THE BIOLOGY AND RESPIRATORY PHYSIOLOGY OF *APHELENCHUS AVENAE* BASTIAN 1865

by

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I dedicate this thesis to

my wife and son

Abstract

Three isolates of Aphelenchus avenae were mass cultured monoxenically and assessed for bio-physiological variation. Isolates E and F [from U.K.] reproduced parthenogenetically; Isolate A [from Malawi] reproduced amphimicticly. The oxygen consumption rates (QO_2) of all three isolates were similar but declined with time of harvest. Arrhenius plots of the QO_2 data [Log QO_2 vs. $1/^oK$] showed "intersecting" type discontinuities at different temperatures depending on isolate.

Isolates differed also in population development rates at 10^oC to 30^oC. Arrhenius plots i.e. Log population development rate vs. $1/^oK$, corresponded very closely with those obtained with the QO_2 data and probably indicates the occurrence of "thermotypes" within the species.

Each isolate differed in response of the QO_2 of the whole worms [cultured at 25^oC] to 3 mM NaCN. The QO_2 of isolate-F was 75-80% inhibited, QO_2 of isolate-E was 0-12% inhibited and that of isolate-A was 17-20% inhibited.

The QO_2 of isolate-A cultured at 30^oC was stimulated by 3 mM NaCN. When cultured at 25^oC, stimulation of the QO_2 was observed only following prolonged incubation at 30^oC.

Mitochondrial fractions of isolates A and F utilised only succinate and α-glycerophosphate to any significant extent. The respiratory control ratios (RCRs) were higher for isolate-F as were the P:O ratios. Antimycin-A had little or no effect on the state-3 QO_2 of isolate-A but caused near complete inhibition of the state-3 QO_2 of isolate-F. The ascorbate-TMPD-oxidase activity of isolate-A was only partially (55-65%) inhibited by 3 mM NaCN while that of isolate-F was completely inhibited. Salicylhydroxamic acid (SHAM) produced 50-60% inhibition of the residual (=cyanide insensitive) QO_2 of isolate-A (i.e. 20-30% of total QO_2) but carbon monoxide (CO) was required for complete inhibition.

Spectrophotometric observations showed the presence of cytochromes b, c and a in mitochondrial fractions of all isolates. Treatment with CO altered the spectra considerably giving almost identical spectra to those described for *Moniezia expansa* and *Halobacterium* sps. as characteristic of cyt.o.

A. avenae probably has three potentially functional terminal oxidases, cyt. aa_3; cyt. o and possibly an α-glycerol-phosphate-oxidase (GPO). The latter complex linked to glycolysis, may be responsible for the production of glycerol detected in isolates A and F by GLC techniques.

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CHAPTER 1

General Introduction

The electron transfer sequences in parasites has been suggested to depart from the classical-mammalian pattern. (Bryant, 1970; Barrett, 1976) The main source of difference, according to Bryant (1970), involves the suppression of an oxygen-dependent terminal oxidase in favour of one or more alternative pathways capable of utilizing different electron acceptors resulting in the development of branched electron transport sequences.

Whether this difference is directly related to the parasitic habit is one of the more interesting questions helminthologists and protozoologists are attempting to clarify, although Barrett (1976) has suggested that the importance of oxidative processes in the overall energy balance of helminths differ in different parasites and may even vary between tissues of the same parasite. Therefore in order to provide an adequate answer to this,a more complete understanding of parasite metabolism might be obtained by reference to the most closely related free-living forms with which they are presumed to have shared a common ancestor. Although a comprehensive literature has accumulated on the respiratory-electron transport systems as obtains in parasitic helminths such as Moniezia expansa, Ascaris and Fasciola, there is relatively little literature on the respiratory-characteristics of free living nematodes.

However spectra obtained from homogenates of a fresh-water turbellarian Cura pinguis (Bryant & Fletcher as quoted by Bryant 1970), and the free-living nematodes Caenorhabditis briggsae (Bryant, et al., 1967) and Turbatrix aceti (Rothstein et al., 1970) were reported to be similar to spectra obtained with M. expansa preparations (Bryant, 1970) suggesting that the branched system may be more widely distributed in nature. On this basis Bryant (1970) suggested that the relationship between the branched respiratory chain and parasitism is not direct, and that groups of organisms which became successful parasites possessed these modifications because they were already adapted to conditions which bore certain resemblances to the parasitic environments. Fundamentally the intestines are special cases of a whole class of environments in which oxygen tension or the amount of available oxygen is low. (Bryant, 1970)

The soil environment with its variety of texture, fluctuating oxygen tension, pH and humidity may well have been the habitat that offered the greatest challenge to the archetypal land-based freeliving nematode, acting as an important evolutionary "spring-board" for the parasitic habit. For this reason it is logically the best habitat in which to seek the predecessors of the parasitic mode.

If the extent of geographical distribution is any measure of evolutionary success, Aphelenchus avenae with its cosmopolitan distribution, its wide host range and

short generation time, would be an ideal model to employ in order to study and gain an insight into the metabolic processes (respiratory or otherwise) associated with parasitic nematode species.

However the same attributes that make A. avenae a model organism for such studies may present some difficulties. For instance, geographically separated populations (or isolates) may have adapted to specific local conditions and therefore different isolates may not be homogeneous in their physio-biochemical response to an externally applied physical or chemical constraint(s). Indeed since the monoxenic cultures of A. avenae may be harvested at any time between 30 and 140 days, a considerable variation in response may be expected even within a single isolate.

In this respect the data on the oxygen consumption rates (QO_2) of whole worms of A. avenae differ considerably according to figures cited by various authors. Cooper (1969) and Cooper and Van Gundy (1970a) quoted a figure of 5.6-5.8 μl O_2/mg dry wt./hour at 28°C; Marks (1971) quoted a rate of 10-12 μl O_2/mg dry wt./hour at 30°C; Awan (1975) obtained a mean rate between 4.64 and 4.89 μl O_2/mg dry wt./hour. Although these workers apparently employed different strains of A. avenae they however did not report the age of the cultures from which these populations were derived. Preliminary investigations into the variation of the QO_2 of mixed stages of one isolate of A. avenae (isolate-E)

clearly indicated a small but noticeable drop in the QO_2 with age of the culture, i.e. harvest-time. Thus a standardised routine was established for harvesting cultures and similar assays were carried out on two newly established isolates of A. avenae in order to assess whether the decline of the QO_2 with harvest time was common in all isolates of A. avenae. Further studies were carried out to assess the variation of the QO_2 of the three isolates with temperature, hyperbaric oxygen regimes, and anaerobic-treatment in an attempt to define the profile of the respiratory physiology of these isolates of A. avenae with respect to post-anaerobic changes in the QO_2, Pasteur and Crabtree effects [Barrett, 1976]. The results of these and related studies are presented in Chapter 4.

The response of ecologically isolated populations of A. avenae to variations in temperature optima has been clearly demonstrated [Evans, 1968]. Investigating the rate at which populations developed he reported that the isolates from South Australia, Port Vincent and Western Australia produced the greatest numbers of nematodes at 30°C whereas a Tasmanian isolate showed best development at 25°C. Furthermore the rate of multiplication of the Tasmanian isolate was very much slower than that of the Western Australianand Port Vincent isolates at 25°C, but was approximately the same as that of the South Australian isolate. Preliminary observations on Melbourne, Broken-Hill and Armidale isolates indicated that these populations developed faster at 30°C than at 25°C or 20°C.

These observations indicated that some isolates can be distinguished from others on the basis of biological differences.

It has been observed (Evans, 1968, 1970b) that temperature appears to also effect other processes such as feeding, oviposition and eclosion of a South Australian isolate of A. avenae. Since these activities are all affected by temperature the rate of development of populations studied in this work were analysed in terms of the Arrhenius equation.

Evans (1968) also investigated population composition at various temperatures for the South Australian isolate and suggested that the A/L ratios may be employed to compare the generation times of different populations, or isolates obtained from different environments. In this context studies were carried out on the time dependent changes of the L/A ratios (as opposed to A/L ratios employed in earlier studies by Evans, 1968) of the three isolates of A. avenae at a range of temperatures. These observations and related studies on the effects of anaerobic-treatment, low temperature regimes, and cyanide treatment, on the post-exposure reproductive capacity of the isolates are reported in Chapter 3.

Observations on the effects of physical factors such as temperature, oxygen/nitrogen regimes on the biology and oxygen consumption of an organism can provide useful information concerning its overall

physiology. However such studies have their limitations since the observed effects are difficult to interpret without some knowledge of the metabolic mechanisms which induce them.

The understanding of electron transport mechanisms which operate in A. avenae would undoubtably contribute towards the interpretation of biological data such as that gathered in Chapter 3 in respect of A. avenae.

The elucidation of such electron transport mechanisms in organisms, in general, has been advanced by experiments dealing with the question of whether or to what extent certain compounds inhibit the respiration. The results of such experiments however must be interpreted with caution especially if entire organisms are used. (Von Brand, 1979) Considerable differences in sensitivity towards a given inhibitor have been reported to occur in closely related species. The oxygen uptake of Caenorhabditis briggsae (whole worms) was only partially sensitive to cyanide and carbon monoxide (Bryant, Nicholas & Jantunen, 1967) while the state-3 respiration of Caenorhabditis elegans mitochondria exhibited $\simeq$ 85% inhibition with 20 μM KCN (Murfitt, Vogel & Sanadi, 1976). The state-3 succinate oxidation by a mitochondrial preparation of Turbatrix aceti in contrast required 111 μM NaCN for complete inhibition while 10 uM azide and up to 74 μM cyanide had little effect (Rothstein, Nicholls & Nicholls, 1970).

In this context, the cyanide sensitivity studies on parasitic protozoa are of particular interest. The respiration of blood stream forms of the pathogenic African trypanosomes is very sensitive to arsenicals and bromoacetate, while trypanosomes of the "Lewisi-group" are much less sensitive to such sulfhydryl inhibitors; but exactly the opposite pattern of sensitivity is shown by them with respect to cyanide, the classical inhibitor of cytochrome oxidase [Von Brand, 1979].

Earlier work on a variety of different parasitic forms showed a wide variety of responses even to classic inhibitors like cyanide [Von Brand, 1966] indicating that different parasites possess different respiratory mechanisms. On this basis Von Brand [1966] separated "for convenience" some of the data gathered by many workers with respect to cyanide sensitivity. These references as quoted by Von Brand [1966] are summarised as follows:

Group I Where the QO_2 is inhibited by both NaCN and where tested by carbon monoxide [CO].
Malarial parasites [McKee et al., 1946; Bovarnick et al., 1946]
Fasciola, Moniezia [van Grembergen, 1944, 1949]
Litomosoides [Bueding, 1949a]

Group II Where the QO_2 is inhibited by cyanide but is insensitive to carbon monoxide.

Culture forms of T. cruzi (Baernstein and Tobie, 1951)

Diphyllobothrium, Triaenophorus (Friedham & Baer, 1933)

Larvae of Trichinella (Stannard et al., 1938)

In the latter carbon monoxide is reported to have brought about a distinct stimulation of the oxygen uptake.

Group III Where the QO_2 is not markedly inhibited by even fairly high concentrations of cyanide if not stimulated by it.

African pathogenic Trypanosomes

Trichomonads

Paramphistomum

Ascaris

In Paramphistomum cyanide stimulated respiration by approximately 100% at an oxygen tension of 160 mm Hg, while at 760 mm Hg only very slight stimulation and even inhibition of up to 30% was observed. (Lazarus, 1950 as quoted by Von Brand, 1966)

Preliminary work on one isolate of A. avenae (isolate-E) showed that its QO_2 was only marginally sensitive to cyanide. Concentrations of up to 10 mM cyanide produced less than 20% inhibition. However

exposure to cyanide for even very short periods significantly affected the fecundity of adult females of this isolate. Thus the effects of NaCN, parachloromercuro-benzoate [pCMB], salicylhydroxamic acid [SHAM] and carbon monoxide [CO] on the oxygen consumption of whole worms of three different isolates of A. avenae were assessed using standardised procedures to obtain information on the respiratory mechanisms of A. avenae. The results of these studies are presented and discussed in Chapter 5.

Cooper and Van Gundy [1970b] have reported that in microaerobic and anaerobic environments the principal glycolytic end product in A. avenae was lactic acid [during the first 12-16 hours] after which it was ethanol. However they reported that some ketones and glycols may have been present in concentrations not detectable in the tests. These substances were analysed by Cooper et al., using gas-liquid chromatography on Porapak columns.

About the same time Rothstein [1969] reported that substantial amounts of labelled glucose, trehalose and glycerol were produced by C. briggsae from ^{14}C-labelled acetate. The quantity of glycerol produced by C. briggsae incubated in "whole" axenic liquid media was greater than that produced by the worms incubated in water.

More recently, Madin, Crowe and Loomis [1978] have shown that glycerol begins to appear in live A. avenae only when masses of A. avenae in pellets are subjected to slow drying. More specifically glycerol synthesis began only when the water content of the worm-pellets reached a critical level, i.e. 2.4 mg water per mg.dry weight. They reported that glycerol synthesis occurred while lipid levels fell probably due to operation of the glyoxylate pathway. This pathway although well documented in micro-organisms is not prevalent among eukaryotes although some key enzymes have been described in nematodes. However glycerol originates, its presence in anhydrobiotic A. avenae and is well documented [Madin, Crowe & Loomis, 1978] but it is possible that A. avenae also synthesizes glycerol during normal metabolism. Therefore GLC analysis of exudates of A. avenae whole-worms for volatile end products, and specifically glycerol was undertaken, especially because there was evidence as will be reported in Chapter 5 and Chapter 7A, that a salicylhydroxamic acid [SHAM] sensitive oxidase viz. α-glycerol-phosphate-oxidase complex [GPO - apparently present in both isolates of A.avenae] could well be involved in the production of glycerol in A. avenae. Similar glycerol production involving the GPO-complex [coupled to glycolysis] has been reported to occur in other organisms [Grant & Fulton, 1957; Grant, 1966 as quoted by Bowman & Flynn, 1976] and at different rates under aerobic and anaerobic conditions.

Thus experiments were designed to assess whether this was so in A. avenae. These investigations are reported in Chapter 6.

The response of A. avenae to nematicides such as ethylene-di-bromide [EDB] has demonstrated that this halogenated hydrocarbon reacts with heme-proteins of the respiratory pathway and with serine residues of esterase[s] or protease[s] causing the intoxication and death of A. avenae [Castro & Belser, 1978].

Earlier observations by Evans and Thomason [1971] and Evans [1973] suggested that the ability of nematodes to survive exposure to EDB was enhanced at low temperatures and under anaerobic conditions and they therefore inferred a connection between aerobic metabolism in the nematode and EDB toxicity. The implication by the latter authors was that either the components of the respiratory chain are only vulnerable when in use by the organism or that some product of anaerobic metabolism conferred protection on the treated nematodes.

Prior to these studies, the toxicity of different nematicides and the differential response of various species of nematodes to them, were generally explained in terms of cuticular-barrier effects. [Chitwood, 1952; Goring, 1957; Hollis, 1961]. Marks, Thomason and Castro [1968] however demonstrated that alkyl halide nematicides readily penetrated the cuticle of various species of both plant parasitic and free living nematodes and established a dynamic equilibrium between the external and internal medium.

However Marks (1971) subsequently showed that the oxygen consumption of third stage larvae of Caenorhabditis sp. exposed to 0.53×10^{-2}M EDB was 120% greater than in controls, but similarly treated third and fourth stage A. avenae exhibited a reduced oxygen consumption, approximating to 40% of the controls at 30°C. Awan (1975) investigating a U.K. isolate of A. avenae found that although EDB caused only 27% inhibition of the oxygen consumption, of this organism, worms pretreated with antibiotics (100 units benzylpenicillin and 100 mg streptomycin sulphate) were refractory to EDB, showing only 4.52% inhibition in the presence of EDB. In view of these conflicting reports, investigations were carried out to assess the sensitivity of the three A. avenae isolates to EDB, and its variation with harvest time. The synergistic effect of EDB and NaCN was also investigated. Further the ability of A. avenae to recover following treatment with NaCN and EDB was also investigated since it has been suggested (Castro & Belser, 1978) that A. avenae like all organisms possesses an endogenous capacity to reverse the effects of EDB by re-reducing the iron centres of heme-proteins back to the functional ferrous state. A comparison of the effect of EDB and cyanide was of interest because the mode of action of cyanide is very similar to that of EDB (Castro & Belser, 1978). These studies are presented in Chapter 5.

Mitochondrial extraction and isolation procedures for small free living nematodes have not met with much success,since it has been found that high quality mitochondria could not be prepared from small free living nematodes (such as T. aceti, C. briggsae) if the cuticle was disrupted by using high speed shearing devices such as the Polytron-disintegrator, Bronwill shaker or the Yeda-press (Murfitt, Vogel & Sanadi, 1976). Manual maceration using acid-washed, graded sand or carborundum in mortar has so far yielded better quality mitochondria (Rothstein, 1970; Murfitt et al., 1976; Evans personal communication).

However, the isolation procedures according to the latter authors Murfitt et al. and Rothstein et al. differ significantly. (See Chapter -7A) Thus a compromise isolation procedure was adopted in the present study.

Mitochondrial fractions isolated employing the modified method were used to study the respiratory characteristics of two different isolates of A. avenae, the whole worms of which responded with extreme difference to the general biocide NaCN, and the fumigant nematicide EDB. The respiratory control ratios (RCR^S), the ADP:O ratios for the two isolates utilizing different substrates are reported together with the response of the mitochondrial oxygen uptake (QO_2) to common respiratory inhibitors antimycin-A,

rotenone, sodium cyanide, salicylhydroxamic acid (SHAM) and carbon monoxide, using polorographic techniques.

The differential response of the mitochondrial preparations to these respiratory inhibitors suggested the probable occurrence in both isolates of A. avenae of a branched chain electron-transfer sequence and possibly a third salicylhydroxamic acid sensitive but carbon monoxide/cyanide insensitive oxidase viz. α-glycerophosphate-oxidase (GPO). This is discussed in more detail in the Introduction to Chapters 7 and 7B. Weinbach and Von Brand (1970) described a glycerol-phosphate oxidase in the mitrochondrial fractions of Taenia taenaeformis which is similar in properties to the oxidase evident in the A. avenae isolates examined in the present study.

Evidence of a somewhat similar cytochrome system, as found in these A. avenae isolates, involving a cyanide insensitive alternate terminal oxidase (cytochrome-O) has been cited as one of three possibilities by Rothstein (1970) for Turbatrix aceti, since spectroscopic evidence showed the presence in that species of a carbon monoxide binding pigment. However Rothstein et al., (1970) was unable to obtain confirmatory evidence of the presence of cytochrome-O and/or cytochrome aa_3 in T. aceti viz., "confirmation of this hypothesis is difficult because the classic criterion - the CO-spectrum - cannot be observed." It was reported therefore that the relation of the Turbatrix pigment to the unusual

cytochrome type pigment was not clear although it bore some resemblance to the O-cytochrome described by Cheah [1967] for the cestode Moniezia expansa. [Rothstein et al., 1970].

Analysis of the optical difference spectra of the mitochondrial fractions of A. avenae and the carbon monoxide difference spectra are reported in Chapter 7B, where evidence suggesting the presence of cytochrome-O is presented.

Discussing the unusual properties of T. aceti mitochondria especially the carbon monoxide binding pigment, Rothstein [1970] suggested that it was premature to draw conclusions about the oxidative metabolism of free living nematodes in general. It will be clear from the present study that the oxidative metabolism of A. avenae while employing the classical cytochrome components, also has two other potentially significant oxidases probably capable of terminal electron transfer themselves.

CHAPTER 2

General Materials and Methods

1. Source of isolates of Aphelenchus avenae

Of several isolates of A. avenae established for the study of intraspecific variation in the physiology and biology of this species, preliminary work on three of these isolates indicated that fundamental differences existed among them. The origins of the three isolates used in the present study were as follows.

1) Field Isolate (hereafter referred to as isolate-F) was obtained from a grassed field at Ashurst Lodge, Silwood Park.

2) Evans Isolate (hereafter referred to as isolate-E) of unknown provenance, has been cultured at Silwood Park since before 1970.

3) Amphimictic Isolate (hereafter referred to as isolate-A) was obtained from Rothamstead and this was an isolate originally extracted from soil brought from Malawi.

Isolates-F and E were parthenogenetic and were established from 5-10 specimens extracted from the soil samples and identified as females of A. avenae.

2. Preparation of A. avenae isolates for sterile culture

Following extraction from soil or non-sterile culture, individuals of A. avenae were collected in sterile tap water. After several further passages through tap water they were transferred to a 10%

solution of Penicillin/streptomycin sulphate [10,000 U Penicillin; 10,000 µgm streptomycin] for 1-2 hours. After several changes in sterile distilled water [SDW] the specimens were introduced individually into 0.09% aqueous Hibitane for 15 seconds and washed in SDW before transfer onto 2-day old actively growing petri-dish cultures of Corticium praticola with 3.8% potato dextrose agar [PDA] as substrate. The inoculum for isolate-F consisted of 6 females and the inoculum for isolate-A consisted of 5 females and five males.

3. Monoxenic culture of isolates on petri-dishes

Stock cultures of all isolates were maintained in 9 cm petri-dishes containing sterile PDA [approx. 10 cc/plate] inoculated with the fungus Corticium praticola, the perfect stage of Rhizoctonia solani, by adding inoculum to the centre of the PDA plate. These were incubated at 25°C for 48 hours before nematode inoculum was added. When populations were well established [i.e. had multiplied about 100 fold] 3 or 4 square blocks of agar [1.5 cm x 1.5 cm] were stacked on a young fungal culture and incubated at 25°C to obtain high densities of the new A. avenae cultures. This process of sub-culture was carried out every 10 days for each of the 3 isolates. Maximal populations were attained by 10 days at 25°C and were used to inoculate the Kilner Jar mass cultures.

4. Mass-Monoxenic culture of *A. avenae* in Kilner Jars

The technique adapted for mass culture of *A. avenae* was a modification of the one developed by Evans (1970); 200 ml of oats (whole) and 150 ml of water were autoclaved at 15 p.s.i. for 20-30 mins in a wide mouth Kilner jar, the lid of which was pierced with a cotton wool plug. When cooled to room temperature the jars were inoculated (in a sterile cabinet) with PDA blocks containing *A. avenae* (and *C. praticola*) in high density.

The blocks were selected from stock sub-cultures. PDA blocks (1 cm x 1 cm) containing vigorously growing *C. praticola* were also added to the jars which were then incubated at 25°C or 30°C (as desired for experiments). When harvested after a minimum period of 3 weeks, each jar yielded 1-2 gms wet weight of *A. avenae*. The worms form a curd on the walls of the jars and can be washed off by a jet of water and collected in suspension.

5. Preparation of whole worm suspension of *A. avenae*

A standardised method was used to prepare suspensions of *A. avenae* for experimental use. The nematodes on the inside walls of the Kilner jars were washed off with a jet of distilled water and filtered under suction on Whatman-50 hardened filter paper on a sintered glass funnel. The nematodes were rinsed several times in sterile distilled water, scraped off the filter paper using a flamed scalpel (in a filtered air

cabinet] and introduced carefully onto a 90 μ nylon sieve supported by a perspex ring [i.e. a miniaturised Whitehead extraction technique - Whitehead & Hemming, 1965]. The sieve fitted inside a 9 cm sterile petri-dish and therefore active nematodes passed through the sieve and were collected in the sterile distilled water after 12 hours at 25°C. The nematodes were refiltered under suction, weighed and made into suspensions containing 20 mg wet wt./ml with sterile distilled water.

Depending on experiments designed, the Kilner jars could be harvested at any time from 21 days to 120-140 days as the worms do not desiccate within this period although the food supply may be low after 56 days.

Suspensions of isolate-F and isolate-E survived storage at 0° - 3°C but there was a high mortality following storage at 0° - 3°C in suspensions of worms of isolate-A so they were used as soon after harvest as possible.

6. Calibration of oxygen electrode for assessment of oxygen consumption

The oxygen electrode [Rank & Co.] was calibrated with 3.0 ml of air-saturated distilled water which had been equilibrated for a minimum of 12 hours at the test temperature. After a steady baseline was obtained, the baseline on the polarograph recorder was set at 9 mV on the scale. The calibrating water was removed and

immediately replaced with 3.0 mls of the test nematode suspension held at room temperature. After the suspension was brought to the test temperature the electrode chamber was sealed and the polarographic recording was obtained. A mercury thermometer was used to check the temperature of the suspension in the electrode cuvette. The solubility of oxygen in water at each temperature and 760 mm Hg pressure was obtained from the International table of constants and corrected for barometric pressure.

After assay the nematode sample was placed in a pre-weighed foil crucible. The electrode vessel was rinsed several times with distilled water to ensure all nematodes were collected. Nematodes were oven-dried at 85°C for 72 hours, then stored over phosphorus pentoxide for 48 hours at 40°C until constant weight was achieved.

CHAPTER 3

Biological Variation Among Three Isolates of Aphelenchus avenae

Introduction

Aphelenchus avenae Bastian, 1863 is a cosmopolitan soil dwelling, mycophagous species and therefore considerable morphometric variation occurs within the species due to geographical location and/or variation of environmental factors. (Evans & Fisher, 1970a; 1970b; Fisher, 1969a; Hechler, 1962) Although Townshend (1964) found that A. avenae reproduced on 54 out of 59 named fungi tested, the population densities attained after 14 days at 25°C (starting from an inoculum of 200 nematodes) varied greatly among the different fungus hosts. While the lowest density observed was on Myrotherium rodium (<500), the highest was on Botrytis fabae (>200,000). The highest densities were observed on Botrytis spp. (i.e. B. fabae > B. cinerea > B. allii) and even on B. allii a mean density of 50,000 was reported. Next to the 3 Botrytis sps. the numbers produced (≃ 40,000) on Corticium praticola, the "perfect" stage of Rhizoctonia solani were the highest while on Rhizoctonia solani (cabbage strain) the numbers were < 25,000. However, although no attempts were made in Townshend's studies to correlate morphometric variation to host fungus he found that the cultures of A. avenae which he studied produced populations exceeding 10,000 on Pythium ultimum Trow, and Trichoderma viride Persoon ex. Fries. These results

he reported were in contrast to those of Mankau and Mankau [1962] who noted that their culture of A. avenae reproduced poorly on Trichoderma viride and not at all on P. ultimum. Thus Townshend [1964] suggested that the differences in these observations may be due to "strains" of A. avenae or fungi or to both.

Individual fungi may show considerable variability in their chemical composition both quantitative and qualitatively. These variations may be influenced by a number of factors such as age of culture, the character of mycelium and sporulation and the ingredients of the substrate [Townshend, 1964]. Cultures of the same or different fungi may be the same chronological age but different physiological ages. The relative level of carbohydrates, nitrogenous compounds and minerals in a substrate has its effect on the content of various substances in the fungus which it supports. Such differences in the chemical composition of a fungus may in turn affect its suitability as a host for A. avenae [Townshend, 1964] and other mycophagous species.

Evans [1970b] studying the factors affecting population densities of a South Australian isolate of A. avenae showed that there were no significant differences in the rate of population increase or in the variations in the adult : larval ratios of the isolate grown on Rhizoctonia solani - 48, growing on two different nutrient agar substrates [Rhizoctonia Medium - RM; Potato Dextrose Agar - PDA]. However the average

length of the females from RM was significantly greater than on PDA at 27 and 28 days in culture (at 25°C) but were similar after 56 days in culture. This was believed to be due to the differences in the nitrogen content of the two media. He speculated that the final population densities on both media were similar despite the deficiency of nitrogen in one, because in the females the demands of egg production takes precedence over other body tissues in the demand for nutrients and only the remainder of the available nitrogen is used for tissue building and PDA supplied less for this process since the nematodes on PDA were smaller. It was also shown (Evans, 1970b) that as the populations developed, the average length of females changed at 30, 25, and 20°C and the decrease in average length was attributed to decrease in amount of available food which occurred sooner where population development was most rapid. The fact that different media, age of fungus and food supply during the test period did not affect the population densities of A. avenae at specific harvest times and temperatures indicates that population growth rates would be useful criteria in assessing differences between isolates of A. avenae.

In synonymizing all the known species of the genus Aphelenchus under Aphelenchus avenae Bastian, 1863, Goodey (1963) drew attention to the need for closer study of various populations of Aphelenchus to determine the extent of variation within the species. This situation

was resolved by Goodey and Hooper [1965] who designated a neotype of A. avenae and described the morphometric variation showing the topotypes. Several species are however tentatively listed as synonyons of A. avenae due to insufficient data. For instance Aphelenchus radicicolus [Cobb, 1913] Steiner, 1931, is considered to be a distinct species by Thorne [1961] and Suryawanshi [1971] because males are more common and functional and since the post-vulval sac is more prominent. However these two criteria have been shown to be influenced by environmental factors [Hansen, Buecher & Harwood, 1970, 1972, 1973].

Evans [1970a] studying the population development among four Australian isolates of A. avenae showed that all isolates except a Tasmanian isolate produced greatest numbers at 30°C while the latter produced more nematodes at 25°C than at the other temperatures. The low temperature optimum for this isolate and the slow reproduction rate [even at its optimum temperature] was explained as being due to the adaptation to a different climate. These differences Evans [1970a] speculated, could be due either to some property of the nematode or to the suitability of the host fungus. Discounting the latter [since the low "vigour" of the Tasmanian was a property which was apparent on several different host fungi] he ascribed the differences in reproduction rate to metabolic differences inherent in the nematodes. This is the only study of a comparative nature in the literature,

conducted to determine intraspecific variation in A. avenae, although isolated observations such as those of Goodey and Hooper [1965] and Hechler [1962] which do not specify conditions used, stage of development of the populations and the time taken to reach a given population density are of limited value. Thus Evans [1970a] drew attention to the need for similar studies on a wide range of isolates of A. avenae in order to collect information on an ecological basis for the classification of isolates.

The ratio between numbers of adults and larvae [A/L ratios] has been shown by Evans [1970b] to be a useful criterion in studying the generation time within a developing population of A. avenae since it has a short life cycle and generation time. Fluctuation in the A/L ratio observed by Evans is attributed to the hatching of eggs to increase larval numbers and the change of larvae to adults. The time between two successive low points in the ratio, Evans [1970b] speculated, may reflect the average generation time of the nematodes in the population. The generation time at 20°C obtained in this way [12 days] agreed with that obtained by Goodey and Hooper [1965]; by making more frequent harvests [every 45 hours] at higher temperatures, [Evans, 1970b] the generation time at 30°C obtained [≃ 4 days] was similar to the 6 days generation time reported by Hechler [1962] at 28°C.

The studies which are reported in this section therefore assessed the effect of temperature on the population development rates, and the variation of L/A ratios of the three isolates of A.avenae. The effects of anaerobic treatment and low temperature regimes on the post-exposure reproductive capacity, mortality/ viability of the nematodes from the 3 isolates were also investigated. The effect of larval adult numbers and its variation in PDA containing NaCN was compared for 2 isolates. A comparative study of this nature was possible because of the rapidity of the reproduction [generally considered to be meiotic-parthenogenesis - Triantaphyllon, 1971] at ambient temperatures between 25-30°C and also because the ageing of the fungal host did not interfere with its suitability to sustain log-phase populations of A. avenae [Kondrollo, this , 1974] especially since the design of the experiments assumed this factor as constant.

Materials and Methods

1. Source of inoculum for experiments on population dynamics

For studies on comparative growth rates of isolates of *A. avenae*, inoculum was obtained from 10 day old cultures grown on *Corticium praticola* in PDA at 25°C, by cutting the culture into small segments and covering with sterile distilled water. After 2 hours extraction, adults were selected under x 40 magnification and removed to sterile water in a cavity block using a nylon fibre sterilized in 0.5% Hibitane.

2. Effect of temperature on population growth rate

To compare population growth rate standardised agar plates were prepared [modified after Evans [1970b]]. 20 mls of PDA were added to a sterile petri-dish. After setting, a small piece of inoculum cut from the advancing edge of a mycelium of *Corticium praticola*, was added. The dishes were incubated at 25°C for 48 hours before being inoculated with the test nematodes. The nematode inoculum consisted of 5 young surface-sterilized females in case of isolates F and E and 5 mature males in addition to the females, in the case of isolate-A. Temperature treatments at 10°C, 15°C, 20°C, 25°C and 30°C were replicated so that at each temperature 6 plates were harvested for each isolate. The plates were harvested at 45 hours [H_{45}], 95 hours [H_{95}], 145 hours

(H_{145}), 165 hours (H_{165}), 195 hours (H_{195}), 240 hours (H_{240}) and 300 hours (H_{300}) after inoculation, and extracted and counted.

3. Extractions of populations at harvest

To obtain a quantitative extraction of nematodes the method described by Evans (1970b) was used with modification. This method was described by Evans (1970a,b) to be superior to extractions obtained by mechanically shaking the agar and extracting the suspension on a modified Baermann funnel. The agar in the plates was cut into segments and covered with distilled water for 24 hours. This was then rinsed onto a wide nylon mesh (sieve size 1.5 mm square) and sieved into a vial. The blocks of agar were rinsed several times with a strong jet of water till no nematodes were seen when examined under the binocular microscope.

4. Counting of extracted populations

The total number of nematodes in the suspension was counted when fewer than 400 were present; if there were more, the suspension was diluted so that between 100 and 200 nematodes were counted. In this case, bubbles of compressed air were used to agitate the suspension and a sample was withdrawn using a truncate pipette. Larval and adult numbers were counted at X 50 magnification in a counting dish (Doncaster, 1962).

To distinguish between fourth stage larvae and adults a higher magnification was used. Since a large number of replicates were harvested at each time, sample populations were killed by hot acetic acid [Seinhorst, 1962], fixed in TAF [Goodey, 1963] and stored in a refrigerator for counting later.

5. Investigation of L/A ratios at various temperatures

The relationship between numbers of adults and numbers of larvae [expressed as L/A x 10^3] and the variation of the ratios at 20°C, 25°C and 30°C were investigated. 5 cm agar plates were prepared using 5 mls of PDA as medium, inoculated with C. praticola. After 48 hours 5 young adult females were added in the case of isolates F and E and 5 males and the females in the case of isolate A. Harvesting was done more frequently at 45, 95, 135, 168, 195, 215, 240, 280, 320 and 360 hours after inoculation, since overlap of generations could distort the actual fluctuation [Evans, 1970b]. When counting, a correction was made for populations of isolate-A since these initially contained 5 males as well. A value of 200 for 10^3 x L/A indicated the hatch of first larvae. The 3 isolates were compared in this manner.

6. Investigation of changes in composition of populations of two isolates, produced in NaCN incorporated medium

The procedure was identical to that described above except that each plate of PDA also contained 3 mM NaCN.

The fungal host C. praticola grew unaffected in this medium and there was no notable difference in mycelial development rate. Nematode inocula were added as described earlier and extracted at harvest times 45, 95, 168, 195, 215, and 240 hours after inoculation. Counting was as described earlier.

7. Effect of low-temperature treatment on population development

Since preliminary studies on population development rates and O_2 consumption rates at various temperatures indicated that the 3 isolates behaved differently, experiments were designed to investigate this aspect further. In one method, 8 day old petri-dish cultures of each isolate were incubated at 0°C (minimum) to 3°C (maximum) for 14 days. Similar plates were kept at 25°C as controls. In the second method, nematodes were extracted from 8 day old cultures and introduced into 3 ml of sterile distilled water in sterile screw top glass vials and kept at 0°C to 3°C for 14 days. Controls were maintained at 25°C. After incubation 5 females (+ 5 males in the case of Isolate-A) from each plate and vial were inoculated onto 5 cm petri-dishes containing C. praticola in 5 ml PDA and the populations were counted after 130 hours at 30°C. Six plates were counted for each treatment and control for each isolate.

8. Effect of low temperature on percentage mortality in antibiotic suspension

Mixed populations of the three isolates of A. avenae were grown at 25°C for 4 days, then harvested. Approximately 0.2 mls of a 20 mg wet wt./ml suspension of each isolate was introduced into a glass vial containing 5 mls of sterile tap water containing 5% penicillin/streptomycin [prepared by adding 5 mls of a stock solution containing 10,000 U penicillin and 10,000 µg streptomycin sulphate to 95 ml sterile tap water]. The suspension contained about 300-400 nematodes. The vials were incubated at 0°C to 3°C and a control group was incubated at 25°C. Every week for 15 weeks, 6 vials were removed and 0.2 ml of 0.1% ploxine B was added. This stained only dead nematodes which were then counted on a Doncaster dish 12 hours later. The percentage mortality was calculated for the test and control groups.

9. Effect of anaerobic treatment on population development

To test the effect of total anaerobic treatment on the reproductive capacity of 2 isolates of A. avenae, O_2 free N_2 was passed through bacterial filters into quick-fit conical flasks each containing 25 mls of sterile distilled water as a 5% penicillin/streptomycin solution [prepared as described earlier]. As a control the same treatment was repeated with air, 1 ml suspensions of A. avenae of isolates F and A were added to each flask,

which was then made airtight and put to incubate at 25°C in a desiccator flushed with O_2 free N_2. The control flasks were kept in air at 25°C with a facility for continuous passage of filtered, humidified air. After 120 hours nematode inocula (5 females ± males) from the test and control flasks were added to PDA plates containing C. praticola. After incubation at 30°C for 120 hours the populations were counted as described before. Six replicates were counted to obtain the mean densities.

10. The effect of anaerobic treatment on survival/viability

A. avenae from isolates F and A were added to sterile distilled water saturated with O_2 free N_2 as before and the control groups were treated in air-saturated sterile distilled water. After 120 hours 50 individuals from each treatment were picked singly onto a disc of potato dextrose agar (3 mm in diameter x 1 mm thick) in which C. praticola had been established for 72 hours. The discs were cut out, by means of a flamed cork borer, from petri-dish cultures grown at 25°C. The discs with fungal mat were arranged singly in each of the twelve cells of a leucocyte migration plate (Sterilin Co.). Each plate was sealed with wide cellotape to prevent dehydration and contamination of the discs. A small perforation was made in each cell to ensure gaseous exchange. The plates were incubated for 3 weeks in the dark before

assessing the results by counting the dead, surviving and viable nematodes under a binocular microscope. [See section 11 below]

11. Assessment of survival/viability/mortality

The final assessment of the survival studies was as follows. Viable females were those that had oviposited viable eggs which eventually hatched into larvae. Surviving females were those single-unreproductive females which did not oviposit or oviposited non-viable eggs from which no larvae emerged. The dead females were those found encroached by fungal mycelium and those only whose cuticular shells were found. The counts [out of a total of 50] were transformed into percentage figures for comparison of control and test groups of the two isolates tested.

Results

1. Effect of temperature on the population development rates of the 3 isolates of *A. avenae*

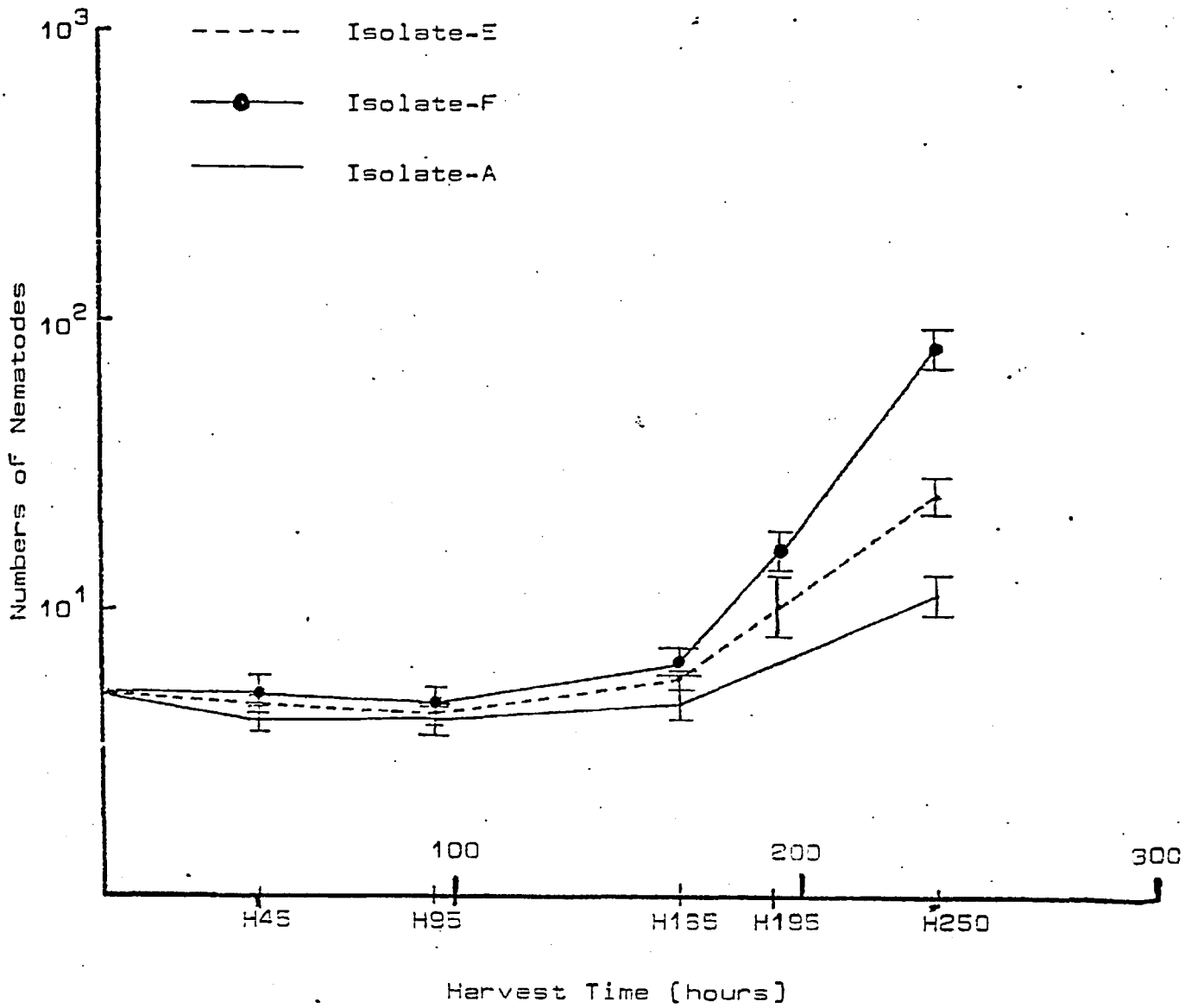
Petri-dishes containing the 3 isolates of *A. avenae* feeding on *C. praticola* on PDA were incubated at 10, 15, 20, 25 and 30°C and the increase in number of nematodes at each temperature was determined. (Appendices 3-1.1 to 3-1.5)

At all temperatures except 10° and 15°C the logarithmic development curve approached a sigmoid shape for all three isolates (Figs. 1, 2, 3, 4 and 5). The 5% confidence limits are indicated in these plots and related t-test comparisons of population counts at each harvest time are given in Appendices 3-2.1 to 3-2.5.

At 10°C (Fig. 1) the numbers produced at H_{240} (≡ Harvest after 240 hours) differed significantly among the three isolates showing very long lag-phases in the development curve up to H_{95}, with densities less than 5.00 for all three isolates. The mean densities of the 3 isolates were similar up to H_{95} ($p \gg 0.05$).

At 15°C the lag-phase was much shorter than at 10°C. (Fig. 2). However even at this temperature the mean densities of the 3 isolates were not significantly different and did not exceed 6.5 at H_{95}, but by H_{145} there were significantly fewer of isolate-A than of E and F and this trend became more pronounced with time. However isolate-E and F only became significantly different at H_{300}.

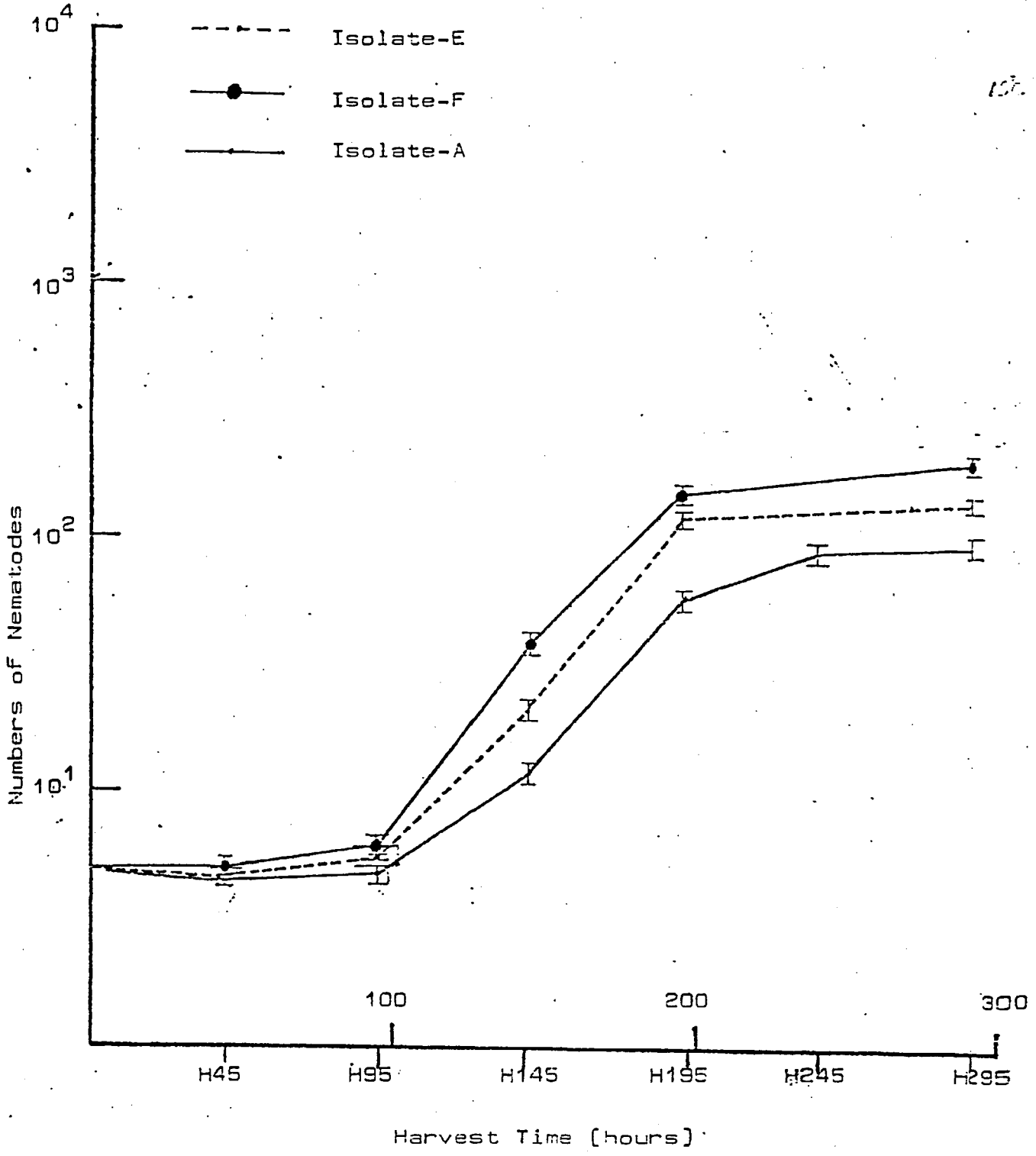
Figure: 1. The population development of the three isolates of A. avenae at 10°C.



The confidence intervals (α=0.05) are indicated in the figure.

Statistical Analysis in Appendix 3-2.1.

Figure: 2. The population develoment of the three isolates of A. avenae at 15°C.



The confidence intervals (α=0.05) are indicated in the figure.

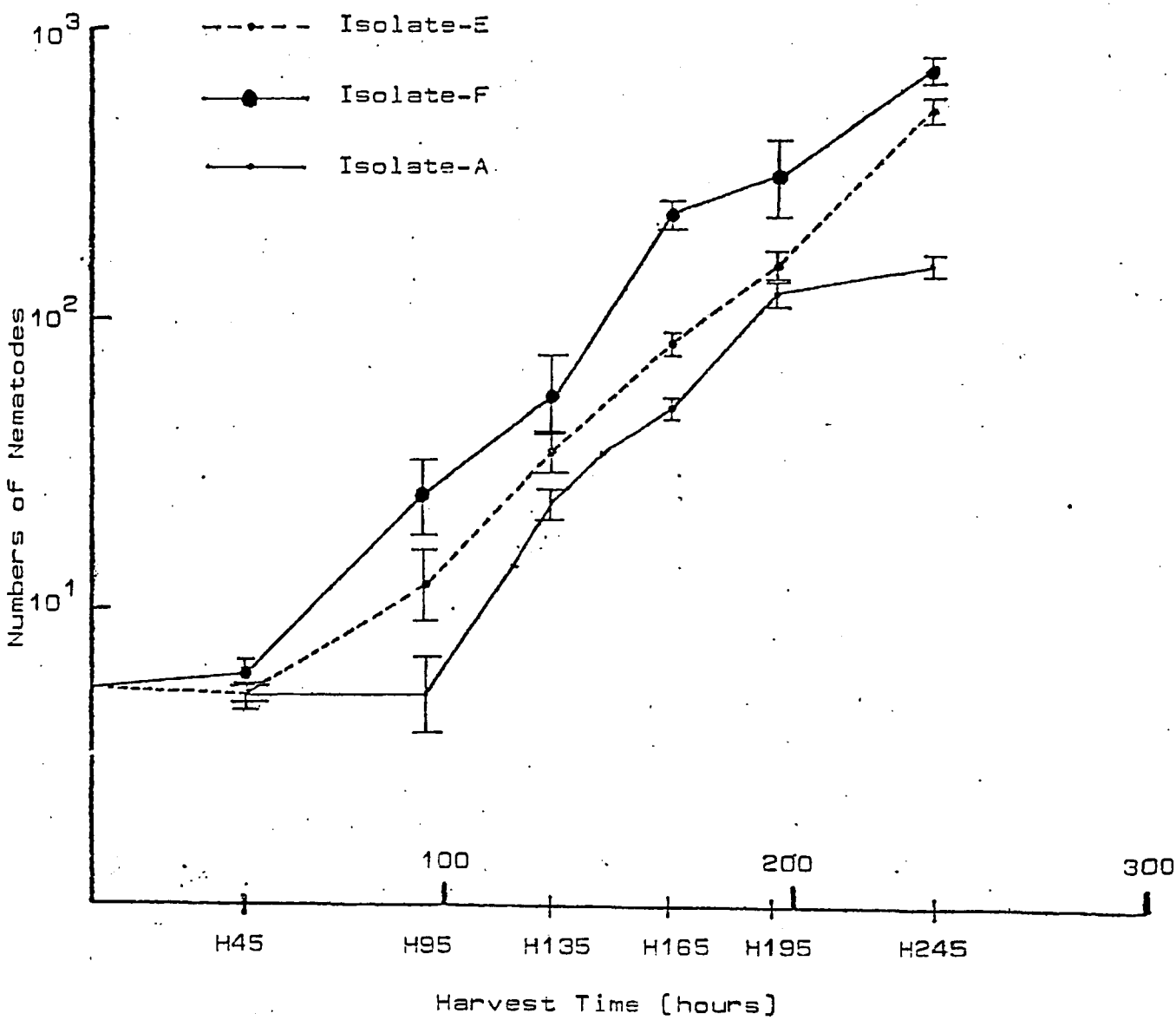
Statistical Analysis in Appendix 3-2.2

Fig. 3 shows the population growth plots at 20°C. The lag-phase for isolate-A is about 40 hours longer than that for isolates E and F. The mean densities of the three isolates were not significantly different at H_{45} but at all other harvest times the differences were significant with isolate-F producing the highest densities and isolate-A producing the lowest density, the differences increasing with harvest time. The time at which a density of 7.0 [i.e. two new individuals] was produced was ≏ 50, ≏ 65 and ≏ 105 hours for isolates F, E and A respectively.

At 25°C, the time taken to achieve a density of 7.0 was still ≏ 100 hours for isolate-A but much less for isolates E and F. At 25°C there was, for the first time, a significant difference between two isolates at H_{45}; isolates- F and E being significantly higher than isolate-A. This trend continued up to H_{240}. Isolates E and F differed significantly at H_{95}, H_{145} and H_{240}. (Fig. 4)

At 30°C all 3 isolates developed rapidly (Fig. 5). It is notable that isolate-A developed similarly to isolate-E at all harvest times (except H_{290}) only at this temperature. Also of interest is the observation that at H_{120} the mean density of isolate-A was not significantly different from the density by isolate-F. This was observed only at this temperature and at this particular harvest time. At all other harvest times isolate-F differed significantly from both isolates A and E.

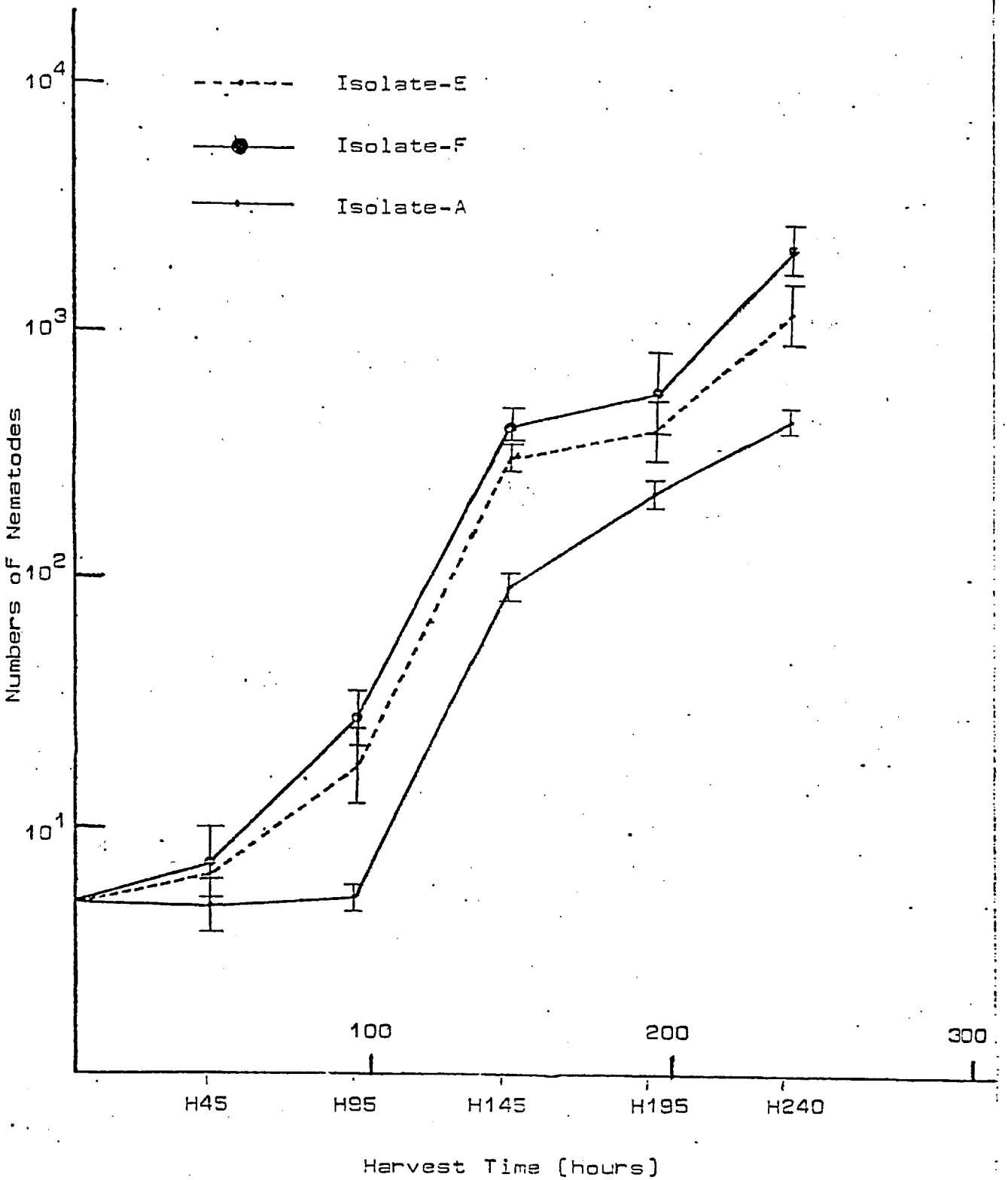
Figure: 3. The population development of the three isolates of A. avenae at 20°C.



The confidence intervals (α=0.05) are indicated in the Figure.

Statistical Analysis in Appendix 3-2.3

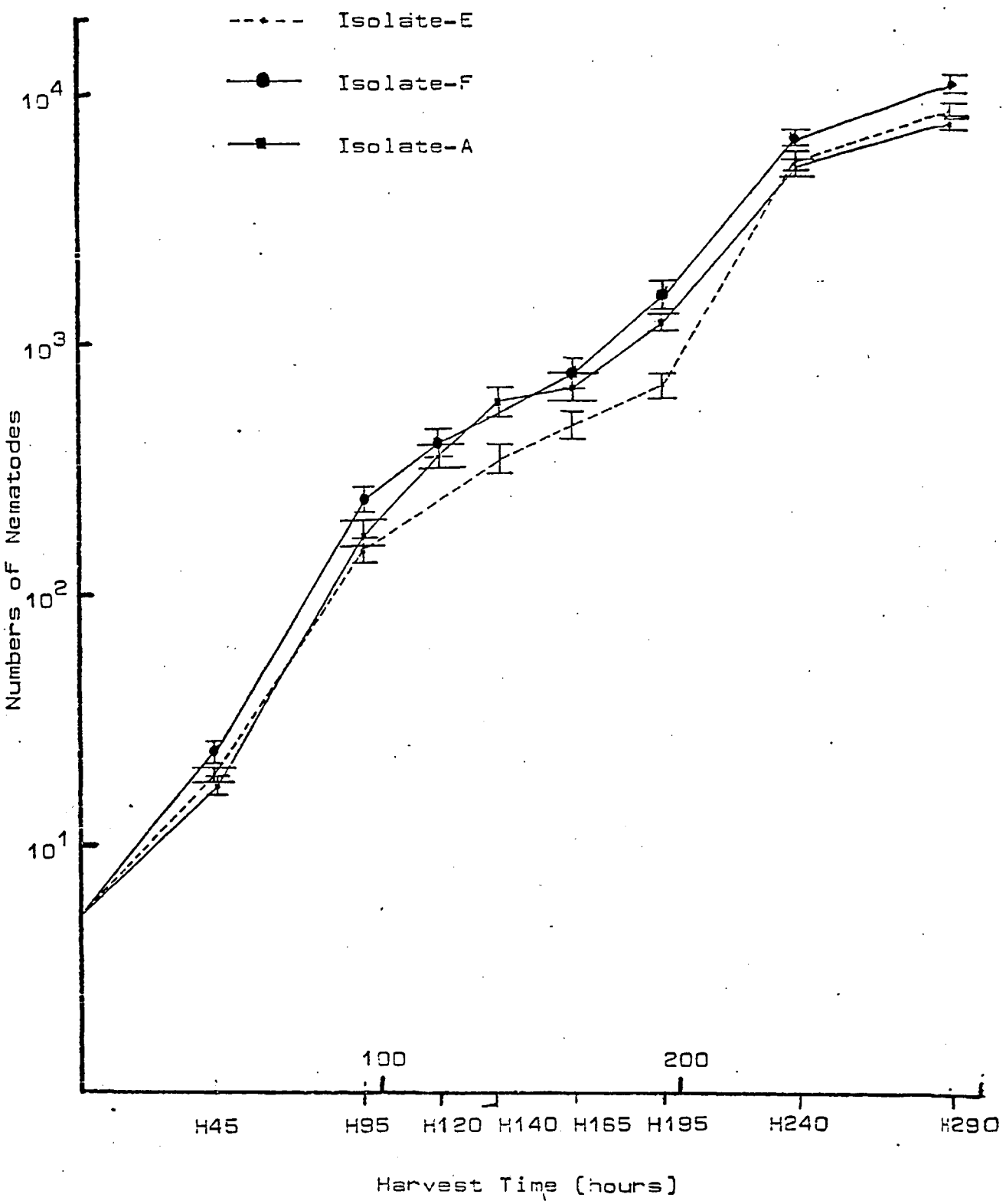
Figure: 4. The poopulation development of the three isolates of A. avenae at 25°C.



The confidence intervals (α=0.05) are indicated in the figure.

Statistical Analysis in Appendix 3-2.4

Figure: 5. The population development of the three isolates of A. avenae at 30°C.



The confidence intervals (α=0.05) are indicated in the Figure.

Statistical Analysis in Appendix 3-2.5

1.b Arrhenius Plots of population growth rates

When rates of population growth were plotted against reciprocals of the absolute temperature [1/°K], the Arrhenius plots showed points of inflection at 20°C for isolates E and F. The plot for isolate A was different, whereas isolates E and F showed positive log-development rates the corresponding value for isolate-A was negative; which corresponds to a rate of population growth of 0.675 nematodes/hour. Isolates E and F in contrast showed rates of +2.3542/hour and + 3.2417/hour which were much higher. Even at 25°C the rate of population growth for isolate-A was lower than that observed for the other two isolates, although they were similar at 30°C. (Fig. 6) [Appendix 3-3.1]

When the Arrhenius activation energy for the observed plots in Fig. 6 was calculated for each section of the curve the following values were obtained.

Table 1 Arrhenius activation energy transitions of the three isolates of A.avenae [Values computed from the Arrhenius plots of the population development rates shown in Figure 6]

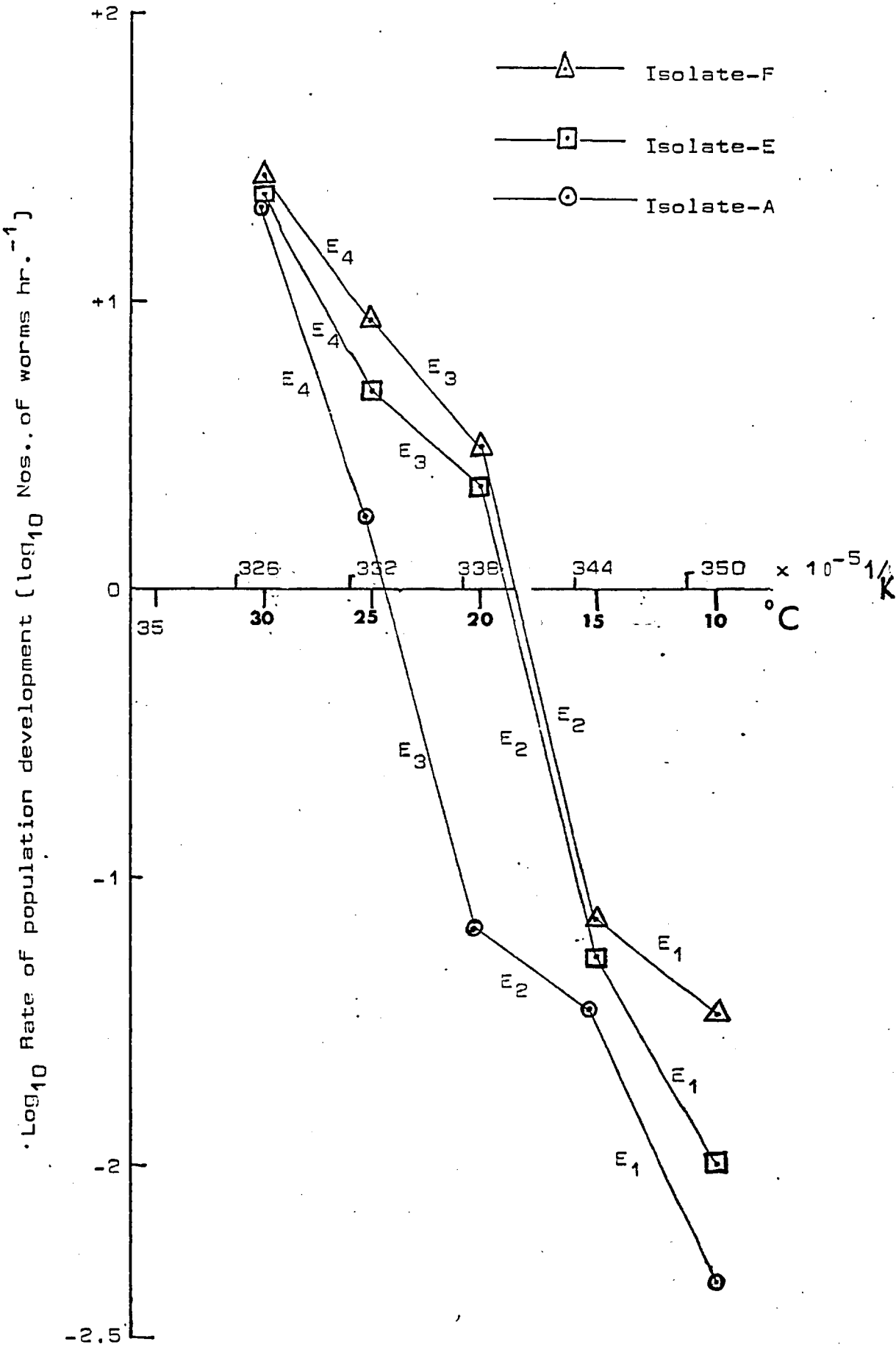
Isolate	E_1^* 10 - 15°C	E_2^* 15-20°C	E_3^* 20-25°C	E_4^* 25 - 30°C
	E^* units:- calories $mole^{-1}$			
Isolate-A	64.50×10^3	21.0×10^3	— 106.5×10^3 —	
Isolate-E	51.75×10^3	122.25×10^3	24.75×10^3	61.5×10^3
Isolate-F	24.0×10^3	123.75×10^3	— 32.25×10^3 —	

Figure: 6. (Facing page)

E_1 - E_4 are the Arrhenius activation energies (cals. $mole^{-1}$) for each limb of the plots.

	Isolate-A	Isolate-F	Isolate-E
E_1	64.5×10^3	24.0×10^3	51.7×10^3
E_2	21.0×10^3	123.7×10^3	122.2×10^3
E_3	106.5×10^3	35.2×10^3	24.7×10^3
E_4	105.0×10^3	35.25×10^3	61.5×10^3

Figure: 6. Arrhenius Plot of population growth rates of the three isolates of A. avenae



The E* value for isolates A and F were the same since no discontinuities occurred in the plots, between 20 and 30°C. There were 2 points of inflection in the Arrhenius curves for isolates A and F while 3 such inflection points were distinctly shown by the plot obtained for isolate-E. Therefore 3 different E* values resulted for isolates A and F i.e. an E* value for each limb following the inflections. Similarly 4 different E* values were obtained for isolate-E.

Linear regressions were computed for the plots in two sets i.e. one for the points on the $^{1}/^{\circ}K$ axis corresponding to temperatures 10°C, 15°C and 20°C (the first limb of the curves), the second regression for the points on the $^{1}/^{\circ}K$ axis corresponding to 20°C, 25°C and 30°C (constituting the second limb) (Fig. 7). These demonstrate the probable significance of the trends which are less apparent in Fig. 6 , because no statistical treatment was applied to substantiate the plots shown in Fig. 6.

2. <u>The effect of temperature on the changes in the L/A ratios with time for the 3 isolates</u>

The ratio of number of larvae to number of adults showed characteristic fluctuations in all developing populations of <u>A. avenae</u> and were more frequent at higher than at lower temperatures. Figs. 8, 9 and 10, and Appendices 3-4.1 to 3-4.9

Figure: 7. (Facing page)

E_1 and E_2 are the Arrhenius activation energies (cals. $mole^{-1}$) for each limb of the plots.

	Isolate-A	Isolate-F	Isolate-E
E_1	43.5×10^3	73.7×10^3	87.5×10^3
E_2	100.7×10^3	37.3×10^3	40.3×10^3

Figure: 7. The regressions computed for the Arrhenius Plots of population growth rates of the three isolates of A. avenae

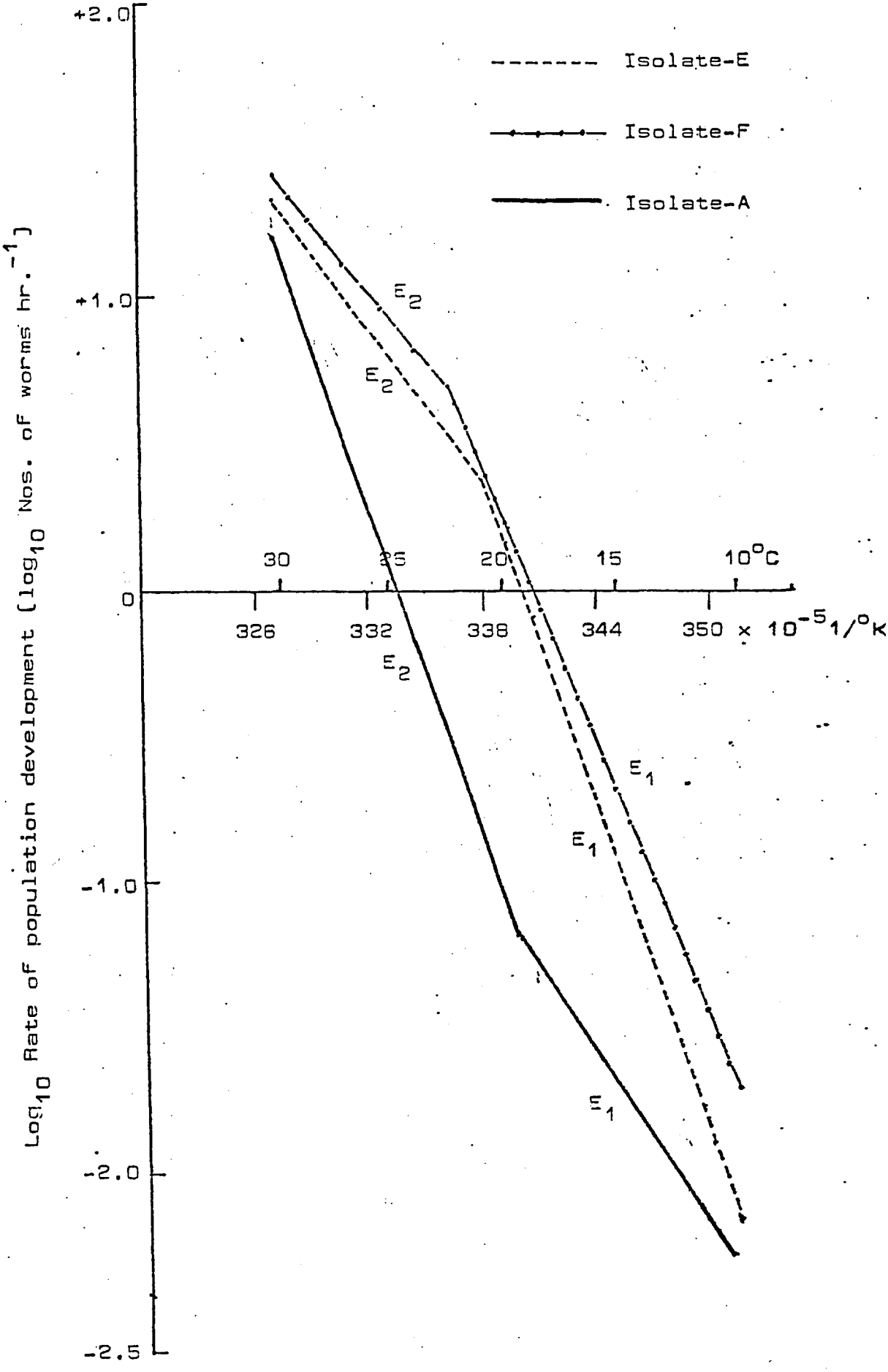
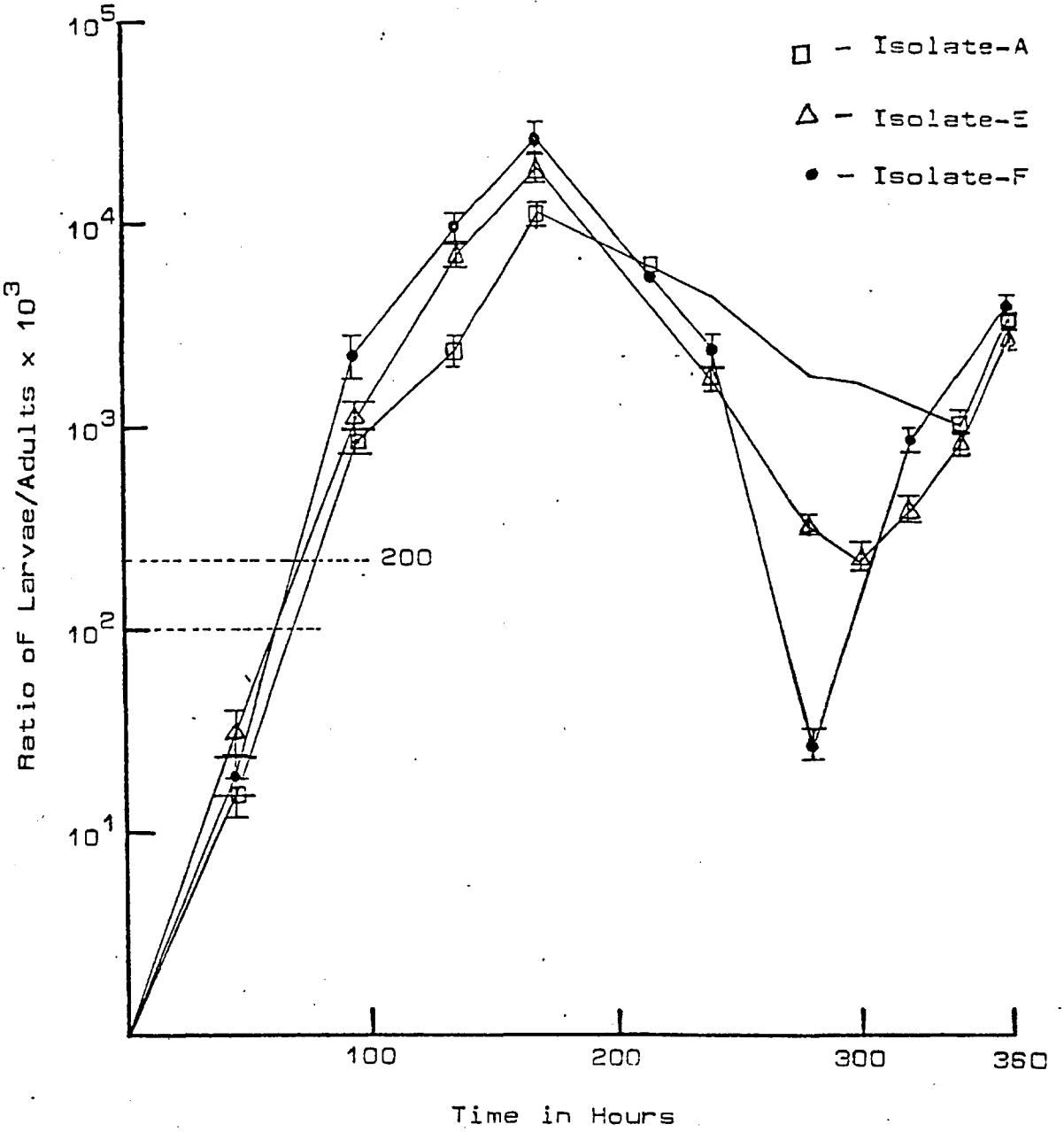


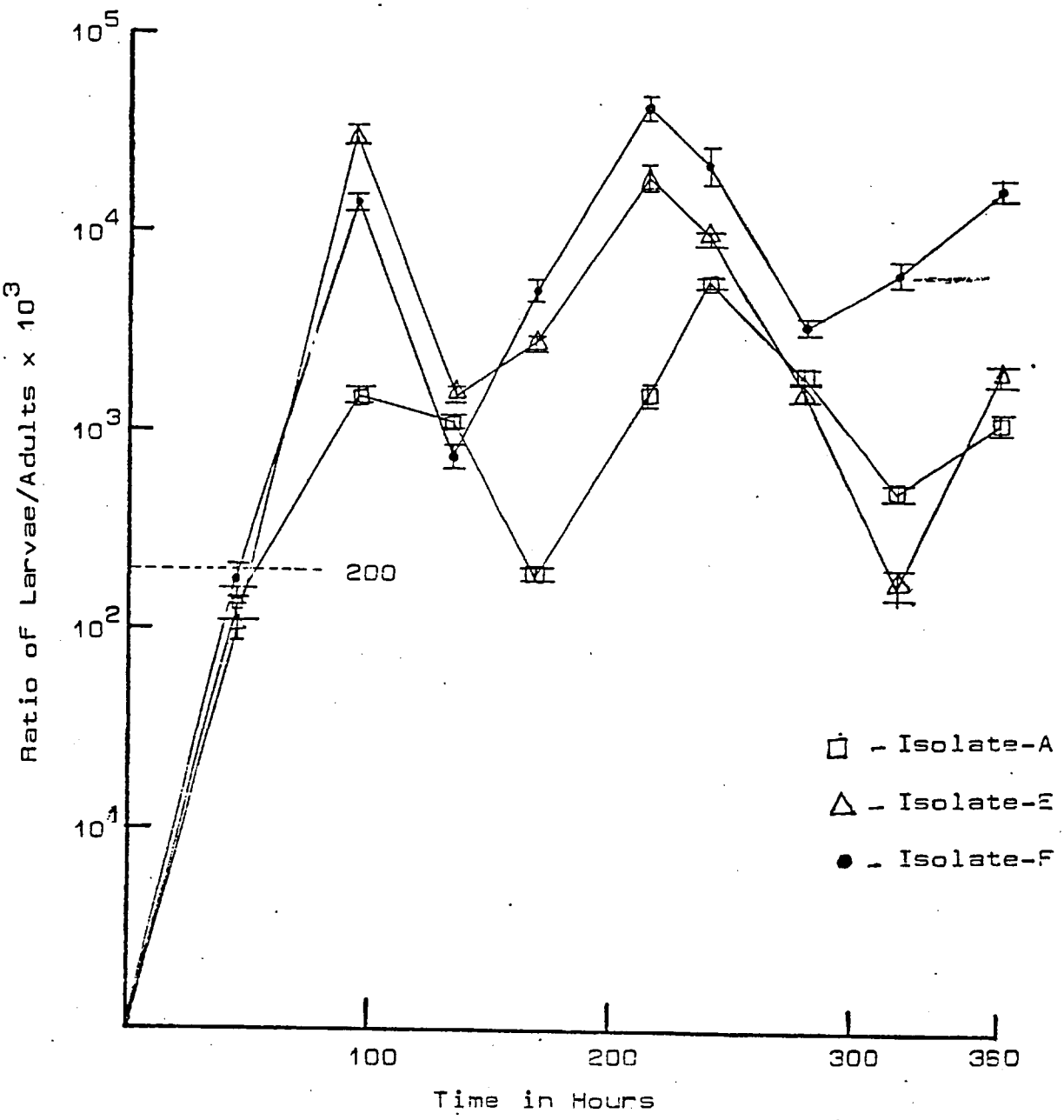
Figure: 8. The changes in the ratio of larvae to adults in the three isolates of A. avenae developing for 15 days at 20°C.



The confidence intervals [α=0.05] are indicated in the figure.

Details in Appendix 3-4.1, 3-4.2 and 3.4.3

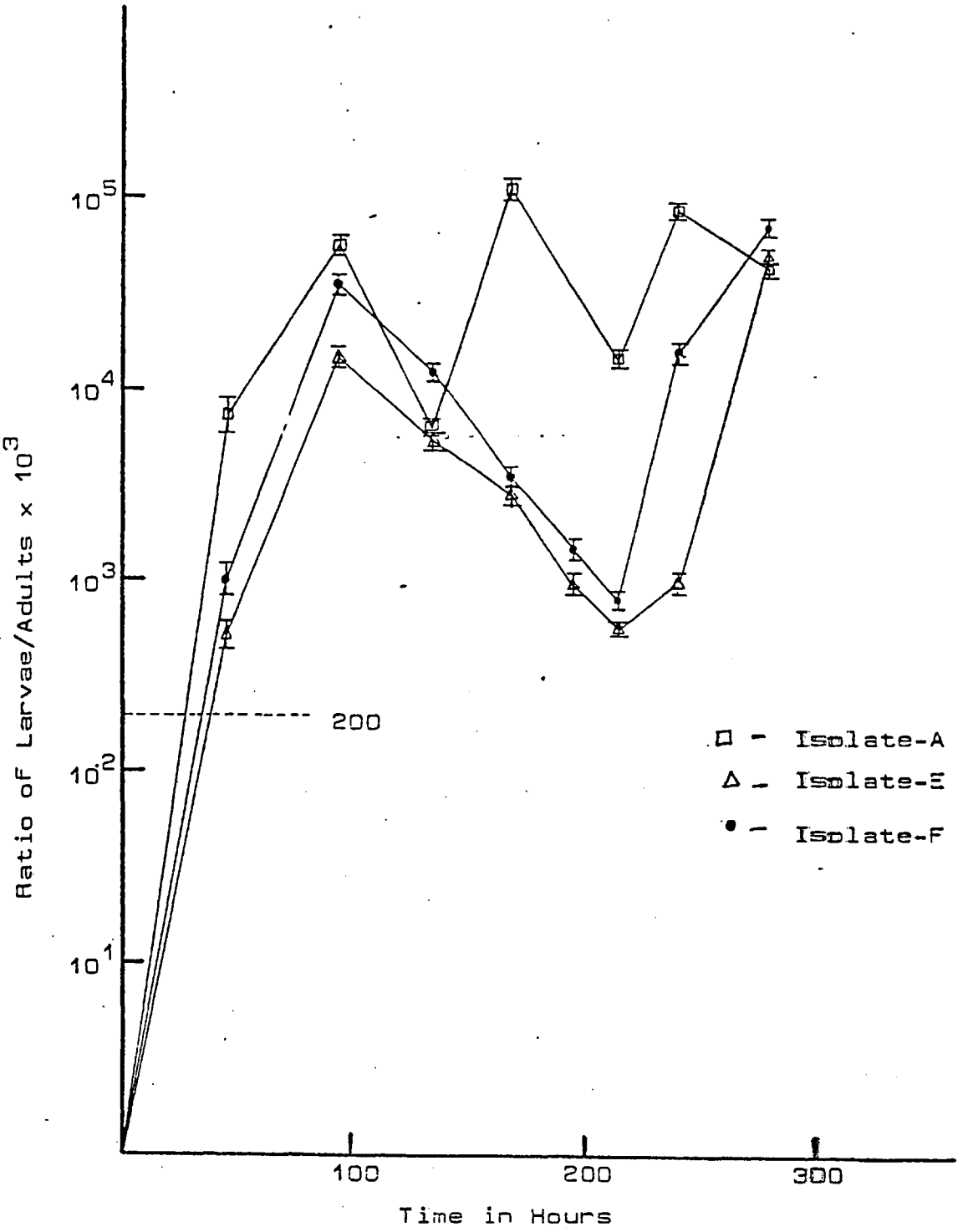
Figure: 9. Variation in the ratio of larvae to adults in the three isolates of A. avenae developing at 25°C.



The confidence intervals (α=0.05) are indicated in the figure.

Details in Appendix 3-4.4, 3-4.5 and 3-4.6

Figure: 10. Variation in the ratio of larvae to adults in the three isolates of A. avenae developing at 30°C.



The confidence intervals (α=0.05) are indicated in the figure.

Details in Appendix 3-4.7, 3-4.8 and 3.4.9

The times between successive minima, measured from the origin of the curve to the first low point and from the latter to the adjacent minimum, if any, and so forth are summarised in the following Table. 2.

Table 2

Isolate	Time (in hours) between successive minima LP_0 to LP_1 to LP_2 etc.		
	20°C	25°C	30°C
A	340	152 and 168 mean = 160 ± 11.3	135, 80, 65 mean 93.33 ± 36.85
F	280	135 and 145 mean = 160 ± 35.3	215
E	295	135 and 185 mean = 140 ± 7.07	215

At 25°C the L/A ratios of all the isolates exhibited 2 minima within the time span of the experiment. The values for isolate E and A may have been better defined if more frequent harvests were made since the points reached at H_{135} and H_{320} may not have been the lowest. The L/A ratios of isolate F were significantly greater than the ratios for isolates E and A. Only isolate-A exhibited 3 minima at 30°C, the time between successive minima decreasing with time and with increase in total numbers. At 30°C the increase in number of adults for isolates F and E was higher than for isolate-A, where the larval numbers increased almost exponentially while the adult numbers lagged behind.

The L/A ratio x 10^3 would in theory be zero till the first larvae hatched at which point the ratio would be 200. The times between production of the first egg and the appearance of the first larva for all 3 isolates at 20°C, 25°C and 30°C are given in the following Table. 3.

Table 3

Isolate — Time in hours between production of first egg (origin of plot) and appearance of 1st larvae (L/A x 10^3 = 200)

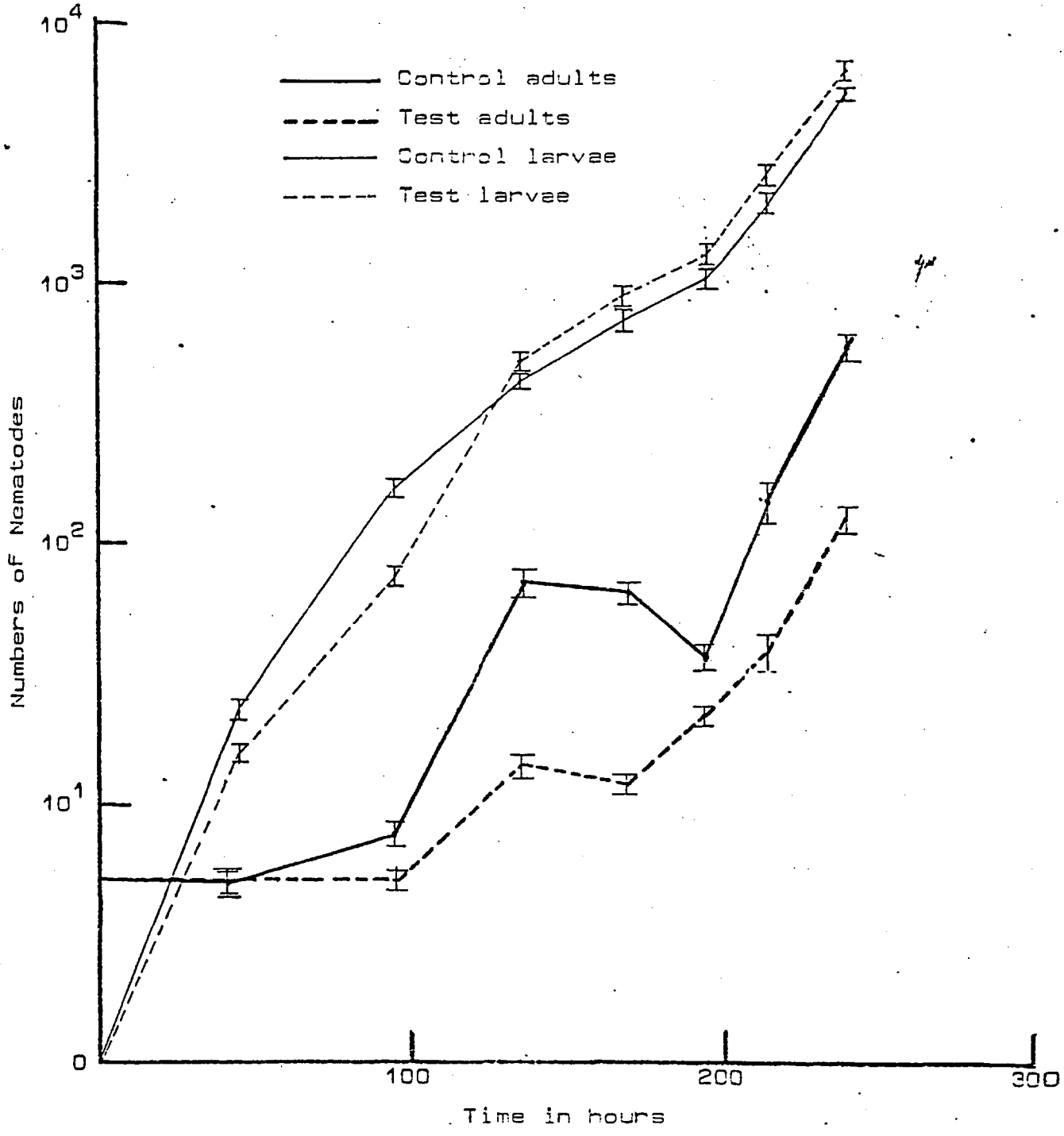
	20°C	25°C	30°C
A	76	51	27
F	69 - 70	47	38
E	70 - 71	49	34

Isolate-A showed abundant males at all temperatures. Males were observed in the parthenogenetic isolates E and F only at 30°C i.e. at H_{138} and H_{215} respectively.

3. Changes in composition of populations of 2 isolates in NaCN media

The increase in number of larvae and adults at 30°C was observed in isolates A and F growing on C. praticola in PDA (controls) and compared with the increase in numbers of larvae and adults developing on C. praticola grown in PDA incorporated with 3mM sodium cyanide. The results are shown in Fig. 11 and 12 and in Appendices 3-5.1 to 3-5.6. In both

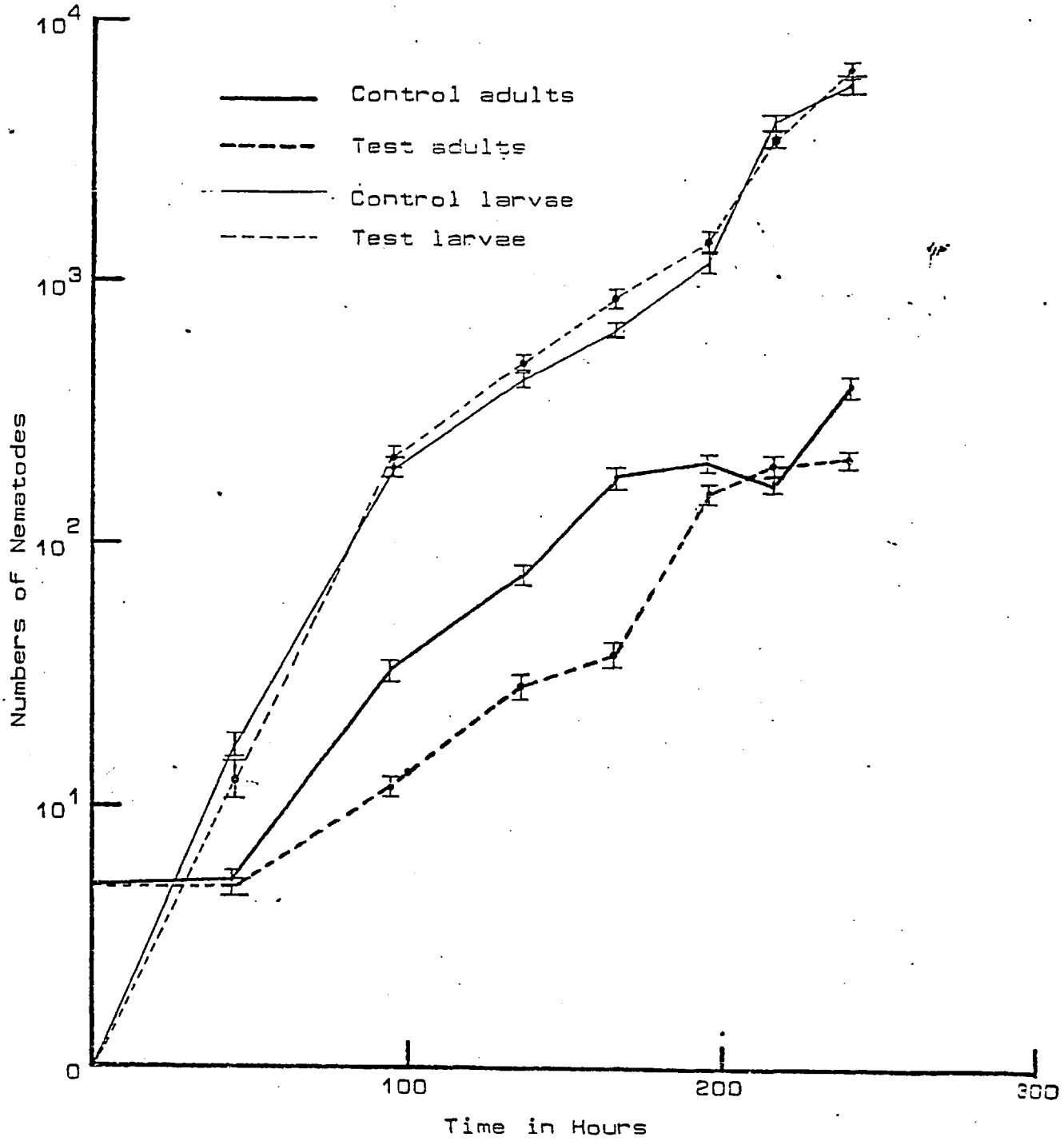
Figure: 11. The effect of 3mM NaCN incorporated agar medium on the change in adult and larval composition of isolate-A at 30°C.



The confidence intervals ($\alpha = 0.05$) are indicated in the plots

See Appendix **3-5.1** for details.

Figure: 12. The effect of 3mM NaCN incorporated agar medium on the change in adult and larval composition of isolate-F at 30°C.



The confidence intervals [$\alpha = 0.05$] are indicated in the plots. See Appendix 3-5.2 for details.

isolates the larval numbers exceeded the adult numbers before H_{45}, in the controls and the test groups. The numbers of larvae in CN-PDA were significantly higher than those observed in the controls at H_{165} and up to H_{240} in isolate-A and at H_{95} up to H_{240} in isolate-F. In both isolates the increase in numbers of adults in CN-PDA was slower than in the controls. The dip in adult numbers at H_{215} in isolate-F [Fig. 11] was due to contamination of the control replicates with an unknown fungus and is of no significance.

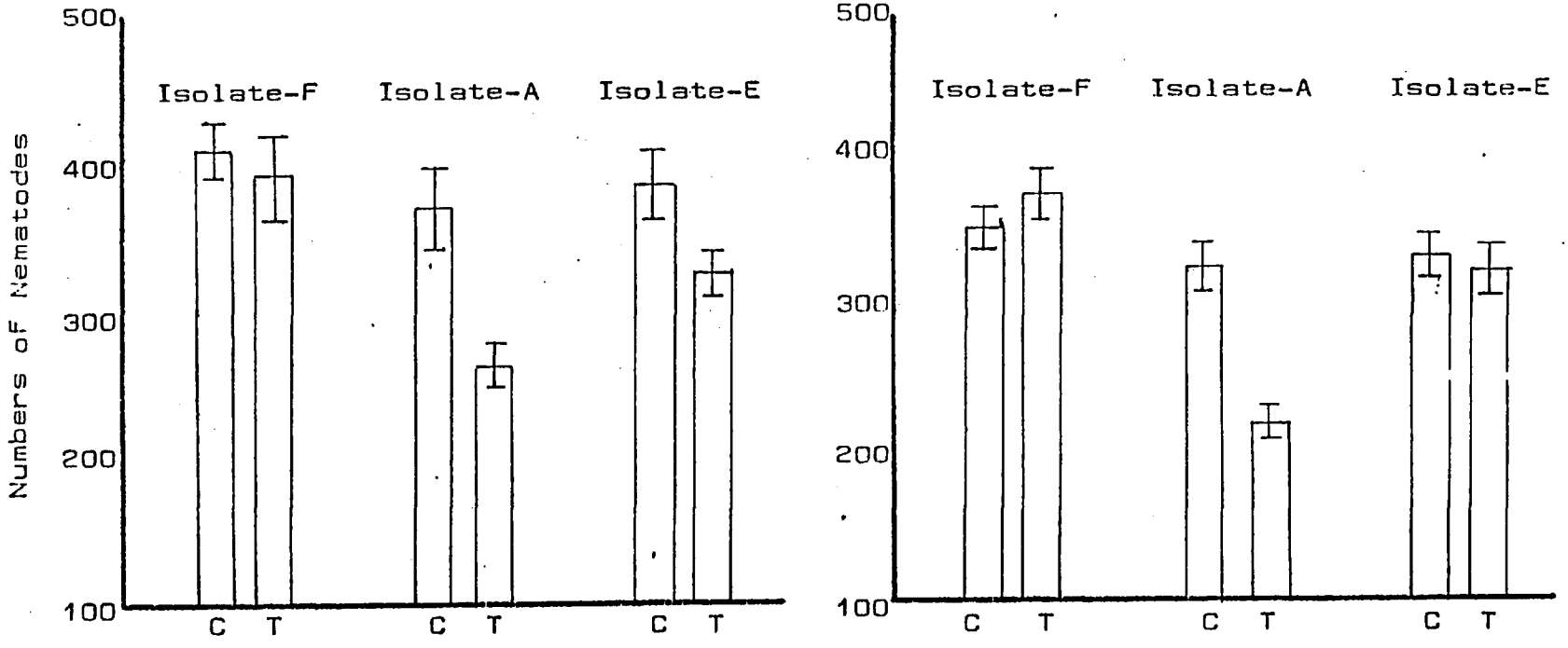
The value of 6 on the y-axis i.e. the production of the first adult in excess of those inoculated was reached by the isolate-A controls in appproximately 68 hours and in 103 hours by the test group. The corresponding figures for isolate-F were 48 hours and 57 hours. The lag-phases of the test groups are thus longer than that of the controls, the lag phases for isolate-F being shorter than those for isolate-A.

4. <u>The effect of low temperature regime on population development</u>

The young non-starving adult females from the 3 isolates subjected to 0-3°C temperature regime for 14 days developed lower densities after incubation at 30°C for 130 hours than the control females which had been kept at 25°C for the same period before incubation. [Fig.13A&B and Appendix 3-6.1].

Figure: 13A. The effect of low temperature (0-3°C.) treatment of "non-starving" young adult females from three isolates of A. avenae on the population growth subsequent to treatment.

Figure: 13B. The effect of low temperature (0-3°C.) treatment of "non-feeding" young adult females from three isolates of A. avenae on the population growth subsequent to treatment.



C - Control Group; T - Test Group.

The confidence intervals [$\alpha = 0.05$] are indicated in the figures.

Table 4a Students 't' test analysis of populations developped by "non-starving" (=feeding) vs. "starving" (non-feeding) females kept at 25°C ambient

Isolate	Feeding		Non-feeding		Significance
	3 Mean (n=6)	S.D.	3 Mean	S.D.	
F	409.0	± 16.90 (17.8)	356.0	± 13.80 (14.5)	$p < 0.001$ ***
A	370.0	± 25.69 (27.0)	352.0	± 16.32 (17.2)	$p > 0.05$ NS
E	384.83	± 20.60 (21.7)	335.0	± 15.03 (15.8)	$0.001 < p < 0.01$ **

The confidence interval ($\alpha = 0.05$, for 5 degrees of freedom two-tailed) is indicated in parenthesis.

Significant values of related 't' test (one-tailed) is indicated.

Student t-test showed that for isolate-F the differences in numbers between control and test groups were not significant but for isolates E and A the differences were significant.

When the nematodes of isolates F, A and E were subjected to the low temperature regime for the same duration, but suspended in distilled water [i.e. in a starving state where the worms had no access to an exogenous food supply] they developed populations of 356.0 $\pm$ 5.63, 352.0 $\pm$ 16.32 and 335.0 $\pm$ 15.03 while the corresponding numbers for the control groups was 378.0 $\pm$ 16.43, 221.50 $\pm$ 11.50 and 326.0 $\pm$ 17.26. The differences were significant only for isolate-A. Therefore, isolate-A showed reduced fecundity following treatment in both the starving and non-starving state. In the case of isolate-E the differences were significant only in the presence of a food supply. The fecundity of isolate-F was unaffected by low temperature in both starving and non-starving states.

5. <u>The effect of starvation at 25°C and 0-3°C on the populations developed by females of the 3 isolates</u>

The populations developed by starving females at an ambient temperature of 25°C were significantly lower than the populations developed by non-starving females kept at the same temperatures in the case of isolates F and E but not in isolate A. [Table 4a].

Table 4b Students 't' test analysis of populations developed by "non-starving" (=feeding) vs. starving (non-feeding) females kept at ambient of 0-3°C

Isolate	Feeding Mean (n=6)	S.D.	Non-Feeding Mean	S.D.	Significance	
F	394.0	± 27.71 (29.2)	378.0	± 16.43 (17.3)	$p > 0.05$	NS
A	263.17	± 15.09 (15.9)	221.50	± 11.50 (12.1)	$p < 0.001$	***
E	326.0	± 14.46 (15.2)	326.0	± 17.26 (18.2)	$p > 0.05$	NS

The confidence interval (α = 0.05, for 5 degrees of freedom two-tailed) is indicated in parenthesis.

Significant values of related 't' test (one-tailed) is indicated.

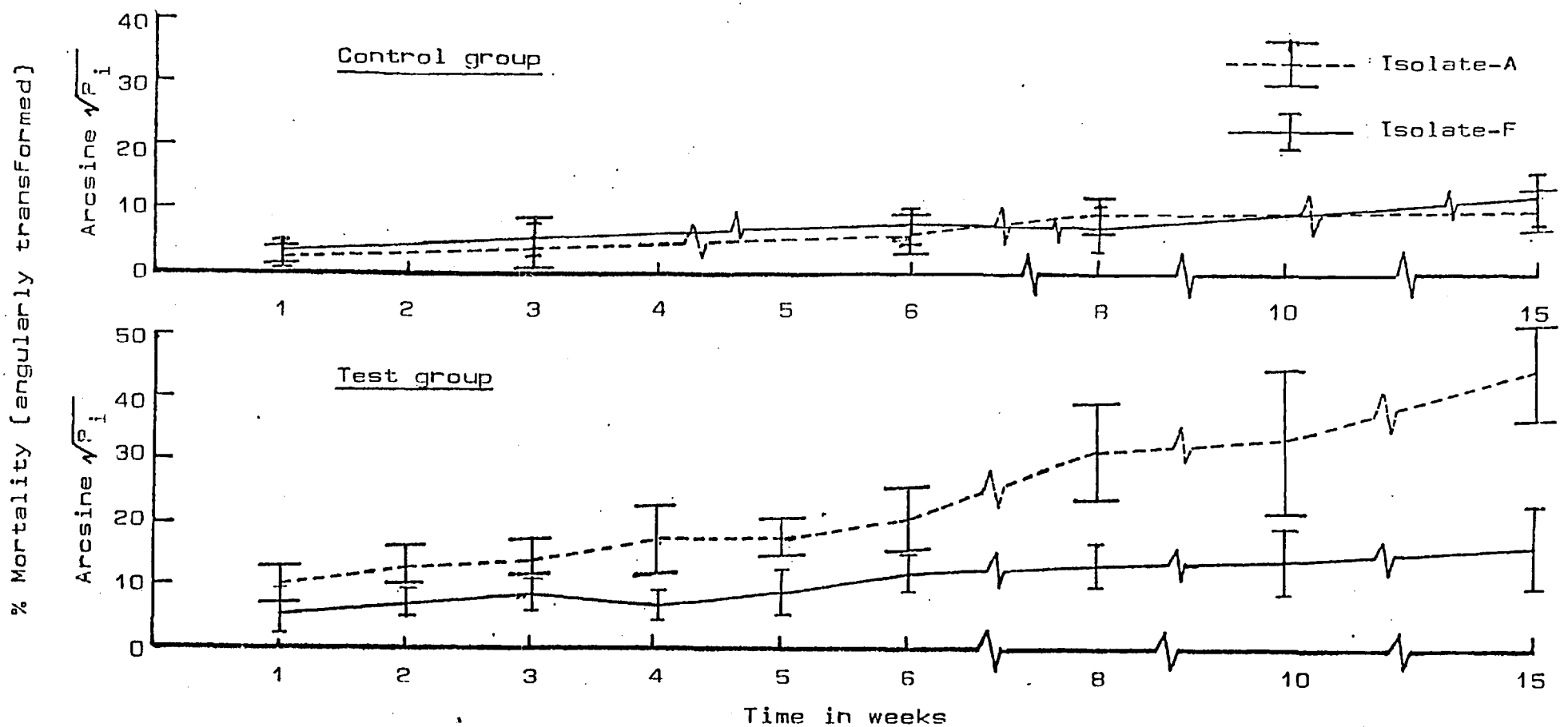
In contrast, starving females of isolate-A subjected to 0-3°C for 14 days showed population densities which were significantly lower than the populations developed by non-starving females subjected to the same temperature regime, while in isolates F and E, the corresponding results were not significantly different (Table 4b).

6. The effect of low temperature on percentage mortality of isolates A and F

Preliminary observations indicated that the nematodes of isolate-A were very susceptible to refrigeration (at temperatures between 0-3°C) while isolates E and F could be stored in suspension for periods of up to 4 weeks and revived with little or no loss of original numbers. The results of an experiment designed to test this are shown in Fig. 14 and in Appendix 3-7.1 to 3-7.3. The corresponding angularly transformed data and students 't' test analysis of the results are given in Appendices 3-7.4 to 3-7.5. Isolate-A showed a higher mean mortality when compared with isolate-F and after 24 days these results were statistically significant. The differences obtained for the controls maintained at 25°C were not significant.

The mortalities for isolate-F following 105 days at 25°C and 0-3°C were not statistically significant at the 5% level. In contrast the corresponding figures for isolate-A were highly significant.

Figure: 14. The effect of low temperature (0 - 3°C.) regime on the mortality of isolates A and F of A. avenae.



The angularly transformed data and statistical analysis are given in Appendices 3-7.1 to 3-7.5

Standard Deviation (n=6) indicated in Figure.

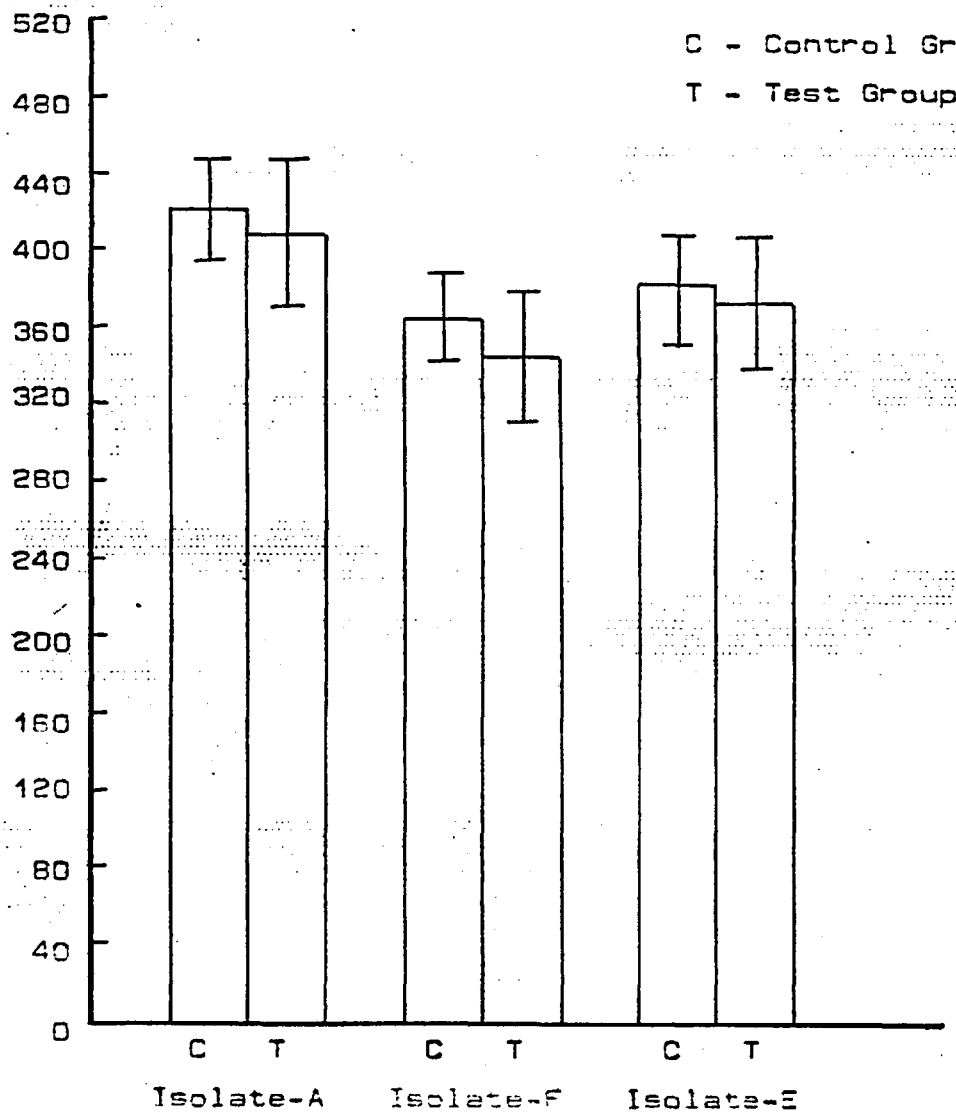
7. The effect of anaerobic treatment on population development

The mean populations developed by 5 females of each isolate after 120 hours in oxygen free N_2 at 25°C was compared with those incubated for the same period in air saturated distilled water (controls). The differences between the control and test groups were not statistically significant (Appendix 3-8.1 and Fig.15)

8. The effect of anaerobic treatment on the viability/survival/mortality of individual nematodes of the 3 isolates

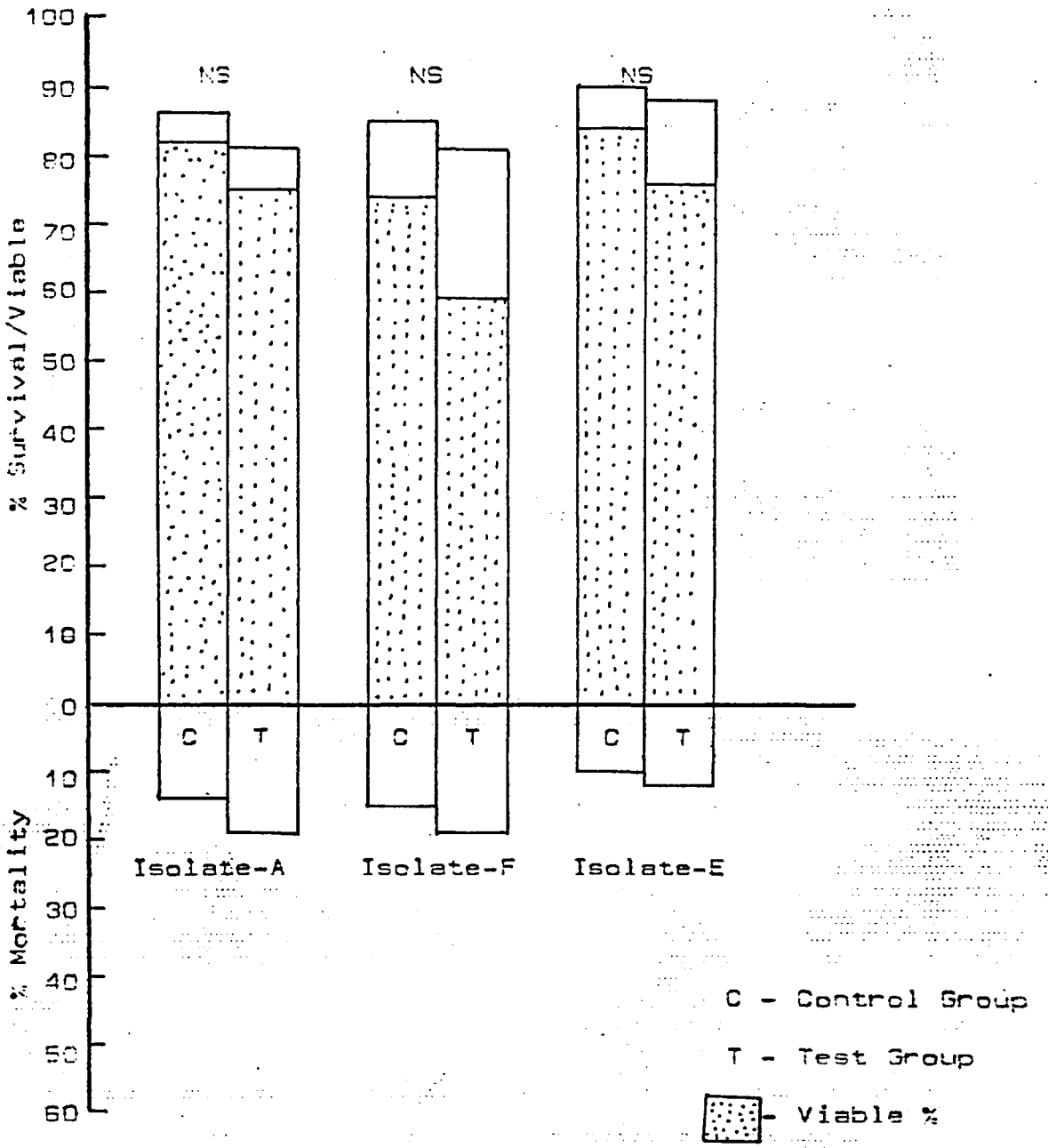
Each of 50 young adult females treated in air saturated distilled water (controls) and oxygen-free-nitrogen saturated distilled water were individually plated onto discs of C. praticola and PDA in leucocyte migration plates and assessed after 3 weeks at 25°C. No significant effects of this treatment were seen in any isolate (Fig. 16 and Appendix 3-9.1 to 3-9.3)

Figure: 15 The population growth (at 30°C) of the three isolates of A. avenae following anaerobic treatment



The confidence intervals (α = 0.05) are indicated in the histogram.

Figure: 16 The effect of anaerobic treatment on the mortality/viability/survival of individual females of the three isolates of A.avenae



NS - Not significant ($p>0.05$)

See Appendix for details. (3-9.1 to 3-9.3)

Discussion

Isolate-F multiplied fastest at all temperatures, isolate-A developed slowest while isolate-E was intermediate between them .

At 30°C however, isolate-A developed as rapidly as isolate-E at all harvest times. Unusually isolate-A produced similar numbers as those produced by isolate-F at H_{120}. These observations suggest that isolate-A, although slower to develop at ambient temperatures below 25°C has a similar capacity to the two parthenogenetic isolates for reproduction at 30°C and is probably adapted to reproduce with the greatest efficiency at temperatures above 30°C. Isolate-A results may also be influenced by time requirement to find a mate. When density gets higher, mating is quicker. Population growth at temperatures above 30°C was not studied as the agar substrate dried too quickly.

The theoretical implications of Arrhenius equation is discussed by Lyons, Kumamota and Raison [1974], but for practical purposes, the changes in E^* values observed signify the occurrence of a phase change [of unit membrane molecular components] which provides for the exclusive functioning of only one of two transition states away from the intersection temperature and is further evidence of differential response of the isolates to temperature. These transition states may represent the operation of two or more isoenzymes [free or membrane bound] which are temperature sensitive.

These data therefore indicate that certain phase changes occurred in isolates E and F at 15°C which equip them with a higher efficiency for reproduction. A similar transition occurs in isolate-A only at 20°C (5°C above that for isolates E and F) above which temperature its reproductive efficiency is far greater than below it.

These 3 isolates therefore represent distinct "thermotypes" within the species A. avenae; isolates E and F exhibit temperature optima for population growth between 15 and 25°C whereas isolate-A shows adaptation to temperatures above the temperature optima shown by isolates E and F. These differences in the reproductive rates can only be attributed to peculari-ities in the isolates probably reflecting inherent metabolic differences between isolates.

These results are similar to those observed for 4 Australian isolates by Evans (1970a) where he showed that the optimum temperature (25°C) for population growth for a Tasmanian isolate was lower than the optimum (30°C) observed for 3 Mainland isolates.

Long lag-phases were observed in the population growth curves at low temperatures. This may be due to one or more of several factors which are temperature dependant and are known to be positively correlated functions of temperature. Examples of such factors are the rate of production of eggs within individual females, the rate of oviposition, the rate of embryonic

development within the egg prior to hatch, the process of hatching itself and in the case of isolate-A, the process of gametogenesis, the behavioural processes leading up to location of mate, copulation and the amphimixis of gametes.

Evans (1970b) suggested that temperature affected all different nematode functions to different extents, citing as evidence the report of Taylor (1962) that embryonic development was fastest at 36°C. Thus Evans (1970b) concluded that population development will be regulated by the rate of the slowest process.

However Fisher (1969) has shown that adverse conditions slowed down the rate of egg-laying but not the total number of progeny produced (about 225 per female (Hechler, 1962). Goodey and Hooper (1965) found that at 20°C well fed females oviposited at the rate of 3 per hour. Their data however is not directly comparable with those in this study since they used different isolates of A. avenae. Assuming that an ambient temperature of 10°C is sub optimal, it nevertheless allows some development.

L/A ratios give useful information regarding generation times (GT) within a population as a whole. They can be counted with care as populations develop and save many individual counts being averaged out. The ratios show fluctuations which reflect change in composition of the larval and adult numbers as one generation completes its life-cycle.

Goodey and Hooper (1965) gave the generation time at 20°C as 11-12 days for their isolate of A. avenae. The generation times obtained for isolates A, F and E at 20°C in the present study were 340 hrs (= 14-16 days), 280 hrs (= 11.66 days) and 295 hrs (= 12.29 days), which agree with that of Goodey and Hooper.

Similarly the generation times at 25°C obtained in this study for isolates A, F and E were 6.67 days (= 160 ± 11.3 hours), 5.83 days (= 140 ± 7.07 hours) and 6.67 days (= 160 ± 35.3 hours). The generation times at 30°C for isolate-A was 3.88 days (= 93.33 ± 36.85 hours) and for isolates F and E 8.95 days (= 215 hours). The GT for isolate-A agrees closely with those obtained by Evans (1970b) for a South Australian isolate (about 4 days at 30°C), but is faster than Hechler's (1962) estimate of "about 6 days at 28°C".

Isolate-A showed significantly longer generation times at lower temperatures and shorter generation time (= GT) at 30°C. Furthermore the GT calculated from 4 different low points showed that it decreased with increasing density. This perhaps was because the probability of location of mates was increased at higher densities (Fig. 10) for this amphimictic population.

The point at which L/A plots exceed 200 on the vertical axis when measured on the time scale would be an estimate of the time required for the hatching of the first larva (e_t). The e_t declined with increasing

temperature for all 3 isolates. The values at 30°C correspond with 28-30 hours hatching time observed at 36°C by Taylor (1962). The short time acquired by isolate-A for hatching at 30°C probably reflects its adaptation to higher ambient temperatures and is further evidence for the presence of "thermotypes" within the species.

The incorporation of NaCN in the agar substrate seems to interfere with the ability of the larvae to moult, since large numbers of larvae were observed in such cultures. The growth of fungus was not physically affected. NaCN was effective in reducing the adult numbers of isolates A and F with respect to the controls. This suggests that NaCN probably interferes with biological processes even with isolate-A whose oxygen consumption rate (QO_2) was insensitive to NaCN [See Chapter 5].

Isolate-F tolerated a temperature regime of 0-3°C for 14 days with or without food with no apparent effect on its reproduction and appears better adapted to survive low ambient temperatures than isolates E and A whose reproduction was depressed after exposure to identical low temperature regimes.

Isolate-E in contrast to isolate-A survived near freezing temperatures better in the starving state than in the non-starving state, since starved females of this isolate multiplied as well as controls starved at 25°C. This may have been due to the quicker induction

of cryptobiosis in response to lack of food and low temperature. On the other hand metabolic differences in neutral lipid mobilization among the isolates in response to stress may also be involved.

However the ability of isolate-F to survive low temperatures for extended periods suggests that the survival of individuals of this isolate is either due to tolerance (passive survival) or possibly altered metabolism (active survival). In this respect Barrett (1968) reported that animals which live at higher temperatures have lipids with a higher melting-point than animals which live at low temperature and that the melting-point of the lipids could be raised by increasing the degree of saturation and/or increasing the chain length of the fatty acids. 'Cold-hardiness' of a species or a strain within a species may therefore be an inherent property of the species or strain which is determined genetically. Thus it may be interesting to analyse the lipid constituents of these isolates. The isolates of A. avenae studied here, especially isolate-F which is probably tolerant of low temperatures, may prove to be different in their lipid composition.

The observations on the mortality of isolates A and F following extended periods at low temperatures confirmed observations from other experiments as to the poor capacity of isolate-A to survive such regimes. The mortality of isolate-A was significantly higher than

that of isolate-F after 21 days at 0-3°C. This is further evidence that isolate-A is a distinct thermotype of A. avenae differing markedly in the ability to survive low temperatures.

The three isolates tested here were homogenous in so far as tolerance to anaerobic conditions were concerned. Following 120 hours of anoxia, females of all 3 isolates reproduced with equal vigour upon return to favourable conditions since the numbers developed by treated nematodes although lower, were not significantly different from those produced by controls kept in air saturated media. Cooper and Van Gundy (1970a) working with a Californian isolate of A. avenae reported that oxygen stress for longer than 120 hours produced a state of cryptobiosis from which 90-95% of the individuals recovered after 90 days. However no information was reported on the reproductive capacity of A. avenae allowed to recover after being subjected to 120 hr of anoxia. In discussing their observations, Cooper and Van Gundy (1970a) suggested that the ability of many soil inhabiting nematodes to survive oxygen-stress for extended periods indicates that their survival is due either to tolerance or altered metabolism.

Observations in Chapter 4 suggested that all 3 isolates behaved as regulators since the ambient oxygen-tension did not seem to affect the oxygen consumption of the three isolates. This observation

and those reported in this Chapter indicate that in these three isolates of A. avenae there is potential for altered metabolism in response to oxygen-stress especially because the survival/viability and mortality of the individuals of the three isolates were not affected to any significant extent by anoxic periods for up to 120 hours.

CHAPTER 4

The effect of physical factors on the respiratory physiology of three isolates of *A. avenae*

Introduction

In studying intraspecific variation especially among a species with wide host and/or geographical distribution, protein and isoenzyme mapping are useful but subject to limitations. To clarify the magnitude of physiological differences, information of a wider range needs to be considered. Comprehending this need, various workers, notably de Connick [1962] and Evans [1970a,b] expressed the value of experimental approach in the elucidation of interspecific and more importantly, intraspecific variation.

The degree of intraspecific variability is likely to be most pronounced in species which possess the capacity to colonize a wide range of ecological and environmental habitats. In this respect, *A. avenae* with its cosmopolitan distribution and wide host range would be an ideal species for such an assessment. Besides the extreme morphological variation [Goodey and Hooper, 1965; Evans and Fisher, 1970] gel-electrophoresis patterns, even between morphologically similar populations, differed significantly for geographically isolated strains of *A. avenae* [Evans, 1971]. Also in this respect the data on the oxygen consumption (QO_2) of *A. avenae* differs considerably according to author. Cooper and Van Gundy [1969] quote a figure of 5.6 - 5.8 µl/mg dry wt./hour at 28°C; Marks [1971] 10-12 µl/mg dry wt./hour at 30°C

varying between 7.42 µl/mg dry wt./hour minimum to 12.85 µl/mg dry wt./hour maximum; Awan [1975] obtained a mean rate between 4.64 - 4.89 µl/mg dry wt./hour at 25°C [range 3.5 to 6.5 µl/mg dry wt./hour].

However these workers did not report the age of the cultures from which these populations were derived. Earlier studies indicated that the QO_2 was lowered as the age of the mass culture [i.e. harvest time] increased. Therefore a standard method was used for the harvesting and extraction of nematodes from mass cultures of the three isolates to assess whether the QO_2 varied with harvest time.

The increase of the QO_2 with temperature is well documented in literature for many organisms, and parasites respond similarly to free living invertebrates [Von Brand, 1979]. The QO_2 of most living systems increases with increasing temperature until a maximum is reached beyond which it declines rapidly ("reversal point" of Von Brand, 1979] because subcellular components of the organisms are damaged. The temperature allowing maximal QO_2 is variable and depends on the "living conditions" of the parasite [Von Brand, 1979]. The "reversal points" of the QO_2 have been reported to be higher in larval nematodes, the adult stages of which inhabit warm blooded hosts. The QO_2 of Cooperia punctata increases between 15 and 40°C [Eckert, 1967] and that of Strongyloides ratti between 10 and 37°C [Barrett, 1969b]. For Panagrellus redivivus the plot

of log QO_2 vs. temperature resulted in two lines which intersected at 20°C. The increase in QO_2 per increment of temperature was greater below 20°C than above and this temperature corresponded to the maximum frequency of wave propogation and change in the respiratory quotient (Santmyer,1956). He stated that "metabolic energy consumed in motion reaches a saturation level at 20-25°C and metabolic energy produced at higher temperatures becomes increasingly inefficient."

The temperature relationships can be expressed in various ways. The Q_{10} was used in earlier literature and was calculated according to the formula:

$$Q_{10} = \left[\frac{K_2}{K_1}\right]^{\frac{10}{t_2-t_1}} \qquad K_1 = QO_2 \text{ at } t_1 \qquad K_2 = QO_2 \text{ at } t_2$$

and it may be calculated from any two temperature intervals.

More recently the formula originally derived by Svante Arrhenius (1889) has been employed to express the exponential increase of reaction rates with temperature according to the formula:

$$K_2 = K_1 \times e^{\frac{E}{2}\left[\frac{1}{T_1} - \frac{1}{T_2}\right]}$$

$$\log K = \frac{-E}{2.303\,R}\,\frac{1}{T}$$

K - Rate of reaction
R - Gas constant
E - Arrhenius activation energy

The expectation for biological systems from Arrhenius theory is a linear relationship from freezing at or below

0°C to denaturation at or above 40°C (Lyons, Raison & Kumamoto, 1974). However plots of log QO_2 vs. the reciprocal of the absolute temperature for most organisms, parasitic or free living, rarely yields a straight line but more often two "bisecting lines" result (Von Brand, 1979) in contrast to the "reversal point" mentioned earlier. In earlier literature such points of intersection were assumed to represent the transition of one "master reaction" to another one.

The biological significance of these observations has been realised (Lyons, Raison & Kumamoto, 1974; Lyons & Raison, 1970). Lyons et al. (1974) refer to these 'bisections' as discontinuities (non-intersecting discontinuities and intersecting discontinuities have been described) and the points of intersection i.e. the points of inflection of the two limbs, correspond to cellular and/or subcellular membrane phase transitions occurring within an organism with response to temperature. The method is based on the observation that the respiratory enzymes of mitochondria undergo a change in conformation at the same temperature at which associated membranes undergo a phase transition. Investigations have shown 4 such bisecting lines for Ascaris lumbricoides (Kruger, 1940a). Replotting of QO_2 data for a variety of parasites, (Strigomonas fasciculata, Trypanosoma cruzi, Ancylostoma caninum and Eustrongylides ignotus) Von Brand, (1979) found that intersection points invariably fell within 33 and 34 units on the temperature axis (units not given) and

except for A. caninum the E values at lower temperature were less than E values for higher temperatures.

Evans (1970a,b) found that temperature appeared to affect the processes of A. avenae (South Australian isolate) such as feeding and egg laying and suggested that this could be a reflection of the effect of temperature on the physiology of A. avenae. Therefore, investigations were carried out on the whole-worms of 3 isolates of A. avenae to verify the effect of ambient temperature on the rates of oxygen consumption.

Among aerobic organisms there are, broadly, two types of response to varying oxygen tensions. "Conformers" have QO_2 dependent on the external oxygen tension, and the critical point at which their respiration begins to decline is high. Parasitic helminths and free living animals such as actinians are represented in this group. The QO_2 of organisms belonging to the second group shows no such dependence on the external oxygen tension, and the QO_2 remains unchanged over a wide range of tensions, that is, the critical point at which their QO_2 begins to decline is low. This group, referred to as the "regulators" (Bryant, Nicholas and Jantunen, 1967) comprises those organisms with organisations facilitating oxygen uptake, and/or oxygen distribution within the body (Von Brand, 1979). The mechanisms involved may vary. Small animals may have surface/mass ratios so favourable to diffusion so as to maintain maximal oxygen influx even at low oxygen tensions.

To the latter group belong parasitic protozoa and some small helminths such as the larvae of Trichinella spiralis, the eggs of Diphyllobothrium latum, eggs of Ascaris lumbricoides.

However Von Brand (1979) suggested that the division discussed above is not absolute as transitional cases do occur. Nevertheless Bryant et al. (1967) made a further generalisation that "regulators" possess some well defined respiratory pigments whose properties in terms of oxygen affinity/loading are similar to those of the organism as a whole and cited haemocyanins, haemoglobin and cytochromes as examples of such pigments and described Caenorhabditis briggsae as a regulator.

Cooper and Van Gundy (1970) found that the exposure of A. avenae to pure nitrogen (N_2) for periods longer than 8 hrs necessitated progressively longer times before initial oxygen consumption was resumed; after 14 hrs of exposure to pure N_2, unstarved A. avenae (< 16 hrs out of culture) and Caenorhabditis sp. both exhibited a small post-anaerobic oxygen debt. However 16-20 hrs non-feeding A. avenae (or Caenorhabditis sp.) exhibited no post-anaerobic rise in the QO_2 (Cooper et al., 1970). They also observed that although 80 hrs of anaerobiosis killed Caenorhabditis sp., A. avenae endured identical treatment for 15 days without decreased viability. A. avenae was suggested to have entered a state of cryptobiosis after 120 hrs in pure nitrogen but the normal QO_2 of such A. avenae was resumed within

24 hrs of exposure to air. Therefore polarographic assays of the behaviour of the QO_2 of 3 isolates of A. avenae were conducted following exposure to anaerobic and hyperbaric oxygen regimes to assess whether the isolates differed in their response to variation in the oxygen tension of the external medium.

Materials and Methods

1. Effect of harvest time on the QO_2 of 3 isolates of A. avenae

Mixed populations from 3 isolates of A. avenae grown under identical conditions in a 25° C.T. room were harvested at 40, 50, 60, 70, 80, 100 and 140 days from mass cultures in Kilner jars. Active nematodes were extracted at each harvest and made into suspensions containing approximately 20 mg wet wt. of nematodes per ml of sterile distilled water [see General Materials and Methods].

The oxygen electrode was calibrated with water which had been equilibrated for 24 hours at 30°C and the nematode suspension was assayed as described in the General Materials and Methods.

Following assay, the dry-weight of the nematode sample was obtained as described in General Materials and Methods.

2. The effect of temperature on the QO_2 of the 3 isolates of A. avenae

Mixed populations of A. avenae from the 3 isolates were harvested at 42 days from Kilner jar monoxenic cultures grown synchronously under identical conditions at 25°C. The harvested nematodes were washed, extracted and made into standard suspensions of 20 mg wet wt./ml as described in General Materials and Methods.

Suspensions of the 3 isolates were assayed at 15°, 20°, 25°, 30° and 35°C, after equilibration at the test temperature in the electrode vessel i.e. without pre-incubation. The O_2-electrode was calibrated as described in General Materials and Methods. The assays were replicated six times at each temperature for each isolate and 3 independant batches of worms from the 3 isolates were used. Dry weights were obtained as described in General Materials and Methods. Calibration was checked frequently for each temperature above 20°C and at temperatures lower than 20°C the calibration was checked between each run as the assays required a longer time.

3. The effect of hyperbaric O_2 and N_2 on the QO_2 of the 3 isolates of A. avenae

Suspensions of A. avenae containing 20 mg wet wt./ml were prepared as described earlier. (See General Materials and Methods). 3 ml of suspension were dispensed into each of 6 sterile Quick-Fit tubes. The tubes were closed with air-tight rubber bungs which had ports through which air from an aquarium pump was passed through an in-line Whatman Grade 10 mini-disposable (bacterial) filter for 15 mins. Each of the six samples was then assayed in the polarograph to obtain the zero time QO_2. After assay each 3 ml sample was withdrawn with a Pasteur pipette and re-introduced into the tubes to which the rubber bungs

were refitted and air bubbled in continuously for 12 hours while being shaken at 80 oscillations per minute, in a water bath at 25°C. The above method was repeated twice using O_2-free-N_2 and pure O_2. The samples were assayed again after 12 hours exposure to air, O_2-free-N_2 and pure O_2 respectively in order to obtain the QO_2 of the nematodes following anaerobic and hyperbaric oxygen treatments. The experiment was repeated twice for each isolate of A. avenae from different batches of mass cultures.

Results

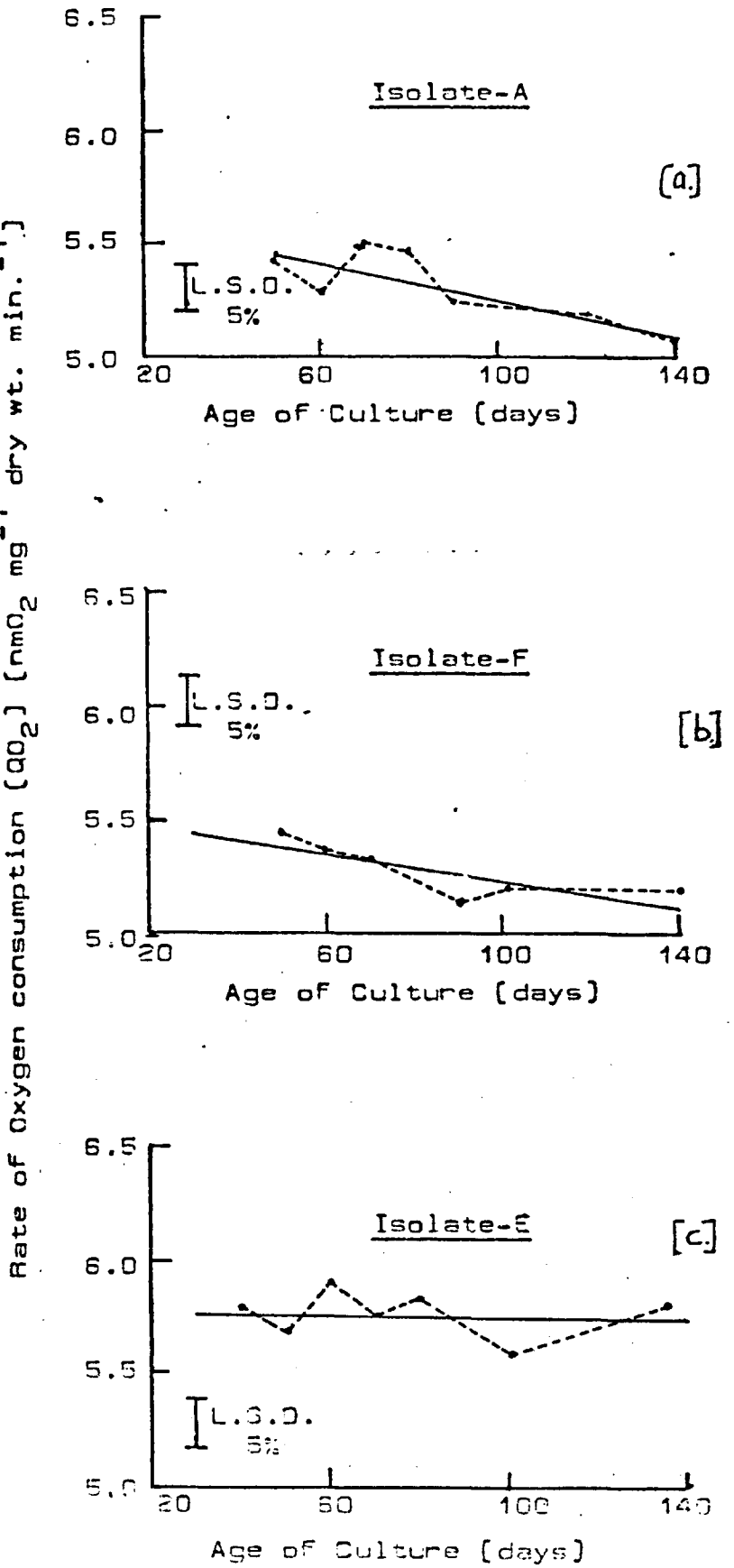
1. The effect of harvest time on the QO_2

The three isolates of A. avenae showed similar QO_2 figures at 50 days harvest [H_{50}] from mass cultures (Fig. 17 and Appendices 4-1.1 to 4-1.3). Isolate-F exhibited a gradual drop in the QO_2 between H_{50} and H_{90} which was almost linear. This was confirmed by the linear regression which showed a negative gradient. Analysis of variance showed that this drop in rate with time was significant [$F = 7.9$, $p < 0.001$].

QO_2 of isolate-A fluctuated between 60 and 90 days harvest (Fig. 17(a) and Appendix 4-1.3) but these values were not significant, although the F-value obtained from the analysis of variance showed that the decline of QO_2 with harvest time was a significant trend, [$p < 0.001$] confirming again the linear regression [$r = 0.79$] and the negative gradient [$m = - 0.003$] obtained for this isolate.

Although isolate-E (Figure 17(c) and Appendix 4-1.1) exhibited the least gradient for the regression QO_2 vs. harvest time, the slope was still a negative one [$m = - 0.0005$] with a regression coefficient approaching 0.15, the lowest for the 3 isolates. [$r = - 0.168$ when compared to -0.790 and -.796 for isolates A and F]. Analysis of variance showed that the decline was significant [$p < 0.01$]. However, in

Figure: 17. The effect of harvest-time (= age of culture) on the QO_2 of three isolates of A. avenae.



none of the isolates was the drop in rates between H_{40} and H_{70} significant ($p < 0.05$).

2. Effect of temperature on the QO_2

The QO_2 of all 3 isolates increased linearly with temperature (Fig. 18 and Appendices 4-2.1 to 4-2.3). Linear regressions of QO_2 on time for isolate-A showed the highest gradient m = + 0.2732 nm O_2/mg dry wt./min/ ^{o}C]; isolate-E was intermediate in magnitude [m = + 0.2104]; and isolate-F showed the lowest response to increasing temperature [m = + 0.2056].

However, Arrhenius plots for isolate-E and F showed points of inflexion in the region of 25^{o}C and isolate-A exhibited a similar inflexion point well below 20^{o}C [Fig. 19] i.e. 341.2 x 10^{-5} on the 1/^{o}K axis. The latter plot remained linear between 20^{o}C and 35^{o}C although rate of increase of QO_2 per unit rise in temperature was lower at temperatures above 20^{o}C than below. In the case of isolates E and F the corresponding values were lower at temperatures above 25^{o}C than below.

The Arrhenius plots of the QO_2 data of all three isolates did not exhibit the expected linear relationship. Instead, distinct inflexion points of the type described as "intersecting-discontinuities" [Lyons et al., 1973] were observed [Fig. 19]. These inflexion points were at the junction of two slopes of varying gradient and represent dual activation energy states. Thus two Arrhenius activation energy [E^{*}] values i.e. one value

Figure: 18 The effect of temperature on the QO_2 of three isolates of A. avenae of identical culture age

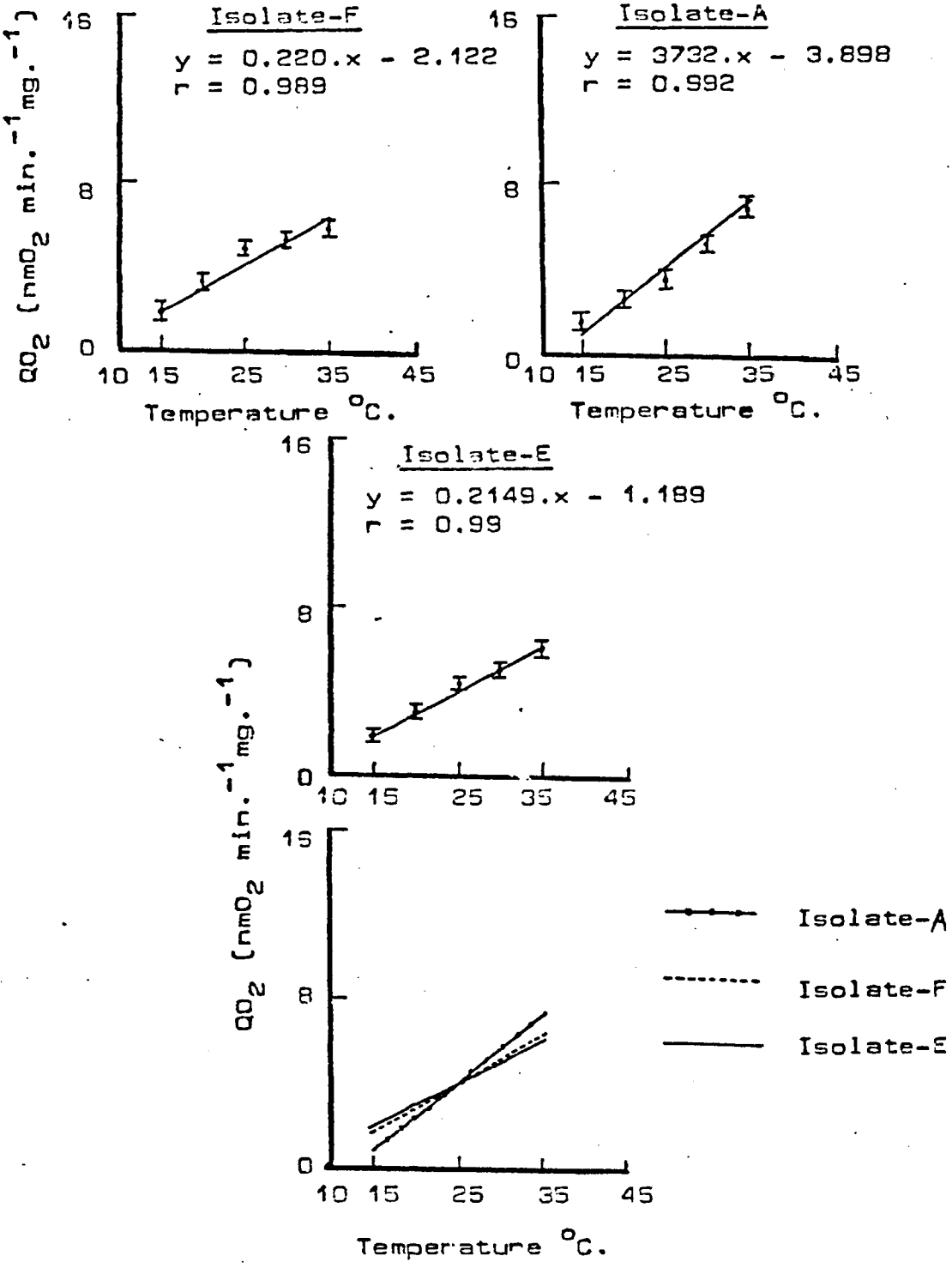
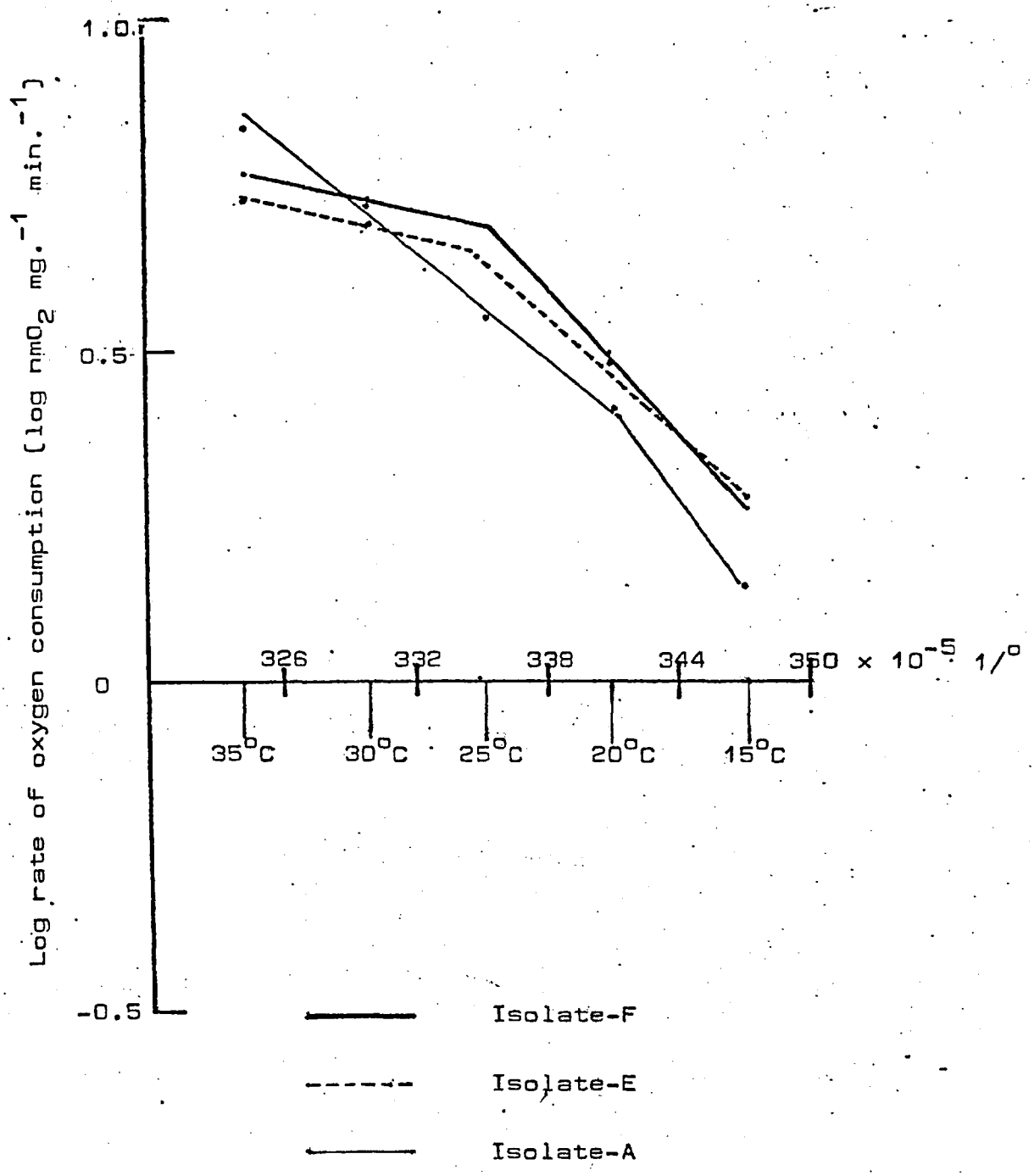


Figure: 19. Arrhenius Plots of the rate of oxygen consumption of the three isolates of A. avenae.



for each slope was calculated for each isolate and these values are given in the following table (Table 5).

Table 5 Activation energy as computed from the Arrhenius plots of oxygen consumption rates for the 3-isolates [Units for E^*:- cals. $mole^{-1}$]

Isolate	E_1	Transition Point	E_2
A	$E^*_{15\text{-}20^\circ C}$ 2.100×10^4	below 20°C	$E^*_{20,25,30,35^\circ C}$ 12.465×10^3
E	$E^*_{15,20,25^\circ C}$ 1.387×10^4	$\geqslant 25^\circ C$	$E^*_{25,30,35^\circ C}$ 3.375×10^3
F	$E^*_{15,20,25^\circ C}$ 1.650×10^4	$\geqslant 25^\circ C$	$E^*_{25,30,35^\circ C}$ 3.385×10^3

A transition from a higher activation energy to a lower one with temperature increase occurred in isolate-A; whereas the transition occurs at a higher temperature for the 2 parthenogenetic isolates. Isolate-A had the highest activation energy of all the 3 isolates at temperature below 15°C and the highest E^* above 25°C.

At 25°C the log QO_2 for isolate-A was lower than the log QO_2 of the other two isolates. But at 35°C, isolate-A had the highest log QO_2 and isolate-E had the lowest log QO_2. At 30°C all 3 isolates had approximately similar log QO_2 values. In fact this was the point at which the Arrhenius plots for the 3 isolates intersected one another, the log QO_2 of isolate E, A and F being 0.69, 0.73 and 0.74 on the vertical axis.

Isolate-A had the greatest difference between log QO_2 values observed among the 3 isolates with isolate E showing least differences.

3. Effect of hyperbaric N_2 on the QO_2

Following 24 hours in total anaerobic medium the QO_2 of all 3 isolates declined but only in isolate-F were these differences significant. [Fig. 20 and Appendix 4-3.1] The QO_2 of isolate-F declined from a zero-time rate of 5.31 $\pm$ 0.22 to 4.52 $\pm$ 0.30 nm O_2 mg^{-1} min^{-1}. This was the largest decrease in the QO_2 [$p < 0.05$] among the 3 isolates subsequent to anaerobic treatment at 25°C. The QO_2 of isolate-A declined [$p > 0.05$] from 5.40 $\pm$ 0.18 to 5.18 $\pm$ 0.31; QO_2 of isolate-E declined from 5.20 $\pm$ 0.20 to 4.78 $\pm$ 0.28 [$p > 0.05$]. In contrast the QO_2 of control groups for isolates E and F incubated for 12 hours in air saturated sterile distilled water increased whereas isolate-A decreased, but none of these changes were statistically significant [$p > 0.05$].

4. The effect of hyperbaric O_2 on the QO_2

Following 12 hours exposure in oxygen saturated water at 25°C, the QO_2 of isolates A and F increased little but not significantly [Fig. 21 and Appendix 4-4.1]. Control groups of isolates F and E increased slightly but not significantly while that of isolate-A decreased.

Figure: 20 (Facing page)

A - QO_2 of test group at zero time.

B - QO_2 of test group following treatment in 100% N_2 for 24 hours.

C - QO_2 of control group following 24 hours in air-saturated medium.

D - QO_2 of control group at zero time.

Standard deviation indicated in figure.

Details given in Appendix 4-3.1

Figure: 20. The effect of 24 hour anaerobic (100% N_2) treatment (at 25°C) on the QO_2 of three isolates of A. avenae.

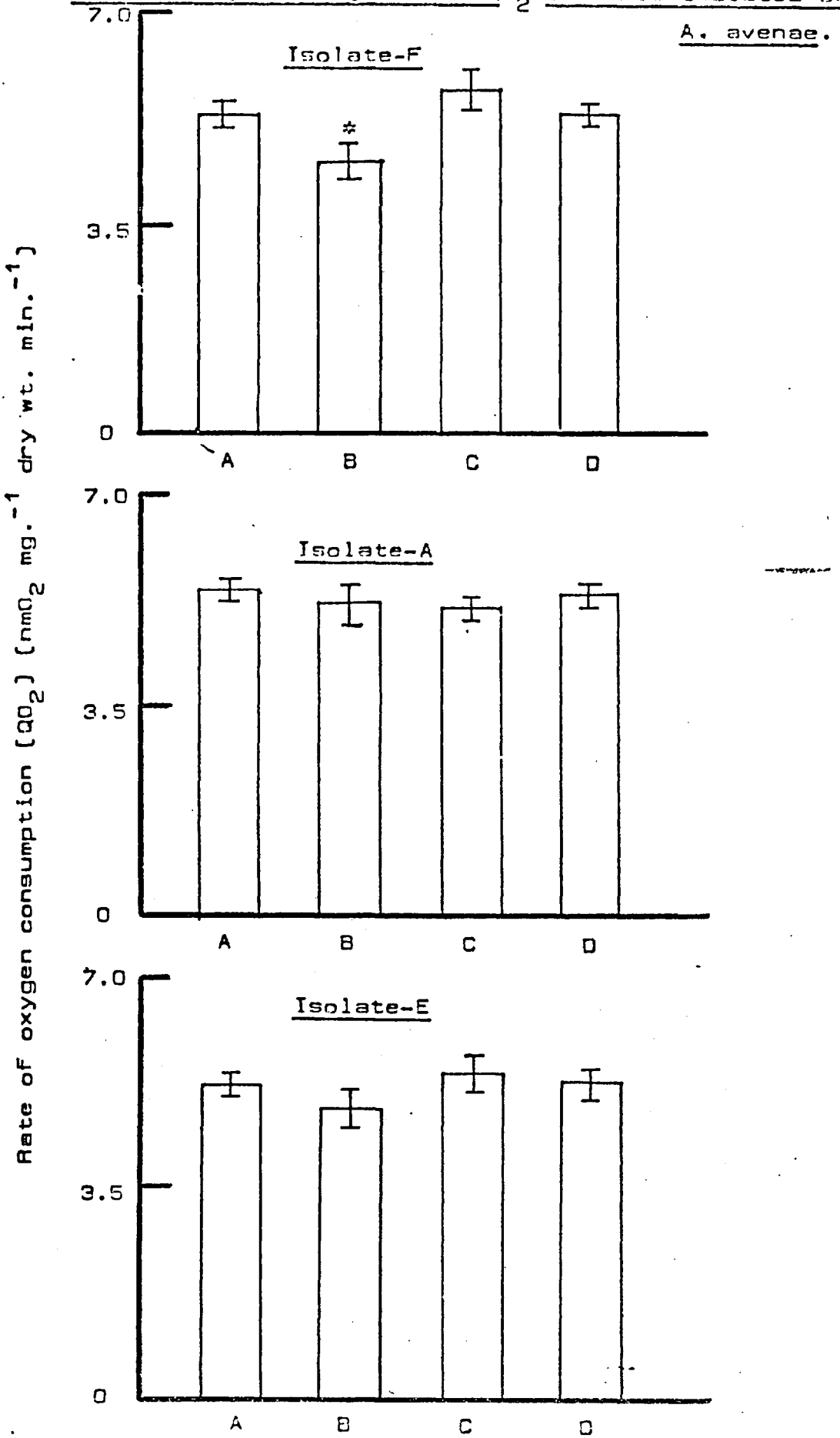


Figure: 21 (facing page)

A - QO_2 of test group at zero time.

B - QO_2 of test group following hyperbaric O_2 treatment for 24 hours.

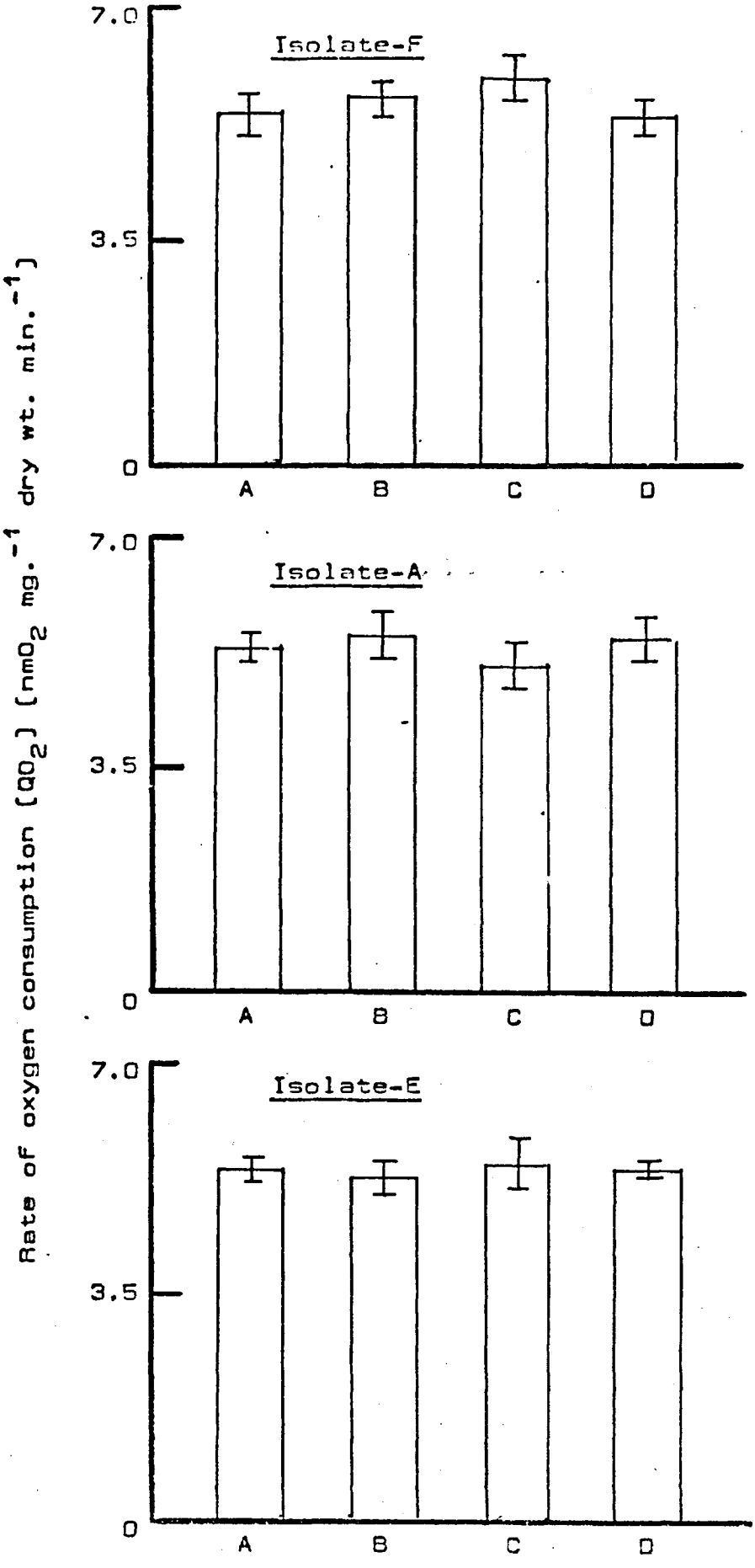
C - QO_2 of control group following 24 hour treatment in air-saturated medium.

D - QO_2 of control group at zero time.

Standard deviation indicated in figure.

Details given in Appendix 4-4.1

Figure: 21. The effect of hyperbaric O_2 on the CO_2 of three isolates of A avenae (incubated for 24 hours at 25°C.).



Discussion

The oxygen consumption rates (QO_2) of the 3 isolates of *A. avenae* (extracted subsequent to different periods in monoxenic culture with *Corticium praticola*) were maximal at 30-60 days and declined very slowly but significantly with later times of harvest. Isolate-A showed the steepest gradient for the regressions when compared with isolates E and F. Nematodes from older cultures appeared smaller and the body contents were less dense compared to young cultures. Exhaustion of the food supply is the likely cause.

Evans (1970b) reported a progressive decrease in average length as population density increased. Whether the change in morphometry is related to the observed decline is debatable but a correlation may exist.

It therefore appears that the age of the culture may give some information relating to the food supply in the culture and indirectly the physiological condition of the nematodes which is also likely to be reflected in QO_2. Thus differences in age of culture might account for variations in QO_2 from Cooper (1968), Cooper and Van Gundy (1970), and Awan (1975) whereas the figures similar to those from Marks *et al.* (1971) were observed with only isolate-F investigated in this study and only in suspensions incubated at 30°C for 24 hours. (See Chapter V).

Phase transitions in biological membranes in response to temperature have been determined by a variety of techniques and assays, including X-ray diffraction, differential calorimetry, enzymatic activity and electron spin resonance spectroscopy. Polarographic measurement of mitochondrial oxidation has also been successfully used to indirectly determine the appearance of membrane phase changes in response to temperature. The method is based on the observation that membrane bound respiratory enzymes in organisms undergo conformation change at the same temperature at which activation energy changes are observed.

Von Brand [1979]

emphasises that these observations do not explain the nature of the temperature dependancy of an organism but are descriptive.

When investigating the change in QO_2 with temperature the nematode samples were not incubated for long periods at the test temperature prior to assay. Barrett [1969b] found that the temperature induced maximal respiration of Strongyloides ratti larvae was sustained only for a short time, the rate declining at the same temperature on prolonged incubation. The effect of prolonged incubation of the isolates at high temperatures will be reported in Chapter V. Therefore, at this stage it was thought that a sudden and rapid change to the test temperature would give a better indication of any changes brought on by purely thermal effects as

opposed to chemically induced effects that could occur due to re-assimilation of excreted metabolites [e.g. ethanol] known to occur in A. avenae [Cooper and Van Gundy, 1971].

When the Arrhenius plots for QO_2 obtained for the 3 isolates were compared, it was clear that the two parthenogetic isolates [i.e. isolates E and F] showed "intersecting-type discontinuities" around 25°C; the gradient above 25°C was less than the gradient below the point of inflextion. Besides indicating transitions in the Arrhenius activation energy these intersections suggest that these 2 isolates were adapted to temperatures of 25°C or below with QO_2 optima around 25°C. Isolate-A on the other hand showed an "intersecting-type discontinuity" below 20°C but the E^* value below 20°C was greater than the E^* value above 20°C [Table 5], which indicates that this isolate has possibly adapted to relatively higher ambient temperatures than the two parthenogenetic isolates. These changes in the magnitude of E^* are significant since they can be explained by the occurrence of a phase-change which provides for the exclusive functioning of only one of the two transition states at any temperature away from the intersection temperature. The high ratio of males in isolate-A may be related to higher temperatures to which this isolate is apparently adapted. Population development rates observed for the 3 isolates [Chapter 3] agree closely with the QO_2 data reported here.

There was no statistically significant decline in the QO_2 of isolates A and E following exposure to pure N_2 for a period of 12 hours at 25°C. But isolate-F showed a significant ($p < 0.05$) decrease in the QO_2 following similar treatment. The behaviour of the latter isolate agrees with the observations by Cooper and Van Gundy (1970) although the drop in the QO_2 of isolates A and E only indicated a trend in these two isolates ($p > 0.05$).

Cooper et al. (1970) used standard Warburg techniques and found that A. avenae exposed to pure N_2 longer than 8 hours required longer times to regain the original QO_2, but even after 16 hours they recovered within 0.5 hours. In my studies suspensions of A.avenae were transferred directly to the polarograph-chamber in the same bathing solution after treatment and the QO_2 was assessed after 15 minutes of equilibration to the assay temperature of 30°C. The method used by Cooper et al. (1971) involved removal of $^2/_3$ of the post-exposure bathing solution and dilution with 1 ml sterile water and 1 ml anti-bacterial/anti-fungal mixture. This latter procedure may have removed and/or diluted metabolic end products excreting into the bathing media during the period of anaerobic treatment. The assessment of the effect of a treatment is best done while the organism is in the same environmental situation rather than in an altered medium.

It was also reported [Cooper et al., 1970] that "unstarved" [i.e. 16 hours non-feeding] A. avenae and Caenorhabditis sp. exhibited a slight post-anaerobic oxygen debt but "starved" [16-20 hours non-feeding] populations showed no such phenomenon. These observations agree with those obtained in the present study since none of the 3 isolates tested exhibited any increase in the QO_2 relative to zero time rates. This may be because the test nematodes were more than 12 hours out of culture at the beginning of the experiment [due to use of a standardized harvest/extraction method - General Materials and Methods, page 16] and thus may have been in the "starving" period, specified by Cooper et al. [1970].

The incubation temperature for control and test groups in this study was 25°C, the same temperature at which the mass-monoxenic cultures of the 3 isolates were grown. However, incubation at 30°C produced significant variations in the QO_2 which were peculiar to each isolate [see Chapter V]. In contrast the isolate of A. avenae used by Cooper et al. [1970] was grown at room temperature which apparently varied between 23°C and 27°C and the QO_2 rates were assayed at 27°C. This difference in the incubation temperature may have contributed to the post-anaerobic QO_2 reported by Cooper et al. [1970].

Many free living and parasitic species accumulate an "oxygen debt" during an anaerobic period which is "paid-off" in an increased QO_2 during the post-anaerobic period, [Von Brand, 1979] and it is often suggested that metabolism of accumulated anaerobic metabolites [lactate, ethanol, succinate and others] is the causative factor. The end products accumulated in the tissues would be degraded oxidatively or partially resynthesized into carbohydrates during the post anaerobic phase. The fact that some helminths such as N. brasiliensis show an oxygen debt while some others do not may be due to their propensity to excrete the end products of anaerobic metabolism [Von Brand, 1979]. Furthermore, there is no clear correlation between the presence or magnitude of an oxygen debt, and either the ability to survive anoxia or the extent of oxygen lack during anaerobiosis [Atkinson, 1973]. According to Rogers [1962] and Von Brand [1966, 1968], species of nematodes differ in their metabolic responses to the absence of oxygen and the extent of excretion of anaerobic by-products.

It is possible that such differences as dicussed above exist between the 3 isolates of A. avenae investigated here and this may explain why no post-anaerobic increase in the QO_2 were observed in these isolates.

The QO_2 of the three isolates were not influenced to any significant degree by hyperbaric levels of oxygen in the bathing medium and suggested that the 3 isolates of A. avenae all behave as "regulators" independant of the external oxygen tension, until a critical oxygen tension is reached. These observations are similar to those obtained by Bryant et al. (1967) for the free-living bacteriophagous nematode Caenorhabditis briggae, the QO_2 of which increased with oxygen tension until $\simeq$ 5% O_2 beyond which point there was little change even in an oxygen saturated medium. Nicholas and Jantunen (1964) have shown that Caenorhabditis sp. can withstand a maximum of 24-48 hours anoxia without dying whereas A. avenae (Cooper et al., 1970) can survive anoxia up to 60 days and regain initial QO_2 when returned to an aerobic state.

CHAPTER 5

The effect of chemical agents on the respiratory oxygen consumption of A. avenae isolates A, F and E

Introduction

In a study of the respiratory physiology of Caenorhabditis briggsae, (Bryant, Nicholas & Jantunen 1967) it was reported that cyanide caused nearly 70% inhibition of the oxygen consumption rate (QO_2) of this free living nematode. Interestingly the effect was not a permanent inhibition since only 14% inhibition of the QO_2 was observed following the removal of the inhibitor. They also observed that C. briggsae "survived for 7-10 days in cyanide with reduced activity before dying."

No work has been reported on the effect of this biocide (sodium cyanide-NaCN) on the rate of oxygen consumption of Aphelenchus avenae. Furthermore a comprehensive investigation of the respiratory physiology of any organism would be incomplete without the assessment of the effect of cyanide - the classical inhibitor of cytochrome oxidase viz. cytochrome aa_3. Therefore studies were carried out to assess the effect of NaCN on the QO_2 of three different eco-strains (isolates) of Aphelenchus avenae to ascertain whether any uniformity of response could be discerned.

On the basis of radio tracer studies, Castro and Belser (1978) postulated that in A. avenae, the two primary modes of intoxication induced by the alkyl halide nematicide ethylene- di-bromide (EDB) are a direct reaction of the

halide with an Fe-II centre in the respiratory sequence and/or the substitution of a serine at the active site of an esterase or protease.

While suggesting that the most sensitive sites to such attack by EDB should be a terminal oxidase, or an oxygen transporting protein, he also pointed out that A. avenae, like most other organisms undoubtably possesses an endogenous capacity to re-reduce the Fe-III in haem-proteins to the functional Fe-II state. Thus Castro, and Belser (1978) envisaged the oxidative destruction by an alkyl halide biocide as representing a gradual flooding out of available Fe-II sites in competition with any endogenous reductant.

Therefore, the assessment of cyanide sensitivity of the QO_2 of A. avenae was of further interest since one of the two primary modes of action of the fumigant nematicide EDB appears to be very similar to the effect cyanide has on cytochrome-oxidase viz. nucleophillic attack of the Fe-II centres of the haem group. A comparison of the effect of cyanide and EDB on the QO_2 of A. avenae was thus carried out. Further, the ability of whole worms to recover normal QO_2 following NaCN and EDB treatment were also investigated with the aim of assessing whether A. avenae has any capacity to negate the oxidant-effect EDB has on its haem-proteins.

Evans (1973) and Evans and Thomason (1971) observed that the ability of nematodes to survive exposure to EDB was enhanced at low temperatures and under anaerobic

conditions, and suggested a connection between aerobic metabolism in the nematode and EDB toxicity. The implication by these authors was that either the components of the respiratory chain are only vulnerable when in use by the organism, or that some product of anaerobic metabolism confers protection on them. Thus the effect of NaCN on the QO_2 of A. avenae (whole worms) subjected to hyperbaric oxygen and anaerobic conditions were assessed.

Marks (1971) studied the gross effects of EDB on nematode metabolism by monitoring the respiratory responses of A. avenae and Caenorhabditis sp. to EDB. He reported that EDB had a stimulatory effect on the QO_2 of third stage larvae of Caenorhabditis sp., conforming with observations on EDB treated cockroaches (Morikawa, 1964).

However in marked contrast Marks (1971) reported that the QO_2 of A. avenae was "not significantly influenced" by the presence of EDB within the temperature range of 10° and 30°C. Since EDB penetrates both these organisms quite readily he speculated that the difference in the respiratory response probably indicated basic physiological differences between these two species.

Awan (1975) in a similar study on A. avenae reported that EDB (1000 ppm) caused an immediate depression of 21.9% of the QO_2 of A. avenae, while halothane depressed the QO_2 only by 17%. These observations by Awan (1975) are contradictory to those of

Marks' (1971) finding that the QO_2 of A. avenae was refractory to EDB.

However these differences in the response of the QO_2 of A. avenae to EDB observed by these authors may have been due to differing metabolic states of the nematodes during the studies or due to genuine intraspecific differences of the Aphelenchus avenae strains investigated, since Awan (1975) studied a strain isolated in the U.K. and Marks (1971) a strain isolated in the U.S.

Therefore the effects of EDB and cyanide were assessed, with reference to the age of the cultures from which the worms were harvested.

The response of the A. avenae isolates to other respiratory inhibitors such as salicylhydroxamic acid (SHAM), parachloromercuribenzoate (p-CMB), carbon monoxide (CO) were also investigated, in order to obtain information on the respiratory physiology of A. avenae whole worms.

Materials and Methods

1. The effect of culture age on the cyanide sensitivity of the QO_2 of three isolates of A. avenae

A. avenae were harvested from cultures of the 3 isolates at 40, 50, 60, 70, 90, 120 and 140 days after inoculation as described in Chapter 2 and extracted for a standard 12 hr period. Suspensions containing 20 mg wet wt./ml were prepared and assayed in the oxygen electrode at 30°C. NaCN (aqueous) was injected via capillary port (using micro-syringe) into the assay chamber to give a final concentration of 3 mM. Assay volume was 3.0 ml. Six replicates were assayed for each batch; and 3 batches from the same harvest time were assessed. Following assay the nematodes were pipetted out into pre-weighed foil crucibles, dried (see Chapter 2) and dry weight assessed. Correction was made for the weight of cyanide contained in 10 ml of a 1 M solution since the worms were dried in cyanide medium.

2. The recovery of isolate-F (mass cultured at 25°C) following cyanide treatment

2.a The recovery (at 30°C) of isolate-F (acclimatized to 30°C) following initial exposure to 6 mM cyanide

A. avenae (isolate-F) from 40 day old mass cultures were harvested in sterile distilled water and extracted for 12 hours and prepared into suspensions (20 mg/ml). 10-12 ml fractions of the suspension were dispensed into sterile quick fit tubes (1.5 cm diameter) and incubated

at 30°C in shaker bath (70 oscillations per minute) for 24 hours. 8-10 such tubes were incubated, each afforded 3-4 replicates. Following this period of acclimatization, the QO_2 of the suspensions (3.0 mls) were assayed in the oxygen-electrode at 30°C. 20 µl of a 1M NaCN solution were injected (effective concentration of 6 mM cyanide) and trace recording obtained for 10 minutes. Each sample of nematodes was subject to cyanide (6 mM) for 15 minutes at the assay temperature (30°C). Following this standard 15 minutes period of exposure, each 3 mls sample was carefully withdrawn from the polarograph chamber using a clean Pasteur pipette, chamber washed to remove all nematodes. The treated worms were filtered dropwise on a Whatman No.1 hardened filter paper (to remove all cyanide) under suction, washed by the dropwise addition of distilled water onto the nematode deposit to further remove all traces of cyanide, and resuspended in 3.0 mls of sterile distilled water. Treated and washed samples of worms were kept in separate quick fit tubes, and were re-incubated at 30°C in shaker bath. Control group of nematodes were similarly acclimatized to 30°C, assayed, but were not treated with cyanide. These were also subject to the filter/wash regime identical to the test group and resuspended in 3.0 ml batches in quick fit tubes. Three to four such tubes from control and test groups were removed at 1, 3, 4, 6, 8 and 24 hours and again assayed at 30°C in the O_2-electrode, and the QO_2

recorded; 6 mM cyanide was applied and the trace obtained again to assess the sensitivity following recovery from the first exposure to cyanide.

2.b The recovery (at 23°C) of isolate-F (acclimatized to 23°C following initial exposure to 6 mM cyanide

The method employed was identical to the earlier experiment (2.a) except that the suspensions of the nematodes were acclimatized to 23°C (instead of 30°C) for 24 hours prior to experiment proper. The QO_2 recordings with and without 6 mM cyanide were obtained at 30°C. Cyanide treatment was for a 15 min (standard) period at 30°C. Following filtration and washing the worms were re-suspended in sterile distilled water as before, but were incubated at 23°C during recovery periods of 2, 4, 6, 8, 24 and 48 hours. Control groups were similarly assayed.

2.c The recovery (at 30°C) of isolate-F (acclimatized to 23°C) following initial exposure to 6 mM cyanide

In this instance, the procedure was similar to 2.a and 2.b except that following washing and resuspension of the treated and control groups of nematodes, they were incubated in quick fit tubes (4 replicates each for each recovery time period) at 30°C for recovery i.e. a temperature higher by 7°C to the temperature they were acclimatized to prior to zero time assays. The sensitivity of the worms (to a second application of 6 mM cyanide)

after 1, 3, 4, 6, 8 and 24 hour periods of recovery were assessed.

3. The variation of the QO_2 of the 3 isolates of *A. avenae* following incubation at 30°C

Forty day old cultures of *A. avenae* from the 3 isolates grown at 25°C were harvested as described in the General Materials and Methods. Suspensions containing 20 mg wet wt./ml of whole worms were dispensed into a series of sterile quick fit tubes. One group of tubes were kept at room temperature (23°C) in a shaker bath while the second group was incubated in a water bath set at 30°C. Each tube afforded 3 replicate assays. The temperature of the nematodes suspensions within each tube took approximately 40 mins. to equilibrate to the temperature of the water bath. Therefore the timing of the incubation period commenced when the temperature of the nematode suspension attained 30°C, which was measured using a mercury thermometer. The assay temperature was 30°C. The experiment was repeated twice on worms of the 3 isolates from two different batches.

4. The effect of prolonged cyanide treatment on the QO_2 of the whole worms of isolates A and F mass cultured at 25°C

Forty day old *A. avenae* mass cultures grown at 25°C were harvested, extracted and made into suspensions (20 mg wet wt./ml) as described in Chapter 2. 10-12 ml

fractions of the suspensions were dispensed into sterile quick fit tubes. 6-10 such tubes were incubated in shaker bath at 30°C, after zero time QO_2 were recorded. This was the control group.

To the second group of suspensions in quick fit tubes, a 1M solution of sodium cyanide was added [via micro syringe] so that a 6 mM final concentration prevailed in the volume of suspension contained within the tube. The latter group of nematodes were thus incubated in a 6 mM solution of NaCN. After zero time sensitivity to 6 mM cyanide was assessed, the test group was also incubated at 30°C in shaker bath. Tubes from both control and test groups were removed at 1, 2, 4, 6, 8, 24 and 48 hours and their oxygen consumption rates were assayed in the oxygen electrode at 30°C. The effect of further 6 mM NaCN applications on the QO_2 of both control groups and cyanide treated groups were assessed by injecting 15µl of a 1M solution of NaCN. Both isolates A and F were separately tested in this manner, to assess the effect of long term treatment of the isolates with NaCN.

5. <u>The response of whole worms of isolates A and F mass cultured at 30°C to NaCN</u>

5.a <u>The response of worms harvested from 31-35 day old ["young"] cultures</u>

Mass cultures of isolates A and F were grown at the higher temperature of 30°C. The harvest, extraction techniques were as described in Chapter 2. The QO_2 was

measured on the oxygen electrode and the response to the application of 20 µl of NaCN was assessed. Both isolates A and F were tested. 3 different batches of cultures were assayed, each was replicated 6 times.

5.b The response of worms harvested from 55 day old ["old"] cultures

Harvest, extraction as described in Chapter 2. The QO_2 was assessed in oxygen electrode and the 20 µl 1M cyanide applied to assess response to NaCN. Worms from 3 different batches of cultures were assessed. Each batch was replicated 6 times.

6. The response of whole worms of isolates A and F [mass cultured at 30°C] to 1000 ppm Ethylene-di-bromide

Worms harvested from "young" [21-35 days after inoculation] and "old" [55-60 days after inoculation] mass cultures were extracted for the standard 12 hour period, made into suspensions and were assayed in the O_2 electrode. EDB in ethanolic solution was applied to 3.0 mls [volume of suspensions used for each assay] of the assay sample of nematodes to give an effective final concentration of 1000ppm. A 600,000 ppm solution of EDB was prepared and 5 µl of such a solution was applied to 3 mls of assay sample. Since the EDB was in ethanolic solution the effect of 5 µl of ethanol on the QO_2 of the nematode suspensions was assessed prior to each experiment.

7. The recovery of isolate F following short term treatment with EDB

Whole worms of isolate F cultured at 30°C, were harvested, extracted from 40 day old mass cultures at 25°C (room temperature) in the standard manner (Chapter 2) and the suspensions were assayed in the O_2 electrode. 5 µl of stock EDB solution (600,000 ppm) was applied and the response was assessed at 30°C. The suspensions were not pre-incubated at 30°C. The polarographic recordings were obtained (six assays) for 10-15 mins. (in the presence and absence of EDB) following which the assayed nematodes were kept in the 1000 ppm EDB solution for a total period of 30 mins. Thereafter they were assayed again in the polarograph. After the second assay they were filtered dropwise through a Whatman No.1 hardened filter paper under suction on a millipore funnel and washed by the dropwise addition of distilled water and resuspended in 3 mls of sterile distilled water, introduced into quick fit tubes and allowed to recover at 25°C in a shaker bath. The QO_2 of the washed nematodes were assessed after a period of 24 hours, and the effect of a second application of 1000 ppm EDB on the post recovery QO_2 of the nematodes was obtained.

8. The effect of hyperbaric oxygen on the QO_2 of A.avenae isolates A and F (cultured at 30°C) and the response to NaCN

Worms from both isolates were harvested at 40 days, extracted (standard 12 hours) and made into

suspensions [20 mg wet wt./ml]. 8 mls samples of the suspensions were dispensed into quick fit tubes and put to incubate at 30°C in a shaker bath. One group [6 tubes] was treated with 100% oxygen prior to dispensation into the tubes, by bubbling 100% oxygen at 25 cc/min for 1 hour. The tubes containing 8 mls suspensions of the worms were fitted with air tight rubber bungs through which 2 disposable type hypodermic needles were inserted and acted as inlets and outlets; through the inlet 100% oxygen [humidified] was bubbled continuously for 24 hours [10 cc per min]. The control group was similarly bubbled with humidified air filtered through a bacterial filter. After 24 hrs both groups were assayed in the O_2 electrode and the response to 6 mM NaCN was assessed.

9. <u>The effect of 100% N_2 treatment on the QO_2 of isolates A and F [cultured at 30°C] and response to NaCN</u>

Whole worms suspensions [20 mg/ml] were prepared from 40 day old mass cultures of isolates A and F grown at 30°C. 10 ml aliquots of the suspensions were introduced into quick fit tubes and six such tubes were incubated at 30°C water bath with facility prepared for the continuous passage of filtered humidified air [10 cc/min] through the suspensions [control group]. The test group was treated with O_2-free-N_2 for 1 hour, divided into quick fit tubes, flushed with N_2 for a further 30 mins, sealed, and put in a desiccator

flushed 3 times with N_2. The sealed desiccator was kept at 30°C in a controlled temperature cabinet. After 24 hrs the 3.0 ml samples were introduced into the O_2 electrode chamber and QO_2 and response to 6 mM NaCN assessed.

10. The concerted effect of NaCN and EDB on the QO_2 of isolate A cultured at 30°C

Mixed populations of isolate A grown at 30°C were harvested between 27 and 35 days after inoculation and subject to 12 hours extraction. Suspensions of the worms were prepared and 3.0 ml aliquots were assayed in the O_2 electrode and the response to 6 mM NaCN and 1000 ppm EDB were ascertained.

11. The effect of salicylhydroxamic acid [SHAM], p-chloro-mercuri-benzoate and carbon monoxide on the QO_2 of isolate A

Mixed populations of isolate A harvested from very young [27-35 day old] cultures were prepared into suspensions [20 mg wet wt./ml] and the immediate effect of 3 mM SHAM, 3 mM pCMB on the QO_2 of the whole worms were assessed. A saturated solution [at 25°C] of CO was prepared and 20 µl of such a solution was applied via micro syringe into the O_2 electrode chamber to assess the effect of carbon monoxide on the QO_2. The final effective concentration of CO in 3 mls of assay-suspension was 298 nm/ml. A second method was employed to assess the effect of CO on whole worms since the

concentration of CO in 20 µl of a saturated solution of CO (at 25°C) would be further diluted in the assay sample contained in the polarograph chamber. Thus 3.0 ml samples of isolate A whole worm suspensions were gassed for 3 minutes (flow rate 20 cc/min) with CO (B.D.H.) (after the normal QO_2 was recorded) re-introduced into the electrode chamber, re-sealed to obtain the QO_2 in the presence of CO (effective concentration = 45 µmoles ml^{-1}). The trace recording was obtained for 7-10 minutes, after which the samples were pipetted out into pre-weighed foil crucibles and dried in an oven (as described in Chapter 2) to obtain the dry weights.

12. The response of A.avenae isolate A, cultured under continous-(forced)-air-flow conditions to the biocides NaCN, EDB and p-CMB

The normal mass-monoxenic culture of A. avenae is carried out in 500 cc Kilner jars which are sealed except for a cotton wool plug to facilitate air exchange. Under these circumstances the accumulation of carbon dioxide and other volatile by-products by both the fungus-host and the nematode may have secondary effects. Thus a system for the continuous aeration of the culture jars was devised. To the metal lids of the jars were attached two glass tubes (3 mm diameter) using an inert adhesive. Sterile, humid air was passed through one inlet into the jars and exited via the

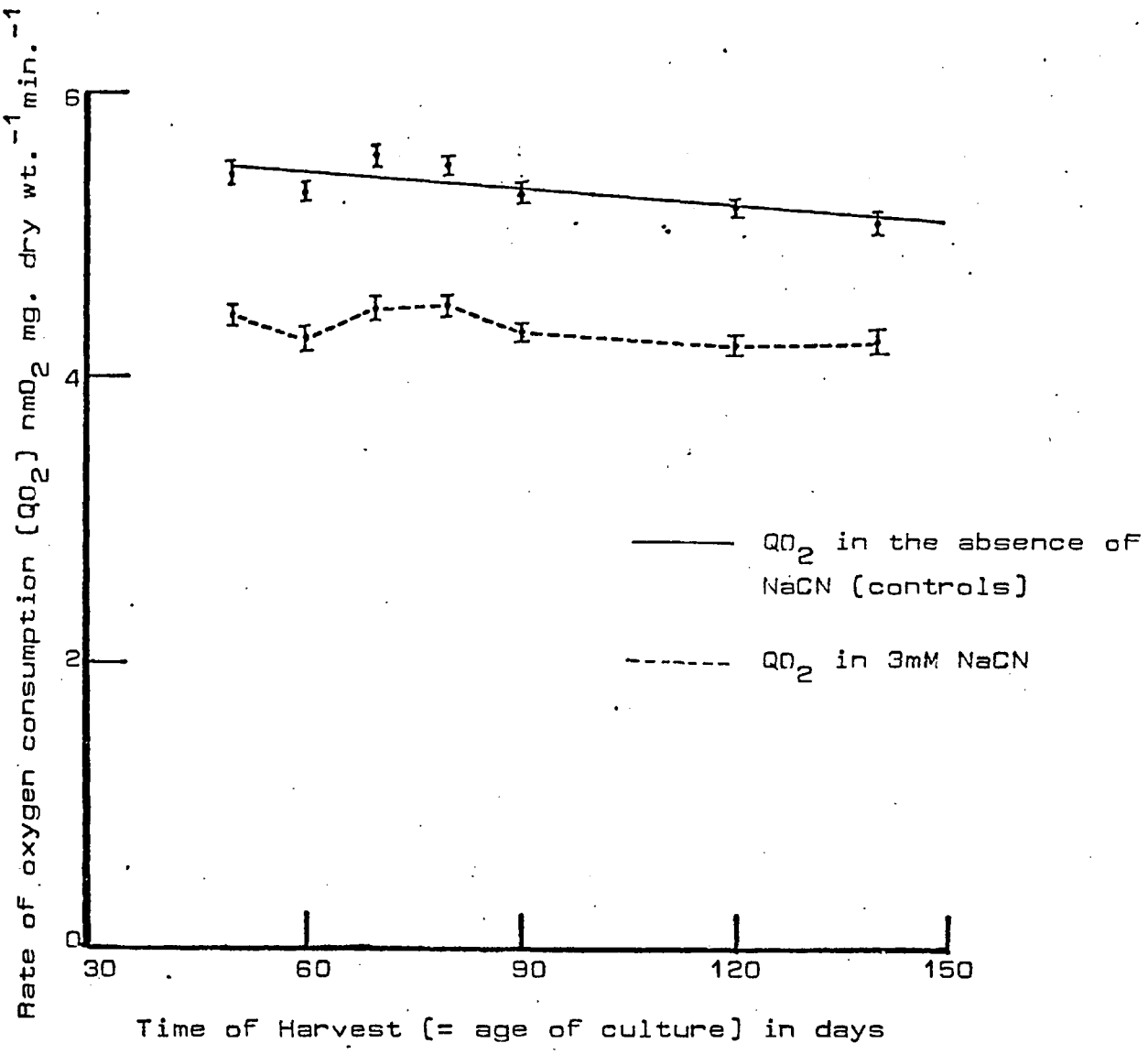
second tube, so that any gases produced in the culture were quickly removed. The cultures were incubated at 30°C and the worms were harvested at 35 days, extracted for standard 12 hour period and suspensions prepared and the QO_2 assessed in the oxygen electrode. The effects of 6 mM NaCN, 3 mM p-CMB and 1000 ppm EDB were assessed as described earlier.

Results

1. The effect of cyanide on the QO_2 of the isolates

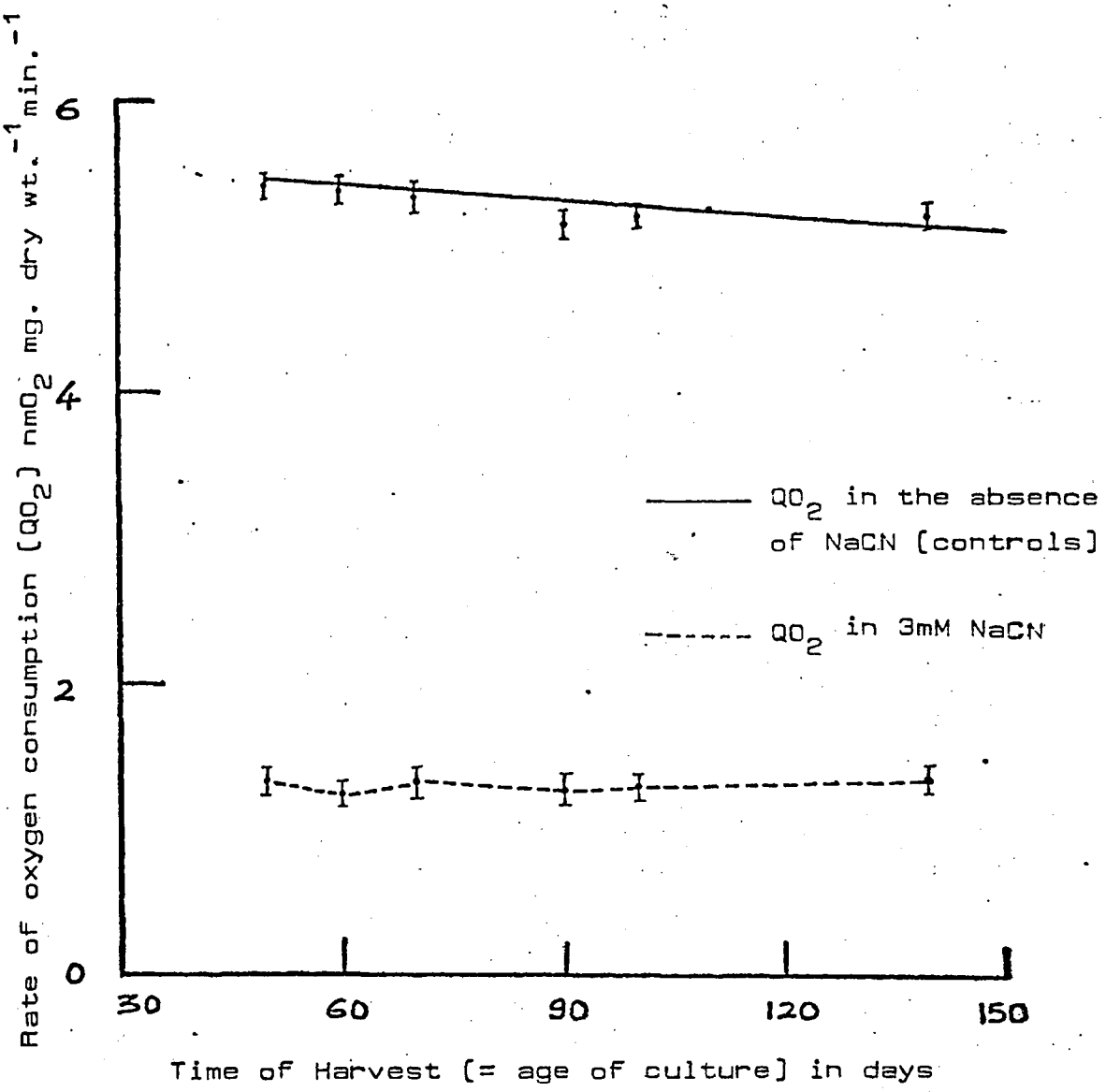
The response of the whole worms of the 3 isolates to 3 mM NaCN and the variation of the response with harvest time (=age of cultures) is shown in Figs. 22, 23 and 24 and in the Appendix Tables 5-1.1 to 5-1.3. There were basic differences among the isolates in the degree of inhibition of the QO_2 produced upon the immediate application of cyanide. Isolate F exhibited a very high sensitivity which varied between 74-78% depending on harvest time. This variation was not significant ($p > 0.005$) between any two harvest times though the nematodes extracted from very old cultures (i.e. $>$ 80 days) appeared more sensitive than those from cultures of intermediate age. Isolate A was less sensitive to identical concentrations of cyanide compared to isolate F, showing in a majority of cases inhibition in the range of 17-20% although a few replicates showed total insensitivity of the QO_2 to cyanide. The most sensitive were the populations extracted from 120 day old cultures. Isolate E exhibited the least degree of inhibition with cyanide in that most samples showed no response at all to 3 mM NaCN and the range varied from 0-12% inhibition; as with isolates A and F the higher sensitivity was observed in worms derived from cultures $\geq$ 90 days in age. In all three isolates the nematodes harvested from older cultures (i.e. $>$ 80-90 days) appeared relatively more sensitive to NaCN (within the range observed for each

Figure: 22. The effect of 3mM NaCN on the rate of oxygen consumption (QO_2) of isolate-A whole worms cultured at 25°C.



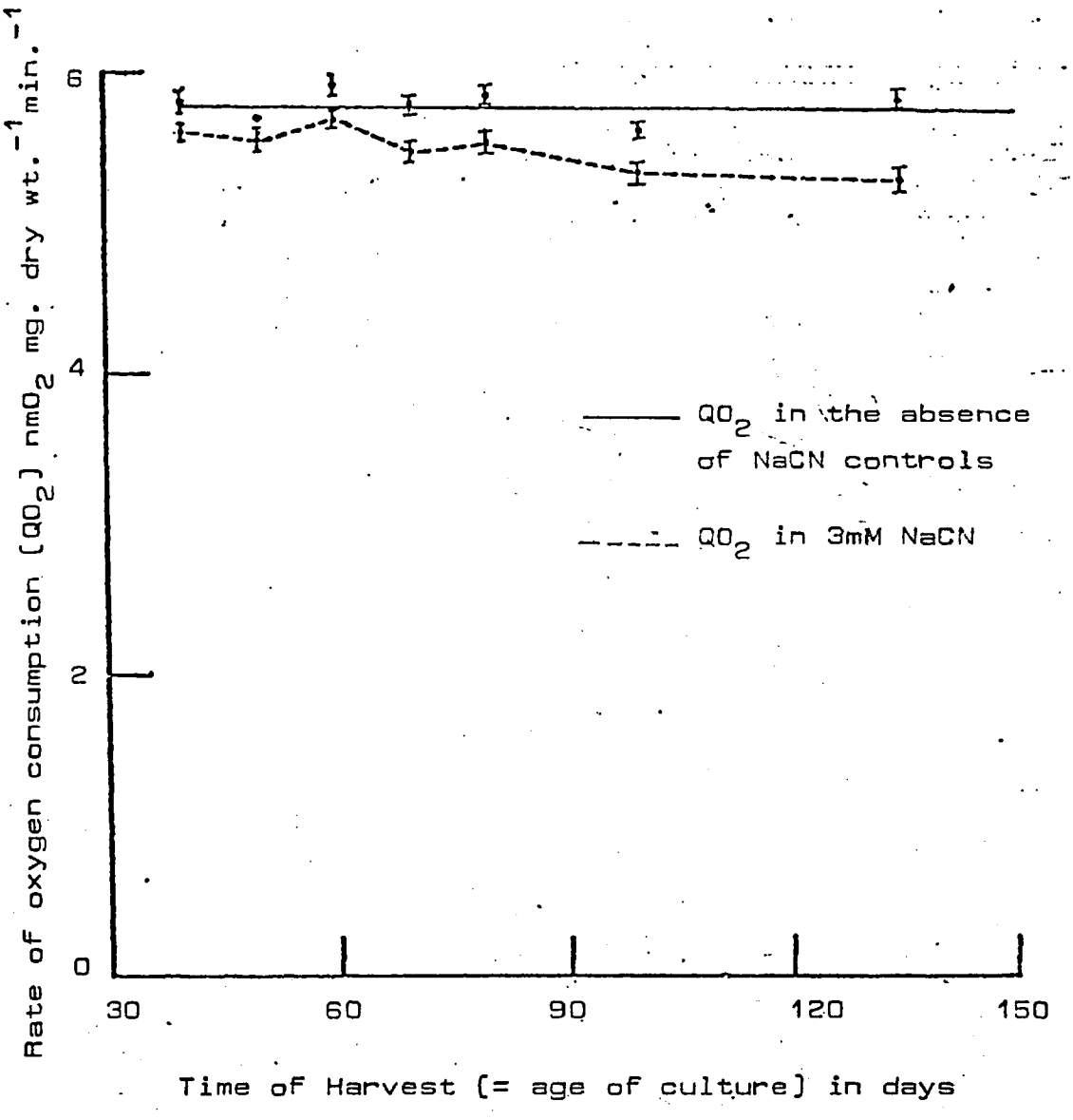
The standard deviation (n=18) is indicated in the figure.

Figure: 23. The effect of 3mM NaCN on the rate of oxygen consumption (QO_2) of isolate-F whole worms cultured at 25°C.



The standard deviation (n=18) is indicated in the figure.

Figure: 24. The effect of 3mM NaCN on the rate of oxygen consumption (QO_2) of isolate-E whole worms cultured at 25°C.



The standard deviation [n=18] is indicated in the figure.

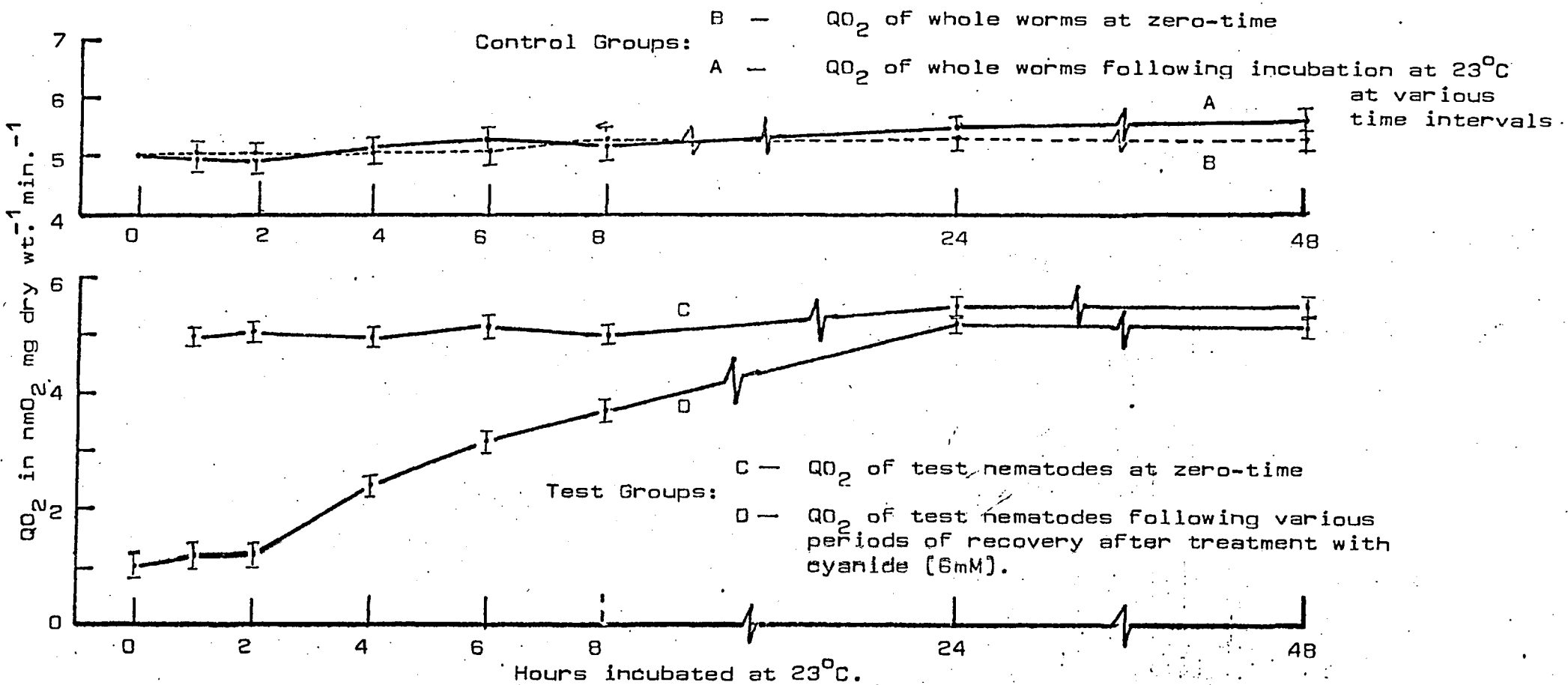
isolate) than those harvested from younger (30-45 days old) cultures. The differences however were not significant ($p > 0.05$). In all three isolates the worms harvested from older cultures appeared to be smaller and somatic contents relatively less dense compared to those harvested from younger cultures.

2. The recovery of isolate F following NaCN treatment

2.a Recovery at 23°C of worms acclimatised to 23°C

Results are shown in Fig. 25 and Appendix 5-2.1. The QO_2 of isolate F acclimatised for 24 hours to 23°C was 5.03 ± 0.15 and 3 mM NaCN caused 78.88% inhibition of the QO_2. The cyanide insensitive QO_2 was ·099 ± 0.16. This was taken to be the general zero time sensitivity of all control groups to cyanide. Initial treatment with 3 mM NaCN produced 74-80% inhibition of the QO_2 of test groups. Following initial exposure, for 15 minutes, the removal of the biocide by washing and re-suspension of the treated worms for 1, 2, 4, 6, 8 and 24 hours at 23°C showed the gradual increase of the 'residual' QO_2 from 1.21 ± 0.12 after 1 hour recovery; through 3.17 ± 0.11 after 6 hour recovery to 5.15 ± 0.19 after 24 hours recovery; following the same periods of recovery, the immediate inhibition obtained with 3 mM NaCN was 41%, 45% and 76% respectively (Fig. 28A and Appendix 5-2.2). Control groups subjected to the identical washing

Figure: 25. The recovery (at 23°C) of the QO_2 of A. avenae - isolate-F - whole worms following cyanide treatment. (Nematodes acclimatized to 23°C.)



The Mean ± S.D. (n=6) is indicated in the figure.

regime, resuspension and incubation at 23°C showed an increase in the QO_2 from 4.95 ± 0.08 (after 1 hr) to 5.46 ± 0.10 (after 24 hrs) and the per cent inhibition by 3 mM NaCN remained around 76-81%. Almost full restitution (96%) of the zero time QO_2 was achieved by the NaCN treated nematode.

2.b <u>The recovery at 30°C of worms acclimatised at 30°C</u>

Fig. 26 and Appendix 5-3.1 show the results obtained. The QO_2 of isolate F acclimatised for 24 hrs at 30°C varied between 7.81 ± 0.16 —— 8.10 ± 0.33, which was greater by approximately 50-60% of the QO_2 of isolate F acclimatised to 23°C. This rise in the QO_2 following incubation at 30°C was characteristic of isolate F. However 3 mM NaCN caused 74-81% inhibition of this increased QO_2, and this was the variation in cyanide sensitivity of the control groups at zero time (Appendix 5-3.2). Initial treatment with 3 mM NaCN caused 74-80% inhibition of the QO_2 of the "test-groups". When washed and resuspended for 1, 6 and 24 hours for recovery, the QO_2 increased with recovery time and exhibited QO_2s of 1.72 ± 0.16, 4.05 ± 0.13 and 7.76 ± 0.09 respectively, showing a steady reactivation of the original zero time QO_2. However following 8 hr recovery from first exposure to cyanide, the sensitivity of the QO_2 to a further 3 mM NaCN was approximately 50% (Fig. 28 b) but after 24 hours recovery (at 30°C) the worms had acquired near zero time sensitivity, i.e.

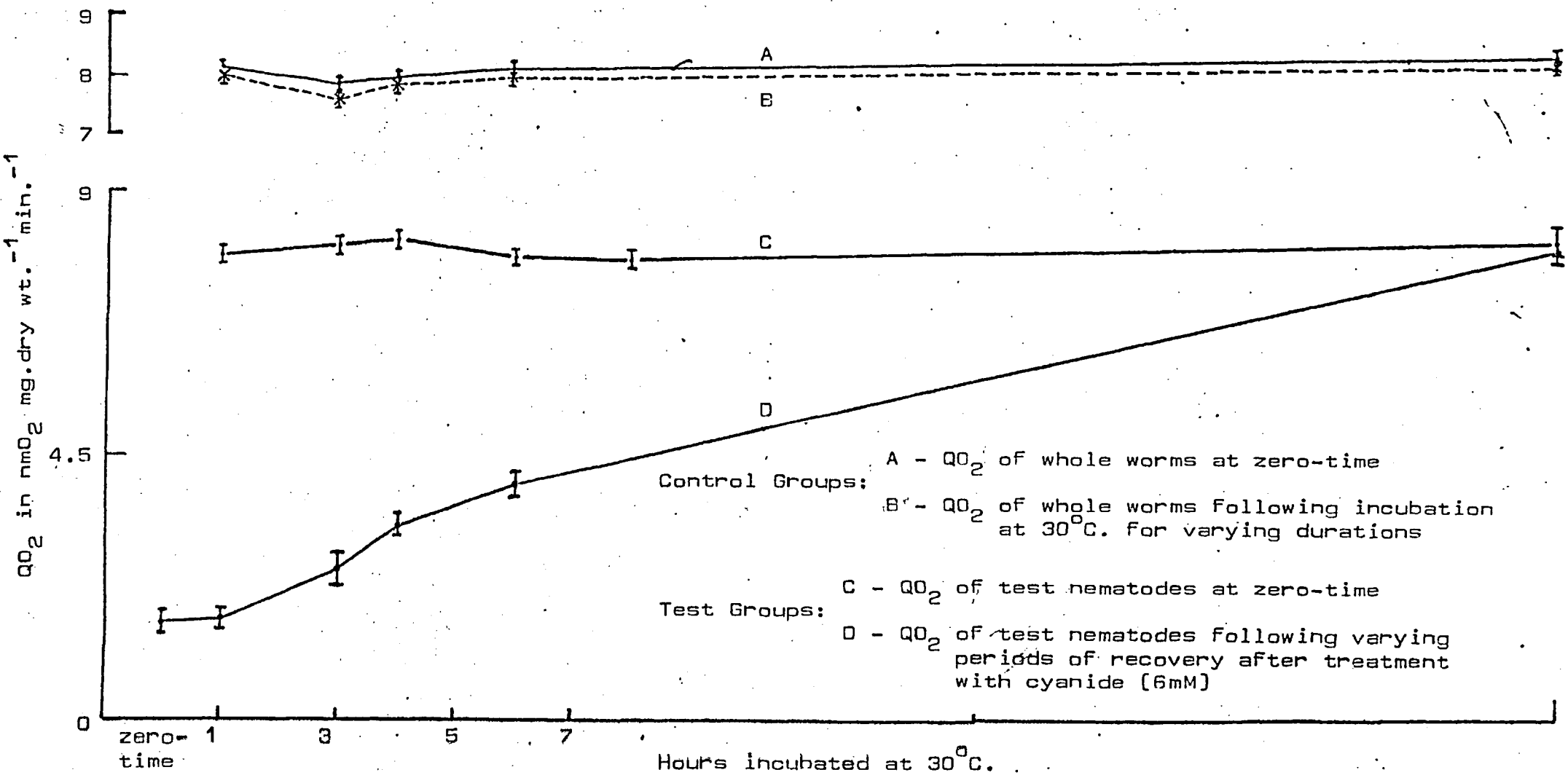


Figure: 26. The recovery (at 30°C.) of the QO_2 of A. avenae (isolate-F) whole worms following treatment with cyanide. (Nematodes acclimatized to 30°C.)

almost the same sensitivity exhibited by control groups, suggesting that almost complete reactivation of the cyanide sensitive QO_2 was achieved within 24 hours following removal of the inhibitor.

2.c <u>The recovery [at 30°C] of isolate F [acclimatised to 23°C]</u>

Fig. 27 and Appendix 5-4.1 and 5-4.2 shows the results obtained. The mean QO_2 after 24 hrs incubation was 5.21 $\pm$ 0.12 and 3 mM NaCN caused 76-80% inhibition and the mean QO_2 in cyanide was 1.10 $\pm$ 0.12. After first exposure, the treated samples washed and re-suspended for 1, 6 and 24 hrs at 30°C showed QO_2s of 1.20 $\pm$ 0.12, 3.31 $\pm$ 0.11 and 7.81 $\pm$ 0.22 respectively. Although the initial zero time QO_2 of 5.25 $\pm$ 0.15 was not attained even after 8 hour recovery, following 24 hour recovery [at 30°C] the QO_2 was approximately 50% greater than the zero time rates. The QO_2 of the controls [after 24 hrs at 30°C] were 7.95 $\pm$ 0.23 compared to 7.81 $\pm$ 0.22 of the samples exposed to NaCN and allowed 24 hrs recovery. However on the second application of 3 mM NaCN, [to the worms which were exposed to cyanide once] the per cent inhibition exhibited after 1, 8 and 24 hrs after recovery was $\simeq$ 40%, 50 and 74% respectively (Fig. 28 c) indicating the complete reactivation of the cyanide sensitive QO_2 of the worms after removal of the inhibitor. It is interesting to note that after 8 hours recovery the QO_2 was approximately 15% lower than the zero time rate

Figure 27: The recovery of the QO_2 of A. avenae, isolate-f, whole worms at 30°C following treatment with cyanide. (Nematodes acclimatized to 23°C.)

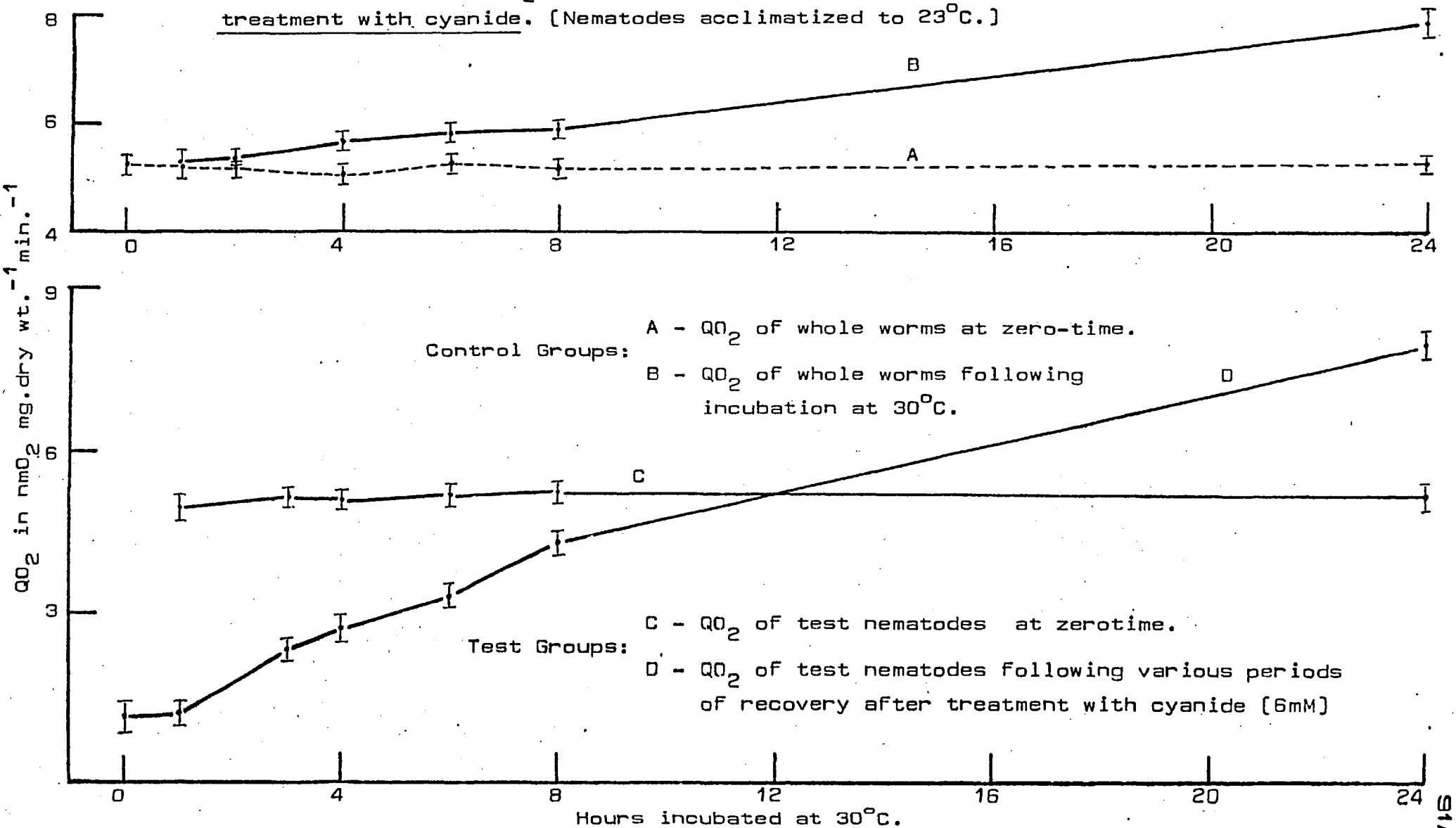


Figure: 28 [A]. The variation in cyanide sensitivity of isolate-F whole worms following an initial treatment with cyanide.

- Worms acclimatized to23°C prior to experiment.
- Initial treatment - 15 mins. in 6mM NaCN.
  Recovery at 23°C (room temperature).
  QO_2 of untreated controls in broken lines (for reference)

Figure: 28[B]. The variation in cyanide sensitivity of isolate-F whole worms following an initial treatment with cyanide.

Worms acclimatized to 30°C. prior to experiment.
Initial treatment - 15 mins. in 6mM NaCN.
Recovery at 30°C.
QO_2 of untreated controls in broken lines.

Figure: 28[C]. The variation in cyanide sensitivity of isolate-F whole worms following an initial treatment with cyanide.

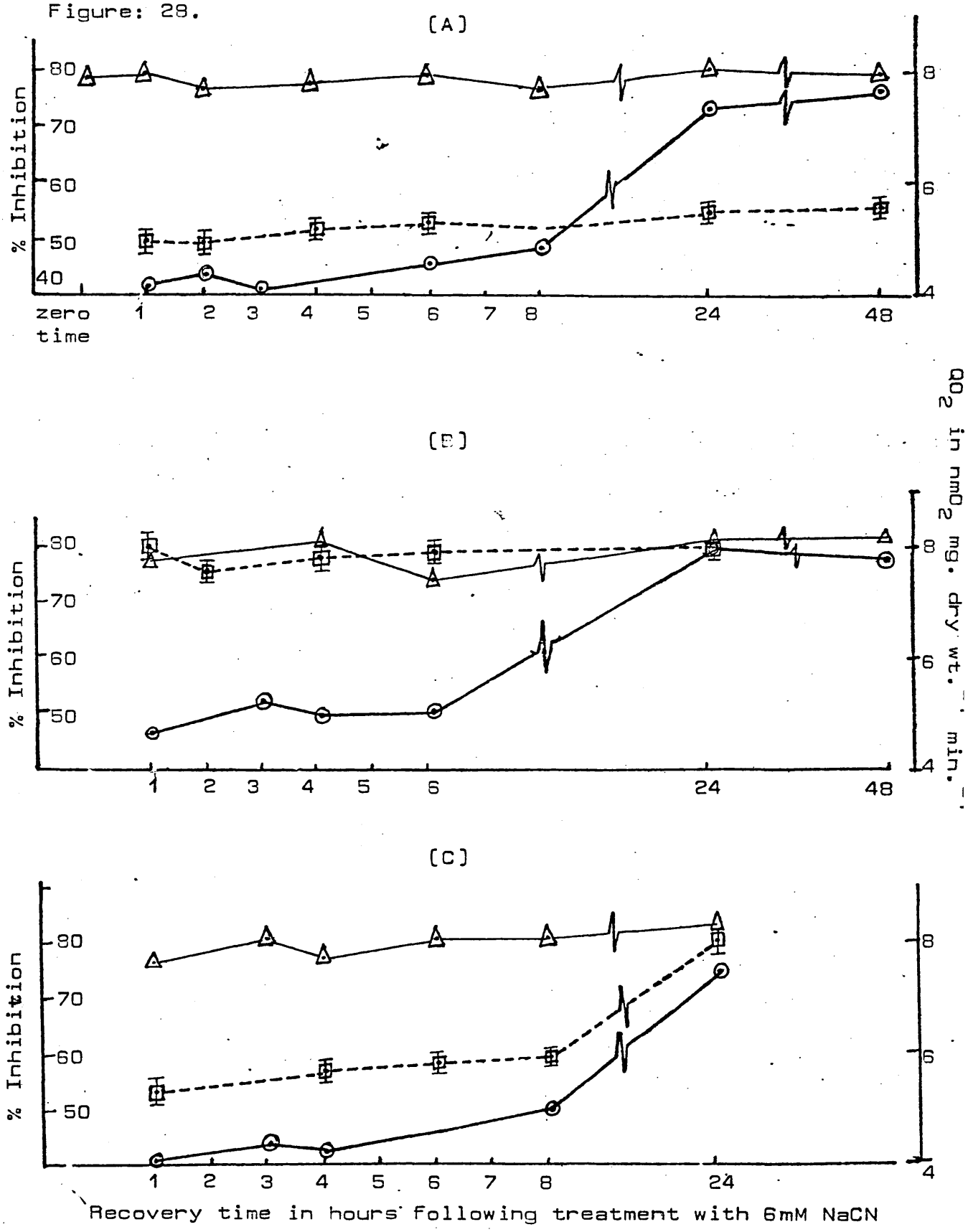
Worms acclimatized to 23°C. prior to experiment.
Initial treatment - 15 mins. in 6mM NaCN.
Recovery at 30°C.
QO_2 of untreated controls in broken lines.

⊙ : - Mean % Inhibition of test groups (by 6mM NaCN) after recovery from initial treatment with NaCN.

△ : - Mean % Inhibition of Control Groups (previously untreated) by 6mM NaCN

⊡ : - Mean QO_2 of Control Groups at test times i.e. following incubation at the specific test temperature for various periods.

Figure: 28.


[A]
% Inhibition
80
70
60
50
40
zero time
1
2
3
4
5
6
7
8
24
48
8
6
4
[B]
% Inhibition
80
70
60
50
1
2
3
4
5
6
24
48
8
6
4
QO_2 in nmO_2 mg. dry wt. min.
[C]
% Inhibition
80
70
60
50
1
2
3
4
5
6
7
8
24
8
6
4
Recovery time in hours following treatment with 6mM NaCN

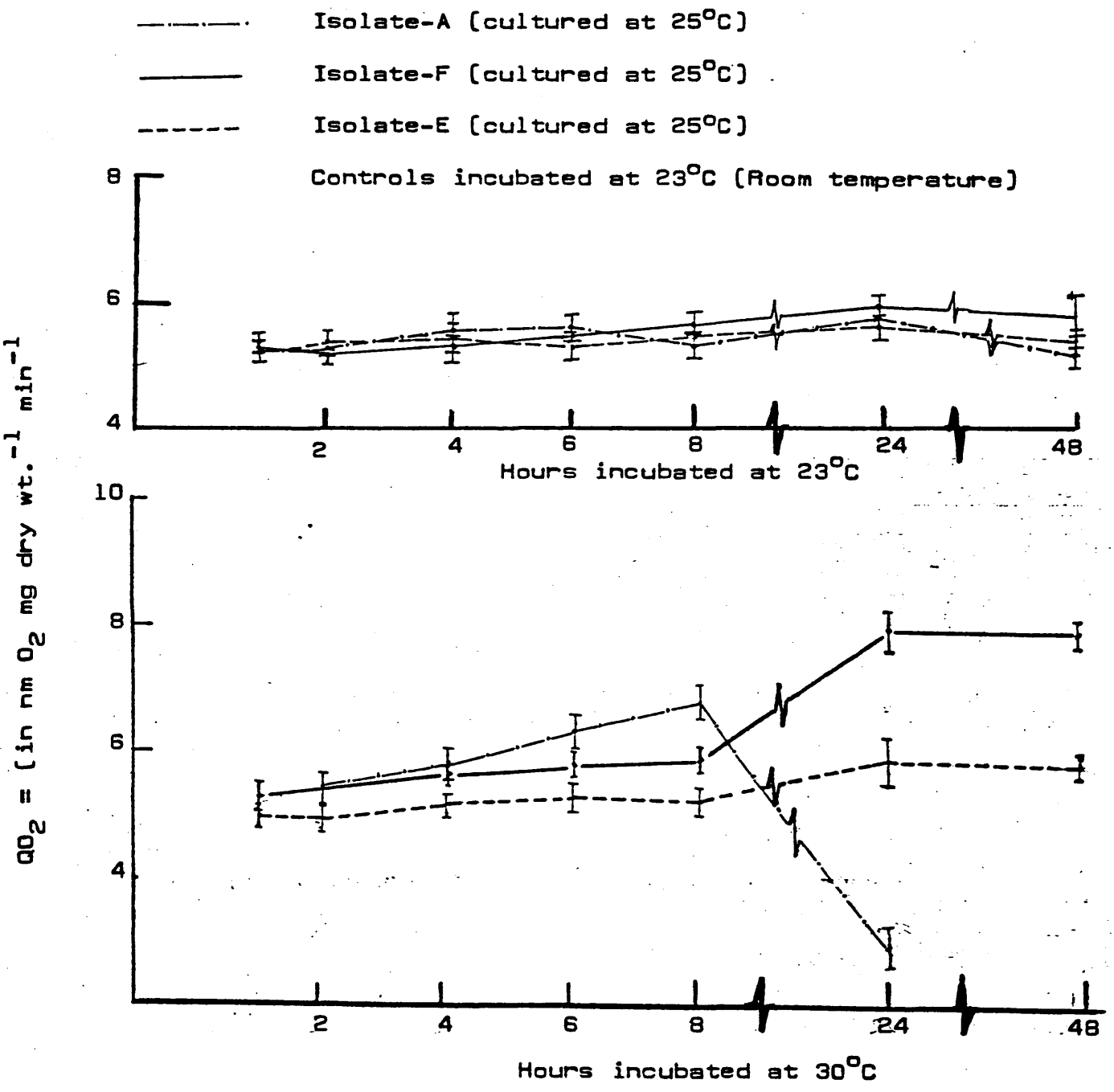
exhibited by the same group and the per cent inhibition achieved was 50.03%. This suggests that isolate-F can recover nearly 85% of its original QO_2 within 8 hours of removal of the inhibitor but that it does so by regaining only 50% of its cyanide sensitive QO_2. In fact suspensions of A. avenae, isolate-F kept for 3 hours in 3 mM NaCN were able to recover their normal QO_2 after washing and re-suspension for 24 hours in distilled water. However after 24 hours intoxication in 3 mM NaCN the worms were unable to recover and showed zero QO_2.

3. The effect of prolonged incubation at 30°C on the QO_2 of whole worms of isolates A, F and E

The results obtained are shown in Fig. 29 and in Appendices 5-5.1 and 5-5.2. The control groups of nematodes incubated at room temperature i.e. 23°C [in sterile quick fit tubes in a shaker bath] showed little increase over a period of 8 hrs. The QO_2 at 24 hrs and 48 hrs was not significantly different from the zero time QO_2.

However when the nematodes from the 3 isolates were incubated under identical conditions but at the higher temperature of 30°C, differences were observed among the 3 isolates. The QO_2 of isolate E increased little up to 8 hrs [Fig. 29] but after 24 and 48 hrs at 30°C the QO_2 of isolate E was significantly different from the zero time and control groups ($p < 0.05$). The

Figure 29 The effect of incubation at 30°C on the QO_2 of whole worms of isolates A, F and E - Controls incubated at 23°C (Room Temp). (All incubations conducted in sterile-distilled wate



The bars indicate the Mean ± S.D. (n = 12)

QO_2 of isolate F whole worms increased by 70-74% ($p<0.01$). In marked contrast the QO_2 of isolate A (cultured at 25°C) decreased dramatically by 50-60% and the difference from the zero time QO_2 and that of the controls was significant ($p<0.01$).

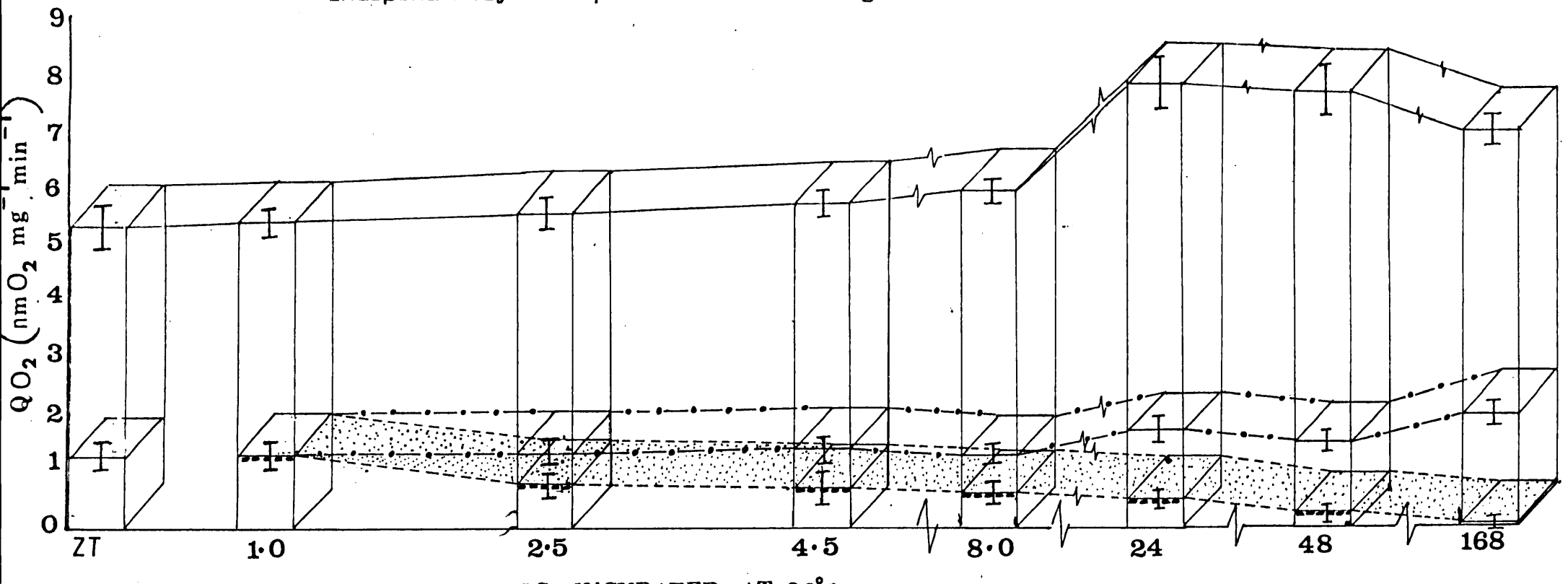
4. <u>The effect of prolonged treatment with NaCN of isolate F and A (cultured at 25°C)</u>

The effect of prolonged incubation of whole worms of isolate F in suspension containing 6 mM NaCN, for various periods of time is shown in Fig. 30 and in Appendix 5-5.1.

Incubation in cyanide for periods longer than 2.5 hours depressed the QO_2 significantly ($p<0.001$) beyond that produced by the immediate application of NaCN. At 24 hours, the QO_2 of the controls was 7.80 ± 0.6 compared to 0.47 ± 0.12 for those incubated in 6 mM cyanide for 24 hours although the application of 6 mM cyanide reduced the rate only to 1.65 ± 0.18, in controls. Incubation at 30°C of controls for 24, 48 and 168 hrs increased the QO_2 to $7.80 \pm .16$, $7.65 \pm .18$, and $6.94 \pm .15$ respectively from a zero time rate of 5.31 ± 0.23. This increase in QO_2 was peculiar to isolate F. However, cyanide application depressed the rate to 1.65 ± 0.18, 1.44 ± 0.31 and 1.15 ± 0.32 respectively while the QO_2 of worms kept continuously in 6 mM cyanide for 24, 48 and 168 hours was $0.47 \pm .12$, 0.36 ± 0.06 and 0.0 respectively.

Figure 30 The effect of short term and long term cyanide [6mM] treatment on the QO_2 of A. avenae isolate-F. Whole worms incubated for various periods at 30°C

Mean $QO_2 \pm$ S.D. [n = 6] for 2 different batches of worms tested independently is represented in the figure.



:- The variation of the QO_2 of isolate-F whole worms with time upon incubation at 30°C.

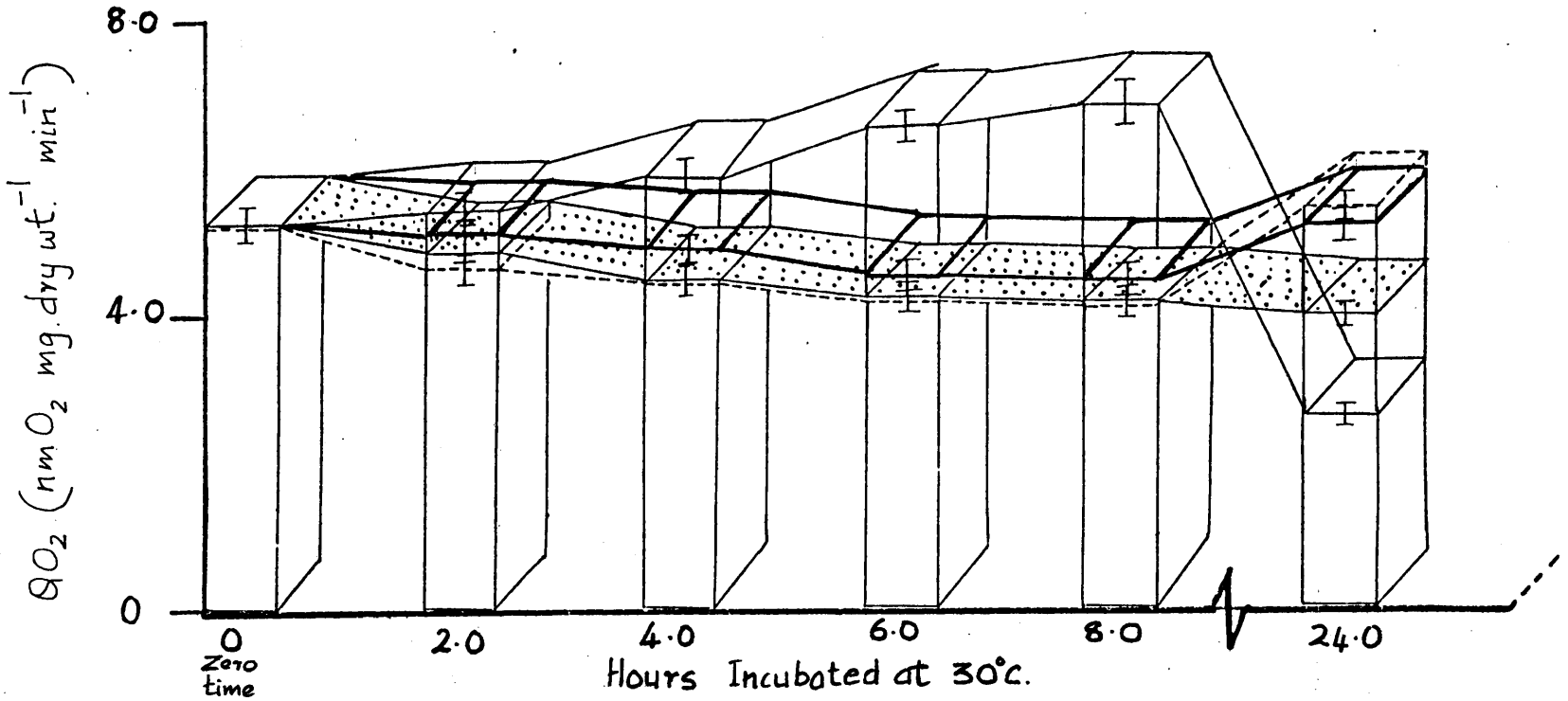
:- The QO_2 of the same whole worm samples immediately after the application of 6mM NaCN.

:- The variation of the QO_2 of a second group of isolate-F whole worms incubated in the continuous presence of 6mM NaCN for identical periods of time at 30°C.

:- The effect of further cyanide [6mM] application on whole worms incubated in the presence of 6mM cyanide.

In Fig. 31 and in Appendices 5-6.1 to 5-6.3 a comparison is made between the effect of prolonged treatment of isolate A whole worms with 6 mM NaCN and the immediate effect of 6 mM NaCN on isolate A worms incubated in sterile distilled water for similar long periods at 30°C. At zero time little or no response (0.56% inhibition) was observed with 6 mM cyanide. The zero time rate was 5.30 ± 0.14. However incubation at 30°C increased the sensitivity up to 8 hours, from 5.32% inhibition (after 2 hours at 30°C) to 35.03% (after 8 hours) at 30°C. Although as expected, gradual increase in the QO_2 of controls from a zero time rate of 5.30 ± 0.14 to a rate of 5.85 ± 0.19 at 8 hours (at 30°C incubation) was observed, the QO_2 after 24 hours in distilled water at (30°C) had dropped to 2.64 ± 0.11 (i.e. a % decrease of 61.4%) but the response of the worms exhibiting this lowered QO_2 to 6 mM NaCN was a dramatic instantaneous stimulation of the QO_2 (Fig. 33) by excess of 100%. (Appendix 5-6.1). This increased QO_2 in cyanide was not significantly different ($p > 0.05$) to the QO_2 originally shown at zero time. The decreased QO_2 observed at 24 hrs was approximately 50% of the zero time rates of the controls, and this decreased rate was maintained up to 48 hours and 168 hours (Fig. 32 and Appendices 5-6.2 to 5-6.3) and the response to cyanide was an immediate stimulation ($\geq$ 100%) of the QO_2. (Appendix 5-6.2) This stimulatory effect of

Figure 31 The effect of short term and long term NaCN (6mM) treatment on the QO_2 of A. avenae isolate-A. Whole worms incubated for prolonged periods at 30°C. Nematodes cultured at 25°C



The immediate effect of 6mM NaCN on worms incubated at 30°C for varying periods.

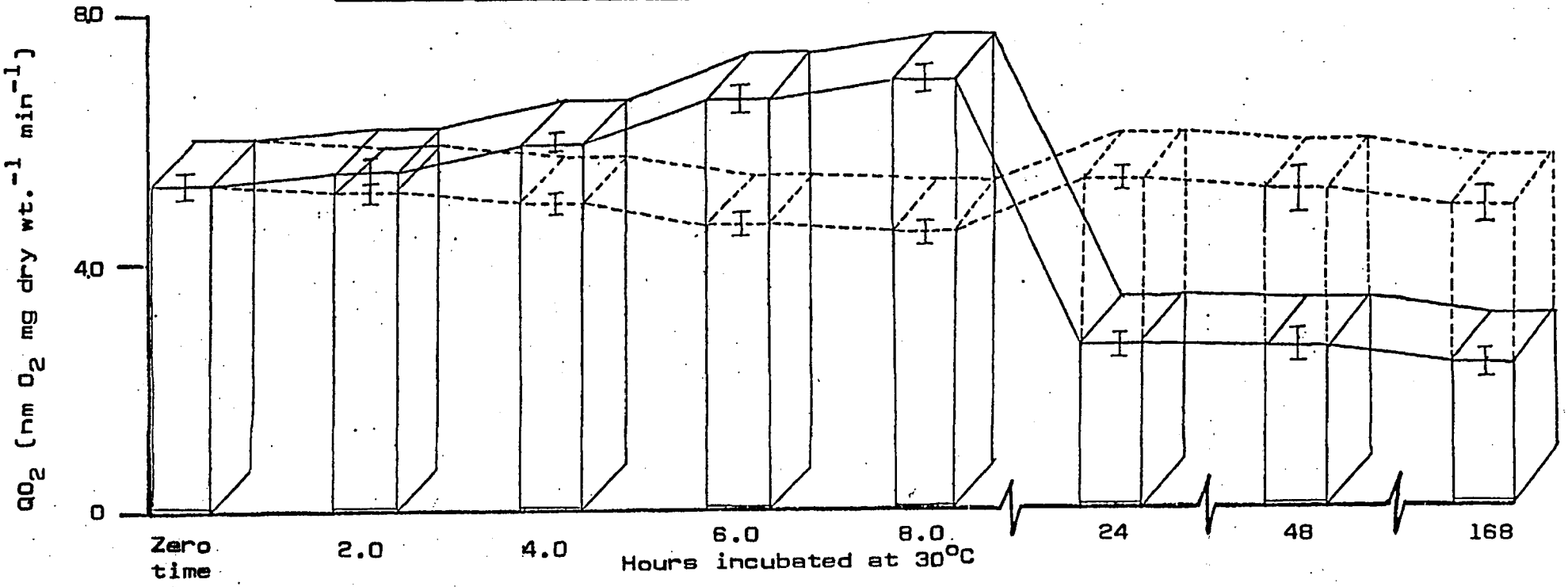
The QO_2 of worms incubated with (6mM) NaCN at 30°C for increasing periods of tim

The immediate effect of a second application of (6mM) NaCN on the QO_2 of the same sample of worms which had already been incubated in 6mM NaCN i.e. final concentration of NaCN-12mM.

Controls i.e. QO_2 of whole worms incubated for increasing periods of time in sterile distilled water at 30°C.

The standard deviation (S.D.) n=6 for each treatment is indicated in the figure.

Figure 32 The immediate effect of NaCN (6mM) on the QO_2 of A. avenae isolate-A whole worms. Nematodes cultured at 25°C and incubated at 30°C for various periods of time.

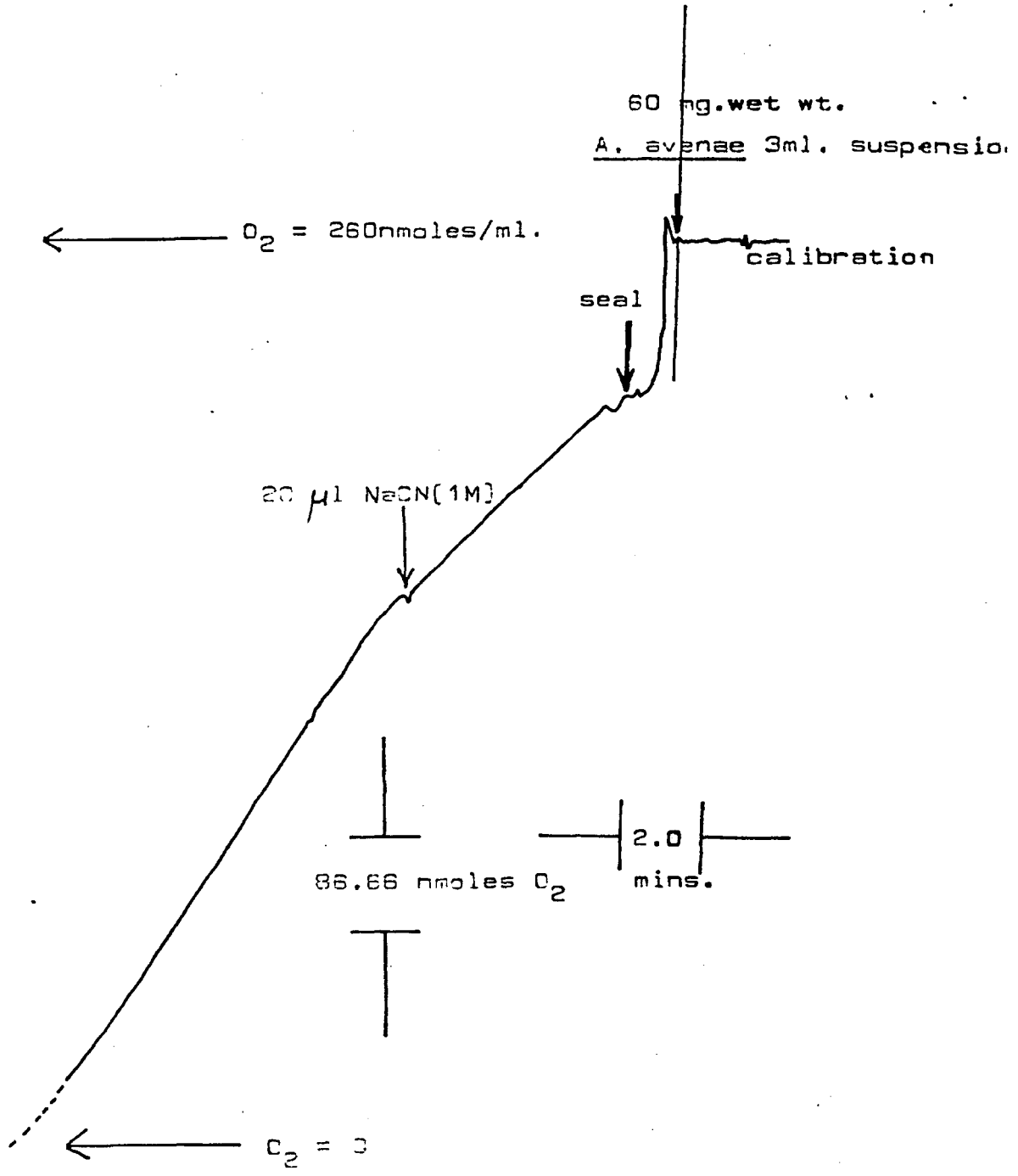


:- The QO_2 of A.avenae isolate-A whole worms incubated in sterile distilled water for prolonged periods at 30°C.

:- The QO_2 of the same samples of worms soon after the application of NaCN (6mM).

The standard deviation (S.D.), n=6, indicated in the figure.

Figure: 33 The effect of NaCN (6mM) on the QO_2 of isolate-A (whole worms) following treatment at 30°C for 24 hrs



NaCN was peculiar to isolate A and was exhibited only if standard suspensions of whole worms of isolate A were incubated for periods greater than 20-24 hrs at an ambient temperature of 30°C, i.e. a temperature 5°C greater than the normal temperature (25°C) at which this isolate was mass cultured. However as will be described later, isolate A cultured at 30°C, exhibited a stimulation of the QO_2 without prior incubation at 30°C. Isolate F whether cultured at 25 or 30°C did not show at any time a positive response to cyanide as exhibited by isolate A.

5. <u>The response of worms harvested from "young" (21-35 days old) and "old" (>55 days old) cultures of isolates A and F (cultured at 30°C) to sodium cyanide</u>

Mixed cultures of isolate A harvested between 21-35 days from mass cultures grown at 30°C showed a stimulation of the normal QO_2 from 5.94 $\pm$ 0.21 to 7.87 $\pm$ 0.24, i.e. a mean per cent increase in the QO_2 of 32.49% (Table 6). Identical populations of worms harvested from old cultures frequently exhibited no response at all to 6 mM NaCN although occasionally a slight stimulation of the QO_2 was observed: thus a mean per cent stimulation of 1.48% was recorded for older cultures of isolate A (Table 6).

The response of worms from isolate F harvested between 21-35 days showed mean QO_2 inhibition of 74.51% (i.e. a reduction of the QO_2 from 5.42 $\pm$ 0.21 ——

Table; 6

The response of whole worms of isolates A and F [mass cultured at 30°C.] to 6mM NaCN.

1. Response of worms at 21-35 days harvest.

	Isolate-A	
Normal QO_2	QO_2 in 6mM NaCN	% Difference in QO_2
5.94 ± 0.21	7.87 ± 0.24	[+] 32.49 [range 28% - 37.2%]
	$p<0.001$	

	Isolate-F	
Normal QO_2	QO_2 in 6mM NaCN	% Difference in QO_2
5.78 ± 0.19	1.47 ± 0.14	[-] 74.51 [range 72% - 77%]
	$p<0.001$	

2. Response of worms at 55days harvest.

	Isolate-A	
Normal QO_2	QO_2 in 6mM NaCN	% Difference in QO_2
5.39 ± 0.18	5.31 ± 0.11	[+] 1.48
	Not significant	

	Isolate-F	
Normal QO_2	QO_2 in 6mM NaCN	% Difference in QO_2
5.47 ± 0.21	1.43 ± 0.19	[-] 73.85 [range 68% - 78%]
	$p<0.001$	

All values are the means ± S.D. [n=6]

Results of related "t"-test conparison given in Table.

QO_2 units: - nmO_2/mg. dry wt./min.

1.43 $\pm$ 0.19] while those harvested from older cultures showed mean QO_2 inhibition in the range of 68-78% with a mean inhibition of 73.85%. There was no significant difference in the response of "young" and "old" populations of isolate F to sodium cyanide.

6. The response of whole worms of isolates A and F to EDB

Mixed populations of isolate A grown at 30°C harvested at 21-35 days exhibited a mean stimulation of the QO_2 by approximately 27% [Table 7] upon immediate application of 1000 ppm EDB, and there was no lag-period, i.e. the respiratory response was instantaneous. The QO_2 in the presence of 1000 ppm EDB was significantly higher [$p<0.001$] than the normal rate of controls.

However the response of mixed populations harvested from older cultures [between 55-65 days] of isolate A exhibited no response whatsoever to identical concentrations of EDB and the QO_2 remained unaffected [Table 7].

In contrast, the QO_2 of mixed populations of isolate F harvested from 21-35 day old cultures were inhibited 21·3 to 31.8% [mean of 26.55%] by 1000 ppm EDB. There was no significant difference in the response of worms isolated from "young" or "old" cultures of isolate F. The application of 1000 ppm ethanol did not alter the QO_2 of either group of worms [$p>0.05$].

Table: 7

The response of whole worms of isolates A and F [mass cultured at 30°C.] to 1000ppm EOB.

1. Response of worms harvested between 21-35 days.

Isolate-A

Normal* QO_2	QO_2 in 1000ppm EOB	% Difference in QO_2
5.91 ± 0.14	7.49 ± 0.18	(+) 26.81% (Range 23% - 31.1%)

$p < 0.001$

Isolate-F

Normal* QO_2	QO_2 in 1000ppm EOB	% Difference in QO_2
5.69 ± 0.21	4.17 ± 0.15	(-) 26.55% (Range 21.3% - 31.8%)

$p < 0.001$

2. Response of worms harvested after 55 days.

Isolate-A

Normal* QO_2	QO_2 in 1000ppm EOB	% Difference in QO_2
5.34 ± 0.18	5.34 ± 0.18	0

Not significant
i.e. no response to EOB

Isolate-F

Normal* QO_2	QO_2 in 1000ppm EOB	% Difference in QO_2
5.39 ± 0.22	4.01 ± 0.12	(-) 25.50% (Range 21.16%-30.73%)

$p < 0.001$

All values are the means ± S.D. [n=6]
Related "t"-test comparison of results given in Table.
* The normal QO_2 was unaffected by 5µl. of ethanol.

7. The recovery of isolate F following short term EOB treatment

The QO_2 of isolate F mixed populations (harvested at 40 days) was significantly reduced upon immediate application of EOB ($p<0.001$ as can be seen from Table 8). Although the QO_2 of the same samples showed a further reduction in the QO_2 when exposed to 1000 ppm EOB for 30 mins, the reduction in rate was not statistically significant (Table 8). Following removal of EOB by washing and filtration and subsequent re-suspension in sterile distilled water for 24 hrs to facilitate recovery, the QO_2 of the worms was not significantly different from the original QO_2 observed. However the worms were less sensitive to a second application of EOB after the 24 hr recovery period following first exposure. The per cent inhibition observed on the second application of EOB varied between 0-7% with a mean inhibition of 2.85%; the mean QO_2 in EOB was significantly different to the post-recovery QO_2 (Table 8). The QO_2 of worms kept in 5 µl of ethanol, washed and assayed 24 hrs after re-suspension in distilled water was unaltered ($p>0.05$) from the normal QO_2.

8. The effect of hyperbaric oxygen on the response of isolates A and F to NaCN

The QO_2 of the controls of isolate A did not differ significantly from those exposed to 100% oxygen

Table 8 The recovery of isolate-F at 25°C following short-term treatment with EDB

(Results are means ± S.D. : n = 6)

	Normal * QO_2	QO_2 immediately after application of 1000 ppm EDB	QO_2 following expsoure to EDB (1000 ppm) for 30 mins	QO_2 after washing and recovery for 24 hrs	QO_2 of "recovered" norms in 1000 ppm EDB (i.e. response to a second application of EDB)
	I	II	III	IV	V
	5.38 ± 0.14	4.14 ± 0.21	3.80 ± 0.27	5.11 ± 0.24	4.96 ± 0.24
		VII	VIII		XI
Mean % Inhibition		23.17% (Relative to normal QO_2)	29.21% (Relative to normal QO_2)		2.85% (Relative to Post-recovery QO_2) (Range 0 -7.1%)

QO_2 units: nmO_2/mg dry wt./min

Related 't' test analysis of the above data

(Percentages were angularly transformed for analysis)

I	v II	$p<0.001$	II	v III	$p>0.05$ (NS)	I	v III	$< p\ 0.001$
I	v IV	$p>0.05$ (NS)	III	v V	$p>0.05$ (NS)	II	v IV	$< p\ 0.001$
VII	v VIII	$p<0.05$	VIII	v XI	$p<0.001$			

*The QO_2 of worms kept in 5 µl ethanol, washed and assayed 24 hrs after resuspension in distilled water was unaltered from the normal QO_2

NS :- Not significant.

saturated medium for 24 hrs (Table 9). The response of both groups to 6 mM NaCN was a stimulation of the QO_2 in the range of 21-25% and there was no difference of the QO_2 of either the controls or the oxygen treated worms in cyanide ($p \geq 0.05$).

The suspensions of isolate F (Table 10) incubated in air saturated media (i.e. controls) exhibited an increase of the QO_2 from 5.40 ± 0.14 at zero time to 6.23 ± 0.21 (which was characteristic of this isolate) which was significant. ($p < 0.001$). However the QO_2 of the controls was inhibited by 78.15% (mean % inhibition) while the worms exposed to hyperbaric oxygen environments showed a much reduced sensitivity (per cent inhibition of 56.8%) to 6 mM NaCN. This reduction in cyanide sensitivity as reflected by the QO_2 inhibition was statistically significant (Table 10).

9. <u>The effect of anaerobic treatment on the response of isolates A and F to NaCN</u>

Incubation of sterile whole worm suspensions of isolate A in 100% nitrogenated medium for 24 hrs at 30°C reduced the QO_2 (Fig. 34) but the reduction was not statistically significant ($p > 0.05$). The worms subjected to the anaerobic period showed no response to 6 mM NaCN and the QO_2 was unaffected. The QO_2 of controls (incubated for 24 hrs in air saturated water) exhibited the characteristic stimulation of the QO_2 with 6 mM NaCN.

Table 9 The effect of hyperbaric oxygen on the QO_2 of isolate-A [cultured at 30°C] and the response to 6 mM NaCN

	QO_2 of Controls at zero time	QO_2 of 'test' group at zero time	QO_2 of 'test' group after 24 hrs in 100% O_2 at 30°C	QO_2 of controls in air sat. water after 24 hrs. at 30° C
	I	II	III	IV
	5.40 ± 0.14	5.41 ± 0.14	5.53 ± 0.17	5.38 ± 0.21
QO_2 in 3mM,NaCN	V	VI	VII	VIII
	6.70 ± 0.16	6.69 ± 0.18	6.71 ± 0.22	6.69 ± 0.23
Mean % change in QO_2	[+]24.15%	[+]23.80%	[+]21.50%	[+]24.51%

Related 't'test analysis of above data

III v IV $p > 0.05$ [NS]

VII & VIII $p > 0.05$ [NS]

Table 10 The effect of hyperbaric oxygen (100%) on the QO_2 of isolate-F (cultured at 30^0C) and the response to NaCN (QO_2 units = nmO_2/mg dry wt/min.)

	QO_2 of controls at zero time	QO_2 of 'test' groups at zero time	QO_2 of 'test' group after 24 hrs. in 100% O_2 at 30^oC	QO_2 of controls in air saturated water after 24 hrs at 30^oC
	A	B	C	D
	5.40 ± 0.14	5.43 ± 0.14	5.84 ± 0.17	6.23 ± 0.21
	E	F	G	H
QO_2 in 3mM NaCN	1.37 ± 0.16	1.01 ± 0.18	2.52 ± 0.22	1.36 ± 0.23
	I	J	K	L
Mean % change in QO_2	-74.52%	-81.38%	-56.80%	-78.15%
% change Range	-72.36% to -76.91%	-78.54% to -83.61%	-48.70% to -58.32%	-74.32% to -80.16%

Related 't' test analysis of the above data (percentages were angularly transformed for statistical treatment)

B v C $.001 < p < 0.01$ C v D $.001 < p < 0.01$ G v H $p < 0.001$

K v L $p < 0.001$ F v G $p < 0.001$

Figure:- 34

T^0:- Zero time QO_2 of test samples.

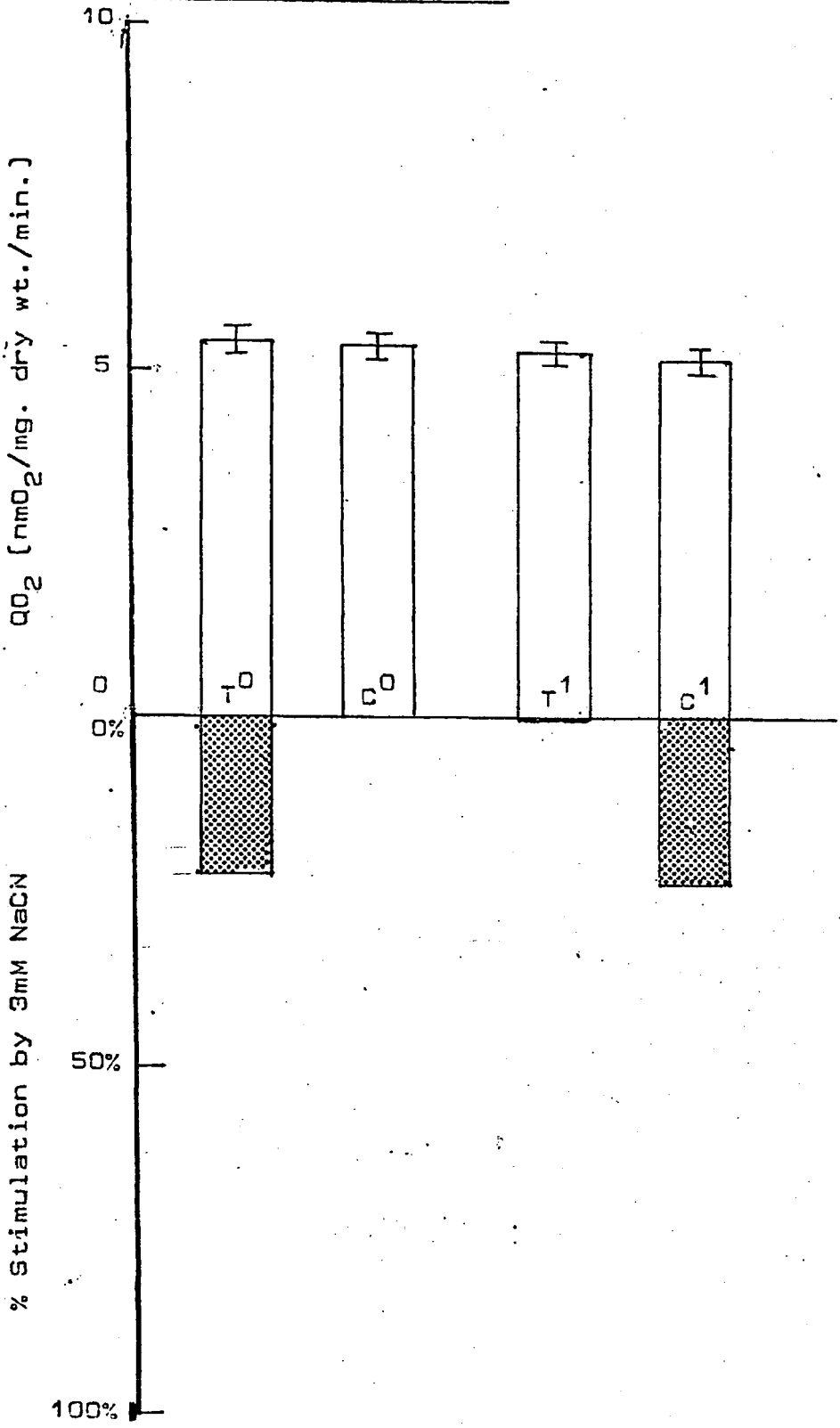
C^0:- Zero time QO_2 of control samples.

T^1:- QO_2 of test samples following anaerobic treatment at 30°C for 24 hours.

C^1:- QO_2 of control samples following incubation at 30°C in air saturated aqueous medium.

Figure:- 34

The effect of anaerobic treatment (of sterile whole-worm suspensions) of isolate-A (cultured at 30°C.) on the QO_2 and response to NaCN (3mM).



The QO_2 of isolate F (Fig. 35) whole worm suspension incubated in O_2-free (100%) nitrogenated medium was reduced significantly compared to that of controls incubated in air saturated medium. The QO_2 of the controls in air was 40-50% greater than the zero time QO_2 of the same samples ($p<0.001$). However the sensitivity to cyanide was not significantly different in the two groups (Fig. 35, Appendix 5-7.1).

Following exposure to 24 hrs anaerobic period, the sensitivity of isolate F to cyanide was significantly reduced (Fig. 35) in that only a mean per cent inhibition of 44.12% was observed and the QO_2 in cyanide was significantly higher than in air-treated controls.

10. <u>The combined effect of EDB and NaCN on isolate A and F cultured at 30°C</u>

The effect of 1000 ppm EDB and 6 mM NaCN (individually) on the QO_2 of isolate F harvested from 30 day old cultures was inhibitory (Table 11). However the application of a mixture of 10 µl of 1M NaCN and 5 ul of 600,000 ppm stock solution of EDB produced almost complete inhibition of the QO_2 of isolate F.

The effect of both these biocides per se on suspensions of isolate A was stimulatory (Figs. 33 and 36 A ; Table 12). However the application of EDB (1000 ppm) after 6 mM NaCN produced an inhibition (Fig. 36B) of the QO_2 of between 19.4 and 26.3% (mean of 23.8%) which was relative to the increased QO2

Table 11 The concerted effect of EDB and NaCN on the QO_2 of isolate-F whole worms extracted from 30 day old cultures (CO_2 units = nmO_2/mg dry wt/min)

All values are the Means ± S.D., n = 6

The normal QO_2 of the test nematodes for all assays was 5.54 ± 0.14 (A)

QO_2 in 6mM NaCN	% Inhibition	QO_2 in 1000 ppm EDB	% Inhibition	QO_2 in 1000 ppm EDB + 6mM NaCN	% Inhibition
(B)	Mean	(C)	Mean	(D)	Mean
1.45 ± 0.12	73.85%	3.96 ± 0.17	28.31%	0.30 ± 0.26	94.47%
	Range 71.81%-76.41%		Range 24.15%-30.61%		Range 91.09%-100%

QO_2 units = nmO_2/mg dry wt./min.

(A) v (B) - $p < 0.001$

(A) v (C) - $p < 0.001$

(A) v (D) - $p < 0.001$

Related 't' test analysis of the above data

(B) v (D) - $p < 0.001$

(C) v (D) - $p < 0.001$

Figure:- 35

T^0:- Zero time QO_2 of test samples.

C^0:- Zero time QO_2 of control samples.

T^1:- QO_2 of test samples following anaerobic treatment at 30°C for 24 hours.

C^1:- QO_2 of control samples following incubation at 30°C in air saturated aqueous medium.

Figure:- 35

The effect of anaerobic treatment [of sterile whole-worm suspensions] of isolate-F [cultured at 30°C.] on the QO_2 and response to NaCN [3mM].

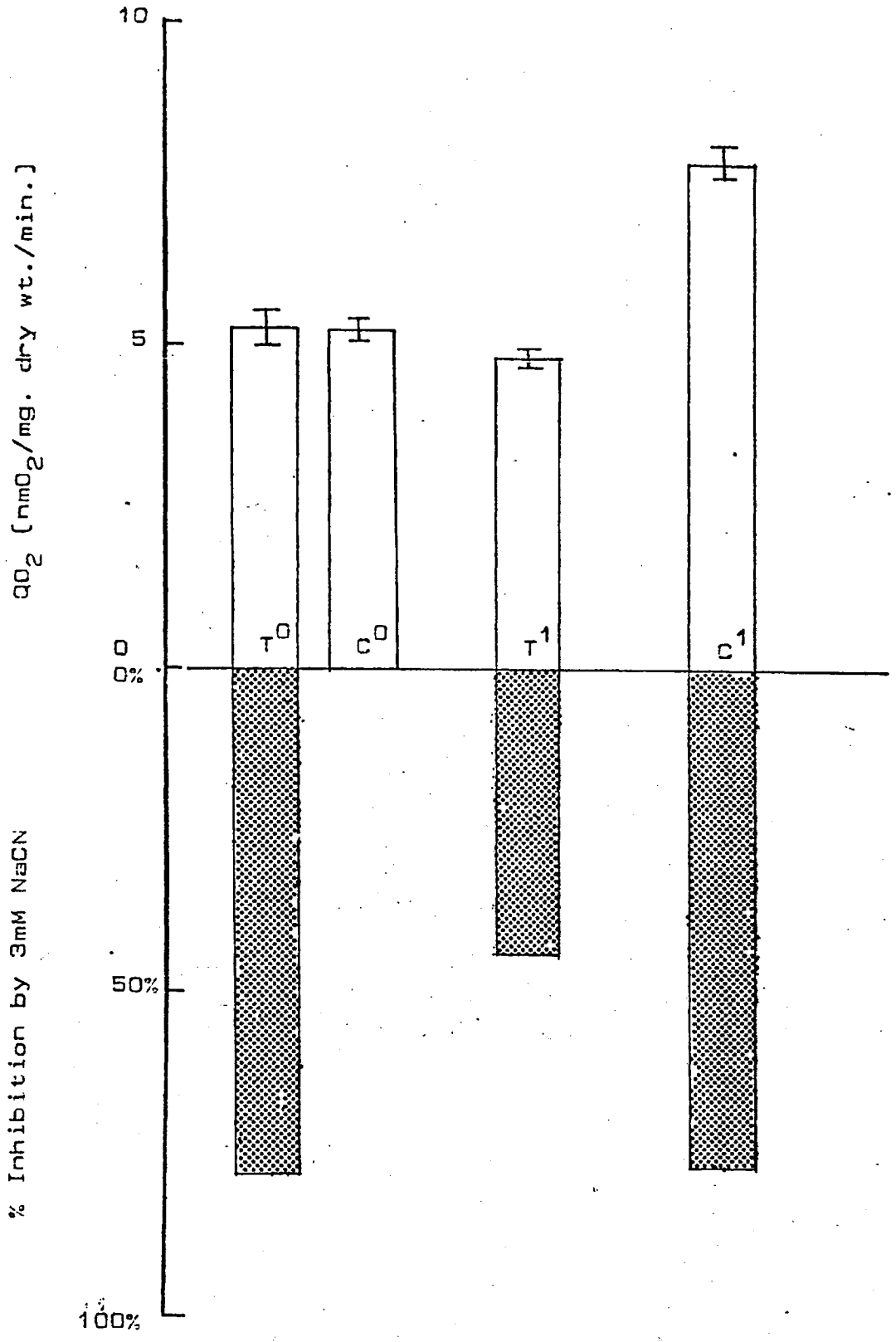


Table 12 The concerted effect of EDB and NaCN on the QO_2 of isolate-A (cultured at 30°C) whole-worms harvested from 25 day old cultures. (QO_2 units = nmO_2/mg dry wt/min)

Effect of NaCN		Effect of EDB		Effect of EDB after NaCN		Effect of EDB + NaCN (Simul. application)	
QO_2 in 6mM NaCN	% Mean change in QO_2	QO_2 in 1000 ppm	% Change in QO_2 Mean	QO_2	% Mean change in QO_2	QO_2	% Change
8.34	(+) 42.7	4.72	(+) 19.2	6.35	(-) 23.8	2.21	(-) 62.10
±	Range	±	Range	±	Range	±	Range
0.34	38.3 - 47.1	0.22	14.3 - 21.9	0.28	19.4 - 26.3	0.14	60.8 - 65.32
(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)

A = The normal QO_2 of the test nematodes for all assays was 5.85 ± 0.24 (Mean ± S.D.)

(+) Stimulation of the QO_2

(-) Inhibition of the QO_2

Related 't' test analysis of the above data

(A) v (B) - $p < 0.001$

(A) v (D) - $p < 0.001$

(D) v (E) - $p < 0.001$

(F) v (H) $p < 0.001$

(A) v (F) $p < 0.001$

(B) v (F) $p < 0.001$

Table 13 The effect of salicylhydroxamic acid [SHAM] p-chloromercuri-benzoate [p-CMB] and carbon monoxide [CO] on the QO_2 of isolate-A [cultured at 30°C and harvested at 25 days]

* The normal QO_2 of the test nemtaodes was 5.92 ± 0.32 [Mean ± S.D; [n=6]

* QO_2 units:- nmO_2/mg dry wt./min.

			Carbon monoxide [in presence of daylight]	
	SHAM [3mM]	p-CMB	25 µl of a saturated sol^n. at 25°C	100% CO treated for 3 minutes
QO_2 [immediately after application]	4.98 ± 0.31	0.63 ± 0.31	3.95 ± 0.60	2.81 ± 0.67
Mean % Inhibition of QO_2	15.63%	89.31%	30.85%	52.77%
% Inhibition Range	11.36% - 17.81%	79.3% - 92.72%	28.87% - 43.71%	42.25% - 63.30%

* Related 't' test proved all values, [except QO_2 observed for the 2 treatments with CO, significant at $0.01 < P < 0.05$] to be significantly different at $p < 0.001$.

* The percentages were angularly transformed for statistical treatment.

produced by NaCN [Fig. 36 b]. When both biocides were applied simultaneously as a mixture, an immediate mean inhibition of 62.10% was observed. Neither biocide produced any inhibitory effect on isolate A when applied independently of each other. [Fig. 33 and 36 A].

11. <u>The effect of SHAM, p-CMB and CO on the QO_2 of whole worms of isolate A</u>

3 mM salicylhydroxamic acid brought about a small but significant inhibition of the QO_2 [11.36% to 17.81%] of isolate A. [Table 13]. 3mM parachloro-mercuribenzoate was the only single compound which per se inhibited the QO_2 of isolate A by 89-90%. 25 µl of a saturated solution of carbon monoxide (at 25°C] produced 30-31% inhibition of the QO_2 of isolate A in the presence of diffused light. Carbon monoxide [saturated assay medium] inhibited the QO_2 of isolate A only by 52.77% in diffused light. [Table 13]

Similar results were obtained for isolate F [cultured at 30°C; harvested at 27 days] the QO_2 of which was only 14-15% depressed by SHAM [3 mM] and only 48.42% inhibited by carbon monoxide. [Table 14]. p-CMB however caused 86-87% inhibition of the QO_2 of isolate F as well.

Figure: 36A The effect of 3000 ppm EOB on the QO_2 of isolate-A whole worms

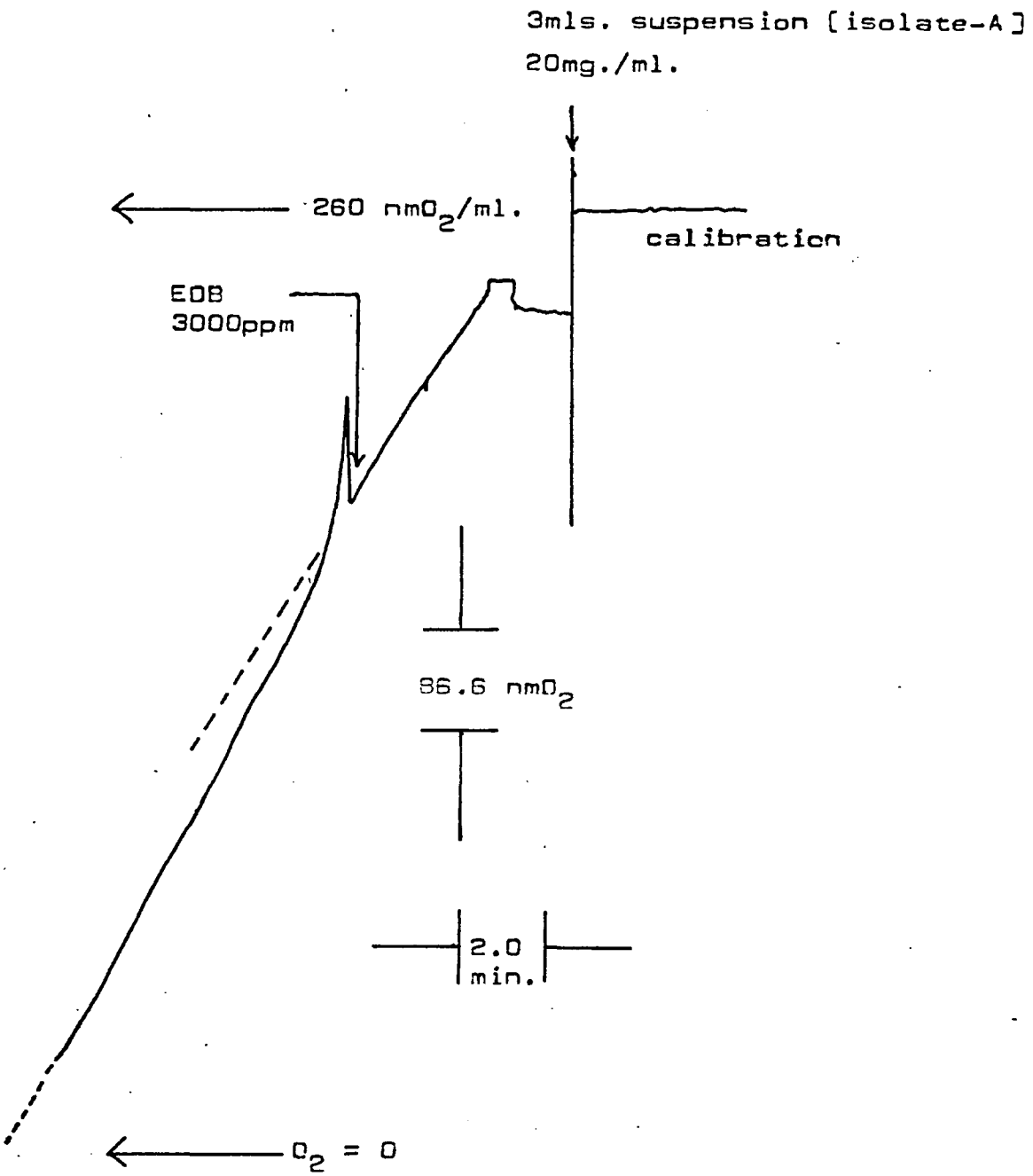


Figure: 36B The effect of EDB (3000 ppm) on the QO_2 of A.avenae, isolate-A whole worms following 6mM NaCN application

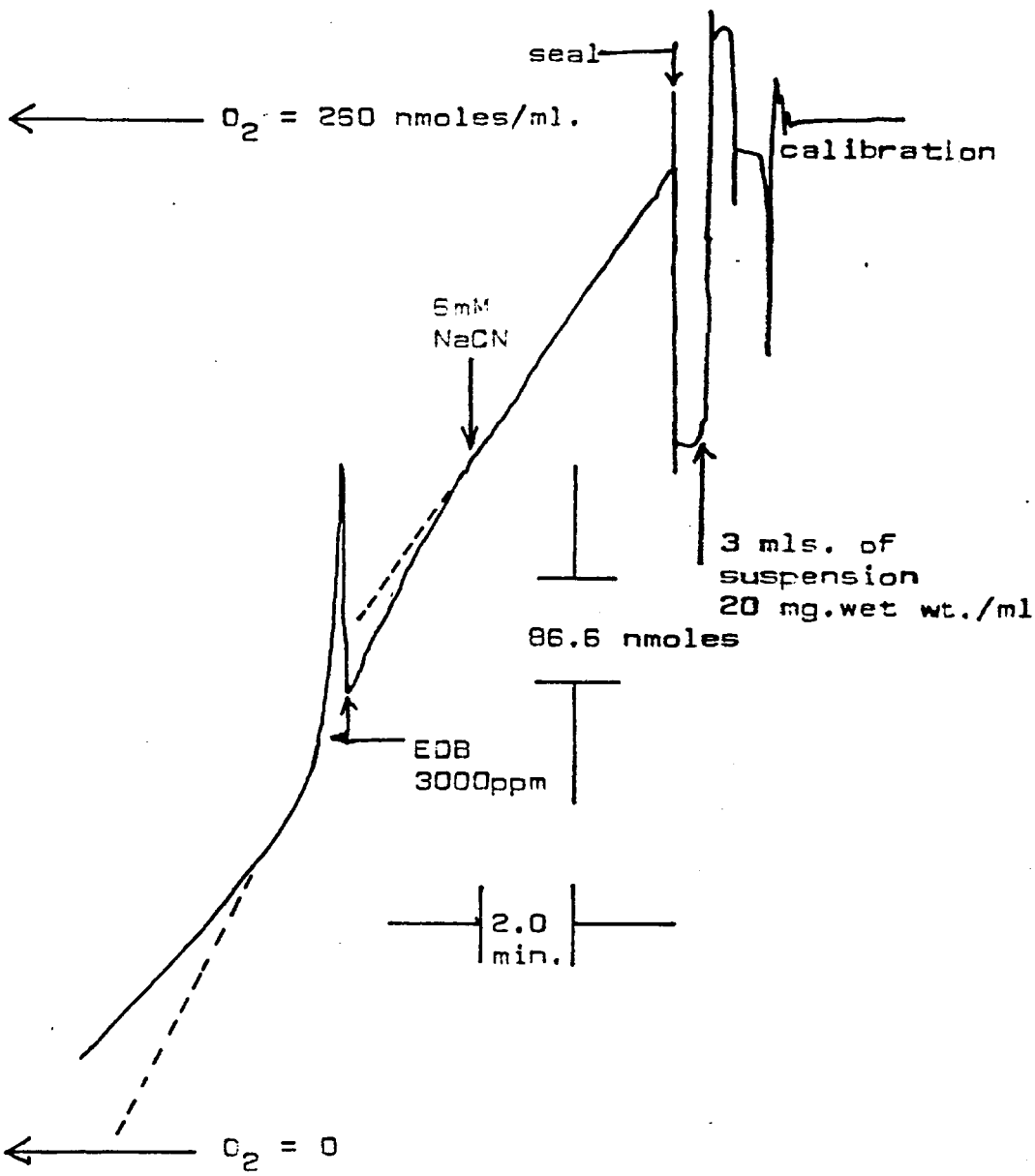


Table 14 The effect of SHAM, p-CMB and CO on the QO_2 of isolate-F [cultured at 30°C and harvested at 27 days]

* The normal QO_2 of the test nematodes was 5.68 ± 0.30 [n = 6]
* QO_2 units:- nmO_2/mg dry wt/min

	INHIBITORS		
	SHAM [3mM]	p-CMB	Carbon Monoxide 100% CO treated at 25°C for 3 mins.
QO_2 immediately after application	4.85 ± 0.26	0.75 ± 0.32	2.82 ± 0.54
Mean % Inhibition	14.59%	86.65%	48.42%
% Inhibition Range	12.15% - 16.01%	77.28% - 94.31%	40.01% - 59.61%

* Related 't' test proved all QO_2 values significantly different at [$p < 0.05$] from normal QO_2.
* Percentages were angularly transformed for statistical treatment.

Table 15 The response of the QO_2 of isolate-A [whole-worms] cultured under continuous ventilation to the application of NaCN, EDB and p-CMB [Nematodes harvested at 27 days]

* The normal QO_2 of the test nematodes was 5.87 ± 0.32
* QO_2 units:- nmO_2 mg dry wt^{-1} min^{-1}

	INHIBITORS		
	NaCN [3mM]	EDB 1000 ppm	p-CMB [3mM]
QO_2 Immediately after application	4.07 ± 0.18	4.38 ± 0.14	0.77 ± 0.18
Mean % Inhibition [-] or Stimulation [+]	+ 30.54 [+32.49]	+ 25.35 [+ 26.31]	- 86.74 [+ 89.31]
Range % Inhibition [-] or % Stimulation [+]	+28.9 to +38.3 [+28.1 to +37.2]	+21.9 to +27.3 [+23 to +31.3]	-74.61 to 94.71 [-79.3 to 92.72]

* The figures in parenthesis are those observed for whole-worms of isolate-A mass cultured in normal Kilner jars and harvested at 27 days.
* All percentages were angularly transformed before statistical treatment.
* Related 't' test showed no significant difference between the response of nematodes cultured under the two different conditions.

12. The response of isolate-A [whole worms] cultured under continuous ventilation to the biocides NaCN, EDB and p-CMB

The response of A.avenae isolate-A mass cultured in the usual Kilner jars under continuous air flow conditions, to the general biocides NaCN, p-CMB and the nematicide EDB is given in Table 15. The response of the nematodes cultured under these conditions did not differ significantly from the response exhibited by isolate-A grown in cotton wool plugged Kilner jars.

Discussion

The differential sensitivity of the three isolates to 3 mM sodium cyanide suggests sub-specific metabolic differences among these ecologically isolated populations of A. avenae. It is unlikely that permeability characteristics or cuticular-barrier effects were the cause of these differences in susceptibility to cyanide because of the rapidity of the response of the QO_2 observed in the polarographic recordings, with whole worms. This was substantiated by the results with mitochondrial preparations as will be presented in Chapter 7 and therefore it is suggested that this differential inhibition of the QO2 of the 3 isolates may be considered a reflection of the different dependencies of the individuals of these isolates on the classical cytochrome oxidase. The high sensitivity of the QO_2 of isolate F to NaCN is possibly an indication of the predominantly cyt. aa_3 mediated terminal oxidation operative in this isolate, with or without a low potential for alternate sequences of electron transfer, dependent or independent of cytochromes. On the other hand the relative insensitivity of the QO_2 of isolates E and A to identical concentrations of NaCN may be due to one of the following. A] the operation of a single cyanide insensitive terminal oxidase which remains unaffected by concentrations of NaCN which wholly inhibit cyt. aa_3 i.e. the presence of an alternative terminal oxidase such as cyt. O; B] the ability to switch over rapidly

from the classical sequence to an alternate sequence - the two being independent of each other; C) the simultaneous operation of a branched-chain respiratory electron transfer sequence where one functions sub-optimally until NaCN inhibits one sequence enabling the other to take over electron transfer; D) the presence of cytochrome independent electron transfer to oxygen such as sequences involving α-glycero-phosphate-oxidase linked glycolysis (Evans & Brown, 1973) or some other non-haem-iron-protein (Bendall & Bonner, 1971) or E) the direct reduction of certain TCA intermediates - such as fumarate - other than involving oxygen. Cyt. o has been recorded occurring with cyt. aa_3 in prokaryotic organisms such as Halobacterium sps. (Cheah 1969, 1970), and in eukaryotic organisms such as Ascaris (Cheah, 1976) Moniezia expansa (Cheah, 1968). However in eukaryotic organisms which possess two terminal oxidases cytochrome aa_3 and cytochrome o, the relative insensitivity of the QO_2 to cyanide is due to the fact that cyt. o is unihibited at concentrations of cyande (0.1 mM to 3 mM) which wholly inhibit cyt. aa_3 (Ray & Cross, 1972).

The recovery (after washing and resuspension) of isolate F following short term exposure to cyanide may be due to the ability for reactivation of the cyanide sensitive oxidase. The increased cyanide sensitivity (70-73% inhibition) of the whole worms after 24 hr recovery period compared to the cyanide sensitivity

[$\simeq$ 50% inhibition] of worms allowed only 8 hr recovery period substantiates this hypothesis, assuming that little or no further synthesis of the oxidase occurs during recovery. Similar observations on Caenorhabditis briggsae [Bryant, Nicholas & Jantunen, 1967] showed that high concentrations of cyanide were required to produce a 70% inhibition of respiration. Furthermore 10^{-4}M NaCN was "not fatal to the worms for at least one week." However the effects of the removal of cyanide on the QO_2 of C. briggsae were not reported. Experiments with carbon monoxide [CO] produced 16% inhibition of the QO_2 in light and 55% inhibition in the dark; the inhibitory effect was "apparently not fatal as worms in a CO atmosphere maintained themselves without reproduction for 3 weeks or more and apparently recovered after the inhibitor was removed." Bryant et al. [1967] stated that the residual respiration rate in the presence of CO may be due to an alternative carbon monoxide resistant pathway which was sufficient to keep C. briggsae alive. On this basis he stated that the terminal oxidase in C. briggsae was probably not of the classical cytochrome oxidase type but may be more similar to the cytochrome-b observed in parasitic helminths. [Cheah, 1969, 1970]. A. avenae isolate F, however, recovered from inhibition by NaCN equally well at 23°C and 30°C; temperature therefore had little or no effect on the rate of reactivation of the cyanide sensitive oxidase, in this isolate which was very susceptible to cyanide.

The slow but steady increase in the QO_2 observed when suspensions of isolate F were incubated at 30°C was linear up to 24 hrs and was more or less constant up to 48 hr. The temperature induced maximal QO_2 (at 24 hrs) was 30-40% higher than the QO_2 at 8 hrs. This increase was peculiar to isolate F and may be due to the production and assimilation of secondary metabolic end products such as organic acids. Cooper and Van Gundy (1970b) showed that succinate production in A. avenae occurred under aerobic/micro-aerobic/anaerobic conditions. Barrett (1978) pointed out the occurrence of glycolysis under aerobic conditions not only in parasitic helminths but also in certain mammalian tissues (such as red blood cells and the retina of eye) as well. This persistence of anaerobic metabolism in parasitic-helminths (aerobically) could be due to either peculiarities in the regulatory mechanisms of their catabolic pathways or the inability to reduce the flux through the glycolytic system during aerobic periods. (Barrett, 1976). If this is also possible for some free living forms, the persistence of anaerobic metabolism in isolates of A. avenae under aerobic conditions is a possibility and hence lactic-acid and ethanol production which Cooper et al. (1970b) found only under anaerobic conditions could also contribute to the increase in the QO_2 observed in isolate F following incubation at higher temperatures.

The prolonged incubation of suspensions of isolate F in cyanide caused a continued decrease in the QO_2 which was directly proportional to the time incubated in 6 mM NaCN. This may be due to any of the following affects; a) the gradual inhibition of the alternate terminal oxidase responsible for the residual QO_2 b) the unspecific inhibition of one or more enzyme complexes by cyanide or c) the retardation of the reactivation of the cyanide-binding-oxidase (probably cyt. $\underline{aa_3}$) due to the continuous permeation of cyanide to the iron centres of the cytochrome-oxidase. Of these, (a) is most probable.

Cytochrome-$\underline{O}$ is the alternate terminal oxidase, since cyt. $\underline{O}$ is partially sensitive to cyanide although much higher concentrations than those which inhibit cytochrome $\underline{aa_3}$ are required for the complete inhibition of cyt.$\underline{O}$.

In contrast to the results obtained with isolate F the QO_2 of isolate A increased (at 30°C) up to 8 hrs, but the QO_2 after 24 hrs at 30°C was 61-62% lower than that at 8 hr. This anomalous behaviour was peculiar to suspensions of isolate A and interpretation is difficult. However Barrett (1969b) reported that although the respiration of Strongyloides ratti larvae increased between 10 and 37°C, the temperature-induced maximal respiration which was sustained only for a short period, declined if the larvae were incubated

for longer periods at the higher temperatures (37°C). Von Brand (1979) refers to these observations but states that the underlying mechanism has not been elucidated. Considering the observations made in the present study it is possible to speculate as to the mechanisms which effect this in A. avenae. The maximal QO_2 of isolate A (observed at 8 hrs at 30°C) was approximately 35% inhibited by 6 mM NaCN. In marked contrast the lowered QO_2 (observed at 24 hrs at 30°C) was stimulated 100% or more by 6 mM NaCN. Similar stimulatory effects of cyanide have been reported by other workers. Von Brand (1979) quoting work by Lazarus (1950) and Rathbone, (1955) pointed out that the QO_2 of Paramphistomum cervi is strongly enhanced by cyanide and that the succinate oxidation by cellular fraction of Ascaris was doubled under the influence of KCN. Cheah (1966) reported that preparations of Moniezia expansa exhibited stimulation of the QO_2 in cyanide, and later (Cheah, 1967b) demonstrated the presence of 2 terminal oxidases, cyt. aa_3 and cyt. O in M. expansa, and showed that cyt. O was a source of hydrogen peroxide. On this basis he concluded that the stimulation of the QO_2 brought about by cyanide was due to the removal by cyanide of inhibitions imposed on certain flavoproteins due to the accumulation of hydrogen peroxide (H_2O_2). Singer and Kearney (1956) described a similar effect in mammalian succinic dehydrogenase (SDH). Cheah (1976) showed that the

cyanide stimulation of succinate oxidation in Ascaris preparations did not occur in the presence of added catalase. It was concluded (Cheah, 1976) that in the absence of added catalase, the H_2O_2 formed as a normal by-product by cyt.O during terminal oxidation, caused the oxidation of sulphydryl groups in the SDH-complex to di-sulphide linkages, bringing about the inactivation of the latter complex which in turn affected the decline in the measured QO_2. Under these conditions, cyanide brought about a stimulation of the QO_2 by relieving the inhibition imposed on the SDH-complex, by re-reducing the di-sulphide linkages (formed by H_2O_2) to the natural -SH state.

Barrett (1976), reviewing bioenergetics in helminths, substantiated these observations in stating that the "cyanide stimulation may be due to the removal of inhibition of the flavoprotein enzymes by virtue of the cyanide ions' carbonyl-combining or metal chelating properties or the more favourable redox-potentials of the cyanochromogens for oxidation". Another possible explanation for the stimulatory action of cyanide according to Barrett (1976) in that when the classical cytochrome sequence is blocked (by cyanide), the drop in ATP production causes an increased flux through the alternate oxidase in order to compensate for the increase in the ratio of ADP:ATP, and this demands a greater O_2 consumption.

However an alternate explanation is possible, i.e. if it is presumed that the mitochondrial membrane proteins and lipids are equally susceptible to attack by H_2O_2 produced by cyt.O (since the latter is also membrane bound), the oxidation of -SH groups of membraner-proteins and perhaps the epoxide formation of the unsaturated lipid components would invariably alter the tertiary conformation of the membrane, causing changes in the innate physico-chemical state of the whole membrane. This could in turn directly affect the permeability properties of the mitochondrial membranes, thus reducing the in-flow of oxidisable substrates to the electron-transport chain and therefore accounting for the lowered O_2 consumption. Cyanide could, as suggested by Cheah (1976), re-reduce the membrane-protein di-sulphide linkages and return the membrane to status quo.

The decline in the QO_2 of isolate-A whole worms on prolonged incubation at 30°C could also be explained employing a similar postulation. The fluid-crystalline phase of the mitochondrial membranes (Lyons et al., 1970) can be easily altered by heat-stress because the physical state, especially of the lipid components, are temperature dependent, and membrane-phase transitions have been demonstrated to occur in response to temperature (Lyons, 1970, 1973). Therefore changes in the lipo-protein complexes could be expected to occur in response to temperature and such changes could alter the

permeability of the mitochondrial membranes to oxidisable substrates and thus account for the reduced QO_2.

In view of these observations, it is suggested that the decline in the QO_2 of isolate-A whole worms on prolonged incubation at 30°C is probably due either to a) the reduced permeability of mitochondrial membranes induced by sustained high temperatures or b) due to the accumulation in the whole worms of an injurious metabolite viz. H_2O_2 which in turn causes the inhibition of other enzymes-complexes directly or indirectly connected with oxidative metabolism, or c) due to a combination of both a and b.

However, as will be presented in Chapter 7B, the mitochondrial fractions and the soluble fractions of isolate-A and F both showed the presence of catalase, i.e. hydrogen-peroxide-oxido reductase activity. Thus, it seems unlikely that H_2O_2 actually accumulates to any appreciable degree. Nevertheless under *in vitro* conditions and high incubation temperatures it is possible that the catalase activity becomes sub-optimal or that the activity of cyt.*O* increases to an extent where labile groups in the catalase enzyme itself is attacked by the increased generation of H_2O_2. These can be verified in future studies by assessing the catalase activity at various temperatures. The difference spectra of mitochondria at high temperatures may show whether cyt.*O* is more active under different temperatures; the

use of specific inhibitors in combination with O_2 electrode studies may be employed to indirectly assess the activity of cyt.*o* at various temperatures.

The mass culture of isolate-A at increased temperatures [$>30^{\circ}C$] apparently produces populations of this isolate which exhibit stimulation of the QO_2 in the presence of cyanide. This could be due to the following- a] the degree to which cyt.*o* mediates in terminal oxidation increases at higher temperatures b] the redox potential of cyt.*o* becomes more favourable for oxidation at temperatures above $30^{\circ}C$ c] the cyt.*aa*$_3$ becomes saturated at higher temperatures allowing electron flow to be directed to cyt.*o*.

However, the fact that older cultures exhibited little if any stimulation of the QO_2 with cyanide could be because the onset of low food supply in the cultures had triggered metabolic changes associated with starvation and perhaps incipient cryptobiosis. This may not however explain the unchanged cyanide sensitivity of populations from 'young' and 'old' cultures of isolate-F, unless it is presumed that fundamental metabolic differences between the two isolates exist.

The fact that EDB [1000 ppm] produced approximately 17% stimulation of the QO_2 of young cultures of isolate-A is of interest because Marks [1971] observed similar stimulatory effects of EDB on *Caenorhabditis* sp. and Morikawa [1964] reported similar effects on the

respiratory O_2 uptake of American cockroaches. However Marks [1971] reported that the strain of A. avenae he examined exhibited no such stimulatory effects in response to 0.52M EDB, but in fact, depressed the QO_2 of A. avenae by 30-40%. Awan [1975] reported approximately 27% inhibition of the QO_2 of A. avenae with 1000 ppm EDB. These latter results agree with the effect of EDB on A. avenae, isolate-F, which showed 21-26% inhibition of the QO_2 with 1000 ppm EDB. However the stimulatory effect of EDB on isolate-A and the inhibitory effect on isolate-F probably indicates basic physiological differences between these two isolates of A. avenae. Evans and Thomason [1971] reported that low dosages of EDB [10 ppm] sometimes increased the viability of A. avenae, whether such biological data can be correlated with observations on the response of oxygen consumption rates in EDB is debatable, though a relationship cannot be denied. Thus further experiments using low EDB dosages is necessary to clarify this further.

The ability of worms of isolate-F to recover their original QO_2 after 30 mins exposure to 1000 ppm EDB agrees with Castro's [1977] suggestion that most organisms possess an endogenous capacity to re-reduce the iron in haemoproteins to the functional Fe-II state since he postulated that EDB interacts with Fe-II porphyrins by oxidising them to Fe-III porphyrins. However the sensitivity to EDB of the worms of isolate-F

following washing and resuspension in water was much reduced showing mean inhibition of 2-3%. This again was similar to observations by Marks [1971] on Caenorhabditis sp., where he found that the re-exposure to a second treatment of EDB, of EDB treated/washed Caenorhabditis L_3s exhibited a QO_2 100% higher than those kept in water after first exposure and washing.

The effect of simultaneous application of 1000 ppm EDB and 3mM NaCN produced near complete inhibition of the QO_2 of isolate-F and the response was immediate with no lag-phase. This indicates that EDB and NaCN act synergistically on the processes leading up to respiratory oxygen uptake, since the inhibition produced by the combination of the two biocides was greater than that produced by either per se. The QO_2 of isolate-A was only 63% inhibited [by NaCN and EDB] suggesting that this isolate is less sensitive to these agents. However in order to assess these different responses elicited by isolates, further work is necessary in order to know the extent of variability which occurs within a single isolate.

Hyperbaric O_2 treatment of isolate-A had little effect on the QO_2 or the stimulatory effect of cyanide. However the reduction in cyanide sensitivity of isolate-F following hyperbaric O_2 treatment suggests that the normally cyanide sensitive oxidase[s] functions below maximum capacity at high O_2-tensions. This may be

because at higher O_2 tensions the competition between cyt.O and cyt.aa_3 favours cyt.O resulting in reduced terminal oxidation mediated by cyt.aa_3.

The failure of cyanide to stimulate the QO_2 of anaerobically treated isolate-A suggests that the effect of cyanide is specifically dependent on some process for which molecular oxygen is required. The presence of cyt.O would explain this observation, since in the absence of oxygen no hydrogen peroxide formation would occur and thus no depression of flavoprotein enzymes would occur and thus cyanide would not bring about a stimulation of the QO_2. Cheah [1969b] postulated that in the absence of O_2 fumarate accepts electrons from cytochrome-O. Whether this occurs in isolate-A is not known but succinate production by A. avenae [Cooper & Van Gundy, 1970b] suggests this as a strong possibility. The lack of any response in isolate-A, following anaerobic treatment, to cyanide may mean that the metabolism under anaerobic conditions is independent of cytochromes and is probably glycolytic, a conclusion arrived at also by Cooper and Van Gundy [1970b].

The reduced QO_2 of isolate-F following anaerobic treatment suggests that isolate-F behaves as a typical "regulator" with regard to external [ambient] oxygen tension. The reduced cyanide sensitivity of post-anaerobic isolate-F is probably because the classical oxidase cyt.aa_3 is operating sub-optimally since under

anaerobic conditions glycolytic [i.e. fermentative] metabolism is bound to predominate for some time after return to oxygen, supporting the suggestions by Cooper and Van Gundy [1970b].

Lowered rates of O_2 consumption are not always the result of the blockage of a component of the respiratory chain since the inhibition can also occur due to the prevention of reactions leading to the formation of the main substrate of the respiratory chain, reduced NAD. Inhibitors such as iodoacetate, arsenicals, and para-chloro-mercuribenzoate [p-CMB] attack enzymes depending for their activity on functional sulphydryl groups [Von Brand, 1979]. Trypanosomes of the Lewisi group [T. lewisi and T. cruzi] are extremely sensitive to cyanide [80-100% inhibition to 3mM] but are much less sensitive [10-55% inhibition] to even weak sulphydryl inhibitors such as bromoacetate [Von Brand, 1979, quoting von Brand & Tobie, 1948]. The Evansi group on the other hand showed an increased QO_2 with 10^{-2}M KCN, but were highly sensitive [> 90% inhibition] to 1mM bromoacetate [Von Brand, 1979]. Because of these reports the effect of p-CMB, a potent sulphydryl inhibitor, was tested on isolate-A. p-CMB was the only compound which singly inhibited 89-90% of the QO_2 of whole worms of isolate-A. Thus the behaviour of whole worms of isolate-A resembles those of the members of the Evansi sub-group of trypanosomes, with regard to the response to NaCN and sulphydryl inhibitors.

It has been known for a long time that cyanides promote germination of many kinds of seeds. [Roberts, 1969]. Thus cyanide resistant respiratory activity has been implicated in germinating seeds [Hendricks & Taylorson, 1972]. Bendall and Bonner [1971] reported that a flavin containing non-haem Iron protein [nhIP] was involved in the cyanide resistant system. Such systems [in mung beans and skunk cabbage mitochondria] were inhibited by aryl-hydroxamates and to a lesser extent by thiocyanates [Schonbaum & Bonner, 1971]. Derivatives of hydroxamic acid have been used in resolving the multiple-oxidase systems in trypanosomes. Hill and Cross [1973] showed that salicyl hydroxamic acid [SHAM] inhibited cyanide insensitive respiration in trypanosomes and the site of inhibition was thought to be between cyt.b and cyt.O. However Opperdoes and Borst [1976] and Hill [1976] have shown that SHAM is a specific inhibitor of the α-glycerophosphate-oxidase [GPO] complex.

SHAM [3mM] caused 11-18% inhibition of the QO_2 of whole worms of isolate-A; if it is assumed that SHAM is a specific inhibitor of the GPO complex, then it is possible that isolates A and F have potential GPO capacity. But the GPO complex in trypanosomes is not inhibited by CO, cyanide, azide, or antimycin A, unlike the GPO of mammalian brain mitochondria and insect flight muscle.

There seem to be no effects on respiratory metabolism which can be attributed to the higher concentration of gases like carbondioxide (CO_2). Isolate-A cultures grown under continuous ventillation in Kilner jars produced the same stimulatory responses of the QO_2 in response to NaCN and EDB and a similar great sensitivity to p-CMB, suggesting that fixation of external gaseous CO_2 is not a necessity for isolate-A in eliciting these peculiarly characteristic responses to biocides.

CHAPTER 6

Analysis of metabolic end-products secreted by whole-worms of A. avenae isolates A and F

Introduction

Caenorhabditis briggsae released a large variety of ^{14}C radio labelled products especially amino acids following incubation with various radio labelled substrates (Rothstein, 1963; 1965). It was later shown that large amounts of labelled glucose, trehalose and glycerol were produced from ^{14}C acetate, by C. briggsae (Rothstein, 1969). Of these glycerol was the major radioactive product if C. briggsae was incubated with 2 ^{14}C acetate in "whole (axenic) medium", whereas incubation in water produced very little glycerol, glucose and trehalose being the major products (Rothstein, 1969). Turbatrix aceti and Panagrellus redivivus behaved similarly when incubated in water, but in "whole medium" other neutral products, "presumably sugars, were produced in addition to glycerol" (Rothstein, 1969).

In microaerobic and anaerobic environments the principal glycolytic end products in A. avenae were lactic acid (during the first 12-16 hrs) after which it was ethanol. (Cooper & Van Gundy, 1970b). Incubation solutions of whole-worms and homogenates were analysed for organic acids, alcohols, glycols and ketones by Cooper et al. (1970b) and it was reported that under anaerobic conditions, incubates contained ethanol which increased to an equilibrium at 40 hours and thereafter

decreased; no acetic acid, or acetaldehyde precursors of ethanol were detected under aerobic or anaerobic conditions. Under aerobic conditions Caenorhabditis sp. produced acetaldehyde [during the first 12-16 hours] which decreased to non-detectable levels after 30 hours, following which only ethanol was detected. Ethanol in the medium came to equilibrium with the homogenates in 34 hours.

Cooper and Van Gundy [1970b] reported the detection of an unidentified 4-carbon alcohol which accounted for 5-10% of catabolized glycogen and also stated that some ketones and glycols may have been present in concentrations not detectable in the tests. Alcohols C_1-C_5, aldehydes C_1-C_5, glycols and ketones were analyzed using gas-liquid chromatography [GLC] using Porapak-Q-columns. [Cooper et al., 1970b]

Investigating the metabolic changes during induction of and recovery from anhydrobiosis in A. avenae, Madin, Crowe and Loomis [1979] reported that trehalose and glycerol increased dramatically during desiccation. Glycerol began to increase in A. avenae after 24 hours. of slow-drying at 97% R.H. They reported a coincidence between the onset of glycerol and trehalose synthesis and the increased ability to survive exposure to dry air. The water content of A. avenae "pellets" at the onset of glycerol and trehalose synthesis was 2.4 mg water/mg dry wt. Survival [during desiccation] was a linear function of water, trehalose and glycerol content when nematode

water content is below 2.5 mg water/mg dry wt., - a value close to 2.4 mg/mg dry wt. at which glycerol appears in drying worms [Madin et al., 1979].

The changes on re-hydration were essentially the reverse of those seen during dehydration. Glycogen was resynthesized while trehalose and glycerol contents in the worms declined and the glycerol which appeared in the water surrounding the worms declined slowly for the next 20 hours. Madin et al. [1979] pointed out that although "considerable heterogeneity existed between individual worms" the correlation between glycerol and trehalose contents in the worms and survival of desiccations was so strong that it is likely that these compounds play important roles in the survival of dehydration.

Madin et al. [1979] concluded that A. avenae synthesizes glycerol and trehalose at the expense of lipid, probably via the glyoxylate pathway. Although this pathway is well known from studies on micro-organisms, it has not been extensively studied in animals, most of whom do not seem to possess the appropriate enzymes [Madin et al., 1979]. However, a number of nematodes have been shown to possess the key enzymes [Madin, Crowe and Loomis, 1979].

Madin et al. [1979] found evidence for the operation of the glyoxylate cycle in A. avenae using ^{14}C acetate as tracer. However there are other pathways which are known to be operative in certain parasitic

protozoa (such as the α-glycerophosphate-oxidase complex of certain Trypanosome sp.) which are also capable of glycerol formation in association with the glycolytic pathway. Although evidence gathered during this project (see Chapter 5 and 7) suggests that the α-GP-oxidase complex may be operative in A. avenae no attempts were made to determine whether glycerol formation occurred via this pathway, since this was not within the scope of the project.

Therefore experiments were planned to assess the volatile organic end products secreted into aqueous media by A. avenae whole-worms of isolates A and F, under aerobic and anaerobic conditions.

Materials and Methods

A. avenae mass cultures of isolates A and F (cultured at 25°C) were harvested at 35 days. Suspensions of mixed stages were prepared from active worms after 12 hours standard extraction period. In order to favour detection of volatile metabolites approximately 3g wet weight of nematodes were suspended in 20 ml of sterile distilled water. Two such groups were prepared for each treatment. To one group 100 units penicillin and 100 µg streptomycin per ml were added to the incubation medium. The suspensions were bubbled with O_2 free N_2 for 1 hour in 150 ml flat bottomed quick fit flasks, which were then sealed and contained in a sealed desiccator flushed with O_2-free N_2 for three 30 min periods. The desiccator was incubated at 27°C in a controlled temperature cabinet for 30 hours.

Similar suspensions of worms from both isolates were bubbled with 100% of humidified/filtered air for 1 hour in flat bottomed 150 ml quick fit flasks which were incubated at 27°C for 30 hours.

Following incubation, the bathing solutions (=incubates) were carefully removed and rapidly introduced into chilled (-20°C) sterile quick fit tubes and stored in deep freeze at -30° to -40°C for further use.

Aliquots of the frozen samples were analysed directly from the aqueous phase using gas liquid chromatography on a 200 cm x 3.2 mm column containing

Porapak-P (80-100 mesh size) in a Perkin Elmer gas chromatograph. A zone temperature of 240°C and an oven temperature of 190°C was employed with flame ionization (F.I.D.) and nitrogen carrier gas. The attenuator was adjusted at 8.

Analysis of the aqueous incubates was possible using Porapak-P because water elutes more rapidly than the compounds of interest (such as ethanol, acetaldehyde, glycerol). Qualitative and quantitative measurements were based on retention times and areas under the curves obtained and were compared using known concentrations of standards (ethanol, butanol, acetaldehyde and glycerol).

Table 16 Analysis of volatile metabolites in whole worm incubates of isolates A and F following 30 hours anaerobiosis at 27°C

Chemical Compound		Retention time [R_t] Porapak-P column (2m x 3.7cm)	Isolate F	Isolate A	Concentration Isolate F	Concentration Isolate A
Solvent [H_2O]		9.6				
Ethanol		19.2 secs	+	++	3880 to 4000 ppm	6700 to 8000 ppm
Unidentified compounds	P	33.6 secs	+	-	-	-
Unidentified compounds	Q	81.6 secs	+	+	-	-
Unidentified compounds	R	158.4 secs	-	+	-	-
Probably a glycol.	S	168.0 secs	+	+	-	-
	T	187.2 secs	-	+	-	-
Glycerol		624.0 secs	+	++	109-114 ppm	1360-1390 ppm

Four to six replicate samples were analysed for each isolate.

Results

The recordings obtained by the GLC analysis are shown in Figs. 37A, 37B, 38A and 38B.

Fig. 37A and Fig. 37B show typical traces obtained with anaerobic incubates of isolate A and isolate F respectively and Table 16 shows the retention times of the compounds under anaerobic conditions and the quantities. Isolate A and F both produced ethanol but isolate A produced almost twice as much as isolate F. Quantities of glycerol detected in isolate A [i.e. 1360-1390 ppm] were nearly 10 fold greater than that detected in isolate F incubates. In addition to these, two unidentified compounds Q and S common to both isolates were detected. Compound P was only found in isolate F and compound R and T only in isolate A. It is likely that S is a glycol. [Table 16]

Fig. 38A and 38B show typical traces obtained with aerobic incubates of isolate A and F respectively. The retention times of compounds are shown in Table 17. No ethanol was detected in either isolate, but an unidentified compound P, [retention time 33.6 secs] detected in anaerobic incubates of isolate F was also seen in aerobic incubates of isolate-A but not in isolate-F. Compounds Q, R, S and T did not occur in aerobic incubates. However two other compounds X and Y were apparent in aerobic incubates of isolate F only. Glycerol occurred in both isolates aerobically although in much smaller quantities than found in anaerobic incubates, and even aerobically

Table 17 Analysis of volatile organic metabolites in whole worm incubates of isolates A and F following aerobiosis at 27°C

Chemical Compound	Retention time (R_t) Porapak-P column (2m x 33mm)	Isolate F	Isolate A	Concentration Isolate F	Concentration Isolate A
Solvent (H_2O)	9.6 secs				
Ethanol*	19.2 secs	-	-	0	0
Unidentified Compounds P	33.6 secs	-	+	-	-
Unidentified Compounds Q*	81.6 secs	-	-		
Unidentified Compounds R*	158.4 secs	-	-		
Unidentified Compounds S*	168.0 secs	-	-		
Unidentified Compounds T*	187.2 secs	-	-		
Unidentified Compounds X	48.0 secs	+	-		
Unidentified Compounds Y	100.8 secs	+	-		
Glycerol	624.0 secs	+	+	11-15 ppm	60-75 ppm

* Only detected in anaerobic incubates

4-6 replicate samples were analysed for each isolate

Fig. 37A GLC trace of volatile products secreted into an aqueous medium by whole worms of isolate-A under anaerobic conditions. Incubation temperature 25°C.

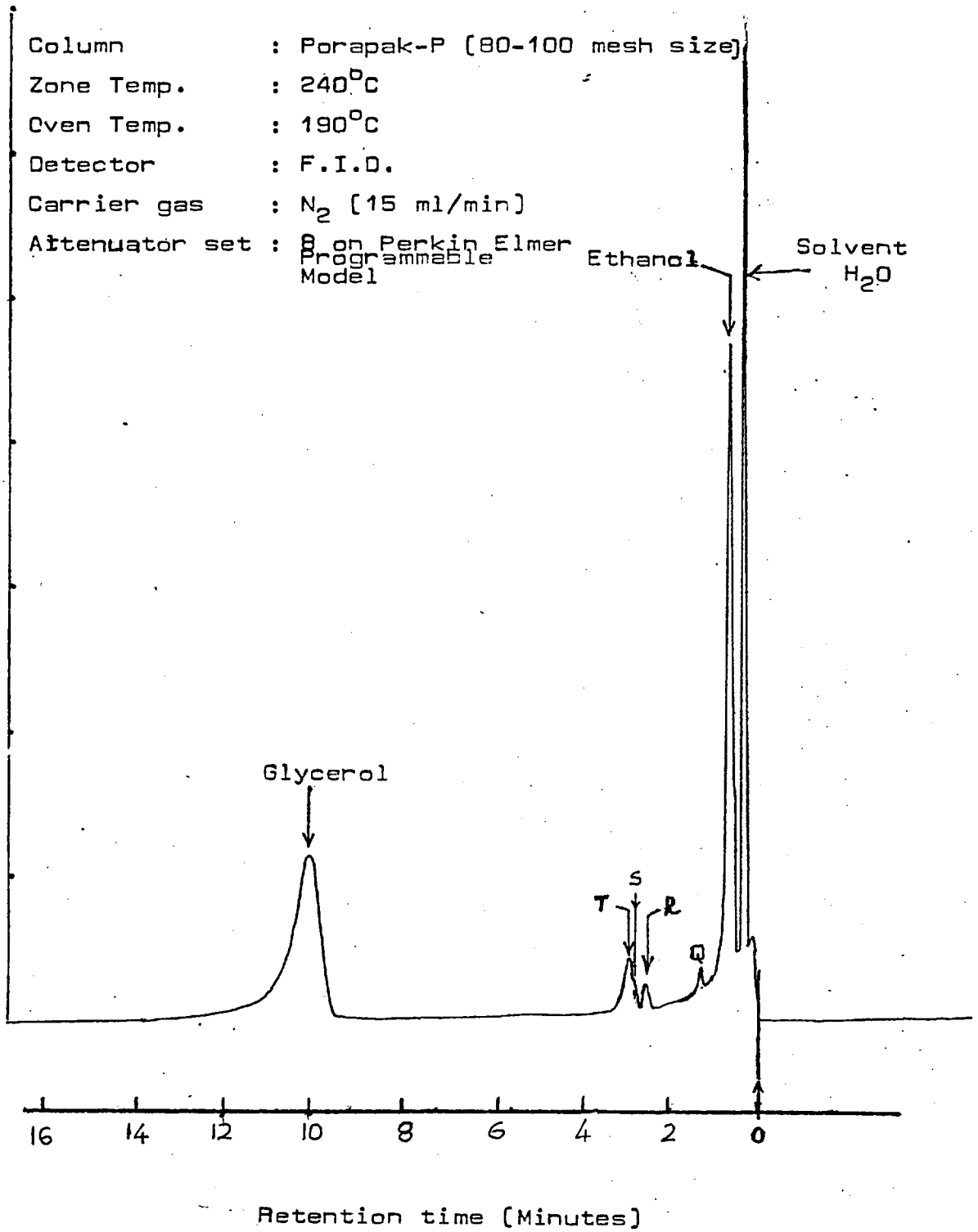


Fig. 37B GLC trace of volatile products secreted into an aqueous medium by whole worms of isolate-F under anaerobic conditions. Incubation temperature 25°C.

Column	: Porapak-P (80-100 mesh size)
Zone temperature	: 240°C
Oven temperature	: 190°C
Detector	: F.I.D.
Carrier gas	: N_2-15 ml/min
Attenuator set	: 8 on Perkin Elmer
Column length	: 200 cm
Column diameter	: 3.3 mm

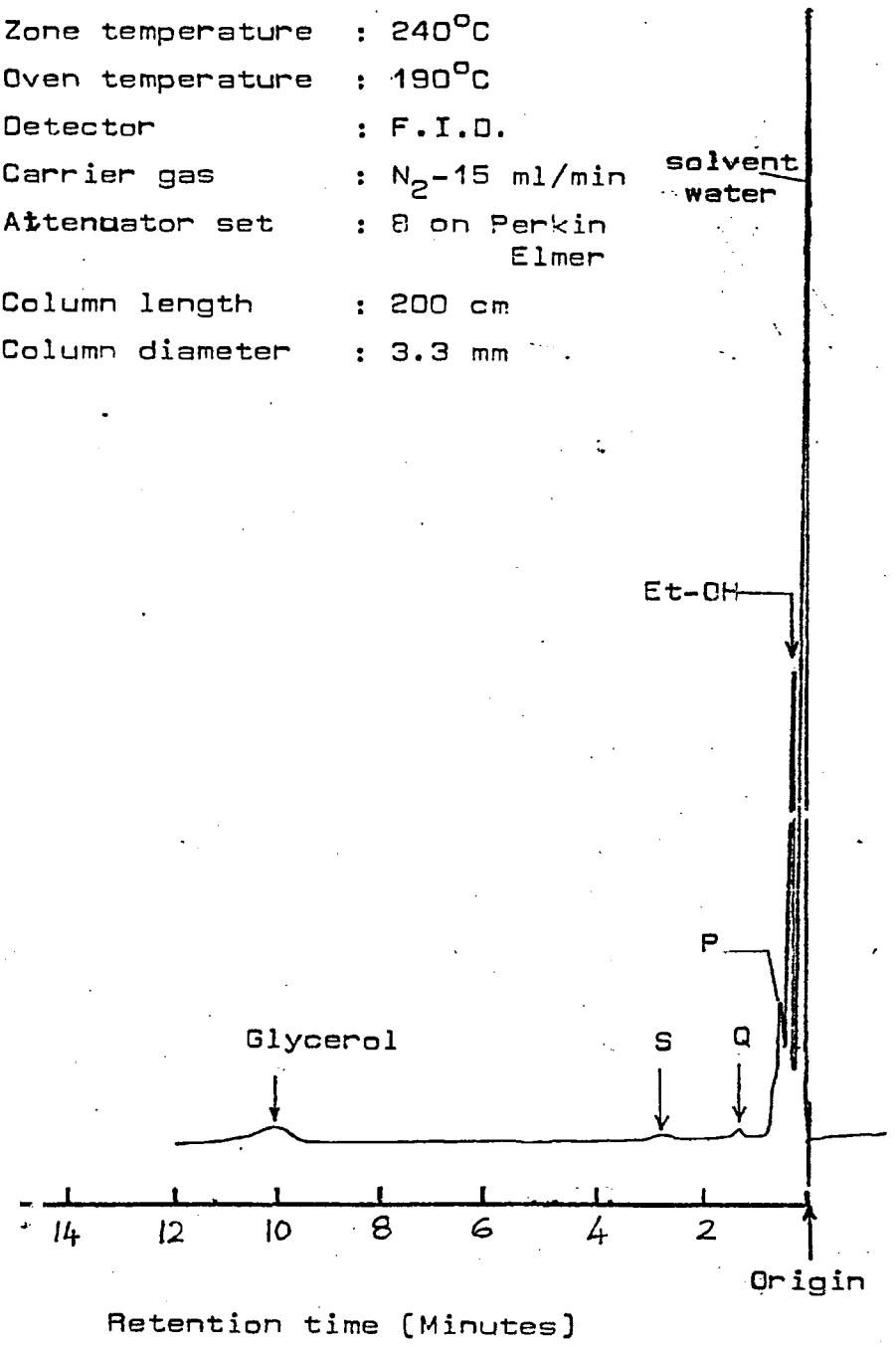


Fig. 38A GLC trace of volatile products secreted into an aqueous medium by whole worms of isolate-A under aerobic conditions. Incubation temperature 25°C.

Column	: Porapak-P (80-100 mesh size)
Zone Temp..	: 240°C
Oven Temp.	: 190°C
Detector	: F.I.D.
Carrier gas	: N_2-15 ml/min
Attenuator set	: 8 on Perkin Elmer
Column length	: 200 cm
Column diameter	: 3.3 mm

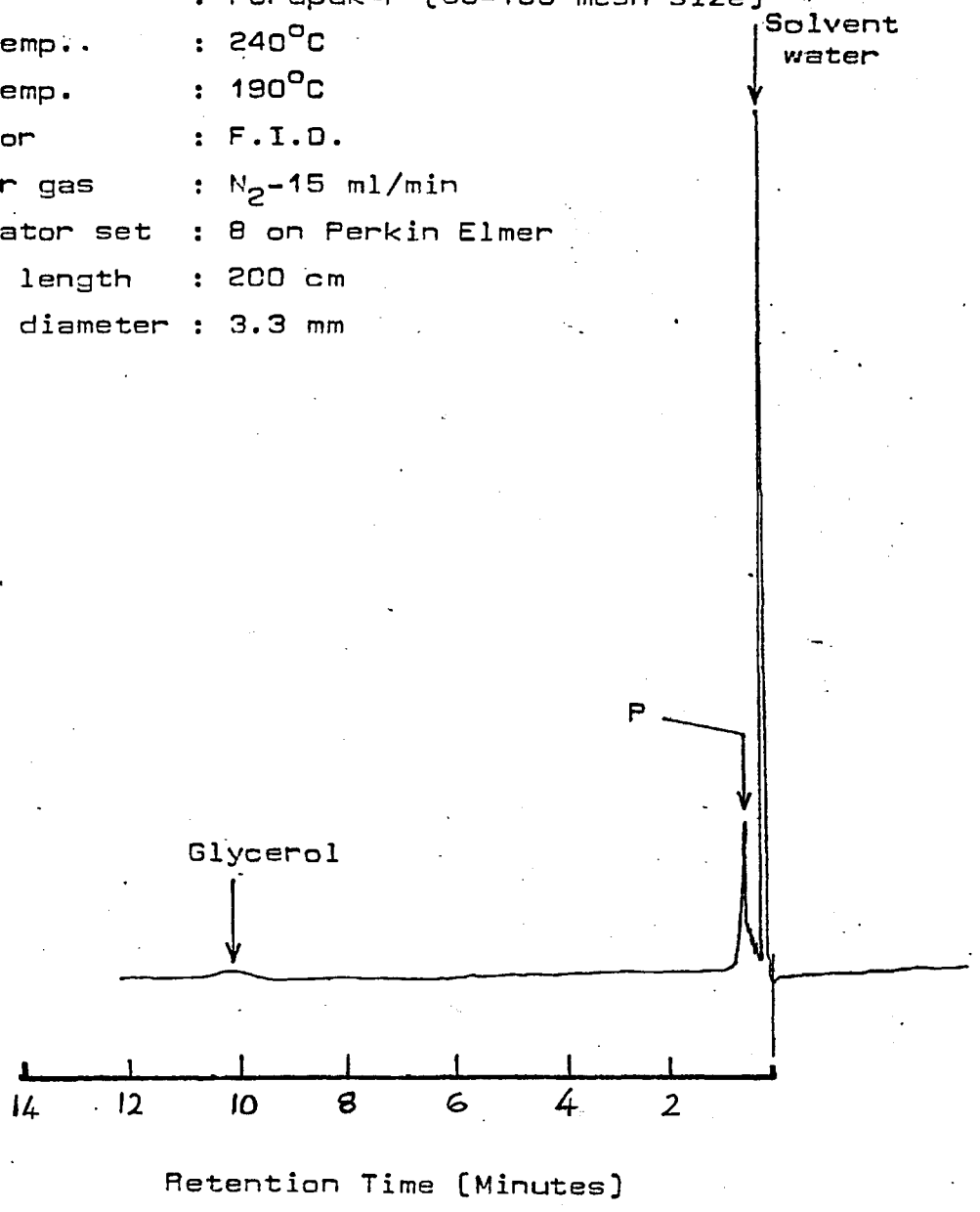
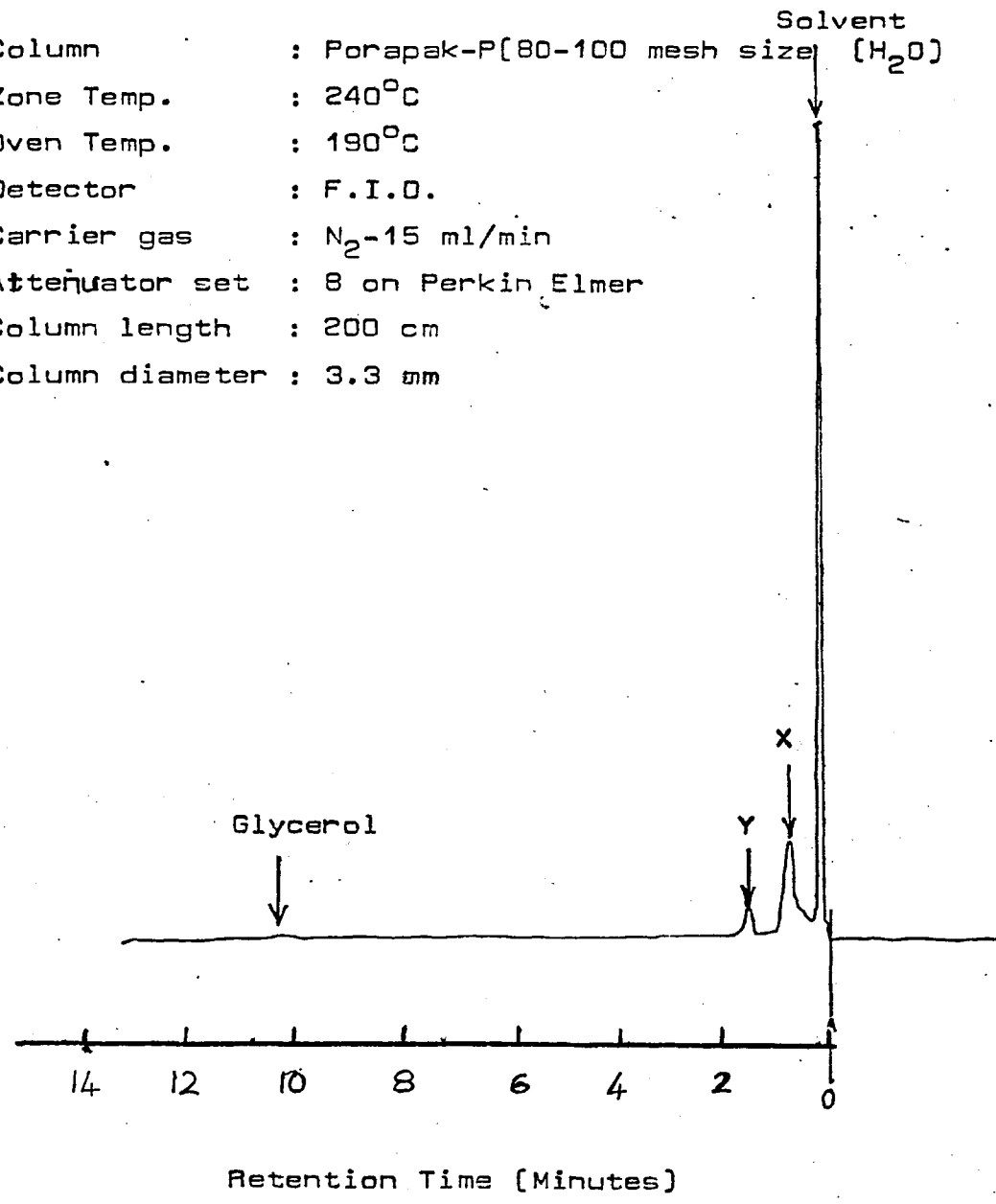


Fig. 38B GLC trace of volatile products secreted into an aqueous medium by whole worms of isolate-F under aerobic conditions. Incubation temperature 25°C.

Column	: Porapak-P[80-100 mesh size]
Zone Temp.	: 240°C
Oven Temp.	: 190°C
Detector	: F.I.D.
Carrier gas	: N_2-15 ml/min
Attenuator set	: 8 on Perkin Elmer
Column length	: 200 cm
Column diameter	: 3.3 mm



isolate A produced 6 - 7 times more glycerol than isolate F. There were no differences when the nematodes were incubated in penicillin/streptomycin containing media.

Discussion

The results are consistent with those of Cooper and Van Gundy (1970b) in that no ethanol was detected in either isolate under aerobic conditions, although the unidentified compound-P could be a 3 or 4 carbon alcohol. However compound-P did not co-chromatograph with propanol or butanol standards.

The differences in the amounts of ethanol detected in the isolates cannot be due to the quantities of nematodes used since equal whole worm wet weights were incubated in identical volumes of sterile distilled water. The fact that isolate A produced almost twice the ethanol of isolate F suggests different glycolytic or fermentative capacities between the isolates, although permeability characteristics may also be involved. The major difference was the amounts of free glycerol produced by isolate A under anaerobic conditions which was nearly 10 fold greater than that produced by isolate-F

Glycerol production and accumulation was observed during rehydration of anhydrobiotic A. avenae (Madin, Crowe & Loomis, 1978) and it has been suggested that glycerol and trehalose are synthesized at the expense of lipid, probably via the glyoxylate pathway. Madin et al. (1978) confirmed the presence of isocitrate-lyase (IL) and malate synthase (MS) and showed that MS activity increases only when A. avenae dried at 97% RH with a concomitant decrease in IL activity. These changes in the activity of IL and MS apparently shifts the metabolism towards glycerol synthesis.

According to Rothstein (1969) the most logical pathway for glycerol synthesis would be one consistent with gluconeogenesis : conversion of acetate ⟶ oxaloacetate (TCA-cycle) and thence to phosphoenolpyruvate (PEP) by the action of phosphoenolpyruvate carboxy-kinase (PEPCK) and the fixing of CO_2. At the stage of 3-phosphoglyceraldehyde a branch point exists at which either glycerol (by reduction) or glucose could be formed. If the medium in which C. briggsae, T. aceti and P. redivivus were incubated contained glucose, the "stress" would be on the synthesis of glycerol; and if incubated in water, glycerol synthesis would be suppressed forcing the reaction towards glucose production. Alternatively he suggested that glucose synthesis could be viewed as being suppressed in "whole medium" which contained substantial amounts of glucose. Salts, vitamins, structural compounds, soy-peptone were the other components of the whole medium. The factor causing conversion of acetate-carbon into glycerol is not known since partially constituted media did not result in glycerol production. Discussing these observations Rothstein (1969) questioned why the glucose in the medium is not the source of glycerol rather than the "energetically unfavourable acetate", since he showed that acetate C^{14} was rapidly metabolized into glycerol. This mechanism for turning glycerol synthesis on or off depending on the constitution of the external medium Rothstein reported was common to all three species of free living nematodes i.e. C. briggsae, T. aceti and P. redivivus.

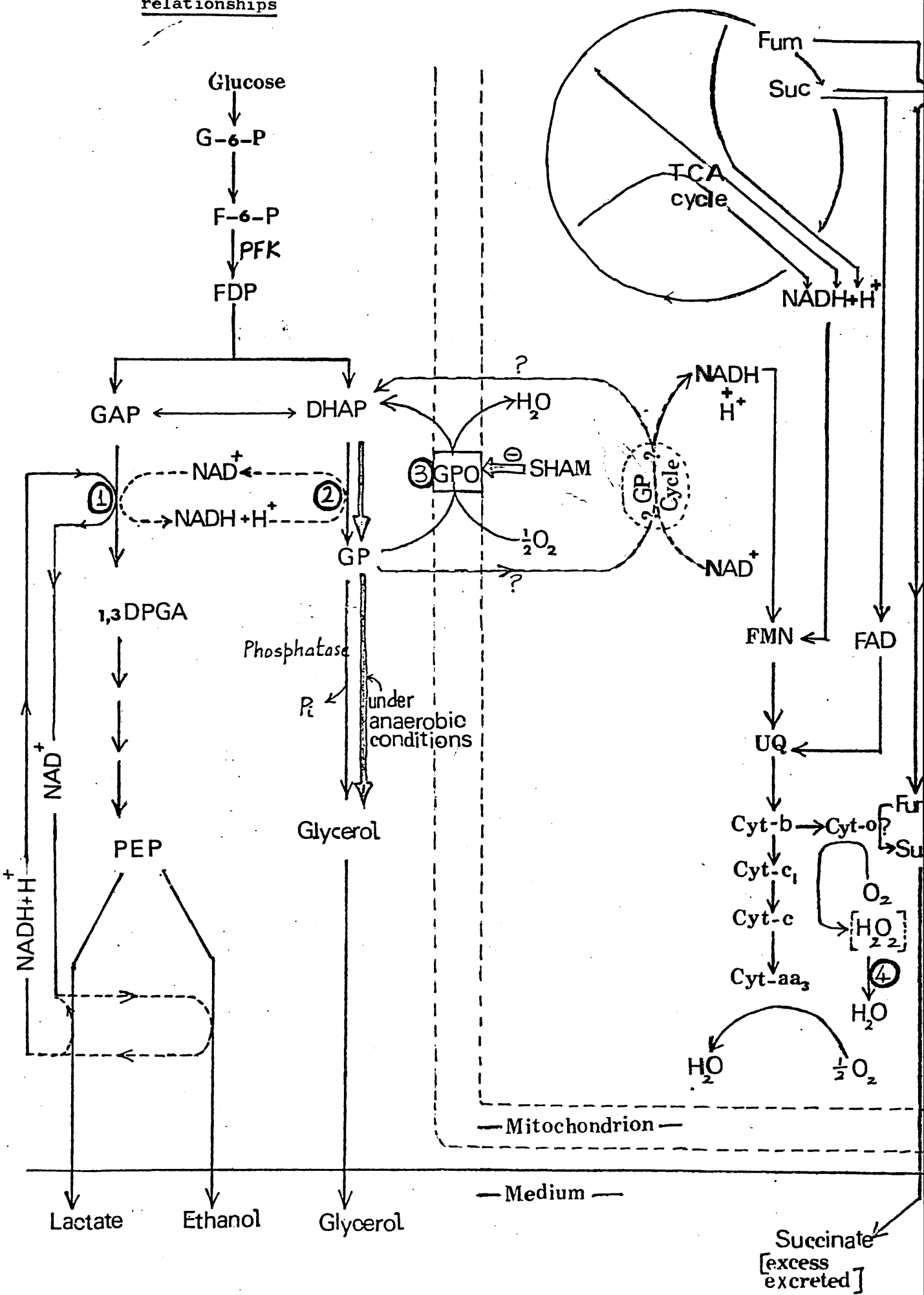
The observations in Chapter 5 of salicylhydroxamic acid (SHAM) sensitive oxygen-uptake of whole worms of isolates F and A, together with evidence of the inhibition of mitochondrial respiration by SHAM (see Chapter 7) suggests the presence in A. avenae of α-glycerophosphate oxidase (GPO). This is an unusual oxidase which is linked to glycolysis for the re-oxidation of $NADH+H^+$, and has been observed in insect-flight muscle, mammalian brain mitochondria and in a majority of trypanosome species. (Bowman & Flynn, 1976). The trypanosome GPO unlike the others reacts with oxygen without the intervention of pyridine nucleotide co-enzymes or of cytochromes and is insensitive to cyanide, azide, amytal and antimycin-A, but is specifically inhibited by SHAM (Opperdoes & Borst, 1976). Iron, thiol-groups and FAD are components of the oxidase which probably consists of a glycerol-3-phosphate:oxygen oxido-reductase and a substrate specific peroxidase. The relationship between glycolysis and GPO is shown in Figure 39.

Reaction-I generates $NADH+H^+$, which is reoxidised in reaction-2 reducing in the process DHAP to GP. GP in turn is reoxidised to DHAP by GPO complex viz. reaction-3. Thus only catalytic amounts of DHAP are required for the reoxidation of $NADH+H^+$ to NAD^+, which is necessary for the maintenance of glycolysis. (Bowman & Flynn, 1976). By such a coupling of glycolysis to GPO by GP-dehydrogenase (enzyme catalysing reaction-2) glucose can be quantitatively converted to pyruvate, which could enter

Abbreviations for Figure 39

G-6-P	-	glucose-6-phosphate
F-6-P	-	fructose-6-phosphate
FDP	-	fructose-1,6-diphosphate
GAP	-	glyceraldehyde-3-phosphate
DHAP	-	dihydroxy-acetone phosphate
1,3 DPGA	-	1,3 diphospho-glycerate
PEP	-	phosphoenol pyruvate
Gly-3-P	-	L-glycerol-3-phosphate
Suc	-	Succinate
Fum	-	Fumarate
SHAM	-	Salicylhydroxamate
PFK	-	Phosphofructokinase
1	-	Glyceraldehyde-3-phosphate NAD^+-oxido reductase
2	-	L-Glycerol-3-phosphate NAD^+ oxidoreductase (=LαGPDH)
3	-	L-α-glycerol-3-phosphate oxidase (GPO)
FMN	-	$NADH+H^+$ dehydrogenase
FAD	-	Succinic dehydrogenase
4	-	Catalase (=Hydrogen peroxide oxido reductase)
UQ	-	Ubiquinone
Cyt	-	cytochrome

Figure 39: The possible metabolic schemes operative in A.avenae and their relationships



the TCA after being converted to acetyl-CoA by the pyruvate-dehydrogenase-complex. Thus the redox-balance could be maintained aerobically.

Under anaerobic conditions, since GPO is not functional in the absence of oxygen, DHAP acts as the terminal electron acceptor and substrate amounts [as opposed to catalytic-amounts] of DHAP are reduced to GP and hence by the action of a phosphatase to glycerol. It is possible that in A. avenae isolate-A especially, anaerobic glycerol synthesis occurs in this manner. However the reoxidation of reduced-NAD could also occur by way of LDH and ADH with concomitant production of lactate and ethanol respectively. Thus A. avenae may have 3 alternate ways in which glycolytic $NADH^{+}H^{+}$ could be reoxidised. The degrees to which they operate may differ among isolates and therefore the anaerobic end products may show variations.

The synthetic pathways for glycerol postulated by Rothstein [1969] for T. aceti, C. briggsae and P. redivivus, envisages the switching off and on of glycerol synthesis in response to glucose in the external medium. In the hypothesis put forward by Madin et al. [1978], the stimulus for glycerol and trehalose synthesis in A. avenae is the loss of water i.e. dehydration [which is also a form of enforced quiescence].

It seems likely that trace amounts of glycerol are synthesized in A. avenae under normal environmental conditions as evident in the results obtained in this

Chapter. In the natural soil habitat of A.avenae, even trace amounts of glycerol would be of significant survival value since it protects against desiccation. It is probable that the slow aerobic rate of glycerol synthesis could be increased under various conditions and it may be argued that GPO-coupled glycolysis would be advantageous over other formentative mechanisms involving the accumulation of lactate or ethanol which may be more toxic than glycerol.

CHAPTER 7

Studies on the mitochondrial fractions of A. avenae isolates A and F

Introduction

The oxidative metabolism of free living nematodes has not been extensively studied. The reported resistance of organisms such as Turbatrix aceti (Ells, 1965 as quoted by Rothstein et al., 1970) and of Caenorhabditis briggsae (Rothstein, 1963) to cyanide toxicity led initially to the question as to whether they possessed a classical respiratory chain. On the basis of the resistance of C. briggsae to (1.0mM) cyanide Rothstein and Tomlinson (1962) tentatively suggested that electron transfer in this organism might be independent of cytochrome systems. However Rothstein (1963) subsequently cited unpublished evidence for the presence of a cytochrome of the b-type in C. briggsae but did not report conclusively on the presence of a complete cytochrome chain.

Bryant, Nicholas and Jantunen (1967) found that carbon monoxide markedly inhibited the respiration (more correctly oxygen uptake) of C. briggsae whole worms in the dark but the effect was largely removed when the worms were exposed to light and the inhibitory effect was not fatal as the worms in a carbon monoxide (CO) atmosphere maintained themselves without reproduction for periods longer than 3 weeks, and recovered following removal of the inhibitor. They suggested that the residual "respiration" may be due to an alternative

CO-resistant pathway since 10^{-4}M cyanide which produced 70% inhibition was also not fatal to the worms for periods of up to one week.

In the same study of C. briggsae (Bryant, Nicholas & Jantunen, 1967) a particulate fraction prepared by subjecting the worms to sonic disruption contained a b-type cytochrome, cytochrome-c and traces of cytochrome-a. The absorption maxima of the b-type cytochrome they reported was similar to the b-type cytochrome demonstrated in Ascaris (Chance & Parsons as quoted by Bryant et al., 1967) and Moniezia (Cheah & Bryant, 1966). The particulate preparation incorporated radio carbon from succinate into only 2 intermediates of the tri-carboxylic acid cycle (TCA cycle) namely fumarate and malate. However they were unsuccessful in reducing the presumptive cytochrome components of C. briggsae with succinate or α-GP even in the presence of antimycin-A, cyanide and carbon monoxide. However, indirect evidence that the pigments may be involved in respiration was obtained by the addition of cyanide, and by passing carbon monoxide through the preparations after reduction with sodium dithionite. They reported that the normal spectrum was considerably altered by both inhibitors although no spectra were published, and suggested that alternative pathways may be present. No polarographic studies of the preparation were presented to substantiate the effect of inhibitors.

It has been reported (Ells & Read, 1961 as quoted by Rothstein *et al.*, 1970) that a number of TCA-cycle related substrates did not increase the oxygen uptake in intact *Turbatrix aceti* or in cell free homogenates from *T. aceti* fortified by various co-factors and neither cytochrome pigments nor cytochrome oxidase could be demonstrated. Ells and Read (1961) as quoted by Rothstein et al. (1970) concluded that *T. aceti* probably lacks a TCA cycle. Rothstein (1963) demonstrated the metabolic conversion of acetate to TCA-cycle related amino acids and concluded that the cycle was operative in *T. aceti*. In a later study Rothstein (1969) demonstrated the conversion of acetate to glycerol in *T. aceti*, *Panagrellus radivivus* and *C. briggsae* suggesting the presence of an active TCA-cycle in these free living nematodes.

In a more recent study of the respiratory metabolism of *T. aceti* (Rothstein *et al.*, 1970) it was demonstrated that the mitochondria derived from this organism readily oxidised succinate showing a respiratory control ratio (RCR) of 2.2. Although α-GP oxidation was not reported most other TCA-cycle related substrates such as glutamate, malate, pyruvate, β-hydroxybutyrate, α-ketoglutarate exhibited RCRs of between 2 and 6.2. (Rothstein et al., 1970). Although the effect of antimycin-A was not reported by Rothstein et al. (1970), it was reported that the *T. aceti* mitochondria were sensitive to azide and cyanide thereby indicating that cytochrome aa_3 was the terminal oxidase. However

37 and 74 μM cyanide produced only partial inhibition of the state-3 oxidation while 111 μM cyanide was required for complete inhibition. Added mammalian cytochrome-c was found to interact with the system stimulating state-3 QO_2. 2,4,dinitrophenol (DNP) also stimulated the succinate oxidation of the mitochondrial preparation of T. aceti.

The difference spectrum of T. aceti mitochondria reduced in the presence of succinate induced a 3-banded spectrum with absorption maxima at 522nm, 550nm, 563nm and 600nm which was qualitatively similar to those obtained with mammalian systems (Rothstein et al., 1970) except that cytochrome-a was lower. Addition of dithionite produced additional bands at 565nm 535nm. When carbon monoxide (CO) was bubbled through the dithionite reduced system a pair of intense absorption maxima were observed at 537 and 568nm, accompanied by a Soret band at 420nm with a concomitant decrease in absorbance at 445nm. In the presence of cyanide the peak at 445 was almost completely abolished when succinate was used to reduce the component cytochromes. Although the latter is cited by Rothstein as evidence that a typical cytochrome system is present in T. aceti mitochondria he reported that confirmation of this hypothesis was difficult because the relatively high amount of CO-binding pigment obscured the possible contribution due to cytochrome-a_3

The CO-binding pigment, Rothstein observed, resembled the cytochrome-*O* described by Cheah [1967] for the cestode *Moniezia expansa*, but was not identical to it. Rothstein *et al*. [1970] showed that the CO-binding pigment was present in unusually high concentrations i.e. 6-fold greater than that of any other component of the respiratory chain, but no maxima in the difference spectra could be found that were attributable to this pigment in the absence of CO, although with dithionite/CO it absorbs at 565nm. Rothstein *et al*. [1970] observed that this pigment with absorption peak at 565nm was different to the *b*-type pigment reported in *C. briggsae* by Bryant, Nicholas and Jantunen [1967] which exhibited a peak at 557nm [at 77°K]. Rothstein further suggested that the *b*-type cytochrome in *T. aceti* which resembled the cytochrome-*O* [also a *b*-type] of *M. expansa* [Cheah, 1967] was not functioning on the main pathway of electron transfer, although he did not rule out the possibility that it can act as a terminal oxidase as it does in *M. expansa* as shown by Cheah [1967, 1968].

Examining the effects of respiratory inhibitors on the oxygen uptake of a mitochondrial preparation of *M.expansa*, Cheah [1966] found that antimycin-A had only a partial inhibitory effect at concentrations which cause complete inhibition of succinate oxidation in mammalian preparations. Similarly azide in concentrations exceeding 1mM stimulated succinate oxidation. Carbon

monoxide had no effect on $NADH+H^+$ oxidation nor on succinate oxidation and the latter was stimulated by CO [Cheah, 1966]. In a series of papers [Cheah, 1967a,b; 1968 and 1975a as quoted by Von Brand, 1979] proposed for M. expansa a branched electron transport chain. The minor branch would essentially correspond to the classical cytochrome system containing, as established, cytochrome-C_{550} and a typical cytochrome oxidase, with oxygen serving as the terminal electron acceptor.

The essential components of the main branch would consist of two cytochromes of the b-type which are designated cytochrome-556 [Moniezia expansa] and cytochrome-552, 556 [Moniezia expansa]. The terminal oxidase is of the O-type. Fumarate is thought to be the biological electron acceptor. In the presence of fumarate no hydrogen peroxide is formed, but succinate is produced probably through the intervention of a fumarate reductase. This process can proceed both in the presence and absence of molecular oxygen. If oxygen, instead of fumarate, is available, electrons are transferred through cytochrome-552, 556 [M. expansa] to oxygen. This process leads to the formation of hydrogen peroxide [Cheah, 1967c as quoted by Von Brand, 1979] which is normally destroyed rapidly by a peroxidase. The initial part of the respiratory chain [Cheah, 1967b as quoted by Von Brand, 1979] is shared by both branches. The connection between flavoproteins and the two branches

is apparently not effected by ubiquinone but by vitamin-K, the respiratory chain resembling in this respect more those of bacteria than of higher animals. Von Brand [1979] stated that branched respiratory chains may be distributed fairly widely among helminths.

Early workers [such as Bueding & Charms, 1952 as quoted by Von Brand, 1979] could not demonstrate an appreciable cytochrome oxidase activity in the tissues of adult Ascaris lumbricoides. Cheah [1976] has demonstrated conclusively that the muscle mitochondria of adult Ascaris contain a branched respiratory chain, the bifurcation occuring at the cytochrome-b level. The minor pathway is apparently identical to the classical mammalian chain and contributes approximately 30% of the oxygen consumption [QO_2] when succinate is the substrate. The major pathway with cytochrome-O as its terminal oxidase is insensitive to low concentrations of antimycin-A and cyanide and is linked with the formation of hydrogen peroxide. The latter pathway is however sensitive to o-hydroxydiphenyl. [Cheah, 1976]. The existence of antimycin - A sensitive and insensitive pathways was confirmed by Cheah [1976] polarographically. Succinate oxidation was inhibited by about 32% by antimycin-A at a concentration of 2.0 µg per mg protein, which was about five times the amount normally required for complete inhibition of the classical mammalian respiratory chain system.

The antimycin-A insensitive pathway could not be completely inhibited even with a very high concentration (35 µg per mg protein) of the inhibitor (Cheah, 1976). Furthermore Cheah (1976) concluded that Ascaris muscle mitochondria contained cyanide sensitive and insensitive pathways.

A somewhat different concept than that developed by Cheah concerning the terminal electron transport of Ascaris emerged from studies by others (Bueding, 1962 as quoted by Von Brand, 1979). Investigating the succinoxidase system of Ascaris mitochondria, Kmetec and Bueding (1961) found that the solubilized, partially purified system was not inhibited by KCN, azide or antimycin-A and led to the formation of hydrogen peroxide. All these properties were in agreement with the assumption that the terminal oxidase is a flavoprotein, since the purified preparations contained little flavin mononucleotides and riboflavine but large amounts of flavin adenine dinucleotide (Bueding, 1963 as quoted by Von Brand, 1979).

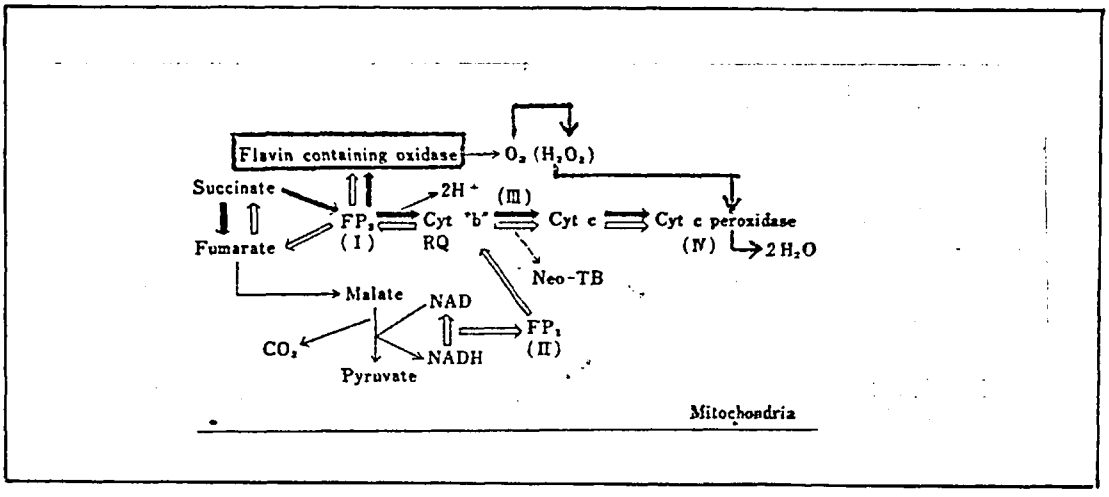
Since the activity of the terminal oxidase depends on the oxygen tension which is very low in the normal habitat of Ascaris, succinate or $NADH+H^+$ oxidation is of minor importance in vivo, the main importance being the re-oxidation of $NADH+H^+$ formed in anaerobic glycolysis, via a reversal of the last reactions of the TCA cycle, namely by the reduction of fumarate to succinate. The succinoxidase system of Ascaris can therefore serve both as

an electron acceptor for succinate or electron donor for fumarate. [Bueding, 1962 as quoted by Von Brand, 1978].

However, according to Cheah [1976] the conflicting findings of Kikuchi, Ramirez and Barron [1961] and of Kmetec and Bueding [1961] regarding the presence [Kikuchi *et al.*, 1961] and absence [Kmetec & Bueding, 1961] of a cyanide sensitive system in *Ascaris* were due to the failure of these workers to eliminate the hydrogen peroxide produced by *Ascaris* mitochondria in their assay systems. Since H_2O and H_2O_2 are the end products of the cyanide sensitive and cyanide insensitive systems, 1 mM cyanide failed to inhibit succinate oxidation in *Ascaris* mitochondria due to the presence of H_2O_2 and under these conditions cyanide occasionally stimulated respiration. However, in the presence of added catalase, 1 mM cyanide inhibited 37% of the succinoxidase activity [Cheah, 1976]. Thus Cheah proposed that the major pathway of succinate oxidation was cyanide insensitive and was linked with the *b*-type cytochrome which was found to be sensitive to o-hydroxy-diphenyl.

The scheme proposed by Cheah [1976] envisaging cyanide sensitive and insensitive pathways i.e. cytochrome aa_3 and cytochrome *O*, mediated electron transfer in *Ascaris*, differs from one postulated by Hayashi and Terada [1973]. These authors suggested that electron transport in *Ascaris* mitochondria consists of an oxygen pressure dependent, cyanide insensitive "flavin-containing-

oxidase" and a cytochrome-c-peroxidase for the re-oxidation of ferro-cytochrome-b via ferrocytochrome c; the H_2O_2 formed by the Flavin-containing-oxidase consumed by cytochrome-c-peroxidase in re-oxidizing ferrocytochrome-c. :-



Scheme for a possible electron transport system in Ascaris muscle mitochondria (After Hayashi & Terada, 1973)

Abbreviations:-

Cyt. - Cytochrome

RQ - Rhodoquinone

FP_1 - NADH dehydrogenase

FP_2 - Succinate dehydrogenase

Sites - In Roman numerals (I to IV)

Sensitivity of the sites

Site I - inhibited by Malonate

Site II - inhibited by Amytal or Rotenone

Site III - inhibited by Antimycin A

Site IV - inhibited by KCN and NaN_3

Cheah [1976] also cautions that the generation of H_2O_2 could be an artefact due to the effect of an increased oxygen pressure on Ascaris muscle mitochondria in the assay systems. Mammalian mitochondria from various tissues when subjected to hyperbaric oxygen produced H_2O_2 during substrate oxidation, the site responsible for H_2O_2 formation was suggested to be at the level of cytochrome-b [Boveris & Chance, 1973 as quoted by Cheah, 1976]. The question whether H_2O_2 is actually produced in vivo by Ascaris and by other parasites living in low oxygen tensions is still to be answered [Cheah, 1976].

The terminal oxidases in Halobacterium sp. [Cheah, 1969, 1970a] Ascaris [Cheah & Chance, 1970b] and Moniezia [Cheah, 1968, 1972] were characterized employing carbon-monoxide difference spectra. Cytochdrome-o and cytochrome-aa_3 were identified by the formation of their respective CO-complexes and the time dependent shift in absorbance maxima of the Soret-peaks.

The α:GP reduced CO-difference spectra of Ascaris muscle mitochondria observed at -196°C [Cheah, 1970b] closely resembled an O-type cytochrome with Soret maximum at 417 nm [corresponds to 420 nm at room temperature]. However Cheah [1970b] was unable to demonstrate cytochrome-a_3, CO-complex [γ-peak at 427-430 nm] due to the fact that the latter was obscured by the strong absorption peak of the O-CO complex at 417 nm [at 77°K] since the CO-binding haemoprotein was 5 times

greater than that of cytochrome-aa_3 [Cheah, 1970b]. However, analysis of the photochemical-action-spectrum showed that Ascaris mitochondria possessed a CO-sensitive and light reversible pigment [i.e. cytochrome-a_3] since the cytochrome-O-CO complex was not light-reversible [Cheah, 1970b].

The α-GP reduced CO-difference spectra of Moniezia expansa [Cheah, 1968, 1972] presented a similar situation in that the a_3-CO complex [γ-peak at 427-430] was effectively obscured by the predominant O-CO complex [γ peak at 419 nm]. However the difference spectrum recorded at 3.0 min; 8 min and 11 min after CO-treatment showed that cytochrome-a_3 binds CO faster than cytochrome-O. Cheah [1968, 1972] employed this criterion to demonstrate the presence of these two terminal oxidases, and showed the shift of the a_3-CO Soret peak [433-436 nm] with time to 420/419 nm after prolonged incubation in CO to facilitate the formation of the O-CO complex. The overall spectral change was due to the sum of these 2 pigments.

Cheah [1969, 1970a] investigated the electron transport particles of the halophile Halobacterium cutirubrum and showed that the ascorbate/TMPD oxidase activity was insensitive to antimycin-A and was only partially inhibited [44%] by 1 mM azide and 84% by 0.5 mM cyanide; CO inhibited only 20% of the oxygen uptake. Spectroscopic analysis showed the presence of cytochromes of the types a, b and c.

The nature of the terminal oxidases of the particles was determined by the formation of the CO-complexes after reduction with dithionite. A peak at 593 nm corresponded to the a_3-CO complex; the peaks observed at 578, 539 and 419 nm and a trough at 560 nm were characteristic of an O-CO complex. [Cheah, 1969]. A trough at 442 nm, was attributed to the combined troughs of the a_3-CO [445 nm] and O-CO [432 nm]. The Soret peak of a_3-CO [430 nm - 431 nm] was not discernible because the predominant O-CO peak obscured it, although the a_3-CO peak could be seen following brief incubation with CO.

Thus in particulate preparations of organisms possessing cytochrome-O and cytochrome-aa_3 as terminal oxidases identification of the overlapping absorption spectra of the CO complexes of the 2 cytochromes in the Soret-region depends on the elimination of the a_3-CO complex which absorbs between 427-430 nm by complexing cytochrome-aa_3 with cyanide to which it binds more strongly. Using this technique Cheah [1970a] demonstrated the presence of cyt.a_3 and cyt.O in Halobacterium halobium. Alternatively the a_3-CO spectrum can be observed free from the interference by the cyt.O-CO complex if the difference spectrum is recorded early. The technique employed by Cheah, is based on both these properties and was used to demonstrate the dual terminal oxidases in Ascaris, Moniezia and Halobacterium sps.

In so far as respiratory data from polarographic measurements are concerned, Cheah [1974] reported that the respiratory control ratio [RCRs] of Ascaris mitochondrial utilizing succinate to be between 1.43 - 3.03, although no clear state-3 to state-4 transitions were observed and thus no ADP.O ratios were quoted. Oligomycin inhibited oxygen uptake due to succinate and was relieved by the uncoupler FCCP. Ascorbate-TMPD oxidase activity was enhanced by ADP. Thus the presence of Site III phosphorylation has been demonstrated [Cheah, 1974] although the state-3 rate for a coupled functional Site III in Ascaris is about 25-30% of the state-3 rate for Site III in mitochondria isolated from aerobic skeletal muscle [Cheah, 1973a as quoted by Cheah, 1974].

Murfitt, Vogel and Sanadi [1976] characterized the mitochondria from Caenorhabditis elegans, and reported that the metabolism and respiratory chain of this organism was similar to the classical mammalian systems. The ADP:O ratios were consistent with those reported for mammalian systems. Essentially complete inhibition of state-3 respiration rate was obtained with cyanide, azide, antimycin A and rotenone. State-3 rates with the substrates succinate, glutamate/malate and β-hydroxybutyrate agree with those reported for T.aceti [Rothstein et al., 1970] although the state-3 rates with pyruvate, α-ketoglutarate and α-glycerophosphate differed. Murfitt et al. [1976] found a larger amount of b-type cytochromes than in mammalian

mitochondria and what appeared to be a loss of cytochrome-c compared to the mitochondrial fraction obtained for T. aceti by Rothstein et al. [1970]. The concentration of b-type cytochromes was 1.6 times that of cytochromes c and c_1. However the carbon monoxide difference spectra of C. elegans mitochondria resulted in peaks which did not correspond to those obtained by Rothstein et al. [1970] for T. aceti.

Cytochrome independent electron transfer and terminal oxidation has been well documented in various organisms. Cheah and Bryant [1966] and Cheah [1967a] have shown that under aerobic conditions the oxidation of $NADH+H^+$ and succinate is mediated by flavoproteins with the accumulation of H_2O_2. Weinbach and Von Brand [1970] in a comprehensive study of the aerobic metabolism of Taenia taeniaeformis mitochondria showed that the respiratory chain of the cestode contained flavoprotein dehydrogenases, non-haem-iron, pyridine nucleotides and a branched chain cytochrome system, one portion sensitive to cyanide and the other insensitive. Of the TCA cycle intermediates, α-glycerophosphate was the substrate most readily oxidised, the others being less effective or inert, as substrates. Antimycin-A and cyanide produced only partial inhibition of the α-GP oxidation although complete inhibition was obtained with dicoumarol.

Weinbach and Von Brand therefore suggested that the oxidation of α-GP by T. taeniaeformis mitochondria was mediated by flavoproteins. This oxidation was

inhibited by several metalchelating agents [salicylaldoxime, thenoyltrifluoroacetone]. They showed that the α-GP oxidation was mediated by a pair of enzymes present in the mitochondria namely in α-GP-dehydrogenase which catalysed the oxidation of $NADH+H^+$ in the presence of 3-dihydroxy acetone phosphate [DHAP] and a α-glycerol-phosphate-oxidase. The product of α-GP oxidation by the mitochondrial α-GP-oxidase [GPO] was DHAP, but no H_2O_2 was detected. These two enzymes working in conjunction, constitute according to Weinbach and Von Brand [1970] the α-GP-cycle. The significance of this cycle was that it provided a mechanism [in the presence of the soluble cytoplasmic NAD-dehydrogenase] for the re-oxidation of $NADH+H^+$ formed during glycolysis since mitochondria are normally impermeable to NADH [Lehninger, 1964, as quoted by Weinbach & Von Brand, 1970].

The α-GP cycle has been also demonstrated in the protozoan T. rhodesiense [Grant & Sargent, 1960 as quoted by Weinbach & Von Brand, 1970]. The trypanosome GPO unlike that of insect flight muscle and mammalian brain mitochondria reacts directly with oxygen and is insensitive to cyanide, azide, amytal and antimycin-A [Bowman & Flynn, 1976]. The product of α-GP oxidation by GPO is DHAP. It has been shown that only catalytic amounts of DHAP are required for the re-oxidation of $NADH+H^+$ to NAD^+ necessary for the maintenance of glycolysis. By this coupling of GPO to α-GP-dehydrogenase

glucose is converted to pyruvate and the redox balance maintained aerobically.

More recently Hill (1976) has shown that GPO is specifically inhibited by salicylhydroxamic acid (non-competitively) and by suramin and diphenylamine. Opperdoes and Borst (1976) has conclusively shown using differential and isopycnic centrifugation that the GPO is localized in the mitochondria and that it is insensitive to cyanide and carbon monoxide (Hill, 1976). Aerobically and especially anaerobically the GPO-comlex is involved in the production of glycerol (in conjunction with a phosphatase) in T. rhodesiense. Weinbach and Von Brand have shown that T. taeniaeformis also utilizes glycerol in preference to glucose.

Since the whole worms of A. avenae from isolates A and F (as discussed in Chapter 5) showed different degreees of inhibition of the oxygen consumption rate with cyanide, azide, and nematicides such as EDB, the mitochondrial respiratory metabolism of the two isolates were assessed. A method for the isolation of mitochondrial fractions of A. avenae is reported in this chapter. Chapter 7A reports the polarographic investigations of the oxidative capacity of the mitochondrial fractions of the 2 isolates with reference to respiratory control ratios (RCR), ADP:O ratios and sensitivity to inhibitors of complexes I, II, III and IV (Whittaker and Danks, 1978). The sensitivity to the inhibitor salicylhydroxamic acid

(SHAM) is also reported. The effect of a common uncoupler of oxidative phosphorylation 2,4 dinitrophenol (DNP) was also investigated.

In Chapter 7B, investigations into the relative composition of cytochrome components of the 2 isolates of A. avenae using optical spectrophotometric techniques is reported.

Investigation of the oxidative capacities of the mitochondrial fractions of A.avenae isolates A and F

Materials and Methods

1. Preparation of mitochondrial fractions

Nematodes from the specific isolates were harvested from 30-40 day old mass cultures and extracted as described in Chapter 2. The extracted worms were washed 3 times, by filtration through a millipore funnel under suction, in sterile distilled water and twice in 0^{b}-4^{o}C isolation medium. The isolation medium [hereafter referred to as IM] consisted of 225 mM mannitol, 75 mM sucrose, 0.2 mM EGTA, and 16 mM Tris-HCl pH 7.0, with 1 mg per ml bovine serum albumin [BSA]. The washed nematode pellet approximately 10 gm wet weight was placed in a 100 mm diameter mortar sterilised and chilled to 0^{o}-4^{o}C. A volume of pre-chilled acid washed sand [BDH] equal in volume to the nematode pellet, and 7 ml of chilled IM were added and the mixture was ground by hand using a pre-chilled pestle for 10 min. The mortar was immersed in crushed ice to maintain the temperature of the homogenate around 1-2^{o}C.

The resulting homogenate was diluted in 15ml of IM and collected in pre-chilled plastic tubes. The remaining sand/nematode mixture was ground for a further 2 min. with 3 mls of IM and the homogenate was diluted with a further 10 ml of IM and pooled with the first homogenate. The mortar was rinsed twice with 2 ml of IM and pooled. The pooled homogenate was centrifuged at 380 g for 7 min. The supernatant was transferred to a pre-chilled tube.

The 380 g pellet was resuspended in 7 mls of cold IM and the mixture was transferred to a pre-chilled all glass [Dounce type] homogeniser and homogenised with 10-12 passes. The resultant homogenate was transferred to pre-chilled tubes and the homogeniser rinsed in 3 ml of IM. The mixture was centrifuged at 380 g for 7 mins [second centrifugation].

The pellet was resuspended in 7ml of IM and the same sequence as described for the first 380 g pellet was repeated once more and the mixture was re-centrifuged at 380 g for 7 mins [third centrifugation]. The pellet from the 3rd centrifugation was discarded. The supernatants from the centrifugations were pooled and centrifuged at 12,000 g for 7 mins.

The resultant pellet was resuspended in 8 ml of IM using a "cold-finger" and re-centrifuged at 12,000 g for 7 min. The supernatants from the 12,000 g centrifugations were discarded. The final pellet was resuspended in 1.5 mls of IM and used in the polarographic assays. All centrifugations were done in an MSE-refrigerated centrifuge whose chamber temperature was maintained at 1-2^{o}C.

2. Reaction medium for respiration measurements

The reaction medium used contained 50 mM Tris, 12 mM NaH_2PO_4, 225 mM sucrose, 1.0 mM EGTA, 10 mM KCl and 5 mM $MgCl_2$ adjusted to pH 7.4, with half strength HCl. 1.8 ml of the reaction medium equilibrated at

25°C and 0.2 ml of the mitochondrial preparation was used throughout the present studies. A saturated oxygen concentration of 240 μM was used for all of the calculations. 2 mls of air saturated reaction medium therefore contained 960 n.Atoms of dissolved oxygen. All assays were carried out at 25°C.

3. Measurement of substrate oxidation and respiratory control ratios

The endogenous rate (if any) was recorded for about 2-3 mins before substrates were added. All substrates were added to a final concentration of 5 mM unless stated otherwise and the first state-4 rate was obtained following which 150 μM aliquot of ADP was added, to obtain state-3 rate. The respiratory control ratios (RCR) were calculated for the second state-4 to state-3 transition following a second addition of 150 μM ADP, i.e. after the first cycle of state-3 oxidation. All assays were carried out within 2-3 hrs after the mitochondria were prepared. The cuvette contained 0.5-0.57 mg mitochondrial protein.

4. Assessment of the effect of respiratory inhibitors

α-glycerophosphate (α-GP) was added (5 mM) to the reaction chamber containing approximately 0.5 mg mitochondrial protein in 2 ml reaction medium following which excess ADP (final concentration 3 mM) was added to obtain state-3 rate. After a steady state-3 rate was achieved 2.0 mM rotenone was added. Succinate (5 mM)

was then added followed by antimycin-A [2 μg per mg protein] and the rate recorded. Finally ascorbate [5 mM] and TMPD [0.5 mM] were added to obtain ascorbate/TMPD oxidase activity followed by NaCN [1 mM] or Azide [3 mM], to assess cytochrome $\underline{aa_3}$ mediated terminal oxidation. If incomplete inhibition was observed, salicylhydroxamic acid [SHAM-3 mM] and/or carbon monoxide [890 nmoles] was added. The CO was prepared by saturating 1 ml of distilled water with pure-CO at 23-25°C [room temperature] and the amount of CO was calculated using the Busen coefficient. 25 μl of such a solution of CO contained 890 nmoles CO which was injected into the reaction chamber via capillary port.

6. <u>The effect of salicylhydroxamic acid [SHAM] and azide on α-GP dependent respiration</u>

The effect of SHAM and azide were recorded in the absence of other inhibitors. 5 mM α-GP was added to obtain a state-4 rate followed by excess ADP [3 mM]. Once a steady state-3 rate was obtained 3 mM SHAM was added. Azide [3 mM] was finally added after a 2-3 min recording was obtained with SHAM.

6. <u>Assessment of the effects of the uncoupler 2,4 DNP</u>

The uncoupling of oxidative phosphorylation was investigated using 2,4 dinitrophenol [DNP]. A steady state-4 rate was obtained by adding 5 mM α GP; 30 μM 2,4 DNP was then added and the rate of oxygen uptake recorded.

7. The effect of exogenous cytochrome-c

a. The effect of cytochrome-c (equine) was assessed by adding 10 μM cytochrome-c to isolate-A and F mitochondrial preparations respiring in the presence of α-GP as substrate. Neither ADP nor any other inhibitors were present in the reaction chamber.

b. The effect of cytochrome-c on the ascorbate/TMPD oxidation rate of the mitochondrial preparations of the 2 isolates was also assessed. Ascorbate (5 mM) and 0.5 mM TMPD were added to the reaction chamber containing 0.5-0.6 mg protein mitochondria. Cytochrome-c was added (10 μM) after a steady rate was obtained with ascorbate/TMPD.

8. Estimation of protein in the mitochondrial preparation

Mitochondrial protein concentration was determined using the method of Lowry, Rosebrough, Farr and Randall (1951). Since the isolation medium employed contained mannitol which interferes with the assay, the method was modified as described by Murfitt, Vogel and Sanadi (1976). The sample of the mitochondrial preparation was precipitated with 5% trichloroacetic acid and centrifuged. The supernatant was discarded. The precipitate was dissolved in 0-1N NaOH which effectively removed the mannitol. The final mixture was neutralized with 0.1N HCl before the Lowry protein assay was accomplished.

Results

1. Substrate utilization and respiratory control in isolate A and F mitochondria

The respiration rates of isolate A and F mitochondrial fractions obtained with various substrates are given in Table 18 A and B, and in Appendices 7-1.1-7-1.4.

The endogenous rate was similar in both isolates. Succinate and α-glycerophosphate [α-GP] were the only TCA-cycle intermediates most readily oxidised by both isolates, under good respiratory control, i.e. showing clear state-3 to state-4 transitions.

Glutamate/malate, β-hydroxybutyrate and pyruvate oxidation rates did not differ significantly from the endogenous rates for both isolates and were oxidsed at substantially lower rates. ADP increased the O_2 utilization little in the presence of these substrates but no state-3 transition to state-4 was observed in response to ADP depletion, indicating poor respiratory control.

The addition of $NADH+H^+$ in substrate amounts resulted in little increase of oxygen uptake over the endogenous rate by both isolates [see Table 18 A and B] but were responsive to succinate and α-GP. The metabolic data shown in Table 18 A and B were obtained from preparations which showed high mitochondrial quality with respect to exogenous $NADH+H^+$ oxidation and were measured immediately after the mitochondria were prepared.

Table 18A :- Oxidation of substrates by isolate-F mitochondrial fractions

Substrate	O_2 utilization (n Atoms/min/mg Protein) Substrate alone	with added ADP	Respiratory Control Ratio (RCR)	ADP:O Ratio
Succinate	*85 ± 8.1[e]	*194 ± 27.4[d]	2.29 ± 0.19[b]	1.88 ± 0.06[a]
Glutamate/malate	29 ± 6.0	31 ± 7.2	-	-
β -Hydroxybutyrate	26 ± 1.8	34 ± 5.8	(1.29 ± 0.18)	-
Pyruvate	29 ± 5.0	36 ± 5.3	(1.27 ± 0.16)	-
NADH	26 ± 4.8	Unaltered	-	-
α-Glycerophosphate	67 ± 7.1[e]	142.0 ± 13.8[f]	2.12 ± 0.18[c]	2.20 ± 0.06
Endogenous	23.1 ± 5.3	Unaltered	-	-

Table 18B :- Oxidation of substrates by isolate-A mitochondrial fractions

Substrate	O_2 Utilization: Substrate alone	O_2 Utilization: With added ADP	Respiratory Control Ratio (RCR)	ADP:O Ratio
Succinate	*119 ± 4.4[e]	*409 ± 25.9[d]	3.43 ± 0.19[b]	1.41 ± 0.06[a]
Glutamate/malate	30 ± 4.8	33 ± 5.9	(1.12 ± 0.04)	-
β-Hydroxybutyrate	26 ± 1.3	27 ± 1.6	-	-
Pyruvate	26 ± 3.4	34 ± 3.8	(1.31 ± 0.19)	-
NADH (1mM)	26 ± 3.7	Unaltered	-	-
α-Glycerophosphate	*118 ± 10.9[e]	*310 ± 40.1[f]	2.62 ± 0.23[c]	2.17 ± 0.07
Endogenous	24.3 ± 3.1	Unaltered	-	-

The results are the means ± S.D. of a minimum of 6 assays from 4 different batches.

For succinate and α-GP the results are the means of 12-14 assays (3 replicates each from at least 4 different batches) - See Appendices 7-1.1 to 7-3.4

Means with superscript "e" differ significantly from the endogenous rate at $p < 0.05$ as indicated by one-tailed - 't' test analysis. (The endogenous rate was not subtracted from data.)

Between isolates the means with the same superscripts differ significantly at $p < 0.01$.

*The RCRs and ADP:O ratios were obtained with reference to the second state 4/state 3 cycle.

The RCRs in parenthesis are not according to the definition proper; since no state-3 to state-4 transition was observed the ratio QO_2 in presence : QO_2 in absence of ADP as opposed to QO_2 in the presence of ADP:QO_2 after ADP was depleted (Definition by Chance)

The state-3 rate of isolate-A preparations with succinate was significantly higher [$p < 0.05$] than that observed with isolate-F preparations as were the respiratory control ratios [RCRs] See Fig. 40 and 41. The ADP:O ratio for isolate-F utilizing succinate [1.88 $\pm$ 0.06] was significantly higher than that observed for isolate-A [1.41 $\pm$ 0.06].

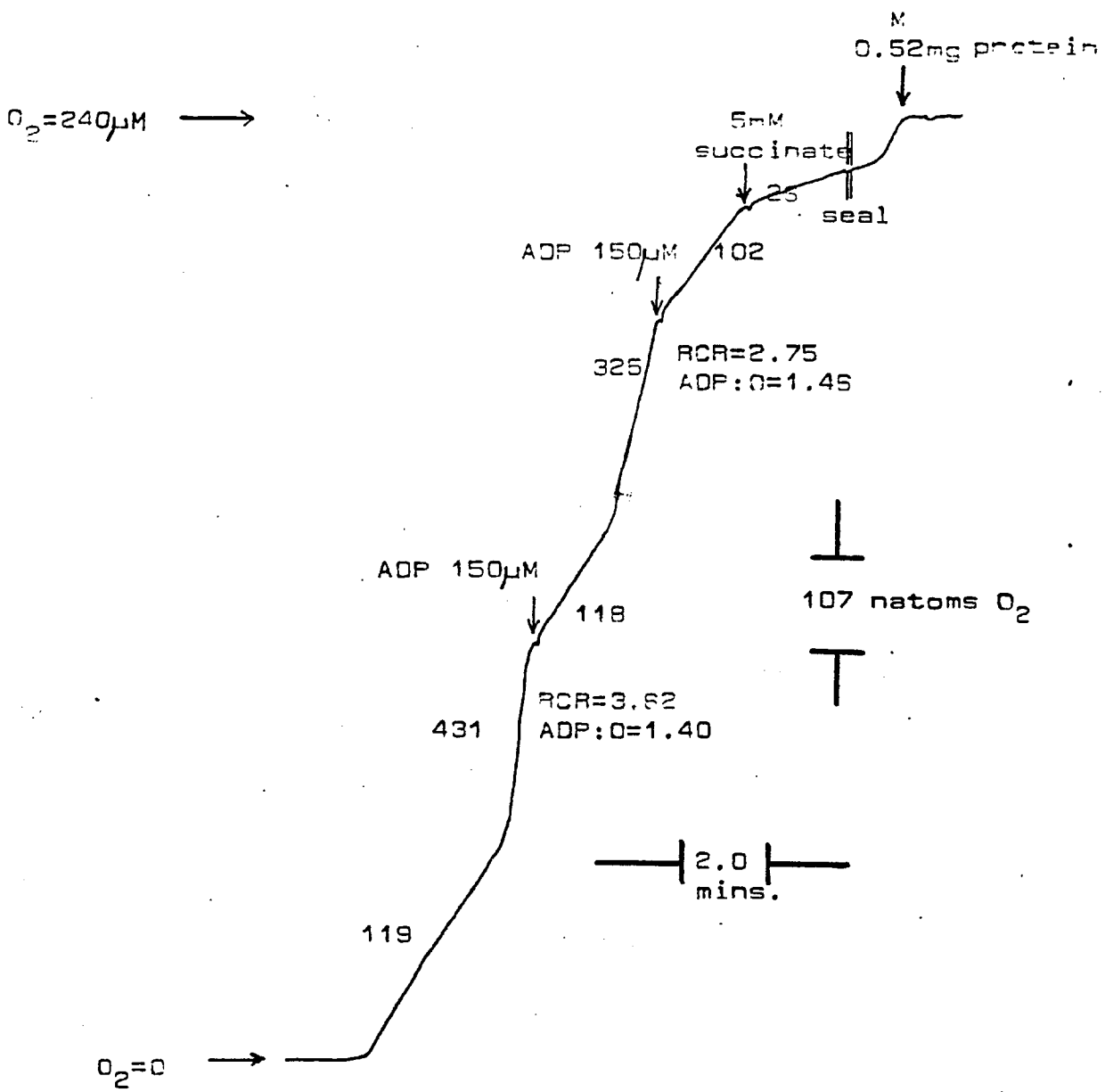
Although the state-3 rate of isolate-A mitochondria respiring in the presence of α-GP, was nearly double that obtained with the isolate-F preparations, the RCRs for the 2 isolates [RCR_{IF} = 2.12 $\pm$ 0.18; RCR_{IA} = 2.62 $\pm$ 0.23] being significantly different [Table 18 A and B], the ADP:O ratios for the two isolates were similar.

The mitochondrial preparations of isolate-F utilizing α-GP and succinate normally exhibited a slightly higher [state-4] rate before the initial addition of ADP than the rate after the ADP was depleted, [state-4 proper according to definition]. Thus the RCR values shown in Table 18 A and B for succinate and α-GP were those obtained after the first cycle of state-3 oxidation. Table 19 shows the effect of addition of the substrates [α-GP and succinate] to state-1 mitochondria of the 2 isolates.

The RCRs for glutamate/malate, p-hydroxybutyrate and pyruvate, were calculated for the change in rate following the initial addition of ADP since no apparent reversal [back] to state-4 rate was observed. The RCR

Figure: 40

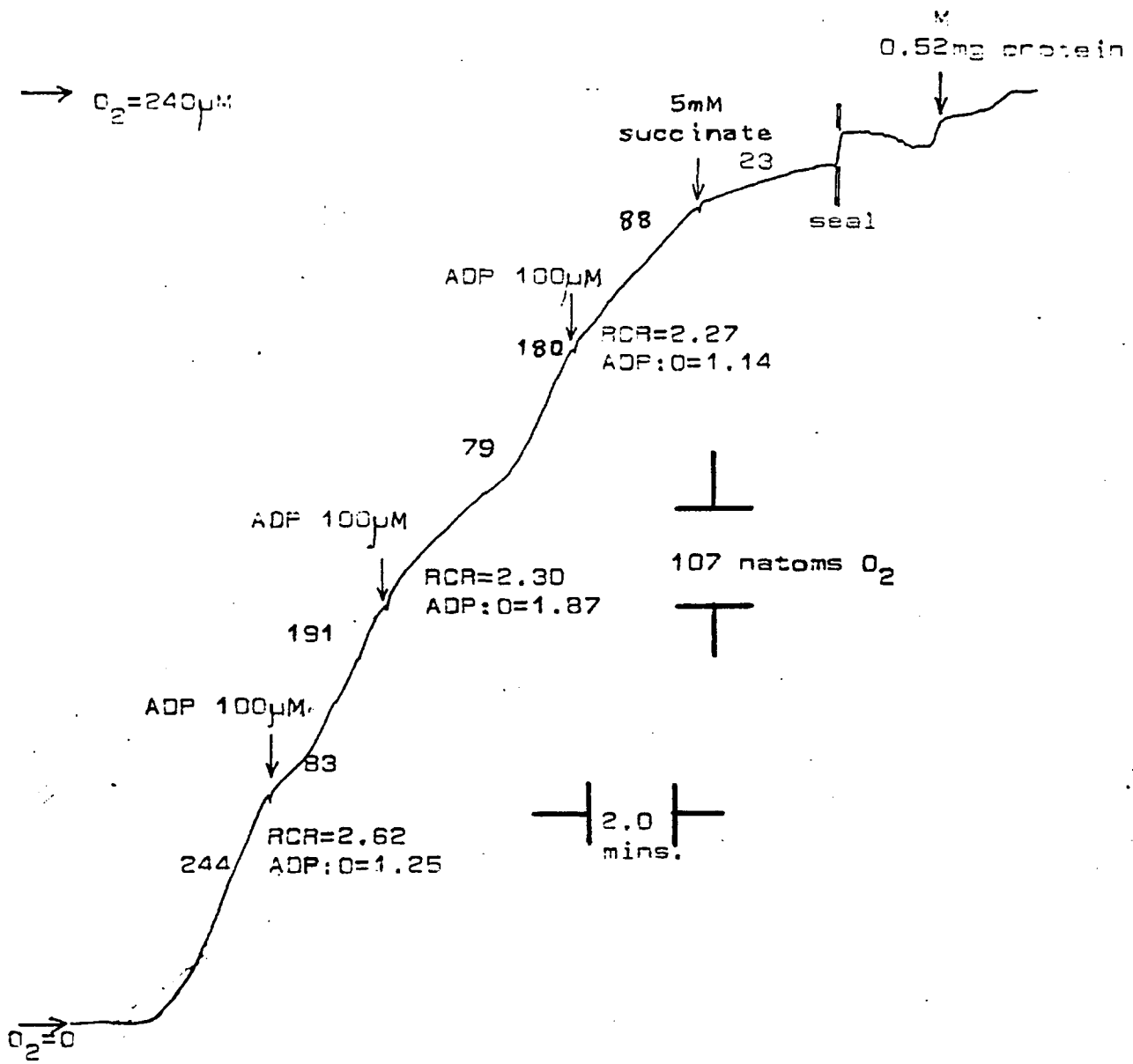
Typical polarographic tracing of A. avenae - isolate-A mitochondrial respiration utilizing succinate as substrate



M :- Mitochondria in 0.2ml isolation medium

Figure: 41

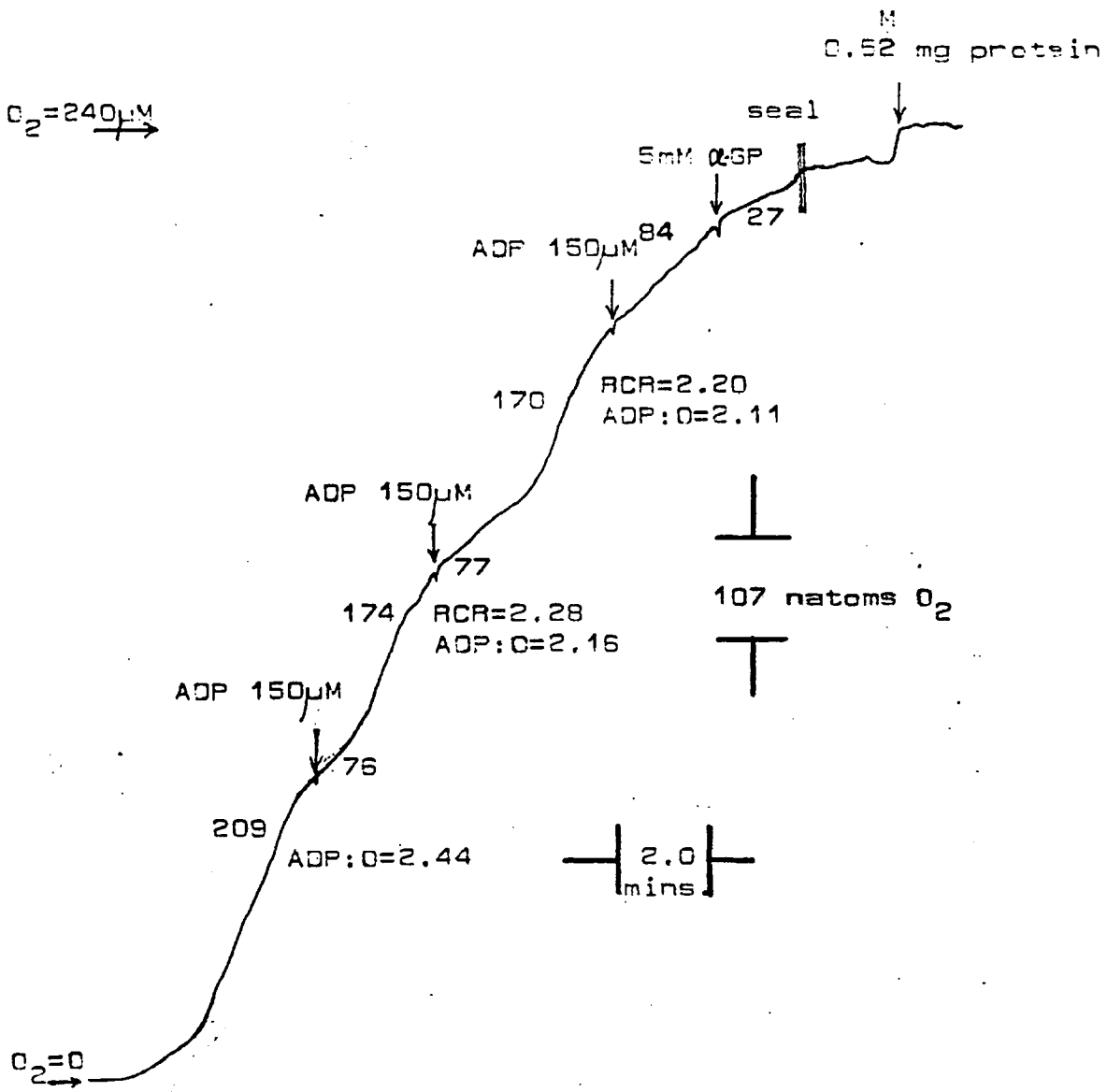
Typical polarographic tracing of A. avenae - isolate-F mitochondrial respiration using succinate as substrate



M :- Mitochondria in 0.2ml isolation medium.

Figure: 42

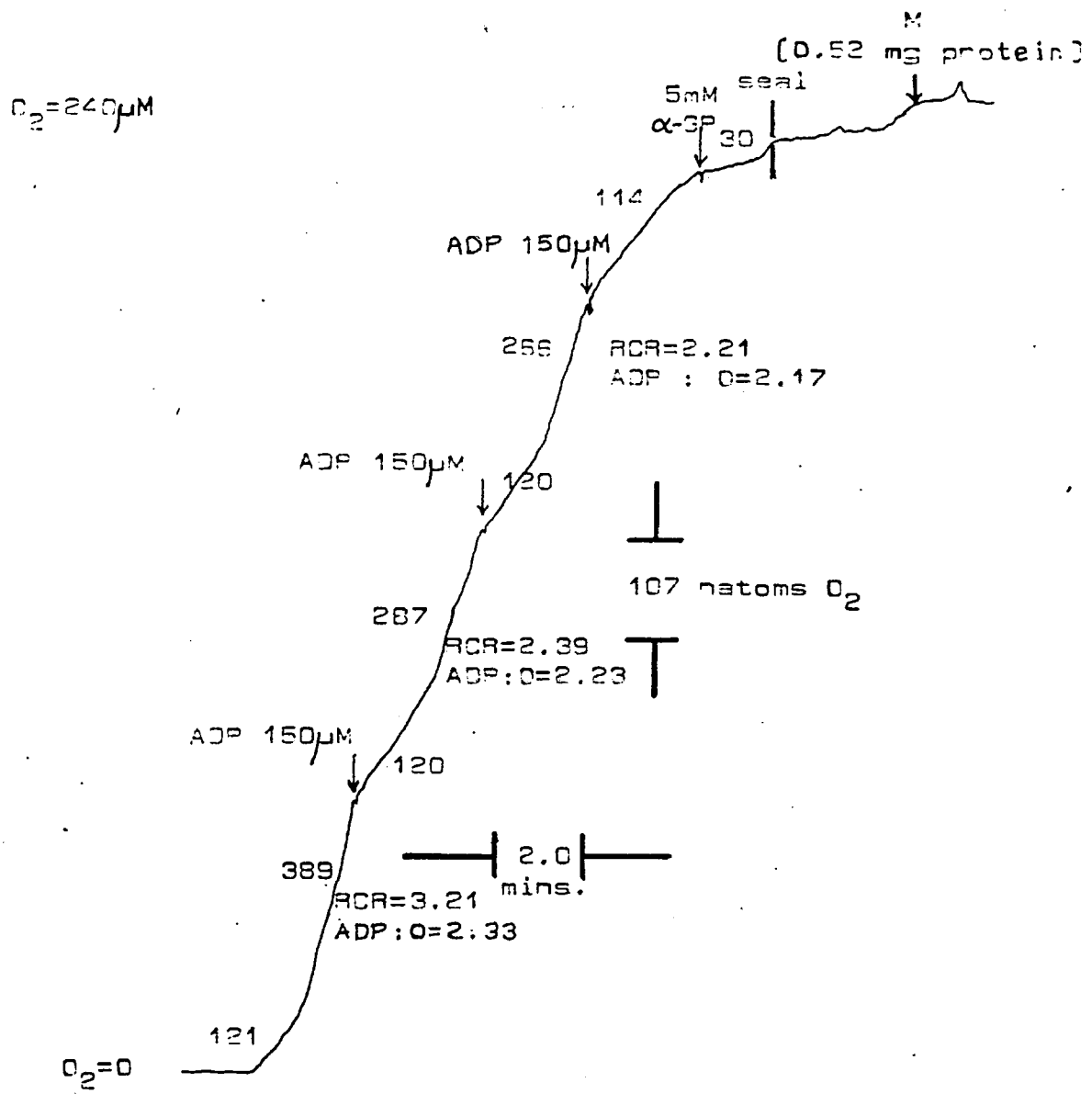
Typical polarographic tracing of A. avenae - isolate-F mitochondrial respiration utilizing α-GP as substrate



M :- Mitochondria in 0.2ml isolation medium.

Figure: 43

Typical polarographic tracing of A. avenae - isolate-A mitochondrial respiration utilizing α-GP as substrate



M :- Mitochondria in 0.2ml isolation medium

Table 19 :- The effect of α-glycerophosphate and succinate on the state-1 QO_2 of mitochondrial fractions of isolates A and F

	Substate	State 1 QO_2	State-4 QO_2	State-4 QO_2 * After first cycle of state-3 oxidation
Isolate-A	α-GP (5mM)	24.3 ± 3.1	116 ± 12.8	118 ± 10.9
	Succinate (5mM)		105 ± 8.9	119 ± 4.4
Isolate-F	α-GP (5mM)	23.1 ± 5.3	82 ± 11.2	67 ± 7.1
	Succinate (5mM)		88 ± 10.8	85 ± 8.1

The values are the means ± S.D. of 14 assays

* The respiratory control ratios (Table 18) were obtained after the first cycle of state-3 oxidation.

The state-4 QO_2s following the first cycle of state-3 oxidation were usually lower than the original state-4 QO_2 obtained upon addition of excess substrate to state-1 mitochondria.

cited for these substrates are the rates in the presence: the rate in the absence of ADP.

The state-4 rate of isolate-F utilizing α-GP as substrate 67 ± 7.1 n.Atoms/min/mg protein was significantly lower than the state-4 rate observed with succinate (85. ± 8.1 n.Atoms/min/mg protein), although the RCRs for the two substrates were similar. The ADP:O ratio with α-GP as substrate was higher than that with succinate.

In the case of isolate-A mitochondria, both α-GP and succinate showed similar state-4 rates, although the RCR for succinate (3.43 ± 0.19) was significantly higher than the RCR for α-GP (2.62 ± 0.23). The ADP:O ratio observed for α-GP was significantly higher than that for succinate. (Table 19).

2. The effect of respiratory chain-inhibitors on the mitochondrial preparations of isolate A and F.

a. Rotenone:

2 mM rotenone inhibited the state-3-QO_2 of both isolates feebly. A mean inhibition of 38.64% (Range 35.1-43.7%) was observed for isolate-A (Fig. 44A shows a typical trace) while a mean inhibition of 39.41% (Range 35.1-44.6%) was observed for isolate-F (Fig.45A shows a typical trace). The means were not significantly different (Table 20). The rotenone inhibition was completely relieved by 5 mM succinate in both isolates.

Table 20

A:- The effect of inhibitors on the mitochondrial respiration of isolate-A

Substrate or electron donor	Inhibitor and Concentration	Number of assays	Inhibition (%) Mean	Range
α-GP	Rotenone (2.0 mM)	10	38.64	34.03 - 43.72
Succinate	Antimycin-A (2 µg/mg protein)	12	7.78 [a]	0 - 19.0
Ascorbate/TMPD	NaCN (1mM)	12	62.95 [b]	60.4 - 66.0
α-GP	SHAM (3mM)	8	21.23	18.9 - 24.2

B:- The effect of inhibitors on the mitochondrial respiration of isolate-F

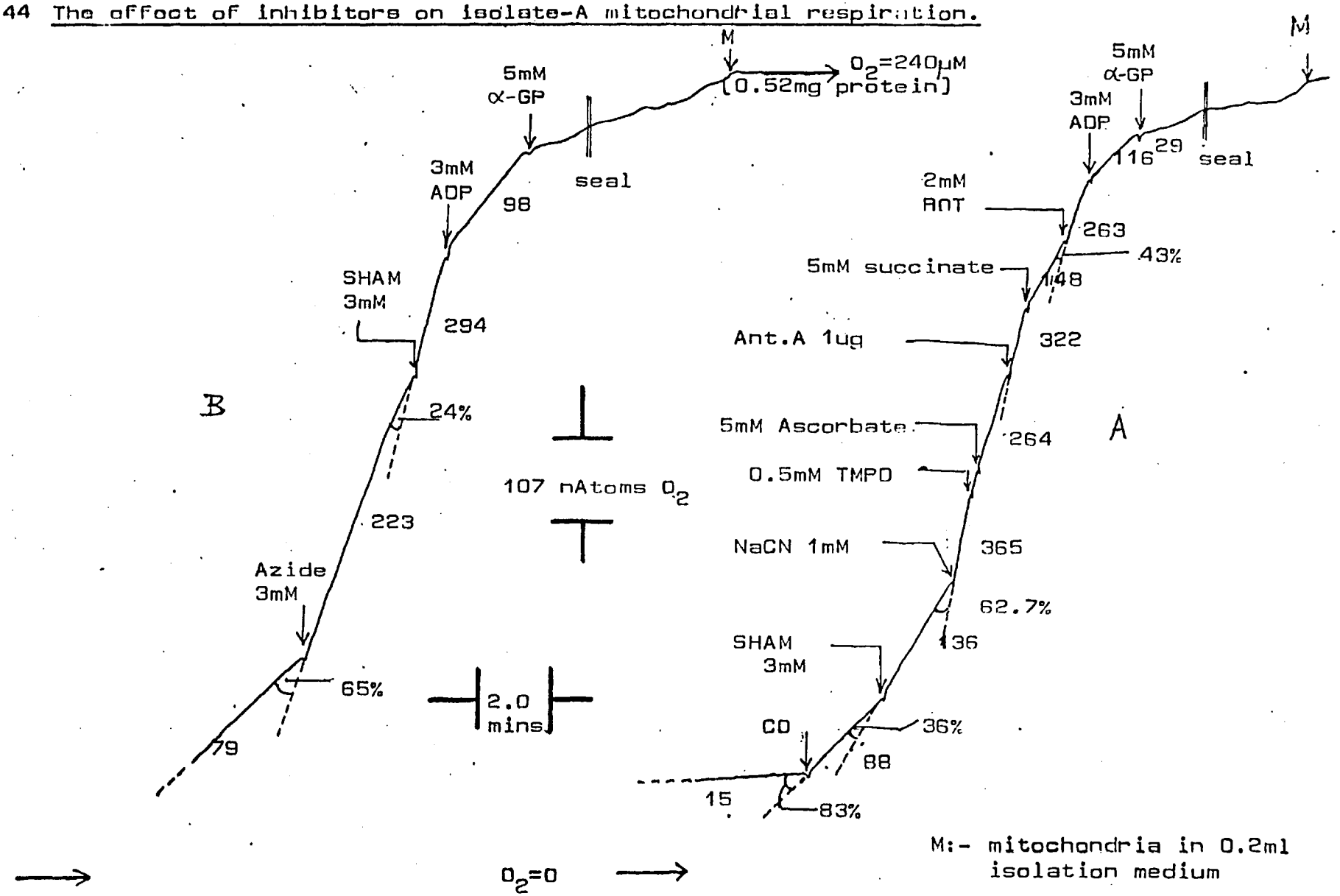
α-GP	Rotenone (2.0 mM)	11	39.41	35.19 - 44.63
Succinate	Antimycin-A (2 µg/mg protein)	9	87.80 [a]	85.6 - 90.6
Ascorbate/TMPD	NaCN (1mM)	11	88.56 [b]	86.66 - 91.25
α-GP	SHAM (3mM)	10	21.84	18.86 - 25.14

Percentage figures angularly transformed for statistical analysis.

Means with same superscripts differ significantly at p = 0.05 according to students 't' test (two-tailed).

Appendices 7-2.1 to 7-2.8 give the detailed data.

Figure: 44 The effect of inhibitors on isolate-A mitochondrial respiration.



The numbers to the right of the tracings are the rates of respiration in nAtoms O_2/min./mg. protein.

b. Antimycin-A

The succinate dependent state-3-QO_2 of isolate-A preparations were little affected by high concentrations [2 μg/mg protein] of antimycin-A, and a mean inhibition of only 7.78% [range 0-19.0%] was observed. This was in marked contrast to a mean inhibition of 87.8% [range 85.6 - 90.6%] of the succinate dependent state-3-QO_2 of isolate-F. [Table 20 and Fig. 45A]. These differences were highly significant [$p < 0.01$].

c. Sodium cyanide

The ascorbate/TMPD oxidase activity of isolate-A was only partially sensitive to 1 mM cyanide showing a mean inhibition of 62.95% [range 60.4 - 66.0%] [Table 20 Fig. 44A]. This was significantly different [$p \leq 0.01$] to the mean inhibition of 88.56% [range 86.66 - 91.25%] observed with 1 mM cyanide with isolate-F mitochondria [Fig. 45A].

d. Salicylhydroxamic acid [SHAM]

Figs. 44B and 45B show the effect of SHAM [3 mM] on the α-GP dependent state-3-QO_2 of isolates A and F. The state-3-QO_2 of both isolates were sensitive to this inhibitor of the α-glycerophosphate-oxidase complex showing approximately 21-22% inhibition [Table 21 and Appendices 7-2.4 and 7-2.5]. The range of inhibition varied between 18-26% and both isolates were apparently equally affected by SHAM.

Table 21 The inhibition of α-glycerophosphate dependent state-3 QO_2 of isolates A and F mitochondrial fractions by SHAM and sodium azide

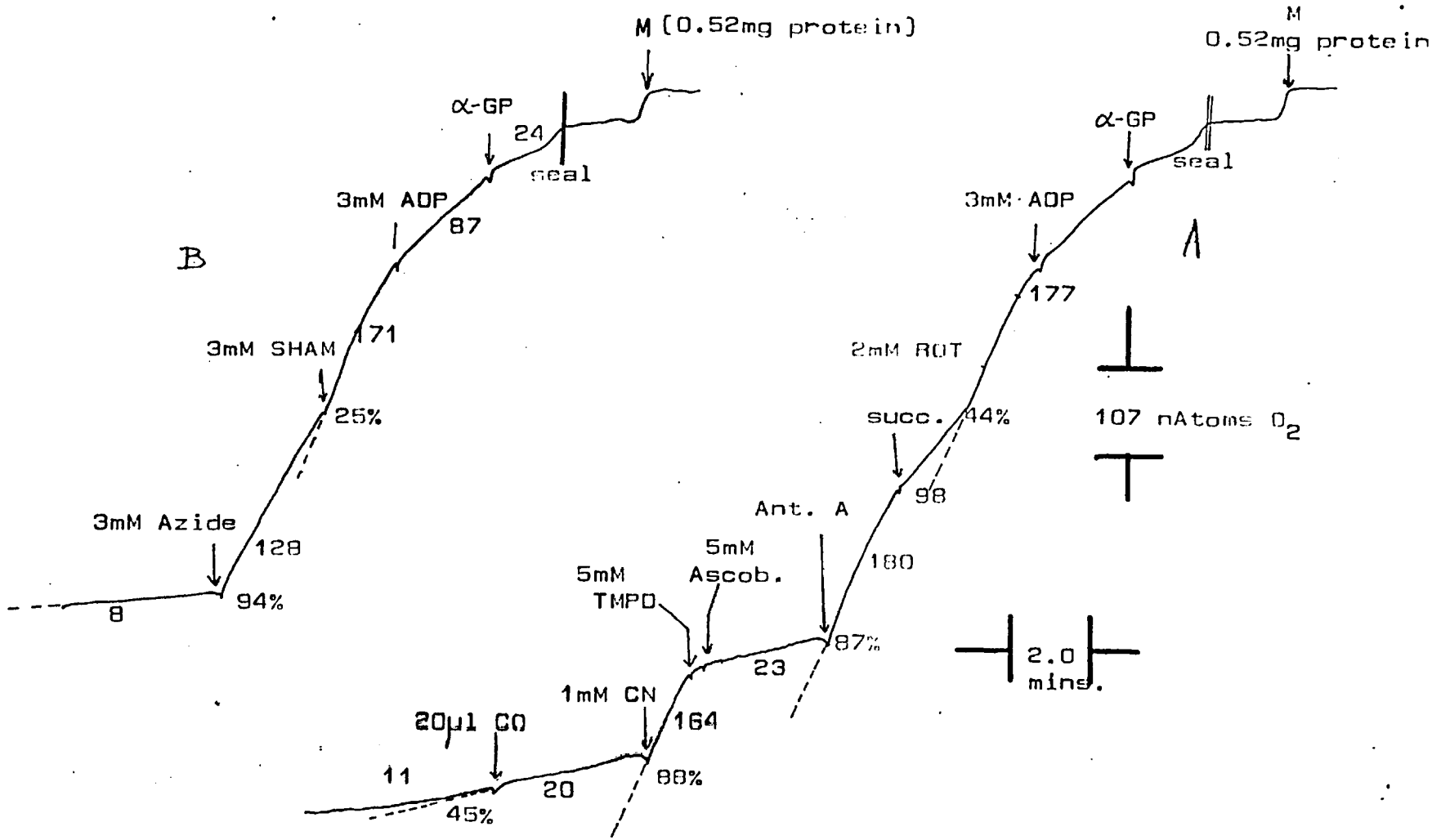
Inhibitor(s)	% Inhibition Range	% Inhibition Mean	Mean % inhibition of SHAM insensitive QO_2 by azide
Isolate A (n = 8)			
SHAM (3mM)	18.92 - 24.22	21.23	
SHAM (3mM) + Azide (3mM)	67.65 - 76.49	73.12 [a]	65.87 [b] (60.00 - 69.40)
Isolate F (n = 10)			
SHAM (3mM)	18.86 - 25.14	21.84	
SHAM (3mM) + Azide (3mM)	83.01 - 95.32	87.85 [a]	84.48 [b] (74.41 - 88.28)

Percentage figures angularly transformed for statistical analysis

Means with same superscript differ significantly at p = 0.05 according to students 't' test (one tailed)

Appendices 7-2.4 and 7-2.5 show the detailed data

Figure: 45 The effect of inhibitors on isolate-f mitochondrial respiration.



M :- Mitochondria in 0.2ml isolation medium.

Table 22 : The inhibition of ascorbate (5mM)/TMPD (0.5mM) dependent QO_2 of isolates A and F mitochondrial fractions by NaCN, SHAM and carbon monoxide (CO)

Inhibitor(s)	% Inhibition Range	% Inhibition Mean (n = 12)	
Isolate-A			
NaCN (3mM)	60.40 - 65.90	62.95 [a]	
NaCN (3mM) + SHAM (3mM)	62.05 - 73.74	71.01	(SHAM inhibited* (23.63% of cyanide (insensitive QO_2
NaCN (3mM) + SHAM (3mM) + CO-(890 nmoles)	92.95 - 95.89	94.37	(CO inhibited* (80.37% of NaCN/SHAM (insensitive QO_2
Isolate-F			
NaCN (3mM)	88.56 - 91.25	88.56 [a]	
NaCN (3mM) + CO (890 nmoles)	91.51 - 94.38	92.99	(CO inhibited 37.71% (of NaCN insensitive (QO_2*

Percentage figures angularly transformed for statistical analysis

Means with same superscript differ significantly at p = 0.05, according to students 't' test (one tailed)

* Appendices 7-2.9 and 7-2.11 gives the detailed data

e. Sodium azide (in the presence of SHAM)

Table 21 shows the effect of 3 mM sodium azide on the SHAM insensitive respiration. Figs. 44B and 45B shows the typical polarographic recordings obtained for the mitochondria of the 2 isolates. 3 mM azide inhibited approximately 66% of SHAM insensitive respiration of isolate-A and approximately 95% of SHAM insensitive respiration of isolate-F.

SHAM and azide inhibited 73% of state-3 α-GP dependent QO_2 of isolate-A and approximately 88% of isolate-F. (Table 21 and Appendices 7-2.4 to 7-2.5).

f. Carbon monoxide (in the presence of NaCN and SHAM)

The QO_2 of isolate-F in the presence of ascorbate/TMPD was inhibited 88.56% (Table 22 and Figs. 44A and 45A) which was enhanced by 890 nmoles of CO. Thus CN/CO produced a mean inhibition of 92.99% (range 91.51-94.38). CO inhibited 37.71% of the cyanide insensitive respiration in the presence of ascorbate/TMPD.

The QO_2 of isolate-A in the presence of ascorbate/TMPD was inhibited 62.95% by 1 mM NaCN; addition of SHAM 3 mM to the preparation after cyanide produced 71.01% inhibition while the NaCN-insensitive QO_2 was inhibited 23.63% by SHAM.

CO inhibited 80.37% of the NaCN/SHAM insensitive respiratory oxygen uptake induced by ascorbate/TMPD. In the presence of all 3 inhibitors the QO_2 of isolate-A mitochondrial QO_2 was 94.37% inhibited. (Table 22).

3. The effect of 2,4 dinitrophenol [DNP]

The state-4 respiration of isolate-F mitochondrial fractions induced by α-GP was increased 182% [range 170-199%] by 30 μM DNP. The state-4 rate of isolate-A preparations were stimulated 151% [range 145-160%] by DNP. [See Table 23A,B and Fig. 47].

4. The effect of exogenous cytochrome-c on α-GP oxidation

Added mammalian cytochrome-c [equine] had no apparent effect on the state-4 rate induced by 5 mM α-GP. 10 μM aliquots added during steady state-4 oxidation had no effect on isolate A and F mitochondrial respiration [Table 24A and B] and Figure 49.

However the ascorbate [5 mM]/TMPD [0.05 mM] induced QO_2 was higher [$p<0.05$] in isolate-F preparations than in isolate-A preparations.

Added cytochrome-c increased isolate-A mitochondrial ascorbate-TMPD activity by 44.6% while that of isolate-F was increased by 77.2%. [Table 24]. No other inhibitors were present in the reaction cuvette, during these assays.

Table 23A The effect of 2,4-DNP on isolate-F mitochondrial fractions respiring in the presence of 5 mM α-GP

QO_2 in 5mM α-GP	QO_2 in 2,4 DNP (30 μM)	% Stimulation of QO_2
87 ± 11.2	246 ± 19.3	182% (Range 170-199%)

Table 23B The effect of 2,4 DNP on isolate-A mitochondrial fractions respiring in the presence of 5 mM α-GP

QO_2 in 5mM α-GP	QO_2 in 2,4 DNP (30 μM)	% Stimulation of QO_2
114 ± 10.8	187 ± 18.7	151% (Range 145-160%)

Values are the mean ± S.D. of 6-8 assays from a minimum of 3 different batches.

Table 24A The effect of equine cytochrome-c on the α-GP dependent respiration of isolate-A and F mitochondrial fractions

	QO_2 in α-GP (5mM)	QO_2 with added (10 μM) cyt. c	% Change in QO_2
Isolate-A	116 ± 11.3	Unaltered (116 ± 11.3)	Zero
Isolate-F	84 ± 9.4	Unaltered (84 ± 9.4)	Zero

Table 24B The effect of equine cytochrome-c on the ascorbate/TMPD-oxidase activity of isolate-A and F mitochondrial fractions

	QO_2 in Ascorbate (5mM) & TMPD (0.5mM)	QO_2 with added (10 μm) cyt.c	% Change in QO_2
Isolate-A	302 ± 8.6 [a]	421 ± 22.6 [b]	44.6% [c] (Range 35.8 - 48.7%)
Isolate-F	318 ± 11.8 [a]	552 ± 27.4 [b]	77.2% [c] (Range 71.1 - 79.3%)

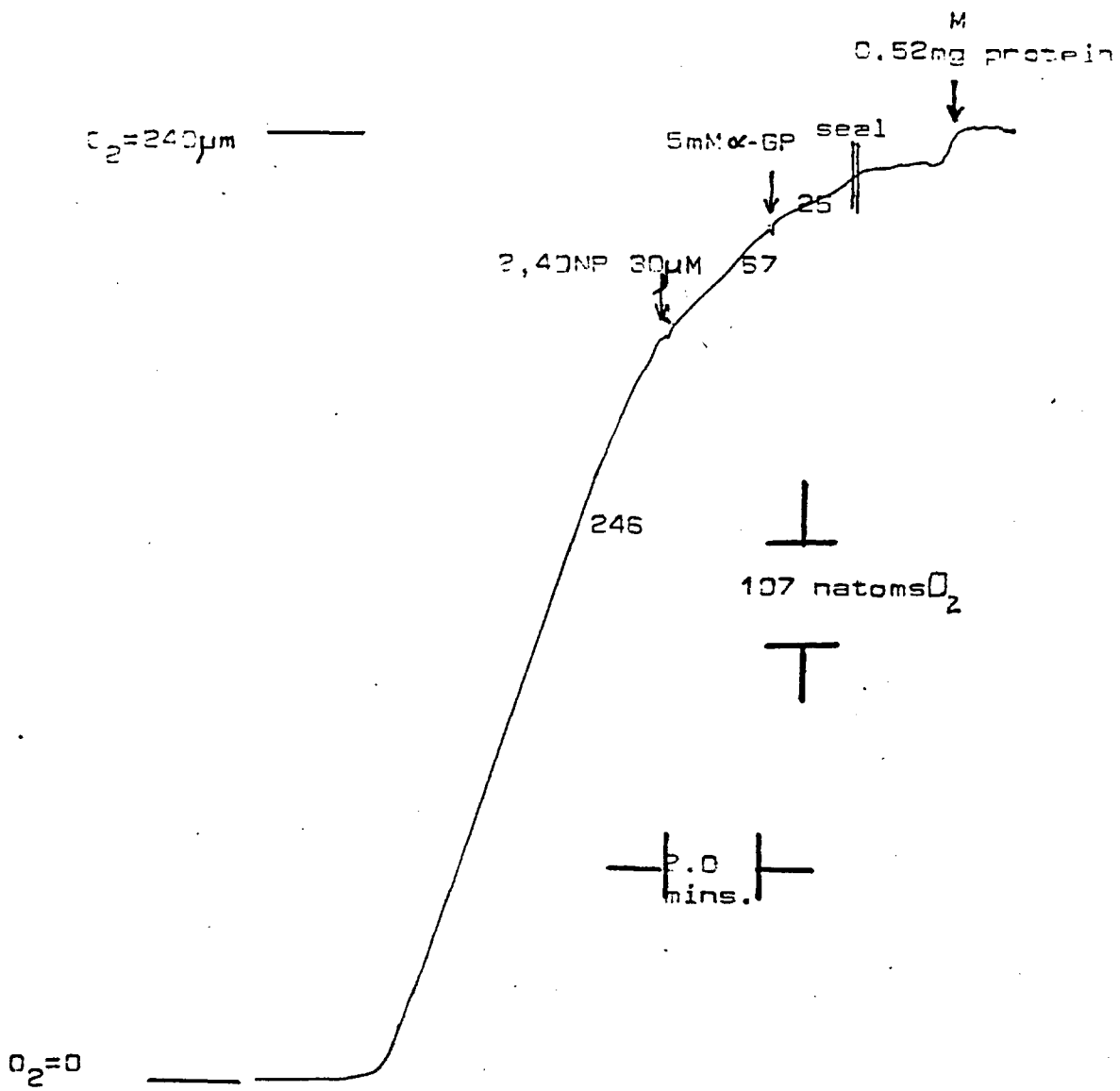
Values are the mean ± S.D. of 6-8 assays from a minimum of 3 different batches.

Ascorbate/TMPD were the only agents in the reaction mixture with 0.2 ml (approx. 0.5 mg protein) mitochondrial preparation.

Means with same superscripts differ significantly ($p < 0.05$) according to students 't' test.

Figure: 46

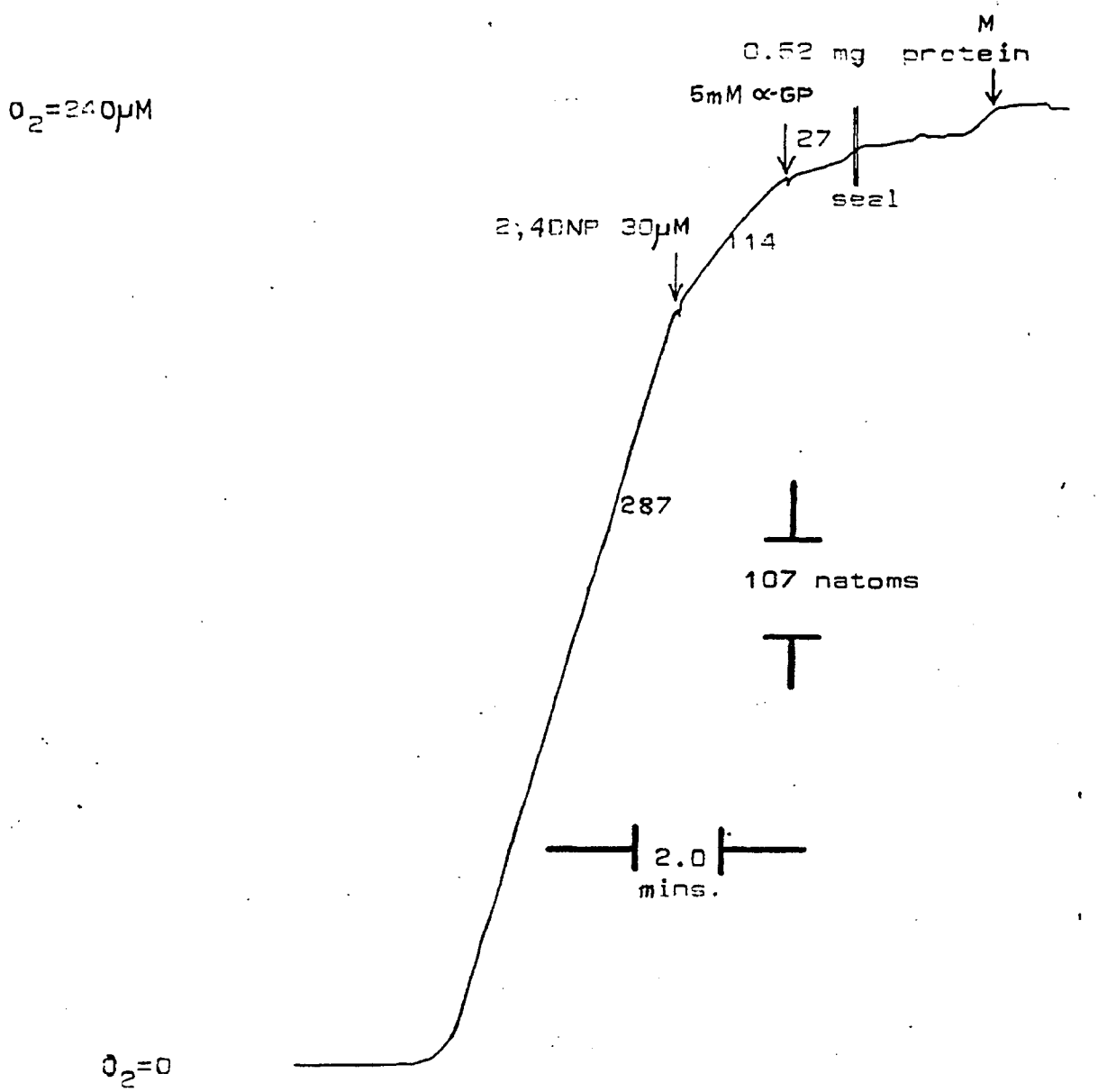
The effect of 2,4 DNP on isolate-F mitochondria respiring in the presence of 5mM α-GP



M ;- Mitochondria in 0.2ml isolation medium.

Figure: 47

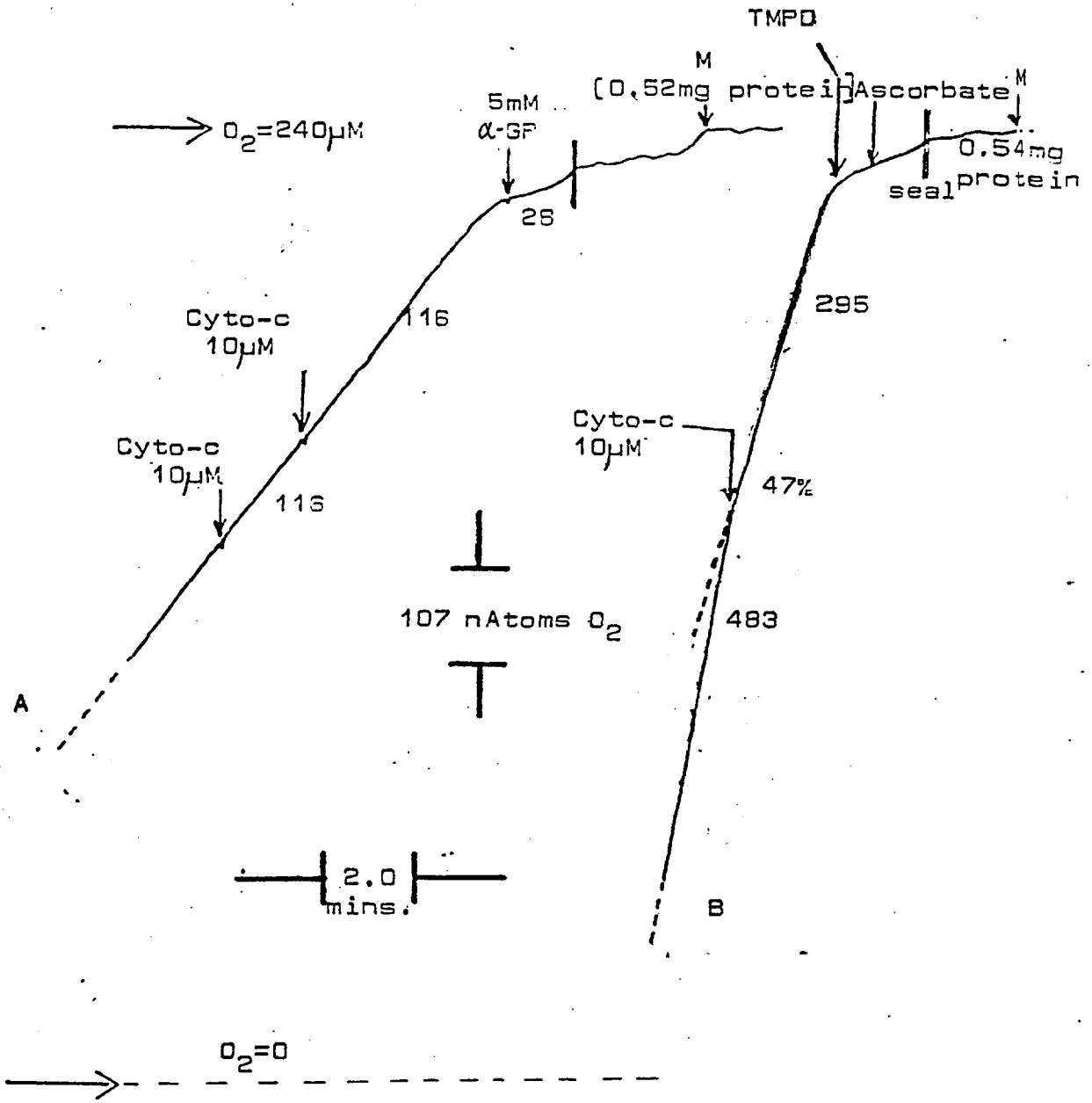
The effect of 2,4 DNP on isolate-A mitochondria respiring in the presence of 5mM α-GP



M ;- Mitochondria in 0.2ml isolation medium

Figure:- 48

The effect of exogenous cytochrome-c on the A)α-GP dependent respiration and B) Ascorbate/TMPD oxidase activity of isolate-A mitochondria.

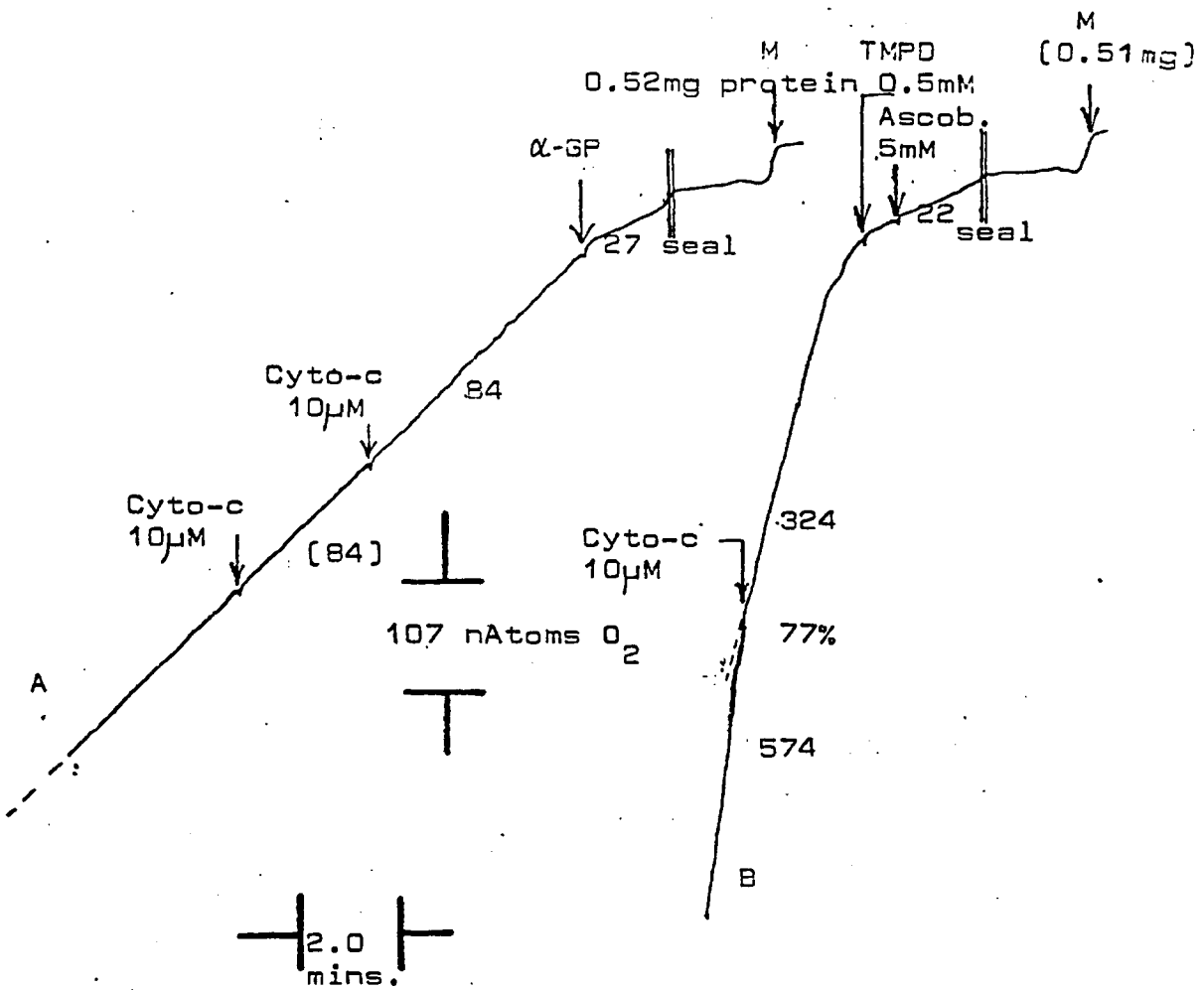


M :- Mitochondria in 0.2ml isolation medium.

The numbers to the right of the tracings are the rates of respiration in nAtoms O_2/min./mg. protein.

Figure:- 49

The effect of exogenous cytochrome-c on the A) α-GP dependent respiration and B) Ascorbate/TMPD oxidase activity of isolate-F mitochondria.



M :- Mitochondria in0.2ml isolation medium.

The numbers to the right of the tracings are rates of respiration in nAtoms O_2/min./mg. protein.

Discussion

Isolation of mitochondria

The methods for the preparation of mitochondria described for *Turbatrix aceti* (Rothstein *et al.*, 1970) and for *Caenorhabditis* (Murfitt, Vogel & Sanadi, 1976) differ noticeably, although the isolation media used were similar and acid washed sea-sand was used as abrasive. Rothstein *et al.* (1970) obtained phosphorylating, intact mitochondria in the following manner. Following the first centrifugation at 480 g for 7 min the pellet was discarded and the supernatant was centrifuged at 17,300 g for 7 min to obtain the mitochondrial pellet.

However, Murfitt *et al.* (1976) obtained *C.elegans* mitochondria from the pellet resulting from the first centrifugation at 380 g for 10 mins and discarded the 380 g supernatant i.e. a procedure exactly opposite to that adopted by Rothstein *et al.* (1970). The 380 g pellet was treated with the proteinase "Nagarse" for 5 min on ice, and then homogenised in a Dounce type unit with 7 passes. Further centrifugations were performed (none exceeding 4500 g) to obtain the final pellet.

Since the two procedures described for the isolation of mitochondria for these free living nematodes differed substantially, a modification of the two methods was developed and employed in the present study to obtain mitochondria from *A. avenae*. However the use of the proteinase Nagarse was omitted since it had been reported

[Weinbach & Von Brand, 1970] that the mitochondria isolated using Nagarse were susceptible to the loss of cyanide sensitivity especially when stored in the cold [0-4°C] for several hours. The isolation medium employed in the present study was the same as that used by Murfitt et al. [1976] except that EGTA was substituted for EDTA. The reaction medium used for polarographic assays of mitochondrial respiration was the same as that described by Murfitt, Vogel and Sanadi [1976].

The effect of added $NADH+H^+$ on the endogenous rate

The mitochondrial fractions of the 2 isolates of A. avenae prepared in the present study showed little or no increase in the rate of respiratory oxygen utilization [QO_2] in the presence of substrate amounts of $NADH+H^+$ indicating the preparations were of good quality and that little disruption of the intact organelles had occurred with the isolation procedure. Murfitt, Vogel and Sanadi [1976] described similar low rates with added $NADH+H^+$ for mitochondria isolated from C. elegans although the endogenous rate increased three to four fold from 7 n atoms/mg/min to approximately 25 n atoms/min/mg, with added NADH. The respiratory QO_2 in the presence of $NADH+H^+$ was similar to the endogenous rate observed with A.avenae mitochondria. The endogenous rate in A. avenae mitochondria was 23-24 n atoms/min/mg which in the presence of $NADH+H^+$ was between 26-30 n atoms/min/mg. The endogenous rate observed with T. aceti mitochondrial preparations by Rothstein et al. [1970] of 3 - 5 μM

oxygen/min/mg corresponds with the endogenous rate, observed in A. avenae preparations in this study.

Interaction with exogenous cytochrome-c

The observation that exogenous mammalian cytochrome-c had no effect on the α-glycerophosphate (α-GP) or succinate oxidation by mitochondrial preparations of both isolates of A. avenae suggests that the preparations were not deficient in this component.

Von Brand (1979) had observed that a lack of response to added ferri-cytochrome-c, by mitochondria respiring in the presence of α-GP and/or succinate, is characteristic of mitochondria with no deficiency of the cytochrome-c component. Weinbach and Von Brand (1970) reached a similar conclusion with mitochondria isolated from the cestode Taenia taeniaeformis which oxidised only α-GP and malate, other TCA intermediates being inert or only feebly oxidised; however the α-GP and malate oxidation was not affected by exogenous mammalian cytochrome-c. On the other hand ascorbate/TMPD induced respiratory QO_2 of mitochondrial preparations of both isolates of A. avenae increased substantially with an addition of exogenous reduced equine-cytochrome-c. (Immediate reduction of the added cytochrome-c occurred in the presence of electron donors ascorbate/TMPD.) This agrees with the finding by Rothstein et al. (1970) with T. aceti mitochondria and is evidence for the presence of cytochrome-oxidase activity in the A. avenae isolates.

However, the ascorbate/TMPD stimulated the respiratory QO_2 of the mitochondrial preparations of the two isolates of A. avenae to different degrees: that of isolate-A was stimulated by approximately 45% while that of isolate-F was increased by nearly 77%. This may be in itself evidence that the cytochrome oxidase activity in the 2 isolates are different. Caution must be exercised in the interpretation of these differences since if it is assumed that both isolates have identical amounts of cytochrome oxidase, it can also be concluded that the differential stimulatory effects of mammalian ferro-cytochrome-c, is due to different abilities of the cytochrome-chains of the 2 isolates to interact with mammalian cytochrome-c. Some bacterial cytochrome-oxidases are intrinsically unable to accept electrons from mammalian cytochrome-c [Smith, 1954; Yamanaka & Okunuki, 1964 as quoted by Weinbach & Von Brand, 1970].

Oxidation of TCA-cycle intermediates and respiratory control

The most distinct finding was that A. avenae mitochondrial preparations of both isolates utilized only succinate and α-glycerophosphate [α-GP] while most other tricarboxylic acid cycle [TCA cycle] intermediates were only feebly oxidised. The failure of most of the TCA-cycle intermediates other than α-GP and succinate to stimulate respiratory QO_2 was apparently not due to the loss of cytochrome-c or nicotinamide nucleotides

since the addition of these substances had little effect with any of the substrates (including α-GP and succinate) tested. Whether this was due to their inability to penetrate the mitochondrial membranes as in the case of insect flight-muscle (Van den Bergh & Slater, 1962 as quoted by Weinbach & Von Brand, 1970) was not assessed in this study since the effect of detergents or sonication on the mitochondrial fractions was not assessed.

The respiratory control ratios (RCRs) of isolate-F mitochondria observed with succinate (2.28 ± 0.19) agrees well with that observed with T. aceti mitochondria (1.9-2.2) by Rothstein et al. (1970), although the succinate RCR for isolate-A mitochondria (3.43 ± 0.19) were very much higher than those observed with T. aceti. However the RCR for succinate observed for C. elegans mitochondria-3.58 (Murfitt, Vogel & Sanadi, 1976) was similar to the succinate-RCR observed with isolate-A mitochondria.

The ADP:O ratios obtained with succinate as substrate for isolates A and F (1.88 ± 0.06 and 1.41 ± 0.06 respectively) were significantly different and were higher than those observed for C. elegans mitochondria (1.26 ± 0.03) by Murfitt et al. (1976). These differences may reflect differences in metabolic activities between the two species or may be due to the differences in the preparative techniques.

The RCR with α-GP observed for isolates A and F mitochondria 2.12 ± 0.18 and 2.62 ± 0.23 respectively, were significantly different ($p < 0.05$). However, the α-GP RCRs for both isolates were considerably higher than that observed with C. elegans mitochondria [1.04 ± 0.04] by Murfitt et al. [1976]. However, Rothstein et al. [1970] did not observe even such low state-3 respiration with α-GP for T. aceti mitochondria. Clear state-3 to state-4 transitions were observed with α-GP utilizing mitochondria in both isolates of A.avenae and the ADP:O ratios obtained with both isolates A and F were similar but were greater than 2.0. The ADP:O ratios obtained with α-GP were significantly greater than the ADP:O ratios observed for each isolate with succinate.

Similar high state-4 respiration rates with α-GP has been reported by Weinbach and Von Brand [1970] for Taenia taeniaeformis mitochondria; they attributed this to the operation of an α-GP oxidase which they showed to be localized in the mitochondria of the cestode. They also reported that $NADH+H^+$ had no effect on the respiratory QO_2 of this cestode in the absence of detergents such as deoxycholate and although malate was the only other TCA-cycle intermediate oxidised by T. taeniaeformis mitochondria the rate of malate-oxidation was 4-fold less than the rate of α-GP oxidation [Weinbach & Von Brand, 1970].

Mitochondrial state-4 QO_2 of both isolates of A. avenae with pyruvate was feebly increased with ADP [isolate-A: 1.27 ± 0.16; isolate-F: 1.31 ± 0.19] although the increased rate did not revert back to the state-4 rate during the periods within which such transitions were commonly observed with α-GP and succinate. Similar small increases with added ADP were observed for β -hydroxybutyrate [RCR = 1.29 ± 0.18] with isolate-A and with glutamate/malate [RCR = 1.12 ± 0.04] with isolate-F preparations. The RCR values were thus calculated for these substrates, as the ratio of the rate in the presence of ADP : the rate in the absence of ADP. Since the depletion of added ADP during α-GP and succinate oxidation was clearly observed by the decrease in the slope of the polarographic recording, the "RCR-proper" as defined by Chance [1959] as quoted by Klingenberg [1967] i.e. the ratio of the QO_2 in the presence of ADP : the QO_2 following depletion of ADP could be calculated.

These differences in the RCR and ADP:O ratios observed between the two isolates of A. avenae may reflect metabolic differences since isolate methods and the media used were identical. Further evidence of differences between isolates A and F observed with the effect of respiratory inhibitors seem to substantiate these differences.

Response to respiratory inhibitors

The partial inhibition [38-39%] of state-3, α-GP oxidation by rotenone which inhibits Complex 1 [i.e. between NADH-dehydrogenase and ubiquinone - Whittaker & Danks, 1978] suggests that α-GP probably has a different mode of entry into the A. avenae respiratory chain than is commonly found in mammalian systems. Although α-GP does not enter via mitochondrial-NAD-dehydrogenase in mammalian systems, this may be possible in the nematode mitochondria. On the other hand since α-GP commonly donates to ubiquinone via FAD flavoproteins [Whittaker & Danks, 1978] these steps may be partially sensitive to rotenone, to account for 38-39% inhibition of α-GP based respiratory QO_2. Cheah [1967b] found similar inhibition of α-GP and $NADH+H^+$ oxidation in Moniezia expansa with amytal, which also inhibits Complex-1. However in M. expansa, the connection between flavoproteins and the branched cytochrome chain is effected by vitamin-K and not by ubiquinone — the latter criterion being more common with bacteria than in higher organisms [Von Brand, 1979]. Whether rotenone inhibits flavoprotein complexes containing FMN rather than FAD, is however not known although in mammalian systems Complex-1, which contains FMN and iron-sulphur compounds is inhibited by it, the transfer of electrons from α-GP $\longrightarrow$ FAD $\longrightarrow$ UQ is not affected by rotenone [Whittaker & Danks, 1978].

Legend and Abbreviations for Figure 50

α-GP	-	α-glycerophosphate	NaCN	-	sodium cyanide
Succ	-	sodium succinate	CO	-	carbon monoxide
β Hy.But.	-	β-hydroxy butyrate	NaN_3	-	sodium azide
Pyru	-	pyruvate	αGPDH	-	αGlycerophosphate dehydrogenase complex
FAD	-	flavin adenine dinucleotide (Complex)	TMPD	-	tetramethylphenylenediamine
FMN	-	flavin mononucleotide (Complex)	Cyt	-	cytochrome
GPO	-	L-α-glycerophosphate oxidase complex	NAD	-	nicotinamide adenine dinucleotide
SHAM	-	salicylhydroxamate (sodium salt)	UQ	-	ubiquinone
Complex II	-	Succinate dehydrogenase complex	IF	-	A.avenae isolate-F
Complex I	-	$NADH+H^+$ dehydrogenase complex	IA	-	A.avenae isolate-A
FP	-	Flavoprotein associated with cyt.O			

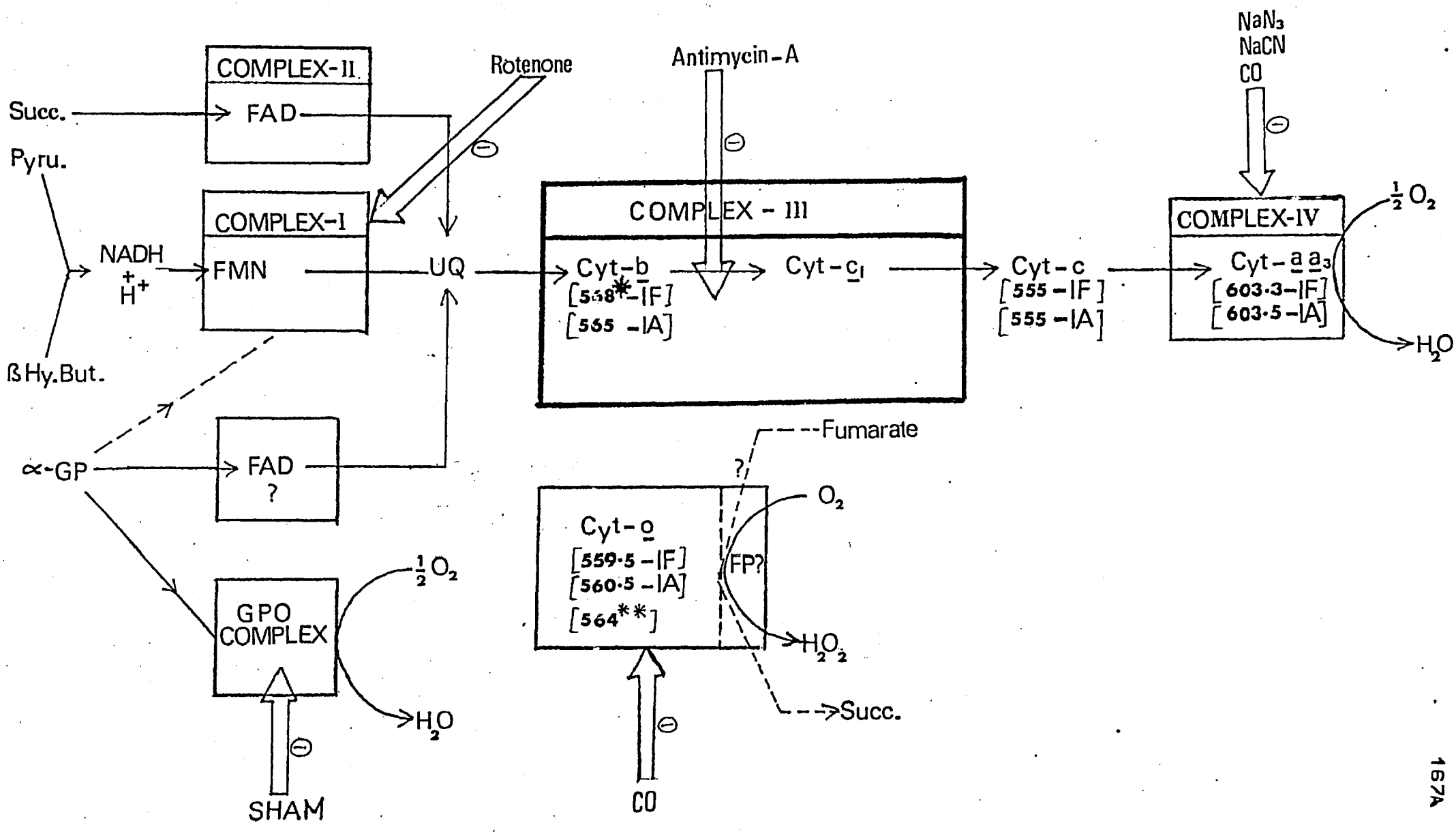
Figures in parenthesis:- Maxima as observed in (ferricyanide)-(ferricyanide + S_2O_4) difference spectra measured at room temperature (24°C)

* Prominent in isolate-F preparations only (See Figure 54)

** Predominant cytochrome species as observed in (ferricyanide Oxidised)-(S_2O_4 reduced) difference spectra

All absorption maxima as observed at room temperature (24°C)

Figure 50 Characteristics of mitochondrial electron transport of A.avenae isolates and the effect of inhibitors.



Antimycin-A

The rotenone inhibition however, was almost completely relieved by succinate in both isolates A and F. The succinate oxidation of isolate-A mitochondria, in the presence of rotenone, was very little affected [0 - 19% inhibition] by concentrations of antimycin-A [2 µg/mg protein] which were about five times the amount required for complete inhibition of the classical mammalian systems [Cheah, 1976].

In contrast the succinate oxidation of isolate-F mitochondrial preparations were more than 85% inhibited by identical concentrations of antimycin-A. The great difference of the effects of this inhibitor suggests the presence of antimycin-A insensitive and sensitive pathways of succinate-oxidation. The partial if not complete insensitivity of succinate-oxidation of isolate-A mitochondrial preparations suggests that in isolate-A, an alternate electron transfer sequence which bifurcates at the cytochrome-b level or at the preceding ubiquinone level [i.e. between Complex-I and Complex-III] operates with a much greater turn-over than in isolate-F. The incomplete inhibition of succinate-oxidation of isolate-F also suggests that this isolate also has potential antimycin-A insensitive respiratory oxygen uptake.

The terminal oxidase of the alternate i.e. antimycin-A insensitive sequence may in all probability be cytochrome-o. Such branched respiratory chain systems, one with the classical-aa_3 type terminal-oxidase and the

other with cytochrome-*O* are widely distributed among helminths (Von Brand, 1979) and has been reported to occur in *Ascaris*, (Cheah, 1976, 1974); *Moniezia expansa* (Cheah, 1966, 1967b, 1972) and in *Fasciola* (as quoted by Barrett, 1976). *Moniliformis dubius* (Bryant & Nicholas as quoted by Von Brand, 1979) mitochondrial preparations were only partially sensitive to antimycin-A. The higher sensitivity of isolate-F mitochondrial preparations to antimycin-A suggests that these mitochondria may be more dependent on the classical cytochrome-oxidase i.e. cytochrome-aa_3 as the terminal oxidase with a small but significant capacity for alternate terminal electron transfer, perhaps mediated by cytochrome-*O*.

Ascorbate TMPD oxidase activity and effect of NaCN

The inhibition by antimycin-A of mitochondrial preparations of both isolates of *A. avenae* were completely relieved by the ascorbate/TMPD combination which donates electrons to cytochrome-*c*, by-passing the antimycin-A inhibition site of Complex-III. However 1 mM cyanide inhibited 88-91% of the ascorbate/TMPD stimulated respiratory O_2 uptake of isolate-F mitochondria, but only 60-66% of that of isolate-A mitochondria. This suggests that the major pathway responsible for electron transfer to oxygen in isolate-F mitochondria is mediated by the classical cytochrome-aa_3 type terminal oxidase. The higher percentage of residual, i.e. cyanide insensitive oxygen uptake in the presence of ascorbate/TMPD in

isolate-A mitochondrial preparations is probably due to an alternate-terminal oxidase(s) which could be cytochrome-*O* and/or other terminal oxidases. It is known that cytochrome-*O* remains unaffected by concentrations of cyanide which wholly inhibit cytochrome-aa_3 (Bowmann & Flynn, 1976; Lemberg & Barrett, 1973). In fact Lemberg and Barrett (1973) reported that "cytochrome-*O* which is less sensitive to cyanide than cytochrome-a_3 and the degree of inhibition varies with the system in which cytochrome-*O* is the terminal electron acceptor".

However it has been shown that the combination ascorbate/TMPD could also donate electrons and thereby reduce the cyt.-*O* of *Mycobacterium phlei* (Revsin & Brodie, 1969 as quoted by Lemberg & Barrett, 1973). Cheah (1969) has shown that ascorbate per se could donate electrons directly to cytochrome-*O* in *Halobacterium cutirubrum*. Therefore in evaluating the effect of cyanide on the ascorbate/TMPD stimulated oxygen uptake of *A.avenae* mitochondria this should be borne in mind since no experiments were conducted to see whether this was the case in the mitochondria of *A. avenae*.

Carbon monoxide as inhibitor

In contrast with the 88% mean inhibition of the ascorbate/TMPD induced oxygen uptake observed with 1 mM cyanide, a 93% mean inhibition was obtained when CO was applied following cyanide treatment. CO inhibited nearly 40% of the cyanide insensitive respiration in the

presence of ascorbate/TMPD. CO binds with cytochrome-*o* and the binding is not photosensitive unlike the light reversible inhibition of cytochrome-a_3 by CO. Thus the increased inhibition of the QO_2 by CO in the presence of light is probably due to the complete or partial inhibition of a relatively cyanide insensitive terminal oxidase which could perhaps be an oxidase similar to cytochrome-*o* or cytochrome-*o* itself.

However, the cyanide insensitive, α-GP induced respiratory state-3 QO_2 of isolate-A mitochondria was approximately 25% inhibited by 3 mM salicylhydroxamic acid (SHAM) and the total inhibition by cyanide and SHAM was increased to a mean 71%. The residual, i.e. cyanide/SHAM insensitive respiratory oxygen uptake was nearly 80% inhibited by CO.

The α-glycerophosphate-oxidase (GPO) system demonstrated in *T. taeniaeformis* mitochondria by Weinbach and Von Brand (1970) is now known to be specifically inhibited by SHAM, (Hill, 1976) and is localized within the mitochondria (Weinbach & Von Brand, 1970; Opperdoes & Borst, 1976). The GPO complex is a flavoprotein oxidase, iron, thiol groups and FAD were initially suggested as components of the oxidase (Grant & Sargent, 1961 as quoted by Bowmann & Flynn, 1976). Recent studies by Fairlamb and Bowman (1975) as quoted by Hill (1976) have shown the presence of FAD, copper, iron, but the absence of acid-labile-sulphur or acid extractable iron; copper and non-haem iron has also been established (Hill,

1976]. The fact that SHAM inhibited nearly 22% of the α-GP induced state-3 respiratory rate of mitochondrial preparations of both isolates in the absence of any other inhibitor suggests the operation of a α-glycerol phosphate-oxidase in these isolates of A. avenae.

Azide inhibition

The SHAM insensitive, α-GP induced state-3 rate of isolate-A mitochondrial preparations were approximately 66% inhibited by [3 mM] sodium azide, and that of isolate-F was approximately 85% inhibited by azide. Since azide is a more specific inhibitor of cytochrome-a_3 than cyanide these percentage inhibition figures probably reflect the dependency of the two isolates on the classical aa_3 type terminal oxidase.

Thus, the information obtained from these observations with inhibitors suggest that a multiple-oxidase system is present in both isolates of A. avenae. However, the relative dependencies on the oxidases seem to vary significantly in each isolate. Barrett [1976] pointed out that there may be other terminal oxidases in addition to cytochrome-a_3 and O, and did not rule out the possibility of flavoprotein oxidases as terminal oxidase agents. The results of the present study suggests that A. avenae possesses an azide-sensitive oxidase; an azide/SHAM insensitive but CO-sensitive oxidase; and a SHAM-sensitive oxidase.

In whole worm suspensions of isolate-A, cyanide (1-3 mM) was found to stimulate oxygen uptake at high temperatures. It has been shown that cytochrome-*O* mediated terminal oxidation results in the production of hydrogen-peroxide (Cheah, 1966; 1967b, 1972, 1975). The GPO-complex is also capable of H_2O_2 generation in an intermediate step. Barrett (1976) pointed out that cytochrome-*O* may not be the only source of H_2O_2 since it could also arise at the level of one of the other *b*-type cytochromes or from flavoprotein oxidases. However Weinbach and Von Brand (1970) demonstrated the GPO-cycle in *T. taeniaeformis* but were unsuccessful in demonstrating the production of H_2O_2 and attributed this to the presence of a catalase in the mitochdonrial preparation. As will be seen in Chapter 7B, *A.avenae* mitochdndrial fractions had catalase (i.e. hydrogen-peroxide-oxido-reductase) activity, but no H_2O_2 formation by the mitochondria could be demonstrated, although spectral evidence strongly suggests evidence of multiple oxidases.

Barrett (1976) while pointing out that there is no satisfactory explanation for the stimulation of the QO_2 by CN^- in helminths (such as *Moniezia*, *Ascaris* and *Fasciola*) speculated that cyanide stimulation may be due to the removal of inhibitions imposed on flavoprotein enzymes by virtue of the cyanide ions' carbonyl-combining, metal-chelating properties; or that the cyanochromogens formed in the presence of cyanide may have a more favourable redox-potential for oxidation. Another possible

explanation cited by Barrett (1976) was that when the classical chain is blocked, the drop in ATP production is reduced and so to compensate for this, the flux (of electrons) through the alternative oxidase is increased. It is of interest to note that in the majority of instances where stimulation of the oxygen uptake of organisms (or by sub-cellular fractions) by cyanide and/or carbon monoxide has been described, multiple-oxidase systems implicating cytochrome-O and cytochrome-a_3 have been demonstrated.

CHAPTER 7B

Spectrophotometric analysis of cytochromes in the mitochondrial fractions of isolates A and F

Materials and Methods

Mitochondrial fractions of the 2 isolates of A. avenae were prepared as described in the Materials and Methods in Chapter 7A. The cytochrome spectra were determined using a Shimadzu Model, MPS 50L split beam spectrophotometer: Low temperature (77°K) spectra were recorded using an Aminco-Chance DW-2 with dual beam facility.

1. Suspension medium for cytochrome spectra

A medium containing 225 mM mannitol, 75mM sucrose, 0.2 mM EGTA and 75 mM Na-PO_4 buffer pH 7.4 with 1 mg/ml. bovine serum albumin (hereafter referred to as MSEB) was used as suspending medium for the mitochondria in order to obtain difference spectra.

Approximately 0.7-0.8 ml of the mitochondrial preparation (in isolation medium) was dispensed into each of two silica micro cells, 1.5 ml volume and 10 mm path length. Adequate MSEB medium (an equal volume) was added to each micro cell to cover the field of the beam. The cells usually contained between 2-3.5 mg mitochondrial protein. This ensured equal amounts of mitochondrial material in reference and test cells. Base lines were obtained using matched cells containing equal quantities of the suspensions.

2. Addition of substrates

Substrates were added to the test cell while the reference cell was oxidised either by bubbling air or by the addition of potassium ferricyanide. NADH, α-GP, succinate were used to reduce the mitochondria in the test cells. When substrates were added to the test cell, equal volumes of buffered medium [MSEB] was added to the reference cell to correspond with the dilution of the test cell.

3. Inhibitors

The effects of Antimycin-A and cyanide on the spectra were obtained by oxidising the reference cell with ferricyanide and adding the inhibitors to the test cells.

4. Dithionite reduced vs. ferricyanide oxidised spectra

A 5.8 mM solution of potassium ferricyanide-$Na_3[Fe(CN)_6]$ was prepared in buffered suspending medium. 20 μl of this was added to the mitochondrial preparation [in isolation medium] and mixed thoroughly. The suspension was divided equally into 2 microcells, and an equal volume of suspending medium [MSEB] was added to each to cover the field of the beam. This ensured that the baseline was recorded in the presence of equal amounts of ferricyanide in both reference and test cuvettes. The application of ferricyanide to both reference and test cuvettes ensures more accurate baseline balancing since

ferricyanide itself absorbs in the visible range (Dr. John Palmer pers. comm.). The cytochromes were quantified from difference spectra obtained in this manner.

5. Carbon monoxide difference spectra

The reference and test cuvettes containing mitochondria suspended in MSEB were used to obtain a baseline, following which 1 mg of sodium dithionite was added to both cells. Pure CO (BDH) was bubbled through the test cell sample for 1-3 mins or as required. The isobestic point at 540 nm was adjusted to correspond with that on the baseline obtained earlier and the difference spectrum i.e. (dithionite reduced)-(dithionite reduced + CO) recorded on slow speed.

When the same preparations of mitochondria were used first to obtain (oxidised)-(reduced) spectra and then the dithionite-reduced CO-difference spectra in order to comparethe spectral changes induced by CO in identical preparations, the following procedure was followed.

Mitochondria prepared from approximately 7-8 g of nematodes were suspended in about 1.2 ml of isolation medium. This was uniformly divided into two micro cells (matched, 1.5 ml capacity, 10 mm light path, silica micro cuvettes) and the minimum volume (usually 0.8 ml) of MSEB medium was added into each cuvette which was adequate

to cover the full field of the beam. A baseline was obtained using these cells, following which 10 µl of 5.8 mM ferricyanide in MSEB medium was added to the reference cell and 1 mg of sodium dithionite was added to the test cell. The isobestic point at 540 nm was fixed to coincide with that on the baseline and the [ferricyanide oxidised]-[dithionite reduced] difference spectrum recorded.

In the next stage pure CO (BOH) was bubbled through the test cell (already reduced with dithionite) for 1.0 min and again the isobestic point (at 540 nm) fixed to that on the baseline and the [ferricyanide oxidised]-[dithionite reduced + CO] difference spectrum recorded.

In the final stage the reference sample was reduced by the addition of 1.0 mg dithionite, the isobestic point at 540 nm fixed to that on the baseline and the [dithionite reduced in the presence of ferricyanide]-[dithionite reduced + CO] difference spectra obtained.

6. Ascorbate reduced CO-difference spectra

To reference and test samples prepared as above, ascorbate was added to a final concentration of 10 mM in each suspension, following which the test cell suspension was bubbled with pure CO for 3 mins and the difference spectrum recorded after adjusting the isobestic point at 540 nm. Following this a solution of sodium cyanide

in Na-PO_4 buffer was added to the test cell to give a final concentration of 1 mM, the isobestic point 540 nm adjusted to coincide with that on the baseline and the [ascorbate-reduced]-[ascorbate + CO + NaCN] difference spectrum recorded.

When more comparative data was necessary from a limited sample of mitochondria the following sequence was followed. The mitochondrial suspensions were uniformly dispensed into the two cuvettes and a baseline recorded. The reference sample was then aerated for 2-3 mins. by bubbling air and to the test cuvette ascorbate was added to give a final concentration of 10 mM. Thus the [endogenous oxidised]-[ascorbate-reduced] difference spectrum was obtained.

Next, the test cuvette [with 10 mM ascorbate] was bubbled with CO for 3 mins and the difference spectrum recorded after aerating the reference sample. This gave the [endogenous oxidised]-[ascorbate + CO] difference spectrum.

In the next stage the reference sample was treated with ascorbate and the [ascorbate reduced]-[ascorbate reduced + CO] difference spectrum recorded. Next the [ascorbate-reduced]-[ascorbate reduced + CO + NaCN] difference spectrum was recorded by adding NaCN [1 mM] to the test cuvette. Finally 1.0 mg dithionite was added to each sample to obtain the [ascorbate + dithionite reduced]-[ascorbate + dithionite reduced + CO + NaCN] difference spectra. The latter could

for all purposes be considered (dithionite reduced)-(dithionite + CO + NaCN) difference spectra since the reducing power of dithionite overrides that of ascorbate. In all cases the isobestic point at 540 nm was first adjusted to coincide with that on the baseline before recording each spectrum. Each scan took approximately 2-3 mins and therefore it was possible to record the difference spectra at short intervals even at low scan speeds.

7. The detection of hydrogen peroxide in mitochondrial incubates of A. avenae

A qualitative test developed by Maehly and Chance (1954) as quoted by Cheah (1966) was employed to detect H_2O_2 in the mitochondrial fractions. Approximately 0.25 mg mitochondrial protein was suspended in 1.0 ml of reaction medium (Materials and Methods, Chapter 7A) and incubated with 5 mM succinate, 5 mM α-glycerophosphate, and 1 mM $NADH+H^+$, for 2 hr at 30^oC. Following incubation, 1.0 ml of 20 mM guaiecol and 0.3 ml of 0.1% horse radish peroxidase was added. The red brown colour (if any resulted) was rapidly extracted with an equal volume of ether and its spectrum i.e. that of tetra-guaiacol was recorded in a Shimadzu spectrophotometer. Ether standard was used as reference.

8. Detection of catalase activity in mitochondria and in the 12,000 g supernatant remaining after mitochondrial precipitate was removed

To 0.1 ml of mitochondrial suspension (approximately 0.25 mg mitochondrial protein) was added 0.2 ml reaction medium, 30 µl of a hydrogen peroxide solution (0.005 mM). After 10 min incubation at 30°C 1.0 ml of a 20 mM guaiacol and 0.3 ml of 0.1% horse radish peroxidase was added. The resulting mixture was immediately extracted with an equal volume of ether and the spectrum of the extract recorded. As a control, an identical volume of the mitochondrial suspension was heated to boiling in 0.2 ml of reaction medium and was treated in an identical manner to the unboiled sample in order to obtain the spectrum.

To obtain the standard tetraguaiacol spectrum, to 30 µl of a hydrogen peroxide solution (0.005 mM) was added 1.0 ml of 20 mM guaiacol, and 0.3 ml of a 0.1% horse radish peroxidase. The red brown colour (tetraguaiacol) was rapidly extracted with an equal volume of ether and the spectrum of the extract recorded. This standard spectrum was compared with the spectrum obtained with the test and control spectra.

9. The difference spectra of whole worms

A. avenae were harvested from mass cultures and extracted as described in the General Materials and Methods. 0.86 ml of live worms containing 60 mg wet wt nematodes/ml

was dispensed into each of 2 silica cuvettes (3 ml volume, 10 mm light path) and an equal volume of 50% (w/v) Ficol was added to each cuvette to prevent the nematodes from sedimenting. After obtaining a baseline the following difference spectra were obtained.
1) (aerated) minus (KCN reduced) 2) (ferricyanide)-(NaCN reduced) and 3) (ferricyanide)-(dithionite + KCN reduced).

Results

1A. The difference spectra of isolate-F whole worms.

When the reference sample was aerated and 3mM KCN was added to the test sample, the difference spectrum showed the appearance of a clear peak at 427 nm. in the Soret region while more diffused peaks one with midpoint at 564-565 nm and another at 600-602 nm. (Fig. 51A) were also suggested.

Addition of dithionite to the test sample and ferricyanide to the reference sample enhanced the peaks and the (ferricyanide oxidised)-(dithionite + NaCN reduced) difference spectra showed a broad peak stretched over 30 nm from about 548-572 nm with the mid point at 564-565 nm and a suggestion of a peak at about 554-555nm (Fig. 51.) which suggest b and c peaks respectively. The combined β-peaks of the b and c types were seen at around 527 nm. A small hump around 600-602 nm suggests the α-peak of an a type.

1B. The difference spectrum of the 380 g precipitate

In the preparative technique employed by Rothstein et al. (1970) for T.aceti mitochondria, following homogenisation of the worms (using graded sand as abrasive) the homogenate was first centrifuged at 480 g and the resulting precipitate was discarded. Murfitt, Vogel and Sanadi in isolating the mitochondria of C.elegans discarded the supernatant of the first (380 g) centrifugation. (Murfitt et al., 1976).

Figure: 51 Difference Spectra of A. avenae isolate-F whole worms.

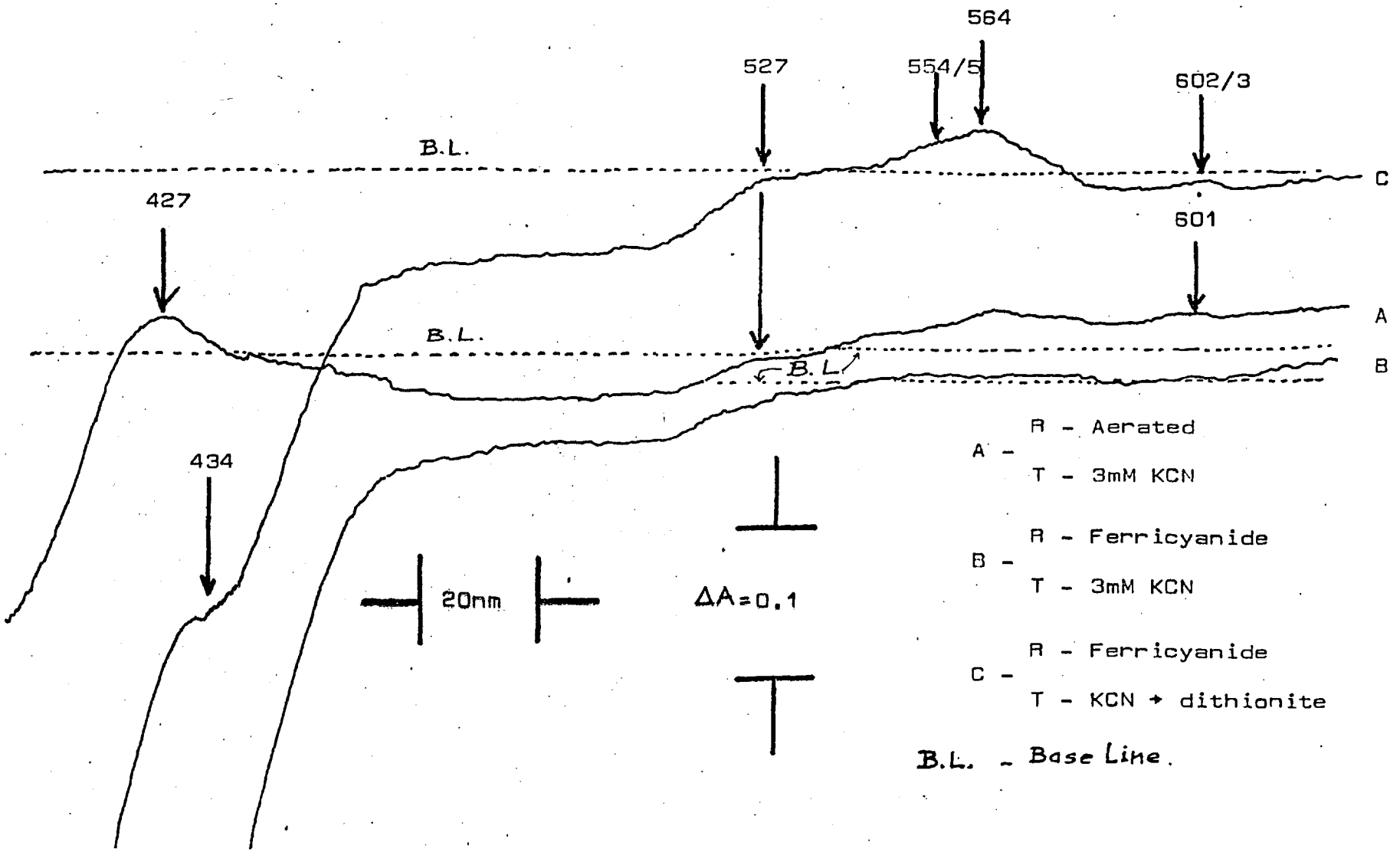


Figure 52 The (ferricyanide-Oxidised) - (dithionite Reduced) differences spectrum of the 380 g sediment resulting from the first centrifugation in the preparative method for the isolation of A. avenae mitochondria. (This fraction was discarded in some isolation procedures, viz., in the preparation of mitochondria from Turbatrix aceti by Rothstein et al., 1970) -

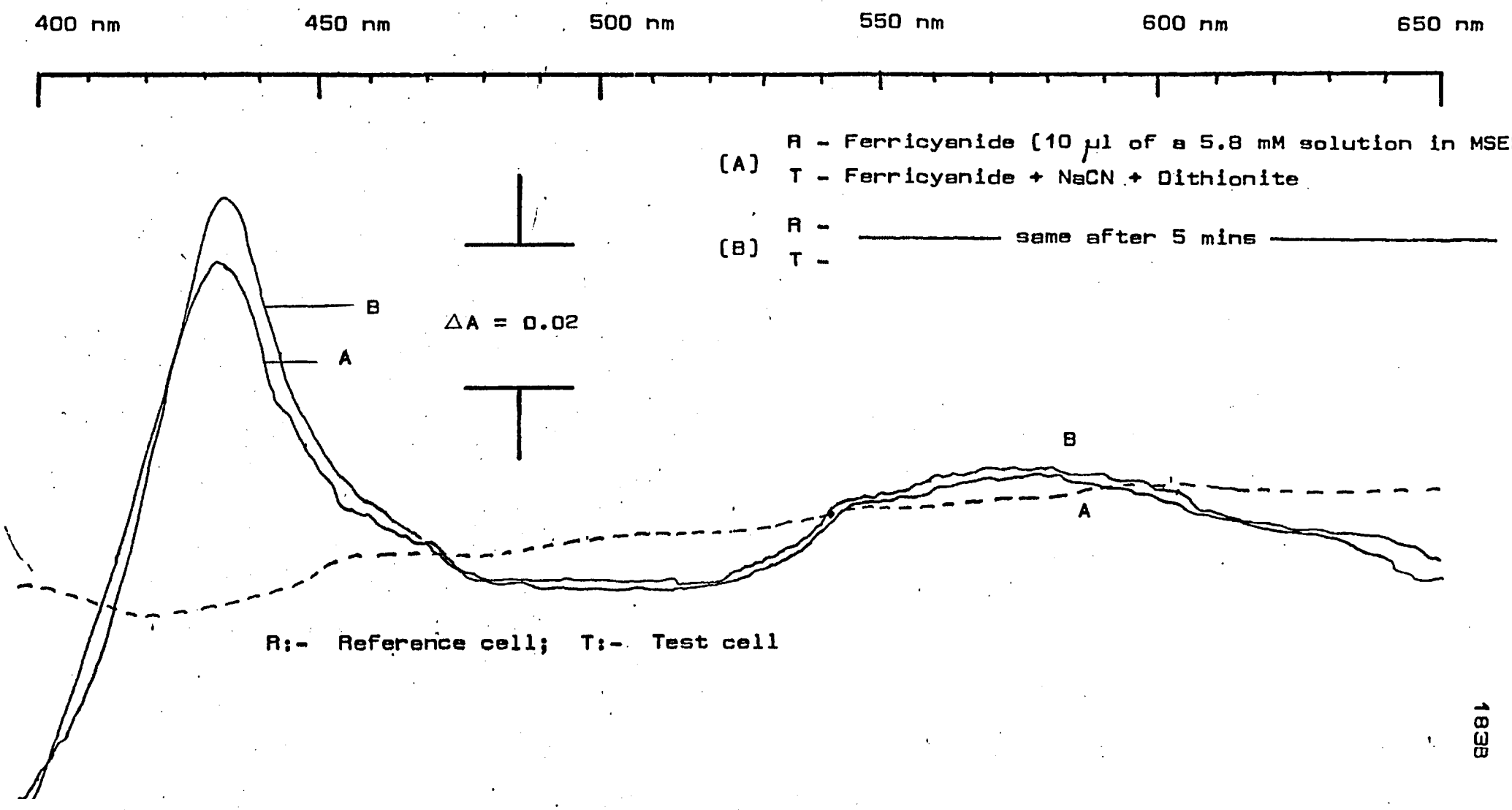
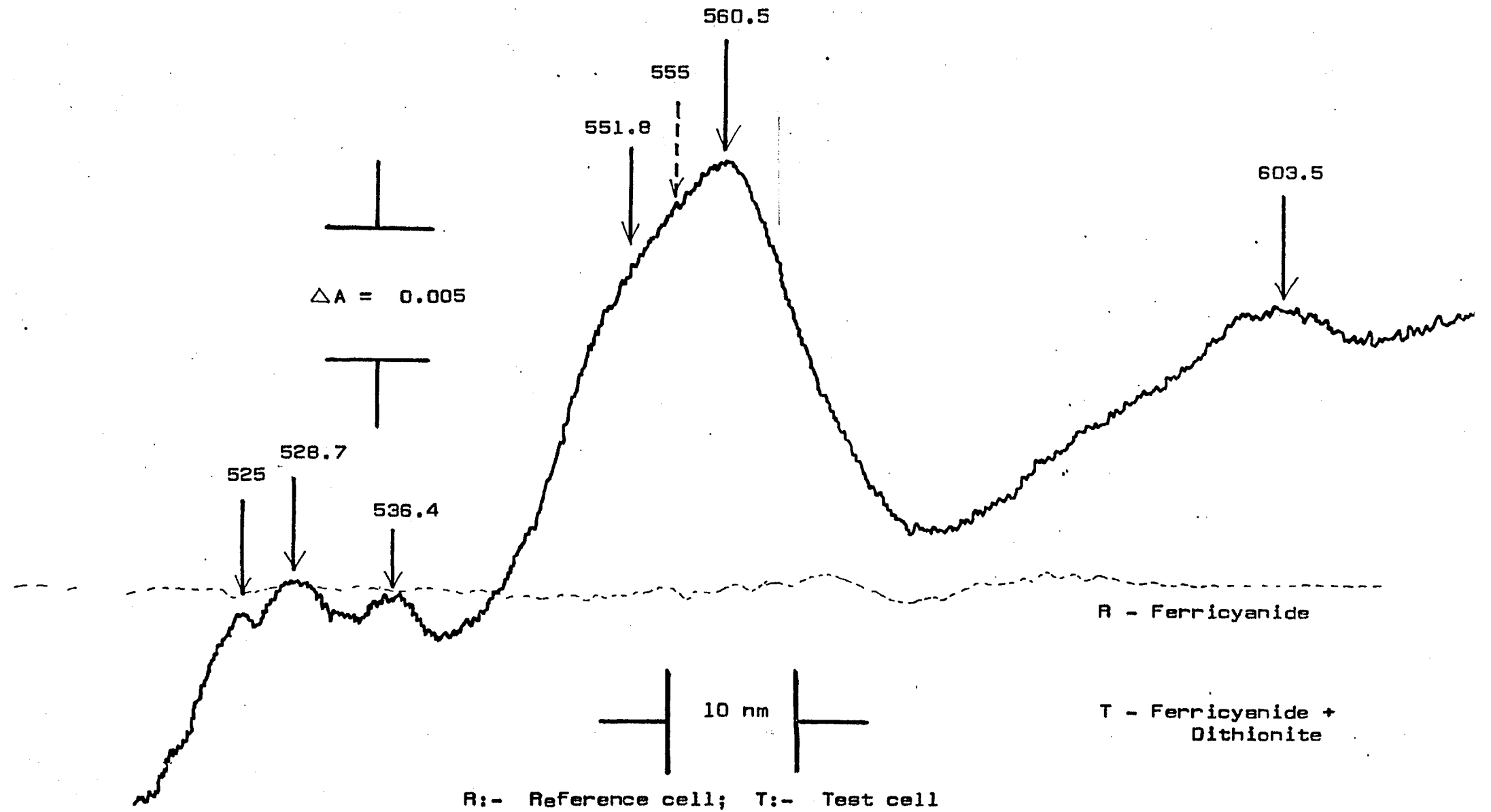


Figure 53 A typical (Oxidised)-(Reduced) difference spectrum of an Isolate-A mitochondrial preparation (Spectrum recorded at room temperature 24°C)



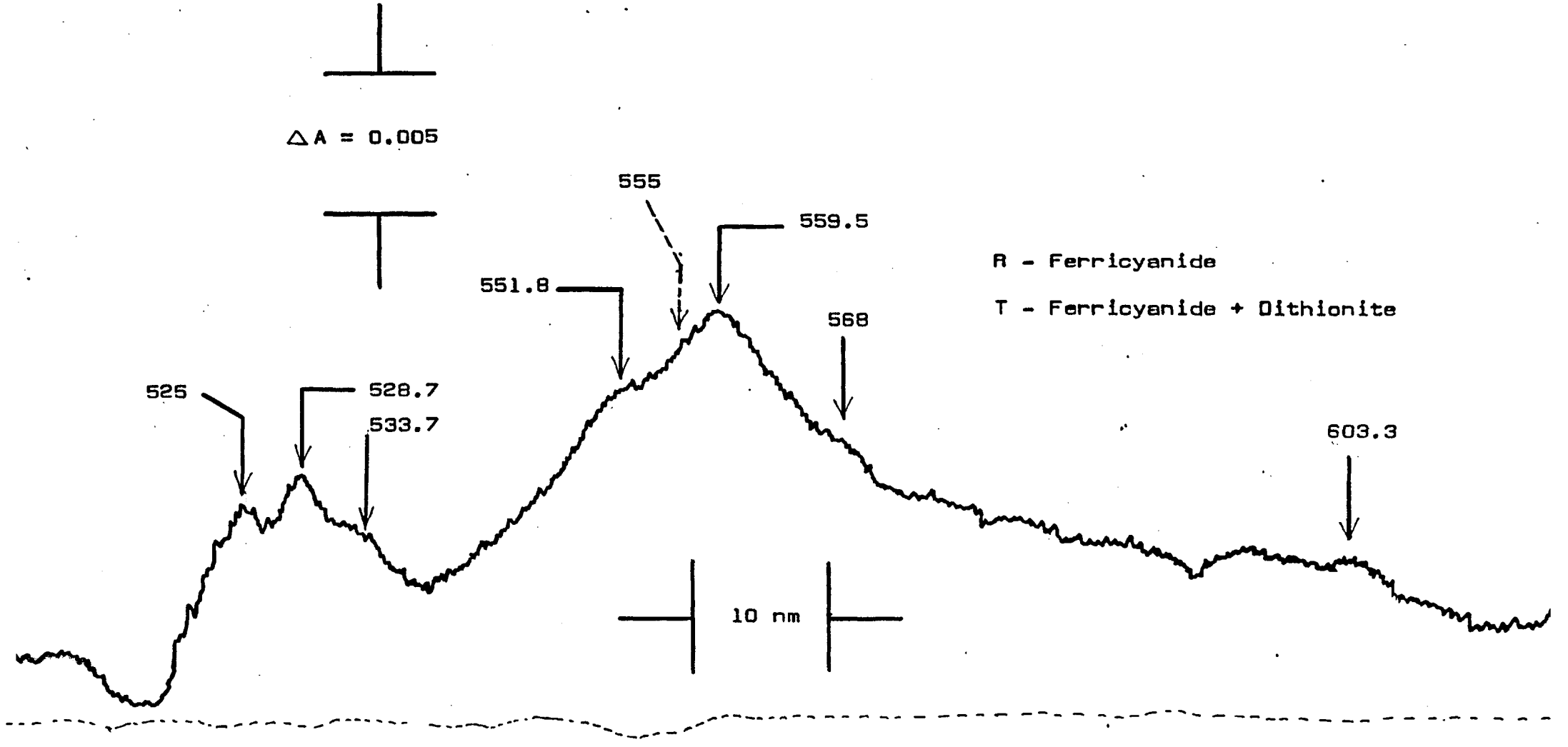
Therefore the homogenates of A.avenae (isolate-F) was centrifuged at 380 g and the pellet was examined in the Shimadzu MPS-50L. The (dithionite reduced)-(ferricyanide oxidised) spectrum of this fraction clearly showed the presence of cytochrome components (Fig. 52).

2. The (oxidised)-(reduced) spectra of mitochondrial fractions of isolates A and F (at room temperature)

The room temperature (ferricyanide oxidised)-(ferricyanide + dithionite reduced) difference spectra of mitochondrial preparations of isolate-A (Fig. 53) exhibited absorbance maxima at 603.5 nm, 560.5 and 551.8 nm. These correspond to the α-peaks of cytochrome aa_3, b and c types respectively. The room temperature difference spectra of isolate-F preparations (Fig. 54) showed similar α-peaks at 603.3 nm, 559.5 nm and 551.8 nm.

The corresponding β-peaks of the b-type and c-type cytochromes of the mitochondria of isolates A and F exhibited minor differences in the R.T. spectra. There were 3 absorption maxima discernible in each isolate. Isolate-A (Fig. 53) exhibited peaks at 536.4, 525 and 528.7 nm; while isolate-F (Fig. 54) preparations showed peaks in the β-range at 533.7, 525, and 528.7 nm. The 536.4 and 533.7 nm maxima represent the β-peaks of the b-type cytochromes.

Figure 54 A typical [Oxidised]-[Reduced] difference spectrum of an Isolate-F mitochondrial preparation [Spectrum recorded at room temperature 24°C]



R:- Reference cell; T:- Test Cell

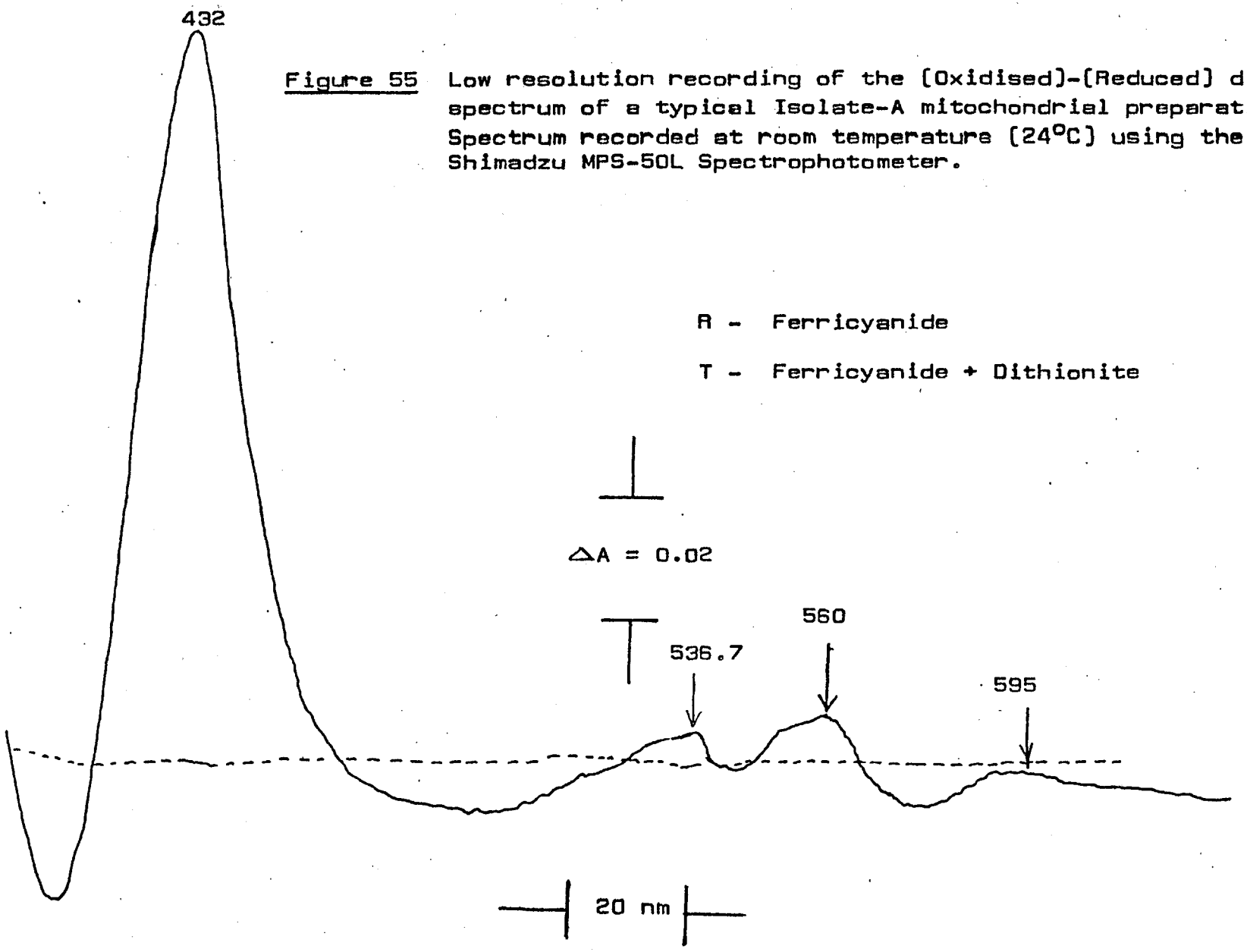


Figure 55 Low resolution recording of the [Oxidised]-[Reduced] difference spectrum of a typical Isolate-A mitochondrial preparation. Spectrum recorded at room temperature (24°C) using the Shimadzu MPS-50L Spectrophotometer.

R:- Reference cell; T:- Test cell

Figure 55 shows the Soret region (peak at 432 nm) in relation to the α and β peaks in a low resolution scan (obtained using the Shimadzu MPS-50L Spectrophotometer) of the (Ferricyanide)-(Ferricyanide + dithionite) difference spectra of isolate-A mitochondria.

3. The difference spectra at 77°K

The -196°C i.e. liquid nitrogen temperature (Ferricyanide)-(Ferricyanide + dithionite) difference spectrum of isolate-A mitochondrial preparations (Fig.56) showed the presence of an α-peak at 557.7 nm which co-related with the 560.5 nm room temperature α peak of the b-type cytochrome. However the absorption peak which at 77°K would co-relate with the room temperature peak at 551.8 nm and which was expected to occur at 549.8 or 548.8 nm i.e. a wavelength 2-3 nm shorter than the wavelength at which the room temperature maximum was observed, was not apparent. Instead a peak was observed at 525 nm which did not co-relate with either the 560.5 nm (b, α) or the 551.8 nm (c, α)peak observed at room temperature.

The absorption maxima in the β region were seen at 520.5 and 528.5 nm. while the α-peak at 596.0 corresponds to the α-peak of cyt.aa_3.

The difference spectra (77°K) of the isolate-F preparations (Fig. 57) showed maxima at 557.5 (α of b type),

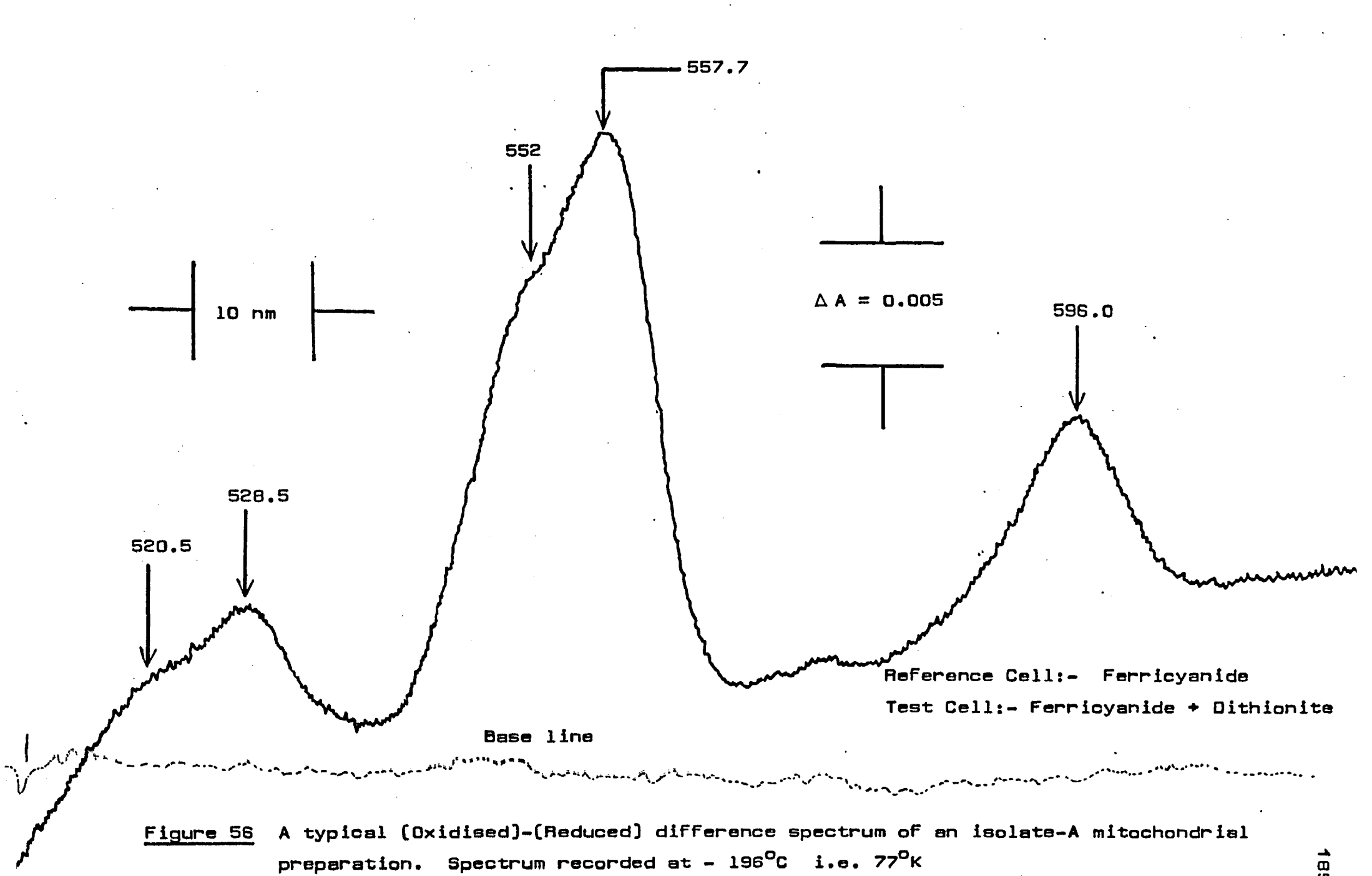


Figure 56 A typical (Oxidised)-(Reduced) difference spectrum of an isolate-A mitochondrial preparation. Spectrum recorded at - 196°C i.e. 77°K

185A

Figure 57 A typical (Oxidised)-(Reduced) difference spectrum of an Isolate-F mitochondrial preparation. Spectrum recorded at -196°C (77°K)

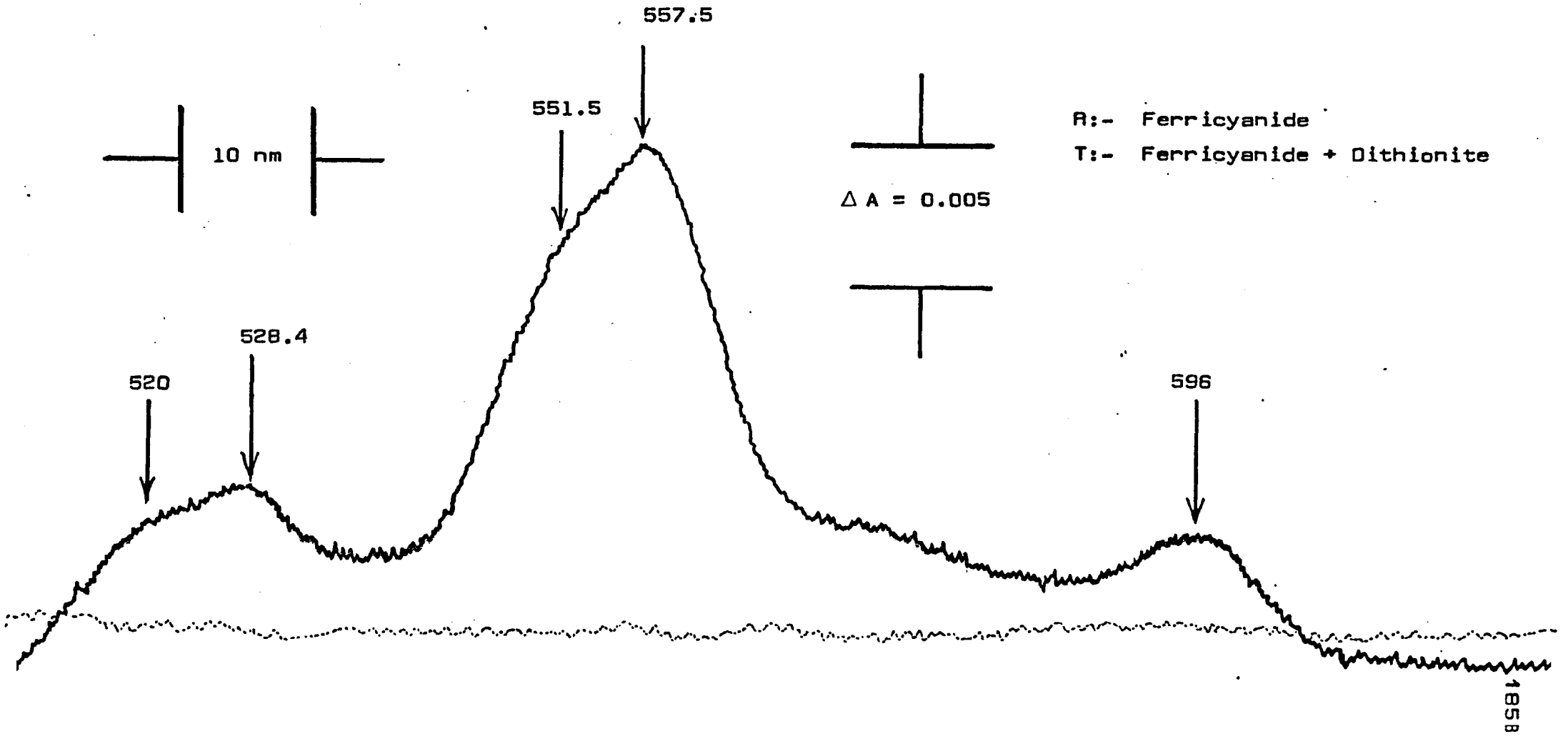
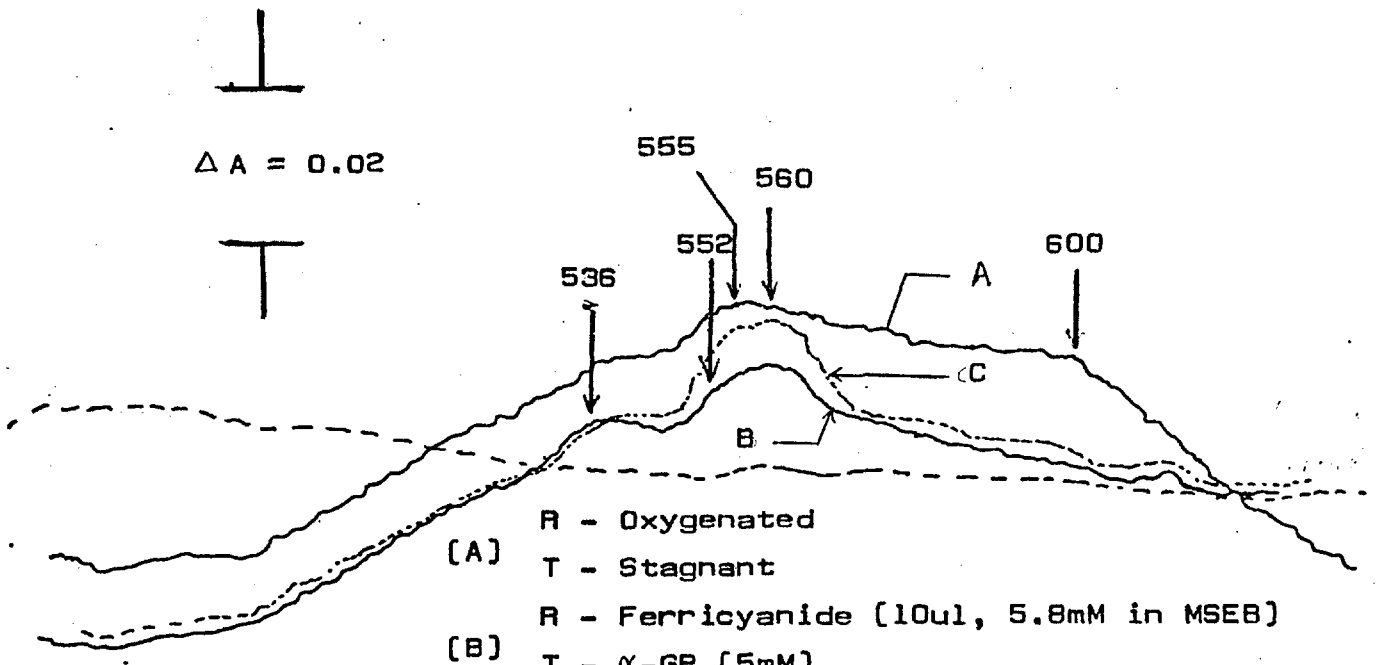


Figure 58 Substrate [α-GP] induced reduction of cytochromes of a typical isolate-F mitochondrial preparation and the effect of antimycin-A. Spectra recorded at 24°C



R:- Reference cell; T:- Test Cell.

551.5 nm. The latter could be the unresolved peak of a b and/or c type cytochrome since a suggestion of a peak at 548 nm (the 77°K α-maximum of the c-type cytochrome which co-relates with the 551.8 room temperature c, α peak) was apparent. In the β-region, maxima were seen at 520 and at 528.4 nm. The α-peak of cytochrome-a was clear at 596 nm.

The concentrations of the cytochrome components for each isolate computed employing the simultaneous equation-matrix of Williams (1963) are given in Table 25. The extinction coefficients used are given in Appendix 7-3.1 and the matrix computation in Appendix 7-3.2.

4. The effect of substrates and antimycin-A on the difference spectra of isolate-A mitochondria

Figure 58A shows the endogenous reduction of isolate-A pigments achieved by aeration of the reference sample while the test sample was kept stagnant. The increase in absorbance in the β-region (maximum at 536 nm) and in the α-region at 555 and at 552 nm indicates the reduction of b and c type cytochromes with a suggestion of a weak maximum at around 600 nm which is probably the α of a-type cytochromes.

On application of 5mM α-glycerophosphate to the test sample while the reference was oxidised using ferricyanide produced a broader peak in the α-region with the mid-point of the peak at 560 nm while the absorption at 536 nm increased little. (Fig. 57A and B).

Addition of antimycin-A to the test sample had little effect on the difference spectrum (Fig. 57C). The absorbance increased at 560 nm, minutely more than at 552 or at 555 nm and a small trough appeared between 555 and 560 nm, suggesting that the b-component at 560 nm was relatively more reduced.

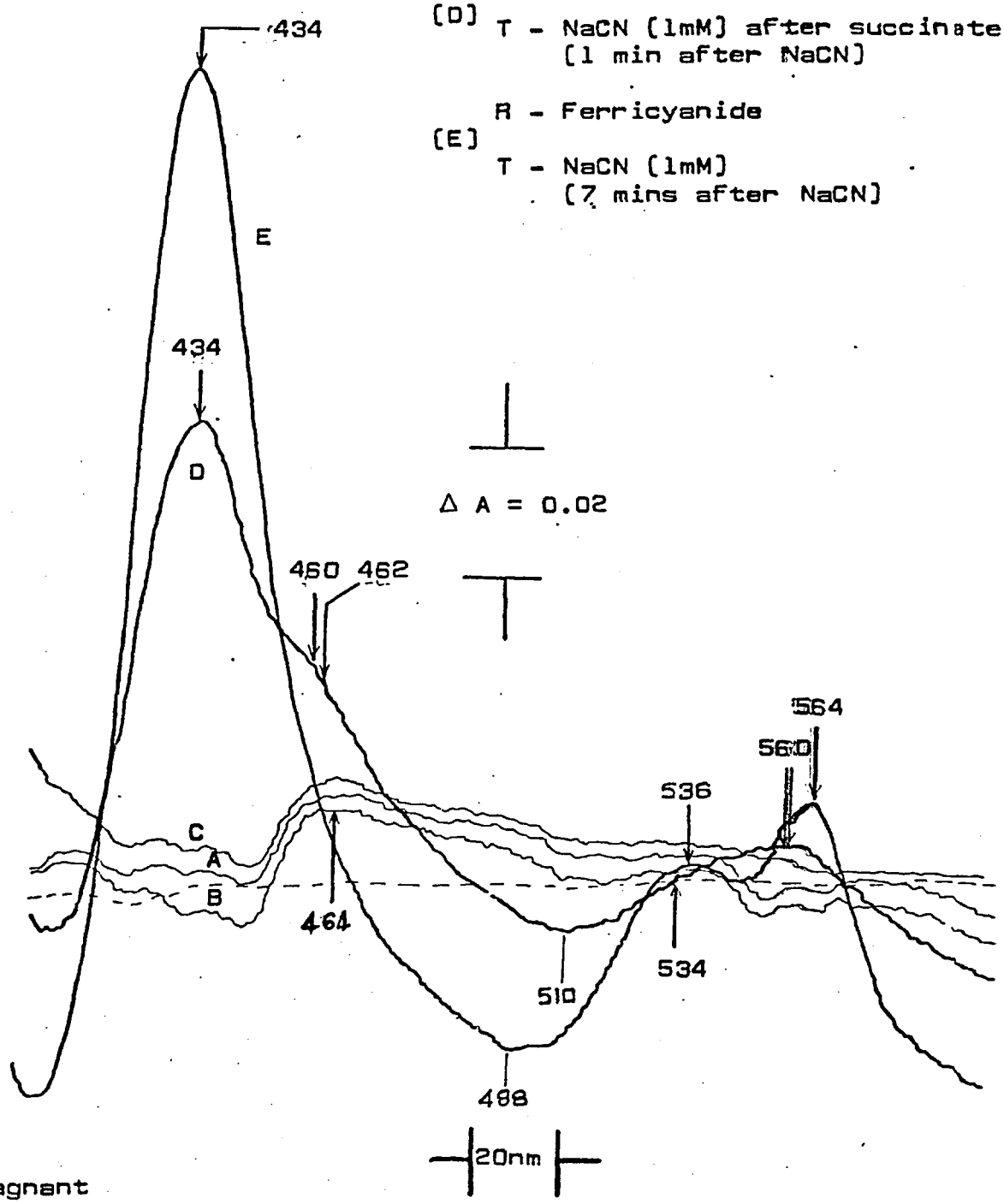
This preparation had the greatest concentration of mitochondria and was prepared from approximately 20 g wet weight of whole nematodes. A sample of this preparation was used to obtain the high resolution spectra (Figs. 53 and 56) at room temperature and at liquid nitrogen temperatures in the Aminco-Chance spectrophotometer.

However the response to substrates and antimycin-A were not so well defined as expected. This may be because the preparation was frozen for some time before analysis due to unavoidable circumstances.

5. The effect of substrates and NaCN on isolate-A mitochondrial preparations

The addition of succinate to the test sample while the reference was stagnant produced little change in the difference spectrum (Fig. 59A,B and C) since only a peak at 465 nm and a diffuse increase around 534 nm was apparent. Little change was apparent even after 5 mins. Oxidation of the reference sample with ferricyanide increased the absorption little between 534 and 560 nm.

Figure 59: The effect of substrates (succinate) and NaCN on the difference spectrum of a typical isolate-A mitochondrial preparation.



[A] R - Stagnant
T - Succinate [5mM] + aeration

[B] R - —— same as [A] after 5 min ——
T -

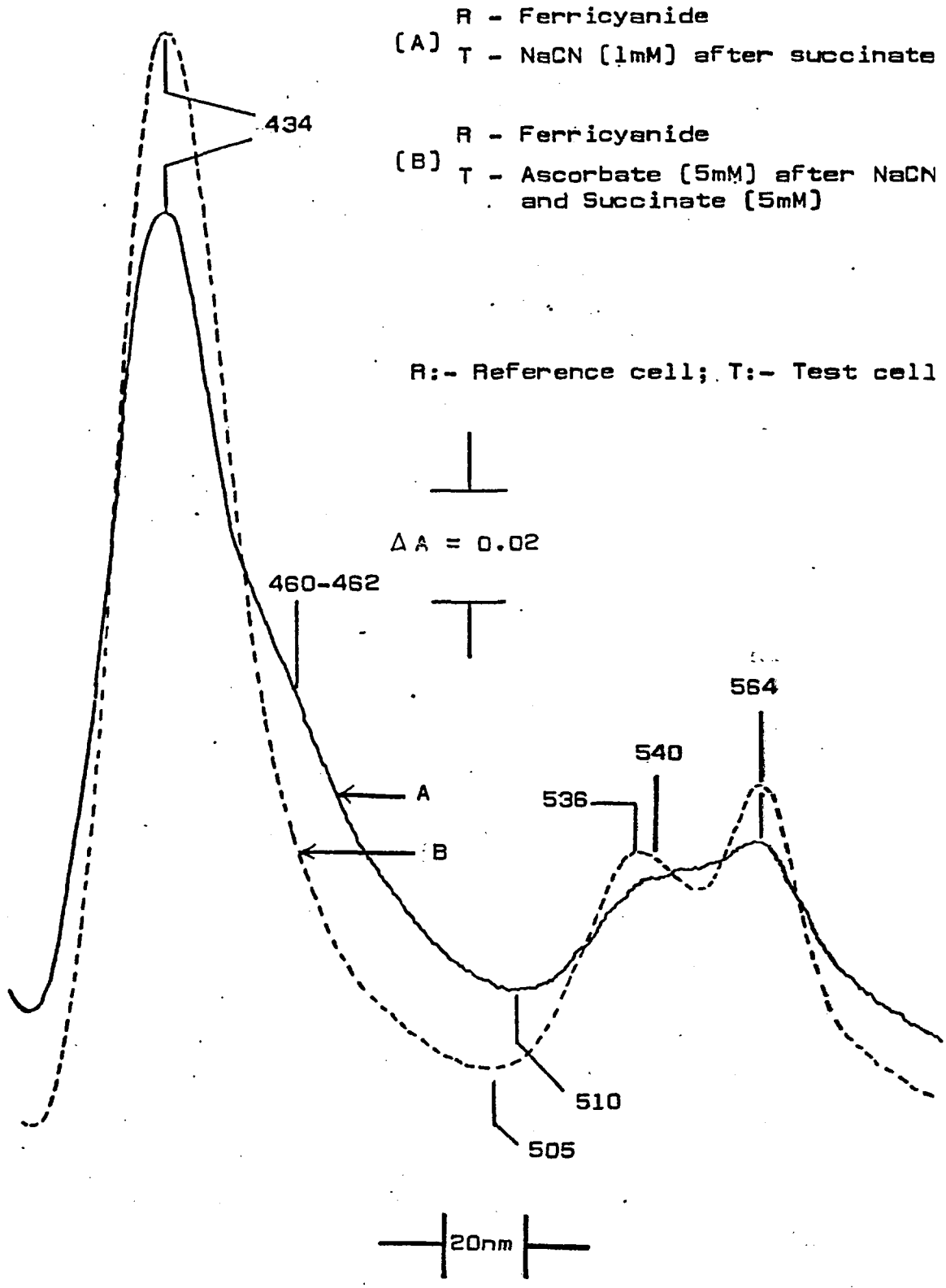
[C] R - Ferricyanide
T - Succinate + aeration

However, addition of 3 mM NaCN to the test sample (while the reference sample was oxidised with ferricyanide) brought about an immediate change in the difference spectrum. Figure 59D shows the spectrum recorded 1 min following cyanide application. A peak appeared at 434 nm in the Soret region, with a prominent shoulder between 460-462 nm. A trough with minimum at 510 nm was followed by a diffuse peak originating at about 534 nm and culminating at 560 nm.

7.0 mins following NaCN application (Fig. 59E) the peak at 434 nm increased sharply by about 0.04 units and the shoulder between 460-462 nm was not discernible. The trough at 510 nm shifted to 498 nm and a clear peak appeared in the β-region at 536.5 and a second sharper peak at 564 nm in the α-region.

Addition of ascorbate (5mM) to the test sample in the presence of NaCN + succinate (Fig.60B) reverted the difference spectrum, in that the absorbance at 434 nm. was reduced (by about 0.02 units) and the shoulder, which appeared upon immediate application of NaCN (Fig.59D) reappeared and the peaks in the α(564 nm) and β(536 nm) regions first observed after 7 mins in NaCN (Fig. 59E) were diminished to a more diffuse α-peak at 540 nm while the β-maximum at 564 nm showing a reduction in O.D. remained.

Figure 60 The effect of ascorbate (5mM) on the difference spectrum produced by a typical Isolate-A mitochondrial preparation in the presence of cyanide.



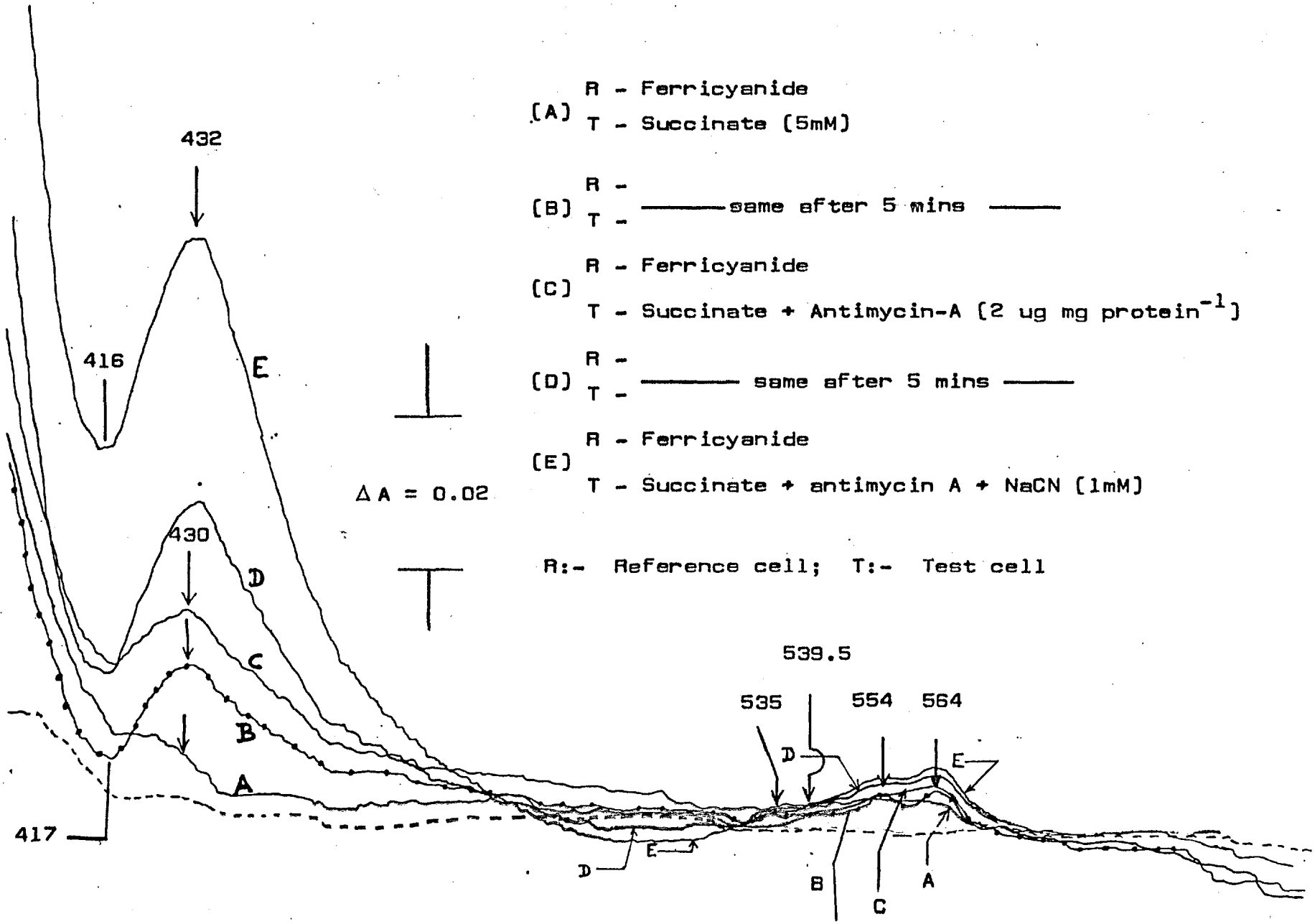


Figure 61 The effect of succinate, antimycin-A, and cyanide on the difference spectrum produced by a typical isolate-F mitochondrial preparation

6. The effect of substrates, antimycin-A, and NaCN on isolate-F mitochondrial preparations

Addition of 5 mM succinate to the test sample while the reference sample was oxidised by ferricyanide [Fig. 61A] produced a small increase in absorbance at 430 nm and a diffuse increase in the α and β regions of the spectrum. 5 min after the treatment, the Soret peak at 430 nm was significantly increased [Fig. 61B] and two clear but small peaks were discernible at 564 and 554 nm in the α-region indicating the reduction of b and c type cytochromes. The corresponding β-region was diffused and unclear.

Addition of antimycin-A (5µg/mg protein) to the test cell reduced with succinate further enhanced the 430 nm Soret peak [Fig. 61C] but in the α-region the trough between the 554 and 564 nm maxima disappeared and the absorbance at 564 nm perceptibly increased 5 mins after antimycin-A treatment, [Fig. 61D] the 564 peak being clearly more enhanced relative to the 554 peak of the c-type. Accompanying this, the Soret peak had shifted approximately 2 nm to ~ 432 nm.

Finally in the [ferricyanide]-[succinate + antimycin-A + NaCN] difference spectrum [Fig. 61E] the Soret maximum at 432 was further enhanced while the 554 and 564 increased little from that seen in the [ferricyanide]-[succinate + antimycin-A] difference spectrum.

7. Difference spectra of the CO-complexes of isolate-F mitochondria

The difference spectrum of the CO-complexes of isolate-F mitochondria is shown in Figure 62 in the sequential form in which it was obtained. Since the Soret peaks of the cytochrome components and their change were to be recorded, a different scale, i.e. the highest resolution afforded by the Shimadzu MPS-50L spectrophotometer was employed.

Figure 63 shows the major changes in the Soret maxima recorded during the sequence followed in obtaining the dithionite reduced CO-difference spectra.

The [Ferricyanide Ox.]-[dithionite Red.] difference spectrum (Fig. 62A) shows peaks at 564 nm [$\underline{b}, \alpha$] and 534 nm (β $\underline{c}$ and $\underline{b}$ type) with the Soret peak at 432 nm.

On treatment of the dithionite reduced sample for 1.0 min with carbon-monoxide (Fig. 62B) the peak at 564 nm decreased in absorbance while the 534 nm peak shifted to 540 nm with a slight reduction in absorbance. Significantly the Soret-peak shifted towards a shorter wavelength by approximately 11.0 nm., to 421.5 nm. with a concomitant increase in absorbance.

On reduction of the reference sample also with dithionite (Fig. 62C) the test sample having been initially reduced with dithionite and treated with CO for 1.0 min., the Soret peak shifted 3 nm towards the shorter wavelength to peak at 419 nm-this time accompanied by $\simeq$ 0.01 reduction in absorbance.

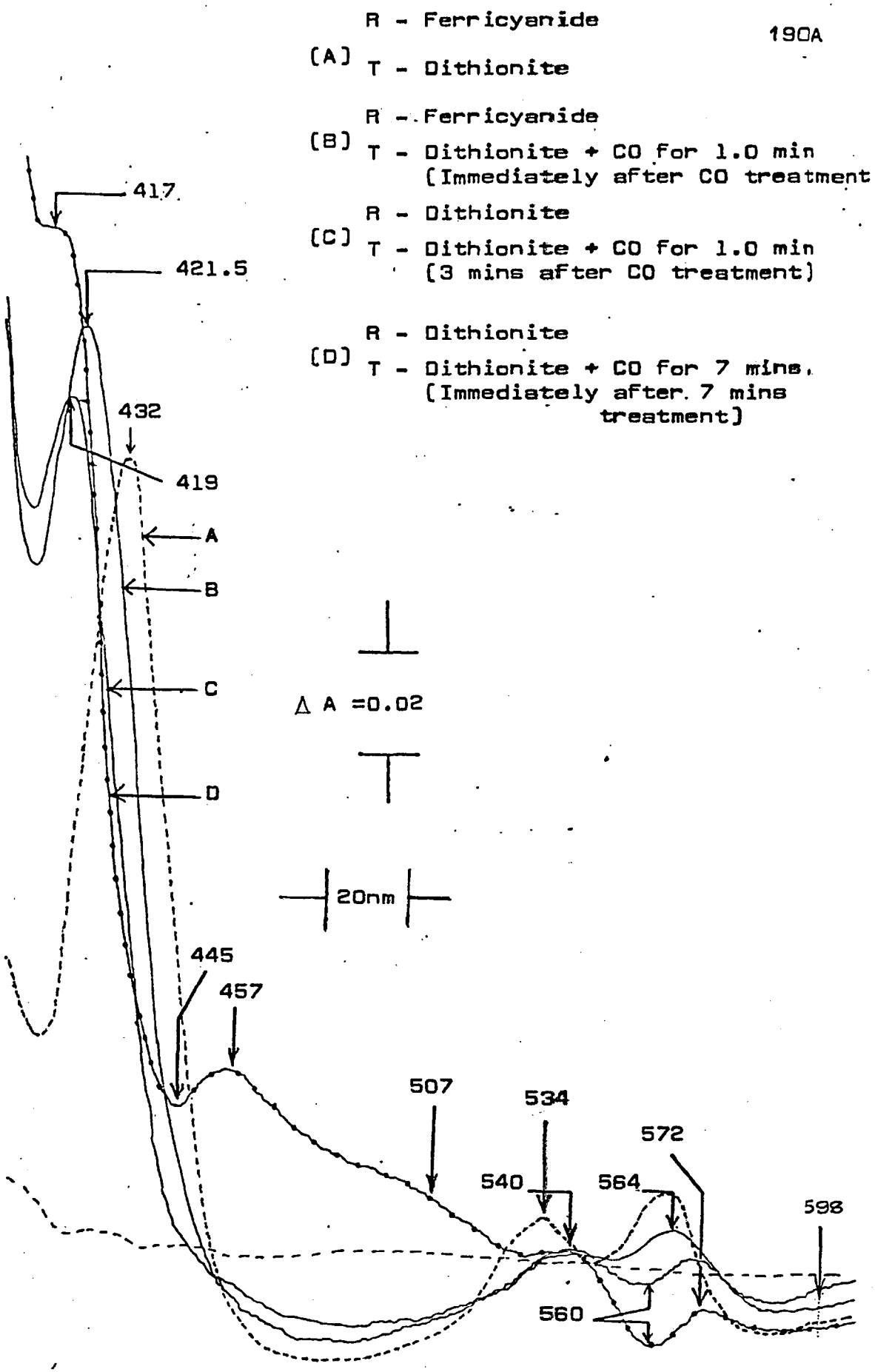
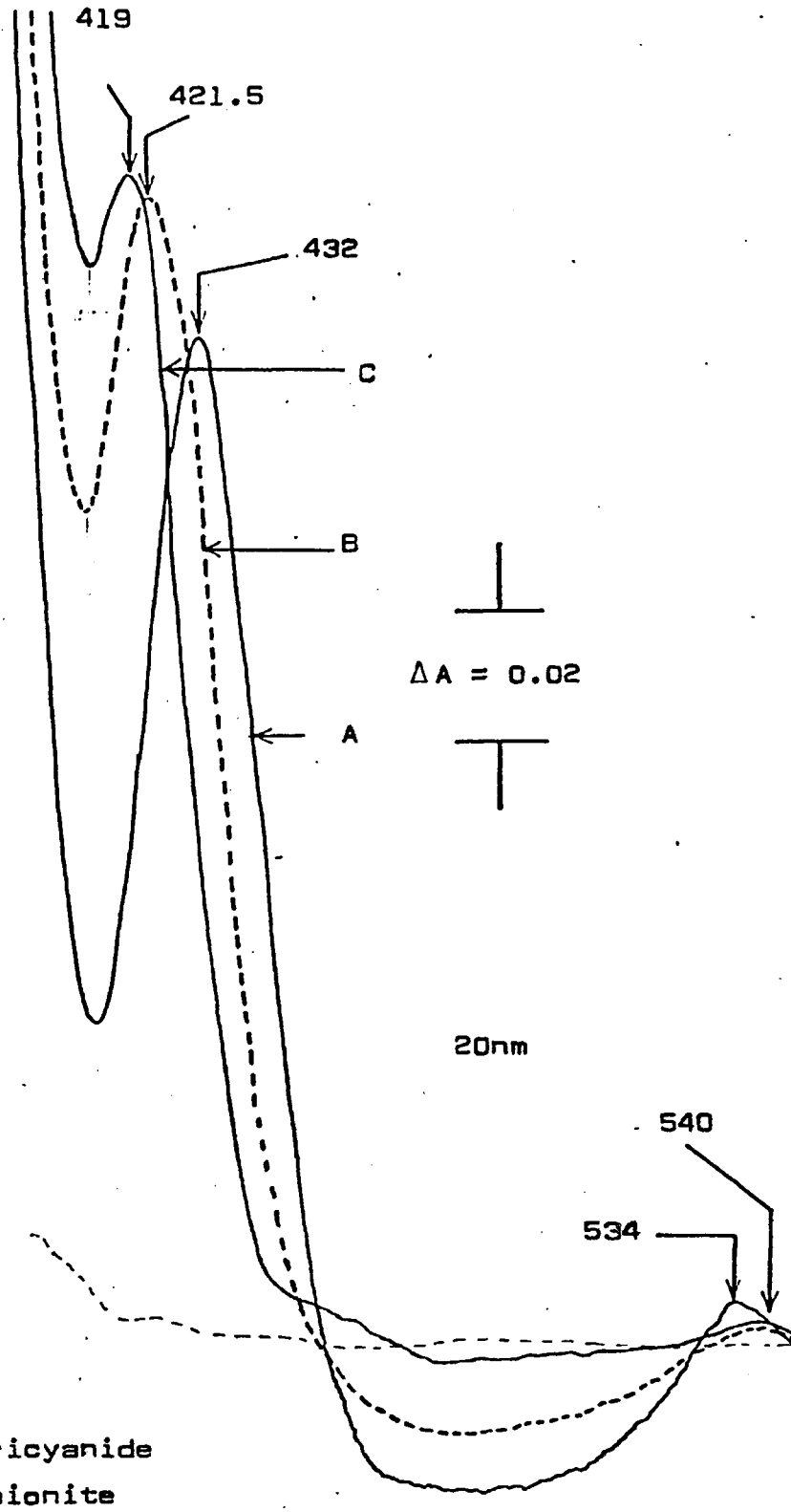


Figure 62 Changes recorded in the (Oxidised)-(Reduced) difference spectrum of a typical mitochondrial preparation of isolate-F during the sequence employed to obtain the final CO-difference spectrum viz.,(Dithionite reduce)-(Dithionite + CO)

Figure 63 Changes in the Soret-maxima recorded during the ISO B. sequence employed to obtain the CO-difference spectrum of an isolate-F mitochondrial preparation



(A) R - Ferricyanide
T - Dithionite

(B) R - Ferricyanide
T - Dithionite + CO for 1.0 mins (immediately after CO treatmen

(C) R - Dithionite
T - Dithionite + CO for 1.0 mins (7 mins after CO treatment)

R:- Reference Cell; T:- Test cell

In the α and β regions, (Fig. 62C) the peak at 564 nm disappeared and a new peak appeared at 572 nm, accompanied by a trough at 560 nm. The peak at 540 nm remained unaltered.

When CO was bubbled continuously for 7.0 mins., the Soret peak at 419 (Fig. 62D) increased significantly in absorbance and a marked trough appeared at 445 nm while two new peaks appeared at 457 nm and at 507 nm. The trough at 560 nm deepened but the peaks at 540 and 572 remained unaltered.

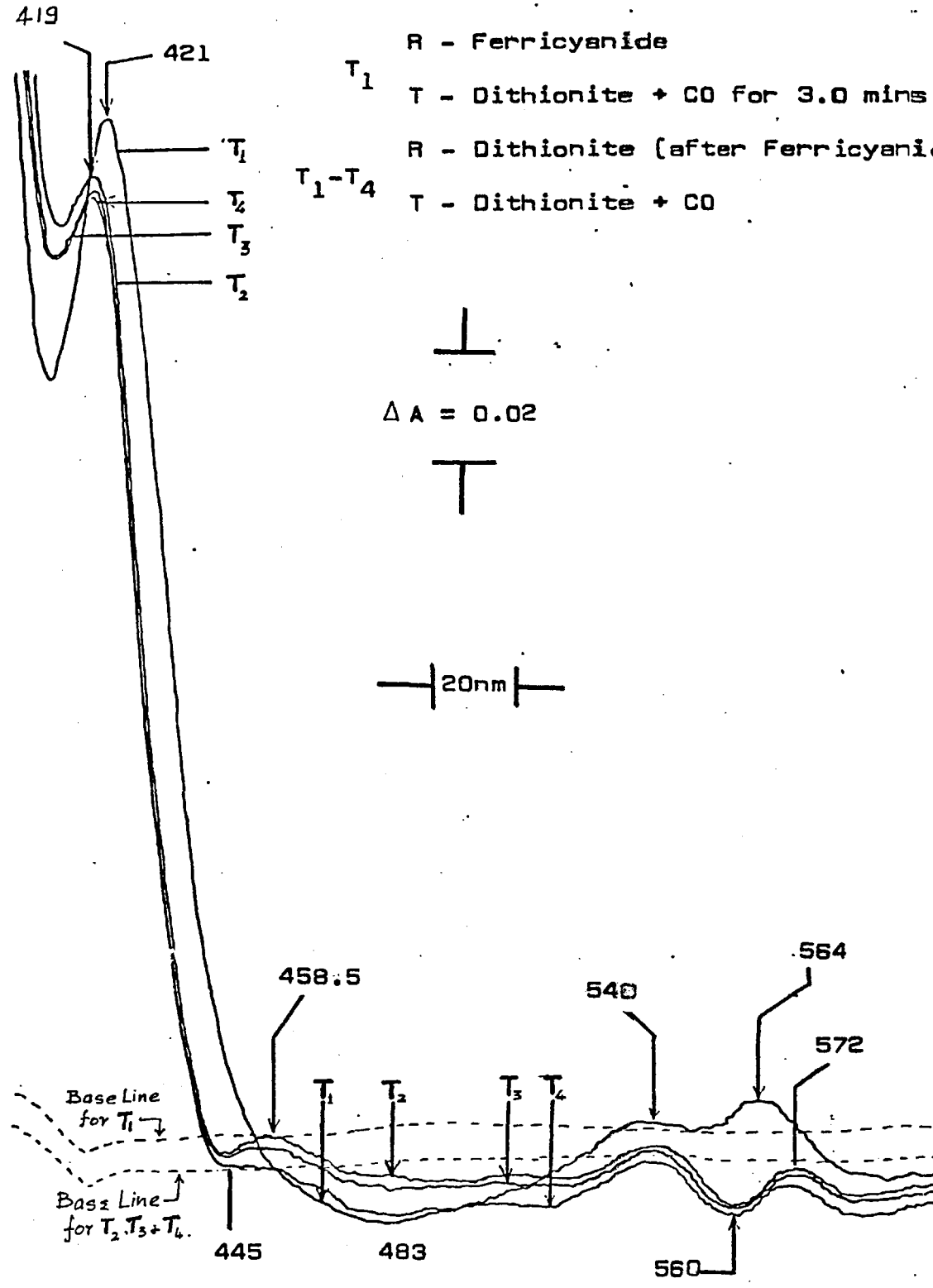
A clear peak corresponding to the α-peak of the a_3-CO complex was not seen at 594 nm although a diffuse peak was observed at 598 nm (Fig. 62D).

8. <u>The time dependent changes in the CO-difference spectra of isolate-F mitochondria</u>

The changes in the absorption maxima of the CO-difference spectra with time following reduction of the reference sample with dithionite is shown in Figure 64.

After 3.0 mins following treatment of the test cell with CO, the Soret peak was observed at 424 nm (Fig. 64T_1). The broad trough with midpoint at 483 nm observed in the (ferricyanide OX.)-(S_2O_4 + CO) difference spectrum was replaced by a peak at 458.5 nm on treatment of the reference sample with dithionite (Fig. 64T_2); in addition the Soret peak at 421 nm shifted to 419 nm with a consequent reduction in absorbance and a new peak (at 572 nm) and trough (minimum at 560 nm) appeared while the 540 nm peak became more prominent.

Figure 64 The time dependent changes in the CO-difference spectrum of an isolate-F mitochondrial preparation



T_2 - 3 mins. after reduction of reference sample with Dithionite i.e. 5 mins after CO-treatment of test sample.

T_3 - 10 mins. after reduction of reference sample with Dithionite i.e. 7 mins after CO-treatment of test sample

T_4 - 15 mins. after reduction of reference sample with Dithionite i.e. 12 mins after CO-treatment of test sample

Table 25

The concentration of cytochromes in mitochondria isolated from isolates-A and F of A.avenae

Cytochrome	Wavelength Pairs (nm)	Major Coefficient (m.molar^{-1} cm^{-1})	Concentration (n.moles/mg protein) Isolate-A	Isolate-F
[a + a_3]*	605 - 630	12.0	0.140	0.148
b-types	563 - 577	14.3	0.272	0.299
c_1	554 - 540	18.8	0.113	0.126
c	550 - 535	21.0	0.057	0.061
[c + c_1]	-	-	0.170	0.187

* The concentration of cytochrome-a_3 in the 2 isolates cannot be calculated according to the Matrix method of Williams (1964). The method of Cheah (1970a) was employed for this and is given in Table 26.

Table 26

Approximate concentrations of the CO-reactive cytochromes in isolates A and F of A. avenae

Cytochrome type	Wavelength pairs	Millimslar Extinction Coefficients EmM	Concentration (n.moles mg.protein^{-1} Isolate-F	Isolate-A
a_3	427 - 4445	148.0 (Cheah,1970a)	0.078	0.060
o	419 - 434	80.0 (Cheah,1970a)	0.18	0.241
Ratio a_3:o			1:2.3	1:4.01

The difference spectrum obtained at time zero following 7.0 mins continuous CO treatment was altered little even after 12 hrs exposure to an artificial light source (Fig. 65). The small changes apparent were a) the shift of Soret peak from 417 - 420 b) the shift of 445 trough to 447 c) shift of 507 peak to 504. The 540 and 572 nm peaks were unaltered as was the 560 nm trough.

Figure 65 compares the CO-difference spectrum 12 and 18 hrs after its inception and the maxima and minima are basically the same except that beyond the 447 nm minimum there is a gradual decrease in absorbance viz. of the 504, 540, 572 and 593 peaks with the troughs at 560 deepening.

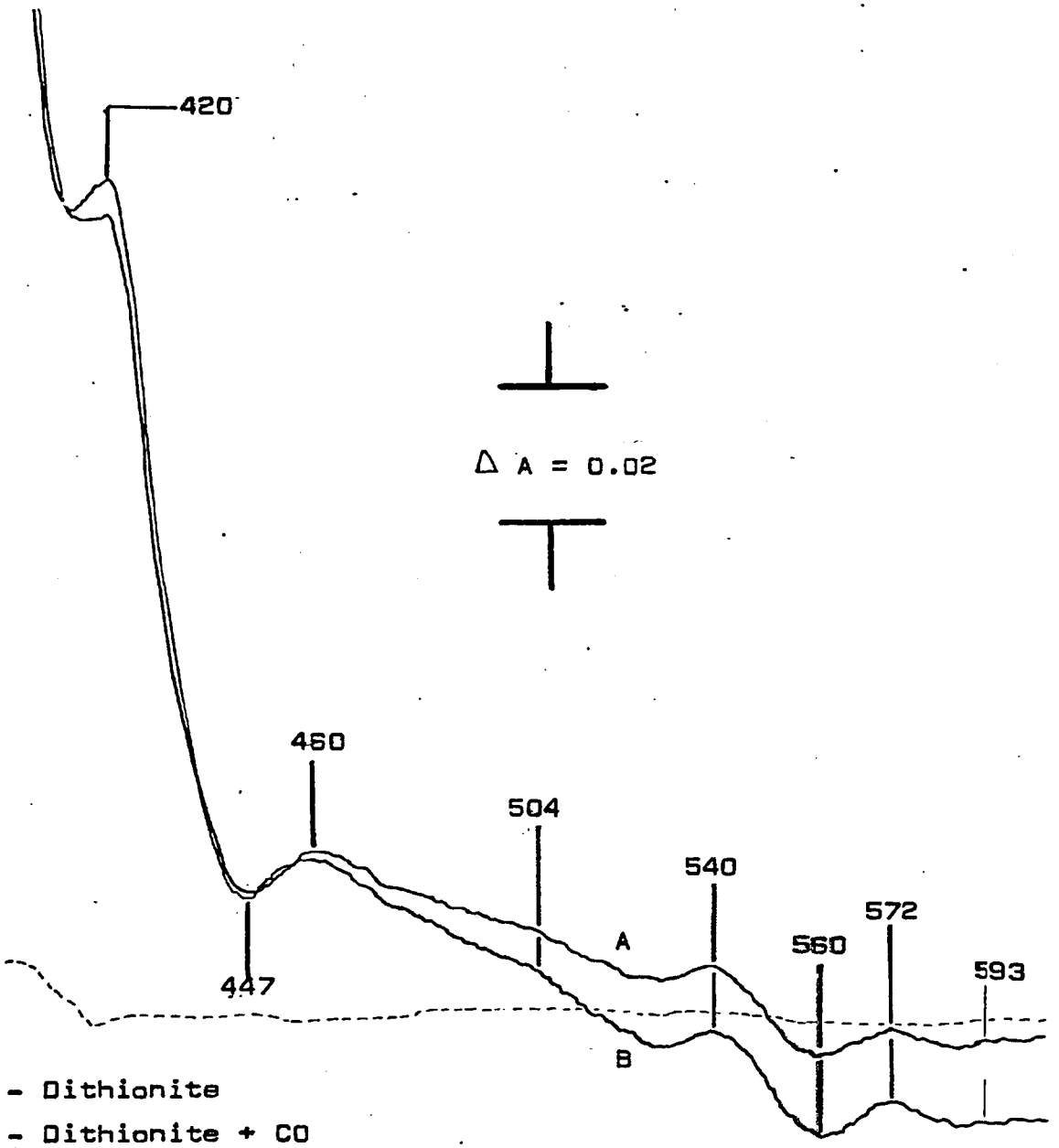
The ratio of the difference in absorbance changes between the wavelength pairs 413-430 nm and 572-558 nm for isolate-F was 15.95:1 which was calculated from Figure 64 T_4 i.e. 15 mins after CO treatment. The ratio is characteristic of cyt.c.

9. Difference spectra of the CO-complexes of isolate-A mitochondria

Figure 66 shows the marked changes in the difference spectra of isolate-A mitochondria brought about by the CO treatment of the dithionite reduced fractions.

Figure 66 trace (A) shows the (ferricyanide Oxd.)-(dithionite Red.) difference spectrum with the Soret

Figure 65 The CO-difference spectrum of an isolate-F mitochondrial preparation following 12 x 18 hrs exposure to a light source



(A) R - Dithionite
T - Dithionite + CO
[12 hrs after formation of CO-complex]

(B) R - Dithionite
T - Dithionite + CO
[18 hrs after formation of CO-complex]

R:- Reference cell; T:- Test cell

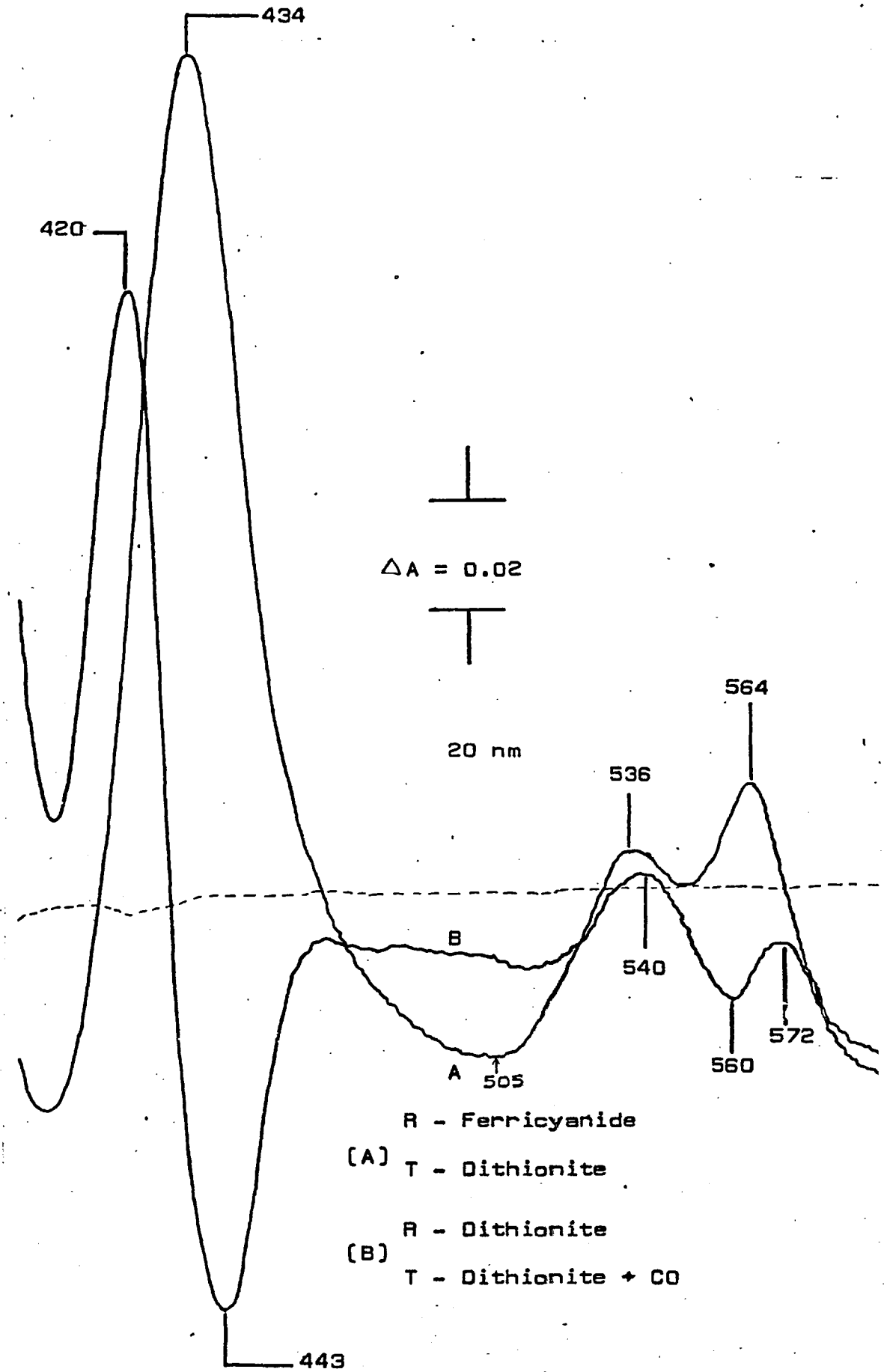


Figure 66 The typical [Oxidised]-[Reduced] difference spectrum of Isolate-A mitochondria compared with the CO-difference spectrum of the same preparation

peak at 432 nm, and the α and β maxima of the combined b and c types appearing at 536 nm and 564 nm at low resolution, (Fig. 53 shows the higher resolution of a similar spectrum) in order to accommodate the Soret peak.

Figure 66 trace (B) shows the (dithionite + CO)-(dithionite + ferricyanide) difference spectrum recorded 3 mins after 1.0 min CO treatment of the test cell. The wide trough with the minimum at 505 nm disappeared completely while a new deep and narrow trough with minimum at 443 nm appeared. At the same time the Soret maximum shifted 14 nm towards the shorter wavelength to peak at 420 nm.

Concomitant with these changes the 536 nm peak disappeared while a new peak at 540 was observed, in its place. The peak in the α-region at 564 nm completely disappeared and in its place a trough appeared almost directly opposite where the peak had been in the (Oxd.)-(Red.) difference spectra (Fig. 66B).

The peaks at 540, 572 nm and the trough at 560 nm are characteristic of the complex cytochrome-O forms with CO. The ratio of the difference in absorbance changes between the wavelength pairs 413-430 nm and 572-558 nm as measured from Fig. 66B was 13.75:1 which is similar to the ratio of cytochrome-O observed for other organisms.

Figure 67 The time dependent changes in the CO-difference spectrum of an Isolate-A mitochondrial preparation

Reference cell :- Dithionite
Test cell :- Dithionite + CO

T_1 - 3.0 min. after CO-treatment
T_2 - 7.0 min. after CO-treatment
T_3 -15.0 min. after CO-treatment
T_4 -30.0 min. after CO-treatment

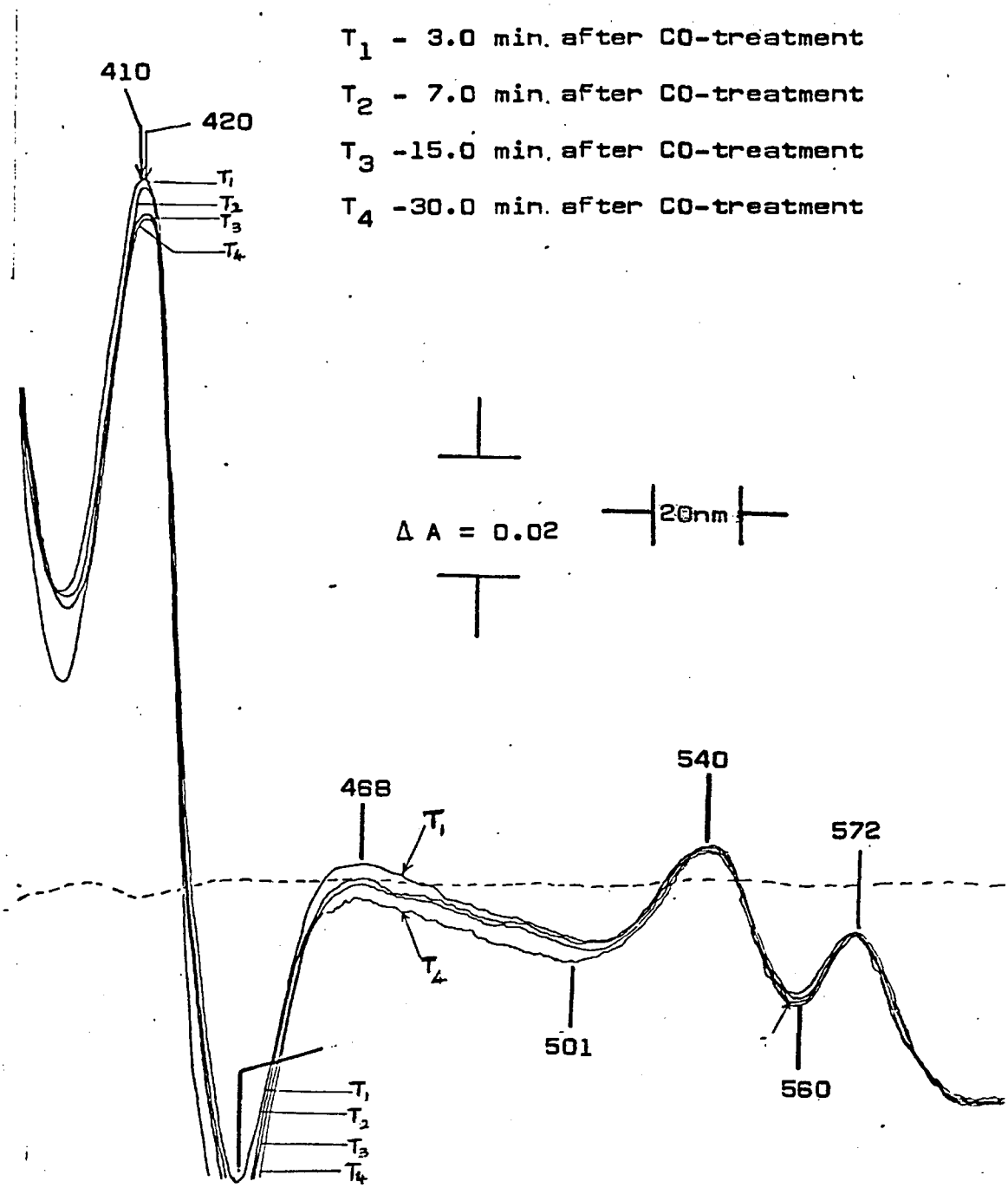
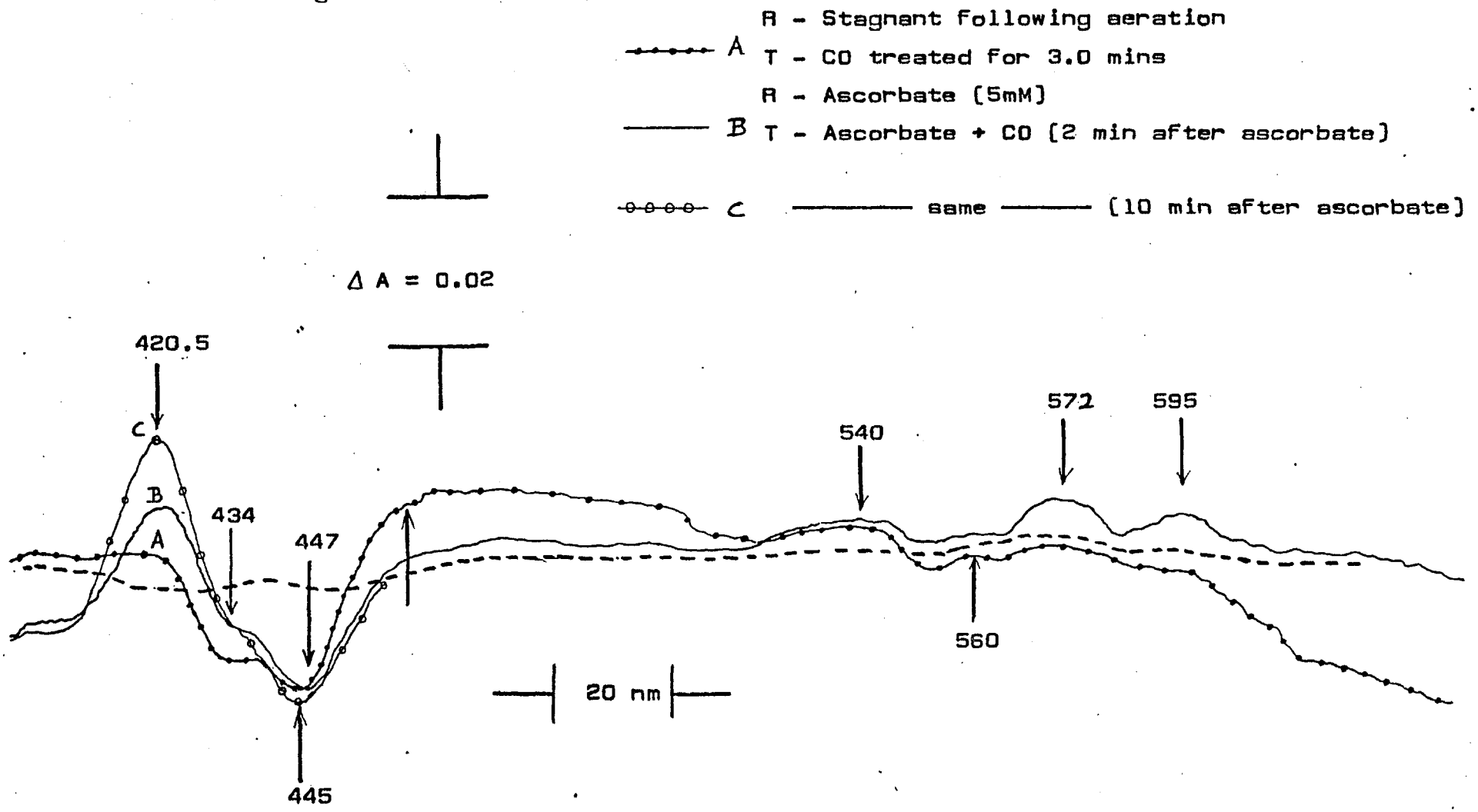


Figure 68 The CO-difference spectra of an Isolate-A mitochondrial preparation following reduction with ascorbate



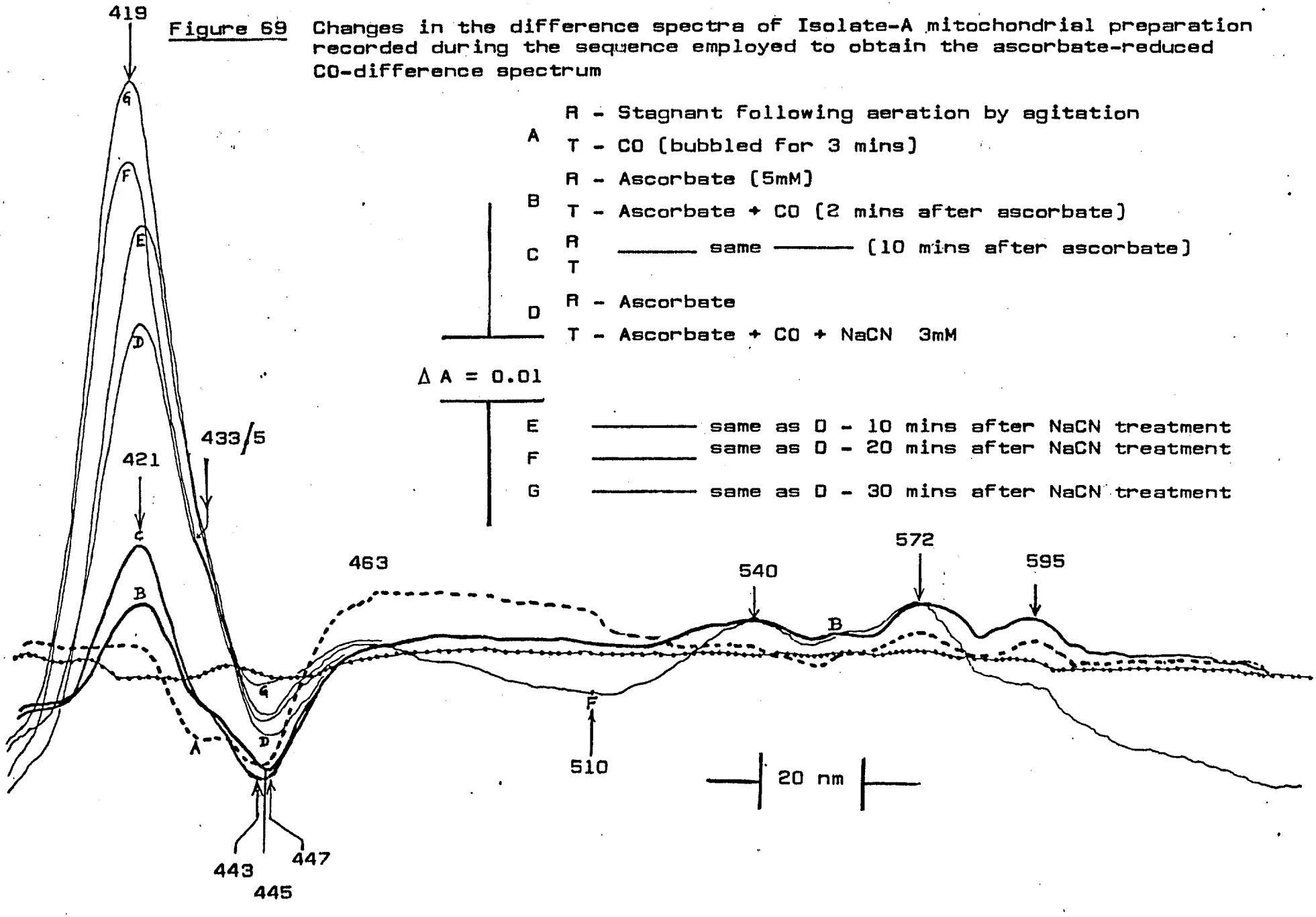
10. The time dependent changes in the CO-difference spectra of isolate-A mitochondria

Figure 67 T_1 to T_4 shows the time dependent changes in the CO-difference spectra of isolate-A mitochondria.

3.0 mins after CO-treatment for 1.0 min. the Soret peak at 410 nm showed the highest absorbance. The trough at 445 nm was shallowest at this time. However 7.0 minutes after CO-treatment the Soret peak shifted from 419-420 nm with decreased absorbance. In contrast the trough at 445 gradually deepened with time up to 30 mins. The peaks at 540, 572 and at 468 nm remained little affected as did the trough at 560 nm.

11. The quantification of cytochrome-O and cytochrome-a_3 in isolates A and F mitochondria using ascorbate as reductant [After the method employed by Cheah 1970a]

Figure 68A shows the difference spectrum obtained for isolate-A preparations upon bubbling CO for 3.0 minutes while the reference sample was aerated. Significant features here are the peaks at 420.5, 433-435 nm and at 445 nm and at 463 nm and the peak in the α region at 559 and at 572. 2 mins. after ascorbate was added to both reference and test samples the [ascorbate red.]-[ascorbate + CO. 3 min.] difference spectra [Fig. 68B] showed that the peak at 420.5 was enhanced while the shoulder at 433-435 remained along with the trough at 445nm. The peak observed in [aerated]-[CO-reduced] difference


Figure 69 Changes in the difference spectra of Isolate-A mitochondrial preparation recorded during the sequence employed to obtain the ascorbate-reduced CO-difference spectrum
A R - Stagnant following aeration by agitation
T - CO (bubbled for 3 mins)
B R - Ascorbate (5mM)
T - Ascorbate + CO (2 mins after ascorbate)
C R
T ——— same ——— (10 mins after ascorbate)
D R - Ascorbate
T - Ascorbate + CO + NaCN 3mM
Δ A = 0.01
E ——— same as D - 10 mins after NaCN treatment
F ——— same as D - 20 mins after NaCN treatment
G ——— same as D - 30 mins after NaCN treatment
419
421
433/5
463
540
572
595
510
443
445
447
20 nm

spectrum disappeared and the 572 nm peak was enhanced, while a prominent new peak appeared at 595.

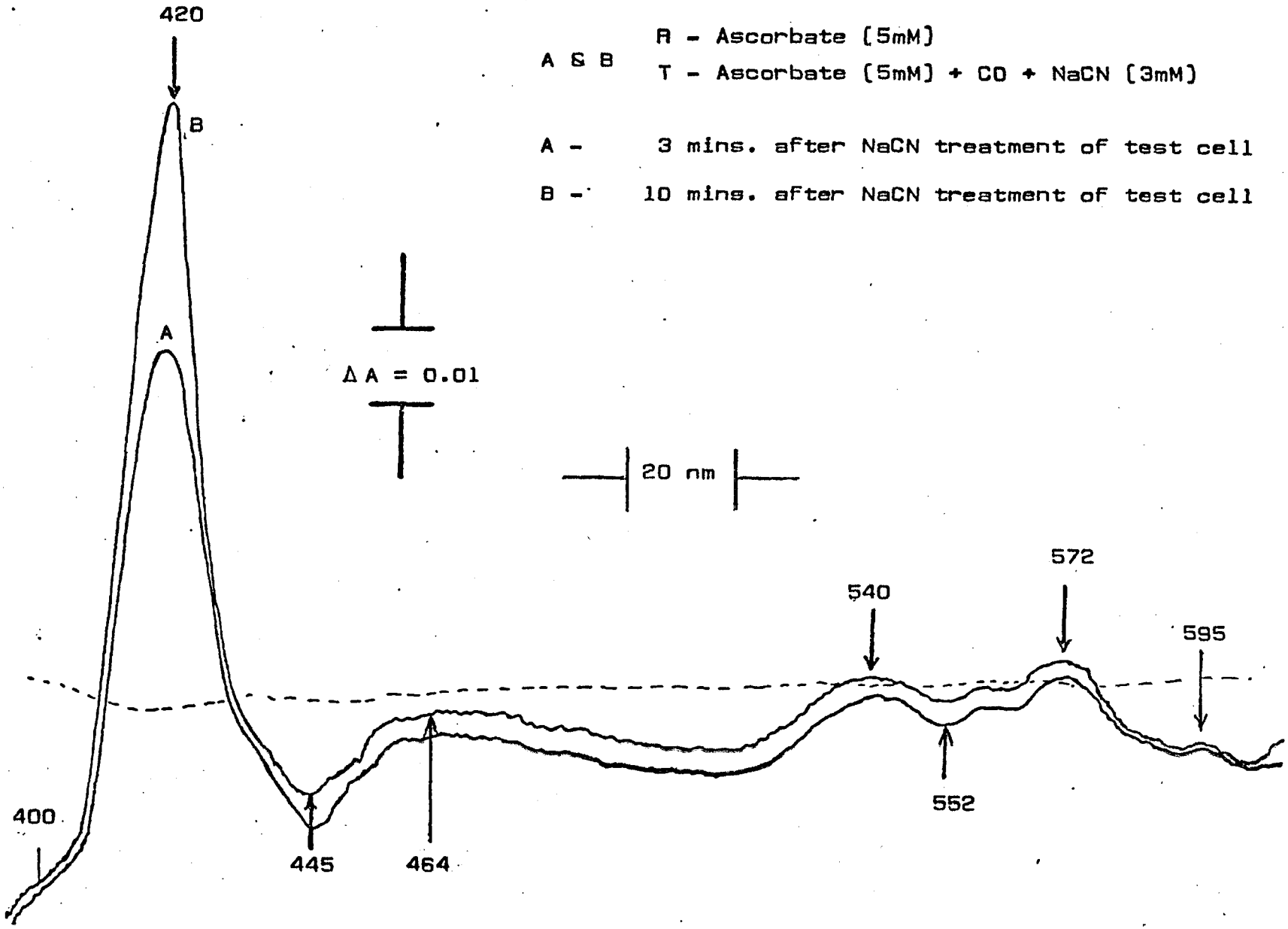
10 mins after the simultaneous addition of ascorbate to both the reference and test samples, (Fig. 68C) only the Soret peak at 420.5 increased while the others remained unaltered.

Figure 69D shows the effect of 3 mM NaCN added to the test sample pre-treated with ascorbate and CO while the reference was reduced with ascorbate i.e. the (ascorbate reduced)-(ascorbate + CO + NaCN) difference spectrum. The Soret peak remained at 420.5 but a large increase in the optical density at this wavelength occurred (Fig. 69D). The shoulder at 433-435 was still present. The minimum at 445 nm shifted towards 443 nm. The maxima at 540, 572 are characteristic of the O-CO complex.

Table 26 shows the quantities of the two cytochromes $\underline{a}_3$ and $\underline{O}$ as computed using the extinction coefficients of Cheah (1970a). It is apparent from these figures that in isolate-A the ratio of cyt.$\underline{a}_3$; cyt.$\underline{O}$ is 1:4.01.

Figure 70 shows the CO-difference spectra obtained with isolate-F. The intermediate stages are omitted. The procedure followed was identical to that described for isolate-A. Figure 70A shows the (ascorbate red.)-(ascorbate + CO + NaCN) difference spectrum recorded 3 min after NaCN application. It shows the characteristic Soret peak of the O-CO complex at 420 nm and the trough

Figure 70 The effect of NaCN on the ascorbate reduced CO-difference spectrum of an isolate-F mitochondrial preparation



A & B
R - Ascorbate (5mM)
T - Ascorbate (5mM) + CO + NaCN (3mM)

A - 3 mins. after NaCN treatment of test cell

B - 10 mins. after NaCN treatment of test cell

at 445 nm formed by the overlapping maxima of the a_3-CO complex with that of the O-CO complex. The maxima at 572, 540 nm are characteristic of the O-CO complex and that at 595 nm in the α peak of the a_3-CO complex.

The concentration of cyt.O and cyt.a_3 calculated from these spectra are given in Table 26. The ratio of cyt.a_3 : cyt-O for isolate-F was 1:2.3.

When dithionite was added to both the reference and test samples of isolate-A mitochondria (Fig. 71C) i.e. (ascorbate + dithionite)-(ascorbate + NaCN + CO + dithionite) difference spectrum the Soret peak shifted to 418.5. The shoulder at 433-435 still remained. The trough at 445 deepened, the maxima at 540 and 572 increased and for the first time a trough appeared at 560 nm which was routinely observed in dithionite reduced CO-difference spectra (but not observed in ascorbate reduced CO-difference spectra). This suggests the dithionite reduces an extra pigment which is not reduced by ascorbate.

12. The detection of hydrogen peroxide on mitochondrial incubates of isolates A and F

No H_2O_2 formation was detected in either of the two isolates. The spectrum of authentic tetraguaiacol is given in Figure 72.

Figure 71 The effect of dithionite on the ascorbate reduced CO-difference spectrum of an Isolate-A mitochondrial preparation

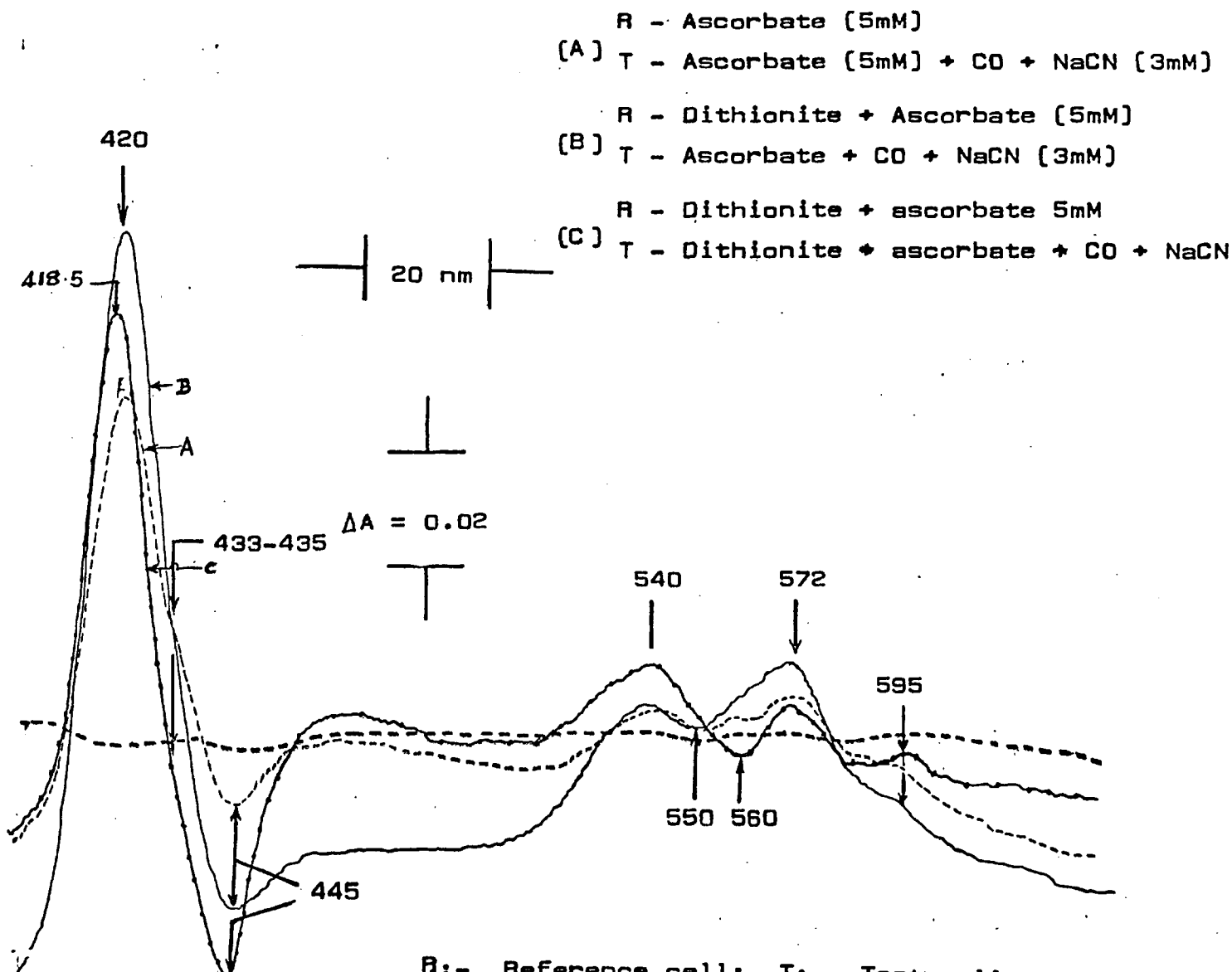
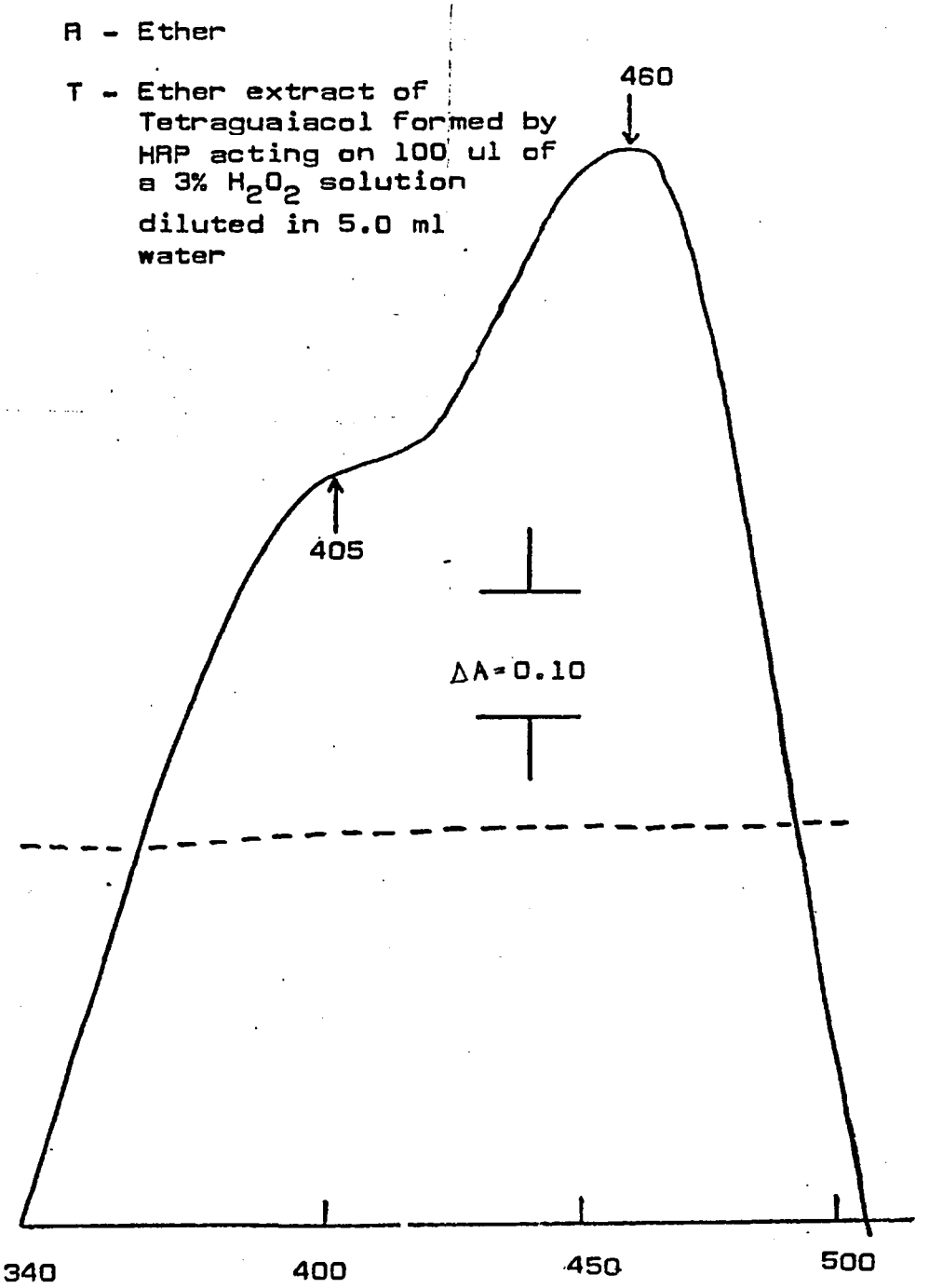


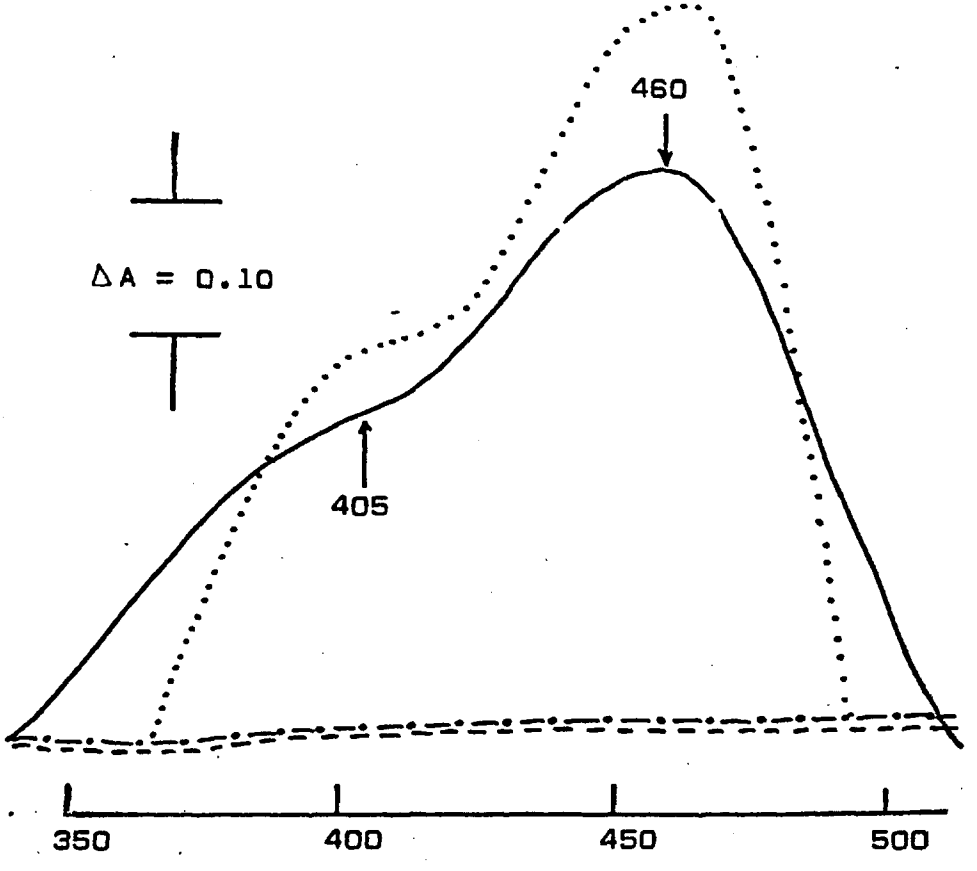
Figure 72 The spectrum of authentic Tetraguaiacol



13. Detection of catalase activity in mitochondrial preparations of isolates A and F

The boiled i.e. heat denatured mitochondrial suspensions of both isolates gave the tetraguaiacol spectrum (Fig. 73) and the light brown-red colour was clearly seen. However the unboiled mitochondrial preparation gave no (brown-red) colour and ether extracts of the post-incubation mixture gave no absorption even suggestive of the presence of tetra-guaiacol. This indicates that the mitochondrial preparations of isolate-A and F have significant catalase activity, while boiling the mitochondrial suspension effectively inactivated the enzyme. Similar experiments on the cytoplasmic fractions remaining after mitochondria were centrifuged out also showed high catalase activity.

Figure 73 Catalase activity in the isolate-A mitochondrial preparations



——— Boiled mitochondrial preparation

—•—•— Unboiled mitochondrial preparation

------ baseline (Ether vs. Ether)

..... Tetraguaiacol formed by authentic H_2O_2

Discussion

The [ferricyanide]-[ferricyanide + dithionite] room temperature difference spectra of isolate-F mitochondria showed the presence of at least one b-type [α-maximum 559.5 nm], c-type [α-maximum at 551.8 nm] and a-type [α-maximum 603.3 nm] cytochromes. The difference spectra of isolate-A mitochondria were very similar showing b-type, c and a-type cytochromes the α maxima of which were observed at 560.5 nm, 551.8 nm and 603.5 nm.

However at liquid nitrogen temperatures the maxima observed for isolate-A mitochondria were at 557.7 nm and 552 nm. The first corresponds to an approximate decrease of 2-3 nm of the room-temperature (R.T.) maximum of 560.5 nm, but the 551.8 nm maximum which should have shifted 2-3 nm to between 548 and 549 nm at 77^{o}K was not apparent; instead a shoulder at 552 nm was observed. This discrepancy suggests the presence of at least one extra pigment at about 554-555nm which was not resolved by the low temperature spectra. A similar discrepancy was seen in isolate-F mitochondria. Although no attempt was made in the present studies to resolve these maxima the discrete use of substrates together with higher concentrations of mitochondria may be employed to clarify this [John M. Palmer pers. comm.]

The α-maximum of the cyt-c of both isolates A and F at 552 and 551.5 nm respectively is different to the α-maximum described for Ascaris muscle mitochondria

which is situated according to Chance and Parsons (1963) at 547 nm; according to Kikuchi and Ban (1961) who placed it at 549 nm. However the cyt.b described in Ascaris by Kikuchi et al (1959), in Moniezia expansa (Cheah, 1966, 1968) and in Moniliformis dubius (Bryant, 1966) has an α-maximum at 553 nm at 77°K which is close to the 552 nm maximum observed at 77°K in both isolates of A. avenae.

On the other hand, the Ascaris soluble cyt.b_{560}, isolated and characterized by Cheah (1972) had dual peaks at 560 nm and 552 nm at 77°K. However in both isolates of A. avenae, although a prominent shoulder was present at 552 nm, the main peak was at 557.7 nm. Cheah (1972) also placed the Ascaris cyt.c at 548 nm which differed with the 547 nm placement by Chance and Parsons (1966) and with that observed by Kikuchi and Ban (1961) at 549 nm. In the 77°K difference spectra of A. avenae isolates A and F there is a suggestion of shoulder in this region which may be the c-cytochromes of A. avenae.

However, the absorption maxima of the major pigment in A. avenae isolates A and F observed at 557.7 and 557.5 nm in (ferricyanide oxidised)-(ferricyanide and dithionite reduced) difference spectra at 77°K, correspond very closely with the 557 nm (77°K) α-maximum of the major b-type pigment observed in Caerorhabditis briggsae mitochondrial preparations by Bryant, Nicholas and Jantunen (1967). The b-type cytochrome in the

[ferricyanide Ox.]-[dithionite red.] difference spectra from another free-living nematode Turbatrix aceti exhibited an absorption maximum at 545 nm at room temperature [Rothstein et al., 1970]

Although the [ferricyanide oxidised]-[ferricyanide + dithionite] difference spectra [recorded at 24°C] indicated the major cytochrome in the mitochondria of isolates A and F absorbed maximally at ≏560 nm, the [ferricyanide oxidised]-[dithionite reduced] difference spectra on every occasion produced the maximal absorption in the α-region at 564 nm, which is close to the 565 nm b-type pigment reported in T. aceti. However while in T. aceti the 565 nm peak represented the second most abundant cytochrome, in A. avenae the b-type cytochrome with the 564 nm peak was the major pigment. In T. aceti the major pigment was a c-type with α-peak at 550 nm.

An interesting feature in the mitochondria of isolates A and F was the presence of significantly more b-type than c-type cytochromes. This agrees with the observations made on Caenorhabditis elegans mitochondrial preparations by Murfitt, Vogel and Sanadi [1976] but are contradictory with observations made on T. aceti mitochondria by Rothstein et al., [1970] where he found the c-cytochromes to be predominant.

The evidence from the polarographic studies [Chapter 7A] showed that this apparent "deficiency" of c-type cytochromes in A. avenae mitochondrial preparations was not due to a loss of cyt.c, since the addition of

exogenous ferrocytochrome-c had no effect on the state-3 QO_2 of the preparations. Furthermore the difference spectrum-[[dithionite reduced]-[ferricyanide oxidised]] of whole-worms of A. avenae isolate-F showed the same proportional absorbance in the α-region of the b and c type cytochromes suggesting the predominance of cyt.b in the intact worms as well. The ratio of c-type: b-type cytochromes for isolate-F was 1:1.6 and that for isolate-A was 1:1.1.

The α-maxima of the a-type cytochromes observed in [ferricyanide oxidised]-[dithionite reduced] difference spectra of isolate A and F at room temperature [R.T.] were respectively at 603.5 and 603.3 nm. However in isolate-A the ratio of b-type: a-type cytochromes was 1:1.84 while the ratio for isolate-F was 1:2.01.

The aeration of the reference [mitochondrial] sample while the test [mitochondrial] sample was left stagnant showed the reduction of b [α 560 nm], c [α 554/5] and a [α 600 nm] type cytochromes in the difference spectrum of isolate-A. This was direct evidence that these component cytochromes were involved in respiratory electron transfer.

The difference spectra obtained with antimycin-A were not ideal since the concentrations of mitochondria used for these studies were inadequate and the spectra relatively poor. The resolution afforded by the Shimadzu [MPS-50L] was not adequate to resolve the spectra beyond

those obtained. Therefore the spectrophotometric data obtained with antimycin-A although discussed here, should be considered with caution until future clarification.

The small although significant increase in absorbance of the b-type (α-560 nm] cytochrome in the presence of antimycin-A suggests that this compound blocks electron transfer between b and c type cytochromes, although the lack of a complete decrement in absorbance in the 554/5 α peak attributable to the c-type cytochrome suggests that the c-cyt. component remains in the reduced state even in the presence of antimycin-A. This may be due either to the relative insensitivity of the b-cytochrome to complete inhibition by antimycin-A or the reduction of the c-type cytochrome by some other compound other than the b α560-cytochrome. This corresponds with the polarographic data which indicated 0-20% inhibition of the state-3 mitochondrial oxygen uptake of isolate-A mitochondria.

The effect of antimycin-A on isolate-F mitochondrial [Fig.57] difference spectra was similar to that observed for isolate-A. Here again antimycin-A was not successful in eliminating the 554 α-peak of the c-type cytochrome although polargraphic data indicated [Chapter 7A] that 80-90% inhibition [with 2 μg antimycin-A/mg protein] of the state-3 mitochondrial oxygen uptake occurred in the presence of antimycin-A.

Thus although the presence of a b-cytochrome which is only partially sensitive to antimycin-A may account for these observations another explanation is possible viz. the presence of a component capable of transferring electrons direct to the c-type and by-passing the b-type, when the latter is blocked by antimycin A.

In fact such a process has been shown to operate in Ascaris muscle mitochondria [Hayashi and Terada, 1973]. They showed that certain flavin oxidases could transfer electrons to Ascaris cyt.c [α 550 nm] to form ferrocytochrome c, which, in the case of Ascaris is re-oxidised by a cytochrome-c peroxidase.

According to the evidence obtained by them the terminal oxidase in Ascaris is an oxygen pressure dependent and KCN insensitive "flavin-containing oxidase" which produces H_2O_2 which is re-used to oxidise ferro-cytochrome-c mediated by the cyt.c-peroxidase. Thus the possibility that the cyt.c component in A.avenae may be able to accept electrons from some flavoprotein component and remain oxidised in the presence of antimycin-A cannot be ruled out without further experimentation, particularly because a] no accumulation of H_2O_2 due to the presence of cyt.c could be shown in A. avenae mitochondria [to be dicussed later in this section] and, b] significant catalase activity was observed in the mitochondrial fractions prepared from both isolates of A. avenae.

The fact that NaCN [1-3 mM] considerably altered the difference spectrum of both isolates A and F is further evidence that the pigments reduced by substrates and by antimycin-A were involved in mitochondrial respiration.

In isolate-F preparations reduced by NaCN a Soret maximum [of an a-type] at 432 nm and α maxima at 534 and 564 nm were observed; the latter indicating that c and b type cytochromes were being reduced. However the α-peaks of a-type cytochromes were not clear in these difference spectra.

The effect of NaCN on isolate-A mitochondria in the presence of succinate as substrate was immediately observed [see Figure 59D] with the development of a Soret peak at 434 nm and a prominent shoulder [unknown] at 460-462 nm. The α and β regions were enhanced with 7.0 min incubation in NaCN [Figure 59E] and showed reduction of the major pigment at 564 nm [α-b-type] with a shoulder in the region 555-560 nm, which may be the unresolved maxima of the c-type and the other b-type pigments. In the β-region a broad peak with mid-point at 536 nm may represent the β-peaks of the b and c type cytochromes.

The shoulder which developed in response to NaCN at 460-462 nm [Figure 59D] diminished with time in the presence of NaCN but appeared on the application of ascorbate to the test-cell, [Figure 60A] with a concomitant decrease in absorbance at 560 and 564 nm

and a shift of the mid-point of the broad β-peak towards 540 nm. This suggests that the major b-type [α-564] can be oxidised by ascorbate.

It is interesting to note that Cheah [1968] observed that the cyt.O of *Halobacterium cutirubrum* could be reduced by ascorbate per se. Since cyt.O is a b-type cytochrome, the α-peak at 564 nm may represent the O-type pigment with the ability to accept electrons directly from ascorbate. However there appears to be a complex of b-cytochromes with one being effectively reduced by α-glycerophosphate [α 560] and the second one being reduced by NaCN [α564] demonstrating the ability to be re-oxidised by ascorbate. In future work these b-types may be resolved by employing different substrates, higher mitochondrial concentrations and high resolution spectra conducted at liquid nitrogen temperatures.

The differential sensitivity of isolate F and isolate-A whole worms to NaCN [Chapter 5] and the partial inhibition of the ascorbate-TMPD induced state-3 oxygen consumption rate in isolate-A mitochondrial fractions is consistent with the spectral changes observed with the mitochondrial fractions of these isolates in response to NaCN. This is because the relative concentrations of the O-cytochrome and the aa_3-cytochromes and/or the degree to which each cytochrome mediates terminal electron transfer will ultimately determine the net response elicited by each isolate

when exposed to sodium cyanide or to any other respiratory inhibitor.

In this context, Lemberg and Barrett [1973] explained - "Respiratory systems in which cytochrome-O is the terminal oxidase differ in their sensitivity to carbon monoxide and cyanide. Cytochrome-O appears to be less sensitive to cyanide than is cytochrome-a_3 and the degree of inhibition varies with the system in which cytochrome-O is the terminal electron acceptor".

Evidence of this come from studies on the respiration of various organisms. The NADH-oxidase of Acetobacter suboxydans was 65% inhibited by 0.75 mM KCN [Lemberg and Barrett, 1973] while that of aerobically-dark-grown Rhodospirillum rubrum was unaffected by 0.3 mM KCN [Taniguchi and Kamen, 1965 as quoted by Lemberg and Barrett, 1973].

In contrast the succinate-oxidation of free-living cells of Rhizobium japonicum werevery sensitive to cyanide, being completely inhibited by 10 μM KCN [Appleby, 1969 as quoted by Lemberg and Barrett, 1973]. Similarly the ascorbate-DCIP system of Acetobacter vinlandii was 95% inhibited by 40 μM KCN [Jones and Redfearn, 1967 - as quoted by Lemberg and Barrett, 1973]. Cheah [1969] reported that the ascorbate-TMPD-oxidase of Halobacterium cutirubrum was 84% inhibited by 0.5 μM cyanide. However the succinate oxidation by Moniezia expansa mitochondrial fractions was not affected by concentrations of cyanide up to 10 mM [Cheah, 1968]

and azide in concentrations greater than 0.1 mM proved stimulatory and antimycin-A in concentrations less than 10^{-5} M had no effect [Cheah, 1968].

Rhodopseudomonas capsulata which had only an O-type cytochrome (α-562 nm; β-525 and γ-426 nm) and no a-type cytochrome, was 50% inhibited by KCN and azide at 10 uM and was active in a gas mixture of 90% CO + 10% O_2 [Klemme and Schlegel, 1969 - as quoted by Lemberg and Barrett, 1973].

Further reports on the 'abnormal' response to CO + cyanide by helminths was discussed in Chapter 5. Therefore although the two isolates of A. avenae investigated here have not very dissimilar cytochrome profiles in their mitochondria preparations, the significant difference in the sensitivity of these preparations and whole worms to respiratory inhibitors [Chapter 7A] may be due to very different metabolic schemes operative [in vivo] -in each isolate.

The CO-binding pigments

The [dithionite reduced]-[dithionite + CO] difference spectra for isolate-F mitochondria after 1.0 min CO treatment shows the formation of more than one CO-complex since the γ-peak at 421.5 nm was at a significantly lower wavelength than that expected for the Soret-peak of the cyt.a_3-CO complex which generally occurs at between 427-430 nm [Cheah, 1970a], and was at a slightly higher wavelength than 425-521 nm, the wavelength at which the Soret maximum of the cyt.O-CO

complex of a number of organisms is known to occur [Lemberg and Barrett, 1973; Daniel, 1970]. In fact the 421.5 nm maximum results from the overlapping maxima of the CO-complexes of cyt.O and cytochrome a_3 [Cheah, 1966, 1968, 1970a].

Cheah [1970a, 1968] obtained a similar Soret-peak at 425 nm for the CO-difference spectrum of Halobacterium salinarium which was due to the dual presence of cyt.a_3 and cyt.O in the membranes of this organism. According to Cheah the ratio of the two cytochrome species and their CO-affinities dictate the location of the Soret-peak in CO-difference spectra of different organisms or different species of the same organism.

The shift of the Soret peak in isolate-A and F preparations with very short [i.e. less than 2.0 min] CO-treatment seem to indicate that the CO-affinity of the A. avenae cyt.O closely parallels that of cyt.a_3. However on prolonged treatment of the isolate-F mitochondria with CO [7.0 min exposure] the Soret-peak was observed to shift to 417 nm indicating that more cyt.O was combining with CO with the increased duration of treatment. The soret-maximum of isolate-A mitochondria [420 nm] observed upon 1.0 min CO treatment did not shift towards the shorter-wavelength on prolonged treatment with CO.

The spectra of both isolates A and F suggests that cyt.a_3 had fully combined with CO following 1.0 min treatment since the trough at 445 nm which represents

the Soret-peak of the $\underline{a}_3$-CO complex (the result of overlap of the 427-430 nm $\underline{a}_3$-CO peak and the 415-421 nm $\underline{O}$-CO peak) was little altered with longer CO treatment.

In this respect the CO-affinity of the A.avenae cyt.$\underline{O}$ seem to be much higher than the cyt.$\underline{O}$ of Bacillus megaterium which required 15 mins bubbling with CO in contrast to the cyt.$\underline{a}_3$ of the same organism which had fully combined with CO in 30 seconds (Broberg and Smith (1967) as quoted by Lemberg and Barrett, 1973). Similar low relative reactivity of the cyt.$\underline{O}$ of Halobacterium cutirubrum was reported by Cheah (1969) On the other hand the dithionite reduced cyt.$\underline{O}$ of Moniezia expansa had fully combined with CO within 11 mins, showing intermediate CO affinity (Cheah, 1966, 1968). In contrast the cyt.$\underline{O}$ in ascorbate-TMPD reduced membrane preparations of Mycobacterium phlei had fully combined with CO as had its cyt.$\underline{a}_3$ after 30 seconds of bubbling with CO (Revsin and Brodie,(1969) as quoted by Lemberg and Barrett, (1973)). Similarly Daniel (1970) investigating the CO-affinity of the cyt.$\underline{O}$ of Acetobacter suboxydans reported 50% of the cytochrome as having combined with CO at a CO-concentration of 3.3 μM.

Regarding the characteristics of the (oxidised)-(reduced) α-absorption maxima of the $\underline{O}$-type cytochromes, this survey of the literature indicates that with a few exceptions $\underline{O}$-type cytochromes can be divided into two groups, one with main α-maximum at 555-558 nm and the other group with the main absorption peak at 564-565 nm

(Daniel, 1970). The (dithionite reduced)-(ferricyanide oxidised) spectra of A. avenae (isolates A and F) show a cytochrome of the b-type which has a maximum at 564 nm. It is most likely that this is the CO-reactive pigment in A. avenae and it is also interesting to note that this is the major pigment in mitochondrial preparation of both isolates of A. avenae.

The characteristics observed in the α and β regions in the (dithionite reduced)-(dithionite reduced + CO) difference spectra in isolate-A and F mitochondria which were attributable to the O-CO complex of the cytochrome-O of A. avenae were maxima at 540 nm, 572 nm and a minimum at 560 nm. The Soret-peak in both isolates were between 417 and 420.

However prolonged treatment of isolate-F mitochondria with CO i.e. 7.0 min treatment, while causing the Soret maximum to shift towards 417 nm (From 420 nm), also produced 2 additional peaks, one at 457nm and another at 507 nm which did not appear with short-term (1.0 min) CO-treatment. Surprisingly no analogous maxima were observed with isolate-A preparations.

This anomaly may be taken to imply the presence of additional co-reactive substances, which understandably have lower affinities to CO than cyt.O and cyt. a_3. On the other hand the data may also be taken to imply that the cyt.O of this isolate has more than one CO-binding site since evidence obtained (Daniel, 1970) strongly suggests the possibility that cyt.O may be

Table 27: Absorbance characteristics of cytochromes-O in various species.

Difference spectra maxima and minima of o-type cytochromes

Organism	Co-reduced minus reduced peaks and troughs nm (tr)			Reduced – oxidized α-peak nm	Ref.
Staphylococcus albus	567–568	535–537	416–418	565	Castor and Chance (1955)
	tr 550				
Acetobacter suboxydans	567–568	535–537	416–418	565	Chance (1953a)
	tr 550				
Achromobacter	572	536	418	563	Arima and Oka (1965)
	tr 554				
Rhodospirillum rubrum	576	540	419–421	564	Taniguchi and Kamen (1965)
	tr 560				
Rhizobium japonicum free cells	574	539	415	562 (559-2, 77°K)	Appleby (1969b)
	tr 556				
Myxococcus Xanthas	575	—	415	562	Dworkin and Niederpruem (1964)
Acetobacter suboxydans	568+560	536	417	557+565	Daniel (1970)
Vitreoscilla	570	553	416	555+565	Webster and Hackett (1966a,b)
	tr 550				
Leucothrix mucor	566	533	416	558	Biggins and Dietrich (1968)
	tr 550				
Leptospira sp.	575	540	417	—	Baseman and Cox (1969)
Bacillus megaterium KM	—	—	410–415	557	Broberg and Smith (1967)
Acetobacter suboxydans (IAM 1828)	567	535	417	558	Iwasaki (1966)
Staphylococcus aureus	568	533	415	555–557	Taber and Morrison (1964)
Halobacterium cutirubrum	578	539	416	559	Cheah (1969)
	tr 560				
Staphylococcus epidermis	573	537	417	558	Jacobs and Conti (1965)
Escherichia coli	567	538	416	—	Revsin and Brodie (1969)
	tr 558				
Haemophilus parainfluenzae	565	532	416	—	White and Smith (1962)
	tr 547				
Mycobacterium phlei	567	538	416	—	Revsin and Brodie (1969)
	tr 557				
Rhodopseudomonas spheroides	570	540	415	—	Sasaki *et al.* (1970)
Tetrahymena pyriformis	572	—	418	—	Perlish and Eichel (1971)
	tr 557				

After Lemberg & Barrett (1973)

a polymeric protein [similar to cyt.*a* + a_3 complex] at least in *Acetobacter suboxydans*.

Pointing out that very few CO-affinity-studies have been undertaken, Daniel [1970] suggested that other O-cytochromes may also exhibit multiple sites of action, i.e. at least two interacting-CO-sensitive sites. Whether *A.avenae* isolate-F and/or isolate-A possesses cytochrome-O with two or more co-operative CO-reactive sites can be clarified in the future, by assessing the slope of the Hill plot for the O-cytochrome.

Thus these CO-difference spectra of *A. avenae* mitochondria unquestionably demonstrates the presence of [at least] two co-reactive cytochromes viz. cyt.a_3 and the other showing all the characteristics of cyt.*O* and resembling the *O*-type pigments described for *Moniezia expansa* [Cheah, 1966, 1968, 1974] and almost identical to the cyt.*O* of *Rhodospirillum rubrum* [Taniguchi and Kamen as quoted by Lemberg and Barret, 1973] See Table 27.

Furthermore the fact that the CO-difference spectrum of isolate-F mitochondria altered little with 16-18 hrs exposure to artificial light sources [see Figure 65A and B] confirms the characteristics of the cyt.*O*-CO complex regarding its stability and resistance to photodissociation unlike the a_3-CO complex. In fact the sharp though small trough at 447 nm which represents the a_3-CO peak formed by the 1 min exposure to CO became less deep with passage of time [see Figure 64 T_1 to T_4] and probably indicates

the slow but steady deterioration of the $\underline{a}_3$-CO complex, in the presence of light.

The CO-difference spectrum 12 and 18 hrs after the 7.0 min CO-treatment of the isolate-F mitochondria was different to the zero-time one showing that most if not all of the $\underline{a}_3$-CO complex and perhaps some of the O-CO complex had broken down and the Soret-peak which was at 417 nm at zero time had shifted to 420 nm with the concomitant reduction in absorbance. However the other maxima characteristic of the O-CO complex at 540 nm, 572 nm and the trough at 560 nm were still evident even after 18 hrs after the formation of the complex.

Quantification of CO-reactive pigments of A.avenae mitochondria

In applying the most comprehensive method developed for the quantitative assessment of cyt.$\underline{a}_3$ and cyt.$\underline{O}$ in situations where these two terminal oxidases occur together, Cheah [1970a] used ascorbate instead of dithionite as the reducing agent. The ratio of cyt.$\underline{O}$ and $\underline{a}_3$ calculated using this technique and the extinction coefficients quoted by Cheah [see Table 26] showed that, of the $\underline{b}$-type cytochromes, in isolate-F fractions approximately 60% constituted the CO-reactive $\underline{b}$-types, i.e. cytochrome[s] $\underline{O}$. Of the $\underline{b}$-types in isolate-A, cytochrome[s] $\underline{O}$ constituted approximately 80%.

Similar occurrences of high concentrations of CO-reactive cytochromes have been reported for another free-living nematode Turbatrix aceti [Rothstein et al., 1970]

They reported that unusually high concentrations of a CO-binding haemoprotein was present in T.aceti which amounted to "some 6-fold greater quantity than that of any single member of the respiratory chain cytochromes". Although Rothstein et al.[1970] could not confirm that the CO-binding haemoprotein was an O-type cytochrome since the CO-spectra could not be observed, they suggested that it bore some resemblance to the O-cytochrome described by Cheah [1966].

It is also interesting to note that in A.avenae isolate-F fractions the CO-reactive-b-type cytochrome i.e. cyt.O was 20% greater than the total a type pigments i.e. [a + a_3] and was only 4% less than the total c type (i.e. c + c_1) cytochromes. However in isolate-A mitochondria cyt.O was present in 70% greater quantity than cyt.[a + a_3] and was 40% greater than the total c-types.

In using ascorbate in the attempt to quantify the O and a_3 cytochromes, another interesting feature was observed. The [aerated]-[CO-reduced] difference spectrum showed the formation of the expected a_3-CO and O-CO complexes [peak 420 and trough 445 nm respectively] in the absence of any other reducing agent, and surprisingly a third Soret-peak i.e. an additional peak at 433-435 nm which was not shown in the dithionite-reduced CO-spectra. This peak at 433-435 nm remained noticeably unchanged in the subsequent [ascorbate + CO]-[ascorbate] difference spectrum as a shoulder on the descending limb of the 420 Soret-peak of the O-CO complex, [see Figures 68 and 69]

and remained even in the presence of NaCN in the [ascorbate]-[ascorbate + CO + NaCN] difference spectrum, although it seemed to gradually diminish on incubation with NaCN.

This shoulder at 433-435 nm cannot be attributed to either the $\underline{a}_3$-CO complex or to the $\underline{O}$-CO complex since these were represented by the minimum at 445 nm and the maximum at 419 nm respectively.

However the only analogous reference to an absorption maximum in this (433-435 nm) region of the spectrum involving an $\underline{O}$-type cytochrome was the absorption maximum observed for an oxygenated form of cyt.$\underline{O}$ in the cyanophyta Vitreoscilla sp. by Webster and Orii (1977). This was Oxy-cyt-$\underline{O}$ and was observed in whole cells of Vitreoscilla and absorbs maximally at 435 nm. Webster and Orii (1977) also showed that although the formation of the oxygenated form of the cyt.$\underline{O}$ was inhibited in the presence of cyanide, the addition of cyanide after the formation of the oxygenated species had little effect on the absorption at 435 nm and suggested that cyanide had a stabilising effect on the oxygenated cyt.$\underline{O}$. These workers also demonstrated the formation of the oxygenated cyt.$\underline{O}$ on the addition of NADH which was replaced by the reduced cyt.$\underline{O}$ when the solution became anaerobic (Fig.74).

While these results by Webster and Orii (1977) were the first evidence for the functional involvement of the oxygenated compound of a cytochrome in living cells, they cited evidence which suggested that the

Figure 74: Cyclic changes of cytochrome-O during terminal respiration of Vireoscilla sp.
[After Webster & Orii, 1977]

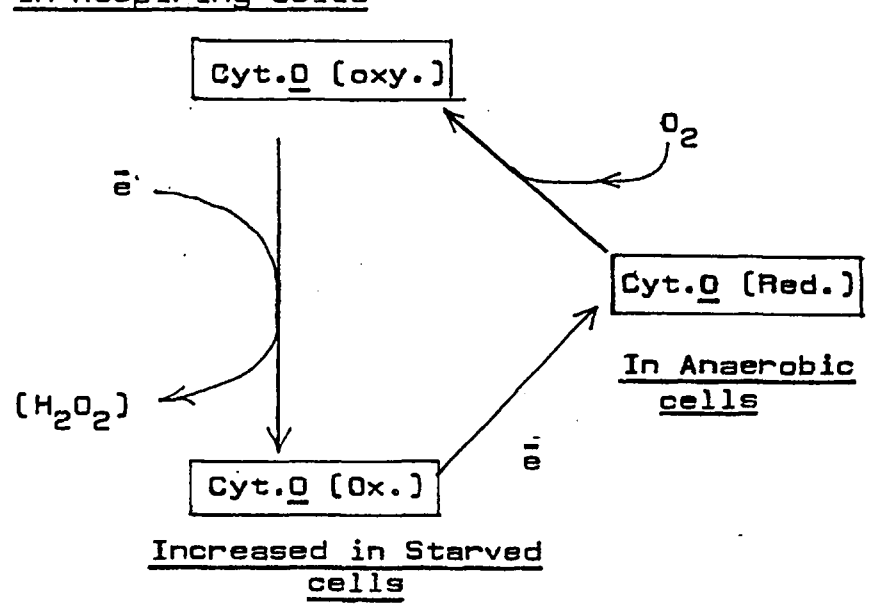
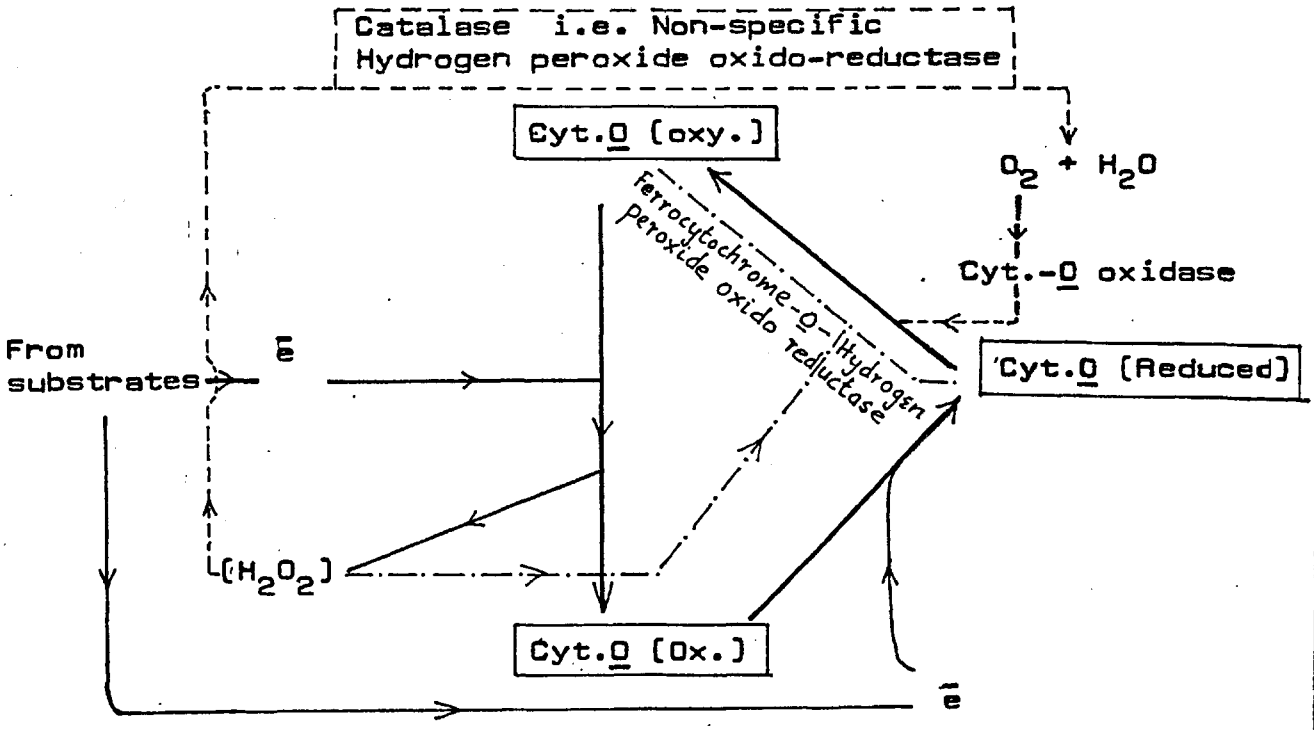


Figure 75: Postulated hypothetical scheme for A.avenae cytochrome-O.



reduced, oxidised and oxygenated form of the O-cytochrome all participated in the cyclic changes of the cytochrome during terminal respiration. Spectra of the oxygenated cyt.O were also obtained when starved cells of Vitreoscilla sp. were used as reference against unstarved cells, and the interpretation was that the amount of the oxygenated form is increased when the supply of electrons to the cyt.O oxidase system is diminished, by starving the cells (Webster & Orri, 1977).

If the cyt.O of A.avenae behaves in a similar manner (see Figure 75) at least one physiological role of cyt.O may be speculated upon. The cyclic-changes in cyt.O would enable it to provide reducing equivalents viz. electrons to maintain electron transport during a temporary absence of oxygen in the surrounding environment, by recycling the O_2 which could be reproduced from H_2O_2 - if the system also includes a substrate specific peroxidase (i.e. a ferrocytochrome-O-hydrogen peroxide oxido reductase) or a catalase (i.e. a hydrogen peroxide oxido reductase) as shown in Figure 75.

Although the evidence for the occurrence of the oxygenated cyt.O in A.avenae isolate-A mitochondria as presented in this study is admittedly not substantial, the presence of catalase activity was unquestionably shown. Evans (1968) has demonstrated catalase activity in some Australian isolates of A.avenae using starch-gel-electrophoresis and also showed quantitative variation among the isolates of this enzyme.

Although the scheme presented here in Figure 75 is hypothetical it must be remembered that where cyt.O mediates electron transfer the synthesis of H_2O_2 is a universal occurrence. Whether this toxic compound is allowed to accumulate in vivo in organisms, possessing cyt.O has been debated extensively. In fact Cheah (1974) while demonstrating the formation of H_2O_2 in Ascaris muscle mitochondria suggested that this may be an artefact of in vitro experiments.

Although in the present studies on A.avenae mitochondria, the production of H_2O_2 could not be detected by spectrophotometric methods, significant catalase (i.e. hydrogen peroxide oxido reductase) activity was evident and thus the scheme postulated is not wholly hypothetical, although further experimentation on the lines employed by Webster and Orii (1977) will be necessary to confirm actual functioning of the system.

CHAPTER 8

Final Discussion

Prior to the commencement of the current study, it was generally accepted that A. avenae possessed attributes such as rapid rate of reproduction, availability of populations reproducing by amphimixis or parthenogenesis, short generation time, and wide host range which made it an ideal test organism. As such A. avenae was employed notably for studies on nematicide-toxicity, effects of anoxia, hypobiosis and for energy-budget assessment.

The most critical short-coming in A. avenae as a model organism is that it is not conducive to genetic analysis by the induction and manipulation of mutant clones (Evans & Womersley, 1980). However the existence of numerous naturally occurring populations (= eco-strains = biotypes = isolates) differing in their eco-biological characteristics whilst retaining homogeneity as a species may alleviate the latter short coming. The aim of the present project was to expand knowledge of the biology of several ecological isolates of A. avenae and to assess whether or to what extent such isolates of this ubiquitous species were homogeneous with respect to respiratory physiological characteristics.

Thus, if it were possible to find an isolate, the respiratory physiology of which paralleled that of animal parasitic helminths, at least in basic features, the value of A. avenae as a convenient, low-cost model-

system for anthelminthic studies (with the long-term aim of the control of such socio-economically important diseases such as filariasis, onchocerciasis) would be enhanced.

In attempting to physiologically characterize several isolates of A. avenae established during the present study, it was apparent that certain important differences existed among three such isolates.

The evidence from the rates of population development, the Arrhenius plots of the population development rates, the generation time studies, and the observations on chilling-tolerance of the isolates of A. avenae (Chapter 3) indicates that biological processes such as oviposition, eclosion, moulting and sexual maturation which dictate the intrinsic fecundity of each isolate are temperature dependent, with each isolate exhibiting transition points at which significant changes in the rate of development are seen.

The different values of Arrhenius activation energy$^{(E*)}$ in the pre-transition and post-transition states probably indicate the approximate energy requirement of an enzyme system, the different isoenzymes of which are activated at the transition points. On the other hand these E^* changes may reflect the cumulative energy requirement of a number of multi-enzyme systems. Thus isolate-F may have iso-enzymes which operate with optimal efficiency at a temperature below that of another.

This was further substantiated by the observations on the Arrhenius plots of the rates of oxygen consumption [Chapter 4] which clearly show that in isolate A [from Malawi] the depression in the QO_2 was much reduced, below 20°C, compared to the two U.K. isolates; while isolate A showed a much greater and sustained increase in the QO_2 - [well above those observed for the two U.K. isolates] from 25°-30°C.

These observations while indicating the occurrence of "thermotypes" within the species, is consistent with the hypothesis that reduced temperatures alter some physical property of the tissue membranes. Thus the direct physical alteration of the membraner phase of cellular components can elicit a great many physiological changes one of which could be the reduction in permeability [below a critical temperature] of the mitochondrial membranes to oxidizable substrates, thus affecting the depression in the oxidative rates reflected in the QO_2.

The decline in the temperature induced maximal QO_2 of isolate-A following sustained incubation in aqueous phase at 30°C is particularly notable, [Chapter 5] since a similar decline in the temperature induced maximal QO_2 of Strongylitis ratti larvae has been reported previously [Barrett 1969b; Von Brand, 1979]. Though the underlying mechanism has not been elucidated [Von Brand, 1979]

it is possible that under sustained high temperatures direct physical alteration of the membraner-phase of the organisms subcellular organelles (particularly those of mitochondria) alters the permeability of the membranes to oxidizable substrates. That this phenomenon was observed in isolate A is curious, but other similarities of this particular isolate to certain respiratory properties of helminths such as Moniezia and Ascaris, probably indicates a close affiliation to parasitic forms, which will be further discussed below.

Under anaerobic conditions both isolates A and F produced ethanol, but trace amounts of glycerol were apparently produced aerobically (by whole worms) although under anaerobic conditions slightly greater quantities were detected in isolate A incubates (Chapter 5B) This observation, coupled with the QO_2 sensitivity of whole worms to SHAM, suggests the possible operation of the relatively "unusual" oxidase - α-glycerophosphate oxidase (GPO) - linked to glycolysis for the reoxidation of $NADH+H^+$ which has been implicated in the production of glycerol, specifically in the absence of oxygen in numerous trypansome species. (T. rhodesiense, T.evansi, T. equinum and T. equiperdum - Bowman & Flynn, 1976). It is of further interest to note that α-GPO has been reported in the parasitic cestode Taenia taeniaeformis by Weinbach and Von Brand (1970). However these observations with SHAM obtained with whole organisms of

A. avenae had to be approached with caution until the effects on mitochondrial fractions were ascertained.

It is remarkable that A. avenae isolates exhibited notable differential sensitivity to cyanide in whole animal assays, though it can be argued, that this was due to different permeability characteristics and/or cuticular barrier effects inherent in the isolates.

However the remarkable stimulatory effect of cyanide on the QO_2 of isolate-A [Chapter 5A] whole worms was reminescent of observations reported on Moniezia expansa [Cheah, 1966, 1968, 1974] and Ascaris lumbricoides [Cheah, 1970b, 1976] where the effect was linked to the occurrence of alternate electron transfer sequences [other than the classical cyt. aa_3] involving cytochrome O. Thus on the one hand, whole animal assays of isolate-F suggest the operation of a typical mammalian type respiratory sequence exhibiting extreme sensitivity to cyanide, while isolate-A is different resembling more the type of system generally observed in parasitic helminths and trypanosomes.

It was therefore deemed important to investigate the possible origins of these differences by examining mitochondrial fractions of these two isolates.

Since existing literature on the isolation and preparative techniques for nematode mitochondria were conflicting [Chapter 7 - Introduction] a modified

technique was devised during this study and the latter standardized isolation procedure was employed to compare the mitochondrial preparations of the two isolates showing the greatest difference in cyanide sensitivity.

The difference spectra of the mitochondria of both isolates indicated the presence of $\underline{a}+\underline{a}_3$, $\underline{b}$ and $\underline{c}$-type cytochromes. Although quantitative differences were apparent, [Chapter 7B - Discussion] the spectra were basically similar at 23°C and 77°K. The [oxidised]-[reduced] difference spectrum of isolate-A fractions were notably similar to those obtained with Arum mitochondrial fractions [J. Palmer pers. comm.]. More significantly the presence of extensive quantities of a CO-binding pigment was apparent in both isolates [Chapter 7B] and furthermore the CO-difference spectra of both preparations were fundamentally similar to those described for Moniezia expansa, Ascaris lumbricoides muscle mitochondria and Halobacterium sp. by Cheah [1966, 1968, 1969, 1970b, 1974] where the dual-occurrence of cytochrome [$\underline{a}+\underline{a}_3$] and cytochrome-$\underline{O}$ were confirmed and quantified in each instance.

However, the sensitivity of the state-3 QO_2 of mitochondrial fractions of the two isolates [as determined polarographically] to the classical respiratory inhibitors were straightforward though diverse. Isolate-F mitochondria were sensitive to rotenone, antimycin-A and the ascorbate-TMPD oxidase activity

was almost completely inhibited by cyanide although CO and SHAM were required for complete inhibition [Chapter 6A]. Isolate-A preparations in contrast were much less sensitive to antimycin-A and cyanide although SHAM and CO were again necessary for complete inhibition. The rotenone sensitivity however was similar in both isolates. The inhibition by SHAM of a minor percentage of the state-3 mitochondrial QO_2 in both isolates [Chapter 7A] may be considered as evidence of the operation of the α-GPO complex, if it is assumed that SHAM is a specific inhibitor of the latter complex.

Thus the respiratory metabolism in A. avenae probably involves multiple oxidases although further work [viz., observation of photochemical action spectra] is necessary in order to confirm the functional involvement of the O-type cytochrome in actual terminal oxidation and the suggested hypothetical involvement of its oxygenated form during anaerobiosis [See Chapter 6B - Discussion].

Regarding the possible operation in A. avenae of the α-GPO complex [in conjunction with glycolysis], the specific inhibitory action of SHAM on this complex as reported by Hill et al., 1976 has to be confirmed by other workers since Palmer [1981] has suggested that SHAM may have other unspecific effects on peroxidases [Schonbaum, 1973, as quoted by Palmer, 1981] and polyphenol oxidases [Rich et al., 1978, as quoted by Palmer, 1981]. However for the present it is justifiable to speculate that glycerol may result from the coupling

of glycolysis to the GPO-complex via GP-dehydrogenase where in the absence of oxygen, DHAP acts as the electron-acceptor and substrate amounts (as opposed to catalytic amounts) of DHAP are reduced to GP and hence to glycerol by the action of a phosphatase.

Although there is ample evidence gathered by other workers (Madin, Crowe and Loomis, 1978) that a Californian isolate of A. avenae synthesizes glycerol, the postulated metabolic scheme put forward by these authors (involving the decline of the specific activity of isocitrate lyase (IL) and malate synthase (MS)) could be an alternative if not the major pathway via which A. avenae synthesizes glycerol upon induction of anhydrobiosis. However, although MS in their postulation is one of the key regulatory enzymes governing glycerol production, at the time of publication they did not "yet have clear data on the IL and MS activities in the 12-36 hour period" and stated that the problem was then still under study. Glycerol synthesis in A. avenae according to these workers, occurs only when the water content of the "pellets" of desiccating worms drop below a critical level (2.4 mg water/mg dry wt.).

However, it is likely that A. avenae has a capacity for the continuous synthesis of glycerol even under normal aerobic conditions, which perhaps may be increased under drying conditions.

If respiring nematodes can only obtain oxygen from the thin film of water they are normally surrounded by, lack of oxygen may be the signal for impending desiccation, leading to a redirection of metabolism (at least in A. avenae) in favour of glycerol accumulation. If this be the case, this hypothesis finds support in the observations on trypanosomes where it has been shown that through the mediation of the α-GPO/glycolysis cycle, one mole each of pyruvate and glycerol accumulated anaerobically per mole of glucose metabolised by T. rhodesiense (Grant and Fulton as quoted by Bowman and Flynn, 1976).

Under aerobic conditions however, glucose was shown to be quantitatively converted to pyruvate by the coupling of glycolysis to GPO by GP-dehydrogenase (Chapter 6). Thus the non-availability of oxygen was apparently the trigger for glycerol accumulation in this organism, although the significance of the latter compound to T. rhodesiense is not known. Whether such a scheme is operative in A. avenae is open to speculation and debate despite the fact that the importance of glycerol to at least one isolate of A. avenae has been proved.

These studies therefore have revealed some of the remarkable metabolic schemes this versatile organism has adopted for survival under different conditions. For instance, the flexibility it shows in the re-oxidation of glycolytic $NADH+H^+$ itself is diverse, i.e. LDH mediated

lactate production; ADH mediated ethanol production and possibly a third - the coupling of α-GP-dehydrogenase to mitochondrial α-GP-oxidase complex; the latter possibly developed for intrinsic-survival value for the synthesis of glycerol.

Thus it seems that collectively, the species A. avenae possesses a diverse metabolic repertoire, the details of which, in any given isolate, are the result of selection pressures. The diverse geographical distribution of A. avenae is probably a consequence of this metabolic diversity.

As a nematologist therefore one may question why the aphelenchoids have not parasitized higher animals whereas other representatives of the group have successfully exploited higher plant-forms [viz., Rhadinaphelenchus cocophilus internally parasitic in the coconut palm; Aphelenchoides besseyi, parasitic on the rice-plant; and Aphelenchoides retzemaboši on ornamentals] and certain groups of insects [viz., Contortylenchus sps.].

Does this inability to parasitize higher-animals invalidate the model? The answer is possibly a negative one because the archetype aphelencoid with its mycophagous habit, and metacorpalheterotopy [Paramonov, 1972] probably left the rhizosphere and followed its fungal host into the root tissues of the higher plants - the phenomenon Paramonov [1972] describes as mycochilophagy. A similar association of fungi with certain insect groups may have

been the evolutionary origins of insect parasitic aphelenchoides. Since higher animals had probably little or no such close association with fungi, the evolutionary process towards parasitism of higher animals, was not available to the aphelenchoides.

From an ecological view point, A. avenae represents a robust type of species drawing on its versatile metabolic repertoire to survive in widely differing habitats and host-fungi some of which produce potentially lethal toxins. Perhaps A. avenae has developed efficient catabolic schemes for the detoxification of such toxins in order to survive while feeding on them.

From a physiological point of view, A. avenae shares at least one common denominator with some prokaryotes (such as Acetobacter suboxydans, Rhodospirullum rubrum, and Halobacterium sps.), the cyanophyta Vittreoscilla sps. and some parasitic helminths (such as Ascaris, Moniezia, Fasciola and Taenia taenaeformis) viz., the occurrence of cytochrome-O. What then is the metabolic advantage to any organism possessing cyt. O as an alternate oxidase? Is it for short term survival in hypoxic or anoxic conditions or is it involved in the lowered metabolism - i.e. hypobiosis associated with incipient cryptobiotic states induced by desiccation (anhydrobiosis), osmotic stress (osmobiosis) and anoxia (anaerobiosis)?

From an eco-biochemical context, the production of ethanol under anoxic conditions can be considered advantageous over lactate production, since it is easily excreted and remetabolised (Cooper, 1971) when favourable conditions return. As suggested earlier, the synthesis of glycerol in response to low-oxygen tensions, although metabolically plausible, needs further research.

From the point of view of pest-control, A.avenae would be an ideal organism for anthelminthic screening programs since some isolates of the species may have greater resistance than others to intoxication. It could be an ideal model for studying induction of resistance to commonly used pesticides/nematicides.

The similarity between the respiratory pigments of Aphelenchus and those of parasitic helminths leads inevitably to speculation on the origins of parasitism. The ancestral helminths may initially have possessed a cytochrome system which suited the organism for the colonization of environments with fluctuating oxygen tensions. Since an intestine of a higher organism would fall into this category (Bryant, 1970) the archetype helminth was most probably pre-adapted for parasitism. A. avenae is probably much closer to the archetype in that it branched off early in the evolutionary process, and although it apparently specialised in one direction -

viz., pharyngeal structure and mycophagous habit - it seems to have retained the original versatile metabolic repertoire so successfully that it is perhaps still evolving - not so much structurally but metabolically - in response to selection pressures which continually operate in various microclimatic conditions.

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Appendix 3-1.1

The population development of the isolates with time at 10°C.

	No. of Hours after inoculation				
	45	95	165	195	240
ISOLATE-A	3	4	4	-	14
	3	6	5	-	10
	4	3	3	-	11
	5	5	4	-	12
	5	3	5	-	8
	4	3	5	-	11
Mean density	4.17	4.0	4.33	-	11.00
S. D.	0.98	1.26	0.82	-	2.0
S. E.	0.40	0.52	0.33	-	0.82
ISOLATE-E	4	4	6	21	32
	5	5	5	19	14
	3	5	5	12	29
	4	4	6	13	21
	5	5	5	22	25
	5	3	7	11	28
Mean density	4.33	4.33	5.67	16.33	24.83
S. D.	0.82	0.82	0.82	4.88	6.49
S. E.	0.33	0.33	0.33	2.0	2.65
ISOLATE-F	3	5	6	18	75
	3	5	7	14	62
	4	5	7	14	59
	5	4	5	17	72
	5	5	8	11	81
	6	3	5	18	79
Mean density	4.66	4.5	6.33	15.33	71.33
S. D.	1.03	0.83	1.21	2.80	9.003
S. E.	0.42		0.49	1.14	3.676

Appendix 3-1.2

The population development of the isolates with time at 15°C.

	No. of Hours after inoculation						
	45	95	145	165	195	240	290
ISOLATE-A	5	4	14	-	49	97	85
	5	5	12	-	68	95	105
	5	5	9	-	65	85	95
	5	5	11	-	58	79	87
	5	5	12	-	52	76	107
	5	5	15	-	56	90	92
Mean density	5	4.83	12.17	-	58.00	87.00	95.17
S. D.	0	0.41	2.14	-	7.45	8.51	9.13
S. E.	0	0.17	0.87	-	3.00	3.47	3.73
ISOLATE-E	4	5	27	-	135	135	151
	5	5	24	-	112	140	144
	5	6	22	-	128	128	148
	4	6	26	-	133	110	123
	5	6	18	-	107	115	130
	5	5	17	-	105	140	126
Mean density	4.67	5.50	22.33	-	120.0	128.0	137.0
S. D.	0.52	0.55	4.13	-	13.54	12.88	12.10
S. E.	0.21	0.22	1.69	-	5.52	5.26	4.95
ISOLATE-F	5	5	31	-	150	186	215
	5	8	38	-	149	198	183
	6	6	45	-	155	175	199
	5	8	47	-	143	153	210
	5	5	39	-	158	159	188
	5	5	29	-	140	167	202
Mean density	5.16	6.16	38.17	-	149.16	173.0	199.50
S. D.	0.41	1.47	7.22	-	6.85	16.91	12.34
S. E.	0.17	0.60	2.95	-	2.80	6.90	5.04

Appendix 3-1.3

The population development of the isolates with time at 20°C.

	No. of Hours after inoculation						
	45	95	130	145	165	195	240
ISOLATE-A	4	3	12	32	57	141	175
	5	5	17	41	64	130	179
	6	7	14	37	49	117	164
	5	5	12	34	44	120	148
	5	5	19	29	41	145	150
	5	6	15	39	55	123	158
Mean density	5.0	5.17	14.83	35.33	51.67	129.33	162.33
S. D.	0.63	1.33	2.79	4.50	8.62	11.50	12.79
S. E.	0.26	0.54	1.14	1.84	3.52	4.69	5.22
ISOLATE-E	5	9	38	-	84	285	443
	6	11	35	-	91	261	665
	5	13	31	-	92	247	584
	4	8	39	-	72	255	476
	5	14	29	-	94	245	530
	5	15	40	-	75	270	693
Mean density	5.0	11.67	35.33	-	84.66	260.50	565.17
S. D.	0.63	2.80	4.50	-	9.33	15.12	100.77
S. E.	0.26	1.14	1.84	-	3.81	6.17	41.13
ISOLATE-F	5	26	61	-	230	303	745
	7	31	56	-	271	398	683
	6	16	40	-	189	295	821
	6	35	63	-	284	415	845
	5	22	45	-	243	385	771
	5	21	59	-	202	320	803
Mean density	5.66	25.17	54.00	-	236.50	352.66	778.00
S. D.	0.81	6.97	9.34	-	37.36	52.62	58.53
S. E.		2.84	3.81	-	15.25	21.56	23.89

Appendix 3-1.4

The population development of the isolates with time at 25°C.

	No. of Hours after inoculation				
	45	95	145	195	240
ISOLATE-A	3	6	80	234	457
	5	5	101	206	440
	4	5	92	222	415
	5	6	83	240	420
	5	5	104	205	455
	6	5	95	227	434
Mean density	4.66	5.33	92.50	222.34	436.83
S. D.	1.03	0.52	9.57	14.40	17.41
S. E.	0.42	0.21	3.90	5.88	7.10
ISOLATE-E	6	23	344	406	1166
	7	11	304	495	1557
	5	18	311	510	1206
	8	13	264	305	895
	8	27	346	400	1586
	5	16	266	290	826
Mean density	6.50	18.0	305.0	401.0	1206.0
S. D.	1.38	6.07	35.88	91.96	319.48
S. E.	0.56	2.48	14.64	37.53	130.40
ISOLATE-F	10	35	504	624	1640
	5	30	404	694	1770
	8	36	358	779	2960
	9	23	433	538	2000
	5	19	385	419	2110
	9	24	388	299	2100
Mean density	7.6	27.83	411.9	558.84	2097.66
S. D.	2.16	6.911	46.28	178.04	462.63
S. E.	0.88	2.82	18.89	72.67	188.83

Appendix 3-1.5

The population development of the three isolates with time at 30°C.

	No. of hours after inoculation								
	45	95	120	140	165	195	240	290	300
ISOLATE-A	18	143	382	621	729	1298	5715	8605	-
	16	148	391	509	737	1326	5210	7905	-
	16	171	363	571	674	1210	4775	7225	-
	15	192	334	518	609	1087	5730	8515	-
	17	187	329	639	628	1105	5165	7285	-
	18	181	385	570	663	1230	4635	7825	-
Mean density	16.67	170.33	364.00	571.33	673.33	1209.33	5205.0	7893.33	-
S. D.	1.21	20.53	26.91	52.49	51.87	97.73	457.64	585.57	-
S. E.	0.49	8.38	10.98	21.43	21.17	39.89	186.79	239.01	-

Appendix 3-1.5 (Contnd.)

No. of hours after inoculation

	45	95	120	140	165	195	240	290	300
ISOLATE-E	13	155	-	438	505	775	6470	-	8980
	20	150	-	520	495	635	5570	-	8500
	18	165	-	436	540	644	5380	-	8440
	23	130	-	522	570	766	4980	-	9490
	20	145	-	475	435	710	6010	-	8610
	20	170	-	483	463	699	6000	-	8660
Mean density	19.00	152.50	-	479.0	503	705.0	5735.0	-	8780.0
S. D.	3.35	14.40	-	37.66	49.26	58.84	530.91	-	395.32
S. E.	1.37	5.88	-	15.37	20.18	24.02	216.7	-	161.42
ISOLATE-F	21	225	351	-	680	1693	5845	12235	-
	25	198	428	-	835	1710	5810	12145	-
	23	275	400	-	705	1550	7155	11050	-
	20	285	439	-	770	1390	6995	9950	-
	25	230	364	-	752	1410	6895	9795	-
	24	255	478	-	822	1607	7025	11215	-
Mean density	23.0	244.67	410.00	-	760.67	1560.0	6620.83	11065	-
S. D.	2.10	32.96	47.93	-	61.73	137.04	620.20	1040.59	-
S. E.	0.86	13.45	19.56	-	25.20	55.93	253.14		

Appendix 3-2.1

Statistical treatment for the comparison of population densities developed by the three isolates at 10°C. Related "t"-test data.

Harvest Time	Isolates subject to test	t-value	p.value	
H45	A and E	0.16	p 0.05	NS
H45	A and F	0.77	p 0.05	NS
H95	A and E	0.69	p 0.05	NS
H95	A and F	1.95	p 0.05	NS
H95	E and F	0.93	p 0.05	NS
H165	A and E	4.88	0.001<p<0.01	**
H165	A and F	4.24	0.001<p<0.01	**
H165	E and F	7.69	p<0.001	***
H195	E and F	4.28	0.001 p 0.01	**
H240	A and E	6.14	p<0.001	***
H240	E and F	8.05	p<0.001	***

Appendix 3-2.2

Statistical treatment for the comparison of population densities developed by the three isolates at 15°C. Related "t"-test data.

Harvest Time	Isolates subject to test	t-value	p.value	
H45	A and E	1.41	$p > 0.05$	NS
H45	A and F	0.51	$p > 0.05$	NS
H95	A and E	0.69	$p > 0.05$	NS
H95	E and F	0.93	$p > 0.05$	NS
H95	A and F	1.95	$p > 0.05$	NS
H145	A and E	4.88	$p < 0.001$	***
H145	E and F	4.24	$0.001 < p < 0.01$	**
H145	A and F	7.69	$p < 0.001$	***
H195	A and E	8.95	$p < 0.001$	***
H195	E and F	4.28	$0.001 < p < 0.01$	**
H300	A and E	6.14	$p < 0.001$	***
H300	E and F	8.05	$p < 0.001$	***

Appendix 3-2.3

Statistical treatment for the comparison of population densities developed by the three isolates at 20°C. Related "t"-test data.

Harvest Time	Isolates subject to test	t-value	p.value	
H45	A and E	0	$p > 0.05$	NS
H45	E and F	1.43	$p > 0.05$	NS
H45	A and F	1.44	$p > 0.05$	NS
H95	A and E	4.67	$p < 0.001$	***
H95	E and F	4.005	$0.001 < p < 0.01$	**
H95	A and F	6.28	$p < 0.001$	***
H130	A and E	8.61	$p < 0.001$	***
H130	E and F	4.008	$0.001 < p < 0.01$	**
H130	A and F	8.96	$p < 0.001$	***
H165	A and E	5.78	$p < 0.001$	***
H165	E and F	5.09	$p < 0.001$	***
H240	A and E	8.78	$p < 0.001$	***
H240	E and F	4.06	$0.001 < p < 0.01$	**

Appendix 3-2.4

Statistical treatment for the comparison of population densities developed by the three isolates at 25°C. Related "t"-test data.

Harvest Time	Isolates subject to test	t-value	p.value	
H45	A and E	2.38	$0.01 < p < 0.05$	*
H45	E and F	0.95	$p > 0.05$	NS
H45	A and F	2.74	$0.01 < p < 0.05$	*
H95	A and E	4.64	$p < 0.001$	***
H95	E and F	2.38	$0.01 < p < 0.05$	*
H95	A and F	7.23	$p < 0.001$	***
H195	A and E	4.27	$0.001 < p < 0.01$	**
H195	E and F	1.75	$p > 0.05$	NS
H195	A and F	4.19	$0.001 < p < 0.01$	**
H240	A and E	5.38	$p < 0.001$	***
H240	E and F	3.53	$0.001 < p < 0.01$	**
H240	A and F	7.98	$p < 0.001$	***

Appendix 3-2.5

Statistical treatment for the comparison of population densities developed by the three isolates at 30°C. Related "t"-test data.

Harvest Time	Isolates subject to test	t-value	p.value	
H45	A and E	1.65	$p > 0.05$	NS
H45	E and F	2.25	$p < 0.05$	*
H45	A and F	5.86	$p < 0.001$	***
H95	A and E	1.58	$p > 0.05$	NS
H95	E and F	5.70	$p < 0.001$	***
H95	A and F	4.26	$0.001 < p < 0.01$	**
H120	A and F	1.98	$p > 0.05$	NS
H140	A and E	5.65	$p < 0.05$	**
H165	A and E	5.29	$p < 0.001$	***
H165	E and F	7.34	$p < 0.001$	***
H165	A and F	2.21		NS
H240	A and E	1.68	$p > 0.05$	NS
H240	E and F	2.41	$0.01 < p < 0.05$	*
H240	A and F	4.08	$0.001 < p < 0.01$	**
H290	A and E	2.79	$0.01 < p < 0.05$	*
H290	E and F	4.56	$0.001 < p < 0.01$	**
H290	A and F	5.91	$p < 0.001$	***

Appendix 3-3.1

Data for Arrhenius plots of population growth rates for isolates A, F and E.

Temperature °C	Temperature °K	Temperature 1/°K	Isolate Code	Nos. of nematodes after 240 hrs. MEAN (n=6)	Rate of population growth (Nos./hr.)	Log Rate log (Nos./hr.)
10°C	283	.003533	F	80.00	0.3333	$\bar{1}$.4771
			E	24.83	0.1035	$\bar{1}$.9852
			A	11.00	0.0458	$\bar{2}$.3388
15°C	288	.003472	F	173.00	0.7208	$\bar{1}$.1422
			E	128.00	0.5333	$\bar{1}$.2730
			A	87.00	0.3625	$\bar{1}$.4407
20°C	293	.003412	F	778.00	3.2417	0.5108
			E	565.17	2.3542	0.3718
			A	162.33	0.6750	$\bar{1}$.1707
25°C	298	.003355	F	2097.66	8.7403	0.9415
			E	1206.00	5.0250	0.7011
			A	436.83	1.8201	0.2601
30°C	303	.003300	F	6620.83	27.5868	1.4407
			E	5735.00	23.8958	1.3783
			A	5205.10	21.6879	1.3362

Appendix 3-3.2

Results of the Linear Regressions computed for the Arrhenius Plots for population growth rate.

ISOLATE	$y = [m]\,x + [c]$ for the temperatures $10^{o}C$, $15^{o}C$ and $20^{o}C$. SLOPE [m]	INTERCEPT [c]	Regression Coefficient [r]
A	$+9.67 \times 10^{3}$	31.91	0.7
E	$+19.45 \times 10^{3}$	66.60	0.7
F	$+16.39 \times 10^{3}$	56.73	0.7

ISOLATE	$y = [m]\,x + [c]$ for the temperatures $10^{o}C$, $15^{o}C$ and $20^{o}C$. SLOPE [m]	INTERCEPT [c]	Regression Coefficient [r]
A	$+22.39 \times 10^{3}$	75.30	0.8
E	$+8.96 \times 10^{3}$	30.90	0.8
F	$+8.29 \times 10^{3}$	28.81	0.8

Appendix 3-4.1

The variation of L/A ratio of isolate-A developing for 15 days at 20°C.

	Harvest Time in Hours										
	45	95	135	168	215	240	280	300	320	340	360
$\log_{10}$ Nos. of Adults	0.69214 ± 0.019	0.52244 ± 0.051	0.82347 ± 0.033	0.66931 ± 0.023	1.32899 ± 0.030	1.46731 ± 0.016	1.84714 ± 0.014	1.91381 ± 0.015	2.01840 ± 0.031	2.13776 ± 0.014	1.84298 ± 0.014
$\log_{10}$ Nos. of Larvae	1.11125 ± 0.025	0.47712 ± 0.050	1.21298 ± 0.031	1.74296 ± 0.020	2.11836 ± 0.029	2.12603 ± 0.010	2.10150 ± 0.011	2.13776 ± 0.015	2.13567 ± 0.033	2.13672 ± 0.017	2.37535 ± 0.015
$\log_{10}$ L/A+3.0	1.19645 ± 0.027	2.93693 ± 0.054	3.38951 ± 0.035	4.07364 ± 0.025	3.78937 ± 0.030	3.65872 ± 0.017	3.25436 ± 0.014	3.22394 ± 0.015	3.11726 ± 0.031	2.99895 ± 0.015	3.53236 ± 0.015

The confidence intervals(C.I.=2.57$S_{\bar{x}}$) For a two-tailed test at =0.05 (n=6;d.F=5) are indicated in the corresponding logarithmic plots on Fig. 8.

Appendix 3-4.2

The variation of L/A ratio of isolate-F developing for 15 days at 20°C.

	Harvest Time in Hours										
	45	95	135	168	215	240	280	300	320	340	360
$\log_{10}$ Nos. of Adults	0.69161 ± 0.021	0.96988 ± 0.051	0.74046 ± 0.033	0.88422 ± 0.069	1.86628 ± 0.051	2.38080 ± 0.054	3.17631 ± 0.014	2.88119 ± 0.015	3.22599 ± 0.020	3.17609 ± 0.015	3.03529 ± 0.016
$\log_{10}$ Nos. of Larvae	1.07935 ± 0.023	1.32899 ± 0.041	1.72015 ± 0.0351	2.36235 ± 0.071	2.60906 ± 0.053	2.74009 ± 0.050	2.59326 ± 0.015	3.15966 ± 0.022	3.15604 ± 0.023	3.43296 ± 0.015	3.63702 ± 0.019
$\log_{10}$ L/A+3.0	1.22891 ± 0.023	3.35910 ± 0.045	3.97979 ± 0.034	4.47812 ± 0.069	3.74277 ± 0.054	3.35928 ± 0.053	1.41697 ± 0.015	3.27847 ± 0.021	2.93004 ± 0.022	3.25686 ± 0.015	3.60168 ± 0.020

The confidence intervals (C.I. = 2.57 $S_{\bar{x}}$) for a two-tailed test at =0.05 (n=6;d.F=5) are indicated in the corresponding logarithmic plots on Fig. 8.

Appendix 3-4.3

The variation of L/A ratio of isolate-E developing for 15 days at 20°C.

	Harvest Time in Hours									
	45	95	135	168	240	280	300	320	340	360
$\log_{10}$ Nos. of Adults	0.71323 ± 0.022	0.80140 ± 0.045	0.66838 ± 0.023	0.88422 ± 0.021	2.34175 ± 0.032	2.86648 ± 0.033	3.23863 ± 0.041	3.00346 ± 0.014	2.99402 ± 0.020	2.80436 ± 0.022
$\log_{10}$ Nos. of Larvae	1.79588 ± 0.023	0.82347 ± 0.045	1.53567 ± 0.027	1.92598 ± 0.029	2.57441 ± 0.033	2.37043 ± 0.030	2.57824 ± 0.035	2.58206 ± 0.011	2.89963 ± 0.020	3.25106 ± 0.027
$\log_{10}$ L/A+3.0	1.49623 ± 0.023	3.02206 ± 0.045	3.86728 ± 0.028	4.04175 ± 0.030	3.23266 ± 0.033	2.50395 ± 0.034	2.33961 ± 0.041	2.57859 ± 0.015	2.90560 ± 0.023	3.44670 ± 0.027

The confidence intervals (C.I.=2.57$S_{\bar{x}}$) for a two-tailed test at =0.05 (n=6; d.f=5) are indicated in the corresponding logarithmic plots on Fig. 8.

Appendix 3-4.4

The variation of L/A ratio of isolate-A developing for 15 days at 25°C.

	Harvest Time in Hours								
	45	95	135	168	215	240	280	320	360
$\log_{10}$ Nos. of Adults	0.65475 ± 0.043	0.41329 ± 0.017	1.41124 ± 0.019	1.83033 ± 0.012	2.08134 ± 0.010	1.80386 ± 0.038	2.37167 ± 0.060	2.96738 ± 0.041	3.25349 ± 0.045
$\log_{10}$ Nos. of Larvae	1.89909 ± 0.021	0.59217 ± 0.015	1.46570 ± 0.020	1.86840 ± 0.022	2.26176 ± 0.012	2.57479 ± 0.041	2.67240 ± 0.054	2.68334 ± 0.043	3.29936 ± 0.048
$\log_{10}$ L/A+3.0	2.25532 ± 0.044	3.17887 ± 0.017	3.05447 ± 0.021	3.03724 ± 0.024	3.18038 ± 0.012	3.77092 ± 0.042	3.30072 ± 0.062	2.71598 ± 0.044	3.04586 ± 0.048

The confidence intervals (C.I. = 2.57 $S_{\bar{x}}$) for a two-tailed test at =0.05 (n=6;d.F=5) are indicated in the corresponding logorithmic plots on Fig. 9.

Appendix 3-4.5

The variation of L/A ratios of isolate-F developing for 15 days at 25°C.

	Harvest Time in Hours								
	45	95	135	168	215	240	280	320	360
$\log_{10}$ Nos. of Adults	0.80140 ± 0.055	0.36735 ± 0.045	2.13987 ± 0.021	1.89019 ± 0.063	1.36172 ± 0.036	1.96217 ± 0.041	2.92582 ± 0.051	2.85712 ± 0.048	2.64081 ± 0.053
Log_{10} Nos. of Larvae	0.12385 ± 0.035	1.34888 ± 0.040	2.00715 ± 0.024	2.60816 ± 0.051	2.98929 ± 0.051	3.30397 ± 0.054	3.47124 ± 0.062	3.66984 ± 0.058	3.87143 ± 0.057
$\log_{10}$ L/A+3.0	2.32244 ± 0.057	3.98153 ± 0.048	2.86726 ± 0.025	3.71796 ± 0.064	4.62757 ± 0.052	4.32149 ± 0.057	3.54541 ± 0.062	3.81271 ± 0.060	4.23061 ± 0.058

The confidence intervals (C.I. = 2.57 $S_{\bar{x}}$) for a two-tailed test at α=0.05 (n=6;d.f=5) are indicated in the corresponding logarithmic plots on Fig. 9.

Appendix 3-4.6

The variation of L/A ratios of isolate-E developing for 15 days at 25°C.

	Harvest Time in Hours								
	45	95	135	168	215	240	280	320	360
$\log_{10}$ Nos. of Adults	0.75281 ± 0.038	$\bar{1}$.18046 ± 0.060	1.81517 ± 0.021	1.96217 ± 0.042	1.50051 ± 0.038	2.03873 ± 0.042	2.77670 ± 0.044	3.51219 ± 0.061	3.33048 ± 0.021
$\log_{10}$ Nos. of Larvae	$\bar{1}$.18045 ± 0.033	1.31513 ± 0.037	2.01140 ± 0.030	2.40879 ± 0.037	0.82170 ± 0.033	3.03848 ± 0.051	2.98196 ± 0.050	2.74636 ± 0.051	3.63393 ± 0.057
$\log_{10}$ L/A+3.0	2.06666 ± 0.040	4.49558 ± 0.063	3.19622 ± 0.033	3.44661 ± 0.044	4.28219 ± 0.040	3.99974 ± 0.053	3.20526 ± 0.053	2.23416 ± 0.063	3.30345 ± 0.061

The confidence intervals [C.I. = 2.57 $S_{\bar{x}}$] for a two-tailed test at =0.05 [n=6;d.f=5] are indicated in the corresponding logarithmic plots on Fig. 9.

Appendix 3-4.7

The variation of L/A ratios of isolate-F developing for 12 days at 30°C.

	Harvest Time in Hours							
	45	95	135	168	195	215	240	280
$\log_{10}$ Nos. of Adults	1.00732 ± 0.018	0.80140 ± 0.024	1.56419 ± 0.021	2.24038 ± 0.022	2.80322 ± 0.015	3.22228 ± 0.016	2.58920 ± 0.042	3.14731 ± 0.037
$\log_{10}$ Nos. of Larvae	1.03462 ± 0.023	2.35088 ± 0.038	2.65705 ± 0.041	2.79685 ± 0.041	2.98242 ± 0.043	3.12683 ± 0.048	3.79465 ± 0.051	4.99409 ± 0.061
$\log_{10}$ L/A+3.0	3.02730 ± 0.026	4.54948 ± 0.038	4.09286 ± 0.042	3.55648 ± 0.045	3.17919 ± 0.047	2.90455 ± 0.051	4.20544 ± 0.057	4.84677 ± 0.064

The confidence intervals [C.I. = 2.57 $S_{\bar{x}}$] for a two-tailed test at =0.05 [n=6;d.f=5] are indicated in the corresponding logarithmic plots on Fig. 10.

Appendix 3-4.8

The variation of L/A ratios of isolate-E developing for 12 days at 30°C.

	Harvest Time in Hours							
	45	95	135	168	195	215	240	280
$\log_{10}$ Nos. of Adults	1.06069 ± 0.033	0.98497 ± 0.017	1.68421 ± 0.032	2.11836 ± 0.023	2.54940 ± 0.015	2.422146 ± 0.019	3.46319 ± 0.031	2.18565 ± 0.025
$\log_{10}$ Nos. of Larvae	0.76566 ± 0.023	2.15481 ± 0.022	2.41692 ± 0.027	2.56506 ± 0.031	2.53677 ± 0.033	3.18516 ± 0.041	3.46209 ± 0.033	3.89553 ± 0.037
$\log_{10}$ L/A+3.0	2.70496 ± 0.035	4.16984 ± 0.026	3.73270 ± 0.035	3.44669 ± 0.034	2.98736 ± 0.041	3.76301 ± 0.045	2.99060 ± 0.033	4.70987 ± 0.039

The confidence intervals [C.I. = 2.57 $S_{\bar{x}}$] for a two-tailed test at =0.05 [n=6;d.f=5] are indicated in the corresponding logarithmic plots on Fig.10.

Appendix 3-4.9

The variation of L/A ratios of isolate-A developing for 12 days at 30°C.

	Harvest Time in Hours							
	45	95	135	168	195	215	240	280
$\log_{10}$ Nos. of Adults	0.68394 ± 0.014	0.93751 ± 0.022	1.84720 ± 0.014	1.81291 ± 0.016	1.56026 ± 0.014	2.16115 ± 0.023	2.76355 ± 0.017	3.23037 ± 0.015
$\log_{10}$ Nos. of Larvae	1.52048 ± 0.023	2.22367 ± 0.031	2.63714 ± 0.029	2.85622 ± 0.040	3.06954 ± 0.043	3.32366 ± 0.046	3.71153 ± 0.048	3.85404 ± 0.052
$\log_{10}$ L/A→3.0	3.83653 ± 0.036	4.66009 ± 0.039	3.78624 ± 0.040	5.04532 ± 0.052	4.50928 ± 0.048	4.16280 ± 0.049	4.94798 ± 0.050	4.62367 ± 0.054

The confidence intervals (C.I. = $2.57S_{\bar{x}}$) for a two-tailed test at =0.05 [n=6;d.f=5] are indicated in the corresponding logarithmic plots on Fig. 10.

Appendix 3-5.1

The effect of NaCN incorporated medium on the change of composition of isolate-A at 30°C.

	Harvest Time in Hours						
	45	95	135	168	195	215	240
Nos. of adults (control group)	4.83 ± 0.98	7.66 ± 2.1	70.33 ± 10.15	65.0 ± 8.15	36.33 ± 3.85	144.83 ± 23.16	580.12 ± 146.74
Nos. of adults in NaCN/PDA	5.0 ± 0.83	5.17 ± 1.12	14.33 ± 1.76	12.17 ± 2.11	22.0 ± 4.1	48.17 ± 15.18	125.33 ± 39.61
Nos. of larvae (control group)	33.17 ± 4.15	167.33 ± 18.78	433.66 ± 40.19	718.17 ± 31.66	1073.66 ± 78.15	2107.0 ± 112.66	5146.8 ± 198.17
Nos. of larvae in NaCN/PDA	16.33 ± 2.15	72.50 ± 9.18	505.17 ± 48.75	779.00 ± 39.17	1200.17 ± 80.55	2403.33 ± 196.15	6601.33 ± 385.52
Total Nos. (control group)	37.0 ± 3.41	174.33 ± 25.38	503.17 ± 49.25	791.33 ± 88.71	1210.0 ± 118.67	2250.83 ± 238.15	5726.66 ± 440.15
Total Nos. (test group)	21.5 ± 3.81	77.66 ± 8.91	519.17 ± 49.46	802.0 ± 98.15	1222.5 ± 143.32	2453.5 ± 271.14	6730.17 ± 585.30

For Statistical Analysis of the data see Appendix 5.3 and Appendix 5.4.

The S.E. obtained for the $\log_{10}$ data of 6 replicates was used to compute the Confidence Intervals (C.I. = 2.56 x $\log_{10}$ S.E. for a two-tailed test at α=0.05;n=6;d.f=5) indicated in figure 11.

Appendix 3-5.2

The effect of NaCN incorporated medium on the change of composition of isolate-F at 30°C.

	Harvest Time in Hours						
	45	95	135	168	195	215	240
Nos.of adults (control group)	5.17 ± 0.83	34.33 ± 5.11	71.33 ± 8.91	179.5 ± 16.87	209.17 ± 18.87	169.33 ± 14.65	410.50 ± 52.51
Nos.of adults in NaCN/PDA	5.0 ± 1.12	12.33 ± 2.66	29.33 ± 4.15	38.50 ± 15.51	161.17 ± 14.18	204.50 ± 19.18	311.66 ± 48.37
Nos. of larvae (control group)	17.17 ± 1.82	196.36 ± 16.55	431.84 ± 11.15	682.17 ± 70.15	1201.33 ± 105.43	4041.27 ± 216.66	6260.0 ± 285.17
Nos. of larvae in NaCN/PDA	13.17 ± 1.98	218.0 ± 22.65	451.33 ± 14.33	893.66 ± 93.61	1284.0 ± 98.61	3680.66 ± 227.76	6704.5 ± 320.98
Total nos. (control group)	22.66 ± 2.43	230.66 ± 30.81	503.17 ± 49.30	861.66 ± 79.18	1710.5 ± 149.15	4210.6 ± 318.61	6670.5 ± 341.15
Total nos. in NaCN/PDA	18.17 ± 3.36	271.50 ± 33.73	480.66 ± 58.15	932.17 ± 108.53	1645.17 ± 161.31	3885.17 ± 320.33	7015.17 ± 419.41

For Statistical Analysis of the data see Appendix 5.5 and Appendix 5.6.

The S.E. obtained for the $\log_{10}$ data of 6 replicates was used to compute the Confidence Intervals [C.I. = 2.56 x $\log_{10}$ S.E. for a two-tailed test at α=0.05;n=6;d.f=5] indicated in figure 12.

Appendix 3-5.3

Statistical treatment for the comparison of larval numbers produced by isolate-A in normal-PDA and cyanide-incorporated-PDA. (Related "t"-test data)

Harvest Time in Hours	Nos. of larvae in controls ± S.D. (n=6)	Nos. of larvae in CN/PDA ± S.D. (n=6)	t-value d.f=10	p-value
H45	33.17 ± 4.15	16.33 ± 2.15	8.05	$p < 0.001$
H95	167.33 ± 18.78	72.50 ± 9.18	10.09	$p < 0.001$
H135	433.66 ± 40.19	505.17 ± 48.75	2.51	$.01 < p < 0.05$
H168	718.17 ± 31.66	779.00 ± 39.17	2.68	$.01 < p < 0.05$
H195	1073.66 ± 78.15	1200.17 ± 80.55	2.50	$.01 < p < 0.05$
H215	2107.0 ± 112.66	2403.33 ± 196.15	2.91	$.01 < p < 0.05$
H240	5146.8 ± 198.17	6601.33 ± 385.52	7.47	$p < 0.001$

Appendix 3-5.4

Statistical treatment for the comparison of adult numbers produced by isolate-A in normal-PDA (controls) and in cyanide-incorporated-PDA. (Related "t"-test data)

Harvest Time in Hours	Nos. of adults in controls ± S.D. (n=6)	Nos. of adults in CN/PDA ± S.D. (n=6)	t-value d.f=10	p-value	
H45	4.83 ± 0.98	5.0 ± 0.83	.298	$p > 0.05$	NS
H95	7.66 ± 2.10	5.17 ± 1.12	2.34	$0.01 < p < 0.05$	
H135	70.33 ± 10.15	14.33 ± 1.76	4.62	$p < 0.001$	
H168	65.00 ± 8.15	12.17 ± 2.11	12.97	$p < 0.001$	
H195	36.33 ± 3.85	22.00 ± 4.10	5.68	$p < 0.001$	
H215	144.83 ± 23.16	48.17 ± 15.18	7.70	$p < 0.001$	
H240	580.12 ± 146.74	125.33 ± 39.61	6.66	$p < 0.001$	

Appendix 3-5.5

Statistical treatment for the comparison of adult numbers produced by isolate-F in normal-PDA (controls) and cyanide-incorporated-PDA. (Related "t"-test data)

Harvest Time in Hours	Nos. of adults in controls ± S.D. (n=6)	Nos. of adults in CN/PDA ± S.D. (n=6)	t-value d.f=10	p-value
H45	5.17 ± 0.83	5.0 ± 1.12	0.274	$p > 0.05$
H95	34.33 ± 5.11	12.33 ± 2.66	2.588	$0.01 < p < 0.05$
H135	71.33 ± 8.91	29.33 ± 4.15	10.21	$p < 0.001$
H168	179.50 ± 16.87	38.50 ± 15.51	13.82	$p < 0.001$
H195	209.17 ± 18.87	161.17 ± 14.18	4.52	$0.001 < p < 0.01$
H215	169.33 ± 14.65	204.50 ± 19.18	3.25	$0.001 < p < 0.01$
H240	410.50 ± 52.51	311.66 ± 48.37	3.082	$0.01 < p < 0.05$

Appendix 3-5.6

Statistical treatment for the comparison of larval numbers produced by isolate-F in normal-PDA (controls) and in cyanide-incorporated-PDA. (Related "t"-test data)

Harvest Time in Hours	Nos. of larvae in controls ± S.D. (n=6)	Nos. of larvae in CN/PDA ± S.D. (n=6)	t-value d.f=10	p-value
H45	17.17 ± 1.82	13.17 ± 1.95	3.33	$0.001 < p < 0.01$
H95	195.36 ± 16.55	218 ± 22.65	1.79	$p < 0.05$
H135	431.84 ± 11.15	451.33 ± 14.33	2.39	$p < 0.05$
H168	682.17 ± 70.15	893.66 ± 93.61	4.02	$0.001 < p < 0.01$
H195	1201.33 ± 105.43	1484.0 ± 98.61	4.35	$0.001 < p < 0.01$
H215	4041.27 ± 216.66	3680.66 ± 227.76	2.55	$0.01 < p < 0.05$
H240	6260.0 ± 285.17	6704.5 ± 320.98	2.30	$0.01 < p < 0.05$

Appendix 3-6.1

The effect of low temperature (0-3°C.) treatment of non-starving young adult females from three isolates of *A. avenae* on the population growth subsequent to treatment.

Nos. of nematodes produced by 5 females (treated at 0-3°C.) after 130 hrs. at 30°C.			MEAN ± S.D. (n=6)	S.E. (n=6)	Nos. of nematodes produced by 5 females (controls) after 130 hrs. at 30°C.			MEAN ± S.D. (n=6)	S.E. (n=6)	significance
ISOLATE-F										
364,	414,		394		420,	398,		409		
369,	421,	A	±	11.35	432,	386,	B	±	6.90	A & B
374,	422,		27.71		417,	401,		16.90		NS
ISOLATE-A										
281,	278,		263.17		390,	353,		370		
245,	259,	C	±	6.18	351,	339,	D	±	10.49	C & D
268,	248,		15.09		387,	401,		25.69		***
ISOLATE-E										
311,	343,		326.0		371,	404,		384.83		
341,	323,	E	±	5.92	399,	363,	F	±	8.41	E & F
309,	329,		14.46		365,	407,		20.60		***

Significant values of "t"-test (d.F = 10) :-

Not significant - NS; $p < 0.05$ - *;

$p < 0.01$ - **; $p < 0.001$ - ***;

Appendix 3-6.2

The effect of low temperature (0-3°C.) treatment of non-feeding young adult females from three isolates of *A. avenae* on the population growth subsequent to treatment.

Nos. of nematodes produced by 5 females (treated at 0-3°C.) after 130 hrs. at 30°C.			MEAN ± S.D. (n=6)	S.E. (n=6)	Nos. of nematodes produced by 5 females (controls) after 130 hrs. at 30°C.			MEAN ± S.D. (n=6)	S.E. (n=6)	significance
ISOLATE-F										
359,	361,		378.0		350,	368,		356.0		
397,	395,	A	±	6.71	342,	372,	B	±	5.63	A & B
383,	373,		16.43		340,	364,		13.80		NS
ISOLATE-A										
210,	207,		221.50		335,	333,		352.0		
235,	219,	C	±	4.69	369,	371,	D	±	6.66	C & D
232,	226,		11.50		348,	356		16.32		***
ISOLATE-E										
311,	340,		326.0		323,	350,		335.0		
341,	344,	E	±	7.05	347,	321,	F	±	6.14	E & F
308,	312,		17.26		320,	349,		15.03		***

Significant values of "t"-test (d.f = 10) :-

Not significant - NS; $p < 0.05$ - *;

$p < 0.01$ - **; $p < 0.001$ - ***

Appendix 3-7.1

The effect of low temperature on the percentage mortality of isolates F and A. (Angularly transformed data.) (Results of 6 replicate treatments.)

Period of incubation at 0-3°C in days	Isolate-F % Mortality (n=6)		Isolate-A % Mortality (n=6)	
7	5.1,	5.4,	12.9,	9.6,
	10.6,	5.5,	10.0,	6.6,
	0.9,	4.8,	12.6,	6.3,
14	9.5,	5.2,	16.4,	16.7,
	7.2,	4.8,	12.6,	9.3,
	4.9,	9.6,	12.0,	8.4,
21	12.2,	4.4,	18.3,	14.2,
	8.3,	8.5,	9.8,	10.0,
	12.0,	5.0,	14.1,	17.9,
28	8.8,	6.7,	23.4,	9.3,
	9.1,	3.9,	21.9,	18.0,
	6.9,	4.3,	17.1,	12.1,
35	6.1,	11.9,	22.0,	13.8,
	9.0,	8.8,	20.4,	16.0,
	12.8,	5.1,	17.8,	14.6,
42	15.6,	15.3,	27.0,	17.2,
	12.1,	8.8,	12.1,	16.3,
	8.6,	11.9,	22.0,	27.8,
56	17.0,	13.0,	21.3,	31.8,
	12.9,	10.0,	24.3,	40.1,
	17.2,	8.8,	39.3,	30.0,
63	-	-	-	-
70	20.5,	19.8,	44.5,	21.3,
	8.3,	14.0,	19.1,	48.3,
	14.4,	7.5,	30.9,	34.8,
105	25.1,	16.6,	54.1,	50.1,
	8.5,	24.9,	46.9,	33.8,
	16.8,	7.7,	39.7,	43.7,

Means are statistically analysed in the following Appendix, Appendix3-7.2.

Appendix 3-7.2

Results of a "t"-test analysis of the mean percentage mortalities of isolates F and A (angularly transformed data) following low temperature (0-3°C.) treatment.
(Results of individual replicates are given in preceding Appendix, Appendix3-7.1)

Period of incubation at 0-3°C in days	Isolate-F Mean % Mortality	± S.D.	Isolate-A Mean % Mortality	± S.D.	p
7	5.38	3.09	9.67	2.83	$p > 0.05$ (NS)
14	6.9	2.26	12.57	3.47	$p > 0.05$ (NS)
21	8.4	3.32	14.05	3.67	$p \geq 0.05$
28	6.62	2.18	16.97	5.46	$p < 0.05$ *
35	8.95	3.05	17.43	3.26	$p < 0.01$ **
42	12.05	3.02	20.40	6.27	$0.01 < p < 0.05$ *
56	13.15	3.47	31.23	7.49	$p < 0.001$ ***
63	-	-	-	-	-
70	14.08	5.49	33.15	11.87	$.001 < p < 0.01$ **
105	16.60	7.56	44.72	7.31	$p < 0.001$ ***

Appendix 3-7.3

Mortality of isolates F and A in suspensions of sterile distilled water at 25°C. ambient. (Angularly transformed data.)

(Results of 6 replicate treatments.)

Period of incubation at 0-3°C in days	Isolate-F % Mortality (n=6)		Isolate-A % Mortality (n=6)	
7	6.0, 3.1, 2.8,	0.1, 0.2, 6.3,	2.7, 0.1, 2.4,	5.1, 1.9, 2.2,
14	-	-	-	-
21	8.8, 0.9, 1.2,	4.8, 4.7, 8.6,	6.4, 0.7, 3.4,	9.6, 0.2, 0.7,
28	-	-	-	-
35	-	-	-	-
42	10.7, 9.8, 7.9,	7.6, 3.1, 5.0,	7.1, 6.9, 7.0,	6.8, 6.2, 6.5,
56	10.6, 10.9, 6.5,	4.1, 6.9, 2.9,	11.5, 8.1, 5.9,	10.5, 6.3, 8.7,
63	-	-	-	-
70	-	-	-	-
105	16.5, 15.9, 11.0,	11.1, 7.5, 7.0,	12.2, 12.7, 9.8,	5.5, 10.3, 6.5,

Means are statistically analysed in the following Appendix, Appendix 3-7.4

Appendix 3-7:4

Results of a "t"-test analysis of the mean percentage mortalities of isolates F and A (angularly transformed data) following incubation at 25°C. ambient.

(Results of individual replicates are given in preceding Appendix, Appendix 3-7.3)

Period of incubation at 0-3°C in days	Isolate-F Mean % Mortality	± S.D.	Isolate-A Mean % Mortality	± S.D.	p
7	3.08	2.69	2.4	1.6	NS
14	-	-	-	-	-
21	4.83	3.42	3.5	3.8	NS
28	-	-	-	-	-
35	-	-	-	-	-
42	7.35	2.87	6.75	0.34	NS
56	6.70	3.63	8.50	2.23	NS
63	-	-	-	-	-
70	-	-	-	-	-
105	11.50	4.03	9.50	2.94	NS

Significant values of "t"-test are given in column p:-

NS - $p > 0.05$

Appendix 3-7.5

Comparison of the percent-mortalities of isolates A and F with time due to low temperature regime. (Related "t"-test data)

(% mortality transformed angularly for statistical treatment)

Duration of incubation at 0-3°C. (days)	t-value (df=10)	p-value	
7	1.88	$p > 0.05$	NS
14	1.86	$p > 0.05$	NS
21	2.229	$p < 0.05$	*
28	2.64	$p < 0.05$	*
35	4.24	$.001 < p < 0.01$	**
42	2.67	$0.01 < p < 0.05$	**
56	4.88	$p < 0.001$	***
70	3.24	$0.001 < p < 0.01$	**
105	5.95	$p < 0.001$	***

Comparison of the percent-mortalities of isolates A and F (controls) at 25°C. ambient. (Related "t"-test data.)

Duration of incubation at 25°C.	t-value (df=10)	p-value	
7	0.483	> 0.05	NS
14	0.580	> 0.05	NS
42	0.465	> 0.05	NS
56	0.942	> 0.05	NS
105	0.892	> 0.05	NS

Appendix 3-8.1

The population growth (at 30°C.) of the three isolates of *A. avenae* following anaerobic treatment.

	Nos. of nematodes at 120 hrs. harvest following anaerobic treatment for 120 hrs. (at 25°C.) (n=6) MEAN ± S.D.		Nos. of nematodes at 120 hrs. harvest following treatment in air saturated medium for 120 hrs. (at 25°C.) (n=6) MEAN ± S.D.
ISOLATE-A	408.50 ± 36.84 (38.65)	NS	420.50 ± 24.52 (25.82)
ISOLATE-F	344.66 ± 34.33 (36.15)	NS	364.33 ± 22.17 (23.35)
ISOLATE-E	372.33 ± 34.17 (35.99)	NS	386.50 ± 28.54 (30.06)

Significant values of "t"-test: $p > 0.05$ = NS

The Confidence Interval for 5 degrees, at $\alpha = 0.05$ i.e. 2.57 x S.E. (two-tailed) is given in parenthesis.

Appendix 3-8.1

The effect of anaerobic (100% N_2) treatment of isolate-A on the Mortality/Viability of individuals

(Analysed using 2 x 2 Chi Square)

	Replicate	Control Group	Test Group	Degrees of Freedom	.	p
Mortality %	1.	14	18	1	0.07	NS
	2.	-	20	1	0.28	
	MEAN	14	19			
Viability %	1.	82	72	1	0	NS
	2.	-	78	1	0	
	MEAN	82	75			
Total Survival %	1.	86	82	1	0	NS
	2.	-	80	1	0	
	MEAN	86	81			

Significant values for 2 x 2 Chi Square test :- $p > 0.05 := NS$

Appendix 3-9.2

The effect of anaerobic (100% N_2) treatment of isolate-F on the Mortality/Viability of individuals (Analysed using 2 x 2 Chi Square)

	Replicate	Control Group	Test Group	Degrees of Freedom	.	p
Mortality %	1.	16	18	1	0.26	NS
	2.	14	20	1	0.07	
	MEAN	15	19			
Viability %	1.	72	58	1	1.11	NS
	2.	76	60	1	2.89	
	MEAN	74	59			
Total Survival %	1.	84	82	1	0	NS
	2.	86	80	1	0	
	MEAN	85	81			

Significant values for 2 x 2 Chi Square test :- $p > 0.05$ = NS

Appendix 3-9.3

The effect of anaerobic (100% N_2) treatment of isolate-E on the Mortality/Viability of individuals

(Analysed using 2 x 2 Chi Square)

	Replicate	Control Group	Test Group	Degrees of Freedom	.	p
Mortality %	1.	8	10	1	0	NS
	2.	12	14	1	0	
	MEAN	10	12			
Viability %	1.	86	74	1	0	NS
	2.	82	78	1	0	
	MEAN	84	76			
Total Survival %	1.	92	90	1	0	NS
	2.	88	86	1	0	
	MEAN	90	88			

Significant values for 2 x 2 Chi Square test :- $p > 0.05 = NS$

Appendix 4-1.1

The effect of harvest time on the QO_2 of isolate-E.

	Time of harvest (days)						
	40	50	60	70	80	100	140
QO_2	5.89	5.78	6.00	5.86	5.87	5.84	6.05
	5.84	5.73	6.15	5.91	6.09	5.62	5.83
Batch	5.99	5.88	6.20	6.01	6.03	5.77	5.90
1	6.04	5.90	6.05	6.06	5.93	5.69	5.98
	6.01	5.76	6.17	6.04	5.92	5.66	6.00
	5.87	5.93	6.03	5.88	6.04	5.80	5.88
	5.82	5.71	5.75	5.60	5.83	5.58	5.79
	5.77	5.66	5.80	5.66	5.67	5.36	5.57
	5.92	5.81	5.90	5.76	5.77	5.52	5.64
Batch	5.97	5.69	5.95	5.82	5.61	5.42	5.72
2	5.94	5.86	5.93	5.79	5.80	5.39	5.74
	5.80	5.83	5.77	5.63	5.64	5.55	5.62
	5.52	5.56	5.88	5.75	5.94	5.94	5.64
	5.57	5.41	5.83	5.53	5.80	5.84	5.54
Batch	5.67	5.46	5.73	5.69	5.74	5.62	5.35
3	5.72	5.61	5.68	5.59	5.88	5.93	5.65
	5.69	5.58	5.86	5.72	5.90	5.97	5.31
	5.55	5.44	5.70	5.56	5.78	5.64	5.56
Mean $\bar{x}$	5.81	5.70	5.91	5.77	5.85	5.67	5.71
S.D. (n=18)	0.16	0.17	0.15	0.16	0.14	0.19	0.21

Appendix 4-1.2

The effect of harvest time on the QO_2 of isolate-F of A. avenae

	Time of harvest [days]					
	50	60	70	90	100	140
QO_2	5.63	5.41	5.37	5.18	5.24	5.10
	5.43	5.55	5.52	5.23	5.39	5.15
Batch	5.59	5.47	5.42	5.33	5.29	5.30
1	5.47	5.61	5.57	5.38	5.44	5.25
	5.57	5.45	5.40	5.35	5.41	5.27
	5.49	5.57	5.54	5.21	5.27	5.13
	5.23	5.48	5.45	4.92	4.98	5.31
	5.33	5.34	5.30	5.07	5.13	5.11
Batch	5.27	5.41	5.36	5.12	5.18	4.92
2	5.53	5.52	5.51	4.97	5.03	5.10
	5.29	5.54	5.47	5.09	5.15	5.29
	5.37	5.38	5.33	4.95	5.01	4.93
	5.61	5.09	5.05	5.11	5.28	5.20
	5.57	5.23	5.21	4.90	5.40	5.00
Batch	5.41	5.15	5.22	4.92	5.07	4.83
3	5.45	5.29	5.25	5.01	5.24	5.18
	5.55	5.13	5.11	5.02	5.44	5.05
	5.47	5.25	5.08	5.18	5.03	4.81
Mean $\bar{x}$	5.45	5.38	5.15	5.11	5.22	5.11
S.D. [n=18]	0.122	0.15	0.16	0.15	0.16	0.17

Appendix 4-1.3

The effect of harvest time on the QO_2 of isolate-A

(QO_2 units:- nmO_2 min^{-1} mg dry wt^{-1})

	Time of harvest (days)					
	50	60	70	80	90	120
QO_2	5.45	5.35	5.54	5.65	5.23	5.29
	5.65	5.50	5.74	5.45	5.43	5.08
Batch	5.47	5.32	5.56	5.47	5.27	5.24
1	5.63	5.52	5.72	5.63	5.39	5.24
	5.46	5.34	5.71	5.48	5.24	5.22
	5.64	5.49	5.57	5.62	5.42	5.16
	5.46	5.13	5.35	5.38	5.29	5.53
	5.26	5.31	5.53	5.58	5.09	5.22
Batch	5.28	5.33	5.37	5.40	5.25	5.34
2	5.44	5.15	5.55	5.56	5.13	5.51
	5.43	5.30	5.38	5.42	5.28	5.31
	5.29	5.16	5.52	5.54	5.10	5.26
	5.25	5.06	5.34	5.45	5.46	5.16
	5.43	5.27	5.54	5.47	5.22	5.05
Batch	5.45	5.29	5.36	5.56	5.10	4.85
3	5.27	5.11	5.52	5.60	5.22	5.03
	5.42	5.12	5.37	5.48	5.41	5.84
	5.28	5.26	5.51	5.36	5.15	5.20
Mean $\bar{x}$	5.42	5.27	5.51	5.51	5.26	5.24
S.D. (n=18)	0.13	0.13	0.14	0.15	0.19	0.21

Appendix 4-2.1

The effect of temperature on the QO_2 of isolate-E

(QO_2 units:- $nmO_2\ min^{-1}\ mg\ dry\ wt^{-1}$)

	Assay temperature				
QO_2.	288°K	293°K	298°K	303°K	308°K
	1.92	3.28	4.03	4.88	6.17
	2.14	3.06	4.25	4.93	5.95
Batch	2.19	3.01	3.98	4.61	6.22
1	1.87	3.33	4.30	4.66	5.90
	2.26	2.94	4.37	5.00	6.29
	1.80	3.40	3.91	4.54	5.83
	2.00	3.20	4.46	5.12	5.84
	1.78	2.98	4.24	4.90	5.62
Batch	1.73	3.25	4.51	4.85	5.89
2	2.05	2.93	4.19	5.17	5.57
	2.12	3.32	4.37	5.24	5.96
	1.66	2.86	3.91	4.78	5.50
	1.58	2.97	4.58	5.18	5.83
	1.80	2.75	4.36	4.96	6.05
Batch	1.85	3.02	4.63	5.23	6.10
3	1.53	2.70	4.31	4.91	5.78
	1.69	2.63	4.70	5.30	6.17
	1.92	3.09	4.24	4.84	5.71
Mean (n = 18)	1.88	3.04	4.30	4.95	5.91
S.D.	0.21	0.22	0.23	0.22	0.227

Appendix 4-2.2

The effect of temperature on the QO_2 of isolate-F

(QO_2 units: $nmO_2\ min^{-1}\ mg\ dry\ wt^{-1}$)

Assay temperature

QO_2	288°K	293°K	298°K	303°K	308°K
	1.578	2.991	4.897	5.466	5.892
	1.827	3.079	4.956	5.271	5.855
Batch	1.802	3.311	4.729	5.208	5.799
1	1.886	3.390	4.714	5.450	5.744
	1.902	3.060	4.970	5.461	5.886
	1.805	3.321	4.980	5.389	5.986
	-	-	4.729	5.460	5.854
	1.871	3.238	4.810	5.416	5.828
	1.850	3.267	5.230	5.354	6.189
Batch	2.180	3.461	5.161	5.726	5.849
2	2.070	3.497	4.880	5.694	6.229
	2.091	3.228	5.150	5.756	5.869
	1.761	3.508	4.890	5.386	6.209
	1.840	3.238	4.481	5.016	5.519
	1.421	2.818	4.582	5.417	5.858
Batch	1.750	3.168	4.880	5.106	5.539
3	1.511	2.887	4.783	5.325	5.834
	1.500	2.925	4.630	5.510	5.487
	1.762	3.127	4.731	4.916	5.887
Mean (n=18)	1.80	3.19	4.85	5.38	5.85
S.D.	0.20	0.22	0.20	0.23	0.21

Appendix 4-2.3

The effect of temperature on the QO_2 of isolate-A

	Assay temperature				
	288°K	293°K	298°K	303°K	308°K
	Batch 1 (n=6)				
Batch 1	1.71 ± 0.12	2.74 ± 0.14	3.42 ± 0.10	5.52 ± 0.98	7.03 ± 0.79
	Batch 2 (n=6)				
Batch 2	1.48 ± 0.11	2.51 ± 0.95	3.81 ± 0.12	5.21 ± 0.11	6.87 ± 0.14
	Batch 3 (n=6)				
Batch 3	1.37 ± 0.13	2.36 ± 0.16	3.27 ± 0.15	5.14 ± 0.14	6.73 ± 0.98
Mean (n=18)	1.52	2.54	3.50	5.29	6.88
S.D	0.22	0.24	0.23	0.21	0.22

Appendix 4-3.1

The effect of hyperbaric N_2 on the QO_2 of 3 isolates of *A. avenae* (QO_2 units: $nmO_2\ min^{-1}\ mg\ dry\ wt^{-1}$)

Isolate	Control Group Zero time in SDW Mean QO_2 [n=6x2]	Control Group Mean QO_2 following 12 hrs in air saturated SDW [n=6x2]	Test Group Zero time Mean QO_2 [n=6x2]	Test Group Mean QO_2 following 12 hrs in N_2 saturated SDW [n=6x2]
F	5.28 ± 0.20	5.71 ± 0.33	5.31 ± 0.22	4.52 ± 0.30
A	5.32 ± 0.23	5.11 ± 0.21	5.4 ± 0.18	5.18 ± 0.31
E	5.22 ± 0.25	5.40 ± 0.30	5.2 ± 0.20	4.78 ± 0.28

Appendix 4-4.1

The effect of hyperbaric O_2 on the QO_2 of 3 isolates of A. avenae. (Incubated for 12 hr at 25°C)

Isolate	Control Group Zero time Mean QO_2 (n=6x2)	Control Group Mean QO_2 after 12 hrs in air saturated SDW (n=6x2)	Test Group Zero time Mean QO_2 (n=6x2)	Test Group Mean QO_2 after 12 hrs in O_2 saturated SDW (n=6x2)
F	5.32 ± 0.30	5.92 ± 0.32	5.41 ± 0.33	5.62 ± 0.28
A	5.43 ± 0.34	5.08 ± 0.35	5.28 ± 0.20	5.46 ± 0.33
E	5.38 ± 0.11	5.45 ± 0.34	5.40 ± 0.18	5.28 ± 0.25

Appendix 5-1.1

The effect of NaCN (3mM) on the QO_2 of isolate-A: derived from mass cultures (culture temperature = 25°C) of varying age

Culture Age	Normal QO_2			QO_2 in 3mM NaCN			% Inhibition			Overall Means ± S.D. (n=18)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Normal QO_2	QO_2 in 3mM NaCN	% Inhibition
50	5.55 ± 0.09	5.36 ± 0.09	5.35 ± 0.09	4.49 ± 0.06	4.44 ± 0.05	4.36 ± 0.23	18.85	17.00	18.28	5.42 ± 0.13	4.43 ± 0.10	18.04
60	5.42 ± 0.09	5.23 ± 0.09	5.18 ± 0.09	4.34 ± 0.09	4.25 ± 0.18	4.24 ± 0.14	19.79	18.50	18.04	5.27 ± 0.13	4.27 ± 0.11	18.77
70	5.64 ± 0.09	5.45 ± 0.09	5.44 ± 0.09	4.61 ± 0.18	4.41 ± 0.11	4.38 ± 0.12	18.16	18.79	19.79	5.51 ± 0.11	4.47 ± 0.12	18.74
80	5.55 ± 0.09	5.48 ± 0.08	5.48 ± 0.08	4.50 ± 0.12	4.50 ± 0.19	4.49 ± 0.11	18.69	17.68	18.02	5.51 ± 0.04	4.49 ± 0.19	18.13
90	5.33 ± 0.09	5.19 ± 0.09	5.26 ± 0.14	4.39 ± 0.12	4.24 ± 0.11	4.33 ± 0.07	17.64	18.30	17.49	5.26 ± 0.12	4.32 ± 0.11	17.81
120	5.24 ± 0.09	5.36 ± 0.12	5.18 ± 0.34	4.22 ± 0.05	4.39 ± 0.10	4.23 ± 0.28	18.69	18.10	18.50	5.24 ± 0.21	4.28 ± 0.18	18.43

Appendix 5-1.2

The effect of NaCN (3mM) on the QO_2 of isolate-F derived from mass cultures (culture temperature = 25°C) of varying age

Culture Age	Normal QO_2 Batch 1	Normal QO_2 Batch 2	Normal QO_2 Batch 3	QO_2 in 3mM NaCN Batch 1	QO_2 in 3mM NaCN Batch 2	QO_2 in 3mM NaCN Batch 3	% Inhibition Batch 1	% Inhibition Batch 2	% Inhibition Batch 3	Overall Means ± S.D. (n=18) Normal QO_2	Overall Means ± S.D. (n=18) QO_2 in 3mM NaCN	Overall Means ± S.D. (n=18) % Inhibition
50	5.53 ± 0.02	5.33 ± 0.10	5.51 ± 0.07	1.21 ± 0.08	1.36 ± 0.09	1.05 ± 0.08	78.11	74.10	81.03	5.45 ± 0.12	1.20 ± 0.15	77.74
60	5.51 ± 0.07	5.44 ± 0.08	5.19 ± 0.07	1.33 ± 0.09	1.14 ± 0.08	1.29 ± 0.09	75.86	79.04	75.14	5.38 ± 0.16	1.25 ± 0.10	76.88
70	5.47 ± 0.08	5.40 ± 0.08	5.15 ± 0.08	1.08 ± 0.07	1.43 ± 0.08	1.28 ± 0.10	80.10	73.44	76.67	5.34 ± 0.16	1.26 ± 0.17	76.73
90	5.28 ± 0.08	5.02 ± 0.08	5.02 ± 0.10	1.15 ± 0.08	1.40 ± 0.09	1.37 ± 0.09	78.22	72.11	72.71	5.10 ± 0.15	1.30 ± 0.13	74.34
100	5.34 ± 0.08	5.08 ± 0.08	5.24 ± 0.16	1.49 ± 0.09	1.11 ± 0.08	1.21 ± 0.04	72.08	78.13	76.81	5.22 ± 0.13	1.27 ± 0.19	75.67
140	5.20 ± 0.08	5.11 ± 0.16	5.01 ± 0.16	1.35 ± 0.08	1.32 ± 0.09	1.25 ± 0.05	74.04	73.97	74.85	5.10 ± 0.12	1.30 ± 0.11	74.28

Appendix 5-1.3

The effect of NaCN [3mM] on the QO_2 of isolate-E, derived from mass cultures [culture temperature = 25°C] of varying age

Culture Age	Normal QO_2			QO_2 in 3mM NaCN			% Inhibition			Overall Means ± S.D. (n=18)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Normal QO_2	QO_2 in 3mM NaCN	% Inhibition
40	5.94 ± 0.08	5.87 ± 0.08	5.62 ± 0.08	5.72 ± 0.27	5.65 ± 0.267	5.48 ± 0.179	3.63	3.63	2.38	5.81 ± 0.16	5.61 ± 0.12	3.21
50	5.83 ± 0.08	5.76 ± 0.08	5.51 ± 0.08	5.68 ± 0.25	5.67 ± 0.26	5.31 ± 0.235	2.53	1.53	3.60	5.70 ± 0.17	5.55 ± 0.21	2.55
60	6.10 ± 0.08	5.85 ± 0.08	5.78 ± 0.08	5.84 ± 0.32	5.69 ± 0.303	5.62 ± 0.198	4.18	2.61	2.55	5.91 ± 0.15	5.71 ± 0.112	3.11
70	5.96 ± 0.08	5.71 ± 0.09	5.64 ± 0.09	5.66 ± 0.34	5.40 ± 0.359	5.35 ± 0.32	5.08	5.28	5.1	5.77 ± 0.16	5.47 ± 0.16	5.15
80	5.98 ± 0.08	5.72 ± 0.09	5.84 ± 0.07	5.67 ± 0.32	5.40 ± 0.31	5.52 ± 0.07	5.11	4.6	5.47	5.85 ± 0.14	5.53 ± 0.13	5.06
100	5.73 ± 0.08	5.47 ± 0.09	5.82 ± 0.15	5.42 ± 0.37	5.22 ± 0.33	5.48 ± 0.34	5.38	4.51	5.78	5.67 ± 0.19	5.37 ± 0.13	5.22
140	5.94 ± 0.08	5.68 ± 0.08	5.50 ± 0.14	5.65 ± 0.32	5.37 ± 0.39	5.22 ± 0.35	5.04	5.46	5.28	5.71 ± 0.21	5.41 ± 0.21	5.26

Appendix 5-2.1

The recovery (at 23°C) of isolate-F (acclimatized to 23°C) following initial exposure to 6mM, NaCN (Data for Control group)

QO_2 of control group at zero time	Incubation (at 23°C) Period (=recovery period) of control group	QO_2 of control group after "recovery" period at 23°C	QO_2 in 6mM,NaCN after "recovery" of controls	% Inhibition by 6mM NaCN, of controls after "recovery" period
5.03 ± 0.15	1	4.95 ± 0.08	0.99 ± 0.16	79.88
5.09 ± 0.13	2	4.92 ± 0.10	1.02 ± 0.21	79.19
5.10 ± 0.11	4	5.12 ± 0.12	1.18 ± 0.12	76.94
5.10 ± 0.16	6	5.26 ± 0.09	1.10 ± 0.14	79.01
5.25 ± 0.10	8	5.17 ± 0.11	1.20 ± 0.17	76.60
5.31 ± 0.15	24	5.46 ± 0.10	1.08 ± 0.22	80.10
5.26 ± 0.19	48	5.58 ± 0.18	1.12 ± 0.24	79.90

All values are Mean ± S.D. (n=6)

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-2.2

The recovery [at 23°C] of isolate-F [acclimatized to 23°C] following initial exposure to 6mM NaCN [Data for "Test" group]

QO_2 of Test group at zero time	QO_2 of Test group at zero time in 6mM NaCN	Recovery period of Test group at 23°C [hrs]	QO_2 of Test group after Recovery at 23°C	QO_2 in 6mM NaCN after recovery	% Inhibition by 6mM NaCN of "Test" group after recovery
4.95 ± 0.14	1.03 ± 0.12	1	1.21 ± 0.12	0.70 ± 0.15	41.52
5.03 ± 0.10	1.16 ± 0.11	2	1.22 ± 0.14	0.68 ± 0.12	43.51
4.92 ± 0.11	1.09 ± 0.14	4	2.41 ± 0.15	1.42 ± 0.14	40.80
5.12 ± 0.14	1.07 ± 0.12	6	3.17 ± 0.11	1.74 ± 0.16	45.03
4.98 ± 0.14	1.16 ± 0.17	8	3.58 ± 0.14	1.86 ± 0.17	48.01
5.47 ± 0.18	1.08 ± 0.21	24	5.15 ± 0.19	1.38 ± 0.19	73.18
5.41 ± 0.21	1.08 ± 0.19	48	5.06 ± 0.18	1.20 ± 0.20	76.15

All values are Means ± S.D. [n=6]

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-3.1

The recovery (at 30°C) of isolate-F (acclimatized to 30°C) following initial exposure to 6mM NaCN (Data for "Test" group)

QO_2 of Test group at zero time	QO_2 of Test group at zero time in 6mM NaCN	Recovery Period of Test group at 30°C (hrs)	QO_2 of Test group after Recovery at 30°C	QO_2 in 6mM NaCN after recovery	% Inhibition by 6mM NaCN of "Test" group after recovery
7.91 ± 0.20	1.61 ± 0.12	1	1.72 ± 0.16	0.92 ± 0.14	46.42
8.11 ± 0.21	1.97 ± 0.13	3	2.63 ± 0.22	1.26 ± 0.12	52.01
8.21 ± 0.21	1.58 ± 0.14	4	3.31 ± 0.15	1.66 ± 0.17	49.65
7.86 ± 0.22	2.10 ± 0.13	6	4.05 ± 0.13	2.02 ± 0.13	50.05
8.03 ± 0.21	1.55 ± 0.16	24	7.76 ± 0.09	1.56 ± 0.12	79.80

All values are the Means ± S.D. (n=6)

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-3.2

The recovery [at 30°C] of isolate-F [acclimatized to 30°C] following initial exposure to 6mM NaCN [Data for Control group]

QO_2 of Control group at zero time	Incubation [at 30°C] period [=recovery period] of Control group	QO_2 of Control group after "recovery" period at 30°C	QO_2 in 6mM,NaCN after "recovery" of Controls	% Inhibition by 6mM,NaCN of Controls after "recovery" period
8.10 ± 0.33	1	7.95 ± 0.13	1.81 ± 0.14	77.18
7.81 ± 0.16	3	7.55 ± 0.19	1.53 ± 0.16	79.65
7.92 ± 0.18	4	7.80 ± 0.12	1.55 ± 0.19	80.08
8.05 ± 0.28	6	7.91 ± 0.14	2.06 ± 0.17	73.91
8.03 ± 0.21	24	7.76 ± 0.09	1.46 ± 0.22	81.10

All values are the Means ± S.D. [n=6]

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-4.1

The recovery (at 30°C) of isolate-F (acclimatized to 23°C) following initial exposure to 6mM cyanide (Data for "Test" group)

QO_2 of Test group at zero time	QO_2 of Test group at zero time in 6mM NaCN	Recovery period of Test group at 30°C (hrs)	QO_2 of Test group after Recovery at 30°C	QO_2 in 6mM NaCN after recovery	% Inhibition by 6mM,NaCN of "Test" group after recovery
4.96 ± 0.21	1.19 ± 0.14	1	1.20 ± 0.12	0.719 ± 0.12	40.07
5.12 ± 0.18	1.02 ± 0.12	3	2.32 ± 0.12	1.33 ± 0.11	42.33
5.09 ± 0.11	1.18 ± 0.11	4	2.71 ± 0.12	1.55 ± 0.14	42.51
5.20 ± 0.12	1.08 ± 0.12	6	3.31 ± 0.11	1.78 ± 0.14	46.01
5.25 ± 0.15	1.04 ± 0.15	8	4.32 ± 0.16	2.15 ± 0.16	50.03
5.19 ± 0.22	1.14 ± 0.19	24	7.81 ± 0.22	2.02 ± 0.16	74.08

All values are Means ± S.D. (n=6)

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-4.2

The recovery [at 30°C] of isolate-F [acclimatized to 23°C] following initial exposure to 6mM, NaCN [Data for Control group]

QO_2 of Control group at zero time	Incubation [at 30°C] period [=recovery period] of Control group	QO_2 of Control group after "recovery" period at 30°C	QO_2 in 6mM, NaCN after "recovery" of Controls	% Inhibition by 6mM, NaCN of Controls after "recovery" period
5.19 ± 0.14	1	5.28 ± 0.13	1.12 ± 0.11	78.71
-	3	-	-	-
5.01 ± 0.11	4	5.62 ± 0.16	1.31 ± 0.14	76.68
5.25 ± 0.15	6	5.80 ± 0.15	1.21 ± 0.12	79.04
5.16 ± 0.17	8	5.87 ± 0.15	1.16 ± 0.14	80.14
5.24 ± 0.21	24	7.95 ± 0.23	1.75 ± 0.17	77.87

All values are Means ± S.D. [n=6]

QO_2 units:- nmO_2/mg dry wt/min

Appendix 5-5.1 The effect of prolonged incubation [at 30°C] of isolate-F in 6 mM NaCN on the QO_2 compared with the immediate effect of 6 mM NaCN

Hrs. incubated	QO_2 of controls	QO_2 upon immediate application of 6mM NaCN	QO_2 following prolonged incubation with 6mM NaCN *
Zero time	5.31 ± 0.23	1.20 ± 0.11	
1.0	5.39 ± 0.17	1.27 ± 0.18 [NS]	1.26 ± 0.12 [a]
2.5	5.50 ± 0.18	1.26 ± 0.08 ***	0.76 ± 0.12 [a]
4.5	5.69 ± 0.20	1.35 ± 0.12 ***	0.68 ± 0.08 [a]
6.0	-	-	-
8.0	5.90 ± 0.20	1.24 ± 0.08 ***	0.58 ± 0.05 [a]
24.	7.80 ± 0.16	1.65 ± 0.18 ***	0.47 ± 0.12 [a]
48.	7.65 ± 0.18	1.44 ± 0.31 ***	0.38 ± 0.06 [a]
168.	6.94 ± 0.15	1.15 ± 0.32 ***	0 [a]

[a] Additional cyanide (10^{-2} M) did not have further inhibitory effect

All values are Means ± S.D. (n = 6)

NS = Not significant; $p < 0.05$ = * ; $p < 0.01$ - **; $p < 0.001$ = ***

QO_2 units : nmO_2/mg dry wt./min

Appendix 5-6.1 The effect of prolonged incubation of isolate-A [whole-worms] in 6mM NaCN, on the QO_2 and the response of treated worms to a further application [10^{-2}M] to NaCN. [Values are the Means ± S.D. of 6 replicates]

Hrs. incubated at 30°C	QO_2 of controls	QO_2 of worms following incubation in 6 mM NaCN at 30°C	QO_2 of the NaCN treated worms following further application of [10^{-2}M final conc.] NaCN	% Inhibition [I]/or %Stimulation[S] upon further application of NaCN to NaCN treated worms
Zero time	5.30 ± 0.14	-	-	-
2	5.45 ± 0.15 ***	4.90 ± 0.15 *	4.68 ± 0.14	4.48% [I]
4	5.85 ± 0.10	4.49 ± 0.13 a	4.49 ± 0.13	0
6	6.56 ± 0.10	4.28 ± 0.16 a	4.28 ± 0.16	0
8	6.85 ± 0.19	4.18 ± 0.10 a	4.18 ± 0.10	0
24	2.64 ± 0.11	4.02 ± 0.21 NS	5.49 ± 0.18	36.56% [S]
48	2.55 ± 0.12	4.23 ± 0.18 NS	5.36 ± 0.11	26.71% [S]

QO_2 units = nmO_2/mg dry wt./min

a - No further response to cyanide in excess of 6 mM

All values are the Means ± S.D. NS - not significant; * - $p < 0.05$; ** $p < 0.01$, $p < 0.001$- ***

Appendix 5-6.2 The immediate effect of 3 mM NaCN on the QO_2 of isolate-A [cultured at 25°C] following incubation for various periods of time at 30°C [Values are the means ± S.D. of 6 replicates]

Hrs. incubated at 30°C	QO_2 of controls	QO_2 in 6 mM NaCN [immediately after application]	% Inhibition [I]/or %Stimulation[S] of QO_2 [Mean of 6]
Zero time	5.30 ± 0.14	5.27 ± 0.11	0.56% [I]
2	5.45 ± 0.15 **	5.16 ± 0.10	5.32% [I]
4	5.85 ± 0.10 ***	4.95 ± 0.11	15.38% [I]
6	6.56 ± 0.10 **	4.61 ± 0.12	29.72% [I]
8	6.85 ± 0.19	4.45 ± 0.11	35.03% [I]
24	2.64 ± 0.11	5.25 ± 0.12 ***	101.14% [S]
48	2.55 ± 0.12	5.11 ± 0.15 NS	100.39% [S]
168	2.32 ± 0.16	4.82 ± 0.13 NS	107.75% [S]

QO_2 units: - nmO_2/mg dry wt./min

Appendix 5-6.3 The effect of prolonged incubation [at 30°C] of isolate-A in 6mM NaCN on the QO_2 compared with the immediate effect of 6 mM NaCN

Hrs. incubated	QO_2 of controls	QO_2 on immediate addition of NaCN 6 mM		QO_2 Following prolonged incubation in NaCN 6 mM
Zero time	5.30 ± 0.14	5.27 ± 0.11		-
2	5.45 ± 0.15	5.16 ± 0.10	**	4.90 ± 0.15
4	5.85 ± 0.10	4.95 ± 0.11	***	4.49 ± 0.13
6	6.56 ± 0.10	4.61 ± 0.12	***	4.28 ± 0.16
8	6.85 ± 0.19	4.45 ± 0.11	**	4.17 ± 0.10
24	2.64 ± 0.11	5.25 ± 0.12	***	4.02 ± 0.21
48	2.55 ± 0.12	5.11 ± 0.15	***	4.23 ± 0.18
168	2.32 ± 0.16	4.82 ± 0.13		

QO_2 units = nmO_2/mg dry wt./min

All values are Means ± S.D. [n = 6]

NS = not significant; * - $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Appendix 5-7.1

The effect of anaerobic (100% N_2) treatment of isolate-F (cultured at 30°C) on the QO_2 and the response to 3 mM NaCN (Values are Means ± S.D.; n = 6)

	QO_2 of 'test' group at zero time	QO_2 of controls at zero time	QO_2 after anaerobic treatment for 24 hrs at 30°C	QO_2 of controls after 24 hrs at 30°C
	5.31 ± 0.18 I	5.28 ± 0.15 II	4.81 ± 0.12 III	7.85 ± 0.22 IV
QO_2 in 3mM NaCN		1.15 ± 0.13 V	2.68 ± 0.18 VI	1.82 ± 0.22 VII
Mean % Inhibition		78.11 %	44.13 %	76.81 %

QO_2 units:- nmO_2/mg dry wt./min

Related t-test analysis of the above data

I v II $p > 0.05$

II v III $p < 0.001$

V v VI $p < 0.001$

Appendix 5-7.2

The effect of anaerobic (100% N_2) treatment of isolate-A (cultured at 30°C) on the QO_2 and the response to 3 mM NaCN (Results are means ± S.D.; n = 6)

	QO_2 of 'test' group at zero time	QO_2 of controls at zero time	QO_2 after 24 hrs anaerobic treatment at 30°C	QO_2 of controls after 24 hrs at 30°C
	5.4 ± 0.15 I	5.32 ± 0.14 II	5.18 ± 0.15 III	5.11 ± 0.18 IV
QO_2 in 3 mM NaCN		6.50 ± 0.23 V	5.18 ± 0.15 VI *No response to NaCN	6.34 ± 0.23 VII
Mean % change in QO_2		(+)22.18%	0	(+)24.07 %

QO_2 units:- nmO_2/mg dry wt./min

Related 't-test analysis of the above data

II	v	III	$p > 0.05$ (NS)
V	v	VI	$p < 0.001$

Appendix 7-1.1 α-Glycerophosphate utilization RCRs and ADP:O ratios of isolate-A mitochondrial fractions

	Replicate	O_2 utilization (n.atoms/min/mg protein)			
		In 5mM α-GP (State-4 QO_2 after cycle 1)	With added ADP (150μM) (State-3 QO_2 cycle-2)	Respiratory Control Ratio (RCR)	ADP:O Ratio
Batch 1					
	1	114	327	2.87	2.10
	2	129	312	2.42	2.24
	3	120	287	2.39	2.09
	4	103	247	2.40	2.11
Batch 2					
	5	122	289	2.37	2.22
	6	110	313	2.85	2.25
	7	124	349	2.82	2.18
Batch 3					
	8	108	305	2.83	2.23
	9	126	351	2.79	2.25
	10	106	261	2.47	2.12
	11	104	250	2.41	2.16
Batch 4					
	12	134	328	2.45	2.09
	13	129	366	2.84	2.25
	14	132	365	2.77	2.08
Mean		118.64	310.71	2.62	2.169
S.D.		10.93	40.14	0.23	0.069

Appendix 7-1.2 Succinate utilization, RCRs and ADP:O ratios of isolate-A mitochondrial fraction

	Replicate	O_2 utilization (n.atoms/min/mg protein)		Respiratory Control Ratio (RCR)	ADP:O Ratio
		In 5mM Succinate QO_2 State-4 after cycle 1	With added (150μM ADP) QO_2 State-3 cycle-2		
Batch 1					
	1	118	431	3.65	1.49
	2	115	396	3.44	1.39
	3	119	431	3.62	1.34
	4	120	380	3.17	1.47
Batch 2					
	5	124	424	3.42	1.35
	6	121	440	3.64	1.34
	7	114	367	3.22	1.48
Batch 3					
	8	110	396	3.60	1.33
	9	121	392	3.24	1.47
	10	124	395	3.19	1.38
Batch 4					
	11	126	459	3.65	1.44
	12	123	397	3.23	1.34
	13	119	430	3.61	1.48
	14	117	398	3.40	1.43
Mean		119.35	409.78	3.43	1.41
S.D.		4.37	25.94	0.19	0.06

Appendix 7-1.3 α-Glycerophosphate utilization, RCRs and ADP:O ratios of isolate-F mitochondrial fraction

	Replicate	O_2 utilization (n.atoms/min/mg protein)		Respiratory Control Ratio (RCR)	ADP:O Ratio
		In 5mM α-GP ($State-4\ QO_2$ after cycle 1)	With added (150μM ADP) QO_2 State-3 cycle-2		
Batch 1					
	1	55	129	2.35	2.18
	2	73	170	2.33	2.27
	3	68	138	2.19	2.16
	4	57	127	2.23	2.13
Batch 2					
	5	69	156	2.26	2.29
	6	72	137	1.90	2.14
	7	75	147	1.96	2.26
Batch 3					
	8	70	161	2.31	2.24
	9	74	142	1.92	2.28
	10	71	153	2.16	2.16
	11	76	142	1.87	2.20
Batch 4					
	12	68	130	1.91	2.15
	13	57	131	2.30	2.27
	14	63	125	1.99	2.15
Mean		67.35	142.0	2.12	2.20
S.D.		7.12	13.80	0.184	0.059

Appendix 7-1.4 Succinate utilization, RCRs and ADP:O ratios of isolate-F mitochondrial fraction

Replicate	O_2 utilization (n.atoms/min/mg protein)		Respiratory Control Ratio (RCR)	ADP:O Ratio
	In 5mM Succinate (State-4 QO_2 after cycle 1)	With added ADP (100μM) (State-3 QO_2 of cycle 2)		
Batch 1				
1	84	193	2.30	1.80
2	75	154	2.06	1.93
3	79	180	2.28	1.79
4	85	211	2.49	1.82
Batch 2				
5	93	193	2.08	1.96
6	90	226	2.51	1.81
7	88	217	2.47	1.95
8	70	146	2.09	1.93
Batch 3				
9	87	215	2.47	1.91
10	89	224	2.52	1.89
11	86	211	2.45	1.92
Batch 4				
12	87	177	2.04	1.81
13	73	154	2.11	1.94
14	92	213	2.32	1.90
Mean	85.14	193.85		
S.D.	8.099	27.48	2.29	1.88
			0.188	0.062

Appendix 7-2.1 The effect of Rotenone on the state-3 QO_2 of isolate-A mitochondrial fraction utilizing α-GP as substrate

	Replicate	State-3 QO_2	QO_2 in 2mM Rotenone	% Inhibition
Batch 1				
	1	271	165	39.11
	2	263	148	43.82
	3	285	188	34.03
	4	292	187	35.95
Batch 2				
	5	291	180	38.14
	6	264	164	37.87
	7	295	174	41.01
	8	286	192	32.86
Batch 3				
	9	294	168	42.85
	10	286	169	40.90
Mean		282.7	173.5	38.64
S.D.		12.18	13.50	-
S.E.		3.85	4.26	-

Appendix 7-2.2 The effect of antimycin-A on the state-3 QO_2 of isolate-A mitochondrial fraction utilizing α-GP as substrate

	Replicate	State-3 QO_2	QO_2 in Ant.A (1µg/mg protein	% Inhibition
Batch 1				
	1	331	268	19.0
	2	310	261	15.8
	3	320	(320)	Zero
	4	322	(325)	Zero
Batch 2				
	5	319	(319)	Zero
	6	332	296	10.8
	7	317	(317)	Zero
	8	319	(319)	Zero
Batch 3				
	9	318	265	16.6
	10	332	(332)	Zero
	11	329	288	12.5
	12	331	269	18.7
Mean		323.33	298	7.78
S.D.		7.36	26.56	-
S.E.		2.12	7.67	-

Appendix 7-2.3 The effect of NaCN on the ascorbate TMPD-oxidase activity of isolate-A mitochondrial fraction utilizing α-GP as substrate

	Replicate	State-3 with Ascorbate+ TMPD	Rate in 3mM NaCN	% Inhibition
Batch 1				
	1	355	126	64.5
	2	362	143	60.4
	3	358	122	65.9
	4	369	140	62.0
Batch 2				
	5	365	136	62.7
	6	370	142	61.6
	7	356	126	64.6
	8	359	122	66.0
Batch 3				
	9	363	140	61.4
	10	367	145	60.4
	11	363	138	61.9
	12	368	132	64.1
Mean		362.91	134.33	62.95
S.D.		5.08	8.40	1.99

Appendix 7-2.4 The effect of SHAM/Azide on the state-3 QO_2 of isolate-A mitochondrial utilizing α-GP as substrate

	Replicate	State-3 QO_2	QO_2 in SHAM (3mM)	% Inhibition	QO_2 in 3mM Azide in presence of SHAM	% In. by SHAM + Azide	% Inhibition of SHAM insensitive QO_2 by Azide 3mM
Batch 1	1	280	227	18.92	70	75.00	69.16
	2	294	223	24.14	79	73.12	64.57
	3	289	234	19.03	74	74.39	68.37
	4	297	234	21.21	86	71.04	63.24
Batch 2	5	285	219	23.15	67	76.49	69.40
	6	303	245	19.14	98	67.65	60.00
Batch 3	7	289	219	24.22	74	74.39	66.21
	8	284	227	20.07	77	72.88	66.07
Mean		290.12	228.5	21.23%	78.12	73.12	65.87
S.D.		7.50	8.84	-	9.87	-	-
S.E.		2.67	3.13	-	3.5	-	-

Appendix 7-2.5 The effect of SHAM/Azide on the state-3 QO_2 of isolate-F mitochondrial utilizing α-GP as substrate

	Replicate	State-3 QO_2	QO_2 in SHAM (3mM)	% Inhibition	QO_2 in 3mM Azide in presence of SHAM	% In. by SHAM + Azide	% Inhibition of SHAM insensitive QO_2 by Azide 3mM
Batch 1	1	183	140	22.65	15	91.80	89.28
	2	162	131	19.14	14	91.35	89.31
	3	159	122	23.27	19	88.05	84.42
Batch 2	4	169	133	21.30	23	86.39	82.70
	5	171	128	25.14	8	95.32	93.75
	6	164	129	21.34	16	90.24	87.59
	7	160	128	20.00	24	85.00	81.25
Batch 3	8	159	129	18.86	27	83.01	79.06
	9	168	129	23.21	33	80.35	74.41
	10	170	130	23.52	22	87.05	83.07
Mean		166.5	129.9	21.84	20.1	87.85	84.48
S.D.		7.41	4.53	-	7.21	4.47	-
S.E.		2.34	1.43	-	2.28	-	-

Appendix 7-2.6 The effect of rotenone on the state-3 QO_2 of isolate-F mitochondrial fraction utilizing α-GP as substrate

	Replicate	State-3 QO_2	QO_2 in 2mM Rotenone	% Inhibition
Batch 1				
	1	169	103	39.05
	2	181	106	41.43
	3	177	98	44.63
	4	186	117	37.09
Batch 2				
	5	179	116	35.19
	6	170	100	41.11
	7	182	111	39.01
	8	163	104	36.19
Batch 3				
	9	184	106	42.39
	10	181	115	36.46
	11	173	102	41.04
Mean		176.81	107.0	39.41
S.D.		7.18	6.72	-
S.E.		2.16	202	

Appendix 7-2.7 The effect of antimycin-A on the state-3 QO_2 of isolate-F mitochondrial fraction utilizing α-GP as substrate

	Replicate	State-3 Rate	Ant.A 1µg/mg protein	% Inhibition	Arc.Sine Pi
Batch 1					
	1	192	25	86.9	78.57
	2	178	23	87.0	78.49
	3	180	23	87.2	78.72
Batch 2					
	4	202	19	90.5	81.02
	5	198	27	86.3	78.02
Batch 3					
	6	195	28	85.6	77.37
	7	201	21	89.5	80.39
	8	183	18	90.1	80.09
	9	196	24	87.7	78.84
Mean		191.66	23	87.8	79.05
S.D.		9.09	3	-	1.19
S.E.		3.03	1	-	0.39

Appendix 7-2.8 The effect of NaCN on the ascorbate-TMPD oxidase activity of isolate-F mitochondrial fraction utilizing α-GP as substrate

	Replicate	Ascor/TMPD State-3	NaCN 3mM	Inhibition %
Batch 1				
	1	160	14	91.25
	2	157	20	87.26
	3	178	17	90.44
	4	164	20	87.80
Batch 2				
	5	167	18	89.22
	6	179	23	87.15
	7	156	18	88.46
	8	165	22	86.66
Batch 3				
	9	158	19	87.97
	10	165	21	87.27
	11	173	19	89.01
	12	176	17	90.34
Mean		166.50	19.2	88.56
S.D.		8.24	2.4	-
S.E.		2.381	0.7	-

Appendix 7-2.9 The effect of CO on the residual QO_2 of isolate-F mitochondria utilizing α-GP as substrate in the presence of NaCN

	Replicate	Ascor/TMPD QO_2 in 3mM NaCN	QO_2 in CO after NaCN	% Inhibition of NaCN insensitive QO_2 by CO	% Inhibition of Ascorbate/TMPD oxidation by CN^- + CO
Batch 1	1	14	9	35.71	94.37
	2	20	11	45.00	92.99
	3	17	10	41.17	94.38
	4	20	13	35.00	92.07
Batch 2	5	18	11	38.88	93.41
	6	23	13	43.47	92.73
	7	18	12	33.33	92.30
	8	-	-	-	-
Batch 3	9	19	12	36.84	92.40
	10	21	14	33.33	91.51
	11	19	12	36.84	93.06
	12	17	11	35.29	93.75
Mean		18.72	11.63	37.71	92.99
S.D.		2.37	1.43		
		0.71	0.43		

Appendix 7-2.10 Effect of CO on the residual QO_2 of isolate-A mitochondria in the presence of (3mM) NaCN and (3mM) SHAM

	Replicate	QO, in the presence of Ascor/TMPD 3mM NaCN & 3mM SHAM	QO_2 in CO after NaCN and SHAM	% Inhibition of Cyanide/SHAM insensitive respiration by CO	% Inhibition of TMPD/Ascorbate oxidation by NaCN/ SHAM and CO
Batch 1	1	88	15	82.95	95.89
	2	98	17	82.65	95.21
	3	105	24	77.42	93.37
	4	94	19	79.78	94.69
Batch 2	5	107	26	75.70	92.95
	6	106	19	82.07	94.86
	7	99	15	84.84	95.78
	8	98	25	74.48	93.03
Batch 3	9	107	20	81.30	94.49
	10	117	21	82.05	94.27
	11	109	23	78.89	93.66
	12	102	21	79.41	94.29
Mean		102.5	20.41	80.37	94.37
S.D.		7.66	3.65	-	-
S.E.		2.21	1.05	-	-

Appendix 7-2.11 Effect of SHAM on the residual QO_2 of isolate-A mitochondria (with Ascorbate/TMPD) in the presence of NaCN (3mM)

	Replicate	QO_2 in 3mM NaCN	QO_2 in presence of Ascor/TMPD, 3mM SHAM after NaCN	% Inhibition	
				of NaCN insensitive respiration by SHAM	of Ascorbate/TMPD oxidation by NaCN+ SHAM
Batch 1	1	126	98	22.22	72.39
	2	143	105	26.57	70.99
	3	122	94	22.95	73.74
	4	140	107	23.57	62.05
Batch 2	5	136	88	35.29	75.89
	6	142	106	25.36	71.35
	7	126	99	21.42	72.19
	8	122	98	19.67	72.70
Batch 3	9	140	107	23.57	70.52
	10	145	117	19.31	68.11
	11	138	109	21.01	69.97
	12	132	102	22.72	72.28
Mean (n=12)		134.33	10.25	23.63	71.01
S.D.		8.40	7.66	-	-
S.E.		2.42	2.21	-	-

Appendix 7-3.2 A values of isolate-A (reduced)-(oxidised) difference spectra as applied in the matrix-technique of Williams (1964)

a_{15} = A 500-535 nm = 0.008 *

a_{25} = A 554-540 nm = 0.120 *

a_{35} = A 563-577 nm = 0.0124 *

a_{45} = A 605-630 nm = 0.002 *

Obtained from spectrum given in Fig.

Volume of suspension was 1.6 ml; protein content was 3.38 mg

Appendix 7-3.3 A values of isolate-F (reduced)-(oxidised) difference spectra as applied in the matrix-equation technique of Williams (1964)

a_{15} = A 500-535 nm = 0.00675

a_{25} = A 554-540 nm = 0.0700

a_{35} = A 563-577 nm = 0.0145

a_{45} = A 605-630 nm = 0.004

Obtained from spectrum given in Fig.

Volume of suspension was 1.6 ml; protein content 2.94 mg

Appendix 7-3.4 Changes in the millimolar extinction coefficients of [Reduced vs. Oxidised] difference spectra of a, b and c type cytochromes employed by Williams, in the matrix computation

Cytochrome	Wavelength Pairs [nm]			
	(605-630)	(563-577)	(554-540)	(550-535)
a	12.0*	-0.326	0.95	0.63
b	0	14.3*	2.55	-3.12
c_1	-0.59	0.91	18.8*	10.3
c	-0.22	-1.16	6.51	21.0*

* Major coefficients used in the simultaneous equation matrix of Williams [1964] for the quantification of cytochromes.

Appendix 7-3.7

The simultaneous-equations matrix technique of Williams [1964] for the calculation of cytochromes in difference spectra

A 550-535 = a_{15} x_1 = [cyt.c] in micromoles/ml in cuvette
" 554-540 = a_{25} x_2 = [cyt.c_1] " " " "
" 563-577 = a_{35} x_3 = [cyt.b] " " " "
" 605-630 = a_{45} x_4 = [cyt.a] " " " "

Equations used [Williams, 1966] Volume = 1.6 ml
Content of Protein = 3.38 mg

$$21.0\,x_1 + 10.3\,x_2 - 3.12\,x_3 + 0.63\,x_4 = a_{15}$$
$$6.51\,x_1 + 18.8\,x_2 + 2.55\,x_3 + 0.95\,x_4 = a_{25}$$
$$-1.16\,x_1 + 0.91\,x_2 + 14.2\,x_3 - 0.326\,x_4 = a_{35}$$
$$-0.22\,x_1 - 0.59\,x_2 + 0\,x_3 + 12.0\,x_4 = a_{45}$$

Solution for b_{15}, c_{25}, d_{35} and e_{45} proceeds as follows

$$b_{15} = a_{15}/21.0$$
$$c_{25} = [a_{25} - 6.51\,b_{15}]/15.6$$
$$d_{35} = [a_{35} + 1.16\,b_{15} - 1.48\,c_{25}/13.8$$
$$e_{45} = [a_{45} + 0.22\,b_{15} + 0.482\,c_{25} - 0.076\,d_{35}]/12.0$$

Then

$x_4 = e_{45}$ i.e. concentration of cytochrome-a micromoles/ml in the cuvette

$x_3 = d_{35} + 0.0263\,x_4$ i.e. concentration of cytochrome-b micromoles/ml in the cuvette

$x_2 = c_{25} - 0.0484\,x_3 - 0.225\,x_3$ i.e. concentration of cytochrome-c_1 micromoles/ml in the cuvette

$x_1 = b_{15} = 0.03\,x_4 + 0.149\,x_3 - 0.491\,x_2$ i.e. concentration of cytochrome c micromoles/ml in the cuvette

Thus if v = volume of suspension in cuvette and p=protein content in sample

$V \times X_4/p$ = cyt.a concentration in nmoles/mg protein
$V \times X_3/p$ = cyt.b concentration in nmoles/mg protein
$V \times x_2/p$ = cyt.c_1 concentration in nmoles/mg protein
$V \times x_1/p$ = cyt.c concentration in nmoles/mg protein