 0.8%, potassium nitrate gave a slightly lower growth rate and a lower maximum for growth (E_{max}) than that found for 0.4% potassium nitrate. Diphasic growth of the organism occurred on 0.05% nitrate. At the conclusion of the growth qualitative tests revealed that nitrite was present in all tubes except the one that had contained 0.05% potassium nitrate.

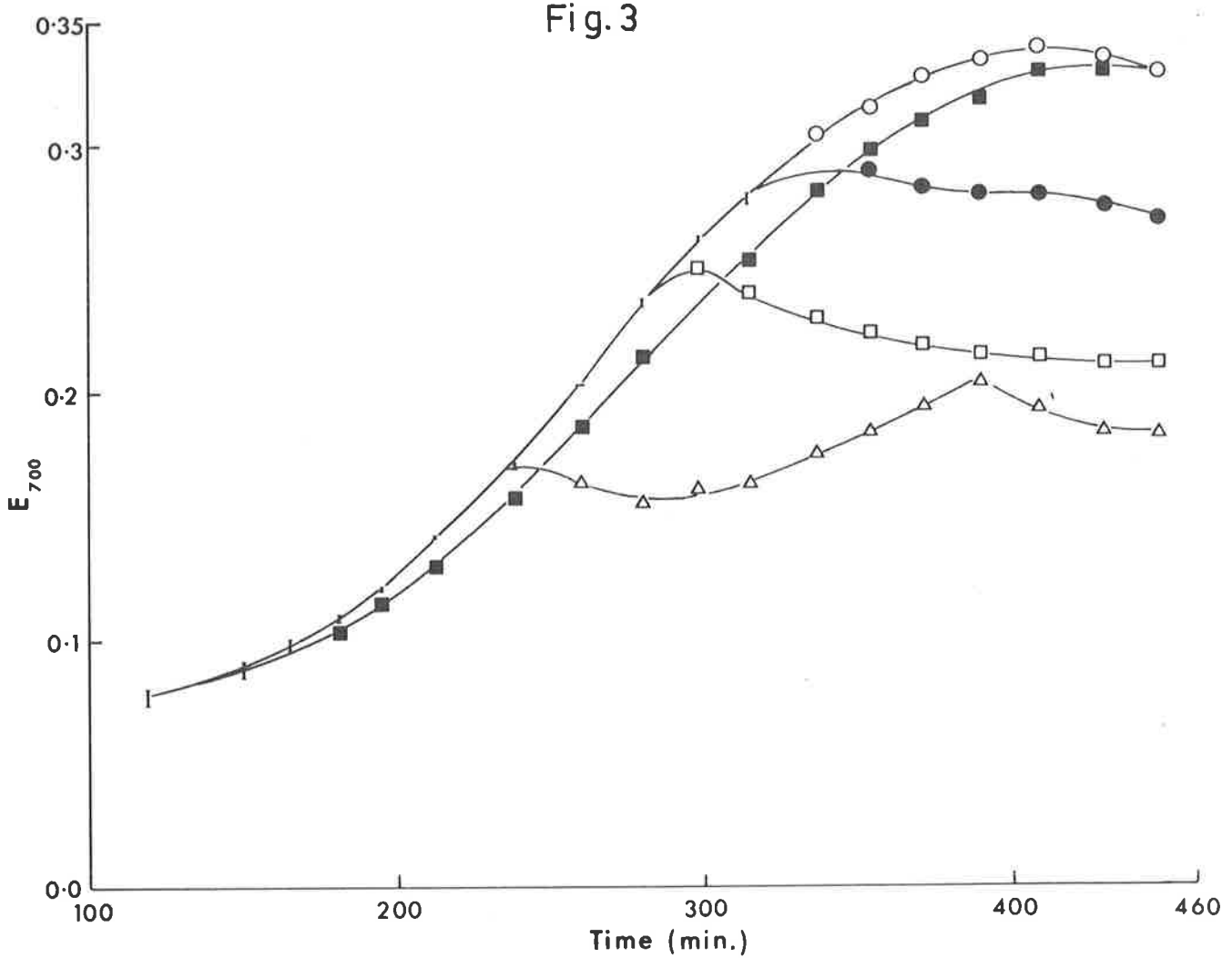
Fig. 3

The Effect of Different Concentrations of Nitrate on Growth in a Glycerol Medium

The polypeptone-basamine medium was used with 0.4% (w/v) glycerol and different concentrations of KNO_3

- △——△ 0.05%(w/v) KNO_3
- □——□ 0.1%(w/v) KNO_3
- ●——● 0.2%(w/v) KNO_3
- ○——○ 0.4%(w/v) KNO_3
- ■——■ 0.8%(w/v) KNO_3

Fig. 3



C. DISCUSSION

The results show that B. stearothermophilus utilized glucose and the glucose-containing carbohydrates, starch and sucrose, under anaerobic conditions in the absence of nitrate. The slight but significant increase in growth rates and the increased E_{max} obtained on glucose and sucrose, in the presence of nitrate, suggests that nitrate caused some stimulation of metabolism and synthesis of cellular constituents. However the decrease in the pH values of the cultures both in the absence and presence of nitrate shows that some acidic end product of metabolism was produced. Wilson (1965) demonstrated that organic acids (mainly lactate) were the end-products of carbohydrate metabolism when this organism was grown anaerobically. Furthermore she reported that, as a result of acid production, growth ceased when the pH reached 5.8. The cessation of growth observed to occur here, in the cultures grown on glucose, starch and sucrose, both with and without nitrate, was probably due to the unfavourable pH. The slightly higher pH values found for the cultures containing nitrate could have been due to:

(i) less acid being excreted into the medium because of its further metabolism by oxidative pathways,

or (ii) cessation of growth before an unfavourable pH was reached because of an accumulation of nitrite.

The former possibility is more likely since cultures containing

glucose and nitrate, in which the pH is maintained at 6.8, grow to much higher cell densities than those reported above.

The growth rate and the final cell density of the fructose-containing culture without nitrate, was approximately half those found for glucose. The final pH of the fructose culture was approximately 0.3 units higher than the pH of the glucose-culture and approximately 0.2 units higher than the fructose culture containing nitrate. Thus it is unlikely that growth ceased, on fructose, because of high acidity.

The 3-fold stimulation of growth rate and 2.4-fold increase in the final cell density caused by the inclusion of nitrate in fructose-medium indicates that the mechanisms for the utilization of fructose were stimulated, by nitrate, to a greater degree than the glucose metabolizing systems. However the values for the final pH and the final cell density of the culture containing both fructose and nitrate were similar to those found for the other carbohydrates discussed above and accumulation of acid appears to be responsible for the cessation of growth.

The lack of growth on glycerol-medium in the absence of nitrate suggests that the organism was unable to metabolise glycerol via the glycolytic pathway under anaerobic conditions. Although the growth rate and the cell density in glycerol plus nitrate were low it is obvious that nitrate was essential for the maintenance of growth on

glycerol under anaerobic conditions. The final pH of the glycerol plus nitrate-grown culture was 0.7 units higher than that of the culture grown on starch plus nitrate - indicating that the glycerol-grown cells produced less acid than the starch-grown cells while reaching almost the same cell density.

The inability of lactate, acetate, glutamate and succinate to support anaerobic growth on nitrate-containing medium was possibly due to both lack of intermediates metabolites from sugar metabolism and the impermeability of the cells to some of these acids. The lack of inhibition, by lactate, of growth on glucose plus nitrate medium indicates that this end-product of glycolysis did not inhibit the metabolic pathway that was being stimulated by nitrate.

Whole cells did not use proline as an energy source either in the presence or absence of nitrate although cell-free extracts, of this organism, actively reduced the low redox-potential dye benzyl viologen with proline as a substrate, under anaerobic conditions (Wilson, 1965).

The growth curves obtained when B. stearothermophilus was grown on medium containing 0.4% glycerol and increasing concentrations of nitrate (fig. 3) indicate that the mechanism responsible for utilizing nitrate, and thus stimulating the growth rate, was saturated with nitrate at the o.1% level. This was shown by the constant initial growth rate that was obtained with 0.1%, 0.2%, and 0.4% nitrate.

Nitrate,or its metabolic product nitrite, appeared to cause some inhibition of growth when concentrations of nitrate greater than 0.1% were initially present. This was indicated by the lack of stoichiometry between the

initial nitrate concentrations and the length of the exponential phase of growth on 0.2%, 0.4% and 0.8% nitrate as well as the lower growth rate obtained on 0.8% nitrate. Further evidence that supports this assumption is the decreasing slopes of the late exponential phases of the growth curves as the nitrate concentration increases.

The diphasic growth obtained with the culture having an initial nitrate concentration of 0.05%, together with the absence of nitrite from the culture after the second phase of growth, was possibly due to the further utilization of nitrite during the second phase of growth. The fact that the cultures that contained initial concentrations of nitrate greater than 0.05% did not exhibit diphasic growth and accumulated nitrite suggests that cells in these cultures were unable to synthesize a nitrite utilizing system. Experiments performed with the aim of devising a quantitative method for measuring nitrate reductase activity provided supporting evidence for the above hypothesis in that there was a complete lack of a nitrite utilizing system in extracts from cells grown on both glucose and glycerol media containing initial concentrations of nitrate ranging from 0.1% to 0.4%.

Because the organism was routinely grown on media containing 0.2% nitrate no attempt was made to determine whether or not a nitrite utilizing system was present in cells grown in the presence of 0.05% nitrate.

Since the completion of the experimental work reported in this thesis Cole and Wimpenny (1968) have shown that E. coli K12 grown anaerobically in the presence of 10mM nitrate released nitrite into the medium during the early logarithmic phase of growth and that this nitrite subsequently disappeared. On the other hand nitrite continued to accumulate when the

initial nitrate concentration in the medium was 100mM.

The highest level of nitrite reductase was found in cells grown on 20mM nitrate; whereas the specific activity was found to be almost zero in cells grown in the presence of 60mM nitrate.

Thus the effects of nitrate on both the accumulation of nitrite in the medium and the levels of nitrite reductase in extracts from E. coli K12 are similar to those obversed incidentally during the anaerobic growth of B. stearothermophilus on media containing nitrate.

No adequate explanation can be suggested for the hypothesis that high concentrations of nitrate prevent the formation of a nitrite utilizing system.

The role of a nitrite utilizing system in the growth of B. stearothermophilus on low initial concentrations of nitrate or nitrite was considered to be outside the scope of this thesis and this topic was not investigated further.

The observations discussed previously suggest that nitrate stimulates the anaerobic growth of B. stearothermophilus, on certain carbon sources by acting as an electron acceptor for a respiratory nitrate reducing system

Further evidence for the involvement of a respiratory nitrate reductase might be provided by a comparison of the activities of some enzymes involved in oxidative metabolism, and a comparison of cytochromes, under aerobic and anaerobic conditions in the presence and absence of nitrate.

Chapter IV is concerned with the results of an investigation made along these lines using both glycerol and glucose as carbon sources.

CHAPTER IV

THE LEVELS OF CYTOCHROMES AND TRICARBOXYLIC ACID CYCLE ENZYMES IN *B. STEAROTHERMOPHILUS* UNDER DIFFERENT GROWTH CONDITIONS.

A. METHODS

1. Spectrophotometric measurements

Measurements of cytochrome concentrations and enzyme activities were made using a Unicam SP.800 (Model B) double beam recording spectrophotometer (Unicam Instruments Ltd., Cambridge, U.K.) in conjunction with a Unicam SP.850 scale expansion accessory. The signal from the scale expansion accessory was fed to an Hitachi QPD 33 recorder (Hitachi, Ltd., Tokyo, Japan) which gave an extinction scale reading of 0 to 0.1, on a 25 cm recorder chart, when the setting on the scale expansion unit was 20.

The SP.800 spectrophotometer was fitted with an SP.820 constant wavelength scanner that allowed measurements of extinction against time to be made at a fixed wavelength setting. This accessory was used, in conjunction with a constant temperature cell housing (SP.874), to measure enzyme activities. The temperature of the cell housing was maintained at 60^{o} with water circulated by means of a temperature controller ('Tempunit'; Techne Ltd., Cambridge, U.K.) that was positioned in an external bath.

A subsidiary sample space (second sample position) situated close to the photomultiplier was used to measure the spectra of light-scattering suspensions such as protoplast membranes.

2. Measurement of Cytochrome Concentrations

Cytochromes were measured in thunberg-type cuvettes (Fig. 4) specially made to fit the second sample position of the Unicam SP.800 spectrophotometer. Protoplast membranes were prepared according to the method described in Chapter II, section 9, p. 63.

The three cytochromes, present in the protoplast membranes, which are investigated here have α absorption bands at 605mμ , 560mμ , and 552mμ . The classification of the cytochromes, with α absorption bands at 560mμ and 552mμ , will be discussed in Chapter V. For the present these cytochromes will be given the names: cytochrome a_3 for the cytochrome with an α absorption band at 605mμ , cytochrome b_1 for the cytochrome with an α absorption band at 560mμ and cytochrome c_1 for the cytochrome with an α absorption band at 552mμ (Morton, 1958).

a. Cytochrome c_1.

The cytochrome c_1 concentration was measured using a thunberg-type cuvette. The cuvette contained: 180 μmoles of potassium phosphate, pH 7.4, and 1ml. of protoplast membrane suspension (approx. 10mg. of protein) in a final volume of 2.9ml. 30 μmoles of sodium ascorbate, in a volume of 0.1ml., was placed in the side arm and the cuvette was alternately evacuated and flushed, ten times, with deoxygenated nitrogen.

After the ascorbate had been tipped, the reduced minus oxidized

Fig. 4

A Thunberg-type Cuvette Manufactured to Fit the Second Sample Position in the Unicam SP.800 Recording Spectrophotometer.

A. 10 mm. light-path, quartz cuvette sealed to a 'halved' B14 graded socket.

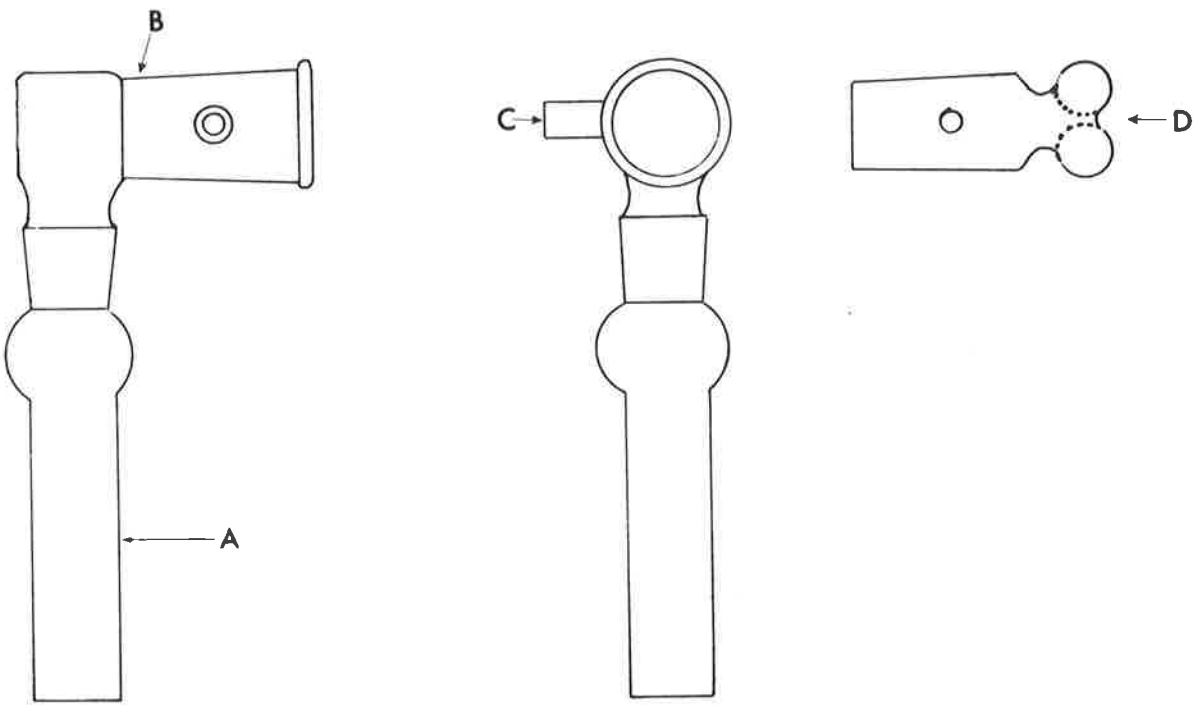
B. Adapter to receive side arm.

C. Outlet for evacuuating and gassing the cuvette.

D. Side-arm for adding substrates after evacuuation.

The drawing is approximately to scale.

Fig. 4



absorption spectrum was recorded several times using an open reference cuvette containing 180 μ moles of potassium phosphate, pH 7.4, and 1ml. of membrane suspension in a final volume of 3ml. When complete reduction of the cytochrome had occurred a final spectrum was recorded.

The cytochrome c_1 concentration was calculated from the difference in extinction between the peak at 552mμ and the trough at 540mμ using the differential molar extinction coefficient of mammalian cytochrome c_1: $\Delta\epsilon = 18.8 \times 10^3$ (Green, Järnefelt & Tisdale, 1959).

The concentration of cytochrome c_1 was expressed as m μ moles of cytochrome c_1 per mg. of membrane protein.

b. Cytochrome b_1.

Cytochrome b_1 was estimated from the difference spectrum obtained by subtracting the spectrum of reduced cytochrome c_1 from the spectrum of membranes reduced with sodium dithionite.

The anaerobic cuvette contained the same amount of membrane suspension as that used for estimating cytochrome c_1 and 180 μ moles of potassium phosphate, pH 7.4, in a final volume of 3ml. Approximately 3mg. of sodium dithionite was placed in the side arm and the cuvette was alternately evacuuated and flushed ten times with deoxygenated nitrogen. After tipping the dithionite the spectrum was drawn several times using the aerobic preparation as a reference. When maximum reduction of the cytochromes was observed the difference

spectrum : dithionite reduced minus ascorbate reduced was recorded from 630mμ to 500mμ .

The cytochrome b_1 concentration was calculated from the difference in extinction between the peak at 560mμ and the trough at 577mμ using the differential molar extinction coefficient for mammalian cytochrome b: $\Delta\epsilon = 14.3 \times 10^3$ (Goldberger, Smith, Tisdale and Bornstein, 1961). The concentration was expressed as mμ moles of cytochrome b_1 per mg. of membrane protein.

c. Cytochrome a_3

The difference spectrum: dithionite reduced minus oxidized was used to calculate the concentration of cytochrome a_3. The difference between the extinction at 605mμ and the extinction at 630mμ was measured and the concentration of cytochrome a_3 was calculated using the differential molar extinction coefficient: $\Delta\epsilon = 12 \times 10^3$ (Griffith & Wharton, 1961).

3. Preparation of Total Lysates for the Estimation of Tricarboxylic Acid Cycle Enzymes

Protoplasts were prepared as described in General Methods (Chapter II, section 8, page 62). 5ml. of protoplast suspension was mixed in a pollyallomer tube with 2ml. of distilled water. The resulting mixture was cooled, in crushed ice, and subjected to

sonic irradiation for 3 one minute periods with a 'Soniprobe' (Soniprobe type 1130A, Dawe Instruments Ltd., London, U.K.) at maximum power output. The resulting lysate (hereafter referred to as cell extract) was used for the estimation of tricarboxylic acid cycle enzymes.

4. The Measurement of the Activities of Tricarboxylic Acid Cycle Enzymes

The activities of the enzymes: isocitric dehydrogenase, succinic dehydrogenase, and malic dehydrogenase were measured in anaerobic cuvettes to prevent oxidation of the reduced products. A slightly negative pressure of nitrogen was maintained in the anaerobic cuvette to allow for the expansion of the gas when the temperature of the cuvette was increased.

The cell extract was either used as prepared, or was appropriately diluted, so that the change in extinction per minute, during the enzyme assay, did not exceed 0.04. All the enzyme activities were measured at 60^{o}.

a. Isocitric Dehydrogenase (D_s-Isocitrate:NADP oxidoreductase Decarboxylating EC 1.1.1.42).

The reduction of NADP to $NADPH_2$ in the presence of isocitrate was used to measure NADP dependent isocitric dehydrogenase (Ochoa, 1955).

The anaerobic cuvette contained in a volume of 2.9ml.: Tris-chloride buffer, pH 6.4, 150µmoles; $MnCl_2$, 0.9µmoles; NADP, 1.2µmoles; 0.1ml. of cell extract. 1.5µmoles of D-L isocitrate, in a volume of 0.1ml., was placed in the side arm and the cuvette was evacuated and flushed ten times with deoxygenated nitrogen. A cuvette containing

the above reaction mixture, without NADP, in a final volume of 3ml. was used as a reference. Both cuvettes wre pre-incubated for 5 min. at 60° before tipping the isocitrate. The change in extinction at 340 mμwas recorded for 2 min. and the molar extinction coefficient of 6.22×10^3 was used to calculate the change in $NADPH_2$ concentration. The activity of isocitric dehydrogenase was expressed as mμmoles $NADPH_2$ formed/min./mg. protein. A cuvette containing 0.9μmole of NADP in the above reaction mixture was incubated at 60° until the extinction at 340mμ remained constant. The extinction remained constant for at least 6 min. indicating that no oxidation of $NADPH_2$ occurred under the conditions employed for measuring isocitric dehydrogenase activity.

b. Aconitase. (Citrate(isocitrate) hydro-lyase, EC 4.2.1.3)

Aconitase activity was measured by the method of Massey (1955). The absorption due to aconitate at 240mμ was measured and the rate of change in concentration of aconitate was calculated using the molar extinction coefficient: $\varepsilon = 3.3\times10^3$. The sample cuvette contained in a volume of 2.9ml.: potassium phosphate buffer, pH 7.4, 150μmoles; cell extract, 0,1ml. The reference cuvette contained the same mixture in a final volume of 3ml. Both cuvettes were pre-incubated at 60° for 5 min. and the reaction was started by adding 150μmoles of trisodium citrate, in a volume of 0.1ml., to the sample cuvette. The rate of the reaction was measured for 2 min. Preliminary experiments showed that the rate of reaction was linear for periods of time up to 12 min. at 60°

The activity of aconitase was expressed as mμmoles of aconitate produced/min./mg. protein.

c. Succinic Dehydrogenase (Succinate: 2,6-dichloro phenol-indophenol oxido reductase E.C. 1.3.99.1)

The method of Green, Mii & Kohout (1955) was used. The anaerobic cuvette contained in a final volume of 2.9ml.: 0.1ml. of cell extract; potassium phosphate buffer, pH 7.4, 100 µmoles; 2,6-dichlorophenol-indophenol, 60 µg. 50 µmoles of potassium succinate, pH 7.4, in a volume of 0.1ml, was placed in the side arm and the cuvette was alternately evacuated and flushed 10 times with deoxygenated nitrogen. The reference cuvette contained the above reaction mixture, without dichlorophenolindophenol, in a final volume of 3ml. After 5 min. pre-incubation at 60^{o} the reaction was started by tipping the succinate. The change in extinction at 600mµ was measured and the rate of reduction of the dye was calculated using the molar extinction co-efficient: $\epsilon = 16.1 \times 10^{3}$. The activity of the enzyme was expressed asmµmoles of dichlorophenolindophenol reduced/min/mg. protein.

d. Malic Dehydrogenase (L-Malate: NAD oxido reductase, E.C. 1.1.1.37)

Malic dehydrogenase activity was estimated by measuring the rate of reduction of NAD at 340mµ in the presence of L-malate (Siegel & Englard, 1960).

The anaerobic cuvette contained in a volume of 2.9ml.: glycine-NaOH buffer, pH 10, 300 µmoles; L-malate, 300 µmoles; 0.1ml. of cell extract. 6 µmoles of NAD, in a volume of 0.1ml, were placed in the side arm and the cuvette was evacuated and flushed 10 times with

deoxygenated nitrogen. The reference cuvette contained the above reaction mixture, without NAD, in a volume of 3ml. The cuvettes were pre-incubated for 5 min. at 60^{o} and the reaction was started by tipping the NAD. Under the anaerobic conditions employed no oxidation of the product of the reaction, reduced NAD, was observed.

The molar extinction coefficient:$\varepsilon = 6.22 \times 10^{3}$ was used to calculate the rate of reduction of NAD. The activity of the enzyme was expressed as mμmoles of $NADH_2$ produced/min./mg. of protein.

e. Fumarase (L-Malate hydro-lyase, EC 4.2.1.2.)

Fumarase activity was measured by following the reduction of L-malate at 240mμ (Massey, 1955).

The assay mixture contained in a volume of 2.9ml.: potassium phosphate buffer, pH 7.4, 150μmoles; 0.1ml. cell extract. The reference cuvette contained the same reaction mixture in a volume of 3ml. After pre-incubation for 5 min. at 60^{o}, the reaction was started by adding 150μmoles of L-malate in a volume of 0.1ml.

The molar extinction coefficient for fumarate,$\varepsilon = 2.4 \times 10^{3}$, was used to calculate the rate of formation of fumarate.

The activity of the enzyme was expressed as mμmoles of fumarate produced/min./mg. protein.

B. RESULTS

The distribution of the cytochromes and nitrate reductase in cells of B. stearothermophilus was determined by centrifuging osmotically-lysed protoplasts at 38,000 x g for one hour. The supernatant was saved and the sediment was washed several times by suspension in 0.1 M potassium phosphate, pH 7.4, and re-centrifugation at 38,000 x g.

The supernatant did not contain any detectable cytochromes. Nitrate reductase was not detected in the supernatant provided it was carefully separated from the membrane precipitate. The spectra of the cytochromes and nitrate reductase activity were found entirely in the protoplast membranes.

Table 2 lists the concentrations of cytochromes a_3, b_1, c_1 and the activity of benzyl viologen-nitrate reductase in protoplast membranes prepared from cells grown on glucose and also on glycerol under different growth conditions. Each line in the table represents the results from a separate growth.

The concentrations of individual cytochromes varied in the different batches of cells produced under the one set of growth conditions. This was probably due to the high growth rate of the organism (the mean generation time at 60° on glucose medium was 4 min. Wilson (1965)) which made it difficult to harvest each batch of cells at the same cell density.

Table 2

The Effects of Different Growth Conditions on the Concentrations of Cytochromes and Nitrate Reductase

The concentrations of cytochromes and the activities of nitrate reductase were measured in protoplast membranes prepared from cells grown under the various growth conditions.

Each line represents the results from a separate growth.

Cells were grown at 60° in 8 litres of medium and the appropriate gas was passed into the medium at the rate of 5 litres per min. The cultures were harvested when the extinction, of each medium, at 700mμ reached 0.5.

The preparation of protoplast membranes is described in the general methods section (Chapter II, p.63). The estimation of cytochromes is described in the methods section of this Chapter.

Table 2

Growth Conditions	Concentration of cytochromes mμ moles per mg. protein			Ratio b_1/c_1	Nitrate Reductase, Specific activity*
	a_3	b_1	c_1		
Glucose, anaerobic		0.884	0.136	6.2	3
		0.742	0.141	5.3	
		0.982	0.245	4.1	
		0.832	0.179	4.6	
Glucose, aerobic	0.112	0.801	0.639	1.25	<.004
	0.309	1.08	0.855	1.26	
	0.125	0.839	0.638	1.38	
Glycerol, aerobic	0.148	0.761	0.662	1.2	<.004
	0.133	1.08	0.754	1.4	
	0.233	0.992	1.11	0.9	
Glucose, anaerobic plus nitrate		1.80	0.985	1.8	12
		2.09	0.990	2.1	
		1.50	0.857	1.8	
Glycerol, anaerobic plus nitrate		2.42	1.29	1.9	12
		2.33	1.08	2.2	
		2.51	1.38	1.8	

* Specific activity of nitrate reductase
= the number of μ moles/min./mg. protein at 60^{o}

Table 3

The Average Ratios of Cytochrome b_1 to Cytochrome c_1 found in Cells Produced under Different Conditions of growth

The results from Table 2 were used to calculate the average ratio of cytochrome b_1 to cytochrome c_1. The figures in parentheses indicate the number of separate growths from which the averages were obtained.

Table 3

Growth conditions	Average ratio Cyt. b_1/Cyt. c_1
Glucose, anaerobic (4)	5
Glucose, aerobic (3)	1.3
Glycerol, aerobic (3)	1.2
Glucose, anaerobic + KNO_3 (3)	1.9
Glycerol, anaerobic + KNO_3 (3)	2

Cytochrome a_3 was not detected in anaerobically grown cells and its concentration was variable in cells grown aerobically on both glycerol and glucose.

Since the relative amounts of cytochrome b_1 and cytochrome c_1 produced under the different growth conditions were of interest, the ratio of cytochrome b_1 to cytochrome c_1 for each experiment was calculated and the averages of these ratios were tabulated for each set of growth conditions (Table 3).

The table shows that when cells were grown anaerobically on glucose the concentration of cytochrome b_1 was five times the concentration of cytochrome c_1. In cells grown aerobically both on glucose and on glycerol the ratio of cytochrome b_1 to cytochrome c_1 was 1.3 and 1.2 respectively. The presence of 0.2% (w/v) potassium nitrate caused a change in the pattern of cytochromes in cells grown anaerobically on glucose - the ratio cytochrome b_1 : cytochrome c_1 decreased from 5:1 to 1.9:1. A similar pattern was found in cells grown anaerobically on glycerol plus 0.2% nitrate - the ratio being 2:1. This change in ratio seemed to be due largely to an increased cytochrome c_1 and in order to demonstrate more clearly the changes in the concentrations of the individual cytochromes under the different growth conditions, the values for the average concentrations of cytochrome b_1 and of cytochrome c_1 for each set of growth conditions were expressed as ratios of the average concentrations of the same cytochromes in cells grown anaerobically on

glucose (Table 4). The table shows that the average concentration of cytochrome b_1 in cells grown anaerobically on glucose, aerobically on glucose and aerobically on glycerol was relatively constant. There was a 2.1-fold increase in cytochrome b_1 in cells grown anaerobically on glucose + nitrate and a 2.9-fold increase in cells grown anaerobically on glycerol + nitrate.

The presence of oxygen increased the average concentration of cytochrome c_1, 4.1-fold and 4.8-fold, in glucose-grown and glycerol-grown cells respectively. Under anaerobic conditions, nitrate caused an even greater increase in cytochrome c_1 concentration; a 5.4-fold increase of cytochrome c_1 occurred in glucose plus nitrate-grown cells and a 7.1-fold increase in glycerol plus nitrate-grown cells.

Benzyl viologen-nitrate reductase activity was not detected in aerobically grown cells either in the presence or absence of nitrate. A low but significant level of activity was found in cells grown anaerobically on glucose (Table 2). The presence of 0.2% (w/v) nitrate caused a 4-fold increase in the level of nitrate reductase activity in cells grown anaerobically on glucose. A similar level was found in cells grown anaerobically on glycerol plus nitrate.

The activities of the enzymes: aconitase, isocitric dehydrogenase, succinic dehydrogenase, fumarase and malic dehydrogenase in extracts from cells grown under aerobic conditions and under

Table 4

The Average Ratio of Cytochrome b_1 and of Cytochrome c_1 Relative to the Average Amount Present in Cells Grown Anaerobically on Glucose

The values for the average concentration of cytochrome b_1 and cytochrome c_1, for each set of growth conditions, are expressed as ratios of the average concentrations of the same cytochromes in cells grown anaerobically on glucose.

Table 4

Growth Conditions	Average concentration mμmoles/mg. protein	
	Cytochrome b_1	Cytochrome c_1
Glucose, anaerobic	1	1
Glucose, aerobic	1.1	4.1
Glycerol, aerobic	1.1	4.8
Glucose, anaerobic plus nitrate	2.1	5.4
Glycerol, anaerobic plus nitrate	2.9	7.1

anaerobic conditions in the presence and absence of nitrate, on both glucose and glycerol are shown in table 5. Each line in the table represents the results from a separate growth.

So that a comparison could be made between the activities of individual enzymes, the activities of the enzymes found in different cell batches, produced under the same conditions, were averaged. Table 6 gives the average activity found for each enzyme expressed as a ratio of the average activity of the same enzyme in extracts from cells grown anaerobically on glucose.

When the conditions for growth on glucose were changed from anaerobic to aerobic a slight increase occurred in the activities of isocitric dehydrogenase, succinic dehydrogenase and fumarase, (1.3-fold to 1.6-fold increases). The activities of aconitase and malic dehydrogenase increased 3.5-fold and 3.8-fold respectively. On the other hand the activities of all the enzymes increased considerably in cells grown aerobically on glycerol (4.8-fold to 13-fold increases).

The activities of aconitase and fumarase in cells grown anaerobically on glucose plus nitrate were lower than the activities found for the same enzymes in cells grown anaerobically on glucose.

The activities of all the enzymes in cells grown anaerobically on glycerol plus nitrate were higher than those found in cells grown anaerobically on glucose plus nitrate. With the exception of

Table 5

The Effects of Different Growth Conditions on the Concentrations of Some Tricarboxylic Acid Cycle Enzymes

The table lists the specific activities at 60° of the tricarboxylic acid cycle enzymes measured in extracts from cells grown under the different conditions.

Each line represents the results from a separate growth.

Cells were grown at 60° in 8 litres of medium and the appropriate gas was passed into the medium at the rate of 5 litres per min. The cultures were harvested when the extinction of each medium at 700mμ reached 0.5.

The preparation of cell extracts and enzyme assays are described in the methods section.

Table 5

Activity: mμ moles substrate or product/min./mg. protein at 60°

Growth Conditions	Aconitase	Isocitric dehydrogenase	Succinic dehydrogenase	Fumarase	Malic dehydrogenase
Glucose, anaerobic	15.8	41.1	1.2	21.3	2.81
	14.8	29.1	1.6	27.0	1.12
	17.5	32.7	1.72	20.8	1.98
Glucose, aerobic	72.0	48.2	3.37	35.4	5.56
	55.0	34.8	1.09	37.8	3.40
	43.8	51.0	2.16	38.4	13.4
Glycerol, aerobic	126	195	12.3	115	27.5
	72.8	116	13.8	91.8	21.9
	52.9	182	12.9	140	24.6
Glucose, anaerobic + KNO_3	6.41	70.1	1.99	5.95	5.58
	6.66	44.8	2.42	4.57	1.88
	7.37	50.8	1.25	11.8	2.73
Glycerol, anaerobic + KNO_3	14.6	85.8	2.48	40.3	33.7
	13.3	137	2.75	26.6	38.7
	21.3	73.5	2.74	60.5	14.3

Table 6

The Levels of Tricarboxylic Acid Cycle Enzymes Found Under Different Growth Conditions Relative to the Levels of Enzymes Found Under Anaerobic Conditions on Glucose

The averages of the individual results tabulated in Table 5 were used to calculate the average ratio of each enzyme activity to the activity of the same enzyme in extracts from cells grown anaerobically on glucose.

The figures in parentheses indicate the number of separate growths from which the averages were obtained.

Table 6

Relative enzyme activities

Growth Conditions		Aconitase	Isocitric dehydrogenase	Succinic dehydrogenase	Fumarase	Malic dehydrogenase
Glucose, anaerobic	(3)	1	1	1	1	1
Glucose, aerobic	(3)	3.5	1.3	1.5	1.6	3.8
Glycerol, aerobic	(3)	5.2	4.8	8.7	5	13
Glucose, anaerobic + KNO_3	(3)	0.4	1.6	1.3	0.34	1.7
Glycerol, anaerobic + KNO_3	(3)	1	2.9	1.8	1.9	14.7

aconitase, the activities of the enzymes in cells grown anaerobically on glycerol plus nitrate were higher than the activities found in cells grown on glucose under aerobic conditions.

Malic dehydrogenase activity was greatly increased in glycerol-grown cells - the greatest increase occurred in the presence of nitrate

C. DISCUSSION

The absence of detectable concentrations of cytochrome a_3 in anaerobically-grown cells indicates that this cytochrome is synthesized only in the presence of molecular oxygen.

The low level of cytochrome c_1 in cells grown anaerobically on glucose resulted in cytochrome b_1 being the main cytochrome present in these cells. The increased concentration of cytochrome c_1 in both aerobic cells and cells grown anaerobically on glucose or glycerol in the presence of nitrate suggests that some phenomenon associated with active electron transport was responsible for the increased synthesis of this cytochrome.

The constant level of cytochrome b_1 found in cells grown anaerobically on glucose and aerobically on both glucose and glycerol indicates that cytochrome b_1 is a so-called constitutive cytochrome in this organism.

The increased concentrations of cytochrome c_1 and cytochrome b_1 in cells grown anaerobically in the presence of nitrate suggests that nitrate played some part in the respiratory electron transport under these conditions. However it is not clear whether one cytochrome alone,or both of the cytochromes, were involved in nitrate respiration

The low but significant levels of tricarboxylic acid cycle enzymes found in cells grown anaerobically on glucose may have been due to their role in anabolic processes. Similar low levels were found in E. coli, that had been grown anaerobically on glucose (Gray, Wimpenny & Mossman, 1966).

The large increases in the specific activities of the enzymes in cells grown aerobically on glycerol and the smaller increases in cells grown aerobically on glucose both indicate that increased synthesis of these enzymes consistently occurred in the presence of oxygen. However the levels of these enzymes was greater in glycerol than in glucose and it would appear that glucose, or a glucose catabolite, exerted an inhibitory effect on the synthesis of tricarboxylic acid cycle enzymes.

The decrease in aconitase and fumarase activities in cells grown anaerobically on glucose plus nitrate is contrary to the slight increase that occurred in the remaining enzymes under the same conditions. The low levels of these two enzymes in glucose plus nitrate-grown cells as well as the low level of aconitase in cells grown anaerobically on glycerol plus nitrate suggests that the synthesi of these enzymes was affected by nitrate or nitrite. However the nitrat (nitrite) effect was less in the presence of glycerol than in the presence of glucose.

The fact that the overall level of enzyme activities (with the exception of aconitase) was higher in cells grown anaerobically on glycerol plus nitrate than in cells grown aerobically on glucose indicates that nitrate was supporting some oxidative metabolism

under the former conditions.

The high level of malic dehydrogenase activity in glycerol-grown cells both in the presence and absence of nitrate was possibly due to the involvement of this enzyme in glycerol catabolism.

Nitrate effectively stimulated the synthesis of cytochrome c_1 and cytochrome b_1, to almost the same extent, both in the presence of glycerol and of glucose whereas the stimulation of the synthesis of tricarboxylic acid cycle enzymes by both nitrate and oxygen was modified by the carbon source present; the enzyme levels were higher in the presence of glycerol than in the presence of glucose.

These findings tenuously support the hypothesis that nitrate acts as an electron acceptor for oxidative metabolism under anaerobic conditions. The roles of cytochrome b_1 and cytochrome c_1 in the reduction of nitrate and the possible sequence of components in the nitrate reducing system will be investigated in the next Chapter.

CHAPTER V

CHARACTERIZATION OF THE NITRATE REDUCTASE SYSTEM OF B. STEAROTHERMOPHILUS

A. METHODS

1. The extraction of protohaem from membranes and the formation of the pyridine haemochrome of protohaem

5ml. of membrane suspension (approximately 30mg. of protein) was pipetted into 15ml. of ice-cold acetone with vigorous stirring. The resulting mixture was immediately centrifuged at 38,000 x g for 15min. at 4^{o}. The clear supernatant was discarded and the precipitate was resuspended in 20ml. of chloroform-methanol (2:1 by vol.) and allowed to stand, with occasional stirring, for 30 min. at 4^{o}. The mixture was centrifuged at 38,000 x g for 15 min. and the supernatant was discarded. The precipitate was washed with 20ml. of ice-cold acetone by resuspension and centrifugation. The precipitate was then suspended in 15ml. of acetone-HCl 36% w/v (99:1 by vol.) and was kept at 4^{o} for 10 min. before being centrifuged at 38,000 x g for 10 min. The supernatant was saved and the precipitate was re-extracted with 5ml. of acetone-HCl (99:1 by vol.).

The acetone-HCl extracts were combined and extracted with 200ml. of ether-water (1:10 by vol.). The ether phase was removed and the aqueous phase was re-extracted with 5ml. of dry ether. The combined ether extracts were dried over solid sodium chloride. The ether extract was removed from the sodium chloride and evaporated to dryness in vacuuo. The residue was dissolved in 3ml. of pyridine and 3ml. of 0.2N-NaOH was added. The mixture was placed in two

cuvettes and to one approximately 3mg. of sodium dithionite was added to form the pyridine ferrohaemochrome. The remaining cuvette was used as a reference. The reduced minus oxidized spectrum was recorded from 620mμ to 400mμ .

2. The formation of the pyridine haemochrome of acetone-HCl insoluble haem

The membrane residue, after extraction with acetone-HCl, was resuspended in 3ml. of pyridine.

The mixture was homogenized in a glass homogeniser with a teflon pestle. 3ml. of 0.2N-NaOH was added and the mixture was homogenized once more. The resulting suspension was added to two cuvettes, one of which was then treated with dithionite in the method described above and the reduced minus oxidised spectrum recorded.

3. Preparation of membranes for spectrophotometric observations

Protoplast membranes were prepared as described previously (Chapter II, p.63) from cells grown anaerobically on glycerol plus nitrate. The membrane-bound cytochromes were reduced under anaerobic conditions as described in the methods section of Chapter IV. Each anaerobic cuvette contained 180 μmoles of potassium phosphate, pH 7.4, and 1ml. of membrane suspension (approximately 10mg. of protein) in a final volume of 2.9ml. The cuvettes were evacuated and filled with deoxygenated nitrogen and the appropriate

substrate was added from the side arm in a volume of 0.1ml. Membranes were reduced with either 1 μmole of $NADH_2$, to give $NADH_2$-reduced membranes, or 10 μ moles of sodium ascorbate, to give ascorbate-reduced membranes.

When it was necessary to add nitrate, under anaerobic conditions, to a reduced cytochrome preparation the following procedure was used. The anaerobic cuvette was attached unopened to the vacuum line and the connecting pressure tubing was evacuated and flushed several times with deoxygenated nitrogen. Nitrogen was admitted to the cuvette and the side arm was quickly removed and replaced with a side arm containing 3mg. of potassium nitrate. During this procedure the adapter was being flushed with deoxygenated nitrogen. The cuvette was then evacuated and flushed several times with deoxygenated nitrogen. The spectrum was recorded and compared with that obtained before removal of the side arm. If the spectra were identical the nitrate was added and a further spectrum was recorded.

All the cytochrome spectra were recorded with the cuvettes in the second sample position of the Unicam SP.800 spectrophotometer.

4. $NADH_2$-Nitrate reductase Assays: Inhibitor studies

$NADH_2$-nitrate reductase was assayed as described in general methods chapter (Chapter II, p.64).

When studying the action of inhibitors the enzymic activity was

Fig. 5

The Pyridine Haemochrome Spectra of the b-type and c-type Cytochromes Present in Membranes from B. stearothermophilus

The organism was grown under anaerobic conditions in glycerol plus nitrate medium and protoplast membranes were prepared.

The extraction of haem and formation of pyridine haemochromes is described in the methods section.

Fig. 5A The dithionite-reduced minus oxidized spectrum of the pyridine haemochrome of haem extracted from the membranes by acetone-HCl.

Fig. 5B The dithionite-reduced minus oxidized spectrum of the pyridine haemochrome of the haemoprotein whose haem was not extracted from membranes by acetone-HCl.

Fig.5A

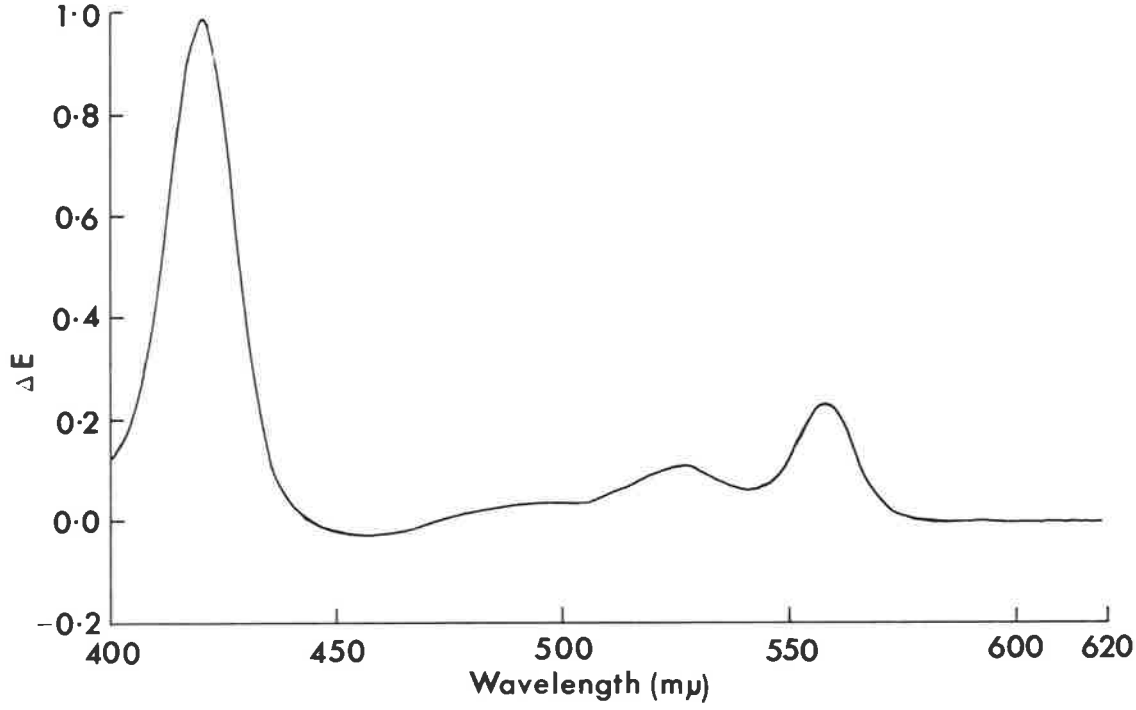
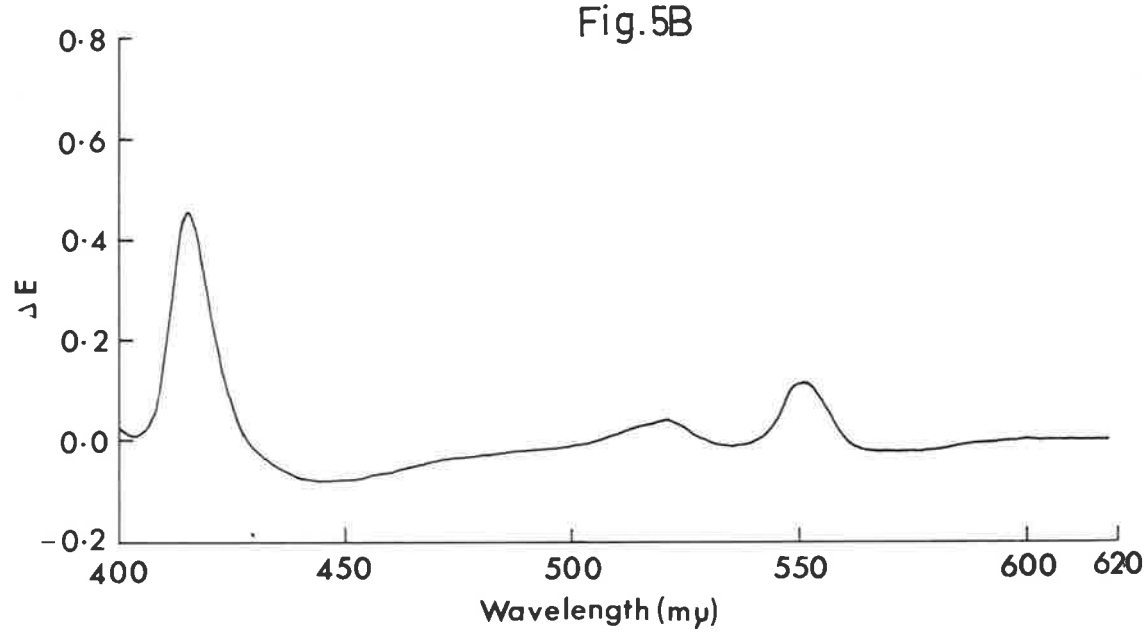

1·0
0·8
0·6
0·4
0·2
0·0
-0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)

Fig.5B


0·8
0·6
0·4
0·2
0·0
-0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)

calculated using a separate standard curve for nitrite in the presence of each inhibitor or co-factor used. When additions to the reaction mixture were made in ethanol or NaOH the same amount of each solvent was included in both an enzymic control and the reaction mixtures used for the nitrite standards.

B. RESULTS

The reduced minus oxidized spectrum of the pyridine haemochrome formed from acetone-HCl-extractable haem had absorption maxima at 557mμ , 527mμ and 420mμ (Fig. 5A). The spectrum is typical of the reduced minus oxidized spectrum of protohaem pyridine haemochrome (Morton 1958).

The pyridine haemochrome spectrum of the cytochromes in membranes that had previously been extracted with acetone-HCl, had maxima at 550mμ , 520mμ and 415mμ (Fig. 5B).

The maxima are similar to those found for the pyridine haemochromes of purified mammalian cytochrome c_1 (Sekuzi, Orii & Okunuki, 1960) and the soluble cytochrome c_{552} from E. coli (Fujita, 1966).

The haem group of the cytochrome present in the acetone-HCl-extracted membranes was not soluble in ether. Thus this cytochrome is classified as a c-type cytochrome. (Florkin & Stotz, 1965).

The ascorbate-reduced, minus oxidized spectrum of membranes had

apsorption maxima at 552mμ , 523mμ and 427mμ (Fig. 6A, dashed line). This cytochrome had been designated cytochrome c_1 in the previous chapter (see Discussion, this chapter). A shoulder on the 552mμ α band had maximum at approximately 560mμ .

When the $NADH_2$-reduced, minus ascorbate-reduced spectrum was recorded, maxima were observed at 560mμ , 530mμ and 430mμ (Fig. 6B, solid line). The cytochrome responsible for this spectrum had been referred to as cytochrome b_1 in the previous chapter.

When membranes, prepared from cells grown anaerobically on glycerol plus nitrate, were reduced with $NADH_2$ (1 μ mole) the reduced minus oxidized spectrum had a broad peak at 554mμ to 560mμ , a peak at 524mμ with a secondary peak at 530mμ and a soret peak at 430mμ (Fig. 6B solid line). This spectrum is characteristic of the combined spectra of b-type and c-type cytochromes. 3mg. of potassium nitrate was added anaerobically to the cuvette containing the $NADH_2$-reduced cytochromes and the spectrum: $NADH_2$-reduced + KNO_3, minus oxidized was recorded (Fig. 6B, dashed-dot line). Maxima occurred at 552mμ , 523mμ and 427mμ ; the 552mμ peak had a shoulder at approximately 560mμ . Thus the spectrum was identical to that of cytochrome c_1 (Fig. 6A-dashed line). In order to record the spectrum of the cytochrome that had been oxidized by nitrate another anaerobic cuvette was prepared in which the cytochromes were reduced with $NADH_2$ (1 μ mole). The spectrum: $NADH_2$-reduced, minus $NADH_2$-reduced plus KNO_3, was recorded (Fig. 6B, dashed line).

Fig. 6

The Effect of Nitrate Upon the Combined Spectrum of Cytochrome b_1 and Cytochrome c_1

B. stearothermophilus was grown anaerobically on glycerol plus nitrate medium and membranes were prepared. Identical membrane suspensions in anaerobic cuvettes were reduced with either $NADH_2$ (1 μmole) or sodium ascorbate (10 μ mole). Where appropriate KNO_3 (3mg.) was added anaerobically as described in methods section.

Fig. 6A ——— $NADH_2$-reduced, minus ascorbate-reduced.

----- Ascorbate-reduced, minus oxidized.

Fig. 6B ——— $NADH_2$-reduced, minus oxidized.

-·-·-·- $NADH_2$-reduced + KNO_3, minus oxidized.

----- $NADH_2$-reduced, minus $NADH_2$-reduced + KNO_3

Fig. 6A

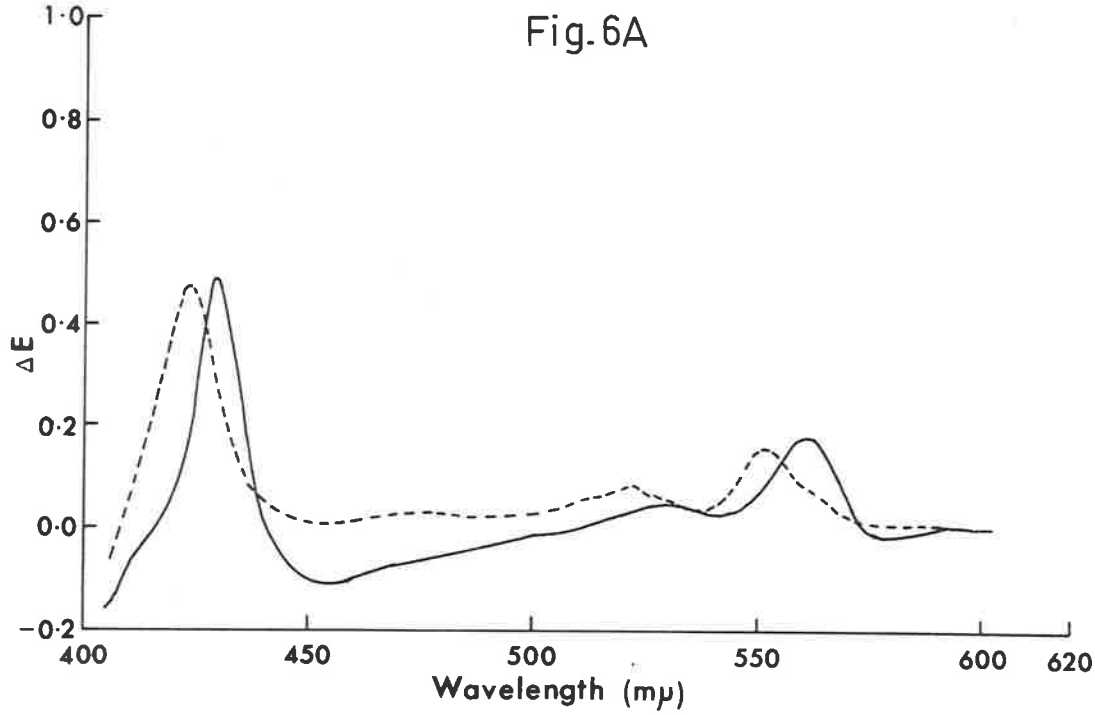

1·0
0·8
0·6
0·4
0·2
0·0
−0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)

Fig. 6B

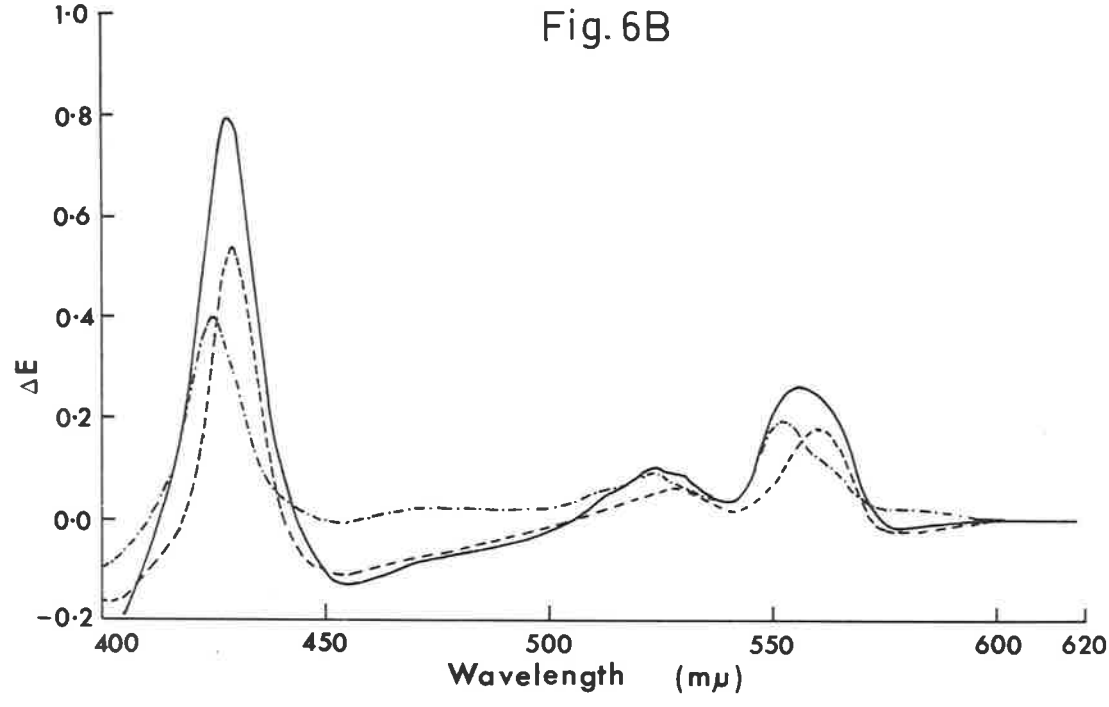

1·0
0·8
0·6
0·4
0·2
0·0
−0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)

Fig. 7

The Effect of Nitrate Upon the Spectra of Reduced Cytochrome b_1 and Reduced Cytochrome c_1 Present in Membranes from B. stearothermophilus Grown Anaerobically on Glycerol plus Nitrate Medium

Membranes were prepared and identical portions placed in anaerobic cuvettes. The membranes were reduced with either $NADH_2$ (1 μmole) or sodium ascorbate (10 μmoles). Where appropriate KNO_3 (3mg.) was added as previously described.

Fig. 7A: 1. $NADH_2$-reduced, minus ascorbate-reduced.

2. $NADH_2$-reduced plus NO_3^-, minus ascorbate-reduced.

Fig. 7B: 1. Ascorbate-reduced plus NO_3^-, minus oxidized.

2. Ascorbate reduced plus NO_3^- plus O_2, minus oxidized.

Fig.7A

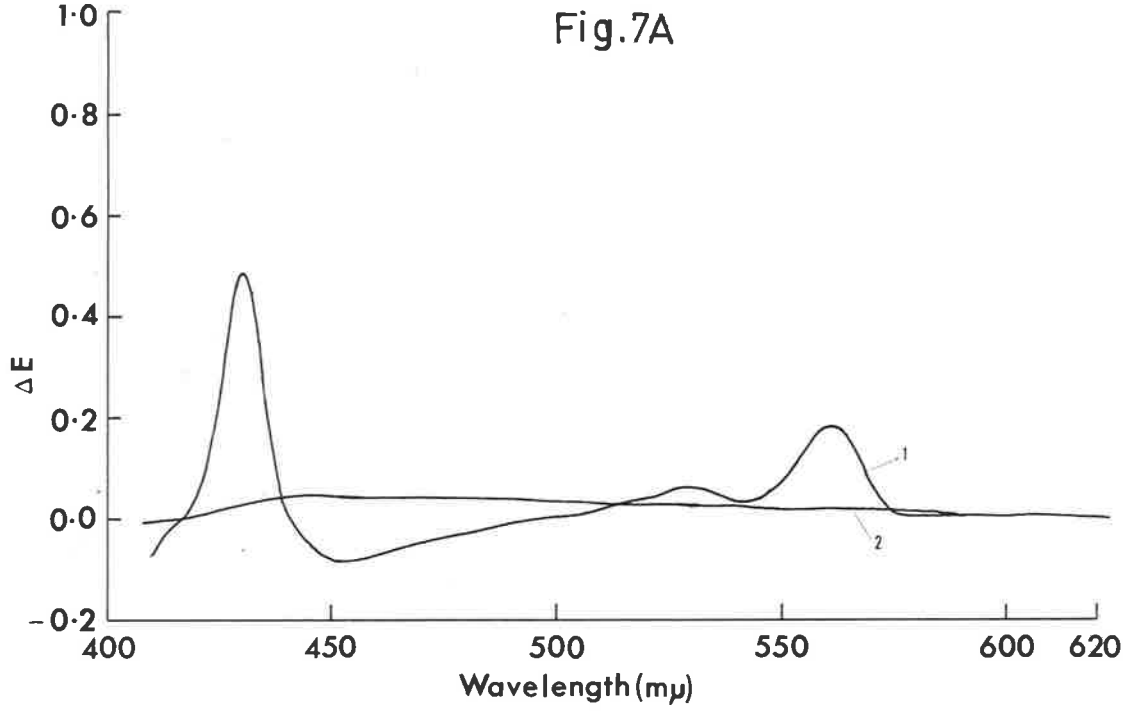
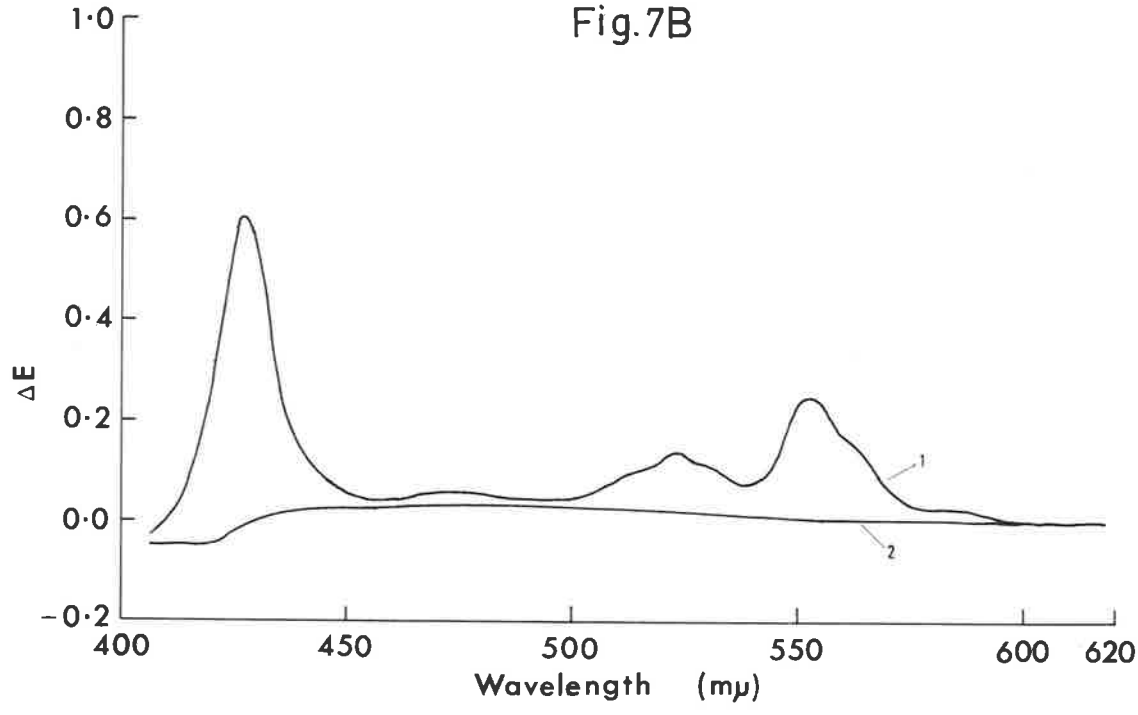

1·0
0·8
0·6
0·4
0·2
0·0
-0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)
1
2

Fig.7B


1·0
0·8
0·6
0·4
0·2
0·0
-0·2
ΔE
400
450
500
550
600
620
Wavelength (mμ)
1
2

Maxima were found at 560mμ , 530mμ and 430mμ . This spectrum was identical to that found for cytochrome b_1 (Fig. 6A solid line).

Additional proof for the role of cytochrome b_1 in nitrate reduction was provided by recording the spectrum: $NADH_2$-reduced, minus ascorbate reduced, (Fig. 7A, 1) which gave the spectrum of cytochrome b_1. 3mg. of potassium nitrate was then added to the cuvette and the spectrum: $NADH_2$-reduced plus KNO_3, minus ascorbate reduced, was recorded. The spectrum of reduced cytochrome b_1 disappeared and a relatively flat base line resulted.

To determine whether cytochrome c_1 was in any way involved in electron transport to nitrate, the ascorbate-reduced, minus oxidized, spectrum was recorded, then 3mg. of potassium nitrate was added anaerobically and the spectrum was re-drawn. The spectra before and after the addition of nitrate to the ascorbate-reduced cytochrome, were identical (Fig. 7B, 1: spectra superimposed). The shoulder at 560mμ was unaffected by the addition of nitrate. When the anaerobic cuvette was opened and gassed with oxygen for 5 min. the reduced spectrum of cytochrome c_1 disappeared to give a flat base line (Fig. 7B, 2).

It was thought that the shoulder present at 560mμ on the α peak of reduced cytochrome c_1 might be due to reversed electron transport from the cytochrome c_1 to a b-type cytochrome. An experiment was designed to test this hypothesis. Two anaerobic cuvettes

were prepared, one containing membranes reduced with ascorbate, the other containing ascorbate-reduced membranes plus 30 µg. 2-n-heptyl-4-hydroxyquinoline-N-oxide.

The spectrum ascorbate-reduced, minus ascorbate-reduced plus hydroxyquinoline-N-oxide, was recorded (Fig. 8,- the reduced minus oxidized spectrum of ascorbate-reduced membranes was included for comparison). The difference spectrum was similar to that of a b-type cytochrome with absorption maxima at 558mµ . Both cuvettes were opened and gassed with oxygen and the spectrum was re-drawn. A flat base line was obtained indicating that hydroxyquinoline-N-oxide did not prevent the oxidation of either cytochrome c_1 or reduced cytochrome b_{558} by oxygen.

An examination of the spectra of $NADH_2$-reduced, membranes reveals a marked trough at approximately 450mµ (Figs. 6A, 6B & 7A) which was probably due to oxidized flavoprotein in the reference cuvette (Chance & Williams, 1956). Addition of nitrate to $NADH_2$-reduced, membranes resulted in the disappearance of the trough (Figs. 6B & 7A) indicating that oxidation of the flavoprotein had occurred.

The effects of a number of inhibitors, metal chelating agents and co-factors on $NADH_2$-nitrate reductase activity were investigated and the results were tabulated in Table 6. The results were expressed as a percentage of the $NADH_2$-nitrate reductase activity

Fig. 8

The Effect of 2-n-heptyl-4-hydroxyquinoline-N-oxide on the Ascorbate Reduced Cytochrome Spectrum

B. stearothermophilus was grown anaerobically on glycerol + nitrate medium and membranes were prepared.

Identical membrane preparations were placed in two anaerobic cuvettes and 30 μg. of hydroxyquinoline-N-oxide was placed in one. The final volume of solution in each cuvette was 2.9ml. Both cuvettes were evacuated and filled with deoxygenated nitrogen before the membranes in each were reduced with 10 μ moles of sodium ascorbate (in 0.1ml.).

——— Ascorbate-reduced, minus oxidized.

----- Ascorbate-reduced, minus ascorbate-reduced, plus hydroxyquinoline-N-oxide.

Fig.8

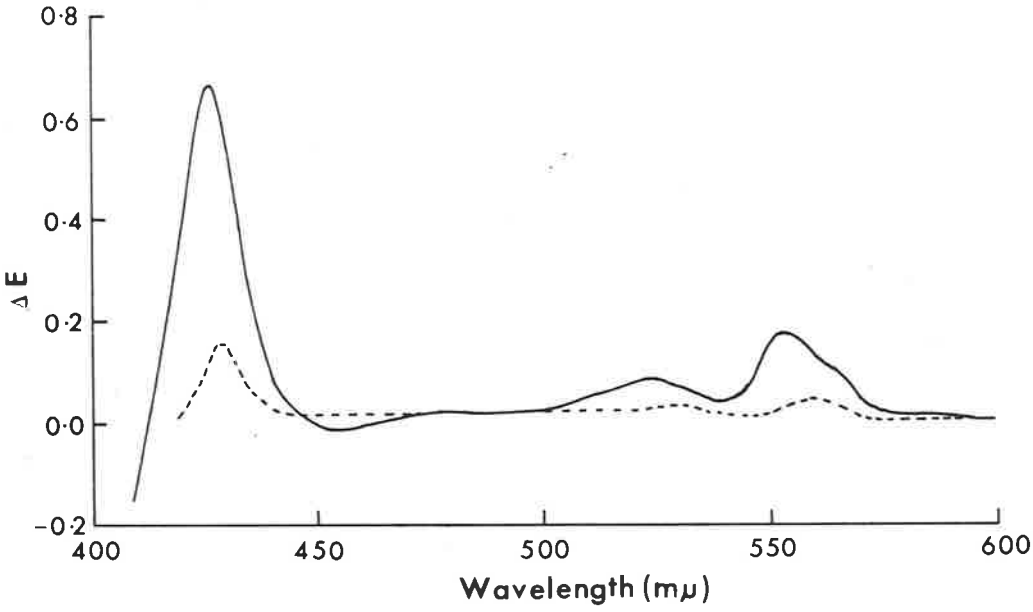


Table 7

The Effects of Inhibitors, Co-factors and Metal Chelating Agents on $NADH_2$-Nitrate Reductase Activity

The $NADH_2$-nitrate reductase system described in General Methods was used to assay nitrate reductase activity in protoplast membranes prepared from cells grown anaerobically on glycerol plus nitrate medium.

The activities, in mμ moles of NO_2^-/min./mg. protein at 60^o, were expressed as a percentage of the activity of the assay with no added inhibitors, additional co-factors or metal chelators.

Nitrite standards were made up in separate reaction mixtures containing each of the additions listed.

Hydroxyquinoline-N-oxide was added in 0.02ml. of 0.01N-NaOH. 8-hydroxyquinoline and thenoyltrifluoroacetone were each added in 0.02ml. of 95% ethanol. Enzyme controls were included containing 0.02ml. of each of these solvents.

Table 7

Addition	Concentration	Relative Activity
$NADH_2$	5×10^{-4} M	100
Sodium Amytal	5×10^{-3} M	105
Sodium Amytal	10^{-2} M	21
Atebrine	2.5×10^{-4} M	0
FMN	2×10^{-4} M	40
FAD	2×10^{-4} M	101
2-n-heptyl-4-hydroxy-quinoline-N-oxide	5×10^{-4} M	2.3
Menadione	10^{-3} M	70
Dicumarol	6×10^{-5} M	78.2
Dicumarol	3×10^{-5} M	72.7
1-10,phenanthroline	2×10^{-3} M	0
8-hydroxyquinoline	2×10^{-3} M	10
Thenoyltrifluoroacetone	10^{-4} M	8
KCN	5×10^{-4} M	25

Substitution of 5×10^{-4} M $NADPH_2$ for 5×10^{-4} M $NADH_2$ resulted in only 1% of nitrate-reductase activity.

obtained in the standard assay. $NADPH_2$ did not effectively support nitrate reduction.

Amytal, at a concentration of 5×10^{-3} M stimulated $NADH_2$-nitrate reductase activity slightly whereas 10^{-2} M amytal caused 79% inhibition. Atebrine completely inhibited $NADH_2$-nitrate reductase activity. These results indicate that flavoprotein was involved in this nitrate-reducing process.

FMN inhibited the $NADH_2$-nitrate reductase system by 60% in contrast to the lack of either inhibition or marked stimulation by FAD.

10^{-3} M Menadione inhibited nitrate reductase activity as did the vitamin K antagonist dicumarol.

The marked inhibition by 2-n-heptyl-4-hydroxyquinoline-N-oxide affords further support for the suggested role of cytochrome b_1 as an electron transfer ring component of the nitrate-reducing system.

The inhibition by KCN and by the metal chelating agents, 1-10, phenanthroline, 8-hydroxyquinoline, and thenoyltrifluoroacetone (2-(4,4,4,-trifluoro-3-oxybutanonyl-2)-thiophine) suggests that an unknown metal participates in electron transfer to nitrate.

C. DISCUSSION

The cytochromes with α absorption bands at 560mμ and 552mμ have been referred to in this thesis as cytochrome b_1 and cytochrome c_1 respectively. It should be noted that these cytochromes were named after reference to published literature on cytochromes with similar absorption maxima (Morton, 1958). Definite confirmation of their classification as a b-type and a c-type cytochrome cannot be made until the respective prosthetic groups of the purified cytochromes have been chemically identified (Florkin & Stotz, 1965).

According to the recommendations on cytochrome nomenclature, suggested by the International Union of Biochemistry, the cytochromes should be named cytochrome 560 (B. stearothermophilus) and cytochrome 552 (B. stearothermophilus).

The purification of these cytochromes and the isolation and identification of their respective prosthetic groups was outside the scope of this thesis. However the pyridine haemochrome spectra presented in this chapter do provide some support for the hypothesis that a b-type and a c-type cytochrome were present in the protoplast membranes. Although the data presented do not demonstrate precisely which of the cytochromes is a b-type or a c-type cytochrome it is reasonable to assume that the cytochrome with an α absorption band at 560mμ is a b-type cytochrome and the cytochrome with an α absorption band at 552mμ is a c-type cytochrome. For convenience, and so that the respiratory chain of B. stearothermophilus may be

compared with those of other nitrate-reducing bacteria, the names cytochrome b_1 and cytochrome c_1 will be used throughout the remainder of this thesis, with the proviso that further proof of their identity is required.

The results of the spectral studies, on membranes from cells grown on glycerol plus nitrate, indicate that cytochrome b_1 is the cytochrome involved in respiratory nitrate reduction in this organism.

Cytochrome c_1, together with the b-type cytochrome possessing an α absorption band at 558mμ , appear to be components of an aerobic electron transport chain.

The spectral studies revealed that flavoprotein became oxidized upon the addition of nitrate to membranes reduced by $NADH_2$ and this observation together with the inhibition of $NADH_2$-nitrate reductase activity by amytal (10^{-2} M) suggest that a flavoprotein mediates hydrogen transfer from reduced NAD to other components of the respiratory nitrate reducing system.

The inhibition of nitrate reduction by FMN and by Menadione might be explained if these substances stimulated an electron transport pathway that was competing with nitrate reductase for electrons. The apparent stimulation of nitrate reductase by 5×10^{-3} M amytal (Table 6) might then be due to a greater sensitivity of the oxygen pathway to the lower concentration of this inhibitor. If one assumes that this hypothesis is correct then a naphthoquinone analogue would

have to occur in both respiratory chains to account for the dicumarol inhibition of nitrate reduction. Downey (1962) has reported that oxidative phosphorylation in B. stearothermophilus coupled to electron transport to oxygen, was dependent on a naphthoquinone derivative and that menadione stimulated nitrate reductase significantly although it was not essential for its activity.

The inhibition of $NADH_2$-nitrate reductase by the metal-chelating agents and cyanide was similar to the inhibition of respiratory nitrate reductases found in other bacteria and was apparently due to the chelation of some metal component of the system.

The results discussed here do not provide any information on the nature of the enzyme responsible for the reduction of nitrate to nitrite. The following chapter is concerned with the solubilization and characterization of the terminal nitrate reductase.

CHAPTER VI

THE SOLUBILIZATION AND PROPERTIES OF NITRATE REDUCTASE

A. METHODS

1. Growth of bacteria and preparation of membranes

B. stearothermophilus was grown anaerobically on glycerol (0.4%) plus potassium nitrate (0.4%) medium in 8 litre batches. Membranes prepared from each batch of cells as described in the General Methods (p. 63) were stored at -10°.

2. Assay of benzyl viologen-nitrate reductase activity

The method was substantially the same as that described in the General Methods (p. 64) except that the pH of the phosphate buffer was 6.0 (see optimum pH of soluble enzyme in the Results section of this Chapter).

B. RESULTS

1. Solubilization of nitrate reductase

The method used to solubilize the nitrate reductase was similar to that used by Lemberg, Pilger, Newton & Clarke (1964) to solubilize cytochrome oxidase from ox heart mitochondria.

The frozen membranes, prepared from 100g. (wet weight) of cells, were thawed and the protein concentration was adjusted to 13mg. per ml. with 0.05M potassium phosphate, pH 7.4. 40% (w/v) sodium cholate was added to give a final concentration of 2% (w/v). Solid ammonium sulphate was added slowly with stirring to make the solution

25% saturated. The pH was adjusted to 7.4 with 3N ammonium hydroxide and the solution was left at 4^{o} for 48 hr. After this time the solution was stirred at room temperature for 2 hr., cooled to 0^{o} and centrifuged at 78,000 x $g_{av.}$ for 2 hr. (Type 30 head, Beckman L2-65).

51% of the initial benzyl viologen-nitrate reductase activity was found in the supernatant. However 51% of the total membrane protein was also solubilized by this procedure and no purification was achieved at this stage.

The supernatant (vol. 74 ml.) was dialyzed against three 10 litre changes of 0.01M potassium buffer, pH 7.4, over a period of 16 hr., to remove cholate and ammonium sulphate. Precipitation of protein occurred during dialysis and this was removed from the non-diffusable material by centrifugation at 78,000 x $g_{av.}$ for 1 hr. 23% of the original nitrate reductase activity, present in the membranes remained in the supernatant.

Chromatography on DEAE cellulose and Biogel P100 failed to increase the purity of the enzyme. Centrifugation of the 78,000 x g supernatant at 220,000 x $g_{av.}$ for 3 hr. (Type 65 head, Beckman L2-65) resulted in 3 fractions in the centrifuge tube: a dark red precipitate, a lighter greenish-brown infranatant, and a colourless supernatant.

Spectral studies made on the three fractions revealed that they

contained cytochrome c_1. Fig. 9 shows the borohydride-reduced, minus oxidized spectra obtained with the supernatant, infranatant, and precipitate, respectively, in 5 mm. light-path cuvettes. The base line for each spectrum has been displaced so that the respective spectra can be compared.

The borohydride-reduced, minus oxidized spectrum of the precipitate had absorption maxima at 552 mμ , 523 mμ and 417 mμ . The corresponding reduced minus oxidized spectrum of the supernatant had no discernable α or β absorption peaks but a small soret peak occurred at approximately 416 to 418 mμ .

The borohydride-reduced, minus oxidized spectra of the three fractions, unlike the $NADH_2$-reduced, minus oxidized spectrum of membranes (fig. 6B) did not exhibit any absorption at 450 mμ due to oxidized flavoprotein.

The absence of absorption bands attributable to cytochrome b_1 in the reduced minus oxidized spectrum of the 220,000 x g precipitate afforded an opportunity to ascertain the principle absorption maxima of both the absolute oxidized and absolute reduced spectra of cytochrome c_1. The respective spectra are shown in fig. 10. The oxidized spectrum had a soret absorption peak at 409 mμ and the borohydride-reduced spectrum had absorption maxima at 552 mμ , 523 mμ and 414 mμ .

Since nitrate reductase activity was detected in all three

Fig. 9

The Borohydride-Reduced minus Oxidized Spectra of the 220,000 x g Supernatant, Infranatant and Precipitate from Cholate-solubilized Membranes.

The 78,000 x $g_{av.}$ supernatant (cholate-free) from solubilized membranes was centrifuged at 220,000 x $g_{av.}$ for 3 hr.

The resulting supernatant, infranatant and precipitate were separated. 15 ml. of the supernatant was freeze-dried, taken up in 3 ml. of 0.1M-NaCl and dialyzed against 0.1M-NaCl.

The respective fractions were placed in 5 mm. light-path stoppered cuvettes and each was reduced with approximately 2 mg. of potassium borohydride. The reduced minus oxidized spectrum of each fraction was recorded using an open cuvette containing an identical amount of the respective fraction as a reference.

The protein concentrations were: supernatant, 1.8mg./ml.; infranatant, 6.5mg./ml.; precipitate, 10.4mg./ml.

1. reduced minus oxidized spectrum of supernatant.
2. reduced minus oxidized spectrum of infranatant.
3. reduced minus oxidized spectrum of precipitate.

Fig. 9

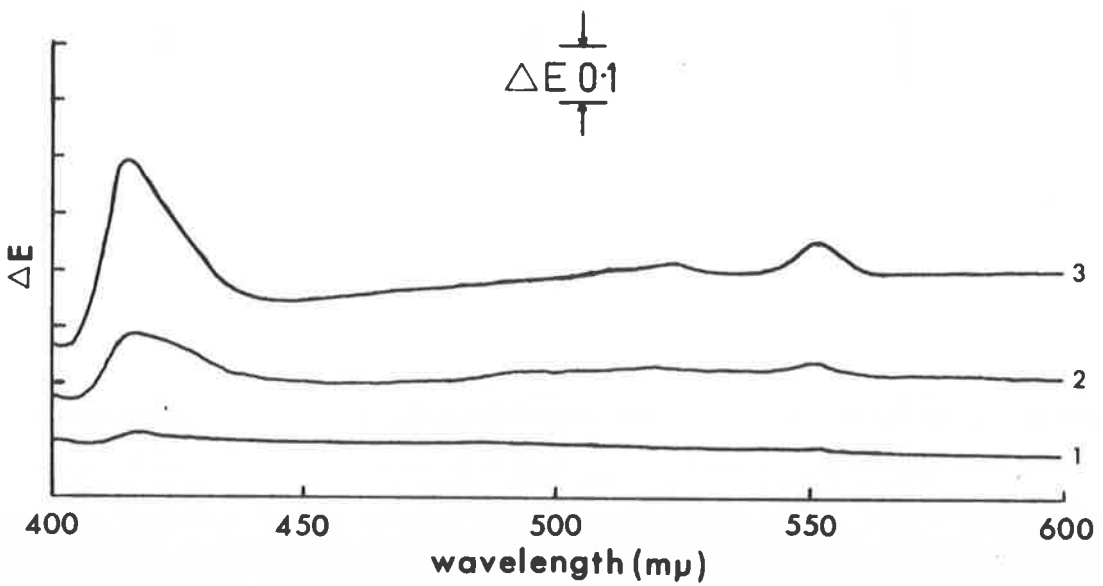


Fig. 10

Absolute reduced and absolute oxidized spectra of cytochrome c_1.

The 0.5 cm. cuvettes containing both reduced and oxidized precipitate, that were used to record the spectrum shown in fig. 9, (3), were used separately to obtain the absolute reduced and absolute oxidized spectra of cytochrome c_1.

——— Borohydride-reduced precipitate with 0.1M-NaCl as a reference.

----- Oxidized precipitate with 0.1M-NaCl as a reference.

Fig. 10

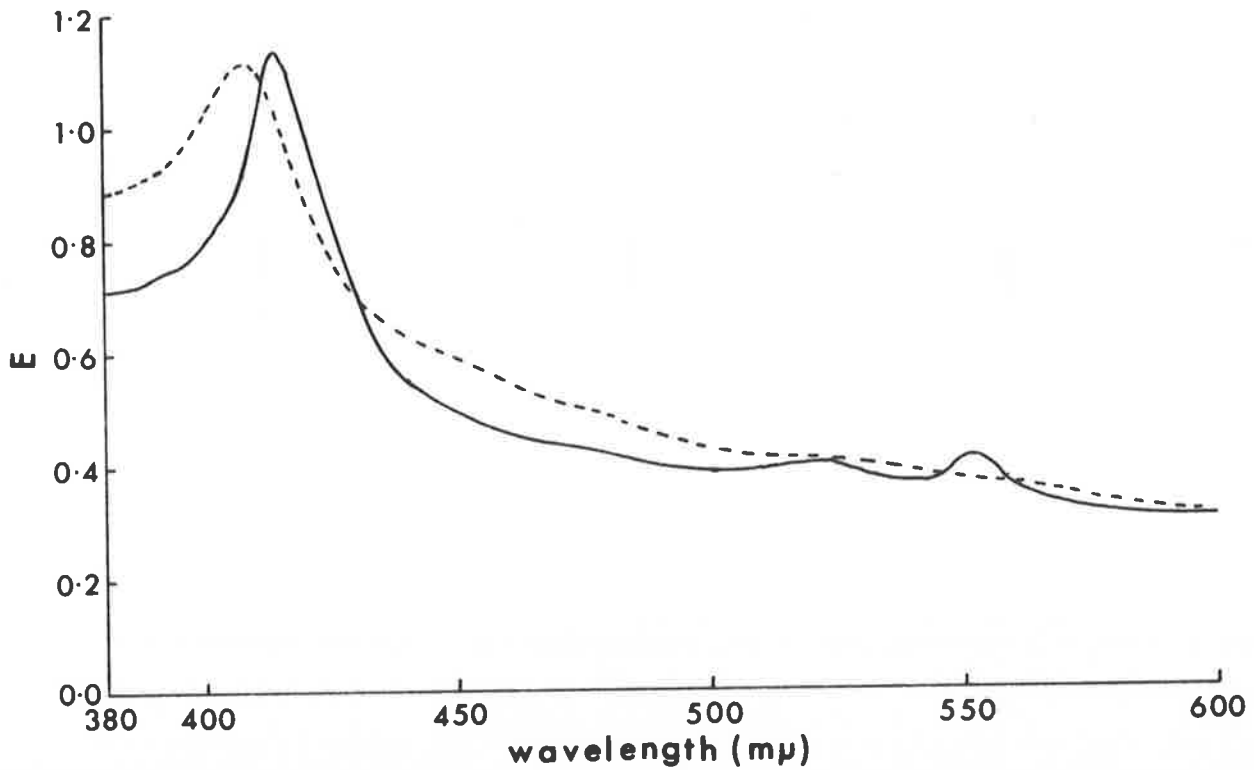


Table 8

The Distribution of Cytochrome c_1 and Nitrate Reductase in Fractions obtained by High Speed Centrifugation of Cholate-Ammonium Sulphate-Solubilized Membranes.

The 78,000 x $g_{av.}$ supernatant obtained after solubilization of membranes with 2.0% sodium cholate-0.25 saturated ammonium sulphate was centrifuged at 220,000 x $g_{av.}$ for 3 hr.

Cytochrome c_1 concentration was calculated from the height of the α absorption peak at 552 mμ after reduction with sodium borohydride (approx. 2 mg.).

The nitrate reductase activity with reduced benzyl viologen as a donor was assayed as described in General Methods.

Table 8

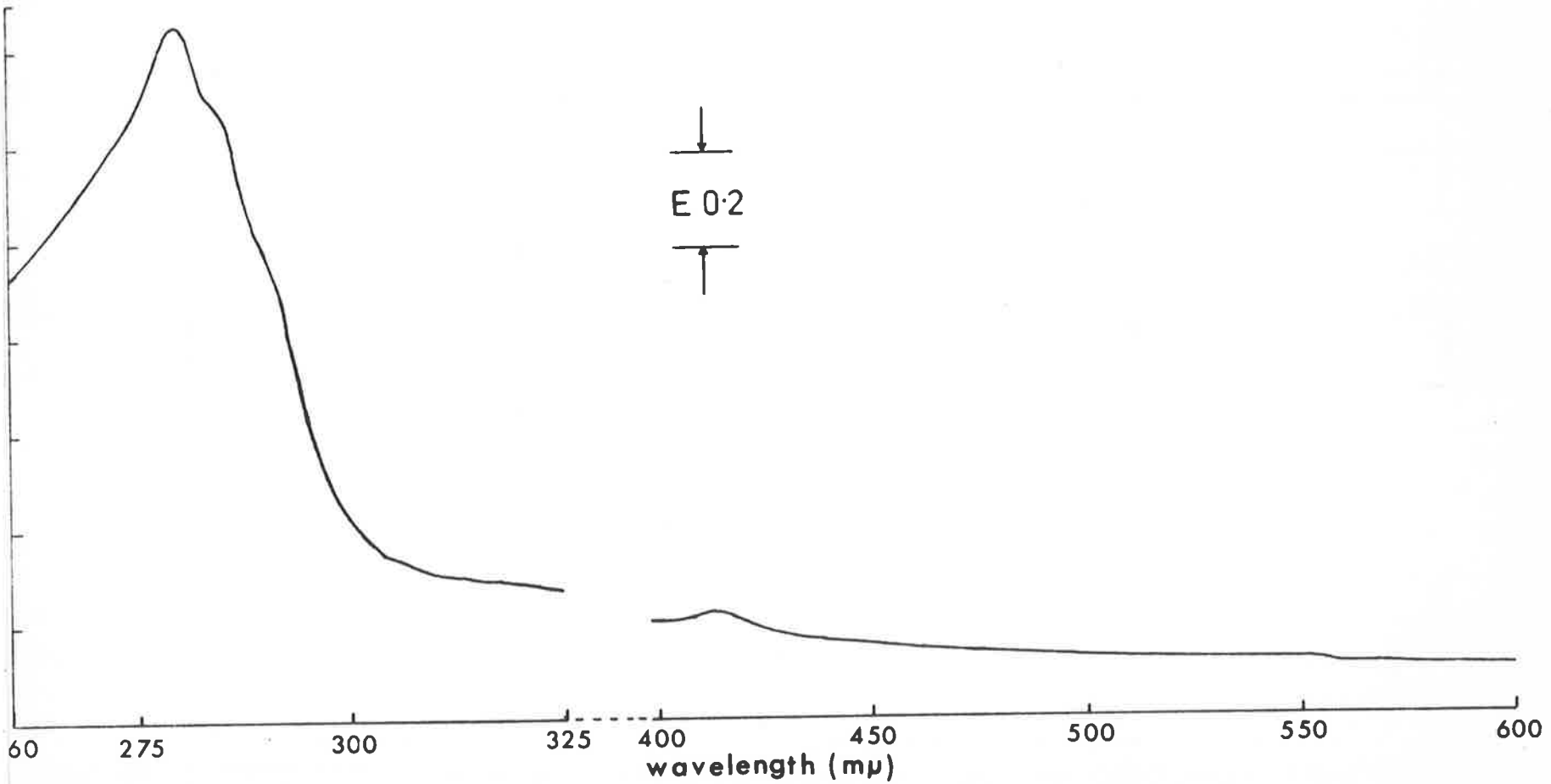
Fraction	Concentration of cytochrome c_1 mμmoles/mg protein	Specific Activity nitrate reductase mμmoles NO_2^-/min/ mg protein
220,000 x $g_{av.}$ supernatant	0.06	4,400
220,000 x $g_{av.}$ infranatant	0.163	3,300
220,000 x $g_{av.}$ precipitate	0.388	1,700

Fig. 11

Absolute Reduced Spectrum of the 220,000 x $g_{av.}$ Supernatant.

The spectrum of borohydride reduced supernatant was recorded between 260 mμ and 600 mμ with 0.1M-NaCl as a reference.

Fig. 11



fractions a comparison was made between the cytochrome c_1 concentration and the specific activity of benzyl viologen-nitrate reductase in each fraction. Table 8 shows that there was an inverse relationship between cytochrome c_1 concentration and the specific activity of nitrate reductase - suggesting that the cytochrome c_1 and the nitrate reductase were present as separate entities.

Although the specific activity of the nitrate reductase in the supernatant (Table 8) was approximately the same as that found in the original membrane preparation, it was felt that since the enzyme activity was not completely sedimented after being centrifuged at 220,000 x $g_{av.}$ for 3 hr. a soluble form of nitrate reductase was present.

The absolute spectrum of borohydride-reduced supernatant, in a 10 mm. light path cuvette, is shown in fig. 11. The principle peaks observed were the soret peak of reduced cytochrome c_1 at 414 mμ and a protein absorption peak at 280 mμ .

The nitrate reductase was not pure enough to enable accurate measurements to be made of its molecular weight. However the sedimentation characteristics of the proteins present in both the supernatant and infranatant were compared in the analytical ultracentrifuge. A portion of the supernatant (15 ml.) was freeze-dried, taken up in 2.5 ml. of 0.1M sodium chloride and dialysed against 5 litres of 0.1M sodium chloride for 12 hr. The contents of the

dialysis sack were removed and made up to 3 ml. with 0.1M sodium chloride. The protein concentration was 1.5 mg. per ml. The concentrated 220,000 x g supernatant was then placed in a single-sector cell of the Beckman-Spinco analytical ultracentrifuge (Model E). The 220,000 x g infranatant, in 0.1M sodium chloride, was placed in a single-sector cell with a positive wedge window and both cells were inserted into the rotor (type An-D) and centrifuged at 59,780 r.p.m. at 4^{o}. Fig. 12 is a positive enlargement of the schlieren pattern images from both cells after 24 min. at 59,780 r.p.m. The upper pattern is that of the infranatant, the lower pattern is that of the supernatant. A small peak was observed in the supernatant 4 min. after reaching top speed. This peak quickly moved across the cell and can be seen as sedimented material in fig. 12 (right hand side of lower image). The movement of the major peak was extremely slow. Broadening of this peak occurred during the run - probably due to diffusion. The approximate uncorrected sedimentation constant of the major protein peak in the supernatant was found to be 2.5×10^{-13} sec.

The infranatant schlieren pattern showed one major peak, at the start of the run, that quickly broke up into the peaks seen in fig. 12. A comparison of the two patterns does not reveal any peaks in the supernatant corresponding to the multiple peaks in the infranatant.

Fig. 12

The Sedimentation Patterns of Proteins Present in the 220,000 x g Supernatant and Infranatant of Cholate-Ammonium Sulphate-Solubilized Membranes.

The supernatant and infranatant fractions were obtained by centrifuging cholate-ammonium sulphate-solubilized membranes at 220,000 x $g_{av.}$ for 3 hr. (type 65 head, Beckman L2-65).

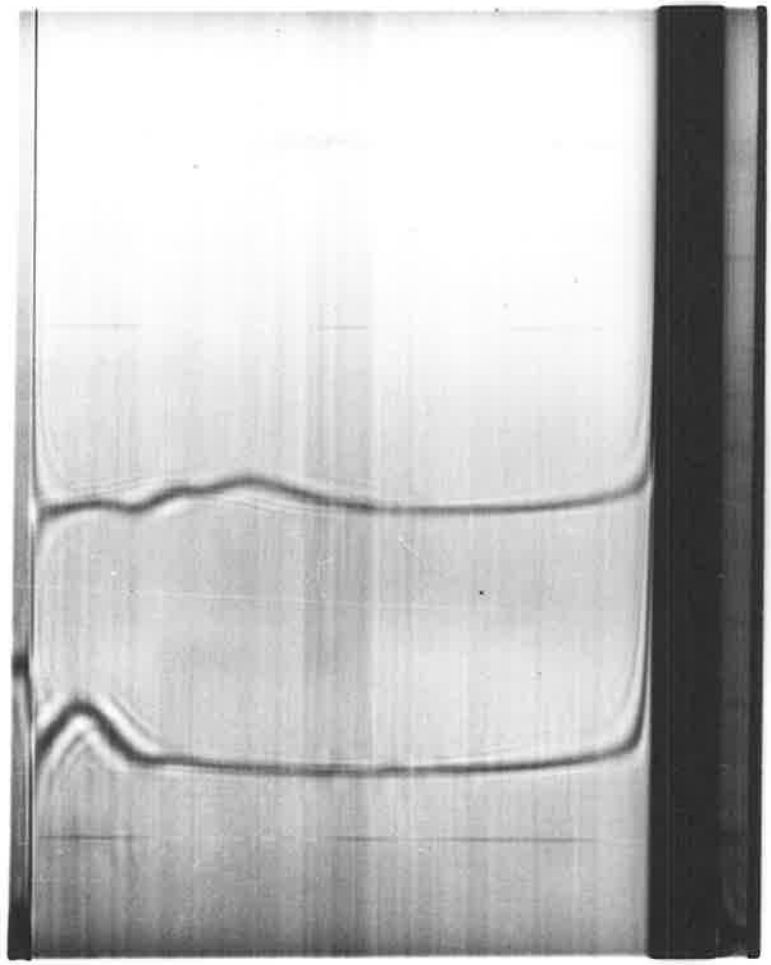
The sedimentation patterns were obtained in a Model E Beckman-Spinco analytical ultracentrifuge using schlieren optics.

Conditions:	solvent:	0.1M NaCl
	temperature:	4^{o}
	speed:	59,780 r.p.m.
	phase plate angle:	60^{o}

The upper trace, produced by using a positive wedge window, is that of the infranatant. The lower trace is the supernatant schlieren pattern.

The direction of sedimentation is from left to right.

Fig. 12



2. The Properties of nitrate reductase

(i) Optimum pH at 60°.

Preliminary investigations revealed that the addition of 0.1 ml. of 0.5% (w/v) sodium dithionite (in 0.25M potassium phosphate, pH 7.4) to the reaction mixtures changed their initial pH values. Therefore all pH measurements were made on samples of the reaction mixture after the addition of enzyme and dithionite.

The final pH of the reaction mixture was plotted against the velocity of the enzymic reaction using two buffers: 0.1M (final concentration) potassium phosphate from pH 7.4 to pH 5.8 (final pH) and 0.1M sodium acetate buffer from pH 6.5 to pH 5.5 (final pH). All the pH values were determined at 25°.

The optimum pH values for benzyl viologen-nitrate reductase activity were 6.1 to 6.3 in phosphate buffer (fig. 13). A broad pH versus activity curve was obtained for acetate buffer with a maximum between pH 5.9 and pH 6.1. The maximum activity of the enzyme in phosphate buffer was greater than that found in acetate buffer. A 50% decrease in enzyme activity occurred between pH 6.3 and pH 7.4 in phosphate buffer.

(ii) Stability to heat.

The heat stability of the soluble enzyme was investigated by incubating the enzyme in the reaction mixture, without nitrate, at 60° for 30, 60 and 120 min. Enzyme activity was subsequently

measured at 40^{o}. The activity of the enzyme decreased by 6.6% after one hour and by 13.7% after two hours (fig. 14).

(iii) Michaelis constants at 40^{o} and 60^{o}.

The variation of the initial velocity of the enzyme with nitrate concentration was determined at both 40^{o} and 60^{o} (figs. 15A, 15B). The values obtained were used to calculate the respective Michaelis constants by the method of Lineweaver & Burk, as described by Dixon & Webb (1958). The plot of the reciprocal of the initial velocity at both temperatures against the reciprocal of the substrate concentration gave straight lines (figs. 16 & 17). The respective Michaelis constant (K_m) was calculated both by finding the intercept of each extrapolated line on the substrate axis (not shown in figs. 16 & 17) and from the slope of each line.

The K_m values were found to be 1.52×10^{-4}M at 40^{o} and 2.03×10^{-4}M at 60^{o}. The V_{max} values, calculated from the intercepts on the velocity axes, were 36.2 mμ moles NO_2^-/min. at 40^{o} and 142.4 mμ moles NO_2^-/min. at 60^{o}.

(iv) Energy of activation.

The variation of initial enzyme velocity with temperature was determined between the temperature limits: 30^{o} and 70^{o} (fig. 18). It is apparent from the figure that the enzyme was partly inactivated at 70^{o}.

The relationship between the energy of activation, E, the

Fig. 13

The Optimum pH for Benzyl Viologen-Nitrate Reductase

The reaction mixture was similar to that described for benzyl viologen-nitrate reductase assays in General Methods, except that the pH values of the buffers were varied.

The pH values of the final reaction mixture after the addition of enzyme and dithionite were measured at 25°. The Radiometer 22 pH meter was standardized against 0.05M potassium hydrogen phthalate at 25°.

0.1M potassium phosphate buffer

0.1M sodium acetate buffer

Fig.13

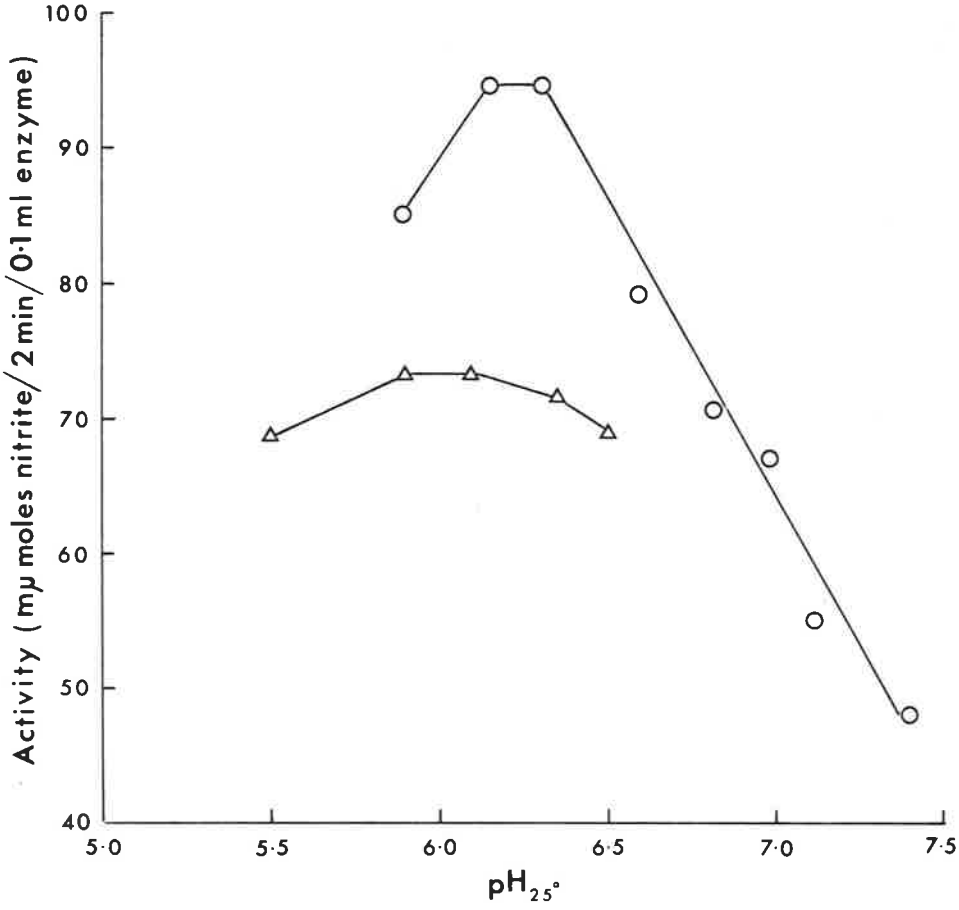

Activity (mμ moles nitrite/2 min/0·1 ml enzyme)
100
90
80
70
60
50
40
5·0
5·5
6·0
6·5
7·0
7·5
pH25°

Fig. 14

The Heat Stability of Benzyl Viologen-Nitrate Reductase

The enzyme (0.5 ml.) was incubated at 60° in the following incubation mixture:

potassium phosphate, pH 6.0, 500 μ moles; benzyl viologen, 2 μ moles; in a final volume of 2.5 ml. An initial 0.4 ml. sample was removed before heating commenced and further 0.4 ml. samples were removed 30, 60 and 120 min. after the start of incubation.

20 μ moles of potassium nitrate were added to each reaction mixture and the volume was made up to 0.8 ml. with distilled water. The tubes were then assayed for benzyl viologen-nitrate reductase activity at 40° as described in General Methods.

Fig. 14

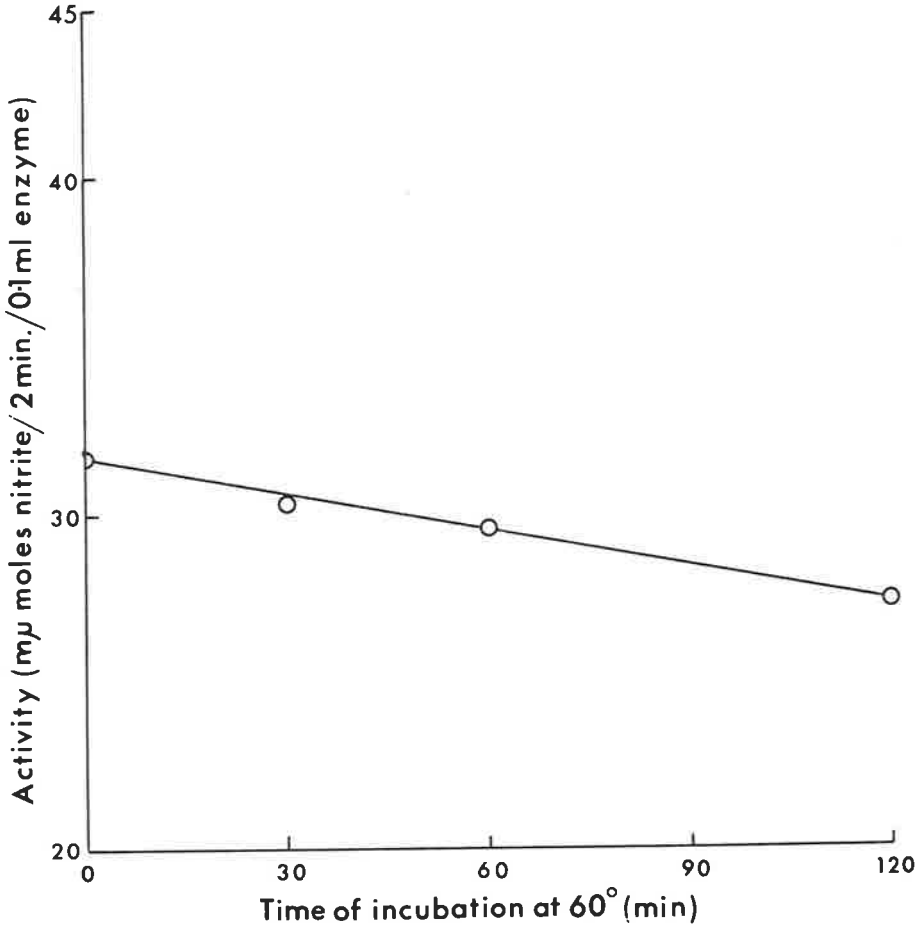

45
40
30
20
0
30
60
90
120
Activity (mμ moles nitrite/2 min./0·1 ml enzyme)
Time of incubation at 60° (min)

Fig. 15

The Variation of Velocity with Nitrate Concentration at 40° and 60° Respectively

The reaction mixture contained: potassium phosphate buffer, pH 6.0, 100 μmoles; benzyl viologen, 0.4 μmoles; enzyme 0.1 ml. and the appropriate amount of potassium nitrate in a final volume of 0.9 ml.

Three tubes were assayed for benzyl viologen-nitrate reductase activity, at 60° and 40° respectively, for 30 sec., 1 min. and 2 min. at each concentration. The initial velocity was determined and plotted against potassium nitrate concentration.

Fig. 15A: The effect of potassium nitrate concentration on the enzyme velocity at 40°.

Fig. 15B: The effect of potassium nitrate concentration, on the enzyme velocity at 60°.

Fig. 15A

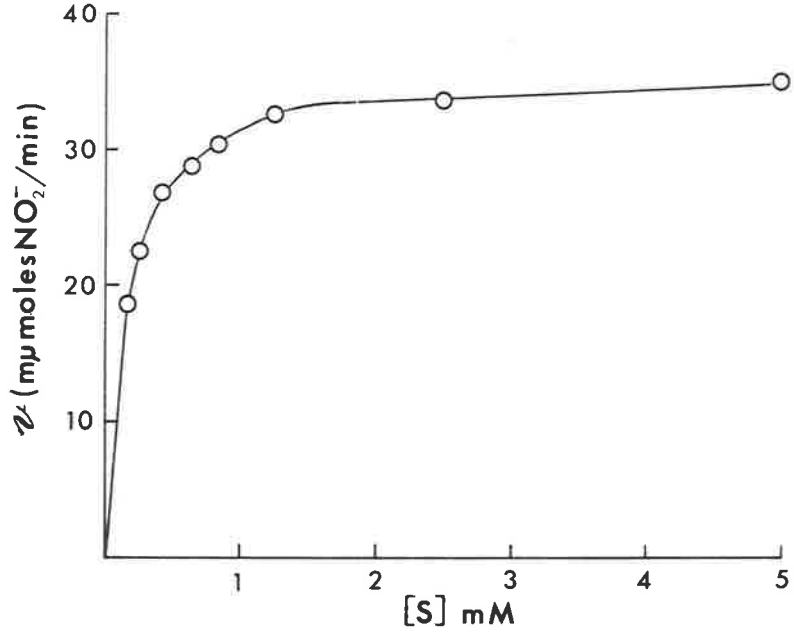


Fig. 15B

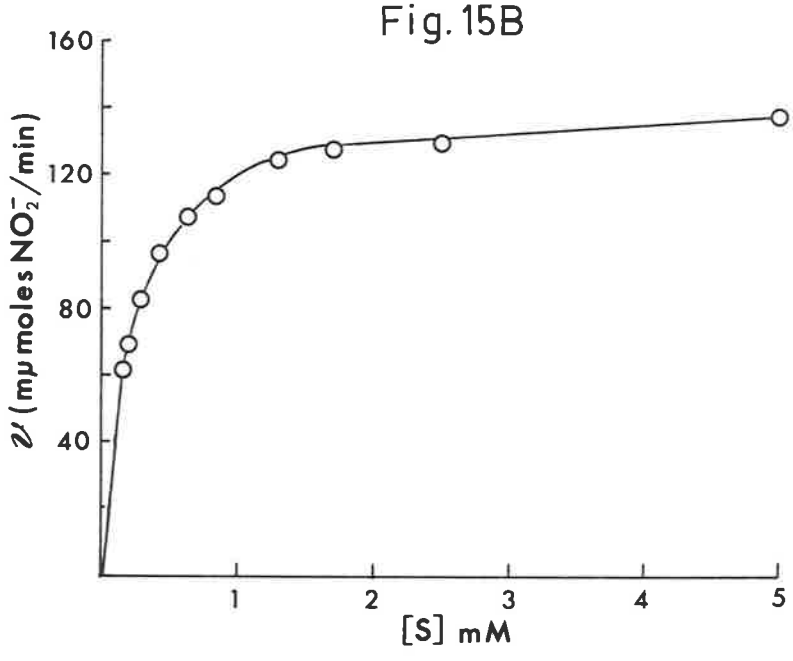


Fig. 16

The Effect of Potassium Nitrate Concentration on the Velocity of Nitrate Reduction by Benzyl Viologen-Nitrate Reductase at 40^{o}: Lineweaver-Burk plot.

The graph was obtained by plotting the reciprocal of the initial velocity against the reciprocal of the potassium nitrate concentration using the values presented in fig. 15A.

$K_m = 1.52 \times 10^{-4} M.$

$V_{max} = 36.2$ mμ moles/min.

Fig. 16

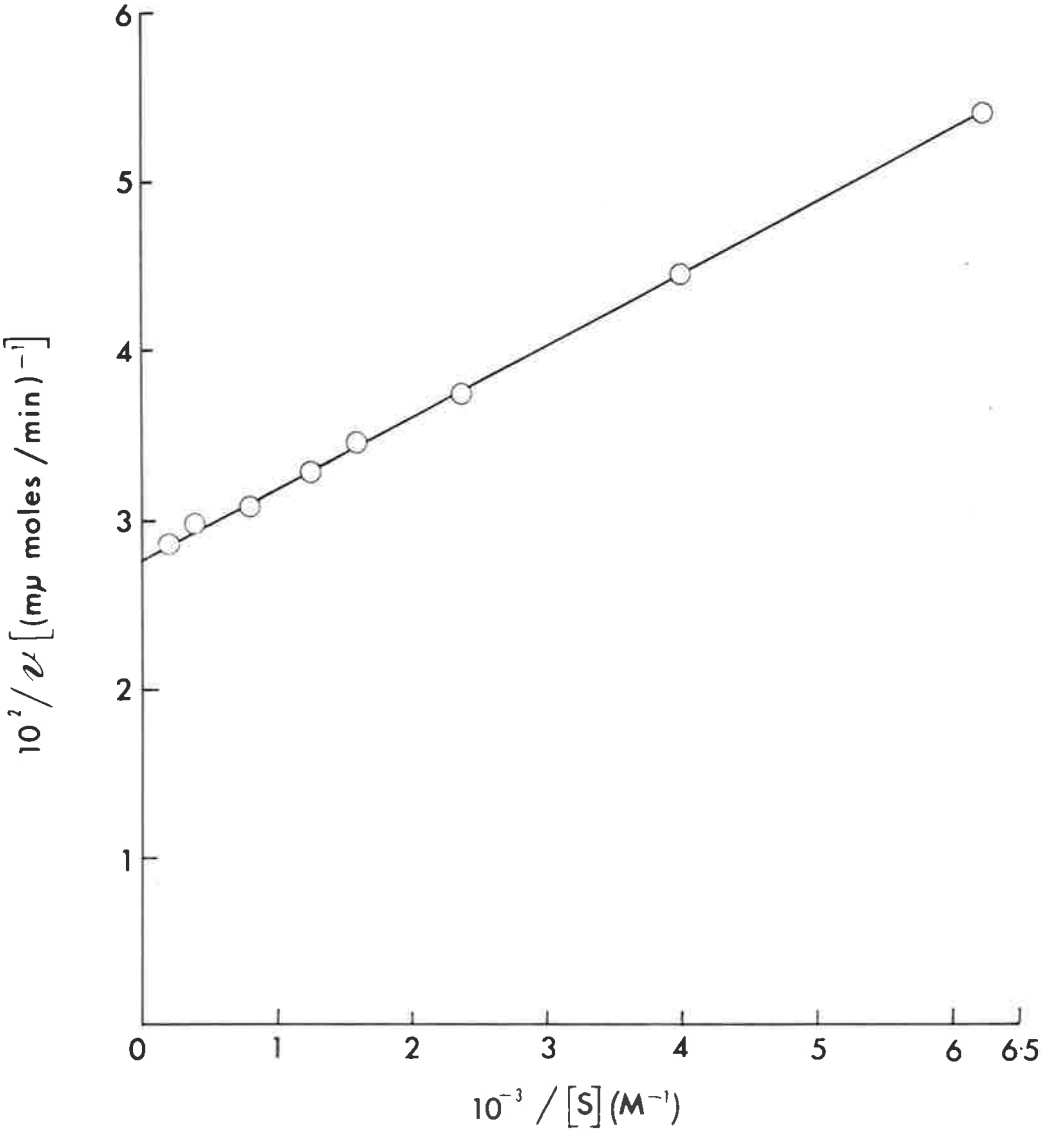


Fig. 17

The Effect of Potassium Nitrate Concentration on the Velocity of Nitrate Reduction by Benzyl Viologen-Nitrate Reductase at 60^{o}: Lineweaver-Burk plot.

The graph was obtained by plotting the reciprocal of the initial velocity against the reciprocal of the potassium nitrate concentration using the values presented in fig. 15B.

$$K_m = 2.03 \times 10^{-4} M.$$

$$V_{max} = 142.4 \text{ m}\mu \text{ moles } NO_2^-/\text{min}.$$

Fig.17

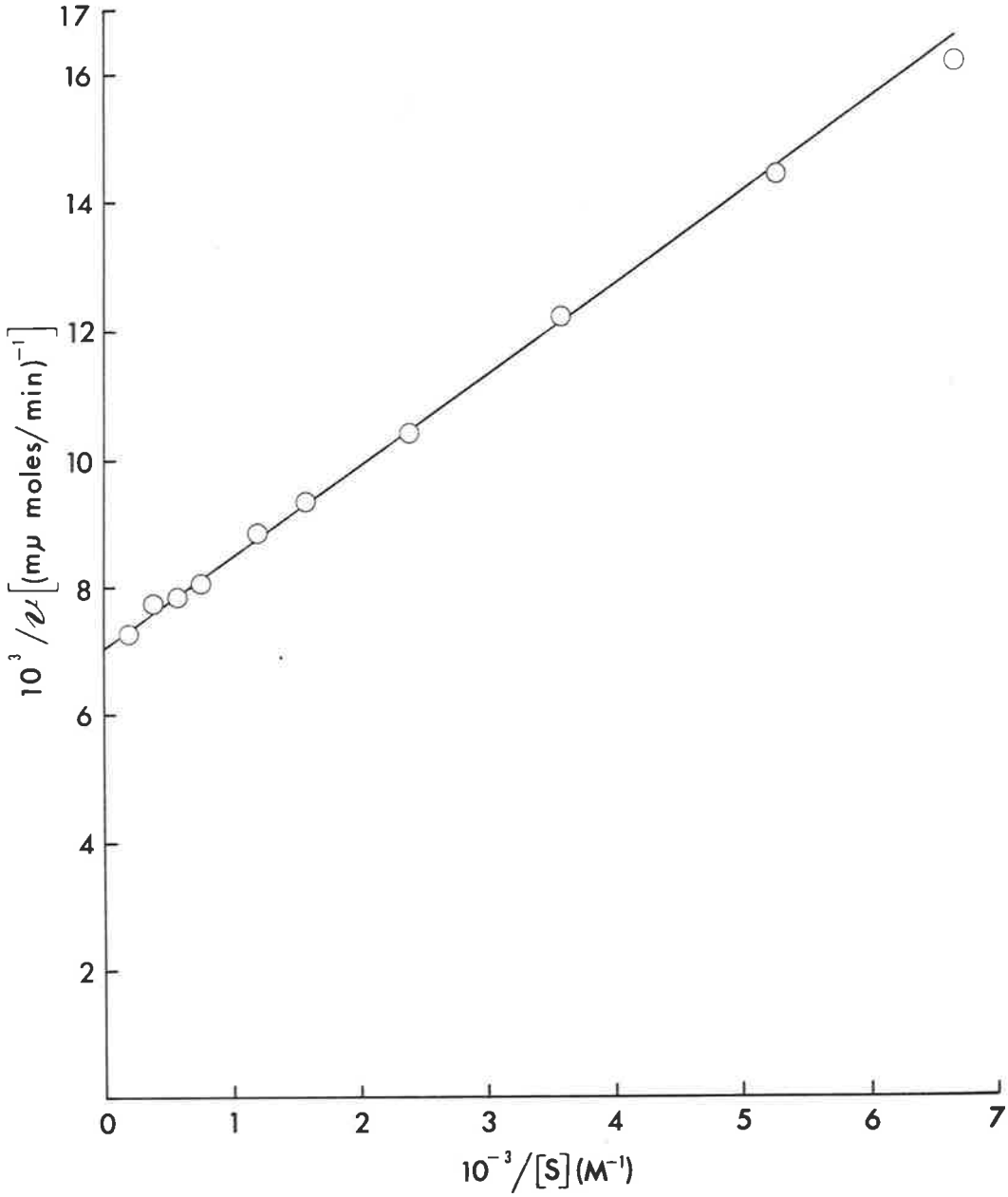


Fig. 18

The Effect of Temperature on the Initial Velocity of Nitrate Reduction by Benzyl Viologen-Nitrate Reductase

The reaction mixture contained in a final volume of 0.9 ml: potassium phosphate buffer, pH 6.0, 100 μ moles; benzyl viologen, 0.4 μ moles; potassium nitrate, 20 μ moles and 0.1 ml. of enzyme. The reaction was started by the addition of 0.1 ml. of 0.5% sodium dithionite in 0.25M potassium phosphate pH 7.4. Three tubes were incubated for 30 sec., 1 min. and 2 min. at each temperature and the initial velocity was calculated. The initial velocity was determined for the reaction between the temperature limits 30^{o} and 70^{o}.

Fig. 18

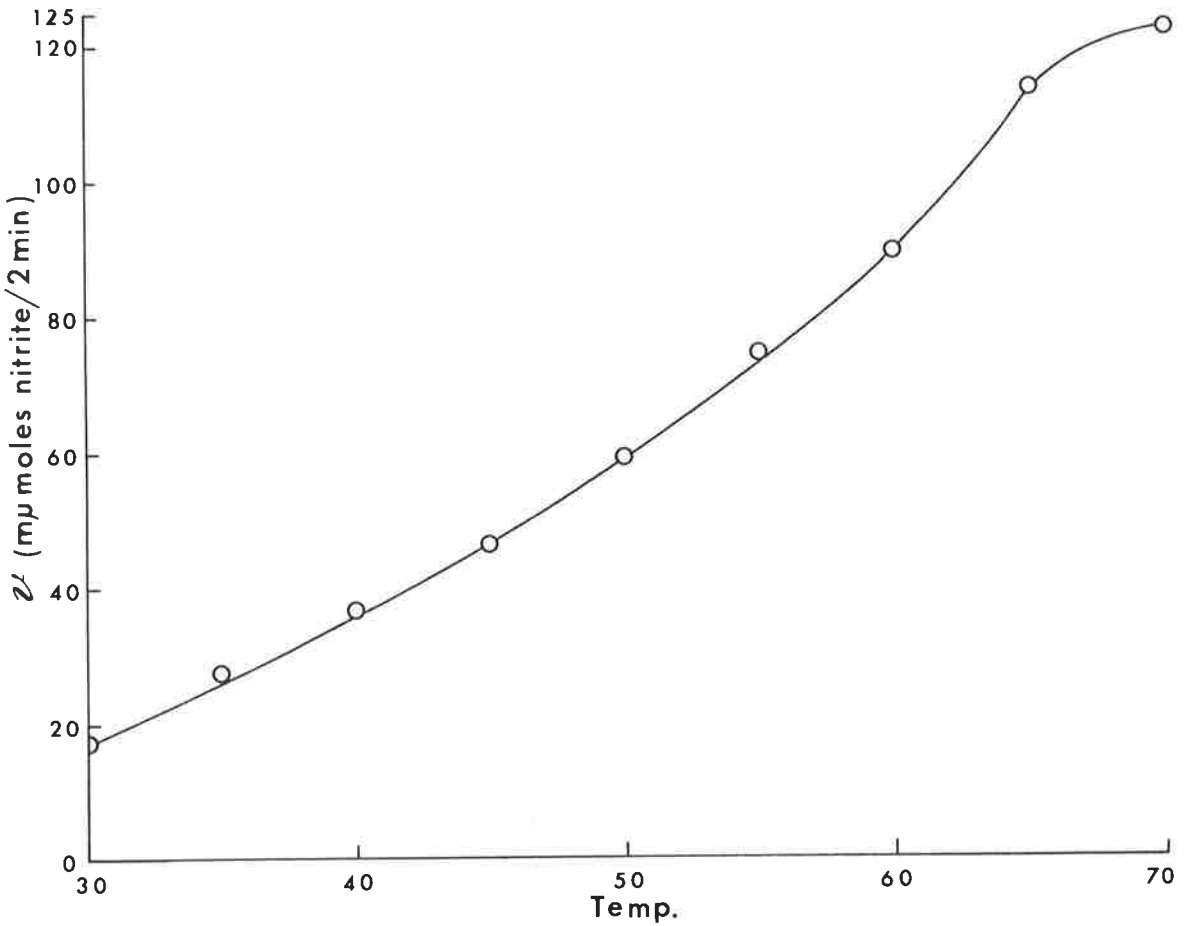
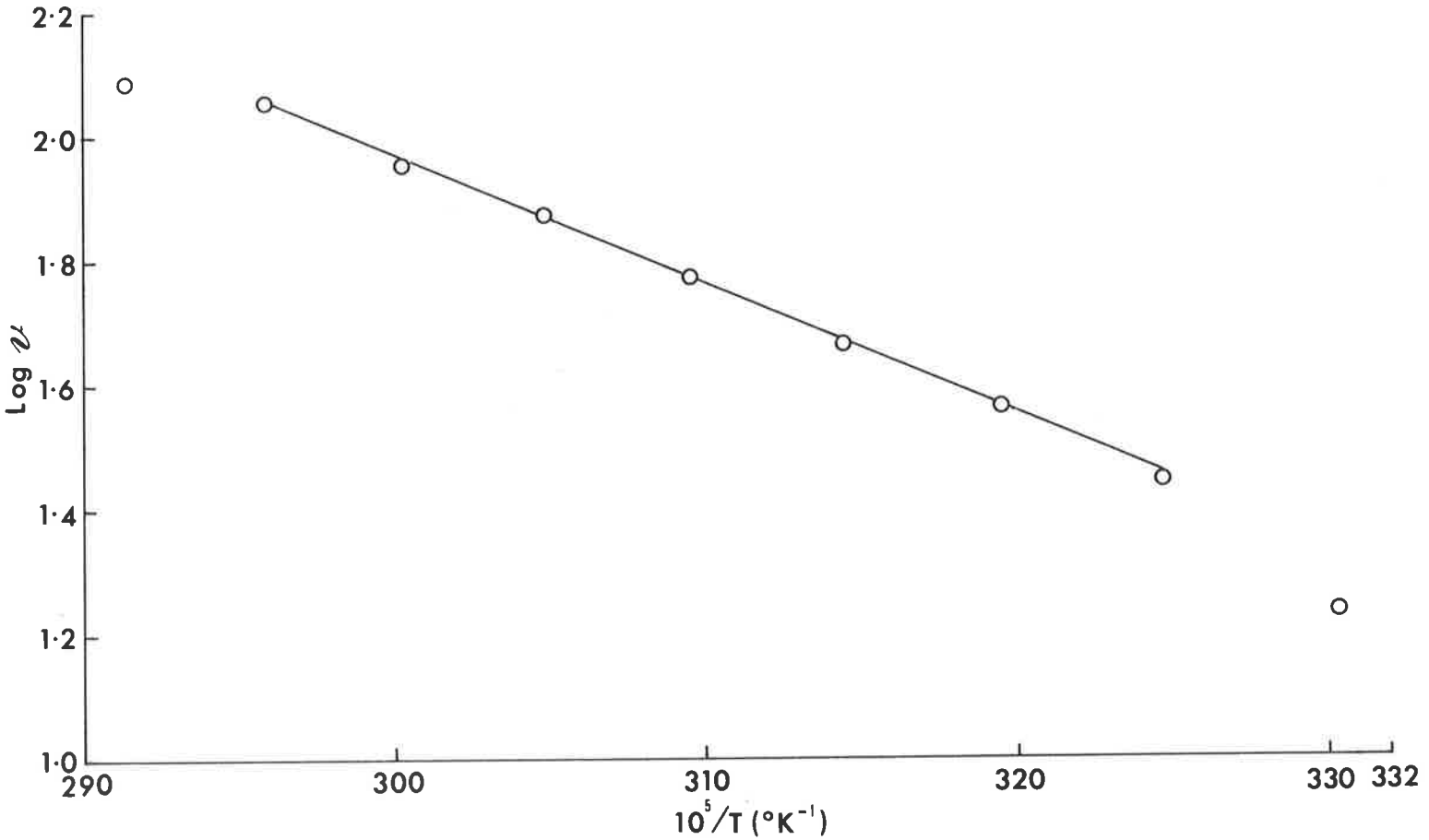

125
120
100
80
60
40
20
0
v (mμ moles nitrite/2 min)
30
40
50
60
70
Temp.

Fig. 19

The Effect of Temperature on the Initial Velocity of Nitrate Reduction by Benzyl Viologen-Nitrate Reductase: Arrhenius Plot

The values presented in fig. 18 were used to draw the graph of the log of the initial velocity against the reciprocal of the absolute temperature.

Fig.19



reaction velocity constant, k, and the absolute temperature, T, is described by the Arrhenius equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}$$

where R is the gas constant (1.987 calories per degree per mole).

This equation simplifies to:

$$d \log k = \frac{E}{2.303R} \cdot \frac{dT}{T^2}$$

$$d \log k = \frac{-E}{2.303R} \cdot d\left(\frac{1}{T}\right)$$

If the reaction velocity (v) is assumed to be directly proportional to k then E can be obtained by plotting log v against $\frac{1}{T}$. The slope of the line is $\frac{-E}{2.303R}$.

The values obtained for fig. 18 were used to draw the graph log v versus $\frac{1}{T}$ (fig. 19). The slope of the resulting straight line was measured and the value for the energy of activation, E, was calculated to be - 9,450 cal.

(v) The effects of inhibitors on nitrate reductase.

The effects of a number of inhibitors, co-factors and metal chelating agents on benzyl viologen-nitrate reductase activity are shown in table 9.

The activity was not affected by amytal, atebrine, 2-n-heptyl-4-hydroxyquinoline-N-oxide, FMN, FAD or menadione.

Table 9

The Effects of Inhibitors, Co-factors and Metal Chelating Agents on Benzyl Viologen-Nitrate Reductase Activity

The benzyl viologen-nitrate reductase assay method described in General Methods was used to assay nitrate reductase activity.

The activities, in mμ moles of NO_2^-/min/mg protein at 60^o, were expressed as percentages of the activity of the assay with no added inhibitors, additional co-factors or metal chelators.

Nitrite standards were made up in separate reaction mixtures containing each of the additions listed.

Hydroxyquinoline-N-oxide was added in 0.02 ml. of 0.01N NaOH. 8-Hydroxyquinoline and thenoyltrifluoroacetone were each added in 0.02 ml. of 95% ethanol.

Enzyme controls were included containing 0.02 ml. of each of these solvents.

Table 9

Addition	Concentration	Relative Activity
Sodium amytal	5×10^{-3}M	100
Sodium amytal	10^{-2}M	100
Atebrine	2.5×10^{-4}M	100
FMN	2×10^{-4}M	100
FAD	2×10^{-4}M	100
Menadione	10^{-3}M	100
2-n-heptyl-4-hydroxy quinoline-N-oxide	10^{-4}M	100
Thenoyltrifluoroacetone	10^{-4}M	55
8-hydroxyquinoline	10^{-3}M	85
Benzoin α-oxime	10^{-3}M	100
1,10-phenanthroline	2×10^{-3}M	40

The metal chelating agents: thenoyltrifluoroacetone, 8-hydroxyquinoline and 1,10-phenanthroline inhibited the activity by 45%, 15% and 60% respectively. Benzoin-α-oxime (cupron) did not cause any inhibition.

$NADH_2$ was tested as a hydrogen donor for nitrate reductase using the $NADH_2$-nitrate reductase reaction mixture described in general methods (p. 64). No $NADH_2$-nitrate reductase activity was found with assay times of up to 5 min. at 60^{o}.

(vi) The flavin content of benzyl viologen-nitrate reductase.

A portion of the soluble nitrate reductase was made 0.1N with respect to HCl to give 3 ml. of solution containing 0.12mg. of protein per ml. The fluorescence of the solution was measured in a 'Beckman Ratio Fluorometer' that had been previously calibrated against FMN standards. The solution was then boiled for 15 min., cooled and the fluorescence was re-measured. The pH was adjusted to 7 with 2N-KOH and 3 μg. of Russel viper venom in 50 μl. of water was added to the neutral solution. The fluorescence was measured after 5 min. incubation at room temperature.

The amount of FMN detected in the soluble preparation, using the above method, was equivalent to 0.025 mole per 100,000 g. of protein. On this basis the molecular weight of the enzyme if it contained 1 mole of either FAD or FMN per mole of enzyme, would be 4×10^{6}. Since the ultracentrifuge data suggest that the enzyme had a much

smaller molecular weight than this it is assumed that the enzyme was not a flavoprotein.

C. DISCUSSION

It is obvious that cholate-ammonium sulphate solubilization of benzyl viologen-nitrate reductase was not an efficient method of obtaining high yields of the soluble enzyme. Purification was probably hampered by the presence of traces of cholate, that were not removed by dialysis, and allowed small particles to remain in 'solution'. Nevertheless the presence of benzyl viologen-nitrate reductase activity in the supernatant of cholate-ammonium sulphate solubilized membranes after centrifugation at 220,000 x g. for 3 hr. indicates that some of the enzyme was solubilized.

The schlieren patterns, for the supernatant and infranatant, observed in the analytical ultracentrifuge suggest that the supernatant was composed largely of soluble protein of one species with a very small amount of quickly sedimenting protein, whereas the infranatant contained a number of proteins with relatively high sedimentation velocities. It is probable that the increasing amounts of cytochrome c_1 found in the infranatant and the precipitate were present in small particles, since it is unlikely that the molecular weight of cytochrome c_1, from B. stearothermophilus is greater than 20,000. The nitrate reductase activity in these fractions may have been due to either its association with particles

or its presence as a contaminant that was carried down with the infranatant and precipitate during centrifugation.

It would be necessary to obtain a much purer preparation of soluble nitrate reductase, free of small particles, before the sedimentation constant and molecular weight could be accurately determined.

The heat stability of the enzyme in the absence of membranous material indicates that this enzyme is intrinsically heat stable. However a definite conclusion on the cause of heat stability of the nitrate reductase cannot be reached until the enzyme is purified further.

The 1.3-fold increase in K_m when the temperature was increased by 20^o could have been due to a conformational change in the protein molecule of the nitrate reductase. However the K_m of the heat-stable enzyme might have been affected in several ways by the increase in temperature.

The increased energy of rotation and vibration of the amino acid side chains, on the protein molecule, might have caused local changes in the spatial relationship of these groups to one another, without changing the overall configuration of the protein molecule. Continual transitory changes in the positions of these groups could be expected to cause an increase in K_m if two or more groups were responsible for binding the substrates to the enzyme in the correct position for the subsequent enzymic catalysis.

K_m is a graphically determined quantity and is dependent upon at least three velocity constants as shown by the equation:

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

where k_1, k_{-1} and k_2 are the respective velocity constants defined by the equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products}$$

where E, S and ES are enzyme, substrate and enzyme-substrate complex respectively. K_m would increase if the rise in temperature caused an increase in either k_{-1} or k_2, or a decrease in k_1. The situation becomes involved when terms for the second substrate, reduced benzyl viologen, are introduced into the above equations.

Other effects which might alter the K_m of the enzyme upon increasing the temperature include: alteration of the ratio of ionized to unionized groups in the active centre of the enzyme due to the effect of temperature on the pK values of the respective groups, and changes in the pH of the buffer solution in which the enzyme is dissolved (Dixon & Webb, 1958).

Thus a definite conclusion cannot be reached about the cause of the increase in K_m, between 40° and 60°, without a careful investigation of the variable factors that might contribute to the final value obtained for K_m.

The values obtained for the energies of activation of a number of enzymes from thermophilic bacteria have varied from -3,700 cal. for malic dehydrogenase (Marsh & Militzer, 1952), to -21,000 cal. for pyrophosphatase (Marsh & Militzer, 1956). The value obtained here for nitrate reductase, namely -9,450 cal., falls within this range.

No definite conclusions can be drawn about the relationship between the activation energy and the temperature for growth since enzymes from mesophilic organisms have been found to have energies of activation ranging from -8,700 cal. to -25,000 cal. (Sizer, 1943). Benzyl viologen-nitrate reductase activity was not inhibited by amytal or atebrine, nor was it stimulated by either FAD or FMN. These findings, together with spectral studies and flavin estimations on the soluble enzyme, suggest that the enzyme was not a flavoprotein.

The inhibition of the enzyme activity by metal chelating agents is strong evidence for the presence of metal in the electron transfer sequence between reduced benzyl viologen and nitrate.

CHAPTER VII

DISCUSSION

The work described in this thesis was undertaken with two main aims: firstly to characterize the nitrate reducing system and determine its role in the anaerobic metabolism of the organism; secondly to isolate the nitrate reductase and to investigate its stability to heat and its other enzymic and kinetic properties in order to gain some information about the mechanisms of thermobiosis.

The good growth obtained under anaerobic conditions on glucose and glucose-containing carbohydrates, as well as the production of acid, indicates that the organism was able to grow anaerobically, on these carbon sources, by using the glycolytic pathway. Wilson (1965) has shown that B. stearothermophilus converts glucose almost quantitatively to lactate except under highly aerobic conditions.

The slight stimulation of anaerobic growth on glucose, starch and sucrose, by nitrate, together with the production of nitrite, suggests that nitrate was being used as an electron acceptor for some energy-producing pathway. However glycolysis was probably the main route for glucose catabolism since the amount of acid produced in the presence of nitrate was only slightly less than that found in its absence.

The anaerobic growth on fructose was interesting in that the growth rate and cell density was only half that found for glucose. Nitrate stimulated the growth rate on fructose to a much greater extent than the growth rate on glucose-containing carbohydrates.

However the similarity between the final cell densities and pH values of the fructose and glucose cultures, in the presence of nitrate, suggests that the cells grown on fructose plus nitrate utilized the glycolytic pathway rather than an oxidative pathway for fructose catabolism . The fact that the organism was unable to grow anaerobically on glycerol medium alone indicated that glycerol was not metabolized via the glycolytic pathway. Nitrate was demonstrated to be an essential requirement for anaerobic growth on this carbon source and since the pH of the glycerol plus nitrate culture, after the cessation of growth, was only slightly lower than the initial pH of the medium it is likely that nitrate was acting as an electron acceptor for oxidative metabolism.

Similar observations were made by Quastel, Stephenson & Whetham (1925) who reported that E. coli would not grow anaerobically, with lactate as a carbon source, unless nitrate was present.

Constant initial growth rates were obtained when B. stearothermophilus was grown on a series of media containing glycerol and concentrations of potassium nitrate ranging from 0.1% to 0.4%. The initial growth rate on 0.05% potassium nitrate was less than that found for 0.1% potassium nitrate which suggests that the nitrate-utilizing system was saturated with nitrate at the latter concentration.

Downey (1966) found that the maximum anaerobic growth rate for

B.stearothermophilus (strain 2184), growing on medium containing sucrose and nitrate, occurred at a concentration of approximately 0.2% potassium nitrate.

The lack of stoichiometry between the initial nitrate concentration and the length of the exponential phase of growth, in glycerol medium, was possibly due to the inhibitory effect of nitrite on growth. Nitrite was detected in all cultures grown on glycerol plus nitrate except that containing an initial concentration of 0.05% nitrate. The growth at this concentration of nitrate was diphasic. No explanation can be given for this observation. Further experiments, considered to be outside the scope of this thesis, would need to be performed in order to explain the absence of nitrite in cultures grown on 0.05% nitrate, the apparent toxic effect of nitrite on growth and the lack of nitrite reductase observed in extracts from cells grown on high concentrations of nitrate.

The results discussed so far provide evidence for the existence of a respiratory nitrate-reducing system in B. stearothermophilus. One of the criteria used to characterize respiratory nitrate reductase is the utilization of nitrate to support respiration, in place of oxygen, under anaerobic conditions. This implies that oxidative catabolism of carbon sources, as distinct from glycolysis, occurs in the cell under conditions of nitrate respiration.

Verhoeven (1956) has proposed a further classification of dissimilatory (i.e. respiratory) nitrate reduction based on the nature of the end-products of nitrate reduction. According to Verhoeven's sub-classification, dissimilatory nitrate reduction that results in an accumulation of nitrite should be classed as incidental dissimilatory nitrate reduction, and nitrate reduction, in which the products are gaseous (denitrification), where nitrate is 'essential for the well-being of the cell', should be classed as true dissimilatory nitrate reduction.

The classification of nitrate reduction by B. stearothermophilus as either incidental, or true, dissimilatory nitrate reduction is difficult. The organism would not grow anaerobically in a complex medium with glycerol as a carbon source unless nitrate was present - thus nitrate reduction under these conditions was essential for growth, but nitrite was excreted into the medium. On the other hand, although nitrate stimulated the anaerobic growth of the organism on glucose, fructose, starch or sucrose, good growth occurred on these carbon sources in the absence of nitrate.

A comparison of the levels of a number of tricarboxylic acid cycle enzymes in extracts from cells of B. stearothermophilus, grown under both anaerobic and aerobic conditions, provided some information about the role of the tricarboxylic acid cycle in oxidative metabolism under conditions of nitrate respiration.

When the levels of the respective enzymes in extracts from cells grown aerobically on glucose and on glycerol were compared it was obvious that although, in both cases, oxygen increased the levels of the enzymes over those found in anaerobically grown cells; the levels of tricarboxylic acid cycle enzymes found in glucose-grown cells were lower than those found in glycerol-grown cells.

A similar comparison of the enzymes in extracts from aerobically-grown cells with those grown anaerobically on glucose plus nitrate and also those grown on glycerol plus nitrate, revealed that nitrate was not as effective as oxygen in increasing the synthesis of these enzymes.

Low levels of the tricarboxylic acid cycle enzymes, were found in cells grown on glucose plus nitrate whereas the levels of all the enzymes, except aconitase, in cells grown anaerobically on glycerol plus nitrate were higher than those found in extracts from cells grown aerobically on glucose.

The levels of aconitase and fumarase in cells grown anaerobically on glucose were lower in the presence of nitrate than in its absence. There was a 2.5-fold increase in aconitase and a 5-fold increase in fumarase when glucose was replaced by glycerol but the levels of these enzymes were much lower than those found in cells grown aerobically on glycerol.

Similar observations on the effect of nitrate upon these two

enzymes have been made by Forget & Pichinoty (1967) using Aerobacter aerogenes and by Wimpenny & Cole (1967) using E. coli. Like B. stearothermophilus both these organisms are facultative anaerobes.

Forget & Pichinoty (1967) demonstrated the existence of a tricarboxylic acid cycle in extracts of Aerobacter aerogenes grown under aerobic conditions. However, when the organism was grown anaerobically decreased levels of condensing enzyme, aconitase, isocitric dehydrogenase, α-ketoglutarate dehydrogenase, succinic dehydrogenase and fumarase were found. The levels of both aconitase and fumarase in cells grown anaerobically on glucose, pyruvate and citrate were lower in the presence of nitrate than in its absence.

Wimpenny & Cole (1967) showed that although nitrate stimulated the anaerobic growth of E. coli both on complex and defined medium with glucose as a carbon source, the fumarase and aconitase levels in E. coli grown anaerobically on glucose and nitrate were lower than the levels found in cells grown anaerobically in glucose without nitrate. These authors demonstrated that the addition of nitrate or nitrite to cells growing aerobically, in the absence of glucose, decreased the initially high levels of fumarase and aconitase. They further demonstrated that nitrate and nitrite inhibited the activity of these enzymes in vitro, but there was a lack of correlation between the amount of nitrate required to cause inhibition in vivo and that necessary for inhibition in vitro.

The results reported in this thesis indicate that nitrate was not solely responsible for the low activity of fumarase and aconitase but that the carbon source present during growth modified the effect of nitrate on the levels of these enzymes. Wimpenny (1966) reported that aconitase and fumarase rapidly disappeared in resting cell suspensions of *E. coli* and that glycerol appeared to maintain the original levels of these enzymes better than glucose.

It is suggested that *B. stearothermophilus* was not utilizing the tricarboxylic acid cycle for oxidative metabolism during anaerobic growth on medium containing glucose and nitrate. The stimulation of anaerobic growth, on glucose-containing carbohydrates, by nitrate might be explained if the nitrate reductase system was acting as a hydrogen acceptor for pathways other than the tricarboxylic acid cycle.

The increased levels of most enzymes in the cycle when the organism was grown anaerobically on medium containing glycerol and nitrate, suggests that some oxidative metabolism via the tricarboxylic acid cycle might have occurred in these cells.

The increased production of cytochromes in nitrate-reducing bacteria grown under anaerobic conditions on nitrate has been observed in several species of bacteria. Verhoeven & Takeda (1956) showed that when the growth conditions for *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Micrococcus denitrificans* were changed from

aerobic to anaerobic, increased amounts of cytochrome c were synthesized. Unfortunately it is not clear if nitrate was present in the respective media.

Vernon (1956b) studying the cytochromes of an unidentified Pseudomonad found that the concentration of cytochrome c_1 was twice that of cytochrome c in cells grown anaerobically on nitrate-containing medium. Under aerobic conditions cytochrome c was the major cytochrome present.

Cole & Wimpenny (1967) showed that the maximum concentration of soluble cytochrome c_{552} in E. coli grown in continuous culture under anaerobic conditions occurred at a nitrate concentration of 10 mM. As the concentration of nitrate was increased the cytochrome c_{552} concentration decreased. The maximum concentration of cytochrome b_1 occurred at a nitrate concentration of approximately 50 mM.

These authors suggested that the response of cytochrome b_1 and cytochrome c_{552} to increasing nitrate concentrations was due to the oxidation-reduction potential of the medium apparently governed by the nitrate-nitrite redox couple. However the discovery by Fujita & Sato (1966) that soluble cytochrome c_{552}, in E. coli was involved in nitrite reduction, suggests that this cytochrome was induced by nitrite that had been produced during growth by the nitrate reducing system of the organism. Increased concentrations of nitrate probably inhibited the synthesis of the nitrite reductase and stimulated the

synthesis of cytochrome b_1 as well as nitrate reductase.

It is possible that the level of cytochrome c_1 in this organism might be controlled by the redox potential of the complete system since the levels of cytochrome c_1, under conditions of nitrate respiration and oxygen respiration were observed to increase by similar amounts when compared with the level found in cells grown in the absence of these electron acceptors. The fact that cytochrome c_1 was not involved in electron transport to nitrate could lend further support to the redox control theory. However the differences in the concentrations of cytochrome c_1 in membranes obtained from cells grown on glucose or glycerol in the presence of both nitrate and oxygen indicate that redox potential alone does not control cytochrome c_1 synthesis in this organism and that the control system is more complex than that proposed for cytochrome c_{552} synthesis in *E. coli* (Wimpenny & Cole, 1967).

The increase in cytochrome b_1 concentration that occurred in the presence of nitrate but not oxygen is indicative of the role played by this cytochrome in the transfer of electrons to nitrate.

When one considers the levels of both tricarboxylic acid cycle enzymes and cytochromes in cells grown anaerobically on glycerol or on glucose, in a number of separate experiments, it is obvious that the synthesis of these proteins in the presence of nitrate is greater in glycerol-grown cells than in glucose-grown cells.

Whether the cells, growing anaerobically in glycerol plus nitrate, were utilizing the tricarboxylic acid cycle for nitrate respiration or whether some other pathway was sharing the oxidative catabolism of glycerol cannot be decided with any certainty. Isotopic tracer studies with radioactively labelled glycerol would be required to solve this problem.

The low anaerobic growth rate obtained on glycerol plus nitrate indicates that whatever pathway was being used the overall metabolic rate must have been slow compared with the high metabolic activity of cells utilizing glycolysis during anaerobic growth on glucose.

This low growth rate is what might be expected if the tricarboxylic acid acid cycle was being used since the activities of two of the key enzymes, aconitase and fumarase, were found to be present at levels that would have some slowing-down effect upon the overall rate of the cycle.

The levels of cytochromes did not mirror the levels of tricarboxylic acid cycle enzymes in cells grown anaerobically on either glucose or glycerol in the presence of nitrate. The mechanism controlling the synthesis of the cytochromes was obviously different from that controlling the synthesis of tricarboxylic acid cycle enzymes. Thus the cytochrome levels do not provide any evidence for nitrate respiration, where this term signifies the coupling of oxidative metabolism to nitrate reduction.

The results discussed so far suggested that the nitrate-reducing

system was of the respiratory type. The distribution and character of the nitrate reductase was investigated in extracts of protoplasts formed from cells grown anaerobically on glycerol plus nitrate.

An $NADH_2$-dependent nitrate reductase system was found to reside wholly in protoplast membranes obtained from osmotically lysed protoplasts. Observations made by Downey (1966) have confirmed the particulate nature of this enzyme system. However the particulate material prepared by Downey (1966) from sonically irradiated protoplast required a centrifugal force of 144,000 x g for its sedimentation - probably because the membranes were fragmented during their preparation.

Spectral studies made on membranes, containing the $NADH_2$-nitrate reductase system, showed that three cytochromes were present: cytochrome b_1, cytochrome c_1 and a b-type cytochrome with absorption maxima at 558 mμ , 530 mμ and 430 mμ (cytochrome b_{558}).

The use of spectrophotometric techniques, involving the anaerobic addition of nitrate to membranes that had been reduced with $NADH_2$, revealed that reduced cytochrome b_1 was oxidized by nitrate whereas both cytochrome b_{558} and cytochrome c_1 remained in the reduced state. The oxidation of reduced cytochrome b_{558} and reduced cytochrome c_1 by oxygen suggests that these cytochromes were involved in an aerobic electron transport system. Downey (1960) reported that, in membranes from B. stearothermophilus, reduced cytochrome b_1 was oxidized by nitrate and that reduced cytochrome c_1 was oxidized only by oxygen.

Spectra produced by Downey did not have any peak or shoulder corresponding to cytochrome b_{558} - however a slight assymetry of the cytochrome c_1 α peak may have been due to the former cytochrome.

One of the criteria used to classify respiratory nitrate reductase is the direct participation of a cytochrome in the electron transfer pathway to nitrate. The evidence presented in this thesis indicates that the $NADH_2$-nitrate reductase of B. stearothermophilus is of the respiratory type with cytochrome b_1 participating in electron transport from $NADH_2$ to nitrate. Cytochromes of the b-type have been found as components of the respiratory nitrate reducing systems in a number of different bacteria (Taniguchi, Sato & Egami, 1956; Vernon, 1956a; Chang & Lascelles, 1963; Katoh, 1963).

Spectral and inhibitor studies made on $NADH_2$-reduced membranes also implicated a flavoprotein as a member of the electron transport chain from $NADH_2$ to nitrate. Downey (1966) reported that atebrine but not amytal inhibited $NADH_2$-nitrate reductase activity. In the present work amytal at a concentration of 5×10^{-3}M apparently stimulated the $NADH_2$-nitrate reductase activity whereas at a concentration of 10^{-2}M, inhibition occurred. It is suggested that the lower concentration of amytal inhibited an aerobic pathway that was competing with $NADH_2$-nitrate reductase for electrons and that the latter system was only inhibited when the concentration of amytal was increased. The apparent inhibition of $NADH_2$-nitrate reductase by FMN might be explained if FMN stimulated this aerobic pathway.

The inhibition of $NADH_2$-nitrate reductase activity by menadione was contrary to the observations made by Downey (1962) who stated that $NADH_2$-nitrate reductase activity was stimulated by a natural naphthoquinone, extracted from B. stearothermophilus, as well as by vitamin K_1 and menadione. Downey showed that the aerobic electron transport pathway required a naphthoquinone derivative for its maximum activity. In a later communication Downey & Sundstrom (1965) reported that the nitrate reductase system required vitamin K_2 for the oxidation of reduced FAD with nitrate as an electron acceptor.

The results reported in this thesis suggest that the stimulation, by menadione, of the aerobic pathway was greater than the stimulation of $NADH_2$-nitrate reductase and that electrons were channeled to oxygen, present in the incubation mixture, at the expense of the nitrate reducing system, thus resulting in the apparent inhibition of $NADH_2$-nitrate reductase activity.

The inhibition of the $NADH_2$-nitrate reductase activity by metal chelating agents and by KCN indicates that an unknown metal was a component of the electron transport chain. The location of this postulated metal component was not investigated. However studies carried out on the inhibition of the terminal nitrate reductase, which will be discussed later, indicate that a metal was involved in the reduction of nitrate by the terminal enzyme.

A tentative scheme for the $NADH_2$-nitrate reductase system and the

associated aerobic system, based both on evidence discussed here, for particulate $NADH_2$-nitrate reductase, and later in the discussion, for the soluble nitrate reductase, is presented in fig. 20. The inclusion of FAD and a naphthoquinone analogue is based upon the work of Downey (1962; 1966) and Downey & Sundstrom (1965).

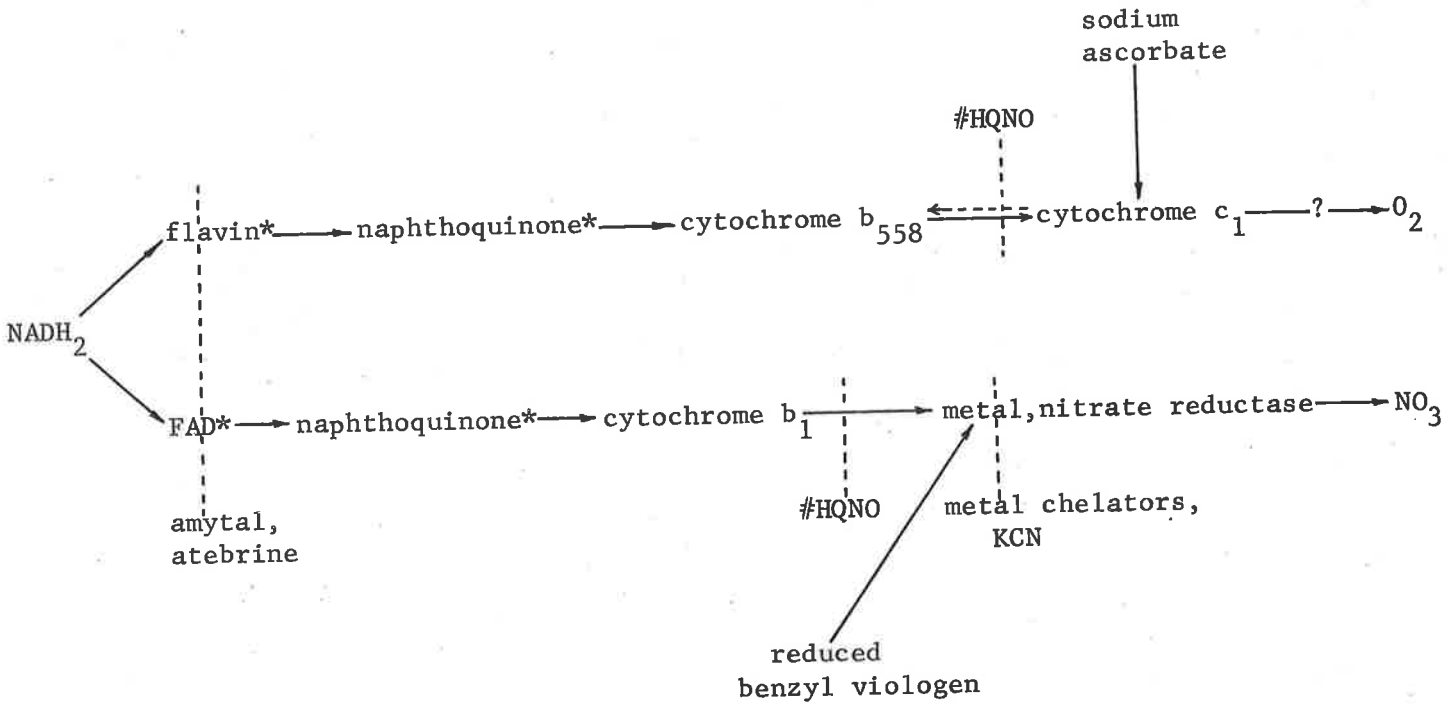
The method used to solubilize the terminal component of the nitrate reducing system, nitrate reductase, was extremely inefficient. The apparent high yield of soluble enzyme obtained after the initial extraction was in fact a mixture of soluble nitrate reductase and fragmented membranes held in 'solution' by cholate. This was shown by the precipitation of much of the nitrate reductase activity after dialysis to remove the cholate. Nevertheless enough soluble enzyme was obtained to enable various studies to be made on it.

The presence of a very small amount of cytochrome c_1 in the soluble enzyme preparation was probably due to the incomplete removal of small particles, containing this cytochrome, during preparation of the soluble nitrate reductase.

The absence of cytochrome b_1 from the soluble enzyme plus the fact that the enzyme was inactive with $NADH_2$ and required reduced benzyl viologen as a hydrogen donor shows that the nitrate reductase had been removed from the remainder of the $NADH_2$-nitrate reductase complex.

Fluorimetric studies excluded flavin as a component of the

Fig. 20



* The inclusion and sequence of these components is based mainly on the work of Downey (1962; 1966) and Downey & Sundstrom (1965).

HQNO = 2-n-heptyl-4-hydroxyquinoline-N-oxide.

soluble enzyme. Inhibition of reduced benzyl viologen-nitrate reductase activity by metal-chelating agents indicates that a metal was involved in the reduction of nitrate by the soluble enzyme (fig. 20). Molybdenum has been implicated as an essential part of nitrate reductase enzymes, both of the assimilatory type (Nicholas & Nason, 1954a; 1954b, Nicholas & Nason, 1955) and of the respiratory type (Taniguchi & Itagaki, 1960). Because of the small amount of soluble enzyme from B. stearothermophilus that was available no attempt was made to determine whether or not the metal component was molybdenum.

Soluble nitrate reductase enzymes have been isolated from E. coli (Taniguchi & Itagaki, 1960), Achromobacter fischeri (Sadana, Roa & Joshi, 1963) and Rhizobium japonicum (Lowe & Evans, 1964).

All of these enzymes were characterized by their lack of activity with reduced pyridine nucleotides and their requirement for reduced redox dyes as electron donors. Spectra of the purified E. coli and Achromobacter fischeri enzymes showed that they were free of cytochromes. Only the E. coli enzyme has been assayed for flavin content and the amount detected was too small to be considered significant.

The major difference between the Achromobacter fischeri enzyme and the E. coli enzyme was in their respective molecular weights. Taniguchi & Itagaki (1960) reported that the molecular weight of the E. coli nitrate reductase was one million, whereas the sedimentation coefficient obtained for the Achromobacter enzyme indicated that it was a much smaller molecule. The molecular weight of the enzyme

from B. stearothermophilus was not determined although an uncorrected S value of 2.5 was calculated from analytical ultracentrifuge patterns. This value suggests that, like the Achromobacter fischeri enzyme ($S_{20,w}$ not greater than 4.5), the enzyme studied here was a relatively small molecule. It is interesting to note that Taniguchi and Itagaki (1960) reported in a footnote to their paper that digestion of their enzyme with chymotrypsin produced an enzyme with approximately the same activity but with a smaller sedimentation coefficient than the untreated enzyme. The presence of 40 atoms of non-haem iron per molecule of E. coli nitrate reductase suggests that the enzyme was not completely free from respiratory chain components. Possibly the nitrate reductase moiety in E. coli has a molecular weight lower than one million.

The K_m values obtained for nitrate reductase at both 40^o and 60^o, with nitrate as the variable substrate, were higher than the values found for the nitrate reductase enzymes from E. coli, Rhizobium japonicum and Achromobacter fischeri. Because of the many variables that contribute to changes in K_m with increase in temperature no definite conclusion can be reached about the increase in K_m that occurred when the temperature was raised from 40^o to 60^o. However the fact that the increase was only 1.3-fold for a 20^o rise in temperature indicates that the structural integrity of the enzyme was not affected.

The energy of activation obtained for the soluble nitrate reductas

from B. stearothermophilus (-9,450 cal.) was similar to that found for nitrate reductase from a mesophile, Aerobacter aerogenes (-9,250 cal.), by Pichinoty & Senez (1959). The activation energies of a number of enzymes from B. stearothermophilus have been determined. They ranged in value from -3,700 cal. for malic dehydrogenase (Marsh & Militzer, 1952) to -21,000 cal. for pyrophosphatase (Marsh & Militzer, 1956). Thus there does not appear to be any correlation between the energies of activation for individual enzymes, from this thermophile, and its high temperature optimum for growth.

The soluble nitrate reductase enzymes discussed earlier varied in their stability to heat. The E. coli enzyme was stable to heat at 60° for 5 min. (Taniguchi & Itagaki, 1960) whereas the enzyme from Rhizobium japonicum was completely inactivated after 20 min. at 38° (Lowe & Evans, 1964). The enzyme from Achromobacter fischeri lost 10 to 15% of its activity after 10 min. at 60° (Sadana & McElroy, 1957).

The enzyme from B. stearothermophilus lost only 13% of its initial activity after being kept at 60° for 2 hr. Thus nitrate reductase is another example of an enzyme that is heat-stable in the absence of any apparent stabilizing factors, that is, it is intrinsically heat stable. The number of such enzymes that have been investigated in this organism are too few to enable any definite conclusions to be reached about the role of intrinsically heat-stable enzymes in maintaining metabolism and growth in thermophilic organisms. Many more enzymes and enzyme

systems both soluble and membrane-bound will have to be investigated in order to solve the problem of thermobiosis.

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